A study of the *INK4A/ARF* and *INK4B* loci in childhood acute lymphoblastic leukaemia using quantitative real time polymerase chain reaction

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For Chloe

Abstract

Background

Childhood acute lymphoblastic leukaemia (ALL) accounts for the largest number of cases of childhood cancer (25-35%) and is the primary cause of cancer related morbidity. Today more than 76% of children with ALL are alive and disease free at 5 years. Approximately one in 900 individuals between the ages of 16 and 44 years is a survivor of childhood cancer. In contrast, those patients who relapse with childhood ALL currently have a 6-year event free survival of 20-30%.

The short arm of chromosome 9p is mutated or deleted in many cancers including leukaemia. Aberrations of the *INK4A/ARF* and *INK4B* loci at the 9p21 band are linked to the development and progression of cancer. In murine cancer models there is evidence to suggest that mutations of *Ink4a/Arf* and *p53* gene loci promote resistance to chemotherapeutic drugs known to trigger apoptosis.

Aim

The initial aim of this project was to develop an accurate, reproducible method to detect deletions at the *INK4A/ARF* locus in patient bone marrow specimens. This technique was then applied to detect the incidence of deletions of this locus in childhood ALL specimens. The hypothesis developed was that deletion at the *INK4A/ARF* locus at diagnosis in childhood ALL is an independent prognostic marker and is involved in disease progression. A secondary aim of this study was to determine which deletions at the *INK4A/ARF* and *INK4B* loci are the most relevant in leukaemogenesis in childhood ALL.

Methods

An original method for assessing *INK4A/ARF* locus deletion, homozygous as well as hemizygous, was developed using quantitative real-time polymerase chain reaction (qPCR). To simulate the presence of normal cells, a calibrated mixing experiment using two cell lines was conducted. This method was then applied to detect deletions in bone marrow

specimens from patients with childhood ALL. A similar technique was applied to explore the incidence of deletion of the individual genes *p15INK4B*, *p14ARF* and *p16INK4A* at diagnosis and at relapse. A study of *TP53* gene mutations was also included using published methodology. These results were correlated with clinical outcomes.

Results

In an unselected study group of 45 patients with childhood ALL, the qPCR technique was applied to diagnostic bone marrow specimens. The incidence of homozygous deletion of *INK4A/ARF* (exon 2) was 25% and the incidence of hemizygous deletion of this exon was 13%. The risk ratio for relapse for hemizygous deletion of this exon compared with no deletion was 6.558 (p=0.00687) and for homozygous deletion was 11.558 (p=0.000539).

From 25 patient specimens at diagnosis and at relapse the incidence of homozygous and hemizygous deletion of *INK4A/ARF* (exon 2) at diagnosis was 32% and 20%, respectively. In contrast, the incidence of homozygous deletion at relapse was 64% for homozygous deletion and 0% for hemizygous deletion. In this paired study group, both hemizygous and homozygous deletion at diagnosis were associated with a significantly decreased median time to relapse compared with no deletion.

Detailed mapping of these loci using three genes (*p15INK4B*, *p14ARF* and *p16INK4A*) was performed on bone marrow specimens at diagnosis and at relapse from 20 patients with childhood ALL. All patient specimens had a deletion of at least one of three genes at diagnosis and at relapse. There was a significant correlation for a deletion of *p16INK4A* and reduced median time to relapse. No *TP53* mutations were detected at diagnosis but three relapse specimens were found to have mutations of *TP53* exon 7.

An exploration as to whether the deletions were somatically acquired was made by measuring the incidence of deletion of *p16INK4A* (exon 1α) in a panel of remission specimens. This was compared to a panel of marrow specimens from normal individuals. Evidence for hemizygous *p16INK4A* deletion was found in 2% of the normal specimens and in 17.6% of those remission marrows from patients with leukaemia.

Conclusions

This study has explored the involvement of the *INK4A/ARF* and *INK4B* loci in childhood ALL using an original, accurate and reproducible method. This qPCR technique can be applied to the study of these loci in other cancers and lends itself to the study of other tumour suppressor genes.

This study has shown that deletion of the *INK4A/ARF* locus is an independent prognostic indicator in childhood ALL. In addition, the frequency of deletion at the *INK4A/ARF* and *INK4B* loci is increased at relapse compared to diagnosis in childhood ALL. In the relapse study group, deletion of the *p16INK4A* gene at diagnosis was associated with a decreased median time to relapse compared to other genes analysed. Murine studies suggest that such deletions may result in an increased resistance to chemotherapy. If the findings from this study are confirmed in a larger cohort, it is expected that therapeutic interventions based on assessment of the *p16INK4A* gene in diagnostic childhood ALL specimens will be implemented to prevent relapse in standard risk patients and help to improve the outcome in high risk patients.

Preface

The regulations of the University of Western Australia provide an option for applicants for the Degree of Philosophy to present their work as a series of manuscripts submitted for publication.

This thesis is presented in the form of a series of manuscripts published or submitted for publication. They relate to the exploration of the *INK4* cluster in childhood ALL and are presented in a logical sequence, which develop the issues raised in the introduction. The discussion integrates the manuscripts and addresses those problems presented in the introduction. It considers the clinical and research implications of this work and suggests directions for the future. Each manuscript is presented with the original figures and tables retained as required by the specific journal the manuscript had been submitted to. However they are numbered in the thesis format for ease of reading. The appendices contain relevant figures, which were not published due to manuscript specification. In this series of papers patient bone marrow specimens were used repeatedly, however DNA was not available in all cases. As a consequence, the numbering of patients is different between the papers.

All of the studies undertaken were of the candidate's own design in collaboration with others where stated and were completed following consultation with supervisors. The candidate was responsible for all data preparation, analysis and presentation. Her supervisors and co-authors assisted with the corrections and proof reading of the manuscripts once the drafts had been prepared. The contribution of others is presented below and in the acknowledgments.

Chapter 2
"Hemizygous *p16(INK4A)* deletion in paediatric acute lymphoblastic leukaemia predicts independent risk of relapse."
Carter TL**,** Watt PM, Kumar R, Burton PR, Reaman GH, Sather HN, Baker DL and Kees UR
(Blood.2001 Jan 15;97(2): 572-4).

Chapter 3
*"INK4a/*ARF deletions are acquired at relapse in childhood acute lymphoblastic leukaemia: a paired study on 25 patients using real-time PCR."
Carter TL, Reaman GH and Kees UR
(Br J Haemtol.2001 May: 113 (2): 323-8).

Chapter 4
"Deletion of one copy of the *p16 (INK4A)* tumour suppressor gene is implicated as a predisposing factor in paediatric leukaemia."
Carter TL, Terry PA, Gottardo N, Baker DB, Kees UR and Watt PM.
(BBRC.2004 June: 318(4): 852-55).
Contribution by others to this paper: DNA extraction and *p16* Exon 1α analysis on specimens from patients with AML and the normal controls was performed by PA Terry (Research Assistant). Clinical details on patient 1 were obtained from patient notes by Dr

Nicholas Gottardo (Oncology Fellow), PMH. These investigations were performed whilst the candidate was in Canada.

Chapter 5

"Targeted therapies should be considered when abnormalities of the *INK4A/ARF* and *TP53* are discovered in childhood acute lymphoblastic leukaemia (ALL)."
Carter TL, Terry PA, Watt PM, Reaman GH, Baker DL and Kees UR.
(Leukaemia. Submitted for publication December 2004).

Contribution by others to this paper: Optimisation experiments for qPCR on all other exons apart from *p16INK4A/ARF* Exon 2, were conducted by P.A.Terry. DNA extraction and qPCR analysis on patient samples was performed by the candidate.

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Abbreviations

ALL
Acute lymphoblastic leukaemia

AML
Acute myeloid leukaemia (Acute non-lymphocytic leukaemia)

B-lineage ALL
B precursor ALL

COG
Children's Oncology Group

CNS
Central nervous system

CDK
Cyclin dependent kinase

CKI
Cyclin dependent kinase inhibitor

EFS
Event free survival

FISH
Fluorescence in-situ hybridisation

***INK4* cluster**
During the evolution of this thesis the accepted description of these loci altered. This was partly due to the unusual nature of the coding of the *INK4A/ARF* locus.
Below is an explanation of the different terms used. As each paper was accepted for publication the accepted nomenclature for these loci changed.

The *INK4* cluster contain the *INK4B* and the *INK4A/ARF* loci

INK4B (*CDNK2B*, *MTS2*, *p15, p15INK4B*)

INK4A/ARF (*CDNK2A*, *MTS1*, *p16*, *p16INK4A/ARF*). This locus codes the genes for the proteins p16INK4A (p16) amd p14ARF(p14)

In mice p19Arf is the protein equivalent of p14.

In mice the loci are *Ink4a*/*Arf* and *Ink4b*

IgH
Immunoglobulin heavy chain gene

RT-PCR
Reverse transcriptase polymerase chain reaction

qRT-PCR
Quantitative real time RT-PCR

qPCR
Quantitative real-time polymerase chain reaction

RB, RB1
The retinoblastoma gene
The protein is written as RB or Rb

TP53
The tumour suppressor gene *TP53* (*p53* in mice)
The protein is TP53 or p53 in humans
It is always p53 in mice

T-ALL
T-cell ALL

TCR
T-cell receptor gene

TSG
Tumour suppressor gene

WBC
White blood count

WCC
White cell count

List of Tables

List of Figures

Contents

Chapter 1

1.1 Leukaemia

1.1.1 Background on childhood leukaemia

Cancer is an umbrella term covering a number of distinct conditions characterised by uncontrolled cellular proliferation. As the average age of the population in many countries steadily rises, so do cancer-related deaths. Approximately one in 900 individuals between the ages of 16 and 44 years is a survivor of paediatric cancer (Toren, Rechavi et al. 1996). Cancer treatments are aggressive and costly, both to the individuals suffering the disease and to the community. It is imperative that cancer research enables treatment to be specifically targeted to the cancer cell and not toxic to the human host.

The leukaemias account for the largest number of cases of childhood cancer and are the primary cause of cancer related mortality. In most populations leukaemia accounts for 25-35% of all childhood malignancies (Sharp, Cotton et al. 1999).

Today, more than 76% of children with acute lymphoblastic leukaemia are alive and disease
free at 5 years, compared to less than 10% in 1960, probably making it the most successfully treated among the disseminated human cancers (Kersey 1997). There have been a number of improvements in treatment during this period, including identification of better methods of central nervous system (CNS) prophylaxis and treatment intensification (Gaynon, Trigg et al. 2000). We are still unsure of the exact aetiology of the most common leukaemia and because of scarcity of paired diagnostic and relapse specimens the biology of relapse is largely unknown.

This thesis will focus on the possible molecular events in the pathogenesis of acute lymphoblastic leukaemia (ALL), the most common childhood leukaemia in developed countries. Clues to understanding the pathogenesis will be provided by a discussion of the research involving the molecular events in the pathway to cancer. The focus of this study will be the *INK 4* cluster on chromosome 9p and its involvement in childhood ALL. The

short arm of chromosome 9 is mutated or deleted in many cancers, including leukaemia (Sharpless and DePinho 1999). In addition aberrations of these loci are linked to the further development and progression of cancer (Kamb, Gruis et al. 1994) (Sherr 2001). Relapsed childhood ALL has a 6 year event free survival (EFS) of 20-30% (Henze, Fengler et al. 1991; Schroeder, Garwicz et al. 1995; Gaynon, Qu et al. 1998). Understanding the molecular events involved at relapse will provide clues to explain chemotherapeutic resistance and hopefully prevent toxicity from unnecessary intense treatment.

1.1.1.1. Definition

Leukaemia is a cancer of the haematopoietic system, involving in most cases, malignant transformation of lymphoid progenitor cells and less commonly transformation of myeloid progenitor cells (Margolin 1997) (Golub, Weinstein et al. 1997)

In developed countries 70-80% of childhood leukaemia is ALL and 15-17% is acute myeloid leukaemia (AML) (Sharp, Cotton et al. 1999). There is considerable geographical variation in the incidence of acute leukaemia worldwide. In North America, Canada, Europe and in Australia the incidence is between 35 and 49 per million. An incidence of less than 25 per million is reported in India and Africa (Sharp, Cotton et al. 1999).

The incidence of leukaemia among children varies considerably with age. In most countries the rate of leukaemia is highest among children under five years and decreases with age. There is a sharp peak in ALL incidence among the 2-3 year old children (80/1,000,000) contrasting with 20/1,000,000 for the 8-10 year group (Ries, Smith et al. 1999). This age peak varies between different communities and has not always been present. Paediatric records from early last century shows that there was no such peak in the incidence of ALL in the UK during this period (Parkin, Stiller et al. 1988). In Africa where the incidence of leukaemia is low the peak is not present (Parkin, Stiller et al. 1988).

The incidence of AML varies with age but with a different pattern. The AML rates are highest in the first 2 years of life, but decrease to a nadir at approximately 9 years of age, followed by slowly increasing rates during the adolescent years. In adults the majority of leukaemia is myeloid (85%), the remainder mostly lymphoid (Ries, Smith et al. 1999).

The differing geographic and age patterns of ALL and AML suggest different aetiological processes. This is supported by molecular studies, which demonstrate different molecular and cytogenetic changes in the two leukaemias.

1.1.1.2 Molecular events in cancer pathways

Molecular abnormalities that arise in cancer pathways can be investigated using a number of different cytogenetic and molecular techniques.

1.1.1.2.1 Chromosomal translocations

The concept of dominant "gain of function" mutations in cancer initially came from experiments involving retroviruses and cell fusion experiments. Genes related in sequence to those in the transforming retroviruses were found in the DNA of normal cells. These genes had functions in the control of normal cell growth or differentiation, but their inappropriate activation by a variety of mechanisms was shown to lead to cancer. The normal cellular genes were termed "proto-oncogenes" and their activated counterparts "oncogenes" (Bishop 1981).

The development of chromosomal banding in the 1970s provided further cytogenetic evidence for a role of activation of specific genes in cancer. In some tumours there were chromosomal translocations with consistent breakpoints and some of these breakpoints proved to be in, or near to, already described oncogenes. This led to the discovery of oncogenes at chromosomal break points eg. the homeobox gene discovered at the translocation breakpoint t(10;14)(q24;q11). This homeobox gene was found to be closely related to developmentally regulated homeotic genes of flies and mammals (Krumlauf 1994).

Hox11 can be detected during murine embryogenesis in different tissues and is essential for development of the spleen (Dear, Colledge et al. 1995). Abnormalities in expression of this gene have been found to be associated with T-cell ALL (T-ALL) (Rabbitts 1994; Kees UR, Heerema NA et al. 2003) .

1.1.1.2.2 Loss of tumour suppressor gene function

The "classical" paradigm of a molecular event in cancer that causes recessive tissue specific loss of function originally was defined by alterations in tumour suppressor genes (TSG). These genes are thought to be the "gatekeepers" in the control of the cell cycle and their loss results in uncontrolled cellular proliferation (Kinzler and Vogelstein 1997). It has been proposed that loss of gatekeeper function is an initiating event in cancer (Cook and McCaw 2000).

The view of all TSGs as being classically recessive is now being challenged as many such genes have been shown to be semidominant or haploinsufficient(Fodde and Smits 2002).

Alterations of TSG are commonly found in inherited forms of cancer (5-10% of human tumours are known to have a hereditary component)(Russo, Zanna et al. 2000). Tumour formation is rapid where there are abnormalities in the somatic cells involving the following genes: *BRAC1* and *BRAC2* especially for hereditary breast and ovarian cancer, *hMLH1* and *hMSH2* for hereditary non polyposis colorectal cancer, *APC* for familial adenomatous polyposis, *ret* for medullary thyroid carcinoma, *TP53* for the Li-Fraumeni syndrome, *p16INK4A* for melanoma and *RB* for the retinoblastoma gene (Russo, Zanna et al. 2000).

A recent study by Hernando and colleagues has shown that loss of the retinoblastoma protein (RB) can contribute to aneuploidy by leading to aberrant Mad2 over-expression. The consequences of this defect resulting in an uncoupling of mitotic control, possibly leading to an increase in mutation rate fuelling further tumour evolution (Hernando E, Nahle Z et al. 2004).

1.1.1.2.3 Abnormal epigenetic regulation

Methylation of CpG residues is the main epigenetic modification in mammals, abnormal methylation of the CpG islands located in the promoter region of genes usually leads to transcriptional silencing. Examples include *p16INK4A, p15INK4B, p14ARF*, Von Hippel-Lindau (*VHL*), the oestrogen and progesterone receptors, E-cadherin, death associated

protein (DAP) kinase and the first tumour suppressor gene described, *RB* (Esteller 2000). Epigenetic regulation of gene expression by methylation is an important mechanism of the determination of cell fate in embryogenesis. Methylation is also linked to histone deacetylation, another key mediator of transcriptional repression. This is followed by the recruitment of histone deacetylase by the methyl CpG binding protein MeCp2 (Gartler SM, Varadarajan KR et al. 2004).

It is not clear whether the epigenetic silencing of particular genes in cancer occurs through a stochastic process followed by selection, or whether certain promoters are predisposed. Certain promoters may be trivially predisposed due to density of CpG residues. Loss of function of the cyclin dependent kinase (CDK) inhibitor, *p16INK4A* may occur through deletion, point mutation or promoter hypermethylation, but the frequency of each mechanism differs between tumour types (Baylin and Herman 2000). Epigenetic regulation may also allow derepression of genes as the promoter region of a oncogene may be hypomethylated as in the case of the *HOX-11* gene in T-cell childhood ALL (Watt, Kumar et al. 2000).

1.1.1.2.4 Abnormal apoptosis

Many oncogenes and TSGs mediate their effects by interfering with or inducing apoptotic signalling. Apoptotic pathways are significantly altered in cancer cells relative to untransformed cells, and these differences present a therapeutic window that can be exploited for development of cancer drugs (Huang and Oliff 2001).

TP53 is the most commonly mutated or deleted gene in human cancer. The consequences of its disruption are profound, either in the germline of patients with Li-Fraumeni syndrome, or in mice with targeted gene deletion. Abundant evidence suggests that *TP53* exerts regulation of cell cycle progression as well as apoptotic cell death, both in response to environmental or metabolic stressors. The specific decision of cell cycle arrest versus death may underlie the ability of *TP53* to trigger death in cancer cells or arrest with repair in non-cancer cells (Fisher 2001). The apoptotic role of *TP53* in chemotherapeutic resistance and its link with cancer will be discussed in a subsequent chapter.

1.1.1.2.5 Genomic instability

Mutational alterations conferring instability are thought to occur early during tumour formation. Genetic instability drives tumour progression by generating mutations in oncogenes and TSGs (Cahill, Kinzler et al. 1999). Colorectal tumours have provided a model for this concept. Lengauer and colleagues have shown that colorectal tumours with microsatellite instability exhibited a striking defect in chromosome segregation. This chromosomal instability reflected a continued cellular defect that persisted throughout the lifetime of the tumour cell and was not simply related to chromosome number. Their data indicated that persistent genetic instability might be critical for the development of colorectal cancers (Lengauer, Kinzler et al. 1997).

In hereditary conditions with genomic instability there is an association with cancer development later in life. Hereditary conditions such as Bloom's syndrome, Fanconi's anaemia and ataxia telangiectasia are associated with abnormal chromosomal fragility, enhanced mutation rates and the development of leukaemia usually in late adolescence and adulthood (Mathur, Chowdhury et al. 2000). This supports the theory of genetic instability leading to further mutations and the development of cancer.

Molecular events that arise in the cancer pathways are complex. They not only provide aetiological clues to the pathogenesis of leukaemia, they can also act as prognostic markers at the time of diagnosis. Knowledge of the aetiology of these events may help to create strategies for more tumour specific treatment and it is hoped eventually for prevention.

1.1.1.3 The aetiology of childhood ALL

The heterogeneity of childhood leukaemia at diagnosis and its differing epidemiology groups has led to the hypothesis that elevated risk in ALL may be determined by high economic status and population mixing (Kinlen 1995). These associations are thought to be related to the relative immunological isolation in infancy in developed countries compared to infants in developing countries. This is thought to influence patterns of exposure to common infectious agents before the appearance of the leukaemia (Greaves and Alexander 1993).

There are some reports of clustering of ALL cases, which is thought to suggest an aetiologic agent such as a virus but this controversial (Kinlen 1995). Human T-cell leukaemia virus-1 and II may be associated with adult, but not paediatric ALL. Epstein-Barr virus (EBV) infection has been linked to a limited number of cases of T-cell lymphoma but not ALL (Johnson, Harrod et al. 2001) (Epstein 2001).

There are a number of known genetic conditions where the incidence of leukaemia is increased; these include Down syndrome, neurofibromatosis, Shwachman syndrome, Bloom syndrome, ataxia telangiectasia, Langerhan cell histiocytosis and Klinefelter syndrome (Janin 1995). All of these syndromes apart from Down syndrome, are associated with leukaemia developing in late adolescent to adult life. Down syndrome is associated with a significantly increased risk of childhood leukaemia and the development of leukaemia in adult life is uncommon (Hasle, Clemmensen et al. 2000). In addition, patients with Down syndrome and leukaemia often have a better than expected outcome compared to similar risk patients who do not have Down syndrome (Hasle, Clemmensen et al. 2000).

What antenatal events could occur which may lead to the initiation of leukaemia in young children? The role of numerous epidemiological factors, including maternal and paternal exposure to radiation, history of maternal foetal loss or fertility problems, higher birth weight and use of exogenous growth hormone, remains controversial in the cause of childhood ALL (Uckun, Sensel et al. 1998).

Studies of maternal and patient genetic polymorphisms in drug and toxin metabolising enzymes provide clues to more subtle predisposing factors. These genetic polymorphisms may result in impaired detoxification of chemotherapy or inefficient repair of drug-induced genetic damage. A potential role for polymorphisms in the genes encoding the enzymes *glutathione-S-transferases*, *quinone oxidoreductase*, *myeloperoxidase*, *N-acetyltransferase*, *cytochrome P450* (*CYP*) *1A1* and *3A4*, *methylenetetrahydrofolate reductase* (*MTHFR*), *cystathionine- beta-synthase* and others has been associated with the aetiology of leukaemia and therapy-related complications (Perentesis 2001). A link between maternal folate enzyme polymorphisms and leukaemia has been suggested following the discovery of an

inverse epidemiological association between maternal folate intake and childhood ALL (Thompson, Gerald et al. 2001). The concept that maternal folate metabolic polymorphisms could influence risk of ALL is intriguing and needs to be explored in a larger study. There is further molecular evidence to support the antenatal initiation of leukaemia.

In infant leukaemia the most common translocation is t(4;11)(q21;q23)(*MLL-AF4*). Translocations involving the *MLL* gene on 11q23 occur in up to 60% of cases in patients diagnosed before 12 months of age (Rubnitz, Behm et al. 1996). More than 30 different translocations involving the *MLL* gene have been described (Faderl, Kantarjian et al. 1998). These partner genes are involved in transcriptional regulation and it is believed that fusion proteins from these translocations are involved in aberrant gene regulation. Translocations affecting the 11q23 region occur in up to 80% of secondary leukaemia arising after chemotherapy with topoisomerase II inhibitors (Faderl, Kantarjian et al. 1998). This molecular abnormality is associated with an aggressive disease and a brief latency period of 18 months. Translocations of 11q23 have been found at birth in monozygotic twins. Using neonatal blood spots, Gale and colleagues demonstrated the presence of the same *MLL-AF4* gene fusion in monozygotic twins who developed acute leukaemia at the age of 5 months and 2 years (Gale, Ford et al. 1997). This brief latency period combined with the neonatal data would suggest that *MLL* abnormalities occur antenatally and are sufficient for predisposing children to develop leukaemia.

The *MLL* fusion genes are found in less than 5% of childhood leukaemia. In childhood ALL the most common cytogenetic abnormality is the t(12;21)(p13;q22)(*TEL-AML1*) translocation. This rearrangement results in the fusion of the oligomerisation domain of *TEL(ETV6)* on chromosome 12 to the entire coding region of *AML1(CBFA2)* on chromosome 21 (Golub, Barker et al. 1997). When analysed by fluorescent in-situ hybridisation (FISH), Southern blot or polymerase chain reaction (PCR), this translocation is found in 16-32% of paediatric B-lineage ALL cases (Shurtleff, Buijs et al. 1995) (Rubnitz, Downing et al. 1997) (Faderl, Kantarjian et al. 1998). This rearrangement is associated with favourable clinical features and a good outcome. Twin studies have also found that this translocation can be detected at birth. Ford and colleagues found evidence of

the same *TEL-AML-1* translocations in identical twins that were diagnosed at different ages, supporting the antenatal presence of this translocation (Ford, Bennett et al. 1998). Using neonatal blood spots in monozygotic twins a latency period of up to nine years between the development of leukaemia in the second twin has been found (Wiemels, Ford et al. 1999). This latency period and the lack of twin concordance in childhood ALL supports the hypothesis that the *TEL-AML-1* fusion is insufficient for overt leukaemia. Unlike the 11q23 abnormalities this translocation is associated with a good prognosis.

A consistent genetic abnormality observed at diagnosis in childhood ALL along with the *TEL-AML1* fusion itself is the *TEL* deletion (Romana, Mauchauffe et al. 1995). Loss of the normal *TEL* allele prevents the TEL to TEL-AML1 heterodimerisation, which is thought to compromise fusion protein activity (McLean, Ringold et al. 1996). One hypothesis put forward by Greaves is that loss of the normal *TEL* allele could represent the critical post-natal event in childhood leukaemia (Greaves 1999). In addition he speculates that the trigger that leads to the *TEL* allelic loss is an "exuberant and unbalanced immune response to infection" (Greaves 1999). This is thought to be more common in developed countries because of the lack of early appropriate "immune-modulating-infectious exposures in infancy". This hypothesis is supported by epidemiological studies and geographic variation (Parkin, Stiller et al. 1988) (Kinlen 1995).

However, these two translocations are not found in over 50% of childhood ALL and therefore it is clear that childhood leukaemia is not always initiated antenatally. Yagi and colleagues used blood spots of infants and children who were diagnosed with B-lineage ALL and analysed a combination of immunoglobulin heavy gene (IgH) rearrangements (Yagi, Hibi et al. 2000). These rearrangements can be used as clonal markers of leukaemia and they will be discussed in a later chapter. They found evidence of the same clonotypic rearrangements in the neonatal blood spots on the Guthrie cards and in the leukaemic cell DNA of patients (Yagi, Hibi et al. 2000). This would indicate that the clonal IgH rearrangements, a marker of malignancy, were present at birth.

These studies provide compelling support for the antenatal origin of some cases of childhood ALL. Progress in understanding the molecular events in cancer pathways has

not only improved our understanding of leukaemogenesis but also allowed the identification of discrete prognostic groups with specific molecular and cellular features (Ferrando and Look 2000). Clinical classifications and molecular data provide important information to adopt risk-adjusted therapies and reduce toxicities.

1.1.1.4 Classification and survival in childhood acute lymphocytic leukaemia (ALL)

ALL represents a heterogeneous group of diseases that vary with respect to the morphological, cytogenetic, and immunologic features of the transformed cells. Technical improvements in immunofluorescence staining and flow cytometry, together with the availability of numerous monoclonal antibodies that recognize lineage-associated membrane molecules has illuminated the heterogeneity in ALL (Miller, Leikin et al. 1981). The malignant clones in patients with ALL are thought to originate from normal lymphoid progenitor cells arrested at early stages of B- or T-lymphocyte development. Although cells from the majority (85%) of paediatric patients express B-lineage-associated antigens, those from 15% of patients express the T-lineage associated antigens. T-lineage ALL (T-ALL) in children is associated with numerous unfavourable presenting features (Uckun, Sensel et al. 1998).

In 1993 the National Cancer Institute (NCI) in the USA sponsored a workshop to define more uniform criteria for risk-based treatment assignment for childhood ALL. Participants included representatives from all major paediatric cancer groups in the USA. The results established the classification of childhood ALL using combined data from all clinical trials (Smith, Arthur et al. 1996). The current classification of ALL is based on these findings and defines patient groups by age, phenotype and tumour burden. For patients with B-precursor (pre-B, B-lineage) ALL the standard-risk category includes patients 1 to 9 years of age with a white blood cell count (WBC) of less than 50,000 $x10^6$ /L. The remaining patients are classified as having high-risk ALL. For patients with T-ALL different treatment strategies have yielded different conclusions concerning the prognostic significance of the T-cell immunophenotype at diagnosis. Some clinical groups classify patients with T-ALL as high risk, while others assign risk based on the age and WBC criteria as above (Smith, Arthur et al. 1996).

A review of Children's Cancer Group (CCG) trials in childhood leukaemia supported the above classification, including additional adverse prognostic factors such as; poor early marrow response at day 7 of induction and abnormal cytogenetics other than hyperploidy. CNS disease at diagnosis was described as a significant risk factor in univariate analyses, although it correlated with WBC count in multivariate analyses (Gaynon, Trigg et al. 2000).

Proposed Children's Oncology Group (COG) (formally the Children's Cancer Group and the Paediatric Oncology Group) B-lineage ALL protocols use further risk-adjusted classification, including good prognostic genetic factors and initial response to induction therapy. Proposed T-ALL protocols recognise the different molecular characteristics and response to therapy in childhood T-ALL.

Treatment based on clinical risk stratification has resulted in 4 year event-free survival (EFS) of 80% for standard risk and 4 year EFS for high risk of 65% (Smith, Arthur et al. 1996). Identification of the molecular markers involved in leukaemogenesis and their relation to clinical outcome help define which genotypes require more intensive therapy and prevent relapse. They have also provided targeted therapies. It is hoped that the understanding of the pathways involved in the development of a leukaemic blast will help prevent chemotherapeutic resistance and relapse.

1.1.1.5 Cytogenetic and molecular prognostic markers of risk in childhood ALL

1.1.1.5.1 Ploidy

The number of chromosomes within a cell is termed its ploidy. Normal human cells have 46 chromosomes while leukaemic cells often have an abnormal number of chromosomes. The DNA content of cells can be estimated by flow cytometry. Using this convenient technique a DNA index in leukaemic cells (ratio of the DNA content of leukaemic cells Go/G1 cells versus that of normal diploid cells) can distinguish two prognostic categories of patients with DNA indices of < 1.16 or ≥ 1.16 (approximately equal to less than 53 or greater than or equal to 53 chromosomes) (Trueworthy, Shuster et al. 1992). Using this classification, a DNA index of greater than or equal to 1.16 is associated with a 90% 4 year

EFS (Trueworthy, Shuster et al. 1992). There is heterogeneity within this group since there is evidence of an associated structural chromosomal abnormality. Trisomy of chromosome 10, 17 and 18 carry favourable univariate prognostic significance and trisomy 5 carries adverse prognostic significance. Multivariate analysis found trisomy 10 the most important prognostic factor in the high hyperploid subset of leukaemias. (Gaynon 2000).

1.1.1.5.2 Chromosomal translocations associated with known clinical outcomes in pre-B ALL

About 50% of new cases of childhood ALL have non-random translocations, although the proportion may be greater as only molecular screening technology allows detection of rearrangements not associated with structural changes apparent by light microscopy (Ferrando and Look 2000).

The most common cytogenetic abnormality in children with ALL (16-32%) is the t(12;21)(p13;q22)(*TEL-AML1)* translocation. Its role in the aetiology of primary leukaemia has been discussed and it is associated with good clinical prognostic features. However, this translocation is also found at relapse and this will be discussed in a later section.

The t(1;19)(q23;p13) translocation, present in 5% to 6% of childhood ALL, fuses the *E2A* gene on 1q23 with the *PBX1* locus on 19p13. This translocation is strongly associated with the B-lineage immunophenotype. Translocation of the basic helix-loop-helix (bHLH) *E2A* gene to the *PBX1* locus generates a fusion gene. *PBX1* is the human homolog of the *Drosophila* extradenticle protein and is thought to participate in the regulation of cell differentiation and development through its interaction with major HOX proteins. (Kamps 1997). Animal models have shown that the leukaemogenic effect of *E2A-PBX1* is mediated, at least in part, by the induction of cell differentiation arrest (Kamps 1997). The balanced translocation remains an adverse prognostic factor and is associated with very poor outcome , with a 5 year EFS of less than 45%. (Gaynon 2000).

The cytogenetic hallmark of chronic myeloid leukaemia (CML), the t(9;22)(q34;q11)(*BCR/ABL)* translocation or “Philadelphia chromosome”, is also present in less than 5% of childhood ALL (Faderl, Kantarjian et al. 1998). The t(9;22) translocation

transposes the 3' portion of the *ABL* gene on chromosome 9 to the 5' region of the *BCR* gene on chromosome 22, generating a chimeric mRNA that encodes a tyrosine kinase oncoprotein (Gordon 1999). This rearrangement is a prognostic indicator in childhood ALL and is associated with a very poor outcome, with a 5 year EFS of less than 45% (Gaynon 2000). Despite consistent use of intensified chemotherapy in CML, remission induction rates rarely exceed 70% and relapse rates are high. Allogenic bone marrow transplantation early in first clinical remission (CR) is the only therapy that has secured any appreciable long-term control of this disease (Ferrando and Look 2000). STI-571, a novel target specific molecule inhibits the tyrosine kinase activity of this chimeric protein and has shown a strong anti-leukaemic effect in adults, however its effect in childhood ALL where the t(9;22)(*BCR-ABL*) translocation is detected has been less successful (Sawyers 2001).

Cytogenetic abnormalities of 11q23 involving the *MLL* gene chromosomal region are present in up to 60-70% of infants with ALL and in 10% of cases in older children and adults (Faderl, Kantarjian et al. 1998) (Rowley 1998). *MLL* rearrangements in paediatric cases are associated with a young age (less than 2 years), high WBC, organomegaly, and CNS involvement (Behm, Raimondi et al. 1996). Infants with 11q23 abnormalities that do not involve chromosome band 4q21 have a better outcome (5-year EFS of 57%) (Gaynon 2000). 11q23 abnormalities in older children and adolescent do appear to define a subgroup with a worse prognosis when compared to other parameters. However it does not appear to be associated with such aggressive disease compared to its presence in infants (Gaynon 2000) (Carroll WL, Bhojwani D et al. 2003).

1.1.1.5.3 Chromosomal translocations in T- ALL

T-ALL is characterised by lymphoblasts expressing maturation stage-related T-cell antigens and is associated with a higher WBC at diagnosis, older age at presentation and a predominance of males (Uckun, Sensel et al. 1998). T-ALL has cytogenetic abnormalities that differ from B-lineage leukaemia. These differences may explain its different clinical behaviour and one may suspect a different aetiology since it is more common in the older age groups (greater than 10 years).

A high percentage of T-ALL cases lack detectable cytogenetic abnormalities (Ferrando and Look 2000). In T-ALL three translocations have been reported frequently t(10;14)(q24;q11), t(1;14)(p32;q11) and t(11;14)(p13;q11) (Rabbitts 1991). They juxtapose enhancer elements responsible for the expression of T-cell receptor (*TCR*) genes next to different oncogenes. This juxtaposition leads to the over expression of specific genes in developing thymocytes and ultimately to leukaemic transformation through disruption of transcriptional pathways involved in normal T-cell proliferation, differentiation and survival (Ferrando and Look 2000).

Based on cytogenetic analysis, translocations involving the *HOX11* locus on 10q24 are found in 5% of T-ALL, although the incidence of deregulation detected using molecular techniques is much higher (Salvati, Ranford et al. 1995) (Ferrando and Look 2000; Kees UR, Heerema NA et al. 2003) (Brake RL, Chatterjee PK et al. 2004). The presence of t(10;14) has been associated with a favourable outcome (Schneider NR, Carroll AJ et al. 2000). The existence of negative regulatory elements in the *HOX11* promoter (Brake, Kees et al. 1998), together with the expression of this oncogene in erythroleukaemia and some T-cell ALL lines lacking 10q abnormalities suggests mechanisms other than *TCR* translocation can lead to aberrant *HOX11* expression (Salvati, Ranford et al. 1995).

The *SCL* (*TAL1*) gene on 1p33 is expressed by hematopoietic progenitors and is essential for haematopoiesis (Begley and Green 1999). Abnormal *SCL* expression has been reported in up to 60% of T-ALL (Bash, Crist et al. 1993), although this frequency of deregulation may be an overestimate and the true proportion may be closer to 30% (Begley and Green 1999) . *SCL* rearrangements may identify a favourable-prognosis sub-group of patients within the T-ALL group (Kikuchi, Hayashi et al. 1993).

LMO1 (11p15) and *LMO2* (11p13) have been implicated in the pathogenesis of T-ALL. *LMO2* is essential both for yolk sac erythropoiesis and for adult haematopoiesis in mice (Warren, Colledge et al. 1994). Evidence from studies using transgenic mice suggest that *LMO* genes may cooperate with *SCL* leading to the formation of aberrant transcription complexes that disrupt normal differentiation programs in T-cell precursors (Baer, Hwang

et al. 1997). A study by the CCG identified the t(11;14) translocation in 13/169 cases of T-ALL but found no correlation with prognosis (Heerema, Sather et al. 1998).

1.1.1.5.4 Loss of tumour suppressor gene function in ALL

Loss of heterozygosity (LOH) on chromosome 12p13 in 17-33% of childhood ALL suggests the presence of a TSG in this region (Stegmaier, Pendse et al. 1995). Chromosome 6q deletions have been found in childhood ALL. A candidate TSG, *GRIK* has been identified at this breakpoint but as yet this has not been associated with differences in prognosis (Sinclair PB, Sorour A et al. 2004). Structural analysis of the *TP53* gene on chromosome 17 has shown abnormalities in only a small subset (2-3%) of ALL (Wada, Bartram et al. 1993).

Inactivation of the *RB* gene has been found in 18% of patients with ALL and in 27% of B-lineage cases with t(9;22)(*BCR/ABL*)(Sauerbrey, Stammler et al. 1998). Defects in the *RB* gene have not been shown to have any clinical prognostic significance in childhood ALL, but their frequent presence in combination with t(9;22) translocation in B-lineage cases is intriguing. Loss of function of a major cell cycle regulator such as the RB combined with the *BCR-ABL* chimeric protein may explain the resistance of this disease to conventional chemotherapies. Loss of RB may represent one of the early events, leading to instability and increased mutation rates (Hernando E, Nahle Z et al. 2004).

The most common non-random chromosomal deletions found in childhood ALL involve chromosome 9p (Heerema, Sather et al. 1999). There is a considerable amount of evidence that the TSGs at the *INK4A/ARF* and *INK4B* loci are deleted in ALL.

1.2 The *INK4A/ARF* and *INK4B* loci on chromosome 9 and their importance in childhood ALL

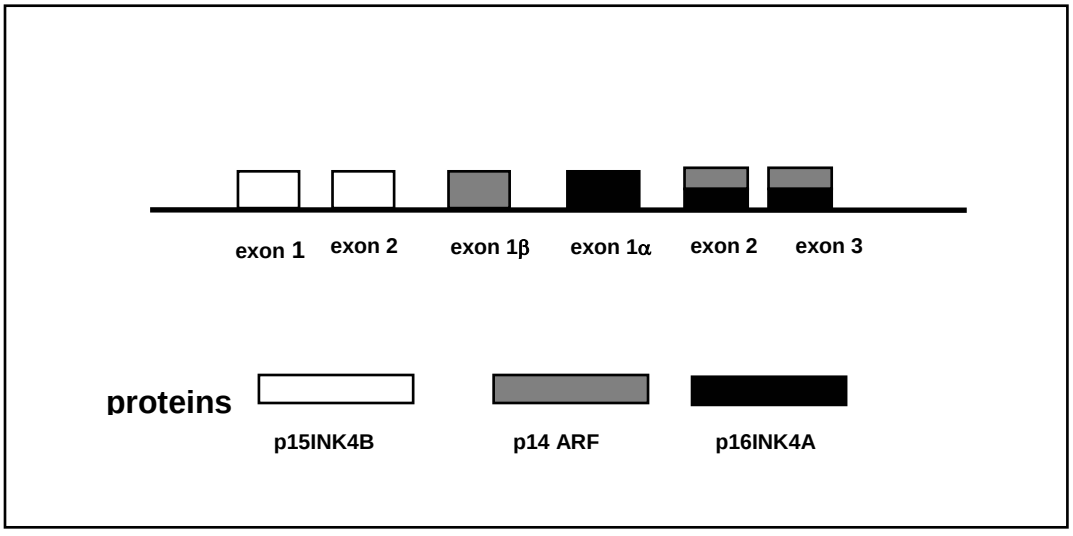
The short arm of chromosome 9 is frequently mutated or deleted in many cancers, including leukaemia, non-Hodgkin's lymphoma, multiple myeloma, malignant gliomas, lung carcinoma, ovarian and bladder cancer (Sharpless and DePinho 1999).

There is substantial evidence that aberrations of the cell cycle, including abnormalities of the *INK4A/ARF* locus, are linked to the development and progression of cancer (Kamb, Gruis et al. 1994) (Cairns, Polascik et al. 1995). Although mutations of *INK4B* have been found in some human malignancies, they are often large deletions that concurrently affect the *INK4A/ARF* locus (Drexler 1998) (Sharpless and DePinho 1999).

1.2.1 Genes at the *INK4A/ARF* and *INK4B* loci and their function in the control of the cell cycle

The *INK4A/ARF* and *INK4B* loci extend over 50kb at the 9p21 chromosomal band and include *INK4A/ARF/ CDKN2A/MTS1* and *INK4B/CDKN2B/MTS2*. *INK4A/ARF* has a complex gene structure as it encodes two unrelated proteins p16INK4A and p14ARF (p19Arf in mice) from two distinct mRNA transcripts. These RNAs are transcribed from individual promoters located 5' to alternative first exons (exon 1α and exon 1β respectively) (Quelle, Zindy et al. 1995). Exon 2 is read in two distinct reading frames to code for p16INK4A and p14ARF proteins (Figure 1).The product of the *p16INK4A* gene (which was shown to reside at 9p21) was identified through its ability to inhibit cyclin dependent kinase 4 (CDK4) (Serrano, Hannon et al. 1993). Frequent point mutations of *p16INK4A* but not *p15INK4B* were found in melanoma-prone kindreds, and homozygous deletions of the *INK4A/ARF* locus were noted in a variety of tumour cell lines (Kamb, Gruis et al. 1994).

Figure 1.1 : the genomic structure of the *INK4* cluster



Mitogen-dependent progression through the first gap phase (G_1) and initiation of DNA synthesis (S phase) during the cell cycle are regulated by several classes of cyclin dependent kinases (CDKs) whose activities are constrained by CDK inhibitors (CKI). The *INK4* proteins, *p16INK4A* and *p15INK4B* bind to CDK4 and CDK6 and act as CKI. RB phosphorylation is directly antagonized by *INK4* mediated inhibition of cyclin D-dependent kinases, thereby maintaining RB in a growth suppressive, hypophosphorylated state (Sherr and Roberts 1999). Evidence suggests that *INK4*-mediated growth arrest may depend on mobilization of other proteins from complexes containing cyclin D1-CDK4 to cyclin E-CDK2 (Sherr and Roberts 1999).

TP53 is activated only when cells are stressed or damaged. These cells pose a threat to the organism because they are more at risk of becoming cancerous. The wild type TP53 protein negatively regulates progress through the cell cycle in these cells and induces intrinsic apoptosis via the mitochondria (Carroll, Bhojwani et al. 2003). p14ARF acts by binding to MDM2, which is a negative regulator of TP53 (Vogelstein, Lane et al. 2000). Thus abnormalities of p14ARF result in a down regulation of TP53 activity.

There is evidence that *INK4A/ARF* mutations promote resistance to chemotherapeutic drugs from a murine transgenic lymphoma model (Schmitt, McCurrach et al. 1999). Findings from this animal model provide direct evidence that mutations at the *INK4A/ARF* locus have a negative impact on the outcome of cancer therapy.

Two murine studies provide evidence to support the theory that genes at the *INK4A/ARF* locus may be haploinsufficient that is loss of one allele may result in loss of function (Krimpenfort, Quon et al. 2001) (Sharpless, Bardeesy et al. 2001). If this is true for leukaemia, this locus may well join a growing class of haploinsufficient tumour suppressor genes, which includes *TP53*, *TGF-β, p27/KIP1, Pten* and *BLM* (Tang, Bottinger et al. 1998) (Fero, Randel et al. 1998) (Kwabbi-Addo, Giri et al. 2001) (Gruszka-Westwood, Hamoudi et al. 2001) (Gruber, Ellis et al. 2002). Where loss of one allele occurs in a TSG, the subtle dosage issues must be understood if pathways to cancer are to be explored (Fodde and Smits 2002). The suggestion that genes at the *INK4A/ARF* locus may be

haploinsufficient has profound implications in cancer. In childhood leukaemia haploinsufficiency at this locus has never been explored.

1.2.2 Deletions of the *INK4A/ARF* and *INK4B* loci in childhood ALL

In a large study of newly diagnosed childhood ALL patient specimens Heerema and colleagues found that 11% had associated cytogenetic abnormalities of the 9p chromosome. They identified a subgroup of standard risk patients with 9p deletions who had a significantly increased risk of treatment failure (Heerema, Sather et al. 1999). The most frequent mode of *INK4A/ARF* inactivation in paediatric ALL occurs by deletion (Kees, Burton et al. 1997) (Drexler 1998). In a review of the literature by Drexler, an incidence of homozygous *INK4A/ARF* deletion in childhood ALL of 23% in B-lineage and 64% in T-ALL was found (Drexler 1998). Despite a number of studies investigating the incidence of *INK4A/ARF* deletions in childhood ALL the clinical significance of such deletions, as an independent prognostic indicator remains controversial (Drexler 1998) (Tsihlias, Kapusta et al. 1999).

In T-ALL the incidence of abnormalities of the *INK4A/ARF* locus is higher. Inactivation of the cell cycle regulatory pathway may be essential in the pathogenesis of T-ALL (Omura-Minamisawa, Diccianni et al. 2000). In a comprehensive study on primary paediatric T-ALL specimens, Omura-Minamisawa and colleagues found the incidence of abnormalities of *p16INK4A* and *p15INK4B* at the DNA, RNA and protein level were 93% and 99%, respectively. This study also explored abnormalities of *p14ARF* and found inactivation at the DNA level occurred at approximately the same rate as *p16INK4A* inactivation (Omura-Minamisawa, Diccianni et al. 2000).

It would appear that other mechanisms apart from deletion are causing inactivation of genes at these loci. In childhood ALL methylation of the CpG islands of *p15INK4B* is more common than methylation of *p16INK4A* and *p14ARF* CpG islands (Iravani, Dhat et al. 1997) (Wong, Ng et al. 2000) (Batova, Diccianni et al. 1997). Promoter methylation of *p16INK4* and *p14ARF* is more frequent in other types of cancers such as in adult ALL, which may suggest different aetiologies (Iravani, Dhat et al. 1997) (Esteller, Tortola et al. 2000).

There is no doubt that abnormalities at the *INK4A/ARF* and *INK4B* loci are frequently found at diagnosis in childhood ALL, although their prognostic significance remains controversial. Whether deletions of these loci effect chemotherapeutic response can be explored in relapse specimens.

1.3 Relapsed childhood ALL

Current risk adjusted therapies for childhood ALL are expected to result in an EFS of greater than 76%. In sharp contrast, relapsed paediatric ALL is a difficult disease to treat. Despite dramatic improvements in the prognosis of paediatric ALL, the overall 6-year EFS for relapse patients is only 20–30% (Henze, Fengler et al. 1991) (Schroeder, Garwicz et al. 1995) (Gaynon, Qu et al. 1998). Relapses are defined as very early (less than 18 months), early (greater than 18 and less than 30 months) and late (greater than 30 months) (Uderzo, Dini et al. 2000). Allogenic bone marrow transplant is superior to conventional chemotherapy for initial relapse, especially if the relapse is very early (Uderzo, Dini et al. 2000) (Wheeler, Richards et al. 2000).

The differing responses to chemotherapy in very early and late relapse leukaemia may imply different aetiologies. Studies in B-lineage ALL indicate that rapid cytoreduction below the threshold of molecular detection during the induction phase of treatment is correlated with long-term survival; however, this information cannot predict exactly which patients would relapse (Wasserman, Galili et al. 1992).

Understanding the genetic alterations critical in the aetiology of relapse in paediatric ALL has been difficult because of the scarcity of paired specimens.

1.3.1 Cytogenetic changes at relapse

Karyotypic abnormalities found at diagnosis such as t(9;22), hypodiploidy and chromosomal 9p deletions are known high risk features, however their role in the development of relapse is not clear (Uckun, Sensel et al. 1998) (Heerema, Nachman et al. 1999) (Heerema, Sather et al. 1999).

Development of additional structural chromosomal changes at relapse has been reported (Berger, Le Coniat et al. 1988). These additional chromosomal changes have been documented in 55-60% of patients with ALL at relapse (Secker-Walker, Alimena et al. 1989) (Heerema, Palmer et al. 1992) (Vora, Frost et al. 1998). In a study by Shikano and colleagues on paired paediatric ALL specimens with abnormal karyotypes at diagnosis, the most frequent changes at relapse found were 6q-, 7p- and 9p- structural abnormalities (Shikano, Ishikakawa et al. 1990).

It is important to note that karyotype analysis underestimates the frequency of changes at the molecular level (Salvati, Ranford et al. 1995) (Shurtleff, Buijs et al. 1995). Several molecular lesions are detected at higher frequency in relapsed childhood ALL, including *TP53* mutation, increased Cyclin-D1, BAX protein expression and *TEL/AML-1* expression and deletion of the genes at the *INK4A/ARF* locus.

1.3.2 Molecular lesions involved at relapse

Relapsed childhood ALL can be grouped clinically by the median time to relapse as discussed above. Molecular lesions at relapse appear to define these groups. *TEL/AML-1* at relapse appears to correlate with a longer period of EFS and a better prognosis post relapse (Harbott, Viehmann et al. 1997) (Loh, Silverman et al. 1998) (Seeger, Adams et al. 1998) . There is some evidence to suggest that failure of eradication of a "pre-leukaemic" *TEL/AML-1* clone by treatments that lack effective doses of asparaginase may lead to emergence of a second leukaemia with *TEL/AML-1* expression (Loh, Silverman et al. 1998) (Ford, Fasching et al. 2001). The effect of chemotherapy on the cancer cell has been investigated using murine models.

Aberrations of the cell cycle are linked to the development and the progression of cancer, including the genes at the *INK4A/ARF* locus (Kamb, Gruis et al. 1994; Cairns, Polascik et al. 1995). Based on limited studies in childhood ALL, progression of disease also appears to be associated with the loss of genes at this locus (Ohnishi, Hanada et al. 1996) (Diccianni, Batova et al. 1997; Maloney, Mcgavran et al. 1999). Abnormalities of

INK4A/ARF locus lead to upregulation of cylin D1. Expression of cyclin D1 was increased in relapsed specimens compared to diagnostic specimens in an unpaired retrospective study and this was associated with a poorer prognosis (Sauerbrey, Hafer et al. 1999). A murine lymphoma model provides independent evidence that *INK4A/ARF* mutations promote resistance to chemotherapeutic drugs (Schmitt, McCurrach et al. 1999). These mice developed highly invasive lymphomas, and the tumour cells displayed apoptotic defects that were resistant to chemotherapy. Further investigation by Schmitt and colleagues has shown a link between this resistance and abnormalities of both the *Ink4a/Arf* and *TP53* loci (Schmitt, Fridman et al. 2002).

The incidence of *TP53* mutations at diagnosis in paediatric ALL is 2-3%, contrasting with a reported incidence at relapse of 0-24% (Wada, Bartram et al. 1993) (Diccianni, Yu et al. 1994) (Marks, Kurz et al. 1996; Blau, Avigad et al. 1997) (Gump J, McGavran L et al. 2001). The *TP53* mutations identified at the time of relapse were associated with a decreased duration of first remission and overall decrease in survival time from diagnosis. *TP53* is involved in cellular apoptosis and defects developing in *TP53* must affect the way a cancer cell responds to chemotherapy. A study on paired diagnostic and relapse ALL specimens explored the expression of apoptotic proteins and found a significant increase in the probability of relapse associated with BAX protein expression (Hogarth and Hall 1999).

The role of critical genetic alterations in relapse leukaemia and their response to treatment is vital to understanding the aetiology of leukaemogenesis. It also provides us with a better understanding of the prognostic significance of genetic alterations at diagnosis. Whether these changes are occurring in the original dominant clone, possibly due to chemotherapy exposure, or reflect the expansion of a subclone more resistant to chemotherapy is important to determine when targeted therapies are sought.

1.3.3 Monitoring disease following exposure to chemotherapy

Developing new sensitive methods for monitoring acute leukaemia disease activity is one of the major tasks of research laboratories, since early detection of relapse may improve clinical outcome. Markers of disease such as translocations can be monitored in an

individual patient and their appearance in the bone marrow, peripheral blood or CNS can be an early indication of relapse prior to the detection of leukaemic blasts. Studies on the t(9; 22) translocation have found early evidence of relapse in this way but cytogenetic studies are not a sensitive indicator (Uckun, Nachman et al. 1998).

Lymphoid malignancies offer a unique opportunity for the investigation of disease evolution and progression at the genetic level as a consequence of recombination within the immune system gene loci. Studies of gene rearrangements in lymphoproliferative diseases have provided information of practical diagnostic value for distinguishing neoplastic from reactive disease, assigning lineage and to some extent determining extent of disease (Trainor, Brisco et al. 1991).

1.3.3.1 Immunoglobulin heavy chain (*IgH*) and *TCR* gene rearrangements

Neoplastic B-cells, like their normal counterparts, undergo *IgH* gene rearrangement before clonal expansion (Arnold, Cossman et al. 1983). In a mature B-cell population a single *IgH* gene rearrangement can be used as a marker of monoclonality and hence a malignant process (Cossman, Uppenkamp et al. 1988). T-cells have a surface antigen receptor (TCR) that can appear either as a αβ configuration or γδ. The genes that encode these proteins are arranged early in T-cell neoplasms and can be used as clonal markers (Steenbergen, Verhagen et al. 1995). Leukaemogenesis is a dynamic process with continued evolution of *IgH* and *TCR* rearrangements (Steenbergen, Verhagen et al. 1995) (Choi, Greenberg et al. 1996). This may be a reflection of more general genetic change giving rise to the outgrowth of more aggressive, treatment resistant tumour subclones.

1.3.3.2 Clonal evolution in leukaemia

A number of studies have used *IgH* and *TCR* gene rearrangements to study disease evolution. Dibenedetto and colleagues, found in bone marrow specimens from children with T-ALL that the persistence of *TCR* rearrangements at a specific time during treatment i.e. 34-40 weeks after diagnosis (when the patient entered maintenance therapy), correlated with subsequent bone marrow relapse (Dibenedetto, Lo Nigro et al. 1997). The absence of detectable *TCR* rearrangements any time after diagnosis was correlated with a favourable

outcome (Dibenedetto, Lo Nigro et al. 1997). A study on B-lineage childhood ALL specimens discovered a similar scenario. High levels of residual disease, as detected by *IgH* rearrangements using semi-quantitative PCR, at the end of induction therapy identified patients at an increased risk of relapse (Wasserman, Galili et al. 1992). In the majority of patient bone marrow specimens in ALL it would appear that these rearrangements both in B-lineage and T-ALL represent major leukaemia clones and can be measured to follow the evolution of that particular clone (Steenbergen, Verhagen et al. 1995).

There is evidence from patient and murine studies to support the presence of subclones, not detected at diagnosis, which could account for relapse disease. Using mice with severe combined immunodeficiency syndrome (SCID) Steenbergen and colleagues found that when cells from patients with B-lineage ALL were injected into mice, different subclones grew out in different mice, represented by the presence of different *IgH* rearrangements (Steenbergen, Verhagen et al. 1996). In a study of specimens from three children with B-lineage ALL, measuring IgH and *TCR* rearrangements at diagnosis and following chemotherapy, subclones were found to respond differently in response to therapy (de Haas, Verhagen et al. 2001). This would support the hypothesis that subclones display differences in respect to their chemotherapeutic resistance. The presence of more than one detectable clone at diagnosis, demonstrated by more than one major rearrangement, could be associated with a worse prognosis. A study by Green and colleagues, on bone marrow specimens from 65 paediatric B-lineage ALL patients supports this theory (Green, McConville et al. 1998). They found that clonal diversity as detected by *IgH* and *TCR* rearrangements was a useful prognostic indicator and was associated with a high probability of relapse in standard risk patients ($p=0.0048$). In addition, patients with clonal diversity had a reduced 5 year EFS (Green, McConville et al. 1998). Detailed analysis of the leukemic cells at diagnosis and at relapse by use of *IgH* and *TCR* rearrangements may give insight into the heterogeneity at diagnosis (process of subclone formation) and at relapse (selection of subclones) possibly related to development of therapy resistance (Beishuizen, Verhoeven et al. 1994).

1.4 Overall objectives of this study

1.4.1 Development of a novel, accurate, reproducible method to detect deletions at the *INK4A/ARF* and *INK4B* loci

Assessing the status of a TSG accurately in a mixed cellular milieu such as human bone marrow can be difficult because of the presence of normal cells. Previous studies of the *INK4A/ARF* and *INK4B* loci in cancer have used Southern blot and conventional PCR. Using these techniques it is difficult to control for normal cell interference when investigating a deletion of one or both alleles. The first aim of this project was to establish a high throughput, sensitive assay to assess tumour suppressor genes at the *INK4A/ARF* and *INK4B* loci. The assay should distinguish homozygous from hemizygous deletion for each gene and control for normal cell contamination.

1.4.2 Establishing the incidence of *INK4A/ARF* and *INK4B* loci deletions in specimens from patients with childhood ALL

Bone marrow specimens were studied using the qPCR technique developed in a group of a patients with childhood ALL.

1.4.2.1 Clarification of the prognostic implications of a *INK4A/ARF* locus deletion at diagnosis in childhood ALL

Abnormalities at the *INK4A/ARF* locus were studied in specimens from childhood ALL patients obtained at the time of diagnosis and their incidence correlated with clinical outcome. This study was expected to clarify the independent prognostic implications of deletions at the *INK4A/ARF* locus at diagnosis in childhood ALL.

1.4.2.2 Incidence of *INK4A/ARF* and *INK4B* loci deletion at diagnosis and at relapse in childhood ALL

Based on limited studies in childhood ALL, progression appears to be associated with the loss of genes at *INK4A/ARF* and *INK4B* loci. A detailed study of the *INK4A/ARF* and *INK4B* loci was conducted on paired diagnostic and relapse paediatric ALL specimens to

elucidate the importance of the *INK4A/ARF* and *INK4B* loci at relapse and their possible relevance in resistance to conventional treatment.

1.4.3 Development of resistance to chemotherapy and deletion of *INK4A/ARF* and *INK4B* in patient specimens

Murine studies have shown that abnormalities of the *Ink4a/Arf* and *p53* loci may affect chemotherapeutic resistance. A detailed study of *TP53* mutations, in the paired study group will also be investigated and this will be correlated with *INK4A/ARF* and *INK4B* deletion.

Chapter 2

Hemizygous *p16INK4A* deletion in paediatric acute lymphoblastic leukaemia predicts independent risk of relapse

2.1. Abstract

The genes at the *INK4A/ARF* locus at 9p21 are frequently involved in human cancer. Virtually all $p16^{INK4A}$ exon 2 (henceforth called *p16)* inactivation in paediatric acute lymphoblastic leukaemia (ALL) occurs by gene deletion. The results of this study illustrate that real-time quantitative polymerase chain reaction is capable of detecting gene deletion in primary patient specimens with a precision not previously achieved by conventional methods. Importantly, this assay includes the detection of hemizygous deletions. The study revealed, strikingly, that the risk ratio for relapse for hemizygous deletion compared with no deletion was 6.558 (P = .00687) and for homozygous deletion was 11.558 (P = .000539). These results confirm and extend the authors' previous findings that homozygous deletion of *p16* in paediatric ALL patients is an independent prognostic indicator of outcome from therapy.

2.2. Introduction

The assessment of deletion of certain genes requires the detection of hemizygosity in primary patient specimens contaminated with normal cells. Meeting this challenge in cancer screening is becoming increasingly critical with the recent identification of several tumour suppressor genes that are haplo-insufficient rather than classically recessive (Cook and McCaw 2000). The genes at the *INK4A/ARF* locus act as tumour suppressors via 2 proteins, $p16^{INK4A}$ and $p14^{ARF}$ ($p19^{ARF}$ in the mouse), while the role of *p15* in leukemogenesis remains unresolved (Cairns, Polascik et al. 1995). The $p16^{INK4A}$ is a cyclin-dependent kinase inhibitor that acts upstream of the retinoblastoma (RB) protein to control cell cycle arrest (Roussel 1999). The $p19^{ARF}$ is translated in an alternative reading frame from $p16^{INK4A}$ and activates p53 by interfering with its negative regulator, MDM2 (Sharpless and DePinho 1999). Hence, mutations at the *INK4A/ARF* locus can disrupt both the *RB1* and *p53* tumour suppressor pathways (Roussel 1999; Sharpless and DePinho 1999). Essentially all $p16^{INK4A}$ inactivation in paediatric acute lymphoblastic leukaemia (ALL) occurs by gene deletion (reviewed in Kees et al and Drexler) (Kees, Burton et al.

1997; Drexler 1998). The evidence for an independent prognostic role of *p16*^{INK4A}, exon 2 (henceforth called *p16*) deletion in paediatric ALL, is inconclusive (reviewed in Drexler and Tsihlias et al) (Drexler 1998) (Tsihlias, Kapusta et al. 1999). All of these studies were bases on detecting *p16* deletion by either Southern blotting or conventional polymerase chain reaction (PCR) analysis. Bone marrow specimens from leukaemia patients at diagnosis invariably contain some normal cells that cause problems with accurate quantitation of deletion of any gene. This prompted us to examine whether gene deletions can be detected by real-time quantitative PCR and whether *p16* zygosity presents a simple and reliable test for leukaemia prognosis.

2.3. Study design

2.3.1. Patients

Diagnosis bone marrow specimens were studies from 45 ALL patients, on the basis of the availability of cryopreserved specimens (Table 2.1). The Princess Margaret Hospital, Perth, Australia, provided 30 patients, and the Children's National Medical Center, Washington, DC, provided 15. There was no selection on the basis of either *p16* genotype or time-to-relapse. Informed consent was obtained from all patients or their guardians to use specimens for research. Specimens were collected between 1981 and 1997. The leukaemia immunophenotype (B-lineage or T-cell ALL) was determined with the use of a panel of monoclonal antibodies (Kees, Burton et al. 1997). Cytogenetic results revealed that one of the patients were known to have either t(4;11) or t(9;22). For all but 2 patients studied, therapy was administered according to risk-adjusted protocols of the Children's Cancer Group, most of which were based upon modifications of the Berlin-Frankfurt-Munster trials; the exception consisted of 2 patients treated on a previously reported intensive therapy protocol for high-risk patients (Gaynon, Bostrom et al. 1998).

2.3.2. Real-time PCR analysis in multiplex format

Genomic DNA was isolated from cryopreserved specimens and control cell lines by standard methodology (Kees, Burton et al. 1997). All primers and probes were designed by means of Perkin-Elmer Primer Express software (Perkin-Elmer, Foster City, CA), and

primers were supplied by Geneworks (Adelaide, Australia). The sequences were are follows: *p16* exon 2 forward (F): ggctctacacaagcttcctttcc; *p16* exon 2 reverse (R) tcatgacctgccagagagaaca; *β-actin* F: agcgcggctacagcttca; and *β-actin* R: cgtagcacagcttctccttaatgtc. The probe for *p16* had the sequence cccccaccctggctctgacca and was labelled with FAM, whereas the probe from *β-actin,* atttcccgctcggccgtggt*,* was labeled with VIC (both probes manufactured by Perkin-Elmer). The reactions were optimized first individually and then for multiplexing. The reaction was performed in a final volume of 50uL. The final concentrations of primers and probes were as follows: p16F 50ng/uL, p16R 50ng.uL, p16 probe 200nM, *β-actin* F 50ng/uL, *β-actin* R 200ng/uL and *β-actin* probe 200nM. Each reaction contained 50ng DNA as template, ad the Taqman Universal Master Mix (Perkin-Elmer) was used. The standard thermal cycling conditions of the ABI PRISM 7700 Sequence Detection instrument were applied. A standard calibration curve was included with each experiment with the use of a range of concentrations of DNA extracted from Raji B cells (range: 1.56-100 ng DNA).

Table 2.1: Clinical characteristics and *p16*$^{INK4A/ARF}$ status of study group

Parameter	Number (Percentage)
Total Number:	45
Sex:	
Female	14 (31)
Male	31 (69)
Age:	
< 1 yr	1 (2)
1 yr - 10 yrs	30 (67)
> 10 yrs	14 (31)
Immunophenotype:	
T-ALL	13 (29)
B-lineage-ALL	32 (71)
WCC at diagnosis:	
<50,000 x 10^6/L	26 (58)
>50,000 x 10^6/L	19 (42)
***P16*$^{INK4A/ARF}$ genotype of leukaemia cells:**	
GG	28 (62)
DD	11 (25)
GD	6 (13)

T-ALL indicates T-lineage acute lymphoblastic leukaemia; WCC, white cell count; GG, germline *p16*$^{INK4A/ARF}$; DD, *p16*$^{INK4A/ARF}$ homozygous deletion; GD, hemizygous *p16*$^{INK4A/ARF}$.

2.3.3. *p16* gene deletion analysis in specimens containing normal cells

We simulated normal cell contamination by using mixtures of DNA from Raji B cells, which are wild type for *p16* (G/G), and K562 cells, which show homozygous deletion of *p16* (D/D). The experimentally determined ratio for *p16/β-actin* was expressed as a function of the input ratio of Raji B/K562 cells. The test yielded a linear graph with a correlation coefficient of 0.9687, indicating that normal cell contamination (here simulated by Raji B cells) in a *p16* D/D sample can be accurately measured by this technique. On the

basis of this result, the bone marrow specimens were interpreted as follows. Ratio for *p16/β-actin* less then 0.4: *p16* deletion (D/D); ratio 0.4 to 0.8: hemizygous *p16* (G/D); ratio exceeding 0.8: germline *p16* (G/G). The method was compared with Southern blot analysis, and the 2 methods agreed in all 11 cases tested, including G/D specimens. All but one of the 45 specimens contained fewer than 25% normal cells, according to an independent review by a haematologist, and the experimentally determined ratio for *p16/β-actin* was used to determine the genotype directly. The remaining specimen contained more than 50% normal cells, a factor that was taken into account.

2.3.4. Statistical analysis

The main analysis was based on methods appropriate for censored failure times. The primary time scale was calendar time from diagnosis; the primary response was relapse. Univariate analysis was based upon Kaplan-Meier survival functions and the Mantel-Cox (log-rank) test statistic (Kalbfleisch and Prentice 1980). Multivariate analysis was based on the Cox proportional hazards regression model and the likelihood-ratio test (McCullagh and Nelder 1983). Covariates known to modulate the risk of relapse were included in the primary model whether they were statistically significant or not. Secondary modeling demonstrated that removal of the nonsignificant covariates did not modify substantive conclusions. Final models were subjected to (and passed) standard tests of goodness of fit (McCullagh and Nelder 1983). Analysis was undertaken in SAS version 6.12 (Cary, NC) for Unix.

2.4. Results and discussion

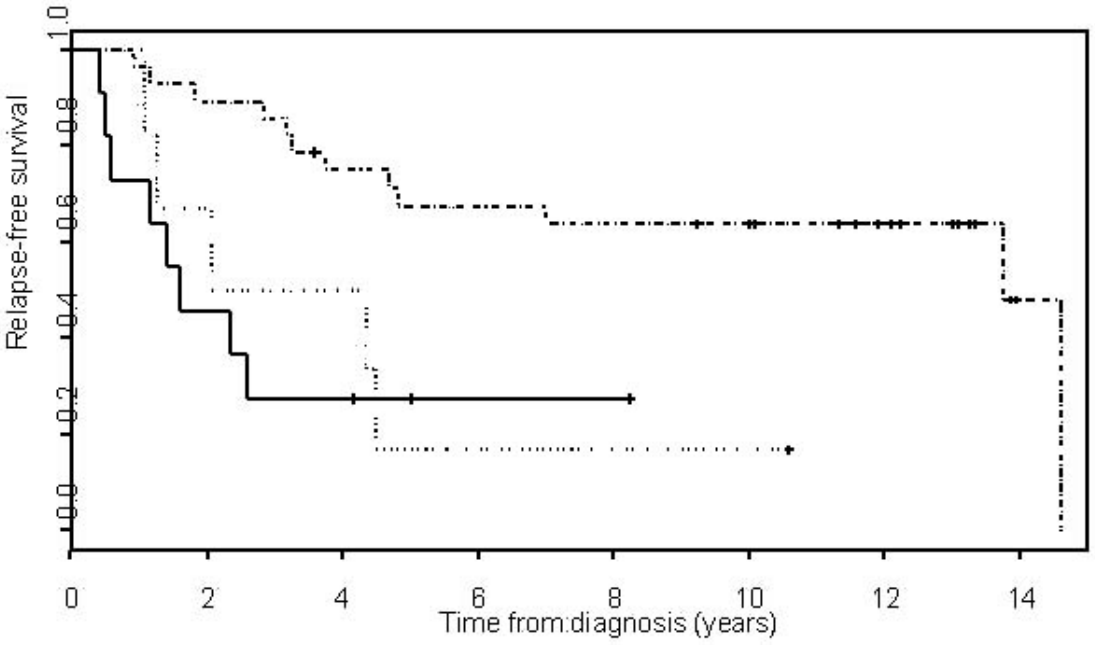
Deletion analysis of *p16* was performed on 45 paediatric ALL patients at diagnosis, and the results are summarized in Table 2.1. Of the 45 patients, 11 (25%) demonstrated a homozygous deletion; 6 (13%) were hemizygous; and 28 (62%) were wild type for the *p16* gene. In a previous study using Southern blot analysis performed in this laboratory, the incidence of homozygous *p16* deletions at diagnosis was 18.3% (9/48) (Kees, Burton et al. 1997). These findings for homozygous deletions are in agreement with the frequency of homozygous deletion reported for paediatric ALL patients: 23% for B-lineage and 64% for T-lineage ALL (T-ALL) (reviewed by Drexler) (Drexler 1998). The combined

frequency for hemizygous add homozygous deletions determined here is 38%. When the distribution of T-ALL versus B-lineage ALL cases in our study was taken into account, the observed frequency is higher than expected, most likely owing to the higher sensitivity of the PCR technique.

2.4.1. Hemizygous and homozygous *p16* deletions are independent prognostic indicators for poor outcome

Figure 2.1 illustrates the Kaplan-Meier curves for relapse-free survival stratified by *p16* status. Among these patients who were "censored" (i.e. had not relapsed at the end of follow-up), the minimum follow-up time was 3 years, and all but 3 such patients were followed for at least 8 years. With the use of the log-rank test for any differences between 3 groups, the P value was .0021. Multivariate analysis was performed by means of Cox proportional hazards regression, including the known risk factors for paediatric ALL patients: gender, immunophenotype, age, and white cell count at diagnosis. When we adjusted simultaneously for the influence of these risk factors, both hemizygous and homozygous deletions remained highly statistically significant predictors of poor outcome; compared with G/G patients, the risk ratio was 11.558 (P = .000539) for patients with homozygous deletions and 6.558 (P = .00687) for hemizygous deletions (Table 2.2). This comprehensive adjustment for potential confounding variables showed that any *p16* deletion (D/D or G/D) is a major independent risk factor for relapse. These results confirm and extend previous findings for our laboratory and others regarding the prognostic significance of *p16* deletion in paediatric ALL patients (Kees, Burton et al. 1997) (Heyman, Rasool et al. 1996). Of the patients in this analysis, 11 were also included in our earlier study (Kees, Burton et al. 1997). If these are excluded, the estimated adjusted-risk ratios for G/D versus G/G and D/D versus G/G are 5.325 (P = .0462) and 9.856 (P = .0027), respectively. This represents a completely independent test of the hypothesis that the *p16* deletion is associated with prognosis in childhood ALL. Nevertheless, a prospective study on a larger number of uniformly treated patients should be conducted to confirm the prognostic significance of the *p16* deletion, and such a study should include a test for loss of *p14*.

Figure 2.1 Kaplan-Meier survivor function



G/G (------), G/D (…...) and D/D (____) patient samples

Hemizygous status as determined in this study could be due to a mixture of leukaemia cells (D/D, G/D, and G/G) or true hemizygosity in all leukaemia cells. Owing to lack of material, it was not possible to study the G/D specimens by means of the fluorescence in situ hybridization technique. However, the clonality could be assessed by analyzing the rearrangement of the T-cell receptor and immunoglobulin heavy chain genes (Yamada, Hudson et al. 1989) (Veelken, Tycko et al. 1991). Examination of the 5 G/D specimens from patients who relapsed revealed that in 3 cases there was clear evidence for clonal disease as only one rearranged band was detected whereas the 2 remaining cases showed 2 bands suggesting biclonal disease showing hemizygous deletion of *p16*, excluding the potential for false hemizygous readout due to a mixture of G/G with D/D leukemic cells in the specimens.

In the current study, we identify hemizygous and homozygous loss of *p16* as a major independent negative prognostic indicator in paediatric ALL. These results are consistent with the reported general sensitivity of paediatric ALL to current chemotherapy. Unlike the majority of good-prognosis paediatric ALL patients, who appear to have an intact apoptosis pathway, the subpopulation that is refractory to treatment with apoptosis-inducing drugs may have bypassed normal regulation by mutation of key regulations such as *p16* (Greaves 1999). Independent evidence that *INK4A/ARF* mutations promote resistance to chemotherapeutic drugs has recently been reported in a transgenic lymphoma model (Schmitt, McCurrach et al. 1999). The findings from this animal model provide direct evidence that mutations at the *INK4A/ARF* locus have a negative impact on the outcome of cancer therapy. The quantitative PCR method used in our study is suitable for high-throughput screening of patient specimens and has many clinical applications as it can be adapted to the deletion analysis of other tumour suppressor genes and other cancers.

Table 2.2 Hemizygous and homozygous *p16$^{INK4A/ARF}$* deletions are independent prognostic indicators for poor outcome

Genotype	Risk ratio*	Confidence interval	P value
GD versus GG	6.558	1.858 - 23.880	0.00687
DD versus GG	11.558	2.825 - 47.284	0.000539

GG, wildtype for *p16$^{INK4A/ARF}$*
DD, homozygous deletion of *p16$^{INK4A/ARF}$*
GD, hemizygous deletion of *p16$^{INK4A/ARF}$*

* Multivariate analysis was performed to determine risk ratios.

Chapter 3

INK4A/ARF deletions are acquired at relapse in childhood acute lymphoblastic leukaemia: a paired study on 25 patients using real-time polymerase chain reaction.

3.1. Summary

Current risk-adjusted intensive therapies for childhood acute lymphoblastic leukaemia (ALL) are expected to result in an event-free survival of greater than 75%. In sharp contrast, relapsed paediatric ALL is a difficult disease to treat. In this study, 25 paediatric patients with ALL were analysed at diagnosis and relapse for their *p16* (exon 2) status using the most accurate method of detection, real-time polymerase chain reaction (PCR). The median time to relapse for the group was 27 months. At diagnosis, the incidence of *p16* homozygous and hemizygous deletion in this group was 32% and 20% respectively. The incidence of homozygous *p16* deletion at relapse was 64%. A large number of patients, eight of 16 (50%), developed *p16* homozygous deletion at relapse. Of those eight patients, four were hemizygous and four were germ-line at diagnosis. At diagnosis, those patients with a homozygous or hemizygous *p16* deletion relapsed sooner than those germline for *p16*. We have shown that *p16* alterations are frequently present in relapsed lymphoblastic leukaemia in children.

3.2. Introduction

Current risk-adjusted therapies for childhood acute lymphoblastic leukaemia (ALL) are expected to result in an event-free survival of greater than 75%. In sharp contrast, relapsed paediatric ALL is a difficult disease to treat. Despite dramatic improvements in the prognosis of paediatric ALL, the overall 6 years event-free survival (EFS) for relapse patients is 20-30% (Gaynon, Qu et al. 1998) (Henze, Fengler et al. 1991) (Schroeder, Garwicz et al. 1995). Understanding the genetic alterations critical in the aetiology of relapse in paediatric ALL has been difficult because of the scarcity of paired specimens.

Karyotypic abnormalities found at diagnosis such as t(9:22), hypodiploidy and chromosome 9p abnormalities, are known high-risk features; however, their role in the development of relapse is unknown (Uckun, Nachman et al. 1998) (Heerema, Sather et al. 1999).Development of new chromosome structure changes at relapse have been reported (Berger, Le Coniat et al. 1988). Clonal evolution has been documented in 55-60% of patients with ALL at relapse (Secker-Walker, Alimena et al. 1989) (Heerema, Palmer et al. 1992) (Vora, Frost et al. 1998). In a study by Shikano *et al* of 21 paired paediatric ALL specimens with abnormal karyotypes at diagnosis, the most frequent changes at relapse were the development of 6q- (3/21), 7p-(2/21) and 9p-(2/21) structural abnormalities (Shikano, Ishikakawa et al. 1990).

It is important to note that macroscopic karyotype analysis underestimates the frequency of changes at the molecular level (Salvati, Ranford et al. 1995) (Shurtleff, Buijs et al. 1995). Several molecular lesions are detected at higher frequency in relapsed childhood ALL, including *p53* mutation, increased cyclin-D1 expression, increased BAX protein expression and deletion of the genes at the *INK4A/ARF* locus.

The incidence of *p53* mutations at diagnosis in paediatric ALL is 2 - 3% (Wada, Bartram et al. 1993) (Marks, Kurz et al. 1996), contrasting with a reported incidence at relapse of 19 - 24% (Diccianni, Yu et al. 1994) (Blau, Avigad et al. 1997). The *p53* mutations identified at the time of relapse were associated with a decreased duration of first remission and overall decrease in survival time from diagnosis.

A study on 10 sequential ALL specimens looked at the expression of apoptotic proteins and found a significant increase in the probability of relapse associated with BAX protein expression (Hogarth and Hall 1999).

In an unpaired retrospective study on 87 patients, expression of cyclin D1 was increased in relapsed compared with diagnostic specimens and this was associated with a poorer prognosis (Sauerbrey, Hafer et al. 1999).

There is substantial evidence that aberrations of the cell cycle are linked to the development and progression of cancer (Kamb, Gruis et al. 1994), including the genes at *INK4A/ARF* locus (Cairns, Polascik et al. 1995). The most frequent mode of *p16* inactivation in paediatric ALL occurs by gene deletion (Kees, Burton et al. 1997) (Drexler 1998). The *INK4A/ARF* locus encodes two proteins $p16^{INK4A}$ and $p19A^{ARF}$ (p14 in humans) using an overlapping reading frame. Both proteins play a role in regulating cell growth, survival and senescence (Quelle, Zindy et al. 1995). Studies in mice have shown that the $p16^{INK4A}$ and $p19^{ARF}$ proteins exert their tumour suppressor activity via the *RB1* and *p53* tumour suppressor pathways, respectively (Roussel 1999) (Sharpless and DePinho 1999).

Based on limited studies in childhood ALL, progression also appears to be associated with the loss of genes at this locus (Ohnishi, Hanada et al. 1996) (Diccianni, Batova et al. 1997) (Maloney, Mcgavran et al. 1999). These observations prompted us to study 25 paediatric ALL patients from whom specimens were available at diagnosis and relapse. In addition, we developed an innovative technique for the detection of *p16* deletion using real-time quantitative polymerase chain reaction (PCR). Our data indicated that deletion of *p16* was acquired at the time of relapse in 50% of patients.

3.3. Patients and methods

3.3.1. Clinical material.

The study group was composed of 25 paediatric ALL patients who relapsed after initial treatment. Ten were treated at the Princess Margaret Hospital (PMH), Perth, Australia, and data for the other 15 patients were supplied by the Children's National Medical Center, Washington, USA. Cases were studied based on the availability of cryopreserved Ficoll-Hypaque-enriched leukaemia blasts from bone marrow aspirates. Informed consent was obtained from all patients or the patients' guardians to obtain specimens for study. Patient specimens were collected between 1981 and 1997. Presenting features are summarized in Table 3.1. The immunophenotypes of the leukaemia were determined using a panel of monoclonal antibodies (Kees, Ranford et al. 1996), and patients were classified as either T-cell ALL (T-ALL) or B-lineage ALL. The median white cell count (WCC) (x 10^9/l) at diagnosis was 58.9 (range 3.7-980). The median age at diagnosis was 9 years 7 months

(range 8 months - 17 years 7 months). Median time to relapse was 27 months. Cytogenetic results revealed that none of the patients were known to have either t(4;11) or t(9;22).

Therapy was administered to all patients studied according to risk-adjusted protocols of the Children's Cancer Group (Gaynon, Bostrom et al. 1998), most of which were based upon modifications of the Berlin-Frankfurt-Munster trials with the exception of two patients treated on a previously reported intensive-therapy protocol for high-risk patients (Gaynon, Bostrom et al. 1998).

3.3.2. Real-time PCR analysis of *p16* in multplex format

Genomic DNA was isolated from cryopreserved specimens and control cell lines by standard methodology (Kees, Burton et al. 1997). The primers for human *p16* exon 2 and for *β-actin* were designed using Perkin Elmer Primer Express and were supplied by Geneworks, Australia and the sequences were as follows: *p16* forward (F): ggctctacacaagcttcctttcc, *p16* reverse (R): tcatgacctgccagagagaaca, *β-actin* F: agcgcggctacagcttca and *β-actin* R: cgtagcacagcttctccttaatgtc. The probe for *p16* had the sequence cccccaccctggctctgacca and was labelled with FAM, whereas the probe for *β-actin*, atttcccgctcggccgtggt, was labeled with VIC (both probes manufactured by Perkin Elmer. Primers and probes were first optimized for each reaction to achieve minimal threshold cycle numbers and maximal delta Rn (fluorescence intensity over background) values. The two reactions were then run in the same well after optimizing the conditions for

Table 3.1

Clinical characteristics and *p16$^{INK4A/ARF}$* status of study group at diagnosis.

Parameter	Number (Percentage)
Total number:	25
Sex:	
Female	5 (20)
Male	20 (80)
Age:	
< 1 yr	1 (4)
1-10 yrs	13 (52)
> 10 yrs	11 (44)
Immunophenotype:	
T-ALL	8 (32)
B-lineage-ALL	17 (68)
WCC at diagnosis:	
<50,000 x 10^6/L	11 (44)
≥50,000 x 10^6/L	14 (56)
NCI Current Classification:	
Standard	4 (16)
High	21 (84)
***p16$^{INK4A/ARF}$* genotype:**	
GG	12 (48)
DD	8 (32)
GD	5 (20)

GG, wildtype for *p16$^{INK4A/ARF}$*
DD, homozygous deletion of *p16$^{INK4A/ARF}$*
GD, hemizygous deletion of *p16$^{INK4A/ARF}$*

multiplexing. The reaction was performed in a final volume of 50 ul. The final concentrations of primers and probes were as follows: *p16* F 50 ng/ul, *p16* R 50 ng/ul, *p16* probe 200 nmol/l, *β-actin* F 50 ng/ul, *β-actin* R 200 ng/ul and *β-actin* probe 200 nmol/l. Each reaction contained 50 ng of DNA as template and the Taqman universal master mix (Perkin Elmer) was used. The thermal cycling conditions of the ABI PRISM 7700 Sequence Detection instrument were set to 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C alternating with 1 min at 60°C. A calibration curve was included with each experiment using a range of concentrations of DNA extracted from Raji B cells (range 1.56-100 ng DNA).

3.3.3. *p16* deletion analysis in specimens containing normal cells.

Mixtures ranging from 5% to 100% of Raji B cells (germline for *p16*, GG) relative to concentrations of K562 cells (homozygous deletion of *p16*, DD) were made. The ratio of *p16/β-actin* for duplicate specimens was determined experimentally in a multiplex reaction, and these values were graphed against the input ratios of Raji B/K562. The test yielded a linear graph with a correlation coefficient of 0.9687. This result clearly indicated that normal cell contamination (here simulated by the Raji B cells) in a *p16* DD sample can be accurately measured using this technique. Based on this result, the bone marrow specimens were interpreted as follows. Ratio for *p16/β-actin* <0.4: *p16* deletion (DD); ratio 0.4-0.8: hemizygous *p16* (GD); ratio >0.8: germline *p16* (GG). The analysis was performed using duplicate samples for all patient specimens and possible hemizygous specimens were independently repeated twice. The reproducibility of the assay was assessed by calculating the interclass correlation coefficient, which was 0.884.

All but one of the 25 specimens contained less than 25% normal cells at diagnosis, based on an independent review by a haematologist who was blinded to the identity of the specimens. In these cases, the experimentally determined ratio for *p16/β-actin* was used directly to determine the genotype. One specimen was confirmed to contain >50% normal cells and this was taken into account (patient 10, Table 3.2). At the time of relapse, there was only one specimen for which we were unable to obtain a histological blast count (patient 20, Table 3.2). There were two extramedullary relapses (patient 9 and 11, Table

3.2), and neither their bone marrow blast count nor their *p16* status could be determined at relapse. The principal investigator was blinded to patient's risk features at diagnosis and estimated blast analysis for bone marrows at diagnosis and relapse until *p16* status was interpreted.

3.3.4 Statistical analysis.

Median relapse times and their associated confidence intervals were estimated using the Kaplan-Meier method and the statistical significance of any differences was assessed by the log-rank test. Median relapse times were estimated from the survival curve at the time when the curve goes below 0.5

3.4 Results

3.4.1. Clinical characteristics and *p16* status of study group at diagnosis

Bone marrow specimens from leukaemia patients invariably contain normal cells. This poses problems with accurate quantification of deletion of any gene, either by Southern blot analysis or conventional end-point PCR analysis. To determine whether real-time quantitative PCR can detect deletion of *p16* exon 2 (henceforth referred to as *p16)* in specimens containing normal cells, we simulated it by using mixtures of DNA from Raji B cells, which are wild type for *p16* (GG) and K562 cells which show homozygous deletion of *p16* (DD). The multiplex CR method we established (see patients and methods) demonstrated that *p16* deletion could be accurately measured.

The *p16* status of patients at diagnosis is summarized in Table 3.1: eight patients (32%) had a homozygous deletion, five (20%) patients were hemizygous and 12 (48%) patients were wild type for *p16*. This study group had a greater number of patients in the high-risk category (based on National Cancer Institute (NCI) classification) compared with studies using unselected specimens (Table 3.1). It consisted of 84% high-risk and 16% standard-risk patients. The male:female ratio was 5:1 and there were 32% T-ALL and 68% B-lineage ALL. Because of small numbers (and strong associations) only sex (P = 0.002) and

phenotype (T-ALL/B-lineage, P = 0.004) remained significant when included together in a regression model.

Table 3.2 : *p16*$^{INK4A/ARF}$ status of study group as quantitated by real-time PCR

No.	Sex	Age at diagnosis	Time to relapse	Phenotype	WCC at diagnosis (x10^6/L)	*p16*$^{INK4A/ARF}$ status at diagnosis	*p16*$^{INK4A/ARF}$ status at relapse
P1	M	8mo	1yr 10 mo	B-lineage	95,000	GG	DD
P2	F	7yr 8mo	1yr 7mo	B-lineage	58,900	DD	DD
P3	M	9yr 7mo	7yr	B-lineage	3,700	GG	DD
P4	M	2yr 9mo	12mo	T-ALL	47,000	GG	GG
P5	M	2yr 9mo	2yr 7mo	T-ALL	200,600	DD	DD
P6	F	3yr 5mo	5mo	B-lineage	128,300	DD	DD
P7	F	8yr 6mo	1yr 2mo	B-lineage	700	GD	DD
P8	M	6yr 7mo	4yr 4mo	B-lineage	73,200	GD	DD
P9	M	10yr 2mo	6mo	T-ALL	42,500	DD	#
P10	F	14yr 3mo	1yr 2mo	B-lineage	7,400	DD	DD
P11	M	17yr 7mo	4yr 6mo	T-ALL	13,400	GD	#
P12	M	12yr 2mo	1yr 3mo	T-ALL	871,000	GD	DD
P13	M	2yr 5mo	14yr 7mo	B-lineage	123,000	GG	DD
P14	M	9yr 8mo	1yr 5mo	T-ALL	183,000	DD	DD
P15	M	14yr 4mo	13yr 9mo	B-lineage	3,700	GG	GG
P16	M	4 yr 6mo	3yr 8mo	B-lineage	657,000	GG	GG
P17	M	15yr 2mo	4yr 10mo	B-lineage	450,000	GG	GG
P18	M	9yr 1mo	2yr	T-ALL	980,000	GD	DD
P19	M	11yr 3mo	3yr 2mo	B-lineage	37,500	GG	GG
P20	M	13yr 11mo	7mo	T-ALL	67,000	DD	++
P21	M	14yr 3mo	2yr 10mo	B-lineage	12,800	GG	GG
P22	M	13yr 7mo	4yr 8mo	B-lineage	15,100	GG	DD
P23	F	7yr 8mo	2yr 3mo	B-lineage	117,600	DD	DD
P24	M	14yr 9mo	1yr 2mo	B-lineage	119,000	GG	GG
P25	M	6yr 5mo	3yr 3mo	B-lineage	15,300	GG	GG

G Germline
D Deletion
CNS relapse
++ see Material and Methods

3.4.2. *p16* status as quantified by real-time PCR

Table 3.2 details the *p16* status as quantified by real-time PCR at diagnosis and relapse. Fourteen patients had a homozygous deletion at relapse. Four of the patients, hemizygous at diagnosis, showed homozygous deletion at relapse (P7, P8, P12 and P18). Four B-lineage patients changed from wild-type status at diagnosis to homozygous deletion at relapse (P1, P3, P13 and P22). We were able to determine the constitutional *p16* status of two patients shown to have hemizygous *p16* status. Both were found to be germline for *p16*, demonstrating that the hemizygous deletion was only present in the leukaemia cells.

3.4.3. Alterations in *p16* status from diagnosis to relapse

Table 3.3 summarizes the genotypic changes from diagnosis to relapse and the time to relapse for each group. Six of the patients had homozygous *p16* deletion at both diagnosis and relapse. Of the remaining 16 patients, 50% developed a deletion of one or both alleles at relapse. There was a significant difference in time to relapse (P = 0.01) for *p16* status at diagnosis: DD and GD relapsed sooner with median relapse times of 17 months (95% CI 11-23 months and 15 months (95% CI 5-25 months), respectively, compared with the median relapse time for GG of 39 months (95%, CI 29-49 months). Of those patients who had a homozygous *p16* deletion at relapse, there was significant heterogenesis in relapse time (P = 0.04) with median relapse times of 17 months (95% CI 11-23 months) for those who remained DD; 15 months (95% CI 5-25 months for those with alteration GD to DD; and 56 months (95% CI 0-117 months) for the alteration GG to DD.

Table 3.3
Alterations of *p16*$^{INK4A/ARF}$ status from diagnosis to relapse in ALL patients.

Diagnostic *p16*$^{INK4A/ARF}$ status	Relapse p16 status	Number of patients(22)	Median Time to Relapse
DD	DD	6	1 year 3.5 mo
GD	DD	4	2 years
GG	DD	4	5 years 10 mo
GG	GG	8	3 years 2.5 mo

Relative time to relapse and diagnostic *p16*$^{INK4A/ARF}$ status: p=0.005
Relative time to relapse and alterations in *p16*$^{INK4A/ARF}$ status: p=0.02

3.3. Discussion

Twenty-five paediatric patients with ALL were analysed at diagnosis and relapse for their *p16* status using the most precise method of detection, real-time PCR. *INK4A/ARF* exon 2 was used as the target for DNA amplification. This is the largest *p16* paired study to date and we found 50% (8/16) of the patients developed a *p16* homozygous deletion at relapse. Southern blot analysis was used in a study on 18 paediatric ALL patients and of the 14 who were germline for *p16* at diagnosis, five (35.7%) developed a homozygous deletion at relapse (Maloney, Mcgavran et al. 1999). Diccianni *et al* (1997) reported that only two of six paediatric T-ALL patients developed loss of *p16* at relapse whereas eight were deleted at both stages (Diccianni, Batova et al. 1997). In our study, six of eight patients who developed *p16* deletions at relapse were B-lineage ALL, contrasting with the findings on seven B-lineage paediatric ALL patients by Ohnishi *et al* (1996) in which only one was deleted at relapse (Ohnishi, Hanada et al. 1996).

To compare the frequency of *p16* deletion reported for paediatric ALL at diagnosis with the frequency found in our study at relapse, we calculated the expected frequencies based upon results for all published investigations. The incidence of *p16* homozygous deletion at diagnosis was found to be 64% in T-ALL and 23% in B-lineage ALL (Drexler 1998). Taking the composition of T-ALL and B-lineage patients in our study into account, the expected incidence of homozygous deletions at diagnosis was 35% and we observed 32%. Hence, the findings from our study group at diagnosis concur with data from unselected ALL patients. In contrast, the result for deletion at relapse in our study was 14/22 (64%) compared with the expected 33%. Thus, our study clearly shows an increased incidence of *p16* homozygous deletion at relapse compared with diagnosis. Data on hemizygous patients are not included in the above comparison because there are no other studies which have been able to assess the incidence of *p16* hemizygous deletions in paediatric ALL.

Clues to the possible aetiology of relapse in these patients may be found when median relapse time is compared among the different groups. Those patients with an altered *p16* status at diagnosis had a significantly shorter time to relapse than those germline at diagnosis. This correlates with our previous findings on an unselected group of paediatric ALL patients at diagnosis in which *p16* homozygous and hemizygous deletions were found to predict an independent risk of relapse (Kees, Burton et al. 1997) (Carter, Watt et al. 2001).

In this study, one of the patients who had altered *p16* status at diagnosis was standard risk (NCI current classification). This is in agreement with a large cytogenetic study on 1839 paediatric ALL patients by Heerema *et al*, (1999), which showed that abnormalities of chromosome arm 9p (the site of the *INK4A* locus) identified a subgroup of standard risk patients with an increased risk of treatment failure (Heerema, Sather et al. 1999).

Two possible explanations may account for the eight patients who showed germline at relapse. First, these patients may harbour *p16* point mutations, although this is rare in paediatric ALL or methylation defects in the promoter region of *p16*. We intend to address both possibilities in future studies. These investigations will include examination of mRNA and protein expression of p16 and p14.

Second, these patients may have acquired mutations in other critical genes. Mutations of *p53* have been found in paediatric ALL at relapse (Diccianni, Yu et al. 1994) (Blau, Avigad et al. 1997). Expression of cyclin D1 has been shown to be increased in relapsed compared with diagnostic specimens in paediatric ALL and this level of expression is associated with a poor prognosis (Sauerbrey, Hafer et al. 1999).

Our study group developed leukaemic relapse with evidence of abnormalities in the key cell cycle regulator *p16*. An *INK4A/ARF* -/- murine model using lymphomas provides independent evidence that *INK4A/ARF* mutations promote resistance to chemotherapeutic drugs (Schmitt, McCurrach et al. 1999). These mice developed highly invasive lymphomas, and the tumour cells display apoptotic defects and are resistant to chemotherapy.

The origin of the leukaemia cells at relapse could be a result of evolution of a leukaemic clone (from GG to DD or GD to DD) or proliferation of a minor subclone that was resistant to chemotherapy. There is evidence that both processes occur in ALL, based on assessment of T-cell receptor and immunoglobulin (Ig) gene rearrangements (Biondi A, Rossi V et al. 1992; Steward, Goulden et al. 1994).

In the future, we envisage real-time PCR being applied to detect diagnostic markers for high-risk patients based on a number of clinically relevant mutations such as *p16* deletions. It is a sensitive, specific and fast method, which requires small amounts of patient material, and it has enabled us to analyse a tumour suppressor gene using a quantitative method, revealing its involvement at relapse.

3.6. Acknowledgments

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Chapter 4

Deletion of one copy of the *p16^{INK4A}* tumour suppressor gene is implicated as a predisposing factor in paediatric leukaemia

4.1 Abstract

The *p16^{INK4A}* tumour suppressor gene is frequently disrupted by mutation or deletion in a wide range of cancer types, ranging from leukaemia to cancers of the bladder, skin, lung, liver, and spleen. We have previously shown that deletion of at least one copy of the *p16^{INK4A}* gene is associated with an increased risk of relapse in paediatric leukaemia. Our data suggest that hemizygous *p16^{INK4A}* deletion may be constitutional, conferring susceptibility to leukaemia. Confirmation of this association is worthy of a larger study. Data from primary leukaemia specimens are also presented here which examined the possibility that the remaining allele of the gene was inactivated by another mechanism such as mutation or was silenced by methylation. These possibilities were formally excluded in a case of hemizygous loss of the *p16^{INK4A}* gene in leukaemia, establishing that in this case the *p16^{INK4A}* deletion was either semidominant or fully halpoinsufficient for relapse susceptibility in this disease. Implementation of high throughput methods such as those used here for detecting hemizygous loss of tumour suppressor genes will become increasingly important for molecular diagnosis of cancer. This is particularly true for the emerging class of tumour suppressor genes where deletion of one allele is sufficient to confer cancer susceptibility or poor prognosis with standard treatment.

4.2 Introduction

We recently reported the accurate detection of *p16^{INK4A}* deletion in primary leukaemia specimens (Carter, Watt et al. 2001). This study employing a high throughput real-time quantitative polymerase chain reaction (PCR) methodology demonstrated that our novel assay had sufficient sensitivity to allow routine detection of hemizygous deletion of exon 2 of the *p16^{INK4A}* tumour suppressor gene, even in the presence of normal cells in leukaemia specimens. This assay has already proved useful for larger patient cohort studies (Ferrando

and Look. 2003). Out study identified multiple cases of homozygous *p16*^{INK4A} deletion in primary acute lymphoblastic leukaemia (ALL) specimens, which powerfully predicted leukaemia relapse in the patients with a risk ratio of 11.6. Our data also showed that hemizygous deletion of *p16*^{INK4A} was similarly associated with a marked 6.5 fold increased risk of relapse (Carter, Watt et al. 2001).

These observations are compatible with *p16*^{INK4A} functioning as a semidominant tumour suppressor gene, rather than being classically recessive as previously believed, since deletion of one allele alone is associated with an observable phenotype; in this case an increased risk of relapse when compared to patients without deletion in their leukaemia cells. However, following this study, the possibility remained that the *p16*^{INK4A} gene was not strictly semidominant for cancer susceptibility but that the remaining wild-type allele was epigenetically silenced by methylation or inactivated by mutation. Our initial study also revealed hemizygous status in leukemic cells from patients diagnosed with acute myeloid leukaemia (AML) (unpublished observation). Since our previous investigation did not address whether the deletions were somatic, we examined this aspect in the study presented here.

4.3 Materials and Methods

4.3.1 Clinical history and specimens.

Clinical history of all leukaemia patients studied is summarized in Table 4.1. Sample size was limited due to availability of germline DNA from patients with AML and ALL (obtained from peripheral blood in remission).

Patient P1 presented at the age of 2 years 9 months with a limp and bone pain (Table 4.1). He was diagnosed with acute myelogenous leukaemia (AML, monoblastic) with testicular involvement. He was treated on Children's Cancer Group (CCG) protocol 2891 therapy which resulted in remission. He had a combine bone marrow and CNS relapse 2 months later and was re-induced on CCG 2004 protocol and subsequently underwent an autologous bone marrow transplant (ex vivo 4HC purged). Two months later he sustained an isolated

CNS relapse and died 1 month later. The 49 normal constitutive DNA specimens were obtained from randomly selected donors. Informed consent for this study was obtained from all patients and parents.

4.3.2 Quantitative PCR (Q-PCR) in multiplex format.

The test was conducted using previously published multiplex methodology (Carter, Watt et al. 2001) with the following primer sequences: p16 (Elα)-F5'-ccaacgcaccgaatagttacg-3', Elα-R 5'-gcacctgattcaattcccct-3', and probe sequence Elα-Pr 5'-ccacctggatcggcctccga-3'

4.3.3 Reverse transcriptase reactions

For reverse transcriptase PCR we used the forward primer 5' -ggctggctggtcacca-3'
And the reverse primer 5'-ctacgaaagcgggggtggg-3' with our published reverse transcriptase and PCR protocols (Kees UR, Heerema NA et al. 2003).

4.3.4 Sequencing

RT-PCR products containing the entire coding region of the *p16*^{INK4A} gene were sequenced directly from flanking primers in the amplified non-coding DNA using an ABI sequencing machine exactly as recommended by the supplier. Sequencing was repeated where reads were ambiguous. Overlapping sequence from both strands was concordant, confirming that no errors existed in the sequence (Appendix B1).

Table 4.1

Clinical features of leukaemia patients

and *p16Exon 1α* status of their somatic DNA

Patient	Age at diagnosis (years)	Diagnosis *	*p16* E1α
1	2.8	AML	GD#
2	4.5	AML	GG
3	9.6	ALL	GD
4	4.2	ALL	GG
5	1.0	ALL	GG
6	12.4	CML	GG
7	8.2	ALL	GG
8	4.1	AML	GG
9	5.3	ALL	GG
10	0.5	ALL	GG
11	13.2	ALL	GG
12	1.5	ALL	GG
13	11.9	AML	GG
14	8.3	AML	GG
15	3.3	JCML	GG
16	11.3	AML	GG
17	4.6	AML	GD

* AML acute myelogenous leukaemia, ALL : acute lymphocytic leukaemia, CML: chronic myelogenous leukaemia, JMML: juvenile myelomonocytic leukaemia

The gene status was determined by Q-PCR. GG: wild type, GD: hemizygous deletion

4.3.5 Statistics

Analysis was undertaken in SAS (Cary, NC) using the Fischer two-tailed exact test.

4.4 Results

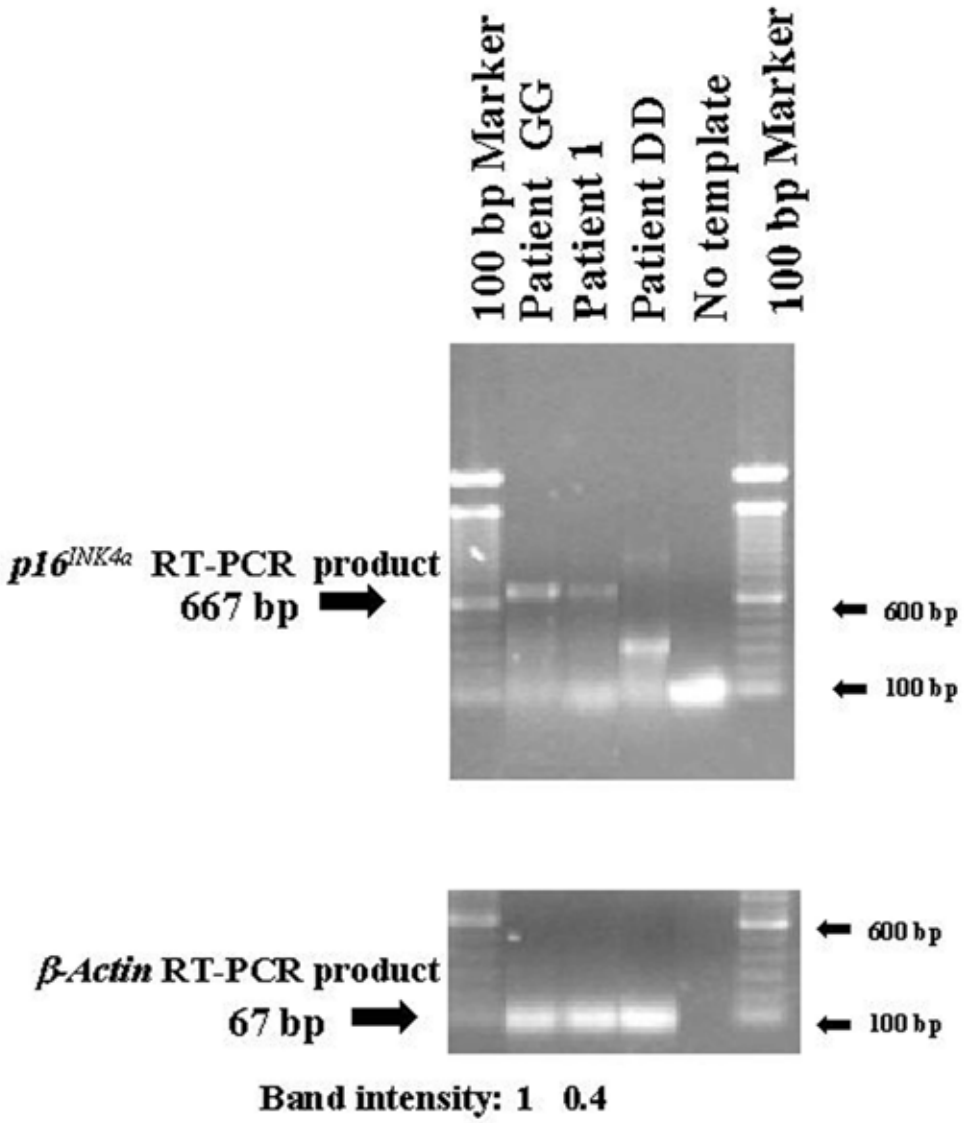
Our previous study of *p16*^{INK4A} status focused on paediatric ALL patients. We extended our survey on *p16*^{INK4A} status to include the diagnosis of leukaemia cells of an AML patient (P1) and a number of other paediatric leukaemia patients (Table 4.1). Using our published multiplex quantitative real-time PCR assay (Carter, Watt et al. 2001), we observed a hemizygous deletion of the *p16*^{INK4A} gene in the leukaemia cells of P1, by virtue of the absence of Exon 1α. This technique has been validated using Southern Blot, Quantitative RT-PCR, and FISH (Kees, Terry et al. 2004).

To formally exclude transcriptional silencing of the remaining *p16*^{INK4A} gene we performed reverse transcriptase PCR (RT-PCR) of the entire coding region, amplified from the leukaemia cells of the patient shown to contain only one allele of *p16*^{INK4A}. The RT-PCR signal (667 bp) was obtained at about half the intensity of the wild-type positive control, while a homozygously detected control was negative, as expected (Fig. 4.1). To further exclude the remaining possibility that despite being expressed, the remaining allele contains a mutation which renders the phenotype of the gene effectively null, we cloned and sequenced the coding region of the *p16*^{INK4A} expressed in the hemizygous specimen. The sequence of the remaining *p16*^{INK4A} allele was confirmed to be identical to a normal control sequence.

To establish the extent of constitutional *p16*^{INK4A} deletion, we surveyed genomic DNA specimens for deletion of *p16*^{INK4A} (Exon 1α) in 49 normal individuals and 16 paediatric leukaemia patients, eight of them diagnosed with ALL and eight with various forms of myeloid leukaemia (Table 4.1). We did obtain evidence of hemizygous deletion of *p16*^{INK4A} Exon 1α in one of 49 normal individuals (2%). In striking contrast, we found that

Figure 4.1

Expression of *p16*^{INK4A} and *βactin* transcripts by RT-PCR. Leukaemia specimen from patient P1 (GD for E1α) obtained at diagnosis shows expression of *p16*^{INK4A} transcript. Two patient specimens of known genotype, GG (wild-type) and DD (deleted on both alleles) were included as controls. Band intensity calculated as a ratio of the *p16*^{INK4A}/*βactin* indicated below panels.



3 of the 17 leukaemia patients (17.6%, including patient P1) showed deletion of one allele in their constitutive DNA; one patient being diagnosed with ALL and two with myeloid forms of the disease (Table 4.1). This statistically significant difference ($P<0.049$) implicates hemizygous $p16^{INK4A}$ deletion as a predisposing factor in leukaemia.

4.4 Discussion

Haploinsufficiency has been previously described in AML affecting other genes such as the *Runx1/AML-1* in familial thrombocytopenia. In this condition a hemizygous point mutation of *Runx1/AML-1* is thought to predispose to the acquisition of additional mutations that cause leukaemia (Song, Sullivan et al. 1999). In a recent murine lymphoma model it has been suggested that the *Runx1/AML-1* gene rather than being haploinsufficient acts as a dominant oncogene (Wotton, Stewar et al. 2002). The incidence of hemizygous *Runx1/AML-1* lesions in human AML specimens is not high but has been described in non-familial MO AML (Langabeer , Gale et al. 2002). In a recent study on specimens from patients with chronic myeloid leukaemia (CML) an incidence of 10-15% of hemizygous deletions flanking the *ABL* and *BCR* translocation points was associated with an unfavorable prognosis compared to those non-deleted for that region (Kolomietz , Marrano et al. 2003).

Taken together, our results provide the first evidence that hemizygous loss of the $p16^{INK4A}$ gene may confer cancer susceptibility in humans, building on the documented role of inherited mutations in melanoma (Kefford, Bishop et al. 2002). A review of parental germline DNA in our study would have also provided information regarding the possibility that this mono allelic deletion is inherited. Unfortunately, this was not possible in this study but a larger future study could include parental germline DNA. A more detailed analysis of individual patient specimens has been performed looking at the extent of these hemizygous deletions at the *INK4* locus. These are the subject of another paper which is currently in progress. The method presented here using quantitative real-time PCR for detection of hemizygous deletions has been validated by three independent methods: Southern Blot, quantitative real-time RT-PCR, and FISH (Kees, Terry et al. 2004).

Our findings are in agreement with a recent animal study in which carcinogen treated $p16^{INK4A}$ +/• mice where shown to be more tumour prone than their wild-type controls (Macdiarmid , Stevenson et al. 2003). Moreover, this study also demonstrated that a further mutation (in this case the remaining $p16^{INK4A}$ allele) had occurred in the tumours.

Hence, the $p16^{INK4A}$ gene may also belong to the growing class of haploinsufficient tumour suppressor genes, which include *p53, TGF-β, p27/KIP1, PTEN,* and *BLM* (Fero, Randel et al. 1998; Tang, Bottinger et al. 1998; Gruszka-Westwood, Hamoudi et al. 2001; Kwabbi-Addo, Giri et al. 2001; Gruber, Ellis et al. 2002). We note, however, that despite evidence of a selection for loss of one allele of the $p16^{INK4A}$ gene in some cancer cells, a further selection may exist for loss of the second allele and/or mutations of other genes, see review by Fodde and Smits (Fodde and Smits 2002). In this scenario, deletion of $p16^{INK4A}$ would be semidominant for leukaemia predisposition rather than strictly haploinsufficient.

To our knowledge this is the first observation in a small study, implicating the role of $p16^{INK4A}$ deletion as a predisposing factor in leukaemia. AML and ALL are different diseases that may not have overlapping risk factors. However, loss of gatekeeper genes such as $p16^{INK4A}$ effect so many different cancers that possible haploinsufficiency or codominance of this gene must be further explored. Regardless, this finding emphasizes the importance of addressing this subtle dosage issue in a range of cancers and requires a much larger study. We believe therefore that the most appropriate way of assessing deletion of $p16^{INK4A}$ in the clinical diagnosis of cancer is to use techniques capable of routinely detecting loss of only one allele of the gene such as real-time quantitative PCR. If validated in larger studies, such an approach will be particularly valuable for tumour suppressor genes shown to be semidominant or haploinsufficient. It is also applicable to the increasingly recognized cancers where deletion of a tumour suppressor gene is of prognostic significance (Gruszka-Westwood, Hamoudi et al. 2001) (Kwabbi-Addo, Giri et al. 2001) (Sasaki , Zlatescu et al. 2001). As molecular profiling gains increased usage in clinical diagnosis, such practical high throughput methods may eventually become routine.

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Chapter 5

Targeted therapies should be considered when abnormalities of *INK4A/ARF* and *TP53* are present in childhood acute lymphoblastic leukaemia

5.1 Abstract

5.1.1 Aim

Abnormalities of the family of proteins encoded by the *INK4* cluster on 9p21 are commonly involved in human cancer, comprising *INK4A/ARF* (*CDNK2A*, *p16INK4A* /*p14ARF*) and *INK4B (CDNK2B, p15INK4B*). Using an overlapping reading frame the *INK4A/ARF* encodes two proteins p16INK4A and p14ARF (p19Arf in mice). Studies in mice showed that p16Ink4a and p19Arf proteins exert their tumour suppressor activity via the *Rb* and *p53* pathways, respectively. The most common mode of *INK4A/ARF* inactivation in childhood acute lymphoblastic leukaemia (ALL) occurs by gene deletion. Using quantitative real time polymerase chain reaction (qPCR) we previously found that deletion of *INK4A/ARF* in leukaemia cells was an independent prognostic indicator in childhood ALL (Carter, Watt et al. 2001) and was involved at relapse (Carter, Reaman et al. 2001). In both studies qPCR was based on primers targeted for *INK4A/ARF* (exon 2) and individual gene frequencies were not measured. In murine models there is evidence to suggest that mutations of *Ink4a/Arf* and *p53* may promote resistance to chemotherapeutic drugs. The *p16Ink4a* gene in mice has been shown to play a more important role following exposure to chemotherapy. These findings suggest that leukemic cells that demonstrate resistance to chemotherapy, for example at the time of relapse, should show such features more prominently than newly diagnosed leukemic cells. This hypothesis was tested in a cohort of patients by detailed mapping of the *INK4* cluster.

5.1.2 Methods

Using qPCR three genes encoded at the *INK4* cluster (*p15INK4B* , *p14ARF* and *p16INK4A*) were analysed in 20 childhood ALL bone marrow specimens at diagnosis and

at relapse. A study of *TP53* (exons 4 to 8), using a fluorescent single strand conformation polymorphism (FSSCP) assay was performed to investigate abnormalities of *TP53*.

5.1.3 Results

Surprisingly, all diagnostic specimens had a deletion of at least one of the genes at the *INK4* cluster. For each gene studied the number of deletion events was higher at relapse. For the *p16INK4A* gene there was a significant overall relationship between median time to relapse and homozygous deletion at diagnosis (p=0.0014). Homozygous deletions of *p15INK4B* and *p14ARF* at diagnosis did not appear to have a significant effect on median time to relapse in this cohort. Three relapse patient specimens were found to have *TP53* (exon 7) mutations and these were associated with *INK4* cluster deletions.

5.1.4 Conclusions

We have shown that deletion events at the *INK4* cluster increase in frequency at relapse in childhood ALL. These findings provide new insights into the biological mechanism of relapse. They suggest that deletions of *INK4A/ARF* in childhood ALL may make cancer cells more resistant to chemotherapy. These findings should be confirmed in a larger patient cohort. Patients with *INK4A/ARF* deletions, particularly when *p16INK4A* is affected, who relapse and thus fail intial therapy, represent a patient subgroup who require urgent consideration for targeted therapies that are not dependent on the function of such tumour suppressor genes.

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5.2 Introduction

Approximately 70-80% of patients with childhood ALL are cured by chemotherapy. In sharp contrast, relapsed ALL has a poor clinical outcome with a 6 year event free survival of 20% (Gaynon, Qu et al. 1998). Relapsed childhood ALL is often sensitive to the same drugs used in induction, suggesting cells at relapse retain some of the features present at diagnosis. These blasts must have acquired some form of resistance to the initial treatment since the prognosis is so different. They may represent the emergence of a subclone different to the dominant leukemic clone present at diagnosis . Leukaemic blasts from

specimens at relapse have been shown to have additional chromosomal features that were not present at diagnosis, whilst also retaining chromosomal features found at diagnosis (Secker-Walker, Alimena et al. 1989; Heerema, Palmer et al. 1992; Vora, Frost et al. 1998). The additional genetic features in the leukaemic cells at relapse may be linked to response to chemotherapy. Understanding the complex mechanisms involved in failure of treatment regimens and why specific anti-tumour drugs succeed or fail is vital if we are to improve the prognosis in cancer (Hanahan and Weinberg 2000). There is experimental evidence that shows that response to chemotherapy may be determined by mutations in certain "key" regulatory genes. Mutations of the *Ink4a/Arf* (*INK4A/ARF* in humans) and *p53* loci in a murine model have demonstrated increased resistance to apoptotic chemotherapeutic drugs compared with controls (Schmitt, Fridman et al. 2002).

The *INK4* cluster is located at 9p21 (Kamb, Gruis et al. 1994) and it codes for a family of proteins that function as inhibitors of CDK4 and CDK6 (Krug, Ganser et al. 2002). Using alternative reading frames the human *INK4A/ARF*(*CDKN2A*)(*Ink4a/Arf* in the mouse) encodes two proteins that both function in tumour suppression, p16INK4Aand p14ARF (p19Arf in the mouse). p16INK4A maintains retinoblastoma protein (RB) in its growth suppressive state through inhibition of cyclin dependent kinase activity (Serrano, Hannon et al. 1993; Quelle, Zindy et al. 1995). p14ARF interacts directly with HDM2 (Mdm2 in the mouse) to stabilize and activate TP53. The activities of RB and TP53 are disrupted in a wide variety of human cancers (Sherr 2001). Recent evidence has shown that loss of RB function leads to cellular aneuploidy and probable genomic instability (Hernando E, Nahle Z et al. 2004). Existing deletions at the *Ink4a/Arf* locus in the mouse result in further genomic instability leading to additional defects at the *Ink4a/Arf* locus and mutations of *p53* (Sherr 2001; Schmitt, Fridman et al. 2002). If this is the case in humans then the leukaemia cells from patients who have relapsed should show such features more prominently than newly diagnosed leukemic cells. This hypothesis was tested in a cohort of patients from whom specimens were available at diagnosis and at relapse.

In a previous study we investigated, gene deletions and the link with response to therapy. Using qPCR, analysis of *INK4A/ARF* (exon 2) on paired patient bone marrow we found an incidence of homozygous deletions at relapse of 64% compared to 32% at diagnosis. Those

patients with deletions of *INK4A/ARF* at diagnosis had a significantly shorter median time to relapse compared with patients who were germline at diagnosis (Carter, Reaman et al. 2001). We were unable to differentiate whether p14ARF or p16INK4A was affected since exon 2 codes for both gene products.

Few studies of diagnostic childhood ALL specimens have assessed both genes at the *INK4A/ARF* locus individually. In a study using immunocytochemical (ICC) analysis of p16INK4A on 126 cases of newly diagnosed childhood ALL the overall survival and event free survival (EFS) was significantly higher in patients positive for p16INK4A than for those negative. This remained significant within the B-lineage ALL subgroup (Dalle, Fournier et al. 2002). In a paediatric ALL study of 194 patient specimens analysed by Southern blot, homozygous deletion of *p16INK4A* and *p14ARF* was found to be a significant independent prognostic risk factor (Calero Moreno, Gustafsson et al. 2002). This concurs with our previous study in which homozygous or hemizygous deletion of *INK4A/ARF* at diagnosis was found to be a statistically independent prognostic indicator of inferior outcome (Carter, Watt et al. 2001).

The relevance of *p15INK4B*, *p14ARF* and *p16INK4A* inactivation in leukaemia cells at the time of diagnosis remains to be resolved (Drexler 1998) (Tsihlias, Kapusta et al. 1999). There are a number of studies, including our own, which support the involvement of *INK4A/ARF* deletion as a marker of progression (Ohnishi, Hanada et al. 1996) (Diccianni, Batova et al. 1997; Maloney, Mcgavran et al. 1999). Few investigations have attempted to correlate inactivation of *p15INK4B* and clinical outcome (Batova, Diccianni et al. 1997) (Quesnel et al, 1998). In addition studies of *Ink4b* null mice have not shown overt developmental anomalies nor increased susceptibility to tumours later in life (Latres E, Malumbres M et al. 2000). Murine exploration is yet to show a link with chemotherapeutic resistance and deletion of *p15INK4B*.

The association between abnormalities of the *Ink4a/Arf* locus and chemotherapeutic resistance has also been studied in conjunction with another key regulator of the cell cycle and apoptosis, the tumour suppressor gene *p53*. Investigations of *TP53* mutations in childhood ALL have found an incidence at diagnosis of 3% (Wada, Bartram et al. 1993)

and at relapse of 0-19% (Gump J, McGavran L et al. 2001) (Zhu YM, Foroni L et al. 1999). To date no studies on human paired patient specimens have investigated both the *INK4* and *TP53* loci simultaneously.

In attempt to discover whether these genes are key regulatory genes in leukaemia we explored the *INK4* cluster in childhood ALL in more detail. Using qPCR we analysed *p14ARF*(exon 1 beta), *p16INK4A* (exon 1 alpha) and *p15INK4B* (exon 1 and 2) in our cohort of childhood ALL patients from whom paired bone marrow specimens were available. The feasibility of using qPCR assay to accurately and reproducibly assess gene deletion in primary patient specimens has been shown in previous studies (Carter, Watt et al. 2001) (Kees, Terry et al. 2004). The hypothesis in the present study predicts that following exposure to chemotherapeutic agents leukaemia cells will demonstrate more deletions at the *INK4* cluster compared to the status at diagnosis. Similarly, the frequency of *TP53* mutations at relapse is expected to be higher than that at diagnosis.

5.3 Patients and methods

5.3.1 Clinical information

The study group was composed of 20 paediatric ALL patients who relapsed. Paired diagnostic and relapse specimens were collected between 1981 and 1997 from patients treated at Princess Margaret Hospital (PMH), Perth, Australia (n=7) and the Children's National Medical Centre, Washington, USA (n=13). Cases were studied based on the availability of cryopreserved Ficoll-Hypaque-enriched leukaemia blasts from bone marrow aspirates. Ethical approval for this study was obtained from the institutional Review Board and informed consent was obtained from all patients or the patients' guardians.

Clinical features of all leukaemia patients studied are summarized in Table 5.1. The immunophenotypes of the leukaemia cells were determined using a panel of monoclonal antibodies (Kees, Ranford et al. 1996). Therapy was administered to all patients studied according to risk-adjusted protocols of the Children's Cancer Group (Gaynon, Bostrom et al. 1998).

5.3.2 Quantitative real-time PCR (qPCR) analysis in multiplex format

Genomic DNA was isolated from cryopreserved specimens and control cell lines by standard methodology (Kees, Burton et al. 1997). qPCR for p16INK4A/ARF (exon 2) was conducted based on our previously published multiplex methodology (Carter, Watt et al. 2001). Additional qPCR for *p14ARF* exon 1 β and *p16INK4A* exon 1α were conducted according to published methods (Kees, Terry et al. 2004). The same design and methodology was used.

Primers and probes for *p15INK4B* were as follows: *p15* exon 1;
Forward ; 5'-GGGCGGCAGCGATGA-3', Reverse; 5'-CCTCCCGAAACGGTTGACT-3' and Probe; 5'-CTTCCAGGAGCTGTCGCACCTTCTCC-3', *p15* exon 2: Forward; 5'-TGCGCACAGCCACGG-3', Reverse; 5'TGGGAAATTGGGTAAGAAAATAAAGT-3' and Probe; 5'-ACTGACGCCAGGTTCCCCAGCC-3'.

Primer and probe concentrations were as follows: (i) *p15* exon 1: E1-F 500 nM, E1-R 500nM, E1-Pr 200nM, β-*Actin* F 100nM, β-*Actin* R 50 nM and β-*Actin* Pr 200nM.
(ii) *p15* exon 2: E2-F 300 nM, E2-R 300nM, E2-Pr 200nM, β-*Actin* F 100nM, β-*Actin* R 50 nM and β-*Actin* Pr 200nM. A standard calibration curve was included in each experiment as detailed before (Carter, Watt et al. 2001; Kees, Terry et al. 2004). After analyzing the data, the ratios obtained were adjusted to take into consideration the level of normal cells present in the bone marrow (Kees, Terry et al. 2004).

5.3.3 Determination of gene status at the *INK 4* cluster

Using the technique described above the status of each exon at the *INK 4* cluster was determined in 20 paired specimens. Gene status was established based on the exon analysis. For example, the functional correlation of DD at exon 1α and GG at exon 2 for *p16INK4A* would be absence of normal p16INK4A protein. Such a specimens was scored as DD for the *p16INK4A* gene.

5.3.4 *TP53* analysis by fluorescence-single strand conformation polymorphism (FSSCP)

Screening for mutations within exons 4 to 8 of the *TP53* gene was carried out using the FSSCP *TP53* mutation protocols published by Iacopetta and colleagues (Soong R and BJ. 1997). Primers were kindly provided by Professor Iacopetta and detection of PCR products was conducted in his laboratory.

5.3.5 Statistical analysis

The main analysis was based upon methods appropriate for censored failure times. The primary time scale was calendar time from diagnosis in months. Univariate analysis was based upon Kaplan Meier survival functions (Kalbfleisch and Prentice 1980). Formal tests of differences between survival functions were based on the Mantel-Cox ('logrank') test statistic (Kalbfleisch and Prentice 1980). Multivariate analysis was based upon the Cox proportional hazards regression model (Kalbfleisch and Prentice 1980). Statistical significance testing was based upon the likelihood-ratio test (McCullagh and Nelder 1983). Covariates known to modulate the risk of relapse were included in the primary model, whether they were statistically significant or not. Secondary modeling was undertaken to demonstrate that removal of these 'non-significant' covariates did not modify substantive conclusions. Secondary modeling was also used to check that conclusions were consistent in key subgroups. Final models were subjected to standard tests of goodness of fit including: the need for nonlinear terms (for example, interactions), an analysis of Martingale residuals and tests for the excessive regression influence of individual data points (McCullagh and Nelder 1983). All reported models satisfy all of these tests of goodness of fit. Analysis was undertaken in SAS version 6.12 for Unix.

5.4 Results

5.4.1 Clinical characteristics of the study group and genotype at the *INK4* and *TP53* loci

The study group comprised 20 childhood ALL patients; 16 were males and 4 were females.

There were 16 B-lineage ALL and 4 T-cell ALL (T-ALL) and the median age at time of diagnosis for the group was 112 months (range: 8-182 months). The median white cell count (WCC) at diagnosis was 106.3 x 10^9 /L (range: 3.7-980 x 10^9 /L). The median time to relapse for the group was 32.5 months (range: 5-175 months). Table 5.1 summarizes the clinical characteristics of the patient group.

Bone marrow specimens were obtained at the time of diagnosis and at relapse. The status of each exon was determined in these 20 paired specimens for the three genes at the *INK4* cluster. Gene status was then determined taking into account the functional correlation of the exon analysis, described in Methods, and the results are presented in Table 5.1. Strikingly, in this cohort of patients, no specimens were normal at the *INK4* cluster at diagnosis or at relapse.

Comparison of gene status of most samples at relapse showed an increased incidence of deletion at this genetic cluster compared with the paired diagnostic sample . There were only four bone marrow specimens at relapse that did not follow this pattern. In the relapse specimens from patients 4 and 17 this observation was made for *p15INK4B* and from patients 11 and 19 for *p14ARF*.

Table 5.1: ***Clinical characteristics of the study group and genotype at the INK4 and TP53 loci in leuakemia cells***

Patient Number	**Gender***	**Age (Mo)**	**Phenotype**	**Stage****	***p15INKB***	***p14ARF***	***p16INK4A***	***TP53* Exon 7 Mutation**
1	M	8	B-lineage	Diagnosis	GG^	GG	GD	No
				Relapse(22)	DD^^	DD	DD	No
2	F	92	B-lineage	Diagnosis	DD	DD	DD	No
				Relapse(19)	DD	DD	DD	No
3	M	115	B-lineage	Diagnosis	GD	GD	GD	No
				Relapse(84)	GD	DD	DD	No
4	M	33	T-ALL	Diagnosis	GD	DD	DD	No
				Relapse(31)	GG	DD	DD	No
5	F	41	B-lineage	Diagnosis	DD	DD	DD	No
				Relapse(5)	DD	DD	DD	No
6	M	79	B-lineage	Diagnosis	GG	GD	DD	No
				Relapse(52)	DD	DD	DD	No
7	F	171	B-lineage	Diagnosis	DD	DD	DD	No
				Relapse(14)	DD	DD	DD	**Yes**
8	M	146	T-ALL	Diagnosis	GG	GD	GD	No
				Relapse(15)	DD	DD	DD	No
9	M	29	B-lineage	Diagnosis	GD	GD	GG	No
				Relapse(175)	DD	DD	DD	No
10	M	116	T-ALL	Diagnosis	DD	DD	DD	No
				Relapse(17)	DD	DD	DD	No
11	M	172	B-lineage	Diagnosis	GD	GD	GG	No
				Relapse(165)	DD	GG	DD	No
12	M	54	B-lineage	Diagnosis	GG	GG	GD	No
				Relapse(44)	DD	DD	DD	No
13	M	182	B-lineage	Diagnosis	DD	DD	DD	No
				Relapse(58)	DD	DD	DD	No
14	M	109	T-ALL	Diagnosis	DD	DD	DD	No
				Relapse(24)	DD	DD	DD	**Yes**
15	M	135	B-lineage	Diagnosis	GG	GG	GD	No
				Relapse(38)	GG	GD	DD	No
16	M	171	B-lineage	Diagnosis	GG	GD	GD	No
				Relapse(34)	DD	GD	DD	**Yes**
17	M	163	B-lineage	Diagnosis	GD	GD	GD	No
				Relapse(54)	GG	DD	DD	No
18	F	92	B-lineage	Diagnosis	DD	DD	DD	No
				Relapse(27)	DD	DD	DD	No
19	M	177	B-lineage	Diagnosis	GD	GD	GG	No
				Relapse(14)	DD	GG	GG	No
20	M	77	B-lineage	Diagnosis	GD	GG	GD	No
				Relapse(39)	DD	DD	DD	No

Gender* : M; male, F; female

** : time to relapse in months in brackets

^G; germline, ^^D; deletion

5.4.2 Gene deletion status at the *INK4* cluster for 20 ALL patients at diagnosis and at relapse

In our previous study of paired patient specimens in childhood ALL we found an incidence of *INK4A/ARF* homozygous deletion (DD) at diagnosis of 32% and of 64% at relapse. We were unable to ascertain the size of the gene deletions, nor were we able to determine the individual frequency for *p14ARF* and *p16INK4A* deletion at diagnosis and at relapse. In this study we investigated the frequency of gene deletion within this specimen group at diagnosis and at relapse for all three genes including *p15INK4B, p14ARF* and *p16INK4A* . Figure (5.1a) shows a summary of genotypic patterns at the *INK4* loci in order of frequency. Interestingly, the incidence of homozygous deletion for all three genes (*p15INK4B* ;DD*, p14ARF* ;DD and *p16INK4A* ;DD) at diagnosis and at relapse was 7/20 (35%) and 13/20(65%), respectively. This is similar to our previous findings using only primers for *INK4A/ARF* (exon 2). Only two specimens were hemizygous for all three genes (*p15INK4B* ;GD*, p14ARF* ;GD and *p16INK4A* ;GD) at diagnosis. Remarkably, no patient specimens were germline across the *INK4* loci (*p15INK4B* ;GG*, p14ARF* ;GG and *p16INK4A* ;GG) at diagnosis or at relapse.

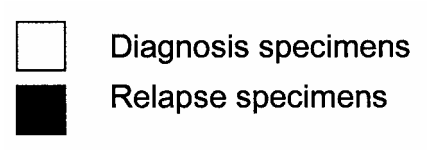
As described previously murine models have found that deletions of both *p16Ink4a* and *p14Arf* may make the genome more unstable. Analysis of the specimens showing homozygous deletion of *INK4A/ARF* (p16INK4A/p14ARF) revealed an incidence at diagnosis and relapse of 8/20(40%) and 16/20(80%), respectively.

Next we analyzed the gene status for each marrow specimen according to immunophenotype. Previous studies have found the incidence of *INK4A/ARF* deletions to be higher in T-ALL compared with B-lineage leukaemia. Figure (5.1b) represents genotypic status by B-lineage in order of frequency. The incidence of homozygous deletion of all three genes in the B-lineage leukaemia group at diagnosis and relapse was 31.25% and 62.5%, respectively. The incidence of hemizygous deletion for all three genes across the *INK4* loci in the B-lineage group at diagnosis was 12.5% and was zero at relapse. Patients with T-ALL account for approximately 15% of childhood ALL. In this study 20% (n=4) of the specimens were T-ALL. The incidence of homozygous deletion across the group at diagnosis and relapse was 50% and 75%, respectively. None of the T-ALL

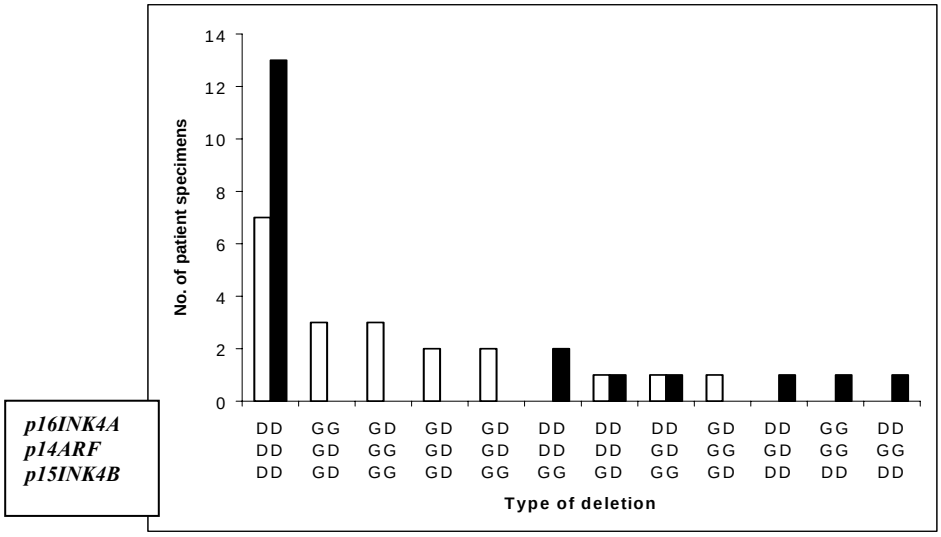
specimens at diagnosis or relapse had hemizygous deletions for all three genes.

Previous studies of paediatric ALL have focused on exon 2 deletion at the *INK4A/ARF* locus. The incidence of *INK4A/ARF (p16INK4A*/*p14ARF*) homozygous deletions in the B-lineage group when analyzed together was 31.25% at diagnosis and 75% at relapse. In the T-ALL group the incidence of *INK4A/ARF* homozygous deletion at diagnosis and relapse was 75% and 100%, respectively.

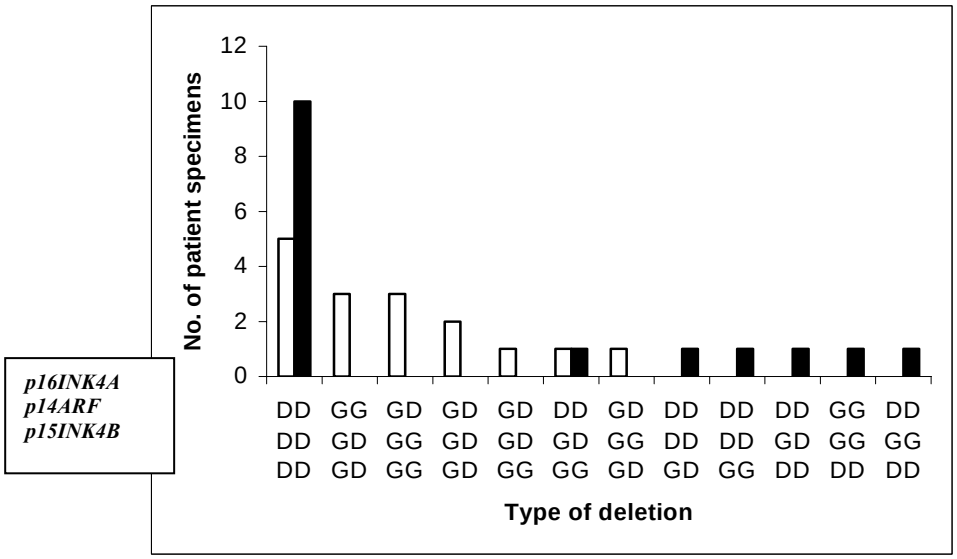
Figure 5.1 (a) Pattern of genotypic status at the *INK4* cluster for three genes (*p15INK4B, p14ARF and p16INK4A*) at diagnosis and at relapse in a group of 20 childhood ALL bone marrow specimens. (b)Pattern of genotypic status at the *INK4* cluster for three genes (*p15INK4B, p14ARF and p16INK4A*) at diagnosis and at relapse in 16 B-lineage childhood ALL bone marrow specimens
GG; Germline
DD; Homozygous deletion
GD; Hemizgous deletion



a



b



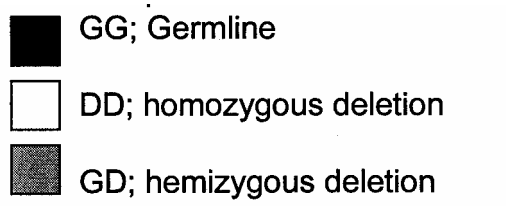
5.4.3 Higher incidence of deletions at the *INK4* cluster at the time of relapse

The purpose of this study was to explore deletion events at the *INK4* cluster in the bone marrow specimens at diagnosis and at relapse. Based on the literature we had anticipated the incidence of deletion events for each gene to be higher at relapse, and this to be associated with a reduced median time to relapse. Deletion status at diagnosis and at relapse with median time to relapse for all three genes at the *INK4* cluster is represented in Figure 5.2. Each pie graph demonstrates the proportion of patient specimens showing the gene status i.e. GG, GD or DD. The frequency of deletions for *p15INK4B* was as follows; 35% (DD), 35% (GD) at diagnosis and 80% (DD), 5% (GD) at relapse. The frequency of deletions for *p14ARF* was as follows; 40% (DD), 40% (GD) at diagnosis and 80%(DD), 10% (GD) at relapse. The frequency of deletions for *p16INK4A* was as follows; 45% (DD), 40% (GD) at diagnosis and 95% (DD), 0% (GD) at relapse. Notably, for each gene there was an increased incidence of deletion at relapse compared to diagnosis.

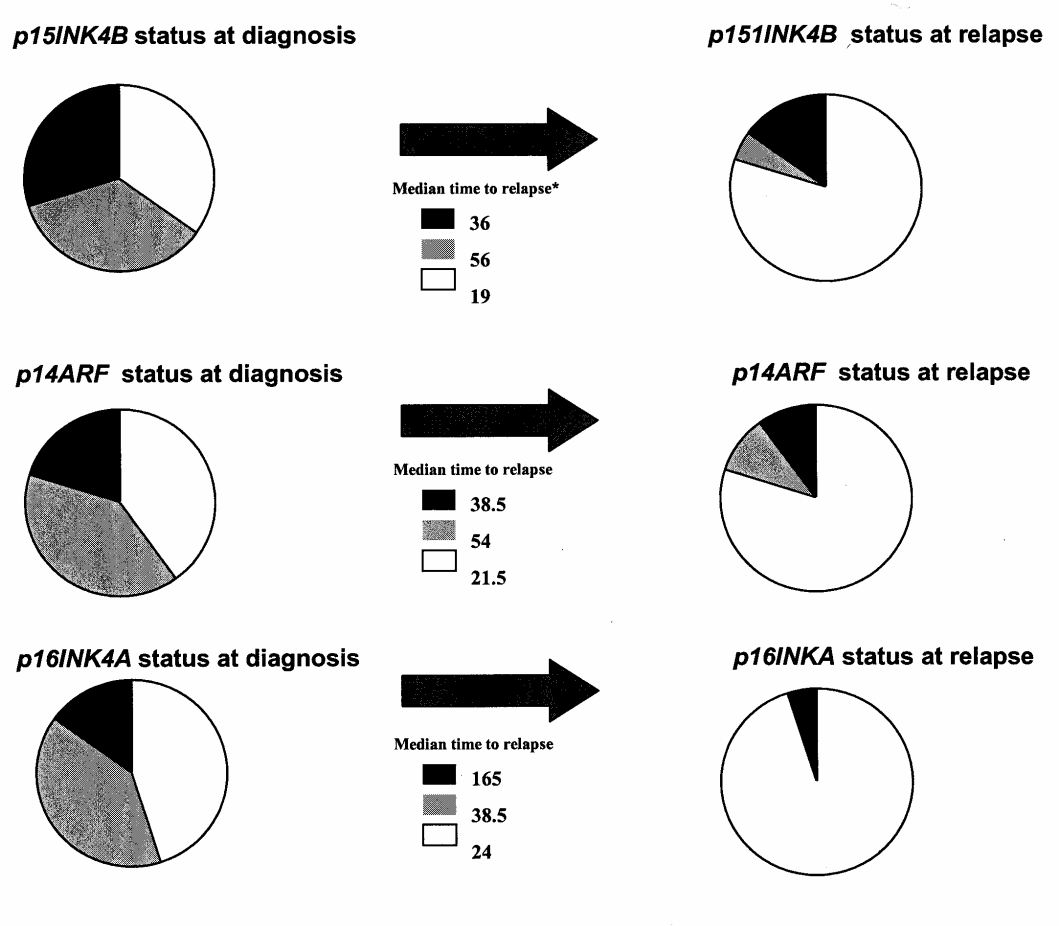
The median time to relapse in months was determined for each particular gene and the three subtypes; i.e. DD, GD or GG at diagnosis. The median time to relapse, recorded in months, is was as follows; *p15INK4B* ; 19 (DD), 56(GD) and 36 (GG), *p14ARF* ; 21.5 (DD), 54(GD) and 38.5 (GG) and *p16INK4A* ; 24 (DD), 38.5 (GD) and 165 (GG). Remarkably, for each gene, the shortest time to relapse was obtained for DD status at diagnosis.

Using a Cox model that included all genes (along with immunophenotype, gender and WBC at diagnosis) a significant overall relationship between time to relapse and *p16INK4A* status was found (p=0.016). There was a significant difference in time to relapse (p=0.02) for *p16INK4A* status at diagnosis. Other genes did not show significance when analyzed alone.

Figure 5.2: Deletions at the *INK4* cluster in relapsed paediatric ALL (n=20). Each pie graph demonstrates the proportion of patient specimens showing the gene status at diagnosis and relapse.



*shows median time to relapse (in months) for each genotypic sub-group.

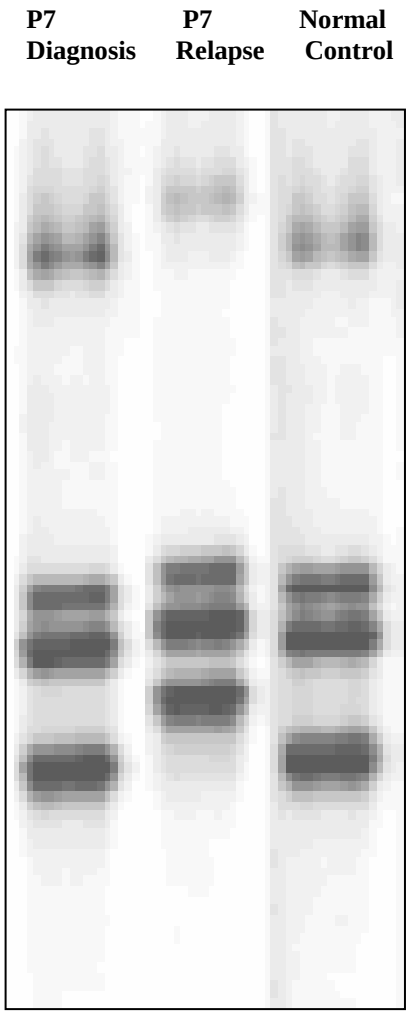


5.4.4 Incidence of *TP53* mutations

Although the incidence of *TP53* mutations at diagnosis in childhood ALL was reported to be low, from 3 to 5%, the incidence at relapse was reported to be higher, up to 19% (Zhu , Foroni et al. 1999; Gump, McGavran et al. 2001). Murine studies have found that *Ink4a/Arf* deletions and *p53* mutations can be present in tumour cells and that a combination of both abnormalities can make the cell more tumourigenic (Zhang et al, Cell, 1998). In the current study group the incidence of *TP53* mutations in patient specimens at diagnosis and at relapse was examined *TP53* was determined for exons 4, 5, 6, 7 and 8 (Wada, Bartram et al. 1993). No mutations were detected in any of the diagnostic specimens. Three relapse bone marrow specimens showed mutations of *TP53* exon 7 (15%). Figure 5.3 shows an example of an exon 7 mutation using FSSCP, at relapse on a bone marrow specimen from patient P7. The relapse specimen shows a different band pattern compared to the diagnostic specimen due to a mutation.

All three patient specimens were associated with deletions of *p16INK4A* and *p14ARF* at diagnosis. The median time to relapse for the three specimens with *TP53* mutations was 24 months (range: 14-34 months). In contrast, the median time to relapse in the specimens showing no *TP53* mutations was 38 months (5-175 months). Information on patient overall survival was available on fifteen of the patients. Ten of those patients died after relapse. Two of the patients that had *TP53* mutations at relapse died within two months of their bone marrow relapse.

Figure 5.3 SSCP analysis of *TP53* (exon 7) of bone marrow specimens from patient P7 at diagnosis and at relapse. The relapse specimen shows a different band pattern due to a mutation.



5.5 Discussion

The data presented in this study on relapsed childhood ALL confirm our initial hypothesis i.e. that the incidence of deletion events at the *INK4* cluster in leukaemic blasts at relapse is increased. These deletion events occurred at both the *INK4A/ARF* and *INK4B* loci. Thus, the leukaemia cells from patients who relapsed, demonstrating resistance to chemotherapy, show these features more prominently than those from newly diagnosed patients. In contrast to unselected study groups, none of the patient specimens were normal at this cluster at diagnosis (Kees, unpublished observation) (Bertin, Acquaviva et al. 2003). In this study group the key gene found to have a significant overall relationship between median time to relapse and homozygous deletion at diagnosis was *p16INK4A*. In addition, three relapse patient specimens were found to have *TP53* (exon 7) mutations. These specimens were associated with *INK4* cluster deletions and a decreased median time to relapse, compared to those specimens with no *TP53* mutations.

Murine research provides an insight into the possible mechanism involved, as the roles of *p16Ink4a* and *p19Arf* in cancer cells and their interaction with each other and chemotherapy are the subject of many studies (Kamijo, Bodner et al. 1999; Krimpenfort , Quon et al.; Sharpless, Bardeesy et al. 2001) (Schmitt, Fridman et al. 2002). They show that abnormalities of both *p19arf* and *p16Ink4a* are associated with tumour development which appears to be worse on exposure to carcinogens and are associated with a more aggressive phenotype (Krimpenfort , Quon et al. 2001; Sharpless, Bardeesy et al. 2001). Based on research by Schmitt and colleagues, using a lymphoma model in the mouse, *p16Ink4a* plays a more prominent role following exposure to chemotherapy(Schmitt, Fridman et al. 2002). They propose that the complex mechanism whereby *p19Arf* is inactivated during tumour development, followed by a *p16Ink4a* deletion upon therapy can disable premature senescence and impair treatment outcome. In mice the *p19Arf* protein binds to the *TP53* negative regulator, Mdm2 . Cells containing an intact p19Arf-mdm2-TP53 pathway senesce prematurely in response to oncogenic Ras stimulation or more dramatically exhibit an apoptotic response to enforced Myc expression (Schmitt, Fridman et al. 2002) (Sherr 2001). A mutation in *TP53* and the *Ink4a/Arf* locus leads to the development of a particularly aggressive tumour (Zhang et al, 1998, et al). *TP53* mutations, involving exon 7, at relapse have been described in association with poor clinical outcome in previous

studies (Diccianni, Yu et al. 1994; Kawamura , Ohnishi et al. 1999; Barnabas, Shurafa et al. 2001). Understanding the critical interaction between *TP53* and deletions of the *INK4* cluster can be better understood if one looks at cell division and the Retinoblastoma protein (RB). A recent study has found evidence to link abnormalities of Rb with aneuploidy (Hernando , Nahle et al. 2004). Deregulation of these critical genes involved in cell division and apoptosis appear to effect the response of the cell to chemotherapy.

In this study we do indeed see further deletion events at the *INK4A/ARF* locus at relapse and this occurred after the leukaemic blasts were exposed to chemotherapy. Deletions within the *p16INK4A* exons were significantly associated with a decrease in median time to relapse. These findings should be confirmed in a larger patient cohort, however it has been suggested that *p16INK4A* may play a more prominent role in human cancers than *p14ARF* (Bartsch , Sina-Frey et al. 2002). In a study on relapse specimens Einsiedel et al found that *p16INK4A* deletions were associated with a shorter median time to relapse compared to undeleted specimens (Einsiedel, Taube et al. 2002). They suggested that this reflected a bias toward more adverse events in the group of patients with *p16INK4A* deletions. Early relapses are often associated with more aggressive disease (Yumura-Yagi , Hara et al. 2002). Murine studies support the role of the *INK4A/ARF* locus in the development of relapse and not just as a marker of aggressive disease. Whether deletions of the *INK4A/ARF* locus are developing following exposure to chemotherapy in human leukaemia cells can be better studied when paired diagnostic and relapse specimens are investigated.

In this paired study the incidence of deletion of all three genes (*p15INK4B*, *p14ARF* and *p16IN4A*) was 35% at diagnosis and 65% at relapse. This finding is similar to our previous investigations of *p16INK4A* where only exon 2 was studied by qPCR (Carter, Reaman et al. 2001). However, the analysis of the *INK 4* cluster in this study reveals the deletions to be much larger, involving both *p15INK4B* and *p14ARF*. These findings are in agreement with cytogenetic studies in paediatric patients who failed therapy. Deletions of chromosome 9p at diagnosis have been reported to be associated with increased risk of treatment failure in both high risk and standard risk patients with childhood ALL (Heerema, Sather et al. 1999). Loss of heterozygosity studies have identified a high incidence (39%) of loss of chromosomal arm 9p at the *INK4* cluster in relapsed childhood

ALL (Takeuchi , Seriu et al. 2003). In addition, loss of chromosome 9p has been described in secondary cytogenetic aberrations associated with childhood Philadelphia positive ALL (Heerema , Harbott et al. 2004). These findings now show that the deletion events in the majority of specimens are larger than those at diagnosis.

Two of the diagnostic specimens were hemizygous for all genes across the *INK4* cluster . The remaining allele could be inactivated by mutation or epigenetic events. Unfortunately, we were unable to explore this further in those patient specimens. The possibility of *p14ARF* and *p16INK4A* haploinsufficiency has been raised in our previous study and is further explored elsewhere (Carter, Terry et al. 2004).

In four patient samples the leukaemia cells at the time of relapse showed a smaller deletion at the locus (affecting *p15INK4B* and *p14ARF*) compared to diagnosis. This has been previously described at other loci in relapsed paediatric ALL. Konrad and colleagues investigated *TEL-AML1* deletions at relapse from children with ALL and two relapse specimens were found to have smaller translocations compared to diagnosis (Konrad, Metzier et al. 2003). This may occur where minor clones present at diagnosis emerge as the major clone at relapse.

In our current study we were not able to ascertain whether the deletion event at the *INK4* cluster or *TP53* mutation occurred in the original dominant leukemic clone. These events detected at relapse may have been due to the emergence of a subclonal population undetected at diagnosis and more resistant to apoptotic agents. Studies of *IgH* and *TCR* gene rearrangements, on paired patient specimens have shown that in the majority of specimens the original leukemic clone was still the dominant clone detected at relapse (Nirmala , Rajalekshmy et al. 2002; Szczepanski , van der Velden et al. 2003). This would suggest that in our study the abnormalities found at the *INK4* and *TP53* loci, in the majority of relapse specimens were occurring in the dominant leukemic clone following exposure to chemotherapy. In an attempt to answer this question we are studying the clonal evolution of this patient group using PCR methodology to determine *IgH* and *TCR* gene rearrangements.

This data provides further evidence that deletions found at the *INK4*A/ARF locus, particularly those affecting *p16INK4A* should be targeted with therapies that restore defective apoptosis and cellular senescence. Murine studies have shown how that the *Ink4a/Arf* locus and *TP53* are altered following exposure to chemotherapy creating an unstable genome (Lee and Schmitt. 2003). Microarray studies of mantle cell lymphoma have shown that deletion of the *INK4A/ARF* locus in combination with *Cyclin D1* expression co-operate to increase cellular proliferation and are associated with patient survival (Rosenwald, Wright et al. 2003). Recent studies of chromosomal division show how abnormal function of the RB leads to aneuploidy and further genomic instability (Hernando , Nahle et al. 2004). The uses of specific CDK inhibitors that are independent of such defects in cellular division and apoptosis must be considered when deletions of the *INK4A/ARF* locus are discovered. Flavopiridol a cyclin-dependent kinase inhibitor is one of a new class of agents being trialed on human cancers where traditional alkylating agents have failed . Flavopiridol induces cell cycle arrest and tumour growth inhibition in solid tumours and leukaemias (Shapiro 2004) (Quiney , Dauzonne et al. 2004). Interactions between flavopiridol and bortezomib (a proteosome inhibitor) have shown synergistic activity in myeloid cells resistant to conventional therapy (Dai , Rahmani et al. 2004).

This study combines clinical patient data and molecular genetic analysis to provide further clues as to the role of the *INK4* and *TP53* loci in leukaemia. In a cohort of twenty patients who relapsed, as distinct from an unselected group, it shows that *INK4* cluster deletions are increased. We also suggest that targeted therapies such as flavopiridol should be urgently considered when *p16INK4A* deletion is found at diagnosis on a bone marrow specimen.

5.6 Acknowledgments

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Chapter 6

6. Discussion

6.1 Cancer

Cancer is a complex disease as demonstrated by the ability of cancer cells to evade therapy. Understanding the balance between cellular proliferation, senescence and apoptosis is the key to cancer therapy. The regulation of "key" genes involved in these processes and the mechanisms effected when they are deleted or mutated are better understood as new molecular tools become available. The human genome project has provided a rich data source for the study of such key regulatory genes. Genomic expression profiling has enabled the exploration of the molecular pathways in which these genes participate.

For both the clinician and research scientist the exciting challenge of this new knowledge is to be able to define which genes are the key regulators in the cancer cell and how to target therapies to encourage apoptosis and senescence, and block cellular proliferation. TSGs involved in cell cycle regulation are often upregulated following DNA damage. By this method they prevent cellular proliferation and lead to cellular senescence. Any mutation or deletion leading to their dysfunction effects the balance of cellular growth and sensitivity to chemotherapy. Are these TSGs the key regulators of the cancer cell and can therapies be designed to cause apoptosis independent of their function when they are mutated or absent? Investigating the loss of TSGs has been a challenging area of molecular research. Expression of abnormal proteins and mRNA can be detected in a normal cellular environment. In contrast, the detection of absence of a product in a cancer cell surrounded by normal stromal cells has been technically difficult. Molecular methods to accurately detect deletion of TSGs in patient specimens are necessary if we are to understand the pathogenesis of this disease. A qPCR technique developed as part of this thesis provides an excellent opportunity to study such deletions.

6.2 Childhood ALL

Childhood leukaemia is a heterogeneous disease and finding the key genes involved in disease progression will enable more accurate risk stratification and hopefully targeted therapies. Microarray analysis of leukaemic blasts at diagnosis and relapse have provided links to cancer pathways (Carroll , Bhojwani et al. 2003) (Hoffmann , Dixon et al. 2004) (Yeoh , Ross et al. 2002; Ferrando and Look. 2003). Microarray gene profiles have shown distinct ALL subtypes, which correlate with known clinical and immunophenotypic subgroups ie. T-ALL, E2A-PBX1, BCR-ABL. TEL-AML1, rearranged MLL, hyperdiploidy and another distinct unknown subgroup (Yeoh , Ross et al. 2002).

6.3 Relapsed childhood ALL

Relapsed cancer provides a link in understanding tumour pathways since relapsed cancer cells must have developed ways of evading apoptosis. To discover the key genes involved in these molecular events we must study the difference between diagnostic and relapse specimens. Relapsed childhood ALL has an event free survival for 6 years of only 20-30% (Gaynon, Qu et al. 1998). In sharp contrast newly diagnosed childhood ALL has an expected overall event free survival of 76% (Gaynon 2000). Relapsed ALL often responds to the same drugs used at diagnosis and most patients enter a clinical remission, however this remission is short-lived (Gaynon, Qu et al. 1998). Historically, paired relapse childhood ALL specimens for study purposes have been scarce. However, clues have been provided by studies where the development of additional chromosomal structural changes at relapse have been reported (Berger, Le Coniat et al. 1988). The most frequent changes to the leukaemic blasts at relapse are the development of structural abnormalities in the chromosomal loci; 6q-, 7p- and 9p- (Shikano, Ishikakawa et al. 1990). In addition, deletions of chromosome 9p are known high risk diagnostic features (Heerema, Sather et al. 1999).

6.4 The *INK4A/ARF* and *INK4B* loci in childhood ALL

The short arm of chromosome 9 is mutated or deleted in many cancers including leukaemia. The *INK4* cluster extends over 50 kb at the 9p21 chromosomal band and includes two loci, *INK4A/ARF* and *INK4B*. The genes at the *INK4A/ARF* locus act as tumour suppressors via two proteins, p16INK4A and p14ARF (Cairns, Polascik et al. 1995). The role of *p15INK4B* in leukaemogenesis remains unresolved. Could loss of these key cell cycle regulators provide further clues to the pathogenesis of leukaemia and the development of chemotherapeutic resistance? Is it possible to provide a therapeutic strategy to prevent relapse when these genes are deleted?

The evidence for an independent prognostic role for loss of *p16INK4A/ARF* has been controversial and its role in recurrence of leukaemia largely unknown (Kees, Burton et al. 1997; Drexler 1998) (Maloney, Mcgavran et al. 1999). Previous studies were based on detecting *p16INK4A/ARF* deletion by either Southern blotting or conventional PCR analysis. Bone marrow specimens from patients with leukaemia invariably contain a proportion of normal cells that cause problems with accurate quantitation of deletion of any gene. This prompted the development of a new technique to measure gene deletion with greater accuracy in patient specimens. The hypothesis tested was that using qPCR to assess *p16INK4A/ARF* deletion would be a simple and reliable test for leukaemia prognosis. The identification of many TSGs found to be haploinsufficient rather than classically recessive made application of qPCR to the robust detection of hemizygosity critical in the development of this technique.

Aberrations of the cell cycle are linked to the development and the progression of cancer. Based on limited studies in childhood ALL progression also appeared to be associated with the loss of genes at the *INK4A/ARF* locus (Diccianni, Batova et al. 1997) (Ohnishi, Hanada et al. 1996) (Maloney, Mcgavran et al. 1999). A murine model using lymphoma provided evidence that *Ink4a/arf* mutations promoted resistance to chemotherapeutic drugs (Schmitt, McCurrach et al. 1999). Further investigations by Schmitt and colleagues have shown a link between this resistance and abnormalities of both the *Ink4a/arf* and *p53* loci (Schmitt,

Fridman et al. 2002). The current study provided an opportunity to address the potential subtle differences between murine models designed to study tumourigenesis and human disease. Previous invitro studies on the *p16INK4A* gene in human osteosarcoma cells in senescence have alerted us to the differences in human cell response compared to murine models (Gombart, Yang et al. 1997; Krimpenfort , Quon et al. 2001; Sharpless, Bardeesy et al. 2001). In accordance with this theory I examined the incidence of *INK4A/ARF, INK4B* and *TP53* loci abnormalities at diagnosis and at relapse of childhood ALL in a paired study. The hypothesis formed was that deletion events at the *INK4* cluster, particularly the *INK4A/ARF* locus, would be increased at relapse following exposure to chemotherapeutic agents.

6.5 A novel method for the accurate detection of *INK4A/ARF* and *INK4B* loci deletion

This research achieved technical advances in the development of a qPCR technique capable of detecting gene deletion in primary patient specimens with a precision not previously achieved by conventional methods. Importantly, this assay allowed for the first time the detection of hemizygous deletions in a robust high throughput assay from patient specimens.

The qPCR technique was developed using a multiplex format (ie. β-Actin was used as an internal control for DNA content and the multiplex analysis was performed in the same PCR tube). A mixing experiment using two cell lines (K562; homozygous deletion of *p16* exon 2 and Raji B; germline *p16* exon 2) was used to calibrate for normal cell detection in the bone marrow specimens. These specimens were independently reviewed by a haematologist. The marrow blast count and the qPCR *p16/β-Actin* ratio were used to determine the genotype. The primers and probes in the multiplex qPCR were initially directed at *p16INK4A/ARF* (exon 2) to compare with the published literature. Previous studies of childhood ALL have used this exon as a target for probes in Southern blot analysis and primers in conventional PCR methods (Heyman, Rasool et al. 1996; Ohnishi, Hanada et al. 1996; Kees, Burton et al. 1997; Drexler 1998).

The results of this study illustrated that qPCR was capable of detecting gene deletion in primary patient bone marrow specimens. We were able to detect homozygous and hemizygous deletion of genes at the *INK4* cluster in cancer cells, within a normal cell background. In contrast, a similar study using qPCR but not using multiplex format was unable to assess hemizygous deletion (Einsiedel, Taube et al. 2002).

The novel technique developed as part of this research has been shown to be highly reproducible and gene specific. The qPCR method used in this study is suitable for high throughput screening of patient specimens and has many clinical applications as it can be applied to the study of these loci in other cancers and lends itself to the study of other TSGs (Carter, Watt et al. 2001). A subsequent investigation by Kees and colleagues has verified the accuracy of this technique in detecting hemizygous deletion by three independent methods(Kees, Terry et al. 2004).

This method for analysing *INK4A/ARF* using qPCR has since been applied by other groups for the detection of deletion in lymphoma and T-ALL (Tort , Hernandez et al. 2002) (Ballerini P, Blaise A et al. 2002). Furthermore, the development of this research technique and its practical application to clinical childhood ALL demonstrate what can be achieved when key cancer questions are posed in the setting of a tertiary paediatric cancer centre that collaborates directly with a scientific institution (Corrigan and Feig. 2004).

6.6 Detection of *INK4A/ARF* and *INK4B* deletions in childhood ALL using qPCR

6.6.1 *p16INK4A/ARF* as an independent prognostic indicator in childhood ALL

To affirm whether or not *p16INK4A/ARF* was an independent prognostic indicator in childhood ALL the qPCR technique developed was applied to an unselected cohort of diagnostic bone marrow specimens from 45 patients with childhood ALL. In a previous study using Southern blot analysis performed in our laboratory, the incidence of homozygous *p16* deletions in childhood ALL specimens at diagnosis was 18.3% (Kees, Burton et al. 1997).

The analysis of *p16INK4A/ARF* exon 2 using qPCR showed that of the 45 patient diagnostic specimens 11 (25%) demonstrated a homozygous deletion, 6 (13%) were hemizygous and 28 (62%) were wild type. These findings were in agreement with the frequency of homozygous deletion reported for childhood ALL patient specimens: 23% for B-lineage and 64% for T-cell ALL (T-ALL) (Drexler 1998). The combined frequency for hemizygous and homozygous deletions of exon 2 in this study was 38% and the increased sensitivity of this method was demonstrated. This analysis showed that the risk ratio for relapse for a homozygous deletion of *p16INK4A/ARF* was 11.558 ($p<0.05$), providing conclusive evidence that deletion of *p16INK4A/ARF* was an independent prognostic indicator for poor outcome at diagnosis in childhood ALL.

The study revealed strikingly, that the risk ratio for relapse for hemizygous deletion of *p16INK4A/ARF* compared with no deletion was 6.558 ($p=0.00687$). This novel discovery raised some further considerations that could have far reaching consequences in many different cancers but needs to be confirmed in a larger cohort. An investigation by Einsiedel and colleagues on 125 relapse childhood ALL specimens using qPCR found that deletions of *p16INK4A* were not independent prognostic indicators of outcome, rather they were associated with other adverse diagnostic features and did not provide additional information (Einsiedel, Taube et al. 2002). This study did not use paired specimens and as stated previously was not able to assess hemizygous deletions. In conclusion, Einsiedel and colleagues suggested immunocytochemical analysis be performed as an independent method of assessment. This approach was adopted by Dalle and colleagues who analysed the expression of p16INK4A in childhood diagnostic ALL specimens using this method and found a significant association between loss of expression and poor outcome (Dalle, Fournier et al. 2002). In a retrospective study on 194 diagnostic patient specimens using the conventional Southern blotting technique, Calero Moreno and colleagues found that bi-allelic deletion of the *p16INK4A* and *p14ARF* genes was an independent poor prognostic risk factor (Calero Moreno, Gustafsson et al. 2002).

Taken together, these studies support the findings that deletion of *p16INK4A* is an independent prognostic indicator of poor outcome at diagnosis in childhood ALL.

6.6.2 Hemizygous deletions of *p16INK4A/ARF* in childhood ALL

Prior to development of qPCR researchers had attempted to detect hemizygous deletions using Southern blot and conventional PCR. It was very difficult to control for normal cell contamination using these techniques (Heyman, Rasool et al. 1996) (Ohnishi, Hanada et al. 1996) (Tsihlias, Kapusta et al. 1999) (Drexler 1998). Using the qPCR technique developed in the present study, hemizygous deletions of *p16INK4A/ARF* at diagnosis in specimens from patients with childhood leukaemia were accurately detected. Surprisingly, those patients with hemizygous deletions detected in diagnostic bone marrow specimens were not only shown to have an increased risk ratio for relapse (6.558), but also a shorter median time to relapse.

This research also raised some technical considerations. Was the apparent deletion suggested by the new assay truly hemizygous or was the second allele not being expressed? This question was addressed critically by analysis of *p16INK4A* expression using conventional RT-PCR (primers specific for the coding region of *p16INK4A* were used) where patient bone marrow mRNA was available. A patient with leukaemia who was found to be hemizygously deleted for *p16INK4A/ARF* at diagnosis was found to have a approximately half the expression of *p16INK4A* compared to those specimens found to be germline. Sequencing of the cDNA did not show evidence of any point mutations. This supported the data that these deletions were truly hemizygous and were not epigenetically silenced by methylation or inactivated by mutation (Carter, Terry et al. 2004). A publication by Kees and colleagues has also subsequently demonstrated confirmation of hemizygous deletions detected by the qPCR method using FISH and real-time RT-PCR on cell lines (Kees, Terry et al. 2004).

The increased risk of relapse associated with hemizygous deletion of *p16INK4A/INK4A* was compatible with *p16INK4A* or *p14ARF* functioning as a semi-dominant tumour suppressor gene, rather than being classically recessive as previously believed, since deletion of one allele alone was associated with an observable phenotype. In this case an increased risk of relapse compared to patients without deletion in their leukaemia cells. There is some murine evidence that *p19Arf* is haploinsufficient but no studies on human

specimens have explored haploinsufficiency of *p16INK4A* (Kamijo, Bodner et al. 1999; Krimpenfort , Quon et al. 2001; Sharpless, Bardeesy et al. 2001). Our previous investigation did not address whether the deletions were constitutive or somatically acquired, this aspect was examined in a panel of leukaemia patient specimens and compared to a panel of normal individuals. In one normal individual evidence of a hemizygous *p16INK4A* deletion was found (2%). However, in contrast 17.6% of the patient remission specimens had hemizygous *p16INK4A* deletion and this disproportionate bias was found to be statistically significant. The DNA was extracted from the peripheral blood lymphocytes (PBLs) of normal controls who had no medical or family history available. DNA extracted from the PBLs of patients in remission could have theoretically contained submicroscopic levels of leukemia cells and thus, reflected their phenotype rather than the constitutional phenotype. Future investigations of constitutional *p16INK4A* hemizygosity should include a non-haematopoietic source of DNA as well as an assessment of other family members. This is the first observation implicating the role of *p16INK4A* hemizygous deletion as a predisposing factor in leukaemia (Carter, Terry et al. 2004).

The importance of addressing the subtle dosage issues of *p16INK4A* in a range of cancers needs further exploration. Confirmation that deletion of this gene may be implicated as a predisposing factor in leukaemia needs further study in larger groups of patient specimens.

6.6.3 The incidence of *p16INK4A/ARF* deletions in relapsed childhood ALL

The findings reported here show that deletions of the *INK4A/ARF* locus are associated with poor outcome and are implicated in the pathogenesis of leukaemia. The hypothesis was formed that deletion events at this locus would be increased at relapse compared to diagnosis. Using the qPCR technique developed for *p16INK4A/ARF* this hypothesis was tested on paired diagnostic and relapse samples from 25 patients with childhood ALL. The findings confirmed our hypothesis, the incidence of homozygous and hemizygous deletion of *p16INK4A/ARF* at diagnosis was 32% and 20%, respectively whilst the incidence of homozygous deletion of this locus at relapse was 64%. Interestingly, in 8 relapse specimens the *p16INK4A/ARF* homozygous deletions were not present in the corresponding diagnostic specimens. In addition this study showed that those patients with

an altered *p16INK4A/ARF* status in their bone marrow specimen at diagnosis had a significantly shorter median time to relapse compared to those specimens not deleted at diagnosis (Carter, Reaman et al. 2001).

Murine lymphoma models have shown that *Ink4a/arf* mutations can promote chemotherapeutic resistance (Schmitt, McCurrach et al. 1999). Studies in mice show evidence that *p19Arf* deletions are associated with progressive disease (Kamijo, Bodner et al. 1999). In the analysis on relapse specimens we were unable to determine whether the effects we observed were due specifically to *p16INK4A* or *p14ARF*. Both genes are involved in cell cycle regulation; the p16INK4A protein is a regulator of the retinoblastoma protein and the p14ARF protein is involved in p53 regulation. The role of *p15INK4B* deletion in relapsed childhood ALL had not been described using qPCR. Previous studies have suggested that deletion of *p15INK4B* never occurs without associated deletion of *p16INK4A*(Heyman, Rasool et al. 1996). Evidence would suggest that epigenetic deregulation of *p15INK4B* may also be an important phenomenon in leukaemia (Iravani, Dhat et al. 1997) (Batova, Diccianni et al. 1997) (Wong, Ng et al. 2000). Epigenetic regulation of *p15INK4B* was not investigated in this study due to limitations in sample availability but exploration of *p15INK4B* deletion in the paired study group was performed.

6.6.4 Analysis of the *INK4A/ARF, INK4B* and *TP53* gene loci in relapsed childhood ALL

In murine cancer models there is evidence to suggest that mutations of *Ink4a/Arf* and *p53* gene loci promote resistance to apoptotic chemotherapeutic drugs (Schmitt, Fridman et al. 2002) (Lee and Schmitt. 2003). Childhood ALL studies of relapsed patient specimens have found an increased incidence of *TP53* mutations in ALL of up to 24% (Wada, Bartram et al. 1993) (Diccianni, Yu et al. 1994) (Marks, Kurz et al. 1996; Blau, Avigad et al. 1997) (Gump , McGavran et al. 2001). Detailed mapping of the *INK4* cluster for the three genes *p15INK4B*, *p14ARF* and *p16INK4A* was conducted in this study using qPCR. Mutations of *TP53* were explored using a published technique (Soong and Iacopetta.

1997). These investigations were performed on a group of paired bone marrow specimens from 20 childhood ALL patients at diagnosis and at relapse.

Surprisingly, all patient specimens had a deletion of at least one of the three genes studied at the *INK4* cluster at diagnosis and at relapse. This is in contrast with unselected groups (Kees, unpublished observation) (Bertin , Acquaviva et al. 2003). The incidence of deletion events increased at relapse for each gene studied. The incidence of homozygous deletion for all three genes studied at diagnosis was 35% and at relapse was 65%. The incidence of hemizygous deletion for all three genes at diagnosis was 10% and at relapse was 0%. There was a significant overall relationship between median time to relapse and *p16INK4A* status. There was however, no significant relationship found for median time to relapse and deletions of *p15INK4B* and *p14ARF*. No *TP53* mutations were detected at diagnosis but three specimens had exon 7 mutations in addition to *INK4* cluster deletions at relapse (Carter, submitted to Leukaemia, 2004).

A murine lymphoma model has shown that tumours with mutations of *p53* or *Ink4a/arf*, but not those lacking *Arf* alone, responded poorly to chemotherapy (Schmitt, Fridman et al. 2002). The results presented in this thesis confirm the observation by Sherr and colleagues, that in humans deletion of *p16INK4A* may be more significant following exposure to chemotherapy (Sherr 2001).

This evidence supports the importance of the *INK4* cluster in childhood ALL, particularly following exposure to chemotherapy. Subtle dosage issues, which affect the function of this gene, may also be important in a number of cancers and novel evidence is presented here that it may be involved in predisposing a person to leukaemia.

6.7 Deletions of the *INK4A/ARF* and *INK4B* loci in childhood ALL and therapeutic interventions

This data provides further evidence that deletions found at the *INK4*A/ARF locus, particularly those affecting *p16INK4A* should be targeted with therapies that restore defective apoptosis and cellular senescence. Murine studies have shown that the *Ink4a/Arf*

locus and *TP53* are altered following exposure to chemotherapy creating an unstable genome (Lee and Schmitt. 2003). Microarray studies of mantle cell lymphoma gene expression have shown that deletion of the *INK4A/ARF* locus and *Cyclin D1* expression co-operate to increase cellular proliferation. These two measurements together assisted in the determination of patient survival (Rosenwald, Wright et al. 2003). Recent studies of mitosis reveal how abnormal function of the retinoblastoma protein can lead to aneuploidy and further genomic instability (Hernando , Nahle et al. 2004). The uses of specific CDK inhibitors that are independent of such defects in cellular division and apoptosis must be considered when deletions of the *INK4A/ARF* locus are discovered. Flavopiridol, a cyclin-dependent kinase inhibitor is one of a new class of agents being trialed on human cancers where traditional alkylating agents have failed. Flavopiridol induces cell cycle arrest and tumour growth inhibition in solid tumours and leukaemia (Shapiro 2004) (Quiney, Dauzonne et al. 2004). Interactions between flavopiridol and bortezomib (a proteosome inhibitor) have shown synergistic activity in myeloid cells resistant to conventional therapy (Dai , Rahmani et al. 2004).

6.8 Future Directions

6.8.1 Monitoring clonal evolution

This study has shown new molecular events at the *INK4* cluster and *TP53* locus at relapse compared to diagnosis in bone marrow specimens from patients with childhood ALL. Were these changes detected occurring in the original dominant leukaemic clone or due to the emergence of a subclonal population undetected at diagnosis and more resistant to apoptotic agents? Studies of *IgH* and *TCR* gene rearrangements, on paired patient specimens have shown that in the majority of specimens the original leukaemic clone is still the dominant clone detected at relapse (Nirmala , Rajalekshmy et al. 2002; Szczepanski , van der Velden et al. 2003). This would suggest that in the majority of relapse specimens investigated in my own study, the deletion events found at the *INK4* cluster and the mutations at the *TP53* locus were most likely to be occurring in the dominant leukaemic clone following exposure to chemotherapy. In accordance with this theory a study of the clonal evolution of this patient group using PCR targets for *IgH* and *TCR* gene rearrangements will be conducted. Detailed analysis of the leukemic cells at

diagnosis and relapse by use of *IgH* and *TCR* rearrangements may give insight into the heterogeneity at diagnosis (process of subclone formation) and at relapse (selection of subclones, possibly related to development of therapy resistance) (Beishuizen, Verhoeven et al. 1994).

Clonal evolution from diagnosis to relapse will be analysed using *IgH* and *TCR* rearrangements. Clonal diversity analysis may provide information regarding prognostic implications at diagnosis. Knowledge of possible subclone selection at relapse will help in our understanding of leukaemogenesis and resistance to treatment. Studies on *IgH* and *TCR* gene rearrangements will be correlated with the analysis of the *INK4* cluster on these paediatric specimens to provide a more comprehensive picture of childhood relapsed ALL. These gene rearrangements are currently being investigated in childhood ALL to monitor minimal residual disease.

6.8.2 Application of qPCR and microarray methods in leukaemia

Microarray methods are currently expensive and require high quality mRNA extraction. Gene expression profile analysis from leukaemic blasts has detected 20 genes that can define the seven subgroups found in the study by Yeoh and colleagues (Yeoh , Ross et al. 2002). Analysis of these discriminating genes can be more cheaply performed using automated real-time reverse transcription PCR (qRT-PCR) (Carroll , Bhojwani et al. 2003). Microarray methods have also been applied in the detection of loss of heterozygosity in cancer cell lines and this method has been corroborated with qPCR (Bignell , Huang et al. 2004). The validation of gene expression profile analysis is being performed using qPCR with increasing frequency (Rosenwald, Wright et al. 2003).

Proteomic analysis may obviate the need for painful and laborious collection of bone marrow specimens. Proteomics allows the analysis of proteins on serum patient specimens at diagnosis and at relapse (Bocchetta and Carbone. 2004). It is evolving as a method of pattern recognition in risk assessment for many cancers including leukaemia (Te Kronnie , Bicciato et al. 2004) (Carroll , Bhojwani et al. 2003). Once pathways are defined analysis of specific genes needs to be performed. Research on tumour pathways that effect patient

response to therapy such as the findings in this study will help further define which proteins will be the most relevant in such assessment These genetic and protein profiles may enable us to decrease intensive treatment when it is unnecessary and use specific tumour targeted therapies.

Currently, qPCR is the most accurate way of assessing gene expression. In the future custom microarray chips may be used in the diagnosis of many cancers including leukaemia. Expression of key regulatory genes may then be validated by qPCR. Hence, the methods described in this thesis using qPCR are expected to be used with greater frequency as new genes are discovered.

6.9 Summary

6.9.1 Relapsed childhood ALL

The failure of chemotherapy in a child with ALL has devastating effects on the patient, family and hospital staff. The bleak prognostic outlook for patients with relapsed childhood ALL in contrast to their prognosis at diagnosis highlights the need for an understanding of drug resistance and leukaemogenesis. Patient treatment is based on clinical, morphological, immunophenotypic and molecular classification. Understanding the prognostic implications of diagnostic molecular abnormalities provides clues to leukaemogenesis and also possible therapeutic interventions. However, scarcity of paired diagnostic and relapse specimens for study purposes has made the exploration of these molecular abnormalities difficult.

Cytogenetic studies on diagnostic relapse specimens have suggested deletion of chromosome 9p are important in the development of relapse. This chromosome is deleted in many cancers including leukaemia. The *INK4A/ARF* and *INK4B* loci located on chromosome 9p code for TSGs that are important in cell cycle control and apoptosis. Abnormalities of this region occur in many cancers and deletions are well described in both adult and childhood leukaemia. The clinical relevance of these deletions in childhood ALL has been controversial. Knowledge of their importance in leukaemogenesis was unknown but murine studies suggested that they were important in chemotherapeutic resistance. This

had far reaching clinical implications as new therapies had emerged which could act independent of these TSGs. Thus detection of a deletion at these loci at diagnosis could change therapeutic practice and possibly prevent relapse.

6.9.2 Analysis of *INK4A/ARF* and *INK4B* deletions in childhood ALL

This thesis has addressed the questions initially posed, but in doing so has raised a number of significant new questions to be addressed in future studies. The project has explored the involvement of the *INK4A/ARF* and *INK4B* gene loci in childhood ALL using a precise and accurate method of detection. The novel qPCR technique developed as part of this study is able to detect tumour suppressor gene deletions in a normal cell milieu. It has shown detection of homozygous deletion of *p16INK4A/ARF* at diagnosis in childhood ALL is an independent indicator of poor outcome. In addition analysis of the *p16INK4A/ARF* locus has confirmed that deletion events occur more frequently at relapse and their presence at diagnosis events is associated with a decreased median time to relapse. More specifically, deletion of the *p16INK4A* gene is important following exposure to chemotherapy and has a significant effect on median time to relapse.

This study also suggests that hemizygous deletions of this gene in somatic cells may predispose a person to leukaemia. Further, deletions of *p16INK4A* gene at diagnosis may increase resistance to apoptotic chemotherapeutic drugs which are cell cycle dependent. Since childhood ALL specimens are limited, larger studies are needed to confirm prospectively the role of these loci in this disease. In addition functional studies on human cell lines and mice are needed to further assess the functional effects of deletions at these loci and pathways effected in response to chemotherapy.

6.9.3 Therapeutic interventions

The results of this study would suggest that the *INK4* cluster contain key regulatory genes involved in leukaemogenesis. Detection of homozygous or hemizygous deletion of *p16INK4A* by qPCR on a bone marrow specimen at diagnosis in childhood ALL should

alert the treating physician to the possibility of therapeutic failure. The addition of drugs that act independently of these TSGs such as flavopiridol should be tested in clinical trials where such deletions are monitored by qPCR.

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Appendix A1 RESPONSE:

P16^{INK4A} gene deletion in paediatric acute lymphoblastic leukaemia

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Response written by UR Kees.

Einsiedel et al comment on the prognostic value of the *p16*^{INK4A} gene deletion in paediatric acute lymphoblastic leukaemia, with particular reference to the methodology used in our recent publication (Carter, Watt et al. 2001). The real-time polymerase chain reaction (PCR) was developed in our laboratory to detect deletion of the *p16*^{INK4A} exon 2 gene since the Southern blotting method used in our previous studies does not allow accurate quantitation of gene deletion. The novel method described by us is performed in a multiplex format where the p16 test gene is amplified in the same tube as the reference gene, *β-actin*. In order to establish that the technique has the capacity to detect gene deletion in specimens containing normal cells, we conducted mixing experiments using DNA from 2 cell lines, we conducted mixing experiments using DNA from 2 cell lines, one showing homozygous deletion of p16 (D/D) and the other being wild type (G/G). This test was performed multiple times using independent samples of DNA mixtures and reproducibly yielded a linear graph with a correlation coefficient of 0.9687 to 0.9742 between the input ratio of mixed DNA and the experimentally determined ratio of p16. The reproducibility of the assay was further examined by repeatedly analyzing patient specimens representing the 3 p16 genotypes G/G, G/D and D/D. These specimens were measured in 6 replicas each, showing ratios of 0.91 $\pm$ 0.03 for the G/G specimen, 0.69 $\pm$ 0.08 for the G/D specimen, and 0.13 $\pm$ 0.02 for the D/D specimen (mean $\pm$ SD). The same specimens were measured in 2 additional independent experiments (in duplicates each), and the values (mean $\pm$ SD) from the 3 tests were 0.95 $\pm$ 0.13, 0.63 $\pm$ 0.07, and 0.07 $\pm$ 0.05 for the G/G, G/D, and D/D specimens, respectively. Taken together, these results indicate that the interclass correlation coefficient measuring the reproducibility of measurements for a given genotype is 0.909.

We elected to conduct the mixing experiment using 2 cell lines, rather than a cell line showing hemizygous deletions. Apart from the fact that we do not have access to a line showing this particular feature, such a cell line may contain submicroscopic lesions in p16 not detected by cytogenetics. The mixing experiment provides much more information as it allows titration over the entire range, from 0 to 100 percent. This is of critical importance as it was necessary to focus on the range between 0% and 25% to assess the suitability of the test for patient specimens containing normal cells. The ratios to determine the p16 genotype of the patient specimens was based on the reproducibility of the assay (see above) and we opted to use conservative values of 0.4 and 0.8. Most importantly, the patient specimens showed a clear triphasic distribution, consistent with discrete populations having G/G, G/D or D/D alleles.

Einsiedel et al were not able to establish a technique as accurate and reproducible as ours, which may be due to the instrument used or/and to the fact that test and reference genes were not measured in a multiplex reaction. Our results on many standard calibration curves showed that the conditions optimized for multiplexing pass the test for the comparative efficiency test, which means that they would allow detection and comparison of the test gene and reference gene in separate tubes. Although we did not expect to achieve the required accuracy to conduct the analysis in separate tubes, we verified whether our conditions would be suitable. Indeed the results confirmed this to be the case, but as expected, the accuracy in repeat tests is not as high as by the multiplexing method.

As stated in our paper, we intended to confirm the status of the G/D specimens by using an independent technique. Due to lack of material, it was not possible to conduct fluorescence insitu hybridization studies. Similarly, we refer to the controversy regarding p16 deletion as a prognostic factor in paediatric ALL. Rather than quoting many individual publications, we referenced the reviews by Drexler et al and by Tsihlias et al, which contain all relevant publications (Drexler 1998) (Tsihlias, Kapusta et al. 1999). Moreover, as stated in our paper, we agree with Einsiedel et al regarding the need for a larger study to assess the significance of *p16* deletion as a prognostic marker, and such a study is currently in progress in our laboratory.

In order to determine the frequency of p16 deletion at relapse, we studied patients from whom we obtained diagnosis and relapse bone marrow specimens (Carter, Reaman et al. 2001). The data showed the rate to be much higher at the time of relapse, which is in agreement with a study by Maloney et al (Maloney, Mcgavran et al. 1999).

We are intrigued by the motivation underlying the statement "The number of patients Carter et al investigated is too low for the use of multivariate Cox regression analysis with so many variables, and neither did they show any odds ratios of the confounding factors included in their analyses nor did they present data about the associated that might exist between $p16^{INK4A}$ loss and relevant prognostic factors." The first part is not substantiated; we would strongly recommend that personal beliefs about the theoretical validity of an analysis might usefully be accompanied by an explanation of theoretical basis of those beliefs. There are plenty of unique failure times to ensure that the number of risk sets underpinning the generation of the partial log likelihood in this case is adequate to permit the number of parameters we use in our analysis to be estimated. Obviously the data set is relatively small, but that is why we quote confidence intervals and why we state that further investigation is essential. As is almost always the case, severe space limitations prevented us from including (1) the results of formal model checking (completeness of linear predictor; analysis of Martingale residuals; checks of leverage and influence), which showed that our primary models fitted well, or (2) the associations between baseline potentially confounding covariates and outcome and between the covariates and $p16^{INK4A}$ loss. We happen to agree that such data ought to be provided, and they were in fact included in earlier longer versions of the paper, but the reality is that requirements for radical abridgment ultimately meant that they had to be removed. It is of relevance to note that, had these results been reported, it would not in any way have changed the conclusions of the paper. In essence, we agree with much of what Einsiedel et al write, and in particular are delighted that they support our call for further research. There is no question that the differing results from the various studies to which we and they refer are intriguing and need to be properly understood. In particular, it would be of interest whether $p16^{INK4A}$ deletion remains of independent prognostic significance within the subgroups of patients with T-cell or B-precursor ALL, respectively.

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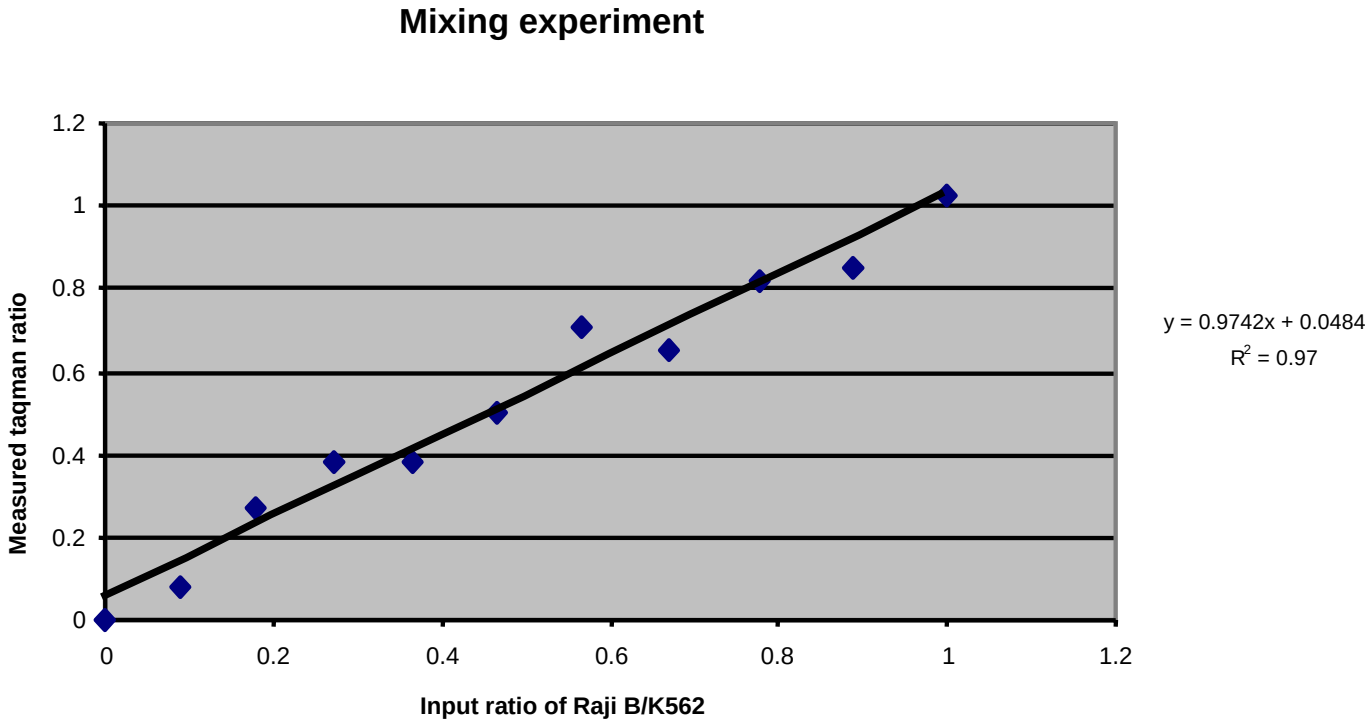
Appendix A2 Simulation of normal cell contamination by using mixtures of DNA from Raji B cells, which are wild type for *p16 INK4A/ARF* (GG), and K562 cells, which show homozygous deletion of *p16 INK4A/ARF* (DD).



Appendix B1: Sequence analysis of *p16*^{INK4A} RT-PCR product for specimen P1 at diagnosis and relapse aligned with published *p16*^{INK4A} *mRNA* sequence. No evidence of point mutations or deletions in patient specimen.