UNIVERSITY OF SOUTHAMPTON

FACULTY OF HEALTH SCIENCES

School of Medicine

Novel insights into the genomic architecture of Chronic Lymphocytic Leukaemia

by

Miqdad RAJABALI

Thesis for the degree of Master of Philosophy

June 2011

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF HEALTH SCIENCES
SCHOOL OF MEDICINE
Master of Philosophy

NOVEL INSIGHTS INTO THE GENOMIC ARCHITECTURE OF CHRONIC LYMPHOCYTIC LEUKAEMIA

Miqdad Rajabali

Sequential karyotypic and FISH analysis in patients with CLL show evidence of genomic evolution in 10 – 20% of cases. In addition, telomere dysfunction has been shown to correlate with genomic instability and thus may be involved in genomic evolution. Since specific deletions have been found to be associated with different disease prognosis, the acquisition of secondary aberrations may impact on disease progression. To investigate this, DNA samples of 29 patients presenting with either stage A0 CLL or mBL were analysed at two different time points, PT and FU (median time between sample: 85 months. Patients were grouped as either stable (for 5 or more years) or progressive (treated within 3 years). High resolution Affymetrix SNP6.0 array was used to investigate copy number aberrations and loss of heterozygosity at both time points and FISH/karyotype data was used for confirmation and validation.. In addition, the high resolution STELA technique was used to measure telomere length at each time point. It was found that patients with progressive as well as stable disease had acquired secondary aberrations at FU, including novel deletions and gains. Telomere loss was also found in both groups of patients. 11q/17p aberrations and large (>2Mb) but not small (<2Mb) 13q deletion (P=0.03) were associated with genomic evolution and telomere loss. Several established biomarkers, including IgVH mutational status shown not such correlation. A number of patients identified as good risk at diagnosis, acquired abverse genomic features at folloe-up. These included the acquisition of a class II 13q deletion and a complex genomic profile. Finally, case studies enabled a more detailed analysis and revealed the presence of secondary aberrations in conjunction with sudden shifts in WBC count that are a marker of disease progression. This study supports the need to detect genomic changes throught the course of the disease, and genomic aberrations can be acquired and effect prognosis.

Table of Contents

DECLARATION OF AUTHORSHIP

I, Miqdad Rajabali declare that the thesis entitled “Novel insights into the genomic architecture of chronic lymphocytic leukaemia” and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where I have consulted the published work of others, this is always clearly attributed;
- 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;
- I have acknowledged all main sources of help;
- 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;
- none of this work has been published before submission

Signed: ……

Date:……

Acknowledgements

In the name of God, the Most Kind, the Most Merciful. All praise belongs to God, the Lord of the worlds, the Creator and Sustainer.

This work has been made possible with the tremendous help from my supervisor, Dr. Jon Strefford as well as the entire team; Dr Helen Parker and Dr Matt Rose-Zerilli. I heartily thank them for all their input.

Many thanks also go to Dr David Oscier, at the Royal Bournemouth Hospital, and his team, specifically Anton Parker for his help in the WBC count data and Anne Gardiner for the FISH data.

Many thanks also go to Dr Duncan Baird for his data on telomere length and for his feedback on my first draft of chapter II.

Finally, I owe everything to my parents who have pushed me to achieve this and my wife for her patience and enthusiasm.

Abbreviations

ABL1 - Abelson tyrosine kinase 1 gene
aCGH – comparative genome hybridisation array
AIHA - Autoimmune haemolytic anaemia
ALL – Acute Lymphocytic leukeamia
AML - Acute Myelogenous Leukemia
ATM – Ataxia telangiectasia mutated gene
Bcl-2 – B-Cell lymphoma 2 gene
BCR – B cell receptor gene
CD38 – Cluster of differentiation 38 protein
CLL – Chronic Lymphocytic leukeamia
CML – Chronic myelogenous leukaemia
CMV – cytomegalovirus infection
CNA – Copy number aberration
CNV – Copy number variable
DLEU2 – Deleted in Leukeamia 2 gene
DNA – Deoxyribonucleic acid
FISH - Fluorescence in situ hybridisation
FOXO – Forkhead box gene
FU – Follow up time point
GC – Germina centre
GSTT1 - Glutathione S-transferase theta-1 gene
IGH – Immunoglobuline heavy chain gene
IgVH – Immunoglobuline heavy chain variable region locus
Kb – Kilobase
LOH – Loss of heterozygosity
Mb - Megabase
mBL - Monoclonal B-cell lymphocytosis
MDR - Minimal Deleted Region
miR-15a – Micro-RNA 15a
miR16-1 – Micro RNA 16-1
Mycn - N-myc myelocytomatosis viral related oncogene
PCR – Polymerase chain reaction
PT – Presentation time point
Rb (RB1) – Retinoblastoma gene
RBH – Royal Bournemouth Hospital
RNA – Ribonucleic acid
SNP – Single-nucleotide polymorphism
SNRPN - Small nuclear ribonucleoprotein-associated protein N gene

SOD1 - Superoxide dismutase 1 gene

SRC – sarcoma gene

STELA - Single telomere length analysis

SYK – Spleen tyrosine kinase gene

TP53 (P53) – Tumour protein 53 gene

TRF - Terminal Restriction fragment

TRPM1 - Transient receptor potential cation channel subfamily M member 1 gene

TTFT – Time to first treatment

WBC – White blood cell count

ZAP70 - Zeta-chain-associated protein kinase 70 protein

1. General Introduction

1.1. DNA, Chromosomes and cell division

Deoxiribonucleic acid, DNA, is the blueprint of all cells, containing all the genetic information, coded by 4 different nucleotides attached together in a double strand helix structure. Packed into chromosomes, the human genome contains more than 3 billion base pairs of which less than 2% code for genes. Genes are nucleotide sequences in the DNA which can be transcribed as RNA by RNA Polymerase and then translated as proteins by Ribosomes outside of the cell nucleus. Genes are made of exons which are the coding regions and introns which are regions between the exons. Gene expression is tightly regulated by a number of mechanism; transcription factors promote or silence the transcription of gene by binding to specific sequences of the DNA, upstream of the gene (promoters or silencer regions), which they can recognise. Another mechanism for gene expression involves the packing of DNA, which, when tightly bound to histones proteins, restricts gene expression.
DNA and chromatin are packed in structures called chromosomes. Each somatic cell has 2 copies of each of the 22 autosomal chromosomes and either two copies of the X sex chromosome (female) or one copy each of the X and Y sex chromosome (Male). Each chromosome is composed of two identical chromatids bound together at the centromere, as shown in figure 1. Hence, each gene has two copies, or two alleles, on each homologous chromosome. During cell division, called mitosis, chromosomes are duplicated and divided to produce 2 identical cells.

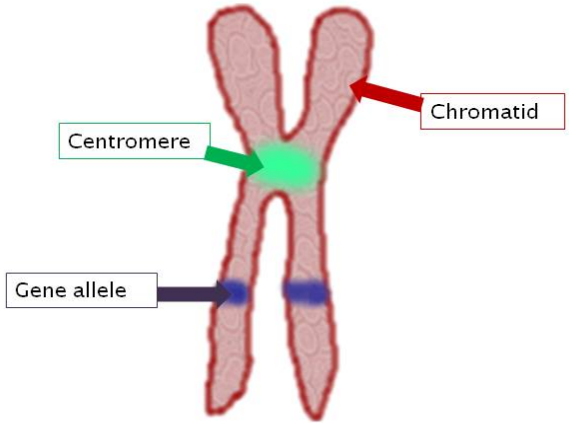


Figure 1: Picture of a chromosome, showing the centromere and two copies of a gene

Cell cycle can be divided into two different phases: Interphase and mitosis. Interphase occurs in three stages: G_1 phase, where the cell begins to grow, S phase where the DNA and chromosome replicate and the G_2 phase, which prepares to cell entry into

mitosis. This latter phase is divided into four stages: prophase, where the DNA is condensely packed and structured, metaphase, where the chromosomes are moved to the centre of the cell and centromeres are lined up, anaphase, where the centromeres of each chromosome are broken and moved to opposite sides of the cell, and telophase, where the cells are divided into two, each with one pair of chromosomes.

Cells division is tightly regulated by a number of proteins, such as cyclins (Vermeulen, Van Bockstaele et al. 2003). Undividing cells enter the G_0 phase of senescence (summarised in figure 2).

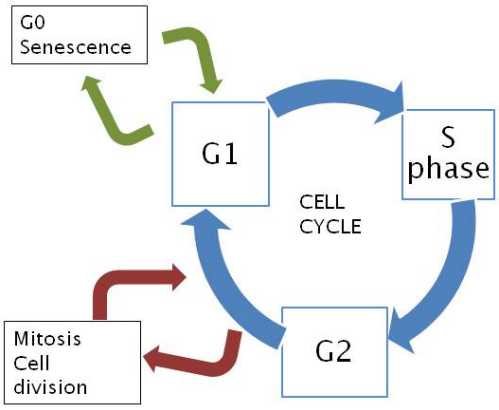


Figure 2: The difference phases of cell cycle include S, G_1 and G_2. Following G_2, cells undergo mitosis or division. Cell cycle arrest can occur and cells enter G_0 senescence phase.

During cell division, the DNA replicates into two identical copies. The replication is initiated by Helicases which separate the two DNA strand and subsequently DNA polymerase uses each strand of the DNA as a template to synthesise a complementray strand. DNA replication does not reach the end of chromosomes due to the presence of telomeres which consist of tandem repeats of TTAGGG sequence, which cannot be replicated by the Polymerase. As DNA gets shorter after every cycle, cells can only divide a certain number of times (Hayflick limit: 40 to 60 times), after which cells enter into senescence (Norrback and Roos 1997).

Errors, such as deletion or addition of a nucleotide, during replication can occur, but are repaired by various mechanisms during a cell cycle pause triggered by DNA damage checkpoints. These checkpoints are controlled by proteins such as *ATM*, which respond to double stranded breaks. If DNA damage is irreparable, signals are triggered downstream of *ATM*, such as P53, to cause cell death. Apoptosis is one of the mechanism by which DNA as well as cell components are fragmented and the entire cells are removed from the system.

1.2. Cancer and genetic alterations

Cancer is a general term used for a group of diseases characterised by an uncontrolled growth of cells. This is due to a malfunction of cell division, which may affect any area of the human body. In Europe alone, 3 million new cases of malignant tumours are recorded every year and worldwide, cancer accounts for 1.7 million deaths per year (WHO 2004)

A number of alterations to the genome can cause cell cycle dysfunction (Evan and Vousden 2001). Alterations involve epigenetic changes (such as methylation), mutations, loss of heterozygosity (LOH), deletions of gains of region of the chromosome, which can target regions of any chromosome or whole chromosomes (monosomy & trisomy) as well as translocations. LOH can be a random event whereby an individual acquires both alleles of a gene (or a region of chromosome) from one parent and not the other (termed as 'Uniparental disomy') or it can be the loss of the only functional allele for a particular gene or chromosome region which targets mostly tumour suppressor genes. LOH is a result of error in recombination following mutation in the first allele, and does not cause loss of copy number as shown in figure 3 (Gondek, Tiu et al. 2008). Translocations involve the swapping of highly homologous parts of the DNA sequence during replication which cause loss of the correct gene sequence and can be sometimes accompanied with loss of chromosomes regions (unbalanced translocation) (Janz 2008). The gain-of-function of proto-oncogenes and/or the loss-of-function of tumour suppressor genes are the main events which can lead to uncontrolled growth and tumour (Hanahan and Weinberg 2000). Proto-oncogenes code for growth factors, their receptors, transcription factors or other proteins involved in the regulation of cell cycle differentiation and proliferation. For instance, the translocation of the chromosome 22 region containing the *BCR* gene with chromosome 9 containing the *ABL1* gene (philadelphia chromosome), results in the overexpression of the *ABL1* oncogene in B-cells, and this has been found to cause chronic myelogenous leukemia (CML) (Quintás-Cardama and Cortes 2009). On the other hand, tumour suppressor genes code for proteins involved in triggering cell cycle arrest and apoptosis. Most common tumour suppressor genes deleted in a large number of cancers are *Rb* (retinoblastoma) and *TP53* (Sherr and McCormick 2002).

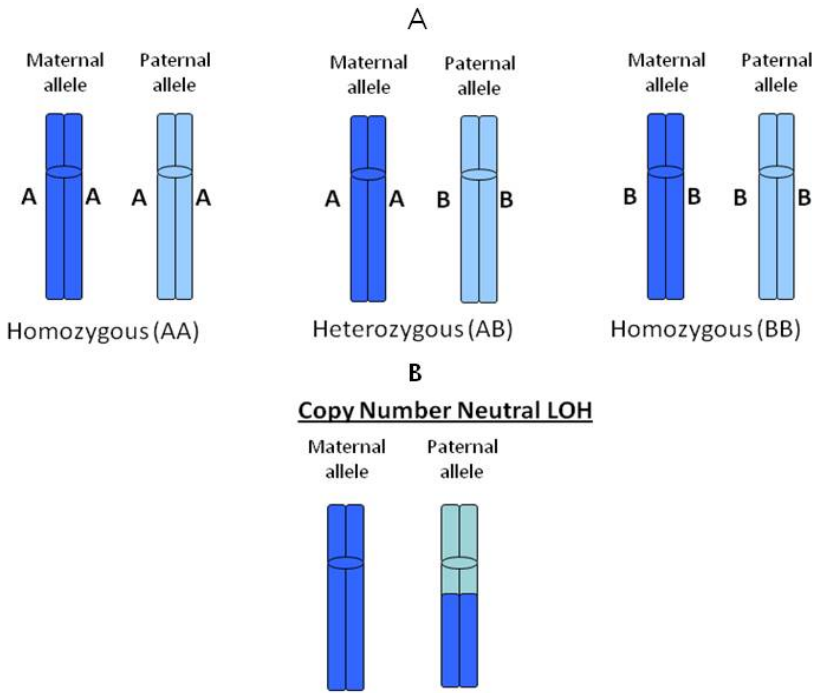


Figure 3: ***A.*** Two copies of each gene, (alleles) on each chromosome, are received from the parent cell, one maternal and one paternal. An individual can be homozygous for a particular gene, whereby both allele are similar, or heterozygous for a particular gene, whereby each allele is different. ***B.*** Loss of heterozygosity occurs as a result of incorrect recombination and causes loss of the function alleles

1.3. Detecting genomic aberrations

A number of techniques have been used to detect genomic aberrations in cancer cells. G-banding is a method which produces a karyotype (figure 4) of chromosomes using a stain called Giemsa. DNA sequence rich in A and T create darker bands and alterations in chromosomes, such as translocation, can be noted by G-Banding (David Burnett 2005).

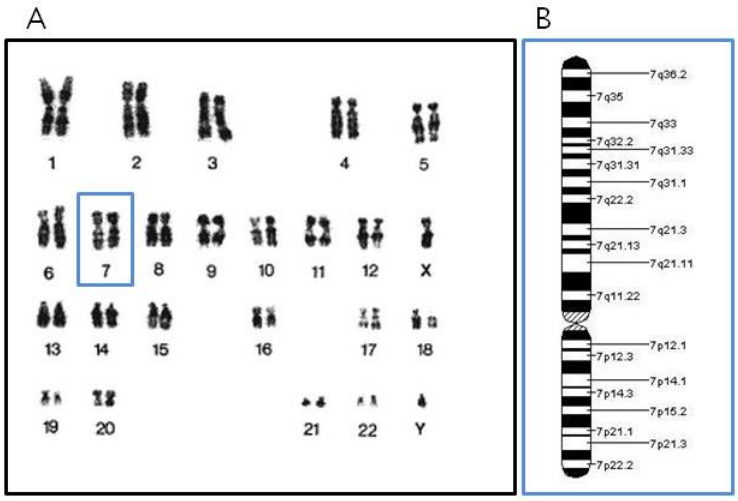


Figure 4: ***A.*** Image of a karyotype showing 22 chromosome and an X and Y sex chromosome. ***B.*** Detailed look at chromosome 7 showing G-bands.

Fluorescence *in situ* hybridisation (FISH) is another cytogenetic technique, with a higher resolution that G-Banding. FISH involves labeling metaphase, or interphase chromosomes with a fluorescent sequence specific probe, which can then be detected using a microscope (Read 2003). Using FISH, changes in chromosomes of at least 1Mb can be detected, and the use of more than one probe enables detection of translocations.

A much higher resolution method for genome wide analysis, has been the comparative genome hybridisation array (aCGH), which involves using a large number of probes to tag cDNA from two different sources and hybridising to a single array for comparison studies in order to changes in copy number (Shinawi and Cheung 2008). The resolution of aCGH varies, as probes can be spaced out between 1 every 1Mb or 1 ever 100kb (Shinawi and Cheung 2008). It is important to note that unlike FISH method, arrays cannot detect balanced translocations.

The highest resolution for detecting copy number changes as well as LOH is the Affymetrix SNP6.0 array (Pfeifer, Pantic et al. 2007), which contains 2.7 million of 26 base pair probes, including nearly 1 million probes for detecting copy number changes. The use of SNP array also allows detection of LOH, since SNP genotype can be compared using patient matched controls (Green, Jardine et al. 2010).

High concordance has been noted between different arrays and other methods for copy change detection (Curtis, Lynch et al. 2009). High resolution array, has been used for cancer studies and suggested to be used for regular clinical use (Ankita, Sung-Hae

et al. 2008). It is clear nonetheless that FISH technique should not be ignored as translocations play an important role in tumours (Janz 2008), including leukeamias.

1.4. Haematopeiesis

Hematopoietic stem cells (HSCs) are pluripotent stem cells in the bone marrow that give rise to myeloid and lymphoid progenitor cells, which give rise to all circulating blood cells in a multistep process known as haematopoiesis. Myeloid cells go on to differentiate into erythrocytes (red blood cells), granulocytes, macrophages and platelets whilst lymphoid cells are progenitor cells for T and B lymphocytes and natural killer cells. Lymphocytes are part of the adaptive immune system, which recognise foreign molecules (antigens) through highly specific structures called antibody, generated after proliferation and maturation (Kuby 2006).

B-Lymphocyte development and maturation occurs in the bone marrow, in a number of steps, where each step represents a change in the expression of antibodies. Antibodies are composed of 2 identical light polypeptide chains (L) and 2 identical heavy polypeptide chains (H), each bound by a disulfide bond (figure 5). The first 110 amino acid of each chain form a highly variable sequence called V regions which give the antibody its high specificity to antigens. The rest of the antibody is a constant sequence (C) which binds to the B cell surface (B-cell receptor) or other immune cells (for soluble antibodies) (Rajewsky 1996).

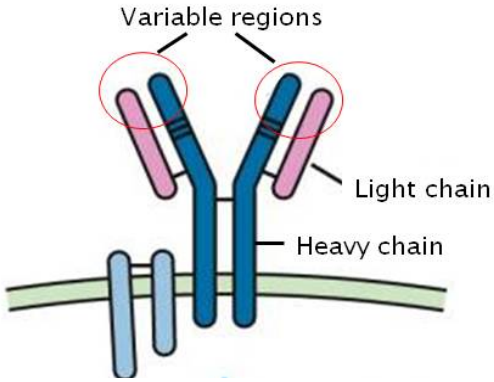


Figure 5: Figure of a membrane bound antibody showing light and heavy chains linked by disulfide bonds.

The heavy chain of the antibody is coded by the IGH gene locus on chromosome 14, whilst the light chain can be from two different gene clusters: IGL ( ) cluster from chromsome 22 or IGK ( ) from the gene clusters on chromosome 2.

The final mRNA for antibodies is derived following somatic recombination of exons of 4 different groups from one heavy chain and either of the light chains: Variable (V), diversity (D), joining (J) and constant (C) (Li, Woo et al. 2004).
The C region is divided into 5 different classes and code for the constant region of the heavy chain: (IgG isotype) , (IgA isotype) , (IgD isotype) , (IgM isotype) and (IgE isotype) .
The variable region is formed of 1 VDJ segment out of a large number of different segments, and this gives antibody the huge diversity and potential for antigen recognition. The somatic recombination occurs during B-cell development as follows:

D-J joining of the heavy chain occurs first, during the phase of pro-B cell, where a segment of the D gene from the heavy chain is spliced (randomly) and joined to a segment of the J gene from the heavy chain. A segment of the V gene is then spliced from the DJ segment and forms a VDJ heavy chain primary RNA. The VJ rearragement of the light chain occurs during the pre-B cell stage. At the next stage, immature B-cell, the antibody with constant antibody region IgM is expressed at the cell surface. Once releases from the bone these B-cells reached the spleen and are differentiated into mature, yet naïve B cells (summarised in figure 6) (Hardy and Hayakawa 2001)

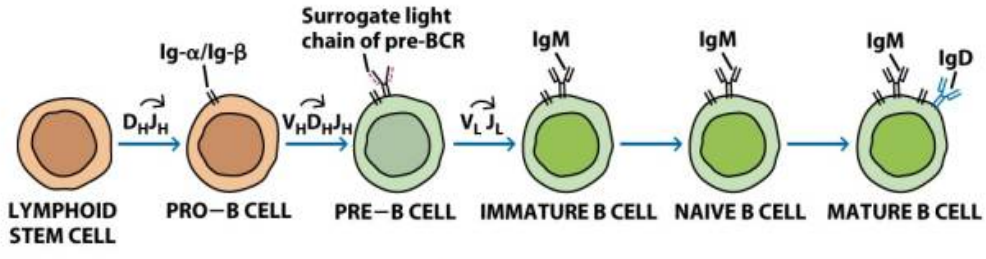


Figure 6: Picture showing stages of B-cell maturation with changes to the antibody and cell surface receptor. *Figure taken from Kuby, IMMUNOLOGY, 6th edition (2007).*

Lymphocytes then circulate in the blood and the lymph node and reside in various lymhpoid organs. Antigen recognition causes naïve B cells to come out of G_0 phase of the cell cycle and undergo maturation and proliferation into either effector cells or memory B cells (Kuby 2006). This step occurs in the germinal centres of the lymph nodes, where naïve B cells undergo hypermutation and class switch as well as extensive proliferation as part of the immune system's deffence against foreign bodies (virus, bacteria) (Natkunam 2007). Hypermutation involves the variable region genes and enables higher affinity to antigens, whereas class switch is the change from constant region IgM to one of the other constant region, mostly IgG, in order to enable interaction with various effector molecules. B-cells come out of the germinal centre as

either plasma cells, involved in the direct protection of the body, or as memory B-cell which retain the antigen specific antibody for future recognitions (Kuby 2006). Plasma cells undergo cell-death after a number of whilst memory B-cells which can survive a number of years.

The regulation of B-cell development, maturation and proliferation is tightly regulated by a complex system involving cytokins, transcription factors, cell cycle checkpoint proteins, etc… (Thomas, Srivastava et al. 2006). For instance, SYK and SRC tyrosine kinase proteins play an important role in activating proliferation of the pre-B cell whilst FOXO and p27 negatively regulated cell cycle (Herzog, Reth et al. 2009). Other important proteins include Bcl-2 family protein which inhibits apoptosis (Cory 1995) and other proteins such as BOX or Fas induce program cell death (Lindsten, Ross et al. 2000; Pasqualetto, Vasseur et al. 2005). The levels of lympocytes, pre and post germinal centre are thus tightly regulated through signals for survival, cell cycle arrest as well as programed cell death (apoptosis) (Osborne 1996). Dysfunction of this sytem may lead to either increased proliferation of B-cells or long term survival.

1.5. Leukemia and lymphoma: Introduction

Leukemia affects 7000 new patients every year in the UK, affecting more men than women, specifically in elderly individuals (CRUK 2007). Chronic Lymphocytic Leukemia (CLL) is the most common form of Leukemia in the west, accounting for 35% of all leukemias, and has an incidence of 3.5/100000 every year in the US (Dighiero and Hamblin 2008). CLL affects mostly elderly individuals, with very rare cases of CLL in individuals below the age of 50 (CRUK 2007).

Leukemia and lymphomas are malignant tumours of hematopoietic cells.
Leukemia proliferate are single cells in the blood stream whilst lymphomas grow as tumour mass within lymphoid tissues such as bone marrow, lymph node or thymus.

Leukemias are classified into two major groups according to their clinical progression: Acute leukemia, such as Acute Lymphoctyic Leukemia (ALL) or Acute Myelogenous Leukemia (AML) appear suddenly and progress rapidly. The tumour cells are also known to arise from non-mature B cells. Chronic Leukemia on the other hand is less aggressive and in general develops sowly, often without any symptoms. Chronic Lymphocytic Leukemia (CLL) is a chronic malignancy which arises from mature B cells.

1.6. CLL

1.6.1. CLL diagnosis

On the clinical stage, CLL is defined by 2 main characteristics, which also distinguish it from other lymphomas such as mantle-cel lymphoma (Dighiero and Hamblin 2008):

- Absolute Lymphocytosis of at least $5x10^9$/L mature B cells (Cheson, Bennett et al. 1996)
- Cell surface markers:
  $CD19^+$ and $CD5^+$ as B cell markers
  $CD23^+$; $CD20^+$; Surface $Immunoglobulin^+$ (or); $CD79b^-$ (Binet, Caligaris-Cappio et al. 2006)
  Low level of CD22 expression (Cheson, Bennett et al. 1996)

CLL is differentiated from monoclonal B-cell lymphocytosis (mBL) by a much smaller lymphocyte count in the blood in mBL (less than $5x10^9$/L) (Marti, Rawstron et al. 2005)

1.6.2. CLL disease course and prognostic markers

Patients with CLL disease have been found to follow a heterogeneous disease course, where some patients have stable disease without a need for treatment and others have an aggressive disease with poor survival, despite numerous rounds of treatment (Shanafelt 2009). For this reason two different staging systems have been established, Rai and Binet (Rai, Sawitsky et al. 1975; Binet, Leporrier et al. 1977). The latter is graded into three stages, A, B, C, with stage B and C being late disease stages with high lymphocyte count and lymph node involvement (Hamblin 2007). Although patients on stage A do not require treatment and have a mean survival of 12 years, patients on stage C have a mean survival of 2 years with treatment for aggressive CLL (Hamblin 2007).

A large number (~35) of prognostic markers to predict the course of CLL disease have been established (Furman 2010), and include IgVH mutation status, CD38 expression, ZAP70 expression as well as genomic aberrations. These markers play an important role clinically in predicting the disease course and assessing high-risk groups of patients (Kay, O'Brien et al. 2007).

1.6.3. IgVH mutation status

An important prognostic marker came in the form of IgVH mutation status. Reports found more than half of CLL patients with evidence of mutation on the IgVH gene, with less than 98% sequence homology with the germline gene and that difference in survival was evident between the two groups (Damle, Wasil et al. 1999). Further research showed a median survival of 8 years in patients with unmutated IgVH status, compared to a median survival of 25 years in patients with a mutated IgVH status (Hamblin, Davis et al. 1999; Jelinek, Tschumper et al. 2001; Hamblin 2007). IgVH mutation status, when recorded at diagnosis and early stage disease, has thus been established as an independent marker for disease progression (Hamblin, Davis et al.

1999; Oscier, Gardiner et al. 2002). It is important to note that an exception has been recognised; B-CLL cells with the IgVH3-21 gene segment, whether mutated or unmutated, have been associated with poor prognosis (Tobin, Thunberg et al. 2003).

Interestingly the IgVH mutation status has given rise to a debate as to whether CLL disease should be regarded as two distinct diseases (Hamblin 2002). The origin of the B-CLL cell is proved to be different, since the tumour cells with mutated IgVH status would arise from post-germinal centre B-cells whereas the tumour cells with unmutated IgVH status would have naïve, pre-GC lymphocytes origin. Nonetheless, regardless of whether it may be two diseases or one, with two distinct disease course and morphology, the treatment and clinical impact remains the same.

Since IgVH mutation screening is not an easy and speedy task, research has attempted to find surrogate markers with easier detection capacities.

1.6.4. CD38 expression

Another prognostic marker of CLL disease has been the expression levels of CD38, a glycoprotein found on most white blood cells (Hamblin 2007). Patients with 30% or more malignant lymphocytes expression cell surface had a significantly poorer median survival compared to patients with less than 30% CD38 expression (Damle, Wasil et al. 1999; Chevallier, Penther et al. 2002). However, despite being far easier to measure clinically compared to IgVH (using flow cytometry), an issue has been raised regarding CD38 expression as surrogate marker for IgVH, as a discordance was noted between IgVH mutation status and CD38 expression as the latter was shown not to highlight the same patients as IgVH status (Hamblin, Orchard et al. 2000; Hamblin, Orchard et al. 2002). Nonetheless CD38 expression has been an established independant marker for disease progression (Damle, Wasil et al. 1999; Chevallier, Penther et al. 2002), with two important obstacles; levels of CD38 expression were not found to be constant during the course of the disease (Hamblin, Orchard et al. 2002), and the cut off for cells expressing CD38 has been questioned with studies using much lower than the initial cut off (7% as opposed to 30%) (Krober, Seiler et al. 2002). Nevertheless, CD38 expression, measured at early disease stage, remains an important prognostic marker in CLL (Van Bockstaele, Verhasselt et al. 2009).

1.6.5. ZAP70 expression

A surrogate marker for IgVH was found in ZAP70 expression, a zeta-associated protein with a molecular weight of 70kD (Rassenti, Huynh et al. 2004). Interestingly, B cells lack ZAP70 expression, whilst B-CLL cells with high levels of ZAP70 expression were found to predict IgVH mutation cases (Wiestner, Rosenwald et al. 2003) as well as time

to treatment (Rassenti, Huynh et al. 2004). ZAP70 was also shown to predict disease outcome and time to treatment (Weinberg, Volkheimer et al. 2007; Rassenti, Jain et al. 2008). Nonetheless, there are still controversies regarding the methods for ZAP70 analysis as discordances have been noted, as well as regarding the cut off for high/low ZAP70 expression (Van Bockstaele, Verhasselt et al. 2009).

Whilst biomarkers play an important part in predicting disease progression, the presence of genomic aberrations in CLL patients has been found to involve numerous genes involved in malignancy which drive different disease course. These will be discussed in the next section.

1.7. Genomic aberrations

The presence of chromosomal abnormalities in CLL has been noted in large studies and shown to correlate with survival and disease progression (Dohner, Stilgenbauer et al. 2000). Dohner et al noted 268 out of 325 patients with chromosome aberrations, using FISH technique (Dohner, Stilgenbauer et al. 2000). The most common aberrations were found to be 13q, 11q and 17p deletions, as well as trisomy 12. These will be introduced below.

1.7.1. 13q deletion

Deletion on the long arm of chromosome 13 (13q14) has been found in a majority of CLL patients (over 55%) and has been linked with a favourable disease course (Dohner, Stilgenbauer et al. 2000). Patients with a sole 13q deletion were found to have a better survival than patients with other aberrations as well as with a normal genome (at FISH resolution) (Dohner, Stilgenbauer et al. 2000). Extensive research has been done on patients with 13q deletion. It was found that deletion in this region can be mono-allelic or bi-allelic (homozygous) although no difference in prognosis was found between these two groups (Fink, Geyer et al. 2004; Van Dyke, Shanafelt et al. 2010). In addition, patients with a higher percentage of nuclei with 13q deletion (>65%) were found to have a worse prognosis than other 13q patients (Van Dyke, Shanafelt et al. 2010). Furthermore, and more importantly, the size of 13q14 deletion was found to impact prognosis.

The size of 13q deleted has been shown to vary, however a Minimal Deleted Region (MDR) has been recognised on 13q14, spanning the locus D13S319 (Liu 1997). This region is known to be deleted in most cases and includes many tumour linked genes such as *DLEU2* which also has within it the microRNA clusters (miR) 15a/16-1 (Bullrich, Fujii et al. 2001; Calin, Dumitru et al. 2002).

13q14 deletions involving the MDR were termed as type I deletions whilst 13q deletion involving the *RB1* (which were also larger than 2Mb), were classed as type II (Ouillette, Erba et al. 2008). Parker et al and others noted a significant difference in the risk of disease progression between patients with class I and class II 13q deletion: Patients with class II 13q deletion were shown to have a higher risk for disease progression (Ouillette, Erba et al. 2008; Parker, Rose-Zerilli et al. 2010) as well as a shorter time to treatment (Dal Bo, Rossi et al. 2011) highlighting the importance of genes outside of the MDR.

Deletion of this region is seen in various other tumours suggesting therefore presence of a tumour suppressor gene, as suggested below:

DLEU2

Not much is known about the function of *Deleted in Leukemia 2* gene, except that it includes the miR-15a/16-1 genes and deletion of this gene has also been found to cause tumour in mice (Klein, Lia et al. 2010) and thus suggested to play a tumour suppressor role.

Mir 15a/16-1

MicroRNA (miRNA) are non coding RNA of approximatily ~21 nucleotides, which are increasingly shown to have vital roles in the regulation of gene expression, through binding on target RNA by complementary base pairing and suppressing translation (Pillai, Bhattacharyya et al. 2007) and downgrading RNA transcript (Lim, Lau et al. 2005). Half of all MiRNAs are located in cancer associated regions of the genome suggesting link with tumour (Calin, Sevignani et al. 2004).

Calin et al have shown that MiR15a/16-1 are expressed in $CD5^+$ B cells and are downregulated in most CLL cases (68%) (Calin, Dumitru et al. 2002). In addition, mutation of this region has been associated with deletion on 13q14 (Calin, Ferracin et al. 2005). Studies have found miR15a/16-1 to negatively regulated apoptosis and proliferation (Calin, Cimmino et al. 2008), downregulate cell cycle entry proteins in B cell and cause lymphoproliferation in mice (Klein, Lia et al. 2010).
Interestingly, a number of genes have been suggested as downstream targets of miR15a/16-1, including cell cycle regulator MCL1 and BCL2 family protein (Calin, Cimmino et al. 2008). The latter has been shown to be overexpressed in MiR15a/16-1 deleted cells, and thus cause cell proliferation (Cimmino Amelia 2005).

Nonetheless, research on understanding the role of MiRNA in CLL is still ongoing, as a number of other genes have been shown to be regulated by miR15a/16-1 (Hanlon Katy

2009), while BCL2 has been found not to always correlate with its deletion (Ouillette, Erba et al. 2008).

DLEU7

Positioned in a number of studies within the MDR of 13q14 deletion (Ouillette, Erba et al. 2008), *DLEU7* has been suggested as a tumour suppressor involved in CLL disease (Palamarchuk, Efanov et al. 2010; Pekarsky, Zanesi et al. 2010). Studies have found low levels of DLEU7 in CLL patients, even in patients without evidence of 13q14 deletion (Hammarsund, Corcoran et al. 2004), and has been shown to be a potent inhibitor of NF- B signal, leading to inihbition of apoptosis (Palamarchuk, Efanov et al. 2010). This gene is thus another target of the recurrent 13q14 deletion in CLL patients.

RB1

RB1 gene has been noted as part of class II deleted 13q patients and found to be involved in the malignant transformation of CLL (Liu, Szekely et al. 1993). *RB1* has been established as a cause for uncontrolled proliferation and genomic instability (Hernando, Nahle et al. 2004) and deletion of this gene has been linked with a worse prognosis than patients with small 13q14 deletions (Dal Bo, Rossi et al. 2011).

LATS2

Lower levels of *LATS2* has been found in patients with class II 13q deletion compared to other patients (Ouillette, Erba et al. 2008), and has been linked with the P53 pathway and thus a tumour suppressor (Visser S 2010).

To conclude, presence of 13q14 deletion in the majority of CLL patients has been linked with a number of potential tumour suppressor genes. Interestingly, deletion of this region can involve either all genes mentioned above or just the *DLEU/miR15a/16-1* region, and prognosis of patients will change accordingly, since large 13q14 deletion have been found to present a higher risk for disease progression (Parker, Rose-Zerilli et al. 2010).

1.7.2. 11q23 deletion

Deletion of the 11q region has been found in approx 10-20% of CLL patients (Dohner, Stilgenbauer et al. 2000). Deletion of this region has been found to be an independent prognostic marker, linked with poor survival, short time to treatment as well as extensive lymphadenopathy (Dohner, Stilgenbauer et al. 2000; Austen, Powell et al. 2005). Whilst most patients have very large deletions, the gene highlighted with

pathogenic impact in 11q deleted patients has been *ATM*, a gene involved in the P53 pathway, as a number of patients have been shown to have mutation of this gene (Schaffner, Stilgenbauer et al. 1999; Austen, Powell et al. 2005).

1.7.3. 17p13 deletion

Patients with deletion on 17p13 have been associated with the worst prognosis, with rapid disease progression and short survival (Dohner, Stilgenbauer et al. 2000). Deletion on this region occurs in approx 5-10% of patients (Dohner, Stilgenbauer et al. 2000) and involves the *TP53* gene. Deletion or mutation of *TP53* have been found to be independent prognostic marker for poor survival (Oscier, Gardiner et al. 2002) and disease progression (Cordone, Masi et al. 1998). Deletion on 17p has also been associated with poor response to alkylating agenst and purine analgoue (el Rouby, Thomas et al. 1993; Dohner, Fischer et al. 1995), relapse (Lozanski, Heerema et al. 2004; Grever, Lucas et al. 2007) and genomic instability (Dicker, Herholz et al. 2008).

Interestingly, Tam et al have shown clinical heterogeneity in patients with 17p and found percentage of nuclei with deletions to be important in the survival rate (Tam, Shanafelt et al. 2009). Furthermore, other studies have shown that presence of 13q deletion in patients with loss of *TP53* have a much better disease progression (Daniel, Tait et al. 2009). These studies highlight the complex nature of 17p aberration as prognostic marker.

1.7.4. Trisomy 12

Gain of the entire chromosome 12, or trisomy 12, is the second most common aberration in CLL patients (Dohner, Stilgenbauer et al. 2000). An intermediate prognosis, between 13q and 17p deletion has been suggested (Dohner, Stilgenbauer et al. 2000), although patients with trisomy 12 have been found to have advanced disease stage (Juliusson, Robert et al. 1985; Knauf, Knuutila et al. 1995). Studies have found short time to treatment in trisomy 12 patients, but longer overall survival (Escudier, Pereira-Leahy et al. 1993). Interestingly, no gene has yet to be singled out in patients with trisomy 12 although research is under way (Porpaczy, Bilban et al. 2009).

The prognosis value of all these aberrations has been shown in figure 7. Patients with 13q deletion had the longest overall survival, followed by trisomy 12 patients. Deletion on 11q or 17p was linked with poor overall survival.

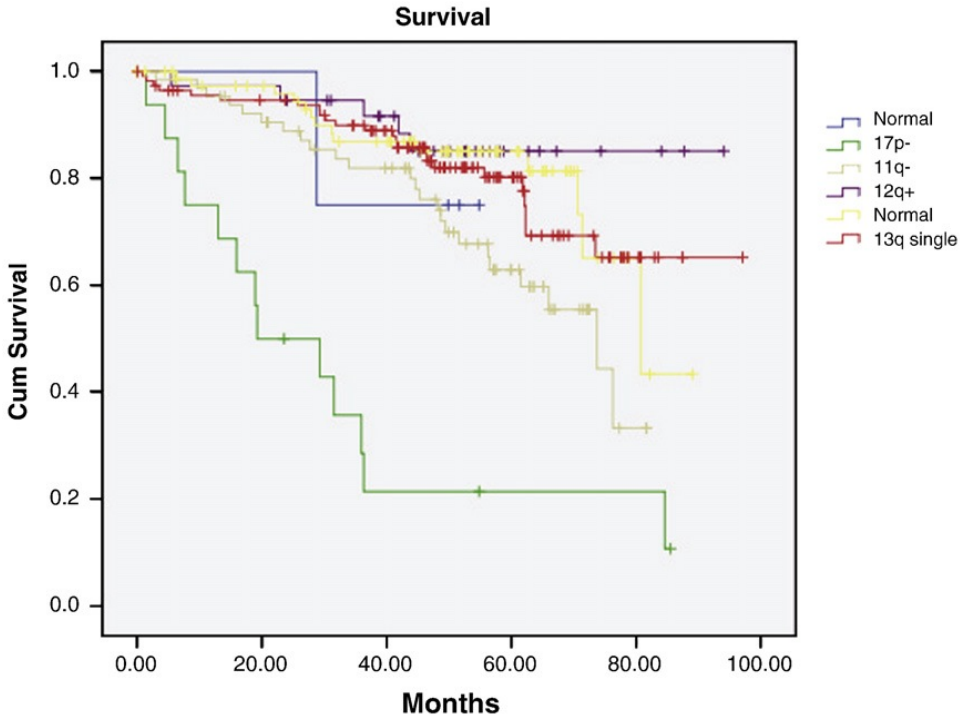


Figure 7: The overall survival of patients with different genomic aberrations highlights shortest survival in patients with 11q and 17p deletion and longest survival in patients with trisomy 12 and 13q deletion. Picture taken from Zenz et al (Zenz, Mertens et al. 2011)

1.7.5. Other aberrations

A number of other chromosomal aberrations have been noted in CLL patients. Deletion on 6q21 was found in low frequency (3-6%) ((Dohner, Stilgenbauer et al. 2000)). Although a number of studies have shown no prognosis value for 6q deletion (Cuneo, Roberti et al. 2000), other research has noticed an association between this deletion and short time to treatment as well as intermediate prognosis with distinct phenotype (Cuneo, Rigolin et al. 2004).

Chapiro et al found that 2p gain was the 2nd most frequent aberration after 13q14, albeit in a small cohort and only in patients with late Binet stage (Chapiro, Leporrier et al. 2009). Other studies have also shown a strong association between 2p gain and unmutated IgVH status (Jarosova, Urbankova et al. 2010). The gain of the short arm of chromosome 2 involves 2 important oncogenes, *rel* and *mycn,* and was associated with poor prognosis (Chapiro, Leporrier et al. 2009).

1.7.6. Genomic complexity

Recent studies have found genomic complexity to be linked with disease progression. Patients with 3 or more aberrations were found to have signifiacntly shorter time to treatment (Kujawski, Ouillette et al. 2008). Whilsts 17p has previously been linked with

genomic instability, Ouillette et al have found 11q deletion as well as type II 13q deletion to be linked with genomic complexity (Ouillette, Fossum et al. 2010). Interestingly, genomic complexity has also been linked with short telomeres at the end of chromosomes (Roos, Krober et al. 2008), as these play an important role in causing genomic instability, further secondary aberrations, as well as disease progression (Lin, Letsolo et al. 2010).

1.8. Summary and aims

In conclusion, it is clear from the above that genomic aberrations play an essential role in the disease progression of CLL, although at different degrees. Patients with type I 13q deletion seem to have the best prognosis, whereas patients with type II 13q or trisomy 12 have an intermediate prognosis and patients with 11q and 17p deletions have the worst prognosis, with short survival and short time to treatment. Numerous genes have been highlighted in each aberrations, included miR15a/16-1, *ATM* and *TP53*.

Most studies have investigated genomic aberrations at a single time point, either diagnosis, early or late disease stage. However, it has been suggested that genomic aberration may not be stable throughout the disease course and secondary aberration may be acquired in patients with CLL (Oscier D 1991). In addition, when investigating the acquisition of secondary aberrations in CLL patients, it has been suggested that telomere length may play an important role, as a short telomere may be associated with genomic instability (Lin, Letsolo et al. 2010).

Therefore, this study aims to investigate the presence of genomic evolution in patients with CLL as well as its role in disease progression. In addition, telomere length will be studied and linked to genomic instability and prognostic markers.

<u>Aim</u>

- Investigate Genomic evolution over time in CLL patients
- Investigate telomere erosion over time in CLL patients
- Explore the effects of secondary aberrations and telomere dysfunction on CLL disease progression.

2. Methods

2.1. Patients

2.1.1. Patient samples

Tumour cells of 29 patients at two different time points were received from The Royal Bournemouth Hospital (RBH). In addition, density gradient separated mononuclear cells from 22 out of 29 patient was received from RBH (patient matched), and were termed 'normal' sample.

2.1.2. Patient data

The 29 patients had an average age of 65 years at presentation and nearly 70% of patients were male.

The patients were diagnosed with either mBL or Binet disease stage A0 and the first DNA sample analysed at time point called 'presentation' (PT) was taken on average 55 months after diagnosis (median: 16 months, range: 0-239 months) and the second DNA sample analysed at time point called 'follow up' (FU) was taken on average 129 months after diagnosis (median: 85 months, range: 17-316 months).

Patients were either termed 'stable' or 'progressive' cases, where disease was either stable for at least 5 years (n=9) or progressive within 3 years (n=20).

2.2. Clinical data

Clinical data was provided by the Royal Bournemouth hospital.

2.2.1. Disease stage

Patients in this cohort had either mBL or early CLL disease stage A/A1 at **presentation** (table 1). However, as shown in table 2, only 50% of patients remained at early stage disease, as 4 patients progressed to late stage A1/A2, and 10 patients progressed to aggressive disease stage B and C. (All progressive patients progressed to late disease stage whilst stable cases remained at mBL or stage A0).

Table 1: Patients at PT had either mBL or early CLL stage

Clinical stage	*Number of cases at PT*
mBL	7 (25%)
A0	17 (61%)
A	3 (11%)
A1	2 (7%)
B/C	0%

Table 2: Half of the patients in this cohort remained at early stage disease whilst the other half progressed to more aggressive disease stage

Disease stage (PT → FU)	*Number of cases*
mBL/A → mBL/A	14 (50%)
A0 → A1/A2	4 (14%)
mBL/A1 → B/C	10 36%)

2.2.2. Treatment and TTFT

Whilst 2 patients received treatment prior to PT sample, a total of 6 patients were treated between the PT and FU sample and 9 other patients received treatment after the FU sample. When analysing the impact of treatment in this study, the 15 patients with treatment after the first time point will be termed as 'treated'.

A total of 6 patient received one round of treatment , 5 patients received 2 rounds of treatment, 2 patients received 3 rounds of treatment and 2 other patients received 4 rounds of treatment after PT.

Time to first treatment (TTFT) was calculated from PT sample to treatment and ranged from 17 to 116 months (median: 46 months).

2.2.3. WBC count

The White Blood Cell (WBC) count, in 10^9/L, was taken at different time points and was ploted as a graph against time in months (figure 8). The circles represent each time WBC count was taken, whilst the green and purple square reprenset PT and FU sample date. The red sign marks treatment date, which would in most cases be followed by a fall in WBC count. Two variables were looked at in this study: the 'maximum WBC count' and the 'rate of WBC count increase per month'. The median 'maximum WBC count' was 120x10^9/L (range: 30 -266 x10^9/L), whereas the median rate of WBC

increase per month was 2.4 x10^9/L/month (range: 0.18 – 30.8 x10^9/L/month). 'Lymphocyte doubling time' was not used due to lack of ressources as well as the size of the cohort which did not allow Cox regression statistical study.

2.2.4. Survival

A total of 4 patients had died as per the end of the year 2009. Due to the small number of patients in this study, overall survival was not looked at.

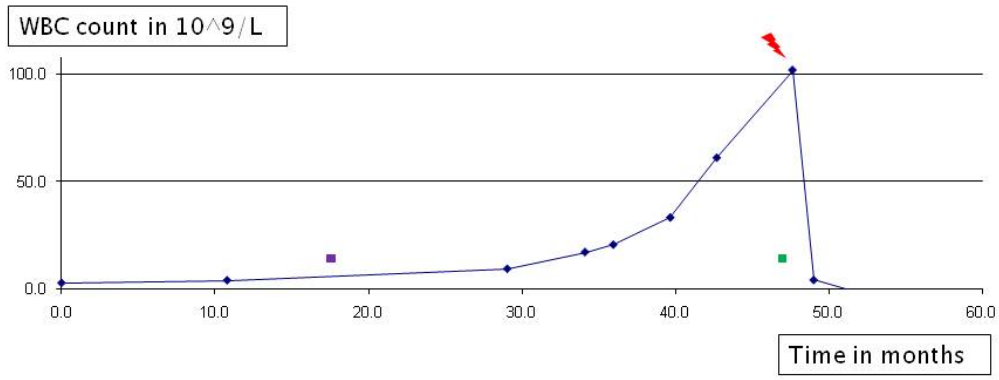


Figure 8: The rise of WBC count over time in one patient

2.3. Biomarkers

IgVH mutations status and CD38 data was provided by the RBH.

2.3.1. IgVH mutation status

Immunoglobulin variable gene was amplified using PCR technique with either 5' primers for all leader sequences of the V_H1 to V_H6 families or 5'framework 1 (FW1) consensus primer together with 3' primers for either the J_H region or 3' primers for the constant region sequence. The PCR products were then sequenced using an automated DNA sequencer and the nucleotide sequences were compared using the EmBL/GenBank database. IgVH status was defined as 'unmutated' or 'mutated' using a 98% germ-line homology cut off.

A total of 8 patients in this study had unmutated IgVH status whilst 21 patients had mutated IgVH status.

2.3.2. CD38 expression

Cell surface expression of CD38 was examined by flow cytometry: Cryopreserved cells were incubated with anti-CD5 (FITC labeled), anti-CD19 (PE labeled) and anti-CD38 (RPE-Cy5 labelled). Each sample was run with an isotype-matched negative control to separate positive and negatively stained cells. The percentage of cells with positive CD38 expression was measured in the $CD19^+/CD5^+$ population (using 'gates' on the

scatter-forward-scatter, SCC-FSC plot). CD38 expression was defined as 'positive' when more than 30% of cells expressed CD38 and 'negative' when less than 30% of cells expressed CD38 marker.
A total of 7 patients in this study had CD38+ whilst 22 patients had negative CD38 expression.

2.4. Cytogenetic and mutation data

Cytogenetic (Karyotype + FISH) and mutation data (on *ATM* and *TP53*) was provided by the RBH.

2.4.1. Karyotype

Karyotype analysis by standard cytogenetic technique was performed on all 29 patients at both time point (PT and FU). The results were described according to the International System for human Cytogenetic Nomenclature (ISCN 2009). Translocations were accepted as real when seen in 2 or more cells. The results are found in appendix1.

2.4.2. FISH

Interphase Fluorescence in situ hybridisation (FISH) probes were used to detect trisomy 12, deletion on chromosome 11q22.3 (*ATM*), 13q13 (*D13S319*) and 17p13 (*TP53*) in all 29 patients at PT and FU. The results are found in appendix 1.

2.4.3. Mutation status

The nonisotopic RNase cleavage assay (NIRCA, AMS Biotechnology,Oxford, United Kingdom) was used to screen for *TP53* mutation in 3 patients. Only one patient (ID=9) was found to have a mutation on *TP53*.

2.5. Genome-wide DNA analysis

Genome-wide analysis using the Affymetrix SNP6.0 array platform was performed on all 29 patients at presentation and follow up as well as normal sample of 22 patients.

The process described below involved DNA extraction, array running and analysis.

2.5.1. DNA extraction

Tumour cells and purified granulocytes from 29 patients with CLL were received from the Royal Bournemouth Hospital and stored at -80°C. DNA was extracted using the QIAGEN kit: The cells were suspended in 200 µl of Phosphate Buffered Saline (PBS) prior to the addition of 20 µl of Proteinase K and 200 µl of Buffer AL to lyse the cells. Subsequent to 10 min incubation at 56°C, 200 µl of Ethanol was added and the

contents were transferred to a DNeasy Mini Spin Column, and centrifuged (Thermo Scientific) at 800 RPM for one minute. 500 l of wash Buffer AW1 was then added, followed by centrifugation at 8000 RPM for 1 minute. 500 l of wash Buffer Aw2 was then added, followed by centrifugation at 8000 RPM for 3 minute for the washing procedure. Finally, 200 of Buffer AE to elute the DNA was added prior to incubation for one minute. Approximately 24ng/ l of DNA were collected in about 50 l, providing a total yield of 1200ng.

The DNA samples were then run on an agarose gel for quality assessment. Figure 9 reveals 12 DNA samples and the presence of a single band showing DNA of good quality (a poor quality sample would have shown a smear of bands suggesting the fragmented DNA).

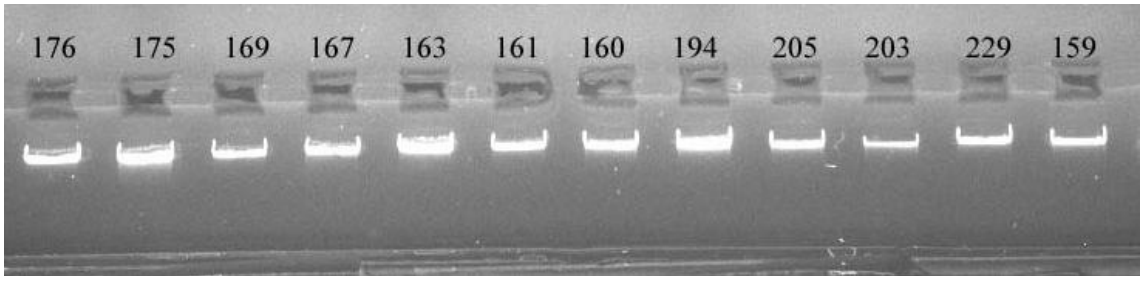


Figure 9: Photo of an Agarose Gel result of 12 DNA sample for quality assessment showing good quality DNA.

2.5.2. SNP6.0 array running

Recent development in technology (Affymetrix Genome-Wide Human SNP Array 6.0) has enabled us to use a high density genomic profiling platform consisting of 1.8 million genetic markers detecting copy number change as well as copy number neutral loss of heterozygosity (CNNLOH), a significantly higher resolution method than previous techniques.

Affymetrix Cytogenetics Copy Number Assay is divided into 7 steps, summarised in figure 10. Six DNA samples were used at a time, with positive (DNA provided by Affymetrix) and negative control (water). The aim was to digest the DNA using two different digestion enzymes and amplify the fragments using PCR. The fragmented DNA were then labeled and placed on the array for reading. All reagents were provided by Affymetrix, unless stated otherwise.

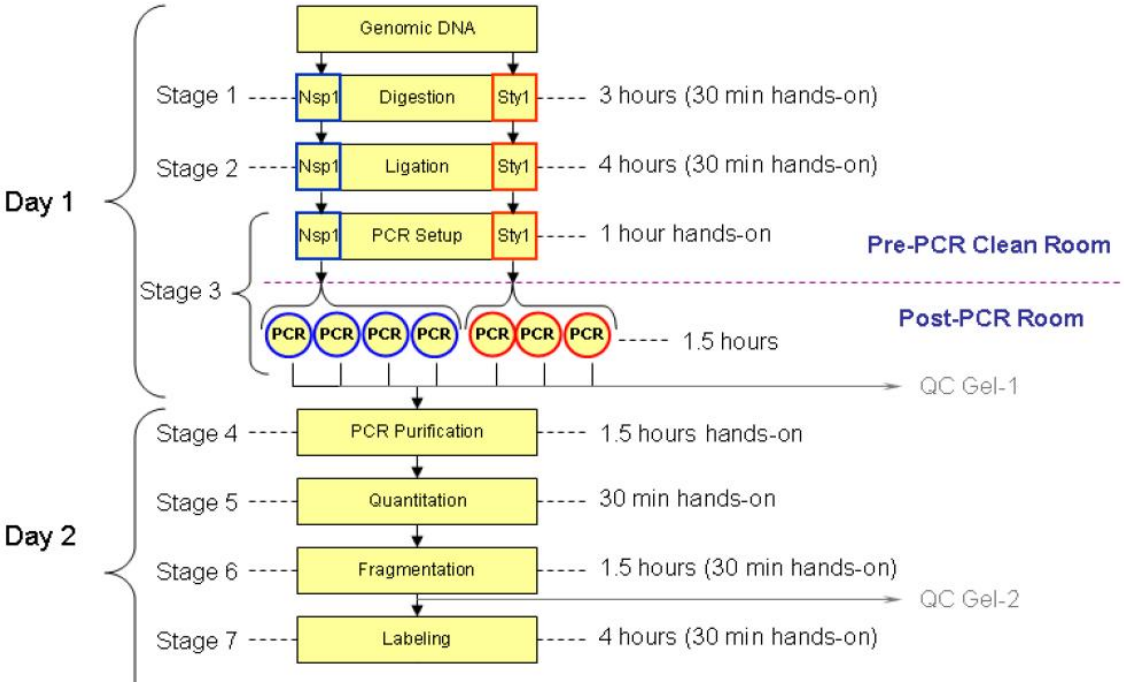


Figure 10: Summary of the different steps for the Affymetrix Protocol. Picture taken from the Affymetrix® Cytogenetics Copy Number Assay User Guide

a) DNA Digestion

The 1st step in the Affymetrix Cytogenetics Copy Number Assay involved DNA digestion using two different enzymes*: Sty and Nsp.* DNA sample was diluted at 50ng/ l with AccuGene water (Affimetrix) and 5 l was placed in 2 wells at either ends of a 96 well plate (figure 11). 5 l (x2) of AccuGene water was used as negative control and 5 l (x2) of Ref103 DNA (provided by Affimetrix as purified DNA at the correct concentration for a successful array) as positive control.

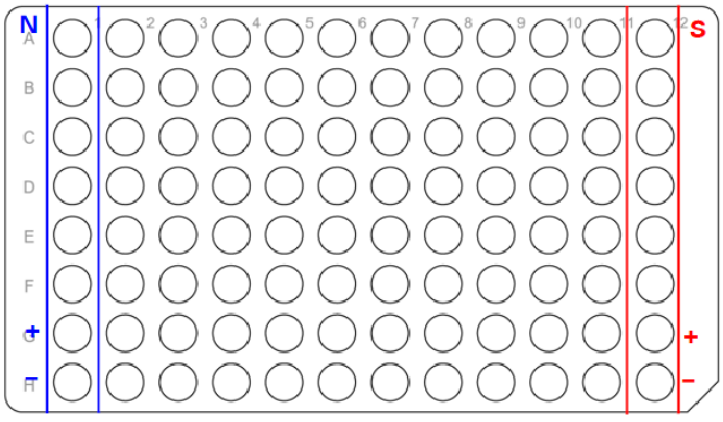


Figure 11: Picture of a 96 well plate, where each DNA sample is mixed with either *Nsp* and *Sty* enzyme

The following reagents were mixed to make 2 digestion master mix with 2 different enzymes, *Sty* and *Nsp*: Water Accugene (103.9 l) and BSA (Bovine serum albumin, to

increase enzyme performance) (1.8 l) in both master mix, as well as either NE Buffer *Nsp* (18 l) and *Nsp* I (9 l) for the *Nsp* enzyme master mix or NE Buffer *Sty* (18 l) and *Sty* (9 l) for the *Sty* enzyme master mix.

14.75 l of each master mix was then added to each well, so that each genomic DNA sample was mixed with *Nsp* enzyme in one well and *Sty* enzyme in the second well as shown in figure above.

An adhesive film was then used to seal the plate in order to avoid evaporation and the plate was then vortexed at maximum speed followed by a quick spin for 15 seconds using a Centrifuge (Sorvall). Finally, the plate was loaded on a thermal cycler (Applied Biosystems) and left to incubate at 37°C for 2 hours (optimal temperature for digestion) followed by incubation at 65°C for 20 min (to preserve the fragments and inhibit the enzyme).

b) DNA ligation

Following digestion, the DNA fragments were mixed with adaptor primers in order to prepare for the PCR reaction. The following reagents were mixed to make 2 different ligation master mixes: T4 DNA ligase buffer (23 l) and T4 DNA ligase (18.4 l) in both master mix, as well as either *Nsp* Adaptor Primer (6.9 l) for the *Nsp* master mix or *Sty* Adaptor Primer (6.9 l) for the *Sty* master mix.

19.75 l of each master mix was then added to each well (corresponding to the digestion enzyme used) in the plate containing digested genomic DNA.

An adhesive film was then used to seal the plate in order to avoid evaporation and the plate was then vortexed at maximum speed followed by a quick spin for 15 seconds using the Sorvall Centrifuge. Finally, the plate was loaded on a thermal cycler (applied Biosystems) and left to incubate at 16°C for 3 hours followed by incubation at 70°C for 20 min.

c) DNA amplification

Following ligation, the DNA samples were amplified by PCR. Samples were thus diluted at 1 in 4 using AccuGene water and 10 l aliquots were transferred into a new 96 well plate in the following manner: from each well, 4 aliquots of *Nsp* sample were transferred into 4 wells and 3 aliquots of *Sty* sample were transferred into 3 wells as shown in figure 12.

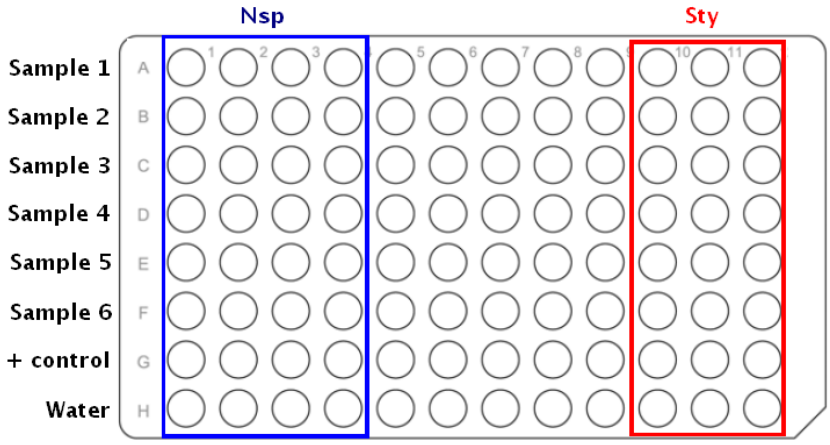


Figure 12: Picture of the PCR step where diluted DNA, following ligation, is transferred into a new plate in which four rows have NSP fragmented DNA and 3 rows have Sty fragmented DNA.

The following reagents, supplied by Clontech, were mixed to make a PCR master mix: Water AccuGENE (2544 l), TITANIUM Taq PCR buffer (644 l), GCMelt (1288 l), dNTP (902 l), PCR primer 002 (290 l) and TITANIUM Taq Polymerase (129 l).

90 l of master mix was then added to each well containing 10 l of either *Sty* or *Nsp* sample. The plate was then sealed, vortexed and placed in a thermal cycler (Applied Biosystems) for the PCR reaction to occur: Incubation at 94˚C for 3min was followed by 30 cycles of 94˚C (to separate the DNA double strand) for 30sec, 60˚C (for primer binding) for 45 sec and 68˚C (optimum enzyme temperature) for 15sec. Finally, the plate was incubated at 68˚C for 7min and then left overnight at 4˚C.

To check if amplification reaction was successful, the PCR product was run on a 2% Agarose gel was made (Agarose powder from FISHer Scientific). Aliquots of 3 l of each reaction (from each *Sty* and *Nsp* samples) were mixed to 3 l of Gel Loading Dye (Sigma), loaded unto the 2% gel and left running at 120 volts for 30min.

Figure 13 shows a successful PCR reaction. There are no distinct single bands as ligation and amplification has given rise to many different fragment sizes in large quantities.

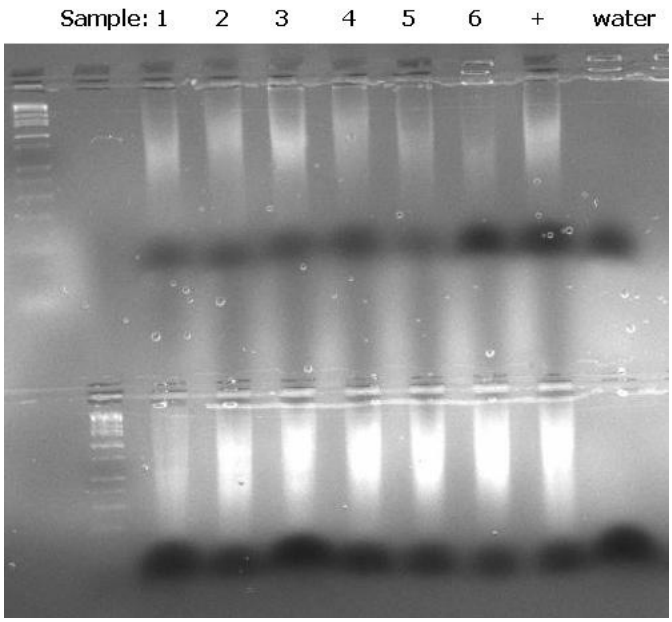


Figure 13: Photo of a gel with amplified DNA sample following successful PCR

d) PCR product purification

To purify DNA samples from buffers and enzymes, all 7 aliquots from each sample were pooled to one 2mL round bottom tube prior to the addition of 1mL of magnetic beads ApmPURE to each sample, which bind strongly to the DNA, allowing the impurities to be filtered out. The tubes were then left to incubate at room temperature for 10min prior to centrifugation at high speed for 3min.

The tubes were then placed on a stand containing magnets which attract the beads (Invitrogen- MagnaRack) and thus the supernatant could be removed. 1.5mL of 75% Ethanol was then added to each tube for purification and vortexed for 2min prior to centrifugation at high speed for 3min.

The tubes were placed on the magnetic stand and supernatant was removed without disturbing the pellet. The tubes were then placed back in the centrifuge for 30 sec and the left over supernatant was removed. To further ensure that no traces of ethanol remained, tubes were left uncapped at room temperature for 12min. 55 l of Buffer EB (10 mM Tris-Cl), which is the elution buffer, was then added to each tube, then vortexed for 10min, and re-suspended prior to centrifugation at maximum speed for 5min. Finally the tubes were placed on the magnetic stand to separate the DNA and the beads and 45 l of the eluted sample was transferred unto a new 96 well plate and 2 l was transferred to a small eppendorf.

e) DNA Quantitation

18 l of AccuGENE water was added to each 2 l of purified PCR product and DNA was quantified using a NanoDrop spectometre. A DNA yield of 4.5 to 7 g/ l was considered acceptable, as shown in figure 14.

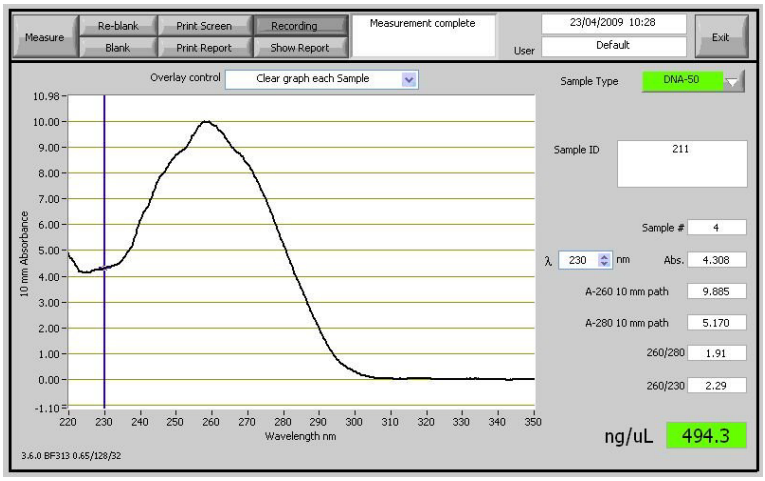


Figure 14: Picture from NanoDrop quantification showing a purified DNA sample.

f) DNA Fragmentation

Genomic DNA should be in small fragments for the hybridisation on the array and thus DNA sample was fragmented in the following manner: 5 l of 10x Fragmentation Buffer was added to each 45 l sample in a new 96 well plate. The following reagents were mixed to make a Fragmentation master mix: Water AccuGENE (118.75 l), 10xFragmentation Buffer (13.75 l) and Fragmentation Reagent (5 l). 5 l of the fragmentation master mix was then added to each well, and the plate was sealed with adhesive film, prior to vortex. Finally, the plate was placed in a thermal cycler block (Applied Biosystems) and left to incubate at 37˚C for 35min followed by 95˚C for 15min.

To check if the fragmentation process was successful, 2 l of each sample was mixed with 4 l of Gel Loading Dye (Sigma) and loaded unto a 4% TBE gel (FISHer Scientific). The gel was run at 120volts for 30min and acceptable results are shown in figure 15.

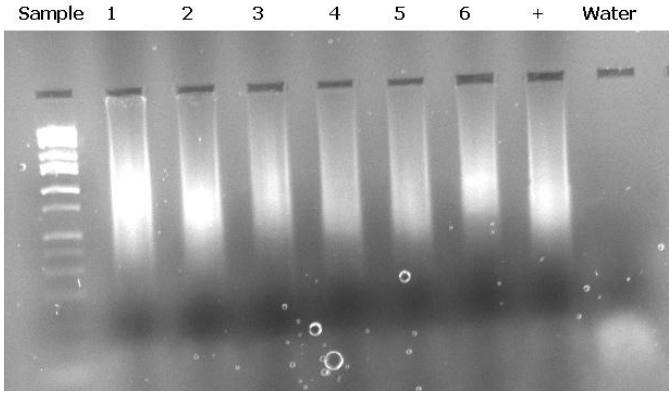


Figure 15: Photo from an agarose gel with successfully fragmented DNA samples

g) DNA Labeling

The following reagents were mixed to make a Labeling master mix: TdT buffer (98 l), DNA labelling Reagent (14 l) and TdT enzyme (24.5 l).

19.5 l of the master mix was then added to each 48 l sample before sealing the 96 well plate and vortex. The plate was then placed in the thermal cycler block (Applied Biosystems) for 4 hours at 37°C (optimal temperature for the enzyme) followed 15min at 95°C (to preserve fragments and inhibit enzyme).

h) DNA hybridisation and scanning

Following DNA labeling, the samples were transferred unto an eppendorf. The following reagents were then mixed to make a Hybridisation master mix: MED (12 l), Denhardt's Solution (13 l), EDTA (3 l), Herring Sperm DNA (3 l), Oligo Control reagent (2 l), Human Cot-1 DNA (3 l), Tween-20 (1 l), DMSO (13 l) and TMACL (140 l).

The DNA samples were then loaded onto the Affymetrix SNP6.0 platform and sent to the London lab for scanning using the GeneChip Scanner 3000 7G (Affymetrix) and processing (using proprietary software GeneChip Operating System software, Affymetrix. The feature-extracted .CEL files were received and quality controlled using Genotyping Console 2.1 software (Affymetrix) and all samples achieved manufacturer's quality control score (MAPD score), although 4 samples were very close to poor quality (cut off: 0.4) (Appendix 2).

2.5.3. Analysis of data

The results were received as .CHP files and imported into Partek Genomic Suite (Partek Inc, MO, USA) to analyse copy number and loss of heterozygosity. The raw fluorescence intensity values for each array feature were aligned onto the human genome sequence (Build 36.3). Copy number gain and copy number loss were defined,

using the naked eye, as deviation of probes from normal value of 2. Paired analysis with patient-matched normal genome was performed when possible. Copy number changes greater than 50 kb or included in a copy number variant (CNV) region (according to the database of genomic variants, http://projects.tcag.ca/variation/) were recorded as CNV and not CNA (copy number aberrations). Large losses of heterozygosity were compared to normal sample and acquired copy number neutral LOH were recorded as 'LOH'. Analysis was performed at two separate dates: First, each sample, PT and FU were analysed independently (unpaired). Then, at a later date, PT and FU sample were paired-analysed to confirm the recorded CNAs. Figure 16 illustrates a chromosomal deletion from one of the patients in the cohort.

It is important to note that Birdseed analysis was performed (comparing 50000 probes) to ensure that each normal, PT and FU sample were patient-matched (appendix 2). Birdseed is an algorithm which assigns AA, AB and BB genotype to each allele specific probe and then produces confidence scores for every individual at every SNP. Hence, matching Birdseed probes between two samples suggest DNA from a same individual (Nishida 2008).

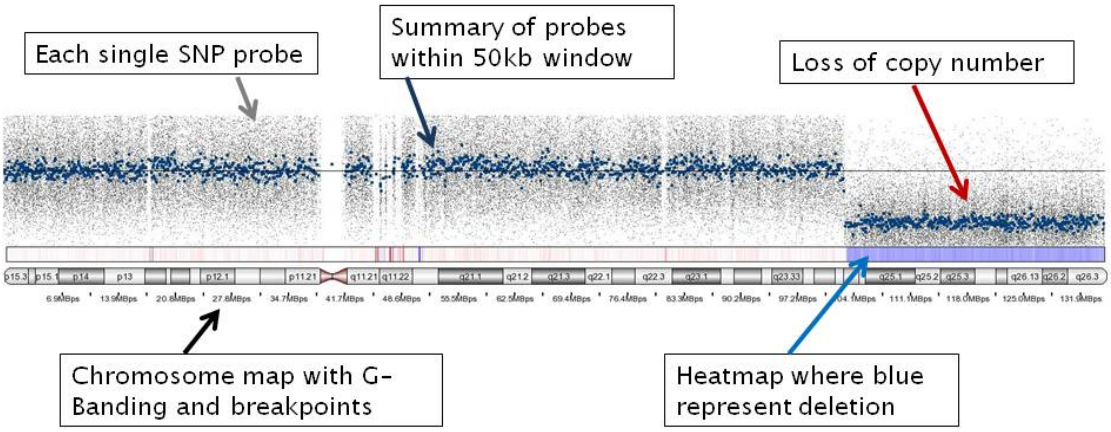


Figure 16: Explanation of a SNP6.0 array data showing deletion of the large part of a chromosome

2.6. Statistical analysis

Statistical analysis was performed using SPSS software (SPSS, Chicago, IL). The variables used were disease status (Stable vs. progressive), IgVH mutation status (mutated vs. unmutated), CD38 expression (positive vs. negative), disease stage (early stage A0/A vs. late stage B/C), poor prognosis aberration (presence vs. absence of deletion or mutation of either *ATM* of *TP53*), 13q deletion size (smaller than 2Mb vs. larger than 2Mb), complexity (≥3 CNA vs <3 CNA), total number of CNA per patient (string variable), total deletion size per patient (string variable), translocation (detected translocation vs. absence of translocation), treatment (treated vs. not treated), TTFT

(string variable), maximum WBC count (string variable) and rate of WBC count rise per month (string variable).

Because of the small number of cases in this study, overall survival was not used and Cox regression or Kaplan-Meier analysis were not performed.
However, when looking at two nominal variables, FISHer's exact test was used and when looking at two string variables, Pearson's Correlation was used. Otherwise, Non-parametric Mann Whitney test or T-Test was used depending on normality test. In each case, probabilities of less than 0.05 were accepted as significant value.

3. Chapter I: Genomic aberrations over time and its impact in CLL disease

3.1. Introduction

The heterogeneous nature of the disease course of CLL has pushed research towards the discovery of prognostic markers, such as IgVH mutation, CD38 expression and genomic aberrations (Damle, Wasil et al. 1999; Hamblin, Davis et al. 1999). However, whilst biomarkers, especially IgVH mutation, are stable and thus used as valid prognosis markers throughout the disease course, the clinical use of genomic aberrations in predicting disease course may be questioned due to the discovery of genomic evolution in CLL patients (Berkova 2009), implying the acquisition of poor prognosis aberration significantly later than diagnosis and thus altering the course of the disease.

3.1.1. Genomic evolution in CLL does occur and has been demonstrated using various techniques

A number of previous studies have used G-banding to demonstrate changes in the genome during the course of CLL disease. Nowell et al looked at a small cohort of 12 patients and showed that, occasionally, small karyotypic changes occurred during the disease (Nowell, Moreau et al. 1988). Juliunsson et al noted 3 out of 41 patients who acquired additional karyotypic aberrations over time (Juliusson, Friberg et al. 1988). Further research focusing on sequential analysis showed a higher rate of genomic evolution and confirmed that patients with CLL could have an unstable genome, for instance; Oscier et al showed that 18 of 112 patients (16%) had evidence of genomic evolution (Oscier D 1991), and Kay et al detected clonal abnormality in 39% of patients (10/28) (Kay NE 1995). Both of these studies performed genomic analysis using the G-banding technique.

The development of Fluorescence *In Situ* Hybridisation (FISH) allowed genomic evolution to be studied at a greater resolution. Hijlmar et al used FISH to investigate trisomy 12 and found 2 patients who acquired trisomy 12 in a cohort of 77 patients (Hjalmar, Hast et al. 2001). Chevalier et al used FISH to look at the common aberrations, deletions of 13q, 17p, 11q and trisomy 12, and found genomic evolution in 42% of patients (13/31) (Chevallier, Penther et al. 2002). This study also found no differences in the time length between two samples with and without evolution. Shanafelt et al conducted a large FISH study with probes on 6q in addition to the

common aberrations and found evidence of genomic evolution in 27% of patients (17/63) after a median time from first sample of 94 months (Shanafelt, Witzig et al. 2006). Stilgenbauer et al used FISH probes for 3q, 8q and 14q in addition to the common aberrations and found 17% of patients with genomic evolution (11/64) after a median time from first sample of 42 months (Stilgenbauer, Sander et al. 2007). Finally, Berkova et al investigated 97 patients with FISH and found 26% of patients with genomic evolution (Berkova 2009).

With the advent of array based comparative genome hybridisation (aCGH), which investigates genomic changes at a higher resolution, further evidence of genomic evolution in CLL patients was provided. Bea et al, in a study on progressed CLL and Richter's syndrome used aCGH and found 41% of patients with genomic evolution (7/17) (Beà, López-Guillermo et al. 2002).

To conclude, it is clear that genomic evolution does occur in CLL patients, although this has mostly been studied at low resolution and often focused solely on recurrent aberrations in CLL. To date, no study has yet to use the latest SNP6.0 technology to investigate evolution across the entire genome at high resolution.

3.1.2. Genomic evolution is not always linked to the various prognostic markers

With the heterogeneous disease course of CLL, it is important to discover the impact of genomic evolution on disease progression. To do this, a number of studies have looked at the association between established prognostic markers and genomic evolution.

Bea et al showed that genomic evolution was associated with clinical progression, specifically to late disease stage C, as only 2 of 6 stable patients showed evidence of genomic evolution, compared to 6 out of 10 progressive patients (Beà, López-Guillermo et al. 2002). Stilgenbauer et al also found a higher proportion of patients who progressed to advanced disease stage with genomic evolution, and revealed acquisition of novel aberrations as an independent prognostic marker for disease progression (Stilgenbauer, Sander et al. 2007).

Shanafelt et al revealed that genomic evolution was associated with high ZAP70 expression but not with CD38 expression or IgVH mutation status (Shanafelt, Witzig et al. 2006). Stilgenbauer et al found that all patients with genomic evolution had an unmutated IgVH status, but could not confirm an association with elevated ZAP70 expression (Stilgenbauer, Sander et al. 2007). Furthermore, Berkova et al revealed no

association with any of the prognostic makers, although genomic evolution was linked with patients who had a combination of all three negative markers (ZAP70+, CD38+ and unmutated IgVH) (Berkova 2009).

Interestingly, Shanafelt et al did show that all patients with a mutated IgVH status acquired favourable 13q deletions whilst half of patients with an unmutated IgVH status acquired a deletion on 11q or 17p (Shanafelt, Witzig et al. 2006). This was confirmed by Berkova et al who showed the acquisition of 11q/17p deletions predominantly in patients with unmutated IgVH status or CD38+ (Berkova 2009).

Hence, although further studies are required to confirm the link between genomic evolution and biomarkers as conflicting results have been shown so far, it has been suggested that genomic evolution targeting deletion on 11q/17p occurred in patients with poor prognosis markers, particularly in patients with unmutated IgVH status.

3.1.3. Genomic evolution and treatment: Cause or effect?

Genomic evolution has been shown to occur in treated as well as untreated patients (Berkova 2009). However, Shanafelt et al and Stilganbauer et al both found the majority of patients (70% and 91% respectively) with genomic evolution received treatment prior to analysis (Shanafelt, Witzig et al. 2006; Stilgenbauer, Sander et al. 2007). Further research is required to explore the presence of genomic evolution before and after treatment, as evolution prior to treatment could suggest a role on disease progression, whereas evolution after treatment may suggest genomic instability as a result of treatment. In addition, follow up at disease progression would be required to further explore the role of genomic evolution in relapse.

3.1.4. Genomic evolution occurs on various chromosomes

FISH studies have noted the occurrence secondary aberrations on all the probes used: 13q, 11q (*ATM*) and 17p (*TP53*), as well as deletions on 6q, 8p (Stilgenbauer, Sander et al. 2007) and trisomy 12 (Hjalmar, Hast et al. 2001; Chevallier, Penther et al. 2002). The evolution of 13q has been interesting, as some studies found the acquisition of new 13q deletions (Chevallier, Penther et al. 2002; Shanafelt, Witzig et al. 2006), whilst others noted that patients only undergo changes in an already acquired 13q deletion (from hemizygous to homozygous) (Stilgenbauer, Sander et al. 2007). FISH studies have thus only been able to demonstrate genomic evolution on recurrent abnormalities. A high resolution array would provide clarity on the extent of genomic evolution occurring throughout the genome. So far, only one study has used high resolution array (aCGH) and it reported secondary gains on 2p, 7p and 1q as well as deletion of 2p, 7q and 8p in follow up samples (Beà, López-Guillermo et al. 2002).

Further studies using a high resolution array are required to confirm the presence of evolution throughout the genome of patients with CLL as well as the recurrent targets of secondary aberrations.

3.1.5. The role of genomic evolution on disease progression

Patients have been found to exhibit poor prognosis after the acquisition of an 11q or 17p deletion as a consequence of genomic evolution, but the impact of genomic evolution on disease progression has yet to be explored in detail. Previous research has suggested that overall survival is worse in patients after genomic evolution (Stilgenbauer, Sander et al. 2007), but the effect of new aberrations acquired during a prospective study of CLL disease has not been investigated. It could, nonetheless, be speculated that genomic evolution targeting tumour suppressor or oncogenes would alter the disease course of CLL, from stable to a more aggressive course of disease.

3.1.6. The present study

The present study is the first to use high resolution SNP6.0 array to investigate evolution in the entire genome, and to correlate the presence of genomic evolution with established prognostic factors (IgVH mutation status, CD38 expression) as well as disease progression and clinical symptoms (disease stage, treatment, WBC count).

3.1.7. Aims

- Confirm the presence of genomic evolution in CLL patients
- Correlate genomic evolution and prognostic marker
- Investigate the consequence of genomic evolution on the disease course
- Explore the genomic evolution targeting copy number variants

3.2. Results

3.2.1. Prognostic biomarkers at PT

The cohort comprised of 20 progressive cases and 9 stable cases. Of the 20 progressive patients, 8 had unmutated IgVH status and 12 had mutated IgVH status, whilst all 9 stable patients had mutated IgVH, and it can thus be seen that IgVH mutation status was associated with disease status (Pearson Correlation P=0.026).

A total of 7 patients had high levels of CD38 expression. However, data suggests this was neither linked with disease status (Pearson correlation P=0.3) nor with IgVH status (Pearson Correlation P=0.2). This does not contradict previous studies and will thus be used as a variable as prognostic marker (Hamblin, Orchard et al. 2002).

Therefore, IgVH but not CD38 expression was associated with disease status in this study.

3.2.2. Genomic aberrations as prognostic markers

Since deletion of 13q, 11q/17p and genomic complexity will be examined as prognostic markers for good/poor prognosis in this study, it is important to establish these markers at the first time point (PT).

13q deletion at PT

At presentation, there were 19 cases (65% of all cases) with a 13q deletion (involving the minimally deleted region or MDR) with a size of deletion ranging from 0.24 Mb to 73.85 Mb. Only 2 patients from the 9 stable cases did not have a 13q deletion but 60% of progressive patients had a 13q deletion and therefore13q deletion at PT was not found to be associated with disease status in this cohort (Chi square; P=0.4). This would be due to the low number of patients in this study.
However, previous papers have shown the importance of 13q deletion size, with poor prognosis associated with larger than 2Mb 13q del (Parker, Rose-Zerilli et al. 2010). It was found in this study that, when small 13q deletions, hemizygous and homozygous, were grouped as “ClassI 13q deletions” and large and complex 13q deletion were grouped as “ClassII 13q deletions”, a significant association between the size of the 13q deletion and disease progression was found, as seen in the table 3 (Chi Squared; **P=0.017**). Patients with large 13q deletion are more likely to be progressive, while patients with small 13q deletion have a more stable disease.

Table 3: Deletion of 13q sorted into 5 different groups and categorized as stable or progressive showed large deletion in progressive patients and small deletions in stable patients.

Aberrations	*Stable*	*Progressive*	*Total*
13q del (≤ 2Mb)	5	5	10
13q homozygous del	2	0	2
13q large del (≥ 2Mb) + MDR homozygous del	0	2	2
13q large del (≥ 2Mb)	0	4	4
13q complex del (more than 2 del in the region)	0	1	1

11q and 17p aberration at PT

Previous research has shown that aberrations on 11q and 17p are linked with poor prognosis (Dohner, Stilgenbauer et al. 2000). In this cohort, 1 patient had deletion on 17p, 1 patient had deletion on 11q, 1 patient had mutation on *ATM*, and 1 patient had LOH of 17p. These 4 patients with aberrations targeting *ATM* and *TP53* were all progressive patients. However, as 16 other patients had progressive disease without *ATM* or *TP53*, this link between poor prognosis aberration and disease status was found to be statistically insignificant (Chi squared P=0.2).
Nevertheless, 3 of 4 patients with 11q/17p had unmutated IgVH status and only 1 of them did not undergo treatment, although this patient did have disease stage B. Therefore it can be seen that 11q and 17p aberrations are present in progressive patients with late stage disease and unmutated IgVH status.

Genome complexity at PT

Patients with a complex genome (≥3 CNA) have been associated with poor prognosis(Kujawski, Ouillette et al. 2008). Patients in this cohort, however, have not shown association between complexity and disease status (chi square P=0.3) or IgVH mutations status (Chi square: P=1) as a number of progressive patients (n=9, 30%) had no, or just one aberration at PT.

However, complexity was associated with CD38 expression, as 71% of patients with high level of CD38 had a complex genome, compared to 86% of patients with CD38- who did not have a complex genome (chi square **P=0.008**).
Nonetheless, patients with progressive disease had significantly larger deletions compared to stable cases (Mann Whitney P=0.06) as shown in table 4.

Table 4: Patients with progressive disease have larger deletions compared to stable patient.

[1]P=0.06; [2]P=0.003; [3]P=0.02

Aberrations	*Stable (9)*	*Progressive (20)*	*CD38 negative (22)*	*CD38 positive (7)*
Mean/Median number of CNA	1.4/1	2.7/2	1.7/1[2]	4.3/4
Complex cases	1 (11%)	7 (35%)	3 (14%)	5 (71%)
Mean/Median CNA deletion size[1]	**0.9/1 Mb**	**18/5 Mb**	**10/1 Mb[3]**	**22/12 Mb**
Normal genome	1 (11%)	4 (20%)	5 (23%)	0%

3.2.3. Genomic evolution in CLL

Genomic evolution occurs in CLL patients

When investigating aberrations at PT and FU, genomic evolution was highlighted between the two time points (Figure 17 and Figure 18):

Presentation time point: A total of 67 acquired copy number changes were found at presentation in the 29 CLL patients (mean: 2.3; range 0-8; median: 2). A number of patients (n=8, 28%) had a complex genome (≥3 CNA) and/or translocation. There were more deletions than gains (56 deletions vs 11 gains). A total of 5 patients (17%) had a normal genome (i.e. with no detectable aberrations). The most common aberrations were deletion of the 13q MDR (n=19; 65%), including 5 patients with homozygous 13q deletion, followed by whole chromosome gains (7 in 3 patients), which included trisomy 12. 5 patients had aberrations of either *TP53* or *ATM*: 2 patients had a deletion of either gene, 2 other patients had mutation on either gene and 1 patient had LOH of 17p. Many non-recurrent aberrations were present on chromosomes 2, 3, 4, 6, 7, 9, 10, 11, 12, 17, 18, 19, 21 and 22. FISH data were concordant with the SNP data and G-banding data identified 6 patients with translocations that could not be detected by SNP 6.0 microarray.

Follow up (FU) time point: A total of 93 acquired copy number changes were found at follow up in the 29 CLL patients (mean: 3.2; range 0-10; median: 2). A number of patients (n=14; 48%) had a complex genome (= ≥3 CNA and/or translocation. There were more deletions than gains (6 times more deletion: 81 deletions vs 12 gains). A total of 3 patients had a normal genome (i.e. with no aberrations). The most common aberrations found were deletion of the 13q MDR (n= 19, 65%) followed by whole chromosome gains (7 in 3 patients), which included trisomy 12. There were 5 patients with aberrations of either *TP53* or *ATM*: 2 patients had deletion of ATM, 1 patient had deletion of P53, 2 patients had either mutation of P53 or ATM, and 2 patients had LOH on 17p. Homozygous deletion was observed in 10 patients (34%) on chromosome 13 mainly (n=9), but also on chromosome 11 (n=1). FISH data were concordant with the SNP data and G-banding data showed 12 patients with translocations.

As shown in table 5, the genome of CLL patients evolved over time, with nearly 1.5 times more CNA at FU than at PT, more homozygous deletions, and more complex cases. A total of 11 patients (40%) underwent genomic evolution. There were also twice as many patients with translocations found at FU.

Table 5: Summary of SNP and FISH data showing genomic aberrations at presentation (PT) and follow up (FU)

Aberrations	*PT*	*FU*
Total number of CNA	67	93
Mean number of CNA	2.3	3.2
CNA count range	0-8	0-10
Homozygous deletions	5 (17%)	10 (35%)
Complex cases	9 (31%)	14 (48%)
Patients with trisomy	3 (10%)	3 (10%)
Normal Genomes	5 (17%)	3 (10%)
Patients with translocation	6 (20%)	12 (41%)

Genomic evolution causes deletion of large portions of DNA but can also target specific genes

Having shown evidence for a significantly larger number of CNA at FU compared to PT, it was further shown that genomic evolution in CLL patients resulted in the overall presence of larger deletions and gains. Patients at FU, had more than twice the number of deletion sized 5-15Mb. It was also found that the size of secondary aberrations due to genomic evolution can range from 0.05Mb to 50Mb (table 6).

Table 6: The genomic aberrations found at PT and FU arranged by size group shows genomic evolution occurs in all sizes, except trisomy

Copy Number acquired size	*PT*	*FU*
0.05 - 0.1Mb	2	5
0.1 - 0.5Mb	7	9
0.5 - 1Mb	8	10
1 - 5Mb	26	32
5 - 15Mb	5	11
15 - 50Mb	9	15
50 Mb +	3	4
Trisomy	7	7
Total	**67**	**93**

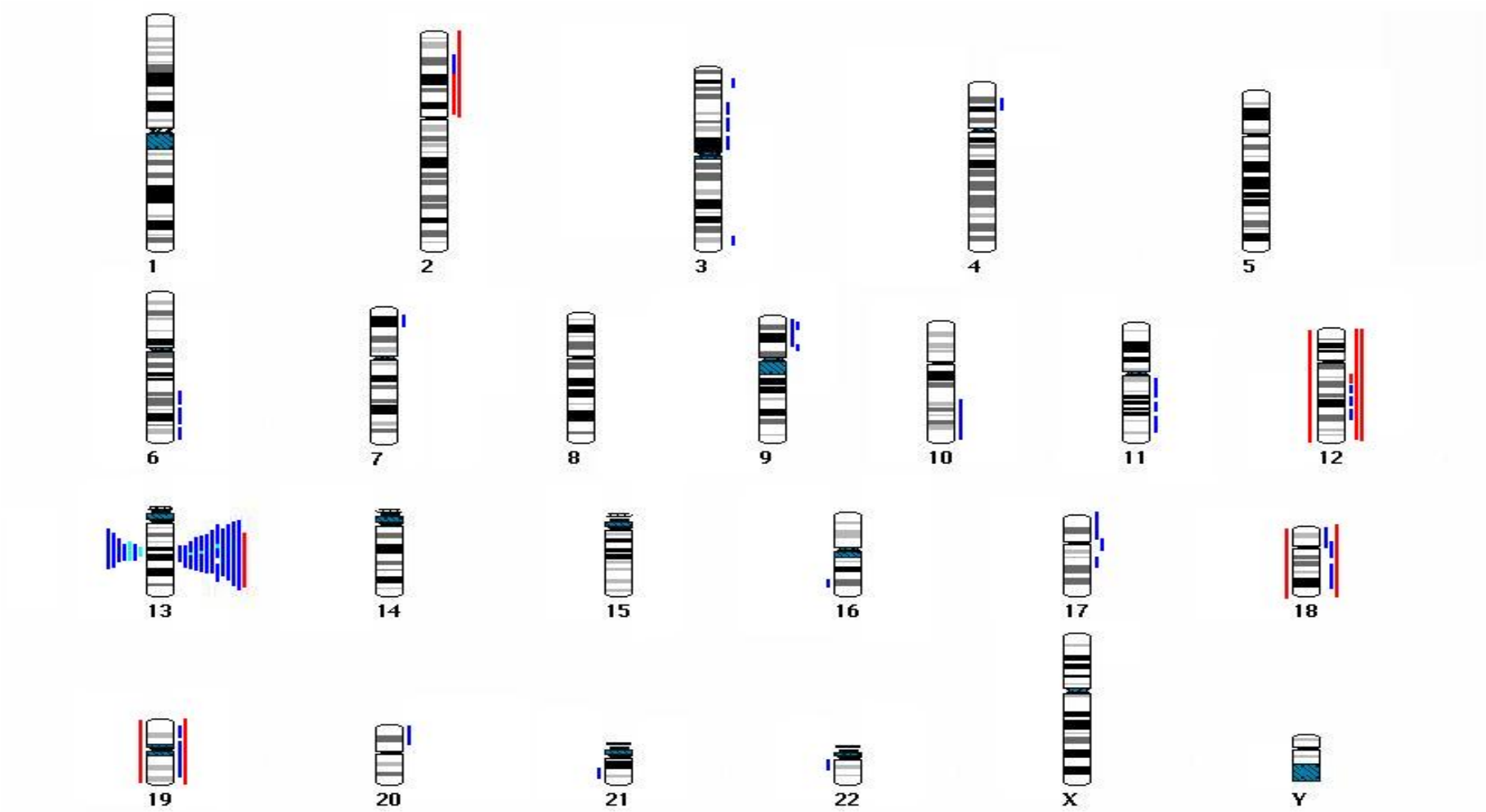


Figure 177: Karyogram showing copy number deletions (dark blue for mono-allelic and light blue for bi-allelic) and gains (red) arranged by size, of all 29 patients (stable cases on the left side of the chromosome and progressive patients on the right side) at **presentation (PT)**

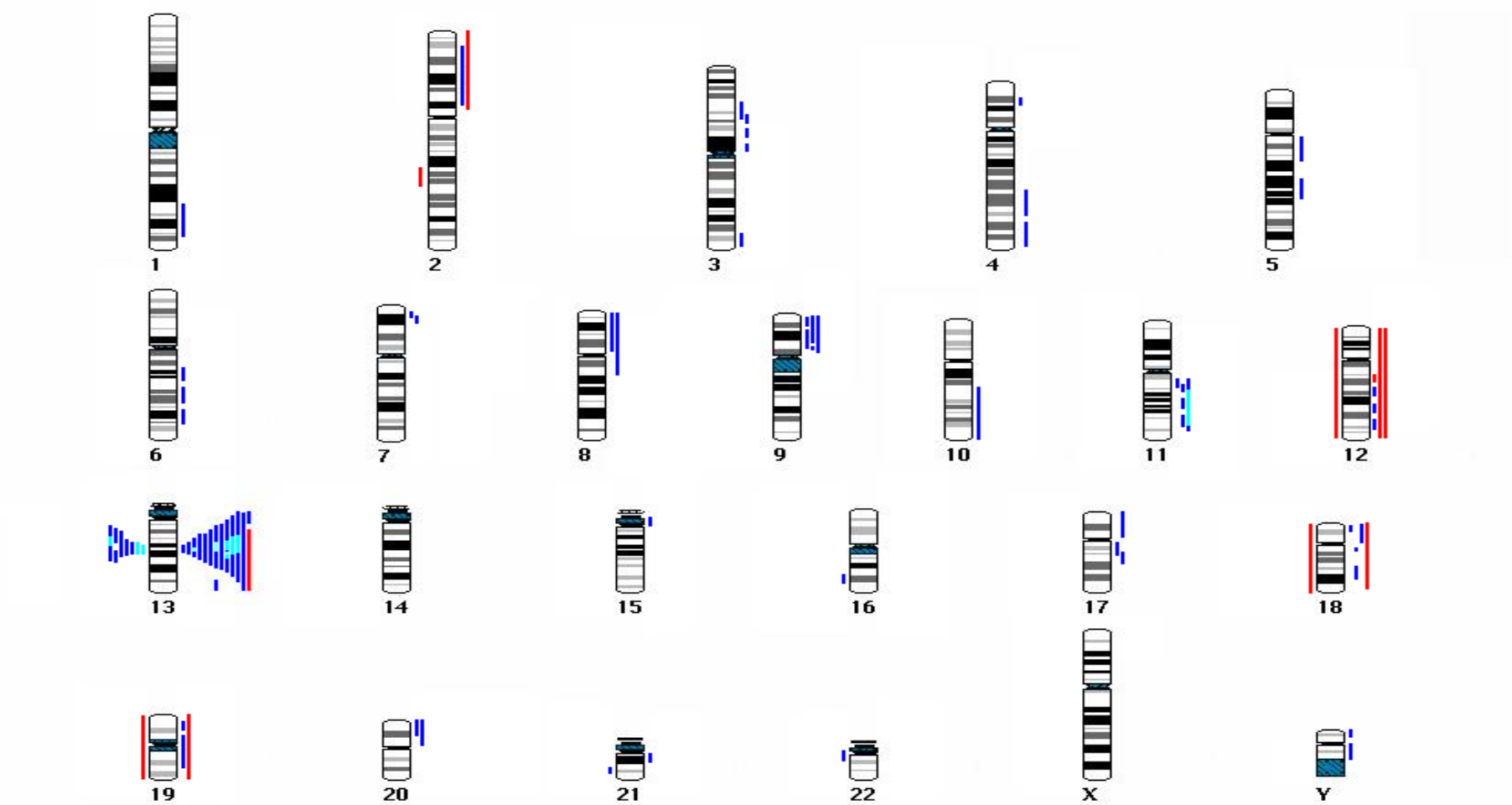


Figure 18: Karyogram showing copy number deletions (dark blue for mono-allelic and light blue for bi-allelic) and gains (red) arranged by size, of all 29 patients (stable cases on the left side of the chromosome and progressive patients on the right side) at **Follow up (FU)**

Genomic evolution occurs throughout the genome

Secondary aberrations were found to occur on various chromosomes, including chromosome 1, 2, 4, 5, 7, 8, 9, 11, 13, 15, 18, 20, 21 and Y. Table 7 shows the list of acquired CNA at FU. Chromosome 13 was found to be most affected by genomic evolution. Deletion on 13q was shown to become larger (n=2) or biallelic (n=4), however no patient was shown to acquire a new deletion of the 13q MDR.

Table 7: List of CNA acquired at FU through genomic evolution. Del = deletion; (x) = chromosome number; (x-x) = specific deleted breakpoint on chromosome

Copy number change	*Size of CNA (in Mb)*
Del(1)(230.76-233.2)	2.4
Enhanced(2)(120.78-120.89)	0.1
Del(4)(131.5-143.0)	11.4
Del(4)(179.53-191.26)	11.7
Del(5)(79.2-91.1)	11.9
Del(5)(59.18-64.26)	5
Del(7)(2.24-2.41)	0.1
Del(8)(0.0-43.9)	43.9
Del(8)(0.0-70.79)	70.7
Del(9)(21.69-29.38)	7.6
Del(9)(0.0-15.15)	15.1
Del(11)(79.3-123.72)	44.4
Del(11)(63.42-65.23)	1.8
Del(13)(51.55-51.65)	0.09
Del(13)(39.31-40.15)	0.8
Del(15)(29.07-29.12)	0.05
Del(18)(1.7-2.86)	1.1
Del(20)(0.0-12.65)	12.6
Del(21)(31.87-31.96)	0.9
Del(Y)(4.61-6.8)	2.1
Del(Y)(7.93-27.18)	19.2
Homozygous Del(11)(81.12-119.91)	38.7
Homozygous Del(13)(48.58-50.37)	17.9
Homozygous Del(13)(49.39-50.39)	1
Homozygous Del(13)(47.25-48.13)	0.8
Homozygous Del(13)(48.71-50.76)	20.4

Do patients with genomic evolution have a longer follow up time?

Follow up samples were taken between 10 and 180 months, and it is therefore important to establish any link between genomic evolution and time between PT and FU. Patients who showed evidence of genomic evolution had an average of 77 months between PT and FU sample, whereas patients without a genomic evolution also had an

average of 77 months between PT and FU. This esablishes that the time difference between PT and FU sample has no influence on genomic evolution (T-Test, P=0.9)

3.2.4. Genomic evolution occurs in progressive as well as stable patients

Since patients with progressive disease have been linked with genomic complexity (Ouillette, Fossum et al. 2010), it could be assumed that genomic evolution would occur in patients with a progressive disease.

However, this cohort shows that there was no association between genomic evolution and disease status (Chi square; P=0.4) as both groups of patients showed evidence of new aberrations at FU (Table 8)

Table 8: Genomic evolution is seen in patient with progressive as well as stable disease status

Sub-group	*Genomic evolution*	*Without evolution*	*Total*
Progressive patients	9 (45%)	11 (55%)	20
Stable patients	2 (22%)	7 (78%)	9

Nonetheless, the scale of genomic evolution was different in the two groups of patients as stable patients go on to acquire less CNA than progressive patients, as shown in figure 19. Approximately 50% (n=4) of progressive patients who undergo genomic evolution acquire 3 or more new CNA, whilst all stable patients that undergo genomic evolution acquire 2 or less new CNA (mean: 1.1 vs 0.3 CNA respectively).

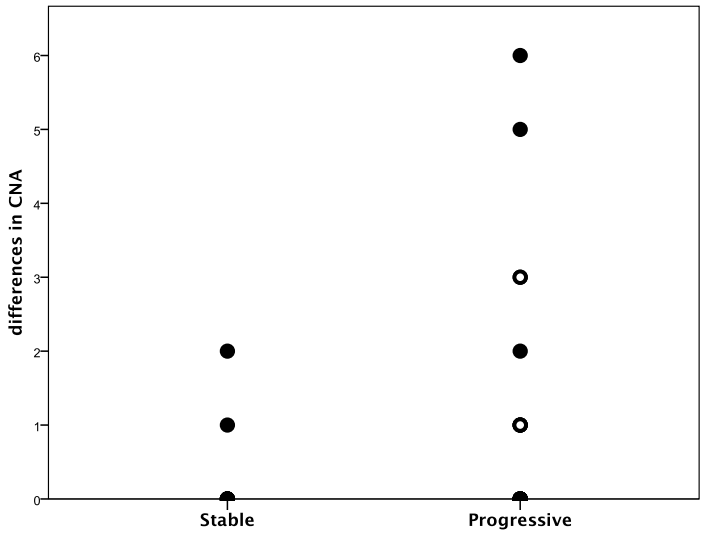


Figure 18: The number of CNA acquired through genomic evolution is higher in progressive patients than in stable patients. The Y axis represents the number of CNA acquired as a result of genomic evolution. The full circles represent a total of 1 patient; empty circles represent a total of 2 patients.

A large difference in the size of CNA acquired between progressive patients and stable patients was also shown (table 9). Statistic analysis demonstrated a trend of larger secondary aberrations on progressive compared to stable patients (T-Test; **P=0.06**). Therefore, it can be seen that genomic evolution occurs in both; stable and progressive patients, however the latter group of patients acquired more, and larger, secondary aberrations.

Table 9: The average deletion and gain size in progressive and stable cases reveals a large difference between the two groups

Sub-group	*Average total deletion size of genomic evolution (range)*	*Average total gain size of genomic evolution (range)*
Stable patients (9)	0.3 Mb (0- 11.2Mb)	0.12 Mb (0- 1.1Mb)
Progressive patients (20)	16.7 Mb (0-117Mb)	0.03 Mb (0-0.1Mb)

3.2.5. Do prognostic markers predict genomic evolution?

Genomic evolution was investigated in relation to prognostic markers in order to established any association and prediction for the occurrence of secondary aberrations in CLL patient. The following was found;

Disease stage

All 29 patients in this cohort had either mBL (n=7), CLL stage A0 (n=17) or A0/1 (n=5). It is therefore clear that Binet disease stage cannot predict genomic evolution, as patients with early stage disease undergo genomic evolution.

IgVH mutation status and CD38 expression

There was no significant correlation between IgVH mutation status or CD38 with genomic evolution (Chi square; P=1 and P=0.6 respectively). As shown in table 10, patients with unmutated IgVH status were as likely to undergo genomic evolution as patients with mutated IgVH status. Furthermore, there was no association between IgVH mutation status and the number of secondary aberrations, (Mann Whitney P=0.8) or the total size of deletions acquired by evolution (Mann Whitney P=0.8). A similar result was found with CD38 expression.

Table 10: The occurrence of genomic evolution and the scale of genomic evolution (average number and size of secondary aberrations) between patients with mutated or unmutated IgVH status, and between patients with low or high CD38 expression. [1]P=0.8; [2]P=0.8; [3]P=0.7; [4]P=0.7

	IgVH mutated (21)	***IgVH unmutated (8)***	***CD38 negative (22)***	***CD38 positive (7)***
Number of cases with genomic evolution	8 (38%)	3 (38%)	9 (41%)	2 (29%)
Number of CNA acquired/patient	0.75[1]	1.25	1[3]	0.86
Range of CNA acquired	1 - 5	1 - 6	1 – 6	2 – 5
Total del size of evolution/patient	7.5 Mb[2]	22.5 Mb	10 Mb[4]	16.9 Mb

It can thus be concluded that genomic evolution cannot be predicted by IgVH mutation or CD38 expression, as patients with either prognostic marker have been shown to acquire secondary aberrations.

13q deletion

13q deletion size was grouped into ClassI and ClassII (i.e. depending on size, where class I deletions are smaller than 2Mb and class II deletions are larger than 2Mb). It is thus examined whether 13q deletion size can predict genomic evolution.
Patients with large 13q deletions had a trend towards genomic evolution, with only 25% of patients with small 13q acquiring secondary aberrations, compared to 70% of patients with large 13q deletions (Chi square P=0.7). In addition, as shown in table 11, patients with large 13q deletion were associated with a high number of CNA through genomic evolution (Mann Whitney test; **P=0.03**), and showed larger deletion sizes (Mann Whitney test; **P=0.07**).

Table 11: The occurrence of genomic evolution and the scale of genomic evolution (average number and size of secondary aberrations) in patients with small and large 13q deletion.

13q deletion groups:	***Small 13q del (12)***	***Large 13q del (7)***
Genomic evolution	3 (25%)	5 (71%)
Number of CNA gained at FU/patient	0.3	1.7
Size of deletion acquired/patient	2.7 Mb	17.7 Mb

17p & 11q aberrations

Patients with 11q and 17p aberrations are linked with poor prognosis as well as genomic instability.

A total of 4 patients had aberrations targeting *ATM* and *TP53* and 3 of these acquired new aberrations at FU. However, 8 other patients, without 11q or 17p aberration also acquired aberrations at FU, and so poor prognosis aberration was not found to be linked with genomic evolution in this study (Chi square: P=0.1).
However, patients with 11q/17p aberration are more likely to acquire a higher number of CNA (Mann-Whitney, **P=0.01**), as well as larger deletion size (Mann-Whitney, **P=0.01**). Large differences in genomic evolution between the two groups are noted in table 12. It can be seen, therefore, that poor prognosis aberrations 11q and 17p were not found to be associated with genomic complexity, although patients with deletion on *ATM* or *TP53* had a large scale genomic evolution.

Table 12: The occurrence of genomic evolution and the scale of genomic evolution (average number and size of secondary aberrations) in patients with and without *TP53/ATM* aberrations

Aberrations	***No* TP53 *or* ATM *aberration (25)***	**TP53 *or* ATM *aberration (4)***
Cases with genomic evolution	8 (32%)	3 (75%)
Number of CNA acquired/ patient	0.5	3
CNA deletion acquired size/patient	1.6 Mb	74 Mb

Genomic complexity

In this cohort, patients with a complex genome at PT are equally likely to acquire new aberrations as patients without a complex genome (Chi square; P=1). Nonetheless, as shown in table 13, patients with a complex genome acquire larger secondary aberrations at FU, although this is statistically insignificant (Mann Whitney; P=0.7). Therefore, complexity does not predict genomic evolution, as patients without a complex genome at PT have been shown to acquire new aberrations at FU.

Table 13: The occurrence of genomic evolution and the scale of genomic evolution (average number and size of secondary aberrations) in patients with and without a complex genome at PT

Aberrations	***No Complex genome at PT (21)***	***Complex genome at PT (8)***
Cases with genomic evolution	8 (27%)	3 (27%)
Number of CNA acquired/ patient	0.7	1.2
CNA deletion acquired size/patient	5.9 Mb	26.4 Mb

Results summary

This study has shown that genomic evolution is not associated with any prognostic markers. However, patients with 11q/17p aberration or a large 13q deletion acquire a significantly higher number, and larger, secondary aberrations.

3.2.6. Does genomic evolution have an impact on CLL disease?

Genomic aberrations have been shown to result in 2 different disease courses; deletion on 13q leads to a different disease progression to deletion on 17p or 11q. Therefore, having noted the presence of secondary aberration, both recurrent and non-recurrent, it is essential to examine the impact of secondary aberration on disease progression. For this, genomic evolution will be analysed in relation to disease stage, treatment, and WBC count. Progression to late stage, requirement for treatment and the rise of WBC count are all symptoms of disease progression.

Genomic evolution and change in disease status

A total of 12 patients progressed from mBL or stage A0 to late stage disease B/C, while the rest of the patients (n=14) remained at early stage A0 or mBL.
Genomic evolution occurred in 5 patients (42%) who progressed to late stage disease. However, 4 patients (29%) who remained at early stage disease also acquired secondary aberrations at FU, and consequently genomic evolution was found not to be associated with disease stage progression (chi square, P=0.6).

Genomic evolution and treatment / TTFT

Genomic evolution was not associated with treatment as 66% of patients with genomic evolution received treatment compared to 50% of patients without genomic evolution (chi square; P=0.6). In addition, there was no association between number or size of secondary aberrations and treatment (Mann Whitney, P=0.4; P=0.4 respectively). Genomic evolution was also not associated with TTFT, as no significant different in TTFT was found between patients who had undergone genomic evolution and patients who did not (mean: 47 vs. 65. T-Test; P=0.2) (figure 20).

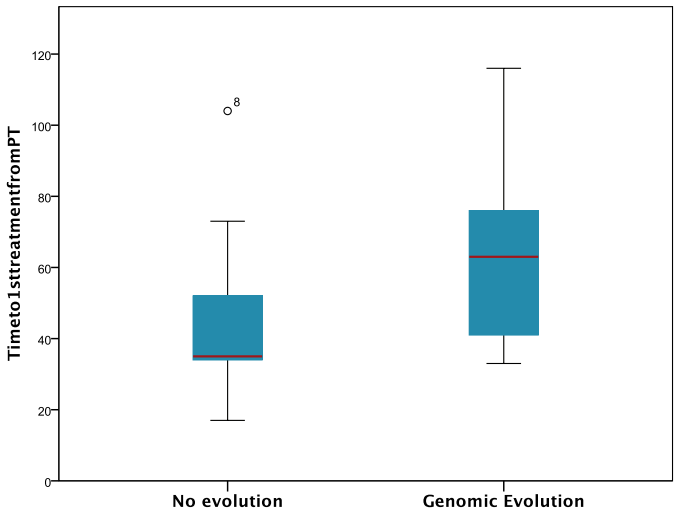


Figure 19: Box-plot showing no significant difference in TTFT between patients who had undergone genomic evolution and patients who had not.

Genomic evolution and White Blood Cell count

Patients with genomic evolution reached an average maximum WBC count of $85x10^9$/L, compared to $92x10^9$/L in the rest of the patients, suggesting no link between genomic evolution and maximum WBC (Mann Whitney, P=0.7).
Furthermore, the average rate of WBC increase was $1.7x10^9$/L/month in patients with acquired secondary aberrations, compared to $2.8x10^9$/L/month in the rest of the cohort, further suggesting no association between the two factors (Mann whitney, P=0.2). Therefore no link was found between genomic evolution and changes in WBC in CLL patients.

Results summary

It can be concluded that genomic evolution does not impact on disease progression, treatment or WBC count increase, as patients with secondary aberrations at FU do not show significant differences in disease progression, TTFT or WBC count.

Table 144: Prognostic markers, clinical data and genomic aberration of all 29 patients in the cohort. [1]S=Stable;P=Progressive; [2]M=Mutated;U=Unmutated;[3]in months; nt=not treated; [4]in x10^9/L/month; T=treated between PT and FU; [5]del=deletion; enh=gain; 17p=deletion on chr 17 including P53; 11q=deletion on chr 11 including ATM; 13q=deletion on 13q MDR; (s)=smaller than 2Mb; (L)=larger than 2Mb; x2=including a small homozygous region; (h)= homozygous 13q deletion; Tri=trisomy; (2)=number of deletion on the chromosome; (g)= smaller than 0.1Mb, targeting gene, N=normal; Bold=only at FU; [6]B=Balanced translocation; U=unbalanced translocation; Bold=only at FU

ID	Disease status[1]	IgVH/ CD38[2]	Alive/ dead	TTFT after PT [3]	Number of treatment	WBC rise[4]	Disease stage at PT	Disease stage at FU	Genomic aberration[5]	Trans-location[6]
1	S	M/-	A	nt	0	0.52	mBL	A0	13q(s); **13q(L)x2**	
5	P	M/+	D	52	3	T	A0	C	Tri12; Tri18; Tri19; 13q(L); del3(2); del9(2)	
7	P	M/-	A	nt	1	0.00	A	A0	13q(L); **13q(2)x2; del18; LOH13q**	B, **U**
8	S	M/-	A	nt	0	0.00	mBL	mBL	13q(s); del16(g)	U
9	P	M/-	A	nt	2	0.00	A	A	13q(L)x2; **11q; LOH17p**	
10	S	M/-	D	nt	0	0.04	A0	mBL	13q(s)	
11	P	M/-	A	46	2	T	A0	B	N	
12	P	M/-	A	104	1	0.00	A0	C	N	
14	P	U/+	A	73	1	0.00	mBL	mBL	Tri12	
15	P	M/+	A	74	2	T	mBL	C	13q(L); del2; enh13; enh2; **del13(g); del15(g)**	U
16	P	U/+	D	35	2	4.21	A0	A2	del2p; del18p	
18	P	U/-	A	31	2	5.26	A1	C	13q(s)	
19	S	M/-	A	nt	0	0.13	A0	A0	(h)13q(s); LOH13q(2)	
21	S	M/-	A	nt	0	0.98	A0	A0	13q(s); del21(g); **enh2(g); del13(g)**	
22	S	M/-	A	nt	0	0.02	A0	A0	N	

23	S	M/-	A	nt	0	0.12	mBL	A0	(h)del13q(s); LOH13q	**U**
25	S	M/+	A	nt	0	0.00	mBL	mBL	Tri12; tri18; tri19	
28	P	M/+	A	nt	0	0.02	A	B	13q(L); 17p; del17(g); del18(2); **11qx2; 20p; delY(2)**	U
29	P	M/-	A	34	2	2.94	A0	A1	13q(s); del4; 9p; 20p;	
30	P	U/-	A	52	1	4.24	A0	B	Del10; del12(3); enh12; del19(2); LOH17p; **8p; del9(2)**	
32	P	M/-	A	116	1	0.53	A0	A1	13q(s)x2; **del21(g)**	
33	P	U/-	A	41	4	T	**A0**	**B**	**Del7(g)**	**B**
34	P	M/-	A	34	4	T	A0	B	13q(s)	
35	P	M/-	D	76	1	0.36	A0	C	13q(L); del7; **13q(L)x2**	**U**
86	S	M/-	A	nt	0	0.00	mBL	mBL	13q(s); del22(g)	B
247	P	U/+	A	17	1	5.13	A0	A	11q(3); del3(3); del17(g)	
248	P	U/-	A	nt	0	0.78	A0	A0	13q(L)	
249	P	M/-	A	nt	0	2.36	A0	A1	13q(L)(3)x2; del6(3)	U
250	P	U/-	A	33	3	n/a	A1	Terminal disease	**Del1; del4(2); del5(2); del8; LOH17p**	**U**

3.3. Case study

It is clear that the number of patients in this cohort is too small to show any statistically significant evidence of the impact of genomic evolution on disease progression. And since this study gathered data on genomic aberration from G-banding, FISH as well as SNP6.0, and had treatment and WBC count data in addition to prognostic marker information (see Appendix 3), each patient was thoroughly examined as case studies to uncover the role of secondary aberration in relation to disease progression, treatment and WBC count (summarised in table 14).

Patients were divided into four groups according to disease status (progressive or stable) and genomic evolution.

Patiens with a stable disease have a stable genome

Patient 10 had a mutated IgVH status, low CD38 expression levels and was diagnosed in 1989 with CLL stage A0. The PT sample was taken in 1993 and showed a small (≤2Mb) 13q deletion which was confirmed by FISH. Clinically, the WBC remained lower than $12x10^9$/L and the disease was stable. The FU sample taken 15 years later showed no evidence of secondary aberration, although the FISH data showed a small population of cells with homozygous deletion of the 13q MDR (6.5%). The patient died a year later (2009) from unrelated causes (Aortic Aneurysm).

Patient 19 had a mutated IgVH status, low CD38 expression level and was diagnosed in 1999 (PT) with CLL stage A0. The PT sample was taken at diagnosis, and showed a homozygous deletion of 13q (≤1Mb) which was confirmed by FISH. In addition, a region of LOH on chromosome 13 (74Mb, from 40.51-114.1) was observed by SNP6.0. Disease and WBC count remained stable (at approx 30 $x10^9$/L), and the FU sample taken 9 years later showed no genomic evolution, although the karyotype showed a small population of cells with an 11q25 deletion (3 cells).

Patient 23 had a mutated IgVH status, low CD38 expression levels, and was diagnosed in 1985 with mBL. The PT sample was taken in 1999 and revealed a small but biallelic deletion of 13q (≤1Mb), which was confirmed by FISH (86% homozygous deletion). In addition a region of LOH on chromosome 13 (74Mb size, from 40.51-114.1) was noted. A slow increase in WBC count ($0.07x10^9$ per month, or from $5x10^9$ to $13x10^9$ in 5 years) resulted in the patient being classified as CLL stage A0 in 2009. The FU sample at that stage (10 years after PT) showed no genomic evolution, although the

karyotype data revealed structural rearrangements and decentric chromosomes, which were not identified by SNP6.0 profiling.

Patient 22 had a mutated IgVH status, low CD38 expression levels, and was diagnosed in 1996 with CLL stage A0. The PT sample was taken in 1998 and the SNP array showed no evidence of genomic aberrations. WBC count remained stable at around $30x10^9$/L. The FU sample was taken 10 years later and showed no genomic evolution by array or FISH.

Patient 8 had a mutated IgVH status, low CD38 expression levels and was diagnosed in 1993 with mBL. The PT sample was taken in 1997, and showed a small (≥1Mb) 13q deletion as well as a small deletion (<0.1Mb) on chromosome 16 involving the *CDH13* gene. The FISH data also revealed a small portion of tumour cells (25%) with a homozygous deletion of 13q. The karyotype suggested a complex genome, with a translocation between chromosome 2, 5 and 13. Nonetheless, the patient remained stable with mBL and very low WBC count ($5x10^9$/L) and the FU sample taken 10 years later showed no evolution, as well as a similar karyotype to the PT sample.

Patient 25 had a mutated IgVH status but high CD38 expression levels, and was diagnosed in 1999 with mBL. The PT sample was taken in 2003 and showed trisomy 12, which was confirmed by FISH, in addition to trisomies of chromosomes 18 and 19. Clinically, the disease was stable and white blood cell count was lower than $10x10^9$/L. The FU sample taken more than 5 years later showed no evolution by array or FISH.

Patient 86 had a mutated IgVH status, low CD38 expression levels and was diagnosed in 1989 with mBL. The PT sample was taken in 2003 and showed deletion of 13q (≥1Mb) which was confirmed by FISH, as well as deletion (0.1Mb) of *GSTT1* on chromosome 22. Clinically, the patient's WBC remained stable at <5x109/L. The FU sample taken 6 years later showed no genomic evolution by array or FISH.

Patients with a stable disease course can exhibit genomic evolution

Patient 1 had a mutated IgVH status, low CD38 expression levels and was diagnosed in 1998 with mBL. The PT sample was taken in 1999 and showed a small (≤1Mb) 13q hemizygous deletion, but FISH data showed 12% of cells having a homozygous deletion. Clinically, the WBC count remained stable at $15x10^9$/L, until 2008 when it suddenly doubled to $36x10^9$/L. The FU sample taken 9 years after PT showed a large (3Mb) deletion of 13q which also involved a homozygous deletion of the MDR region

(figure 21). The FISH results showed a larger proportion of tumour cells (45%) with a homozygous 13q deletion.

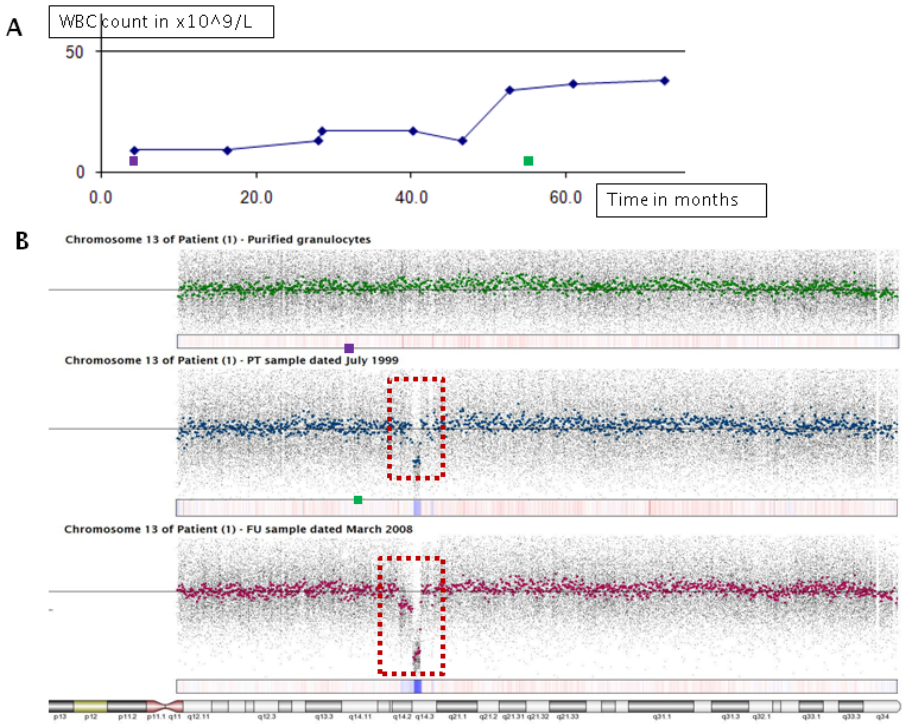


Figure 20: ***A.*** graph showing WBC count (x109/L) against months. PT and FU sample are represented by small squares (purple and green respectively). ***B***. SNP array data showing small 13q deletion at PT and large deletion of 13q with homozygous deletion of the MDR.

Patient 21 had mutated IgVH status, low CD38 expression levels, and was diagnosed in 1998 with mBL. The PT sample taken in 2006 showed a small (≥1Mb) 13q deletion involving the MDR, which was confirmed as hemizygous by FISH, as well as deletion (<0.1Mb) of *RUNX1* on chromosome 21. The WBC count rose between PT and FU at a rate of $1x10^9$/L/month and up to a maximum of 76 $x10^9$/L. The FU sample taken 2 years after PT showed no change in the 13q deletion by the array, but FISH data showed evolution of 13q deletion from hemizygous to homozygous. In addition, the array showed acquisition of a small deletion (0.1Mb) on chr 13 involving the *NEK3* gene (figure 22).

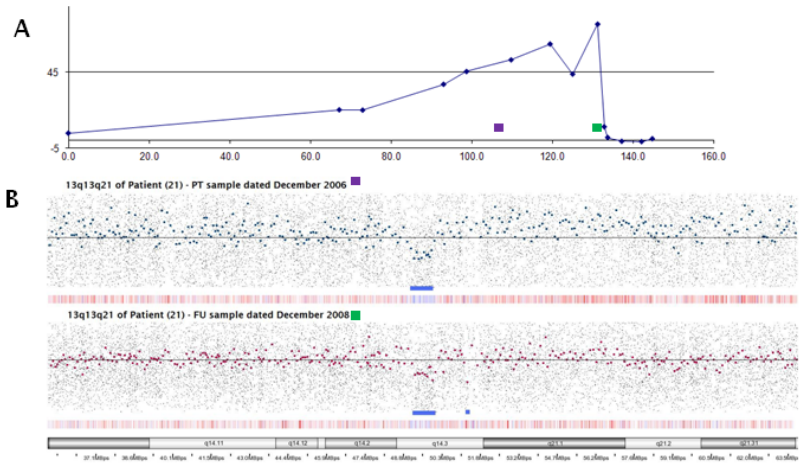


Figure 21: ***A.*** graph showing WBC count (x10^9/L) against months. PT and FU sample are represented by small squares (purple and green respectively). ***B.*** SNP array data showing deletion on chr13 at both time points as well as secondary aberration at FU involving the *NEK3*. ***C.*** Map of chromosome 13 with breakpoints.

Patients with progressive disease show genomic evolution

Patient 9 had a mutated IgVH status and low CD38 expression levels. The patient was diagnosed in 1990 with CLL stage A0 and was treated two years later, and again in 2006, due to high WBC. The patient was, however, intolerant to treatment and WBC count was not affected. The PT sample was taken in 2007, when patient was at stage A with a WBC count of 130x10^9/L, and showed a large 13q deletion, (4.6 Mb) with homozygous deletion of the MDR region, which was confirmed by FISH, as well as mutation of *TP53*. The FU sample taken a year later, showed genomic evolution with deletion on chromosome 11 (1.8 Mb) involving many genes, amongst which was REL A. Furthermore, the FU sample also showed LOH of 17p, which was not seen at PT. Clinically, however, the patient was still at CLL stage A, but with a rising WBC count (rate of 2.2x10^9/L/month).

Patient 28 had a mutated IgVH status but high CD38 expression levels, and was diagnosed with mBL in 1994. The PT sample was taken in 2000 and showed a complex genome with large deletion of 13q, loss of 17p and two deletions on 18p. The FISH results also showed a small population of cells (6%) with ATM and P53 deletions, and karyotype revealed that deletions on chromosome 18 were due to translocations. The patient progressed to stage A2 within 3 years with very aggressive clinical symptoms (groin nodes), but a very low and stable WBC count (less than 14x10^9/L). The patient reached stage B in 2007. The FU sample taken 9 years after PT (in 2009) showed large genomic evolution, with deletion of 11q, including homozygous deletion of the ATM

region, as well as deletions on chromosome 20 and 17 (involving the *BRIP1* cancer gene). The FISH results were taken at various periods between PT and FU and showed a progressive increase in the population that had *TP53* as well at *ATM* deletion (table 14). The karyotype showed complex translocations.

Table 15: Disease progression (clinical stage), the progressive acquisition of *ATM* and *TP53* deletion shown by FISH, and the SNP array data of patient 28

Date	***1996***	***2000 (PT)***	***2001***	***2004***	***2009 (FU)***
CLL stage	mBL	mBL	A0	A2	B
FISH result TP53	6%	60%	.	.	75%
FISH result ATM	.	3%	6%	29%	39%
Genomic (SNP)	.	13q, 17p, 18q	.	.	+ 11qx2; 20p; 17q

Patient 30 had an unmutated IgVH status, low CD38 expression levels, and was diagnosed in 2000 with CLL stage A0. The PT sample was taken in 2004, when patient was still at clinical stage A0, and showed a very complex genome; multiple deletions on chromosome 12 and 19, deletion on chromosome 10 as well as LOH on 17p. The WBC increased between 2004 (PT) and 2007 at a rate of 0.65×10^9/L/month, but this suddenly changed in 2007 to 13.21×10^9/L/month, reaching 221×10^9/L in 2008. The FU sample taken 4 years after PT (in 2008), when patient was at stage B and just before treatment, showed large genomic evolution with deletions on chromosome 8 and 9p (figure 23). The karyotype also showed a complex genome with unbalanced translocation (which was not seen at PT). The WBC count has been stable since treatment.

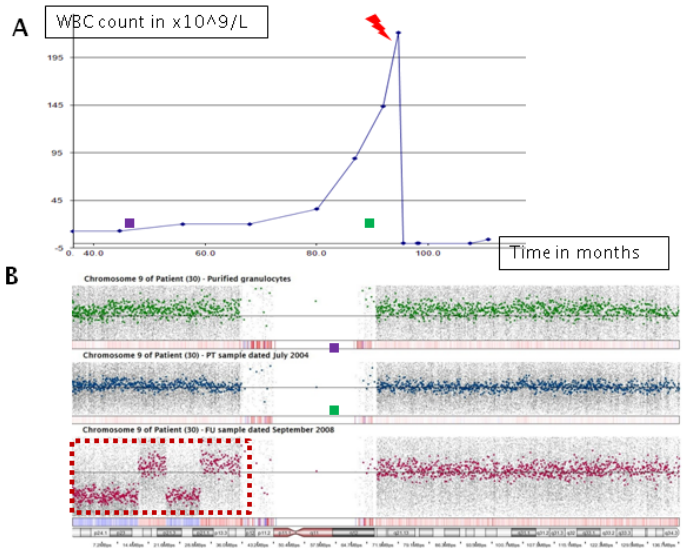


Figure 22: ***A.*** graph showing WBC count (x10^9/L) against months. PT and FU sample are represented by small squares (purple and green respectively). ***B.*** SNP array data showing deletion on chr8 occurring only at FU and map of chromosome 8 with breakpoints.

Patient 250 had an unmutated IgVH status, low CD38 expression levels, and was diagnosed in 1989 with mBL. The PT sample was taken in 2001, when patient was at stage A1, and showed mutation of *ATM* (but not of *TP53*), but no genomic aberrations were shown on the array (normal genome). Within 3 years, the patient required treatment, which was followed by relapse and complications (Hodgkins disease) and further treatments, followed again by relapse. The follow up sample taken 9 years after PT (in 2009) showed large genomic evolution with deletions on chromosome 1, 4, 5 and 8 as well as LOH on 17p. The WBC count data is not available. The patient is at a terminal stage.

Patient 33 had an unmutated IgVH status, low CD38 expression levels. and was diagnosed in 1997 with CLL stage A0. The PT sample was taken in 1998 and showed no aberrations (normal genome) by array or FISH. The patient progressed to stage B in 2002, with a WBC count of 200x10^9/L, and was given a first round of treatment. The disease remained stable until 2005, when suddenly a rise in WBC count which reached (x10^9/L) was noted, and patient underwent a second round of treatment in 2007. Just before treatment, the FU sample (8 years after PT) showed no large aberrations, but only a small deletion (0.1Mb) on chromosome 7 which involved a number of genes. Furthermore, the karyotype showed a small population with translocation between chromosome 4 and 15. Two years later, in 2009, the disease progressed to Richters, and the patient required a further round of treatment.

Patient 7 had a mutated IgVH status, low CD38 expression levels, and was diagnosed in 1981 with CLL stage A0. The patient underwent multiple treatments from 1982 to 1997 due to high WBC count and swollen cervical nodes. The PT sample was taken in 2000 and revealed a large deletion (3Mb) on chromosome 13, which was confirmed by FISH. From then, the WBC count decreased (not treated), despite the fact that FISH data in 2005 and FU genomic data in 2007 (7 years after PT) revealed genomic evolution: patient acquired 2 homozygous deletion in the 13q, LOH (40.6-68.7) on chromosome 13, as well as a deletion on 18p (1Mb).The karyotype data revealed that these deletions were due to translocation between chromosome 13 and 18. The WBC count and disease course has remained stable.

Patient 35 had a mutated IgVH status, low CD38 expression levels, and was diagnosed in 1998 with CLL stage A0.The PT sample was taken in 2001 and revealed a large deletion (2.4 Mb) on chromosome 13, which was confirmed by FISH, and a small (0.1Mb) deletion on chromosome 7 (a region devoid of any known genes). Clinically, changes occurred between PT and FU; Between diagnosis and 2004 the WBC increase rate was $0.12x10^9$/L/month whilst between 2004 and 2007 (FU) the WBC increase rate was 0.36 $x10^9$/L/ month, reaching a maximum of 44 $x10^9$/L before being treated. Furthermore, the disease progressed to stage A1 in 2004 and then to stage C in 2007 (FU). The FU sample taken 6 years after PT, revealed a much larger 13q deletion (3Mb) as well as homozygous deletion of the MDR region in addition to the aberrations seen at PT (figure 24). Also, the karyotype data showed translocation between chromosome 9 and chromosome 13. Clinically, treatment did not reduce WBC count and patient acquired different complications such as severe Pancytopenia (very low number of blood cells) and CMV reactivation. Patient died in 2009.

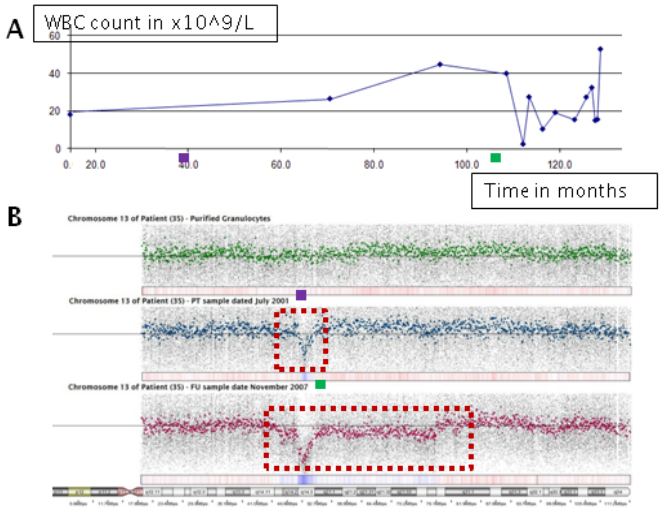


Figure 23: ***A.*** graph showing WBC count (x109/L) against months. PT and FU sample are represented by small squares (purple and green respectively). ***B.*** SNP array data showing deletion on chr13 with evolution at FU, and map of chromosome 13 with breakpoints.

Patient 15 had a mutated IgVH status but high CD38 expression levels, and was diagnosed in 2001 (PT) with mBL. The PT sample was taken at diagnosis and showed deletions on 2p and 13q as well as gain of the large arm of chromosome 13, and gain on the short arm of chromosome 2. The karyotype data revealed translocation between chromosome 2 and 13, as well a balanced translocation between chromosome 5 and 15. The patient progressed to CLL stage A0 in 2006, and the stable WBC count seen since diagnosis suddenly turned into a high rise, from $28x10^9$/L to $76x10^9$/L as well as progression to stage C in 2008, which resulted in a first round of treatment. The follow up sample was taken in 2009 or 7 years after PT, and just before a second round of treatment. At this stage, genomic data showed the acquisition of two new small aberrations; deletions on chromosome 13 (0.8Mb) involving *FOXO1* and on chromosome 15 (<0.1Mb) involving *TRPM1*. After the FU sample and a second round of treatment, the WBC count was seen to rise again.

Patient 32 had a mutated IgVH status, low CD38 expression levels, and was diagnosed in 1997 with CLL stage A0. The PT sample was taken in 1998 and revealed deletion (1.2Mb) on 13q, with homozygous deletion of the MDR, which was confirmed by FISH. The WBC count was stable at $40x10^9$/L until 2006, when it suddenly rose to $110x10^9$/L, and the patient, at clinical stage A1, underwent treatment in 2009 (FU). The FU sample taken 9 years after PT and just before the treatment showed the acquisition of a new small aberration; deletion on chromosome 21 (<0.1Mb; involving *SOD1*). The FISH data showed no large genomic evolution on the 13q deletion. The WBC count remained stable after FU.

Patients can develop progressive disease without genomic evolution

Patient 11 had a mutated IgVH status, low CD38 expression levels, and was diagnosed in 2002 (PT) with CLL stage A0 and high WBC ($53x10^9$/L). The PT sample was taken at diagnosis and revealed no aberrations (normal genome), although FISH data showed hemizygous deletion on 13q (10%). After two stable years, the WBC count suddenly started to rise and reached 115 x 10^9/L. Patient also progressed to clinical stage C, and finally underwent a first round of treatment in 2006. However, the WBC count continued to rise, and at a higher rate than before treatment. The Follow up sample taken 5 years after PT (2008), when patient was at stage B and a WBC count of 136 $x10^9$/L, showed no genomic aberrations, although the FISH data at this stage again showed hemizygous deletion of 13q, and the karyotype revealed large deletion of the long arm of chromosome 13. The patient progressed to stage C in 2009, and reached a WBC count of 233 x 10^9/L, before being treated again.

Patient 12 had a mutated IgVH status, low CD38 expression levels, and was diagnosed in 1998 (PT) with mBL. The PT sample was taken at diagnosis and showed no aberrations (normal genome) by array or FISH. Clinically, there was an increase in WBC count at a rate of $0.95x10^9$/L/month, from $7x10^9$/L at PT to $105x10^9$/L in 2007 (FU). The follow up sample taken 9 years later (2007) showed no aberrations (normal genome) by array or FISH. The patient underwent treatment at FU.

Patient 34 had a mutated IgVH status, low CD38 expression levels, and was diagnosed in 1991 with CLL stage A0. The PT sample was taken in 1998 and showed a sole deletion of the 13q MDR (0.2Mb), which was confirmed by FISH. In 2001, the patient progressed to stage C with a WBC count of less than $40x10^9$/L and underwent treatment. However, following complications (CMV infection), relapse and high WBC count (up to $50x10^9$/L), the patient underwent a second round of treatment in 2003. The Follow up sample was taken in 2006 (7 years after PT) when patient was at clinical stage B, with a WBC count of $90x10^9$/L and before a third round of treatment. At this stage, genomic data showed no genomic evolution, by array or by FISH (figure 25). Two years after FU, patient progressed to Richters disease and was treated a fourth time.

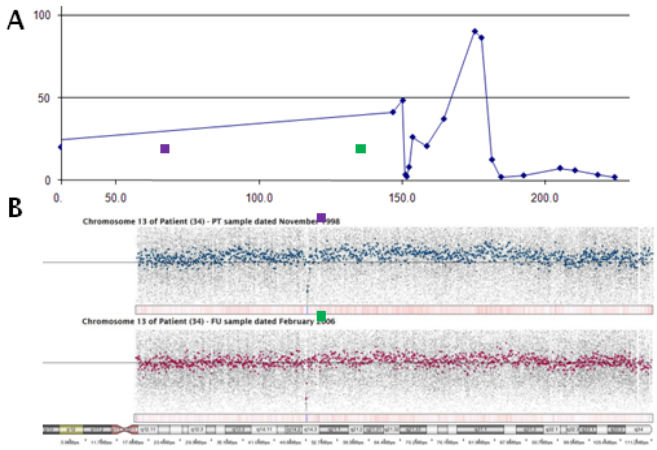


Figure 24: ***A.*** graph showing WBC count (x109/L) against months. PT and FU sample are represented by small squares (purple and green respectively). ***B.*** SNP array data showing deletion on chr13 with no evidence of evolution at FU, and map of chromosome 13 with breakpoints.

Patient 18 had an unmutated IgVH status, low CD38 expression levels, and was diagnosed in 2001 with CLL stage A1. The PT sample was taken in 2004 and showed a sole deletion of 13q (1.1Mb), which was confirmed by FISH. From 2004 to 2007 (FU), a high increase of WBC was seen, from 81 $x10^9$/L to 266$x10^9$/L and progression to stage C which resulted in treatment. The FU sample taken 3 years after PT and just before the treatment showed no evolution, although FISH data showed a small population of cell (20%) with homozygous deletion of 13q MDR (i.e. evolution of 13q from monoallelic deletion to biallelic). Clinically, the response to treatment was partial and patient remained at stage C before a second round of treatment in 2008, and a third in 2009. The patient progressed to Richter's disease in 2010.

Patient 248 had an unmutated IgVH status, low CD38 expression levels and was diagnosed in 2001 (PT) with CLL stage A0. The PT sample was taken at diagnosis and revealed a large deletion on chromosome 13q (>12Mb). The WBC count rose from diagnosis/PT at a rate of 0.6 $x10^9$/L/month, reaching 82$x10^9$/L in 2008 (FU). The FU sample taken 7 years after PT, showed no evolution by array or FISH. The patient remains at clinical stage A0 and is yet to be treated.

Patient 247 had an unmutated IgVH status, high CD38 expression levels, and was diagnosed in 2006 with mBL. Clinically, the patient had a low and stable WBC count (<10$x10^9$/L). The PT sample was taken in 2007, when patient was classed as A0 CLL, and showed a very complex genome with multiple deletions on chromosome 3 and 11, one of which included the *ATM*, as well as deletion on chromosome 17 which included *NF1*. In 2008, the rate of WBC count suddenly rose, from 0.2$x10^9$/L/month to 5.8$x10^9$/L/month, reaching 102$x10^9$/L in 2009 as well as progression to stage A. The

FU sample taken 1 year after PT (in 2009) and just before treatment, showed no genomic evolution, by array or by FISH.

Patient 5 had a mutated IgVH status but high CD38 expression levels, and was diagnosed before 1999. The PT sample was taken in 2000, when patient was at stage A0, and showed a very complex genome; Deletion of 13q, multiple deletions on chromosome 3 and 9 as well as trisomy of chromosome 12, 18 and 19. The disease progressed to stage C in 2004 with a WBC count of $143x10^9$/L and patient received treatment. After treatment, the WBC count increased again and patient was treated a second time in 2006. The FU sample taken 8 years after PT (in 2008) revealed no genomic evolution, by array or by FISH. Further treatments in 2008 were followed by infections and death.

Patient 29 had a mutated IgVH status, low CD38 expression levels, and was diagnosed before 2004. The PT sample was taken in 2004 when patient was at stage A0, and showed a complex genome with deletions on chromosome 4, 13q, 9 and 20. The disease progressed to clinical stage A1 and patient had a high rate increase of WBC count ($2.94x10^9$/L/month), reaching $120x10^9$/L, which resulted in treatment in 2007. The FU sample taken 3 years after PT (in 2007), and just before the treatment, showed no genomic evolution, by array or by FISH. After the treatment, WBC counts rose again at a similar rate, until a second treatment in 2010.

Patient 249 had a mutated IgVH status, low CD38 expression levels, and was diagnosed with CLL stage A0 in 2007. The PT sample was taken at diagnosis, when WBC count was high ($80x10^9$/L) and showed multiple deletions on chromosome 6 and 13, which karyotype data revealed as translocation between the two chromosomes. The FU sample taken a year later, when patient had progressed to stage A1 with a higher WBC count ($141x10^9$/L) showed no genomic evolution, by array or by FISH.

Patient 14 had an unmutated IgVH status, high CD38 expression levels, and was diagnosed in 2001 (PT) with mBL. The PT sample was taken at diagnosis and revealed a sole trisomy of chromosome 12, which was confirmed by FISH. The FU sample taken 4 years after PT (in 2005) when patient was at stage A0, showed no genomic evolution. The WBC count remained stable at $5x10^9$/L until 2005 but then suddenly rose to reach $30x10^9$/L in 2007. At this stage patient went from clinical stage A2 to Richters within two months and was treated.

Patient 16 had an unmutated IgVH status, high CD38 expression levels, and was diagnosed in 2001 (PT) with CLL stage A0. The PT sample was taken in 2002 and

showed deletions of 2p and 19p. The WBC count rose at a rate of $3.2x10^9$/L/month, reaching $172x10^9$/L, and the patient progressed to stage A2 in 2004. The FU sample taken 2 years after PT (in 2004) showed no genomic evolution, by array or by FISH. Patient underwent a first round of treatment in 2005 and a second in 2007. Following complications (pneumonia), patient died in 2009.

3.4. Evolution targeting 15q11.2

In this study, genomic evolution in CLL patients was noted also in Copy Number Variants (CNV), as only 3 patients out of 29 had the same number of CNV.

However, the most interesting observation was made on chromosome 15 (15q11.2), where 15 patients had acquired 2 small secondary aberrations. As figure 26 reveals, these aberrations are present at FU only in 14 patients and at PT and FU in 1 patient.

There was no correlation between gain in 15q11.2 and any of the variables in this study. The secondary aberration was present in stable and progressive cases, mutated and unmutated IgVH cases, treated and non treated cases. In addition, no link was discovered between recurrent aberrations or genomic evolution and gain at 15q11.2

SNP data of one of these patients is shown in figure 27. A closer look at the SNP array, shows a first gain, 40kb in size, at breakpoint 22847230-22887446. The second gain is 64kb at breakpoint 22966682-23030848.

As shown in figure 28, the gain region involves the *SNORD1-116* non coding RNA, as well as the *IPW/SNRPN* gene.

It was clear that this aberration was not due to noise on a particular batch of array as PT and FU sample were run at different time and, in addition, as shown in Appendix 4, the FU samples of the 15 patients with this aberration were also run on different batches at different dates.

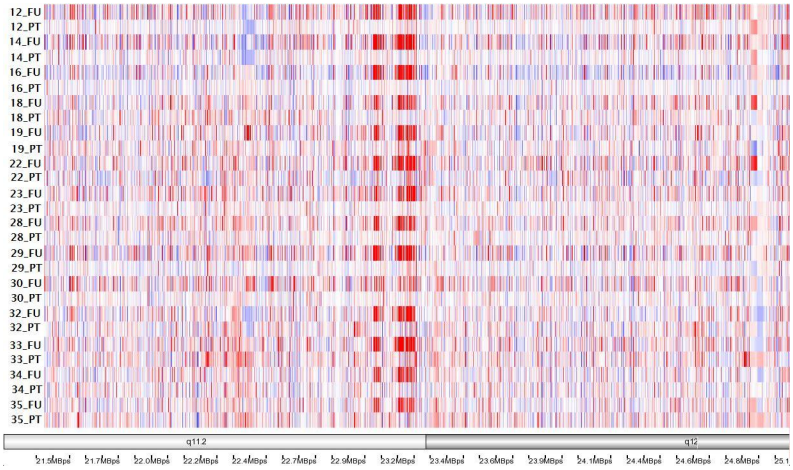


Figure 25: Heatmap of PT and FU patients with gain at 15q11.2. Bright red suggests deletion. A significant difference is noted between PT and FU samples in this region.

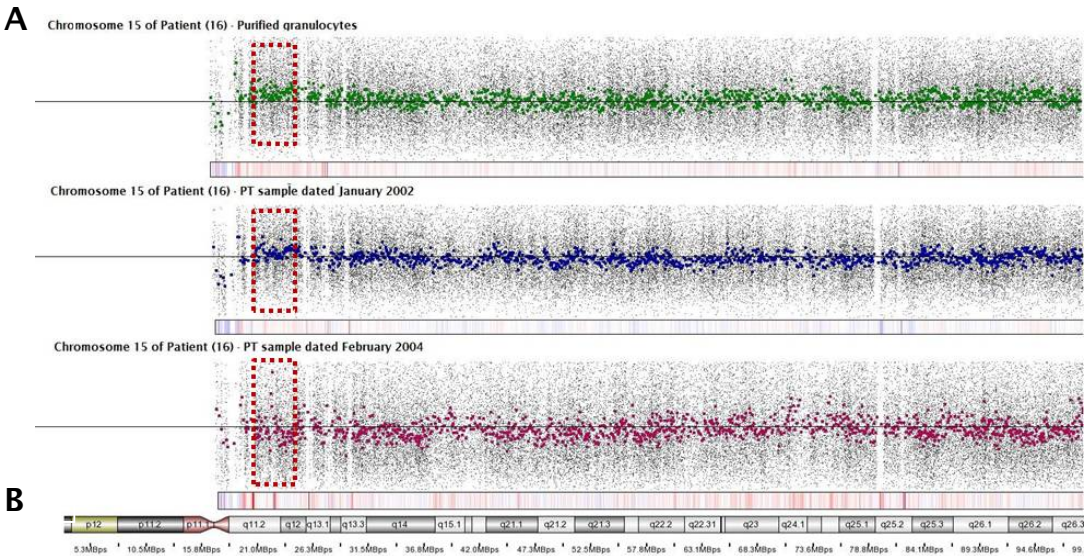


Figure 26: ***A.*** SNP array data and heatmap of normal, PT and FU sample of patient 16 showing gain at 15q11 present only at FU. ***B.*** Map of chromosome 15 with breakpoints

Figure 27. ***A.*** Map of chromosome 15. ***B.*** Genes in the 15q11.2 region between 22,8-23,5 (according to UCSC browser, NCBI36). ***C.*** Specific gained region in 15 patients in this cohort. ***D.*** map of SNP probes

3.5. Discussion

Genomic aberrations in CLL are thought to play an important role in disease progression, and have also been shown to act as prognostic markers. This study aimed to examine the genome of 29 patients, using SNP6.0 high resolution array, to confirm previous research which suggested that CLL patients have an evolving genome, and this could alter the way aberrations are looked at clinically.

3.5.1. CLL patients do have an evolving genome

Our cohort showed evolution in nearly 40% of patients (n=11), which corresponds to previous studies looking at genomic evolution, for instance Chevalier et al who found evolution, using FISH, in 42% of patients (Chevallier, Penther et al. 2002), and Bea et al who showed evolution, using aCGH, in 41% of CLL patients(Beà, López-Guillermo et al. 2002).

This is the first study which observed the evolution in CLL patients using the SNP6.0 high resolution array. As a result, although evolution of the recurrent abnormalities was noted in 13q and 11q, a number of uncommon regions were either deleted or gained in 6 patients (20%). These included small deletions on chr13, chr15, chr21 and gains on chr2 which targeted specific genes. Other large deletions were also noted on chr1, chr4 and chr5. Furthermore, loss of heterozygosity was shown exclusively in FU samples of 3 patients, which targeted 17p in 2 of them. Finally, patients in this cohort were also shown to acquire translocations (1 Balanced, 4 unbalanced).

This data clearly confirms that CLL patients undergo evolution, not only on recurrent aberrations, but also on other regions on the genome, and in many cases these target genes of interest, which will be discussed shortly.

The ever changing nature of the genome in a large proportion of patients from a small cohort such as ours shows its significance CLL disease. Patients such as ID 250 in our study clearly demonstrate that genomic analysis at diagnosis or even 11 years after diagnosis (PT sample) does not reveal prognosis, as 9 years after PT (FU sample), patient acquired 6 large (between 2Mb and 43Mb) aberrations on 5 different chromosomes which resulted in treatment and terminal disease stage. Presence of secondary aberrations in nearly half of CLL patients suggests therefore that clinically, genomic screening should be done more than just at diagnosis (Dal-Bo, Bertoni et al. 2009).

3.5.2. Genomic evolution was not linked with prognosis markers

This study, in accordance to what was previously shown (Shanafelt, Witzig et al. 2006; Berkova 2009), found that genomic evolution was not associated with IgVH mutation or CD38 expression.

In addition, there was no association between biomarkers and secondary high-risk aberration, as deletion on 11q as well as LOH on 17p was acquired by patients with mutated and unmutated IgVH, as well as both CD38 positive and CD38 negative patients. This conflicts with previous reports which suggested genomic evolution on 11q/17p in unmutated IgVH patients predominantly (Shanafelt, Witzig et al. 2006).

Nonetheless, patients with either 11q/17p deletion or class II 13q deletion in this study were associated with secondary aberrations, suggesting that these high-risk deletions can predict genomic evolution. Previous reports have shown the role of *ATM* and *TP53* deletion in genomic instability (Zenz, Krober et al. 2008) and hence it was not surprising to note genomic evolution in patients with 11q/17p aberration in our cohort. However, the role of large 13q deletion in genomic evolution would be an interesting lead for further research. Genes deleted in large 13q deletion which are not involved in patients with small 13q deletion may play a role in genomic instability. Ouillette et al have highlighted retinoblastoma (*Rb*) gene as deleted in a subset of 13q14 deleted patients (Ouillette, Erba et al. 2008) and this gene has recently been shown to cause secondary aberrations (van Harn, Foijer et al. 2010). This strengthens the research by Parker et al, which showed large 13q deletions in progressive cases (Parker, Rose-Zerilli et al. 2010).

3.5.3. Genomic evolution may alter prognosis

Genomic aberrations have been used clinically as prognostic markers; for instance class I 13q deletions have been linked with stable disease whilst deletions on 17 or 11q as well as genomic complexity have been associated with poor prognosis (Dohner, Stilgenbauer et al. 2000; Ouillette, Fossum et al. 2010). We found in this study that patient acquired secondary aberration that altered the prognosis risk evaluated at the first time point: genomic evolution resulted in two stable patients (ID =1 and 21) to pass from low-risk aberrations to poor prognosis aberrations. The first patient acquired a large 13q aberration and the second acquired a complex genome (≥3 CNA), both of which are associated with poor prognosis. Both patients had sudden change in the WBC count between PT and FU sample suggesting an impact of the secondary aberrations on the WBC count. Both patients are nonetheless stable cases, although patient 21 received treatment for AIHA (autoimmune haemolytic anaemia) which reduced the WBC count.

Hence patients with good prognosis aberrations at diagnosis, such as class I 13q deletion may acquire secondary aberrations, such as class II aberrations which could increase the risk of disease progression. Further research in genomic evolution is thus important as clinically, it may be necessary to monitor the progress of the genome to predict changes in disease progression.

3.5.4. Genomic evolution impacts disease progression

This study initially suggested that genomic evolution has no impact on disease progression, as secondary aberrations occurred in 4 patients who remained at early stage disease. However, 2 of these patients had been treated prior to PT sample and thus disease was stable despite evolution. The other two patients had changes in their WBC count, despite not progressing in Binet staging.

Furthermore, case studies enabled a detailed look and highlighted the impact of secondary aberrations in a number of patients.

Patient 28, between PT and FU, progressed firstly from mBL to stage A0, then to stage A2, and finally to stage B, and this was associated with a number of secondary aberrations at FU (from 5 CNA at PT to 10 CNA at FU), including homozygous deletion of a smaller region on 11q involving *ATM*. This patient also strenghtens the idea of monitoring genomic evolution clinically, as Austen et al have shown worse prognosis for patients with biallelic deletion of *ATM* (Austen, Skowronska et al. 2007). The implication for treatment is also quite significant as patients with biallelic deletion of *ATM* have responded poorly to cytotoxic chemotherapeutics (Austen, Skowronska et al. 2007). In addition, since aberrations can alter treatment choice, for instance patient with p53 deletion would respond poorly to purine analogues (eg. fludarabine) (Dohner, Fischer et al. 1995), it has been suggested, and our study has confirmed, that genomic aberrations should be screened for at regular intervals, including prior to treatment, in order to ensure correct choice of treatment for good response (Dal-Bo, Bertoni et al. 2009).

A number of other patients in this study, such as patient 30 and 35, were found to have sudden changes in WBC count and progression to late disease stage which was associated with presence of large secondary aberrations (>3Mb) on chromosome 8, 9 and 13. These regions may involve tumour suppressor or oncogenes which play a role in the progression of CLL. A number of studies have shown for instance the presence of tumour suppressor genes, such as *LZTS1*or *TRAIL-R1* on the small arm of chromosome 8 in various cancers including B-cell lymphoma (Ishii, Vecchione et al.

2001; Armes, Hammet et al. 2004; Knowles, Aveyard et al. 2005; Rubio-Moscardo, Blesa et al. 2005). The role of reccurent abnormalities in CLL disease has been extensively researched, but the influence of other rare deletion and gains has also been highlighted (Crowther-Swanepoel, Broderick et al. 2010; Dalemari, Mahmoud et al. 2010) and hence cannot be ignored, especially when occurring as secondary aberration and altering the disease course.

Therefore, genomic evolution was found to occur in parallel with progression to late stage disease, and change in the rate of WBC count increase in a number of patients suggesting an important role of secondary aberration in CLL disease progression.

3.5.6. Genomic evolution targets genes and impacts disease progression

Whilst genomic evolution targeted large and recurrent deletions and gains, 6 small aberrations targeting genes were noted as secondary aberrations in 4 CLL patients.

Genomic evolution in progressive patient ID 15 involved *FOXO1* and *TRPM1* (these aberrations were added to an already complex genome with large 13q deletion). The transcription factor, *FOXO1,* is known to play a tumour suppressor role in cell cycle regulation (Nakamura 2000), whereas downregulation of *TRPM1* has been involved in melanocytic tumours (Deeds, Cronin et al. 2000) and malignant melanoma (Duncan, Deeds et al. 2001), and also shown to act as tumour suppressor in melanoma, due to miR-211 being encoded within the 6th intron (Mazar, DeYoung et al. 2010). These targets could thus play a role in disease progression, especially since there is a change in the WBC count between PT and FU samples, which could indicate a link with the secondary aberrations. In addition, patient had aggressive disease after the FU sample with relapse after each of the 2 rounds of treatment.

Progressive patient 32 is an interesting case study, where PT sample showed a small deletion on 13, whilst FU sample suggested secondary aberration targeting *SOD1*. A sudden change in WBC count and progression to A1 stage disease is also found between PT and FU sample. The Superoxide Dismutase 1 gene has been shown to remove superoxide radicals, and hence prevent free-radical mediated DNA damage (Huang, Feng et al. 2000), and has been suggested as a target for therapy, since inhibition of *SOD1* would induce apoptosis (Huang, Feng et al. 2000). In addition, *SOD1* has previously been reported to be downregulated following *Bmi-1* overexpression in CML (Merkerova, Bruchova et al. 2007) whilst other studies have shown an overexpression of *SOD1* in CD34+ subpopulatin of CML cells, which had impact on treatment (Liu 2010). Therefore, further research in deletion of this gene, especially its impact on treatment, is required.

Secondary aberration on 7p22 was the sole aberration, at SNP6.0 resolution, in progressive patient 33. High blood cell count, treatment and relapse were noted between PT and FU, and further research in the exact time in which this aberration appeared would be useful in order to precisely show its impact in the disease progression. Nonetheless, deletion of this region involved 4 genes, and was found to play a part in tumorigenesis in non-small cell lung cancer (Campbell 2008). *NUDT1* is the most interesting gene in this region, and has been shown to be overexpressed in a number of tumours, protecting from oxidative damage, including NSCLC (Speina, Arczewska et al. 2005) and breast cancer (Wani, Milo et al. 1998), although other research has shown deletion of *NUDT1* to cause tumours in mice (Tsuzuki 2001). Further research in the role of aberration of this region in CLL may reveal genes of interest.

Secondary aberrations targeting specific genes have been discovered in this study, and found to be associated with changes in the disease progression in CLL patients. Further research on the smaller and non-recurrent aberrations occuring at PT and FU in CLL patients may be significant, as these could play crucial role in tumorigenesis and need for treatment.

3.5.7. Secondary aberration not always necessary for disease progression

Interestingly, a number of patients with progressive disease did not acquire any secondary aberrations at FU, despite changes in WBC count and disease between PT and FU.

In addition, out of the 3 patients who had no evidence of any aberrations at both time points (normal genome) and 2 of these were progressive cases with high WBC count and late disease stage B/C. In addition, other patients like case ID 18 and case ID 34, had small 13q deletion, but a high WBC count, late stage disease C, and transformation to Richter's disease.

This suggests that there may be other factors driving CLL disease, which are not detected by the high resolution SNP6.0. Genetic mutations which cause loss of protein function would be the most probable cause in these cases. Monitoring the mutation in key genes in these patients would be interesting. Having observed the sudden changes in disease progression in these patients, it may be possible that genomic evolution can target mutations.

3.5.8. Genomic evolution on 15q11.2

The use of high resolution array has enabled the discovery of small changes in the genome of patients between two time points. The presence of a secondary gain in the 15q11.2 region of 15 patients was significant, and noted for the first time in CLL.

SNRPN is an imprinted gene in the 15q11.2 involved in Prader-Willi syndrome (Nicholls, Saitoh et al. 1998). However, deletion on this gene has also been found in renal cell carcinoma (Dotan, Dotan et al. 2000), neurocytoma (Korshunov, Sycheva et al. 2007), gliobastoma (Korshunov, Sycheva et al. 2006) and gastric cancer (Takada, Imoto et al. 2005), while methylation of this gene was noted in germ-cell tumours (Bussey, Lawce et al. 2001) as well as AML (Benetatos, Hatzimichael et al. 2010). In addition, gain in this region has been highlighted in Barett's adenocarcinoma(Albrecht, Hausmann et al. 2004). However, not much research has been done regarding the role of *SNRPN* in tumours, most likely due to its presence in a CNV region. However, our study has shown a striking difference of copy number change in this region over time in a number of patients, and having noted aberration of this region in other cancers, it would be of great interest to extent the research further on 15q11. In addition, Leung et al suggested that this gene may be involved in pre-mRNA splicing (Leung, Nagai et al. 2011). Therefore, upon confirming aberration of this gene using PCR in a large cohort, further research into genomic targets of *SNRPN* would be of great interest.

3.5.9. Conclusion

In conclusion, this study has used the high resolution SNP6.0 array to confirm genomic evolution in patients with CLL disease. Secondary aberrations were shown in progressive as well as stable patients and were not associated with biomarkers. Patients with 11q/17p aberrations were more likely to acquire secondary aberrations, as were patients with a large 13q deletion. Genomic evolution occurred on recurrent and non-recurrent regions, and with the high resolution array, a number of small deletion and gains were also found exclusively in the FU sample, including changes 15q11.2, a CNV region encompassing the *SNRPN* gene. Individual case studies enabled a more comprehensive outlook on all patients and using this, it was found that genomic evolution occurred in a number of patients in parallel to sudden changes in WBC count and disease progression. Many patients nonetheless had aggressive CLL disease in absence of copy number changes PT or genomic evolution at FU, suggesting the involvement of other factors, most probably genetic mutations.
Therefore, genomic copy number changes as well as mutations need to be monitored, at high resolution, more often than just at diagnosis in CLL patients, as cases with low-risk aberration may acquire high-risk deletion/gains/mutations at later stage which could alter the course of the disease.

4. Chapter II: Telomere length over time and its impact in CLL disease

4.1. Introduction

4.1.1. Telomere and telomerase in human cells

Telomeres are tandemly repeated DNA sequence of up to 25kb at the end of chromosomes. In humans, a number of proteins interact with the TTAGGG repeat sequence, such as TRF1 and TRF2 (Broccoli, Smogorzewska et al. 1997), and this structure plays a key role in preventing the end of chromosomes to be recognised as double-stranded DNA break, and thus avoiding loss of DNA during replication (Harley, Futcher et al. 1990) as well as preventing end-to-end fusion as a result of non-homologous end joining (NHEJ) (van Steensel, Smogorzewska et al. 1998; van Gent and van der Burg 2007).

Telomeres are maintained by the telomerase enzyme, a reverse transcriptase, which uses an RNA template to add the repeat sequence (Kim, Piatyszek et al. 1994). However, human somatic cells express low levels of telomerase and due to the fact that DNA replication occurs in a semiconservative manner (Harley, Futcher et al. 1990), end of chromosomes are lost at a rate of 60/120bp with every cell division (Baird, Rowson et al. 2003). Telomere loss leads to a DNA damage response (Fagagna, Reaper et al. 2003), which can cause either cell senescence or apoptosis, and this prevents somatic cells to transform into tumour cells (Pepper and Baird 2010).

Therefore, when this system is overcome, and telomerase is overexpressed, the process of apoptosis is not triggered and cells acquired immortality leading to malignancies (Kim, Piatyszek et al. 1994).

4.1.2. Telomere and telomerase in cancer cells

A number of evidence of loss of telomere function has been shown in tumours. Short telomeres have been observed in many cancers, including CLL, as a result of heavy cell division and tumorigenesis (Bechter, Eisterer et al. 1998; Meeker, Hicks et al. 2002; Meeker and Argani 2004). In addition, telomerase knockout mice, and hence with short telomeres, have shown to be associated with higher rate of tumour formation (Rudolph, Chang et al. 1999) and a number of tumours have shown downregulation of telomerase enzyme through a number of different pathways (Kanzawa 2003; Lin and

Elledge 2003), or even deletion of the *TERT* gene, encoding the catalytic subunit of the enzyme (Baird 2010). Finally, loss of telomere has been shown to be associated with non-reciprocal translocations and dicentric chromosomes, and these aberrations have been noted in carcinomas (Sawyer, Husain et al. 2000; Gisselsson, Jonson et al. 2001).

Therefore, short telomeres as well as loss of telomerase enzyme have been widely implicated in tumour progression.

4.1.3. The role of telomere dysfunction in driving malignancy

Research has yet to find a conclusive role of telomeres in driving tumour formation. Nonetheless, as suggested by Pepper et al, the loss of DNA damage response, through deletion or mutations of the P53 pathway, leads to cell division beyond the point at which apoptosis would be initiated (Pepper and Baird 2010). This results in further loss of telomeres and consequently, telomere fusion occurs causing translocations, rearrangement, deletions and overall large genomic instability, a situation termed as "crisis" (Maser and DePinho 2002; Lin, Letsolo et al. 2010). The final step and key to ensure cell survival and tumour progression, is the upregulation of telomerase enzyme (Norrback and Roos 1997; Greider 1998), caused most probably by the genomic changes. This results in the regeneration of telomere length and stabilization of the genome, stopping any new alterations to the DNA, but at the same time promoting survival of cells with an aberrant genome. This process is summarised in figure 29.

Research in the field has thus looked at the length of telomeres and the activity of telomerase to understand the crucial impact of telomere dysfunction in tumour progression.

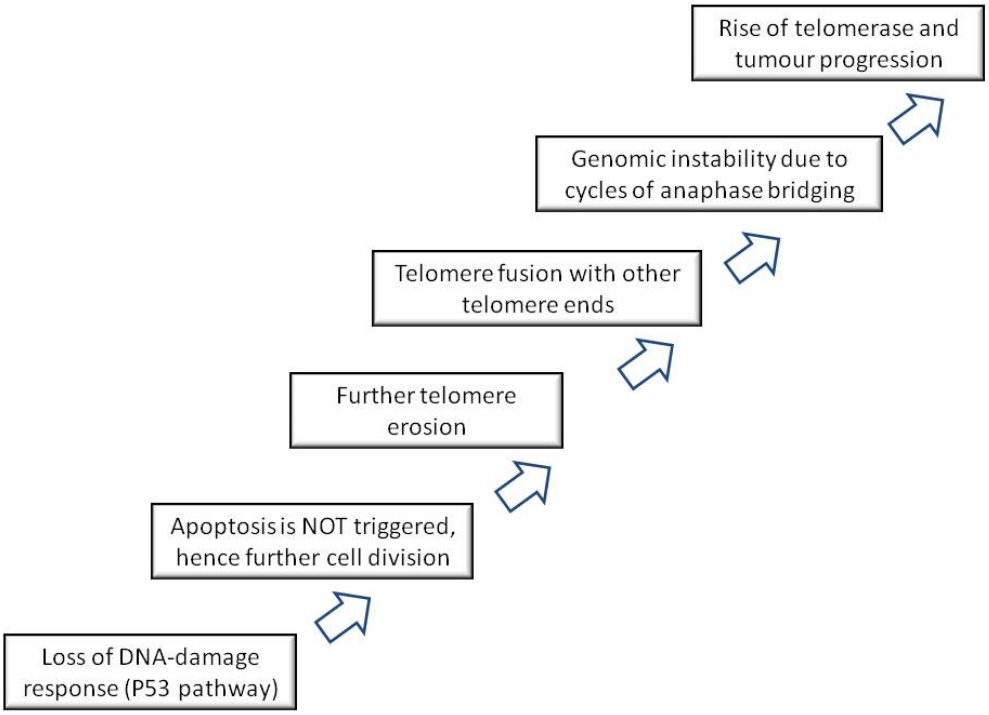


Figure 28: Figure showing the 6 stages to tumour progression, highlighting the role of telomere dysfunction (Pepper and Baird 2010)

4.1.4. Different techniques are used to monitor telomere length

Telomere length can be measured using a number of techniques. The primary method used when telomere was first discovered was Terminal Restriction fragment (TRF) analysis, which involves using telomere repeat probes on fragmented DNA and detecting it via Southern blot hybridisation. However, due to the presence of TTAGGG sequences at other than chromosome ends, TRF analysis is very imprecise (Moyzis, Buckingham et al. 1988; Baird 2005).

A second technique for measuring telomere length has used Fluorescence in situ hybridisation (FISH). The most common and precise method using FISH to detect telomere length is flow-FISH, which consist of hybridising fluorescently labelled peptide nucleic acid probes and using flow cytometry to detect telomere length. Although this provides a higher resolution than TRF, it does come with high levels of background noise and require chromosomes in metaphase stage (Rufer, Dragowska et al. 1998; Baird 2005).

The recently invention of Single telomere length analysis (STELA) from Baird et al's lab has brought the most high resolution and precise tool for telomere length

investigation (Baird, Rowson et al. 2003; Baird 2005). This technique uses single stranded linker structures at the telomere end for single molecule PCR assay, which are then resolved by gel electropheresis and detected by southern hybridisation with a telomere specific probe. This method enables precise measurement of telomere length and can be chromosome specific. A number of recent studies have used STELA and reported very small telomere length in CLL patients (Lin, Letsolo et al. 2010), which would not have been possible with the other techniques.

4.1.5. Telomere dysfunction in CLL

Patients with CLL have short telomeres

Damle et al used flow-FISH method to measure telomere length of purified B-lymphocytes from patients with CLL, age-matched healthy donors, and healthy donors aged 60 or above. The results revealed significantly shorter telomeres CLL patients compared to both, aged matched as well as over 60 subjects, confirming the presence of short telomere in CLL, as seen in other malignancies (Damle, Batliwalla et al. 2004).

However, as was discussed in the main introduction, the disease course of CLL is very heterogeneous and a number of markers have been established to distinguish patients with stable disease and patients with more aggressive disease. Hence, research has compared telomere length in patients with different bio-markers and disease progression.

Patients with late stage disease have shorter telomeres

Patients with progressive disease were shown to have much shorter telomeres compared to patients who had a more stable disease course. Bechter et al, with the use of Southern hybridisation technique, showed that patients with Binet stage C had shorter overall survival rate as well as significantly shorter telomeres, compared to patients at Binet stage A (median 5.3kb vs. 6.6 kb respectively) (Bechter, Eisterer et al. 1998). Lin et al confirmed previous results by using STELA technique and looking at telomere length of the sex chromosomes as well as chromosome 17, as shown in table 16 (Lin, Letsolo et al. 2010). Interestingly, the use of high resolution technology revealed patients, at late disease stage, with complete absence of telomere (Lin, Letsolo et al. 2010). Other studies have also shown telomere length as an independent prognosis marker for transformation to Richter's disease (Rossi, Lobetti Bodoni et al. 2009). Therefore, patients with late-stage and aggressive disease have shorter telomeres.

Table 16: Summary of results from Lin et al (2010) comparing XpYp and 17p telomere length in patients at different Binet disease stage.

Clinical stage	*Mean XpYp (kb)*	*Mean 17p (kb)*
Binet stage A	5.1	4.8
Binet stage B	3.8	4
Binet stage C	2.3	3.1

Telomere length and prognostic markers

IgVH mutation status is an established marker for disease progression, as patients with late stage disease were shown to have unmutated IgVH status, whilst patients with mutated IgVH had a much better prognosis.

Hultdin et al used southern blotting to compare telomere length between 27 patients with mutated and 34 with unmutated IgVH status and found a significant different between the 2 groups (5.4kb vs. 4.3kb respectively) (Hultdin, Rosenquist et al. 2003). Damle et al, using flow-FISH method, also found a significant different between mutated and unmutated IgVH patients (mean 4.4kb vs. 2.5kb respectively), and interestingly reported a correlation between the number of Ig V gene mutations and telomere length in CLL patients (Damle, Batliwalla et al. 2004). This was confirmed by Roos et al, in an extensive study of 152 patients, using RT-PCR technique, who also went on to show that patients with high level of CD38 expression (7% cut off used) had significantly shorter telomeres compared to patients with low CD38 expression ($P<0.000$) and patients with high level of ZAP70 expression (20% cut off used) had significantly shorter telomeres compared to patients with low levels of ZAP70 expression ($P<0.0001$) (Roos, Krober et al. 2008). In addition, Roos et al also found an association between telomere length and genomic aberrations: Patients with either 11q or 17p deletion, targeting *ATM* or *TP53*, had shorter than the median telomere length, whilst patients with 13q deletion had longer than the median telomere length (significant P values, but median telomere length not given) (Roos, Krober et al. 2008).

Therefore, the length of telomere has been shown to be different in patients grouped by different biomarkers, and short telomeres are linked with poor prognosis markers such as unmutated IgVH status and high levels of CD38 and ZAP70 expression. In addition, patients with poor prognosis deletions such as 11q and 17p have short telomeres, whilst patients with good prognosis 13q deletion have overall longer telomeres (Roos, Krober et al 2008).

Telomere length and survival/treatment

A number of studies have linked short telomere to poor survival and short time to treatment (Hultdin, Rosenquist et al. 2003; Damle, Batliwalla et al. 2004). Rossi et al, using Southern blot analysis in a large cohort of CLL patients (n=191), showed that when a 5kb telomere length cut off was used, patients with short telomere had significantly shorter overall survival as well as shorter time to first treatment compared to patients with long telomere (Rossi, Lobetti Bodoni et al. 2009). Roos et al also showed poor survival and shorter time to treatment in patients with shorter than median telomere length (Roos, Krober et al. 2008).

In conclusion, research has shown that patients with early stage disease and good prognosis marker are associated with significantly longer telomere length compared to patients with late-stage disease or with poor prognosis markers.

4.1.6. Impact of short telomere in CLL disease progression

Having discussed the presence of short telomere in patients with CLL, it is vital to understand the dynamics which occur at the telomeric level and look at how short telomeres occur in B-CLL cells as well as their consequence on the disease progression and genomic aberrations.

Origin of short telomere in B-CLL cells

Loss of telomere length in normal B cells is a result of cell division, as well as stochastic telomeric deletion, as suggested by Baird et al (Baird, Rowson et al. 2003). However, research has suggested that telomere length in B-CLL cells is pre-defined from the original clone (Michele Dal-Bo 2009).

As discussed in the main introduction, research has suggested that B-CLL cells from patients with unmutated IgVH status arise from pre-germinal centre, whilst B-CLL cells from patients with mutated IgVH status arise from post-germinal centre (Hamblin 2002). And since, telomere length is associated with the IgVH mutation status, it is suggested that differences in telomere length occurs prior to transformation into malignant lymphocytes (Dal-Bo, Bertoni et al. 2009). This would mean that telomere length seen in B-CLL cells would be pre-defined, i.e. patients with aggressive disease would have short telomere from the onset, rather than a result of prolific cell division.

Weng et al have studied the length of telomere and the levels of telomerase at different stages of B-cell development and found that increase in telomerase activation

occurs as cells enter into the germinal centre but this decreases post-GC as memory B-cells emerge with mutated IgVH but also long telomeres, necessary for their survival, and low telomerase activity, and thus low probability of tumour progression (Weng, Granger et al. 1997). Therefore, as summarised in figure 30, B-CLL cells with a pre germinal centre orgine have unmutated IgVH status as well as short telomeres, whilst B-CLL cells from post-GC or memory lymphocyte origin have mutated IgVH as well as long telomeres. Interestingly, Trentin et al showed differences in telomerase activity between stable and progressive patients just one year after diagnosis (Trentin, Ballon et al. 1999). This confirmed the idea that differences in telomere length as well as telomerase activity between good and poor prognosis patients are present in lymphocytes prior to tumour transformation.

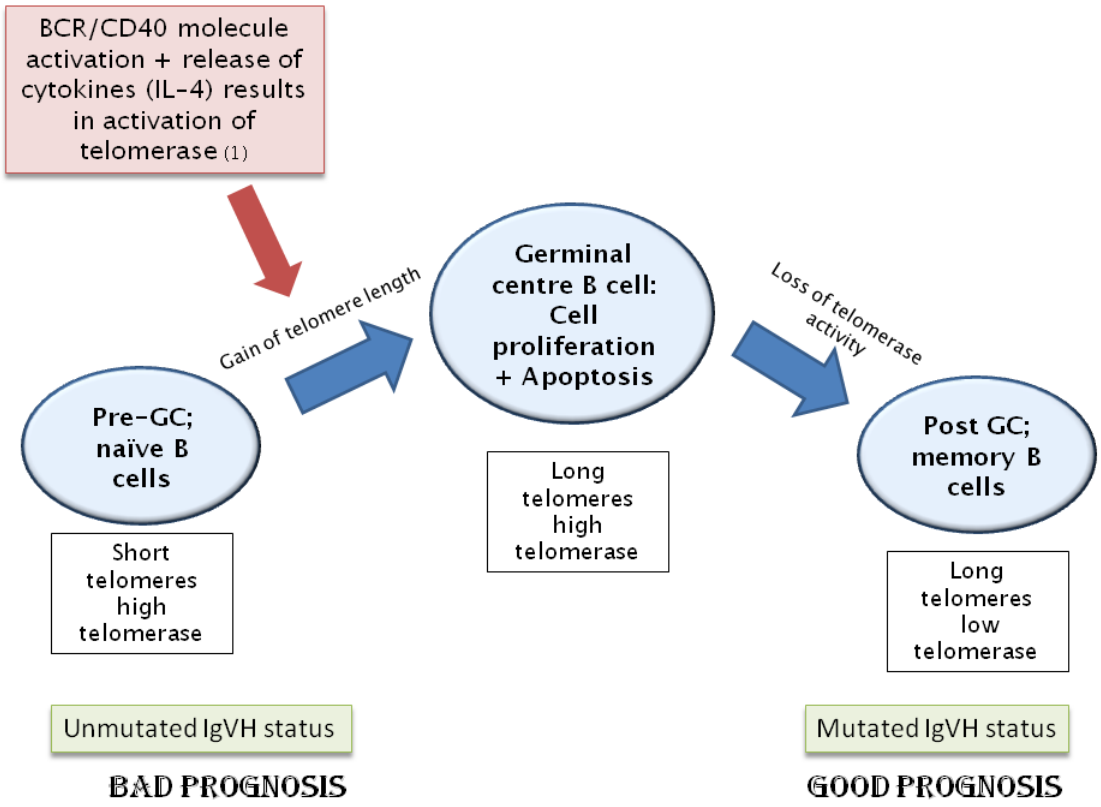


Figure 29: B-CLL cells of patients with short telomere, high telomerase and unmutated IgVH status arise from pre-GC and are associated with poor prognosis whilst B-CLL cells of patients with long telomeres, low telomerase activity and mutated IgVH status arise from post-GC and are associated with good prognosis. (1) Trentin 1999

The impact of telomere dysfunction on disease progression

The progress from normal B cell to a tumour B-CLL cell has been suggested to occur in two steps, M1 and M2 (Shay and Wright 2005). A primary aberration, either deletion or

mutation, will cause proliferative cell division (Pepper and Baird 2010) which will result in loss of telomere length and as a result, B cells will enter into a senescent stage, termed M1, characterized by cell cycle arrest but low level of apoptosis. From then, it has been suggested that loss of DNA damage induced apoptosis pathways (P53) or cell cycle checkpoints (p16/Rb) would cause cells to enter a second stage, termed M2, in which further rounds of cell division can occur (Shay and Wright 2005). As a result, telomere length reaches to quasi non-existence and cells enter into "crisis". Loss of telomere length causes fusions of the ends of chromosomes which results in genomic instability fuelled by breakage of anaphase-bridges, as observed in CLL patients with aggressive disease stage (Lin, Letsolo et al. 2010). Therefore, telomere dysfunction in B-CLL cells drives disease progression by causing genomic instability (Lin, Letsolo et al. 2010; Pepper and Baird 2010). This has been summarised in the figure 31.

It is important to note that this theory has been contested (Jahrsdorfer and Weiner 2008). Research has for instance shown that patients with poor prognosis have a higher lymphocyte proliferative rate compared to good prognosis patients (Messmer, Messmer et al. 2005; Longo, Laurenti et al. 2006). This would suggest that genomic aberrations drive extensive cell division and cause as a result shortening of telomere. Hence, telomere length would be a consequence rather than a cause for disease progression. It is clear that further research is essential in order to understand the impact of short telomere in CLL disease.

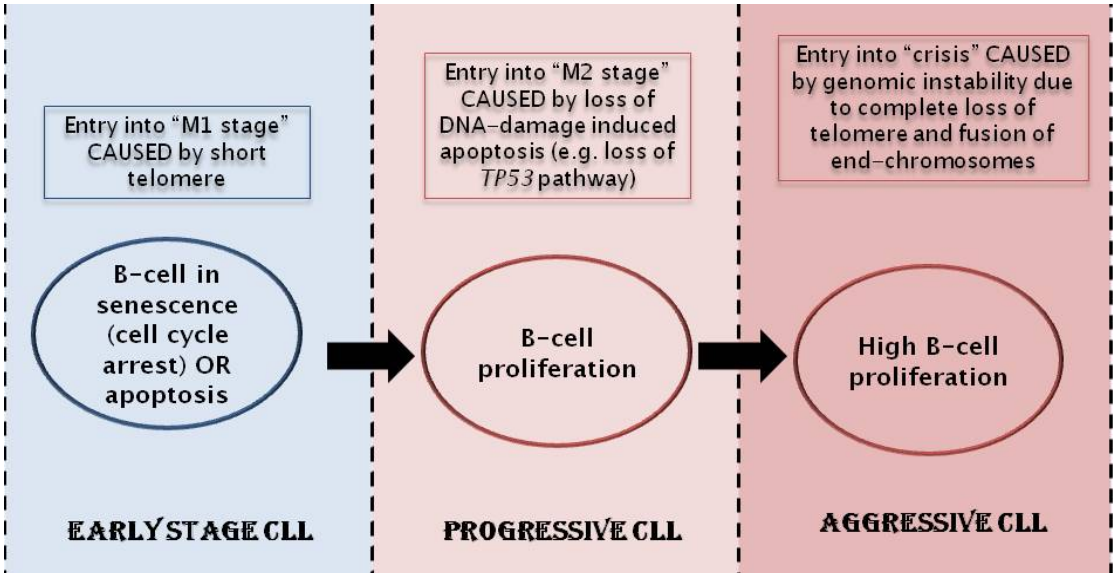


Figure 30: The significant involvement of telomere dysfunction in the progress from early stage to aggressive CLL disease

The role of telomerase in CLL disease progression

As mentioned above, patients with unmutated IgVH status and aggressive CLL disease have been shown to have high levels of telomerase activity as well as short telomeres. However, knowing the role of telomerase, the presence of short telomeres in cells with elevated activity of the enzyme is conflicting. Lin et al have suggested that although telomerase is unregulated it is not sufficient to maintain telomere length (Lin, Letsolo et al. 2010). Interestingly, Remes et al as well as other reports have suggested that there is no correlation between telomere length and telomerase activity in B-CLL, especially in very aggressive disease (Remes, Norrback et al. 2000; Hultdin, Rosenquist et al. 2003). Finally, it has been shown that telomerase is not a lone element in telomere repair but rather a number of complex proteins are required to ensure telomere maintenance, such as the shelterin complex (amongst which are TRF1 and TRF2) as well as other factors (Poncet, Belleville et al. 2008). Poncet et al have shown low level of telomeric proteins in B-CLL cells compared to normal B cells, which suggests that whilst telomerase may be elevated in poor prognosis cases with short telomere, defects in telomeric proteins may cancel the effects of telomerase on telomere repair (Poncet, Belleville et al. 2008). In addition, Augereau et al showed down-regulation of shelterin proteins in newly diagnosed CLL patients (Augereau, T'kint de Roodenbeke et al. 2011). These studies highlight the role of a number of proteins, other than telomerase, in telomere maintenance, and hence explain how B-CLL cells can have short telomere at the same time as high telomerase activity.

4.1.7. Conclusion

In conclusion, the absence of telomerase in somatic cells results in age-related loss of telomere followed by apoptosis. However, loss of telomere in lymphocytes with aberrant DNA damage pathway results in genomic instability and chromosome rearrangement which drives disease progression. Results have shown that telomere length is defined by B cell origin, where short telomere are shown in pre-germinal centre B cell (unmutated IgVH status) whilst long telomere are present in post-GC lymphocytes (mutated IgVH), and the former is associated with poor prognosis.

4.1.8. Aims

The aim of this study is to look at telomere length at two different time points and in relation to the various prognosis marker, as well as in correlation with genomic aberrations, clinical data and WBC count in order to not only confirm its ability to predict disease outcome and genomic instability, but also to explore its role in CLL progression.

- Confirm telomere length in relation to disease status and clinical markers (treatment and WBC count)
- Confirm telomere length in relation to prognostic markers
- Investigate telomere length in relation to genomic instability
- Explore telomere erosion between PT and FU

4.2. Methods

4.2.1. Telomere length was detected using STELA technique

A maximum of 300ng/ul of DNA of each of the 29 patients at two different time points were sent to Dr Duncan Baird at the University of Cardiff to analyse telomere length using their newly developed Single Telomere Length Analysis technique (STELA), a new PCR-based method to measure telomere length at higher resolution than previous techniques (Baird 2005).

The DNA was first ligated in presence of a telorette linker, DNA ligase and ligation buffer. The ligated DNA was then diluted and multiple PCRs were carried out in presence of telomere-adjacent and teltail primers, NTPs, Tris-HCL, $(NH_4)_2SO_4$, Tween-20, $MgCl_2$ and a 25:1 mixture of *Taq* and *Pwo* polymerase. PCR was done in the following conditions: 25 cycles of 94˚C for 15s, then 65˚C for 30s and finally 68˚C for 10 minutes.

DNA fragments were then resolved by agarose gel electrophoresis (0.5%Tris-acetate-EDTA) and were detected by Southern hybridisation with ^{32}P-labelled telomere adjacent probes generated by PCR using primers XpYpE2 and XpYpB2. The hybridised fragments were detected by a phosphorimager (Molecular Dynamics Storm 860) and the molecular weight of the fragments was calculated using the Phoretix 1D quantifier. The mean XpYp as well as 17p telomere length of each sample at each time point, in Kb, was received on an excel sheet.

4.3. Results

4.3.1. Telomere length overview

Telomere length at presentation (PT) and follow up (FU)

Telomere data of XpYp and 17p at PT was available on 24 patients. As summarised in table 17, the mean XpYp telomere length at PT was 4kb compared to 3.7kb at FU whilst the mean 17p telomere length at PT as well as FU was 4 kb.

Table 17: XpYp and 17p telomere length mean, median and range at Presentation and Follow up

Telomere length	*XpYP (PT)*	*XpYP (FU)*	*17p (PT)*	*17p (FU)*
Mean (kb)	4	3.7	4	4
Median (kb)	3.7	4	3.5	3.7
Range (kb)	2.3 - 7.8	1.4 – 5.9	2.2 - 7.2	2.2 - 7.3
Standard deviation	1.44	1.39	1.31	1.44

No correlation was found between telomere length and the age of patients

As telomere shorten over time, it was important to establish any correlation with telomere length. It was thus shown that in this cohort, telomere length at either PT or FU was not proportional to age (Pearson's correlation; P=0.6 and P=0.8 at PT and FU respectively). In addition, the extent of telomere erosion was not proportional to the time elapsed between PT and FU (Pearson's correlation; P=0.7 and P=0.2 for XpYp and 17p respectively) (figure 32), confirming that loss of telomere is not age-related.

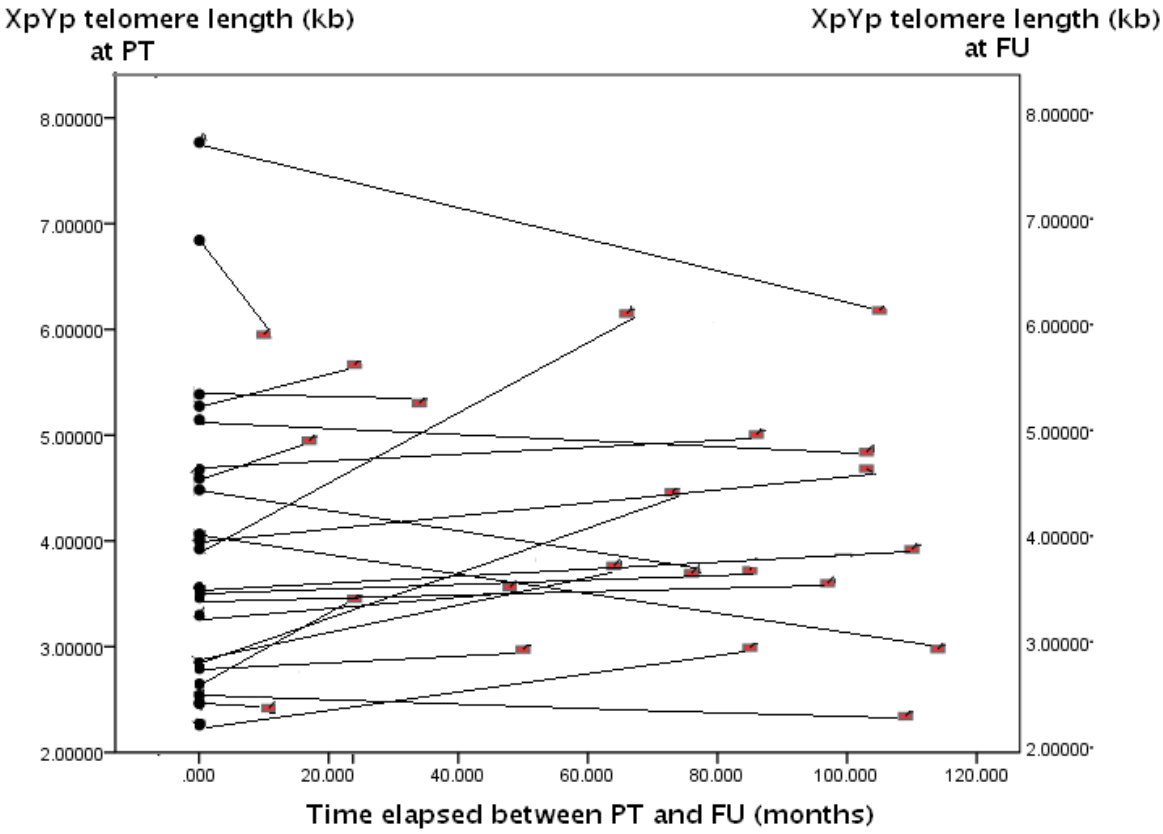


Figure 31: Graph showing XpYp telomere length at PT and FU in relation to time elapsed between the two time points. The Y axis on the left represents telomere length in kb for PT samples, each represented by full black circles. The Y axis on the right represents telomere length in kb for FU samples, each represented by red squares. The distance between the PT and FU sample (X axis) represents time, in months between both samples. No correlations was found between telomere erosion and time between two samples (P=0.2)

4.3.2. Telomere length and disease status

Telomere length has been shown previously to be much shorter in patients with progressive CLL disease.

At both time points (PT and FU), patients with progressive disease had a shorter mean XpYp and 17p telomere length but also the shortest as well as the longest telomere length (FU data shown in table 18). However, as shown in table 18, at FU, telomere length of stable cases was longer and telomere length of progressive cases was shorter than the median telomere length of the entire cohort (XpYp: 4kb and 17p: 3.7). Nonetheless, no correlation was found between disease status and telomere length at either time (T-Test: XpYp at PT, P=0.6; XpYp at FU, P=0.1; 17p at PT, P=0.7; 17p at FU, P=0.3).

Table 18: Comparing mean, median and range of XpYp and 17p telomere length at FU between stable and progressive patients. [1]P=0.1; [2]P=0.3

Telomere length	*XpYp (FU) Stable*	*XpYp (FU) Progressive*	*17p (FU) Stable*	*17p (FU) Progressive*
Mean (kb)	4.6	3.5	4.3	3.9
Median (kb)	4.3	3	4.2	3.1
Range (kb)	2.1 – 5.8	1.4 – 5.9[1]	2.8 – 6.4	2.2 – 7.3[2]

4.3.3. Telomere length and prognostic markers

Previous studies have shown correlation between telomere length and various prognosis markers (Roos, Krober et al. 2008). This section will investigate telomere length in relation to biomarkers, disease stage and genomic aberrations.

Telomere length was associated with IgVH mutation and CD38 expression

Similar to what has been noted in previous research, patients with unmutated IgVH mutation status were associated with short telomere, both XpYp and 17p, at each time point, PT (T-Test; **P=0.0001** and **P=0.013** respectively) and FU (T-test; **P=0.004** and **P=0.03** respectively). At PT, patients with unmutated IgVH status had a mean XpYP telomere length of 2.7kb compared to 4.5kb in mutated IgVH patients. Similar results were shown at FU (2.5kb vs. 4kb respectively).

Furthermore, patients with high CD38 expression had shorter XpYp (T-Test, **P=0.05**) and shorter 17p (T-Test, **P=0.05**) telomere length at PT compared to CD38 negative patients. This was also shown at FU (T-test; **P=0.04** XpYp and **P=0.08** 17p). At PT, patients with CD38+ had a mean XpYP telomere length of 3.1kb compared to 4.3kb in CD38-. Similar results were shown at FU (2.9kb vs. 4kb respectively).

IgVH mutation status and CD38 expression were therefore shown to be good markers for short telomere throughout the disease course.

Telomere length was not linked with clinical stage or progression to late stage disease

Since patients at PT have either mBL or early disease stage A0, telomere length at PT cannot be analysed in relation to disease status.

When looking at telomere length at FU, in accordance with what was previously suggested in various studies (Lin, Letsolo et al. 2010), patients with clinical stage B/C

had the shortest telomere (table 19). However, unexpectedly, patients with mBL or stage A0 at FU also have relatively short telomeres, and therefore, telomere length was not statistically linked with disease stage (T-test; P=0.2 and P=0.4 respectively).

Table 19: Comparing XpYp and 17p mean and range (in kb), at FU, between patients with either early stage mBL/A0 disease, stage A1/A2 or late stage disease B/C. [1]P=0.2; [2]P=0.4

Clinical stage at FU	*XpYp (FU) Mean*	*XpYp (FU) Range*	*17p (FU) Mean*	*17p (FU) Range*
mBL/A0 (12)	3.85	2.1 - 5.8	4.03	2.5 – 6.4
A1/A2 (6)	4.19	1.7-5.9	4.74	2.2 – 7.3
B/C (8)	3.19[1]	1.4 – 5.4	3.45[2]	2.5 - 5.3

Finally, patients with the shortest XpYp and 17p telomere at PT were shown to progress to advanced clinical stage, from mBL to stage B/C (table 20). However, stable patients who remained at stage mBL and A0 also had short telomeres and therefore, telomere length at PT could not predict progression to aggressive clinical stage. Interestingly, patients who progressed from stage A0 to A1 or A2 had the longest 17p telomere at FU whilst patients who remained at stage A0 or A had the shortest 17p telomere at FU. Case studies in the next section will give more details on these patients.

Table 20: Comparing XpYp and 17p mean (in kb), at PT and FU, between patients who either remained at an early stage disease or who progressed to late stage disease

Clinical stage PT → FU	*XpYp (PT) Mean*	*XpYp (FU) Mean*	*17p (PT) Mean*	*17p (FU) Mean*
mBL → mBL/A0 (5)	3.9	4	4.1	4
A/A0 → A/A0 (6)	4.4	4.1	3.6	2.9
A0 → A1/A2 (4)	4.9	4.6	6.7	6.4
mBL-A1 → B/C (9)	3.5	3	3.1	3

Telomere length was not associated with 13q deletion size

Patients with small 13q deletion had an average XpYp telomere of 4.1kb at PT and 4.2kb at FU while patients with large 13q deletion had an average XpYp telomere of 4.6kb at PT and 4.3kb at FU. However, as shown in figure 33, there was a notable difference in the median XpYp telomere length between patients with different 13q size. However 13q deletion size was not statistically linked with XpYp telomere at

either time point (T-Test, P=0.5; P=0.4, respectively). A similar result was shown for 17p deletion (T-test, P=0.8; P=0.4, respectively)

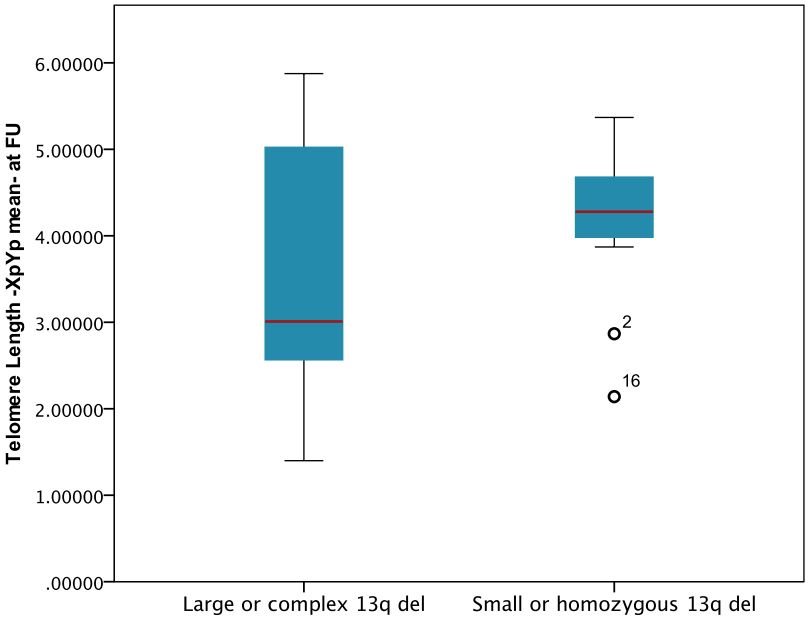


Figure 32: Box plot showing XpYp telomere length (Y axis, in kb) in patients with small 13q deletion or large 13q deletion. The circle represents outliers ('2' is patient ID 5 and '16' is patient ID 23)

Telomere length was associated with 11q/17p aberration

Patients with either 11q or 17p aberrations were shown to have significantly shorter telomere at FU, but not at PT. Patients with poor prognosis aberration had an average XpYp and 17p telomere of 2.81kb and 2.65kb at FU compared to an average of 3.95kb and 4.34kb in the rest of patients, suggesting that XpYp as well as 17p telomere at FU was linked with poor prognosis aberration (T-Test **P=0.08** and **P<0.0001** respectively). However, at PT, no significant association was shown between XpYp or 17p telomere and poor prognosis aberration (T-Test P=0.4 and 0.2 respectively)

Summary of results

From the above result, it was found that telomere length was associated with IgVH mutation status, CD38 expression and poor prognosis aberration 11q/17p. In addition, patients with progressive disease or with late stage disease B/C had the shortest telomere length, although this was not significantly different from stable or early stage disease. Finally, a large but not significant, difference in telomere length was noted between patients with small and large 13q deletion size.

4.3.4. Telomere length and clinical progression

Rossi et al suggested telomere length as an independent predictor of treatment requirement in CLL patients (Rossi, Lobetti Bodoni et al. 2009). This section will thus investigate telomere length in relation to treatment, time to first treatment (TTFT) as well as WBC count.

Telomere length was linked with TTFT but not with requirement for treatment

Telomere length at PT or FU was not associated with treatment, as both treated and untreated patients had very short XpYp and 17p telomere at both time points: XpYp mean at PT for treated and non treated was 3.8kb and 3.7kb respectively, whilst at FU the telomere length was 3.5kb and 3.8 respectively (Mann Whitney, P-.04 and P=0.6 respectively). A similar result was found for 17p telomere length (PT: 3.8kb vs. 4.2kb. FU: 3.9kb vs. 4.2kb, Mann Whitney P=0.4 and P=0.6 respectively)

However, XpYp telomere length at PT as well as at FU was shown to correlate with Time to first treatment (TTFT): Patients with the shortest XpYp telomere length had shorter TTFT treatment (Pearson Correlations; **P=0.012**) (figure 34) and this was also shown as a trend for 17p telomere (Pearson Correlation; **P=0.06**).

When telomere length at FU was looked at in relation to TTFT, it was found that only short XpYp telomere length had a trend towards shorter TTFT whilst no association was found between 17p telomere and TTFT (Pearson Correlation; **P=0.06** and P=0.3 respectively).

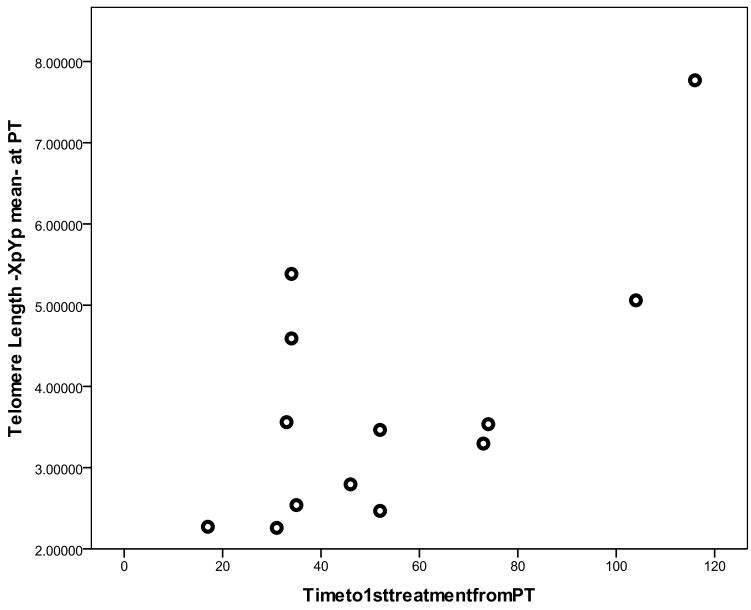


Figure 33: XpYP telomere length at PT (Y axis, in kb) against time to first treatment (X axis, in months) where each circle represents one patient. Patients with short XpYp telomere length have shorter TTFT. R2 linear=0.44

Telomere length and White blood cell count

Telomere length was investigated in relation to the rate of WBC count increase per month and was found to predict the clinical symptom: Patients with short XpYp telomere at PT as well at FU was associated with a high rate of WBC count rise (Pearson correlation; **P=0.043** and **P=0.044** at PT and FU respectively) (figure 35). There was, however, no difference in the rate of WBC count increase between patients with short or long 17p telomere.

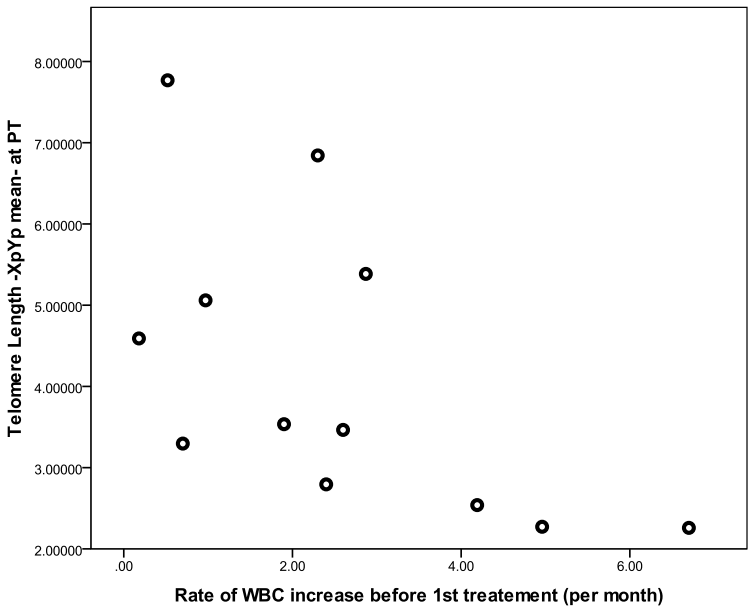


Figure 34: XpYp telomere length at PT (Y axis, in kb) against the rate of WBC count increase (X axis, in x10/L/month), where each circle represents one patient. Patient ID 30 was not included (outlier).

4.3.5. Telomere length and genomic aberration

Short telomeres have been previously shown to cause genomic instability in CLL patients. This section will examine telomere length at the two time points (PT and FU) in relation to genomic complexity, translocations and terminal aberrations

Telomere length at PT is not linked with genomic complexity at PT

This cohort found no correlation between telomere length and complexity (≤3 CNA) at PT: Patients with a complex genome at PT had a mean XpYp telomere length of 3.5kb compared to 4.2kb in patients without a complex genome (T-Test P=0.3). A similar result was shown for 17p telomere (3.9kb vs. 4.1kb respective, T-Test, P=0.8)

In addition, there was no correlation between the number of CNA and the size of XpYp or 17p telomere length (Pearson correlation, P=0.3 and P=0.7 respectively).

This was due to a number of patients with 1 or no aberration at PT who had shorter than average telomere, whilst a number of patient with a complex genome at PT who had longer than average telomere.

Nevertheless, patients with a *sole* 13q deletion at PT did have a trend towards longer telomere compared to the rest of the cohort (T-Test; **P=0.08** for XpYP and MannWhitney; **P=0.07** for 17p).

Telomere length at PT does not predict genomic evolution

Having shown no association between telomere length at PT and genomic aberration at the same time, genomic evolution was looked at to evaluate the impact of short telomere on the genome over time.

Telomere length, XpYp or 17p, was not associated with genomic evolution (T-Test; P=0.07 for XpYp and P=0.9 for 17p). Surprisingly, patients who go on to acquire secondary aberrations had longer XpYp telomere at PT compared to patients with a more stable genome (table 21).

Table 21: The mean, median and range of XpYp and 17p telomere length of patients with or without genomic evolution showed no association between telomere length at PT and genomic evolution. [1]P=0.07; [2]P=0.9

Telomere length	*XpYp No evolution*	*XpYp Genomic evolution*	*17p NO evolution*	*17p Genomic evolution*
Mean (kb)	3.6	4.7	4	4
Median (kb)	3.5	4.7	3.4	3.6
Range (kb)	2.3 - 5.4	2.5 – 7.8[1]	2.2 – 7.2	2.3 – 6.2[2]

Telomere length at PT does predict scale of genomic evolution

When looking solely at patients who undergo genomic evolution, it became clear that patients with a larger scale evolution have much shorter XpYp telomere at PT compare to patients who have a much smaller evolution: Patients who acquired 3 or more CNA had a median XpYp telomere length of 3kb whilst patients who acquired less than 3 CNA had a median of 5.3Kb XpYp telomere length (T-Test; **P=0.04**).
The size of deletion acquired through genomic evolution was also linked with XpYp telomere length as patients with the shortest XpYp telomere at PT acquired the largest secondary aberrations (Pearson's Correlation, **P=0.02**). (figure 36).

However, this was not significantly different with 17p telomere (T-test; P=0.1 – Median of 3.5kb for 3 or more CNA acquired compared to 4.4kb).

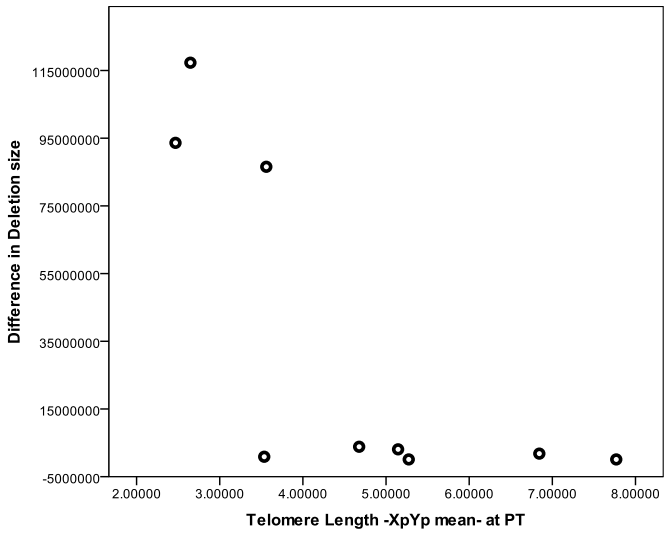


Figure 35: Total deletion size of secondary aberration (Y axis, in base pair) against XpYp telomere length at PT (X axis, in kb), were each circle represents one patient with genomic evolution. Patients with the shortest telomere at PT acquire the largest secondary aberration (P=0.04). R^2=0.54

Telomere length at FU and complexity

When looking at the telomere length at the second time point (FU) in relation to genomic complexity, it was found that there was no association between the two, as patients with complex genome had a mean XpYp telomere of 3.9kb compared to a mean of 3.5kb in patients with less than 3 CNA (Mann Whitney, P=0.4). Similar result was found with 17p telomere (3.9kb vs. 4kb, Mann Whitney; P=0.4).

However, when the number and size of CNA was investigated, it was found that patients with short XpYp telomere length had a higher number of CNA as well as larger total deletion size compared to the rest of the cohort (Pearson's Correlation: **P=0.06** and **P=0.02** respectively), although this was not true for 17p telomere length (Pearson's Correlation: P=0.1 and P=0.4 respectively).

Telomere length is linked with terminal aberrations

Since loss of telomere leads to end-chromosome fusion, it was suggested that short telomeres could be linked with terminal aberrations. These were defined as copy number changes targeting either end of chromosomes.

A total of 7 patients were found to have terminal aberrations at FU which included deletions of 2p, 3q, 8p, 9p, 17p, 18p and 20p as well as gain of 13q.

A significant different in telomere length at FU was noted between patients with terminal aberrations (mean 2.9kb) and the rest of the cohort (mean 4kb) (T-Test,

P=0.03). However, as shown in table 22, this was not significant when looking at 17p telomere length (Mann Whitney, P=0.1).

Table 22: Mean, median and range of XpYp and 17p telomere length at FU in patients with terminal aberrations and patients without terminal aberrations (rest of cohort). A significant difference was noted in XpYp but not in 17p. [1]P=0.03; [2]P=0.1

Telomere length (FU)	*XpYp rest of cohort (FU)*	*XpYp Terminal aberration (FU)*	*17p rest of cohort (FU)*	*17p Terminal aberration (FU)*
Mean (kb)	4	2.9	4.2	3.2
Median (kb)	**4.3**	**2.9**	4	3
Range (kb)	1.7 – 5.9	1.4 – 4.9[1]	2.2 – 7	2.5 – 7.3[2]

Telomere length was not associated with translocation

In this cohort, patients with an unbalanced translocation had a mean XpYp telomere length of 3.3kb, below the average of the entire cohort, compared to 3.9kb in the rest of patients, which was above the average of the entire cohort. Nonetheless, the difference in telomere length between patients with and without unbalanced translocation was not significant (T-Test P=0.2).

There was also no association between unbalanced translocations and the length of 17p telomere (mean 4kb vs. 4kb).

Summary of results

Short telomere length at FU but not at PT was linked with genomic instability when looking at copy number changes, but not when looking at unbalanced translocations. In addition, short telomere did not predict genomic evolution, although a significant difference in telomere length was noted between patients who acquire less than 3 and patients who acquire more than 3 CNAs. Finally, XpYp but not 17p telomere length was shown to be linked with terminal aberrations in CLL patients.

4.3.6. Telomere erosion

Telomeres were previously shown to be unstable over the course of the disease (Brugat, Nguyen-Khac et al. 2011), with the occurrence of telomere loss or 'erosion', which was linked with chromosome fusion and genomic instability (Lin, Letsolo et al. 2010). This section will aim to investigate telomere erosion between PT and FU sample

in relation in relation to prognostic markers, genomic aberration as well as clinical features (treatment and WBC count).

Overview

A look at telomere erosion in our cohort shows that 16 patients have a shorter XpYp and 14 patients have a shorter 17p telomere, whilst 8 and 10 patients have longer XpYp and 17p telomere respectively at the second time point (FU). The average loss of XpYp telomere was 0.38kb whilst the average loss of 17p telomere was 0.26kb (table 23).

Table 23: The mean, median and range of XpYp and 17p telomere loss (in kb) in this cohort

Telomere length	*XpYP erosion*	*17p erosion*
Mean (kb)	- 0.38	- 0.26
Median (kb)	- 0.33	- 0.08
Range (kb)	(- 1. 89) – (+1.84)	(- 1.49) – (+0.65)

Progressive but not stable patients lose telomere length over time

Most patients with gain of telomere are stable cases whereas a majority of patients with loss of telomere are progressive cases (66% of stable cases gain telomere whilst 86% of progressive have telomere erosion). In addition, the largest loss of XpYp is seen in progressive patients, whilst the larger gain of XpYp is seen in a stable case. The median XpYp as well as 17p telomere erosion is loss of 0.5 Kb in progressive cases but no change (0 Kb) in stable cases (figure 37). Telomere erosion was thus shown to be associated with the disease status: Mann Whitney; **P=0.06** for XpYp and **P=0.02** for 17p (The outlier seen in the figure, patient 23, was no included and will be discussed in telomere case studies).

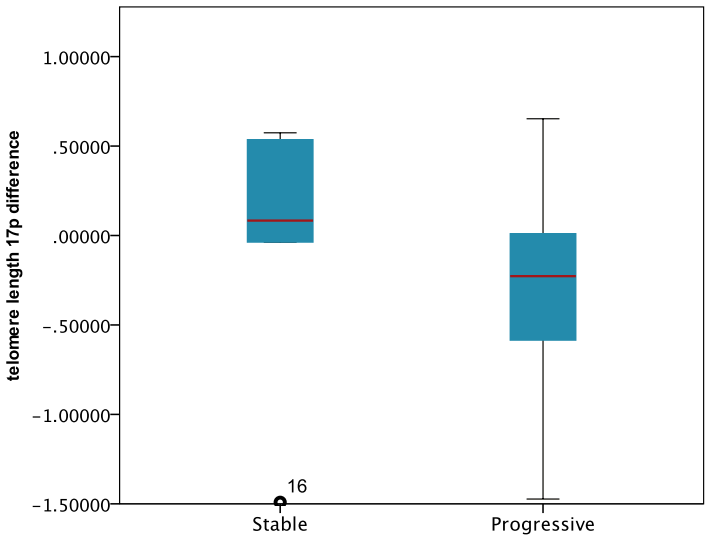


Figure 36: Box plot shows difference in 17p telomere erosion (Y axis, in kb) between stable and progressive patients. Outlier "16" is patient ID 23, a stable case with large telomere erosion.

Telomere erosion was not associated with biomarkers

Telomere erosion was not associated with prognostic markers IgVH status or CD38 expression. Patients with unmutated IgVH status had an average loss of 17p telomere of 0.35kb compared to a loss of 0.22kb in patients with mutated IgVH status (T-Test, P=0.4). A similar result was found with XpYp telomere (loss of 0.22kb vs. loss of 0.45kb, T-Test, P=0.6).

However, when looking at CD38 expression, a surprising difference was noted, where patients with positive CD38 expression had on average no change in 17p telomere length whilst patients with negative CD38 expression had an average loss of 0.34kb. Nonetheless, 17p telomere loss was not significantly associated with CD38 expression (T-test, P=0.07). A similar result was found for XpYp telomere (Mean loss of 0.2kb vs. loss of 0.4kb respectively; T-test; P=0.5)

Telomere erosion was not associated with progressive disease stage

As noted in table 24, patients who remained at mBL or CLL stage A0 had on average acquired XpYp and 17p telomere length, whilst patients who progressed to late stage disease B or C had lost XpYp and 17p telomere. However, patients who remained at early stage A/A0 also lost telomere length, and thus XpYp or 17p telomere erosion was no associated with progression in disease stage (T-Test P=0.9 and P=0.8 respectively).

Table 24: The average XpYp and 17p telomere loss in patients grouped by disease stage progression

Clinical stage PT → FU	*XpYp difference Mean*	*17p difference Mean*
mBL → mBL/A0 (4)	+0.39	+ 0.18
A/A0 → A/A0 (6)	- 0.63	- 0.51
A0 → A1/A2 (4)	- 0.58	- 0.12
mBL-A1 → B/C (8)	- 0.32	- 0.21

Telomere erosion was associated with poor prognosis deletions but not complexity

When telomere erosion was analysed in relation to different aberrations with prognosis values, it was found that telomere erosion was associated with 13q deletion: Patients with large 13q deletion had an average loss of XpYp and 17p telomere length of 0.9 kb and 0.6kb respectively while patients with small 13q deletion had on average the same XpYp telomere and gained an average 0.1kb of 17p telomere (0.1kb). Patients with large 13q deletion were thus associated with telomere erosion (T-Test; **P=0.01** for XpYp and **P=0.007** for 17p).

In addition, it was interesting to note that telomere erosion on 17p but not XpYp was associated with aberration of *TP53* or *ATM:* Patients with a poor prognosis aberration had an average loss of 0.68kb compared to 0.13kb in the rest of the cohort (Mann Whitney; **P=0.04**). A difference in XpYp telomere erosion was also noted between patients with and without poor prognosis aberrations, although this was not significant (average loss of 0.74kb vs. 0.28kb respectively, Mann Whitney P=0.1)

Finally, in this cohort, it was found that patients with a complex genome at FU had a higher than average loss of telomere length (0.5kb of XpYp and 0.3kb of 17p) while patients without a complex genome had a much lower than average loss of telomere length (0.01kb of XpYp and 0.1kb of 17p). Nonetheless, the difference in telomere loss between patients with and without complex genome was not significant (T-test, P=0.3 for XpYp; P=0.5 for 17p).

Telomere erosion was not linked with treatment

It was interesting to note that patients who received treatment between PT and FU had on average no difference in 17p telomere compared to an average loss of 0.33kb in patients who did not receive treatment. A similar result was shown with XpYp telomere, where treated patients had a much smaller telomere erosion compared to

patients who weren't treated, although in both cases, treatment was not significantly associated with loss of telomere (T-Test, P=0.8 and P=0.3 respectively).

In addition, XpYp or 17p telomere loss was also not associated with TTFT (Pearson correlation P=0.4 and P=0.67 respectively).

Telomere erosion was associated with genomic evolution

Short telomeres have been found to cause genomic instability, and thus it is suggested that telomere erosion would be linked with complexity as well as genomic evolution, since significant loss of telomere would drive secondary aberrations.

In this cohort, telomere erosion was shown to be linked with genomic evolution: Patients with large telomere erosion on XpYp and 17p were more likely to have acquired secondary aberrations (T-Test; **P=0.004** for XpYp and **P=0.04** for 17p) compared to the rest of the cohort (table 25)

Table 25: Mean, median and range of loss of XpYp and 17p telomere length (in kb) between patients with and without genomic evolution.

Telomere length	*XpYp No evolution*	*XpYp Genomic evolution*	*17p NO evolution*	*17p Genomic evolution*
Mean (kb)	0	- 0.9	0	- 0.5
Median (kb)	0	- 0.8	0	- 0.5
Range (kb)	(- 0.5) – (+1.8)	(- 1.9) – (+0)	(- 0.7) – (+0.7)	(- 1.5) – (+0.5)

Furthermore, patients with a gain of translocation, balanced and unbalanced, at FU had undergone telomere erosion on 17p but not XpYp, and statistically, translocation at follow up was associated with telomere erosion on 17p but not XpYp (Mann-Whitney, P=0.1 for XpYp and **P=0.04** for 17p). The median telomere loss of 17p was 1.2kb in patients who had acquired a translocation; whilst the rest of the cohort had a median loss of 17p telomere of 0.03kb. A large difference was also noted in the XpYp erosion between the two groups (loss of 1.7kb and loss of 0.3kb respectively) despite this not being statistically significant.

4.4. Case studies

The results from the above statistical analysis would be difficult to interpret as conclusive due to the very small cohort of patient. Therefore, this study also looked at patients individually to investigate the role of telomere in genomic instability and disease progression. Case study will also explain why a number of results noted above were unexpectedly insignificant, as this study will uncover some rare cases of short telomere in stable cases and long telomeres in progressive patients (summarised in table 26).

4.4.1. Stable cases have long telomere

Stable patients were shown to have longer than average telomere, without any erosion between PT and FU, as witnessed in patients 10, 19, 21 and 22. This was true even when patients had an unbalanced translocation, such as patient 8, or telomeric aberrations such as patient 25. The latter's long telomere despite a complex genome with trisomies of chromosome 12, 18 and 19 can be explained when looking at its karyotype data (49,XX,+12,+18,+19 [7]/46,XX [23]) which reveals a large population of cells with a normal genome, and since telomere length is measure as a mean from a population of cell it can be speculated that the population with a normal genome would skew the telomere length towards a long telomere result. Finally, patient 1 also has relatively long telomere, although large telomere erosion is seen between PT and FU, which coincides with genomic evolution (gain of a larger deletion of 13q as well as biallelic deletion of MDR region).

4.4.2. Stable cases can also have short telomeres

However, two stable cases have shown to have very small telomere: Patient 23, who has short telomere at FU following large telomere erosion, acquires an unbalanced translocation. And, patient 86 has very short telomere at PT and short 17p telomere at FU (as patient undergoes XpYp gain of telomere between the two time points), has a balanced translocation as well as deletion of GSTT1 at PT, a gene shown to be linked with short telomere (Broberg, Björk et al. 2005). It will be interesting to follow the disease progression in these patients.

4.4.3. Progressive cases have short telomeres linked with genomic instability

Progressive cases, with a complex genome, telomeric aberrations, deletion of *TP53* or *ATM*, genomic evolution as well as unbalanced translocations, such as patients 5, 28, 30 and 247, have very short telomere and undergo erosion between PT and FU. This was true even for patients who become somewhat "stable" after rounds of treatments but still acquired new aberrations, such as patient 7 and 9 who despite having a stable

WBC count, undergo genomic evolution, aberration on 17p and 11q (patient 9), gain of unbalanced translocation (patient7) and have very short telomere at FU in addition to a large erosion.

4.4.4. Progressive cases, despite complex genomes, can have long telomeres

However, not all progressive patients with unstable genome had short telomeres: Patient 29 is a progressive case with a complex genome, including 2 telomeric aberrations, but has long telomeres and no erosion. Patient 249 is a progressive case with unbalanced translocation and patient 250 is an aggressive CLL case with the largest genomic evolution, including gain of telomeric aberration, but both have long telomere and no erosion. Finally patient 35 has long telomeres at FU (PT n/a) despite a large 13q deletion which undergoes genomic evolution, in addition to an unbalanced translocation.

4.4.5. Progressive cases have short telomeres even without an unstable genome.

Furthermore, progressive cases even without complex genome have short telomeres. Patient 15 has short 17p telomere and undergoes erosion on XpYp whilst genomic data reveal an unbalanced translocation and acquisition of new aberrations at FU. Patient 16 has short telomere at both time points which coincides with telomeric aberrations on chromosomes 2 and 18. Patient 14 had short telomeres at FU as well as trisomy 12. Patient 248 has short telomere at PT and FU and undergoes large 17p telomere erosion, although genomic data reveal a sole large 13q deletion (>12Mb). Patient 18 has shorter than the median telomeres at PT, despite having only a small 13q deletion. Even patient 11 has a very short telomere at PT despite a normal genome.

4.4.6. Progressive cases with a stable genome have long telomeres

Nonetheless, patient 12 was shown to have a normal genome and relatively long telomere despite an aggressive CLL disease. In addition, patient 34 also had long telomeres and a sole 13q deletion but aggressive disease. Finally patient 32, despite large telomere erosion, had relative long telomere at both time points.

Table 26: Telomere length, clinical data and genomic aberration of all 29 patients in the cohort. [1]S=Stable;P=Progressive; [2]M=Mutated;U=Unmutated;[3] in Kb, bold=shorter than median; [4]in months; nt=not treated; [5]in x10[9]/L/month; T=treated between PT and FU; [6] minus=loss of telomere; [7]del=deletion; enh=gain; 17p=deletion on chr 17 including P53; 11q=deletion on chr 11 including ATM; 13q=deletion on 13q MDR; (s)=smaller than 2Mb; (L)=larger than 2Mb; x2=including a small homozygous region; (h)= homozygous 13q deletion; Tri=trisomy; (2)=number of deletion on the chromosome; (g)= smaller than 0.1Mb, targeting gene, N=normal; Bold=only at FU; [8]B=Balanced translocation; U=unbalanced translocation; Bold=only at FU

ID	CLL status[1]	IgVH/ CD38[2]	XpYp (PT)[3]	17p (PT)[3]	TTFT after PT [4]	WBC rise[5]	XpYp (FU)[3]	17p (FU)[3]	XpYp erosion[3,6]	17p erosion[3,6]	Genomic aberration[7]	Trans Location[8]
1	S	M/-	5.14	4.92	nt	0.52	4.31	5.08	-0.83	0.16	13q(s); **13q(L)x2**	
5	P	M/+	**3.46**	**2.16**	52	T	**2.87**	**2.82**	-0.60	0.65	Tri12; Tri18; Tri19; 13q(L); del3(2); del9(2)	
7	P	M/-	4.68	3.85	nt	0.00	**2.98**	**2.62**	-1.70	-1.23	13q(L); **13q(2)x2; del18; LOH13q**	B, **U**
8	S	M/-	.	.	nt	0.00	4.10	4.58	.	.	13q(s); del16(g)	U
9	P	M/-	6.84	4.40	nt	0.00	5.61	**2.93**	-1.23	-1.47	13q(L)x2; **11q; LOH17p**	
10	S	M/-	.	.	nt	0.04	4.28	**3.57**	.	.	13q(s)	
11	P	M/-	**2.79**	**3.37**	46	T	**3.06**	**3.48**	0.26	0.11	N	
12	P	M/-	5.06	**3.12**	104	0.95	**.**	.	.	.	N	
14	P	U/+	**3.30**	4.06	73	0.00	**2.82**	4.10	-0.47	0.04	Tri12	
15	P	M/+	**3.54**	**3.06**	74	T	**3.01**	**2.97**	-0.53	-0.09	13q(L); del2; enh13; enh2; **del13(g); del15(g)**	U

16	P	U/+	**2.54**	**3.28**	35	4.21	**2.70**	**3.26**	0.16	-0.02	del2p; del18p	
18	P	U/-	**2.26**	**2.47**	31	5.26	.	.	.	.	13q(s)	
19	S	M/-	4.06	4.22	nt	0.13	4.13	4.23	0.07	0.01	(h)13q(s); LOH13q(2)	
21	S	M/-	5.27	**3.35**	nt	0.98	5.28	3.89	0.00	0.54	13q(s); del21(g); **enh2(g); del13(g)**	
22	S	M/-	.	.	nt	0.02	4.38	6.42	.	.	N	
23	S	M/-	3.92	5.39	nt	0.12	**2.14**	3.90	-1.78	-1.49	(h)del13q(s); LOH13q	**U**
25	S	M/+	4.00	4.14	nt	0.00	5.84	4.71	1.84	0.57	Tri12; tri18; tri19	
28	P	M/+	**2.65**	**2.96**	nt	0.02	**1.40**	**2.51**	-1.25	-0.45	13q(L); 17p; del17(g); del18(2); **11qx2; 20p; delY(2)**	U
29	P	M/-	5.39	7.15	34	2.94	4.85	7.26	-0.53	0.11	13q(s); del4; 9p; 20p;	
30	P	U/-	**2.47**	3.56	52	4.24	**2.13**	**2.52**	-0.33	-1.04	Del10; del12(3); enh12; del19(2); LOH17p; **8p; del9(2)**	
32	P	M/-	7.77	6.22	116	0.53	5.88	5.74	-1.89	-0.48	13q(s)x2; **del21(g)**	
33	P	U/-	.	.	41	T	.	.	.	.	**Del7(g)**	**B**
34	P	M/-	4.59	5.35	34	T	4.51	4.96	-0.09	-0.39	13q(s)	
35	P	M/-	.	.	76	0.36	5.37	5.27	.	.	13q(L); del7; **13q(L)x2**	**U**
86	S	M/-	**2.85**	**2.86**	nt	0.00	**3.87**	**2.82**	1.02	-0.04	13q(s); del22(g)	B
247	P	U/+	**2.27**	**2.38**	17	5.13	**1.68**	**2.20**	-0.59	-0.18	11q(3); del3(3); del17(g)	
248	P	U/-	**2.46**	**3.15**	nt	0.78	**2.15**	**2.46**	-0.31	-0.69	13q(L)	
249	P	M/-	4.48	7.16	nt	2.36	4.44	7.07	-0.04	-0.08	13q(L)(3)x2; del6(3)	U
250	P	U/-	**3.56**	**3.39**	33	n/a	**3.24**	**3.12**	-0.32	-0.27	**Del1; del4(2); del5(2); del8; LOH17p**	**U**

4.5. Discussion

Research in telomere dynamics has uncovered many associations with disease progression and genomic instability, particularly in CLL. This study aimed to look at telomere length at two different time points and understand its role in CLL disease.

4.5.1. Telomere length overview, in relation to previous studies, highlights STELA technique

The length of XpYp and 17p telomere in this cohort was shorter than other studies: We found a mean telomere length of 3.7kb and 4 kb for XpYp and 17p respectively whilst Bechter et al for instance had a mean of 6kb (Bechter, Eisterer et al. 1998) and Rossi et al had a median of 6.3kb (Rossi, Lobetti Bodoni et al. 2009). However, both these studies used hybridisation technique, which would be less precise because STELA but not hybridisation assay can detect presence of very short telomere (Baird 2005).

Interestingly, studies using FISH-Flow or RT-PCR had similar average telomere length to our study: Roos et al used an RT-PCR technique and found an average telomere length of 4kb (Roos, Krober et al. 2008), whilst Damle et al used FISH methods and showed an average telomere length of 3.7kb (Damle, Batliwalla et al. 2004).

The shortest telomere in our study was 2.2kb. However, other studies have shown telomere even shorter than found in our study: Lin et al for instance reported an XpYp telomere of 0.9kb in a patient who transformed to Richters disease (Lin, Letsolo et al. 2010). Nonetheless, we found large scale aberrations and genomic rearrangement in patients with small telomere, which highlights the difficulty to find a cut off for when short telomere are poor prognosis and cause genomic instability, despite reports on the importance of ‘short telomere’ over ‘average telomere length’ (Hemann, Strong et al. 2001). Although a 2kb telomere length has been found to trigger cell cycle arrest signal (Levy, Allsopp et al. 1992), more research is needed, especially with high resolution technology, to define ‘short telomere’ and establish a telomere length cut off at which B-CLL cells be termed poor prognosis, or be linked with an unstable genome.

4.5.2. Telomere length and biomarkers

Telomere length was shown here to be associated with biomarkers IgVH mutation and CD38 expression, confirmed previous findings (Hultdin, Rosenquist et al. 2003; Roos, Krober et al. 2008). Interestingly, our study found a median XpYp telomere of 2.5kb for unmutated and 4.5kb for mutated IgVH patients, which is a much larger difference compared to Huldting et al’s research who reported a median of 4.2kb and 5kb in

unmutated and mutated IgVH patients respectively (Hultdin, Rosenquist et al. 2003). This is could be due to Huldtin et al using the less precise hybridisation technique.

Our study however did not confirm Lin et al's report of a link between telomere length and disease stage (Lin, Letsolo et al. 2010), although patients with late disease stage B and C were found to have the shortest telomere. Yet, the presence of short telomere in stable and early stage A0 disease, such as in our study, has been previously reported: Lin et al found similar telomere length between patients with stage C and a number of patients with stage A (Lin, Letsolo et al. 2010). In addition, Ricca et al also could not find a correlation between binet stage and telomere length (Ricca, Rocci et al. 2007). The presence of short telomere in early stage disease, just as the presence of unmutated IgVH status in stable patients, brings strength to the idea that telomere length is not necessarily a result of proliferative cell division, as would be found in late stage disease, but is defined from the orgine B-cell clone, whether pre-GC or post-GC.

4.5.3. Telomere length may not be a suitable prognostic marker

Only few studies have referred telomere length as a prognostic marker for disease progression (Rossi, Lobetti Bodoni et al. 2009; Sellmann, de Beer et al. 2011). Even then, Sellman et al used delta telomere length (telomere data from CLL patients in relation to healthy aged-matched donor) and found a threshold of -4.2kb to predict disease outcome (Sellmann, de Beer et al. 2011), whilst Rossi et al used a cut off of 5kb (based on their cohort) to showed prognosis value (Rossi, Lobetti Bodoni et al. 2009), and Roos et al found telomere length as independent marker only when disease stage was excluded (Roos, Krober et al. 2008).

Our study strongly suggests that it is difficult to class telomere length as a prognosis marker, because although it was found to correlate with IgVH mutation, which is predictable since telomere length has been found to depend of cell origin (Pre or Post GC lymphocyte) (Weng, Granger et al. 1997), and genomic instability, which is again predictable since short telomere drive genomic instability (Lin, Letsolo et al. 2010), no correlation was found between disease stage or treatment and telomere length in our cohort. We showed that telomere length at PT could not predict disease progression nor was telomere erosion linked with progression to late disease stage. In addition, this study uncovered 3 stable patients (out of 9) with critically short telomere: two patients underwent genomic evolution and large telomere erosion and the third patient had shorter than the median telomere length at both time points, whilst all three had stable disease, with no treatment, no CLL symptoms and low WBC count ($15< x10^9$/L). The presence of short telomere in a large percentage of stable cases, which has also

been reported previously (Lin, Letsolo et al. 2010) questions its possible usage as prognostic marker.

In addition, Rossi et al found short telomere to predict Richter's transformation (Rossi, Lobetti Bodoni et al. 2009). However, our study included 4 patients who progressed to Richter's disease and 2 out of 3 of these patients had longer than the median telomere length as well as very little evidence of telomere erosion (data on 1 patient was not available).

Furthermore, our study found no correlation between short telomere and treatment requirement, although a significant association was noted between TTFT and telomere length.

Finally, telemore length detection is a tedious as well as lenghty process which makes it a difficult prognostic marker for clinical usage.

Hence, the presence of short telomere in early stage disease and the lack of significant evidence associating telomere length to disease progression and treatment, calls for the need of further research before telomere length can be classed as a marker for disease progression

4.5.4. Telomere dysfunction was linked with unstable genome

As shown in previous studies (Lin, Letsolo et al. 2010), our study found that patients with short telomeres were associated with significantly more copy number changes as well as a larger scale genomic evolution. We also, for the first time in CLL, found a link between terminal aberrations and short telomeres. As suggested by Gisselsson et al in other tumours, short telomere cause chromosome fusion and loss of terminal aberration through formation of anaphase bridges (Gisselsson, Jonson et al. 2001). All patients in our cohort with short telomere and terminal aberrations (n=7) had aggressive disease, whilst 70% of them received more than 2 round of treatment, 70% of them had a higher than average WBC count increase rate and 3 of them had large scale genomic evolution between PT and FU. Loss of telomere function plays thus an important role in genomic instability which drives disease progression.

However, we found no link between unbalanced translocation and telomere length. Although a majority of patients with translocation had a much lower than average telomere length, confirming previous reports of short telomere driving chromosome rearrangement (Lin, Letsolo et al. 2010), a number of patients with translocations also had long telomeres, such as, patient ID 8 or ID 86, who had stable disease, stable WBC

count as well as mutated IgVH. This suggest that not all translocations may be telomery driven, as mutated IgVH and stable WBC count in these patients suggests that long telomere were found from the original cell. Although not much has been established as to the cause of translocations, factors other than telomere dysfunction have been suggested as mechanism for translocation, such as non-homologous end-joining due to specific susceptible target sequences or DNA mutations (Aplan 2006; Lieber, Yu et al. 2006). In addition, as reviewed by Aplan et al, translocation do not necessarily give rise to malignancy and may require additional dysfunctions for disease progression, which would explain the translocation in stable cases found in our cohort (Aplan 2006).

The absence of any association between translocation and telomere length in our study was also due to a number of patients with shorter than average telomere but no translocations. However, when reviewing these patients, it was found that they had large and complex deletion and gains, including terminal aberrations. This suggests that telomere dysfunction drives genomic instability through both, terminal aberrations and translocations.

4.5.5. Telomere dysfunction was linked with 11q/17p as well as 13q deletion size

It was interesting to note the link between 11q/17p aberration and telomere dysfunction, which confirmed previous study looking at specific aberrations and telomere length (Roos, Krober et al. 2008). Salin et al also found genomic instability and short telomere in B-CLL cells resistant to irradiation-induced apoptosis (P53 pathway aberration) (Salin 2009). This association however could be expected as Pepper et al suggested that primary aberration targeting DNA-damage pathway results in uncontrolled cell division and thus loss of telomere (Pepper and Baird 2010).

What was interesting however, and shown in our study for the first time, was the link between 13q deletion size and telomere length. The size of 13q deletion and its prognosis value in CLL patient was highlighted in a recent study by Parker et al (Parker, Rose-Zerilli et al. 2010). In our small cohort of patients, cases with poor prognosis large 13q deletion were associated with short telomere whilst patients with good prognosis small 13q deletion had longer than average telomeres. It could be speculated that the sequence deleted in large but not small 13q deletions encompass a gene involved in the P53 pathway which would drive cell division, and explain its association with short telomere. In addition, the first chapter of our study showed an association between 13q deletion size and genomic evolution. With strong evidence of telomere dysfunction driving secondary aberration, it would be exciting to investigate

the role of genes present in the large 13q deletion, especially in relation to cell cycle control.

4.5.6. Telomere erosion was found in CLL patients with progressive disease and was linked with genomic instability

To our knowledge, we report here the largest study on the changes in telomere length over time in CLL patients. Damle et al investigated telomere erosion in 12 patients (Damle, Batliwalla et al. 2004) whilst Brugat et al used hybridisation technique on a case study to found loss of 0.8kb of telomere length over time which correlated with the gain of secondary aberrations targeting DNA damage repair pathway (Brugat, Nguyen-Khac et al. 2011). Our study found that patients with progressive disease were more likely than stable cases to lose telomere length over time, which suggest a role of telomere dysfunction in disease progression.

However, we confirmed Damle et al study showing no association between telomere erosion and IgVH mutation (Damle, Batliwalla et al. 2004). This suggests that telomere loss is independent of the length of telomere at the onset, since both mutated and unmutated IgVH status patients undergo erosion. Our study showed that aberration on 11q and 17p targeting *ATM* and *TP53* as well as large 13q (but not small 13q) were linked with loss of telomere over time. In addition, we also found a strong link between loss of telomere over time and the acquisition of secondary aberrations well as gain of translocation over time. These data confirm the idea that telomere dysfunction occurs following a primary aberration targeting DNA damage repair checkpoint and causes further secondary aberrations, which drive disease progression.

4.5.7. Telomere dysfunction in absence of aberration on DNA-damage pathway

Nonetheless, the role of telomere dysfunction in CLL disease progression has been debated. Whilst Lin et al and others have suggested that short telomere cause genomic instability which results in cell division and aggressive CLL disease (reviewed by (Ladetto 2010), other have proposed short telomere as a mere consequence of proliferative B-CLL cell division (Jahrsdorfer and Weiner 2008). Our study shows a number of patients with short telomere or even large telomere erosion despite no apparent (by SNP6.0 array, FISH or G-banding) deletion of P53 pathway genes. For instance, patient 23 in our study, who is a stable case with mutated IgVH status with a sole small 13q deletion and no evidence of other aberrations, shows evidence of large loss of telomere over time, which even results in the acquisition of an unbalanced translocation. In addition, patient 11 in our study has a normal genome (no detected aberration using array or FISH), yet shorter than average telomere length. This may

question the theory that telomere loss occurs following aberrations on DNA-damage checkpoints (Brugat, Gault et al. 2009) and thus support Johrndorfer's theory that telomere may be a result of cell division.

Whilst most studies discuss telomere dysfunction as implicated in disease progression, it is clear that more research is required to confirm this especially looking at patients with a normal genome or small 13q deletion.

4.5.8. Role of telomerase and further research

Our study uncovered a number of patients with long telomere despite high WBC count and translocation. For example, progressive patient 249 had mutated IgVH status, significant rise in WBC count as well as a translocation between chromosome 6 and 13. Nonetheless, this patient had the longest telomere in our study at either time points. The possible explanation for long telomere despite cell division and translocation would be a rise in telomerase activity in these cells. Lo et al found fluctuation of telomerase activity in cancer cells (Lo, Sabatier et al. 2002), whilst Bechter et al as well as other studies have shown that progressive CLL patients have significantly higher telomerase activity (Counter, Gupta et al. 1995; Bechter, Eisterer et al. 1998). However Lin et al suggested that this would not be sufficient to maintain telomere length in aggressive CLL cases (Lin, Letsolo et al. 2010). A look at the activity of telomerase in our study would have shed more light in the presence of telomere in progressive cases with translocations and high WBC count. Nonetheless, it is clear that changes in telomere as well as telomerase occur over time and play a role in genomic instability and disease progression.

4.5.9. Conclusion

In conclusion, it can be said that although short telomeres have been associated with advanced stage CLL patients, it is difficult to confirm it as a prognostic marker as a large proportion of stable and early-stage patients have been noted with short telomere. Furthermore, short telomere were predominantly found in patients with unstable genome, although it was shown that not only translocations occurred in patients with 'long telomere' but also a proportion of patients with short telomere had no complex genome. Our study also highlighted the changes in telomere length between two time points, which was strongly associated with genomic evolution, but not disease stage or IgVH status. Finally, it was suggested that although short telomere, as previously hypothesised, would drive disease progression, the primary aberration targeting DNA-damage apoptosis pathway (P53) may be essential for B-CLL cells to enter further proliferation and immortal state.

5. General Discussion

Chromosomal aberrations have been associated with different disease prognosis in CLL patients. Numerous studies have looked at patients at a single time point and found chromosomal aberrations to predict either a stable disease or a progressive disease, with short time to treatment and requirement for treatment (Dohner, Stilgenbauer et al. 2000). However, tumours in general have been shown to be very unpredictable, acquiring genomic changes which aid tumoregenisis and cell survival (Heng, Bremer et al. 2006; Heng, Stevens et al. 2010). Patients with CLL have also been previously shown to have an unstable genome (Stilgenbauer, Sander et al. 2007). In addition, recurrent aberrations such as deletion of *TP53* as well as telomere dysfunction, noted in CLL patients, have been linked with genomic instability (Schwartz, Jordan et al. 2001; Salin 2009; Lin, Letsolo et al. 2010). For these reasons, research should turn towards the investigation of changes in the genome throughout the disease course.

Our study has shown that secondary aberrations in CLL is not a rare occurrence, nor is the loss of telomere length. The former has been found, using high resolution to target a large number of regions throughout the human genome, which has also been noted before (Kim, Jung et al. 2010) and hence may play an important role in disease progression.

The presence of secondary aberrations questions the validity of an initial prediction of disease course, or even choice of treatment, since different treatments are used according to chromosomal aberrations (Lozanski, Heerema et al. 2004; Butler and Gribben 2010). This is of great significance as secondary aberrations have also been recently found to significantly target 17p (Zainuddin, Murray et al. 2011) and thus the necessity for genetic tests prior to treatment. Clearly, our study has shown that patients can move from low-risk to high-risk aberrations, and this was associated with disease progression (rise in WBC count and need of treatment). This is not the first time that alteration of prognostic significance has been noted (Gunn, Mohammed et al. 2008).

In addition, having noted the association between telomere dysfunction and genomic instability in previous research (Lin, Letsolo et al. 2010), our study noted the occurrence of telomere erosion in CLL patients, which was not linked to any prognostic group, suggesting that patients with good prognosis may move to poor prognosis following loss of telomere. Although the prognosis impact of telomere length has not

been established, it is clear that telomere dysfunction results in genomic instability, or evolution as noted in our cohort.

It is therefore clear that monitoring genomic changes, change in copy number, mutation as well as telomere length, during the course of the disease is essential to ensure correct prognosis and choice of treatment.

5.1. Conclusions

In conclusion, this study looked at the changes in copy number and telomere length in 29 patients with CLL disease and found that secondary aberrations as well as loss of telomere occurred in progressive as well as stable cases and was not linked to prognostic markers. Nevertheless, patients with 11q/17p predictably acquired larger aberrations and had short telomeres, but so did patients with class II but not class I 13q deletion. In addition, intriguing changes were noted in copy number variants, particularly at 15q11. It was suggested from this study that the genome of CLL patient may be monitored throughout the disease course as a number of patients had a sudden change in disease course in parallel to the acquisition of poor prognosis aberrations. This was also linked with the loss of telomere length, which was shown to drive genomic instability.

5.2. Future research

Since the cohort in this study was relatively small, results would need to be confirmed in larger studies. Nonetheless, below is some key further research:

- The use of high resolution enabled a detailed genome-wide analysis and revealed a number of small aberrations at either time point or just as secondary aberration. In addition, a number of these deletions were found to be sole deletions or linked with small 13q deletion in progressive cases. Further research would involve confirming these changes at the genome level, using PCR, as well as investigate changes in the RNA/protein expression. Since genes in these regions involved tumour suppressor geness, it would be interesting to examine the role of these aberration on disease progression.

- A number of patients were found to acquire secondary aberration on 13q deletion, including homozygous deletions. In addition, class II 13q deletion were found to correlate with disease progression and telomere dysfunction. Further research in the common secondary deleted region may reveal genes involved in DNA repair.

- The exciting deletions noted on 15q11 need to be confirmed at the DNA level. In addition, it is highly credible that large cohort have recorded this deletion but classed it as CNV. Going back to large cohorts to confirm the presence of this deletion would enable a closer look as to its link with prognostic markers.

- This study did not analyse telomerase levels. Future research in the levels of telomere maintenance enzyme in each patient may reveal more information on the cause of short/long telomere, especially in patients with long telomere alongside translocation, or stable patients with short telomere.

- Further research in measuring the rate of telomere loss per year, using the WBC count data, would uncover new data and group patients with similar loss rate.

6. Appendix 1

Table showing FISH and karyotype data of all 29 patients at both time points

ID	FISH (PT)	FISH (FU)	Karyotype (PT)	Karyotype (FU)
1	D13S319[Deletion] 23% hemizygous 12% homozygous	ATM[Normal], TP53[Normal]; D13S319[Deletion]: 40% hemizygous 45% homozygous	46,XY,?add(10)(q?26) [2]	46,XY [30]
5	D13S319[Deletion]: 68% hemizygous; 12C[Deletion]: 47% trisomy	ATM[Normal],TP53[Normal]; D13S319[Deletion]: 98% hemizygous ; 12C[Deletion]: 78% trisomy	49,XY,+12,add(13)?dup(13)(q12q14),+18,+19[5]	49,XY,+12,add(13)?dup(13)(q12q14),+18,+19[5] /49,XY,idem,del9p13[2] /46XY,add(1q?32),del13q12-q14[3]
7	12C[Normal], ATM[Normal]; TP53[Normal]; D13S319[Deletion]: 91% hemizygous	12C[Normal], ATM[Normal]; TP53[Normal]; D13S319[Deletion]: 93% hemizygous	46,XY,t(1:2)(p36:p13)[2]	46,XY,del(13)(q14q22)[1]/ 46XY,der13del13(q14q22) t(13:18)(q22:p13),der(18)t (13:18)[11]
8	D13S319 [Deletion]:44% Hemizygous and 25% homozygous	12C[Normal], ATM[Normal]; TP53[Normal]; D13S319[Deletion]: 22% homozygous	'46,Xyder(13)inv(13)(q14 .1q21.1)t(2:5:13)(q37:q3 3:q14),der(2)t(2:5:13)[3]	'46XY,der(2)t(2:5:13)(q37: q32:q14),der(5)t(2:5:13),d er(13)inv(13)(q14q21)t(2:5 :13)[10]
9	12C[Normal], ATM[Normal],TP53[Normal], D13S319[Deletion]: 99% Homozygous	12C[Normal], ATM[Normal],TP53[Normal], D13S319[Deletion]: 78% hemizygous 6.5% Homozygous	'45,X-X[4]	complex,unstable,fragmen ts,dicentrics
10	done in 1999: D13S319 [Deletion]:93% Hemizygous	12C[Normal], ATM[Normal],TP53[Normal], D13S319[Deletion]: Hemizygous at in 78% and homozygous in 6.5%	46,XY	46,XY [30]
11	D13S319[Deletion]: 10% hemizygous	done in 2005: 12C[Normal],	46,XY	46,XY,del(13)(q14q22)[3],i dem,add(8)(p?23)[1]

		ATM[Normal],TP53[Normal], D13S319[Deletion]: 13% hemizygous		
12	12C[Normal], ATM[Normal],TP53[Normal], D13S319[Normal]	12C[Normal], ATM[Normal],TP53[Normal], D13S319[Normal]	46,XY	46,XY [29]
14	D13S319[Normal]; 12C[Trisomy], ATM[Normal],TP53[Normal]	D13S319[Normal]; ATM[Normal],TP53[Normal]; 12C[Trisomy]: 64% trisomy	47,XY,+12[30]	47,XY+12
15	ATM[Normal],TP53[Normal];D13S319[Deletion]: 26% hemizygous at with 2 signals 39% hemizygous at with 3 signals	12C[Normal], ATM[Normal],TP53[Normal]; 13q NOT DONE	47,XY,t(2:13)(p21:q14),+i(2)(p10)der(2)t(2:13),del(3)(q?),t(5:15)(q33:q15)[11]	47,XY,t(2;13)(p23;q14)del(13)(q12q14),+der(2)(13q34->::2p23->2q31::->13q34),t(5;15)(q33;q13)[23]/47,sl,add(20)(p13)[1]/46,XY [2]
16	ATM[Normal],TP53[Normal]; D13S319[Normal]	12C[Normal], ATM[Normal],TP53[Normal],D13S319[Normal]	46,XY,add(18)(p11)[12]	46,XY,add(18)(p11)[12]
18	ATM[Normal],TP53[Normal]; D13S319[Deletion]: 95% hemizygous	12C[Normal]; ATM[Normal],TP53[Normal], D13S319[Deletion]: 60% hemizygous 20% homozygous	46,XY	46,XY,del(13)(q12q14) [6]/46,XY [19]
19	ATM[Normal],TP53[Normal]; D13S319[Deletion]: 74% homozygous	12C[Normal]; ATM[Normal],TP53[Normal]; D13S319[Deletion]: 94% homozygous	‘46,XY	'46,XY,del(11)(q23q25)[1]/46,XY,?del(11)(q23q25) [3]/46,XY [26]
21	D13S319[Deletion]: 28% hemizygous	ATM[Normal],TP53[Normal]; 12C[Normal]; D13S319[Deletion]: 37% Homozygous	'45,X-X	'45,X-X
22	ATM[Normal],TP53[Normal]; D13S319[Normal]	12C[Normal]; ATM[Normal],TP53[Normal]; 13Q NOT	'46,XY	'46,XY [29]

		DONE		
23	12C[Normal]; ATM[Normal],TP53[Normal];D13S319[Deletion]: 65% homozygous	12C[Normal]; ATM[Normal],TP53[Normal];D13S319[Deletion]: 86% homozygous	'46,XX	45,XX,der(17)t(17;20)(p?;p?),-20 [inc 5]/46,XX [11]NB non clonal sructural rearrangementd including dicentric chromosomes also seen
25	D13S319[Normal]; 12C[Trisomy], ATM[Normal],TP53[Normal]: Trisomy 12 in 14% of cells	D13S319[Normal]; 12C[Trisomy], ATM[Normal],TP53[Normal]: Trisomy 12 in 26% of cells	'49,XX,+12,+18,+19	'49,XX,+12,+18,+19 [7]/46,XX [23]
28	[Deletion], D13S319[deletion] 6% hemizygous of P53 ; ATM[Normal] 3% of ATM	ATM[Deletion] P53[Deletion]: 76% hemizygous of P53 39% hemizygous at ATM; D13S319[deletion] 72% hemizygous	45,XY,der(18)t(17:18)(q?11:q23) [2]	'45,XY,der(18)t(17;18)(q21;q23) [4]/44,sl,-Y,del(13)(q12q3?2) [2]/44,sdl1,del(11)(q14q25) [6]/44,sdl2,t(6;11)(q13;q14) [7]/43,sdl1,add(16)(p13),-20 [2]/46,XY [13]
29	12C[Normal], ATM[Normal],TP53[Normal]; 13Q NOT DONE	12C[Normal]; ATM[Normal],TP53[Normal]; D13S319[Deletion]: 53% hemizygous 10% homozygous	'46,XX,del(13)(q14q22) [1]/46,XX,?del(13)(q14q22)[1]/46,XX[27]	'46,XX,?del(13)(q14q22 [2]/47,XX,+?r [1]/44,XX,t(1;2)(q32;q21),-13,-19 [1]/46,XX [26]
30	ATM[Normal],TP53[Normal]; D13S319[Normal]	12C[Normal];ATM[Normal],TP53[Normal]; D13S319[Normal]	'46,XX	'45,XX,?2(q),add(10q)del(12)(q13q21),-19[7],idem,-8,-9,der(12:9:8)(???)[15]
32	ATM[Normal],TP53[Normal];D13S319[Deletion]: 24% hemizygous 65% homozygous	12C[Normal];ATM[Normal],TP53[Normal]; 13q NOT DONE	'46,XY	46,XY,del(13)(q14q22) [1]/46,XY [29]
33	ATM[Normal],TP53[Normal]; D13S319[Normal]	12C[Normal];D13S319[Normal] ATM AND P53 NOT RECENTLY DONE	'46,XX	46,XX,t(4;15)(p?16;q?15) [4]/46,XX [21]
34	ATM[Normal],TP53[Normal];D13S319[Deletion]: 60% hemizygous	12C[Normal]; ATM[Normal],TP53[Normal];D13S319[Deletion]: 32%	'46,XX	'46,XY [30]

		hemizygous		
35	ATM[Normal],TP53[Normal],D13S319[Deletion]: 35% hemizygous	12C[Normal]; ATM[Normal],TP53[Normal];D13S319[Deletion]: 91% hemizygous	46,XY	46,XY,t(9;13)(q32;q14)[6], 46,XY,del(13)(q14q22) [1], 46,XY [38]
86	ATM[Normal],TP53[Normal],D13S319[Deletion]: 31% hemizygous	ATM NOT DONE; TP53[Normal],12C[Normal];D13S319[Deletion]: 22% hemizygous	46,XY,t(5:6)(q35:q21) [2]	46,XY,t(5;6)(q35;q21) [2]/46,XY [38]
247	ATM[Deletion],TP53[Normal],D13S319[Normal]: 77% of ATM	12C[Normal]; ATM[Deletion],TP53[Normal],D13S319[Normal]: 77% hemizygous ATM	'46,XY,del(11)(q21q23)[7]	46,XY,del(11)(q23q25) [16]/45,-X,Y,i(17)(q10) [1]/46,XY [13]
248	ATM[Normal],TP53[Normal],D13S319[Deletion]: 90% hemizygous	12C[Normal], ATM[Normal],TP53[Normal],D13S319[Deletion]:83% hemizygous	'46,XY.del(13)(q12q14)	'46,XY.del(13)(q12q14)
249	ATM[Normal],TP53[Normal],D13S319[Deletion]:9% hemizygous 86% homozygous	12C[Normal], ATM[Normal],TP53[Normal],D13S319[Deletion]:9% hemizygous 86% homozygous	'46,Xyt(6:13)(q26:q14)[30]	'46,Xyt(6:13)(q26:q14)[4]
250	13Q NOT DONE; ATM[Normal],TP53[Normal]	12C[Normal], ; ATM[Normal],TP53[Normal]; D13S319[Normal]	'45,x-x[2]	'46,XX,t(1:2)(p13q13),t(3:5)(p25p13),-8[cp4]

7. Appendix 2

MAPD score for normal, PT and FU array showing positive scores for all samples, except thos underlined. Birdseed data is also showed, confirming patient matched samples.

ID	*Normal MAPD score*	*PT MAPD score*	*FU MAPD score*	*Birdseed analysis (based on 50000 probes) in %*
1	0.2026644	0.3032022	?	99
5	0.2343662	0.3208943	0.3330209	99
7	0.2404362	0.245983	0.3249855	99
8	0.2655746	0.2348108	0.3846466	99
9	0.2416852	0.2662187	0.3593588	99
10	0.2875923	0.2564836	0.3517643	99
11	0.2932868	0.2382123	0.3368792	99
12	0.2267051	0.2127029	0.4041316	99
14	0.254896	0.2451761	0.3741238	99
15	0.2403825	0.3267927	0.2891427	99
16	0.2563863	0.2240404	0.3298258	99
18	0.2498528	0.2736083	0.3219639	99
19	0.2543191	0.3473347	0.383017	99
21	n/a	0.3830189	0.3322433	99
22	0.3307168	0.4019882	0.4140458	99
23	0.2551951	0.2505109	0.3668374	99
25	0.3361719	0.4148286	0.331688	99
28	0.270712	0.2374334	0.358154	99
29	0.3517024	0.1997756	0.3439162	99
30	0.3606648	0.216813	0.3366632	98
32	0.6290085	0.2863686	0.3307582	99
33	0.304495	0.3064315	0.4077737	99
34	n/a	0.3420789	0.291683	99
35	0.3899631	0.3012755	0.3196116	99
86	n/a	0.3391409	0.2773856	99
247	0.3508173	0.311726	0.3028514	99
248	0.3851296	0.3606753	0.3470268	99
249	n/a	0.3600878	0.2721696	99
250	n/a	0.3605579	0.3023126	98

8. Appendix 3

Complete data of all 29 patients

Patient data: 1_RB

ID:	1

Disease Status:	Stable
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	4
%ZAP70:	1
Alive:	yes

Patient clinical information	Diagnosed in 1998 with mBL. 2005: CLL stage A0. Stable since

Genomic data in brief:	13q deletion at presentation. Larger 13q and homozygous at Follow up

WBC count graph:

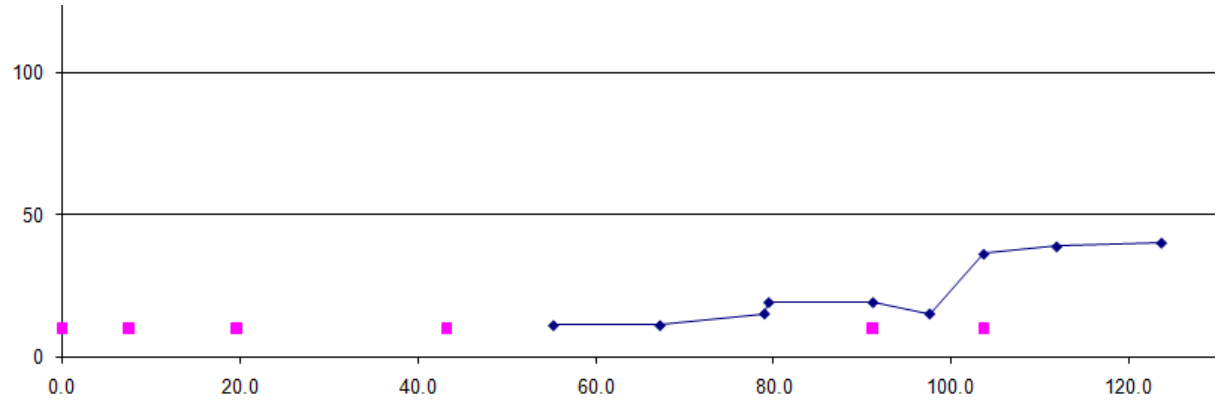
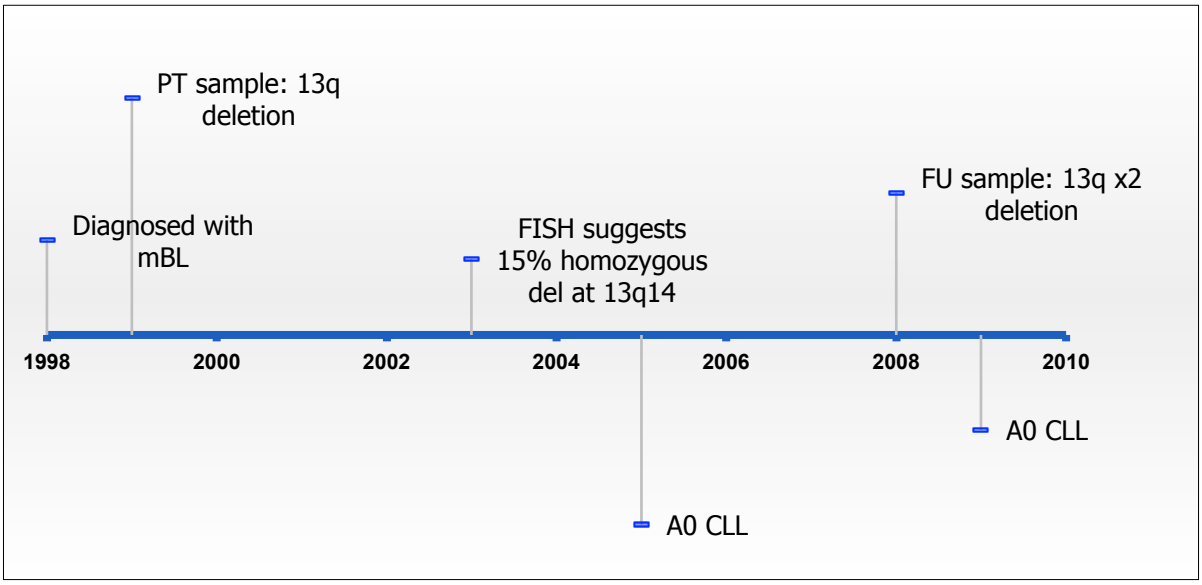


Figure 37:WBC count of RB (1) : PT sample at 0 and FU sample at 103

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	30/07/1999	
FISH data:	D13S319[Deletion] 23% hemizygous 12% hmozygous	
Karyotype	46,XY,?add(10)(q?26) [2]	
Number of CNV:	30	
CNA <1Mb:		
CNA >1Mb:	Diminished(13)(49.39-50.39)	13q14.3

Sample date	07/03/2003	
Karyotype	46, XY, del(7)(q?32q?36) [5] 46,XY,idem,t(1,6)(p22,q?) [1] 46,XY, ?add(10)(q?26) [1] 46,XY,t(1,19)(p22,p13) [1]	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal],D13S319[Deletion]:	58%hemizygous and 15%homozygous

Follow up Sample	14/03/2008	
FISH data	ATM[Normal], TP53[Normal]; D13S319[Deletion]:40% hemizygous 45% homozygous	40% hemizygous 45% homozygous
Karyotype	46,XY [30]	
Number of CNV:	31	
CNA <1Mb:		
CNA >1Mb:	**Diminished(13)(47.52-50.59)** **Diminished X2(13)(49.39-50.39)**	13q14.2 + 13q14.3 13q14.3

Patient data: 5_LL

ID:	5

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	100
%ZAP70:	1
Alive:	No [DOD:24/06/2008]

Patient clinical information	Patient in 2004 has CLL at stage C and is treated with CHLOR 2006: No presence of nodes but large spleen. Treated with FC x4. WBC goes down but large spleen (4cm) and marrow shows multiple foci. Patient does however improve. 2008: WBC goes back up and is treated first with mPred x2 then with mp+Rit Gains an infection and death.

Genomic data in brief:	Complex genome with deletions at chr 3, 13q deletion and trisomy 12, 18 and 19 at both PT and FU.

WBC count graph:

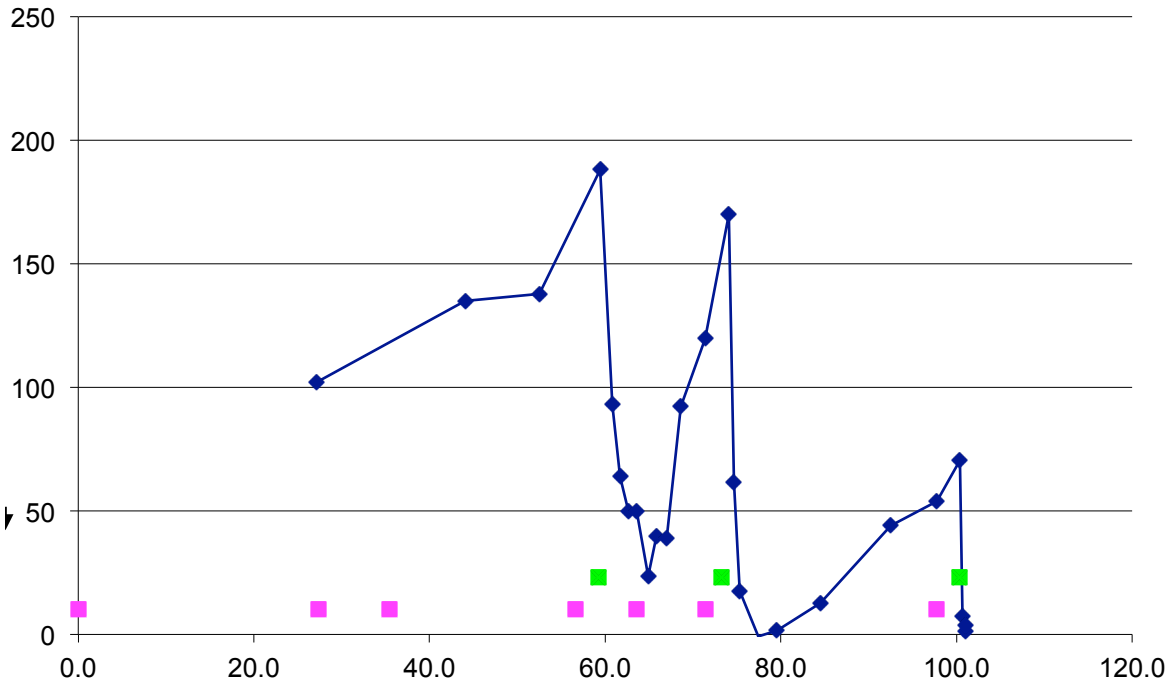
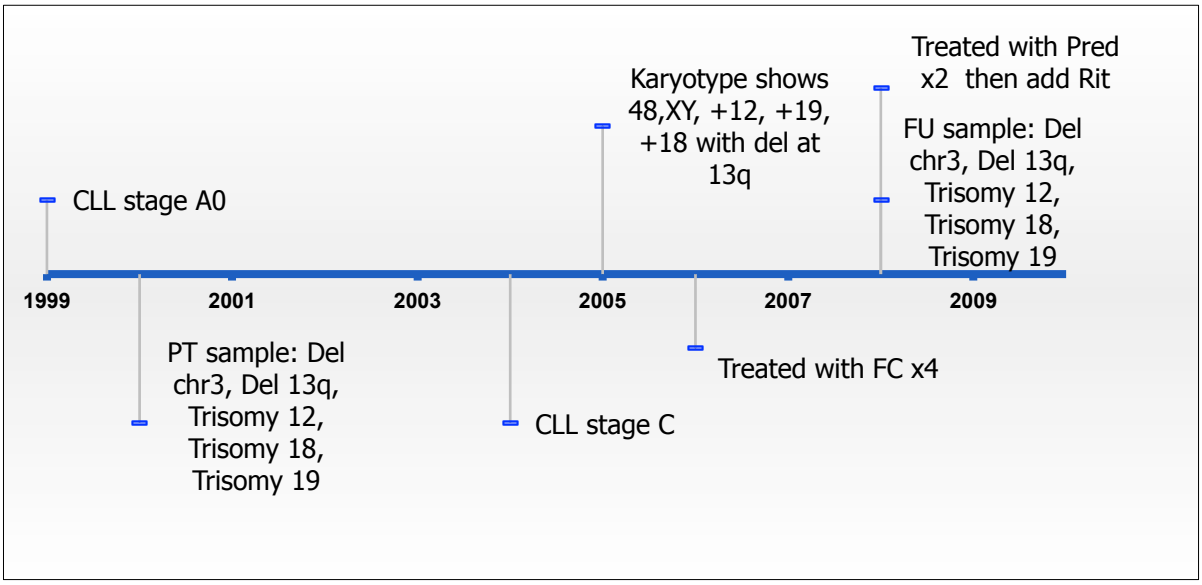


Figure 38: WBC of LL (5). PT sample at 0 and FU sample at 97

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	26/01/2000	
FISH data:	D13S319[Deletion]: 68% hemizygous; 12C[Deletion]: 47% trisomy	
Karyotype	49,XY,+12,add(13)?dup(13)(q12q14),+18,+19[5]	
Number of CNV:	27	
CNA <1Mb:	Diminished(3)(46.94-47.39) Diminished(9)(37.0-37.48) Diminished(3)(196.76-197.75)	
CNA >1Mb:	Diminished(9)(4.69-5.93) Trisomy(12)(0.02-132.28) Diminished(13)(48.68-50.7) Trisomy(18)(0.0-76.11) Trisomy(19)(0.04-63.79)	

Sample date	11/05/2005	
Karyotype	48,XY, +12,+19,add(13)?dup(q12q14) [7] 49,idem +18 [11] 49,idem, +18, del(9)(p13) [5]	
FISH data:	ATM[Normal],TP53[Normal]	

Follow up Sample	17/03/2008	
FISH data:	ATM[Normal],TP53[Normal]; D13S319[Deletion]: 98% hemizygous ; 12C[Deletion]: 78% trisomy	
Karyotype	49,XY,+12,add(13)?dup(13)(q12q14),+18,+19[5] /49,XY,idem,del9p13[2] /46XY,add(1q?32),del13q12-q14[3]	
Number of CNV:	26	
CNA <1Mb:	Diminished(3)(46.94-47.39) Diminished(9)(37.0-37.48) Diminished(3)(196.76-197.75)	
CNA >1Mb:	Diminished(9)(4.69-5.93) Trisomy(12)(0.02-132.28) Diminished(13)(48.68-50.7) Trisomy(18)(0.0-76.11) Trisomy(19)(0.04-63.79)	

Patient data: 7_PB

ID:	7

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	2
%ZAP70:	1
Alive:	yes

Patient clinical information	Diagnosed in 1981 as CLL stage A0. 1982: High WBC and cervical nodes (Stage A) and is given Intermittent treatment CHLOR between 1982 and 1997. No treatment since 1997 and stage A0 since

Genomic data in brief:	Large 13q deletion since 1988. Kary on 2005 suggests translocation between chr13 anc chr18. Array at FU (2007) shows a complexe homozygous 13q deletion as well as large chr 18 deletion

WBC count graph:

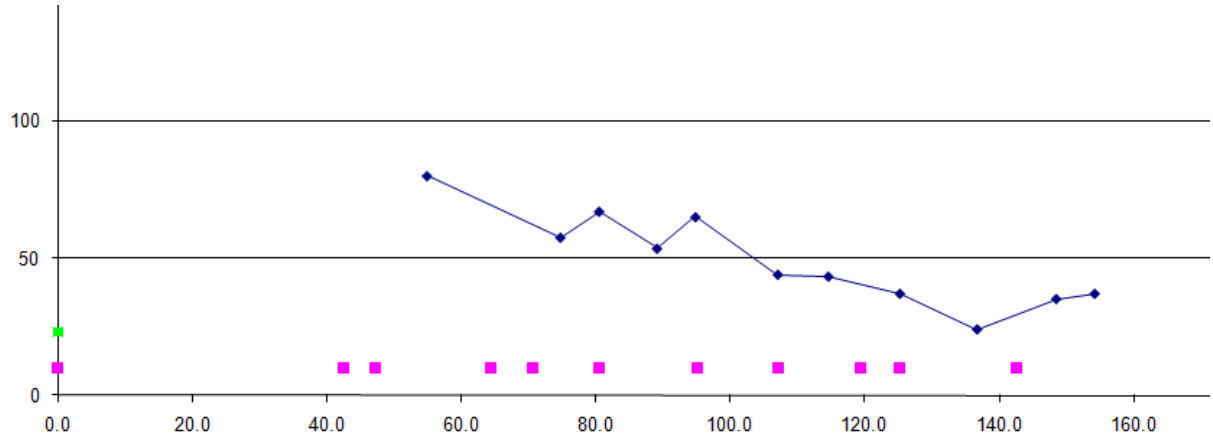
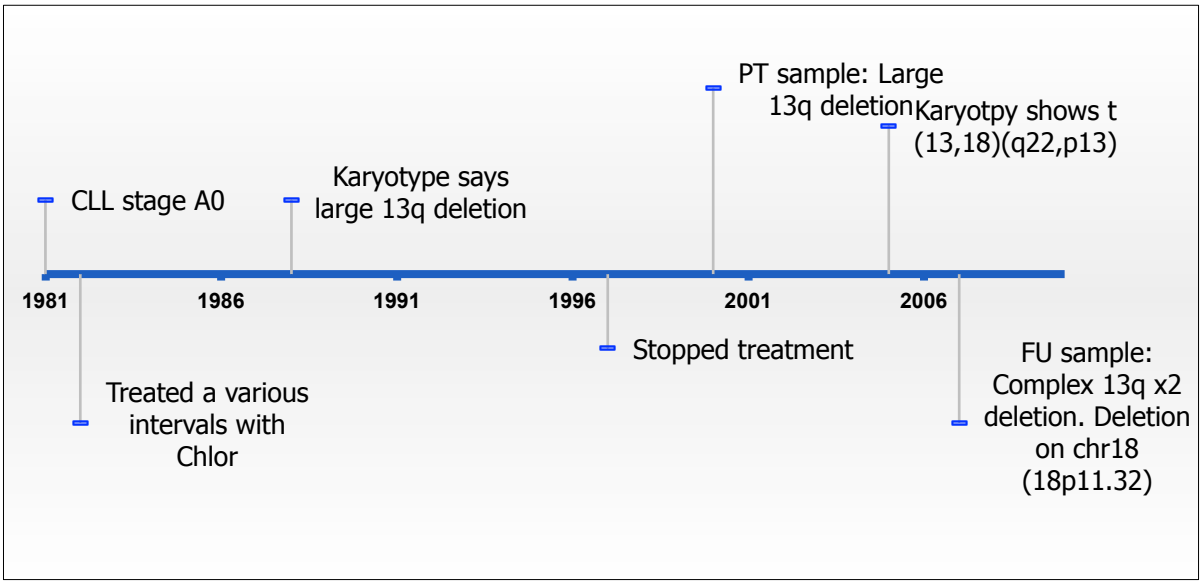


Figure 39: WBC of PB (7). PT sample at 42 and FU sample at 80

Timeline of Samples available with DATA from Array + FISH data:



Sample date	06/07/1998	
Karyotype	46,XY,del(13)(q14q22)	

Presentation Sample	18/12/2000	
FISH data:	12C[Normal], ATM[Normal]; TP53[Normal]; D13S319[Deletion]:91% hemizygous	91% hemizygous loss at 13q14
Karyotype	46,XY,t(1:2)(p36:p13)[2]	
Number of CNV:	26	
CNA <1Mb:		
CNA >1Mb:	Diminished(13)(41.07-69.04)	13q14.11-13q21.33

Sample date	17/02/2004	
FISH data	12C[Normal], ATM[Normal],TP53[Normal], D13S319[Deletion]	88% hemizygous loss at 13q14

Sample date	25/10/2005	

Karyotype	46,XY,del(13)(q14,q22) [1] 46,XY,der(13)del(13)(q14q22)t(13;18)(q22;p13),der(18)t(13,18) [11]	

Follow up Sample	14/05/2007	
FISH data	12C[Normal], ATM[Normal]; TP53[Normal]; D13S319[Deletion]: 93% hemizygous	
Karyotype	46,XY,del(13)(q14q22)[1]/ 46XY,der13del13(q14q22)t(13:18)(q22:p13),der(18)t(13:18)[11]	
Number of CNV:	27	
CNA <1Mb:	**Diminished X2(13)(47.25-48.13)**	
CNA >1Mb:	Diminished(13)(41.1-69.07) **Diminished X2(13)(48.58-50.37)** **Diminished(18)(1.7-2.86)** **LOH(13)(40.59-68.69**)	18q11.32

Patient data: 8_AL

ID:	8

Disease Status:	Stable
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	16
%ZAP70:	6
Alive:	yes

Patient clinical information	mBL patient since 1993

WBC count graph:

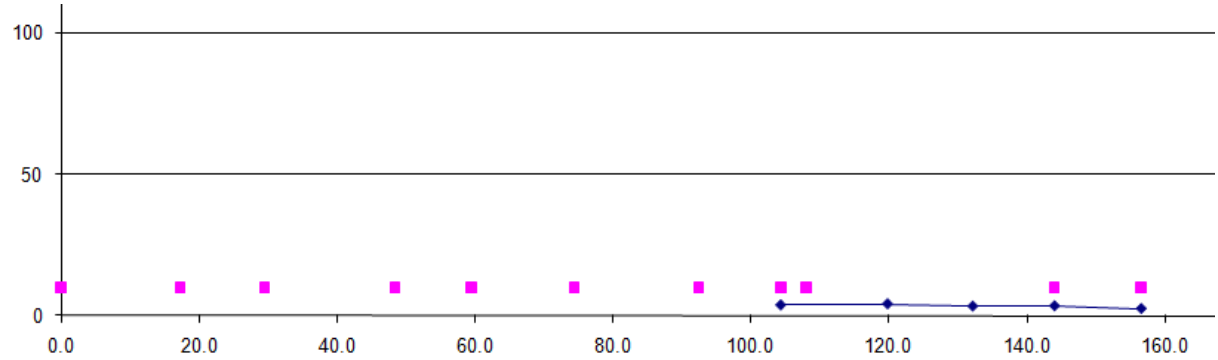
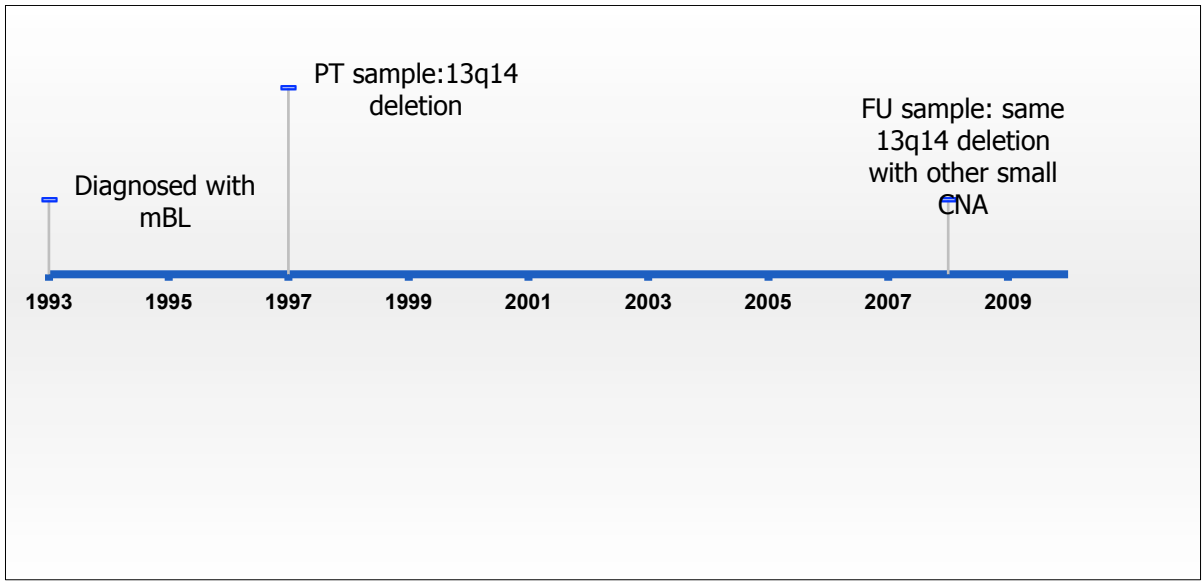


Figure 40: WBC of AL (8). PT sample at 29 and FU sample at 156

Timeline of Samples available with DATA from Array + FISH data:



Sample date	05/01/1994	
Karyotype	46,XY, t(2,5)(q35,q22) [1]	
FISH data:	D13S319 [Deletion]	44% Hemizygous and 25% homozygous loss at 13q14

Sample date	12/01/1995	
Karyotype	46,XY,t(2,5,13)(q35,q33,q14) [8]	

Presentation Sample	10/09/1997	
FISH data	D13S319 [Deletion]:44% Hemizygous and 25% homozygous	
Karyotype	'46,Xyder(13)inv(13)(q14.1q21.1)t(2:5:13)(q37:q33:q14),der(2)t(2:5:13)[3]	
Number of CNV:	30	
CNA <1Mb:	Diminished(16)(81.54-81.62)	
CNA >1Mb:	Diminished(13)(49.34-50.47)	

Sample date	05/10/1999	
Karyotype	46,XY,der(13)inv(13)(q14q21)t(2,5,13) t(2,5,13)(q37,q32,q14), der(2)t(2,5,13) [1]	

Sample date	03/12/2003	
FISH data:	D13S319 [Deletion]	22% homozygous loss at 13q14

Follow up Sample	07/04/2008
FISH data	12C[Normal], ATM[Normal]; TP53[Normal]; D13S319[Deletion]: 22% homozygous
Karyotype	'46XY,der(2)t(2:5:13)(q37:q32:q14),der(5)t(2:5:13),der(13)inv(13)(q14q21)t(2:5:13)[10]
Number of CNV:	31
CNA <1Mb:	Diminished(16)(81.54-81.62)
CNA >1Mb:	Diminished(13)(49.34-50.47)

Patient data: 9_DC

ID:	9

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	21
%ZAP70:	1
Alive:	yes

Patient clinical information	Diagnosed in 1990, CLL stage A0 1992 CHLOR treatement due to high WBC. 2006: Stage A, CHLOR treatement due to high WBC, but intolerant. 2007: Stage A. CYCLO x2 then FC x2 treatment (then P53 discovered) Stage A since then

Genomic data in brief:	Homozygous 13q deletion at PT is same at FU. 11q deletion seen at FU along with other small CNA. Follow up also shows LOH(17)(0.0-17.85)

WBC count graph:

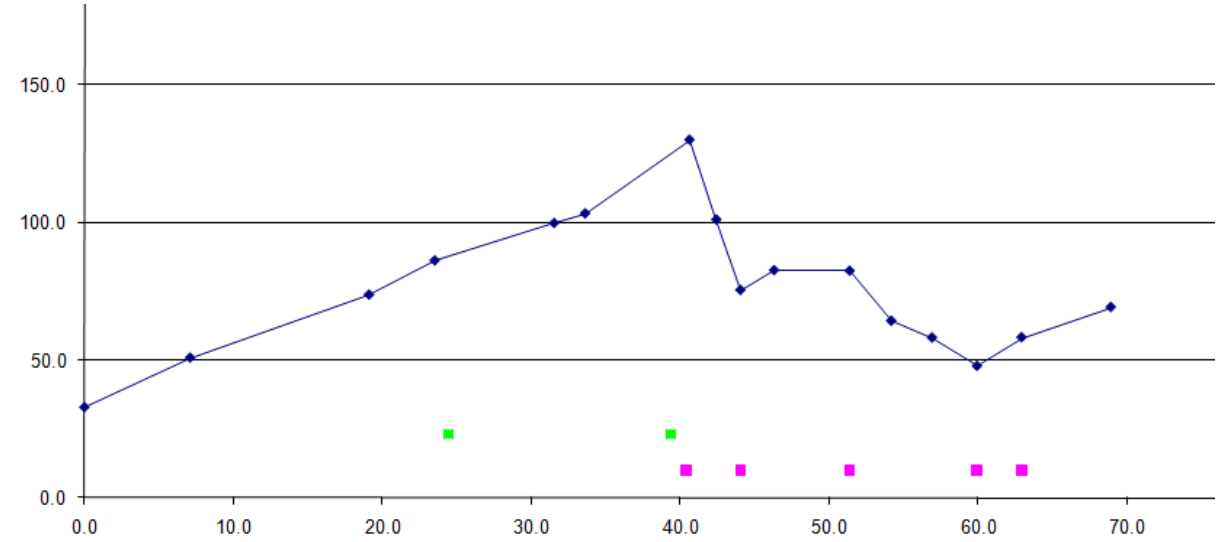
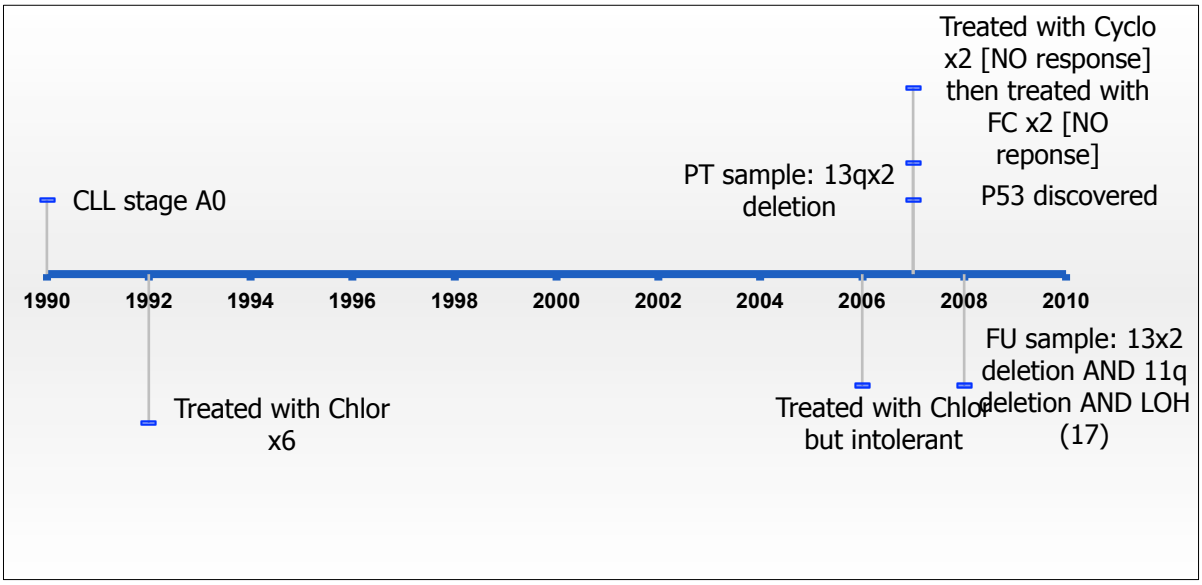


Figure 41: WBC of DC (9). PT sample at 40 and FU sample at 53

Timeline of Samples available with DATA from Array + FISH data:



Sample date	03/03/2004	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal], D13S319[Deletion]	95% Homozygous deletion at 13q14

Presentation Sample	31/05/2007	
FISH data	12C[Normal], ATM[Normal],TP53[Normal], D13S319[Deletion]:99% Homozygous	99% Homozygous deletion at 13q14
Karyotype	'45,X-X[4]	
Number of CNV:	24	
CNA <1Mb:	Diminished X2(13)(49.41-49.83)	
CNA >1Mb:	Diminished(13)(45.8-50.45)	

Follow up Sample	30/04/2008	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal], D13S319[Deletion]: 78% hemizygous 6.5% Homozygous	
Karyotype	complex,unstable,fragments,dicentrics	
Number of CNV:	25	
CNA <1Mb:	Diminished X2(13)(49.44-49.85)	
CNA >1Mb:	**Diminished(11)(63.42-65.23)** Diminished(13)(45.82-50.44) **LOH(17)(0.0-17.85)**	11q13.1 13q14.12q14.3 13q14.3

Patient data: 10_FL

ID:	10

Disease Status:	Stable
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	1
%ZAP70:	3
Alive:	No (19/09/2009) CONFIRM?

Patient clinical information	Diagnosed in 89 with CLL stage A0 (Full Blood Count abnormal due to Aortic Aneurysm). mBL from 2006

Genomic data in brief:	Stable patient with small 13q14 deletion

WBC count graph:

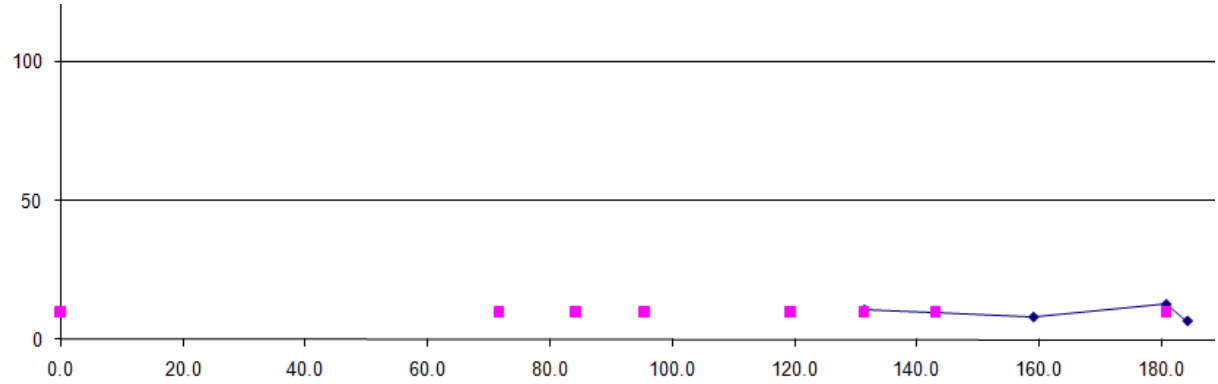
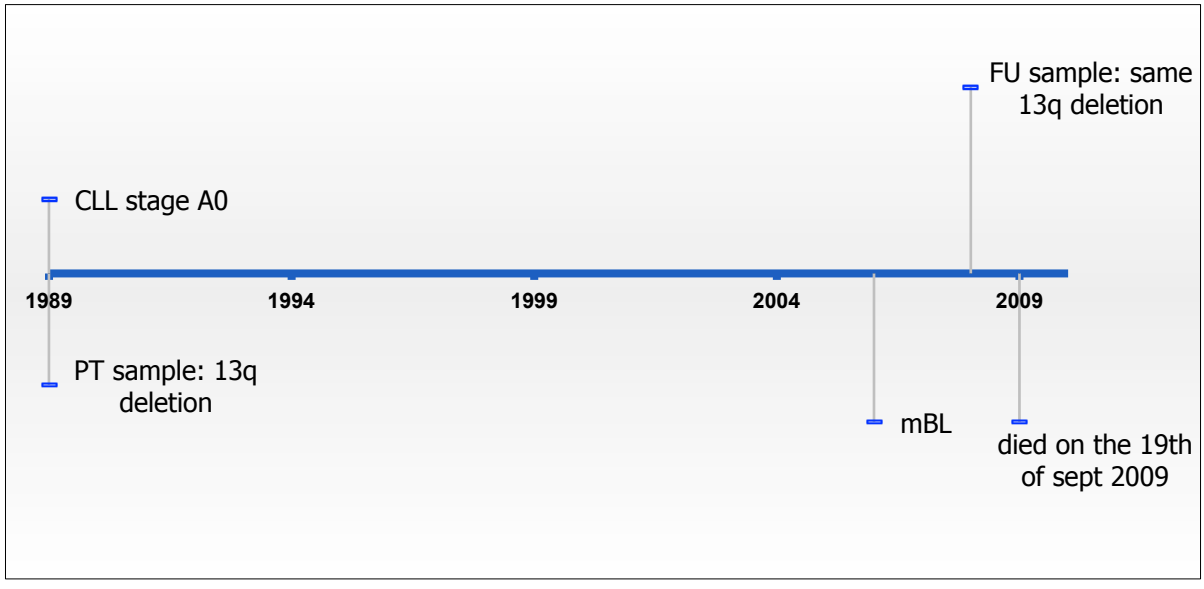


Figure 42: WBC of FL (10). PT sample at 0 and FU sample at 180

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	31/03/1993	
FISH data	done in 1999: D13S319 [Deletion]:93% Hemizygous	
Karyotype	46,XY	
Number of CNV:	26	
CNA <1Mb:		
CNA >1Mb:	Diminished(13)(48.72-50.66)	13q14.3

Follow up Sample	14/04/2008	
FISH data	12C[Normal], ATM[Normal],TP53[Normal], D13S319[Deletion]:Hemizygous at in 78% and homozygous in 6.5%	Hemizygous loss at 13q14 in 78% and homozygous loss in 6.5%
Karyotype	46,XY [30]	
Number of CNV:	24	
CNA <1Mb:		
CNA >1Mb:	Diminished(13)(48.7-50.68)	13q14.3

Patient data: 11_ES

ID:	11

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	3
%ZAP70:	3
Alive:	yes

Patient clinical information	Diagnosed in 2002 with CLL stage A0. Progressed to stage C by 2006 and therapy given (CHLOR x3). WBC went down but no increase in HG. 2008, stage B, WBC increasing. 2009, reached stage C and therapy given (CHLOR x6). Stage A since then.

WBC count graph:

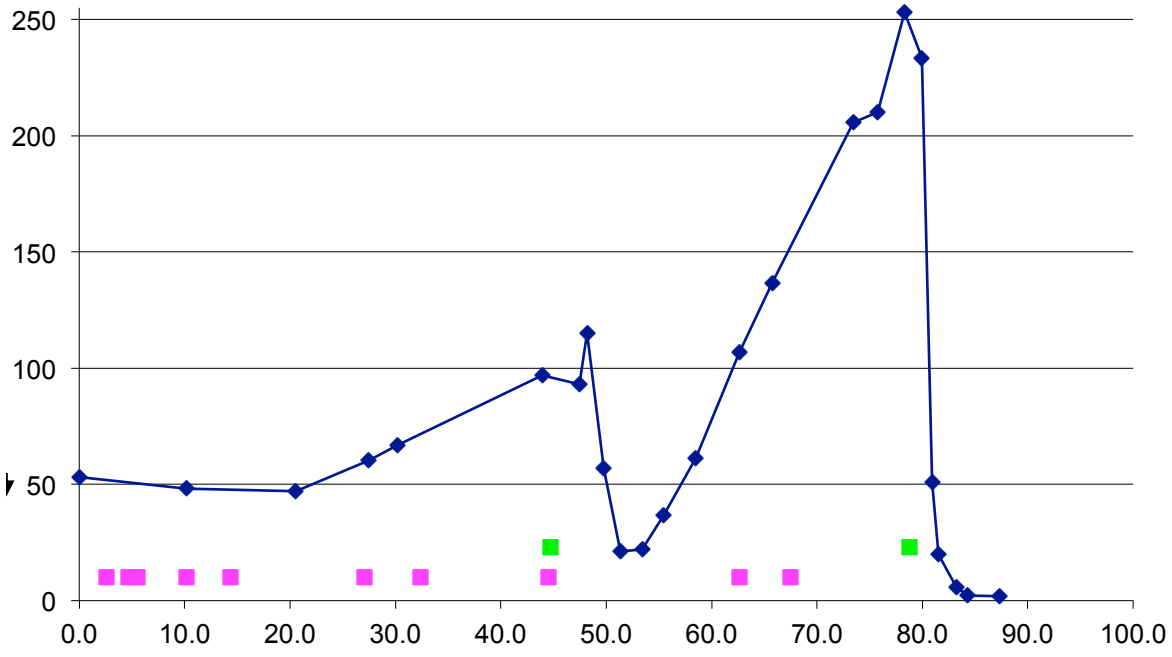
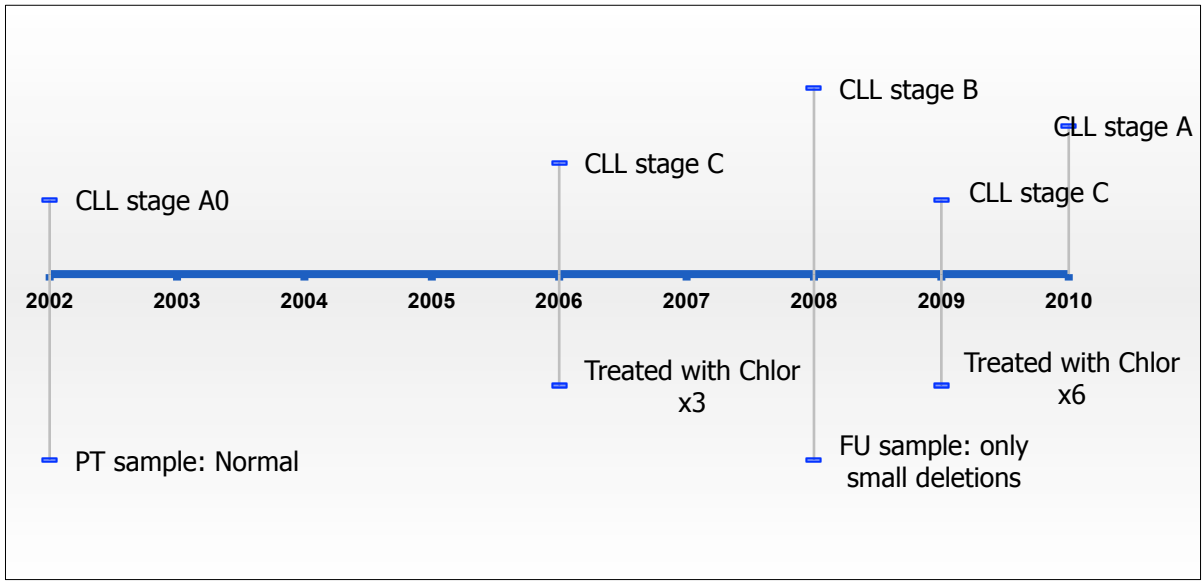


Figure 43: WBC of ES (11). PT sample at 2 and FU sample at 67

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	28/11/2002	
FISH data:	D13S319[Deletion]: 10% hemizygous	10% hemizygous loss at 13q14
Karyotype	46,XY	
Number of CNV:	33	
CNA <1Mb:		
CNA >1Mb:	Normal	

Follow up Sample	23/04/2008	
FISH data	done in 2005: 12C[Normal], ATM[Normal],TP53[Normal], D13S319[Deletion]: 13% hemizygous	
Karyotype	46,XY,del(13)(q14q22)[3],idem,add(8)(p?23)[1]	
Number of CNV:	35	
CNA <1Mb:		
CNA >1Mb:		

Patient data: 12_MC

ID:	12

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	1
%ZAP70:	3
Alive:	yes

Patient clinical information	Diagnosed in 1998 with mbl/A0 CLL. Progressed to stage C CLL in 2007 and treatement given: FC x6. Stage A since 2009

Genomic data in brief:	Normal genome

WBC count graph:

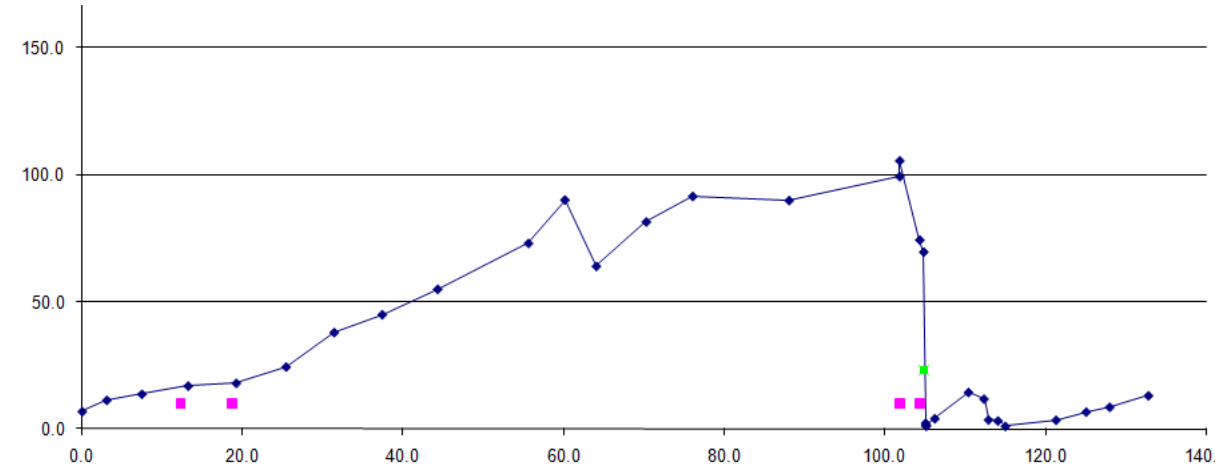
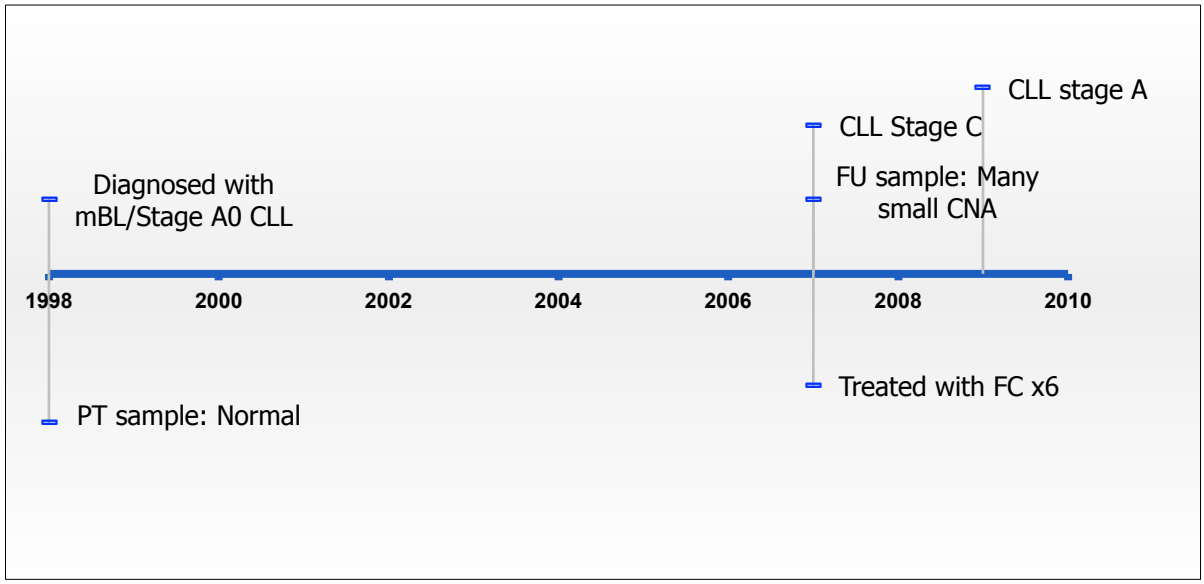


Figure 44: WBC of MC (12). PT sample at 0 and FU sample at 104

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	05/11/1998	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal], D13S319[Normal]	
Karyotype	46,XY	
Number of CNV:	33	
CNA <1Mb:		
CNA >1Mb:	Normal	

Sample date	19/11/1999	
Karyotype	46,XY, t(1;21;3)(p13;q22;p23), t(7,14)(q11;q22) [1]	
FISH data:	12C[Normal],	

Sample date	01/06/2000	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal],D13S319[Normal]	

Follow up Sample	18/07/2007	
FISH data	12C[Normal], ATM[Normal],TP53[Normal], D13S319[Normal]	
Karyotpe	46,XY [29]	
Number of CNV:	30	
CNA <1Mb:		
CNA >1Mb:		

Patient data: 14_AS

ID:	14

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Unmutated
%CD38:	95
%ZAP70:	80
Alive:	yes

Patient clinical information	Diagnosed with mBL in 2001. CLL stage A0 in 2006. Stage A2 in 2007 which turned into Richters 2 months later. Treatement given: R-CHOP x8. CLL stage A0 in 2010

Genomic data in brief:	Trisomy 12 patients

WBC count graph:

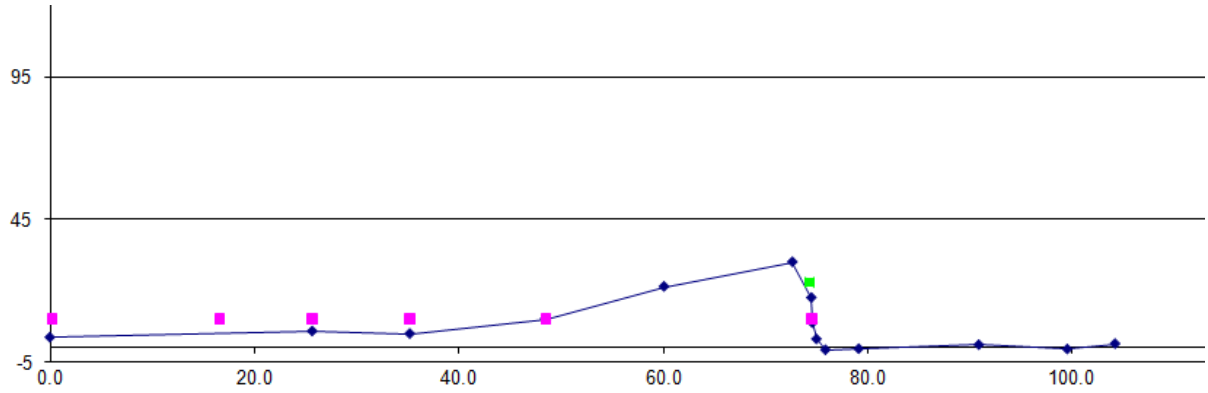
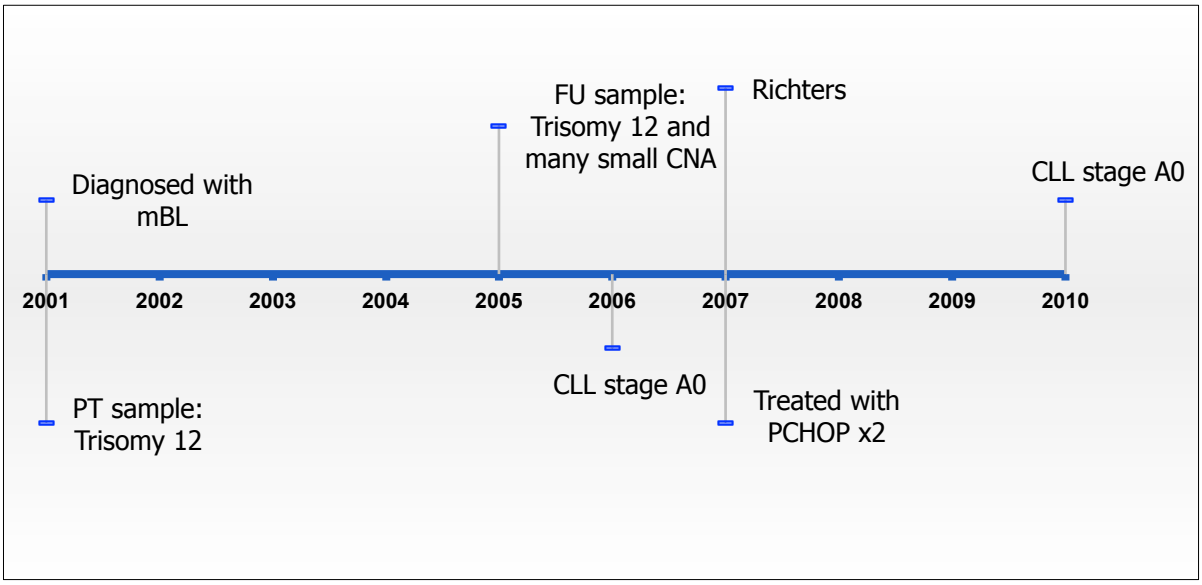


Figure 45: WBC of AS (14). PT sample at 0 and FU sample at 74

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	07/06/2001	
FISH data:	D13S319[Normal]; 12C[Trisomy], ATM[Normal],TP53[Normal]	Trisomy 12 in 64% of cells
Karyotype	47,XY,+12[30]	
Number of CNV:	32	
CNA <1Mb:		
CNA >1Mb:	Trisomy(12)(0.02-132.28)	

Sample date	06/05/2004	
FISH data:	12C[Trisomy], ATM[Normal],TP53[Normal],D13S319[Deletion]	Trisomy 12 in 57% of cells

Follow up Sample	14/06/2005	
FISH data	D13S319[Normal]; ATM[Normal],TP53[Normal]; 12C[Trisomy]: 64% trisomy	
Karyotype	47,XY+12	
Number of CNV:	25	
CNA <1Mb:		
CNA >1Mb:	Enhanced(12)(0.0-132.28)	

Sample date	13/08/2007	
FISH data:	ATM[Normal],TP53[Normal] C-myc [Normal]	
Karyotype	47, XY, +12 [30]	

Patient data: 15_VC

ID:	15

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	48
%ZAP70:	1
Alive:	yes

Patient clinical information	Diagnose with mBL in 2001 (35% clonal B cells) In 2006, stage A0 CLL. 2008: Progresses to stage C (large 9cm spleen) and treatement given (CHLOR x5). Further treatement in 2009 (CHLOR x6)

Genomic data in brief:	Deletion + gain at chr 2 and chr 13. Karyotype suggests translocation.

WBC count graph:

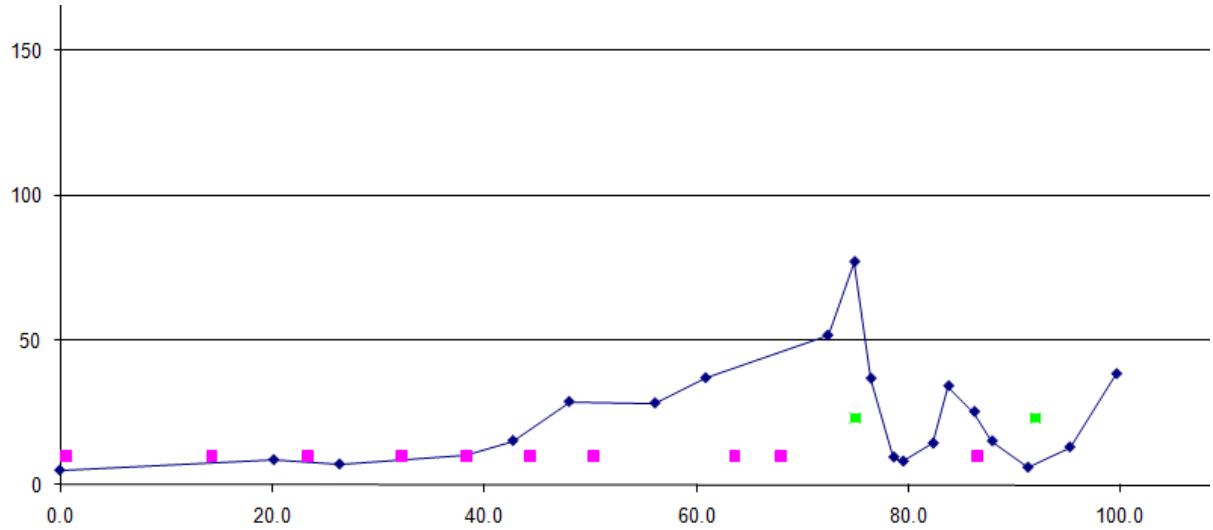
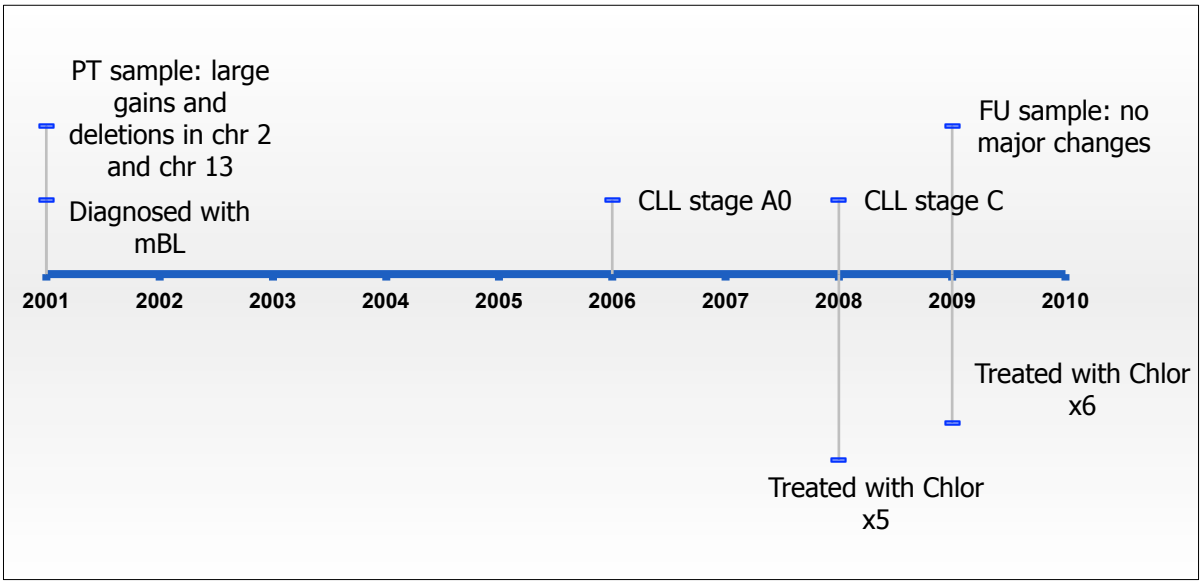


Figure 46: WBC of VC (15). PT sample at 0 and FU sample at 84

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	22/11/2001	
FISH data:	ATM[Normal],TP53[Normal];D13S319[Deletion]: 26% hemizygous at with 2 signals 39% hemizygous at with 3 signals	26% hemizygous loss at 13q14 with 2 signals 39% hemizygous loss at 13q14 with 3 signals
Karyotype	47,XY,t(2:13)(p21:q14),+i(2)(p10)der(2)t(2:13),del(3)(q?),t(5:15)(q33:q15)[11]	
Number of CNV:	21	
CNA <1Mb:		
CNA >1Mb:	Enhanced(2)(44.01-88.89) Diminished(2)(36.85-44.01) Diminished(13)(45.64-50.53) Enhanced(13)(50.53-114.13)	2p21-p11 2p22p21 13q14 13q14-q34

Follow up Sample	15/01/2009	
FISH data	12C[Normal], ATM[Normal],TP53[Normal]; 13q NOT DONE	
Karyotype	47,XY,t(2;13)(p23;q14)del(13)(q12q14),+der(2)(13q34->::2p23->2q31::->13q34),t(5;15)(q33;q13)[23]/47,sl,add(20)(p13) [1]/46,XY [2]	
Number of CNV:	21	
CNA <1Mb:	**Diminished(13)(39.31-40.15)** **Diminished(15)(29.07-29.12)**	
CNA >1Mb:	Diminished(2)(36.92-44.01) Enhanced(2)(44.03-88.91) Diminished(13)(45.91-50.53) Enhanced(13)(50.53-114.13)	2p21-p11 2p22p21 13q14 13q14-q34

Patient data: 16_CR

ID:	16

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Unmutated
%CD38:	86
%ZAP70:	34
Alive:	No (30/01/2009)

Patient clinical information	CLL stage A0 in 2001. High wbc and stage A2 in 2004. Treated with CHLOR in 2005 [PARTIAL response]. Stage A0 but high WBC in 2007, treated with CHLOR [PARTIAL response]. Died in 01/2009 (pneumonia)

Genomic data in brief:	Gain of 2p and deletion of chr18 at both PT and FU

WBC count graph:

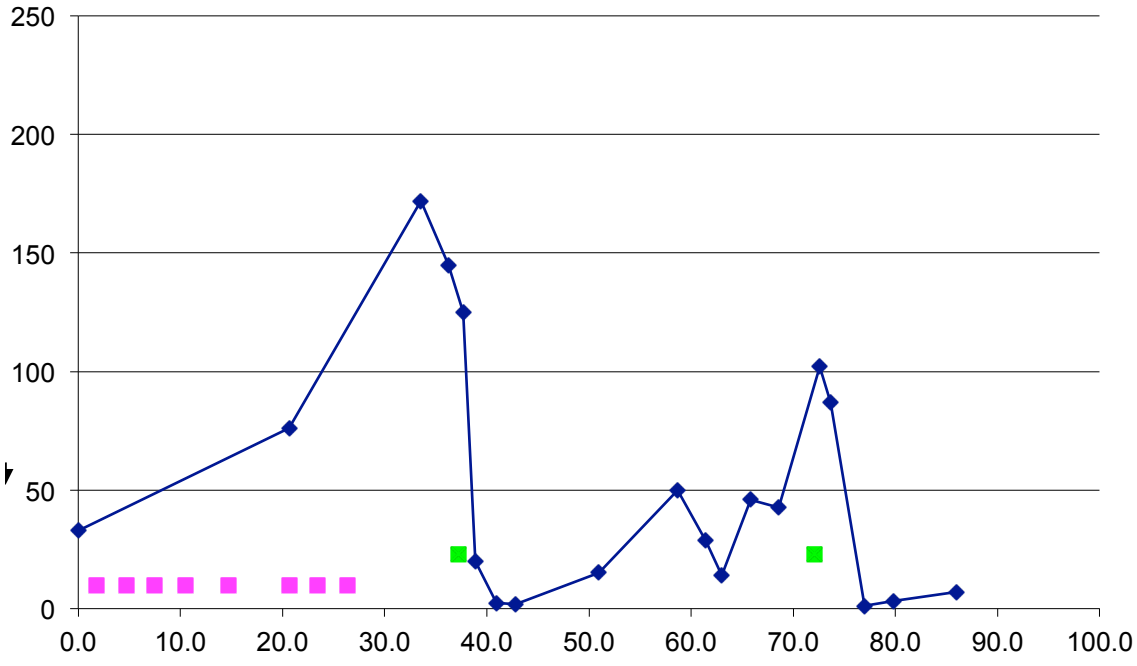
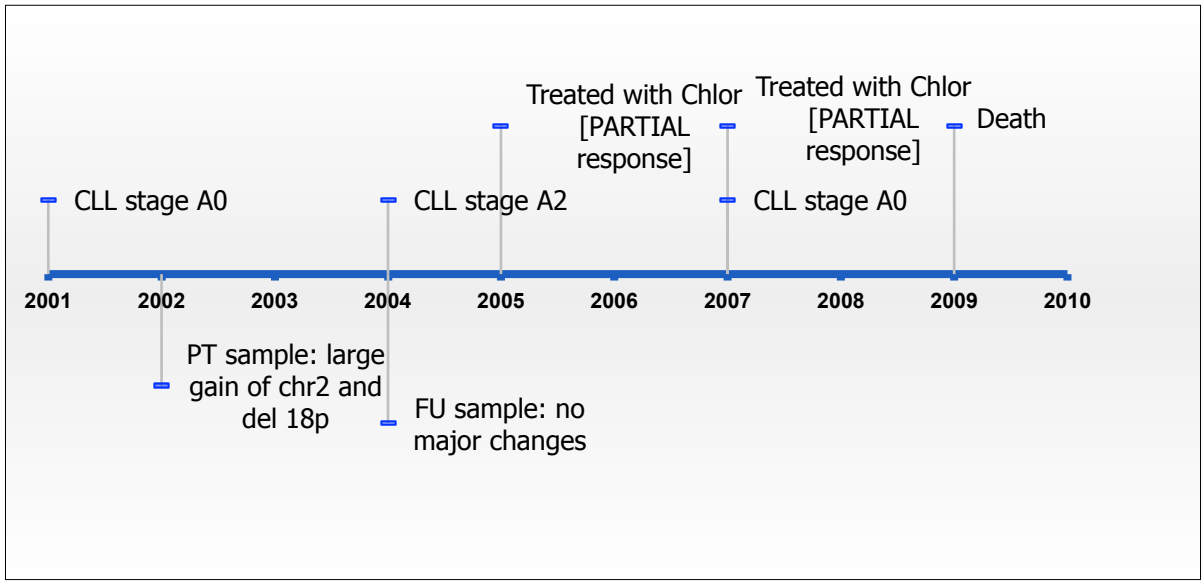


Figure 47: WBC of CR (16). PT sample at 1 and FU sample at 26

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	23/01/2002	
FISH data:	ATM[Normal],TP53[Normal]; D13S319[Normal]	
Karyotype	46,XY,add(18)(p11)[12]	
Number of CNV:	31	
CNA <1Mb:		
CNA >1Mb:	Enhanced(2)(0.0-94.71) Diminished(18)(0.0-15.39)	2p24-p11 18p11

Follow up Sample	10/02/2004	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal],D13S319[Normal]	
Karyotype	46,XY,add(18)(p11)[12]	
Number of CNV:	32	
CNA <1Mb:		
CNA >1Mb:	Enhanced(2)(0.0-94.71) Diminished(18)(0.0-15.39)	2p24-p11 18p11

Patient data: 18_G

ID:	18

Disease Status:	Progressive
Disease stage at Diagnosis:	A1
IgVH status:	Unmutated
%CD38:	21
%ZAP70:	18
Alive:	yes

Patient clinical information	2004 diagnosed with stage A1 CLL. Progressed to stage C and given treatement in 2007 (CHLOR x6 [PARTIAL response]. Remained at stage C in 2008 and was given treatement again (FC x6). Alentuzimab given in 2009 (as consolidation therapy because of minimal residual disease). Disease progressed to Richters in 2010

Genomic data in brief:	Small 13q deletion. Stable genome.

WBC count graph:

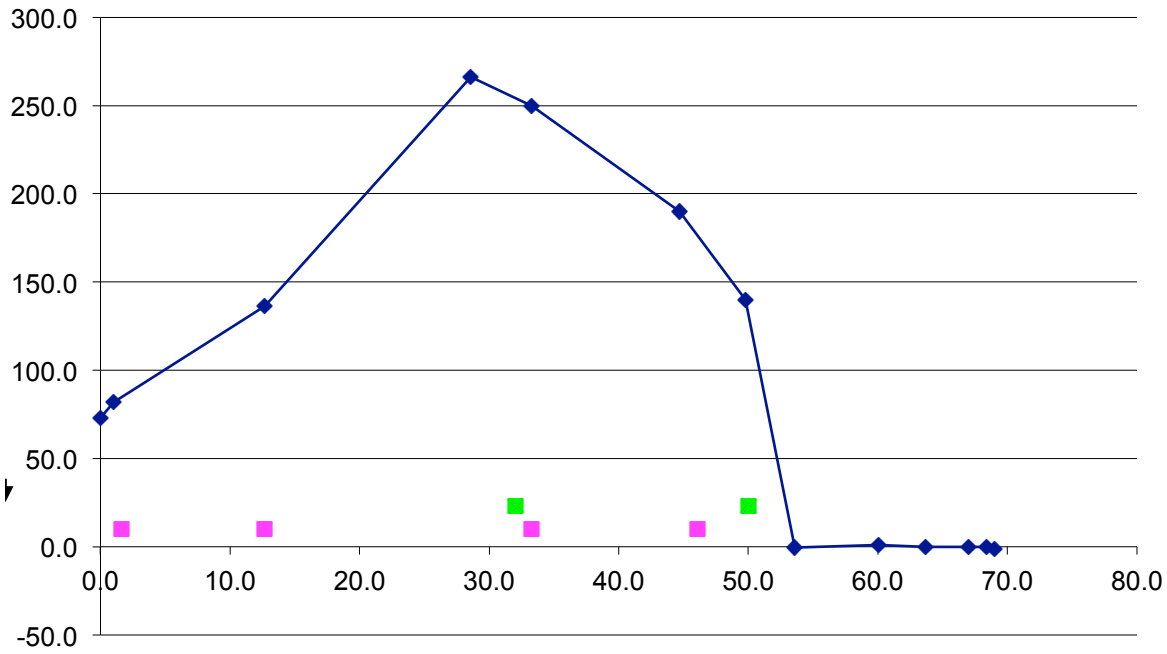
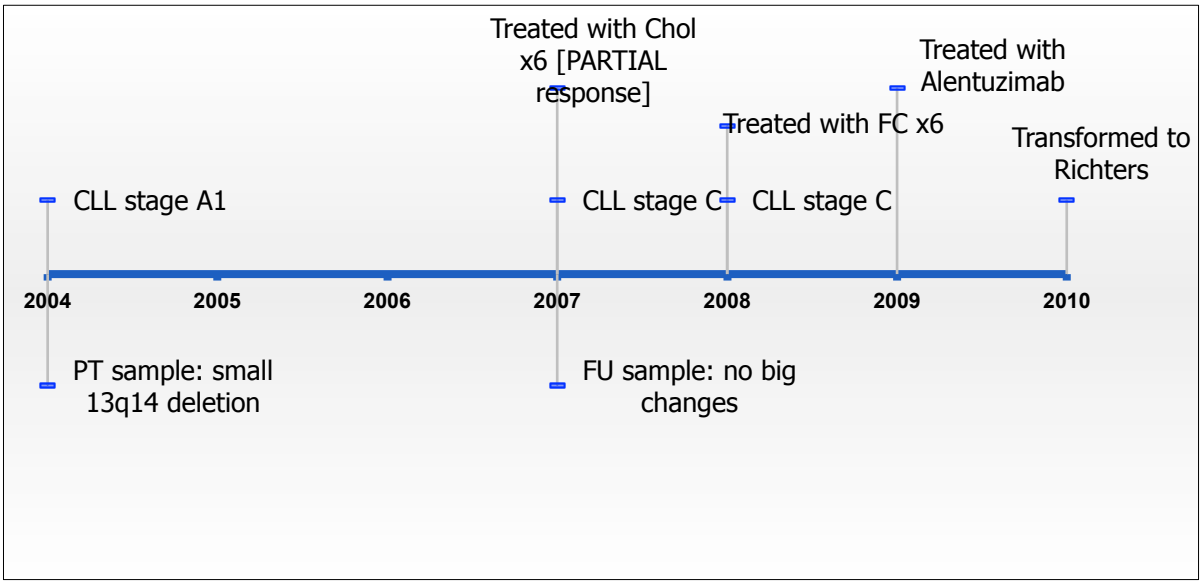


Figure 48: WBC of DG (18). PT sample at 1 and FU sample at 33

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	22/07/2004	
FISH data:	ATM[Normal],TP53[Normal]; D13S319[Deletion]: 95% hemizygous	95% hemizygous loss at 13q14
Karyotype	46,XY	
Number of CNV:	26	
CNA <1Mb:		
CNA >1Mb:	Diminished(13)(49.43-50.56)	

Follow up Sample	12/03/2007	
FISH data	12C[Normal]; ATM[Normal],TP53[Normal], D13S319[Deletion]: 60% hemizygous 20% homozygous	
Karyotype	46,XY,del(13)(q12q14) [6] /46,XY [19]	
Number of CNV:	27	
CNA <1Mb:		
CNA >1Mb:	Diminished(13)(49.45-50.54)	

Sample date	04/04/2008	
FISH data:	D13S319[Deletion]	60% hemizygous 20% homozygous loss at 13q14

Patient data: 19_GG

ID:	19

Disease Status:	Stable
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	1
%ZAP70:	2
Alive:	yes

Patient clinical information	Diagnosed in 1999 with CLL stage A0. Stable since.

Genomic data in brief:	13q14 homozygous deletion with a large LOH.

WBC count graph:

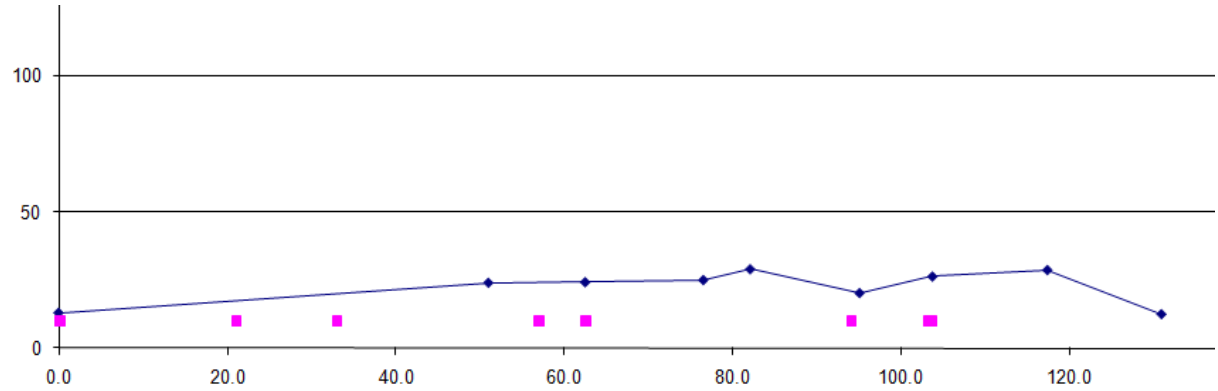
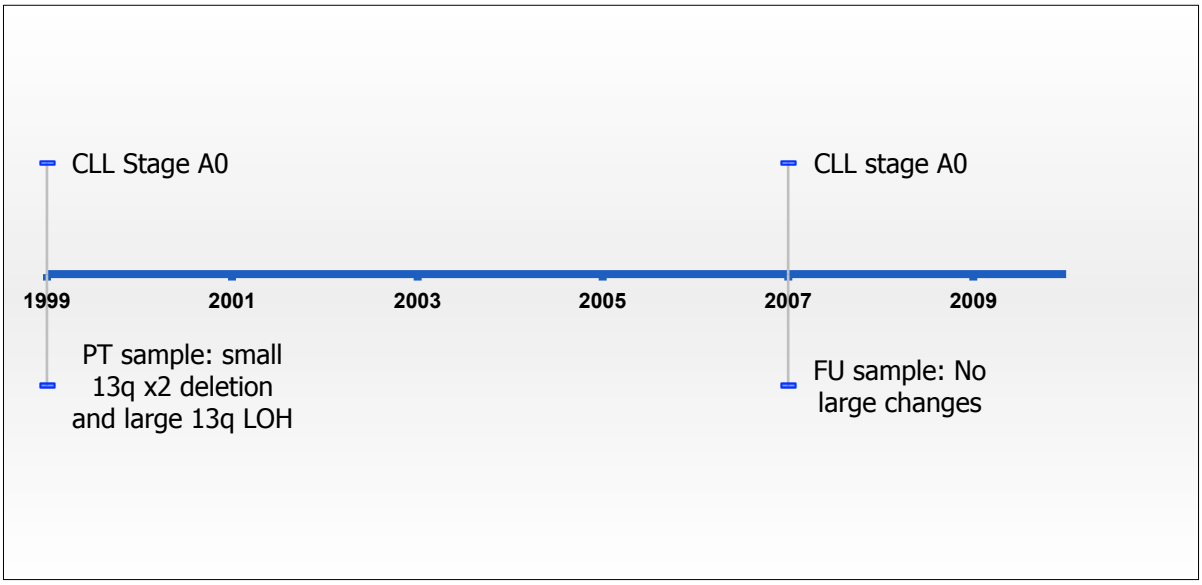


Figure 49: WBC of GG (19). PT sample at 0 and FU sample at 103

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	24/03/1999	
FISH data:	ATM[Normal],TP53[Normal]; D13S319[Deletion]: 74% homozygous	74% homozygous loss of 13q14
Karyotype	'46,XY	
Number of CNV:	24	
CNA <1Mb:	Diminished X2(13)(49.5-50.37)	
CNA >1Mb:	LOH(13)(21.04-114.1)	

Sample date	19/06/2001	
Karyotype	46,XY,t(2;17)(q1?3;q21?) [1]	
FISH data:	ATM[Normal],TP53[Normal]	

Follow up Sample	06/11/2007	
FISH data:	12C[Normal]; ATM[Normal],TP53[Normal]; D13S319[Deletion]: 94% homozygous	94% homozygous deletion
Karyotype	'46,XY,del(11)(q23q25) [1]/46,XY,?del(11)(q23q25) [3]/46,XY [26]	
Number of CNV:	25	
CNA <1Mb:	Diminished X2(13)(49.5-50.37)	
CNA >1Mb:	LOH(13)(21.04-114.1)	

Patient data: 21_AA

ID:	21

Disease Status:	Stable
Disease stage at Diagnosis:	A
IgVH status:	Mutated
%CD38:	3
%ZAP70:	9
Alive:	yes

Patient clinical information	Diagnosed with mBL in 1998 with AIHA. 2006, CLL Stage A0. Stable since.

Genomic data in brief:	Small 13q deletion no major changes between PT and FU

WBC count graph:

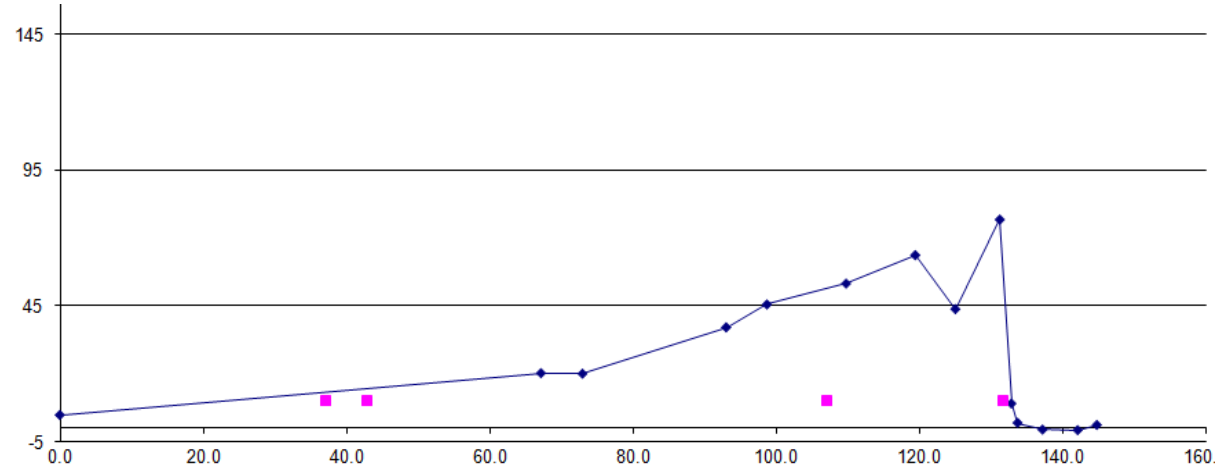
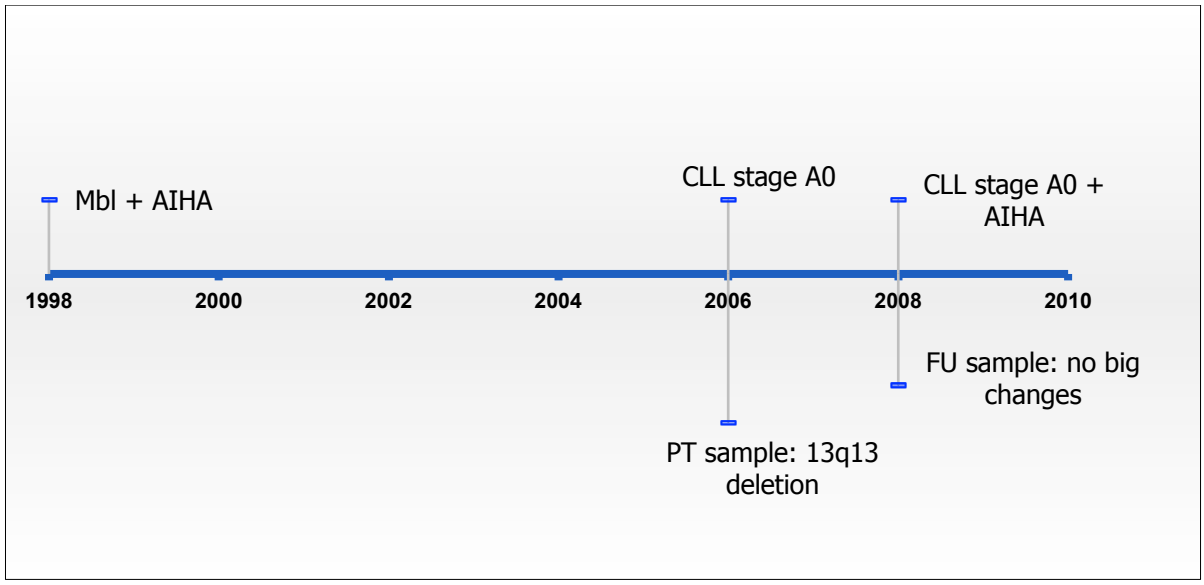


Figure 50: WBC of AA (21). PT sample at 107 and FU sample at 130

Timeline of Samples available with DATA from Array + FISH data:



Sample date	01/08/2001	
Karyotype	45,X, -X [2]	
FISH data:	D13S319[Deletion]	28% hemizygous loss at 13q14

Presentation Sample	07/12/2006	
FISH data:	D13S319[Deletion]: 28% hemizygous	
Karyotype	'45,X-X	
Number of CNV:	19	
CNA <1Mb:	Diminished(21)(35.85-35.93)	
CNA >1Mb:	Diminished(13)(49.34-50.4)	

Follow up Sample	24/12/2008	
FISH data	ATM[Normal],TP53[Normal]; 12C[Normal]; D13S319[Deletion]: 37% Homozygous	
Karyotype	'45,X-X	
Number of CNV:	23	
CNA <1Mb:	Diminished X2(13)(49.5-50.37) **Enhanced(2)(120.79-120.89)** **Diminished(13)(51.55-51.65)** Diminished(21)(35.85-35.93)	
CNA >1Mb:	Diminished(13)(49.6-50.45)	

Patient data: 22_LL

ID:	22

Disease Status:	Stable
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	5
%ZAP70:	5
Alive:	yes

Patient clinical information	Diagnosed 1996 with CLL stage A0. Stable since

WBC count graph:

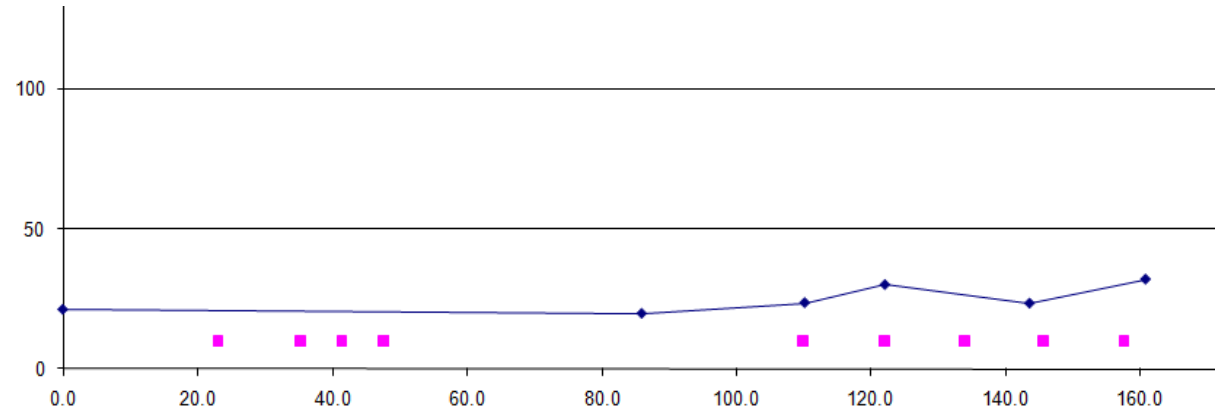
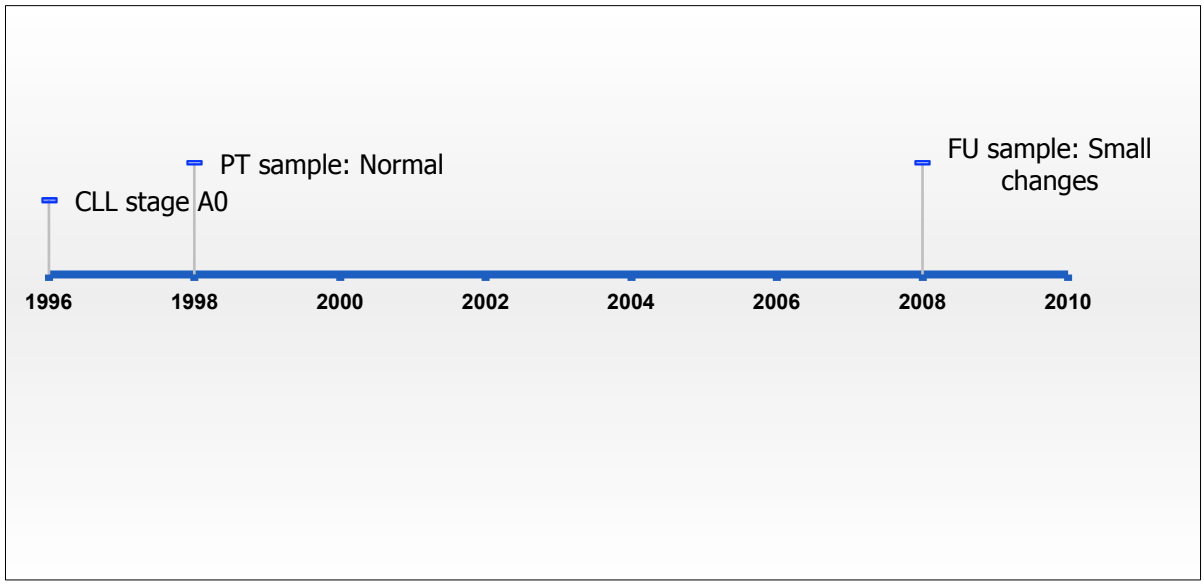


Figure 51: WBC of CG (22). PT sample at 0 and FU sample at 145

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	20/05/1998	
FISH data:	ATM[Normal],TP53[Normal]; D13S319[Normal]	
Karyotype	'46,XY	
Number of CNV:	20	
CNA <1Mb:		
CNA >1Mb:	Normal	

Sample date	26/08/2004	
Karyotype	45,X, -Y [1]	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal],D13S319[Normal]	

Follow up Sample	30/07/2008	
FISH data	12C[Normal]; ATM[Normal],TP53[Normal]; 13Q NOT DONE	
Karyotype	'46,XY [29]	
Number of CNV:	22	
CNA <1Mb:		
CNA >1Mb:		

Patient data: 23_LK

ID:	23

Disease Status:	Stable
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	2
%ZAP70:	4
Alive:	yes

Patient clinical information	Dignosed as mBL in 1985. CLL stage A0 in 2009 and stable since

WBC count graph:

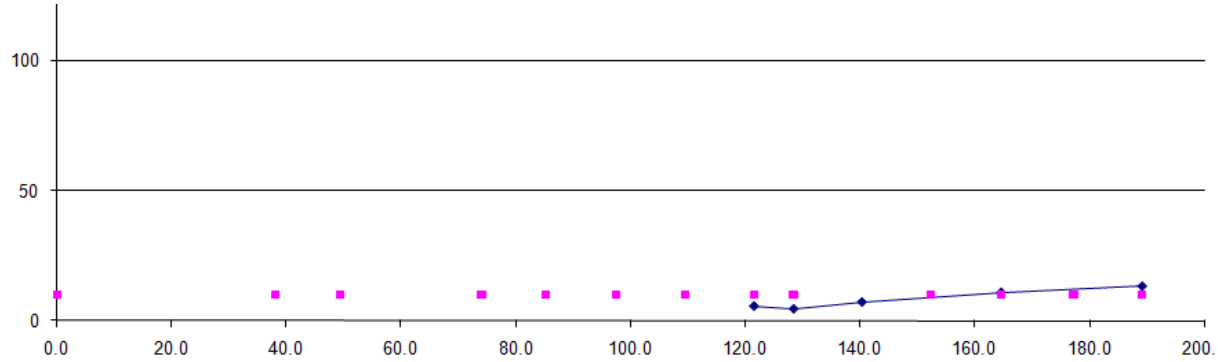
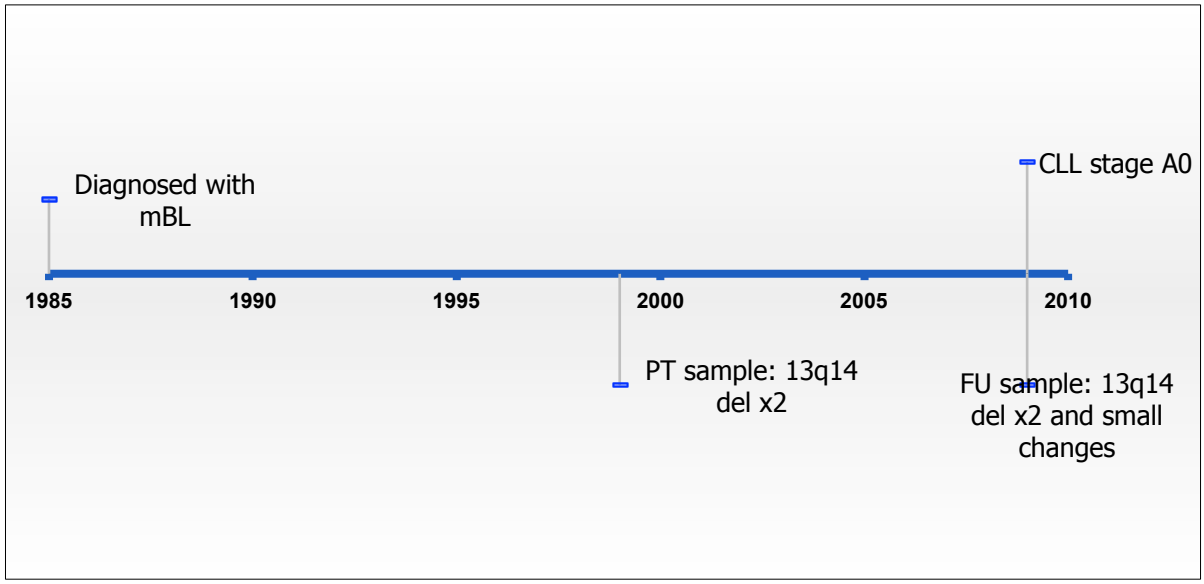


Figure 52: WBC of LK (23). PT sample at 74 and FU sample at 189

Timeline of Samples available with DATA from Array + FISH data:



Sample date	15/09/1993	
Karyotype	46,XX,t(9;15)(p11;q13) [1]	

Sample date	14/11/1996	
Karyotype	46,XX,t(9;15)(p15;q13), 7(qter), del(13)(q?) [2]	
FISH data:	D13S319[Deletion]	66% homozygous loss at 13q14

Presentation Sample	11/11/1999	
FISH data	12C[Normal]; ATM[Normal],TP53[Normal];D13S319[Deletion]: 65% homozygous	
Karyotype	'46,XX	
Number of CNV:	24	
CNA <1Mb:		
CNA >1Mb:	Diminished X2(13)(49.41-50.41) LOH(13)(40.51-114.1)	13q14

Follow up Sample	09/06/2009	
FISH data	12C[Normal]; ATM[Normal],TP53[Normal];D13S319[Deletion]: 86% homozygous	
Karyotype	45,XX,der(17)t(17;20)(p?;p?),-20 [inc 5]/46,XX [11]NB non clonal sructural rearrangementd including dicentric chromosomes also seen	
Number of CNV:	24	
CNA <1Mb:	Diminished X2(13)(49.42-50.4)	
CNA >1Mb:	LOH(13)(40.51-114.1)	

Patient data: 25_SR

ID:	25

Disease Status:	Stable
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	95
%ZAP70:	2
Alive:	yes

Patient clinical information	Diagnosed with mBL in 1999. Stable since

Genomic data in brief:	Patient with trisomy 12, 18 and 19. (no other major CNA)

WBC count graph:

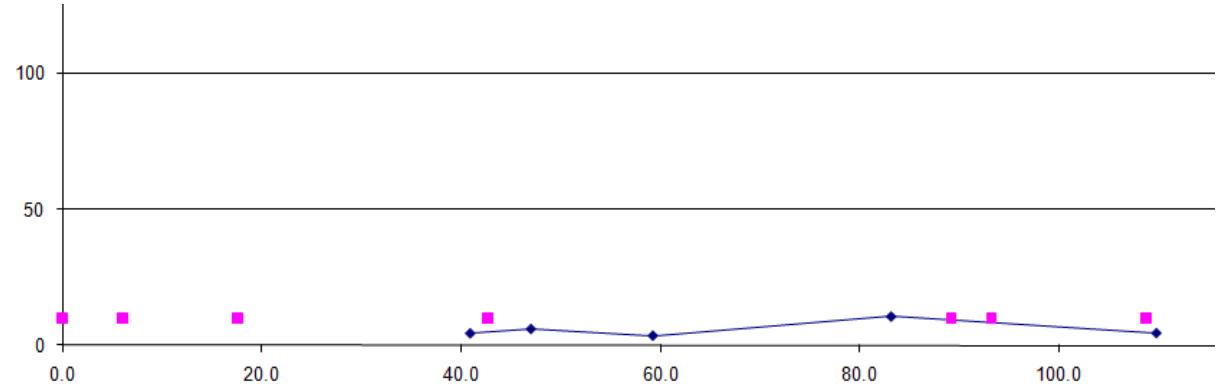
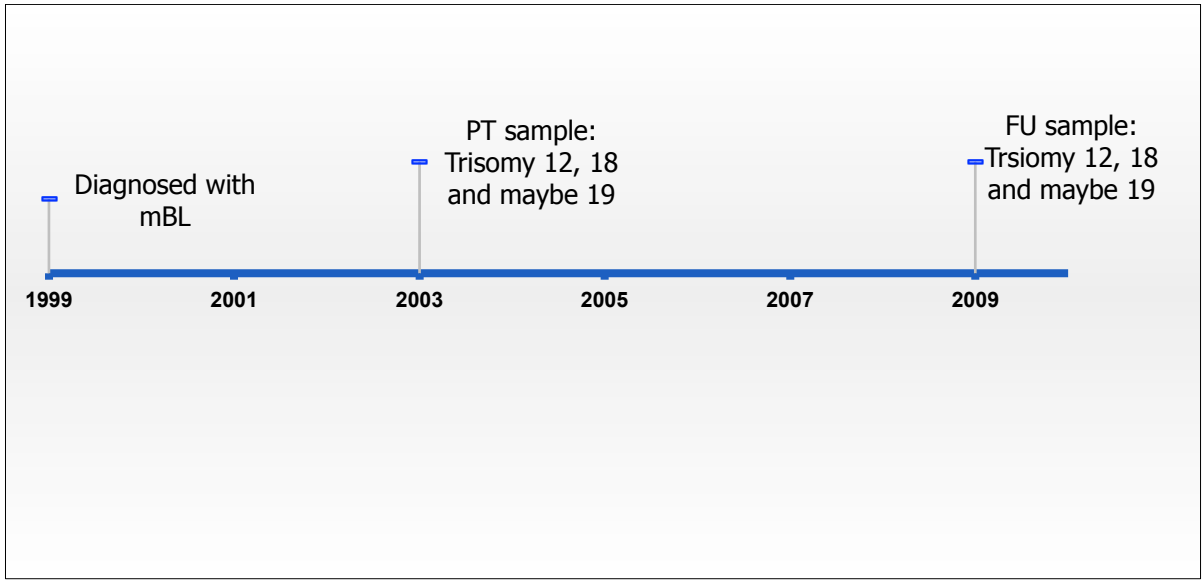


Figure 53: WBC of RS (25). PT sample at 42 and FU sample at 108

Timeline of Samples available with DATA from Array + FISH data:



Sample date	30/06/1999	
Karyotype	49, XX, +12, +18, +19 [17]	

Presentation Sample	05/09/2003	
FISH data	D13S319[Normal]; 12C[Trisomy], ATM[Normal],TP53[Normal]: Trisomy 12 in 14% of cells	
Karyotype	'49,XX,+12,+18,+19	
Number of CNV:	15	
CNA <1Mb:		
CNA >1Mb:	Trisomy(12)(0.0-132.29) Trisomy (18)(0.0-76.02) Trisomy (19)(0.0-63.75)	

Sample date	17/02/2004	
Karyotype	49, XX, +12, +18, +19 [7]	
FISH data:	12C[Trisomy], ATM[Normal],TP53[Normal],D13S319[Normal]	

Follow up Sample	06/03/2009	
FISH data	D13S319[Normal]; 12C[Trisomy], ATM[Normal],TP53[Normal]: Trisomy 12 in 26% of cells	
Karyotype	'49,XX,+12,+18,+19 [7]/46,XX [23]	
Number of CNV:	17	
CNA <1Mb:		
CNA >1Mb:	Trisomy(12)(0.0-132.29) Trisomy (18)(0.0-76.02) Trisomy (19)(0.0-63.75)	

Patient data: 28_EW

ID:	28

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	30
%ZAP70:	1
Alive:	yes

Patient clinical information	Diagnosed in 1994 with mBL. Progressed to mBL/A in 2000 and then CLL stage A0 in 2002. 2003: Stage A2 with left and right groin nodes 2005: Same nodes but bigger 2006, progresses to stage B2 .. 2008 stage B

Genomic data in brief:	P53 deletion at PT with large deletion on chr 13 and chr 18. Follow up shows homozygous deletion at 11q (ATM) and deletions on chr 20 and loss of Y.

WBC count graph:

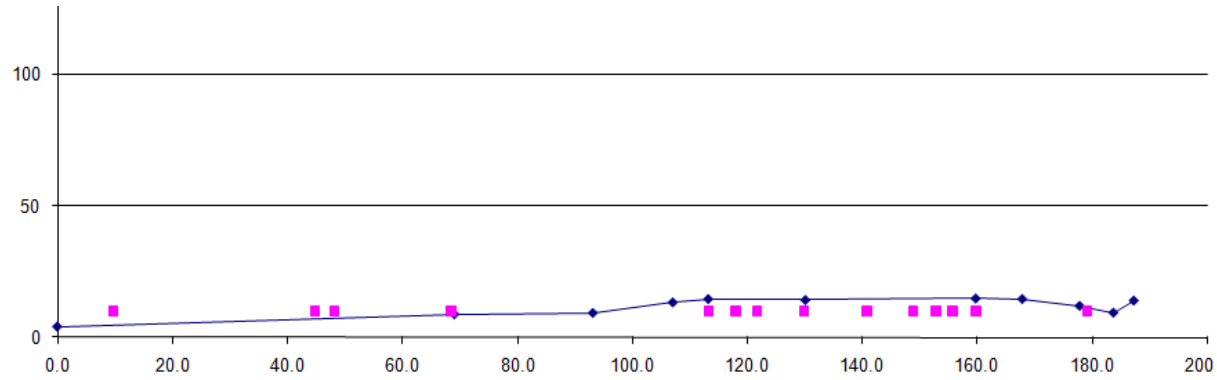
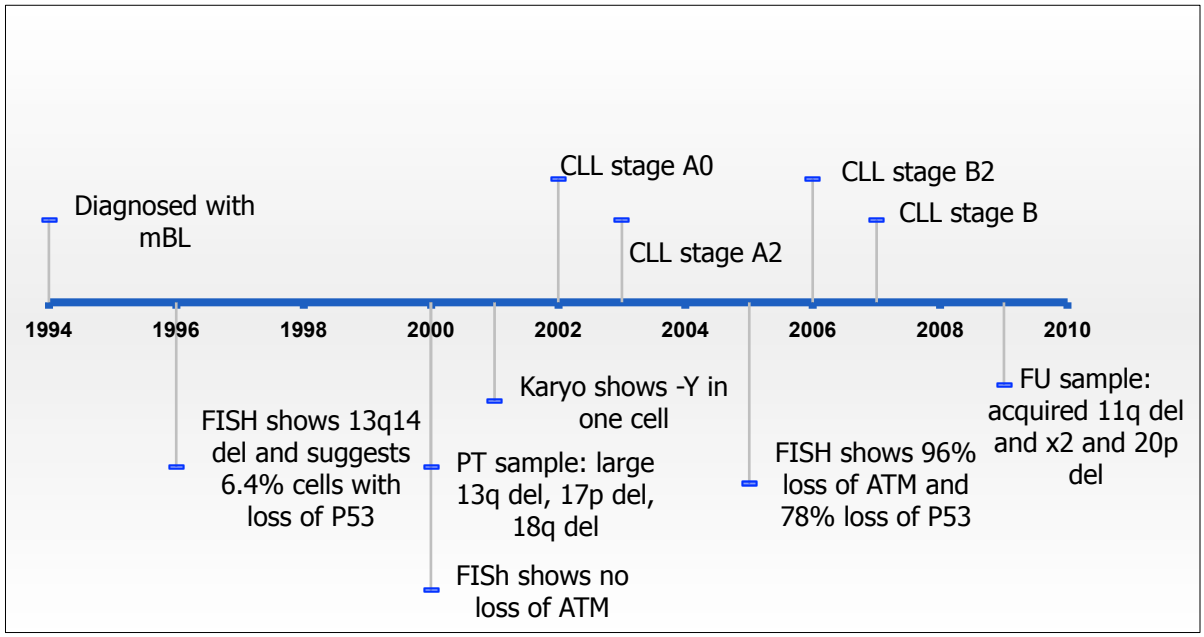


Figure 54: WBC of EW (28). PT sample at 69 and FU sample at 179

Timeline of Samples available with DATA from Array + FISH data:



Sample date	13/06/1996	
Karyotype	45,XY,del(13)(q14), der(18)t(17;18)(q11;q23) [3]	
FISH data:	P53[Deletion], D13S319[deletion]	6% hemizygous loss of P53

Presentation Sample	12/05/2000	
FISH data:	[Deletion], D13S319[deletion] 6% hemizygous of P53 ; ATM[Normal] 3% of ATM	3% loss of ATM
Karyotype	45,XY,der(18)t(17:18)(q?11:q23) [2]	
Number of CNV:	30	
CNA <1Mb:	Diminished(17)(57.26-57.47)	
CNA >1Mb:	Diminished(13)(40.27-114.13) Diminished(17)(0.01-22.3) Diminished(18)(22.05-23.63) Diminished(18)(65.72-72.46)	13q14.11 to 13q34 17p13.3 to 17q11.1 18p11.32 to 18q12.1 18q22.2 to 18q23

Sample date	07/06/2000	
FISH data:	P53[Deletion], ATM[Normal], 12C[Normal]	60% hemizygous loss of P53

Sample date	06/06/2001	
Karyotype	45,XY,der(18)t(17;18)(q11;q23) [2] 44,idem,-Y,del(13)(q14) [14] 42,idem,-Y,del(13)(q14),der(11)t(6;11)(q21;q23),-22 [1] 43,idem,-Y,t(13;16)(q14;p13) [1]	
FISH data:	ATM[Deletion]	6% hemizygous loss of ATM

Sample date	02/02/2004	
FISH data:	ATM[Hemizygous loss], 12C[normal], D13S319[deletion]	29% hemizygous loss of ATM 72% hemizygous loss at 13q14

Sample date	22/06/2005	
FISH data:	ATM[Hemizygous loss], 12C[normal], P53[deletion]	78% hemizygous loss of P53 96% hemizygous loss at ATM

Follow up Sample	26/06/2009	
FISH data:	ATM[Deletion] P53[Deletion]: 76% hemizygous of P53 39% hemizygous at ATM; D13S319[deletion] 72% hemizygous	76% hemizygous loss of P53 39% hemizygous loss at ATM
Karyotype	'45,XY,der(18)t(17;18)(q21;q23) [4]/44,sl,-Y,del(13)(q12q3?2) [2]/44,sdl1,del(11)(q14q25) [6]/44,sdl2,t(6;11)(q13;q14) [7]/43,sdl1,add(16)(p13),-20 [2]/46,XY [13]	
Number of CNV:	30	
CNA <1Mb:	Diminished(17)(57.26-57.47)	
CNA >1Mb:	**Diminished(11)(79.3-123.72)** **Diminished X2(11)(81.12-119.91)** Diminished(13)(40.28-114.13) Diminished(17)(0.0-22.15) Diminished(18)(22.14-23.61) Diminished(18)(65.71-72.47) **Diminished(20)(0.0-12.65)** **Diminished(Y)(4.61-6.8)** **Diminished(Y)(7.93-27.18)**	11q14.1 to 11q24.2 11q14.1 to 11q23.3 13q14.11 to 13q34 17p13.3 to 17q11.1 18p11.32 to 18q12.1 18q22.2 to 18q23 20p13 to 20p12.1 Yp11.2 Yp11.2 to Yq11.23

Patient data: 29_JG

ID:	29

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	0
%ZAP70:	1
Alive:	yes

Patient clinical information	CLL stage A0 in 2004, progresses to stage A1 in 2007 and is given treatement (CHLOR x2). WBC goes down and then back up slowly. Treated again in 2010 with CHLOR.

Genomic data in brief:	Deletion of 9p and 20p as well as small 13q deletion. No major changes at FU

WBC count graph:

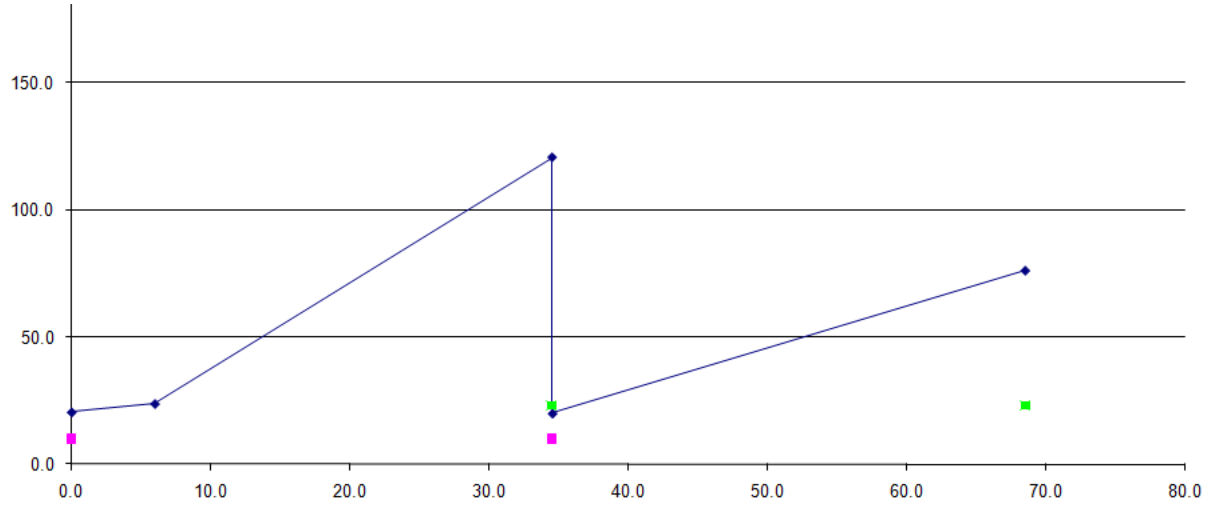
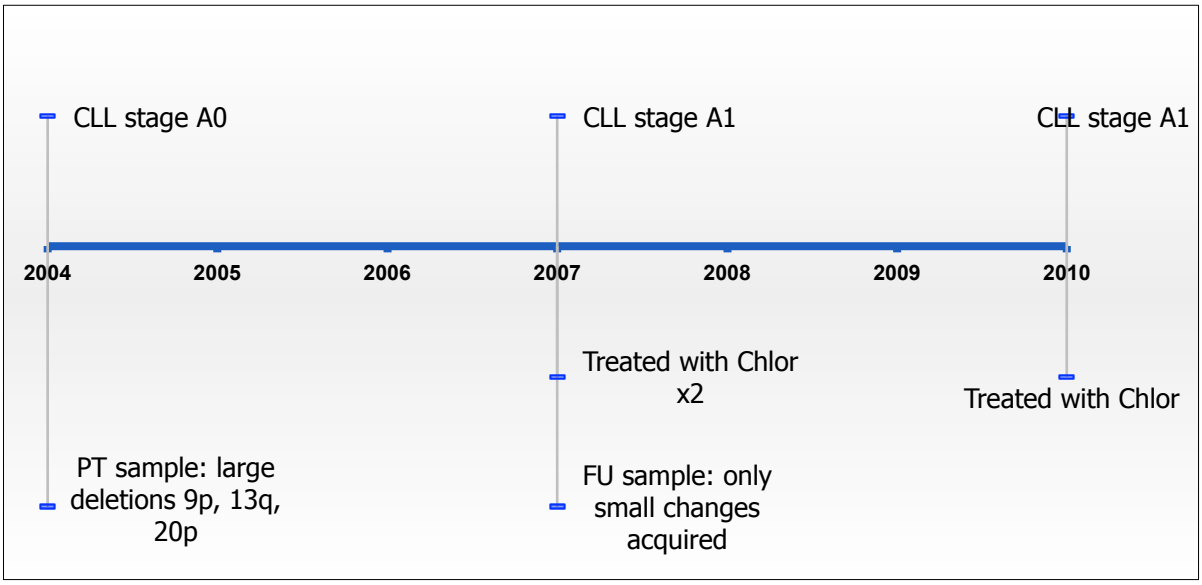


Figure 55: WBC of JG (29). PT sample at 0 and FU sample at 34.5

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	16/06/2004	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal]; 13Q NOT DONE	
Karyotype	'46,XX,del(13)(q14q22) [1]/46,XX,?del(13)(q14q22)[1]/46,XX[27]	
Number of CNV:	28	
CNA <1Mb:		
CNA >1Mb:	Diminished(4)(10.17-11.24) Diminished(9)(0.21-34.24) Diminished(13)(49.33-50.53) Diminished(20)(0.01-17.92)	9p24p13.3 13q14 20p13p11.23

Follow up Sample	02/05/2007	
FISH data:	12C[Normal]; ATM[Normal],TP53[Normal]; D13S319[Deletion]: 53% hemizygous 10% homozygous	53% hemizygous loss 10% homozygous loss
Karyotype	'46,XX,?del(13)(q14q22 [2]/47,XX,+?r [1]/44,XX,t(1;2)(q32;q21),-13,-19 [1]/46,XX [26]	
Number of CNV:	29	
CNA <1Mb:		
CNA >1Mb:	Diminished(4)(10.17-11.24) Diminished(9)(0.0-34.44) Diminished(13)(49.33-50.46) Diminished(20)(0.0-17.92)	9p24p13.3 13q14 20p13p11.23

Patient data: 30_IS

ID:	30

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Unmutated
%CD38:	16
%ZAP70:	35
Alive:	yes

Patient clinical information	CLL stage A0 in 2000 (or mBL) but progresses to stage B in 2008 and is treated with Campred [COMPLETE response]. CLL stage A0 in 2010

Genomic data in brief:	Large deletion on chr 10, 12 and 19 with LOH on 17p. Folow up shows acquired deletions on chr 8 and 9. Karyotype suggests a translocation involving 8, 9 and 12.

WBC count graph:

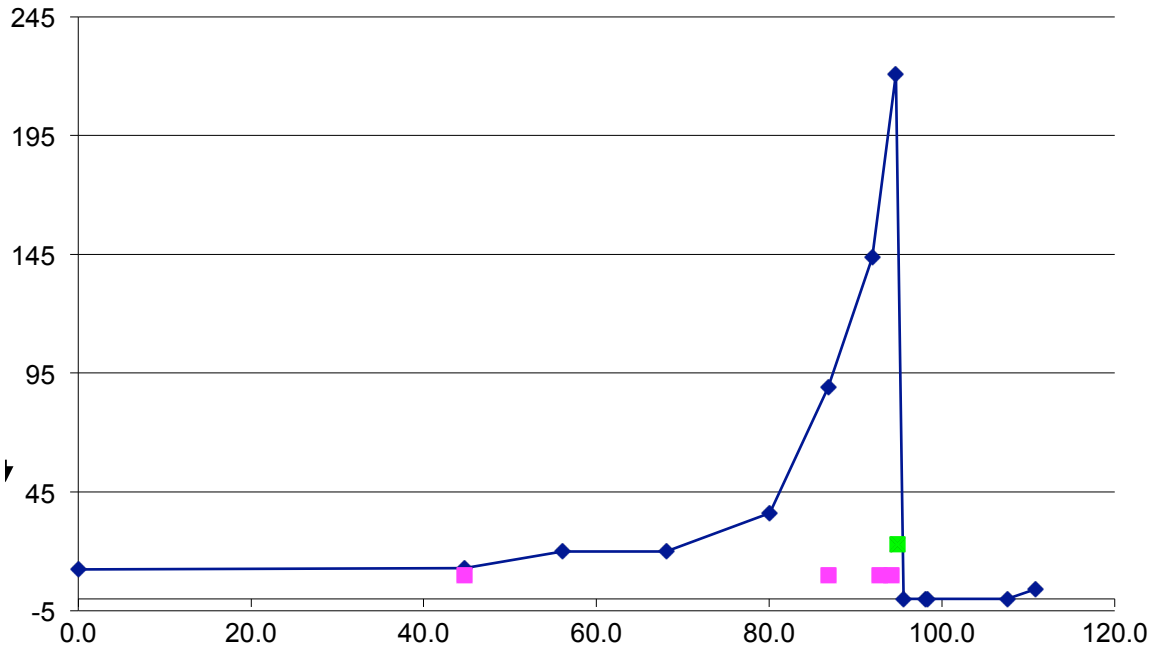
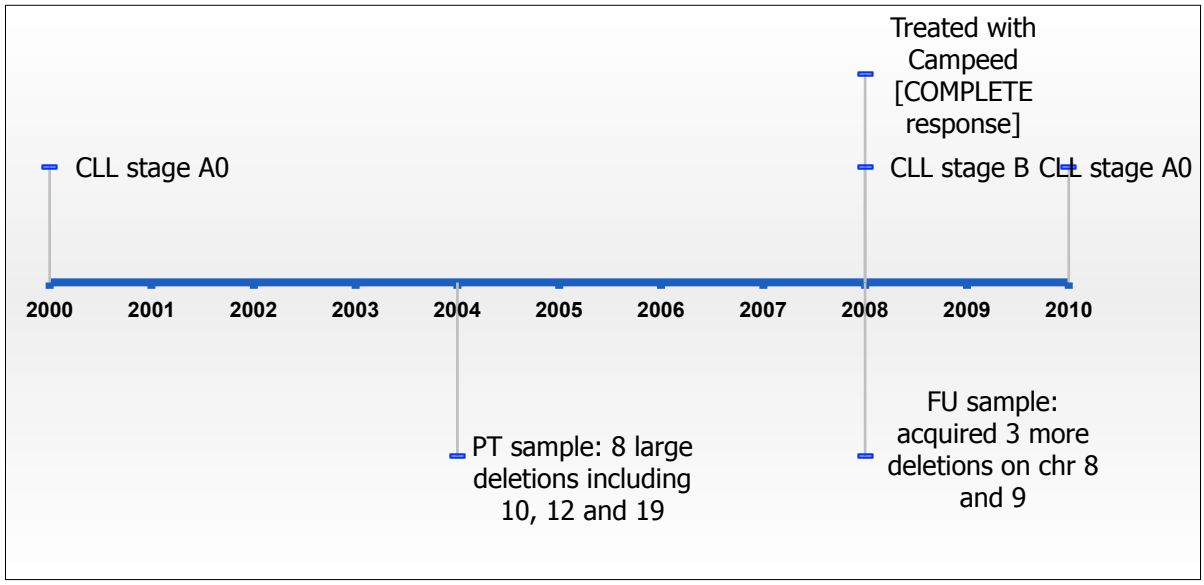


Figure 56: WBC of IS (30). PT sample at 44 and FU sample at 92

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	22/07/2004	
FISH data:	ATM[Normal],TP53[Normal]; D13S319[Normal]	
Karyotype	'46,XX	
Number of CNV:	25	
CNA <1Mb:		
CNA >1Mb:	Diminished(10)(103.34-135.31) Enhanced(12)(56.05-57.25) Diminished(12)(59.14-63.15) Diminished(12)(69.22-70.41) Diminished(12)(78.6-89.79) Diminished(19)(11.31-12.46) Diminished(19)(22.13-43.07) LOH(17)(0.01-18.8)	10q24.32 to 10q26.3 12q13.3 + 12q14.1 12q14.1 + 12q14.2 12q15 + 12q21.1 12q21.2 to 12q21.33 19p13.2 19p12 to 19q13.2

Sample date	24/01/2008	
Karyotype	45,XX,del(12)(q13q21),-19 [2]	
FISH data:	ATM[Normal],TP53[Normal]	

Follow up Sample	22/09/2008	
FISH data:	12C[Normal];ATM[Normal],TP53[Normal]; D13S319[Normal]	
Karyotype	'45,XX,?2(q),add(10q)del(12)(q13q21),-19[7],idem,-8,-9,der(12:9:8)(???)[15]	
Number of CNV:	23	
CNA <1Mb:		
CNA >1Mb:	**Diminished(8)(0.0-70.79)** **Diminished(9)(21.69-29.38)** **Diminished(9)(0.0-15.15)** Diminished(12)(59.18-63.0) Diminished(10)(103.81-135.28) Enhanced(12)(56.13-57.23) Diminished(12)(69.31-70.4) Diminished(19)(11.31-12.66) Diminished(12)(77.97-89.82) Diminished(19)(22.15-43.08) LOH(17)(0-18.8)	8p23.3 to 8q13.3 9p21.3 to 9p21.1 9p24.3 to 9p22.3 10q24.32 to 10q26.3 12q13.3 + 12q14.1 12q14.1 + 12q14.2 12q15 + 12q21.1 12q21.2 to 12q21.33 19p13.2 19p12 to 19q13.2

Patient data: 32_MH

ID:	32

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	4
%ZAP70:	1
Alive:	yes

Patient clinical information	Diagnosed with 1997 with CLL stage A0. Progressed to stage A1 in 2007 (6cm spleen). Treated in 2008 with CHLOR R [good PARTIAL response]. 2009 Detected minimal residual disease

Genomic data in brief:	13q deletion including a small homozygous deleted region. Stable genome.

WBC count graph:

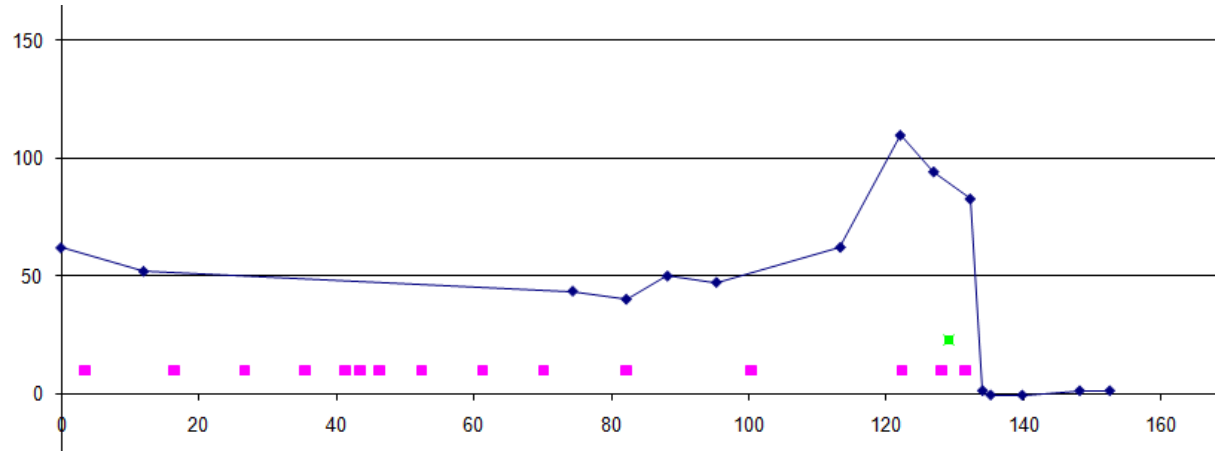
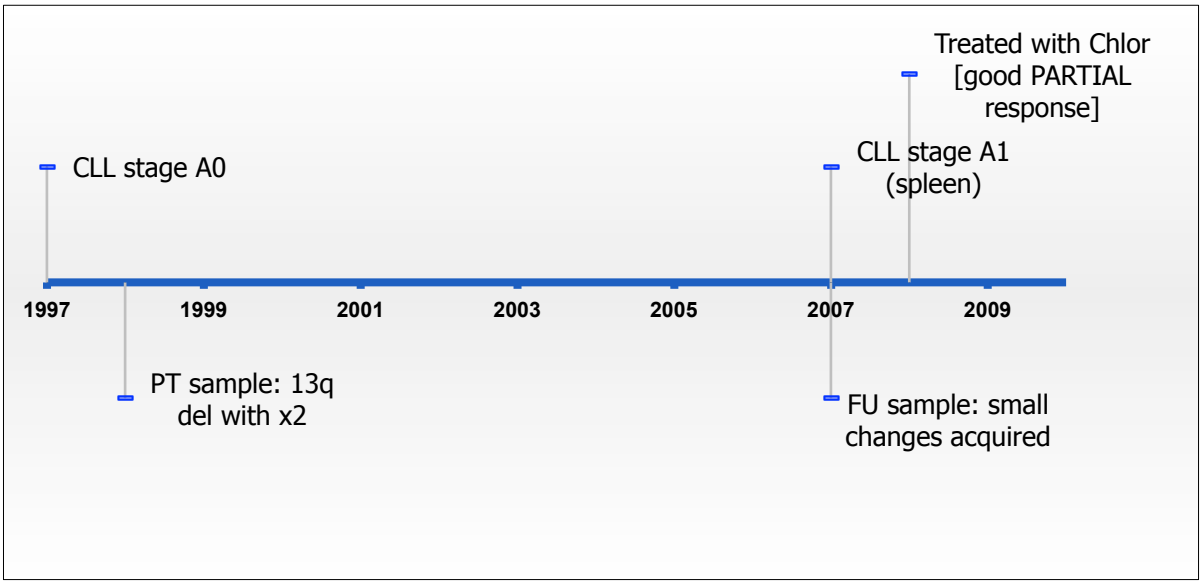


Figure 57: WBC of MH (32). PT sample at 12 and FU sample at 122

Timeline of Samples available with DATA from Array + FISH data:



Sample date	02/09/1997	
FISH data:	D13S319[Deletion]	24% hemizygous loss 65% homozygous loss

Presentation Sample	29/09/1998	
FISH data	ATM[Normal],TP53[Normal];D13S319[Deletion]: 24% hemizygous 65% homozygous	
Karyotype	'46,XY	
Number of CNV:	28	
CNA <1Mb:	Diminished X2(13)(49.49-50.36)	
CNA >1Mb:	Diminished(13)(49.19-50.44)	

Follow up Sample	26/07/2007	
FISH data	12C[Normal];ATM[Normal],TP53[Normal]; 13q NOT DONE	
Karyotype	46,XY,del(13)(q14q22) [1]/46,XY [29]	
Number of CNV:	29	
CNA <1Mb:	**Diminished(21)(31.87-31.96)** Diminished X2(13)(49.52-50.35)	
CNA >1Mb:	Diminished(13)(49.18-50.44)	

Patient data: 33_PF

ID:	33

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Unmutated
%CD38:	14
%ZAP70:	1
Alive:	yes

Patient clinical information	Diagnosed in 1997 with CLL stage A0. Progressed to stage B in 2002 and given treatment FC x5. Still at stage B in may 2007 and given further treatment FC x3 [COMPLETE response] + AIHA (autoimmune haemolytic anaemia) July 2007 further treatment with Rituximab x4 [Anaemic responded?] 2009, left cervical node, diseases progresses to Richters (DLBCL). Treated with CHOP x8 [COMPLETE response]

Genomic data in brief:	No large CNA. Acquired small changes at FU

WBC count graph:

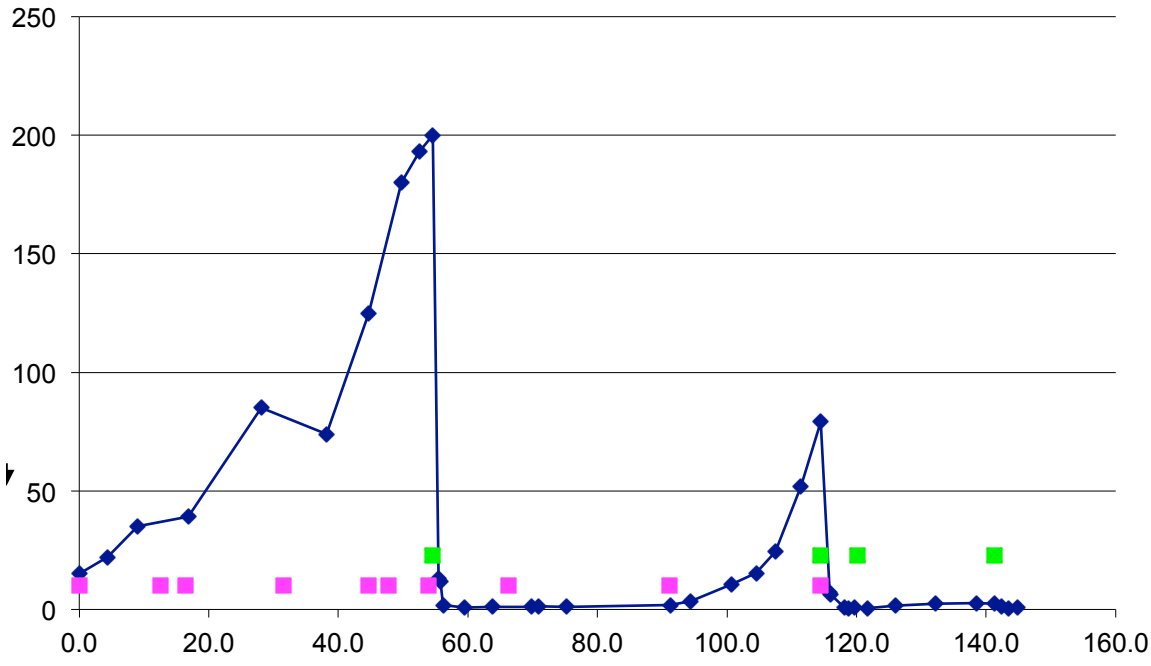
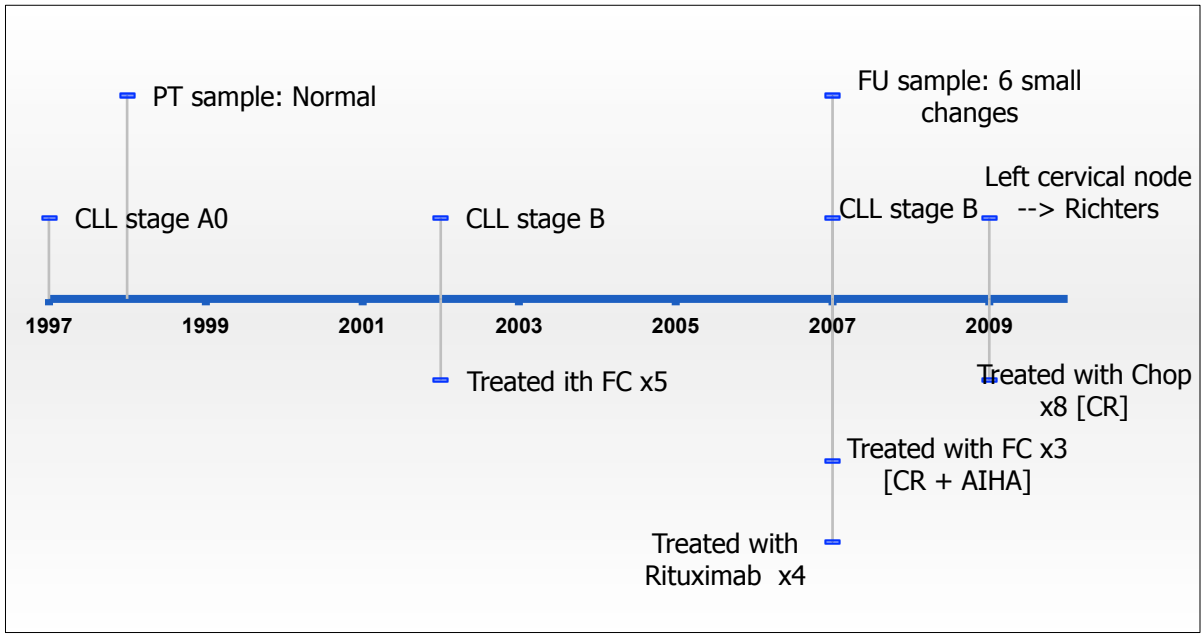


Figure 58: WBC of PF (33). PT sample at 9 and FU sample at 114

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	16/11/1998	
FISH Data	ATM[Normal],TP53[Normal]; D13S319[Normal]	
Karyotype	'46,XX	
Number of CNV:	25	
CNA <1Mb:		
CNA >1Mb:	Normal	

Sample date	01/06/2005	
Karyotype	45,XX,t(4;20)(q11q13),-19 [1] 45,XX,t(2;7)(q3?;q22?),-19 [1] 46,XX,?del(12)(q?),t(13;15)(q?) [1] 46,XX,t(5;9)(q3?;q22?) [1]	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal]	

Follow up Sample	09/05/2007	
FISH data	12C[Normal];D13S319[Normal] ATM AND P53 NOT RECENTLY DONE	
Karyotype	46,XX,t(4;15)(p?16;q?15) [4]/46,XX [21]	
Number of CNV:	30	
CNA <1Mb:	**Diminished(7)(2.24-2.41)**	
CNA >1Mb:		

Patient data: 34_RC

ID:	34

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	3
%ZAP70:	4
Alive:	yes

Patient clinical information	Diagnosed in 1991 with CLL stage A0. Progressed to stage C in 2001 and treated with CHLOR x3 (Infection). Treated again in 2003 with FC x1 (cmv). Stage B in 2006 and further treatement (CHLOR R x6). Progresses to Richters H/D in 2009 and is treated again with R Chop x 6

Genomic data in brief:	Stable genome with no large CNA. Has a 13q deletion

WBC count graph:

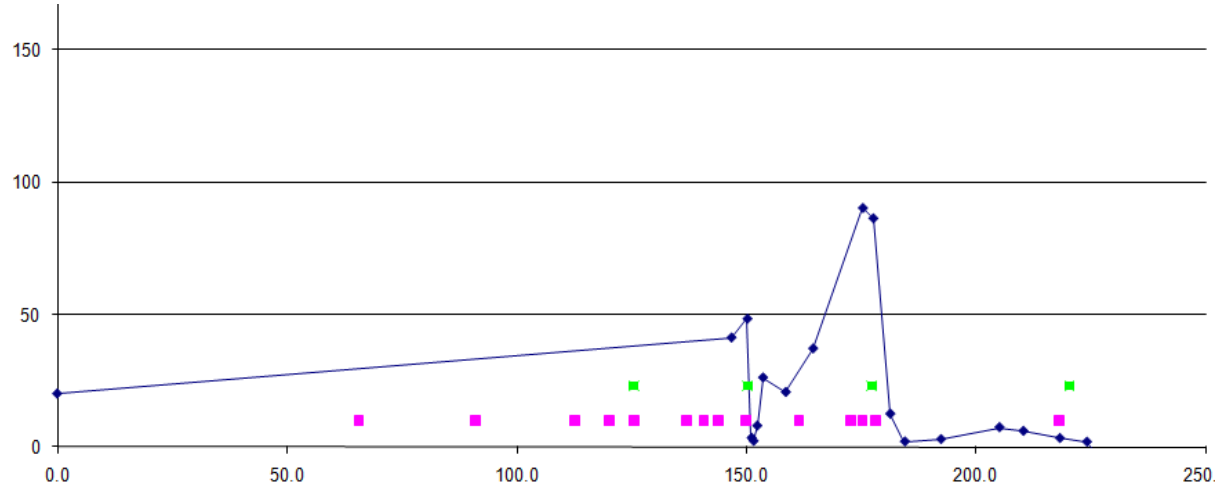
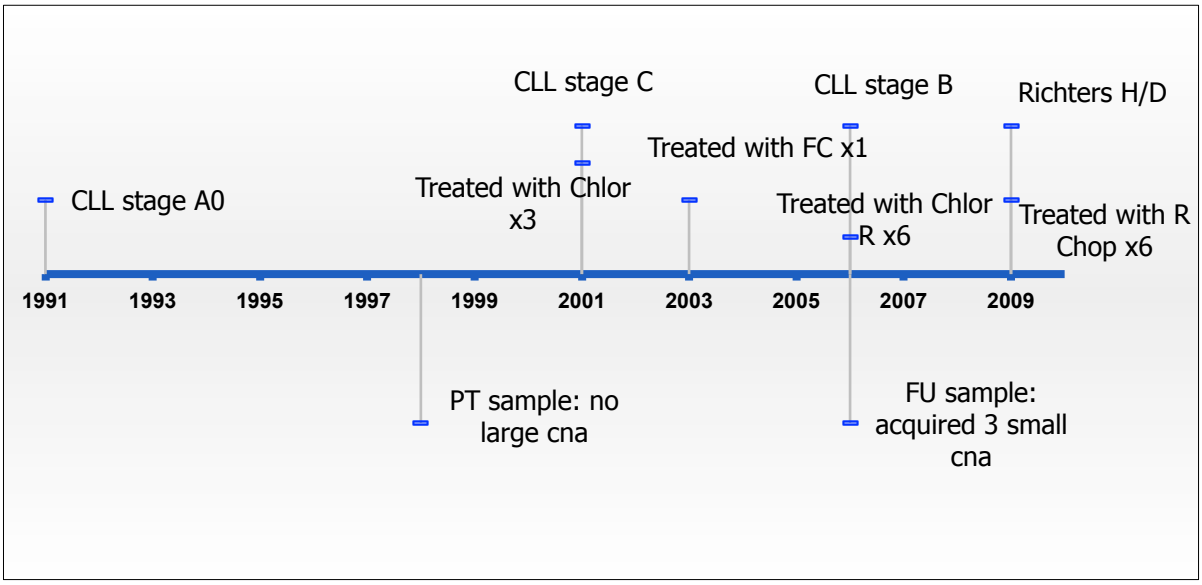


Figure 59: WBC of RC (34). PT sample at 90 and FU sample at 178

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	25/11/1998	
FISH data	ATM[Normal],TP53[Normal];D13S319[Deletion]: 60% hemizygous	
Karyotype	'46,XY	
Number of CNV:	31	
CNA <1Mb:	Diminished(13)(49.58-49.83)	
CNA >1Mb:		

Sample date	08/10/2004	
FISH data:	12C[Normal], ATM[Normal],D13S319[Deletion]	81% hemizygous loss at 13q14

Follow up Sample	23/02/2006	
FISH data	12C[Normal]; ATM[Normal],TP53[Normal];D13S319[Deletion]: 32% hemizygous	
Karyotype	'46,XY [30]	
Number of CNV:	37	
CNA <1Mb:	Diminished(13)(49.54-49.83)	
CNA >1Mb:		

Patient data: 35_BR

ID:	35

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	6
%ZAP70:	2
Alive:	No (2009)

Patient clinical information	Diagnosed with CLL stage A0 in 1998. Progressed to stage A1 in 2004 (spleen 2cm) and further to stage C in 2007 (spleen 14mm) and treated with CHLOR R x1. CMV reactivation and severe pancytopenia (reduction in WBC and rbc) 2008: Stage A1 with 8cm spleen 2009 death

Genomic data in brief:	PT shows a small 13q14 deletion which becomes larger and homozygous in 6 years

WBC count graph:

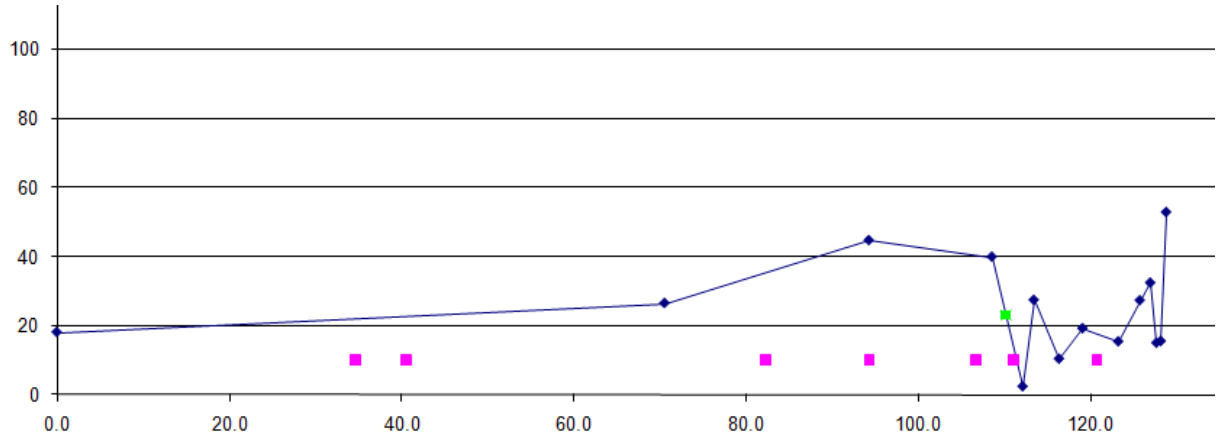
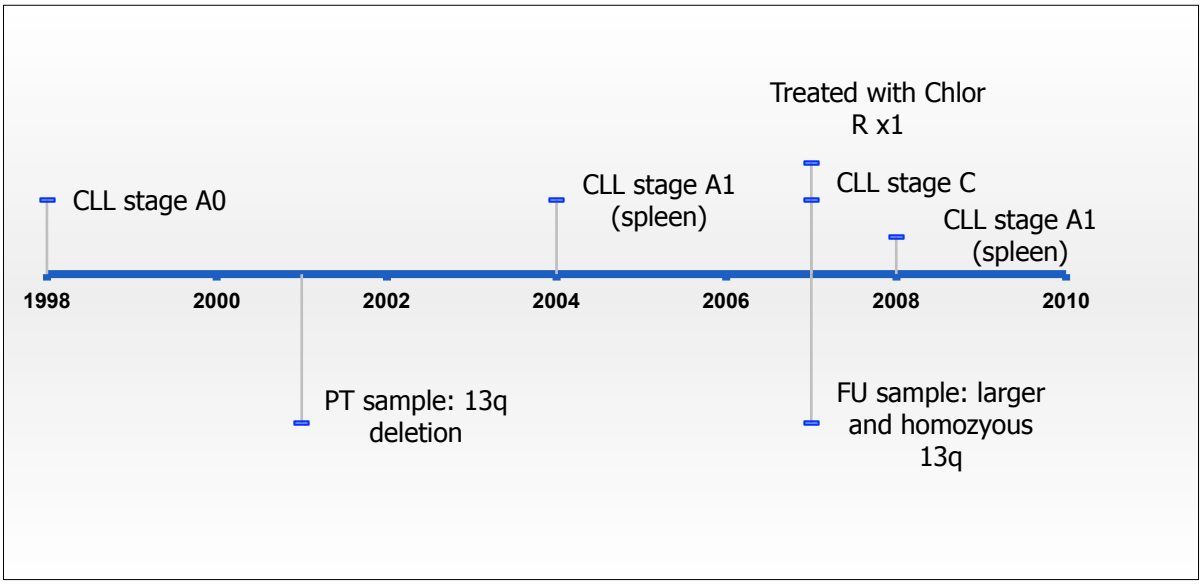


Figure 60: WBC of BR (35). PT sample at 34 and FU sample at 111

Timeline of Samples available with DATA from Array + FISH data:



Sample date	29/11/2000	
Karyotype	46,XY,?add(12)(q24?) [1]	
FISH data:	ATM[Normal],TP53[Normal],D13S319[Deletion]	35%hemizygous loss

Presentation Sample	19/07/2001	
FISH data	ATM[Normal],TP53[Normal],D13S319[Deletion]: 35% hemizygous	
Karyotype	46,XY	
Number of CNV:	31	
CNA <1Mb:	Diminished(7)(13.13-13.24)	
CNA >1Mb:	Diminished(13)(48.84-51.64)	

Follow up Sample	28/11/2007	
FISH data:	12C[Normal]; ATM[Normal],TP53[Normal];D13S319[Deletion]: 91% hemizygous	
Karyotype	46,XY,t(9;13)(q32;q14)[6], 46,XY,del(13)(q14q22) [1], 46,XY [38]	
Number of CNV:	32	
CNA <1Mb:	Diminished(7)(13.13-13.25)	
CNA >1Mb:	**Diminished(13)(45.06-75.76)** **Diminished X2(13)(48.71-50.76)**	13q14.12q22.2 13q14.2q14.3

Patient data: 86_WR

ID:	86

Disease Status:	Stable
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	2
%ZAP70:	27
Alive:	yes

Patient clinical information	Diagnosed in 1989 with mBL. Stable case since.

Genomic data in brief:	Small 13q14 deletion with stable genome. GSTT1 gene deletion is interesting.

WBC count graph:

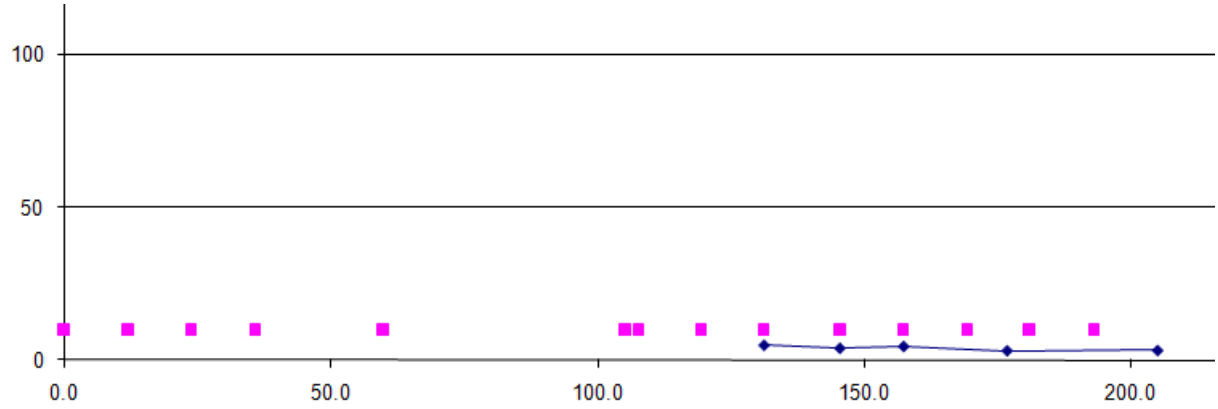
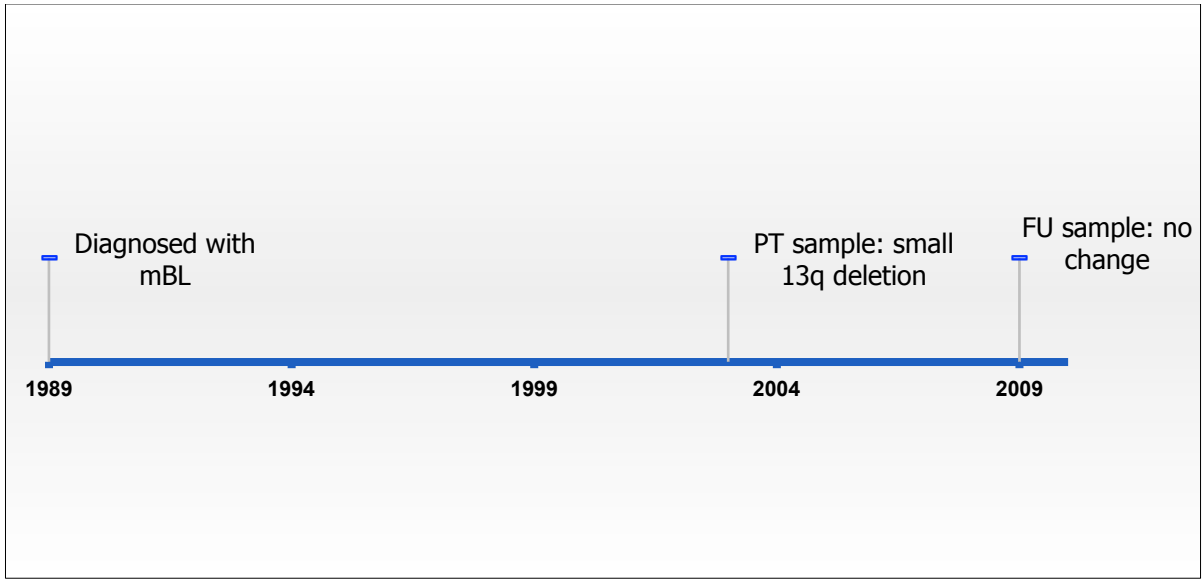


Figure 61: WBC of WR (86). PT sample at 119 and FU sample at 192

Timeline of Samples available with DATA from Array + FISH data:



Sample date	08/02/1990	
Karyotype	46,XY,t(5;6)(q35;q21) [12 46,XY,del(14)(q24) [1]	

Sample date	05/02/1998	
Karyotype	46,XY,t(5;6)(q35;q21) [5]	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal]	

Presentation Sample	23/01/2003	
FISH data	ATM[Normal],TP53[Normal],D13S319[Deletion]: 31% hemizygous	
Karyotype	46,XY,t(5:6)(q35:q21) [2]	
Number of CNV:	17	
CNA <1Mb:	Diminished (22) (22.65-22.77)	22q11.23
CNA >1Mb:	Diminished(13)(49.39-50.49)	13q14

Sample date	21/03/2005	
Karyotype	46,XY,t(5;6)(q35;q21) [2] 47,XY, +6 [1]	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal]	

Follow up Sample	09/03/2009	
FISH Data	ATM NOT DONE; TP53[Normal],12C[Normal];D13S319[Deletion]: 22% hemizygous	
Karyotype	46,XY,t(5;6)(q35;q21) [2]/46,XY [38]	
Number of CNV:	18	
CNA <1Mb:	Diminished(22)(22.65-22.76)	22q11.23
CNA >1Mb:	Diminished(13)(49.39-50.49)	13q14

Patient data: 247_DC

ID:	247

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Unmutated
%CD38:	50
%ZAP70:	6
Alive:	yes

Patient clinical information	Diagnosed with mBL in 2006, progressed to CLL stage A0 in 2007 then stage A in 2009 and given treatment CHLOR R.

Genomic data in brief:	

WBC count graph:

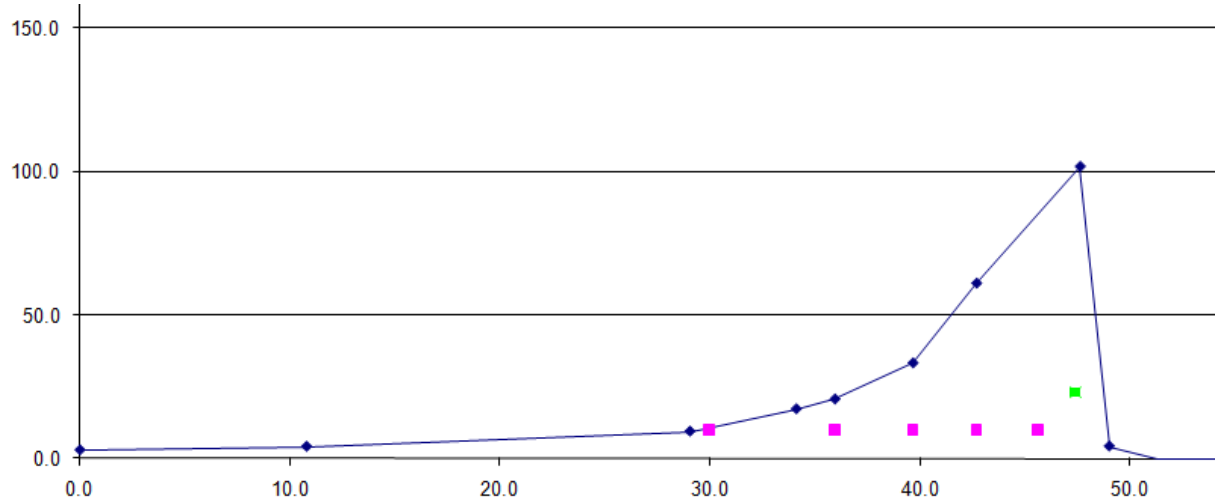
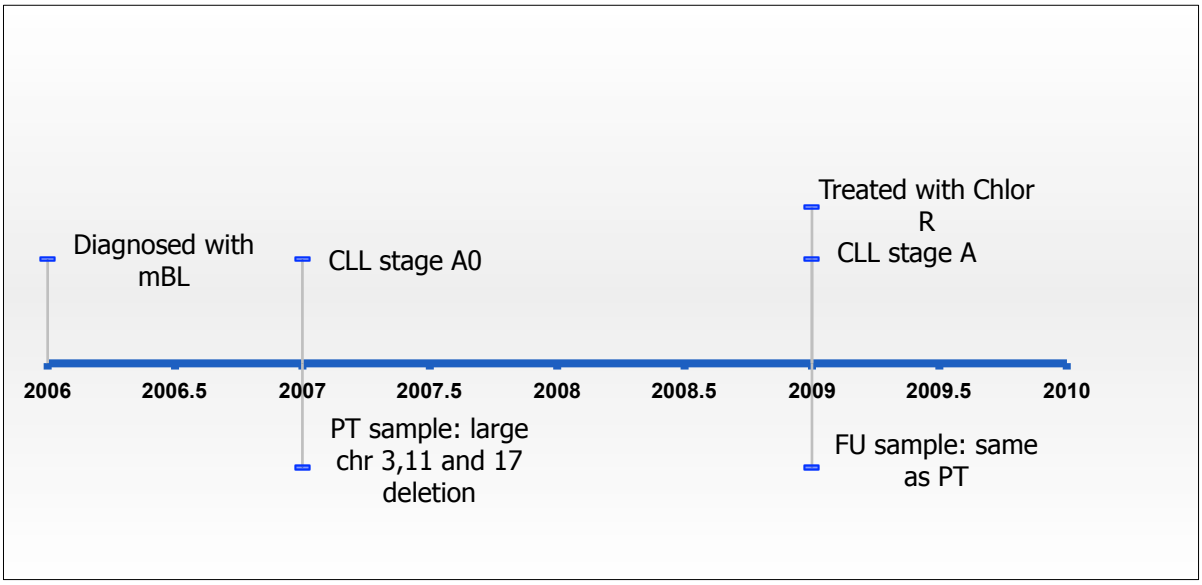


Figure 62: WBC of DC (247). PT sample at 29 and FU sample at 45

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	19/12/2007	
FISH data:	ATM[Deletion],TP53[Normal],D13S319[Normal]: 77% of ATM	77% loss of ATM
Karyotype	'46,XY,del(11)(q21q23)[7]	
Number of CNV:	16	
CNA <1Mb:	Diminished(11)(109.09-109.66) Diminished(3)(80.78-81.43)	
CNA >1Mb:	Diminished(3)(76.36-77.46) Diminished(3)(88.36-89.65) Diminished(17)(25.94-27.47) Diminished(11)(119.31-121.65) Diminished(11)(99.25-108.26)	

Follow up Sample	08/04/2009	
FISH data	12C[Normal]; ATM[Deletion],TP53[Normal],D13S319[Normal]: 77% hemizygous ATM	
Karyotype	46,XY,del(11)(q23q25) [16]/45,-X,Y,i(17)(q10) [1]/46,XY [13]	
Number of CNV:	15	
CNA <1Mb:	Diminished(11)(109.15-109.48) Diminished(3)(80.8-81.41)	
CNA >1Mb:	Diminished(3)(76.37-77.49) Diminished(3)(88.37-89.53) Diminished(11)(119.29-121.64) Diminished(17)(25.94-27.47) Diminished(11)(99.25-108.33)	3p12.3 3p12.3 3p11.1p11.2 11q22 11q22 11q23 17q11.2

Patient data: 248_AS

ID:	248

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Unmutated
%CD38:	16
%ZAP70:	3
Alive:	yes

Patient clinical information	Diagnosed in 2001 with CLL stage A0. WBC rising since.

Genomic data in brief:	Large 13q deletion. No change between PT and FU

WBC count graph:

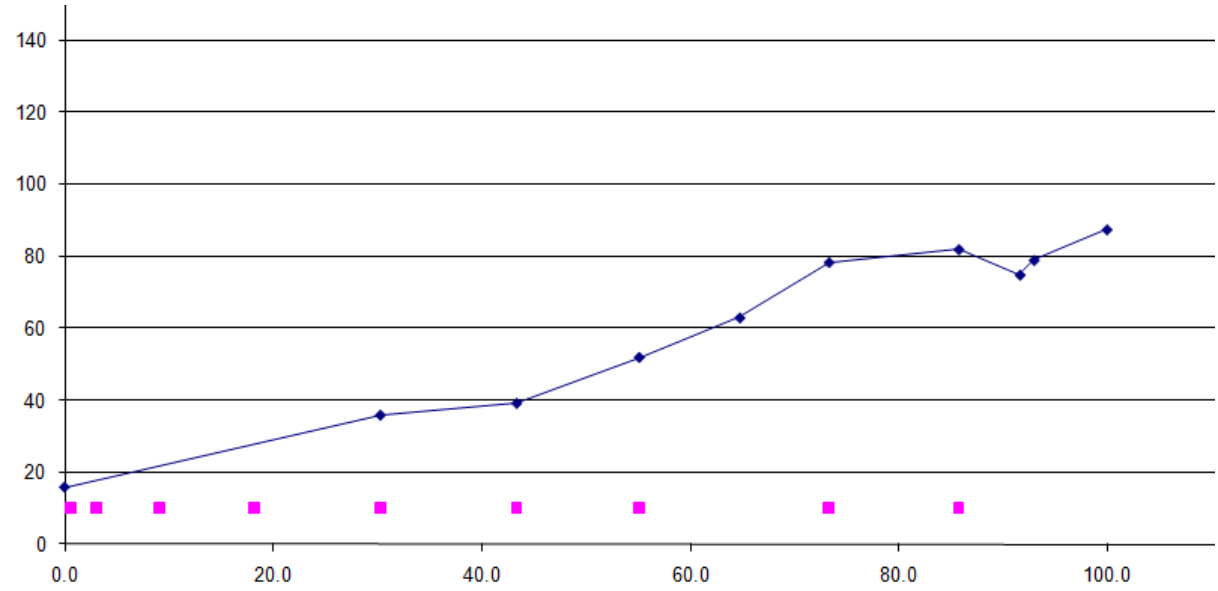
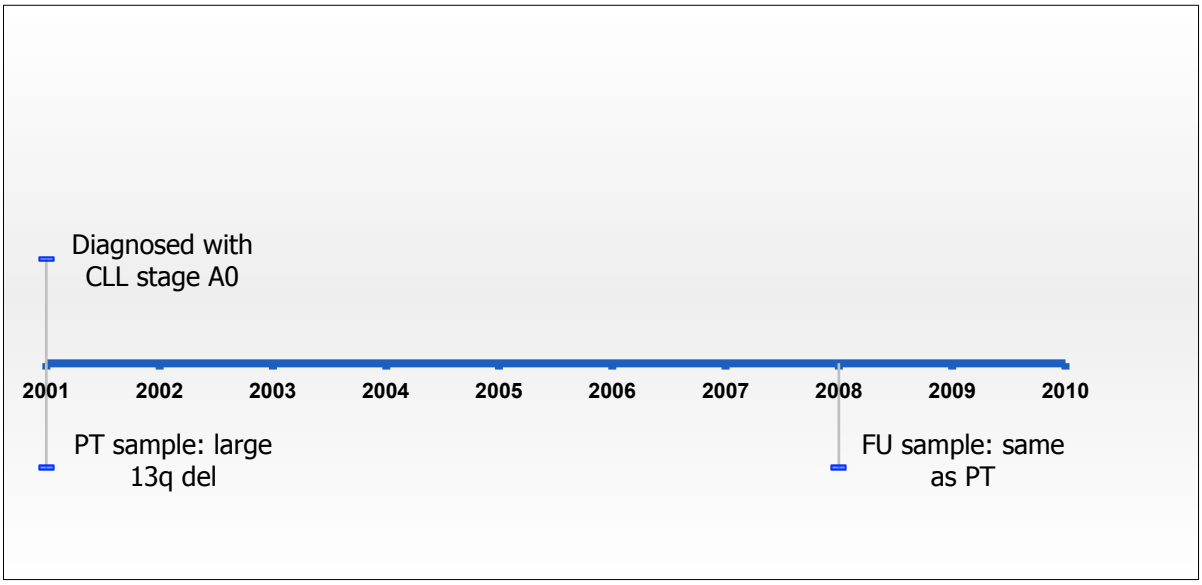


Figure 63: WBC of DC (248). PT sample at 0.5 and FU sample at 85

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	28/11/2001	
FISH data:	ATM[Normal],TP53[Normal],D13S319[Deletion]: 90% hemizygous	90% hemizygous loss at 13q14
Karyotype	'46,XY.del(13)(q12q14)	
Number of CNV:	31	
CNA <1Mb:		
CNA >1Mb:	Diminished(13)(40.42-53.35)	

Sample date	26/06/2005	
Karyotype	46,XY,del(13)(q12q14) [9]	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal],D13S319[Deletion]	92% hemizygous loss at 13q14

Follow up Sample	30/12/2008	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal],D13S319[Deletion]:83% hemizygous	83% hemizygous loss at 13q14
Karyotype	'46,XY.del(13)(q12q14)	
Number of CNV:	32	
CNA <1Mb:		
CNA >1Mb:	Diminished(13)(40.36-53.43)	

Patient data: 249_HV

ID:	249

Disease Status:	Progressive
Disease stage at Diagnosis:	A0
IgVH status:	Mutated
%CD38:	1
%ZAP70:	5
Alive:	yes

Patient clinical information	Diagnosed in 2007 with CLL stage A0. Progressed to stage A1 with 3cm spleen.

Genomic data in brief:	6 large deletions on chr6 and 13. Involved in Translocation

WBC count graph:

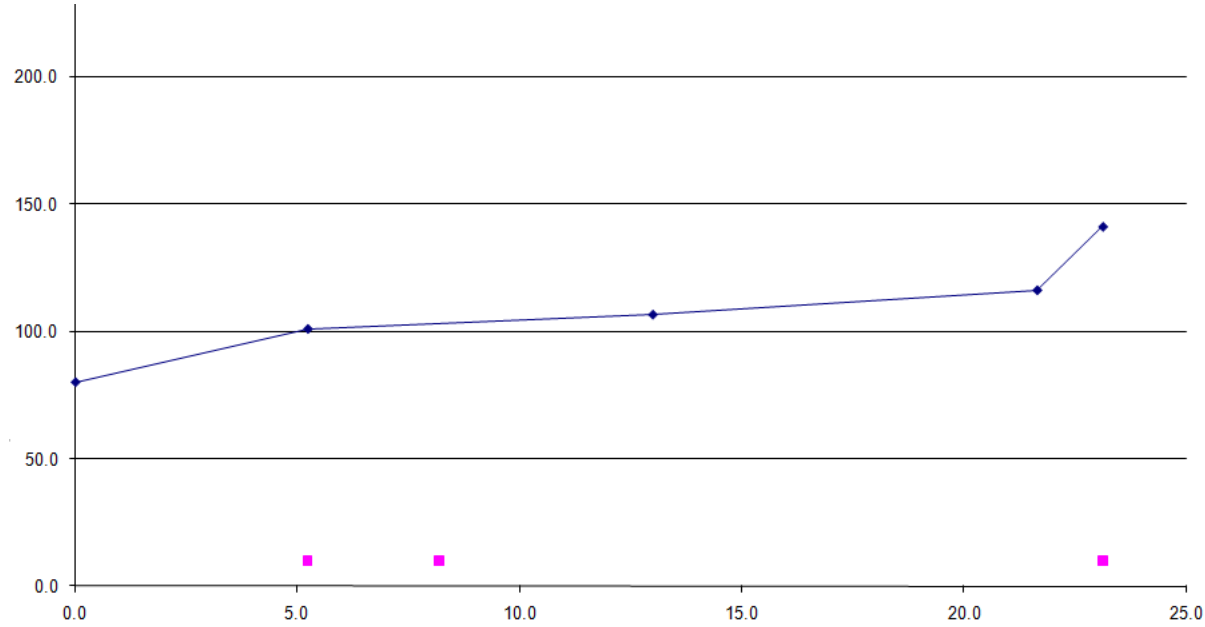
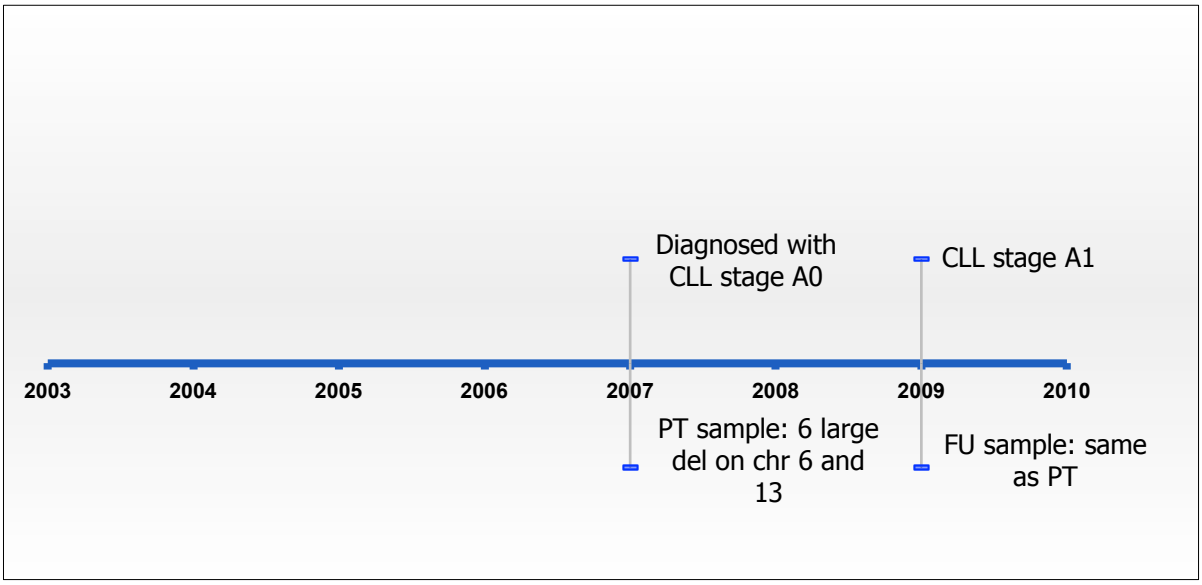


Figure 64: WBC of HV (249). PT sample at 5.1 and FU sample at 23

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	29/08/2007	
FISH data:	ATM[Normal],TP53[Normal],D13S319[Deletion]:9% hemizygous 86% homozygous	9% hemizygous 86% homozygous
Karyotype	'46,Xyt(6:13)(q26:q14)[30]	
Number of CNV:	24	
CNA <1Mb:	Diminished(6)(136.98-137.62) Diminished X2(13)(49.46-50.24)	
CNA >1Mb:	Diminished(6)(144.77-146.29) Diminished(6)(146.99-150.48) Diminished(13)(47.46-50.98) Diminished(13)(52.74-70.25)	

Follow up Sample	23/02/2009	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal],D13S319[Deletion]:9% hemizygous 86% homozygous	
Karyotype	'46,Xyt(6:13)(q26:q14)[4]	
Number of CNV:	26	
CNA <1Mb:	Diminished(6)(136.98-137.62) Diminished X2(13)(49.51-50.23)	
CNA >1Mb:	Diminished(6)(144.73-146.27) Diminished(6)(147.03-150.46) Diminished(13)(47.46-50.96) Diminished(13)(52.79-70.27)	

Patient data: 250

ID:	250

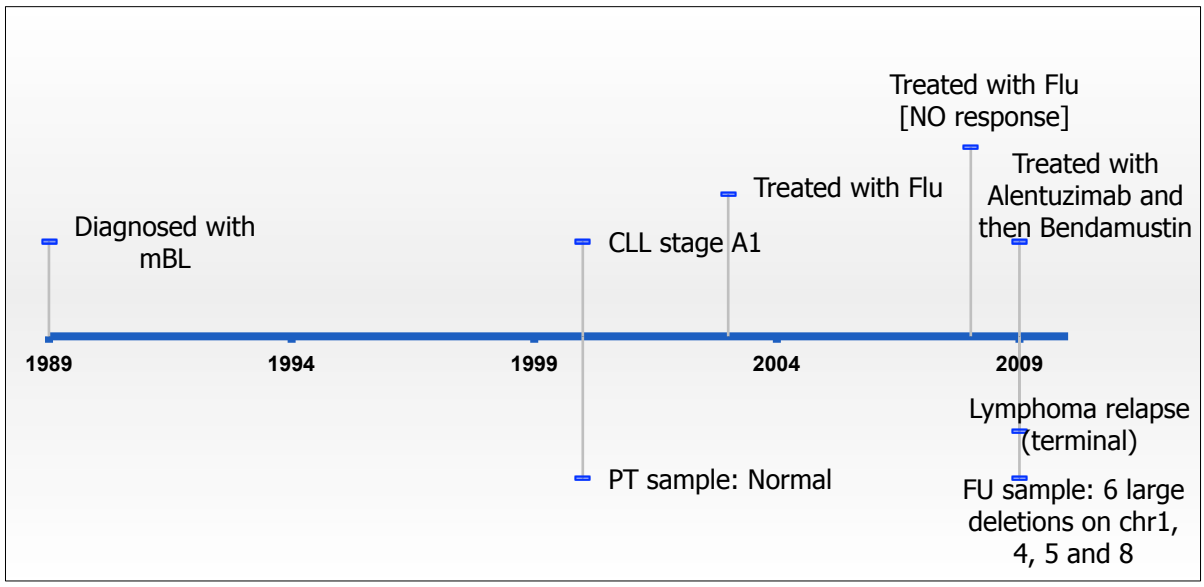
Disease Status:	Progressive
Disease stage at Diagnosis:	A
IgVH status:	Unmutated
%CD38:	4
%ZAP70:	32
Alive:	yes

Patient clinical information	Diagnosed in 1989 with mBL. Progressed to CLL stage A1 (cervical node) in 2000. Treated in 2003 with Flu. Relapsed after 1 year (treated again?). 2007, enlarged glands, develops Hodgkins disease. 2008, CLL progresses and is treated with FLU x1 [NO reponse]. 2009, treated with Alemtuzumab for 4 months, but quick relapse so treated with Bendamustine [GOOD response]. 2009, lymphoma relapse (terminal)

Genomic data in brief:	PT sample has a normal genome. FU sample has 6 large deletions on chr1, 4, 5 and 8

WBC count graph: n/a

Timeline of Samples available with DATA from Array + FISH data:



Presentation Sample	30/03/2000	
FISH data:	13Q NOT DONE; ATM[Normal],TP53[Normal]	
Karyotype	'45,x-x[2]	
Number of CNV:	31	
CNA <1Mb:		
CNA >1Mb:	normal	

Sample date	22/02/2006	
Karyotype	46,XX,t(1;3)(p?21;q?12) [1]	
FISH data:	12C[Normal], ATM[Normal],TP53[Normal],D13S319[Normal]	

Sample date	13/01/2009	
FISH data:	ATM[Normal],TP53[Normal]	No disruption of c-myc

Follow up Sample	17/06/2009	
FISH data	12C[Normal], ; ATM[Normal],TP53[Normal]; D13S319[Normal]	
Karyotype	'46,XX,t(1:2)(p13q13),t(3:5)(p25p13),-8[cp4]	
Number of CNV:	32	
CNA <1Mb:		
CNA >1Mb:	**Diminished(1)(230.76-233.2)** **Diminished(4)(131.5-143.0)** **Diminished(4)(179.53-191.26)** **Diminished(5)(59.18-64.26)** **Diminished(5)(79.2-91.1)** **Diminished(8)(0.0-43.9)** **LOH(17)(0.0-22.62)**	1q42.2q42.3 4q28.3 to 4q31.21 4q34.3 to 4q35.2 5q12.1 to 5q12.3 5q14.1 to 5q14.3 8p23.3 to 8p11.1

9. Appendix 4

Table confirming patients with 15q11 deletion were not run on the same array batch and thus deletion was not 'noise'

Patient ID	*Date of array*
12	28/05/09
14	28/05/09
16	28/05/09
29	28/05/09
32	28/05/09
33	28/05/09
34	28/05/09
35	28/05/09
18	19/11/09
19	19/11/09
22	19/11/09
23	19/11/09
25	19/11/09
28	19/11/09
15	19/06/09

10. List of References

Albrecht, B., M. Hausmann, et al. (2004). "Array-based comparative genomic hybridization for the detection of DNA sequence copy number changes in Barrett's adenocarcinoma." The Journal of Pathology **203**(3): 780-788.

Ankita, P., K. Sung-Hae, et al. (2008). "Validation of a targeted DNA microarray for the clinical evaluation of recurrent abnormalities in chronic lymphocytic leukemia." American Journal of Hematology **83**(7): 540-546.

Aplan, P. D. (2006). "Causes of oncogenic chromosomal translocation." trends Genet **22**(1): 46-55.

Armes, J. E., F. Hammet, et al. (2004). "Candidate tumor-suppressor genes on chromosome arm 8p in early-onset and high-grade breast cancers." Oncogene **23**(33): 5697-5702.

Augereau, A., C. T'kint de Roodenbeke, et al. (2011). "Telomeric damage in early stage of chronic lymphocytic leukemia correlates with shelterin dysregulation." Blood.

Austen, B., J. E. Powell, et al. (2005). "Mutations in the ATM gene lead to impaired overall and treatment-free survival that is independent of IGVH mutation status in patients with B-CLL." Blood **106**(9): 3175-3182.

Austen, B., A. Skowronska, et al. (2007). "Mutation Status of the Residual ATM Allele Is an Important Determinant of the Cellular Response to Chemotherapy and Survival in Patients With Chronic Lymphocytic Leukemia Containing an 11q Deletion." Journal of Clinical Oncology **25**(34): 5448-5457.

Baird, D. M. (2005). "New developments in telomere length analysis." Experimental Gerontology **40**(5): 363-368.

Baird, D. M. (2010). "Variation at the TERT locus and predisposition for cancer." Expert Reviews in Molecular Medicine **12**(16).

Baird, D. M., J. Rowson, et al. (2003). "Extensive allelic variation and ultrashort telomeres in senescent human cells." Nat Genet **33**(2): 203-207.

Beà, S., A. López-Guillermo, et al. (2002). "Genetic Imbalances in Progressed B-Cell Chronic Lymphocytic Leukemia and Transformed Large-Cell Lymphoma (Richter's Syndrome)." The American Journal of Pathology **161**(3): 957-968.

Bechter, O. E., W. Eisterer, et al. (1998). "Telomere Length and Telomerase Activity Predict Survival in Patients with B Cell Chronic Lymphocytic Leukemia." Cancer Research **58**(21): 4918-4922.

Benetatos, L., E. Hatzimichael, et al. (2010). "CpG methylation analysis of the MEG3 and SNRPN imprinted genes in acute myeloid leukemia and myelodysplastic syndromes." Leukemia Research **34**(2): 148-153.

Berkova, Z. Z., M. Trneny, J. Schwarz, J. Karban, E. Cmunt, l. Pavlistova, J. Brezinova, K. Michalova (2009). "clonal evolution in chronic lymphocytic leukemia studied by interphase fluorescence in-situ hybridization." Neoplasma **56**(5): 455-458.

Binet, J.-L., F. Caligaris-Cappio, et al. (2006). "Perspectives on the use of new diagnostic tools in the treatment of chronic lymphocytic leukemia." Blood **107**(3): 859-861.

Binet, J. L., M. Leporrier, et al. (1977). "A clinical staging system for chronic lymphocytic leukemia. Prognostic significance." Cancer **40**(2): 855-864.

Broberg, K., J. Björk, et al. (2005). "Constitutional short telomeres are strong genetic susceptibility markers for bladder cancer." Carcinogenesis **26**(7): 1263-1271.

Broccoli, D., A. Smogorzewska, et al. (1997). "Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2." Nat Genet **17**(2): 231-235.

Brugat, T., N. Gault, et al. (2009). "Aberrant telomere structure is characteristic of resistant chronic lymphocytic leukaemia cells." Leukemia **24**(1): 246-251.

Brugat, T., F. Nguyen-Khac, et al. (2011). "Concomitant telomere shortening, acquisition of multiple chromosomal aberrations and in vitro resistance to apoptosis in a single case of progressive CLL." Leukemia Research **In Press, Corrected Proof**.

Bullrich, F., H. Fujii, et al. (2001). "Characterization of the 13q14 Tumor Suppressor Locus in CLL." Cancer Research **61**(18): 6640-6648.

Bussey, K. J., H. J. Lawce, et al. (2001). "SNRPN methylation patterns in germ cell tumors as a reflection of primordial germ cell development." Genes, Chromosomes and Cancer **32**(4): 342-352.

Butler, T. and J. G. Gribben (2010). "Biologic and clinical significance of molecular profiling in Chronic Lymphocytic Leukemia." Blood Reviews **24**(3): 135-141.

Calin, G. A., A. Cimmino, et al. (2008). "MiR-15a and miR-16-1 cluster functions in human leukemia." Proceedings of the National Academy of Sciences **105**(13): 5166-5171.

Calin, G. A., C. D. Dumitru, et al. (2002). "Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia." Proceedings of the National Academy of Sciences of the United States of America **99**(24): 15524-15529.

Calin, G. A., M. Ferracin, et al. (2005). "A MicroRNA Signature Associated with Prognosis and Progression in Chronic Lymphocytic Leukemia." New England Journal of Medicine **353**(17): 1793-1801.

Calin, G. A., C. Sevignani, et al. (2004). "Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers." Proceedings of the National Academy of Sciences of the United States of America **101**(9): 2999-3004.

Campbell, L. W., Buys TP, Chari R, Coe BP, Lam S, Lam WL (2008). "Integrative genomic and gene expression analysis of chromosome 7 identified novel oncogene loci in non-small cell lung cancer." Genome **51**(12): 1032-1039.

Chapiro, E., N. Leporrier, et al. (2009). "Gain of the short arm of chromosome 2 (2p) is a frequent recurring chromosome aberration in untreated chronic lymphocytic leukemia (CLL) at advanced stages." Leukemia Research **34**(1): 63-68.

Cheson, B., J. Bennett, et al. (1996). "National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment." Blood **87**(12): 4990-4997.

Chevallier, P., D. Penther, et al. (2002). "CD38 expression and secondary 17p deletion are important prognostic factors in chronic lymphocytic leukaemia." British Journal of Haematology **116**(1): 142-150.

Cimmino Amelia , G. A. C., *† Muller Fabbri,* Marilena V. Iorio,* Manuela Ferracin,‡ Masayoshi Shimizu,* Sylwia E. Wojcik,* Rami I. Aqeilan,* Simona Zupo,§ Mariella Dono,§ Laura Rassenti,¶ Hansjuerg Alder,* Stefano Volinia,* Chang-gong Liu,* Thomas J. Kipps,¶ Massimo Negrini,*‡ and Carlo M. Croce (2005). "miR-15 and miR-16 induce apoptosis by targeting BCL2." PNAS **102**(39): 13944-13949.

Cordone, I., S. Masi, et al. (1998). "p53 Expression in B-Cell Chronic Lymphocytic Leukemia: A Marker of Disease Progression and Poor Prognosis." Blood **91**(11): 4342-4349.

Cory, S. (1995). "Regulation of Lymphocyte Survival by the BCL-2 Gene Family." Annual Review of Immunology **13**(1): 513-543.

Counter, C., J. Gupta, et al. (1995). "Telomerase activity in normal leukocytes and in hematologic malignancies." Blood **85**(9): 2315-2320.

Crowther-Swanepoel, D., P. Broderick, et al. (2010). "Common variants at 2q37.3, 8q24.21, 15q21.3 and 16q24.1 influence chronic lymphocytic leukemia risk." Nat Genet **42**(2): 132-136.

CRUK (2007). Retrieved 9th June 2011, from http://www.cancerhelp.org.uk/type/cll/.

Cuneo, A., G. M. Rigolin, et al. (2004). "Chronic lymphocytic leukemia with 6q- shows distinct hematological features and intermediate prognosis." Leukemia **18**(3): 476-483.

Cuneo, A., M. G. Roberti, et al. (2000). "Four novel non-random chromosome rearrangements in B-cell chronic lymphocytic leukaemia: 6p24–25 and 12p12–13 translocations, 4q21 anomalies and monosomy 21." British Journal of Haematology **108**(3): 559-564.

Curtis, C., A. Lynch, et al. (2009). "The pitfalls of platform comparison: DNA copy number array technologies assessed." BMC Genomics **10**(1): 588.

Dal-Bo, M., F. Bertoni, et al. (2009). "Intrinsic and extrinsic factors influencing the clinical course of B-cell chronic lymphocytic leukemia: prognostic markers with pathogenetic relevance." Journal of Translational Medicine **7**(1): 76.

Dal Bo, M., F. M. Rossi, et al. (2011). "13q14 Deletion size and number of deleted cells both influence prognosis in chronic lymphocytic leukemia." Genes, Chromosomes and Cancer: n/a-n/a.

Dalemari, C.-S., M. Mahmoud, et al. (2010). "Verification that common variation at 2q37.1, 6p25.3, 11q24.1, 15q23, and 19q13.32 influences chronic lymphocytic leukaemia risk." British Journal of Haematology **9999**(9999).

Damle, R. N., F. M. Batliwalla, et al. (2004). "Telomere length and telomerase activity delineate distinctive replicative features of the B-CLL subgroups defined by immunoglobulin V gene mutations." Blood **103**(2): 375-382.

Damle, R. N., T. Wasil, et al. (1999). "Ig V Gene Mutation Status and CD38 Expression As Novel Prognostic Indicators in Chronic Lymphocytic Leukemia." Blood **94**(6): 1840-1847.

Daniel, L. V. D., D. S. Tait, et al. (2009). "A comprehensive evaluation of the prognostic significance of 13q deletions in patients with B-chronic lymphocytic leukaemia." British Journal of Haematology **148**(4): 544-550.

David Burnett, D. B., John Crocker (2005). The science of laboratory diagnosis, Wiley-Blackwell.

Deeds, J., F. Cronin, et al. (2000). "Patterns of melastatin mRNA expression in melanocytic tumors." Human pathology **31**(11): 1346-1356.

Dicker, F., H. Herholz, et al. (2008). "The detection of TP53 mutations in chronic lymphocytic leukemia independently predicts rapid disease progression and is highly correlated with a complex aberrant karyotype." Leukemia **23**(1): 117-124.

Dighiero, G. and T. J. Hamblin (2008). "Chronic lymphocytic leukaemia." The Lancet **371**(9617): 1017-1029.

Dohner, H., K. Fischer, et al. (1995). "p53 gene deletion predicts for poor survival and non-response to therapy with purine analogs in chronic B-cell leukemias." Blood **85**(6): 1580-1589.

Dohner, H., S. Stilgenbauer, et al. (2000). "Genomic Aberrations and Survival in Chronic Lymphocytic Leukemia." N Engl J Med **343**(26): 1910-1916.

Dotan, Z. A., A. Dotan, et al. (2000). "Modification in the inherent mode of allelic replication in lymphocytes of patients suffering from renal cell carcinoma: A novel genetic alteration associated with malignancy." Genes, Chromosomes and Cancer **27**(3): 270-277.

Duncan, L. M., J. Deeds, et al. (2001). "Melastatin Expression and Prognosis in Cutaneous Malignant Melanoma." Journal of Clinical Oncology **19**(2): 568-576.

el Rouby, S., A. Thomas, et al. (1993). "p53 gene mutation in B-cell chronic lymphocytic leukemia is associated with drug resistance and is independent of MDR1/MDR3 gene expression." Blood **82**(11): 3452-3459.

Escudier, S., J. Pereira-Leahy, et al. (1993). "Fluorescent in situ hybridization and cytogenetic studies of trisomy 12 in chronic lymphocytic leukemia." Blood **81**(10): 2702-2707.

Evan, G. I. and K. H. Vousden (2001). "Proliferation, cell cycle and apoptosis in cancer." Nature **411**(6835): 342-348.

Fagagna, F. d. A. d., P. M. Reaper, et al. (2003). "A DNA damage checkpoint response in telomere-initiated senescence." Nature **426**(6963): 194-198.

Fink, S., S. Geyer, et al. (2004). "Clinical Significance of Homozygous D13S319 Deletion in B-Cell Chronic Lymphocytic Leukemia (B-CLL)." ASH Annual Meeting Abstracts **104**(11): 2799-.

Furman, R. R. (2010). "Prognostic Markers and Stratification of Chronic Lymphocytic Leukemia." Hematology **2010**(1): 77-81.

Gisselsson, D., T. Jonson, et al. (2001). "Telomere dysfunction triggers extensive DNA fragmentation and evolution of complex chromosome abnormalities in human malignant tumors." Proceedings of the National Academy of Sciences **98**(22): 12683-12688.

Gondek, L. P., R. Tiu, et al. (2008). "Chromosomal lesions and uniparental disomy detected by SNP arrays in MDS, MDS/MPD, and MDS-derived AML." Blood **111**(3): 1534-1542.

Green, M., P. Jardine, et al. (2010). "A new method to detect loss of heterozygosity using cohort heterozygosity comparisons." BMC Cancer **10**(1): 195.

Greider, C. W. (1998). "Telomerase activity, cell proliferation, and cancer." Proceedings of the National Academy of Sciences **95**(1): 90-92.

Grever, M. R., D. M. Lucas, et al. (2007). "Novel agents and strategies for treatment of p53-defective chronic lymphocytic leukemia." Best Practice & Research Clinical Haematology **20**(3): 545-556.

Gunn, S. R., M. S. Mohammed, et al. (2008). "Whole-Genome Scanning by Array Comparative Genomic Hybridization as a Clinical Tool for Risk Assessment in Chronic Lymphocytic Leukemia." J Mol Diagn **10**(5): 442-451.

Hamblin (2002). "Chronic lymphocytic leukaemia: one disease or two?" Annals of Hematology **81**(6): 299-303.

Hamblin, T. J. (2007). "Prognostic markers in chronic lympocytic leukaemia." Best Practice & Research Clinical Haematology **20**(3): 455-468.

Hamblin, T. J., Z. Davis, et al. (1999). "Unmutated Ig VH Genes Are Associated With a More Aggressive Form of Chronic Lymphocytic Leukemia." Blood **94**(6): 1848-1854.

Hamblin, T. J., J. A. Orchard, et al. (2000). "Immunoglobulin V genes and CD38 expression in CLL." Blood **95**(7): 2455-2457.

Hamblin, T. J., J. A. Orchard, et al. (2002). "CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease." Blood **99**(3): 1023-1029.

Hammarsund, M., M. M. Corcoran, et al. (2004). "Characterization of a novel B-CLL candidate gene - DLEU7 - located in the 13q14 tumor suppressor locus." FEBS Letters **556**(1-3): 75-80.

Hanahan, D. and R. A. Weinberg (2000). "The Hallmarks of Cancer." Cell **100**(1): 57-70.

Hanlon Katy, C. E. R., Lorna W. Harries (2009). "Investigating the Targets of MIR-15a and MIR-16-1 in Patients with Chronic Lymphocytic Leukemia (CLL)." PLoS One **4**(9).

Hardy, R. R. and K. Hayakawa (2001). "B CELL DEVELOPMENT PATHWAYS." Annual Review of Immunology **19**(1): 595-621.

Harley, C. B., A. B. Futcher, et al. (1990). "Telomeres shorten during ageing of human fibroblasts." Nature **345**(6274): 458-460.

Hemann, M. T., M. A. Strong, et al. (2001). "The Shortest Telomere, Not Average Telomere Length, Is Critical for Cell Viability and Chromosome Stability." Cell **107**(1): 67-77.

Heng, H. H. Q., S. W. Bremer, et al. (2006). "Cancer progression by non-clonal chromosome aberrations." Journal of Cellular Biochemistry **98**(6): 1424-1435.

Heng, H. H. Q., J. B. Stevens, et al. (2010). "The evolutionary mechanism of cancer." Journal of Cellular Biochemistry **109**(6): 1072-1084.

Hernando, E., Z. Nahle, et al. (2004). "Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control." Nature **430**(7001): 797-802.

Herzog, S., M. Reth, et al. (2009). "Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling." Nat Rev Immunol **9**(3): 195-205.

Hjalmar, V., R. Hast, et al. (2001). "Sequential fluorescence in situ hybridization analyses for trisomy 12 in chronic leukemic B-cell disorders." Haematologica **86**(2): 174-180.

Huang, P., L. Feng, et al. (2000). "Superoxide dismutase as a target for the selective killing of cancer cells." Nature **407**(6802): 390-395.

Hultdin, M., R. Rosenquist, et al. (2003). "Association between telomere length and VH gene mutation status in chronic lymphocytic leukaemia: clinical and biological implications." Br J Cancer **88**(4): 593-598.

ISCN (2009). An International System for Human Cytogenetic Nomenclature. Recommendations of the International Standing Committee on Human Cytogenetic Nomenclature, S.Karger: Basel.

Ishii, H., A. Vecchione, et al. (2001). "FEZ1/LZTS1 gene at 8p22 suppresses cancer cell growth and regulates mitosis." Proceedings of the National Academy of Sciences **98**(18): 10374-10379.

Jahrsdorfer, B. and G. J. Weiner (2008). "Short telomeres in B-CLL: the chicken or the egg?" Blood **111**(12): 5756-.

Janz, C. S. R. a. S. (2008). "Mechanisms and Consequences of Chromosomal Translocation." Cancer Epidemiol Biomarkers Prev **17**(8): 1849 - 1851.

Jarosova, M., H. Urbankova, et al. (2010). "Gain of chromosome 2p in chronic lymphocytic leukemia: significant heterogeneity and a new recurrent dicentric rearrangement." Leukemia & Lymphoma **51**(2): 304-313.

Jelinek, D. F., R. C. Tschumper, et al. (2001). "Analysis of clonal B-cell CD38 and immunoglobulin variable region sequence status in relation to clinical outcome for B-chronic lymphocytic leukaemia." British Journal of Haematology **115**(4): 854-861.

Juliusson, G., K. Friberg, et al. (1988). "Consistency of chromosomal aberrations in chronic B-lymphocytic leukemia. A longitudinal cytogenetic study of 41 patients." Cancer **62**(3): 500-506.

Juliusson, G., K. Robert, et al. (1985). "Prognostic information from cytogenetic analysis in chronic B- lymphocytic leukemia and leukemic immunocytoma." Blood **65**(1): 134-141.

Kanzawa, T. K., Satoru Kyo, Isabelle M. Germano, Yasuko Kondo, Seiji Kondo (2003). "Down-regulation of telomerase activity in malignant glioma cells by p27KIP1." International Journal of Oncology **23**(6): 1703-1708.

Kay, N. E., S. M. O'Brien, et al. (2007). "The role of prognostic factors in assessing /`high-risk/' subgroups of patients with chronic lymphocytic leukemia." Leukemia **21**(9): 1885-1891.

Kay NE, R. E., Peterson LC. (1995). "Tumor suppressor genes and clonal evolution in B-CLL." Leuk Lymphoma **18**: 41-49.

Kim, N., M. Piatyszek, et al. (1994). "Specific association of human telomerase activity with immortal cells and cancer." Science **266**(5193): 2011-2015.

Kim, Y., Y.-C. Jung, et al. (2010). "Evidences showing wide presence of small genomic aberrations in chronic lymphocytic leukemia." BMC Research Notes **3**(1): 341.

Klein, U., M. Lia, et al. (2010). "The DLEU2/miR-15a/16-1 Cluster Controls B Cell Proliferation and Its Deletion Leads to Chronic Lymphocytic Leukemia." **17**(1): 28-40.

Knauf, W. U., S. Knuutila, et al. (1995). "Trisomy 12 in B-Cell chronic Lymphocytic Leukemia: Correlation with Advanced Disease, Atypical Morphology, High Levels of sCD25, and with Refractoriness to Treatment." Leukemia & Lymphoma **19**(3-4): 289-294.

Knowles, M. A., J. S. Aveyard, et al. (2005). "Mutation analysis of the 8p candidate tumour suppressor genes DBC2 (RHOBTB2) and LZTS1 in bladder cancer." Cancer Letters **225**(1): 121-130.

Korshunov, A., R. Sycheva, et al. (2006). "Genetically distinct and clinically relevant subtypes of glioblastoma defined by array-based comparative genomic hybridization (array-CGH)." Acta Neuropathologica **111**(5): 465-474.

Korshunov, A., R. Sycheva, et al. (2007). "Recurrent cytogenetic aberrations in central neurocytomas and their biological relevance." Acta Neuropathologica **113**(3): 303-312.

Krober, A., T. Seiler, et al. (2002). "VH mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia." Blood **100**(4): 1410-1416.

Kuby, T. J. K., Barbara A. Osborne, Richard Goldsby (2006). Immunology, W. H. Freeman.

Kujawski, L., P. Ouillette, et al. (2008). "Genomic complexity identifies patients with aggressive chronic lymphocytic leukemia." Blood **112**(5): 1993-2003.

Ladetto, M. (2010). "Telomere disrupts, CLL progresses." Blood **116**(11): 1821-1822.

Leung, A. K. W., K. Nagai, et al. (2011). "Structure of the spliceosomal U4 snRNP core domain and its implication for snRNP biogenesis." Nature **473**(7348): 536-539.

Levy, M. Z., R. C. Allsopp, et al. (1992). "Telomere end-replication problem and cell aging." Journal of Molecular Biology **225**(4): 951-960.

Li, Z., C. J. Woo, et al. (2004). "The generation of antibody diversity through somatic hypermutation and class switch recombination." Genes & Development **18**(1): 1-11.

Lieber, M. R., K. Yu, et al. (2006). "Roles of nonhomologous DNA end joining, V(D)J recombination, and class switch recombination in chromosomal translocations." DNA Repair **5**(9-10): 1234-1245.

Lim, L. P., N. C. Lau, et al. (2005). "Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs." Nature **433**(7027): 769-773.

Lin, S.-Y. and S. J. Elledge (2003). "Multiple Tumor Suppressor Pathways Negatively Regulate Telomerase." Cell **113**(7): 881-889.

Lin, T. T., B. T. Letsolo, et al. (2010). "Telomere dysfunction and fusion during the progression of chronic lymphocytic leukaemia: evidence for a telomere crisis." Blood: blood-2010-2002-272104.

Lindsten, T., A. J. Ross, et al. (2000). "The Combined Functions of Proapoptotic Bcl-2 Family Members Bak and Bax Are Essential for Normal Development of Multiple Tissues." Molecular cell **6**(6): 1389-1399.

Liu, C. R., Huang S, Wu Y, Li G, Liu Q, Yin D, Liang Y. (2010). "Knockdown of SOD1 sensitizes the CD34+ CML cells to imatinib therapy." Medical Oncology **Ahead of Print**.

Liu, Y., Martin Corcoran2, Omid Rasool1, Ganka Ivanova1, Rachel Ibbotson2, Dan Grandér1, Arati Iyengar2, Anna Baranova1,3, Vladimir Kashuba4, Mats Merup1,5, Xiushan Wu1, Anne Gardiner2, Roman Mullenbach6, Andrew Poltaraus7, Anna Linda Hultström1, Gunnar Juliusson8, Robert Chapman2, Mary Tiller2, Finbarr Cotter6, Gösta Gahrton5, Nick Yankovsky3, Eugene Zabarovsky4, Stefan Einhorn1,a and David Oscier2 (1997). "Cloning of two candidate tumor suppressor genes within a 10 kb region on chromosome 13q14, frequently deleted in chronic lymphocytic leukemia." Oncogene **15**(20): 2463-2473.

Liu, Y., L. Szekely, et al. (1993). "Chronic lymphocytic leukemia cells with allelic deletions at 13q14 commonly have one intact RB1 gene: evidence for a role of an adjacent locus." Proceedings of the National Academy of Sciences of the United States of America **90**(18): 8697-8701.

Lo, A. W., L. Sabatier, et al. (2002). "DNA amplification by breakage/fusion/bridge cycles initiated by spontaneous telomere loss in a human cancer cell line." Neoplasia (New York, N.Y.) **4**(6): 531-538.

Longo, P. G., L. Laurenti, et al. (2006). "The Akt signaling pathway determines the different proliferative capacity of chronic lymphocytic leukemia B-cells from patients with progressive and stable disease." Leukemia **21**(1): 110-120.

Lozanski, G., N. A. Heerema, et al. (2004). "Alemtuzumab is an effective therapy for chronic lymphocytic leukemia with p53 mutations and deletions." Blood **103**(9): 3278-3281.

Marti, G. E., A. C. Rawstron, et al. (2005). "Diagnostic criteria for monoclonal B-cell lymphocytosis." British Journal of Haematology **130**(3): 325-332.

Maser, R. S. and R. A. DePinho (2002). "Connecting Chromosomes, Crisis, and Cancer." Science **297**(5581): 565-569.

Mazar, J., K. DeYoung, et al. (2010). "The Regulation of miRNA-211 Expression and Its Role in Melanoma Cell Invasiveness." PLoS One **5**(11): e13779.

Meeker, A. K. and P. Argani (2004). "Telomere Shortening Occurs Early During Breast Tumorigenesis: A Cause of Chromosome Destabilization Underlying Malignant Transformation?" Journal of Mammary Gland Biology and Neoplasia **9**(3): 285-296.

Meeker, A. K., J. L. Hicks, et al. (2002). "Telomere Shortening Is an Early Somatic DNA Alteration in Human Prostate Tumorigenesis." Cancer Research **62**(22): 6405-6409.

Merkerova, M., H. Bruchova, et al. (2007). "Bmi-1 over-expression plays a secondary role in chronic myeloid leukemia transformation." Leukemia & Lymphoma **48**(4): 793-801.

Messmer, B. T., D. Messmer, et al. (2005). "In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells." The Journal of Clinical Investigation **115**(3): 755-764.

Michele Dal-Bo, F. B., Francesco Forconi, Antonella Zucchetto, Riccardo Bomben, Roberto Marasca, Silvia Deaglio, Luca Laurenti, Dimitar G Efremov, Gianluca Gaidano,

Giovanni Del Poeta, and Valter Gattei (2009). "Intrinsic and extrinsic factors influencing the clinical course of B-cell chronic lymphocytic leukemia: prognostic markers with pathogenetic relevance." Journal of Translational Medicine **7**(76).

Moyzis, R. K., J. M. Buckingham, et al. (1988). "A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes." Proceedings of the National Academy of Sciences **85**(18): 6622-6626.

Nakamura, S. R., Francisca Vazquez Sabina Signoretti, Massimo Loda, and William R. Sellers (2000). "Forkhead Transcription Factors Are Critical Effectors of Cell Death and Cell Cycle Arrest Downstream of PTEN." Mol. Cell. Biol. **20**(23): 8969–8982.

Natkunam, Y. (2007). "The Biology of the Germinal Center." Hematology **2007**(1): 210-215.

Nicholls, R. D., S. Saitoh, et al. (1998). "Imprinting in Prader-Willi and Angelman syndromes." Trends in Genetics **14**(5): 194-200.

Nishida, A. K., Atsushi Tajima, Yuko Ogasawara, Yoshimi Ishibashi, Yasuka Uehara, Ituro Inoue, and Katsushi Tokunaga (2008). "Evaluating the performance of Affymetrix SNP Array 6.0 platform with 400 Japanese individuals." BMC Genomics **9**: 431.

Norrback, K. F. and G. Roos (1997). "Telomeres and telomerase in normal and malignant haematopoietic cells." European Journal of Cancer **33**(5): 774-780.

Nowell, P. C., L. Moreau, et al. (1988). "Karyotypic stability in chronic B-cell leukemia." Cancer Genetics and Cytogenetics **33**(2): 155-160.

Osborne, B. A. (1996). "Apoptosis and the maintenance of homoeostasis in the immune system." Current Opinion in Immunology **8**(2): 245-254.

Oscier D, F. M., Herbert T, Lambert R (1991). "Karyotypic evolution in B-cell chronic lymphocytic leukaemia." Genes Chromosomes Cancer **3**(1): 16-20.

Oscier, D. G., A. C. Gardiner, et al. (2002). "Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors." Blood **100**(4): 1177-1184.

Ouillette, P., H. Erba, et al. (2008). "Integrated Genomic Profiling of Chronic Lymphocytic Leukemia Identifies Subtypes of Deletion 13q14." Cancer Research **68**(4): 1012-1021.

Ouillette, P., S. Fossum, et al. (2010). "Aggressive Chronic Lymphocytic Leukemia with Elevated Genomic Complexity Is Associated with Multiple Gene Defects in the Response to DNA Double-Strand Breaks." Clinical Cancer Research **16**(3): 835-847.

Palamarchuk, A., A. Efanov, et al. (2010). "13q14 deletions in CLL involve cooperating tumor suppressors." Blood **115**(19): 3916-3922.

Parker, H., M. J. J. Rose-Zerilli, et al. (2010). "13q deletion anatomy and disease progression in patients with chronic lymphocytic leukemia." Leukemia.

Pasqualetto, V., F. Vasseur, et al. (2005). "Fas receptor signaling is requisite for B cell differentiation." Journal of Leukocyte Biology **78**(5): 1106-1117.

Pekarsky, Y., N. Zanesi, et al. (2010). "Molecular basis of CLL." Seminars in Cancer Biology **In Press, Corrected Proof**.

Pepper, C. and D. M. Baird (2010). "Shortened telomeres: a driving force behind leukemia?" Future Oncology **6**(11): 1681-1686.

Pfeifer, D., M. Pantic, et al. (2007). "Genome-wide analysis of DNA copy number changes and LOH in CLL using high-density SNP arrays." Blood **109**(3): 1202-1210.

Pillai, R. S., S. N. Bhattacharyya, et al. (2007). "Repression of protein synthesis by miRNAs: how many mechanisms?" Trends in Cell Biology **17**(3): 118-126.

Poncet, D., A. Belleville, et al. (2008). "Changes in the expression of telomere maintenance genes suggest global telomere dysfunction in B-chronic lymphocytic leukemia." Blood **111**(4): 2388-2391.

Porpaczy, E., M. Bilban, et al. (2009). "Gene expression signature of chronic lymphocytic leukaemia with Trisomy 12." European Journal of Clinical Investigation **39**(7): 568-575.

Quintás-Cardama, A. and J. Cortes (2009). "Molecular biology of bcr-abl1–positive chronic myeloid leukemia." Blood **113**(8): 1619-1630.

Rai, K., A. Sawitsky, et al. (1975). "Clinical staging of chronic lymphocytic leukemia." Blood **46**(2): 219-234.

Rajewsky, K. (1996). "Clonal selection and learning in the antibody system." Nature **381**(6585): 751-758.

Rassenti, L. Z., L. Huynh, et al. (2004). "ZAP-70 Compared with Immunoglobulin Heavy-Chain Gene Mutation Status as a Predictor of Disease Progression in Chronic Lymphocytic Leukemia." New England Journal of Medicine **351**(9): 893-901.

Rassenti, L. Z., S. Jain, et al. (2008). "Relative value of ZAP-70, CD38, and immunoglobulin mutation status in predicting aggressive disease in chronic lymphocytic leukemia." Blood **112**(5): 1923-1930.

Read, T. S. a. A. P. (2003). Human molecular genetics 3, Garland Science.

Remes, K., K. F. Norrback, et al. (2000). "Telomere length and telomerase activity in malignant lymphomas at diagnosis and relapse." Br J Cancer **82**(3): 601-607.

Ricca, I., A. Rocci, et al. (2007). "Telomere length identifies two different prognostic subgroups among VH-unmutated B-cell chronic lymphocytic leukemia patients." Leukemia **21**(4): 697-705.

Roos, G., A. Krober, et al. (2008). "Short telomeres are associated with genetic complexity, high-risk genomic aberrations, and short survival in chronic lymphocytic leukemia." Blood **111**(4): 2246-2252.

Rossi, D., C. Lobetti Bodoni, et al. (2009). "Telomere length is an independent predictor of survival, treatment requirement and Richter's syndrome transformation in chronic lymphocytic leukemia." Leukemia **23**(6): 1062-1072.

Rubio-Moscardo, F., D. Blesa, et al. (2005). "Characterization of 8p21.3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes." Blood **106**(9): 3214-3222.

Rudolph, K. L., S. Chang, et al. (1999). "Longevity, Stress Response, and Cancer in Aging Telomerase-Deficient Mice." Cell **96**(5): 701-712.

Rufer, N., W. Dragowska, et al. (1998). "Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry." Nat Biotech **16**(8): 743-747.

Salin, M. R., L. Morat, and L. Sabatier (2009). "Increased genomic alteration complexity and telomere shortening in B-CLL cells resistant to radiation-induced apoptosis." Cytogenetic and Genome Research **122**(3-4): 343–349.

Sawyer, J. R., M. Husain, et al. (2000). "A role for telomeric and centromeric instability in the progression of chromosome aberrations in meningioma patients." Cancer **88**(2): 440-453.

Schaffner, C., S. Stilgenbauer, et al. (1999). "Somatic ATM Mutations Indicate a Pathogenic Role of ATM in B-Cell Chronic Lymphocytic Leukemia." Blood **94**(2): 748-753.

Schwartz, J. L., R. Jordan, et al. (2001). "TP53-dependent chromosome instability is associated with transient reductions in telomere length in immortal telomerase-positive cell lines." Genes, Chromosomes and Cancer **30**(3): 236-244.

Sellmann, L., D. de Beer, et al. (2011). "Telomeres and prognosis in patients with chronic lymphocytic leukaemia." International Journal of Hematology **93**(1): 74-82.

Shanafelt, T. D. (2009). "Predicting clinical outcome in CLL: how and why." Hematology **2009**(1): 421-429.

Shanafelt, T. D., T. E. Witzig, et al. (2006). "Prospective Evaluation of Clonal Evolution During Long-Term Follow-Up of Patients With Untreated Early-Stage Chronic Lymphocytic Leukemia." J Clin Oncol **24**(28): 4634-4641.

Shay, J. W. and W. E. Wright (2005). "Senescence and immortalization: role of telomeres and telomerase." Carcinogenesis **26**(5): 867-874.

Sherr, C. J. and F. McCormick (2002). "The RB and p53 pathways in cancer." Cancer cell **2**(2): 103-112.

Shinawi, M. and S. W. Cheung (2008). "The array CGH and its clinical applications." Drug Discovery Today **13**(17-18): 760-770.

Speina, E., K. D. Arczewska, et al. (2005). "Contribution of hMTH1 to the Maintenance of 8-Oxoguanine Levels in Lung DNA of Non–Small-Cell Lung Cancer Patients." Journal of the National Cancer Institute **97**(5): 384-395.

Stilgenbauer, S., S. Sander, et al. (2007). "Clonal evolution in chronic lymphocytic leukemia: acquisition of high-risk genomic aberrations associated with unmutated VH, resistance to therapy, and short survival." Haematologica **92**(9): 1242-1245.

Takada, H., I. Imoto, et al. (2005). "Screening of DNA copy-number aberrations in gastric cancer cell lines by array-based comparative genomic hybridization." Cancer Science **96**(2): 100-110.

Tam, C. S., T. D. Shanafelt, et al. (2009). "De novo deletion 17p13.1 chronic lymphocytic leukemia shows significant clinical heterogeneity: the M. D. Anderson and Mayo Clinic experience." Blood **114**(5): 957-964.

Thomas, M. D., B. Srivastava, et al. (2006). "Regulation of peripheral B cell maturation." Cellular Immunology **239**(2): 92-102.

Tobin, G., U. Thunberg, et al. (2003). "Chronic lymphocytic leukemias utilizing the VH3-21 gene display highly restricted V 2-14 gene use and homologous CDR3s: implicating recognition of a common antigen epitope." Blood **101**(12): 4952-4957.

Trentin, L., G. Ballon, et al. (1999). "Telomerase activity in chronic lymphoproliferative disorders of B-cell lineage." British Journal of Haematology **106**(3): 662-668.

Tsuzuki, A. E., Hisato Igarashi, Tomoo Iwakuma, Yoko Nakatsuru, Yohei Tominaga, Hisaya Kawate, Kazuki Nakao, Kenji Nakamura, Fumio Ide, Shinobu Kura, Yusaku Nakabeppu, Motoya Katsuki,Takatoshi Ishikawa, and Mutsuo Sekiguchi (2001). "Spontaneous tumorigenesis in mice defective in the MTH1 gene encoding 8-oxo-dGTPase." Proc Natl Acad Sci USA **98**(20): 11456–11461.

Van Bockstaele, F., B. Verhasselt, et al. (2009). "Prognostic markers in chronic lymphocytic leukemia: A comprehensive review." Blood Reviews **23**(1): 25-47.

Van Dyke, D. L., T. D. Shanafelt, et al. (2010). "A comprehensive evaluation of the prognostic significance of 13q deletions in patients with B-chronic lymphocytic leukaemia." British Journal of Haematology **148**(4): 544-550.

van Gent, D. C. and M. van der Burg (2007). "Non-homologous end-joining, a sticky affair." Oncogene **26**(56): 7731-7740.

van Harn, T., F. Foijer, et al. (2010). "Loss of Rb proteins causes genomic instability in the absence of mitogenic signaling." Genes & Development.

van Steensel, B., A. Smogorzewska, et al. (1998). "TRF2 Protects Human Telomeres from End-to-End Fusions." Cell **92**(3): 401-413.

Vermeulen, K., D. R. Van Bockstaele, et al. (2003). "The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer." Cell Proliferation **36**(3): 131-149.

Visser S, X. Y. (2010). "LATS tumor suppressor: A new governor of cellular homeostasis." Cell cycle **9**(19): 3892-3903.

Wani, G., G. E. Milo, et al. (1998). "Enhanced expression of the 8-oxo-7,8-dihydrodeoxyguanosine triphosphatase gene in human breast tumor cells." Cancer Letters **125**(1): 123-130.

Weinberg, J. B., A. D. Volkheimer, et al. (2007). "Clinical and molecular predictors of disease severity and survival in chronic lymphocytic leukemia." American Journal of Hematology **82**(12): 1063-1070.

Weng, N.-p., L. Granger, et al. (1997). "Telomere lengthening and telomerase activation during human B cell differentiation." Proceedings of the National Academy of Sciences of the United States of America **94**(20): 10827-10832.

WHO (2004). from http://www.euro.who.int/en/what-we-do/health-topics/noncommunicable-diseases/cancer/facts-and-figures.

Wiestner, A., A. Rosenwald, et al. (2003). "ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile." Blood **101**(12): 4944-4951.

Zainuddin, N., F. Murray, et al. (2011). "TP53 Mutations are infrequent in newly diagnosed chronic lymphocytic leukemia." Leukemia Research **35**(2): 272-274.

Zenz, T., A. Krober, et al. (2008). "Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia: results from a detailed genetic characterization with long-term follow-up." Blood **112**(8): 3322-3329.

Zenz, T., D. Mertens, et al. (2011). "Importance of genetics in chronic lymphocytic leukemia." Blood Reviews **25**(3): 131-137.