UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH & LIFE SCIENCES

School of Medicine

The molecular pathogenesis of myeloproliferative neoplasms

by

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ABSTRACT

FACULTY OF MEDICINE, HEALTH & LIFE SCIENCES, SCHOOL OF MEDICINE

Doctor of Philosophy

THE MOLECULAR PATHOGENESIS OF MYELOPROLIFERATIVE NEOPLASMS

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Myeloproliferative neoplasms (MPNs) are a heterogeneous group of haematological stem cell malignancies characterised by proliferation of one or more cells of the myeloid lineage. The molecular investigation of MPN was revolutionized in 2005 by the finding that approximately 95% of cases with polycythaemia vera (PV) and 50-60% of cases of essential thrombocythaemia (ET) and primary myelofibrosis (PMF) are characterised by a single acquired mutation, *JAK2* V617F. My study has focused on four principal areas:

(i) Involvement of V617F in other myeloid disorders. After developing sensitive methods to detect and quantify V617F, this mutation was identified in 17% of cases of atypical chronic myeloid leukaemia (17/99) as well as other atypical MPN, thus demonstrating that it was more widely involved in myeloid disorders that initially thought. Homozygosity of V617F was shown to have arisen by acquired uniparental disomy (UPD) and examination of two cases with V617F plus either *KIT* D816V or *BCR-ABL* demonstrated that the mutations had arisen in independent clones.

(ii) In vitro assays to predict imatinib sensitivity. Haemopoietic colony and liquid cultures were used to determine if peripheral blood or bone marrow cells from atypical MPN cases (n=200) were sensitive to imatinib. Of those that responded in one or both cultures (n=185) some had known abnormalities of *PDGFRA* or *PDGFRB*, but a significant minority proved negative for all molecular tests suggesting the presence of uncharacterised imatinib-sensitive mutations.

(iii) V617F as a marker of response to therapy. JAK2 V617F was used as a molecular marker to monitor the response of PV patients (n=21) to therapy with imatinib and interferon-α. Neither therapy eradicated V617F but there was a modest reduction in %V617F which correlated with haematological response. By contrast, in those patients that did not respond (n=13) the %V617F marginally increased.

(iv) Genetic predisposition to MPN. Whilst investigating the possible contribution of *JAK2* single nucleotide polymorphisms to the phenotypic diversity associated with V617F, marked skewing of alleles associated with the mutation was observed. Further investigation revealed that V617F-associated disease is strongly associated with a specific constitutional *JAK2* haplotype, designated 46/1, in all three disease entities compared to healthy controls (PV, n=192, $P=2.9\times10^{-16}$; ET, n=78, $P=8.2\times10^{-9}$ and MF, n=41, $P=8.0\times10^{-5}$). Furthermore, allele-specific PCR demonstrated that V617F specifically arises on the 46/1 allele in most cases. The 46/1 JAK2 haplotype thus predisposes to the development of V617F associated MPNs (OR=3.7; 95% CI 3.1-4.3) and provides a model whereby a constitutional genetic factor is associated with an increased risk of acquiring a specific somatic mutation.

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Declaration Of Authorship

I, Amy Victoria Jones, declare that this thesis entitled:

The molecular pathogenesis of myeloproliferative neoplasms

and the work presented in it are my own and has been generated by me as the result of my own original research. I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Either none of this work has been published before submission, or parts of this work have been published as:
    - Jones AV, Chase A, Silver RT, Oscier D, Zoi K, Wang YL, et al. *JAK2* haplotype is a major risk factor for the development of myeloproliferative neoplasms. Nat Genet. 2009;41(4):446-9
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List of Abbreviations

ABI	Applied Biosystems
aCML	atypical CML
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
AS-PCR	Allele Specific Polymerase Chain Reaction
ATP	Adenosine Triphosphate
BFU-E	Blast Forming Unit-Erythroid
BM	Bone Marrow
bp	base pair
cDNA	complementary DNA
CEL	Chronic Eosinophilic Leukaemia
CFU-Baso	Colony Forming Unit-Basophil
CFU-E	Colony Forming Unit-Erythroid
CFU-G	Colony Forming Unit-Granulocyte
CFU-GM	Colony Forming Unit-Granulocyte/Megakaryocyte/Macrophage
CFU-GEMM	Colony Forming Unit-Granulocyte/Erythroid/Megakaryocyte/ Macrophage
CFU-M	Colony Forming Unit-Macrophage
CI	Confidence Interval
CLL	Chronic Lymphocytic Leukaemia
CML	Chronic Myeloid Leukaemia

CMML	Chronic Myelomonocytic Leukaemia
CMPD	Chronic Myeloproliferative Disorder
CNL	Chronic Neutrophilic Leukaemia
CR	Compete haematological response
CSF1R	Colony Stimulating Factor 1 Receptor
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
DD	Dimerisation Domain
dGTP	Deoxyguanosine 5'-triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide 5'-triphosphate
ddNTP	Di-Deoxynucleotide 5'-triphosphate
dTTP	Deoxythymidine 5'-triphosphate
EEC	Endogenous Erythroid Colony
EPO	Erythropoietin
ET	Essential Thrombocythaemia
FAM	6-carboxyfluorescein
FISH	Fluorescence *in situ* hybridization
FO	Forward outer
Fwt	Forward Wild-type
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase

GMP	Granulocyte-Macrophage progenitor
GTP	Guanosine Triphosphate
GWAS	Genome-wide Association Study
HES	Hypereosinophilic Syndrome
HIF	Hypoxia inducible factor
HLA	Human Leukocyte Antigen
HRM	High Resolution Melt
HSC	Haematopoetic Stem Cells
HU	Hydroxyurea
IE	Idiopathic Erythrocytosis
IFNα	Interferon Alpha
IGF-1	Insulin-like Growth Factor-1
IL	Interleukin
IMF	Idiopathic Myelofibrosis
ITD	Internal Tandem Duplication
JM	Juxtamembrane
JMML	Juvenile Myelomonocytic Leukaemia
KI	Kinase Insert
lacZ	β-galactosidase
LD	Linkage disequilibrium
LNA	Locked Nucleic Acid
LOH	Loss of Heterozygosity

LREC	Local Regional Ethics Committee
Maf	minor allele frequency
MAPK	Mitogen-activated Protein Kinase
MCD	Mast Cell Disease
MEK	Map-Erk Kinase
MEP	Megakaryocyte Erythroid Progenitor
MDS	Myelodysplastic Syndrome
Mg	Milligram
MLPA	Multiple Ligation Probe Amplification
MNC	Mononuclear Cell
MPD	Myeloproliferative Disorder
MPN	Myeloproliferative Neoplasm
MPN-U	Myeloproliferative Neoplasm-Unclassifiable
MQDA-PCR	Quantitative Multiplex DNA Array based PCR
mRNA	messenger RNA
MU	Mega Units
NR	No Haematological Response
NRTK	Non-receptor Tyrosine Kinase
OR	Odds Ratio
PAR	Population Attributable Risk
PB	Peripheral Blood
PCR	Polymerase Chain Reaction

PDGF	Platelet Derived Growth Factor Receptor
Peg-rIFNα	pegylated recombinant interferon alpha
PFCP	Primary Familial and Congenital Polycythaemia
Ph	Philadelphia Chromosome
PI3K	Phosphoinositide-3-kinase
PKC	Protein Kinase C
PKD	Protein Kinase D
PR	Partial Haematological Response
PTP	Protein Tyrosine Phosphatase
PV	Polycythaemia Vera
rIFNα	recombinant interferon α
RNA	Ribonucleic Acid
Rmt	Reverse mutant
RO	Reverse outer
Rpm	revolutions per minute
RR	Relative Risk
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcriptase PCR
SAP	Shrimp Alkaline Phosphatase
SCF	Stem Cell Factor
SD	Standard Deviation
SE	Secondary Erythrocytosis

SH	Src Homology
SHP	SH2-containing Phosphatases
siRNA	silent interfering RNA
SM	Systemic Mastocytosis
SNP	Single Nucleotide Polymorphism
SOCS	Suppressors of Cytokine Signalling
STAT	Signal Transducers and Activators of Transcription
TK	Tyrosine Kinase
TM	Transmembrane
TPO	Thrombopoietin
UCMPD	Unclassified Chronic Myeloproliferative Disorder
UPD	Uniparental Disomy
UV	Ultraviolet
VHL	von Hippel-Lindau
WBC	White Blood Cell
WGA	Whole Genome Amplification
WHO	World Health Organisation
WTB	Wild-type blocking
WTCCC	Wellcome Trust Case Control Consortium
XCIP	X chromosome inactivation pattern

1 Introduction

1.1 Normal haematopoiesis

The process of normal haematopoiesis originates in haematopoietic stem cells (HSCs), cells with the unique ability to undergo self-renewal and generate progenitor cells for all haematopoietic lineages on demand. Haematopoiesis is delicately regulated, giving rise to cells with functions that vary considerably, from immunity to the transport of oxygen. HSCs give rise to committed progenitor cells, which in turn proliferate and differentiate into functional mature cells. The formation of new committed progenitors is balanced against the rate of cell death by apoptosis, as most of these cells have a limited life span. Moreover, the haematopoietic system is also adapted to greatly amplify cell numbers in response to biological stresses such as bleeding or infection, which then return to basal levels once the stress has resolved. The numbers and distribution of the different cell types in the peripheral blood exhibit little variation under steady-state conditions, an observation that indicates the production of differentiated haematopoietic cells is tightly regulated [1].

In adults, the major site of haematopoietic cell formation is the bone marrow, where an associated stroma of endothelial cells, fat cells and fibroblasts provide a supporting role for HSCs and progenitors. As illustrated in Figure 1.1, a HSC makes an early important fate-determining decision to commit to one of two major pathways, myeloid or lymphoid [2,3]. After this point, the progenitor cell progressively differentiates, taking on characteristics of its chosen lineage and losing the ability to proliferate and self renew [4-6]. Mature cells arising from the myeloid lineage include erythrocytes, neutrophils, monocytes/macrophages, eosinophils, basophils and megakaryocytes/platelets, whereas mature cells arising from the lymphoid lineage become either B cells, T cells or natural killer cells.

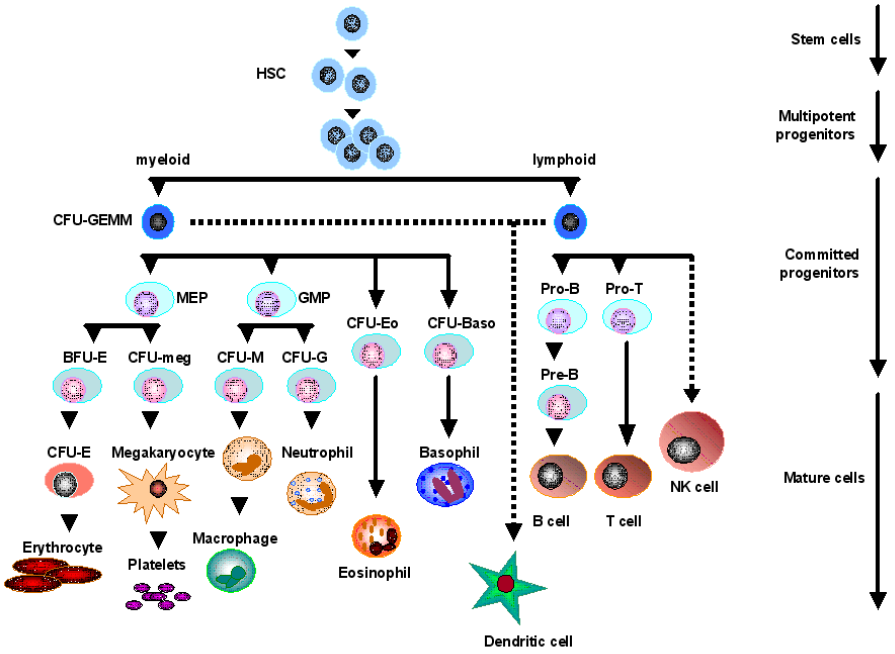


Figure 1-1. Haematopoiesis. Every mature blood cell is derived from a multipotent haematopoietic stem cell (HSC). CFU-GEMM ; colony forming unit granulocyte erythroid megakaryocyte/macrophage, MEP; megakaryocyte erythroid progenitor, GMP; granulocyte-macrophage progenitor, BFU-E; burst forming unit- erythroid, CFU-E; colony forming unit-erythroid, CFU-M; colony forming unit macrophage, CFU-G; colony forming unit granulocyte, CFU-Baso; colony forming unit basophil.

Haematopoiesis is subject to a variety of control mechanisms, influenced by inherited and environmental factors, which ultimately operate to maintain a balanced production of diverse blood cells [7]. Within the cell, a series of transcription factor networks programme progenitor cell fate and diversity [8]. Differentiation is also controlled by paracrine stimulation by growth factors and cytokines secreted by other cells and cells in the microenvironment within which the progenitor cell resides. Indeed, cytokines and their cognate receptors play a critical role by fine tuning regulatory pathways that mediate both basal and emergency haematopoiesis. Once cytokines have bound their target cell via cell-surface receptors, they stimulate biological responses such as proliferation, survival, and differentiation through a variety of signal transduction pathways which all serve to modulate gene expression [9]. Signalling networks that contribute to myeloid

differentiation are well characterised [10]; some of the most prominent pathways include *JAK-STAT, RAS/MEK/ERK, PI3K-AKT,* and p38 *MAPK/JNK/SAPK* [11]. Although defined here as discrete pathways, interactions and cross-regulation also contribute to the complexity of intracellular signalling. Excessive stimulation may be controlled by several mechanisms, for example, the *JAK-STAT* pathway is direct targeted by specific antagonists such as SH2-containing phosphatases (SHPs), and the suppressors of cytokine signalling (SOCS)[12,13].

1.2 Leukaemia

Haematological malignancies are characterised by alterations in the balance between stem cell proliferation, lineage commitment and differentiation. One definition of leukaemia is that of a 'multi-hit' model, whereby critical cellular pathways are deregulated by accumulating genetic damage, resulting in an autonomous, proliferating clone. The phenotype of the malignancy is determined by the pathways that are subverted, and sub-types of leukaemia are classified partly by the predominant types of blood cell. Clonal proliferation of abnormal cells within the bone marrow not only leads to an accumulation of blood cells in major organs like the liver and spleen, but normal haematopoiesis also becomes disrupted, so conditions such as anaemia, thrombocytopenia, and immunological dysfunction can also develop.

As with other neoplasias, leukaemia is believed to arise from a single ancestral cell which has acquired a selective growth advantage due to an acquired somatic mutation. Normal individuals display polyclonal haematopoiesis, demonstrated by Lyon's hypothesis of chromosome X inactivation [14]. In females, early in embryogenesis, each cell inactivates at random one of its two X chromosomes. This pattern of inactivation is passed on to all progeny cells, with the result that all women are mosaic with regard to X inactivation. In contrast, since leukaemia arises from a single cell, all the cells of a patient's leukaemia will carry the same inactive X. A number of assays to detect clonality have been developed, many depending on differential methylation patterns between active and inactive X chromosomes at polymorphic regions in the genome. Determining clonality is a robust feature used for characterising, defining and diagnosing leukaemia [15-17]. Importantly,

acquired cytogenetic or molecular changes can also serve as markers of clonality. An example is the Philadelphia (Ph) chromosome in chronic myeloid leukaemia (CML), which can be isolated in both myeloid and lymphoid cells, suggesting the mutation is acquired early in a HSC, before lineage commitment [18].

After the initial mutation, or 'hit', progeny acquire more mutations in a sequential and random fashion. Each new hit may confer an additional selective advantage leading to further clonal expansion and disease progression. There is some evidence that certain mutations can themselves increase genetic instability (Figure 1.2), for example, by the generation of reactive oxygen species [19] or inhibition of mismatch repair which gives rise to further mutations [20].

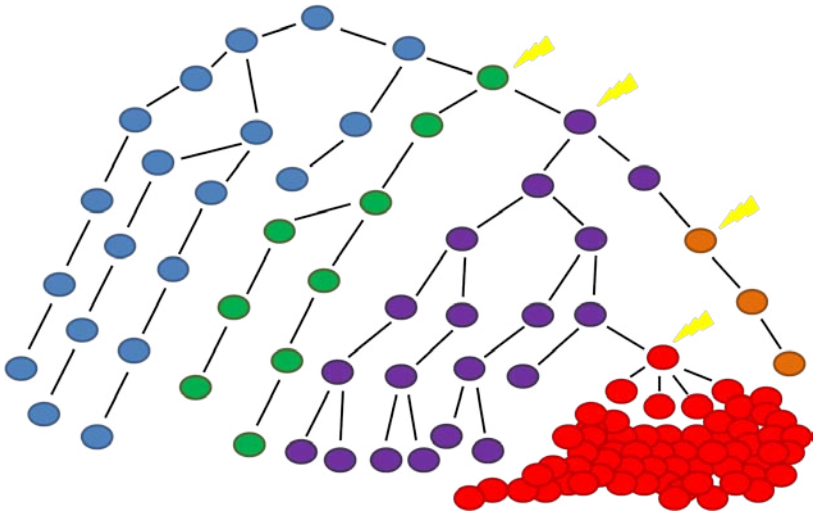


Figure 1-2. Clonal proliferation in leukaemia. Normal haematopoiesis (blue cells) is subverted when one cell (green) acquires a mutation (yellow lightning bolt) which confers a small selective advantage. Over time, progeny of this cell acquire further mutations (purple, orange, red). Some mutations may be deleterious and cause the clone to die out (orange), whereas other mutations result in an increasingly aggressive phenotype as each mutation is acquired successively. Overt malignancy is characterised by the presence of a heterogeneous group of subclones (green, purple) plus a dominating clone (red). All neoplastic cells will share some overlapping mutations, but some cells may carry additional mutations acquired late in clonal evolution.

1.2.1 Classification of leukaemia

The World Health Organisation (WHO) has classified leukaemia into many distinct groups primarily based on the cell type of the presenting clone (lymphoid or myeloid), and whether the disease is acute or chronic in nature. Other features such as cell morphology, blood counts, symptoms, immunophenotype, clinical course of disease, clonal origins and any underlying genetic or cytogenetic abnormalities are also considered in making an accurate diagnosis. Acute leukaemia is characterised by a predominance of clonally derived precursor cells that have acquired a block preventing differentiation, plus a proliferative advantage, resulting in accumulation of immature blast cells. Acute

leukaemia is often aggressive, and progresses rapidly with fatal consequences. Conversely, chronic leukaemia is generally slower to develop and progress. The predominant clone is typically comprised of fully differentiated cells that have undergone relatively normal maturation (Figure 1.1). Myeloid disorders are broadly divided into three clincopathological categories; acute myeloid leukaemia (AML), myelodysplastic syndrome (MDS) and myeloproliferative neoplasms (MPNs). This thesis focuses on the molecular pathogenesis of chronic leukaemias of myeloid origin, in particular MPNs and myelodysplastic/myeloproliferative neoplasms (MDS/MPNs).

1.2.1.1 Myeloproliferative neoplasms (MPNs) and myelodysplastic/myeloproliferative neoplasms (MDS/MPNs)

The concept of myeloproliferative disorders (MPDs) was first proposed by William Dameshek in 1951 [21], who noticed there were strong similarities between the diseases chronic myeloid leukaemia (CML), polycythaemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). The classification of chronic myeloproliferative disorders (CMPDs), recently re-termed 'myeloproliferative neoplasms' (MPNs) [22], groups CML, PV, ET and PMF into the same category as chronic neutrophilic leukaemia (CNL), chronic eosinophilic leukaemia/hypereosinophilic syndrome (CEL/HES), mast cell disease (MCD) and unclassifiable MPD [23].

Although these diseases are defined as distinct entities they share similar biological features. It is now well established that CMPDs are haematological diseases that share a common stem cell-derived clonal origin, arising in a primitive bone marrow progenitor cell [24-27]. Clonal myeloproliferation leads to an excess of abnormal cells in peripheral blood, and together with extramedullary haemopoiesis often causes splenomegaly and hepatomegaly. Although the cells mature relatively normally, there is potential for further evolution of the neoplastic clone, which may result in bone marrow failure or transformation to an aggressive blast phase of disease.

Another category of chronic myeloid neoplasms are the myelodysplastic/myeloproliferative neoplasms (MDS/MPNs) [23] which exhibit both proliferative and dysplastic features. On one hand there is evidence in peripheral blood

of effective clonal myeloproliferation, for example, eosinophilia or monocytosis. However the cells are often morphologically dysplastic, and occasionally there is cytopenia of one or more of the other cell lineages, due to ineffective proliferation or a decrease in the number of precursors. A hypercellular, fibrotic bone marrow and organomegaly are also associated features. Diseases included in the MDS/MPN category are chronic myelomonocytic leukaemia (CMML), juvenile myelomonocytic leukaemia (JMML), atypical CML (aCML), and MDS/MPN unclassifiable (MDS/MPN-U) [23]. How the MPNs and MDS/MPNs are currently classified into categories and their relationship to one another is depicted in Figure 1.3.

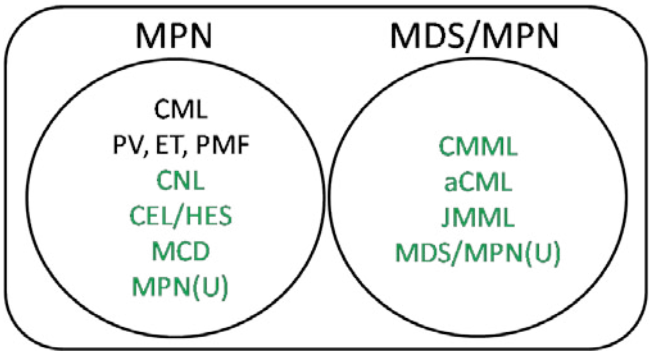


Figure 1-3. Classification of adult MPN and MDS/MPN. 'Classic' MPNs are indicated in black and 'atypical' MPNs in green.

Discrimination between the MPN and MDS/MPN categories is made difficult by overlapping clinical, laboratory and morphological findings. An incomplete knowledge surrounding the underlying pathogenesis means diagnosis of these neoplasms is based mainly on the lineage of the predominant proliferating cells and in some cases the level of bone marrow fibrosis, but this is tentative in the absence of a pathognomonic marker. I will refer to both the MPN and MDS/MPN groups broadly as MPNs for simplicity. To date there in no single genetic abnormality associated with this category of chronic myeloid neoplasms, although a pathognomonic marker, *BCR-ABL* characterises CML, and *JAK2* V617F was initially isolated in the majority of cases with PV as well as other classic MPN.

1.2.2 CML and the Philadelphia chromosome - the paradigm for deregulated tyrosine kinases

CML is the most extensively characterised classical MPN, with an incidence of 1 to 1.5/100 000 population per year [28]. CML typically presents in the chronic phase and, in the absence of intervention, progresses over the course of several years to more aggressive stages, accelerated phase and ultimately to a blast phase which resembles acute leukaemia. The natural course of disease progression from chronic phase to blast crisis is within three to five years, and it is thought this is due to the acquisition of further genetic abnormalities [29]. Chronic phase CML is characterised by an abnormal expansion of granulocyte progenitors; neutrophils that are at various stages of maturation predominate the haematological profile of peripheral blood. As observed on blood smears, the myeloid mass primarily consists of granulocytes, but also erythroid, megakaryocyte, and monocyte lineages that are derived from the malignant clone [18,30,31].

1.2.2.1 Molecular pathogenesis of CML

The first chromosomal abnormality to be associated with a haematological malignancy was the Philadelphia chromosome (Ph). In 1960, Nowell and Hungerford [32] first defined the consistent association between CML and the presence of a 'minute' chromosome, later named the Ph chromosome after its place of discovery. The cytogenetic abnormality was subsequently defined as a reciprocal translocation between the long arm of chromosomes 9 and 22 [33]. The t(9;22)(q34;q11) translocation was finally characterised at the molecular level in the 1980s, as a fusion between the breakpoint cluster region (*BCR*) gene at chromosome 22q11 and the Abelson (*ABL*) gene at chromosome 9q34. This gives rise to a novel chimaeric *BCR-ABL* gene, which when translated produces a BCR-ABL fusion protein with prominent tyrosine kinase activity [34]. Fusion to BCR constitutively activates the ABL tyrosine kinase moiety, and this activation depends on the BCR N-terminal coiled-coil domain [35]. The net result is deregulated autophosphorylation of the protein and activation of a number of downstream signalling pathways, which prolong cell survival and impact on cell proliferation [36,37]. Expression of *BCR-ABL* is sufficient to

cause chronic phase CML *in vivo*, whilst disease progression to blast phase is thought to depend on additional cytogenetic changes [38].

BCR-ABL has now become the defining marker of CML, and by definition is present in all cases [39]. However, *BCR-ABL* is also isolated in a significant proportion of acute leukaemias; 30% of cases with adult lymphoblastic leukaemia (ALL) [40], 3 to 5% of childhood ALL [41] and in 2% of cases with AML [42]. Variations in the breakpoint in the *BCR* gene create three oncogenic fusion proteins; p190, p210 and p230. All variants have been shown to generate leukaemia in murine models [38,43]. The p190 protein is found more commonly in ALL [44] whereas the p210 protein is the main form isolated in CML. The p230 protein is much rarer, having been described in CML plus cases with CNL [45]. The mechanisms that underlie the different phenotypes induced by the different splice forms are still largely unknown, but attempts have been made to define distinct groups depending on the type of BCR-ABL fusion protein [46,47]. The Ph chromosome is now detected by routine cytogenetic techniques in 85 to 90% of CML patients at diagnosis. Apparent Ph negative CML cases are generally found to harbour cryptic *BCR-ABL* fusions, formed by insertion of *ABL* material onto the *BCR* region without the reciprocal translocation [48,49]. Although not visible cytogenetically, *BCR-ABL* transcripts formed by sub-microscopic insertions and complex translocations can be detected by fluorescence *in situ* hybridisation (FISH) and by polymerase chain reaction (PCR) [50-52].

1.2.3 Other classical MPN

The three other classic MPNs, PV, ET and PMF, share several common features, namely an origin in a multipotent haematopoietic stem cell, a relatively normal cellular maturation, and in cases with PV and ET, a striking overlap in clinical presentation and the propensity to evolve into post-polycythemic or post-thrombocythemic myelofibrosis [53]. All carry the potential to transform into AML [54], although this is much less common than in CML. Classic MPNs are among the most frequent haematological neoplasms, and usually affect the adult elderly population, but may be found at a much younger age in rare cases. Similarities between the three neoplasms suggest that each may share a similar

underlying molecular pathogenesis, which will be discussed in more detail in later sections.

1.2.3.1 Polycythaemia vera (PV)

Primary PV is principally characterised by the clonal expansion of the erythroid lineage [24] which results in an elevated level of haemoglobin and a raised haematocrit, a measure of the proportion of blood volume that is occupied by erythrocytes. The elevated red cell mass causes a range of symptoms, most importantly the risk of thrombotic events due to an increased blood viscosity, but also splenomegaly, hypertension, pruritus, gout, stomach ulcers, headaches and fatigue. If left untreated, patients are at substantial risk of developing Budd-Chiari syndrome (hepatic vein thrombocytosis) [55] and myelofibrosis [56]. Treatment currently focuses on targeting symptoms and reducing the potential for thrombotic complications. The removal of blood by phlebotomy is effective at reducing the blood volume and haematocrit, and is often combined with myelosuppressive agents such as hydroxyurea (HU) and recombinant interferon α (rIFNα) [57]. Approximately 2.6 cases per 100 000 population are diagnosed per year [58] and up to 10 to 15% have an abnormal karyotype at diagnosis. The most common abnormalities include trisomies of 1q, 8, and 9, together with deletions of 20q [59-61]. An abnormally low serum erythropoietin (EPO) level is one diagnostic requirement for 'true' primary PV. Measurement of EPO levels helps determine if the erythrocytosis is a reactive consequence of either malignant or benign tumour tissue (hypoxia-independent EPO production), haemoglobinopathies or environmental factors. For example, serum EPO levels are increased by high altitude and carbon monoxide poisoning [62,63]. In cases with erythrocytosis and normal or elevated EPO a diagnosis of secondary PV or idiopathic erythrocytosis may be considered. Alternatively, marked erythrocytosis may be caused by congenital PV which results ultimately from either an abnormally elevated set point for EPO production, or abnormal oxygen homeostasis. In this situation, raised serum EPO levels are caused by mutations in the von Hippel-Lindau protein (VHL), an important component of the polyubiquitin pathway that degrades a transcription factor (hypoxia-inducible factor, HIF-1) responsible for the response to hypoxia [64]. Mutations in *VHL* are also responsible for hereditary Chuvash polycythaemia [65-67]. A different part of the oxygen-sensing pathway is targeted in primary familial and congenital polycythaemia (PFCP), where truncating mutations in the EPO

receptor abrogate negative regulatory domains resulting in upregulation of EPO-responsive signalling pathways [68]. Cases with this form of PV have a low serum EPO and when cultured *in vitro,* erythrocyte progenitors display hypersensitivity to stimulation with EPO [69,70].

Research into the molecular pathogenesis of PV suggested a defect in the signalling pathways that lead to autonomous erythrocytosis. In 1974, Prchal and Axelrad observed that primary PV progenitors form erythroid colonies *in vitro* in the absence of exogenous EPO [71]. In contrast, bone marrow cells from healthy individuals do not form these endogenous erythroid colonies (EECs). EECs can be isolated in 97 to 100% of PV cases who have not been exposed to cytoreductive therapy [72-74], and may also be cultured from a subset of cases with ET and MF [71,75]. PV progenitor cells are highly sensitive to stimulation with cytokines (for example granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interleukin-3 (IL-3), insulin growth factor-1 (IGF-1), and thrombopoietin (TPO), but are surprisingly unresponsive to stimulation with EPO [76-80]. Similarly, megakaryocyte progenitors are hypersensitive to TPO, in ET and MF [81]. Since the principal feature of PV was autonomous formation of EECs without exogenous stimulation with EPO, it was logical to infer a defect in the normal erythropoietic pathways in the pathogenesis of PV. Based on this and findings of EPOR mutations in familial erythrocytosis, the EPOR and components of its downstream signalling pathway were investigated for abnormalities in primary PV, but the receptor was found to be structurally and functionally intact [82,83]. Similarly, no mutations were identified in another candidate, SHP-1, a tyrosine phosphatase previously shown to interact with the EPOR [84] and be downregulated in progenitor cells isolated from PV patients [85]. Erythrocytes from most PV patients have elevated levels of signalling intermediates STAT3 [86], and PKC [87], plus upregulation of the *PRV-1* gene [88] and an abundance of anti- apoptotic proteins like BCL-X_L [89]. Unlike other malignancies where the INK4a locus is inactivated, PV erythroid progenitors exhibit increased expression at this gene [90]. It was only relatively recently that understanding of the molecular pathogenesis behind PV and other classic MPNs was furthered by the finding of a single mutation targeting the *JAK2* gene.

1.2.3.2 Essential thrombocythaemia (ET)

ET is characterised by the clonal expansion of megakaryocytes, resulting in the overproduction of platelets. For accurate diagnosis the megakaryocytes should display a large and mature morphology, with little or no granulocyte or erythroid proliferation. There should also be no evidence of reactive thrombocytosis, and diagnoses of other myeloid neoplasms such as CML, PV, and MDS should be ruled out. Patients with ET typically suffer bleeding and thrombosis, plus weakness, headaches, pruritus and splenomegaly. A large proportion of patients remain asymptomatic throughout the course of disease, but some will progress into a myelofibrotic or even a leukaemic phase. As not all patients with ET will require treatment at presentation, cytoreductive therapy is only given to those who are at an increased risk of thrombocytosis or bleeding. Similar to other MPNs, myelosuppressive agents such as HU and rIFNα may be administered, alongside low-dose aspirin. Thrombosis is more specifically addressed by anagrelide, which appears to affect megakaryocyte development more specifically than other agents, and is a first line therapy for younger patients and those with a greater risk of transformation [91]. In ET, chromosomal abnormalities are found in <5% of cases, and the majority of these are represented by numerical gain of chromosome 9, but trisomy 8 and 13q- are also seen [92,93].

1.2.3.3 Primary Myelofibrosis (PMF)

PMF, previously known as chronic idiopathic myelofibrosis or myelofibrosis with myeloid metaplasia, is characterised by excessive clonal proliferation of megakaryocytes, with a leucoerythroblastic blood profile. The megakaryocytes may display abnormal morphology, such as an aberrant nuclear/cytoplasmic ratio, or hyperchromatic, bulbous or irregularly folded and dense nuclei, and are called poikylocytes [22]. Moreover, deposition of reticulin and collagen in the extracellular spaces of the bone marrow (myelofibrosis) is a typical feature, and this impairs the patient's ability to generate new blood cells, resulting in a progressive pancytopenia. Stem cells that normally reside in the bone marrow are forced into circulation, so haematopoiesis is shifted to extramedullary sites such as the spleen and liver.

Additional diagnostic criteria are an increased serum lactate dehydrogenase, anaemia, palpable splenomegaly and hepatomegaly and there must be no evidence of reactive fibrosis [63]. Myelofibrosis may occur *de novo*, where it is termed PMF, or arise as a secondary event following PV or ET. Myelofibrosis leads inevitably to bone marrow failure, and the median survival is only five years. Other complications include cardiac failure due to fibrosis, haemorrhage, infection and thrombocytosis, and a significant minority transform to AML. Apart from stem cell transplantation, which is the only potential curative therapy, there are few successful maintenance therapies available [94]. Cytogenetic abnormalities are common in PMF and are similar to those seen in PV, such as 20q-, 13q-, partial trisomy 1q and trisomy 8 [63].

1.3 Tyrosine kinases in normal and malignant haematopoiesis

Within the human genome there are documented to be 518 putative protein kinases [95], of which 90 are tyrosine kinases. Tyrosine kinases are enzymes that catalyse the transfer of γ-phosphate from a purine nucleotide triphosphate, such as adenosine triphosphate (ATP) or guanosine triphosphate (GTP) to hydroxyl groups of specific amino acid residues of their protein substrates. In contrast to the serine/threonine kinases, the substrates of tyrosine kinases are tyrosine residues. Tyrosine phosphorylation activates enzyme activity, but also allows the recruitment of downstream signalling proteins, by changing conformation and creating binding sites for proteins with Src homology 2 (SH2) and other phosphotyrosine binding domains. This process is a fundamental mechanism for the reversible regulation of protein activity and function. Phosphorylation by protein kinases is a central mechanism for intracellular signal transduction, mediating a range of cell functions from cell cycle progression, differentiation, metabolism, cytoskeletal rearrangement, apoptosis and DNA synthesis. Tyrosine kinases are categorised based on cellular location as either non-receptor or receptor, and there are 32 and 58 of each present in the human genome, respectively [96]. The receptor tyrosine kinases (RTKs) bind extracellular ligand, and span the cell membrane. The non-receptor tyrosine kinases (NRTKs) are located within the cell. Both RTKs and NRTKs and their ligands are important

mediators of normal haematopoiesis, and as a consequence deregulation of these signalling pathways by intergenic or intragenic mutation can result in leukaemogenesis. The following sections discuss tyrosine kinases involved in myeloid haematopoiesis, with emphasis on mutations implicated in the pathogenesis of MPN. These mutations fall into two broad categories:

- Balanced chromosomal translocations, insertions or deletions that target tyrosine kinases generate fusion proteins, in which the extracellular ligand binding domain is replaced by the N-terminal portion of the fusion partner. The chimaeric protein retains the full catalytic kinase domain but is no longer responsive to normal mechanisms of activation and regulation. Instead, fusion proteins are constitutively activated, driving cell proliferation and promote cell survival by overriding apoptosis-related signalling [97].

- Point mutations that occur in the activation loop alter the conformation of the kinase domain, switching it to an active state, a change normally induced by phosphorylation of an activation loop tyrosine residue. Alternatively, regulatory regions that hold the protein in an inactive state can be targeted by mutation, causing destabilisation of protein conformation, and release of normal constraints on kinase activation. Extracellular domain mutations can also result in aberrant receptor dimerisation. In all situations, the tyrosine kinase is then constitutively activated in the absence of natural ligand or rendered hypersensitive to normal stimulation by ligand.

1.3.1 Receptor tyrosine kinases (RTKs)

Examples of RTK families with relevance for leukaemogenesis are given below.

1.3.1.1 Class III RTKs

The class III RTKs are characterised by five immunoglobulin-like domains in the extracellular ligand binding region, a single transmembrane domain and two intracellular

tyrosine kinase domains, separated by a kinase insert domain [98]. This family includes the receptors KIT, FLT3R, PDGFRα, PDGFRβ and FMS. All of these except PDGFRα are believed to play important roles in maintaining a steady state of haematopoiesis, and are subject to tight regulation. As illustrated in Figure 1.4, inactive class III RTKs are thought to exist as monomers, embedded in the plasma membrane. Ligand binding promotes receptor dimerisation, juxtaposing the two catalytic domains to induce a conformational change, partially activating enzyme activity. Autophosphorylation of a key tyrosine residue in the activation loop heightens enzymatic activity and additional tyrosine residues are then autophosphorylated. Individual phosphorylated tyrosines serve as docking sites for an array of intracellular signalling molecules which subsequently recruit other downstream signalling intermediates to mediate a physiological response. Induction of signalling pathways JAK/STAT, PLCγ, PI3K and Ras-Raf-MEK/ERK are all observed in class III RTK activation [99].

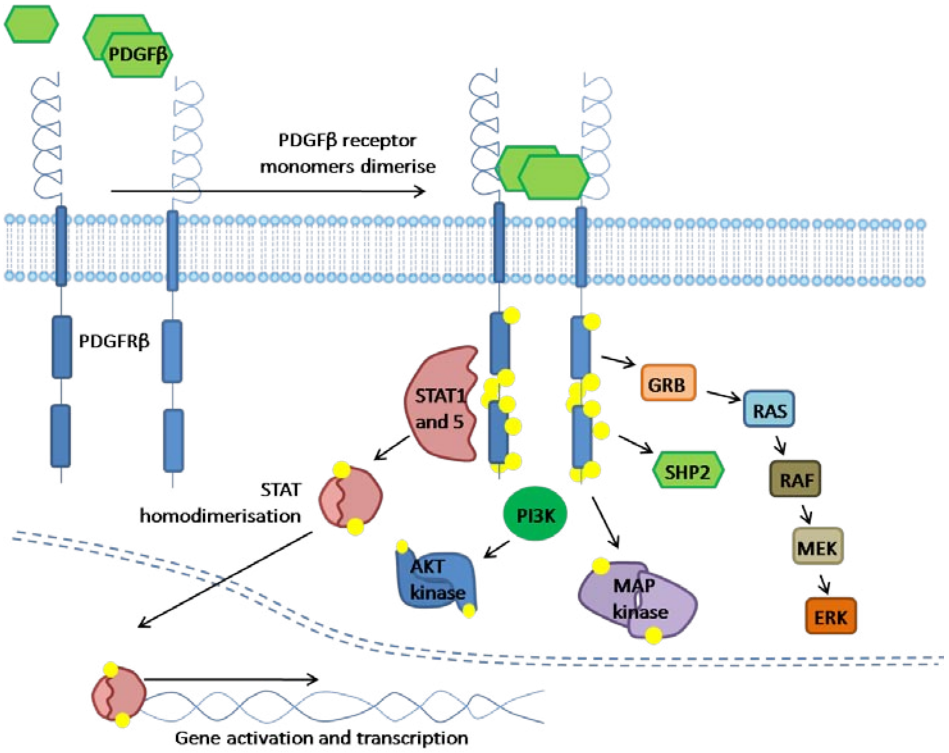


Figure 1-4. An illustration of activation and signalling from the RTK PDGFRβ. Platelet-derived growth factor (PDGF) ligand binding induces PDGFRβ receptor dimerisation, and the conformational change causes activation of two kinase domains located on the intracellular portion of the protein. Auto and transphosphorylation at key tyrosine residues creates docking sites for signalling molecules (yellow circles represent phosphorylated tyrosines). A cascade involving various signalling pathways is initiated, resulting in changes in expression of genes involved in the control of cell proliferation, differentiation and survival.

1.3.1.1.1 Platelet derived growth factor receptors (PDGFRs)

The PDGF receptors, PDGFRα and PDGFRβ, are two highly related proteins [100], that bind a total of four ligands (PDGF A, B, C, and D), which exist in dimeric isoforms linked by disulphide bonds. Both receptors have a role in mesenchymal cell migration and proliferation [101], but their involvement in the pathogenesis of MPN is better understood.

The most common *PDGFRA* fusion gene is now known to be *FIP1L1-PDGFRA* arising by a cytogenetically invisible interstitial deletion at chromosome 4q12 [102]. The resulting oncoprotein was found to transform Ba/F3 cells to growth factor independence, similar to

BCR-ABL. Moreover, the association between *FIP1L1-PDGFRA* and clonal eosinophilia has allowed unified molecular characterisation of a subset of patients with HES and CEL into one entity based on the presence of *FIP1L1-PDGFRA* [23,103]. Additional *PDGFRA* fusion genes have also been reported, for example *BCR-PDGFRA* was isolated in very rare cases with aCML [104]. Other rare *PDGFRA* fusions have strengthened the notion that deregulation of PDGFRα is linked to clonal eosinophilia [105-107].

PDGFRB is also targeted by balanced chromosomal translocation, and more than 20 different fusions involving this gene have been recorded in patients with various MPNs [108]. The best characterised abnormality is the t(5;12)(q33;p13) resulting in a *ETV6-PDGFRB* fusion gene [109]. In an analogous manner to the BCR-ABL oncoprotein, pathogenicity is dependent on both the ETV6 dimerisation motif and the tyrosine kinase activity of PDGFRβ. However fusions involving PDGFRβ remain extremely rare [110].

1.3.1.1.2 KIT

The RTK KIT and its ligand stem cell factor (SCF) are essential for the development and differentiation of mast cells, plus other cells of the haematopoietic system [111,112]. Originally identified in a series of mast cell lines [113], constitutively activating point mutations in *KIT* have been isolated in a number of neoplastic conditions, most notably in the MPN systemic mastocytosis (SM) [114] and gastrointestinal stromal tumours (GISTs) [115]. *KIT* mutations have also been identified in patients with other MPNs and AML [113,116]. Approximately 80% of all cases with SM carry the *KIT* D816V point mutation, and examination of the bone marrow for this change is recommended in all suspected cases of SM [117]. The mutated amino acid D816 is located in the catalytic domain of the tyrosine kinase, but mutations also target the juxtamembrane and extracellular domains.

1.3.1.1.3 FMS

FMS (also known as CSF1R) is the receptor for the macrophage colony-stimulating factor (M-CSF), and is important for the growth and differentiation of the monocyte, macrophage, osteoclast lineage [118]. A missense mutation in *FMS* was identified in the imatinib-sensitive cell line GDM1 [119], and mutations targeting various regions of this gene are activating when expressed in cell lines *in vitro* [120]. However, a recent screen of the tyrosine kinase and juxtamembrane domain of *FMS* in cases with CMML did not reveal any activating mutations [121], so it is unlikely disruption of this RTK is involved in the pathogenesis of MPN.

1.3.1.1.4 FLT3

FLT3 (Fms-like tyrosine kinase) and FLT3 ligand play an important role in the development and expansion of multipotent haematopoietic stem cells and progenitors [122]. Constitutive activation of FLT3 occurs either by internal tandem duplication (*FLT3-ITD*) of the juxtamembrane domain region or by point mutations usually involving the kinase domain [123-125]. Both types of mutation constitutively activate FLT3. ITD mutations are a common finding in 25% of younger adults with AML [126], and approximately 7% of AML cases have point mutations in the activation loop of the kinase [127,128]. In AML patients, *FLT3-ITDs* are associated with reduced survival, leukocytosis, a high percentage of blast cells in the bone marrow, and an increased risk of relapse from complete remission [123,126]. Additionally, duplication of the *FLT3-ITD* allele by acquired uniparental disomy (UPD) and subsequent loss of the wild-type allele occurs in approximately 10% of cases, and this carries an additional poor prognosis with a high rate of relapse [129,130]. Cases carrying *FLT3-ITDs* often have a normal karyotype and a mutation in the *NPM1* gene [131], and it was recently shown that those positive for *FLT3-ITD* but negative for an *NPM1* mutation were associated with a particularly bad outcome [132].

1.3.1.1.5 Class IV RTKs, the FGF receptor family

The fibroblast growth factor receptor (FGFR) family members *FGFR1* and *FGFR3* are also targets of chromosomal rearrangement. Translocations that disrupt *FGFR1* on chromosome 8p11 are closely associated with a defined 8p11 syndrome that present as an MPN with eosinophilia and other features such as T-lymphoblastic lymphoma, granulocyte sarcoma and rapid progression to acute leukaemia, usually of myeloid phenotype. The three most common translocations involving *FGFR1* are t(8;13)(p11-12;q11-12), t(8;9)(p11;q34), and t(6;8)(q27;p11) which result in the formation of *ZNF198-FGFR1, CEP110-FGFR1,* and *FOP-FGFR1,* respectively [133-135]. The closely related *FGFR3* gene is transcriptionally upregulated by a t(4;14) translocation in 5 to 10% of cases with multiple myeloma (MM) [136], and a small subset of cases with MM can also harbour activating *FGFR3* mutations.

1.3.1.2 Non RTKs (NRTKs)

Signalling cascades from extracellular stimuli also utilise intracellular NRTKs. After ligand binding, a stimulated receptor activates an associated NRTK, and tyrosine phosphorylation subsequently recruits additional signalling molecules by providing binding or docking sites. Lack of transmembrane domains situates NRTKs either in the cytosol or nucleus. NRTKs are kept in an inactive state by intramolecular inhibition, or by association with inhibitory proteins or lipids [137]. NRTK activation may also occur by phosphorylation as a downstream target of signalling mediators, or by dissociation of inhibitor proteins.

1.3.2 JAK2

The mammalian genome encodes four JAK (Janus-Associated Kinase) family members, JAK1, JAK2, JAK3 and TYK2. Whereas JAK1, JAK2, and TYK2 are expressed ubiquitously in mammals, JAK3 is primarily expressed in haematopoietic tissue [138,139]. Haematopoietic cytokines, interferons, and growth factors all use JAK family members for signal transduction, placing JAKs in an important position for cell growth, survival, development,

and differentiation of immune cells, plus the mounting of effective innate and adaptive immune response. All family members share a unique structure, with a relatively high degree of homology. As illustrated in Figure 1.5, there are seven so called JAK homology domains (JH) denoted JH1 to JH7 that characterise the JAK family. From the C to the N terminus, JH1 represents the kinase domain, JH2 the pseudokinase domain, JH3 and JH4 contain an SH2-like domain and linker regions, whereas JH5 to JH7 contain a FERM (band 4.1, ezrin, radixin, moesin) domain [138,140]. The most distinguishing feature characterising JAKs is the bipartite protein structure. The kinase domain, JH1, catalyses tyrosine phosphorylation of substrates and thus is responsible for signal propagation. The N terminus binds and stabilises other proteins, mediating the interaction of JAK with receptors and regulatory proteins [141-143]. The FERM domain of JAK2 is important for promoting cell surface localisation of particular cytokine receptors, such as the EPO receptor [141] and thrombopoietin receptor [144]. The JH2 domain, despite carrying most of the conserved amino acids that are characteristic of a functional kinase, lacks all tyrosine kinase ability due to a lack of key residues required for effective catalysis and nucleotide binding. In fact, the JH2 domain, also referred to as the pseudokinase [138], is critical for regulation of JAK activity, as it is predicted to inhibit the basal activity of the kinase domain [145,146]. Deletion of the JH2 domain in both JAK2 and JAK3 has been shown to result in increased autophosphorylation as well as increased phosphorylation of the downstream substrate STAT5 [146,147]. Furthermore, a more detailed analysis of the JAK2 JH2 region using recombinant proteins indicated that this domain suppressed basal JAK2 activity by lowering the activation threshold of the kinase without affecting its affinity for a substrate peptide [148]. While no complete three-dimensional structure for any JAK currently exists, the crystal structure of the JH1 domain has been solved for JAK2 and JAK3 in an active form, complexed with specific inhibitors [149,150].

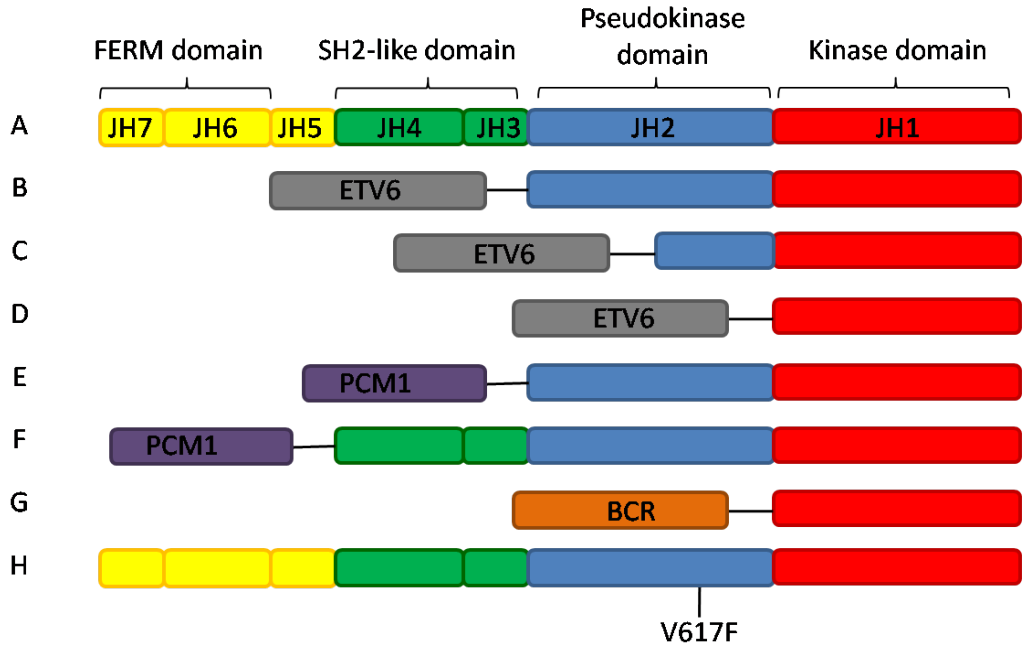


Figure 1-5. Schematic representation of the primary structure of JAKs, JAK2 fusion proteins and point mutation isolated in MPN. The primary structure of JAK is comprised of seven domains, JH1 to JH7 (panel A). The ETV6-JAK2 fusion proteins contain the entire JH1 domain, but variable portions of JH2, and were first identified in patients with atypical CML (panel B) [151], and ALL (panel C and D) [151,152]. The PCM1-JAK2 fusion proteins all contain the entire JH1 and JH2 domains, and in some cases parts of the SH2-like domain, and have been found in cases with T-cell lymphoma, aCML and CML/ALL (panel E and F) [153-155]. The fusion BCR-JAK2 was identified in a case with aCML (panel G) [156].The *JAK2* V617F point mutation is located in the JH2 domain (panel H).

Similar to most kinases, JAKs require phosphorylation of residues in their activation loop for full activity. A YY motif is present in the activation loop of all four JAK kinases [148], and phosphorylation at this site is the first event in the activation cascade required for signal transduction [157,158]. The JAKs have different receptor affinities, for example, JAK3 appears to associate with cytokine receptors that include the γc chain of interleukin-2 (IL-2) receptor (i.e. IL-4R, IL-7R) whereas JAK2 is associated with a wide range of cytokine receptors, including those activated by growth hormone [159,160], EPO [161], prolactin [162], G-CSF [163], and IL-3 [164], as well as some G protein-coupled receptors. JAKs play an essential physiological role, underscored by the observation that murine knockouts of JAK2 result in ineffective erythropoiesis [165], and JAK3 deficiency is embryonically lethal [166]. It is not

fully known by which precise mechanism ligand binding results in the activation of JAKs, however a model has been inferred based on the results from various studies, and is illustrated in Figure 1.6. In an unstimulated, latent state, JAKs form a complex with a native unliganded receptor, via non-covalent interaction with the receptor and the SH2 domain of JAK. Receptor oligomerisation/dimerisation due to ligand binding results in the juxtapositioning of the JAKs, which are in the vicinity through either homo- or heterodimeric interactions. Recruitment of JAKs is likely to result in their phosphorylation, either by autophosphorylation or by cross-phosphorylation by other JAKs or other families of tyrosine kinases. The heightened JAK activity results in phosphorylation of receptors on target tyrosine kinase sites. These phosphorylated tyrosines serve as docking sites which allow the binding of other signaling proteins, such as STATs, Src-kinases, protein phosphatases, Shc, Grb2, MAP kinase, AKT kinase and PI3K [167]. In the JAK/STAT signalling pathway, phosphorylation of STATs causes dimerisation, followed by translocation to the nucleus where they interact with specific regulatory elements on target genes, modulating gene transcription [168,169]. This process is tightly controlled at multiple levels by protein tyrosine phosphatases, suppressors of cytokine signalling (SOCS), and protein inhibitors of activated STAT (PIAS) [170-173]. There is mounting evidence to show that STAT3 and STAT5 play an important role in growth factor induced myeloid differentiation [174,175]. G-CSF stimulation activates STAT3, and this interaction is critical for differentiation of granulocytes [176]. Mice with a conditional knockout of STAT3 show embryonic lethality, indicating a requirement for STAT3 in early development [177]. Results from a conditional knockout study, where STAT3 is not expressed in myeloid cells, showed STAT3 deficiency impairs immune responses, and has an important role in the function of mature myeloid cells [178]. Evidence suggests STAT3 may promote cellular differentiation by upregulating the expression and enhancing the transcriptional activity of CCAAT/enhancer binding protein alpha (C/EBPα), a key transcription factor that drives myeloid differentiation [179]. STAT5 is also important in cytokine-induced myeloid differentiation [180]. Successful differentiation of neutrophils, induced by G-CSF, is perturbed by expression of a dominant negative form of STAT5 [181]. It has been suggested that STAT5 may be responsible for promoting the survival of myeloid progenitors via transcriptional upregulation of the anti-apoptotic protein Bcl-X_L, thereby allowing myeloid differentiation to proceed [182]. Activating mutations of the EPO receptor, found in familial

erythrocytosis, are also associated with constitutive phosphorylation of JAK2 and STAT5 [183].

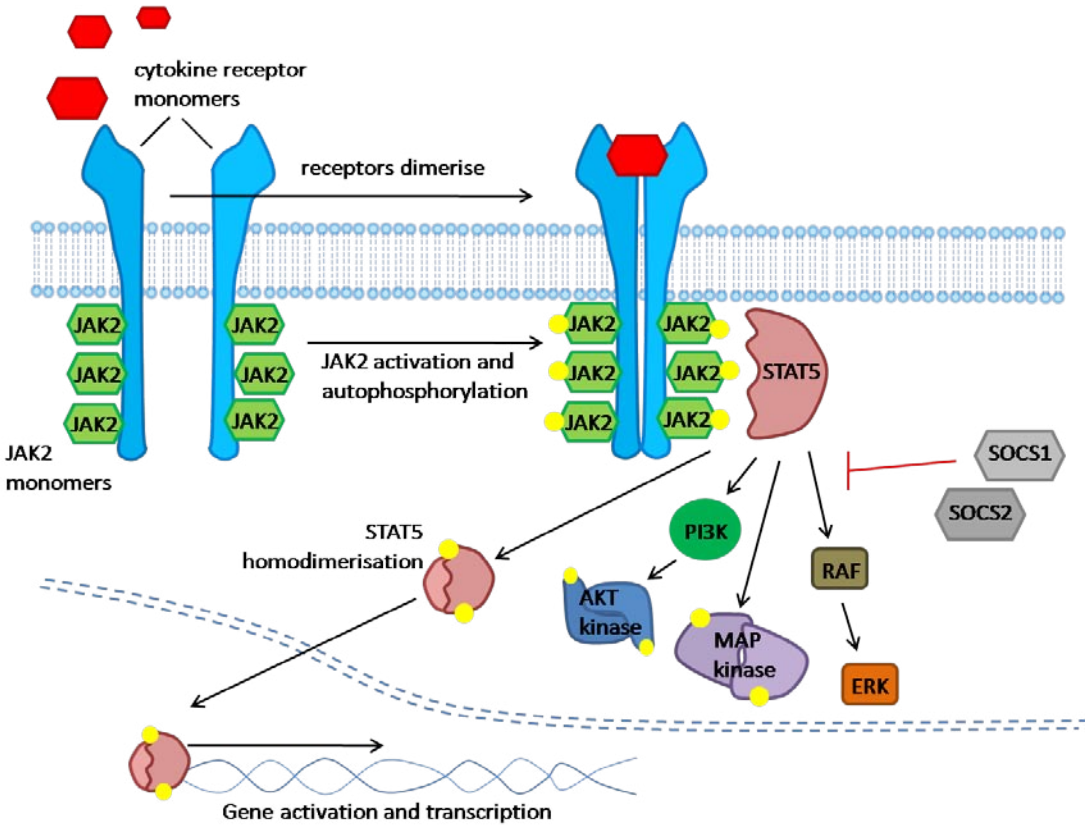


Figure 1-6. Summary of the JAK2 signalling pathway. Binding of cytokine ligand to the respective receptor induces receptor dimerisation, and transphosphorylation of receptor-associated JAK2. JAK2 subsequently phosphorylates signalling intermediates, namely STATs (yellow circles represent phosphorylated tyrosines). Activated STATs dimerise, translocate to the nucleus whereby they activate or repress target gene promoters. Other signalling intermediates are scaffolded onto the receptor-JAK2 complex and become activated, including PI-3-kinase, AKT kinase, MAP kinase and the Ras/Raf/MEK/ERK pathway. A negative feedback loop involving SOCS 1 and 2 contributes to attenuation of the signalling cascade.

Although key to growth factor and cytokine signalling, the JAK/STAT pathway is not the only means by which receptors transmit signals to the nucleus. Other pathways, equally important in driving proliferation and preventing apoptosis, include the Ras/Raf/MEK/ERK pathway [184,185], and the PI3K/PTEN/Akt/mTOR pathway [11] . Each pathway may regulate

the other at various levels by cross-interactions, heightening the complexity of intracellular signalling. For example, there is evidence to show STATs may be regulated by threonine phosphorylation [186], mediated by ERK [187], indicating a point of interaction between the JAK/STAT and Raf/MEK/ERK pathways.

Constitutive activation of different JAKs and STATs are believed to mediate neoplastic transformation and promote abnormal cell proliferation in various tumour phenotypes, including haematological malignancies. For example, wild-type JAK2 is implicated in abnormal cell growth induced by *BCR-ABL* in CML [188] but more importantly recent developments have implicated *JAK2* as a principle target for activation in MPN.

1.3.2.1 Mutations that target *JAK2*

The first evidence that *JAK2* could contribute to the pathogenesis of leukaemia came from the characterisation of various chromosomal translocations all involving the fusion of *JAK2* to different transcription factors (Figure 1.5). The first to be identified, *ETV6-JAK2,* was isolated in a T-cell ALL patient [152], and later in a child with early B-precursor ALL and in adult aCML [109,189]. Like other tyrosine kinase fusion proteins involving *ETV6* [109,189], the oligomerisation domain of *ETV6* facilitates constitutive activation of the kinase domain of the fusion partner, in this case the JH1 domain of JAK2. *ETV6-JAK2* is capable of transforming a murine cell line to factor independence [152], and reproducing features of malignancy when expressed in transgenic mice [190-192]. This oncogene has been shown to signal through the JAK/STAT, PI3K, Ras/ERK pathways [193,194], plus upregulate NF-κB and STATs 1 and 5 [190,195]. Another oncogenic fusion protein involving *JAK2* is *PCM1-JAK2* [155], identified in several cases of chronic and acute leukaemia, such as aCML [154], acute erythroid leukaemia [196] and T-cell lymphoma [153]. A single case of aCML was also found to carry a *BCR-JAK2* fusion gene [156]. Nevertheless, *JAK2* translocations remain rare in MPN, especially when compared to the incidence of *JAK2* V617F.

In 2005 it emerged that *JAK2* plays a very important role in the molecular pathogenesis of MPN, by the finding of a consistent, acquired point mutation, isolated in a significant

proportion of cases with classical MPN. Approximately 80 to 95% of cases with PV and roughly 40 to 60% of cases with ET and MF were found to carry *JAK2* V617F [197-201], the discovery of which was simultaneously reported in a series of remarkable publications. Several groups claim discovery of the *JAK2* mutation following quite different research strategies to arrive at the same result.

(i.) The observation that the spontaneous formation of EECs grown from PV progenitors could be blocked by JAK inhibitors led one group of researchers to sequence the entire *JAK2* gene in a small group of PV cases, revealing V617F [197]. EEC formation was inhibited by short-interfering RNA (siRNA) directed against *JAK2*, but not siRNAs against other various candidate genes. James et al. (2005) also performed several functional studies to define the role *JAK2* V617F plays in the pathogenesis of PV. When expressed *in vitro*, *JAK2* V617F is capable of inducing constitutive STAT5-mediated signalling independently of any stimulation by EPO. Furthermore, expression of the mutant *JAK2* (but not wild-type) induced EPO hypersensitivity and EPO-independent survival of cultured cell lines *in vitro.* When expressed in mice, *JAK2* V617F induced a PV-like phenotype with splenomegaly and marked erythrocytosis.

(ii.) A similar approach was undertaken by Zhao et al. (2005) [201] who isolated *JAK2* V617F in 20/24 cases with PV, and confirmed the mutant JAK2 protein had heightened kinase activity and cells transfected with *JAK2* V617F displayed hyper-activated signalling after stimulation with EPO.

(iii.) Recurrent abnormalities of chromosome 9 have been previously linked to classic MPN, and include trisomy 9, unbalanced translocations involving chromosome 9, amplification of 9p [93,202] but the finding of loss of heterozygosity (LOH) at 9p proved to be the most significant finding. Using microsatellites analysis, chromosome 9p was found to be a region where there was recurrent LOH in 33% of PV cases [203]. Cases with 9pLOH retained two copies of chromosome 9, indicating that LOH had occurred by acquired UPD following somatic recombination [203,204]. Markers for the 9pLOH region were subsequently

investigated in four families with PV, but as they were found not to cosegregate with the PV phenotype, this suggested a somatic event was responsible for the LOH [199]. By increasing the number of microsatellite markers, Kralovics et al. (2005) mapped a minimal region shared by all patients carrying 9pLOH and MPN, and this common 6.2Mbp genomic interval contained the *JAK2* gene. With this knowledge, the DNA encoding *JAK2* was sequenced to reveal V617F. Furthermore, homozygosity for V617F correlated with 9pLOH, and the incidence of this was 34% in PV, 22% in MF and 3% in ET.

(iv.) Two further publications reported discovery of *JAK2* V617F after a high-throughput sequencing strategy that focused on screening a set of PV cases for mutations in the activation loops and autoinhibitory domains of tyrosine kinases [198,200]. Both groups confirmed *JAK2* V617F is acquired, by showing only the wild-type allele was present in the vast majority of T cells [198] and buccal cells [200] sampled from cases carrying V617F.

1.3.2.2 Structure and possible mechanism of *JAK2* V617F

The *JAK2* mutation, located in exon 14, changes a valine to a phenylalanine at amino acid residue 617 (nucleotide substitution 2343 G>T RefSeq ID NM_004972 www.ensembl.org). At the protein level, the amino acid change is located in a highly conserved region, in the pseudokinase or JH2 domain. As previously mentioned, the JH2 domain is predicted to interact directly with the true kinase domain JH1, and negatively regulate signalling [148]. A homology model of the JH1 and JH2 domains of JAK2 based on the crystal structure of the FGF receptor kinase has been proposed, and is shown in Figure 1.7. In this model, residues V617 to E621 form a loop connecting two beta strands of the N-terminal lobe of the JH2 domain, with C618 contacting the kinase activation loop. It has been postulated that V617F, C618 and other local residues hinder the movement of the activation loop from an inactive to an active conformation [205]. Based on this it may be inferred that V617 plays a direct role in negatively regulating JAK2 signalling. Substitution of valine at residue 617 with the large aromatic amino acid phenylalanine is likely to disrupt this highly

balanced negative regulation, although this remains to be proven biochemically. Interestingly, the first evidence suggesting that the JH2 domain played a direct role in a leukaemic phenotype came from studies describing a point mutation in the JH2 domain of *Drosophila melanogaster* Janus kinase, Hopscotch [206]. When overexpressed in *Drosophila* cells, Hopscotch carrying the E695K mutation resulted in hyper-phosphorylation and constitutive activation of D-STAT, the STAT equivalent in the fly.

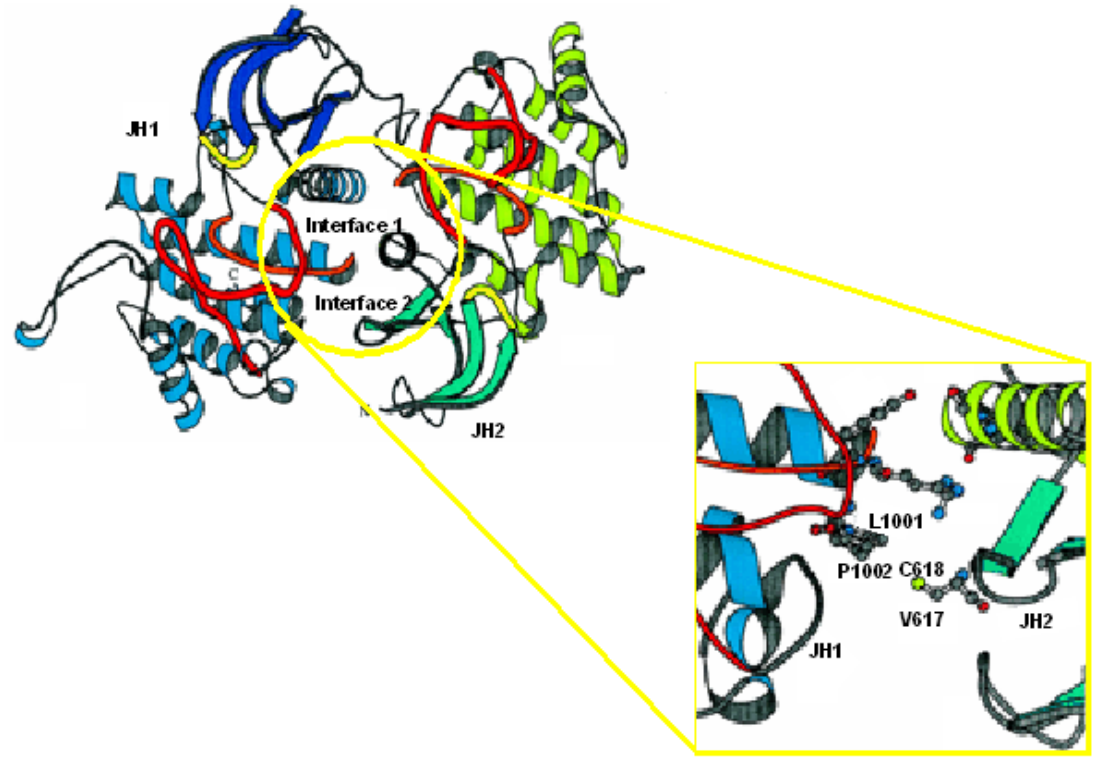


Figure 1-7. Predicted structural model of JAK2 domains JH1 and JH2. In an inactive state, the JH1 kinase domain is held in close spatial proximity to the JH2 domain, which performs an inhibitory function. On closer inspection (yellow box), the L1001 and P1002 residues of the activation loop are held closest to residues V617 and C618 of the JH2 domain (adapted from Lindauer et al. (2001) [205].

1.4 Inhibition of tyrosine kinase signalling by imatinib

For CML and other aggressive forms of MPN, allogeneic stem cell transplantation is believed to be the only curative therapy currently available, but only a minority of patients are eligible for this procedure due to a limited number of HLA-matched donors and the fact that only relatively young patients can tolerate the procedure. Characterisation of the protein structure of kinases enabled researchers to rationally develop drugs which inhibited tyrosine kinase activity [207]. Like other tyrosine kinases, the kinase domain of the ABL moiety catalyses tyrosine phosphorylation by binding ATP and transferring the γ-phosphate to target tyrosine residues. Competitive inhibition with ATP for the activation domain was postulated as an effective mechanism to prevent signalling from the aberrant kinase [208,209]. A synthetic compound, imatinib mesylate, also known as Glivec® (Novartis Pharmaceuticals, Basel, Switzerland) proved to be strikingly effective at inhibiting proliferation of malignant cells and inhibiting apoptosis induced by BCR-ABL *in vitro* [208] and in experiments performed in primary CML progenitors *in vivo* [209,210]. Imatinib lacks the essential phosphate groups provided by ATP, thus phosphorylation of substrate proteins cannot occur and ultimately downstream signalling is prevented [209]. The efficacy of imatinib was confirmed in a large randomised trial of chronic phase CML patients, where over 1000 cases were given imatinib, or rIFNα plus cytarabine. After 19 months over 75% of patients receiving imatinib achieved complete cytogenetic response, compared to less than 35% of those who received rIFNα. In addition imatinib was more easily tolerated that rIFNα [211]. However, despite the high rates of haematological and cytogenetic response with imatinib, one drawback has been the emergence of acquired resistance in a minority of patients brought about by mechanisms such as point mutations in the kinase domain [212-217], overexpression of the *BCR-ABL* [218], and overexpression of the multidrug resistance P-glycoprotein [219]. Furthermore, imatinib is not effective in treating advanced stages of CML in blast crisis [209], or *BCR-ABL* positive ALL where resistance develops rapidly [220]. In an attempt to overcome some of these drawbacks, a series of second generation ATP-competitive ABL kinase inhibitors were developed. Compounds such as dasatinib (Sprycel®) and nilotinib (Tasigna®) are more active against BCR-ABL than imatinib [221-223]. However, neither compound is able to overcome the resistance displayed by the T315I mutation in BCR-ABL [222], and have demonstrated off-target effects through

non-specific protein binding [224]. This might be circumvented by other inhibitors such as the aurora kinase inhibitor MK-0457, which has shown activity against the T315I mutation in patients with CML [225].

1.4.1 Imatinib and other MPNs

Analogous to *BCR-ABL* positive CML, it was suggested patients carrying other deregulated tyrosine kinases could also be amenable to treatment with targeted inhibitors, like imatinib. In fact, imatinib has been shown to occupy the ATP binding site and inhibit phosphorylation from kinases other than ABL, including ABL2, KIT, PDGFRα, PDGFRβ and FMS [226]. Tyrosine kinases FGFR1, FGFR3, FLT3 and JAK2 are not sensitive to imatinib. Although rare, MPN patients carrying translocations that deregulate *PDGFRA* [102,103,227,228] and *PDGFRB* [229-237] have been treated with imatinib, with a large degree of success [238,239]. Moreover, the recurrent but cytogenetically invisible *FIP1L1-PDGFRA* deletion was only identified after a series of HES cases were found to be sensitive to imatinib *in vitro*, and were investigated for deregulated imatinib sensitive tyrosine kinases [102,240]. A small number of HES cases that lack *PDGFRA* and *PDGFRB* rearrangements have been reported [102,227,241] , and together with data from our laboratory, this suggests that additional imatinib-sensitive lesions remain uncharacterised.

Imatinib has also been shown to have a positive effect in PV, by reducing the need for phlebotomy [242]. Investigations *in vitro* showed imatinib strongly inhibited or even blocked spontaneous erythrocytosis, a hallmark of PV [243]. It was first suggested imatinib was acting on an unknown mutated tyrosine kinase, but now it is thought that imatinib reduced signalling from normal KIT [244], which is known to play a role in erythropoiesis [76].

1.5 Aims of this study

Initially, the general aim of this study was to further the understanding of the molecular pathogenesis of PV and other MPNs, focusing on the hypothesis that they were driven by deregulated tyrosine kinases. After the discovery of *JAK2* V617F, my research then focused on:

- Characterisation of *JAK2* V617F in different MPNs.
- The development of robust, sensitive molecular techniques to detect *JAK2* V617F and exon 12 mutations.
- The development of culture assays to predict the response of patients to imatinib.
- Evaluation of the response of PV patients to imatinib and interferon using *JAK2* V617F as a molecular marker.
- Identification of genetic factors that predispose to V617F positive MPNs.

2 Materials and methods

2.1 Patient samples

Patient samples were either referred from hospitals throughout the UK for diagnostic analysis or provided by collaborators from Europe and the US. Research described here was approved by the Salisbury and South Wiltshire Research Ethics Committee in the study entitled 'Mechanisms and consequences of tyrosine kinase activation in chronic myeloproliferative disorders and related conditions' (LREC study number O5Q2008/6), either following informed consent or anonymisation. The cohorts of patients used for specific projects are described in their respective sections.

2.2 Separation of cells from peripheral blood and bone marrow samples

The precise procedure for cell separation varied from sample to sample depending on cell count and reason for referral. Typically, mononuclear cells and granulocytes were separated using lymphoprep and red-cell lysis. Cell pellets, GTC lysates, cytogenetic cultures or cryopreserved samples were then prepared depending on requirements.

2.3 Cell counting and assessment of viability

To count nucleated cells in PB or BM, the sample was diluted 1:10 in 2% acetic acid (Sigma) to lyse the red blood cells, and cell number was then counted using a Neubauer haemocytometer (BDH, Poole, UK). Cell viability was assessed using trypan blue, which stains dead cells blue but is excluded from live cells. Typically, cells were mixed 1:1 with trypan blue and then counted using a haemocytometer.

2.3.1 Separation of mononuclear cells

Mononuclear cells (MNCs) were separated by density gradient centrifugation over Lymphoprep^{TM} (Axis Shield, Norway). Two x10^7 cells were diluted in Hank's Balanced Salt Solution (HBSS, Invitrogen, Paisley, UK) to a maximum volume of 25ml. The cell suspension was layered slowly over an equal volume of Lymphoprep prior to centrifugation for 30 minutes at 1800 rpm (revolutions per minute) in a Centra CL3 bench-top centrifuge without braking. After centrifugation, MNCs were removed from the interface using a sterile pasteur pipette (BDH) and transferred to a clean tube. The recovered cells were washed twice in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with L-glutamine, penicillin, streptomycin and 10% fetal calf serum, before counting.

2.3.2 Separation of granulocytes

Granulocytes are sedimented to the bottom of the tube during lymphoprep centrifugation along with the red cells. After removing mononuclear cells and discarding the supernatant, cold red-cell lysis buffer (see Appendix I for recipes of all buffers and solutions) was added to the cell pellet. Red blood cells were then removed by selective osmotic lysis after incubation on ice for 10 minutes. After centrifugation for 5 minutes at 1500rpm, the cell pellet was washed twice in supplemented RPMI 1640, and then counted.

Depending on the total cell number, MNC and granulocyte fractions were divided for different purposes. Cells were stored for future DNA extraction in 1- 2 x 10^7 cell aliquots at -70°C. For RNA extraction 1x 10^7 cells were lysed with guanidium thiocyanate (GTC) solution mixed with 7µl β-mercaptoethanol (Sigma, Poole, UK) per 1ml GTC. MNCs and granulocytes were also used fresh in the imatinib sensitivity *in vitro* assays.

2.4 Nucleic acid extraction

2.4.1 RNA extraction and cDNA synthesis

350µl of GTC lysed cells for RNA extraction were mixed with an equal volume of 70% ethanol and applied to an RNeasy kit column (Qiagen, West Sussex, UK). Following the manufacturer's guidelines, the column was subject to several washes by centrifugation and the RNA was then eluted in 20µl deionised, RNase-free water. The RNA was then heated to 65°C for 5 min to destroy any tertiary structures and placed on ice. For cDNA synthesis, the RNA (1 to 5µg approximate yield) was incubated with 21µl cDNA mix (50mM Tris pH 8.3, 75mM KCl, 3mM $MgCl_2$, 1mM DTT, 1mM dATP, 1mM dCTP, 1mM dTTP and 1mM dGTP), 100µg/µl of random $pd(N)_6$ hexamers (Amersham Pharmacia, Amersham, UK), 150U of Murine Moloney Leukaemia Virus (MMLV) reverse transcriptase (Invitrogen, Paisley, UK) and 15U of RNase inhibitor (Promega, Southampton, UK) for 2 hours at 37°C. The reaction was terminated by enzyme denaturation at 65°C for 10 minutes. All cDNA samples were stored at -20°C.

2.4.2 Salt extraction of DNA from cell pellets

Cells were thawed prior to addition of 1ml resuspension buffer (RSB, see Appendix I), 30µl 10% sodium dodecyl sulphate (SDS) and 200µg Proteinase K (Roche, UK) per $1x10^7$ cells. The sample was mixed and incubated overnight at 37°C. Three hundred microlitres of 6M sodium chloride was added and the tube was shaken for 20 sec. After centrifuging at 13000 rpm for 30 minutes, the supernatant was collected into a 1.5ml centrifuge tube (Sarstedt). An equal volume of 100% ethanol was added to precipitate the DNA, and the tube was inverted until the DNA visibly collected as a 'hair ball.' DNA was removed using a sterile hypodermic needle. After washing the pellet in 1ml 70% ethanol, the DNA was resuspended in 50 to 500µl of 1x Tris-EDTA (TE, pH 7.4) depending on yield.

2.4.3 DNA extraction from BM and PB slides

Unstained BM and PB smears were scraped free using a sterile scalpel into 200µl sterile PBS, and genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, West Sussex, UK) following manufacturer's guidelines. DNA was eluted in 40µl 1x Tris-EDTA (TE, pH 7.4).

2.4.4 Whole Genome Amplification (WGA)

WGA was performed on samples where only small quantities of DNA were available for mutation screening, using the GenomiPhi DNA Amplification kit (GE Healthcare, Amersham) according to the manufacturer's instructions. Following this method, microgram quantities of genomic DNA up to 10Kb in length can be amplified from as little as 1ng of genomic DNA. The strand displacement amplification reaction uses Phi29 DNA polymerase, random hexamer primers, dNTPs, and input DNA to amplify DNA representative of the original sample. WGA DNA was diluted to 50ng/µl for use in PCR, and stored at -20°C.

2.5 Cell culture

2.5.1 Cell lines

Cell lines were cultured in supplemented RPMI 1640 medium (Invitrogen) and maintained at 0.5 - 1.0 x10^6 cells/ml. Cultures were incubated at 37°C in a humidified atmosphere with 5% carbon dioxide. Typically, cells were split twice weekly or as required. In general, a change in the medium from pink or orange to yellow indicates that more medium was required.

2.5.2 Cryopreservation of myeloid cell progenitors

MNCs were stored for future use by cryopreservation, a method that enables the cells to retain all biological activity, when thawed out after a long duration at very low

temperatures. Cells are mixed with the cryoprotectant dimethyl sulfoxide (DMSO) which prevents ice crystal formation before storage in liquid nitrogen. MNCs in 2-5x10^7 cell aliquots per vial were cryopreserved, by the gentle addition of the same volume of ice-cold freezing mixture. The cell mixture was then dispensed into vials, wrapped in paper towel and placed in a polystyrene box, before being placed at -70°C. The cells are thereby cooled slowly for a minimum of one night before storage in liquid nitrogen at -196°C.

2.5.3 Thawing of myeloid cell progenitors

A thawing solution, warmed to 37°C, was required to thaw cryopreserved cells (Appendix I). Once removed from storage, vials were thawed quickly by 37° C incubation before transferring the cells to a 50ml tube. Dropwise, 20ml of pre-warmed thawing solution was then added. The tube was topped up to 50ml with pre-warmed (37°C) supplemented RPMI 1640, and then centrifuged at 1,500rpm for 5 minutes. The supernatant was discarded and 200,000U of DNase I was added dropwise to the cell pellet. The tube was gently shaken to disrupt the pellet and to prevent the formation of clumps. Ten mililitres of supplemented RPMI 1640 was then added and the cells centrifuged as before. The DNaseI and supplemented RPMI 1640 wash was then repeated before finally discarding the supernatant and resuspending the cells in 10ml supplemented RPMI 1640. To the resuspended cells 200,000U DNaseI was added and the cells were left for one hour at room temperature. The cells were finally spun and resuspended in supplemented RPMI 1640 to determine cell count and viability. The cells were counted using a haemocytometer and live and dead cells were differentiated by staining with an equal volume of trypan blue.

2.5.4 Growth of myeloid progenitors in semi-solid medium (colony assay)

The clonogenic potential of haematopoietic myeloid progenitors was studied by culturing the cells in Methocult (H4434; Stem Cell Technologies, London, UK) a semi-solid medium containing recombinant cytokines (EPO, IL-3, SCF, GM-CSF, SCF) which stimulate single cell progenitors to divide and differentiate into microscopically visible colonies. The cells

are immobilised within the medium allowing counting of cell number using an inverted microscope. Reagents, such as growth inhibitors or cytokines can be added to the medium as required.

Prior to use, one bottle of 200ml Methocult was defrosted, vigorously mixed and left to stand for 30 minutes at 4°C for air bubbles to disperse. The medium was then aliquoted into 3ml volumes into bijoux, and stored at -20°C until required. MNCs isolated from fresh PB were cultured in Methocult at a density of $2x10^5$ to $4x10^5$ cells per 40mm dish (Fisher Scientific, Leicestershire, UK) (in a final volume of 1ml), in triplicate, following the manufacturer's instructions. Cells derived from BM were plated at a lower concentration of up to $1.3x10^5$ cells /ml. The optimum concentration of cells produced between 25 and 300 colonies per ml. Cells were mixed with the medium gently using a 1ml syringe, avoiding the introduction of air bubbles. Dishes were incubated at 37°C and 5% CO_2 in a humidified atmosphere, alongside a small water reservoir to prevent medium dehydration. Colonies arising from a single myeloid progenitor are visible after 7 to 14 days incubation and were characterised based on morphology, as described by Sutherland et al. (1989) [245]. The colonies were defined as either CFU-GM, CFU-GEMM, CFU-E (one to two clusters containing up to 200 erythroblasts) and BFU-E (produces a single: colonies containing greater than 200 erythroblasts). CFU-GM are typically pale with dispersed cells, and CFU-GEMM colonies have a concentrated nucleus which is brown-red in colour also surrounded by pale dispersed cells. BFU-E and CFU-E contain haemoglobinised erythroid precursors and are therefore predominantly red in colour, more so after 10 days incubation when the cells have had longer to differentiate. Normally, BFU-E and CFU-E require the presence of EPO in the medium to stimulate division and differentiation, but in MPN patients they can arise spontaneously.

2.6 Polymerase chain reaction (PCR)

PCR is a technique widely used in molecular biology for the amplification of DNA sequences from a template at low concentration [246]. A DNA polymerase is used to amplify target DNA by *in vitro* enzymatic replication, and the product is formed in an exponential fashion. The heat-stable polymerase *Taq,* originally isolated from the bacterium *Thermus aquaticus,* uses single stranded DNA as a template and DNA oligonucleotides (primers) to initiate DNA synthesis. Primers are designed at either end of the amplicon, and are complementary to the DNA sequence flanking the target DNA. The vast majority of PCR methods use thermal cycling, alternately heating and cooling the PCR sample over a defined number of temperature steps. The target DNA is sequentially denatured at 96°C and then cooled to allow hybridisation of primers. The temperature the primers anneal to their target is determined by primer length, the G/C and A/T ratio and the buffer that is used. The reaction is then heated to 72°C to allow polymerisation, forming two new strands that act as templates for subsequent cycles and thus products accumulate exponentially during the course of the process. PCRs were performed in a programmable thermal cycler (MJ Research) with heated lid, in 0.5ml tubes. The sequences of all primers used can be found in Appendix III.

2.6.1 Amplitaq GOLD PCR

Unless otherwise stated, all PCRs were performed using Amplitaq Gold (Applied Biosystems, Warrington, UK) in a volume of 25µl. Primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3_www.cgi) with an optimal melting temperature of 60°C. A PCR mix was typically prepared using final conditions as follows:

1 x Amplitaq Gold buffer

1.5mM $MgCl_2$

0.2 mM each of dCTP, dTTP, dATP, dGTP

0.5 µM of each primer

1U Amplitaq Gold

Typical cycling conditions for PCR with Amplitaq GOLD were:

Step	Temp (°C)	duration
1	95	10 min
2	94	1 min
3	60	1 min
4	72	1 min
5	29 times to step 2	
6	72	10 min
7	15	hold

2.6.2 Pyrosequencing PCR

Pyrosequencing PCRs were made using Amplitaq Gold polymerase and were identical to standard PCRs except that the total reaction volume per sample was 50µl, and primers were designed using the pyrosequencing software (Assay Design Software 1.0, Biotage). The cycling conditions are described in Appendix II.

2.6.3 High Fidelity PCR

In some cases it was necessary to amplify regions in the range of 3 to 5kb in size, which is out of the usual range achievable using Amplitaq Gold. Here I used the Expand High Fidelity PCR System (Roche Applied Science, Uppsala, Sweden) which in addition to *Taq* DNA polymerase also contains a proofreading enzyme to increase the fidelity of replication. On ice, PCRs were set up with 10µl of Mix 1 (Roche) (containing 2µl High Fidelity buffer with 15mM $MgCl_2$, 1U Enzyme mix and 7.7µl water) 7µl Mix 2 (final concentration of 200µM dNTPs), 500nM of forward and reverse primers and 1µl DNA to give 50 to 100ng DNA. The cycling programme is described in Appendix II.

2.7 Agarose gel electrophoresis

Agarose gel electrophoresis was routinely used to assess the size and quantity of DNA after various procedures such as DNA extraction or PCR. An electrical current causes negatively charged DNA to migrate towards a positively charged anode through an

agarose gel. DNA is thereby separated according to size as the distance of migration is proportional to the size of the DNA, with smaller products being able to move a further distance through the agarose. DNA is visualised using ethidium bromide, a compound that intercalates with DNA helices causing them to fluoresce under ultra violet (UV) light. Gels were made with 1.5% to 3% agarose, depending on the required resolution.

Two percent gels were made by heating 1g of agarose in 50ml 1x tris-borate (TBE) buffer (Sigma) in a microwave, cooled to "hand-hot" before the addition of 0.4µg ethidium bromide then poured into the gel tank with an appropriate sized comb. Before loading gels with PCR product, the gels were immersed in 1xTBE buffer and the combs were removed. Typically, 10µl of a PCR reaction was mixed with 2µl of 6 x loading buffer (see Appendix I) before loading into the well. The loading buffer enables monitoring of the approximate location of PCR product as the gel is run, and the high sucrose concentration facilitates loading into the well. A 1kb plus ladder, 100bp to 12kb (Invitrogen) was run alongside PCR products, to aid sizing of bands (see Figure 2.1). Gels were run at 80V for approximately 30 min, and viewed using a UV transilluminator.

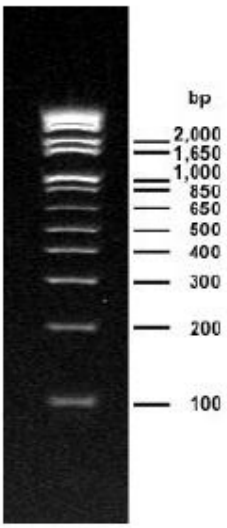


Figure 2-1. Gel electrophoresis of the Invitrogen 1kb plus DNA ladder. All PCR products were run alongside this ladder in order to determine product size.

2.8 DNA Sequencing

PCR products and plasmids were sequenced in this study using dideoxynucleotide chain termination, originally developed by Sanger et al. (1977) [247]. The PCR-based sequencing reaction generally contains a PCR product as template, dideoxynucleotides (ddNTPs), each base of which is labelled with a different fluorochrome, deoxynucleotides, DNA *Taq* polymerase, and one primer. The ddNTPs lack a 3' hydroxyl group, required for the formation of 3'-5' phosphodiester bond and thereby chain extension is halted when a ddNTP is incorporated into the product. The product will therefore be labelled with a single fluorochrome-labelled ddNTP. A mixture of DNA molecules of different lengths is thereby created. The products are separated by capillary gel electrophoresis, and the nucleotide sequence, indicated by the terminating ddNTP, is read by a laser.

2.8.1 ExoSAP

To prepare PCRs for fluorescent-based sequencing, each product was first incubated with a combination of two hydrolytic enzymes: exonuclease I (New England Biolabs), which

digests excess primers, and shrimp alkaline phosphatase (SAP, Promega) which removes the phosphate group from nucleotides, thereby preventing them from incorporation during the sequencing reaction. Two microlitres of PCR product was incubated with 1µl of exonuclease I and SAP, (made up in a ratio of 1:4), incubated at 37°C for 15 minutes, then heated to 80°C for 15 minutes to inactivate the enzymes. The DNA from this reaction was then diluted by 5 to 10µl with sterile distilled water (depending on the intensity of the PCR product), and then used as a template for sequencing.

2.8.2 Sequencing reaction

The reaction mix for a typical sequencing reaction was as follows:

1.5µl 5x v1.1 buffer (Applied Biosystems)
200ng Primer
0.5µl v1.1 Big Dye Terminator (Applied Biosystems)
1µl template
dH_2O to 10 µl

Cycling conditions are described in Appendix II.

2.8.3 Removal of unincorporated dye terminators

Unincorporated fluorescent ddNTPs were removed using the montage SEQ_{96} sequencing reaction clean up kit (Millipore, Watford, UK) following the manufacturer's instructions. DNA from the cleaned sequencing reaction was resuspended in deionised formamide and loaded onto an ABI 3100 or 3130 sequencer (Applied Biosystems).

2.8.4 Pyrosequencing

Pyrosequencing is a modified sequencing technique that provides accurate quantification of allele ratios [248]. In order for a sample to be genotyped for a particular allele or acquired

mutation, an assay must be designed using the manufacturer's software (Assay Design Software 1.0, Biotage). This programme designs two primers to generate an amplicon flanking the target allele, plus a third primer used to detect the polymorphism, called the sequencing primer. In general, the sequencing primer is designed so that the first base to be added in the sequencing reaction is the site of the polymorphism to be detected. One of the amplification primers must be biotinylated to hold the amplicon to a small filter during the sequencing process. Amplification was checked by running 5µl product on a 1.5% agarose gel. The PCR products were then subject to sequencing. The basic principles of the pyrosequencing reaction are shown in Figure 2.2.

First, the amplicon is denatured to become single stranded, so only the biotinylated strand remains in the reaction. The sequencing reaction occurs when dNTPs are added sequentially to a mix containing sequencing primer, template, enzymes and substrates. If the dNTP added is complementary to the base in the single stranded template, it will be incorporated. This causes the release of pyrophosphate which initiates a chemical cascade. The substrate adenosine 5' phosphosulfate (APS) (plus pyrophosphate) is converted to ATP by the enzyme ATP sulfurylase. Luciferase then catalyses a reaction between ATP and luciferin. Light is generated proportional to the amount of dNTP incorporated. An enzyme apyrase degrades unincorporated nucleotides. The flashes of light determine the sequence read by the pyrosequencing machine.

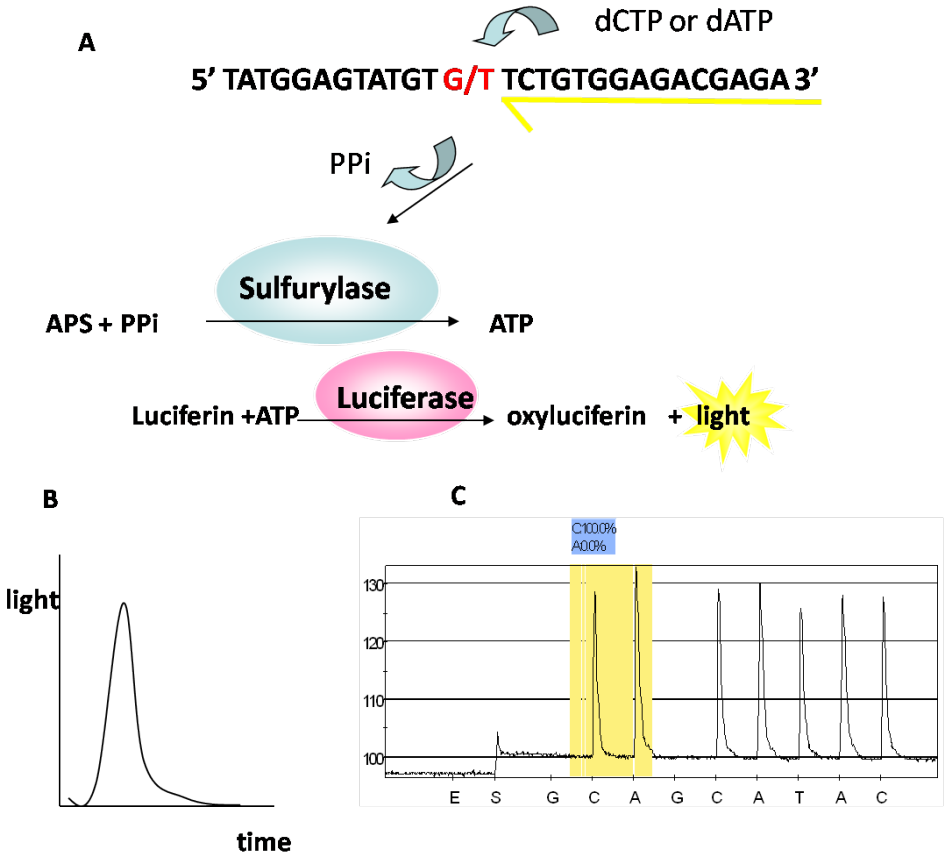


Figure 2-2. The principles of pyrosequencing. (A) A single-stranded amplicon spanning the polymorphism or mutation is quantified by the sequential addition of nucleotides onto a sequencing primer, and pyrophosphate released by polymerisation starts an enzymatic cascade resulting in the release of light. (B) The amount of light (y axis) is proportional to the amount of dNTP incorporated into the growing oligonucleotide (x axis). (C) The reaction is displayed in real-time as a pyrogram. Here the addition of enzymes (E), substrates (S) are shown alongside addition of the C and A nucleotides being measured (highlighted in yellow). The enzyme mix (E) and substrate (S) are added into the reaction first, and are then followed by nucleotides. The variant bases being genotyped are highlighted in yellow, and are flanked by bases not part of the sequence that control for non specific emission of light. This is followed by a run of invariant nucleotides, necessary for allele quantification. The software provides the allele ratio in a blue box above the graph.

To carry out the pyrosequencing reaction, details such as specific assay type, location of samples on plate, sample identifier, reagent type are entered onto a computer connected to the Pyrosequencing machine (PSG MA 2.1, Biotage). The process was performed

according to manufacturer's instructions, using the Pseq 96 SNP Reagent Kit (Biotage), which contained the enzymes, substrate mixture and nucleotides. Firstly, the PCRs were prepared. One PCR was performed per sample in a volume of 50µl, and this provided two aliquots of 20µl for subsequent analysis in the pyrosequencing process. Each reaction was made up with 3µl Streptavidin Sepharose^{TM} HP (Amersham Biosciences, Chalfont St. Giles, UK), 37µl binding buffer and 20µl PCR product and 20µl sterile distilled water. The mixtures were vigorously shaken for 10 minutes using a Variomag Monoshaker (Camlab, Over, UK).

- The sequencing primer was then diluted and aliquoted into custom 96 well plates with low wells (Pseq 96 Plate Low, Biotage), in the corresponding location to the PCR reaction. Each well contained 45µl annealing buffer and 0.3µM sequencing primer.

- Single stranded biotinylated PCR products were then prepared for sequencing using the Vacuum Prep Tool. The streptavidin beads adhere to the biotinylated amplicons and both are captured onto the filter paper probes on the Vacuum Prep Tool when the vacuum was applied. The Vacuum Prep Tool holding the bead-amplicons was then washed with:

    - 70% ethanol for 5 seconds
    - denaturation solution (0.2M sodium hydroxide, Sigma) for 5 seconds
    - washing buffer for 5 seconds

- The vacuum was then stopped and the beads were released into the plate containing the sequencing primer and annealing buffer.

- The plate containing the bead-bound amplicons was heated to 80°C for 2 minutes and then cooled to allow the sequencing primer to hybridise to the single stranded PCR product.

- The plate was then placed into the pyrosequencing machine with a specialized pyrosequencing cartridge containing dNTPs, enzymes and substrates (all from

Biotage), and the sequencing run was started. A luminometer within the machine then captures the amount of light produced by the luciferase-catalysed reactions that take place in each well.

- After the run had finished, the sample genotypes were determined using the Allele Frequency Quantification function in the software, which calculates the allele ratio using the peak heights of the invariant preceding nucleotides. Allele values derived from the same PCR were averaged to give one genotype. An example of the sequencing trace, or pyrogram obtained from a pyrosequencing reaction is depicted in figure 2.2c.

2.9 Multiple ligation-dependent probe amplification

Multiple ligation-dependent probe amplification (MLPA) is a PCR-based technique that provides quantification of multiple genomic regions in a single reaction. [249]. The basic principle is shown in figure 2.3.

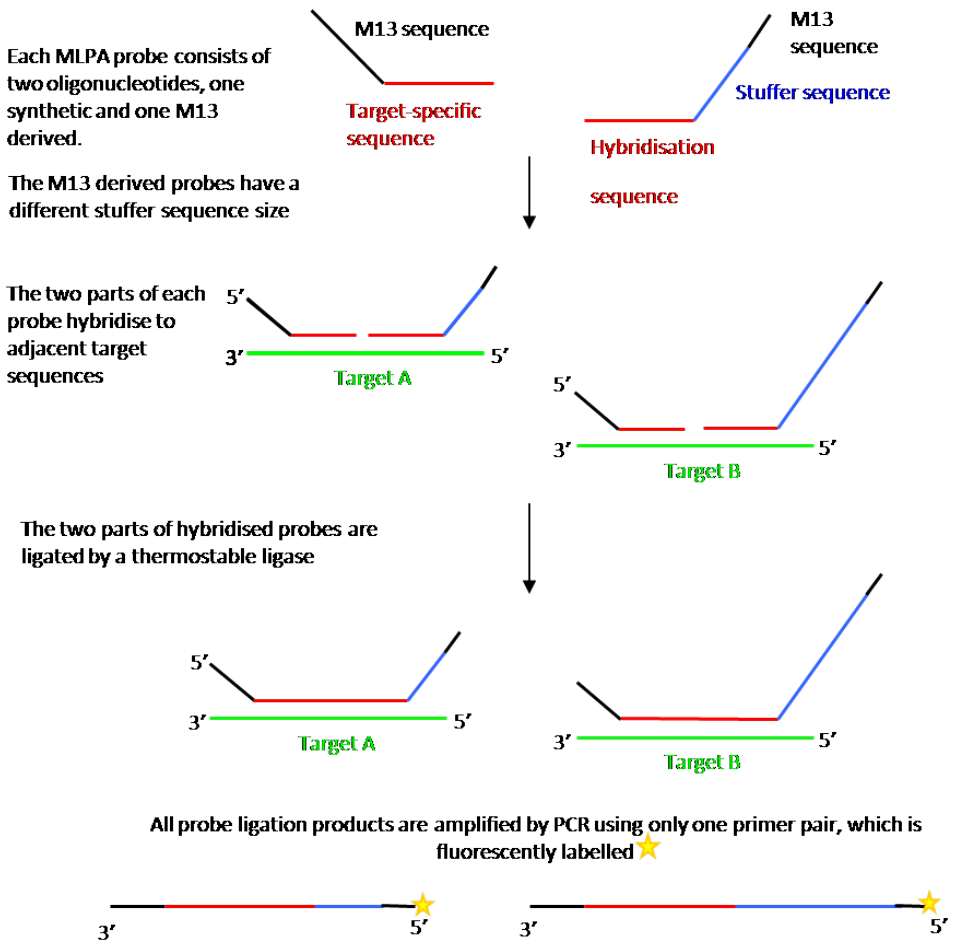


Figure 2-3. The MLPA reaction.

Each MLPA probe consists of two components; a target-specific probe (red portion in figure 2.3) and a M13 phage derived tagging sequence (black). Mixtures of several primer pairs (up to 45) are added to the same reaction along with DNA template. When both probe oligonucleotides are hybridized to their respective targets, they become ligated into a complete probe by the action of a thermostable ligase. The outer phage-derived portions of the primer pair allow simultaneous amplification of all sequences in the reaction by FAM-labeled M13 primers. Only primers that correctly bind to their targets and are ligated will be amplified thus conferring a high degree of specificity. The 'stuffer' sequences (blue), which vary in length to give a unique size final product, allow identification of each amplicon by gel electrophoresis. The MLPA reaction was performed on approximately 50ng DNA, following the protocol devised by the MLPA kit supplier,

MRC-Holland (Amsterdam, Holland). Briefly, the DNA was denatured to a single-stranded state by heating at 98°C for 5 min, then left to cool to 25°C for 2 min. Unless otherwise stated, 1.5µl of the probe mix (each primer was used at 1.33fmol working concentration) was carefully mixed with an equal volume of MRC probe buffer (MRC-Holland) and added to the denatured sample. After an initial heating at 95°C for 1 min, the reaction was incubated overnight (minimum 16 hours) at 60°C with a thin layer of mineral oil (Sigma) covering the reaction surface to prevent evaporation. Incubation at 60°C overnight allowed the primers to bind the target sequence. The temperature of the reaction was then reduced to 54°C and 32µl ligase-65 mixture was added under the oil layer. The Ligase-65 mixture was made up on ice immediately before use, and consisted of 3µl Ligase-65 buffer A (MRC-Holland) and 3µl Ligase-65 buffer B (MRC-Holland) and 1µl Ligase-65 (MRC-Holland) made up to 32µl with distilled water. The ligation reaction was left at 54°C for 15 min, and the ligase was inactivated by heating at 98°C for 5 min. The PCR mixture was made up on ice immediately before use. One microlitre of MLPA primer mix, 2µl PCR red buffer (MRC-Holland), 2µl PCR blue buffer (MRC-Holland), and 0.25µl Polymerase (MRC-Holland) were made up to 20µl with distilled water and mixed well. The ligation reaction was then brought down to 60°C and 10µl of the ligation mix was added to the PCR reaction. The PCR conditions were as follows; 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72°C, then a final 20 minute incubation at 72°C. Amplification was conducted in a thermal cycler with a heated lid (MJ Research). One microlitre of MLPA PCR product was mixed with 9µl Hi-Di Formamide (Fisher Scientific) and 0.1µl of ROX500 size standards (Applied Biosystems). The products were then separated on an ABI 3100 genetic analyser and interpreted using Genotyper version 2.0. Peak heights from each patient were then exported to an Excel spreadsheet, which was designed to assess the ratios of each test peak relative to all other peaks for that individual. The relative amounts of the probe amplification products reflect the relative copy number of the target sequences. By comparing the peak pattern of a given sample with that obtained on various reference samples, the relative quantity of each amplicon can be determined.

2.10 Cloning of PCR products

In some instances, it was necessary to clone a PCR product in a plasmid vector prior to sequencing. Essential features of a cloning plasmid are i) independent replication within a host cell, ii) antibiotic resistance which aids future selection, iii) multiple cloning sites allowing insertion of foreign DNA. PCR products were cloned by insertion into a plasmid vector using the TOPO cloning kits (Invitrogen), and Figure 2.4 shows a map of the pCR4-TOPO vector.

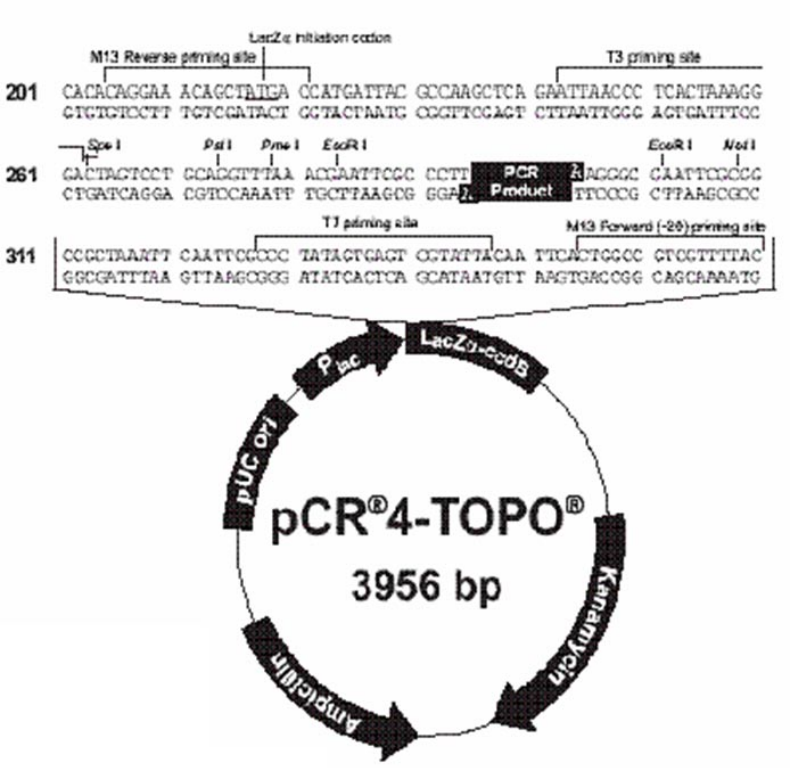


Figure 2-4. The plasmid pCR4-TOPO.

PCR-derived fragments less than 4kb in size were cloned using the TOPO TA Cloning kit (Invitrogen). *Taq* polymerase has template-independent terminal transferase activity, which adds dATP to the 3' end of PCR products. PCR products can then be ligated into linearised pCR4-TOPO vector, which has complementary single 3’ T overhangs.

Two features of the vector allow selection of bacteria that have been transfected with a vector that carries an insert. Firstly, the vector carries a gene for ampicillin resistance;

bacteria that are not transformed are therefore killed by ampicillin in the medium. Secondly, the pCR4-TOPO vector carries a DNA fragment derived from the *Lac* operon of *E.coli*, which encodes the β-galactosidase amino-terminal fragment, the *LacZ* gene. The fragment complements itself *in trans* with a defective form of β-galactosidase that is present in the host bacteria for transformation. When plated onto X-gal agar plates, bacteria containing the religated vector lacking an insert form blue colonies as the transformed cells contain a functional *LacZ* gene that can convert the colourless substrate X-gal into a blue coloured precipitate. If a PCR product is successfully ligated into the cloning site of the vector, the *LacZ* gene reading frame is disrupted so no active enzyme can be produced and the colonies remain white. These colonies were then picked and grown before plasmid extraction.

2.10.1 Ligations

One microlitre TOPO TA Cloning kit salt solution (Invitrogen), 2-3μl fresh PCR product, and sterile water to a volume of 5μl were mixed before the addition of 1μl PCR-4 vector (Invitrogen), on ice. After gentle mixing, the ligation was incubated at room temperature for five minutes, then placed on ice to await transformation.

2.10.2 Chemical transformation

For each transformation, one tube of TOP10 *E. coli* chemically competent cells was thawed on ice. Two μl of the TOPO cloning reaction was added and mixed gently, and left on ice for 20 min. The cells were then heat shocked by placing the tubes in a 42°C water bath for 30 seconds, then put onto ice. 250μl of room temperature SOC medium was added to each vial of cells, followed by incubation at 37°C in a shaking incubator (225rpm) for 30 minutes. The transformed cells were then spread in volumes of 10μl-100μl onto pre-warmed (to 37°C) LB agar plates, supplemented with ampicillin (100mg/ml, Sigma) and spread with 40μl of X-gal (50mg/ml, Promega, Madison, USA). The plates were inverted and incubated at 37°C overnight. Two negative control plates were included:

untransformed competent *E.coli* and a control plate without bacteria. After incubation, plates were placed at 4°C to aid colour development.

2.10.3 Culturing and extraction of plasmids

Only white colonies were picked for culturing. Each colony was grown up in 2.5ml LB broth, containing 50µg/ml ampicillin, overnight at 37°C in a shaking incubator (320 rpm). A control culture of LB and antibiotic was also set up. Plasmid DNA was then extracted from 2ml of culture using the Qiagen Miniprep kit (Qiagen, West Sussex, UK), following the manufacturer's instructions. The remaining 500µl culture was mixed with equal volume of 50% glycerol for storage at –70°C.

Plasmids were extracted using an 'alkaline lysis' method according to the manufacturer's instructions. Firstly, a high pH lyses the cell walls, then when the lysate is then neutralised and the salt concentration is reduced, proteins and bacterial DNA are precipitated and removed by centrifugation. The plasmid DNA remains in the supernatant, which is then applied to a silica column where the plasmid DNA is selectively adsorbed and after a series of ethanol washes is eluted with 50 to 80µl distilled sterile water.

2.10.4 Restriction enzyme digests

Restriction enzymes recognise specific nucleotide sequences that are usually 4bp to 6bp long. They cleave the DNA at these sites, generating either blunt ends or ends with short single stranded overhangs that are referred to as sticky ends. 1U of enzyme was used to digest approximately 1µg of DNA in the appropriate buffers, and then incubated at 37°C overnight. Digestion was checked by running on an agarose gel.

3 Characterisation of *JAK2* V617F in myeloproliferative neoplasms

3.1 Introduction

The original aim of my work was to investigate the molecular pathogenesis of MPN, focusing specifically on PV and pursuing the hypothesis that these diseases may be driven by activated tyrosine kinases. Shortly into my research, an acquired point mutation was discovered in the *JAK2* gene in the vast majority of cases with PV and henceforth my attention was then focused on this abnormality. The initial reports identified *JAK2* V617F (2343 G>T, NM_004972) in 73% to 97% of cases with PV, 23 to 57% of cases with ET and between 9% and 45% of cases with MF [197-201]. It was also noted in these initial studies that subsets of patients were homozygous for the *JAK2* V617F allele, and it was suggested that this was associated with acquired UPD at the *JAK2* locus.

3.1.1 Aims

Following the initial reports of V617F I then conducted my own screen to verify the presence of *JAK2* V617F in our cases with MPN. My specific aims were to:

1- Investigate the involvement of *JAK2* V617F in cases with classic and atypical MPN.
2- Develop sensitive and quantitative methods of screening for the mutation in patient DNA, for use in a diagnostic setting.
3- Investigate the relationship between the mutant allele burden, *JAK2* copy number and acquired UPD.
4- Examine cases with both *JAK2* V617F plus *KIT* D816V or *BCR-ABL* to determine whether the mutations were mutually exclusive.

3.2 Materials and methods

3.2.1 Patient samples

For the characterisation of *JAK2* V617F, samples from a total of 679 individuals were studied retrospectively. Of these, 480 were patients with a known or suspected diagnosis of an MPN (268 males, 212 females) referred for analysis in Salisbury or laboratories of collaborating groups in New York, Mannheim and Athens. In total, patients were referred for PV (n=72, including one case who also had SM), ET (n=59), IMF (n=35), idiopathic HES (n=134), SM (n=28), CML-like diseases (aCML, CMML and related atypical MPNs (n=99) or unclassified MPN cases (n=53). All HES, SM, CML-like diseases and atypical unclassified MPNs were tested for *BCR-ABL, FIP1L1-PDGFRA* and other gene fusions as indicated by karyotype; SM cases were tested for *KIT* D816V. Samples from patients with AML (n=17), *BCR-ABL* positive CML (n=18) plus controls from normal individuals (n=160) and patients with secondary erythrocytosis (n=4) were also studied.

3.2.2 *JAK2* V617F genotyping by allele-specific (AS) PCR

After initially verifying the presence of the *JAK2* mutation in a small set of PV samples by sequencing, I devised an allele specific PCR (AS-PCR) to detect the mutation. From then on patients and controls were screened for *JAK2* V617F using AS-PCR. This test employs two primer pairs which specifically amplify the normal and mutant sequences, alongside amplification of a control band, all within a single reaction. Primers were designed using an AS-PCR design programme (http://cedar.genetics.soton.ac.uk/public_html/primer1.html) [250] and included mismatches of two bases close to the site of the mutation in order to maximise discrimination of the two alleles. Amplification was performed as described in the Methods section, following standard conditions except that the final concentrations of the outer primers and the mutant/wild-type specific inner primers were 1µM and 0.5µM, respectively. Figure 3.1 shows the strategy of AS-PCR. The PCR contains four primers; primers Forward Outer (FO) and Reverse Outer (RO) flank exon 14 and the 463bp product serves as a control for successful amplification. Primers RO and Forward wild-type (Fwt)

generate a small wild-type specific product (229bp), and primers FO and Reverse mutant specific (Rmt) generate a slightly larger mutant specific product (279bp). Control experiments indicated that the assay gave identical results using 20 to 200ng input DNA (data not shown). A gel picture of AS-PCR performed on some V617F-positive cases is shown in Figure 3.2.

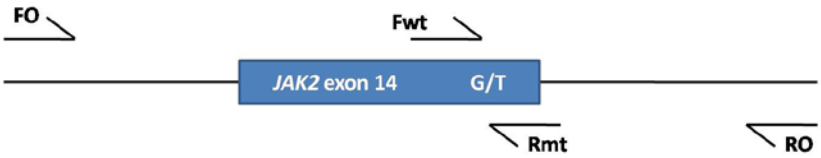


Figure 3-1. AS-PCR strategy to detect *JAK2* V617F.

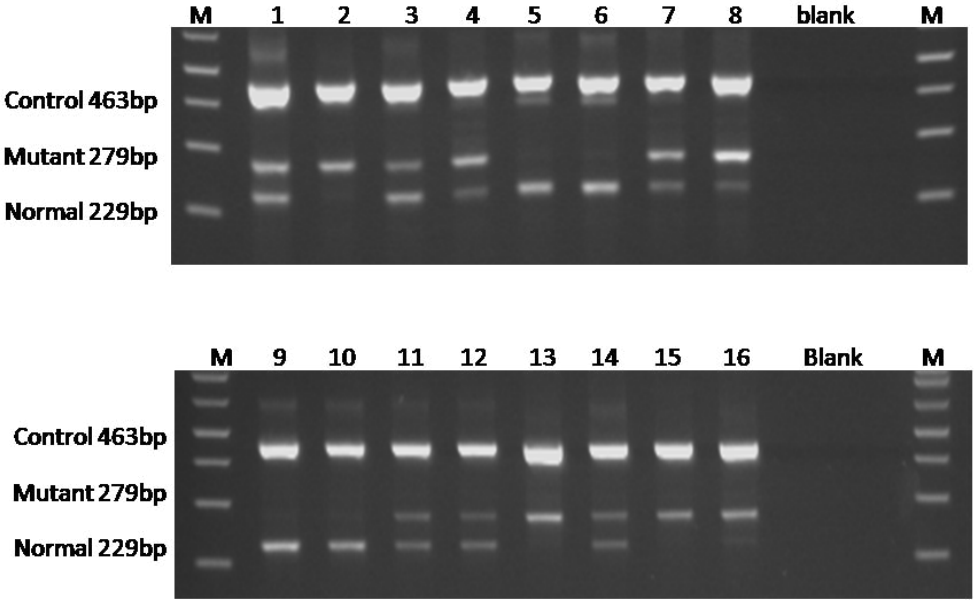


Figure 3-2. Representative results of AS-PCR to detect *JAK2* V617F in genomic DNA. Tracks 5, 6, 9 and 10 show a normal genotype; tracks 1, 3, 11, 12 and 14 show a mutant band that is weaker or similar intensity to the normal band and were therefore scored as heterozygous for the 2343G>T mutation; tracks 2, 4, 7, 8, 13, 15 and 16 show a mutant band that is stronger than the normal band and were therefore scored as homozygous for the mutation.

3.2.3 *JAK2* V617F genotyping and allele quantification by pyrosequencing

In addition to AS-PCR, I also applied pyrosequencing for the detection of *JAK2* V617F. This technique allows accurate quantification of the proportion of mutant alleles. The sequences of the PCR primers (JAK2_pyro_V617F_F and JAK2_pyro_V617F_R) and the sequencing primer (JAK2_pyro_V617F_seq) are listed in Appendix III. The results from pyrosequencing are displayed in a picture called a pyrogram and some examples are shown below. In this situation, pyrosequencing quantified the proportion of the mutant T allele, out of the total peak heights produced by the normal G allele and mutant T allele combined, to express the results as a percentage. Each test was performed in duplicate, and scored as homozygous if the proportion of mutant allele was greater than 50%, the maximum expected if a heterozygous mutant clone had expanded to include all cells in the sample. This was likely to underestimate the number of homozygotes in practice as our samples included lymphocytes (the majority of which are probably not part of the mutant clone). Based on the analysis of a series of normal control samples (detailed below), if the result fell below 5% the specimen was scored as normal and between 5% and 50% samples were recorded as heterozygous for *JAK2* V617F.

3.2.4 Measuring the copy number of chromosome 9p by multiple ligation probe amplification (MLPA)

It was necessary to determine whether our patients with a homozygous *JAK2* mutation acquired this genotype through deletion of one allele on chromosome 9 or duplication of the mutant allele via acquired UPD. To do this I devised an MLPA-based test to measure the copy number of the *JAK2* locus. By comparing peak areas of homozygous *JAK2* V617F patients to that of other peaks amplified elsewhere in the genome, and to that from normal controls, the dosage of the *JAK2* locus could be determined. The finding of only one copy of the *JAK2* locus in homozygous mutant cases would indicate hemizygosity, whereas two copies suggest copy-number neutral LOH, i.e. acquired UPD.

The set of MLPA probes used here were originally designed by Kathy Waghorn to measure tyrosine kinase copy number changes in patients with MPN. Primers were

designed to non-polymorphic exons of *FES* (15q26), *JAK2* (9p24), *SOAT1* (1q25), *BTK* (Xq22), *TYK2* (19p13), *MST1R/RON* (3p21) and *HCK* (20q11) with a single primer pair for each gene. Hybridisation of probe pairs to genomic DNA, ligation and amplification procedures were performed following a procedure originally described by Schouten et al. (2002)[249]. Here, the primers were used at 1.33fmol working concentration, and 1.5µl of the primer mix was combined with an equal volume of MRC probe buffer (MRC-Holland) and added to the 2µl containing each sample DNA. MLPA was then performed as described above. Using the Genotyper programme, the peak area data was exported to an Excel spreadsheet, and the ratios of each test peak relative to all other peaks for that individual were calculated. Ratios of the *JAK2* peak to the other peaks of each patient sample were compared to the same ratios obtained for two normal individuals, which were included in each run. For normal sequences a dosage quotient of 1.0 is expected, whereas if a deletion or duplication is present the dosage quotient was expected to be 0.5 and 1.5 respectively [251]. The gene *BTK* is located on the X chromosome and provided an internal control to check the specificity of the assay (i.e. a dosage quotient of 0.5 in males and 1.0 in females when compared to a female control).

3.2.5 Development of MLPA-based test to detect copy number changes in *JAK2*

In order to detect subtle changes in *JAK2* copy number, I developed a second MLPA-based assay which contained more probes targeting the *JAK2* gene than the probe set described above. To do this I modified a commercially available MLPA probe mix designed for detecting copy number changes in the telomeres of all chromosomes (P036B MRC-Holland, Amsterdam). Four MLPA probe pairs targeting *JAK2* exons 3, 9, 16 and 20 were designed so that they could be combined with the telomere probe mix, and tested on each case simultaneously. The *JAK2* probes consisted of adjacent 5' and 3' oligonucleotides which, when amplified by universal primers following the hybridisation and ligation steps, generated products in the size range 98 to 126bp. The 3' oligonucleotides for each *JAK2* probe pair were modified with a phosphate group at their 5' end, and each oligonucleotide pair contained a tag sequence recognised by the MLPA Salsa primers, so that the *JAK2* probes were amplified alongside the telomere probes in

one reaction. 1.5µl of the *JAK2* primer mix (primers used at a final concentration of 1.33fmol), was combined with 1.5µl of the MRC telomere mix (MRC, Holland) and 3µl MRC probe buffer (MRC, Holland), and added to the 2µl containing each sample DNA. The four *JAK2* peak area ratios per sample were averaged to give a relative dosage quotient for *JAK2* as a representation of copy number.

3.2.6 Extracting DNA from myeloid progenitor cells cultured *in vitro*

To investigate the genotypes of individual myeloid progenitor colonies, MNCs were cultured *in vitro* in semi-solid medium. After ten days incubation, colonies containing more than 100 cells were characterised based on their morphology and then plucked from the growth medium for DNA analysis. The pipette was set to 3 to 5µl and, using an inverted microscope, a well-separated single colony was plucked from semi-solid medium, placed into 100µl sterile distilled water and immediately vortexed to disperse the cells. DNA was extracted by boiling for 10 minutes, then vortexed for 10 seconds. Regions of semi-solid medium not supporting cell growth were also removed in a similar way to provide a measure of the background level of DNA. The amount of DNA obtained from approximately 200 cells was expected to be relatively low, so for DNA-based investigations 10µl from each colony prep was added to a PCR that was adjusted to accommodate this increased volume of DNA. The PCR products were then treated as normal. The remaining colony-preps were stored at -20°C.

3.3 Results

3.3.1 The V617F *JAK2* mutation is widespread in PV, ET and IMF

Initially I aimed to confirm the presence of V617F in classic MPN as well as testing if it was also seen in other, atypical disorders. I started by using the AS-PCR test as this was the most rapid and cost effective screen. A total of 480 samples with a known or suspected diagnosis of MPN were screened for *JAK2* V617F: 129 (27%) were positive using the AS-PCR assay and 351 (73%) were negative.

The results for classic MPN are listed in Table 3.1. The proportion of positive cases for each disease subtype ranged from highly prevalent in PV [58/72 (81%)], to less common in ET [24/59 (41%)] and MF [15/35 (43%)]. The incidences of V617F in our classical MPN cases are similar to those reported elsewhere [197-201].

Reference	PV	ET	IMF	Normal controls
James et al. [197]	40/45 (89%)	9/21 (43%)	3/7 (43%)	0/15
Kralovics et al. [199]	83/128 (65%)	21/93 (23%)	13/23 (57%)	0/71
Baxter et al. [198]	71/73 (97%)	20/51 (39%)	8/16 (50%)	0/90
Levine et al. [200]	121/164 (74%)	37/115 (32%)	16/46 (35%)	0/269
Zhao et al. [201]	20/24 (83%)	-	-	-
This study [252]	58/72 (81%)	24/59 (41%)	15/35 (43%)	0/160
Total	373/482 (77%)	110/339 (32%)	55/127 (43%)	0/605

Table 3-1. Early publications reporting the occurrence of *JAK2* V617F in PV, ET and IMF and its absence in normal controls.

3.3.2 *JAK2* V617F is also found in other MPN

Strikingly, in addition to PV, ET and IMF, I also detected the mutation in a substantial number of patients with a CML-like disease (aCML, CMML and related atypical MPDs; now classified as MDS/MPN) and MPN-U. Of those with a CML-like disease, 17/99 (17%) were V617F positive, and 13/53 (25%) of those with atypical MPN were positive for V617F. The *JAK2* mutation was also found in 2/6 (33%) of patients with CNL, 2/134 (1.5%) patients with HES (Table 3.2).

These findings had not been previously reported and served to establish the importance of *JAK2* V617F in a heterogeneous series of MPN. *JAK2* V617F was not detected in any of the cases that also tested positive for rare tyrosine kinase fusion genes (n=24), nor in individuals with CML (n=18), AML (n=17) or healthy controls (n=160). Out of the eight *KIT* D816V positive cases with SM, one individual also had the *JAK2* mutation but was considered clinically to have both SM and PV. Of the 14 *JAK2* V617F mutation negative PV patients, 13 were male ($p=0.005$, χ^2) but no other significant associations between sex and mutation status were identified.

Disease subtype	number	V617F positive number (% of cases)	Gender/age of V617F positive cases m/f; median age/yrs (range)	V617F negative number (% of mutants)	Gender/age V617F negative cases m/f; median age/yrs (range)	V617F homozygotes number (% of mutants)
PV	72[a]	58 (81%)	31m/27f; 57 (26-76)	14 (19%)	13m/1f; 57 (33-78)	24 (41%)
ET	59	24 (41%)	15m/9f; 65 (5-88)	35 (59%)	23m/12f; 56 (24-88)	4 (17%)
IMF	35	15 (43%)	10m/5f; 65 (44-78)	20 (57%)	15m/5f; 62 (41-92)	10 (67%)
Idiopathic HES	134[b]	2 (1.5%)	1m/1f; (64 (63-65)	132 (98.5%)	51m/81f; 54 (3-89)	2 (100%)
Systemic mastocytosis	28[c]	0 (0%)	n/a; n/a	28 (100%)	16m/12f; 53 (4-90)	n/a
CML-like MPN	99[d]	17 (17%)	13m/4f; 62 (17-76)	82 (83%)	60m/22f; 66 (2-95)	8 (47%)
MPN-U	53	13 (25%)	7m/6f; 63 (17-77)	40 (75%)	17m/23f; 61 (21-87)	7 (54%)
Total	480	129 (27%)	77m/52f; 58 (2-95)	351 (73%)	195m/156f; 62 (5-88)	55 (43%)

Table 3-2. Summary of results and patient details for the characterisation of the *JAK2* V617F mutation. [a] Includes one case with PV plus systemic mastocytosis who was positive for *JAK2* V617F and *KIT* D816V. [b] Includes seven *FIP1L1-PDGFRA* positive CEL cases, all of whom were negative for *JAK2* V617F. [c] Includes seven *KIT* D816V positive cases, all of whom were negative for *JAK2* V617F. [d] Includes 17 cases with rare tyrosine kinase fusions (*ETV6-PDGFRB* n=9, *ZNF198-FGFR1* n=2, *BCR-FGFR1* n=2, *PCM1-JAK2* n=2, *BCR-PDGFRA* n=1, *TP53BP1-PDGFRB* n=1), all of whom were negative for *JAK2* V617F. n/a indicates not available or not applicable.

3.3.3 Determining the sensitivity of the molecular techniques used to characterise *JAK2* V617F

The presence of *JAK2* V617F was verified by sequencing, and some traces showing the point mutation are shown in Figure 3.3. As had been suggested by the AS-PCR, some cases appeared to be homozygous for the mutation and some were heterozygous. In an initial analysis of PV cases (n=51) sequencing was fully concordant with the AS-PCR result. It soon became apparent however that some cases had a low proportion of mutant allele which would have been difficult or impossible to detect by sequence analysis alone. This technique is generally accepted to have a sensitivity of only 20 to 30% depending on the sequencing context.

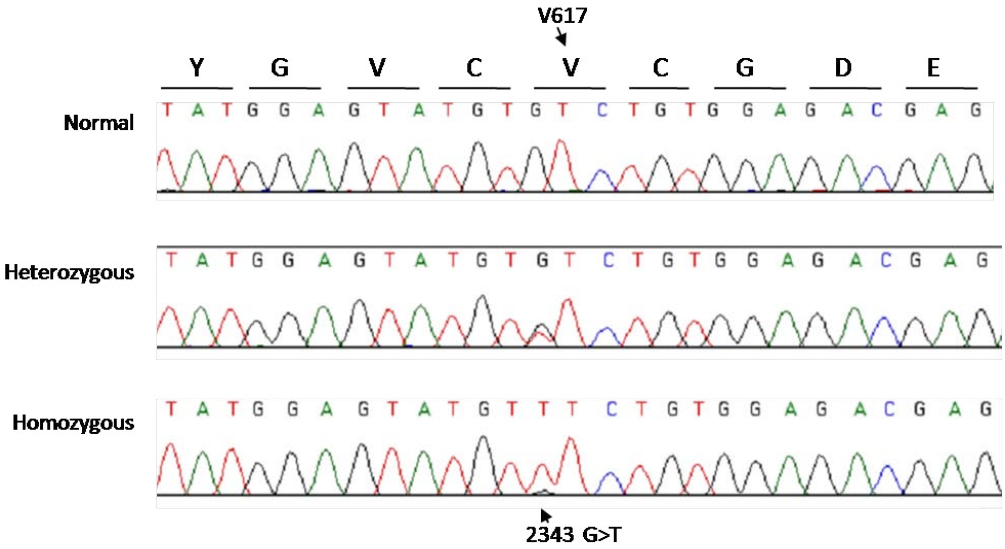


Figure 3-3. Sequence traces of three individuals found to be normal, heterozygous and homozygous by AS-PCR.

In occasional cases the intensity of the mutant band by AS-PCR was very weak and it was difficult to decide if the patient was positive or negative. It became necessary therefore to clarify the sensitivity of the AS-PCR in detecting the *JAK2* V617F. This was achieved using a dilution series made with DNA from a homozygous patient diluted into DNA from a healthy normal control. AS-PCR was found to reproducibly detect down to 2 to 5% mutant allele (Figure 3.4). Following this a more quantitative assay for V617F was developed using pyrosequencing.

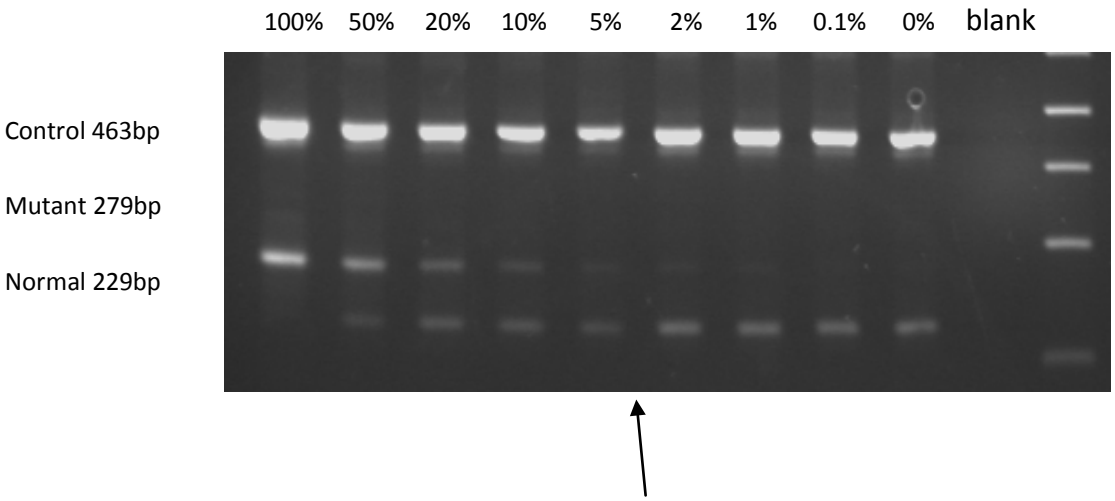


Figure 3-4. Sensitivity of the AS-PCR assay. The arrow indicates the AS-PCR PCR is routinely capable of detecting a level of 2 to 5% mutant allele.

Pyrosequencing for the *JAK2* mutation was performed initially on a set of 29 normal DNAs. The mean level of mutant alleles measured was 0.53% (range 0 to 2.90%, standard deviation 0.83%). From this a figure of equal to or below 5% mutant allele (average + 5S.D.) was used to define a sample as normal for the *JAK2* mutation. Next, 23 MPN cases found to be normal by AS-PCR were tested and all were found to be also negative by pyrosequencing.

The accuracy of pyrosequencing to quantify V617F levels was then tested using a dilution series created by mixing PB granulocyte DNA from a 98.3% V617F positive patient with PV with normal DNA derived from PB total leukocytes. The dilution series was extended to include a wider range of dilutions, and the data is shown in Table 3.3 and Figure 3.5. The pyrosequencing results accorded well with the dilution level of mutant allele into wild-type.

Although there was general consensus in the initial reports that many patients with classic MPN were positive for V617F, inspection of Table 3.1 shows quite marked discrepancies in the frequency of positive cases reported for different subgroups, e.g. 65-97% of PV and 23-43% of ET. The varying degrees of sensitivity for each molecular

method used to detect the *JAK2* mutation are likely, at least in part, to explain the different frequencies of mutation found by different groups. In particular, those groups who just performed sequencing, for example, Kralovics et al. (2005) [199] and Levine et al. (2005) [200] are likely to have underestimated the true incidence of *JAK2* V617F in their cohorts. Results from this study suggest a combination of AS-PCR and pyrosequencing is sufficient to detect and quantify *JAK2* V617F routinely in a diagnostic setting.

% *JAK2* V617F	% mutant allele determined by pyrosequencing				Mean average % mutant allele
	A	B	C	D	
100	100	97.5	97.4	98	**98.23**
80	79.6	77.3	77.8	77.6	**78.08**
60	58.7	58	57.3	59.5	**58.38**
50	49.1	48.2	51.9	48.7	**49.48**
40	39.5	39.5	36.4	38.1	**38.38**
20	21.8	17.7	20.4	19.2	**19.78**
10	10.4	10.6	9	11.5	**10.38**
5	4.9	4.2	4.6	3.4	**4.28**
2	2.9	2.5	2.1	1.4	**2.23**
1	3.3	0.9	0.2	5.3	**2.43**
0.1	4.3	2.3	0	1.7	**2.08**
0	1.8	1.2	2.1	1.5	**1.65**

Table 3-3. Pyrosequencing results from a dilution series of *JAK2* V617F homozygous DNA and normal DNA. The % *JAK2* V617F indicates the proportion of mutant JAK2 alleles based on the dilution factor. A-D are quadruplicate replicates of the pyrosequencing analysis with the mean of these values shown in the last column.

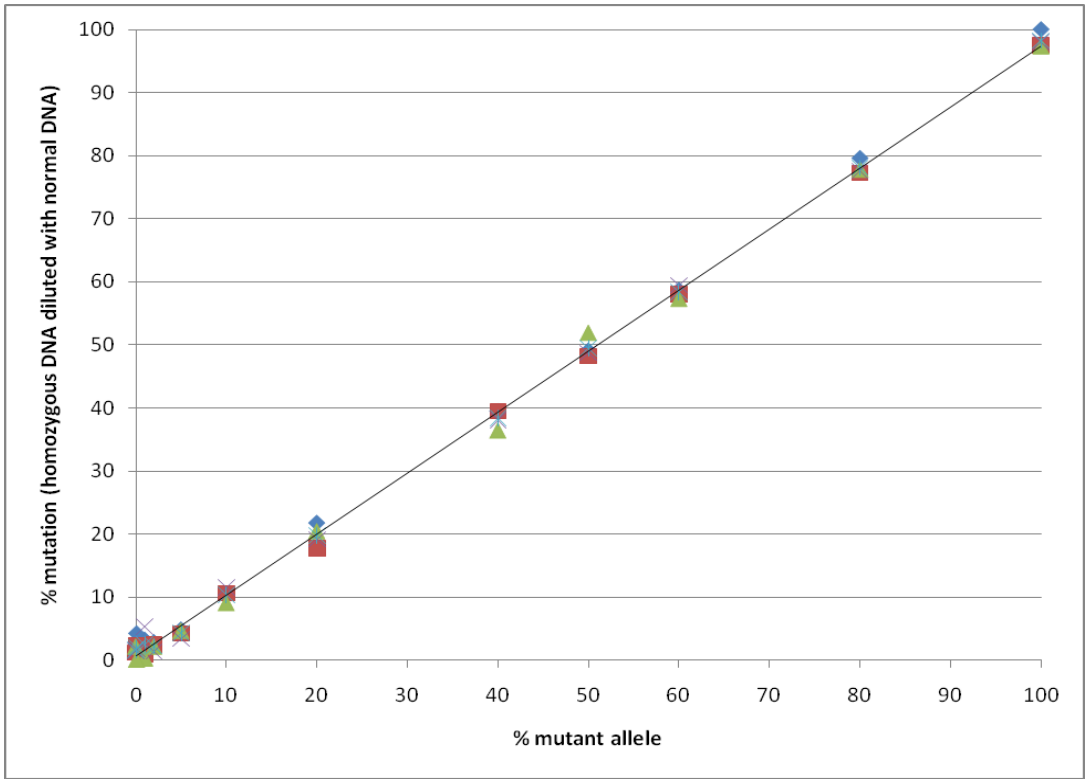


Figure 3-5. Line graph showing %V617F as determined by pyrosequencing performed on four separate *JAK2* V617F dilution series.

3.3.4 Pyrosequencing analysis confirms that *JAK2* V617F levels vary widely in MPN

Pyrosequencing provides accurate quantification of allele ratios and provided a more accurate measurement of the size of the mutant clone in our cases. If the pyrogram gave a percentage of mutant allele above 50%, that sample was scored as homozygous. Below and at 50%, and above 5% mutant percentage, that sample was termed heterozygous for the *JAK2* mutation. Samples with no evidence of mutation (i.e. <5%) were classed as normal. Pyrosequencing was performed on 90 mutant samples with a stronger mutant band by AS-PCR, and confirmed homozygosity in the majority of samples (%V617F >50%). Examples of normal, heterozygous and homozygous V617F pyrograms are shown in Figure 3.6.

For some samples analysed by AS-PCR there was insufficient DNA available for pyrosequencing. These cases were scored as homozygous if the ratio of wild-type to

mutant bands was at least as strong as that seen in the 50% dilution control (Figure 3.4, 50% track) as determined by visual inspection.

Overall 55 out of the 129 (43%) mutant samples were homozygous, and results for each patient subgroup are summarised in Table 3.2. The ratio of homozygotes to heterozygotes was not significantly different from the average of all cases in any of the subgroups, apart from ET where the proportion of homozygous mutants was significantly lower than that seen in other subgroups (P=0.009, χ^2). Other publications report comparable levels of homozygosity for PV, ET and IMF [197-201].

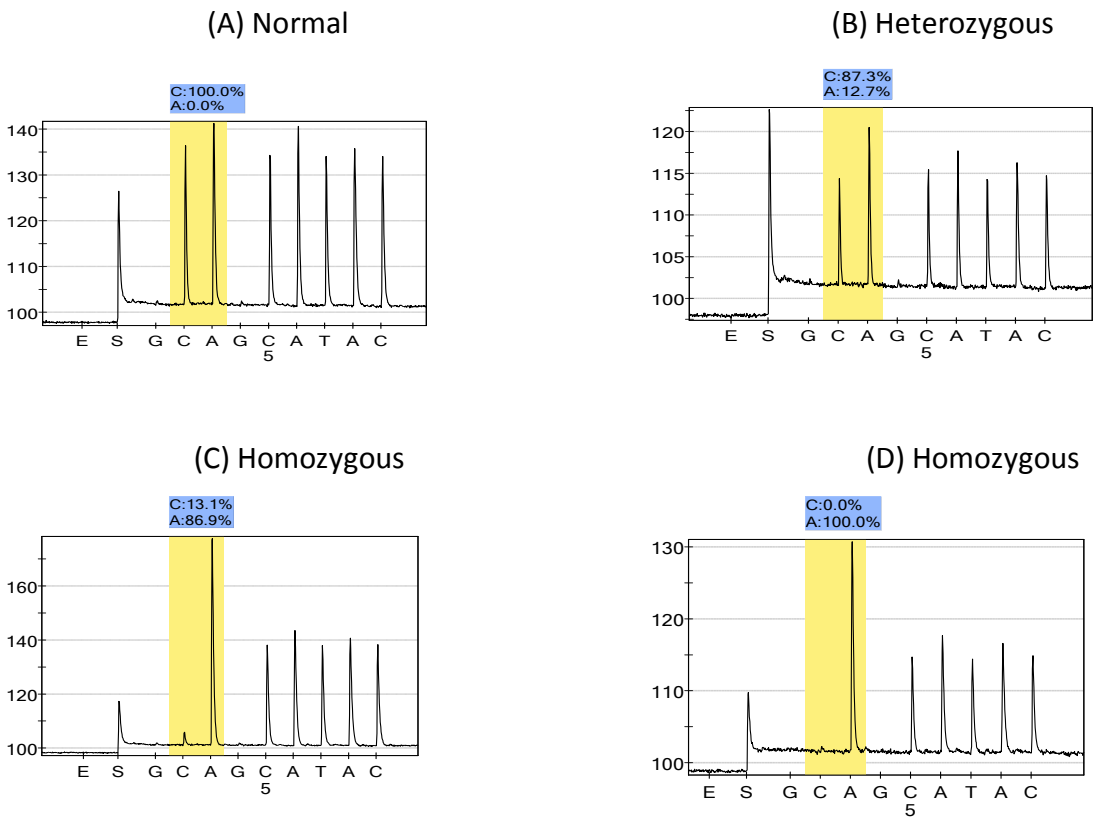


Figure 3-6. Representative pyrograms quantifying the *JAK2* V617F mutation. Pyrograms from normal (A), one heterozygous (B) and two homozygous (C and D) V617F-positive individuals are shown. The sequencing primer is in reverse orientation (immediately abutting the site of the mutation) and the dispensation order GCAGCATAC was used (the two Gs are internal controls that should give no peak, E and S indicate enzyme and substrate, respectively). In a normal individual (sequence CACATAC) the peak heights of the first C and A are similar. In a heterozygous individual (sequence (C/A)ACATAC) the C peak is reduced in height and the A peak increased above all other peaks as both the mutant A and the following A are being read in the same direction. In a homozygous individual (sequence AACATAC) the first peak is either much lower than the A peak (trace C) or can be absent (trace D). The ratio of the normal (C) and mutant (A) alleles is calculated by the Pyrosequencing SNP analysis software.

3.3.5 *JAK2* V617F and chromosome 9p acquired UPD

Homozygosity for *JAK2* V617F was established in 24/72 (33%) cases of PV, 4/59 (7%) cases of ET and 10/35 (29%) cases of IMF. This is strikingly similar to the frequencies of acquired chromosome 9 UPD that has been described in these diseases [199]. To determine whether UPD for chromosome 9 was associated with homozygosity for the *JAK2* V617F, a colleague (Claire Curtis) performed microsatellite analysis on 57 cases (normal n=30, homozygous V617F n=27). In normal individuals, most microsatellites were expected to be heterozygous. Consecutive tracts of homozygous markers usually indicate LOH due to complete or partial chromosomal loss (i.e. hemizygosity), or acquired UPD.

Significant tracts of homozygosity in the vicinity of *JAK2* was observed in 4 (13%) of the normal cases and 25 (93%) of the *JAK2* V617F homozygotes ($P<0.0001$) [252]. To determine whether this homozygosity arose through loss of the wild-type allele, I then measured the number of copies of the *JAK2* gene relative to control genes in patient DNA using MLPA. Seven cases with a homozygous *JAK2* V617F mutation and at least three consecutive 9p microsatellite markers were tested and all were found to possess two copies of *JAK2,* consistent with homozygosity as a consequence of acquired UPD for chromosome 9p (Figure 3.7). These findings are consistent with those reported by Kralovics et al. (2005) [199]. An example of the calculations used to determine *JAK2* copy number is included in Appendix IV.

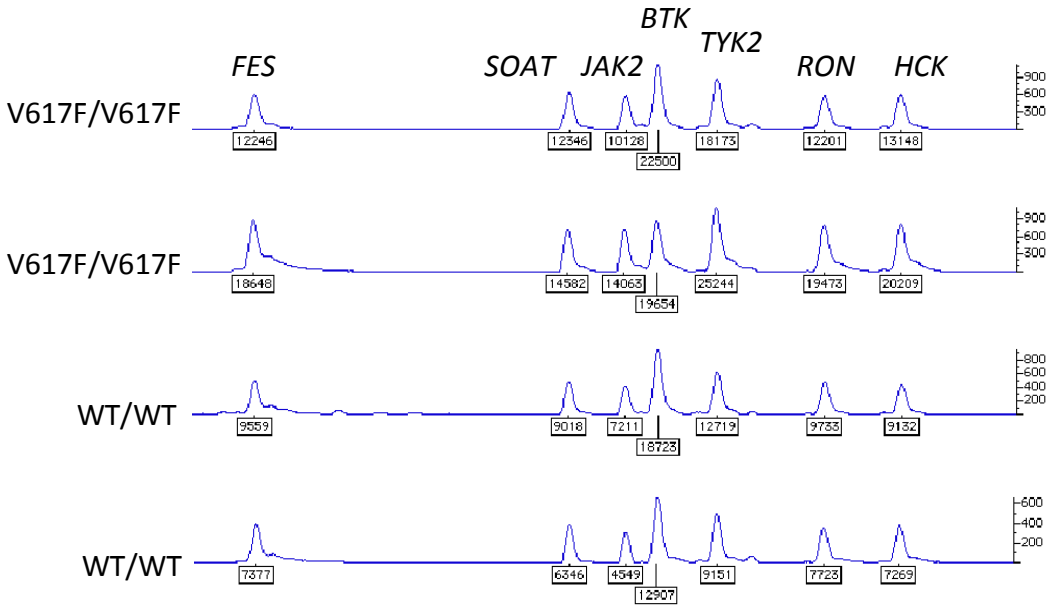


Figure 3-7. Representative results of MLPA analysis measuring copy number of the *JAK2* gene. The relative peak areas for *JAK2* are the same for two homozygous V617F cases with 9pLOH (V617F/V617F) and two normal controls (WT/WT), indicating the presence of two copies of *JAK2.* The second case is male, and shows a reduced peak height for the X-linked *BTK* gene compared to the three other cases, all of which were female.

3.3.6 Is evolution of V617F-positive disease associated with amplification of *JAK2*?

Acquired UPD of chromosome 9 is responsible for homozygosity for V617F in a significant proportion of cases with MPN. It is widely acknowledged that this mechanism of mitotic recombination arises as a consequence of genomic instability [253]. Recently it was suggested that this instability may also be responsible for amplification of *JAK2* by tandem duplication in some V617F-positive cases, accounting for disease evolution [254]. It was proposed that cells with more than two copies of mutant *JAK2* carry an additional signalling advantage. In a follow-up study the same group found that this phenomenon was most prevalent in PV, with more than one-third of mutation-positive cases apparently having more than two V617F copies per cell [255]. While increased V617F copy

number has been described in cell lines [256], this was the first suggestion that *JAK2* might be amplified in patients. Classical MPN is characterised by cytogenetically visible abnormalities of chromosome 9 which alter the copy number of *JAK2*, for example +9, +9p, del(9), and translocations involving chromosome 9, but these cases are relatively infrequent. In my MLPA analysis above no amplification of *JAK2* was detected in 7 of 7 cases tested and thus the findings of Hammond et al. were surprising.

Here I investigated whether elevated *JAK2* copy number could be detected with an alternative methodology to the quantitative PCR based method used by Hammond et al. (2007) [255]. For this investigation I developed a second MLPA based test. This was designed to be more accurate than the method above by inclusion of multiple *JAK2* probes and building the assay onto an established commercially available subtelomere kit (MRC-Holland) that was known to produce reliable and reproducible results.

3.3.6.1 Validation of MLPA-based test to detect copy number changes in *JAK2*

The assay was first tested on a series of control samples. Firstly, 21 haematologically normal individuals gave a median *JAK2* dosage quotient of 1.01 (range 0.71-1.28). The dosage quotient for a fibroblast cell line carrying a der(9)t(6;9) was 0.50, corresponding to deletion of one copy on *JAK2*, which was further supported by deletion of one copy of the 9p telomere probe. To control for increased copy number of the *JAK2* locus, the V617F-positive cell lines SET-2 and HEL were analysed. SET-2 has approximately 75% V617F mutant allele per cell, as determined by pyrosequencing, while MLPA here revealed five copies of the 9p telomere (dosage quotient was 2.47) and five copies of *JAK2* per cell (dosage quotient was 2.50). This suggests that SET-2 has four mutant and one wild-type copy of *JAK2*. HEL had 100% V617F by pyrosequencing and MLPA indicated nine to ten copies of *JAK2* (dosage quotient was 4.72), plus total deletion of the 9p telomere and other abnormalities. MLPA traces are shown in Figure 3.8, and accord well with previous estimates of *JAK2* copy number in these cell lines, as determined by fluorescence *in situ* hybridisation [256].

3.3.6.2 Investigation of *JAK2* copy number in V617F-positive MPN

Next I tested DNA isolated from PB total leukocytes from 63 MPNs (PV, n=45; MF, n=5; other MPN, n=13) that were homozygous for V617F as determined by pyrosequencing (median %V617F=87%; range 63 to 99%). The *JAK2* dosage quotients observed (median 0.99; range 0.67-1.25) ascertained by MLPA were no different from the normal control group and no case was above the previously defined cut-off required to define a duplication [251,257]. An example of the MLPA traces, *JAK2* dosage quotient and calculated copy number is shown for four homozygous V617F-positive cases in Figure 3.8. Analysis of a further 16 heterozygous V617F-positive cases (median %V617F = 31%), yielded similar results with again no evidence for increased *JAK2* copy number.

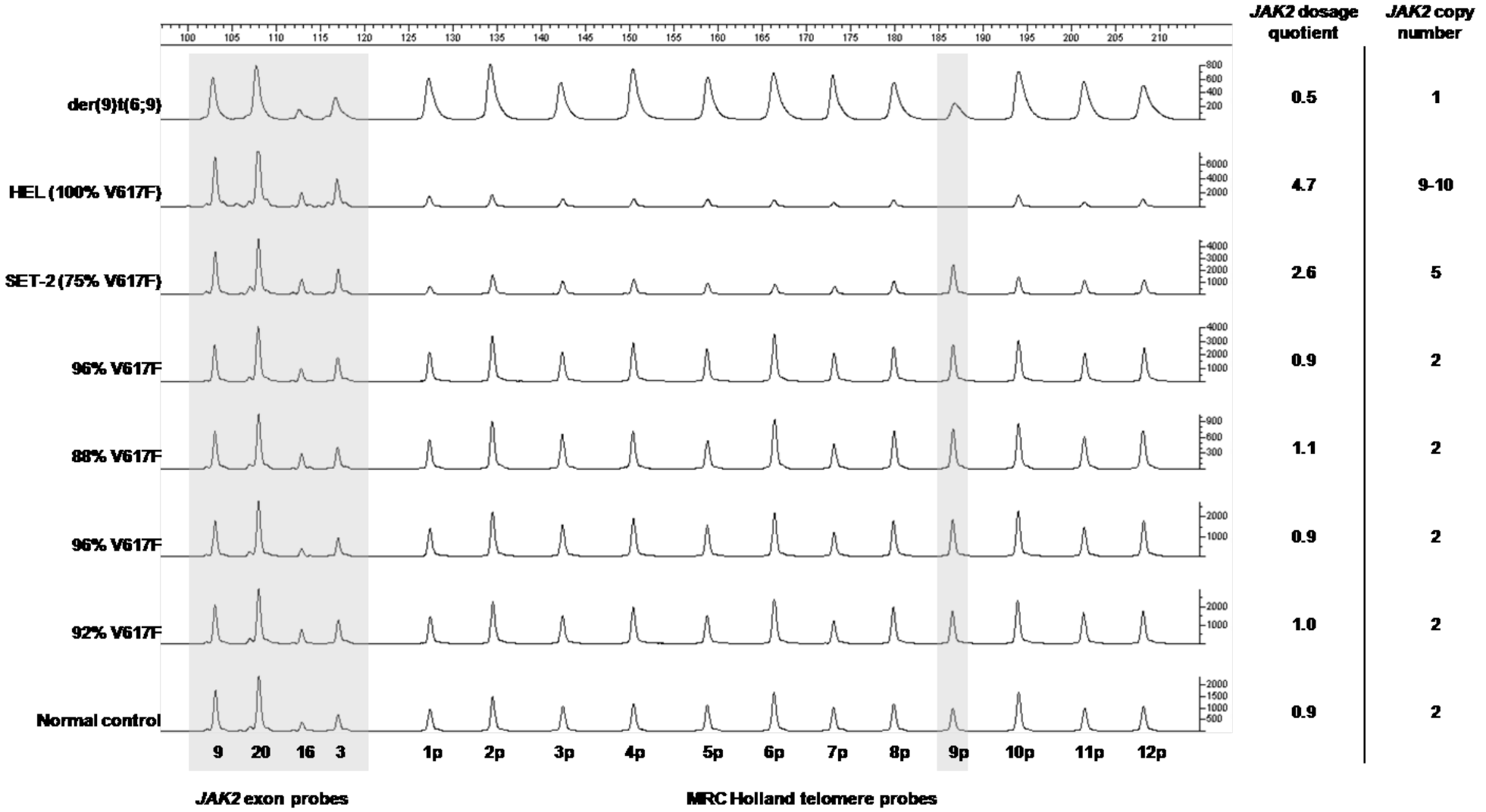

JAK2 dosage quotient
JAK2 copy number
der(9)t(6;9)
0.5
1
HEL (100% V617F)
4.7
9-10
SET-2 (75% V617F)
2.6
5
96% V617F
0.9
2
88% V617F
1.1
2
96% V617F
0.9
2
92% V617F
1.0
2
Normal control
0.9
2
9
20
16
3
1p
2p
3p
4p
5p
6p
7p
8p
9p
10p
11p
12p
JAK2 exon probes
MRC Holland telomere probes

Figure 3-8 (previous page). Representative MLPA genotyper traces for measuring *JAK2* copy number per cell. The *JAK2* probes for exons 9, 20, 16, and 3 result in smaller bands than those from the MRC-Holland subtelomere probe mix, of which 1p to 12p are shown here. The *JAK2* dosage quotient and estimated copy number are shown on the right. The der(9)t(6;9) cell line, carrying only one copy of *JAK2*, shows reduced *JAK2* and 9p subtelomere peaks relative to the other peaks. HEL and SET-2 show an increase in the relative size of these peaks, while no *JAK2* copy number alterations were detected in four illustrative patient samples with a high %V617F.

3.3.7 *JAK2* V617F-positive MPN and other genetically distinct clones provide insights into a multi-step pathogenesis

In the initial study group of 480 cases I found one case that tested positive for both *KIT* D816V and *JAK2* V617F. During routine screening, a further three cases with *KIT* D816V and one case with *FLT3* ITD were also found to be *JAK2* V617F positive. Anecdotal reports in the literature have described similar cases, but it was unclear whether the mutations arise in the same cell lineage [258] or in independent clones [259]. Here I investigated two cases in more detail.

3.3.7.1 *JAK2* V617F and *KIT* D816V

Firstly, it was possible to examine the molecular origin of clones carrying *KIT* D816V and *JAK2* V617F mutations in one case with overt SM and thrombocytosis, for whom a fresh PB sample was available. Analysis of the whole blood showed that the levels of both mutations were low, with *JAK2* V617F detectable only by AS-PCR and not by pyrosequencing indicating a mutation burden in the 2-5% range. Pyrosequencing for the KIT mutation indicated a level of 15%. The PB sample was separated by density gradient centrifugation following standard conditions and MNCs were cultured in semi-solid medium and exposed to cytokines that promote division and differentiation of myeloid cells, in the absence of erythropoietin. Colonies which are assumed to arise from single colony-forming progenitor cells were picked after ten days incubation, and DNA was extracted and genotyped for *KIT* D816V and *JAK2* V617F mutations by pyrosequencing.

The results from the *JAK2* V617F assay were considered negative if they had a mutant allele level of less than 5% [252] and less than 10% for the *KIT* D816V pyrosequencing assay (data provided by Kathy Waghorn, personal communication). Mutant allele values above these limits were scored as positive. Additionally, mutant allele levels greater than 50% were termed homozygous, and mutant allele levels below 50% were termed heterozygous. DNA was also extracted by standard procedures from total PB leukocytes removed prior to density gradient centrifugation. This DNA sample was also screened for both mutations using pyrosequencing, and additionally by AS-PCR for *JAK2* V617F.

In total 43 colonies were picked and screened for both mutations, and the results are listed in table 3.4. Seven colonies carried *JAK2* V617F, 13 colonies carried *KIT* D816V, and 23 colonies were negative for *KIT* D816V and *JAK2* V617F. The mutations were mutually exclusive, i.e. there was no evidence for both mutations occurring in the same clone. The remaining 23 colonies were normal for each mutation, producing pyrosequencing results that ranged from 0 to 5% mutant allele for *JAK2* V617F, and 0 to 8.6% mutant allele for *KIT* D816V. In addition to the colonies, aliquots were removed from the area of semi-solid medium not supporting cell growth to ascertain the background level of DNA that may influence mutation screening. Eight blank preps were genotyped and four failed to amplify on PCR, and four were found to contain DNA that was negative for either mutation on mutant allele genotyping. The pyrosequencing values for these blank aliquots ranged from 0 to 3.1% mutant allele for *JAK2* V617F, and ranged from 7.9 to 10.4% mutant allele for *KIT* D816V, and were thought to have resulted from amplification of low level DNA from background wild-type cells.

Mutation status	colony number
Negative for *KIT* D816V and *JAK2* V617F	23
JAK2 V617F positive	7
KIT D816V positive	13
KIT D816V and *JAK2* V617F positive	0

Table 3-4. Genotyping of myeloid colonies for *JAK2* V617F and *KIT* D816V mutations.

The mutant allele levels for each mutation-positive colony are listed in table 3.5. Out of the seven colonies carrying *JAK2* V617F, five had mutant allele levels approaching homozygosity, and two colonies were heterozygous. For the colonies with V617F levels between 50- 90%, it was suspected that contamination with a background of normal cells lowered the overall level of mutant allele down from the expected value of 100%. Similarly there is evidence of contamination with normal cells in a series of heterozygous colonies 6 and 7 for *JAK2*, plus 12, 17 and 18 for *KIT*. Although care was made to select single colonies for analysis, the picking of overlapping clones cannot be ruled out. *KIT* D816V was isolated in 13 individual colonies, and all colonies were heterozygous for this mutation. Ten of these colonies had values close to 50%, whereas the other three were lower, again probably due to a mixture of normal and mutant cells. Despite this uncertainly it was clear that no colony with both mutations was detected. Given the low level of both mutations isolated in total PB leukocytes, it was surprising to find this number of mutant-positive colonies, but this may be due to differential stimulation of mutant and normal CFU-GM by myeloid growth factors present in the semi-solid medium.

Colony identifier	*JAK2* V617F %mutant allele	*KIT* D816V %mutant allele	
1	72.4	5.2	*JAK2* mutated colonies
2	82.1	8.4	
3	82.8	0.0	
4	84.6	6.0	
5	91.8	8.0	
6	26.4	0.0	
7	34.2	8.6	
8	1.6	50.1	*KIT* mutated colonies
9	3.5	53.9	
10	1.4	51.8	
11	1.7	45.5	
12	1.5	21.1	
13	0.4	54.1	
14	0.3	52.8	
15	0.0	50.3	
16	0.1	51.0	
17	2.3	14.0	
18	2.2	16.0	
19	3.5	50.4	
20	3.5	55.0	

Table 3-5. Mutant allele percentages for *KIT* D816V and *JAK2* V617F positive colonies.

3.3.7.2 *JAK2* V617F and *BCR-ABL*

The second investigation was performed in collaboration with Dr Kramer (Heidelberg, Germany), who was responsible for the care of a patient with *BCR-ABL* positive CML who also tested positive for *JAK2* V617F. It is widely reported that patients with chronic phase CML respond rapidly to treatment with imatinib, but this patient, who responded well to imatinib as determined by a reduction in *BCR-ABL* levels by quantitative PCR, developed splenomegaly and myelofibrosis. This prompted a screen for *JAK2* V617F, and the patient was found to carry the mutation. Using pyrosequencing, I measured the level of the mutant allele in DNA samples taken at different time points over the clinical course. As shown in Figure 3.9, the *JAK2* mutation was present at the initial diagnosis of *BCR-ABL* positive CML at a level of approximately 40% and this remained roughly constant during

imatinib treatment. It was postulated therefore that *BCR-ABL* had arisen on the background of a pre-existing *JAK2* V617F positive disease [260].

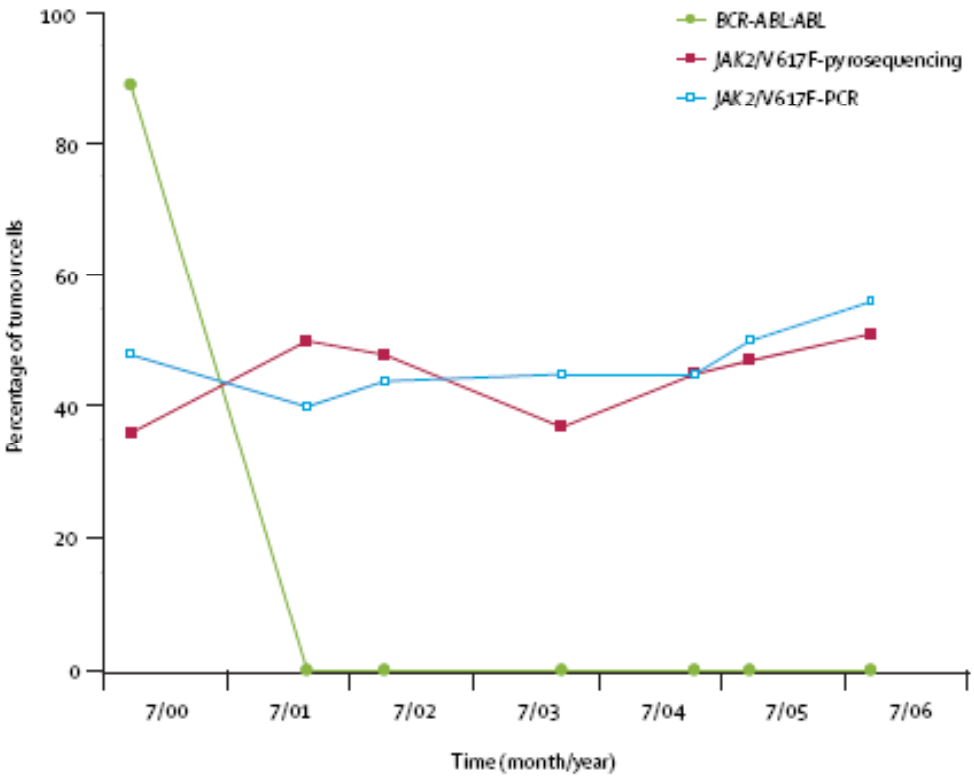


Figure 3-9. *BCR-ABL* ratio and *JAK2* V617F allele frequency during treatment with imatinib in a patient with *BCR-ABL* positive CML. *BCR-ABL* and V617F levels were determined by quantitative RT-PCR, indicated by the green and blue lines, respectively. The V617F mutation burden measured by pyrosequencing is indicated in red (taken from Kramer et al. (2007) [260]).

3.4 Discussion

This part of my study was focused on characterising an acquired point mutation, *JAK2* V617F in MPN. I confirmed early reports that this mutation is seen in the majority of patients with PV and a significant number of patients with ET and MF. I also found V617F in more heterogeneous atypical MPN, leading to the understanding that *JAK2* V617F is more prevalent than initially thought. The implications of a singular point mutation arising in diseases that are clinically diverse but clearly related remains unclear, but one possibility is that V617F serves to provide a molecular definition for a clinical entity with diverse features at presentation.

3.4.1 The development of sensitive and quantitative methods of detecting *JAK2* V617F

In order to screen cases with MPN efficiently, it became clear that sequencing alone would not detect all positive cases, due to dilution of positive cells with an excess of normal cells. Therefore I designed an AS-PCR assay capable of detecting down to approximately 2% mutant allele, which was sufficient to detect V617F in the majority of cases. However I did encounter a small minority of cases with very low levels of mutation which proved difficult to categorically define as low level heterozygous or normal. The technical difficulties I experienced in measuring the *JAK2* mutation may account for the published differences in the proportions of positive cases within MPN subtypes, where sequencing alone was used [199,200]. Baxter et al. (2005) [198] state that direct sequencing underestimates the proportion of patients carrying the *JAK2* mutation as sequencing will only reproducibly detect a heterozygous mutation if it is present in more than 40% of cells, or approximately greater than 20% of alleles. V617F may be present in PB at levels that range from low level heterozygous upward to complete homozygosity, and this may reflect progression of the disease from initiation to clonal expansion.

JAK2 V617F was also detected using pyrosequencing, which provided an accurate measurement on the level of the mutant allele in positive cases. It was noted that the majority of cases affected by *JAK2* V617F are heterozygous, i.e. the V617F-positive cells

carry one copy of the mutation and retain one wild-type copy, and this is then superimposed on a background of normal cells. However, in PV, the mutant allele was found to predominate in 41% of cases. Investigations using microsatellite markers around the *JAK2* locus and measurement of *JAK2* copy number indicates that in these individuals mitotic recombination leading to acquired UPD has bought about homozygosity for the mutation. Whilst these results concur with the findings reported by others [197-200], the incidence of homozygosity in PV also accords with results reported prior to the discovery of V617F, where loss of heterozygosity on chromosome 9p was shown to occur in approximately 30% of PV patients [203]. Such findings imply that, at least in a subset of patients, a minimum of two sequential changes are required for pathogenesis: acquisition of V617F and then mitotic recombination followed by selection for V617F homozygosity.

3.4.2 *JAK2* V617F is seen in diverse MPN, but not in disorders of the lymphoid lineages

The heterogeneous group of diseases that are classified as myeloproliferative in nature now appear to be united by a single molecular marker, the *JAK2* V617F mutation. In this study I found the mutation in HES/CEL, CNL, atypical CML, CMML and unclassified MPNs. Subsequently others reported similar findings [261-263] . Table 3.6 shows a comparison of the frequencies of the *JAK2* mutation in atypical MPN from these publications.

Disease	This study	Steensma et al. (2005)[261]	Levine et al. (2005) [263]	Jelinek et al. (2005) [262]
HES/CEL	2% (n=134)	0% (n=11)	-	-
SM	0% (n=28)	25% (n=8)	-	-
CNL	33% (n=6)	17% (n=6)	-	-
aCML /CMML	17% (n=99)	-	8% (n=116)	15% (n=68)
MPN-U	25% (n=53)	-	-	

Table 3-6. A comparison of published mutation frequencies of the *JAK2* V617F mutation in MPN.

Currently we have screened the largest number of HES/CEL cases and found a very small minority with *JAK2* V617F. Steensma et al. (2005) [261] failed to find any evidence of the

mutation in their HES cases, but this may be explained by their small sample size. However, this group reported 25% (2/8) of patients with SM carry the *JAK2* mutation. Interestingly, we also screened a patient with PV and evidence of mast cell disease, and found both the *JAK2* V617F and *KIT* D816V mutations. Both *JAK2* V617F positive SM cases reported by Steensma et al. (2005) were negative for *KIT* D816V. Cases carrying two mutations are rare, and provide a unique opportunity to further understand the pathogenic nature of these oncogenic changes. This case was subsequently investigated to try to understand whether the *JAK2* and *KIT* mutations occurred in two different or one single clone (discussed below).

CNL is a very rare myeloproliferative disease characterised specifically by an expansion of neutrophils [264]. We found *JAK2* V617F in 2 out of 6 (33%) cases with CNL. Steensma et al (2005) [261] reported a similar number; V617F was present in 1 out of 6 (17%) of CNL cases. Regarding atypical CML, we found 17% (n=99) carried *JAK2* V617F. The *JAK2* mutation was identified in 25% unclassified MPNs (n=53). Similar findings for aCML were also reported by Jelinek et al. (2005) [262] and Steensma et al. (2005) [261].

Other studies have shown that V617F is almost always restricted to the myeloid lineage diseases. Studies on ALL [262], both T cell and B cell lineage as well as CLL [263] have failed to find any evidence of *JAK2* V617F in lymphoid leukaemias. Regarding AML, we failed to find the *JAK2* mutation in 17 cases. This was verified by Jelinek et al. (2005) [262] who tested a range of patients with AML (M0 to M6) and failed to find *JAK2* V617F in 28 cases. However, when AML was preceded by PV, ET or IMF, the *JAK2* mutation was found in 12 out of 22 (55%) cases. Levine et al. (2005) [263] also report 4 out of 222 (1.8%) cases of AML with the *JAK2* mutation, where 3 of these 4 positive AML cases had a preceding MPN. Campbell et al. (2006) [265] subsequently described three cases with V617F-positive myeloproliferative disease which transformed to acute leukaemia. Interestingly, the leukaemic cells of these patients were V617F negative, suggesting that the leukaemia arose in a V617F negative cell. These observations suggest that acquisition of V617F might be a secondary event, following an initial pre-*JAK2* phase of disease, which resulted from an unidentified initial mutation.

In this study, *JAK2* V617F was found in 81% of cases with PV, the highest frequency of all MPNs tested. Although it may be argued that some cases were diagnosed incorrectly, these results suggest that not every patient with PV carries V617F. Investigations into these rare cases subsequently revealed a series of mutations that target a different region of the *JAK2* gene in V617F negative PV and idiopathic erythrocytosis [266]. My investigations into the prevalence and methods of detecting *JAK2* exon 12 mutations are described in Chapter 4.

As *JAK2* V617F continued to be characterised, its diagnostic importance clarified and it has been incorporated into algorithms for diagnosis and classification of MPN and related disorders including the new WHO criteria [22]. The utility of these revised criteria have contributed significantly to an apparent increase in the incidence of ET in a well-defined population in France [267].

3.4.3 The role of *JAK2* V617F in development of MPN

Introduction of V617F into mice gives rise to a PV-like phenotype but there are several lines of evidence to suggest that the mutation might be a secondary event, at least in some cases. Analysis of X chromosome inactivation patterns [268,269], transformation of *JAK2* V617F-positive MPN to V617F negative leukaemia [265,270], growth of *JAK2* V617F negative EECs from V617F-positive patients [259,271] and an apparent familial tendency to develop an MPN [204,272] all provide evidence that support this notion. On the other hand, introduction of V617F into mice reproducibly results in a PV-like disease suggesting that a single hit may be sufficient [197].

Although it is not known whether V617F is a true disease-initiating event, and what other molecular events are implicated in disease progression, it is reasonable to suggest that *JAK2* V617F does account for some aspects of a MPN but not necessarily the complete phenotype. Figure 3.10 shows two possible pathogenic mechanisms that may account for the development of myeloproliferative disease. Kralovics et al. (2005) [199] proposed, in model A that the MPN phenotype is initiated by a mutation such as *JAK2* V617F arising on one allele of chromosome 9p, either alone or in combination with a hypothetical pre-

existing somatic mutation in an unknown gene. In model B, MPN is induced by one or more unknown mutations with *JAK2* V617F as a secondary event. The proliferative advantage of *JAK2* V617F causes the mutant clone to expand at a faster rate than the wild-type clone. In both models, a heterozygous V617F-positive cell then undergoes recombination at chromosome 9p so the V617F allele is duplicated by UPD to produce a cell with two copies of V617F. With no wild-type *JAK2* now present, the homozygous V617F positive cell out-competes the heterozygous mutant cells and the wild-type cells towards full clonal dominance.

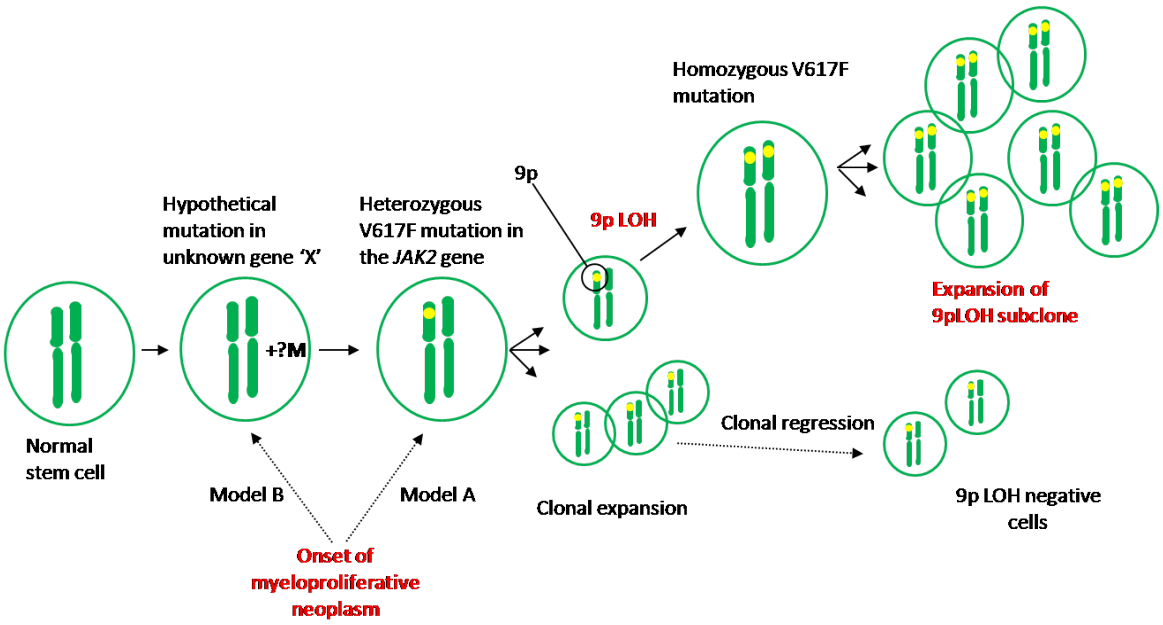


Figure 3-10. Hypothetical role of the *JAK2* V617F mutation in myeloproliferative diseases. Adapted from Kralovics et al. (2005) [199].

Accordingly, MPN patients without the mutation should be in the early stages of disease, or have a shorter disease duration that those with the *JAK2* mutation. It would then be predicted that patients who are homozygous for *JAK2* V617F should have a longer disease duration, a theory supported by preliminary findings from Kralovics et al. (2005) [199]. Clinical data showed a significant correlation between the presence of the *JAK2* mutation and frequency of disease complications (secondary fibrosis, haemorrhage, and

thrombosis). In addition, patients with the *JAK2* mutation were more likely to be older and have received cytoreductive therapy, than those with normal *JAK2*. These findings have been substantiated in more recent studies, which provide evidence of a linear relationship between %V617F and haemoglobin concentration, spleen size, leukocyte count [273-277], and an inverse relationship regarding platelet count. This last point is in keeping with the low level of mutant alleles found in ET patients, whose clinical phenotype is dominated by thrombocytosis [278,279]. Additionally it has been proposed that patients positive for V617F have a higher risk of post-PV MF than those who are wild-type for the mutation, and that disease progression occurs more often in cases with a homozygous mutation burden [273,276]. Concerning MF, there are conflicting studies as to whether V617F level can predict progression to acute leukaemia [276,277]. However it is highly likely transforming MF cases with wild-type or low level V617F carry other mutations that dominate myeloproliferation.

The isolation of one mutation in a range of myeloid disorders was not anticipated, but as each disease sub-type shares many overlapping features, perhaps this is indicative of the underlying signalling pathways behind each disorder. The V617F mutation does not occur in non-haematological cancers [280], but it is present in myeloid malignancies other than classical *BCR-ABL* negative MPNs. The question of how one mutation can apparently give rise to such distinct but related diseases remain largely unexplained. One clear correlation however is the level of mutant JAK2 signalling. Here I described a statistically higher level of homozygosity in patients with PV (41%) and MF (67%) compared to patients with ET (17%). The observation that mitotic recombination at chromosome 9 is a rare event in ET was later investigated at the haematopoietic progenitor level [281]. When myeloid progenitors were cultured and genotyped for V617F, colonies carrying a homozygous *JAK2* mutation were only isolated from PV patients, not those with ET. All 17 PV patients were found to carry progenitor colonies with heterozygous, homozygous and normal *JAK2,* whereas progenitors from ET patients were only heterozygous V617F positive or normal. One explanation for this observation may be that PV patients may have a longer pre-diagnosis phase of their disease, allowing more time for homozygosity to occur. Alternatively, mitotic recombination around the *JAK2* locus may occur more frequently in patients with PV, perhaps as a result of an unknown mutation or genetic predisposition.

Dose of V617F has been linked to the disease severity in mouse models, with a low level of mutation producing an ET-like phenotype, and a higher level resulting in erythrocytosis and fibrosis [282,283].

3.4.4 The *JAK2* gene is not amplified in V617F-positive MPN

Recently it was suggested that cells carrying *JAK2* V617F could increase their copies of mutant *JAK2* not only by mitotic recombination and acquired UPD, but also by frequent complete or partial duplication of the chromosome [255]. This mechanism associated with disease progression has been previously described, for example mutant *EGFR* [284], *MET* [285] and *RET* [286] are either duplicated, or the wild-type allele is lost, and all result in increased tumourigenic potential.

Here I investigated the copy number of the *JAK2* gene using MLPA, a technique used widely to provide sensitive quantification of DNA copy number [249]. To do this, DNA from homozygous V617F positive cases was hybridised to four probes targeting different regions of *JAK2,* plus a series of probes that detect every chromosome telomere provided by a commercial kit available from MRC Holland. My results showed no amplification of *JAK2* in 63 homozygous V617F positive cases, or 16 heterozygous cases, clearly suggesting amplification of the *JAK2* locus by tandem duplication is rare in V617F-positive MPN. This conclusion conflicts with results described previously by Hammond et al. (2007) [255]. Hammond et al. (2007) used a novel TaqMan assay to detect and measure average copies of *JAK2* V617F per cell in absolute terms, as opposed to a ratio of mutant to wild-type alleles. The V617F signal was compared to that generated by a series of plasmid standards, and the results were combined with an additional test that determined cell number. I chose to test these findings using an alternative methodology capable of detecting minor changes in copy number of DNA. The reasons for these discrepant findings are not immediately obvious. Perhaps tandem duplication of *JAK2* only occurs in specific cell types, such as differentiated erythrocytes and granulocytes. Hammond et al. (2007) examined granulocyte DNA, whereas only total leukocyte DNA was available for this study. However as previous investigations into the variability of mutation burden between different cell subsets did not reveal any major differences, this point may not be

significant. Moreover, the main clone in PV is predominantly granulocytic and/or erythrocytic, especially in cases with high level V617F burden, so if there was any amplification of *JAK2* it should have been detected. Hammond et al. (2007) also suggests their results may be attributed to in-frame internal tandem duplication of the V617F region, analogous to mutations observed in *FLT3*. To test this I determined whether amplification of exon 14, the location of V617, could be selectively amplified in homozygous V617F-positive cases. Using cDNA-specific primers to exons 13 and 15, I screened 15 homozygous V617F-positive cases by RT-PCR, and did not find any evidence of aberrant sized bands (data not shown). It is likely therefore that the discrepancy between the two studies is purely technical. The ability to accurately detect and quantify V617F is clearly important from a diagnostic perspective and this might be facilitated in the future by the availability of quantitative standards as well as consensus on the best techniques to use.

3.4.5 Evidence of oligoclonality in MPN

Mutations resulting in hyperactive signalling are generally mutually exclusive because of functional redundancy and so the finding of cases that tested positive for *JAK2* V617F and *KIT* D816V, *FLT3* ITD or *BCR-ABL* was a surprise. The first patient I found had symptoms of both SM and thrombocytosis, and was found to be positive for *KIT* D816V and *JAK2* V617F. My results showed the mutations appear to have arisen in separate myeloid progenitor clones. Whether the clonal populations share a preceding unifying mutation remains undetermined. This result contrasts with data published recently by Sotlar et al. [258] who reported the finding of both *KIT* D816V and *JAK2* V617F mutations in a small minority of patients with SM with myelofibrosis. Using laser microdissection to separate neoplastic mast cells from $CD15^{+}$ myeloid cells, both mutations were apparently isolated in both cell fractions. The authors argued both mutations existed in the same cell lineage, but concluded investigations at a single cell level were necessary to define this observation.

The data described here contributed to a recent report by Beer et al. (2009) [287] where four other cases carrying two mutations in different signalling pathways were

investigated in detail at the progenitor cell level. The results showed the existence of two separate clonal expansions in all cases studied; *JAK2* V617F-positive clones were found to be distinct from clones carrying other tyrosine kinase mutations; *MPL* W515L, and *JAK2* exon 12. However additional cytogenetic abnormalities, del(20q), trisomy 9, and trisomy 8, tended to occur in the same clone as the activated kinase. X-chromosome inactivation patterns of 2 out of 3 female patients carrying both *MPL* and *JAK2* mutations showed the clones had arisen from independently related progenitors, arguing against the presence of an unknown, preceding mutation, at least in these few cases. The clonality of cases carrying del(20q) plus *JAK2* V617F were also investigated using colony assays by Schaub et al. (2008) [288]. Interestingly, there was no particular pattern for the occurrence of either clonal marker; each existed either in a mutually exclusive pattern, or within the same clonal population, and did not follow a strict temporal order of occurrence. It remains difficult to determine, however, at what point of development the mutations arose, and whether the *JAK2* point mutation precedes other mutations.

The next case was diagnosed with *BCR-ABL* positive CML, who after treatment with imatinib, developed myelofibrosis later found to be caused by *JAK2* V617F. Samples taken over a period of six years showed V617F was present at a constant level but symptoms of an MPN only developed after the drastic reduction of *BCR-ABL* positive cells. It was concluded that the *JAK2* V617F mutation preceded *BCR-ABL*, however, as this case was not investigated at a single cell level this remains unclear. Certainly there was a substantial clone present that was positive for V617F but negative for *BCR-ABL*.

Isolation of *BCR-ABL* and *JAK2* V617F has been previously been recorded in cases with CML with underlying myeloproliferative features [289-292], however the incidence of both mutations occurring in a single case is very rare [262]. A CML patient with a history of PV was subsequently studied at the single haematopoietic progenitor level by Bocchia et al. (2007) [293]. The results showed *BCR-ABL* transcript and *JAK2* V617F allele were isolated together in the majority of erythroid and myeloid colonies cultured, at the time of diagnosis of CML. There were also a small number of colonies that carried only V617F, but there were none that were singly positive for *BCR-ABL.* Whilst this is evidence from only one case, it does suggest that *JAK2* V617F and *BCR-ABL* can co-exist in an early myeloid-

erythroid committed progenitor, and that the two oncogenic tyrosine kinases may provide cooperative proliferative advantages to a neoplastic clone.

Study into these cases with biclonal expansions has provided some interesting information into the phenotypic heterogeneity of MPN. In most cases that have been analysed it appears that V617F and the other mutation are in different clones, although co-existence in the same clone is possible. The finding that mutations are in different clones indicates that the patient literally has two diseases, e.g. ET and CML, ET and SM. Since these are such are such rare disorders the chance that they both arose completely independently is remote. Although one possibility might be a pre-existing clonal expansion caused by an unknown precursor mutation, the X-chromosome inactivation analysis by Campbell et al. [265] indicates that this is not always the case. Another possibility is an inherited predisposition to the acquisition of mutations, something that is explored further in Chapter 6.

4 Predicting and monitoring the responses of MPN patients to treatment

4.1 Introduction

The most important reason for understanding the pathogenic origin(s) of MPN is to develop effective treatments that may alleviate symptoms and induce a sustained cure. MPNs are disorders that once initiated in a haematopoietic stem cell, cause uncontrolled proliferation of one or more myeloid lineages, forming a clone of mature neoplastic cells. MPNs often lead to thrombohaemorrhagic complications and in many cases to progressive marrow myelofibrosis, anaemia, splenomegaly and risk of transformation to acute leukaemia.

Stem cell transplantation remains the only curative treatment [294], but it is associated with substantial morbidity and is limited to younger patients with suitable donors. The main treatment strategies for MPN are to try to redress the phenotypic characteristics of the disorder, i.e. cytoreductive therapy and phlebotomy to reduce the number of abnormal cells and other treatments to ameliorate symptoms. The effectiveness of different treatment strategies are determined by measurement of haematological parameters such as the haematocrit, platelet count, phlebotomy requirement and spleen size. Response to treatment may also be measured at the cytogenetic level if the aberrant clone carries a karyotypic abnormality which can be used as a marker. However as clonal cytogenetic abnormalities occur at different frequencies ranging from 3% to 40% depending on the subtype of MPN [92,295], this approach is not widely applicable.

By contrast the situation in CML allows for both cytogenetic and molecular monitoring of patients treated with highly effective targeted therapy. The Ph chromosome not only proved to be a useful clonal marker, but the oncogenic fusion protein BCR-ABL created by the translocation is now the main target of therapy with imatinib [296]. The efficacy of imatinib may be measured accurately cytogenetically by reduction in the number of Ph positive chromosomes, and also by monitoring the level of *BCR-ABL* transcripts by real-time PCR [297,298]. Thus, the isolation of *BCR-ABL* in CML serves as the paradigm for successful targeted therapy in MPN, a pathognomonic marker of disease that is

effectively inhibited by imatinib. It was subsequently discovered that imatinib is also active against other tyrosine kinases activated by chromosomal translocation, namely PDGFRα [102] and PDGFRβ [299]. Thus, identifying patients that carry PDGFRα and PDGFRβ oncogenic fusion proteins is important for their optimal management. On the other hand, the identification of imatinib sensitive patients may help identify new pathogenetic abnormalities, as was the case for *FIP1L1-PDGFRA* [102].

Aberrant activation of tyrosine kinases accounts for a significant proportion of causative pathogenic lesions in MPN, but the molecular basis of disease for many other patients remains obscure. The identification of *JAK2* V617F in nearly all cases with PV, and approximately half of those with ET and MF not only furthered understanding of the pathogenetic mechanisms behind the disease, but also has the potential as a useful molecular marker of clonality and a target for therapy.

4.1.1 Aims

The aims of this part of my study were to develop and evaluate procedures to predict or assess the response of MPN patients to treatment. Specifically I sought to:

1. Identify MPN cases that were sensitive to imatinib using *in vitro* assays as a route to help identify novel imatinib responsive abnormalities.
2. Use *JAK2* V617F as a molecular marker of clonal proliferation to measure the response of PV patients undergoing treatment with imatinib and rIFNα.

4.2 Materials and methods

4.2.1 The *in vitro* imatinib sensitivity assays

The colony and liquid culture assays were established initially by Dr B. Schultheis (Hammersmith Hospital, London) and Dr. A. Chase (WRGL, Salisbury). Cells from *BCR-ABL* positive, untreated CML patients were used as positive controls and found to be significantly inhibited by imatinib in both semi-solid medium and liquid culture. By contrast, the cell growth and survival of all normal controls tested were affected by imatinib to a much lesser degree. A 'positive response' was classified as that which mirrors the pattern of samples with an established sensitivity to imatinib, in this case *BCR-ABL* positive CML. A measure of 'responsiveness' called the 'index of response' was devised for the colony assay, based on data recorded from positive and negative controls. A positive or negative response on liquid culture was determined by visual inspection only. Any deviation from the pattern of a *BCR-ABL* positive CML sample is regarded as unresponsive to imatinib, and was often similar to the results from normal controls.

Imatinib was obtained from Novartis (Basel, Switzerland) and was used at concentrations ranging from 1μM to 10μM. It has been previously shown 1μM imatinib selectively induces apoptosis in fresh *BCR-ABL* positive CML cells with little effect on normal haematopoiesis [220]. The concentration of imatinib required to inhibit cells transformed by BCR-ABL by 50% (IC_{50}) was 582nM [300]. Imatinib was also shown to inhibit signalling at a lower concentration for PDGFRα and PDGFRβ fusion proteins; the IC_{50} for ETV6-PDGFRβ was 15nM [301] and FIP1L1-PDGFRα was 3.2nM [102].

4.2.1.1 The *in vitro* imatinib colony assay

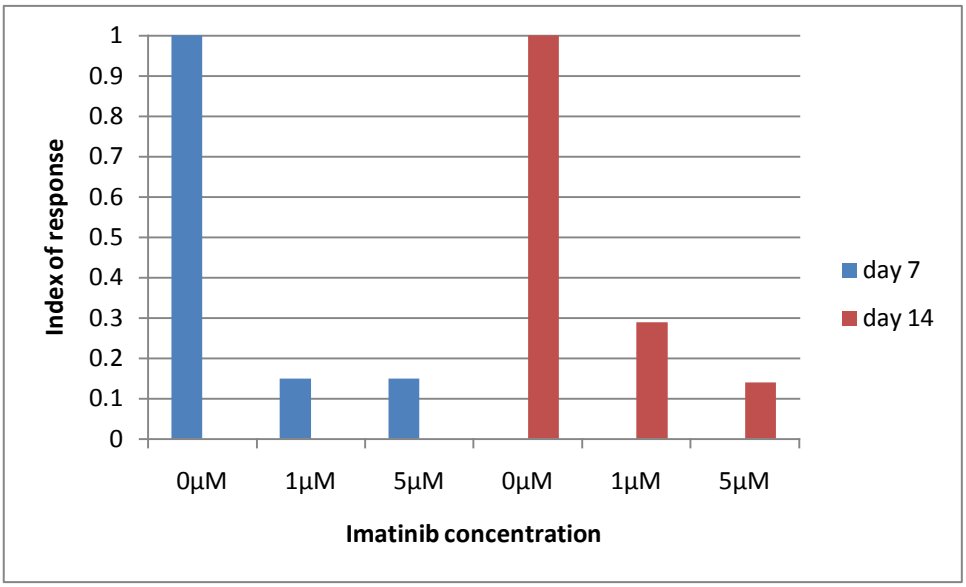
Colony assays were performed in triplicate using fresh MNCs isolated from PB or BM cultured in semi-solid media, as described in Chapter 2. Additionally, imatinib was added prior to plating, so that the cells were exposed to a final concentration of 0μM, 1μM and 5μM inhibitor, respectively. The dishes were incubated at 37°C, 5% CO_2, and colonies

were scored at 7 and 14 days incubation. Individual colonies were counted if the total cells per colony exceeded approximately 50 cells at day 7 and 100 cells at day 14.

The measure of inhibition by imatinib, or 'index of response,' was calculated as the mean response at 1 µM and 5µM imatinib at days 7 and 14 compared to the untreated control. The numbers of colonies for each level of imatinib exposure were averaged and divided by the average of the untreated control. An example of the calculations used to formulate the index of response is included in Appendix V. In this manner, the varying levels of inhibition by imatinib between different patients could be compared.

Figure 4.1A shows the colony assay results from a case carrying *ETV6-PDGFRB* who showed a positive response to imatinib. Myeloid colony formation was inhibited markedly by exposure to imatinib, as shown by the colony numbers from the imatinib treated 1µM and 5µM dishes expressed relative to the untreated colony counts. The index of response for this case was 0.18. This contrasts to the weaker effect imatinib has on cells from a healthy normal control, shown in Figure 4.1B where the index of response was higher at 0.45. Based on experiments performed by Dr. A. Chase using a series of normal controls and *BCR-ABL* positive CML patients [302], an index of response of 0.2 or below determines a positive response to inhibition with imatinib.

A



B

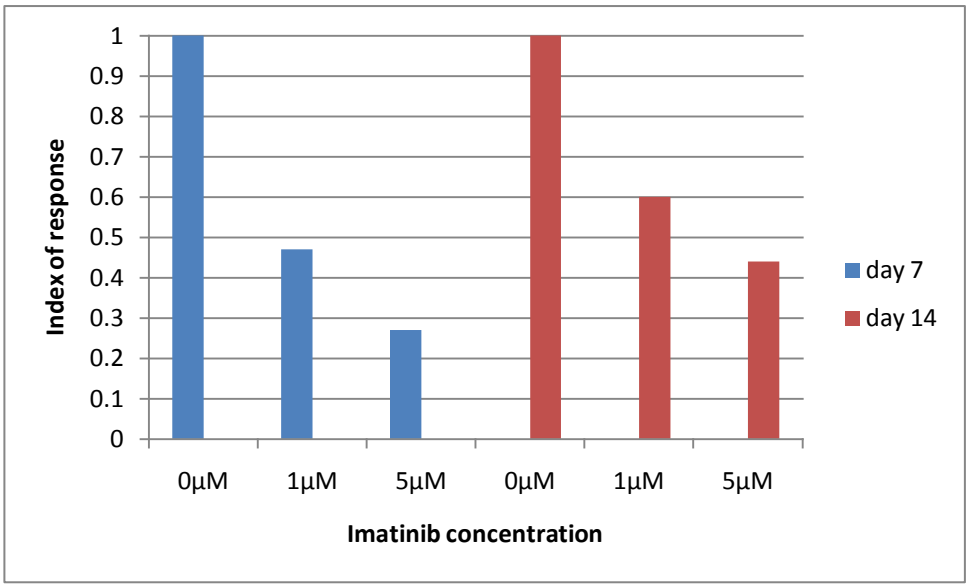


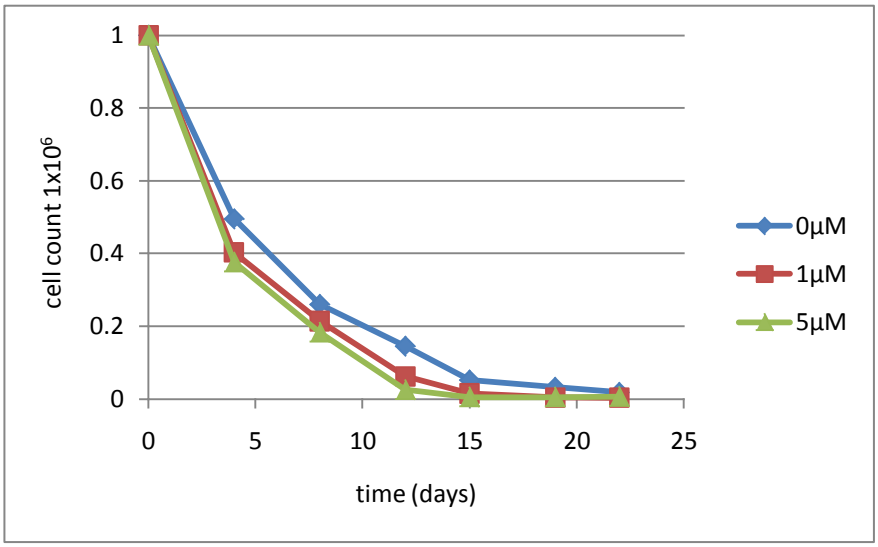
Figure 4-1. The *in vitro* imatinib sensitivity colony assay. Example A shows an example of a positive response to imatinib from a case with *ETV6-PDGFRB* (index of response; 0.18), and example B shows a negative response to imatinib from a normal control (index of response; 0.45). Myeloid mononuclear cells were cultured in semi-solid medium and exposed to two concentrations of imatinib to measure the degree of inhibition on colony formation. Colonies were scored on days 7 and 14, and colony numbers from the imatinib-treated dishes were expressed relative to the untreated colony counts.

4.2.2 The in vitro imatinib liquid culture assay

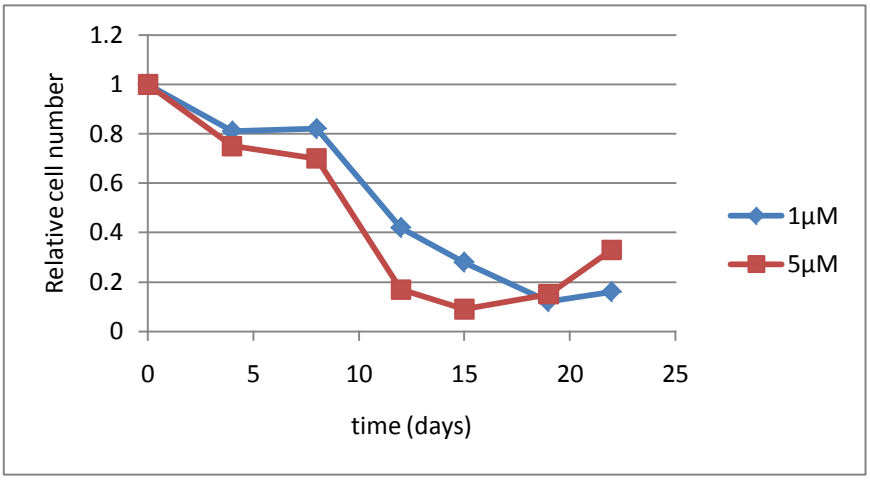
To examine the effect of exposure to imatinib on the survival and proliferation of mature MPN cells, either granulocytes or MNCs were cultured in supplemented RPMI 1640 with 0μM, 1μM and 5μM imatinib, for two weeks. The liquid culture assay was set up using a starting concentration of $1x10^6$ to $5x10^6$cells/ml in 24 well plates, in duplicate, and surrounding empty wells filled with sterile water to prevent evaporation. The plates were incubated at 37°C, 5% CO_2 over a period of 2 to 3 weeks, and cells in each well were counted twice weekly, using trypan blue staining to discriminate alive from dead cells. Medium from each well was changed weekly.

Similar to the *in vitro* colony assay, the liquid culture assay was first tested on cells from a series of normal controls and *BCR-ABL* positive CML patients to determine parameters for measuring the inhibition by imatinib (data provided by Dr A. Chase). An example of cell counts and the calculations from a liquid culture assay performed on granulocytes from an imatinib-sensitive MPN case are described in Appendix V. A positive response is shown by a reduction in growth and survival in the imatinib treated cultures compared to the untreated cultures (Figure 4.2). Although the judgement as to whether the treated cultures differed significantly from controls was subjective, the results of these assays clearly correlated with patient genotype as detailed below.

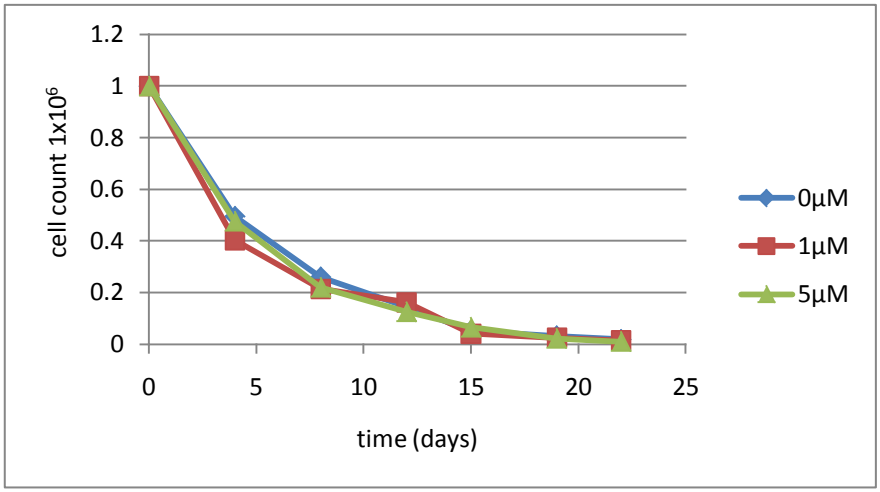
A(i)


cell count 1x10^6
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
time (days)
0µM
1µM
5µM

A(ii)


Relative cell number
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
time (days)
1µM
5µM

B(i)


cell count 1x10^6
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
time (days)
0µM
1µM
5µM

B(ii)

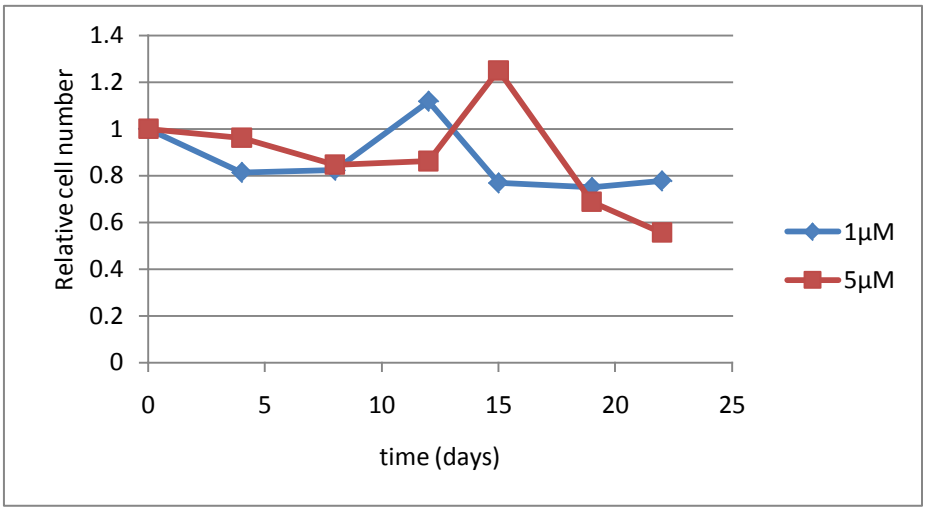


Figure 4-2. The *in vitro* liquid culture imatinib sensitivity assay. Liquid culture results for an imatinib-responsive *BCR-ABL* positive patient (A) and an unresponsive case (B), expressed as total cell number (i) and relative cell number (ii). The degree of sensitivity to imatinib was more easily interpreted by expressing the counts from the treated cultures relative to the untreated cultures for each time point.

4.2.3 Measurement of *JAK2* V617F in PV cases undergoing treatment with imatinib and rIFNα

DNA was extracted from total leukocytes, granulocytes separated from PB samples or from pre-treatment, unstained BM slides. *JAK2* V617F was initially detected using AS-PCR PCR. The proportion of mutant allele (%V617F) was quantified using pyrosequencing using primers JAK2_pyro_V617F_F and JAK2_pyro_V617F_R and sequenced with primer JAK2_ pyro_V617F_seq. Comparison of the %V617F between control and treated patients was performed by the Mann-Whitney test.

4.3 Results

4.3.1 Identification of candidate imatinib-sensitive MPN patients *in vitro*

As outlined above, a minority of MPN patients carry oncogenic tyrosine kinase fusion proteins that can be effectively targeted by imatinib therapy *in vivo* [102,220,296,301]. Although most of these cases have rearrangements of *PDGFRA* or *PDGFRB*, occasional imatinib sensitive cases have been reported in the absence of any known molecular or cytogenetic marker. Here I investigated whether cells isolated from the PB or BM of 200 MPN patients could be cultured *in vitro* with imatinib to determine whether they are likely to be responsive to this inhibitor. Further investigation by other members of the group was then focused on finding acquired genetic abnormalities in imatinib-sensitive kinases.

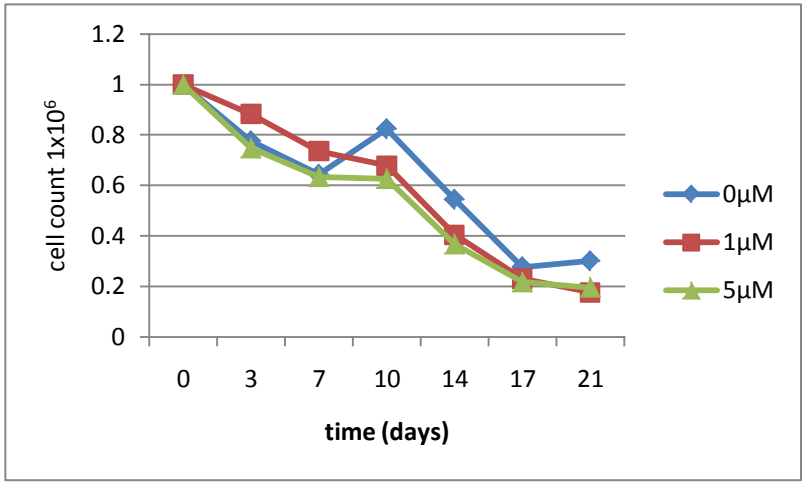
4.3.1.1 Results of the in vitro imatinib sensitivity assays on MPN cases

The colony assay was performed on 157 MPN samples, of which 131 gave an evaluable response; 26 showed a positive response to imatinib as defined above, 17 were borderline responders and 88 did not respond to imatinib. The imatinib sensitivity status of the remaining 26 samples was not determined, either because the MNCs failed to divide in culture, or the colonies were uncountable due to over-proliferation.

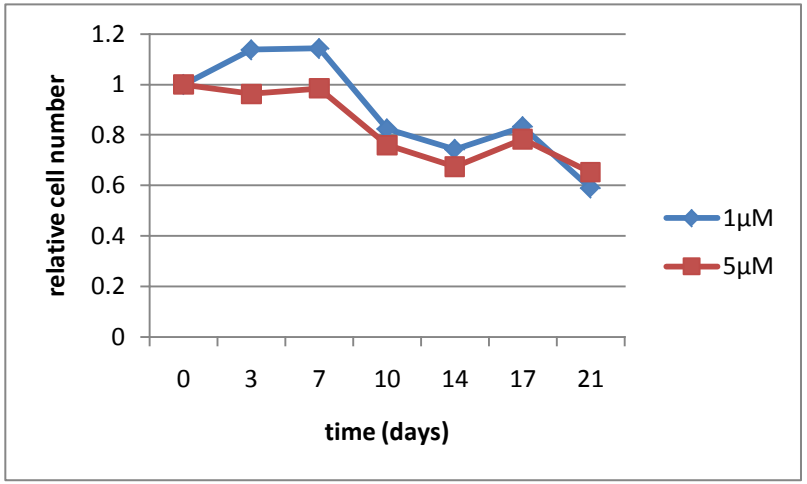
Of the 171 MPN samples subject to liquid culture assay, 145 gave an evaluable result, with 24 showing marked inhibition to imatinib, 16 samples were borderline responders and 105 did not show any significant inhibition to the inhibitor. Twenty six samples were not successfully evaluated by liquid culture as all cells died very early in culture.

Figure 4.3 shows an example of one sample, E111, which displayed borderline inhibition to imatinib on liquid culture, and a tentative positive response on colony assay, with an index of 0.182. This case proved negative for all molecular tests.

A



B



C

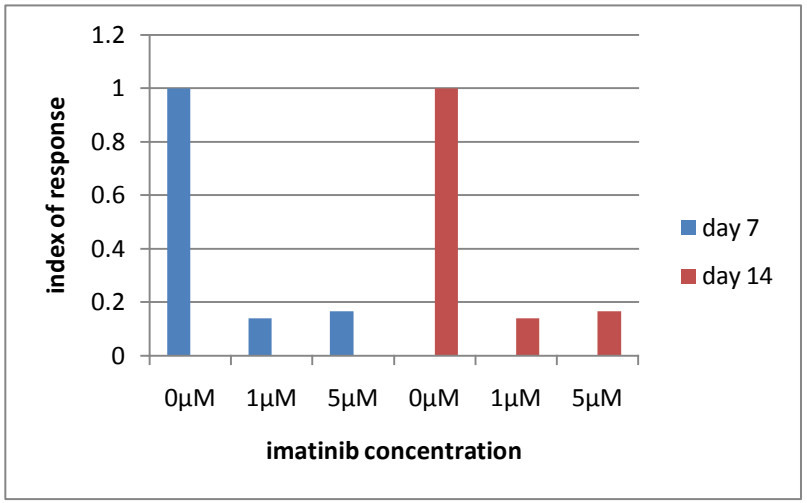


Figure 4-3. Case E111 showed a borderline response to imatinib exposure in liquid culture and colony assay. For the liquid culture assay, line graphs depicting total cell number (A) and relative cell number (B) some evidence of inhibition. In the colony assay (C) the cells were inhibited by imatinib, but this effect was marginal (index = 0.182).

There were 15 samples that did not give a result on either assay. In total 185 out of 200 MPN samples tested yielded a result for one or both *in vitro* assays. Six cases responded to imatinib in both assays, whereas a significant number of samples (n=38) were inhibited by exposure to imatinib in one assay, but not the other. This is shown in more detail in Table 4.1, where the results for each sample in liquid culture and corresponding colony assay are listed as positive, borderline, negative, not done or not informative. A full table of results for each case alongside any molecular abnormality identified is listed in Appendix VI.

		colony assay					
		positive	borderline	negative	not done	not informative	total
liquid culture assay	positive	6	3	12	2	1	**24**
	borderline	4	1	1	5	5	**16**
	negative	7	6	51	30	11	**105**
	not done	4	3	13	0	9	**29**
	not informative	5	4	11	6	0	**26**
	total	**26**	**17**	**88**	**43**	**26**	**200**

Table 4-1. A summary of results from the *in vitro* liquid culture and colony assay.

4.3.2 Molecular results of cases subject to imatinib sensitivity assay

All samples tested negative for *BCR-ABL* by RT-PCR. Other fusions were tested for as indicated by karyotypic abnormalities. All samples were also screened for *FIP1L1-PDGFRA* by RT-PCR, and 15 were shown to harbour the fusion gene. Out of the 200 MPN samples set up for either *in vitro* assay, three had *ETV6-PDGFRB* translocations, two had rare *PDGFRA* translocations (*BCR-PDGFRA* and *KIF5B-PDGFRA*), and one sample had a *BCR-FGFR1* translocation. 146 out of the 200 samples were screened for *JAK2* V617F by allele specific PCR and pyrosequencing and 11 tested positive. Of 18 samples with a confirmed or suspected diagnosis of SM, 9 were found to be positive for the *KIT* D816V mutation. The main findings are summarised in Table 4.2.

		Number of positive responders/number tested	
Tyrosine kinase mutation	Number with tyrosine kinase mutation (out of 200 unless stated)	Colony assay	Liquid culture assay
PDGFRB translocations	2	2/2	1/2
FIP1L1-PDGFRA	15/199	3/14	11/12
PDGFRA translocations	2	0/2	0/2
BCR-FGFR1	1	0/1	0/1
KIT D816V	9	0/8	1/6
JAK2 V617F	11/146	4/7	0/10
Uncharacterised mutation	161	16/118 (13.6%)	11/133 (8.3%)

Table 4-2. Results of *in vitro* imatinib sensitivity assays in MPN patients. One patient carried *KIT* D816V plus *JAK2* V617F.

Figure 4.4 shows results of the colony assay for all cases investigated in this study. Individual indeces of response are plotted vertically, and cases are grouped according to molecular abnormality status. Colony growth of healthy normal controls was not inhibited by imatinib, and all indices lie above 0.2. Conversely, the indeces for the *BCR-ABL* positive controls all fall below 0.2. MPN cases carrying *PDGFRB* translocations displayed very low indeces, however cases with *FIP1L1-PDGFRA* fusions showed a more varied level of imatinib sensitivity, with the majority proving unresponsive to the drug by this method.

MPN cases with *KIT* D816V were not inhibited by imatinib in this assay, a finding compatible with the known resistance of this mutation to imatinib. *JAK2* V617F positive cases displayed varied imatinib sensitivity, ranging from cases with acute sensitivity, to others that were unresponsive. Finally, uncharacterised MPN cases displayed a range of sensitivities, with some being classed as positive responders.

The remaining samples proved negative for all PCR based tests, and 16/118 (13.6%) of these showed a response by colony assay and 11/133 (8.3%) were positive by liquid culture. These figures include three cases that significantly responded to imatinib on both assays. Overall, 24/161 (14.9%) of samples without known mutations responded to imatinib. If all borderline responders were included, this value rises to 29.8% (48/161) patients displaying some degree of imatinib sensitivity.

It was not possible to actually treat any of these positive responders with imatinib *in vivo*, so whether these cases harbour true imatinib-sensitive tyrosine kinase mutations remains to be determined. However, it seems likely that at least some of them may harbour mutations of imatinib sensitive tyrosine kinases.

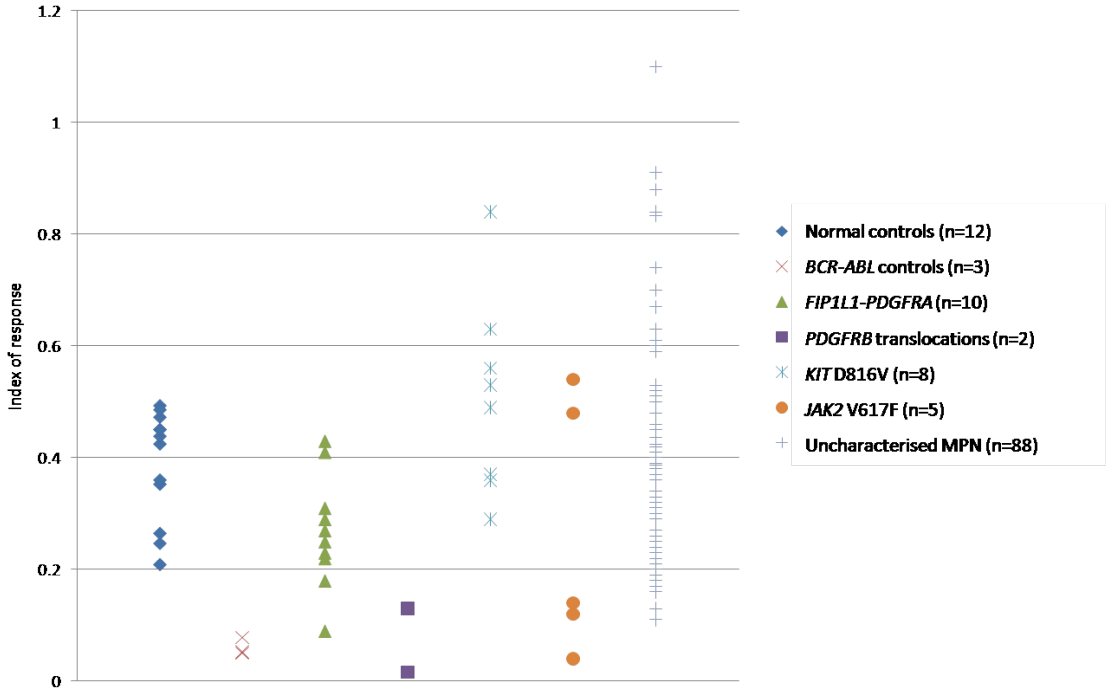


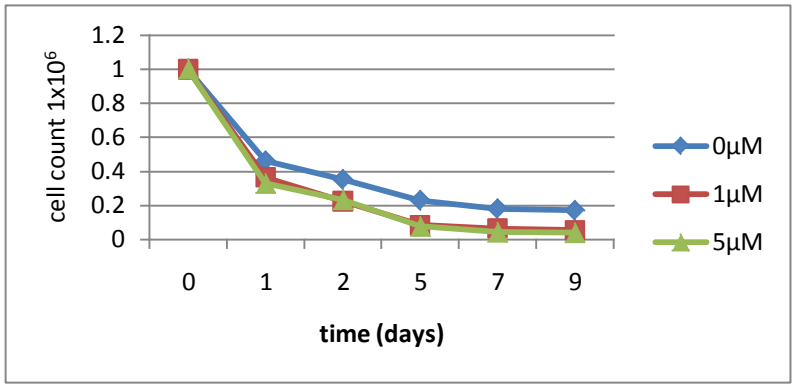
Figure 4-4. *In vitro* imatinib sensitivity colony assay results (indeces) plotted for MPN cases for the controls and each mutation category.

4.3.3 Deregulated tyrosine kinases may be identified in MPN patients that display sensitivity to imatinib *in vitro*

Of the 185 cases who gave an evaluable response in either or both assays, a total of 44 cases displayed sensitivity to imatinib in one or more assay. Of the 44 cases, 20 were found to carry tyrosine kinase mutations, whereas 24 proved negative for all molecular tests. There were a total of 39 cases identified with a tyrosine kinase mutation, confirming signalling from some deregulated tyrosine kinases is not inhibited by imatinib.

Out of 15 *FIP1L1-PDGFRA* positive cases studied, 14 were examined for imatinib sensitivity by colony assay and only three displayed significant reductions in colony numbers. In contrast, out of the 12 *FIP1L1-PDGFRA* positive cases tested by liquid culture, 11 were positive (an example of the liquid culture on a *FIP1L1-PDGFRA* case that responded is shown in Figure 4.5). Imatinib sensitivity was therefore much more obvious for this fusion in the liquid culture set up with granulocytes compared to the colony assay set up with MNCs. The two assays primarily assess two different biological processes: the colony assay measures proliferation of progenitor cells whilst the liquid culture assay primarily assesses survival of non-proliferating cells. Two possible explanations for these discrepant results are therefore that *FIP1L1-PDGFRA* may activate survival rather than proliferation pathways, or that CFU-GM may not be part of the malignant clone. Further work by Dr Andy Chase revealed that only a minority of CFU-GM from *FIP1L1-PDGFRA* positive cases harbour the fusion gene. This data, along with my results, contributed to a study reporting that *FIP1L1-PDGFRA* is found predominantly in more advanced stage of differentiation compared to *BCR-ABL* which is present in both myeloid and lymphoid lineages, and the great majority of CD34+ cells as well as the great majority of CFU-GM [302].

A



B

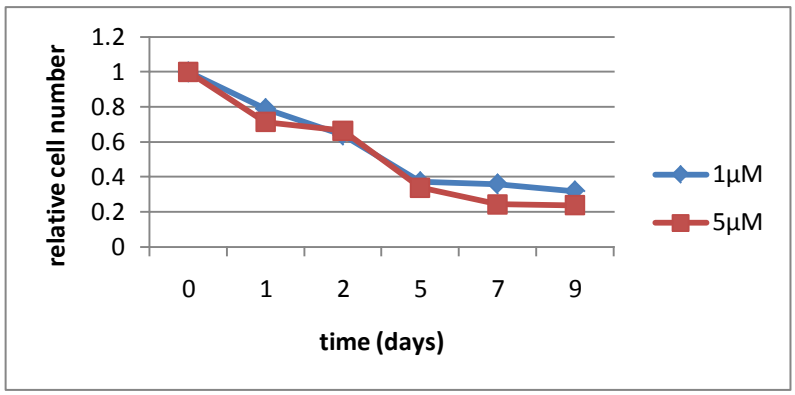


Figure 4-5. The liquid culture imatinib sensitivity assay results from a *FIP1L1-PDGFRA* positive case E107.The results are shown as total cell number (A) and relative cell number (B). Cell culture was stopped at day 9 after marked inhibition was observed.

Samples from MPN patients found to harbour rarer tyrosine kinase translocations were investigated using both *in vitro* assays where possible. Two out of three patients carrying *ETV6-PDGFRB* were significantly inhibited by exposure to imatinib in liquid culture. The third case exhibited borderline sensitivity, but it subsequently emerged that this patient was undergoing imatinib therapy at the time the PB sample was taken. One *ETV6-PDGFRB* positive case also underwent colony assay and yielded a clear positive result. A recently characterised *PDGFRA* translocation, *KIF5B-PDGFRA* [105], was marginally inhibited by imatinib on colony assay, and did not display any sensitivity in liquid culture, however this patient did respond to imatinib when treated. One patient carrying *BCR-PDGFRA* did not

show imatinib sensitivity in either assay, moreover, this case did not respond to imatinib when administered *in vivo,* despite literature reports stating *BCR-PDGFRA* is responsive to imatinib [104,303].

Of the nine *KIT* D816V positive SM samples, eight were set up for colony assay and six were set up in liquid culture. Only one case was significantly inhibited by imatinib in liquid culture, and all cases were unresponsive by colony assay. Overall, this is consistent with previously reported resistance of this mutation to imatinib.

Of the eleven MPN cases carrying *JAK2* V617F set up in either liquid culture or colony assay, 4/7 displayed a significant sensitivity to imatinib on colony assay, but no *JAK2* V617F positive samples (0/10) clearly responded to imatinib in liquid culture. Of note, all *JAK2* V617F positive samples that responded by colony assay were homozygous for the mutation, plus a further two homozygous cases were also classed as borderline responders in liquid culture. One MPN case carried both *KIT* D816V and *JAK2* V617F point mutations, but did not display any sensitivity to imatinib in either assay. The results for *JAK2* V617F positive cases are at first sight inconsistent with the fact that JAK2 is not responsive to imatinib, but it is possible that the colony growth from these cases included EPO-independent BFU-Es as a consequence of *JAK2* V617F rather than the expected CFU-GMs. Growth of BFU-Es is known to be inhibited by imatinib, probably as a consequence of inhibition of normal KIT.

Collectively these results indicate that the assays in their current form are far from perfect: they do not identify all genuine imatinib responders and several cases that most likely have imatinib resistant disease were identified as responders in one or both assays. Nevertheless, it is possible that some of the 24 cases with no known mutation that were responsive in one or both assays did genuinely have imatinib responsive disease. However due to the high cost of treatment none of these individuals actually received imatinib and so this remains speculative. Subsequent analysis of these cases by other members of the group have thus far failed to reveal any cryptic tyrosine kinase fusion genes or imatinib responsive point mutations in these cases and so the reasons for the *in vitro* responsiveness remains unclear.

Certainly it would be highly desirable to have a simple assay that reliably predicted imatinib responsive MPNs. In addition to the problems I observed with false positive and false negative results, the assays were often difficult to interpret and were very time consuming to perform. Potentially some other techniques could be explored, e.g. total phosphotyrosine levels have been used to predict response to imatinib therapy in CML patients [304], and measurement of expression levels of the tumour suppressor *WT1* have been used with some success to predict imatinib sensitivity in CML [305]. More broadly, downstream signalling components that are targeted by ABL, PDGFRα and PDGFRβ, could perhaps be monitored and used to identify cases with activated tyrosine kinases. An example could be phosphorylation of STAT5, which is known to be downstream of many activated tyrosine kinases [301,306] and can be rapidly assessed by flow cytometry [307]. However I did not develop any of these techniques or attempt to refine the culture assays any further as it became apparent that true imatinib responsive cases that were negative for known responsive fusions were in fact very rare. For example in the study that first identified *FIP1L1-PDGFRA*, Cools et al. analysed 16 imatinib responsive cases of which only 9 (56%) tested *FIP1L1-PDGFRA* positive. However in a more recent trial of imatinib in HES, Baccarani et al. (2007) found that some *FIP1L1-PDGFRA* negative cases did appear to respond haematologically to imatinib but the responses were transient and all relapsed within approximately one year, strongly suggesting that the responses were a consequence of non-specific myelosuppression. In previous studies these cases may well have been considered erroneously as true imatinib responders [308].

4.4 The utility of *JAK2* V617F as a molecular marker for predicting treatment response in PV

The seminal discovery of *JAK2* V617F furthered the understanding of the molecular basis of MPN, and its presence confirms the diagnosis of a clonal disorder instead of a reactive process. Here I describe one of the first studies to use V617F as a molecular marker of disease in PV as a means to gauge response to treatment. V617F is a very suitable candidate for this role, as nearly all PV cases are V617F positive. Furthermore, the level of V617F is also associated with more severe disease; specifically higher haematocrit, lower mean cell volume, higher white blood cell count, vascular complications and myelofibrosis [273,275]. Measurement of *JAK2* V617F levels is thus prognostically important throughout the clinical course of affected patients.

Traditionally, many PV patients receive phlebotomy to reduce red cell mass. Because patients treated only by venesection are more likely to suffer thrombosis or haemorrhage which is frequently fatal, myelosuppressive therapy is also administered. Drugs such as chlorambucil and radioactive phosphorus are rarely used nowadays due to the potential for promoting leukaemic transformation [309]. Hydroxyurea (HU) is currently the cytoreductive treatment of choice, because of its effectiveness, convenience and low cost, but a potential for leukaemia may still exist [310 311] especially in patients who develop post-polycythaemic myelofibrosis and who have been treated with other agents [312-314]. Despite conventional treatment with phlebotomy and HU, many patients can experience night sweats, pruritus, and develop iron deficiency [315]. Anagrelide and rIFNα may be prescribed in certain cases. It has been reported that rIFNα may induce long term remission in PV but at the cost of appreciable side effects [316-319]. More recently, experimental trials have indicated that the majority of PV patients treated with imatinib achieved complete or partial haematological responses. Imatinib has been shown to be effective in reducing phlebotomy requirements in PV and often results in a reduction of spleen size, but it has not been reported to lower elevated platelet counts [242,244,320].

Occasional reports have shown that rIFNα or imatinib may induce cytogenetic responses in some PV cases [316] however most individuals do not present with a chromosome abnormality [265,295,321], and thus it has not been generally possible to gauge the depth of

any treatment-related responses. Therefore, the isolation of *JAK2* V617F in the majority of PV cases, together with the use of relatively sensitive methods of detection, makes monitoring treatment responses at the molecular level a real possibility.

4.4.1.1 Study design

To undertake this study, we collaborated with Dr R. Silver, a clinician based in New York, US, who provided the samples from PV patients undergoing treatment with imatinib and rIFNα. I established the *JAK2* V617F status of each case, and samples from *JAK2* V617F-positive patients were then quantified before and during treatment by pyrosequencing.

In total 111 patients were studied, of whom 21 had undergone treatment in two sequential phase II single institution studies. Seven cases received rIFNα in the first study, where IFNα was administered as an initial dose ranging from 1MU three times weekly to 3MU daily. These patients had a median follow up of 60 months ranging from 13 to 132 months. Fourteen cases received imatinib in the second study, where the initial dose administered ranged from 400 to 800mg daily, and these patients had a median follow up of 17 months, ranging from 5 to 31 months. The 90 remaining patients comprised the control group and were either untreated or treated by phlebotomy only, HU and/or anagrelide.

Clinical responses were assessed by Dr R. Silver, who classified the haematological response as either complete haematological response (CR), partial haematological response (PR) or no haematological response (NR). The criteria used to determine response to treatment were based on that set by the Polycythemia Vera Study Group, and are listed in table 4.3.

(A)

	CR	PR
Phlebotomy	No requirement	No requirement
Haematocrit	Sustained at <45% for men and <42% for women	Sustained at <45% for men and <42% for women
Platelet count	≤ $600x10^9$/L	600 to $1000x10^9$/L

(B)

	CR	PR
Phlebotomy	No requirement within first 18 months of treatment	No requirement within first 18 months of treatment
Haematocrit	Sustained at ≤45% for men and ≤42% for women	Sustained at ≤45% for men and ≤42% for women
Platelet count	≤ $400x10^9$/L	> $400x10^9$/L
Spleen size	Absent spleen, if initially palpable	Spleen reduced to ≤50% original size, if initially palpable

Table 4-3. Criteria used to measure haematological response of PV patients undergoing treatment with (A) rIFNα and (B) imatinib.

4.4.1.2 Results

AS-PCR detected *JAK2* V617F in 82/90 (82%) of the control PV cases, and in all 21 cases undergoing treatment with imatinib and rIFNα indicating that neither therapy eradicated *JAK2* V617F. To quantitate the level of disease I then used a pyrosequencing assay, which measures the proportion of alleles that have the V617F mutation. A description of the clinical details and V617F measurement for each patient who received imatinib and rIFNα is shown in table 4.4.

(A) imatinib

Patient identifier	sex	Age at diagnosis (years)	Dose (mg/day)	F/U (months)	Response	%V617F on treatment	%V617F pre-treatment
1	M	26	800	5	NR	44	nd
2	F	60	700	5	PR	41	34
3	M	49	800	9	NR	89	71
4	F	67	500	9	PR	91	88
5	M	58	400	10	PR	72	nd
6	M	48	800	15	PR	57	40
7	M	31	800	17	PR	76	63
8	F	48	500	17	CR	8	25
9	F	28	800	19	PR	83	82
10	M	72	400	21	NR	72	nd
11	M	54	300	24	CR	21	44
12	M	43	400	25	PR	60	40
13	M	30	600	25	PR	19	nd
14	M	53	700	31	PR	30	nd

(B) rIFNα

Patient identifier	sex	Age at diagnosis (years)	Dose	F/U (months)	Response	%V617F on treatment	%V617F pre-treatment
15	F	32	3MU three times/week	13	CR	19	nd
16	F	39	3MU/5 days/week	45	CR	39	nd
17	M	53	3MU/day	50	CR	29	nd
18	F	48	4MU/alternate days	60	PR	87	nd
19	M	42	2MU three times/week	60	CR	27	nd
20	M	52	4.25MU three times/week	108	CR	22	nd
21	M	39	3.5MU/day	132	CR	25	nd

Table 4-4. Summary of patient characteristics and results for cases undergoing therapy with (A) imatinib and (B) rIFNα. Abbreviations: nd, not determined; NR, no response; PR, partial response; CR, complete response; MU, million units; F/U, follow up (equals time of sampling) after starting treatment with imatinib or rIFNα.

As shown in Figure 4.6, the results showed that the median percentage of mutated *JAK2* alleles (%V617F) did not differ significantly between the imatinib treated cases (median 59%, range 8-91) and the V617F positive cases from the control group (median 53%, range 5-100). The %V617F was lower in the rIFNα treated cases (median 27%, range 19-87) compared to controls (p=0.03). This apparent difference may simply reflect the high proportion of cases in this selected group who had achieved CR, and not necessarily any preferential inhibitory effect of rIFNα over imatinib.

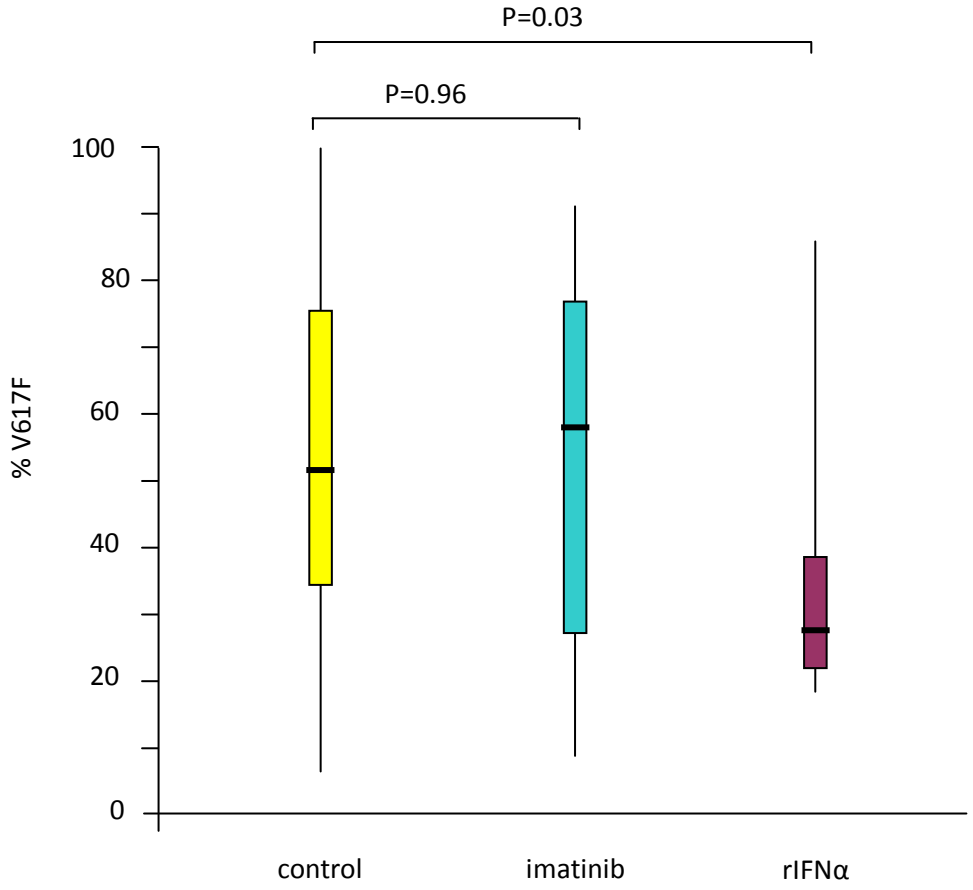


Figure 4-6. Levels of *JAK2* V617F in treated and control PV cases. (A) Comparison of %V617F in control (n=82), mutation-positive PV cases who were not treated with imatinib or rIFNα and patients who were treated with imatinib (n=14) or rIFNα (n=7). Vertical lines indicate the range of results, open boxes indicate the interquartile range and thick horizontal lines indicate median values.

The results from molecular analysis on the imatinib treated patients revealed the median %V617F for individuals with NR, PR and CR were 72% (44-89; n=3), 60% (30-91; n=9) and 15% (8 and 21; n=2), respectively. In the rIFNα treated group the single case with a PR had 87% V617F compared to a median of 26% (19-39) for the six cases in CR. Individuals who achieved CR on imatinib or rIFNα (n=8) had a lower %V617F (median 24%, range 20-29) than those patients who did not achieve CR (n=13, median=72, range 19-91) and also the control group (P=0.001 and P=0.0007, respectively) (Figure 4.7).

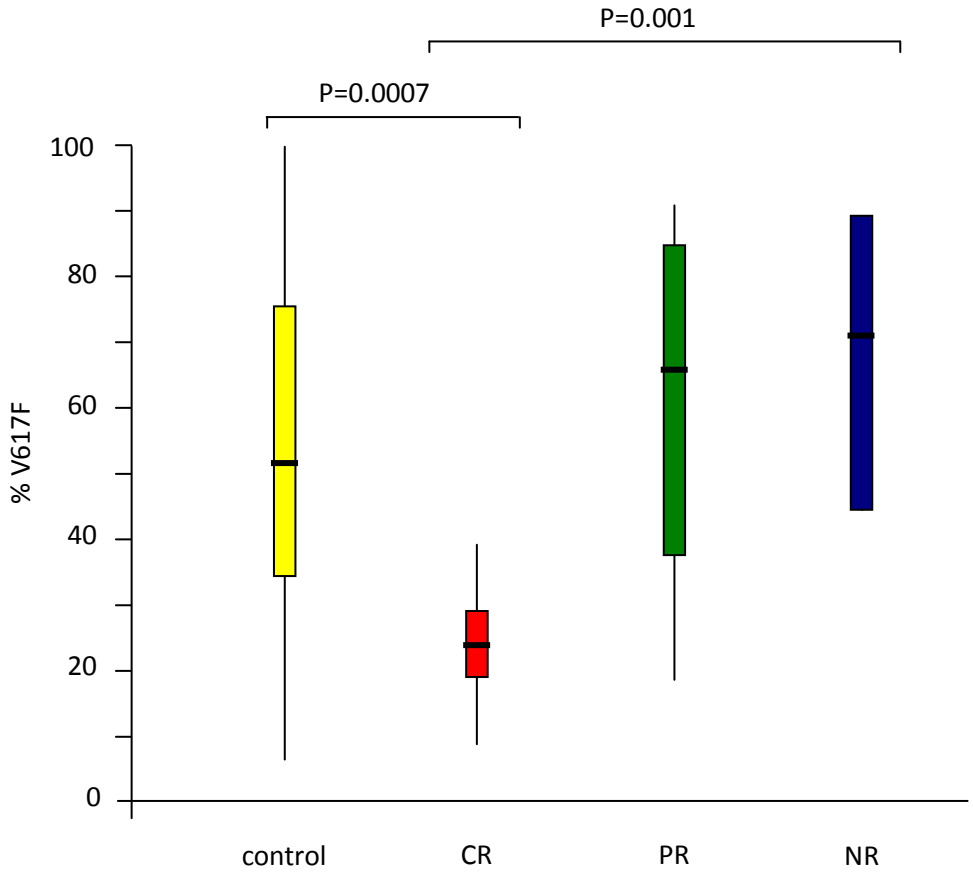


Figure 4-7.Comparison of %V617F levels in control PV cases (n=82) and patients who showed CR (n=8), PR (n=10) or NR (n=3) following therapy with imatinib or rIFNα.

To determine more precisely the magnitude of any change as a consequence of therapy, we compared %V617F levels in pre- and post-treatment samples for 9 of the imatinib treated cases. Pre-treatment samples (stored bone marrow slides) were obtained retrospectively and not available for the other cases. Of these nine cases, the seven who showed NR or PR on imatinib showed a marginal increase (median 1.2 fold, range 1.0-1.5) in the percentage of V617F alleles on treatment. In contrast, the two patients who achieved CR showed a two to three fold reduction in %V617F on treatment (Figure 4.8).

Subsequent to the publication of this study we identified one patient who achieved CR on rIFNα for whom a pre-treatment sample was available. In this case there was also a fifteen-fold reduction (from 61% to 4.1%) in %V617F levels upon treatment.

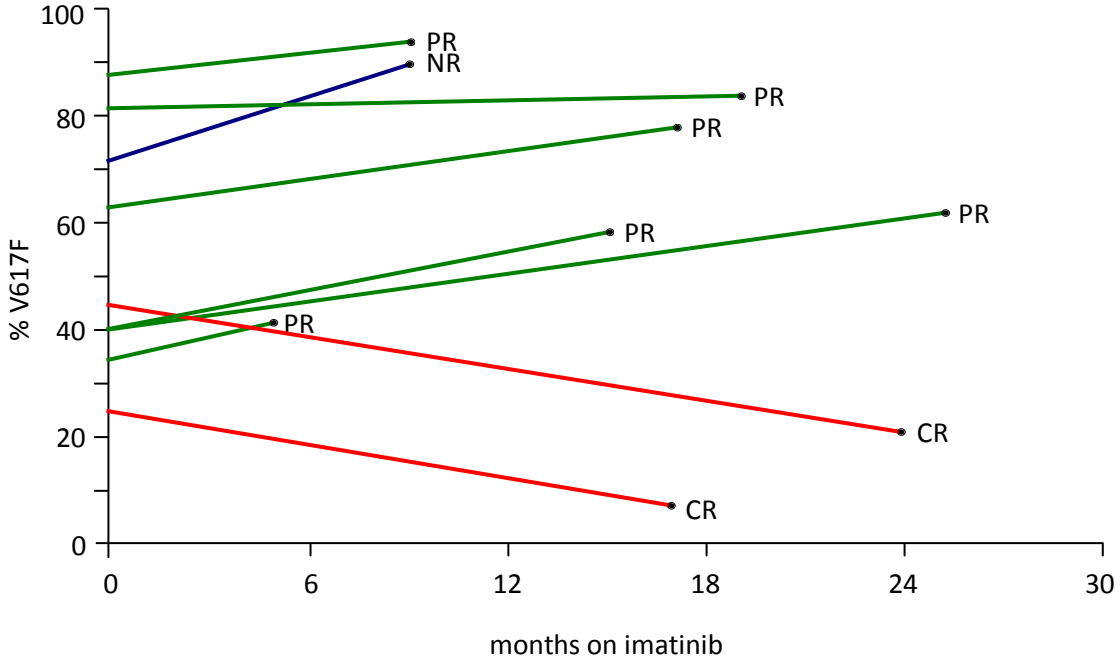


Figure 4-8.Changes in %V617F on imatinib therapy. %V617F was measured before starting imatinib and at various times whilst on therapy for 7 patients who showed NR (blue line) or a PR (green line), and two cases who showed a CR (red line).

To confirm that the changes observed in this study were not due to differences in %V617F between PB and BM, I compared the %V617F levels in contemporaneous PB and BM

specimens taken from 11 control PV patients. Very similar levels V617F mutant alleles were observed in blood and marrow in all cases, with an overall correlation coefficient of 0.97 (Figure 4.9).

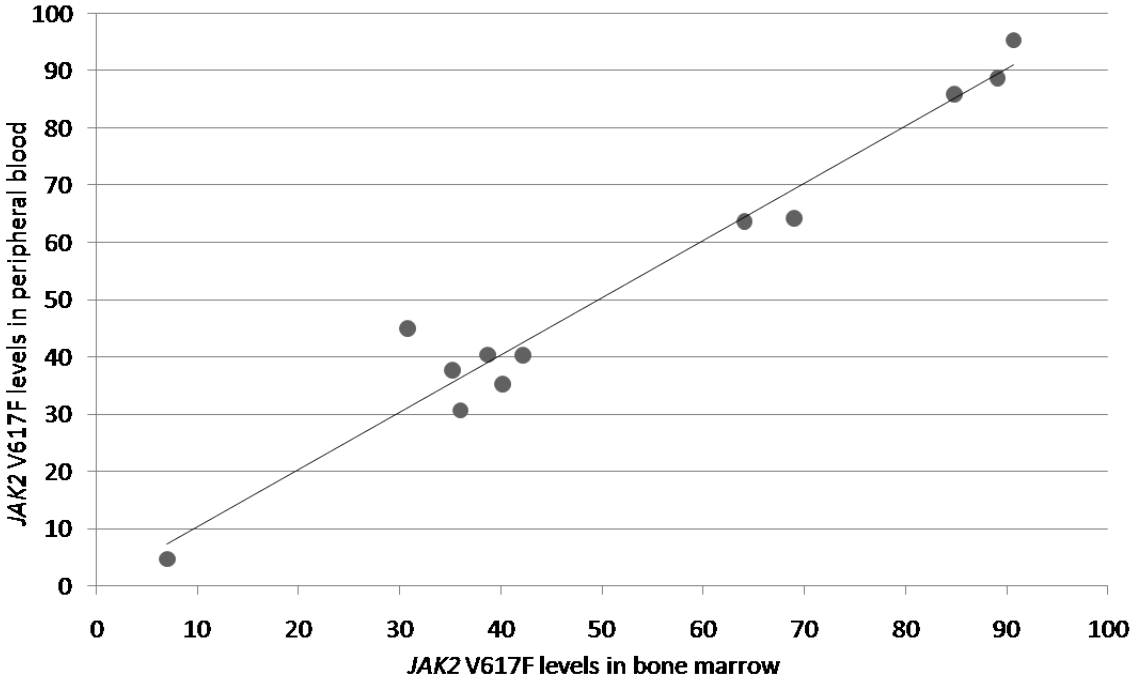


Figure 4-9.Comparison of *JAK2* V617F level in PB and BM from 11 control PV cases.

4.5 Discussion

4.5.1 *JAK2* V617F is a useful molecular marker for monitoring PV patients treated with imatinib and rIFNα

The discovery of *JAK2* V617F prompted a revision of the World Health Organisation classification of Hematopoietic and Lymphoid Neoplasms (2008) [22] which now quotes V617F as a major diagnostic marker of myeloproliferative disease. Although the precise pathogenetic contribution of the mutation remains unclear, V617F is an attractive candidate with which to monitor minimal residual disease and evaluate treatment efficacy. Here I described the first attempts at using V617F to gauge the depth of response of PV patients treated with imatinib and rIFNα. Both these therapies have been reported to induce remission in PV patients, but the responses have not previously been investigated at the molecular level. I found all patients undergoing imatinib or rIFNα therapy remained strongly positive for *JAK2* V617F, although there was a significant reduction in the median percentage of mutant alleles that correlated with haematological response. In addition, individuals who achieved complete haematological remission had lower levels of V617F than those who did not. Pre-treatment samples were available for nine imatinib-treated cases, and of these seven with no or partial haematological responses showed a marginal increase in the percentage of V617F alleles on treatment (median 1.2 fold, range 1.0 to 1.5), whereas the two patients who achieved complete haematological remission showed a 2 to 3 fold reduction. These results indicate that although PV patients may benefit from therapy with rIFNα and imatinib, patient responses at the molecular level are relatively modest.

The magnitude of these responses contrasts strongly with CML, in which many individuals experience a 1000 fold or greater reduction in *BCR-ABL* levels, following treatment [298,322]. Imatinib is a selective inhibitor of ABL, PDGFR, KIT and FMS tyrosine kinases but is not active against JAK2. It is known that KIT signal transduction pathways are required for erythropoiesis [323,324] and it is possible that the observed clinical benefits of imatinib in PV are a consequence of KIT inhibition.

Similar findings were subsequently reported by the Nordic MPN study group who described a limited effect on V617F levels during two years of pegylated rIFNα therapy [325]. In this study, eight V617F-positive patients receiving pegylated rIFNα, achieved complete haematological remission after 24 months. Of these eight cases, only five demonstrated a 1.2 to 3.6 fold reduction in V617F burden.

The results in both this study and my work were taken from a limited group of available patients investigated retrospectively and it cannot be excluded that other PV patients might exhibit more substantial molecular responses. Indeed, Kiladjian et al. (2006) [326] undertook a prospective evaluation of V617F as a molecular marker in a phase 2 study of pegylated rIFNα (peg-IFNα) in PV patients, with a median followed up time of 11 months. In 24 out of 27 PV patients, a mean average decrease from 49% to 27% V617F levels was observed following treatment, and in one case mutant *JAK2* was no longer detectable after 12 months. Furthermore, the authors recently published results from a multicenter phase 2 trial of treatment of V617F-positive PV with peg-IFNα-2a, a modified form of rIFNα, with higher efficacy and better tolerance [327]. Out of 37 evaluable patients, all displayed a haematological response at 12 months follow up, including 94.6% who achieved a complete haematological response. V617F levels were monitored in sequential samples from 29 patients, and the level of V617F decreased progressively in 26 cases, from a median average of 45% V617F before peg-IFNα-2a, to 3% after 36 months. In seven cases, the levels of V617F were reduced below the limits of detection. The authors conclude peg-IFNα-2a is very effective at inducing high rates of haematological and molecular response in PV, and may even eliminate the *JAK2* mutant clone in selected cases. However, the real-time PCR-based method of detecting *JAK2* V617F employed in this study was sensitive to only approximately 1% mutant level, so the potential for a persisting low level of mutation-carrying cells that survive peg-IFNα-2a cannot be ruled out. Indeed, one patient with PV who achieved complete molecular response with peg-IFNα-2a after two months treatment, discontinued treatment and V617F reappeared shortly afterwards [328].

Altogether, these preliminary data suggest that even in sustained haematological remission under rIFNα treatment, the malignant myeloproliferative clone remains present in the majority of *JAK2* V617F positive cases, and treatment should continue even if

molecular remission appears to be reached. IFNα is known to exert a general anti-myeloproliferative effect by targeting cytokine and haematopoietic cells and exerting a non-specific influence on cell-mediated and humoural immune responses, but its precise mechanism of action remains to be understood.

Measurement of *JAK2* V617F has also been used to assess the molecular effect of V617F-positive MPN following other therapeutic strategies. The efficacy of HU has been evaluated in a large series of V617F-positive ET cases. Campbell et al. (2005) reported a superior haematological response in V617F-positive ET patients compared to patients with wild-type JAK2 receiving HU treatment [329]. Furthermore, a recent study also describes a two-fold reduction in V617F levels in three quarters of V617F-positive PV and ET patients being treated with HU [330].

Similar studies have also provided insights into the correlations between *JAK2* V617F mutant allele burden and disease phenotype. A significant decrease of BM *JAK2* V617F allele burden was associated with haematologic improvement in three patients with post-PV MF and a del(5)(q31) abnormality, who underwent treatment with lenalidomide [331]. However, a case with V617F-positive chronic eosinophilic leukaemia achieved a complete molecular response obtained after IFNα-2a treatment but did not display evidence of any significant haematological improvement [332]. The effect of dose-reduced allogeneic stem cell transplantation for cases with *JAK2* V617F-positive MF was also evaluated at the molecular level, using a real time PCR assay to detect V617F that claimed to have a sensitivity of down to 0.01%. A total of 21 *JAK2* V617F positive cases underwent stem cell transplantation procedures, and 78% became negative for V617F suggesting complete molecular remission. Furthermore, in those patients who eventually relapsed, variation of V617F allele burden correlated with the clinical course [333].

Taken together therefore, these observations indicate that the clinical manifestation of MPN and changes in V617F allele burden are associated in most instances. However, it is premature to conclude that modest reductions in V617F allele burden indicate clinical benefit although some studies, including mine, demonstrate that molecular responses correlate with haematologic improvement following treatment. Some people have argued that since the majority of cases that have achieved haematological response

remain V617F-positive, this suggests that alternative pathogenic mutations may co-exist with JAK2 V617F. Together with studies comparing clonality based on X chromosome inactivation patterns and co-existing cytogenetic abnormalities alongside the *JAK2* mutation, this implies V617F could be a secondary genetic event [268,271,334]. More recently, a variety of JAK2 inhibitors have been trialled in MPN. Early results indicate that patients with advanced disease may benefit from treatment, i.e. they feel better and experience reductions in spleen size, but disappointingly any reductions in the clone size as determined by the %V617F are very modest [335-338].

5 Assessment of methods for detecting *JAK2* exon 12 mutations

5.1 Introduction

Investigations into the molecular pathogenesis of the 5% of PV cases that are V617F negative revealed that a significant proportion carry mutations in exon 12 of the *JAK2* gene [266]. Mutations in exon 12 span nucleotides 1606 to 1635 and are significantly different to V617F in that typically more than one base is altered by insertion, deletion and substitution, or combinations of all three. The net effect appears to be either amino acid substitution, deletion of one or more residues, or duplication of a small number of residues, all of which conserve the reading frame of the protein. Exon 12 mutations affect residues lying approximately 80 amino acids before V617, a region which contributes to the SRC homology (SH2) domain and beginning of the JH2 domain. Although the structure of the JAK2 protein has yet to be fully elucidated [149], homology-based molecular modelling suggests these regions are near the predicted loop that carries V617 in a theoretical model of the full-length JAK2 protein [339]. Furthermore, like V617F, *in vitro* analysis has shown that *JAK2* exon 12 mutations activate pathways associated with erythropoietin signalling, and in a murine model expression of one of these exon 12 mutations resulted in a MPN phenotype [266]. Data has emerged suggesting exon 12 mutations are associated with a particular clinical phenotype, namely a relatively isolated erythrocytosis, with normal levels of leukocytes and platelets[340]. This marked erythrocytosis is associated with low serum erythropoietin levels [341,342], however these features are not sufficiently distinctive for individuals with exon 12 mutations to be recognised by clinical phenotype alone. Indeed, exon 12 mutations have also been found in cases of idiopathic erythrocytosis (IE) as well as PV.

Subsequent to the initial report describing mutations in exon 12, further cases with novel sequence changes have been described, summarised in Figure 5.1. The significance of each particular sequence change is not yet known, but mutations F537_K539delinsL, H538QK539L, N542_E543del and K539L all induce IL3-independent growth of the factor dependent cell line Ba/F3/EPOR, with very similar proliferation kinetics to one another, and to V617F [266]. Interestingly, these experiments showed all exon 12 mutations were

more strongly activating (i.e. higher phosphorylation levels of substrate proteins) than the V617F allele. This supports the hypothesis that stronger signalling induced by, for example, homozygous V617F and now exon 12 mutations, is associated with a more erythroid phenotype, whereas weaker signalling is associated with a phenotype with elevated platelets [281,343].

Amino acid position		535	537	539	542	543	545	547
Amino acid		M V	F H	K I	R N	E D	L I	F
Wild-type DNA sequence		ATG GTG	TTT CAC	AAA ATC	AGA AAT	GAA GAT	TTG ATA	TTT
Mutation	**No. cases reported**	**nucleotide sequence**						
N542_E543del	17	ATG GTG	TTT CAC	AAA ATC	AGA ---	--- GAT	TTG ATA	TTT
F537_K539delinsL8		ATG GTG	TT- ---	--A ATC	AGA AAT	GAA GAT	TTG ATA	TTT
K539L	5	ATG GTG	TTT CAC	TTA ATC	AGA AAT	GAA GAT	TTG ATA	TTT
H538QK539L	1	ATG GTG	TTT CAA	TTA ATC	AGA AAT	GAA GAT	TTG ATA	TTT
R541_E543delinsK	6	ATG GTG	TTT CAC	AAA ATC	A-- ---	-AA GAT	TTG ATA	TTT
H538_K539delinsL	3	ATG GTG	TTT TT-	--A ATC	AGA AAT	GAA GAT	TTG ATA	TTT
E543_D544del	7	ATG GTG	TTT CAC	AAA ATC	AGA AAT	--- ---	TTG ATA	TTT
I540_E543delinsMK	3	ATG GTG	TTT CAC	AAA AT-	-GA ---	-AA GAT	TTG ATA	TTT
V536_I546dup11	1	ATG GTG	TTT CAC	AAA ATC	AGA AAT	GAA GAT	TTG ATA,	
		GTG	TTT CAC	AAA ATC	AGA AAT	GAA GAT	TTG ATA	TTT
F537_I546dup10+F547L	1	ATG GTG	TTT CAC	AAA ATC	AGA AAT	GAA GAT	TTG ATA,	
		TTT	CAC AAA	ATC AGA	AAT GAA	GAT TTG	ATA TTG	
547insL+I540+F537dup8	1	ATG GTG	TTT CAC	AAA ATC	AGA AAT	GAA GAT	TTG ATA	TTT,
		TTA	ATC AGA	AAT GAA	GAT TTG	ATA TTT		

Figure 5-1. Different *JAK2* exon 12 mutations identified to date. DNA, amino acid sequence and position, followed by a list of the different exon 12 mutations identified, and the number of cases reported in the literature are indicated [266,341,342,344,345]. Nucleotide deletions are indicated by a dash, substitutions are in blue and nucleotide insertions are in red. Continuation of sequence to the line underneath is indicated by a comma.

Exon 12 mutations were originally discovered by a sequencing screen of DNA extracted from EECs cultured from V617F negative PV patients, as this greatly enriches for mutant alleles that may not be detectable by normal sequencing [266]. The culturing of patient material prospectively to find exon 12 mutations is very time consuming and thus not widely applicable in a diagnostic setting. A more sensitive method, AS-PCR, was therefore developed to detect the four mutant alleles that were first identified (K539L, N542_E543del, F537_K539delinsL and H538QK539L) in order to screen more cases [266]. A number of subsequent publications used a combination of direct sequencing and AS-PCR to screen V617F negative PV cases for exon 12 mutations [341,346,347]. AS-PCR is a relatively sensitive method [341], but four individual PCRs must be performed per sample and only pre-defined mutations are detected, precluding the isolation of most other sequence changes. Direct sequencing is potentially able to detect all mutations, but is a relatively insensitive technique that is not generally able to detect mutations when present in less than 20% of alleles. Since exon 12 mutation-positive cells are often present at low levels in PB compounded by the finding that, in contrast to V617F, cells carrying exon 12 mutations are nearly always heterozygous [266], alternative methods of detection are needed.

5.1.1 Aims

To help address the shortcomings of presently available methods, the aim of this part of the study was to develop and apply alternative sensitive and generally applicable PCR-based techniques to identify exon 12 mutations in V617F negative PV and IE. To this end I developed and evaluated two methods: HRM analysis and wild-type blocker PCR.

5.2 Methods

5.2.1 HRM reaction conditions

A 126 bp amplicon was generated using primers located in *JAK2* exon 12 and intron 12 (JAK2_ex12_HRM_F and JAK2_ex12_HRM_R, primers sequences listed in Appendix III). Each 20µL PCR contained 20-40ng DNA or $1x10^6$ plasmid copies, 0.5U Platinum *Taq* polymerase (Invitrogen, Paisley, UK), 2.5mM $MgCl_2$, 1x Platinum *Taq* polymerase buffer (Invitrogen), 200µM dNTPs, 0.5µM of each primer, and 1x LC Green Plus (Idaho Technologies, Salt Lake City, UT, USA) as the intercalating dye. PCRs were performed in duplicate, and the cycling and HRM analysis conducted on a RotorGene 6000™ real-time analyser (Corbett Life Sciences, Mortlake, NSW, Australia). The PCR profile was as follows; an initial hold at 95°C for 10 minutes, 40 cycles of 95°C for 15s, 58°C for 30s, and 72°C for 20s, followed by 50°C for 30s, then a melt from 70°C to 95°C rising at 0.1°C per second (the speed of data acquisition was up to 1000 data collection points per °C transition).

Normalisation bars were between 72°C and 75°C for the leading range, and 92°C and 93°C for the tailing range. The resulting data were analysed using the associated custom software (RotorGene Series Software V1.7.25). All samples and mutation-carrying plasmids were run alongside a series of at least six normal controls whose combined melt profile characteristics were used as a standard reference that the software used to distinguish normal from variant melt profiles, with a confidence of 90%.

5.2.2 Wild-type blocking PCR (WTB-PCR)

WTB-PCR was performed in duplicate on 25ng DNA or 1µl plasmid dilution ($1x10^6$ copies) using 0.5µM *JAK2* exon 12 forward and reverse primers (JAK2_ex12_WTB_F and JAK2_ex12_WTB_R), 1U Amplitaq DNA polymerase Stoffel fragment (Applied Biosystems, Foster City, CA, USA), 10x Stoffel buffer (Applied Biosystems), 0.2mM dNTPs (Amersham), 3.75mM magnesium chloride, 1µM *JAK2* exon 12 LNA oligonucleotide (JAK2_ex12_wtLNA_oligo), made up to 25µl with sterile distilled water. WTB-PCR was conducted on a Tetrad thermocycler (MJ Research), under the following conditions; 95°C

for 10 minutes, 30 cycles of 94°C for 1 minute, 57°C for 1 minute and 72°C for 1 minute, and a final extension of 72°C for 10 minutes. Ten microlitres of each PCR was run on a 1.5% gel to check for amplification. PCR products were sequenced directly depending on band intensity. If the PCR product was weak or not visible, a second round of PCR was carried out by diluting the WTB-PCR product 1:50 in sterile distilled water, and nested into the second PCR. The second PCR conditions were 1µl diluted product from the first PCR, 0.5µM forward primer (JAK2_ex12_WTB_F2), 0.5µM reverse primer (JAK2_ex12_WTB_R2), 1U Amplitaq Gold (Applied Biosystems), 10x Amplitaq buffer (Applied Biosystems), 0.2mM dNTPs, 1.5mM $MgCl_2$ and distilled water to a final volume of 25µl. PCR amplification was conducted as described above, except the melting temperature was 60°C.

5.2.3 Sequencing HRM products

HRM products were treated with ExoSAP, purified and sequenced.

5.3 Results

5.3.1 Application of high resolution melt (HRM) analysis to *JAK2* exon 12

5.3.1.1 Rationale and background

HRM analysis is an established method of detecting mutations in a short region of DNA, and involves the precise monitoring of the progressive change in fluorescence caused by the release of a saturating intercalating DNA dye from a DNA duplex as that duplex is denatured by increases in temperature [348]. DNA duplexes each have a characteristic melting behaviour that is subtly altered by nucleotide substitutions, deletions and insertions, and these melting differences are resolved by the release of an appropriate intercalating DNA dye, such as LC Green. This methodology has been successfully used to screen patient samples for *JAK2* V617F [349,350], plus other clinically relevant genes such as *KIT* [258], *BRAF,* [351], *EGFR, HER2,* [352] *RET* [353] and *KRAS* [354]. The HRM approach to mutation detection is advantageous in that it can theoretically detect any nucleotide changes within the amplicon being analysed. Furthermore, once established it is a simple and quick method whereby the melt analysis is performed immediately following PCR amplification. The PCR product can then be sequenced to confirm the presence of a mutation, and to identify the affected nucleotides.

5.3.2 HRM analysis validation and determination of sensitivity level

For optimal sensitivity, HRM amplicons must span the minimal region of interest using primers that amplify exponentially during PCR, whilst avoiding if possible any inherited polymorphisms that also affect the melt behaviour of the amplicon. In this study, PCR primers were designed to amplify a 126 bp region that spanned the entire coding region of exon 12.

Initially HRM was evaluated on a series of 30 normal controls plus a patient carrying F537-K539delinsL, at a level that was known to be detectable by sequencing and AS-PCR. The normal controls produced similar melt profiles whereas the case carrying a mutation produced a clearly variant melting profile. As only limited material from patients positive for exon 12 mutations was available, optimisation of the method was subsequently

performed on plasmids encoding the various mutant alleles, and sensitivity levels were determined on dilution series of mutant plasmids into a plasmid carrying the wild-type allele. A series of pGEM-T plasmids containing cloned inserts for several exon 12 mutation were provided by Dr Linda Scott, Cambridge. For the dilution series to test assay sensitivity, I created a wild-type plasmid using primers JAK2_ex12_WTB_F and JAK2_ex12_WTB_R, to amplify normal human genomic DNA. The amplicon was cloned into PCR-4 for propagation. Plasmids were used at a concentration of $1x10^6$ copies/μL diluted in 0.1X TE containing 50μg/ml tRNA.

HRM analysis was first trialled on plasmids diluted 50:50 into wild-type, as at this concentration the test sample should contain the highest possible ratio of heteroduplexes. Mutant alleles tested were F537-K539delinsL, H538-K539delinsL, H538QK539L, K539L, R541-E543delinsK, N542-E543del or E543-D544del, and all produced variant melt profiles on HRM. HRM data is presented in two formats: the first is a normalised plot (Figure 5.2A), in which the amount of intercalating dye remaining at temperature point is expressed as a fraction of the amount prior to data acquisition. The second is a difference plot, where the average HRM profile of the control samples was used by the genotype function of the machine software as the standard wild-type profile for subsequent comparison to each of the test samples (Figure 5.2B). Analysis of these seven mutant samples demonstrated that each mutant allele has its own characteristic melting curve that was clearly distinct from those obtained when wild-type exon 12 samples were analysed. The individual nature of the mutant melting curves became more apparent when the data were represented in a difference plot. Interestingly, those mutations that had in common a K539L substitution had similar difference plots, with the greatest divergence between mutant and wild-type profiles occurring at approximately 79^oC. The difference plots for the R541-E543delinsK, N542-E543del and E543-D544del mutants had a different slope to those associated with a K539L substitution, and the greatest divergence between mutant and wild-type occurred at approximately 77.5^oC. These differences were seen consistently over multiple runs.

The ability of HRM analysis to detect low levels of exon 12 mutations in a background of normal DNA was evaluated by titrating each of the mutant alleles with wild-type exon 12 to produce using a range of mutant allele dilutions (100%, 50%, 20%, 10%, 5% and 1%).

HRM was then applied to the dilution series made from each of the mutant alleles. An example of the difference plots obtained for the F537-K539delinsL and N542-E543del mutations are depicted in Figure 5.3.

The lower limit for detecting the nucleotide changes in *JAK2* exon 12 was calculated by the RotorGene Series Software (using a confidence interval of 90%) to be 5% for F537-K539delinsL and N542-E543del alleles, 7% for the R541-E543delinsK and E543-D544del alleles, 10% for the K539L allele, and 20% for the H538-K539delinsL and H538QK539L alleles. These data were also used as a guide to estimate the mutation level for two patients with a F537-K539delinsL or a N542-E543del mutation. Patient 1 (PT1; labelled 'query' in Figure 5.3A) appeared to have a mutant allele burden of approximately 50%, consistent with all granulocytes being heterozygous for the F537-K539delinsL mutation, whereas Patient 2 (PT2; labelled 'query' in Figure 5.3B) had a mutant allele burden greater than 20%, but less than 50%. The estimates obtained using HRM analysis accorded well with the estimates independently obtained from granulocyte DNA sequence traces (data not shown).

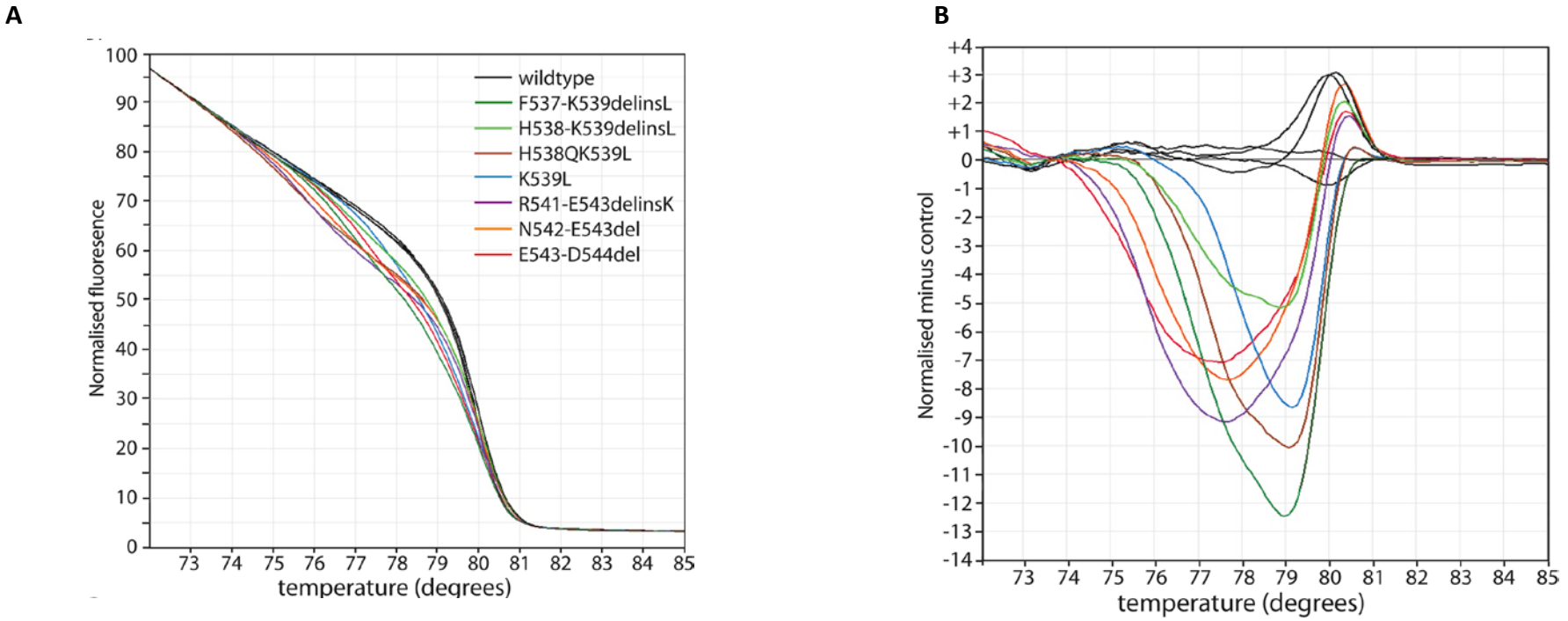


Figure 5-2. Development of a high-resolution melting-curve (HRM) assay to detect *JAK2* exon 12 mutations. A) HRM analysis of six wild-type exon 12 samples and 50:50 mixtures of wild-type and each exon 12 mutation. All mutant alleles have a melting curve profile that is distinct from that of the wild-type allele. B) Difference plots demonstrate more clearly that each mutant allele has its own characteristic melting curve, with those alleles with deletions of residues that include E543 (red; E543_D544del, purple; R541_E543delinsK, orange: N542_E543del) having a similar curve that is distinct from those alleles containing a K539L substitution (blue; K539L, brown; H538QK539L, dark green; F537_K539delinsL and light green; H538_K539delinsL).

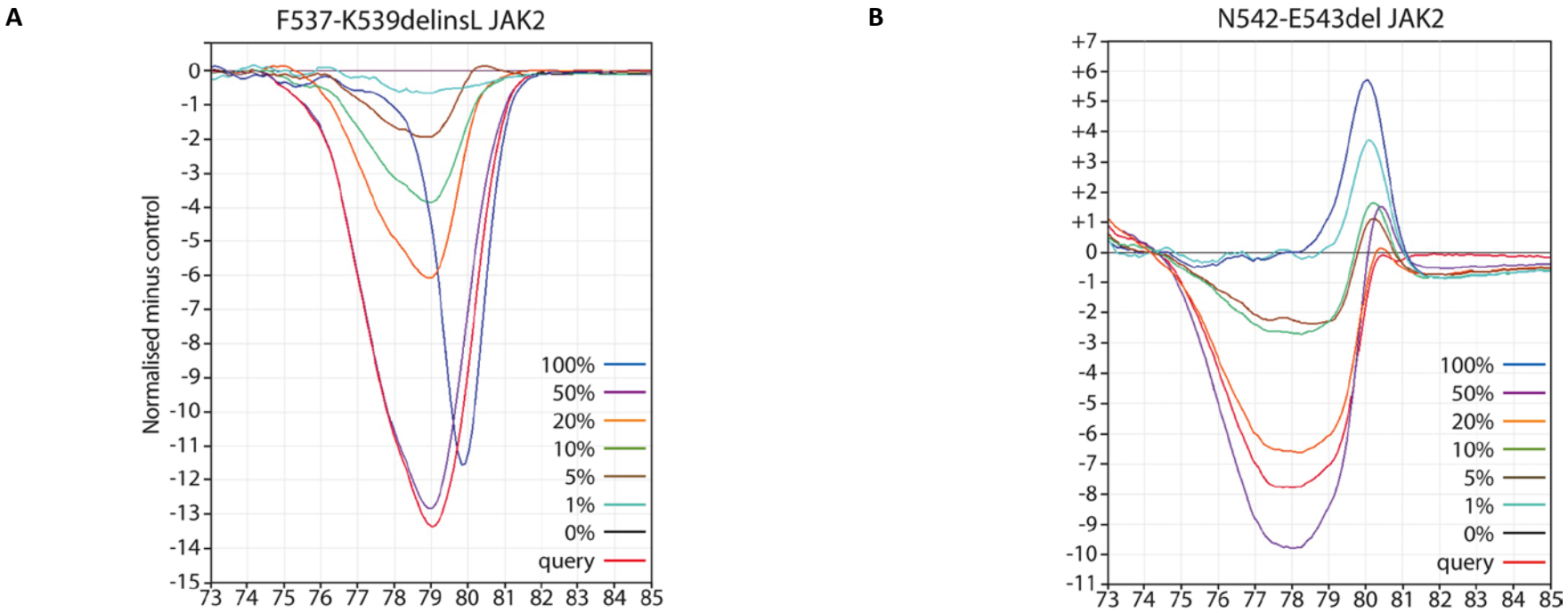


Figure 5-3. HRM assays detect mutant *JAK2* alleles present at low frequency. HRM analysis of mixtures of wild-type and mutant *JAK2* exon 12 alleles demonstrate that these assays can detect relatively low levels of mutant allele. Examples are shown for the F537_K539delinsL (A) and the N542_E543del mutations (B). Also included are the HRM traces of two granulocyte DNA samples from patients previously shown to carry the respective exon 12 mutation (indicated on each figure as 'query').

5.3.3 Development of a novel wild-type blocking PCR for the detection of existing and novel *JAK2* exon 12 mutations

The sensitivity with which some mutations could be detected by HRM was disappointingly low, e.g. only 20% for the H538-K539delinsL and H538QK539L alleles. For V617F it is generally considered that a sensitivity of 1-2% is required for routine analysis and presumably therefore it would be desirable for exon 12 mutation assays to have a similar sensitivity. An alternative method of detecting exon 12 mutations was therefore investigated, with a focus on improving the sensitivity of detection above that of the HRM methodology, whilst retaining the capacity to detect novel sequence changes. I investigated a wild-type blocker (WTB) assay, previously described to amplify *BRAF* mutations in DNA from melanoma samples containing a large proportion of wild-type DNA [355]. Central to the WTB-PCR method is a non-extendable oligonucleotide probe with locked nucleic acid (LNA)-substituted bases that binds a specific sequence (in this case the wild-type allele) with high affinity such that high temperatures do not melt the duplex during PCR cycling. The Stoffel fragment of *Taq* DNA polymerase is used for amplification, which lacks the 5'-3' exonuclease activity of the parent enzyme and thus cannot degrade the blocker oligonucleotide. If mutations are present in the target sequence, the affinity of the LNA-oligonucleotide and target DNA heteroduplex is reduced sufficiently such that the LNA-oligonucleotide is displaced and amplification can proceed. Thus, the LNA-oligonucleotide specifically blocks amplification of the wild-type sequence; any mutations are enriched in the PCR product and can be identified by sequencing. An overview of the WTB-PCR principle is outlined in Figure 5.4.

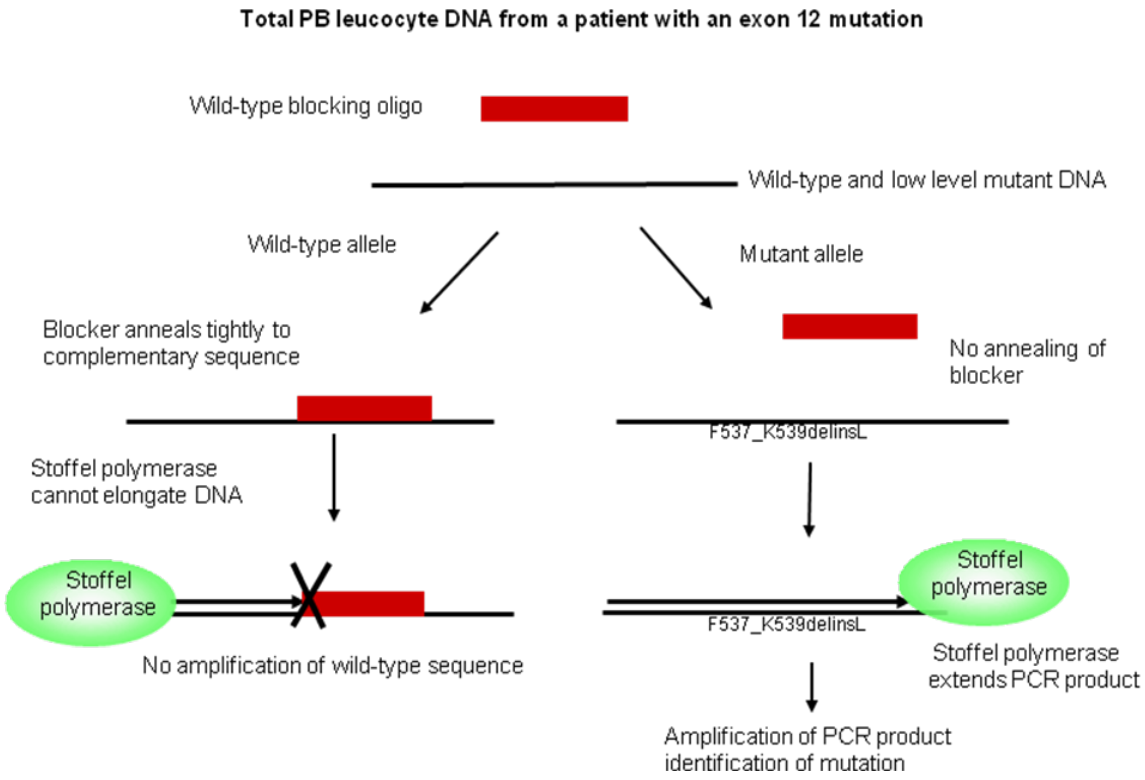


Figure 5-4. WTB-PCR strategy. The LNA-oligonucleotide probe anneals to wild-type sequence preventing amplification of wild-type DNA. Mismatches in the target sequence prevent the LNA-oligonucleotide blocker binding and amplification by Stoffel-fragment polymerase can proceed.

In this study, I adapted WTB-PCR for the detection of exon 12 mutations in PB total leukocyte or granulocyte DNA samples. An oligonucleotide that contains six LNA-modified nucleotides was designed to be complementary to wild-type *JAK2* exon 12 sequence affected by mutations described in the original report by Scott et al. (2007) [266]. Six LNA-modified bases were incorporated into the oligonucleotide as this provided optimal rigidity for binding target sequence with high affinity, whilst retaining a melting temperature so high that the resulting heteroduplex was not destabilised during thermal cycling. The position of this LNA-oligonucleotide probe is shown in Figure 5.11. New mutations in exon 12 have subsequently been identified after the LNA-oligonucleotide probe was designed, and all apart from the relatively rare large insertion mutations,

V536_I546dup11, F537_I546dup10+F547L and 547insL+I540+F547dup8 [342], are covered by the LNA-oligonucleotide sequence.

5.3.3.1 Determining WTB-PCR sensitivity in detecting *JAK2* exon 12 mutations

WTB-PCR was optimised on genomic DNA previously identified as positive for F537-K539delinsL mutation by AS-PCR and by normal sequencing. A concentration of 1µM wild-type *JAK2* exon 12 LNA-oligonucleotide was found to optimally block amplification from 25ng of wild-type DNA. WTB-PCR was also tested on DNA from 20 healthy normal controls, and did not result in the amplification of any false positive /erroneous sequence changes in any case.

The ability of WTB-PCR to detect low levels of exon 12 mutation was evaluated using a series of plasmid-dilution experiments for seven different mutations. WTB-PCR was performed with and without the LNA-oligonucleotide, and the latter PCR served as a control for amplification by Stoffel-fragment polymerase. WTB-PCR products were visualised on a 1.5% agarose gel, and those that produced weak bands (those at the lowest dilutions of mutant plasmid) were re-amplified by nesting into a second PCR. Sequencing confirmed that the presence of the LNA-oligonucleotide enriched the proportion of mutant allele on the chromatogram for each exon 12 mutation tested, whether the mutation consisted of deletions, substitutions or both. An example of a gel picture following first step amplification with and without the LNA-oligonucleotide probe on a plasmid dilution series of the F537_K539delinsL allele into wild-type is shown in Figure 5.5.

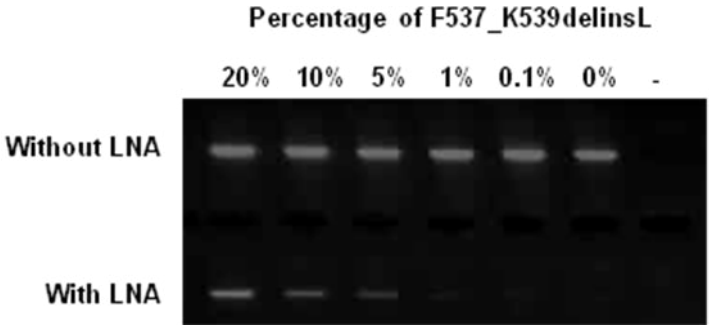


Figure 5-5. Gel of WTB-PCR amplification (first step) on plasmid dilutions of wild-type *JAK2* exon 12 and the F537_K539delinsL mutation. PCR amplification with and without the LNA-oligonucleotide blocker is shown. Without the LNA-oligonucleotide, mutant and wild-type sequences are amplified indiscriminately. In the presence of the LNA-oligonucleotide, amplification from wild-type exon 12 is blocked and only mutant alleles are replicated by Stoffel-fragment polymerase.

The LNA-oligonucleotide was shown to inhibit amplification of wild-type sequence thus enhancing mutant alleles even at dilutions containing relatively low levels of mutant plasmid. Mutant alleles were visible and discernable on the resulting sequencing chromatograms down to levels of 1% for all exon 12 mutant alleles tested. An example of the sensitivity of WTB-PCR at detecting the F537_K539delinsL allele is shown in Figure 5.6.

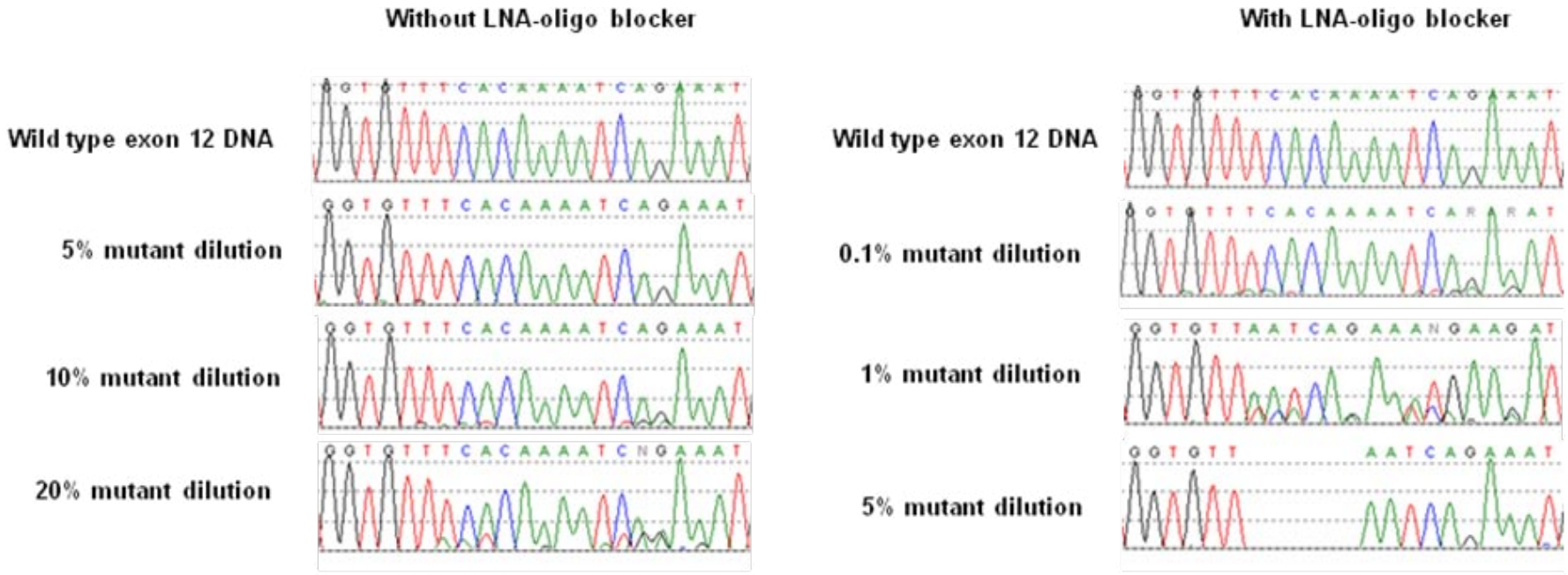


Figure 5-6. Sequence traces of WTB-PCR products following amplification of different dilutions of F537_K539delinsL plasmid with and without the LNA-oligonucleotide blocker. In the absence of the LNA-oligonucleotide blocker the mutation was not detectable below a 10% dilution of mutant into wild-type plasmid. However, in the presence of the LNA-oligonucleotide, sequencing revealed the presence of the F537_K539delinsL mutation even at a dilution of 1%.

5.3.4 Screening *JAK2* V617F negative PV and IE patients for mutations in exon 12

The ability of both HRM analysis and WTB-PCR to detect exon 12 mutations was evaluated in a direct comparison with AS-PCR in three V617F negative patient cohorts: 37 cases with IE (Group 1; supplied by Dr Melanie Percy, Belfast), 19 cases with PV (Group 2; supplied by Dr Richard Silver, New York) and a further 211 cases (Group 3) acquired locally, with a suspected or confirmed diagnosis of PV or IE. Most of these cases provided by Dr Percy and Dr Silver were known to have low erythropoietin levels. Due to lack of specific consent, detailed clinical information was not available for the local cases.

The analysis also included positive control DNA from four cases with F537_K539delinsL, N542_E543del, and R541_E543delinsK (2 cases) kindly provided by Dr Linda Scott, plus plasmid dilution series described above (plasmids carrying F537-K539delinsL, H538-K539delinsL, H538QK539L, K539L, R541-E543delinsK, N542-E543del and E543-D544del mutations).

5.3.4.1 Preliminary screen for *JAK2* exon 12 mutations by AS-PCR

Control plus Group 1 and 2 samples were initially screened using four separate AS-PCRs that detect the F537_K539delinsL, K539L, H538QK539L and N542_E543del alleles, as described [266]. Of the Group 1 samples, 6 were positive by AS-PCR. Case M2 was positive using the H538QK539L allele-specific primer and case M29 was positive for K539L. Cases M18 and M39 were positive for both H538QK539L and K539L mutation detecting AS-PCRs. Cases M42 and M44 were positive by the AS-PCR for F537_K539delinsL. None of the Group 2 samples tested positive (results summarised in Table 5.1).

5.3.4.2 HRM analysis of *JAK2* exon 12 in V617F negative cases

The HRM methodology established using cloned exon 12 alleles was then used to screen the PV and IE cases. Each case was run in duplicate, alongside a series of four normal controls and the four mutation positive controls. HRM analysis was successful in discriminating DNA samples wild-type for *JAK2* exon 12 from those carrying an exon 12

mutation. Figure 5.7 shows the results from an HRM screen on eight cases including the four positive controls, each of which produced clearly divergent melt profiles.

Of the test cases in the three patient groups, just four (M7, M18, M40 and E2871) produced melt profiles that were consistently variant to that produced by the normal controls, suggesting the presence of a mutation (data not shown). Sequencing of the HRM product using the primers used for amplification revealed nucleotide changes in each case except M40 (results summarised in Table 5.1).

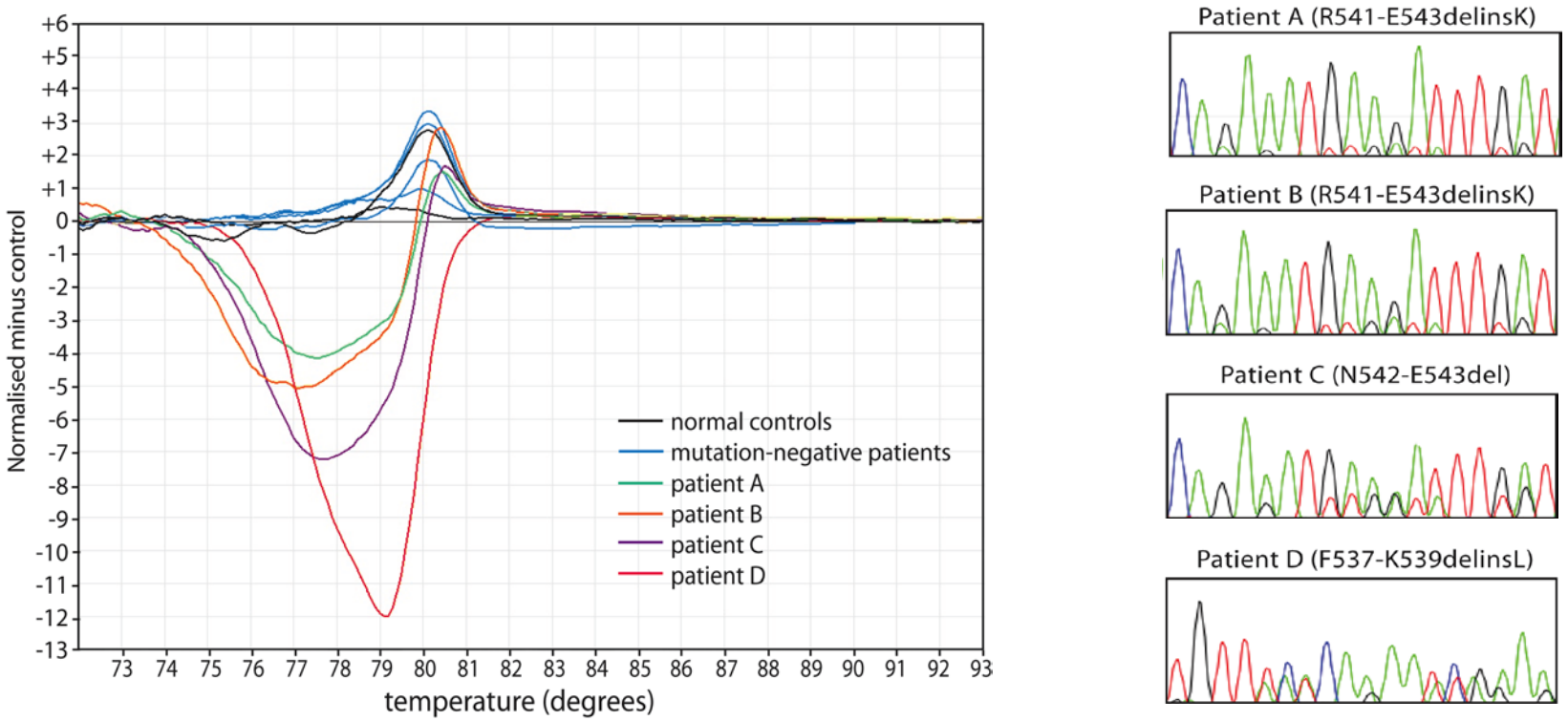


Figure 5-7. HRM analysis on *JAK2* V617F negative patients with PV and IE. The difference plot shows the melting profile of exon 12 amplified from 4 normal controls, 4 mutation-positive cases (patients A-D)and 4 cases with V617F negative IE (indicated as mutation-negative patients). Granulocyte DNA sequence chromatograms from the four mutation-positive cases are shown, with patients A and B having an R541-E543delinsK mutation, patient C having a N542-E543del mutation, and patient D having a F537-K539delinsL mutation.

5.3.4.3 *JAK2* exon 12 mutations detected by WTB-PCR

WTB-PCR was then conducted on all samples. Fourteen cases were confirmed to carry mutations in exon 12 on sequencing of the products. The results are summarised in Table 5.1 and nucleotide sequence changes are listed in Figure 5.11. An agarose gel picture displaying the products following first step amplification by WTB-PCR with and without the oligonucleotide probe, on one normal control and six mutation-positive cases is shown in Figure 5.8.

Lane identifier	Sample	*JAK2* exon 12 mutation identified
a	E2871	H538_K539delinsL
b	M2	H538QK539L
c	M7	I540_E543delinsKK
d	E596	E543_D544del
e	M18	K539L plus other clones
f	M35	N542D_E543X

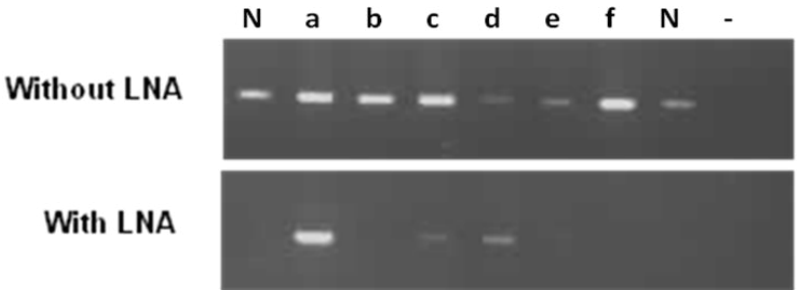


Figure 5-8. Gel picture of WTB-PCR on six *JAK2* exon 12 mutation positive cases. Gel electrophoresis of exon 12 PCR products from first step amplification with and without the LNA-oligonucleotide blocker for two normal cases (N) and six mutation-positive cases (a to f), with corresponding mutation identified listed in Table 5.11. Mutations were identified in cases M2, M18 and M35 following sequencing the product from a second round of PCR amplification.

Of the 14 mutation positive cases identified on sequencing of the WTB-PCR product, three had novel nucleotide changes that have not been reported elsewhere. Case E2871 had an H538_K539delinsL change and case M7 had I540_E543delinsKK but for case M18, the sequencing trace following WTB-PCR was difficult to interpret (Figure 5.9).

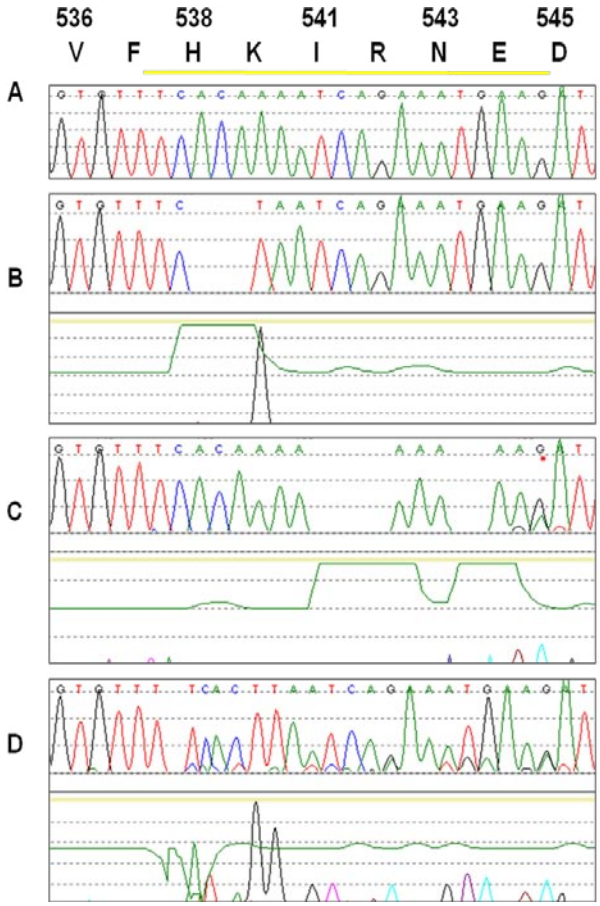


Figure 5-9. WTB-PCR sequence traces from cases carrying novel mutations in *JAK2* exon 12. (A) Amino acid number and residue corresponding to the wild-type DNA sequence targeted by the blocking LNA-oligonucleotide (indicated by yellow line). The novel sequencing changes are easily identifiable as H538_K539delinsL in case E2871 (B) and I540_E543delinsKK in case M7 (C), however for case M18 (D), the trace was abnormal but uninterpretable. A comparison of mutant sequences to wild-type sequence produced by the Mutation Surveyor 3.1 software is indicated below the sequencing trace for each case. A flat central green line indicates sequence homology; deletions are deviation above this line and substitutions are indicated by a peak.

I therefore cloned the PCR product from case M18 for further investigation. Sequencing of 24 individual colonies identified five different clones, each of which carried the AA to TT substitution that corresponds to the K539L allele. Additionally, four of these clones also carried deletions and/or insertions of one or two nucleotides immediately preceding the K539L, resulting in a frame shift that theoretically causes creation of STOP codons downstream (Figure 5.10 and 5.11).

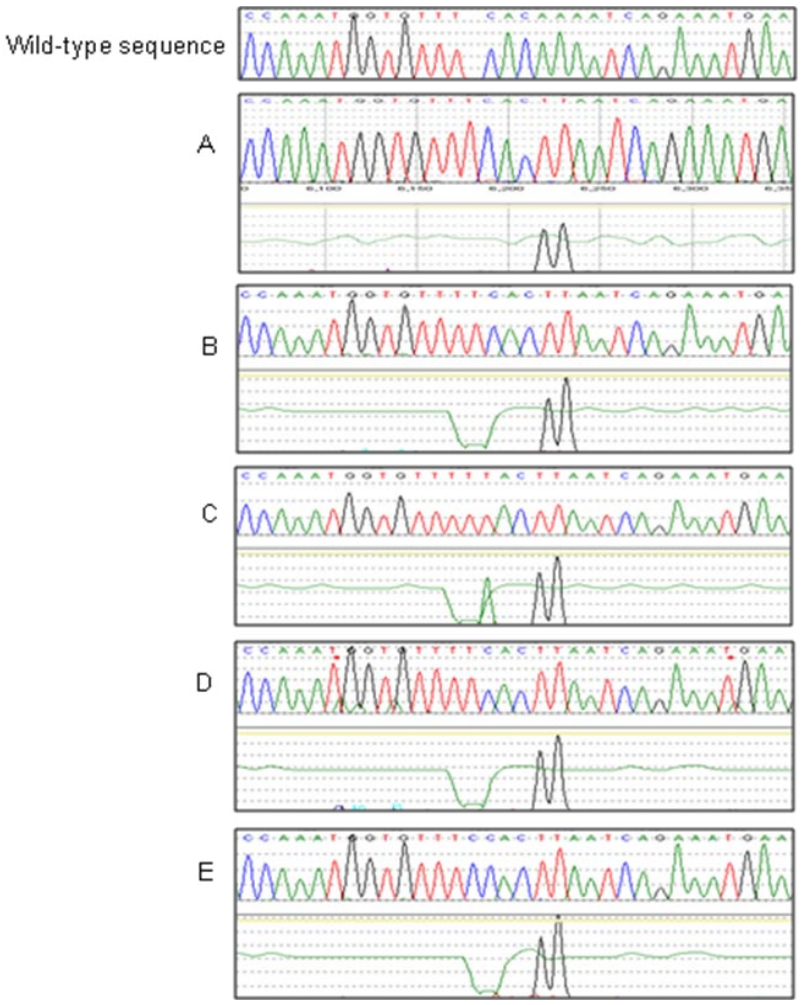


Figure 5-10. Clones isolated from WTB-PCR product from case M18. Five different clones were identified (panels A to E), each carrying the AA to TT substitution that causes K539L.

5.3.4.4 Summary of *JAK2* exon 12 mutations detected by AS-PCR, HRM analysis and WTB-PCR

In total 267 cases were analysed for mutations in exon 12 of *JAK2* by AS-PCR, HRM analysis and WTB-PCR, and 10 cases tested positive for a mutation (in addition to the four positive controls), of which 8 came from Group 1, 2 were from Group 2 and none were from Group 3. The results are summarised in Table 5.1 and the sequence changes for all cases found to carry exon 12 mutations in this study are shown in Figure 5.11.

Overall, both HRM and WTB detected all four positive controls, whereas AS-PCR only picked up two of them. Of the ten mutant cases detected by WTB, six were detected by AS-PCR and only three by HRM.

group	case identifier	AS-PCR	HRM analysis	WTB-PCR	*JAK2* exon 12 mutation identified
Controls	A	negative	variant	mutation visible	R541_E543delinsK
	B	negative	variant	mutation visible	R541_E543delinsK
	C	N542_E543del	variant	mutation visible	N542_E543del
	D	F537_K539delinsL	variant	mutation visible	F537_K539delinsL
Group 1	M2	H538QK539L	negative	mutation visible	H538QK539L
	M7	negative	variant	mutation visible	I540_E543delinsKK
	M18	H538QK539L and K539L	variant	mutation visible	K539L background, some clones with STOP codons
	M29	K539L	negative	mutation visible	K539L
	M38	negative	negative	mutation visible	F537_K539delinsL
	M39	H538QK539L and K539L	negative	mutation visible	H538_K539del
	M42	F537_K539delinsL	negative	mutation visible	F537_K539delinsL
	M44	F537_K539delinsL	negative	mutation visible	F537_K539delinsL
Group 2	E2871	negative	variant	mutation visible	H538_K539delinsL
	E596	negative	negative	mutation visible	E543-D544del

Table 5-1. Summary of *JAK2* exon 12 mutations found in *JAK2* V617F negative PV and IE cases. Each case was screened by AS-PCR, HRM and WTB-PCR for sequence changes in exon 12.

Amino acid sequence		Q	M	V	F	H	K	I	R	N	E	D	L	I
Wild-type DNA sequence		CAA	ATG	GTG	TTT	CAC	AAA	ATC	AGA	AAT	GAA	GAT	TTG	ATA
Case	**Mutation**													
A, B	R541_E543delinsK	CAA	ATG	GTG	TTT	CAC	AAA	ATC	A--	---	-AA	GAT	TTG	ATA
C	N542_E543del	CAA	ATG	GTG	TTT	CAC	AAA	ATC	AGA	---	---	GAT	TTG	ATA
D, M38, M42, M44	F537_K539delinsL	CAA	ATG	GTG	TT-	---	--A	ATC	AGA	AAT	GAA	GAT	TTG	ATA
E596	E543_D544del	CAA	ATG	GTG	TTT	CAC	AAA	ATC	AGA	AAT	---	---	TTG	ATA
M2	H538QK539L	CAA	ATG	GTG	TTT	CAA	TTA	ATC	AGA	AAT	GAA	GAT	TTG	ATA
E2871	H538_K539delinsL	CAA	ATG	GTG	TTT	C--	-TA	ATC	AGA	AAT	GAA	GAT	TTG	ATA
M7	I540_E543delinsKK	CAA	ATG	GTG	TTT	CAC	AAA	A--	--A	AA-	-AA	GAT	TTG	ATA
M29	K539L	CAA	ATG	GTG	TTT	CAC	TTA	ATC	AGA	AAT	GAA	GAT	TTG	ATA
M18	K539L	CAA	ATG	GTG	TTT	CAC	TTA	ATC	AGA	AAT	GAA	GAT	TTG	ATA
	H538delinsSLNQKX	CAA	ATG	GTG	TTT	TCA	CTT	AAT	CAG	AAA	TGA	STOP		
	H538delinsPLNQKX	CAA	ATG	GTG	TTT	CCA	CTT	AAT	CAG	AAA	TGA	STOP		
	H538delinsLPX	CAA	ATG	GTG	TTT	TTA	CCT	TAA	STOP					
M39	H538_K539del	CAA	ATG	GTG	TTT	---	---	ATC	AGA	AAT	GAA	GAT	TTG	ATA

Figure 5-11. Nucleotide sequence alterations in *JAK2* exon 12 identified in this study. Wild-type exon 12 DNA and amino acid sequence are shown, with the mutation-positive cases underneath. Nucleotide deletions are indicated by a dash and substitutions are blue. The wild-type blocking LNA-oligonucleotide target sequence (underlined).

5.4 Discussion

5.4.1 Assessment of AS-PCR, HRM and WTB-PCR at detecting *JAK2* exon 12 mutations

Exon 12 mutations in the *JAK2* gene are unusual in that they typically comprise a combination of small intragenic deletions, insertions, and substitutions that conserve the reading frame. Nevertheless, these diverse abnormalities result in altered growth factor responses *in vitro* and a myeloproliferative phenotype when expressed in a murine BM transplant model [266]. Compared with patients with *JAK2* V617F-positive PV, patients with PV or IE carrying an exon 12 mutation have a clinical phenotype mainly characterised by isolated erythrocytosis and low serum EPO [341,356]. Analogous to V617F, the finding of an exon 12 mutation strongly indicates the presence of an MPN, so it is important to have robust methods of detection to aid diagnosis.

Since the initial discovery of mutations in exon 12 of the *JAK2* gene [266], other mutations affecting the same region have been described. Some of these mutations, e.g. I540_E543delinsMK were discovered by enriching for the mutation-carrying cells by the expansion of EECs *in vitro,* then by sequencing across exon 12 [344]. In other cases the mutations were visible by directly sequencing exon 12 amplified from PB granulocyte DNA, e.g. V536_I546dup11 and F537_I546dup10+F547L, indicating a relatively large proportion of mutant-allele in the specimen [342]. Application of the four AS-PCRs originally designed by Scott et al. (2007) [266] has contributed to the identification of the majority of exon 12 mutation-positive cases described in the literature. Although AS-PCR is more sensitive than direct sequencing it targets specific mutations and will therefore miss most novel sequence changes. However due to the primer design, some novel variants may be detected, for example the E543_D544del mutation was originally identified by sequencing, but was also detected by AS-PCR for the N542_E543del mutation [341]. It is likely that other cases found to be positive by AS-PCR may carry subtly different mutations to those that the PCR was designed to detect. However the opposite may be true in some instances: in one case direct sequencing revealed an AAA to CTA substitution which caused a K539L mutation, but this was not detected by AS-PCR for K539L, as this PCR was specifically designed for the AA to TT substitution [356].

HRM analysis was used in this study to detect exon 12 mutations, based on the premise that this technique detects any possible sequence changes in the amplicon being studied, as detection is based on differences in melting behaviour. In theory, HRM analysis should be capable of detecting the V536_I546dup11 and F537_I546dup10+F547L mutant alleles, as these target bases within the amplicon region, but DNA was not available to assess this. I evaluated the sensitivity of HRM using plasmids carrying a series of exon 12 mutations diluted into wild-type plasmid, and found that HRM was only capable of detecting mutation level down to 5% for some mutant alleles, but only 20% mutant level for other alleles. Furthermore, whist HRM analysis did detect mutations in all four positive controls plus three cases in the screen, there were seven that went undetected by this method.

One likely explanation, as determined by the experiments with mutation-carrying plasmids, is the unexpectedly poor sensitivity of HRM, especially in the case of mutations affecting histidine 538. The case (M2) that carried a H538QK539L mutation was not detected by HRM, consistent with the fact that the detection level for this particular mutation is above 15%. Furthermore, the application of HRM as only a pre-screening method was demonstrated by the false identification of one case (M40). This case was later found to be negative for exon 12 sequence changes by WTB-PCR and sequencing, and the false-positive result was attributed to an abnormally high salt concentration in the DNA sample, which was later resolved by cleaning the DNA using a Microcon filter (following manufacturer's instructions, Millipore, Watford, UK).

Because of the low sensitivity of HRM, I sought to develop a PCR based method that reduced the amount of wild-type sequence whilst selectively amplifying any mutant alleles present. For this I designed a wild-type blocking (WTB) PCR based on an existing method published by Dominguez and Koloney (2005) [355], where this technique was used to amplify *BRAF* mutations in DNA from melanoma samples containing a large proportion of wild-type DNA. I designed an oligonucleotide probe containing LNA substituted bases that selectively blocked amplification of wild-type exon 12 sequence whilst amplification of other regions can proceed. This increases the proportion of mutant alleles so they become more visible on a sequencing chromatogram. The sensitivity of WTB-PCR at detecting various exon 12 mutations was tested using plasmids carrying a series of exon

12 mutations diluted into wild-type plasmid described above, and all different mutant alleles tested were detected readily at a level of 1% mutant. WTB-PCR was then used to screen all groups of *JAK2* V617F negative PVs and ten cases were positive for an exon 12 mutation, plus all four controls. Furthermore, WTB-PCR detected novel sequence changes in three cases (M7, M18 and E2871):

- WTB-PCR on case M7 revealed two small deletions in exon 12 that resulted in an I540_E543delinsKK mutation. This was detected by HRM but not by any AS-PCR. In addition, the nucleotide deletions are different to those that create the previously described I540_E543delinsMK (figure 5.9C).
- Case M18 was positive by AS-PCR for both H538QK539L and K539L mutations and had a variant melt profile on HRM. However, sequence analysis on the trace from WTB-PCR proved difficult to interpret so the WTB-PCR product was cloned for further investigation. Sequencing of individual clones revealed five different mutations (Figure 5.10). Each clone carries an AA to TT substitution that when translated individually corresponds to the K539L mutation, but additionally there were also insertions and/or substitutions of one or two T or C nucleotides that shift the reading frame introducing a STOP codon immediately downstream. It seems likely that the out-of-frame variants are non-functional since premature truncation of the JAK2 protein in the pseudokinase JH2 domain would result in loss of the entire catalytically active JH1 kinase domain. The presence of these multiple mutations, however, is highly unusual and suggests the possibility of underlying genetic instability.
- The mutation in case E2871 was readily visible upon sequencing the product following HRM analysis as H538_K539delinsL. This mutation was then confirmed by WTB-PCR. Despite being present at a relatively high level in PB, this mutation would not have been detected by an AS-PCR screen alone. In addition, the bases mutated to create H538_K539delinsL are different to those reported previously [346].
- The WTB-PCR revealed an E543_D544del mutation in patient E596, plus five DNA samples from this patient taken over a period of approximately two years (data

not shown). The mutation was not detected by HRM, nor by AS-PCR, suggesting a particularly low level of mutation.

This last point raises the question what level of mutation is clinically significant? Although there is no definitive answer to this question, it is generally considered that assays designed to detect V617F should have a sensitivity approaching 1% and therefore it seems reasonable that exon 12 mutation assays should have a similar sensitivity. My study indicates that WTB-PCR is the most suitable method for detecting exon 12 mutations since it can detect relatively low level mutations and identify novel sequence changes. However there are two important technical issues of the application of WTB-PCR. Firstly, WTB-PCR works optimally on a low DNA concentration (below 20ng/µl per PCR), to avoid saturation of the LNA-oligonucleotide with a high wild-type DNA concentration and therefore it is important to accurately quantify test DNAs. Secondly, only exon 12 mutations within the region covered by the oligonucleotide blocker may be identified by this method. Subsequent to the initial design of WTB-PCR, other mutations have been reported in the literature and the wild-type DNA region of all apart from the two cases involving the 36 base pair duplications [342] are covered (at least in part) by the oligonucleotide blocker. It is probable other mutations may be found in exon 12 that lie outside this region and will not be detected by my WTB-PCR but they could be covered by designing additional WTB oligonucleotides. A further weakness is that the test relies on the somewhat subjective judgement of visualising an amplification product on a gel after the WTB blocker PCR. A more robust approach may be to routinely perform nested PCR on all samples, and to sequence the products in each case. Obviously though this approach would be more time consuming and expensive.

Although the identification an exon 12 mutation provides evidence of a clonal disorder and is therefore diagnostically and potentially clinically relevant, the significance of each particular sequence alteration remains unclear. When four exon 12 mutant alleles, K539L, N542_E543del, F537_K539delinsL and H538QK539L, were expressed in Ba/F3 cells *in vitro*, they induced similar levels of IL3-independent cell division and activated signalling pathways involved in erythrocytosis. Furthermore, expression of K539L in a murine model induced MPN associated with marked erythrocytosis [266]. It is likely that the other (in-frame) mutations would behave in the same way, but this remains to be formally tested.

Furthermore, it is possible that different sequence changes may be associated with clinical variability, however given the rarity of these changes it is unlikely that any consistent differences could be recognised.

There is considerable discrepancy in the frequency of exon 12 mutations in patients with V617F negative PV reported in the literature. Some reports describe exon 12 mutations in virtually all patients studied [266,340,344,347] whereas exon 12 mutations were much rarer in other studies [346,356]. Apart from clinical phenotype, an obvious reason for this discrepancy was the methods of detection used, where studies that relied only on direct sequencing detected fewer mutations. Moreover, the source of DNA used for screening affects the sensitivity of detection. It is suggested that exon 12 mutations preferentially cause expansion of erythroid progenitors within the bone marrow [266]. Screening of BM cells may be preferable to PB granulocytes, however this has not been proven. Analysis of EEC colonies may result in detection of further cases but it is impractical on a routine basis [344]. Treatment is likely to impact on the level of circulating neoplastic cells, so obtaining samples at diagnosis is also important in the detection of exon 12 mutations.

One important conclusion drawn from my study was that the incidence of exon 12 mutations in *JAK2* V617F negative cases referred with unexplained erythrocytosis is very rare, even when using sensitive methods of detection. While it is conceivable there may be cases with extremely low levels of exon 12 mutation that remain undetected, WTB-PCR, AS-PCR and HRM failed to isolate any examples of exon 12 mutations in the 211 local cases. Although detailed clinical information was not available for these cases, it is likely that the great majority did not have an MPN but rather had marginally abnormal blood counts that may have been caused by environmental factors such as excess drinking and/or smoking (Dr David Oscier, Bournemouth, personal communication). In contrast, the analysis of a clinically well-characterised cohort known to have low EPO levels (Group 1) revealed mutations in 8/37 (22%) of cases, which suggests that mutation testing should be highly targeted towards individuals who fulfil the diagnostic criteria for PV, have low serum EPO levels and EECs, and do not carry V617F. The pick-up rate of exon 12 mutations in Group 2 was somewhat lower at 2/19 (11%), however since several of these cases had been treated with interferon or imatinib it is possible that the clone size may have been reduced to below the sensitivity of detection.

6 *JAK2* haplotype is a major risk factor for the development of myeloproliferative neoplasms

6.1 Introduction

6.1.1 The genetics of *JAK2* in MPN

The presence of the *JAK2* V617F mutation has proven to be a definitive pathogenetic marker for a subset of Philadelphia chromosome negative MPN. However unlike CML, where expression of *BCR-ABL* drives a largely uniform disease phenotype, V617F has been isolated in distinct MPN subtypes that share important features but have different clinical characteristics. Isolation of *JAK2* V617F in over 90% of cases with PV, roughly half of cases with ET and MF [197-201], plus other, rarer forms of atypical MPN [252] was unexpected, but several lines of evidence have been proposed to account for the phenotypic diversity associated with V617F.

(i) Levels of V617F expression. In many cases of PV and MF, the level of V617F is relatively high as a consequence of heterozygous cell undergoing mitotic recombination at chromosome 9p to become homozygous (acquired UPD), but this event is rare in ET [198,199,252,281]. Clinical studies have indicated that disease phenotype is correlated with dosage of the V617F mutant allele, where a higher mutation burden is associated with longer disease duration, splenomegaly, raised haematocrit, and the propensity to transform to AML [275,357-359]. Furthermore, when expressed retrovirally in transgenic mice, the ratio of *JAK2* wild-type to mutant allele strongly affects the outcome of disease phenotype. Relatively low levels of *JAK2* V617F induced thrombocythemia, characteristic of an ET-like phenotype, whereas polycythaemia was present in animals with high degree of transgene expression [283].

(ii) Additional acquired mutations. There are several lines of evidence that indicate other, largely uncharacterised, acquired abnormalities influence disease phenotype, either in combination with or independently of V617F [360]. The observation that in some cases only a proportion of clonal PV cells are V617F positive [271,361], and the demonstration of clonal

haematopoiesis by X chromosome inactivation pattern (XCIP) analysis in informative females with JAK2V617F negative ET [269,334], are both strong indicators for the presence of alternative mutations to *JAK2* V617F. Furthermore, it has been shown in rare cases with *JAK2* V617F-positive PV, that progression to AML is associated with expansion of a *JAK2* V617F negative clone [262].

(iii) Genetic background. Mouse models have provided evidence to show that the genetic background, i.e. the mouse strain may modify phenotype induced by *JAK2* V617F. When the *JAK2* V617F transgene was expressed in the BM of C57Bl/6 mice, the animals displayed erythrocytosis with mild leukocytosis. However, when *JAK2* V617F was expressed in Balb/c mice, a different genetic background, the mice displayed erythrocytosis with dramatically increased leukocyte count and bone marrow fibrosis [282,362,363]. In humans, both epidemiological data and familial MPN studies indicate that inherited factors may predispose to the development of MPNs. Familial MPN is characterised by a pattern of autosomal dominant inheritance with incomplete penetrance, variable presence of the three MPN entities PV, ET and MF in a single family, and with clinical and molecular features indistinguishable from sporadic MPN [204]. In all cases examined so far, *JAK2* V617F and *JAK2* exon 12 mutations were acquired somatically in the haematopoietic cells of some (but not all) affected familial cases, but these mutations were never inherited through the germ-line [364,365]. Therefore, in these families an unknown inherited mutation predisposes carriers to somatically acquire *JAK2* mutations and presumably other uncharacterised mutations. With regard to sporadic MPN, Landgren et al. (2008) showed, in a population-based approach, an increased risk of PV, ET and MF in first degree relatives of patients with known MPNs [272]. If the predisposing factor has low penetrance then it might be relatively common in the population. Recently, Pardanani et al. (2008) have suggested that inherited single nucleotide polymorphisms (SNPs) within *JAK2* are associated with specific MPN subtypes, providing the first evidence in patients that constitutive host factors contribute to MPN phenotype [366]. In this study I aimed to examine these recent findings more closely, and further characterise the role of inherited factors in *JAK2* V617F-positive MPN.

6.1.2 Genetic association studies

In order to investigate variation within the *JAK2* locus I used methods employed for the genetic mapping of somatically inherited traits and phenotypes. The smallest unit of variation is the SNP, a DNA sequence variant that occurs when a nucleotide differs between individuals. Almost all common SNPs have only two alleles, and within a given population, SNPs are assigned a minor allele frequency (maf), which is the lowest allele frequency at a locus observed in that particular population. SNPs are the most common type of genetic variation, and because they are often conserved across populations they are useful markers for use in association studies. Genetic association studies aim to test whether SNPs are associated with a disease, by comparing results from two different groups; disease-carrying subjects and healthy controls. SNPs found to have a significant association may themselves be in a biologically relevant locus or may be a marker of another nearby genetic change i.e. the association may be due to linkage disequilibrium (LD) with the true causal variant. LD describes the observation that alleles that are close together in the genome tend to be inherited together. A carefully selected group of these SNPs, called tag SNPs, can be used to capture all genetic variation in an LD block. When grouped together, SNPs are referred to collectively as a haplotype, and within haplotype blocks recombination is rare and LD is high. Therefore, the SNPs within a haplotype block are inherited together and the genotype of a single tag SNP can be used to infer the genotypes of other SNPs within the block. The strength of the correlation between tag SNPs is described using several statistics, but the most useful in association studies is the r^2 value. The Pearson correlation coefficient, r^2, is a measure of LD between two genetic markers, and values equalling 1 indicate the two SNPs have not been separated by recombination, whereas lower values indicate a lesser degree of LD. The size of LD blocks depends on the rate of recombination: in areas of low recombination LD blocks may extend for hundreds of kilobases whereas in regions of high recombination LD blocks may only be a few Kb or less. The definition of LD blocks, haplotypes and tag SNPs across the genome have enabled genome wide association studies (GWAS) to be performed. These studies have been very successful in detecting common, low penetrance predisposition alleles to a variety of disorders [367].

In epidemiological studies it is possible to define statistically the degree of risk associated with a particular variant, calculated as a ratio of the probability of the event occurring in a group with the phenotype in question versus a control group. There are two expressions that define this risk, the Odds-Ratio (OR) and Relative Risk (RR), and the formulae used to determine these expressions are described below.

6.1.3 Distortion of SNP ratios in cases with acquired UPD

Quantification of SNP allele ratios in constitutional DNA samples usually results in a polymorphism being defined as homozygous, heterozygous, or nullizygous for a particular allelic variant. Haplotypes are typically determined by long range PCR, cloning and sequencing to genotype the SNPs, or by statistical inference [368]. However the *JAK2* mutation provided a unique opportunity to read haplotypes directly in cases with acquired UPD.

The mitotic recombination that gives rise to acquired UPD in many patients with PV and MF typically involves all or a substantial region of chromosome 9p and thus SNPs within this region are also reduced to homozygosity [199]. V617F positive cases that have a high level of V617F mutation carry a large homozygous V617F clone, and it is this population of neoplastic cells that distorts SNP allele ratios away from the expected 50:50 value that is seen in normal and heterozygous V617F cells (Figure 6.1). It should be possible therefore to directly read *JAK2* haplotypes in cases with homozygous V617F as a consequence of distorted SNP allele ratios (Figure 6.2). Haplotypes provide more power for genetic association studies since they more accurately describe the genetic variation in any region whereas tag SNPs are often strongly but not completely associated with any particular haplotype.

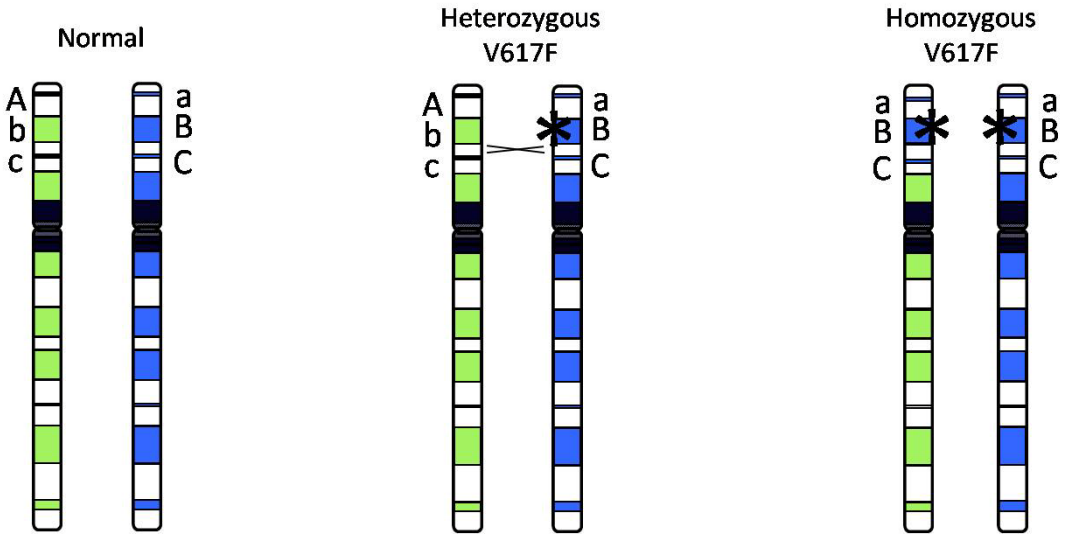


Figure 6-1. Acquired UPD duplicates *JAK2* V617F and flanking heterozygous SNPs to homozygosity. Many heterozygous SNPs span chromosome 9 (heterozygosity is illustrated here as A:a, B:b, C:c at each locus). V617F (indicated by an asterisk) arises on one chromosome (blue), without affecting the ratio of heterozygous SNP allele ratios close by. If the region carrying V617F undergoes mitotic recombination, the mutant allele becomes duplicated onto the other (green) chromosome and all flanking SNPs are also reduced to homozygosity.

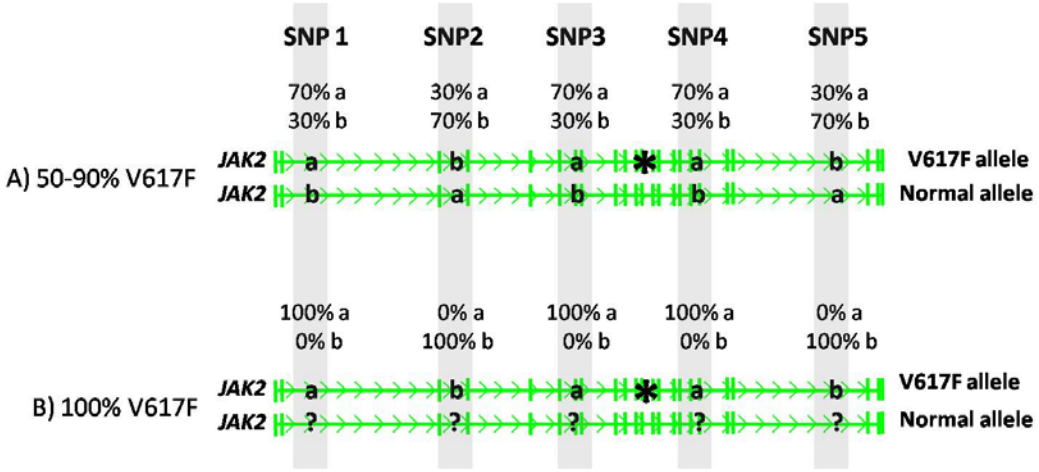


Figure 6-2. Heterozygous SNP allele ratios are skewed by high levels of acquired UPD. Many heterozygous SNPs span wild-type *JAK2*, but it is not possible to say which allelic variants are associated with one another, only that the ratio is 50:50. If *JAK2* V617F (illustrated by the asterisk) is present at levels ranging from 50 to 90% (panel A), clonal expansion will skew heterozygous SNP ratios so that the all SNP alleles linked in *cis* to the mutation are selected for. By measuring SNP allele ratios quantitatively a combination of linked SNP alleles (haplotype) can be read from the distorted SNP allele ratios. In this example, the haplotype associated with V617F is abaab, and the residual wild-type haplotype can also be determined (babba). In some cases with a very high level of mutation (≥90% V617F) (panel B), all heterozygous SNP ratios are reduced to homozygosity by the mutant positive clone, so the normal background cannot be seen.

6.1.4 Aims

Initially, the aim of this study was to try and confirm or refute the association between particular *JAK2* variants and certain MPN subtypes proposed by Pardanani et al. (2008). In doing this, it became apparent that there was a strong association between *JAK2* V617F positive diseases and a particular constitutional haplotype of this gene. The study then focused on defining this association, specifically:

1. Characterising *JAK2* haplotypes in more detail.
2. Evaluating the risk of developing MPN associated with *JAK2* haplotype.
3. Preliminary investigations into the reasons for *JAK2* haplotype skewing.
4. Investigating the impact of *JAK2* haplotype in V617F negative MPN.
5. Investigating the impact of *JAK2* haplotype in familial MPN.

6.2 Materials and methods

6.2.1 Patient samples

Genomic DNA or cDNA was extracted from PB or BM samples from local cases and also cases provided by Dr R. Silver (New York, USA). DNA was also provided by Dr L. Wang (New York, USA), Dr A. Reiter (Mannheim, Germany), Dr K. Zoi (Athens, Greece) and Dr H. Cario (Ulm, Germany).

A total of 775 cases with MPN were analysed. Of these, 592 were V617F positive (PV, n=203; ET, n= 224; MF, n=41; unclassified MPN, n=124), and 183 had V617F negative disease (locally obtained ET, n=47; Greek ET, n=136) as determined by AS-PCR and pyrosequencing. Individuals from a previously described MPN family were also analysed [364]. The controls included healthy individuals from the UK (n=188, first degree relatives of individuals referred for variety of genetic conditions) and Greece (n=108, provided by Dr K. Zoi). Data generated by the Welcome Trust Case Control Consortium (WTCCC) from the UK blood donor cohort (n=1500) [369] was also used.

6.2.2 Genotyping by pyrosequencing

In this study all genetic variation at SNP loci and *JAK2* V617F burden was measured in PB leukocyte DNA using pyrosequencing. Because the allelic ratio (i.e. the ratio of allele A to allele B at any SNP) for any heterozygous SNPs were distorted away from the value of 0.5 in cases with a sizeable homozygous V617F clone, I adopted the following scoring criteria for all SNPs: (i) if one allele had an allelic ratio ≥0.9, the sample was scored as homozygous for that SNP, (ii) where allelic ratios were 0.11-0.89, samples were scored as heterozygous. These cut offs were at least 3 standard deviations more than background, (i.e. values read for allele B in healthy controls who were A/A homozygotes). Similarly, for homozygous V617F cases (V617F >50%), SNPs with allelic ratios ≥0.6 or ≤0.4 were considered to be derived from the V617F-mutated or residual wild-type alleles, respectively. For V617F homozygous cases in which the %V617F was ≥90%, no information could be obtained about the residual wild-type *JAK2* allele and thus these cases were considered to contribute only one allele to the analysis (Figure 6.2).

6.2.3 Allele-specific PCR (AS-PCR)

AS PCR was performed using forward primers that were specific for V617F (JAK2_rs867_T_F) or the corresponding wild-type sequence (JAK2_rs867_G_F) in combination with a common reverse primer (JAK2_i14.15_R3), producing a 565bp product that included the 46/1 tag SNP rs12343867. The AS-PCR strategy to capture the 46/1 haplotype is described in figure 6.3, alongside an example of the PCR products and sequencing traces for some heterozygous V617F-positive cases. PCR was optimised on DNA from the HEL cell line (100% V617F) and normal healthy controls, and tested on homozygous V617F-positive cases where the mutation was known to be in *cis* to the 46/1 haplotype. Products were sequenced to determine whether V617F was on the 46/1 allele.

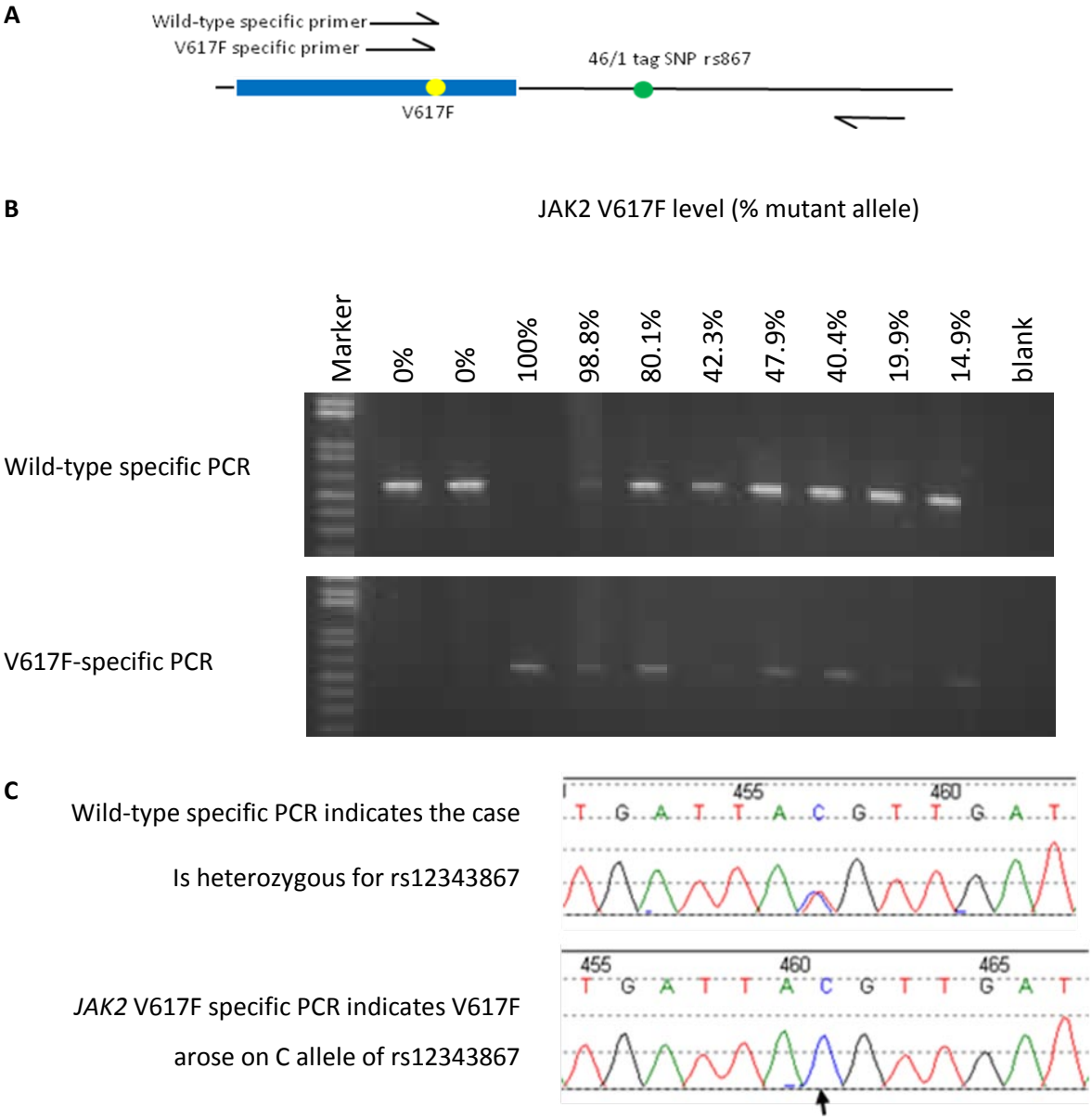


Figure 6-3. AS-PCR for tag SNP rs12343867 captures the 46/1 haplotype in heterozygous V617F-positive cases. (Panel A) AS-PCR between V617F (yellow circle) located in *JAK2* exon 14, captures the 46/1 tag SNP (green circle). The positions of the mutant specific and wild-type specific primers are indicated. (Panel B) Wild-type and mutant specific PCR products from AS-PCR performed on normal controls (0%), a 100% V617F positive cell line, and seven V617F positive cases with varying levels of mutation. (Panel C) Sequencing to identify the tag SNP (SNP nucleotide indicated by arrow) genotype of each PCR product confirms the wild-type is heterozygous (CT heterozygous tag SNP) for the 46/1 haplotype, whereas the V617F mutation has arisen on the 46/1 haplotype (C tag SNP allele only).

6.2.4 Colony analysis

Haemopoietic colonies from normal controls comprising a minimum of 100 cells were counted on day 14, and characterised based on morphology as either CFU-GM, CFU-GEMM, CFU-E, or BFU-E. *JAK2* haplotype status was determined after colonies were counted using DNA extracted from the MNC or granulocyte cell fractions.

6.2.5 Expression analysis

RNA was extracted from peripheral blood leukocytes of V617F negative MPN cases that were known to be heterozygous for rs10429491 and/or rs2230724 (*JAK2* exonic SNPs) as well as heterozygous for 46/1, as determined by rs12340895 genotype. RNA was reverse transcribed with random hexamer primers and the ratio of the two alleles for each SNP in genomic DNA and cDNA was determined by specific pyrosequencing assays.

6.2.6 Statistical analysis

The proportion of 46/1 alleles for each patient subgroup was compared to controls using Fisher's exact test (2-tailed). Colony numbers were compared to genotype using the Mann-Whitney U test.

Odds ratios and relative risk were calculated as follows:

A=number of 46/1 alleles in cases

B=number of 46/1 alleles in controls

C=number of non-46/1 alleles in cases

D=number of non-46/1 alleles in controls

The OR and RR can then be calculated as:

OR= (A/B)/(C/D)

RR = (A/(A+B)(C/(C+D))

These values and 95% confidence intervals were calculated using a 2-way Contingency Table Analysis html applet supplied by Paul Strike, Salisbury Research & Development Support Unit.

The population attributable risk level % (PAR%) was calculated as f(RR-1)/(1+f(RR-1) *100, where ‘f’ is the frequency of risk factor (46/1 allele in population of WTCCC controls) [370].

6.3 Results

6.3.1 Defining genetic variation at the *JAK2* gene locus

Pardanani et al. identified three *JAK2* SNPs (rs7046736, rs10815148, and rs12342421) that were significantly but reciprocally associated with PV and ET [366]. Three additional *JAK2* SNPs (rs10758669, rs3808850, and rs10974947) were also significantly associated with PV compared to controls. However there were two obvious weaknesses in this study: (i) they had not taken into account the fact that acquired UPD in many PV cases will distort the ratios of *JAK2* SNPs and (ii) the study used only HapMap samples as controls. I therefore set out to perform a more detailed analysis to determine if the associations could be replicated.

In order to map the genetic variation of *JAK2,* Pardanani et al. (2008) genotyped a total of 13 SNPs. Analysis of the resultant allele frequencies led the authors to define six major haplotypes, which accounted for 67.7% of *JAK2* alleles in the MPN population. Table 6.1 shows the haplotypes proposed by Pardanani et al. (2008), listed 1 to 6, alongside the tag SNPs studied and the frequency of each haplotype for the 179 MPN cases genotyped in their study. These haplotypes could be captured by just six SNPs as highlighted in blue in Table 6.1, and the reduced haplotype produced from these six SNPs is listed in the last column.

	JAK2 tag SNP														
haplotype number	rs7864782	rs1887427	rs10758669	rs3808850	rs1887429	rs2274471	rs7849191	rs7046736	rs10815148	rs12342421	rs10974947	rs2031904	rs10815160	haplotype frequency	Revised haplotype
1	A	A	A	A	C	T	C	A	A	G	G	G	T	0.015	AAAGGG
2	A	A	A	T	C	T	T	C	T	C	G	G	T	0.148	AACCGG
3	A	A	C	A	C	T	C	A	A	G	G	G	T	0.192	ACAGGG
4	G	A	A	A	C	T	T	C	T	C	A	G	G	0.014	GACCAG
5	G	A	C	A	C	T	C	A	A	G	G	G	T	0.178	GCAGGG
6	G	G	A	A	A	C	C	C	T	C	A	A	G	0.13	GACCAA

Table 6-1. *JAK2* haplotypes defined by Pardanani et al. (2008). Genotyping of 13 SNPs predicted six haplotypes that accounted for 67.7% *JAK2* alleles. A minimal set of six tag SNPs that can capture these haplotypes are indicated in blue along with haplotype frequency and reduced haplotype genotypes.

I first investigated the genetic diversity of *JAK2* in our cohort of homozygous PV cases. I selected only homozygous cases with a mutant burden greater than 50% because skewing of heterozygous SNP allele ratios would aid the haplotyping of alleles that were either linked or not linked to V617F. Pyrosequencing assays were designed for the six SNPs that comprise the revised haplotype listed in table 6.1 (rs7864782, rs10758669, rs7046736, rs12342421, rs10974947, and rs2031904) and used to screen 73 homozygous PV cases. Whilst it was possible to read the haplotype associated with V617F in most cases with 60-90% V617F, the residual haplotype (i.e. the haplotype on which V617F had not arisen) could also be read by the finding of heterozygous allele ratios in the range 0.1-0.4. In cases with ≥90% V617F, however, information about alleles on the non-V617F chromosome were lost (Figure 6.2). This was because it was not possible to determine whether a homozygous SNP allele pyrosequencing result was due to complete skewing from a heterozygous allele ratio, or the SNP was constitutionally homozygous.
Table 6.2 shows an example of how high level V617F positive cases were haplotyped for these six SNPs by allele skewing.

case	%V617F	rs7864782		rs10758669		rs7046736		rs1234241		rs10974947		rs2031904		V617F haplotype	Wild-type haplotype
		A	G	A	C	A	C	G	C	A	G	A	G		
E659	99	100	0	7	93	100	0	100	0	6	94	2	98	ACAGGG	?
E2433	80	14	86	4	96	82	18	85	15	0	100	0	100	GCAGGG	ACCCGG
E2513	62	71	29	31	69	68	32	74	26	8	92	0	100	ACAGGG	GACCGG
E1186	49	46	54	9	91	48	52	45	55	51	49	49	51	?	?

Table 6-2. Examples of four *JAK2* V617F positive PV cases quantified for six *JAK2* tag SNPs using pyrosequencing. The V617F level (% mutation burden) is listed alongside the allele frequencies for each SNP (%allele) measured by pyrosequencing. The V617F associated haplotype and the wild-type haplotype is listed last in the row. In many cases that harboured a homozygous V617F clone it was possible to directly read the haplotype on which the mutation arose by the finding that one allele at each SNP predominated (allelic ratio ≥0.6, e.g. cases E659, E2433 and E2513). In cases with a homozygous clone and %V617F <90%, it was usually possible to read the residual haplotype (i.e. the haplotype of the chromosome that had not acquired V617F) by the finding of allelic ratios between 0.1-0.4 (e.g. cases E2433 and E2513). Where the homozygous clone was small or nonexistent (most cases with %V617F <60%), neither the V617F nor wild-type haplotype could be read (e.g. case 1186).

The results for 73 V617F positive cases with a homozygous V617F burden (>50%) are listed in table 6.3. It was possible to identify each of the six haplotypes predicted by Pardanani et al. (2008) in many cases, plus I was able to identify nine further haplotypes that were designated 7 to 15. In 27 cases the allele skewing due to a high mutation burden was such that residual wild-type haplotype could not be determined. In total therefore I was able to read 73 V617F associated haplotypes and 45 residual wild-type haplotypes.

							V617F			wild-type	
haplotype number	rs7864782	rs10758669	rs7046736	rs12342421	rs10974947	rs2031904	number (n=73)	f		number (n=45)	f
1	A	A	A	G	G	G	2	0.03		2	0.04
2	A	A	C	C	G	G	1	0.01		11	0.24
3	A	C	A	G	G	G	26	0.36		4	0.09
4	G	A	C	C	A	G	2	0.03		2	0.04
5	G	C	A	G	G	G	29	0.40		0	0.00
6	G	A	C	C	A	A	2	0.03		5	0.11
7	A	C	C	C	G	G	2	0.03		7	0.16
8	G	A	C	C	G	G	3	0.04		7	0.16
9	A	C	A	C	G	G	2	0.03		1	0.02
10	A	A	A	C	G	G	0	0.00		2	0.04
11	A	A	C	C	A	A	0	0.00		1	0.02
12	A	C	C	C	A	G	1	0.01		0	0.00
13	G	C	C	C	G	G	0	0.00		3	0.07
14	A	C	C	C	A	A	0	0.00		1	0.02
15	G	A	A	G	G	G	3	0.04		0	0.00
undefined							0	0.00		27	0.60

Table 6-3. *JAK2* haplotypes found in homozygous (>50% V617F) cases. Haplotypes were determined from the genotype status of 6 *JAK2* SNPs. Haplotype numbers 1-6 were defined by Pardanani et al; haplotypes 7-15 were identified in this study. The number and frequencies (f) of each haplotype carrying the V617F allele and the background wild-type allele are indicated.

The genotyping data reveals a striking difference between the V617F and residual wild-type haplotypes: 55 of the 73 V617F alleles (75%) were accounted for by just two haplotypes, numbers 3 and 5. In contrast only 4 (9%) of the wild-type alleles were haplotype 3 and none were haplotype 5. This striking difference is highly significant ($P=7x10^{-13}$; Fishers exact test). Haplotypes 3 and 5 are highly related and only differ by rs7864782 genotype. This SNP lies just 5' of the *JAK2* gene, and is in a separate LD block according to HapMap data (Figure 6.4).

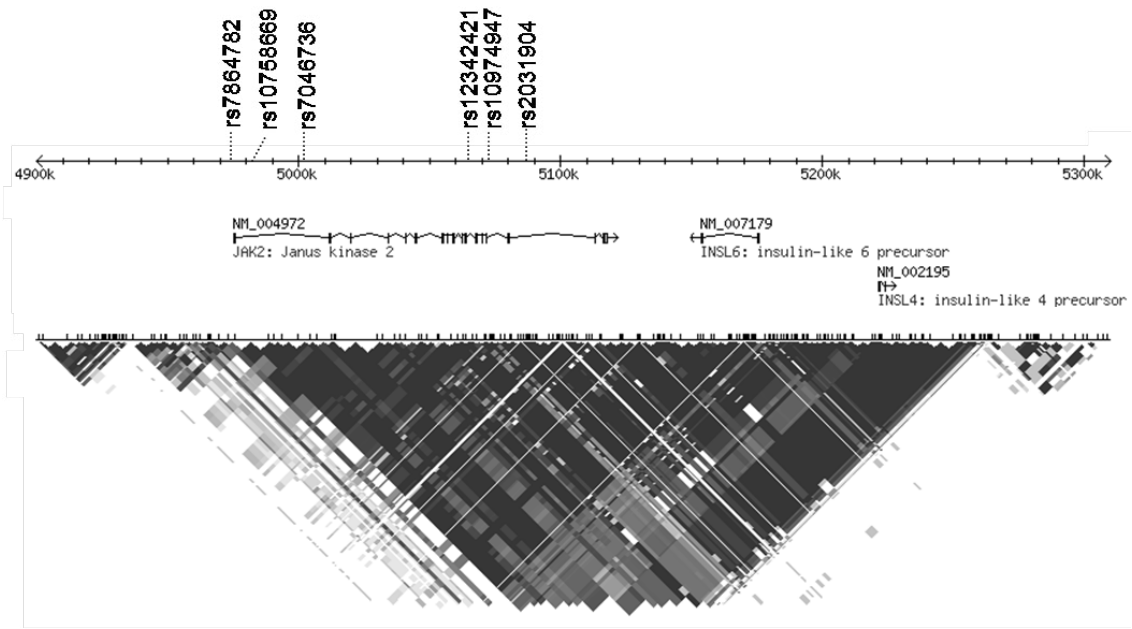


Figure 6-4. Genetic variation spanning the *JAK2* gene. The r^2 values, a measure of marker to marker LD, are plotted pairwise as boxes, and dark shading indicates high LD. This plot was generated using genotype data from the CEU population (diagram taken from the HapMap website, www.hapmap.org). The relative position of the six tag SNPs used to define variation across the *JAK2* gene are indicated.

In summary therefore, these results indicated that homozygosity for V617F was not seen randomly, but rather occurred preferentially when this mutation was present on the related *JAK2* haplotypes 3 and 5.

6.3.2 *JAK2* haplotypes predicted by SNP data from the WTCCC

To explore this observation in more detail, a more accurate haplotype structure of *JAK2* was determined using 14 SNPs genotyped by the Wellcome Trust Case Control Consortium (WTCCC) in 1500 UK healthy blood donors [369]. The allele frequency data was subject to analysis using a computer programme called PHASE. PHASE implements a Bayesian statistical method for reconstructing haplotypes from population genotype data [368]. The programme inferred a total of 92 haplotypes, of which nine accounted for 94% of *JAK2* alleles (Figure 6.5).

Haplotype number	**rs7864782**	**rs10124001**	rs10758669	**rs1327493**	**rs6476934**	**rs10815144**	rs7046736	**rs2149556**	rs12342421	**rs10974944**	**rs10119004**	rs10974947	rs12343867*	**rs12340895***	rs2031904	**rs10491652**	**rs11793659**	**rs17425637**	**rs17425819**	**rs10815160**	WTCCC frequency
1	G	C	C	C	T	A	A	T	G	G	A	G	C	G	G	C	G	T	T	T	0.124
8	G	C	A	C	T	A	C	T	C	C	A	A	T	C	A	C	A	C	C	G	0.165
32	G	C	.	C	T	G	.	C	.	C	G	.	T	C	.	C	A	C	C	T	0.083
38	G	C	A	G	G	A	C	T	C	C	A	A	T	C	G	C	A	C	C	G	0.021
46	A	C	C	C	T	A	A	T	G	G	A	G	C	G	G	C	G	T	T	T	0.117
55	A	C	.	C	T	A	.	T	.	C	A	.	T	C	.	C	A	C	C	G	0.035
71	A	C	A	C	T	G	C	C	C	C	A	G	T	C	G	C	A	C	C	T	0.023
76	A	C	A	C	T	G	C	C	C	C	G	G	T	C	G	C	A	C	C	T	0.359
88	A	T	A	C	T	G	A	T	G	C	G	G	T	C	G	C	G	T	T	T	0.011

Figure 6-5. The nine most common *JAK2* haplotypes in the UK population. The 14 SNPs in bold were analysed by the WTCCC in 1500 blood donors from which the frequencies were determined; asterisks indicate SNPs that tag 46/1. Rs78644782, rs10124001 and rs10758669 are immediately upstream of *JAK2*; all other SNPs are within *JAK2* introns.

I was then able to match up the six haplotypes predicted by Pardanani et al. (2008) [366] with the WTCCC haplotypes using SNPs genotyped in both studies where an allelic variant was unique to one particular haplotype. To aid the matching process, I designed pyrosequencing assays to genotype four additional SNPs, (rs6476934, rs2149556, rs10815160 and rs17425819). These SNPs were genotyped in homozygous V617F positive cases who displayed evidence of allele skewing following previous genotyping, and who

carried a haplotype (defined by Pardanani et al., 2008) other than haplotypes 3 or 5. This analysis revealed that WTCCC haplotype numbers 46 and 1 were identical to haplotypes 3 and 5 defined in Pardanani et al. (2008). As previously mentioned, this haplotype is identical within the region of the LD block that includes the *JAK2* gene (Figure 6.4) and are therefore henceforth referred together as '46/1.' Haplotype 46/1 has a combined frequency of 0.24 in the WTCCC population. Figure 6.5 shows that rs12343867 and rs12340895 both tag 46/1, i.e. the genotype for these SNPs is different for 46/1 compared to other haplotypes. Genotyping either of these SNPs can therefore be used as a surrogate to determine 46/1 status. SNP rs12340895 was chosen for this purpose as it performed better in pyrosequencing assays. For any individual, a G/G genotype for this SNP indicates homozygosity for 46/1; G/C indicates heterozygosity for 46/1 and C/C indicates that neither allele is 46/1.

6.3.3 Confirmation that homozygous V617F-positive MPN cases are reciprocally associated with the 46/1 haplotype

As described above, the association of the 46/1 haplotype with V617F positive disease was first determined in 73 PV cases with a homozygous level of mutation. These cases, plus an additional 69 cases of homozygous V617F-positive MPN (mutation burden >50%), were genotyped for the tag SNP rs12340895 to confirm the association of 46/1 with V617F. It was found that out of the 142 alleles that harboured V617F, 109 (77%) were 46/1 haplotype whereas this haplotype was seen for only 9 of the 74 (12%) residual wild-type alleles that could be read ($P = 1.4 \times 10^{-20}$, Fisher's exact test, 2-tailed). The frequency of 46/1 was significantly greater for V617F alleles compared to that seen in normal WTCCC controls ($P = 1.4 \times 10^{-34}$), and the frequency of residual 46/1 wild-type alleles was significantly less than WTCCC controls (P=0.007). These data confirm a huge excess of 46/1 alleles in homozygous V617F mutated MPNs, and that the *JAK2* mutation in these cases is usually on the 46/1 allele.

6.3.4 Genotyping of rs12340895 in healthy normal controls

In genetic studies it is important to compare genotypes between ethnically matched cases and controls. All patients in the MPN disease categories PV, MF, ET and unclassified MPN analysed in this study were drawn from the UK or US, and thus were assumed to be predominantly Caucasian. The WTCCC controls are therefore likely to be sufficient, however it was important to check that these data were representative and therefore a local cohort for 188 healthy controls (anonymised first degree relatives of individuals referred for a variety of non-malignant conditions) were genotyped for rs12340895. As described below, some MPNs were from Greece and so it was also necessary to determine allele frequencies in healthy normal cases from this country. The results (Table 6.4) show that there is no significant difference between the frequency of 46/1 in local controls and the WTCCC and that there is no difference between the UK and Greece.

	number of cases	genotype			alleles		allele frequency	
		CC	CG	GG	C	G	C	G
HapMap CEU European population							0.742	0.258
local healthy normal controls	188	103	78	7	284	92	0.755	0.245
WTCCC	1500				2280	720	0.760	0.240
Greek normal controls	108	60	41	7	161	55	0.745	0.255

Table 6-4. Allele genotypes and frequency of the *JAK2* SNP rs12340895 in healthy normal controls used in this study.

6.3.5 Heterozygous V617F-positive MPNs are also associated with the 46/1 haplotype

The 46/1 haplotype status was then determined in further cases carrying a lower level of V617F (<50% V617F). Heterozygous V617F-positive cases with a confirmed diagnosis of PV, ET and MF, plus other cases with an unclassified MPN, were subject to pyrosequencing for rs12340895. In some cases with PV and MF carrying a heterozygous level of V617F (i.e. %V617F ≤50%), it was possible to see the effect of a small homozygous

V617F positive clone, manifested as an allele distortion of the tag SNP, if the tag SNP was originally heterozygous. For these cases it was possible to infer the presence of the 46/1 haplotype by skewing of the allele ratio to the G allele of the tag SNP. The results from cases that showed allele skewing are shown in Table 6.5. Again, there is a marked excess of 46/1 associated with the V617F allele compared to the wild-type allele ($P=3.1x10^{-17}$ for all 163 cases).

	V617F allele		WT allele	
	46/1	not 46/1	46/1	not 46/1
PV	67	32	17	82
MF	10	10	1	19
Unclassified MPN	25	19	10	34
total	102	61	28	135

Table 6-5. Determining the haplotype associated with V617F in cases heterozygous (<50%) *JAK2* V617F-positive MPN disease entities, by tag SNP (rs12340895) genotyping. Allele frequencies that were either homozygous or skewed were used to determine the haplotype associated with V617F.

In total, analysis of 177 heterozygous V617F positive MPNs showed that the 46/1 haplotype was more frequent (135/354 alleles, 38%) compared to that seen in 188 locally sourced healthy controls (92/376 alleles, 24%; P = 0.0001) as well as the WTCCC cohort ($P =3.3x10^{-8}$). This confirmed that 46/1 is enriched in V617F positive MPNs irrespective of whether they harbour a homozygous or heterozygous mutant clone.

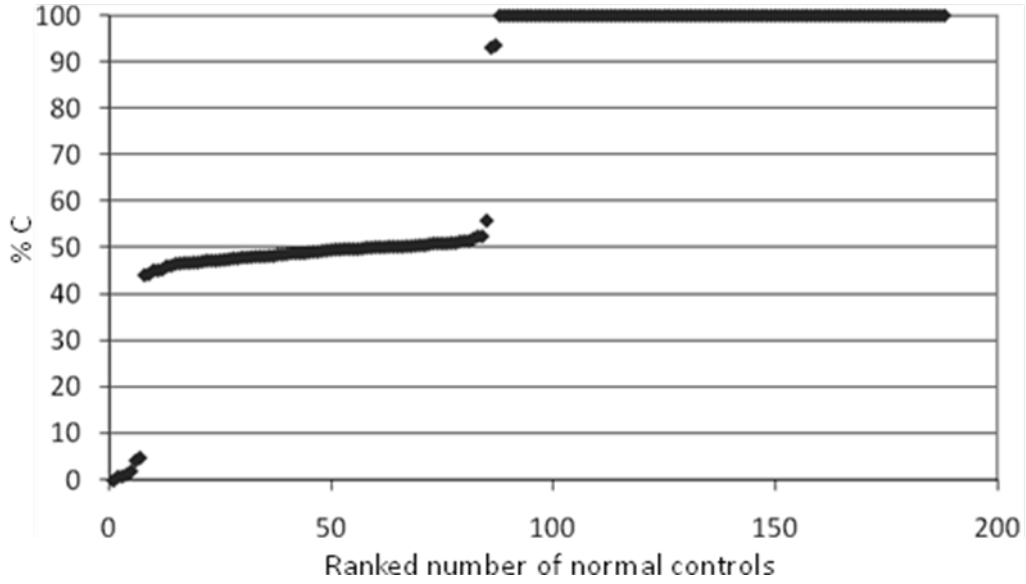
Table 6.6 shows the total allele frequencies of the 46/1 haplotype in the V617F-positive MPN disease subtypes and normal controls. Haplotype 46/1 was more frequent in all V617F positive disease entities regardless of origin (UK or USA) and the excess of 46/1 was highly significant for all disease subtypes. The allele frequencies seen in PV, ET and MF were very similar and the fact that the P value is higher in PV and lower in MF just reflects the number of cases that underwent analysis. There was no difference between allele frequencies seen in controls and cases with idiopathic erythrocytosis (n=76), a disorder characterised by marked erythrocytosis with a reactive origin.

	total cases	genotypes			allele frequencies (i)		minus cases >90%		allele frequencies (ii)		F of 46/1	P value (versus local controls)	OR (95% CI)	P value (versus WTCCC controls)	OR (95% CI)
		CC	CG	GG	C	G	C	G	N non 46/1 alleles	N 46/1 alleles					
PV[a] (UK)	192	36	100	56	172	212	1	15	171	197	0.54	2.88E-16	3.6 (2.6-4.8)	7.56E-30	3.6 (2.9-4.6)
ET (UK)	78	18	41	19	77	79	0	0	77	79	0.51	8.24E-09	3.2 (2.1-4.7)	4.27E-13	3.2 (2.4-4.5)
MF[a] (UK)	41	10	21	10	41	41	1	4	37	40	0.48	8.00E-05	2.9 (1.7-4.7)	6.12E-06	2.9 (1.9-4.6)
unclassified MPN[a] (UK)	124	23	62	39	108	140	2	5	106	135	0.56	3.33E-15	3.9 (2.8-5.6)	3.40E-24	4.0 (3.1-5.3)
V617F positive ET (GR)	143	42	78	23	162	124	n/a	n/a	162	124	0.43	4.51E-07[b]	2.4 (1.7-3.3)[b]	9.18E-12	2.4 (1.9-3.1)
Idiopathic erythrocytosis	76	40	31	5	111	41	n/a	n/a	111	41	0.27	0.58	1.1 (0.7-1.7)	0.437	1.2 (0.8-1.7)
UK controls	188	103	78	7	284	92	n/a	n/a	284	92	0.24	-	-	8.48E-01	1.0 (0.8-1.3)
WTCCC controls	1500	-	-	-	2280	720	n/a	n/a	2280	720	0.24	8.48E-01	1.0 (0.8-1.3)	-	-
GR controls	108	60	41	7	161	55	n/a	n/a	161	55	0.25	8.43E-01	1.1 (0.7-1.6)	6.22E-01	1.1 (0.8-1.5)

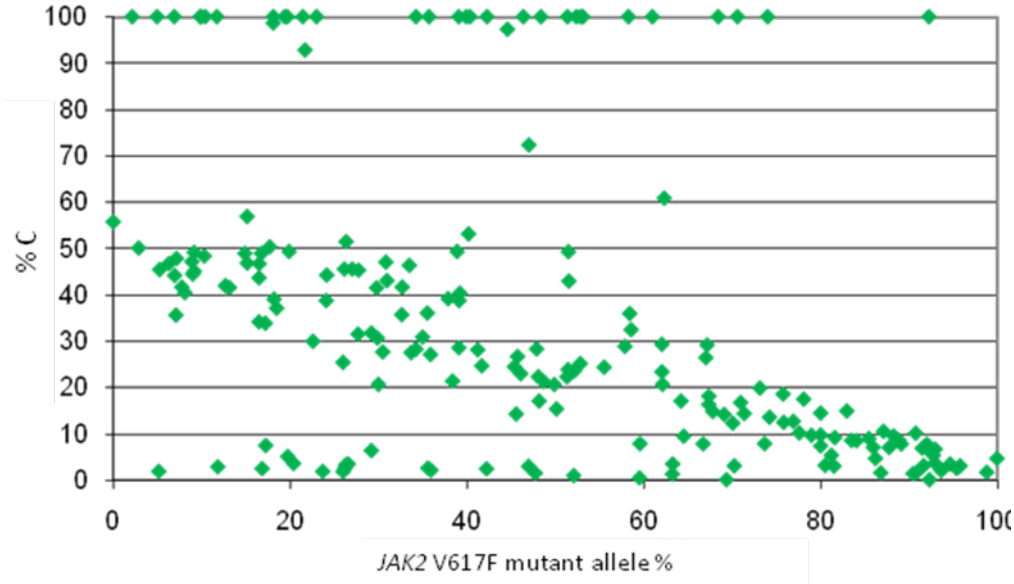
Table 6-6. (Previous page) Summary of genotyping results. Genotypes and allele frequencies (i) for SNP rs12340895 (G allele tags the 46/1 haplotype, C allele tags all other haplotypes) shown for each *JAK2* V617F-positive MPN disease category; PV, MF, ET and unclassified MPN. Groups marked [a] contain some cases with ≥90% V617F and thus the residual wild-type allele could not be assigned as 46/1 or not 46/1. Number (N) of the allele frequencies (ii) of V617F arising on the 46/1 haplotype and V617F arising on all other backgrounds are indicated, alongside levels of significance achieved in comparison to the UK local controls and the WTCCC controls, and the respective OR of these comparisons. Frequency (F) of 46/1 for each group is indicated. P values were calculated using Fisher's exact test, 2-tailed. GR, Greek samples; UK, United Kingdom samples; [b] values versus healthy Greek controls.

To illustrate the association of V617F with the 46/1 haplotype, Figure 6.6 depicts allele distributions of the tag SNP rs12340895 for controls and the V617F positive cases. In the 188 local normal controls, the SNP allele frequency is plotted in a ranked fashion, and this plot shows the common allelic variant is the C, with very few cases homozygous for the G allele (Figure 6.6A). When the allele frequencies for the 192 PV cases are plotted against level of V617F, it is apparent that the normal distribution is not observed, but there is an asymmetrical distortion towards the G allele, the allele that tags the 46/1 haplotype (Figure 6.6B). Indeed, this bias for the G allele is emphasised by the observation that many V617F positive cases were homozygous for the G allele, in stark contrast to the matched population of normal controls. There was also an over-representation of the G allele in ET (Figure 6.6 C), and MF (Figure 6.6 D). Allele skewing resulting from reduction to homozygosity is a rare event in ET, indicated by the relatively lower level of V617F in this disease.

A. Normal controls



B. PV



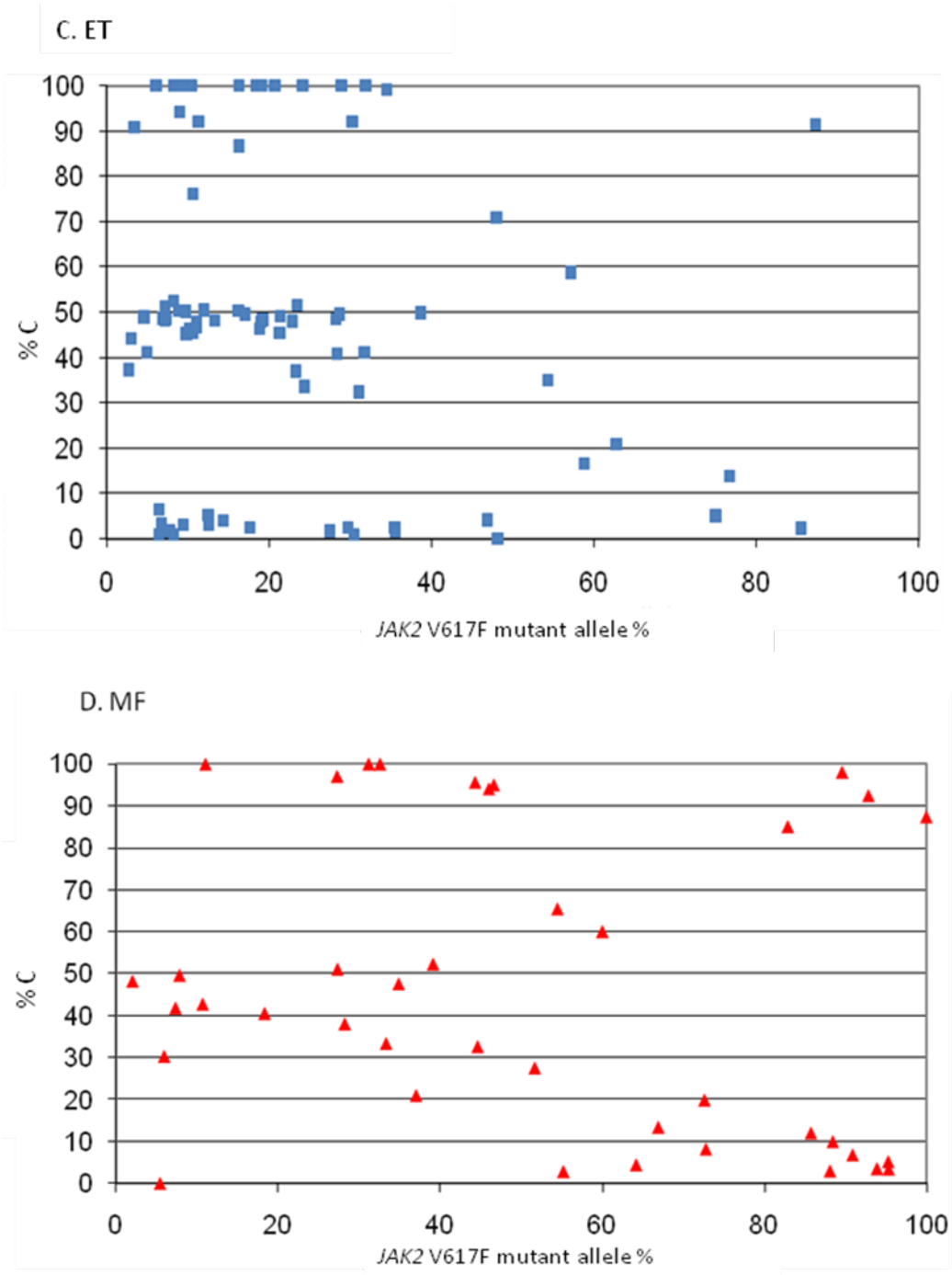


Figure 6-6. Scatter plots showing the allele distribution of the *JAK2* 46/1 tag SNP rs12340895. The graphs show genotype of the SNP (% C) in ranked order for 188 local normal controls (A), and against level of the *JAK2* mutation (% mutant allele) for V617F positive PV cases (B), V617F positive ET (C) and V617F positive MF (D).

6.3.6 Determining the risk of developing MPN conferred by the 46/1 *JAK2* haplotype

The fact that 46/1 is more frequent in V617F positive MPNs compared to controls indicates that this haplotype predisposes to these diseases. The magnitude of this predisposition is indicated by the OR and RR. The ORs for each disease entity are shown in table 6.6 and the data for all V617F positive disease is summarised in table 6.7. Based on the results from these analyses, it was determined that *JAK2* 46/1 confers an OR of 3.7 (95% CI 3.1-4.3), and a RR of 2.6 (95% CI 2.3-2.9), indicating that 46/1 is a strong predisposition factor for development of V617F associated MPNs.

	G	C	total
V617F positive cases	465	405	870
WTCCC	720	2280	3000
totals	1185	2685	3870

Table 6-7. 2 X 2 contingency table showing the allele frequencies for the tag SNP rs12340895 from 435 cases with *JAK2* V617F positive MPN and 1500 normal controls from the WTCCC. The risk allele G tags the 46/1 haplotype.

The counts of 46/1 alleles in cases together with corresponding counts in WTCCC controls and the population frequency of 46/1 from the WTCCC data were then used to determine the population attributable risk (PAR). Put simply, PAR measures the reduction in incidence that would be observed if the population had no 46/1 alleles, compared with its actual haplotype distribution. When compared to the frequency of the risk factor (46/1 allele; 53.4%) in the general population (WTCCC; 24%), the PAR for the 46/1 allele was calculated to be 27.8% [370]. Recently results from a Swedish epidemiological study have demonstrated that there is a 5.7 to 7.5 fold elevated risk of MPN among first degree relatives of MPN patients, suggesting there are common, strong, shared susceptibility genes predisposing to PV, ET and MF [272]. Data provided by Landgren et al. (2008) (RR 5.7 to 7.5) shows the range of PAR in their study was 53% to 61%. Assuming no difference between the UK and Swedish populations, the 46/1 haplotype thus accounts for 50% of the increased risk in first degree relatives.

6.3.7 Allele-specific PCR capturing a second tag SNP to determine if V617F arose on the 46/1 haplotype

The results so far have shown that both homozygous and heterozygous *JAK2* V617F positive MPNs are linked to an excess of the 46/1 haplotype. In homozygous V617F cases it was clear that V617F was generally on the 46/1 allele in cases that were heterozygous for 46/1, however in cases without any allele distortion it was not clear if V617F was on the 46/1 or non-46/1 allele.

To determine if V617F was generally in *cis* or *trans* to the 46/1 allele, cases were selected that were heterozygous for both the mutation and the haplotype for analysis. The SNP rs12343867 C allele tags the presence of the 46/1 haplotype (Figure 6.5) and lies just 410 bp from the *JAK2* V617 codon. An AS-PCR could therefore be designed which detected both the tag SNP and V617F (Figure 6.3).

In total AS-PCR was performed on 66 informative heterozygous V617F positive cases and the results are shown in table 6.8. Sequencing the products revealed that 49 (74%) V617F alleles arose on a 46/1 allele whereas only 17 (26%) residual wild-type alleles were 46/1 ($P=2.1x10^{-8}$). In both heterozygous and homozygous V617F cases, it is clear therefore that the V617F mutation tends to be on the 46/1 allele.

	V617F allele		WT allele	
	46/1	not 46/1	46/1	not 46/1
PV	17	6	6	17
ET	16	2	2	16
MF	4	3	3	4
Unclassified MPN	12	6	6	12
totals	49	17	17	49

Table 6-8. Results from AS-PCR for tag SNP rs12343867 on informative V617F heterozygotes without allele distortions.

6.3.8 Does the 46/1 haplotype affect expression of *JAK2*?

One possible reason for the observed association might be that *JAK2* on the 46/1 haplotype is expressed at a different level to *JAK2* on other haplotypes. To explore this possibility, pyrosequencing primers were designed to quantify the allele ratios of two *JAK2* exonic SNPs (rs10429491 and rs2230724) in matched cDNA and genomic DNA from control V617F negative MPN cases that were heterozygous for at least one of the exonic SNPs, as well as being heterozygous for 46/1.

In total 46 cases were studied; 29 cases heterozygous for rs2230724, and 40 cases heterozygous for rs10429491 (some cases were heterozygous for both SNPs). The cDNA specific primers were designed with one amplification primer crossing over an exon-exon boundary and were confirmed to be cDNA-specific by testing on genomic DNA. In DNA the allele ratio is expected to be normally distributed around 50%, whereas in cDNA any expression difference associated with 46/1 compared to other haplotypes would be expected to result in deviations from 50%, e.g. if *JAK2* on 46/1 was expressed 1.5 fold higher than *JAK2* on other haplotypes then the cDNA allele ratio would be 60%, if 2 fold higher then 67% etc.

A comparison of the variances (F-test) or means of the allele ratios (t-test) between cDNA and genomic DNA for each individual with either SNP did not reveal any significant differences (rs2230724, p=0.16; rs10429491, p=0.98). This indicates that 46/1 is not

associated with either increased or decreased *JAK2* expression, at least in peripheral blood leukocytes from individuals with *JAK2* V617F negative MPN (Figure 6.7).

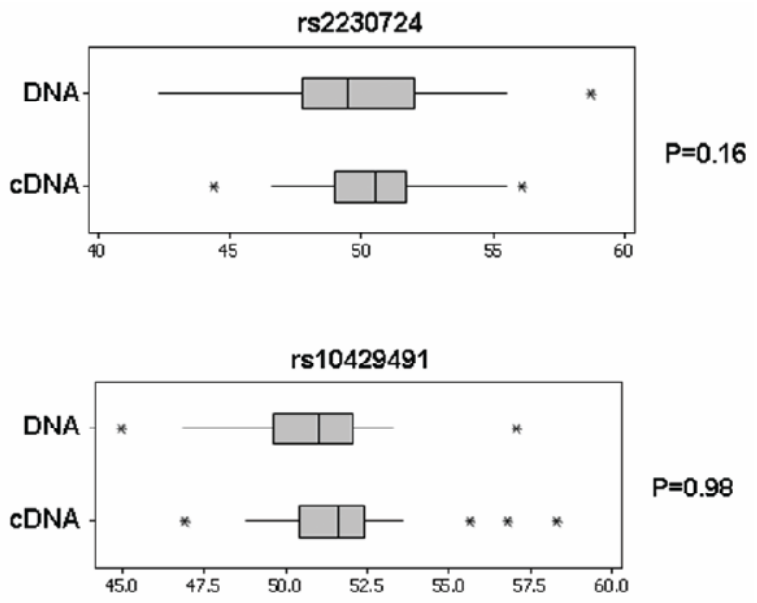


Figure 6-7. No expression differences between *JAK2* on 46/1 compared to other alleles. Box plots showing allele ratios in V617F negative, 46/1 heterozygous controls (n=46) that were also heterozygous for rs2230724 (n=29, top panel) and/or rs10429491 (n=40, bottom panel). There was no difference between the allele ratio variances (F-test, P values shown) or means (t-test) between cDNA and DNA for either SNP indicating no detectable expression differences associated with 46/1.

It may be postulated any functional variant which predisposes to V617F positive MPN may exist only on a subset of 46/1 alleles, and perhaps any discernable *JAK2* expression differences would be missed by examining V617F negative cases. However as the frequency of 46/1 is 0.24 in normal controls, and approximately 0.5 in V617F positive MPNs, this means an excess of 0.26 in MPNs which may carry the 46/1 allele with the predisposing factor. Based on this, this subset is more likely to be enriched in V617F positive cases. *JAK2* expression differences were then investigated in V617F positive MPNs, and only heterozygous mutation positive cases with no evidence of a homozygous clone were selected for analysis. As above, the variances (F-test) or means of the allele ratios (two sample t-test) between cDNA and DNA for 14 cases were compared, for exonic SNPs rs10429491 and rs2230724. Interestingly, the difference between DNA and cDNA mean allele ratios for SNP rs10429491 was just significant (t-test p=0.04), but not significant for rs2230724 (t-test p=0.191) (Figure 6.8). This statistical test assumes a normal distribution of data; if the non-parametric Mann-Whitney test is used the difference for rs10429491 falls just below the level of significance (p=0.065).

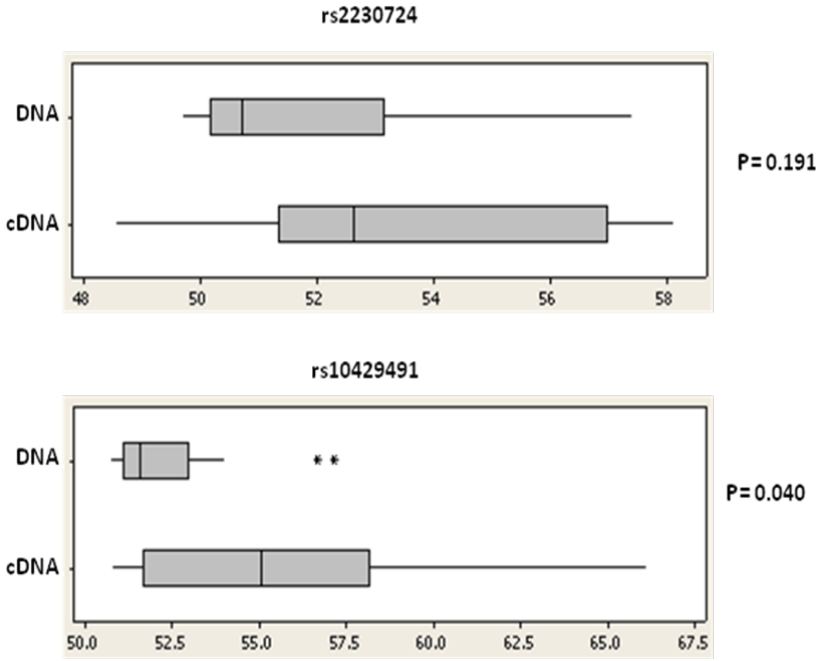


Figure 6-8. Expression differences between DNA and cDNA for two exonic *JAK2* SNPs in MPN cases with *JAK2* V617F on 46/1. Boxplots showing the mean allele variances between DNA and cDNA were compared in *JAK2* V617F positive cases carrying one or more copies of 46/1, for rs2230724 and rs10429491. A small difference between DNA and cDNA was observed for rs10429491, but not rs2230724 (t-test p values are shown).

The finding of a significant difference between DNA and cDNA for rs10429491 could allude to subtle functional differences conferred by 46/1 in only a subset of V617F positive cases carrying 46/1. The rs10429491 mean allele ratios for DNA and cDNA were 52.4 and 55.8, respectively, indicating a difference of only 3.4%. However, this was not the case for rs2230724, and it would be expected both SNPs would show a difference if there was a real effect of 46/1 on expression. The rs10429491 expression increase is very marginal, and although the data is far from convincing, it does provide the rationale for examining further cases.

6.3.9 Is the sequence of *JAK2* on 46/1 the same as that on other haplotypes?

To determine if the primary sequence of *JAK2* on 46/1 was the same as other haplotypes all 25 exons were sequenced, including those encoding the 5' and 3' untranslated regions, in eight MPN cases with V617F on the 46/1 allele and eight healthy controls that were negative for 46/1. No sequence variants were detected, except for the two previously described silent polymorphisms rs10429491 and/or rs2230724 in exons 6 and 19, respectively. To determine if these exonic SNPs or other unknown intronic variants might affect splicing overlapping RT-PCRs were performed to cover all exon-exon junctions. Sequencing of the products did not reveal any differences between *JAK2* mRNA in MPNs with V617F on 46/1 (n=8) and controls without 46/1 (n=8) (primers listed in Appendix III).

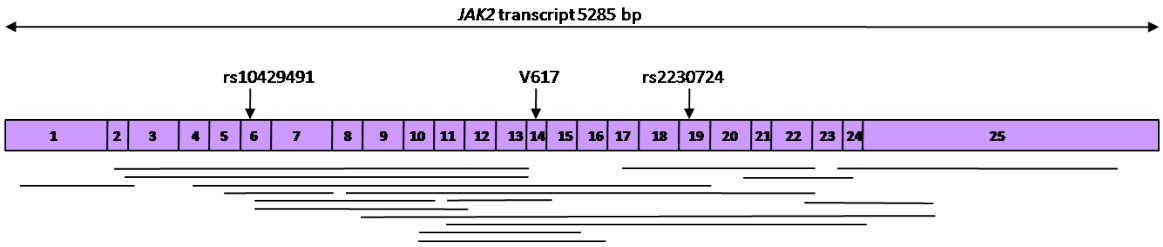


Figure 6-9. Illustration of overlapping RT-PCR products covering the *JAK2* transcript. *JAK2* contains 25 exons and all exon-exon boundaries were amplified by a series of RT-PCRs (horizontal lines) designed to detect any alternative splice variants. Location of V617, and coding SNPs rs10429491 and rs2230724 are shown.

6.3.10 Does *JAK2* haplotype status influence myeloid colony formation in haematopoietically normal individuals?

To investigate the possibility that *JAK2* on the 46/1 haplotype is different functionally from other *JAK2* alleles, a preliminary experiment was performed to determine if its effect was visible *in vitro.* JAK2 is required for signaling downstream of a variety of receptors for cytokines that are critical for myelopoiesis (e.g. IL-3, G-CSF, GM-CSF, EPO) as well as other receptors that are active in lymphoid and non-haemopoietic cells [371]. It is

widely known that myeloid colony formation varies widely between individuals, so I investigated whether *JAK2* haplotype status had a measurable effect on myeloid colony formation in haematologically normal individuals. In a prospective analysis, PB samples were obtained that were less than 4 days old from 104 anonymised haematologically normal controls. MNCs were plated in semi-solid medium containing myeloid cytokines and EPO and after 14 days the number of colonies were counted and characterised based on morphology as CFU-GM or BFU-E. The *JAK2* haplotype status was determined after colonies were counted using DNA extracted from the MNC or granulocyte cell fractions by genotyping the 46/1 tag SNP rs12340895.

Out of 104 cases studied, 102 successfully grew myeloid colonies and were included in the analysis. Table 6.9 shows a summary of the results from this investigation. The allele frequency for the tag SNP was C: 0.72, G: 0.27 which was not significantly different to the normal controls.

	CFU-GM	BFU-E	total colony number
range	0-146	3-489	7-602
median	30.0	102.0	122.5
mean	36.2	129.4	162.4

Table 6-9. Summary of myeloid colony type and number from 102 haematopoietically normal individuals.

The colony number and type were then grouped according to *JAK2* haplotype status. The numbers of CFU-GM and BFU-E colonies without the 46/1 haplotype was compared to the colony numbers from those that carried either one or two copies of the 46/1 haplotype (the number of cases that were homozygous for 46/1 was too small to analyse as a single entity). The median colony numbers of CFU-GM, BFU-E and total colony count for the two genotype groups were compared using the Mann-Whitney U test (Table 6.10 and Figure 6.10).

	not 46/1 (54)			one or two copies of 46/1 (48)			
	range	mean	median	range	mean	median	P value
CFU-GM	1-137	39.9	37.0	0-146	32.0	26.0	0.03
BFU-E	3-489	132.8	111.5	4-378	125.6	98.5	0.947
total colony number	7-602	172.6	138.0	7-484	157.5	121.0	0.594

Table 6-10. Myeloid colonies grown in semi-solid media from PB from haematopoietically normal individuals, grouped according to 46/1 *JAK2* haplotype status. The table shows the number (range), mean and median CFU-GM and BFU-E colonies counted after 14 days incubation, for cases nullizygous or heterozygous/homozygous for the 46/1 allele. Number of cases for each category is shown in brackets.

Despite a wide range of values, this data showed that individuals who carried at least one 46/1 allele grew significantly fewer CFU-GM than those who did not (p=0.03, Mann Whitney U test). There was no effect, however, on BFU-E growth (Figure 6.10). Although it is not obvious why this should be, this result is consistent with the hypothesis that *JAK2* on 46/1 is indeed functionally different from other *JAK2* alleles.

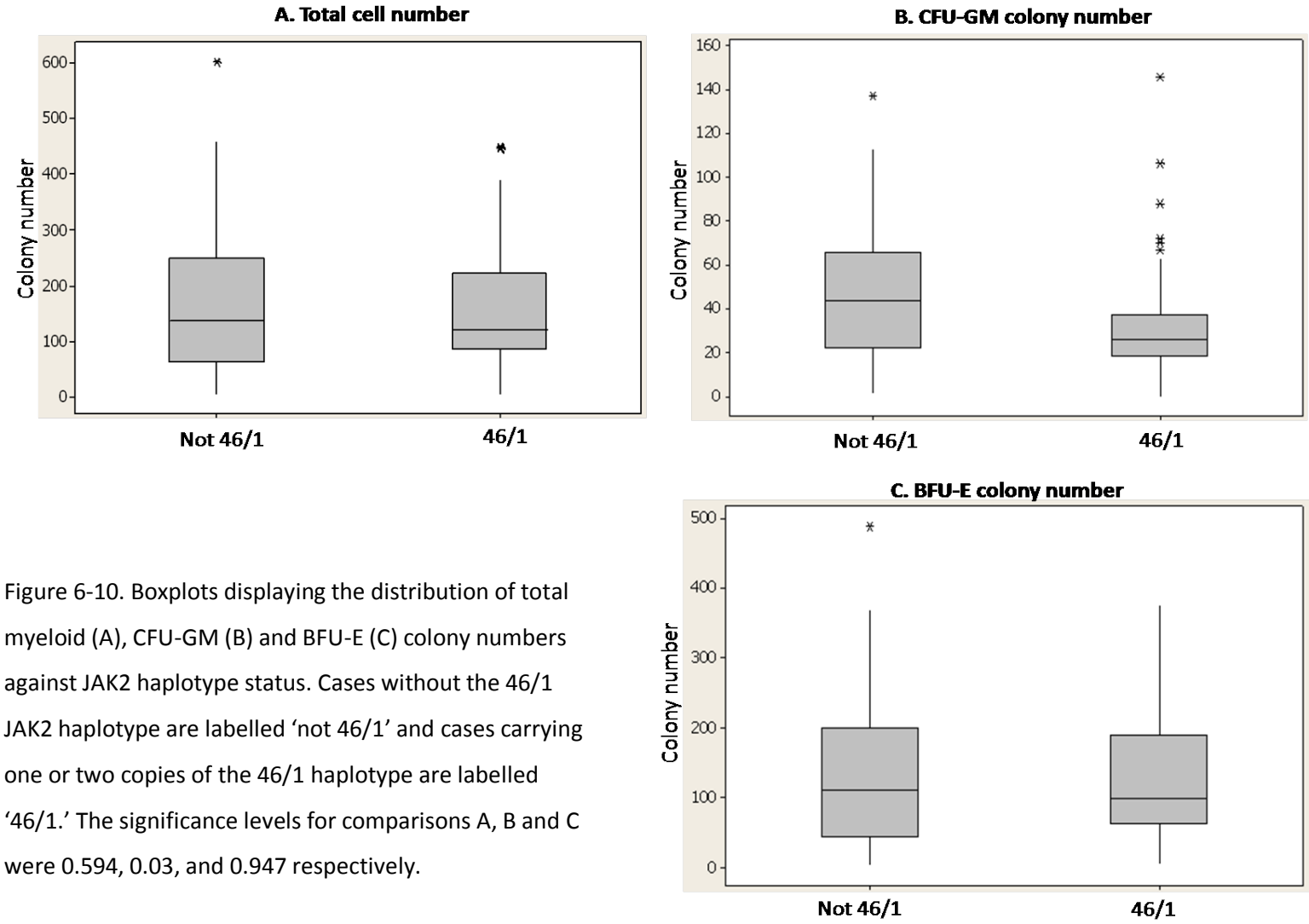


Figure 6-10. Boxplots displaying the distribution of total myeloid (A), CFU-GM (B) and BFU-E (C) colony numbers against JAK2 haplotype status. Cases without the 46/1 JAK2 haplotype are labelled 'not 46/1' and cases carrying one or two copies of the 46/1 haplotype are labelled '46/1.' The significance levels for comparisons A, B and C were 0.594, 0.03, and 0.947 respectively.

6.3.11 The relevance of the 46/1 haplotype in *JAK2* V617F negative MPN

The possibility that a functional variant might also be relevant to the pathogenesis of V617F negative MPNs was explored. I genotyped tag SNP rs12340895 in V617F negative ET and MF cases from the UK (n=47) and found 37/94 alleles were 46/1, which was significantly greater than the frequency observed in locally sourced controls (P=0.009) as well as the WTCCC cohort (P=0.002) (Table 6.11).

This apparent association was then investigated in a larger group of V617F negative ETs, provided by Dr K. Zoi (Athens, Greece). Rs12340895 allele frequencies of 136 V617F negative ETs were compared to matched controls (n=108) from Greece. This analysis failed to confirm a significant association between 46/1 and V617F negative MPN, however the low P value (P=0.09) suggests that the relevance of 46/1 to V617F negative cases warrants further investigation.

The *JAK2* 46/1 haplotype was also investigated using tag SNP genotyping in further V617F negative MPN entities (n=497); HES, *FIP1L1-PDGFRA* positive CEL, *KIT* D816V-positive and negative SM and *BCR-ABL*-positive CML cases sampled at chronic phase. As shown in Table 6.11 there was no significant association between 46/1 and any of these diseases.

		SNP genotypes			SNP allele frequencies		P value (versus matched controls)
	Number of cases	CC	CG	GG	C	G	
V617F negative MPN (UK)	47	17	23	7	57	37	0.009
V617F negative ET (GR)	136	58	67	11	183	89	0.09
HES	88	46	38	4	130	46	0.67
FIP1L1-PDGFRA positive CEL	32	17	11	4	45	19	0.44
SM	88	49	34	5	132	44	0.92
KIT D816V positive SM	42	21	19	2	61	23	0.58
*BCR-AB*L positive CML	247	70	53	7	193	67	0.09
UK controls	188	103	78	7	284	92	-
WTCCC controls	1500				2280	720	-
Greek controls	108	60	41	7	161	55	-

Table 6-11. Distribution of the 46/1 haplotype in *JAK2* V617F negative MPN. Genotype and allele frequencies for rs12340895 (G allele tags the 46/1 haplotype, C allele tags all other haplotypes) shown for each *JAK2* V617F negative MPN disease category. All MPN cases were obtained from local centres and the levels of significance achieved in comparison to UK controls are indicated, except the Greek *JAK2* V617F negative ETs were compared to matched controls from Greece. P values were calculated using Fisher's exact test, 2-tailed.

6.3.12 Investigating *JAK2* haplotype status in familial MPN

It has been previously shown that *JAK2* V617F is not inherited in affected individuals from families with MPN, but that an unknown factor predisposing to acquiring the mutation must be responsible instead [204,364,372]. Through collaboration with Dr Holger Cario and Dr Heike Pahl, I was able to investigate the *JAK2* haplotype status of a PV pedigree in which V617F arose independently in two affected individuals [373]. Family members were analyzed for the 46/1 haplotype tag SNP rs12340895 and, as shown on Figure 6.11, one affected individual (UPN 534) was heterozygous for 46/1, but the second (UPN 533) was negative for this haplotype. Allele-specific PCR for UPN 534 revealed that V617F had arisen on the 46/1 allele, confirming the association between this haplotype and the

mutation. This shows that 46/1 is not solely responsible, at least in this family, for predisposition to PV.

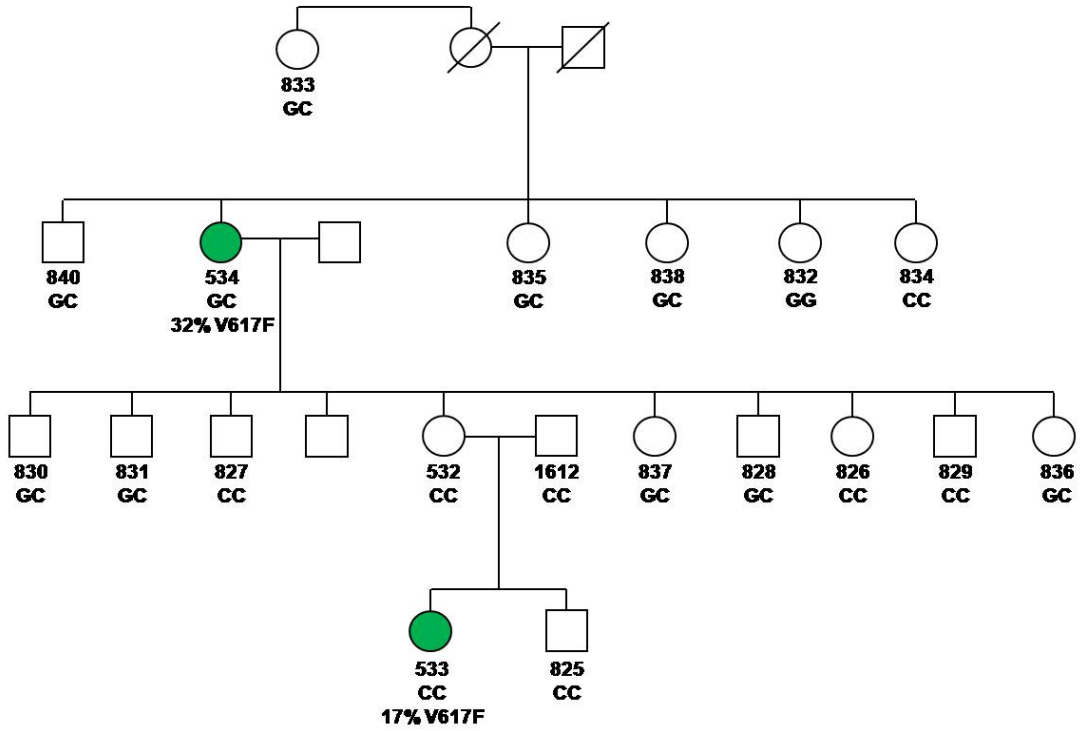


Figure 6-11. Familial PV pedigree genotyped for the *JAK2* 46/1 haplotype. The two affected individuals (UPNs 534 and 533) are shown as green circles. The genotype for rs12340895 is shown (G = 46/1 allele; C = non-46/1 allele), plus the %V617F in affected cases. Allele-specific PCR for UPN 534 showed that V617F arose on the 46/1 allele. All other cases were negative for V617F, *PRV1* expression, endogenous erythroid colony growth and had normal blood counts [373].

6.4 Discussion

6.4.1 Estimation of homozygous clone size using flanking SNPs

Many blood samples from MPN cases will comprise a mixture of three types of cell: normal *JAK2*, heterozygous V617F and homozygous V617F. The %V617F measures the total proportions of mutant and wild-type alleles but gives limited information with regard to the relative proportions of heterozygous and homozygous cells. The maximum %V617F for a heterozygous clone is 50% and so levels higher than this must indicate that a homozygous clone is present. For %V617F levels <50%, however, the situation is less clear. As illustrated in Figure 6.12A and B, the %V617F may be identical in cases with a large heterozygous clone and those with a small homozygous clone. These two situations may be distinguished by an analysis of flanking SNPs. Pyrosequencing is readily able to distinguish differences in allele ratio of 10% and thus analysis of flanking SNPs can detect homozygous clones that constitute only 20% of the total cell population (Figure 6.12C).

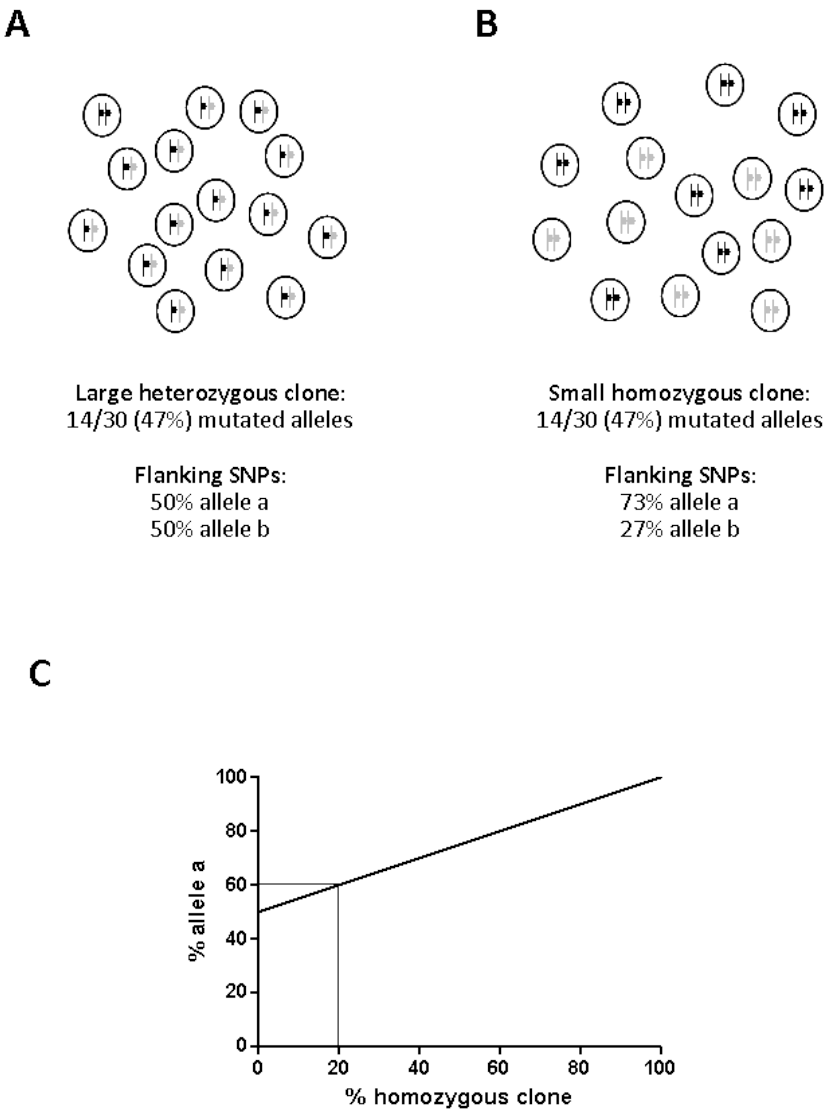


Figure 6-12. Detection of small homozygous clones using flanking SNPs. Panels A and B illustrate a large heterozygous clone and small homozygous clone, respectively. Fifteen cells with two chromosomes (wild-type, black; V617F, grey) each are shown for each clone. The proportion of mutant chromosomes (%V617F) is identical in both scenarios. Measurement of the allele ratios of flanking SNPs, however, clearly distinguishes the two. C. Graph illustrating the relationship between homozygous clone size and skewed allele ratios of heterozygous flanking SNPs. Pyrosequencing can reliably detect differences in allelic ratios of 10% and therefore a homozygous clone that comprises at 20% or more of the total cell population can be detected. The percentage of mutant alleles depends on the size of both the homozygous and the heterozygous mutated clones, but in the extreme situation of 80% normal cells, 20% homozygous and 0% heterozygous mutated cells the total percentage of mutated alleles is only 20%. Thus, measurement of flanking SNPs can detect a small homozygous clone in cases with only 20% V617F.

The time scale of the progression from heterozygosity to homozygosity is not well defined, but there are two theoretical extremes, outlined in figure 6.13. Firstly evolution to homozygosity may occur slowly so that a heterozygous clone expands steadily and achieves a large size before the homozygous clone begins to grow out and eventually predominate. In the second situation, the transition to homozygosity occurs rapidly, without the significant expansion of the heterozygous clone.

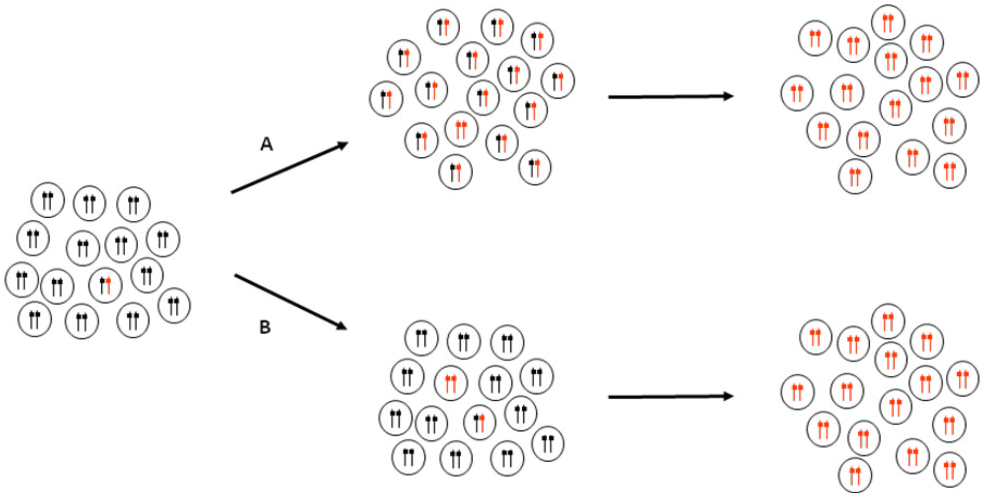


Figure 6-13. Clonal cell populations in *JAK2* V617F positive PV and MF. The *JAK2* mutation arises in one cell, (red) and confers a selective advantage, driving clonal expansion. If a heterozygous V617F-positive cell then acquires a second hit (acquired UPD at 9p), the new homozygous clone may expand at a faster rate than the heterozygous clone. The transition to may occur slowly (A) giving time for a heterozygous clone to expand, or rapidly (B) with minimal chance of a heterozygous clone building before the homozygous clone takes over.

The theoretical effect of progression of V617F from a heterozygous to homozygous state on flanking SNPs is illustrated in Figure 6.13. Considering the situation B in Figure 6.13 where transition to homozygosity occurs early in the evolution of the disease, the allele ratio of heterozygous SNPs in the region affected by acquired UPD would be expected to steadily change from 50% to either 100% or 0% over time depending on which chromosome V617F arose (lines 'N + Homozygous V617F' on Figure 6.14). For situation A in Figure 6.13, the heterozygous clone increases is size until it reaches a maximum of 50%

V617F when all cells in the sample are heterozygous for the mutation. During this time there is no change in flanking SNP ratios (line 'N + Heterozygous V617F' on Figure 6.14). A homozygous clone then begins to grow out and the flanking SNP ratios change steadily to 100% or 0% (lines 'Heterozygous + Homozygous V617F on Figure 6.14). For any given sample, therefore, it is expected that that flanking heterozygous SNP ratios will fall somewhere in the blue arrowhead. This theoretical scenario accords very well with that actually seen in patients (Figure 6.6), although due to the preference for V617F to arise on 46/1, one half of the arrowhead predominates (seen most clearly for PV in Figure 6.6b).

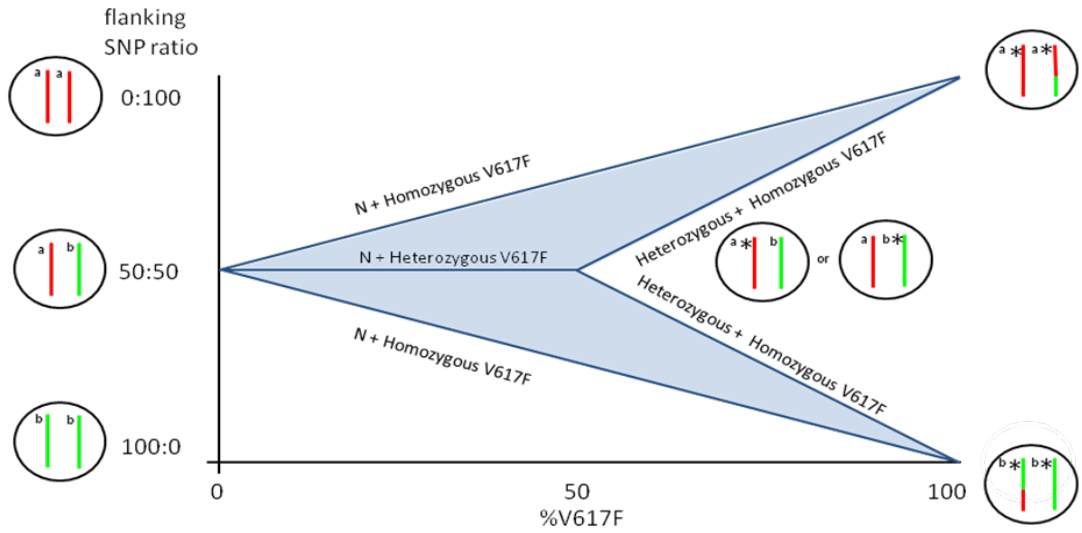


Figure 6-14. Homozygous *JAK2* V617F distorts heterozygous SNP allele ratios. See text for details.

6.4.2 V617F-associated disease is preferentially associated with the 46/1 *JAK2* haplotype

This study describes the molecular characterisation of an inherited predisposition to myeloproliferative disease, specifically the finding that the risk of developing an MPN positive for the *JAK2* V617F point mutation is associated with a particular constitutional *JAK2* haplotype. Moreover, this association is comparatively strong, in comparison to other predisposition alleles identified in genome-wide association studies. Overall there was a three- to fourfold greater chance of developing V617F-positive MPN in individuals that carry the haplotype compared to those that did not (OR of 3.7 [95% CI 3.1-4.3] and RR of 2.6 [95% CI 2.3-2.9; n=435]), both calculated using cases versus WTCCC controls. Initially the analysis focused on homozygous V617F cases, exploiting the fact that distorted flanking SNP allele ratios could be used to directly read the haplotype on which V617F resided as well as the residual haplotype that did not acquire V617F. Seventy seven percent of homozygous V617F-positive cases carried V617F on the 46/1 haplotype and using tag SNPs it was subsequently shown that this haplotype was also overrepresented in heterozygous V617F positive cases. This result was highly significant, and contrasted strongly with results initially published by Pardanani et al. (2008) who did not see this association [366]. There are two principal factors that may account for this difference. First, although *JAK2* V617F status was considered as an independent variable for the statistical analysis in the Pardanani study, the V617F mutation burden and the SNP allele skewing brought about by acquired UPD in homozygous cases was not considered. Second, the results from SNP allele frequencies for each test group were compared statistically to the relatively small Caucasian population defined by HapMap, not a matched group of controls with a similar ethnicity. A 46/1 tag SNP (rs10758669) was reported by Pardanani et al. to be significantly associated with PV but not with ET or MF compared to haplotype controls. This result is presumably explained by the relatively high prevalence of V617F in PV compared to the other two subtypes.

Far from providing evidence for phenotypic pleiotropy in MPN as first suggested by Pardanani et al. (2008), the findings in this study provide the first evidence of a constitutional factor predisposing to acquisition of a specific mutation. Furthermore, the counts of 46/1 alleles in cases, along with corresponding counts in WTCCC controls and

the population frequency of 46/1 from the WTCCC data, suggest that 46/1 accounts for approximately 28% of the population attributable risk [370]. A recent study from Sweden has demonstrated a RR of 5.7-7.5 in first degree relatives of MPN patients corresponding to an attributable risk of 53-61% [272]. Assuming no difference between the UK and Swedish populations, the 46/1 haplotype thus accounts for 50% of the increased risk in first degree relatives, indicating that additional factors predisposing to these diseases remain to be identified. As the association of the 46/1 haplotype with V617F-positive disease may account for only half of this risk of sporadic MPN, these finding provide a good basis for undertaking a wider investigation to identify other common low penetrance predisposition alleles, particularly for V617F negative disease. This could be accomplished in a genome-wide association study.

The finding that certain SNPs and haplotypes are associated with common disease has been well defined in the literature. Many publications report the localisation of common SNPs associated with a wide range of malignant and non-malignant diseases and diverse clinical conditions, such as type 1 and type 2 diabetes, obesity, colorectal and breast cancer [374-378]. Some associations with leukaemia have also been described such as a particular haplotype of the pro-apoptotic gene *BCL2* was linked to increased susceptibility of developing CML [379], and polymorphisms in apoptosis- and immunoregulation-related genes have been shown to be associated with the risk of CLL [380-382]. Characterisation of disease-associated loci may lead to the identification of causal variants that are in LD with the significant SNPs.

In some instances the causal mechanism behind the association between trait and a particular SNP has been elucidated. Polymorphisms in the promoter regions of a gene may affect expression, whereas polymorphisms in the gene may alter mRNA stability or protein structure. For example, functional polymorphic loci are well characterised in cytokine and cytokine receptor SNPs [383]. Additionally, the functional consequence of a common polymorphism in the complement factor B gene was recently found to confer a disease-protective effect against age-related macular degeneration [384].

6.4.3 How is the 46/1 haplotype associated with *JAK2* V617F disease?

These results show that the 46/1 haplotype predisposes to V617F-positive disease, but underlying mechanism is unclear. There are two hypotheses that may account for the association of V617F disease with 46/1:

1. V617F may arise randomly on all haplotypes but 46/1 is in LD with an unknown constitutional functional variant that interacts with V617F in a manner that makes the development of clinically manifest disease more likely compared to V617F on a non-46/1 haplotype (fertile ground hypothesis).
2. There is a specific mutational mechanism by which V617F preferentially arises on a 46/1 haplotype (hypermutability hypothesis).

These hypotheses are not necessarily mutually exclusive and are considered below.

Inspection of the HapMap data (www.hapmap.org; Figure 6.4) indicates that the entire *JAK2* gene is contained within a 280kb LD block that includes two other genes located at the 3' end; *INSL4* and *INSL6.* In principle, 46/1 would be linked to a functional SNP that affects any of these three genes. The expression of both *INSL4* and *INSL6* was investigated by RT-PCR performed on cDNA from PB granulocytes from two V617F-positive PV cases and two normal controls. No product was amplified, strongly suggesting that both genes are not expressed in haemopoietic cells. Indeed, a search using the Gene Expression Omnibus website managed by NCBI (http://www.ncbi.nlm.nih.gov/geo/) revealed both genes are not expressed in haematopoietic tissue. It is highly likely therefore, that any functional variant within 46/1 directly affects *JAK2*.

6.4.4 Evidence for a functional variant linked to the *JAK2* 46/1 haplotype

The first hypothesis suggests that V617F arises at the same rate on different *JAK2* haplotypes, but if the mutation is on 46/1, the cell gains a stronger selective advantage compared to V617F on other haplotypes. Alternatively it is possible that the selective advantage is the same but there is a greater chance of abnormal blood counts developing if V617F is on 46/1. Either scenario might be explained by a functional difference of *JAK2* on 46/1, e.g. altered gene expression or protein function.

To look for a possible functional difference, myeloid colonies were cultured from 102 haematopoietically normal individuals and grouped according to *JAK2* haplotype status in a prospective investigation. Surprisingly, I did see a difference with regards to haplotype status; normal cases with one or two copies of the 46/1 allele grew significantly fewer CFU-GM, that those who did not carry any 46/1 alleles. There was no impact, however on BFU-E colony formation. Whilst this result supports the notion that the 46/1 haplotype is functionally different to other backgrounds, exactly how this result fits with the phenotype of V617F-positive disease is not immediately obvious. Marked erythrocytosis with variable amplification of other myeloid cell lineages [385], and hypersensitivity to myeloid cytokines [386] characterise the majority of cases with *JAK2* V617F. Indeed it has been shown *in vitro* that the level of V617F expression correlates with greater hypersensitivity to EPO [197]. It could be hypothesised that the 46/1 *JAK2* variant, through altered binding affinity to one or more myeloid cytokine receptors, indirectly subverts signalling to reduce granulocyte progenitor numbers, leaving the signalling pathway stimulated by the EPO receptor unaffected, so that the numbers of erythroid progenitors remains unchanged, but expand via an autocrine or paracrine mechanism.

Further evidence for a functional variant comes from the finding that SNP rs10758669, which also tags 46/1, was identified as being significantly associated with Crohn's disease and ulcerative colitis in a recent genome wide association study [387-389]. Crohn's disease and ulcerative colitis are believed to result from aberrant inflammatory responses, and it has been suggested that JAK2 may be relevant due to its role in IL23 signalling in T-cell subsets [388]. The finding that *JAK2* 46/1 (and also variants of *STAT3* and *IL23*) are associated with these diseases strongly suggests that constitutional signalling differences in this pathway influence susceptibility to Crohn's.

In this study, experiments were undertaken initially to detect expression differences in *JAK2*, using V617F negative cases that were heterozygous for one of two *JAK2* exonic SNPs as well as being heterozygous for the 46/1 haplotype. By quantifying the allele ratios for both SNPs in matched DNA and cDNA it should have been possible to detect deviations in the level of *JAK2* transcripts between haplotypes. Since pyrosequencing can reliably detect differences in allele ratios of 10%, expression differences of only 1.5 fold between 46/1 and non 46/1 haplotypes should be detectable. My data showed there was an apparent difference for one exonic SNP but not the second, but the difference of 3.4%

was very minor. The discordant results between the two SNPs analyses suggest more cases should be examined to see if there is a real difference. Although it is possible that expression differences less than 1.5 fold might be relevant, this seems unlikely. Moreover, as this experiment was conducted on DNA and cDNA from total leukocytes, it is possible that relevant expression differences that are confined to early stages of myeloid cell development might be missed. Expression differences may be better analysed in a detailed investigation of flow-sorted myeloid progenitors from V617F positive cases carrying one or two copies of 46/1.

Sequence analysis of all *JAK2* exons failed to identify any sequence differences between this gene on 46/1 and non 46/1 haplotypes. Since it is possible that intronic variants could lead to altered splicing, overlapping RT-PCRs to cover all exon-exon junctions were performed but again no differences were detected. These findings suggest that if there is any functional difference of *JAK2* on 46/1, it must be very subtle. This is perhaps not surprising since roughly a quarter of V617F positive cases are negative for 46/1, and so this haplotype is clearly not required for disease development.

6.4.5 Evidence for a mutational mechanism linked to the *JAK2* 46/1 haplotype

The alternative hypothesis is that the 46/1 haplotype could confer a hypermutable property on the *JAK2* locus. Carriers of the 'genetically unstable' haplotype would more frequently acquire mutations in *JAK2,* and those mutations confer a selective advantage that results in a clonal disorder.

Evidence for this theory was proposed by Olcaydu et al. (2009) [390], who also discovered the association of V617F-positive MPN with the 46/1 *JAK2* haplotype (which they termed 'GGCC' haplotype). In this study, haematopoietic myeloid colonies were cultured from three individuals with MPNs who carried clones of V617F-positive cells plus other cytogenetically visible abnormalities, del(20q) and del(13q). Analysis of individual colonies revealed patterns that indicated V617F must have originated at least twice. Further analysis was performed on a larger series of cases using an allele specific PCR plus genotyping for the nearby SNP rs12343867 (which happens to tag 46/1). Of 109 cases analysed, three had V617F on both alleles suggesting that the mutation had arisen twice. A case of PV positive for both *JAK2* V617F and an exon 12 mutation was also recently

described, where the mutations defined separate clonal populations [259]. Since the acquisition of V617F is a rare event and the probability of an exon 12 mutation is considerably less, the chance of acquiring two completely independent *JAK2* mutations is miniscule. Olcaydu et al. conclude there must be a DNA sequence variant present on the risk allele that confers susceptibility to DNA damage or unfaithful replication or repair. A recent study by Plo et al. (2008) [391] reported that *JAK2* V617F expression *in vitro* stimulates homologous recombination (HR) and thus genetic instability, but also wild-type JAK2 was also capable of inducing HR, but to a lesser extent. Mutations preceding acquisition of V617F may also account for, or contribute to the genetic instability observed in MPN [268].

Exactly how the *JAK2* 46/1 allele might create this 'mutator phenotype' is unclear. If the 46/1 haplotype is more predisposed to acquiring mutations, the mechanism responsible must presumably be able to confer hypermutability from a distant location since analysis of the exons revealed no sequence differences. One area worthy of investigation described recently is that insertion-deletion polymorphisms present in heterozygous form increase the mutation rate in regions located in close proximity [392]. This could perhaps be tested by undertaking deep resequencing experiments [393] to determine whether there are any differences in spontaneous mutation rates close to *JAK2* between normal individuals carrying the risk haplotype, and those with other haplotypes. Results from this may provide an explanation as to why a minority of healthy normal individuals were found to apparently carry a low level of the *JAK2* V617F mutation [394].

In addition to the specific association described here for MPN, the finding that 46/1 predisposes to a somatic mutation may have wider relevance. Genome-wide association studies are identifying increasing numbers of loci that predispose to diverse malignancies. The findings in this study suggest that such loci should be considered as candidates for the acquisition of somatic mutations. At least two other relevant examples are present in the literature. First, the *APC* polymorphism I1307K, found in 6.1% of people of Ashkenazi-Jewish origin, accounts for an approximate 2-fold increased risk of colorectal cancer. This rare allele creates an unstable sequence that is hypermutable, leading to somatic, truncating mutations occurring in adjacent sequence which predispose to cancer [395]. More recently, research into common genetic variation that modifies susceptibility to lung cancer has revealed particular polymorphisms and haplotypes within the *TP53* gene

that are associated with adverse prognosis and an increased risk (OR 2.32) of developing lung cancer in African-Americans, but not Caucasians [396]. Whether this is a result of a functional interaction or propensity to acquire mutations is unclear.

6.4.6 The *JAK2* 46/1 does not explain V617F-positive familial MPN

Examples of MPN segregating in families has provided firm evidence that that *JAK2* V617F is an acquired somatic mutation, and not passed through the germ-line. Firstly, V617F was shown to be absent in T lymphocytes of familial patients carrying the mutation in granulocytes [372]. Furthermore, a different mutation burden was reported in affected relatives, whereas if the mutation was transmitted in the germ-line then V617F should be found in 50% of alleles [365].

My study included an investigation of the 46/1 haplotype in one family with MPN, previously characterised by Cario et al. (2005)[364]. This family included two *JAK2* V617F-positive relatives with PV, but the obligate carrier was healthy with wild-type *JAK2.* Genotyping of this family revealed only one affected individual carried V617F on the 46/1 haplotype. This indicates that 46/1 does not provide a genetic predisposition to acquiring V617F, at least in this family. Indeed, linkage of disease to 9p has not been described in any MPN family to date [204]. Further characterisation of *JAK2* haplotypes in other families with members with V617F-positive MPN will clarify the influence of the 46/1 allele, however it seems likely that these are different situations. To be seen as a familial trait, any genetic variant must be highly penetrant, i.e. the chance of developing an MPN is high if the variant is inherited. The 46/1 haplotype on the other hand is very common: a gene frequency of 0.24 predicts that 5.8% of normal individuals will be homozygous for 46/1 and 36.5% will be heterozygous (p^2 and 2pq in the Hardy-Weinberg equation, respectively). Although very common and associated with an increased risk of developing an MPN, the absolute risk is still very low because of the very low penetrance. If the incidence of a V617F positive MPN is roughly 4 per 100,000 per annum [23], and the risk of developing an MPN is three fold higher for individuals with at least one copy of 46/1, then it would be expected that the incidence of V617F positive MPN would be 3 per 100,000 per annum for individuals with 46/1 and 1 per 100,000 per annum for individuals without

46/1. The contribution of 46/1 to understanding genetic predisposition to MPN is illustrated in Figure 6.15.

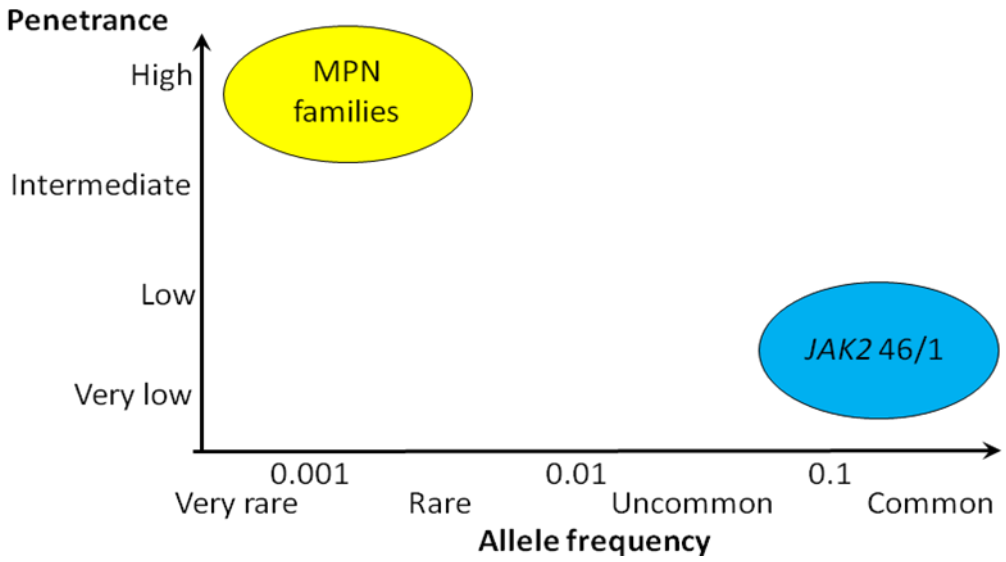


Figure 6-15. Genetic predisposition to MPNs. This illustration shows the two ends of the genetic spectrum. On the top left side are rare examples of families with members with MPN displaying Mendelian inheritance, where the penetrance is high. Conversely, in the bottom right hand side there are the common alleles, like the *JAK2* 46/1 haplotype, where the effects on risk of disease contributed by each gene locus may be modest, but when combined they may substantially increase the risk of disease.

7 Conclusions and discussion

Much of the discussion of the work in this thesis is presented in the preceding chapters. This chapter discusses my work in a wider context, with reference to some of the work I have undertaken subsequently and areas for future research.

The original aim of this study was to investigate how deregulated tyrosine kinases contribute to the molecular pathogenesis of MPNs. Shortly after starting my investigation, *JAK2* V617F was discovered, and my research then focused on characterising this very interesting mutation. Since starting this project, understanding of the molecular biology of MPN has been furthered substantially, and recent findings will be discussed alongside my results where relevant.

7.1 Detection and characterisation of *JAK2* V617F

In 2005, the finding of *JAK2* V617F was a highly significant step forward in the understanding of the pathogenesis of MPN, and this mutation became a new disease marker when the WHO revised the diagnostic criteria for PV, ET and MF [397]. Research presented in this thesis described the first evidence that V617F was not restricted to cases with classical MPN, but may also be in atypical MPNs, specifically MPN-U, aCML, and rare cases with CNL and HES [252]. Other studies confirmed my findings, and wider screens isolated V617F in 1% MDS and 18% megakaryocytic AML. However, V617F was not found in patients with *BCR-ABL* positive CML (except for rare cases that appear to have co-incident CML and MPN), AML, or ALL [261,262]. More intensive investigations for V617F in *de novo* AML isolated the mutation in 3% of patients with M1 and M2 subtypes, suggesting a correlation of V617F with less differentiated leukaemias with a normal karyotype [398]. V617F was also identified in approximately 6% of AMLs carrying a t(8;21) translocation, suggesting oncogenic co-operation between the translocation product RUNX1-CBFA2T1 and *JAK2* V617F, analogous to that proposed for *FLT3* ITD mutations in this subtype of AML [399].There have also been extensive investigations looking for V617F in

non-haematological cancers and lymphoid malignancies, but V617F was entirely absent [280].

At the beginning of my investigations into *JAK2* V617F, it was quickly determined that standard sequencing techniques would miss a significant proportion of V617F positive cases due to an inadequate level of sensitivity. To circumvent this, I used two alternative methods of detection; AS-PCR and pyrosequencing, which attained sensitivity levels of 2% and 5%, respectively. Although AS-PCR was semi-quantitative, accurate quantification of the mutation level was achieved using pyrosequencing. After validation in my hands, both methods were then used by the Wessex Regional Genetics Laboratory to test for *JAK2* V617F as part of a diagnostic service to confirm a diagnosis of MPN. Over 3500 cases have now been screened for V617F. A very small minority of samples carry low levels of mutation detected by AS-PCR, but not by pyrosequencing due to its slightly higher detection threshold. In these circumstances a provisional report is issued with a request for a new sample in six months time to see whether the mutation becomes more readily detected. During routine screening, it was observed that one individual was strongly positive for V617F on AS-PCR but then apparently negative by pyrosequencing. Following direct sequencing, it transpired the patient carried V617F plus a C618R mutation, a very rare change reported previously in only two individuals [400]. The C618R mutation is thought to have destabilised the pyrosequencing sequencing primer oriented in the reverse direction, whereas in the AS-PCR the mutation-detecting primer is in the forward orientation so could detect V617F. There are now a range of methods published in the literature claiming accurate detection of *JAK2* V617F [401], and some semi-quantitative real-time PCR assays report sensitivity levels of down to 0.01% mutation level [402]. However, the clinical significance of a such a low mutation burden in patients at diagnosis remains to be determined, especially given recent anecdotal reports that nearly 1% of haematologically normal individuals apparently tested positive for V617F [394]. Such sensitive techniques, however, may be important for monitoring therapeutic responses as discussed below.

One issue with using *JAK2* V617F diagnostically is that in the context of myeloid neoplasms, V617F cannot be used to distinguish one MPN from another. The presence of V617F does complement laboratory and clinical markers, including histology in the

diagnosis of both ET and MF, by excluding the possibility of reactive thrombocytosis or myelofibrosis. Where a diagnosis of PV is suspected, clinicians now generally choose to screen for V617F in PB rather than measure red cell mass in the patient, which is an unpleasant and expensive procedure [22]. However V617F or exon 12 mutations are not detected in occasional PV cases, either because they are wholly absent, or are present beneath the limits of detection [347]. These observations together with the fact that only half of ET and MF cases are positive for *JAK2* V617F have led to V617F being only one of a series of criteria in the diagnostic algorithms defined by the WHO [22].

The finding of one single mutation in several phenotypically related, but clearly distinct diseases was unexpected. Explanations for this are far from confirmed, but do provide tentative evidence for how V617F can be associated with different neoplasms. One hypothesis relates to the level of *JAK2* V617F the patient carries, that is the disease phenotype is correlated with V617F mutant allele burden. *In vitro* evidence for this comes from experiments expressing variable levels of mutation in mouse models. Low levels of V617F induce an ET-like phenotype dominated by thrombocytosis, whereas a higher level of mutant allele leads to a PV-like phenotype, characterised by erythrocytosis and eventually myelofibrosis [282,283,363,403]. As I have shown in this thesis, *JAK2* V617F allele burden is a measurable variable being determined by the frequency of mitotic recombination events and the expansion of mutant positive clones. In humans the average V617F mutation burden of ET cases is statistically lower than that seen in PV and MF, consistent with the fact that acquired UPD is an infrequent event in ET [198,199,252]. This was later explored at the progenitor level by Scott et al. (2006), who grew EECs from patients with ET, PV and MF and investigated the presence of V617F in each colony. Even in cases with <50% V617F in total granulocytes, a mixture of heterozygous and homozygous V617F erythroid colonies was present in most PV and MF cases, but only heterozygous V617F positive colonies were found in the vast majority of ET cases [281]. It has been speculated that as PV cases have a lower serum EPO level than ET cases, homozygous V617F positive erythroid colonies have a stronger selective advantage in a low-EPO environment. Alternatively, PV cases may have a longer subclinical prediagnosis phase of disease than ET cases, allowing more time for homozygosity to occur. Mitotic recombination at the *JAK2* locus may be more frequent in PV perhaps due to a

haematopoietic microenvironment that is more genetically damaging, or the presence of other mutations or inherited differences that influence genetic stability. Whatever the reason, it is highly likely that *JAK2* V617F alone cannot account for the MPN phenotypes seen clinically.

A recent study correlated *JAK2* V617F allele burden in cases with MPN with gender, and showed women had significantly lower allele burdens than men at diagnosis. Furthermore, on follow up the increase in allele burden per year was significantly less in females compared to males, and women were 4.5 times more likely to evolve from ET to PV. This suggests sex is an independent factor that may go some way to account for the variability in V617F allele burden, and that females have a lower frequency of mitotic recombination events compared to males [404].

Another highly plausible explanation for why *JAK2* V617F is linked to more than one neoplasm is that V617F may not be the disease-initiating lesion. Other undiscovered mutations may precede the acquisition of V617F, and directly influence the neoplastic phenotype [360,405]. Evidence for preceding mutations comes from studies focusing on markers of clonal proliferation. For example, it has been shown that in patients with a V617F mutation burden of <25%, the granulocytes that did not carry V617F were often clonal, as determined by the X chromosome inactivation pattern in female cases, or by the presence of deletions on chromosome 20q [268]. Furthermore, when the level of clonal granulocytes was measured and compared against the level of the V617F allele, a direct correlation was observed for PV, but not ET or MF [269]. Another study showed that V617F-positive MPN cases carry a small number of EECs which were wild-type for *JAK2,* alongside other V617F-positive colonies [271]. Subsequently, other mutations have been shown to be acquired in MPN, but as yet not one has been strictly associated with a particular phenotype.

Mutations in the thrombopoietin receptor gene, *MPL*, are associated with a more distinctive clinical picture; having been isolated only in a small proportion of MF and ET cases [406]. *MPL* W515L mutations constitutionally activate JAK-STAT signalling, induce ligand independent proliferation in cell lines and reproduce many phenotypic characteristics of MF when expressed in mouse models, e.g. atypical megakaryocytic

hyperplasia, splenomegaly due to extramedullary haematopoiesis and thrombocytosis. Inactivating mutations of the E3 ubiquitin ligase, *CBL,* have been isolated in a small minority of cases with MF, CMML and aCML and are thought to act by a general increase in the level of signalling from tyrosine kinases [407,408]. Other mutations have been identified for which the mechanism of transformation is currently less clear. Acquired mutations of *TET2* have been isolated in approximately 15% of patients with various forms of myeloid disease; 19% of MDS, 12% MPN (*JAK2* V617F positive and negative cases), 22% CMML and 24% of cases with secondary AML [409]. Likewise, 11% of MDS and 43% of cases with CMML carry mutations in *ASXL1* [410]. The very newly indentified *IDH1* and *IDH2* mutations have, as yet, only been isolated in 31% of *JAK2* V617F positive MPNs that have evolved to acute leukaemia [411], and not in PV or ET cases that were in chronic phase.

7.2 MPN cases may have more than one clonal proliferation

There is mounting evidence to suggest an increasing degree of clonal heterogeneity in patients with MPN. It has been shown previously that the clonal burden is not accounted for by burden of *JAK2* V617F alone, and in some cases the disparity is made up by other markers of clonal proliferation, like del(20q) or other unidentified mutations [268]. More detailed study of progenitor colonies has shown that del(20q) [288], and mutations in *TET2* [409] may follow or precede acquisition of a *JAK2* mutation. In this thesis I studied myeloid progenitors from a patient carrying both *JAK2* V617F and *KIT* D816V *in vitro*. The mutations were found to be mutually exclusive, an observation mirrored in a larger series of similar MPNs with biclonal mutations or cytogenetic abnormalities [287]. It is currently hypothesised that (i) either the different clonal populations are phylogenetically related, having risen from a shared founder clone, or (ii) the different clones are unrelated, and reflect transformation of independent stem cells. Although transformation of independent stem cells might at first sight seem implausible, it is worth pointing out that the molecular basis for disease predisposition in MPN families has not been elucidated and it is possible that these individuals have inherited a genetic variant that increases the probability of acquiring mutations in haemopoietic cells.

This issue of biclonality is elegantly illustrated in those cases with MPN that transform to acute leukaemia. It was observed that in those cases that transform from V617F positive MPN to acute phase, the level of V617F frequently drops and even disappears as transformation progresses [265,270]. This suggests a clonal V617F negative cell carries the potential to acquire various leukaemogenic mutations and proliferate independently of V617F. It was recently observed that the majority of cases that transformed to *JAK2* V617F positive AML were preceded by primary MF or myelofibrotic transformation, implying that *JAK2* V617F AML is preceded by mutations that give rise to a 'myelofibrosis-like' phenotype [412]. In contrast, other cases with additional mutations in *TP53, CBL, TET2,* and now *IDH* mutations [411], were present in *JAK2* wild-type leukaemic blasts but absent from *JAK2* V617F positive MPN. This implies different pathogenetic mechanisms may underlie transformation to *JAK2* wild-type and *JAK2* mutant AML.

There have been speculations that the cell in which *JAK2* V617F arises may go some way to explain some of the phenotypic pleiotropy of MPN. It has been shown, at least in PV cases that *JAK2* V617F arises in multipotent HSCs and their myeloid-restricted progeny, and predisposes towards erythroid differentiation [413,414]. The observation that V617F was absent in T cells from PV patients first suggested V617F was a myeloid-specific mutation [197,199]. However more investigations have isolated V617F in T and B cells in small groups of MPN patients [415 416], and some individuals with MF have a mutation burden in lymphocytes that equals that measured in granulocytes [417,418]. Moreover, the notion that V617F may favour different cellular targets, and that this may define the clinical phenotype of MPN remains a weak hypothesis, given the observation that CD34+ cells carry V617F at very similar levels to that found in granulocytes [418]. Conversely, enforced expression of *JAK2* V617F in human HSCs and myeloid progenitors directed differentiation towards the erythroid lineage, along with increased expression and phosphorylation of GATA-1 and PU.1 transcription factors [419-421]. Altogether this suggests that V617F arises in a stem cell and directs myeloid proliferation, rather than being restricted to myeloid cells.

Recently a very interesting, novel role of JAK2 was identified; JAK2 may directly regulate gene expression by the phosphorylation of histones in the nucleus [422]. It is well characterised how JAK2 functions in the cytoplasm as a critical signalling component for diverse receptors at the cell surface [371]. This new nuclear function of JAK2 revealed a

novel epigenetic mechanism by which JAK2 may function in an altogether different setting. Both wild-type and constitutively active (V617F positive) JAK2 were shown to phosphorylate histone H3 at position Y41. Phosphorylation prevents binding of heterochromatin protein 1 alpha (HP1α), the effect of which is likely to include increased mitotic recombination and increased expression of heterochromatic genes. Whether this has any relevance to MPN is currently unclear, but these findings indicate that the cellular consequences of deregulated JAK2 are likely to be complex.

7.3 Acquired uniparental disomy

One recurrent theme of this thesis has been acquired UPD for chromosome 9, observed in a significant proportion of cases with *JAK2* V617F positive MPN. Brought about by somatic recombination at mitosis, this mechanism ultimately results in duplication of one chromosome or chromosomal region and concurrent loss of the other allele. In this thesis I used a PCR-based method, MLPA, to show MPN cases with a high V617F burden carried two copies of *JAK2.* This complemented microsatellite analysis to show that the observed stretches of homozygosity had arisen by mitotic recombination and not by deletion or duplication of chromosome 9. Furthermore, the observation that acquired UPD not only reduces one mutation, but also all the heterozygous SNP alleles linked *in cis,* to homozygosity, provided a key tool in identifying a particular constitutional *JAK2* haplotype associated with the acquisition of V617F.

Acquired UPD has been best described in solid tumours, usually with the effect of inactivating tumour suppressor genes [423-425]. As with *JAK2* V617F [197], acquired UPD allows malignant cells to multiply gene mutations and to dispose of wild-type alleles, thus obtaining a selective growth advantage over cells without acquired UPD. The advent of high-density SNP genotype arrays has shown that approximately 17% of cases with AML have evidence of acquired UPD [426]. Moreover, homozygously mutated genes *CEBPA, WT1, FLT3* and *RUNX1* are all associated with acquired UPD in AML [129 427], suggesting that acquired UPD is a relatively common mechanism of disease progression. More recently, mutations of *MPL* [406], *CBL* [407], and *TET2* [409,428] have been discovered in MPN, and all are

associated with varying levels of acquired UPD. It is unclear why loss of heterozygosity of mutations at some loci tend to occur by acquired UPD and others by deletion of the wild-type allele. LOH for *JAK2* and *MPL* mutations is almost always associated with acquired UPD whereas LOH of *TP53* and *RB* mutations are usually a consequence of deletion of the wild-type allele. One reason for a preference for acquired UPD might be the presence of one or more neighbouring dosage sensitive genes, deletion of which would confer a proliferative disadvantage or even lethality to the cell. It is also possible that the expression of imprinted genes may either be silenced or enhanced by acquired UPD, depending on which allele is methylated and which one is duplicated. Alternatively, acquired UPD may serve to duplicate a minor disease-prone allele present in the germ-line, which may have a deleterious effect. Most likely, the acquisition of an additional copy of the mutant allele results in increased levels of a protein with pathogenetic potential. This might be of relevance for the activity of oncogenes such as *JAK2* V617F but not for tumour suppressor genes such as *TP53.*

The molecular basis of a significant proportion of MPN cases still remains to be determined. Identifying recurrent regions of acquired UPD has proven very useful in the past for selecting candidate genes, but as the regions of homozygosity are often greater than 10 megabases in size, this is still an arduous task. Alternatively, new acquired mutations are likely to be identified as a result of 'next-generation' sequencing, which is set to become cheaper and easier to use.

7.4 *JAK2* V617F is a suitable molecular marker of clonal disease and response to treatment

Following the successful application of imatinib as a BCR-ABL tyrosine kinase inhibitor for the treatment of CML, the identification of JAK2 mutations in MPNs has generated extensive interest in developing JAK2-specific inhibitors to treat these diseases. Although it may or may not be the disease-initiating lesion [265,268-271], expression of *JAK2* V617F in murine models is singularly sufficient to cause a myeloproliferative phenotype, where the mice recapitulate many clinicopathologic features observed in human PV, ET and MF

[282,283]. Therefore, mutant JAK2 remains a valid target for therapeutic intervention in MPNs. As I have investigated in this thesis, *JAK2* V617F is a reliable and sensitive marker of disease, and may be used to monitor response to therapy. Other groups have reported the use of %V617F as a molecular marker, and the majority correlate a fall in %V617F with haematological improvement [277,279,326-328,330-333].

Numerous JAK2 inhibitors have been identified, based on the experience gained from the development of tyrosine kinase inhibitors in CML. Although no single small-molecule JAK2 inhibitor is available yet for broad clinical use, there are already a number compounds reported to have significant activity against *JAK2* V617F *in vitro* [429-431] and some have been tested in animal models [432] and *in vitro* cultures of *JAK2* V617F positive patient cells [433]. A small number are under evaluation in phase I and II studies in patients with primary and secondary MF. Some V617F positive patients with MF and post PV/ET MF treated with compounds INCBO18424 and XL019 displayed significant reduction of spleen size, reduced leukocytes and improvement of symptoms [434]. However, there was very limited reduction in proportion of V617F in PB granulocytes indicating minimal impact on the size of the malignant clone.

Exactly how these compounds exert their effects in not understood, but it is possible there may be significant off-target effects, e.g. on other JAK family members or possibly as a consequence of inhibiting normal JAK2. Unlike BCR-ABL, the V617F mutation is localised in a region outside of the ATP-binding pocket of the JAK2 enzyme. Therefore ATP-competitive inhibitors are not likely to distinguish between wild-type and mutant JAK2 proteins. The JAK family play an important role in coordinating haematopoiesis, making it pertinent to specifically target JAK2 to avoid undesirable long-term effects. JAK3, like JAK2, is expressed in haematopoietic cells, and plays a vital role in immunity, so any unwarranted inhibition may be damaging, especially as *JAK3*-deficient mice are viable but display severe combined immunodeficiency [435]. Despite the failure to reduce the size of the mutant clone, it has been suggested JAK inhibitors do have significant therapeutic benefit by controlling debilitating symptoms of disease, like splenomegaly or the effects of high levels of circulating cytokines, e.g. pruitus [434].

There is mounting clinical evidence correlating *JAK2* V617F allele burden to disease phenotype, complications and evolution to myelofibrosis. As mentioned above, ET cases tend to have a low allele burden, those with PV and MF carry an intermediate level, and it has been shown elsewhere those with post-PV MF have a very high level of V617F [278,279,405]. To investigate the clinical relevance of *JAK2* mutation status, a large series of ET cases entered into the Primary Thrombocythaemia (PT-1) trial were screened retrospectively for V617F, and status was correlated to various clinical, laboratory and diagnostic parameters [329]. Roughly half of the ET cases were positive, and those that carried V617F were more likely to have a PV-like phenotype characterised by increased haemoglobin, elevated neutrophils and erythrocytes, more venous thrombosis, and higher rate of polycythaemic transformation. V617F-positive ET cases also had a low serum EPO level, and were significantly more sensitive to hydroxyurea compared to anagrelide. Furthermore, due to the numerous shared features, it has been proposed that *JAK2* V617F positive PV and *JAK2* V617F positive ET could be better viewed as a continuum, with each neoplasm at either ends of the model, with the difference being accounted for by transition to V617F homozygosity, acquired or inherited genetic modifiers, gender, and constraints of physiology, like suppression of EPO production and depletion of iron stores. There are now numerous clinical studies correlating %V617F to disease characteristics, and the majority agree that there is a linear relationship between mutation burden and severity of disease [273-279,436]. However the differences are relatively subtle and are probably of limited value for the assessment of individual patients.

7.5 *JAK2* exon 12 mutations are very rare in MPN

A small proportion of cases with PV (some with an initial diagnosis of IE) acquire mutations in exon 12 of *JAK2*, and these mutations are thought to have a similar pathogenic effect as V617F by inducing a myeloproliferative phenotype, characterised by marked erythrocytosis [266]. However, unlike V617F, exon 12 mutations may be found almost anywhere in the exon, exist as nucleotide insertions, deletions, substitutions or a mix of all three changes. These features together with the observation that exon 12 mutations predominantly exist in heterozygous form only, and positive cells are relatively

infrequent in PB and BM, make detecting them technically challenging. Having been unable to find an exon 12 mutation in a large series of local V167F negative cases with a tentative referral of MPN using existing methods of standard sequencing and AS-PCR, I developed and applied two alternative PCR-based methods for detecting mutations in exon 12. These were HRM analysis and WTB PCR. Both methods proved more sensitive than standard sequencing, were capable of detecting novel mutations, but the WTB PCR was the most sensitive at uncovering low level mutations. Despite these improved methods, no exon 12 mutations were detected in our local group, suggesting the incidence of this particular mutation is extremely low, and screening should only be undertaken after careful clinical characterisation.

To date mutations in exon 12 and V617F of *JAK2* have only ever been isolated in patients with a myeloid disease [197,266]. Recently, mutations at JAK2 residue R683 have been identified in 18% of patients with Down's syndrome (DS)-associated ALL [373,437]. Further investigation revealed *JAK* mutations in a small minority of paediatric ALL cases, where mutations in *JAK2* mainly targeted R683 [438]. The finding that mutations affecting R683 have been identified almost exclusively in DS and non-DS high-risk ALL, and mutations in exon 12 and exon 14 are entirely myeloid-associated, suggests the nature of the *JAK2* mutation may play a direct role in establishing the disease phenotype. Additionally, a T875N *JAK2* mutation was identified at in an AMKL cell line [439], and a deletion of five amino acids starting at residue I682 (IREED deletion) was found in a single case of DS-ALL [440]. The reasons for why different mutations within the *JAK2* gene are linked to different phenotypes are unclear, but this does suggest there is something functionally different about *JAK2* in each biological setting. As mentioned previously, V617F has been isolated at varying levels in both myeloid and lymphoid cell types, so it would be interesting to determine what other lineages carry these other *JAK2* mutations.

One study has investigated the question of why valine at position 617 mutates to phenylalanine in MPN and not another amino acid. In an *in vitro* system, several other mutations at position 617 could, to varying extents, induce constitutive signalling but only V617W was comparable to V617F in stimulating STAT5 activation. However, the codon that codes for tryptophan (TGG) could only be obtained by three nucleotide substitutions from the wild-type valine (GTC), whereas phenylalanine (TTC) only required one

nucleotide substitution [441]. Consequently V617W is expected to be very uncommon in patients.

As discussed above, patients with MPN may carry more than one activating mutation, and the combination of these could influence disease phenotype. There is already some precedence for the idea of cooperating mutation(s) contributing to pathogenesis in relation to JAK2. In cases with paediatric ALL carrying the *JAK2* R683 mutations, there were also often concomitant alterations in genes involved in lymphoid development (*IKZNF1*) and tumour suppression (*CDKN2A/B*), suggesting that co-occurrence of these lesions is important for pathogenesis of this particular disease, or that these other abnormalities are lineage-specific secondary mutations [438]. A minority of V617F positive MPNs have also been shown to carry mutations in genes with myeloid growth factor signalling roles, e.g. *KIT* D816 [114], *MPL* W515 [406], and *CBL* [407,408] however in most of these that have been analysed the *JAK2* and other mutations occur in separate clones. A new set of mutations have been identified in MPN which may disrupt chromatin remodelling with consequent effects on the regulation of gene expression. These include mutations in *TET2* [409] and *ASXL1* [410] but it is not yet clear if these co-operate in any way with deregulated JAK2 signalling.

Interestingly, the phenotype and clinical course of *JAK2* activated by mutation (MPN being a relatively indolent, chronic disease), is distinctly different to disease associated with *JAK2* fusion genes (e.g. *ETV6-JAK2*, *PCM1-JAK2*), which more closely resembles leukaemias associated with *ABL1* fusion genes (aggressive CML and ALL). This observation highlights the phenotypic importance of the mechanism leading to kinase activation. Moreover, both *JAK2* fusions above have been isolated in both myeloid and lymphoid proliferative phenotypes, suggesting that they do themselves not specify either myeloid or lymphoid differentiation.

7.6 Inherited predisposition to acquiring MPN

In this thesis I describe the finding that *JAK2* V617F is not acquired randomly, but instead preferentially arises on a specific constitutional *JAK2* haplotype which was termed '46/1.'

At least one allele of 46/1 is found in approximately 50% of normal individuals, and so this haplotype is a common low penetrance predisposition allele, similar to those typically identified by GWAS. The association between V617F and 46/1 is relatively strong, with the odds of developing an MPN three to four fold higher in individuals carrying the haplotype than those who did not. Furthermore, it was possible to determine that the 46/1 haplotype accounts for 50% of the MPN risk attributable to genetic factors. The 46/1 haplotype is associated with homozygous and heterozygous levels of V617F. The 46/1 haplotype block has a tag SNP in close proximity to the V617F mutation, so it was possible to determine in cases heterozygous for 46/1 and V617F that the mutation was significantly more likely to arise on the 46/1 chromosome.

46/1 was seen at comparable frequencies in different MPN subtypes, so does not explain the phenotypic diversity associated with *JAK2* V617F. Nor does 46/1 account for familial MPNs, which are expected to be caused by rare, highly penetrant variants in genes that remain to be identified. It is not clear why an acquired mutation as prevalent as *JAK2* V617F is associated with a particular inherited background. The first hypothesis considers 46/1 is inherently more genetically unstable, acquiring V617F at a faster rate than other haplotypes (hypermutability hypothesis). The second hypothesis proposes V617F may arise on all haplotypes at equal rates but 46/1 may carry an additional factor that either provides a selective advantage to the V617F positive clone, or interacts in some way to increase the likelihood of abnormal blood counts (fertile ground hypothesis). These hypotheses are not necessarily mutually exclusive, and there is evidence to support each theory.

The hypermutability hypothesis is favoured by Olcaydu et al. (2009) [390], who observed that V617F was seen in *cis* to both alleles of a nearby heterozygous SNP in 3 out of 109 (3%) MPNs. In a recent screen of 46/1 in 207 ET cases, I found 2 (1%) carried V617F on 46/1 plus a different *JAK2* haplotype, thus confirming the findings of Olcaydu et al. Additionally, rare cases with both *JAK2* V617F and *JAK2* exon 12 mutations have been recorded [259]. The finding of double mutated cases might therefore in theory be explained by hypermutability of *JAK2* on 46/1, but other factors could account for this. For example, other acquired or inherited genetic variants may predispose to the acquisition of *JAK2* mutations, thus greatly increasing the probability of two separate mutations in the same

individual. This enhanced propensity to acquire *JAK2* mutations is already accepted in the context of familial MPNs, where multiple affected family members acquire V617F or an exon 12 mutation as a result of inheriting an as yet uncharacterised, highly penetrant genetic variant [364,372]. Although documented MPN families are relatively uncommon, a study recently published estimated that as many as 8% of cases have a family history of MPN [442], and thus a substantial minority of affected individuals may have an elevated, inherited propensity to acquire multiple *JAK2* mutations. The *JAK2* V617F mutation itself was recently reported to increase the rate of homologous recombination as well as mutation rate [391] and thus *JAK2* mutant cells may be intrinsically more likely to acquire additional *JAK2* mutations. There may be acquired or inherited weaknesses of various components of DNA repair or mitotic spindle machinery, or telomere shortening. The incidence of acquired UPD increases with age in MPN, and also MDS and AML, so perhaps cumulative acquisition of genetic and epigenetic lesions may contribute to development of disease [443].

If there is a specific mutational mechanism by which V617F preferentially arises on the 46/1 haplotype, then other mutations in *JAK2* may also be associated with this allele. An association between 46/1 and *JAK2* exon 12 mutations was described recently [444], a finding I replicated recently in 69 cases (p=0.002, manuscript submitted). Similar to my findings for V617F, in 31 cases heterozygous for 46/1, 71% of exon 12 mutations were acquired in *cis* to 46/1. Nevertheless, an association of different *JAK2* mutations with 46/1 is equally compatible with the alternative hypothesis, fertile ground.

Our group marginally prefer the fertile ground hypothesis, based on the observations presented in this thesis that 46/1 is associated with lower numbers of myeloid colony forming units in normal individuals. Furthermore, a 46/1 tag SNP showed robust association with Crohn's disease and ulcerative colitis, non-malignant disorders believed to result from a perturbed inflammatory response [387-389]. GWAS studies in Crohn's disease also detected significant associations with genes encoding the IL-23 receptor and STAT3, thereby strongly implicating functional differences in the IL23/JAK2/STAT3 pathway in the pathogenesis of this disorder. Moreover, it has subsequently been shown that there is a 20% increased incidence of MPN in individuals that have had a preceding history of an autoimmune disorder, and for Crohn's disease this risk is 2 to 3 fold [445]. These findings

strengthen the hypothesis that common functional genetic variants impacting on JAK2 signalling predispose to these clinically very diverse conditions. With this in mind, I recently investigated whether the STAT3 SNP rs744166, identified in Crohn's GWAS studies also predisposes to MPN. Using pyrosequencing, I genotyped PB DNA from our cases with *JAK2* V617F positive PV (n=136) and *JAK2* V617F negative ET (n=338), and compared the results to control data obtained from the WTCCC blood cohort. No significant differences were observed between cases and controls, and we concluded therefore that there was no evidence to indicate that this particular variant of *STAT3* predisposes to MPN.

Since finishing the work for this thesis I have focused mainly on trying to distinguish between the fertile ground and hypermutability hypotheses. If, according to the fertile ground hypothesis, the 46/1 haplotype is functionally different to *JAK2* on other haplotypes, it might be expected to influence the clinical phenotype or be associated with specific features of disease. To explore this possibility I genotyped a 46/1 tag SNP in patients entered into the Primary Thrombocythaemia 1 (PT-1; n=751) study, a clinical trial that included newly diagnosed and previously treated patients aged 18 years or over who met the Polycythaemia Vera Study Group (PVSG) criteria for ET [446]. Results were then compared to clinical, laboratory and demographic data by the trial statistician, Dr Peter Campbell (Cambridge, UK) for both V617F-positive and V617F negative cases. No significant associations were seen with any clinical or laboratory feature (for example, age at diagnosis, blood counts, haemoglobin, erythropoietin and spleen size). Furthermore, there was no association with survival, arterial thrombosis, major haemorrhage, or transformation to MF or acute leukaemia (manuscript submitted). Similar findings were recently published by Pardanani et al. (2009) [447]. By contrast absence of 46/1 has been associated with inferior survival in MF [448].

Analysis of the PT-1 cohort indicated that the *JAK2* 46/1 haplotype was more prevalent in cases with an exon 10 *MPL* mutation, although this did not achieve statistical significance (P=0.06). This observation was explored further in an additional 176 cases recruited from the UK, Greece, Italy and Germany, all proven to be negative for V617F. After controlling for population stratification by using controls from each of the four countries, the

association between 46/1 and *MPL* exon 10 positive cases achieved significance (p=0.004, manuscript submitted).

MPL requires JAK2 for signalling and thus the finding of an excess of 46/1 alleles in *MPL* mutated cases suggests a functional difference of *JAK2* on 46/1 compared to other alleles, rather than the 46/1 allele being more genetically unstable. There is already some precedence for a functional SNP variant in AML pathogenesis. Investigations into patients with AML and a complex karyotype revealed an association between a SNP variant located in an enhancer region of the PU.1 transcription factor, which through altered binding of a transcriptional regulatory protein (SATB1), was shown to reduce PU.1 expression significantly in specific myeloid progenitor subtypes [449]. Regarding 46/1, however, I have not found evidence of any expression differences between 46/1 and non 46/1 alleles, nor have I found any variants after sequencing all coding regions and untranslated regions of *JAK2* in eight homozygous V617F positive MPNs with 46/1.

I conclude that if a functional difference is present on 46/1, then its effect is relatively subtle. For example, a difference in expression of *JAK2* may only be evident in a haematopoietic precursor cell (similar to the PU.1 SNP above), and not apparent in my experiments on total leukocytes isolated from PB. Alternatively, 46/1 may impact on cell cycle progression. A subtle functional difference would also be consistent with the observation that 46/1 is not present in approximately 25% of *JAK2* V617F positive MPNs, and thus the haplotype is clearly not required for disease development.

46/1 has great potential for further experimental investigation. My preliminary results using myeloid progenitors isolated from the PB of healthy normal controls provided tentative evidence that 46/1 was functionally different to other *JAK2* alleles. However, to define exactly how 46/1 influences cell proliferation, differentiation, and other parameters, a more detailed approach will be required. If the effects of 46/1 are restricted to a particular lineage or stage of differentiation, then this requires comparison of highly purified cell subtypes isolated from the PB, and probably BM, of both normal and V617F positive cases. Obtaining these samples is likely to prove difficult, plus the numbers of each cell type may limit what experiment can be performed. *In vitro* experiments into 46/1 are confounded by the fact that *JAK2* V617F positive cases carry

variable levels of mutant allele making direct comparison difficult. Since the causal variant linked to 46/1, whether fertile ground or hypermutability, is unknown it is not possible to use cell lines or mouse models to further understand the underlying mechanism. One experimental alternative might be the creation of immortal B cell lines from *JAK2* V617F positive cases by transfection with Epstein-Barr virus. By selection of those positive for V617F and homozygous for 46/1, then it could be assumed that the functional variant may be present in the cell line. The growth factor IL-6 receptor signals via JAK2, so it could be hypothesised that stimulation of this pathway could reveal measurable differences attributed to the 46/1 allele. If a more phenotypically similar renewable cell source is required then one possibility is the use of human induced pluripotent stem (iPS) cells derived from *JAK2* V617F positive patients [450]. These human iPS cells exhibit characteristics similar to human ES cells and can be expanded in culture. The cells can be induced to differentiate into CD34+ progenitors that recapitulate features of *JAK2* V617F positive progenitors, e.g. increased erythropoiesis, and a characteristic gene expression profile.

It is not known whether the association of *JAK2* and *MPL* mutations with the 46/1 haplotype reflects something unique about the *JAK2* locus, or whether other acquired driver mutations in cancer also arise on specific inherited haplotypes. In a preliminary investigation I found no relationship between *MPL* mutations and *MPL* haplotype (manuscript submitted). Whilst this does not preclude the possibility that somatic mutations in other genes might occur preferentially on particular haplotypes, it does suggest that this is not a general phenomenon. I am currently looking at relationship between acquired mutations in AML and their respective haplotypes.

As previously mentioned, the 46/1 haplotype accounts for 50% of the population attributable risk of developing MPN, but it only explains a very minor component of V617F negative disease. A small GWAS performed on V617F-positive MPNs by Kilpivaara et al. (2009) [451] was able to detect 46/1, plus the frequency of three SNPs at 3q21, 4q31 and 7p11 were reported to differ between PV and ET, suggesting a potential role in phenotypic diversity. However, I failed to replicate these findings in a larger series of cases suggesting these findings were either spurious or much weaker than originally proposed. Nevertheless, it is highly likely that there are other common, low penetrance

predisposition alleles to be identified and MPNs, particularly V617F negative cases, seem ripe for a comprehensive GWAS analysis.

Before 2005 the study of MPN was generally considered to be rather a scientific backwater. I consider myself fortunate to have started my studies at the time of the *JAK2* revolution and participate in a field that is now at the cutting edge of molecular haematology.

Appendix I

Solutions

Freezing mix: 4ml RPMI supplemented with 10% foetal calf serum, L-glutamine and penicillin-streptomycin, 4ml DMSO and 12ml foetal calf serum.

Guanidium thiocyanate (GTC): 4M guanidium thiocyanate, 5mM EDTA, 25mM sodium citrate pH 7.0, 0.5% sarcosyl. 7µl β-mercaptoethanol per 1ml of GTC was added fresh prior to use.

LB broth: 25g LB broth per litre of distilled water, and autoclaved before use.

LB agar: 3g agar per 200ml LB broth, and autoclaved before use.

Loading buffer (for DNA gel electrophoresis): 0.05% bromophenol blue, 40% sucrose, 0.5% sodium dodecyl sulphate, 0.1M EDTA, pH8.0.

Lysis buffer (for removing red cells from PB and BM samples): 7M urea, 0.3M NaCl, 10mM EDTA, 10mM Tris-Cl, pH 7.5.

Phosphate buffered saline (PBS): PBS was prepared as instructed by the manufacturer (Oxoid, Basingstoke, UK). One tablet of PBS was dissolved in 100ml water and autoclaved.

Red cell lysis buffer: 155mM $KHCO_3$, 0.1mM EDTA, pH 7.4 at 4°C.

Resuspension buffer (RSB): 4.38g NaCl, 48ml EDTA made up to 1 L with sterile water.

Thawing Solution for cryopreserved cells: 20mls HBSS (Invitrogen), 100,000U DNase I (Invitrogen) and 200U sodium heparin (CP Pharmaceuticals, Wrexham, UK).

Appendix II

PCR programmes

High fidelity PCR programme

Step	Temp (°C)	duration
1	95	2 min
2	94	20 sec
3	64	2 min
4	68	14 min
5	10 times to step 2	
6	94	20 sec
7	64	40 sec
8	68	14 min
9	22 times to step 6	
10	72	9 min
11	15	forever

Pyrosequencing PCR

Step	Temp (°C)	duration
1	94	7 min
2	94	30 sec
3	58	30 sec
4	72	45 sec
5	39 times to step 2	
6	72	7 min
7	15	forever

Sequencing programme

Step	Temp (°C)	duration
1	96	1 min
2	96	10 sec
3	50	30 sec
4	60	2 min
5	25 times to step 2	
6	4	forever

Appendix III

Primers sequences

JAK2 V617F AS-PCR primers

Primer name	Sequence (5'-3')
Forward outer (FO)	TCCTCAGAACGTTGATGGCAG
Reverse outer (RO)	ATTGCTTTCCTTTTTCACAAGAT
Forward wild-type specific (Fwt)	GCATTTGGTTTTAAATTATGGAGTATaTG
Reverse mutant specific (Rms)	GTTTTACTTACTCTCGTCTCCACAaAA

Pyrosequencing primers

F = forward primer, R = reverse primer, SEQ = sequencing primer, BIO = biotinylated

Primer name	Sequence (5'-3')
JAK2_pyro_V617F_F	5'BIO-GAAGCAGCAAGTATGATGAGCA
JAK2_pyro_V617F_R	TGCTCTGAGAAAGGCATTAGAA
JAK2_pyro_V617F_seq	TCTCGTCTCCACAGA
KIT_pyro_D816V_F	5'BIO -CCTTACTCATGGTCGGATCAC
KIT_pyro_D816V_R	GCAGAGAATGGGTACTCACGT
KIT_pyro_D816V_seq	CACATAATTAGAATCATTCTTG
JAK2_RS7864782_F	5'BIO-TTGTAATTATGCATGTCGGCTTCC
JAK2_RS7864782_R	TAAGAGCTCAAGACAGGGCTGTTT
JAK2_RS7864782_SEQ	GCTGTTTGTGGCTCAGT
JAK2_RS10758669_F	5'BIO-GGTTTTCAAGCACCAAAGTTTAAT
JAK2_RS10758669_R	GGTTGGCTATGGCAAAGAAA
JAK2_RS10758669_SEQ	AAATTTTCCAATACCTCC
JAK2_RS6476934_F	ACTCTTTCCTCCCCTAAGCTCTGT
JAK2_RS6476934_R	5'BIO-CAAATAAAAAGGGCCTCAGAGCA
JAK2_RS6476934_SEQ	TTCGTCTGAAAGGATCTA
JAK2_RS7046736_F	5'BIO-AAAAAGCCAATTCGAGTCACTTA
JAK2_RS7046736_R	AACTGGAAATCACACTGCCTTAAA
JAK2_RS7046736_SEQ	AAAATGCATTGATGCTT
JAK2_RS2149556_F	5'BIO-CATTCCTTTTTATTGCTGGGTAGT
JAK2_RS2149556_R	AAGTGAAAGCAACCCAAATGTCC
JAK2_RS2149556_SEQ	GAATACATTTAAAATTTGAG
JAK2_RS12342421_R	TTTTCCCTTTGCTCGAGTTATTC
JAK2_RS12342421_F	5'BIO-AACTATCACATTGGCTCACACAG
JAK2_RS12342421_SEQ	TGTCAACATAAACCACATC
JAK2_RS10974947_F	5'BIO-AATTACAGGGTTTGAAAATTACCG
JAK2_RS10974947_R	TCTTTGGCTTGAACTTTTCACA
JAK2_RS10974947_SEQ	CAGAAATGTAATAACACTCC

JAK2_RS10119004_F	5'BIO-TTGTCATTTTTAACTGCGCTTCT
JAK2_RS10119004_R	CAATGCTTTTATCTGTGGGAATTA
JAK2_RS10119004_SEQ	GAGACTCCTTTTCCTCAC
JAK2_RS12343867_F	TGGCAGGTTCAACATAACATTGG
JAK2_RS12343867_R	5-BIO-TGCCGTAAACCTTCAATATGTAAA
JAK2_RS12343867_SEQ	GGAACTGACAGAAATGATT
JAK2_RS12340895_F	TGGTAATATCTTCGAGGTATGCC
JAK2_RS12340895_R	5'BIO-CCAAACCACTGCTTTTGAGAAGA
JAK2_RS12340895_SEQ	GGTATGCCTTTATTTTAG
JAK2_RS2031904_F	5'BIO-GTACGGGAACAATACAACTTAGCA
JAK2_RS2031904_R	TGCAGCGTGAGAACAGACTAATA
JAK2_RS2031904_SEQ	GAGTCATAAGTGCCTAACC
JAK2_RS17425819_F	GGCTCTGGCGTCTTAGTCTTCA
JAK2_RS17425819_R	5'BIO-AGCCCTTCCCACCACACA
JAK2_RS17425819_SEQ	CGACTGGCTCACAGAC
JAK2_RS10815160_F	CCCCAGTGATACATGAATTTAAT
JAK2_RS10815160_R	5'BIO-TTTGGAGCACAGTAGAGTCTTAAT
JAK2_RS10815160_SEQ	TATGAAGATTCAGAGATTTG
JAK2_rs2230724_DNA_F	5'BIO-ACTCACGATTATTTTGGTCAACTT
JAK2_rs2230724_DNA_R	AACTGTGTAGGATCCCGGTCT
JAK2_rs2230724_DNA_seq	GGCACCAGAAAACCC
JAK2_rs2230724_cDNA_F	5'BIO-CCTTCTTTCAGAGCCATCATACGA
JAK2_rs2230724_cDNA_R	GCACATCTCCACACTCCCAAAAT
JAK2_rs2230724_cDNA_seq	GGCACCAGAAAACCC
JAK2_rs10429491_DNA_F	AGCCTGGCCAATTTGTATCTTGTA
JAK2_rs10429491_DNA_R	5'BIO-CCATCCCAAGACATTCTTCCT
JAK2_rs10429491_DNA_seq	GGCATGATTTTGTGCA
JAK2_rs10429491_cDNA_F	GAAGCTCCTCTTCTTGATGACTTT
JAK2_rs10429491_cDNA_R	5'BIO-TTTGGTAAGAATGTCTTGTAGCTGATAGAG
JAK2_rs10429491_cDNA_seq	GGCATGATTTTGTGCA
EPOR_rs318699_F	5'BIO-AGCACATCTCATTCCCCTGAA
EPOR_rs318699_R	CAGCTCCAGCAAAAAGTCCC
EPOR_rs318699_seq	TGTGTCATGTGTCCACC

JAK2 MLPA primers

Primer name	Sequence (5'-3')
JAK2_exon3_MLPA_A	GGGTTCCCTAAGGGTTGGATGAAGCAAATAGATCC AGTTCTTC
JAK2_exon3_MLPA_B	5'Phosphate-AGGTGTATCTTTACCATTCCCTTGAAGGGCTCAGAATGAGCCGC TCAGTTCCCTTCTAGATTGGATCTTGCTGGCAC
JAK2_exon9_MLPA_A	GGGTTCCCTAAGGGTTGGAATAGATTAACTGCAGATGCACATC
JAK2_exon9_MLPA_B	5'Phosphate-

	ATTACCTCTGTAAAGAAGTAGCACTCTTGATTACATCCAGGGGC GCGTGTCTAGATTGGATCTTGCTGGCAC
JAK2_exon16_MLPA_A	GGGTTCCCTAAGGGTTGGATCTGCTTATCAGAGAAGAAGACAG
JAK2_exon16_MLPA_B	5'Phosphate-GAAGACAGGAAATCCTCCTTTCATTGGGTCCGACAGCTTTCTAG ATTGGATCTTGCTGGCAC
JAK2_exon20_MLPA_A	GGGTTCCCTAAGGGTTGGAACTTGGGTAATTTTGGTGTG
JAK2_exon20_MLPA_B	5'Phosphate-GAGATGTGCCGTGGTCGCTGTAAAGGATCCTGATGCG AGGGCCAGGGCTTCAGGGCTTCTAGATTGGATCTTGCT GGCAC

MLPA primers used to determine JAK2 copy number

Gene name, GenBank Accession number and size of PCR product	Primer name	Sequence (5'-3')
FES NM_002005 90bp	FES_Exon 2 _F	GGGTTCCCTAAGGGTTGGACTGGAGGGC ATGAGAAAGTGGATG
	FES_Exon 2 _R	5'Phosphate-GCCCAGCGGGTCAAGAGTGACAGGTCTA GATTGGATCTTGCTGGCAC
JAK2 NM_004972 109bp	JAK2_Exon 1_F	GGGTTCCCTAAGGGTTGGAGCTAGCTGC ACGGTGATATTTCTGAAATGCCAATT
	JAK2_Exon 1_R	5'Phosphate-CTATGAAGCAAATAGATCCAGTTCCAGCT TCTAGATTGGATCTTGCTGGCAC
SOAT1 NM_003101 112 bp	SOAT1_Exon 4_F	GGGTTCCCTAAGGGTTGGAGACGCATTG CAGGTCAGCATCATTAGATAATGGTGG
	SOAT1_Exon 4_R	5'Phosphate-GTGCGCTCTCACAACCTTTTCTGTGTCAA GCAGCTCTAGATTGGATCTTGCTGGCAC
BTK NM_000061 115bp	BTK_Exon 2_F	GGGTTCCCTAAGGGTTGGACTTCAAGAA GCGCCTGTTTCTCTT
	BTK_Exon 2_R	5'Phosphate-GACCGTGCACAAACTCTCCTACGTCAGGT TAAACGCTGTGCGTGATCATTCTAGATTG GATCTTGCTGGCAC
TYK2 NM_003331 118bp	TYK2_Exon1_F	GGGTTCCCTAAGGGTTGGAGCATAACGT ATCAGCAGTGAGTCATCGCTGACAGCT
	TYK2_Exon1_R	5'Phosphate-GAGGAAGTCTGCATCCACATTGCACTGC AACTAGGATCCGTCTAGATTGGATCTTGC TGGCAC

RON NM_002447 124bp	RON_Exon1_F	GGGTTCCCTAAGGGTTGGAGTCAATGAT GTCCAGTCCGCAATCGCCTGCATGTGCTT GGGC
	RON_Exon1_R	5'Phosphate-CTGACCTGAAGTCTGTCCAGAGCCGTCAT CCAGTACGGCGTCTAGATTGGATCTTGCT GGCAC

<u>*JAK2* exon 12 HRM and WTB-PCR primers</u>

Primer name	Sequence (5'-3')
JAK2_ex12_HRM_F	AATGGTGTTTCTGATGTACC
JAK2_ex12_HRM_R	AGACAGTAATGAGTATCTAATGAC
JAK2_ex12_WTB_F	CTCCTCTTTGGAGCAATTCA
JAK2_ex12_WTB_R	GAGAACTTGGGAGTTGCGATA
JAK2_ex12_wtLNA_oligo*	TTCA+C+A+AAATCAGAA+A+T+GAAG
JAK2_ex12_WTB_F2	GGAGCAATTCATACTTTCAGTGT
JAK2_ex12_WTB_R2	AACAGATGTTGTTTTAAAAGGAC

*LNA modified nucleotides are preceded with an '+'

<u>AS-PCR primers to detect *JAK2* SNP rs12343867</u>

Primer name	Sequence (5'-3')
JAK2_rs867_T_F	TGGTTTTAAATTATGGAGTATATT
JAK2_rs867_G_F	TGGTTTTAAATTATGGAGTATATG
JAK2_i14.15_R3	GCAAGGTGCAATAAAATGAGG

***JAK2* cDNA specific primers**

Transcript position (bp)	Primer name	Forward sequence (5'-3')	Primer name	Reverse sequence (5'-3')
9-475	JAK2_ex1_F	GGAGAGAGGAAGAGGAGCAGA	Jak2_ex2.3_R	CAGAACATTTGCCGTCGCG
465 -2210	JAK2_ex2_F4	GGCAACAGGAACAAGATGTGAACTG	JAK2_ex13.14_R4	ACTTGCTGCTTCAAAGAAAGACTCTGAAT
557 - 2205	JAK2_ex2.3_F4	CGACGGCAAATGTTCTGAAAAAGA	JAK2_ex13.14_R4	ACTTGCTGCTTCAAAGAAAGACTCTGAAT
771-1438	JAK2_ex4_F	TCTGGTATCCACCCAACCAT	JAK2_ex7.8_R	AAATCCTGTTCTGTCAGTGTCTCA
771-3075	JAK2_ex4_F	TCTGGTATCCACCCAACCAT	JAK2_ex19.20_R	TACCCTTGCCAAGTTGCTGT
885-1438	JAK2_ex5_F2	GAGCCTATCGGCATGGAATA	JAK2_ex7.8_R	AAATCCTGTTCTGTCAGTGTCTCA
1023-1721	JAK2_ex6_F	GAATGTCTTGGGATGGCAGT	JAK2_ex9.10_R	AATGGCAAAATCCATCGAAA
1023-2270	JAK2_ex6_F	GAATGTCTTGGGATGGCAGT	JAK2_ex11.12_R	TTTGATTTATCTTTTGGCTTTGG
1485-3561	JAK2_ex8_F	GCAAACCAAGAGGGTTCAAA	JAK2_ex22.23_R	TCTGGAGCATACCAGAATATG
1536-3832	JAK2_ex8.9_F3	GGTAAAAATCTGGAAATTGAACTTA	JAK2_ex25_R	AAGGAGGGGCGTTGATTTAC
1866-2370	JAK2_ex11_F	GAGAATGAAGAGTACAACCTC	JAK2_ex14.15_R	AAGGGTGTTTTCTTCTAGAAAAT
2033 - 3190	JAK2_ex11_F4	TTACCAGATGGAAACTGTTCGCTCA	JAK2_ex25.3UTR_R4	GGTCATTTCTTTCATCCAGCCATGT
1731-2498	JAK2_ex10_F	AGAAAGCAGGTAATCAGACTGGA	JAK2_exon15/16_R	TGTAGCTTTCTTTGAGAATCCAGA
1731 -2725	JAK2_ex10_F	AGAAAGCAGGTAATCAGACTGGA	JAK2_exon16/17_R	TAGATTACGCCGACCAGCA
2685-3561	JAK2_ex17_F	TTTGGCAACAGACAAATGGA	JAK2_ex22.23_R	TCTGGAGCATACCAGAATATG
3247-3689	JAK2_20.21_F	CAGTGCTGGTCGGCGTAAT	JAK2_ex24_R	TTGTTTGTCATTGCCAATCATACGC

3543-3832	JAK2_ex22.23_F	CCCATATTCTGGTATGCTCCA	JAK2_ex25_R	AAGGAGGGGCGTTGATTTAC
3661-4778	JAK2_ex23.24_F2	CCAGCGGAATTTATGCGTATGAT	JAK2_ex25_R2	TTATTGGCAGTCAGCAGCTC

Appendix IV

Calculations performed on MLPA data to determine *JAK2* copy number of homozygous V617F positive MPNs

Peak areas from each patient were exported to an Excel spreadsheet, which was designed to assess the ratio of each peak relative to all other peaks for that individual, plus equivalent peak areas obtained from other V617F positive cases or normal controls run alongside each other in the assay. As illustrated below, values for each peak area were entered into the corresponding control or test column in the spreadsheet, which then applied a series of calculations to normalise each test peak area against one other peak area from the same run and the equivalent values derived from the control sample. The tables below show examples of data derived from two female homozygous *JAK2* V617F case normalised together (Table A1), a male homozygous *JAK2* V617F case normalised against a female equivalent, with one copy of X-linked gene *BTK* (Table A2), and a female case carrying a constitutional del(9p) normalised to a different normal female sample, showing only one copy of *JAK2* (Table A3). An example of the calculations used to normalise test peaks in example A to the control, and determine the dosage quotient for *JAK2,* is:

> *JAK2* copy number = (test *FES* peak area/test *JAK2* peak area)/ (control *FES* peak area/control *JAK2* peak area)
>
> or (9559/9018)/ (7795/8320) = 1.1314

If the resulting dosage quotient falls between the range 0.8 to 1.2 this denotes the presence of two copies of the target region (MLPA copy number ranges defined previously by Bunyan et al. [251]). A deletion was scored if the dosage quotient was less than 0.7 (coloured red by the spreadsheet), and a duplication was scored if the quotient was 1.3 or greater (coloured green). Thus a full row of dosage quotients all below 0.6 highlighted red by the spreadsheet indicates a deletion of one copy of that gene, and a row of dosage quotients all above 1.3 and highlighted green indicates a copy number increase. Any red-coloured differences should be consistent across different markers, as shown in Tables A2 and A3.

	peak area		Exons						
Exons	**Control**	**Test**	***FES***	***JAK2***	***SOATIA***	***BTK***	***TYK2***	***RON***	***HCKA***
FES	7795	9559	1.0000	1.1314	0.8663	0.9547	1.0427	1.0736	1.1429
JAK2	8320	9018	0.8839	1.0000	0.7657	0.8438	0.9216	0.9489	1.0102
SOATIA	5094	7211	1.1544	**1.3060**	1.0000	1.1020	1.2037	1.2393	**1.3193**
BTK	14576	18723	1.0475	1.1851	0.9074	1.0000	1.0922	1.1246	1.1972
TYK2	10815	12719	0.9590	1.0850	0.8308	0.9156	1.0000	1.0296	1.0961
RON	8521	9733	0.9315	1.0538	0.8069	0.8892	0.9712	1.0000	1.0646
HCKA	8511	9132	0.8750	0.9899	0.7580	0.8353	0.9123	0.9394	1.0000

Table A1.

	peak area		Exons						
Exons	**Control**	**Test**	***FES***	***JAK2***	***SOATIA***	***BTK***	***TYK2***	***RON***	***HCKA***
FES	7034	18648	1.0000	1.0738	0.8457	**1.6653**	0.9691	0.9559	0.8215
JAK2	5906	14582	0.9313	1.0000	0.7876	**1.5510**	0.9026	0.8902	0.7651
SOATIA	4486	14063	1.1825	1.2697	1.0000	**1.9692**	1.1460	1.1303	0.9714
BTK	12346	19654	**0.6005**	**0.6448**	**0.5078**	1.0000	**0.5819**	**0.5740**	**0.4933**
TYK2	9228	25244	1.0319	1.1080	0.8726	**1.7184**	1.0000	0.9863	0.8477
RON	7021	19473	1.0462	1.1233	0.8847	**1.7422**	1.0139	1.0000	0.8594
HCKA	6262	20209	1.2173	**1.3071**	1.0295	**2.0272**	1.1797	1.1636	1.0000

Table A2.

	peak area		Exons						
Exons	**G1654**	**Test**	***FES***	***JAK2***	***SOATIA***	***BTK***	***TYK2***	***RON***	***HCKA***
FES	4300	9479	1.0000	**2.0773**	1.2782	1.2463	0.9995	1.2667	1.0154
JAK2	4494	4769	**0.4814**	1.0000	**0.6153**	**0.6000**	**0.4811**	**0.6098**	**0.4888**
SOATIA	3214	5543	0.7824	**1.6252**	1.0000	0.9751	0.7820	0.9910	0.7944
BTK	6517	11527	0.8024	**1.6668**	1.0256	1.0000	0.8020	1.0164	0.8147
TYK2	5984	13198	1.0005	**2.0784**	1.2788	1.2469	1.0000	1.2674	1.0159
RON	6310	10981	0.7894	**1.6399**	1.0091	0.9839	0.7890	1.0000	0.8016
HCKA	3754	8150	0.9848	**2.0458**	1.2588	1.2274	0.9843	1.2475	1.0000

Table A3.

Appendix V

***In vitro* colony assay calculations to determine response to imatinib**

Shown here is an example of the myeloid colony counts and calculations used to assess the response to imatinib exposure for one patient with MPN. Colonies from each triplicate dish a, b and c were counted at days 7 and 14, and the values for each triplicate were averaged (table B1).

	0µM				1µM				5µM			
	a	b	c	av	a	b	c	av	a	b	c	av
day 7	7	9	11	9.00	3	1	0	1.33	2	1	1	1.33
day 14	10	10	8	9.33	3	1	4	2.67	1	2	1	1.33

Table B1. *In vitro* colony assay colony numbers measured in triplicate at day 7 and day 14, exposed to 0, 1 and 5 µM imatinib.

The 1µM and 5µM values were then expressed relative to the 0µM value (table B2):

	No of colonies		
	0µM	1µM	5µM
Day 7	1	0.15	0.15
Day 14	1	0.29	0.14

Table B2. *In vitro* colony assay colony number expressed as a fraction of the value of the unexposed measurement.

The index of response was then calculated as the average of the relative 1µM and 5µM values at days 7 and 14:

$$\frac{0.15+0.15+0.29+0.14}{4} = 0.18$$

In this instance the result would be considered as borderline as it is only marginally below the response threshold (0.2) defined by the analysis of CML cases and normal controls.

***In vitro* liquid culture assay calculations to determine response to imatinib**

In this example of the *in vitro* imatinib liquid culture assay, PB granulocytes from a patient carrying an imatinib-sensitive lesion (*BCR-ABL*) were exposed to 0µM, 1µM and 5µM imatinib and viable cells were counted over a period of 22 days. Each culture was set up in duplicate (dishes a and b), and the total cell numbers (recorded here in table B3 as multiples of $1x10^6$) were averaged.

Day	0µM			1µM			5µM		
	a	b	average	a	b	average	a	b	average
0	5	5	5.00	5	5	5.00	5	5	5.00
4	2.45	2.5	2.48	1.98	2.05	2.02	1.78	1.98	1.88
8	1.3	1.3	1.30	0.98	1.16	1.07	0.83	1	0.92
12	0.8	0.65	0.73	0.33	0.29	0.31	0.1	0.15	0.13
15	0.37	0.15	0.26	0.09	0.06	0.08	0.02	0.03	0.03
19	0.12	0.2	0.16	0.03	0.01	0.02	0.04	0.01	0.03
22	0.08	0.1	0.09	0.01	0.02	0.02	0.05	0.01	0.03

Table B3. *In vitro* liquid culture assay cell numbers ($1x10^6$cells) counted in duplicate over time (days) in response to 0, 1, and 5 µM imatinib.

The results were then expressed relative to the count at day 0, which is given a value of 1 (table B4).

Day	0µM	1µM	5µM
0	1	1	1
4	0.495	0.403	0.376
8	0.26	0.214	0.183
12	0.145	0.062	0.025
15	0.052	0.015	0.005
19	0.032	0.004	0.005
22	0.018	0.003	0.006

Table B4. *In vitro* liquid culture assay cell counts normalised to the cell counts of the untreated control.

These values were then plotted on a line graph. The response, determined by visual inspection, is defined as a reduction in cell survival in the imatinib treated cultures, compared to the untreated culture (Figure 4.2). Inhibition of cell growth and survival by

imatinib exposure was also determined by expressing the counts from the treated cultures relative to the untreated cultures for each time point (table B5).

Day	1µM	5µM
0	1	1
4	0.81	0.75
8	0.82	0.70
12	0.42	0.17
15	0.28	0.09
19	0.12	0.15
22	0.16	0.33

Table B5. *In vitro* liquid culture assay cell counts of the imatinib-exposed cultures, normalised to the untreated control.

Appendix VI

***In vitro* imatinib sensitivity liquid culture and colony assay results alongside molecular abnormality where identified.**

Sample identifier	referral	Colony assay result	Liquid culture assay result	mutation identified
E199	?HES	negative	negative	*JAK2*V617F
E300	aMPN	negative	negative	*BCR-FGFR1*
E367	AML with SM	negative	negative	*KIT* D816V
E464	MPN	negative	negative	*JAK2*V617F
E662	SM	negative	negative	*KIT* D816V
E673	MPN	negative	negative	*JAK2*V617F
E726	SM	negative	negative	*KIT* D816V
E784	SM	negative	negative	*KIT* D816V
E177	aCML	not informative	negative	*JAK2*V617F
E213	HES	not informative	negative	*JAK2*V617F
E610	MPN	not informative	negative	*JAK2*V617F
E640	SM	not informative	negative	*KIT* D816V
E664	MDS/MPN	not informative	negative	*JAK2*V617F
E513	T cell lymphoma	negative	not done	*FIP1L1-PDGFRA*
E683	MPN	borderline	not informative	*KIF-PDGFRA*
E251	SM	negative	not informative	*KIT* D816V
E386	MPN	negative	not informative	*BCR-PDGFRA*
E685	SM	negative	not informative	*KIT* D816V
E776	SM	negative	not informative	*KIT* D816V
E359	HES	borderline	positive	*FIP1L1-PDGFRA*
E614	SM	borderline	positive	*FIP1L1-PDGFRA*
E630	?MPN	borderline	positive	*FIP1L1-PDGFRA*
E176	HES	negative	positive	*FIP1L1-PDGFRA*
E44	HES	negative	positive	*FIP1L1-PDGFRA*
E458	HES	negative	positive	*FIP1L1-PDGFRA*
E566	HES	negative	positive	*FIP1L1-PDGFRA*
E606	HES	negative	positive	*FIP1L1-PDGFRA*
E758	SM	negative	positive	*KIT* D816V
E817	HES	negative	positive	*FIP1L1-PDGFRA*
E156	MPN	positive	borderline	*JAK2*V617F
E350	MPN	positive	borderline	*JAK2*V617F
E521	MPN	positive	borderline	*ETV6-PDGFRB*
E583	MPN	positive	negative	*JAK2*V617F
E536	HES	positive	not informative	*FIP1L1-PDGFRA*
E537	HES	positive	not informative	*JAK2*V617F
E688	HES	positive	not informative	*FIP1L1-PDGFRA*
E107	MPN	positive	positive	*FIP1L1-PDGFRA*

E273	AML with t(5;12)	positive	positive	*ETV6-PDGFRB*
E905	?MPN	positive	positive	*FIP1L1-PDGFRA*
E194	CNL	negative	positive	none identified
E552	HES	negative	positive	none identified
E950	HES	negative	positive	none identified
E219	HES	negative	positive	none identified
E57	HES	negative	positive	none identified
E117	AML with eosinophilia	not done	positive	none identified
E250	MDS/MPN	not done	positive	none identified
E667	MPN	not informative	positive	none identified
E649	HES	positive	positive	none identified
E111	MPN	positive	borderline	none identified
E402	HES	positive	negative	none identified
E595	HES	positive	negative	none identified
E650	HES	positive	negative	none identified
E897	MPN	positive	negative	none identified
E1009	MPN	positive	negative	none identified
E74	SM	positive	negative	none identified
E492	HES	positive	not done	none identified
E495	HES	positive	not done	none identified
E988	HES	positive	not done	none identified
E1045	HES	positive	not done	none identified
E281	MPN	positive	not informative	none identified
E508	HES	positive	not informative	none identified
E689	HES	positive	positive	none identified
E61	aCML	positive	positive	none identified
E504	HES	borderline	borderline	none identified
E531	HES	negative	borderline	none identified
E106	MPN	not done	borderline	none identified
E173	MPN	not done	borderline	none identified
E180	aCML	not done	borderline	none identified
E201	MPN	not done	borderline	none identified
E245	HES	not done	borderline	none identified
E68	AML with eosinophilia	not informative	borderline	none identified
E178	SM	not informative	borderline	none identified
E210	HES	not informative	borderline	none identified
E228	MPN	not informative	borderline	none identified
E378	CMML	not informative	borderline	none identified
E316	HES	borderline	negative	none identified
E317	HES	borderline	negative	none identified
E394	HES	borderline	negative	none identified
E608	HES	borderline	negative	none identified

E721	MPN	borderline	negative	none identified
E970	HES	borderline	negative	none identified
E568	HES	borderline	not done	none identified
E580	IMF	borderline	not done	none identified
E989	HES	borderline	not done	none identified
E682	HES	borderline	not informative	none identified
E777	MPN	borderline	not informative	none identified
E862	HES	borderline	not informative	none identified
E42	HES	not done	negative	none identified
E62	HES	not done	negative	none identified
E63	HES	not done	negative	none identified
E70	MPN	not done	negative	none identified
E71	MPN	not done	negative	none identified
E72	aCML	not done	negative	none identified
E85	MPN	not done	negative	none identified
E99	aCML	not done	negative	none identified
E101	HES	negative	negative	none identified
E103	aCML	not done	negative	none identified
E110	CMML	not done	negative	none identified
E115	MPN	not informative	negative	none identified
E116	HES	not done	negative	none identified
E158	aCML	not done	negative	none identified
E160	aCML	not done	negative	none identified
E163	CMML	not done	negative	none identified
E167	CMML	not done	negative	none identified
E195	HES	not done	negative	none identified
E203	HES	not done	negative	none identified
E258	HES	not done	negative	none identified
E282	MPN	not informative	negative	none identified
E284	HES	negative	not informative	none identified
E287	HES	negative	negative	none identified
E288	HES	not done	negative	none identified
E290	MDS	negative	negative	none identified
E305	MPN	not done	negative	none identified
E310	aCML	negative	negative	none identified
E311	HES	negative	negative	none identified
E332	HES	negative	negative	none identified
E333	HES	negative	not done	none identified
E337	HES	negative	negative	none identified
E347	aCML	negative	negative	none identified
E351	HES	negative	negative	none identified
E353	HES	negative	negative	none identified
E372	HES	negative	negative	none identified
E373	SM	not informative	negative	none identified
E374	HES	not informative	negative	none identified

E393	HES	negative	negative	none identified
E416	HES	negative	negative	none identified
E417	HES	not informative	negative	none identified
E430	HES	negative	not informative	none identified
E435	MPN	negative	negative	none identified
E443	MPN	negative	not done	none identified
E467	MPN	negative	negative	none identified
E469	HES	negative	negative	none identified
E471	HES	negative	negative	none identified
E472	aCML	not done	negative	none identified
E480	MPN	negative	negative	none identified
E484	aCML	negative	negative	none identified
E487	HES	negative	negative	none identified
E491	HES	negative	negative	none identified
E497	HES	negative	not done	none identified
E512	HES	negative	negative	none identified
E514	CMML	negative	negative	none identified
E518	MDS/MPN	negative	not done	none identified
E520	HES	negative	negative	none identified
E523	HES	not done	negative	none identified
E532	MPN	negative	negative	none identified
E542	HES	negative	negative	none identified
E543	MPN	negative	negative	none identified
E560	HES	negative	not done	none identified
E567	HES	negative	not informative	none identified
E587	HES	negative	negative	none identified
E597	HES	negative	negative	none identified
E605	MDS	negative	not informative	none identified
E607	MPN	not done	negative	none identified
E609	HES	negative	negative	none identified
E622	HES	negative	not informative	none identified
E626	MPN	negative	negative	none identified
E628	HES	not done	negative	none identified
E634	HES	negative	negative	none identified
E636	HES	negative	negative	none identified
E641	ET transforming to MF	negative	not done	none identified
E643	AML with eosinophilia	not done	negative	none identified
E651	HES	negative	not done	none identified
E660	SM	negative	not done	none identified
E663	MPN	negative	negative	none identified
E697	MPN	negative	negative	none identified
E709	HES	negative	not done	none identified
E720	HES	negative	negative	none identified

E728	HES	not done	negative	none identified
E730	HES	negative	negative	none identified
E748	MDS/MPN	negative	not done	none identified
E755	HES	negative	not informative	none identified
E781	HES	negative	negative	none identified
E793	SM	negative	not done	none identified
E795	M	not done	negative	none identified
E816	HES	negative	negative	none identified
E818	MPN	negative	not informative	none identified
E831	aCML	negative	negative	none identified
E899	aCML	negative	negative	none identified
E920	JMML/MPN	negative	negative	none identified
E1010	HES	negative	negative	none identified
E1031	HES	not done	negative	none identified
E1044	HES	negative	not done	none identified
E1072	HES	not done	negative	none identified
E67	HES	not done	negative	none identified
NP99	MPN	not informative	negative	none identified
E74	SM	not informative	not done	none identified
E81	MPN	not informative	not done	none identified
E205	MPN	not informative	not done	none identified
E207	HES	not informative	not done	none identified
E209	HES	not informative	not done	none identified
E240	SM	not done	not informative	none identified
E241	SM	not done	not informative	none identified
E242	HES	not done	not informative	none identified
E257	HES	not informative	not done	none identified
E267	HES	not informative	not done	none identified
E118	aCML	not done	not informative	none identified
E121	HES	not done	not informative	none identified
E168	MPN	not informative	not done	none identified
E170	HES	not informative	not done	none identified
E185	HES	not done	not informative	none identified

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JAK2 haplotype is a major risk factor for the development of myeloproliferative neoplasms

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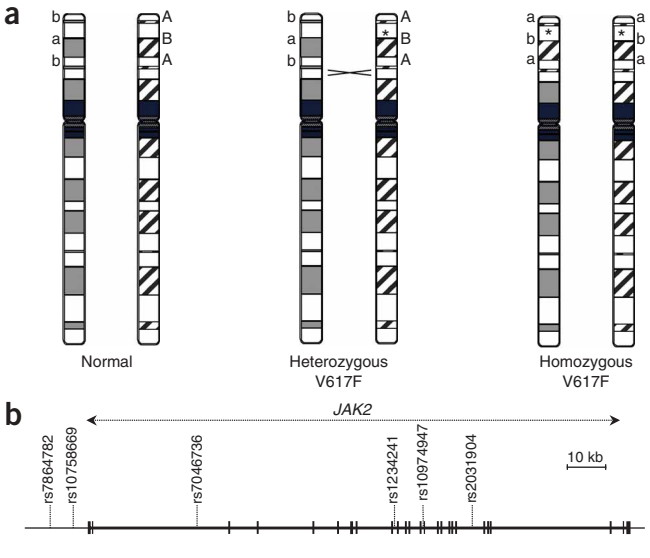
Chronic myeloproliferative neoplasms (MPNs) are a group of related conditions characterized by the overproduction of cells from one or more myeloid lineages. More than 95% of cases of polycythemia vera, and roughly half of essential thrombocythemia and primary myelofibrosis acquire a unique somatic 1849G>T *JAK2* mutation (encoding V617F) that is believed to be a critical driver of excess proliferation[1–4]. We report here that *JAK2*^{V617F}-associated disease is strongly associated with a specific constitutional *JAK2* haplotype, designated 46/1, in all three disease entities compared to healthy controls (polycythemia vera, $n = 192$, $P = 2.9 \times 10^{-16}$; essential thrombocythemia, $n = 78$, $P = 8.2 \times 10^{-9}$ and myelofibrosis, $n = 41$, $P = 8.0 \times 10^{-5}$). Furthermore, *JAK2*^{V617F} specifically arises on the 46/1 allele in most cases. The 46/1 *JAK2* haplotype thus predisposes to the development of *JAK2*^{V617F}-associated MPNs (OR = 3.7; 95% CI = 3.1–4.3) and provides a model whereby a constitutional genetic factor is associated with an increased risk of acquiring a specific somatic mutation.

The finding of *JAK2*^{V617F} was a major step forward in understanding the pathogenesis of MPNs, but it remains unclear how this single abnormality gives rise to distinct clinical entities. Clinical phenotype is clearly associated with *JAK2*^{V617F} dosage: in many PV and MF cases *JAK2*^{V617F} is reduced to homozygosity as a consequence of acquired isodisomy (generally referred to as acquired uniparental disomy; aUPD) at chromosome 9p, but this is rare in ET[2,5,6]. Several lines of evidence indicate that other, largely uncharacterized, acquired abnormalities also have a role in specifying disease phenotype either in combination with or independently of *JAK2*^{V617F} (refs. 7,8).



Case	%V617F	rs7864782		rs10758669		rs7046736		rs1234241		rs10974947		rs2031904		V617F haplotype	Wild-type haplotype
		A	G	A	C	A	C	G	C	A	G	A	G		
E659	99	100	0	7	93	100	0	100	0	6	94	2	98	ACAGGG	Unknown
E2433	80	14	86	4	96	82	18	85	15	0	100	0	100	GCAGGG	ACCCGG
E2513	62	71	29	31	69	68	32	74	26	8	92	0	100	ACAGGG	GACCGG
E1186	49	46	54	9	91	48	52	45	55	51	49	49	71	Unknown	Unknown

Figure 1 Allele distortions due to aUPD enable direct reading of *JAK2* haplotypes. (**a**) The *JAK2*^{V617F} mutation (indicated by an asterisk) and flanking SNPs are reduced to homozygosity in a proportion of cells following mitotic recombination. (**b**) SNPs and *JAK2*^{V617F} were quantified by pyrosequencing. In many cases that harbored a homozygous *JAK2*^{V617F} clone, it was possible to directly read the haplotype on which the mutation arose by the finding that one allele at each SNP predominated (allelic ratio ≥0.6, for example, cases E659, E2433 and E2513). In cases with a homozygous clone and %V617F <90%, it was usually possible to read the residual haplotype (that is, the haplotype of the chromosome that had not acquired *JAK2*^{V617F}) by the finding of allelic ratios between 0.1–0.4 (for example, cases E2433 and E2513). Where the homozygous clone was small or nonexistent (most cases with %V617F <60%), neither the *JAK2*^{V617F} nor wild-type haplotype could be read (for example, case E1186).

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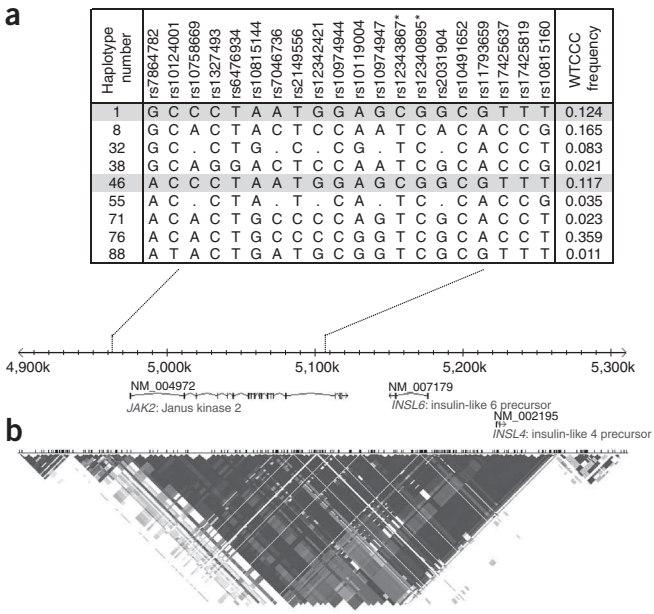

Figure 2 SNPs, haplotypes and LD around *JAK2*. (**a**) The nine most common *JAK2* haplotypes in the UK population. The 14 SNPs in bold were analyzed by the WTCCC in 1,500 blood donors from which the frequencies were determined; asterisks indicate SNPs that tag 46/1, which are highlighted in gray. rs78644782, rs10124001 and rs10758669 are immediately upstream of *JAK2*; all other SNPs are within *JAK2* introns. (**b**) LD in the *JAK2* region (HapMap data release 23a/phase II March 2008).

Furthermore, both epidemiological data and family studies indicate that inherited factors may predispose to MPNs[9,10] and it has also been suggested that inherited SNPs within *JAK2* are associated with specific MPN subtypes[11].

To determine the role of inherited factors, we initially analyzed six *JAK2*-spanning SNPs (rs7864782, rs10758669, rs7046736, rs12342421, rs10974947, rs2031904) in MPN cases with a homozygous $JAK2^{V617F}$ clone (%V617F allele >50%; $n = 142$) using pyrosequencing, which provides a quantitative readout of allele ratios. The mitotic recombination that gives rise to aUPD typically involves most of chromosome 9p[2,6] and thus SNPs within this region are also reduced to homozygosity; consequently, the haplotype on which $JAK2^{V617F}$ arose could be read directly from allele ratios that were significantly greater than the expected value of 0.5. In most cases with 60–90% V617F, the residual haplotype (that is, the haplotype on which V617F had not arisen) could also be read by the finding of heterozygous allele ratios in the range 0.1–0.4. In cases with ≥90% V617F, however, information about alleles on the non-V617F chromosome was lost (**Fig. 1** and Methods). Notably, of the 142 alleles that harbored V617F, 109 (77%) had an identical haplotype (subsequently designated 46/1) within the *JAK2* gene, whereas this haplotype was seen for only 9 of the 74 (12%) residual wild-type alleles that could be read ($P = 1.4 \times 10^{-20}$, Fisher's exact test, two-tailed). These results indicated that homozygosity for $JAK2^{V617F}$ was not random, but rather occurred preferentially when this mutation was present on a specific *JAK2* haplotype.

To explore this observation in more detail, we first determined the haplotype structure of *JAK2* using 14 SNPs genotyped by the Wellcome Trust Case Control Consortium (WTCCC) in 1,500 healthy blood donors from the UK[12]. PHASE analysis[13] inferred 92 haplotypes, of which nine accounted for 94% of *JAK2* alleles (**Fig. 2**). Two haplotypes (numbers 46 and 1; referred to henceforth together as 46/1) were identical except for rs7864782 and had a combined frequency of 0.24. A tagged SNP (rs12340895) that was in complete linkage disequilibrium (LD) with 46/1 was then used to screen for this haplotype in further cases. In an initial analysis of 177 heterozygous $JAK2^{V617F}$-positive MPNs, 46/1 occurred more frequently (135/354 alleles) than in 188 locally sourced healthy controls (92/376 alleles; $P = 0.0001$) as well as the WTCCC cohort ($P = 3.3 \times 10^{-8}$). The 46/1 haplotype was more frequent in all $JAK2^{V617F}$-positive disease entities regardless of origin (UK or United States); however, there was no difference in the frequency of 46/1 between controls and cases with idiopathic erythrocytosis (**Table 1**). To determine whether $JAK2^{V617F}$ was in *cis* or *trans* to the 46/1 allele in cases that were heterozygous for both the mutation and the haplotype, we carried out allele-specific PCRs for $JAK2^{V617F}$ to amplify products that included a second 46/1 tag SNP (rs12343867) in intron 14. Sequencing of the products in 66 informative cases showed that 49 (74%) $JAK2^{V617F}$ alleles arose on a 46/1 allele, whereas only 17 (26%) residual wild-type alleles were 46/1 ($P = 2.1 \times 10^{-8}$).

We have previously described a polycythemia vera pedigree in which $JAK2^{V617F}$ was not inherited but arose independently in two affected individuals[14]. Family members were analyzed for rs12340895:

Table 1 Summary of genotyping results

Category	Number of cases	Number of 46/1 alleles	Number of non-46/1 alleles	Frequency 46/1	*P* value (versus UK local controls)	OR (95% CI)	*P* value (versus WTCCC controls)	OR (95% CI)
$JAK2^{V617F}$-positive PV	192[a]	197	171	0.54	2.88E−16	3.6 (2.6–4.9)	7.56E−30	3.6 (2.9–4.6)
$JAK2^{V617F}$-positive ET	78	79	77	0.51	8.24E−09	3.2 (2.1–4.7)	4.27E−12	3.2 (2.4–4.5)
$JAK2^{V617F}$-positive MF	41[a]	37	40	0.48	8.00E−05	2.9 (1.7–4.7)	6.12E−06	2.9 (1.9–4.6)
$JAK2^{V617F}$-positive unclassified MPN	124[a]	135	106	0.56	3.33E−15	3.9 (2.8–5.6)	3.40E−24	4.0 (3.1–5.3)
Idiopathic erythrocytosis	76	41	111	0.27	5.80E−01	1.1 (0.7–1.7)	4.37E−01	1.2 (0.8–1.7)
$JAK2^{V617F}$-negative MPN (UK)	47	36	58	0.38	9.00E−03	1.9 (1.2–3.1)	2.00E−03	2.0 (1.3–3.0)
$JAK2^{V617F}$-positive ET (GR)	143	124	162	0.43	4.51E−07[b]	2.4 (1.7–3.3)[b]	9.18E−12	2.4 (1.9–3.1)
$JAK2^{V617F}$-negative ET (GR)	136	89	183	0.33	9.00E−02[b]	1.5 (1.1–2.1)[b]	2.00E−03	1.5 (1.2–2.0)
UK controls	188	92	284	0.24	–	–	8.48E−01	1.0 (0.8–1.3)
WTCCC controls	1,500	720	2,280	0.24	8.48E−01	1.0 (0.8–1.3)	–	–
GR controls	108	55	161	0.25	8.43E−01	1.1 (0.7–1.6)	6.22E−01	1.1 (0.8–1.5)

PV, polycythemia vera; ET, essential thrombocythaemia; MF, myelofibrosis; MPN, myeloproliferative neoplasm (all samples from the first six categories were from the UK and United States). GR, Greek samples; UK, UK samples; WTCCC, Wellcome Trust Case Control Consortium analysis of 1,500 UK blood donors.
[a]These groups included some cases with ≥90% V617F and thus the residual wild-type allele could not be assigned as 46/1 or not 46/1. [b]Values versus healthy Greek controls. All *P* values were calculated using Fisher's exact test, two-tailed.

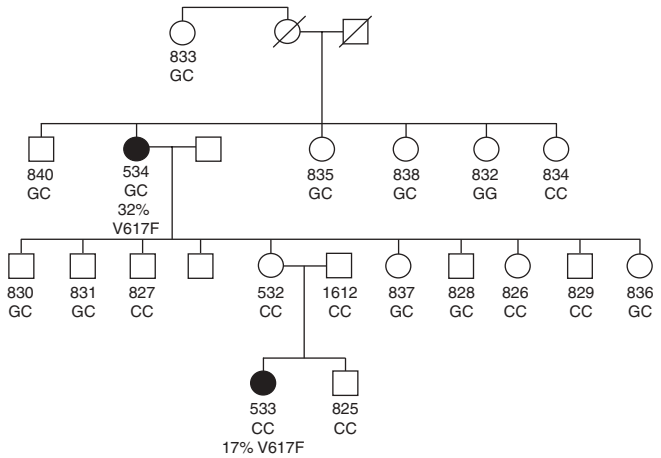

Figure 3 Familial polycythemia vera pedigree. The two affected individuals (UPNs 534 and 533) are shown as black circles. The genotype for rs12340895 is shown (G = 46/1 allele; C = non-46/1 allele), as is the %V617F in affected cases. Allele-specific PCR for UPN 534 showed that *JAK2*^{V617F} arose on the 46/1 allele. All other individuals had normal blood counts and were negative for *JAK2*^{V617F}, *PRV1* overexpression and endogenous erythroid colony growth[14].

one affected individual (UPN 534) was heterozygous for 46/1 but the second (UPN 533) was negative for this haplotype (**Fig. 3**). Allele-specific PCR for UPN 534 showed that *JAK2*^{V617F} had arisen on the 46/1 allele, confirming the association between this haplotype and the mutation. However, this pedigree illustrates that 46/1 is not solely responsible, at least in this family, for predisposition to polycythemia vera. Indeed, linkage of disease to 9p has not been described in any family with MPN.

We suggest two hypotheses to account for the association of MPNs with 46/1: (i) *JAK2*^{V617F} may arise randomly on all haplotypes but 46/1 is in LD with an unknown constitutional functional variant that interacts with *JAK2*^{V617F} in a manner that makes the development of clinically manifest disease more likely compared to *JAK2*^{V617F} on a non-46/1 haplotype, or (ii) there is a specific mutational mechanism by which *JAK2*^{V617F} preferentially arises on a 46/1 haplotype. These hypotheses are not necessarily mutually exclusive. Inspection of the HapMap data (**Fig. 2**) indicates that the entire *JAK2* gene is contained within a 280-kb LD block that includes two other genes (*INSL4* and *INSL6*) that are not expressed in hemopoietic cells, as verified by RT-PCR analysis. It is highly likely therefore that any functional variant within 46/1 directly affects *JAK2*. Notably, rs10758669, a SNP that also tags 46/1, was identified as significant in a recent genome-wide association study of Crohn's disease[15], thus supporting the hypothesis of a functional *JAK2* variant on that allele. This SNP was also reported to be significantly associated with polycythemia vera but not with essential thrombocythemia or myelofibrosis[11], a result that is presumably explained by the relatively high prevalence of *JAK2*^{V617F} in polycythemia vera compared to the other two subtypes.

JAK2 is required for signaling by diverse myeloid cytokine receptors (for example, IL-3, G-CSF, GM-CSF, EPO) as well as other receptors in lymphoid and nonhemopoietic cells[16]. To investigate the possibility that *JAK2* on the 46/1 haplotype is different functionally from other *JAK2* alleles, we tested whether 46/1 influences myeloid colony formation in hematologically normal individuals ($n = 56$). In a prospective analysis, we counted the numbers of granulocyte-macrophage colony-forming units (CFU-GM) and erythroid burst-forming units (BFU-E) in peripheral blood and compared the results to *JAK2* haplotype. Individuals that carried at least one 46/1 allele grew significantly fewer CFU-GM, consistent with the hypothesis that *JAK2* on 46/1 is indeed functionally different from other *JAK2* alleles. There was no effect, however, on BFU-E growth (**Fig. 4**).

One possible reason for the observed association might be that *JAK2* on 46/1 is expressed more or less than *JAK2* on other haplotypes. To explore this possibility, we used pyrosequencing to quantify the allele ratios of two *JAK2* exonic SNPs (rs10429491 and rs2230724) in matched cDNA and genomic DNA from control *JAK2*^{V617F}-negative cases that were heterozygous for at least one of the exonic SNPs as well as heterozygous for 46/1 ($n = 46$). We found no differences in allele ratios in cDNA and genomic DNA with either SNP, indicating that 46/1 is not associated with either increased or decreased *JAK2* expression, at least in peripheral blood leukocytes (**Supplementary Fig. 1** online).

Finally, we explored the possibility that a functional variant might also be relevant to the pathogenesis of *JAK2*^{V617F}-negative MPNs. We genotyped rs12340895 in *JAK2*^{V617F}-negative essential thrombocythemia and myelofibrosis cases ($n = 47$) from the UK and found that 36 or 94 alleles were 46/1, significantly higher than the frequency in locally sourced controls and the WTCCC cohort ($P = 0.009$ and $P = 0.002$, respectively). However, we failed to confirm this association in an additional series of *JAK2*^{V617F}-negative essential thrombocythemia cases ($n = 136$) and controls ($n = 108$) from Greece, although the P value of 0.09 and slightly elevated odds ratio suggests that the relevance of 46/1 to *JAK2*^{V617F}-negative cases warrants further investigation (**Table 1**).

Our data thus demonstrate that both homozygous and heterozygous *JAK2*^{V617F}-associated disease is preferentially associated with 46/1, and that this haplotype seems to be in LD with an as-yet-uncharacterized functional variant. However, this does not exclude the possibility that *JAK2* on 46/1 allele might be also be hypermutable. Whatever the mechanism, our data indicate that 46/1 is a strong predisposition factor for development of *JAK2*^{V617F}-associated MPNs (OR = 3.7; 95% CI = 3.1–4.3; relative risk = 2.6; 95% CI = 2.3–2.9; $n = 435$ cases versus WTCCC controls). The counts of 46/1 alleles in cases and WTCCC controls and the population frequency from the WTCCC data (**Table 1**), suggest that 46/1 accounts for 28% of the population attributable risk[17]. A recent Swedish study demonstrated a relative risk of 5.7 in first-degree relatives of individuals with polycythemia vera[10], corresponding to an attributable risk of 53%. Assuming no difference between the UK and Swedish populations, 46/1 thus accounts for slightly over 50% of the increased risk in first-degree relatives. For essential thrombocythemia and

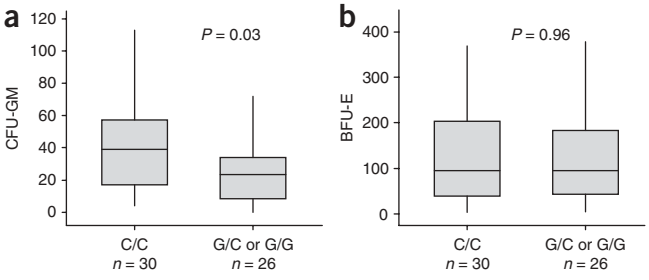

Figure 4 Association between *JAK2* haplotype and numbers of hemopoietic colonies. CFU-GM and BFU-E colony growth per 4×10^5 peripheral blood mononuclear cells from 56 healthy controls that were 46/1 nullizygous (C/C at rs12340895, $n = 30$) or had at least one 46/1 allele (G/C, $n = 21$; or G/G, $n = 5$). Box plots illustrate the 95% range (vertical lines), median (horizontal lines) and interquartile range (boxes). Colony numbers were compared by the Mann-Whitney U test.

myelofibrosis, the contribution of 46/1 is less clear, as it is unknown what proportion of the risk in first-degree relatives[10] is attributable to cases that are *JAK2*^{V617F} positive and those that are *JAK2*^{V617F} negative.

In addition to the specific association we describe here in MPNs, our findings may have wider relevance. Genome-wide association studies are identifying increasing numbers of loci that predispose to diverse malignancies[18–20]; our findings suggest that these loci should be considered as candidates for the acquisition of somatic mutations.

METHODS

Subjects. We analyzed a total of 775 subjects with MPN, of whom 183 had *JAK2*^{V617F}-negative disease and 592 were *JAK2*^{V617F} positive (PV, $n = 203$; ET, $n = 224$; MF, $n = 41$; unclassified MPN, $n = 124$). Subjects were recruited from clinics in the UK, United States and Greece. We also analyzed a previously described family with MPN from Germany[14]. For controls we analyzed healthy individuals from the UK ($n = 188$) and Greece ($n = 108$), and we also used data generated by the WTCCC from the UK blood donor cohort ($n = 1{,}500$)[12]. The study was approved by the relevant internal review boards and ethics committees and informed consent was provided according to the Declaration of Helsinki.

Genotyping. Total peripheral blood leukocyte DNA was analyzed by pyrosequencing for SNPs and *JAK2*^{V617F} as described[21]. Primer sequences are provided in **Supplementary Table 1** online. Because the allelic ratios (the ratio of allele A to allele B at any SNP) for any heterozygous SNPs were distorted away from the expected value of 0.5 in cases with a sizeable homozygous *JAK2*^{V617F} clone, we adopted the following scoring criteria for all SNPs: (i) if one allele had an allelic ratio ≥ 0.9, the sample was scored as homozygous for that SNP; (ii) where allelic ratios were 0.11–0.89, samples were scored as heterozygous. These cutoffs were at least 3 s.d. more than background (that is, values read for allele B in healthy controls who were A/A homozygotes). Similarly, for homozygous *JAK2*^{V617F} cases with V617F $>50\%$, SNPs with allelic ratios ≥ 0.6 or ≤ 0.4 were considered to be derived from the V617F-mutated or residual wild-type alleles, respectively. For *JAK2*^{V617F} homozygous cases in which the V617F was $\geq 90\%$, no information could be obtained about the residual wild-type *JAK2* allele and thus these cases were considered to contribute only one allele to the analysis.

Allele-specific PCR. Allele-specific PCR was performed using forward primers that were specific for *JAK2*^{V617F} (VF-ASF) or the corresponding wild-type sequence (WT-ASF) in combination with a common reverse primer (ASR), producing a 565-bp product that included the 46/1 tag SNP rs12343867. Amplification conditions were optimized on DNA from the HEL cell line (100% V617F) and normal healthy controls. Products were sequenced to determine whether *JAK2*^{V617F} was on the 46/1 allele or not.

Colony analysis. Mononuclear cells (MNCs) from peripheral blood of hematopoietically normal controls were isolated by centrifugation over lymphoprep (Axis-Shield) and cultured in methylcellulose medium (H4434; Stem Cell Technologies) at a density of 4×10^5 cells per 30-mm plate (in a final volume of 1 ml), in triplicate, following the manufacturer's instructions. Colonies comprising a minimum of 100 cells were counted on day 14, and characterized on the basis of morphology as either CFU-GM, CFU-GEMM, CFU-E or BFU-E, as described by StemCell Technologies. *JAK2* haplotype status was determined after colonies were counted using DNA extracted from the MNC or granulocyte cell fractions.

Expression analysis. RNA was extracted from peripheral blood leukocytes of *JAK2*^{V617F}-negative MPD cases that were known to be heterozygous for rs10429491 and/or rs2230724 (*JAK2* exonic SNPs) as well as heterozygous for 46/1, as determined by rs12340895 genotype. RNA was reverse transcribed with random hexamer primers and the ratio of the two alleles for each SNP in genomic DNA and cDNA was determined by specific Pyrosequencing assays (**Supplementary Table 1**).

Statistical analysis. The proportion of 46/1 alleles for each case subgroup was compared to controls using Fisher's exact test (two-tailed). Colony numbers were compared to genotype using the Mann-Whitney *U* test. For the expression analysis, the mean and variance of SNP allelic rations were compared by *t* and *F* tests, respectively. Odds ratios (OR) were calculated as (number of 46/1 alleles in cases/number of 46/1 alleles in controls)/(number of non-46/1 alleles in cases/number of non-46/1 alleles in controls). Relative risk (RR) was calculated as (number of 46/1 alleles in cases/number of 46/1 alleles in cases plus controls)/(number of non-46/1 alleles in cases/number of non-46/1 alleles in cases plus controls). Population attributable risks were calculated as $(f(RR - 1)/1 + f(RR - 1))100$, where f is allele frequency[17].

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

The study was designed by A.V.J., A. Chase., F.G. and N.C.P.C. A.V.J. performed the laboratory analysis. R.T.S., D.O., K.Z., Y.L.W., H.L.P., H.C. and A.R. provided clinical samples and associated information. A.V.J., A. Chase, A. Collins and N.C.P.C. analyzed the data. N.C.P.C. wrote the first draft of the manuscript and all authors contributed to and approved the final version.

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bjh short report

Clonal diversity in the myeloproliferative neoplasms: independent origins of genetically distinct clones

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Summary

This study looked for clonal diversity in patients with a myeloproliferative neoplasm associated with more than one acquired genetic lesion. A tyrosine kinase mutation and a cytogenetic lesion were present in the same clone in six of seven patients. By contrast, the genetic lesions were present in separate clones in all six patients with two tyrosine kinase pathway mutations. Moreover, in two patients the clones were genetically unrelated by X-chromosome inactivation studies. These data demonstrated clonal diversity in a subset of patients with early stage haematopoietic malignancy and showed, for the first time, that such clones may arise independently.

Keywords: myeloproliferative disorder, cytogenetics, clonality, leukaemia, tyrosine kinases.

Neoplasia is thought to be initiated in a single cell, with stepwise accumulation of genetic lesions leading to a malignant phenotype. However, divergent clones bearing dissimilar patterns of somatic mutation have been observed in both multifocal and premalignant epithelial tumours. In some cases, an overlap in somatic mutation pattern was observed, indicating that divergent clones were the phylogenetically related progeny of a shared founder clone (Sidransky *et al*, 1992). In other cases, no such overlap could be identified, raising the possibility that some tumours are comprised of more than one genetically unrelated clone (Leedham *et al*, 2008).

The human myeloproliferative neoplasms (MPNs) are premalignant clonal stem cell disorders often driven by activated tyrosine kinases, including *BCR-ABL1* in chronic myeloid leukaemia and *JAK2* mutations in polycythaemia vera and essential thrombocythaemia. Recurrent genetic alterations, other than those activating a tyrosine kinase, are uncommon in early disease, but genetic events accumulate with progression through an accelerated phase of disease to acute leukaemia (Campbell & Green, 2006; Melo & Barnes, 2007). As such, MPNs serve as an experimentally tractable paradigm for the study of early stage tumorigenesis. Circumstantial evidence suggests that some MPN patients harbour genetically dissimilar clones: cytogenetically abnormal but *BCR-ABL1* negative clones have been reported following imatinib therapy (Deininger *et al*, 2007); patients with a *JAK2* mutant MPN can progress to a *JAK2* wild-type acute leukaemia (Campbell *et al*, 2006); and two different tyrosine kinase mutations may be found in the same patient (Bocchia *et al*, 2007; Li *et al*, 2008).

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With respect to the latter observation, analysis of individual progenitor colonies has been reported in only two cases, and although one case suggested the presence of biclonal disease, evolution from a shared founder clone could not be excluded (Bocchia *et al*, 2007; Li *et al*, 2008). We have therefore studied MPN patients with more than one acquired genetic lesion to look for the presence of clonal diversity and to assess the genetic relationship of such clones.

Materials and methods

Patients met the Polycythemia Vera Study Group criteria for essential thrombocythaemia or polycythaemia vera or the World Health Organisation criteria for primary myelofibrosis or systemic mastocytosis and were recruited from MPN clinics in Cambridge, Belfast, London, Birmingham and Salisbury. Institutional and Multi-Region Ethics Committee approval was obtained and the study was carried out in accordance with the principals of the Declaration of Helsinki.

For culture of progenitor colonies, peripheral blood mononuclear cells were plated in Methocult (Stem Cell Technologies, Vancouver, Canada) and incubated for 14 days at 37°C and 5% CO_2. Individual colonies were placed either in water and heated to 98°C for DNA extraction or into RLT buffer for RNA extraction according to the manufacturer's protocol (Qiagen, Hilden, Germany).

Results and discussion

Haematopoietic colonies were grown from peripheral blood obtained from 13 MPN patients with two or more acquired genetic lesions (Table I). Individual colonies, each derived from a single progenitor, were genotyped for the relevant genetic lesions. Using quantitative pyrosequencing, all three *JAK2* V617FF-positive patients with trisomy 9 harboured colonies with a 1:2 ratio of wild-type to mutant allele (Fig S1A and B), indicating (i) the *JAK2* mutation and trisomy 9 were in the same clone; (ii) trisomy 9 reflected duplication of the chromosome carrying the mutant *JAK2* allele; and (iii) loss of wild-type *JAK2* is not essential for the development of polycythaemia vera (Scott *et al*, 2006). Quantitative pyrosequencing also demonstrated that in two patients trisomy 8 and *JAK2* V617F were present within the same clone (Fig S1C and D). Loss of heterozygosity analysis was used to study three patients with a 20q deletion (Fig S2A and B). In Patient 5, the 20q deletion preceded acquisition of the *BCR-ABL1* fusion. Although 20q deletions have been reported in chronic myeloid leukaemia, to our knowledge this is the first report of a 20q deletion preceding acquisition of *BCR-ABL1*. In Patient 6, the 20q deletion coexisted with the *JAK2* mutation in all colonies, but in Patient 7 the two lesions were present in separate clones. Taken together, our results demonstrate that cytogenetic lesions frequently reflect evolution within the clone carrying an oncogenic tyrosine kinase. However in one of seven cases the cytogenetic abnormality reflected the presence of a separate clone (Fig 1A).

We also studied six patients carrying *JAK2* V617F together with a second tyrosine kinase pathway mutation (four *MPL* W515L, one *KIT* D816V and one *JAK2* E543-D544del; patients 8–13, Table I). In all six cases, analysis of individual colonies demonstrated that *JAK2* V617F and the second mutation were mutually exclusive, indicating that the two lesions were present in separate clones (Figs 1B and S2C). This could result from the two genetic lesions occurring as

Table I. Clinical features of patients with two or more acquired genetic lesions.

			At diagnosis							
Patient	MPN	Sex	Age years	Hb g/l	WCC $\times 10^9$/l	Plts $\times 10^9$/l	Prior Therapy	Disease duration (years)	Lesion 1	Lesion 2
1	PV	F	61	158*	20·2	671	HC	3	*JAK2* V617F	Trisomy 9
2	PV	F	65	198*	7·2	542	HC	8	*JAK2* V617F	Trisomy 9 Trisomy 8
3	PV	M	71	182	10·4	977	HC	1	*JAK2* V617F	Trisomy 9
4	PV	M	66	177*	10·6	553	HC	4	*JAK2* V617F	Trisomy 8
5	CML	M	52	134	26·8	167	IM	9	*BCR-ABL*	del(20q)
6	PMF	M	59	94	3·3	68	None	9	*BCR-ABL1*	del(20q)
7	ET	M	69	135	7·9	1016	HC	12	*JAK2* V617F	del(20q)
8	ET	F	92	134	10·8	1240	HC	2	*JAK2* V617F	*MPL* W515L
9	ET	F	28	140	12·8	1221	HC, AN	20	*JAK2* V617F	*MPL* W515L
10	ET	M	72	128	6·7	1258	HC	1·5	*JAK2* V617F	*MPL* W515L
11	ET	M	74	140	6·6	979	None	0	*JAK2* V617F	*MPL* W515L
12	SM	M	43	147	10·1	306	NL, PK, HC	3·5	*JAK2* V617F	*KIT* D816V
13	PV	F	73	172*	5·3	314	Ven	8	*JAK2* V617F	*JAK2* E543-D544del

MPN, myeloproliferative neoplasm; Hb, haemoglobin; WCC, white cell count; Plts, platelet count; PV, polycythaemia vera; CML, chronic myeloid leukaemia; PMF, primary myelofibrosis; ET, essential thrombocythaemia; SM, systemic mastocytosis; HC, hydroxycarbamide; IM, imatinib; AN, anagrelide; NL, nilotinib; PK, PKC412; Ven, venesection alone.
*Increased red cell mass confirmed by radio-isotope studies.

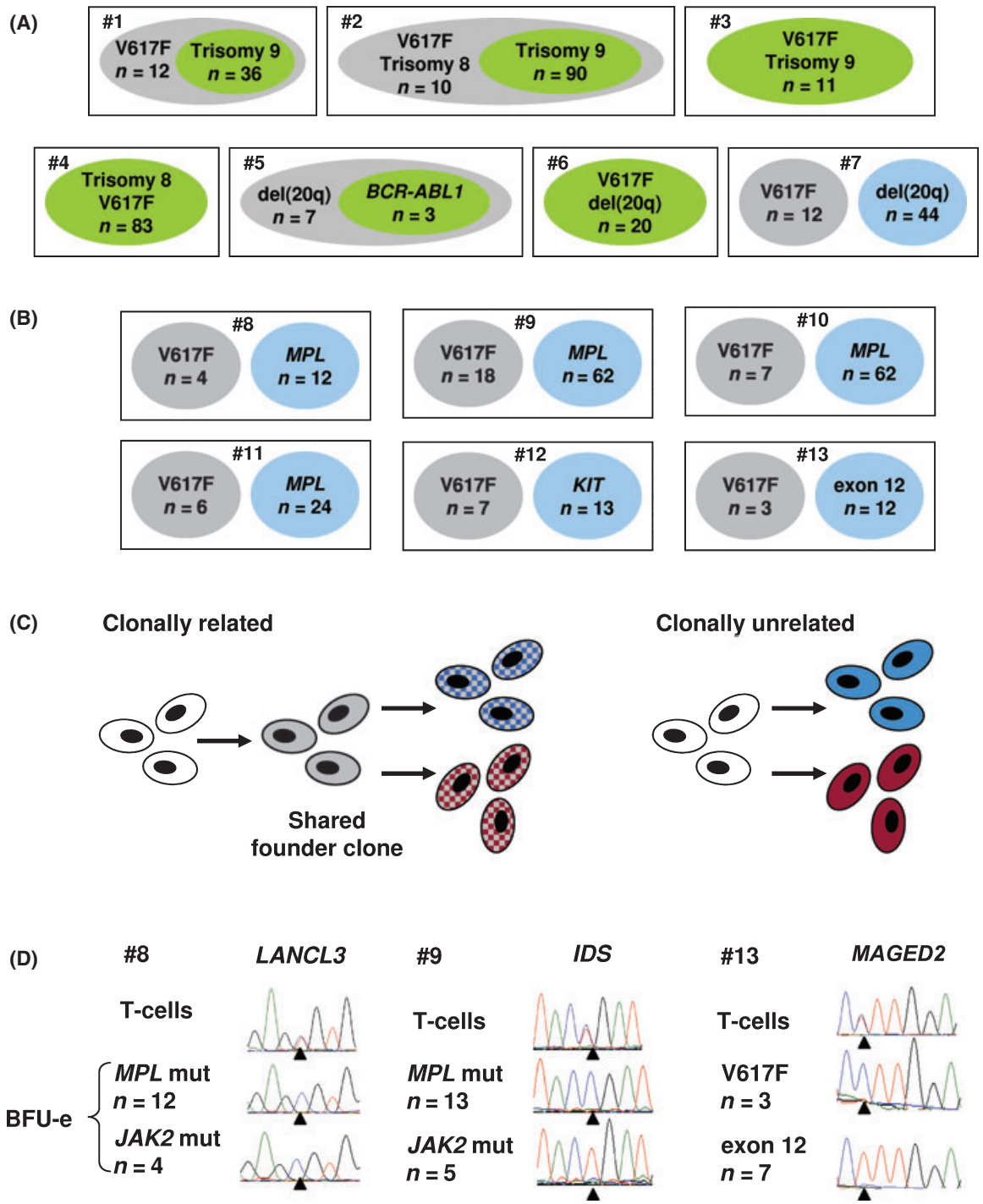

Fig 1. Analysis of progenitor colonies from myeloproliferative neoplasm (MPN) patients with two acquired genetic lesions. (A) Summary of individual colony analysis from seven patients (1–7) with a tyrosine kinase pathway mutation and an additional cytogenetic abnormality, showing the number of individual colonies bearing each genotype and illustrating the relationship of the two clones in each patient. (B) Summary of individual colony analysis from six patients (8–13) with two tyrosine kinase pathway mutations, showing the number of individual colonies bearing each genotype and illustrating the relationship of the two clones. (C) Models to explain the coexistence of two separate mutation-bearing clones: either the mutation bearing clones are clonally related, having arisen from a shared founder clone, or the mutation-bearing clones are clonally unrelated, having arisen in independent haematopoietic stem cells. (D) X-chromosome inactivation patterns in the mutation-bearing clones from female patients (8, 9 and 13). cDNA prepared from single erythroid colonies was amplified by reverse-transcription polymerase chain reaction followed by direct sequencing of an informative expressed single nucleotide polymorphism (SNP) in a gene known to undergo X-inactivation (rs12009345 in *LANCL3*, rs11549009 in *IDS* and rs2071932 in *MAGED2*). In Patients 9 and 13 the mutation-bearing clones expressed different alleles of the X-linked SNP, indicating that the two mutations had arisen in unrelated haematopoietic stem cells.

separate events within a pre-existing founder clone; alternatively the two genetic lesions may represent independent clonal expansions (Fig 1C). To distinguish between these possibilities, X-chromosome inactivation patterns (XCIP) were studied in the three female patients (Fig 1D). In Patient 8, both *MPL*-mutant and *JAK2*-mutant colonies expressed the same allele of the X-linked *LANCL3* gene, a result consistent with either model in Fig 1C. By contrast, in Patient 9, the *MPL*-mutant and *JAK2*-mutant colonies expressed different alleles of the X-linked *IDS* gene. Furthermore, in Patient 13 the *JAK2* V617F-positive and *JAK2* E543-D544del-positive colonies expressed different alleles of the *MAGED2* gene. These data provide formal proof that Patients 9 and 13 both harboured two clones that arose independently and not from a shared founder clone.

Three aspects of our data are worth emphasising. Firstly, whereas cytogenetic abnormalities tended to occur in the same clone as a tyrosine kinase mutation, detection of a second tyrosine kinase pathway mutation reflected the presence of a separate clonal expansion in all six cases studied. It is possible that additional activated tyrosine kinases within the same clone would not confer any further selective advantage, consistent with the observation that tyrosine kinase pathway mutations are generally mutually exclusive in myeloid leukaemias (Kelly & Gilliland, 2002). Secondly, we have identified seven patients with biclonal disease from around 1 000 MPN patients screened. Although the precise denominator is unclear, this seems much greater than expected given the prevalence of each individual MPN (McNally *et al*, 1997). Thirdly, our data demonstrated that patients may harbour diverse clones that have arisen independently and not from a shared founder clone. This phenomenon has been observed in multifocal thyroid carcinoma (Shattuck *et al*, 2005), but to our knowledge this is the first demonstration in haematological malignancies. These data suggest a role for either an inherited allele or an environmental mutagen in promoting the acquisition or survival of such clones. A familial association with MPN has been reported (Landgren *et al*, 2008), often showing incomplete penetrance (Bellanne-Chantelot *et al*, 2006), and a recent report implicated environmental exposure in MPN pathogenesis (Hoffman *et al*, 2007). In conclusion, our data indicate that patients with early stage haematological malignancy may harbour distinct clones, and that such clones may arise independently.

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Author contribution

PAB designed and performed experiments and co-wrote the manuscript. AVJ designed and performed experiments and contributed to the manuscript. AJB, AGF, EMB and KJV performed mutation analysis under the supervision of WNE. BO, CW, MFM, JC, BJPH and CNH provided patient samples and clinical information. NCPC designed experiments and contributed to the manuscript. ARG directed the study and co-wrote the manuscript. All authors have reviewed and approved the manuscript.

Conflict of interest disclosure

We have no conflict of interest to declare.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Genotyping of haematopoietic colonies for the presence of trisomy 9 and trisomy 8.

Fig S2. Assays used for detection of mutations and loss of heterozygosity.

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Rapid identification of *JAK2* exon 12 mutations using high resolution melting analysis

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ABSTRACT

Diverse *JAK2* exon 12 mutations have been described in patients with V617F-negative polycythemia vera. Development of a sensitive detection assay capable of identifying any of these mutations is required for medium-throughput diagnostic screens. Non-mutated and mutant *JAK2* exon 12 alleles were amplified from patient samples and cloned into plasmid vectors, then used to determine the sensitivity of a novel high-resolution melting-curve assay designed to detect all mutant *JAK2* exon 12 alleles tested. High resolution melting analysis was more sensitive than direct sequencing and capable of detecting exon 12 mutations in granulocytes at moderate levels. In a blinded analysis of DNAs from V617F-negative erythrocytosis patients, with direct sequencing and allele-specific PCR used in one laboratory and high resolution melting analysis in another, high resolution melting successfully identified *JAK2* exon 12 mutations in all 4 mutation-positive patients. High resolution melting analysis is a rapid, sensitive and high-throughput technique that is suitable for screening for *JAK2* exon 12 mutations.

Key words: *JAK2*, high resolution melting curve analysis.

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Introduction

The presence of an acquired V617F *JAK2* mutation has been reported in the majority of patients with a myeloproliferative disorder (MPD);[1-5] the mutant allele can be detected in 95% of patients with polycythemia vera (PV), and in half of those with essential thrombocythemia (ET) or primary myelofibrosis (PMF).[1,6,7] The molecular pathogenesis of the disorder in most MPD patients lacking the V617F mutation remains largely unclear. Alternate mutations are absent in the *JAK* or *STAT* gene family members in patients with V617F-negative ET or PMF,[8] although acquired mutations in *JAK2* exon 12 have been identified in cases of V617F-negative PV.[9] In contrast to patients with V617F-positive PV, patients with a *JAK2* exon 12 mutation present with erythrocytosis, but have white cell and platelet counts within the normal range. These mutations may also be detected in patients with erythrocytosis that fail to fulfill the Polycythemia Vera Study Group (PVSG) diagnostic criteria for PV,[10] and are instead classified as having idiopathic erythrocytosis (IE).[9,11]

Individual allele-specific PCR reactions have been developed to detect the first four exon 12 mutations to be described.[9] However, additional mutations have subsequently been identified,[11-16] including duplications that might not easily be identified using an allele-specific PCR strategy.[14] It is possible that additional mutant alleles exist. There is, therefore, a need to develop more generally applicable, sensitive assays to detect *JAK2* exon 12 mutations for use in a diagnostic setting.

Design and Methods

Patients

DNA samples from 50 erythrocytosis patients attending the Johns Hopkins Medical Institutes (Baltimore, MD, USA) and from exon 12 mutation-positive patients attending Addenbrooke's Hospital (Cambridge, UK) were used. Institutional Ethics Committee approval was obtained at both institutions, written informed consent obtained from each patient, and the study was carried out in accordance with the principals of the Declaration of Helsinki. Clinical features of the Baltimore patients have been published elsewhere.[13]

Mutant and wildtype JAK2 exon 12 alleles

Patient genomic DNA was prepared from density gradient-purified granulocytes using the Puregene Cell kit (Gentra Systems, Minneapolis, MN, USA). Presence of the V617F JAK2 mutation was excluded by sequencing and allele-specific PCR[1] (*data not shown*). DNA was amplified using primers flanking *JAK2* exon 12 (forward: 5'-CTCCTCTTTGGAG-

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CAATTCA-3'; reverse: 5'-GAGAACTTGGGAGTTGC-GATA-3'). PCR products were treated with ExoSAP-IT (GE Healthcare, Amersham, UK), then cloned into pGEM-T (Promega, Southampton, UK).

HRM reaction conditions

A 126bp amplicon was generated using primers in *JAK2* exon 12 (5'-AATGGTGTTTCTGATGTACC-3') and intron 12 (5'-AGACAGTAATGAGTATCTAATGAC-3'). Each PCR contained 20-40 ng DNA or 1×10^6 plasmid copies, 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of each primer, 1× Platinum *Taq* polymerase buffer and 0.5U Platinum Taq polymerase (Invitrogen, Paisley, UK), and 1× LC-Green Plus (Idaho Technologies, Salt Lake City, UT, USA). Duplicate PCRs and the HRM analysis were conducted on a RotorGene 6000™ real-time analyzer (Corbett Life Sciences, Mortlake, Australia). The PCR profile was: an initial hold at 95°C for 10 mins., 40 cycles of 95°C for 15 secs., 58°C for 30 secs., and 72°C for 20 secs., followed by 50°C for 30 secs., then a melt from 70°C to 95°C rising at 0.1°C/second (data acquisition was up to 1,000 data collection points per °C transition). Normalization bars were between 72-75°C for the leading range, and 92-93°C for the tailing range.

Sequencing HRM products

HRM product was treated with ExoSAP-IT, then used as the template for sequencing with the Big Dye Terminator kit (Applied Biosystems, Warrington, UK). Each 10 μL reaction mix consisted of 10 ng primer, 0.5 μL of Big Dye Terminator premix and 1.5 μL 5x sequencing buffer. Sequencing reactions were performed using 24 cycles of 96° C for 30 secs., 50° C for 15 secs., and 60° C for 2 mins. on a MJ Research Tetrad thermocycler (Biorad, Hemel Hempstead, UK). The Montage SEQ96 sequencing reaction clean-up kit (Millipore, Watford, UK) was used to purify sequencing reactions, and the resultant DNA resuspended in deionised formamide and loaded onto an ABI-3100 sequencer (Applied Biosystems). Sequences were analyzed using Mutation Surveyor 3.1 software (SoftGenetics, State College, PA, USA).

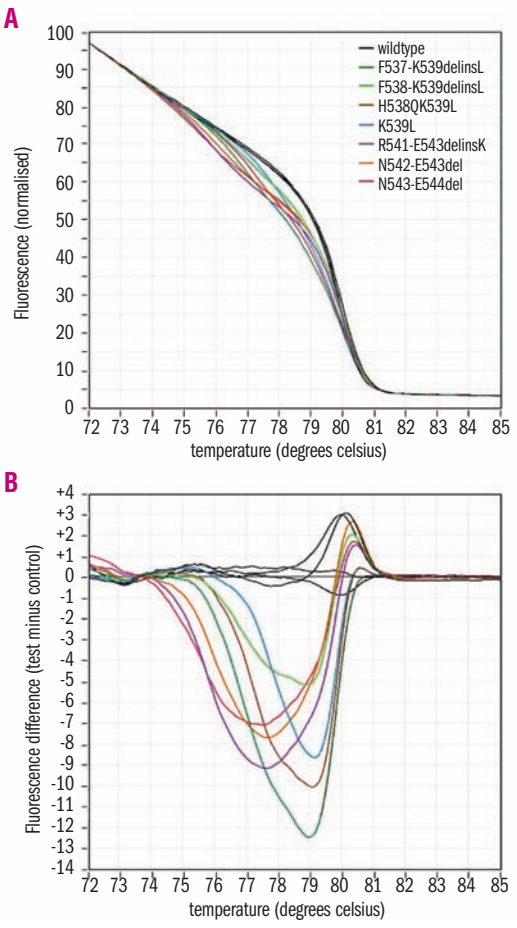

Figure 1. Development of a high-resolution melting-curve (HRM) assay to distinguish between the various *JAK2* exon 12 mutated alleles. (A) Analysis of six wildtype exon 12 samples (black line) and 50:50 mixtures of wildtype and F537-K539delinsL (green), H538-K539delinsL (lime), H538QK539L (brown), K539L (cyan), R541-E543delinsK (purple), N542-E543del (orange) or E543-D544del (red) alleles reveal that all mutant alleles had a melting curve profile distinct from that of the wildtype allele, and each with its own characteristic profile. (B) Difference plots demonstrate more clearly that each mutant allele has a characteristic melting curve, with those alleles with deletions of residues including E543 (purple, orange, red lines) having a curve distinct from those alleles with a K539L substitution (green, blue, brown lines).

Results and Discussion

High-resolution melt-curve analysis assay validation and sensitivity

One drawback to employing allele-specific PCR assays to screen large numbers of DNAs has been that multiple individual PCR reactions would be needed for each sample. Moreover, novel mutations would escape detection. Given these concerns, we sought to establish a single sensitive screening method capable of detecting all possible *JAK2* exon 12 mutations; one candidate approach is high-resolution melt-curve analysis (HRM), which has been successfully applied to the detection of the V617F mutation within *JAK2* exon 14 in patients diagnosed with an MPD.[17-20] PCR primers were redesigned to generate an amplicon suitable for HRM analysis of *JAK2* exon 12. HRM involves precise monitoring of the progressive fluorescence change caused by the release of an intercalating DNA dye from a DNA duplex as that duplex is denatured by increasing temperature.[21] Base substitutions, deletions and insertions produce subtle differences in the melting behavior of individual DNA duplexes, which can be detected by the use of an appropriate fluorescent dye, such as LC-Green.[21] Since this methodology will be affected by the presence of inheritable polymorphisms, a candidate region containing *JAK2* exon 12 and intron 12 (chromosome 9: 5,059,925-5,060,831; genome build 2006) was selected on the basis of an absence of polymorphisms in the NCBI database (*http://www.ncbi.nlm.nih.gov/sites/entrez/*). However, we subsequently identified a previously unreported polymorphism within this region in 85 out of 128 tested individuals; this variation involves the presence or absence of a pentanucleotide sequence (position 5,060,231-5,060,235) that would significantly affect any HRM profile. Accordingly, this region was excluded from

the final amplicon. HRM analysis was performed on a RotorGene 6000™ real-time analyzer, and the resulting data analyzed using the associated RotorGene Series Software (V1.7.25). Figure 1 shows typical results obtained when equal amounts of wildtype and mutation-carrying plasmid were used. The data are presented in two formats: a normalized plot (Figure 1A), in which the amount of intercalating dye remaining at any temperature point is expressed as a fraction of the amount prior to data acquisition; and a difference plot, where the average HRM profile of the control samples was used by the genotype function of the machine software as the standard wildtype profile for subsequent comparison to each of the test samples (Figure 1B). Analysis of six control and seven mutant samples demonstrated that each mutant allele had its own characteristic melting curve that was distinct from those obtained when wildtype exon 12 samples were analyzed (Figure 1A). The individual nature of the mutant melting curves became more apparent when the data were represented in a difference plot (Figure 1B). Mutations that had in common a K539L substitution had similar difference plots, with the greatest divergence between mutant and wildtype profiles occurring at 79° C. Difference plots for the R541-E543delinsK, N542-E543del and E543-D544del mutants had slopes different to those associated with a K539L substitution, and the greatest divergence between mutant and wildtype occurred at 77.5° C.

The ability to detect low levels of a *JAK2* exon 12 mutation in a background of non-mutated DNA was evaluated by titrating each of the mutant alleles with wildtype exon 12 to produce a range of mutant allele dilutions. The lower limit for detecting nucleotide changes in exon 12 was calculated by the RotorGene software to be 5% for F537-K539delinsL (Figure 2) and N542-E543del alleles, 7% for the R541-E543delinsK and E543-D544del alleles, 10% for the K539L allele, and 20% for the H538-K539delinsL and H538QK539L alleles (*data not shown*). Titration data were also used to estimate the level of mutant allele in the granulocyte DNA sample of a F537-K539delinsL-positive PV patient (PT1; Figure 2).[9] Estimates obtained using HRM analysis agreed closely with those independently obtained from granulocyte DNA sequence traces.

JAK2 exon 12 mutation detection in V617F-negative patients with erythrocytosis

The HRM methodology established using cloned exon 12 alleles was next assessed using a set of granulocyte DNA samples obtained from 50 V167F-negative erythrocytosis patients,[13] only 10 of whom fulfilled PVSG diagnostic criteria.[10] Since exon 12 mutation-positive patients often present with an isolated erythrocytosis and might not fulfil these criteria,[9,11] this patient cohort was selected for analysis as it was considered to be similar to sample populations being assessed for the presence of an exon 12 mutation in a

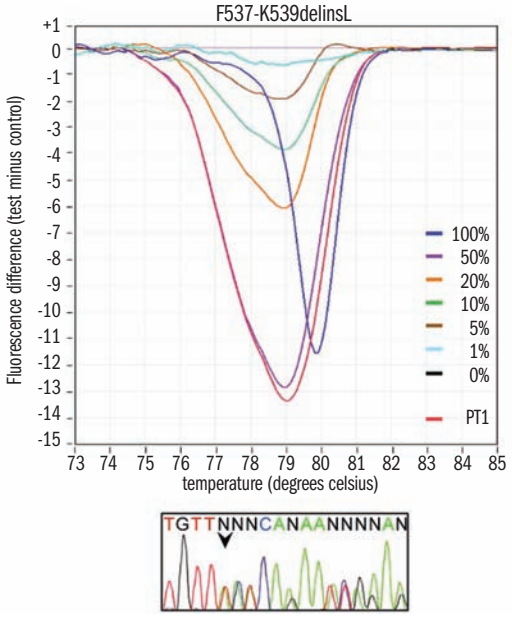

Figure 2. High resolution melting assays detect mutant *JAK2* alleles present at a low frequency. High resolution melting analysis of mixtures of wildtype and F537-K539delinsL *JAK2* exon 12 alleles (0% mutant, black line; 1%, cyan; 5%, brown; 10%, lime; 20%, orange; 50%, purple; 100%, blue) demonstrates that these assays can detect relatively low levels of mutant allele. Also included is the high resolution melting trace (red) and sequence chromatogram of a granulocyte sample from a patient previously shown to carry an exon 12 mutation.

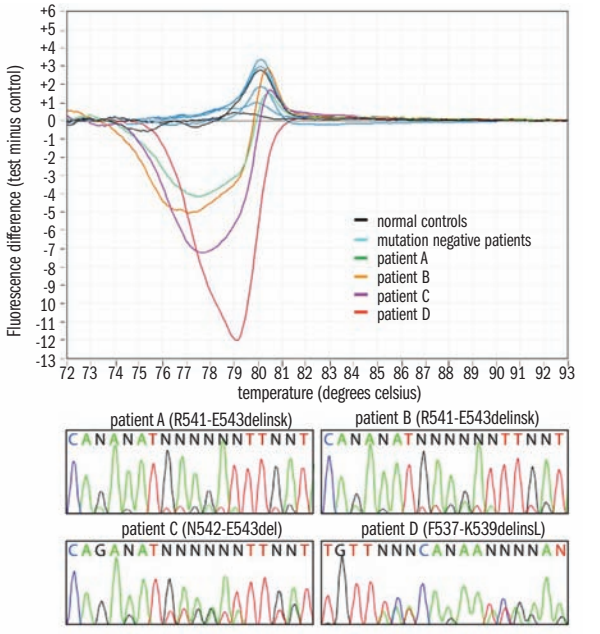

Figure 3. Screening of 50 V617F-negative patients with erythrocytosis by high resolution melting assays and sequencing independently identifies 4 mutation-positive cases. Sequencing, allele-specific PCR, and high resolution melting analysis was independently used to screen granulocyte DNAs from 50 V617F-negative erythrocytosis cases. Difference plots are shown for 4 of the 8 samples from healthy controls (black traces), 4 samples from mutation-negative patients within this cohort (cyan traces), and for the 4 samples suggestive of a *JAK2* exon 12 mutation (Patients A to D: lime, orange, purple and red lines respectively). Granulocyte DNA sequence chromatograms from each of these mutation-positive cases are also shown, with Patients A and B having an R541-E543delinsK mutation, Patient C having a N542-E543del mutation, and Patient D having a F537-K539delinsL mutation.

diagnostic laboratory setting. Allele-specific PCR and sequence analysis of each sample (*data not shown*) had been performed 1-2 months earlier by an investigator not involved with the HRM analysis, and four samples were found to be positive for a *JAK2* exon 12 mutation by each method. Subsequent HRM was successful in discriminating DNA samples with a wildtype *JAK2* exon 12 genotype from those including an exon 12 mutation (Figure 3), and correctly identified all positive samples in this group with no false-positive results. Direct sequencing of HRM products confirmed the presence of an exon 12 mutation in these cases. When compared with the titration experiment results (Figure 2), these data suggested mutant allele burdens of 20%, 25%, 35% and 50% respectively. All 4 patients had mild splenomegaly, and serum erythropoietin levels below the normal range, consistent with previous observations.[9,11] Two patients had not fulfilled the PVSG criteria at diagnosis; however, subsequent identification of *JAK2* exon 12 mutations permits reclassification of these cases as PV using the recently modified WHO criteria.[22] None of the 5 patients with a first- or second-degree relative with an MPD was mutation-positive.

Several mutation screening methods have been used so far to detect *JAK2* exon 12 mutations, each with its particular disadvantages. Dideoxy sequencing is rarely sensitive below a 10% mutant allele frequency, corresponding to a threshold of 20% granulocytes heterozygous for an exon 12 mutation. Allele-specific PCR requires performing multiple amplification reactions in order to ensure that all potential mutations can be excluded. A PCR-based technique with sensitivity comparable to that of allele-specific PCR has been recently described,[15] although this relies upon the presence of a deletion within exon 12, thereby precluding detection of the duplication mutations or the nucleotide substitution mutants present in some individuals. Single-strand conformation polymorphism, denaturing high performance liquid chromatography or HRM methodologies might prove useful, as they make the detection process more cost effective by reducing the amount of sequencing ultimately required. However, the HRM approach described here has the added advantage of increased simplicity and rapid turn-around time, being an in-tube method in which the melting analysis is performed immediately after PCR amplification.

Mutation analysis of granulocyte DNA samples from V617F-negative erythrocytosis patients using sequencing, allele-specific PCR and HRM allowed us to assess the sensitivity and positive predictive value of the HRM methodology. Mutations were not detected by allele-specific PCR or sequencing in samples scored as wildtype by HRM, resulting in 100% sensitivity and 100% positive predictive value for this methodology in the patient sample set tested. Four samples had aberrant melting profiles that suggested the presence of an exon 12 mutation; independently-obtained allele-specific PCR and sequencing data confirmed the presence of these alterations (Figure 3, and *data not shown*). In instances of patients with a low clonal burden, however, exon 12 mutations identified by HRM may not be confirmable by dideoxy sequencing; allele-specific PCR may be required for mutation confirmation in these instances. Alternatively, as all patients positive for *JAK2* exon 12 mutation have erythropoietin-independent erythroid colonies (EECs),[9,11] individual colonies could be analyzed by dideoxy sequencing to confirm the mutation suspected.

We observed two limitations to this HRM methodology: one was its inability to identify the H538QK539L and H538-K539delinsL *JAK2* alleles when their abundance was less than 20%. However, these are both relatively rare mutant alleles, occurring in only 4 of the 50 cases reported in the literature.[9,11-14,16,23] In contrast, the most common exon 12 mutations (F537-K539delinsL, N542-E543del, E543-D544del) were all detectable at a relative abundance of 7% or less. Secondly, patient samples with purely mutant DNA may pose a problem due to an absence of heteroduplex formation. However, homozygous exon 12 mutations are relatively rare in patients, with only 2 cases reported.[11,14] In both instances, non-mutated exon 12 sequence was apparent within the granulocyte DNA sequence trace, and we would predict that HRM analysis of these DNAs would provide a melting curve significantly different from that of a control sample.

In conclusion, we successfully used HRM to identify somatic mutations in *JAK2* exon 12 in 4 of 50 erythrocytosis patients lacking the V617F *JAK2* mutation. These results correlated exactly with allele-specific PCR and sequencing results obtained independently.

Authorship and Disclosures

AVJ designed research, performed research, analyzed data, wrote the paper; NCPC designed research, wrote the paper; HEW performed research; ARG designed research, wrote the paper; LMS designed research, performed research, analyzed data, wrote the paper.

The authors reported no potential conflicts of interest.

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LEADING ARTICLE

FIP1L1-PDGFRA in chronic eosinophilic leukemia and *BCR-ABL1* in chronic myeloid leukemia affect different leukemic cells

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We investigated genetically affected leukemic cells in *FIP1L1-PDGFRA*+ chronic eosinophilic leukemia (CEL) and in *BCR-ABL1*+ chronic myeloid leukemia (CML), two myeloproliferative disorders responsive to imatinib. Fluorescence *in situ* hybridization specific for *BCR-ABL1* and for *FIP1L1-PDGFRA* was combined with cytomorphology or with lineage-restricted monoclonal antibodies and applied in CML and CEL, respectively. In CEL the amount of *FIP1L1-PDGFRA*+ cells among CD34+ and CD133+ cells, B and T lymphocytes, and megakaryocytes were within normal ranges. Positivity was found in eosinophils, granulo-monocytes and varying percentages of erythrocytes. *In vitro* assays with imatinib showed reduced survival of peripheral blood mononuclear cells but no reduction in colony-forming unit growth medium (CFU-GM) growth. In CML the *BCR-ABL1* fusion gene was detected in CD34+/CD133+ cells, granulo-monocytes, eosinophils, erythrocytes, megakaryocytes and B-lymphocytes. Growth of both peripheral blood mononuclear cells and CFU-GM was inhibited by imatinib. This study provided evidence for marked differences in the leukemic masses which are targeted by imatinib in CEL or CML, as harboring *FIP1L1-PDGFRA* or *BCR-ABL1*.
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Introduction

BCR-ABL1+ chronic myeloid leukemia (CML) is a paradigm for the multilineage involvement of an imatinib-sensitive chronic myeloproliferative disorder in which an affected stem cell ordinately differentiates toward both myeloid and lymphoid hematopoietic pathways.

Chronic eosinophilic leukemia (CEL), is a chronic myeloproliferative disorder whose diagnosis is based on persistent eosinophilia ($>1.5\times10^9$/l), organ involvement and increased blast cells in peripheral blood or bone marrow and/or clonality, as shown by cytogenetics and X-inactivation.[1–4] Cools *et al.*[5] first described a genetic clonal hallmark for a specific CEL subgroup. The *CHIC2* gene deletion at 4q12 results in a fusion between the *FIP1L1* and *PDGFRA* genes. As a consequence the *PDGFRA* tyrosine kinase is activated. Interestingly, imatinib mesylate, a tyrosine kinase inhibitor, rapidly induces remission in the majority of patients, although disease is not fully eradicated.

In order to compare the hematopoietic cell lineages affected by *FIP1L1-PDGFRA* in CEL and by *BCR-ABL1* in CML, we combined fluorescence *in situ* hybridisation (FISH) with cytomorphology (MISH), as well as with immunostaining fluorescence immunophenotyping and interphase cytogentics as a tool for the investigation of neoplasms (FICTION). Furthermore, we tested *in vitro* imatinib sensitivity both on peripheral blood mononuclear cells and in colony-forming unit growth medium (CFU-GM) from patients with CEL and CML. Results showed that *FIP1L1-PDGFRA*+ cells are limited to advanced stages of myeloid cell differentiation, whereas *BCR-ABL1* is present in both myeloid and lymphoid lineages. Accordingly, imatinib inhibited peripheral blood mononuclear cells and CFU-GM in *BCR-ABL1* +CML, but only peripheral blood mononuclear cells in *FIP1L1-PDGFRA*+ CEL. In this study, we assessed a restricted lineage affiliation of hematopoietic cells labeled by *FIP1L1-PDGFRA* in CEL.

Materials and methods

Patients and controls

A total of 16 *FIP1L1-PDGFRA*-positive CEL cases were included in this study. Bone marrow aspirates were used to investigate lineage affiliation in eight patients. In eight additional cases, peripheral blood was used for *in vitro* studies on imatinib sensitivity. Patients were selected from the Hematology Departments of the Universities of Perugia and Bari (Italy), from the Hematology Department and Centre for Human Genetics of the University of Leuven (Belgium) and from the Wessex Regional Genetics Laboratory of the Salisbury District Hospital, Salisbury (UK). Four cases of CML were selected from patients referred to the Hematology Department of the University of Perugia (Italy). Twelve healthy donors were used as controls. The study was approved by the Hematology Department IRB (Reg. no. 00003450; FWA 00005268) of University of Perugia, Italy.

Genomic probes and monoclonal antibodies

The *FIP1L1-PDGFRA*/4q12 was studied with clone RP11-3H20 labeled with biotin alone or in combination with clone RP11-120K16 labeled with digoxigenin (RP11 belongs to the Roswell Park Cancer Institute (RPCI)11 library, http://bacpac.chori.org/).

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The t(9;22)(q34;q11)/*BCR-ABL1* was investigated with the LSI *BCR-ABL* dual-color/dual-fusion translocation probe (*BCR* clone in green and *ABL1* clone in orange) (Vysis, Olympus, Milan, Italy).The following monoclonal antibodies were used: anti-CD13, anti-CD14, anti-CD33, anti-CD3, anti-CD7, anti-CD19, anti-CD20, anti-glycophorin C, anti-glycophorin A (Dako, Milan, Italy), anti-CD34 (Becton-Dickinson, Milan, Italy), anti-CD133 (Miltenyi Biotec S.r.l., Bologna, Italy).

FICTION and MISH

A FICTION published methodology[6] was slightly modified. Briefly, cytospins were prepared from bone marrow mononuclear cells after centrifugation on Lymphoprep (AXIS-SHIELD, Norway) using 100 μl from a cell suspension of 1×10^6/ml. Slides were air-dried at room temperature for 24 h fixed in acetone for 10 min and incubated with monoclonal antibodies for 30 min at room temperature. The three-step staining technique used the following Cy3-conjugated polyclonal antibodies: goat anti-mouse, rabbit anti-goat and donkey anti-rabbit (Jackson Immunoresearch/Li StarFISH, Milano, Italy). After immunostaining, slides were fixed in Carnoy's fixative (metanol:acetic acid, 3:1) for 1 min and in 1% paraformaldehyde for 10 min, washed in distilled water and dehydrated in an ethanol series. For MISH investigations, bone marrow smears were air-dried and fixed in methanol:acetic acid (3:1) for 5 min. Slides and probes were co-denaturated on a hot plate at 76°C and incubated overnight at 37°C in a humidified chamber. To detect biotinylated DNA, slides were incubated three times: with fluorescein isothiocyanate (FITC)-conjugated avidin, with biotinylated goat anti-avidin antibody and with FITC-conjugated avidin (Vector Laboratories, DBA Italia, Milan, Italy).

Immunophenotype and hybridization signals were simultaneously identified and counted under an Olympus fluorescence microscope with filter sets for Cy-3 and FITC equipped with a CCD camera (Sensys-Photometrics, Tucson, AZ, USA) run from image analysis software (Vysis, SmartCapture, Olympus, Milan, Italy). For each antibody, at least 13 (range 13–94) cells were checked in CEL and CML patients and at least 145 cells in normal controls (range 145–375).

MISH on eosinophils, and on megakaryocytes was carried out after morphological identification of single cells on bone marrow smears applying a set of DNA clones described previously (Figures 1 and 2). All the experiments were evaluated by two independent observers.

CD34$^+$ cell selection

CD34$^+$ stem cells were labeled with CliniMACS CD34 microbeads (Miltenyi Biotec S.r.l., Bologna, Italy) and passed through the magnetic field of a Mini MACS separator (CD 34 Progenitor Cell Isolation Kit, Miltenyi Biotec S.r.l., Bologna, Italy). Purity was analyzed on a FACscan (Cytomics FC 500, Beckman Coulter, Milan, Italy) using a monoclonal antibody specific for CD34 conjugated with FITC (Becton Dickinson, Milan, Italy). After suspension in phosphate-buffered saline, cell cytospins were prepared and fixed: (1) 5 min in 30% fixative (3:1 methanol:acetic acid) diluted with 0.075 M KCl, (2) 10 min in 20% ethanol diluted with 0.075 M KCl and (3) 10 min in fixative (3:1 methanol:acetic acid). After fixation interphase FISH was performed using clone RP11-3H20 and clone RP11-120K16, as described previously.[7]

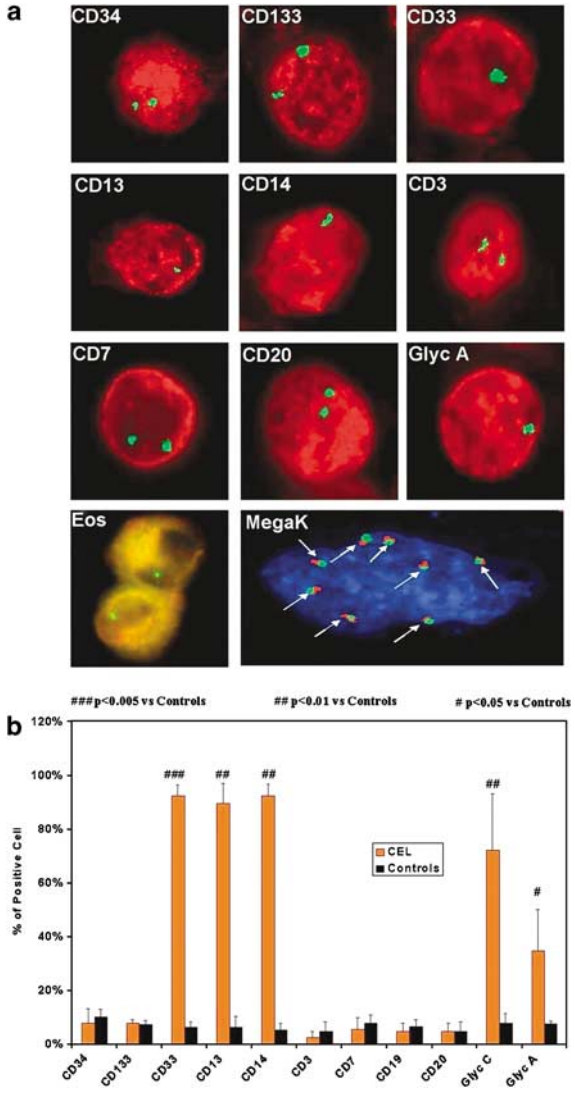

Figure 1 Fluorescence *in situ* hybridization on intact cells in *FIP1L1-PDGFRA*+ CEL. (**a**) FICTION with monoclonal antibodies against CD34, CD133, CD33, CD13, CD14, CD3, CD7, CD20, glycophorin A (top three lines). Red staining detects positive intact cells expressing the specific antigen. Green spots indicate FISH signals in the nuclei using a FITC-labeled genomic probe (RP11-3H20) for *CHIC2*. Two green signals indicate normal disomic cells; one shows *CHIC2* deletion, that is, *FIP1L1-PDGFRA* fusion (original magnification $\times 1000$). Bottom line MISH (FISH on morphologically identified cells). Left: autofluorescent eosinophils without counterstain; in each nucleus, one green signal indicates *CHIC2* deletion. Right: one megakaryocyte with polyploid nucleus with DAPI (blue) counterstaining. Green signals indicate 3H20 probe (*CHIC2* gene); red signals indicate 120K16 probe (internal control). Eight overlapping red–green signals (arrows) show no *CHIC2* deletion (original magnification $\times 1000$). (**b**) Histogram with FICTION results from eight CEL cases (orange) and eight healthy controls (black). In CEL, CD33+, CD13+, CD14+, glycophorin C+ and A+ cells bearing *CHIC2* deletion were significantly greater ($P<0.05$ vs controls). Non-parametric *t*-test, Mann–Whitney test.

Progenitor cell colony assay

Thawed bone marrow mononuclear cells were washed in Iscove-modified Dulbecco's medium (IMDM; StemCell Tecnologies, Vancouver, Canada). Cells were counted and 1×10^5 were plated in 1 ml aliquots in 30 mm Petri dishes in a semisolid assay. At least six Petri dishes were prepared for each patient.

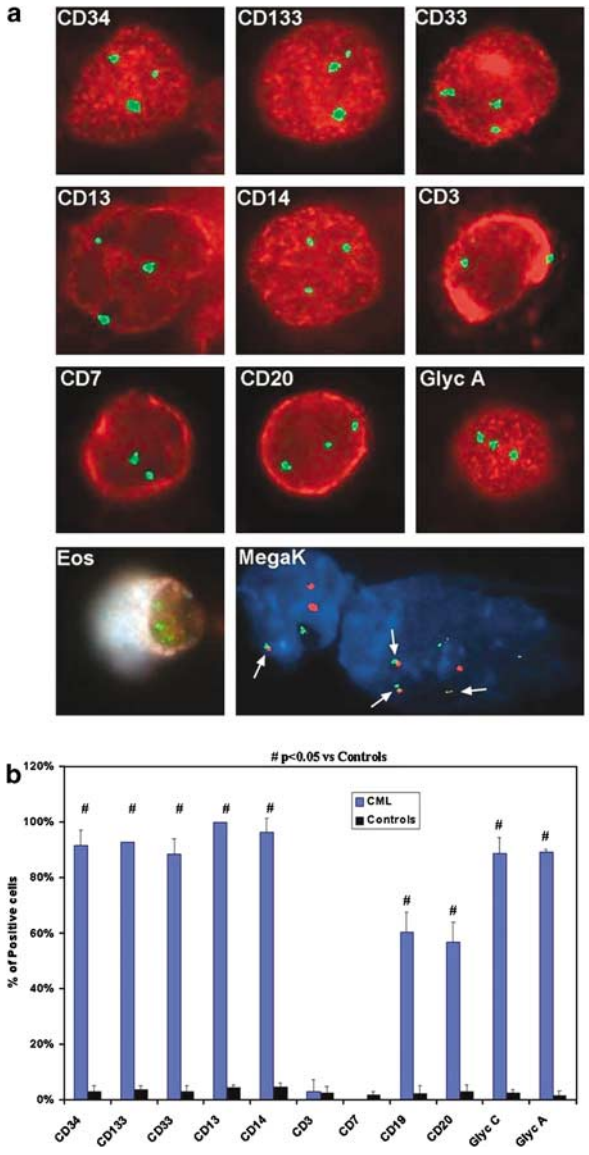

Figure 2 Fluorescence *in situ* hybridization on intact cells in *BCR-ABL1*+ CML. (**a**) FICTION with monoclonal antibodies against CD34, CD133, CD33, CD13, CD14, CD3, CD7, CD20, glycophorin A (top three lines). Red staining detects positive intact cells expressing the specific antigen. Green spots indicate FISH signals in the nuclei using a Spectrum Green-labeled genomic probe (Vysis, Olympus, Milan, Italy) for *BCR*. Two green signals indicate normal disomic cells; three show *BCR* split because of Philadelphia translocation (original magnification ×1000). Bottom line MISH (FISH on morphologically identified cells). Left: autofluorescent eosinophil without counterstain; three green signals indicate Philadelphia translocation. Right: one megakaryocyte with polyploid nucleus with DAPI (blue) counterstaining. *ABL1* gene is indicated by red signals and *BCR* gene by green. Four overlapping red–green signals (arrows) indicate Philadelphia *BCR-ABL1* fusion. (**b**) Histogram with FICTION results from four CML cases (blue) and four healthy controls (black). In CML, the numbers of CD34+, CD133+, CD33+, CD13+, CD14+, CD19+, CD20+, glycophorin C+ and A+ cells bearing *BCR-ABL1* fusion signals were significantly greater ($P<0.05$ vs controls). Non-parametric *t*-test, Mann–Whitney test.

The medium contained 30% FCS (HyClone, Logan, UT, USA), 3 U/ml rhuEpo, 50 ng/ml stem cell factor, 10 ng/ml GM-CSF, 10 ng/ml IL-3 (all from PeproTech, UK), 0.9% methylcellulose (StemCell Inc., Canada) and IMDM. Cultures were incubated for 14 days, at 37°C, 5% CO_2, in a humidified incubator. CFU-GM colonies were scored under an inverted microscope according to standard criteria.[8] Colonies (40–50) were transferred singly into a microtiter well containing 40 μl hypotonic solution (0.075 M KCL) for each cytospin. Cytospins were fixed following the same protocol described before for FISH on CD34+ cells. After fixation interphase FISH was carried out applying a set of DNA clones described previously in the genomic probe section. All the experiments were evaluated by two independent observers.

Clonality by X-chromosome inactivation

Both peripheral blood and buccal epithelial samples were obtained from the single CEL woman included in the study. Mononuclear cells were isolated by Ficoll–Hypaque density gradient centrifugation (Lympholyte-H, CEDARLANE, HORNBY, Canada). T lymphocytes were purified from the mononuclear cell fraction using magnetic immunobeads coated with an anti-CD3 antibody (Miltenyi Biotech GmbH, Bergish Gladbach, Germany). Polymorphonuclear leukocytes (PMN) were isolated from the red cell pellet following erythrocytes removal by hypotonic lysis.

The X-chromosome inactivation patterns were established by polymerase chain reaction (PCR) analysis of DNA methylation at the human androgen receptor locus (HUMARA) as previously published.[9]

In vitro *imatinib sensitivity*

Test 1: Liquid culture assay. Peripheral blood mononuclear cells or granulocytes from CEL, CML and normal controls were cultured in 24-well plates in 1 ml aliquots of $2–5 \times 10^6$ cells in Roswell Park Memorial Institute medium 1640 medium supplemented with 10% serum. Duplicate wells were treated with 1 and 5 μM imatinib. Cells were counted with a hemocytometer twice weekly for 3 weeks. The medium was supplemented on days 7 and 14. A decrease in cell numbers in imatinib-treated wells compared with untreated control wells indicated a positive response.

Test 2: CFU-GM assay. Peripheral blood mononuclear cells from CEL, CML and normal control were separated using lymphoprep (Axis-Shield, Oslo, Norway) and cultured in methylcellulose supplemented with growth factors (Stem Cell Technologies Ltd, Vancouver, Canada) at a cell density of 2×10^5 cells/ml in 3 cm Petri dishes. Imatinib (Novartis, Basel, Switzerland) was added to final concentrations of 1 and 5 μM. Colony numbers were scored on days 7 and 14 from triplicate plates. The response index was calculated as the mean reduction in colony numbers in 1 and 5 μM treated dishes on days 7 and 14 compared with untreated dishes. After control experiments using normal individuals and patients with *BCR-ABL1*-positive CML, an index below 0.2 was established as indicating positive response.

Results

The outcomes of FICTION and MISH studies on CEL patients were summarized in Table 1 (see also Figure 1a and b). CD34 was investigated in 5/8 cases (patients 1, 2, 4, 5, 6, Table 1). In all of them the percentage of *CHIC2* deletion was equal to or below the cutoff (13%) established at the upper limit on normal control (Table 1). In patient 8, purified CD34+ cells were

investigated with microbeads conjugated to an anti-CD34 monoclonal antibody. FISH analysis showed one signal in 15/400 cells (4%) of the enriched CD34+ population (normal experiment control 5%, i.e., 18/400). We used the anti-CD133 monoclonal antibody as an additional marker for a totipotent stem cell. This antibody was studied in patients 2 and 6 and the percentage of deletion was 9 and 7%, respectively (Table 1). For this antibody, the cutoff was set at 10%.

CD33 was investigated in 6/8 patients. The percentage of cells bearing *CHIC2* deletion ranged from 84 to 95%. CD13 was evaluated in 7/8 patients showing a percentage of deletion between 80 and 98%. The anti-CD14 antibody also showed that 88–97% cells were affected by the *CHIC2* deletion in the five patients studied (Table 1).

Glycophorin C was investigated in 5/8 (no. 1, 2, 3, 4, 6, Table 1) patients in whom *CHIC2* deletion was detected in 37–88% of cells (normal cutoff 11%). As glycophorin C may crossreact with some granulocytes, glycophorin A was also tested. Positivity confirmed that erythroid cells were affected by *CHIC2* deletion. However, *CHIC2* deletion in glycophorin A-positive cells was 48% (38/79), 46% (43/93) and 15% (14/94), respectively in patient 1, 2 and 6, suggesting inter-individual variance in the malignant erythrocyte component of CEL.

Interphase fluorescence in situ hybridization (I-FISH) on megakaryocytes from healthy controls showed colocalization of green and red signals as expected in the absence of 4q12 deletion. Results in 30 megakaryocytes in patient 2 showed between 6 and 26 copies of colocalized red–green signals (Table 1 and Figure 1a).

Eosinophils were identified by autofluorescence granules. In all patients, the positivity ranged between 88 and 98% of elements (Table 1).

Results on both T and B lymphocytes were within the cutoff limits established in normal controls (Figure 1a and b, and Table 1).

In four cases of *BCR-ABL1*+ CML, the *BCR-ABL1* rearrangement was observed on totipotent CD34+ and CD133+ stem cells and in committed lineages downstream, that is, granulo-monocytes, erythrocytes, megakaryocytes and B lymphoid cells (Table 2 and Figure 2a and 2b). In patient 2, 80% of eosinophils were positive for *BCR-ABL1* rearrangement (Table 2).

Table 1 FICTION and MISH experiments for *CHIC 2* deletion in eight CEL cases

	Patient								
Antibody	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*Controls cu offs*[a]
CD34	3/30 (10)	2/40 (5)	—	4/30 (13)	3/25 (12)	0/30 (0)	—	—	13
CD133	—	2/22 (9)	—	—	—	2/28 (7)	—	—	10
CD33	40/42 (95)	40/42 (95)	27/32 (84)	35/38 (92)	—	40/43 (93)	40/42 (95)	—	9
CD13	49/50 (98)	40/49 (82)	40/42 (95)	30/32 (94)	20/25 (80)	30/36 (83)	38/40 (95)	—	11
CD14	30/33 (91)	32/33 (97)	—	28/31 (90)	—	37/42 (88)	34/35 (97)	—	9
CD3	1/41 (2)	0/40 (0)	1/40 (2)	1/31 (3)	2/30 (7)	1/32 (3)	0/14 (0)	—	9
CD7	2/26 (8)	3/28 (11)	—	0/35 (0)	—	2/32 (6)	1/31 (3)	—	12
CD19	2/29 (7)	—	—	—	—	1/35 (3)	—	—	11
CD20	—	2/28 (7)	0/16 (0)	2/26 (8)	—	2/32 (6)	1/31 (3)	—	9
Glyc C	42/52 (81)	30/42 (71)	15/17 (88)	11/13 (85)	—	18/48 (37)	—	—	11
Glyc A	38/79 (48)	43/93 (46)	—	—	—	14/94 (15)	—	—	14
Megak	—	0/30 (0)	—	—	—	—	—	—	0
Eos	50/54 (93)	40/44 (91)	45/51 (88)	35/39 (90)	30/32 (94)	41/42 (98)	40/42 (95)	40/43 (93)	—

Abbreviations: CEL, chronic eosinophilic leukemia; Eos, eosinophils; Glyc A, glycophorin A; Glyc C, glycophorin C; Megak, megakaryocytes.
In each case, the number of positive cells/total cells (with percentage in parenthesis) is show.
[a]Highest percentage of normal cells with one CHIC 2 signal.

Table 2 FICTION and MISH experiments for *BCR/ABL1* fusion gene in four CML cases

	Patient				
Antibody	*1*	*2*	*3*	*4*	*Controls cutoffs*[a]
CD34	33/35 (94)	30/36 (83)	40/42 (95)	30/32 (94)	5
CD133	28/30 (93)	45/50 (90)	19/20 (95)	19/21 (90)	5
CD33	30/34 (88)	26/32 (81)	40/43 (93)	36/39 (92)	5
CD13	25/25 (100)	30/30 (100)	—	—	5
CD14	24/24 (100)	30/32 (93)	—	—	5
CD3	0/30 (0)	2/32 (6)	—	—	5
CD7	0/26 (0)	0/13 (0)	—	—	3
CD19	14/28 (50)	21/33 (64)	27/40 (67)	21/35(60)	6
CD20	26/42 (62)	16/31 (52)	—	—	5
Glyc C	22/26 (85)	30/32 (93)	—	—	4
Glyc A	—	27/30 (90)	—	—	3
Megak	20/20 (100)	—	—	—	—
Eos	—	12/15 (80)	—	—	—

Abbreviations: CML, chronic myeloid leukemia; Eos, eosinophils; Glyc A, glycophorin A; Glyc C, glycophorin C; Megak, megakaryocytes.
In each case, the number of positive cells/total cells (with percentage in parenthesis) is shown.
[a]Highest percentage of normal cells with three BCR signals.

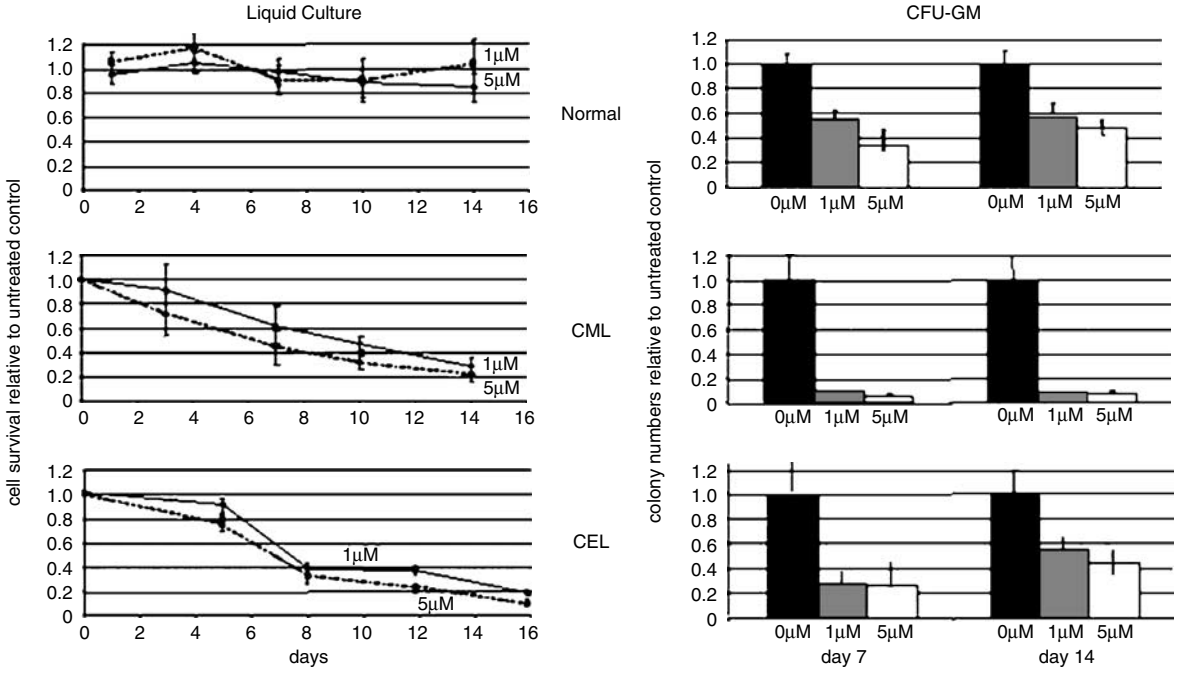

Figure 3 Imatinib mesylate sensitivity assay. Left: liquid culture. Curves indicate mean of duplicate cultures. Broken line indicates 1 μM concentration; full line indicates 5 μM concentration: Upper: results in healthy subjects. Middle: results in CML. Lower: results in CEL. CML and CEL patients show a marked reduction in relative cell numbers at both imatinib concentrations. Right: triplicate CFU-GM cultures. Upper: results in healthy subjects (response index = 0.49). Middle: results in CML (response index = 0.08). Lower: results in CEL (response index = 0.41).

FISH on clonogenic cells, that is, CFU-GM colonies, recovered at day 14 in methylcellulose, and was carried out in CEL (no. 1, 2, 6, Table 1) and in CML (no. 2 and 3 Table 2). Results showed no CHIC2 deletion in CEL cases (the percentage of CHIC2 deletion was equal to or above the cutoff of 5%) and positivity for *BCR-ABL1* in CML cases (respectively 100% in one case, and 50% in the other).

Clonal analysis in the CEL female patient (case no. 2, Table 1) showed skewed X-chromosome inactivation patterns in both granulocytes and T lymphocytes. To distinguish between the monoclonal nature of these cells and skewed Lyonization, we examined the clonality pattern of buccal mucosa cells as non-hematological control for the myeloid stem cell disorder. The band pattern was opposite to that of PMN and T lymphocyte in keeping with a true clonal hematopoiesis affecting both granulocytes and T cells.

In vitro tests for sensitivity to imatinib in CEL showed that peripheral blood mononuclear cells in liquid cultures were inhibited (Figure 3, bottom left), but no significant response was seen in CFU-GM (median response index: 0.33 *vs* 0.43 in controls) (Figure 3, bottom right). By contrast, three newly diagnosed *BCR-ABL1*+ CML patients showed a significant response in both liquid cultures (Figure 3, left) and CFU-GM assay (median response index = 0.05) (Figure 3, right).

Discussion

This study provided us with new insights on the characterization of leukemic populations from two imatinib-sensitive myeloproliferative disorders, namely *BCR-ABL1*+ CML and *FIP1L1-PDGFRA*+ CEL.

As expected, in CML the *BCR-ABL1* rearrangement consistently labeled multipotential stem cells expressing CD34 and CD133 antigens. Moreover, both myeloid and lymphoid cells were affected, mirroring a predicted cascade of hematopoietic differentiation.[10] These findings concur with results of conventional cytogenetics, G6PDH enzymatic activity, X-inactivation and detection of the *BCR-ABL1* transcript[11–14] and validate our FISH approach for the assignment of a typical leukemic genetic lesion to different hematopoietic lineages.

From this study, in CEL the CD34+ stem cells were not affected, although in one case (patient 4, Table 1) with a high value of *CHIC2* deletion we cannot exclude the presence of *FIP1L1-PDGFRA* in some CD34+ cells. Moreover, as a low amount of positive nuclei from CD34+ sorted cells were reported by Robyn *et al.*,[15] we also checked *FIP1L1-PDGFRA* in CD34+ enriched cells after microbeads in patient 8 confirming our FICTION results. Furthermore, in two additional cases (patients 2 and 6, Table 1), *CHIC2* deletion was also absent in CD133-positive cells. Thus, in contrast with *BCR-ABL1* in CML, CD34+ and CD133+ stem cells are virtually spared by the *FIP1L1-PDGFRA* genetic lesion in CEL.

B and T lymphocytes were not affected by *FIP1L1-PDGFRA*, as shown by identical results in CEL and normal samples (Table 1 and Figure 1). However, in our female CEL patient in whom X-inactivation could be performed (patient 2, Table 1), we found that clonality included T lymphocytes, despite the absence of *FIP1L1-PDGFRA* fusion, suggesting that, at least in some cases, *FIP1L1-PDGFRA* recombination occurs after a still unknown clonal mutation in a multipotential stem cell. Similar results have been recently shown in a subset of chronic myeloproliferative disorders in which clonality included cells with and without the typical $JAK2^{V617F}$ mutation.[16] Interestingly, as far as it concerns lymphocyte cells, analogies are emerging between *FIP1L1-PDGFRA* in CEL and *PDGFRB* recombinations in other Ph-negative chronic myeloproliferations. In a previous publication on a case of atypical CML, we showed that the *H4-PDGFRB* fusion affected myeloid progenitors but neither CD3+ nor CD19+ lymphocytes.[17] More recently we obtained similar

results in a case of *ETV6-PDGFRB*+ chronic myelomonocytic leukemia in which both CD3+ and CD19+ cells were normal (Crescenzi B and Mecucci C, unpublished). Whether *PDGFRA* and *PDGFRB* recombinations affect a myeloid committed progenitor, or if an affected multipotential stem cell undergoes only restricted and disparate myeloid maturation, when targeted by *PDGFRA* or *PDGFRB* recombinations, remains to be determined. With respect to *PDGFRB*, the last hypothesis is supported by Shigematsu *et al*. (*Blood* 2004; **104**, abstract no. 387), who reported that an *ETV6-PDGFRB* fusion gene was able to address only myeloid lineage differentiation when transfected in lymphoid progenitors.

In this study we found that, in addition to eosinophils, the leukemic mass in *FIP1L1-PDGFRA*+ CEL was limited to erythrocytes and granulomonocytes. Strikingly, CFU-GM growth was inhibited by imatinib in CML, but not in CEL (Figure 3) and, accordingly, FISH on CFU-GM from CML was positive for *BCR-ABL1*, whereas FISH on CFU-GM from three CEL cases was negative for *FIP1L1-PDGFRA*. Whether *FIP1L1-PDGFRA* behaves as a genetic lesion able to impart self-renewal properties to mature precursors remains to be investigated. Alternatively, the restricted *FIP1L1-PDGFRA*+ hematopoiesis in CEL might be supported by microenvironmental conditions, such as overexpression of specific cytokines. Interestingly, a critical role of extrinsic factors in the pathogenesis of a full CEL phenotype has been recently emphasized in a murine model published by Yamada *et al*.[18] who showed that *FIP1L1-PDGFRA* was not able to generate organ infiltration by eosinophils in the absence of overexpression of interleukin 5.

In conclusion, for the first time we identified major differences between *BCR-ABL1*+ cells in CML and *FIP1L1-PDGFRA*+ cells in CEL. Altogether our results show that the *BCR-ABL1*+ model of affected CD34+ and CD133+ stem cell ordinately undergoing multilineage differentiation does not apply to CEL whose leukemic mass, labeled by *FIP1L1-PDGFRA* fusion and targeted by imatinib is restricted to advanced stages of myeloid hematopoiesis.

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JAK2-V617F mutation in a patient with Philadelphia-chromosome-positive chronic myeloid leukaemia

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In July, 2000, a 50-year-old man presented with leukocytosis and splenomegaly (21 cm). Leucocyte concentration was 93×10^9/L, haemoglobin 150 g/L, and platelets 345×10^9/L (figure 1). A differential blood count showed 54% neutrophils, 2% lymphocytes, 13% myelocytes, 7% metamyelocytes, 2% promyelocytes, 1% blasts, and 7% basophils. Lactate dehydrogenase (LDH) concentration was increased at 484 U/L. 17 months previously, the blood count had been in the normal range. Bone-marrow aspiration was dry and bone-marrow biopsy showed marked myeloid hyperplasia, increased megakaryopoiesis, and the beginning of fibrosis. Cytogenetic analysis revealed a chromosome translocation (t[9;22][q34;q11]) in all ten metaphases examined. Expression of B3A2 *BCR-ABL* mRNA was detected by reverse transcriptase polymerase chain reaction (RT-PCR) in peripheral-blood leucocytes. A diagnosis of *BCR-ABL*-positive chronic myeloid leukaemia (CML) was made and treatment with hydroxycarbamide was started, resulting in rapid normalisation of peripheral-blood leucocytes. After 3 weeks, treatment was changed to imatinib (400 mg/day). Within 1 month, a complete haematological response was recorded. Cytogenetic and interphase fluorescence in-situ hybridisation at 6 months after the start of imatinib showed a complete cytogenetic response. The *BCR-ABL*:*ABL* ratio dropped from 90% at diagnosis to 0·021% after 6 months. 16 months after starting imatinib, *BCR-ABL* transcripts became undetectable by real-time and nested RT-PCR (figure 2).[1] Repeated cytogenetic and quantitative *BCR-ABL* RT-PCR analyses showed a continuing complete cytogenetic and molecular response, as shown in figure 2. However, in 2003, an unexpected decrease in platelet count and a continuous increase in serum LDH concentration was noted (figure 1). In 2004, immature myeloid cells began to appear in the peripheral blood and a bone-marrow biopsy in May, 2004, revealed advanced fibrosis. In 2005, leucocyte concentration continually rose above the upper normal limit. The spleen was enlarged to 18 cm. Cytogenetic analysis identified a normal karyotype and *BCR-ABL* transcripts were still undetectable. Therefore, a *JAK2*-V617F mutational analysis was done, which highlighted the presence of a heterozygous *JAK2*-V617F mutation, leading to the diagnosis of idiopathic myelofibrosis (IMF) according to the WHO diagnostic criteria. For *JAK2* genotyping, DNA was amplified and PCR products were directly sequenced using the PCR primers *JAK2*-1F (5'-TGCTGAAAGTAGGAGAAAGTGCAT-3') and *JAK2*-1R *(5'-TCCTACAGTGTTTTCAGTTTCAA-3')*.

The proportion of mutant *JAK2*-V617F alleles was quantified using pyrosequencing as described.[2] Additionally, the allelic ratio of *JAK2*-V617F was confirmed by a newly established quantitative real-time (RQ) PCR assay based on LightCycler technology (Roche Diagnostics, Mannheim, Germany). DNA was extracted from peripheral blood leucocytes after red-cell lysis by standard procedures. Total *JAK2* was established using a forward primer, 5'-AGGCTACATCCATCTACCTCAG-3', a reverse primer, 5'-CCTAGCTGTGATCCTGAAACTG-3' (MWG Biotech, Ebersberg, Germany), and the hybridisation probes 5'-ACAGGCTTGACCCATAA-CACCTGAAATA GAG-3' and 5'-GAGTGGTACAGGAA-TCATGAATAATGCCAGTCA-3' (Tib Molbiol, Berlin, Germany). Mutant *JAK2*-V617F alleles were quantified using the same forward primer and probes in combination with a mutation-specific reverse primer, 5'-TTTTACTTACTCTCGTCTCCACAGAA-3' (MWG Biotech, Ebersberg, Germany). The allele copy number was identified using a plasmid standard curve. *JAK2*-V617F positivity was expressed as the ratio between mutant *JAK2*-V617F and total *JAK2*. Dilution experiments showed an assay sensitivity of 1%.

Retrospective analysis of frozen samples of peripheral-blood leucocytes showed that the *JAK2*-V617F mutation was already present at initial diagnosis of *BCR-ABL*-positive CML (figure 3). Consistent with the presence of an acquired somatic mutation, the *JAK2*-V617F mutation was absent in purified CD3+ T lymphocytes (figure 3)

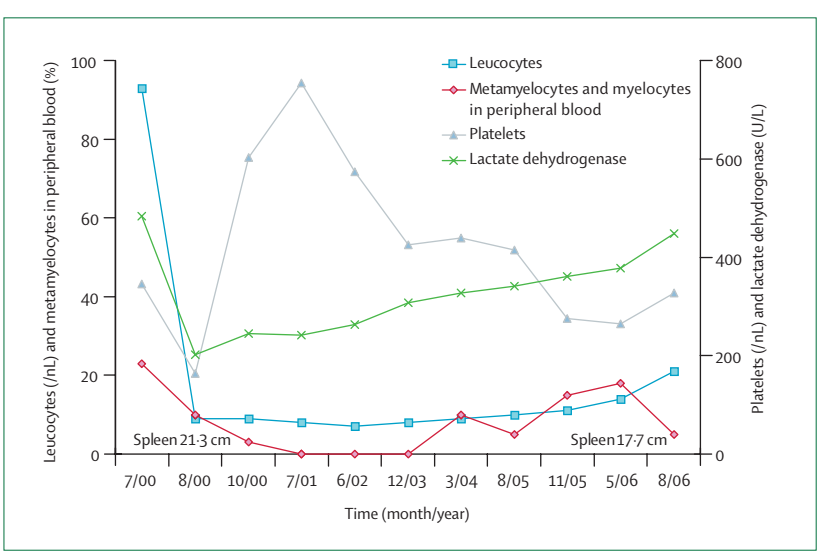

Figure 1: **Course of peripheral-blood parameters during treatment with imatinib in a patient with *BCR-ABL*-positive chronic myeloid leukaemia**

and buccal cells (data not shown). As demonstrated by two independent methods—*JAK2*-V617F pyrosequencing and PCR—the proportion of the mutant allele stayed roughly constant from diagnosis of CML in July, 2000, to February, 2006 (figure 2). Repeated cytogenetic analysis in May, 2006 again showed a normal karyotype.

JAK2 is a tyrosine kinase that has an important role in the signalling pathways of many haemopoietic growth-factor receptors. The single acquired point mutation V617F (1849G>T) in *JAK2* occurs in 50–97% of patients with IMF, essential thrombocytosis, and polycythemia vera.[2–6] By contrast, the *JAK2*-V617F mutation has never been identified in a patient with *BCR-ABL*-positive CML.[7] This is the first description of a patient, to our knowledge, in whom the *BCR-ABL* fusion gene and an acquired somatic *JAK2*-V617F mutation could be detected contemporaneously, with IMF becoming clinically relevant after successful treatment of CML by imatinib.

Several conclusions can be drawn from these data. First, bone-marrow fibrosis was not a consequence of progressive CML. Rather, myelofibrosis developed as a second myeloproliferative disorder (MPD) during complete cytogenetic and molecular remission of CML. Although the *BCR-ABL* fusion gene might represent the initiating lesion in most cases of CML, clinical manifestations of the disease can be variable. Specifically, bone-marrow fibrosis can occur, sometimes varying greatly during the course of the disease, and has been used to categorise patients into different groups with distinctive survival caracteristics.[8] Fibrogenesis in MPD is generally assumed to be mediated by the abnormal release of transforming growth factor-β and platelet-derived growth factor (PDGF). Imatinib, a selective inhibitor of ABL, KIT, and PDGF receptor tyrosine kinases, and which is not active against JAK2, has been seen to reduce the content of bone-marrow fibre in patients with CML.[9] Regression of myelofibrosis by imatinib is thought to be caused either by a direct PDGF-antagonistic effect or by normalisation of megakaryopoiesis from which abnormal concentrations of PDGF are released. However, expression of *JAK2*-V617F in murine haematopoietic cells leads to MPD associated with myelofibrosis.[10] Moreover, expression of a *BCR-JAK2* fusion gene has been described in a patient with atypical CML and bone-marrow fibrosis,[11] and the *PCM1-JAK2* fusion is associated with the development of myelofibrosis in patients with chronic and acute leukaemias.[12] These findings suggest that *JAK2*-V617F contributes to the development of myelofibrosis in MPD,[3,10] and might have been responsible for the induction of myelofibrosis in our patient which, consequently, was independent of the presence of BCR-ABL and did not respond to imatinib treatment. This assumption is supported by the absence of clinical and molecular responses of patients with IMF and polycythemia vera treated with imatinib.[13,14] If treatment with imatinib had not been initiated, the clinical course of the patient could only have been speculated upon. Treatment and response to imatinib might have accelerated the outgrowth of the IMF.

Second, our data suggest that in our patient, the *JAK2*-V617F mutation had occurred before the acquisition of the Philadelphia (Ph)-chromosome. Development of a Ph-positive CML has rarely been reported in patients with MPD.[15] In the few cases that have been reported,

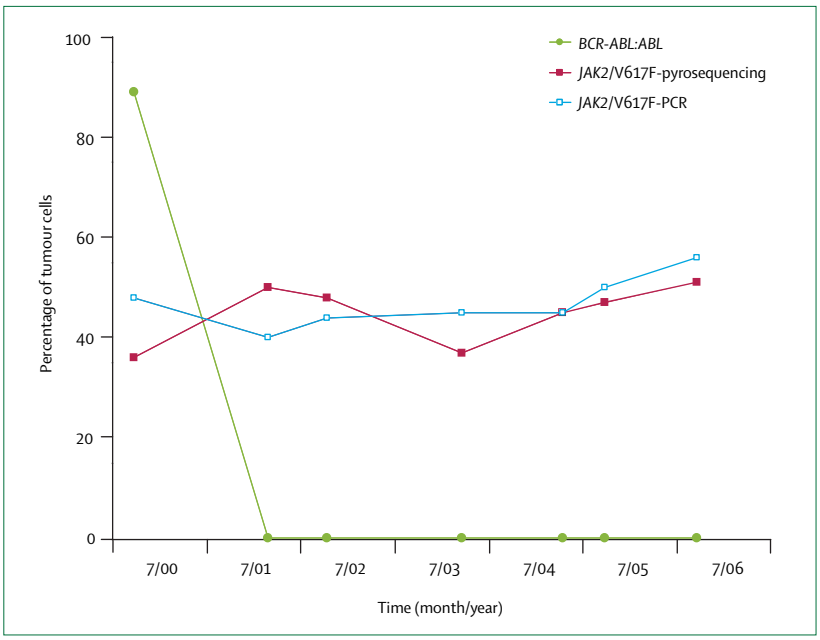

***Figure 2:* BCR-ABL:ABL ratio and *JAK2*-V617F allele frequency during treatment with imatinib in a patient with *BCR-ABL*-positive chronic myeloid leukaemia**
Parameters established by pyrosequencing and PCR.

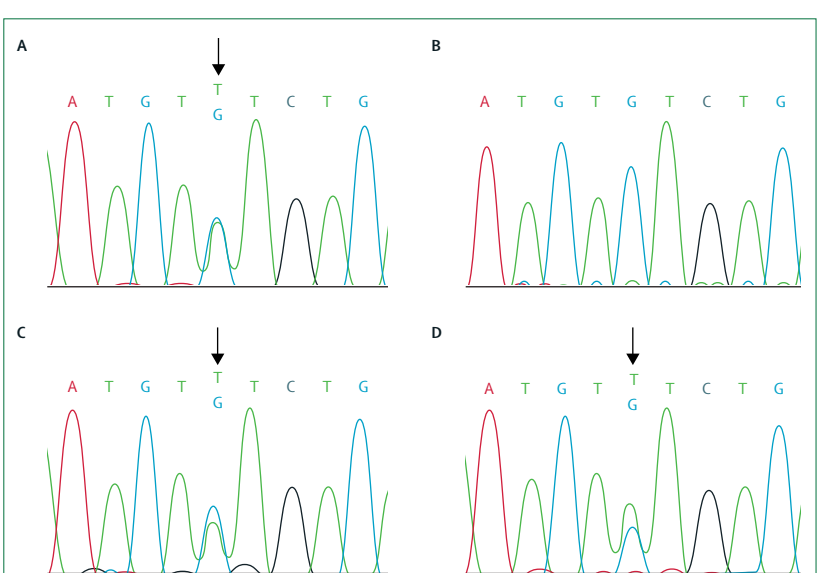

***Figure 3:* Presence of the *JAK2*-V617F mutation in a patient with *BCR-ABL*-positive chronic myeloid leukaemia**
A heterozygous G→T transversion in *JAK2* (arrows) is present in DNA from leucocytes (A,C,D) at different time points (A: July, 2000; C: November, 2001; D: May, 2006) during the course of the disease, but absent in T cells (B; May, 2006), consistent with the existence of an acquired somatic origin of the mutation.

there is a debate as to whether MPD and CML arise separately from each other, representing independent transformation of a normal stem cell, or whether the Ph-chromosome arises in a stem cell that was part of the MPD clone. In our patient, *JAK2*-V617F was detectable at an almost constant allele frequency in all peripheral-blood samples examined (figure 2). The most likely explanation for this finding is the presence of a heterozygous *JAK2*-V617F mutation in most of the peripheral-blood leucocytes, implicating the simultaneous presence of the *BCR-ABL* fusion gene and the *JAK2*-V617F mutation in white blood cells. Because *BCR-ABL* became undetectable after treatment with imatinib, but the *JAK2*-V617F allele frequency remained virtually unchanged, the conclusion can be drawn that the Ph-chromosome was acquired from a haematopoietic cell carrying the *JAK2*-V617F mutation. Several findings suggest that MPD are caused by a mutation in an, as yet, unknown gene that precedes the acquisition of either a *JAK2*-V617F mutation or a *BCR-ABL* fusion gene.[3,16] The most reasonable explanation for the findings presented here is that *BCR-ABL* can, in rare cases, occur on the background of the *JAK2*-V617F mutation, possibly augmented by an elusive initial mutation predisposing to the acquisition of both a *JAK2*-V617F mutation and a *BCR-ABL* translocation.

Conflicts of interest

The authors declared no conflicts of interest.

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Brief report

Minimal molecular response in polycythemia vera patients treated with imatinib or interferon alpha

Amy V. Jones, Richard T. Silver, Katherine Waghorn, Claire Curtis, Sebastian Kreil, Katerina Zoi, Andreas Hochhaus, David Oscier, Georgia Metzgeroth, Eva Lengfelder, Andreas Reiter, Andrew J. Chase, and Nicholas C. P. Cross

Imatinib and recombinant interferon alpha (rIFNα) can induce remission in polycythemia vera (PV) patients, but gauging the depth of responses has not been possible due to lack of a specific disease marker. We found that patients undergoing imatinib (n = 14) or rIFNα (n = 7) therapy remained strongly positive for V617F *JAK2,* although there was a significant reduction in the median percentage of mutant alleles that correlated with hematologic response (*P* = .001). Furthermore, individuals who achieved complete hematologic remission had lower levels of V617F than those who did not (*P* = .001). Of 9 imatinib-treated cases for whom pretreatment samples were available, 7 with no or partial hematologic responses showed a marginal increase (median, 1.2-fold; range, 1.0-1.5) in the percentage of V617F alleles on treatment, whereas the 2 patients who achieved complete hematologic remission showed a 2- to 3-fold reduction. Our data indicate that, although PV patients may benefit from imatinib or rIFNα, molecular responses are relatively modest. (Blood. 2006;107: 3339-3341)

Introduction

Polycythemia vera (PV) is a myeloproliferative disease characterized by an increased red-cell mass and usually an increase in megakaryocytes in the bone marrow and platelets in the peripheral blood. Untreated, the disease leads to thrombohemorrhagic complications and in most cases to progressive marrow myelofibrosis, anemia, and splenomegaly.[1] Long-term remission has been reported with recombinant interferon alpha (rIFNα),[2-7] and, more recently, a number of patients have achieved complete or partial responses after treatment with imatinib mesylate.[8-11]

rIFNα has been reported to induce cytogenetic remission in occasional patients and/or result in conversion of monoclonal to polyclonal hematopoiesis.[3] Such events have not been reported as yet following imatinib treatment. However, since only 20% of patients present with a chromosomal abnormality, it has not been possible, in general, to gauge the depth of responses to treatment. Here, we have exploited the recently described V617F *JAK2* mutation[12-18] as a disease marker to measure the response of PV patients to rIFNα or imatinib.

Study design

Patients

We studied 111 PV patients, of whom 21 had undergone treatment in 2 larger sequential phase 2 single-institution studies. Seven patients received rIFNα in the first study (initial doses 1 MU 3 times weekly to 3 MU daily; median follow-up, 60 months; range, 13-132), and 14 received imatinib in the second study (initial doses 400 to 800 mg daily; median follow-up, 17 months; range, 5-31). The 90 remaining patients, constituting the control group, were either untreated or treated by phlebotomy only, hydroxyurea, and/or anagrelide. For rIFNα-treated cases, complete hematologic response (CR) was defined as no need for phlebotomy, a sustained hematocrit level of less than .45 (45%) for men and less than .42 (42%) for women, and platelet count of 600×10^9/L or less. Partial hematologic response (PR) was the aforementioned but platelet count ranged between 600 and 1000×10^9/L (Polycythemia Vera Study Group criteria). For imatinib-treated patients, CR was defined as phlebotomy-free within the first 18 months of treatment, hematocrit level of .45 (45%) or less for men and .42 (42%) or less for women, platelet count of 400×10^9/L or less, and absence of splenomegaly if spleen was initially palpable. PR was defined as phlebotomy-free within

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A.V.J., K.W., C.C., S.K., and A.J.C. performed or supervised the laboratory analysis and contributed to the interpretation of results. R.T.S. provided samples from all imatinib- or interferon alpha–treated cases plus some controls, provided patient care, and contributed to the design of the study. K.Z., A.H., D.O., G.M., E.L., and A.R. contributed control patient samples and supplied essential data. N.C.P.C. designed the study and wrote the first draft of the paper. All authors commented on and contributed to the final paper.

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the first 18 months, hematocrit level of .45 (45%) or less for men and .42 (42%) or less for women, platelet count more than 400×10^9/L, and spleen reduced to 50% or less of the original size, if initially palpable. All responses were thus considered quantitative, not qualitative. The studies had been approved by the internal review boards from participating institutions and informed consent was provided according to the Declaration of Helsinki.

Molecular studies

DNA was extracted from peripheral-blood samples (n = 111) by standard procedures after isolation of total leukocytes or granulocytes following red-cell lysis or by density gradient centrifugation over Histopaque 1077 (Sigma-Aldrich, Ayrshire, United Kingdom). DNA was also extracted from pretreatment, unstained bone marrow slides (n = 9). V617F was detected initially using an amplification refractory mutation system (ARMS) assay as described.[17] The proportion of mutant alleles (%V617F) was quantified using pyrosequencing as detailed,[17] except that samples were amplified using primers 5′-TCTTTGAAGCAGCAAGTATGA-3′ and 5′-biotin-GCCTGTAGTTTTACTTACTCTCG-3′ and sequenced with primer 5′-TTTTAAATTATGGAGTATGT-3′. Comparison of the %V617F between control and treated patients was performed using the Mann-Whitney test.

Results and discussion

Using ARMS, V617F was detected in 82 (91%) of the control PV cases and in all 21 cases undergoing treatment with imatinib or rIFNα. The sensitivity of the assay is approximately 5%,[17] indicating that neither therapy had reduced the percentage of mutant cells below this level. Patient details and results are summarized in Table 1.

To quantify the level of disease, we used pyrosequencing, which measures the proportion of mutant alleles.[17] As shown in Figure 1A, we found that the median percentage of mutated *JAK2* alleles (%V617F) did not differ significantly between the imatinib-treated cases (median, 59%; range, 8%-91%) and the V617F-positive cases from the control group (median, 53%; range, 5%-100%). The %V617F was lower in the rIFNα-treated cases (median, 27%; range, 19%-87%) compared with controls ($P = .03$), which probably reflects the high proportion of cases in this selected group who had achieved CR rather than any preferential inhibitory effect of rIFNα over imatinib.

For imatinib-treated patients, the median %V617F for individuals with NR, PR, and CR were 72% (range, 44%-89%; n = 3), 60% (range, 30%-91%; n = 9), and 15% (range, 8%-21%; n = 2), respectively. In the rIFNα-treated group, the single case with a PR had 87% V617F compared with a median of 26% (range, 19%-39%) for the 6 cases in CR. Individuals who achieved CR on imatinib or rIFNα (n = 8) had a lower %V617F (median, 24%; range, 20%-29%) than those patients who did not achieve CR (n = 13; median, 72%; range, 19%-91%) and also the control group ($P = .001$). There was a significant trend in reduction of %V617F values on comparison of patients who showed NR, PR, or CR to therapy ($P = .001$, Figure 1B).

To determine the magnitude of any change that occurred as a consequence of therapy, we compared %V617F levels in pretreatment bone marrow and posttreatment peripheral-blood samples for 9 of the imatinib-treated cases (pretreatment samples were not available for the other cases). Of these 9 cases, the 7 with NR or PR showed a marginal increase (median, 1.2-fold; range, 1.0- to 1.5-fold) in the percentage of V617F alleles on treatment. In contrast, the 2 patients who achieved CR showed a 2- to 3-fold reduction in %V617F on treatment (Figure 1C). To confirm that these changes were not distorted by differences in the size of the V617F clone between bone marrow and peripheral blood, we compared the %V617F in contemporaneous specimens taken from 11 control PV patients. As shown on Figure 1D, similar %V617F levels are seen in blood and marrow.

Interferon-α exerts a general antimyeloproliferative effect, although its precise mechanism of action is not understood. Since pretreatment samples were not available for the rIFNα-treated cases in our study group, we cannot be certain that this therapy resulted in a reduction of %V617F levels. However, this seems likely to be the case since the rIFNα-treated patients (all but one of

Table 1. Summary of patient characteristics and results for cases undergoing therapy with imatinib or rIFNα

Patient no.	Sex	Age at diagnosis, y	Rx	Dose	F/U, mo	Response	% V617F on treatment	% V617F before treatment
1	M	26	Imatinib	800 mg/d	5	NR	44	ND
2	F	60	Imatinib	700 mg/d	5	PR	41	34
3	M	49	Imatinib	800 mg/d	9	NR	89	71
4	F	67	Imatinib	500 mg/d	9	PR	91	88
5	M	58	Imatinib	400 mg/d	10	PR	72	ND
6	M	48	Imatinib	800 mg/d	15	PR	57	40
7	M	31	Imatinib	800 mg/d	17	PR	76	63
8	F	48	Imatinib	500 mg/d	17	CR	8	25
9	F	28	Imatinib	800 mg/d	19	PR	83	82
10	M	72	Imatinib	400 mg/d	21	NR	72	ND
11	M	54	Imatinib	300 mg/d	24	CR	21	44
12	M	43	Imatinib	400 mg/d	25	PR	60	40
13	M	30	Imatinib	600 mg/d	25	PR	19	ND
14	M	53	Imatinib	700 mg/d	31	PR	30	ND
15	F	32	rIFNα	3 MU 3 times/wk	13	CR	19	ND
16	F	39	rIFNα	3 MU 5 days/wk	45	CR	39	ND
17	M	53	rIFNα	3 MU/day	50	CR	29	ND
18	F	48	rIFNα	4 MU, alternate d	60	PR	87	ND
19	M	42	rIFNα	2 MU 3 times/wk	60	CR	27	ND
20	M	52	rIFNα	4.25 MU 3 times/wk	108	CR	22	ND
21	M	39	rIFNα	3.5 MU/d	132	CR	25	ND

Rx indicates treatment; F/U, follow-up (= time of sampling) after starting rIFNα or imatinib; NR, no response; ND, not determined; PR, partial response; CR, complete response; and MU, million units.

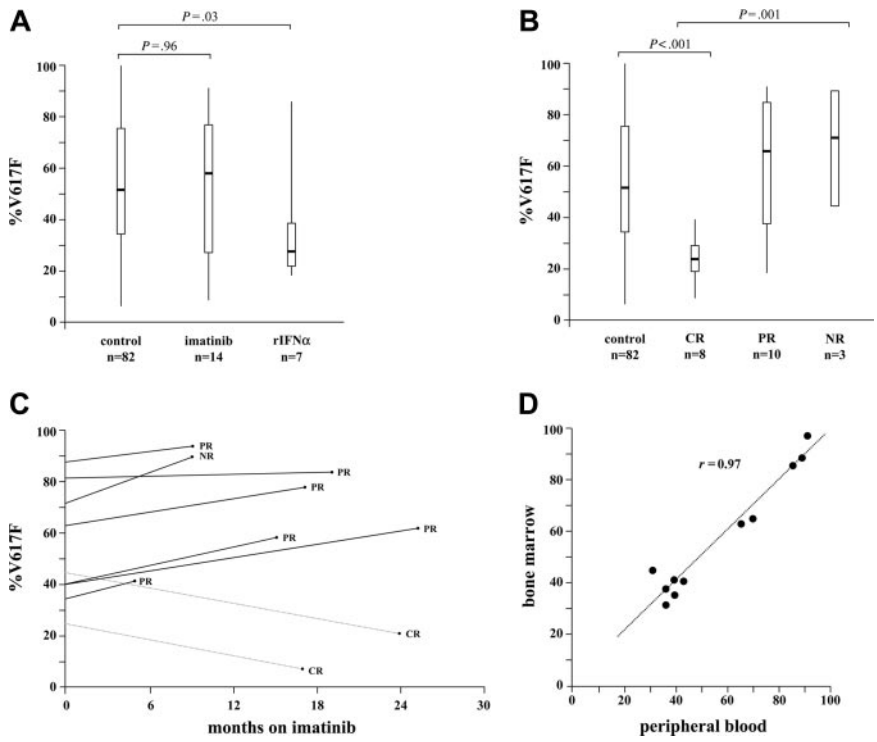

Figure 1. Levels of *JAK2* V617F in treated and control PV cases. (A) Comparison of %V617F in control, mutation-positive PV cases who were either treated or not treated with imatinib or rIFNα. (B) Comparison of %V617F in control PV cases and patients who showed CR, PR, or NR following therapy with imatinib or rIFNα. Vertical lines indicate the range of results; boxes, the interquartile range; and thick horizontal lines, median values. (C) Changes in %V617F on imatinib therapy. The %V617F was measured before starting imatinib and at various times while on therapy for 7 patients who showed no response (NR) or a partial response (PR), and 2 patients who showed a complete response (CR). (D) Comparison of %V617F level in peripheral blood and bone marrow from 11 control PV cases.

whom were in CR) had significantly lower %V617F levels than controls, and, furthermore, these levels were comparable with those seen in CR cases on imatinib. Indeed, subsequent to this study, we have identified a further PV patient who showed a reduction in the %V617F from a pretreatment level of 70% to 35% in CR on rIFNα.

Imatinib is a selective inhibitor of ABL, PDGFR, KIT, and FMS tyrosine kinases but is not active against JAK2. It is known that KIT signal transduction pathways are required for erythropoiesis,[19,20] and it is possible that the observed clinical benefits of imatinib in PV are a consequence of KIT inhibition. Although our study demonstrates that molecular responses can be discerned in PV patients that correlate with hematologic improvement, the magnitude of these responses contrasts starkly with chronic myeloid leukemia, wherein many individuals experience a 1000-fold or greater reduction in *BCR-ABL* levels.[21,22] While we cannot exclude the possibility that occasional PV patients might exhibit more substantial molecular responses, we conclude that the response of PV to imatinib or rIFNα is relatively modest.

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has been reported that, in contrast to $p190^{BCR-ABL}$ that is normally found in Ph^+ ALL, CML associated $p210^{BCR-ABL}$ by virtue of its unique Dbl/Plecstrin-homology domain, can bind to small GTPases of the Rho family both *in vitro* and *in vivo*.[3] This in turn gives rise to Rho activation, which might be responsible for promoting cellular motility. Taken together, it need to be substantiated whether or not these events do indeed contribute to malignant transformation; however, considering BCR-ABL-induced abundant pool of p27 in the CML cytoplasm, one can at least speculate the involvement of BCR-ABL-p27-RhoGTPase axis in CML blast transformation (Figure 1).

Past few decades have witnessed considerable advancement in identifying putative phosphorylation hotspots in p27 that are important for cell cycle regulation. The N-terminus portion of p27 is recruited to cyclin–cdk complexes and results in cell cycle arrest and it is the C-terminus portion that can promote cellular motility and thus metastatic potential. Intriguingly, these two functions are virtually partitioned by a single parameter: the address of p27 at a given point of time. Future studies could be directed to unravel the individual domain contribution by using respective knockout animal models, and it would be important to look upon whether tampering p27 in such a manner can have any consequences on cancer predisposition. Furthermore, different CDKIs control diverse array of functions for HSC maintenance. However, we know very little about how these different CDKIs differentially regulate quiescence, self-renewal and homing, three important aspects of regenerative medicine, of normal and malignant HSCs. To conclude, we are only beginning to appreciate the underlying importance of p27 and other cell cycle inhibitors in the context of LSCs. Indeed, how such an intrinsically unstructured p27 protein governs CML biology need to be explored further.

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No evidence for amplification of V617F *JAK2* in myeloproliferative disorders

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Acquired uniparental disomy following mitotic recombination results in homozygosity for the V617F *JAK2* mutation in a proportion of patients with myeloproliferative disorders (MPD), particularly polycythemia vera (PV) and myelofibrosis (MF).[1] Recently, Hammond *et al.*[2] in a letter in *Leukemia* indicated that a proportion of MPDs harbor more than two copies of V617F *JAK2* and suggested that disease evolution may involve further gene duplication associated with genetic instability. In a follow-up study, the same group found that this phenomenon was most prevalent in PV, with more than one-third of mutation-positive cases apparently having more than two V617F copies/cell.[3] Increased V617F copy number has been described in cell lines,[4] but has not thus far been verified in patients.

To determine if elevated *JAK2* copy number could be detected with an alternative methodology, we developed a test based on multiplex ligation-dependent probe amplification (MLPA), an established technique for detecting DNA copy number changes.[5–7] Four MLPA probe pairs targeting *JAK2* exons 3, 9, 16 and 20 were designed, so that they could be combined with the commercially available telomere MLPA probe mix (P036B

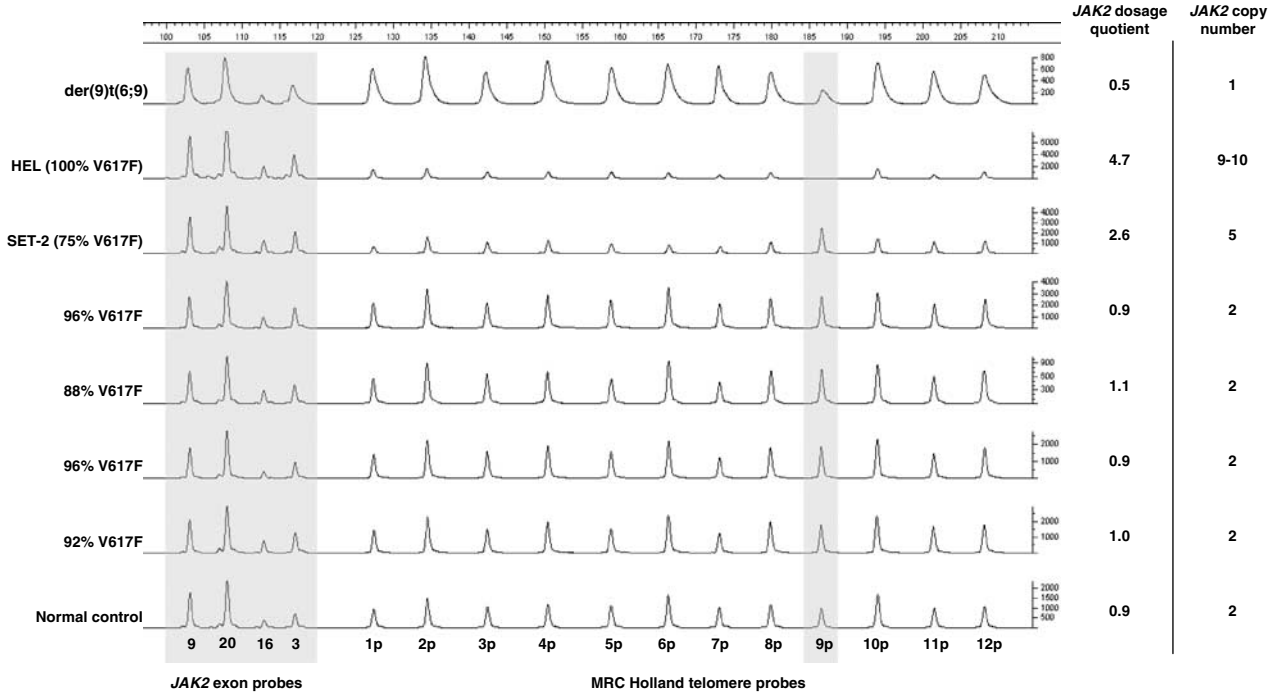

Figure 1 Representative MLPA genotyper traces for measuring *JAK2* copy number per cell. The *JAK2* probes for exons 9, 20, 16 and 3 result in smaller bands than those from MRC-Holland telomere probe mix, of which 1p–12p are shown here. The *JAK2* dosage quotient and estimated copy numbers are shown. The der(9)t(6;9) cell line, which harbors only one copy of *JAK2*, shows reduced *JAK2* and 9p subtelomere peaks relative to the other peaks. HEL and SET-2 show an increase in the relative size of these peaks, while no JAK2 copy number alterations were detected in four illustrative patient samples with a high %V617F.

MRC-Holland, Amsterdam). The *JAK2* probes consisted of adjacent 5′ and 3′ oligonucleotides which, when amplified by universal primers following the hybridization and ligation steps, generated products in the size range 98–126 bp. The 3′ oligonucleotides for each probe pair were modified with a phosphate group at their 5′ end, and each oligonucleotide pair contained a tag sequence recognized by the MLPA Salsa primers, so that the *JAK2* probes were amplified alongside the telomere probes in one reaction. MLPA was performed and results were analyzed as described.[7,8] Ratios of test peaks to control peaks and control peaks to other control peaks in each patient sample were compared to the same ratios obtained for two normal individuals that were included in each run. The four *JAK2* peak area ratios per sample were averaged to give a relative dosage quotient for *JAK2* as a representation of copy number per cell. For normal sequences, a dosage quotient of 1.0 is expected; if a deletion or duplication is present, the dosage quotient should be 0.5 and 1.5, respectively.

To validate the assay, we initially analyzed a series of control samples. Twenty-one hematologically normal individuals gave a median *JAK2* dosage quotient of 1.01 (range 0.71–1.28). The dosage quotient for a fibroblast cell line carrying a der(9)t(6;9) was 0.50, corresponding to deletion of one copy of *JAK2*, which was further supported by deletion of one copy of the 9p telomere probe. To control for increased copy number of the *JAK2* locus, the V617F-positive cell lines SET-2 and HEL were analyzed. SET-2 has approximately 75% V617F, as determined by pyrosequencing,[8] while MLPA revealed approximately five copies of the 9p telomere (dosage quotient = 2.47) and five copies of *JAK2* per cell (dosage quotient = 2.50). HEL has 100% mutant allele by pyrosequencing and MLPA indicated 9–10 copies of *JAK2* (dosage quotient = 4.72), plus total deletion of the 9p telomere and other abnormalities. These results are illustrated in Figure 1, and accord well with previous estimates of *JAK2* copy number in these cell lines determined by fluorescence *in situ* hybridization.[4]

Next, we investigated peripheral blood leukocyte DNA from 63 MPDs (PV, $n = 45$; MF, $n = 5$; other MPD, $n = 13$) that were homozygous for V617F as determined by pyrosequencing (median %V617F = 87%; range 63–99%). The *JAK2* dosage quotients (median 0.99; range 0.67–1.25) ascertained by MLPA were no different from the normal control group and no case was above the previously defined cut-off required to define a duplication.[6,7] Analysis of a further 16 heterozygous V617F cases (median %V617F = 31%) yielded similar results with again no evidence for increased *JAK2* copy number. Although Hammond *et al*. suggested that their results could be caused by an internal tandem duplication of the V617F region rather than a complete duplication of *JAK2*, such abnormalities have never been reported on amplification of *JAK2* exon 14 from genomic DNA and, in addition, we have not detected any aberrant size bands using cDNA-specific primers to exons 13 and 15 in 15 homozygous V617F-positive cases analyzed by RT-PCR. We conclude therefore that amplification of the mutant *JAK2* allele is not a common factor in the evolution of *JAK2* V617F-positive disease.

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Rituximab with chemotherapy improves survival of non-germinal center type untreated diffuse large B-cell lymphoma

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Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous disorder that can be subdivided into several subtypes based on DNA microarray-based gene expression profiling: germinal center B-cell-like (GC) DLBCL and activated B-cell-like (ABC) DLBCL. Patients with GC DLBCL have been shown to exhibit a more favorable clinical outcome than patients with non-GC DLBCL.[1] As with cDNA microarray, immunohistochemistry can also be used to distinguish the GC/non-GC subtype of DLBCL and to predict survival,[2] but there have been few reports comparing clinical outcomes in cases of GC DLBCL and non-GC DLBCL treated with rituximab.[3,4] We retrospectively analyzed the treatment effect of rituximab with conventional chemotherapy on these two DLBCL subtypes by means of immunohistochemistry.

We analyzed patients with untreated DLBCL who were diagnosed according to the WHO classification.[5] Patients who were diagnosed in our hospital between 2001 and 2005 were treated with rituximab plus TCOP (R-TCOP) (cyclophosphamide, therarubicin, vincristine and prednisolone), and those who were diagnosed in our hospital between 1997 and 2002 were treated with TCOP alone. R-TCOP consisted of a combination of rituximab (375 mg/m^2 administered on day 1 of each chemotherapy cycle) and TCOP (cyclophosphamide 750, therarubicin 50 and vincristine 1.4 mg/m^2 administered on day 2 and prednisolone 100 mg/m^2 administered on days 2–6). TCOP consisted of TCOP alone. We reduced the chemotherapeutic dosage to two out of three patients who were more than 65 years of age. Tumor response was assessed at the end of treatment according to the International Workshop criteria.[6]

Immunohistochemical staining was performed using heat-induced antigen retrieval, as described previously.[7,8] Details of the immunohistochemical staining are provided in Table 1. The scoring was based on the algorithm described by Hans *et al.*[2] Bcl-6, CD10 and melanoma associated antigen (mutated) 1 (MUM1) were considered positive if 30% or more of tumor cells were stained with the respective antibody, and bcl-2 was considered positive if 30% or more tumor cells were stained. Given that bcl-6 and CD10 are markers of GC B cells, a case was considered to be a case of GC DLBCL if CD10 alone was positive or if both bcl-6 and CD10 were positive. If both bcl-6 and CD10 were negative, the case was considered to be a case of non-GC DLBCL. If bcl-6 was positive and CD10 was negative, the expression of MUM1 was used to determine the group, that is if MUM1 was negative, the case was considered to be a case of GC DLBCL, and if MUM1 was positive, the case was considered to be a case of non-GC DLBCL.

The results of immunohistochemical staining and response rates were compared between groups using χ^2 test and Fisher's exact test. Event-free survival (EFS) was calculated from the time of diagnosis to the date of disease progression, death or last contact. Overall survival (OS) was calculated as the time from diagnosis to the date of death or last contact. Survival curves were constructed using the Kaplan–Meier method and compared by log-rank test. Differences were considered significant at $P<0.05$. A general-purpose statistical software package, StatFlex Version 5.0 (Artech Co., Osaka, Japan), which provides results compatible with SPSS, was used for the data analyses.

The R-TCOP group comprises 38 patients. The median follow-up time for surviving patients in this group was 13 months. The TCOP group comprises 31 patients. The median follow-up time for surviving patients in this group was 25 months. Of the 69 cases in total, 26 (38%) and 43 (62%) were considered cases of GC DLBCL and non-GC DLBCL, respectively, on the basis of immunohistochemical analysis. In the R-TCOP group, 18 (48%) and 20 (52%) cases were classified as cases of GC DLBCL and non-GC DLBCL, respectively. In the TCOP group, eight (26%) and 23 (74%) cases were classified as cases of GC DLBCL and non-GC DLBCL, respectively. Bcl-2 was expressed in 63% of cases in the R-TCOP group, that is in 61% of GC DLBCL and 65% of non-GC DLBCL. Bcl-2 was expressed in 53% of cases treated with TCOP alone, that is in 50% of GC DLBCLs and in 70% of non-GC-DLBCLs. The patient characteristics (age, sex, performance status, Ann Arbor clinical stage, B symptoms, bone marrow involvement, extranodal disease, International Prognostic Index (IPI) and lactate dehydrogenase (LDH)) did not differ significantly between groups (that is, between patients grouped by treatment or by DLBCL subtype) (Table 2).

Myeloproliferative Disorders

Chronic neutrophilic leukemia with an associated V617F JAK2 tyrosine kinase mutation

Chronic neutrophilic leukemia (CNL) is a rare disease and can cause considerable diagnostic difficulty. Although the *V617F JAK2* mutation has been described by several groups to be associated with classical myeloproliferative disorders (MPD), this same mutation has been detected with a low incidence in atypical MPD, such as CNL. Here we report the presence of the *V617F* mutation in a CNL patient, who is unusual for having survived for more than 96 months, with little disease progression. It remains to be established what role this mutation, which gives cells a proliferative advantage, might play in the pathogenesis and prognosis of rare atypical MPD.

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(*http://www.haematologica.org/journal/2005/12/1696.html*)

Chronic neutrophilic leukemia (CNL) is a rare disease categorized under the World Health Organization (WHO) classification[1] as a chronic myeloproliferative disease. In essence it is a diagnosis of exclusion, in a patient presenting with neutrophilia and splenomegaly with no evidence of chronic myeloid leukemia or a reactive neutrophilia. Diagnostic difficulties are not uncommon and it is likely that many previous cases considered to be CNL were misclassified. The stated figures for prognosis vary widely. Reilly[2] performed a survival analysis of 33 cases of patients felt to have '*true chronic neutrophilic leukemia*' and suggested an overall median survival of 30 months and a 5-year survival of 28%. Treatment approaches to individuals with this disorder appear to be heterogeneous.

It is unusual for CNL individuals to survive for long, although it has been described,[3] and we have one such individual attending our clinic. This patient presented at the age of 61 years with a 2- month history of fatigue and an influenza-like illness. Examination revealed only moderate hepatosplenomegaly. Hematologic findings were a hemoglobin of 14.8 g/dL, total white cell count of 54×10^9/L (with neutrophils 48×10^9/L), and a platelet count of 316×10^9/L. Examination of blood films revealed a large population of segmented neutrophils with few immature granulocytes and no circulating myeloblasts. Bone marrow aspirate demonstrated a hypercellular marrow secondary to granulocytic proliferation with neither overt dysplastic features nor an increase in the blast percentage. The trephine biopsy did not indicate any fibrosis, increase in plasma cells or megakaryocytic proliferation. Conventional cytogenetic analysis demonstrated a normal male karyotype and the patient was negative for the *BCR/ABL* transcript by polymerase chain reaction analysis. Since no cause was established for a secondary neutrophilia he fulfilled the WHO[6] diagnostic criteria for CNL and the management strategy at this stage was one of observation only. Recently published data[4-8] have shown the acquired *V617F* Janus Kinase (*JAK*) 2 mutation is widespread amongst *classical* myeloproliferative (MPD) disorders, albeit in a heterogeneous manner. JAK2 is a member of the JAK non-receptor protein tyrosine kinase family and this mutation is located in the highly conserved JAK homology 2 (JH2) pseudokinase region. Loss of valine at position 617 in this region would result in constitutive tyrosine kinase phosphorylation activity by disruption of the intrinsic auto-inhibitory activity of the JH2 domain.[9] Consequently, cells possessing the *V617F JAK2* mutation would exhibit cytokine hypersensitivity and possess a proliferative advantage.

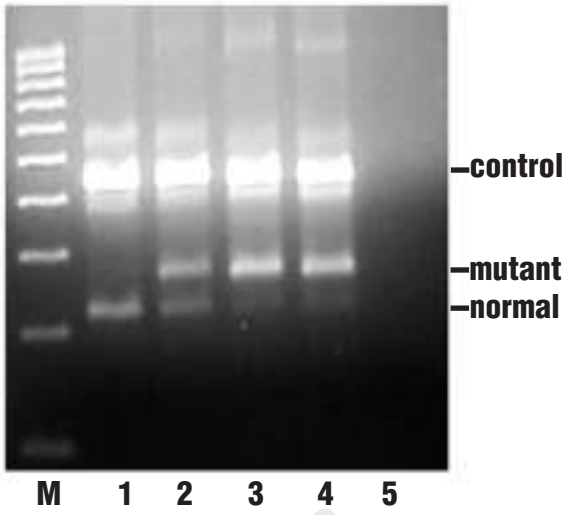

Figure 1. ARMS assay for the detection of the *JAK2* 2343 G→T base change. The wild type allele *JAK2* allele and the mutant *V617F* allele are indicated by the presence of 229bp and 279bp bands. The PCR amplification control band is located at 463bp. The CNL patient, (Lane 4) strongly exhibited the 279bp band and was thus scored as homozygous. Lane 1: homozygous wild type control; Lane 2: heterozygous control; Lane 3: 2343 G→T homozygous control; Lane 5: PCR negative control; M: 1kb DNA ladder (ABgene, Epson, Surrey, UK).

Intriguingly, the *V617F JAK2* mutation has also been described in both *atypical* MPD and myelodysplastic syndromes,[5,10] albeit at a low the incidence. Steensma *et al.*[10] analyzed six CNL patients and only one was found to possess a homozygous *V617F JAK2* mutation. Of particular interest, this patient also had a history of B-cell lymphoma. This was not the case in our patient. Another study[5] indicated that 20% of atypical or unclassified MPD were also positive for the *V617F JAK2* mutation.

We wanted to establish whether our patient possessed the *V617F JAK2* mutation we, therefore collected a blood sample, after gaining informed consent according to the Declaration of Helsinki. The sample was fractionated and genomic DNA isolated using a Nucleon BACC 1 DNA extraction kit (Nucleon Biosciences, Manchester, UK). The 2343 'G to T' *JAK2* mutation (*V617F*) was by detected byan amplification refractory mutation system (ARMS) as described elsewhere[4] except Thermo-Start DNA Polymerase (ABene, Epson, Surrey) was used. ARMS PCR allows the detection of both the wild type *JAK2* allele and the mutant *V617F* allele, as indicated by the presence of 229bp and 279bp bands on a 3% agarose gel (Figure 1). Our patient proved homozygous for the mutation in the granulocytes and macrophages. Sequencing data is shown in Figure 2.

At present, our patient remains symptomatically well and is maintained on hydroxycarbamide (also known as hydroxyurea) 500 mg daily which was commenced because his absolute neutrophil count continued to rise

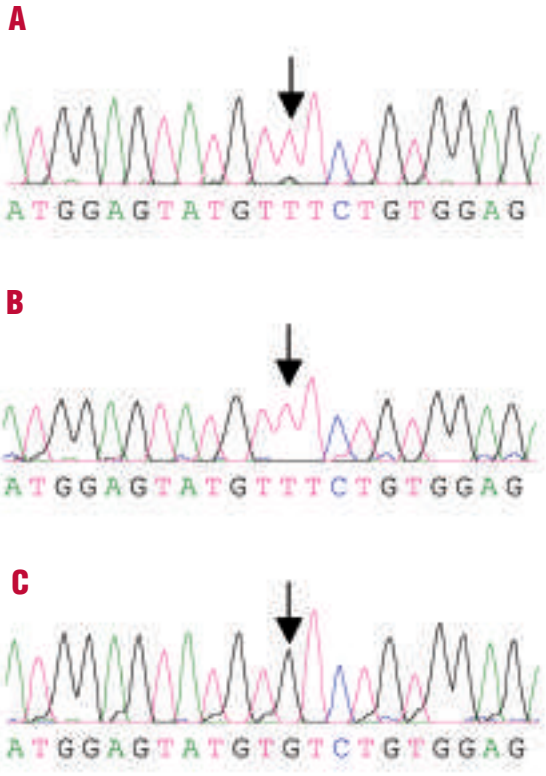

Figure 2. Detection of the G1849T mutation in the JAK2 gene. Sequencing of exon 12 of the JAK2 gene detected a base change of G to T at nucleotide 1849 in unfractionated blood (B), macrophages/granulocytes (C) as compared to wild type sequence (D). Bases are as follows: G: black; A: green; T: red; C: blue.

above 100×10^9/L. His neutrophil count now averages around $30\text{-}45\times10^9$/L. A trial of imatanib mesylate, 400 mg daily, produced no hematologic response.

As mentioned previously, this patient with CNL has had an unusually long survival (now 96+ months). Interestingly, it appears that in other MPD the *V617F JAK2* mutation is associated with a longer median disease duration.[7]

An analysis of all cohorts of patients with CNL will enable an evaluation of the prevalence and prognostic relevance of the *V617F JAK2* mutation in this disease. However, it remains to be established how the presence of a *JAK* mutation, which provides a proliferative advantage, affects the pathogenesis of an atypical MPD.

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Widespread occurrence of the *JAK2* V617F mutation in chronic myeloproliferative disorders

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The analysis of rare chromosomal translocations in myeloproliferative disorders has highlighted the importance of aberrant tyrosine kinase signaling in the pathogenesis of these diseases. Here we have investigated samples from 679 patients and controls for the nonreceptor tyrosine kinase *JAK2* V617F mutation. Of the 480 myeloproliferative disorder (MPD) samples, the proportion of positive cases per disease subtype was 30 (20%) of 152 for atypical or unclassified MPD, 2 of 134 (2%) for idiopathic hypereosinophilic syndrome, 58 of 72 (81%) for polycythemia vera, 24 of 59 (41%) essential thrombocythemia (ET), and 15 of 35 (43%) for idiopathic myelofibrosis. V617F was not identified in patients with systemic mastocytosis (n = 28), chronic or acute myeloid leukemia (n = 35), secondary erythrocytosis (n = 4), or healthy controls (n = 160). Homozygosity for V617F was seen in 43% of mutant samples and was closely correlated with chromosome 9p uniparental disomy. Homozygosity was significantly less common in ET compared with other MPD subtypes. In 53 cases analyzed, the median level of *PRV1* expression was significantly higher in V617F-positive cases compared with cases without the mutation. We conclude that V617F is widespread in MPDs. Detection of this acquired mutation is likely to have a major impact on the way patients with MPD are diagnosed, as well as serving as an obvious target for signal transduction therapy. (Blood. 2005;106:2162-2168)

Introduction

Chronic myeloproliferative diseases (CMPDs) are clonal hematopoietic stem cell disorders characterized by proliferation of one or more myeloid cell lineages in the bone marrow and increased numbers of mature and immature cells in the peripheral blood. CMPDs include polycythemia vera (PV), essential thrombocythemia (ET), idiopathic myelofibrosis (IMF) and chronic myeloid leukemia (CML), plus rarer subtypes such as chronic neutrophilic leukemia (CNL), hypereosinophilic syndrome (HES), and chronic eosinophilic leukemia (CEL). These diseases overlap with myelodysplastic/myeloproliferative diseases (MDS/MPDs) such as atypical CML (aCML) and chronic myelomonocytic leukemia (CMML), in which proliferation is accompanied by dysplastic features or ineffective hematopoiesis in other lineages.[1] We refer here broadly to all these groups as myeloproliferative disorders (MPDs).

Although there are strict diagnostic criteria for MPD subtypes, precise categorization remains a subject of debate[2] and furthermore, it can be difficult to differentiate some cases from reactive disorders. Only CML is characterized by a pathognomonic molecular marker, the *BCR-ABL* fusion, and the primary abnormalities driving excess proliferation in most other cases have been obscure. However, several lines of evidence have implicated aberrant tyrosine kinase signaling as the root cause of MPDs. Breakpoint cluster region–abelson (BCR-ABL) itself is a constitutively active tyrosine kinase that is believed to be the primary, and probably the only, driving force behind chronic-phase CML.[3] Other gene fusions have been identified in rare cases of aCML, CMML, and HES/CEL that involve the tyrosine kinases *PDGFRA*, *PDGFRB*, *FGFR1*, and *JAK2*.[4,5] In addition, the *KIT* receptor is activated by point mutation in the majority of cases of systemic mastocytosis, a disease that is classified separately by the World Health Organisation but which is clearly myeloproliferative in nature.[1,6]

Observations in other MPD subtypes have also implicated aberrant signaling in their pathogenesis, albeit indirectly. Myeloid cells from patients are hypersensitive to a number of growth factors and cytokines, with PV and IMF in particular being characterized by the presence of erythroid and megakaryocytic precursor cells, respectively, that grow spontaneously in culture.[7-10] This factor-independent growth and/or terminal differentiation can be blocked by a range of signal transduction inhibitors.[11,12] Finally, some patients show increased expression of the antiapoptotic factor B-cell leukemia–XL (Bcl-XL) and activation of signal transducer activator of transcription (STAT) 3 or STAT5,[11,13,14] all of which are downstream elements of tyrosine kinase signaling. To investigate the molecular pathogenesis of MPDs we therefore initiated a mutation screen for genes encoding tyrosine kinases and downstream signaling components.

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Patients, materials, and methods

Patients

We studied samples from a total of 679 individuals. Of these, 480 were patients with a known or suspected diagnosis of an MPD (268 males, 212 females) referred for analysis in Athens, Mannheim or Salisbury. Patients were referred for PV (n = 72, including one case who also had systemic mastocytosis), ET (n = 59), IMF (n = 35), idiopathic HES (n = 134), systemic mastocytosis (n = 28), CML-like diseases (aCML, CMML and related atypical MPDs, n = 99) or atypical, unclassified MPD (n = 53). All HES, systemic mastocytosis, CML-like diseases, and atypical, unclassified MPD cases were tested for *BCR-ABL*, *FIP1L1-PDGFRA*, *KIT* D816V (mastocytosis cases only), and other gene fusions as indicated by karyotype. None were *BCR-ABL*–positive, but 24 had rare tyrosine kinase fusion genes involving *PDGFRA*, *PDGFRB*, *FGFR1*, or *JAK2*, and *8* had the *KIT* D816V mutation. We also studied samples from patients with AML (n = 17), BCR-ABL–positive CML (n = 18) plus controls from healthy individuals (n = 160) or individuals with secondary erythrocytosis (n = 4). The study was approved by the Internal Review Boards from participating institutions and informed consent was provided according to the Declaration of Helsinki.

Mutation screening

Initially, we studied a set of 40 atypical, CML-like MPD patients by heteroduplex analysis (either denaturing high-performance liquid chromatography or conformation-sensitive capillary electrophoresis) to screen candidate genes encoding tyrosine kinase and downstream signaling components for mutations. We focused on known mutational hotspots seen in other malignancies or regions that were homologous to mutational hotspots in other genes. We screened *KIT* (exons 10, 11 and 17), *PDGFRA* (exons 12 and 18), *PDGFRB* (exons 12, 17 and 18), *FMS* (exons 7, 9, 12, 13, 18, 21 and 22), *JAK2* (exon 20), *CSK* (exon 11), *FES* (exon 17), *SYK* (exon 11), *STYK1* (exon 8), *TIE1* (exons 18 and 19), *PTPN11* (exons 3 and 13), and *BRAF* (exons 11-14), but no abnormalities were detected apart from known polymorphisms. All exons of *HRAS*, *KRAS*, and *NRAS* were also screened and 5 (12.5%) cases were found to have activating mutations of N-Ras (G12S, n = 2; G12D, n = 1; G12C, n = 1; and G13S, n = 1). Polymerase chain reaction (PCR) primer sequences and assay conditions are available on request. Following the recent report of the *JAK2* V617F mutation (2343G > T; *JAK2* exon 12; RefSeq ID NM_004972[15]) in patients with PV, ET, and IMF,[16-19] we focused specifically on this abnormality.

V617F genotyping by amplification refractory mutation system (ARMS)

DNA was extracted by standard procedures after isolation of total leukocytes from peripheral blood following red cell lysis or mononuclear cells by density gradient centrifugation over Histopaque 1077 (Sigma-Aldrich, Ayrshire, United Kingdom). Patients and controls were genotyped initially by a DNA tetra-primer ARMS assay, a method that uses 2 primer pairs to specifically amplify the normal and mutant sequences plus a positive control band in a single reaction. Primers were designed using a ARMS design program[20] and include mismatches to maximize discrimination of the 2 alleles (shown in lowercase) and mutant/wild-type–specific bases (underlined). PCR primers were: forward outer (FO), 5′-TCCTCAGAACGTTGATGGCAG-3′; reverse outer (RO), 5′-ATTGCTTTC-CTTTTTCACAAGAT-3′; forward wild-type–specific (Fwt), 5′-GCATTTG-GTTTTAAATTATGGAGTATaTG-3′; reverse-mutant–specific (Rmt), 5′-GTTTTACTTACTCTCGTCTCCACAaAA-3′. Amplifications were performed for 30 cycles with HotStar Taq polymerase (Qiagen, Crawley, United Kingdom), an annealing temperature of 60°C, 25 ng genomic DNA, and standard amplification conditions, except that the final concentrations of the outer primers and the mutant/wild-type–specific inner primers were 1 μM and 0.5 μM, respectively. Products were resolved on 3% agarose gels and visualized after staining with ethidium bromide. Control experiments (not shown) indicated that the assay gave identical results using 20 to 200 ng input DNA.

V617F genotyping and allele quantitation by Pyrosequencing

DNA samples were amplified using primers 5′-biotin-GAAGCAGCAAG-TATGATGAGCA-3′ (forward; *JAK2* exon 12) and 5′-TGCTCT-GAGAAAGGCATTAGAA-3′ (reverse; *JAK2* intron 12). Amplicons were generated in a 50 μL reaction volume with 15 pmol of forward and reverse PCR primers, 0.2mM dNTPs (deoxyribonucleoside triphosphates; Promega, Southampton, United Kingdom), 1.5 mM $MgCl_2$, 1 ×Buffer II (Applied Biosystems, Warrington, Cheshire, United Kingdom), 1 unit AmpliTaq Gold (Applied Biosystems) using 10 ng genomic DNA. PCR conditions were 94°C for 7 minutes; 50 cycles with denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and elongation at 72°C for 30 seconds; 1 cycle at 72°C for 7 minutes; and a final hold at 15°C. Single-stranded biotinylated PCR products were prepared for sequencing using the Pyrosequencing Vacuum Prep Tool (Biotage, Uppsala, Sweden). Three microliters Streptavidin Sepharose HP (Amersham Biosciences, Chalfont St Giles, United Kingdom) was added to 37 μL Binding buffer (10 mM Tris [tris(hydroxymethyl)aminomethane]–HCl, pH 7.6; 2 M NaCl; 1 mM EDTA [ethylenediaminetetraacetic acid]; 0.1% Tween 20) and mixed with 20 μL PCR product and 20 μL high-purity water for 10 minutes at room temperature using a Variomag Monoshaker (Camlab, Over, United Kingdom). The beads containing the immobilized templates were captured onto the filter probes after applying the vacuum and then washed with 70% ethanol for 5 seconds, denaturation solution (0.2 M NaOH) for 5 seconds, and washing buffer (10 mM Tris-acetate, pH 7.6) for 5 seconds. The vacuum was then released and the beads released into a PSQ 96 Plate Low (Biotage) containing 45 μL annealing buffer (20 mM Tris-acetate, 2 mM magnesium acetate, pH 7.6), 0.3 μM sequencing primer (5′-TCTCGTCTC-CACAGA-3′; *JAK2* exon 12, reverse orientation). The samples were heated to 80°C for 2 minutes and then allowed to cool to room temperature. Pyrosequencing reactions were performed according to the manufacturer's instructions using the PSQ 96 single nucleotide polymorphism (SNP) Reagent Kit (Biotage), which contained the enzyme and substrate mixture and nucleotides. The sample genotypes were determined using the Allele Frequency Quantification function in the SNP Software (Biotage, Uppsala, Sweden). Samples were scored as homozygous if the proportion of the mutant allele was greater than 50%, the maximum expected if a heterozygous mutant clone had expanded to include all cells in the sample. In practice this is likely to underestimate the number of homozygotes since our samples included lymphocytes (a proportion of which are probably not part of the mutant clone).

Quantification of *PRV1* mRNA

Mononuclear cell RNA was extracted and reverse transcribed into cDNA with random hexamer primers using standard procedures. Real-time quantitative (RQ)–PCR was performed in 12.5 μL mastermix (TaqMan Universal PCR Master Mix, Applied Biosystems); using 0.25 μM PRV1-TM probe (5′-FAM[6-carboxy fluorescein]–TTGTCTGGTGTGGT*TCAACAAGAAGCT-3′, where * = 6-carboxy-tetramethylrhodamine [TAMRA]), 0.5 μM of each 3′ and 5′ oligonucleotide primers (PRV1-se 5′-CTCTCAGGAGGTGGGCTGT-3′ and PRV1-as 5′-GCGCAGAGAAGATCCCGA-3′ respectively), 5 μL cDNA in a final volume of 25 μL. Amplification was performed in a 2-step cycle (denaturation, 95°C for 15 seconds, annealing/extension at 62°C for 60 seconds) for 50 cycles. Amplification and postprocessing calculations were performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The values obtained were normalized using the *ABL* gene as endogenous control[21] using the deltadeltaCt method.[22]

Microsatellite analysis

DNA samples were amplified with a series of fluorescently labeled primer pairs flanking highly polymorphic microsatellite markers on chromosome 9p (D9S1779, D9S1858, D9S288, D9S1813, D9S286, D9S254, D9S157, D9S171). Standard conditions were used with 25 to 50 ng input genomic DNA, a final volume of 10 μL, 32 amplification cycles, and 55°C annealing temperature. Products were analyzed on an ABI 3100 genetic analyser using the Genotyper 2.0 program (Applied Biosystems, Foster City, CA). Peak heights were compared with controls and scored as heterozygous for each marker if there were 2 clear peaks of similar intensity, or the ratio of

the 2 peaks were similar to healthy controls. Patients were scored as homozygous if only one peak was visible or, to allow for the presence of background normal cells, if one peak was one third or smaller than the expected size compared with controls. Uniparental disomy (UPD) was scored if 4 or more consecutive markers abutting or encompassing *JAK2* were homozgygous ($P < .05$ of this occurring in the absence of UPD based on published rates of heterozygosity[23]).

Copy number determination by multiplex ligation probe amplification (MLPA)

MLPA primers were designed to nonpolymorphic exons of *FES* (15q26), *JAK2* (9p24), *SOAT1* (1q25), *BTK* (Xq22), *TYK2* (19p13), *MST1R/RON* (3p21), and *HCK* (20q11), and obtained from Biomers (Ulm, Germany). Primers were between 43 and 69 bases in length and consisted of M13 tags, a random stuffer sequence of variable length in order to generate gene-specific products of different sizes and 20 bases homologous to the target of interest (primer sequences available on request). Primers were designed in pairs that would abut each other when hybridized to genomic DNA, and the right-hand primer in each pair was synthesised with a 5′phosphate group to enable ligation to the left oligonucleotide. Hybridization of probe pairs to genomic DNA, ligation, and amplification was performed as described,[24] with the PCR products being separated on an ABI 3100 genetic analyzer. Peak areas were exported to an Excel spreadsheet (Microsoft, Redmond, WA), which was designed to assess the ratios of each test peak relative to all other peaks for that individual. Ratios of the *JAK2* peak to other peaks in each patient sample were compared with the same ratios obtained for 2 healthy individuals, which were included in each run. For normal sequences a dosage quotient of 1.0 is expected; if a deletion or duplication is present the dosage quotient should be 0.5 and 1.5, respectively.[25] *BTK* is on the X-chromosome and therefore provided an internal control that the assay was working (dosage quotient of 0.5 in males and 1.0 in females).

Statistical analysis

Observed and expected frequencies were compared by χ^2 analysis. Mutation status was compared with *PRV1* mRNA levels using the Mann-Whitney test.

Results

Incidence of V617F *JAK2*

Of the 480 samples with a known or suspected diagnosis of an MPD, 129 (27%) were positive for the V617F *JAK2* mutation by the ARMS assay and 351 (73%) were negative. Representative results are shown on Figure 1A. Sequence analysis of selected cases (n = 51) was fully concordant with the ARMS results (Figure 2A), although in some cases the proportion of the mutant allele was low and would have been difficult or impossible to detect by sequence analysis alone. Analysis of a dilution series indicated the sensitivity of the ARMS assay to be 1% to 2% (Figure 1B), whereas sequencing is generally accepted to have a sensitivity of only 20% to 30%, depending on the sequence context.

Using the ARMS assay, we found that the proportion of positive cases per disease subtype ranged from 58 (81%) of 72 for PV to 0 (0%) of 28 for mastocytosis (Table 1). Strikingly, V617F was detected in a substantial proportion of patients with CML-like diseases and atypical, unclassified MPD. V617F was not detected in any of the 480 MPD cases with rare tyrosine kinase fusion genes (n = 24), nor in any individual with CML (n = 18), AML (n = 17), or in healthy controls (n = 160). V617F and *KIT* D816V were found in one individual who had both PV and systemic mastocytosis, but not in any of the other 7 D816V-positive cases. Of the 14 V617F mutation–negative patients with PV, 13 were male ($P = .005$, χ^2) but no other significant associations between sex and mutation status were identified. Mutation analysis of *JAK2* exon 12 in V617F-negative cases did not reveal any additional sequence variants.

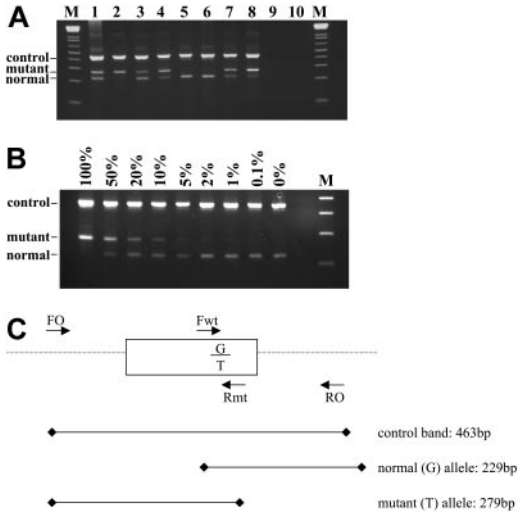

Figure 1. ARMS assay to detect the *JAK2* 2343G > T in genomic DNA. (A) Representative results. Tracks 5 and 6 show a normal genotype; tracks 1 and 3 show a mutant band that is weaker or similar intensity to the normal band and were therefore scored as heterozygous for the 2343G > T mutation; tracks 2, 4, 7, and 8 show a mutant band that is stronger than the normal band and were therefore scored as homozygous for the mutation. Tracks 9 and 10 are negative controls and M is the 1-kilobase (kb)+ DNA ladder (Invitrogen, Paisley, United Kingdom). (B) Sensitivity of the ARMS assay. DNA from a homozygous patient with minimal residual wild-type allele was diluted with normal DNA and amplified. The ARMS assay is routinely capable of detecting V617F at a dilution of 1% to 2%. (C) Schematic outline of the assay. Primers FO and RO flank *JAK2* exon 12 and should generate a control 463-bp band in all cases. Primers Fwt and RO generate a 229-bp wild-type (2343G)–specific product and primers FO and Rmt generate a 279-bp mutant (2343T)–specific product.

Relative ratios of V617F and normal *JAK2* alleles

The ARMS and sequencing results for several patients showed mutant bands much stronger than the wild-type bands (Figures 1A and 2A) and in some cases the wild-type band was not visible at all, suggesting that the mutation may be homozygous in the mutant clone. Since ARMS and sequencing results are not always quantitative, we designed a Pyrosequencing assay for the V617F mutation, as this method is capable of providing robust allele ratios.[26] Pyrosequencing was performed on the 90 mutant samples for which DNA was available, and confirmed homozygosity in most of the samples with a stronger mutant band by ARMS analysis (Figure 2B). Samples analyzed by ARMS only were therefore scored as homozygous if the ratio of mutant to wild-type bands was at least as strong as that seen in the 50% dilution control (Figure 1B, 50% track), as determined by visual inspection. Overall, homozygosity was seen in 55 (43%) of 129 mutant samples and results for each patient subgroup are summarized on Table 1. The frequency of homozygotes relative to heterozygotes was not significantly different from the average of all cases in any of the subgroups apart from ET, in which the proportion of homozygous mutants was significantly lower than average ($P = .009$, χ^2). We found evidence of a residual wild-type signal in all but 2 homozygous cases by Pyrosequencing, with dilution experiments (not shown) indicating that the sensitivity of the assay was approximately 5%. Pyrosequence analysis of 23 cases that were normal genotype by the ARMS test yielded normal (100% wild-type allele) in all cases.

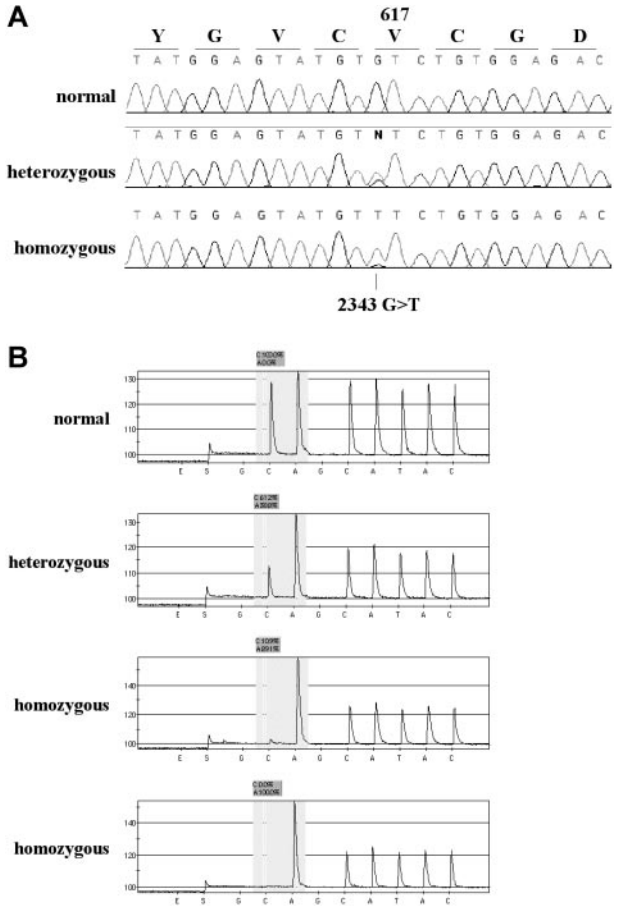

Figure 2. Detection of the *JAK2* by sequence analysis, and Pyrosequencing. (A) Sequence traces of 3 individuals found to be wild-type, heterozygous, and homozygous by the ARMS assay. (B) Representative pyrograms for one wild-type, one heterozygous, and 2 homozygous individuals. The sequencing primer is in reverse orientation (immediately abutting the site of the mutation) and the dispensation order GCAGCATAC was used (the 2 Gs are internal controls that should give no peak; E and S indicate enzyme and substrate, respectively). In a wild-type individual (sequence CACATAC) the peak heights of the first C and A are similar. In a heterozygous individual (sequence (C/A)ACATAC) the C peak is reduced in height and the A peak increased above all other peaks as both the mutant A and following A are being read in the same reaction. In a homozygous individual (sequence AACATAC) the first C peak is either much lower than the A peak (third trace) or, occasionally, absent (fourth trace). The ratio of the healthy (C) and mutant (A) alleles is calculated by the Pyrosequencing SNP analysis software. Values are $\log_{10}$ delta-delta Ct*PRV1/ABL*.

V617F and chromosome 9p uniparental disomy

A homozygous V617F clone was seen in 24 (33%) of 72 cases of PV, 4 (7%) of 59 cases of ET, and 10 (29%) of 35 cases of IMF. This is strikingly similar to the frequencies of acquired chromosome 9p UPD (autosomal regions for which both copies are derived from a single parent) that has been described in these diseases. Furthermore, the minimal disomic region contains *JAK2*.[27,28] To determine if UPD9 was associated with homozygosity for V617F, we initially performed microsatellite analysis on 57 MPD cases (wild type, n = 30; V617F homozygous, n = 27). Microsatellites were scored as homozygous or heterozygous and the results are summarized on Figure 3. In wild type individuals, most microsatellites are expected to be heterozygous. Consecutive tracts of homozygous markers usually indicate loss of heterozygosity (LOH) due to complete or partial chromosome loss, or UPD. We found significant tracts of homozygosity in the vicinity of *JAK2* in 4 (13%) of the wild type cases and 25 (93%) of the V617F homozygotes ($P < .001$). To determine whether this homozygosity arose through LOH or UPD, we measured the number of copies of the *JAK2* gene relative to control genes by MLPA. One copy of *JAK2* would indicate LOH, whereas 2 copies would indicate UPD. All 7 cases analyzed that were homozygous for both V617F and at least 3 consecutive 9p markers had 2 copies of *JAK2*, consistent with UPD9 (Figure 4A).

Correlation between the presence of V617F and overexpression of *PRV1*

Overexpression of the *PRV1* gene has been reported in the great majority of patients with PV and a subset of patients with ET and IMF.[29-32] Although the mechanism of overexpression is not known, many groups consider quantification of *PRV1* mRNA to be a useful supplementary test in the diagnosis of MPDs. To determine if *PRV1* expression is associated with abnormalities of *JAK2*, we compared mRNA levels to mutational status in 53 cases (PV, n = 15; ET, n = 32; IMF, n = 6). Although there was considerable overlap between the range of *PRV1* levels in normal *JAK2* (n = 30, median = 293, range = 6-17 500) and V617F cases (n = 23, median = 2700, range = 59-208 000), the difference between the 2 groups was significant ($P < .001$, Mann-Whitney test). The median *PRV1* level for homozygous V617F cases (median = 4940, n = 11) was greater than the median for heterozygous cases (median = 1650, n = 12), but the difference was not significant ($P = .1$, Mann-Whitney). Results are summarized in Figure 4B.

Table 1. Patient details and summary of results

Disease subtype	No. patients	V617F-positive			V617F-negative			V617F homozygotes no. (% of mutants)
		No. patients (%)	No. male/no. female	Median age, y (range)	No. patients (%)	No. male/no. female	Median age, y (range)	
PV	72*	58 (81)	31/27	57 (26-76)	14 (19)	13/1	57 (33-78)	24 (41)
ET	59	24 (41)	15/9	65 (5-88)	35 (59)	23/12	56 (24-88)	4 (17)
IMF	35	15 (43)	10/5	65 (44-78)	20 (57)	15/5	62 (41-92)	10 (67)
Idiopathic HES	134†	2 (1.5)	1/1	64 (63-65)	132 (99)	51/81	54 (3-89)	2 (100)
Mastocytosis	28‡	0 (0)	NA	NA	28 (100)	16/12	53 (4-90)	NA
CML-like MPDs	99§	17 (17)	13/4	62 (17-76)	82 (83)	60/22	66 (2-95)	8 (47)
Unclassified MPD	53	13 (25)	7/6	63 (17-77)	40 (75)	17/23	61 (21-87)	7 (54)
Total	480	129 (27)	77/52	58 (2-95)	351 (73)	195/156	62 (5-88)	55 (43)

NA indicates not applicable.

*Includes one case with PV plus systemic mastocytosis who was positive for V617F and *KIT* D816V.

†Includes 7 *FIP1L1-PDGFRA*-positive CEL cases, all of whom were negative for V617F.

‡Includes 7 *KIT* D816V-positive cases, all of whom were negative for V617F.

§Includes 17 cases with rare tyrosine kinase fusions (*ETV6-PDGFRB,* n = 9; *ZNF198-FGFR1,* n = 2; *BCR-FGFR1,* n = 2; *PCM1-JAK2,* n = 2; *BCR-PDGFRA,* n = 1; *TP53BP1-PDGFRB,* n = 1), all of whom were negative for V617F.

Patient	V617F	D9S1779	D9S1858	D9S288	D9S1813	JAK2 D9S286	D9S254	D9S157	D9S171	LOH/UPD
1	WT/WT									NO
2	WT/WT									NO
3	WT/WT									NO
4	WT/WT									NO
5	WT/WT									NO
6	WT/WT									YES
7	WT/WT									NO
8	WT/WT									NO
9	WT/WT									NO
10	WT/WT									NO
11	WT/WT									NO
12	WT/WT									NO
13	WT/WT									NO
14	WT/WT									NO
15	WT/WT									NO
16	WT/WT									NO
17	WT/WT									YES
18	WT/WT									NO
19	WT/WT									NO
20	WT/WT									NO
21	WT/WT									NO
22	WT/WT									NO
23	WT/WT									NO
24	WT/WT									YES
25	WT/WT									NO
26	WT/WT									NO
27	WT/WT									NO
28	WT/WT									NO
29	WT/WT									YES
30	WT/WT									NO
31	M/M									YES
32	M/M									YES
33	M/M									YES
34	M/M									YES
35	M/M									YES
36	M/M									YES
37	M/M									YES
38	M/M									YES
39	M/M									NO
40	M/M									YES
41	M/M									YES
42	M/M									YES
43	M/M									YES
44	M/M									YES
45	M/M									YES
46	M/M									YES
47	M/M									YES
48	M/M									YES
49	M/M									YES
50	M/M									YES
51	M/M									YES
52	M/M									YES
53	M/M									YES
54	M/M									YES
55	M/M									YES
56	M/M									YES
57	M/M									YES

Figure 3. Microsatellite analysis to identify regions of homozygosity. The V617F genotype of the patients is indicated as either homozygous normal (WT/WT; patients 1-30) or homozygous mutant (M/M; patients 31-57). Microsatellite markers on chromosome 9p are shown in order from the most telomeric (D9S1779) to the most centromeric (D9S171) and are scored as homozygous (black), heterozygous (white), or not done (gray). Based on published rates of heterozygosity,[23] significant tracts of chromosome 9p homozygosity (LOH/UPD) were scored if 4 consecutive markers above or below *JAK2* were homozygous.

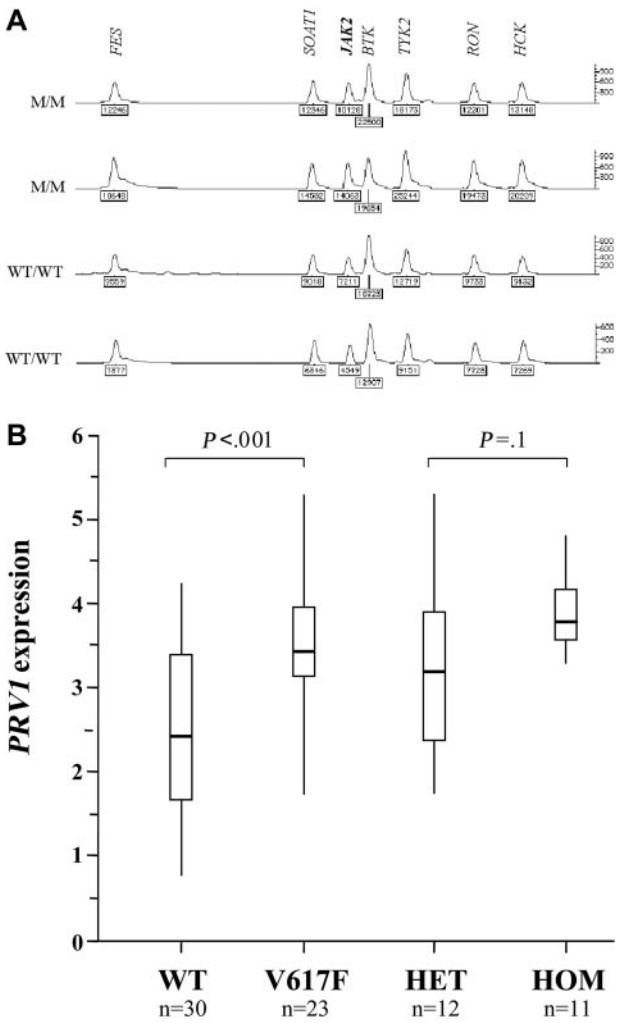

Figure 4. *JAK2* copy number and correlation of status with *PRV1* expression. (A) Representative results of MLPA analysis to measure *JAK2* copy number. The relative peak areas for *JAK2* are the same for 2 homozygous V617F cases with 9p LOH (M/M) and 2 healthy controls (WT/WT), indicating the presence of 2 copies of *JAK2*. The second case is male and shows a reduced peak height for the X-linked gene *BTK* compared with the 3 other cases, all of which were female. (B) *PRV1* mRNA levels ($\log_{10}$deltadeltaCt*PRV1*/*ABL*) determined by real-time PCR in 30 MPD patients with normal *JAK2* (WT) and 23 MPD patients with the V617F mutation. These 23 patients are also shown split into the 12 heterozygous (HET) and 11 homozygous (HOM) cases. Vertical lines indicate the range of results, open boxes indicate the interquartile range, and thick horizontal lines indicate median values.

Discussion

We have identified the *JAK2* V617F mutation in approximately one-fifth of patients with an atypical MPD, as well as confirming the presence of this mutation in the great majority of patients with PV and nearly half of cases with ET or IMF. The finding of an identical mutation in such clinically diverse (although clearly related) cases is quite remarkable. There is nothing in the sequence context to suggest that position 2343G might be particularly mutable and, since no other mutations were found in *JAK2* exon 12, it seems likely that the V617F substitution must have very specific functional consequences. The mutation occurs in a highly conserved region of the pseudokinase (JH2) domain, a region that is homologous to the true tyrosine kinase domain but lacks key catalytic residues, and has been shown to constitutively activate JAK2.[16,17,19] The pseudokinase domain is

believed to negatively regulate JAK2 signaling by direct interaction with the kinase domain.[33] Structural modeling has suggested that residues V617 to E621 form a loop connecting 2 β-strands of the N-terminal lobe of the pseudokinase domain, with C618 contacting the kinase activation loop. It has been postulated that V617, C618, and other local residues inhibit movement of the activation loop from its inactive to its active conformation (ie, the V617 region plays a direct role in negatively regulating JAK2 signaling).[34] The substitution of V617 by the large aromatic amino acid phenylalanine is likely to disrupt this negative regulation, although this remains to be proven biochemically. However, evidence that amino acid substitutions in the pseudokinase domain can activate *JAK2* comes from the *Drosophilia* mutation hopscotch, in which a mutation corresponding to E695K in the human protein results in hematopoietic hyperplasia.[35] In addition, JAK2 has been directly implicated in human malignancy, first by fusion to *TEL/ETV6* and *PCM1* as a consequence of chromosome translocations in leukemia[36-38] and, second, by aberrant signaling in a wide range of solid tumors.[39]

JAK2 is a nonreceptor tyrosine kinase that plays a major role in myeloid development by transducing signals from diverse cytokines and growth factor receptors, including those for interleukin (IL)–3, IL-5, erythropoietin, granulocyte-macrophage–colony-stimulating factor (GM-CSF), G-CSF, and thrombopoietin.[39,40] It seems likely that a mutation which interferes with the negative regulation of JAK2 could account for the observed hypersensitivity of myeloid cells from MPD patients to growth factors. However it is not clear why different individuals with V617F show preferential expansion of erythroid, granulocyte, megakaryocyte, monocyte, or eosinophil lineages. Potentially, this could be due to the identity of the cell in which the mutation arises, the constitutional genetic background of the individual, or to other, secondary acquired changes.

In addition to secondary or constitutional differences, V617F itself may also play a role in specifying disease phenotype since we found homozygosity to be significantly less common in ET compared with other MPD subtypes. However, definitive identification of V617F zygosity in individual cases is complicated by the fact that it is not possible to distinguish between a relatively small homozygous clone and a larger heterozygous clone when mixed populations of cells are analyzed. Nevertheless, we found a wide range (5%-100%) in the relative proportion of mutant alleles in V617F-positive cases by Pyrosequence analysis. The fact that (1) the mutation was present at a level substantially less than the wild-type allele in many heterozygous cases, (2) the wild-type allele was detectable in the great majority of homozygous patients, and (3) the age of homozygous cases (median, 63 years; range, 17-69 years) was not significantly different from heterozygous cases (median, 62 years; range, 5-88 years) strongly suggests that the mutation was acquired. Other studies have shown that V617F is absent in T cells and the great majority of buccal epithelial samples, clearly indicating that the mutation was acquired in these individuals.[16-19] However, we cannot exclude the possibility that V617F might be inherited in occasional cases, although this seems unlikely as linkage to chromosome 9p has been excluded in MPD families.[30,41]

Although V617F accounts for an important subset of cases with CML-like diseases and atypical MPDs, the pathogenesis of the majority of these cases remains unknown. In our original series of 40 CML-like cases, only one-third of those that were negative for rare tyrosine kinase fusion genes were found to have the V617F *JAK2* mutation or an activating *NRAS* mutation (no case had a *JAK2* and *RAS* mutation). PV and IMF are both considered to be clonal diseases, suggesting that the molecular basis of roughly 20% and 60%, respectively, of these diseases remain to be elucidated. However, these figures may be overestimates since it is likely that some of the cases in our study had an unrecognized reactive condition. It is known that the clinical diagnostic precision varies between clinicians and it is possible therefore that the true incidence of V617F in PV and IMF is somewhat higher than we found in this study.[42] On the other hand, it is likely that technical differences relating to the sensitivity of V617F detection are also a contributory factor to the published differences in the proportions of positive cases within MPD subtypes. As for ET, approximately half of cases have clonal disease and half are polyclonal, and therefore presumably reactive.[43] Although we did not perform clonality assays, we found the V617F mutation in 41% of ET cases, suggesting that the great majority of clonal ET is V617F positive. We did not observe V617F in patients with CML or AML; however, the numbers of cases analyzed were relatively small and therefore we cannot exclude the possibility that a small subset of cases might carry this mutation. Interestingly, 6 of the patients in our series had chronic neutrophilic leukemia, 2 of which were V617F positive.

We found that homozygosity for V617F in all disease subtypes was closely associated with chromosome 9 UPD, confirming previous results and suggesting a selective advantage for 2 copies of mutant *JAK2*.[17] We also found significant 9p homozygosity in 4 MPD cases without V617F, which could indicate a mutation in a different region of *JAK2*. Although gain of chromosome 9p is known to be associated with MPDs,[44-46] UPD for this region is much more common. Recently, UPD for diverse chromosomal regions has been identified in acute leukemia[47,48] and, although most of these regions have not yet been associated with specific acquired mutations, it is possible that reduction of oncogenic mutations to homozygosity by cytogenetically cryptic mitotic recombination is widespread in malignancy.

The fact that V617F was found in the great majority of cells and was homozygous in many cases suggests that it is probably the primary abnormality driving myeloproliferation. As such, it is clearly a very attractive target for signal transduction therapy with small molecule inhibitors, although at the time of writing no clinically available JAK2 inhibitors have been described. JAK family members play a crucial role in the immune system; for example, inherited JAK3 deficiency causes severe combined imunodeficiency,[49,50] and JAK2 also plays an important role in cardiovascular signaling systems.[51] Development of inhibitors that inhibit V617F without undesirable side effects may therefore be challenging.

Diagnosis of MPDs is often complex, expensive and in the case of ET, based solely on exclusion criteria. Since the detection of acquired V617F is simple to perform and unambiguously establishes the presence of a clonal disorder, we believe that *JAK2* mutation testing will rapidly become a frontline test for individuals with a suspected diagnosis of an MPD.

Acknowledgment

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