Chromosome 6q16-21 deletions in acute lymphoblastic Leukaemia

A thesis submitted for the degree of Doctor of Philosophy by

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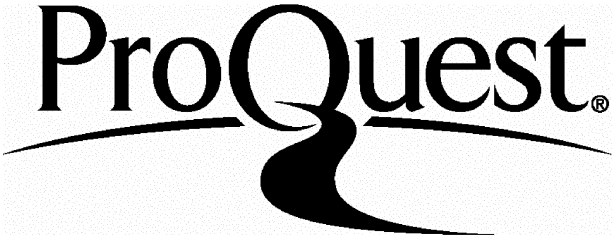

Abstract

The identification of chromosomal abnormalities in acute leukaemia has become an important prognostic parameter for the evaluation of outcome both in adult and childhood patients. However, the prognostic role of some abnormalities, such as deletion of 6q remains to be proven.

The aim of the work described in this thesis was threefold. First, to define the region of minimal deletion (RMD) on chromosome 6, band q16-21, deleted, as by cytogenetics analysis, in 5-13% of patients with acute lymphoblastic leukaemia (ALL). Second, to evaluate the incidence of 6q deletion in a homogenous group of adult and childhood ALL patients. Third, to investigate the prognostic value of 6q deletion in a homogenously treated group of patients.

Genetic mapping and loss of heterozygosity (LOH) analysis were used to expand from our preliminary data. We used highly informative polymorphic microsatellite markers which are available centromeric and telomeric to 6q21 on 69 paired tumour and remission DNA samples from patients with ALL (34 adults and 35 children).

A panel of five microsatellite markers recommended by the national cancer institute (NCI) was also applied to further assess microsatellite instability (MSI).

Wild type (WT) was detected in 44 (64%) patients; LOH was demonstrated in 16 (23%) patients and (MSI) was detected in 9 (13%) patients.

Our study showed that a RMD is identified by marker D6S1709 on the centromeric side and marker D6S278 on the telomeric side with markers D6S283 and D6S1592 being the most frequently deleted markers. This is to date a region of approximately 3.9MB. Our RMD overlaps with the RMDs previously detected by cytogenetics and fluorescence in situ hybridisation (FISH) analysis.

The occurrence of LOH did not appear to segregate with gender, age and immunophenotype or influence clinical outcome in the cohort of patients studied. MSI, however, occurred more frequently in children (17%) than adults (8.8%) but equal frequency was observed in B and T-lineage ALL. Adults with MSI pattern were older than adults with either wild type or LOH pattern. Overall survival was not influenced by the occurrence of either LOH or MSI in B-ALL but conferred worse prognosis in T-ALL.

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Finally, I would like to express my warm thanks to my children who have shared the difficult moments with me and endured my ups and downs. Thank you for your love and understanding... sorry if I was not myself at times.

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List of abbreviations

RMD	Region of minimal deletion
ALL	Acute lymphoblastic leukaemia
LOH	Loss of heterozygosity
NCI	National cancer institute
MSI	Microsatellite instability
WT	Wild type
FISH	Fluorescence in situ hybridisation
B-ALL	B-lineage acute lymphoblastic leukaemia
T-ALL	T-lineage acute lymphoblastic leukaemia
TSG(s)	Tumour suppressor gene(s)
UV	Ultraviolet light
IR	Ionising radiation
FAB	French- American-British classification
AML	Acute myeloid leukaemia
WHO	World Health Organisation
WBC count	White blood cell count
Ig	Immunoglobulin gene
TCR	T- cell receptor gene
NHL	Non- Hodgkin's lymphoma
PCR	Polymerase chain reaction
B-CLL	B-cell chronic lymphocytic leukaemia
RFLP	Restriction fragment length polymorphism
HCC	Hepatocellular carcinoma
M6P/IGF2R	Mannose 6 -phosphate/insulin-like growth factor 2 receptor
cM	Centimorgan
Kb	Kilobase
AI	Allelic imbalance
CGH	Comparative genomic hybridisation
IGF2R	Insulin growth factor receptor-2
LCM	Laser capture microdisection
PEP	Primer extension preamplification
MM	Malignant mesothelioma
SRO	Smallest region of overlap
9p	Short arm of chromosome 9

CML	Chronic myeloid leukaemia
RB	Retinoblastoma
DCC	Deleted in colon carcinoma
WARG	Wilm's tumour/aniridia/genitourinary anomaly/mental retardation
HNPCC	Hereditary non-polyposis colorectal carcinoma
LFS	Li- Fraumeni syndrome
FAP	Familial adenomatous polyposis
APC	Anaphase promoting complex
FPD	Familial platelet disorder
XP	Xeroderma pigmentosa
AA	Amino acid
BER	Base excision repair
NER	Nucleotide excision repair
HR	Homologous recombination
EJ	End joining
MMR	Mismatch repair
UDG	Uracil DNA glycolase
XRCC1	X- ray cross complementing 1
PCNA	Proliferating cell nuclear antigen
GG-NER	Global genome nucleotide excision repair
TCR	Transcription coupled repair
RPA	Replication protein A
CS	Cokayne syndrome
TDD	Trichothiodystrophy
DSB	Double strand break
VDJ	Variable- diversity-joining
NHEJ	Non homologous end joining
DNA-PK	DNA- Dependent Protein Kinase
XRCC4	X- ray cross complementing 4
AT	Ataxia teleangiectasia
NBS	Nijmegen breakage syndrome
IDLs	Insertion deletion loops
CTCL	Cutaneous T-cell lymphoma
MSI-H	Microsatellite instability of high frequency type
MSI-L	Microsatellite instability of low frequency type
MSS	Microsatellite stable

MSS	Microsatellite stable
SSCP	Single stranded conformational polymorphism
PTT	Protein truncation test
YAC(s)	Yeast artificial chromosome(s)
PAC	P1-derived artificial chromosome
BAC	Bacterial artificial chromosome
Tm	Melting temperature
ddw	Double distilled water
APS	Ammonium per sulphate
bp	Base pair
HGMP	Human Genome Mapping Project Resource Centre
G+C	Guanine and Cytosine
F	Forward primer
R	Reverse primer
CCR	Continuous clinical remission
MNC	Mononuclear cell
DFS	Disease free survival
ATL	Adult T-cell leukaemia
LTP	Long term potentiation
MDS	Myelodysplastic syndrome
IWCCLL	International Working Party on Chromosomes in CLL
MMR	Mismatch repair
T-PLL	T-Prolymphocytic leukaemia
LGL	Large granular lymphocyte
T-CLL	T-chrononic lymphocytic leukaemia
CON	Colcemid overnight
PHA	Phytohaemagglutinin
TPA	Phorbol ester
MCL	Mantle cell lymphoma
FGFR-1	Fibroblast growth factor receptor-1
T-CLL	T-cell chronic lymphocytic leukaemia

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Chapter 1

Introduction

1.1. Cancer genetics

Cancer is the result of a multistep process that involves sequential acquisition of mutations that drive the progressive transformation of a normal cell into a malignant one (Hanahan D. and Weinberg R. A., 2000). These mutations may result from a variety of events. Different aspects of the multi-step process leading to the formation of cancer will be discussed in this introduction. An attempt will be made to extensively cover all of the topics involved but, for reason of space and relevance, some individual topics may only be briefly mentioned while others will be more extensively discussed. Throughout, I will refer the reader to extensive reviews, when appropriate.

The transformation of a normal to a malignant cell may involve steps during which regulatory genes that govern the processes of proliferation and homeostasis undergo mutations or structural changes (as consequence of chromosomal translocations and amplifications) resulting in increased proliferation. These genes are referred to as oncogenes.

In other instances, deletion of genetic material is associated with loss of function of genes involved in suppressing cell proliferation. These genes are referred to as tumour suppressor genes (TSG).

Cancer cells may develop following the loss of regulatory mechanisms controlling cell death (apoptosis) and replicative senescence, to become immortal.

The molecular changes leading to any of the above events are frequently recognised as a failure to repair DNA damage resulting in permanent structural or functional changes and subsequent tumour formation. DNA damage *per se* may result from endogenous processes such as DNA replication errors, toxic metabolic products such as free radicals that attack DNA or inherent chemical instability of some DNA bases. Exogenous DNA damage can be induced by exposure to environmental agents such as ultraviolet light (UV), ionising radiation (IR) and genotoxic chemicals. If unrepaired, this may lead

to mutations and increased cancer risk (Bertram J. S., 2000; Loeb K. R. and Loeb L. A., 2000).

Transformation of the cancer cell to an immortal cell requires an adequate supply of nutrients and oxygen needed for the maintenance of the high rate of proliferation, which is also a hallmark of malignancy (Bertram J. S., 2000).

Changes in one or several of the above pathways result in the acquisition of novel functional capabilities such as self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan D. and Weinberg R. A., 2000). As a consequence, a cell can survive and proliferate to form a clone or a population of cells that is genetically distinct from a "normal" cell.

This thesis will report on the investigation of tumours predominantly in the haematopoietic tissue but, whenever relevant, reference to tumours in other tissues will be used.

1.2. Leukaemia

Leukaemias are clonal proliferations affecting the haematopoietic tissue. They emerge as a result of acquired changes (translocations, deletions and insertions) of a variety of genes that confer either proliferative capability or survival advantages (Gilliland D. G., 1998). The cell in which most common leukaemic transformation occurs may be a lymphoid or myeloid precursor or a pluripotent stem cell capable of differentiating into both myeloid and lymphoid cells.

Leukaemias are broadly classified into acute leukaemias (characterised by continued expansion of immature cells) and chronic leukaemias (characterized by an expanded pool of proliferating cells that retain their ability to differentiate to end-cells). Leukaemias are further divided into lymphoid, myeloid and biphenotypic depending on the expression of lymphoid, myeloid or both differentiation markers (Bain B. J., 1999). Classification of acute leukaemia depends primarily on morphology and cytochemistry but essential information is gained from immunological classification, cytogenetic and molecular analysis of both DNA and RNA. The definition of acute leukaemia according to the French, American and British classification (FAB) remains the most widely used

worldwide (Bennett J. M. *et al.*, 1976; 1980; 1985; 1991) despite further modifications and expansions by other groups (Bloomfield C. D. and Brunning R. D., 1985; Cheson B. D. *et al.*, 1990; Hayhoe F. G., 1988).

Acute lymphoblastic leukaemias (ALL) are the most common malignancy in childhood, accounting for 80% of cases of acute leukaemia in children, but only 20% of adult leukaemia, whilst acute myeloid leukaemia (AML) comprises around 80% of adult cases (Greaves M., 1999)

Chronic lymphoid leukaemias are broadly classified into B and T-lineage leukaemias with a minority being of natural killer cell lineage. The FAB classification includes among chronic leukaemias some lymphomas that may involve bone marrow and peripheral blood (Bennett J. M. *et al.*, 1989).
According to the World Health Organisation (WHO) both B-cell and T-cell neoplasms are divided into precursor B-cell or T-cell neoplasms, mature B-cell or T-cell neoplasms and B-cell or T-cell proliferations of uncertain malignant potential.
The neoplasms of the myeloid lineage are classified into: Chronic myeloproliferative diseases, Myelodysplastic/Myeloproliferative diseases, Myelodysplastic syndromes and, acute myeloid leukaemias (Jaffe E. S. *et al.*, 2001).

This thesis will mainly deal with the following cases of acute leukaemias:

1. Precursor B lymphoblastic leukaemia/lymphoblastic lymphoma (Acute lymphoblastic leukaemias, FAB: L1 and L2).
2. Precursor T lymphoblastic leukaemia/lymphoblastic lymphoma

Leukaemias emerge frequently as a result of genetic deregulations associated with chromosomal abnormalities that will be briefly described in the next paragraphs.

1.3. Cytogenetic abnormalities in leukaemia

Cytogenetic analysis of leukaemic cells shows recurring and consistent chromosomal abnormalities that are specific for certain types of proliferations. Chromosomal abnormalities can arise from numerical or structural changes, or both, particularly in

ALL (Ferrando A. A. and Look A. T., 2000).

Numerical chromosomal changes lead to clones with increased or decreased copies of one or more chromosomes and are classified into groups according to the chromosome number.

Structural abnormalities include translocations, deletions, inversions and other rearrangements involving genes with oncogenic potential. These lead to disruption of specific differentiation or proliferative pathways and the progression of leukaemogenesis. Some structural changes are associated with B-lineage ALL whilst others are specifically found in the T-cell lineage.

Figure 1.1 and Figure 1.2 show the distribution of numerical chromosomal abnormalities and the distributions of chromosomal translocations in paediatric and adult ALL cases, respectively. Both figures are adapted from (Ferrando A. A. and Look A. T., 2000).

1.4. Translocations and associated genetic changes

In haematological malignancies translocations are generally more frequent than deletions that are, in turn, more frequent than inversions, as detected by conventional cytogenetic analysis. Chromosomal/molecular abnormalities in B and T lineage precursor ALL in children and adults are not only important for understanding the mechanisms involved in the process of leukaemogenesis but also for their prognostic value. For instance, the t(12;21)/(*TEL/AML*) and the hyperdiploid karyotypes each identifies subgroups of children who respond better to chemotherapy treatment and have lower relapse rate, whilst the t(9;22) (*BCR-ABL)*, 11q23 abnormalities (involving the *MLL1* gene) and near haploidy identify subgroups of ALL with poor clinical outcome (Harrison C. J., 2000).

These chromosomal/molecular markers are being incorporated into risk classification as they can introduce prognostic information that would not otherwise be gained from conventional risk factors such as white blood cell (WBC) count, age, blast cell count at day 14 or 28 post-induction and immunophenotyping (Ferrando A. A. and Look A. T., 2000).

Molecular characterisation of different types of translocations has shown that the

molecular breaks occur in the intronic sequences between exons of genes located on two non-homologous chromosomes.

Two major types of resulting abnormalities have been identified. In the first type, translocations involve the fusion between an antigen receptor gene (immunoglobulin, *Ig*, or T cell receptor genes, *TCR*) and different transcription factors that will be activated by the *Ig* or *TCR* control elements as a result of the translocation (Rabbitts, 1991; 1994). These occur in both B and T cell malignancies and some of the most common of such translocations are listed in the two tables below (Table 1.1 and Table 1.2). Classic examples are *MYC* activation following t(8;14) in Burkitt's type lymphoma, *HOX11* activation following t(10;11) or *LMO2* activation in the t(11;14) in T-ALL.

In the second type, the translocation results in a "chimeric" fusion gene as exons of both genes contribute to the production of a new and chimeric protein, such as *BCR-ABL* following t(9;22); *E2A-PBX*1 in t(1;19) or *TEL-AML1* following the t(12;21).

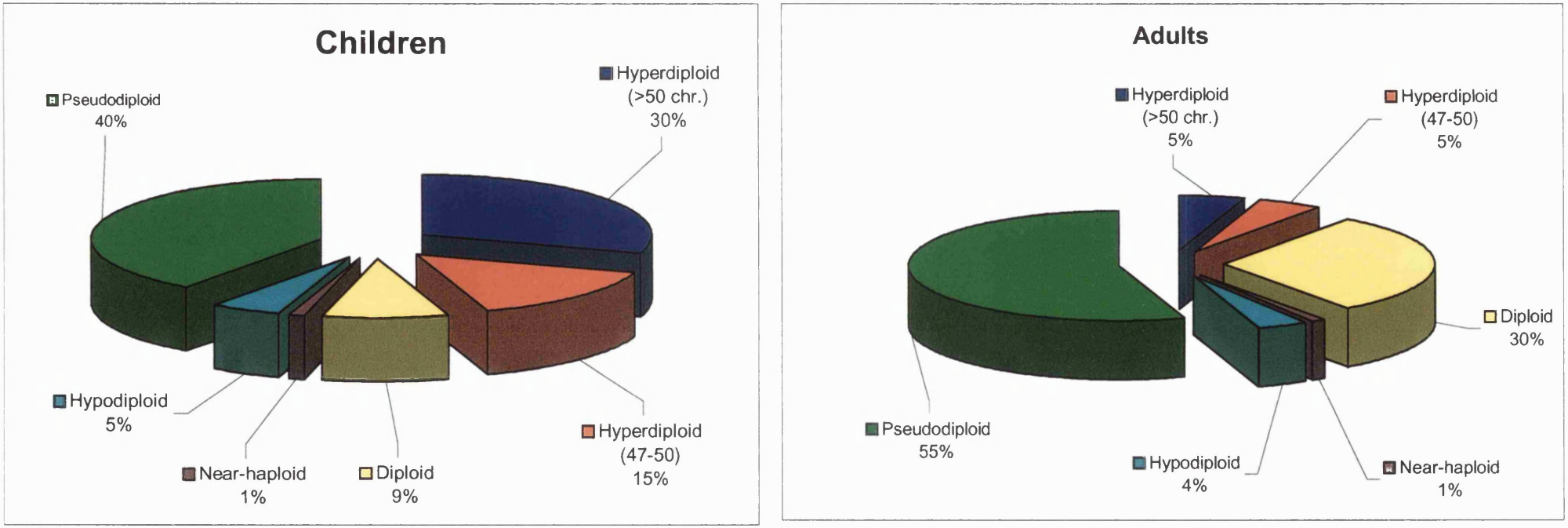


Figure 1.1. Adapted from (Ferrando A. A. and Look A. T., 2000). *Distribution of numerical chromosomal abnormalities in paediatric and adult ALL cases. In children there is a high frequency of the prognostically favourable hyperdiploid subgroup (>50 chromosomes). In adults this abnormality is less frequent.*

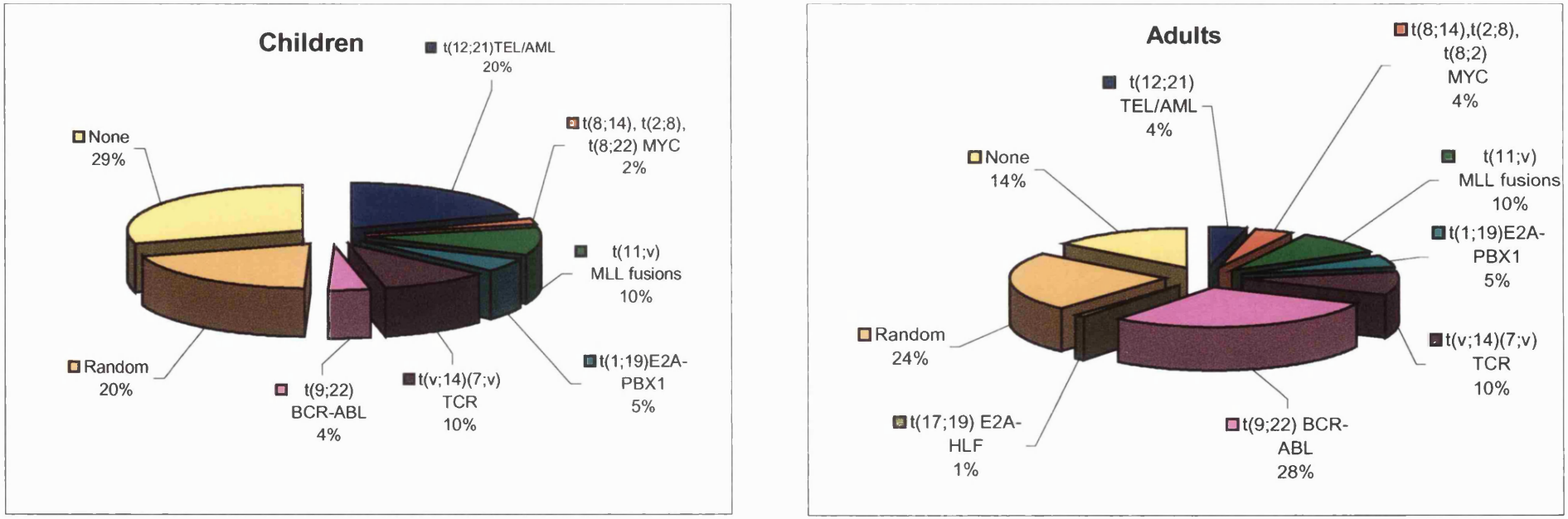


Figure 1.2. Adapted from (Ferrando A. A. and Look A. T., 2000). *Distribution of chromosomal translocations in paediatric and adult ALL cases. t(12;21)/TEL-AML shows higher frequency in children versus adults whereas t(9;22)/BCR-ABL shows higher incidence in adults versus children.*

Rearrangement	Gene	T cell receptor
inv(14)(q11q32)	*IGH*	*TCRA/D*
inv(14)(q11q32)	*TCL1*	*TCRA/D*
t(14;14)(q11;q32)	*TCL1*	*TCRA/D*
t(10;14)(q24;q11)	*HOX11*	*TCRD*
t(7;10)(q35;q24)	*HOX11*	*TCRB*
t(1;14)(p33;q11)	*TAL1*	*TCRD*
t(7;9)(q34;q32)	*TAL2*	*TCRB*
t(7;19)(q34;p13)	*LYL1*	*TCRB*
t(11;14)(p15;q11)	*LMO1*	*TCRDA*
t(11;14)(p13;q11)	*LMO2*	*TCRDA*
t(7;11)(q35;p13)	*LMO2*	*TCRB*
t(8;14)(q24;q11)	*MYC*	*TCRA*
t(1;7)(p34;q34)	*LCK*	*TCRB*
t(7;9)(q34;q34.3)	*TAN1*	*TCRB*
t(8;14)(q24;q32)	*MYC*	*IGH*
t(2;8)(p13;q24)	*MYC*	*IGL kappa*
t(8;22)(q24;q11)	*MYC*	*IGL lambda*

Table 1.1. *Translocations leading to Antigen receptor-oncogene activation most commonly detected in ALL.*

Translocation **Fusion gene type**	**Partner genes**
t(12;21)(p13;q22)	*TEL/AML1*
t(7;12)(q36;p13)	*HLXB9/TEL*
t(9;12)(q11;p13)	*PAX5/TEL*
t(9;12)(q34;p13)	*ABL/TEL*
t(9;22)(q34;q11)	*ABL/BCR*
t(4;11)(q21;q23)	*AF4/MLL*
t(6;11)(q27;q23)	*AF6/MLL*
t(9;11)(p21;q23)	*AF9/MLL*
t(10;11)(p12;q23)	*AF10/MLL*
t(11;19)(q23;p13)	*MLL/ENL*
t(1;19)(q23;p13)	*PBX/E2A*
t(17;19)(q22;p13)	*HLF/E2A*

Table 1.2. *Significant Non-Random Structural Chromosomal Abnormalities leading to fusion gene-type translocations in ALL.*

Genes affected by the translocations listed above are conventionally referred as oncogenes.

Oncogenes were first discovered through studies of tumours caused by viruses in avian and rodent models. These viruses are called acute transforming viruses and shown to incorporate a copy of a particular mammalian gene into their genome (Stehelin D. *et al.*, 1976). The viral oncogenes make the acute transforming viruses capable of stimulating the infected cell to proliferate and consequently help viral replication. The normal cellular counterparts of the oncogenes are known as proto-oncogenes and they have roles in diverse signalling pathways that regulate normal development and differentiation. These proto-oncogenes can be converted into oncogenes by various molecular mechanisms including point mutation, chromosomal translocation and gene amplification (Butturini A. and Gale R. P., 1990; Haber D. A. and Fearon E. R., 1998). They may play their role in malignant transformation by mutations that are usually dominant, as they become negative regulators of cell death and positive regulators of proliferation. It is sufficient for one copy of the gene to be affected for a phenotype to appear (Grander D., 1998).

Because this thesis reports primarily on deletions associated with human lymphoid malignancies I will refer to more extensive reviews for a comprehensive coverage of translocations associated with human malignancies (Bergsagel P. L. and Kuehl W. M., 2001; Boxer L. M. and Dang C. V., 2001; Kuppers R. and Dalla-Favera R., 2001; Pandolfi P. P., 2001; Pekarsky Y. *et al.*, 2001; Rabbitts T. H., 1994) while deletions will be more extensively described below.

1.5. Deletions and associated genetic changes

Chromosomal deletions occur in approximately 15-20% of ALL patients and several different types have been described in the past few years and defined at the molecular level. Only the most common deletions will be described in the following paragraphs.

1.5.1.Cytogeneticaliy identifiabie deietions

1.5.1.1. Chromosome 6 q deletions

Deletions of chromosome 6 will be discussed in greater detail because they represent the main topic of the investigation presented in this thesis. Both their involvement in haematological and solid tissue cancers will be presented, as more information is available at present on the latter than the former type of cancer. There are several commonly deleted regions on the long arm of chromosome 6 (6q) that vary in different tumours and different studies. Not all regions showing allelic loss/deletions overlap suggesting that several tumour suppressor genes on 6q may be associated with carcinogenesis. These different issues will be discussed whenever pertinent to this presentation.

A. Chromosome 6q deletions in haematological malignancies

In haematological malignancies, deletions on the long arm of chromosome 6 are frequently found in ALL and non-Hodgkin's lymphomas (NHL). Cytogenetic analysis has shown 6q deletions in 4%-13% in ALL (Hayashi Y. *et al.*, 1990), and in 13%-15% in NHL (Offit K. *et al.*, 1991), while loss of heterozygosity (LOH) analysis has shown 6q deletions in higher proportion (7%-13%) of ALL (Takeuchi S. *et al.*, 1995b), (Gerard B. *et al.*, 1997) and NHL (22%) (Gaidano G. *et al.*, 1992).

The higher frequencies of 6q deletions detected by LOH studies may be attributed to the ability of the technique to detect small deletions that are beyond the resolution of cytogenetic analysis. In addition, the polymerase chain reaction (PCR) employed for LOH analysis overcomes the problems associated with cytogenetic analysis such as

poor metaphases, especially in ALL cases increasing the number of cases for testing. Three distinct and non-overlapping regions of minimal deletions (RMD) have been identified on 6q. RMD1, at 6q25-27, is more commonly detected in NHL (Merup M. *et al.*, 1998); RMD2, at 6q21, is identified mainly in ALL (Takeuchi S. *et al.*, 1998) while FISH analysis identified a commonly deleted region on 6q11-21 (Guan X. Y. *et al.*, 1996). A study done by Gaidano et al using restriction fragment length polymorphism (RFLP) detected 6 q deletions in 22.5% in a series of 71 patients with NHL and highlighted two regions of deletions, one on 6q21-23 and one in 6q25-27 (Gaidano G. *et al.*, 1992) in support of heterogeneity in 6q deletions in lymphoid malignancies (Offit K. *et al.*, 1993). I refer to Figure 3.1. and 3.2. (Chapter 3) for a diagrammatic representation of these regions.

The three reported regions are found to be associated with different pathological subtypes at least in NHL. RMD1 at 6q25-27 is most frequently associated with intermediate grade NHL; RMD2 at 6q21 is associated with high grade NHL and RMD3 at 6q23 is associated with low-grade NHL without t(14;18)(Offit K. *et al.*, 1993). Also, deletion of chromosome 6q21-q23 was found to be the most common cytogenetic abnormality by chromosome banding technique and accounting for 26% of small lymphocytic NHL (Offit K. *et al.*, 1994).

Studies focused on the RMD2 in ALL revealed 2 sub-regions both on 6q21. The first one, RMD-2A is between markers D6S1709 and D6S301 with marker D6S283 being the most frequently deleted marker within this region (Merup M. *et al.*, 1998). The second sub region, RMD-2B, is defined between markers D6S301 and D6S302 (Takeuchi S. *et al.*, 1998). This region has been further narrowed down to approximately 2Mb between marker D6S447 and marker D6S246 (Sherratt T. *et al.*, 1997). Jackson and colleagues (2000) also confirmed the presence of 2 RMD on 6q in ALL and further reduced the region of deletion within RMD-2B to less than 1.8Mb (Jackson A. *et al.*, 2000). A detailed description of these data will be presented in Chapter 3 of this thesis.

In B-cell chronic lymphocytic leukaemia (B-CLL) conflicting data have been reported regarding the incidence of 6q abnormalities. Chromosomal banding analysis has shown 6q abnormalities in 6% of cases (Juliusson G. and Gahrton G., 1993).

Higher incidence of 6q deletions has been detected in B-CLL using FISH with probes mapping to 6q23-q26 (Zhang Y. *et al.*, 1997). However, in another study using interphase FISH to analyse a large group of B-CLL patients only 7% of the studied cases have shown deletions on 6q21 (Stilgenbauer S. *et al.*, 1999). This latter study could not confirm the high frequency detected by Zhang et al (Zhang Y. *et al.*, 1997), but showed an incidence consistent with that obtained by other groups (Juliusson G. and Gahrton G., 1993; Merup M. *et al.*, 1998).

B. Chromosome 6q deletions in solid tumours

6q deletions have been detected in hepatocellular (De Souza A. T. *et al.*, 1995a), pancreatic (Abe T. *et al.*, 1999), breast (Devilee P. *et al.*, 1991), prostate (Visakorpi T. *et al.*, 1995), ovarian (Lee J. H. *et al.*, 1990), (Saito S. *et al.*, 1992), gastric (Ochi H. *et al.*, 1986), small cell lung carcinoma (Merlo A. *et al.*, 1994), malignant melanoma (Ray M. E. *et al.*, 1996), salivary gland adenocarcinoma (Stenman G. *et al.*, 1989) and parathyroid adenoma (Tahara H. *et al.*, 1996).

1. Hepatocellular carcinoma (HCC)

In hepatocellular carcinoma (HCC) most LOH studies have focused on 6q26-27. The deletions detected ranged between 30%-70%. 6q26-27 is the location for a putative candidate tumour suppressor gene encoding mannose 6-phosphate/insulin-like growth factor 2 receptor (*M6P/IGF2R*) (De Souza A. T. *et al.*, 1995a; De Souza A. T. *et al.*, 1995b; Piao Z. *et al.*, 1997).

Other minimally deleted regions in HCC have been identified on 6q23 within 1-cM interval (Koyama M. *et al.*, 2000) and on 6q14 within 2 centimorgans (cM) (Huang S. F. *et al.*, 2000).

2. Pancreatic cancer

Three commonly deleted regions on 6q have been identified in exocrine adenocarcinoma human pancreatic cancer. Region A, a less than 500 kilobase pairs (Kb) region on 6q21 in 69% of the cases, region B, a 7-cM region on 6q23-24 in 60%, and region C, a 13-cM region with 51% frequency of LOH between D6S305 and D6S264 in 6q26 (Abe T. *et al.*, 1999). It is interesting that region A is in the middle of the 2 regions detected by a study of LOH analysis in ALL (Takeuchi S. *et al.*, 1998).

In sporadic endocrine pancreatic tumours about 60% of the cases showed losses on 6q22.1 and 6q23-24 where TSGs are suggested to exist in these regions. These TSGs might be of importance not only in initiation of the tumour but also in malignant metastatic progression (Barghorn A. *et al.*, 2001). Moreover, it has been found that 6q losses occur in several other endocrine tumours including parathyroid adenoma (Tahara H. *et al.*, 1996), adrenocortical carcinoma (Zhao J. *et al.*, 1999) and pheochromocytoma (Dannenberg H. *et al.*, 2000), suggesting that the same TSG may be involved in these tumours. There is a possible link between the presence of several genes on the long arm of chromosome 6 coding for hormones and hormonal receptors and the occurrence of tumours in these endocrine organs (Barghorn A. *et al.*, 2001).

3. Breast lesions

Analysis of breast lesions by cytogenetic and FISH techniques has shown that deletions in 6q24-qter are common in breast lesions including benign breast tumours (83.9%), premalignant lesions (64%) and breast carcinoma (77.4%) (Tibiletti M. G. *et al.*, 2000). A study that combined the use of allelotyping and comparative genomic hybridisation (CGH) of 178 paired breast tumour and normal tissues DNAs using 30 CA repeat markers showed that 76% of the cases displayed allelic imbalance (AI) of at least one locus. Five distinct domains of AI were identified from centromere to telomere, by D6S300 (domain 1), D6S443 (domain 2), D6S261 (domain 3), D6S314-D6S409 (domain 4) and D6S441- D6S415 (domain 5). CGH analysis of 34 tumours showing allelic alterations on 6q showed that the 6q21-22 region was the most commonly involved in gains, whereas 6q13-14 and 6q25-27 were frequently lost have been detected. (Rodriguez C. *et al.*, 2000).

4. Prostate cancer

Using CGH allelic loss has been detected in 22% of primary prostate cancer and 44% of recurrent cancers with a minimal deletion region extending from 6 cen-6q21 (Visakorpi T. *et al.*, 1995). This proximal 6q deletion had been confirmed by a second study and the region of minimal deletion narrowed down to 6q14-21 with evidence of a second region at 6q16.3-q23.2. The same study showed that distal 6q deletions in prostate cancer are uncommon, representing about 4%. This excludes the insulin growth factor receptor –2 (*IGF2R*) as playing a role in prostate cancer (Cooney K. A. *et al.*, 1996). A study of allelic loss on 6q in primary prostate cancer has revealed a region of minimal deletion on 6q in a 1.5 Megabase interval between markers D6S1056 and D6S300 at 6q16.3-21(Srikantan V. *et al.*, 1999).

5. Ovarian cancer

Non-random deletions of 6q have been reported in primary ovarian cancer both by FISH (Pejovic T. *et al.*, 1992; Thompson F. H. *et al.*, 1994) and LOH analysis (Dodson M. K. *et al.*, 1993; Ehlen T. and Dubeau L., 1990; Lee J. H. *et al.*, 1990; Zheng J. P. *et al.*, 1991). One of the studies concentrated on the terminal 6q region and showed LOH at 6q27 in 59%-73% of cases (Foulkes W. D. *et al.*, 1993). A second region of deletion has been identified at 6q21-23 (Cliby W. *et al.*, 1993).

Using Southern blot analysis, 34 primary ovarian epithelial tumours were examined for the presence of tumour-specific allelic loss. Six genomic probes for chromosomes 6q, 11p, 13q, 16q, and 17p were used. LOH on chromosome band 6q was detected in only 15% of primary ovarian tumours at the oestrogen receptor site. (Gallion H. H. et al., 1992). This is in contrast to the findings by other groups (Lee J. H. et al., 1990; Zheng J. P. et al., 1991) who detected allelic loss at the same site in > 50% of ovarian tumours. Furthermore, detailed mapping of 6q25-26 identified a 4-cM region of minimal deletion on 6q25.1-q25.2 (Colitti C. V. et al., 1998). Suzuki et al found LOH at 6q27 in 54% of cases studied without significant relationship to the different histological types of the tumour (Suzuki M. et al., 1998). Another study showed LOH at 6q27 in 5 out of 7 cases of ovarian tumours (Rey J. M. et al., 2000). Recently a study in which combined laser microdisection (LCM) and primer extension preamplification (PEP) had been used, identified three distinct regions on chromosome 6. The three identified regions were on 6p24-25, 6q21-23 and 6q25.1 –27 and these may be associated with the early onset of ovarian cancer (Wang V. W. et al., 2001).

6. Gastric carcinoma

Deletions of 6q are common in gastric carcinomas. Consistent deletions of 6q21-qter have been observed in 27%-45% of cases, (Ochi H. *et al.*, 1986; Panani A. D. *et al.*, 1995; Seruca R. *et al.*, 1993). LOH analysis showed 32% allelic losses at 6q (Seruca R. *et al.*, 1995). LOH at 6q is detected in all histological types (Gleeson C. M. *et al.*, 1997; Seruca R. *et al.*, 1995) and in both early and advanced cases (Seruca R. *et al.*, 1993). Deletion mapping of 6q identified two regions of deletion; an interstitial one on 6q16.3 spanning 12-16 cM, and another, distal one, at 6q23-24 (Queimado L. *et al.*, 1995). The interstitial region has been narrowed down from 12-16 cM to a region of 2 cM on 6q16.3-q23.1 (Carvalho B. *et al.*, 1999). Genetic aberrations associated with the development of gastric high-grade large B-cell lymphoma have not been fully

identified, to date. LOH on 6q has been detected in 42% of cases. Two regions were identified on 6q21-22.1 and 6q23.3-25 (Starostik P. *et al.*, 2000).

7. Other solid tumours

Parathyroid adenomas are benign monoclonal tumours associated with hypercalcaemia and increased parathyroid hormone secretion (Arnold A. *et al.*, 1988).
Two regions of allelic loss on 6q have been identified in parathyroid adenoma. These regions of deletions are on 6q22-23 and 6q26-27 (Tahara H. *et al.*, 1996). It is of interest that the 6q22-23 region overlaps with the commonly deleted regions observed in B-cell NHL (Gaidano G. *et al.*, 1992), and malignant melanoma (Millikin D. *et al.*, 1991). Region 6q26-27 has been found deleted in other tumours including renal cell carcinoma (Morita R. *et al.*, 1991), malignant melanoma (Millikin D. *et al.*, 1991), ovarian cancer (Saito S. *et al.*, 1992), and in lymphoma (Gaidano G. *et al.*, 1992).

Malignant mesothelioma (MM) is a rare tumour of mesodermal origin that arises from mesothelial cells lining the pleura, peritoneum and pericardium (Cavenee W. K. *et al.*, 1983). Cytogenetic studies revealed losses of 6q15-21 in about 40% of cases (Taguchi T. *et al.*, 1993). The presence of multiple tumour suppressor loci in 6q is suggested as four non-overlapping regions of losses have been identified in MM. These regions of chromosomal loss were identified as *S*mallest *R*egions of *O*verlap (SRO) 1-4, where SRO1 on 6q14-21, SRO2 on 6q16.3-6q21, SRO3 on 6q21-23.2 and SRO4 on 6q25 (Bell D. W. *et al.*, 1997). Each of these regions overlaps with the reported regions of deletion in breast cancer (Fujii H. *et al.*, 1996; Orphanos V. *et al.*, 1995a; Sheng Z. M. *et al.*, 1996; Theile M. *et al.*, 1996). The SRO1 overlaps with deletions reported in ovarian (Orphanos V. *et al.*, 1995b; Saito S. *et al.*, 1992) and prostate cancer (Cooney K. A. *et al.*, 1996) and SRO4 overlaps with deletions occurring in the serous type of ovarian cancer (Orphanos V. *et al.*, 1995b).
Moreover, the deleted regions reported in MM overlap with the minimally deleted regions in 6q21, 6q23 and 6q25-27 reported in NHL (Offit K. *et al.*, 1993).
Taken altogether it seems that one or more candidate TSG genes on 6q may be involved in different types of malignancies. Figure 1.3 shows the deleted regions on 6q reported in some solid tumours.

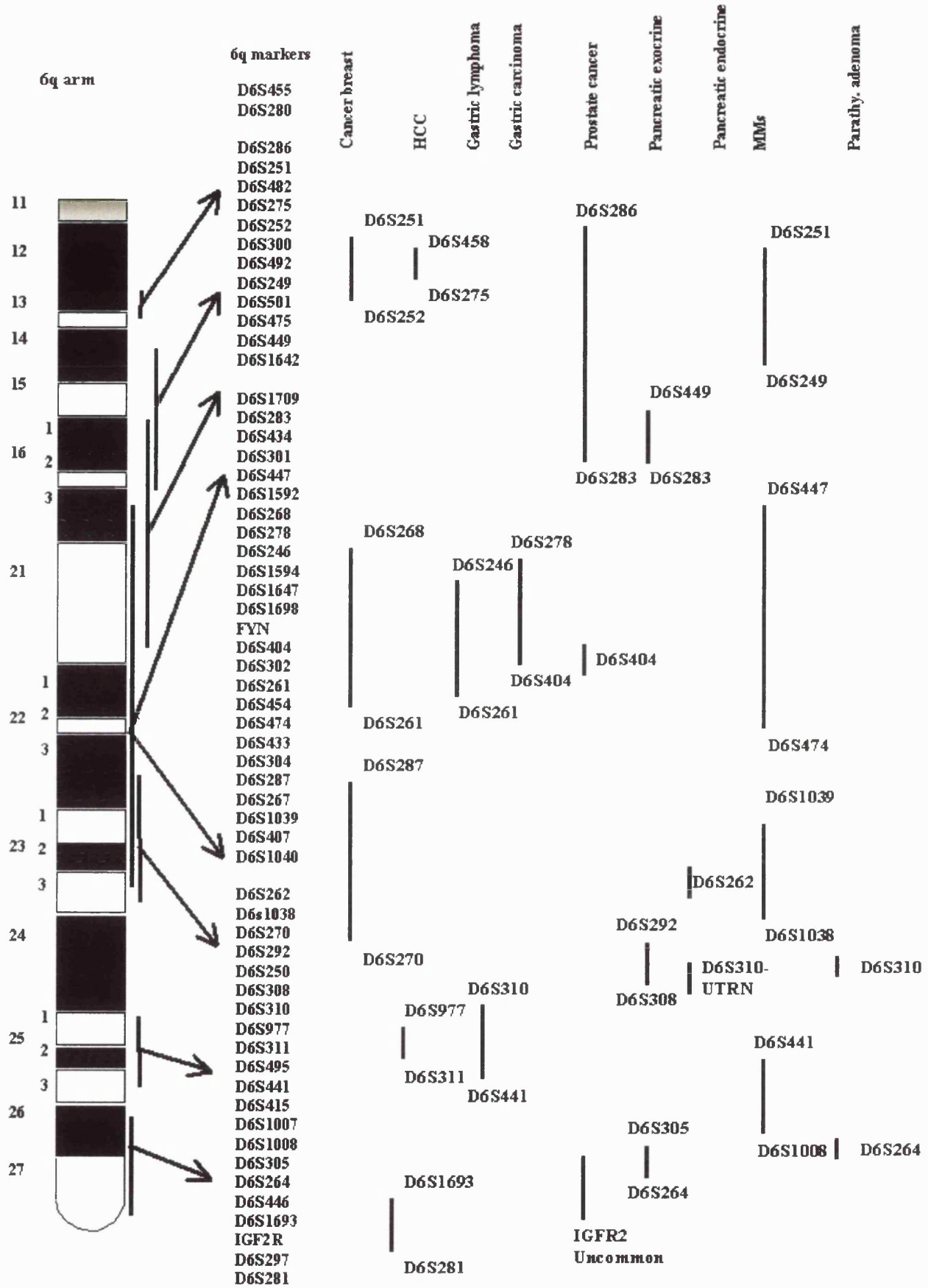


Figure.1.3. *illustrates some of the reported regions of 6q deletions in solid tumours. See Figure 3.1. and 3.2. for comparable diagrammatic representation of region of deletions in lymphoid human malignancies .HCC: hepatocellular carcinoma, MMs: malignant mesotheliomas, Parathy.adenoma: parathyroid adenoma.*

1.5.2. Other non-random deletions in human lymphoid acute malignancies

1.5.2.1. 9p deletions

Abnormalities involving the short arm of chromosome 9 (9p) have been reported in approximately 10% of both adults and childhood ALL (Heerema N. A. *et al.*, 1999), and higher percentage in T ALL (Secker-Walker L. M., 1997). The majority of 9p abnormalities result in a deletion of this arm, which usually include the cell cycle regulatory genes $p16^{INK4A}$, $p15^{INK4B}$ *and* $p14^{ARF}$ (Drexler H. G., 1998; Ferrando A. A. and Look A. T., 2000; Zhou M. *et al.*, 1997). The main target appears to be $p16^{INK4A}$. Deletions of $p16^{INK4A}$ have been detected by molecular analysis and FISH in approximately 80% of childhood T ALL and 20% of c-/pre-B ALL (Ferrando A. A. and Look A. T., 2000; Morison I. M. *et al.*, 2002; Rubnitz J. E. *et al.*, 1997). It has been reported that the maternal allele is more commonly deleted than the paternal allele in childhood ALL (Morison I. M. *et al.*, 2002). Deletions of 9p have been shown to be an adverse risk factor in B-lineage, but not T-lineage ALL (Heerema N. A. *et al.*, 1999), except that the homozygous form of this deletion is associated with significantly poorer evevt free survival (EFS) in T ALL (Kees U. R. *et al.*, 1997). Indeed, a homozygous deletion of *p16* is associated with increased rates of relapse and death in childhood ALL, even when other prognostic factors are taken into account (Heyman M. *et al.*, 1996), but no clinical relationships were detected for *p14* and *p16* deletions in adult ALL (Faderl S. *et al.*, 1998).

P15 promoter methylation is associated with loss of gene expression in both adults and childhood ALL and AML, whereas *p16* methylation is rare in all primary leukaemias (Drexler H. G., 1998). This suggests that promoter-methylation may also result in gene silencing without deletion ultimately leading to oncogenesis.

1.5.2.2. 12p deletion

Deletions of 12p12-13 bands occur in 17%-33% of cases studied and may indicate the presence of a tumour- suppressor gene in this region (Stegmaier K. *et al.*, 1995). Two

genes recently identified in the region are the *TEL (ETV6)* and the cyclin dependant-kinase inhibitor *p27* gene (Aissani B. *et al.*, 1999). In childhood ALL cases with 12p LOH, no point mutation has been detected in these genes on the retained allele suggesting the presence of a third tumour suppressor gene on 12p, yet unidentified. Alternatively *TEL* and *p27* haploinsufficiency may have a role in ALL transformation (Stegmaier K. *et al.*, 1995) but this remains to be demonstrated.

1.5.2.3. 13q deletions

Deletions of chromosome 13 occur in approximately 2-10% of acute leukaemias affecting both adults (5-10%) and childhood ALL (2-5%) (Chung C. Y. *et al.*, 2000). This deletion is more frequent, however, in other chronic lymphoid malignancies such as B-CLL and NHL, with the most common deletion detected within 13q12-16 region.
In a recent study of a large series of childhood ALL patients; breakpoints of 13q12-14 were identified in 36 (2%) of the 1,946 cases with available cytogenetic data (Heerema N. A. *et al.*, 2000). Eight cases had balanced rearrangements of 13q12-14, but 27 patients had a partial loss of 13q, and one had both a partial gain and a partial loss, suggesting that translocations can associated in a proportion of patients with loss of material.
Association between translocations and deletions has also been recently described for chronic myeloid leukaemia (CML) patients where loss of chromosome 9 in patients carrying t(9;22)(Sinclair P. B. *et al.*, 2000) negatively influences the overall clinical outcome.
With regards to chromosome 13q deletions, however, no individual TSG has yet been identified in ALL within the region spanning the D13S262 and D13S25, in a region telomeric to the *BRCA2* gene (13q14).

Chromosomal deletions are commonly indicative of the presence of a tumour suppressor gene within the deleted regions. A brief description of the main molecular, functional, and structural analysis of tumour suppressor genes will follow in the next paragraphs.

1.6. Tumour suppressor genes

Tumour suppressor genes (TSGs) or anti-oncogenes are the negative regulators of cell division. Loss of function of TSGs can be the result of germline or somatic mutations resulting in loss-of-function. Most commonly, the function of both copies of the TSG in a cell needs to be affected for a phenotype to manifest itself. For this reason, people carrying heterozygous germline mutations of TSGs are more predisposed to cancer, as they only require loss of one remaining allele for the phenotype to appear.
This mechanism of action also relates the hereditary and non-hereditary forms of cancer. In the hereditary form the first mutation required is germinal while in the non-hereditary form it is a somatic mutation. Again in the hereditary case all the somatic cells carry an initial event, whereas in non-hereditary cases a clone of somatic cells would do so (Knudson A. G., Jr., 1971).

The discovery of TSGs in human cells came from the several lines of studies, briefly summarised below.

1.6.1. Cell fusion experiments

The first line of evidence came from the cell fusion experiments between normal and tumour cells, where the normal phenotype was found to be dominant (Harris H., 1988) over the abnormal phenotype and suggested that tumour cells might lack the expression of one or more of those genes that act as negative regulators of cell growth.

1.6.2. Chromosome transfer techniques

A second line of evidence came from the use of chromosome transfer techniques in which microcells with a single human chromosome were fused with a tumour cell, allowing the identification of a particular chromosome that suppressed the tumour formation suggesting that this chromosome contained a tumour suppressor gene (Marshall C. J., 1991).

1.6.3. Familial cancer studies

A third line of evidence came from studies of familial cancers. Several inherited predispositions to cancer were genetically mapped. In some cases the corresponding chromosomal regions showed deletions which implied that one copy of the gene in question had been lost and a cell without a functional gene would emerge, if the other copy of the gene were mutated. The functioning copy of the gene could be inactivated by several mechanisms including those genetic events that lead to homozygosity of the corresponding chromosomal regions. Candidate TSGs were identified by investigating specific groups of tumours with chromosomal regions that had been converted to homozygosity. Retinoblastoma (*RB), p53*, deleted in colon carcinoma (*DCC*) and Wilm's tumour/aniridia/genitourinary anomaly/mental retardation (*WAGR*) genes are examples of such genes (Marshall C. J., 1991).

It is worth noting that inactivation of a TSG can occur both by loss of function mutations that result in inactivation of the protein or deletion of the gene such as mutations occurring in *RB* and *p53*, as well as by gain of function mutations, which convert the protein into a dominant negative form that can suppress the activity of the wild type protein such as mutations of *p53* (Hunter T., 1991).

1.6.4.Loss of heterozygosity (LOH)

LOH can occur as a consequence of multiple events including:

(i) Loss of the chromosome carrying the normal allele.

(ii) Loss of the normal chromosome and duplication of the defective or mutant chromosome.

(iii) Somatic cell recombination in which the sister chromatid exchange results in loss of the normal allele and the presence of two chromosomes each with defective allele.

(iv) Deletion or point mutation of the normal allele can also occur (Lasko D. *et al.*, 1991).

LOH can be assayed by a comparison of polymorphic loci in DNA in normal and tumour DNA in the same individual and the finding of contiguous regions of tumour DNA where on allele is lost. It is useful when cytogenetic abnormalities are not available via standard cytogenetic test or FISH analysis. However, it requires tumour cell purity greater than 50-60% to be informative. Table 1.3 shows examples of identified TSG using the different approaches described above.

Tumour suppressor gene	Chromosomal location	Human tumours associated with sporadic mutation	Associated cancer syndrome
RB1	13q14	Retinoblastoma, osteosarcoma	Familial retinoblastoma
WT1	11p13	Nephroblastoma	Wilm's tumour
P53	17q11	Sarcomas, breast/brain tumours	Li-Fraumeni
NF1	17q11	Neurofibromas, sarcomas, gliomas	Von Recklinghausen neurofibromatosis
NF2	22q12	Schwannomas, meningiomas	Neurofibromatosis type 2
VHL	3p25	Haemangioma, renal pheochromocytoma	Von-Hipple Lindau
APC	5q21	Colon cancer	Familial adenomatous polyposis
INK4a	9p21	Melanoma, pancreatic	Familial melanoma
PTC	9q22.3	Basal cell carcinoma, medulloblastoma	Gorlin syndrome
BRCA1	17q21	Breast/ovarian tumours	Familial breast cancer
BRCA2	13q12	Breast/ovarian tumours	Familial breast cancer
DPC4	18q21.1	Pancreatic, colon, hamartomas	Juvenile polyposis
FHIT	3p14.2	Lung, stomach, kidney, cervical carcinoma	Familial clear cell renal carcinoma
PTEN	10q23	Glioblastoma, prostate, breast	Cowden syndrome
TSC2	16	Renal, brain tumours	Tuberous sclerosis
NKX3.1	8p2.1	Prostate	Familial prostate carcinoma
LKB1	19p13	Hamartomas, colorectal, breast	Peutz-Jeghers
E-Cadherin	16q22.1	Breast, colon, skin, lung carcinoma	Familial gastric cancer
MSH2	2p22	Colorectal cancer	HNPCC
MLH1	3p21	Colorectal cancer	HNPCC
PMS1	2q31	Colorectal cancer	HNPCC
PMS2	7p22	Colorectal cancer	HNPCC
MSH6	2p16	Colorectal cancer	HNPCC

Table 1.3. *Tumour suppressor genes and associated human cancers. Modified from (Macleod K., 2000). This table shows examples of tumour suppressor genes defined strictly by the observation that germline mutation of these genes predisposes to human cancer. HNPCC; hereditary non- polyposis colorectal cancer.*

Some of the classic TSGs, and their mechanisms of action will be briefly described below.

1.7. Some classic tumour suppressor genes

1.7.1. The Retinoblastoma gene (*RB*)

Retinoblastoma is the most common malignant eye tumour in children. Approximately 40% of the cases are of the familial form while the remaining 60% of cases occur sporadically. In the familial form the tumour appears during the neonatal period or shortly after birth. It is usually bilateral with a high incidence of developing other cancers later in life such as osteosarcoma, carcinomas of the bladder, the lung, the prostate and the breast. In sporadic cases there is no previous family history. The tumour occurs in one eye only and appears later in life, with low incidence of involvement of the second orbit or developing secondary malignancies as in the familial form. The two hit hypothesis of carcinogenesis (Knudson A. G., Jr., 1971) is based on this pattern of inheritance (Figure 1.4). In familial cases, children are born with one defective allele due to germ line mutation and one normal allele, which then become damaged after birth. This also explains the high incidence of bilateral tumours in the familial cases. The low frequency of bilateral involvement of both orbits in the sporadic cases can be explained by the inheritance of two normal alleles with low probability that the two alleles will be damaged in both retinas (Knudson A. G., Jr., 1978; Knudson A. G., Jr. *et al.*, 1975). About 5-10% of the hereditary cases show a constitutional deletion of part or all chromosome band 13q14. The use of polymorphic genetic markers as reflection fragment length polymorphisms (RFLPs) has demonstrated the recessiveness of the *RB* in oncogenesis (Cavenee W. K. *et al.*, 1983) and confirmed the two hits hypothesis with the need for a second event such as mutation, deletion, chromosomal non-disjunction, and somatic recombination (Knudson A. G., Jr., 1978). Furthermore it was demonstrated that normal cDNA of *RB* causes reversion of the tumourigenic effects of cultivated tumour cells that were mutant for *RB* (Knudson A. G., 1993). *RB* thus became the first human tumour suppressor gene to be discovered.

The *RB* gene has been mapped to chromosomal band 13q14.2. It codes for a protein, the RB protein, which is expressed in the nucleus of all cells and regulates important processes such as cell cycle, differentiation and apoptosis.

The RB protein is phosphorylated during the cell cycle. In its unphosphorylated form it blocks the passage through G1 to S phase by complexing with the transcription factor E2F that normally activates cell cycle genes (Knudson A. G., 1993).

1.7.2.*P53* Gene

P53 has been mapped to human chromosome 17, band p13. Loss of heterozygosity of chromosome arm 17p specific markers in many human tumours implicated it as a tumour suppressor gene. It was found that *p53* is consistently mutated in most case of Li-Fraumeni syndrome (LFS). LFS is a dominantly inherited syndrome (Malkin D. *et al.*, 1990) that has been discovered during the studies of familial rhabdomyosarcoma in children (Li F. P. and Fraumeni J. F., Jr., 1969). The families of these children have a high incidence of developing tumours such as breast carcinoma, osteosarcoma and leukaemia. P53 functions by monitoring any DNA damage induced by stress such as anoxia, insufficient nucleotides for DNA synthesis and single strand breaks and responds by directing the cell towards either cell cycle arrest or apoptosis depending on the degree of DNA damage and the ability of the cell to repair the damage before the next cycle. 70% of human cancers show defective *p53* gene, which indicates its important role in eliminating DNA damage (Levine A. J., 1997).

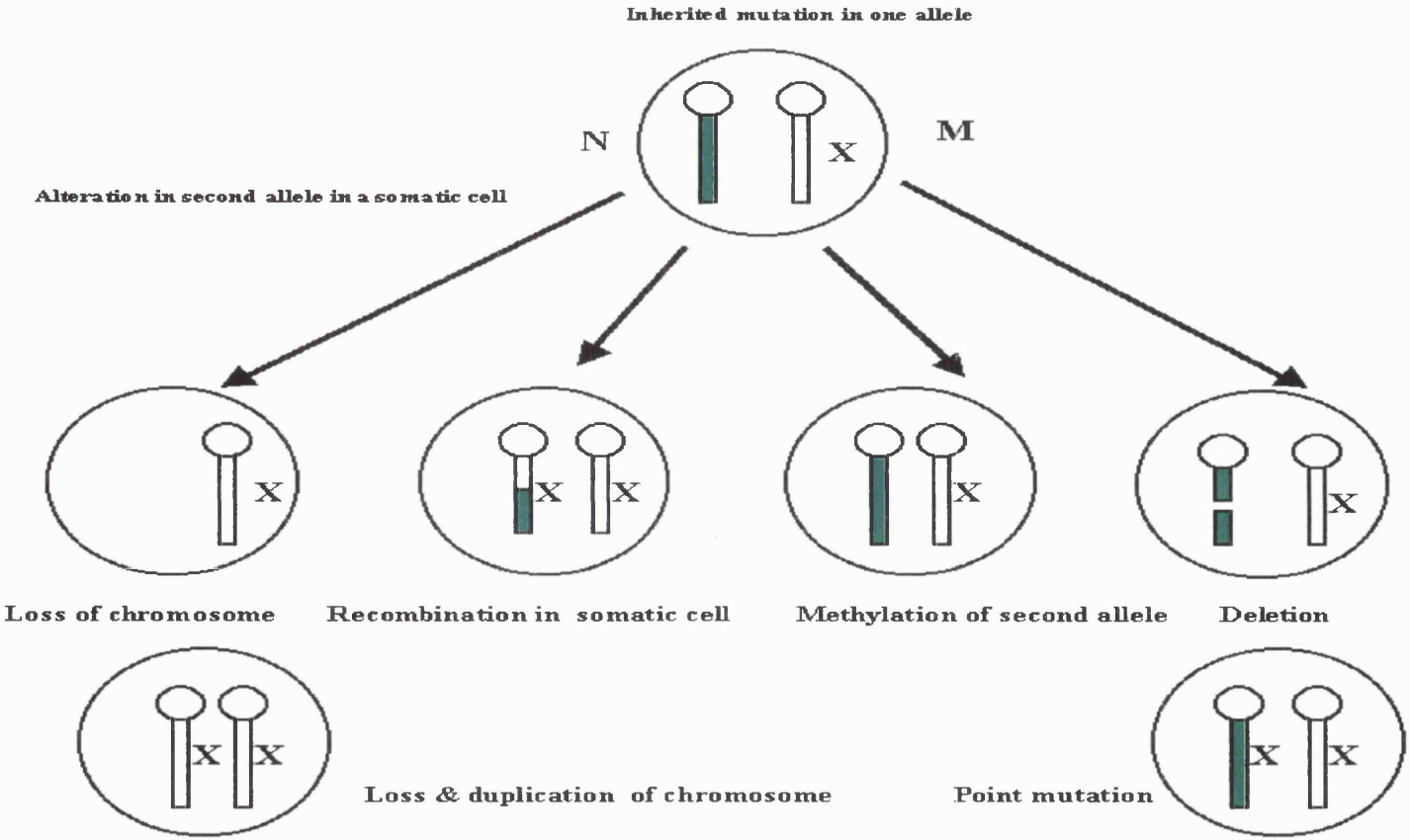


Figure 1.4. Two hits hypothesis of Knudson. Adapted from (Lasko D. et al., 1991)
A tumour suppressor mutation of a single allele of a gene is inherited as a dominant mutation and that a second mutation in the other allele occurs by a variety of mechanisms in a somatic cell to give the homozygous defective state. N: Normal; M: Mutated.

1.8. Mechanisms by which a TSG is inactivated

1. Somatic mutations
2. Haploinsufficiency
3. Epigenetic silencing

1.8.1. Somatic mutations

Somatic mutation is defined as a mutation that occurs in any cell of the body other than a germ-line cell, and thus it is not heritable. This is the most common mechanism that leads to alteration of gene function. Several genes are found to be inactivated by somatic mutations such as anaphase promoting complex (*APC)*, implicated in familial adenomatous polyposis (FAP). It is mutated in about 50% of sporadic colorectal tumours; most mutations are frameshift mutations (Fearon E. R. and Vogelstein B., 1990). *P53*, the guardian of the genome that prevents the cells from dividing before DNA damage is repaired, is another example of a tumour suppressor gene which can be inactivated by missense mutations, non sense mutation, deletions and insertions (Baker S. J. *et al.*, 1989; Lane D. P., 1992; Olschwang S. *et al.*, 1997). Other examples are mutations affecting the *Rb* gene (Dryja T. P. *et al.*, 1984) and *INK4a* (Kamb A. *et al.*, 1994).

1.8.2. Haploinsufficiency

Although many regions of chromosomal loss have been identified using LOH analysis (Osborne R. J. and Hamshere M. G., 2000), the number of TSGs carrying inactivation somatic mutations on the partner allele is small. Therefore, other mechanisms leading to "function-inactivation" are required. Among those recently proposed, haploinsufficiency (Fero M. L. *et al.*, 1998) and epigenetic silencing of genes are the most popular (Baylin S. B. and Herman J. G., 2000).

Haploinsufficiency is a situation in which the protein produced by a single copy of an otherwise normal gene is not sufficient to assure a normal phenotype and this may

contribute to tumourigenesis by pathways that do not require inactivation of the normal retained allele of a gene and this is supported by several examples in the literature (Cook W. D. and McCaw B. J., 2000). These include *NF1* (Jacks T. *et al.*, 1994), *PTEN* (Podsypanina K. *et al.*, 1999), $p27^{Kip1}$ (Pietenpol J. A. *et al.*, 1995) and *p53* (Nigro J. M. *et al.*, 1989), (Venkatachalam S. *et al.*, 1998), particular in animal models.

For example, in heterozygous *p53* knockout mice, some tumours retained a functional *p53* allele (Venkatachalam S. *et al.*, 1998) and have a high predisposition to tumours. Similarly, Fero et al have shown that both *p27* nullizygous and *p27* heterozygous mice were predisposed to tumours in multiple tissues when treated with γ irradiation or chemical carcinogens. Tumours in heterozygous mice were found to be associated with neither mutation nor silencing of *p27* (Fero M. L. *et al.*, 1998).

Haploinsufficiency was recently found to be a plausible explanation to justify the association of familial platelet disorder (FPD) and AML associated with intragenic deletion or heterozygous mutations of the *CBFA2/AML1* gene (Song W. J. *et al.*, 1999). Analysis of bone marrow or peripheral blood cells from affected FPD/AML individuals showed a decrement in megakaryocytic colony formation, demonstrating that *CBFA2* dosage affects megakaryopoiesis. The authors' findings support a model for FPD/AML in which haploinsufficiency of *CBFA2* causes an autosomal dominant congenital platelet defect and predisposes to the acquisition of additional mutations that cause leukaemia.

1.8.3. Epigenetic silencing

Epigenetic silencing, described several years ago in a case of t(8;16) translocation, has become a frequent finding in human malignancies. Silencing of promoter regions can occur via hypermethylation, histone deacetylase activity or via the recruitment of DNA methyltransferases to target promoters, as recently described for the PML-RARα fusion protein. Retinoic acid treatment was found to induce promoter demethylation, gene expression, and reversion of the transformed phenotype (Di Croce L. *et al.*, 2002).

Also, the *p73* gene on 1p36.2-3, (frequently deleted in human cancer) encodes for a protein that is both structurally and functionally homologous to the p53 protein. To date, however, mutations of *p73* have not been found. However, the 5' region upstream of exon 1 was aberrantly methylated in approximately 30% of primary ALL and Burkitt's

lymphomas patients (Corn P. G. *et al.*, 1999). In both leukaemia cell lines and primary ALL, methylation was associated with transcriptional loss of *p73* by reverse transcription-PCR.

1.9. Molecular pathogenetic events associated with cancer

As described in the first paragraphs of this chapter, there are mounting evidence that all the genetic changes associated with the chromosomal abnormalities described above are caused by errors in DNA damage repair machinery. As this thesis will demonstrate in the results paragraph, this may also be a common event in chromosome 6q deletions. Therefore, a paragraph of this Introduction will be dedicated to the description of these events.

1.9.1. Failure to repair DNA damage

DNA damage can be either spontaneous or arise after exposure to exogenous agents.

A. Spontaneous DNA damage

Spontaneous DNA damage may be induced as a result of replication errors, or inherent instability of the DNA molecule. This leads to the dissociation, even under normal physiological conditions, of some chemical bonds in the DNA molecule leading to deamination of cytosine, adenine, guanine and 5-methyl cytosine with the production of uracil, hypoxanthine, xanthine and thymine, respectively. In addition to these spontaneous changes, DNA damage occurs as the result of chemical attack by products of normal cellular metabolism that contain reactive products derived from oxidative respiration and lipid peroxidation, i.e. superoxide anions, hydroxyl radicals and hydrogen peroxide. Over 100 oxidative modifications have been identified in DNA. Cells have well developed anti-oxidant defences to minimize cellular damage. This antioxidant defence system comprises enzymatic pathways (superoxide dismutase, catalase, glutathione peroxides and peroxyredoxins) and low molecular mass scavengers such as glutathione (Hoeijmakers J. H. J., 2001). Reactive oxygen species (ROS) are generated as by-products of metabolic processes of all growing cells. It is proposed that ROS play a key role in cancer development and ROS are believed to contribute to cancer initiation, promotion and progression phases (Wiseman, H. and Halliwell, B. 1996).

B. Exogenous DNA damage

Exogenous damage can result from the exposure of DNA to chemical or physical carcinogens or from anti-cancerous chemotherapeutic agents.

1. Chemical carcinogens

The link between exposure to environmental carcinogens and tumour development has been known for a long time, for instance, in workers exposed to coal tar. Other examples are clear from the induction of bladder carcinoma and exposure to 2-naphthylamine or from ethylnitrosamine and development of liver carcinoma (Magee P. N., 1972). Realising that some human cancers are caused by direct exposure to chemicals resulted in the publication of a list of carcinogens (Doll R. and Peto R., 1981) and in a change of working and environmental conditions in several countries worldwide.

2. Physical carcinogens

Exposure to ionising or ultraviolet irradiation can induce mutations. DNA damage resulting from ionising-radiation may be direct damage by causing single or double DNA strand breaks or indirect damage by radiolysis of water and production of free radicals (Hall J. and Angele S., 1999). Although it has not enough energy to produce ions, ultraviolet irradiation can be absorbed by DNA bases and induce chemical reactions. These lead to base pairing disruption and, consequently, if not repaired can give rise to mutations. The inability to repair UV-induced DNA damage has been found associated with skin cancer. The mutations affect genes involved in specific signal transduction pathways regulating cell cycling, differentiation and apoptosis (Cleaver J. E. and Crowley E., 2002). Identifying the molecular pathways of ultraviolet-induced carcinogenesis has resulted in a greater awareness of the danger of exposure to sunlight (Sarasin A., 1999). Sunlight and in particular its UV component, is the major environmental trigger that underlies the clinical signs of skin cancer. The UV spectrum can be divided depending on the wavelength into UV-A, UV-B and UV-C. As atmospheric ozone prevents UV-C from reaching the surface of the earth, the major skin cancer risk is thought to come from exposure to UV-B. Two major types of DNA lesions are formed upon UV light absorption. The photoproducts are either in the form of cyclobutane pyrimidine dimers (CPDs) or 6 pyrimidine-4- pyrimidone photoproducts [6-4(PPs)]. The major consequences of these events are the CC $\rightarrow$ TT double transition mutations, which are considered UV premutagenic lesions. These mutations affect particularly the *TP53* gene leading to its inactivation and tumour formation (van Steeg, H. and Kraemer, K. H. 1999) and are referred to as "signature type mutations".

Xeroderma pigmentosa (XP) is a rare autosomal recessive disease characterised by inability of the skin cells to repair DNA damage caused by exposure to UV light. The disorder arises from a mutation in one of seven genes (XPA-XPG) that have been mapped to specific sites on human chromosomes and their role in nucleotide excision repair has been identified (Hoeijmakers J. H. J., 2001; Sarasin A., 1999).

3. Chemotherapy and cancer

Many chemotherapeutic agents used in cancer treatment are carcinogens including alkylating agents such as cyclophosphamide (Fraser M. C. and Tucker M. A., 1989). Most of the therapy-related cancers are leukaemias with a peak of incidence of 8 years post therapy. Solid tumours are also common but they have a longer incubation period (ten years) after completion of therapy (Tucker M. A. *et al.*, 1988). Radiotherapy is also associated with increased cancer risk but it is rarely associated with secondary cancer due to the localised nature of the applied dose.
Mutations are permanent change in the nucleotide sequence of a DNA molecule. Mutations can affect the germinal cells (germline mutations) or are acquired during the life span of a cell (somatic mutations). The latter, by far the commonest, and include point mutations, frame shifts, deletions, and stop codon mutations.
In a broad sense, mutations can also include chromosomal abnormalities such as translocation duplication, amplification. They may result, (depending on the event) in gene-fusions leading to a chimeric protein with altered function, loss of gene function through extensive chromosomal deletions. Point mutations are caused by single base pair changes, which can be "silent" (resulting in no aminoacid, AA, change) or "non-silent" (results in AA change). A point mutation can produce missense mutation (AA replacement) or nonsense mutation, when a termination codon appears, leading to the premature termination of protein synthesis. Mutations of the *RAS* gene are the commonest example of some of these changes occurring in both haematological and solid tumour malignancies (Minamoto et al., 2000). Addition or deletion of one or two base pairs within a coding sequence of a gene can affect the reading frame of the gene and result in an altered or absent gene product. These mutations are called frame shift mutations as they result in shifting of the reading frame of an mRNA. Large numbers of base pairs can be either deleted or inserted into the middle of the gene resulting in loss of function.
Neither the chemical nor physical damage to DNA alone is, however, mutagenic, as this damage should be converted to a permanent change and result in DNA "mutation". To achieve that, DNA replication and subsequent cell divisions are necessary. Thus, proliferation is an important factor in the process of mutations and for the expansion of the clones of cells carrying these mutations. We refer to the "initiated cell" as the cell that carries a mutation in an important gene due to unrepaired DNA damage. However, other events normally take place facilitating both growth advantage and loss of regulatory control mechanisms on proliferation and apoptosis (Ames B. N. *et al.*, 1993).

1.9.2. DNA repair mechanisms

Different processes are involved in DNA repair depending on the nature of the damage induced. The outcome of unrepaired DNA damage is diverse and it involves both acute and long-term effects. Acute effects of the damage can lead to transient cell cycle arrest at specific checkpoints in the G1, S, G2 and M phases to allow repair of the damage or can lead to inhibition of important processes such as transcription, replication and chromosomal segregation that may trigger apoptosis. Long-term consequences of DNA damage can induce permanent changes in DNA sequence such as point mutations or chromosomal aberrations with the possibility of cancer or inborn diseases, (Figure 1.5) (Hoeijmakers J. H. J., 2001). The main DNA maintenance mechanisms operating in mammals are base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR), end joining (EJ) (Lindahl T. and Wood R. D., 1999) and mismatch repair (MMR) (Kolodner R. D. and Marsischky G. T., 1999) in addition to the role of telomeric DNA sequences in the maintenance of the genomic stability (McEachern M. J. *et al.*, 2000).

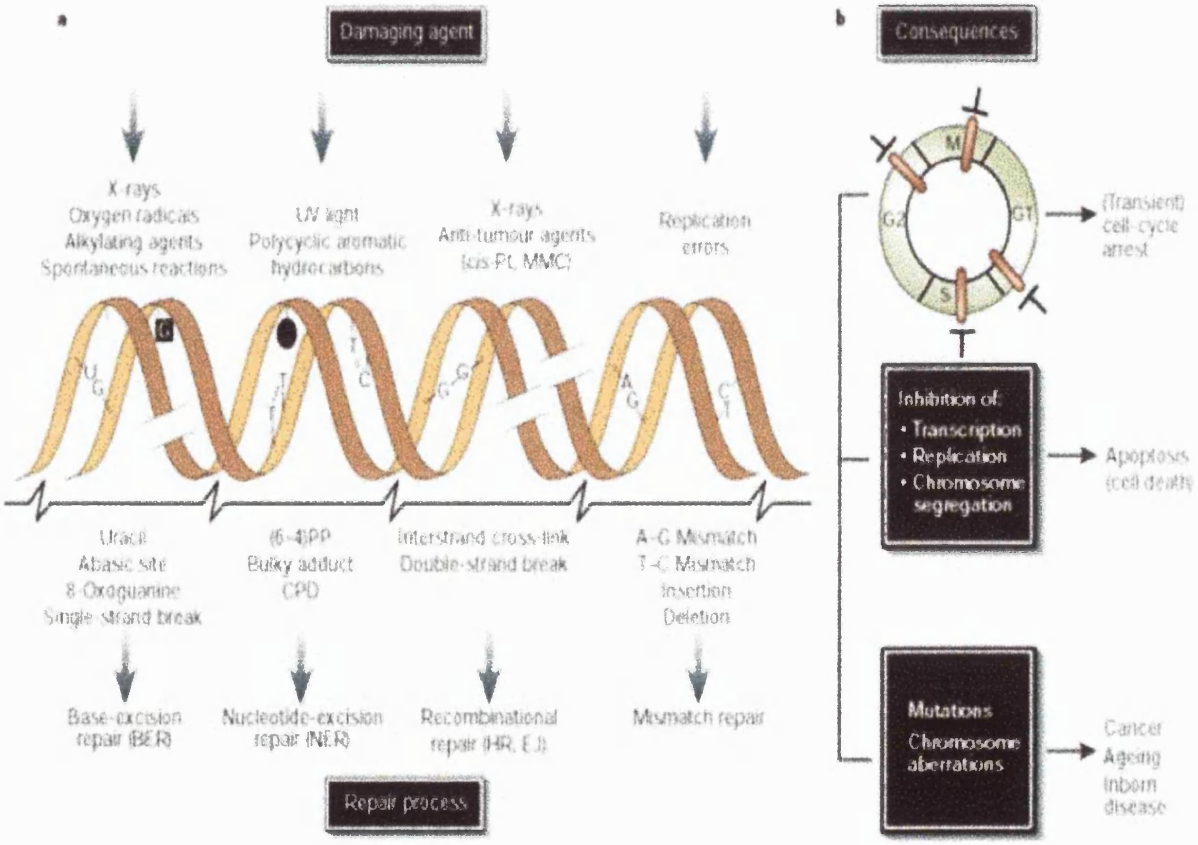


Figure 1.5. Adapted from (Hoeijmakers J. H. J., 2001). *DNA damage, repair mechanisms and consequences.* ***a****, Common DNA damaging agents (top); examples of DNA lesions induced by these agents (middle); and most relevant DNA repair mechanism responsible for the removal of the lesions (bottom).* ***b****, Acute effects of DNA damage on cell-cycle progression, leading to transient arrest in the G1, S, G2 and M phases (top), and on DNA metabolism (middle). Long-term consequences of DNA injury (bottom) include permanent changes in the DNA sequence (point mutations affecting single genes or chromosome aberrations which may involve multiple genes) and their biological effects. Abbreviations: cis-Pt and MMC, cisplatin and mitomycin C, respectively (both DNA-crosslinking agents); (6–4) PP and CPD, 6–4 photoproduct and cyclobutane pyrimidine dimer, respectively (both induced by UV light); BER and NER, base- and nucleotide-excision repair, respectively; HR, homologous recombination; EJ, end joining.*

1. Base excision repair (BER)

BER is mostly but not exclusively concerned with damage of endogenous origin including spontaneous hydrolytic depurination of DNA, deamination of cytosine and 5 methylcytosine residues and hydroxyl free radicals generated as a by-products of normal oxygen metabolism. The main strategy of correcting such DNA damage is BER, in which an altered DNA base is excised by a DNA glycolase and the resulting abasic site is corrected by an endonuclease, DNA polymerase and DNA ligase (Lindahl T. *et al.*, 1997). The high resolution structure of uracil-DNA glycolase (UDG), which is required for the removal of deaminated cytosine residues from DNA has been solved in 1995 (Mol C. D. *et al.*, 1995). Knockout mice deficient in BER enzymes such as AP endonuclease, DNA polymerase β and DNA ligase *XRCC1* (X ray cross complementing 1) resulted in an early embryonic lethality, illustrating the importance of the repair of endogenous lesions and providing an explanation of the apparent absence of human disorders caused by inherited BER deficiencies (Lindahl T. *et al.*, 1997).

The BER mechanism involves the replacement of a single damaged nucleotide in the DNA with a normal one. This reaction requires the presence of UDG, AP endonuclease, DNA polymeraseβ and a DNA ligase III/XRCC1 heterodimer. In addition to this major pathway of BER (called the short patch BER), a second minor pathway (referred to as long patch BER), which differs in the latter stages of the repair processes has been identified in mammals (Frosina G. *et al.*, 1996). It involves patches of several nucleotides instead of just a single nucleotide, DNA Polymerase β, δ or ε and proliferating cell nuclear antigen (PCNA) for repair synthesis as well as the FEN1 endonuclease to remove the displaced DNA strand and DNA ligase 1 for sealing (Hoeijmakers J. H. J., 2001; Lindahl T. *et al.*, 1997).

2. Nucleotide- excision repair (NER)

Most NER lesions arise from exogenous sources. The main function of this pathway in humans is the removal of UV-induced DNA photoproduct caused by sunlight exposure (Lindahl T. *et al.*, 1997). NER can act on a wide variety of adducts in DNA and is most

effective on bulky lesions. It can deal with any lesion that interferes with base pairing and obstructs transcription and replication. Two NER pathways exist; global genome nucleotide excision repair (GG-NER) which functions to survey the entire genome for detecting disrupted base pair instead of lesions *per se* (Sugasawa K. *et al.*, 2001) and transcription coupled repair (TCR) that focuses on damage that blocks elongating RNA polymerases (Tornaletti S. and Hanawalt P. C., 1999). The subsequent steps of GG-NER and TCR may be identical. The XPB and XPD helicases of the transcription factor TFIIH open about 30 base pairs of DNA around the damage. The replication protein A (RPA) stabilises the opening by binding to the undamaged strand. Then the endonucleases of the NER system cleave the 3′ and 5′ borders of the opened stretch only in the damaged strand the DNA replication machinery then complete the repair by filling the gap (Jan H J Hoeijmakers, 2001). Xeroderma pigmentosa (XP), Cockayne syndrome (CS) and Trichothiodystrophy (TTD) are syndromes associated with inborn defective NER. XP arises from mutations in one of seven genes (XPA-XPG). CS is caused by a mutation in the CSA or CSB genes and is dissimilar from XP as no predisposition to cancer is observed. TTD is sharing many symptoms with CS but with additional symptoms such as brittle hair, nail and scaly skin (Lehmann A. R., 2001).

3. Double strand break (DSB) repair, homologous recombination and end joining

Double strand break (DSB) is probably the most dangerous type of DNA damage. Since both strands are damaged the cell has no normal template to provide the information needed to reconstitute the damaged strand (Bertram J. S., 2000). The damage can result from exogenous agents such as IR and certain chemotherapeutic agents, from endogenously generated reactive oxygen species or during single strand replication. It can also occur at the termini of the chromosomes due to defective metabolism of telomeres or may be generated to initiate recombination between homologous chromosomes during meiosis and finally can occur as an intermediate during V-D-J (variable-diversity-joining) immunoglobulin rearrangement and immunoglobulin class switch recombination (Khanna K. K. and Jackson S. P., 2001).

Repair of DSB is more difficult than any other type of DNA damage. Errors during

joining of broken ends may occur leading to loss or amplification of chromosomal segments and sometimes to chromosomal translocations. Loss of a chromosomal segment encoding a tumour suppressor gene, amplification of segments encoding an oncogene or translocations leading to a gene fusion may lead to tumourigenesis (Khanna K. K. and Jackson S. P., 2001). There are two distinct mechanisms for DNA DSB repair namely, homologous recombination (HR) and non-homologous end joining (NHEJ) (Haber J. E., 2000; Karran P., 2000). NHEJ has been considered the major pathway in DSB repair in mammalian cells, but it is now established that HR also has a crucial role (Moynahan M. E. *et al.*, 1999; Sonoda E. *et al.*, 1999). NHEJ of the two DNA ends does not require an undamaged partner and does not rely on extensive homology between the DNA ends to be joined. In this pathway, sometimes after a limited degradation at the two DNA termini, the two ends are ligated together. Hence, NHEJ is often prone to error and small sequence deletions are always introduced (Khanna K. K. and Jackson S. P., 2001). NHEJ involves a DNA end binding heterodimer of two proteins (Ku70) and (Ku 80), which activate the catalytic subunit (DNA-PKcs) of DNA-dependant protein kinase (DNA-PK). Repair by NHEJ is then completed by the DNA ligase IV/Xrcc4 (X-ray cross complementing 4) complex (Karran P., 2000). In HR repair pathway, the DNA ends are first resected in the 5′ to 3′ direction by nucleases. The resulting 3′ single-stranded tails then invade the double helix of a homologous, undamaged partner molecule and are extended by polymerase, which copies information from the partner. In mitotic cells the ends are ligated by DNA ligase1 and the interwound DNA strands (Holiday junctions) are resolved (Khanna K. K. and Jackson S. P., 2001).

Ataxia teleangiectasia (AT) (Rotman G. and Shiloh Y., 1998), ataxia teleangectasia-like disorder and Nijmegen breakage syndrome (NBS) (Petrini J. H., 2000) are three conditions associated with cancer predisposition, immunodeficiency, sensitivity to x-rays and chromosomal instability all are caused by defective DSB repair modes (Digweed M. *et al.*, 1999; Rotman G. and Shiloh Y., 1998).

4. Mismatch repair (MMR)

MMR system removes nucleotides mispaired by DNA polymerases and insertion/deletion loops (IDLs) which result from slippage that might occur during replication of some repetitive sequences. Defective MMR genes are associated with

increased mutation rates and predisposition to cancer. Mammalian MMR involves multi-member families of the E.coli prototype factors known as MutS and MutL (Kolodner R. D. and Marsischky G. T., 1999). Heterodimers of hMSH2/6 called hMutSα recognises mismatches whereas dimers of hMSH2/3 called hMutSβ recognise insertion/ deletion loops. Heterodimers of hMutL-like proteins hMLH1/hPMS2 (hMutLα) and hMLH1/hPMS1 (hMutLβ) are also important as they interact with MSH complexes and with the other factors in the replication machinery. Some other proteins are also implicated in the excision and resynthesis processes. These proteins include polymerases (polδ/ε), RPA, PCNA, RFC, Exonuclease1 and endonuclease FEN1 (Jiricny J., 1998; Kolodner R. D. and Marsischky G. T., 1999). In human there are at least six known MMR genes; *hMSH2, hMSH6, hMSH3, hMLH1, PMS2 and hMLH3.*

Hereditary non-polyposis colorectal carcinoma (HNPCC) is a hereditary disorder resulting from mutations of *hMLH1* and *hMSH2* genes (Hoeijmakers J. H. J., 2001; Wheeler J. M. *et al.*, 1999).

Three major steps are involved in the correction of mismatch repair (Jiricny J. and Nystrom-Lahti M., 2000).

- **Mismatch recognition and assembly of repairosome.**
- **Degradation of the strand containing the mismatch.**
- **Repair synthesis.**

For mismatch recognition, hMSH2 heterodimerising with hMSH6 or hMSH3 depends on the nature of the mismatch to be corrected. For repairing base-base mismatches hMSH6 is required but for insertion deletion loops the hMSH3and hMSH6 have redundant functions (Jiricny J., 1998). The heterodimers hMSH2 and hMSH6 is known as hMutSα, and that formed of hMSH2 and hMSH3 is called hMutsβ and it preferentially recognises IDLs.

Mismatched DNA induces ADP→ATP resulting in conformational changes (Fishel R. and Wilson T., 1997) that converts the hMSH2/hMSH6 complex into a sliding clamp that leaves the mismatched site and diffuses along the DNA backbone (Blackwell L. J. *et al.*, 1998; Gradia S. *et al.*, 1999; Iaccarino I. *et al.*, 2000). This conformational change is thought to be followed by the assembly of the repairosome. Some other

heterodimers such as MLH1 and PMS2 or MLH1and MLH3 or MLH1and PMS1are thought to be required together with other proteins necessary for MMR.
These proteins include the PCNA, exonucleases, DNA polymerases and helicases (Jiricny J., 1998; Kolodner R. D. and Marsischky G. T., 1999). Figure 1.6 shows MMR of base/base mismatches and insertion loop.

Microsatellites are repetitive DNA sequences consisting of oligonucleotide units that are repeated in tandem. They are distributed through out the whole genome. They are more prone to slippage during DNA replication and this results in errors that normally corrected by MMR machinery (Dib C. *et al.*, 1996; Litt M. and Luty J. A., 1989; Weber J. L. and May P. E., 1989). Tumours with defective MMR genes show a microsatellite length change, which is known as microsatellite instability (MSI).

1.10. Microsatellite Instability (MSI)

MSI is defined as a change of any length due to either insertion or deletion of repeating units in a microsatellite within a tumour when compared to normal tissue. It is caused by a failure of the DNA MMR system to repair errors that occur during DNA replication with accumulation of mutations and alteration in the length of microsatellite sequences (Boland C. R. *et al.*, 1998).

It has been found that germline mutations of the MMR genes gives rise to hereditary non-polyposis colon cancer (HNPCC). Individuals from HNPCC families are predisposed to a high risk of developing colon cancer, endometrial carcinoma and other cancers (Aarnio M. *et al.*, 1999; Vasen H. F. *et al.*, 1996). Most of the cases of HNPCC are associated with germline mutations in *MSH2* or *MLH1* while *MSH6, PMS2* and *PMS1* are less frequently involved (Peltomaki P. and de la Chapelle A., 1997; Peltomaki P. and Vasen H. F., 1997)

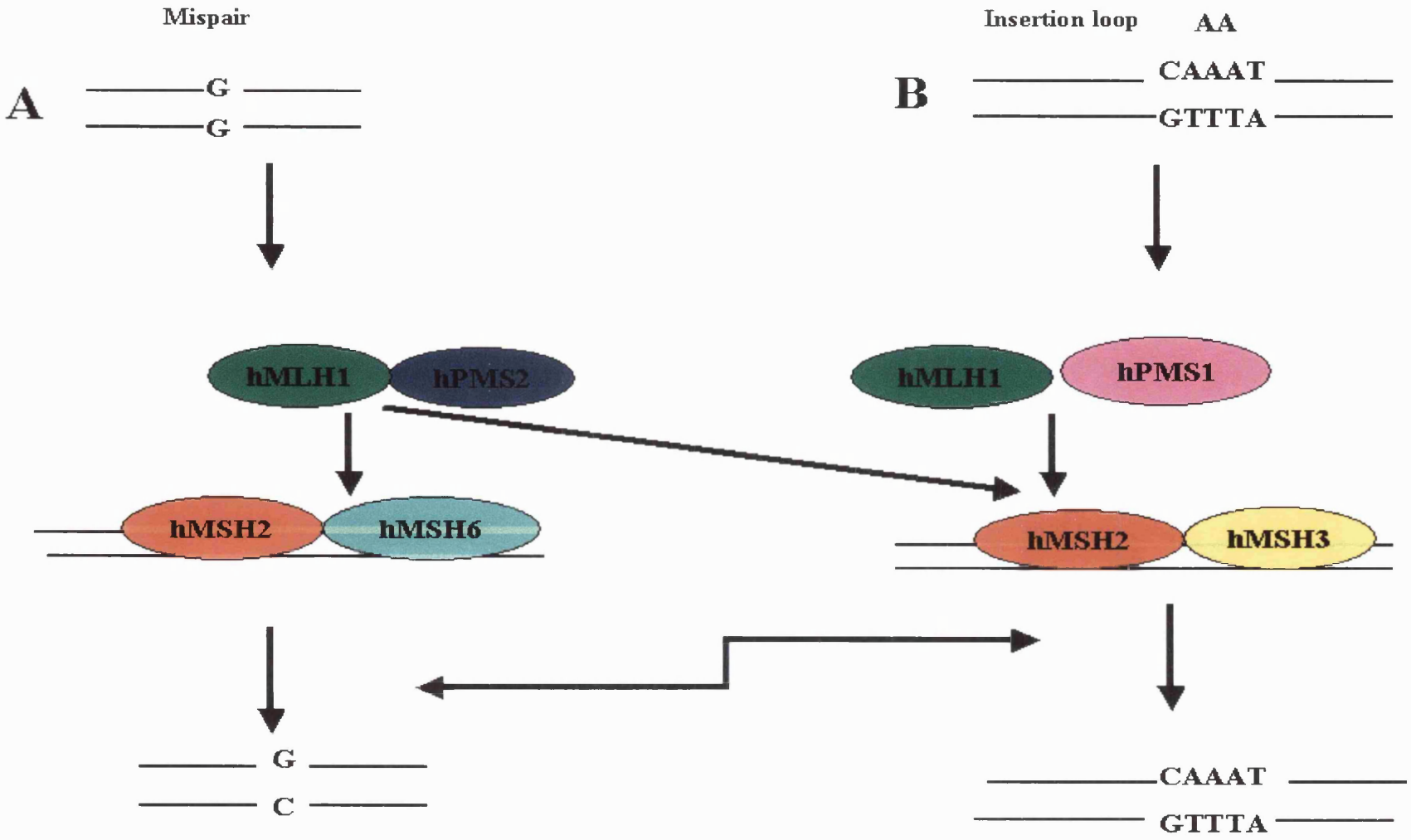


Figure 1.6. Adapted from (Wheeler J. M. et al., 1999). *Mismatch repair (MMR). A mispaired base is corrected by hMSH2/hMSH6 complex, insertion/ deletion loop (IDL) is corrected by hMSH2/hMSH3 complex. MutL related proteins (hMLH1/hPMS2 and hMLH1/ hPMS1 complexes) then interact with MutS proteins that are already bound to the mispaired bases. The hMSH2/hMSH6 complex may also supports the repair of insertion/ deletion loops.*

1.10.1. Microsatellites

Although MSI is seen in about 90% of all HNPCC, it is only seen in about 15% of sporadic cancer (Ionov Y. *et al.*, 1993; Thibodeau S. N. *et al.*, 1993). Despite the similar phenotype of MSI in HNPCC and sporadic colon cancers the pathways involved in the biallelic inactivation of MMR genes and subsequently, the development of MSI are different (Peltomaki P., 2001). MSI is due to inactivation of both alleles of a MMR gene, in keeping with the Knudson's hypothesis (Knudson A. G., Jr., 1971). In HNPCC, a single mutation is inherited in the germline as a first hit. Inactivation of the second allele either by somatic mutation or LOH then follows as a second hit. This differs from the majority of sporadic tumours in which an "epigenetic" mechanism is involved resulting in promoter hypermethylation of *MLH1* gene (Kuismanen S. A. *et al.*, 2000). It has been proposed that the Knudson's hypothesis should be expanded to include these recently described epigenetic mechanisms of gene inactivation (Jones P. A. and Laird P. W., 1999) which were described above.

MSI has been also described in childhood ALL (Takeuchi S. *et al.*, 1997b), AML (Tasaka T. *et al.*, 1997), adult T-cell leukaemia/lymphoma (Hayami Y. *et al.*, 1999), mucosa associated lymphoid tissue lymphoma (Peng H. *et al.*, 1996), in primary cutaneous T-cell lymphoma (CTCL) (Scarisbrick J. J. *et al.*, 2000), and rarely in B-cell non-Hodgkin's lymphomas (Gamberi B. *et al.*, 1997).

1.10.2. Microsatellite instability (MSI) testing

1. Molecular testing

MSI can be detected by examining microsatellite repeat fragments as the number of these repeats can be changed if MMR is defective. A panel of five microsatellite markers (BAT25, BAT26, D5S346, D2S123and D17S250) were selected by the national cancer institute (NCI) for detection of MSI, and they were firstly recommended for assessing patients with colorectal carcinoma. These markers can be analysed using PCR. In the NCI guidelines, the tumours were classified according to the total number of these markers showing MSI: MSI high frequency (MSI-H), MSI low frequency (MSI-L) or microsatellite stable (MSS). MSI-H tumours are tumours with MSI in more than 30%-40% of the markers analysed. MSI-L classifies tumours showing MSI in

less than 30%-40% of the tested markers. If none of the markers showed MSI then it would be considered as MSS (Boland C. R. *et al.*, 1998). These guidelines have extended to other types of cancer and have been used in the study reported here.

2. Immunohistochemistry

Immunostaining of MMR genes can be detected immunohistochemically by using specific antibodies for hMSH2, hMLH1, hMSH6, hPMS1 and hPMS2 (Frazier M. L. *et al.*, 2000). Evaluation of the usefulness of immunohistochemistry as a method for identifying colorectal carcinoma with MMR defects due to inactivation of *MLH1* or *MSH2* gene has been found to show a sensitivity ranging from 72-90% (Dieumegard B. *et al.*, 2000; Marcus V. A. *et al.*, 1999; Stone J. G. *et al.*, 2001; Thibodeau S. N. *et al.*, 1996). Because of the requirement for suitable tissue /material for investigation this may have limited application and was not available for our investigation.

3. Single strand conformation polymorphism (SSCP)

SSCP is one of the most commonly used techniques for mutational scanning. Although it has the advantage of being rapid and less expensive than nucleotide sequence analysis, both techniques are complementary. Any mutation detected using SSCP screening is confirmed by using sequence analysis.
This technique has been used successfully to identify mutations in *hMLH1*and *hMSH2* genes (Wijnen J. *et al.*, 1996; Wijnen J. *et al.*, 1995). SSCP has commonly now been replaced by using Gene Scanning when equipments are available in laboratories for the analysis of the PCR products obtained using specially-labelled primers.

4. Other techniques

Some other techniques can be used for MSI assays such as Protein truncation test (PTT) (Powell S. M. *et al.*, 1993). For instance, end-to-end exon specific nucleotide sequence analysis is a useful approach for identifying gene mutations, where primers can be designed flanking each of the exons of the gene to generate PCR products encompassing the entire exon. These PCR products can then be sequenced and abnormalities identified.

1.11. Telomere metabolism

In the process of DNA maintenance mechanisms operating in mammals, increasing relevance has been given to the protective role of telomeres, telomerases and general mechanisms involved in maintaining the chromosomal integrity. As a matter of completeness, a brief mention will be made in the following paragraph.

Telomeres are the physical ends of the chromosomes. They represent the caps of the chromosome ends that protect the underlying genomic sequences from being lost during replication process. These caps are formed of 5-15 kb of repetitive sequence TTAGGG in mammals (Hoeijmakers J. H. J., 2001). The progressive erosion of the chromosome ends controls the life span of the normal cells. In germ cells and haemopoietic stem cells which need to keep their replicative abilities and maintenance of the telomere length is essential. It has been found that these cells express an enzyme, telomerase, which plays an important role in maintaining telomere length (McEachern M. J. *et al.*, 2000). It is of interest to note however, that this enzyme is not expressed in somatic cells (Shay J. W. and Bacchetti S., 1997). This results in "replicative senescence" in somatic cells, after repeated replication cycles, with progressive telomere shortening (Counter C. M. *et al.*, 1992). This event triggers expression of the *p53* gene and results in cell cycle arrest or alternatively apoptosis when a critical degree of telomere shortening has been reached (Vaziri H. and Benchimol S., 1999). In the absence of telomere maintenance, the malignant cell would undergo senescence or apoptosis. Malignant cells are characterised by a reactivation of the telomerase enzyme in order to overcome senescence and apoptosis. It is not clear whether telomerase-positive tumour cells reactivate the enzyme or whether tumours arise from a self-renewal stem cell, which originally possesses the activity. In addition, the extent of the telomerase activity correlates with the aggressiveness of the disease. This is shown when small lung carcinoma is compared with the less aggressive carcinoid tumours (Sarvesvaran J. *et al.*, 1999), invasive breast carcinoma compared with ductal carcinoma in situ (Umbricht C. B. *et al.*, 1999) or in melanoma (Ramirez R. D. *et al.*, 1999) and in colon carcinoma (Tatsumoto N. *et al.*, 2000).

Distinct molecular mechanisms control telomere function; interaction between the molecular components of these mechanisms can define telomere behaviour. Telomeres can switch between capping and uncapping states depending on the cell type, species and a combination of some of the previously mentioned mechanisms (Blackburn E. H.,

2001).

1.12. Remarks

As the data are presented in the next few chapters, the reader will notice that additional information relevant to each part of the investigation is given in the introduction of each chapter. This was considered necessary for the reason of not prolonging this first chapter unnecessarily but to provide a comprehensive coverage of each topic somewhere in this thesis. Great effort was made to reduce the incidence of repetitions and duplications.

Aims of this study

The aims of this study were:

1. To extend the mapping of the RMD-2B region between the markers D6S447 and D6S246. This area had been identified as an area of common deletion in ALL patients with cytogenetically detectable 6q deletion in a previous investigation in our laboratory.

2. To analyse the incidence and extent of 6q deletion in a larger cohort of ALL patients using LOH.

3. To analyse the association of 6q deletion and clinical outcome in patients with ALL.

4. To identify and characterise candidate tumour suppressor genes on 6q21 that could be involved in the pathogenesis of ALL in patients with 6q deletions.

Plan of investigation

This project was divided into two parts.

1. Investigate the extension and characterisation of the map of the uncloned region between Yeast artificial chromosomes (YACs) 860f10/860e12 and 36CB11.

   Approach

   - Vectorette cloning of ends of these YACs.

   - PCR screening of ICI/Zeneca libraries using primers from available STS in the region of interest.

   - Using probes from available sequences in the region proximal to the gap to screen CEPH libraries to obtain more clones and to test these clones for overlapping.

2. The use of LOH to investigate deletions on 6q21. A series of microsatellite markers on the 6q21 were used on paired DNA samples from patients of ALL to identify 6q deletions as most of these deletions are difficult to define by FISH and cytogenetics and frequently they represent interstitial deletions.

In summary we planned to define a region minimal deletion on 6q21 with the aim to identify a tumour suppressor gene/s involved in the development of ALL in patients with 6q deletions.

Chapter 2

Material and Methods

Details for the preparation of solutions are provided in Appendix 1. In some instances, description of procedures has been included in some of the result chapters if they were considered more pertinent to some paragraph of the investigation.

2.1. DNA Preparation

2.1.1.Preparation of DNA from transformed yeast cells (YAC DNA maxiprep)

Individual YAC clones were streaked onto an AHC plate and grown for 48-72 hours in a 37°C incubator. When colonies were visible (within 2-3 days of growth), 5 ml of AHC media was inoculated with a single yeast colony and grown at 37°C in a shaking incubator (2-3 days). The cultures were then centrifuged at 3,000 rpm for 15 minutes to pellet the cells. The pellet was washed with 500µl of distilled water and then spun again using the same conditions. The pellet was resuspended in 200µl of GDIS, 0.35g of glass beads- acid washed (Sigma, UK) and 200µl of phenol (BDH, UK) were added. The tubes were vortexed vigorously for 5 minutes. 200 µl of distilled water were added to the suspension, mixed well and then spun for 4 minutes in a microfuge. The aqueous layer was then removed and treated with 5µl of 10mg/ml DNAse free RNAse (Sigma, UK) and 1µl of Proteinase K (25mg/ml) (Boehringer Ingelheim) for 20 minutes at 37°C. The DNA was extracted once with phenol and then once with phenol: chloroform: isoamyl alcohol (PCIA)(25:24:1/v:v:v). The YAC DNA was recovered by centrifugation for 10 min at 13,000 rpm in a microfuge, transferred to a new eppendorf and then precipitated with 2 volumes of cold ethanol and 10% Na acetate, 3M pH 4.8. After incubation at -70°C for several hours or overnight, the DNA was recovered by centrifugation for 10 min. at 13,000 rpm and the pellet was then washed with 70% cold ethanol and air-dried. The pellet was then resuspended in 50-200 µl of TE depending on DNA recovery. The DNA yield was determined by comparing the yeast DNA

concentration with known different concentrations of uncut λ DNA (50ng/μl) after running them side by side on 0.8% minigel and visualised by ethidium bromide staining on an UV transilluminator.

2.1.2. Small-scale plasmid preparation (minipreps); method 1

A single bacterial colony containing the desired plasmid construct was used to inoculate each of 1.5ml aliquots of 2xTY plus ampicillin (100μg/ml). These were incubated at 37°C overnight in a shaking incubator. The 1.5ml culture was transferred to Eppendorf tubes and spun at 13,000 rpm in a microfuge for 5 minute to pellet the cells. Stocks were kept by streaking out from each tube onto fresh 2xTY/ampicillin plates, which were then grown at 37°C overnight and stored at 4°C. The cell pellets were resuspended in 100μl ice cold GTE and then left at room temperature for 10 minutes. 200μl of freshly prepared 0.2 M NaOH /1% SDS was then added to lyse the cells. The tubes were rapidly inverted and incubated at room temperature for 5 minutes. The bacterial DNA was precipitated by adding 250μl of 3M Na Acetate pH 4.8. The tubes were shaken and incubated on ice for 15 minutes. The cell debris and E. coli DNA was then pelleted by spinning in a microfuge for 5 minutes, at maximum speed (13,000 rpm). The supernatant was then poured into a fresh tube and plasmid DNA was precipitated with 2 volumes of 100% cold ethanol. The tubes were incubated on ice for 15-30 minutes or kept at -20°C for 10 minutes to maximise the amount of plasmid DNA precipitated.

The DNA was then pelleted by centrifugation, washed with 70% ethanol, dried in a vacuum dessicator for 5 minutes and resuspended in 30-50μl of TE. 5μl were analysed by restriction endonuclease digestion with the addition of 0.5μl DNAse free RNAse (10mg/ml)(Sigma, UK). The plasmid inserts were then characterised by agarose gel electrophoresis and visualised by ethidium bromide staining on a UV light transilluminator.

2.1.3.Alternative method (Qiagen column spin miniprep kit); method 2

The miniprep cultures were set up as for method 1 in 1.5ml cultures with appropriate antibiotics. The following day the pellets were collected by centrifugation and cell stocks were prepared as described below (paragraph 2.2.2). DNA from a culture cell pellet was prepared using a Qiagen spin column miniprep kit. The manufacturer's instructions were followed and the DNA was eluted in 30-50µl of TE. The plasmid inserts were characterised as described in below.

2.1.4.Large scale plasmid preparations (Maxi preps)

A single bacterial colony containing the plasmid of interest was inoculated into 100ml of 2xTY plus100µg/ml ampicillin and was grown overnight in a shaking incubator at 37°C. The following morning, 600µl of the culture was taken to prepare a 15% glycerol stock that was stored at -70°C. The remaining culture was spun at 3,000 rpm in a bench centrifuge for 15 minutes. The supernatant was discarded and 10 ml of cold GTE was added to the pelleted cells and incubated on ice for 10 minutes. 20ml of freshly prepared 0.2M Na OH/ 1% SDS was added to each tube to lyse the cells. The tubes were then quickly shaken and incubated at room temperature for a further 5 minutes. To precipitate the Escherichia coli DNA, 10ml of 3M Na Acetate pH 4.8 was added to each tube and the tubes were shaken vigorously, and then placed on ice for 15-60 minutes. The tubes were then spun as before and the supernatant was strained through gauze into a new Falcon tube.

The DNA was precipitated by the addition of an equal volume of isopropanol. After incubating on ice for 10 minutes the DNA was pelleted by centrifugation, as before. The pellet was dissolved in 2ml of TE and 2ml of 5M Li CL was added to precipitate the Escherichia Coli DNA. The tubes were incubated on ice for 10 minutes and then were spun. The supernatant was then collected and transferred to a fresh Falcon tube. Two volumes of ice cold 100% ethanol were added to precipitate DNA. The pellet was collected by centrifugation after incubation on ice for 5 minutes. The pellet was then resuspended in 2ml of TE and incubated with 4µl of 10mg/ml DNAse free RNAse (Sigma, UK) to destroy the Escherichia coli RNA, for 15 minutes at 37°C.

One ml of 2.5 M NaCl, 20% PEG was added to collect the plasmid DNA from solution and the reaction was incubated on ice for 5 minutes. The DNA was then pelleted by centrifugation at maximum speed in a microfuge for 5 minutes, having been

transferred to 1.5ml Eppendorf tube. The supernatant was then discarded, all excess PEG was removed. The pellet was resuspended in 500µl of TE. Two phenol: chloroform: isoamyl (PCIA) extractions and one chloroform extraction were performed. The plasmid DNA was precipitated with 2 volumes of 100% ethanol and 10% 3M Na Acetate (as above) at -20°C for 20 minutes. The precipitated DNA was collected by centrifugation, washed with 70% ethanol, and then dried in vacuum dessicator for 5-10 minutes. The pellet was resuspended in 100-200µl of TE depending on its size. The DNA yield was then determined by spectrophotometry at OD_{260} nm.

2.1.5. Large scale PAC preparations

A single bacterial colony was inoculated into 50ml 2xTY containing 25µg/ml kanamycin. The culture was grown overnight in a shaking incubator at 37°C.The following morning; 600µl were taken to prepare 15% glycerol stock and stored at -70°C. The cells were then spun down at 3,000 rpm for 15 minutes. The DNA was extracted following the same steps used for plasmid DNA maxipreps (see paragraph 2.1.4).

2.2. Restriction endonuclease digestion of DNA

For mapping and analysis of plasmid, PAC and YAC DNA with restriction endonuclease 500ng-3µg of DNA were digested in a total volume of 30-50µl with a 5-10 fold of excess of enzyme. All digests were set up in 1x restriction enzyme buffer (Carlo's buffer) with 1mM DTT and 3mM Spermidine. Digests were incubated at 37°C for a minimum of 3 hours. When specific fragments were required for probe preparation or subcloning, the digests were scaled up accordingly with the volume of enzyme added not exceeding 10% of the total volume.

2.3. Agarose gel electrophoresis of DNA

All DNA samples were mixed with 0.2 volumes of tracking-dye before electrophoresis. Most restriction digests of YAC DNA were resolved onto a 0.8% agarose gels containing 1µg/ml ethidium bromide. All digests were run in 0.5x TBE (12.5cm)

buffered gels at 100 volts for 1-1.5 hours in a Hybaid gel electrophoresis tank. Small PCR products were resolved on higher concentration agarose gels ranging from 1-2% gels. The molecular weight markers used were either λ bacteriophage cut Hind III (NBL Gene Sciences) or the phage ϕX174 cut with Hae III (Gibco BRL).

2.4. Preparation of DNA probes

Following restriction enzyme digestion of BAC or PAC clones containing fragments used as probes, the fragments were resolved on agarose gel. The fragment of interest was then excised under UV light with a scalpel blade. The DNA was then purified from the agarose slice using a Jetsorb DNA extraction Kit (Genomed). The manufacture's instructions were followed and the DNA was eluted in a final volume of 40μl of TE.
1μl of probe DNA (approximately 20–40ng) was diluted to 48μl with distilled water and boiled for 5 minutes before placing on ice. The 48μl was then collected by brief centrifugation and added to a pre-prepared Rediprime (Random primer labelling Amersham Life Sciences) tube for labelling DNA. 2μl of [α-^{32}P] dCTP (370MBq/ml, 10mCi/ml) (Amersham Bioscience, Little Chalfton, UK) was added and the reaction was incubated for 30-60 minutes at 37C°. The reaction was diluted with water to 100μl and unincorporated nucleotides were separated from the DNA probe by centrifugation through a G50 Sephadex column (Pharmacia). This was prepared in 1ml syringe plugged with glass wool and equilibrated with distilled water. The labelled probe was then diluted again to 500μl and boiled for 5 minutes to denature the DNA before being added directly to the hybridisation solution.

2.5. Kinase end labelling of oligonucleotides

Oligonucleotide 1028 and oligonucleotide 1207 (see Chapter 3 for details of these primers) were used as probes for hybridisation of the filter lifts obtained from the vectorette cloning method. These were end-labelled using T4 Kinase and [γ-^{32}P] ATP. A 20μl reaction was set up 100ng of oligonucleotide using 2μl of 10x kinase buffer, 2μl of [γ-^{32}P] ATP (370MBq/ml, 10mCi/ml) (Amersham Bioscience, Little Chalfton, UK), and 1μl of T4 kinase (New England Biolabs 10,000U/ml). This mixture was incubated at 37°C for one hour. The reaction was diluted to 100μl with distilled water and

unincorporated nucleotides were separated by centrifugation through a G50 Sephadex column. The labelled probe was then added directly to the hybridisation solution. Hybridisations were carried out at room temperature overnight in 6xSSC following a 1-2 hours of prehybridisation of the filters in "Church buffer" at 65°C. The following day, filters were washed in 6xSSC/0.1% SDS. Washing was started at room temperature and then the temperature of the washing solution was increased gradually by 5°C each wash. The final washing temperature was determined by the melting temperature (TM) of the oligonucleotide minus 12°C (Sambrook J. *et al.*, 1989). The signal was detected by autoradiography as described, below.

2.6. Filter hybridisation

Filters were prehybridised in a hybridisation glass bottle at 65°C for 1-2 hours in Church buffer. Labelled probes were then added to the hybridisation buffer and incubated for a time ranging between 4 hours to an overnight. The stringency of the washes depended on the hybridisation that has been performed. Filter lifts from plasmid colonies were washed in 2xSSC/0.5% SDS, initially at room temperature, then at 65°C for 10 minutes. The CEPH YAC library filters were washed twice each for 30 minutes with 0.5xSSC/0.2% SDS at 65°C. If following the washes the filters showed residual background radioactivity (i.e. above 15-20 counts per second as detected by a Geiger counter), they would be washed further at a higher stringency. Filters were then exposed to Fuji Medical X-ray film (RX) at -70°C, between 2 hours to 14 days depending on the intensity of the signal. The position of positive signal could be identified using orientation marks.

2.7. Cloning of YAC ends using the "Vectorette Method"

For additional information on this procedure see Chapter 3.

1µg of YAC DNA was digested in a total volume of 100µl .The enzymes used were DraI, EcoRV, FspI, HincII, NaeI, PvuII, RsaI and ScaI. The standard digestion reaction was as following

1µg of YAC DNA

1/10 digestion buffer (x10 stock)

1/100 DTT (100mM stock)

1/100 Spermidine (300mM stock)

3µl enzyme

Double distilled (ddw) water to final volume 100µl

The tubes were incubated at 37°C for overnight. 5µl of each digest were run on 1% agarose minigel to check for complete digestion. The digests were heat inactivated at 65°C for 10 minutes.

The YAC digests were then ligated to vectorette unit, where the tubes were spun briefly and the following was then added to each tube:

4µl of 1M Tris pH 7.6

1µl of 100 mM ribose ATP pH 7.0

5µl of 2 pmol /ml annealed bubble (see below).

1µl of T4 DNA ligase

The tubes were incubated at 37°C for 1-2 hours. Then, 380µl TE was added, the contents of the tubes were mixed well and aliquoted into 2 sets of tubes, one set to be used and the other set was kept at -20°C until use. 5µl of each digest were used for PCR amplification of vectorette libraries.

Standard PCR reaction was as follows:

5µl	224 primer (Universal vectorette primer)
5µl	1089/1091 primer (Left/right arm primers)
5µl	Buffer
5µl	2mM dNTPs
0.5µl	Taq polymerase
24.5µl	ddH2O

Negative control was done with water, a drop of mineral oil was added, and the reaction was carried out as follows:

94°C	5 minutes	1 cycle
93°C	1 minute	
65°C	1 minute	38 cycles
72°C	1 minute	
72°C	5 minutes	1 cycle

Nested PCR reaction was carried out where 6µl of each of the first round of PCR products were used for vectorette library amplification with a second set of primers, the

PCR reaction was as follows:

6µl	224 primer (Universal vectorette primer)
6µl	1201/20359primer (Left/right arm primers)
6µl	Buffer
6µl	2mM dNTPs
0.6µl	Taq Polymerase
29.4µl	ddH2O

Nested negative control was also done and the reaction was carried out.

94°C	5 minutes	1 cycle
93°C	1 minute	
58°C	1 minute	30 cycles
72°C	1 minute	
72°C	5 minutes	1 cycle

15µl of the second PCR reaction was digested with 1µl of EcoRI in a total volume of 20µl at 37°C for 1-2 hours.

The digested PCR products were run alongside with the undigested ones onto a 1.5-2% agarose minigel. Any undigested product with a band size more than 300 bp and showing a difference of 90 basepairs (bp) from the digested product was excised using a scalpel under UV light and the DNA was purified using a Jetsorb (Genomed) Kit following the manufacturer's recommendations. The resulting pure fragments were eluted in 30-40µl of TE.

The purified products were then digested with BamHI and HindIII in total volume of 50µl, incubated at 37°C for 1-2 hours, heat inactivated at 65°C for 10-20 minutes and then ligated to a Bluescript plasmid vector (pBSK, Stratagene, UK). The vector had been digested with the same restriction enzymes used to digest the PCR products followed by phosphorylation to reduce number of false positive colonies as described in paragraph 2.8. The ligation was incubated at 37°C for 1-2 hours.
Competent cells were prepared by Hannahan's method (see paragraph 2.9.1 below) and transformation of 4 and 8µl of each reaction was performed according to the protocol described in 2.9.

The transformed cells were then spread on 2 xTY with 100µg/ml ampicillin plates and incubated at 37°C for overnight. Colonies were then transferred onto nylon filters and hybridised with oligonucleotide 1208 for right arm products and oligonucleotide 1207 for left arm products. The positive clones were picked and sequenced.

2.7.1.Preparation of Vectorette bubble mix (Stock& working solution)

3.2 nmol of top primer and 3.3nmol of bottom primer were transferred into an eppendorf then made up to 100µl with water containing 25mM $MgCl_2$ (0.5µl of 5M $MgCl_2$), this gives a concentration of 65pmol/µl (stock).

The mix was then heated to 65°C for 5 minutes and then allowed to cool slowly to room temperature. A working solution of the bubble mix was then prepared by diluting the stock solution 1:30 with water to give a concentration of 2pmol/µl.

Alternatively, a tenfold concentrated annealing buffer was prepared containing 10mM TRIS and 10mM $MgCl_2$ to prepare the annealed vectorette unit.

300pmol of top primer and 300pmol of bottom primer were transferred into an Eppendorf tube and 10µl of x10 annealing buffer were added and completed to a final volume of 100µl with double distilled water. This gives a concentration of 3pmol/µl of the vectorette unit.

This mix was then heat inactivated at 60°C for 10 minutes and transferred to cool at room temperature for 1 hour, then kept at –20°C until use. 2µl of this unit (6pmol) were then ligated to YAC DNA in a final volume of 100µl.

2.8. Cloning of DNA into plasmid vector

2.8.1.Vector preparation

1µg of uncut vector, Bluescript plasmid (Boehringer Ingelheim) was digested in 50µl digest with the appropriate restriction endonuclease enzyme/s required. After 3 hours of incubation at 37°C a 5µl aliquot was electrophoresed on a 0.8 % agarose gel with 50ng of uncut vector in parallel with a molecular weight marker, to check for complete

digestion of the vector. The reaction was then placed at 65°C for 10 minutes to inactivate the enzyme before being placed briefly on ice. To dephosphorylate the vector ends, 2-4µl of calf intestinal alkaline phosphatase (1U/µl, Boehringer Ingelheim) was added and the reaction was incubated again at 37°C for 1 hour. The reaction was then diluted with TE to100µl and mixed with 100µl of phenol: chloroform: isoamyl alcohol (PCIA). The aqueous phase was then collected by centrifugation and was centrifuged through a G50 Sephadex column that had been equilibrated with TE, to remove residual phenol. The vector was stored at -20°C and 2µl of vector was used for each ligation reaction (see paragraph 2.8.3).

2.8.2.Insert preparation

Depending on the size of the fragments to be cloned, 5-20µg of DNA was digested with 5-10 fold excess of appropriate restriction enzyme under optimal conditions. The digested products were run on an agarose gel of a concentration appropriate to separate the fragments of interest and visualised by ethidium bromide staining. The band to be cloned was then excised using a scalpel under UV light and the DNA was purified using a Jetsorb (Genomed) Kit. The resulting pure fragments were eluted in 30-40µl of TE.
If the fragment to be cloned was a PCR product, then the PCR primers were designed with restriction enzyme sites at the 5' end. 5µl of the PCR product was analysed by gel electrophoresis on an agarose ethidium bromide gel. The rest of the PCR product was then purified using Qiagen PCR clean up kit, following the manufacturer's recommendations. The DNA to be cloned was eluted in 40µl of sterile water and the total DNA was digested in a volume of 15-20µl of the digested insert was then electrophoresed on an agarose gel, and the DNA band to be cloned was excised under UV light and purified as previously described.

2.8.3.Ligation

Two ligation reactions were set up for each insert, usually with 1-3 molar excess of insert DNA and 2µl (10ng/µl) of vector, plus a control ligation with vector only, to check for background false positive religation. Ligations were carried out in total

volume of 20µl using 200-400 U of T4 DNA ligase (New England Biolabs, 400U/ml) in 1x ligase buffer (New England Biolabs) containing 1mM ATP. The ligations were incubated in a water bath at 14°C overnight and then stored at -20°C until required.

2.9. Transformation of bacterial cells with recombinant clones

2.9.1.Preparation of Hannahan's competent cells

A single colony of TG1 strain of Escherichia coli cells was inoculated in 5ml of SOB medium containing 2% glucose and 10mM $MgCl_2$. This was then grown overnight to stationary phase in a shaking incubator at 37°C. 500µl of the overnight culture was diluted in 50ml of SOB medium in a sterile 250ml flask. 500µl of 20% glucose and 500µl of 1M $MgCl_2$ were added and the flask was returned to the 37°C incubator. The culture was then grown to $OD_{600\ nm}$ of 0.5-0.55. The cells were placed on ice for 15 minutes and then the culture was transferred to a 50ml Falcon tube. The cells were pelleted by centrifugation at 1,600 rpm for 10 minutes. The pellet was then resuspended in $1/3^{rd}$ of the total culture medium (16.6ml) of cold TFB and left on ice for 10 minutes. The cells were then spun as before and the pellet resuspended in $1/12.5^{th}$ (4ml) of TBF. A final 7% in volume of DnD was added in two 3.5% aliquots with 10 minutes incubation between the two aliquots. After a further 15 minutes incubation on ice the cells were ready for transfection.

2.9.2.Transfection of competent cells

200µl of competent cells were then mixed with 8µl of the overnight ligation in a 1.5ml Eppendorf, incubated on ice for 45 minutes and then heat shocked in a 42°C water bath for 2 minutes. For Bluescript transfections, 2 volumes (400µl) of 2x TY were added and the cells were incubated at 37°C for 30-60 minutes to allow expression of the ampicillin resistance gene prior to plating. 30µl of each IPTG and X-Gal (or 60µl of a mixture of the two) was added to each tube. The whole culture was then plated onto 2xTY agar plates supplemented with ampicillin (100µg/ml). The plates were left to dry for 20

minutes at room temperature and then incubated overnight at 37°C.

2.9.3.In situ colony hybridisation

In order to select recombinants containing the desired insert, plaques or colonies were screened by in situ hybridisation as follows. Lifts were taken from each plate onto Whatman 1MM paper filters or nylon filters. The filters were laid over the plate surface for 1-2 minutes. Orientation marks were made using a 19-gauge needle and black ink. The filters were then carefully removed from the plates. For Bluescript colonies the filters were placed on 3MM Whatman paper soaked in 2xSSC/5%SDS for 2 minutes to lyse the cells. DNA was then transferred and fixed to the filters by microwaving on the highest setting for 2.5 minutes. Further fixing was carried out by UV cross-linking. After rinsing the filters in 3xSSC, the filters were ready for prehybridisation. The filters were then hybridised to the appropriate labelled DNA probe.

2.10. Screening Yeast artificial chromosome (YAC) libraries

ICI (Anand et al, 1990) and CEPH YAC (Chumakov et al, 1992) libraries utilised during this study were obtained from Human Genome Mapping Project Resource Centre (UK HGMP Resource Centre) (Hinxton, Cambridge, UK).

2.10.1. PCR screening of ICI YAC libraries

ICI libraries are available as DNA pools for PCR screening. In the initial step, the 40 primary pools, consisting of DNA in agarose and numbered 1-40, and letters A-I were screened. When positive pool(s) were identified, the appropriate secondary pools were ordered. The positive clone(s) were identified directly from the secondary step where both the plates containing the positive clone and the plate co-ordinate would be determined. Additional details of this procedure are given in Chapter 3.

The DNA pools were sent out embedded in agarose plugs in 5mM EDTA solution. The plugs were washed in TE buffer for 2 hours, with shaking and 2 changes of buffer. They were left in TE at 4°C overnight (this is to make sure that the EDTA was fully eluted

from the plugs). TE was then replaced with 100µl of distilled water. The plugs were then melted at 65°C for 15-20 minutes. 3µl was used in a 30µl PCR reaction.

2.10.2. CEPH libraries screening

The CEPH libraries were available to us as filters containing all the clones and these filters were screened by hybridisation. Additional details of this procedure are provided in Chapter 3.

The CEPH filters were soaked in 2x SSC for 5 minutes prior to prehybridisation. They were then prehybridised in Church buffer for 2 hours at 65°C. The labelled probe was added and incubated overnight at 65°C. The following day the filters were washed twice each for 30 min in 0.5 SSC/0.2% SDS at 65°C. The filters were exposed to Fuji Medical X- ray film (RX) at -70°C for 7-14 days. The position of positive signal was identified using orientation marks.

2.11. Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was performed using many different templates and primers. All reactions were carried out in 50µl volumes with 1x PCR buffer (Promega) plus 1.5 mM $MgCl_2$. Unless otherwise mentioned the Taq polymerase used was Promega and 0.5-1µl was used per reaction. 10% of 2mM dNTPs stock was added with 250ng-600ng of each primer. Between 50ng and 1µg of total DNA was used routinely in the PCR reactions. The volume was then made up with sterile water to the required final volume. A drop of mineral oil (Sigma, UK) was laid over the reaction to prevent evaporation. A standard program was used for all PCRs unless otherwise stated, with the annealing temperature being altered depending on the T_m of the primers used.

PCR conditions were as follows:

Hot start	94°C	5 minute	1cycle
Denaturation	94°C	1 minute	
Annealing	Variable	1 minute	35 cycles
Extension	72°C	2 minutes	
Extension	72°C	10 minutes	1 cycle

PCR products were analysed by electrophoresis on an agarose gel containing 1μg/ml of ethidium bromide. The agarose percentage varied depending on the expected size of the PCR products.

2.12. Detection of loss of heterozygosity (LOH) and microsatellite instability (MSI)

2.12.1. Radioactive polymerase chain reaction

PCR was performed in a reaction mixture of 30μl. 250ng of each primer was used. 10% 2mM of dNTPs, 0.2μl of [α-^{32}P] dCTP (370MBq/ml, 10mCi/ml) (Amersham Bioscience, Little Chalfton, UK), 50-100ng of patient's genomic DNA, 1× PCR buffer (Promega) containing 1.5mM/L $MgCl_2$ and 0.3-0.5μl of Taq polymerase (Promega) was added to the reaction and the final volume was adjusted to 30μl using sterile double distilled water, a drop of mineral oil (Sigma, UK) was laid over each reaction to prevent evaporation.
PCR conditions were as follows:

Hot start	94°C	5 minute	1cycle
Denaturation	94°C	1 minute	
Annealing	Variable	1 minute	30 cycles
Extension	72°C	2 minutes	
Extension	72°C	5 minutes	1 cycle

The annealing was done at each primer specific annealing temperature according to the melting temperature (Tm) for each oligonucleotide primer pair. A negative control reaction containing no DNA was examined for each PCR reaction.

2.12.2. Acryiamide gel electrophoresis for LOH and MSI detection

Electrophoresis was performed using 40x50x0.4cm gels. Before pouring the gel, the

glass plates were siliconised using Dimethyl dichlorosilane solution (BDH) The plates were separated by 0.4mm spacers and fastened together with tape and clips. 140µl of 25% ammonium persulphate solution (APS) and 100µl of Temed (Amresco, UK) was added to 75ml of 1XTBE acrylamide gel mix and the reaction was poured into the plates using a 50ml syringe. A comb to form the wells was placed in position and the gel was allowed to set for 30 minutes. The tape and the clips were then removed and the gel was then placed in a Gibco BRL S2 gel tank. 1 litre of 1xTBE was used as buffer and 1µl of formamide stop solution was loaded into each well to verify that wells were free of leakage. Ten µl of each sample PCR reaction was mixed with 5µl of formamide stop solution in a microtiter tray and was denatured at 80°C for 15-20 minutes. 5µl of each sample mix was then loaded into each well. The gel was run at 60 Volts for 2-5 hours depending on the size of the PCR product. The plates were then disassembled and the back plate was removed. The acrylamide gel was transferred to 3MM Whatman paper and covered with Saran wrap before being dried under vacuum at 80°C on a gel dryer for 2 hours. The dried gel was then exposed for 24-36 hours at -70°C to a Fuji Medical autoradiography film. Shorter exposures were performed when needed.

Chapter 3

Physical mapping a region of deletion on chromosome 6 and q16-21

3.1. Introduction

Deletion of chromosome 6 is a non-random chromosomal abnormality occurring in approximately 5-10% of human lymphoid malignancies, although an incidence of 22-26% can be detected in some subgroups of B cell malignancies, such as NHL (Gaidano G. *et al.*, 1992; Offit K. *et al.*, 1993). Among the most frequently observed chromosomal abnormalities in haematological and solid malignancies are deletions affecting the 6q and a more extensive description of deletions affecting both haematological and solid tumours has been provided in chapter 1 of this thesis.

Conflicting data have been published regarding the incidence and regions of minimal deletion on 6q in the different subsets of the lymphoid malignancies (Hatta Y. *et al.*, 1999). At least two distinct and non-overlapping regions of minimal deletions (RMD) have been identified on chromosome 6q (Figure 3.1).

RMD-1 at 6q25-27 is predominantly found in non-Hodgkin's lymphoma (NHL) (Gaidano G. *et al.*, 1992; Menasce L. P. *et al.*, 1994a; Merup M. *et al.*, 1998). RMD-2 is located at 6q21 and is predominantly found in acute lymphoblastic leukaemia (ALL) (Gerard B. *et al.*, 1997; Hayashi Y. *et al.*, 1990; Menasce L. P. *et al.*, 1994b; Sherratt T. *et al.*, 1997; Takeuchi S. *et al.*, 1998).

Studies on the RMD-2 have revealed two sub-regions (Figure 3.2). The first one, RMD-2A, has been positioned between markers D6S1709 and D6S301 with marker D6S283 being the most frequently deleted marker within this region (Merup M. *et al.*, 1998).

The second sub region, RMD-2B, has been positioned between markers D6S301 and D6S302 (Gerard B. *et al.*, 1997; Takeuchi S. *et al.*, 1998). This region has been further narrowed down to approximately 2Mb between marker D6S447 and marker D6S246 (Menasce L. P. *et al.*, 1994b; Sherratt T. *et al.*, 1997).

Despite extensive mapping, the region telomeric to D6S283 and centromeric to D6S268 was still incomplete at the time this project started and the presence of gaps clearly resulted in an underestimation of the size of these regions and distance between them

and made the assignment of any candidate TSG difficult. Limited information was also available on the number of genes located within this region and a schematic representation is given in (Figure 3.3).

3.2. Preliminary data and work on 6q deletion previously carried out in our laboratory

Analysis of 6q deletion in lymphoid malignancies has been the topic of investigation in our laboratory for approximately three years prior to the beginning of my study. This had resulted in the generation of a partial directional map using ICI/Zeneca and CEPH yeast artificial chromosome (YAC) libraries (see Figure 3.4) and some of these results have been previously published (Jackson A. *et al.*, 2000). The YAC clone 830d9, containing marker D6S447 and YAC clone 28CD7 had been previously identified (Figure 3.4) as well as clones 916c5 and 856c1 containing marker D6S268 and D6S278. Using vectorette cloning (see Material and Methods, paragraph 2.7) the ends of these and other clones telomeric (28CD7, 916c5, 3GA3 and 6EH12) and centromeric (830d9, 853b7) to the identified region of deletion were used to generate primers/probes for successive screenings of ICI/Zeneca and CEPH libraries. The aim was to cover the region between these clones and, through their sequence analysis, identify candidate TSGs.

Data on a group of 25 patients previously investigated by FISH was also available from our laboratory at the time my investigation started, to help to define the region of minimal deletion localised between markers D6S447 and D6S268 (Jackson A. *et al.*, 2000). We intended to extend this analysis to additional cases to verify the extent of the region of deletion using a molecular approach (see Chapter 4).

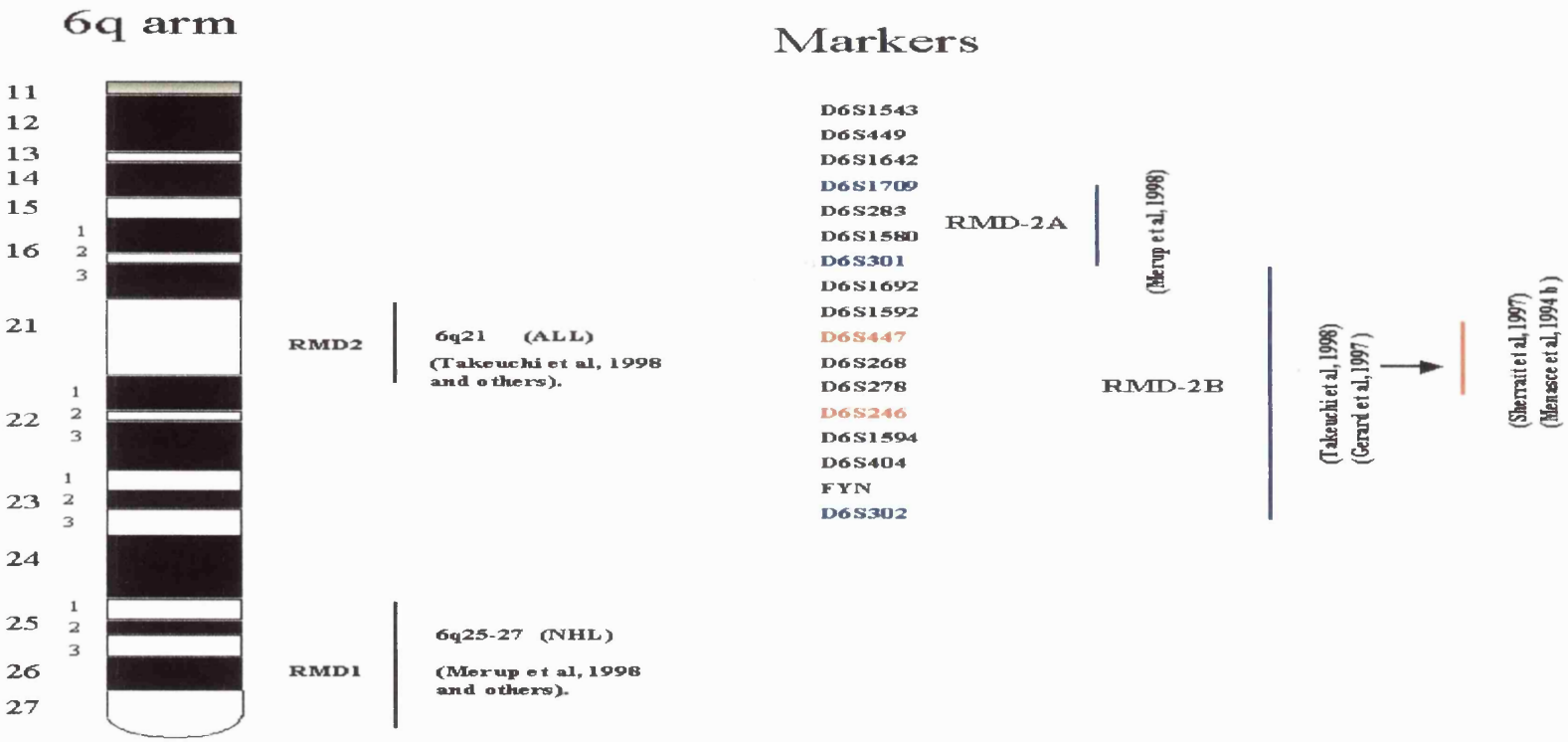


Figure 3.1. *Shows the two regions of minimal deletion (RMD) on 6q. RMD-1 on 6q25-27, mainly in non-Hodgkin's lymphoma (NHL), and RMD-2 on 6q21 mainly in acute lymphoblastic leukaemia (ALL). Two subregions of RMD-2 are illustrated; RMD-2A between markers D6S1709 and D6S301 and RMD-2B between markers D6S301 and D6S302 (blue coloured lines and text). RMD-2B was narrowed down to a region between D6S447 and D6S246 (red coloured line and text).*

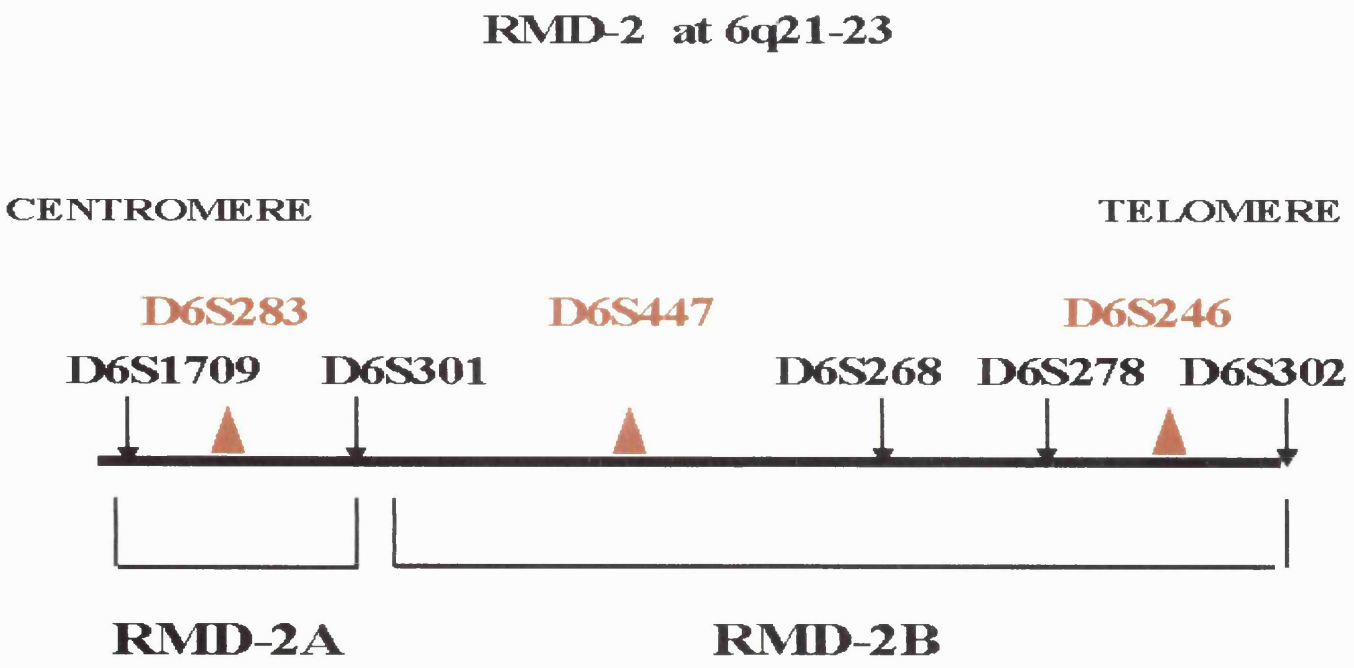


Figure 3.2. *Shows the two subregions of RMD-2 on chromosome 6q21-23; RMD-2A between markers D6S1709 and D6S301 and RMD-2B between markers D6S301 and D6S302.*

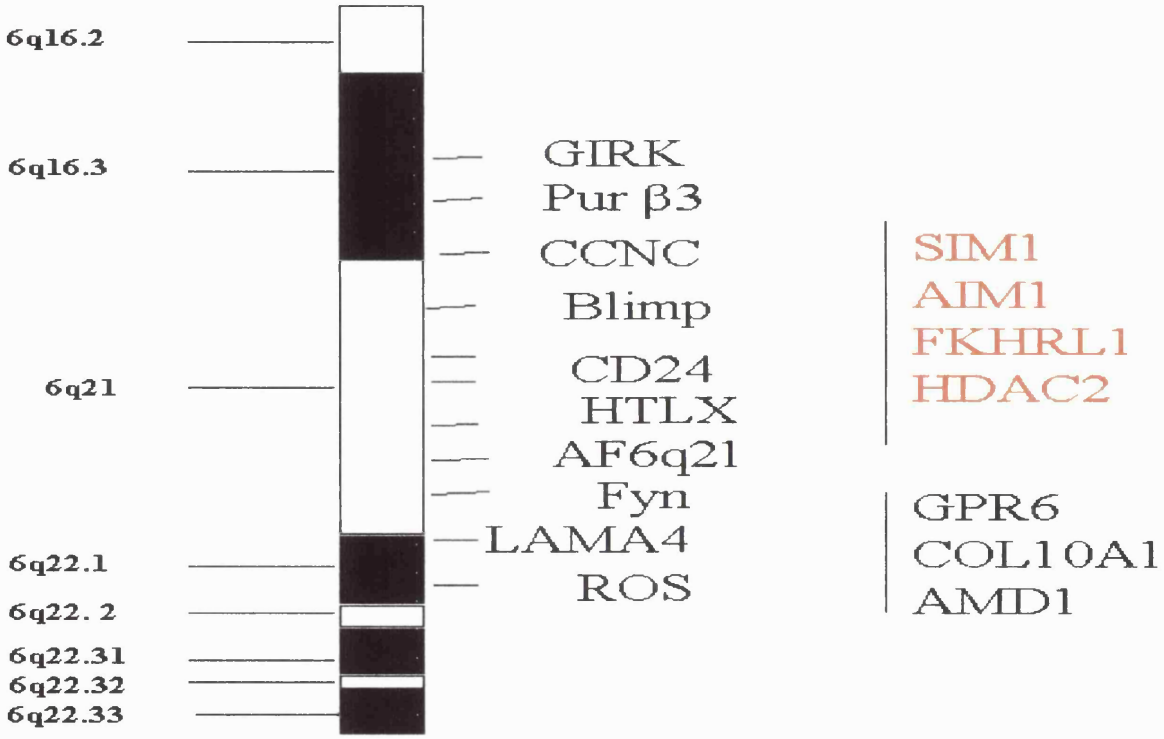


Figure 3.3. *Shows a list of the genes that have been identified on 6q16.3-21 at the time the investigation of 6q deletions in lymphoid malignancies in our laboratory was started. The position of genes on the far right end of the figure had not been more precisely defined with respect to the other genes.*

Finally, collaboration with the chromosome 6-project group at Sanger Centre in Hinxton had been established in order to integrate any progress in this work with their chromosome 6 sequence data and to integrate the position of our clones with their contigs.

This chapter will describe the approach used to investigate the minimal region of deletion corresponding to RMD-2B (in Figure 3.2), which at the beginning of my investigation appeared to be the focus of deletion in patients with ALL.

3.3. Strategy

We aimed to identify novel clones between YACs 860f10/860e12 (centromeric) and 36CB11 (telomeric) by using:

1. Vectorette cloning of ends of YACs from within the region of deletion.
2. PCR screening of ICI/Zeneca libraries using primers from available STS in the region of interest or sequences available from newly sequenced clones (YACs, BACs or PACs).
3. Screening of CEPH libraries using probes from DNA segments in the region proximal to the gap to obtain more clones and to test these clones for overlapping.

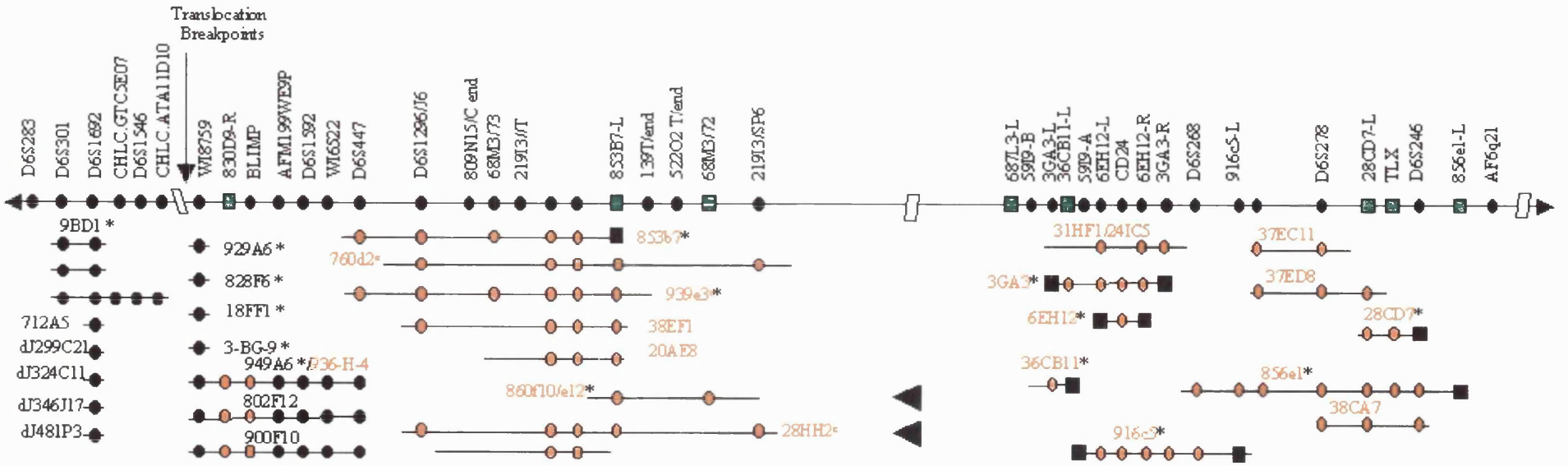


Figure 3.4. *Shows the partial directional map generated by colleagues from the previous work, the map in this figure shows YACs 3GA3, 36CB11 (telomeric) and YACs 860f10/860e12 (centromeric) defining the boundary of a new previously uncloned region. Symbols are as follows: Black solid circles: data from contig WC6.13; red solid circles: data confirmed by our group; green squares: probes available; Black squares: ends identified; c: chimeric clones; dJ: PACs from Pieter de Jong PAC library; *: YAC/PAC clone localised by FISH.*

3.4. Material and Methods

For YAC DNA preparation see Chapter 2 (paragraph 2.1.1.).

3.4.1.The vectorette cloning system

This technique is used to isolate sequences adjacent to the YAC vector and therefore results in the cloning of the YAC termini (Riley J. *et al.*, 1990). Some details of this approach have already been described in Chapter 2 (paragraph 2.7). Additional information, however, more pertinent to the use of this technique in this chapter is provided in the following paragraphs.

As previously mentioned, YAC DNA is digested with a range of restriction enzymes suitable to generate relatively short fragments (ranging approximately between 300-1000 bp) containing short stretches of the YAC cloning vector. After digestion with one of the restriction enzymes listed in Chapter 2 (paragraph 2.7 and also below), the resulting DNA fragments are ligated to synthetic oligonucleotide duplexes, termed vectorettes (and referred to as "vectorette bubble"), resulting in the generation of a vectorette library. A wide range of restriction enzymes can be used including those creating a blunt or overhang ends. The only enzymes that cannot be used are those which cut at the YAC cloning site or those which cut between the cloning site and the primer annealing region of the YAC vector. The synthetic duplex contains a region of non-complementarity. A primer (universal vectorette primer 224) is designed to be identical to the non-complementarity region of the bottom strand of the duplex. Among the several different fragments generated, some will contain part of the vector sequences and the adjacent first YAC insert segment. When amplification is performed using YAC vector arm specific primers (right and left YAC arm specific primers) and primer 224 (from within the non-complementary sequence of the vectorette bubble), it is expected that only terminal fragments containing vector sequences will be amplified (as graphically illustrated in Figure 3.5). All primers are listed below in Table 3.1.

Primer name	Sequence
Vectorette bubble top strand primer	5′-NAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGACGAGAGAA-3′
Vectorette bubble bottom strand primer	5′CTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATCGCTGTCCTCTCCT TN-3′
YAC Right arm primer 1st round (1091)	5′-ATATAGGCGCCAGCAACCGCACCTGTGGC-3′
YAC Right arm primer 2 nd round (20359/H3)	5′-TAGAAGCTTCTTGCAAGTCTGGGAAGTGAATGGAGAC-3′
YAC Left arm primer 1st round (1089)	5′-CACCCGTTCTCGGAGCACTGTCCGACCG-3′
YAC Left arm primer 2nd round (1021/H3)	5′-TAGAAGCTTAGTATACTCTTTCTTCAACAATTAAATACTCTCGGT-3′
Universal vectorette primer (224/BamHI)	5′- TAGGGATCCCGAATCGTAACCGTTGGTACGAGAATCGCT-3′

Table 3.1. *Vectorette primer sequences. YAC; yeast artificial chromosome, H3; Hind III restriction enzyme; N: redundant the presence of all four bases.*

Figure 3.5 is a schematic representation of the vectorette cloning system. As part of my investigation, this method was applied to YAC 36CB11 and 860f10, 860e12 and 28HH2. See Chapter 2 (paragraph 2.7) for further details of the cloning method of vectorette products.

In brief, one μg of DNA was digested with a range of restriction enzymes including DraI, EcoRV, FspI, HincII, NaeI, PvuII, RsaI and ScaI. All these enzymes generated a blunt digest to facilitate the use of a single vectorette bubble. Following inactivation of digested DNA, the DNA was ligated to the vectorette units (linker) at 15°C, as described in Chapter 2. Following ligation DNA was diluted to 500μl final volume. A two-rounds (nested) PCR amplification was carried out using primers 1089 (I round) and 1201 (II round) for the right arm, and primers 1091 (I round) and 20359 (II round) for the left arm in combination with primer 224 as a universal antisense primer for both rounds of PCR in a semi-nested PCR using 5μl of the total vectorette library.

The PCR product was then checked on a 1.5-2% agarose gel. The obtained PCR products were then digested with EcoRI and loaded on 2% agarose gel to run in parallel with uncut PCR products. As the YAC vector contained an EcoRI at the cloning site, this step helped to identify true from false PCR amplification products. Consequently,

any undigested product with a band size greater than 300 base pair (bp) and with a difference between the digested and undigested product of 90bp was considered a true product. This was then excised from the gel, purified and eluted into TE. The eluted products were then digested with BamHI (a restriction site included in the universal primer 224) and Hind III (a restriction site included into the vector primer). The product was heat inactivated and ligated into a Bluescript plasmid vector (pBSK, Stratagene, UK), which has been digested with Hind III and Bam HI. Following transformation, positive colonies were identified using blue-white selection or by hybridisation to a probe specific for the right or left YAC vector as described in Chapter 2 paragraphs 2.5 and 2.6.

3.4.2. BAC and ICI library screening

The ICI and CEPH libraries are available from the Human Genome Mapping Project Resource Centre (HGMP) as DNA pools for PCR screening (ICI) (Anand R. *et al.*, 1990) or libraries filters for hybridisation screening using radiolabelled probes (CEPH) (Chumakov I. *et al.*, 1992).

3.4.3. ICI library screening

Several of the PCR primer combinations required optimisation for both annealing temperature and $MgCl_2$ concentration to obtain a clean PCR product on screening the ICI libraries. Each set of primers derived from sequences of the YAC ends, was tested for amplification of chromosome 6-only sequence using polymerase chain reaction (PCR) on chromosome 6 hybrid cell DNA (obtained from the Sanger Centre), total human DNA (positive control) and mouse DNA (negative control) to rule out chimeric amplification of non-human or non-chromosome 6 sequences.

All new YACs were then analysed by FISH to verify their localisation to chromosome 6 and to exclude chimeric YACs. Other members of the laboratory had carried out this part of the work, when required. In the initial step of screening the ICI/Zeneca libraries, the primary pools, 1-40 was screened. When positive pool(s) were identified, the second step was to screen the positive secondary pool(s) 1-20 and A-I pools. In this way the

plate and its coordinates were determined giving the YAC (s) name(s). See Chapter 2, paragraph 2.10.1.

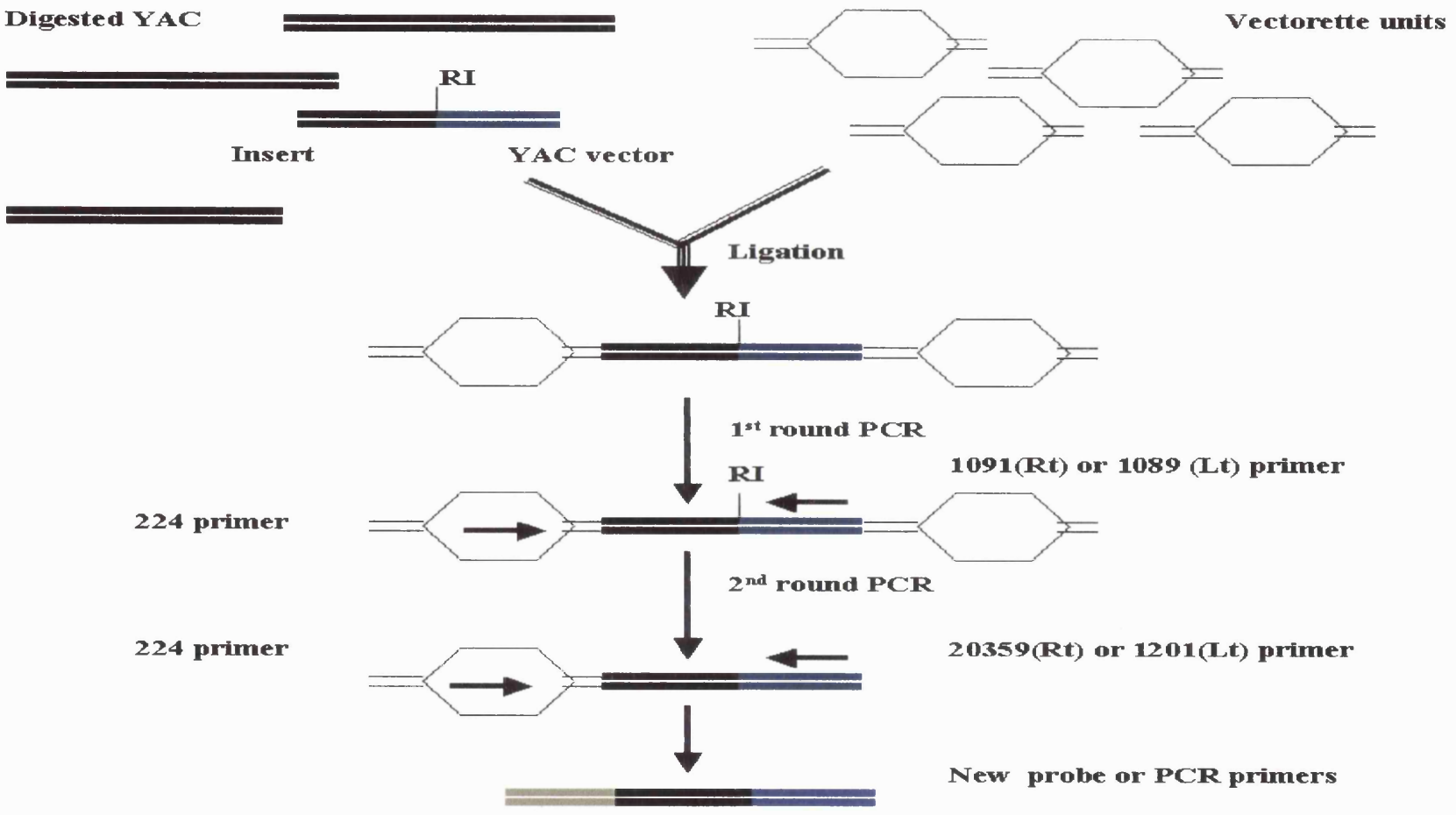


Figure 3.5. *Summary of vectorette cloning system. YAC: yeast artificial chromosome, RI: EcoRI restriction enzyme cloning site, Rt: right YAC arm primer, Lt: Left YAC arm primer, Primer 224: universal vectorette primer.*

3.4.4.CEPH library screening

For the CEPH libraries we had access to filters containing all clones which could be screened by hybridisation using probes derived from the YAC end-vectorette cloning step or genomic probes available through this investigation. See Chapter 2, paragraph 2.10.2 for hybridisation procedures.
Figure 3.4 shows YACs 3GA3, 36CB11 (telomeric end) and 860f10, 860e12 (centromeric) defining the boundary of a contig available at the beginning of this investigation and a new previously uncloned region for which no information is available from any public database.

3.5. Results

3.5.1.Vectorette cloning

The vectorette method has been applied extensively to YACs 36CB11, 860f10, 860e12 and 28HH2. Although this method has worked previously in our laboratory, no true PCR product (PCR product with a band size greater than 300 base pair and with a difference between the digested and undigested product of 90bp) was amplified from any of the tested YACs. In several instances, both cloning and screening of colonies were carried out but failed to yield any fragment or sequence corresponding to a true YAC end, according to the criteria described above. For this reason we moved to the screening of the CEPH library, suspecting that the ICI libraries failed to contain any new clone overlapping with YAC 36CB11, 860f10 or 860e12. Also, through our collaboration with the Sanger Centre a number of PAC and BAC clones had become recently available and these were also used for investigation.

3.5.2.Combined BAC and CEPH library screening

Using information available from partial or full sequence of BAC clones (through the collaboration with the Sanger Centre) several STS sequences in the region of interest were used and these are summarised in Table 3.3. In details, end sequences were

available from each end of the following bacterial artificial chromosome: bA59I9 (bA59I9A and bA59I9B), bA182N12 (bA182N12A and bA182N12B) and one end only for marker bA254J7. PCR primers were designed from these sequences and were tested on human genomic DNA and chromosome 6-only. Marker bA182N12 has been ruled out from any further investigation after being proved not to map on chromosome 6.

Primary screening of ICI/Zeneca libraries using primers for bA59I9/A detected several positive clones (clones 6, 7, 8, 14, 17, 20, 21, 26 and also few others but showing a much weaker signal). The second round screening of clone 6 yielded two positive pools (8, 20) rather than the three required and thus could not be translated into a YAC name. The same pattern was observed during the screening of the library using primers for bA59I9/B where clones 6, 7, 8, 14, 37 were also positive. Screening of the secondary clones, however, failed to detect any positive YACs. A summary of the screening is given below.

Primer combination*	Library	Round I	Round II
bA59I9A F/R	ICI	**6**, 7, 8, 14, 17, 20, 21, 26	8, 20
bA59I9B F/R	ICI	6, 7, 8, 14, 37	Negative

: Sequences of individual primers are given in Table 3.2. **F: forward primer; **R**: reverse primer.*

Primary screening of the libraries using primers for marker bA254J7 gave no positive clones.

Primer combination*	Library	Round I	Round II
bA254J7F/R	ICI	Negative	Negative

Using the sequences of these BACs and searching human genome databases (DNA, expressed sequence tags, ESTs, or STSs databases) to obtain novel overlapping clones resulted in the identification (via BLAST sequence homology search) of other markers (bA68M3, bA687L3, bA809N15 and bA139N14 and P1-derived artificial chromosome dJ219I3 and dJ439N14). Marker bA68M3 was used to screen ICI /Zeneca libraries and revealed no positives from the primary screening.

Primer combination*	Library	Round I	Round II
bA68M3-72F/R	ICI	Negative	Negative

A genomic DNA probe was generated from this marker (using the cloning procedure for PCR products described in Chapter 2 (paragraph 2.4) and used to screen CEPH library filters. YACs 860f10, 860e12 and 760d2 were found to be positive. These YACs were already known to us as they had been identified from the previous work in our laboratory.

However, no novel clones were identified. Of these, clone 760d2 was known to be chimeric while the clones from the 860 series were chromosome 6-genuine clones.

Primer combination*	Library	Results
bA68M3-72F/R probe	CEPH	**860f10, 860e12 and 760d2** were positive

PCR screening of ICI/Zeneca libraries using primers specific for marker bA687L3 identified two positive primary clones: 17 and 19, while secondary screenings, however, were negative.

Primer combination*	Library	Round I	Round II
bA687L3 F/R	ICI	17, 19	Negative

A genomic probe generated using the same marker showed a background signal when tested on control filters, and failed to provide the quality standard required for a filter library screening.

Primer combination*	Library	Result
bA687L3 F/R probe	CEPH	Failed to provide quality standard for CEPH screening

ICI/Zeneca libraries screening using primers specific for marker dJ219I3/SP6 end, has yielded clone **28** as positive clone. Secondary screening of clone **28** resulted in the identification of pools 8, 10 and H, which translated in the identification of clone YAC 28HH2 as final positive clone.

Primer combination*	Library	Round I	Round II

dJ219I3-SP6F/R	ICI	**28**	8, 10, H **YAC 28HH2**

ICI/Zeneca libraries screening using primers specific for marker bA809N15 (reference sequence 104) end, yielded primary clones **20**, 22, 28 and 33 as positive. Secondary screening of clone 20 yielded clones 5, 6, 9, 12, 16, A and C. These corresponded to YACs 20AE8, 20AF1 and 20CF4 as final positive YACs. YAC 20AE8 was already known to us from previous work and all the other three identified YACs were known to lye on the centromeric side of the gap.

Primer combination*	Library	Round I	Round II
bA809N15 (104) F/R	ICI	**20**, 22, 28, 33	5,6, 9, 12, 16, A, C **YAC 20AE8, 20AF1, 20CF4**

Primers were designed from the sequences of markers bA809N15 (160) end, bA139N14 and dJ439I14 and were used to test if these clones overlaps with any of the YACs or other newly identified clones in the region of interest see Table 3.3.

At each stage when a YAC or new clone was identified, the clone was screened by PCR with all available set of primer combinations (as illustrated in Table 3.3) to confirm its orientation and extent of overlap with the other clones from the same region. Using this approach we could verify that despite several clones available from both the centromeric and telomeric end of the gaps no overlap between the region containing the D6S268 marker and the region containing the D6S447 markers had been established at this stage of the screening. Data from this part of the investigation is summarised in the Table 3.3 below. White circles indicate negative PCR while the black circles indicate positive PCR signal for the combination of primers (left list) on individual clones (list at the top).

In conclusion, despite extensive use of vectorette as well as CEPH library screening collaboration with the Sanger Centre no further information of the region of minimal deletion on 6q21 was obtained. The gap between markers D5S246 and D6S447 was reduced in size but not completely covered by overlapping clones. Therefore, the real extent of this gap remained undetermined. The extent of the new clone coverage is

illustrated in Figure 3.6 below.

Most of the effort of this part of the investigation aimed at reducing the physical gap within a region of minimal deletion as indicated by the FISH analysis carried out previously to the beginning of this investigation. Unfortunately we appeared to have encountered a region of genomic DNA which gave rise to limited number of clonable fragments both within YAC, PAC and BAC libraries.

Marker	Primers	Sequence	Annealing Temp °C	Size bp	$MgCl_2$	1° ICI screening	2° ICI screening	YAC
bA59I9A	bA59I9/AF	GGCCGGGCGGGGGCTCAACC	55	215	1mM	6, 7, 8, 14, 17, 20, 21, 26	Negative	-
	bA59I9/AR	CCTCTAGGGTTGAAGTGATTC						
bA59I9B	bA59I9/BF	CAGCGTCTCGCTCTGTCAGCC	64	280	1mM	**6**, 7, 8, 14, 37	8, 20	-
	bA59I9/BR	GCCTGGGTACGGTGGCTCAC						
bA254J7	bA254J7/F	GACTTCTTCCATAACCACCAC	55	180	1.5mM	Negative	Negative	-
	bA254J7/R	CCTCATTAACAATCTAATTAATCATT						
bA182N12A	bA182N12/F	CTGGCTCCACGGAATGCTGCG	60	430	1.5mM	Excluded, as it is found not to be on chromosome 6	Not done	-
	bA182N12/AR	GTGCAGGCCTGGAGAGAGAA						
bA182N12B	bA182N12/BF	ATGATCTTCTTGGAGGCAGAG	55	127	1.5mM	Excluded, as it is found not to be on chromosome 6	Not done	-
	bA182N12/BR	CAGAAGGAAGGAGTATTGATGC						
bA68M3/72	bA68M3/72F	CTCAGAGTTTCCATTCAGCTC	53.4	559	1.5mM	Negative	Negative	-
	bA68M3/72R	GGCTTAATGACTTCAGAATTTC						
bA68M3/72 Encore	bA68M3/72F2 EcoRI	TAGGAATTCGCAAATGCAAACG	66	320	1.5mM	Probe generated to Screen CEPH libraries		**860f10** **860e12** **760d2**
	bA68M3/72R2 EcoRI	ATGCCATTTTTGCCTATGTGG TAGGAATTCGGCTTAATGACTTC AGAATTTCATAGAATGC						
bA68M3/73	bA68M3/73F	AACTTGATTTCCAGGGAAAGGGAAT	63	380	1.5mM	Used to detect overlapping clones	-	-
	bA68M3/73R	CTGGGCAAAGAGTGACCCTTACT						
bA687L3	bA687L3/F	CATGGATGATGATGGGCACCTGTAA	66	290	1.5mM	17, 19	Negative	-
	bA687L3/R	TCACCATGTTAGCCAGGCTGATCAC						

Table 3.2. *Shows primers used and the results of the screening of ICI/Zeneca or CEPH libraries.*

Continuation of table 3.2.

bA687L3 (EcoRI)	bA687L3/F2 (EcoRI)	TAGGAATTCACTCAGGAGGCT GAGGCAGAAGAATTGCTT	66	340	1.5mM	Probe generated, but was of poor quality	-	-
	687L3/R2 (EcoRI)	TAGGAATTCCTGTAGCCTCGAACTCCCTGGCTTAAGC						
dJ219I3/SP6	dJ219I3/SP6/F	TTCCAAGACCCCCGAGTCAATGCCTAAAACC	68	380	1.5mM	**28**	8, 10, H	**28HH2**
	dJ219I3/SP6R	GACTGATCAGGTGTTTTGTGGATTCCCTT						
dJ219I3/T7	dJ219I3/T7F	GCAAAATGTCTTCATGACCATGAGGTAGAC	66	360	1.5mM	Used to detect overlapping clones	-	-
	dJ219I3/T7R	ACTGGGGAACTTAACAAGGTGTGTTTGTT						
bA809 (104)	bA809 (104) F	CATTGTGGGGAGAGTTTAGG	54	248	1.5mM	**20**, 22, 28, 33 also used to detect overlapping clones	5, 6, 9, 12, 16, A and C	**20AE8** **20AF1** **20CF4**
	bA809 (104) R	TAAGCCCATGGGCTAAGCCTA						
bA809 (160)	bA809 (160) F	AGGGGAGATTAAGTGCAAGT	56	310	1.5mM	Used to detect overlapping clones	-	-
	bA809 (160) R	TTGAAGGCTGTCAGCATGAG						

Table3.3. *Summarises the results of testing the different available clones to detect their overlapping relationship.*
Empty circles indicate negative PCR while the solid black circles indicate positive PCR signal for the combination of primers (left list) on individual clones (list at the top). W: indicates weak PCR signal.

Table 3.3

MarkersYACs	900f10	830d9	844h1	936e3	853b7	38ef1	12DH10c	20AE8	28HH2	939e3	860e12	860f10	760d2	522o2	b139N14	439I14	68M3	809N15	219I3	20AE8	687L3	20CFU	59I9	36CB11	3GA3	6EH12	916c5	856e1
Blimp																												
D6S1592																												
D6S447																												
830d9-R																												
D6S1296/J6																												
CHLC.GGAT14A05/J5					●				●	●	○	○	●				●	●	●	●		●		○				
CHLC.GGAT16C02/J4																												
68M3/73					●					●	○	○	○			●	●	●	○	○		●		○				
853b7-L					●				●	●	●	●	●				●	●	●	●		●						
68M3/72					○					○	●	●	○				●	●	●	●		○		○				
522O2/Cent end																												
522O2/Telom end																												
b139N14																												
139N14/Tel end																												
139N14/Cent end																												
b439I14																												
809N14/Cent end 104					○				○		○	○	○			○	○	●	○	●		●						
219I3/SP6 end					w				●	w			●			○	○	●	●	●		●						
219I3/T7 end					●					●	●	●	●			●	●	●	●									
809N14/Cent end 160					○				○		○	○	○			○	●	●	●									
687L3/cent end																												
59IAB											○	○												○	●	●	○	○
59I9A																								●	○	○	○	○
3GA3-R																												
6EH12-L																												
36CB11-L																												
CD24																												
6EH12-R																												
3GA3-L																				○		○						
D6S268																												
916c5-L																												

Figure 3.6. *Shows the final picture and the extension of the partial directional map from the previous work. Blue boxes represent the newly identified clones from this study, dJ: PACs from Pieter de Jong PAC library, * denotes YAC/PAC clone localised using FISH, green squares: probes available, black square: ends available, solid black circles: data from contig WC6.13 and solid black and blue circles: data confirmed by our group*

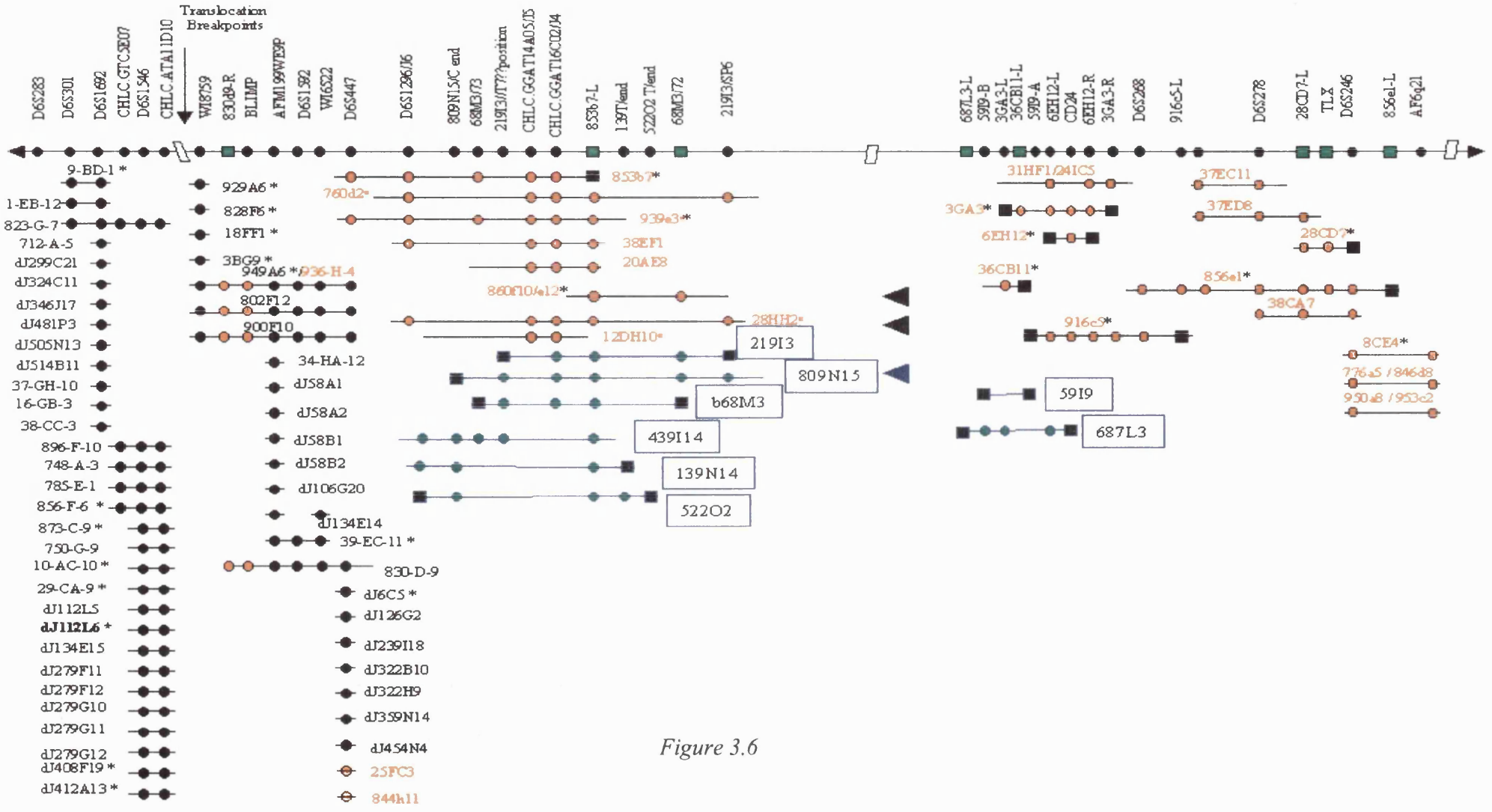

Translocation Breakpoints
D6S283
D6S301
D6S1692
CHLC.GTC3E07
D6S1546
CHLC.ATA11D10
WI8259
830d8-R
BLIMP
AFM199WB9P
D6S1592
WI6522
D6S447
D6S1296/J6
809N15/C end
68M3/73
219I3//T??position
CHLC.GGAT14A05/J5
CHLC.GGAT16C02/J4
853b7-L
139T/end
522O2 T/end
68M3/72
219I3/SP6
687L3-L
59I9-B
3GA3-L
36CB11-L
59I9-A
6EH12-L
CD24
6EH12-R
3GA3-R
D6S268
916c5-L
D6S278
28CD7-L
TLX
D6S246
856e1-L
AF6q21
9-BD-1 *
1-EB-12
823-G-7
712-A-5
dJ299C21
dJ324C11
dJ346J17
dJ481P3
dJ505N13
dJ514B11
37-GH-10
16-GB-3
38-CC-3
896-F-10
748-A-3
785-E-1
856-F-6 *
873-C-9 *
750-G-9
10-AC-10 *
29-CA-9 *
dJ112L5
dJ112L6 *
dJ134E15
dJ279F11
dJ279F12
dJ279G10
dJ279G11
dJ279G12
dJ408F19 *
dJ412A13 *
929A6 *
828F6 *
18FF1 *
3BG9 *
949A6 */936-H-4
802F12
900F10
34-HA-12
dJ58A1
dJ58A2
dJ58B1
dJ58B2
dJ106G20
dJ134E14
39-EC-11 *
830-D-9
dJ6C5 *
dJ126G2
dJ239I18
dJ322B10
dJ322H9
dJ359N14
dJ454N4
25FC3
844h11
760d2*
853b7*
939e3*
38EF1
20AE8
860f10/e12*
28HH2*
12DH10*
219I3
809N15
b68M3
439I14
139N14
522O2
31HF1/24IC5
3GA3*
6EH12*
36CB11*
916c9*
37EC11
37ED8
28CD7*
856e1*
38CA7
8CE4*
776a5 / 846d8
950a8 / 953c2
59I9
687L3

Figure 3.6

3.6. Recent mapping

By the end of September 2000, no additional information was available from any of the databases which could enhance our investigation and therefore the decision was taken to concentrate our effort into the definition of the area of minimal deletion by using LOH analysis. The results of this investigation will be presented in Chapter 4. However, we continued searching the databases available from the UK and the international web sites in order to monitor the availability of any clone which could have become available from the extensive genome sequencing project and that mapped in our region of interest.

Through our collaboration with the Sanger Centre (as beginning of 2002) analysis of chromosome 6-sequence project (http://www.sanger.ac.uk/) showed that a gap still existed between marker bA809N15 and marker bA59I9, as showed above. More recently, however, investigators at the Centre decided then to return to YAC library screening. This is normally avoided, whenever possible, because of the very high incidence of chimerism detected among YAC clones. Their investigation was, however, successful and two YAC clones were identified. These are yR39EC11 on the centromeric end of the gap and yX28F10 on the telomeric end of the gap. With the help of these clones one contig was placed within the gap, as illustrated in Figures 3.7 and 3.8.

Figure 3.7 which is derived from the Sanger Centre most recent update (http://www.sanger.ac.uk/cgi-bin/humace/SCMAPS.cgi?chr=6) shows that there are to date 3 intervals (numbered 54, 55 and 56) on chromosome 6 contig 2 (chr6_ ctg2), details of the sequenced clones in these three intervals can be accessed at:

http://www.sanger.ac.uk/cgi-bin/humace/SCMAPS.cgi?chr=6&interval=interval54,
http://www.sanger.ac.uk/cgi-bin/humace/SCMAPS.cgi?chr=6&interval=interval55,
http://www.sanger.ac.uk/cgibin/humace/SCMAPS.cgi?chr=6&interval=interval56,

respectively.

These are joined through the YACs mentioned above and shown in Figure 3.7 and Figure 3.8 (A. Mungall's personal communication).

However, the two YAC clones are at present only very partially sequenced. This in turn, limits the estimate of the full size of the region, the gap previously identified and candidate genes within the area, which still remain to be defined.

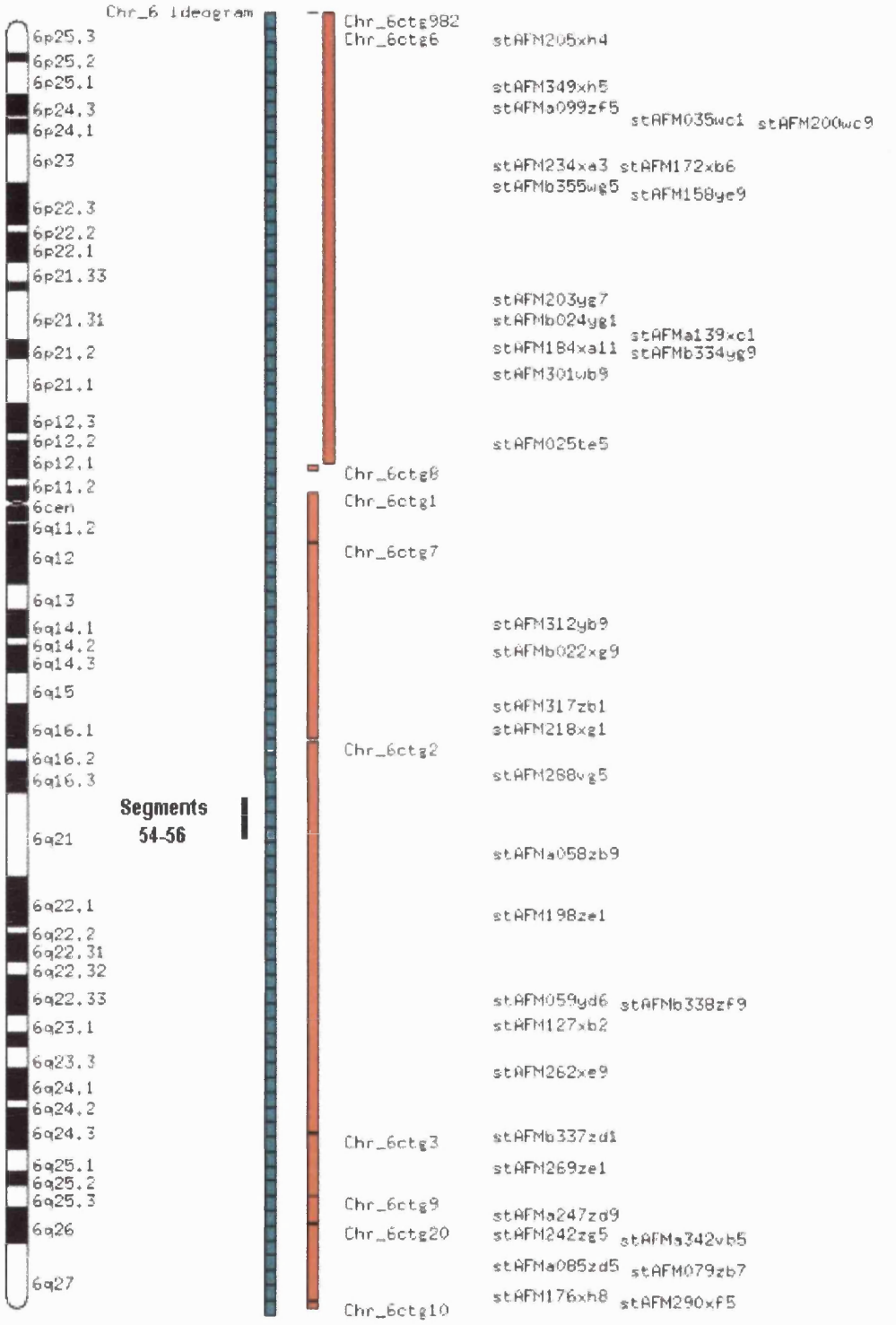


Figure 3.7. *Shows the most recent update derived from the Sanger Centre data, Black bar: segments number (54, 55, 56) which are in our region of interest. Chr_6ctg 2 summarises the different contigs on chromosome 6 according to the Sanger Centre data.*

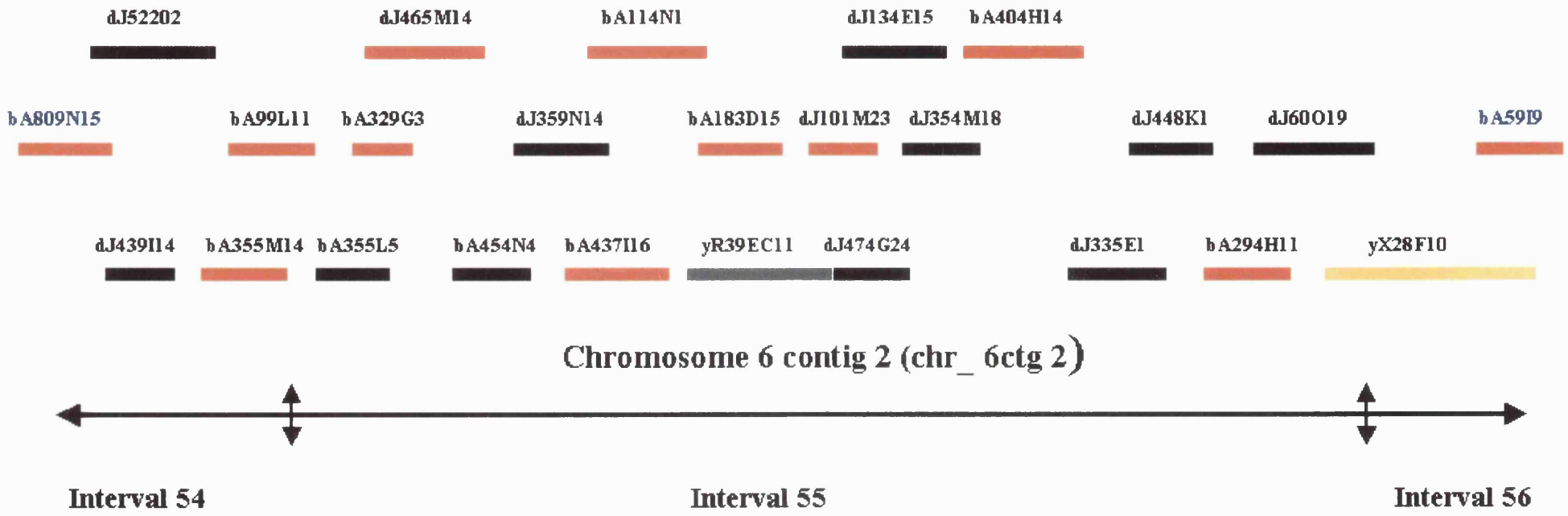


Figure 3.8. *Shows the different clones that have been completely sequence by the Sanger Centre. YACs; yR39EC11 and yX28F10 are the joining clones that link other fully sequenced clones in our region of interest on segments 54, 55, 56 located on Chr_6ctg2.*

3.7. Discussion

This chapter summarises the work carried out in order to further characterise a region of minimal deletion previously identified on chromosome 6 band q16-21. FISH analysis carried out in our laboratory by Dr. Carrara on a series of 25 ALL patients (Jackson A. *et al.*, 2000) and work published by a close collaborator (Dr CJ Harrison; Sherrat et al, 1997)(Sherratt T. *et al.*, 1997) had indicated that this was a region of common deletion in acute leukaemias. For this reason most of our effort had been concentrated within this region. At the time the investigation started, the region was poorly mapped and the extent of the area was extremely poor and estimated to be in the region of 4-4.5 Mb between D6S447 and D6S268.

Extensive screening using vectorette system (on 4 YACs), CEPH, ICI library screening and information using clones from the Sanger Centre (7 clones) had failed to yield any new clone. However, as recently as February 2002, new information had become available through our collaboration with Dr. Mungall at the Sanger Centre. This part of the study revealed that 2 YAC clones (yR39EC11 on the centromeric end of the gap and yX28F10 on the telomeric end of the gap) had been identified. To date we have still limited information on these YAC clones.

When the full sequence will become available, it will be of great interest to reveal molecular features of this region to explain the difficulty encountered by both us and the Sanger Centre in identifying clones from this region. It is likely that either extensive repeats or secondary structure of this region may explain the difficult in cloning. This has been previously encountered in other human regions, which have resulted in some gaps.

The publications of the complete DNA sequence of chromosome 22 (Dunham I. *et al.*, 1999), chromosome 21 (Hattori M. *et al.*, 2000) and chromosome 20 (Deloukas P. *et al.*, 2001), have revealed the presence of unclonable gaps in these sequences.

About 3% of the complete DNA sequence of the long arm of chromosome 22 (22q), the first chromosome to be completely sequenced, proved to be unclonable and it was concluded that the gaps contain sequences the are unclonable to host vector used in this study (Dunham I. *et al.*, 1999). It was shown that the completed sequence covers 33.4 MB of a total region spanning 34.491 kilobases on 22q leaving 11 gaps uncloned (Dunham I. *et al.*, 1999).

The complete sequence of human chromosome 21 has left three small clone gaps and seven sequence gaps comprising about 100 kilobases on 21q (Hattori M. *et al.*, 2000).
The complete DNA sequence of chromosome 20, the third chromosome to be completely sequenced revealed that four physical gaps spanning about 320 kilobases remained in the clone map of the 20q. It was suggested that the sequences in these gaps are unclonable to host vector systems used in this study, probably due to the high Guanine and Cytosine (G+C) content of the sequence in this region (Deloukas P. *et al.*, 2001).

Chapter 4

The use of Loss of heterozygosity (LOH) to investigate deletion on 6q21

4.1. Introduction

Loss of genetic material from one or both chromosomes results in changes in the organisation of the genomic structure of a particular gene. If this loss results in the suppression of normal cell differentiation and development of the malignant state, the gene is referred to as being a TSG. In these circumstances when one allele of the gene is grossly abnormal or absent, suppression of the malignant state no longer occurs and the cell transforms into a cancer cell. The gene is said to have a tumour suppressor activity. Cancer formation may be associated with loss of function of both alleles or of only one allele.

Such events can be assayed by LOH (here as a technique), which involves a comparison of polymorphic loci adjacent to the gene of interest or located within the region of interest. The polymorphisms analysed using LOH are based on individual patterns of repeats (mono-di, tri or tetra-nucleotides) and they almost represent a "fingerprint" of each individual patient. Consequently, the DNA from tumour and non-tumour in the same individual are required for analysis. LOH analysis has therefore become almost synonymous of tumour suppressor gene analysis and several areas investigated for LOH might be expected to contain tumour suppressor genes (Ponder B. A., 2001) in many cancers (Osborne R. J. and Hamshere M. G., 2000).

The main aim of the second part of this project was to investigate deletion of 6q21 using LOH. This approach had a dual aim:

1. To allow the analysis of a larger cohort of patients ALL cases than possible by FISH because of lack of material available for FISH analysis.
2. To expand the analysis of deletion to the more proximal region (6q16-21) which

had also emerged as a common region of deletion for investigation in our laboratory (Jackson A. *et al.*, 2000).

We used highly informative polymorphic microsatellite markers which are available centromeric and telomeric to 6q21 on paired genomic DNA samples from patients with ALL.

The markers used on this study were selected depending on the data available from:

1. FISH analysis performed in our laboratory.
2. LOH studies published by other groups and showing 6q21 as a frequently deleted region in ALL.

The panel of the microsatellite markers used in this study were as follows (in a centromeric to telomeric orientation): BACH2, D6S1709, D6S283, D6SS301, D6S1592, D6S268, D6S278, D6S246, D6S302 and D6S304 (Table 4.1 and Figure 4.1) (Genome Database at http:// www.gdb.org).

In addition a set of other markers (D6S443, D6S1664, D6S447) were also tested but subsequently excluded from analysis because found to fall short of the quality of amplification required for this analysis (background, false amplification or poorly informative).

Paired DNA samples (tumour and non-tumour from samples collected at time of remission from the same individual) from ALL patients were analysed for LOH on 6q21 by PCR amplification of the above mentioned microsatellite sequences. Patients were selected for availability of material and no other clinical or biological factors. Patients' material had been collected within the Department of Haematology as part of the investigation on minimal residual disease in adult or childhood ALL. Ethical approval to the use of material had been provided by the patient when undertaking the treatment for acute lymphoblastic leukaemia using UKALLXII trial treatment.

All non-tumour samples were obtained from patients assessed on basis of morphological and clinical criteria

For the investigation described in this chapter, the markers used, their sequences and the PCR conditions applied are shown in Table 4.1.

Marker	Primers sequence	Ann. Temp.°C.	Time of Electroph. run	PCR conditions		
BACH2	F: CTGCAGGGAATAGGTAACTGT R: AAAGTGCAGGACCCCTCTGA	58	3h 30min			
D6S1709	F: GCTACAGAATGCCATAGAATTAC R: ACTTGAAAAATTATTAGCCAATG	55	3 h	94°C	5 min	1 cycle
D6S283	F: TTTACAAAATCCTGTCCTGC R: TTGATAGTTGTCTTGATACAACAGA	54	5 h			
D6S301	F: CACAACCATATGTNCCAATT R: ATTAAATGTGCATACGCAAA	55	4h 30 min	94°C	1 min	
D6S1592	F GCTACTCAGAAGGCCG R: GCAAATGATTTTGTTTAATCC	52	4h 30 min	Ann. temp	1 min	30 cycles
D6S268	F: CTAGGTGGCAGAGCAACATA R: AAAAGGAGGTCATTTTAATCG	55	2 h	72°C	2 min	
D6S278	F: GCCTGGTGCATGATCTTTTT R: CAGACAGAGTAGGGCATTCC	60	2h 30 min			
D6S246	F: GTCGCTTGAGTCTGGGAGGTCAAT R: GGGTTTGAAAGAAGACTGCAGCCT	64	4h 30 min	72°C	5 min	1 cycle
D6S302	F: TTCACAATGACAAGTCGAGG R: TTCTTTAGGATAAGCCAATACACG	58	4 h			
D6S304	F: TTCCACTCTGCTCCAGACAG R: TTGAAAGTTTGAGAAGCACTTGTTA	58	4h 30 min			

Table 4.1. *6q21 microsatellite markers used for LOH study. F; forward primer, R; backward primer, Ann.temp; annealing temperature, min; minute, h; hour.*

4.2. Microsatellite instability (MSI) assessment

During this investigation a number of patients showed a pattern that did not comply with LOH but was compatible with a MSI pattern. These patients will be analysed separately in the result section of this chapter as they have been investigated using a specific panel of markers, according to the NCI parameters.

The panel of markers used for MSI investigation included two mononucleotides repeats (BAT-25 and BAT-26) and three dinucleotide repeats (D5S346, D2S123, D17S250)(Table 4.2). Markers BAT-25 and BAT-26 are characterised by the presence of quasi-monomorphic profile in the population studied using these markers so that the availability of a matching normal DNA is not an absolute requirement (Hoang J. M. *et*

al., 1997; Zhou X. P. *et al.*, 1997). BAT-26 is located within intron 5 of the *hMSH2* (Liu B. *et al.*, 1994), and BAT-25 locus is in the intron 16 of the *c-KIT* gene (Parsons R. *et al.*, 1995). Using this panel of primer combinations tumours were classified as tumour with high frequency of instability (MSI-H) if 2 or more markers showed instability and with low frequency instability (MSI-L) if instability was detected in one marker only, as mentioned in Chapter 1 (paragraph 1.10.2).

Methodologically, the same approach used for the assessment of LOH (described in Chapter 2, paragraph 2.12) was applied to assess MSI. All the 5 markers were used on both normal (remission) and tumour (presentation/relapse) DNA samples of the patients who showed MSI using the markers listed in Table 4.1. BAT-25 and BAT-26 were also used to screen the first 60 tumour samples without comparison to non-leukaemic/remission material, but not on the remaining nine cases due to limited tumour DNA material from these patients. Table 4.2 summarises the sequence of the primers used, PCR conditions and their chromosomal locations.

Marker	**Primers sequence**	**Ann. temp.°C**	**Chromosomal localisation**
BAT-25	F: TCGCCTCCAAGAATGTAAGT	58	4q12-4q12
	R: TCTGCATTTTAACTATGGCTC		
BAT-26	F: TGACTACTTTTGACTTCAGCC	56	2p16-2p16
	R: AACCATTCAACATTTTTAACCC		
D5S346	F: ACTCACTCTAGTGATAAATCGGG	53	5q21-5q 22
	R: AGCAGATAAGACAGTATTACTAGTT		
D2S123	F: AAACAGGATGCCTGCCTTTA	60	2p16-2p 16
	R: GGATTTCCACCACCTATGGGAC		
D17S250	F: GGAAGAATCAAATAGACAAT	52	17q11.2-17q12
	R: GCTGGCCATATATATATTTAAAC		

Table 4.2. *Markers used to assess for microsatellite instability (MSI). Abbreviations are as follows: Ann. temp; annealing temperature, F; forward primer, R; reverse primer.*

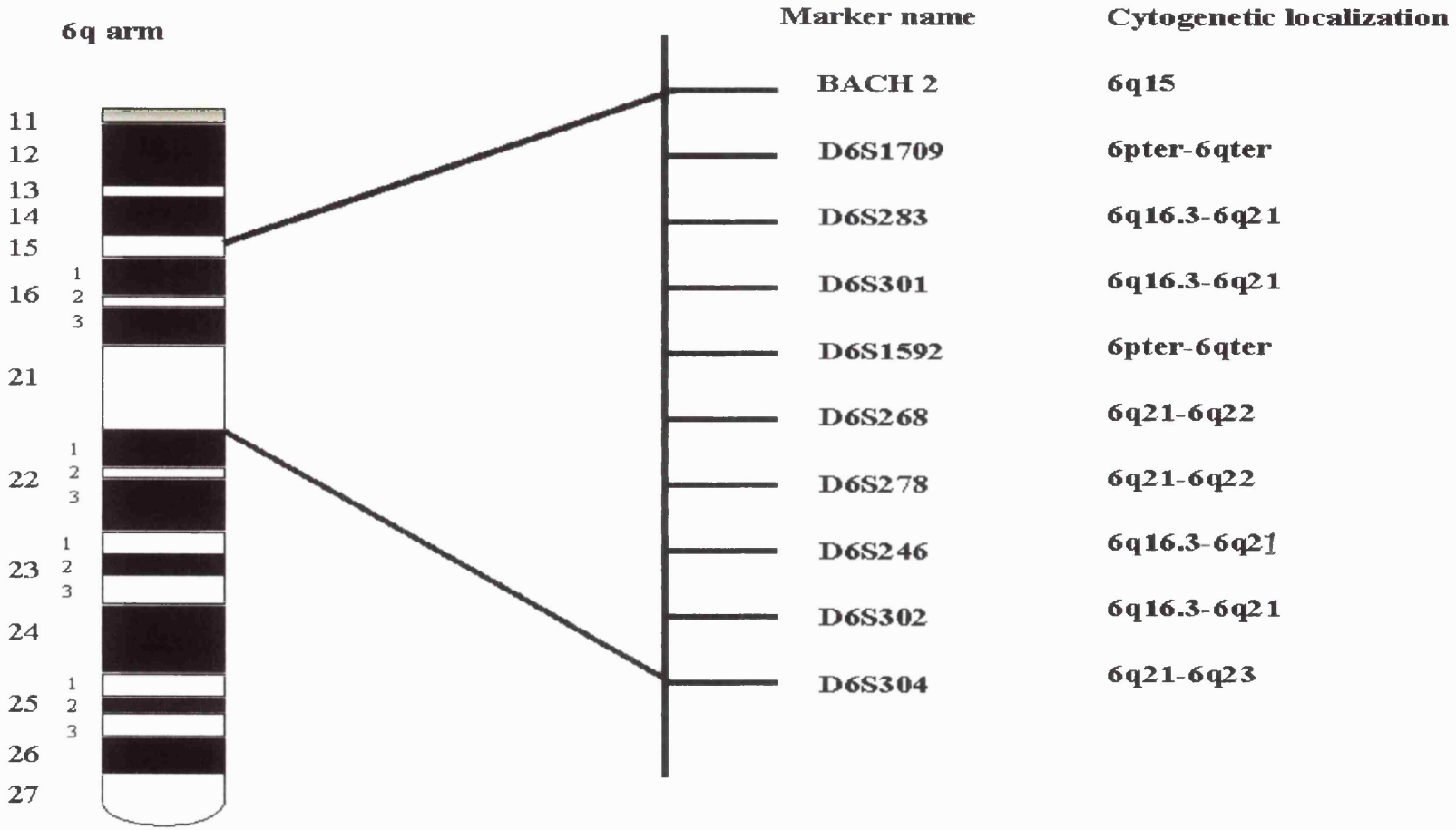


Figure 4.1. *Graphic distribution of microsatellite markers used for loss of heterozygosity (LOH) of 6q21.*

4.3. Patients and Methodology

4.3.1.Patients

Patients enrolled in this study had *de novo* ALL diagnosed at the Royal Free Hospital or few other UK centres. Patients were treated according to the UKALLX, XI (for childhood ALL cases) or XII MRC protocols (adult ALL cases).

Patients' age ranged between 2 and 48 years (median: 15 years). Thirty-five were children (median age 6; range: 2-14 yrs of age) and thirty-four were adults (median age: 24 yrs; range: 16-48 yrs of age). Twenty-two were females (11 children and 11 adults) and the remaining were males (24 children; 23 adults) (Table 4.3, Figure 4.2). Median WBC count at diagnosis was $23.8x10^9$/L (range: 1.7-565 $x10^9$/L), comparable between adults (median $23.8x10^9$/L) and children ($28.7x10^9$/L).

Patients were grouped immunophenotypically according to their FAB classification (Bennett J. M. *et al.*, 1976). The majority of cases analysed were B-cell lineage ALL (25 cALL and 18 pre B) and T- lineage ALL (16 patients). The remaining 10 patients included 1 CML in B cell BC and 1 L3 ALL, 2 null ALL, 1 pre/cALL, 1 B cell of unknown phenotype and 4 mixed (1) or biphenotypic (3).

Four patients first presented in relapse while the rest were all de novo ALL patients.

Eighteen children and 16 adults remained in complete clinical remission (CCR) (including one patient who died in CCR) (median period: 43.4 months for the children and 71 months for the adults). Eighteen adults and 17 children relapsed between 3 and 48 months (median: 42.8 months; children: 29 months; adults: 12 months median). The interval between presentation and time of relapse was statistically different ($p=0.033$) between adults and children.

Table 4.3

Serial No	Sex	Adult or Child	Age (yrs)	Disease	WBC ($x10^9/l$)	Presentation	Cytogenetic abnormality		Event	Event (time)
1	M	C	4	CALL	10.2	De novo	Hyperdiploidy		Relapse	41.3
2	M	C	3	Pre-B	40.9	De novo	Normal		CCR	23.6
3	F	A	22	Mixed	91.7	De novo	Failed		Relapse	29
4	F	C	13	CALL	Low	De novo	Hyperdiploidy	t(12;21)	CCR	20.6
5	F	A	16	Pre-B	5	De novo			Relapse	12
6	M	A	19	CALL	39.3	De novo			CCR	34
7	F	C	11	ALL Biphen	110	De novo	46,XX		CCR	38.8
8	M	C	12	Pre B	154	De novo	del 6q	t(12;21)	CCR	23.8
9	F	C	5	CALL	52	De novo	t(X;6)(?;q15)	t(12;21)	CCR	42.1
10	F	C	8	CALL	10.8	De novo			CCR	6
11	M	A	16	T-ALL	104	De novo	6q (q15-23)		Relapse	9
12	F	A	25	null ALL	24.6	De novo	Other		CCR	52
13	M	C	3	CALL	250	De novo			CCR	52.6
14	M	A	17	Pre-B	3	De novo	Other		Relapse	17
15	M	C	2.5	Pre-B*	Low	De novo	Other		CCR	49.2
16	F	A	39	Pre-B	Na	De novo	Other		CCR	32.5
17	M	A	18	CALL	97.4	De novo	Other		Relapse	13.5
18	M	C	9	T-ALL	Na	De novo			Relapse	6

Continuation of Table 4.3

19	M	C	6	Pre- B	13.1	De novo	del 6q		Relapse	35.3
20	M	C	14	T-ALL	Na	De novo			Relapse	17.5
21	F	A	17	T-ALL	239	De novo	Other		CCR	8
22	M	A	16	T-ALL	101	De novo	abn 9p		Relapse	9
23	M	A	19	T-ALL	37	De novo			CCR	82
24	M	A	48	Pre-B	7.1	De novo			Relapse	17
25	M	C	7	CALL	2.8	De novo			Relapse	11.1
26	M	C	6	Pre- B	53	De novo	t(9;22)		CCR	42.8
27	M	C	4	CALL	40	De novo			CCR	91.8
28	M	A	26	CALL	10.2	De novo	Other		CCR	80
29	M	A	24	null ALL	565	De novo	t(4;11)		CCR	75
30	M	C	3	CALL	7.2	Relapse	47XY, 21+		Relapse	39.4
31	M	A	20	CALL	Na	Relapse			Relapse	48
32	M	A	21	CALL	38	De novo	Normal		CCR	84
33	F	A	24	Pre-B	15.6	De novo	del 6q	t(1;19)	CCR	13
34	F	C	10	CALL	38	De novo	t(12;21) PCR	45,XX	Relapse	14.5
35	F	A	31	Pre-B	10.6	De novo			CCR	73
36	M	C	2.5	CALL	19	De novo	Other		CCR	47.7
37	M	A	39	Pre-B	Na	De novo			CCR	17.6
38	M	C	2	Pre-B	47.3	De novo	Complex		CCR	110
39	F	A	28	CALL	45	De novo	del 6q		CCR	82

Continuation of Table 4.3

40	M	A	45	T-ALL	Na	De novo			Relapse	7.5
41	M	C		B- ALL (L3)	19.5	De novo			CCR	13.5
42	M	C	4	Pre-B/cALL	170	De novo			Relapse	36.2
43	F	A	16	Pre-B	23	De novo			CCR	89
44	M	A	25	Pre-B	8.9	De novo	t(12;21)		Relapse	22
45	F	C	3	CALL	4.1	De novo	del 12		CCR	51.4
46	M	A	16	CALL	4.3	De novo	Other		CCR	73
47	M	C	11	null biphen	7.3	De novo	del 6q	t(12;21)	Relapse	39.5
48	M	A	25	CALL	1.7	De novo	Normal		CCR	69
49	M	C	3	CALL	3	De novo	del 6q	t(12;21)	Relapse	31.05
50	F	C	13	CALL	39	De novo			Relapse	36
51	F	C	9	CALL	48	De novo			CCR	44
52	M	A	17	CALL	Na	De novo	Other		Relapse	11
53	F	C	5	Pre-B	4.2	De novo	Add 9; t(8;9)		CCR	67.4
54	M	C	12	CALL	3.8	Relapse			Relapse	48
55	M	C	12	CML/Bc	90	De novo			Relapse	10.5
56	F	C	6	Pre-B	19.3	De novo	del 9(p12)	47, X	Relapse	16.7
57	F	A	24	CALL	Na	De novo			Relapse	42
58	M	C	13	B-ALL	na	De novo			Relapse	24
59	F	C	4	Pre-B	16	De novo			Relapse	29
60	M	A	27	T-ALL	97	De novo	Other		Relapse	10

Continuation of Table 4.3

61	M	A	21	T-ALL	33	De novo			Relapse	3
62	M	A	44	T-ALL	4	De novo			Relapse	7
63	M	A	30	T-ALL	8	De novo			Relapse	24
64	M	A	36	T-ALL	100	De novo			Died in CR	11
65	M	C	2	T-ALL	12.7	De novo			Relapse	9.1
66	M	C	10	T-ALL	84	De novo			CCR	10
67	M	A	31	T-biphen	5	De novo			Relapse	11.9
68	F	A	17	T-ALL	7	Relapse			Relapse	5
69	M	C	7	T-ALL	98	De novo			CCR	98

Table 4.3. *Abbreviations are as follows: M: male; F: female; A: adult ALL; C: childhood ALL; Na: non analysed; De novo: patients presented as de novo ALL; only cytogenetic abnormalities referring to the presence of known and common abnormalities are given; i.e. t(9;22); del6 or del9; t(12;21) and t(4;11) are highlighted; CCR: continuous clinical remission; time to event is expressed in months.*

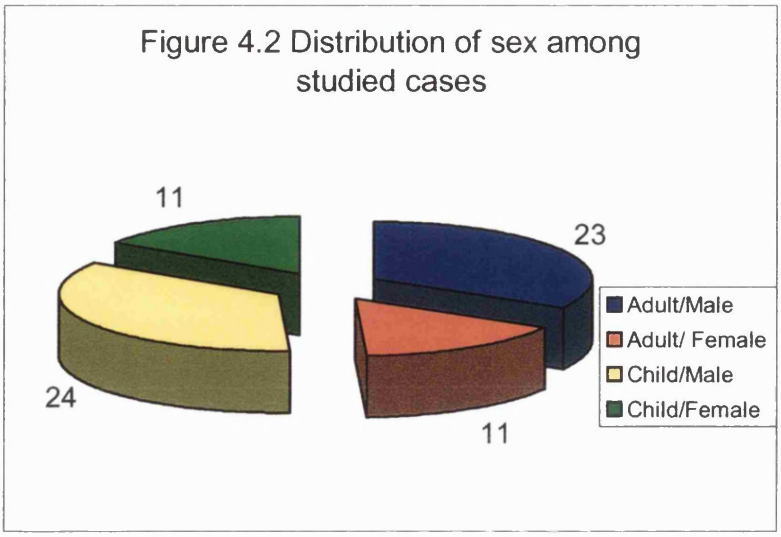


Figure 4.2. *Shows distribution of sex among studied cases, of the 35 children investigated, 11 were females and 24 were males, of the 34 adults, 23 were males and 11 were females.*

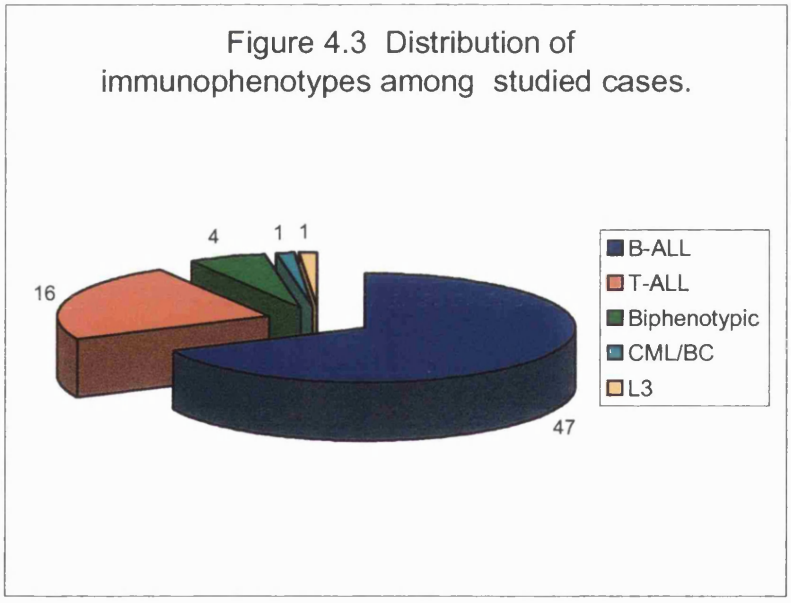


Figure 4.3. *Shows the distribution of immunophenotypes among the studied 69 cases; 47 were of B-lineage ALL, 16 were of T-lineage All, 4 were biphenotypic, 1 was chronic myeloid leukaemia in blast crises (CML/BC) and 1 was of L3 type.*

4.3.2. DNA samples

Mononuclear cells (MNC) were obtained from fresh bone marrow samples by Ficoll-

Hypaque (Nycomed Pharma AS, Oslo, Norway) centrifugation and either stored at -20°C or –70°C for subsequent extraction or were processed immediately. DNA was extracted from the mononuclear cell suspensions using a DNA extraction kit (Gentra-Flowgen, UK) according to the manufacturer's instructions. This part of the work had been carried out by colleagues in the laboratory prior to the commencement of my investigation and DNA was already available for use.

DNA from archival material (stained or unstained slides) was extracted using Dexpat™ suspension (Takara Shuzo Co., Ltd, Japan), as previously described (Mortuza F. Y. *et al.*, 2001). All DNA samples were amplified for the constitutional β-actin gene to ensure DNA integrity (Porfiri E. *et al.*, 1993).

Paired DNA samples from presentation/relapse and remission were analysed for LOH on 6q16-21 by using oligonucleotide primers for the selected highly polymorphic dinucleotide repeat microsatellite markers listed in Table 4.1.

4.3.3.PCR conditions

PCR was performed with the incorporation of deoxycytidine triphosphate [α-^{32}P] dCTP. The final volume of the reaction mixture was 30μl, which included 250ng of each oligonucleotide primer, 0.2mM of dNTPs, 0.2μl of [α-^{32}P] dCTP (370MBq/ml, 10mCi/ml) (Amersham), 50-100ng of patient's genomic DNA, 1x PCR buffer (Promega) containing 1.5 mM $MgCl_2$, 0.3-0.5μl of Taq polymerase (Promega) was added to the reaction and the final volume was adjusted to 30μl using sterile double distilled water. The choice of using ^{32}P-dCTP instead of ^{33}P-dCTP was based almost exclusively on financial ground.

Thirty cycles of PCR were performed in a DNA thermal cycler (Techne). Each cycle consisted of denaturing at 94°C for 1 minute, annealing at each primer specific annealing temperature (according to the melting temperature for each oligonucleotide primer pair). A hot start at 94°C for 5 minutes and a final extension cycle at 72°C for 5 minutes were performed. A negative control reaction containing no DNA was performed for each PCR reaction (see also Chapter 2, paragraph 2.12.1).

4.3.4.Polyacrylamide gel electrophoresis

PCR products were diluted with a formamide stop solution in a ratio of 2:1. The diluted PCR products were denatured for 20 minutes at 80°C. 5µl of each of the denatured PCR products were then loaded on 6% denaturing polyacrylamide gel containing 8 M urea. Electrophoresis was performed at 60 Volts in a manual sequencing gel apparatus using 1x TBE buffer. The time of the gel run ranged from 2-5 hours depending on the expected size of the PCR product (see Table 4.1). The gel was then dried onto a 3MM Whattman paper in a vacuum gel dryer, and then exposed to an X-ray film at –70°C for 24-72 hours depending on the intensity of the PCR signals. See also Material and Methods paragraph 2.12.2.

4.3.5.Interpretation of data: LOH analysis

Samples were independently evaluated for LOH at each of the 10 highly polymorphic microsatellite markers used in this study. For each particular locus of the tested microsatellite markers, LOH was assessed in the matching tumour DNA sample if it was informative in the non-tumour DNA sample. Genetically informative samples are those showing a pattern compatible with two distinguishable alleles. Signal intensities on the radiographs were analysed visually by myself and two other colleagues with no previous knowledge of the patient's clinical data. When signal intensities of alleles of tumour DNAs were compared to those of the corresponding normal DNAs, a reduction in the signal intensity of more than 50% was scored as LOH. Attempts made to use densitometry measurements were made for nearly all markers but showed to give inconsistent and misleading results.

4.3.6.Interpretation of data: MSI analysis

During the analysis a pattern compatible with MSI has been detected in a number of patients. Such pattern was assigned to an individual patient when novel alleles in the tumour DNA were detected when compared with the matching non-tumour DNA sample upon testing with each individual primer combination.
MSI in ≥30-40% of the markers tested (≥4 markers out of 10 markers used for 6q21 investigation) was scored as MSI of the high frequency (MSI-H), whereas MSI in <30-

40 % of the markers used (<4 markers out of 10 markers used) was considered as an indicator of low frequency of instability (MSI-L). This scoring system was also applied to the NCI panel of microsatellite markers that used to further assess the MSI. MSI-H tumours were defined if ≥2 markers of 5 loci analysed showed instability, whereas MSI-L tumours were defined as having instability in one marker of the 5 loci analysed. Following the NCI guidelines evaluation of MSI using the reference panel markers, tumours showing no apparent instability may be defined as having MSI/L or as microsatellite stable (MSS) tumours. Samples that showed apparent LOH in addition to MSI were scored as non-informative for LOH. In general a sample was scored as either showing LOH or MSI for a specific marker, but not both, in accordance with the recommendations by the NCI workshop (Boland C. R. *et al.*, 1998).
Finally, all samples that showed LOH or MSI were subjected to repeated PCR amplification and analysis for confirmation.

4.3.7.Statistical analysis

Standard statistical tests were carried out (Fisher's exact test, χ^2 contingency tests, and parametric and non-parametric t-tests) using statistics programs GraphPad Prism, and SPSS. Disease-free survival (DFS) curves were generated using the Kaplan-Meier method (Peto R. *et al.*, 1977), and compared with the log-rank or, in the case of more than two logically ordered survival curves, log-rank test for linear trend.

4.4. Results

4.4.1. Incidence of LOH in ALL

Wild type (no LOH or MSI) was detected in 44 (64%) of 69 ALL patients analysed. In 16 (23%) patients LOH at one or more loci was demonstrated (8 adults and 8 children) (Figure 4.4). In details, 9 (13%) patients showed LOH at 1 site, 3 (4.3%) cases at 2 sites, 1 (1.4%) patient at 3 sites and 3 (4.3%) cases at 4 sites (Table 4.6). Contiguous deletion was detected in only 3 of these patients (number 5, 9 and 25), while overall the most common pattern was that of interstitial deletion (Table 4.6 and Figure 4.9).

Among adults with *de novo* ALL, 21 (65.6%) of 32 patients showed a wild-type pattern while this pattern was detected among 20 (60.5%) of childhood *de novo* ALL patients. Eight *de novo* adults (25%) and eight children (24.2%) showed a LOH pattern. There was no difference in the incidence of LOH or wild type in the two age groups (see Table 4.4).

Age group	De novo*	WT	LOH	MSI
Adult ALL (no=34)		23 (67.5%)	8 (23.5%)	3 (MSI-L)(9%)
De novo*	32	21 (65.6%)	8 (25%)	3 (9%)
Relapse*	2	2 (100%)	0	0
Childhood ALL (no=35)		21 (60%)	8 (23%)	6 (17%)
De novo*	33	20 (60.5%)	8 (24.2%)	5 (15%)
Relapse*	2	1 (50%)	0	1 (50%)

Table 4.4. *Wild type; LOH and MSI incidence according to age. *: According to presentation status; Percentages are calculated for each individual subgroup.*

Patients with a LOH did not differ from the group showing wild type pattern for age and total WBC. All tests revealed no significant differences between the two groups. However, it may be worth only noticing that the median WBC count both in children and adults was higher (71 and $57x10^9$/L) than in the wild-type groups (19 and $20.5x10^9$/L) but this difference did not reach statistical significance.

4.4.2. Incidence of MSI in ALL

In total 9 patients (6 children and 3 adults) showed a MSI pattern. One child had presented in relapse but all other cases were de novo ALL (Table 4.4).

MSI was detected in 7 (10%) patients as the sole abnormality, and in 2 (3%) patients MSI was accompanied by LOH (Figure 4.4; Table 4.5; Table 4.6).

In 3 of these 7 patients (number 26, 34 and 47) one marker in each patient showed a LOH pattern that, however, was excluded from the overall calculation of LOH incidence because of the high level of MSI (MSI/H) in these three patients (according to the NCI recommendations).

MSI-H and MSI-L were equally represented among children. However, MSI-L was the only pattern observed in adults (Table 4.4). In 3 (4.3%) of the 7 patients (number 26, 34

and 47) high MSI level (MSI/H) was detected while the remaining 4 (5.7%) patients (number 30, 38, 39, and 40) showed a low MSI level (MSI/L) (see Table 4.5).

In the 2 (3%) cases (number 62 and 65) that showed LOH/MSI pattern, MSI detected was of the low level. LOH was detected in only 1 locus and these patients have been excluded from the calculation of LOH as I classified them, while they were included in a separate group referred to as LOH/MSI. These results are summarised in Table 4.5.

MSI occurred twice more frequently in children (6 of 35 cases, 17%) than in adults (3 of 34, 8.8%). Among children with *de novo* ALL, however, when patients showing MSI, LOH or WT were compared, there was not difference in age (6 years median in MSI positive compared to wild-type ALL) or WBC count ($38x10^9$/L median WBC count in MSI positive children compared to $19.4x10^9$/L in wild type and to $71x10^9$/L in children with LOH) among the groups.
Among the adults, however, the adults with MSI were older (median: 44 years of age) than patients with LOH (20 years) and wild type (22 years), although these differences were not significant. The median WBC count was $24.5x10^9$/L in patients with MSI compared to $20.5x10^9$/L in the WT group and $56.3x10^9$/L in adults with LOH.

Subgroups Total no=69	**Category**	**No of samples**	**Percentage**
Group I	Wild type	44	64%
Group II	LOH 1 marker/>1 marker	16 9/7	23% 13%/10%
Group III	MSI MSI-H/MSI-L	7 3/4	10% 4.3%/5.7%
Group VI	LOH/MSI	2	3%

Table 4.5. LOH and MSI analysis of 69 cases of ALL investigated in this study. *Abbreviations are as follows. LOH: loss of heterozygosity, MSI: microsatellite instability, MSI-H: microsatellite instability of high frequency and MSI-L: microsatellite instability of low frequency.*

In total 25 (36%) out of the 69 investigated patients showed a pattern of instability on

chromosome 6q (either in the form of LOH or MSI). This indicates a high incidence of chromosome 6 instability which will be discussed further in the final chapter of this thesis.

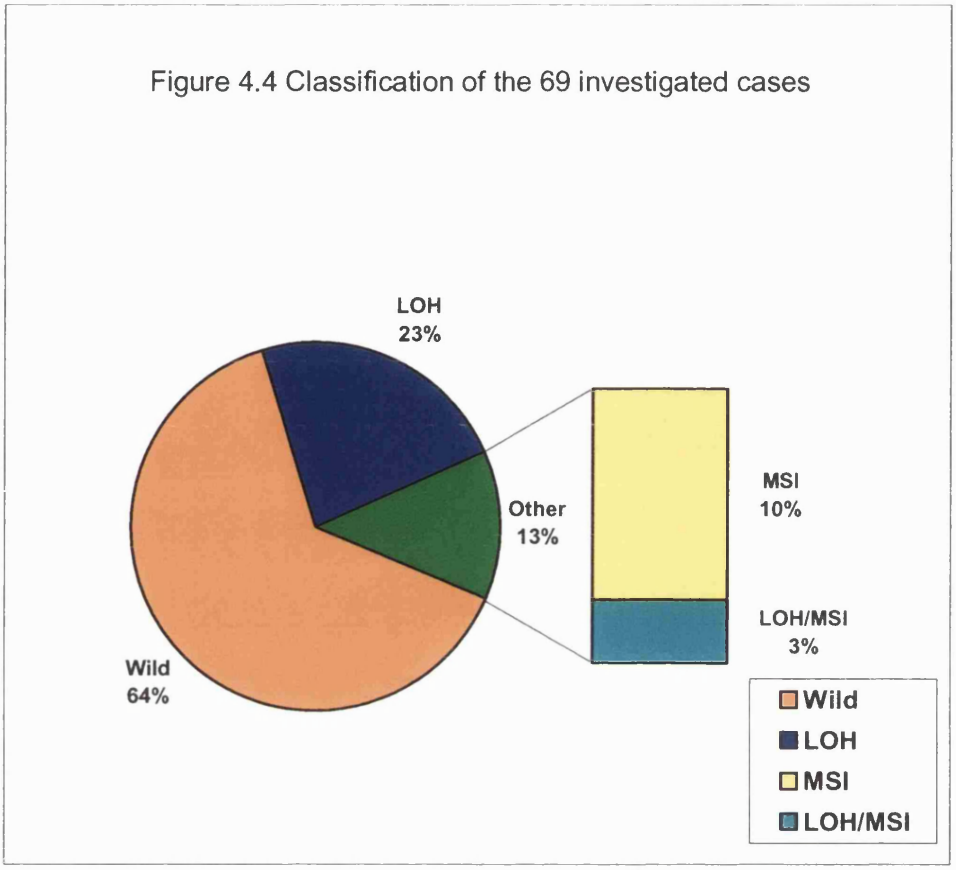


Figure 4.4. *Shows the incidence of Loss of heterozygosity (LOH) and microsatellite instability (MSI) among the analysed patients.*
Wild type (no LOH or MSI) was detected in 44 (64%) of 69 ALL patients analysed (LOH) was detected in 16 (23%) and 13% of the cases showed (MSI).

The incidence of LOH and MSI was analysed in the B and T cell ALL patients, irrespective of the status at presentation. In the B lineage ALL (50 patients), twice as many patients showed LOH (12 patients; 24%) than MSI (6 patients; 12%; 3 MSI-H and 3 MSI-L). Among T-ALL patients, the incidence of LOH was comparable to the incidence observed in B cell ALL. Four patients (23%) showed LOH while 3 (17%) showed MSI-L. In the two immunophenotypic groups, 32 patients (64%) and 10 patients (59%) showed wild-type pattern. The biphenotypic and mixed phenotype patients showed only a WT phenotype. The microsatellite pattern did not show any

statistically significant association with immunophenotype (T-TEST).

Finally, 7 patients showed 6q deletion by cytogenetics (numbers: 8, 11, 19, 33, 39, 47 and 49) and one patient (number 9) carried a translocation involving chromosome 6 (X;6)(?;q15). LOH and MSI were detected in 5 of 7 patients with cytogenetically detectable chromosome 6 abnormality and a LOH pattern was detected in the patient with a translocation. Two patients with cytogenetically detectable deletion showed no LOH with the markers used.

The association between translocations and deletions has been previously demonstrated in t(9;22) (Sinclair *et al*, 2000) and our investigation would appear to suggest that this might be detected in other abnormalities.

In two cases, a wild type pattern only was detected. This may be related to a low percentage of cells carrying the del (6q) (below the threshold of detection by LOH) or the deletion may be localised to a different region than the area investigated by the markers used in our study.

On the other hand, in another 11 patients 6q abnormalities was shown by microsatellites but no cytogenetics abnormality has been reported. Seven of them (numbers: 15, 22, 29, 46, 48, 53 and 60) showed LOH only; 2 patients (numbers: 26 and 34) showed MSI-H and LOH (in at least one marker) and 2 patients (numbers: 30 and 38) showed MSI only as an indication of chromosome 6q instability. The discrepancy between cytogenetics and microsatellite analysis may be related to (i) the presence of small interstitial deletions that would be undetectable by cytogenetics; (ii) the reduced capability of some cells to go into metaphase during culture and therefore go undetected. Interphase FISH may help to resolve this problem but deletion analysis of interphase cells is associated with high risk of false negative tests and therefore not very reliable.

Number	BACH	D6S1709	D6S283	D6S301	D6S1592	D6S268	D6S278	D6S246	D6S302	D6S304
1	○	○	○	○	○	○	○	○	○	○
2	○	○	○	○	○	○	○	○	○	○
3	○	○	○	○	○	○	○	○	○	○
4	○	○	○	○	○	○	○	○	○	○
5	○	○	○	●	○	○	○	○	●	●
6	○	○	○	○	○	○	○	○	○	○
7	○	○	○	○	○	○	○	○	○	○
8	●	○	●	○	●	○	○	○	●	○
9	○	○	●	●	●	○	●	○	○	○
10	○	○	○	○	○	○	○	ND	○	○
11	●	○	○	○	○	○	○	ND	●	○
12	○	○	○	○	○	○	○	○	○	○
13	○	○	○	○	●	○	○	○	○	○
14	○	○	○	○	○	○	○	○	○	○
15	○	○	○	○	●	○	○	○	○	●
16	○	○	○	○	○	○	○	○	○	○
17	○	○	○	○	○	○	○	○	○	○
18	○	○	●	○	○	○	○	○	○	○
19	○	○	○	○	○	○	○	○	○	○
20	○	○	○	○	○	○	○	○	○	○
21	○	○	○	○	○	○	○	○	ND	○
22	●	○	○	○	○	○	○	○	○	○
23	○	○	○	○	○	○	○	○	○	○
24	○	○	○	○	○	○	○	○	○	○
25	○	○	●	●	●	●	○	○	○	○
26	○	MSI	●	MSI	○	MSI	○	MSI	MSI	MSI
27	○	○	○	○	○	○	○	○	○	○
28	○	○	○	○	○	○	○	○	○	○
29	○	○	○	●	○	○	●	○	○	○
30	○	ND	○	MSI	○	MSI	○	○	MSI	NI
31	○	○	○	○	○	○	○	○	○	○
32	○	○	○	○	○	○	○	○	○	○
33	○	○	●	○	○	○	○	○	○	○
34	MSI	MSI	●	MSI	MSI	MSI	MSI	MSI	MSI	MSI
35	○	○	○	○	○	○	○	○	○	○
36	○	○	○	○	○	○	○	○	○	○
37	○	○	○	○	○	○	○	○	○	○
38	MSI	○	○	○	○	○	○	MSI	MSI	○
39	○	○	○	○	○	○	○	○	MSI	ND
40	○	○	○	○	MSI	○	○	MSI	ND	ND
41	○	○	○	○	○	○	○	○	○	○
42	○	○	○	○	○	○	ND	○	○	○
43	○	○	○	○	○	○	○	○	ND	○
44	○	○	ND	○	○	○	○	○	○	○
45	○	○	○	○	○	○	○	○	○	○
46	○	○	○	●	○	○	○	○	○	○
47	MSI	MSI	MSI	MSI	●	MSI	MSI	MSI	○	MSI
48	○	○	○	○	○	○	○	○	●	○
49	○	○	○	○	○	○	○	○	○	○
50	○	○	○	○	○	ND	○	○	○	○
51	○	○	○	○	○	○	○	○	NI	○
52	○	○	○	○	○	○	○	○	○	○
53	○	○	○	○	○	○	●	○	○	○
54	○	○	○	○	○	○	○	○	○	○
55	○	○	○	○	○	○	○	○	○	●
56	○	○	○	○	○	○	○	○	○	ND
57	○	○	○	ND	○	○	○	○	○	○
58	○	○	○	○	○	○	○	○	○	○
59	○	○	ND	○	○	○	○	○	○	○
60	○	○	○	○	○	○	○	●	○	○
61	○	NI	○	○	○	○	○	○	○	○
62	○	○	○	○	○	MSI	○	●	○	ND
63	○	○	○	○	○	○	○	○	○	○
64	○	○	○	○	○	○	○	○	○	○
65	○	○	○	○	●	○	MSI	○	○	○
66	○	○	○	○	○	○	○	○	○	○
67	○	○	○	○	○	○	○	○	○	○
68	○	○	NI	○	○	○	○	○	○	○
69	○	○	NI	○	○	○	○	○	○	○

Table 4.6. *Patients' analysis using 6q16-6q21 microsatellite markers. Sixty-nine paired DNA samples from ALL patients were screened for LOH/MSI on 6q16-21. Solid circles: LOH for the tested locus; open circle: retained heterozygosity for the tested locus; MSI: microsatellite instability; NI: non-informative test for the marker analysed, ND: not done.*

4.4.3. Region of instability

Among the markers used, D6S283 and D6S1592 were the most commonly deleted (deleted in 7 loci of 35 loci each), followed by D6S301 (5 loci), D6S302 (4 loci) and BACH2, D6S278 and D6S304 (3 loci each) (see Table 4.6 and Figure 4.5). When MSI is taken into account, markers D6S283 and D6S1592 that were the most commonly involved markers by LOH, were the least involved by MSI (see Table 4.6; Figures 4.6; 4.7 and 4.8). The most frequently affected were markers D6S268, D6S246 and D6S302 (5 loci each) followed by D6S301 (4 loci), BACH2, D6S1709, D6S278 and D6S304 (3 loci each), D6S1592 (2 loci) and D6S283 (1 locus only).

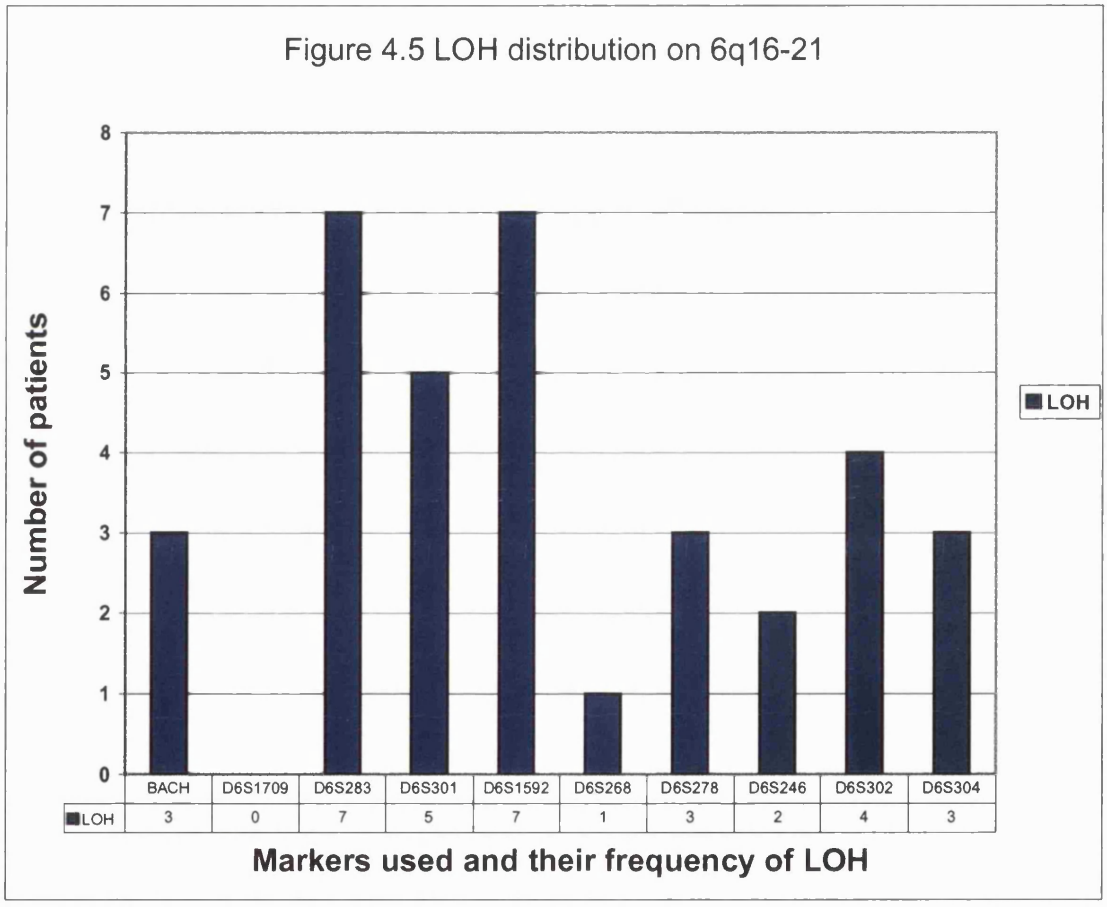


Figure 4.5. *Shows the frequency of 6q16-21 microsatellite markers involvement by loss of heterozygosity (LOH). Markers D6S283 and D6S1592 were the most frequently deleted markers where as marker D6S1709 was always retained.*

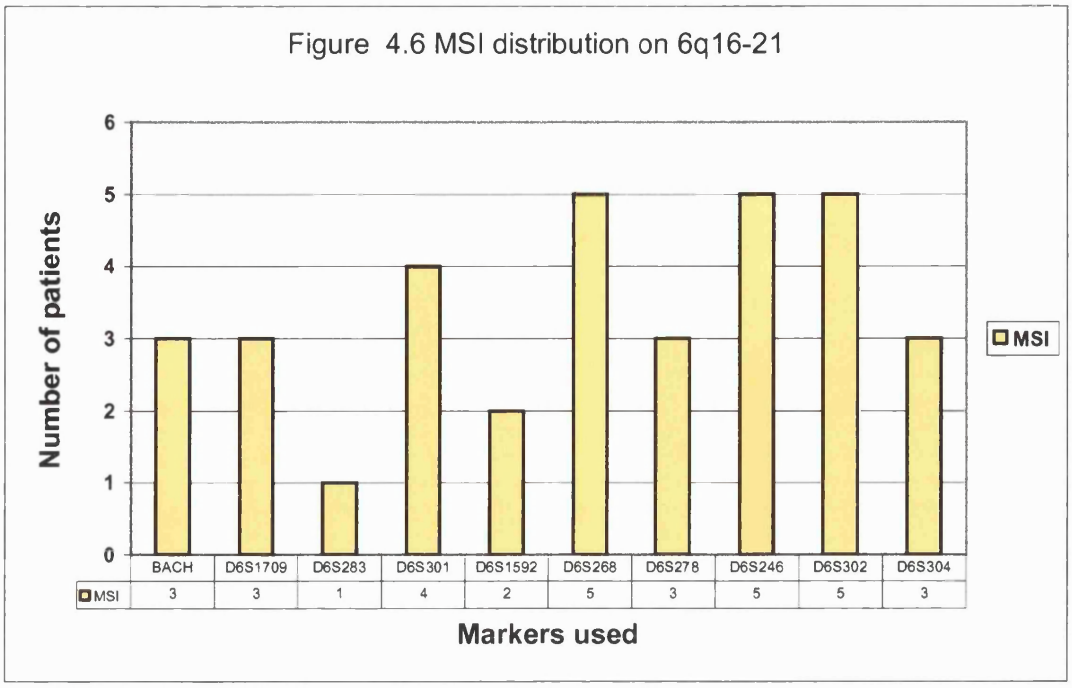


Figure 4.6. *Shows the frequency of 6q16-21 markers involvement by microsatellite instability (MSI). Markers D6S268, D6S246 and D6S1592 were the most frequently involved markers (5 loci each), whereas marker D6S283 was the least involved by MSI (1 locus only).*

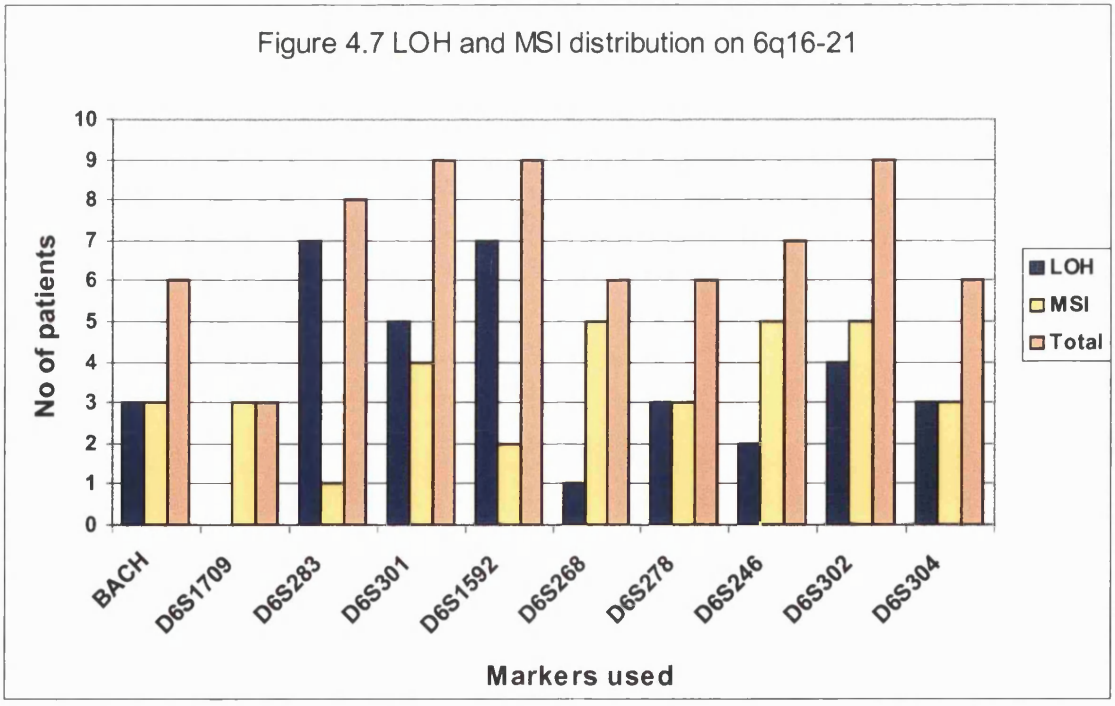


Figure 4.7. *Shows the frequency of 6q16-21 markers involvement by loss of heterozygosity (LOH) and microsatellite instability (MSI) and the total number of loci involved by instability. Markers D6S283 and, and D6S1592 were the most frequently involved markers by LOH but the least involved by MSI.*

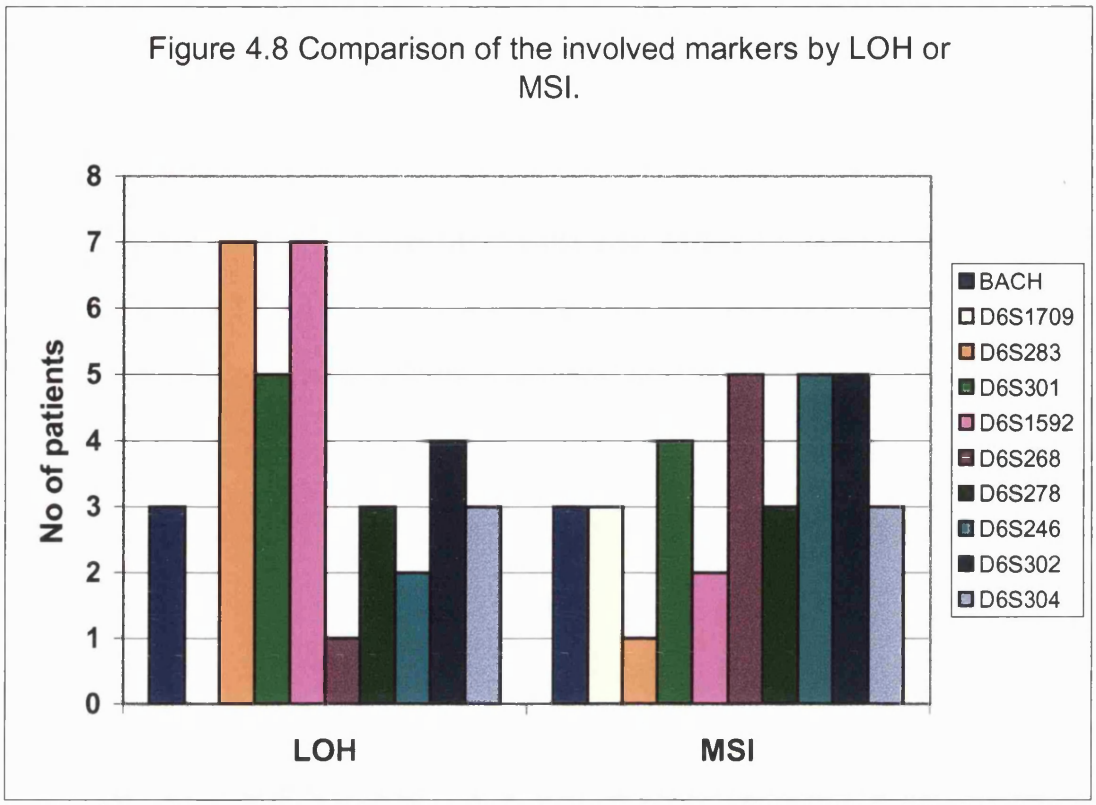
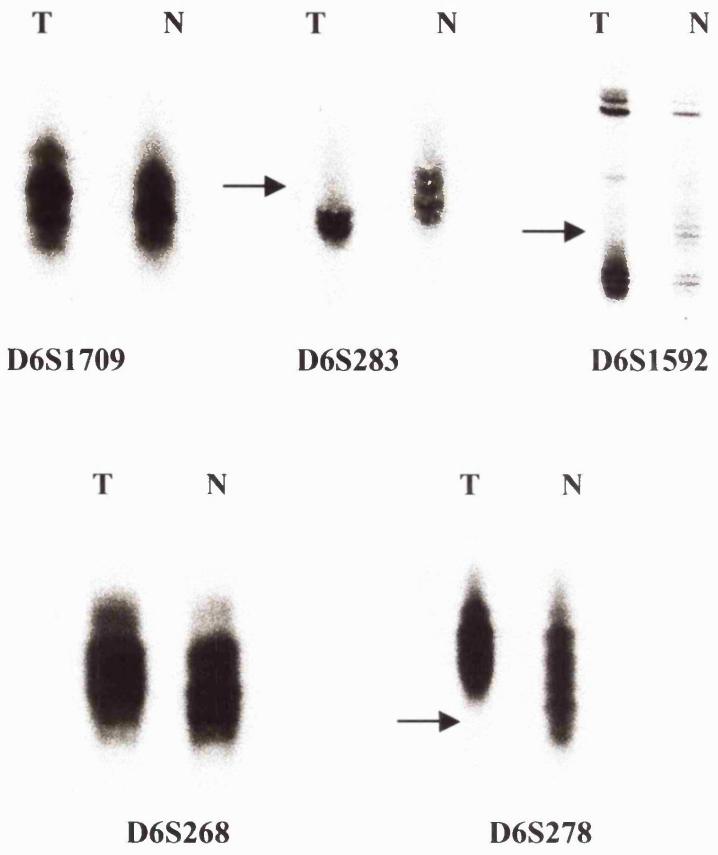


Figure 4.8. *Shows a comparison of 6q16-21 microsatellite markers and their frequency of involvement by loss of heterozygosity (LOH) presented on the left side of the graph and microsatellite instability (MSI) on the right side of the graph.*

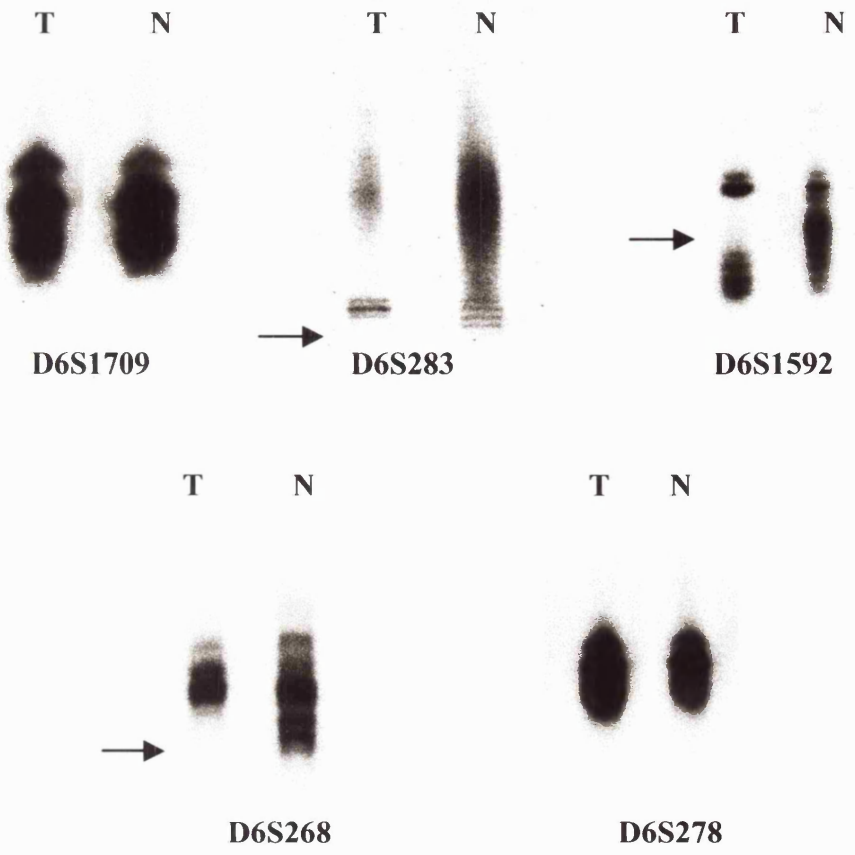
The cluster of markers D6S283, D6S301 and D6S1592 identified a contiguous region with frequency of LOH higher than other parts on 6q16-21 region. The region of minimal deletion (RMD) is identified by two cases (case number 9 and case number 25) (Table 4.6 and Figure 4.13). Cases no 9 showed deletions of markers D6S283, D6S301, D6S1592 and D6S278 whereas markers D6S1709 and D6S246 were retained. Case number 25 showed deletions of markers D6S283, D6S301, D6S1592 and D6S268 but retained both markers D6S1709 and D6S278. These data suggest that the minimally deleted region on 6q16-21 in our studied cases of ALL has been identified between markers D6S1709 and D6S278 (Figure 4.9). Other examples of the distinct pattern of LOH of the analysed cases are shown in Figure 4.10 and 4.11, whereas Figure 4.12 shows the pattern of MSI of the 6q21 markers as an example of a case of MSI/H (cases number 34).

Figure 4.9. Patterns of LOH of 6 q markers in the cases that determined the RMD in our study (case number 9 and case number 25).



Case number 9

Patterns of LOH of 6 q markers incase number 9. Arrows point at lost alleles. Case 9 has evidence of allelic loss at D6S283, D6S1592 and D6S278, with retention of D6S1709. Marker D6S268 was also retained.



Case number 25

Patterns of LOH of 6 q markers in case number 25. Arrows point at lost alleles. Case 25 has evidence of allelic loss at D6S283, D6S1592 and D6S268, with retention of D6S1709 proximally and D6S278 distally.

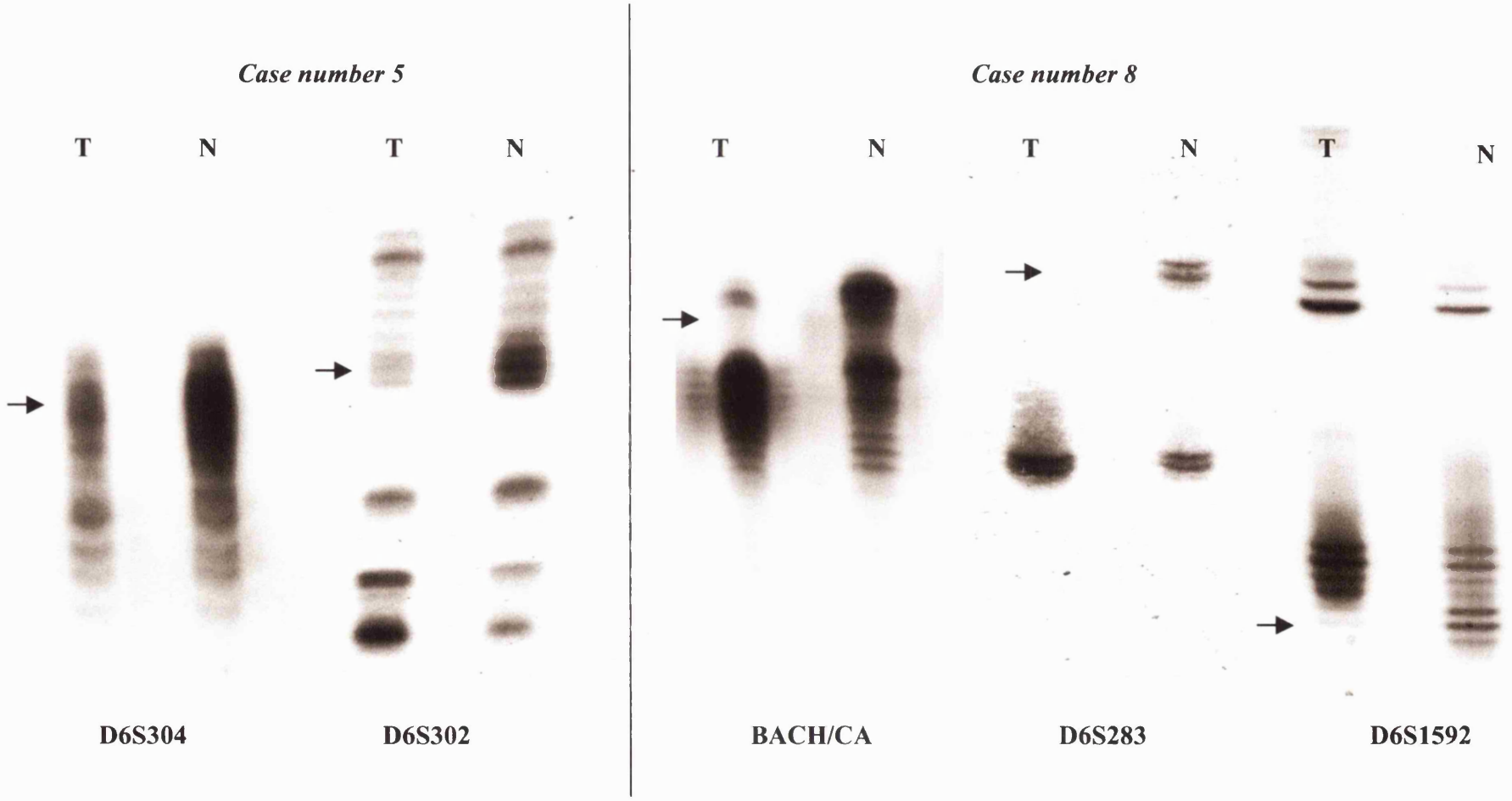


Figure 4.10. *Representative autoradiographs showing examples of Loss of heterozygosity (LOH) of 6 q markers. Examples with distinct pattern of 6q LOH are shown. Case number and marker name are indicated above and below each autoradiograph respectively. Arrows point at lost alleles. T; tumour DNA, N; non-tumour DNA.*

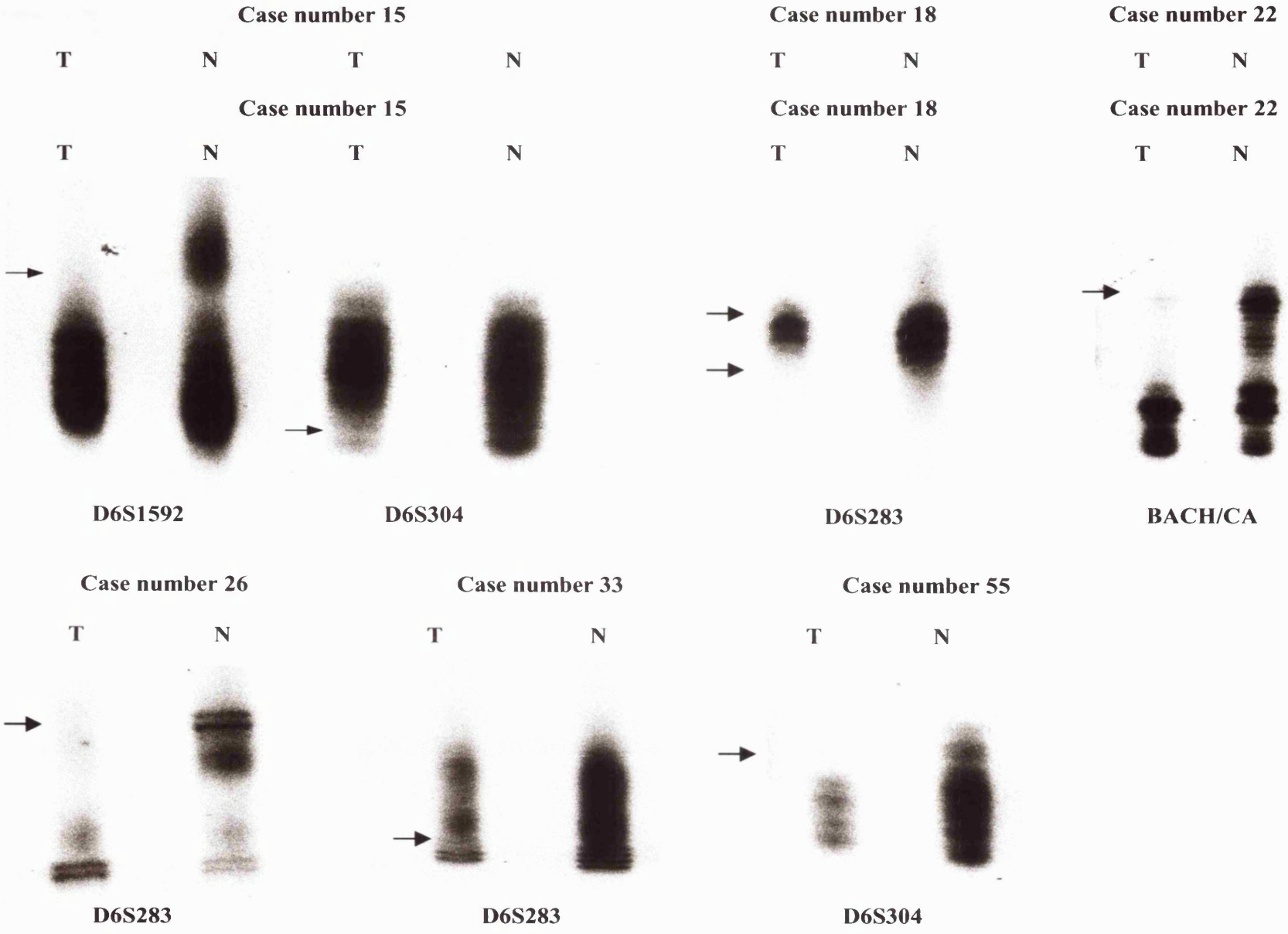


Figure 4.11. Representative autoradiographs showing examples of LOH of 6q markers. Arrows point at lost alleles. Case numbers are indicated above and marker name is indicated below each autoradiograph. Abbreviations are as follows: T; tumour DNA, N; non-tumour DNA.

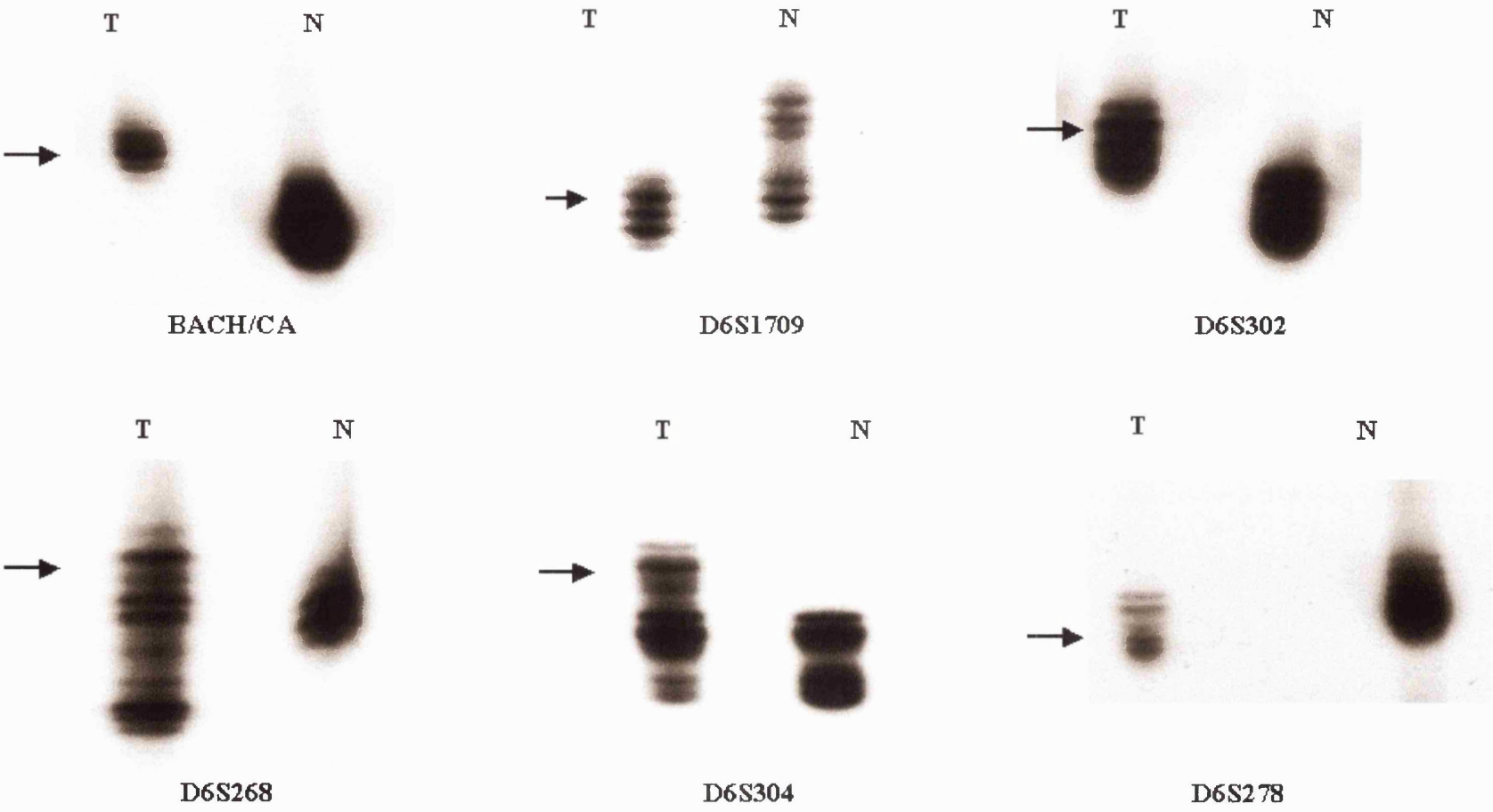


Figure 4.12. *Representative autoradiography showing Microsatellite instability (MSI) in case number 34 using the 6q markers. T; tumour, N; non- tumour. Arrows point at the novel fragments or the shifted band of the tumour sample when compared to the non-tumour sample.*

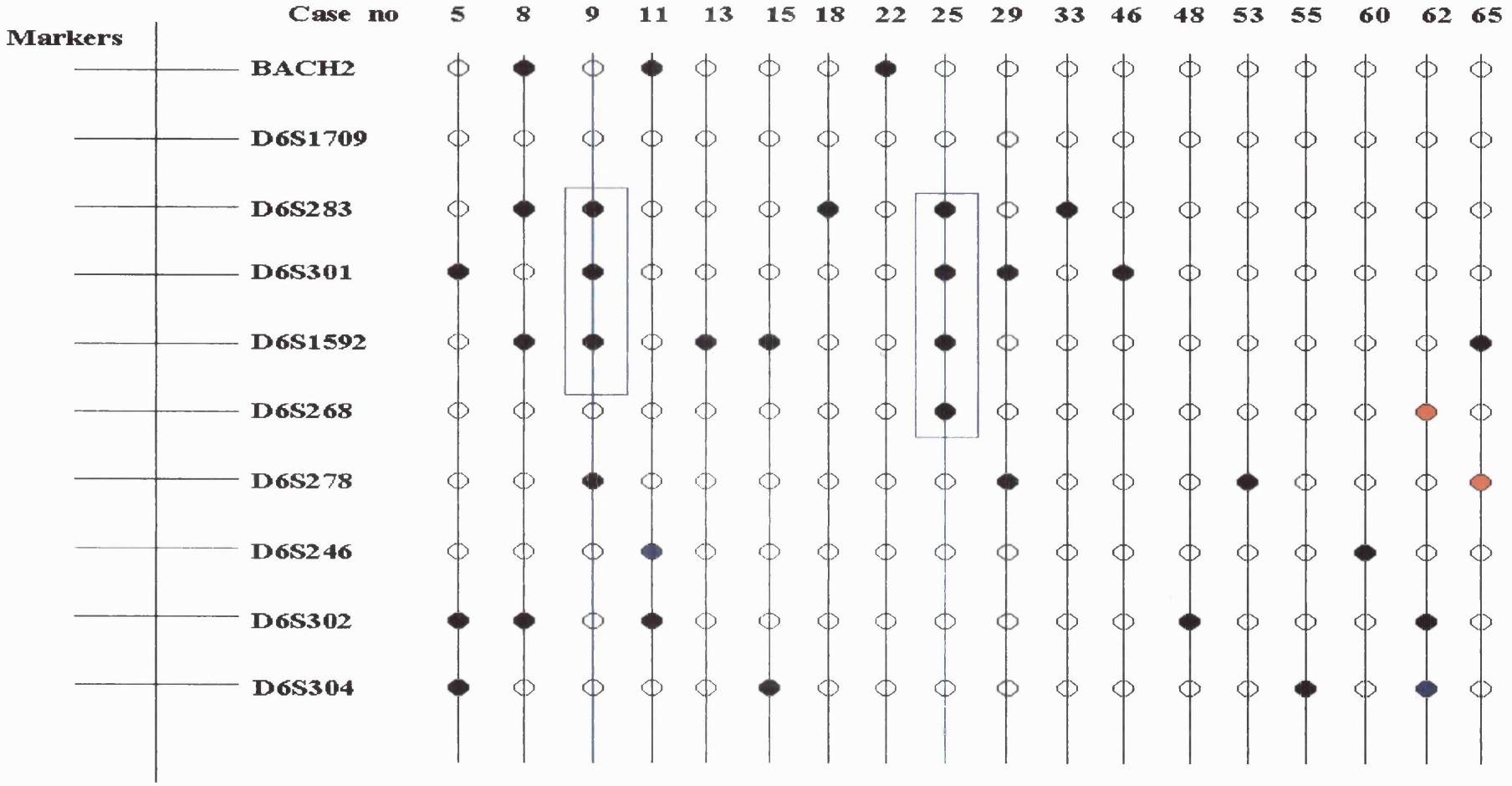


Figure 4.13. *Schematic representation of loss of heterozygosity (LOH) results for microsatellite markers on 6q16-21. Solid black circle: LOH; open circle: retained heterozygosity; solid red circle: MSI and solid blue circle not done. Blue-coloured boxes represent the pattern of LOH that determined the region of minimal deletion (RMD) in this study.*

4.5. Additional studies done on patients with MSI on 6q21

As described previously nine patients showed a pattern compatible with MSI. Confirmation of MSI pattern was then further assessed by investigating these patients (number 26, 30, 34, 38, 39, 40, 47) and 2 controls (patients number 1 with a wild-type phenotype and patient no 8 with a LOH phenotype) with a panel of additional markers according to the recommendation of NCI (Boland C. R. *et al.*, 1998)(Table 4.2). Not enough DNA was available to carry out the same investigation on two other additional patients (number 62 and 65). Table 4.7 contains the results of the investigation carried out on patients 1, 8, 26, 30, 34, 38, 39, 40 and 47. Three patients (number 26, 34 and 47) that showed a high level of MSI (MSI/H) using the 6q21 microsatellite markers, showed a pattern consistent with high frequency of microsatellite instability at all the loci investigated except for BAT-25 and BAT-26. Marker D5S346 was selected and PCR amplification was performed using 6 normal samples (E4-E9) from normal volunteers and on 10 samples from our collection of patients showing the wild phenotype (number 1, 2, 3, 4, 6, 7,12, 14, 16, 17). None of these samples showed a pattern diverging from the expected wild-type pattern. BAT-25 and BAT-26 markers showed the same pattern in all 60 tumour DNA samples and in the normal samples (numbers E4-E9). Figure 4.14 shows examples of MSI pattern using markers D5S346 and D2S123.

Marker	**Control patients Wild/LOH**		**MSI/H patients**				**MSI/L patients**		
	Patient's serial numbers								
	1	8	26	30	34	47	38	39	40
BAT-25	N	N	N	N	N	N	N	N	N
BAT-26	N	N	N	N	N	N	N	N	N
D2S123	N	N	N	MSI	MSI	MSI	N	N	N
D5S346	N	N	MSI	N	MSI	MSI	N	N	N
D17S250	N	N	MSI	N	MSI	MSI	N	N	N
Scoring	-	-	MSI/H ≥2/5	MSI/L <2/5	MSI/H ≥2/5	MSI/H ≥2/5	MSI/L or MSS <2/5	MSI/L or MSS <2/5	MSI/L or MSS <2/5

Table 4.7. *Summarises the results of the additional studies carried out on the patients with MSI. N; Normal pattern, MSI/H; Microsatellite instability of high frequency type, MSI/L; Microsatellite instability of low frequency type, MSS; Microsatellite stable LOH; Loss of heterozygosity.*

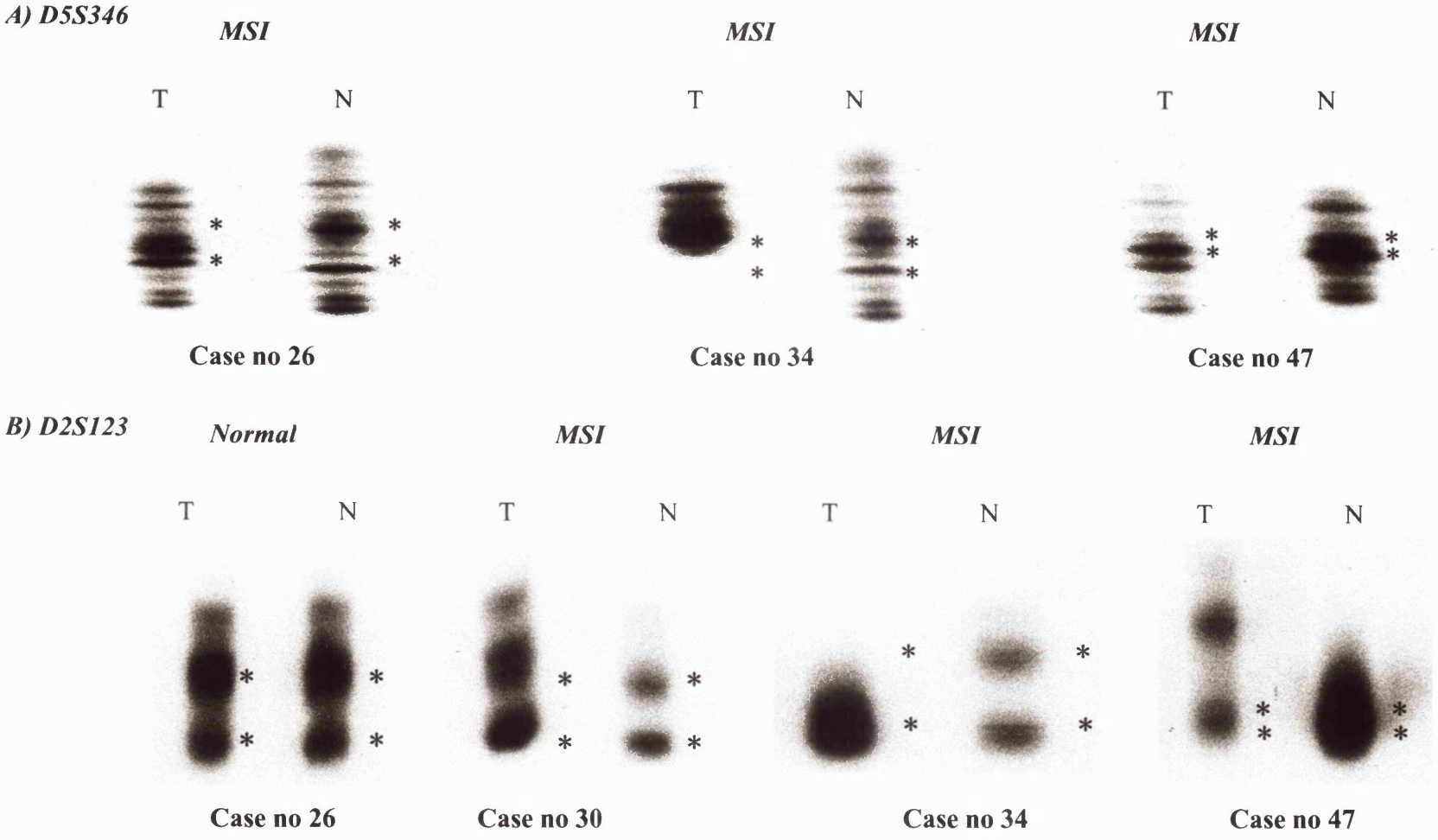


Figure 4.14. *Representative autoradiographs of markers D5S346 and D2S123 (NCI markers panel).* ***A)*** *Marker D5S346 showing MSI pattern in nos 26, 34 and 47.* ***B)*** *Marker D2S123 showing normal pattern in case 26 and MSI pattern in nos 30, 34 and 47. An asterix indicates the position of the wild type bands for the individual markers in different patients' DNA samples.*

4.6. Analysis of patients' cilnicai data using survival curves

The impact of LOH or MSI or LOH/MSI pattern was assessed in patients carrying these patterns compared to the wild-type group by looking at overall survival. Survival curves are a way of graphically displaying survival rates. On these graphs, vertical axis denotes the survival rates and horizontal axis denotes time.

The analysis was performed only for patients with B-lineage and T-lineage ALL while the patients belonging to other subgroups were excluded because of their small numbers. Also, only patients with *de-novo* ALL were considered while relapse ALL patients were excluded, to keep the group of patients analysed more homogenous. Table 4.8 summarises the results of this analysis, while Figures 4.15-4.26 illustrate graphically the survival curves on the different subgroups.

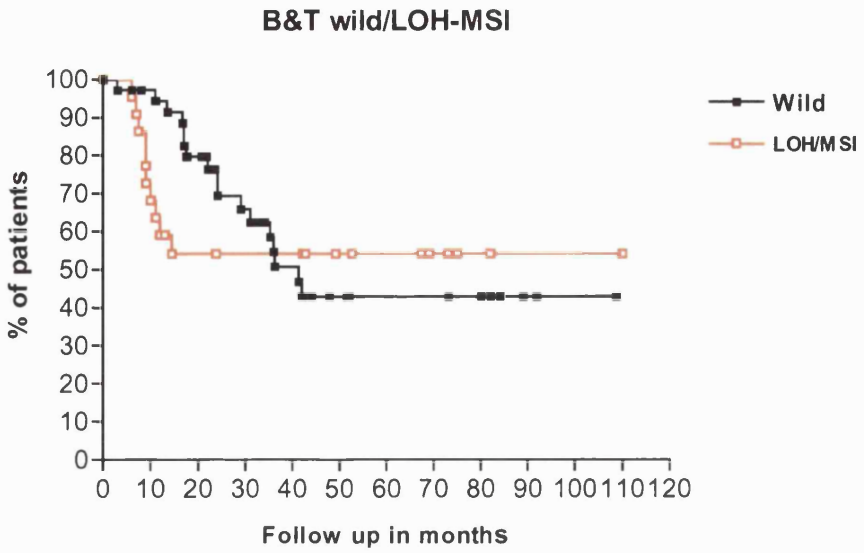
The presence of a LOH pattern/MSI appears to have a significant impact only in the T-ALL subgroup (Figure 4.24, 4.25 and 4.26 and *$p<0.0001$, <0.0001 and 0.0026,* respectively*)*.

LOH or/and MSI appear to have no impact in the overall outcome of patients with B lineage ALL, although larger groups of patients will needed to be analysed for these correlations to be further corroborated.

Further discussion about the significance of these results will be presented in the final chapter of this thesis.

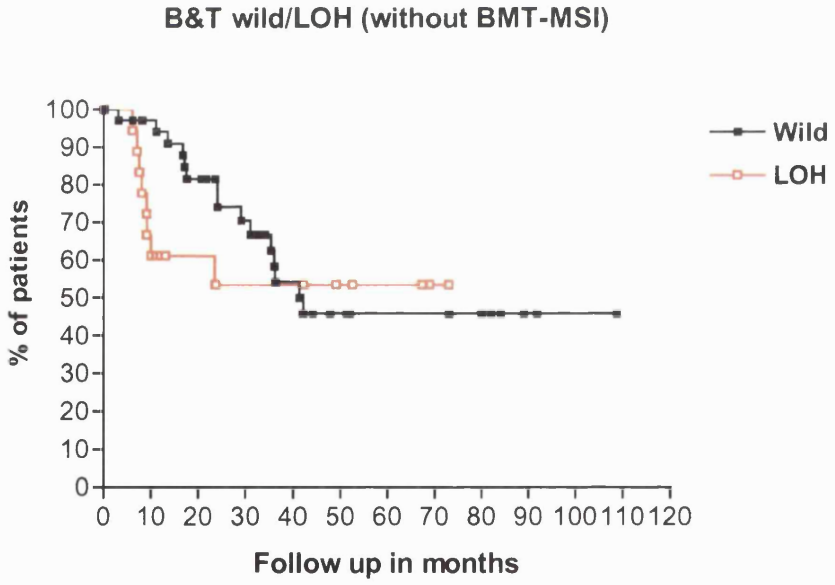
Graph no	Group of patients tested	χ^2 test	P value	P value summary
1	B&T lineage wild versus LOH-MSI (2 parameters)	0.1271	0.7214	Not significant
2	B&T lineage wild versus LOH excluding BMT & MSI (2 parameters)	0.6168	0.4323	Not significant
3	Adults only B-lineage wild versus LOH-MSI (2 parameters)	0.7956	0.3724	Not significant
4	Adults only B-lineage wild versus LOH excluding BMT&MSI (2 parameters)	0.1496	0.6989	Not significant
5	B-lineage wild versus LOH-MSI (2 parameters)	2.767	0.0962	Not significant
6	B-lineage wild versus LOH excluding BMT& MSI (2 parameters)	1.841	0.1748	Not significant
7	Children only B-lineage wild versus LOH excluding BMT& MSI (2 parameters)	1.972	0.1602	Not significant
8	Children only B-lineage wild versus LOH -MSI (2 parameters)	1.941	0.636	Not significant
9	B-lineage adults, children wild versus LOH-MSI (4 parameters)	2.894	0.4083	Not significant
10	B-lineage adults, children & T-lineage altogether wild/LOH-MSI (6 parameters)	66.08	<0.0001	Significant***
11	B&T lineage adults& children wild versus LOH-MSI (8 parameters)	66.95	<0.0001	Significant***
12	T- lineage wild versus LOH-MSI (2 parameters)	9.090	0.0026	Significant***

Table 4.8. *Summarises the obtained results of the analysed survival curves of the investigated group of ALL cases.*



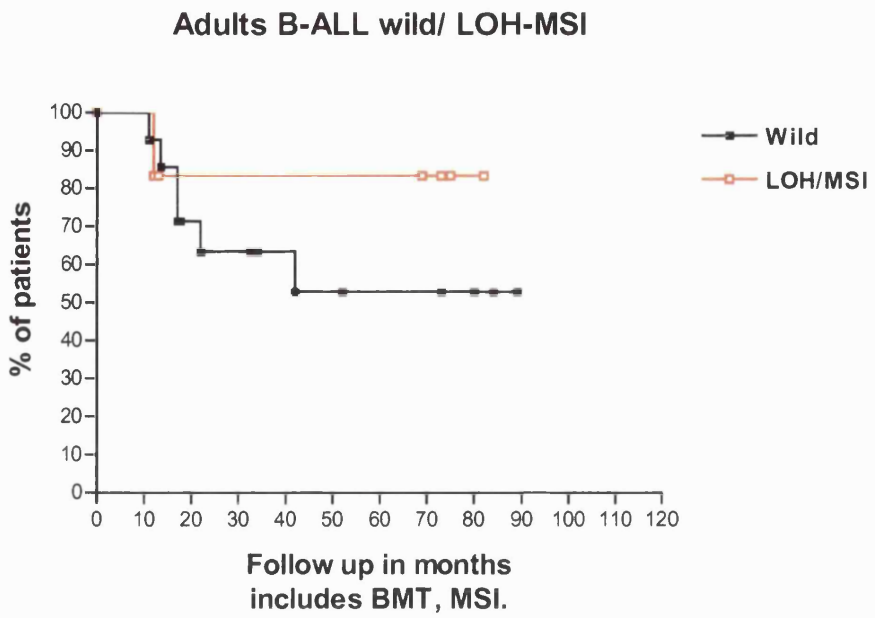
Graph number 1

Figure 4.15. *Survival curve analysis of B and T lineage wild versus LOH-MSI, P value=0.7214; not significant*



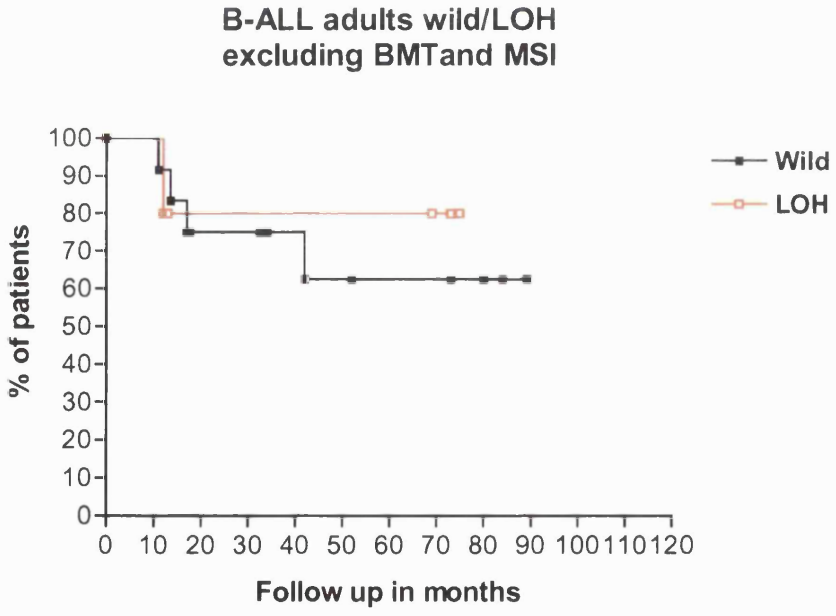
Graph number 2

Figure 4.16. *Survival curve analysis of B&T lineage wild versus LOH-MSI excluding BMT and MSI, P value=0.4323; non significant*



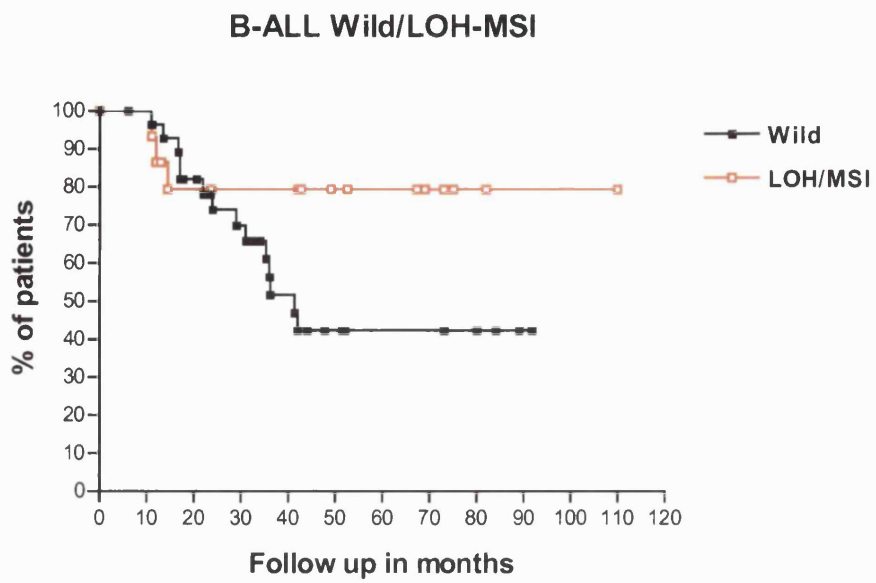
Graph number 3

Figure 4.17. *Survival curve analysis of adults only B- lineage wild versus LOH-MSI P value=0.3724; not significant.*



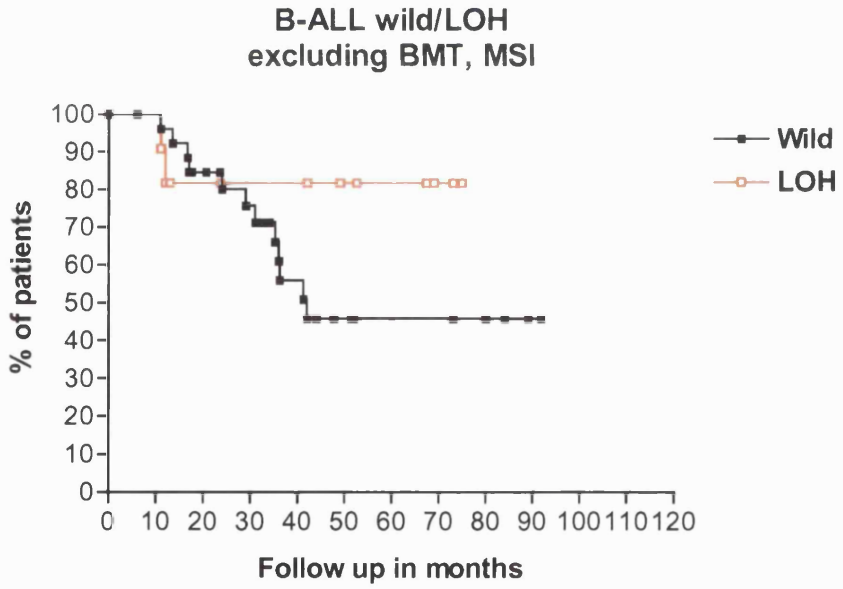
Graph number 4

Figure 4.18. *Survival curve analysis of adults only B- lineage wild versus LOH-MSI excluding BMT and MSI; p value=0.6989; not significant.*



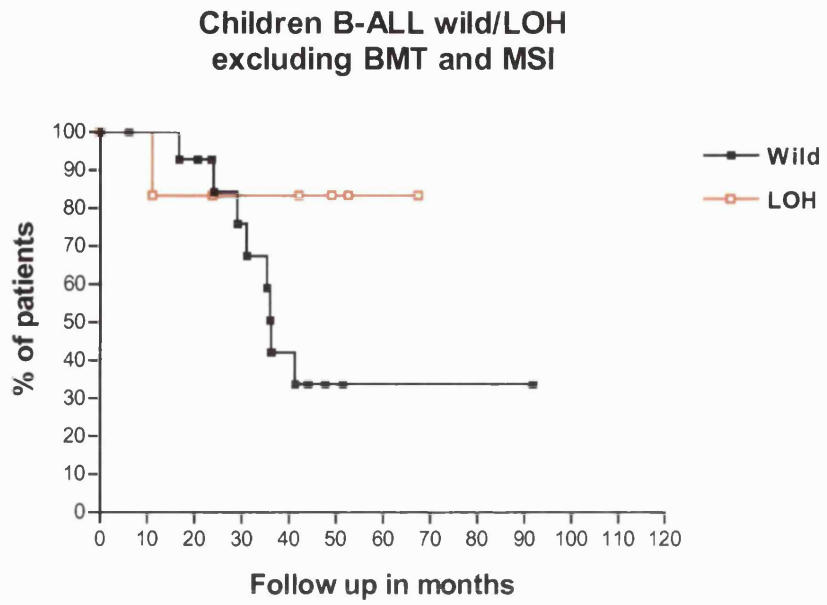
Graph number 5

Figure 4.19. *Survival curve analysis of B- lineage wild versus LOH-MSI P value=0.0962; not significant.*



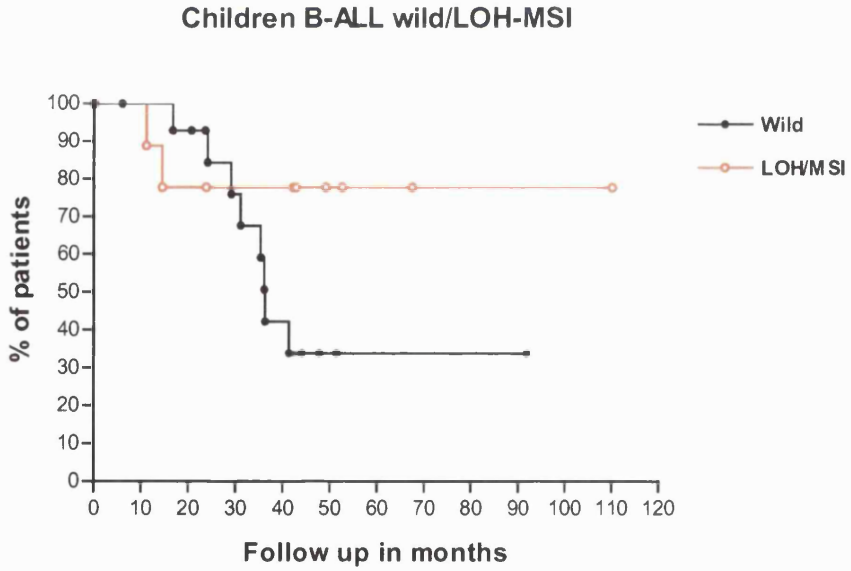
Graph number 6

Figure 4.20. *Survival curve analysis of B- lineage wild versus LOH excluding BMT and MSI, p value= 0.1748 not significant.*



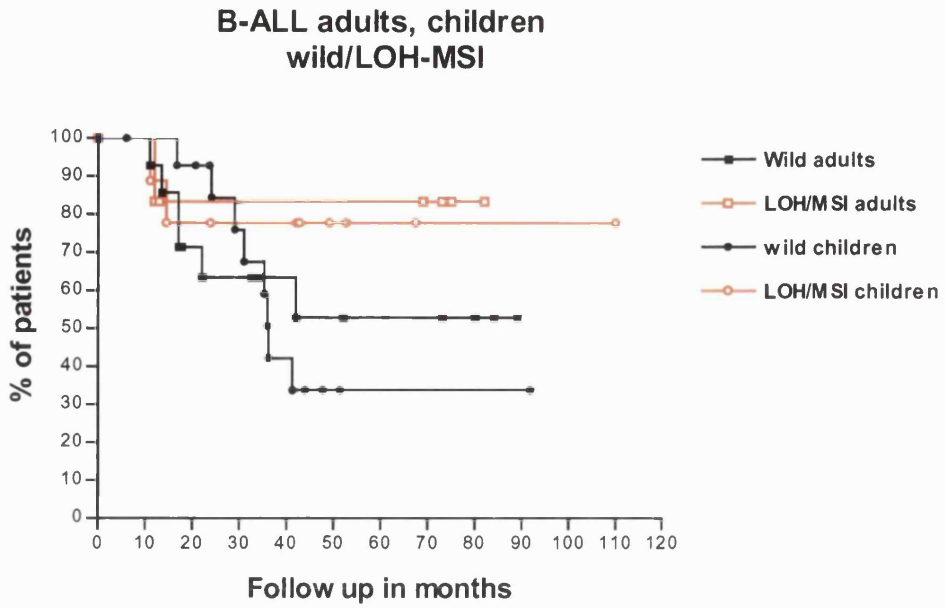
Graph number 7

Figure 4.21. *Survival curve analysis of children only B- lineage wild versus LOH, excluding BMT and MSI; p value=0.1602; non significant.*



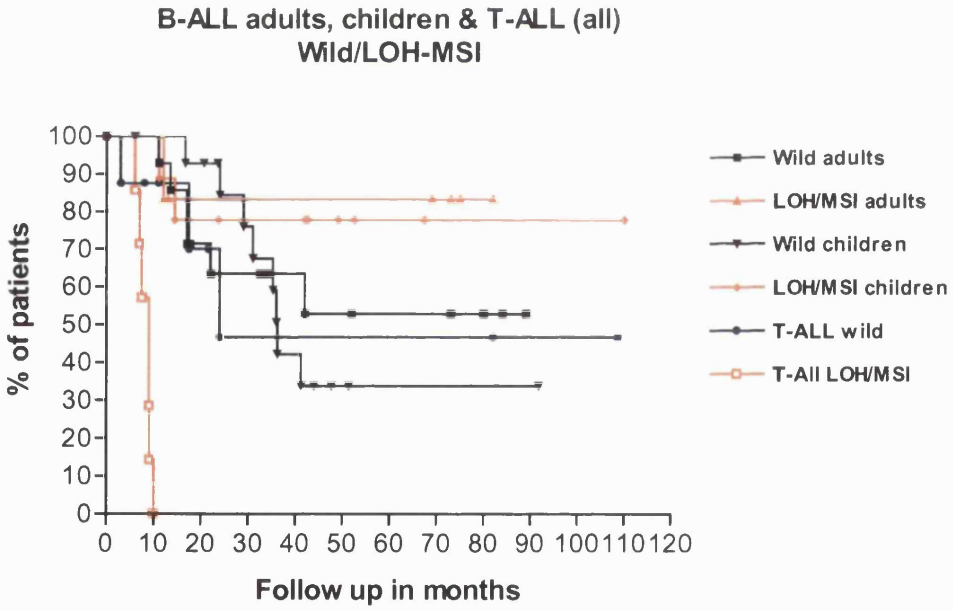
Graph number 8

Figure 4.22. *Survival curve analysis of children only B-lineage wild versus LOH-MSI P value=0.636; not significant.*



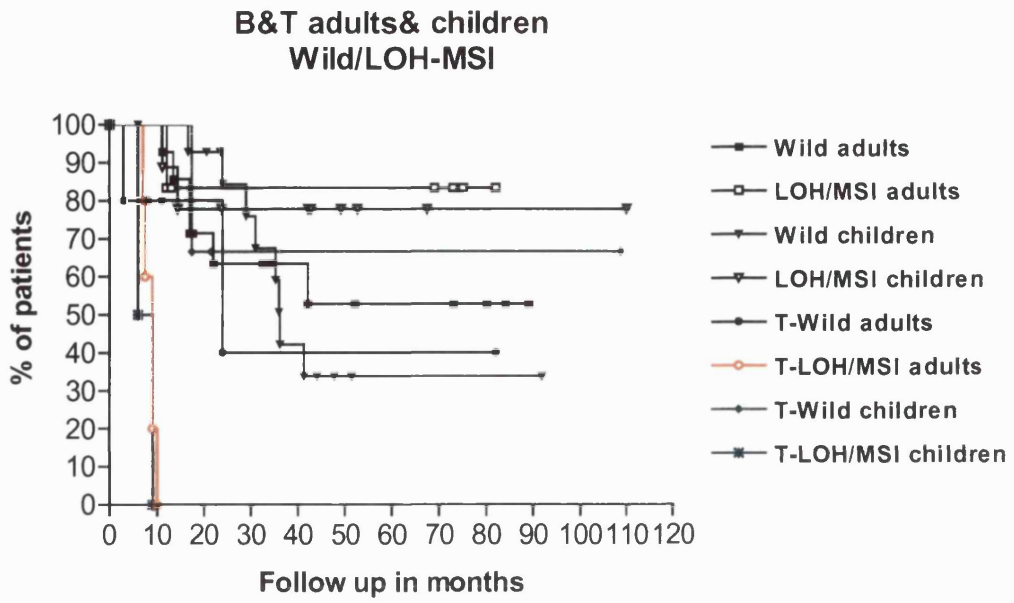
Graph number 9

Figure 4.23. *Survival curve analysis of B- lineage adults and children wild versus LOH-MSI, P value=0.4083; not significant*



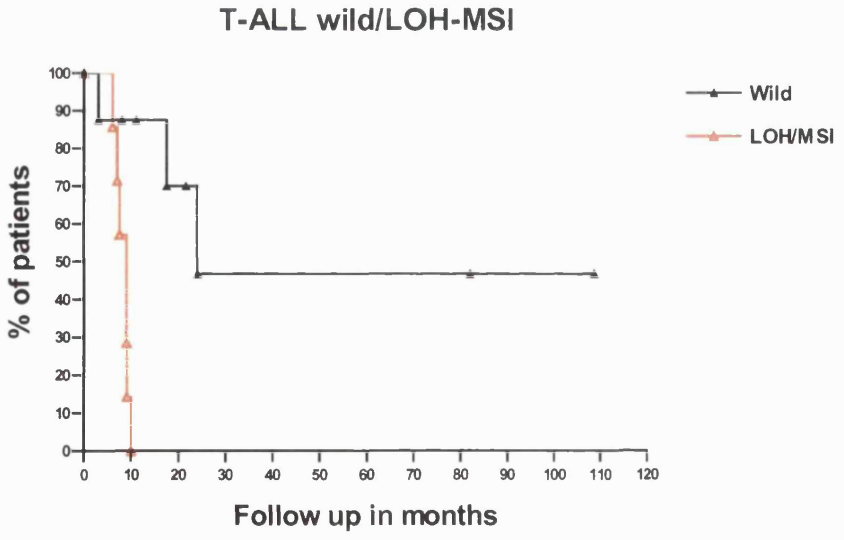
Graph number 10

Figure 4.24. *Survival curve analysis of B- lineage adults and children and T-linage (alltogether) wild versus LOH-MSI, p value<0.0001 significant.*



Graph number 11

Figure 4.25. *Survival curve analysis of B&T lineage adults and children wild versus LOH-MSI, p value<0.0001 significant.*



Graph number 12

Figure 4.26. *Survival curve analysis of T- lineage wild versus LOH-MSI P value=0.0026 significant.*

Chapter 5

Discussion

Previous cytogenetic studies have identified deletions on chromosome 6, band q16-21 in approximately 4-13% of acute leukaemias suggesting the existence of a candidate tumour suppressor gene/s within this region. Investigation into the frequency of 6q deletions in ALL, expansion of the genomic map of 6q16-21 and sequence analysis of these regions aiming to identifying candidate genes in the minimally deleted area represented the main targets of the investigation presented in the previous chapters of this thesis.

A review of the results obtained in the context of previous published work and future studies will be presented in this chapter.

5.1. Chromosome 6q deletion investigation

Several studies have shown heterogeneity in frequencies of 6q deletions depending on several factors. First, the approach used. Cytogenetic measurements show frequencies varying from 4% to 13% in ALL (Hayashi Y. *et al.*, 1990) (Raimondi S. C., 1993) or sometimes as high as 23% in adults T-cell leukaemia/lymphoma (Kamada N. *et al.*, 1992). FISH detects a frequency between 6% and 44% in B-CLL and ALL (Merup M. *et al.*, 1998; Stilgenbauer S. *et al.*, 1999; Zhang Y. *et al.*, 1997) while frequency based on LOH also varies greatly between studies: 13% (Takeuchi S. *et al.*, 1995b), 2% (Cave H. *et al.*, 1996), 6.7% (Gerard B. *et al.*, 1997), 15% (Takeuchi S. *et al.*, 1998) and 34% (Merup M. *et al.*, 1998).

Second, different clinical cohorts of patients (from ALL to NHL to CLL) show differences in incidence.

Third, frequencies vary in different age groups (adults and children). The majority of studies focused on childhood patients and even within this group incidence varied extensively from 34% (Merup M. *et al.*, 1998) to 6.4% (Gerard B. *et al.*, 1997) to 15% (Takeuchi S. *et al.*, 1998). Little information has been available on 6q deletions in adult ALL.

Fourth, the use of randomly selected microsatellite markers and their density within a

region may affect final results, as different studies have used different markers.
Finally, frequencies also vary between different ALL subgroups depending on immunophenotypes (B or T cell lineages) with T-ALL probably showing the highest incidence, from 23% by cytogenetic analysis (Theile M. *et al.*, 1996) to 41% by LOH studies (Hatta Y. *et al.*, 1999; Hatta Y. *et al.*, 1998).

Discrepancies, in incidence between LOH and cytogenetic might be attributed to the use of PCR as a basis for analysis in the LOH studies which overcomes the common problems associated with cytogenetic analysis of leukaemia and lymphoma samples, mainly the poor yield of the mitoses and fuzzy appearance of the chromosomes. Furthermore, detection of the small interstitial deletions may be beyond the resolution of the cytogenetic analysis whereas LOH is a powerful technique for detection of such small deletions, as mentioned above.
These discrepancies are not restricted to chromosome 6. LOH analysis of chromosome 9p and 12p in childhood ALL showed that deletions of those two arms by LOH were much more frequent than those reported from the cytogenetics studies (Takeuchi S. *et al.*, 1996; Takeuchi S. *et al.*, 1995a; Takeuchi S. *et al.*, 1995b; Takeuchi S. *et al.*, 1997a).

At the time the investigation presented in this thesis started, FISH studies were being conducted to investigate 6q deletion by a colleague (Mr Paul Sinclair) while I was assigned the task to investigate 6q deletions by using LOH. This approach has the advantage of being easily applicable to large cohort of patients, multiple sites can be analysed simultaneously and small deletions undetected by other techniques can be detected easily.
In addition, at the time this study commenced some information was already available on the region of minimal deletion while less was known about the extent of the region, in term of size and sequence. This was mainly due to the lack of a finalised genomic map of the area of interest because of gaps in the region of 6q16-21.
FISH analysis of 6 cases of ALL had defined a common region of deletion between M6P1 locus at 6q14-15 and the FYN locus at 6q21 (Menasce L. P. *et al.*, 1994b) and this region covered several MB in size. This RMD had been narrowed down by another FISH study by the same group, which defined the RMD between markers D6S447 and

D6S246 (Sherratt T. *et al.*, 1997) and estimated the distance between the two markers to be approximately 2 MB.

This represented the starting point of our investigation through collaboration with Dr Harrison (the principal Investigator of Sherrat's study, at the time based in Manchester, UK). In 1998 the scientists in our laboratory began FISH investigation using YAC clones and redefined a RMD telomeric to D6S447 and centromeric to marker D6S268 (Jackson A. *et al.*, 2000) (see Chapter 3, Figure 3.4) and redefined the region of deletion to approximately 1.8MB. At the time, the physical distance between markers D6S447 and D6246 had been estimated to be larger than 3.5MB, using YAC clones identified in the process (Jackson A. *et al.*, 2000).

However, because of a gap between markers D6S447 and D6S268 the correct estimate of the size and gene in the region were yet uncertain. I have described in Chapter 3 the effort made to identify clones from within this region.

Several clones were isolated in the process (Chapter 3) and most recently two new YAC clones from the ICI and ICRF libraries have allowed bridging the gap between D6447 and D6S268 (A. Mungall and colleagues, Sanger Centre, Personal communication). The distance between D6S447 (in clone dJ359N14) and D6S268 (in clone dJ67A8) has now been estimated to be 1,848,070 bp (1.8MB). The specific co-ordinates of this region are listed below (courtesy of A. Mungall at the Sanger Centre, Hixton, Cambridge, UK). This is smaller than we previously estimated. However, it is apparent from other studies resulting from accurate mapping and sequencing of the human genome that overall the size of the genome is in fact "shrinking" from a postulated 3.9GB to 3.2GB according to recent estimations (Dr M Ross, Sanger Centre, personal communication) and therefore this finding is very much in line with similar observations on other chromosomes.

Subsequence "dJ359N14" 2863749 3000047 ***contains D6S447***

Subsequence "bA437I16" 2999948 3063753

Subsequence "bA114N1" 3061754 3180774

Subsequence "bA183D15" 3178775 3252682

Subsequence "yR39EC11" 3250683 3379675

Subsequence "dJ101M23" 3393688 3377676

Subsequence "dJ134E15" 3393589 3570751

Subsequence "dJ474G24" 3570652 3594201

Subsequence "dJ354M18" 3628873 3594106

Subsequence "bA404H14" 3658736 3628774

Subsequence "dJ335E1" 3802441 3658637

Subsequence "dJ448K1" 3856246 3802342

Subsequence "bA294H11" 3994152 3856147

Subsequence "dJ60O19" 4155407 3994053

Subsequence "yX28F10" 4153408 4165164

Subsequence "bA59I9" 4163165 4348475

Subsequence "bA300D11" 4346476 4430756

Subsequence "bA412O4" 4428756 4558901

Subsequence "dJ67A8" 4711819 4556902 contains D6S268

In addition, another gap between a region containing markers D6S283 and D6S301 on the centromeric side and D6S447 on the telomeric side had also been recently completely mapped and found to encompass 2.3MB region (http://www.ensembl.org/Homo_sapiens/mapview?chr=6).

For both regions, however, the correct estimate will become available in the next few months as soon as the sequence of chromosome 6 is completed (A Mungall, personal communication). Analysis of candidate genes within these regions will be presented below, in the following paragraphs.

5.2. Region of deletion identified by LOH analysis

In addition to the mapping analysis I also concentrated on LOH analysis to further define the region of minimal deletion on 6q16-21. In our study we included patients from a homogenous group (ALL) with efforts made to include comparable (in number) cohorts of adult and childhood patients. Ten markers were selected as those found to be most informative for this investigation and with the highest incidence of polymorphisms as derived from published data and database analysis (Genome Database at http:// www.gdb.org).

In our cohort of patients, using LOH deletion was detected in 16 (23%)(Chapter 4 paragraph 4.4.1). MSI was detected in 9 patients (13%). Twenty-five patients (36%) in total showed instability on chromosome 6 by using microsatellite analysis. The

occurrence of LOH did not appear to segregate with age, immunophenotype, gender or clinical outcome. Overall survival was not influenced by the occurrence of either LOH or MSI in B lineage ALL but conferred worse prognosis in T-ALL. We only observed that adults with MSI patterns were older than adults with either a WT or LOH pattern. The numbers are however, too small at present and needs to be expanded in future studies.

Our data differs slightly from other LOH studies of 6q deletions. Some studies have detected lower 13% (Takeuchi S. *et al.*, 1995b), 26% (Takeuchi S. *et al.*, 1998), 2% (Cave H. *et al.*, 1996), 6.7% (Gerard B. *et al.*, 1997) and others have detected higher incidence; 41% of adult T-cell lymphoma (Hatta Y. *et al.*, 1999; Hatta Y. *et al.*, 1998), while some studies have show frequencies similar to ours; 32% (Merup M. *et al.*, 1998).

Our study showed that the RMD is limited by marker D6S1709 on the centromeric side and marker D6S268 on the telomeric side with markers D6S283 and D6S1592 being the most frequently deleted markers (Figure 5.1)(see also Chapter 4 for more detailed analysis). This is to date a region of approximately 3.9MB, at least according to the most recent official map from Ensembl. Our RMD overlaps with the RMDs previously detected by cytogenetics and FISH analysis (Figure 5.1).

LOH analysis studies on 6q in ALL have identified the RMD between markers D6S301 and D6S1594 (Gerard B. *et al.*, 1997). The same region was then identified by Takeuchi (Takeuchi S. *et al.*, 1998) as one of two commonly deleted regions they observed in their study. The first region was identified between markers D6S283 and D6S302 and the second one more centromeric, between D6S283 and D6S275 (Takeuchi S. *et al.*, 1998). Marker D6S283 represented the boundary between these two regions and defined the RMD in ALL to less than 500kb (as estimated in their study) between markers D6S1709 on the centromeric side and D6S434 on the telomeric side.

This was also the RMD defined by the clustering of deletions around D6S283 in a study by Merup (Merup M. *et al.*, 1998). It is now known that this interval is only 77.31 kb in size.

Our RMD is also consistent with the RMD identified by Gerard (Gerard B. *et al.*, 1997) and the first region of minimal deletion identified by Takeuchi (Takeuchi S. *et al.*, 1998). Marker D6S1709 which represents the centromeric limit of the RMD identified by Merup (Merup M. *et al.*, 1998) is also the most centromeric marker of our RMD

and marker D6S283 which showed clustering of deletions by the same study was also identified as the most commonly deleted marker in our study see Figure 5.1.

A region of minimal deletion within 2- MB on 6q21 has been identified in a study using FISH analysis of 6q16-21 in lymphoid malignancies. Six yeast artificial chromosome probes were used to define deletion breakpoints within chromosome bands 6q16-21 in cases of ALL and NHL. Of the 32 case studied, 30 showed deletion of D6S246 and, in the two cases in which D6S246 was retained, the adjacent marker, D6S447 was deleted. Thus the region of minimal deletion identified by this study is located on 6q21 between D6S447 and D6S246 (Sherratt T. *et al.*, 1997) see also (Figure 5.1).

In a more recent study done by FISH on patients with ALL and NHL, the commonly deleted region of 3cM was identified on 6q21 between markers D6S1565 on the centromeric side and D6S434 on the telomeric side (Zhang Y. *et al.*, 2000). Our RMD overlaps with the telomeric part of this region (Figure 5.1).

Detailed deletion mapping of chromosome 6q in 22 cases of acute/lymphomatous adult T-cell leukaemia (ATL) using LOH identified the smallest commonly deleted region on 6q15-21 between markers D6S1652 and D6S1644 (Hatta Y. *et al.*, 1999).

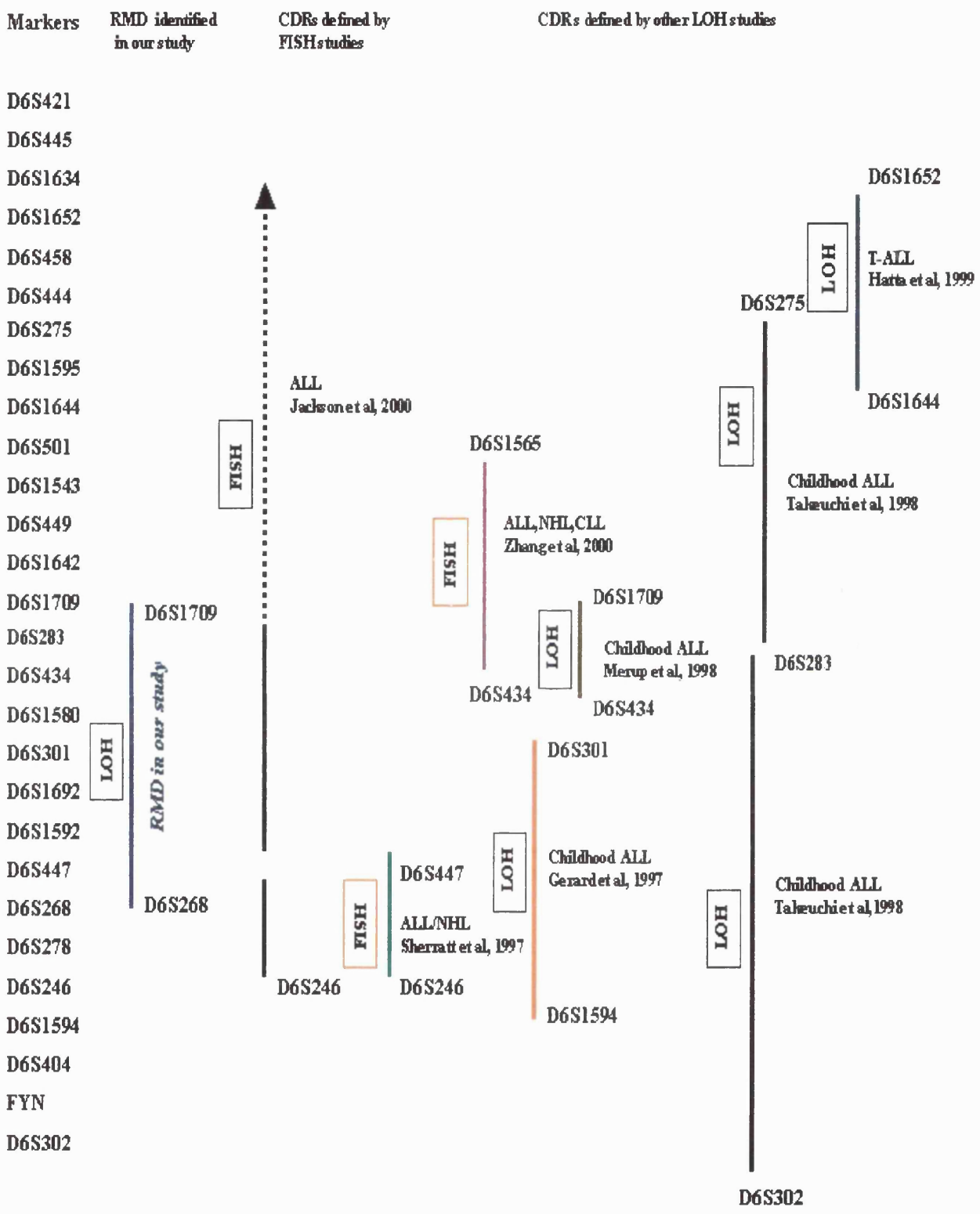


Figure 5.1. *Illustrates a comparison between the region of minimal deletion (RMD) in our study and the commonly deleted regions (CDRs) derived from other studies. References are displayed alongside each region. Abbreviations: RMD; region of minimal deletion, FISH; fluorescence in situ hybridisation, LOH; loss of heterozygosity.*

In conclusion, the RMD deletion identified in our study shows extensive overlap with other RMD identified by others. Despite different approaches, a common region of overlap can be identified between D6S1709 and D6S434 and a second region of deletion between D6S447 and D6S268. Using the Ensembl information the two regions are at present estimated to encompass 3.99 MB and 1.8 MB, respectively.
Within these regions at present further smaller deletions have been identified by some investigators (Merup M. *et al.*, 1998), for instance between markers D6S1709 and D6S434, a segment only 77.4 kb long, as mentioned above.

In the next paragraphs I will now present the analysis carried out within these different regions and the identification of genes from within these regions.

5.3. Candidate genes mapped to 6q15-21

Part of my investigation has helped to define a region of minimal deletion using LOH and previously published work. It was therefore of great interest as a final part of this analysis to annotate genes within the region of deletion as one or more of them may in the future be identified as important players in acute leukaemia associated to deletion of chromosome 6q. Several genes are located in our region of interest on 6q15-21 between markers BACH2 and D6S304. Table 5.1 lists these genes positioned according to the sequencing data from the Ensemble database. This data can be accessed at http://www.ensembl.org/Homo_sapiens/mapview?chr=6. In Table 5.1 I have provided the names, synonyms, localisation and functions of these genes according to the data available from Gene Cards (Weizmann Institute of Science). Figure 5.2 is schematic presentation of the genes on 6q15-21.

In summary, genes mapped to the region between 6q16.3 and 6q21 in a centromeric to telomeric orientation are *GRIK2, BVES (POPEYE protein1), POPEYE protein 3, PREP, PRDM1/BLIMP, APG5L, AIM1, NR2E1/TLX, SNX3, AF6Q21-Forkhead gene* (Table 5.1). Several other genes have been identified on 6q16-21. However, the list above contains the core of the genes that fall within the RMD identified in my study (Table 5.1, labelled in blue). The features of these genes will be briefly described below.

Other gene identified on the same genomic bands but outside the RMD are also listed in Table 5.1 but are not described in greater details here. In addition, there are at present several other genes the existence of which is predicted on sequence analysis, but that still remain speculative and require further functional and structural studies in the future before they are granted a more credible status.

GRIK2 (GLUR6) *or glutamate receptor ionotropic kainate 2* is located at cytogenetic band 6q13.6-6q21; it functions mainly as an excitatory neurotransmitter in the brain. It is highly expressed in cerebellum and cerebral cortex (Paschen W. *et al.*, 1994). *GRIK2* contains markers D6S1555, D6S1543, D6S449, D6S1642, D6S1709/D6S283 and D6S434 (from centromere to telomere). The position of *GRIK2* gene in relation to our RMD and to the published common regions of deletion (CDRs) in ALL suggests the possibility of *GRIK2* as being a tumour suppressor gene. Figure 5.1.

Kainate receptors alter the excitability of mossy fiber axons and have been reported to play a role in the induction of long-term potentiation (LTP) at mossy fiber synapses in the hippocampus. Additionally, short-term synaptic facilitation was impaired in Glur6 knockout mice, suggesting that kainate receptors act as presynaptic autoreceptors on mossy fiber terminals to facilitate synaptic transmission. Contractor and colleagues concluded that their data demonstrate that kainate receptors containing the *GRIK2* subunit are important modulators of mossy fiber synaptic strength (Contractor A. *et al.*, 2001).

According to Knudson hypothesis (tumour suppressor gene model), biallelic inactivation of a gene results from deletion of one allele and inactivation of the second allele by mutation. Further investigations and mutational analysis of the cases that showed deletion of one or more markers of those contained within the gene are required in order to confirm or to rule out the possibility that *GRIK2* is a candidate tumour suppressor gene.

BVES (POP1) *or blood vessel epicardial substance* has been cytogentically localised to 6q21 band, it is expressed in developing and adults heart and skeletal muscles (Reese D. E. and Bader D. M., 1999; Reese D. E. *et al.*, 1999). It belongs to a family of homologous genes with the *POP3* gene lying distal to *POP1* also on chromosome 6.

***PREP**, PEP or prolyl endopeptidase*, is localised to band 6q16-21. It hydrolyses peptide bonds on the C-terminal side of proline residues and found to be expressed in several tissues including colon, gall bladder, uterus, endometrium, B-cells from Burkitt's lymphoma, thymus and human retina (Shirasawa Y. *et al.*, 1994; Vanhoof G. *et al.*, 1994).

PRDM1, BLIMP1 is located proximal to D6S447. It was characterised as the B-lymphocyte-induced maturation protein1 and a repressor of *beta-interferon* gene expression. The protein binds specifically to the PRDI (positive regulatory domain I element) of the *beta-IFN* gene promoter. It functions as a transcription factor, promotes B cell maturation, and represses human beta-IFN gene expression. The specific high level of expression of *BLIMP1* in late B-cells and plasma cells, its induction during B-cell differentiation and its ability to drive maturation of B-cells suggest that this gene may play a role in the differentiation and pathogenesis of B-cells. It was suggested that this gene could play a tumour suppressor role in B-NHL tumours (Mock B. A. *et al.*, 1996) but further evidence of this role has not been produced.

***APG5L**, Autophagy protein 5 like (APG5-like) or apoptosis-specific protein* is localised to 6q21 it may play a role in the apoptotic process (Mock B. A. *et al.*, 1996) (Hammond E. M. *et al.*, 1998).

***AIM1** or absent in melanoma 1* is localised to 6q 21, its expression is associated with the experimental reversal of tumourigenicity of human malignant melanoma. It is a good candidate tumour suppressor gene of malignant melanoma and possibly exerting its function through interactions with cytoskeleton (Ray M. E. *et al.*, 1996; Ray M. E. *et al.*, 1997).

***NR2F1, nuclear receptor TLX** or tailless homologue* is localised at band 6q21. The gene is a member of steroid nuclear receptor superfamily, it is mainly expressed in the brain, it maps to 6q21 between *FYN* and D6S447 and contains marker D6S246 (Jackson A. *et al.*, 1998). (See Figure 5.3) Although this gene is localised within a region of deletion described by our study and several others, our laboratory has already ruled out the role of this gene as candidate TSG based on the lack of mutations on the retained allele (Jackson A. *et al.*, 2000).

Table 5.1. *Lists the known genes mapped to 6q15-21, gene name, synonyms, cytogenetic localisation and known functions.*

Gene	Synonyms	Localization	Function
BACH2	BTB and CNC homolgue1 Basic Leucine transcription factor 2	6q15	Human B- cell specific transcription factor.
MAP3K7	Mitogen- activated protein kinase 7 Transforming growth factor Beta- Activated Kinase 1; TGF Beta Activated Kinase 1	6q16-q16.3	Phosphoactivate undefined map kinase. Mediator of TGF-Beta signal transduction Stimulates NF Kappa B activation
GPR63	G protein coupled receptor 63 PSP24 (beta) PSP24 B	6q16.1-6q16.3	
FUT9	Fucosyltransferase 9 (alpha (1,3) Fucosyltransferase) FUC-TIX Fuc-TIX	6q16	Abundantly expressed in brain and stomach also detected in spleen, Peripheral blood leukocytes.
POU3F	Nervous system specific octamer- binding transcription factor N-Oct 3. Brain specific Homeobox POU Domain Protein 2. BRN-2 Protein contains N-Oct 5A, N OCT 5B. POU domain, class 3, transcription factor 2.	6q16	Transcription factor that positively regulates the genes under control of corticotropin releasing hormone.
FBX24	FBL4 FBL5 F-box and leucine repeat protein	6q16.1-6q16.3	

CCNC	Cyclin C	6q16	May play a role in transcriptional regulation Binds and activates cyclin dependant kinase cdk2 that phosphorelates the ctd (carboxyl terminal domain) of the large subunit of rna polymerase ii (rnap ii).
PRDM13	PR Domain Zinc finger Protein PR domain containing 13	6q16-6q21	
SIM1	Single Mutated Homologue 1 Single minded (Drosophila) homologue 1	6q16.3-6q21	Transcription factor that may have pleiotropic effect during embryogenesis and in the adults.
GRIK2	Glutamate Receptor, Ionotropic Kinate 2 Precursor. Glutamate receptor 6 (GLUR6) Excitatory Amino Acid Receptor 4 (EAA4)	6q16.3-6q21	L-glutamate acts as an excitatory neurotransmitter at many synapses in the CNS.
BVES	Blood vessel epicardial substance POP1 HBVES HBVES bves protein	6q21	
PREP	Prolyl endopeptidase Post proline cleaving enzyme (PE)	6q21	Cleaves peptide bonds on the C-terminal side of prolyl residues within peptides that are up to approximately 30 amino acids long.
PRDM1	Beta interferon gene positive regulatory domain 1 binding factor BLIMP 1 B-lymphocyte induced maturation protein 1PRD1-BF1	6q21-22.1	Repressor of β-interferon gene expression.

APG5L	Autophagy protein 5 like (APG5-like) ASP HAPG5 HAPG5 Apoptosis specific protein APG5 POT1 APG5	6q21	Apoptosis specific protein
AIM1	Absent in melanoma 1	6q21	May function as suppressor of malignant melanoma, it may exerts its effects through interactions with the cytoskeleton
NR2E1	Nuclear receptor. TLX Tailless homologue XTL TLX TLL	6q21	Binds DNA as a monomer to hormone receptor elements (hre) containing an extended core motif half site sequence-aaggtca.
CD164	CD164 antigen Sialomucin MGC-24 MUC-24	6q21	Carcinoma associated protein, probably a mucin CD164 (MGC-24 v) is an adhesion glycoprotein expressed by human haematopoietic progenitors and bone marrow stromal cells that serve as a potent negative regulator of haematopoiesis.
SMPD2	NSMase Sphingomyeline phosphodiesesterase2 Neutral membrane Neutral sphingomyelinase	6q13-6q22.3	

GPR6	Probable G protein- coupled receptor Neutral membrane	6q21-q22.1	Integral membrane protein (orphan receptor)
WASF1	WASF1 WAVE Similar to plant extensin like protein SCAR1 WAS Protein family, member 1 WAVE1 Wiskott-Aldrich Syndrome Protein Family member 1		Downstream effector molecules involved in the transmission of signals from tyrosine kinase receptors to act in cytoskeleton
FYN	FYN oncogene related SRC, FGR, YES	6q21	Implicated in control of cell growth

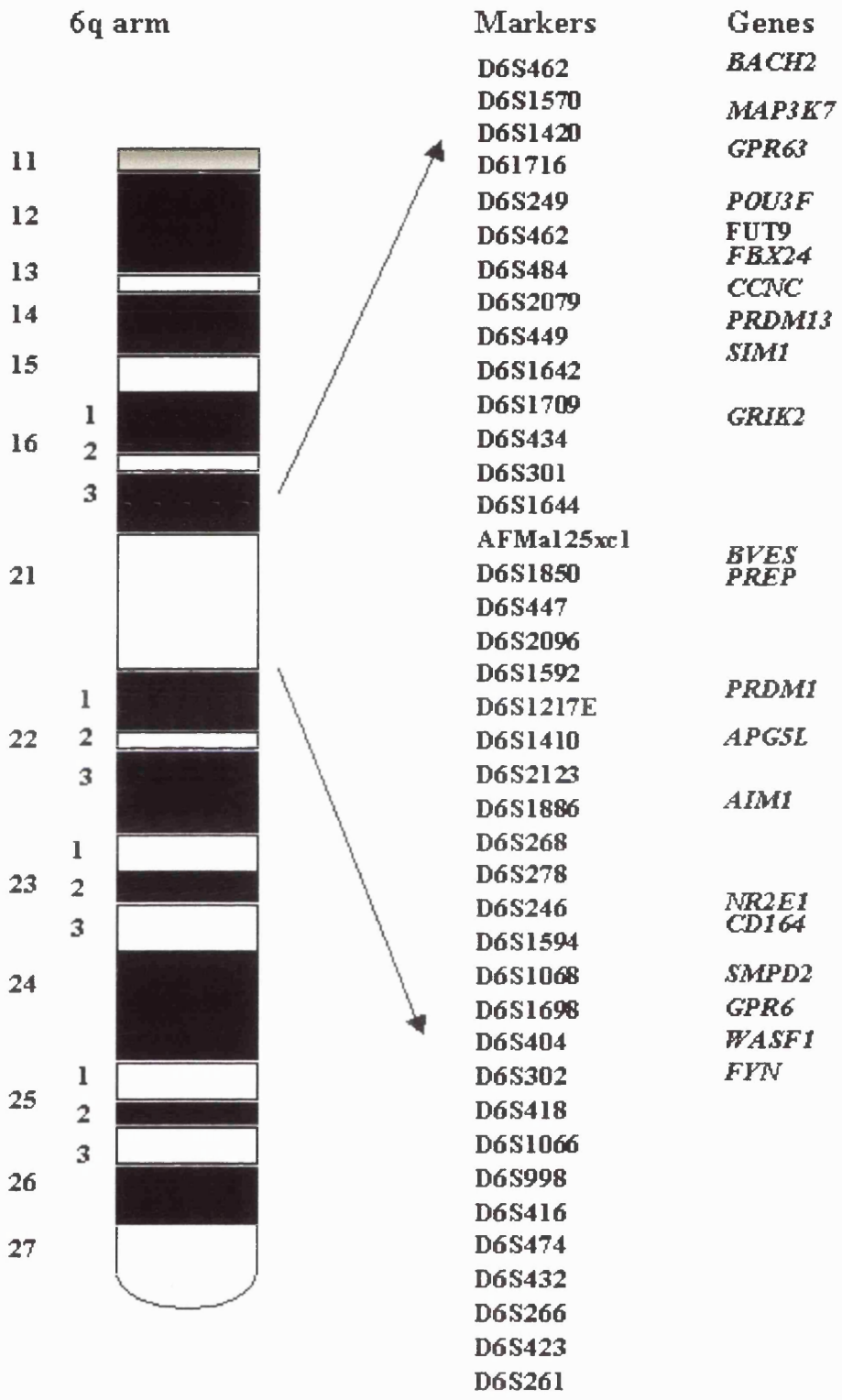


Figure 5.2. *Shows the position of the known genes that mapped to 6q15-21.*

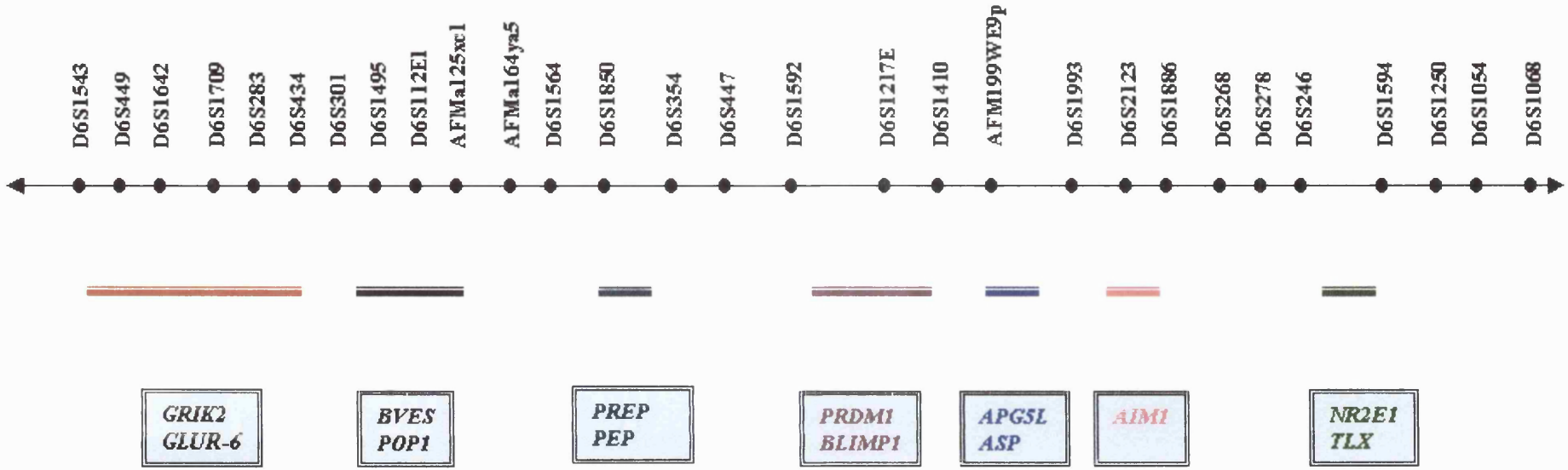


Figure5.3. *Illustrates known genes mapped to our region of minimal deletion on 6q16.3-21.GRIK2; glutamate receptor ionotropic kainate 2, BVES; Blood vessel epicardial substance, PREP (PEP) Prolyl endopeptidase, PRDM1 (BLIMP1); B-lymphocyte-induced maturation protein 1, APG5L (ASP); apoptosis specific protein, NR2E1 (TLX); tailless homologue (Drosophila). This figure is not drawn to scale. The sizes of the lines that represent the genes do not correlate with the size of the genes.*

Finally, while investigation was carried out for LOH analysis several samples showed a pattern compatible with MSI that will now be briefly discussed below.

5.4. Incidence of MSI

The same methods for detection of LOH can be applied to detect MSI. MSI is due to either expansion or retraction of microsatellite repeat sequences as a consequence of slippage of one DNA strand against the other strand. This DNA slippage occurs during DNA replication. Because MMR genes are responsible for correction of such errors, MSI is attributed to defective MMR genes. MSI was detected in 9 (13%) of 69 ALL cases; in 7(10%) cases MSI was detected as the only abnormality and in 2 (3%) cases it was accompanied by LOH of other markers.

MSI was initially reported in HNPCC (Loeb L. A., 1994) as well as other kinds of solid tumours including sporadic colorectal (Fleischhacker M. *et al.*, 1995), gastric, pancreatic (Han H. J. *et al.*, 1993), prostatic (Egawa S. *et al.*, 1995), cervical carcinomas (Larson A. A. *et al.*, 1996) and renal cell carcinoma (Uchida T. *et al.*, 1994).

MSI has been reported previously in several haematological malignancies but it does not appear to be a very common event. It also appears to vary in incidence between myeloid and lymphoid cells. MSI has been infrequently described in the progression from myelodysplastic syndrome (MDS) to AML (Tasak T. *et al.*, 1996); in 8% of the tested cases of MDS with karyotypic alterations (Maeck L. *et al.*, 2000), in a subset of CLL (Gartenhaus R. *et al.*, 1996), mucosa associated lymphoid tissue lymphoma (Peng H. *et al.*, 1996), and in primary cutaneous T-cell lymphoma (Scarisbrick J. J. *et al.*, 2000).

Studies on leukaemia/lymphoma cell lines have detected MSI in 21% of lymphoid leukaemia/lymphoma cell lines but not in myeloid leukaemia cell lines (Kodera T. *et al.*, 1999). In a further study on 117 leukaemia/lymphoma cell lines (44 B lymphoid lineage cell lines, 30 T-cell lymphoid cell lines and 43 myeloid cell lines) MSI was detected in 20% of the lymphoid cell lines and only in 5% of the myeloid cell lines. In these lymphoid cell lines the incidence of MSI was higher in T-cell acute lymphoblastic leukaemia (T-ALL) than in the B-cell lineage malignancies which might indicate that MSI is an important event in the development or progression of lymphoid leukaemia especially of T-ALL (Inoue K. *et al.*, 2000).

This was consistent with the investigation of MSI in human haematopoietic neoplasms, rather than cell lines, which showed a higher prevalence of MSI in ALL (27%) than in AML (10%) (Indraccolo S. *et al.*, 1999) confirming the lineage restriction of the phenomenon. In a series of 48 primary childhood leukaemias, the presence of MSI was investigated using 85 microsatellite makers localized to 12 different chromosomes. MSI was reported in 10% of the cases and interestingly it was localised to the three most frequently deleted chromosome arms 9p, 12p and 6q in ALL (Takeuchi S. *et al.*, 1997b).

Our reported incidence of MSI in ALL is consistent with that obtained from this latter study. MSI occurred more frequently in children (6 out of 35; 17%) than adults (3 out of 34; 8.8%). Although slightly more frequent in T lineage ALL (3 of 17 cases overall; 17%) than in B lineage (6 of 50; 12%) (Chapter 4 paragraph 4.2.2), these differences were not significant. Our study however, restricted its LOH analysis to a small region of chromosome 6 and this may explain some of the differences. This differs from the higher incidence of MSI observed in T than in B-lineage ALL in cell lines studies (Inoue K. *et al.*, 2000). A higher incidence of MSI (45%) has been reported in other haematological malignancies such as ATL (Hatta Y. *et al.*, 1998) that also shows a high incidence of LOH.

As there are no defined criteria for the scoring of LOH and MSI in non-colonic malignancies, we followed the criteria suggested by NCI (Boland C. R. *et al.*, 1998).

- MSI in ≥30-40% of the markers was classified as high frequency type (MSI-H) and MSI in <30-40% of the markers was classified as low frequency type (MSI-L).
- In situations where a marker shows allelic loss (LOH) associated with MSI it was suggested to score those markers showing MSI as non-informative for LOH in tumours with MSI-L, whereas all markers should be scored as non-informative for LOH in tumours with MSI-H phenotype.
- Markers were scored as having either LOH or MSI but not both.
- Cases showing instability in 2 or more of the recommended reference panel markers are defined as having MSI-H, those showing instability in 1 marker only are having MSI-L where as cases with no apparent instability may either be classified as MSI-L or MSS.

It is worth mentioning that in 3 cases that scored as MSI-H (case number 26, 34 and 47) one marker showed LOH. This case, however, was excluded from our calculation of

LOH in keeping with the criteria of MSI and LOH evaluation. This however, may result in an underestimation of the total impact of LOH.

All the microsatellite markers used in order to detect 6q deletions in our study were dinucleotide repeat microsatellite markers. It is known that the dinucleotide repeats are more stable than the trinucleotide and tetranucleotide repeats hence they are less sensitive to detect MSI-L. However, they are less stable than mononucleotide repeat markers, which have been proven to be useful for the detection of MSI-H, such as BAT-25, BAT 26, BAT-40 and some other markers.

5.5. Additional studies to assess MSI

We have used the working reference panel of the 5 markers (BAT-25, BAT-26, D5S346, D2S123 and D17S250) recommend by NCI to define and distinguish MSI-H from MSI-L tumours (Chapter 4 paragraph 4.2). Although this panel is recommended for characterisation of MSI in colorectal cancer primarily, this panel of markers can also be applied to other malignancies as recommended (Boland C. R. *et al.*, 1998). We used this panel to screen 7 of 9 cases that showed MSI by using the 6q21 microsatellite markers. All 3 samples with MSI-H using the 6q21 markers showed a consistent MSI-H with 3 out of 5 markers of the reference panel showing allelic alteration. Three other cases were scored as MSI-L using the 6q21 markers and did not show any apparent instability with the reference panel markers; hence can be either scored as MSI/L or microsatellite stable (MSS) cases (Chapter 4 paragraph 4.4.3). BAT–25 and BAT-26 only showed a wild-type pattern when used to screen 60 of 69 of our patients including those showing MSI-H and MSI-L with other microsatellite markers (by NCI) and 6q markers.

Our results are consistent with the data from a study of the allelic status of both BAT-25 and BAT-26 in 34 primary leukaemias, where no allelic alterations were detected at these two loci in these samples (Inoue K. *et al.*, 2000). This is in contrast to the detection of unstable alleles for BAT-25 and BAT-26 loci in 5 out of 24 (Kodera T. *et al.*, 1999) and in 17 out of 117 leukaemia/lymphoma cell lines (Inoue K. *et al.*, 2000). This discrepancy of the results between cell lines and primary leukaemia samples might be due to the late occurrence of MSI in haematological malignancies (Inoue K. *et al.*, 2000) or changes occurring in cell line under different culture conditions.

BAT-25 and BAT-26 are known to have a quasimorphic profile that facilitate the identification of MSI as the shortened unstable alleles can be easily identified and distinguished from those of normal size. However, it was reported that allelic variations could occur at both loci in African–American people (Pyatt R. *et al.*, 1999) BAT-25 was found to be polymorphic, whereas no allelic variants were detected at the BAT-26 locus in Japanese (Ichikawa A. *et al.*, 2001).

In spite of that some authors have suggested that given the small allelic profile at these 2 loci, MSI can be characterized without the availability of the matched normal DNA for comparison (Hoang J. M. *et al.*, 1997; Zhou X. P. *et al.*, 1997). It was recommended that all studies assessing MSI using these markers should use matched normal /tumour DNA (Butturini A. and Gale R. P., 1990; Pyatt R. *et al.*, 1999). Alternatively, the tumour tissue alone can be used in routine screening of MSI using BAT-25 and BAT-26 Loci, but normal tissue should then be investigated when abnormal alleles are detected (Ichikawa A. *et al.*, 2001). For assessing MSI in our 7 cases we used both tumour and normal DNA, but tumour only was used to screen the 60 cases for MSI.

The previously mentioned reference panel is believed to identify tumours characterised by defective MMR genes mainly in the two major MMR genes, *hMSH2 and hMLH1* (Dietmaier W. *et al.*, 1997). BAT-26 locus is contained within the fifth intron of the *MSH2* gene (Hoang J. M. *et al.*, 1997) and BAT-25 locus is located within intron 16 of the *c-kit* oncogene (Parsons R. *et al.*, 1995).

Recent studies have suggested that immunohistochemical assessment of MLH1 and MSH2 proteins may be an alternative way of MSI identification (Cawkwell L. *et al.*, 1999; Terdiman J. P. *et al.*, 2001). It was reported that immunohistochemistry of MMR protein has a high negative predictive value (i.e. lack of expression is associated with no detectable disease). This is in contrast to molecular detection of MSI using BAT-25 and BAT-26 (Perrin J. *et al.*, 2001) that was shown to provide a less reliable assay according to other investigators (Hoang J. M. *et al.*, 1997).

MSI is seen in more than 90% of HNPCC (Aaltonen L. A. *et al.*, 1994) and only in 10- 15% of sporadic colorectal cancers (Thibodeau S. N. *et al.*, 1996). In HNPCC a germline mutation in *MLH1* and *MSH2* (Liu B. *et al.*, 1996) accompanied by loss of the second allele is the predominant mechanism of MMR inactivation, whereas in sporadic colorectal cancers with MSI, somatic mutations of MMR genes are uncommon (Borresen A. L. *et al.*, 1995) and the hypermethylation of *MLH1* promoter has been recently described as the predominant cause of inactivation of MMR genes (Kane M. F. *et al.*, 1997; Wheeler J. M. *et al.*, 1999).

In haematological malignancies, abnormal expression of *MSH2* was found in 14 of 43 (32.6%) cases of adult acute leukaemia mainly in elderly (over 60 years of age) and in therapy related acute leukaemia suggesting that abnormal expression of MMR genes may play an important role in leukaemogenesis especially in AML of the elderly and secondary to chemotherapy

(Zhu Y. M. *et al.*, 1999). An abnormal expression of *MSH2* has also been reported in leukaemic cell lines (Brimmell M. *et al.*, 1998). Mutational analysis of the *MLH1* in 133 patients with acute and chronic myeloid leukaemia has not detected any mutation in any of the samples. The author suggested that the major MMR gene, *MLH1* has no role in the pathogenesis or progression of myeloid leukaemia (Auner H. W. *et al.*, 1999). However, loss of function may be due to other mechanisms such as promoter hypermethylation that cannot be detected by mutational analysis.

5.6. Impact of LOH and MSI in overall survival

MMR genes play an important role in maintaining the genetic stability of DNA. Inactivation of these genes is associated with "mutator-phenotype" which is thought to lead to accumulation of mutations in oncogenes and tumour suppressor genes. MSI is the hallmark of defective MMR genes. Tumours with MSI have been found to potentially inactivate certain target genes by permitting an increased frequency of mutations in short repeat tracts in the DNA encoding the expressed portions of these genes. Most of the microsatellite alterations result in frame shift mutations that lead to inactivation of the affected allele. Several potential target genes have already been identified including; *TGF-β type II receptor, IGF-II R, BAX, MSH3, MSH6, PTEN, APC* and *β2-microglobulin* (Boland C. R. *et al.*, 1998). In leukaemia lymphoma cell lines with MSI, frameshift mutations were most frequently detected in the *BAX* gene (82%), while the *MSH3, MSH6, TGF-β type II receptor, IGF-II R* genes were less frequently mutated (24-47%). This might indicate that MSI is involved in the development and/or progression of lymphoid malignancies, through the inactivation of *BAX* and other genes (Inoue K. *et al.*, 2000).

A unique clinical and pathological phenotype is associated with tumours showing a MSI/H pattern (about 15% of colorectal cancer), whereas MIS/L and MSS appear to be phenotypically similar. MSI/H colorectal tumours are found predominantly in the proximal colon and show less aggressive clinical course than are stage matched MSI/L or MSS tumours (Boland C. R. *et al.*, 1998). The implication of MSI/H versus MSI/L in haematological malignancies as well as the relationship between replication error and tumour progression in lymphoid neoplasms have not been extensively investigated and additional studies will be required to evaluate the impact of these pattern as prognostic factors in haematological malignancies.

The event-free survival of patient with ALL showed that the survival rate of patients with LOH at 6q was similar to that of patients with a normal 6q (Takeuchi S. *et al.*, 1998). Analysis of the clinical significance of 6q deletions in NHL (Offit K. *et al.*, 1993; Offit K. *et al.*, 1991) showed previously a correlation between NHL subtypes and localisation of 6q deletion. For instance, deletions within 6q25-27 bands were associated with intermediate grade NHL, deletions of 6q23

band were found in low grade NHL lacking a t(14;18) and 6q21 deletions were observed in high grade NHL.

While Tilly et al showed that 6q23-26 deletion is a negative prognostic factor in follicular NHL with high risk of transformation to high grade lymphoma and with significantly shorter survival times (Tilly H. *et al.*, 1994), Whang-Peng et al reported that 6q deletion was associated with shorter survival rates in NHL without t(14;18) (Whang-Peng J. *et al.*, 1995). Offit et al reported no significant difference between the clinical course in the patients with small lymphocytic NHL with or without 6q deletions (Offit K. *et al.*, 1994). Also conflicting data have been reported regarding the prognostic significant of 6q deletions in B-CLL. Oscier et al found a shorter treatment free survival for B-CLL patients with abnormal 6q (Oscier D. G. *et al.*, 1990) whereas no adverse prognostic effect of 6q deletions was found by a banding study compiled by the International Working Party on Chromosomes in CLL (IWCCLL) (Juliusson G. and Gahrton G., 1993). In an interphase FISH study conducted on 285 patients with B-CLL 6q21 deletion was found in a subgroup of patients characterized by a large tumour mass but no inferior outcome (Stilgenbauer S. *et al.*, 1999). No significant association between LOH of 6q and treatment failures in a uniformly treated series of ATL (Hatta Y. *et al.*, 1999) was reported.

Although many studies have described MSI in different haematological malignancies, its relationship to the prognosis has not been fully explored yet. This is mainly due to the requirement to recruit large number of patients with different subtypes of leukaemia and have a long follow up period (Takeuchi S. *et al.*, 1997b). In contrast to the finding that MSI seems to be a good prognostic factor in colorectal carcinoma (Thibodeau S. N. *et al.*, 1993), MSI seemed to be an adverse prognostic indicator in ALL and NHL. It has been reported that 2 of the 6 leukaemic patients with MSI detected at diagnosis relapsed within 15 months and the relapse was refractory to chemotherapy, and that NHL patients with MSI had poor response to chemotherapy. This suggests that MSI in leukaemia might be associated with resistance to chemotherapy (Indraccolo S. *et al.*, 1999).In conclusion MSI for markers on 6q16-21 appears to be a common finding in patients with ALL. The telomeric region of 6q21 (around marker D6S268) appears to be more affected than the centromeric region (around marker D6S283) in an inverted relationship with LOH. This region, overall, shows a rather high incidence of chromosomal instability whether because of LOH or MSI and it clearly indicates a hot spot for chromosomal deletions or translocations.

5.7. Advantages and limitations of Cytogenetics, FISH and LOH for detection of chromosomal deletions.

Knowledge of chromosomal deletions has significantly contributed to the identification of tumour suppressor genes. This follows from the observation that deletion of one allele is the first step towards loss of function that can be achieved by the several mechanisms discussed in several section of this thesis. Massive deletions often obliterate entire chromosomes (monosomy) or chromosome arms in tumour tissue. A typical example of this underlying event led to the discovery of the *RB1* (Knudson, A. G., Jr. 1971).

Such deletions can be identified by cytogenetic analysis, which is sensitive enough to detect chromosomal abnormalities in small population of cells and if deletion is present in more than 5% of the cell population. Although cytogenetic analysis is highly informative in the majority of the cases, its interpretation may be difficult if the karyotype is complex or multiple clones are present. Failure to produce successful metaphases may occur due to problems associated with low mitotic index or poor chromosome morphology.

The development of fluorescence *in situ* hybridisation (FISH) has improved the sensitivity, accuracy and reliability of cytogenetic investigation. Interphase FISH, in particular has contributed to the screening of a large number of non-dividing cells (Harrison, C. J. 2000). The sensitivity and specificity can improve greatly by the use of 2 or 3 probes simultaneously.

However, both cytogenetic and FISH analyses have the problem of being unable to detect small interstitial deletions that might be beyond the resolution of these techniques. Cytogenetic studies have identified non-random 6q deletions in ALL and other tumours including malignant melanoma, renal cell carcinoma, and salivary gland adenocarcinoma (Stenman, G. *et al.* 1989; Hayashi, Y. *et al.* 1990; Millikin, D. *et al.* 1991; Morita, R. *et al.* 1991). FISH studies on 6q deletions helped to identify and the refine the region of minimal deletion on 6q in ALL (Menasce, L. P. *et al.* 1994; Menasce, L. P. *et al.* 1994; Sherratt, T. *et al.* 1997; Jackson, A. *et al.* 2000; Zhang, Y. *et al.* 2000).

As methodological problems in the cytogenetic analysis of solid tumours have restrained attempts to apply standard techniques to screening for deleted chromosomal areas, comparative genomic hybridisation (CGH) has more recently proven to be a powerful genome-wide screening method. Comparative genomic hybridisation (CGH) allows DNA copy number losses and gains to be studied in one hybridisation experiment (Kallioniemi, A. *et al.* 1994). CGH is sensitive for detecting deletions that are 10 to 20 megabases in size (Bentz, M. *et al.* 1998). However, as cytogenetics and FISH, CGH requires cultured cells in addition to its limited ability of detecting small deletions.

In view of the difficulty in obtaining suitable material for cytogenetics and FISH analysis, because of the limited ability to identify small deletions, but thanks to the progress in genome mapping and sequencing, LOH analysis has also made a great contribution to the identification of region of deletions using closely linked microsatellite markers. LOH analysis has helped to identify regions of deletions and direct the search for candidate tumour suppressor genes to specific area of the human genome. As a consequence, several TSGs have been identified from regions showing frequent LOH in tumours (Fearon, E. R. and Vogelstein, B. 1990) both in solid and haematological cancers.

Several types of polymorphic markers have been used in LOH studies including restriction fragment length polymorphism (RFLP) where LOH can be analysed by Southern blot hybridisation using the chromosome specific RFLP probes (Gaidano, G. *et al.* 1992). However the use of RFLP markers is restricted by the need of large amount of DNA, the need for tedious and time consuming Southern hybridisation assays and their relatively low level of informativeness.

Microsatellite markers are short tandem repeats of DNA that are distributed throughout the genome. Repetition of a microsatellite may be based on single base (A)n or poly A, two bases (CA)n or dinucleotide, or three or more bases. Microsatellites may be either monomorphic and show no variation in size between cells or individuals, or polymorphic showing a variety of sizes. They are stable and very occasionally the length of a microsatellite marker can be seen to change when it passes from one individual to another. The polymorphic nature of the microsattellite markers together with the use of PCR based technique helped to overcome the problems associated with RFLP. The amount of DNA is very limited and the markers are numerous and most of

them are highly informative. PCR amplification using oligonucleotide primers is rapid an can be automated. Analysis of LOH can be either done by separating isotipically labelled (^{32}P- or preferably ^{33}P) PCR products on denaturing polyacrylamide gels followed by autoradiography or one primer of each PCR primer pair can be labelled with a fluorescent dye then after amplification the PCR products are analysed on an automated DNA sequencer using the software programme (Gene Scanning).

LOH was identified only when substantial reduction was measured in the ratio of allele radiographic signal intensities in the tumour sample relative to that in the corresponding normal sample (Takeuchi, S. *et al.* 1995; Gerard, B. *et al.* 1997; Merup, M. *et al.* 1998; Takeuchi, S. *et al.* 1998).

Although LOH analysis using microsatellite markers is a powerful technique that identified higher frequencies of chromosomal deletions than the reported frequencies by cytogenetic studies but it still show some limitations.

These limitations include the requirement of tumour cell purity that exceed 70-80% as the presence of contaminating non-tumour DNA may affect the interpretation. In our study on 6q deletions in ALL we have selected our samples with the percentage of blasts of at least 80-90% in cases of the tumour samples and the non-tumour samples correspond to remission specimen collected at time of morphological and clinical complete remission. Additional limitations include uninformative markers with limited polymorphism or lack of markers within a critical region when aiming at accurate identification of deletions.

In the definition of RMD, interstitial deletions can be difficult to interpret but are common to every study, which has implemented LOH, and 6q deletion analysis is no exception (Gerard, B., Cave, H. *et al.* 1997; Takeuchi, S. *et al.* 1998). In the study presented here we have obtained similar pattern of interstitial deletions with some cases showing loss of some markers and retention of others. Only 3 out of 16 cases showed contiguous deletions and 9 out of 16 cases studied showed loss of only one marker which was not consistent with one specific microsatellite marker. The presence of such interstitial deletions may be due to the power of LOH technique to pick small deletions that would be missed by cytogenetics.

Another potential interpretation pitfalls may occur when one of the alleles of a certain microsatellite marker is missing and no other novel fragment are present. This case is usually not easy to interpret, as this might represent true LOH or MSI in which the shifted allele has migrated with the wild type allele. The NCI workshop recommended

that such a finding should be scored as negative for MSI. However, the frequency that a shifted allele would co-migrate with the remaining wild allele is believed to be low.
Also, difficult interpretation is encountered when a specific marker shows both apparent LOH and the presences of novel fragments (MSI). In such situations it is difficult to distinguish whether the apparent allelic loss is due to MSI or it is truly present in addition to MSI. Those markers showing MSI should be scored as non-informative for LOH in tumours with MSI/L, whereas all markers should be scored as non-informative for LOH in tumours with MSI/H (Boland, C. R. *et al.* 1998).

5.8. Future studies

The results discussed above suggest that cytogenetics alone may be insufficient in the characterisation of 6q deletions. FISH on the other hand may fail to identify small interstitial deletions which may be relevant for the definition of RMD. LOH although very sensitive, may only provide information with regards to the identification of region of minimal deletion, but may be insufficient for the identification of candidate TSGs and the definition of RMD may be complicated by interstitial deletions.

For a comprehensive evaluation of the extent and role of 6q deletion future studies may therefore include:

1. Expansion of the total number of patients analysed by LOH.
2. Narrow down the region of minimal deletion to smaller genomic region by using novel polymorphic sites identified through human genome project.
3. Sequence analysis of genes such as *GRIK2* for the identification of somatic mutations responsible for the inactivation of the gene on the retained allele in patients with heterozygous deletions
4. Analysis of other genes like *PRDM1*, as it was previously suggested that this gene could play a TSG role in B-NHL tumours but confirmation of this role has not been obtained yet. No mutation analysis has yet been reported for the *BLIMP* gene and this investigation should be performed in the future.
5. Investigation on the role played by other candidate genes including: *AIM1* (a candidate TSG in malignant melanoma) and *NR2F1/TLX* gene. The role of the latter gene as a TSG gene has been previously ruled out as based on lack of mutations of the retained allele. However, epigenetic mechanisms should also be further investigated although this may depend on the correct identification of promoter sequences.
6. Expansion on the number of cases showing MSI pattern to achieve a better evaluation on the role of MSI in both childhood and adult ALL as this is a novel finding of this study.

7. Investigation of the MMR in the cases that showed MSI. Investigation of the protein expression of MMR genes by immunohistochemical staining would be possible as monoclonal antibodies to at least 4 MMR genes (*MLH1*, *MSH2*, *PMS1* and *PMS2*) are now available. Although no previous reports on using this technique for haematological malignancies with MSI, paraffin embedded sections of trephine bone marrow biopsies may be the suitable material for the future investigations. Newly diagnosed patient's material should be stored for this type of investigation in the future. In addition mutational and epigenetic studies of MMR genes are required to identify possible mechanisms responsible for MSI.
8. Analyse the sequence of the YAC clones (yR39EC11 and yX28F10 presently partially sequenced) to identify the structural characteristics that may explain the difficulty in the cloning of these regions.
9. Use sequence analysis of the two above YACs to identify candidate genes within these YACs that may be relevant to this investigation.

All the above aspects will be important in the planning of the future studies on chromosome 6 deletion in ALL and may help to redefine the involvement of novel genes in the pathogenesis of acute leukaemia in humans.

Chapter 6

A study of chromosome 8 abnormallties in T-Prolymphocytlc leukaemia (T-PLL) with special reference to band 8p11-12

6.1. Introduction

Based on the immunological together with the morphological characteristics, lymphoid malignancies are classified into B-cell and T-cell malignancies. T-cell disorders are broadly divided into thymic-derived (immature) and postthymic-derived (mature) types. Thymic derived leukaemias and lymphomas are lymphoblastic proliferations that occur most frequently in children and young adults (Bennett J. M. *et al.*, 1989).

T-prolymphocytic leukaemia (T-PLL) and large granular lymphocytic (LGL) leukaemia are the only two primary leukaemias of the mature or postthymic-derived malignancies (Bennett J. M. *et al.*, 1989). The French-American-British cooperative group (FAB) recognised T-PLL as an entity distinct from LGL leukaemia (Bennett J. M. *et al.*, 1989). Studies by other groups have established and justified the separation of these two diseases (Matutes E. *et al.*, 1991).

T-PLL is a rare mature T-cell disorder (Matutes E. *et al.*, 1991) characterised by an aggressive clinical course resistant to therapy. It is usually associated with lymphadenopathy, splenomegaly, skin lesions, and rapidly increasing lymphocytosis (Hoyer J. D. *et al.*, 1995; Matutes E. *et al.*, 1991). T-PLL is also called T-cell chronic lymphocytic leukaemia (T-CLL) due to the maturity of the proliferating cell (Hoyer J. D. *et al.*, 1995; Wong K. F. *et al.*, 1996), but morphologically it is well distinguished from typical CLL. T-PLL is morphologically characterised by irregular nuclear shape, deep basophilic cytoplasm, cytoplasmic protrusions, and the presence of a prominent nucleolus that suggested immaturity, and hence the term T-PLL which remains in the FAB proposals (Bennett J. M. *et al.*, 1989; Foon K. A. and Gale R. P., 1992; Matutes E. and Catovsky D., 1993; 1996; Matutes E. *et al.*, 1986).

A very striking karyotypic similarity exists between T-PLL and an aggressive mature T-cell leukaemia, which affects patients with Ataxia Telangiectasia (AT), a rare familial disease with predisposition to chromosomal breakage and an elevated risk of cancer at several sites (Brito-Babapulle V. and Catovsky D., 1991; Taylor A. M. *et al.*, 1996).

The link between AT-associated T-cell disorder and sporadic T-PLL has been supported by the demonstrations of frequent AT gene biallelic mutations in sporadic T-PLL (Stilgenbauer S. *et al.*, 1997; Vorechovsky I. *et al.*, 1997). Specific abnormalities found in T-PLL and in premalignant T-cell clones in AT patients are inv(14)(q11q32), t(14;14)(q11q32) and t(X;14)(q28;q11). These rearrangements juxtaposes the TCL-1 (located at 14q32) or MTCP-1 (located at Xq28) genes to TCR α/δ locus on 14q11 (Stern M. H. *et al.*, 1993; Virgilio L. *et al.*, 1994), resulting in overexpression of $p14^{TCL1}$ and $p13^{MTCP1}$ (Fu T. B. *et al.*, 1994; Stern M. H. *et al.*, 1993). $p13^{MTCP1}$ has been found to be restricted to T-PLL (Madani A. *et al.*, 1996). Cytogenetic data in the reported cases of T- cell leukaemia in AT patients revealed that additional chromosomal abnormalities are present in the same clone with the development of overt leukaemia. This suggests that the transition from premalignant to malignant phase is dependent on additional abnormalities such as i(8q), which is a frequent abnormality in T-PLL cases. (Brito-Babapulle V. and Catovsky D., 1991; Maljaei S. H. *et al.*, 1998).

The common types of chromosome 8 abnormalities in T-PLL are idic(8)(p11), t(8;8)(p11;q12) and 8p+. These abnormalities are usually associated with polysomy for 8q. In a study conducted on 21 T-PLL cases, it has been found that about 80% of the cases have chromosome 8 abnormalities, of which 57% had either i(8q) or t(8;8)(p11;q12). However the formation of chromosome 8 abnormalities in T-PLL consistently involve 8p.

6.2. Aim of study

The main aim of this study was to investigate any available cases of T-PLL for:

1. Characterisation of chromosome 8 abnormalities by G-banding and whole chromosome 8 paint.
2. Examination of the distribution of breakpoints using FISH with a series of YAC probes mapping to 8p11-p12.
3. Identification of recurrent breakpoints by determining the presence or absence of YAC signals on metaphase and interphase cells.
4. Detection of any translocated or splitted YAC signals on metaphase chromosomes.
5. Testing few candidate genes such as MOZ, FGFR1, and ECT for rearrangement in T-PLL.

6.3. Material and methods

Peripheral blood samples were obtained from untreated patients diagnosed with T-PLL at the Royal Marsden Hospital NHS Trust based on the clinical, morphological and immunological criteria. All the cases were first subjected to conventional cytogenetic analysis and, chromosome 8 specific paint by FISH. The cases that showed any of the chromosome 8 abnormalities mentioned in the introduction were subjected to further study by FISH using a series of 11 YAC probes mapping to 8p11-p12, to define the breakpoint.

6.3.1. Cytogenetics analysis

1-2x10^6cells was seeded in 10ml of RPMI-1640 medium with supplements; four 10 ml cultures were prepared for each sample and incubated at 37°C as follows:

- Colcemid overnight (CON) culture (50µl colcemid; final concentration 0.05µg/ml).
- 3 days phytohemagglutinin (PHA) culture (0.2ml PHA/10ml; final concentration 20µg/ml).
- 5 days PHA culture as above.
- 5 days PHA+ phorbolester (TPA) (0.1ml PHA+0.1ml TPA; final concentration 2.5µg/ml).

6.3.1.1. Harvesting protocol

1 hour prior to harvesting, 0.1ml colcemid/10ml culture (final concentration 0.1µg/ml) was added (except for the CON culture). The culture was gently mixed and returned to the incubator. At the end of the hour the contents of the culture flask were transferred to a 10ml round bottomed tube and centrifuged at 1500rpm for 10 minutes.

The supernatant was removed using plastic Pasteur pipette and the pellet was resuspended. 10 ml hypotonic solution (0.075M KCL at 37°C) was added and the tube was incubated at 37°C for 10 minutes. The tube was then centrifuged at 1,500rpm for 10 minutes. The supernatant was removed, discarded and the pellet was resuspended.

10 ml of freshly prepared ice-cold fixative (3 parts methanol: 1 part glacial acetic acid) was slowly drop-wise added whilst gently shaking the tube to prevent clumping.
Centrifugation was then done at 1,500rpm, the supernatant was removed and the pellet was resuspended, 10ml of fixative was added and centrifugation was then carried out as before.
The previous step was repeated once more or until the fixative was no longer brown/yellow.
After the final wash the supernatant was removed and the pellet was gently resuspended by rolling between palms. The fixative was added drop-wise until the cell suspension was slightly cloudy.
Using a plastic Pasteur pipette a drop of cell suspension was placed on a pre-cleaned slide just removed from 90% ethanol and wiped dry.
The slide was then examined under a phase contrast objective. If the chromosomes were poorly spread the cell suspension was then diluted and another slide was prepared. If there were very few cells the sample was centrifuged again and the pellet was resuspended in smaller volume of fixative than previously used and another slide was prepared.
Slides for banding were placed on a slide heater at 50-60°C for 24 hours or at 37°C for 3-5 days, and slides for FISH were left to dry for 1 hour then wrapped in aluminium foil and stored at –20°C until use.

6.3.2. Fluorescence in situ hybridisation (FISH) protocol

6.3.2.1. Slide preparation

The slide was pre-treated with 100µl RNase A working solution (10µl of RNAse A 10mg/ml of stock solution in 1ml 2xSSC, pH 7.0), covered with 22x50mm coverslip and incubated in a pre-warmed humidified chamber at 37°C for 1 hour.
The slide was then washed once in 2xSSC and once in PBS each for 5 minutes at room temperature.
Pepsin pre-treatment was then done at 37°C for 10 minutes. 0.1mg/ml pepsin (100µl of pepsin 10% stock solution in 100 ml SQ water, pH 2-2.5) was added just before the slide was placed in the coplin jar and the pH of the SQ water was adjusted to pH2-2.5

before it was warmed to 37°C.

The slide was washed in PBS for 5 minutes, and incubated at 37°C for 30 minutes in 2xSSC pH 7.0.

Dehydration was performed in ascending concentrations of ethanol series 70%, 90% and 100% for 2-5 minutes each and then left to dry at room temperature.

6.3.2.2. Probe preparation

Commercial probes were prepared and denatured as instructed by manufacturer, and home-grown probes were diluted when necessary with hybridisation buffer so that the volume would give 10µl probe/slide.

The probe was denatured for 5-10 minutes using a hotplate pre-warmed to 70°C, and then preannealed at 37°C for 30 minutes.

6.3.2.3. Slide denaturation

100µl of denaturing solution was applied and the slide was covered with a 22x50mm coverslip.

The slide was denatured for 1 minute on a hotplate pre-warmed to 70°C, and then washed in 2xSSC pH 7.0 at room temperature for 5 minutes.

Dehydration was then done through 70%, 90% and 100% ethanol series, 2-5 minutes each and the slide was then left to dry.

6.3.2.4. Hybridisation

10µl of denatured probe was added to the slide, which then covered with 22x22mm coverslip, the edges of the coverslip were then sealed with vulcanising rubber solution.

The slide was then incubated in a prewarmed humidified chamber at 37°C for at least 12-16 hours.

6.3.2.5. Post hybridisation washes

The following day, the slide was washed three times in 1x SSC at 45°C, three times in

0.1xSSC at 60°C, 5 minutes each and finally in 4xSSC/0.05% Triton X, pH 7.0 at room temperature for 5 minutes.

A. For the directly labelled probes

The slide was dehydrated through 70%, 90%, 100% ethanol series, 2-5 minutes each, and left to dry then mounted in vectashield with DAPI and stored at 4°C.

B. For the indirectly labelled probes

100µl of blocking solution prewarmed at 37°C was applied to the slide, and the slide was covered with 22x50 mm cover slip and, incubated in a prewarmed humidified chamber at 37°C for 15 minutes.
The slide was then washed briefly in 4xSSC/0.05%Triton X, pH 7.0.
100µl of the first antibody layer was applied to the slide, which was then covered with 22x50 mm coverslip and incubated in a prewarmed humidified chamber at 37°C for 15 minutes.
The slide was then washed twice in 4xSSC/0.05% Triton X, pH 7.0 with gentle agitation at room temperature, 5 minutes each.
100 µl of the second antibody layer was applied to the slide with the same washing steps done as before.
The slide was dehydrated through 70%, 90%and 100% ethanol series, 2-5 minutes each, left to dry, mounted in Vectashield with DAPI and stored at 4°C until use.

6.3.2.6. Preparation of antibody layers for the detection of biotin and digoxigenIn labelled probes

The antibody diluent (10% Marvel, 4xSSC/0.05% TritonX pH 8.5) was centrifuged for 2 minutes at 13,000 rpm.
The supernatant was filtered using a 13mm, 0.2µm syringe filter and stored at 4°C until use.
The antibodies were removed from the fridge and centrifuged at 13,000rpm for 2 minutes to prepare the antibody cocktail:

First layer	Volume
FITC-Avidin (stock 5mg/ml)	0.5µl
Rhodamine anti-Digoxigenin (stock 200mg/ml)	10µl
Antibody diluent	90µl
Second layer	
Anti- Avidin (stock 0.25mg/ml)	4µl
Texas red anti-Sheep (stock 0.3mg/ml)	5µl
Antibody diluent	90µl

6.3.3.Labelling of DNA for indirect detection (Nick translation)

All reagents and tubes were placed on ice.

The following were added to a tube labelled with the name of the probe to be indirectly labelled:

Reagent	Volume
Distilled water	27-n* µl, where n is the volume of probe containing 1µg of DNA
10XNT buffer	5µl
DDT (0.1M)	5µl
dNTP mix (0.5mM dATP, dCTP, dGTP, 0.1mM dTTP)	4µl
Biotin-16-dUTP or Digoxigenin –11-dUTP	2µl
Probe DNA	n*µl
DNase I 1mg/ml stock diluted 1:1000 in double distilled water	5µl
DNA Polymerase I	2µl
Total volume	50µl

The tube contents were then mixed, centrifuged for 2-5 seconds at 13,000rpm and, then incubated at 15°C for 2 hours.
The tube was then placed on ice and the following reagents were added:

Cot DNA (1mg/ml)	100µl
Salmon sperm DNA (10mg/ml)	5µl
Yeast RNA (10mg/ml)	5µl
3M Na acetate	1/10 volume
Ice cold 100% Ethanol	2.5 volume

The tube contents were vortexed to mix and incubated at –20°C overnight or at –80°C for 1 hour.
The reaction was centrifuged at 13,000rpm for 10 minutes, the supernatant was discarded and, the pellet was left to dry.
The pellet was resuspended in 25µl hybridisation buffer and stored at –20°C.

6.4. Results

Frozen fixed material for cytogenetics and frozen slides were available from 6 cases (cases number 1-6) that obtained prior to the start of this project, partial cytogenetic data of these cases showed evidences of clonal abnormalities specific for T-PLL. Some other new T-PLL cases were obtained (cases number 7-13) during the course of this study .The latter cases were analysed cytogentically using G-banding. Whole chromosome 8 painting using FISH was performed on all the 13 cases in order to confirm the presence or absence of chromosome 8 abnormalities. Table 6.1 shows combined cytogenetic and chromosome 8 paint findings of the studied cases.
In cases 1, 2, 3, 4, 7 and 8 an idic(8)(p11) was present with absence of one normal chromosome homologue see (Figure 6.1.A). In case 5, idic(8)(p11) with loss of normal chromosome 8 and again of a marker chromosome containing chromosome 8 material; add(8) was observed see (Figure 6.1.B). In case 6, three subclones were detected. Subclone A showed idic(8)(p11) with loss of one normal chromosome 8; subclone B

had both normal chromosome 8 homologues replaced by an idic(8)(p11), a derivative der(8)t(8;13)(p11;q10?) and a marker containing chromosome 8 material add(8); subclone C had a derivative and the marker but no idic(8)(p11) see Figure 6.1.C.

Case number	Partial or complete karyotype	Whole chromosome 8 paint
1	inv(14)(q11q32), i(8)(q10)	idic(8)(p11)
2	inv(14)(q11q32), i(8)(q10)	idic(8)(p11)
3	inv(14)(q11q32), i(8)(q10)	idic(8)(p11)
4	inv(14)(q11q32), i(8)(q10)	idic(8)(p11)
5	inv(14)(q11q32), i(8)(q10), der(8)t(8;?)	Idic(8)(p11), add(8)
6	inv(14)(q11q32), i(8)(q10)	6A: idic(8)(p11) 6B: idic(8)(p11), der(8)t(8;13)(p11;q10?), add(8) 6C: der(8)t(8;13)(p11;q10?), add(8)
7	42-45,XX, -X[2], add(1p)[3], add(6q)[1], -8, i(8)(q10)[5], -12, add(12p)[1], -13[5], -14, t(14;14)(q11;q32)[5],17,add(17p)[2], -21[2], -22[2], 3-5 mar.[cp5]	idic(8)(p11)
8	43-44,Y, -X, -7[3], i(8)(q10)[3], -11[10], -12, add(12p)[3]/-12, dup(12)(q13q24)[3] , inv(14) (q11q32)[10], -22, -22[9],rx 1-3, mar1, mar2, mar3.[cp10]	idic(8)(p11)

9	44-45, XX, -X[2], -1, add(1p)[2], -5, add(5p)[3],-9[2], -10[2],-12[1], -13[4],-14, t(14;14)(q11;q32)[4], i(17)(q10)[2], -22[2], 2 -3 mar[4].[cp4]	No chromosome 8 abnormalities detected
10	43-47, XX, dup(7)(q11-12q32)[9],-14, inv(14)(q11q32)[10], +17[9], -18[10], 1-3 mar.[cp10]	No chromosome 8 abnormalities detected
11	44 -47, XX, -1, -13, t(1;13)(p22;q12), inv(14)(q11q32)[9], 2-3 mar[9].[cp9]	No chromosome 8 abnormalities detected
12	46, XY[4], 46, X, -Y, -16, mar[6][cp6]	No chromosome 8 abnormalities detected
13	41-44, XY, add(2q), -5, -7, add (8p), add(11q)[3] -12, add(13q), inv(14)(q11q32), add(18)[7].[cp7]	No chromosome 8 abnormalities detected

Table 6.1. *Combined cytogenetics and FISH using whole chromosome 8 paint.*

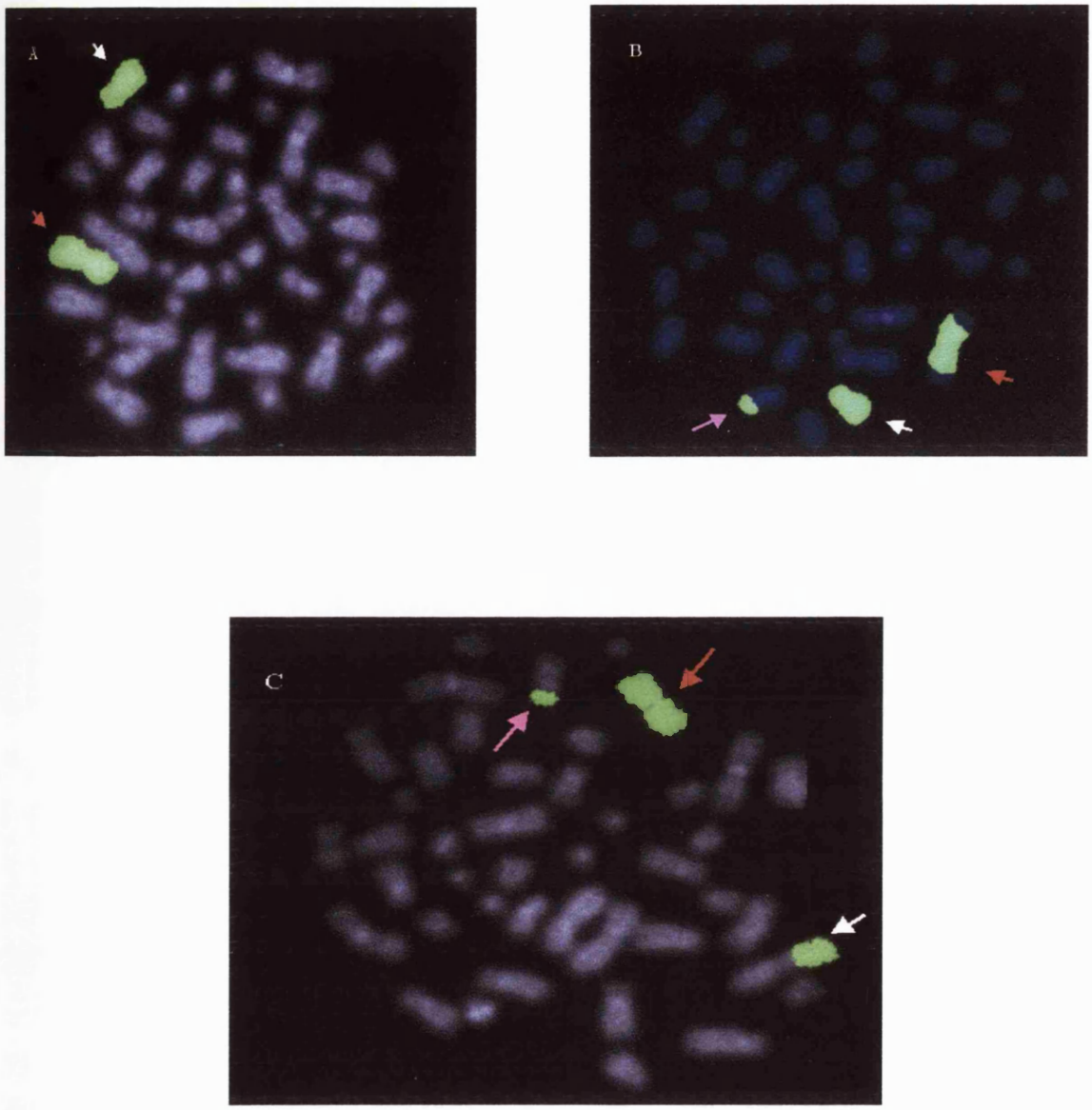


Figure 6.1. *Summarises the different patterns of whole chromosome 8 paint.*
A*. Shows the characteristic pattern in case numbers 1, 2, 3, 4, 7 and 8 .Red arrow points at idic(8)(p11), white arrow points at normal chromosome 8.*
B*. Shows the characteristic pattern in case number 5.Red arrow points at i(8)(p11), white arrow points at normal chromosome 8 and pink arrow points at add(8).*
C. *Shows the characteristic pattern in case number 6. Red arrow points at i(8)(p11), white arrow points at der(8) and pink arrow points at add(8).*

FISH was carried out on these 8 cases using 11YAC clones mapped to 8p11-p12 from CEPH Megayac library (Genethone Corporation, Paris, France) in order to localize 8p11-p12 breakpoints in those T-PLL cases with involvement of 8p.

These YAC clones were selected from the Whitehead institute YAC contig database (http://www.genome.wi.mit.edu). YAC names, ordered from telomere to centromere on 8p11-p12, were as follows (size in kilobase pairs (Kb) is indicated in parentheses): 896f4 (1630), 750h12 (1190), 938b2 (1260), 955b4 (930), 807a2 (1550), 899e2 (1610), 856b8 (1000), 949a12 (1480), 940f10 (1440), 806e9 (1770) and 910d7 (810).

The 8p region was tested using each of these YAC probes with a chromosome 8-specific centromeric probe in the same hybridisation. Control slides with normal metaphases from healthy individuals were hybridised at the same time with each of the YACs used to exclude chimerism and to check quality of the hybridisation conditions used, and hybridisation of YACs to normal chromosome 8 acted as an internal control. In the controls and T-PLL cases, the localisation of the YACs to 8p11-p12 was confirmed on normal chromosome 8. In T-PLL cases 1 and 3 YAC 899e2 and all of the centromeric YACs were present on the isodicentric chromosome (Figure 6.2 A-C), further characterisation was undertaken with YAC 807a2 (which forms an overlapping contig with both YACs 955b4 and 899e2). This YAC gave a signal on the normal chromosome 8 and on idic(8)(p11) in both cases, however the signal was smaller in size on the idic(8)(p11) than on the normal 8, consistent with each case having a breakpoint within this 1.5 MB region (Figure 6.2 D).

The most telomeric YAC hybridising to the idic(8)(p11) in case 4 was YAC 910d7 see (Figure 6.3), and in case 5 was YAC 806e9 (Figure 6.4).

Although karyotyping and whole chromosome 8 paint in cases 2 and 7 showed a single clone carrying 8, idic(8)(p11), probing with the YACs has identified 2 subclones in each case that differed in the number of YACs, which hybridised to the idic(8)(p11). Three different clones were identified by karyotyping and whole chromosome 8 paint in case 6. In the idic(8)(p11) in subclone A, YAC 955b4 and those telomeric to it were deleted; however, a small signal was present with YAC 807a2, as in case 1 and 3. The idic (8)(p11) in subclone B retained YAC 806e9, while the 8p11 breakpoint in the t(8;13) in subclone B and C left YAC 910 intact. The marker chromosome with chromosome 8 material, present together with t(8;13)(p11;q10?) in clones B and C, had YACs 940f10

and all YACs telomeric to it Figure 6.5. Thus it seems that an interstitial loss of the segment between YACs 910d7 and 940f10 have occurred during the translocation. In case 8 all the tested YACs were retained in all the tested metaphases without identification of any breakpoint at 8p11-12.

Table 6.2 summarises the dual colour FSH results in the 8 cases.

Case No	1	2	2	3	4	5	6	6	6	7	7	8
Subclones	-	A	B	-	-	-	A	B	C	A	B	-
YAC	20	2/15	13/15	20	15	15	10/20	6/20	4/20	17/20	3/20	20
896f4	▫	▫	▫	▫	▫	▫	▫	▫	▫	▫	▫	▪
750h12	▫	▪	▫	▫	▫	▫	▫	▫	▫	▫	▫	▪
938b2	▫	▪	▫	▫	▫	▫	▫	▫	▫	▫	▫	▪
955b4	▫	▪	▫	▫	▫	▫	▫	▫	▫	▫	▫	▪
807a2	▪	▪	▪	▪	▫	▫	▪	▫	▫	▫	▪	▪
899e2	▪	▪	▪	▪	▫	▫	▪	▫	▫	▫	▪	▪
865b8	▪	▪	▪	▪	▫	▫	▪	▫	▫	▫	▪	▪
949a12	▪	▪	▪	▪	▫	▫	▪	▫	▫	▫	▪	▪
940f10	▪	▪	▪	▪	▫	▫	▪	▫	▫	▫	▪	▪
806e9	▪	▪	▪	▪	▫	▪	▪	▪	▫	▫	▪	▪
910d7	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪	▪

Table 6.2. *Shows the presence (▪) or absence (▫) of the signal of the 11 YACs used in the study of the 8 T-PLL cases, subclones are indicated by A, B, and C with the number of metaphases per clone shown beneath them.*

This FISH study revealed that 8p11-p12 breakpoints in the studied cases of T-PLL have occurred in two regions. In clones and subclones 1, 2B, 3, 6A, 7B the 8p11-p12 breakpoint on idic(8)(p11) was in the region flanked by YACs 955b4 and 899e2. Using another YAC which overlaps the above two YACs a significant difference in size of the obtained signal was seen between normal 8 and idic(8)(p11) in these clones. This suggested that the breaks occurred within YAC 807a2 telomeric to FGFR1 gene. In clones and subclones 4, 5, 6B, 6C and 7A, the breakpoints were found to be on both sides of the region defined by YAC 806e9 and flanked by centromerically and YAC940f10 telomerically. It is of interest that case number 6 showed all the kinds of breakpoints obtained in this study, and also showed that the breakpoint in t(8;13)(p11;q10?) is different from that obtained in the myeloproliferative disorder with a translocation involving 8p11(Aguiar R. C. *et al.*, 1997; Reiter A. *et al.*, 1998; Smedley D. *et al.*, 1998a; Smedley D. *et al.*, 1998b).

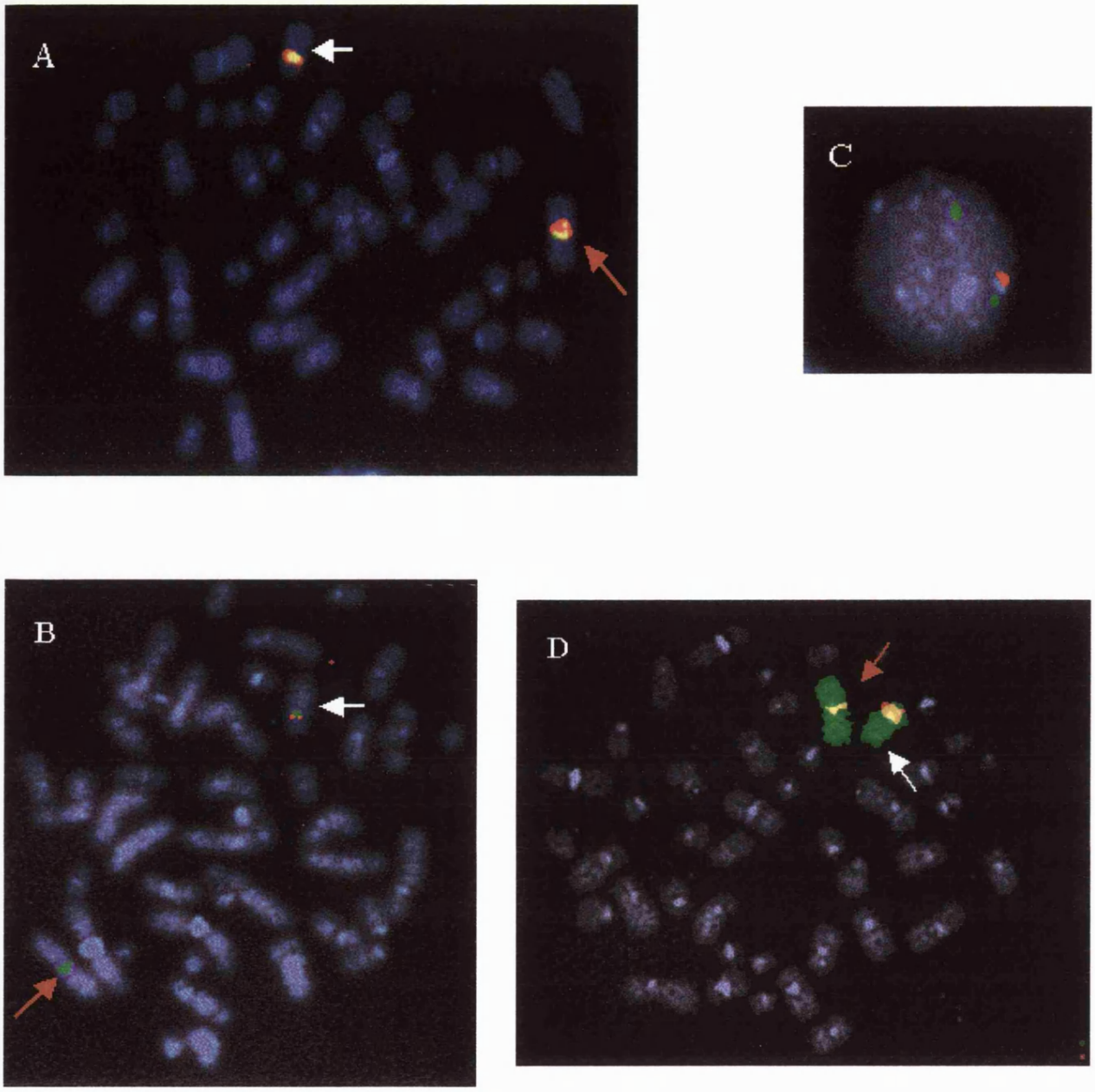
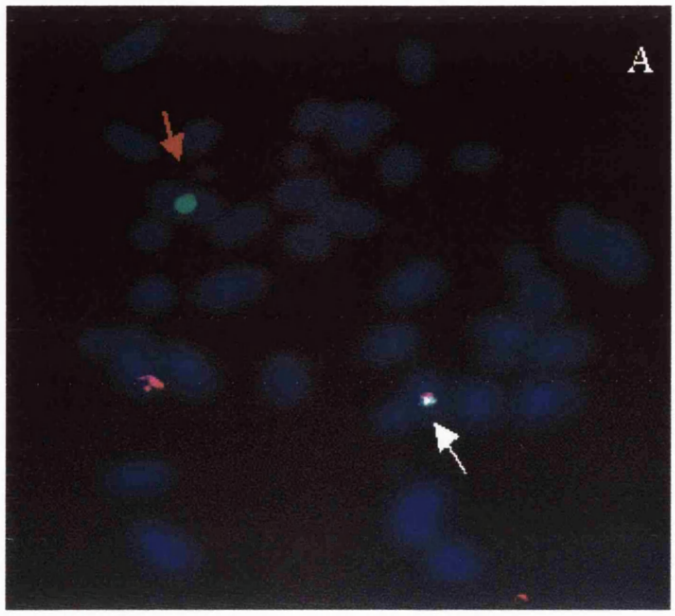


Figure 6.2. *Fluorescence in situ hybridisation (FISH) using chromosome 8 specific centromeric probe (green) and 8 p11-p12 YAC probes (red) representing the pattern obtained in cases number 1, 2 (clone B), 3, 6 (clone A) and 7 (clone B). Red arrows point at i(8)(p11) and white arrows point at normal chromosome 8.*
A. *Shows positive YAC 899e2 signal on i(8)(p11), normal 8*
B. *Shows negative YAC 955b4 signal on i(8)(p11).*
C. *An interphase cell showing 2 green signals for chromosome 8 specific centromere probe while showing only one red signal of YAC955b4.*
D. *Whole chromosome 8 paint (green) and YAC807a2 probe (red) showing that the signal on the idic(8)(p11) is smaller than its counterpart on the normal chromosome 8.*



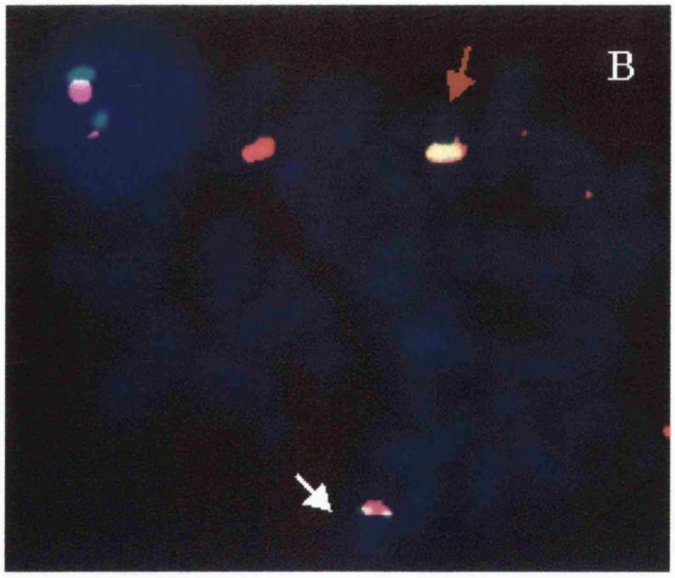


Figure 6.3 *Fluorescence in situ hybridisation (FISH) using chromosome 8 specific centromeric probe (green) and 8p11-12 YAC probes (red) showing breakpoint localisation in cases number 4 and in case number 7 (clone A). Red arrows point at i(8)(p11) and white arrows point at normal chromosome 8.*
A. *Shows negative YAC 806e9 signal on i(8)(p11).*
B. *Shows positive YAC 910d10 signal on i(8)(p11)*

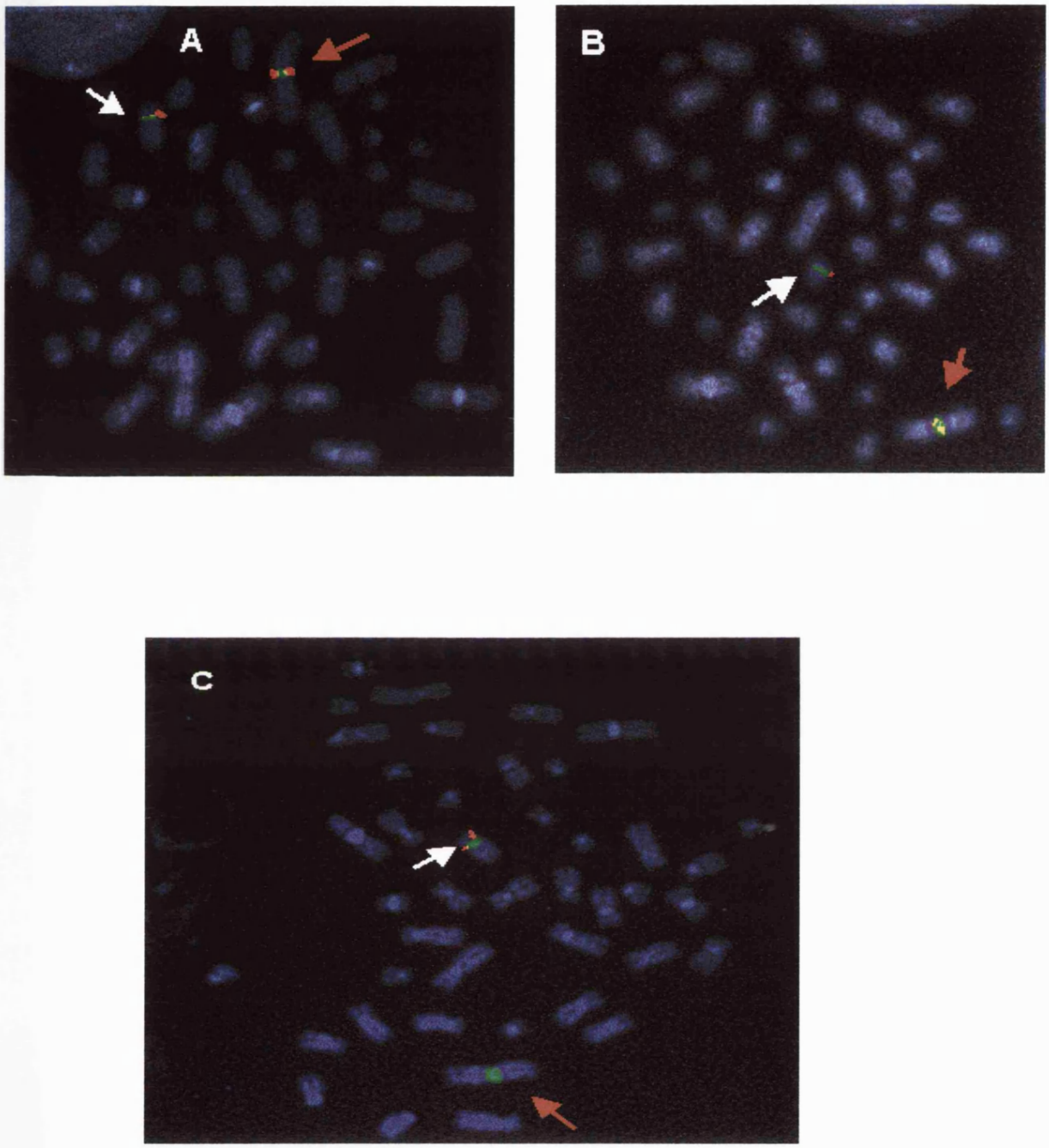


Figure 6.4. *Fluorescence in situ hybridisation (FISH) using chromosome 8 specific centromeric probe (green) and 8p11-12 YAC probes (red) in case number 5. Red arrows point at i(8)(p11) and white arrows point at normal chromosome 8.*
A. *Shows positive YAC 910d7 signal on i(8)(p11)*
B. *Shows positive YAC 806e9 signal on i(8)(p11)*
C. *Shows negative YAC 940f10 signal on i(8)(p11)*

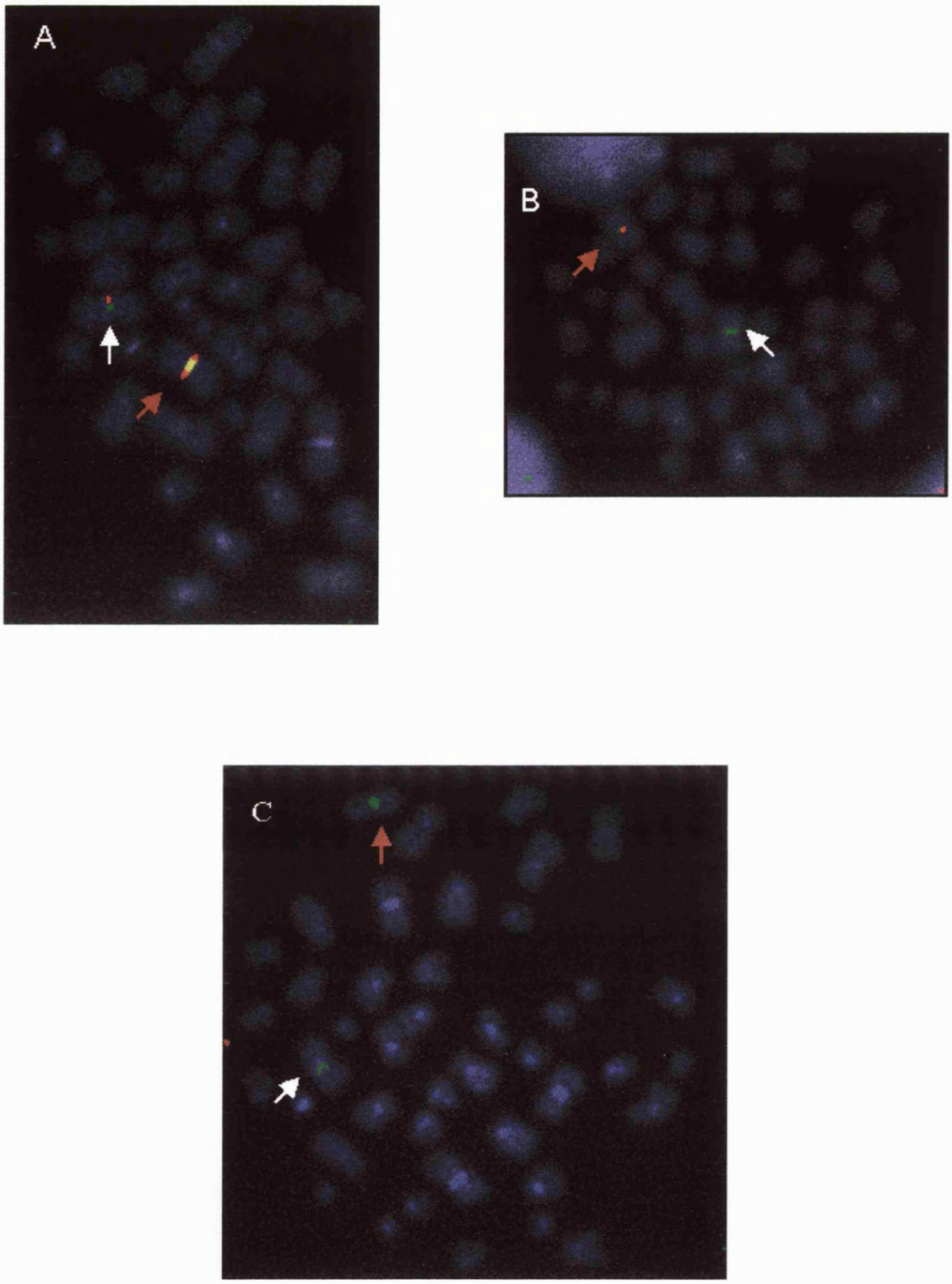


Figure 6.5. *Fluorescence in situ hybridisation (FISH) using chromosome 8 specific centromeric probe (green) and 8p11-p12 YAC probes (red) in case number 6 (clone B). Red arrows point at i(8)(p11) and white arrows point at der(8).*
A. *Shows positive YAC 910d7 signal on i(8)(p11), der(8)*
B. *Shows positive YAC 806e9 signal on i(8)(p11) but not on der(8)*
C. *Shows negative YAC 940f10 signal on both i(8)(p11) and der(8)*

6.5. Discussion

8p is suggested to harbour TSGs, as frequent non random LOH at polymorphic markers mapping to 8p has been reported in several solid tumours, including colorectal carcinoma (van der Bosch K. *et al.*, 1992), lung (Ohata H. *et al.*, 1993), liver (Emi M. *et al.*, 1992), bladder (Knowles M. A. *et al.*, 1993), prostate (Bergerheim U. S. *et al.*, 1991), head and neck (Li X. *et al.*, 1994) breast (Pykett M. J. *et al.*, 1994) and kidney cancers (Schoenberg M. *et al.*, 1995). It was also suggested that a tumour suppressor gene may be located in the subtelomeric region of chromosome 8p, and associated with increased ability to metastasise in hepatocellular carcinoma (Sunwoo J. B. *et al.*, 1999).
It was found that genomic loss of 8p was associated with the leukaemic forms of mantle cell lymphoma (MCL) because it was detected in 79% versus 11% of patients with nodal mantle cell lymphoma. Narrowing of the deleted segment on 8p resulted in the identification of 8p21-23 as the commonly deleted segment in MCL (Martinez-Climent J. A. *et al.*, 2001). The same genetic abnormality has been reported in other malignancies including T-PLL, and bladder, breast, prostate, lung, head and neck, and colorectal carcinoma (Knuutila S. *et al.*, 1999).
As the formation of chromosome 8 abnormalities in T-PLL consistently involve 8p, this has lead to the suggestion that loss of a tumour suppressor gene or activation of an oncogene on 8p could cooperate with increased dosage of the q arm and/or the expression of TCL-1/MTCP-1in allowing the malignant phenotype to emerge (Maljaei S. H. *et al.*, 1998).
Involvement of 8p11 in other haematological malignancies has been reported. Cloning of t(8;13)(p11;q12) which is associated with myeloproliferative syndrome with eosinophilia, lymphadenopathy and high incidence of T-cell non-Hodgkin's lymphoma, which is rapidly progressing to acute myeloid leukaemia has shown that the ZNF198 gene (a novel gene) at 13q12 is fused to fibroblast growth factor receptor-1 (FGFR1) gene at 8p11(Reiter A. *et al.*, 1998; Smedley D. *et al.*, 1998b).
Another translocation t(8;16)(p11;p13) has been cloned from cases of acute myeloid leukaemia (AML) with M4/M5 morphology and showed the involvement of MOZ gene on 8p11 (Aguiar R. C. *et al.*, 1997).

The results presented in this chapter support the existence of two breakpoint regions

within 8p11-12 in T-PLL. The first region is telomeric to YAC 899e2 (contains the fibroblast growth factor receptor 1 gene) and clustered within YAC 807a2. The second region was more telomeric and the breakpoints fell on both sides of the region identified by YAC 806e9 and were flanked by YAC 940f10 on the telomeric side and YAC 910d7, (containing the MOZ gene) on the centromeric side. Some of the studied cases showed that 8p abnormalities with breakpoints falling to either region could be present simultaneously in the same sample with 50% of the patients showing breakpoints within YAC 807a2.

The clustering of two regions of breakpoints shown in this study was usually accompanied by an increase in the dosage of the q arm. The amplification of the q arm of chromosome 8 associated with deletion of 8p sequences distal to the breakpoints could mean that the 2 events may act synergistically to produce the malignant phenotype.

Abnormalities affecting two separate regions on chromosome 8 have been described in other types of cancer such as prostate and bladder (Bova G. S. *et al.*, 1993; Kerangueven F. *et al.*, 1995; Macoska J. A. *et al.*, 1994). Distal sequences on 8pter-8p21 and proximal sequences on 8p11-p12 have been found to be affected in these and other cancers and these findings have led to the suggestion that at least two tumour suppressor genes exist on 8p that may be important for the development and/or progression of these malignant disorders.

Finally, the frequent occurrence of idic(8)(p11) in T-PLL together with clustering of the breakpoints may suggest that 8p abnormalities could arise as a result of hot spots for recombination at these sites. This may in turn be due to abnormal function of other genes located on other chromosomal sites that enhance/influence recombination rate. Mutations of the ATM gene have been extensively described in T-PLL (Saito S. *et al.*, 1992; Stilgenbauer S. *et al.*, 1997; Yuille M. A. *et al.*, 1998). ATM loss of function is known to increase rates of recombination (Meyn M. S., 1993) and this could account for the higher rates of recombination at these breakpoints.

The suggestion that loss of a tumour suppressor gene plus increased dosage of the q arm and/or the high expression of TCL1/MTCP-1 [resulting from inv(14)/t(14;14)] may

therefore play a role in the development of T-PLL (Maljaei S. H. *et al.*, 1998; Maljaie S. H. *et al.*, 1995). The findings reported in this study greatly support this hypothesis. It is also possible to suggest that loss of a tumour suppressor gene within the deleted segments on 8p11-12 would be implicated in the pathogenesis of T-PLL, although further investigations will be necessary to corroborate our preliminary data.

Appendix 1. Solutions

AHC	1.7g yeast nitrogen base w/o amino acids 5g $(NH4)_2SO2$ 10g casein hydrolysate 10ml 2mg/ml adenine sulphate take to 900ml with double distilled water After autoclaving, add 100ml of filter-sterilised 20% glucose
GDIS	2ml of 10% Triton 1ml of 10% SDS 333µl of 3M NaCl 100µl 1M Tris EDTA pH 8 20µl 0.5M EDTA To 10 ml with double distilled water
2xTY	16g bacto-tryptone 10g yeast extract 5g NaCl to 1 litre with double distilled water
Phenol/chloroform/isoamyl alcohol (PCIA)	25 parts phenol 24 parts chloroform 1 part isoamyl alcohol
Chloroform/isoamyl alcohol (CIA)	24 parts chloroform 1 part isoamyl alcohol
TE	10mM Tris pH 8.0 1mM EDTA pH 8.0

"Carlo's" buffer	3ml 1M Tris acetate pH 7.5 3ml 2M K acetate pH 7.5 1ml 1M Mg acetate 1 ml1mg/ml BSA fraction V For 10 ml final volume of stock solution.
1x TBE acrylamide solution	75ml 40% (19:1) acrylamide/bis-acrylamide 100ml 5xTBE 240g Urea to 500ml with double distilled water.
6% acrylamide gel	75ml of 1 xTBE acrylamide solution 140µl 25% APS 100µl Temed Final volume 75ml.
SOB	20g bacto-tryptone 5g bacto-yeast extract 0.5g NaCl 10ml of 250mM KCl, adjust to pH 7.0 to 1 litre with double distilled water.
GTE	12.5ml 1M Tris 10.0 ml 0.5M EDTA 25 ml 20%glucose to 500ml with double distilled water

20 x SSC	175.3 g NaCl 2g KCl, 26.8g $Na2HPO_4$-$7HO_2$ 2.4g KH_2PO_4 in 800ml H_2O. Adjust to pH to 7.4 with HCl to 1 litre with double distilled water. Sterilise by autoclaving and store at room temperature.
Church buffer	500ml of 1 M Na_2HPO_4 pH 7.2 10g Bovine serum albumin 70g SDS 2ml of 0.5 M EDTA pH 8.0 to 1 litre with double distilled water
Salmon sperm DNA	5mg/ml DNA in distilled water add 10.5mM of NaOH boil for10 minutes
5 x TBE	27g Trisma base 13.75g Boric acid 10 ml of 0.5M EDTA pH 8.0 to 500ml with double distilled water
1M (Dithiothreitol) DTT	3.09g DTT in 20ml double distilled sterile water pH 5.2 with 0.01M Na acetate
0.1M DTT used for all reactions	Dilute with double distilled water
Loading buffer	30% glycerol 0.25% bromophenol blue 0.25% xylene cyanol 0.1M EDTA pH8.0

TFB	20 ml of 0.5M(2(N-morpholine) ethane sulphonic acid pH 6.3 7.4g potassium chloride 8.9g manganese chloride 1.5g calcium chloride 0.8g hexamincobaltic chloride to 1 litre with double distilled water.
DND	1.35g DTT 9ml DMSO 90% v/v 100µl of 1M pH 7.5 potassium acetate To 10 ml with double distilled water
Stop solution	95% formamide 20mM EDTA 0.05% bromophenol blue 0.05% xylene cyanol
Lambda DNA Hind III digest (240ng/µl)	125µl of Hind III (Total 30µg) 195µl of TE 80 µl of tracking dye Store at –20°C
ϕX174 Hae III	50µl of ϕX174 Hae III (approximately 20µg) 590µl of TE 160µl of tracking dye Store at -20°C
IPTG	25mg/ml dissolved in double distilled water Store in 500µl aliquots at -20°C

X-Gal	25mg/ml dissolved in Dimethyl formamide Store in 500µl aliquots at -20°C

Appendix 2. Solutions

Solutions used for cytogenetics and FISH analysis

1. Cell preparation

Hypotonic solution (0.075M KCL)	Dissolve 5.6g KCL in 1 litre of distilled water, store at 37°C.
Fixative (Carnoy's Fixative)	3 parts methanol: 1 part glacial acetic acid, prepare freshly just before use.

2. Slide pre-treatment

2xSSC	100ml 20xSSC + 900ml SQ water. Adjust to pH7.0 with 1M citric acid, store at 4°C.
Denaturation solution (70% Formamide; 2xSSC; 50mM Na buffer)	7ml Formamide + 1ml 20xSSC + 1ml 0.5 M Na buffer + 1ml SQ water. Store at –20°C as 1.5ml aliquots.

3. Post hybridisation

1xSSC	20ml 20xSSC + 380ml SQ water, store at 4°C.
0.1xSSC	20ml 2xSSC + 380ml SQ water, store at 4°C.
4xSSC/0.05% TritonX	1L 20xSSC + 4L SQ water + 2.5 ml TritonX. Adjust to pH7.0 with1M citric acid, store at room temperature.

Blocking solution

Stock solution	Dissolve Boehringer Mannheim Blocking Reagent (cat no: 1096 176) in Maleic acid buffer (100mM Maleic acid; 150mM NaCL, pH7.5) to give a final concentration of 10% (w/v), autoclave and store at –20°C.
Working solution	Dilute stock solution with sterile Maleic acid buffer to give a final concentration of 1%(w/v). Store as 1ml aliquots at –20°C.
Antibody diluent	(10% Marvel; 4xSSC/0.05% Triton X). 10 g Marvel dissolved in 100ml SQ water Aliquot in 1.5 ml eppendorf tubes Centrifuge for 1 hour at 13,000rpm Transfer supernatant into 50ml Falcon tube Dilute 1:2 and adjust to pH8.5 Filter through 0.45µm syringe filter Filter through 0.22µm syringe filter Store at –20°C as 5ml aliquots.

4. Probe preparation

Hybridisation buffer	(50% Formamide; 2xSSC; 50mM Na phosphate buffer; 10% Dextran sulphate) 7ml Formamide 1.4ml 20xSSC 1.4ml 0.5 Na phosphate buffer 2.8ml 50% Dextran sulphate 1.4ml SQ water Store as 1.5ml aliquots at –20°C.

0.5M Na buffer	0.5M Na_2HPO_4-solution A 0.5M Na H_2PO_4-solution B Add approximately 15ml of solution A to 40ml of solution B Adjust pH to 7.0 using solution B.
5. Mitogens	
Phytohaemagglutinin (PHA)	Abbott Diagnostic (HA-15) Reconstitute lyophilised powder with 5ml sterile distilled water. Store at 4°C.
TPA	Sigma (P-1585)
Stock solution	Dissolve 1mg of TPA in acetone and then rapidly transfer to 9.9ml RPMI 1640 to give a concentration of 0.1mg/ml. Store as 0.5ml aliquots at –20°C.
Working solution	Add 0.5ml TPA stock solution to 19.5ml Hanks BBS to give a final concentration of 2.5µg/ml. Store as 0.5ml aliquots at –20°C. TPA is carcinogenic, and is destroyed by light.
6. Antibody stock solutions	All antibodies are used as supplied by manufacturer and are stored as the following aliquots at -20°C.
Avidin FITC (5mg/ml)	Vector Labs (A-2001) Store as 50µl aliquots at –20°C.
Anti-Avidin FITC	Vector Labs (SP-2040) Dilute 1mg/ml stock 1 in 4 in antibody diluent to give 250µg/ml (i.e. 2.5µg/µl) Store as 200µl aliquots at –20°C.
Rhodamine Anti-Digoxigenin (200µg/ml)	Boehringer Mannheim (1207 750) Store as 100µl aliquots at –20°C.

Texas Red Anti-Sheep (1.5mg/ml)	Vector Labs (TI-6000) Dilute the stock 1 in 5 with antibody diluent to give a concentration of 300µg/ml. Store as 500µl aliquots at –20°C.

7. Solutions required for nick translation

10X NT Buffer	(0.5M Tris-HCLpH7.8, 50mM $MgCL_2$, 0.5mg BSA/ml) 5ml 1M Tris-HCL 0.5ml 1M MgCL2 5mg BSA Make up volume to 10ml with sterile water. Store as 1ml aliquots at –20°C.
dNTP mix	(0.5mM dATP, 0.5mM dCTP, 0.1mM dGTP, 0.1mMdTTP).

Prepare on ice	Cat No (BoehringerMannheim)
25µl 100mM dATP	1051440
25µl 100mM dCTP	1051458
25µl 100mM dGTP	1051466
25µl 100mM dTTP	1051482

Make up volume to 5ml with sterile water. Store as 1ml aliquots at –20°C.

0.1M DTT (Sigma) Stock solution	Prepare it on ice in a fume hood; Dissolve 0.77g DDT in 5 ml sterile water to obtain 1M concentration.
Working solution	Dilute stock solution 1 in 10 to give 0.1M concentration. Store as 1ml aliquots at –20°C.

DNase I Boehringer Mannheim 100mg Cat no: 104159	Prepare: 1mg/ml (in 20mM Tris-HCL pH7.8, 50mM NaCL, 1mM DTT, 0.1mg BSA/ml, 50% glycerol).

Prepare on ice

1ml	1M Tris-HCL pH7.8
0.5 ml	0.1M DDT
2.5ml	1M NaCl
50mg	DNase 1
5mg	BSA
25ml	Glycerol

Make up volume to 50ml with sterile water. Store as 1 ml aliquots at –20°C. Immediately before use dilute 1 in 1000 (i.e. 1µl of 1mg/ml DNAse I in 1ml sterile water).

Yeast RNA (Sigma 10mg/ml)	Dissolve in TE buffer pH7.4. Store as 1ml aliquots at –20°C.
Salmon sperm DNA (Sigma 10mg/ml)	Dissolve in T.E buffer pH 7.4. Store as 1ml aliquots at –20°C.

The following reagents were used as supplied and were stored at –20°C

Reagent	**Manufacturer**	**Cat Number**
DNA Polymerase I	Boehringer Mannheim	6427110
Biotin-16-dUTP	Boehringer Mannheim	1093070
Digoxigenen-11-dUTP	Boehringer Mannheim	1558706
Cot-1 DNA	Gibco-BRL	15279-011

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Sorour A., Brito-Babapulle V., Smedley D., Yuille M. and Catovsky D. (2000). "Unusual breakpoint distribution of 8p abnormalities in T-prolymphocytic leukemia: a study with YACS mapping to 8p11-p12." Cancer Genet Cytogenet 121(2): 128-132.

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Conference papers

Author	*Title*	*Full Reference*
Brito Babapulle V; Sorour AF; Matutes E; Catovsky D.	Chromosome 12p abnormalities in T-prolymphocytic leukaemia: A study by fluorescent in situ hybridisation.	Blood 94: Suppl 1 1228, 1999
Sorour A; Brito Babapulle V; Smedley D; Yuille M; Catovsky D.	Chromosome 8p11-12 break points in T-prolymphocytic leukaemia.	Br J Haematol 105: Suppl 1 222, 1999

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