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CRYPTIC TELOMERIC REARRANGEMENTS IN INDIVIDUALS WITH IDIOPATHIC MENTAL RETARDATION

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ABSTRACT

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CRYPTIC TELOMERIC REARRANGEMENTS IN INDIVIDUALS WITH IDIOPATHIC MENTAL RETARDATION

by Christine Angela Joyce

The cause of mental retardation, present in approximately 3% of the population, is unexplained in the majority of cases. A pilot study to assess the prevalence of cryptic telomeric rearrangements, which result in segmental aneuploidy and gene dosage imbalance, has shown that such imbalances could account for at least 6% of highly selected cases and may be a significant cause of idiopathic mental retardation (IMR).

Two populations of patients with unexplained developmental delay (unselected and selected), and a population of control individuals have been investigated to determine the frequency of submicroscopic telomeric rearrangements associated with IMR and the frequency within the normal population.

In contrast to current thinking, our data have shown that true cryptic telomeric rearrangements are not a significant cause of IMR. No fully cryptic abnormalities were detected in our IMR study population, although a semi-cryptic unbalanced telomeric translocation was identified in one selected patient by high resolution G-band analysis. This abnormality was confirmed and characterised by fluorescence *in situ* hybridization (FISH) using telomere-specific probes. A further eighteen cytogenetically detected subtle terminal rearrangements were characterised using multi-telomere FISH. Nine of these had previously been reported as normal. These results raise the possibility that cryptic telomeric rearrangements reported by other groups may also be visible with the aid of high resolution analysis. A review of cryptic, semi-cryptic, and subtle but visible terminal abnormalities in the literature provided further support for this hypothesis.

Unexpectedly, two cryptic telomeric abnormalities were detected among our control individuals, suggesting that submicroscopic telomeric abnormalities may be a not uncommon finding in the general population. A possible explanation for these unexpected findings in apparently phenotypically normal individuals is that the particular genetic imbalances in these two cases involve regions of the genome with few functional genes. These abnormalities indicate the existance of a subset of non-pathogenic, telomeric imbalances. Hence our data have important implications when defining the significance of cryptic telomeric rearrangements detected during screening programmes.

In addition, a site of cross hybridization for the 11p telomere-specific probe was identified at the telomeric region of 17p. This result is indicative of sequence homology and suggests a mechanism for the origin of a familial 11p;17p translocation.

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Contribution of the author to the collaborative work presented in this thesis

The work presented in this thesis was initiated by the author but is the product of collaborative research between cytogenetic and molecular colleagues from the Wessex Regional Genetics Laboratory (WRGL), clinical colleagues from the Wessex Clinical Genetics Service (WCGS), and Dr. Jonathan Flint and Dr. Lyndal Kearney from the Institute of Molecular Medicine, Oxford.

Conventional cytogenetic analyses were carried out either by the author or by the clinical cytogeneticists working in the post-natal cytogenetic section of this laboratory. The fluorescence PCR based DNA studies presented in Chapters 3-5 were carried out by Dr. Simon Thomas at the WRGL. The clinical examinations of the majority of patients presented in Chapter 5 were carried out by Dr. Nick Dennis, Dr. Karen Temple, or Dr. Mandy Collins at the WCGS.

The initial probe preparation for *in situ* hybridisation, including growth, extraction, and labelling of the whole panel of telomere-specific probes was carried out by the author. Mrs. Mary Hart and Miss Rebecca Protheroe provided excellent technical support and maintained this library of labelled probes. All of the *in situ* experiments and subsequent analysis and interpretation of the results were carried out by the author. Advice on statistical analysis was provided by Mr Paul Strike from the Research and Development Support Unit, Salisbury.

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I am indebted to Professor Patricia Jacobs for her enthusiastic support and supervision. Without her encouragement and her 'bullying' in the early days of my career in genetics I very much doubt that I would have found the courage to venture from the relative safety of the diagnostic bench to undertake this research project.

This thesis would not have been possible without the excellent training I have received over the years from many of my colleagues at the Wessex Regional Genetics Laboratory, especially from Dr John Crolla. Furthermore, I wish to thank the many colleagues who have contributed to the work in this thesis, in particular Mary Hart, Rebecca Protheroe and Sarah Beal. They have provided not only excellent technical support but also a friendly working atmosphere. I would also like to thank my collaborators, Dr Lyndal Kearney, Dr Jonathan Flint and Regina Regan for the very generous donation of probes used in this study.

I am also enormously grateful to my friend and partner, Tim Waterman. Not only has he taught me how to cope with the intricacies of Word Template documents but he has been there throughout with support, encouragement, and most importantly a sense of perspective and humour. Thank you.

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ABBREVIATIONS USED IN THE TEXT

ASD	atrial septal defect
ATR-16	Alpha-thalassemia/mental retardation syndrome mapped to chromosome 16
BAC	bacterial artificial chromosome
CATCH 22	cardiac defect, abnormal facies, thymic hypoplasia, cleft palate, hypocalcaemia, 22q11 deletions
CCD	charged coupled device
CHD	congenital heart disease
CISS	chromosome *in situ* suppression hybridization
CNS	central nervous system
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate
dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate
EDTA	ethylene diamine tetra acetic acid
FdU	fluorodeoxyuridine
FISH	fluorecence *in situ* hybridization
FITC	fluorescein isothiocyanate
FSHD	facioscapulohumeral muscular dystrophy
G-bands	Giemsa bands
GTG	G-bands by trypsin using Giemsa
H1, H2 and H3	GC-rich isochore classes

Hb H mental retardation	form of alpha thalassemia where only one alpha globin chain is present and the beta chains form haemoglobin homotetramers
IMR	idiopathic mental retardation
IMS	industrial methylated spirit
IQ	intelligence quotient
ISCN	International System for Human Cytogenetic Nomenclature
IUGR	intra-uterine growth retardation
kb	kilobase
KCl	potassium chloride
L1 and L2	AT-rich isochore classes
LB	Lennox broth
LINES	long interspersed elements
Mb	megabase
MCA	multiple congenital anomalies
MDS	Miller-Dieker syndrome
M-FISH	multi-fluor fluorescence *in situ* hybridization
p	chromosome short arm
PAC	P1 artificial chromosome
PAR	pseudoautosomal region
PCR	polymerase chain reaction
PDA	patent ductus arteriosus
PEV	position effect variegation
PHA	phytohaemagglutinin
PND	prenatal diagnosis
q	chromosome long arm
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SINES	short interspersed elements
SMS	Smith-Magenis syndrome
STS	sequence tagged sites
SRY	sex determining region of the Y chromosome
TE	tris-ethylene diamine tetra acetic acid

TRITC	tetramethyl-rhodamine isothiocyanate
UV	ultraviolet
VNTRs	variable number tandem repeats
VSD	ventricular septal defect
WAGR	Wilms tumour, aniridia, genital anomalies, mental retardation
WCGS	Wessex Clinical Genetics Service
WHS	Wolf-Hirschhorn syndrome
WRGL	Wessex Regional Genetics Laboratory
YAC	yeast artificial chromosome

CHAPTER 1

INTRODUCTION

1.1 The Role of Chromosome Abnormalities in the Aetiology of Mental Retardation

Mental retardation is present in approximately 3% of the childhood population of the UK. The majority of these individuals have an IQ between 50-70 (mild mental retardation) while 10% have an IQ <50 (moderate and severe mental retardation). The cause of mental retardation can be established in less than half of cases. An important cause has been shown to be chromosomal rearrangements that result in segmental aneuploidy and alter the dosage of developmental genes. Chromosome abnormalities have been reported in up to 40% of individuals with severe mental retardation (Connor and Ferguson-Smith, 1985; Raynham *et al.,* 1996; Schaefer *et al.,* 1992) but in only 5-10% of patients with mild or moderate mental retardation (Hagberg *et al.,* 1981; Lamont *et al.,* 1986).

Conventional banded chromosome analysis at an ISCN 400-550 band level (ISCN, 1995) has been successful in detecting abnormalities in approximately 1% of unselected newborns, and of these about a third result in some degree of mental impairment (Jacobs *et al.,* 1992). The majority of abnormalities resulting in mental retardation are numerical (79%), *ie* there is an additional or missing chromosome. The remaining abnormalities are structural, either unbalanced (19%) or balanced (2%). Unbalanced structural chromosome rearrangements, where regions of chromosome are deleted or duplicated, are almost invariably associated with mental retardation and dysmorphic features. In contrast, carriers of balanced rearrangements, where there is no apparent loss or gain of genetic material, are usually clinically normal, although they are at risk of producing chromosomally unbalanced offspring.

There is, however, an increasing number of families with nonpathogenic euchromatic imbalances at the cytogenetic level (reviewed by Barber *et al.*, 1998; Gardner and Sutherland, 1996). Many of these are unique deletions, duplications or unbalanced translocations. Others, such as the euchromatic variants of the proximal long arm of chromosome 15 (Browne *et al.,* 1997; Jalal *et al.,* 1994), the distal short arm of chromosome 8 (Barber *et al.,* 1998; Joyce *et al.,* 1996), the proximal long arm of chromosome 9 (Macera *et al.,* 1995), the proximal short arm of chromosome 9 (Calabrese *et al.,* 1994), and the proximal short arm of chromosome 16 (Barber *et al.,* 1999; Bryke *et al.,* 1990) have been reported in multiple families. Although rare, these

cases highlight the importance of family studies and literature review before concluding that a specific cytogenetic abnormality is causative of a patient's phenotype.

Many individuals with mental retardation have a recognisable pattern of malformations suggestive of a specific syndrome and chromosome abnormality, for example Down syndrome caused by trisomy for chromosome 21 (Lejeune *et al.,* 1959). However, in the majority of patients no such pattern is present and the cytogenetic analysis of the whole genome is necessary. Amongst those cases where the cause of mental retardation has been identified, approximately two-thirds are due to chromosome abnormalities. It therefore seems likely that many unexplained cases are also chromosomal in origin but that the abnormalities are beneath the resolution of conventional cytogenetic techniques.

1.1.1 Detection of submicroscopic chromosome abnormalities using fluorescence *in situ* hybridization

Banded chromosome analysis can detect abnormalities greater than 2-4 megabases (Mb) of DNA. However, with the increased sensitivity and specificity of fluorescence *in situ* hybridization (FISH) techniques, smaller abnormalities can now be detected by hybridizing molecular genetic probes to metaphase chromosomes.

Non-isotopic *in situ* hybridization was first described by Manning *et al.* (1975). The probes were labeled with biotin and sites of hybridization detected with avidin-coated microspheres using electron microscopy. In the late 1970's the use of fluorescent detection reagents was introduced (Bauman *et al.,* 1980; Rudkin and Stollar, 1977) resulting in increased ease of use and spatial resolution. Originally only repetitive sequences, such as centromeric alphoid DNA sequences, could be visualized. The introduction of 'chromosome *in situ* suppression hybridization' (CISS) (Jauch *et al.,* 1990; Hulten *et al.,* 1991), where repetitive sequences which are interspersed between the unique sequences within each probe are competed out, has enabled the detection of single-copy sequences using probes of only 1-2 kilobases (kb) (Lawrence *et al.,* 1990; Lichter *et al.,* 1990; Trask *et al.,* 1989) and entire chromosomes using forward and reverse chromosome painting probes (Carter *et al.,* 1992; Cremer *et al.,* 1988; Pinkel *et al.,* 1988). This advance has dramatically expanded the applications of FISH.

The FISH technique is both powerful and simple. DNA sequences are first labelled with reporter molecules and broken into 200-400 bp fragments. Although probes may be directly conjugated with fluorescent molecules, the most widespread approach is to use nick translation to incorporate reporter molecules such as biotin or digoxigenin as labelled nucleotides and, after hybridization, detect the reporter molecules with immunofluorescent reagents. Both the probe and target chromosomes are denatured then complementary sequences in the probe and target are allowed to reanneal. After stringent washing and detection, a discrete fluorescent signal is visible at the site of probe hybridization. A variety of probe-labelling schemes are available for simultaneous detection, in different colours, of two or more sequences in the same nucleus or metaphase.

Since its introduction FISH has rapidly become indispensable in clinical cytogenetics. In particular there are many published reports documenting the diagnostic value of FISH in the characterisation of chromosome abnormalities which cannot be resolved by conventional cytogenetic methods and importantly in the detection of submicroscopic abnormalities. In recent years it has become clear that an increasing number of genetic conditions, such as WAGR (Wilms tumour, Aniridia, Genital anomalies, mental Retardation) (Fantes *et al.,* 1992), Williams syndrome (Ewart *et al.,* 1993) and the CATCH-22 syndromes (Cardiac defect, Abnormal facies, Thymic hypoplasia, Cleft palate, Hypocalcaemia, 22q11 deletions) (Scambler *et al.,* 1991), are caused by chromosomal imbalances which are beneath the resolution of conventional cytogenetic analysis but can be readily detected using FISH.

Many of these submicroscopic imbalances involve deletion of so called 'haploinsufficiency' genes - where two copies are required for a normal phenotype. In all of these syndromes the specific pattern of malformations in the patients will direct the investigations to a particular region of the genome. However, for patients with idiopathic mental retardation (IMR) but without a distinctive pattern of dysmorphic features the search for an underlying genetic cause remains a challenge. Practical tools for screening the whole genome for imbalances are not yet available. Instead efforts are being focused on identifying rearrangements of the terminal (telomeric) regions of chromosomes.

1.1.2 Terminal rearrangements resulting in mental retardation

The majority of unbalanced structural rearrangements result in deletion (partial monosomy) or duplication (partial trisomy) of terminal regions of chromosomes. These mostly arise due to malsegregation at meiosis of either a familial or *de novo* reciprocal translocation to give a derivative deletion/duplication chromosome. Terminal (single breakpoint) deletions are apparently rare, although telomere healing can occur, stabilizing the broken chromosome end (Flint *et al.,* 1994; Lamb *et al.,* 1993; Wilkie *et al.,* 1990a; Wong *et al.,* 1997). The extent and content of the genetic imbalance in these terminal partial aneuploidy cases are generally unique making phenotype/genotype correlations difficult.

However, some terminal deletions are associated with well-defined mental retardation/multiple congenital anomaly syndromes. These include Wolf-Hirschhorn syndrome (WHS) caused by a deletion of the region 4p16.3 (Estabrooks *et al.,* 1994; Tommerup *et al.,* 1993; Wolf *et al.,* 1965) and cri-du-chat syndrome caused by deletion of 5p15 (Lejeune, 1963; Overhauser *et al.,* 1994). Deletions of the terminal band 16p13.3 have been associated with two independent disorders, Rubinstein-Taybi syndrome (Rubenstein and Taybi, 1963; Breuning *et al.,* 1993; Wallerstein *et al.,* 1997) and alpha-thalassemia/mental retardation syndrome (ATR-16) (Wilkie *et al.,*1990b). Miller-Dieker syndrome (MDS), first described by Miller in 1963 and later by Dieker *et al.* (1969), is a contiguous gene disorder caused by monosomy for the region 17p13.3 which contains the lissencephaly gene LIS1 (Dobyns *et al.,* 1993; Ledbetter *et al.*, 1989; Mantel *et al.,* 1994; Pilz *et al.,* 1995). Terminal deletions of both the short arm and long arm of chromosome 18 are also associated with well-defined clinical phenotypes. De Grouchy *et al* first described 18p monosomy in 1963 and 18q partial monosomy, 'carp-mouth syndrome' in 1964. Preliminary correlations between phenotype and 18q deletion size have been made by Kline *et al.* (1993). In all of these syndromes both cytogenetically detectable and more subtle deletions requiring detection with critical region FISH probes occur.

In addition to these well defined, classic syndromes, high resolution G-banded analysis and FISH techniques have led to the delineation of other subtelomeric monosomy 'syndromes'. Watt *et al.* (1985) were the first to propose the existence of a monosomy 22q syndrome. Since their initial description, a total of 24 cases of 22q13.3

monosomy have been reported. Of these, two were the products of gross cytogenetic rearrangements (Romain *et al.,* 1990; Watt *et al.,* 1985), 11 were detected using conventional cytogenetics (Herman *et al.,* 1988; Narahara *et al.,* 1992; Nesslinger *et al.,* 1994; Phelan *et al.,* 1992; Yong *et al.,* 1997; Zwaigenbaum *et al.,* 1990), three were subtle suspected deletions confirmed by FISH (Doheny *et al.,* 1997; Praphanphoj *et al.,* 2000), and six were submicroscopic deletions (Anderlid *et al.,* 1999; Flint *et al.,* 1995; Knight *et al.,* 1999; Smith *et al.,* 1998; Vorsanova *et al.,* 2000). Two further deletions were detected serendipitously by Precht *et al.* (1998) whilst using a distal 22q FISH probe as an internal control. Similarly 41 distal deletions of chromosome 1p, including submicroscopic deletions, have been reported (Blennow *et al.,* 1996; Faivre *et al.,* 1999; Giraudeau *et al.,* 1997; Kearney *et al.,* 1998; Knight *et al.,* 1999; Reish *et al.,* 1995; Riegel *et al.,* 1999; Shapira *et al.,* 1997; Slavotinek *et al.,* 1999a; Slavotinek *et al.,* 1999). These reports have also led to the delineation of a syndrome.

In summary, the role of terminal deletions in the classic haploinsufficiency syndromes, WHS, cri-du-chat, ATR and MDS, is well established. High-resolution G-banding, FISH and molecular techniques have led to identification of the critical regions for these disorders and this in turn has enabled further delineation of the associated phenotypes. These modern cytogenetic and molecular techniques have also identified further syndromes caused by deletions in the telomeric regions of other human chromosomes. It therefore seems likely that cryptic imbalances of these and other telomeric regions underlie many further cases of IMR.

1.2 Telomeres

Telomeres are the specialised protein/DNA complexes that cap the ends of linear eukaryotic chromosomes. Telomeres ensure the complete replication of chromosome ends during the process of discontinuous strand synthesis and prevent end to end fusions and exonucleolytic degradation (Blackburn, 1990 and 1991; Zakian, 1989). They also play an important role in chromosome movement and pairing during meiosis (Chikashige *et al.,* 1994).

1.2.1 Telomere structure

In most eukaryotes the chromosome ends terminate in tandemly repeated simple G-rich sequences. Moyzis *et a.* (1988) identified and characterised a human telomere consensus sequence, (TTAGGG)*n,* and suggested that this sequence is a functional human telomere. It was found to be tandemly repeated (between 3 and 20 kb) at the ends of all human chromosomes (Allshire *et al.*, 1989) and showed strong evolutionary conservation among vertebrate species (Meyne *et* al., 1989).

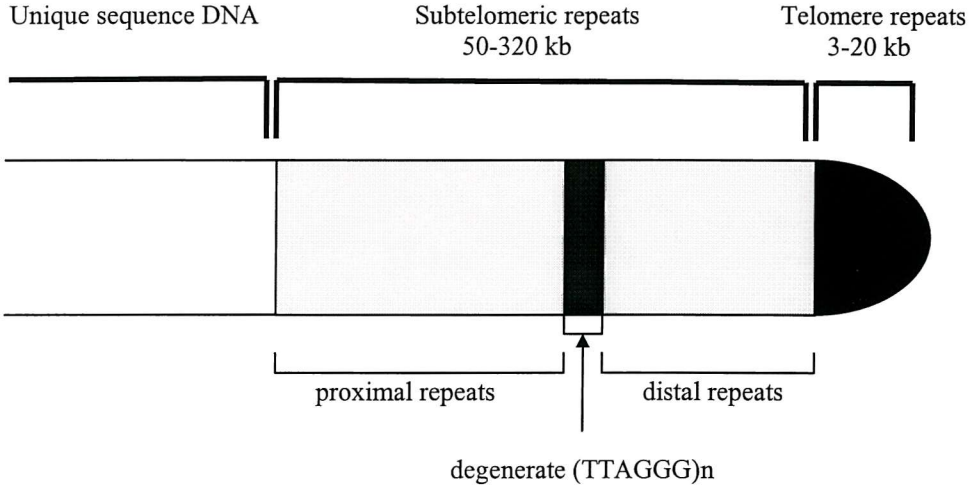

Figure I.1. Schematic diagram showing the organization of the telomeric regions of human chromosomes.
The distal subtelomeric repeats include short (<2 kb) sequences shared by many non-homologous chromosomes. The proximal subtelomeric repeats contain longer (~10-40 kb) sequences shared by fewer chromosomes.

Immediately proximal to this simple repeat, most chromosomes have additional complex subtelomeric repeat sequences (also called telomere associated repeats - TAR). Subtelomeric sequences include SINES (*Alu* elements), LINES (*Kpn* family elements), short tandemly reiterated repeats, CA repeats and more complex families of moderately repeated sequences (Flint *et al.*, 1996). Although some of these repeats appear to be unique (for example 7q and the pseudoautosomal region (PAR) of Xp and Yp), most have shared homology between different subsets of human telomeres (Brown *et al.*, 1990; Cross *et al.*, 1990). One such sequence is TelBam3.4 , a 3.4 kb *Bam*HI fragment immediately adjacent to the (TTAGGG)*n* repeat, which was shown by Brown *et al.* (1990) to be shared by a number of non-homologous subtelomeric regions. Other

examples of subtelomeric homology include sequences within the most telomeric 60 kb of chromosome 4p which are homologous to telomeric regions on 13p, 15p, 21p and 22p (Youngman *et al.,* 1992) and a 20p telomere-associated DNA which shares sequences with the subtelomeric regions of chromosomes 4q and 18p (Chute *et al.,* 1997).

Subtelomeric repeats consist of two sub-domains (Flint *et al.,* 1997a). The distal sub-domain contains a mosaic of very short (<2 kb) sequences which are shared by many chromosomes, whereas the proximal sub-domain contains longer (~10-40 kb) sequences which are shared by fewer chromosomes. The two sub-domains are separated by a block of degenerate (TTAGGG)*n* repeats and sequences with homology to putative origins of replication. This same organization is also observed in yeast telomeres. Such conservation suggests an important functional role for the organization of telomere repeats.

In addition to sequence homology, subtelomeric repeats are polymorphic in both their length, ranging in size from 50 to 320 kb, and position, occurring in different combinations of telomere ends in different individuals (Brown *et al.,* 1990). The human 16p telomeric region has been analysed in the most detail and has been shown to contain a subtelomeric length polymorphism (Wilkie *et al.*, 1991). At least three 'telomere alleles' exist in the population in which the alpha-globin genes (α1 and α2) lie 170 kb, 350 kb or 430 kb from the telomere. The two most common alleles, allele A (170 kb) and allele B (350 kb), share no sequence homology with each other. However, allele A is clearly related to the subtelomeric regions on Xq and Yq, and allele B is related to the subtelomeric regions of 9q, 10p and 18p. Three different length alleles have also been described at 16q (Harris and Thomas, 1992). Furthermore, Macina *et al.* (1994) have shown that a telomere length polymorphism of 55 kb also exists on 2q and a DNA segment containing two CpG islands 100 kb from the 2q telomere is duplicated at distal 8p23.

Polymorphism in subtelomeric regions is thought to arise via two pathways (Flint *et al.*, 1996). Intrachromosomal events are believed to be responsible for structural alterations, such as length polymorphisms, within individual subtelomeric regions whereas interchromosomal rearrangements, mediated by regions of shared homology, transfer subtelomeric sequences between chromosomes.

Unique, chromosome specific DNA for each telomere is located proximal to the subtelomeric repeats, approximately 100-300 kb from the end of each chromosome (National Institutes of Health *et al.*, 1996). This region not only contains the most distal genes for each chromosome arm but is also the most gene-rich region of the genome. The human genome is made up of long DNA segments (>300 kb), the isochores, which are compositionally homogeneous and can be fractionated on caesium sulphite gradients. Fractions have been grouped into five isochore classes, the AT-rich L1 and L2 classes, and the increasingly GC-rich H1, H2 and H3 classes (Saccone *et al.,* 1993). In general, G-positive bands are AT-rich and G-negative bands GC-rich. The H3 isochore family has by far the highest gene and CpG island concentration, and the highest transcriptional and recombinational activity. Saccone *et al.* (1992) have shown that the H3 isochore family is predominantly localized to the telomeric (T-band) regions of human chromosomes.

The H3 isochores represent approximately 3% of the genome but are believed to contain over a quarter of all genes (Gardiner, 1996). Despite the recent release of the draft human genome sequence the number of genes in the human genome is unknown. Published estimates range from 30,000 (Ewing and Green, 2000; Crollius *et al.,* 2000) to 120,000 (Liang *et al.,* 2000). Assuming the recent figure of 29,472 genes published by the Sanger Centre to be the most accurate (http://www.ensembl.org, 09-10-2000) and a human genome length of $3x10^9$ bp, on average 1 Mb of DNA would contain approximately 10 genes. The gene density of the H3 isochores is expected to be in the region of one gene per 12 kb, compared to one gene per 120 kb in AT-rich regions. This high gene density has subsequently been confirmed by Flint *et al.* (1997b) who, whilst sequencing the distal 284 kb of the short arm of chromosome 16, identified ~1 functional gene per 20 kb within the most distal unique sequence DNA. The first functional gene was found only ~8 kb from the subtelomeric region and ~44 kb from the telomeric repeats. Imbalances of these telomeric regions would therefore be expected to have disproportionately greater clinical consequences than similar interstitial imbalances. However the H3 isochores do not appear to be evenly distributed among telomeres. Some telomeric regions such as 1p, 4p, 16q and 19p have a higher concentration of H3 isochores than others such as 3q, 4q, 6p and 15q. Other human telomeres, namely 3p and 19q, appear to contain no H3 isochores. These regions are G-band positive and would therefore be expected to be AT-rich and gene-

poor. It is reasonable to assume that the clinical significance of a telomeric imbalance would be related to the H3 isochore concentration of the given telomere.

1.2.2 Role of Telomeres

The primary role of telomeres is believed to be ensuring the complete replication of chromosome ends. While DNA polmerases are capable of replicating double stranded DNA they are unable to replicate the 3' tail which is present at the ends of all eukaryote chromosomes. The process of telomere replication is instead carried out by the ribonucleoprotein telomerase which contains essential RNA and protein subunits. The reverse transcriptase subunit of telomerase catalyzes the addition of a species-specific telomere repeat by copying part of its intrinsic RNA to make one strand of the telomeric repeat DNA, thus restoring the 3' tail. Telomerase was first described in the ciliated protozoan, *Tetrahymena* (Greider and Blackburn, 1987; Greider and Blackburn, 1989) but has subsequently been identified in the human cell line, HeLa (Morin, 1989).

Recent work has led to the concept of a telomere homeostasis system (reviewed by Lingner and Cech, 1998). Whilst telomerase activity is detected in fetal ovaries, fetal and adult testes, the majority of tumour cells and immortal cell lines, it is undetectable in most somatic cells. Consequently telomere repeats shorten with age in somatic cells. Harley and Villeponteau (1995) proposed that this shortening, associated with senescence, acts as a molecular clock to prevent oncogenesis. The reactivation of telomerase in most tumours suggests that telomere elongation might be an important, if not essential, step in tumour formation (de Lange, 1994). However, in tumour cells the telomere repeat tract length does not increase exponentially but is instead stabilized.

Several proteins that bind duplex telomeric DNA repeats have been identified, including the human proteins TTAGGG Repeat Factor 1 (TRF1) (Bilaud *et al.,* 1996; Broccoli *et al.,* 1997a; Chong *et al.,* 1995; Zhong *et al.,* 1992) and TRF2 (Bilaud *et al.,* 1997; Broccoli *et al.,* 1997b). These proteins play a key role in the protective activity of telomeres, preventing exonucleolytic degradation and end to end chromosome fusions (van Steensel *et al.,* 1998). In addition they are believed to negatively regulate the activity of telomerase to limit the elongation of individual chromosome ends (Marcand *et al.,* 1997). Marcand *et al.* (1997) demonstrated a protein-counting mechanism for the

regulation of telomere length in yeast. They suggested that in the case of *S. cerevisiae* the binding of ~15 Rap1p proteins alters the shape of the telomere so that telomerase can no longer bind. The telomere repeat tract then shortens due to a combination of incomplete replication, nucleolytic degradation and recombination. When the number of bound Rap1p molecules falls to below 15 the telomerase complex can bind and elongate the telomere again. The structural and functional properties of telomeres appear to be highly conserved, and a similar feedback mechanism has been proposed for length regulation of human telomeres (Griffith *et al.,* 1999; van Steensel and de Lange, 1997).

It is, however, the role of telomeres in homologous chromosome pairing at meiosis which is of most relevance to this thesis. Telomeres are believed to be the first regions of the chromosome to pair (Zakian, 1995). Studies in a variety of species have demonstrated highly conserved telomere behaviour in the early stages of meiosis. Chikashige *et al.* (1994) demonstrated the role of yeast telomeres in premeiotic chromosome movement; Bass *et al.* (1997) have shown that maize telomeres cluster at the nuclear envelope prior to the initiation of synapsis; Scherthan *et al.* (1996) reported that in both mouse and man telomeric movement to the nuclear periphery during early meiotic prophase is associated with the onset of chromosome pairing.

This clustering of telomeres is consistent with the process of homology searching and pairing being initiated at the telomeres. Barlow and Hultén (1996) have shown with combined immunocytogenetic and molecular cytogenetic analysis of meiosis I human spermatocytes that the telomeric regions are the first regions to pair at the onset of meiosis. Further evidence to support this theory has been provided by the many reports of increased recombination for both sexes at human telomeres (Laurie and Hultén., 1985; reviewed by Lese and Ledbetter, 1998). The telomeric array and/or subtelomeric repeats may be responsible for this increase in recombination. For most regions of the human genome, female recombination rates are generally higher than male recombination rates (Ashley, 1994). At telomeres this pattern is apparently reversed with significantly higher male recombination rates (Blouin *et al.,* 1995; Donis-Keller *et al.,* 1987; Rouyer *et al.,* 1990). The only known exception to date is the telomeric region of human 14q, where female recombination is more frequent than male recombination (Wintle *et al.,* 1997). The biological basis for meiotic recombinational differences between males and females is unknown. It has been suggested that the actual recombination mechanisms differ

between the sexes. (Ashley, 1994). It may also differ between the telomeres and the rest of the genome where meiotic exchange is more evenly dispersed.

1.2.3 The role of telomeres in unequal exchange and gene dosage imbalance.

It is these unique aspects of telomere structure, shared homology, polymorphism and increased recombination rates detailed above which suggest that telomeres may play a leading role in mediating non-homologous chromosome pairing and unequal cross-over events, which could lead to gene dosage imbalance.

Homologous recombination at meiosis is necessary for the formation of chiasmata which ensure proper chromosome segregation and increases genetic diversity in gametes. However, illegitimate recombination between non-homologous chromosomes rearrangements is also of evolutionary significance. The telomeric regions of primate chromosomes have undergone rapid changes over relatively short periods of time (Royle *et al.,* 1994) and duplications including many non-functional pseudogenes (Kermouni *et al.,* 1995; Rouquier *et al.,* 1998) are frequent and widespread among the subtelomeric regions. As suggested by Trask *et al.* (1998), inter-chromosomal telomeric exchanges of large DNA sequence blocks suggests that the subtelomeric zones may serve as an evolutionary test-bed.

In addition, non-homologous recombination at the telomeres appears to play an important role in stabilizing chromosomes which have lost telomeres and subtelomere repeats (Flint *et al.,* 1996). Two mechanisms exist to rescue such broken chromosomes. The first, telomere healing, involves the direct addition of simple telomeric repeats to the broken end (Flint *et al.,* 1994; Lamb *et al.,* 1993; Wilkie *et al.,* 1990a; Wong *et al.,* 1997). The second, telomere capture, involves the acquisition of a pre-existing telomere by recombination between shared repetitive elements (Meltzer *et al.*, 1993; Flint *et al.,* 1996). Internal (TTAGGG)*n* repeat arrays, as described by Chute *et al.* (1997) on human 20p and by Louis *et al.* (1994) in yeast, may act as a pool of telomeric repeats for the rescue of broken telomeres.

However, such a high degree of homology at telomeres which provides opportunity for mispairing and exchange events has obvious disadvantages. Firstly, unequal exchange between subtelomeric repeats may result in a change in the structure of

the subtelomere which in turn could affect the expression of genes nearby by 'position effect variegation' (PEV) (Kleinjan and van Heyningen, 1998). Such a mechanism has been proposed in the condition facioscapulohumeral muscular dystrophy (FSHD). Both inter- and intrachromosomal subtelomeric rearrangements have been associated with FSHD. A variable length 3.3 kb *Kpn*I tandem-repeat unit (D4Z4) at human chromosome 4q35, adjacent to subtelomeric sequences, is duplicated on the telomere of 10q (Bakker *et al.*,1995; Deidda *et al.*, 1995). The 4q35 and 10qter sequences share 98-100% homology (Cacurri *et al.*, 1998) and a subtelomeric exchange of the 3.3 kb repeat units between 4q35 and 10qter occurs in at least 20% of the population (van Deutekom *et al.*, 1996). FSHD is caused by a deletion of an integral number of these 3.3 kb repeat units, irrespective of the origin of the repeated units (van Deutekom *et al.*, 1993 and 1996; Wijmenja *et al.*, 1992). A *de novo* 4q;10q interchromosomal exchange generating a short 4q35 *Kpn* repeat fragment as well as a 4q intrachromosomal *Kpn* deletion will cause FSHD (van Deutekom *et al.*, 1996; Lemmers *et al.*, 1998). This truncation of the subtelomeric region is believed to affect the expression of the as yet unidentified critical gene in distal 4q by PEV.

Secondly, telomere associations between non-homologous chromosomes that share common subtelomeric repeats may occasionally result in translocation of unique sequence DNA adjacent to the subtelomere repeats. Indeed, unequal recombination leading to gene dosage imbalance has been observed at both the short arm and long arm telomeric PARs of the human sex chromosomes. Unequal cross-over events between highly homologous loci resulting in translocation of the SRY (sex determining region of the Y chromosome) gene to Xp has been shown to account for 80% of XX males and some XY females (Levilliers *et al.*, 1989; Rouyer *et al.*, 1987; Weil *et al.*, 1994). Similar unequal exchange between the Xq PAR and Yq heterochromatic region giving rise to a Yq- chromosome that contains 5-10 Mb of Xqter has been reported in three unrelated boys with severe mental retardation, generalized hypotonia and microcephaly (Lahn *et al.*, 1994).

In summary the high rate of meiotic recombination at telomeres and the existence of polymorphic telomere alleles could predispose to translocations and unequal cross-over events. Non-homologous telomeric translocations and telomere healing/capture may result in truncated telomeres which could influence expression of adjacent genes or result in gene dosage imbalance. The unique sequence DNA adjacent to the subtelomere

repeats has the highest gene density in the human genome. Genetic imbalances in these regions would therefore be expected to have disproportionately greater clinical significance. Collectively, these aspects of telomere structure and function have made these regions targets for intensive mapping and cloning efforts in the search for genes involved in idiopathic mental retardation.

1.3 Cryptic Telomeric Imbalances In Idiopathic Mental Retardation

1.3.1 Cryptic and semi-cryptic telomeric rearrangements

The telomeric regions of human chromosomes pose a major challenge in clinical cytogenetics. Translocations and deletions of these gene rich regions of the genome are particularly difficult to detect by conventional banding methods because most of the terminal bands are G-negative and morphologically similar.

When discussing cryptic telomeric rearrangements it is important to distinguish between those which are fully cryptic, where no abnormality is detected using conventional cytogenetic techniques, and those where one of the two reciprocal products can be identified by G-banding but the other is below cytogenetic resolution, termed 'semi-cryptic' (Kuwano *et al.,* 1991).

Cryptic deletions detected by either FISH or molecular techniques have been reported in WHS, cri-du-chat, Rubenstein-Taybi, ATR and MDS as well as in cases of 1p and 22q monosomy, detailed in section 1.1.2. In addition, both cryptic and semi-cryptic telomeric translocations have been identified. Unbalanced maternal semi-cryptic translocations have been reported in patients with WHS (Altherr *et al.,* 1991) and MDS (Kuwano *et al.,* 1991), 4p;19p and 3q;17p respectively. The characterisation of these two 'deletions' and the identification of parental semi-cryptic translocations has obvious implications for the recurrence risk in these families. A 'simple' *de novo* deletion would confer no increased recurrence risk whereas when shown to be familial the risk of a further child with an unbalanced telomeric rearrangement would be as great as 50%.

Fully cryptic familial telomeric rearrangements have been described in cri-du-chat, ATR and MDS. One of the first cryptic translocations was described by Lamb *et al.* (1989). Although the proband's karyotype was reported as normal, Lamb *et al.* were directed to investigate the alpha-globin loci on 16p because of his haemoblobin H (Hb H)

disease, mental retardation and dysmorphic features. A maternal deletion in the alpha-globin region was identified. Subsequent FISH analysis showed the mother carried a cryptic 1p;16p translocation. The proband's sister, who also has mental retardation but normal haematological findings, was shown to have inherited the reciprocal unbalanced form of this translocation. Similarly, directed referrals have led to the detection of a cryptic 4q;5p telomeric translocation in a large family with multiple affected individuals with cri-du-chat syndrome (Overhauser *et al.,* 1989), a cryptic 8q;17p telomeric translocation in the father of a patient with MDS (Kuwano *et al.,* 1991), a maternal cryptic 10q;17p translocation in a further case of MDS (Masuno *et al.,* 1995), and a cryptic 4p;10q translocation in the mother of a patient with WHS (Goodship *et al.,* 1992).

Other cryptic telomeric rearrangements have been detected in individuals with mental retardation but less distinctive clinical phenotypes. These include a duplication of the terminal segment of 22q (Biesecker *et al.,* 1995), familial cryptic translocations between 18q and 21q (Bartsch *et al.,* 1997) and between 5q and 6q (Groen *et al.,* 1998), and an unbalanced translocation between 2p and 18p (Horsley *et al.,* 1998).

1.3.2 Screening for cryptic telomeric rearrangements

The reports of cryptic and semi-cryptic telomeric rearrangements in patients with WHS, cri-du-chat, ATR, 18p monosomy and MDS suggest that karyotypically normal patients presenting with features of any of these syndromes should be investigated further. In addition simple 'terminal' deletions may be semi-cryptic unbalanced translocations. Identification of a familial translocation would have important implications for genetic counselling and recurrence risk estimation. However, in light of these reported cases and the high meiotic recombination rates of the telomeric regions, it is suspected that cryptic telomeric imbalances may be responsible for other cases of mental retardation. A screen of a population of IMR patients for such rearrangements has therefore been proposed (Flint *et al.,* 1995; Ledbetter, 1992; Lese and Ledbetter, 1998; National Institutes of Health *et al.,* 1996; Wilkie, 1993).

Two approaches for screening an IMR population have been evoked. The use of hypervariable DNA polymorphisms (also called variable number tandem repeats,

VNTRs) to detect subtelomeric imbalances was first proposed by Wilkie (1993). VNTR loci are concentrated at the terminal bands of human chromosomes (Royle *et al.,* 1988). Telomeric imbalances are detected by the observation of abnormal inheritance of parental alleles. Flint *et al.* (1995) utilized this approach to screen 99 patients with IMR. Using highly polymorphic probes from the subtelomeric regions of 28 chromosome ends they identified three cases of monosomy, two cases of 22q partial monosomy and one case of 13q partial monosomy. After correcting for the use of probes for only 28 telomeres, the authors calculated that at least 6% of IMR cases may be caused by cryptic telomeric rearrangements. In a more recent study, Slavotinek *et al.* (1999) screened 27 highly selected patients with IMR and a minimum of three dysmorphic features using microsatellite markers from the subtelomeric regions of all chromosome arms. They identified two deletions giving a frequency of 7.5% and an estimated frequency of 18% after adjusting for marker informativeness. This approach has the disadvantage of being unable to identify balanced rearrangements such as parental translocations or inversions which may have given rise to the imbalance detected in a proband. In addition samples from both parents are required to undertake investigation of a proband, and the VNTR or microsatellite polymorphisms used must be informative in the family.

A second approach is to use FISH probes for each telomere (Ledbetter, 1992). To be informative each probe must be unique to a single telomere. The National Institutes of Health and Institute of Molecular Medicine Collaboration (1996) have generated such a panel of telomeric probes.

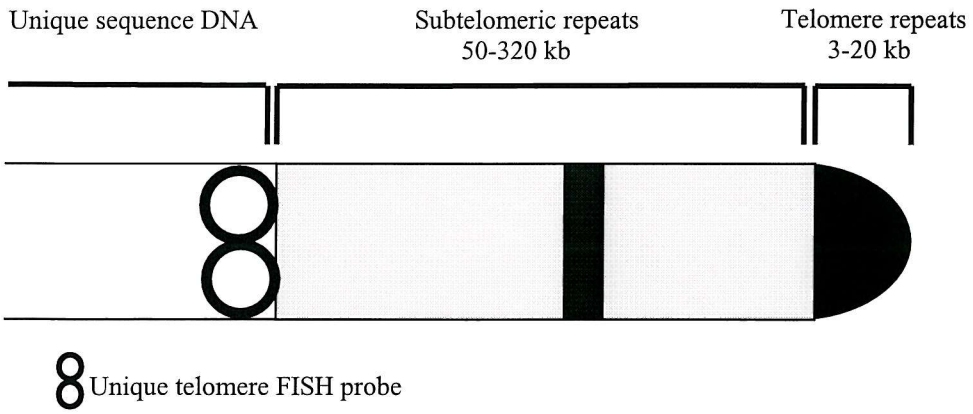

Figure I.2 Schematic diagram illustrating the position of the telomeric FISH probes in relation to the structure of human telomeres.

The total number of probes in the set is 41, rather than 48, because the X and Y chromosomes share homologous sequences at both their short and long arm PARs and unique sequence telomeric probes for the acrocentric short arms have not been cloned. These probes, generated from half-YACs containing human telomeric fragments (Riethman *et al.,* 1989), map to the most distal unique sequence DNA adjacent to the subtelomeric repeats, 100-300 kb from the chromosome end. Seven of the probes, isolated using the most distal known marker, are at an unknown distance from the chromosome end.

The advantages of a FISH approach include the ability to screen a proband without the need for parental samples and the ability to detect balanced parental rearrangements. However, even allowing for dual colour FISH, where two probes labelled with different haptens or fluorophores are hybridized together, the time involved in analysing probes from each chromosome for a deletion or duplication event would make this strategy unfeasible.

A number of techniques have recently been developed to enable the simultaneous detection of multiple chromosomes including combinatorial multi-fluor FISH (Speicher *et al.,* 1996; Schrock *et al.,* 1996) and ratio-labelling strategies (Dauwerse *et al.,* 1992). These methods can be time consuming and require expensive image capturing equipment and M-FISH software for the analysis and interpretation of results. In 1994, Larin *et al.* (1994) reported a method for the analysis of multiple independent hybridization reactions on a single slide, and more recently, Knight *et al.* (1997) utilized the principle of this technique to develop a multiprobe device for the independent detection of the majority of subtelomeric chromosome regions. It is this technique together with the panel of telomeric probes (National Institutes of Health *et al.,* 1996) that was used in this project for the identification of cryptic telomeric rearrangements.

1.4 Aims Of Research

Several groups are undertaking screening programmes to detect cryptic telomeric rearrangements in populations of individuals with IMR and data from six of these studies have been published. On the basis of these results it has been postulated that such cryptic rearrangements represent a significant cause of IMR. However, to date all IMR populations investigated, with the exception of a population of community-based referrals reported by Knight *et al.* (1999), have been highly selected.

Importantly, there is currently very little information concerning the frequency of cryptic telomeric rearrangements in the normal population. In order to rule out polymorphisms, most of the telomeric FISH probes have been tested on 5-10 unrelated normal individuals (National Institutes of Health *et al.,* 1996) and Flint *et al.* (1995) have excluded cryptic telomeric deletions of chromosomes 13q and 22q in 160 and 186 control families respectively. The only systematic screen for telomeric rearrangements in a control population has recently been reported by Knight *et al.* (1999). As discussed in section 1.1.1, whilst the majority of chromosomal imbalances identified to date have been associated with mental retardation and dysmorphic features, there are reports of euchromatic imbalances in individuals with normal phenotypes. Although the telomeres of human chromosomes are known to be gene rich regions of the genome, the relatively small nature of the imbalances detected by screening programmes and the fact that these abnormalities may not have been previously described will necessitate caution when making phenotype/genotype correlations. It is therefore vital that in addition to screening a population of patients with IMR, the frequency of cryptic telomeric abnormalities in a control population is established.

The literature has led some to propose that screening for cryptic telomeric abnormalities should be considered in all cases of IMR. The intention of our study is to investigate whether this premise is justifiable by screening an unselected population of IMR patients and an unbiased population of control individuals.

Specifically the aims of this research project were:

1. To develop a panel of efficient, labelled telomeric FISH probes from the set of probes cloned into cosmids, plasmid artificial chromosomes and bacterial artificial

chromosomes generously donated by our collaborators, Lyndal Kearney and Jonathan Flint (Institute of Molecular Medicine, UK).

2. Using the multiprobe FISH approach (Knight *et al.,* 1997), to determine the frequency and significance of telomeric rearrangements by screening two populations of individuals with IMR (unselected and selected) as well as a population of control individuals.

3. To investigate whether a specific sub-set of patients with IMR are more likely to have an underlying telomeric imbalance and whether some telomeres are 'hot-spots' for deletion or translocation events.

4. To investigate the origin of telomeric rearrangements by screening subtle terminal abnormalities for cryptic telomeric imbalances.

CHAPTER 2

METHODOLOGY

2.1 Introduction

For all cases investigated the numbering system used in this thesis is based on the chronological recruitment of individuals to the study. Patients within the IMR study population were given the prefix 'P', individuals within the control population the prefix 'C', and cases where a terminal structural chromosome rearrangement was detected using conventional cytogenetic analysis the prefix 'S'.

2.2 Conventional Cytogenetic Methods

2.2.1 Blood Culture

Peripheral blood samples were received in lithium heparin, an anti-coagulating agent. Blood was cultured for conventional cytogenetic and *in situ* hybridization analysis using a PHA-stimulated, FdU-synchronized lymphocyte culture regime adapted from the method published by Webber and Garson (1983). All procedures were carried out in a Class II laminar flow safety cabinet. Peripheral blood (0.3 ml) was added to 5 ml of complete RPMI medium in a sterile 10 ml culture tube. After 48 hours incubation at 37 °C, the cultures were arrested in S-phase by the addition of 0.05 ml of FdU ($1x10^{-5}$ M). FdU is an antagonist of thymidilate synthetase which is necessary for the conversion of dUMP to dTMP. Blocking this reaction prevents dTTP being produced which in turn inhibits DNA synthesis. Uridine (0.05 ml at $4x10^{-4}$ M) was also added to enable RNA synthesis to continue. After a further 17 hours incubation at 37 °C, the FdU block was released by the addition of thymidine at physiological levels (0.1 ml of a 0.125 mg/ml working solution). The cultures were incubated at 37 °C for a further 4.5 hours. Colchicine (0.1 ml of a 40 μg/ml working solution) was added for the final 15 minutes of incubation. Colchicine binds to tubulin dimers preventing polymerization and thereby inhibiting the spindle dependent alignment of chromosomes on the metaphase plate. The effect of adding colchicine is to arrest the cultured lymphocytes in mitosis. The cultures were then centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 5 ml of hypotonic solution (1% KCl). The cultures were centifuged at 1200 rpm for 5 minutes and the supernatant discarded. The cell pellet was then resuspended in 5 ml of fresh 3:1 methanol:glacial acetic acid fixative. Centrifugation and fixing was repeated twice. The cell suspension was stored at –20 °C until required for slide preparation.

2.2.2 GTG-Banding And Cytogenetic Analysis

The cell suspensions were centifuged for 5 minutes at 1200 rpm and the supernatant discarded to leave a cell pellet in approximately 1.5 ml of fixative. Metaphase spreads were prepared for conventional cytogenetic analysis by applying one drop of cell suspension onto a cleaned microscope slide. As the cell suspension spread over the slide 'Newton rings' appeared. At this point a drop of fresh fixative was applied and the slide allowed to air dry. The cell and metaphase density was observed using phase-contrast microscopy and the cell suspension concentration adjusted accordingly. Slides were 'aged' by UV transillumination for 50 seconds prior to banding. GTG banding was adapted from the method published by Seabright (1971). Slides were pretreated with a hydrogen peroxide solution for 1-2 minutes then rinsed in isotonic saline. Slides were dipped in a 0.25% trypsin solution for 5-10 seconds then immediately rinsed and stained with Leishman stain (diluted 1:2 with Sorenson's buffer pH 6.8). The slides were stained for approximately 1 minute then blotted and left to air dry. A test slide was examined and timings for subsequent slides were adjusted accordingly. Slides were mounted in histamount under a 1.5 mm thick coverslip and examined under high power (x1200 magnification). Analysis was carried out at to a minimum ISCN 550 band level (figure II.1). In general, for each case four cells were analysed and a further six cells counted and sexed.

Figure II.1 Ideogram adapted from the International System for Human Cytogenetic Nomenclature (ISCN 1995).

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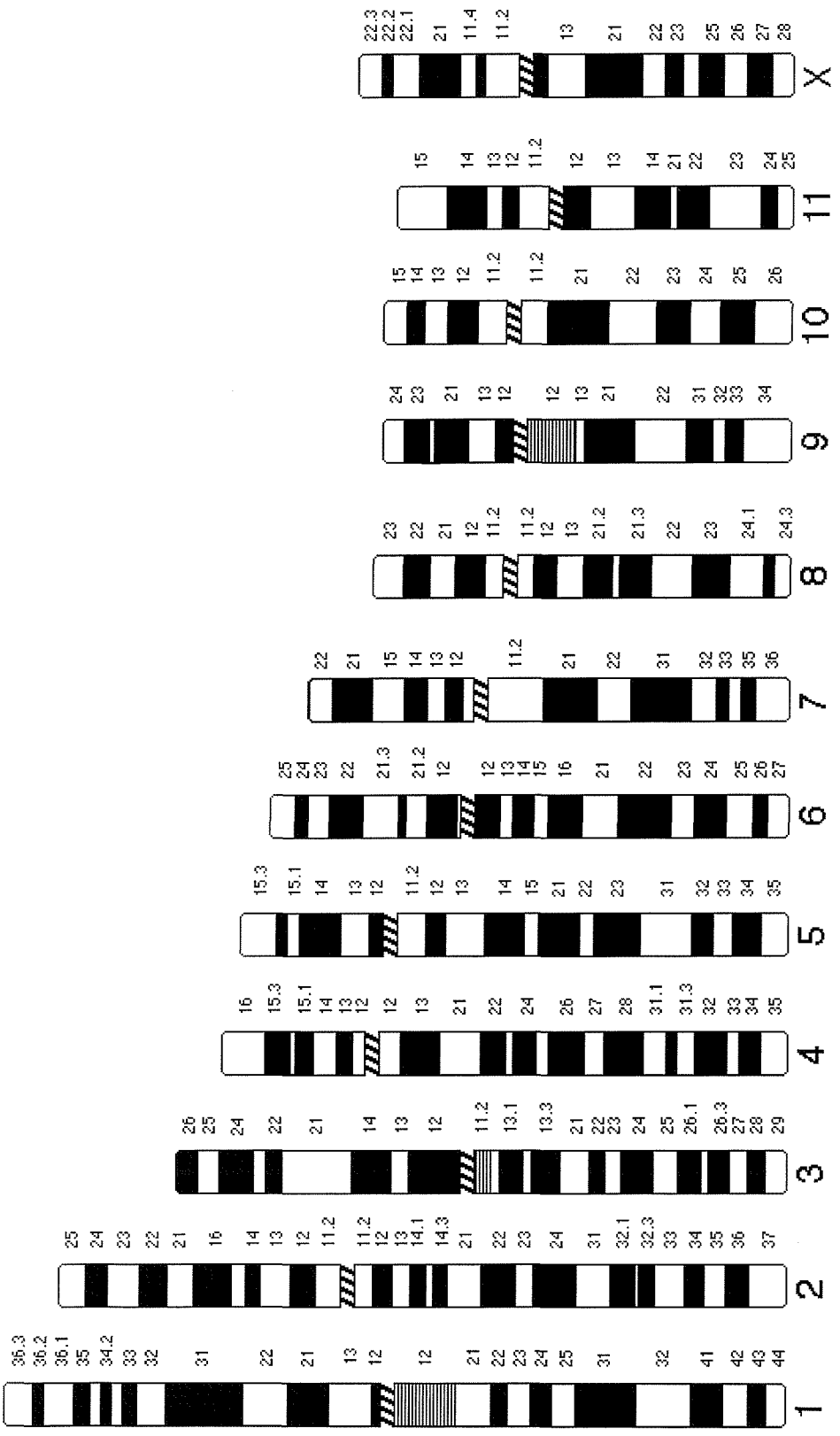
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Figure II.1 continued

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2.3 Molecular Cytogenetic Methods

2.3.1 Culture, Extraction And Labelling Of Probe DNA

The telomeric and centromeric probes used in this study were non-commercial and generously donated by collaborators. Probe details are given in Table III.1 and their cytogenetic locations are shown in figure II.2.

Probes were cloned into plasmid, cosmid, bacteriophage P1, P1 artificial chromosome (PAC) and bacterial artificial chromosome (BAC) vectors. All vectors with kanamycin resistance were cloned into Lawrist 4 or 6 and cultured in LB medium, whereas vectors with ampicillin resistance were cloned into Supercos which preferred 2x YT medium. Agar plates streaked with bacteria containing the vector were received at the laboratory. All procedures were carried out in a Class II laminar flow safety cabinet. For each probe two individual colonies were selected from the agar plates and used to inoculate two tissue culture flasks containing 100 ml of broth + appropriate selective antibiotic. The cultures were grown to saturation in a shaking incubator at 37 °C.

Two glycerol stocks were prepared from each saturated culture (750 µl sterile glycerol:750 µl bacterial medium) and stored at –70 °C for future probe growth and extraction. The probe DNA was extracted from the bacterial cultures using a rapid alkaline lysis protocol. Each culture was transferred to 50 ml centrifuge tubes and centrifuged for 10 minutes at 3000 rpm. The supernatant was discarded using a glass Pasteur pipette with a vacuum aspirator and the pellet resuspended in 1.8 ml of P1 solution. The EDTA removes magnesium ions that are essential for preserving the overall structure of the cell envelope and also inhibits cellular enzymes that could degrade DNA. The RNAse A degrades the bacterial RNA within the cell. 1.8 ml of P2 solution was added, the tubes shaken gently and left at room temperature for 5 minutes. P2 contains another cell membrane disrupting agent, sodium dodecyl sulphate (SDS). SDS is a detergent which aids the process of lysis by removing lipid molecules. In addition, P2 contains sodium hydroxide which adjusts the pH of the cell extract to 12.0-12.5. At this narrow pH range the non-supercoiled bacterial DNA is denatured, whereas the supercoiled vector DNA is not. P3 solution (potassium acetate,1.8 ml) was then added and the tubes placed on ice for 5 minutes. The addition of acid neutralises the cell extract, causing the denatured bacterial DNA strands to reaggregate in a random manner

forming a white precipitate. Any remaining RNA and protein also becomes insoluble. The tubes were then centrifuged at 10000 rpm at 4 °C for 10 minutes to pellet the insoluble bacterial DNA, RNA and protein, leaving pure vector DNA in the supernatant. Using a Gilson pipette, the supernatant was transferred to 1.5 ml eppendorf tubes containing 800 µl of ice-cold isopropanol. This precipitates the DNA, which was then pelleted by centrifugation at 14000 rpm for 15 minutes. The pellet was then washed twice with 70% ethanol before being dried and resuspended in 10 µl of TE buffer (pH 8.0). The tubes for each probe were then pooled.

The DNA concentration was measured using ultraviolet absorbance spectrophotometry. The dye Hoechst 33258 was added to a 2 µl sample of the final DNA extract. The dye binds to the DNA and subsequent ultraviolet radiation absorption is directly proportional to the amount of DNA present. By using a standard DNA solution of known concentration, the concentration of the extracted DNA was established. In order to check for the presence, integrity and quality of probe DNA in the vector DNA solution a restriction enzyme digest was performed using the appropriate enzyme. DNA (500 ng) was added to 2 µl restriction enzyme, 2 µl appropriate enzyme buffer, 1 µl spermidine and sterile dH_2O in a total volume of 20 µl. The reaction was incubated at 37 °C for 1 hour and then the DNA was run on a 0.8% agarose gel to separate the fragments. Ethidium bromide was added to the agarose before the gel was poured. This stains the DNA fragments enabling them to be visualized/photographed under UV light. The size and pattern of restriction fragments was then compared to reference data.

Approximately 10 µg of DNA was used in a nick translation reaction using a method derived from the Sanger Centre (Carter, pers. com.). Probe DNA was labelled with either biotin-16-dUTP (q-arm probes) or digoxigenin-11-dUTP (p-arm and centromeric probes), (Boehringer). Biotin, vitamin H, is found abundantly in egg white and digoxigenin is a steroid that occurs naturally in the plants *Digitalis purpurea* and *D. lanata*. Each of the nucleotides is modified at a position that does not interfere with hydrogen bonding between the probe and the target DNA. The linker arm of 11 or 16 carbon atoms ensures access of the detection reagents and minimises steric hinderance during hybridization. The nick translation reaction employs two enzymes, DNaseI and DNA polymerase which incorporate labelled nucleotides along both strands of the DNA

duplex. The most critical parameter in the reaction is the activity of DNaseI, which 'nicks' double-stranded DNA. Too little nicking leads to inefficient incorporation of the label and probes that are too long; too much nicking results in probes that are too short. The optimum size of labelled probe DNA is ~500bp. Probe DNA (10 μg) was added to 25 μl 10x nick translation buffer, 19 μl 0.5 mM dNTP mix (2 μl each 100 mM dATP, dCTP and dGTP, Pharmacia and 1194 μl sterile dH_2O), 7 μl biotin-16-dUTP or digoxigenin-11-dUTP, 19 μl enzyme mix (14 μl DNaseI working solution, Sigma + 5 μl DNA polymerase, Sigma), and sterile dH_20 in a total volume of 250 μl. The reaction was incubated at 14 °C for 40-45 minutes. The reaction was then paused by placing on ice while the size of the labelled DNA was checked. 1 μl of the reaction mix was run on an ethidium bromide stained 0.8% agarose gel along with lambda/*Hind*III (Gibco BRL) as a size marker. The reaction was then quenched by the addition of 25 μl EDTA which inactivates the enzymes. Salmon sperm carrier DNA (25 μl), 3 M sodium acetate pH 7 (50 μl), and 100% ethanol (900 μl, ice-cold) were added to the mix which was then incubated at –20 °C overnight to precipitate the labelled probe DNA. After centrifugation (13000 rpm for 10 minutes) the DNA pellet was washed in 80% ethanol (900 μl, ice-cold) then dried. The pellet was resuspended in 75 μl TE buffer (pH 8.0) and then diluted with 125 μl sterile dH_20. This labeled probe preparation was stored at –20 °C until required.

TABLE II.1 Details of FISH probes

Probe	Cytogenetic Location	Vector	Antibiotic Resistance[1]	Medium	Enzyme	Source
CEB108	1p	cosmid	kanamycin	LB	*Eco*RI	NIHIMMC[2]
2123.2a1	1q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
160H23	1q	PAC	kanamycin	LB	*Eco*RI	NIHIMMC[2]
2052f6	2p	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
210E4	2q	P1	kanamycin	LB	*Eco*RI	NIHIMMC[2]
B47a2	3p	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
dj11286B18	3p	PAC	kanamycin	LB	*Eco*RI	NIHIMMC[2]
B35c1	3q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
196F4	3q	PAC	kanamycin	LB	*Eco*RI	NIHIMMC[2]
B31	4p	cosmid	kanamycin	LB	*Eco*RI	NIHIMMC[2]
CT55	4q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
84c11	5p	cosmid	kanamycin	LB	*Eco*RI	NIHIMMC[2]
B22a4	5q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
36I2	6p	PAC	kanamycin	LB	*Eco*RI	NIHIMMC[2]
62L11	6p	PAC	kanamycin	LB	*Eco*RI	NIHIMMC[2]
2158e3	6q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
57H24	6q	PAC	kanamycin	LB	*Eco*RI	NIHIMMC[2]
109A6	7p	cosmid	kanamycin	LB	*Eco*RI	NIHIMMC[2]
2000a5	7q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
3K23	7q	PAC	kanamycin	LB	*Eco*RI	NIHIMMC[2]
2205a2	8p	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
2053b3	8q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
34H2	9p	PAC	kanamycin	LB	*Eco*RI	NIHIMMC[2]
2241c1	9q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
2189b6	10p	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
2136a1	10q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
2209a2	11p	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
2072c1	11q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
90I5	12p	PAC	kanamycin	LB	*Eco*RI	NIHIMMC[2]
2196b2	12q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]

221-K18	12q	PAC	kanamycin	LB	*Eco*RI	NIHIMMC[2]
L1.26	13/21 cen	plasmid	ampicillin	LB	*Eco*RI	Devilee[3]
2002e1	13q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
p14.1	14/22 cen	plasmid	ampicillin	LB	*Eco*RI/ *Sal*I	Rocchi[4]
2006a1	14q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
pTRA25	15 cen	plasmid	ampicillin	LB	*Eco*RI	Choo[5]
154P1	15q	PAC	kanamycin	LB	*Eco*RI	NIHIMMC[2]
cGG4	16p	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
D3b1	16q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
240G10	16q	PAC	kanamycin	LB	*Eco*RI	NIHIMMC[2]
2111b1	17p	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
B37c1	17q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
52M11	18p	P1	kanamycin	LB	*Eco*RI	NIHIMMC[2]
2050a6	18q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
F20643	19p	cosmid	kanamycin	LB	*Eco*RI	NIHIMMC[2]
F21283	19q	cosmid	kanamycin	LB	*Eco*RI	NIHIMMC[2]
2005a4	20p	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
1061-I1	20p	PAC	kanamycin	LB	*Eco*RI	NIHIMMC[2]
204A16	20q	P1	kanamycin	LB	*Eco*RI	NIHIMMC[2]
197A4	20q	P1	kanamycin	LB	*Eco*RI	NIHIMMC[2]
C9a1	21q	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
N85A3	22q	cosmid	kanamycin	LB	*Eco*RI	NIHIMMC[2]
CY29	X/Yp	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]
c8.1/2	X/Yq	cosmid	ampicillin	2YT	*Eco*RI	NIHIMMC[2]

[1]Kanamycin at 30 μg/ml; Ampicillin at 50 μg/ml.

[2]National Institutes of Health and Institute of Molecular Medicine Collaboration (1996).

[3]Dr P Devilee, Dept of Human Genetics, University of Leiden, The Netherlands.

[4]Dr M Rocchi, Institute of Genetics, University of Bari, Italy.

[5]Dr K Choo, The Murdoch Institute, Royal Childrens Hospital, Melbourne, Australia.

Figure II.2 Cytogenetic locations of FISH probes.

Where more than one probe is given at any one telomere the position does not indicate order. Short arm telomere probes and centromeric probes are illustrated in red, long arm telomeric probes in green.

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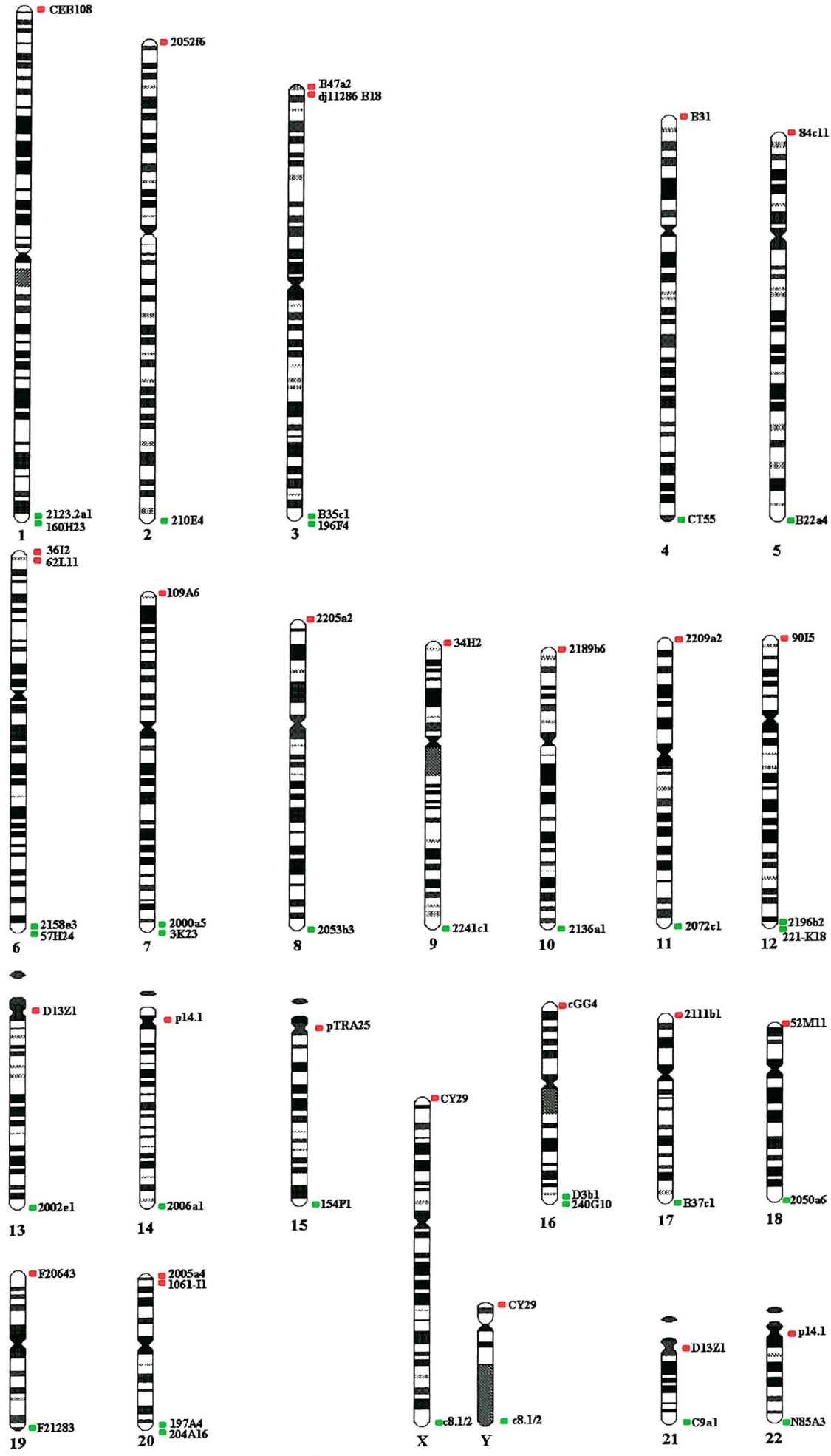
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160H23
1
2052f6
210E4
2
B47a2
dj11286 B18
B35c1
196F4
3
B31
CT55
4
84c11
B22a4
5
36I2
62L11
2158e3
57H24
6
109A6
2000a5
3K23
7
2205a2
2053b3
8
34H2
2241c1
9
2189b6
2136a1
10
2209a2
2072c1
11
90I5
2196b2
221-K18
12
D13Z1
2002e1
13
p14.1
2006a1
14
pTRA25
154P1
15
cGG4
D3b1
240G10
16
2111b1
B37c1
17
52M11
2050a6
18
F20643
F21283
19
2005a4
1061-I1
197A4
204A16
20
CY29
c8.1/2
X
CY29
c8.1/2
Y
D13Z1
C9a1
21
p14.1
N85A3
22

2.3.2 Preparation Of Multiprobe

The plastic multiprobe device (Cytocell) consists of 24 raised bosses, 3 rows of 8 squares. To each of these bosses 0.4-1 μg of Cot-1 DNA (Gibco), 2-5 μl of the appropriate p-arm or centromeric probe preparation and 2-5 μl of the appropriate q-arm probe preparation were added and allowed to air dry. The multiprobe device was then stored at 4 °C until required.

2.3.3 Slide Preparation And Pretreatments

The template microscope slide was demarcated into 24 numbered squares. The fixed cell suspensions were centifuged for 5 minutes at 1200 rpm and the supernatant discarded to leave a cell pellet in approximately 1.5 ml of fixative. A 2 μl drop of cell suspension was applied to a cleaned microscope slide. When dry the spot was examined by phase-contrast microscopy and the concentration of the suspension adjusted to give at least 15 good quality metaphase spreads in a 2 μl drop. The template slide was prepared by soaking in 70% industrial methylated spirit (IMS) for 2 minutes then wiped clean and allowed to air dry. Cell suspension (2μl) was pipetted onto each of the 24 areas of the slide, allowing each spot to dry before the next was applied so that they did not interfere with each other as they spread. For optimum FISH results the metaphases should be free of cytoplasm, therefore prepartions which were cytoplasmic were pretreated with 100 μl of RNase/pepsin solution (5 mg/ml) under a coverslip for $2^1/_2$ minutes at room temperature. The RNase removes cytoplasmic and nuclear RNA, preventing hybridization with the probe and the pepsin digests proteins, increasing probe and detection reagent accessibility. All slides were washed in 2x SSC for 2 minutes then dehydrated through an alcohol series (70%, 90% and 100% IMS, each for 2 minutes). Dehydrating the slides prevented the probe being diluted during the hybridization. The slides were air dried and stored in slide trays wrapped in silver foil at –20 °C until required.

2.3.4 *In Situ* Hybridization

Both the template slide and multiprobe device were placed in an incubation chamber in a 37 °C waterbath for 15 minutes. Hybridization solution (3 µl, consisting of 65% deionized formamide, 1x SSC, 10% dextran sulphate) was applied to each of the multiprobe panels and left for 10 minutes to alllow the dried probes to resuspend. The multiprobe device was then inverted onto the template slide, matching the probe panels with their corresponding squares of the template slide. The multiprobe/template slide was then placed onto a preheated 74 °C hotplate for 2.5 minutes, slide undermost. The formamide in the hybridization solution lowers the melting point of DNA allowing denaturation of both the double stranded target and probe DNA. The multiprobe/template slide was transferred to a hybridization chamber and incubated for 3-5 hours at 37 °C in an uncovered waterbath. The SSC regulates the ionic strength of the hybridization solution and helps to stabilize the nucleic acid duplexes that form during hybridization. The dextran sulphate increases the hybridization reaction rate by forming a matrix in the hybridization mixture which concentrates the probe. The unlabeled cot-1 DNA which was added to the multiprobe device is used to block hybridization of the probe to repetitive sequences within the target (competitive *in situ* suppression, CISS hybridization). The multiprobe device was removed and the template slide washed for 2 x 5 minutes in a prewarmed stringent wash solution (0.4x SSC/0.05% Tween 20, Sigma) at 65 °C. The slide was then rinsed in 2x SSC followed by 4x SSC + Tween 20 before being incubated in a light-proof chamber for 15 minutes with 150 µl of blocking reagent (Boehringer) under a coverslip. The coverslip was removed and the slide incubated for 20 minutes with 150 µl detection reagent (1:1000 sheep anti-digoxigenein-tetramethyl-rhodamine (TRITC, Sigma) + 1:1000 avidin fluorescein-isothiocyanate (FITC, Vector) diluted in blocking reagent). The slide was then washed for 2 x 3 minutes in 4x SSC/0.05% tween 20 and mounted in 80 µl of antifade solution containing DAPI (4',6-diamidino-2-phenylindole; Vector). After blotting the coverslip was sealed with clear nail varnish. Slides were stored in a light-proof container until required. Slides were examined using a Zeiss Axiophot epifluorescence microscope, and images captured using a cooled CCD camera with Digital Scientific Smart Capture or PSI MacProbe software.

2.4 Molecular Methods

DNA was extracted from peripheral blood by a salt precipitation technique as described by Miller *et al.* (1988). Polymorphic microsatellite markers and sequence-tagged sites (STSs) were amplified by the polymerase chain reaction (PCR), and the products were resolved by electrophoresis in 4% polyacrylamide gel using an ABI 377 DNA sequencer. Data were then analysed using Genescan and Genotyper software. Primer sequences were obtained from the Genome Database (GDB: www.gdb.org), Location Database (LDB: http://cedar.genetics.soton.ac.uk/public_html/ldb.html), Reed *et al.* (1994), Rosenberg *et al.* (1997), Slavotinek *et al.* (1999a), and Vocero-Akbani *et al.* (1996).

PCR conditions for microsatellite markers consisted of a 15 minute initial denaturation step at 94 °C followed by 32 cycles of 94 °C, 55 °C, and 72 °C for 30 seconds each, followed by 7 minutes at 72 °C and a final incubation at 60 °C for 1 hour. PCR reactions were conducted in a Perkin-Elmer GeneAmp 9600 thermocycler in 10 μl reaction volumes containing 50 ng DNA, 1x PCR buffer (Qiagen), 200 μM dNTPs, 33 ng of each primer and 0.5 units HotStar *Taq* polymerase (Qiagen).

Quantitative PCR for the subtelomeric regions of 4q and 17q utilised the STSs U57851, WI5353, U57870, and STSG16001 which were co-amplified with a sequence from the adenomatous polyposis coli (APC) gene on chromosome 5q21 to act as an internal control within each reaction (adapted from Poropat and Nicholson, 1998). The peak height of each allele was quantified using Genotyper software and the ratio of test:control calculated to yield a relative measurement of sequence copy number in each individual, compared to normal controls. PCR conditions were the same as those described above except that only 21 cycles were performed to maintain the amplification within the linear range.

2.5 Statistical Analysis

Comparisons of the frequencies of cryptic telomeric abnormalities detected among selected and unselected patients with IMR and among control individuals were made using Fisher's exact probability calculation. Confidence intervals were calculated following the method of Wilson (1927). To compensate for multiple comparisons

(between selected IMR patients and control individuals, and between unselected IMR patients and control individuals) a Bonferroni correction was applied, and $p<0.025$ was taken as significant.

In light of the estimated prevalence of cryptic telomeric abnormalities among patients with IMR reported by Flint *et al.* (1995), sample sizes of 150 were chosen for both our unselected IMR and control poulations. This sample size would enable a frequency of 6% among unselected patients to be defined as statistically significant should a cryptic rearrangement be identified in one control individual. Due to the nature of their referral, it was not possible to obtain a sample size of 150 for our selected probands.

CHAPTER 3

CRYPTIC TELOMERIC ABNORMALITIES IN IDIOPATHIC MENTAL RETARDATION POPULATIONS

3.1 Study Populations

Two hundred patients have been included in the IMR study population. These individuals fall into two categories. Firstly, an unbiased population of 150 patients with developmental delay referred to the Wessex Regional Genetics Laboratory (WRGL), in which conventional cytogenetic, molecular cytogenetic and molecular investigations had failed to find a cause of the developmental delay. Secondly, a highly selected population of 50 patients referred by Clinical Geneticists where inclusion in this study was specifically requested. Again no cause of the patients' developmental delay had been identified.

In the vast majority of cases no indication of the nature or severity of the patient's developmental delay was provided with the referral information. In order for comparisons to be made between the results of the present and other published studies a retrospective questionnaire for each proband was sent to the referring clinician. Details of the severity of developmental delay, any dysmorphic features or major congenital abnormalities, and any relevant family history were requested. Completed questionnaires were returned for 114 (76%) of unselected probands and for 46 (92%) of selected probands. A summary of the clinical findings among the IMR populations are given in Table III.1.

3.2 Results

All patients were screened for the presence of deletion, duplication or translocation of each telomeric probe. A summary of the cytogenetic and telomeric FISH results are given in Tables III.2 and 3. Details of the PCR results are given in Appendix 1.

An apparently normal karyotype was reported using conventional G-banded analysis in 149 of the 150 unselected probands and in 49 of the 50 selected probands. The excess of males in the study population reflects an increased male:female sex ratio among IMR referrals. A normal result for each of the telomeric probes was obtained in all of these patients (Figure III.1-3).

TABLE III.1 Clinical Findings in IMR Study Populations

	All probands		Unselected		Selected	
	n/200	(%)	n/150	(%)	n/50	(%)
QUESTIONNAIRE RETURNED	160	(80)	114	(76)	46	(92)
DEVELOPMENTAL DELAY						
none	11	(6)	9	(6)	2	(4)
unspecified	61	(30)	53	(35)	8	(16)
mild	46	(23)	39	(26)	7	(14)
moderate/severe	82	(41)	49	(33)	33	(66)
DYSMORPHIC FEATURES						
Major congenital anomalies	47	(24)	23	(15)	24	(48)
Minor congenital anomalies	91	(46)	52	(35)	39	(78)
Either	107	(54)	63	(42)	44	(88)
FAMILY HISTORY						
FH of dev delay	57	(28)	41	(27)	16	(32)
FH of dysmorphism	11	(5)	9	(6)	2	(4)
Either	65	(32)	48	(32)	17	(34)
REFERRING CLINICIAN						
Clinical Geneticist	85	(43)	36	(24)	50	(100)
Consultant Paediatrician	88	(44)	87	(58)		
Consultant Psychiatrist	19	(9)	19	(13)		
Consultant Neurologist	5	(2)	5	(3)		
Consultant Obstetrician	1	(0.5)	1	(0.7)		
G.P.	2	(1)	2	(1.3)		

TABLE III.2 Cytogenetic and Telomere FISH Results in Unselected IMR Study Population

Conventional Cytogenetic + FISH Analysis	Telomere FISH Analysis	No of Cases
Apparently normal male karyotype	No cryptic telomeric rearrangement detected	103
Apparently normal female karyotype	No cryptic telomeric rearrangement detected	46
Balanced reciprocal translocation 46,XY,t(2;15)(q13;q22.31)mat	No cryptic telomeric rearrangement detected	1
TOTAL		150

TABLE III.3 Cytogenetic and Telomere FISH Results in Selected IMR Study Population

Conventional Cytogenetic + FISH Analysis	Telomere FISH Analysis	No of Cases
Apparently normal male karyotype	No cryptic telomeric rearrangement detected	23
Apparently normal female karyotype	No cryptic telomeric rearrangement detected	26
Subtle abnormality suspected 46,XY,add(13)(q34)*de novo*	Semi - cryptic unbalanced translocation der(13)t(13;19)(q34;qter)*de novo*[1]	1
TOTAL		50

[1] See text for full karyotype

One patient (P78) from the unselected group was shown to have an apparently balanced reciprocal translocation between the long arm of one chromosome 2 and the long arm of one chromosome 15. This translocation was also carried in an apparently identical form by the proband's mother, three brothers and maternal aunt. Although the translocation appeared to be segregating with developmental delay no genetic imbalance had been detected. It therefore remained a possibility that this rearrangement was coincidental to the proband's delay. FISH with the complete set of telomeric probes confirmed this balanced translocation but failed to identify an additional, cryptic rearrangement. His karyotype is therefore 46,XY,t(2;15)(q13;q22.32) *mat*.ish t(2;15)(2052f6,154P1;pTRA25,210E4).

The remaining patient (P72), included in the selected group, had previously been reported to have an apparently normal male karyotype and a deletion of the DiGeorge/ CATCH 22 critical region at 22q11.2 had been excluded by FISH. However high resolution G-banded analysis (ISCN 850 band level) identified a previously undetected subtle abnormality of the terminal region of the long arm of one chromosome 13. FISH with the whole panel of telomeric probes confirmed the suspected monosomy of the subtelomeric region of 13q and detected trisomy for the subtelomeric region of 19q . The additional 19q telomeric probe (F21283) was observed at the long arm subtelomeric region of the abnormal chromosome 13. P72 was therefore shown to have a semi-cryptic unbalanced translocation between the subtelomeric regions of the long arms of chromosomes 13 and 19. Parental karyotypes were shown to be normal by both high resolution G-banded analysis and FISH with the telomeric probes 2002e1 (13q) and F21283 (19q). The unbalanced telomeric rearrangement in P72 had therefore arisen *de novo* and his karyotype can be given as 46,XY,add(13)(q34)*de novo*.ish der(13)t(13;19)(q34;qter) (F21283+,2002e1-), 22q11.2(cH748x2) (figure III.4). Fluorescence PCR analysis with polymorphic microsatellite markers showed that P72 had inherited two maternal and one paternal D19S218 and D19S890 alleles. A normal biparental pattern of inheritance was observed for D13S1295. This *de novo* rearrangement was therefore of maternal origin and involved trisomy for 19q of at least 150 kb and monosomy for 13q of less than 450 kb.

3.3 Polymorphisms and Cross Hybridization

A number of polymorphisms and anomalous findings were detected using the panel of telomeric FISH probes. Firstly, in 42 of the 200 (21%) patients in the IMR study population a variation in signal strength was noted with the probe CY29 (Xp/Yp telomeric probe) between either the two X chromosomes or between the X and Y chromosomes. A similar variation in signal strength was observed with the probe c8.1/2 (Xq/Yq telomeric probe) in 9/200 (4.5%) patients and with the probe 210E4 (2q) in one patient (P79) (figure III.5).

Finally two telomeric probes were found to cross hybridize. The probe 2209a2 (11p telomeric probe) was found to hybridize weakly to the short arm telomeric region of chromosome 17 (figure III.6a) and the probe F21283 (19q telomeric probe) was found to hybridize to the region 2q13 (figure III.6b).

Figure III.1 Normal FISH results with the telomeric probes for chromosomes 1-8. Numbers to the left of each image indicate the chromosome to which the probes are specific. Short arm telomeric probes are labelled with digoxigenin and detected with anti-digoxigenin TRITC (red). Long arm telomeric probes are labelled with biotin and detected with avidin FITC (green).

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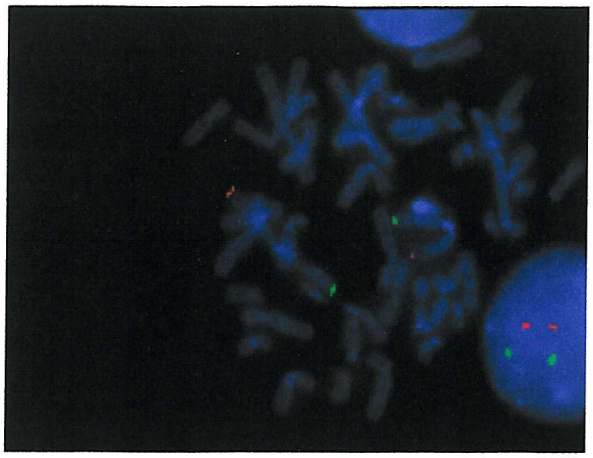

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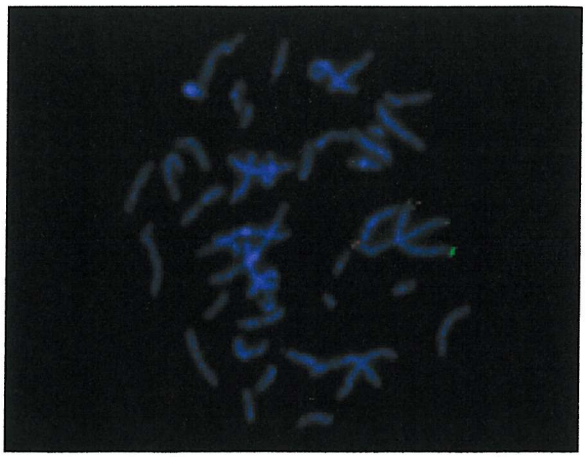

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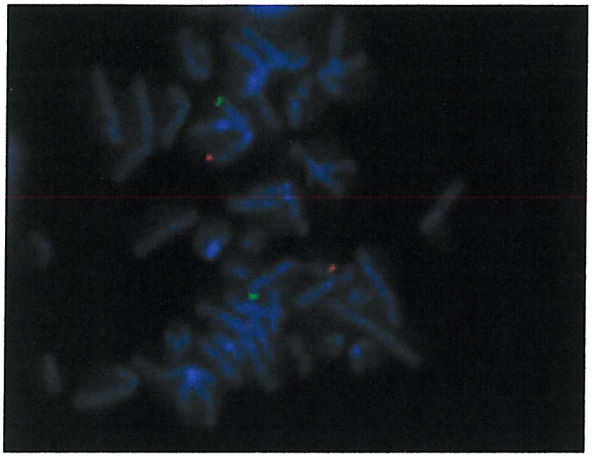

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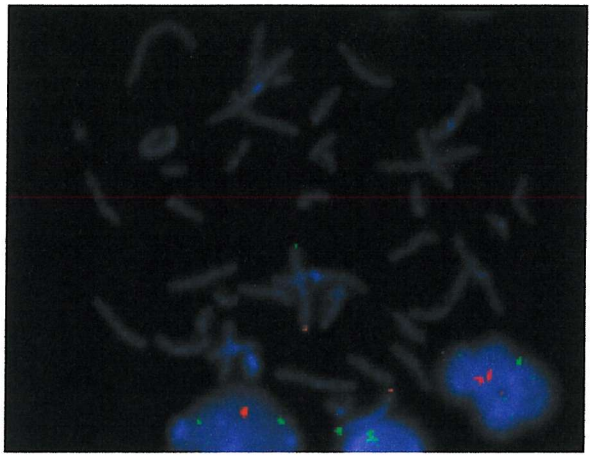

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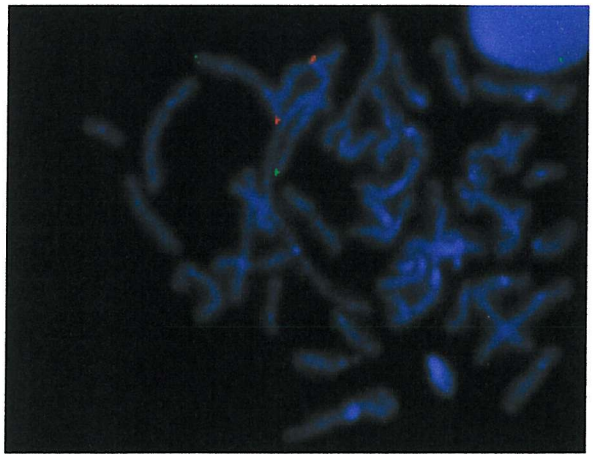

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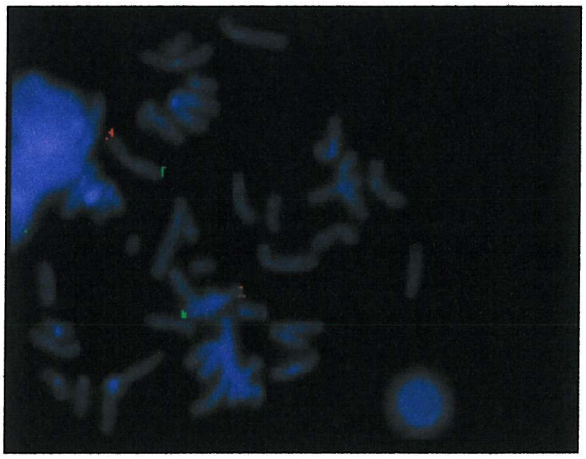

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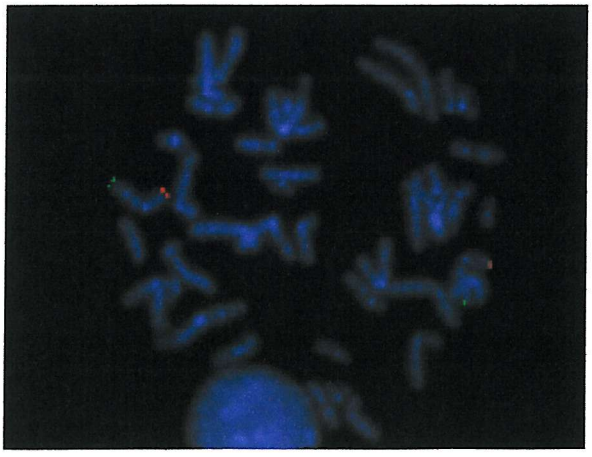

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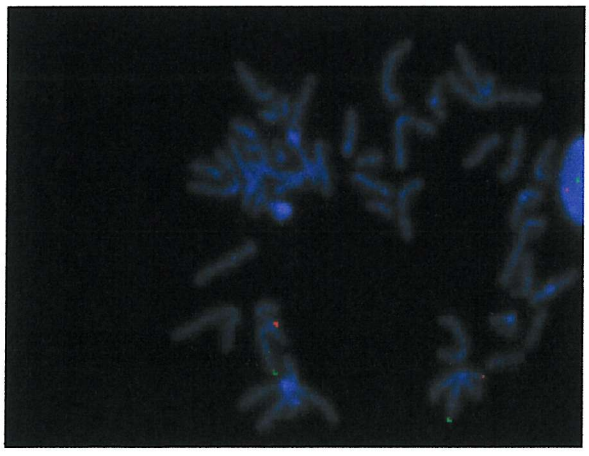

Figure III.2 Normal FISH results with the telomeric probes for chromosomes 9-16. Numbers to the left of each image indicate the chromosome to which the probes are specific. Short arm telomeric probes and centromeric probes are labelled with digoxigenin and detected with anti-digoxigenin TRITC (red). Long arm telomeric probes are labelled with biotin and detected with avidin FITC (green). Note the cross hybridization of the centromeric probes for chromosome 13 (D13Z1) with the 21 centromere and chromosome 14 (p14.1) with the 22 centromere.

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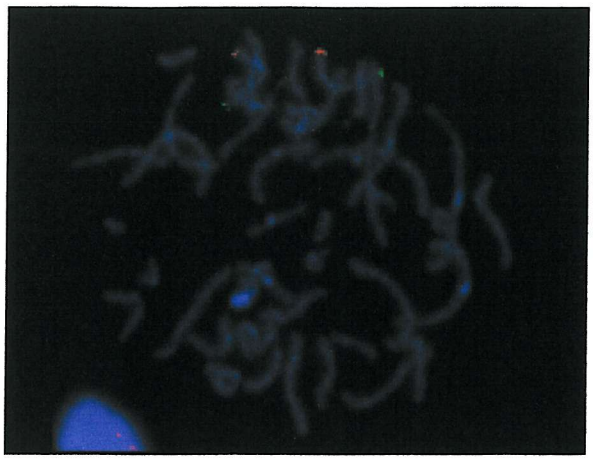

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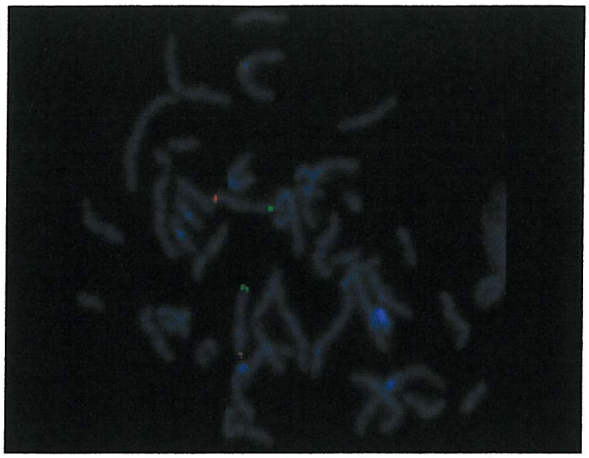

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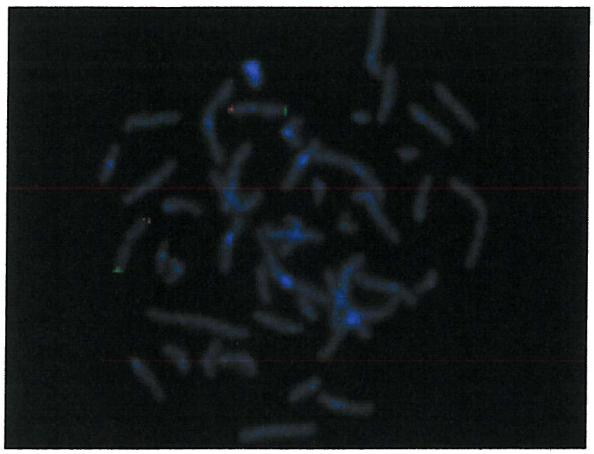

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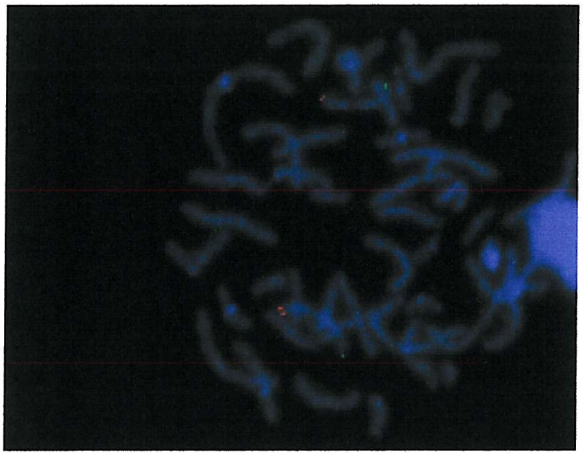

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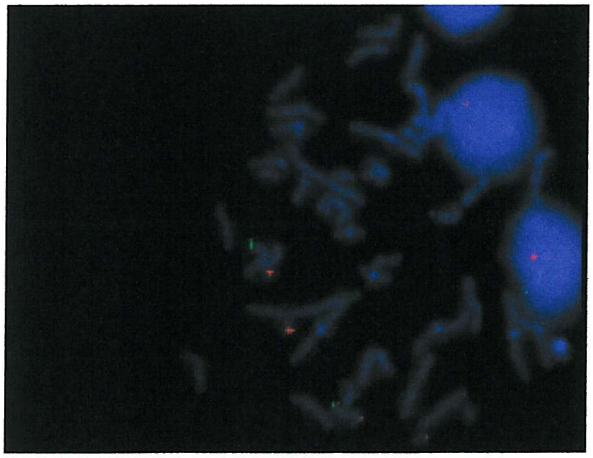

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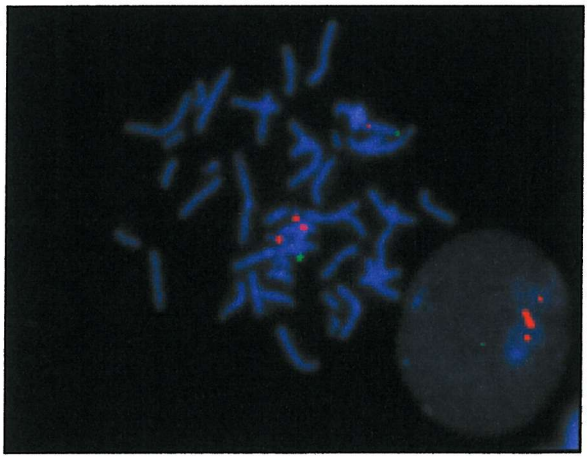

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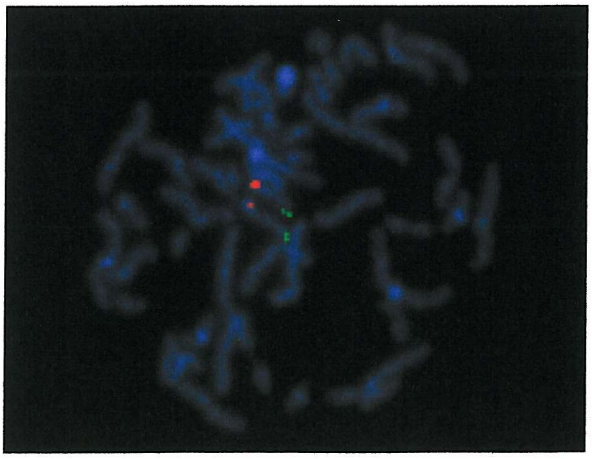

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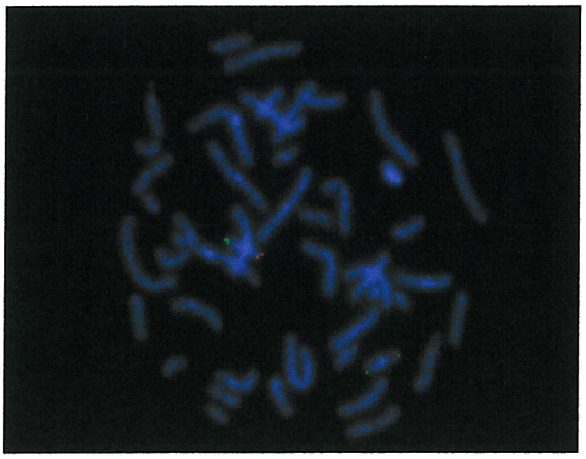

Figure III.3 Normal FISH results with the telomeric probes for chromosomes 17-22 and the X and Y chromosomes.

Numbers to the left of each image indicate the chromosome to which the probes are specific. Short arm telomeric probes and centromeric probes are labelled with digoxigenin and detected with anti-digoxigenin TRITC (red). Long arm telomeric probes are labelled with biotin and detected with avidin FITC (green). Note the cross hybridization of the centromeric probes for chromosome 21 (D13Z1) with the 13 centromere and chromosome 22 (p14.1) with the 14 centromere.

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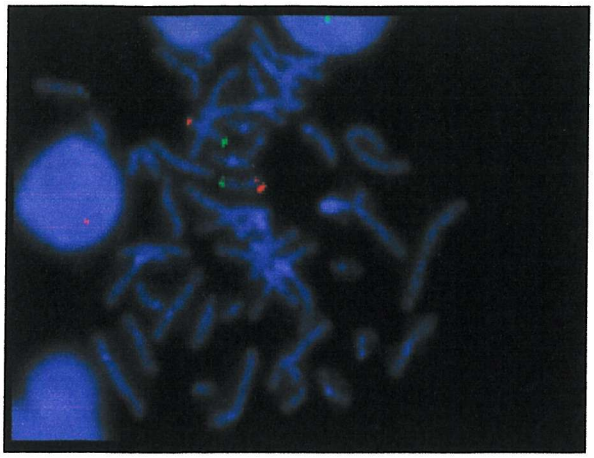

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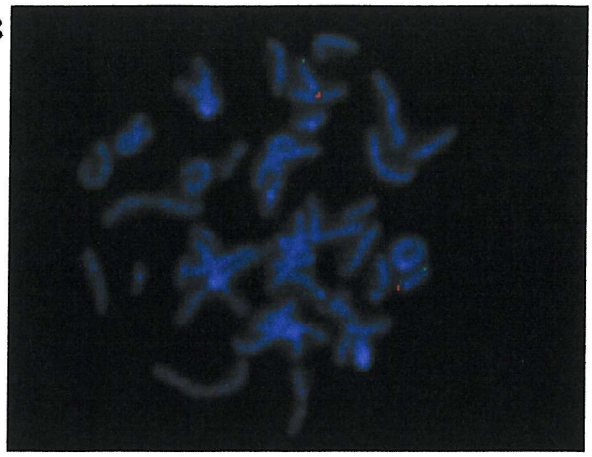

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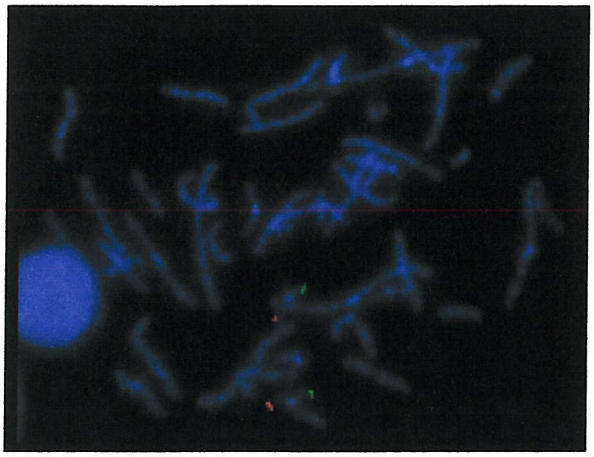

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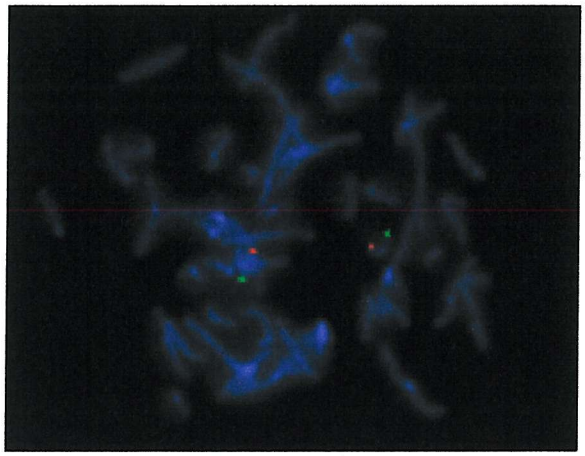

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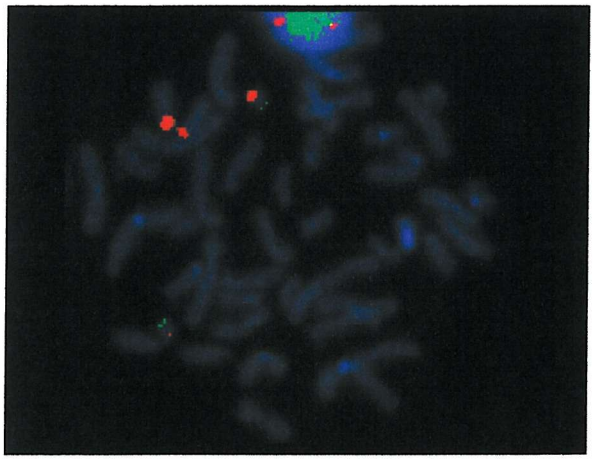

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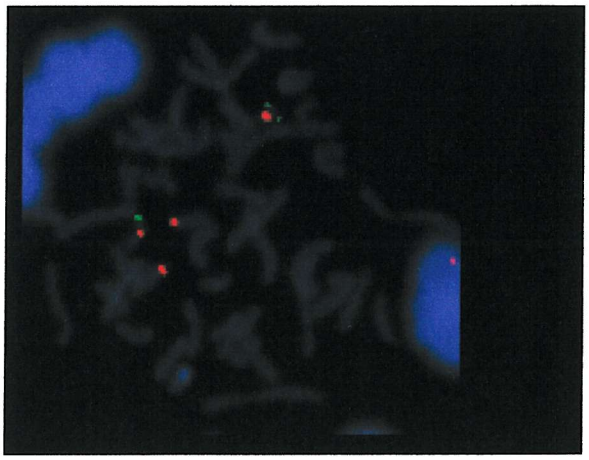

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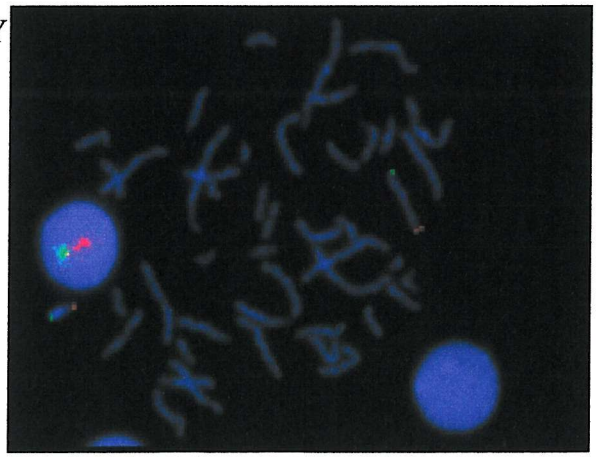

Figure III.4a FISH result in patient P72 with the 13q telomeric probe (2002e1) (green) and the 13/21 centromeric probe (D13Z1) (red).
The arrow indicates the absence of a 2002e1 signal on the derived chromosome 13.

Figure III.4b FISH result in patient P72 with the 19q telomeric probe (F21283) (green) and the 19p telomeric probe (F20643) (red).
The arrow indicates the presence of an additional F21283 signal on the derived chromosome 13.

Figure III.4c Partial karyotype of patient P72.
The arrow indicates the 13q breakpoint.

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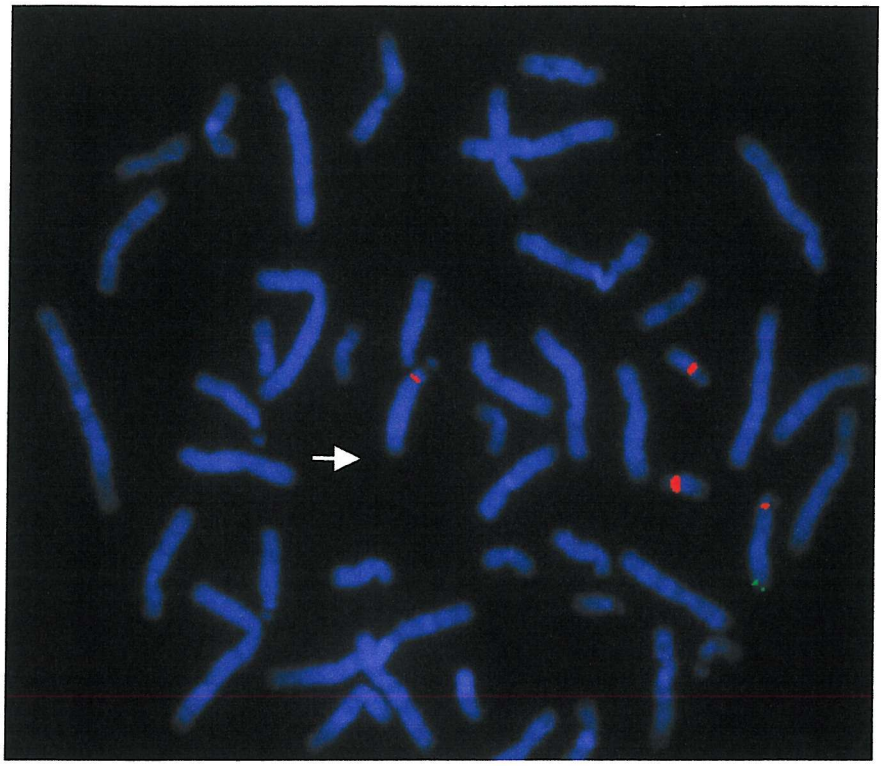

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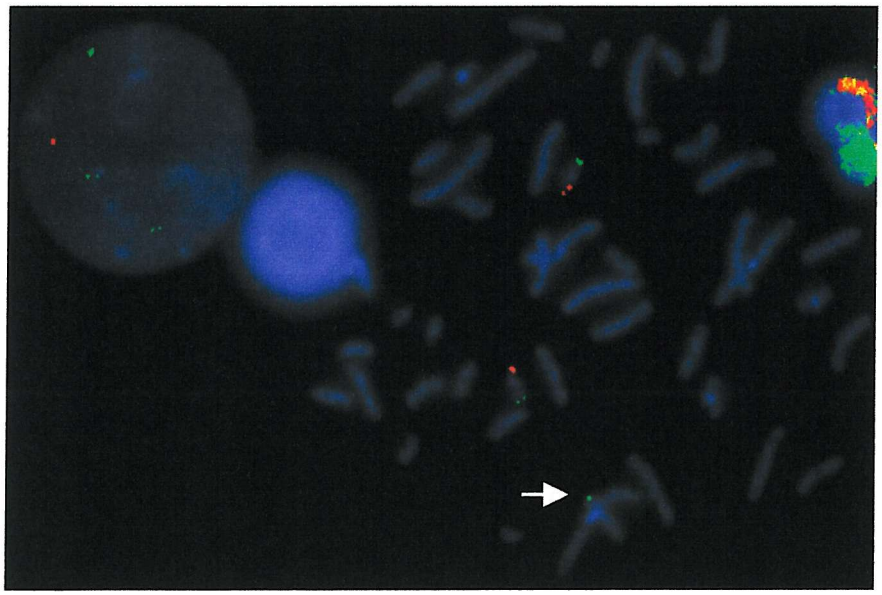

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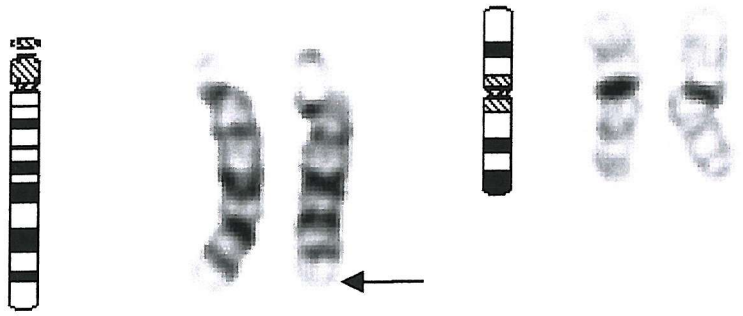

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Figure III.5a FISH result with the Xp/Yp telomeric probe (CY29) (red) and the Xq/Yq telomeric probe (c8.1/2) (green).
The arrow indicates a weak CY29 signal on an X chromosome.

Figure III.5b FISH result with the Xp/Yp telomeric probe (CY29) (red) and the Xq/Yq telomeric probe (c8.1/2) (green).
The short arrow indicates a weak CY29 signal on a Y chromosome and the long arrow a weak c8.1/2 signal on an X chromosome.

Figure III.5c FISH result in patient P79 with the 2p telomeric probe (2052f6) (red) and the 2q telomeric probe (210E4) (green).
The arrow indicates a weak 210E4 signal on one chromosome 2.

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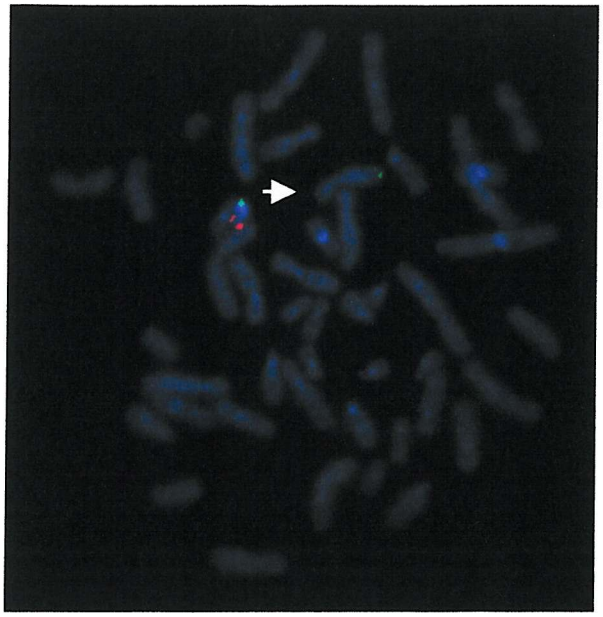

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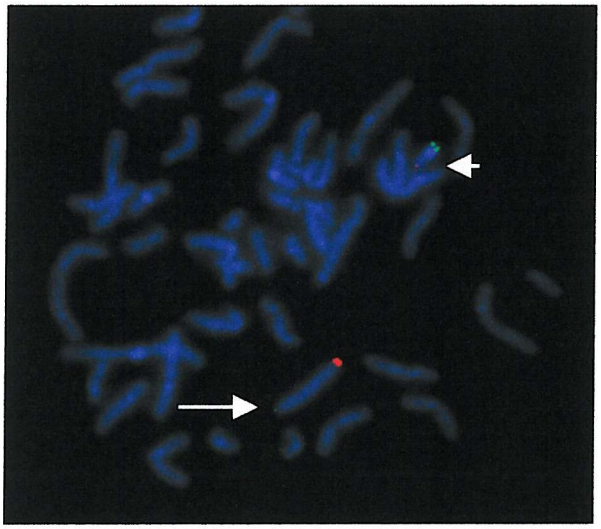

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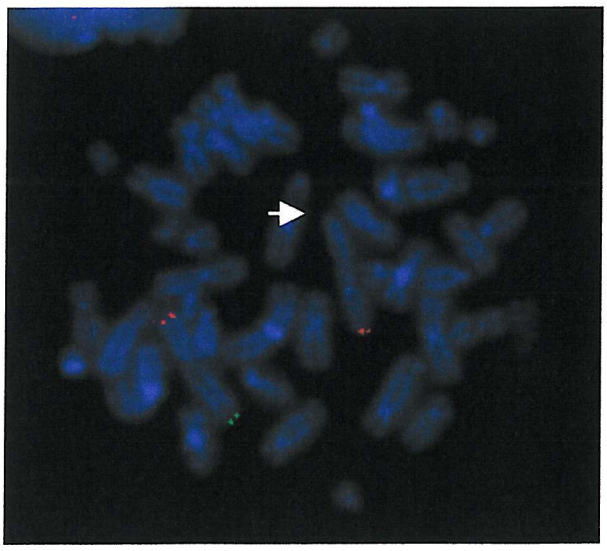

Figure III.6a FISH result with the 11p telomeric probe (2209a2) (red) and the 11q telomeric probe (2072c1) (green).
The arrows indicate the sites of cross hybridization of 2072c1 at the telomeric regions of the short arms of both chromosomes 17.

Figure III.6b FISH result with the 19p telomeric probe (F20643) (red) and the 19q telomeric probe (F21283) (green).
The arrows indicate the sites of cross hybridization of F21283 at 2q13.

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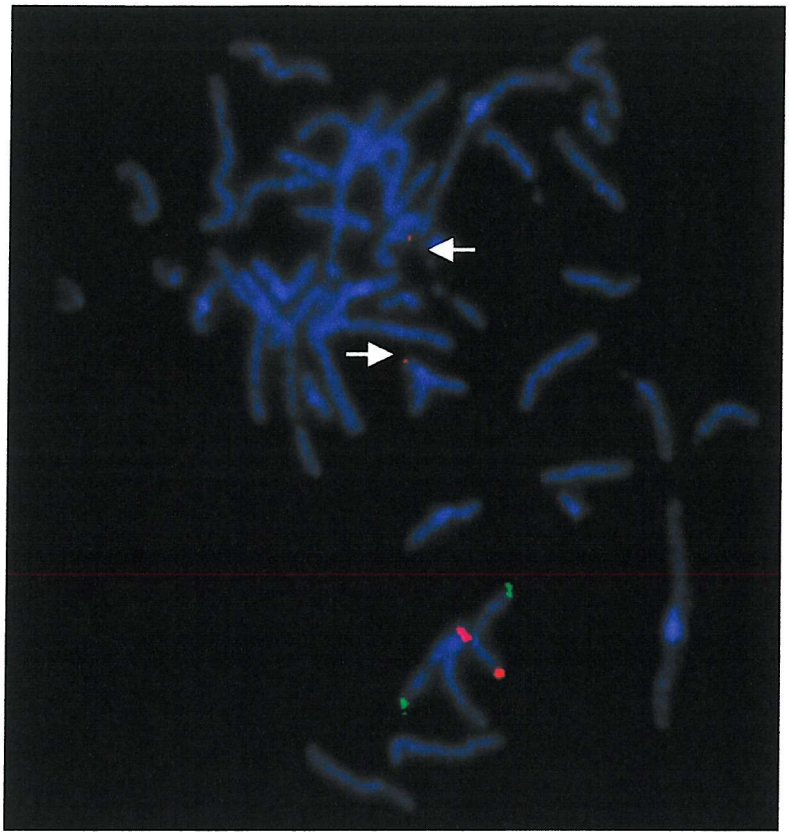

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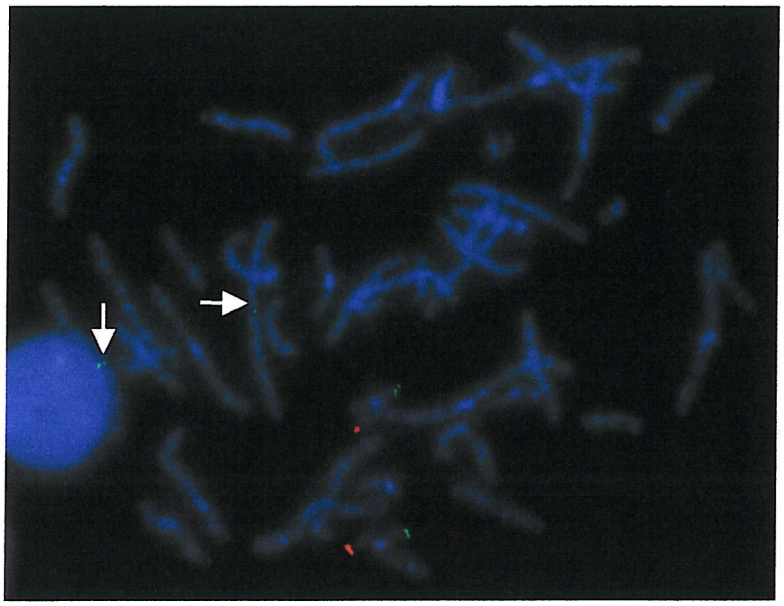

CHAPTER 4

CRYPTIC TELOMERIC ABNORMALITIES IN A CONTROL POPULATION

4.1 Control Population

The control population consisted of 150 individuals referred to the WRGL as relatives of patients with either numerical or structural chromosome abnormalities. Conventional cytogenetic analysis had shown that all these individuals had apparently normal karyotypes. Seventy-five of these individuals were relatives of probands with gross chromosome imbalances. These were believed to be the cause of the phenotypes for which the probands were referred, either because the imbalance segregated with MR in the family, or because a review of the literature had identified previous cases with similar imbalances in association with similar phenotypes. Fourteen individuals were relatives of probands ascertained because of MR or dysmorphism in whom an apparently balanced chromosome rearrangement had been identified, no further family history of MR or dysmorphism was recorded. Of these, seven were *de novo* rearrangements. The remaining 61 individuals were relatives of probands ascertained for reasons other than MR, including infertility and prenatal genetic diagnosis.

4.2 Results

All individuals were screened for the presence of deletion, duplication or translocation of each telomeric probe. A summary of the cytogenetic and telomeric FISH results are given in Table IV.1. Details of the PCR results are given in Appendix 1.

An apparently normal karyotype was reported using conventional G-banded analysis in all 150 individuals. A normal result for each of the telomeric probes was obtained in 148 of the 150 control cases (98.7 %).

One control individual (C19) was referred for analysis during her sixth pregnancy when a trisomy 7 cell line was detected at amniocentesis. One previous, deceased child had Smith-Lemli-Opitz syndrome. One pregnancy was terminated following biochemical confirmation of a diagnosis of Smith-Lemli-Opitz syndrome and another spontaneously miscarried. The remaining two children were healthy. Conventional G-banded analysis showed an apparently normal female karyotype. However, multi-telomere FISH detected a deletion of the terminal region of the long arm of chromosome 4. No other telomeric FISH probe was found to hybridize to the deleted chromosome. The abnormal chromosome 4 was identified by retrospective blind analysis of the

original G-banded slide by an experienced cytogeneticist (figure IV.1). However, quantitative PCR analysis using the distal 4q STSs WI-5353 and U57851 failed to confirm the deletion.

TABLE IV.1 Cytogenetic and Telomere FISH Results in Control study Population

Conventional Cytogenetic + FISH Analysis	Telomere FISH Analysis	No of Cases
Apparently normal male karyotype	No cryptic telomeric rearrangement detected	70
Apparently normal female karyotype	No cryptic telomeric rearrangement detected	78
Apparently normal female karyotype	Cryptic unbalanced telomeric rearrangement del(4)(pter→q35.2:)[1]	1
Apparently normal male karyotype	Cryptic unbalanced telomeric rearrangement der(Y)t(Y;17)(pter;q25.3)[1]	1
TOTAL		150

[1] See text for full karyotype

The remaining individual from the control population (C31) was found to have an additional signal with the 17q telomeric probe (B37c1). This signal was at the distal tip of the short arm of the Y chromosome. The probe for the telomeric region of the short arms of the X and Y chromosome (CY29) also hybridized to the distal short arm of this abnormal Y chromosome. This individual is therefore trisomic for the subtelomeric region of the long arm of chromosome 17 and his karyotype is 46,XY.ish 17qter(B37c1x2),der(Y)t(Y;17) (pter;q25.3)(B37c1+,CY29+) (figure IV.2). Retrospective cytogenetic analysis failed to detect this abnormality and it is therefore classed as a cryptic telomeric imbalance. C31, one member of an extensive family

(figure V.5), was referred for exclusion of a subtle translocation between the distal short arms of one chromosome 11 and one chromosome 17. Both parents of C31 were deceased so the inheritance pattern of this rearrangement could not be formally established. However, several other family members, including the brother of C31, were examined using the 17q telomeric probe (B37c1) and all were found to have a normal hybridization pattern. Fluorescence PCR analysis with the polymorphic minisatellite markers D17S928 or 17qtel13 (D17S2200) failed to identify evidence of a third allele in C31. Quantitative PCR using the STSs U57870 and STSG16001 also failed to confirm duplication of distal 17q.

4.3 Polymorphisms and Cross Hybridization

A variation in signal strength was noted in 29 of the 150 (19%) individuals in the control population with the probe CY29 (Xp/Yp telomeric probe) and in 8/150 (5%) with the probe c8.1/2 (Xq/Yq telomeric probe).

Figure IV.1a FISH result in control individual C19 with the 4q telomeric probe (CT55) (green) and the 4p telomeric probe (B31) (red).
The arrow indicates the absence of a CT55 signal on the deleted chromosome 4.

Figure IV.1b Partial karyotype of C19.

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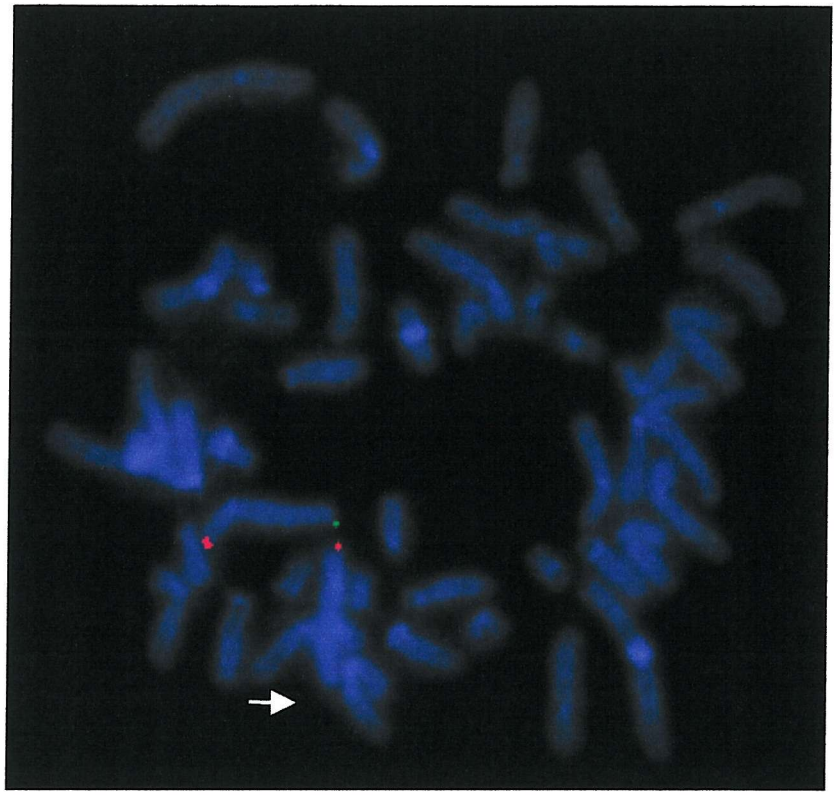

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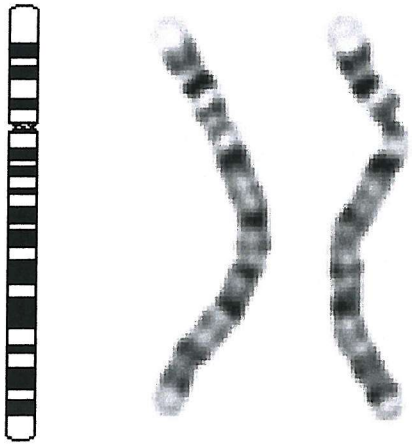

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Figure IV.2a FISH result in control individual C31 with the 17q telomeric probe (B37c1) (green) and the 17p telomeric probe (2111b1) (red).
The arrow indicates the presence of an additional B37c1 signal on the derived Y chromosome.

Figure IV.2b FISH result in C31 with the 17q telomeric probe (B37c1) (green) and the Xp/Yp telomeric probe (CY29) (red).
The arrow indicates the presence of co-hybridization of both probes on the derived Y chromosome.

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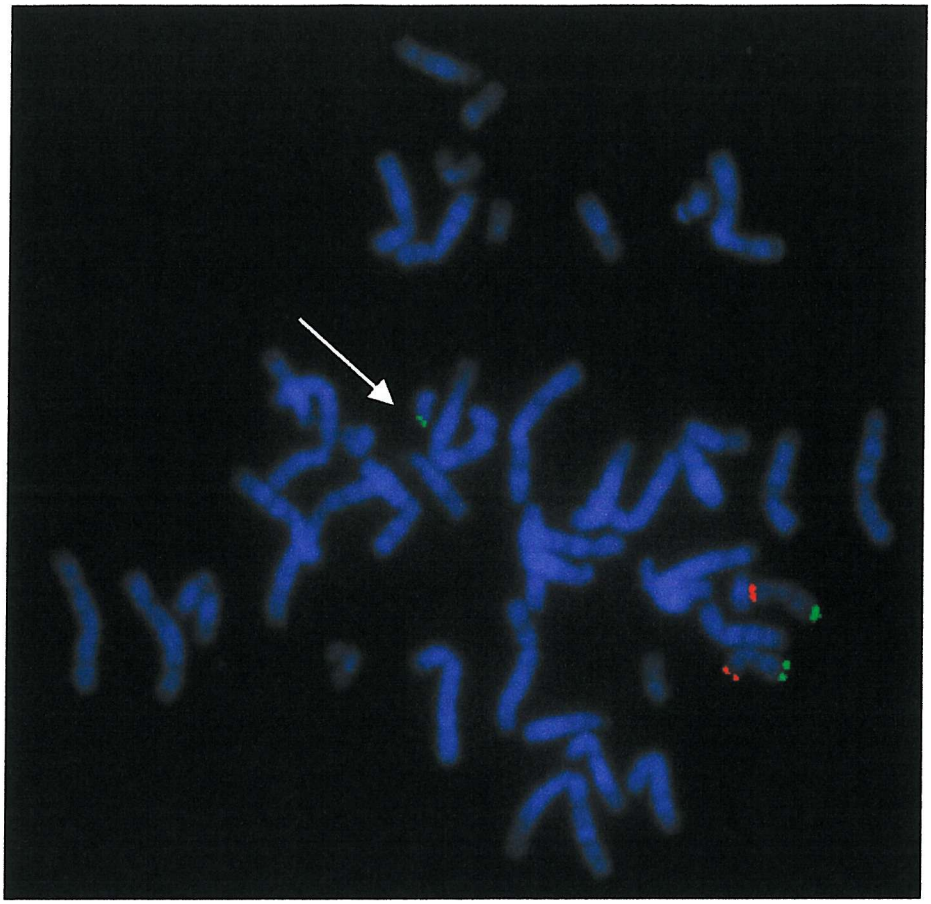

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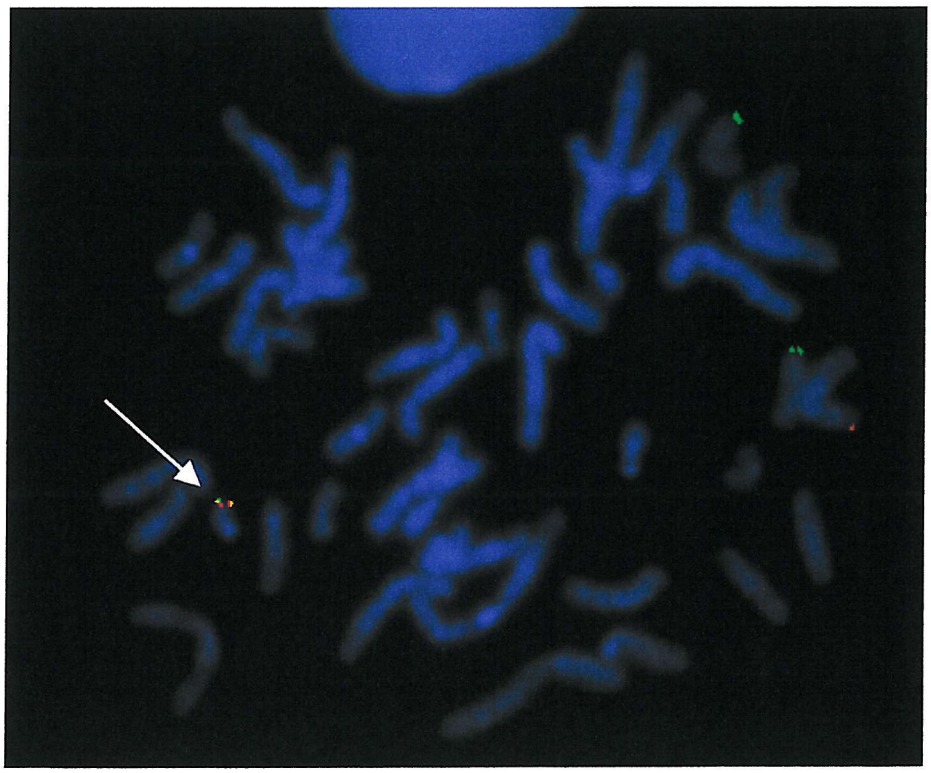


CHAPTER 5

A TELOMERE FISH AND MOLECULAR STUDY OF SUBTLE TERMINAL CHROMOSOME REARRANGEMENTS

5.1 Introduction

Eighteen individuals with subtle or cryptic terminal rearrangements detected using either high resolution cytogenetic analysis or FISH were reinvestigated using telomeric FISH probes. In three cases (S5, S8, and S16) telomere specific FISH probes were used to confirm subtle unbalanced telomeric translocations which had been inherited from apparently balanced parental translocations. In all three cases the rearrangement had gone undetected using conventional analysis on at least one occasion. In the remaining 15 cases multi-telomere FISH was used to further characterise the abnormal chromosome and screen for an underlying semi-cryptic telomeric exchange.

5.2 Clinical Information

Unbalanced Translocations

Case S3

This patient was referred with developmental delay and a suspected diagnosis of fragile X syndrome. In addition she was noted to have long slender hands and long flat feet.

Case S5

Soon after birth this patient was found to have transposition of the great arteries, a ventricular septal defect (VSD) and an atrial septal defect (ASD). He had obvious redundant skin which hung in folds, a linear raw area in the neck, at the base of a fold of skin, and a low posterior hair line. He had a dysmorphic appearance with ridging of the coronal suture and a high forehead. The eyebrows were arched and both eyes were small with obvious iris hypoplasia and bilateral colobomas. He had epicanthic folds, a depressed nasal bridge, anteverted nares, a beaked nose and a small chin. He was also noted to have syndactyly of the fingers and toes, and abnormal genitalia. He was reviewed at 6 months of age following corrrective cardiac surgery at which time he was hypotonic with minimal head control and was not sitting. The mother of the proband was also noted at birth to have redundant skin, a linear raw area at the neck, a markedly low posterior hair line and complete tissue syndactyly of all digits. She did not have a

dysmorphic facial appearance. At the age of 9 years she presented with glaucoma and was diagnosed as having a congenital abnormality resembling Reiger's anomaly.

Case S9

This patient was referred at three days of age with a suspected diagnosis of Wolf Hirschhorn syndrome (WHS). He had a cleft lip and palate, hypertelorism, epicanthic folds, left iris coloboma and bilateral exomphalos.

Case S6

This individual was first referred at two days of age with epicanthic folds, single palmar creases, hypotonia and a small, low set left ear. Following physical examination by a Clinical Geneticist at 5 months of age, he was re-referred for cytogenetic investigation with a suspected diagnosis of WHS. At this time he was noted to have hypertelorism, midline defects, and short stature.

Case S1

This patient was referred for cytogenetic analysis at nine months of age because of minor dysmorphic features. She was recorded as having delayed visual maturation, left ptosis, fish-like mouth, and talipes.

Case S12

This child was born at home following no antenatal care to a mother dependent on heroin IV, valium, and methadone. At birth the child was not breathing and required ventilation in the special care baby unit in addition to morphine for neonatal abstinence syndrome. He was noted to have multiple dysmorphic features including micrognathia, a hypoplastic nose, hypoplastic nipples, tapering fingers, a small jaw, hypertrophied gums, low set posteriorly rotated ears, a short neck and down-slanting eyes. Ultrasound investigation showed an abnormal dysplastic left kidney with duplex ureters and a ureterocoele. He continued to have seizures and was found to have profound metabolic acidosis. At 3 weeks and 2 days he was first referred for cytogenetic analysis. Subsequent ultrasound scans of the head at two months of age showed significantly

enlarged ventricles and featureless cerebral hemispheres with little in the way of gyral folds. The child died aged 12 weeks following a seizure. At post mortem he was noted to have profound growth retardation and numerous dysmorphic features, including thin lips with downturned corners and a long philtrum, in addition to those already recorded. Examination of the patient's brain showed lissencephaly. The cause of death was given as natural causes with severe congenital abnormalities of the cerebral hemispheres and the urinary system as the main contributing factors. A suspected diagnosis of Miller-Dieker syndrome (MDS) was given.

Case S8

This was the first child born to healthy, unrelated parents. The pregnancy had been uneventful and prenatal cytogenetic analysis following amniocentesis showed an apparently normal female karyotype. The parents had also been investigated cytogenetically because of six previous spontaneous miscarriages. The patient was born at 34 weeks gestation and immediately referred for re-investigation with a suspected diagnosis of either MDS or Pallister Killian syndrome. She had tapering fingers with overlapping 3rd and 4th fingers and bilateral clinodactyly, widespaced 1st and 2nd toes, hypotonia, and was suffering from respiratory distress. In addition, ultrasound examination showed dilated lateral ventricles and agenesis of the corpus callosum. The patient died in the neonatal period.

Case S11

This was the first child born to healthy, unrelated parents. The pregnancy had been uneventful and prenatal cytogenetic analysis following amniocentesis showed an apparently normal female karyotype. The parents had also been investigated cytogenetically because of previous spontaneous miscarriages. The patient was referred for re-investigation at two days of age with a suspected diagnosis of either MDS or Pallister Killian syndrome. She was noted to have a vertical forehead crease, micrognathia, microcephaly and a squeaky cry.

Case S10

This child was referred at 6 months of age because of failure to thrive. In addition, he was noted to have a prominent forehead, downward slant to eyes, and visual inattention.

Case S7

This child was born prematurely at 30 weeks gestation. She was referred for cytogenetic investigation at 6 days of age following an ultrasound scan which showed absence of the corpus callosum. She was receiving ventilation. Hypertelorism, low set ears, and a patent ductus arteriosus (PDA) were noted.

Case S16

This child was first referred for cytogenetic analysis at 9 days of age. At this time he was hypotonic and his occipital head circumference was greater than the 97th centile. An anti-mongoloid slant and a beaked nose were also noted. Exclusion of a diagnosis of fragile X syndrome was requested. He was re-referred aged 9 years at which time he had developmental delay with little expressive language, a triangular face with a prominent chin, down slanting eyes, protuberant ears, soft skin, and hypermobile joints. His height was on the 97th centile and his weight on the 95th centile.

Terminal Deletions

Case S13

This child was referred at birth with a suspected diagnosis of Fanconi's anaemia. Symmetrical intra-uterine growth retardation, a flattened broad nasal bridge, bilateral thumb anomalies, radial dysplasia, and rockerbottom feet with overlapping toes were noted.

Case S4

This patient was referred at 17 years 9 months of age with a suspected diagnosis of fragile X syndrome. He had developmental delay and epilepsy but no dysmorphic features.

Case S17

This child was referred aged 1 year 8 months because of short stature. His height and weight were both below the 0.4^{th} centile.

Case S14

This patient was referred to the Cytogenetics Laboratory, Queens Hospital, Croydon in the neonatal period. He had congenital heart disease, low set ears, and skin tags.

Interstitial Deletions

Case S18

This child was first referred for cytogenetic investigation at 9 months of age. At this time she was noted to have developmental delay and microcephaly. She was subsequently referred with a suspected diagnosis of Angelman syndrome at 5 years 2 months because of spastic diplegia.

Case S2

This child was first referred at three years of age because of learning difficulties and unspecified dysmorphic features. He was re-referred for cytogenetic analysis aged 12 years and 7 months with a suspected diagnosis of either Smith-Magenis syndrome (SMS) or Treacher-Collins syndrome. At this time he was noted to have developmental delay, bilateral deafness, and behavioural problems including hyperactivity, poor sleep patterns, and nail-picking.

Case S15

This child was referred aged 4 years 4 months with developmental delay and a suspected diagnosis of fragile X syndrome.

5.3 Results

A summary of the cytogenetic, telomeric FISH and parental origin results are given in Table V.1. Details of the PCR results are given in Appendix 1.

Unbalanced Translocations

Case S3 46,XX,add(2)(q37)*pat.*ish der(2)t(2;14)(q37;p11)

This patient was found using high resolution G banded analysis (ISCN 850 band level) to have a terminal abnormality of the long arm of one chromosome 2 (figure V.1a). It was suspected that additional material of unknown origin had replaced the telomeric region of 2q. The same abnormality was detected in the proband's twin sister, who was similarly affected, and in both her phenotypically normal father and brother (figure V.1b). FISH with the 2q telomeric probe (210E4) confirmed the deletion of this region in the proband and her sister and identified a semi-cryptic apparently balanced reciprocal translocation between the subtelomeric region of 2q and the short arm of chromosome 14 in the father and brother (figure V.1c).

Case S5 46,XY,der(2)t(2;7)(q37.2;q36.3)*mat.* ish der(2)t(2;7)(2000a5+,210E4-).

An apparent terminal deletion of the distal long arm of one chromosome 2 was detected in this individual following high resolution chromosome analysis. The proband's mother had previously been reported to have an apparently normal karyotype when referred with a diagnosis of focal dermal hypoplasia. However, following the discovery of a cytogenetically small imbalance of chromosome 2 in her son, high resolution analysis showed a subtle balanced translocation between the distal tips of the long arms of chromosomes 2 and 7. FISH with the telomeric probes 210E4 (2q) and 2000a5 (7q) confirmed this translocation (figure V.2a).

TABLE V.1 Summary of Cytogenetic, Telomere FISH and Parental Origin Results of Terminal Rearrangements

Case No	Karyotype following conventional analysis	Karyotype following telomere FISH[3]	Parental origin
	Unbalanced translocations		
S3	46,XX,add(2)(q27.3)pat	46,XX,der(2)t(2;14)(q27.3;p11) pat	Paternal balanced translocation
S5	46,XY,der(2)t(2;7)(q37.2;q36.3)mat[2]	46,XY,der(2)t(2;7)(q37.2;q36.3)mat	Maternal balanced translocation
S9	46,XY,add(4)(p16.1)de novo	46,XY,der(4)t(4;8)(p16.1;p23.1)de novo	Maternal - Interchromosomal
S6	46,XY,del(4)(p16.3) de novo[1]	46,XY,der(4)t(4;11)(p16.3;p15.5) de novo	Unknown
S1	46,XX,add(5)(p14.2)de novo	46,XX,del(5)(p14.2p15.32)t(5;6)(p15.32;p25.1)de novo	Unknown
S12	46,XY.ish del(17)(p13.3p13.3)[1]	46,XY.ish der(17)t(5;17)(p15.33;p13.3)[1]	Unknown
S8	46,XX,der(17)t(11;17)(p15.5;p13.3)mat[1,2]	46,XX,der(17)t(11;17)(p15.5;p13.3)mat	Maternal balanced translocation
S11	46,XX. ish del(17)(p13.3p13.3)[1]	46,XX.ish der(17)t(11;17)(p15.5;p13.3)mat[2]	Maternal balanced translocation
S10	46,XY,add(18)(q21)de novo	46,XY,rea(18)(pter→q21.2::p11.1→pter)de novo	Maternal - Intrachromosomal
S7	46,XX/46,XX,add(18)(q23)de novo	46,XX/46,XX,der(18)t(4;18)(p16.3;q23)de novo	Post Zygotic Mitotic
S16	46,XY,der(22)t(14;22)(q32.33;q13.31) pat[1]	46,XY,der(22)t(14;22)(q32.33;q13.31) pat	Paternal balanced translocation

Continued...

Case No	Karyotype following conventional analysis	Karyotype following telomere FISH[3]	Parental origin
	Terminal deletions		
S13	46,XY,del(1)(p36.2)de novo	46,XY,del(1)(p36.2)de novo	Maternal
S4	46,XY,del(8)(p23.3)pat	46,XY,del(8)(p23.3)pat	Paternal deletion
S17	46,XY,del(15)(q26.2)de novo	46,XY,del(15)(q26.2)de novo	Paternal
S14	46,XY,del(16)(p13.3)de novo	46,XY,del(16)(p13.3)de novo	Unknown
	Interstitial deletions		
S18	46,XX,del(1)(p36.21p36.22)[1]	46,XX,del(1)(p36.21p36.22)	Unknown
S2	46,XY,del(10)(p15.1)de novo[1]	46,XY,del(10)(p15.1p15.3)de novo	Unknown
S15	46,XY,del(22)(q13.3)[1]	46,XY,del(22)(q13.3q13.3)	Unknown

[1] Previously reported to have an apparently normal karyotype
[2] Parental balanced translocation previously reported as normal
[3] See text for full karyotype

Case S9 46,XY,add(4)(p16.3)*de novo*.ish der(4)t(4;8) (p16.1;p23.1)(2205a2+,B31-)

High resolution analysis of this individual showed a subtle abnormality of the distal short arm of one chromosome 4. The distal tip of the short arm, including the Wolf-Hirschhorn critical region, appeared to have been replaced by a small chromosomal segment of similar size but unknown origin. FISH using a probe for the locus D4S96 confirmed that the WHS critical region was deleted. FISH with the whole panel of telomeric probes confirmed the suspected monosomy of the subtelomeric region of 4p and detected trisomy for the subtelomeric region of 8p. The additional 2205a2 signal (8p) was observed at the distal short arm of the abnormal chromosome 4. S9 was thus shown to have a semi-cryptic unbalanced translocation between the subtelomeric regions of the short arms of chromosomes 4 and 8. Parental karyotypes were shown to be normal by high resolution G-banded analysis. Fluorescence PCR analysis showed that S9 had inherited one paternal and both maternal D8S504 and D8S307 alleles. A normal biparental inheritance pattern was observed for the minisatellite marker D4S2936. This *de novo* rearrangement was therefore of maternal origin and involved trisomy for 8p of at least 2.6 Mb and monosomy for 4p of approximately 1.5 Mb (figure V.3).

Case S6 46,XY,del(4)(p16.3)*de novo*.ish der(4)t(4;11) (p16.1;p15.5)(2209a2+,B31-,D4S96-).

This individual was originally reported to have an apparently normal male karyotype. However, when re-referred by a Clinical Geneticist with a suspected diagnosis of Wolf-Hirschhorn syndrome, high resolution analysis showed a subtle terminal deletion of the distal short arm of one chromosome 4. FISH with the cosmid D4S96-, which hybridizes to the WHS critical region confirmed the deletion. Re-investigation with the whole panel of telomeric probes identified a semi-cryptic unbalanced translocation between the subtelomeric regions of the short arms of chromosomes 4 and 11 (figure V.2b). Although cytogenetic analysis of parental samples has shown this semi-cryptic rearrangement to be *de novo*, repeat samples for DNA extraction have not been received. It has not therefore been possible to determine the parental origin of this abnormality. Fluorescence PCR analysis showed that S6 had inherited three alleles at the loci 11p tel 03 and D11S922, indicating that at least 340 kb of distal 11p was duplicated. A normal heterozygous pattern was observed for the

minisatellite marker D4S2936, indicating that the extent of the 4p monosomy was less than 1.5 Mb.

Case S1 46,XX,add(5)(p15.2)*de novo*.ish del(5)(p14.2p15.32)t(5;6)(p15.32;p25.1) (36I2+,wcp6+,84C11-,D5S23-,wcp5+ ;84C11+,wcp5+,D5S23-,36I2-,wcp6+).

This individual was shown by conventional cytogenetic analysis to have an abnormality of the short arm of one chromosome 5. The distal tip of 5p, including the cri-du-chat critical region, appeared to have been replaced by a small chromosomal segment of similar size but unknown origin. FISH with the cosmid D5S23, which hybridizes to the cri-du-chat critical region confirmed a deletion of this region. FISH using the commercially available whole chromosome paint (WCP) multiprobe identified a semi-cryptic unbalanced but reciprocal translocation between chromosomes 5 and 6. Telomeric FISH probes 84c11 (5p) and 36I2 (6p) confirmed a reciprocal translocation between the regions 5p15.33→5pter and 6p15.1→6pter. Retrospective high resolution analysis failed to identify the abnormal chromosome 6. Parental karyotypes were shown to be normal by high resolution G-banded analysis.

Case S12 46,XY. ish der(17)t(5;17) (p15.33;p13.3)(84c11+,2111b1-,D17S379-)

Conventional analysis undertaken in the neonatal period failed to identify any abnormality in this individual. However, the patient subsequently died and a post mortem suggested a diagnosis of Miller-Dieker syndrome. FISH was performed on metaphases cultured from a skin biopsy and a deletion of the MDS critical region was detected using a cosmid specific for the locus D17S379. FISH with the whole panel of telomeric probes identified a cryptic unbalanced translocation between the subtelomeric regions of the short arms of chromosomes 5 and 17. This abnormality was not visible with retrospective G band analysis. Parental blood samples were unavailable and thus it has not been possible to determine the inheritance of this abnormality.

Case S8 46,XX,der(17)t(11;17)(p15.5;p13.3)*mat*. ish der(17)t(11;17) (2209a2+,2111b1-,D17S379-).

Prenatal cytogenetic analysis failed to identify any abnormality in this individual. However, after birth the suspected diagnosis of MDS directed a high resolution analysis

and a subtle terminal deletion of the short arm of one chromosome 17 was identified. FISH with a cosmid from the locus D17S379 confirmed a deletion of the MDS critical region. High resolution analysis of parental blood samples detected an apparently balanced maternal translocation between the distal short arms of chromosomes 11 and 17. Parental karyotypes had previously been reported as normal following referral for recurrent miscarriages (ISCN 550 band level). FISH with the telomeric probes 2209a2 (11p) and 2111b1 (17p) confirmed this translocation (figures V.4). Further carriers of this balanced translocation were identified in this family using the telomeric FISH probes (figure V.5). Despite a detailed investigation of the family histories of cases S8 and S11, there was no evidence that these two families were related.

Case S11 46,XX.ish der(17)t(11;17)(p15.5;p13.3)(2209a2+,2111b1-,D17S379-)*mat.*

In remarkably similar circumstances to case S8, a diagnosis of MDS was suspected at birth following the report of an apparently normal karyotype at amniocentesis. However, despite a directed high resolution analysis no cytogenetically visible abnormality of 17p was identified. FISH with a cosmid from the locus D17S379 detected a submicroscopic deletion of the MDS critical region in the proband. Parental karyotypes had previously been reported to be normal following referral for recurrent miscarriages and repeat high resolution examination failed to identify an abnormality. However, FISH analysis of parental blood samples using the same D17S379 cosmid showed that the deletion in S11 was the result of unbalanced segregation of a maternal rearrangement involving 11p. FISH with the telomeric probes 2209a2 (11p) and 2111b1 (17p) confirmed the presence of an apparently balanced cryptic translocation between the distal short arms of chromosomes 11 and 17.

Case S10 46,XY,add(18)(q21)*de novo*.ish rea(18)(pter→q21.2::p11.1→pter) (52M11+,2050a6-,52M11+).

Conventional banded analysis identified an undefined structural abnormality involving the distal long arm of chromosome 18. FISH with the telomeric probes 52M11 (18p) and 2050a6 (18q) allowed this rearrangement to be further characterised. As expected, the abnormal chromosome 18 was deleted for the probe 2050a6. However an additional 52M11 signal was detected at the distal long arm. S10 therefore has a

rearranged chromosome 18 in which the distal half of the long arm has been replaced by an additional copy of the majority of the short arm, resulting in monosomy for the region 18q21.2→qter and trisomy for the region 18p11.1→pter. Parental karyotypes were normal. Fluorescence PCR analysis showed that S10 had inherited two copies of one of the maternal D18S59 and D18S62 alleles and only one paternal 18qtel11 and 18qtel69 (D18S70) allele. This *de novo* rearrangement therefore arose from an intrachromosomal event of maternal origin and involved trisomy for 18p of at least 4.7 Mb and monosomy for 18q of at least 1.8 Mb (figure V.6).

Case S7 46,XX/46,XX,add(18)(q23)*de novo*.ish der(18)t(4;18)(p16.3;q23) (B31+,2050a6-)

Cytogenetic analysis showed the presence of two cell lines in S7. Whilst 34/55 cells had an apparently normal female karyotype, the remaining 21/55 cells had a structurally abnormal chromosome 18 which had additional material of unknown origin on the distal region of the long arm. Using the whole panel of telomere probes the add(18) was further characterised as a semi-cryptic unbalanced translocation between the distal short arm of chromosome 4 and the distal long arm of chromosome 18. The abnormal cell line is therefore monosomic for the region 18q23→qter and trisomic for the subtelomeric region of 4p. Parental karyotypes were normal. Since S7 has a mosaic karyotype with a normal cell line this semi-cryptic unbalanced translocation must be of post zygotic mitotic origin. Fluorescence PCR analysis showed two copies of the paternal 4ptel04, D4S2936 and D4S12 alleles. A normal biparental pattern of inheritance was observed at 18qtel69 (D18S70). This *de novo* rearrangement therefore involved trisomy for 4p of at least 4 Mb (paternal) and monosomy for 18q of less than 1.8 Mb (unknown origin).

Case S16 46,XY,der(22)t(14;22)(q32.33;q13.31)*pat*.ish der(22)(N85A3-)

Case S16 was previously reported to have an apparently normal male karyotype. However, re-analysis at a higher banding resolution than was possible in the neonatal period revealed a small terminal deletion of the long arm of one chromosome 22. High resolution analysis (ISCN 850 band level) of parental blood samples showed that this 'deletion' was the result of unbalanced segregation of a paternal balanced translocation

between the subtelomeric regions of 22q and 14q. FISH with the telomeric probes 2006a1 (14q) and N85A3 (22q) confirmed the subtle unbalanced translocation in S16 (figure V.7a).

Terminal Deletions

Case S13 46,XY,del(1)(p36.2)*de novo*.ish del(1)(CEB108-).

Case S13 was found using high resolution banded analysis to have a small deletion of the distal region of the short arm of one chromosome 1. FISH with the telomeric probe CEB108 (1p) confirmed a deletion of the subtelomeric region. No other telomeric probes hybridized to the distal short arm of this deleted chromosome. Parental karyotypes were normal. Fluorescence PCR analysis showed that S13 had inherited only a paternal D1S243 allele but both a maternal and paternal allele at D1S2825. This *de novo* rearrangement was therefore of maternal origin and involved monosomy for 1p of at least 3.9 Mb (figure V.8a)

Case S4 46,XY,del(8)(p23.3)*pat*.ish del(8)(2205a2-).

Cytogenetic analysis showed this individual to have a small deletion of the terminal region of the short arm of one chromosome 8. Parental chromosome analysis confirmed that S4 had inherited this deletion in an apparently identical form from his father. FISH with the telomeric probe 2205a2 (8p) confirmed the deletion. FISH with the whole panel of telomeric probes confirmed the unbalanced nature of this abnormality in both S4 and his father. Fluorescence PCR analysis showed that S4 had inherited only a maternal D8S504 allele. Biparental inheritance was observed at D8S307. These results indicate that this paternally inherited deletion is between 1.2 and 2.6 Mb.

Case S17 46,XY,del(15)(q26.2)*de novo*.ish del(15)(154P1-).

Case S17 was found using high resolution banded analysis to have a small deletion of the distal region of the long arm of one chromosome 15. FISH with the telomeric probe 154P1 (15q) confirmed a deletion of the subtelomeric region. No other telomeric probes hybridized to the distal long arm of this deleted chromosome. Parental karyotypes were normal. Fluorescence PCR analysis showed that S17 had inherited only

a maternal allele at the loci D15S642, D15S966, and D15S87. This *de novo* deletion was therefore of paternal origin and involved monosomy for 15q of at least 140 kb.

Case S14 46,XY,del(16)(p13.3)*de novo*.ish del(16)(cGG4-,RT1+).

Cytogenetic analysis carried out at the Cytogenetics Laboratory, Queen's Hospital, Croydon identified a small deletion of the distal region of the short arm of one chromosome 16. FISH on cell suspensions received from Queen's Hospital with the telomeric probe cGG4 (16p) confirmed a deletion of the subtelomeric region. By contrast, the probe RT1, derived from the 3' end of the CBP (CREB-binding protein) gene, showed signal on both 16 homologues. RT1 maps centromeric to cGG4 but still within the distal segment of 16p13.3. No other telomeric probes hybridized to the distal short arm of this deleted chromosome. Normal parental karyotypes were reported by Queens Hospital.

Interstitial Deletions

Case S18 46,XX,del(1)(p36.21p36.22)or(p36.22p36.23).ish del(1)(CEB108+, 6K215D11-).

Case S18 had previously been reported to have an apparently normal female karyotype. However, re-analysis at a higher resolution of banding revealed a small deletion of the distal short arm of one chromosome 1. FISH using the telomeric probe CEB108 (1p) and the probe 6K215D11 from the 1p36 region confirmed the deletion and showed that the deletion is interstitial rather than terminal. Unfortunately since the precise cytogenetic locus for the deleted probe 6K215D11 is not known, it has not been possible to determine the exact cytogenetic breakpoints (figure V.8b). Blood samples from the parents of case S18 have not been received and therefore we have been unable to determine the inheritance pattern of this deletion.

Case S2 46,XY,del(10)(p15.1)*de novo*.ish del(10)(p15.1p15.3)(2189b6+).

Case S2 had previously been reported to have an apparently normal karyotype. However, high resolution repeat analysis identified a small deletion of the distal short arm of one chromosome 10. Although cytogenetically this appeared to be a terminal deletion, FISH using the telomeric probe 2189b6 (10p) gave a clear signal on both 10

homolgues, showing that the deletion is interstitial rather than terminal. Parental karyotypes were shown to be normal by high resolution G-banded analysis.

Case S15 46,XY,del(22)(q13.3).ish del(22)(q13.3q13.3)(N85A3+).

Case S15 was found using conventional banded analysis to have a terminal deletion of the long arm of one chromosome 22. However, FISH using the telomeric probe N85a3 (22q) gave a clear signal on both 22 homolgues, showing that the deletion is interstitial rather than terminal (figure V.7b). Blood samples from the parents of case S15 have not been received and therefore we have been unable to determine the inheritance pattern of this deletion.

Figure V.1a Partial karyotype of Case S3

The arrow indicates the derived chromosome 2.

Figure V.1b Pedigree of Case S3.

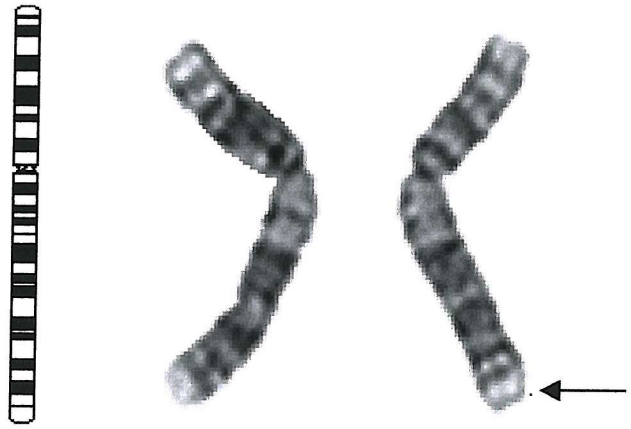
The proband S3 is indicated by the arrow.

Figure V.1c FISH result in the father of Case S3 with the 2q telomeric probe (210E4) (green) and the 14/22 centromeric probe (D14Z1) (red).

The arrow indicates the presence of the 210E4 signal on the derived chromosome 14.

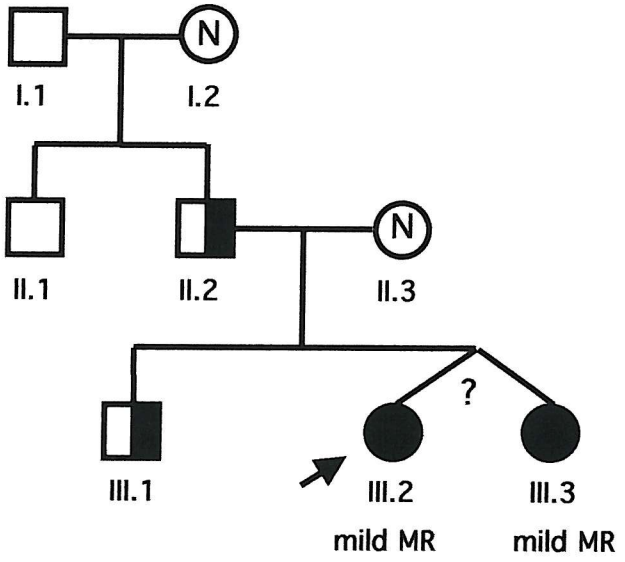
Facing page

a



2 2 add(2)(q37.2)

b



c

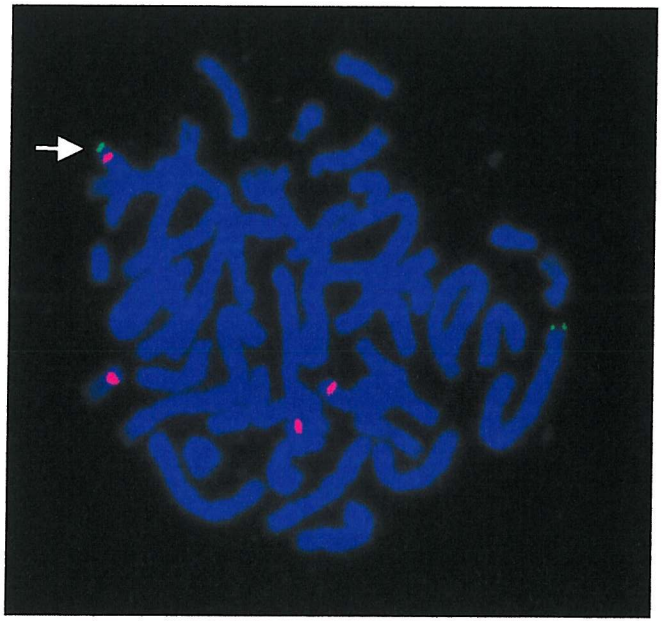
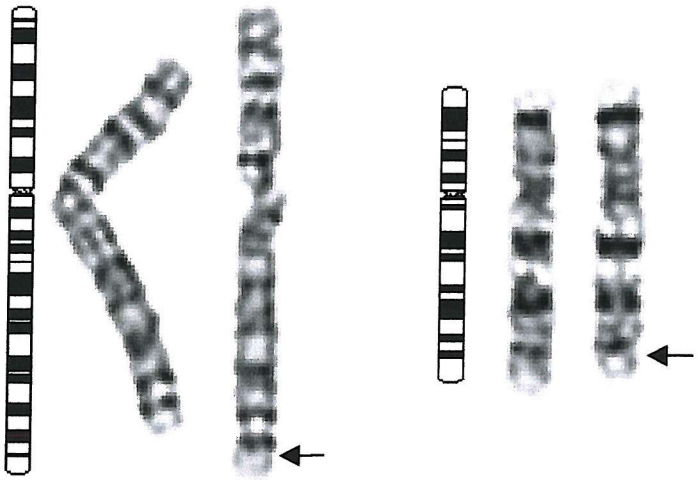


Figure V.2a Partial karyotype of the mother of Case S5.
The arrows indicate the breakpoints on the derived chromosomes 2 and 7.

Figure V.2b Partial karyotype of Case S6.
The arrow indicates the breakpoint on the derived chromosome 4.

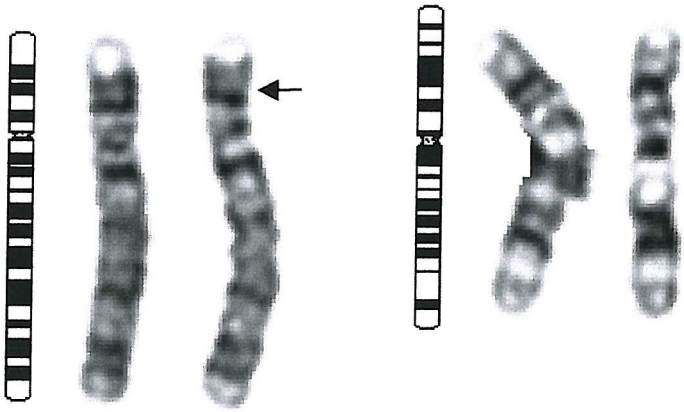
Facing page

a



2 2 der(2) 7 7 der(7)

b



4 4 der(4) 11 11 11

Figure V.3a FISH result in Case S9 with the 4p telomeric probe (B31) (red) and the 4q telomeric probe (CT55) (green).
The arrow indicates the missing B31 signal on the derived chromosome 4.

Figure V.3b FISH result in Case S9 with the 8p telomeric probe (2205a) (red) and the 8q telomeric probe (2053b3) (green).
The arrow indicates the additional 2205a2 signal on the derived chromosome 4.

Figure V.3c Partial karyotype of Case S9.
The arrow indicates the breakpoint on the derived chromosome 4.

Figure V.3d Fluorescence PCR results with the polymorphic marker D8S307 in S9.
The proband has inherited one paternal and both maternal alleles indicating that this rearrangement has arisen from a maternal inter-chromosomal event.

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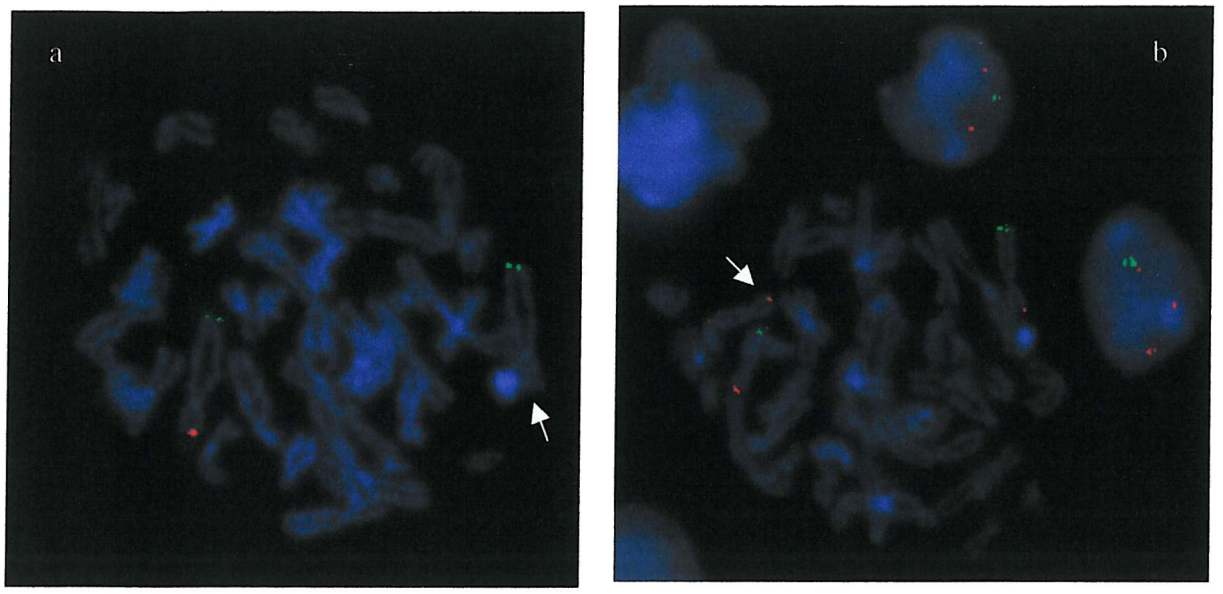
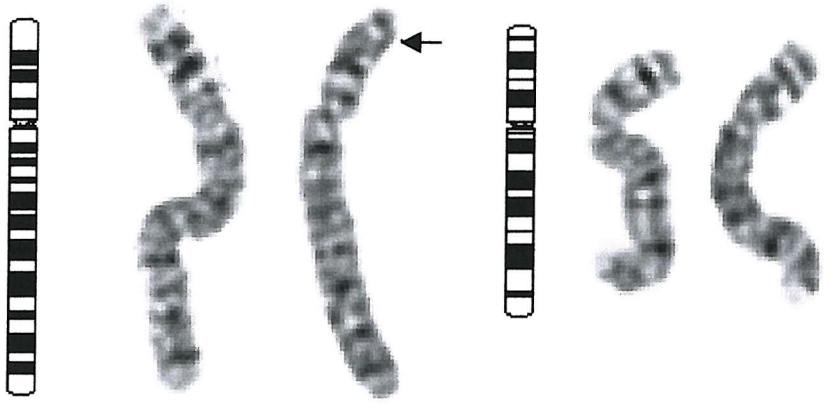

a
b

c


4
4
der(4)
8
8
8

d

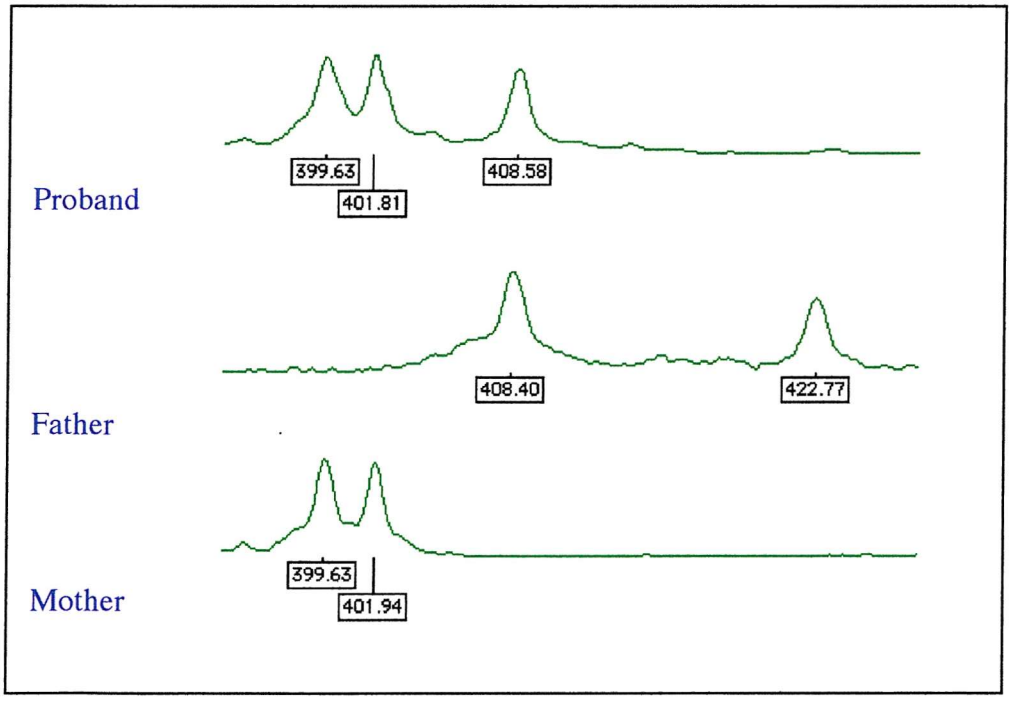
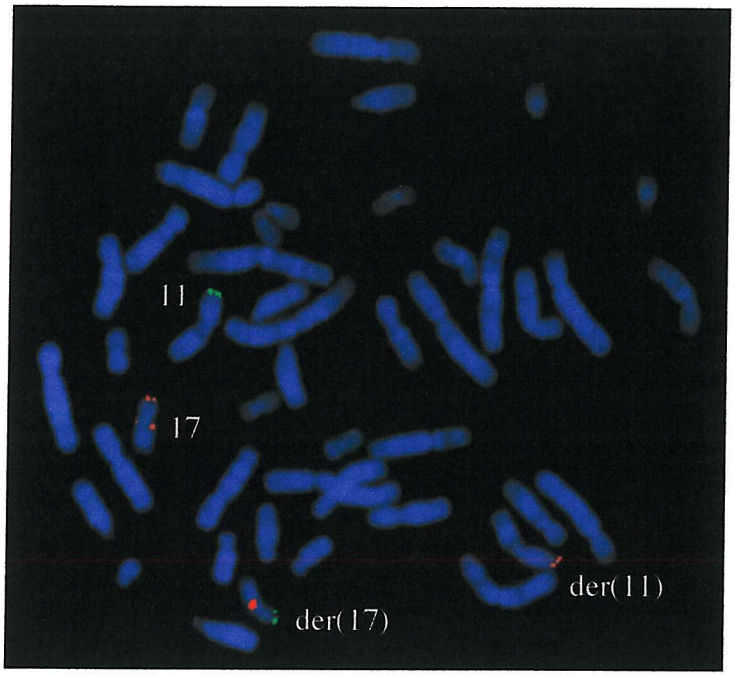

Proband
399.63
401.81
408.58
Father
408.40
422.77
Mother
399.63
401.94

Figure V.4a FISH result in the mother of Case S8 with the 11p telomeric probe (2209a2) (green) and the Miller-Dieker syndrome critical region probe (D17S379) (red).

Figure V.4b Partial karyotype of the mother of Case S8.
The arrows indicate the breakpoints on the derived chromosomes 11 and 17.

Facing page

a



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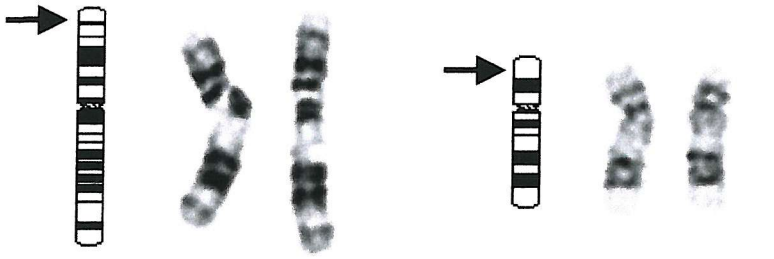

11 11 der(11) 17 17 der(17)

Figure V.5 Pedigree of Case S8.

The proband S8 is indicated by the arrow.

V.5 is the control individual C31.

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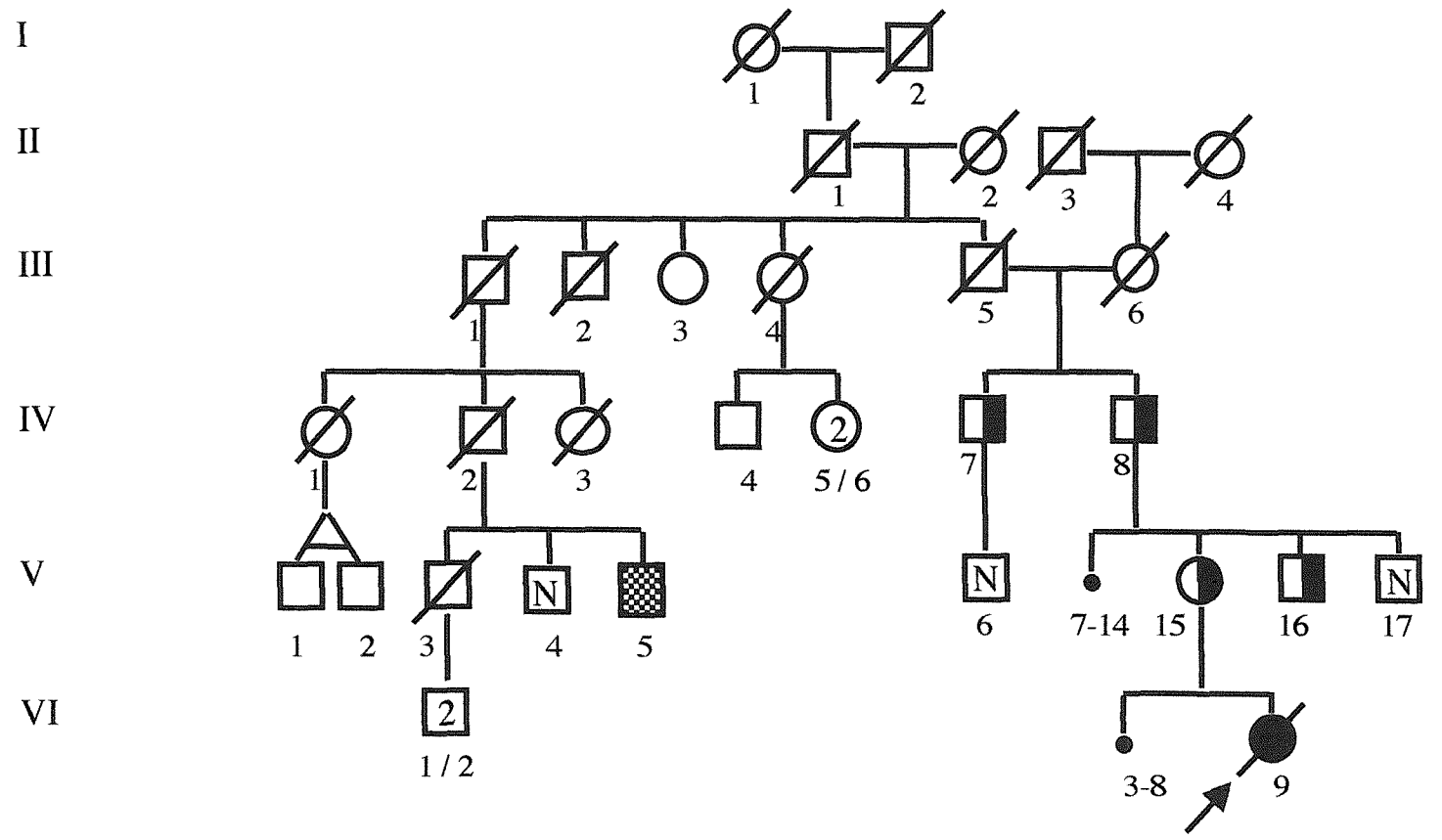
I
II
III
IV
V
VI
1
2
1
2
3
4
1
2
3
4
5
6
1
2
3
4
2
5 / 6
7
8
1
2
3
4
5
N
6
7-14
15
16
N
17
2
1 / 2
3-8
9

Figure V.6a FISH result in Case S10 with the 18p telomeric probe (52M11) (red) and the 18q telomeric probe (2050a6) (green).
The rearranged chromosome 18 with a 52M11 signal at each end is indicated by the arrow.

Figure V.6b Partial karyotype of Case S10.
The brackets indicate the duplication of most of the short arm of chromosome 18.

Figure V.6c Fluorescence PCR results with the polymorphic marker D18S59 in S10.
The proband has inherited one paternal and two copies of one of the maternal alleles indicating that this rearrangement has arisen from a maternal intra-chromosomal event.

Figure V.6d Fluorescence PCR results with the polymorphic marker 18q tel 69 in S10.
The proband has inherited only one paternal allele.

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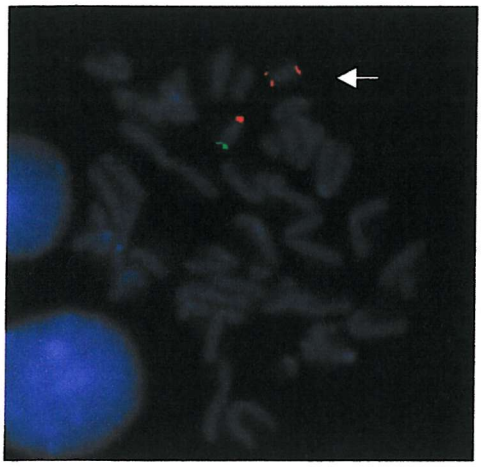
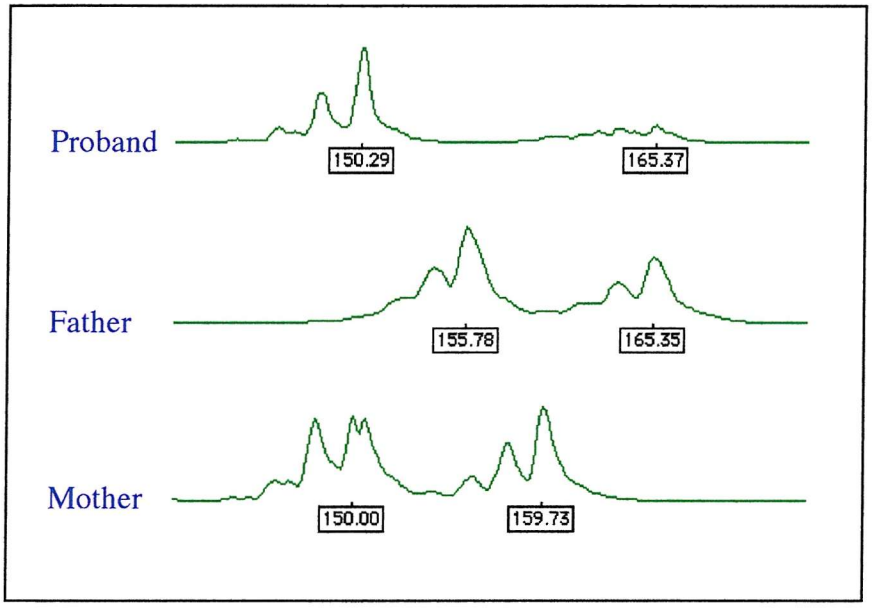
a



b

18 18 rea(18)

c



d

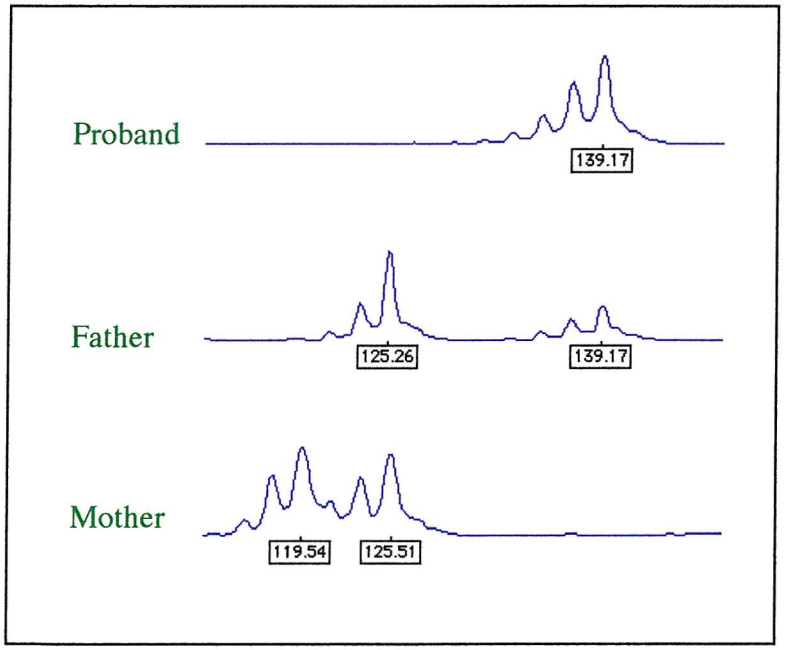
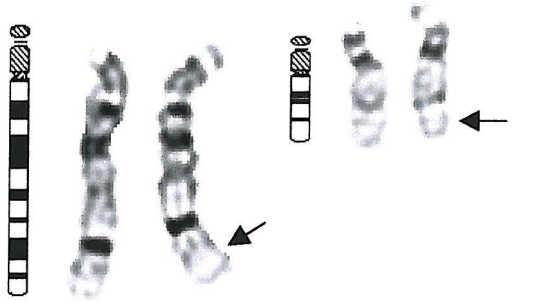


Figure V.7 Partial karyotypes showing the 22q deletions in Cases S16 (a) and S15 (b).

Figure V.8 Partial karyotypes showing the 1p deletions in Cases S13 (a) and S18 (b).

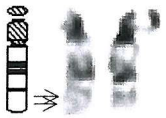
Facing page

a Case S16



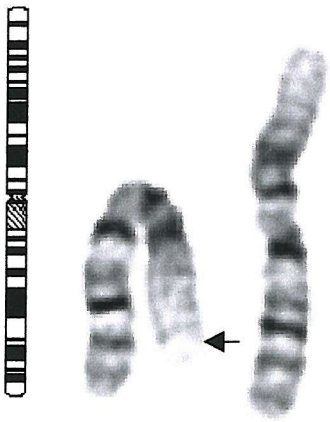
14 14 der(14) 22 22 der(22)

b Case S15



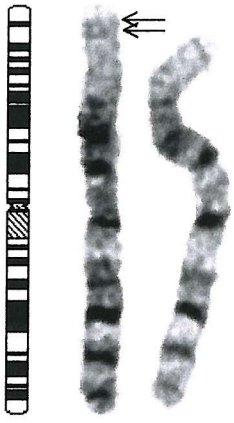
22 22 del(22)

a Case S13



1 1 del(1)

b Case S18



1 1 del(1)

CHAPTER 6

DISCUSSION

6.1 Introduction

A major challenge in clinical cytogenetics is the search for new causes of mental retardation, which is unexplained in more than half of all cases. In cases without a recognisable pattern of dysmorphic features efforts are being focused on screening for submicroscopic terminal rearrangements. Abnormalities in these regions are thought to be particularly difficult to detect using conventional cytogenetic methods because the ends of most human chromosomes are morphologically indistinguishable.

There are several features of telomere structure and function that suggest that cryptic rearrangements of these regions may underlie many cases of IMR. Firstly, chromosome pairing and homology searching initiates at the telomeres. Secondly, the subtelomeric regions are rich in pseudogenes and repeat sequences which share homology between non-homologous chromosomes resulting in mispairing at early meiotic prophase. Finally, increased recombination rates have been observed at the telomeres. Collectively, these aspects of telomere structure and function provide the opportunity for non-homologous telomere pairing which could lead to exchange events and gene dosage imbalance. Since the telomeric regions of human chromosomes are the most gene-rich regions of the genome, such gene dosage imbalance at the chromosome ends would be expected to have a disproportionately greater clinical significance than interstitial imbalances of similar size.

Cytogenetically detectable rearrangements of some specific chromosome ends are associated with well-defined mental retardation syndromes. Recently high resolution G-banded analysis, together with FISH and molecular techniques, has resulted in the delineation of further syndromes caused by terminal rearrangements.

Screening individuals with IMR for telomeric imbalances was first proposed by Ledbetter in 1992. This publication was followed by several others confirming the value of such a screen and outlining practical approaches to performing the investigation (Flint *et al.,* 1995; Lese and Ledbetter, 1998; National Institutes of Health *et al.,* 1996; Wilkie, 1993).

The first screen was carried out by Flint *et al.* (1995) and, although only 28 chromosome ends were examined in 99 IMR patients, their results indicated that at least 6% of IMR cases may be caused by cryptic telomeric imbalances. This frequency was later refined as 7.4% after including a marker for the telomeric region of 1p (Giraudeau

et al., 1997). Based on these results the authors postulated that cryptic telomeric rearrangements could represent a major cause of IMR. However, in this pilot study the vast majority of patients were highly selected. Importantly, there were no reported systematic screens to determine the incidence of cryptic telomeric rearrangements in a control population with which to compare these findings. If the significance of telomeric imbalances in the aetiology of mental retardation is to be determined, it is vital that the frequency of such imbalances among individuals with no phenotypic abnormality is known. It was therefore the intention of this study to investigate the prevelance of cryptic telomeric rearrangements in both unselected and selected populations of IMR patients as well as in an unbiased population of control individuals.

A further aim of the study was to investigate a population of individuals with cytogenetically visible, apparently terminal rearrangements for an underlying semi-cryptic telomeric exchange. It was hoped that the data obtained would provide an insight into how and when telomeric rearrangements are formed.

6.2 Frequency of cryptic telomeric rearrangements

6.2.1 Frequency of cryptic telomeric rearrangements in IMR populations

The results of the research presented in this thesis indicate that cryptic telomeric imbalances are not as frequent in patients with IMR as the pilot study undertaken by Flint *et al.* (1995) suggested. Among 200 individuals (150 unselected and 50 selected) with IMR no fully cryptic telomeric rearrangements were detected, giving a frequency of 0%. However, the upper limit of the 95% CI for unselected patients is 2.5% and for selected patients 7.1%.

To date, data from only six studies have been reported. Only the molecular investigation by Slavotinek *et al.* (1999a) and the FISH study by Knight *et al.* (1999) have been published in full. The remaining studies have been published in abstract form only and therefore only limited clinical information is available. The results of these studies are given in Table VI.1. The prevalence of cryptic telomeric rearrangements in these studies varies between 0.5 and 23.5%, with 95% CI range of 0.1-47.3%. It is clear that the variation in the number of patients entered into the studies explains some of the differences. However, there are several other real and potential sources of bias which

could contribute to the variation in prevalence estimates. These are (i) selection criteria, (ii) sex ratios of the study populations, and (iii) the resolution of conventional analysis used to exclude a chromosome abnormality prior to entry into the study.

TABLE VI.1 Reported Prevalence of Cryptic Telomeric Rearrangements Among IMR Populations

Study	Selection Criteria	Subtelomeric rearrangements detected	Prevalence (%)	95% CI
Viot *et al.* 1998	IMR + dysmorphic features +/- FH	4/17	23.5	9.6-47.3
Anderlid *et al.* 1999	IMR : 61% severe, 70% dysmorphic, 32% FH	6/44	13.6	6.4-26.7
Lamb *et al.* 1999	IMR + dysmorphic features	1/43	2.3	0.4-12.1
Knight *et al.* 1999	Mod/severe IMR Mild IMR	21/284 1/182	7.4 0.5	4.9-11.0 0.1-3.0
Slavotinek *et al.* 1999a	IMR + 3^{+} dysmorphic features	2/27		2.1-23.4
Vorsanova *et al.* 2000	IMR – severe + mild	14/407	3.4	2.1-5.7
Present Study	IMR - Selected IMR – Unselected	0/50 0/150	0.0 0.0	0.0-7.1 0.0-2.5

Figure VI.1 Summary graph of published cryptic telomeric rearrangements identified via telomere screening

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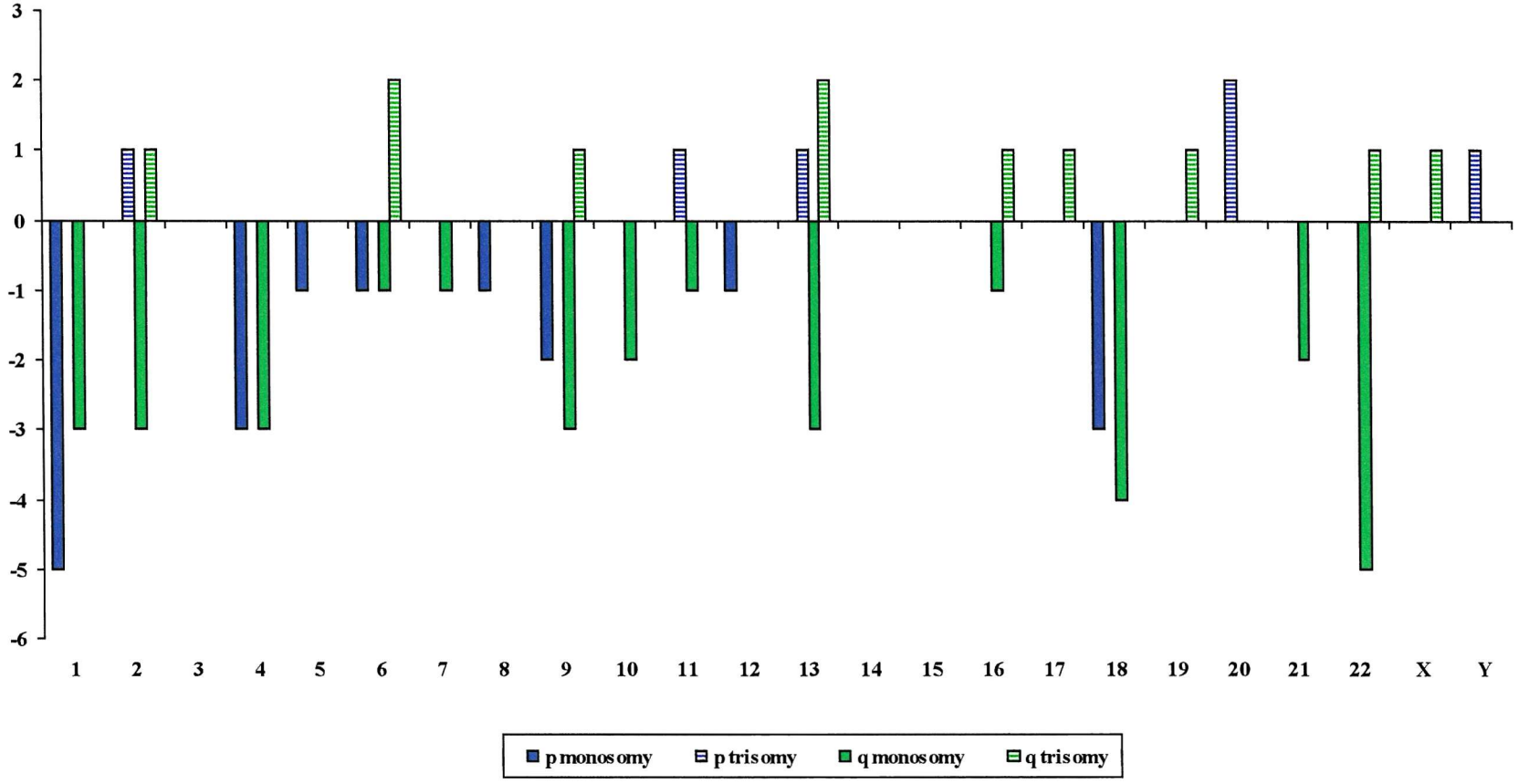
Figure VI.1 Summary Graph of Telomeric Rearrangements Identified by Telomere Screening



Anderlid *et al.,* 1999; Bacino *et al.,* 2000; Giraudeau *et al.,* 1997; Knight *et al.,* 1999; Lamb *et al.,* 1999; Riegel *et al.,* 1999; Slavotinek *et al.,* 1999a; Shaffer and Benke, 1999; Viot *et al.,* 1998; Vorsanova *et al.,* 2000

Selection Criteria

In Knight *et al.*'s large study a significant difference in frequency of cryptic telomeric abnormalities was observed between those patients with moderate to severe MR and those with mild MR (p=0.0004, Fisher's exact test). Their results indicate that the degree of mental impairment is a major predictor of the probability of finding a cryptic telomeric imbalance as the cause of the child's phenotype.

Further contributing factors include the presence of congenital anomalies in the proband and a positive family history. In all positive cases published to date, where clinical details have been provided, physical anomalies have been reported in addition to IMR. All of the 22 cases with cryptic rearrangements reported by Knight *et al.* (1999) had minor anomalies, most notably facial dysmorphism, and 16/22 (73%) had a major congenital anomaly such as microcephaly, agenesis of the corpus callosum, or hypospadias. Twelve of the imbalances were *de novo* but 10 resulted from malsegregation of a balanced parental translocation. In 9 of these 10 families, at least one other affected relative was reported; relatives with a concordant phenotype were shown to have inherited the same telomeric imbalances, whereas those with a discordant phenotype had inherited the reciprocal telomeric imbalances.

Since neither the degree of developmental delay, a positive family history or the presence of dysmorphic features or congenital anomalies were used as selection criteria for our unselected IMR population, it initially appears unsurprising that no cryptic abnormalities were detected. Furthermore, the small sample size of our selected IMR referrals may explain the lack of abnormalities in this group. However, the results of our retrospective questionnaire requesting further clinical details for all our IMR cases has shown that the selected and, to a lesser degree, the unselected study populations were, in fact, enriched for cases with moderate to severe IMR, dysmorphic features and a positive family history (Table III.1). Eleven patients, out of a total of 160 (6%) where a completed questionnaire was returned, were reported to no longer have developmental delay. Of these 11 cases, 6 were reported to have behavioural problems and 5 were initially assessed in the neonatal period but subsequently developed normally. However, moderate to severe MR was recorded for 49/150 (33%) unselected and 33/50 (66%) selected patients. Given Knight *et al.*'s estimated frequency of 7.4% for such cases, we

would have expected to identify at least 3 cryptic telomeric rearrangements among our unselected group and at least 2 among the selected patients.

Furthermore, among the 50 selected patients 44 (88%) were reported to have additional dysmorphic features and 17 (33%) had a positive family history. This phenotypic profile is remarkably similar to that of the study population reported by Anderlid *et al.* (1999) where 6 out of 44 patients were found to have a cryptic telomeric imbalance. Although there is some overlap between the 95% CI for these two populations there is a significant difference between the estimated frequencies of cryptic rearrangements (p=0.0087, Fisher's exact test).

A summary of cryptic telomeric imbalances identified during telomere screening is given in Figure VI.1. A further 3 cases of unbalanced translocations were reported by Viot *et al.*(1998) but insufficient karyotypic information meant they have been excluded from this graph. It can be seen that 25/52 abnormalities reported have included telomeric imbalances associated with recognised clinical phenotypes (1p, 4p, 9p, 13q, 18p, 18q, and 22q deletions). It is possible that a more rigorous clinical assessment could have led to a diagnosis in these cases thus excluding them from the multi-telomere screening populations. Knight *et al.* (1999) considered this possibility but state that the clinical phenotypes of such cases identified in their study were atypical. Whether this is also true for the other studies is uncertain. Nevertheless, this remains a source of potential bias.

Sex Ratios

Another factor, which may have biased the observed prevalence estimates, is the sex ratios of the study populations. Unfortunately no data regarding the proportions of male and female patients are available from any of the published screens. Among the selected cases in this study a 1:1 ratio between males and females was observed. In contrast, only 46/150 (31%) of unselected patients were female. The high proportion of male probands is a reflection of both genetic and social bias. Males are more frequently affected by mental retardation than females. Penrose (1938) and Priest *et al.* (1961), as well as others, found more males in state institutions for the mentally retarded, and Priest *et al.* also found that affected sibs were more often male. More than 30 X-linked MR genes have been identified to date. Yet routine screening is only carried out to exclude a diagnosis of fragile X syndrome. It is likely that the underlying cause of the MR in at least some of our male probands is a mutation in one of these X-linked MR genes. There

is also believed to be a bias when a patient is being considered for genetic investigation, due in part to the gross over-estimate of the frequency of fragile X syndrome when originally determined by cytogenetic evaluation. Recent molecular studies have shown that the true frequency of fragile X is in the region of 1/4000-6000 males (Crawford *et al.*, 1999; de Vries *et al.*, 1997; Youings *et al.*, 2000). Nevertheless, the exaggerated frequency of 1/1000 males has resulted in a bias whereby a male child with borderline mild MR is more likely to be referred for genetic analysis than a female child. Hence it is possible that a study population with a more balanced sex ratio than observed in our unselected group might yield a higher rate of cryptic telomeric abnormalities.

Cytogenetic Resolution

The third factor believed to be contributing to the variation in reported frequency estimates for cryptic telomeric rearrangements is the unavoidably subjective nature of conventional G band analysis, which is dependent not only on the quality of chromosome preparations and banding resolution, but also on the proficiency of the analyst. Support for this comes not only from the results of this study but also from a literature review of subtle terminal abnormalities.

Firstly, one patient (P72) among the selected IMR referrals, who had previously been reported to have an apparently normal karyotype at an ISCN 550 band level, was found to have a semi-cryptic unbalanced 13q;19q translocation using high-resolution G-banded analysis (ISCN 850 band level) and telomeric FISH investigation. Secondly, 9 of the 18 subtle terminal abnormalities described in chapter 5 were originally reported as normal. Although 4 were subsequently identified because a clinical diagnosis of either WHS or MDS directed the analysis (S6, S8, S11, and S12), the remaining 5 were identified due to a repeat analysis at a higher resolution of banding (S2, S5, S15, S16, and S15).

Thirdly, in order to compare those cases identified during telomere screening or directed FISH investigation with those detected using conventional banding, 167 subtle telomeric abnormalities have been reviewed and considerable overlap was found between visible and cryptic rearrangements (Appendix 2). Excluding the 4 cases with apparently no phenotypic effect (cases 45, 64, 109, 167), 58 (36%) were classed as visible, 26 (16%) were initially identified by conventional means but were classed as semi-cryptic because FISH with telomeric probes revealed additional complexity, and 79 (48%) were cryptic.

These figures are obviously subject to publication bias. Nevertheless, the karyotypes given in Appendix 2 do show that subtle telomeric rearrangements with apparently identical breakpoints (ISCN, 1995) are found in all three classes.

As already discussed, the morphological similarity of most terminal bands can make cytogenetic detection of telomeric imbalances difficult. In cases with small translocations this difficulty can be compounded especially if the size and banding patterns of the exchanged segments are similar. Among such cases reviewed, several are worthy of note. Firstly, the three 4p;11p unbalanced translocations (cases 60-62, Appendix 2). Two cases were reported to be cryptic (Knight *et al*, 1999; Reid *et al*, 1996), whilst the third (present study, S6) was identified during repeat analysis following a suspected diagnosis of WHS. Secondly, three cases with unbalanced segregation of a parental 2q;7q translocation are recorded (cases 51, 52, 79, Appendix 2). Of these, two cases were found to have the derived chromosome 2. The first of these was visible at the 850 band level (present study, S5) although the balanced maternal translocation had previously been missed when analysed at the 550 band level. The second was identified using routine cytogenetic analysis but was fully characterised using FISH (Speleman *et al*, 2000). The third case carried the derived chromosome 7 and was identified during telomere screening (Knight *et al.* 1999). Finally, there are two cases with MDS due to unbalanced segregation of a maternal balanced 11p;17p translocation (present study S8 and S11). In both cases the balanced maternal translocation had been missed during routine analysis, as had the derived chromosome 17 during prenatal cytogenetic diagnosis. Whilst the first case was subsequently identified during directed conventional analysis, the second was only identified by FISH. Similarly, among terminal deletion cases it can be seen that deletions involving the short arms of chromosomes 1 and 18 and the long arms of chromosomes 2, 18 and 22 have been reported to be both cryptic and visible. It is suspected that many of these rearrangements are identical, although differences between the breakpoints at the molecular level cannot be excluded.

These results raise the possibility that cryptic telomeric rearrangements reported by other groups, might be detectable by an experienced cytogeneticist with high-resolution banded analysis. Indeed, 4 of the 22 'cryptic' telomeric imbalances reported by Knight *et al.* (1999) and 1 of the 2 reported by Slavotinek *et al.* (1999a) were visible at the 850 band level with hindsight. Similarly, several other 'cryptic' abnormalities identified using FISH or molecular techniques have retrospectively been found to be

visible at the 550 band level (Bartsch *et al.,* 1997; Biesecker *et al.,* 1995; Groen *et al.,* 1998).

It is difficult to give a definitive size for imbalances which should be visible with G banded analysis. However, imbalances of ~4 Mb in proximal 15q, deletions associated with Prader-Willi and Angelman syndromes and duplications associated with MR / autistic spectrum disorder (Christian *et al.,* 1999; Amos-Landgraf *et al.,* 1999), are in the majority of cases visible at the resolution afforded by routine cytogenetic analysis, as are deletions of ~5 Mb in proximal 17p associated with Smith-Magenis syndrome (Chen *et al.,* 1997). In contrast, deletions associated with Williams syndrome and DiGeorge/ CATCH22, of ~1.5 Mb in proximal 7q (Tassabehji *et al.,* 1999) and 1.5-3 Mb in proximal 22q (Carlson *et al.,* 1997) respectively, can rarely be identified with conventional banding. This gives us an estimate of the resolution of cytogenetics in the order of ~3-4 Mb.

Knight *et al.* (1999) have attempted to size the telomeric imbalances in their cases. However, in 15/22 rearrangements the extent of the imbalances are given in centimorgans. These results are very ambiguous since at the telomeres there is known to be both increased recombination and large differences in the male to female recombination rates. Of those 7 cases where a size is given in megabases, only two involved imbalances of less than 4 Mb. Both of these were deletions of chromosome 22q, one previously described by Flint *et al.* (1995) of 130 kb and one estimated to be between 0.1-1.0 Mb. The other 5 cases appear to involve imbalances within the range presumed to be visible with conventional cytogenetics.

However, with the availability of the Human Genome Project sequence data, it has become apparent that the linkage maps used to order molecular markers and size imbalances are somewhat inaccurate. For instance, when compiling a sequence-based integrated map of chromosome 22, Tapper *et al.* (pers. com.) have found that only 42% of molecular markers are within 1 Mb of their correct location, 71% within 2 Mb, and 93% were within 4 Mb. The maximum discrepancy was 6.4 Mb. This makes correlating size estimates derived from molecular investigations with polymorphic markers to the 'visibility' of any particular abnormality difficult.

For this reason exact size determination has not been attempted for the telomeric imbalances detected during this study using high resolution G banding. However, whilst

investigating their parental origin, it has been found that some of the imbalances are apparently below the size previously thought to be detectable using G band analysis. The paternally inherited terminal deletion of 8p (S4) was estimated to be between 1.2 and 2.6 Mb, and the *de novo* unbalanced 13q;19q rearrangement (P72) involved monosomy of 13q of less than 450 kb.

Nevertheless, although it may be suspected that some of the abnormalities reported by other groups are not truly 'cryptic', it cannot be denied that all of the telomeric abnormalities detected during multi-telomere screening had been reported normal by routine cytogenetic analysis on at least one previous occasion. The same is true for the semi-cryptic rearrangement identified in the selected population and 9/18 subtle terminal rearrangements in the third population of structural chromosome aberrations. So although the case for more detailed cytogenetic analysis can be made, it can also be argued that the multi-telomere FISH approach is a more reliable way to identify cryptic and semi-cryptic telomeric rearrangements.

Among the pathologically significant subtle terminal rearrangements reviewed 84/163 (52%) were reported as terminal deletions and 69 (42%) were shown to be unbalanced terminal rearrangements. The former is certainly an over estimate since only 36/84 (43%) of terminal deletions had been fully investigated with the whole panel of telomere probes. Importantly, the remaining 10 abnormalities (6%) have been shown to be interstitial rather than terminal. Caution must therefore be observed if multi-telomere FISH is to be relied upon to detect subtle terminal chromosome abnormalities. Not only would these 10 cases have gone undetected by such an approach but other more proximal interstitial chromosome imbalances, also a significant cause of MR, may be missed if sub-optimal G banded analysis is considered acceptable in conjunction with a telomere screen.

In summary the results of this study indicate that true cryptic telomeric abnormalities are not a significant cause of IMR in unselected populations. Whilst submicroscopic telomeric rearrangements undoubtedly exist it is likely that the prevalence estimates reported by other groups have been biased by clinical selection and possibly also by sub-optimal conventional analysis.

6.2.2 Frequency of cryptic telomeric rearrangements in control populations

In contrast to the IMR study populations, 2/150 (1.3%) of individuals in the control population were found to have cryptic telomeric imbalances. This frequency is not statistically different to that observed in either our unselected IMR population (p=0.249, Fisher's exact test) or our selected IMR population (p=0.562, Fisher's exact test).

The only other systematic multi-telomere screen in control individuals with which to compare these findings was carried out by Knight *et al.* (1999). They identified no cryptic telomeric abnormalities among 25 healthy male volunteers or 50 healthy male relatives of probands attending a genetics clinic (Table VI.2). Although there was a significant difference between the frequencies observed among their moderate to severe MR cases and their control population (p=0.006, Fisher's exact test), the difference in prevalence between their mild MR group and their controls did not reach statistical significance (p=0.708, Fisher's exact test).

TABLE VI.2 Reported Prevalence of Cryptic Telomeric Rearrangements Among Control Populations

Study	Selection Criteria	Subtelomeric rearrangements detected	Prevalence (%)	95% CI
Knight *et al.* 1999	Healthy male volunteers or male relatives attending genetics clinic	0/75	0.0	0.0-4.9
Present Study	Relatives of probands with numerical or structural chromosome abnormality	2/150	1.3	0.4-4.7

The potential for bias in the selection of control individuals for this study should be considered. All were karyotypically normal parents or relatives of probands in whom a chromosome abnormality had been detected. There was no known family history of MR for 61 control individuals. The families of the remaining 89 controls had been ascertained because of MR or dysmorphism in the proband. However, in 75 of these the chromosome imbalance was believed to be the sole cause of the proband's MR, either

because the imbalance segregated with MR in the family, or because a review of the literature had identified previous cases with similar imbalances in association with similar phenotypes. A further seven were apparently balanced *de novo* chromosome abnormalities which may have been responsible for the probands' phenotypes either as a result of a microdeletion or disruption of gene(s) at one or more of the breakpoints. In the last 7 cases the apparently balanced chromosome rearrangement was believed to be coincidental to the probands' phenotype. Although inclusion of these last 14 individuals could have biased the control population, it is worth noting that the two cryptic telomeric abnormalities were not identified from among this group. One was identified in an individual referred following detection of a numerical abnormality at amniocentesis, and the other in a relative of a proband with MDS due to unbalanced segregation of a maternal translocation.

The particular telomeric imbalances in the two cases identified during this study will be discussed below (section 6.3.2). However, chromosomal imbalances without phenotypic effect are not without precedence. There is an increasing number of reports of nonpathogenic euchromatic imbalances which are visible at the cytogenetic level of resolution (reviewed by Barber *et al.*, 1998; Gardner and Sutherland, 1996). It is reasonable therefore to assume that nonpathogenic euchromatic imbalances detectable at the higher resolution afforded by FISH analysis also exist and may even be more frequent. The results of this study suggest that cryptic telomeric rearrangements may be a not uncommon finding in control individuals and that their prevalence among unselected IMR cases is not significantly different to the general population frequency. This has important implications when determining the significance of a cryptic telomeric imbalance to a patient's phenotype. It is possible that some rearrangements identified by telomere screening are coincidental to the patients' mental retardation. The frequency of cryptic telomeric imbalances detected in IMR populations may not therefore represent the incidence of pathological imbalances.

6.3 Significance of telomeric rearrangements

The telomeric regions of human chromosomes are believed to have the highest concentration of genes in the genome and therefore imbalances of these regions are likely to have disproportionately greater clinical significance than interstitial imbalances of

similar size. However it has been shown that telomeres vary in their H3 isochore concentration (Saccone *et al.*, 1992) and hence in their gene concentration. Thus it is reasonable to surmise that the clinical significance of a cryptic telomeric imbalance will depend on the particular telomere involved.

Furthermore, it has been shown that many non-functional pseudogenes map to the subtelomeric regions of chromosomes (Kermouni *et al.*, 1995; Rouquier *et al.*, 1998). These pseudogenes would contribute to the GC-rich H3 isochores and therefore a high H3 isochore concentration may not necessarily indicate an enrichment of functional genes. Hence the need for caution when interpreting the significance of telomeric abnormalities.

As well as causing gene-dosage imbalance it is possible that cryptic and subtle telomeric rearrangements could have phenotypic consequences as a result of another pathological mechanism. Changes in the length and structure of the subtelomeric region might affect the expression of nearby genes by position effect variegation (PEV). PEV has been proposed in FSHD where rearrangements involving subtelomeric repeats have been implicated (van Deutekom *et al.*, 1993 and 1996). Evidence for position effects, whereby chromosomal rearrangements may effect the regulation of gene(s) either upstream or downstream of a breakpoint, has also been reported for WAGR on chromosome 11p (Fantes *et al.*, 1995) and camptomelic dysplasia on chromosome 17q (Wirth *et al.*, 1996). More recently, Barbour *et al.* (2000) have shown that inactivation of a structurally normal α-globin gene by a negative chromosomal position effect was the cause of alpha-thalassaemia in one family. It is possible that other telomeric rearrangements may result in structural modification of the telomere which in turn might result in altered expression of nearby genes and consequential pathogenic effect(s).

6.3.1 Clinical significance of cryptic and subtle telomeric abnormalities in patients with IMR.

When attributing the cause of a patient's phenotype to a chromosome abnormality, two points should be considered. Firstly, is there a relevant family history and does the phenotype segregate with the chromosome imbalance. Secondly, if the imbalance has arisen *de novo* or has been inherited from a balanced parental rearrangement but no other relatives carry an unbalanced form, are there precedents in the literature which could corroborate an association between the imbalance and the

phenotype. Such cases can therefore be considered as two groups, those where there is substantial evidence to establish a causal association, and those where the relationship between the telomeric imbalance(s) and the patient's phenotype is uncertain.

Causal Association Suggested by Family History

Among the cytogenetically identified subtle terminal chromosome rearrangements we have investigated during this study, although six had been inherited, in only one case was there a family history which could support a causal association between the imbalance and the proband's phenotype. This was the terminal deletion of 8p described in patient S4 and in his father. Both had moderate to severe MR and the proband also had epilepsy. A review of 8p deletions (Wu *et al.,* 1996) has shown that the majority of patients with terminal 8p23.1 deletions have mental retardation and behavioural problems, with a proportion of patients also having seizures, congenital heart defects and genitourinary anomalies. However, Reddy (1999) has reported two terminal 8p23.1 deletions in association with an apparently normal phenotype. The first was ascertained prenatally but shown to be carried by the phenotypically normal father. The second was detected when the proband was being investigated for recurrent miscarriages. In this particular case the deletion was mosaic and thus its clinical effect may have been tempered. However, these cases do raise the possibility that the deletion in the present case is coincidental to the patient's MR. Molecular analysis using polymorphic minisatellite markers has indicated that the extent of the deletion in father and son is between 1.2 and 2.6 Mb. Furthermore, the deletion breakpoint in this case was determined by high resolution analysis to be at 8p23.3. Since this deletion is therefore smaller than previously reported 8p monosomies the possibility that it is a coincidental finding and that the proband and his father share an independent MR condition cannot be excluded.

Causal Association Supported by Evidence in the Literature

The only telomeric imbalance identified among the IMR populations of the present study was a derived chromosome 13 from a *de novo* 13q;19q telomeric translocation (P72). The abnormality was detected by high resolution G-banded analysis and confirmed and characterised by multi-telomere FISH. The proband was monosomic for the region 13q34→qter and trisomic for the very distal region of 19qter. This 6 year

old boy was referred with mild developmental delay, adducted thumbs and suspected diagnoses of fragile X syndrome or CATCH 22.

Owing to the very small imbalances involved it is difficult to be certain of the phenotypic consequences of this rearrangement. However, there are five reports in the literature of a deletions of 13q34 with which to compare our case (de Die-Smulders *et al.*, 1999; Flint *et al.*, 1995; Guichet *et al.*, 1998; Knight *et al.*, 1999; Vorsanova *et al.*, 2000). Of these, four are classed as cryptic, two of which are pure monosomies. The other three abnormalities are unbalanced translocations involving concomitant trisomies for Yp, 5q, and 20p. All five cases were ascertained due to MR. Unfortunately further clinical details are only available for two cases. The first, a *de novo* unbalanced Yp;13q translocation, was identified during a screen of patients with IMR for telomeric rearrangements. The proband, a 3 year old boy, presented with developmental delay, mild facial dysmorphism and microcephaly (Flint *et al.*, 1995). The second, a paternally derived unbalanced 13q;20p translocation, presented with severe MR, growth retardation, and dysmorphic features (de Die-Smulders *et al.*, 1999). No deletions of 13q were detected in the 150 control individuals in the present study, in 160 control families in Flint *et al.*'s (1995) study, or in 75 control individuals in Knight *et al.*'s (1999) study. It is therefore probable that this telomeric partial 13q monosomy is the cause of the developmental delay in this patient.

In contrast, it is difficult to assess the impact that the small distal 19q trisomy will have on the phenotype of P72. There are three other cryptic duplications of distal 19q in the literature. The first, a paternally derived unbalanced 1p;19q translocation, was reported by Knight *et al.* (1999). The proband presented with moderate MR, short stature and conductive hearing loss. In the second case, an unbalanced 17p;19q translocation, the proband presented with MDS (Alvardo *et al.*, 1993). The third cryptic 19q trisomy was identified in a patient with features consistent with the 22q terminal deletion syndrome. This patient was found to have an unbalanced 19q;22q translocation as a result of malsegregation of a maternal balanced translocation (Praphanphoj *et al.*, 2000). In all of these three cases, the clinical phenotype of the probands can be accounted for by the concomitant monosomy of either 1p, 17p, or 22q. It is therefore uncertain whether such a small trisomy for distal 19q has any pathological effect. Saccone *et al.* (1992) have shown that chromosome 19 appears to consist almost entirely of H3 isochores. However the telomeric region of 19q (q13.4→qter) is G-band positive

and, in contrast to the rest of the chromosome, does not appear to contain any H3 isochores. It is possible, therefore, that this duplication does not contain any functional genes and may not contribute to the phenotype of P72.

Two cases were referred with a suspected diagnosis of WHS and were found to be monosomic for distal 4p, including the WHS critical region (S6 and S9). There is therefore little doubt that monosomy of 4p was the cause of their phenotypes. However, in both cases a concomitant cryptic trisomy was detected, 11p and 8p respectively. Similarly, three cases of MDS were investigated and found to be unbalanced translocations involving monosomy for distal 17p. In two of these cases the unbalanced karyotype in the proband was a result of malsegregation of a balanced maternal 11p;17p translocation (S8, S11). In the third case the proband was shown to have a cryptic trisomy of distal 5p (S12). The contibution of these trisomies to the phenotype of these patients with WHS and MDS is less clear. No precedents were found in the literature for a cryptic duplication of distal 8p and although there are two reported cases of cryptic 11p trisomy, both were found in patients with WHS and deletions of 4p (Knight *et al.,* 1999; Reid *et al.,* 1996). There appeared to be no significant phenotypic impact associated with a cryptic trisomy 11p in these cases. Duplications of distal 5p have been reported in association with a phenotype characterised by minor facial anomalies, developmental delay and seizures (Chia *et al.,* 1987; Qumsiyeh *et al.,* 1993). Although masked by the more severe clinical features of MDS, it is possible that the cryptic 5p trisomy contributed to the phenotype of S12.

In case S1 of this study, although initially reported to have a terminal abnormality of 5p, further investigation has shown this girl to have a semi-cryptic, *de novo*, reciprocal but unbalanced rearrangement involving distal 5p and distal 6p resulting in monosomy for 5p14.2-5p15.32. This interstitial deletion includes the cri-du-chat critical region, and although not initially suspected, her phenotype has since been confirmed to be consistent with this diagnosis.

Two *de novo* cases of distal 1p monosomy were investigated (S13, S18). Although one was shown to be a terminal deletion the other was found to be a distal interstitial deletion. Deletions of the distal short arm of chromosome 1 represent a recently defined syndrome with a distinct pattern of phenotypic features, including growth delay, moderate to severe MR, cardiac malformations and dysmorphic facies

(Faivre *et al.,* 1999; Slavotinek *et al.,* 1999b). In case S13 although the patient was too young to assess any mental impairment, his symmetrical IUGR, facial dysmorphisms and congenital anomalies are consistent with the terminal 1p deletion. The majority of patients reported with distal 1p monosomy have a terminal 1p36 deletion. However, two previously reported cases have been shown to have distal interstitial deletions (Biegel *et al.,* 1993; Howard and Porteous 1990). In both cases there was some phenotypic overlap with terminal deletion cases and the developmental delay and microcephaly reported in S18 are also consistent with the 1p36 deletion syndrome.

Two apparently terminal deletions of 22q were characterised (S15, S16). One was found to be the result of malsegregation of a paternal balanced 14q;22q translocation, while the other was shown to be a distal but interstitial 22q deletion. Deletions of distal 22q have previously been reported and patients share a common phenotype which includes generalised developmental delay, normal or accelerated growth, hypotonia, delayed expressive speech, and mild facial dysmorphism (Doheny *et al.,* 1997; Nesslinger *et al.,* 1994). The overgrowth, delayed development and speech, and facial dysmorphism recorded in patient S16 are therefore consistent with the terminal 22q monosomy. The extent of the concomitant trisomy of distal 14q was minimal such that it may have no discernable effect on the patient's phenotype. No precedent in the literature was found. In contrast, although patient S15 was referred with developmental delay, no additional features associated with monosomy of distal 22q were recorded. It is possible that he does have some of these features but they were overlooked when the clinical details were entered on the patient's referral form. Only one other interstitial deletion of this region of 22q has been reported (Romain *et al.,* 1990). This patient presented with developmental delay, hypotonia and facial dysmorphism. As with distal 1p deletions it is likely that there is at least some phenotypic overlap between terminal and interstitial 22q13 deletions.

Loss of distal 18q also represents a well-established syndrome (Stathdee *et al.,* 1995; Kohonen-Corish *et al.,* 1996) but with variable clinical severity. Although the majority of cases reported have involved monosomy greater than that identified in one of the two cases of terminal 18q rearrangements in this study, there are precedents in the literature for cryptic and semi-cryptic 18q deletions. Vorsanova *et al.* (2000) reported a case with pure 18q23 deletion in association with MR, and Knight *et al.* (1999) described a further patient with pure terminal 18q deletion, MR and dysmorphic features. Other

cases have been reported with 18q monosomy and trisomy for distal Xq (Knight *et al.*, 1999), 2p (Lamb *et al.*, 1999), 5p (Reddy and Fugate 1999), and 21q (Bartsch *et al.*, 1997). All patients presented with MR, although in the last case any phenotypic effect of the 18q monosomy would have been masked by the manifestation of Down syndrome due to the concomitant 21q trisomy. Neither of the two present cases presented with MR since they were referred in the neonatal period. However, they did manifest dysmorphic features which have been reported in some cases of 18q monosomy (Buyse, 1990). In case S10 the rearrangement was also shown to involve trisomy for most of the short arm of chromosome 18. It is unlikely that this 18p duplication would have significant additional phenotypic consequences. Evidence for this comes from Wolff *et al.* (1991) who reported a duplication of 18p in a mother and child with apparently normal phenotypes. They also reviewed 14 additional cases of trisomy 18p which were all either phenotypically normal or manifested non-specific abnormalities which may have been the result of concomitant chromosomal imbalances. In patient S7 the mosaic terminal rearrangement of 18q was shown to be an unbalanced 4p;18q translocation. There does not appear to be any previous reports of cryptic telomeric duplications of 4p. The closest parallel found is a report by Partington *et al.* (1997) of a duplication of the distal region of 4p associated with overgrowth, dysmorphic features and mild to moderate MR. However, the phenotypic consequences in the present case are uncertain because of (i) the small size of both imbalances - molecular investigations have indicated that the extent of the 18q monosomy is less than 1.8 Mb, whilst the 4p trisomy is greater than 4 Mb; and (ii) the presence of the normal cell line which may ameliorate the phenotypic effects which might otherwise have been found.

Two cases with terminal rearrangements of 2q were investigated. The first (S3) was shown to be a paternally derived 2q;14p translocation. Both the proband and her monozygotic twin sister had inherited an unbalanced form resulting in monosomy for 2q37.3 to 2qter and trisomy for the terminal region of the acrocentric short arm of chromosome 14. The phenotypically normal father and brother carried the balanced form of the translocation. The duplication of 14p would not be expected to have any phenotypic consequences since satellited chromosomes with no other euchromatic imbalance have been reported in phenotypically normal individuals (Reddy and Sulcova, 1998). Deletions of distal 2q have previously been reported and Reddy *et al.* (1999) have recently reviewed 36 cases of terminal 2q37 deletions. Several patients presented

clinically with features of Albright's hereditary osteodystrophy (eg Phelan *et al.*, 1995; Power *et al.*, 1997) or with brachydactyly type E (Wilson *et al.*, 1995). Deletions of this region of chromosome 2 have also been associated with developmental delay, hypotonia, minor facial dysmorphism, and foot anomalies. It is therefore likely that the telomeric 2q deletion is the cause of the developmental delay and foot anomalies described in both S3 and her sister.

Finally, a suspected terminal deletion of 15q (S17) was confirmed as a 'pure' deletion resulting in monosomy for the region 15q26.2→qter. There does not appear to be any previously reported cases of such a small 'pure' deletion of this region of chromosome 15. Roback *et al.* (1991) described a slightly larger deletion of the region 15q26.2→qter which included the insulin-like growth factor 1 receptor gene (IGF1R) in an infant with intra-uterine growth retardation. It is possible the short stature reported in S17 also results from hemizygosity at the IGF1R locus. Notably, no developmental delay was recorded in patient S17. Since the deletion in the present case is smaller than that reported by Roback *et al.* it is not surprising that it has resulted in a less severe phenotype.

Clinical Significance Uncertain

In the following three cases where a subtle telomeric chromosome abnormality was identified no categorical evidence in the literature for a causal association between the imbalance(s) and the patients' phenotypes was found. However, given that (i) all these abnormalities were visible with conventional analysis, with a presumed resolution of approximately 3 Mb, and that (ii) on average in the human genome, 1 Mb of DNA contains approximately 10 genes and in the subtelomeric regions the gene density is estimated to be ~one gene per 10-20 kb (Flint *et al.*, 1997b), it is highly unlikely that these chromosomal imbalances would be without a significant phenotypic effect.

The second case of 2q monosomy (S5) investigated during this study was shown to be an unbalanced 2q;7q translocation resulting from malsegregation of an apparently balanced maternal translocation. The proband had complex congenital heart disease, post axial polydactyly, hypotonia, and global developmental delay. However, redundant skin, a linear raw area at the neck, syndactyly, and eye abnormalities were all recorded in both S5 and his mother. None of these features have been previously associated with

deletions of distal 2q. Duplication of 7q36→7qter is associated with MR but not with major malformation or distinct dysmorphism (Schinzel, 1990; Verma *et al.,* 1992). Furthermore, it is likely that the duplication detected in the present case is smaller than those previously reported and therefore may have little additional effect. The relationship between the telomeric imbalances detected in S5 and the major phenotypic abnormalities found in both S5 and his mother are unclear. It is possible the translocation breakpoint(s) interrupts and important gene(s), but also possible that a second independent genetic condition is present in both of them.

Case S14 was investigated and found to be deleted for the telomeric region of 16p. It was shown that the deletion in this patient did not extend to the Rubinstein-Taybi locus at 16p13.3. Congenital heart disease, low set ears and skin tags were recorded in this newborn boy. There have been many reports of terminal deletions of 16p in patients with ATR-16 (Lamb *et al.,* 1993; Wilkie *et al.,* 1990b). It is thought that this is a contiguous-gene syndrome resulting from deletion of several genes including the α-globin genes and a putative mental retardation gene. However, the phenotypic range of ATR-16 syndrome is still poorly defined. The most frequently observed features, besides thalassaemia and MR, include hypertelorism, talipes equinovarus, down-slanting palpebral fissures, a broad flat nasal bridge, and epicanthic folds (Lindor *et al.,* 1997; Wilkie *et al.,* 1990b). None of these features were recorded in this patient and he was too young to be assessed for developmental delay. Horsley *et al.* (1999) have studied a series of 35 patients with deletions of 16p13.3. Of these, 20 had thalassaemia with associated MR, and the remaininng 15 had thalassaemia but were otherwise normal. One of these latter patients was shown to have a deletion encompassing at least 13 genes. From these data, Horsley *et al.* concluded that none of these genes are recessive or dosage sensitive. Although it is likely that this deletion is the cause of this patient's phenotype, an assessment at a later stage of development will be necessary to determine if this deletion encompasses the ATR-16 critical region.

The final case (S2) was initially reported to have a *de novo* subtle terminal 10p detetion. Re-investigation has shown that this deletion is in fact interstitial, resulting in monosomy for 10p15.1→p15.3. This child was referred with developmental delay, bilateral deafness, and behavioural disturbances including hyperactivity and nail picking. No precedent was found in the literature for such a small deletion of this region of 10p

with which to compare his phenotype. However, deletions of 10p have been reviewed by Shapira *et al.* (1994) and it is interesting to note that deafness was reported in 2/14 cases with larger 10p deletions.

Cryptic Telomeric Imbalances Detected During Screening

To date 52 telomeric rearrangements detected during screening programmes of patients with IMR have been reported (Anderlid *et al.,* 1999; Bacino *et al.,* 2000; Giraudeau *et al.,* 1997; Knight *et al.,* 1999; Lamb *et al.,* 1999; Riegel *et al.,* 1999; Slavotinek *et al.,* 1999a; Shaffer and Benke, 1999; Viot *et al.,* 1998; Vorsanova *et al.,* 2000). Unfortunately, in three cases insufficient karyotypic information excludes them from further discussion (cases 69, 76, and 90 in appendix 2). As already discussed, from Figure VI.1 it can be seen that 25 of these have involved telomeric imbalances with well-established clinical phenotypes (1p, 4p, 9p, 13q, 18p, 18q, and 22q deletions).

A further seven cases were the result of malsegregation of a parental balanced translocation, and in six of these it was shown that unbalanced derivatives of the translocations segregated with MR in the families (Knight *et al.,* 1999). In these cases, as in those cases where the telomeric imbalances have been associated with well-defined clinical phenotypes, there is substantial evidence that the rearrangements are the cause of the patients' disabilities.

Of the remaining 16 cases, 14 involved cryptic terminal deletions of 1q, 2q, 4q, 5p, 6p, 6q, 9q, 10q, 11q, 16q, and 21q. A further case was found to have an unbalanced 9q;21q rearrangement resulting in 9qter trisomy and 21q monosomy. In these cases there is need for caution when attributing pathogenicity to the imbalances. Knight *et al.* (1999) state that in their cases mapping data have shown that the rearrangements either include regions previously found to be deleted in patients with MR, or are so large that they are almost certain to have an associated phenotype. However, whether the same is also true for the cryptic rearrangements detected by other groups has yet to be established. Of particular note are the two cryptic 2q deletions (Anderlid *et al.,* 1999; Viot *et al.,* 1998) and the cryptic 4q deletion (Vorsanova *et al.,* 2000) which will be discussed in sections 6.3.3 and 6.3.2 respectively.

In the final case, an unbalanced 1q;13q rearrangement resulting in partial 1q monosomy and partial 13q trisomy was detected in a child with developmental delay and

moderate cerebral palsy. Although initially thought to account for the proband's phenotype, this unbalanced telomeric rearrangement was later found in the phenotypically normal father (Shaffer and Benke, 1999). This case highlights the uncertainty surrounding the pathogenicity of cryptic telomeric imbalances, since an apparently identical 1q;13q rearrangement was reported by Knight *et al.* (1999) in a family where telomeric imbalances segregated with an abnormal phenotype.

6.3.2 Clinical significance of cryptic telomeric abnormalities in a control population.

Among the control individuals examined in this study two cryptic telomeric imbalances have been identified. The first, a deletion of the distal region of 4q was detected in the mother (C19) of a fetus examined prenatally where a trisomy 7 cell line was identified. There was no indication of any developmental problems or congenital malformations in the referral details supplied with the blood sample or in the patient's notes held at the Clinical Genetics Centre. This deletion of the terminal sequences of 4q does not therefore appear to have a pathogenic effect in this individual. Although quantitative PCR failed to confirm this deletion, a retrospective, blinded high resolution G banded analysis did identify the abnormal chromosome. Importantly, in light of this result, it is possible that the cryptic deletion of 4q reported by Vorsanova *et al.* (2000) was coincidental to the proband's MR.

Tupler *et al.* (1996) described a similar deletion of 4q35 in three members of a family who were found to have a satellited chromosome 4 resulting from an unbalanced translocation between the distal long arm of a chromosome 4 and the short arm of a D or G group chromosome. The proband, a 9 year old male, presented with hypogenitalism but no developmental delay. The mother and maternal grandfather were also found to carry the same abnormal unbalanced chromosome 4. Both were phenotypically normal. It is therefore most likely that the deletion is coincidental to the hypogenitalism reported in their proband. Deletions of an integral number of 3.3 kb *Kpn*I tandem repeats within the 4q telomeric locus D4Z4 have been implicated in the etiology of FSHD. However, the control individual C19 showed no sign of dystrophy supporting the hypothesis that deletions of this region do not necessarily result in FSHD (Tupler *et al.*, 1996).

The second cryptic telomeric imbalance detected in the control population was an unbalanced rearrangement between the distal regions of 17q and Yp identified in a cytogenetically normal relative (C31) in a family with a subtle 11p;17p telomeric translocation. This 31 year old phenotypically normal male was trisomic for the region 17q25.3→qter but with no detectable monosomy in Yp. Duplications of the distal portion of 17q have been reported in at least 15 cases (Buyse, 1990). All of these duplications have resulted from unbalanced segregation of a parental rearrangement, either translocation or peri-or paracentric inversions and all patients are mentally retarded. Other reported associated findings include facial dysmorphism, syndactyly, polydactyly, scoliosis, shortening of proximal limbs, renal defects, genital anomalies and congenital heart defects. However all of these cytogenetically visible duplications involve significantly greater genetic imbalances than the submicroscopic duplication detected in C31. The only precedents in the literature of cryptic duplications of the telomeric region of 17q were reported by Bacino *et al.* (2000) and Ghaffari *et al.* (1998) (cases 54 and 55, Appendix 2). Both were unbalanced 2q;17q rearrangements resulting from malsegregation of a paternal balanced translocation. In the former case the proband presented with severe mental retardation and multiple minor anomalies, some of which have been described in patients with trisomy for different regions of 17q. In the latter case the abnormality was found in association with craniofacial dysmorphism, tetralogy of fallot, laryngomalacia, and inguinal hernia. It is possible that the cryptic 17q trisomy had no phenotypic consequences in this patient, with the phenotype resulting from the 2q monosomy. It is also possible that the 17q trisomy detected in this study is smaller than in either of the two previous cryptic cases. We have been unable to confirm this imbalance by either retrospective high resolution G banded analysis or by quantitative PCR.

Although there was no detectable imbalance of Yp, it is the involvement of this region of the genome which may result in a phenotypic effect in this individual. It is possible that the presence of this additional 17q telomeric DNA at the end of the Yp PAR in C31 may disrupt X-Y pairing (sex vesicle formation) at meiosis causing spermatogenic arrest and infertility.

In addition to these two cases and the familial 1q;13q imbalance reported by Shaffer and Benke (1999), there is another telomeric imbalance in the literature with apparently no phenotypic effect (Killos *et al.*, 1997). This imbalance was detected in an

individual with a satellited chromosome 17. The satellite material was found at distal 17p and when investigated with a 17p telomeric FISH probe a terminal deletion was detected.

It is also interesting to note that in some patients with WHS, cri-du-chat syndrome, ATR-16 and MDS and cryptic or semi-cryptic telomeric rearrangements, concomitant partial telomeric trisomies of 3q, 4q, 8q, 10q and 19p have not confounded the clinical diagnosis (Altherr *et al.*, 1991; Flint *et al.,* 1996; Goodship *et al.,* 1992; Kuwano *et al.,* 1991; Lamb *et al.,* 1989; Masuno *et al.,* 1995; Overhauser *et al.,* 1989). Although it is likely that the severity of the phenotypes resulting from the 'primary' imbalances in these patients have masked any additional features resulting from these partial trisomies, it remains a possibility that some of these additional cryptic telomeric imbalances have resulted in little or no pathogenic effect.

In conclusion, the two cryptic abnormalities detected in the systematic screen of control individuals do not appear to have an associated phenotypic effect. It is proposed that these rearrangements are representative of a subset of nonpathogenic telomeric imbalances. As more cases of cryptic telomeric imbalances are detected and reported, correlation of genotype to phenotype will become more detailed and informative. At present it is clear that whilst cryptic imbalances of some telomeres will produce a severe phenotype, such as WHS, MDS, severe mental retardation and/or multiple congenital anomalies, some may result in only mild mental retardation and others, such as a deletion of the telomeric sequences of 4q and a duplication of the telomeric region of 17q, may have no pathogenic effect. Consequently a combination of family studies, literature review, and genetic mapping should be employed when determining the pathogenicity of any given cryptic telomeric imbalance identified during screening programmes.

6.3.3 Polymorphisms

In addition to the genetic imbalances described so far, other anomalous results have been detected using telomeric FISH probes. During this study signals of variable intensity were observed with both the XpYp and XqYq telomeric FISH probes in IMR patients (21% and 4.5% respectively) and in control individuals (19% and 5% respectively). In some cases it was necesssary to repeat the probe hybridization in order to demonstrate the presence of a signal. This polymorphism was also noted by Knight

and Flint (1999). Recombination between the short arm PARs ensures proper sex chromosome segregation (Page *et al.,* 1987; Rouyer *et al.,* 1986). As a consequence, the nucleotide sequences of the most distal segments of Xp and Yp are indistinguishable. The sequences of the most distal 320 kb of Xq and Yq are also indistinguishable (Weil *et al.*, 1994), although the crossing-over frequency of the long arm PAR is much lower (Freije *et al.,* 1992; Lin *et al.,* 1995). It is interesting to note that in the present study the signal intensity variation was more frequent at the XpYp telomeric region than at the XqYq telomere, consistent with this known variance in recombination frequencies. The presence of these anomalous findings in similar proportions of both IMR patients and control individuals leaves little doubt that they represent telomere polymorphisms.

In this study, a consistently reduced fluorescent signal was observed with the 2q telomeric probe (210E4) in one patient with familial IMR (P79). The same phenomenon was observed by Horsley *et al.* (1998) in a patient with a semi-cryptic unbalanced 2p;18p translocation and a mild phenotype with no mental retardation. Although this polymorphism was not observed in any of the control individuals in the present study, it has been reported by Knight and Flint (2000). It therefore seems unlikely that this finding is related to the IMR of P79. Horsley *et al.* (1998) postulated that the reduced fluorescent signal was most likely a consequence of the 55 kb subtelomeric repeat length polymorphism at the 2q telomere reported by Macina *et al.* (1994).

It is therefore plausible that the two cryptic 2q telomeric deletions detected during screening of patients with IMR (Anderlid *et al.,* 1999; Viot *et al.,* 1998) were in fact polymorphisms and coincidental to the patients' MR. Until a significant number of control individuals have been screened using these techniques, anomalous findings such as these will continue to pose a diagnostic dilema.

6.4 Some telomeres are hot-spots for cryptic rearrangements

As already discussed cytogenetically visible terminal deletions of some human chromosome arms are associated with specific mental retardation/multiple congenital anormality syndromes. Deletions of the terminal regions of 4p, 5p, 16p, 17p, 18p and 18q accompany well defined syndromes, whereas terminal deletions of 1p and 22q are associated with less distinctive syndromes. Directed referrals have led to the identification of cryptic and semi-cryptic rearrangements of all of these telomeric

regions. Hence it is not remarkable that 22/52 submicroscopic rearrangements detected in IMR patients during telomere screening have involved deletion or duplication of these same regions. A further four patients have been found to have deletions of the telomeric region of 13q (present study P72; Flint *et al.,* 1995; Knight *et al.,* 1999; Vorsanova *et al.,* 2000). During this study one cryptic and one subtle 11p;17p unbalanced translocation (S11 and S8 respectively), and one semi-cryptic 4p;11p unbalanced translocation have been identified. Two further cryptic unbalanced 4p;11p translocations were found in the literature (Knight *et al.,* 1999; Reid *et al.,* 1996).

These cases indicate that the telomeric regions of 1p, 4p ,5p, 16p, 17p, 18p, 18q, 22q, and possibly 13q and 11p, are foci for cryptic telomeric rearrangements and therefore targeted investigations to detect imbalances of these regions should be considered. The associated clinical phenotypes of deletions of 4p, 5p, 16p, 17p, 18p, and 18q are well defined, and the phenotypes of 1p and 22q monosomy syndromes are becoming better delineated as more patients are identified and described. Consequently, detailed clinical analysis will play an increasingly important role in the diagnosis of pathogenic cryptic telomeric abnormalities.

One case in this study was found to have a telomeric imbalance resulting from malsegregation of a paternal 2q;14p translocation. Cytogenetic evaluation of this derived chromosome 2 gave no indication of the presence of satellite material. However, in reviewing the subtle terminal chromosome rearrangements in the literature, 4 further telomeric rearrangements that have involved the short arms of the acrocentric chromosomes (cases 40, 41, 89, 166, Appendix 2) were found. In addition, Katz *et al.* (1999) have reported evidence that an 18q- chromosome was also formed by an illegitimate (non-homologous), cryptic unbalanced translocation with satellite III DNA normally located on the short arm (p11) of the acrocentric chromosomes. The telomeric regions of the acrocentric chromosomes have not been cloned because imbalances of these regions, which code for ribosomal RNA, are known to produce no phenotypic effect. It is therefore possible that an undetected cryptic rearrangement involving the short arm of an acrocentric chromosome underlies many apparently terminal rearrangements.

6.5 Origin of telomeric rearrangements

As already discussed in section 5.1 the high degree of homology of the subtelomeric sequences and the increased rate of recombination at the telomeres provides the opportunity for non-homologous telomere pairing which could lead to exchange events and gene dosage imbalance. This assumption has led to the search for cryptic telomeric rearrangements as underlying causes in cases of IMR.

Different mechanisms have been proposed for the formation of telomeric rearrangements, including (i) illegitimate telomere recombination between non-homologous chromosomes promoted by sequence homology and polymorphic telomere alleles, (ii) telomere healing, and (iii) telomere capture. In support of the first mechanism, Wilkie *et al.* (1991) have identified three polymorphic alleles at the telomeric region of 16p. The two most common alleles share no sequence homology with each other. One is related to the subtelomeric regions on Xq and Yq, and the other shares homology with the subtelomeric regions of 9q, 10p and 18p. An individual heterozygous for two different 16p telomere alleles would be predisposed to non-homologous telomere pairing and translocation events. Furthermore, the high degree of sequence homology at the telomeric regions of 4q and 10q (Cacurri *et al.,* 1998) has been implicated in illegitimate unequal crossing-over events which result in truncation of the 4q subtelomeric region and ensuing FSHD phenotype (van Deutekom *et al.,* 1996). Finally, the presence of hot-spots for telomeric rearrangements suggests that sequences adjacent to the telomeres of these chromosome arms are particularly prone to illegitimate recombination and are therefore likely to have polymorphic telomere alleles.

Several findings in the present study concur with this mechanism and in addition suggest that sequence homology is not limited to the subtelomeric repeats but extends into the unique sequence telomeric DNA. Firstly, the probe 2209a2 which hybridizes to the telomeric region of 11p was found to hybridize weakly to the short arm telomeric region of chromosome 17, implying that these unique sequence telomeric regions share sequence homology. This finding is of particular interest given the two familial subtle telomeric 11p;17p translocations identified during this study. It is possible that during early meiotic prophase, when the telomeres cluster at the nuclear periphery and homology searching and pairing initiates, this sequence homology promoted a cross-over event resulting in the 11p;17p telomeric rearrangement.

Secondly, the 19q telomeric probe gave clear signals on both chromosomes 2 at the region q13 in addition to signals at distal 19q. This observation can be explained by the fact that the region 2q13 contains an ancient fusion site of chimpanzee chromosomes 12 and 13 and is known to be flanked by sequences with homology to some subtelomere repeats (Hoglund *et al.,* 1995). However, due to the interstitial site of the 2q sequences, this homology is less likely to result in aberrent recombination.

Thirdly, as already discussed the telomeric probes specific for both the short and long arms of the X and Y chromosomes were found to produce signals of varying intensities. This is believed to be the result of unequal crossing over between the most telomeric unique sequence DNA of both the long and short arms of the X and Y chromosomes. The high degree of homology at both PARs is known to promote ectopic recombination between the two sex chromosomes (Lahn *et al.,* 1994; Levilliers *et al.,* 1989; Rouyer *et al.,* 1987; Weil *et al.,* 1994).

Among the subtle terminal rearrangements reviewed 71/167 (43%) were shown to be unbalanced terminal rearrangements and 10 (6%) have been shown to be interstitial rather than terminal deletions. Whilst the remaining 86 (51%) were reported as terminal deletions, this is certainly an over-estimate since only 37 of these (43%) had been fully investigated with the whole panel of telomere probes. Of the 21 subtle or cryptic telomeric rearrangements identified in this present study, 13 (62%) were unbalanced translocations, 3 (14%) interstitial deletions, and 5 (24%) terminal deletions. It is postulated that all of these unbalanced translocations arose via illegitimate telomere pairing.

Whilst many other cryptic and semi-cryptic telomeric imbalances have been shown to result from telomeric translocations and therefore endorse the mechanism proposed by Wilkie *et al.* (1991), others appear to be either interstitial or terminal deletions. Recent evidence indicates that illegitimate recombination between duplicated blocks of homologous sequences is the underlying mechanism in the formation of pericentromeric interstitial deletions associated with a number of disorders. These disorders include Williams syndrome at 7q11.23 (Perez Jurado *et al.,* 1998), Prader-Willi and Angelman syndromes at 15q11.2 (Christian *et al.,* 1999), Smith-Magenis syndrome at 17p11.2 (Chen *et al.,* 1997), Charcot-Marie Tooth disease type 1A and hereditary neuropathy with liablity to pressure palsies also at 17p11.2 (Lopes *et al.,* 1998; Pentao *et*

al., 1992), and DiGeorge/velo-cardio-facial syndrome at 22q11.2 (Edelmann *et al.,* 1999). Since the subtelomeric regions, like pericentromeric regions, are known to contain repeat sequences and pseudogenes, it is reasonable to suppose that telomeric interstitial deletions may also be formed by illegitimate recombination.

Chromosomes with terminal deletions must acquire telomeric repeats if they are to be stably transmitted through successive cell divisions. Chromosomes without telomeres are highly unstable (Zakian, 1995) because telomeres protect the chromosome from end to end fusions and permit the complete replication of the ends of chromosomes. There are two proposed mechanisms for rescuing such broken chromosomes. The first, telomere healing, involves the addition of (TTAGGG)n repeats by the ribonucleoprotein telomerase. Although telomerase is undetectable in most somatic cells, telomerase activity has been demonstrated in fetal ovaries and in fetal and adult testes. The reactivation of telomerase in the germ line may enable chromosomes broken during meiosis to be rescued. This mechanism has been implicated in the stabilisation of terminal deletions of 16p in patients with ATR-16 (Flint *et al.,* 1994; Lamb *et al.,* 1993; Morin, 1991; Wilkie *et al.,* 1990a).

The second proposed method of rescuing broken chromosomes is 'telomere capture'. This involves the acquisition of a pre-existing telomere via recombination. Flint *et al.* (1996) reported a patient with ATR-16 in whom a broken chromosome 16p had been stabilised with a telomere acquired by recombination between subtelomeric *Alu* repeat elements. There is little to distinguish this mechanism from that already outlined whereby telomeric translocations arise via non-homologous crossing over. The difference lies in the timing of events, whether a telomeric breakage occurs and is subsequently rescued by recombination with another telomere sharing homologous sequences, or whether recombination between two telomeres which share sequence homology is the initial event and is followed by malsegregation to give an unbalanced rearrangement. Hence, it is possible that terminal telomeric deletions are also formed by illegitimate recombination.

Also of interest is the parental origin of *de novo* cryptic telomeric rearrangements. Genetic recombination is increased at the telomeres in both males and females. However, whilst for most regions of the human genome female recombination rates are generally higher than male recombination rates (Ashley, 1994), at telomeres this pattern

is apparently reversed (Blouin *et al.,* 1995; Donis-Kellar *et al.,* 1987; Rouyer *et al.,* 1990). The only known exception to date is the telomeric region of 14q, where female recombination is more frequent than male recombination (Wintle *et al.,* 1997).

Relatively higher telomeric recombination rates in males than in females might initially suggest that *de novo* cryptic telomeric rearrangements are more likely to be paternal in origin. This theory is supported by evidence of preferential involvement of the paternal homologue in the classic non-cryptic terminal haploinsufficiency syndromes WHS (Dallapiccola *et al.,* 1993; Quarell *et al.,* 1991; Tupler *et al.,* 1992), cri-du-chat syndrome (Overhauser *et al.,* 1990), and monosomy 18p and 18q syndromes (Cody *et al.,* 1997). However, of the six *de novo* semi-cryptic telomeric abnormalities identified in this study where parental origin has been established, four were of maternal origin, one was of paternal origin, and the remaining case was mosaic indicating a post-zygotic mitotic origin. We were unable to formally demonstrate the origin of the cryptic Yp;17q rearrangement since both parents are deceased. Nevertheless, the fact that neither the brother of C31 or any other relatives examined carry this rearrangement and the fact that the abnormality involves the Y chromosome indicates that this cryptic telomeric imbalance is a *de novo* rearrangement of paternal origin. Among the cases of *de novo* cryptic telomeric rearrangements published to date where parental origin has been determined, 15 are of paternal and 19 of maternal origin (Flint *et al.,* 1995; Flint *et al.,* 1996; Giraudeau *et al.,* 1997; Knight *et al.,* 1999; Lamb *et al.,* 1993; Precht *et al.,* 1998; Riegel *et al.,* 1999; Shapira *et al.,* 1997; Slavotinek *et al.,* 1999).

These cases, although few, do not appear to corroborate the hypothesis that increased male recombination at the telomeres results in a prevalence of paternal *de novo* cryptic telomeric rearrangements. Furthermore, there does not appear to be any correlation between parental origin and the mechanism which may have formed the telomeric abnormality since both apparently terminal rearrangements and unbalanced translocations are represented in both the paternal and maternal origin categories. Instead, these cases indicate that control and repair mechanisms, which must be in place to limit non-homologous telomeric recombination, are as likely to fail during female meiosis as during male meiosis. Whilst the relatively low frequency of such rearrangements, even among IMR populations, is an indication of the robustness of these meiotic checkpoints.

5.6 Project Summary

Recent reports have suggested that cryptic telomeric rearrangements resulting in gene dosage imbalance might represent a significant cause of IMR. However, these frequencies have been based on screens of highly selected populations of IMR patients. The incidence in unselected individuals with IMR may be similar but is likely to be much lower. Furthermore, although genetic imbalances at the gene rich telomeric regions of chromosomes would be likely to result in a phenotypic effect, nonpathogeneic imbalances have been reported in an increasing number of individuals and families. The frequencies of cryptic telomeric abnormalities in previous studies may not therefore reflect the more important frequency of pathogenic telomeric rearrangements. The aim of this project was to address both these points by determining the frequency and significance of cryptic telomeric rearrangements by screening two population samples of patients with IMR (unselected and selected) and a population sample of unbiased control individuals.

In contrast to current information, the data from this study have shown that true cryptic telomeric rearrangements are not a significant cause of IMR. Among 200 individuals with IMR no fully cryptic abnormalities were detected. However, one individual was found to have a semi-cryptic 13q;19q unbalanced telomeric translocation which was not detected on original cytogenetic investigation. The abnormality in this patient was identified by high resolution G-banded analysis (ISCN 850 band level) and confirmed and characterised by multi-telomere FISH. Among 18 cytogenetically detected terminal abnormalities we have characterised, nine had also been previously reported as normal. These results raised the possibility that cryptic telomeric rearrangements reported by other groups may also be visible with the aid of high resolution cytogenetic analysis. A review of cryptic, semi-cryptic, and subtle but visible telomeric rearrangements in the literature has shown considerable overlap between these classes of abnormality, further supporting the belief that previous estimates of the frequency of cryptic imbalances in populations with IMR have been biased by sub-optimal conventional analysis. Other potential sources of bias, such as sex ratios and selection criteria, were considered. Whilst it is probable that cryptic rearrangements are more frequent in patients with moderate to severe MR and multiple congenital anomalies

the phenotypic profile of the selected IMR population was not significantly different from other study populations. It is therefore concluded that the main source of bias is likely to be the level of resolution of cytogenetic analysis used to exclude a chromosome abnormality before inclusion in a multi-telomere screening programme.

Importantly this study has begun to address the lack of information regarding the frequency of cryptic telomeric rearrangements in a normal population. Among 150 control individuals two cryptic telomeric imbalances were identified, indicating that cryptic telomeric rearrangements may be a not uncommon finding in the general population. This result has important ramifications when determining the significance of such rearrangements and is supported by two further case studies in the literature of telomeric imbalances without proven phenotypic effect. To date in all reports of multi-telomere screening , the presence of a genetic imbalance has been assumed to be the cause of the proband's phenotype. The results of this study suggest that such assumptions cannot be made. In all future cases of telomeric imbalance, it will be important to map the extent of the deletion/duplication.

It is also proposed that certain telomeric regions are hot spots for cryptic rearrangements and that detailed clinical analysis will become increasingly important in the diagnosis of IMR. Three cases with subtle distal but interstitial chromosome deletions were identified by conventional analysis, two of which shared the well-defined clinical phenotype associated with terminal deletions. These cases raise the possibility that others could go undetected if too much emphasis is placed on the role of multi-telomere FISH screening in the diagnosis of IMR.

Different mechanisms have been proposed for the formation of telomeric rearrangements, including (i) illegitimate telomere recombination between non-homologous chromosomes promoted by sequence homology and polymorphic telomere alleles, (ii) telomere healing, and (iii) telomere capture. Cross hybridization was found between the 11p telomere-specific FISH probe and the telomeric region of 17p, indicative of a previously unreported sequence homology between the unique sequence DNA adjacent to these telomeres. It is postulated that this homology may have given rise to the subtle telomeric 11p;17p translocations which were identified in the mothers of two children with MDS, thus supporting the first proposed mechanism. Further evidence was provided for this mechanism by the high proportion of unbalanced

translocations among subtle and cryptic telomeric abnormalities in both this study and a review of the literature. Finally despite the higher level of male recombination at the telomeres, there is as yet no evidence for a sex bias in the parental origin of *de novo* telomeric abnormalities.

APPENDIX 1

MOLECULAR RESULTS

IMR Study populations

CASE P72

	PROBAND	MAT	PAT	Origin
CEN				
D13S1295	101 . 115	101 . 111	115	NORMAL
13q tel 10	150	150 . 152	150	not informative
D13S293	92	92	92	not informative
13qter				

CEN				
D19S210	138	138	132 . 138	not informative
D19S218	242 . 242 . 257	242	257	MAT DUP
D19S890	190 . 190 . 198	190 . 198	190 . 198	DUP
19qter				

Control Individuals

CASE S19

	PROBAND	MARKER	RESULT	Origin
CEN				
U57851		dosage	NORMAL	
WI-5353		dosage	NORMAL	
4qter				

CASE S31

	PROBAND	MARKER	RESULT	Origin
CEN				
D17S914	FAIL			
D17S928	154 . 156		NORMAL	
17q tel 13	231 . 233		NORMAL	
U57870		dosage	? normal	
STSG160001		dosage	? normal	
17qter				

Unbalanced Translocations

CASE S9

	PROBAND	MAT	PAT	Origin
4pter				
4p tel 02	153	153 . 157	153 . 155	not informative
4p tel 04	180	174 . 180	174 . 180	not informative
D4S2936	172 . 180	172 . 180	172 . 180	NORMAL
D4S412	238	238 . 244	238	not informative
CEN				

8pter				
D8S504	399 . 401 . 408	399 . 401	408 . 422	MAT DUP
D8S307	130 . 139 . 139	139	128 . 130	MAT DUP
CEN				

CASE S6

	PROBAND	MAT	PAT	Origin
4pter				
4p tel 02	244			not informative
4p tel 04	155			not informative
D4S2936	172 . 180			NORMAL
D4S412	173			not informative
CEN				

11pter				
11p tel 03	(187) . 187 . 195			DUPLICATED
D11S922	225 . 239 . (239)			DUPLICATED
CEN				

CASE S10

	PROBAND	MAT	PAT	Origin
18pter				
D18S59	150 . 150 . 165	150 . 160	155 . 165	MAT DUP
D18S52				FAIL
D18S62	303 . 303 . 309	303	309	MAT DUP
CEN				
18q tel 69	139	119 . 125	125 . 139	MAT DEL
18q tel 11	162	170	162 . 170	MAT DEL
18qter				

CASE S7

	PROBAND	MAT	PAT	Origin
4pter				
4p tel 02	153	153	153 . 155	not informative
4p tel 04	180 . 188 . 188	180	174 . 188	PAT DUP
D4S412	246 . 246 . 250	244 . 250	246	PAT DUP
CEN				

CEN				
18q tel 69	133 . 137	131 . 137	133 . 135	NORMAL
18q tel 11	162	162 . 164	162 . 164	not informative
18qter				

Terminal Deletions

CASE S13

	PROBAND	MAT	PAT	Origin
1pter				
D1S243	166	164	164. 166	MAT DEL
D1S468				FAILED
D1S2845	207	207 . 223	201 . 207	not informative
CEN				

CASE S4

	PROBAND	MAT	PAT	Origin
8pter				
D8S504	136		140	PAT DEL
D8S307	399 . 445		391 . 399	NORMAL
CEN				

CASE S17

	PROBAND	MAT	PAT	Origin
CEN				
D15S87	76	76 . 78	78 . 80	PAT DEL
D15S966	250	234. .250	234 . 236	PAT DEL
D15S642	220	198 . 200	206 . 210	PAT DEL
15qter				

APPENDIX 2

REVIEW OF SUBTLE TERMINAL REARRANGEMENTS

APPENDIX 2 Review of Telomeric Rearrangements

Case	Imbalance Monosomy	Imbalance Trisomy	Class	FISH result	Clinical presentation	Reference
1-30	1p		V	del(1)(p36)*de novo*[1]	Developmental delay, hypotonia, seizures, multiple congenital anomalies, dysmorphism	Slavotinek *et al* 1999b (review)
31-35	1p		C	del(1)(p36)*de novo*	MR, dysmorphism	Giraudeau *et al* 1998; Knight *et al* 1999; Riegel *et al* 1999; Slavotinek *et al* 1999a
36	1p		V	del(1)(p36.2)*de novo*	IUGR, skeletal anomalies, dysmorphism	Present study S13
37	1p		V	del(1)(p34.1p36.1)	Dysmorphism, congenital heart disease	Howard + Porteous 1990
38	1p		V	del(1)(p36.1p36.2)	Developmental delay, dysmorphism	Biegel *et al* 1993
39	1p		V	del(1)(p36.21p36.22)*de novo*	Developmental delay, microcephaly, spastic diplegia	Present study S18
40	1p	15p	S	46,XX,der(1)t(1;15)(p36.31;p11.2)pat	Physical anomalies, psychomotor retardation, seizures	Blennow *et al* 1996
41	1p	15p	S	der(1)t(1;15)(p36;p11.2)	Hypotonia, minor anomalies	Barbi *et al* 1992
42	1p	19q	C	46,XX,der(1)t(1;19)(p36.3;q13.4)pat [sib with same imbalance]	Moderate MR, short stature, conductuive hearing loss	Knight *et al* 1999
43	1p	21q	S	der(1)t(1;21)(p36;q13)	Developmental delay	Yunis *et al* 1981
44	1q		C	46,XY,del(1)(q44)*de novo*	MR	Knight *et al* 1999
45	1q	13q	C	der(1)t(1q;13q)pat[2]	Developmental delay, moderate cerebral palsy; father phenotypically normal	Shaffer + Benke 1999
46	1q	13q	C	46,XY,der(1)t(1;13)(q44;q34)mat [cousin with der(13)t(1q;13q)mat]	Severe MR, micrognathia, Pierre Robin sequence, thoracic hemivertebrae	Knight *et al* 1999
47	2q		C	del(2)(q37.2)*de novo*	MR, dysmorphic features	Viot *et al* 1998
48	2q		V	46,XX,del(2)(q37.2)	Albright hereditary osteodystrophy-like syndrome	Reddy *et al* 1999
49	2q		V	46,XY,del(2)(q37.2)	Behavioural problems, mild MR, foot abnormalities, abnormal situs inversus	Reddy *et al* 1999
50	2q		C	del(2q)	Mild MR, dysmorphic features	Anderlid *et al* 1999
51	2q	7q	V	46,XY,der(2)t(2;7)(q37.2;q36.3)mat	CHD, bilateral colobomata, syndactyly, abnormal genitalia	Present study S5
52	2q	7q	S	46,XY,der(2)t(2;7)(q37;q35)pat	MR, failure to thrive, dysmorphic features	Speleman *et al* 2000
53	2q	14p	S	46,XX,der(2)t(2;14)(q37;p11)pat [sister with same imbalance]	Developmental delay, long hands and feet	Present study S3
54	2q	17q	C	46,XY,der(2)t(2q;17q)pat	Severe MR, minor anomalies	Bacino *et al* 2000

C cryptic; S semi-cryptic; V cytogenetically visible; [1] 6 cases required FISH confirmation; [2] non-pathogenic variant

APPENDIX 2 continued

Case	Imbalance Monosomy	Imbalance Trisomy	Class	FISH result	Clinical presentation	Reference
55	2q	17q	C	46,XY,der(2)t(2q;17q)pat	Craniofacial dysmorphism, tetralogy of fallot, laryngomalacia, inguinal hernia	Ghaffari *et al* 1998
56	4p		C	del(4p)	Severe MR, epilepsy, dysmorphic features	Anderlid *et al* 1999
57	4p	8p	S	46,XY,der(4)t(4;8)(p16.1;p23.1)*de novo*	WHS	Present study S9
58	4p	10q	C	46,XX,der(4)t(4p;10q)mat	WHS	Goodship *et al* 1992
59	4p	11p	C	der(4)t(4;11)(p16.3;p15.5)	WHS	Reid *et al* 1996
60	4p	11p	S	46,XY,der(4)t(4;11)(p16.3;p15.5)*de novo*	WHS	Present study S6
61	4p	11p	C	46,XY,der(4)t(4;11)(p16;p15.5)pat	WHS	Knight *et al* 1999
62	4p	19p	S	46,XX,der(4)t(4p;19p)mat	WHS	Altherr *et al* 1991
63	4p	22q	C	46,XX,der(4)t(4;22)(p16;q13.3)mat [nephew with same imbalance, niece with der(22)]	Severe MR, prominent nasal bridge, microcephaly, short stature	Knight *et al* 1999
64	4q		C	del(4)(q35.2)	MR	Vorsanova *et al* 2000
65	4q		C	46,XX,del(4)(q35.3)[2]	CONTROL	Present study C19
66	4q	6q	C	46,XY,der(4)t(4;6)(q35;q27)pat [cousin with der(6)t(4q;6q)mat]	Severe MR, facial dysmorphism, syndactyly, genital anomalies	Knight *et al* 1999
67	4q	20p	C	46,XX,der(4)t(4q;20p)pat	Mild gross motor + speech delay, VSD, minor facial dysmorphism	Ghaffari *et al* 1998
68	4q	20p	C	46,XX,der(4)t(4;20)(q35;p13)mat [cousin with same imbalance]	Severe MR, VSD, low set ears, facial dysmorphism, growth retardation	Knight *et al* 1999
69	4	22	C	der(4)t(4;22)familial	MR, dysmorphic features	Viot *et al* 1998
70	5p		C	del(5p)	MR	Vorsanova *et al* 2000
71	5p	4q	C	der(5)t(4q;5p)	Cri-du-chat	Overhauser *et al* 1989
72	5p		S	46,XX,del(5)(p14.2p15.32)t(5;6)(p15.32; p25.1)*de novo*	Dysmorphic features, delayed visual maturation	Present study S1
73	5p	7p	C	46,XX,der(5)t(5;7)(p15.3;p21)mat	Cri-du-chat	Bernstein *et al* 1993
74	5p	10p	C	46,XX,der(5)t(5;10)(p14.2;p12.2)mat	Cri-du-chat	Catrinel *et al* 1999
75	5q	6q	C	46,XYder(5)t(5;6)(q35.2;q27)mat	Dandy-Walker anomaly, dysmorphic features	Groen *et al* 1998
76	5	11	C	der(5)t(5;11)	MR, dysmorphic features	Viot *et al* 1998
77	6p		C	46,XY,del(6)(p25)*de novo*	MR	Knight *et al* 1999

C cryptic; S semi-cryptic; V cytogenetically visible; [2] non-pathogenic variant

APPENDIX 2 continued

Case	Imbalance		Class	FISH result	Clinical presentation	Reference
	Monosomy	Trisomy				
78	6q		C	del(6q)	Severe MR, microcephaly, hyperactivity, skeletal malformation	Anderlid *et al* 1999
79	7q	2q	C	46,XX,der(7)t(2;7)(q37;q36)pat	Severe MR, microcephaly, hypotonia, eye abnormalities, dysmorphism	Knight *et al* 1999
80	8p		V	46,XY,del(8)(p23.1)pat	PND, father phenotypically normal	Reddy 1999
81	8p		V	46,XX,del(8)(p23.1)/46,XX	Recurrent spontaneous abortions	Reddy 1999
82	8p		V	46,XY,del(8)(p23.3)pat [father with same imbalance]	Developmental delay, epilepsy	Present study S4
83	8p		V	46,XY,del(8)(p23.1p23.1)	Developmental delay, behavioural problems, ASD	Reddy 1999
84	8p	20p	C	46,XY,der(8)t(8;20)(p23;p13)mat [cousin with same imbalance]	Moderate MR, epilepsy, undescended testes	Knight *et al* 1999
85	9p		C	46,XY,del(9)(p24)*de novo*	MR	Knight *et al* 1999
86	9p	16q	C	46,XX,der(9)t(9;16)(p24;q24)*de novo*	MR	Knight *et al* 1999
87	9q		C	del(9)(q34.3)	MR	Vorsanova *et al* 2000
88	9q		C	del(9q)	MR, autism	Anderlid *et al* 1999
89	9q	13p	C	46,XX,der(9)t(9;13)(q34;p11.1)mat [sister with same imbalance]	Severe MR, hypotonia, sensorineural deafness, coarse facies, joint laxity	Knight *et al* 1999
90	9	8	C	der(9)t(8;9)	MR, dysmorphic features	Viot *et al* 1998
91	10p		S	46,XY,del(10)(p15.1p15.3)*de novo*	Developmental delay, behavioural problems, deafness	Present study S2
92	10q	20q	C	46,XY,der(10)t(10q;20q)	Moderate MR	Ghaffari *et al* 1998
93-94	10q		C	del(10)(q26.3)	MR	Vorsanova *et al* 2000
95	11q		C	del(11)(q25)	MR	Vorsanova *et al* 2000
96	12p	6q	C	46,XY,der(12)t(6;12)(q27;p13.3) mat [brother with same imbalance + sister with der(6)]	Moderate MR, microcephaly, growth retardation, facial dysmorphism	Knight *et al* 1999
97	13q		C	del(13)(q34)	MR	Vorsanova *et al* 2000
98	13q		C	46,XY,del(13)(q34)*de novo*	MR	Knight *et al* 1999
99	13q	Yp	C	46,XY,der(13)t(Y;13)(p11.3;q34)*de novo*	MR, microcephaly, mild facial dysmorphism	Flint *et al* 1995; Knight *et al* 1999
100	13q	5q	V	46,XY,der(13)t(5;13)(q35.2;q34)	MR	Guichet *et al* 1998
101	13q	19q	S	46,XY,der(13)t(13;19)(q34;q13.43)*de novo*	Mild developmental delay, adducted thumbs	Present study P72

C cryptic; S semi-cryptic; V cytogenetically visible

APPENDIX 2 continued

Case	Imbalance		Class	FISH result	Clinical presentation	Reference
	Monosomy	Trisomy				
102	13q	20p	C	46,XY,der(13)t(13;20)(q34;p13)pat	Severe MR, growth retardation, dysmorphic features	de Die-Smulders *et al* 1999
103	15q		V	46,XY,del(15)(q26.2)*de novo*	Short stature <0.4thcentile	Present study S17
104	16p		V	46,XY,del(16)(p16.3)		Present study S14
105	16p	1p	C	46,XX,der(16)t(1;16)(p36.33;p13.3)pat [sister with same imbalance]	Multiple congenital anomalies, developmental delay	Warburton *et al* 2000
106	16p	1p	C	46,XY,der(16)t(1p;16p)mat [sister with der(1)t(1p;16p)]	HbH disease, dysmorphism	Lamb *et al* 1989
107	16p	3q	C	der(16)t(3;16)(q29;p16.3)	Mild-moderate MR, dysmorphic features	Holinski-Feder *et al* 2000
108	16p	8q	C	46,XY,der(16)t(8q;16p)pat	α thalassaemia trait, tuberous sclerosis complex	Eussen *et al* 2000
109	16q		C	del(16)(q24.3)	MR	Vorsanova *et al* 2000
110	17p		S	del(17)(p13.3)[2]	Normal	Killos *et al* 1997
111	17p	3q	S	46,XX,der(17)t(3;17)(q28;p13.1)mat	MDS	Kuwano *et al* 1991
112	17p	5p	C	46,XY,der(17)t(5;17)(p15.33,p13.3)	MDS	Present study S12
113	17p	8q	C	46,XX,der(17)t(8;17)(q24.3;p13.3)pat	MDS	Kuwano *et al* 1991
114	17p	9p	S	46,XX,t(9;17)(p24.2;p13.3)mat	FH of MDS	Kohler *et al* 1994
115	17p	9q	C	46,XY,der(17)t(9;17)(q34.3;p13.3)	Developmental delay, dysmorphic features	Estop *et al* 1995
116	17p	10q	C	46,XX,der(17)t(10;17)(q26.3;p13.3)mat	MDS	Masuno *et al* 1995
117	17p	11p	V	46,XX,der(17) t(11;17)(p15.5;p13.3)mat	MDS	Present study S8
118	17p	11p	C	46,XX,der(17) t(11;17)(p15.5; p13.3)mat	MDS	Present study S11
119	17p	19q	C	46,XY,der(17)t(17;19)(p13.3;q13.3)	MDS	Alvardo *et al* 1993
120	17p	20p	C	46,XY,der(17)t(17;20)(p13.3;q13.3)pat	MDS	Van Zelderen-Bhola *et al* 1997
121-123	18p		C	del(18)(p11.32)	MR	Vorsanova *et al* 2000
124	18p	2p	S	46,XX,der(18)t(2;18)(p25;p11.2)	Mild distal spinal atrophy, abnormal chest shape, keratosis pilaris	Horsley *et al* 1998
125	18p	5p	S	46,XX,der(18)t(5;18)(p15.3;p11.32)pat	Learning difficulties	Reddy + Fugate 1999
126-130	18q		S	3 interstitial deletions 2 der(18)t(18q;?)	18q- deletion syndrome [review of 35 18q- cases]	Brkanac *et al* 1998

C cryptic; S semi-cryptic; V cytogenetically visible; [2] non-pathogenic variant

APPENDIX 2 continued

Case	Imbalance Monosomy	Imbalance Trisomy	Class	FISH result	Clinical presentation	Reference
131	18q		C	del(18)(q23)	MR	Vorsanova *et al* 2000
132	18q		C	del(18q)	MR, dysmorphic features	Slavotinek *et al* 1999a
133	18q	Xq	C	46,XY,der(18)t(X;18)(q28;q23)*de novo*	MR	Knight *et al* 1999
134	18q	2p	C	der(18)t(2p;18q)familial	MR, dysmorphic features	Lamb *et al* 1999
135	18q	4p	S	46,XX/46,XX,der(18)t(4;18)(p16.3;q23)*de novo*	Agenesis of corpus callosum, hypertelorism, low set ears, PDA	Present study S7
136	18q	18p	S	46,XY,rea(18)(pter->q21.1::p11.1->pter) *de novo*	Failure to thrive, visual inattention, dysmorphism	Present study S10
137	18q	21q	C	46,XX,der(18)t(18;21)(q23;q22.1)	Down syndrome	Bartsch *et al* 1997
138	21q		C	del(21)(q22.1)	MR	Vorsanova *et al* 2000
139	21q	9q	C	der(21)t(9q;21q)	Severe MR, short stature, CNS malformation	Anderlid *et al* 1999
140-141	22q		C	46,XX,del(22)(q13.3)	Developmental delay, hypotonia, expressive language delay	Precht *et al* 1998
142-144	22q		C	46,XY,del(22)(q13.3)	MR	Flint *et al* 1995; Knight *et al* 1999
145	22q		C	del(22)(q13.33)	MR	Vorsanova *et al* 2000
146	22q		C	del(22q)	MR, autism, epilepsy, dysmorphic features	Anderlid *et al* 1999
146-157	22q		V	del(22)(q13.3)	Developmental delay, hypotonia, expressive language delay	Herman *et al* 1998; Narahara *et al* 1992; Nesslinger *et al* 1994; Phelan *et al* 1992; Yong *et al* 1997; Zwaigenbaum *et al* 1990
158	22q		V	del(22)(q13.3q13.33)*de novo*	Development delay, hypotonia, facial dysmorphism	Romain *et al* 1990
159	22q		S	46,XY,del(22)(q13.3q13.3)	Developmental delay	Present study S15
160	22q	1q	S	der(22)t(1;22)(q44;q13.32)mat	Developmental delay, hypotonia, expressive language delay	Doheny *et al* 1997
161	22q	6p	S	der(22)t(6;22)(p25;q13)	Developmental delay, hypotonia, expressive language delay	Doheny *et al* 1997; Praphanphoj *et al* 2000
162	22q	9q	C	46,XX,der(22)t(9q;22q)	MR, dysmorphic features	Flint *et al* 1995
163	22q	14q	V	46,XY,der(22)t(14;22)(q32.33;q13.31) pat	Developmental delay, hypotonia, expressive language delay	Present study S16

C cryptic; S semi-cryptic; V cytogenetically visible

APPENDIX 2 continued

Case	Imbalance		Class	FISH result	Clinical presentation	Reference
	Monosomy	Trisomy				
164	22q	17p	C	46,XY,der(22)t(17;22)(p13.3;q13.3)	Multiple congenital anomalies	Smith *et al* 1998
165	22q	19q	S	46,XX,der(22)t(19;22)(q13.42;q13.31)mat	22q deletion syndrome	Praphanphoj *et al* 2000
166	Xp	14p	C	46,Y,der(X)t(X;14)(p22.31;p11.1)mat	Short stature, developmental delay, dry skin	de Vries *et al* 1999
167		17q	C	46,X,der(Y)t(Y;17)(pter;q25.3)[2]	CONTROL	Present study C31

C cryptic; S semi-cryptic; V cytogenetically visible; [2] non-pathogenic variant

APPENDIX 3

REAGENTS AND BUFFERS

BLOOD CULTURE

RPMI-1640 medium:

100 ml RPMI-medium (Sigma)

2 ml penicillin/streptomycin solution

2 ml L-glutamine (Imperial laboratories)

12.5 ml 10% fetal calf serum (Sigma)

1 ml phytohaemagglutinin (Murex Biotech LTD)

1% KCl:

5.6 g 0.075 M potassium chloride (Sigma)

1 litre dH_2O

GROWTH AND EXTRACTION OF PROBE DNA

LB Broth (GibcoBRL):

20 g per litre dH_2O

2 YT Broth (GibcoBRL):

31 g per litre dH_2O

Agar (Dibco):

2 g /100 ml Media

Appropriate antibiotic at desired concentration

P 1 solution:

15 mM Tris Base pH 8.0 (1.5 ml 1 M Tris Base pH 8.0)

10 mM EDTA (2 ml 0.5 M EDTA)

Made up to 100 ml

100 μg/ml RNase A (added just before use)

P 2 solution:

4 ml NaOH (Sigma)

2 ml 10% SDS

14 ml dH_2O

P 3 solution:

60 ml 5 M KAC

11.5 ml Glacial Acetic Acid

28.5 ml dH_2O

TE (pH8.0):

10 ml 1 M Tris (pH 8.0)

2 ml 0.5 M EDTA (pH 8.0)

up to 1000 ml dH_2O

0.8% Agarose Gel:

1.2 g Agarose

150 ml 1 x TBE (dilute 5x TBE accordingly)

5 x TBE Buffer (Sigma):

Contents of container dissolved in 4 litres of dH_2O. Then the 5 x TBE buffer is made up to 20 litres with dH_2O to make a stock of 1 x TBE Buffer.

Loading Buffer:

8 g Sucrose

1 ml 5% Orange G

40 µl 0.5 M EDTA

Made up to final volume of 20 ml with dH_2O.

NICK TRANSLATION OF PROBE DNA

Stock DNase I - 1 mg (Sigma):

1 mg/ml Stock - 1 mg/ 1 ml of Enzyme Diluent - Store at -20 ^{0}C

Enzyme Diluent:

400 µl Sterile dH_2O

100 µl 10 x Nick Translation Buffer

500 µl Sterile Glycerol

DNase I Working Solution (1 µg/ml):

1 µl of Stock DNase I in 1 ml of Enzyme Diluent

10 x Nick Translation Buffer - 1 ml

0.5 M Tris pH 7.5 - 500 µl of 1 M Tris pH 7.5 Stock

0.1 M $MgSO_4$ -100 µl of 1 M $MgSO_4$ Stock

1 mM Dithiothreitol (Mw 154.25) - 0.1542 mg

500 µg/ml Bovine Serum Albumin - 50 µl of 10 mg/ml Stock

3 M Sodium Acetate pH 7.0:

24.61 g dissolved in 200 ml sterile dH_2O.

IN SITU HYBRIDIZATION

Complete Hybridization Mix:

5 ml de-ionised formamide + dextran sulphate

2 ml 10x SSC

3 ml dH_2O

20 x SSC:

175.3 g NaCl (Sigma)

88.2 g $C_6H_5Na_3O_7.2H_2O$ (Sigma)

Made up to 1 litre dH_2O.

4 x SSC/Tween 20:

800 ml dH_2O

200 ml 20x SSC

500 µl Tween (Sigma)

2 x SSC:

450 ml dH_2O

50 ml 20 x SSC

RNase A (Sigma):

1 mg/ml in RNase Buffer

RNase Buffer - 10 mM Tris/15 mM NaCl pH 7.5

Pepsin (Sigma):

50 mg/ml in 0.01 M HCl

Blocking Reagent (Boehringer):

1 g in 100 ml 4 x SSC/Tween 20

0.4 x SSC 0.1% Tween 20:

10 ml of 2 x SSC

40 ml of dH_2O

50 µl of Tween 20.

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