Investigation of genetic alterations in paediatric patients with T-cell acute lymphoblastic leukaemia

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DECLARATION

I, Sarah Jenkinson, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

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ABSTRACT

Approximately 25% of paediatric patients with T-cell acute lymphoblastic leukaemia (T-ALL) develop recurrent disease and post-relapse prognosis remains poor. Identification of molecular prognostic markers at diagnosis is needed so that earlier intervention with more intensive therapy can be targeted at those at the greatest risk of relapse, and dose reduction considered for those at a lower risk. Denaturing HPLC (dHPLC) was used to screen selected regions of the *NOTCH1* and *FBXW7* genes in samples from 162 paediatric T-ALL patients. Overall, 101 (62%) had *NOTCH1* mutations (*NOTCH* ^{MUT}) and 29 (18%) were *FBXW7*^{MUT}. The cohort was divided into three genotype groups for analysis: wild-type (WT) for both genes (*NOTCH1*^{WT}*FBXW7*^{WT}), a single *NOTCH1* mutation (*NOTCH1*^{Single}*FBXW7*^{WT}), and either *NOTCH1*^{Double}*FBXW7*^{WT} or *NOTCH1*^{MUT}*FBXW7*^{MUT} (*NOTCH1±FBXW7*^{Double}). Patients with *NOTCH1±FBXW7*^{Double} mutations were significantly associated with negative minimal residual disease (P=.01) and an excellent overall survival (P=.005), and should not be considered for more intensive therapy in first remission. *PTEN* mutation status was determined by dHPLC and the mutant level quantified by fragment analysis. Overall, 21 (13%) were *PTEN* ^{MUT} and median mutant level was 48% (range 10%-96%). Loss of genomic *PTEN* was investigated by quantification of two SNP loci in 76 informative patients and Illumina SNP array analysis in 139 patients with sufficient DNA. Of 145 patients, 15 (10%) had *PTEN* deletions, and combining the mutation and deletion status, 32 (22%) harboured a *PTEN* abnormality. *PTEN* genotype was not a significant prognostic indicator of response to therapy or clinical outcome; therefore it is not warranted for use in risk-adapted therapy at the present time. *PTEN* genotype had no impact on the favourable outcome of the patients with *NOTCH1±FBXW7*^{Double} mutations; nor did it further stratify the *NOTCH1*^{Single}*FBXW7*^{WT} or *NOTCH1*^{WT}*FBXW7*^{WT} groups. This work provides insight into the biology of *NOTCH1*, *FBXW7* and *PTEN* mutations and their use as clinical markers.

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COMMONLY USED ABBREVIATIONS

AA	Amino acid
ALL	Acute lymphoblastic leukaemia
BM	Bone marrow
bp	Base pairs
CD	Cluster of differentiation
cDNA	Complementary DNA
CI	Confidence intervals
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CNS	Central nervous system
dHPLC	Denaturing high-performance liquid chromatography
DNA	Deoxyribonucleic acid
EFS	Event-free survival
ETP	Early T-cell precursor
G-CSF	Granulocyte-colony stimulating factor
GMP	Granulocyte/macrophage progenitor
GSI	Gamma secretase inhibitor
HD	Heterodimerisation
HSC	Haemopoietic stem cell
HLA	Human leukocyte antigen
HR	Hazard ratio
IL	Interleukin
LIC	Leukaemia initiating cell
LSC	Leukaemic stem cell
MEP	Megakaryocyte/erythroid progenitors
MRC	Medical Research Council
MRD	Minimal Residual Disease
NCI	National Cancer Institute
NGS	Next generation sequencing
nt	Nucleotide
OR	Odds ratio
OS	Overall survival

PB	Peripheral blood
PCR	Polymerase chain reaction
RED	Restriction enzyme digest
RFS	Relapse-free survival
SCT	Stem cell transplantation
SNP	Single nucleotide polymorphism
TAD	Transactivation domain
TRM	Treatment-related mortality
WBC	White blood cell count
WGA	Whole genome amplified
WHO	World Health Organization
WT	Wild-type

CHAPTER 1: INTRODUCTION

Acute lymphoblastic leukaemia (ALL) is an aggressive malignant disease of haematopoietic cells derived from the transformation of lymphoid progenitors. The disease is characterised by the accumulation of lymphoblasts in the bone marrow (BM) and peripheral blood (PB), and the expansion of the blast cells within the BM, resulting in the failure of normal haematopoiesis. ALL is a very heterogeneous disease in terms of disease biology, and this heterogeneity is observed in the varied immunophenotypes and gene mutations found in patients at diagnosis, and reflected in the difference in prognosis and response to therapy observed between patients.

The work presented in this thesis describes the investigation of mutations in the *NOTCH1*, *FBXW7* and *PTEN* genes in paediatric patients with T-cell acute lymphoblastic leukaemia (T-ALL), a subtype of ALL. This chapter provides an overview of the classification, treatment and prognostic factors of the disease in order to put this work into context. The specific biology and clinical relevance of the mutations in the genes mentioned above is provided in the introductions to Chapters 3-6.

1.1 Haematopoiesis

The blood contains many different cellular compartments with a wide range of functions, from the transport of oxygen to the production of an immune response to fight infection. Haematopoiesis is the formation of these components of the blood system, all derived from a haematopoietic stem cell (HSC). The main site of haematopoiesis in adults is the BM, however during embryonic and foetal development it also occurs in the liver, spleen, thymus and lymph nodes. Pluripotent HSCs are able to differentiate into all cells of the blood system including red blood cells (erythropoiesis), lymphocytes (lymphopoiesis), myeloid cells (myelopoiesis) and platelets (megakaryopoiesis), and they reside in small numbers in the BM. An overview is given in Figure 1.1. HSCs are also characterised by their capacity for self-renewal. Studies suggest that HSCs generate either multipotent common myeloid progenitor (CMP) or common lymphoid progenitor (CLP) cells, which can differentiate into cells of the myeloid and lymphoid lineages, respectively (Orkin & Zon, 2008). The CLP produces B- and T-lymphocytes, and the CMP megakaryocyte/erythroid progenitors (MEPs), which then give rise to erythrocytes and megakaryocytes, and granulocyte/macrophage progenitors (GMPs) which can differentiate

Figure 1.1 Haematopoiesis. Haematopoiesis is the formation of the components of the blood system, all derived from an HSC. HSCs generate either the multipotent CMP or CLP cell, which can differentiate into cells of the myeloid and lymphoid lineages, respectively This process requires a number of different transcriptions factors, indicated by red bars. Abbreviations: HSC, haematopoietic stem cell; LT, long-term; ST, short-term; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor; RBCs, red blood cells. Taken from Orkin and Zon *et al* (2008).

+

through more lineage-restricted committed precursor cells into terminally differentiated cells, such as neutrophils and macrophages. Committed lymphoid progenitors differentiate under the tightly regulated influence of various growth factors such as interleukin-2 (IL-2), IL-7 and SDF-1 (Orkin & Zon, 2008). Transcription factors are also involved in lineage determination including Ikaros, E2A and PU.1 for lymphoid differentiation (Orkin, 2000) and PAX5 for B-cell development (Nutt & Kee, 2007). As cells become more differentiated, they lose self-renewal capacity and pluripotency, characteristics of the HSC.

1.2 ALL

1.2.1 Leukaemogenesis

Transformation of a normal haematopoietic cell to a leukaemic cell requires not only the capacity to cause a block in differentiation while maintaining self-renewal properties, but also an aberrant proliferation and survival signal (Kelly & Gilliland, 2002). Therefore unless a single genetic event is sufficient to confer all the above properties, it is clear that the acquisition of contributory genetic events is necessary for malignant transformation and the development of acute leukaemia. Cooperating events such as chromosomal translocations or gene mutations are acquired over time in a multistep process (Gilliland *et al.*, 2004), and several studies suggest that these acquired changes confer a survival advantage to tumour cells (Anderson *et al.*, 2011;Greaves, 2007;Merlo *et al.*, 2006;Nowell, 1976;Stratton *et al.*, 2009). For example, in ALL, a study of syngeneic twins was able to identify a pre-natal *ETV6-RUNX1* gene fusion that represented a pre-leukaemic clone likely to predate the development of ALL, suggesting that additional genetic events are required (Bateman *et al.*, 2010).

Newer technologies such as next generation sequencing (NGS) have revealed a complexity and diversity of human cancer at the DNA sequence level, and sequencing of individual cancer genomes has enabled the detection of a large number of somatic genetic alterations (Stratton *et al.*, 2009). However, it is likely that only a small number of the somatic mutations identified confer a selection advantage on the cells that acquire them, and these mutations are defined as driver mutations (Stratton *et al.*, 2009). The driver mutations are generally in cancer-associated genes and it has been suggested that most cancers have more than one. They disrupt genetic

pathways and are thought to contribute to the "hallmarks of cancer" described by Hanahan and Weinberg *et al* (2011), which include the ability to sustain proliferative signalling, evade growth suppressors, resist apoptosis, enable replicative immortality, induce angiogenesis and activate invasion and metastasis. Exactly how many genetic 'hits' are required for leukaemia development is unclear but their frequency varies between cancers, with an estimate of between 5 and 20 in an individual cancer type (Beerenwinkel *et al.*, 2007;Rangarajan *et al.*, 2004;Sjoblom *et al.*, 2006). The large majority of somatic mutations are therefore thought to be unlikely to confer a selection advantage and are defined as passenger mutations (Stratton *et al.*, 2009).

The specific mutations or genetic events that confer a selection advantage over normal cells cause the expansion of the leukaemic clone and subsequent accumulation of leukaemic blasts in the PB and BM. In ALL, these genetic hits are discussed further in section 1.4 and include the disruption of cell differentiation by the aberrant expression of *TAL1*, a key regulator of HSC development, or an increase in cell proliferation and/or survival by activating mutations in signal transduction pathway components such as *IL-7R* and *NRAS* (Van Vlierberghe & Ferrando, 2012). In recent years there have been major advances in the identification of the malignant populations responsible for the maintenance of leukaemia and initiation of relapse. Early studies examined the leukaemia blast cell proliferation kinetics in in vivo models of acute leukaemia and demonstrated that two proliferating populations existed within the tumour: a larger fast cycling subset with a 24hr cell cycle time, and a smaller slow cycling dormant subset able to repopulate the fast cycling cells (Clarkson et al., 1975). However, it was only in later xenotransplantation assays that leukaemia stem cells (LSCs) were first described when transplantation of an immature $CD34^{+}CD38^{-}$ fraction of tumour cells purified from a patient with acute myeloid leukaemia (AML) into severe combined immunodeficient (SCID) mice was shown to initiate leukaemic engraftment and produce large numbers of mature blast cells (Lapidot *et al.*, 1994). The LSC has been defined as a leukaemic cell distinct from the bulk of the tumour which, like an HSC, possesses the capacity to self-renew and replenish the heterogeneous cell populations of the leukaemia, and may also be quiescent (Dick, 2008). The immature phenotype of the LSC in some types of leukaemia suggests that the normal haematopoietic cell from which the LSC is derived may be a multipotent HSC which has undergone leukaemic transformation. The identification of the LSC in ALL has been more difficult to define. In contrast to AML, self-renewal was shown to not be restricted to an HSC-like, $CD34^{+}CD19^{-}$ population, but was found in a range of normal B precursor populations

including a mature $CD34^{-}CD19^{+}$ population that was able to replenish the entire disease phenotype including the $CD34^{+}CD19^{-}$ subset (Kong *et al.*, 2008;le Viseur *et al.*, 2008). These studies suggest that in ALL the cell of origin may be a more committed lymphoid progenitor cell that has acquired some stem-cell properties, in particular self-renewal capacity, during leukaemogenesis (Bomken *et al.*, 2010).

1.2.2 Clinical presentation and incidence

The accumulation of leukaemic blasts in the BM and PB leads to the ablation of normal haematopoiesis, known as BM failure, and resultant PB cytopenia. This may cause an increased susceptibility to infections due to neutropenia, fatigue or shortness of breath as a result of anaemia, and pain in the bone and joints. Thrombocytopenia may also lead to bleeding and a propensity for the skin to bruise easily. An enlargement of the lymph nodes, liver and spleen may also be seen in some patients.

The incidence of ALL has two apparent peaks, one in early childhood between two and five years of age and a second peak after middle age. There is an estimated annual incidence of two cases per 100,000 individuals in the population, and approximately 60% of cases occur at <20 years of age (Inaba *et al.*, 2013). Childhood ALL accounts for approximately 23% of cancers in children (Pui & Evans, 1998).

1.2.3 Epidemiology

ALL is considered to arise from multiple factors including genetic susceptibility, environmental exposures and chance. In both children and adults there is a male predominance with a ratio of 1.5:1. Cases are highest in non-Hispanic whites, and Switzerland has the highest worldwide incidence. There has been a steady increase in the number of cases diagnosed worldwide, with an average reported increase of 0.7% per year between 1970 and 1999 (Dores *et al.*, 2012). There are a number of risk factors for ALL. The chromosomal disorder Down's syndrome (trisomy 21) confers a 20 fold increased risk of developing ALL. Other inherited or congenital conditions such as neurofibromatosis type 1, Schwachman syndrome, Bloom syndrome and ataxia telangiectasia also increase the risk of ALL. Studies have demonstrated the risk of both foetal and postnatal X-ray exposure. For example, X-ray therapy was used in the treatment of children with tinea capitis and many cases developed brain and skin cancers and ALL (Shore *et*

al., 2003). Exposure to other agents including radiation, pesticides and low electromagnetic fields are also risk factors and are listed on the National Institute of Health website: http://www.cancer.gov/cancertopics/pdq/treatment/childALL/. It has also been shown that specific single nucleotide polymorphisms (SNPs) in the *ARID5B* gene are linked to ALL susceptibility (Xu *et al.*, 2012).

1.3 Classification

ALL can develop from lymphoid cells blocked at any stage of differentiation. Early classification was based on the French-American-British (FAB) system which defined the leukaemia on the basis of morphological appearance and cytochemistry (Bennett *et al.*, 1981). On this basis, ALL was categorised into three subtypes, L1, L2 or L3, however distinguishing between the L1 and L2 subgroups did not provide any prognostic information so the system is no longer used. Therefore, a panel of monoclonal antibodies against different cluster-of-differentiation (CD) molecules is used to diagnose by immunophenotype (Pui, 1995). Cases are classified as either T-ALL or B-ALL depending on the expression of specific markers, cytoplasmic CD3 with CD7 plus CD2 or CD5 for T-cell lineage and cytoplasmic CD79a, CD19 and HLA-DR for B-cell lineage leukaemia (Swerdlow et al., 2008).

1.3.1 European Group for the Immunological Characterisation of Leukaemias (EGIL) classification of ALL

The EGIL classification system allows the grouping of leukaemias within the B and T-cell lineage according to the level of cell differentiation (Table 1.1) and is widely used. The immunophenotype of the leukaemic cells is assessed by flow cytometry. For the B-cell lineage the four immunological subtypes are Pro-B-ALL, Common ALL, Pre-B-ALL and Mature B-ALL. The four subgroups of T-cell leukaemia are Pro-T-ALL, Pre-T-ALL, Cortical/Thymic T-ALL and Mature T-ALL. The most common subtype of ALL is Common ALL, which accounts for 50% of ALL cases. Cortical/Thymic T-ALL is the most common T-cell leukaemia, 17% of total ALL cases are classified into this subgroup (Bene *et al.*, 1995). In some instances, the

Table 1.1 EGIL classification of immunophenotype of ALL using flow cytometric markers

	% ALL cases	Positive markers	Negative markers
B-ALL			
Pro B-ALL	10%	$HLA^{-}DR^{+}$, TdT^{+}, $CD19^{+}$	$CD10^{-}$, $CyIg^{-}$
Common ALL	50%	$CD10^{+}$, TdT^{+}	$CyIg^{-}$
Pre-B-ALL	10%	$CyIg^{+}$, $CD10^{+}$, TdT^{+}	
Mature B-ALL	4%	$SmIg^{+}$	TdT^{-}, $CD34^{-}$
T-ALL			
Pro-T-ALL	7%	TdT^{+}, $CD7^{+}$, $CyCD3^{+}$	$CD2^{-}$, $CD4^{-}$, $CD8^{-}$
Pre-T-ALL	1%	TdT^{+}, $CD7^{+}$, $CyCD3^{+}$, $CD2^{+}$	$CD4^{-}$, $CD8^{-}$
Cortical/thymic T-ALL	17%	TdT^{+}, $CD7^{+}$, $CyCD3^{+}$, $CD1a^{+}$, $CD2^{+}$, $CD5^{+}$, $CD4^{+}$, $CD8^{+}$	
Mature T-ALL	1%	$CD7^{+}$, $SmCD3^{+}$, $CD2^{+}$, CD4 /8^{+}	TdT^{-}, $CD1a^{-}$

Abbreviations; HLA-DR, human leukocyte antigen DR; TdT, terminal deoxynucleotidyl transferase, Cy, cytoplasmic; Ig, immunoglobulin; Sm, surface. Taken from Bene *et al*., (1995).

subgroups can be of prognostic use, for example *MLL* rearrangements are most frequently seen in Pro-B-ALLs and are associated with a poor outcome (Cimino *et al.*, 1995;Pui *et al.*, 1994). However this classification system does not incorporate cytogenetic abnormalities and is unhelpful in cases that do not show an immunophenotype characteristic of the outlined subgroups.

1.3.2 World Health Organisation (WHO) classification of ALL

In recent times more detailed genetic information has been obtained and, combining this with cytogenetic, morphological, and immunophenotypic information, the WHO has developed a classification system that was first reported in 2001 (Jaffe *et al*, 2001) and recently updated (Campo *et al.*, 2011). There are three main groups within this classification: B-lymphoblastic leukaemia/lymphoma not otherwise specified, B-lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities, and T-lymphoblastic leukaemia/lymphoma (Table 1.2).

1.3.3 Classification of biphenotypic leukaemia

In a small subset of ALLs, the lineage of origin is unclear; either two separate leukaemic blast populations of different lineages are present, or a single blast population co-expressing both myeloid and lymphoid markers. The WHO classification refers to the former as bilineage leukaemia and the latter as biphenotypic leukaemia (Swerdlow et al., 2008). Accurate diagnosis of biphenotypic leukaemia is essential as it constitutes a rare subgroup of ALL with a poor prognosis. It is not uncommon for leukaemias to aberrantly express a single marker from an alternate lineage, therefore it is important to distinguish these from the biphenotypic subgroup. The EGIL points classification system was devised to do this and is based on the number and degree of specificity of the myeloid and lymphoid markers expressed by the leukaemic blasts (Table 1.3). Biphenotypic leukaemia is considered to be present if there is a score of greater than two points for myeloid markers together with greater than one point for lymphoid markers (Bene *et al.*, 1995).

1.3.4 Early T-cell precursor (ETP) ALL

ETP-ALL is characterised by the lack of expression of T-cell lineage markers CD1a and CD8, weak or absent expression of CD5, and expression of one or more myeloid or stem cell marker.

Table 1.2 World Health Organisation classification of ALL

Precursor Lymphoid neoplasms
B-cell lymphoblastic leukaemia/lymphoma, not otherwise specified
B-cell lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities
B-cell lymphoblastic leukaemia/lymphoma with t(9;22)(q34;q11.2) *BCR-ABL1*
B-cell lymphoblastic leukaemia/lymphoma with t(v;11q23) *MLL* rearrangement
B-cell lymphoblastic leukaemia/lymphoma with t(12;21)(p13;q22) *TEL-AML1(ETV6-RUNX1)*
B-cell lymphoblastic leukaemia/lymphoma with hyperploidy
B-cell lymphoblastic leukaemia/lymphoma with hypoploidy
B-cell lymphoblastic leukaemia/lymphoma with t(5;14)(q31;q32) *IL3-IGH*
B-cell lymphoblastic leukaemia/lymphoma with t(1;19)(q23;p13.3) *E2A-PBX1*
T-cell lymphoblastic leukaemia/lymphoma

Taken from Swerdlow *et al*., (2008).

Table 1.3 EGIL classification for the diagnosis of biphenotypic leukaemia

	B-lineage	T-lineage	Myeloid lineage
2 points	$CD79^+$, IgM^+, $CD22^+$	$CD3^+$ Anti TCR^+	MPO^+
1 point	$CD19^+$ $CD10^+$ $CD20^+$	$CD2^+$, $CD5^+$, $CD8^+$ $CD10^+$	$CD13^+$ $CD33^+$ $CD65^+$
0.5 point	TdT^+	TdT^+, $CD17^+$, $CD10^+$	$CD14^+$ $CD15^+$ $CD64^+$ $CD117^+$

Abbreviations; Ig, immunoglobulin; TCR, T-cell receptor; TdT, terminal deoxynucleotidyl transferase. Taken from Bene *et al*., (1995).

This subset of T-ALL also has a gene expression pattern similar to that of normal ETP cells, with overexpression of myeloid genes including *GATA2*, *CEBPA* and *CD34*. ETP blast cells derive from an immature haematopoietic progenitor cell arrested at an early stage of maturation, which retains the ability to differentiate into myeloid and T-cell lineages (Zhang *et al.*, 2012).

1.4 T-ALL

Of the total number of ALL cases, 10-15% of paediatric and 25% of adult patients have a T-cell phenotype, which derives from the transformation of T-cell progenitors in the thymus (Pui & Evans, 1998). T-ALL is more frequent in males than females, and patients tend to be associated with a higher white blood cell count, older age and the presence of a mediastinal mass when compared to those with a B-cell phenotype. T-ALL is also associated with an increased risk of central nervous system (CNS) infiltration at diagnosis (Marks *et al.*, 2009;Pui & Evans, 1998).

1.4.1 Cytogenetics

The most common cytogenetic abnormality is the deletion of chromosome 9p21, which is seen in >70% of patients with T-ALL (Ferrando *et al.*, 2002) (Table 1.4). The deletions encompass the cell cycle regulator *CDKN2A* locus which encodes the *p16INK4a* and *p14ARF* tumour suppressor genes. Chromosomal rearrangements in T-ALL frequently involve genes encoding transcription factors and place the transcription factor under the control of a T-cell receptor (TCR) gene such as *TCRB* (7q34) or *TCRA-D* (14q11), where the strong TCR gene enhancers and promoters drive their aberrant expression (Van Vlierberghe *et al.*, 2008).

1.4.1.1 bHLH transcription factors

Frequently rearranged transcription factors in patients with T-ALL include members of the basic helix-loop-helix (bHLH) family, *TAL1*, *TAL2*, *LYL1* and *BHLHB1*. The proteins contain two α-helices connected by a loop, required for homodimer and heterodimer formation, and a basic domain that mediates DNA binding. The most frequently rearranged bHLH transcription factor is the *TAL1* gene located on chromosome 1p32, and aberrant expression is seen in 60% of patients with T-ALL (Ferrando *et al.*, 2002). TAL1 is essential for HSC development and survival (Aifantis *et al.*, 2008). It is expressed on haematopoietic progenitors and plays a role in megakaryocyte/erythrocyte differentiation. It binds E-box motifs following heterodimerisation

Table 1.4 Cytogenetic aberrations reported in patients with T-ALL

Gene category	Gene	Rearrangement	Incidence	Impact on outcome	Reference
Translocations involving TCR genes					
bHLH family	*TAL1*	t(1:14)(p32;q11)	3%	Good	Cave *et al*., (2004)
		t(1;7)(p32;q34)	3%	Good	Cave *et al*., (2004)
	TAL2	t(7;9)(q34;q32)	<1%	Unknown	Van Grotel *et al*., (2006)
	LYL1	t(7;19)(q34;p13)	<1%	Unknown	Hwang *et al*., (1995)
	bHLHB1	t(14;21)(q11.2;q22)	<1%	Unknown	Wang *et al*., (2000)
LMO family	*LMO1*	t(11;14)(q15;p11)	2%	Unknown	Graux *et al*., (2006)
	LMO2	t(11;14)(p13;q11)	7%	Unknown	Graux *et al*., (2006)
		t(7;11)(q35;p13)	3%	Unknown	Graux *et al*., (2006)
Homeobox family	*TLX1 (HOX11)*	t(10;14)(q24;q11)	5%-10%	Good	Cave *et al*., (2004)
		t(7;10)(q34;q24)			
	TLX3 (HOX11L2)	t(5;14)(q35;q32)	24%	Unknown	Cave *et al*., (2004)
		t(5;7)(q35;q21)	24%	Poor/No impact	Cave *et al*., (2004)
	HOXA cluster	t(7;7)(p15;q34)	3%	Unknown	Cave *et al*., (2004)
Proto-oncogenes	*NOTCH1*	t(7;9)(q34;q34.3)	<1%	Unknown	Ellisen *et al*., (1991)
	C-MYB	t(6;7)(q23;q34)	3%	Unknown	Clappier *et al*., (2007)
	C-MYC	t(8;14)(q24;q11)	2%	Poor	Erikson *et al*., (1986)
Cell cycle	*CCND2*	t(7;12)(q34;p13)	<1%	Unknown	Clappier *et al*., (2006)
		t(12;14)(p13;q11)	<1%	Unknown	Clappier *et al*., (2006)
Formation of fusion genes					
bHLH family	*STIL/TAL1*	1p32 deletion	16%-30%	Good	Cave *et al*., (2004)
Homeobox family	*HOXA (MLL-ENL)*	t(11;19)(q23;p13)	1%	Unknown	Ferrando *et al*., (2003)
	HOXA (SET-NUP214)	9q34 deletion	3%	No impact	Van Vlierberghe *et al*., (2008)
		inv(14)(q11.2q13)			
	HOXA (CALM-AF10)	t(10;11)(p13;q14)	5%-10%	Poor	Asnafi *et al*., (2003)
Signal transduction	*NUP214-ABL1*	Episomal amplification of 9q34	4%	Poor	Graux *et al*., (2006)
		t(9;12)(q34;p13)			
	ETV6-ABL1	t(9;14)(q34;q32)	<1%	Unknown	Graux *et al*., (2006)
	EML-ABL1	t(9;22)(q34;q11)	<1%	Unknown	Graux *et al*., (2006)
	BCR-ABL1		<1%	Poor	Raanani *et al*., (2005)

Table 1.4 Continued

Gene category	Gene	Rearrangement	Incidence	Impact on outcome	Reference
Deletions					
Cell cycle	*CDKN2A/2B* *CDKN1B* *RB1*	9p21 deletion 12p13 deletion 13q14 deletion	70% 2% 4%	Good Unknown No impact	Ferrando *et al*., (2002) Graux *et al*., (2006) Remke *et al*., (2009)

Abbreviations: *TAL*, T-cell acute lymphoblastic leukaemia gene; *LMO*, Lim-only domain gene; *TLX*, T-cell leukaemia homeobox gene; *HOXA*, homeobox; *MYB*, myeloblastosis viral oncogene homolog gene; *MYC*, myelocytomatosis viral oncogene homolog gene; *CCND*2, Cyclin D2 gene; *STIL*, *SCL/TAL1* interrupting locus; *NUP214*, nucleoporin 214kDa gene; *ABL1*, c-abl1oncogene; *CDKN2A/B*, cyclin-dependent kinase inhibitor; *RB1*, retinoblastoma-1 gene.

with one of the class I bHLH E-proteins such as E2A or HEB, and forms a transcriptional complex with other factors including GATA3, LIM-only (LMO) domain proteins LMO1/2 and runt-related transcription factor 1 (RUNX1) (O'Neil *et al.*, 2004;Ono *et al.*, 1998). In patients with T-ALL with aberrant *TAL1* expression, there is a block in differentiation at the late double-positive (DP) stage of thymocyte development. The t(1;14)(p32;q11) translocation, which juxtaposes the *TAL1* gene to the *TCRα/δ* gene, is present in 3% of paediatric cases, and between 16%-30% of patients with T-ALL harbour a small deletion at chromosome location 1p32 which places *TAL1* next to and under the subsequent control of the *STIL* gene (Van Vlierberghe & Ferrando, 2012).

1.4.1.2 LMO proteins

Frequent translocations are also seen involving the LMO domain genes, *LMO1*, *2* and *3*. The LMO proteins form transcriptional complexes with TAL1 and LYL1, therefore activation of the *LMO1* and *LMO2* genes is frequently found in conjunction with aberrant *TAL1* expression (Ferrando *et al.*, 2002). In a transgenic mouse model, leukaemogenesis was enhanced in mice overexpressing both the *Tal1* and *Lmo1/2* genes (Aplan *et al.*, 1997;Larson *et al.*, 1996). Another study demonstrated that overexpression of *Lmo2* resulted in the initiation of leukaemia by conferring the capacity to self-renew to developing thymocytes (McCormack *et al.*, 2010). Translocations of the *LMO* genes are seen in approximately 10% of paediatric T-ALL cases (Van Vlierberghe & Ferrando, 2012). The most frequent is the t(11;14)(p13;q11) rearrangement where the *LMO2* gene is placed under the control of the *TCRα/δ* receptor.

1.4.1.3 HOX transcription factors

Members of the *HOX* family of transcription factors are also rearranged in T-ALL. HOX transcription factors play an essential role in organogenesis, such as TLX1 (Hox11), which is required for spleen development, and TLX3 (Hox11L2) for neural development. The oncogenic role of *TLX1* was recently demonstrated in a transgenic mouse model, where aberrant expression resulted in the induction of leukaemia (De Keersmaecker *et al.*, 2010). The t(10;14)(q24;q11) rearrangement, in which the *TLX1* gene is translocated to the *TCRα/δ* receptor locus, occurs in approximately 5%-10% of paediatric and 30% of adult patients with T-ALL (Ferrando *et al.*, 2002). Frequent rearrangements are also seen in *TLX3*, the most common, the t(5;7)(q35;q21) juxtaposes the gene to the *TCRα/δ* receptor and is seen in 25% of T-ALL cases (Bernard *et al.*, 2001). Approximately 3% of patients with T-ALL harbour rearrangements in the

HOXA cluster of *HOX* genes, which are essential for the regulation of axial patterning during development (Soulier *et al.*, 2005). The t(7;7)(p15;q34) translocation places the *HOXA9* and *HOXA10* genes in the *TCRβ* receptor locus.

1.4.1.4 Fusion genes

A number of chromosomal rearrangements generate fusion transcripts encoding distinct transcription factors. The *MLL-ENL* gene fusion is seen in 1%, the *SET-NUP214* in 3% and the *CALM-AF10* in approximately 5%-10% of cases of T-ALL (Van Vlierberghe & Ferrando, 2012). All three of these fusion genes result in the upregulation of the *HOXA* cluster. The *ABL1* gene is rearranged in 8% of cases, and 4% of cases harbour episomal amplification and expression of the *NUP214-ABL1* fusion gene (Graux *et al.*, 2004;Hagemeijer & Graux, 2010), commonly those with *TLX1* and *TLX3* rearrangements. Other *ABL1* rearrangements include the *ETV6-ABL1* and *EML-ABL1* fusion transcripts (De Keersmaecker *et al.*, 2005).

1.4.1.5 Proto-oncogenes

The leucine zipper transcription factor *c-MYB* is overexpressed as a result of the t(6;7)(q23;q32) translocation in 3% of cases of T-ALL, predominantly in patients aged two years and under (Clappier *et al.*, 2007). Duplications of the gene are also found in approximately 8%-15% of cases (Lahortiga *et al.*, 2007). The *MYC* gene is also rearranged in 2% of cases, where the t(8;14)(q24;q11) translocation places the gene under the control of the *TCRα* receptor (Van Vlierberghe & Ferrando, 2012).

1.4.2 Gene mutations

There are number of genes that have been found to be recurrently mutated in the leukaemic cells of patients with T-ALL, which, in combination with the variety of chromosomal abnormalities seen in transcriptional regulators and signal transducers, is the basis of the heterogeneity seen in this disease (Table 1.5). Further study of the reported chromosomal translocations and rearranged oncogenes in patients with T-ALL led to the identification of frequent mutations in genes including the *NOTCH1* receptor, which is discussed further in chapter 3 (Weng *et al.*, 2004). One group engineered lymphoma-prone mice with chromosomal instability to assess disease-associated copy number aberrations by array-based comparative genome hybridisation (a-CGH) (Maser *et al.*, 2007). The authors identified recurrent deletions of *FBXW7* and *PTEN*,

and targeted re-sequencing of the genes in patients with T-ALL revealed inactivating mutations, and these are discussed further in chapters 3 and 5 respectively.

A number of other studies utilised copy number analysis by SNP array or aCGH to identify candidate genes for mutation screening. Heterozygous frameshift mutations of *WT1* are present in approximately 10% of cases of T-ALL and are frequently associated with aberrant *TLX1* and *HOXA9* expression (Tosello *et al.*, 2009). LEF1 is a member of the lymphoid enhancer factor/T-cell factor family of transcription factors and interacts with nuclear β-catenin in the WNT signalling pathway (van Noort & Clevers, 2002). Inactivating mutations of the *LEF1* gene have been reported in 7% of paediatric patients (Gutierrez *et al.*, 2010b). Loss of function mutations have also been detected in the *BCL11B* gene in 9%-13% of paediatric patients with T-ALL (De Keersmaecker *et al.*, 2010;Gutierrez *et al.*, 2011). The *BCL11B* gene encodes a zinc finger transcription factor that plays a critical role in the differentiation and survival of T-cell progenitors in the thymus (Wakabayashi *et al.*, 2003). Other transcription factors have been shown to be mutated in cases of early immature T-ALL including *ETV6* and *RUNX1*. The t(12;21) *ETV6-RUNX1* translocation is frequently described in patients with pre-B-ALL, however inactivating mutations of *ETV6* have been reported in 13% of paediatric cases of T-ALL (Van Vlierberghe *et al.*, 2011) and *RUNX1* loss of function mutations in 10% of cases (Della Gatta *et al.*, 2012).

Further advances in technology such as whole genome and exome NGS have led to the detection of mutations in numerous other genes in leukaemic cells. A study examining the increased incidence of T-ALL in male cases performed targeted capture and NGS of genes located on the X chromosome and identified mutations of the plant homeodomain finger 6 gene (*PHF6*) in 16% of paediatric and 38% of adult cases (Van Vlierberghe *et al.*, 2010). Mutations were associated with cases with aberrant TLX1/3 and TAL1 expression. PHF6 is suggested to have a role in transcriptional regulation and DNA repair, and the loss of function mutations suggest that *PHF6* may be a tumour suppressor gene.

In the first report of whole-genome sequencing (WGS) of samples from patients with ALL, 12 cases of ETP-ALL were sequenced (Zhang *et al.*, 2012). ETP-ALL is characterised by the absence of T-lineage cell surface marker expression and cases commonly harbour a high number of genetic alterations but lack a common abnormality. There was a marked diversity from case to case in the frequency and location of the alterations identified; however the

Table 1.5 Somatic mutations in T-ALL

Gene category	Gene	Incidence	Impact on outcome	Reference
Activating mutations				
NOTCH1 pathway	*NOTCH1*	60%	Good/No impact	Weng *et al*., (2004), Breit *et al*., (2006)
Signal transduction	*IL-7R*	10%	No impact	Shocat *et al*., (2011)
	JAK1	1%	No impact	Zhang *et al*., (2012)
	JAK3	5%	No impact	Zhang *et al*., (2012
	STAT5B	6%	Poor	Bandapelli *et al*., (2014)
	NRAS	5%-10%	No impact	Zhang *et al*., (2012)
	FLT3	2%	No impact	Zhang *et al*., (2012)
Inactivating mutations				
NOTCH1 pathway	*FBXW7*	8%-30%	Good/No impact	Maser *et al*., (2007)
Signal transduction	*PTEN*	10%	Poor/No impact	Palomero *et al*., (2007), Jotta *et al*., (2010)
	NF1	3%	No impact	Zhang *et al*., (2012)
Transcription factors	*WT1*	10%	No impact	Tosello *et al*., (2010)
	LEF1	10%-15%	Unknown	Gutierrez *et al*., (2010)
	ETV6	13%	No impact	Zhang *et al*., (2012)
	BCL11B	10%	No impact	Guterriez *et al*., (2011)
	RUNX1	10%-20%	Poor/No impact	Zhang *et al*., (2012)
	GATA3	5%	Poor	Zhang *et al*., (2012)
	CNOT3	4%	Unknown	De Keermaeker *et al*., (2013)
Chromatin regulators	*EZH2*	10%-15%	Poor	Zhang *et al*., (2012)
	SUZ12	10%	No impact	Zhang *et al*., (2012)
	EED	10%	No impact	Zhang *et al*., (2012)
	PHF6	20%-40%	No impact	Van Vlierberghe *et al*., (2010)

Abbreviations: *IL-7R*, interleukin-7 receptor; *JAK*, Janus kinase 1; *STAT5B*, signal transducer and activator of transcription; *NRAS*, neuroblastoma-RAS gene; *FLT3*, FMS-related tyrosine receptor 3; *FBXW7*, F-box and WD40 domain-containing protein 7; *PTEN*, phosphatase and tensin homolog; *NF1*, neurofibromatosis 1 gene; *WT1*, Wilms Tumour 1 gene; *LEF1*, lymphoid enhancer factor 1 gene; *ETV6*, ETS translocation variant 6 gene; *BCL11B*, B-cell CLL/lymphoma 11B; *RUNX1*, runt-related transcription factor 1; *EZH2*, enhancer of zeste homolog 2; *EED*, embryonic ectoderm development; *PHF6*, planthomeodomain finger 6.

majority harboured abnormalities that could be classified into three groups. The first group were mutations in genes predicted to result in abnormal cytokine receptor or RAS signalling including *N-RAS, K-RAS, FLT3, JAK1, JAK2* and *IL-7R*. The second group were mutations in genes encoding regulators of haematopoietic development including *ETV6*, *GATA3*, *IKZF1* and *RUNX1*. Finally, the third group were mutations targeting genes involved in histone modification, including components of the polycomb repressor complex 2, *EED*, *EZHZ* and *SUZ12*. Novel somatic mutations were also identified in the study; inactivating mutations of the *DNM2* and *RELN* genes were described in patients with both ETP and non-ETP ALL and in the *ECT2L* gene in cases of ETP only (Zhang *et al.*, 2012).

A recent study of whole exome sequencing of 67 samples from patients with T-ALL identified mutations in 15 candidate driver genes (De Keersmaecker *et al.*, 2013). Of the 15 genes, eight had already been previously described, *NOTCH1*, *FBXW7*, *WT1*, *BCL11B*, *JAK3*, *PTEN*, *DNM2* and *PHF6*. The seven newly identified genes were *CNOT3*, *RPL10*, *RPL5*, *ODZ2*, *TET1*, *KDM6A* and *MAGEC3*. The *CNOT3* gene was mutated in 4% of cases and was associated with adult T-ALL. Mutations were truncating or missense, resulting in weak or absent expression of the CNOT3 protein, which is part of the CCR4-NOT complex that plays a role in the assembly of the proteosome (Collart & Panasenko, 2012). Mutations of the ribosomal genes *RPL5* and *RPL10* were reported in 2% and 5% of the cohort respectively and *RPL10* mutations were associated with paediatric T-ALL. Mutations in the *STAT5B* gene have also recently been identified in 6% of paediatric patients with T-ALL (Bandapalli *et al.*, 2014) and somatic heterozygous mutations in non-coding sites that introduce MYB binding motifs and create a super-enhancer upstream of the *TAL1* gene in a subset of cases of T-ALL (Mansour *et al.*, 2014).

1.5 Factors associated with patient outcome

The survival rate of paediatric patients with T-ALL has risen to approximately 90% in recent trials (Inaba *et al.*, 2013), but for adults, long-term remission rates are currently at approximately 50% (Pui & Evans, 2006). Historically, the prognosis of patients with T-ALL was particularly poor in comparison to patients with B-ALL. However improvements in therapeutic approaches including landmark advances such as the introduction of high-dose methotrexate, have led to T-ALL survival rates equalling those of B-ALL in recent years (Pui &

Evans, 2013). This improvement in treatment protocols has been paralleled by increased toxicity, and a wide variation in the response to the same treatment regimen is seen between patients. This has led to the development of a risk-based approach to therapy to avoid over- or under-treatment of individuals. The prognostic factors currently used to stratify patients are detailed below.

1.5.1 Clinical features

Age and white blood cell count (WBC) at diagnosis are robust prognostic factors when stratifying patients for risk-adapted therapy. In current paediatric trials, age ≥10 years is considered to be an adverse risk factor, with a better prognosis in patients aged between 1 and 10 years (Inaba *et al.*, 2013). In paediatric treatment protocols the latter are therefore assigned to a less intense treatment regimen providing that they have no other poor risk factors (Clappier *et al.*, 2010;Kox *et al.*, 2010;Qureshi *et al.*, 2010;Zuurbier *et al.*, 2010). There have been a number of studies demonstrating the inferior outcome of adolescent patients and those in their early 20s when treated on adult rather than paediatric regimens (Boissel *et al.*, 2003;de Bont *et al.*, 2004;Ramanujachar *et al.*, 2007). Whether this difference is a reflection of the biological differences in susceptibility to therapy, clinical differences in treatment tolerability or compliance issues decreasing the effectiveness of treatment remains unclear however, and recent paediatric trials have raised the age of accrual to 25 years accordingly (Nachman, 2005). In adults, increasing age is associated with a worse prognosis. A recent study demonstrated that patients aged between 55-65 years had a lower complete remission and survival rate, and more infections during induction therapy (Sive *et al.*, 2012). The association of older age with a worse outcome in B-ALL is in part due to the higher incidence of the t(9;22) *BCR-ABL* translocation in this age group, which is linked to resistance to chemotherapy (Ribeiro *et al.*, 1997). An elevated WBC indicates a high tumour load and proliferation rate, and for T-ALL the cut-off point for high-risk disease is a presenting WBC $>100x10^9$/L (Bassan & Hoelzer, 2011;Hunger *et al.*, 2012). In paediatric patients, boys historically have a slightly worse prognosis than girls as a result of testicular relapse; therefore current treatment regimens extend the maintenance therapy for boys by one year. Sex has no effect in adult trials (Marks *et al.*, 2009).

There is limited evidence to suggest that the maturation level of the T-cells is associated with prognostic significance, unlike in B-ALL, where a $CD10^-$ pro-B-ALL immunophenotype is associated with high-risk disease (Bassan & Hoelzer, 2011). In an adult study, cases of T-ALL

expressing CD1a with an immunophenotype similar to that of early cortical thymocytes seemed to respond better to therapy (Marks *et al.*, 2009). However, ETP-ALL is a recently identified subgroup of T-ALL which accounts for 15% of cases and is associated with an increased risk of treatment failure in the context of some trials (Coustan-Smith *et al.*, 2009;Ma *et al.*, 2012), although this association has not been replicated in the UKALL 2003 trial (Patrick *et al.*, 2014). A recent study has suggested that CD45 expression may be a potential prognostic marker in T-ALL (Cario *et al.*, 2014). The authors demonstrated an association between high CD45 expression and a higher rate of relapse and worse survival in paediatric patients treated on the ALL-BFM 2000 protocol. They hypothesised that this was due to the decreased proliferation of cells as a result of the CD45-mediated inhibition of Src and JAK kinases. A reduced proliferation gene expression profile has previously been shown to be linked to in vivo treatment resistance in primary samples from patients with T-ALL (Cario *et al.*, 2005;Chiaretti *et al.*, 2004). Genes encoding proteins shown to function in cell proliferation, including *TTK* and *FGFR1*, were expressed at low levels in the blast cells of relapsed patients.

1.5.2 Cytogenetics

A number of recurrent chromosomal abnormalities have been shown to have prognostic significance in B-ALL. Some chromosomal abnormalities are associated with a more favourable outcome such as hyperdiploidy (Paulsson & Johansson, 2009) and the *ETV6-RUNX1* fusion (Attarbaschi *et al.*, 2004). Others are associated with a poorer prognosis including the Philadelphia chromosome t(9;22) (Ribeiro *et al.*, 1997), rearrangements of the *MLL* gene (Pui *et al.*, 2003) and the intrachromosomal amplification of the *AML1* gene (iAMP21) (Moorman *et al.*, 2007). However this has not been the case in T-ALL. One possible reason for this is the relatively small proportion of T-ALL compared with B-ALL patients in ALL clinical trials, meaning that the cohort sizes for impact studies are small and outcomes vary between treatment protocols. One study of 153 paediatric patients with T-ALL reported an association between a favourable outcome and presence of either the *STIL/TAL1* fusion gene or *TLX1* or *TAL1* rearrangements (Cave *et al.*, 2004). However, in other studies a poor prognosis was found to be associated with overexpression of the *TLX3* gene and *NUP214-ABL1* episomal amplification (Burmeister *et al.*, 2006;Cave *et al.*, 2004;Ferrando *et al.*, 2004;van Grotel *et al.*, 2006). In one study, patients harbouring *CDKN2A* deletions were associated with a shortened survival after relapse (Diccianni *et al.*, 1997).

1.5.3 Response to therapy

Early response to glucocorticoid-based induction therapy is morphologically assessed from BM aspirates and the tumour load at this time point is a robust prognostic marker. The specific glucocorticoid administered varies between trials, the UKALL 2003 protocol uses dexamethasone whereas a number of other paediatric treatment protocols use prednisone (Clappier *et al.*, 2010;Kox *et al.*, 2010;Zuurbier *et al.*, 2010). Induction response is assessed on day 8 or 15 of treatment for high- and low-risk patients respectively. Patients with more than 25% blasts present in the BM at this time are classed as high risk and receive a more dose-intensive induction and prolonged consolidation therapy.

The measurement of minimal residual disease (MRD) at the end of induction therapy is also used to stratify treatment of ALL. Although complete remission is considered to be the presence of <5% blast cells in the BM, this is based on microscopic assessment. However, more than half of relapses occur from patients who had a rapid early response based on morphological criteria and it has been calculated that these patients may still have up to 10^{10} leukaemic cells (Bassan & Hoelzer, 2011). As a result, more sensitive techniques were developed to quantify the residual leukaemic cells, and the level of disease persisting after induction therapy is currently the most powerful predictor of relapse in modern treatment protocols as it defines submicroscopic ALL present in patients in morphological remission. For the clinical use of MRD, the technique used is required to be sensitive, able to distinguish leukaemic cells from normal cells, available within a dedicated time frame for a therapeutic decision, reliable, and applicable to routine laboratories worldwide. The two main methods to track the leukaemic cells are flow cytometry and PCR.

Using flow cytometry, aberrant phenotypes are identified on leukaemic cells on the basis of the combination of expression and variable intensity of staining of T-cell markers such as terminal deoxynucleotidyl transferase (TdT), CD2 and CD7, together with markers of other lineages. These may include B-cell markers such as CD19 or markers of the myeloid lineage such as CD13 or CD33 (Campana & Coustan-Smith, 1999). This technique is challenging due to the need for fresh cells and it is generally less sensitive than PCR, 0.01% versus 0.001%. However, improved methods using multi-colour combinations of additional leukaemia markers in B-ALL have improved the sensitivity (Coustan-Smith *et al.*, 2011).

Quantitative PCR analysing 'leukaemia –specific' junctional regions of rearranged TCRγ and δ genes (Szczepanski *et al.*, 1999) can detect leukaemic cells with a sensitivity as low as 1 leukaemic cell in 10^6 normal cells, and this method is widely used. During normal T-cell development, lymphocytes must first rearrange the variable diversity joining (VDJ) segments of the *TCRγ* and *TCRδ* genes to form a *γδTCR*. The *TCRβ* and *TCRα* loci are then rearranged to form the *αβTCR* (Dadi *et al.*, 2009), the process being mediated by RAG recombinase genes (*RAG1* and *RAG2*). In order to produce a polyclonal population of T-cells with diverse antigen specificity, TdT introduces random DNA insertions and deletions; therefore, the sequence of individual VDJ regions varies between each normal T-cell. PCR across the VDJ region of cells from a normal individual would not produce a specific band as a result of this diversity, whereas the expansion of a dominant clone with a specific VDJ rearrangement would produce a positive result. PCR primers are therefore designed for the specific clonal rearrangement identified in an individual at leukaemic presentation, and presence of the clone can be tracked during the course of treatment.

Several studies have shown that there is a relationship between the risk of relapse and persistence of high MRD levels, and the measurement of residual disease is now incorporated into most modern treatment protocols (Cave *et al.*, 1998;van Dongen *et al.*, 1998;Schrappe *et al.*, 2011). The definition of positive and negative disease varies between trials. In the UKALL 2003 protocol, levels of MRD of more than 1 leukaemic cell in 1000 cells ($>10^{-3}$) are classed as MRD-positive, levels below this as MRD-negative. In the ALL-BFM and EORTC protocols, the level of MRD-positive disease is defined as $>10^{-4}$ and $>10^{-2}$ respectively (Clappier *et al.*, 2010;Kox *et al.*, 2010).

1.5.4 Other genetic alterations

As cytogenetic alterations do not seem to accurately predict the risk of treatment failure and recurrent disease, there has been a focus on the relationship between other genetic alterations and outcome in ALL. In B-ALL an emerging candidate is the *IKZF1* gene, which encodes the zinc-finger containing transcription factor IKAROS. Loss of function deletions and mutations of this gene are a common feature of high-risk *BCR-ABL* ALL and are associated with an increased risk of treatment failure and relapse (Mullighan *et al.*, 2009;van der Veer *et al.*, 2014). There are few cohort studies of adequate size to significantly address the prognostic value of most molecular markers identified in patients with T-ALL or their use in targeted therapy. This

is particularly true if a marker is only present in a small proportion of patients, and large cohorts of patients are therefore required for meaningful subgroup analyses. However, with the introduction of NGS and array-based technology, potential targets are now beginning to emerge. The complexity caused by the variety of mutations and the way in which they may interact with other markers means that determining the prognostic value of gene mutations remains a challenge.

The correlation between outcome and *NOTCH1* mutations is the most widely studied molecular marker in patients with T-ALL, and the prognostic use of mutations in this gene is discussed further in chapter 4. Other gene alterations associated with outcome include *GATA3*, *RUNX1* and *EZH2* mutations, hallmarks of ETP-ALL which has been associated with a poor 5-year EFS of <50% in the context of some trials (Grossmann *et al.*, 2011;Zhang *et al.*, 2012). A recent study used targeted sequencing based genotyping of 25,000-34,000 SNPs to identify genome profiles associated with relapse risk (Wesolowska-Andersen *et al.*, 2014). Eleven SNPs were found to be associated with relapse, in genes including *MPO*, *MMP7* and *ESR1*. When correlating SNP status to clinical features such as MRD status and WBC, the authors were able to define three risk groups, and using these groups to stratify patients, the lowest relapse rate was 4% in contrast to 76% for the high-risk group. Studies like these may allow for future individualised therapy. Other potential markers include the absence of biallelic *TCR*γ deletion (ABD), which indicates that cells have not undergone complete *TCR*γ rearrangement, a characteristic of early thymocyte precursors. One study demonstrated that a subset of paediatric T-ALL patients with ABD had a biological overlap with ETP-ALL as defined by gene expression (Gutierrez *et al.*, 2010a). The clinical course of the patients with ABD was aggressive and associated with a poor response to induction therapy.

1.6 Treatment of ALL

The current treatment protocols for paediatric ALL are largely based on the Berlin-Frankfurt-Munster (BFM) regimens. There are three phases of treatment: induction, consolidation and maintenance, with patients assigned to one of three intensities of treatment at diagnosis. These regimens have become increasingly risk-based, with factors such as age, WBC and MRD used to stratify patients in order to reduce treatment in low-risk patients and direct intensification for those at the greatest risk.

1.6.1 Induction therapy

Four to five weeks of induction chemotherapy eliminates the initial tumour cell burden and restores normal haematopoiesis in almost 99% of cases of paediatric and 78%-92% cases of adult ALL (Bassan & Hoelzer, 2011;Hunger *et al.*, 2012). Induction therapy includes the administration of a glucocorticoid such as prednisone or dexamethasone, with the latter being used in the UKALL 2003 trial as it has been shown to provide better control of CNS leukaemia (Mitchell *et al.*, 2005). In the UKALL 2003 trial, induction therapy is allocated according to risk factors. Patients with low-risk disease (age 1-10 years and WBC $<50x10^9$/L) are allocated to a three drug induction regimen with vincristine, dexamethasone and asparaginase (regimen A). High-risk patients (age >10 years and/or WBC $>50x10^9$/L) are allocated to receive an additional anthracycline such as daunorubicin (regimen B). Patients who are *BCR-ABL* positive, hypodiploid, *MLL* gene rearranged, *RUNX1* amplification positive, or who have a morphological slow early response (SER, defined as >25% bone marrow blasts at day 8 or 15 of induction therapy for high- and low-risk patients respectively), and are less than 16 years of age are assigned to 'augmented BFM' therapy (regimen C), which is described below and involves a more intense Capizzi interim maintenance protocol using intravenous methotrexate.

1.6.2 Consolidation and maintenance therapy

Consolidation or intensification therapy is administered after induction treatment to eliminate the persistent residual leukaemic blast cells, and involves cyclic courses of interim maintenance and delayed intensification phases. In regimen A of the UKALL 2003 trial, consolidation is given for a duration of 32 weeks and consists of two courses of interim maintenance using dexamethasone, vincristine, 6-mercaptopurine and oral methotrexate and two courses of delayed intensification with dexamethasone, vincristine, asparaginase, cytarabine, cyclophosphamide and 6-mercaptopurine. However, these drugs have limited specificity for leukaemic cells over normal BM cells, and their toxic effects cause periods of severe marrow failure and pancytopenia. A recent report from the UKALL 2003 trial reported that there was no difference in EFS between MRD-negative patients who received either one or two delayed intensifications, suggesting that treatment reduction is feasible in this subgroup (Vora *et al.*, 2013). Regimen B is similar to A but includes a ‘standard BFM consolidation’ phase immediately after induction using vincristine, asparaginase, cyclophosphamide, cytarabine and 6-mercaptopurine, whereas regimen C replaces the interim maintenance course with a more intense Capizzi interim

maintenance protocol using intravenous methotrexate. Patients then receive maintenance chemotherapy, consisting of daily 6-mercaptopurine and weekly methotrexate with pulses of vincristine and dexamethasone for a duration of two years for girls and three years for boys.

1.6.3 Adult treatment protocols

In adult ALL trials such as the ECOG2993/UKALLXII trial, treatment consists of two phases of induction therapy. The first course uses prednisolone, vincristine, asparaginase and daunorubicin, and the second phase uses cyclophosphamide, cytarabine and 6-mercaptopurine. Three courses of high dose methotrexate are then administered followed by consolidation with more chemotherapy, autologous or allogeneic transplantation. However, despite the same drugs being used in both paediatric and adult treatment protocols, the outcome between these two sets of patients remains very different. Factors included in this difference include the higher rate of induction deaths seen in adult patients, a decreased drug tolerance to agents such as asparaginase which can cause liver toxicity, and other treatment-related mortalities (Fielding, 2008). A more recent adult trial UKALL14 is currently testing a new drug, nelarabine, a purine nucleoside analog with selected cytotoxicity, for the treatment of patients with T-ALL in this age group.

1.6.4 Treatment-related mortality

The improvement in treatment protocols in recent years and the reduction in relapse rates in paediatric patients with ALL has been paralleled by an increased toxicity. This has raised the importance of decreasing treatment-related mortality (TRM) (Rabin, 2014). Infection is one of the main causes of TRM and a recent report on UKALL 2003 revealed that 30% of total deaths and 64% of treatment-related deaths were due to infectious causes (O'Connor *et al.*, 2014). Infection-related mortality (IRM) was associated with Downs Syndrome and patients with high-risk factors at diagnosis. In the non-Downs Syndrome patients there was a heightened risk of IRM during induction therapy and the more intensive phases of treatment. The infections were predominantly respiratory or catheter-linked infections of the blood, and in 55% of IRM cases death occurred within 48 hours from the onset of infection. The identification of low-risk patients would spare them from aggressive induction therapy, therefore reducing the risk of toxicity.

1.6.5 CNS-directed therapy

T-ALL is associated with an increased risk of CNS infiltration at diagnosis and the control of this aspect of the disease is an important part of treatment. Prophylatic cranial irradiation has been shown to effectively manage CNS disease and is used in the context of some paediatric treatment protocols including BFM-ALL regimens (Kox *et al.*, 2010), however its use has been removed from other trials in order to prevent complications such as neurotoxicity and secondary malignancies (Pui & Howard, 2008). These protocols, including the UKALL 2003 trial, replaced cranial irradiation with intrathecal chemotherapy with agents such as methotrexate, as studies had demonstrated that CNS relapse risk was equivalent using either approach (Veerman *et al.*, 2009).

1.6.6 Stem cell transplantation

Haematopoietic stem cell transplantation (SCT) is an established treatment in childhood ALL and is an alternative option to further chemotherapy. It can be effective at preventing relapse by inducing a graft-versus-leukaemia (GVL) effect. However, this is an aggressive therapy associated with significant TRM, due to both the intensive treatment required to ablate the recipients' BM as well as the effects of the donated cells, with complications including graft-versus-host disease (GVHD), graft failure and severe infections. More recent SCT protocols with the use of high-resolution human leucocyte antigen (HLA)-typing and improved conditioning and supportive care have reduced TRM and relapse (Leung *et al.*, 2011). Furthermore, the prognosis of patients receiving HSCs from an HLA-matched unrelated donor, cord blood or a haploidentical donor is now comparable to that in patients receiving HSCs from an HLA-matched sibling donor (Eapen *et al.*, 2007;Leung *et al.*, 2011). Allogeneic SCT is considered for paediatric patients who have recurrent disease or high-risk factors at first remission (Balduzzi *et al.*, 2005). These risk factors include *BCR-ABL*, *MLL* rearrangement, *iAMP21* or *E2A-HLF* positive ALL, or MRD-positive disease at the end of induction therapy (Bader *et al.*, 2009;Leung *et al.*, 2012).

1.7 Aims of this thesis

The identification of molecular markers with prognostic relevance is advantageous in patients with T-ALL, as they may guide future risk stratification and aid clinical decision making, such

as whether SCT is necessary. The significance of mutations in the *NOTCH1* and *FBXW7* genes remains unclear in patients with T-ALL. The incidence of *NOTCH1* and *FBXW7* mutations was therefore investigated in a cohort of paediatric patients with T-ALL and these results are presented in chapter 3. To address the prognostic implications of the *NOTCH1* and *FBXW7* mutations, the characteristics and clinical outcome of patients treated on the UKALL2003 protocol was correlated to mutation status (chapter 4). To further elucidate the molecular profile of the paediatric patients with T-ALL and to investigate other candidate markers, mutations in the *PTEN* gene were also explored in terms of their incidence and mutant level (chapter 5). To address the prognostic use of the mutations and copy number changes of the *PTEN* gene, the effect of the *PTEN* genetic alterations on clinical outcome was evaluated in the paediatric patients (chapter 6).

CHAPTER 2: MATERIALS AND METHODS

2.1 Molecular Biology

2.1.1 Reagents

Acetonitrile (ACN) (VWR International Ltd., Lutterworth, UK)

Agar (Sigma-Aldrich Company Ltd, Poole, UK)

Agarose (Bioline, London, UK)

Betaine (Sigma-Aldrich Company Ltd, Poole, UK)

BIOTAQ DNA polymerase (Bioline, London, UK)

BIOTAQ DNA polymerase buffer and magnesium chloride (Bioline, London, UK)

Boric acid (VWR International Ltd, Lutterworth, UK)

Bromophenol blue (Merck, Frankfurt, Germany)

Carbenicillin (Melford Laboratories Ltd, Ipswich, UK)

Chloroform (VWR International, Lutterworth, UK)

DNA size standard kit – 600 (Beckman Coulter UK Ltd, Buckinghamshire, UK)

dNTPs (Bioline, London, UK)

Dodecyl-trimethyl ammonium bromide (DTAB) (Sigma Aldrich Company Ltd, Poole, UK)

Ethylenediamine tetraacetic acid disodium salt (EDTA) (VWR International Ltd, Lutterworth, UK)

Ethanol 100% (VWR International Ltd, Lutterworth, UK)

Ethidium bromide solution (Sigma-Aldrich Company Ltd, Poole, UK)

Glycerol (VWR International Ltd, Lutterworth, UK)

Hyperladder I and IV (Bioline, London, UK)

Luria-Bertani (LB) broth capsules (MP Biomedicals, London, UK)

Molecular Biology water (Sigma Aldrich Company Ltd, Poole, UK)

One Shot Max Efficiency DH5α-T1 competent *E. coli* (Invitrogen Life Technologies, Paisley, UK)

Optimase buffer and magnesium sulphate (Transgenomic Ltd, Glasgow, UK)

Optimase DNA polymerase (Transgenomic Ltd, Glasgow, UK)

Phosphate buffered saline (PBS) (Sigma Aldrich Company Ltd, Poole, UK)

Phusion Hot Start High-Fidelity DNA Polymerase (New England Biolabs UK Ltd, Hitchin, UK)

Phusion HF buffer (New England Biolabs UK Ltd, Hitchin, UK)

Primers (Integrated DNA Technologies, Leuven, Belgium; Invitrogen, Paisley, UK)

QIAquick Gel Extraction kit (QIAGEN, Crawley, UK)

QIAquick PCR Purification kit (QIAGEN, Crawley, UK)

Restriction enzymes and buffers (New England Biolabs UK Ltd, Hitchin, UK)

Sample loading solution (SLS) (Beckman Coulter UK Ltd, Buckinghamshire, UK)

Super optimal broth with catabolite repression (SOC) (Invitrogen Life Technologies, Paisley, UK)

TOPO TA cloning kit (Invitrogen Life Technologies, Paisley, UK)

Tri-ethylene ammonium acetate (TEAA) (Transgenomic Ltd, Glasgow, UK)

Tris base (Tris(hydroxymethyl)aminomethane) (VWR International Ltd, Lutterworth, UK)

WellRED oligos (Sigma-Aldrich Company Ltd, Poole, UK)

X-Gal (Invitrogen Life Technologies, Paisley, UK)

2.1.2 Buffers

DNA lysis buffer: 8% DTAB Solution. For 100ml: 4g DTAB, 4.4g NaCl, 0.6055g Tris-hydrochloride, 0.93g EDTA disodium salt dissolved in distilled water

10x TBE (pH8.3): For 1 litre. 108.9g Tris, 55.7g Boric acid, 7.4g EDTA dissolved in distilled water

Loading buffer: 30% glycerol, 0.025% bromophenol blue in 1xTBE

2.1.3 DNA Extraction

Cells were suspended at a concentration of 1 x 10^6 cells per 100µl in PBS. Two times the volume of DNA lysis buffer was added, mixed thoroughly by inversion and incubated at 68^{o}C for 5 minutes. After cooling, an equal volume of chloroform was added and mixed thoroughly and the sample was centrifuged at 3000g for 20 minutes. The upper aqueous layer containing the DNA in solution was removed, and an equal volume of 100% ethanol was added and the solution was gently mixed until the DNA precipitated. The precipitated DNA was centrifuged at 3000g for 5 minutes. The DNA pellet was transferred to a clean tube and washed with 70% ethanol, then resuspended in molecular biology water. Samples were quantified using a Nanodrop spectrophotometer and were stored at 2-8^{o}C.

2.1.4 Polymerase Chain Reaction (PCR)

The PCR was used to amplify specific regions of genomic DNA. The DNA is amplified by successive cycles of denaturation of the template DNA, sequence-specific forward and reverse primer annealing to define the region to be amplified, followed by an extension step of DNA polymerisation. In order to ensure that there was no variation in the conditions in each reaction tube, a master mix of all required reagents was made and aliquoted prior to the addition of DNA template or water for the negative control. Three different Taq polymerases were used during this work, BIOTAQ, Optimase and Phusion Hot Start High-Fidelity DNA polymerase, each with specific conditions as recommended by the manufacturer.

For amplification using BIOTAQ DNA polymerase, a standard reaction mix for a 20µl PCR contained 2µl 10x NH_4 reaction buffer (670nM Tris-HCl, 160mM $(NH_4)_2SO_4$, 100mM KCl, 0.1% stabiliser), 1mM $MgCl_2$, 200µM of each dNTP, 0.5µM of each forward and reverse primer, 0.5U BIOTAQ DNA Polymerase and 10-100ng DNA template. The standard cycling conditions were 35 cycles of denaturation at 95°C for 30 seconds, annealing of primers for 30 seconds at a temperature specific to the primers (this information is given in the Appendices) and extension at 72°C for 30 seconds, followed by a final extension incubation of 5 minutes at 72°C to ensure complete extension of all the primers. For amplification using Optimase DNA polymerase, a standard 20µl PCR contained 2µl 10x Optimase reaction buffer, 1.5mM $MgCl_2$, 200µM of each dNTP, 0.5µM of each forward and reverse primer, 1U Optimase DNA Polymerase and 10-100ng DNA template. An initial denaturation step was added (95^{o}C for 5

minutes) and the primer extension step increased to 1 minute. The standard reaction mix for a 20µl PCR using Phusion Hot Start High-Fidelity DNA Polymerase contained 4µl 5x Phusion HF buffer (containing 7.5 mM $MgCl_2$, providing 1.5mM $MgCl_2$ in the final reaction), 200µM of each dNTP, 0.5µM each of the forward and reverse primers, 0.2U Phusion Hot Start High-Fidelity DNA Polymerase and 10-100ng DNA template. The standard cycling conditions used were an initial denaturation step of 98°C for 2 minutes, then 35 cycles of denaturation at 98°C for 42 seconds, annealing of primers for 42 seconds and extension at 72°C for 42 seconds, followed by a final extension incubation of 5 minutes at 72°C. As indicated in the relevant chapters, where required the PCR additive betaine was also added to PCR reaction mixes to enable amplification of difficult templates.

2.1.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used to both confirm the presence of PCR products and to size separate different fragments of DNA following restriction enzyme digestion, or to purify specific bands for sequencing. The required amount of agarose (between 1% and 2.5% weight/volume depending on the application) was dissolved in 35ml of 1xTBE by heating in a microwave oven and cooled slightly before the addition of 3.5µl of a 1mg/ml ethidium bromide solution and pouring into a mould. After the gel had set, it was covered in running buffer of 1x TBE containing the same concentration of ethidium bromide as the gel (0.1µg/ml). An aliquot of each PCR product or restriction enzyme digest to be analysed was mixed with loading buffer before loading into a well in the gel and where required, an aliquot of ladder was also loaded. Samples were electrophoresed at approximately 70V until they had ran across approximately two thirds of the gel. PCR products were visualised on a UV transilluminator by fluorescence of ethidium bromide intercalated in the DNA, and polaroid images were made.

2.1.6 Denaturing HPLC using the WAVE platform

Denaturing high-performance liquid chromatography (dHPLC) on the WAVE platform (Transgenomic Ltd., Glasgow, UK) was used to detect nucleotide changes in DNA PCR products. An overview of the process is shown in Figure 2.1. In WAVE analysis, the sample is injected into the flow path of buffer containing triethylammonium acetate (TEAA) and acetonitrile (ACN) and then flows through a polystyrene-divinyl benzene copolymer DNA separation column located in an oven at the temperature required for analysis. Navigator

software (Transgenomic Ltd) was used to determine the temperature or multiple temperatures at which to analyse the sample, which is dependent on the melting profile predicted by the software from the sequence of the amplicon. Representative amplicons melting curves are given in chapter 3. In general, a temperature was chosen at which the proportion of the PCR product predicted to be helical was between 50% and 95%. More than one temperature may be required to analyse the same sample depending on the percentage helicity across the amplicon. TEAA acts as an ion-pairing agent and forms TEA+ ions in solution, and the positively charged TEA+ associates with the negatively charged phosphate backbone of the DNA, thereby creating a hydrophobic layer on the fragment. The PCR products then bind to the column as the hydrophobic layer is attracted to the hydrophobic beads of the DNA separation column. Over time, the proportion of ACN in the buffer is gradually increased, and as the ACN concentration increases, the ion-pairing property of the TEAA decreases, causing the DNA to be eluted from the column. The presence of heteroduplexes containing mismatched bases leads to the DNA binding less strongly to the column than the homoduplexes, and so they are eluted from the column first. The DNA passes through a UV detector, which records the absorbance at 260 nm, and the results are displayed in the form of a chromatogram of absorbance over time. A sample containing only homoduplexes should produce a single peak on the chromatogram. The presence of heteroduplexes will create additional peaks on the chromatogram, for example the four peaks shown in Figure 2.1, however the number of peaks is dependent on the particular sequence.

Prior to WAVE analysis, PCR was used to amplify the region of interest from a template DNA using a proof-reading enzyme where possible, to limit errors in base inclusion to reduce potential false mutant peaks. The presence of PCR products was checked by agarose gel electrophoresis. The PCR products were then denatured by incubation at 95°C for 5 minutes, followed by 40 cycles of 1 minute incubations starting at 92°C and reducing by 1.5°C per cycle, to allow heteroduplex formation if a mutation was present, as shown in Figure 2.1. If only one type of allele, either wild-type (WT) or mutant, was present in the sample then just one homoduplex was produced.

The fraction collector facility of the WAVE instrument was used where purification of a particular heteroduplex peak was required, which enabled specific fractions eluted from the

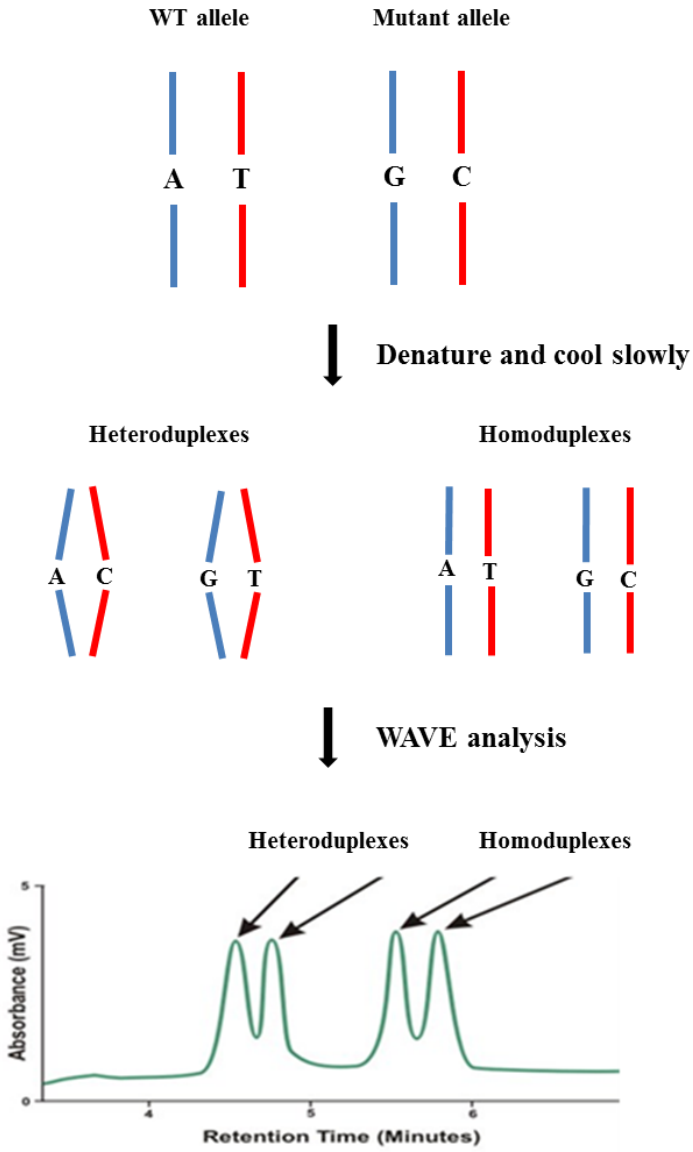

Figure 2.1 Mutation detection by dHPLC analysis. The region of interest was amplified by PCR and the PCR products were denatured and then gradually re-annealed slowly to enable heteroduplex formation. These were then analysed by dHPLC at a specific temperature. Heteroduplexes were eluted from the column first, followed by the homoduplexes.

column to be collected into separate vials. The fractions could then be further analysed by re-amplification by PCR and sequencing. For samples where the heteroduplex was present at a low level, fragment collection was performed at a denaturing analysis temperature that enabled the collection and enrichment of the heteroduplex.

2.1.7 DNA sequencing

PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Crawley, UK). The NanoDrop 1000 Spectrophotometer (Fisher Scientific UK Ltd., Loughborough, UK) was used to quantify the concentration of DNA in the purified product and the product was then diluted with water to a concentration of 1ng/µl per 100 bp to be sequenced. This diluted product was sent, along with the correct primer at a concentration of 5pmol/µl, to the Scientific Support Service at UCL Cancer Institute for direct nucleotide sequencing.

2.1.8 Restriction enzyme digestion

All restriction enzymes were obtained from New England Biolabs (Hitchin, UK) and PCR products were digested for four hours using the appropriate buffer and temperature specified by the manufacturer. Further details are given in the relevant chapters.

2.1.9 LB broth and plates

LB broth was made by adding 13 LB tablets to 500ml distilled water followed by autoclaving. For LB-agar plates, 7.5g of agar was added to the above mix and then autoclaved. Once the reagents for both the broth and plates had cooled to below 55^{o}C, carbenicillin (final concentration 100µg/ml) was added. Plates were then poured, set for one hour and stored at 2-8^{o}C until required.

2.1.10 TOPO TA Cloning of PCR products

PCR products were cloned using the TOPO TA cloning kit (Invitrogen Life Technologies, Paisley, UK) which utilises the polyadenosine (A) overhang at the 3' end of PCR products that is produced by non-proofreading Taq polymerases, such as BIOTAQ DNA Polymerase. PCR products were ligated with the pCR2.1-TOPO vector, which contains single 3'-thymidine (T)

overhangs, in a reaction mix of 0.5-4µl of PCR product, 1µl vector, 1µl salt solution (22mM NaCl; 10mM $MgCl_2$) in a final volume of 6µl. The mix was incubated at room temperature for 5 minutes.

The ligation mix was then transformed into One Shot Max Efficiency DH5α-T1 *E. coli* cells. One vial of cells was thawed on ice for each transformation, then 1µl of ligation reaction was added and incubated on ice for 30 minutes. The cells were then heat-shocked for 30 seconds at 42°C and placed on ice for 2 minutes before 250µl of SOC medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) was added per vial. The cells were then incubated at 37°C in a shaking incubator for 1 hour. LB agar plates containing 100µg/ml carbenicillin and pre-coated with 40µl of a 40mg/ml X-gal solution in dimethylformamide to allow blue/white screening of colonies, were pre-warmed to 37°C. Between 20µl and 100µl of transformed bacteria were spread per plate and the plates were then incubated overnight at 37°C to allow colony formation. A successfully ligated PCR sequence was indicated by the presence of a white colony due to disruption of the *LacZα* gene in the pCR 2.1-TOPO vector. Colonies without an insertion were blue. To screen individual bacterial colonies for the presence of PCR inserts, white colonies were picked and expanded in wells of a 96-well plate containing 200µl of LB broth and incubated at 37°C overnight. Products for sequencing were then obtained by PCR amplification, using 2µl of the cultured broth as a template.

CHAPTER 3: SCREENING FOR MUTATIONS IN THE *NOTCH1* AND *FBXW7* GENES

3.1 Introduction

Early genetic studies investigating *Drosophila melanogaster* that harboured characteristic notches on the ends of their wings led to the cloning and subsequent identification in the 1980s of the Notch receptor gene (Wharton *et al.*, 1985). Since then, numerous studies have explored the role of Notch signalling in various cellular process including apoptosis, proliferation and differentiation. In mammalian embryos, Notch receptors and ligands are expressed during organogenesis and play a role in the development of a wide variety of tissues derived from the endoderm (pancreas [(Lammert *et al.*, 2000)]), the mesoderm (haematopoietic system [(Milner & Bigas, 1999)]) and the ectoderm (nervous system [(Yoon & Gaiano, 2005)]). In developmental systems, it has been shown that Notch-controlled interactions between cells play a role in the regulation of the cell-fate decisions of various multipotent precursors (Artavanis-Tsakonas *et al.*, 1999). Notch regulates lineage specification at developmental decision points. An example of this is during peripheral neurogenesis in the fly, where an equipotent precursor can give rise to one of two alternative cell fates depending on whether it expresses Notch or its ligand. The outcome of Notch signalling is diverse and is likely to be dependent on factors such as signal strength and specific developmental environment (Pear & Radtke, 2003). Studies have shown that Notch receptors and their ligands are expressed on haematopoietic cells, and that activation of the signalling pathway plays an important role in the lineage decisions at various stages of T-cell development (Ogawa, 1993).

3.1.1 NOTCH1 structure

In mammals, the Notch family consists of four Notch receptors, NOTCH1-4. The *NOTCH1* gene, found on human chromosome 9q34.3, has 34 exons and encodes a type 1 single-pass transmembrane protein that binds to ligands belonging to either the Delta-like or the Jagged/Serrate-like family of ligands (Delta-like ligands 1, 3 or 4, Jagged1 or Jagged2). The extracellular portion of the NOTCH1 receptor consists of 36 N-terminal epidermal growth factor (EGF)-like repeats which mediate ligand binding, and a negative regulatory region comprised of three Lin-12/Notch repeats A-C (LNR) which are responsible for maintaining the

receptor in an inactive state (Figure 3.1). The heterodimerisation (HD) domain is responsible for stable subunit association and comprises a 103 amino acid N-terminal extracellular region (HD-N) and a 65 amino acid C-terminal transmembrane region (HD-C). The intracellular portion of NOTCH1 consists of the juxtamembrane (JME) domain, a RAM (RBP-Jκ associated molecule) domain and 7 ankyrin repeats which mediate interaction with transcription factors. The C-terminal end consists of a proline- and glutamine-rich transcriptional activation (TAD) domain and a C-terminal PEST (proline-, glutamic acid-, serine- and threonine- rich) domain where the receptor is targeted for proteolytic degradation by the E3 ubiquitin ligase FBXW7 (Weng *et al.*, 2004).

Maturation and signalling through the NOTCH1 receptor involves three cleavage steps. The first is by a furin-like convertase, which cleaves the receptor just external to the transmembrane domain at site S1 during intracellular trafficking in the Golgi complex (Logeat *et al.*, 1998) (Figure 3.2). This leads to expression of the receptor subunits as a heterodimer on the membrane, where ligand binding occurs. The strength and affinity of the receptor-ligand interaction is regulated by a critical modification in which the ligand binding site in the EGF repeats is glycosylated by the protein O-fucosyltransferase (O-fut1) (Kopan & Ilagan, 2009;Stanley & Okajima, 2010). Ligand binding initiates two more proteolytic cleavages, the first by the ADAM-family metalloprotease TNFα converting enzyme (TACE). In the inactive state, the LNR domain is folded over the HD domain protecting the TACE cleavage site, and this protective conformation is only shifted upon ligand binding to the EGF repeats (Sanchez-Irizarry *et al.*, 2004). TACE cleaves the protein just outside the membrane at site S2, which is followed by a final cleavage by γ-secretase at site S3. This liberates the intracellular portion of NOTCH1 (ICN-1) to translocate to the nucleus, where it binds to members of the CSL family of transcription factors (Bray, 2006;Brou *et al.*, 2000;Struhl & Greenwald, 1999) (Figure 3.2). CSL binds motifs in promoter and enhancer regions, and in an inactivated state it represses its target genes (Tun *et al.*, 1994) and is bound to co-repressors including nuclear receptor co-repressor-2 (NCOR2, also known as SMRT) and co-repressor interacting with RBPJ-1 (CIR-1). When ICN-1 translocates to the nucleus and binds to CSL, the co-repressors are displaced and co-activators including Mastermind-like-1 (MAML-1) are recruited, leading to the transcription of downstream targets.

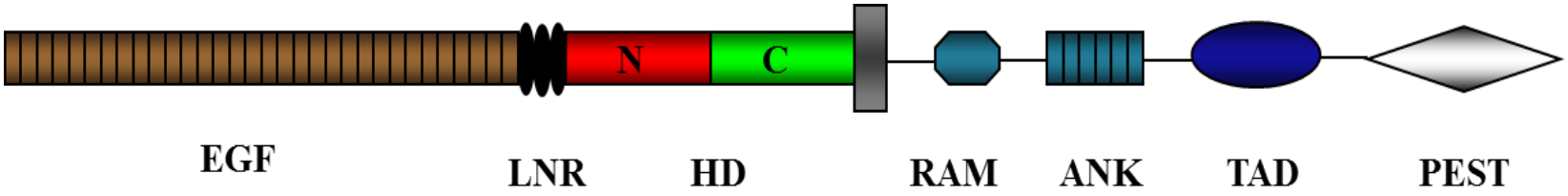

Figure 3.1 Functional domains of NOTCH1. The N-terminal extracellular region consists of 36 Epidermal Growth Factor (EGF) like repeats, the NOTCH1 Lin12/Notch repeats (LNR) domain and the heterodimerisation (HD) N and C-terminal domains. The intracellular region consists of the juxtamembrane (JME) domain, the RAM/ankyrin (ANK) repeats, and the C-terminal transcriptional activation (TAD) and PEST domains. Adapted from Weng *et al* (2004).

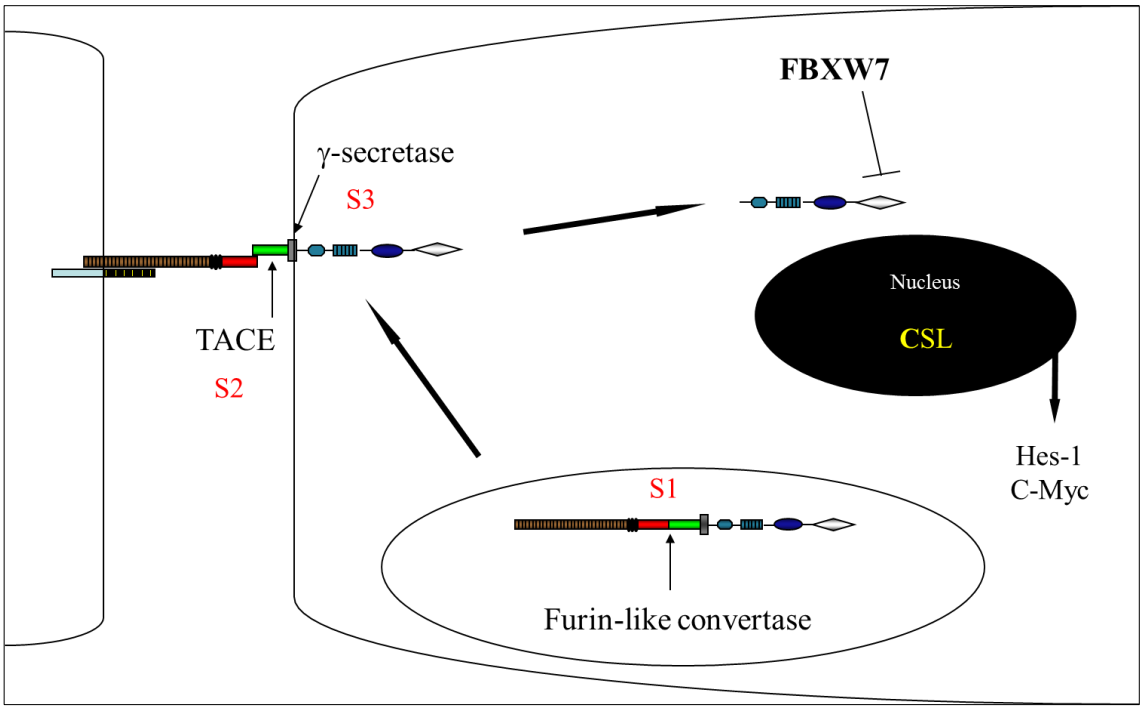

Figure 3.2 NOTCH1 signalling pathway. Signalling through the NOTCH1 receptor involves three cleavage steps. The first is by a furin-like convertase which cleaves the receptor just external to the transmembrane domain at site S1. This leads to expression of the receptor subunits as a heterodimer on the membrane, where ligand binding initiates two more proteolytic cleavages. First by TACE at site S2, and finally by γ-secretase at site S3, liberating the intracellular portion of NOTCH1 (ICN-1) to translocate to the nucleus where it binds to members of the CSL family of transcription factors**.**

3.1.2 NOTCH signalling and haematopoiesis

Haematopoietic stem cells give rise to common lymphoid progenitors (CLPs) which are directed from the bone marrow towards the thymus, where they differentiate through the double-negative $CD4^-CD8^-$ (DN) stage to become double positive (DP) cells expressing both CD4 and CD8, before entering the circulation as single positive (SP) $CD4^+$ or $CD8^+$ T-cells (Dadi *et al.*, 2009;Krangel, 2009) (Figure 3.3). When the CLPs enter the thymus the first lineage decision is made, T-versus B-cell fate (Zuniga-Pflucker, 2004). Signalling through the NOTCH1 receptor has been shown to be essential for the commitment of CLPs to the T-cell lineage. This was demonstrated in Notch-1 deficient mice that showed a block in T-cell development at the earliest stage, with CLPs adopting a B-cell fate by default (Radtke *et al.*, 1999). This was also shown in a complementary study where murine haematopoietic precursors were transduced with a constitutively activated ICN-1. In mice receiving the ICN-1 transduced cells, T-cell development occurred outside the thymus in the bone marrow (Aster & Pear, 2001;Izon *et al.*, 2002;Pear *et al.*, 1996;Pui *et al.*, 1999). Once the T-cell fate has been adopted, T-cells must subsequently commit to either the αβ or γδ lineage where they express either the αβ receptor, and become CD4 and CD8 cells, or the γδ receptor, which occurs in only a small subset of T-cells expressed in the epithelium and epidermal tissues. NOTCH1 signals have been shown to promote the development of αβ T-cells, which must first successfully rearrange the T-cell receptor (TCR) β gene. The β-chain is then expressed on the cell surface, and this leads to formation of the pre-TCR by association with the pTα chain (Washburn *et al.*, 1997). Thymocytes which do not successfully rearrange their β-chain genes do not pass the β-selection point and die by apoptosis. T-cells then go on to rearrange the α-chain and become single positive T-cells expressing either CD8 or CD4 (Robey *et al.*, 1996). NOTCH1 is downregulated after β-selection (Taghon *et al.*, 2006;Witt *et al.*, 2003).

Lineage commitment of T-cells is coordinated in the nucleus, where ICN-1 interacts with the transcriptional co-activator MAML. This leads to displacement of the co-repressor complex made up of SMRT and CIR-1 from its association with the DNA-binding protein CSL, and induction of the transcriptional activation of T-cell lineage-specific NOTCH1 downstream targets. One of these targets is the basic helix-loop-helix transcription factor Hes1. Studies in knockout mouse models have demonstrated the importance of Hes1 in the expansion of early T-cell progenitors (Tomita *et al.*, 1999), with this need diminishing in the later stages of T-cell development (Kaneta *et al.*, 2000). In 2006, the basic region-helix-loop-helix-leucine zipper

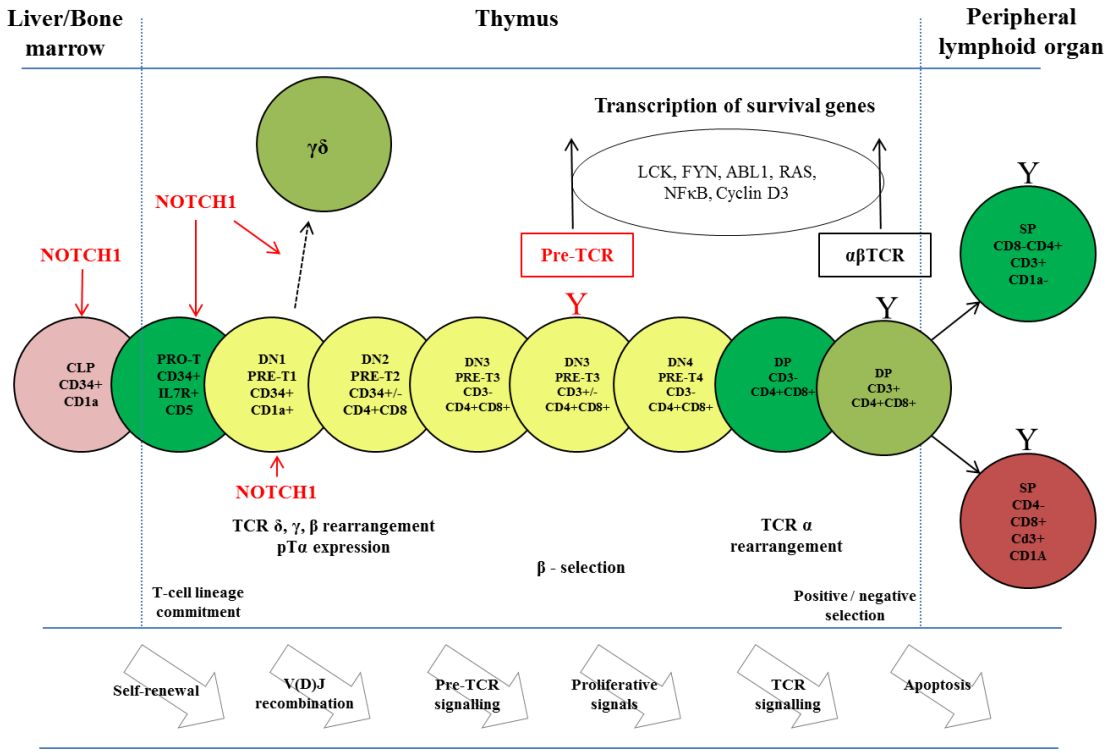

Figure 3.3 Stages of T-cell development. Haematopoietic stem cells give rise to common lymphoid progenitors (CLPs), which are directed from the bone marrow towards the thymus where they differentiate through the double-negative (DN) stage to become double positive (DP) cells expressing both CD4 and CD8, before entering the circulation as single positive (SP) $CD4^+$ or $CD8^+$ T-cells. Red arrows indicate the stages where NOTCH1 is required.

protein c-Myc was identified as a direct NOTCH1 target (Palomero *et al.*, 2006;Sharma *et al.*, 2007;Weng *et al.*, 2006). Studies have shown that at the β-selection checkpoint during T-cell development, Notch-dependent c-Myc transcription in DN3 cells promotes the proliferation of T-cell progenitors that successfully rearrange their TCRβ chain and produce a pre-TCR (Liu *et al.*, 2010;Weng *et al.*, 2006). It has also been suggested that c-Myc coordinates signals for cell survival, growth and proliferation of post β-selection thymocytes as they develop to DP cells.

Another critical role of NOTCH1 signalling in haematopoiesis is in driving cell cycle progression by regulating genes which are involved in the G1 to S-phase transition (Liu *et al.*, 2011). NOTCH1 directly activates the transcription of cyclin D1 and induces CDK2 activity, promoting the entry of cells into S-phase (Ronchini & Capobianco, 2001). Cyclin D3 has also been identified as a NOTCH1 target (Joshi *et al.*, 2009). During T-cell development, NOTCH1 signalling activates the PI3K/AKT pathway (Palomero *et al.*, 2008). It is not clear how this is done, but possible mechanisms have been hypothesised. The first is by the expression of the pTα gene, which is a direct target of NOTCH1; the human pTα contains at least one conserved DNA binding site for CBF-1 (Deftos & Bevan, 2000;Fehling *et al.*, 1995;Reizis & Leder, 2002). This results in the assembly of the pre-TCR and AKT then becomes activated via pre-TCR signalling (Sade *et al.*, 2004). The second mechanism is through HES1, which represses phosphatase and tensin homolog (PTEN), a negative regulator of the PI3K/AKT pathway (Palomero *et al.*, 2007). It has been shown that activation of this pathway can substitute for NOTCH1 signalling at the β-selection checkpoint (Ciofani & Zuniga-Pflucker, 2005).

3.1.3 Oncogenic NOTCH1 signalling in haematopoietic malignancies

NOTCH1 signalling is essential for normal T-cell development, but aberrant signalling has been linked to various human cancers. The first evidence implicating the NOTCH1 signalling pathway in leukaemogenesis came in 1991 from the identification of a rare t(7;9)(q34;q34.3) translocation in less than 1% of paediatric T-ALL cases, juxtaposing the TCRβ promoter with the intracellular portion of the *NOTCH1* receptor gene. The translocation led to the expression of a constitutively activated truncated NOTCH1 protein, formerly known as translocation-associated NOTCH1 (TAN1) (Ellisen *et al.*, 1991). Further studies in murine models showed that the forced expression of a similar constitutively active truncated form of NOTCH1 induced a highly aggressive form of T-ALL (Aster *et al.*, 1994;Pear *et al.*, 1996). It was also demonstrated that sustained NOTCH1 signalling was required for the growth in vitro of murine

T-ALL cell lines (Weng *et al.*, 2003). Further evidence implicating NOTCH1 in the pathogenesis of T-ALL came when deletions of either the 5' or the 3' domain of NOTCH1 were identified in over half of radiation-induced murine T-cell thymic lymphomas (Tsuji *et al.*, 2003).

3.1.4 Activating mutations in the *NOTCH1* gene

From the growing evidence of the role of NOTCH1 in the pathogenesis of T-ALL, Weng *et al* (2003) tested human T-ALL cell lines lacking the t(7;9)(q34;q34.3) translocation for dependency on NOTCH1. This was done by treating the cells with a γ-secretase inhibitor (GSI) which would block the final cleavage of the NOTCH1 receptor and thereby inhibit translocation of ICN-1 to the nucleus. Thirty cell lines were tested and, of these, five showed a G0/G1 cell cycle arrest which was reversed by retroviral expression of ICN-1 (Weng *et al.*, 2003), suggesting that the growth of these cell lines was NOTCH1-dependent. The HD and PEST domains of the NOTCH1-dependent cell lines were then sequenced, revealing that four of the five harboured mutations in these regions. The mutations found in the HD domain were missense mutations and the PEST mutations were short frameshift insertions and deletions predicted to result in deletion of the C-terminal domain. One of the cell lines harboured mutations in both the HD and PEST domains, which were found to be in cis on the same allele of *NOTCH1* by sequencing of cDNA. The cell lines which were insensitive to the GSI were subsequently sequenced and nine of the 19 were *NOTCH1* mutant-positive.

This led to the same group investigating the *NOTCH1* HD and PEST mutational status of a cohort of 96 paediatric T-ALL patients. They demonstrated mutations in 56% of the patients. Mutations in both the HD-N and HD-C domain were short in-frame size changes or missense mutations, and the PEST domain mutations were frameshift size changes or point mutations resulting in premature stop codons. Of the 96 patients, 17 (18%) had dual HD and PEST mutations. This mutation incidence has since been confirmed in several other studies (Table 3.1). Mutations tend to cluster in the HD and PEST domains, but have also been reported at a lower frequency in the TAD, LNR and JME domains. Overall, it has been found that mutations occur at a frequency of between 22-57% in paediatric and 42-62% in adult T-ALL patients. Putting the paediatric studies together, of 1115 cases examined, 548 (49%) had at least one mutation in *NOTCH1*. The most commonly mutated region across the studies was the HD

domain, with 429 (38%) patients harbouring a mutation in either exon 26 or 27. Of these, 105 (9%) patients also had a co-incident PEST mutation (Table 3.1).

3.1.5 Functional consequences of *NOTCH1* mutations

To investigate the functional consequence of the mutations identified, Weng *et al* (2004) carried out reporter gene assays in human U20S cells, known to have a low level of NOTCH1 expression, transfected with either full-length *NOTCH1* plasmids or those bearing various deletions and mutations, and a NOTCH1-sensitive luciferase reporter gene, *CBF1*. Missense mutations in the HD domain resulted in a 3-9 fold increase in luciferase activity, and deletion in the PEST domain resulted in a 1.5-2 fold increase. There was a striking 20-40 fold increase for plasmids harbouring both an HD and a PEST domain mutation in cis, indicating that the two mutations lead to synergistic NOTCH1 activation, a finding that has also been shown by others (Chiang *et al.*, 2008). Together with crystallography data, these studies have given insights into the mechanisms of pathway activation by *NOTCH1* mutations (Gordon *et al.*, 2009).

Mutations in the HD domain are classified into two distinct groups that were established in a study by Malecki *et al* (2006). They confirmed the activating potential of 15 representative HD point mutations and an insertion by introducing them into a full length NOTCH1 cDNA which was then transiently expressed in U20S cells. Of these mutations, all except one resulted in the increased activation of a NOTCH1–sensitive luciferase reporter gene containing CSL binding sites as compared to the NOTCH1–WT. Furthermore, they demonstrated that HD mutations were capable of activating NOTCH1 signalling independently of ligand using a NOTCH1 ΔEGF construct lacking the N-terminal EGF repeats, where the same level of activation as before was seen in the mutants. The effect of the HD mutants on NOTCH1 heterodimer stability was then investigated when authors introduced the mutations into a soluble polypeptide consisting of the LNR and HD domains. The HD mutations correlated with an increased sensitivity to cleavage at site S2 and when transiently expressed in mammalian cells, only the WT polypeptide underwent furin processing and was secreted into conditioned media as a heterodimer with the LNR-HD domains intact. Using all this data, HD mutants were then assigned to two distinct classes (Malecki *et al.*, 2006). Class I mutations are missense point mutations or small in-frame size changes that enhance subunit dissociation and destabilise the NOTCH1 heterodimers, leading to exposure of the metalloprotease cleavage site. Common class I mutations include leucine to proline point mutations, where the introduction of proline

Table 3.1 Studies of *NOTCH1* mutation detection in patients with T-ALL

Reference	Age group	Screening method	Exons screened	Total in study	Total *NOTCH1* mutant patients (%)	Type of mutations and number of mutated patients
Weng *et al*., (2004)	Paediatric	dHPLC	26, 27, 34	96	54 (57%)	25 (26%) missense or in-frame insertions/deletions in HD domain 6 (13%) missense or frame-shift insertions/deletions in PEST domain 6 (18%) mutations in both HD and PEST domains
(Breit *et al.*, 2006)	Paediatric	Sequencing	26, 27, 34	157	82 (52%)	55 (35%) missense or in-frame insertions/deletions in HD domain: of these 84% were in the HD-N, 16% were in the HD-C 13 (8%) missense /frame-shift insertions/deletions in PEST domain 14 (9%) mutations in both HD and PEST domains
(van Grotel *et al.*, 2008)	Paediatric	dHPLC	26, 27, 34	70	40 (57%)	28 (40%) missense or in-frame insertions/deletions in HD domain 6 (15%) missense or frame-shift insertions/deletions in PEST domain 6 (15%) mutations in both HD and PEST domains
(Park *et al.*, 2009)	Paediatric	dHPLC	26, 27, 34	69	24 (35%)	16 (23%) mutations in HD domain$^{\Delta}$ 8 (12%) mutations in PEST domain
(Zuurbier *et al.*, 2010)	Paediatric	Sequencing	25-34	141	79 (56%)	47 (33%) missense or in-frame insertions/deletions in HD domain 17 (12%) nonsense or missense mutations in PEST domain 5 (4%) in-frame insertions in JME domain 9 (6%) mutations in both HD and PEST domains 1 (1%) mutations in both HD and JME domains
(Clappier *et al.*, 2010)	Paediatric	Sequencing + Fragment Analysis	26-28, 34	134	77 (57%)	45 (33%) missense or in-frame insertions/deletions in HD domain 17 (13%) frame-shift insertions/deletions in PEST domain 15 (11%) mutations in both HD and PEST domains
(Kox *et al.*, 2010)*	Paediatric	Sequencing	26, 27, 34	301	150 (50%)	94 (31%) mutations in HD domain$^{\Delta}$ 29 (10%) mutations in PEST domain 27 (9%) mutations in both HD and PEST domains
(Erbilgin *et al.*, 2010)	Paediatric	dHPLC	26, 27, 34	87	19 (22%)	11 (12%) missense or in-frame insertions in HD domain: of these 73% were in the HD-N, 27% were in the HD-C 6 (7%) missense or frame-shift insertions/deletions in PEST domain 2 (3%) mutations in both HD and PEST domains

Table 3.1 Continued

Reference	Age group	Screening method	Exons screened	Total in study	Total *NOTCH1* mutant patients (%)	Type of mutations and number of mutated patients
(Mansur *et al.*, 2012)	Paediatric	Sequencing	26, 27, 34	138	60 (43%)	42 (30%) missense or in-frame insertions/deletions in HD domain 11 (8%) nonsense or frame-shift insertions/deletions in PEST domain 7 (5%) mutations in both HD and PEST domains
Fogelstrand *et al*., (2014)	Paediatric	Sequencing	26-28, 34	79	45 (57%)	29 (37%) missense or in-frame insertions/deletions in HD domain 8 (10%) missense or frame-shift insertions/deletions in PEST domain 8 (10%) mutations in both HD and PEST domains
(Zhu *et al.*, 2006)	Paediatric and adult	Sequencing	26, 27, 34	77 (53 paediatric 24 adult)	32 (42%)	23 (30%) missense or in-frame insertions/deletions in HD domain: of these 70% were in the HD-N, 30% were in the HD-C 5 (7%) missense or frameshift insertions/deletions in PEST domain 3 (4%) had missense or frameshift insertions/deletions in TAD domain 1 (1%) missense mutation in ANK domain
(Asnafi *et al.*, 2009)	Adult	Sequencing	26-28, 34	141	88 (62%)	59 (42%) missense or in-frame insertions/deletions in HD domain 9 (6%) missense or frameshift insertions/deletions in PEST domain 16 (10%) mutations in both HD and PEST domains 1 (2%) mutation in both HD and TAD domains 3 (2%) mutations in JME domain
(Mansour *et al.*, 2009)	Adult	dHPLC	26-28, 34	88	53 (60%)	36 (41%) missense or in-frame insertions/deletions in HD domain 8 (9%) nonsense or missense mutations PEST domain 3 (3%) mutations in JME domain 6 (7%) mutations in both HD and PEST domains

Abbreviations: dHPLC, denaturing high performance liquid chromatography; HD, heterodimerisation; JME, juxtamembrane; TAD, transcriptional activation; ANK, ankyrin repeats.
* extended cohort of original study by Breit *et al* (2006).
Δ details of the type of mutation not available.

residues into the protein are thought to destabilise the structure (Weng *et al.*, 2004). Class II mutations are larger in-frame insertions which either duplicate the S2 site, identified as residues A1710-V1711 (Mumm *et al.*, 2000;Brou *et al.*, 2000), or are adjacent to and enhance access of TACE to the cleavage site. This class of mutation was found to be the most potent activating mutation in studies where a 14 amino acid insertion duplicating the S2 cleavage site resulted in the highest level of activation in reporter gene assays (Chiang *et al.*, 2008) (Malecki *et al.*, 2006). This mutation also resulted in the onset of leukaemia in murine models, whereas the L1593P and L1600P mutants were only able to accelerate leukaemia induced by constitutive K-ras signalling (Chiang *et al.*, 2008). Disruption of the HD domain leads to ligand hypersensitivity or ligand-independent NOTCH1 activation (Sanchez-Irizarry *et al.*, 2004).

Mutations reported in the C-terminal PEST domain generally truncate the protein, resulting in increased levels of activated ICN-1 due to impaired degradation by the proteasome (Weng *et al.*, 2004;Gupta-Rossi *et al.*, 2001). Studies have shown that these mutations leave FBXW7 unable to bind and therefore ICN-1 is stabilised in the nucleus (Chiang *et al.*, 2008;Thompson *et al.*, 2007). Approximately 20% of patients have mutations in both the HD and PEST domains, which, as previously mentioned, leads to synergistic NOTCH1 activation (Weng *et al.*, 2004;Malecki *et al.*, 2006).

Another group of mutations have recently been identified in the NOTCH1 JME domain, usually large insertions which are thought to be functionally similar to HD domain mutations. Sulis *et al* (2008) showed that the JME domain mutations are internal tandem duplications of the exon and adjacent intronic sequences. Functional studies on these mutations analysed the levels of activated ICN-1 in cells transfected with the pcDNA3 *NOTCH1 Jurkat JME17* plasmid containing only exons 19 to 29 of *NOTCH1*and an internal tandem duplication of 51 bases within exon 28. The results demonstrated that expression of these mutant alleles caused a 2000-fold activation of the NOTCH1 reporter compared with controls. The JME mutations found in T-ALL patient samples all coded for a NOTCH1 receptor with an intact but significantly expanded extracellular JME region, which resulted in the S2 cleavage site and HD/LNR complex being displaced away from the membrane, thus making the S2 site more accessible to metalloprotease cleavage. The JME insertion mutants all contained a QLHF sequence, which was initially hypothesised to be an alternate S2-like site to increase signal activation. However, upon testing of mutants with insertions of 5, 8, 11, 12, 13 and 14 amino acids, the activation was only moderately increased (1.5–12 fold) in the mutants with 5, 8 or 11 amino acid insertions but

increased 700-2000 fold in mutants with 12 or 14 amino acid insertions. This suggests that the activation of NOTCH1 in JME mutants is dependent on the length of the space inserted between the HD-LNR complex, and is independent of the specific amino acid sequence the mutation introduces (Sulis *et al.*, 2008). Mutations in the LNR domain are uncommon, but may lead to the activation of NOTCH1 cleavage by selectively releasing the inhibitory effect of the LNR repeats on the S2 cleavage site (Sulis *et al.*, 2008).

Aberrant NOTCH1 signalling has also been implicated in other cancers, including solid tumours where activating mutations have been found in non-small cell lung carcimoma (NSCLC) (Westhoff *et al.*, 2009). Two studies have also recently identified *NOTCH1* activating mutations in chronic lymphocytic leukaemia (CLL) (Fabbri *et al.*, 2011;Puente *et al.*, 2011). In both NSCLC and CLL, the mutations reported were in the PEST domain and correlated with reduced patient survival. Loss of function *NOTCH1* mutations have also been reported in head and neck squamous cell carcinoma (HNSCC), suggesting a tumour-suppressive role of NOTCH1 in some cancers (Agrawal *et al.*, 2011;Stransky *et al.*, 2011). The mutations found were missense substitutions and mostly within the EGF-like repeats, resulting in inefficient ligand binding. A role for NOTCH signalling has also been implicated in multiple myeloma, where overexpression of both the NOTCH1 and NOTCH2 receptors and the Jagged-1 and Jagged-2 ligands has been reported in primary samples (Houde *et al.*, 2004;Jundt *et al.*, 2004).

3.1.6 Other mechanisms of NOTCH1 activation

Studies in mice have suggested that it is likely that other mechanisms of ligand-independent NOTCH1 activation remain to be discovered in human T-ALL, despite initially seeming to refute the theory that HD domain mutations are required for leukaemogeneic NOTCH1 signalling. This is owing to the fact that the most common *NOTCH1* mutations in human T-ALL are clustered in the HD domain, whereas in murine T-ALL, HD mutations are rare, and the most commonly mutated region is the PEST domain (Aster *et al.*, 2008). The PEST mutations found in murine T-ALL commonly arise in transgenic mice with genetic backgrounds that predispose to tumourigenesis such as p53, E2A or Ikaros deficiency or constitutive K-ras signalling (O'Neil *et al.*, 2006;Reschly *et al.*, 2006;Dumortier *et al.*, 2006;Chiang *et al.*, 2008) . HD mutations are present in only a small proportion of these tumours, leaving it unclear how NOTCH1 becomes activated. However, investigations using retroviral mutagenesis had shown that the *NOTCH1* gene was an insertion site when the Murine Leukaemia Virus (MuLV) was

used to accelerate leukaemia onset in *myc* transgenic mice (Hoemann *et al.*, 2000). Many of these insertions occurred in close proximity to the intronic breakpoint described in the t(7;9) translocation (Girard *et al.*, 1996), and integrated in both the HD and LNR domains of *NOTCH1* leading to increased expression of ICN-1. The characterisation of radiation-induced thymic lymphomas in ataxia telangiectasia mutated knock-out (*Atm* $^{-/-}$) mice identified deletion breakpoints at the 5' end of the *NOTCH1* gene, with the tumours generating short NOTCH1 transcripts through errors in V(D)J recombination (Tsuji *et al.*, 2004;Tsuji *et al.*, 2009). Subsequent screening of a panel of murine T-ALL cell lines showed that the majority harboured 5' region deletions in the *NOTCH1* gene (Ashworth *et al.*, 2010;Jeannet *et al.*, 2010). The deletions either encompassed exon 1 and the proximal promoter and were RAG-dependent, or removed the region between exon 1 and exons 26-28 and were RAG-independent. The more frequent RAG-dependent deletions activate an internal promoter which lies in or near exon 25, driving expression of NOTCH1 transcripts with 5' deletions. Both types of deletion result in the initiation of translation at a conserved methionine residue in the transmembrane domain, leading to the production of truncated, activated NOTCH1 protein (Ashworth *et al.*, 2010). Ikaros loss can also lead to increased transcription of *NOTCH1* alleles with 5' deletions. The induction of T-ALL was markedly shortened when Ikaros-deficient mice were crossed with a conditional mouse model in which the 5' end of *NOTCH1* was deleted upon expression of T-cell specific Cre recombinase (Gomez-del Arco *et al.*, 2010;Jeannet *et al.*, 2010). The deletion also activated the same internal promoter in or near exon 25 as in the RAG-dependent deletions (Ashworth *et al.*, 2010). This suggests that Ikaros and possibly other proteins act as tumour suppressors. Whether these mechanisms are features of human T-ALL is yet to be determined.

3.1.7 NOTCH1 activated signalling pathways in T-ALL

Various groups have identified some of the downstream targets of the NOTCH1-CSL activation complex in T-ALL cells (Wang *et al.*, 2011a;Palomero *et al.*, 2006;Weng *et al.*, 2006), and some of these are also regulated by NOTCH1 in normal thymocytes. In the context of the transformation of T-cell precursors, the NOTCH1 signalling pathway supports cell growth. The best characterised targets include the bHLH transcriptional repressor HES1 and the transcription factor c-Myc (Palomero *et al.*, 2006;Weng *et al.*, 2004), and key pathways activated include the PI3K/AKT (Palomero *et al.*, 2007;Ciofani & Zuniga-Pflucker, 2005;Sade *et al.*, 2004) and mTOR pathways (Chan *et al.*, 2007;Cullion *et al.*, 2009). Conditional loss-of-function studies in mice and lentiviral knockdown in human T-ALL cell lines have demonstrated that NOTCH1-

induced T-ALLs are HES1-dependent (Wendorff *et al.*, 2010). The role of HES1 in the pathogenesis of the disease remains unclear, although it has been shown to bind to and repress the *PTEN* promoter, suggesting that aberrant NOTCH1 signalling could upregulate signalling through the PI3K/AKT pathway (Palomero *et al.*, 2007). Normal thymocytes express c-Myc, which is a consistently expressed downstream target of ICN-1 in human and mouse T-ALL lines, and expression directly correlates with NOTCH1 signal strength and peaks during β-selection. Inhibition of NOTCH1 signalling with GSI treatment in these cell lines resulted in the rapid downregulation of c-Myc and retroviral expression of c-Myc alone rescued some of them from NOTCH1 inhibition, suggesting that NOTCH1 dependency is mediated through c-Myc in these cell lines (Weng *et al.*, 2006;Chan *et al.*, 2007;Sharma *et al.*, 2007). Weng *et al* (2006) transduced murine marrow with a c-Myc transgene in which expression could be turned off in the presence of doxycycline and demonstrated, that upon doxycycline administration, the cells proliferated rapidly and subsequently underwent apoptosis. However, upon retroviral transduction with ICN-1, an increase in endogenous c-Myc expression was seen along with the inhibition of cell death. This suggests that NOTCH1-mediated c-Myc expression is crucial for the maintenance of T-ALL.

More recently, other direct NOTCH1 signalling targets which activate the PI3K/AKT pathway have been implicated in T-ALL. The IL7-Rα is transcriptionally regulated directly by NOTCH1 and is required for normal lymphoid development. Activating mutations have been described in approximately 10% of samples from patients with T-ALL, leading to ligand-independent IL7-R homodimerisation and activation of STAT5 and JAK1, promoting cell transformation and tumour formation (Shochat *et al.*, 2011;Zenatti *et al.*, 2011). The insulin growth factor receptor-1 (IGF1R) is also a NOTCH1 target, and NOTCH1 signalling is required to maintain IGF1R expression at high levels in T-ALL cells (Medyouf *et al.*, 2011;Wang *et al.*, 2011a). Pharmacologic inhibition or genetic deletion of the IGF1R was found to block the growth and viability of T-ALL cells, and a reduction of IGF1R signalling decreased the transplantability of the tumours in secondary recipients, thereby compromising leukaemia initiating cell (LIC) activity (Medyouf *et al.*, 2011).

3.1.8 F-box and WD40 domain-containing protein 7 (FBXW7)

Studies elucidating the mechanism of the proteasome-dependent degradation of ICN-1 in *Caenorhabditis elegans* identified FBXW7 in this process, as it targets the NOTCH1 PEST

domain for ubiquitination and subsequent degradation by the proteasome (Hubbard *et al.*, 1997). The *FBXW7* gene is located on human chromosome 4q31.3, has 12 exons and encodes an E3-ubiquitin ligase which is abundantly expressed in haematopoietic cells (Mao *et al.*, 2004). F-box and WD40 domain containing protein 7 (FBXW7) is a constituent of the highly conserved SCF complex comprised of SKP1, CUL1, RBX1 and FBXW7 (Figure 3.4). The F-box protein of FBXW7 is approximately 40 amino acids long and binds to the protein targeted for ubiquitination independently of the complex, by interacting with the bridging protein SKP1 which is anchored to the scaffold structure CUL1 (Nakayama & Nakayama, 2005). This allows the protein to be brought into proximity of the functional E2-ubiquitin which is bound to the RBX1 zinc-binding domain, and results in transfer of the ubiquitin to a lysine residue on the target protein. FBXW7 also contains eight WD40 repeats which are short structural motifs that typically fold together to form a type of beta propeller structure, providing a rigid scaffold for interaction with various proteins (Orlicky *et al.*, 2003;Li & Roberts, 2001;Smith *et al.*, 1999). FBXW7 forms homodimers through a domain just upstream of its F-box domain, but the requirement for this is unclear as monomeric forms still bind to most substrates (Welcker & Clurman, 2008). The SCF complex covalently attaches ubiquitin groups to proteins that contain the consensus sequence I/L-I/L/P-T-P-XXXX (Welcker & Clurman, 2008). This consensus sequence is known as a phosphodegron as a conserved residue in the substrate must first be phosphorylated to allow the complex to bind. In the NOTCH1 PEST domain, this has been identified as T2512, which is phosphorylated by GSKβ and CDK8 (Foltz *et al.*, 2002;Fryer *et al.*, 2004).

Cyclin E was the first identified target of FBXW7, although its importance in T-ALL remains unclear (Hao *et al.*, 2007;Koepp *et al.*, 2001;Orlicky *et al.*, 2003). Other substrates of FBXW7 include c-Myc, mTOR and c-Jun (Mao *et al.*, 2008;Koepp *et al.*, 2001;Wei *et al.*, 2005;Welcker *et al.*, 2004). As previously mentioned, c-Myc is a direct downstream target of NOTCH1, and FBXW7 further regulates NOTCH1 signalling by degrading c-Myc (Palomero *et al.*, 2006). The oncogenic potential of this pathway was demonstrated in an *fbxw7* conditional knockout mouse model, which led to the mice developing thymic lymphomas with high expression of c-Myc (Onoyama *et al.*, 2007). Mao *et al* (2008) carried out a genome-wide search for FBXW7 targets in human breast cancer cell lines and identified the enzyme mTOR as one of these. The cell lines and primary tumours showed that loss of FBXW7 correlated with deletion of *PTEN*, which also activates mTOR.

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Figure 3.4 Structure of the SCF complex. (A). Overall architecture of the SCF complex where the F-box protein binds to the target protein then interacts with SKP1. (B). Ribbon diagram showing the eight WD40 repeats fold together to form a β propeller structure where target proteins bind. Taken from Hao *et al* (2007) and Magori *et al (*2011).

FBXW7 gene mutations and deletions have been reported in various types of human cancer. In a study using a triple knockout model ($TP53^{-/-}$, $Terc^{-/-}$ and $ATM^{-/-}$) where mice developed thymic lymphomas, analysis of the tumours demonstrated *FBXW7* deletions encompassing the whole gene (Maser *et al.*, 2007). The same group then sequenced a cohort of 23 human T-ALL cell lines and 38 clinical samples and found that 48% of the cell lines and 29% of the clinical samples were deleted or mutated in *FBXW7* (Maser *et al.*, 2007). Deletions of the *FBXW7* gene have not been widely reported, although a recent study identified 14% of T-ALL patients harboured a deletion (Rudner *et al.*, 2011). The mutations found in the study in human *FBXW7* were predominantly missense mutations and clustered in the arginine residues of the WD40 domains, which are required for substrate interaction (Orlicky *et al.*, 2003). The most common mutations observed were those involving R465 located in exon 9, and R479 in exon 10, and sequencing of matched bone marrow samples from patients in remission showed that these mutations were acquired somatically (Maser *et al.*, 2007). Since then a number of paediatric and adult studies have extended these findings and these are detailed in Table 3.2. In paediatric T-ALL, the incidence of *FBXW7* mutations was 16% (range 10% to 31%). The majority of the mutations were located in the mutational hotspot exons 9 and 10, and three studies screened these exons alone (Clappier *et al.*, 2010;Kox *et al.*, 2010;Mansur *et al.*, 2012).

FBXW7 mutants have been shown to be unable to bind to the NOTCH1 PEST domain or to c-Myc, therefore stabilising ICN-1 and c-Myc in the nucleus. Recent studies have also shown that FBXW7 mutation can modulate leukaemia-initiating cell (LIC) activity (King *et al.*, 2013). The heterozygous mutation R465C mutation was found to abolish the capacity of Fbxw7 to ubiquitinate c-Myc, and the presence of the R465C mutation in $Fbxw7^{mut/+}$ Mx1-Cre mice did not compromise normal HSC function but led to an increase in the LIC population. This increase was as a result of the stabilisation of c-Myc, as the expression of c-Myc directly correlated with the LIC population in vivo. Studies have indicated that this stabilisation of c-Myc by *FBXW7* mutations may also confer GSI resistance in some T-ALL lines (O'Neil *et al.*, 2007).

Mutations in *FBXW7* are thought to mimic *NOTCH1* PEST mutations; therefore mutations in the *NOTCH1* PEST domain and *FBXW7* in the same patient are rare. This was demonstrated by sequence analysis of *NOTCH1* in T-ALL cell lines and primary T-ALL samples harbouring *FBXW7* mutations. None of the cell lines were mutated in the PEST domain whereas all of them either had an HD mutation or WT *NOTCH1* alleles (Thompson *et al.*, 2007). As with *NOTCH1*

Table 3.2 Studies of *FBXW7* mutation detection in patients with T-ALL

Reference	Age group	Screening method	Exons screened	Total in study	Total *FBXW7* mutant patients (%)	Type of mutations and number of patients with mutations
Malyukova *et al*., (2007)	Paediatric	PCR-SSCP	2-12	26	8 (31%)	All missense mutations; 3 in exon 9, 4 in exon 10 and 1 in exon 12 2 (25%) also had a *NOTCH1* mutation
(Park *et al.*, 2009)	Paediatric	Dhplc	2-12	69	11 (16%)	9 (12%) missense mutations in exons 9,10 and 12 1 31bp insertion in exon 9, 1 deletion in exon 12 and 1 missense mutation in exon 9 + indel in exon 3 5 (7%) also had a *NOTCH1* mutation
(Zuurbier *et al.*, 2010)	Paediatric	Sequencing	5, 7+11	141	23 (16%)	22 (15%) missense mutations: 1 in exon 7, 1 in exon 8 and 2 in exon 11, 18 in exons 9 and 10 1 nonsense mutation in exon 11 10 (7%) also had a *NOTCH1* mutation
(Clappier *et al.*, 2010)	Paediatric	Sequencing + Fragment Analysis	9+10	134	20 (15%)	19 (14%) missense mutations 1 short frameshift insertion introducing a premature stop codon 17 (13%) also had a *NOTCH1* mutation
(Kox *et al.*, 2010)	Paediatric	Sequencing	9+10	301	42 (14%)	42 (14%) mutations in exons 9 or 10
(Erbilgin *et al.*, 2010)	Paediatric	dHPLC	7-11	72	7 (10%)	5 (7%) missense mutations in exons 8, 9 and 10 1 insertion in exon 12 2 (3%) also had a *NOTCH1* mutation
(Mansur *et al.*, 2012)	Paediatric	Sequencing	9+10	110	21 (19%)	21 (19%) mutations in exon 9 or 10 13 (12%) also had a *NOTCH1* mutation
Fogelstrand *et al*., (2014)	Paediatric	Sequencing	9-14	79	17 (22%)	15 (18%) missense mutations: 9 in exon 9, 3 in exon 10 and 2 in exon 12, 1 in exons 9 +10 1 insertion in exon 12 and 1 deletion in exon 8 15 (7%) also had a *NOTCH1* mutation
O'Neil *et al*., (2007)	Adult	Sequencing	7-11	81	7 (9%)	All missense mutations in exons 9 and 10
Thompson *et al*., (2007)	Adult	Sequencing	9+10	95	15 (16%)	All missense mutations in exons 9 and 10

Table 3.2 Continued

Reference	Age group	Screening method	Exons screened	Total in study	Total *FBXW7* mutant patients (%)	Type of mutations and number of patients with mutations
(Asnafi *et al.*, 2009)	Adult	Sequencing	9, 10+12	141	34 (24%)	5 (7%) missense mutations in exons 9 and 10 21 (19%) also had a *NOTCH1* mutation
(Mansour *et al.*, 2009)	Adult	dHPLC	8-12	88	16 (18%)	16 (18%) missense mutations in exons 9 and 10 11 (13%) also had a *NOTCH1* mutation

Abbreviations: dHPLC, denaturing high performance liquid chromatography; SSCP, single strand conformation polymorphism; indel, insertion/deletion..

HD and PEST domain mutations, co-incident *NOTCH1* and *FBXW7* mutations are also thought to act in synergy to amplify signal strength. In reporter assays with a HD mutation coupled with a mutation disrupting FBXW7 binding, the loss of *NOTCH1*/*FBXW7* interaction produced a five-fold increase in NOTCH1 activity (Malyukova *et al.*, 2007;Thompson *et al.*, 2007). More recently, in vivo experiments where a truncated Notch1 construct constitutively cleaved in a ligand-independent manner, was retrovirally expressed on an $Fbxw7^{+/+}$, $Fbxw7^{\Delta/+}$ or $Fbxw7^{mut/+}$ background, showed that the mice harbouring the heterozygous mutation developed leukaemia at a much shorter latency to the WT mice or mice harbouring loss of one Fbxw7 allele. This indicates that *NOTCH1* and *FBXW7* mutations synergise to confer a greater leukaemia initiating capacity (King *et al.*, 2013).

3.1.9 Techniques used to detect *NOTCH1* and *FBXW7* mutations

There are two main approaches that different groups have used to screen for mutations in the *NOTCH1* and *FBXW7* genes, these are summarised in Tables 3.1 and 3.2. Sanger sequencing of PCR products, either directly or after cloning, has been the most commonly used technique. The main advantage of sequencing is that it can detect all types of mutation whether they are size changes, point mutations, heterozygous or homozygous. For a large number of samples this technique can, however, be expensive and labour-intensive. The sensitivity of nucleotide sequencing is also an issue as it can be easy to miss a low level somatic mutation. A number of studies have used the technique of denaturing high performance liquid chromatography (dHPLC) on the WAVE platform (Transgenomic Ltd, Glasgow, UK) for mutation detection, which relies on heteroduplex analysis (Erbilgin *et al.*, 2010;Park *et al.*, 2009;van Grotel *et al.*, 2008;Weng *et al.*, 2004). A previous study in our laboratory identifying *NOTCH1* and *FBXW7* mutations in an adult cohort of T-ALL patients had used the latter technique (Mansour *et al.*, 2009) and more details of the technique are given in section 2.1.6. There are a number of advantages to dHPLC; both size changes and point mutations can be detected with a higher sensitivity than sequencing and with a reasonably high throughput. Analysis of a single PCR product takes between 3 and 7 minutes depending on the DNA separation column used. There is little sample handling, and only samples with a chromatogram that is different to that of a known WT control sample need to be investigated by sequencing. dHPLC on the WAVE platform was therefore chosen to detect mutations in the *NOTCH1* and *FBXW7* genes in the studies presented in this thesis.

NOTCH1 mutations were first described in exons encoding the HD and PEST domains, i.e. exons 26, 27 and 34, and the majority of studies have only screened for mutations in these exons (Table 3.1). More recently, mutations have been described at a lower frequency in

exons 25 and 28 encoding the LNR and JME domains respectively, therefore in the present study patient samples were screened for mutations in exons 25-28 and 34 of *NOTCH1*. The reported mutational hotspots in the *FBXW7* gene are in exons 9 and 10 (Table 3.2). A lower frequency of mutations has been reported in other exons encoding the WD40 domains, exons 8, 11 and 12. It was therefore decided that the patient samples in this cohort would be screened for mutations in exons 8-12 of the *FBXW7* gene.

3.2 Materials and Methods

3.2.1 Patients and samples

Diagnostic samples were available from the United Kingdom Leukaemia and Lymphoma Research Childhood Leukaemia Cell Bank from 162 of the 388 (42%) T-ALL patients entered into the United Kingdom Medical Research Council ALL 2003 (UKALL 2003) trial between 2003 and 2011. Ethical approval for the trial was obtained from the Scottish Multi-Centre Research Ethics Committee. Informed consent was obtained in accordance with the Declaration of Helsinki. Samples were from peripheral blood or bone marrow and were received as frozen cells or genomic DNA. DNA was prepared using detergent lysis and chloroform extraction (section 2.1.3) or, if the number of cells was less than $1x10^6$, using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Crawley, UK), and dissolved in TE buffer.

3.2.2 *NOTCH1* mutation screening

NOTCH1 mutation screening of the LNR-B (exon 25), HD-N (exon 26), HD-C (exon 27), JME (exon 28), TAD and PEST (exon 34) domains was carried out by PCR and dHPLC, followed by sequencing of samples with abnormal chromatograms. Samples were amplified by 35 cycles of PCR using the primers and appropriate annealing temperature shown in Appendix Table 1. Wherever possible, PCR products were obtained using the proof-reading enzyme Optimase (Transgenomic). The standard Optimase DNA Polymerase reaction mix and cycling conditions are detailed in chapter 2 (section 2.1.4). Due to a high GC content of the HD-C, JME and TAD domains, 1mol/L betaine was also included in the reaction mix for these amplicons to enhance PCR efficiency. For the *NOTCH1* HD-N, PEST and LNR-B domains, where adequate PCR products could not be obtained using Optimase, the non-proof-reading enzyme BIOTAQ DNA polymerase (Bioline) was used. Details of the standard BIOTAQ DNA Polymerase reaction mix and cycling conditions are given in

section 2.1.4. PCR products were checked on a 2% agarose gel stained with ethidium bromide (section 2.1.5).

PCR products were screened by dHPLC on the WAVE DNA Fragment Analysis System (Transgenomic, Glasgow, UK) as detailed in section 2.1.6. They were denatured and cooled slowly to enable the formation of heteroduplexes, then analysed on the WAVE at optimal melting temperatures which were calculated using Transgenomic Navigator software (Appendix Table 1). Representative amplicon melting curves for three exons are given in Figure 3.5. Each WAVE run included a known wildtype (WT) case for each particular exon and, where possible, a known mutant control to allow for comparison.

3.2.3 Confirmation of common *NOTCH1* polymorphisms

A common synonymous SNP in the HD-C domain of *NOTCH1,* (C5094T, rs10521 MAF=0.417) interfered with mutation detection (Figure 3.6A). Therefore the presence of the SNP was investigated by PCR and restriction enzyme digestion and/or sequencing of all samples. For the PCR and digest, products were obtained with 35 cycles using the mismatch forward primer SNP-F (Appendix Table 1) and the screening reverse primer 27-R with the BIOTAQ enzyme (see section 2.1.4 for standard reaction mix and cycling conditions) with the addition of 1mol/L betaine. The mismatch primer introduced a BsaA1 digestion site that would distinguish between the polymorphic alleles, giving bands of 158 and 29bp for the C-alleles and a single uncut band of 187bp for the T-alleles. PCR products were digested for 3hrs at 37^{o}C with the restriction enzyme BsaA1 and analysed by agarose gel electrophoresis (Figure 3.6B). A second SNP in the HD-C domain (G5073A, rs61751538) and two SNPs in the TAD domain of *NOTCH1,* G6853A (rs61751490 MAF=0.006) and C6870T (rs61751488 MAF=0.004), were confirmed by direct sequencing.

3.2.4 Confirmation of *NOTCH1* mutations

For samples with abnormal chromatograms, fresh PCR products were obtained using the BIOTAQ enzyme with standard reaction mix and conditions and sequenced in one direction. Where a low-level mutant was evident from the WAVE chromatogram, direct sequencing was sometimes not sufficiently sensitive to characterise the nucleotide change. The fragment collector function of the WAVE was therefore used to purify the heteroduplex peaks at the analysis temperature at which the peak was most visible. Heteroduplex peaks were collected, re-amplified and sequenced. For more complex mutations, the exon was amplified and the products cloned using the TOPO TA cloning kit (Invitrogen, Paisley, UK) as described in

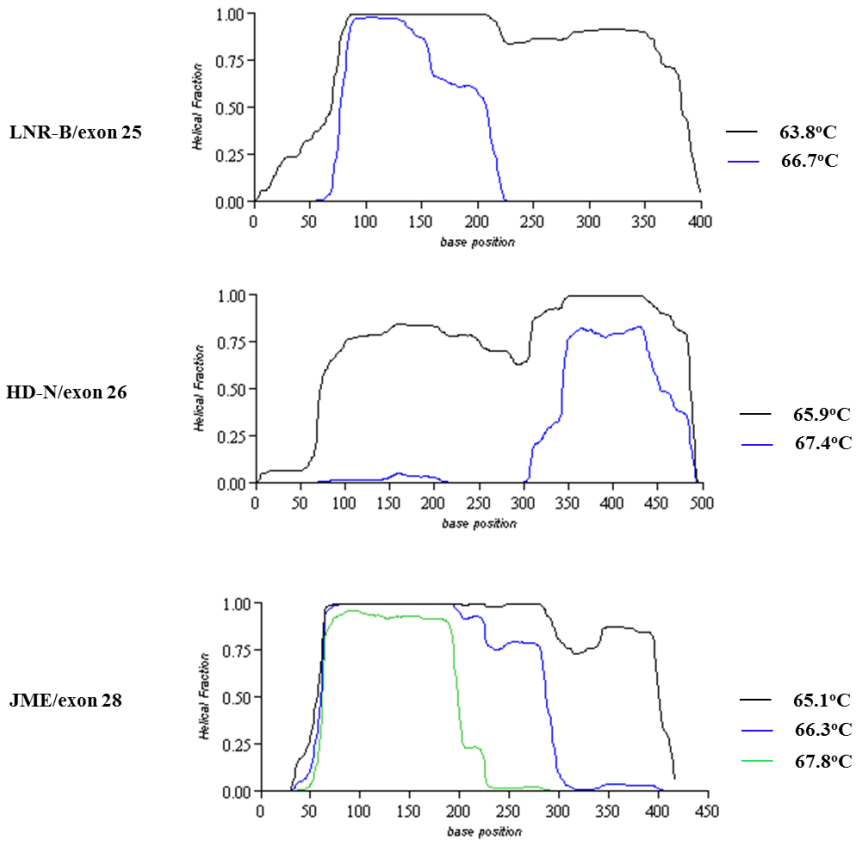

Figure 3.5 Amplicon melting curve profiles. Melting curve profiles for the LNR, HD-N and JME domains at the temperatures selected for WAVE analysis, displayed as the helical fraction against the base position in the PCR product.

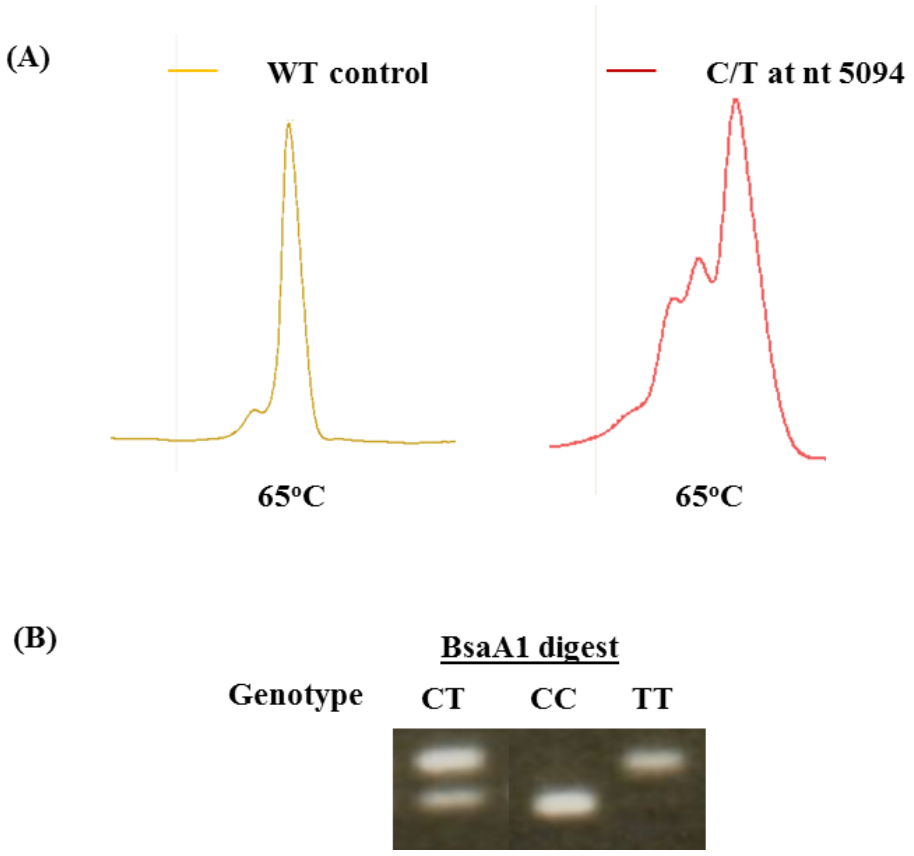

Figure 3.6 Identification and confirmation of a common *NOTCH1* polymorphism. (A). *NOTCH1* HD-C exon 27 WAVE chromatograms of a WT control and a case that is heterozygous (C/T) for the synonymous SNP at nucleotide (nt) 5094. (B). Genotyping of C/T alleles at nt 5094 by amplification with a mismatch primer designed to introduce a BsaA1 digestion site in C-alleles but not T-alleles. The result of the BsaA1 digest is shown as visualised on a 2% agarose gel stained with ethidium bromide.

section 2.1.10. For the JME-mutant positive samples, PCR products were size separated by gel electrophoresis using 1% agarose. Mutant bands were excised from the gel using the Qiagen Gel Extraction kit (Qiagen, Crawley, UK) and then sequenced.

3.2.5 *FBXW7* mutation screening

Patient samples were screened for *FBXW7* mutations in exons 8-12 which encode the WD40 domain. PCR products were obtained using the Optimase enzyme (Transgenomic) with standard reaction mix and cycling conditions as detailed in section 2.1.4 and amplified by 35 cycles of PCR, followed by dHPLC analysis on the WAVE. PCR primers, annealing temperatures and WAVE analysis temperatures are given in Appendix Table 1. Fresh PCR products from samples with abnormal chromatograms were sequenced. The presence of a polymorphism in the intron between exons 11-12 was confirmed by 35 cycles of PCR with the BIOTAQ enzyme with primers and annealing temperatures shown in Appendix Table 1, followed by direct sequencing of the products.

3.3 Results

Genomic DNA was available for analysis from peripheral blood or bone marrow samples taken at diagnosis from 162 T-ALL patients entered into the MRC UKALL 2003 trial.

3.3.1 *NOTCH1* mutation detection

WAVE analysis was used to screen the LNR-B, HD-N, HD-C, JME, TAD and PEST domains of *NOTCH1*. Of the 162 patients investigated, 133 (82%) had one or more exons with abnormal WAVE chromatograms. In 29 cases, the abnormal chromatograms were due to the presence of a common polymorphism (C5094T, G5073A, G6853A or C6870T), which were confirmed as detailed in section 3.2.3. In a further three cases, direct nucleotide sequencing showed a deletion in intron 26-27. All patients in whom these were the only changes were scored as *NOTCH1*-WT.

A total of 119 mutations were detected in the *NOTCH1* gene in 101 (62%) of the 162 patients analysed (*NOTCH1*^{MUT}). Figure 3.7 shows the location and type of the mutations identified, with an overview in Table 3.3. A full list of nucleotide and amino acid changes for all patients is given in Appendix Table 2. Representative WAVE chromatograms are shown in Figure 3.8. It was not possible to predict the specific mutation based on a

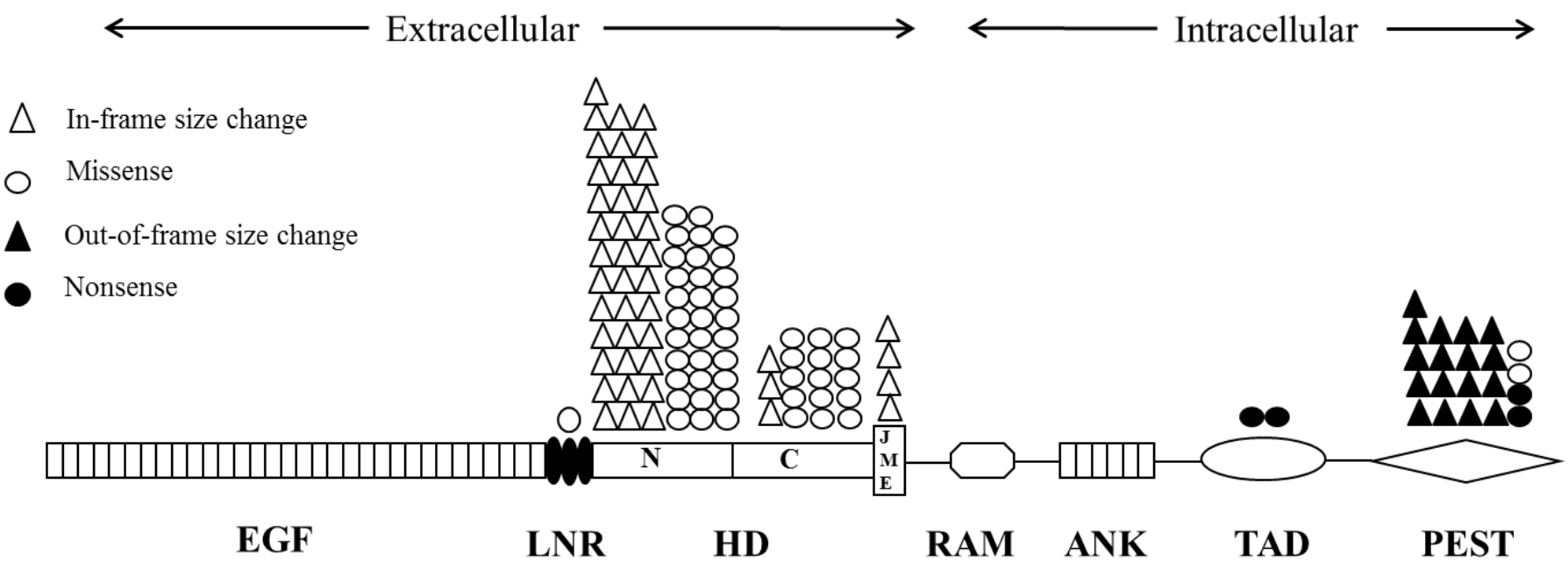

Figure 3.7 Schematic representation of the location and type of *NOTCH1* mutations detected in 162 paediatric T-ALL patients. Mutations identified in the NOTCH1 Lin12/Notch repeats (LNR), heterodimerisation (HD) N and C- terminal domains, juxtamembrane (JME), transcriptional activation (TAD) and PEST domains.

characteristic WAVE chromatogram; therefore all samples with an abnormal chromatogram were sequenced. In the case of most mutants (89%), it was possible to determine the change by direct nucleotide sequencing. This approach was unsuccessful for 13 (11%) mutations due to more complex mutations or the presence of a low-level mutant indicating either non-leukaemic cell contamination in the sample or a mutation in a subclone. Using the fraction collector facility on the WAVE, three of these mutants were purified at a denaturing temperature and one was size-separated at a non-denaturing temperature. For the remaining nine mutations, the exon was amplified by PCR to obtain a fresh PCR product, which was then cloned using the TOPO TA cloning kit and clones were screened as described in section 2.1.10.

Of these 119 mutations, 87 (73%) were found in the HD domain, 70 of them (80%) in the HD-N region of the domain and 17 (20%) in the HD-C region. The mutations in the HD-N region tended to cluster in the highly conserved areas between amino acid residues 1533-1602 (50 mutations) and 1605-1618 (19 mutations). Approximately half of the mutations found in the HD-N domain were missense amino acid substitutions (33 mutations, 47%), of which 20 were leucine to proline amino acid changes. The remaining mutations were short, in-frame insertions, deletions or indels, generally of up to 15bps, although larger in-frame insertions, one of 30bps and two of 39bps, were also identified. The HD-C domain mutations were all located in the region spanning amino acid residues 1674-1723 and comprised 14 (82%) missense amino acid substitutions, 12 of them leucine to proline amino acid changes, one 3bp indel, one 3bp deletion and a larger in-frame insertion of 78bps which consisted of a 10bp insertion plus a 68bp duplication. A total of 25 mutations (21% of all mutations) were found in the PEST domain. These included eight insertions, nine deletions and four indels all causing a frameshift, with the introduction of a premature stop codon resulting in a C-terminally truncated protein. Two nonsense amino acid changes were detected, Q2406X and S2487X, also resulting in a C-terminally truncated protein, and two missense amino acid changes, F2510L and P2459T. In the TAD domain, two nonsense amino acid substitutions were identified, Q2392X and Q2395X, which would result in C-terminal protein truncation. Four mutations were found in the JME domain, all of them large in-frame insertions of 24bps or more. One missense amino acid substitution, C1528R was also found in the LNR-B domain.

Of the 101 patients with a mutated *NOTCH1* gene, 84 patients (83%) had a single mutation (*NOTCH1*^{Single}) (Table 3.3) and 17 patients (17%) had more than one *NOTCH1* mutation, herein denoted *NOTCH1*^{Double}. Of the *NOTCH1*^{Double} patients, 14 (82%) were mutated in both the HD (either the HD-N or HD-C) and PEST domains, one of them also had an LNR-B

Table 3.3 Summary of *NOTCH1* mutations detected

Mutant Status	Mutation 1					Mutation 2				No. of patients
	HD-N	HD-C	JME	TAD	PEST	LNR-B	HD-N	HD-C	PEST	
Single	In-frame size change									32
	Missense									25
		Missense								11
		In-frame size change								1
			In-frame size change							4
				Nonsense						1
					Out-of-frame size change					8
					Missense					1
					Nonsense					1
Total										**84**
Double	Missense							Missense		2
	Missense								Out-of-frame size change	6
	In-frame size change								Out-of-frame size change	2
	In-frame size change					Missense			Out-of-frame size change	1
	In-frame size change								Nonsense	1
	In-frame size change								Missense	1
		In-frame size change							Out-of-frame size change	2
		Missense							Out-of-frame size change	1

Table 3.3 Continued

Mutant Status	Mutation 1					Mutation 2				No. of patients
	HD-N	HD-C	JME	TAD	PEST	LNR-B	HD-N	HD-C	PEST	
				Nonsense					Out-of-frame size change	1
Total										**17**

Abbreviations: dHPLC, denaturing high performance liquid chromatography; HD, heterodimerisation; JME, juxtamembrane; TAD, transcriptional activation; ANK, ankyrin repeats; LNR, Lin-12/Notch repeats.

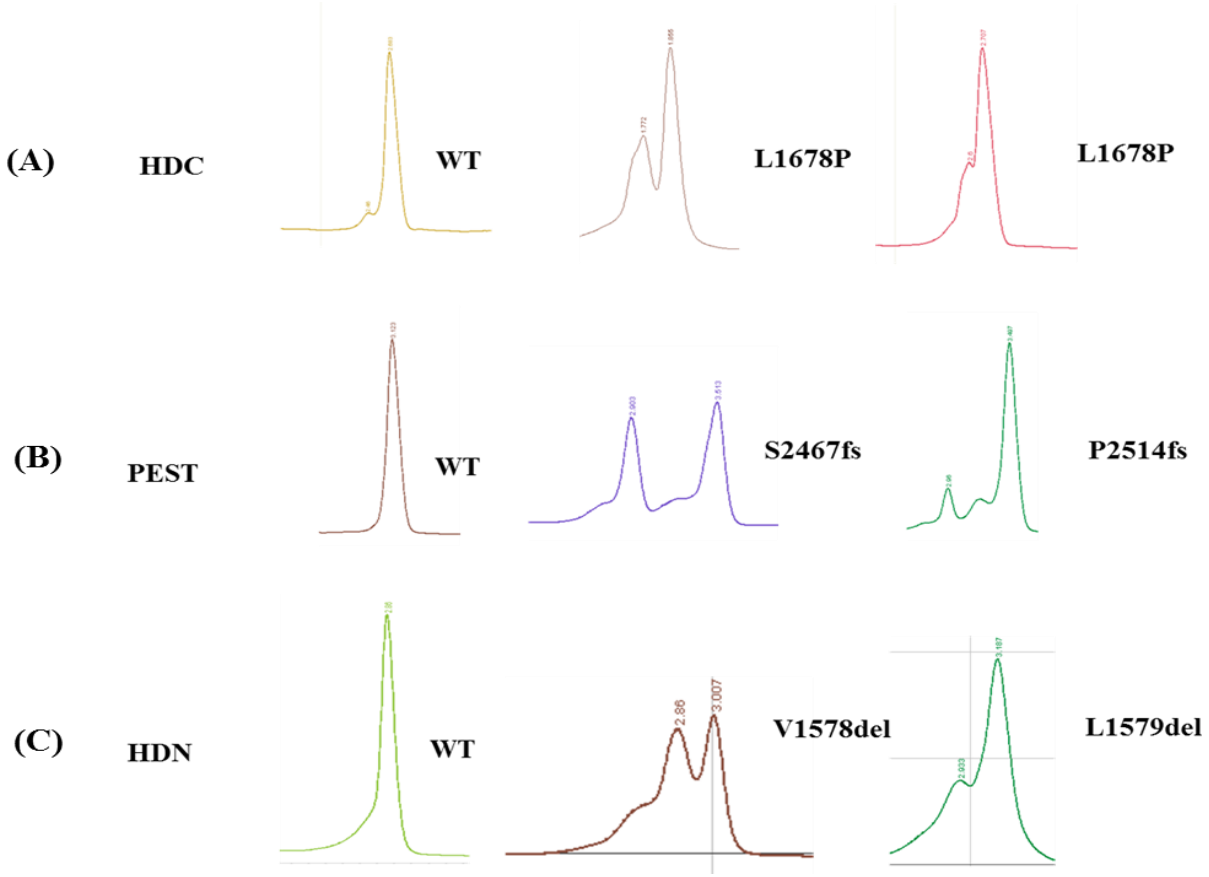

Figure 3.8 Detection of *NOTCH1* mutations by WAVE analysis. Representative WAVE chromatograms of patient samples normalised and compared to that of a known WT control. (A). High level and low level heterozygous point mutations in exon 27. (B). High level and low level insertions in exon 34. (C) High level and low level deletions in exon 26.

mutation, two patients harboured mutations in both the HD-N and HD-C subunits, and one patient in both the PEST and TAD domains.

3.3.2 Confirmation of common *NOTCH1* polymorphisms

Of the 162 patients screened for HD-C mutations in exon 27, 75 (45%) had a WAVE chromatogram suggestive of the common C/T SNP at nt 5094 in exon 27. Heterozygous presence of the SNP was confirmed in all cases, 21 by direct nucleotide sequencing, 31 by PCR with a mismatch primer and BsaA1 digestion (Figure 3.6), and 23 cases by both methods. The incidence of patients that were heterozygous for the SNP did not differ between *NOTCH1*-WT and *NOTCH1*-mutant cases (27 of 61 [44%] vs. 48 of 101 [46%], *P*=.67). Direct sequencing of three other patients with an abnormal chromatogram for exon 27 showed the presence of an A/G SNP at nt 5073. Five cases with an abnormal chromatogram in the TAD domain were scored as heterozygous for the A/G SNP at nt 6853 and two cases for the C/T SNP at nt 6870 by direct sequencing.

3.3.3 *FBXW7* mutation detection

WAVE analysis was used to screen exons 8-12 of the *FBXW7* gene. Of the 162 patients investigated, 42 (26%) had one or more exons with abnormal WAVE chromatograms. In 11 cases, the abnormal chromatograms were due to the presence of a common C/T polymorphism in intron 11-12, which were confirmed by direct sequencing. All patients in whom these were the only changes were scored as *FBXW7*-WT.

A total of 31 mutations were detected in the *FBXW7* gene in 29 (18%) of the 162 patients analysed (*FBXW7*^{MUT}). Full details are given in Appendix Table 2. In the case of all mutants it was possible to determine the change by direct nucleotide sequencing. All mutations were missense amino acid substitutions (Figure 3.9). The most commonly mutated region was exon 9 with 21 (68%) mutations; nine of them were R465H, seven R465C, two R465L, two R465P and one R441Q. Of the eight (26%) exon 10 mutations identified, five were R479Q, two R505C and one G517V. Two patients harboured the R689W mutation in exon 12. Of note, all mutations in *FBXW7* except one, G517V in exon 10, disrupted the conserved arginine residues in the WD40 domain of the gene.

All except two patients had a single *FBXW7* mutation. The patient harbouring the G517V mutation in exon 10 also had an R465C mutation in exon 9, and one patient had a R505C mutation in exon 9 in conjunction with a R689W mutation in exon 12.

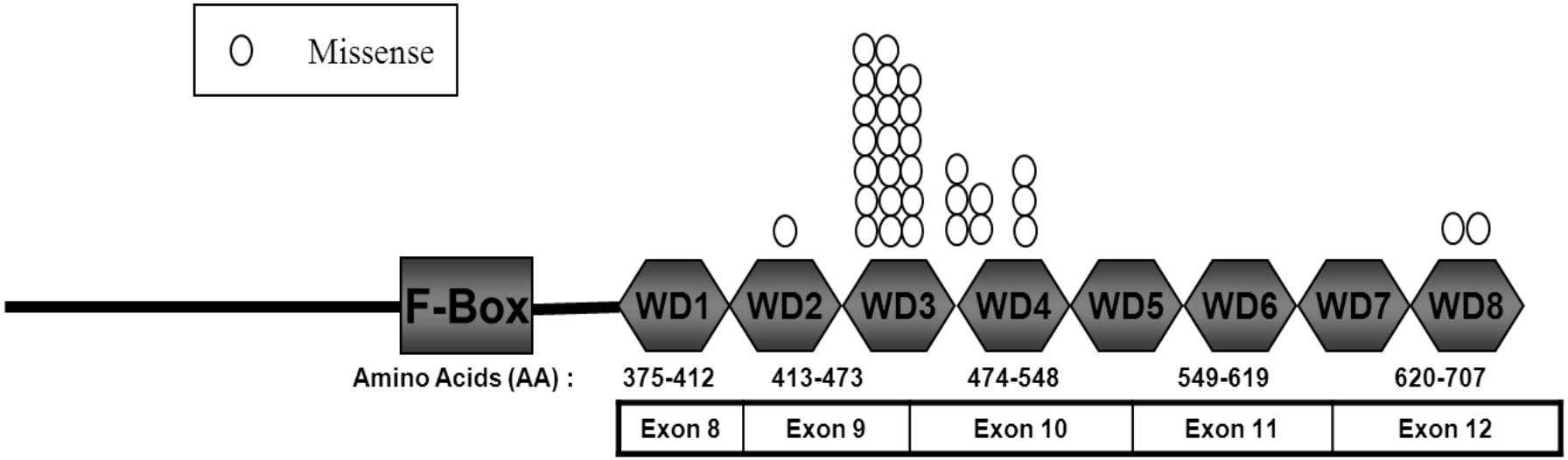

Figure 3.9 Schematic representation of the location and type of *FBXW7* mutations detected in 162 paediatric T-ALL patients. Mutations identified in exons 9, 10 and 12 of the WD40 domain repeats.

Table 3.4 *NOTCH1* and *FBXW7* mutation status of cohort studied

***NOTCH1* domain**	***FBXW7* WT (% of total patients)**	***FBXW7* MUT (% of total patients)**
NOTCH1 WT	57 (35%)	4 (3%)
NOTCH1 MUT	76 (47%)	25 (15%)
HD only	48 (30%)	23 (14%)
JME	4 (3%)	0 (0%)
TAD	1 (<1%)	0 (0%)
PEST only	10 (13%)	0 (0%)
HD+PEST	11 (7%)	2 (1%)
HD+PEST+LNR-B	1 (<1%)	0 (0%)
PEST + TAD	1 (<1%)	0 (0%)

Abbreviations: WT, wild-type; MUT, mutant; HD, heterodimerisation domain; JME, juxtamembrane domain; TAD, transcriptional activation domain; LNR-B, Lin12/Notch repeats.

3.3.4 *NOTCH1* and *FBXW7* genotypes

Considering the mutational status of both the *NOTCH1* and *FBXW7* genes together for all 162 patients, 57 (35%) patients were wild type in both genes, 76 (47%) were mutated in *NOTCH1* only, of these 62 (38% of all mutated cases) were $NOTCH1^{Single}FBXW7^{WT}$ and 14 (9%) were $NOTCH1^{Double}FBXW7^{WT}$, four patients (3%) had an *FBXW7* mutation only and 25 patients (15%) were $NOTCH1^{MUT}FBXW7^{MUT}$ (Table 3.4). The latter all had a *NOTCH1* HD domain mutation coupled with the *FBXW7* mutation; two patients also had a mutation in the *NOTCH1* PEST domain. This data indicates a positive association between a *NOTCH1* HD domain mutation and an *FBXW7* mutation, 23 of 71 (32%) patients with HD-only mutations were $FBXW7^{MUT}$ versus 6 of 91 (7%) other patients (*P*=.0003). Conversely, cases with a *NOTCH1* PEST domain mutation were less likely to have an *FBXW7* mutation, 24 of 133 (18%) $FBXW7^{WT}$ patients had a *PEST* domain mutation versus 2 of 29 (6%) $FBXW7^{MUT}$ patients, but this difference did not reach statistical significance (*P*=.19).

3.4 Discussion

Data is presented in this chapter on the screening of a cohort of paediatric T-ALL patients for mutations in the *NOTCH1* and *FBXW7* genes. The standard mutational screening technique utilised by many groups is direct Sanger sequencing of PCR products, but for a large number of samples this is expensive and labour-intensive and, due to the limited sensitivity of the technique, low level mutants are not easily detected. In the present study, dHPLC was used to screen denatured PCR products from the chosen exons. It is a medium throughput screening method for the sensitive detection of both point mutations and size changes. The technique involves very little sample handling and is more sensitive than Sanger sequencing as studies in our laboratory have shown that it is sensitive enough to pick up mutations which are present at a level of less than 5% of total alleles. The protocols had already been optimised in the laboratory in a previous study identifying *NOTCH1* and *FBXW7* mutations in an adult cohort of T-ALL patients (Mansour *et al.*, 2009). One of the limitations of dHPLC is the requirement for heterozygous mutation as the technique relies on the formation of heteroduplexes that bind less strongly to the column and are therefore eluted first. It is not possible to detect homozygous mutations by dHPLC unless the PCR products to be screened are mixed with wildtype control PCR products prior to denaturation. This was not carried out in the present study as homozygous mutations have not been reported in the *NOTCH1* or *FBXW7* genes in previous paediatric T-ALL studies.

In total, of the 162 patients analysed by dHPLC on the WAVE platform, 101 (62%) had one or more *NOTCH1* mutations. Of the nine studies investigating *NOTCH1* mutations in paediatric T-ALL, the median mutation incidence is 56% (range 22% to 57%). The mutation incidence in the present study is higher than the previous studies combined, but the difference is not significant (548 of 1115, 49%, *P*=.08) (Clappier *et al.*, 2010;Erbilgin *et al.*, 2010;Fogelstrand *et al.*, 2014;Kox *et al.*, 2010;Mansur *et al.*, 2012;Park *et al.*, 2009;van Grotel *et al.*, 2008;Weng *et al.*, 2004;Zuurbier *et al.*, 2010). Possible explanations for the difference in mutation rate between the studies include the exons screened. Six of the nine studies only investigated the HD and PEST domains; however the frequency of mutations in domains outside of these exons, e.g. the JME and LNR domains, is low and therefore would not be predicted to increase the mutation rate significantly. Another factor could be the method used to screen for the mutations. Five of the previous studies used Sanger sequencing, which can miss low level mutations due to lesser sensitivity. However, of the four studies that utilised dHPLC, as in the present study, the mutation rate ranges from 22% to 57%, suggesting that the difference is as a result of reasons other than technique used. Racial origin of the cohorts could also explain the difference. A Japanese study reported a 35% mutation incidence, suggesting that *NOTCH1* mutations are not as prevalent in Asian populations (Park *et al.*, 2009). T-ALL is also a relatively rare disease, and the low numbers of patients in the respective cohorts makes comparison difficult.

The location of the mutations identified in this cohort is comparable to other studies, and although the regions are common, there is heterogeneity amongst individual mutations. The most frequently mutated region is the HD domain, with mutations in 85 of the 101 (84%) *NOTCH1*-mutated patients located here. This is comparable to other studies; the median HD domain mutation rate across the paediatric studies, as a percentage of the total number of mutations, is 78% (range 67% to 85%). The HD domain is essential for stable subunit association, and crystallography data has shown that conserved amino acid residues in the domain form the LNR-HD interaction platform (Gordon *et al.*, 2009). Mutations in this region are likely to disrupt this interaction, and have previously been assigned to two distinct groups predicted to either lead to ligand-independent S2 cleavage or hypersensitivity to ligand binding (Malecki *et al.*, 2006). Mutations found in the present study would be expected to fall into these groups and therefore be functionally significant. The first group are missense point mutations or small in-frame size changes that enhance subunit dissociation and destabilise the NOTCH1 heterodimers, resulting in exposure of the metalloprotease cleavage site. Of the 47 missense mutations identified in the HD domain, 34 (72%) introduced a proline residue into the protein structure which is likely to be structurally disruptive (Weng *et al.*, 2004). The functionality of eight of the 14 missense mutations found

in this cohort (L1678P, R1598P, V1578E, I1616N, I1680T, F1592S, L1600P and L1593P) have been tested and found to result in increased activation (Malecki *et al.*, 2006;Chiang *et al.*, 2008). Of the other point mutations, three are leucine to proline changes, and the remaining three are located in close proximity to the missense mutations investigated in previous studies so would be predicted to be functionally significant. The size changes identified in the present study of up to 15bps may expose the S2 cleavage site by repositioning it away from the LNR domain and the protective conformation. The second distinct class of HD mutations are larger in-frame insertions which either duplicate the S2 site or are adjacent to and enhance access of TACE to the cleavage site. This class of mutation was uncommon in the present cohort as only one duplication spanning amino acids 1710-1711 was identified in the HD-C domain. Of note, the mutations in the HD domain are all in-frame, which suggests the need for an intact ICN for leukaemogenesis.

Another group of mutations have recently been identified in the JME domain (Sulis *et al.*, 2008). They occur less frequently than HD domain mutations, with other studies reporting a 2-6% incidence (Sulis *et al.*, 2008;Zuurbier *et al.*, 2010;Asnafi *et al.*, 2009;Mansour *et al.*, 2009), which was confirmed in our cohort with 3% of patients harbouring this type of mutation. Of the four mutations found in this domain in the present study, three introduce the peptide sequence QLHF that has been previously reported (Sulis *et al.*, 2008). The same group demonstrated that the increase in the level of activation of the signalling pathway as a result of JME mutation is dependent on the number of amino acids that are inserted. An insertion of 12-17 amino acids or more was found to lead to a 200-2000 fold increase in activation above the baseline. Two of the mutations found in the present study would be predicted to be functionally equivalent to this; one had an insertion of 19 amino acids and the other 42 amino acids. In the remaining two mutations, eight and ten amino acids were inserted, which would be predicted to result in only a 1.5-12 fold increase in activation.

The PEST domain was also found to be a mutational hotspot in the present study, with mutations in 25 of the 101 (25%) *NOTCH1*-mutated patients located here. This is comparable to other studies; the median PEST domain mutation rate across the paediatric studies is 33% (range 20% to 42%). As with other studies, the PEST domain mutations found in the cohort were predominantly frameshift size changes leading to the introduction of a premature stop codon, which would result in the synthesis of a C-terminally truncated protein. Two nonsense mutations were also identified which would also result in a truncated receptor. Nineteen of the mutations cause the introduction of a stop codon before the PEST phosphodegron region on or around T2512, which would lead to a truncated receptor being expressed with no FBXW7 binding site. In the remaining six mutations, the mutational start

site is located in amino acids 2514-2517. The PEST phosphodegron spans amino acids 2510-2517, therefore these mutations would also be predicted to disrupt FBXW7 binding. Two missense mutations were also identified in the present cohort, F2510L and P2459T. F2510L would be predicted to affect the FBXW7 binding site, but the functional significance of P2459T is unclear as it is not located near the binding site and so would not be predicted to lead to stabilisation of ICN-1 in the nucleus. A possible explanation is that the mutation is a rare SNP, but remission material was not available to confirm this.

Two TAD domain nonsense mutations were identified which would result in a truncated NOTCH1 receptor lacking the PEST domain. Previous studies have demonstrated that the TAD domain associates with transcriptional coactivators during signalling and that T-cell transformation requires an intact TAD domain, as constructs encoding polypeptides lacking amino acids 2155 to 2374 of the TAD domain failed to induce T-ALL (Aster *et al.*, 2000). The truncating mutations identified in the TAD domain in the present study were located outside of this region at amino acids 2391 and 2394 respectively, so would be predicted to be functionally similar to PEST domain mutations. One mutation was identified in the LNR domain, C1528R, which would be predicted to destabilise the structure by inhibiting calcium binding (Aster *et al.*, 1999;Gordon *et al.*, 2009). Studies in *D.melanogaster* demonstrated that three cysteine residues that form disulphide bonds are critical for the stability of the LNR-HD interface (Tien *et al.*, 2008). It is possible that certain cysteine residues in the LNR domain of the human NOTCH1 receptor mutation are also crucial for disulphide bond formation, and the mutation found in the present study may disrupt this process.

Of the 17 *NOTCH1*^{Double} patients, 14 (82%) were mutated in both the HD (either the HD-N or HD-C) and PEST domains, which is the most frequently reported combination of mutations in *NOTCH1*. One of these patients mutated in both the HD and PEST domain also had an LNR-B mutation. Previous studies have demonstrated that mutations in the HD and PEST domains are in cis on the same allele (Weng *et al.*, 2004). This could not be confirmed in the present study as only genomic DNA was available for analysis. The intronic and exonic sequence between exons 26 and 34 is 10kb in size, therefore although possible, amplification and cloning of a PCR product from genomic DNA would have been challenging and this was not pursued. For the same reason, it could not be determined whether this was also the case in two patients who harboured mutations in both the HD-N and HD-C subunits. One of the patients also has an *FBXW7* mutation indicating the requirement to further increase the activation signal. One patient had a mutation in both the PEST and TAD domains, both mutations are predicted to truncate the protein and be functionally comparable. In the paediatric studies, only one other combination of co-incident

NOTCH1 mutations has been reported, a patient who harboured both an HD and a JME domain mutation (Zuurbier *et al.*, 2010). Six of the nine paediatric studies screened for mutations in the HD and PEST domains only, which could explain the low incidence of other double mutations in their respective cohorts.

In total, of the 162 patients analysed by dHPLC on the WAVE platform, 29 (18%) had an *FBXW7* mutation. Of the eight studies investigating *FBXW7* mutations in paediatric T-ALL, the median mutation incidence is 16% (range 10% to 31%). The mutation incidence in the present study is in line with the previous studies combined (149 of 932, 16%, *P*=.6) (Clappier *et al.*, 2010;Erbilgin *et al.*, 2010;Fogelstrand *et al.*, 2014;Kox *et al.*, 2010;Malyukova *et al.*, 2007;Mansur *et al.*, 2012;Park *et al.*, 2009;Zuurbier *et al.*, 2010). Of the 31 mutations found in 29 patients in this study, 30 (97%) disrupt the conserved arginine residues of the WD40 domain, which has been demonstrated to lead to increased levels of activated ICN-1 as mutant FBXW7 is unable to bind to the NOTCH1 PEST domain and degradation by the proteasome is impaired (Chiang *et al.*, 2008;Thompson *et al.*, 2007). The location of the mutations identified in this cohort fits with other studies, with the mutations mainly clustering in exons 9 and 10. Functional studies have demonstrated that the R465C mutation not only renders FBXW7 unable to bind to the NOTCH1 PEST domain, but also unable to ubiquitinate c-Myc, and the subsequent stabilisation of c-Myc results in an increased population of LICs. The mutants formed heterodimers and exerted a dominant effect over the WT protein (King *et al.*, 2013). R465 is the most frequently mutated amino acid in the present study, with 20 (65%) of the 29 mutations in exons 9 and 10 located here, therefore these mutations are predicted to be functionally significant. Two missense mutations disrupting the arginine residues in exon 12 were also reported in this study, 6% of the total *FBXW7* mutations. Four other groups have reported missense mutations in arginine residues in exon 12 (Fogelstrand *et al.*, 2014;Malyukova *et al.*, 2007;Park *et al.*, 2009;Zuurbier *et al.*, 2010). These mutations are predicted to be functionally similar to the exon 9 and 10 mutations, as exon 12 also encodes part of the WD40 domain which forms the propeller-like structure for interaction with various substrates. Less common missense mutations affecting residues other than arginine have been reported in previous studies, including G498A (Erbilgin *et al.*, 2010), G477S and S516G (Asnafi *et al.*, 2009) in exon 10 and G423V (Mansour *et al.*, 2009;Asnafi *et al.*, 2009) in exon 9. Only one such mutation was found in the present study (3%), G517V located in exon 10. This incidence is in line with other studies (9 of 83, 10%, *P*=.2). Of the eight studies investigating *FBXW7* mutations in paediatric T-ALL, three screened exons 9 and 10 only. In the studies where more exons were investigated, mutations were reported at a lower frequency in exon 7 (Zuurbier *et al.*, 2010), exon 8 (Erbilgin *et al.*, 2010;Fogelstrand *et al.*, 2014) and exon 3 (Park *et al.*, 2009),

however no mutations in exon 8 were identified in this cohort and exons 7 and 3 were not screened. No nonsense mutations or size change mutations were identified in this cohort, however frameshift insertions, deletions and indels have been reported in other studies (Park *et al.*, 2009;Clappier *et al.*, 2010;Erbilgin *et al.*, 2010;Fogelstrand *et al.*, 2014).

Studies have shown that co-incident mutations in the *NOTCH1* HD domain and the *NOTCH1* PEST domain synergise to increase NOTCH1 activation and signal strength, and that mutations in the *FBXW7* gene also have a synergistic effect when coupled with *NOTCH1* mutations (Maser *et al.*, 2007;Weng *et al.*, 2004;Chiang *et al.*, 2008;O'Neil *et al.*, 2007;Thompson *et al.*, 2007). These findings have recently been extended in in vivo mouse models, which demonstrated that *FBXW7* and *NOTCH1* mutations co-operate to stabilise c-Myc, thereby increasing leukaemia initiating capacity (King *et al.*, 2013). The frequency of co-incident *NOTCH1* and *FBXW7* mutations in the present study is 15%, which is comparable to other studies combined (94 of 932, 10% P=.07). There was a positive association between mutations in the *NOTCH1* HD domain only and the *FBXW7* gene. Mutations in the *NOTCH1* PEST domain and *FBXW7* in the same patient are infrequent. Only one patient had an HD mutation with both a PEST and an *FBXW7* mutation. This is consistent with other studies (Clappier *et al.*, 2010;Zuurbier *et al.*, 2010), and supports the hypothesis that in aberrant NOTCH1 signalling, if the *NOTCH1* PEST domain is already mutated and the activation signal is increased, there is no further advantage in acquiring a mutation in *FBXW7*, or conversely, if *FBXW7* is already mutated, there is no further advantage in acquiring a mutation in the *NOTCH1* PEST domain. The greater number of single mutations in the HD domain than the PEST domain suggests that the HD domain becomes mutated prior to the PEST domain in the sequence of acquisition. In a mouse model of radiation-induced T-cell acute lymphoblastic lymphoma, reduced *Fbxw7* activity precluded the requirement for *Notch1* PEST domain mutations for tumour development (Jen *et al.*, 2012).

The data presented in this chapter reports the screening and identification of mutations in the *NOTCH1* and *FBXW7* genes. The association of the mutations with clinical characteristics and the impact of genotype on patient outcome is studied in chapter 4.

CHAPTER 4: PROGNOSTIC IMPACT OF *NOTCH1* AND/OR *FBXW7* MUTATIONS IN PAEDIATRIC T-ALL PATIENTS WITH T-ALL

4.1 Introduction

As described in chapter 1, a risk-adapted approach is used to treat patients with T-ALL, and risk stratification of ALL patients on the UKALL 2003 trial is initially based on age and WBC. Patients are then further stratified by early response to induction therapy and levels of MRD. Several studies have shown the relationship between the risk of relapse and persistence of high MRD levels, and the measurement of residual disease is now incorporated in most modern treatment protocols (Cave *et al.*, 1998;van Dongen *et al.*, 1998). Nevertheless, the delay in only identifying high-risk patients based on MRD levels at day 29 of induction therapy, and the subsequent switch of treatment regimen at this time point, may be a disadvantage for these patients as they may have benefited from earlier more intensive treatment. Identification of additional molecular prognostic markers at diagnosis is therefore needed to discriminate between the lower-risk and higher-risk patients, so that earlier intervention with more intensive therapy such as haematopoietic stem cell transplantation can be targeted at those at greatest risk of relapse, and dose reduction considered for those at lower risk of relapse. In other haematological malignancies such as AML and B-ALL, recent advances have allowed the stratification of patients based on cytogenetic and molecular characterisation. A number of recurrent chromosomal abnormalities have been shown to have prognostic significance in B-ALL. Some chromosomal abnormalities are associated with a more favourable outcome, such as hyperdiploidy (Paulsson & Johansson, 2009) and the *ETV6-RUNX1* fusion (Attarbaschi *et al.*, 2004). Others are associated with a poorer prognosis, including the Philadelphia chromosome t(9;22) (Ribeiro *et al.*, 1997), rearrangements of the *MLL* gene (Pui *et al.*, 2003), and the intrachromosomal amplification of the *AML1* gene (iAMP21) (Moorman *et al.*, 2007). This has not been the case in T-ALL as discussed in chapter 1, and there is a lack of informative prognostic markers. However, recent studies have indicated that abnormalities in the NOTCH1 pathway may be suitable candidates.

4.1.1 Therapeutic targeting of the NOTCH1 signalling pathway

The high frequency of activating *NOTCH1* mutations in T-ALL, as described in chapter 3, implies a central role for NOTCH1 signalling in the pathogenesis of the disease, and the pathway has therefore been identified not only as a potential prognostic marker, but also as a

therapeutic target (Weng *et al.*, 2004). The presence of a *NOTCH1* mutation at diagnosis could identify patients who may benefit from NOTCH1 pathway inhibition. Clinical compounds initially tested were small molecule inhibitors of the γ-secretase cleavage complex, which block the final cleavage in the maturation of the NOTCH1 receptor and therefore the translocation of the intracellular domain to the nucleus (Seiffert *et al.*, 2000). However, the γ-secretase inhibitors tested were not selective for the NOTCH1 receptor and also the blocked signalling through the NOTCH2-4 receptors, and animal studies showed that this resulted in gastrointestinal toxicity due to the accumulation of secretory goblet cells in the intestine (Milano *et al.*, 2004;van Es *et al.*, 2005;Wong *et al.*, 2004;Searfoss *et al.*, 2003). Analysis of these inhibitors in relapsed and refractory T-ALL in a phase-1 clinical trial by Deangelo *et al* (2006) demonstrated that this was also the case in patients. In addition, there was no significant clinical response, indicating the weak anti-leukaemic activity of these agents in T-ALL. More recent studies have shown that the inhibition of NOTCH1 with γ-secretase inhibitors restored glucocorticoid–sensitivity in glucocorticoid–resistant T-ALL cell lines and primary samples (Real *et al.*, 2009). The glucocorticoids inhibited the gut toxicity and this suggests that the use of γ-secretase inhibitors in combination with glucocorticoids may offer a new treatment option in the future (Real & Ferrando, 2009).

However, other studies have focused on the association of the mutations with the clinical characteristics of T-ALL and their prognostic significance.

4.1.2 Clinical characteristics of T-ALL patients with *NOTCH1* and/or *FBXW7* mutations

Several groups have investigated the characteristics of *NOTCH1* and/or *FBXW7* mutant T-ALL since the identification of *NOTCH1* and *FBXW7* mutations in patients with T-ALL in 2004 and 2007 respectively, and these studies are summarised in Table 4.1. Most paediatric studies showed no association of *NOTCH1* and/or *FBXW7* mutation status with age, sex or WBC count, although of seven studies investigating the impact of *NOTCH1* mutations alone on clinical characteristics, three reported the mutations were more frequently found in T-ALL patients with a low WBC count (Mansur *et al.*, 2012;Erbilgin *et al.*, 2010;Park *et al.*, 2009). Of these paediatric reports, six correlated the T-cell immunophenotype of the patients to genotype, and one group showed a significantly higher incidence of *NOTCH1* mutations in the cortical subgroup (CD1a positive) compared with the immature pro/pre-T phenotype (Breit *et al.*, 2006). Another reported that *NOTCH1* and/or *FBXW7* mutations were significantly less frequently associated with mature T-ALL (Zuurbier *et al.*, 2010).

Table 4.1 Studies reporting on the clinical characteristics of T-ALL patients with *NOTCH1* and *FBXW7* mutations

Reference	Total in study	Median age, years (range)	*NOTCH1* mutated (%)	*FBXW7* mutated (%)	Clinical Characteristics and Immunophenotype	Cytogenetics
(Breit *et al.*, 2006)	157	8.8 (2-18)	52	N/A	**No difference:** age, sex, WBC *NOTCH1* mutations less frequently associated with immature pro/pre-T phenotype and more frequently associated with cortical phenotype (*P*=.02)	N/A
(van Grotel *et al.*, 2008)	70	N/A	57	N/A	**No difference:** age, sex, WBC Non-significant trend towards lesser frequency of *NOTCH1* mutations in mature T-ALL cases	No difference
(Park *et al.*, 2009)	55	9.5 (2-15)	37	20	**No difference:** age, sex, T cell immunophenotype **Decreased:** WBC $<100x10^9$/l associated with *NOTCH1* and/or *FBXW7* mutations (*P*=.03)	No difference
(Clappier *et al.*, 2010)	134	N/A	57	15	**No difference:** age, sex, WBC *NOTCH1* and/or *FBXW7* mutations less frequently associated with immature pro/pre-T phenotype and more frequently associated with cortical phenotype (*P*=.06)	No difference
(Zuurbier *et al.*, 2010)	141	N/A	56	16	**No difference:** age, sex, WBC *NOTCH1* and/or *FBXW7* mutations less frequently associated with mature T-ALL cases (*P*=.05)	*NOTCH1* and/or *FBXW7* mutations less frequently associated with *TAL/LMO* rearranged cases and more prevalent in *TLX3* rearranged cases
(Erbilgin *et al.*, 2010)	87	7 (6-16)	22	10	**No difference:** age, sex, T cell immunophenotype **Decreased:** WBC $<50x10^9$/l associated with *NOTCH1* and/or *FBXW7* mutations (*P*=.03)	N/A
(Mansur *et al.*, 2012)	138	8 (1-18)	44	19	**No difference:** age, sex, T cell immunophenotype **Decreased:** WBC associated with missense mutations in *NOTCH1* (*P*=.035)	No difference

Table 4.1 Continued

Reference	Total in study	Median age, years (range)	*NOTCH1* mutated (%)	*FBXW7* mutated (%)	Clinical Characteristics and Immunophenotype	Cytogenetics
(Fogelstrand *et al.*, 2014)	79	9 (1-17)	57	19	**No difference:** sex, WBC *NOTCH1* and/or *FBXW7* mutations more frequently associated with older patients (*P*=.007)	N/A
(Zhu *et al.*, 2006)	77 (53 paediatric 24 adult)	42 (18 paediatric 24 adult)	38	N/A	**No difference:** age, sex, T cell immunophenotype **Increased:** WBC >$10x10^9$/l associated with *NOTCH1* mutations (*P*=.04)	No difference
(Asnafi *et al.*, 2009)	141	27.5 (15-58)	62	24	**No difference:** age, sex, WBC, T cell immunophenotype	Mutation more frequently associated with TLX1 expression (*P*=0.004)
(Mansour *et al.*, 2009)	88	30.5 (16-60)	60	16	**No difference:** age, sex, WBC	N/A

Abbreviations: WBC, white blood cell count; N/A, not available.

Four paediatric studies related cytogenetic abnormalities to mutational status, and one demonstrated that *NOTCH1* and/or *FBXW7* mutations were more frequently associated with rearrangements of *TLX3* and less frequently associated with *TAL/LMO* rearranged cases (Zuurbier *et al.*, 2010).

4.1.3 Impact of *NOTCH1* and/or *FBXW7* mutations on response to therapy and patient outcome in T-ALL

A number of different groups have also reported the impact of the mutations on response to therapy and patient outcome, and these studies are summarised in Table 4.2. The association between a favourable effect on outcome and the presence of *NOTCH1* mutations was first suggested by Breit *et al* (2006), indicating that they might be a suitable candidate prognostic marker in T-ALL. This study, comprising 157 paediatric T-ALL patients from the ALL-Berlin-Frankfurt-Munster (BFM) 2000 trial, showed that those with a *NOTCH1* mutation were more likely to be MRD-negative at the end of induction therapy, and also to be associated with a lower relapse rate (RR) and improved relapse-free survival (RFS). In multivariate analyses, *NOTCH1* mutation status was found to be a favourable prognostic marker independent of age, sex, WBC count at diagnosis and T-cell immunophenotype. Since then, a number of studies have extended these findings. Of eight paediatric studies investigating the association of *NOTCH1* mutation alone on patient outcome, only two could replicate the favourable effect seen in the original study, including an extended series of 301 patients treated on the ALL-BFM 2000 protocol (Kox *et al.*, 2010;Park *et al.*, 2009). In the remaining reports, most found no association between the presence of *NOTCH1* mutations and long term outcome (Erbilgin *et al.*, 2010;Fogelstrand *et al.*, 2014;Larson Gedman *et al.*, 2009;Mansur *et al.*, 2012;van Grotel *et al.*, 2008). However, one study demonstrated the favourable effect of *NOTCH1* mutation on early response to therapy, but this did not transcend into long term outcome (Zuurbier *et al.*, 2010).

The identification of *FBXW7* mutations in T-ALL in 2007, and the finding that they synergise with *NOTCH1* mutations to increase the activation of the NOTCH1 signalling pathway, led to the grouping together of the *NOTCH1* and/or *FBXW7* mutated patients for analysis of outcome in a number of studies of paediatric T-ALL. Of the three paediatric studies where early response to therapy (as assessed by morphology at day 8 or 15 of induction therapy, where a good response was defined as <1000 leukaemic blood blasts/μl) was related to mutational status, all agreed that the presence of *NOTCH1* and/or *FBXW7* mutations was associated with a favourable early response to treatment (Clappier *et al.*, 2010;Zuurbier *et al.*, 2010;Kox *et al.*, 2010). Two of these studies also correlated the

Table 4.2 Studies reporting on the impact of *NOTCH1* and *FBXW7* mutations on clinical outcome in T-ALL

Reference	Age group	Protocol/trial	Total in study	*NOTCH1* mutated (%)	*FBXW7* mutated (%)	Outcome compared to *NOTCH1*^{WT}and/or *FBXW7*^{WT}
(Breit *et al.*, 2006)	Paediatric	ALL-BFM 2000	157	52	N/A	RER and favourable MRD in *NOTCH1*^{MUT} group Lower RR and improved RFS in *NOTCH1*^{MUT} group
(van Grotel *et al.*, 2008)	Paediatric	DCOG-ALL7/8/9	70	57	N/A	No association with outcome in *NOTCH1*^{MUT} group
(Larson Gedman *et al.*, 2009)	Paediatric	POG 8704/9086/9295/9296/9297/9398	47	34	11	No association with outcome in *NOTCH1* and/or *FBXW7* group
(Park *et al.*, 2009)	Paediatric	JACLS-ALL-97	55	37	20	Improved EFS and OS in *NOTCH1*^{MUT} alone and *NOTCH1* and/or *FBXW7* groups
(Zuurbier *et al.*, 2010)	Paediatric	DCOG-ALL7/8/9; COALL-97	141	56	16	RER in *NOTCH1* and/or *FBXW7* group No association with EFS in *NOTCH1* and/or *FBXW7* group
(Clappier *et al.*, 2010)	Paediatric	EORTC 58881/58951	134	57	15	RER and favourable MRD in *NOTCH1* and/or *FBXW7* group No association with EFS and OS in *NOTCH1* and/or *FBXW7* group
(Kox *et al.*, 2010)*	Paediatric	ALL-BFM 2000	301	50	14	RER and favourable MRD in *NOTCH1*^{MUT} alone and *NOTCH1* and/or *FBXW7* group Improved EFS and OS in *NOTCH1*^{MUT} alone group
(Erbilgin *et al.*, 2010)	Paediatric	ALL-BFM 2000	87	22	10	No association with outcome in *NOTCH1*^{MUT}, *FBXW7*^{MUT} or *NOTCH1* and/or *FBXW7* groups
(Mansur *et al.*, 2012)	Paediatric	GBTL1-99	138	44	19	No association with outcome in *NOTCH1*^{MUT}, *FBXW7*^{MUT} or *NOTCH1* and/or *FBXW7* groups
(Fogelstrand *et al.*, 2014)	Paediatric	NOPHO ALL-1998/2000	79	57	19	No association with outcome in *NOTCH1* and/or *FBXW7* group
(Zhu *et al.*, 2006)	Paediatric and adult	VDCP regimen	77 (53 paediatric 24 adult)	38	N/A	Paediatric: No association with outcome in *NOTCH1*^{MUT} Adults: Inferior OS and RFS in *NOTCH1*^{MUT} group
(Asnafi *et al.*, 2009)	Adult	LALA-94; GRAALL-2003	141	62	24	Association with improved EFS and OS in *NOTCH1*^{MUT} alone and *NOTCH1* and/or *FBXW7* group but only significant in multivariate analysis in *NOTCH1* and/or *FBXW7* group

Table 4.2 Continued

Reference	Age group	Protocol/trial	Total in study	*NOTCH1* mutated (%)	*FBXW7* mutated (%)	Outcome compared to *NOTCH1*^{WT}and/or *FBXW7*^{WT}
(Mansour *et al.*, 2009)	Adult	UKALLXII/ECOG2993	88	60	16	No association with outcome in *NOTCH1* and/or *FBXW7* group

Abbreviations: N/A, not available; RER, rapid early response; MRD, minimal residual disease; RR, relapse rate; RFS, relapse-free survival; EFS, event-free survival; OS, overall survival.
***** extended cohort of original ALL-BFM study by Breit *et al* (2006).

presence of *NOTCH1* and/or *FBXW7* mutations to MRD status (assessed at day 29 of induction therapy, with MRD-negative disease defined according to the specific study), both reported a positive association between the presence of mutations and MRD-negativity (Clappier *et al.*, 2010;Kox *et al.*, 2010).

Of the eight paediatric studies that investigated the long term outcome of patients with *NOTCH1* and/or *FBXW7* mutations, only one demonstrated the favourable effect of mutations on EFS and OS (Park *et al.*, 2009), with the favourable effect of the *NOTCH1* mutations being lost on the addition of *FBXW7* mutations in one study (Kox *et al.*, 2010). However, the remaining studies found no association between the presence of *NOTCH1* and/or *FBXW7* mutations and long term survival (Clappier *et al.*, 2010;Erbilgin *et al.*, 2010;Fogelstrand *et al.*, 2014;Larson Gedman *et al.*, 2009;Mansur *et al.*, 2012;Zuurbier *et al.*, 2010;Kox *et al.*, 2010). The presence of mutations in the NOTCH1 signalling pathway therefore only translates into improved overall survival in some trials, and it has been suggested that this is most probably as a result of differences in therapy (Ferrando, 2010).

The impact of mutations on the prognosis of high-risk T-ALL patients has also been controversial. Two studies reported that the effect of *NOTCH1* and/or *FBXW7* mutations differed between the standard- and intermediate-risk groups and the high-risk groups that were defined as poor early responders to induction therapy and/or MRD-positive (Clappier *et al.*, 2010;Kox *et al.*, 2010), In the ALL-BFM study, presence of a *NOTCH1* mutation was associated with an improved outcome in the standard- and intermediate-risk groups, but had no effect on long term outcome in the high-risk group (Kox *et al.*, 2010). Whereas in the EORTC protocol, where there was no reported effect of *NOTCH1* and/or *FBXW7* mutations in the standard- and intermediate-risk groups, high-risk patients had a dismal prognosis, with a significantly poorer outcome and more central nervous system (CNS) relapses when compared to *NOTCH*-WT patients (Clappier *et al.*, 2010).

A recent study has shown in a mouse model that the NOTCH1 receptor positively controls expression of the chemokine receptor CCR7, which is required for targeting T-ALL cells to the CNS (Buonamici *et al.*, 2009). Mutations in *NOTCH1* resulting in constitutive activation of the NOTCH1 pathway upregulated CCR7 expression, therefore leukaemic cells were targeted to the CNS at a higher rate. This could explain the higher rate of CNS relapse in *NOTCH1*-mutated high-risk patients treated on the EORTC trial and their subsequent dismal prognosis. However, CNS relapses are rare in BFM-treated patients in general but also in the high-risk group (Kox *et al.*, 2010). The major difference in the treatment protocols in these two studies is the use of prophylactic cranial radiation in the BFM trial, suggesting that this

may play a role in the prevention of CNS relapse in the *NOTCH1* mutant high-risk T-ALL patients (Kox *et al.*, 2010).

This chapter reports the results of an investigation into the impact of *NOTCH1* and *FBXW7* mutations in a cohort of paediatric patients with T-ALL treated on the UKALL 2003 trial. Other studies have investigated the impact according to the presence of individual *NOTCH1* mutations or the combined *NOTCH1* and/or *FBXW7*-mutated group. However, as described in chapter 3, double mutations in *NOTCH1*, such as an HD domain mutation coupled with a PEST domain mutation, also synergise to increase NOTCH1 signal strength. Therefore in the present study, the $NOTCH1^{Double}FBXW7^{WT}$ group was considered jointly with the $NOTCH1^{MUT}FBXW7^{MUT}$ group as they both lead to synergistic *NOTCH1* activation and are predicted to be functionally equivalent. The cohort was therefore divided into three defined genotype groups prior to analysis, WT for both genes ($NOTCH1^{WT}FBXW7^{WT}$), single *NOTCH1*-mutated alone ($NOTCH1^{Single}FBXW7^{WT}$), and $NOTCH1^{Double}FBXW7^{WT}$ or $NOTCH1^{MUT}FBXW7^{MUT}$ (hereafter called $NOTCH1{\pm}FBXW7^{Double}$).

4.2 Materials and Methods

4.2.1 Patients

As detailed in chapter 3, diagnostic samples from 162 patients entered into the MRC UKALL2003 trial were available for *NOTCH1* and *FBXW7* mutation screening. The trial was opened to patients aged 1-18 years in 2003. The age limit was increased to 20 years in 2006, and 25 years in 2007. Median age of the patients studied was 9 years (range 1-23); 23 were over 16 years, 3 of these over 20 years; 123 were male and 39 female. Patients with bi-phenotypic leukaemia or T-cell lymphoma were excluded. The clinical information for this study was made available by the Clinical Trial Service Unit, Richard Doll Building, University of Oxford, and all statistical analyses were performed by the trial statisticians, Rachel Wade and Sue Richardson.

4.2.2 MRC UKALL 2003 trial protocol

Details of the trial protocol have been published elsewhere (Qureshi *et al.*, 2010). There are three phases of treatment: induction, consolidation and maintenance, with patients assigned to one of three regimens at diagnosis. Details of the drugs used and timing schedules for the three regimens are shown in outline in Figure 4.1. At trial entry, induction therapy was

Regimen A

Induction	Consolidation	Maintenance
Vincristine Dexamethasone Asparaginase	6-Mercaptopurine Dexamethasone	6-Mercaptopurine Dexamethasone
1-5 weeks	**6-38 weeks**	**39-112 weeks girls** **39-164 weeks boys**

Regimen B

Induction	Consolidation	Maintenance
Vincristine Dexamethasone Asparaginase Daunorubicin	6-Mercaptopurine Dexamethasone	6-Mercaptopurine Dexamethasone
1-5 weeks	**6-40 weeks**	**41-114 weeks girls** **41-166 weeks boys**

Regimen C

Induction	Consolidation	Maintenance
Vincristine Dexamethasone Asparaginase Daunorubicin	6-Mercaptopurine PEG asparaginase Methotrexate	6-Mercaptopurine Dexamethasone
1-5 weeks	**6-46 weeks**	**47-118 weeks girls** **47-170 weeks boys**

Figure 4.1 Outline of the MRC UKALL 2003 trial protocol. Drugs used and timing schedules for the three regimens.

allocated according to the National Cancer Institute (NCI) risk score. Patients with low-risk disease (age 1-10 years and WBC $<50x10^9$/L) were allocated to a 3-drug induction regimen with vincristine, dexamethasone and asparaginase (regimen A); high-risk patients (age >10 years and/or WBC $>50x10^9$/L) were allocated to receive additional daunorubicin (regimen B). Patients who had a morphological slow early response (SER, defined as >25% bone marrow blasts at day 8 or 15 for high- and low-risk patients respectively), and were less than 16 years of age were then assigned to 'augmented BFM' therapy (regimen C) and received additional asparaginase during the consolidation and delayed intensification phases. At day 29, patients not in morphological remission but with <25% bone marrow blasts were also reallocated to regimen C. If resistant disease was present (>25% bone marrow blasts), patients were taken off protocol. MRD was assessed in those with a rapid early response (RER) in morphological remission at day 29 using PCR analysis of leukaemia clone-specific rearranged TCRγ and δ genes as previously described (Goulden *et al.*, 1998). If MRD of more than 1 leukaemic cell in 1000 cells ($>10^{-3}$) was present, patients were classed as MRD-positive and were then randomised to either remain on their assigned regimen (A or B) or to be reallocated to regimen C. Of the 162 patients included in this study, 14 patients were treated on regimen A, 92 on B and 56 on C (Figure 4.2).

4.2.3 Cytogenetic and FISH analysis

Cytogenetic and FISH analyses were made available by the Clinical Trial Service Unit and were performed on pre-treatment samples using standard methodologies. They were reported and reviewed as previously described (Moorman *et al.*, 2010;Harrison *et al.*, 2001). FISH testing for *CDKN2A/B, MLL, AF10/CALM, TLX1, TLX3, BCL11B, TAL1, LMO1, LMO2, LYL1* and *TCR* α/δ, γ/β abnormalities were performed using a combination of commercial and home-grown probes as previously reported (Harrison *et al.*, 2005;Sulong *et al.*, 2009;Marks *et al.*, 2009). Classification of patients into the four genetic T-ALL subgroups (rearrangements of *HOXA, TAL1/LMO, TLX1* and *TLX3*) was based on a combination of cytogenetic and FISH data and according to the definition outlined by Van Vlierberghe *et al* (2008).

4.2.4 Clinical end points

Outcome was analysed according to overall survival (OS), defined as the time to death, event-free survival (EFS), which was the time to relapse, secondary tumour or death, and rate of relapse (RR), which was the time to relapse for those that achieved remission, censoring at death in remission.

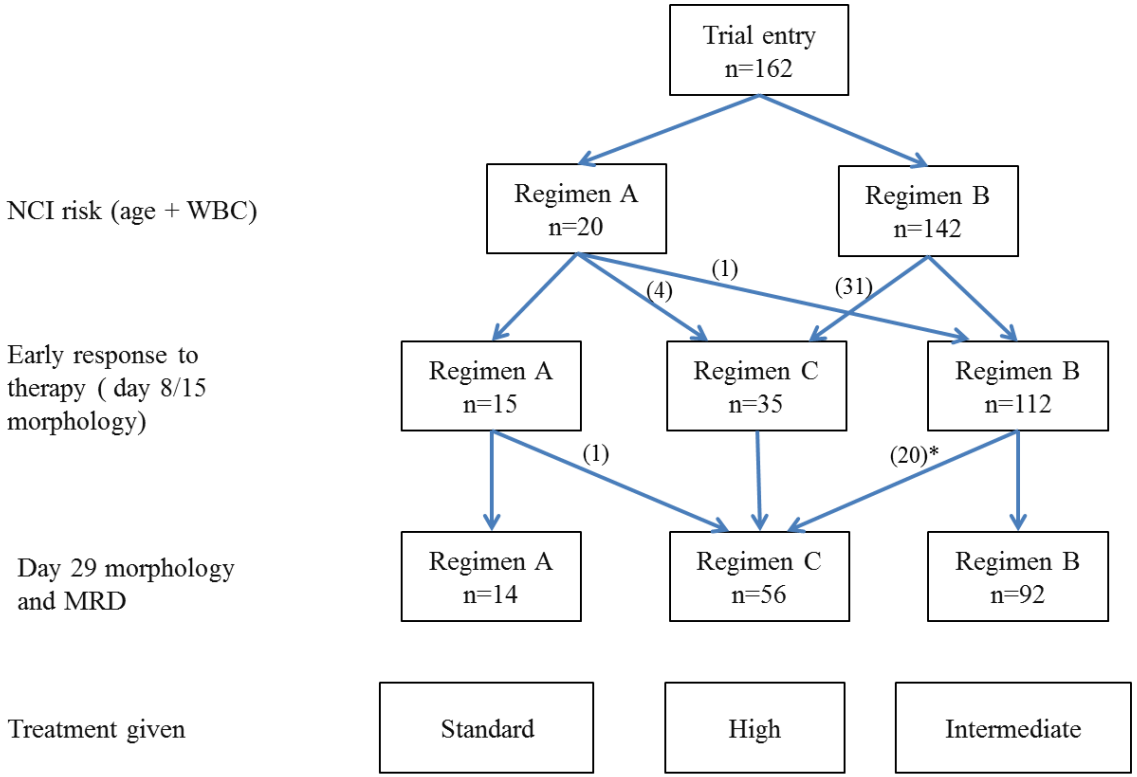

Figure 4.2 Schema of the therapy given to the 162 patients included in this study. NCI, National Cancer Institute; WBC, white blood cell count; MRD, minimal residual disease.

4.2.5 Statistical analysis

Statistical analysis of the UKALL2003 cohort was performed by Rachel Wade and Sue Richards at the Clinical Trial Service Unit, Oxford UK. Chi-squared tests were used to test for differences across the patient molecular groups in categorical subgroups such as gender, genetic subgroup, NCI risk group, SER and MRD, excluding any missing data categories. *P*-values are quoted for heterogeneity and when determining the statistical significance in response to therapy across the three genotype groups, *P*-values are also quoted for trend. Due to small sample size Fisher's exact test was used to test for differences in the incidence of CNS relapse according to genotype. The non-parametric Kruskall-Wallis test was used to determine statistical significance between patient genotype and continuous variable subgroups including WBC and age. Kaplan-Meier curves were used to assess survival, and differences between groups were compared using the log-rank test. Multivariate logistic regression models were used to analyse SER and MRD, and time to event was modelled using Cox regression. *P* values are for Wald chi-squared tests for the effect of *NOTCH1/FBXW7* genotype, excluding OS where the likelihood ratio test is also presented due to no deaths in the *NOTCH1±FBXW7*^{Double} group. Hazard ratios (HRs) and 95% confidence intervals (CI) are quoted for all main endpoints. In all cases, a ratio <1 indicates a benefit for a *NOTCH1/FBXW7* mutation. All *P* values quoted are two-sided, $P<0.5$ was considered statistically significant. The decision to divide the patients according to the three defined genotype groups was made prior to analysis.

4.3 Results

Surviving patients in the cohort of 162 analysed for *NOTCH1* and *FBXW7* mutations were censored on 31st October 2011. Median follow up for survivors in this cohort was 4 years and 6 months (range 2 years 4 months to 7 years 9 months). When the cohort was divided into the three defined genotype groups, 57 patients (35%) were WT for both genes (*NOTCH1*^{WT}*FBXW7*^{WT}), 62 patients (38%) were single *NOTCH1*-mutated alone (*NOTCH1*^{Single}*FBXW7*^{WT}), and 39 patients (24%) were either *NOTCH1*^{Double}*FBXW7*^{WT} (n=14) or *NOTCH1*^{MUT}*FBXW7*^{MUT} ie. *NOTCH1±FBXW7*^{Double}. There were also four patients (3%) with an *FBXW7* mutation only.

4.3.1 Characteristics of T-ALL patients according to genotype

Compared with the 226 T-ALL patients treated on the UKALL 2003 trial who did not have molecular data, there were no significant differences in sex, WBC, NCI risk group or CNS disease at diagnosis (Table 4.3). In the 226 T-ALL patients entered into the trial without molecular data the median age was 11 years (range 1-24 years); the 162 patients with molecular data in the present cohort were slightly younger (*P*=.005), the median age was 9 years (range 1-23 years).

The characteristics of the patients studied are given in Table 4.4. There was no significant difference in sex, WBC, age group, CNS disease or NCI risk group between the three combined genotype groups, excluding the four patients with *FBXW7* mutations alone, nor in cytogenetic characteristics.

4.3.2 Response to glucocorticoid therapy by *NOTCH1/FBXW7* genotype

After the initial stratification at the time of trial entry based on age and WBC count, patients are further stratified by the level of clearance of the blasts in the blood or marrow after one or two weeks of induction therapy. This response to glucocorticoid therapy is assessed morphologically on day 8 for patients initially treated on Regimen B and day 15 for patients on Regimen A. A rapid early response (RER) is defined as ≤25% blasts in the marrow, a slow early response (SER) as >25% blasts present in the marrow.

Early response to therapy was first correlated with the presence or absence of mutations in individual genes. Of the 101 patients with a *NOTCH1* mutation, 80 (79%) had an RER and 21 (21%) an SER. Of the 61 patients lacking a *NOTCH1* mutation, 40 (66%) had an RER and 21 (34%) an SER (Table 4.5). This difference was of borderline significance (*P*=.06), which may suggest that patients with a *NOTCH1* mutation were more likely to be associated with an RER than those without a *NOTCH1* mutation. *FBXW7* mutational status alone had no significant effect on early response. Of the 29 patients with an *FBXW7* mutation, 25 (86%) had an RER and 4 (14%) had an SER, whereas of the 133 patients without an *FBXW7* mutation, 95 (71%) had an RER and 38 (29%) had an SER (Table 4.5). This was not statistically significant (*P*=.1) however, the number of patients with an *FBXW7* mutation was low.

Table 4.3 Characteristics of T-ALL patients with and without molecular data

Characteristic	Patients with data	%	Patients without data	%	*P*[1]
Gender					0.2
Male	123	76%	159	70%	
Female	39	24%	67	30%	
Age Group (years)					0.1
<10	88	54%	99	44%	
10-15	51	32%	87	38%	
≥16	23	14%	40	18%	
Median	9	N/A	11	N/A	0.005[2]
Range	1-23	N/A	1-24	N/A	
WBC at diagnosis ($x10^9$/L)					0.2
<50	50	31%	88	39%	
50-99	25	15%	37	16%	
≥100	87	54%	101	45%	
NCI risk group					0.2
Low	20	12%	38	17%	
High	142	88%	188	83%	
CNS disease at diagnosis					0.4
No	154	95%	210	93%	
Yes	8	5%	16	7%	
5 year event-free survival, % (95% CI)	84% (79%-90%)	N/A	80% (74%-80%)	N/A	0.3[3]

P values: [1]Chi-squared test, [2]Kruskal-Wallis test, [3]Log-rank test.
Abbreviations: WBC, white blood cell count; NCI, National Cancer Institute; CNS, central nervous system; N/A, not applicable; CI, confidence intervals.

Table 4.4 Characteristics of T-ALL patients investigated, grouped according to *NOTCH1/FBXW7* genotype

Subgroup	Total[#]	*NOTCH1*^{WT}*FBXW7*^{WT}		*NOTCH1*^{Single}*FBXW7*^{WT}		*NOTCH1±FBXW7*^{Double}		*P**
		Total no.	%	Total no.	%	Total no.	%	
Gender								0.5
Male	119	40	70%	48	77%	31	79%	
Female	39	17	30%	14	23%	8	21%	
WBC (x10^9/L)								0.8
<50	47	16	28%	17	28%	14	36%	
50-99	25	7	12%	12	19%	6	15%	
≥100	86	34	60%	33	53%	19	49%	
Median count	110.5	131.0	N/A	104.8	N/A	90.0	N/A	0.5**
Range	0.5-881.0	0.5-881.0	N/A	2.5-783.1	N/A	6.4-777.0	N/A	
Age group (years)								0.8
<10	85	31	54%	35	56%	19	49%	
10-15	50	16	28%	19	31%	15	38%	
≥16	23	10	18%	8	13%	5	13%	
Median	9	9	N/A	8	N/A	11	N/A	0.3**
Range	1-23	1-23	N/A	1-23	N/A	2-18	N/A	
CNS disease								0.7
No	150	55	96%	58	94%	37	95%	
Yes	8	2	4%	4	6%	2	5%	
NCI risk group								0.6
Low	18	7	12%	6	10%	5	13%	
High	140	50	88%	56	90%	34	87%	

Table 4.4 Continued

Subgroup	Total[#]	*NOTCH1*^{WT}*FBXW7*^{WT}		*NOTCH1*^{Single}*FBXW7*^{WT}		*NOTCH1*±*FBXW7*^{Double}		*P**
		Total no.	%	Total no.	%	Total no.	%	
Cytogenetics								0.4[†]
Normal	31	10	18%	14	23%	7	18%	
Abnormal	94	41	72%	30	48%	23	59%	
Failed	27	4	7%	16	26%	7	18%	
Missing	6	2	3%	2	3%	2	5%	
Genetic subgroup								0.6
HOXA	9	4	7%	2	3%	3	8%	
TAL/LMO	30	11	19%	12	19%	7	18%	
TLX1	3	1	2%	2	3%	0	0%	
TLX3	8	3	5%	1	2%	4	10%	
Missing	108	38	67%	45	73%	25	64%	
CDKN2A/B deletion								0.99
No	38	14	25%	15	24%	9	23%	
Yes	37	13	23%	15	24%	9	23%	
Missing	83	30	52%	32	52%	21	54%	

[#]Excludes 4 patients with *NOTCH1*^{WT}*FBXW7*^{MUT} genotype.

**P* values: unless otherwise indicated these are given for chi-squared test for categorical subgroups across all 3 molecular groups, excluding any missing data categories. **Kruskal-Wallis test for continuous variable subgroups for WBC and age. [†]Normal versus abnormal.

Abbreviations: WT, wild-type; MUT, mutant; WBC, white blood cell count; CNS, central nervous system; NCI, National Cancer Institute; N/A, not applicable.

Early response to therapy was then correlated to the three genotype groups, excluding the four patients with mutations in *FBXW7* alone. The frequency of an RER was highest in *NOTCH1*±*FBXW7*^{Double} patients and this was of borderline significance (82%, 77%, 40% for *NOTCH1*±*FBXW7*^{Double}, *NOTCH1*^{Single}*FBXW7*^{WT} and *NOTCH1*^{WT}*FBXW7*^{WT} respectively, *P* for trend across all groups =.08) (Table 4.5). When multivariate analysis was performed considering the known prognostic factors for T-ALL, i.e. age and WBC, genotype was not a significant prognostic marker of early response to therapy (Table 4.6). Although the hazard ratios (HR) in both the *NOTCH1*^{Single}*FBXW7*^{WT} group (HR=0.65, 95% confidence intervals (CI) = 0.27-1.40) and the *NOTCH1*±*FBXW7*^{Double} group (HR=0.45, 95% CI = 0.16-1.21) suggest that patients with a mutation are less likely to have an SER, the 95% CI for each group are broad and there is no statistical difference when compared to patients with no mutation.

4.3.3 MRD status according to *NOTCH1/FBXW7* genotype

The MRD status at day 29 post diagnosis was provided by Dr. Nick Goulden at Great Ormond Street Hospital, one of the UKALL2003 trial co-ordinators, and was available for 151 of the 162 paediatric patients. Patients with residual disease at the level of one positive cell in 1000 cells were considered MRD-positive, i.e. unfavourable disease, and those with levels of disease below this were considered MRD-negative, i.e. favourable disease. MRD status was first correlated with the presence or absence of mutations in individual genes. Of the 97 patients with a *NOTCH1* mutation, 57 (59%) had favourable and 40 (41%) unfavourable disease, whereas of the 54 patients lacking a *NOTCH1* mutation, 21 (39%) had favourable and 33 (61%) unfavourable disease (Table 4.5). This difference was statistically significant (*P*=.02), indicating that patients with a *NOTCH1* mutation were more likely to be associated with a low level of MRD than those without a *NOTCH1* mutation. *FBXW7* mutational status alone had no significant effect on MRD. Of the 29 patients with an *FBXW7* mutation, 17 (58%) had favourable and 12 (41%) unfavourable disease, whereas of the 122 patients without an *FBXW7* mutation, 61 (50%) had favourable and 61 (50%) unfavourable disease (Table 4.5). This was not statistically significant (*P*=.4), but there were only a small number of patients with an *FBXW7* mutation. MRD status was then assessed according to the number of *NOTCH1* mutations or the combination of *NOTCH1* and *FBXW7* mutations, excluding the four patients with mutations in *FBXW7* alone.

Table 4.5 Response to chemotherapy and survival status according to *NOTCH1/FBXW7* genotype

		***NOTCH1* genotype**			***FBXW7* genotype**			***NOTCH1+FBXW7* genotype[#]**				
	Total	WT	MUT	*P**	WT	MUT	*P**	Total	*NOTCH1*^{WT} *FBXW7*^{WT}	*NOTCH1*^{Single} *FBXW7*^{WT}	*NOTCH1*± *FBXW7*^{Double}	*P**
Slow early response												
No	120	40 (66%)	80 (79%)	0.06	95 (71%)	25 (86%)	0.1	118	38 (67%)	48 (77%)	32 (82%)	0.2
Yes	42	21 (34%)	21 (21%)		38 (29%)	4 (14%)		40	19 (33%)	14 (23%)	7 (18%)	0.08**
MRD at day 29												
Negative	78	21 (39%)	57 (59%)	0.02	61 (50%)	17 (58%)	0.4	77	20 (40%)	30 (51%)	27 (71%)	0.01
Positive	73	33 (61%)	40 (41%)		61 (50%)	12 (41%)		70	30 (60%)	29 (49%)	11 (29%)	0.005**
CNS relapse												
No	153	60 (98%)	93 (92%)	0.2$^{\Delta}$	125 (94%)	28 (97%)	0.99$^{\Delta}$	149	56 (98%)	56 (90%)	37 (95%)	0.2
Yes	9	1 (2%)	8 (8%)		8 (6%)	1 (3%)		9	1 (2%)	6 (10%)	2 (5%)	0.4**
Outcome at 5 years, % (95% CI)												
RR	161	16% (6%-26%)	11% (5%-17%)	0.4†	15% (9%-21%)	4% (0%-10%)	0.08†	157	17% (7%-28%)	15% (6%-24%)	5% (0%-12%)	0.1†
EFS	162	80% (69%-90%)	87% (80%-94%)	0.1†	82% (76%-89%)	93% (84-100%)	0.1†	158	78% (67%-89%)	84% (75%-93%)	92% (84%-100%)	0.04†
OS	162	83% (73%-93%)	93% (88%-98%)	0.06†	87% (81%-93%)	100%	0.02†	158	82% (71%-92%)	88% (80%-96%)	100%	0.005†

[#]Excludes 4 patients with *NOTCH1*^{WT}*FBXW7*^{MUT} genotype.**P* values: unless otherwise indicated these are for chi-squared test for heterogeneity across all 3 molecular groups. **Trend for *NOTCH1+FBXW7* genotype. $^{\Delta}$Fisher's exact test due to small numbers. †Log-rank test. Abbreviations: WT, wild-type; MUT, mutant; MRD, minimal residual disease; CNS, central nervous system; RR, relapse risk; EFS, event-free survival; OS, overall survival; CI, confidence interval.

Favourable and unfavourable disease were observed in 27 (71%) and 11 (29%) of the 38 *NOTCH1±FBXW7*^{Double} patients respectively, 30 (51%) and 29 (49%) of the 59 *NOTCH1*^{Single}*FBXW7*^{WT} patients, and 20 (40%) and 30 (60%) of the 50 patients with mutations in neither gene (*NOTCH1*^{WT}*FBXW7*^{WT}). The frequency of MRD-negativity was highest in *NOTCH1±FBXW7*^{Double} patients and *P* for trend =.005 (Table 4.5). Of the 11 patients without MRD data, seven were *NOTCH1*^{WT}*FBXW7*^{WT}, three *NOTCH1*^{Single}*FBXW7*^{WT} and one *NOTCH1±FBXW7*^{Double}. When multivariate analysis was performed with the variables age and WBC included in the analysis, the genotype was a highly significant prognostic indicator of MRD status at day 29 (*P*=.0003) (Table 4.6).

4.3.4 Clinical outcome by *NOTCH1/FBXW7* genotype

Outcome data was available for all 162 patients. There was no significant difference in the EFS at 5 years from the 226 excluded patients without molecular data (84% versus 80%, *P*=.3) (Table 4.3).

There was no significant difference between *NOTCH1*^{MUT} and *NOTCH1*^{WT} patients in RR and EFS at 5 years, 11% versus 16% (log-rank *P*=.4) and 87% versus 80% (*P*=.1) respectively, although *NOTCH1*^{MUT} patients did show a trend for improved OS (93% versus 83%, *P*=.06) (Figure 4.3 A-C). Outcome for *FBXW7*^{MUT} patients was generally better than *FBXW7*^{WT} patients, RR 4% versus 15% respectively (*P*=.08), EFS 93% versus 82% (*P*=.1), and OS 100% versus 87% (*P*=.02) (Figure 4.4 A-C). Of 29 *FBXW7*^{MUT} patients, only one relapsed and none have died. In the combined genotype groups, there was a non-significantly lower RR according to the number of mutations, 17%, 15%, 5% for *NOTCH1*^{WT}*FBXW7*^{WT}, *NOTCH1*^{Single}*FBXW7*^{WT} and *NOTCH1±FBXW7*^{Double} patients respectively (*P* for trend =.1), and significantly improved EFS (78%, 84%, 92%; *P*=.04) and OS (82%, 88%, 100%; *P*=.005) (Figure 4.5 A-C). In a multivariate analysis, this significantly better OS was independent of age and WBC (*P*=.004), HRs and CIs for the *NOTCH1*^{Single}*FBXW7*^{WT} and *NOTCH1±FBXW7*^{Double} were 0.06 (95% CI = 0.007-0.54) and 0.02 (95% CI =0.002-0.16) respectively, therefore genotype is a significant prognostic indicator of improved OS (Table 4.6). Many of the 39 *NOTCH1±FBXW7*^{Double} patients had characteristics associated with high-risk disease, 20 (51%) were 10 years or older, 25 (64%) had a presenting WBC >50x10^9/L, with 34 (87%) having NCI high-risk disease (Table 4.4). Furthermore, six (15%) of this group, were NCI high-risk and had an SER, and 12 (31%) had high-level MRD at day 29. Nevertheless, despite the presence of these poor risk factors, only two of the 39 relapsed and both remained alive at the analysis end-point, one had a cord blood transplant, the other was transferred to the EURO-LB 02 protocol.

Table 4.6 Multivariate analysis of outcome with age and WBC as continuous variables

Outcome Variable	Hazard ratio (95% CI)			*P**
	NOTCH1^{WT}*FBXW7*^{WT}	*NOTCH1*^{Single}*FBXW7*^{WT}	*NOTCH1±FBXW7*^{Double}	
SER	1.00	0.65 (0.27-1.40)	0.45 (0.16-1.21)	0.2
MRD	1.00	0.06 (0.007-0.54)	0.02 (0.002-0.16)	0.0003
RR	1.00	0.93 (0.37-2.39)	0.29 (0.06-1.35)	0.3
EFS	1.00	0.68 (0.30-1.56)	0.31 (0.09-1.08)	0.2
OS	1.00	0.61 (0.23-1.60)	0 (No events)	0.6 0.004**

**P* values: unless otherwise indicated these are for Wald chi-square tests for the effect of *NOTCH1/FBXW7* genotype. **Likelihood ratio due to no deaths in *NOTCH1±FBXW7*^{Double} genotype group. Abbreviations: WT, wild-type; SER, slow early response; MRD, minimal residual disease; RR, relapse risk; EFS, event free survival; OS, overall survival; CI, confidence interval.

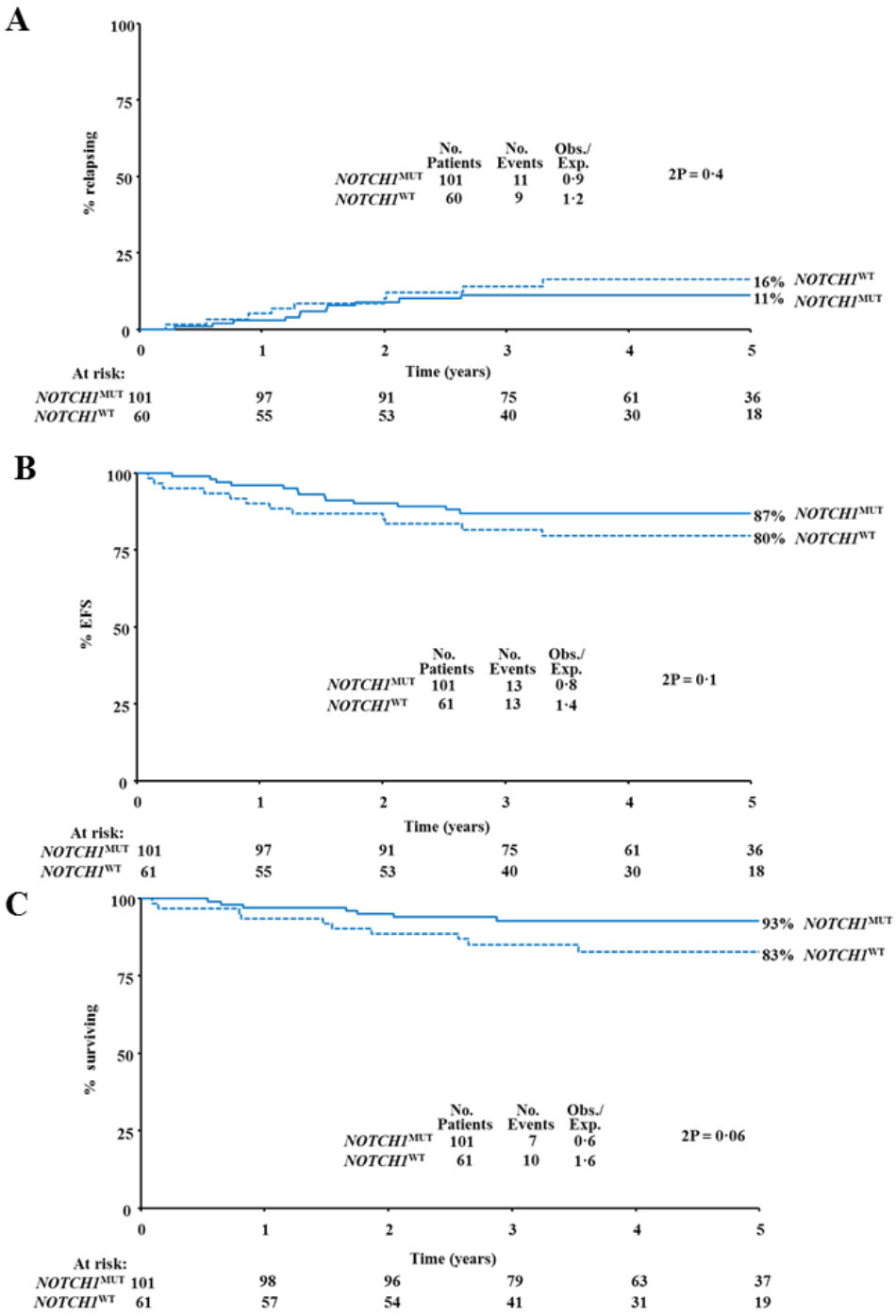

Figure 4.3 The impact of *NOTCH1* mutant status on clinical outcome. Kaplan-Meier curves stratified by *NOTCH1*-mutant (MUT) versus *NOTCH1*-wildtype (WT) status. (A) Relapse rate, (B) Event-free survival, (C) Overall survival.

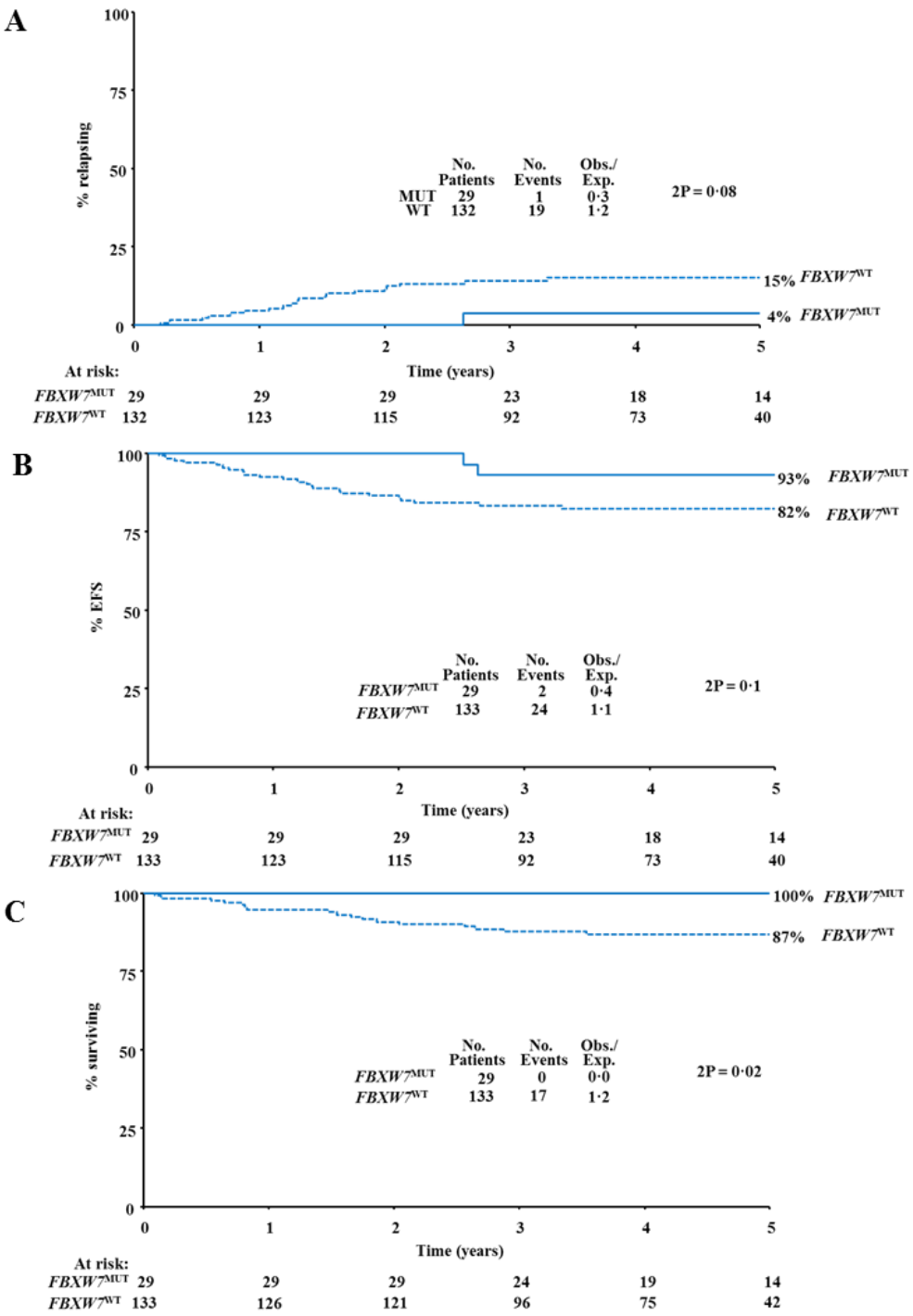

Figure 4.4 The impact of *FBXW7* mutant status on clinical outcome. Kaplan-Meier curves stratified by *FBXW7*-mutant (MUT) versus *FBXW7*-wildtype (WT) status. (A) Relapse rate, (B) Event-free survival, (C) Overall survival.

In other studies, the impact of a *NOTCH1* mutation has differed according to risk group. In the ALL-BFM study, the favourable outcome associated with *NOTCH1* mutations was restricted to those patients with a good response to induction therapy, with no difference in the high-risk group of patients who had either an SER or were MRD-positive (Kox *et al.*, 2010). The EORTC study reported a very poor prognosis in *NOTCH1*^{MUT} high-risk patients, and a high number of CNS relapses in *NOTCH1*^{MUT} patients (Clappier *et al.*, 2010). To examine this in our cohort, outcome was analysed according to genotype in a high-risk group of 85 patients with either an SER or MRD-positive disease and a low risk group of 73 patients with an RER and either MRD-negative disease or MRD status unknown. The OS for the *NOTCH1*±*FBXW7*^{Double} patients did not differ according to risk group, all patients were still alive (Figure 4.6 A-B), and there was some evidence that the favourable impact of the genotype was greater in the high-risk than the low-risk group (*P* for heterogeneity =.05). This difference was not seen for RR or EFS. Of the 14 high-risk double-mutated patients, none had received a transplant; one was treated on regimen A (low/standard risk), five on regimen B (intermediate-risk) and eight on regimen C (high-risk). There was no difference in the incidence of CNS relapse across the three genotype groups (*P*=.2); the highest incidence (10%) was in the *NOTCH1*^{Single}*FBXW7*^{WT} group (Table 4.5).

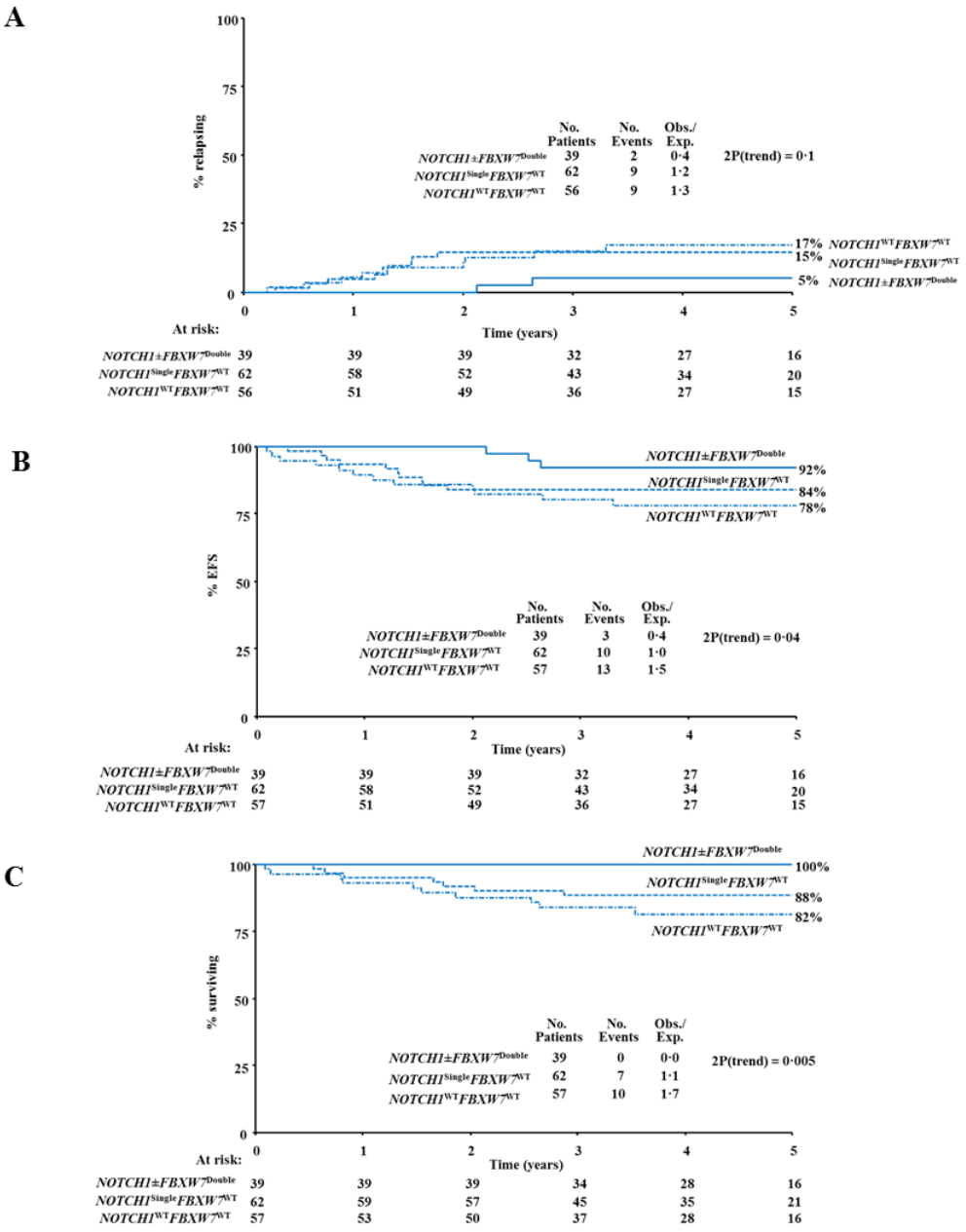

Figure 4.5 The impact of *NOTCH1 /FBXW7* mutant status on clinical outcome. Kaplan-Meier curves stratified by *NOTCH1* and *FBXW7* genotype . (A) Relapse rate, (B) Event-free survival, (C) Overall survival.

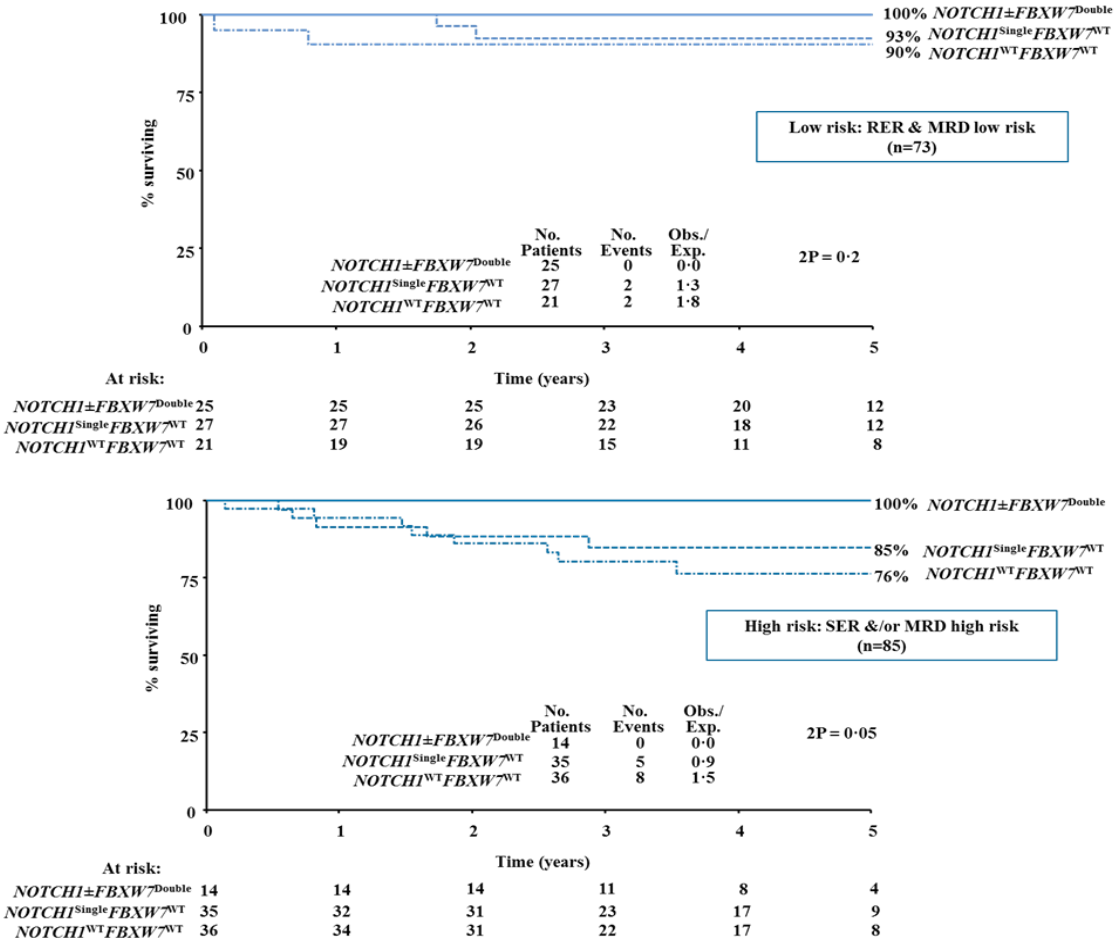

Figure 4.6 The impact of *NOTCH1/FBXW7* genotype on overall survival. Low-risk patients with an RER to induction therapy and MRD-negative, High-risk patients with an SER to therapy and/or MRD-positive.

4.4 Discussion

Data is presented in this chapter on the investigation of *NOTCH1*- and *FBXW7*-mutated T-ALL, in terms of clinical characteristics and the impact of the mutations on response to therapy and long-term outcome, in a cohort of 162 paediatric patients with T-ALL treated on the UKALL 2003 trial. As previously described in chapter 3, *NOTCH1* and *FBXW7* mutations are a frequent occurrence in T-ALL, with a mutation incidence in the present cohort of 62% and 18% respectively. In the eight paediatric studies that have investigated the impact of mutations on patient outcome, all divided their respective cohorts according to the presence of mutations in the *NOTCH1* gene only or in a combined *NOTCH1* and/or *FBXW7*-mutated group (Clappier *et al.*, 2010;Erbilgin *et al.*, 2010;Fogelstrand *et al.*, 2014;Kox *et al.*, 2010;Larson Gedman *et al.*, 2009;Mansur *et al.*, 2012;Park *et al.*, 2009;Zuurbier *et al.*, 2010). In these studies therefore, patients with double mutations in the HD and PEST domains of *NOTCH1* were grouped with patients harbouring single mutations in the gene. However, co-incident mutations in the HD and PEST domain of *NOTCH1*, and mutations in both the *NOTCH1* and *FBXW7* genes, synergise to increase NOTCH1 signal strength and these combinations of mutations are predicted to be functionally equivalent. Therefore in the present study, the cohort was divided into three defined genotype groups prior to analysis, 57 patients (35%) WT for both genes ($NOTCH1^{WT}FBXW7^{WT}$), 62 patients (38%) single *NOTCH1*-mutated alone ($NOTCH1^{Single}FBXW7^{WT}$), and 39 patients (24%) $NOTCH1{\pm}FBXW7^{Double}$. There were also four patients (3%) with an *FBXW7* mutation only which were excluded from analysis due to low numbers.

When comparing the clinical characteristics across the three genotype groups, there was no significant difference in sex, WBC, age group, CNS disease or NCI risk group. This is in line with five of the eight paediatric studies (Breit *et al.*, 2006;Clappier *et al.*, 2010;Fogelstrand *et al.*, 2014;van Grotel *et al.*, 2008;Zuurbier *et al.*, 2010), however three reported the association of *NOTCH1* and/or *FBXW7* mutations with decreased WBC count at diagnosis (Erbilgin *et al.*, 2010;Mansur *et al.*, 2012;Park *et al.*, 2009). Of note, these studies consisted of non-European cohorts and reported an overall lower frequency of mutations in patients, suggesting racial origin could explain the differences. The T-cell immunophenotype was not available for patients in the present cohort. Others have shown a significantly higher incidence of mutations in the cortical subgroup (CD1a positive) patients compared with the mature immunophenotype cases, suggesting that the oncogenic role of the NOTCH1 pathway is less prominent in T-ALL cases arrested at a later stage in T-cell development (Breit *et al.*, 2006;Zuurbier *et al.*, 2010). There was no association between genotype and

cytogenetic characteristics in the present cohort. However, the proportion of patients in which the test results either failed or were missing for cytogenetics, genetic subgroup and *CDKN2A/B* deletion analysis was 21%, 68% and 53% respectively. Therefore, the number of patients harbouring the cytogenetic abnormalities in each group was small, making comparison between the genotypes difficult. In other studies, *NOTCH1* and/or *FBXW7* mutations were more frequently associated with rearrangements of the *TLX3* gene and less frequently associated with *TAL/LMO* rearranged cases (Zuurbier *et al.*, 2010). In one study where *NOTCH1*-mutated patients in an adult cohort had an improved outcome, the mutations were associated with high expression of *TLX1* (20 of 21 patients with high expression of *TLX1* had a *NOTCH1* mutation) (Asnafi *et al.*, 2009). Up-regulation of *TLX1* has also been reported to be associated with an improved prognosis independently of *NOTCH1* mutations (Ferrando *et al.*, 2004), indicating that the importance of NOTCH1 activation during leukaemogenesis and its influence on the outcome of patients may also depend on other factors and collaborating genetic rearrangements.

Knowledge of clearly defined prognostic markers at diagnosis in T-ALL could help to refine current risk stratification strategies that are based on response to therapy, and inform clinical decisions, in particular which patients should proceed to allogeneic stem cell transplantation. When correlating mutational status to response to glucocorticoid therapy in the present cohort, there was a borderline significant trend for patients with a *NOTCH1* mutation to be more likely associated with a RER than those without a *NOTCH1* mutation. *FBXW7* mutational status alone had no significant effect on response, most likely because the patient numbers in the group were low. In the three defined genotype groups, the frequency of a RER was highest in *NOTCH1*±*FBXW7*$^{\text{Double}}$ patients, and again this was borderline significant. However, in other studies, *NOTCH1* and/or *FBXW7* mutations were significantly associated with an RER (Clappier *et al.*, 2010;Kox *et al.*, 2010;Zuurbier *et al.*, 2010). One reason why the difference did not reach significance in the present study could be the specific glucocorticoid used in induction therapy. Glucocorticoids are a class of steroid hormone that play an important role in the treatment of lymphoid malignancies because of their ability to induce apoptosis in lymphoid progenitor cells (Real *et al.*, 2009;Tissing *et al.*, 2003). Dexamethasone is the glucocorticoid used in the UKALL 2003 protocol, whereas other trials use prednisone, and therefore *NOTCH1* and/or *FBXW7* mutated T-ALLs may be less sensitive to dexamethasone.

The association of *NOTCH1* activating mutations with a good response to glucocorticoid therapy in patients is unexpected. It has previously been shown that activated NOTCH1 can prevent certain T-ALL cell lines from undergoing apoptosis induced by dexamethasone by

upregulating anti-apoptotic proteins (Deftos *et al.*, 1998;Sade *et al.*, 2004). More recently, NOTCH1 signalling has been linked to glucocorticoid resistance in a study showing that γ-secretase inhibitors can sensitise for response to glucocorticoids in glucocorticoid-resistant cells in which HES1, a downstream target of NOTCH1, represses glucocorticoid receptor auto-upregulation (Real *et al.*, 2009). This favours resistance as it decreases the number of surface receptors, thereby reducing the apoptotic response. However, the opposite effect is seen in *NOTCH1* mutated T-ALL patients, where mutation is associated with an RER to glucocorticoid treatment, suggesting that the drug effectively kills the leukaemic cells in induction therapy. The effect may be due to the interaction of NOTCH1 with other drugs used. A recent study has also suggested that the glucocorticoid receptor is a novel substrate of FBXW7, and that mutation of the *FBXW7* gene renders it unable to sequester the receptor, resulting in increased apoptosis (Malyukova *et al.*, 2013). This may explain the RER seen in patients with *NOTCH1* and/or *FBXW7* mutations in other trials, as *FBXW7* mutant leukaemic cells are predicted to harbour more glucocorticoid receptors on the surface.

In the present study, patients in the *NOTCH1*±*FBXW7*^{Double} group had a significantly higher frequency of MRD-negativity at day 29 of induction therapy, suggesting that the level of NOTCH1 pathway activation is an influencing factor in response to treatment. The genotype was a highly significant prognostic indicator for response to therapy, independent of age and WBC. Two other paediatric studies also correlated genotype to levels of MRD and both reported the association of *NOTCH1* and/or *FBXW7* mutations with favourable levels of MRD at day 29 of induction therapy (Clappier *et al.*, 2010;Kox *et al.*, 2010). However, the results are not comparable between studies as different parameters are used to define the levels of MRD-positive and MRD-negative disease across the various trials. In the UKALL 2003 trial, MRD-negative disease is defined as $<10^{-3}$ leukaemic cells, whereas in the ALL-BFM and EORTC protocols it is classified as $<10^{-4}$ and $<10^{-2}$ leukaemic cells respectively, therefore a patient who has MRD-negative disease in the context of one trial, may not necessarily in another. For patients where the measurement of MRD is not available, it is possible that knowledge of the genotype could help to inform the clinical decision as to whether a switch of regimen is required. However, this information should be used with caution as approximately one third of patients with a *NOTCH1*±*FBXW7*^{Double} mutation were MRD-positive at day 29, and the excellent OS reported in this group could be as a result of the more intensive treatment in this subset of patients. Of note, five patients were treated on regimen A, 26 on regimen B and eight on regimen C.

When correlating genotype to long term outcome, analysed alone, *NOTCH1* mutations could not predict for improved outcome, but in combination with *FBXW7* mutations, patients in the

NOTCH1±*FBXW7*^{Double} genotype group had a significantly improved EFS and OS. Of the nine paediatric studies reporting the impact of *NOTCH1* and/or *FBXW7* mutations, only two reported an association between mutation and favourable long term outcome (Kox *et al.*, 2010;Park *et al.*, 2009), and in one of the studies this effect was seen in the *NOTCH1* mutated group only (Kox *et al.*, 2010). This suggests that the prognostic significance of the mutations in paediatric T-ALL may be trial-dependent. There was some evidence in the present cohort that the favourable impact on long term outcome of the genotype was greater in the high-risk than the low-risk group, which is in contrast to the outcome reported in high-risk *NOTCH1*^{MUT} patients on other trials (Clappier *et al.*, 2010;Kox *et al.*, 2010). In addition, there was no evidence in the present cohort that lack of cranial irradiation leading to increased CNS relapse was a contributory factor to outcome, as has been suggested by others (Kox *et al.*, 2010).

When outcome for these patients was stratified according to individual gene mutant status, only the presence of an *FBXW7* mutation had a significant effect on OS. This effect was not seen in other studies correlating long term outcome to *FBXW7* mutation alone (Clappier *et al.*, 2010;Erbilgin *et al.*, 2010;Fogelstrand *et al.*, 2014;Kox *et al.*, 2010;Park *et al.*, 2009), however the numbers of patients with an *FBXW7* mutation are relatively small across the studies. A recent study has shown that FBXW7 interacts with glucocorticoid receptor α by GSK3β mediated phosphorylation, and targets the receptor for proteosomal degradation (Malyukova *et al.*, 2013). Mutation of *FBXW7* promotes the stabilisation and activity of the glucocorticoid receptor, thereby enhancing glucocorticoid sensitivity. This could explain the trend towards a favourable outcome for the patients with an *FBXW7* mutation in the present study, only one patient relapsed however the total number of patients in the subgroup was small. The mutation could sensitise the leukaemic cells for dexamethasone, which is used throughout the duration of treatment in the induction, consolidation and maintenance therapies.

Therefore, in the present cohort of paediatric patients with T-ALL treated on the UKALL 2003 trial, the double-mutated group were associated with a much better response than either the *NOTCH1*^{Single}*FBXW7*^{WT} or *NOTCH1*^{WT}*FBXW7*^{WT} patients, in terms of clearance of leukaemic cells, EFS and OS. This could not be attributed to more favourable characteristics at diagnosis, as 87% were classified as NCI high-risk. Furthermore, despite the presence of adverse risk factors post-induction in about one-third of this genotype group (SER and/or MRD-positive in 14 of 39, 35%), only two high-risk *NOTCH1*±*FBXW7*^{Double} patients relapsed and all remain alive. The variable effects have been attributed to differences in treatment in the various trials (Ferrando, 2010), but differences in the combination of

mutations in the genotype groups may also be a significant factor. This may account for the differences in the present study, which is the first to consider patients that were either *NOTCH1*^{Double}*FBXW7*^{WT} or *NOTCH1*^{MUT}*FBXW7*^{MUT} as a single genotype group, as these combinations are thought to be functionally equivalent and induce synergistic NOTCH1 activation (Chiang *et al.*, 2008;O'Neil *et al.*, 2007;Weng *et al.*, 2004;Thompson *et al.*, 2007). Other studies have grouped the patients in a *NOTCH1* and/or *FBXW7* group, which could mean that the favourable effect on outcome of the patients with either a double mutation in *NOTCH1* or a mutation in both *NOTCH1* and *FBXW7* is masked by the patients with only a single *NOTCH1* mutation.

Current whole genome analyses have demonstrated that the genomic landscape for individual T-ALL patients is generally highly hetereogeneous (Remke *et al.*, 2009;Rudner *et al.*, 2011;Zhang *et al.*, 2012), and it is possible that the *NOTCH1*±*FBXW7*^{Double} patients have other collaborating mutations that are associated with better outcome or lack prognostically adverse factors. Nevertheless, determining the contribution of coincident mutations to outcome in T-ALL is likely to be challenging due to the relative rarity of the disease. Recent evidence has also indicated that significance of the mutation is context-dependent, with reports of activating *NOTCH1* mutations, frequently in the TAD and PEST domains, in both chronic lymphocytic leukaemia and mantle cell lymphoma being associated with aggressive disease and poor OS (Puente *et al.*, 2011;Kridel *et al.*, 2012;Fabbri *et al.*, 2011;Villamor *et al.*, 2013).

The results presented in this chapter suggest that knowledge of *NOTCH1/FBXW7* genotype may play a role in refining risk stratification by adding valuable information to morphological and MRD studies, to identify those patients in which intensification of treatment may be unnecessary. However, screening for *NOTCH1* mutations for clinical use presents a challenge. Replicating the method in the present study requires an individual PCR to screen each exon, followed by further separate analysis of each fragment by dHPLC and Sanger sequencing of abnormal chromatograms, meaning that this approach is time consuming. Also there are no amino acid hotspots in *NOTCH1* such as R465 in FBXW7, therefore the whole of each exon requires screening. Nevertheless, NGS technologies are also problematic as the technique generates relatively short sequence reads which are aligned to a reference sequence and highly efficient algorithms are employed to perform the mapping. This approach is successful for point mutations but the algorithms struggle particularly to accurately align reads containing small indels, which occured in 5% of patients in the present study, and can miss large insertions or duplications, which accounted for approximately 6% of mutations. NGS has been used to screen for *NOTCH1* mutations in

other studies, in particular CLL, where a group performed whole exome sequencing (WES) of all *NOTCH1* exons, and reported *NOTCH1* mutations in 4% of patients (Wang *et al.*, 2011b). This is contrast to three other studies who utilised Sanger sequencing to screen only the PEST domain in their respective cohorts of patients with CLL, all reported PEST mutations in 11% of patients (Balatti *et al.*, 2012;Rossi *et al.*, 2013;Villamor *et al.*, 2013). Whether or not the differences in the incidence of *NOTCH1* mutations between the respective studies are as a result of mutations being missed using NGS algorithms, or whether they are due to other factors such as the racial origin of the cohort is unclear. Screening could potentially be limited to patients who are classified as high-risk post-induction therapy, with *NOTCH1*±*FBXW7*$^{\text{Double}}$ patients not considered for further intensification therapy, including allogeneic stem cell transplantation. However, further investigation is clearly required to validate this finding in a larger cohort.

The data presented in this chapter shows the association of mutations in the *NOTCH1* and *FBXW7* genes with the clinical characteristics and long-term outcome of patients with T-ALL. Data on the association of the mutations with other molecular markers is studied in chapter 6.

CHAPTER 5: SCREENING FOR MUTATIONS IN THE *PTEN* GENE AND QUANTIFICATION OF MUTANT LEVEL

5.1 Introduction

In the 1980s, genetic studies on brain cancers showed the complete or partial loss of chromosome 10 (Bigner *et al.*, 1984). However, it was not until 1997, when homozygous deletions of chromosome 10 in glioma cell lines were mapped, that a novel candidate tumour suppressor gene was identified at the 10q23 locus named phosphatase and tensin homolog (*PTEN*) (Li *et al.*, 1997;Steck *et al.*, 1997). The role of PTEN as a tumour suppressor was further established in reports demonstrating frequent mutations of the *PTEN* gene in glioblastoma, prostate and breast cancer cell lines, and in studies where the presence of germline *PTEN* mutations in patients with Cowden disease, an autosomal dominant multiple hamartoma syndrome, increased the risk of the development of several cancers including breast and thyroid (Liaw *et al.*, 1997). Since then, much knowledge has been gained about the structure and function of PTEN and its roles in normal cellular processes.

5.1.1 Structure of PTEN

The *PTEN* gene has 9 exons; the N-terminus encodes the phosphatase domain and consists of exons 1-6, with exons 5 and 6 encoding the WPD, P and TI loops which make up the active site pocket. The C-terminus consists of exons 7-9 and encodes the C2 and PEST domains (Figure 5.1.A). The PTEN lipid phosphatase is ubiquitously expressed and is a 403 amino acid protein containing a catalytic signature motif HCXXGXXR, which is also present in the active sites of other protein tyrosine phosphatases (PTPs) (Denu *et al.*, 1996). However, PTEN has little sequence homology to PTPs outside of this motif, instead the N-terminal region containing the signature has homology to the actin-binding protein tensin, and the molecular chaperone regulating protein auxilin (Li *et al.*, 1997;Steck *et al.*, 1997).

Crystallography data has provided an insight into the structure of the PTEN protein (Lee *et al.*, 1999) (Figure 5.1B,C). The 179 amino acid N-terminal end contains the phosphatase domain, and the structure consists of a five-stranded β-sheet packaged with two α-helices on one side and four on the other. The signature motif at residues 123 to 130 forms the phosphate binding loop (P loop), which is structurally important and positioned at the bottom of the active site pocket. The other walls of the pocket are made from the WPD (TrpProAsp) loop at residues 91-94 and the TI (ThrIIe) loop at residues 42-52 and 163-166. The

Figure 5.1 Structure of PTEN. (A) The N-terminal domain of PTEN consists of the phosphatase domain. The C-terminal region consists of the C2 domain, PEST sequences and PDZ binding domain. (B) The phosphatase domain of PTEN contains the active site pocket composed of the P, TI and WPD loops. (C) The phosphatase and C2 domains are tightly linked at a phosphatase-C2 domain interface. (A) Taken from Chow *et al* (2006). (B) and (C) Taken from Song *et al* (2012).

incorporation of conserved threonine and isoleucine residues in the TI loop widens the active site pocket, which is critical for PTEN to be able to bind its substrates (Figure 5.1B). Mutagenesis studies on the active site pocket residues, H93 on the WPD loop and T167 and Q171 on the TI loop, showed a 75%, 60% and 70% reduction in phosphatase activity respectively, suggesting that the TI loop plays an important role in substrate binding. Mutation of K128 on the signature motif resulted in an 85% reduction of phosphatase activity (Lee *et al.*, 1999). In the signature motif of PTPs, the cysteine and arginine residues are essential for catalysis (Barford *et al.*, 1994), and the histidine and glycine residues are important for the conformation of the P loop (Stuckey *et al.*, 1994). In PTEN these have been identified as C124, R130, H123 and G127 (Lee *et al.*, 1999).

The 170 amino acid C-terminal end consists of two antiparallel β-sheets with two α-helices between the strands to form a β sandwich. It contains the C2 domain that plays a central role in mediating and regulating phospholipid membrane binding via the CBR3 loop, which has a net positive charge resulting from exposed lysine residues at positions 260, 263, 266, 267 and 269 (Essen *et al.*, 1996;Perisic *et al.*, 1998). The phosphatase and C2 domains are tightly linked at a phosphate-C2 interface, suggesting that another function of the C2 domain is to place the active site in the position which is the most optimal to bind to the membrane-bound substrate (Figure 5.1C) (Lee *et al.*, 1999). The C-terminal end of PTEN also contains two PEST sequences and a PDZ binding motif, which binds to PDZ domains on substrates to help recruit PTEN to the membrane (Teng *et al.*, 1997), although this domain is not essential as studies have shown that if the sequence is deleted, PTEN function is not abrogated (Furnari *et al.*, 1997;Georgescu *et al.*, 1999). Studies mapping the minimal *in vivo* functional domain of PTEN using deletion mutations of the N and C termini showed that a protein consisting of residues 10 to 353 maintained its phosphatase activity and was able to induce G1 arrest in cells (Vazquez *et al.*, 2000). This indicated that the last 50 amino acids, the C-terminal tail outside the C2 domain, were not necessary for the phosphatase activity of PTEN, and deletion of the C-terminal tail decreased protein stability and actually increased PTEN activity. The phosphorylation of residues S380, T382 and T383 maintains PTEN in an inactive state and is essential for stability (Raftopoulou *et al.*, 2004). Together these studies indicate that the C-terminal tail exerts inhibitory activity.

Until recently, PTEN was thought to function as a monomer. However, a study has shown that it can homodimerise (Papa *et al.*, 2014). This was demonstrated in co-immunoprecipitation assays where GFP-tagged PTEN and Myc-tagged PTEN were co-transfected into the PTEN-null PC3 cell line. PTEN dimers were found to be more catalytically active than monomers in a phosphatase assay, and the highest level of

dimerisation was apparent when the C-terminal tail was removed. Therefore the phosphorylation of the C-terminal tail regulates PTEN dimerisation.

5.1.2 PTEN and the PI3K/AKT pathway

PTEN is a lipid phosphatase and is the main negative regulator of the PI3K/AKT signalling pathway. It influences multiple cellular functions, including cell growth, survival and proliferation (Maehama & Dixon, 1998). The PI3K/AKT signalling pathway becomes activated upon ligand binding to receptor tyrosine kinases (RTKs). This results in both the dimerisation and autophosphorylation of receptors, allowing interaction of the RTKs with Src homology 2 (SH2) domain-containing molecules, including PI3K (Pawson & Nash, 2003;Schlessinger, 2002) (Figure 5.2). PI3K can be activated by three different mechanisms. The first is upon direct binding of the regulatory subunit of PI3K, p85, to the RTK, which results in the activation of the catalytic subunit of PI3K, p110 (Domchek *et al.*, 1992). The second route is via GRB2 binding to the scaffolding protein GAB, which in turn binds to p85 and activates p110 (Pawson, 2004). The final mechanism involves the binding and activation of SOS by GRB2 resulting in the activation of RAS, which can then activate p110 independently of p85 (Ong *et al.*, 2001). Activation of RAS also initiates signalling down the RAS/RAF/MEK/ERK pathway (Castellano & Downward, 2011). The activation of PI3K results in the generation of phosphatidylinositol triphosphate (PIP_3) in the plasma membrane, followed by recruitment of the serine-threonine kinase AKT to the membrane via its pleckstrin homology (PH) domain. AKT is then phosphorylated and activated, leading to the phosphorylation of many downstream targets. PTEN dephosphorylates PIP_3, thereby deactivating AKT and down-regulating the PI3K/AKT pathway.

An overview of the pathway is given in Figure 5.3. During signalling, AKT isoforms are activated by phosphorylation at two different residues, by phosphatidylinositol-dependent kinase-1 (PDK-1) at T308 (Manning & Cantley, 2007), and at S473 by the mammalian target of rapamycin (mTOR) complex 2 (Zoncu *et al.*, 2011). The activation of AKT isoforms AKT1-3 results in the phosphorylation of many targets including GSK3, FOXO, BCL-2, BAD, MDM2 and p27, which are crucial for cell survival, cell proliferation, angiogenesis and cellular metabolism (Manning & Cantley, 2007). Another protein directly targeted by AKT is tuberous sclerosis protein 2 (TSC2), which forms a heterodimer with TSC1. Phosphorylation of TSC2 by AKT inhibits the activation of RAS-related small GTPase homologue enriched in brain (RHEB), which, when activated, stimulates mTORC1 to phosphorylate its downstream targets (Guertin & Sabatini, 2007). The mTORC1 complex phosphorylates p70 ribosomal protein S6 kinase (S6K) and inhibitory 4E-binding protein 1

Figure 5.2 Function of PTEN. The main function of PTEN lipid phosphatase is the regulation of PI3K/AKT signalling. PTEN dephosphorylates $PIP_{3,}$ thereby deactivating AKT and inhibiting signalling down the pathway. Taken from Chow *et al* (2006).

(4EBP1), resulting in the activation of protein translation of specific mRNAs that are required for cell growth and proliferation. Two groups have recently identified a novel mTORC1 substrate that mediates feedback inhibition of the PI3K/AKT pathway, growth factor receptor bound protein 10 (GRB10) (Hsu *et al.*, 2011;Yu *et al.*, 2011). Phosphorylation of GRB10 via mTORC1 stabilised the protein, resulting in inhibition of the PI3K/AKT pathway. GRB10 expression is frequently downregulated in various cancers, including myeloma and breast carcinoma, and loss of the GRB10 protein and PTEN appear to be mutually exclusive events (Yu *et al.*, 2011).

5.1.3 Functions of normal PTEN

The identification of numerous mutations of the *PTEN* gene in various cancers has given insights into the cellular functions of PTEN, and many of these are through PI3K/AKT signalling. The most studied functions are cell survival and proliferation. Loss of PTEN leads to an accumulation of PIP_3 at the plasma membrane which recruits and activates AKT, thereby promoting the survival of cells by blocking the function of proapoptotic proteins including BAD and BIM (Datta *et al.*, 1997). AKT directly phosphorylates and inactivates BAD, thereby releasing the pro-survival protein, Bcl-2, from inhibition. AKT also phosphorylates FOXO proteins including FOXO1 and FOXO3a, blocking the FOXO-mediated transcription of target genes such as BIM (Manning & Cantley, 2007). Cell survival is also promoted by phosphorylation of the E3 ubiquitin ligase MDM2 by AKT, which then translocates to the nucleus where it negatively regulates p53 and down-regulates p53-mediated apoptosis (Mayo & Donner, 2001). Increases in cell proliferation are seen following cytosolic sequestration of the cyclin-dependent kinase inhibitor p27 as a result of phosphorylation by AKT; p27 is then prevented from localising to the nucleus where it exerts its cell cycle function.

Studies have also identified more novel functions of PTEN and the PI3K/AKT pathway. One of these functions is cell metabolism. Through inhibition of the RAB-GTPase-activating protein (GAP), AKT stimulates glucose uptake by associating with glucose transporter GLUT4-containing vesicles in response to insulin stimulation, leading to the translocation of GLUT4 to the plasma membrane (Eguez *et al.*, 2005). The signalling pathway also inhibits gluconeogenesis by blocking FOXO and peroxisome proliferator-activated receptor-γ (PPARγ) co-activator 1α (PGC1α) (Li *et al.*, 2007;Sundqvist *et al.*, 2005). AKT also phosphorylates GSK3β, resulting in the inhibition of GSK3β-mediated degradation of the transcription factor sterol-regulatory element-binding protein 1C (SREBP1C), which regulates genes involved in cholesterol and fatty acid biosynthesis (Horie *et al.*, 2004).

Figure 5.3 The PI3K/AKT signalling pathway. Abbreviations: GF, growth factor; RTK, receptor tyrosine kinase; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2. Taken from Song *et al* (2012).

PTEN also plays a role in cell migration, and inactivation of *Pten* in the developing brain of mice has been shown to lead to defects in neuronal migration (Fraser *et al.*, 2004;Groszer *et al.*, 2001;Marino *et al.*, 2002). It has also been shown that the loss of *Pten* in mouse embryo fibroblasts caused increased motility, which could be inhibited by the expression of dominant-negative forms of Rac1 and Cdc42, key mediators of cell motility (Liliental *et al.*, 2000). One study reported that PTEN can inhibit the migration of human glioma cells via its C2 domain (Raftopoulou *et al.*, 2004). The same group also confirmed that the activity of the C2 domain is controlled by the phosphorylation state of T383; it is inactive when phosphorylated, and becomes activated upon dephosphorylation and therefore able to inhibit migration of cells.

It has also been demonstrated that PTEN can function outside of the cytoplasm. Localisation of the PTEN protein has been found within the nucleus, and the finding that only cytoplasmic and not nuclear pools of PIP_3 are dephosphorylated by PTEN, suggested a function of PTEN other than its lipid phosphatase activity (Lindsay *et al.*, 2006). Subsequent studies have shown that nuclear PTEN functions to control genomic stability and cell cycle progression. Nuclear PTEN has been found to be important in DNA repair by upregulating the double-strand break repair protein RAD51 (Shen *et al.*, 2007). The loss of PTEN, leading to activation of the PI3K/AKT pathway, results in cytoplasmic degradation of the cell cycle regulator checkpoint kinase 1 (CHEK1). This abrogates the G2-M cell-cycle checkpoint, reduces the nuclear localisation of CHEK1 and promotes double-strand breaks in tumour cells (Puc *et al.*, 2005).

5.1.4 Mechanisms leading to loss of PTEN function in cancer

5.1.4.1 Copy number changes/Genomic loss

As previously mentioned, *PTEN* was first identified as a tumour suppressor gene when homozygous deletions of chromosome 10 were mapped in glioma cell lines (Li *et al.*, 1997;Steck *et al.*, 1997), since then genomic loss of *PTEN* has been reported in a wide range of cancers. In vivo reports have shown that total loss of the *PTEN* gene led to embryonic lethality (Trotman *et al.*, 2003), and more recent studies in a hypomorphic allelic series of mice with decreasing levels of Pten expression demonstrated that even a small reduction of Pten promoted cancer susceptibility (Alimonti *et al.*, 2010). Heterozygous deletion of the *PTEN* gene is frequently seen in breast, prostate, melanoma, bladder, kidney and colon cancer with incidences ranging from 20%-70% (Cairns *et al.*, 1998;Cairns *et al.*, 1997;Guldberg *et al.*, 1997;Schade *et al.*, 2009;Shao *et al.*, 2007), and loss of both alleles of the gene has been identified in thyroid and bladder cancer (Cairns *et al.*, 1998;Dahia *et al.*,

1997). Deletion of *PTEN* has also been reported in T-ALL and this is discussed further in chapter 6.

5.1.4.2 Mutations

Another mechanism for the loss of PTEN function in cancer is as a result of gene mutation. Germline mutations have been reported in approximately 80% of patients with Cowden Syndrome (Liaw *et al.*, 1997;Zhou *et al.*, 2003). Somatic loss-of-function *PTEN* mutations also occur in multiple cancer types, including approximately 40% of endometrial carcinomas (Maxwell *et al.*, 1998;Risinger *et al.*, 1997), 44% of gliomas (Chiariello *et al.*, 1998;Duerr *et al.*, 1998;Wang *et al.*, 1997), 23% of bladder tumours (Cairns *et al.*, 1998;Wang *et al.*, 2000) and 10-20% of melanomas (Birck *et al.*, 2000;Celebi *et al.*, 2000;Guldberg *et al.*, 1997). The mutations occur throughout the nine exons of the gene in these tumours and are either frameshift or nonsense mutations that would be predicted to result in a truncated protein, or in-frame or missense mutations which abrogate the phosphatase activity of PTEN. However, there are reported mutational hotspots; for example, 20% of the mutations found in endometrial tumours disrupt the amino acid R130 in exon 5. This arginine residue is also mutated in 4% of CNS tumours, a cancer in which 6% of mutations also cluster in the amino acid R173 in exon 6 (Hollander *et al.*, 2011).

Two of the most studied *PTEN* missense mutations in cancer are located in exon 5. C124S abolishes the catalytic activity of PTEN and has been found to be associated with endometrial cancer (Bonneau & Longy, 2000;Myers *et al.*, 1997), and G129E abrogates lipid phosphatase activity and has been associated with Cowden disease (Liaw *et al.*, 1997;Myers *et al.*, 1998). Both mutants were able to homodimerise and to form heterodimers with WT PTEN (Papa *et al.*, 2014). In a phosphatase assay, the mutant/WT heterodimers dephosphorylated PIP_3 at a reduced rate compared to the WT dimers, indicating that the mutants exert a dominant-negative effect over the WT PTEN protein. Generation of $Pten^{C124S/+}$ and $Pten^{G129E/+}$ mouse models demonstrated that the missense mutations accelerated the onset of tumourigenesis and the mice had an exacerbated tumour spectrum compared to the *Pten* WT mice. The missense mutations do not, however, affect the levels of PTEN protein, as expression was comparable to WT mice.

The majority of the C-terminal mutations in exons 7-9, encoding the C2 and PEST domains, result in the introduction of premature stop codons leading to a C-terminally truncated protein (Bostrom *et al.*, 1998;Chiariello *et al.*, 1998;Levine *et al.*, 1998;Maxwell *et al.*, 1998;Risinger *et al.*, 1997). When C2 domain mutant constructs introducing a premature stop codon and a construct harbouring the N-terminal missense mutation H123Y were

expressed in the U87 glioblastoma cell line, both N and C-terminal mutant constructs demonstrated larger colony formation compared to WT constructs. This suggested that the consequence of the *PTEN* mutations was abrogated tumour suppressor function (Georgescu *et al.*, 1999). The C2 domain mutants were expressed at a lower level compared with the WT or N-terminal mutant constructs, and had a significantly higher degradation rate. They mapped to regions predicted to form β-strands, suggesting that the C2 domain mutants could disrupt the secondary structure of the protein and that the decreased expression was as a result of impaired folding. They were also found to result in the ablation of phosphatase activity, indicating that the impaired folding of the C2 domain also affects the folding of the phosphatase domain. A later study investigating the functional role of C-terminal mutant R233X, confirmed that this nonsense mutation generated an unstable PTEN protein which was expressed at an almost undetectable level (Papa *et al.*, 2014).

5.1.4.3 Promoter methylation

PTEN expression can also be affected by epigenetic silencing through aberrant methylation of its promoter. Promoter hypermethylation is frequent in breast cancer where it occurs in more than 50% of patients, and in the presence of additional alterations such as the *Wnt* or *Errb2* transgenes, can lead to a decreased tumour latency (Hollander *et al.*, 2011). More than half of thyroid, melanoma and lung cancers also harbour aberrant methylation at the *PTEN* promoter and it has been shown that high levels of 5-methylcytosine in the promoter region correlate with lower levels of mRNA (Alvarez-Nunez *et al.*, 2006;Lahtz *et al.*, 2010;Soria *et al.*, 2002). However, recent studies have suggested that the methylation may correspond to the *PTEN* pseudogene *PTENP1* rather than *PTEN* (Hesson *et al.*, 2012;Zysman *et al.*, 2002). *PTENP1* is a retrotransposed or processed pseudogene located at chromosome 9p13.3 which shares 98% sequence homology with the coding region and 3' UTR of PTEN cDNA, with only 19 nucleotides different between the two sequences. It is transcribed, however a missense mutation of the initiation methionine prevents translation (Fujii *et al.*, 1999).

5.1.4.4 miRNAs

Various miRNAs, non-coding RNAs that mediate gene expression by targeting mRNAs, have been shown to downregulate PTEN expression leading to the promotion of tumourigenesis. These include miR-19 in leukaemia and Cowden disease and miR-21 in a number of other cancers (Mavrakis *et al.*, 2010;Meng *et al.*, 2007;Olive *et al.*, 2009). Recent evidence has also suggested that *PTENP1* behaves as a competitive endogenous RNA (ceRNA) that acts as a decoy for PTEN-targeting miRNAs and inhibits the negative regulatory effects of miRNAs on the expression of PTEN (Poliseno *et al.*, 2010). Where loss

of genomic *PTENP1* has been described in colon and breast cancer, the miRNAs instead bind to *PTEN*, which results in a decrease in protein abundance (Salmena *et al.*, 2011).

5.1.4.5 Post-translational modifications

Post-translational modifications can also result in the loss of PTEN function. The phosphorylation of a number of residues at the C-terminal end of PTEN can reduce plasma membrane localisation and phosphatase activity (Rabinovsky *et al.*, 2009;Vazquez *et al.*, 2001), including residues 380-385 which are phosphorylated by casein kinase 2 (CK2), and S370 and T366 which are phosphorylated by both CK2 and GSK3β (Al-Khouri *et al.*, 2005;Liang *et al.*, 2010). Other mechanisms of post-translational modification include oxidation by reactive oxygen species (ROS) in which a disulphide bridge is formed between residues C71 and C124, resulting in the inactivation of PTEN (Lee *et al.*, 2002). One study demonstrated that the PTEN protein was stabilised as a result of CK2 overexpression and increased intracellular ROS (Silva *et al.*, 2008). A combination of both CK2 inhibitors and ROS scavengers restored PTEN activity.

5.1.5 *PTEN* abnormalities in T-ALL

In early reports, mutations and deletions of the *PTEN* gene were reported in the T-ALL cell lines *Jurkat* and *CEM* (Gronbaek *et al.*, 1998;Sakai *et al.*, 1998;Shan *et al.*, 2000). However, in studies of a triple knockout model to replicate the chromosomal instability of human cancers (*TP53*$^{-/-}$, *Terc*$^{-/-}$, *Atm*$^{-/-}$), the mice developed thymic lymphomas due to telomere dysfunction, impaired DNA damage checkpoints and defective DNA repair, and array-CGH revealed that 43% of the tumours harboured *Pten* deletions encompassing the whole gene (Maser *et al.*, 2007). As a consequence, they performed array-CGH analysis on samples from 26 paediatric T-ALL patients and found that 4 (15%) had a *PTEN* deletion. Furthermore, when the *PTEN* gene was sequenced in samples from a separate cohort of 38 paediatric T-ALL patients, two (5%) had a mutation in the *PTEN* gene. The mutations were located in exon 7 and were frameshift insertions resulting in the introduction of a premature stop codon.

Further evidence for a role of *PTEN* deficiency in T-ALL came from oligonucleotide microarrays to identify disrupted genes associated with resistance to GSIs in a panel of GSI-resistant and GSI-sensitive cell lines (Palomero *et al.*, 2007). They demonstrated that PTEN was consistently found to be down-regulated in the GSI-resistant cell lines, the PTEN protein was absent or reduced, and the cell lines had increased levels of AKT phosphorylation associated with loss of PTEN function. Sequencing of the coding region of the *PTEN* gene

revealed that each of the resistant cell lines harboured mutations in the *PTEN* gene. Of the six mutations identified, one was a missense mutation in exon 5, the rest were frameshift truncating mutations, two in exon 7 and one each in exons 2, 5 and 8. They then went on to screen 111 patient samples and found eight mutations in seven (6%) patients. All mutations were frameshift size changes, five in exon 7, one each in exons 3 and 4 and one homozygous mutation in exon 7.

Since then, a number of other groups have extended these findings in paediatric T-ALL patients, and these studies are detailed in Table 5.1. Overall, mutations have been reported at a frequency of between 5% and 27%. One study reported a 63% mutation incidence rate, which is significantly higher than the other reports (Larson Gedman *et al.*, 2009). However, in this study investigators sequenced cDNA samples and it has been suggested that some of the mutations reported correspond to the *PTEN* pseudogene, *PTENP1*, which is likely to have been amplified by the exonic primers required to screen the cDNA, owing to the high sequence homology it shares with *PTEN*. Therefore this study has been excluded from the cumulative analysis. Combining the data from six studies, of 698 cases, 100 (14%) had at least one mutation in *PTEN.* Of these, 86 patients (86%) harboured exon 7 mutations alone and ten (10%) exon 7 mutations in conjunction with mutations in other exons. Only four (4%) patients did not harbour a mutation in exon 7. The mutations were predominantly frameshift size changes that would result in the introduction of a premature stop codon and loss of the C2 domain.

The majority of mutations found in the *PTEN* gene in T-ALL are heterozygous (85%) and therefore monoallelic, affecting only one allele. However, a small number of biallelic mutations have been identified (15%), including homozygous mutations and compound heterozygous mutations (Gutierrez *et al.*, 2009;Jotta *et al.*, 2010;Palomero *et al.*, 2007;Zuurbier *et al.*, 2012). Absent protein expression has been shown in samples from patients with exon 7 *PTEN* mutations in a number of these studies (Jotta *et al.*, 2010;Palomero *et al.*, 2007;Zuurbier *et al.*, 2012). However, it was shown both in a patient sample and in cell lines that harboured an exon 5 mutation, the protein was present but at a reduced level (Zuurbier *et al.*, 2010). Expression was found to be lower in the biallelic mutated patients compared with monoallelic cases (Jotta *et al.*, 2010;Zuurbier *et al.*, 2012).

Together these studies provide evidence that *PTEN* mutations are a relatively frequent occurrence in paediatric T-ALL. This chapter therefore presents data on the screening for mutations in the *PTEN* gene, in the cohort of UKALL 2003 T-ALL paediatric patients described in the previous chapters.

Table 5.1 Studies of *PTEN* mutation detection in patients with T-ALL

Reference	Total in study	Age group	Screening method	Exons screened	Total *PTEN* mutant patients (%)	Type of mutations
Maser *et al* (2007)	38	Paediatric	Sequencing	1-9	2 (5%)	2 HET frameshift (exon 7)
Palomero *et al* (2007)	111	Paediatric	Sequencing	1-9	7 (6%)	8 size changes in 7 patients: all frameshift 5 (5%) 1 HET (4 exon 7, 1 exon 3) 1 (1%) > 1 HET (exons 4 and 7) 1 (1%) HOM (exon 7)
Jotta *et al* (2009) & Silva *et al* (2008)	62	Paediatric	dHPLC + sequencing	1+7	11 (18%)	13 size changes in 11 patients: 10 frameshift, 3 in-frame 7 (12%) 1 HET (exon 7) 2 (3%) > 1 HET (1 exons 1 and 7, 1 with 2 exon 7) 2 (3%) HOM (exon 7)
Gutierrez *et a*l (2009)	44	Paediatric	Sequencing	1-9	12 (27%)	15 size changes in 12 patients: all frameshift 9 (20%) 1 HET (exon 7) 3 (7%) > 1 HET (exon 7)
(Clappier *et al.*, 2010)	43	Paediatric	cDNA Sequencing	1-9	27 (63%)	27 size changes in 27 patients: 22 frameshift, 5 in-frame 19 (21%) 1 HET (9 exon 7, 10 exons 2-8) 8 (19%) HOM
Zuurbier *et al* (2012)	142	Paediatric	Sequencing	1-9	16 (11%)	26 mutations in 16 patients: 24 frameshift size changes, 2 missense 5 (3%) 1 HET (4 exon 7, 1 exon 4) 10 (7%) > 1 HET (5 with 2 exon 7, 4 exons 5 and 7, 1 exons 6 and 8) 1 (1%) HOM (exon 7)
(Erbilgin *et al.*, 2010)	301	Paediatric	Sequencing	1-9	52 (17%)	58 size changes in 52 patients: all frameshift 47 (15%) 1 HET (46 exon 7, 1 exon 4) 5 (2%) > 1 HET (1 with 2 exon 7, 1 exon 4 and 7, 2 exon 5 and 7, 1 exon 1 and 2 exon 7)

Abbreviations: HET, heterozygous; HOM, homozygous.

5.2 Materials and Methods

5.2.1 Patients

Diagnostic samples were available from the United Kingdom Leukaemia and Lymphoma Research Childhood Leukaemia Cell Bank from 162 of the 388 (42%) T-ALL patients entered into the United Kingdom Medical Research Council ALL 2003 (UKALL 2003) trial between 2003 and 2011, as described in chapter 3 (section 3.2.1).

5.2.2 *PTEN* mutation screening

Mutation screening of the entire coding sequence (exons 1-9) of the *PTEN* gene was carried out by PCR and dHPLC, followed by sequencing of samples with abnormal chromatograms. Samples were amplified by 35 cycles of PCR using the primers and appropriate annealing temperatures shown in Appendix Table 1. Wherever possible, PCR products were obtained using the proof-reading enzyme Optimase (Transgenomic). For exons 1 and 7 where adequate PCR products could not be obtained using Optimase, the proof-reading enzyme Phusion High-Fidelity DNA Polymerase (New England Biolabs) was used. The standard Optimase DNA Polymerase and Phusion High-Fidelity DNA Polymerase reaction mixes and cycling conditions are detailed in chapter 2 (section 2.1.4). PCR products were checked on a 2% agarose gel stained with ethidium bromide.

PCR products were screened by dHPLC on the WAVE DNA Fragment Analysis System (Transgenomic, Glasgow, UK) as detailed in section 2.1.6. As homozygous mutations and hemizygosity have been reported in this gene, amplified products from the patient samples were mixed in equal quantities with products from a known WT control before denaturation. PCR products were denatured and cooled slowly to enable the formation of heteroduplexes, then analysed on the WAVE at optimal melting temperatures which were calculated using the Transgenomic Navigator software (Appendix Table 1). Each WAVE run included a known WT case for each particular exon and, where possible, a known mutant control to allow for comparison.

5.2.3 Confirmation of *PTEN* mutations

For samples with abnormal chromatograms, fresh PCR products were obtained using BIOTAQ DNA Polymerase with standard reaction mix and cycling conditions as detailed in

section 2.1.4, and sequenced in one direction. Where the presence of either multiple mutations or a low-level mutation was evident from direct sequencing, the exon was amplified and the products cloned using the TOPO TA cloning kit (Invitrogen, Paisley, UK) as described in section 2.1.10. Clones were harvested and grown overnight in LB broth before PCR amplification. PCR products from clones were then screened by gel electrophoresis.

5.2.4 Whole Genome Amplification of genomic DNA

There was limited DNA in the majority of the patient samples post mutation screening, therefore whole genome amplification (WGA) was performed using the REPLI-g Mini Kit (Qiagen, Crawley, UK), which enables highly uniform WGA from small samples across the entire genome with negligible sequence bias. The method is based on Multiple Displacement Amplification (MDA), which carries out isothermal genome amplification using a DNA polymerase capable of replicating up to 100kb without dissociating from the genomic DNA template. The DNA polymerase has 3'-5' exonuclease proofreading activity to maintain high fidelity during replication, and is used alongside exonuclease resistant primers to achieve high yields of DNA product. For the reaction, 1-10ng of patient DNA was amplified following the kit instructions.

5.2.5 Quantification of *PTEN* mutant level by fragment analysis

For quantification by fragment analysis, exon 7 PCR products were obtained from both the original non whole genome amplified (non-WGA) DNA and the whole genome amplified (WGA) DNA using BIOTAQ DNA polymerase with a fluorescently labelled forward primer and unlabelled reverse primer (Appendix Table 1). Due to the sensitivity of the assay, the standard BIOTAQ DNA Polymerase reaction mix and cycling conditions given in section 2.1.4 were adjusted. The concentration of each primer was decreased from 0.5µM to 0.25µM, and in each of the denaturation, annealing and extension steps, the temperature was ramped at 0.5^{o}C/second and the time extended to 1 minute. The number of cycles of amplification was reduced from 35 to 28, to limit preferential amplification of smaller PCR products, and also to ensure that the amount of fluorescently labelled PCR product did not saturate the fragment analyser. The final extension step, 72^{o}C for 5 minutes in the standard protocol, was extended to 15 minutes to ensure full extension of all PCR products.

After amplification, 2µl of PCR product was added to 38µl of sample loading solution (Beckman Coulter UK Ltd, Buckinghamshire, UK) containing a DNA size standard ladder

(DNA Size Standard Kit, 400PA, Beckman Coulter, High Wycombe, UK). Samples were then run on the CEQ 8000 Genetic Analysis System (Beckman Coulter) and analysed by capillary electrophoresis. Each run included a WT control to identify the WT peak in the patient samples. In patients with a size change mutation, peaks representing either an insertion or deletion were identified by their different size to the WT peak as called by the instrument software. The fragment analysis software calculated the area under the WT and mutant peaks, and mutant level was determined by expressing the area under each peak as a percentage of total alleles. Wherever possible, quantification of mutations in exon 7 was performed in both non-WGA and WGA samples and a mean total mutant level was determined.

5.2.6 Quantification of mutant level by restriction enzyme digest

In two patients harbouring multiple mutations, where a 1bp size change could not be resolved from the WT peak by fragment analysis, a restriction enzyme recognition site was identified in either the WT or 1bp mutant alleles that enabled differential separation of the alleles by size but did not affect the other mutant alleles. For one patient harbouring the mutation c.696delCinsGG (p.R233fs), exon 7 amplicons were generated as detailed in section 5.2.5, and 8µl of PCR product was digested at 37°C for 4 hours in a reaction mix with 1µl *Hpy99I* and 1µl of manufacturer's CutSmart buffer (New England Biolabs, Hitchin, UK). After digestion, 2µl of the reaction was analysed as described above (section 5.2.5) on the CEQ 8000 Genetic Analysis System. Mutant alleles were undigested giving a 267bp fragment and WT alleles were digested to a labelled 121bp fragment. For another patient harbouring the mutation c.696insT (p.R233fs), 8µl of PCR product was digested at 65°C for 4 hours in a reaction mix with 1µl *Taqα1* and 1µl of manufacturer's buffer 4 (New England Biolabs, Hitchin, UK), and 2µl of the reaction was analysed as described above (section 5.2.5). WT alleles were uncut giving a 267bp fragment and mutant alleles were digested to a labelled 115bp fragment.

5.3 Results

5.3.1 *PTEN* mutation detection

WAVE analysis was used to screen the entire coding sequence of the *PTEN* gene. Of the 162 patients investigated, 89 (55%) had one or more exons with abnormal WAVE chromatograms. In 76 cases, the abnormal chromatograms were due to the presence of a

common polymorphism, rs1903858 (A/G) in intron 1-2, which could be identified from the characteristic WAVE chromatogram. Presence of the SNP was confirmed by fluorescently labelled PCR and restriction enzyme digestion and further details of this are provided in chapter 6 (section 6.3.2). In a further four cases, direct nucleotide sequencing showed another polymorphism in intron 1-2, rs190707033 (C/T). The 68 patients, in whom these were the only changes, were scored as *PTEN*^{WT}.

Mutations were detected in the *PTEN* gene in 21 (13%) of the 162 patients analysed (*PTEN*^{MUT}). Of these, 19 (90%) harboured mutations in exon 7, two of these patients had co-incident exon 6 mutations, and the remaining two patients harboured mutations just in exon 5. Representative WAVE chromatograms are shown in Figure 5.4. Direct sequencing identified the nucleotide change in seven of the patients (33%), including the mutations in exons 5 and 6. However, the presence of either a low level mutant or multiple mutations in exon 7 meant that this approach was unsuccessful for the majority of patients (67%). Therefore, fluorescently labelled PCR products from patients with an abnormal chromatogram in exon 7 were size separated by fragment analysis to determine the number of mutations present in each sample. It was found that the size of the PCR product from a known *PTEN* WT sample, as called by the instrument software, was 2bp larger than the expected 265bp WT product size. However, nucleotide sequencing of the WT PCR product confirmed that the expected 265bp sequence was present with no extra nucleotides. Similar discrepancies have been previously reported in other studies in the laboratory, and are most probably due to the high GC content (60%) of the amplicon causing secondary structure that interferes with electrophoresis of the PCR product.

Fragment analysis revealed a total of 37 exon 7 mutations in the 19 patients. Five patients had a single mutation whereas 14 patients harboured between two and four mutations, with the mutant level of the individual mutations ranging from 2% to 47% of total alleles (see section 5.3.2 for further details). Therefore, the 14 patient samples with uncharacterised exon 7 mutations were amplified to obtain a fresh PCR product, which was cloned using the TOPO cloning kit as described in section 2.1.10. Between 15 and 20 clones per sample were harvested and grown in LB broth overnight, followed by PCR amplification. PCR products from clones were size separated by gel electrophoresis, and where a size change was evident, PCR products from at least two clones of the same size were sequenced. Where the size change was too small to be identified on the agarose gel, between 5 and 10 of the amplified clones were sequenced. Screening of the clones did not reveal the nucleotide sequence of seven mutations identified by fragment analysis. All mutations were present at a level of less than 10% of total alleles, and therefore they were not pursued as their detection would have

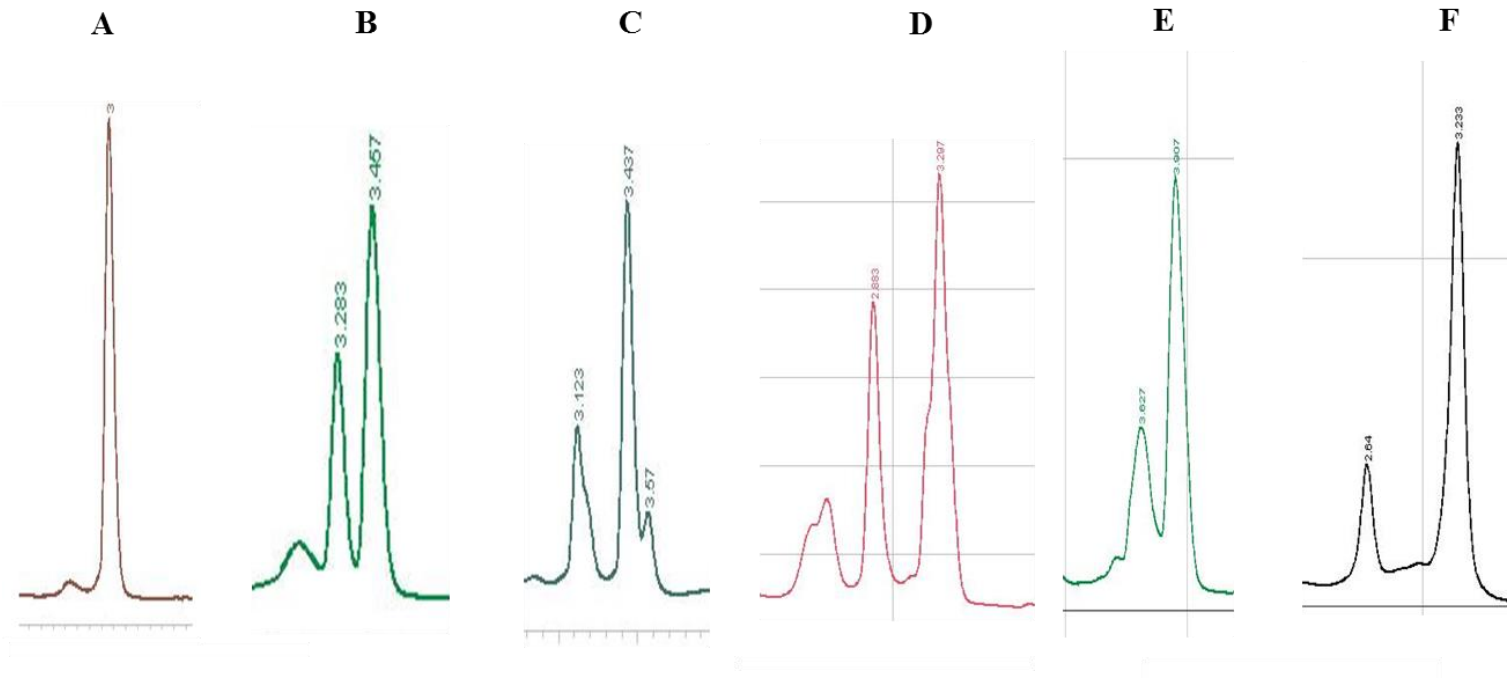

Figure 5.4 Detection of *PTEN* mutations by WAVE analysis. Representative WAVE chromatograms of patient samples normalised and compared to that of a known WT control. (A) Exon 7 WT control (B) Patient with one exon 7 mutation. (C) Patient with two mutations. (D) Patient with three mutations. (E) Patient with an exon 5 mutation. (F) Patient with an exon 6 mutation.

required the further screening of a large number of clones. For seven mutants, the size of the insertion or deletion called by the fragment analysis instrument software differed by 1bp from the size change obtained from nucleotide sequencing. As previously mentioned, this is likely to be due to the GC-rich sequence causing secondary structure, which may interfere with the capillary electrophoresis. All clones where the sequence showed a size change greater than 2bp were detected by fragment analysis. In five patients, cloning and sequencing identified single base pair insertions which could not be seen in fragment analysis. Retrospective inspection of the original sequence showed evidence of the 1bp change in all but one of these cases. In two of the five patients, the 1bp change could be identified upon fragment analysis of the WGA sample, and the mutations were present at a level of 47% and 39% of total alleles, which was compatible with the sequence. This was not the case in the other three patients; therefore direct mutant level quantification by fragment analysis was not possible for these three cases. In two of them, the 1bp size change could be quantified after restriction enzyme digestion as described in section 5.2.6, and the mutations were present at a level of 28% and 2% of total alleles respectively (Figure 5.5). It is therefore not surprising that the 1bp change quantified at 2% was not apparent on either the original sequence or the fragment analysis. No suitable restriction digest site was available that would distinguish the WT from mutant alleles in the remaining case, therefore an estimated mutant level of 17% was determined by measuring ten peak heights in the direct sequence and calculating an average.

A total of 44 mutations were therefore detected in the *PTEN* gene, 40 in exon 7, two in exon 6 and two in exon 5. Figure 5.6 shows the location and type of the mutations and a full list of nucleotide and amino acid changes is given in Appendix Table 3. The mutations in exon 7 included 20 insertions, 17 indels and three deletions, with overall size changes ranging between 1bp and 20bps and, of the characterised mutations, all clustered in the region between amino acid residues 219-256. Of the 33 characterised mutations, 25 of these size changes (75%) caused a frameshift, with the introduction of a premature stop codon resulting in a C-terminally truncated protein. Three (10%) mutations were in-frame, but were indels where the inserted sequence introduced a premature stop codon (Figure 5.7). Five mutations (15%) were in-frame and non-truncating. The two mutations found in exon 5, were a 1bp frameshift insertion resulting in the introduction of a premature stop codon and a 9bp in-frame indel. The two mutations in exon 6 were both short frameshift insertions of 8bp and 13bps respectively, introducing a premature stop codon. Missense mutations were rare with only one found in the cohort, P246L. However, this was scored as WT as the functional

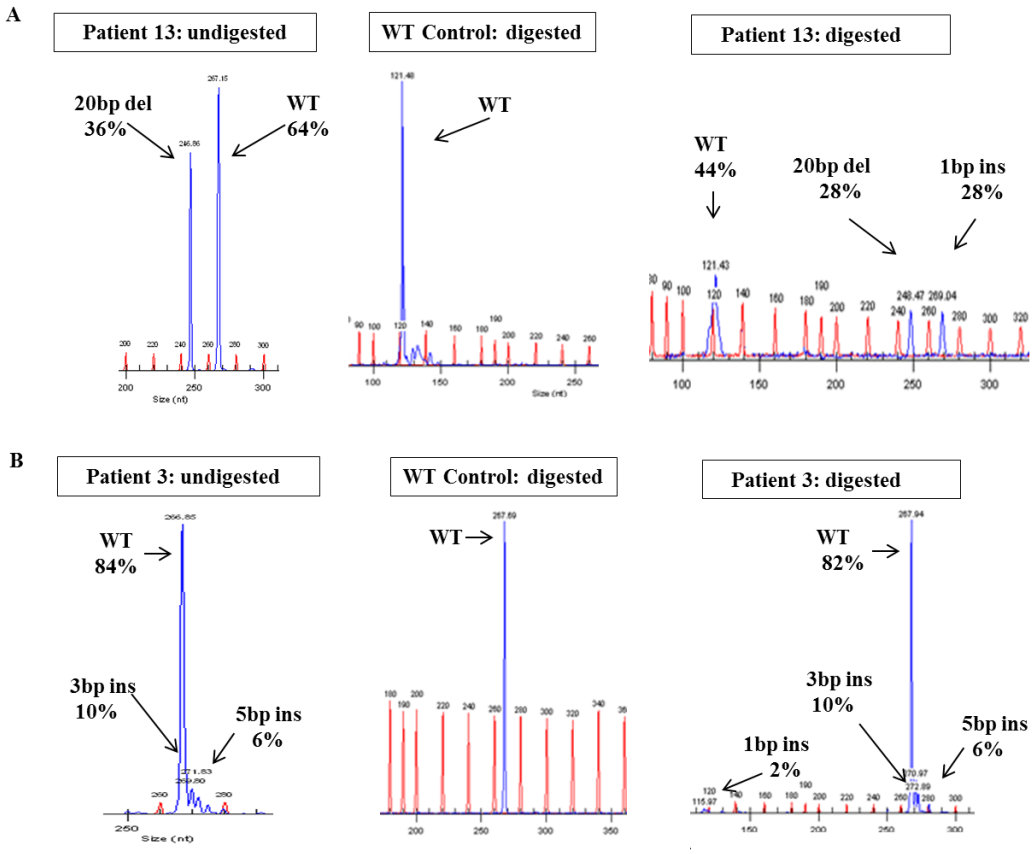

Figure 5.5 Restriction enzyme digest to distinguish 1bp change from WT peak and quantification of mutant level. (A) Hpy99I digest. WT alleles were digested to a labelled 121bp fragment and 1bp ins and 20bp del mutant alleles were not digested giving fragments of 267bp and 247bps. (B) Taqα1 digest. 1bp ins mutant alleles were digested to a labelled 115bp fragment and WT alleles and 3bp and 5bps ins were uncut giving fragments ar 267bp, 270bp and 272bps.

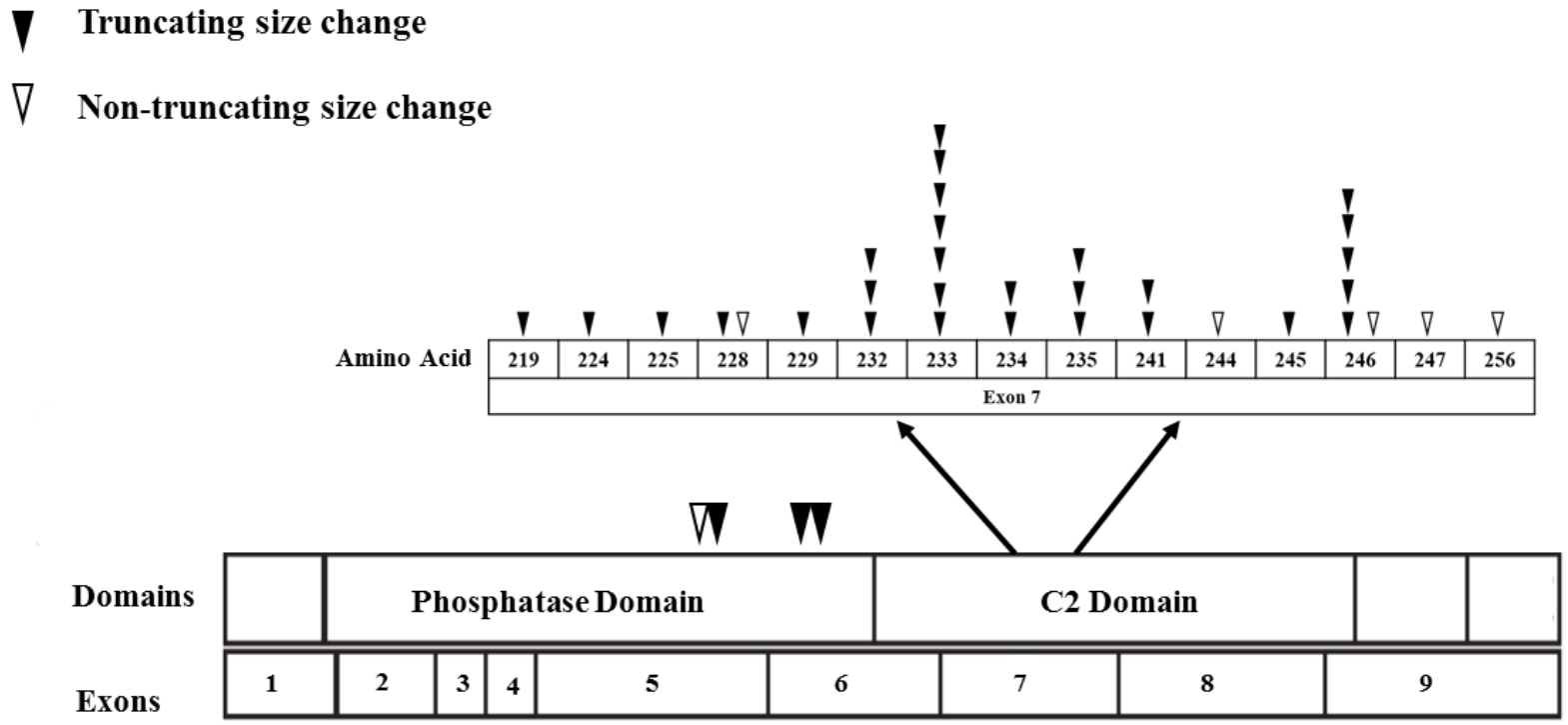

Figure 5.6 Schematic representation of the location and type of *PTEN* mutations detected in 162 paediatric T-ALL patients.

consequence of this mutation is unknown and there is a possibility that the mutation is a rare SNP.

Therefore 44 mutations were identified in 21 (13%) of the 162 patients analysed. Seventeen patients (80%) had exon 7 mutations only, two (10%) exon 6 and 7 mutations, and two (10%) exon 5 mutations only. Seven patients (34%) had one mutation, eight (38%) had two mutations, three (14%) had three mutations and three (14%) had four mutations (Table 5.2).

5.3.2 Quantification of *PTEN* exon 7 mutant level by fragment analysis.

Quantification of mutations in exon 7 of the *PTEN* gene was performed in both non-WGA and WGA samples. Of the 40 individual mutations identified in exon 7, 32 were successfully quantified in both non-WGA and WGA samples and the comparative data obtained from the mutations is shown in Figure 5.8. Comparison was not possible for eight mutations. In one mutation there was no suitable restriction enzyme digest to quantify the 1bp size change, therefore quantification was not performed in either sample, and in two mutations the restriction enzyme digestion was only carried out in the non-WGA sample. In a further two mutations the 1bp size change could only be distinguished from the WT peak in the WGA sample, and in one mutation the PCR product was of poor quality after amplification of the WGA sample, therefore the mutant peak was of an insubstantial height to be included by the software. In the case of the remaining two mutations, the non-WGA sample revealed a 6bp insertion which constituted 78% of the total alleles; however the peak was very broad. In the WGA sample, the 6bp insertion peak resolved into two peaks corresponding to a 6bp and 8bp insertion, each at a level of 37% and 41% respectively, therefore these two mutations had no comparison in the non-WGA sample. Reassessment of the original sequence revealed the presence of the additional mutation, which was originally mistaken for the WT sequence.

Of the 32 mutations in which comparison between the non-WGA and WGA DNA was possible, there was a strong correlation between the mutant levels of the individual mutations in the two samples, $r^2 = 0.92$. The difference in 31 was $\leq 5\%$, the median difference was 1% (range 0% to 21%). In the mutation where the difference was 21%, the mutation was quantified at 51% and 30% in the non-WGA and WGA samples respectively. In the original sequence, the mutant and WT traces are of equivalent heights, suggesting that the mutant level has been underestimated in the WGA sample.

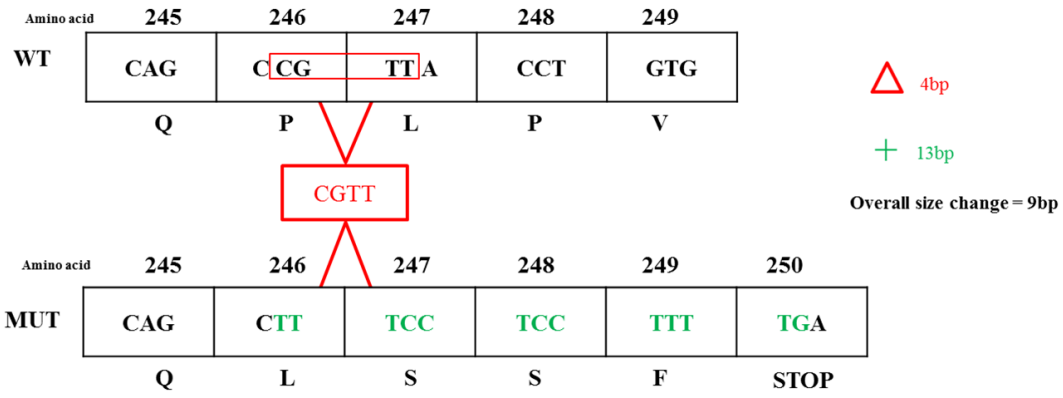

Figure 5.7 In-frame indel mutation resulting in the introduction of a premature stop codon.

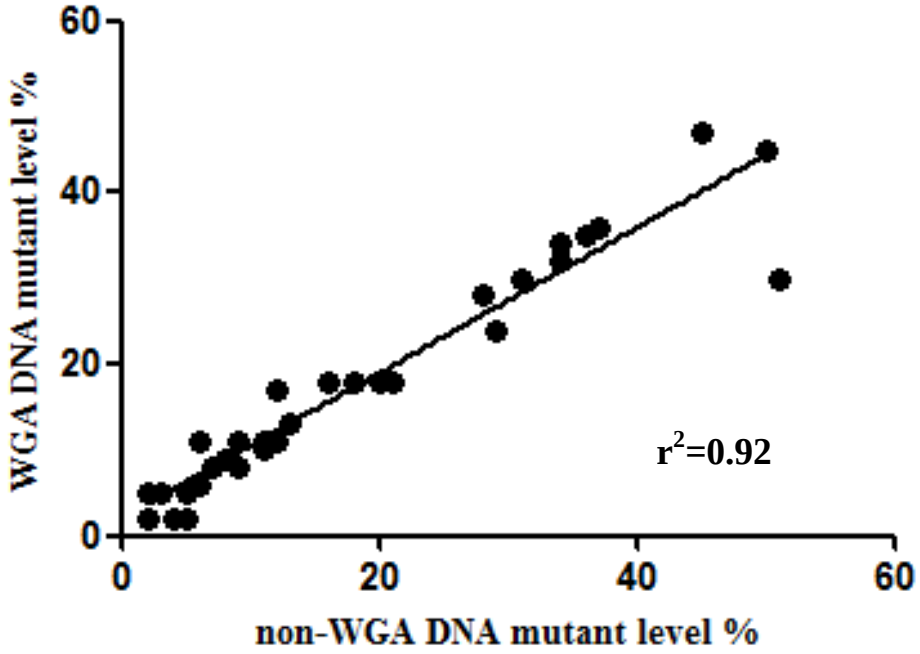

Figure 5.8 Comparison of exon 7 mutant levels quantified in non-WGA and WGA DNA.

An average mutant level of each individual mutation was determined where possible from the mean of the values from the non-WGA and WGA samples. There was a wide variation in the level of the individual mutations, ranging from 2% to 48% of total alleles, with a median of 16%. Examples of mutant level quantification for patients with either one, two, three or four mutations are shown in Figure 5.9. A total mutant level for each patient was determined, and the median total mutant level for the 19 patients with *PTEN* exon 7 mutations was 41% (range 10% to 96%). Fragment analysis to quantify the mutant level in exons 5 and 6 was not performed as there were only two patients with mutations in either exon. An estimated mutant level for these mutations was therefore determined by measuring the peak heights in the direct sequence. Taking into account the estimated levels, the median mutant level for the 21 *PTEN*^{MUT} cases was 48% (range 10% to 96%). Five (24%) patients had a total mutant level of <25%, six (28%) patients had a total mutant level of between 25%-50% and ten (48%) patients had a total mutant level of >50% (Figure 5.10).

5.3.3 Classification of patients with *PTEN* mutations

It was not possible to determine whether the multiple mutations detected in the majority of the mutant-positive patients were present in the same cell, as genomic DNA was the only available material from the patients. However, the level and/or the number of mutations in a given patient may indicate whether there is evidence of monoallelic mutation, where only one allele in a cell is affected, or biallelic mutation, where both alleles in a cell are affected. Therefore, each of the *PTEN* mutant cases were scored according to the following criteria: where one or more mutations were present and the total mutant level was <50%, patients were scored as monoallelic. Patients with either one or multiple mutations totalling >50% were scored as biallelic on the assumption that at least a proportion of cells must have more than one allele affected.

Of the 21 *PTEN*^{MUT} cases, 11 (52%) patients were considered to harbour monoallelic mutations, and the remaining 10 (48%) patients, biallelic mutations. Of the monoallelic-mutated patients, five had a single mutation which constituted nearly 50% of total alleles (37%, 41%, 45%, 47% and 48% respectively) suggesting the presence of a heterozygous mutation in the majority of cells. An example is given in Figure 5.11A where the patient has a mutation at a level of 48% of total alleles, consistent with a heterozygous mutation in 96% of cells. For the remaining six patients, the number of mutations per patient ranged from one to four. The mean mutant level of these mutations was only 8% (range 2% to 21%), therefore it is likely that the mutations are heterozygous and in different subclones, although the possibility that a significant proportion of non-leukaemic cells are present in the sample,

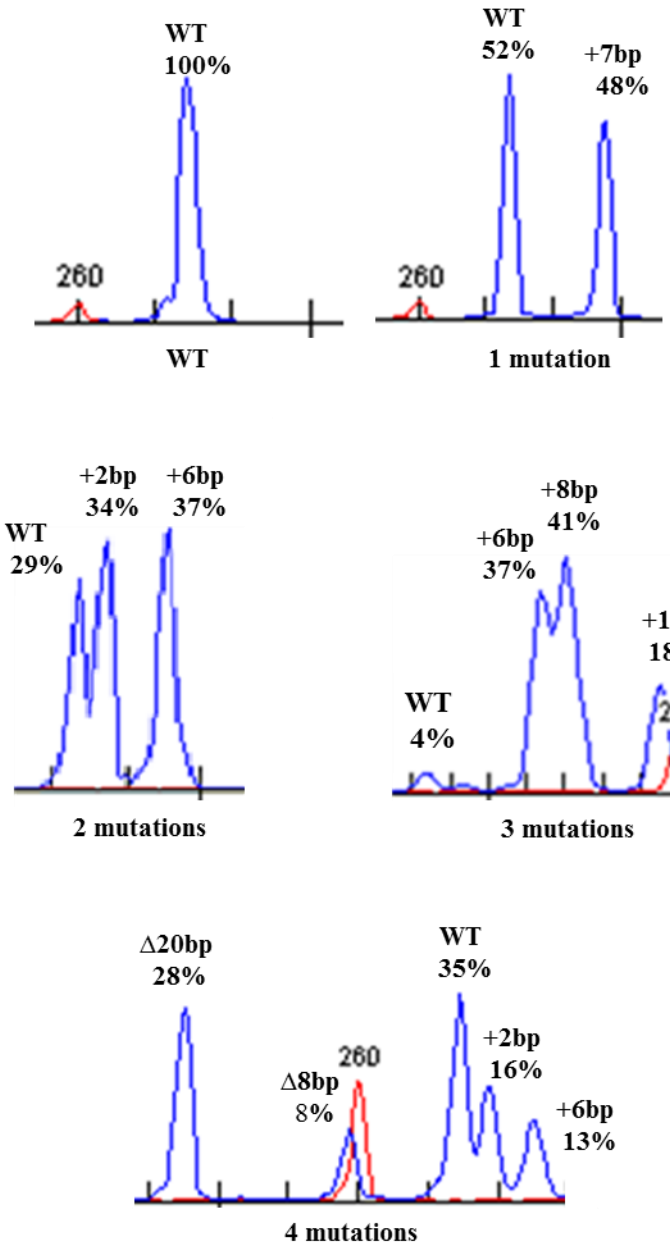

Figure 5.9 Quantification of mutant level by fragment analysis. (A) WT control. (B) Patient with one mutation. (C) Patient with two mutations. (D) Patient with three mutations. (E) Patient with four mutations.

Table 5.2 *PTEN* mutant levels in investigated patients

Patient	No. muts	Exon 5 mutation	Exon 6 mutation	Exon 7 mutation	Total Mutant Level	Monoallelic/ Biallelic
1	1			2bp indel (41%)	41%	Monoallelic
2	1			10bp indel (10%)	10%	Monoallelic
3	1			1bp ins (47%)	47%	Monoallelic
4	1			7bp indel (48%)	48%	Monoallelic
5	1			12bp indel (37%)	37%	Monoallelic
6	2			14bp indel (11%), 13bp del (2%)	13%	Monoallelic
7	2			5bp indel (12%), 4bp ins (11%)	22%	Monoallelic
8	2			8bp ins (21%), 4bp ins (6%)	27%	Monoallelic
9	2			9bp indel (6%), 11bp ins (5%)	11%	Monoallelic
10	3			1bp ins (2%), 5bp ins (6%), 3bp ins (10%)	18%	Monoallelic
11	4			4bp indel (15%), 13bp del (9%), 11bp ins (4%), 7bp ins (6%)	34%	Monoallelic
12	1	1bp ins (70%)*			70%	Biallelic
13	1	9bp indel (60%) *			60%	Biallelic
14	2			4bp ins (45%), 1bp ins (17%)*	62%	Biallelic
15	2			2bp ins (34%), 6bp indel (37%)	71%	Biallelic
16	2			20bp indel (28%), 1bp indel (28%)	56%	Biallelic
17	2			16bp ins (34%), 1bp indel (39%)	73%	Biallelic
18	3		8bp ins (29%)*	18bp ins (29%), 2bp ins (2%)	60%	Biallelic
19	3			12bp indel (18%), 6bp ins (37%), 8bp ins (41%)	96%	Biallelic
20	4			20bp indel (28%), 6bp ins (13%), 2bp indel (16%), 8bp del (8%)	65%	Biallelic
21	4		13bp ins (24%)*	12bp indel (31%), 4bp ins (18%), 14bp ins (4%)	77%	Biallelic

*Mutant level estimated from the sequence. Abbreviations: indel, insertion/deletion.

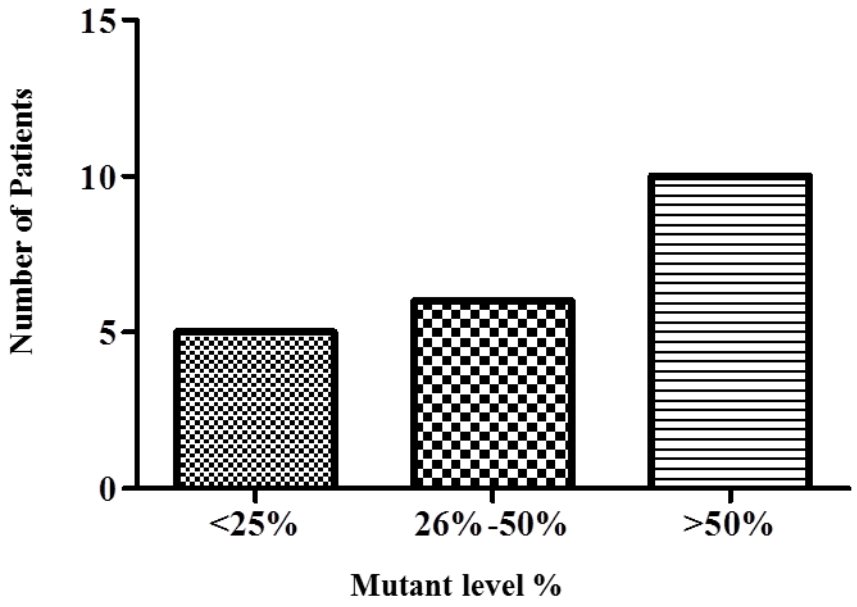

Figure 5.10 Distribution of total *PTEN* mutant level detected in mutant positive patients.

reducing the apparent mutant level, cannot be excluded. An example is given in Figure 5.11B, in which the patient has two mutations at mutant levels of 11% and 2% respectively. The most likely explanation in this case is that the mutations are heterozygous and constitute different cell populations.

Of the biallelic-mutated patients, two patients each had a single mutation in exon 5 constituting more than half of total alleles (60% and 70%). This mutant level is consistent with either a homozygous mutation or hemizygosity, where there is loss of one allele and the remaining allele in the cell is mutated. In the remaining eight cases, all patients harboured more than one mutation and the mean total mutant level was 70% (range 56% to 96%). As the total mutant level for all cases was over 50%, this would be consistent with compound heterozygosity, where each allele in a cell harbours different mutations. Cloning of all the patient samples in which more than one exon 7 mutation was present (as described in section 5.3.1), confirmed that the mutations were not present on the same allele, although it was not possible to determine whether this was the case in the two patients harbouring co-incident exon 6 and 7 mutations. The possibility of hemizygosity or that the individual mutations are homozygous also cannot be excluded. An example is given in Figure 5.12A where the patient has a single mutation at a level of 70% of total alleles. There are two possibilities, one being the mutation is homozygous, and therefore in 70% of cells, both alleles harbour the mutation. The second possibility is that a proportion of the cells are hemizygous and the remaining allele in these cells is mutated. As the mutant level in the patient is 70%, in this scenario 83% of cells would have lost one allele and harbour the mutation. Either way both alleles are affected. Further investigation demonstrated that the patient showed evidence of heterozygous deletion and this is presented in the next chapter. The example in Figure 5.12B is of a patient with two mutations A and B, at levels of 45% and 17% respectively. One possible explanation is that mutation B was acquired in a proportion of cells that already harboured mutation A, meaning 34% of cells are compound heterozygous. The other population of *PTEN*-mutated cells (21%) would therefore be heterozygous for mutation A. Alternatively, it is possible that both A and B are homozygous mutations in separate cell populations, or that a proportion of cells are hemizygous. Further investigation demonstrated that the patient showed no evidence of heterozygous deletion and this is presented in the next chapter.

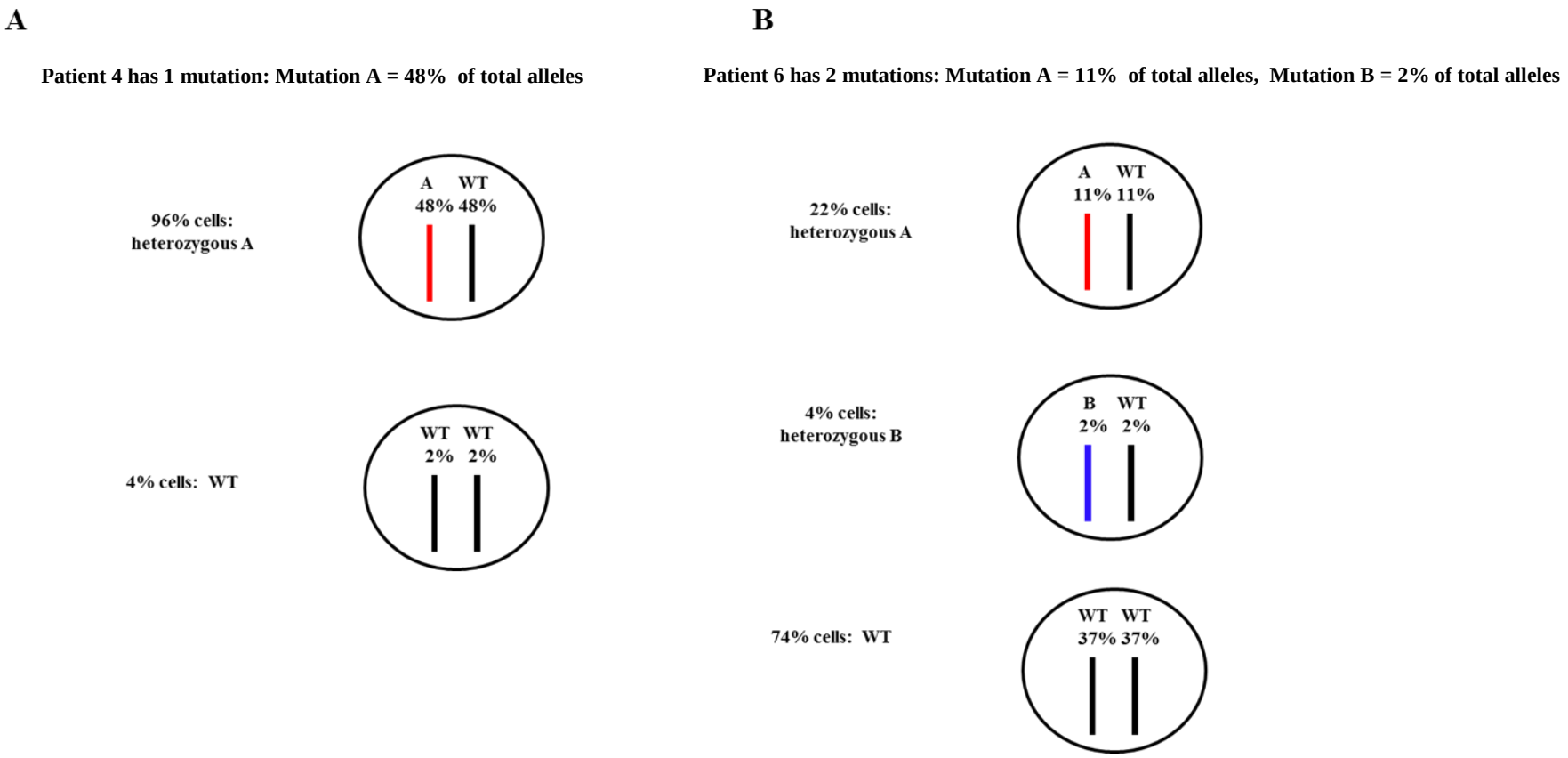

Figure 5.11 Monoallelic *PTEN* mutations. (A) Heterozygous mutation in the majority of cells. (B) Two heterozygous mutations most likely to be in different subclones. Mutant level is given as a % of total alleles. Each cell is assumed to have both chromosomes therefore the percentage shown above each represents the contribution of the individual allele.

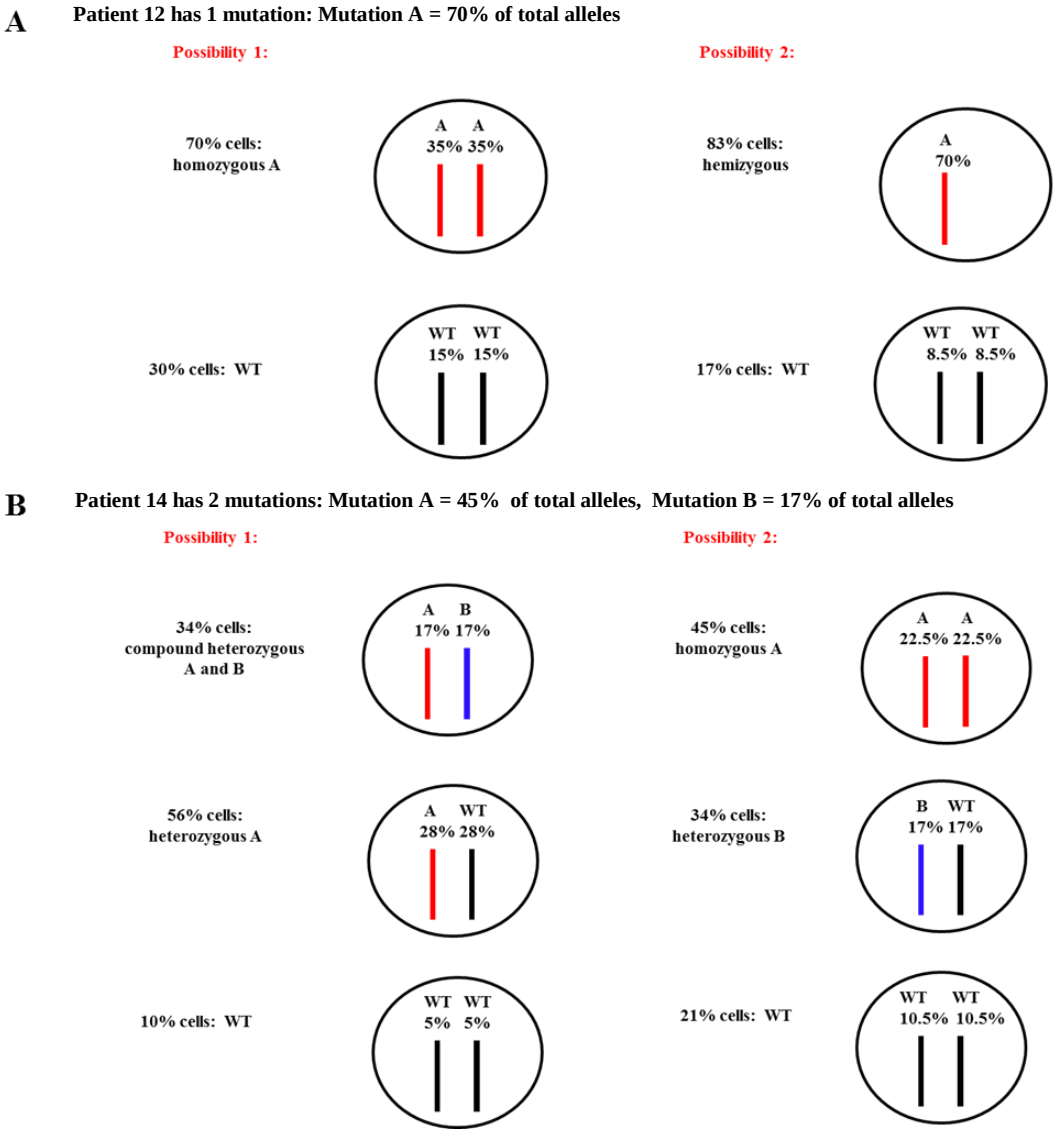

Figure 5.12 Biallelic *PTEN* mutations. (A) Evidence of homozygous mutation or deletion of one allele in a proportion of cells. (B) Evidence of a proportion of cells either harbouring different exon 7 mutations or a homozygous mutation, each with a co-incident population of cells harbouring a heterozygous mutation.

5.4 Discussion

The data presented in this chapter shows the screening of a cohort of paediatric patients with T-ALL for mutations in the *PTEN* gene, and quantification of the mutant level. In total, of the 162 patients analysed, 21 (13%) had one or more mutations in *PTEN*. This incidence is in line with the combined data from other paediatric studies (100 of 698 cases, 14%, *P*=0.6) (Table 5.1). In one study there was a significantly higher incidence of *PTEN* mutations (27 of 43 cases, 63%) compared with the other paediatric studies (Larson Gedman *et al.*, 2009). However, many of the cDNA mutations detected probably were in the *PTEN* pseudogene, *PTENP1*, which is known to be transcribed. *PTENP1* lacks the intronic sequences of the *PTEN* gene and has 98% homology to functional *PTEN* cDNA (Dahia *et al.*, 1998). In the present study, primers for mutational screening were designed to anneal to the intronic sequences flanking the exons, thereby eliminating the possibility of amplification of the pseudogene.

There was a striking clustering of the mutations in exon 7. Of the 21 *PTEN*-mutated patients, 19 (90%) harboured a mutation in this exon. This mutation location has been previously identified and other studies have reported a high frequency of mutations in this region (97 of 102 mutations, 95%, compared to 40 of 44 mutation in the current study, 91%, *P*=0.9). Although mutations were found in other exons, 5 and 6 in the present study and 1, 3, 4, 5, 6 and 8 in other studies (Table 5.1), all but four of these patients also harboured co-incident exon 7 mutations. In other studies, patients harbouring mutations outside of exon 7 alone were infrequent, and this was confirmed in the current study where only 2 of 21 (10%) patients harboured exon 5 mutations alone. Other studies identified mutations in exons 1, 3, 4 and 8. This was not the case in the present cohort, but due to the low frequency of the mutations in these exons in other studies, seven in 100 mutant-positive patients, it is not surprising that none were identified. In the two patients in the present study harbouring mutations in both exons 6 and 7, it was not possible to determine whether the mutations are in cis on the same allele, as RNA was not available. The intron between exons 6 and 7 is 7kb in size, although possible, amplification and cloning of a PCR product from genomic DNA would have been challenging. Therefore, due to the limited material available this was not pursued. Of note, one case with exon 5 and 7 mutations occuring in cis on the same allele has been reported (Zuurbier *et al.*, 2012).

The majority of mutations detected (85%) were size changes predicted to result in the introduction of a premature stop codon, and this is in line with the combined data reported

from other studies (117 of 122 total mutations, 96%, *P*=.6) (Table 5.1). The truncating mutations in exon 7, resulting in the loss of the C2 domain and C-terminal PEST sequences, all targeted the exon in the region between amino acids 219-261, and R233 was the amino acid most frequently disrupted by the mutations. The introduction of a premature stop codon would be likely to result in the induction of nonsense-mediated decay (NMD), leading to a lack of functional PTEN protein being produced. During splicing, exon-exon junction complexes (EJC) are deposited on the mRNA, approximately 20-24 nucleotides from the splice junction (Le Hir *et al.*, 2000). Translation begins upon ribosome binding to the transcript, and amino acid elongation continues until the EJC is displaced, and is complete when a termination codon is reached (Cheng & Maquat, 1993). In NMD, if the mRNA contains a premature stop codon prior to the location of the EJC, mRNA decay is triggered (Cui *et al.*, 1995). Therefore, the clustering of mutations in exon 7 and the high frequency of premature stop codon introduction could suggest that the functional consequence of these mutants in T-ALL is the induction of NMD. However, studies have shown that the truncating mutations R233X (Papa *et al.*, 2014) and PTEN-254, a mutation that introduces a stop codon at residue 254 (Georgescu *et al.*, 1999), did generate a PTEN protein, although it was expressed at low levels and rapidly degraded. Defective folding of both the C-terminal and phosphatase domains also reduced the phosphatase activity of the truncated PTEN protein (Georgescu *et al.*, 1999). In a recent study investigating the PTEN protein expression of a number of mutations that were also detected in the present study, T232fs, R233fs, R234fs and P246fs, the protein was found to be absent in all cases (Zuurbier *et al.*, 2012). The study also reported lack of the PTEN protein in one patient harbouring a heterozygous T232fs mutation, but no details were given as to why the remaining WT allele did not generate a functional protein; a possible explanation is that the other allele has been silenced via another unknown mechanism. This heterozygous mutation was also seen in the present study, as was the combination of an R233fs and P246fs mutation reported by Zuurbier *et al* (2012). Therefore, these patients would be predicted to lack PTEN protein in the affected cells. The remaining truncating mutations detected in the current study, Q219fs, I224fs, Y225fs, N228fs, S229fs, E235fs and F241fs, all clustered in close proximity to the other mutations so would also be predicted to result in a similar functional consequence, but as there were no cells available from the patients, this cannot be confirmed. The functional significance of the in-frame mutations is unclear; all five of these mutants were size changes ranging from 6bp to 18bps. The addition of extra amino acids leading to the generation of an unstable protein is a possible explanation. One mutation in exon 5 and both mutations found in exon 6 introduced a premature stop codon. They are thought to be functionally equivalent to exon 7 mutations as the C2 domain is removed, however the C-terminal end of the phosphatase domain is also truncated. Therefore if these mutants do produce a functional

protein, this suggests the phosphatase activity may be diminished compared to the exon 7 mutants.

A particular feature of the *PTEN* mutations identified in the present cohort is the presence of multiple exon 7 mutations per patient. Of 19 patients with mutations in exon 7, 14 (74%) had more than one mutation in the exon. This is significantly higher than in data reported from other studies combined (11 of 91, 12%, P=0.0005). A possible explanation for this difference is the screening technique used. The mutational screening method utilised by many groups is Sanger sequencing, with six of the seven paediatric studies using this approach. Studies in our laboratory have shown that Sanger sequencing is less effective at detecting mutations that are present at a level less than 10-20% of total alleles. Therefore if there were multiple mutations in a sample, the mutations present at a lower level may not have been identified on the sequence. In the present study, dHPLC was used to screen PCR products, which is more sensitive than sequencing, although it does not indicate the number of mutations present in the sample. Fragment analysis of fluorescently labelled PCR products is a robust way of screening and quantifying mutations, therefore it was utilised to determine mutant level(s) in the present study. This quantification method has been successfully used in the department for other genes and has been shown to be a straightforward and sensitive technique. However, problems with this technique included the inaccurate calling of the fragment size of 18% of the mutants when compared to the nucleotide sequence, and difficulty in quantifying mutants with size changes of 1bp. Therefore, the combined use of dHPLC and fragment analysis enabled the detection of mutants present at a level of less than 20% of total alleles, directing further investigation to characterise the low level mutants, which may otherwise have been undetected, by TOPO cloning and sequencing. There were 14 mutations present at a level ≤10% of total alleles, 32% of all the mutations detected. A further nine (20%) mutations were quantified between 10-20% of total alleles. Therefore of all mutations detected, 52% were <20%. It is possible other mutations have been missed in the present study, as demonstrated by the example given, in which a 1bp size change that quantified at 2% was only detected after restriction enzyme digestion (Figure 5.5B). This mutation was masked by two other mutations present in the patient. Studies in the laboratory have shown mutations quantified at a level of <5% of total alleles can be identified by dHPLC, therefore any missed mutations are only likely to have been in cases with other *PTEN* mutations, as had it been the sole mutant, it is likely it would have been detected.

Of the 44 mutations detected in the present study, approximately 52% were <20% of total alleles and were therefore likely to be present in subclones, although the possibility of non-leukaemic cell contamination cannot be excluded. The presence of normal cells in the

sample would dilute the leukaemic cells which may result in the mutant level being underestimated. This high frequency of subclonal mutations raises the question of whether a small population of subclones are driving resistance to therapy, some of which may not have been detected at diagnosis by insensitive screening techniques. Relapse samples from the patients in the present study were not available for investigation. However, intraclonal heterogeneity at diagnosis and clonal evolution at relapse are known to occur in T-ALL (Anderson *et al.*, 2011;Mullighan *et al.*, 2007;Yang *et al.*, 2008), and in a report where the study of paired diagnostic and relapse samples was possible, the authors demonstrated that *PTEN* mutations were acquired in two of 35 (6%) patients upon relapse (Palomero *et al.*, 2007). It is possible that a PTEN-inactivated subclone that was undetected at presentation was selected for during the progression of disease and expanded, giving rise to relapse. However, as most of the *PTEN* mutations described in T-ALL are predicted to be functionally equivalent, determining the contribution of coincident genetic events is important in understanding the biology of relapse. NGS techniques will facilitate the investigation of other collaborating abnormalities which could be explored for targeted mutation analysis in T-ALL.

It has been shown that *PTEN* is a haploinsufficient tumour suppressor gene, therefore loss of function would be expected in patients in whom one allele is mutated (Di Cristofano *et al.*, 1998;Podsypanina *et al.*, 1999;Suzuki *et al.*, 1998). Subsequent studies have shown that a further decrease of *PTEN* gene dosage below that of one functional allele leads to an accelerated progression to a more aggressive cancer (Alimonti *et al.*, 2010;Trotman *et al.*, 2003). It is unknown whether the loss of one or both alleles is more tumourigenic in T-ALL. One study of paediatric T-ALL reported that patients with biallelic *PTEN* mutations had the lowest survival rate compared to monoallelic and WT patients (Jotta *et al.*, 2010), suggesting that the level of PTEN loss is an important factor in T-ALL but this finding has not been replicated in other studies. Studies in other cancers have shown that the importance of the level of loss of PTEN during tumourigenesis may also depend on other factors and collaborating genetic abnormalities. In an in vivo prostate cancer model, complete loss of *Pten* and WT p53 resulted in the induction of *Pten*-loss-induced cellular senescence (PICS), whereas monoallelic loss led to the progression of cancer (Chen *et al.*, 2005). Therefore, in the absence of a p53 abnormality, monoallelic loss of *PTEN* may be more detrimental than biallelic loss.

As only genomic DNA was available for each patient in the current study, single cell analysis was not possible to determine whether the mutations were present in the same cell to indicate the level of PTEN loss. In two separate studies, authors have classified patients

harbouring more than one mutation as compound heterozygous by re-sequencing of cloned PCR products. Gutierrez *et al* (2009) stated this was the case in three patients, each with two exon 7 mutations. Zuurbier *et al* (2012) reported compound heterozygosity in eight of nine patients with either more than one mutation in exon 7 or mutations in both exons 5 and 7. However, the technique utilised by both groups would only be able to demonstrate whether the mutations are on the same allele, not in the same cell. Therefore, the true incidence of compound heterozygosity is unclear. In the absence of single cell analysis, the mutant level of the individual mutations in a patient may indicate whether the mutations are likely to be monoallelic or biallelic. In the present study, patients with one or more mutations with a total mutant level was <50% were assumed to be monoallelic, and patients with either one or multiple mutations totalling >50%, to be biallelic on the assumption that at least a proportion of the cells must have more than one allele affected. However, using this approach it is not possible to determine whether the biallelic mutations are compound heterozygous or homozygous. The possibility of hemizygoisity also cannot be excluded and this is addressed further in chapter 6. Of the 21 *PTEN*-mutated patients, 11 (52%) patients were therefore classified as harbouring monoallelic mutations and, where more than one mutation was present, the mutations were considered most likely to be heterozygous and in different subclones. In these patients, the total number of cells affected by mutation ranged from 22% to 96%, demonstrating that in some patients, nearly all the cells were affected by *PTEN* mutation. The low mutant level in other patients could be as a result of non-leukaemic cell contamination of the sample or true subclones. Ten patients (48%) were classified as having biallelic mutations. Therefore monoallelic and biallelic mutations were identified at an equivalent frequency, suggesting that the presence of other collaborating mutations may be an important factor in disease progression.

The data presented in this chapter shows the screening, identification and quantification of mutations in the *PTEN* gene. Data on the association of the mutations with clinical characteristics and the impact of the mutations on patient outcome is studied in chapter 6.

CHAPTER 6: COPY NUMBER ALTERATIONS IN *PTEN* AND THE PROGNOSTIC IMPLICATIONS OF *PTEN* ABNORMALITIES ON PAEDIATRIC PATIENTS WITH T-ALL

6.1 Introduction

6.1.1 *PTEN* gene dosage

The identification of tumour suppressor genes led to the two-hit hypothesis in cancer, which was based on the analysis of a childhood cancer, retinoblastoma (Knudson, 1971). Germline deletion or mutation of one allele of the *RB1* gene can be inherited from the parent (the first hit), which leads to retinoblastoma following the acquisition of a mutation in or loss of the other allele (the second hit). However, recent studies attempting to apply the principles of this hypothesis to identify novel tumour suppressors in sporadic cancers have demonstrated that for some genes where the loss of function of one allele has been reported, a second hit on the remaining allele is not required for tumour initiation and progression. As previously mentioned in chapter 5 (section 5.4.1), *PTEN* has been described as a haploinsufficient tumour suppressor gene as loss of only one allele can elicit a cancer phenotype. A recent study has further demonstrated that a more subtle reduction in the level of *Pten* can accelerate cancer progression (Alimonti *et al.*, 2010). The authors generated a murine *Pten* '*hyper*' model by targeting intron 3 of *Pten* with a neomycin cassette under the control of the CMV promoter, which resulted in the disruption of transcription. $Pten^{hy/+}$ cells expressed 80% of normal *Pten* mRNA and protein levels, and were used to generate a hypomorphic allele series of mice with decreasing *Pten* expression: $Pten^{+/+}$, $Pten^{hy/+}$, $Pten^{+/-}$ and $Pten^{hy/-}$. Notably, $Pten^{hy/+}$ mice showed an increased susceptibility to the development of cancers including mammary tumours and epithelial cancers. A further decrease of *Pten* dosage below monoallelic loss was associated with decreased survival. These studies led to *PTEN* being described as an obligate haploinsufficient tumour suppressor gene, where tumour progression may favour partial loss of *PTEN* over complete loss (Figure 6.1).

It has been hypothesised that the cancer susceptibility and disease severity associated with a decrease in *PTEN* gene dosage is tissue-specific and context dependent (Hollander *et al.*, 2011). Studies have shown that the genetic background of the individual or tumour has an impact on the phenotype caused by the loss of *PTEN*. For example, in a mouse model of prostate cancer, loss of one allele of *Pten* in the presence of WT p53 was more tumourigenic than biallelic loss (Chen *et al.*, 2005). Complete loss of *Pten* resulted in the induction of p53-

Figure 6.1 Tumour suppressor genes. (A) Example of a gene which follows the two-hit hypothesis, retinoblastoma (*RB*). (B) Example of a haploinsufficient tumour suppressor gene, *p53*. (C) Example of an obligate haploinsufficient gene, *PTEN*. Abbreviations: PICS, PTEN-induced cellular senescence. Taken from Berger *et al* (2011).

dependent *Pten*-loss-induced cellular senescence (PICS), whereas monoallelic loss led to the progression of cancer. In the murine haematopoietic compartment, complete loss of *Pten* on a WT p53 background induced HSC exhaustion or bone marrow failure and only led to leukaemias, including AML and ALL, when co-incident genetic events occurred, such as loss of p53 (Lee *et al.*, 2010;Yilmaz *et al.*, 2006). Differences in the cancer phenotype of various monoallelic abnormalities have also been reported, with heterozygous loss-of function *PTEN* mutations being associated with higher levels of AKT phosphorylation compared to those with heterozygous (+/-) PTEN loss (Papa *et al.*, 2014). The authors showed that the mutant *Pten* proteins exerted a dominant-negative effect by dimerisation with the WT protein, and suggest that this may result in a more exacerbated tumour spectrum.

6.1.2 Genomic loss of *PTEN* in T-ALL

PTEN deletions were first associated with T-ALL when a homozygous deletion was identified in the T-ALL cell line CCRF-CEM (Gronbaek *et al.*, 1998). It was then shown that thymic lymphomas from *TP53*$^{-/-}$, *Terc*$^{-/-}$, *Atm*$^{-/-}$ triple knockout mice also harboured *Pten* deletions encompassing the whole gene (Maser *et al.*, 2007). As a consequence, array-CGH analysis was performed on samples from 26 paediatric T-ALL patients and four (15%) were found to have a *PTEN* deletion. Since then, a number of other groups have extended these findings, and these studies are detailed in Table 6.1. Overall, deletions have been reported at a frequency of between 5% and 15% in paediatric studies. Combining the data from three studies, of 183 cases, 14 (8%) harboured *PTEN* deletions (Gutierrez *et al.*, 2009;Maser *et al.*, 2007;Zuurbier *et al.*, 2012). Biallelic and monoallelic deletions have been reported, however the functional advantage of one versus the other has not been defined. In the two studies where this information was available, of 157 patients, seven (4%) had a heterozygous deletion and three (2%) a homozygous deletion, which was too few for further analysis (Gutierrez *et al.*, 2009;Zuurbier *et al.*, 2012).

In one study where protein level was assessed, no PTEN protein was detected in patients with either a heterozygous or homozygous deletion, in a patient in which the deletion was subclonal, and in three patients with WT *PTEN* genomic level and sequence (Zuurbier *et al.*, 2012). As a result the group extended their previous findings and used multiplex ligation-dependent probe amplification (MLPA) to screen samples from 146 patients for *PTEN* microdeletions (Mendes *et al.*, 2014) (Table 6.1). They were identified in four (3%) patients: three spanning exons 2-3, two of them heterozygous and one homozygous, and one heterozygous deletion of exons 4-5. The deletions ranged from 11kb to 65kb and all resulted

Table 6.1 Studies of *PTEN* deletion in patients with T-ALL

Reference	Total in study	Age group	Screening method	Total *PTEN* deleted patients	*PTEN* Deletion	Total *PTEN* mutant patients (%)	Total *PTEN* abnormalities	Co-incident *NOTCH1/FBXW7* mutations
Maser *et al* (2007)	26	Paediatric	aCGH +qPCR	4 (15%)	No details	N/A	N/A	No association with *NOTCH1* and/or *FBXW7* mutations
Gutierrez *et al* (2009)	44	Paediatric	aCGH + FISH	4 (9%)	2 HOM 2 HET No deleted patients harbored co-incident mutations	12 (27%)	16 (36%)	N/A
Zuurbier *et al* (2012)	142	Paediatric	aCGH + FISH	6 (5%)	3 HET 1 HOM 2 SUB 3 patients had co-incident mutations	16 (11%)	19 (13%)	*NOTCH1* mutated patients had a significantly lower incidence of *PTEN* mutation (*P*=.006)
Mendes *et al* (2014)*	146	Paediatric	aCGH+FISH MLPA+ genomic breakpoint PCR	16 (11%)	3 HET 2 SUB 1 SUB + MICRO HET 2 MICRO HET 1 MICRO HOM 7 MICRO SUB 8 patients coincident mutations	16 (11%)	26 (18%)	*PTEN* mutation significantly associated with absence of *NOTCH1* mutation (*P*=.05)

Table 6.1 Continued

Reference	Total in study	Age group	Screening method	Total *PTEN* deleted patients	*PTEN* Deletion	Total *PTEN* mutant patients (%)	Total *PTEN* abnormalities	Co-incident *NOTCH1/FBXW7* mutations
Trinquand *et al* (2013)	175	Adult	aCGH+qPCR	6 (3%)	3 HET 1 HOM 2 MICRO 2 patients coincident mutations	17 (10%)	21 (12%)	*PTEN* mutation significantly associated with absence of *NOTCH1*and/or *FBXW7* mutation (*P*=.002)

Abbreviations: aCGH, array-CGH; FISH, fluorescent in-situ hybridisation; HOM, homozygous; HET, heterozygous; SUB, subclonal; MICRO, microdeletion; N/A, not available; MLPA, multiplex ligation probe-amplification.
* extended study of Zuurbier *et al* (2012).

in an out-of-frame transcript. Further screening of the samples for similar breakpoints to those identified revealed subclonal microdeletions in an additional seven patients. In one patient, a clonal microdeletion had already been detected by MLPA and in another patient, a heterozygous deletion had already been characterised in their previous study. Therefore microdeletions were identified in 11 (8%) patients, although only four of these were considered to be clonal. Only two of the 11 deletions had been detected in their previous study using array-CGH. Analysis of the sequences directly surrounding the breakpoints revealed that the microdeletions were as a result of illegitimate RAG-mediated recombination events, mediated by cryptic recombination signal sequences.

Overall therefore, of the two studies where results of both *PTEN* mutations and deletions have been reported, 42 of 190 patients (22%) were considered to have abnormal *PTEN*. Of these, seven (4%) patients had both co-incident mutation and deletion, 14 (7%) harboured deletion only and 22 (12%) mutation only (Gutierrez *et al.*, 2009;Mendes *et al.*, 2014).

6.1.3 Techniques to detect *PTEN* copy number changes

Earlier studies of copy number changes in *PTEN* utilised Southern blotting (Wang *et al.*, 1998). However, the more recent development of high-throughput sequencing and microarray platforms has enabled the detection of copy number changes in large numbers of samples. The three studies that have screened for *PTEN* deletions in samples from paediatric patients with T-ALL all utilised microarray based-comparative genomic hybridisation (array-CGH) (Gutierrez *et al.*, 2009;Maser *et al.*, 2007;Zuurbier *et al.*, 2012). In this assay, DNA from a reference and patient sample are differentially labelled with fluorophores and used as probes to hybridise to genome-wide or more defined nucleic acid targets. Newer approaches to array-CGH have enabled size changes of 200bp to be resolved. However, the technique is expensive, time consuming and it cannot identify copy-neutral aberrations such as acquired uniparental disomy (UPD). More recently, array-CGH platforms incorporating a number of SNP markers have become available to overcome this problem. It also requires 2-5µg of genomic DNA per sample for analysis.

Other approaches examining just the *PTEN* gene have included genomic allele quantification by quantitative PCR (qPCR) to determine a gene dosage ratio of *PTEN* compared to a reference gene, *ALBUMIN* (Trinquand *et al.*, 2013), fluorescence in-situ hybridisation (FISH) analysis, although this technique requires cells from the patient (Gutierrez *et al.*, 2009;Zuurbier *et al.*, 2012), and MLPA (Mendes *et al.*, 2014).

However, a number of studies of paediatric ALL have used SNP arrays to detect copy number changes (Kuiper *et al.*, 2007;Mullighan & Downing, 2009;Mullighan *et al.*, 2008;Kawamata *et al.*, 2008). Here, unlabelled genomic test DNA is fragmented and hybridised to labelled allele-specific oligonucleotide probes that are attached to a beadchip. This is followed by single-base extension of the probes to incorporate detectable labels, generating intensity values in each of the two colour channels corresponding to the two alleles. SNP array panels consist of a uniform distribution of SNP markers to limit large gaps across the entire genome and are used for high resolution copy number aberration detection as the probes can be spaced at intervals of 500bps. The technique can also identify areas of copy-neutral change, and <1 µg DNA/sample is required. There are a number of SNP array platforms available. The most widely used are Affymetrix arrays, for example, the Affymetrix SNP 6.0 array interrogates over 1.8 million genome-wide genetic markers including approximately 900,000 SNPs and 900,000 intensity-only-non-polymorphic probes in regions of known copy number variation and has over 400 probes covering the *PTEN* gene.

To identify *PTEN* copy number changes in the present study, SNP array was chosen over array-CGH as less DNA is required. Due to the high cost of the Affymetrix protocol, the Illumina platform was selected. The Illumina CytoSNP-850K array examines 850,000 empirically selected SNPs spanning the entire genome. It is enriched for coverage of 3262 genes of known cytogenetic relevance in cancer applications and includes 230 *PTEN* probes. It requires only 200ng of DNA per sample, and the long 50-mer probes offer a high signal-to-noise ratio.

6.1.4 Impact of *PTEN* abnormalities on clinical characteristics and outcome in T-ALL

Several groups have focused on the association of *PTEN* mutations and deletions with the clinical characteristics of patients with T-ALL and their prognostic significance, and these are shown in Table 6.2. Most paediatric studies showed no association of *PTEN* abnormalities with age, sex or WBC count, although of three studies correlating genotype to clinical characteristics, one reported that patients harbouring abnormalities were more likely to be associated with a younger age (Mendes *et al.*, 2014) and another showed an association between patients with biallelic mutations and a higher WBC count at diagnosis (Jotta *et al.*, 2010). No association was observed between T-cell immunophenotype and *PTEN* mutations and deletions, and only one study demonstrated an association with cytogenetic aberrations, where patients with *PTEN* mutations and deletions were significantly more likely to have a

TAL/LMO rearrangement and to be negatively associated with *TLX3* rearrangements (Mendes *et al.*, 2014). Only one group has correlated *PTEN* genotype with glucocorticoid response to therapy and MRD status (Bandapalli *et al.*, 2013). The authors demonstrated that patients harbouring *PTEN* mutations were significantly associated with a poor prednisone response when compared to $PTEN^{WT}$ patients (P=0.0007). Similarly, at both day 33 and day 78 of induction therapy, $PTEN^{MUT}$ patients were more likely to be MRD-positive than $PTEN^{WT}$ patients (P=0.0007 and P=0.005 respectively). In a multivariate analysis with factors including age, sex and WBC, *PTEN* mutations remained an independent negative prognostic marker for early response to therapy and MRD status. Of five studies investigating the association of either *PTEN* mutations alone or *PTEN* mutations and deletions on long-term outcome, most agreed that there was no significant difference in long-term outcome between *PTEN*-abnormal ($PTEN^{ABN}$) and $PTEN^{WT}$ patients (Bandapalli *et al.*, 2013;Gutierrez *et al.*, 2009;Larson Gedman *et al.*, 2009;Mendes *et al.*, 2014). However, when Gutierrez *et al* (2009) analysed the outcome of mutated and deleted patients separately, *PTEN* deletions were significantly associated with early treatment failure although this was based on only four patients harbouring deletions. In another study, patients with biallelic *PTEN* mutations had a significantly worse OS than monoallelic and WT patients (Jotta *et al.*, 2010).

6.1.5 *PTEN* abnormalities and *NOTCH1* mutations in T-ALL

A number of studies have suggested that there is a NOTCH-PTEN-AKT regulatory axis which, during normal thymocyte development, mediates upregulation of P13K/AKT signalling but upon aberrant NOTCH1 signalling in T-ALL, switches to promote the growth of leukaemic cells (Palomero *et al.*, 2007). Chromosome immunoprecipitation (ChIP)-on-chip analysis of promoter occupancy in the HPB-ALL T-ALL cell line demonstrated that two transcription factors directly controlled by NOTCH1, HES1 and MYC, are negative regulators of the *PTEN* gene promoter (Jarriault *et al.*, 1995;Satoh *et al.*, 2004;Weng *et al.*, 2006). Further evidence linking NOTCH1 and PTEN came from a study to identify disrupted genes associated with resistance to GSIs in a panel of GSI-resistant and GSI-sensitive cell lines, where PTEN was consistently found to be down-regulated in the GSI-resistant cell lines (Palomero *et al.*, 2007).

The role of co-incident *PTEN* and *NOTCH1* abnormalities in T-ALL has been investigated in two paediatric studies. Both describe a negative association between the incidence of *NOTCH1* and *PTEN* mutations but report different outcomes in their respective cohorts. In the study investigating a cohort of 301 patients treated on the ALL-BFM protocol, where

Table 6.2 Studies reporting on the impact of *PTEN* abnormalities on clinical outcome in patients with T-ALL

Reference	Total in study	Age group	Trial	Total *PTEN* abnormal patients	Co-incident *NOTCH1/FBXW7* mutations	Outcome compared to $PTEN^{WT}$
Gutierrez *et al* (2009)	44	Paediatric	COG 9404	16 (36%) MUT +DEL	N/A	No association with outcome in $PTEN^{ABN}$ group $PTEN^{DEL}$ group alone significantly associated with worse EFS (*P*=.028)
Jotta *et al* (2009)	62	Paediatric	GBTLI ALL-99	11 (18%) MUT	N/A	$PTEN^{BI}$ group associated with inferior OS compared to $PTEN^{MONO}$ and $PTEN^{WT}$ (*P*=.04)
Zuurbier *et al* (2012)	142	Paediatric	DCOG + COALL	19 (13%) MUT+DEL	*NOTCH1* mutated patients had a significantly lower incidence of *PTEN* mutation (*P*=.006)	Association with increased RR in $PTEN^{ABN}$ group in presence or absence *NOTCH1/FBXW7* mutation (*P*=.002)
(Erbilgin *et al.*, 2010)	301	Paediatric	ALL-BFM	52 (17%) MUT	*PTEN* mutation significantly associated with absence of *NOTCH1* mutation (*P*=.05)	SER and unfavourable MRD in $PTEN^{MUT}$ group No association with EFS in $PTEN^{MUT}$ group $NOTCH1^{MUT}$ with/without $PTEN^{MUT}$ associated with improved outcome $PTEN^{MUT}$ alone associated with worse outcome
Mendes *et al* (2014)*	146	Paediatric	DCOG + COALL	26 (18%) MUT+DEL	*PTEN* mutation significantly associated with absence of *NOTCH1* mutation (*P*=.05)	No association with outcome in $PTEN^{ABN}$ group alone Association with reduced EFS in $PTEN^{ABN}$ group in presence or absence *NOTCH1/FBXW7* mutation

Table 6.2 Continued

Reference	**Total in study**	**Age group**	**Trial**	**Total *PTEN* abnormal patients**	**Co-incident *NOTCH1/FBXW7* mutations**	**Outcome compared to *PTEN*^{WT}**
Trinquand *et al* (2013)	175	Adult	GRAALL-2003	21 (12%) MUT+DEL	*PTEN* mutation significantly associated with absence of *NOTCH1*and/or *FBXW7* mutation (*P*=.002)	*PTEN*^{ABN} group associated with reduced RFS and OS *NOTCH1*^{MUT} without *PTEN*^{ABN} associated with improved outcome *NOTCH1*^{MUT} with *PTEN*^{ABN} associated with worse outcome

Abbreviations: MUT, mutated; DEL, deleted; N/A, not available; SER, slow early response; MRD, minimal residual disease; RFS, relapse-free survival; EFS, event-free survival; OS, overall survival; BI, biallelic; MONO, monoallelic; ABN, abnormal; WT, wildtype; RR, relapse rate.
***** extended study of Zuurbier *et al* (2012).

NOTCH1-mutated patients were previously reported to be associated with a favourable outcome (Kox *et al.*, 2010), patients with a *PTEN* mutation in either the presence or absence of a *NOTCH1* mutation had an unfavourable prednisone response and MRD status at day 33 (Bandapalli *et al.*, 2013), suggesting that *PTEN* ablates the benefit of a *NOTCH1* mutation at this early time point. However, by day 78 of induction therapy, *NOTCH1*-mutated patients either with or without a *PTEN* mutation were more likely to be associated with MRD-negativity, suggesting that *NOTCH1* is clinically dominant at this time point and neutralises the negative effect of a *PTEN* mutation. When correlating genotype to long-term outcome, *NOTCH1*-mutated patients, either with or without a *PTEN* mutation, had the more favourable outcome, with the worst outcome seen in the patients with a *PTEN* mutation only. However, the unfavourable effect of a *PTEN* mutation was restricted to the medium risk group of 154 patients with a good prednisone response and an intermediate MRD status, although there were only 14 patients with a *PTEN* mutation in this group.

In the study of the cohort treated on the DCOG and COALL trials, in which there was a borderline significant trend towards *NOTCH1*-mutated patients being more likely to be associated with a worse outcome (Zuurbier *et al.*, 2010), *PTEN* abnormalities (mutations and deletions) alone had no significant effect on RFS (Mendes *et al.*, 2014). However, when analysed with *NOTCH1* genotype, patients with *PTEN* and/or *NOTCH1* mutations demonstrated reduced RFS when compared to those with wild-type *PTEN* and *NOTCH1*. In multivariate analysis including male gender and the presence of *TLX3* rearrangements, *PTEN* and/or *NOTCH1* abnormalities were independent predictors for increased risk of relapse.

This chapter reports the investigation of *PTEN* gene deletions and the impact of *PTEN* mutations and deletions on clinical outcome in the cohort of paediatric patients treated on the UKALL 2003 trial.

6.2 Materials and Methods

6.2.1 qPCR to detect *PTEN* gene deletion

A commercially available assay from Applied Biosystems was used to assess *PTEN* copy number by Taqman qPCR. The reaction mix for a 20µl PCR contained 10µl 2x Taqman Genotyping Master Mix, 1µl of FAM-labelled Taqman *PTEN* Copy Number Assay, 1µl of VIC-labelled Taqman *TERT* Copy Number Reference Assay and 20ng DNA template. The standard cycling conditions were an initial denaturation step at 95°C for 10 minutes followed

by 40 cycles of denaturation at 95°C for 15 seconds, and annealing of primers for 60 seconds at 60°C.

For amplification by SYBR green qPCR, the reaction mix for a 20µl PCR contained 10µl 1x Universal SYBR Green Mix (Sigma, UK), 0.2µM of appropriate forward and reverse primers (Appendix Table 1), and 20ng DNA template. The standard cycling conditions were an initial denaturation step at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing of primers for 60 seconds at 60°C.

6.2.2 SNP identification

The presence of a common A/G SNP in intron 1-2, rs1903858, with a Minor Allele Frequency (MAF) of 0.425, was detected when screening exon 2 of the *PTEN* gene by dHPLC (section 5.3.1). Ensembl Genome Browser was therefore used to search for another SNP at the 3' end of the *PTEN* gene with a similar frequency, and the T/G SNP rs555895 in intron 8-9 (MAF=0.434) was identified. Screening for rs555895 was carried out by PCR and dHPLC. Samples were amplified by 35 cycles of PCR using the primers and appropriate annealing temperatures shown in Appendix Table 1. PCR products were obtained using the proof-reading enzyme Optimase (Transgenomic) and the standard Optimase DNA Polymerase reaction mix and cycling conditions are detailed in section 2.1.4. PCR products were checked on a 2% agarose gel stained with ethidium bromide and were then denatured and cooled slowly to enable the formation of heteroduplexes. Denatured products were screened by dHPLC on the WAVE DNA Fragment Analysis System (Transgenomic, Glasgow, UK) as detailed in section 2.1.6 at optimal melting temperatures calculated using the Transgenomic Navigator software (Appendix Table 1). Each WAVE run included a known WT case to allow for comparison.

6.2.3 SNP Quantification

For quantification by fragment analysis, rs1903858 and rs555895 PCR products were obtained using BIOTAQ DNA polymerase with a fluorescently labelled forward primer and unlabelled reverse primer (Appendix Table 1). Due to the sensitivity of the assay, the standard BIOTAQ DNA Polymerase reaction mix and cycling conditions were adjusted as described in section 5.2.5. After amplification, 2µl of PCR product was added to 38µl of sample loading solution (Beckman Coulter UK Ltd, High Wycombe, UK) containing a DNA size standard ladder (DNA Size Standard Kit, 400PA, Beckman Coulter UK Ltd, High Wycombe, UK), then run on the CEQ 8000 Genetic Analysis System (Beckman Coulter UK

Ltd, High Wycombe, UK) to check that PCR products were obtained. Each run included a WT control to identify the WT peak in the patient samples (Figure 6.2).

A restriction enzyme recognition site was identified on one of the polymorphic alleles in each of the SNPs that would allow separation of the two alleles by size after digestion. For rs1903858, 8µl of PCR product was digested at 37°C for 4 hours in a reaction mix with 1µl *HindIII* and 1µl of manufacturer's buffer 4 (New England Biolabs, Hitchin, UK). After digestion, 2µl of the reaction was analysed as described above on the CEQ 8000 Genetic Analysis System. The A alleles were undigested giving a 314bp fragment and the G alleles were digested to a labelled 281bp fragment. For rs555895, 8µl of PCR product was digested at 37°C for 4 hours in a reaction mix with 1µl *HincII* and 1µl of manufacturer's buffer 3 (New England Biolabs, Hitchin, UK), and 2µl of the reaction was analysed as described above. G alleles were uncut giving a 201bp fragment and the T alleles were digested to a labelled 110bp fragment. The fragment analysis software calculated the area under the peak corresponding to each allele, and the contribution of each was determined by expressing the area under each peak as a percentage of total alleles.

6.2.4 SNP array

The Infinium CytoSNP-850k Beadchip array (Illumina, Essex, UK) was chosen for analysis (see section 6.1.3). In the Infinium protocol, genomic DNA is whole-genome amplified and fragmented then hybridised to 50-mer probes on the beadchip. Enzymatic single base extension of the probes then incorporates detectable labels, thereby determining the genotype call at the specified locus. Prior to analysis, the quality of the patient and control WGA DNA (as described in section 5.2.4) was checked on a 1% agarose gel stained with ethidium bromide. High quality DNA appeared as a high molecular weight band, without a smear of lower molecular weight DNA that indicated degradation or shearing of the sample. For samples where the DNA was of sufficient quality, 750ng was sent for SNP array analysis by Kerra Pearce, UCL Genomics, Institute of Child Health.

Analysis of the Infinium BeadChip data was performed using the GenomeStudio Genotyping Module of the GenomeStudio software. Copy number values and confidence scores for each region were calculated using the cnvPartition plug-in algorithm, which is able to detect copy number changes from the log R intensities and B-allele frequencies of each SNP marker (Figure 6.3). Chromosome 10 was viewed in the Illumina Chromosome browser, and for each patient the *PTEN* gene (location 10:89612850-89721667) was scored as WT, HET (heterozygous deletion) or HOM (homozygous deletion). Detected regions of copy number

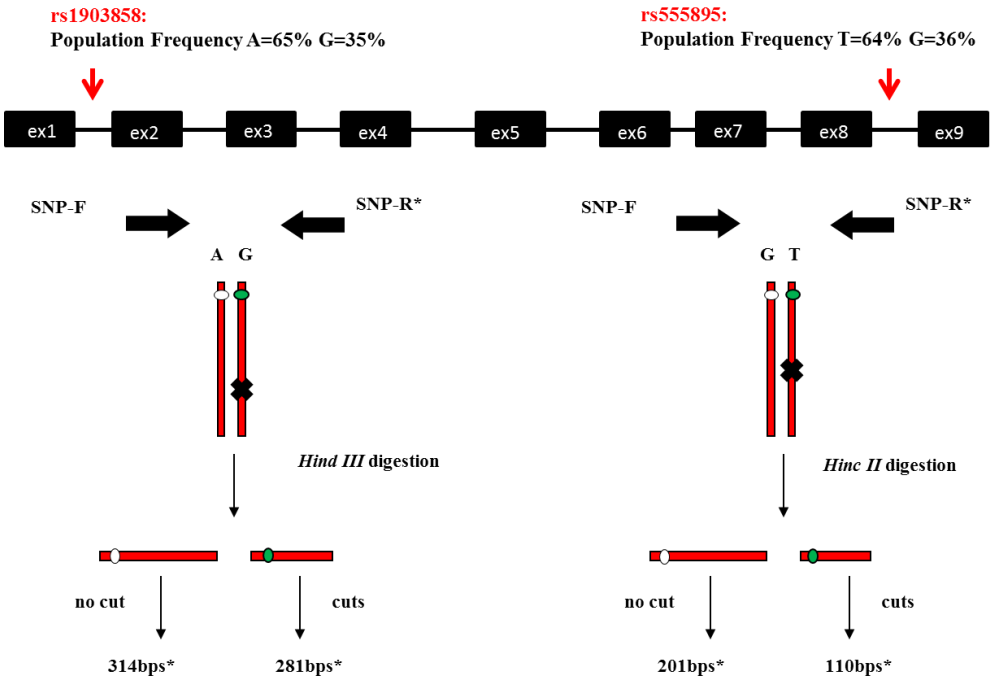

Figure 6.2 SNP allele quantification. Relative contribution of each allele quantified by fluorescently labelled PCR followed by allele-specific restriction enzyme digestion and size separation of labelled PCR products.

loss were shaded red and orange in the chromosome browser, regions of copy number gain were shaded blue and purple. The calculated confidence scores of the known controls, cell lines harbouring a heterozygous deletion (LOUCY cells) or homozygous deletion (CEM cells) were used to determine a scoring threshold and samples with a score of <80 were scored as WT.

6.2.5 Cytogenetic and FISH analysis

Cytogenetic and FISH results were made available by the Clinical Trial Service Unit and were performed at the Northern Institute for Cancer Research, University of Newcastle as described in chapter 4 (section 4.2.3).

6.2.6 Clinical End Points

Outcome was analysed according to overall survival (OS), defined as the time to death, event-free survival (EFS), which was the time to relapse, secondary tumour or death, and relapse-free survival (RFS), which was the time to relapse for those that achieved remission, censoring at death in remission.

6.2.7 Statistical Analysis

Statistical analysis of the UKALL2003 cohort was performed by Amy Kirkwood at the UCL Cancer Trials Centre. Chi-squared tests, or in the instance of small sample size Fisher's exact test, were used to test for differences across the patient molecular groups in categorical subgroups such as gender, genetic subgroup, NCI risk group, SER and MRD, excluding any missing data categories. *P*-values are quoted for heterogeneity or trend where appropriate. Kaplan-Meier curves were used to assess survival, and differences between groups were compared using the log-rank test. All *P* values quoted are two-sided.

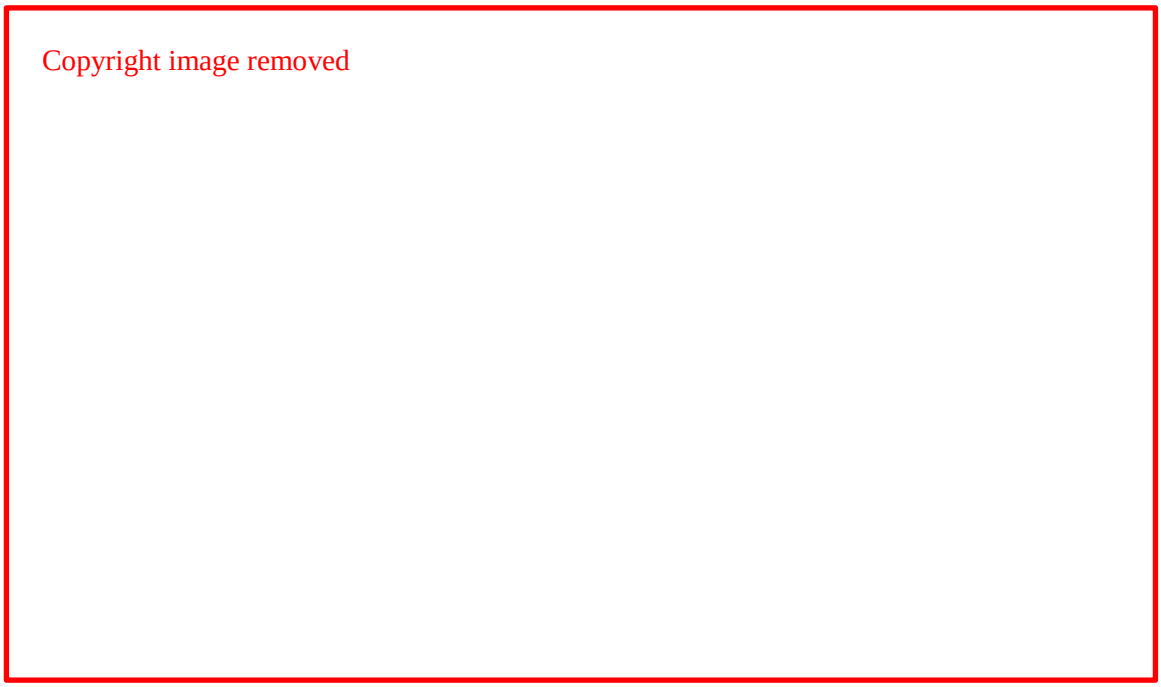

Figure 6.3 Assessment of copy number changes by SNP array. At each SNP locus, the B-allele frequency (BAF) and Log R intensities for the three standard population clusters, AA, AB and BB are scored and plotted. For normal copy-number, CN=2, presence of the three populations is visible on the BAF plot, with probes clustering at 0 on the LogR ratio. For copy number loss, CN=1, the heterozygous AB population is absent from the BAF plot, with probes clustering below 0 on the LogR ratio. For copy number gain, CN=3, extra AAB, ABB populations on the BAF plot are present, with probes clustering above 0 on the LogR ratio. Taken from Illumina Technical Note.

6.3 Results

A number of methods were attempted to detect genomic loss of *PTEN* in the cohort of paediatric T-ALL patients.

6.3.1 qPCR

Initial attempts to screen for *PTEN* gene deletion utilised a commercially available Taqman Copy Number assay with primers for probing *PTEN* in intron 5-6, and a reference gene *TERT*, in a multiplex qPCR. To determine the sensitivity of the assay for discriminating between cells with normal *PTEN* gene copy number, heterozygous or homozygous deletion, various mixes were prepared using cell lines with known *PTEN* genotype, either WT (HL60 cells), heterozygous deletion (LOUCY cells) or homozygous deletion (CEM cells). DNA was extracted and the samples were assayed in triplicate. Despite attempts to optimise the assay by varying the concentration of genomic DNA and adjusting the threshold Ct and baseline parameters, the products obtained were usually inadequately amplified for accurate quantification, and the copy number results obtained were neither reproducible nor consistent with the expected values. Therefore this approach was not pursued any further. Subsequent attempts using SYBR green and custom designed *PTEN* and *TERT* primers with the titration mixes gave similar results. Therefore another strategy to detect loss of genomic *PTEN* was devised.

6.3.2 SNP screening and allele quantification

A common SNP at the 5' end of the gene, A/G rs1903858 in intron 1-2, was detected as part of the mutation screening for exon 2 (see section 5.3.1). Of the 162 patients investigated, 76 (47%) had an abnormal WAVE chromatogram and the chromatograms of four patients indicated an allelic imbalance (Figure 6.4). Presence of the SNP was confirmed by PCR and *Hind*III restriction enzyme digestion as detailed in section 6.2.3 (Figure 6.2). In order to determine whether the genomic loss encompassed the whole gene, an additional SNP at the 3' end in intron 8-9, the T/G rs555895 polymorphism, was identified in Ensembl Genome browser and the samples screened by WAVE analysis for the presence of the SNP as detailed in section 6.2.2. Of the 162 patients investigated, 76 (47%) had an abnormal WAVE chromatogram. There were six patients where the chromatograms suggested an allelic imbalance. Presence of the SNP was confirmed by PCR and *Hinc*II restriction enzyme digestion (see section 6.2.3) (Figure 6.2).

The contribution of each polymorphic allele for both the rs1903858 and rs555895 SNPs was quantified by fluorescently labelled PCR and allele specific restriction enzyme digestion using both non-WGA and WGA samples. At any given SNP site, the expected allele ratio was 50%:50%, i.e. there is one copy of each polymorphic allele. Therefore, to determine the normal range for the assay, samples from a series of 20 SNP-positive normal controls were quantified and a mean allele ratio and range calculated, any deviation from this was considered to indicate loss of genomic material. For rs1903858, the mean percentage for the A allele was 53%±2% (range, 50%-58%), and for rs555895 the mean percentage for the T allele was 53%±2% (range, 49%-57%).

Of the 76 SNP-positive patients informative for one/both SNPs, 72 were successfully quantified using both the non-WGA and WGA samples, and the comparative data obtained from the allele ratios is shown in Figure 6.5. Comparison was not possible for four patients. In one patient there was insufficient original genomic DNA to whole-genome amplify and in three patients the PCR product from the WGA sample was of too poor quality after digestion for the peaks to be called by the software. There was a strong correlation between the levels of the individual SNPs in the non-WGA and WGA samples. For rs1903858, r^2=0.88 and the median difference was 2% (range 0% to 11%) and for rs555895, r^2=0.89 and the median difference was 2% (range 0% to 12%). Only three cases had >10% difference between the two values (two for rs1903858 and one for rs555895). These differences are most likely to be as a result of incomplete digestion of the PCR product, therefore the uncut allele was overestimated in the ratio.

An average allele ratio for each individual SNP level was determined from the non-WGA and WGA samples. Of the 76 patients informative for rs1903858, the allele ratio of 69 (91%) patients was within the normal range. In seven cases (9%) the A/G ratio was 7:93, 17:83, 68:32 (three cases) and 73:27 (two cases) (Table 6.3), which would be consistent with genomic loss in at least a proportion of the cells. An example is given in Figure 6.6B. For rs555895, there were nine (12%) patients with an allelic imbalance, T/G ratio 5:95, 21:79, 22:78, 62:38, 66:34, 68:32 (two cases), 75:25 and 80:20. Seven of these patients had equivalent loss at the 5' and 3' SNP loci, suggesting that the deletion encompassed the whole gene. The remaining two patients' harboured loss at the 3' end only, suggesting that the heterozygous deletion was intragenic (Figure 6.6C).

The allele ratio was then used to calculate the proportion of cells that would harbour a deletion. In patients where there was equivalent loss at both loci, the mean was determined from the 5' and 3' SNP ratios. In the seven patients with heterozygous deletion spanning the

whole gene, the proportion of cells affected were calculated to be 46%, 51%, 53%, 57%, 65%, 75% and 96% respectively, although three of these cases were subsequently found to be amplifications of the *PTEN* gene (see below) (Table 6.3). Of the two patients harbouring loss at the 3' end only, this was the case in 73% and 75% of cells respectively. Of note, the technique only detects heterozygous loss. In a sample harbouring homozygous loss, the proportion of non-deleted cells or contaminating non-leukaemic cells would instead be amplified, giving a false normal allele ratio.

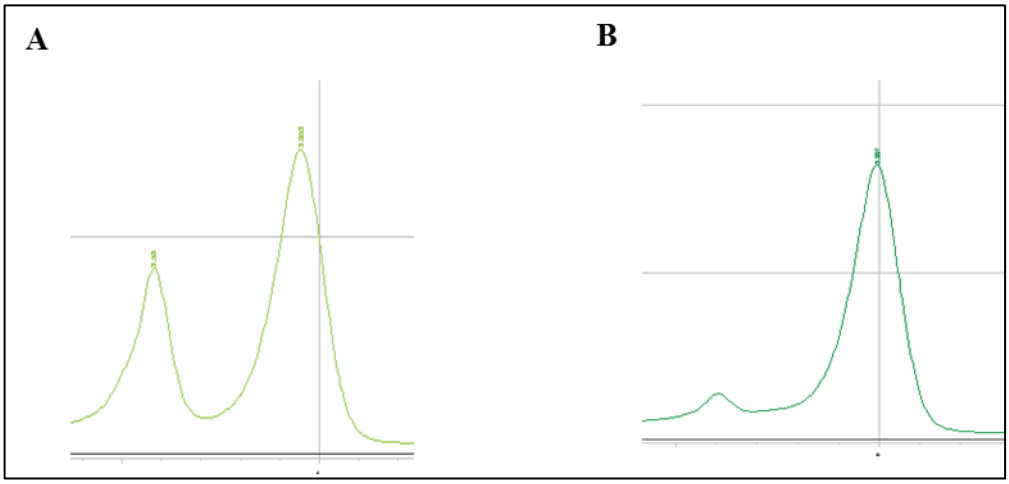

Figure 6.4 Detection of unbalanced SNP levels in patients with T-ALL by dHPLC. Representative WAVE chromatograms of the rs1903858 polymorphism. (A) Patient with a normal SNP level. (B) Patient with an unbalanced SNP level.

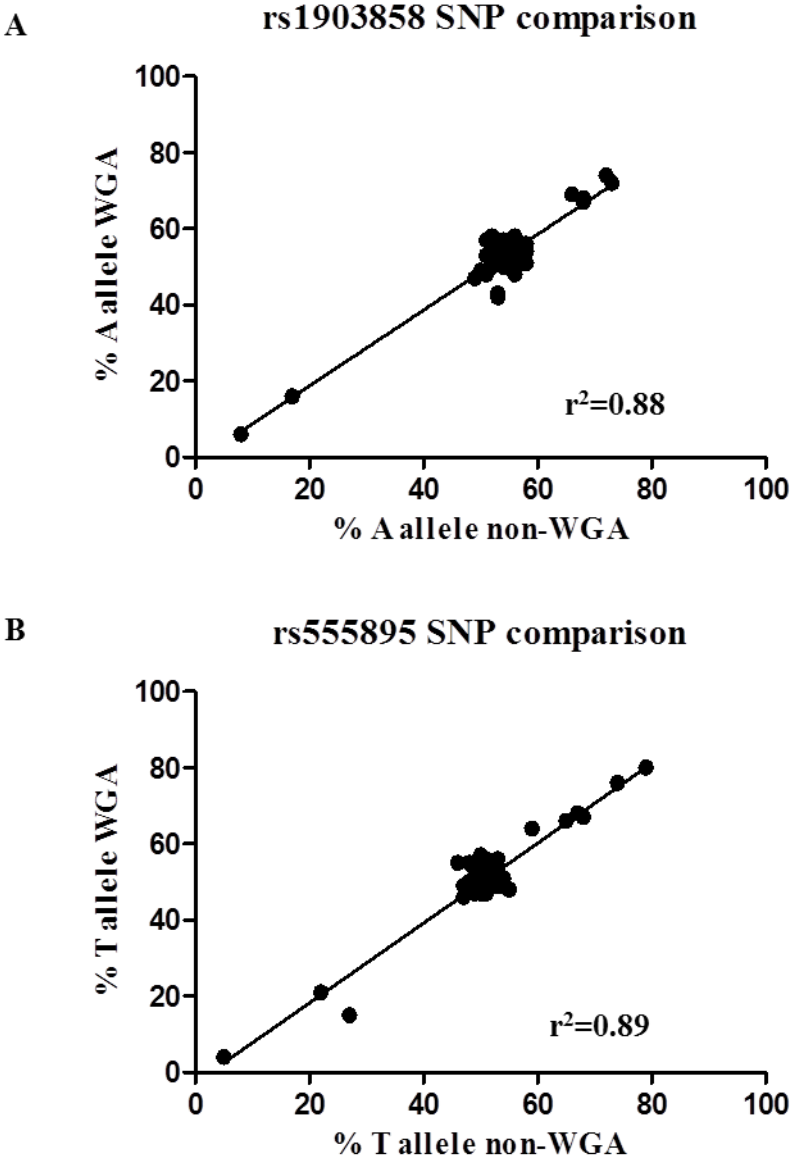

Figure 6.5 Comparison of SNP levels quantified in non-WGA and WGA DNA from patients with T-ALL. Abbreviations: WGA, Whole genome amplified.

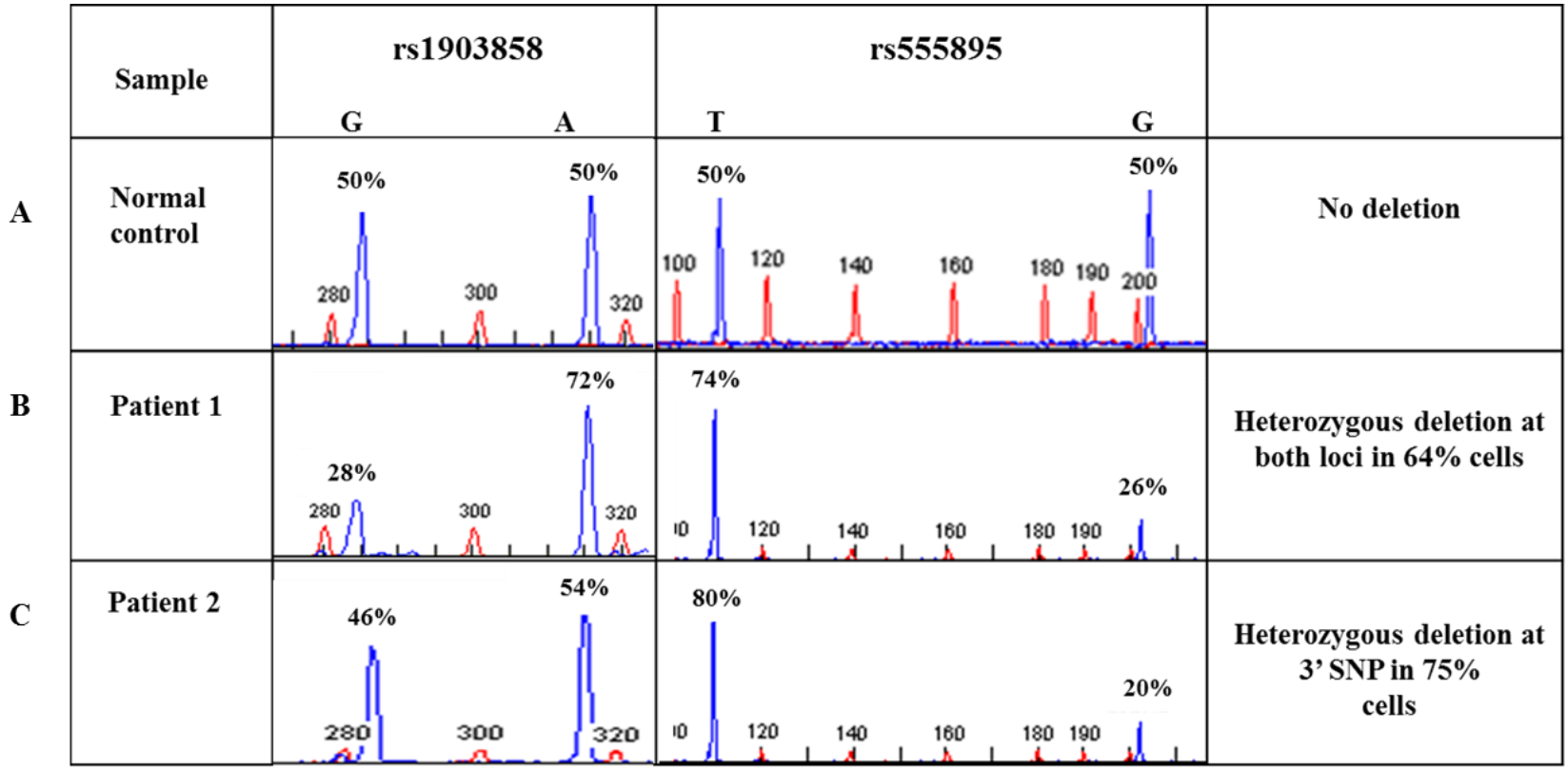

Figure 6.6 Detection of unbalanced SNP levels by capillary electrophoresis in patients with T-ALL. Representative fragment analyses showing (A) Haematologically normal control. (B) Patient with heterozygous loss at both SNP loci in 64% of cells. (C) Patient with heterozygous loss at the 3' SNP locus in 75% of cells.

6.3.3 CytoSNP-850k SNP array analysis

Of the 162 patient samples in the present cohort, there were 13 for which the WGA DNA sample was of insufficient quality to proceed with SNP array analysis. Therefore the CytoSNP-850k arrays were performed on these WGA samples from 149 patients. Ten samples failed the assay, producing unreadable chromosome traces, and were subsequently excluded. The investigation of *PTEN* gene deletion by array analysis was therefore possible in a total of 139 patient samples.

The *PTEN* gene is 108kb in size and located at chromosome 10:89612850-89721667. The CytoSNP-850k Beadchip contains 230 probes to cover this region, distributed approximately one probe per 400-500bps. Analysis of the BeadChip data was performed using the GenomeStudio Genotyping Module of the GenomeStudio software. Chromosome 10 was viewed in the Illumina Chromosome browser and, for each patient, plots corresponding to the Log_r ratio and B-allele frequencies were used to determine the *PTEN* genotype call for the sample, either WT, HET (heterozygous deleted) or HOM (homozygous deleted). Overall, 125 (90%) were scored as WT and partial or complete loss of the gene was detected in 14 (10%) of the 139 samples. Of these, 11 (79%) demonstrated heterozygous loss and three (21%) homozygous loss. Representative examples of the Log_r ratio and B-allele frequency plots of samples harbouring either a heterozygous or homozygous deletion are shown in Figure 6.7. Of the samples with heterozygous deletion, the deletion looked to encompass the whole gene. The homozygous deletions also spanned the entire *PTEN* locus in all cases. It was not possible to determine the level of deletion in each sample. The Log_r ratio and B-allele frequency plots of four patients were consistent with amplification of the entire q arm of chromosome 10 and were therefore scored as WT for the present study, as they do not result in the loss of function of the *PTEN* gene.

The CytoSNP-850k array data was scored for copy number changes in *PTEN* independently of the quantitative SNP allele ratios for the informative patients. Comparison with the SNP quantification data was possible for 72 of the 76 informative patients, as three SNP-informative samples failed the CytoSNP-850k assay and one sample did not have sufficient DNA for array analysis. Eight samples demonstrating an allelic imbalance at both SNP loci gave a corresponding abnormal SNP array trace (Table 6.3). In five of these cases the imbalance was consistent with heterozygous deletion of *PTEN*. In the remaining three cases, the variation of the allele ratio was likely to be as a result of amplification of one allele of *PTEN* in the majority or every cell. Of note, the two cases where the deletion was only seen

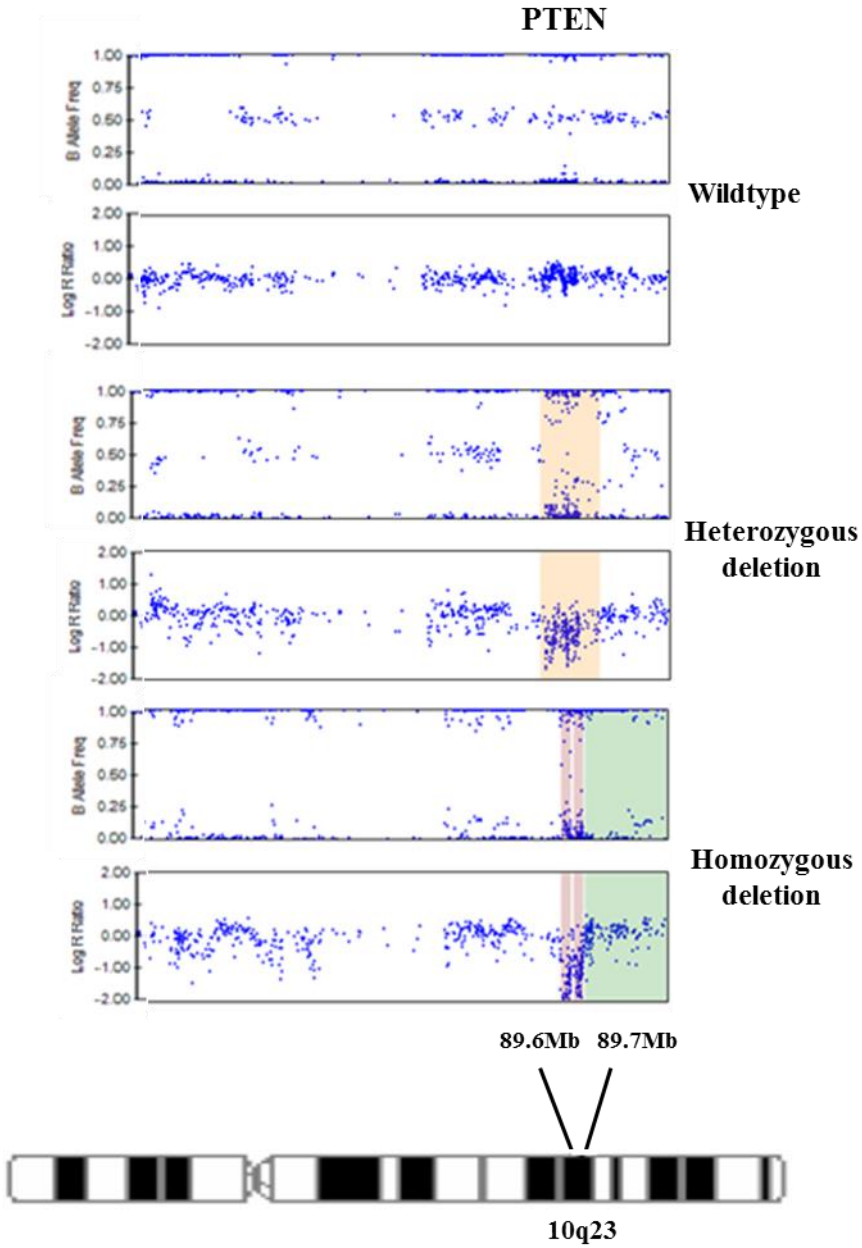

Figure 6.7 Identification of *PTEN* deletions by SNP array. B-allele Frequency (BAF) and LogR ratio plots for samples with wildtype *PTEN*, heterozygous deletion of *PTEN* and homozygous deletion of *PTEN*.

Table 6.3 Comparison of techniques to detect copy number changes in patients with T-ALL

Patient[#]	Copy number status	*PTEN* copy number change (SNP quantification)		% total cells harbouring copy number change	Array
		rs1903858 A%:G%	rs555895 T%:G%		
9	Het Del	68:32	62:38	46%	Het Del
12	Het Del	N/A	N/A	-	Het Del
13	Het Del	54:46	80:20	75% at 3' end	Het Del
21	Het Del	N/A	N/A	-	Het Del
22	Het Del	N/A	N/A	-	Het Del
23	Het Del	7:93	5:95	96%	Het Del
24	Het Del	N/A	N/A	-	Het Del
25	Het Del	N/A	N/A	-	Het Del
26	Het Del	17:83	22:78	75%	Het Del
27	Het Del	73:27	75:25	65%	N/A
28	Het Del	54:46	21:79	73% at 3' end	Het Del
29	Het Del	N/A	N/A	-	Het Del
30	Hom Del	N/A	N/A	-	Hom Del
31	Hom Del	N/A	N/A	-	Hom Del
32	Hom Del	N/A	N/A	-	Hom Del
10	Amp	68:32	68:32	100%	Amp
33	Amp	73:27	68:32	100%	Amp
34	Amp	68:32	66:34	100%	Amp
20	Amp	N/A	N/A	-	Amp

Abbreviations: N/A, not applicable; Het Del, heterozygous deletion; Hom Del, homozygous deletion; Amp, amplification.

[#] Patient number corresponds to and follows on from Table 5.2.

at the 3' end on the SNP allele quantification were initially scored as WT on the array. However, re-examination of the array plots confirmed the presence of a 3' end heterozygous deletion of the gene in both patient samples.

Therefore combining the two techniques, there were 143 patients with *PTEN* deletion status. Of these, 128 (90%) patients were scored as WT, 62 cases by array alone, 63 cases by both SNP quantification and array analysis and three cases by SNP quantification alone. Partial or complete loss of the gene ($PTEN^{DEL}$) was detected in 15 (10%) patients (Table 6.3). In nine patients this was identified by array analysis alone, in five patients by both SNP quantification and array analysis and in one case by SNP quantification alone.

6.3.4 *PTEN* mutation and deletion genotype

Putting together the mutation and deletion data, 143 patient samples had a complete genotype. A further two patients, who failed the CytoSNP-850k assay and were SNP-uninformative and therefore had an undetermined deletion status, were added to the cohort as they harboured a loss-of-function *PTEN* mutation. Considering the mutation and deletion status of these 145 patients, 113 (78%) were *PTEN* WT ($PTEN^{WT}$) and 32 (22%) had abnormalities in the *PTEN* gene ($PTEN^{ABN}$) (Table 6.4). The $PTEN^{ABN}$ group included 17 patients (53%) harbouring mutations ($PTEN^{MUT}$), 11 patients (34%) harbouring gene deletion ($PTEN^{DEL}$) and 4 patients (13%) with co-incident mutations and deletions ($PTEN^{MUT+DEL}$).

Each of the $PTEN^{ABN}$ cases was further classified as harbouring either monoallelic or biallelic abnormalities and was scored according to the following criteria: patients were scored as monoallelic where one or more mutations were present and the total mutant level was <50%, or where there was evidence of heterozygous deletion. Patients with either one or multiple mutations totalling >50%, or homozygous deletion, were scored as biallelic, on the assumption that at least a proportion of cells must have more than one allele affected.

Of the 32 $PTEN^{ABN}$ cases, 19 (59%) patients were considered to harbour monoallelic abnormalities ($PTEN^{MONO}$), and 13 (41%) biallelic abnormalities ($PTEN^{BI}$) (Table 6.4). Of the $PTEN^{MONO}$ patients, ten had one or multiple mutations which constituted <50% of total alleles (total mutant level ranged from 10% to 48%) suggesting the presence of a heterozygous mutation or, if more than one mutation was present, heterozygous mutations in different cell populations. Eight patients harboured heterozygous deletion, of these the

Table 6.4 Details of *PTEN* abnormalities in patients with T-ALL

Patient[#]	No. *PTEN* mutants	*PTEN* Mutations Size change (% mutant)	Total mutant level	*PTEN* deletion (array)	*PTEN* deletion (SNP quantification)		% Cells deleted
					rs1903858 A%:G%	rs555895 T%:G%	
Biallelic							
14	2	4bp ins (45%) 1bp ins (17%)*	62%	Normal	N/A	N/A	-
15	2	2bp ins (34%) 6bp indel (37%)	71%	Normal	53:47	52:48	-
18	3	8bps ins (29%)* 18bp ins (29%) 2bp ins (2%)	60%	Normal	N/A	N/A	-
16	2	20bp indel (28%) 1bp indel (28%)	56%	Normal	55:45	47:53	-
17	2	16bp ins (34%) 1bp indel (39%)	73%	N/A	N/A	N/A	-
19	3	12bp indel (18%) 6bp ins (37%) 8bp ins (41%)	96%	Normal	54:46	50:50	-
20	4	20bp indel (28%) 6bp ins (13%) 2bp indel (16%) 8bp del (8%)	67%	Normal	N/A	N/A	-
30	-	-	-	Hom Del	N/A	N/A	-
31	-	-	-	Hom Del	N/A	N/A	-
32	-	-	-	Hom Del	N/A	N/A	-
12	1	1bp ins (70%)	70%*	Het Del	N/A	N/A	-
13	1	9bp indel (60%)	60%*	Het Del	54:46	80:20	3'end in 75%
21	4	13bp ins (24%)* 12bp indel (31%) 4bp ins (18%) 14bp ins (4%)	77%	Het Del	N/A	N/A	-
Monoallelic							
6	2	14bp indel (11%) 13bp del (2%)	13%	Normal	52:48	50:50	-
10	3	1bp ins (2%) 5bp ins (6%) 3bp ins (10%)	18%	Normal	N/A	N/A	-

Table 6.4 Continued

Patient[#]	No. *PTEN* mutants	*PTEN* Mutations Size change (% mutant)	Total mutant level	*PTEN* deletion (array)	*PTEN* deletion (SNP quantification)		% Cells deleted
					rs1903858 A%:G%	rs555895 T%:G%	
1	1	2bp indel (41%)	41%	Normal	52:48	54:46	-
2	1	10bp indel (10%)	10%	Normal	N/A	N/A	-
3	1	1bp ins (47%)	47%	Normal	51:49	50:50	-
4	1	7bp indel (48%)	48%	Normal	52:48	52:48	-
11	4	4bp indel (15%) 13bp del (9%) 11bp indel (4%) 7bp ins (6%)	34%	Normal	N/A	N/A	-
7	2	5bp indel (12%) 4bp ins (11%)	22%	Normal	N/A	N/A	-
5	1	12bp indel (37%)	37%	Normal	57:43	51:49	-
8	2	8bp ins (21%) 4bp ins (6%)	27%	N/A	N/A	N/A	-
9	2	9bp indel (6%) 11bp ins (5%)	11%	Het Del	68:32	62:38	46%
22	-	-	-	Het Del	N/A	N/A	-
23	-	-	-	Het Del	7:93	5:95	96%
24	-	-	-	Het Del	N/A	N/A	-
25	-	-	-	Het Del	N/A	N/A	-
26	-	-	-	Het Del	17:83	22:78	75%
27	-	-	-	N/A	73:27	75:25	65%
28	-	-	-	Het Del	54:46	21:79	3' end in 73%
29	-	-	-	Het Del	N/A	N/A	-

Abbreviations: N/A, not available; Het Del, heterozygous deletion; Hom Del, homozygous deletion; ins, insertion; del, deletion; indel, insertion/deletion. [#] Patient numbers correspond to and follow on from Tables 5.2. and 6.3.* Mutant level estimated from the sequence.

proportion of cells harbouring the deletion could be determined in four patients and ranged from 65% to 96%. The remaining patient harboured both mutations and a deletion. The two mutations accounted for 11% of total alleles (6% and 5%), and SNP quantification indicated that the heterozygous deletion was present in 46% of cells. In this case it was difficult to determine the possible cell populations but it is likely that the mutated and deleted cells constitute different cell populations. As the quantifications suggested a borderline level, the case was counted as monoallelic.

Of the *PTEN*^{BI} patients, seven patients each had multiple mutations and the mean total mutant level was 69% (range 56% to 96%), which is consistent with compound heterozygosity, although the possibility that individual mutants were homozygous also cannot be excluded. Three patients had homozygous deletions. The remaining three patients had co-incident mutations and deletions. One case had only one mutant at a level of 70% of total alleles and a heterozygous deletion. Therefore it is likely that in approximately 80% of cells, one allele has been lost and the remaining allele is mutated. This example is shown in Figure 5.11A. One patient had a single mutation in exon 5 at a level of 60% of total alleles, and a heterozygous deletion of the 3' end in approximately 75% of cells. It was not possible to determine from the SNP array the size of the intragenic deletion and therefore whether or not it encompassed exon 5. One possible explanation is that 75% of cells harbour a 3' end heterozygous deletion that includes exon 5, and the other allele in these cells carries the exon 5 mutation. Alternatively, it is possible that a homozygous mutation was acquired in a proportion of cells (60%) that already harboured a 3' end heterozygous deletion not encompassing exon 5. The other population of *PTEN*-abnormal cells (15%) would therefore only be heterozygous for the 3' deletion. The remaining patient had four mutations with a total mutant level of 77% (25%, 31%, 18% and 4% respectively) and a heterozygous deletion was detected by array analysis. This suggests that there are various populations of subclones in the patient which may be either compound heterozygous with distinct mutations on different alleles, or hemizygous with the remaining allele in the cell mutated.

6.3.5 Clinical outcome according to *PTEN* genotype

6.3.5.1 Characteristics of T-ALL patients according to *PTEN* genotype

The characteristics of *PTEN*^{ABN} patients were compared to *PTEN*^{WT} patients. The *PTEN*^{ABN} group includes patients harbouring mutations (*PTEN*^{MUT}), patients harbouring gene deletion (*PTEN*^{DEL}) and patients with co-incident mutations and deletions (*PTEN*$^{MUT+DEL}$). There was no significant difference in sex, WBC, age group or NCI risk group between the genotype groups, nor in cytogenetic characteristics (Table 6.5). However, patients with an abnormality

Table 6.5 Characteristics of T-ALL patients according to *PTEN* genotype

Subgroup	Total	*PTEN*^{WT}		*PTEN*^{ABN}		*P**
		Total no.	%	Total no.	%	
Gender						0.47
Male	111	85	75%	26	81%	
Female	34	28	25%	6	19%	
WBC (x10^9/L)						0.15
<50	47	41	36%	6	19%	
50-99	22	17	15%	5	16%	
≥100	76	55	49%	21	66%	
Median count	110.5	95.0	N/A	135.7	N/A	
Range	0.5-881.0	0.5-881.0	N/A	0.7-777	N/A	
Age group (years)						0.09
<10	79	56	50%	23	72%	
10-15	47	40	35%	7	22%	
≥16	19	17	15%	2	6%	
Median	9	10	N/A	8.5	N/A	
Range	1-23	1-23	N/A	1-18	N/A	
CNS disease						0.04
No	135	110	97%	28	88%	
Yes	7	3	3%	4	12%	
NCI risk group						0.6
Low	18	14	12%	4	12%	
High	127	99	88%	28	88%	
Cytogenetics						0.86$^{\dagger\Delta}$
Normal	28	21	19%	7	22%	
Abnormal	90	69	61%	21	66%	
Failed	25	21	19%	4	12%	
Missing	2	2	2%	0	0%	
Genetic subgroup						N/A
AF10-CALM	3	3	3%	0	0%	
LMO2	10	8	7%	2	6%	
MLL	3	3	3%	0	0%	
TAL1	14	8	7%	6	19%	
TLX1	5	4	4%	1	3%	
TLX3	15	14	12%	1	3%	
Unknown	95	73	66%	22	69%	
CDKN2A/B deletion						
No	45	36	31%	9	28%	0.27
Yes	79	56	50%	23	72%	
Missing	21	21	19%	0	0%	

Abbreviations: WT, wild-type; ABN, abnormal; WBC, white blood cell count; CNS, central nervous system; NCI, National Cancer Institute; N/A, not applicable.
* *P* values: unless otherwise indicated these are given for Fisher's exact test. †Chi squared test. $^{\Delta}$Missing/failed excluded.

in the *PTEN* gene had a significantly higher incidence of CNS disease than $PTEN^{WT}$ patients (P=.04).

6.3.5.2 Response to glucocorticoid therapy according to *PTEN* genotype

Early response to therapy was first correlated with the presence or absence of abnormalities in the *PTEN* gene. Of the 32 $PTEN^{ABN}$ patients, 24 (75%) had an RER (≤25% blasts in the marrow at day 8 or 15 of induction therapy) and 8 (25%) an SER (>25% blasts). Of the 113 $PTEN^{WT}$ patients, 84 (74%) had an RER and 29 (26%) an SER (Table 6.6). The difference was not statistically significant (P>.99), suggesting that abnormalities in the *PTEN* gene did not have an effect on response to glucocorticoid therapy.

Early response to therapy was then correlated to presence of either a monoallelic or biallelic abnormality. There was no difference in the frequency of an RER between the $PTEN^{MONO}$, $PTEN^{BI}$ and $PTEN^{WT}$ patients (74%, 77% and 74% respectively, P>.99), suggesting that there was no association between the level of PTEN loss of function, either by gene mutation or genomic deletion, and response to therapy (Table 6.6).

6.3.5.3 MRD status according to *PTEN* genotype

The MRD status at day 29 post diagnosis was available for 134 of the 145 paediatric patients with a complete *PTEN* genotype. Of the 27 $PTEN^{ABN}$ patients, 12 (44%) had favourable disease (<1 positive cell in 1000 cells) and 15 (56%) had unfavourable disease (≥1 positive cell in 1000 cells). Of the 107 $PTEN^{WT}$ patients, 60 (56%) had favourable and 47 (44%) unfavourable disease (Table 6.6). This difference was not statistically significant (P=.28), suggesting that abnormalities in the *PTEN* gene did not have a major effect on MRD.

MRD status was then assessed according to the presence of either a monoallelic or biallelic abnormality. Favourable and unfavourable disease were observed in five (31%) and 11 (69%) of the 16 $PTEN^{MONO}$ patients respectively, seven (64%) and four (36%) of the 11 $PTEN^{BI}$ patients respectively (Table 6.6). There was no difference in the frequency of favourable disease between the $PTEN^{MONO}$, $PTEN^{BI}$ and $PTEN^{WT}$ patients (P=.69), however there was a borderline trend for an association between monoallelic abnormalities and unfavourable disease at day 29 (P=.14).

Table 6.6 Response to chemotherapy and survival status according to *PTEN* genotype

		***PTEN* genotype**				**Level of *PTEN* loss**			
	Total	**WT**	**ABN**	***P****	**Total**	**WT**	**Monoallelic**	**Biallelic**	***P*†**
Slow early response									
No	108	84 (74%)	24 (75%)	>.99	108	84 (74%)	14 (74%)	10 (70%)	>.99#
Yes	37	29 (26%)	8 (25%)		37	29 (26%)	5 (26%)	3 (30%)	
MRD at day 29									
Negative	72	60 (56%)	12 (44%)	0.28	72	60 (56%)	5 (31%)	7 (64%)	0.14Δ
Positive	62	47 (44%)	15 (56%)		62	47 (44%)	11 (69%)	4 (36%)	0.69*
Outcome at 5 years, % (95% CI)									
RFS	144	87% (80%-92%)	84% (66%-93%)	0.55†	144	87% (80%-92%)	83% (57%-94%)	85% (51%-96%)	0.83
EFS	145	85% (77%-90%)	78% (60%-90%)	0.37†	145	84% (77%-90%)	74% (48%-88%)	85% (51%-96%)	0.46
OS	145	91% (84%-95%)	81% (63%-91%)	0.1†	145	91% (84%-95%)	79% (53%-92%)	85% (51%-96%)	0.23

**P* values: unless otherwise indicated these are for chi-squared test. †Log-rank test. # Fisher's exact test. Δ Chi squared test. * Trend for *PTEN* genotype.
Abbreviations: WT, wild-type; MUT, mutant; ABN, abnormal; MRD, minimal residual disease; RFS, relapse-free survival; EFS, event-free survival; OS, overall survival; CI, confidence interval

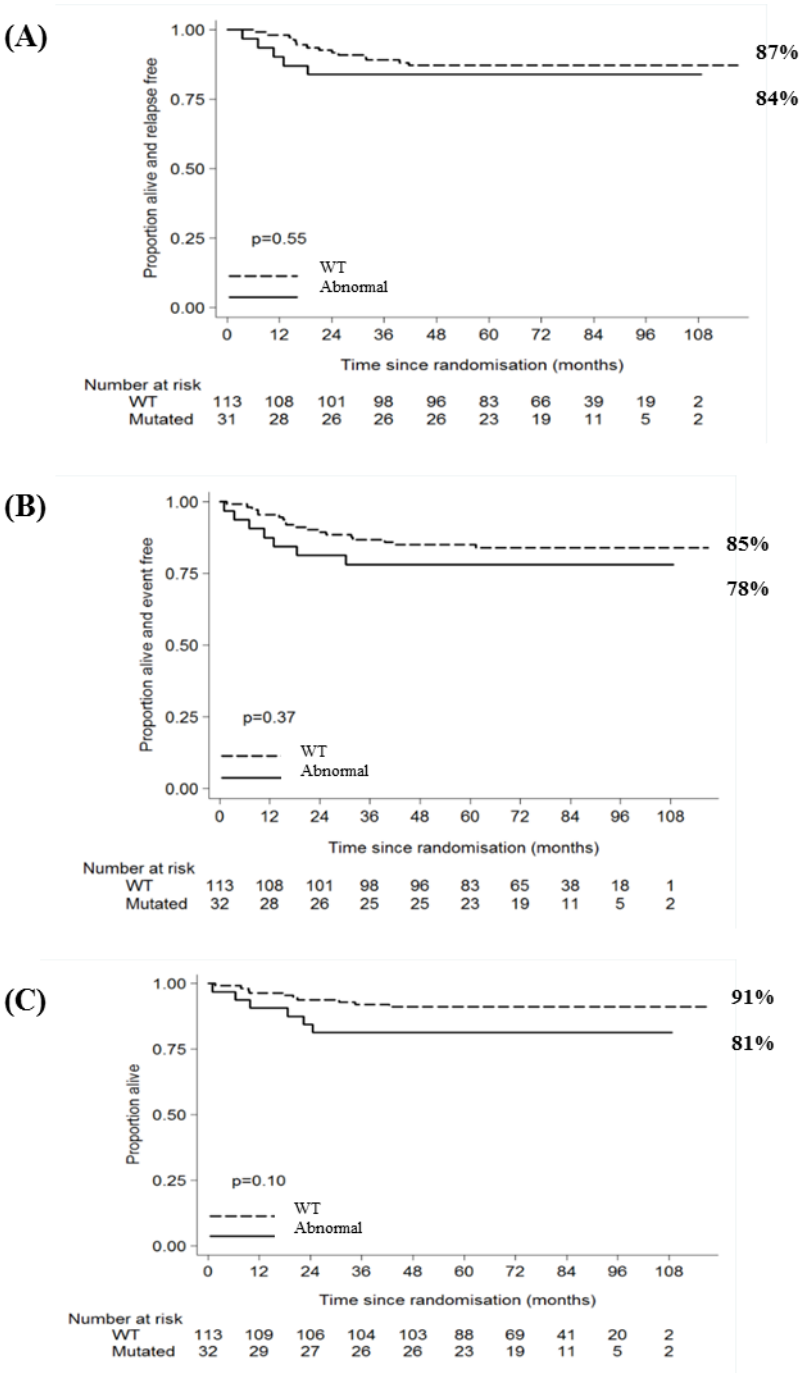

Figure 6.8 The impact on clinical outcome stratified according to *PTEN* genotype. Kaplan-Meier curves for patients with abnormal (ABN) or wildtype (WT) *PTEN* . *PTEN* ^{ABN} classified as *PTEN*^{MUT} and/or *PTEN*^{DEL} patients. (A) Relapse-free survival, (B) Event-free survival, (C) Overall survival.

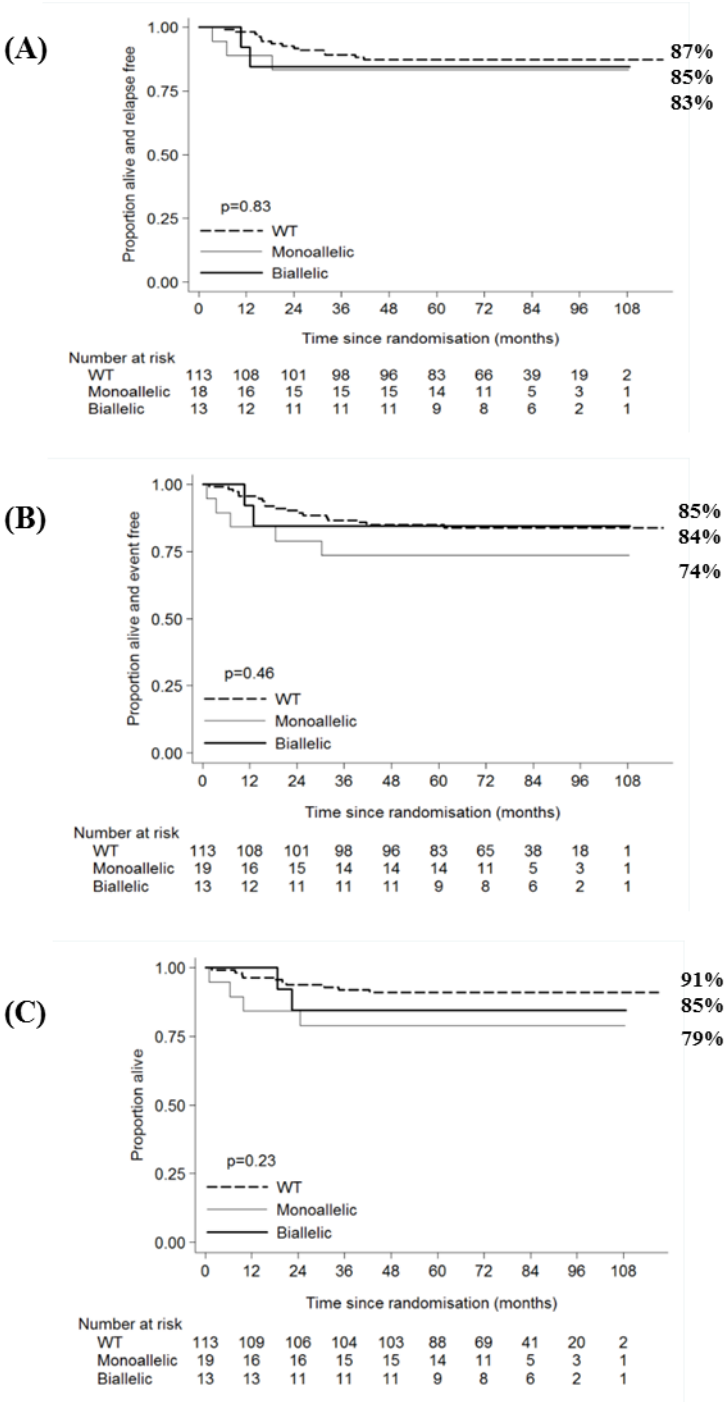

Figure 6.9 The impact on clinical outcome stratified according to the level of *PTEN* loss. Kaplan-Meier curves for patients with monoallelic *PTEN* abnormalities (MONO), biallelic *PTEN* abnormalities (BI) and wildtype *PTEN* (WT). (A) Relapse-free survival, (B) Event-free survival, (C) Overall survival.

6.3.5.4 Long-term outcome stratified by *PTEN* genotype

There was no significant difference between $PTEN^{ABN}$ and $PTEN^{WT}$ patients in either RFS or EFS, 84% versus 87% (*P*=.55) and 78% versus 85% (log-rank *P*=.37) respectively, although $PTEN^{ABN}$ patients did show a trend for a worse OS (81% versus 91%, *P*=.1) (Figure 6.8) (Table 6.6). However, although the HR for OS is 2.27, which suggests that $PTEN^{ABN}$ patients are more likely to have a poorer OS, the 95% CI (0.82-6.24) are broad and there is no statistical difference when compared to WT patients. This was also the case when $PTEN^{MONO}$, $PTEN^{BI}$ and $PTEN^{WT}$ patients were stratified for RFS, 83%, 85% and 87% respectively (*P*=.83) and EFS, 74%, 85% and 84% (*P*=0.46) respectively (Figure 6.9) (Table 6.6). Again there was a borderline trend for a worse OS in patients with monoallelic abnormalities, 79%, 85% and 91% for $PTEN^{MONO}$, $PTEN^{BI}$ and $PTEN^{WT}$ patients respectively (*P*=.23).

6.3.6 *NOTCH1*/*FBXW7*/*PTEN* genotype of T-ALL patients

In order to determine whether *PTEN* abnormalities impact on the good outcome seen in the $NOTCH1{\pm}FBXW7^{Double}$ cases in the present study, and whether they can refine stratification of cases with single *NOTCH1* mutations ($NOTCH1^{Single}$) or wild-type *NOTCH1* ($NOTCH1^{WT}$), *PTEN* genotype was correlated with outcome in the different *NOTCH1*/*FBXW7* subgroups. The incidence of *PTEN* abnormalities in the three genotype groups is shown in Table 6.7. The frequency of *PTEN* abnormalities alone did not differ according to *NOTCH1*/*FBXW7* genotype, 59% $PTEN^{ABN}$ patients had a *NOTCH1*/*FBXW7* mutation compared to 68% $PTEN^{WT}$ patients (*P*=.67).

6.3.7 Long-term outcome according to *NOTCH1*/*FBXW7*/*PTEN* genotype

Patients in the $NOTCH1{\pm}FBXW7^{Double}$ group studied in this cohort have an excellent outcome with 100% OS (see 4.3.4). When the 37 patients in the $NOTCH1{\pm}FBXW7^{Double}$ group were stratified by *PTEN* genotype, there was no significant difference between abnormal and WT patients in OS, as all patients were alive at 5 years. Similarly there was no significant difference in RFS, 100% versus 94% (*P*>.99) for $PTEN^{ABN}$ and $PTEN^{WT}$ patients respectively (Figure 6.10). Of note, there were only two patients who relapsed in the $NOTCH1{\pm}FBXW7^{Double}$ group and both remain alive. Both relapses occurred in patients in the $PTEN^{WT}$ group. These results therefore suggest that *PTEN* genotype had no impact on the favourable outcome of the $NOTCH1{\pm}FBXW7^{Double}$ patients.

Table 6.7 *NOTCH1/FBXW7/PTEN* status of patients with T-ALL

	Total[#]	***PTEN* genotype**	
	145	***PTEN*^{ABN}**	***PTEN*^{WT}**
***NOTCH1*±*FBXW7*^{Double}**	37	5 (14%)	32 (86%)
***NOTCH1*^{Single}*FBXW7*^{WT}**	55	14 (25%)	41 (75%)
***NOTCH1*^{WT}*FBXW7*^{WT}**	49	13 (27%)	36 (73%)

Abbreviations: ABN, abnormal; WT, wildtype.
[#] excludes four patients with an *FBXW7* mutation only.

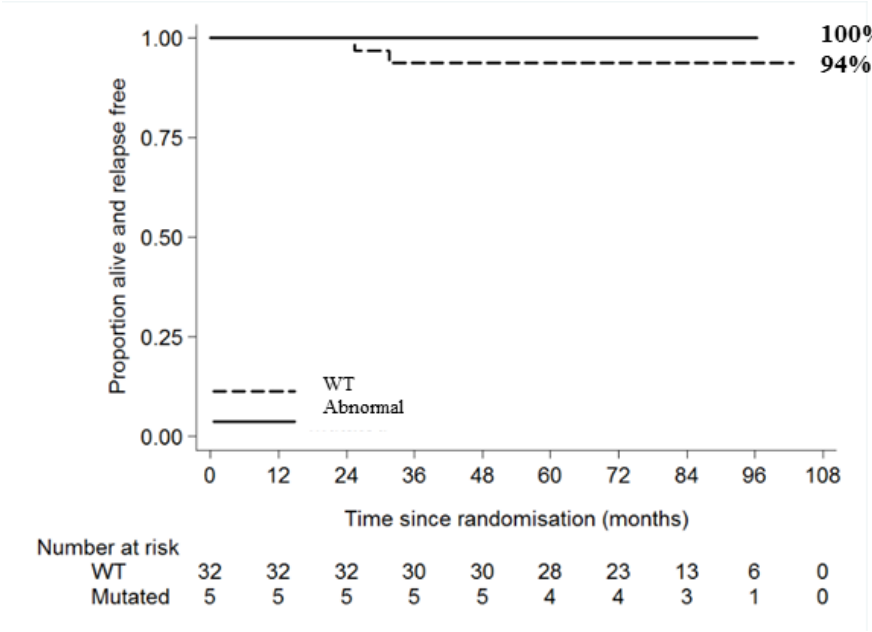

Figure 6.10 The impact on clinical outcome of the *NOTCH1*±*FBXW7*^{Double} group stratified according to *PTEN* genotype. Kaplan-Meier curve for Relapse-free survival for patients with abnormal *PTEN* (ABN) and wildtype *PTEN* (WT).

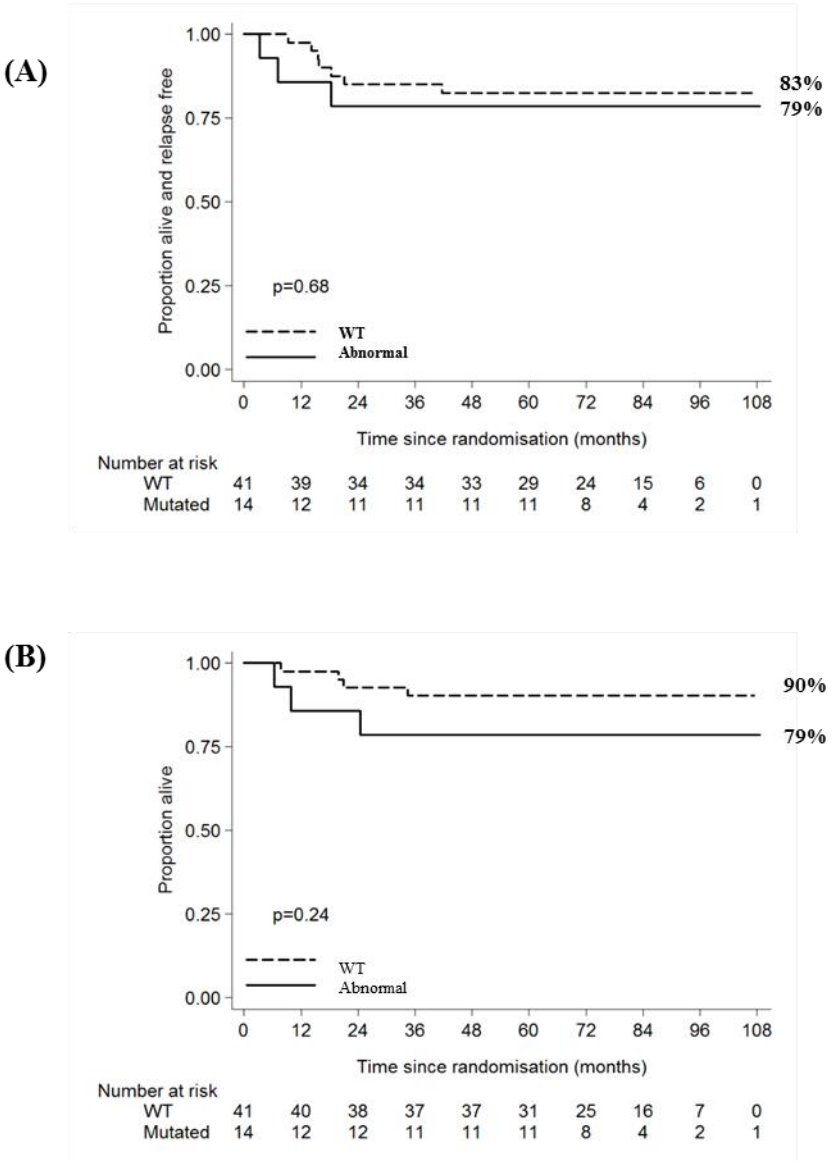

Figure 6.11 The impact on clinical outcome of *NOTCH1*$^{\text{Single}}$*FBXW7*$^{\text{WT}}$ group stratified according to *PTEN* genotype. Kaplan-Meier curves for patients with abnormal *PTEN* (ABN) and wildtype *PTEN* (WT). (A) Relapse-free survival, (B) Overall survival.

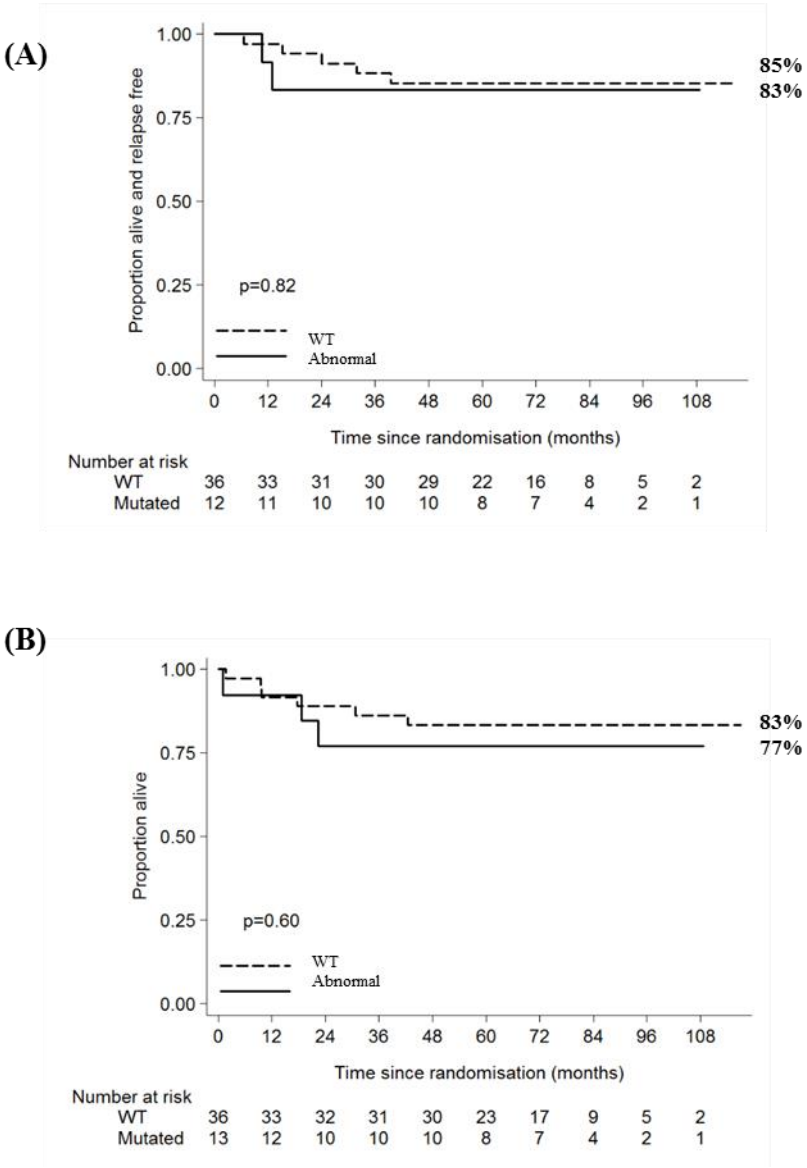

Figure 6.12 The impact on clinical outcome of *NOTCH1*^{WT}*FBXW7*^{WT} group stratified according to *PTEN* genotype. Kaplan-Meier curves for patients with abnormal *PTEN* (ABN) and wildtype *PTEN* (WT). (A) Relapse-free survival, (B) Overall survival.

There was similarly no difference in the outcome of the *NOTCH1*^{Single}*FBXW7*^{WT} and the *NOTCH1*^{WT}*FBXW7*^{WT} groups when stratified by *PTEN* genotype. In *NOTCH1*^{Single}*FBXW7*^{WT} patients, RFS and OS for *PTEN*^{ABN} and *PTEN*^{WT} patients were 79% versus 83% (*P*=.68) and 79% versus 90% (*P*=.24) respectively (Figure 6.11). In *NOTCH1*^{WT}*FBXW7*^{WT} patients, for *PTEN* genotype alone, RFS and OS were 83% versus 85% (*P*=.82) and 77% versus 83% (*P*=.6) for *PTEN*^{ABN} and *PTEN*^{WT} patients respectively (Figure 6.12). There was a suggestion therefore that there may be association between *PTEN* abnormalities and a worse prognosis in the *NOTCH1*^{Single}*FBXW7*^{WT} and *NOTCH1*^{WT}*FBXW7*^{WT} groups. However, the number of patients in these groups was low and although the HR (2.39) suggested that *PTEN*^{ABN} patients were more likely to have a worse OS in the *NOTCH1*^{Single}*FBXW7*^{WT} group, the 95% CI (0.54-10.70) were broad and there was no statistical difference when compared to *PTEN*^{WT} patients.

6.4 Discussion

The data presented in this chapter shows the investigation of *PTEN* deletions in T-ALL and, when analysed in combination with mutations in the *PTEN* gene, the impact of the abnormalities on response to therapy and long-term outcome. Screening for genomic loss of *PTEN* was possible in 145 of the 162 patients with mutation status, and of these, *PTEN* deletion was identified in 15 (10%) patients. This incidence is in line with the combined data from other paediatric studies (24 of 216 cases, 11%, *P*=.84) (Gutierrez *et al.*, 2009;Maser *et al.*, 2007;Mendes *et al.*, 2014) (Table 6.1).

The initial approach to identify copy number changes in *PTEN* applied a qPCR-based strategy using a commercially available Taqman Copy Number assay. However, the copy number results obtained from control samples were both unreproducible and inconsistent with the expected values as a result of inadequate amplification of the genomic DNA. Further attempts using SYBR green and custom designed primers gave similar results; therefore this approach was not pursued any further. Subsequently, a SNP allele quantification method was devised utilising the presence of two common SNPs at opposite ends of the gene, in 76 heterozygous patients. Samples were amplified using a fluorescently labelled primer then underwent allele-specific restriction enzyme digestion and fragment analysis to separate the two polymorphic alleles by size. The contribution of each allele was then calculated by expressing the area under each peak as a percentage of total alleles. An imbalance was identified in nine (12%) of 76 patients. Seven of these patients had equivalent loss at the 5' and 3' SNP loci, suggesting that the deletion encompassed the whole gene. The

remaining two patients' harboured loss at the 3' end only, suggesting that the heterozygous deletion was intragenic. Nevertheless, this method was only applicable in 76 (52%) of 145 patients, could only detect heterozygous loss and was unable to definitely ascertain whether the allelic imbalance was as a result of deletion or amplification of the *PTEN* gene. Therefore further attempts to screen the entire cohort were warranted.

This was achieved using the Illumina CytoSNP-850k array, which is a high-throughput technique enabling copy number analysis across the whole genome. This array platform was chosen as it had a better coverage of the *PTEN* gene and contained more *PTEN* probes than other similarly priced arrays. Furthermore, the array results could be validated in 72 of the 76 patients in which SNP allele quantification was possible and eight samples demonstrating an allelic imbalance at both SNP loci gave a corresponding abnormal SNP array trace. No deletions detected were calculated to be present in less than approximately half of cells in the 72 patients with both array and SNP allele quantification data, the lowest proportion of cells with heterozygous deletion was quantified at 46% by SNP allele quantification. Therefore, it was not possible to determine the cut-off and true sensitivity of the array. Of note, only deletions present in more than 27% of cells for rs1903858 and 26% of cells for rs555895 would give an allelic ratio outside of the normal range using the SNP allele quantification technique. It is therefore likely that some deletions present in subclonal populations have been missed in the present study. Other groups investigating *PTEN* deletion in paediatric cohorts have utilised array-CGH (Gutierrez *et al.*, 2009;Maser *et al.*, 2007;Remke *et al.*, 2009;Zuurbier *et al.*, 2012). One study of 146 patients detected subclonal deletions in two patients using array-CGH, and in one of the patients for whom material was available, the deletion was validated by FISH and quantified to be present in 40% of the leukaemic blasts (Zuurbier *et al.*, 2012). However, the definition of a subclonal population is not actually stated in the study.

A further limitation of the use of SNP arrays to screen for *PTEN* deletions is that the presence of microdeletions is unlikely to be picked up by the array. This was demonstrated in a recent study to further investigate patient samples defined as WT by array-CGH, but in whom PTEN protein expression was absent (Mendes *et al.*, 2014). MLPA and PCR of the common breakpoints were used to detect microdeletions in a cohort of 146 patients, with an overall frequency of 8% of patients harbouring these deletions. In the present study, two samples with deletions at the 3' end of the gene detected by SNP allele quantification were initially scored as WT in the SNP array plots. It was only upon comparison of the array results with the SNP allele quantification data, and subsequent re-examination of the array plots, that the deletions were included. Therefore, the incidence of small deletions, including

microdeletions, may be underestimated in the current study as it is possible that more were present in the 69 patient samples that could not be investigated by SNP allele quantification. Further analysis of the cohort utilising the genomic breakpoint PCR approach, is required in the present study. The Mendes *et al* (2014) study was also able to identify multiple deletions in two patients; one case harboured a heterozygous deletion of exons 4 and 5 with a heterozygous deletion of intron 1-3. The second case harboured a heterozygous deletion of exons 3-9 with a heterozygous deletion of intron 1-3. It is not known whether these deletions were in the same cell or constituted different cell populations. Given the incidence of multiple mutations detected in the present cohort, it is highly likely that some patients could harbour more than one smaller deletion.

When combining the mutation and deletion status, of the 145 patients analysed in the present study, 32 (22%) had one or more abnormality in the *PTEN* gene. This is equivalent to the combined data from two paediatric cohorts in which the *PTEN* mutation and deletion data was available (42 of 190 cases, 22%, *P*=.85) (Gutierrez *et al.*, 2009;Mendes *et al.*, 2014) (Table 6.1). The abnormalities are predicted to result in the loss of the PTEN function either by genomic loss of one or both *PTEN* alleles or by mutation leading to the production of a truncated protein. *PTEN* has been described as a haploinsufficient tumour suppressor gene as loss of one allele can elicit a cancer phenotype in murine models. Therefore, the abnormalities identified in samples in the current study would be predicted to play a role to the pathogenesis of the disease. Both biallelic and monoallelic abnormalities were detected in the present cohort at a frequency of 9% and 13% of patients respectively. Of 19 patients with a monoallelic abnormality, in ten patients the loss of PTEN function could be attributed to the presence of one or more heterozygous truncating mutations, eight patients harboured genomic loss of one allele and one patient demonstrated both mutation and heterozygous deletion. Of the 13 patients with a biallelic abnormality, seven patients harboured populations of cells where both alleles were affected by truncating mutations, three patients had a homozygous deletion and three patients demonstrated both mutation and deletion. Whether or not complete loss of *PTEN* is more tumourigenic than partial loss in T-ALL is unknown, but the incidence of both suggests that the acquisition of a second hit on the remaining *PTEN* allele may not be relevant in some tumours. However, the possibility that the remaining *PTEN* allele in the monoallelic cases is silenced by other unknown mechanisms such as aberrant splicing, mutation or hypermethylation of the *PTEN* promoter, cannot be excluded. The importance of PTEN inactivation during leukaemogenesis and the influence of this on the clinical outcome of patients may also depend on other factors and collaborating genetic abnormalities. For example, studies have shown in an in vivo prostate cancer murine model that in the absence of a p53 abnormality monoallelic loss of *PTEN* may

be more detrimental than biallelic loss, which may explain the frequency of loss of only one allele in T-ALL (Chen *et al.*, 2005). Therefore, further investigation of other potential abnormalities, for example the characterisation of p53 status, is required to address this, and the use of NGS techniques will facilitate this.

Intraclonal heterogeneity at diagnosis and clonal evolution at relapse are known to occur in T-ALL (Anderson *et al.*, 2011;Mullighan *et al.*, 2007;Yang *et al.*, 2008). Two reports in which the study of paired diagnostic and relapse samples was possible demonstrated that *PTEN* deletion was acquired in 12% of patients upon relapse (Clappier *et al.*, 2011), and *PTEN* mutation in 6% of patients (Palomero *et al.*, 2007). Clappier *et al* (2011) injected mice with diagnostic leukaemic cells from a patient who relapsed, and the resulting xenograft sample harboured a *PTEN* deletion, which was the same as in the relapse sample. The deletion is likely to have been present in a subclone at diagnosis that was undetectable due to an insensitive screening technique. This suggests that the *PTEN* inactivated subclone was selected for during the progression of disease and expanded, giving rise to relapse. However, in another study where a patient was found to harbour a subclonal microdeletion, the authors demonstrated that this subclone was not clonally selected for following xenotransplantation, and the microdeletion was still present in a subclonal population (Mendes *et al.*, 2014). These studies raise the question as to which abnormalities are selected for and contribute to relapse, whether cells harbouring subclonal biallelic or monoallelic *PTEN* abnormalities are selectively expanded and which other collaborating mutations are required to further destabilise the cell. Paired relapse samples were not available in the present cohort therefore it was not possible to determine which, if any, of the identified *PTEN*-abnormalities were present at relapse. The unknown level of non-leukaemic cell contamination of the samples also presented an issue with determining accurate subclonal populations. It is possible that a mutation quantified to be present in only a small proportion of cells may actually have been a dominant clone diluted by normal cells. Nevertheless, the presence of subclones was evident in the present cohort by the identification of multiple mutations per patient. Of the total mutations, 14 were present at a level of <10% total alleles, nine were between 10-20% and a further five mutations were between 20-30%. There was no evidence of subclonal deletions in the present study, of the six cases quantified with heterozygous deletions, the proportion of cells affected ranged from 46% to 96%. Of note, there were nine cases with deletions that could not be quantified in the present study because it was not possible to determine this from the SNP array and the patients were not SNP-informative. Therefore of the 15 deletions and 44 mutations detected in the present study, approximately 47% were likely to be subclonal. One patient in the present cohort showed evidence of heterozygous deletion (level unknown) and also harboured four mutations with mutant levels ranging from

4-31%. This implies that even in the presence of an inactivating event such as heterozygous genomic loss that would be predicted to drive clonal selection, there is still a need for the further acquisition of mutations leading to clonal diversity.

The knowledge of well-defined molecular prognostic markers at diagnosis in T-ALL could identify patients who are the highest risk of relapse and help to guide risk stratification and inform clinical decisions. When correlating the presence of *PTEN* abnormalities to response to glucocorticoid therapy in the present cohort, there was no significant difference in the incidence of an SER in patients with abnormal or WT *PTEN* genotype. This is in contrast to a report from patients treated on the ALL-BFM protocol, where *PTEN*-mutated patients were significantly associated with an SER when compared to WT patients. This difference could possibly be attributed to the specific glucocorticoid used in the various trials; patients in the present study treated on the UKALL 2003 trial were administered dexamethasone, whereas patients treated on the ALL-BFM trial received prednisone. The lack of association with a *PTEN* abnormality and an SER was unexpected as mutational loss of *PTEN* has been associated with increased AKT1 phosphorylation in T-ALL (Morishita *et al.*, 2012), which has been linked with glucocorticoid resistance (Piovan *et al.*, 2013). The authors reported that AKT is able to bind to and phosphorylate the NR3C1 glucocorticoid receptor, which resulted in the impairment of responses required to mediate glucocorticoid-induced cell death such as glucocorticoid receptor auto-upregulation and the transcriptional regulation of proapoptotic *BCL2L11*. The effect was reversed upon AKT inhibition. A later study also showed that AKT pathway activation was acquired in T-ALL clones in a zebrafish transgenic model, leading to an increased leukaemia-propagating cell population through activation of mTORC1, rendering the cells resistant to dexamethasone (Blackburn *et al.*, 2014). Therefore loss of *PTEN* function, by deletion or mutation, would be expected to play a role in resistance to therapy. However, the effect on response to glucocorticoid therapy seen in the present cohort may be due to the interaction of PTEN inactivation with other drugs used.

In the present study, there was no association between *PTEN* genotype and MRD status at day 29 of induction therapy. However, when stratified by the level of *PTEN* loss there was a borderline trend for patients with a monoallelic abnormality to be associated with a higher frequency of MRD-positivity, although the numbers in the subgroups are small. This association was unexpected as complete loss of *PTEN* would be thought to be more detrimental to the glucocorticoid response as further increases in AKT activation would increase inhibition of the glucocorticoid receptor, therefore reducing the response to the drug. In the only other study of 301 patients correlating *PTEN* abnormalities to MRD status,

PTEN-mutated patients were significantly associated with MRD-negativity when compared to WT patients (Bandapalli *et al.*, 2013), however the authors did not group the patients by monoallelic or biallelic loss. Nevertheless, the number of patients in the cohort in the present study is smaller (n=134), and a significant effect could possibly be seen if validated in a larger cohort.

When correlating *PTEN* genotype to long term outcome, there was no significant association between *PTEN* abnormalities and prognosis, however there was a borderline trend for $PTEN^{ABN}$ patients to have a worse OS. When furthered stratified by the level of *PTEN* loss, the worst outcome was seen in the monoallelic patients, possibly indicating that the loss of one allele is more detrimental than complete loss, which has been shown on certain genetic backgrounds to lead to the induction of PICS. However, this difference was non-significant and the number of patients in the subgroups was very low. The present study is in agreement with four of the five paediatric studies investigating the association of *PTEN* abnormalities with long-term outcome, in that none found a significant difference between *PTEN*-abnormal and WT patients (Bandapalli *et al.*, 2013;Gutierrez *et al.*, 2009;Mendes *et al.*, 2014;Zuurbier *et al.*, 2012). However, when Gutierrez *et al* (2009) analysed the outcome of mutated and deleted patients separately, patients with *PTEN* deletions were significantly associated with early treatment failure. One other study also reported a significantly worse outcome in $PTEN^{ABN}$ patients, although the number of patients in the respective cohort was small (n=62), therefore the significance of this study should be viewed with caution (Jotta *et al.*, 2010). In the present study, $PTEN^{ABN}$ patients were significantly associated with a higher incidence of CNS disease, which has not been reported in other studies. Increased CNS disease and subsequent CNS relapse could be a possible explanation for the trend for a worse outcome seen in *PTEN* abnormal patients.

The association between resistance to NOTCH1 pathway inhibition with loss of *PTEN* and the resulting constitutive activation of the PI3K/AKT signalling pathway led to the suggestion of dual inhibitors of the NOTCH1 and PI3K pathways as a rational therapeutic approach in T-ALL (Palomero *et al.*, 2007). Subsequent studies have shown that increased NOTCH1 signalling and Myc expression is dispensable for the growth of T-ALL by demonstrating that PTEN loss or activation of the PI3K/AKT signalling pathway can overcome the loss of Myc. Using an inducible zebrafish model of T-ALL, Gutierrez *et al* (2011) demonstrated that *pten* haploinsufficiency significantly promoted the loss of MYC dependence and that AKT pathway activation could replace the MYC signals required for the maintenance of T-ALL. Bonnet *et al* (2011) also demonstrated a striking difference in MYC protein abundance between paired primary diagnostic and relapse samples. A-CGH of

the samples revealed a monoallelic *PTEN* gene deletion at diagnosis which had evolved to a biallelic deletion at relapse, and the absence of PTEN protein correlated with an accumulation of MYC. The MYC transcripts were unaffected, suggesting posttranscriptional deregulation of MYC by PTEN. In a more recent study using an in vivo murine model, it was found that a population of T-ALL clones resistant to PI3K inhibition had reduced levels of ICN-1 and Myc, demonstrated cross-resistance to γ-secretase inhibitors and upregulated PI3K/AKT signalling (Dail *et al.*, 2014). The authors demonstrated that the resulting PI3K inhibitor-resistant primary T-ALLs that emerged in vivo did not harbour the somatic *Notch1* mutations present in the parental leukaemia and generally had reduced PTEN expression, suggesting that the loss of aberrant Notch1 signalling provided a selection advantage to the PI3K inhibitor-resistant leukaemic cells. This raises the possibility that inhibitors of NOTCH1 signalling, when used in combination with PI3K-inhibitors, may actually promote drug resistance in T-ALL by inadvertently promoting the survival and expansion of PI3K inhibitor-resistant clones. As mutations in both *NOTCH1* and *PTEN* are common in patients with T-ALL, these studies therefore pose an interesting question of how the NOTCH1 and PI3K/AKT signalling pathways interact clinically.

When combining the *PTEN* genotype with *NOTCH1/FBXW7* mutational status, the incidence of *PTEN* abnormalities did not differ according to *NOTCH1*/*FBXW7* genotype. This is in contrast to two other paediatric studies, which both reported a significant association between a *NOTCH1* mutation and the absence of *PTEN* abnormalities (Bandapalli *et al.*, 2013;Mendes *et al.*, 2014). This difference may be chance, although there was a slightly lower incidence of *NOTCH1* mutations in the two studies (50% and 56% of the respective cohorts) compared to 62% in the current study. In the present cohort, *NOTCH1*±*FBXW7*$^{\text{Double}}$ patients had an excellent outcome, with all patients alive at 5 years (section 4.3.4). Although there was a borderline trend for a worse OS in the *PTEN*$^{\text{ABN}}$ group, when the *NOTCH1*±*FBXW7*$^{\text{Double}}$ group were further stratified by *PTEN* abnormality, there was no evidence that the abnormalities impacted on the good outcome seen in this group of patients. This suggests that the increased activation of the NOTCH1 pathway is dominant over the loss of PTEN function in the context of the treatment protocol, and that it potentially neutralises the negative effect of a *PTEN* abnormality. This could not be attributed to more favourable characteristics of the *NOTCH1*±*FBXW7*$^{\text{Double}}$ patients at diagnosis, as 87% were classified as NCI high-risk. Furthermore, despite the presence of adverse risk factors post-induction in about one-third of this genotype group (SER and/or MRD-positive in 13 of 37, 35%), there were only two patients who relapsed and both were *PTEN*$^{\text{WT}}$. This is line with Bandapalli *et al* (2013) who, although patients were not stratified into the same genotype groups as in the present cohort, demonstrated that *NOTCH1* activation antagonised the

unfavourable effect of a *PTEN* abnormality, as *NOTCH1*-mutated patients with or without a *PTEN* abnormality had the most favourable outcome, with the worst outcome seen in the patients with only a *PTEN* abnormality. However, the data differs from that reported in two other studies. One was in a paediatric cohort where the authors reported that *NOTCH1*-mutated/*PTEN*-abnormal patients demonstrated a significantly reduced RFS when compared to *NOTCH1/PTEN*-WT patients, and *PTEN* and/or *NOTCH1* abnormalities were independent predictors for increased risk for relapse (Zuurbier *et al.*, 2012). The other was an adult cohort where the presence of *NOTCH1* and/or *FBXW7* mutations and absence of *PTEN/RAS* alterations identified low-risk patients, and all other patients were defined as high-risk (Trinquand *et al.*, 2013). The variable effects seen between trials have been attributed to differences in the treatment regimens between the various protocols (Ferrando, 2010).

When the *NOTCH1*$^{\text{Single}}$*FBXW7*$^{\text{WT}}$ group were stratified by *PTEN* abnormality, the *NOTCH1*$^{\text{Single}}$*FBXW7*$^{\text{WT}}$ patients with a *PTEN* abnormality appeared to have a reduced overall survival rate, but this was not significant. This suggests that the neutralising effect of the *NOTCH1* mutations is limited to the *NOTCH1*±*FBXW7*$^{\text{Double}}$ group, and that the dominant effect of *NOTCH1* is only seen when NOTCH1 activation is increased, either by a second mutation in the gene or by a mutation in *FBXW7*. This would imply that there is a threshold of NOTCH1 activity which must be reached in order to antagonise PTEN. The worst outcome in the present cohort was seen in the patients in the *NOTCH1*$^{\text{WT}}$*FBXW7*$^{\text{WT}}$ group with a *PTEN* abnormality. This is in line with one study which reported that the worst outcome was seen in patients who were WT for *NOTCH1* and harboured a *PTEN* abnormality (Bandapalli *et al.*, 2013).

The data presented in this chapter demonstrates that *PTEN* loss is common in T-ALL; however it appears to have no effect on the prognosis of patients treated on the UKALL 2003 trial. Therefore at present, screening of *PTEN* would not be warranted for use in risk adapted therapy. However the numbers in the subgroups are small, suggesting validation in a larger cohort is required. Both biallelic and monoallelic loss of *PTEN* were found at an equivalent rate in the present study. Further studies are required to determine whether the presence of *PTEN* loss in the majority of cells at diagnosis or in a subclone which is then preferentially selected, is functionally relevant for relapse. For example, investigation of the collaborating mutations required for the expansion of *PTEN*$^{\text{ABN}}$ subclones, as previous studies have demonstrated that in the murine haematopoietic compartment, complete loss of *Pten* only led to leukaemia when co-incident genetic events occurred, such as loss of p53 (Lee *et al.*, 2010;Yilmaz *et al.*, 2006). In view of this, it would be interesting to determine

the incidence of copy number changes in the *p53* gene, located on human chromosome 17p13.1, utilising the available CytoSNP-850k array data. The use of NGS techniques will be able to define the mutational landscape to elucidate which abnormalities collaborate with *PTEN* loss to further destabilise the cell, giving a wider picture of the biology of relapse.

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

Overall survival rates in paediatric patients with T-ALL have steadily improved in recent years as a result of more intense regimens, to a current rate of approximately 90% (Inaba *et al.*, 2013). The increases in survival are largely attributed to lower relapse rates, however, for those patients who do develop recurrent disease, prognosis post-relapse remains poor. Therefore, risk-adapted therapy and the development of personalised medicine is useful to target the most intensive treatment at patients with high-risk, rather than low-risk, features. This is particularly pertinent as the intensification of treatment has also raised concerns about the consequences of treatment-related toxicity, which, in low-risk patients, is now responsible for an equal number of deaths as relapse (Rabin, 2014). A recent report from the UKALL2003 trial reported that there was no difference in EFS between low-risk patients who received either one or two delayed intensifications, suggesting that treatment reduction is feasible in this subgroup (Vora *et al.*, 2013). The identification of molecular markers with prognostic relevance is advantageous in patients with T-ALL, as they may identify subgroups in which treatment reduction is viable, guiding future risk stratification and clinical decision making, such as whether SCT is necessary. At present, age, WBC at diagnosis and response to therapy are used to stratify patients by risk, and molecular features of the disease are not incorporated into risk refinement strategies of current trials (chapter 1). Therefore questions remain regarding the application of biomarkers to therapeutic strategy in T-ALL, and greater understanding is required. Knowledge of this and the interaction between different markers is important for understanding the underlying biology of the leukaemia.

This thesis presents an investigation of *NOTCH1* and *FBXW7* mutations and abnormalities in the *PTEN* gene, which have been reported in other paediatric cohorts but their prognostic significance remains unclear. The incidence of *NOTCH1* and *FBXW7* mutations was determined in a cohort of paediatric patients with T-ALL treated on the UKALL 2003 trial (chapter 3), and the biological characteristics and clinical outcome of the patients according to genotype were explored in chapter 4. Mutations in another candidate gene, *PTEN*, were also investigated in terms of their incidence and mutant level (chapter 5). Copy number changes in the *PTEN* gene and the association of the *PTEN* abnormalities with the clinical characteristics and the outcome of patients was also determined (chapter 6).

Mutations in the *NOTCH1* and *FBXW7* genes were detected using dHPLC of PCR products from selected exons. This technique was shown to be effective at detecting both point mutations and size changes in the cohort of 162 patients. The incidence of the mutations in

both genes and the mutational hotspots, the HD and PEST domain of *NOTCH1* and exons 9 and 10 of the WD40 repeats of *FBXW7*, were comparable to other studies (Figures 3.7 and 3.9). Overall, 76 patients (47%) were mutated in *NOTCH1* only, of these 62 were *NOTCH1*^{Single}*FBXW7*^{WT} and 14 were *NOTCH1*^{Double}*FBXW7*^{WT}, four patients (3%) had an *FBXW7* mutation only and 25 patients (15%) were *NOTCH1*^{MUT}*FBXW7*^{MUT} (Table 3.4). Double mutations in *NOTCH1*, such as an HD domain mutation coupled with a PEST domain mutation, synergise to increase the activation of the NOTCH1 signal, and co-incident *NOTCH1* and *FBXW7* mutations are also thought to act in synergy to amplify signal strength (Malyukova *et al.*, 2007;Thompson *et al.*, 2007). Therefore in the present study, the *NOTCH1*^{Double}*FBXW7*^{WT} group was considered jointly with the *NOTCH1*^{MUT}*FBXW7*^{MUT} group as they both lead to synergistic *NOTCH1* activation and are predicted to be functionally equivalent. The cohort was divided into three defined genotype groups, WT for both genes (*NOTCH1*^{WT}*FBXW7*^{WT}), single *NOTCH1*-mutated alone (*NOTCH1*^{Single}*FBXW7*^{WT}), and *NOTCH1*^{Double}*FBXW7*^{WT} or *NOTCH1*^{MUT}*FBXW7*^{MUT} (*NOTCH1±FBXW7*^{Double}).

There were several differences between patients in the *NOTCH1±FBXW7*^{Double} group and those in the *NOTCH1*^{Single}*FBXW7*^{WT} and *NOTCH1*^{WT}*FBXW7*^{WT} groups, including a significantly higher frequency of MRD-negative disease in the *NOTCH1±FBXW7*^{Double} cases. Therefore, the double-mutated group were associated with a much better response than either the *NOTCH1* single-mutated or WT patients, in terms of clearance of leukaemic cells, EFS and OS (Table 4.5), and genotype was an independent favourable factor for OS and EFS in multivariate analysis (Table 4.6). At the time this work was completed eight studies had been published on the impact of *NOTCH1* and *FBXW7* mutations on outcome in paediatric patients. The present study is the only report to have grouped the patients harbouring double *NOTCH1* mutations with those harbouring mutations in both the *NOTCH1* and *FBXW7* genes, all other studies divided their cohorts according to the presence of mutations in the *NOTCH1* gene only or in a combined *NOTCH1* and/or *FBXW7*-mutated group (Clappier *et al.*, 2010;Erbilgin *et al.*, 2010;Fogelstrand *et al.*, 2014;Kox *et al.*, 2010;Larson Gedman *et al.*, 2009;Mansur *et al.*, 2012;Park *et al.*, 2009;Zuurbier *et al.*, 2010). Of these studies only two reported an association between the presence of a mutation and favourable long term outcome (Kox *et al.*, 2010;Park *et al.*, 2009); in one of the studies this effect was seen in the *NOTCH1*-mutated group only (Kox *et al.*, 2010). The variable effects have been attributed to differences in treatment in the various trials, but differences in the combination of mutations may also be a significant factor.

The present data suggests that knowledge of the *NOTCH1/FBXW7* genotype may have a role in refining the risk stratification by adding valuable information to morphological and MRD studies, in particular that *NOTCH1*±*FBXW7*^{Double} patients would not be considered for further intensification of therapy in first remission, including allogeneic stem cell transplantation. Given that, detecting *NOTCH1* mutations for clinical use would present a challenge as replicating the method in the present study requires an individual PCR to screen each relevant exon or domain, followed by further separate analysis of each fragment by dHPLC, meaning that this approach is labour-intensive and time-consuming. Therefore, screening could be performed in patients classified as high-risk post-induction therapy, which, in the current cohort, would limit analysis to approximately half of the patients.

The incidence of *PTEN* mutations in the paediatric cohort was determined by dHPLC of PCR products from the entire coding sequence of the gene (chapter 5). Direct Sanger sequencing was shown to be ineffective at characterising the mutations in a number of cases where more than one alteration was present in the same exon. Therefore, fragment analysis, cloning and re-sequencing of PCR products was utilised and 84% of the identified mutations were successfully characterised. The incidence of patients with mutations and the clustering of mutations in exon 7 was comparable to other studies, overall, 21 (13%) patients were classified as *PTEN*^{MUT} (Figure 5.6). However, the present study reports a significantly higher overall number of mutations than others, owing to 81% of patients harbouring multiple mutations (Figure 5.9). This difference is probably due to a more sensitive screening approach used in the current study. The mutant level of all mutations detected was quantified either by fragment analysis of fluorescently labelled PCR products or by estimation from the sequence, and 52% of mutations were subclonal. The mutant level and number of mutations indicated the level of *PTEN* loss per patient and monoallelic and biallelic mutations were identified at an equivalent frequency. This data reflects the complexity of the genetic hits to the *PTEN* gene in patients with T-ALL.

The possibility of loss of genomic *PTEN* was also considered in the present study and was investigated using a combination of SNP array and SNP allele quantification (chapter 6). The techniques were shown to be effective at identifying copy number changes, and the incidence of *PTEN* deletion detected is comparable to data from other paediatric studies. Screening for genomic loss of *PTEN* was possible in 143 of the 162 patients with mutation status, and of these, *PTEN* deletion was identified in 15 (10%) patients. When combining the mutation and deletion status, of the 145 patients analysed in the present study, 32 (22%) had one or more abnormality in the *PTEN* gene (Table 6.4). Approximately 53% of the identified *PTEN* abnormalities were likely to be clonal, and both biallelic and monoallelic

abnormalities were detected in the present cohort at a frequency of 9% and 13% of patients respectively. Taken together, the mutation and deletion status of paediatric patients with T-ALL demonstrates the wide spectrum of *PTEN* loss of function, and determining which clones are likely to be responsible for disease progression together with the influence of other collaborating mutations are important factors if *PTEN* status is to be used to aid clinical decisions.

There were no differences in the clinical characteristics, response to therapy or long-term outcome of $PTEN^{ABN}$ patients when compared to $PTEN^{WT}$ patients, however there was a borderline trend for $PTEN^{ABN}$ patients to have a worse OS (Figure 6.8). Increased CNS disease and subsequent CNS relapse could be a possible explanation for the trend for the worse outcome seen in patients with abnormal *PTEN*. When furthered stratified by the level of *PTEN* loss, the worst outcome was seen in the monoallelic patients, although this difference was non-significant, possibly indicating that the loss of one allele is more detrimental than complete loss (Figure 6.9). At the time this work was completed, five other paediatric studies had been published analysing patients by their *PTEN* genotype, with four groups reporting no difference between *PTEN*-abnormal and WT patients (Bandapalli *et al.*, 2013;Gutierrez *et al.*, 2009;Larson Gedman *et al.*, 2009;Mendes *et al.*, 2014). One study reported a significantly worse outcome in $PTEN^{ABN}$ patients, although the number of patients in this cohort was small and therefore the significance of this study should be viewed with caution (Jotta *et al.*, 2010). Therefore at present, screening of *PTEN* would not be warranted for use in risk-adapted therapy.

A further important consideration in the present study was the interaction between the *PTEN* abnormalities and *NOTCH1*/*FBXW7* mutation status. The favourable outcome of the $NOTCH1{\pm}FBXW7^{Double}$ group could be observed in patients with or without a *PTEN* abnormality (Figure 6.10), and the worst outcome was seen in the $NOTCH1^{WT}FBXW7^{WT}$ patients with a *PTEN* abnormality. This suggests that the increased activation of the NOTCH1 pathway is dominant over the loss of PTEN function in the context of the treatment protocol, and that it potentially neutralises the possible negative effect of a *PTEN* abnormality. The negative impact of a *PTEN* abnormality in the *NOTCH1*-mutant cases was reported in a recent study (Bandapalli *et al.*, 2013). Therefore, the possibility that *PTEN* abnormalities in patients who are either $NOTCH1^{Single}FBXW7^{WT}$ or $NOTCH1^{WT}FBXW7^{WT}$ could potentially identify those who require further intensification of treatment and needs to be investigated in a larger study.

There are an ever-increasing number of recurrently mutated genes identified in patients with T-ALL and identifying which markers collaborate with *NOTCH1/FBXW7* mutations and *PTEN* abnormalities, and should therefore be screened in new T-ALL patients, will be increasingly challenging. The use of NGS techniques will be able to further elucidate the mutational landscape. Therefore, studies of this kind are important to aid in rationalisation of which markers should be analysed.

7.1 Future directions

If the *NOTCH1*/*FBXW7* mutant status is to be included in the molecular investigation of patients with T-ALL to aid clinical decisions, a fast, accurate and sensitive method of mutation detection will be required. Replicating the method in the present study requires an individual PCR to screen each relevant exon or domain, followed by further separate analysis of each fragment by dHPLC, meaning that this approach is labour-intensive and time-consuming. Also there are no amino acid hotspots in the *NOTCH1* gene equivalent to the R465 hotspot in the *FBXW7* gene, meaning that the whole exon or domain requires screening. In *NOTCH1*, screening could be limited to the HD and PEST domains only, where approximately 94% of the total mutations detected in the present study are located. However, one double case harboured both a PEST domain and a TAD domain mutation, therefore omitting the TAD domain from the screening panel could potentially lead to 3% of double cases being missed. Targeted NGS technology would be quick and sensitive but could be problematic as the technique generates relatively short sequence reads which are aligned to a reference sequence and can therefore miss large insertions or duplications, which accounted for approximately 6% of mutations in the present study. Furthermore, although highly efficient algorithms have been developed that are highly successful for detecting point mutations, they still struggle to accurately align reads containing small indels, which occurred in 5% of patients in the present study. NGS has been used to screen for *NOTCH1* mutations in other studies, in particular CLL, where a group performed WES of all exons of *NOTCH1* and reported a 4% frequency of *NOTCH1* mutations (Wang *et al.*, 2011b). This is contrast to three other studies that utilised Sanger sequencing to screen only the PEST domain in their respective cohorts of patients with CLL, and all detected mutations at a frequency of 11% (Balatti *et al.*, 2012;Rossi *et al.*, 2013;Villamor *et al.*, 2013). Whether or not the differences in the incidence of *NOTCH1* mutations between the respective studies are as a result of mutations being missed using NGS algorithms, or whether they are due to other factors such as the racial origin of the cohort is unclear, but this does highlight the difficulties for developing NGS assays for the detection of *NOTCH1* mutations. However,

streamlining the molecular work-up of samples is desirable, and the inclusion of *NOTCH1*/*FBXW7* in targeted multiplexed NGS-based assays may have an important impact on future treatment-related decisions.

The data presented in this thesis demonstrated a favourable prognosis for *NOTCH1*±*FBXW7*^{Double} patients. Of the 14 *NOTCH1*±*FBXW7*^{Double} patients with either an SER or MRD-positive disease post-induction, none received a BMT in first remission and all were alive 5 or more years after diagnosis. Therefore, determining the *NOTCH1*/*FBXW7* mutation status of the patient could be limited to patients who are classified as high-risk post-induction therapy, with *NOTCH1*±*FBXW7*^{Double} patients not considered for further intensification of therapy in first remission, including allogeneic SCT. Further investigation is clearly required to validate this finding in a larger cohort. However, this remains challenging due to the relatively small proportion of T-ALL compared with B-ALL patients in ALL clinical trials. The frequency of *NOTCH1*±*FBXW7*^{Double} mutations was also only 24% of the current cohort, meaning that the cohort sizes for impact studies will be small, and a meta-analysis to address this issue is therefore warranted.

Alternative ways in which *NOTCH1*/*FBXW7* mutation status may guide risk-adapted therapy could also be explored. For example, in patients where the measurement of MRD was not available, it is possible that knowledge of the genotype could help to inform the clinical decision as to whether a switch of regimen is required at the end of induction therapy. However, this information should be used with caution as approximately one third of patients with a *NOTCH1*±*FBXW7*^{Double} mutation were MRD-positive at day 29 and the excellent OS reported in this group could be as a result of the more intensive treatment in this subset of patients. Of note, the use of *NOTCH1* mutations as MRD markers is limited by the lack of stability of the mutations as loss at relapse has been demonstrated (Mansour *et al.*, 2007).

The loss of PTEN function has been reported to occur by a variety of mechanisms including copy number changes, mutation, promoter methylation and post-translational modifications (chapter 5). The present study focused on mutations and genomic loss of the *PTEN* gene. However, further investigation is required to detect intragenic *PTEN* deletions and to explore the other mechanisms of loss of function in order to gain a wider picture of the role of PTEN in T-ALL. A recent study used MLPA to screen samples from 146 patients for *PTEN* microdeletions (Mendes *et al.*, 2014). They were identified in four (3%) patients: three spanning exons 2-3, two of them heterozygous and one homozygous, and one heterozygous deletion of exons 4-5. The deletions ranged from 11kb to 65kb and all resulted in an out-of-

frame transcript. Further screening of the samples for similar breakpoints to those identified revealed subclonal microdeletions in an additional seven patients. Microdeletions were therefore detected in 11 (8%) patients, and only two of the 11 deletions had been detected in their previous study using array-CGH (Zuurbier *et al.*, 2012). In view of this, it would be interesting to explore whether these type of microdeletions occur in the present cohort. Preliminary work to investigate this issue has been carried out by screening the breakpoint region of the previously described Type 1 microdeletions, a 65kb region encompassing exons 2 and 3 using PCR of genomic DNA followed by gel electrophoresis. Patient samples harbouring the deletion produced a 306bp amplicon visible on a 2% agarose gel. Evidence of Type 1 microdeletion was identified in four of 145 patients (3%). The deletions had not been detected by the SNP array however all four cases already harboured known *PTEN* abnormalities and this additional information would therefore not change their *PTEN* genotype. Continued investigation of the other described microdeletions (Type II and III) (Mendes *et al.*, 2014), will further determine the extent of loss of genomic *PTEN* in the cohort.

Knowledge of *NOTCH1* and *PTEN* genotype could also be useful in guiding future treatment strategies such as the use of PI3K inhibitors. A recent study using an in vivo murine model, demonstrated that some PI3K inhibitor-resistant cases of primary T-ALL did not harbour the somatic *Notch1* mutations present in the parental leukaemia and generally had reduced PTEN expression (Dail *et al.*, 2014). This suggests that the loss of aberrant Notch1 signalling provided a selection advantage to the PI3K inhibitor-resistant leukaemic cells. Therefore, loss of *NOTCH1* mutations and the presence of *PTEN* abnormalities at relapse may identify patients who have become resistant to PI3K-inhibitor treatment and would benefit from a different therapeutic approach.

Investigations of other molecular markers that can be correlated to the *NOTCH1/FBXW7/PTEN* molecular profile of patients with T-ALL are warranted to further refine risk-stratification. RAS is known to activate the PI3K/AKT pathway and mutations of the gene have been described in T-ALL (Kawamura *et al.*, 1999;Zhang *et al.*, 2012). Studies have also shown that activation of the PI3K/AKT pathway via RAS can lead to the induction of leukaemia (Kong *et al.*, 2013;Shieh *et al.*, 2013). A recent study in an adult cohort of T-ALL patients reported that patients harbouring *PTEN* and/or *RAS* mutations had a significantly worse OS when compared to *PTEN*/*RAS*-WT cases (Trinquand *et al.*, 2013). The authors devised an oncogenetic risk classifier whereby patients with either a *PTEN* mutation or deletion were grouped with patients with a *RAS* mutation, and the presence of *NOTCH1* and/or *FBXW7* mutations in the absence of *PTEN/RAS* alterations identified low-

risk patients, and all other patients were defined as high-risk. This study suggests that the evaluation of the role of *RAS* mutations in the present cohort could provide valuable prognostic information. Subsequent to the work presented in this thesis, the *RAS* mutant status has been determined in the patients from the present study. Of 145 patients, 13 (9%) had a *RAS* mutation (*RAS*^{MUT}). Of these, eight (62%) harboured an *N-RAS* mutation and five (38%) a mutation in *K-RAS*. Putting together the *PTEN* and *RAS* genotype of the 145 patients, 101 (70%) patients were scored as *PTEN*/*RAS*^{WT} and 44 (30%) had either an abnormality in the *PTEN* gene or a *RAS* mutation (*PTEN*/*RAS*^{ABN}). When correlating to clinical outcome, the addition of the *RAS* genotype made no difference to the *NOTCH1±FBXW7*^{Double} cases that retained their excellent OS. Interestingly, in the *NOTCH1*^{Single}*FBXW7*^{WT} patients, the addition of *RAS*^{MUT} to the *PTEN*^{ABN} group improved both the RFS and OS when compared to *PTEN*^{ABN} alone, albeit by a small amount. However, the opposite was seen in the *NOTCH1*^{WT}*FBXW7*^{WT} group, where patients with a *PTEN/RAS* abnormality had a worse outcome than *PTEN*^{ABN} patients alone. These results therefore differ from the recent adult study (Trinquand *et al.*, 2013) and suggest that *RAS* mutations may play a distinct role from *PTEN* abnormalities in the context of some cases of T-ALL. NOTCH1 activation may therefore also neutralise the negative effect of *RAS* mutations in the context of the treatment protocol. However, the numbers in the subgroups are low therefore analysis in a larger cohort is required to determine if *PTEN* and *RAS* abnormalities are significant in paediatric T-ALL.

A recent study of adult patients with T-ALL reassessed the value of conventional risk factors such as WBC count, and new risk factors including MRD response and genetic markers (Beldjord *et al.*, 2014) Authors used the oncogenetic classifier proposed by Trinquand *et al* (2013) in which a high-risk genetic profile was defined by the absence of a *NOTCH1/FBXW7* mutation and/or the presence of a *PTEN/RAS* abnormality. They demonstrated that patients with high-risk genetics had a higher incidence of relapse irrespective of MRD status, suggesting that genotype was the predominant predictor in the cohort which could be further refined by MRD response. The oncogenetic classifier does not translate to the present paediatric cohort, as previously mentioned there was no evidence that the presence of a *PTEN* abnormality impacted on the highly favourable outcome reported in the *NOTCH1±FBXW7*^{Double} patients. The addition of *RAS* genotype also made no difference to the *NOTCH1±FBXW7*^{Double} cases that retained their excellent OS. Approximately one third of this genotype group were MRD-positive at day 29, suggesting that the genetic profile may be the dominant predictor over MRD response. To address the work of Beljord *et al* (2014), further investigation could assess the utility of an adjusted classifier in the present paediatric cohort, in which all *NOTCH1±FBXW7*^{Double} cases, including those with a *PTEN/RAS*

abnormality, be classed as low-risk. High-risk patients would therefore be defined as *NOTCH1*^{Single} cases with a *PTEN/RAS* abnormality and/or MRD-positive disease, and all *NOTCH1/FBXW7*^{WT} cases.

In the present study, only the *PTEN* locus on chromosome 10 was investigated on the CytoSNP-850k array. Further characterisation of genome-wide copy number changes present in each patient is therefore required. A particular candidate for study is cyclin C, which was recently identified as a haploinsufficient tumour suppressor in T-ALL, with heterozygous deletions of the cyclin C encoding gene, CCNC, located on human chromosome 6q21, being detected in 17% of patient samples (Li *et al.*, 2014). The authors demonstrated that the cyclin C-CDK8 complex phosphorylates ICN-1 in vivo, allowing FBXW7 to bind the PEST domain to target ICN-1 for proteolytic degradation. Many of the phosphorylation residues were located within the PEST domain of NOTCH1, including three residues, T2512, S2514 and S2517, which are localised in the phosphodegron. Therefore, ablation of cyclin C resulted in decreased phosphorylation leading to the stabilisation of ICN-1 in the nucleus, thereby increasing the NOTCH1 activation signal. Deletion of CCNC may therefore underlie another mechanism for constitutive NOTCH1 signalling. The cyclin C deletion status of the patients in the present study could be determined from the array data and added to the *NOTCH1/FBXW7* mutation status for prognostic use.

The acquisition of collaborating genetic hits is likely required for the leukaemic phenotype of the patients harbouring *NOTCH1/FBXW7/PTEN* abnormalities in the present study. Therefore, exploration of what the cooperating events are is important. There is a wealth of information still to be explored from future studies using genome-wide technology. For example, WES to attempt to identify those genetic events predicting *NOTCH1*±*FBXW7*^{Double} cases with an excellent outcome. However, issues of complexity and heterogeneity, as shown in the *PTEN* analysis in the current study where the presence of multiple *PTEN* abnormalities was identified in some patients, will present a challenge for the interpretation of such studies. Further study is therefore warranted in order to determine which factors influence the outcome of paediatric patients with T-ALL and should be used to determine future therapeutic strategies.

7.2 Conclusions

This thesis describes an investigation of *NOTCH1* and *FBXW7* mutations and *PTEN* abnormalities in paediatric patients with T-ALL, clarifying their impact on the prognosis of

patients. Novel findings were made with regard to the presence of *NOTCH1±FBXW7*^{Double} mutations correlating to an excellent outcome in paediatric patients with T-ALL. This data has made a significant contribution to the current understanding of both the clinical and biological role of *NOTCH1, FBXW7* and *PTEN* in T-ALL, as well as provided areas for further investigation.

PUBLICATIONS ARISING FROM THE WORK IN THIS THESIS

Jenkinson, S., Koo, K., Mansour, M.R., Goulden, N., Vora, A., Mitchell, C., Wade, R., Richards, S., Hancock, J., Moorman, A.V., Linch, D.C. & Gale, R.E. (2012). Impact of *NOTCH1/FBXW7* mutations on outcome in pediatric T-cell acute lymphoblastic leukemia patients treated on the MRC UKALL 2003 trial. *Leukemia,* **27,** 41-7.

Jenkinson, S., Kirkwood A.A., Goulden, N., Vora, A., Linch, D.C. & Gale, R.E. (2015). Impact of *PTEN* abnormalities on outcome in pediatric patients with T-cell acute lymphoblastic leukemia treated on the MRC UKALL2003 trial. *Leukemia,* Jul 29 [Epub ahead of print]

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APPENDIX

Appendix Table 1 Primer sequences

Gene	Domain/exon	Primer	Sequence	Annealing temperature (^{o}C)	WAVE temperature (^{o}C)
NOTCH1	LNR-B/ exon 25	25B-F 25B-R	5'-CGAGAGCCCCTTCTACCGTTG-3' 5'-CTCCCTCAGCCCCATGAGC-3'	64	63.8, 66.7
NOTCH1	HD-N/ exon 26	26-F 26-R	5'-GGAAGGCGGCCTGAGCGTGTC-3' 5'-ATTGACCGTGGGCGCCGGGTC-3'	70	65.9, 67.4
NOTCH1	HD-C/ exon 27	27-F 27-R SNP-F	5'-GCCTCAGTGTCCTGCGGC-3' 5'-GCACAAACAGCCAGCGTGTC-3' 5'TCCTCGCAGTGCTTCCAGAGTGCCACCCA-3'	Touchdown	65
NOTCH1	JME/ exon 28	28-F 28-R	5'TGATTAATCGCGTAGAAAATCACCT-3' 5'-CACCGGGGACCCAGAAGC-3'	Touchdown	65.1, 66.3, 67.8
NOTCH1	TAD/ exon 34	TAD-F TAD-R	5'-GCTGGCCTTTGAGACTGGC-3' 5'-GCTGAGCTCACGCCAAGGT-3'	61	63.5, 64.5
NOTCH1	PEST/ exon 34	PEST-F PEST-R	5'-CAGATGCAGCAGCAGAACCTG-3' 5'-AAAGGAAGCCGGGGTCTCGT-3'	62	65.7
FBXW7	8	8-F 8-R	5'-AGATAGACTACAAATTACTGTTCCTG-3' 5'-CTTTGTGAAGTGTAGGAAGAGTAAAC-3'	56	56, 58
FBXW7	9	9-F 9-R	5'-TCTACCCAAAAGTAATCATCTTAAGTG-3' 5'-ATAGACGAACAAGTCCCAACCAT-3'	56	58.6
FBXW7	10	10-F 10-R	5'-GTTTTTCTGTTTCTCCCTCTGCA-3' 5'-ACCTTATGATTCATCAGGAGAGC-3'	58	56, 59.6
FBXW7	11	11-F 11-R	5'-GTAATTGATAGGAAGAGTATCCATAC-3' 5'-AACCATTCTGTATGAGGTTGACTC-3'	60	56.6, 59
FBXW7	12	12-F 12-R	5'-CAAATTATAATGTAACTAACTCATAGCCA-3' 5'-GAGTATATAATGTAACTAACTCATAGCCA-3'	56	57.6

Appendix Table 1 continued

Gene	Domain/exon	Primer	Sequence	Annealing temperature (^{o}C)	WAVE temperature (^{o}C)
PTEN	1	1-F 1-R	5'-AGAGCCATTTCCATCCTGCAGA-3' 5'-AACTACGGACATTTTCGCATCCG-3'	63	59.6
PTEN	2	2-F 2-R	5'-CACCTTTTATTACTGCAGCTAT-3' 5'-CACAAAGTATCTTTTTCTGTGG-3'	57	54.1
PTEN	3	3-F 3-R	5'-CAAATGTTAGCTCATTTTTGTT-3' 5'-GTTAAAATGTATCTTAACTCT-3'	51	54.7
PTEN	4	4-F 4-R	5'GTACTTTTTTTTCTTCCTAAGTGCAAAAG-3' 5'-TCACTCGATAATCTGGATGACTCA-3'	62	56
PTEN	5	5-F 5-R	5'-GAGTTTTTTTTTCTTATTCTGAGGTTATC-3' 5'-CTCAGATCCAGGAAGAGGAAAG-3'	62	55.5, 57.2
PTEN	6	6-F 6-R	5'-GGCTACGACCCAGTTACCATAG-3' 5'-CTTCTAGATATGGTTAAGAAAACTGTTC-3'	62	57.1
PTEN	7	7-F 7-R	5'-GACAGTTAAAGGCATTTCCTG-3' 5'-GTCCTTATTTTGGATATTTCTCCCAATG-3'	63.5	56.1, 58.8, 60.0
PTEN	8	8-F 8-R	5'-GCAAATGTTTAACATAGGTGACAG-3' 5'-GATAACTCAGATTGCCTTATAATAGTC-3'	61	52.8, 55.3
PTEN	9	9-F 9-R	5'-GTTTAAGATGAGTCATATTTGTGGGT-3' 5'-CAAGTTTATTTTCATGGTGTTTTATCC-3'	61.5	54.1, 56.9, 57.8
PTEN	Intron 8-9	Int8-9-F Int8-9R	5'-TGATCTTGACAAAGCAAATAA-3' 5'-ACTGCTACGTAAACACTGCTT-3'	64	55.5

F and R indicate forward and reverse primers, respectively. Abbreviations: HD, heterodimerisation domain; JME, juxtamembrane domain; TAD, transcriptional activation domain; LNR-B, Lin12/Notch repeats.

Appendix Table 2 *NOTCH1* and *FBXW7* sequence alterations detected

Patient	Mutation 1			Mutation 2			Single/ Double
	Gene	DNA change	Predicted AA change	Gene	DNA change	Predicted AA change	
1	*NOTCH1*	C7171T	Q2391X				Single
2	*NOTCH1*	4735_4737delCTG	L1579del	*FBXW7*	G1394A	R465H	Double
4	*NOTCH1*	4732_4734delGTG	V1578del				Single
5	*NOTCH1*	T5039C	I1680T				Single
6	*NOTCH1*	4817_4818insTGTCGC	F1606_K1607insVA				Single
8	*NOTCH1*	T4754C	L1585P				Single
12	*NOTCH1*	4775_4776insTGCGGAGTGGGC	F1592_L1593insFAEWA	*NOTCH1*	7368delT	I2456fs	Double
13	*NOTCH1*	T4799C	L1600P				Single
15	*NOTCH1*	T4799C	L1600P				Single
16	*NOTCH1*	G4793C	R1598P				Single
17	*NOTCH1*	T4799C	L1600P	*NOTCH1*	7430_7457dup7457_7458insCCGGACCTAGCGGGG	S2486fs	Double
35	*NOTCH1*	T4847A, 4849_4851delinsCCA	I1615N, F1616P	*FBXW7*	G1394A A1330G	R465H K444E	Double
36	*NOTCH1*	4775_4776insGAA	F1592delinsLN				Single
37	*NOTCH1*	T4754C	L1585P				Single
38	*NOTCH1*	T5033C	L1678P				Single
39	*NOTCH1*	4732_4734delGTG	V1578del	*FBXW7*	G1394A	R465H	Double
40	*NOTCH1*	G4793C	R1598P				Single
41	*NOTCH1*	T4733A	V1578E				Single
42	*NOTCH1*	7541_7545delinsGAA	P2514fs				Single
43	*NOTCH1*	T4778C	L1592P	*FBXW7*	G1394T	R465L	Double
44	*NOTCH1*	C5099A	A1700D	*NOTCH1*	7318_7327delinsGGGGGGGCGTGA	V2443fs	Double

Appendix Table 2 continued

Patient	Mutation 1			Mutation 2			Single/ Double
	Gene	DNA change	Predicted AA change	Gene	DNA change	Predicted AA change	
45	*NOTCH1*	7525_7535delTTCCTCACCCC	F2509fs				Single
46	*NOTCH1*	T4721C	L1574P	*FBXW7*	G1394C	R465P	Double
47	*NOTCH1*	7451delC	Q2459fs				Single
48	*NOTCH1*	T5033C	L1678P				Single
49	*NOTCH1*	G4793C	R1598P	*FBXW7*	G1394A	R465H	Double
50	*NOTCH1*	4816_4818delTTC	F1606del				Single
51	*NOTCH1*	G4793C	R1598P	*NOTCH1*	7625_7626delCT	P2517fs	Double
52	*NOTCH1*	G4790T	S1597I	*FBXW7*	G1394C	R465P	Double
53	*NOTCH1*	5202_5203ins57nt	H1735delinsPLAPVP AGETVEPPPPAQLH				Single
54	*NOTCH1*	4775_4776insTGGAAC	F1592_L1593insGT				Single
55	*NOTCH1*	T5033C	L1678P	*FBXW7*	C1393T	R465C	Double
56	*NOTCH1*	G4793C	R1598P	*FBXW7*	G1394A	R465H	Double
57	*NOTCH1*	4732_4734delGTG	V1578del				Single
58	*NOTCH1*	7475_7478delinsAAGCCC CCAGCTTAGCC	S2492fs				Single
59	*NOTCH1*	T5033C	L1678P				Single
60	*NOTCH1*	7541_7542delCT	P2514fs				Single
61	*NOTCH1*	4816_4818delTTC	F1606del	*FBXW7*	G1436A	R479Q	Double
62	*NOTCH1*	T5033C	L1678P				Single
63	*NOTCH1*	T5033C T4721C	L1678P L1574P	*FBXW7*	C2065T	R689W	Double
64	*NOTCH1*	4775_4778delinsCACCCG CCAAGTTAA	F1592_L1593delinsS PAKLM				Single
65	*NOTCH1*	4816_4817insGGGGGGTTT	F1606delinsLGGF	*NOTCH1*	C7213T	Q2406X	Double
66	*NOTCH1*	G4793C	R1598P	*NOTCH1*	T5033C	L1678P	Double

Appendix Table 2 continued

Patient	Mutation 1			Mutation 2			Single/ Double
	Gene	DNA change	Predicted AA change	Gene	DNA change	Predicted AA change	
67	*NOTCH1*	T4721C	L1574P				Single
68	*NOTCH1*	4811_4812insCTTCTGGAC	V1604_V1605insLLD				Single
69	*NOTCH1*	4732_4734delGTG	V1578del				Single
70	*NOTCH1*	4817_4818insGGA T4582C	F1606delinsLD C1528R	*NOTCH1*	7541_7542delCT	P2514fs	Double
71	*NOTCH1*	G4793C	R1598P	*NOTCH1*	7327_7328insTGAATCC	V2443fs	Double
72	*NOTCH1*	C7183T	Q2394X	*NOTCH1*	7546_7547insAGTACTGAGT	S2516fs	Double
73	*NOTCH1*	7399_7400insATAACCGT	S2467fs				Single
74	*NOTCH1*	T4754C	L1585P				Single
75	*NOTCH1*	T4847A	I1616N	*FBXW7*	G1322A	R441Q	Double
76	*NOTCH1*	4775_4776insGGTGTCTGC	F1592delinsLVSA				Single
77	*NOTCH1*	T7525C	F2509L				Single
78	*NOTCH1*	4746_4747insCCG	P1582_E1583insP	*FBXW7*	C1393T	R465C	Double
79	*NOTCH1*	T4778C	L1593P	*NOTCH1*	7541_7542delCT	P2514fs	Double
80	*NOTCH1*	5077_5145dupinsTCATCTCGGGG	1693_1715dupinsSSRG				Single
81	*NOTCH1*	T4721C	L1574P				Single
82	*NOTCH1*	T5033C	L1678P				Single
83	*NOTCH1*	T4775C	F1592S				Single
84	*NOTCH1*	4810_4811insGCG	N1603_V1604insG	*FBXW7*	C1393T	R465C	Double
85	*NOTCH1*	T5126C	L1709P	*FBXW7*	G1394A	R465H	Double
86	*NOTCH1*	4775_4776insTGA	F1592_L1593insD				Single
87	*NOTCH1*	T4778C	L1593P	*NOTCH1*	7468_7469insGGATGACACCGTTTTAATCCGGTACT	Y2490fs	Double

Appendix Table 2 continued

Patient	Mutation 1			Mutation 2			Single/ Double
	Gene	DNA change	Predicted AA change	Gene	DNA change	Predicted AA change	
88	*NOTCH1*	7524_7534delTTCCTCACCCC	F2509fs				Single
89	*NOTCH1*	4848_4849insCTCCTC	I1616delinsLL	*FBXW7*	G1436A	R479Q	Double
90	*NOTCH1*	4774_4775insTGAAAAATA TCGCGC	F1592delinsLKNIAL	*NOTCH1*	7366_7367insAC, 7327_7328insGGGAC ACCTCGTCT	I2456fs V2443fs	Double
91	*NOTCH1*	4851_4852insGGT	G1619ins				Single
92	*NOTCH1*	7312_7313insAATAATAAT GTTTGTC	P2438fs				Single
93	*NOTCH1*	4776_4778del CCT	F1592del	*FBXW7*	C1393T	R465C	Double
94	*NOTCH1*	T4799C	L1600P	*NOTCH1*	7541_7542delCT	P2514fs	Double
95	*NOTCH1*	G4793C	R1598P				Single
96	*NOTCH1*	5155_5156insGAC	E1719delinsGQ	*NOTCH1*	7398_7399insGCTCC	S2467fs	Double
97	*NOTCH1*	T4721C	L1574P				Single
98	*NOTCH1*	5214_5215ins132nt	Y1738_V1739ins44aa				Single
99	*NOTCH1*	4731_4733delCTG	V1578del	*FBXW7*	C1393T G1550T	R465C G517V	Double
100	*NOTCH1*	4742_4743insGCCCGG	P1582insGP				Single
101	*NOTCH1*	C7457A	S2486X				Single
102	*NOTCH1*	4816_4817insTGGGGACTT	F1606insGDF				Single
103	*NOTCH1*	T4754C	L1585P	*FBXW7*	C1513T	R505C	Double
104	*NOTCH1*	4596_4597ins39nt	K1632_R1633insRPV LQGPLQRRAL				Single
105	*NOTCH1*	4850_4853delinsCTCCCCCG CG	F1617delinsSPPR				Single
106	*NOTCH1*	T5033C	L1678P				Single
107	*NOTCH1*	T4799C	L1600P				Single
108	*NOTCH1*	4821_4822insGTCTTCCAG	K1607_R1608insVFQ				

Appendix Table 2 continued

Patient	Mutation 1			Mutation 2			Single/ Double
	Gene	DNA change	Predicted AA change	Gene	DNA change	Predicted AA change	
109	*NOTCH1*	G4723C, T4724C	V1575P	*FBXW7*	G1436A	R479Q	
110	*NOTCH1*	C7372A, 4816_4817insGGGT TCACCACAGCC	P2458T, F1606delinsLG SPQP	*FBXW7*	C1393T	R465C	
111	*NOTCH1*	T4721C	L1574P				
112	*NOTCH1*	T5033C	L1678P	*FBXW7*	G1394A	R465H	
113	*NOTCH1*	4779_4780insGGCCCC	F1592_L1593insGP				
114	*NOTCH1*	4847_4853delins22nt	I1616_P1618delinsTGPVS PFL				
115	*NOTCH1*	4810_4811insGGG	G1604ins	*FBXW7*	G1436A	R479Q	
116	*NOTCH1*	T4778C	L1593P				
117	*NOTCH1*	4817_4818ins30nt	F1606delinsLSVKEPFVGCL				
118	*NOTCH1*	7531_7542delinsT, 5025_5026delGTC	T2511fs, V1676del	*FBXW7*	C1513T C2065T	R505C R689W	
119	*NOTCH1*	4818delinsGCTCACT	F1606delinsLLT				
120	*NOTCH1*	5220_5221ins30nt	A1740_A1741insPAQLHFM YVA				Single
121	*NOTCH1*	T4721C	L1574P	*FBXW7*	G1394A	R465H	Double
122	*NOTCH1*	5216_5217ins41nt	V1739_A1740insWAGAFGA SVLRMYW				Single
123	*NOTCH1*	T5033C	L1678P				Single
124	*NOTCH1*	4776delinsAGGCCCT	F1592delinsLGP				Single
125	*FBXW7*	C1393T	R465C				Single
126	*FBXW7*	G1436A	R479Q				Single
127	*FBXW7*	G1394T	R465L				Single
128	*FBXW7*	G1394A	R465H				Single

Nucleotides numbered from the major translational start codon at nucleotide position 1. Abbreviations: AA, amino acid; ins, insertion; del, deletion; dup, duplication; nt, nucleotide.

Appendix Table 3 *PTEN* sequence alterations detected

Patient	Mutations		
	Number	DNA change	Predicted AA change
1	1	696_700delinsGGGGCCT	R233fs
2	1	703_709delinsCCTTCCAGGGCATACAG	E235fs
3	1	703_704insG	E235fs
4	1	693_700delinsTCCCGAGAAAGACT	T232fs
5	1	696_702insAAAAACCATTGACGGATGT	R233fs
6	2	722insAGCGAGTTCCCCTCA 13bp del: N/A	F241fs 13bp del: N/A
7	2	700delinsTATGCCdelG 700delinsCCTCA	R234fs R234fs
8	2	722_723insCGGATGGT 671_672insCCCC	F241fs I224fs
9	2	736_739delinsTTTCCTCCTTTTG 11bp ins: N/A	P246fs 11bp ins: N/A
10	3	696_697insT 698_699insGGAAG 3bp ins: N/A	R233fs R233fs 3bp ins: N/A
11	4	695_700delinsCCAGGGAGTA 685_695del 693_699delinsTCAGCTTACCCCACTCCT 7bp ins: N/A	T232fs S229fs T232fs 7bp ins: N/A
12	1	491_492insA	K164fs
13	1	445_446delinsGGCCTTGGGTT	Q149RPPV
14	2	736_737insCCCC 696insA	P246fs R233fs
15	2	702_703insCG 730_735delinsTCCCGTTAAGAT	E235fs P244_Q245delinsLPL RS
16	2	682_701delinsC 696delinsGG	N228fs R233fs
17	2	736_737insGAAGAGAGGACCGAGG 736delinsGG	P246fs P246fs
18	3	765_766insCCCCAACCGCCTACCAGC 546_547insGATGATTA 2bp ins: N/A	E256_F257insPQLPT S K183fs 2bp ins: N/A
19	3	732_735delinsTCTCCTCTAGTCCCGG 738_739insGCCCCG 662_663insGGATGGTA	Q245_P246SPLVPA L247_P248AP Y225fs
20	3	656_674delinsTTTATCGTC 735_736insATCCCT 698_703insGGGGGCG 8bp del: N/A	Q219fs P246_L247IP R233fs 8bp del: N/A
21	4	682_696delinsCAT 737_738insAGTG 536_537insAGTATTATTATAG 14bp ins: N/A	N228fs P246fs S179fs 14bp ins: N/A

Nucleotides numbered from the major translational start codon at nucleotide position 1.
Abbreviations: AA, amino acid; ins, insertion; del, deletion.