1-1-2010

Molecular And Therapeutic Implications Of Notch1 Signaling In Pediatric T-Cell Acute Lymphoblastic Leukemia

Amanda Larson Gedman
Wayne State University,

MOLECULAR AND THERAPEUTIC IMPLICATIONS OF NOTCH1 SIGNALING IN PEDIATRIC T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

by

AMANDA LARSON GEDMAN

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2010

MAJOR: CANCER BIOLOGY

Approved by:

_____

Advisor Date

_____

_____

_____

_____

DEDICATION

This work is dedicated to those who have made a positive impact on my life. First and foremost, to my husband Sam, for his enormous support and encouragement during this entire process. This would have been nearly impossible to do without you. To my parents, Bob and Jackie Larson, for encouraging me to do my very best, and always being there for me whenever I needed them. To my in-laws, Ray and Evelyn Gedman, for their unwavering support. To my grandparents, John and Olive Helka, for their love. They have help shaped who I am today. Last and not least, to the remainder of my family, whose kind words and support will stay with me forever.

ACKNOWLEDGMENTS

I would like to thank Dr. Larry Matherly for giving me the opportunity to work in his lab and to grow as a scientist under his guidance. I believe the skills that I have learned here will carry me throughout my entire scientific career. I would also like to thank my committee members, Drs. Pauley, Taub, Ge and Boerner for their support and dedication to this process. I am thankful for the entire Matherly Lab, past and present members, for their help. You all have made this lab a joy to be apart of. A special thank you to Dr. Qing Chen for her work on the diagnosis and relapse project, and to Sita Desmoulin for her work on the NOTCH1 mutagenesis project. Lastly, I would like to thank the Cancer Biology Program for its dedication and commitment to my training. I truly feel that my training from this program has set me up for a fruitful and fulfilling scientific career.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ANK	Ankyrin
B-ALL	B-cell acute lymphoblastic leukemia
BP-ALL	B-cell precursor acute lymphoblastic leukemia
BCL2	B-cell leukemia/lymphoma 2
BCL-XL	B-cell leukemia/lymphoma X long isoform
bHLH	Basic helix-loop-helix
BSA	Bovine serum albumin
cDNA	Copy deoxyribonucleic acid
CDS	Coding sequence
CK2	casein kinase
CLL	Chronic lymphoid leukemia
CNS	Central nervous system
COG	Children's Oncology Group
CompE	Compound E (([(2S)-2-([3,5-difluorophenyl)acetyl]amino)-*N*-[(3S)-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl]propanamide]))
CPD	conserved phosphodegron
CSL	CBF-1/Su(H)/Lag-1
DLL	Delta-like
DMSO	Dimethylsulfoxide

DNA	Deoxyribonucleic acid
DNR	Daunorubicin
DSL	Delta-Serrate-Lag
DTX1	Deltex
EC	Extracellular subunit
EFS	Event-free survival
EGF	Epidermal growth factor
EX	Exon
FBW7	F-box and WD repeat domain-containing 7
FS/SS	Forward scatter/side scatter
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
GSI	γ-secretase inhibitor
h	Hours
HD	Heterodimerization Domain
HERP	HES-related protein
HES1	Hairy and enhancer of split
HSCs	Hematopoietic stem cells
ICN	Intracellular subunit
IRS1	insulin receptor substrate 1
JAG	Jagged
JAK	Janus kinase
JME	Juxtamembrane region

kDa	Kilodalton
L-ASP	L-asparaginase
MAML	Mastermind-like
$MgCl_2$	Magnesium chloride
min	Minutes
miR	microRNA
MRD	Minimal residual disease
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltretrazolium bromide
MTX	Methotrexate
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor κB
NR3C1	Glucocorticoid receptor
ntc	Non-targeted control
OS	Overall survival
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent protein kinase 1
PE	Phycoerythrin
PEST	Polypeptide enriched in proline, glutamate, serine and threonine domain
PI	Propidium iodine
PI3K	Phosphatidylinositol-3-kinase

PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
POG	Pediatric Oncology Group
PTEN	Phosphatase and tensin homolog
RAM	RBPJκ Associated Molecule
RHEB	Ras homologue enriched in brain
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
S6K1	Ribosomal S6 kinase 1
SCF	SKP1,CUL1,F-box
shRNA	Short-hairpin RNA
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TAD	Transactivation domain
T-ALL	T-cell acute lymphoblastic leukemia
TCR	T-cell receptor
TM	Transmembrane subunit
TSC	Tuberous sclerosis
UTRs	Untranslated region
WBC	White blood cell
WCE	Whole cell extract

CHAPTER 1

THE ROLE OF NOTCH1 IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

1.1 Introduction to Leukemia

Cancer is the number one cause of death from disease for American children. The most common type of pediatric cancer is leukemia, accounting for nearly 1/3 of all cancer cases. Leukemia generally describes the clonal proliferation and accumulation of malignant blast cells in the bone marrow and peripheral blood and is often associated with chromosomal abnormalities and genetic mutations. Leukemia can arise in either the myeloid or lymphoid lineages. Regardless of the origins, the disease is generally classified into two categories, either acute or chronic. Chronic leukemia is the excessive accumulation of fairly mature but abnormal cells, which may take months to years for progression. Acute leukemia is the rapid growth of immature cells with low levels of differentiation.

Although the direct cause of leukemia in children is unknown, some studies suggest that leukemia may be a consequence of *in utero* exposures to ionizing radiation, pesticides and/or solvents[1]. There is a higher frequency of pediatric leukemia in Down Syndrome, Bloom Syndrome, Neurofibromatosis type I and Ataxia-telangiectasis patients[2, 3]. Evidence also suggests that leukemia arises more frequently in Caucasian children, and in those from more affluent societies and urban areas, suggesting that some socioeconomic factors may play a role in the etiology of the disease[4-6]. Inherited genetic alterations in drug metabolism, DNA repair and cell-cycle checkpoints are thought to interact with environmental, dietary, maternal and other factors to influence the development and progression of leukemia, as well as its response to chemotherapy[7-9].

While leukemia (both acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL)) was the leading cause of cancer death in children in both 1975 and 2006, the percentage of death due to leukemia has decreased from 38.9% (1975) to 30.4% (2006)[1]. Despite this decrease in death, the overall incidence of pediatric lymphoid leukemia has increased significantly, with an annual percentage change of 0.8%[1]. The reason for this increase is unknown, however there are several hypotheses exploring the relationships between pediatric leukemic risk and delayed exposure to infectious agents, as well as the relationship to birth weight[1, 10-13]. Despite this alarming increase in incidence rate, the mortality rate for pediatric leukemia has decreased by nearly 64% between 1975 and 2006[1]. This is evident by increases in 5-year survival rates, from 61% during 1975-1978 to nearly 88.5% during 1999-2006 in children younger than 15 years old. Similar improvements are seen in adolescents and young adults (15-19 years old), but their 5-year survival rate was only 50.1% during 1999-2006[1]. This lag in survival improvement is thought to be due to differences in tumor biology and overall treatment between these two age groups[14, 15]. Adolescents and young adults with ALL typically have more prognostically poorer disease characteristics, including advance age and T-cell ALL HOX abnormalities[16,17] (see below). However, it is believed that treatment has more of an impact on the difference in disease survival between pediatric and adult T-ALL patients than the underlying difference in disease characteristics[17]. Children under the age of 15 are usually treated with pediatric protocols, while older adolescents may be treated with either pediatric protocols or adult protocols depending upon their physician. Adult ALL patients face an even worse prognosis. With modern therapies, adults have a long term disease-free survival rate of only 40%[16]. As mentioned

above, the discrepancy in survival rate for children, adolescents and adults may be due to biological differences in the disease at these life stages, as well as the types of therapies administered. That is, children under the age of 10 tend to have more favorable prognostic indicators than adolescents and adults[15]. Treatment protocols for adolescents and young adults have been shown to be far more inferior to pediatric protocols[17].

1.2 Classification of ALL

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease with diverse morphologic, immunologic and genetic features. Immunophenotypically unique ALL subgroups exhibit characteristic biochemical, clinical and cytogenetic features that are typically associated with different prognoses[18-21]. B-cell ALL (B-ALL) accounts for up to 80% of ALL cases. It arises in both precursor (BP) and mature B-cells. Nearly 80% of pediatric BP-ALL patients experience long-term survival (>5 years) with modern therapies[19-21]. B-ALL is usually accompanied by chromosomal abnormalities, which play a key role in the development of the disease. These abnormalities serve as biomarkers which are used to predict prognosis and determine the most optimal therapeutic regimen. Acquired chromosomal abnormalities occur in ~90% of pediatric ALLs and nearly 2/3 of these are directly relevant to prognosis[18, 20, 21]. Hyperdiploidy (>50 chromosomes) is found in ~25% of BP-ALL cases and is a good prognostic indicator for children treated with antimetabolite-based therapy[18, 20, 21]. Chromosomal structural alterations frequently involve balanced or reciprocal translocations leading to recombination of gene loci and deregulated expression of proto-oncogenes or expression of fusion proteins with properties distinct from their wild-type counterparts. These gene fusions frequently lead to the constitutive activation of kinases [e.g., BCR-ABL in t(9;22)] or altered

transcriptional regulation [e.g., MLL in t(4;11); AML1 or TEL in t(12;21)] that are either initiating events in leukemogenesis or greatly influence the chemotherapeutic response[18].

T-cell ALL (T-ALL) is an aggressive and malignant disease of thymocytes[22], and accounts for nearly 15% of pediatric ALLs and nearly 25% of adult ALL cases[21, 23]. It arises in the thymus and can quickly spread through out the entire body. The prognosis of T-ALL has improved in that nearly 80% of children and 50% of adults are now cured with aggressive multi-agent therapies and is quickly approaching cure rates for BP-ALL[20, 21, 24]. However, long-term survival rates for pediatric T-ALL still lag behind those for BP-ALL by up to 20%[20, 21, 24], and such aggressive treatment has numerous late-in-life effects, including secondary cancers. Relapse is a very common feature of T-ALL and is one of the reasons why this subtype of ALL has an inferior clinical outcome. Relapse typically occurs in about 30% of childhood and 50% of adult T-ALL cases[25]. Relapses are the result of outgrowth of residual leukemic cells that were present below the limit of detection following induction therapy. This outgrowth can arise from the original diagnostic leukemic clone that acquired genetic abnormalities that promoted chemoresistance, or may be an entirely different clone that was already predisposed to be chemoresistant[26-28]. Regardless of how relapses occur, the prognosis of T-ALL patients with primary resistant or relapsed disease is very poor[29-32]

T-ALL is associated with far fewer genetic alterations than BP-ALL, most of which involve the juxtaposition of oncogenic transcription factors (HOX11, TAL1, LYL1, LMO1 and LMO2) to the T-cell receptor (TCR) enhancer and/or promoter elements[20-22, 24], and some gene mutations. Genetic abnormalities involving TCR genes, basic helix-loop-helix genes (TAL1, TAL2, LYL1, MYC), cysteine-rich LIM domain-

containing genes (LMO1, LMO2) or homeodomain genes (HOX11/TLX1, HOX11L2/TLX3, HOXA gene cluster) can block differentiation, thus resulting in an more immature phenotype, and promote transformation of normal thymocytes into malignant blasts[25]. Genetic mutations in key genes (CDKN2A/2B, CCND2, LCK, RAS, PTEN, ABL1, JAK2, FLT3) are believed to promote self-renewal of the malignant cells or leukemic stem-cells, alter responses to extracellular signals that allow for constitutive activation, which results in enhanced cell survival, and/or block apoptosis[9, 33] Recent studies have shown that improper activation of multiple signal transduction pathways are involved in the initiation and progression of T-ALL[34]. Some of the signaling pathways known to be involved are:

- NOTCH1
- Phosphatidylinositol 3-kinase(PI3K)-Akt and mammalian target of rapamycin (mTOR)
- Janus kinase (JAK)-signal transducer and activator of transcription (STAT)
- Nuclear factor-κB (NFκB)
- Calcineurin/nuclear factor of activated T-cells (NFAT)[34].

For example, studies have shown that T-ALLs with elevated levels of NFκB family members[35],including RelB, can promote T-cell leukemogenesis and accelerate leukemia onset and increased disease severity[36]. In another example, murine studies have demonstrated how activated calcineurin can enhance the aggressiveness of T-ALL cells and promote leukemia progression[37]. Although we do not know the exact mechanism that causes leukemic transformation, we do know that it involves a multistep process in

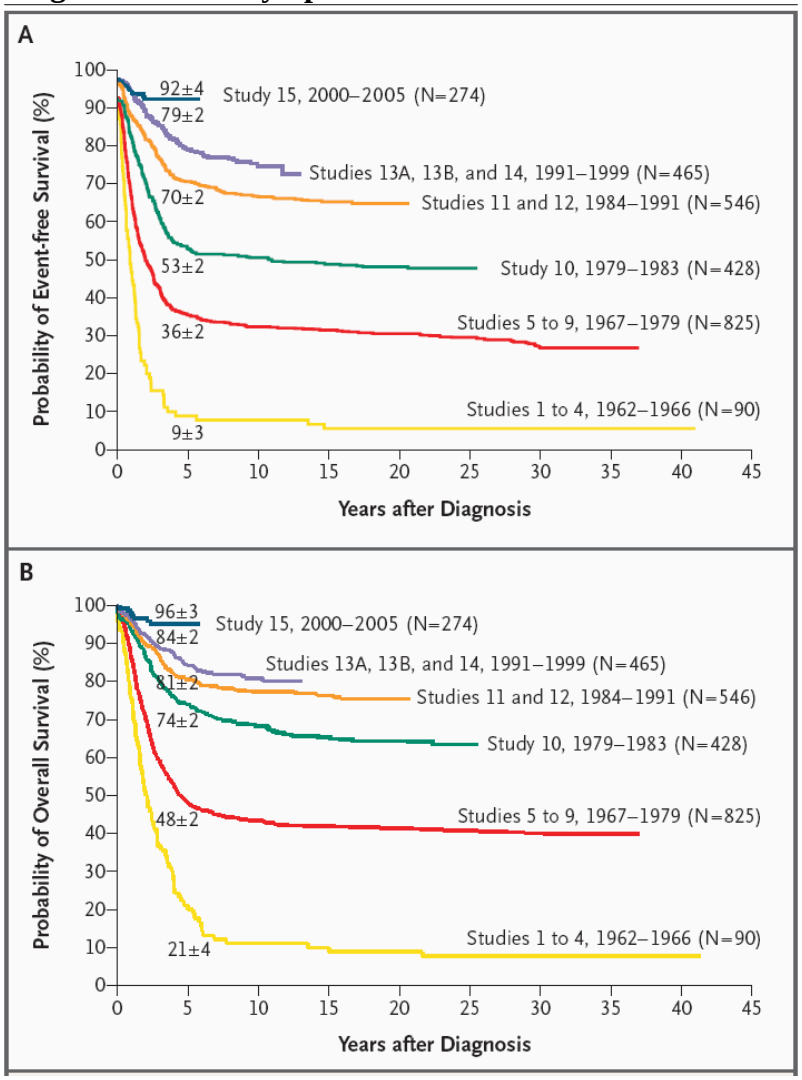
which numerous genetic alterations shift the normal thymocyte into uncontrolled growth and clonal expansion[38].

1.3 Treatment and Risk Stratification of ALL

The treatment of pediatric leukemia is an incredible success story. The treatment for ALL changed drastically when Sydney Farber, in the late 1940's, discovered folic acid given to ALL patients appeared to stimulate the proliferation of ALL[39]. Soon after this breakthrough, Farber and collaborators began synthesizing the antifolates aminopterin and amethopterin (methotrexate) and administered them to children with ALL[40]. These antifolates were successfully able to induce remission of the disease[39]. This soon lead to the discovery of other antileukemic agents in the 1950's, including 6-mercaptopurine[41, 42]. Even with these discoveries, only 5-10% of leukemia patients survived in the early 1960's[43]. It wasn't until 1965 that combinational therapy was introduced. James Holland and colleagues found that a combination of methotrexate, vincristine, 6-mercaptopurine and prednisone could induce long-term remission in pediatric ALL[44]. Today, nearly 80% of all pediatric leukemia patients are cured[19-21] (Figure 1).

The continued success of treating and curing leukemia is not the result of new and innovative drugs. Rather, it's due to better and more efficient use of existing drugs, including methotrexate, vincristine, 6-mercaptopurine and corticosteroids[43, 45]. Today's therapy, often referred to as Risk-Adaptive Therapy, is tailored to the predicted risk for relapse in each patient[19]. The intensity of treatment is based on the likelihood that patients will relapse, as leukemic relapse is the most common cause of treatment failure[46]. Patients are grouped into risk categories based on key presenting factors, which

Figure 1: Event-free Survival and Overall Survival of Children with Newly Diagnosed Acute Lymphoblastic Leukemia



These patients were apart of 15 consecutive studies at St. Jude Children's Research Hospital from 1962 to 2005. The probability of event-free and overall survival were calculated using Kaplan-Meier analysis. This figure was taken from Pui, C.H. and Evan, W.E. (NEJM; 2006)[20].

include sex, age, presenting white blood count (WBC), central nervous system (CNS) involvement status, testicular involvement, leukemia characteristics (lineage, subtype) and initial therapeutic response[19-21, 47, 48]. Initial response to glucocorticoid/prednisone treatment has been identified as a strong prognostic factor in childhood ALL[49-51]. Resistance to glucocorticoids *in vitro* is associated with an unfavorable prognosis[52, 53], as the majority of patients with relapsed ALL have increased resistance to glucocorticoid therapy[53, 54]. Another prognostic marker is the monitoring of minimal residual disease (MRD) at various times after initial induction therapy[55]. MRD tracks the clearance of leukemic cells by RT-PCR and/or flow cytometry techniques[2, 56]. Patients with MRD levels $>10^{-3}$ (i.e., at least 1 leukemic cell is detected out of every 1000 cells) are considered to be at a high risk of relapse, while patients with MRD levels $<10^{-4}$ (i.e., at most, 1 leukemic cell is detected out of every 10,000 cells) are assumed to be at a low risk of relapse[56]. Those patients that fall between the high and low levels are considered to be at a standard risk for relapse. Despite knowing prognostic predictors, treatment outcome still depends on therapy and the underlying biology of the patient and their disease[45].

ALL is a heterogeneous disease and is comprised of malignant blasts arrested at different stages of differentiation, associated with expressing characteristic markers[19-21]. As a result, risk standards for therapy are also based on immunophenotype and the absence and/or presence of genetic alterations[18-21]. There are many genetic alterations in BP-ALL that confer either a favorable or unfavorable prognosis. For example, t(12;21) in BP-ALL results in a TEL-RUNX fusion gene and protein and predicts a favorable outcome. T-ALL is associated with fewer unique features than BP-ALL upon which to

base therapy[20, 21, 24]. This is partly due to the fact that this is a relatively rare disease (only about 0.54 cases per 100,000 children per year[6]) and overall there are too few cases in which the usefulness of these biomarkers can be effectively tested. What is known is that these patients are prone to early initial relapse and inferior outcome, and as a result, their long term survival rates lag behind BP-ALL patients nearly 15-20%[20, 21, 24]. Some T-ALL subtypes have been associated with treatment outcomes. As mentioned earlier, these subtypes generally involve a translocation between the T-cell receptor (TCR) promoter and/or enhancer region and oncogenic transcription factors such as HOX11, TAL1, LYL1, LMO1 and LMO2[19-21, 24]. For example, translocations involving TAL1 and LYL1 are associated with a poor outcome, while HOX11 translocations are associated with a much more favorable outcome[22]. Some genetic mutations have been shown to be associated with treatment outcomes. For examples, activating mutations in TAL1 (~50% of T-ALLs) and mutations in LYL1 are associated with inferior outcomes and survivals[22, 57], whereas mutations in MLL (4-8% of T-ALLs) appear to have no impact on prognosis[57]. Mutations in NOTCH1 occur in over 50% of T-ALLs, and its impact on prognosis and survival has yet to be determined. This is an important focus of our study.

Patients thought to be at higher risk of relapse are treated with more intensive therapeutic regimens, and are considered candidates for allogeneic hematopoietic stem-cell transplantation[2]. Typically, older patients, more often male than female, and patients with presenting WBC > 50,000/μL are at a higher risk of relapse, and thus are treated more aggressively. Low risk patients are typically treated with antimetabolite therapies and standard risk patients are treated with intensive multiagent chemotherapies[2].

Contemporary treatment of ALL typically lasts 2 to 2.5 years, and can be divided into 3 periods, remission induction therapy, intensification (consolidation) therapy and continuation (maintenance) therapy[20]. The goal of remission induction therapy is to eliminate at least 99% of the leukemic cells and restore normal hematopoiesis[20]. During this period, patients are given glucocorticoid, vincristine and either asparaginase, an anthracycline, or both. Such treatment has been able to induce complete remission for nearly 98% of children and 85% of adult ALL patients[20]. With consolidation therapy, patients are given high doses of methotrexate with 6-mercaptopurine and high doses of asparaginase. Often, induction therapy is repeated[20]. Maintenance therapy lasts for 2 or more years, and this amount of time has been proven critical for curing the disease[20]. This therapy utilizes a combination of daily 6-mercaptopurine and weekly methotrexate at low doses. Intrathecal chemotherapy has replaced the need of cranial irradiation to prevent/eliminate CNS leukemia in standard risk patients. Radiation is only used for very high risk patients, along with allogeneic hematopoietic stem-cell transplantation[20]. While many of the same agents and principles are used throughout the world, chemotherapeutic regimens can vary substantially. Even here in the United States, treatment protocols can vary among cancer treatment centers. This is most evident by the treatment of ALL in adolescents and young adults. Depending on the treating facility, this subset of patients can be treated on either pediatric or adult protocols. Current studies are focusing on the development of molecular therapeutic agents that can target specific genetic alteration products, similar to that of imatinib targeting the BCR-ABL fusion product[9].

It is not uncommon for patients to develop resistance to chemotherapy and molecularly-targeted drugs[58-60]. Resistance may be due to (i) poor drug uptake or

enhanced drug metabolism that both result in lower, ineffective intracellular concentration of the chemotherapeutic drug[31]. Leukemic cells (ii) may acquire new genetic abnormalities that can inhibit the drug from interacting with its specific molecular target, or (iii) they just stop responding to therapy by adapting to the persistent biochemical activity of the molecules and their targeted pathways[31]. Another concern is treatment side effects. Side effects of chemotherapy (e.g., secondary leukemias, cardiomyopathy, neuropsychological impairment, infertility) may have a major impact on the quality of life of patients, years after therapy completion.

1.4 The Biology of NOTCH1

The NOTCH receptor was first discovered in *Drosophila* (dNotch) where it caused notches at the end of the wing blade[61], resulting in the partial loss of function of the wing[62, 63]. While there is only a single NOTCH protein and two ligands (Delta (D1) and Serrate (Ser)) in *Drosophila*, mammals, including humans, have 4 NOTCH proteins (NOTCH1, NOTCH2, NOTCH3 and NOTCH4) and 5 ligands[62]. NOTCH signaling is vital to the development of multicellular organisms, as it controls cell fate by regulating cell proliferation, survival and differentiation[64]. NOTCH signaling is also important in adult organisms, where it regulates stem-cell maintenance, binary cell-fate decisions (T- versus B-lymphocyte lineage), and differentiation in self-renewing organs[65]. All four human homologues of the NOTCH receptor share the same overall structure, but have slight differences in extracellular and cytoplasmic domains[62]. Some NOTCH receptors appear to have redundant functions in certain contexts (i.e. NOTCH1 and NOTCH4 in vasculogenesis), while others appear to have unique and essential functions[66]. For example, loss of either NOTCH1 or NOTCH2 is embryonic lethal in mice[67]. There are 5

human NOTCH ligands: Delta-like-1, -3, -4 (DLL1, DLL3, DLL4) and Ser-like ligands Jagged 1 and Jagged 2 (JAG1 and JAG2)[68-72]. Much like the receptor, these are transmembrane proteins that are expressed on the surface of signaling cells[73].

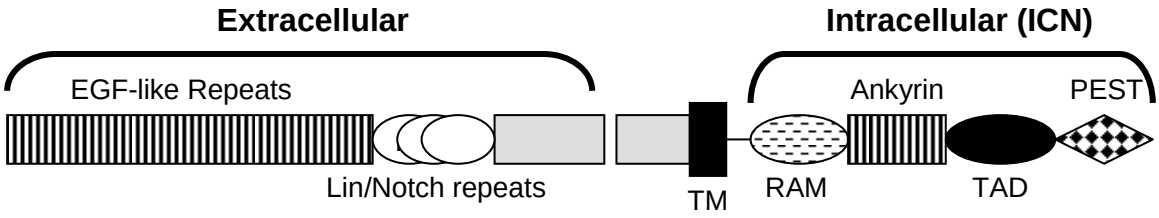
The thymus is the site of T-cell development. Progenitor cells, which are derived in bone marrow, are released into the bloodstream and travel to the thymus. Interactions with thymic stromal cells induce signals within progenitors that direct T-cell commitment, migration, proliferation and differentiation[74, 75]. NOTCH1 signaling is absolutely necessary for T-cell development. In the thymus, progenitor cells bind to NOTCH1 ligands that are embedded in the membrane of thymic stromal cells[74, 75], triggering NOTCH1 signaling and subsequent T-cell commitment. Different NOTCH1 signaling thresholds stimulate different cellular processes. Low signaling is responsible for the inhibition of B-cell development, while high signaling promotes progenitor proliferation and progression into the double positive stage[75]. Mouse models have shown that NOTCH1 inactivation results in the generation of B-cells and the inhibition of T-cell development[76, 77]. Similar studies have shown constitutive NOTCH1 signaling promotes the inhibition of B-cell development[24].

1.5 Overview of NOTCH1 Signaling

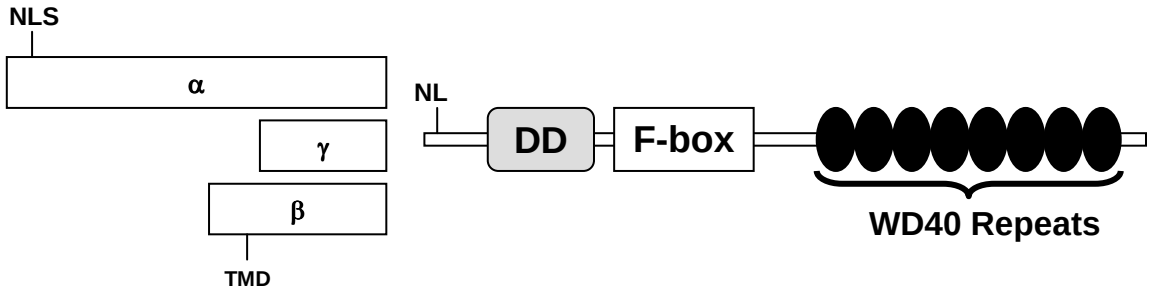
NOTCH1 is a 2550 amino acid single pass transmembrane receptor with a molecular weight of 350kDa[38, 78, 79]. NOTCH1 is comprised of 3 subunits: an extracellular (EC) subunit, a transmembrane (TM) subunit and an intracellular (ICN) subunit (Figure 2). The EC is comprised of 36 epidermal growth factor (EGF)-like repeats that bind to membrane-embedded ligands of the Delta-Serrate-Lag2 (DSL) family on neighboring cells[80-82]. There are three iterated Notch/Lin12 repeats that maintain the

Figure 2: The Structures of NOTCH1 and FBW7

A: NOTCH1



B: FBW7



(A) The heterodimeric NOTCH1 receptor consists of extracellular, transmembrane and intracellular subunits. (B) The E3-ubiquitin ligase FBW7 has 3 isoforms, a, b, and g. Abbreviations: EGF, epidermal growth factor; HD, heterodimerization; TM, transmembrane; RAM, RBP-jκ-associated molecule; TAD, transactivation domain; NLS, nuclear localization sequence; DD, dimerization domain.

receptor in the "off" state in the absence of ligand. At the C-terminus of the EC subunit, there is a 103 amino acid span that is responsible for the dimerization of the EC to a 65 amino acid region in the TM region of ICN. This dimerization is mediated via the heterodimerization (HD) domain. ICN mediates NOTCH1 signaling. It contains a RAM domain that binds to the transcription factor CSL (CBF-1/Su(H)/Lag-1), seven iterated ankyrin (ANK) repeats, and a C-terminal PEST sequence that regulates protein turnover[80-82].

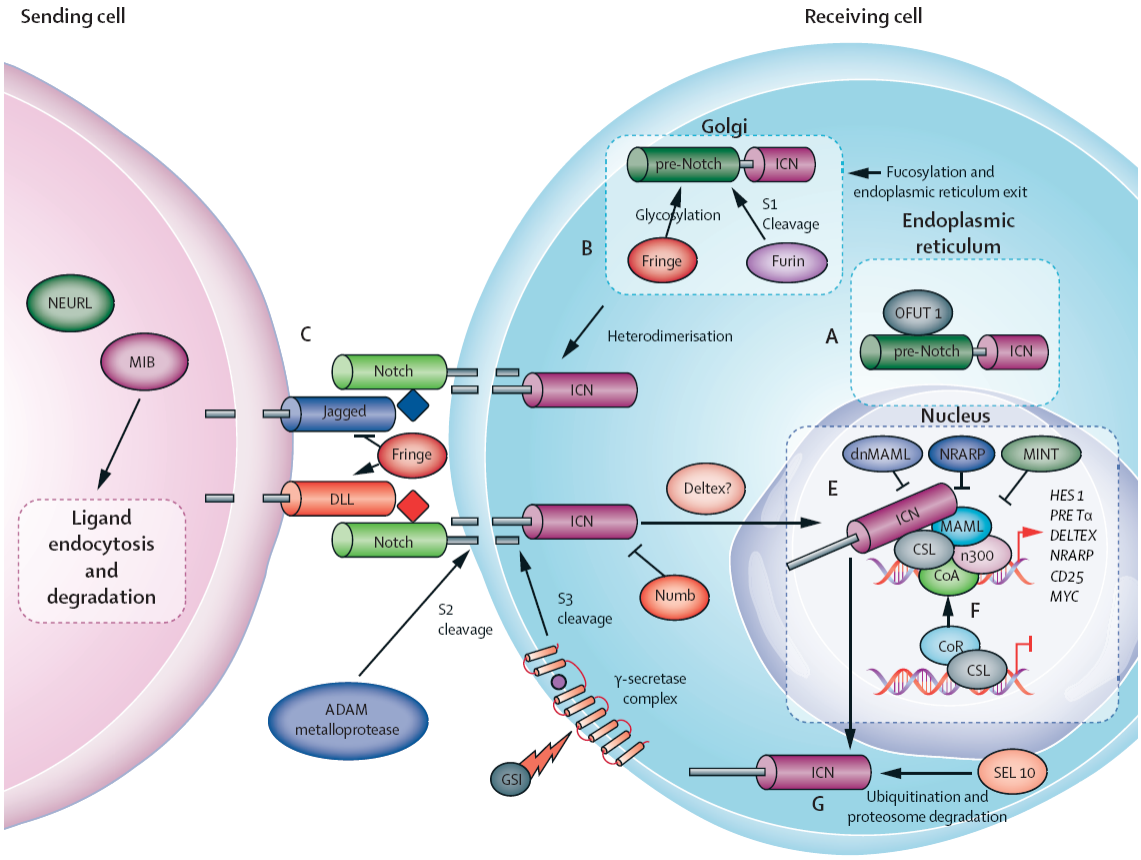
The receptor is initially translated into a single protein in the Endoplasmic Reticulum[83, 84]. Upon transport to the Golgi, it undergoes several posttranslational modifications, including cleavage by a furin-like protease and glycosylation by fringe proteins[83, 84]. The glycosylation status of the receptor determines ligand specificity[74]. The two receptor halves dimerize at the HD domain prior to insertion into the cell membrane. Following ligand binding, the receptor undergoes two additional cleavages in the TM region[85-87]. The first cleavage is carried out by an Adam protease at the cell surface, which removes the EC. The second cleavage is carried out by γ-secretase, and results in the release of ICN. The free ICN translocates to the nucleus, where it binds to CSL, converting it to a co-activator[78, 79, 85]. ICN also recruits additional co-activators, such as Mastermind-Like (MAML) and histone acetyltranferases[78, 79, 85] (Figure 3). NOTCH1 signaling is regulated by the ubiquitin/proteasome degradation pathway[88]. Itch, an E3 ubiquitin ligase, can ubiquitinate membrane-associated NOTCH1[89]. Itch can also cooperate with NUMB, another E3 ubiquitin ligase, to enhance the ubiquitination of NOTCH1, and ultimately prevent the nuclear localization of ICN, thus inhibiting NOTCH1 signaling[90].

The activated form of NOTCH1, ICN, is regulated by the tumor suppressor F-box and WD repeat domain-containing 7 (FBW7; also known as hCDC4, FBXW7, and hAGO)[91-93]. FBW7 is an E3 ubiquitin ligase and a component of SCF (SKP1, CUL1, F-box) type ubiquitin ligases[94]. It can target ICN for proteasomal degradation by binding to a conserved phosphodegron motif (CPD) in the PEST domain that is anchored by T2515[92-94]. This CPD motif in ICN is hyperphosphorylated by cyclin-dependent kinase 8[91-93, 95].

FBW7 is located on chromosome 4 and has three alternative transcripts (α, β, γ) that are the result of alternative splicing[96]. All three isoforms share the same functional domains (Figure 2). FBW7 is comprised of multiple protein-protein interacting domains. The f-box domain recruits SCF via binding to SKP1[97]. The eight WD40 repeats bind to the substrate at the CPD motif[94, 98, 99]. WD40 repeats 3 and 4 contain three highly conserved arginines that mediate substrate binding[98, 99]. The D domain, which lies in front for the f-box, regulates dimerization[98, 100-102].

Few NOTCH1 transcriptional target genes have been identified. They include regulators of apoptosis and cell cycle, including cMYC[103-105], Hairy and Enhancer of Split (HES1), HES-Related Protein (HERP) and Deltex1 (DTX1). HES1, HERP and DTX1 are all basic helix-loop-helix (bHLH) family members[106-108] that act as NOTCH1 effectors by negatively regulating the expression of downstream target genes[106, 109-111]. HES1 and HERP bind to the promoter of its target genes as a dimer with themselves or other family members[106]. HES1 has several target genes, including itself, the proneural gene Mash1, CD4, and the cyclin-dependent kinase inhibitor p21[WAF 109, 112-114]. The downstream effects of DTX1 are controversial, as this E3 ubiquitin ligase[115] has been

Figure 3: An Overview of NOTCH1 Processing and Signaling Activities



This figure was taken from Pui, C.H. *et al.* (Lancet; 2008)[9].

shown to be both a positive [116, 117] and negative regulator[118-120] of NOTCH1 signaling activity.

cMYC was first identified as a direct downstream target of NOTCH1 in a study by Weng *et al.*[105]. Expression profiling with the T6E murine T-ALL cells identified cMYC as a direct transcriptional target of NOTCH1 signaling. Palomero *et al.*[104] used expression profiling with 7 T-ALL human cell lines with constitutively active NOTCH1 that had been treated with a small molecule inhibitor of γ-secretase (GSI), called compound E, to identify 38 upregulated genes and 201 downregulated genes that included biosynthetic pathway genes. By integrating gene expression array results and ChIP-on-ChIP analysis of promoter sequences, cMYC was further identified as a major target of NOTCH1 signaling. It does appear that the downstream effects of NOTCH1 signaling are context-dependent and vary in different cell types and under different conditions[38, 78, 79]. Thus, depending on the cell type, NOTCH1 signaling can exert positive or negative effects on proliferation, differentiation and apoptosis.

1.6 Discovery of NOTCH1 in T-ALL

The role of NOTCH1 in T-ALL was first suggested when a translocation between the receptor and TCRβ was discovered. In t(7;9)(q34;134.3), the promoter region of TCRβ is fused to the intracellular subunit of NOTCH1[121] and results in constitutively active NOTCH1 signaling that alters its downstream effects on gene transcription. In mice, constitutively active NOTCH1 has been shown to inhibit B-cell development and significantly induce T-ALL[122-124]. However, t(7;9) occurs in less than 1% of T-ALL cases. In 2004, the significance of NOTCH1 in T-ALL grew considerably. Weng *et al.*[125] tested human T-ALL cell lines lacking the t(7;9) for NOTCH1 dependency by

inhibiting NOTCH1 signaling with a GSI. GSIs inhibit γ-secretase from cleaving ICN, thus preventing its translocation to the nucleus. Of the 30 T-ALL cell lines examined, only 5 (ALL-SIL, DND-41, HPB-ALL, KOPT-K1, TALL-1) showed G0/G1 cell cycle arrest, suggesting that these cells were dependent upon NOTCH1 signaling for survival[125]. Sequencing of the NOTCH1 receptor across the HD and PEST domains revealed that NOTCH1 was mutated in 4 of the 5 GSI-sensitive T-ALL cell lines, as well as in 9 of the 10 GSI-resistant T-ALL cell lines. The clinical relevance of NOTCH1 mutations was confirmed when they screened 96 pediatric diagnostic T-ALL specimens for NOTCH1 mutations in both the HD and PEST domains and found that 54 (56.2%) patients harbored such mutations. There did not appear to be any association between the presence of mutations and T-ALL subtypes, which has been confirmed by subsequent studies. Since these mutations are not present in the remission bone marrow samples of NOTCH1 mutant patients, it appears that these mutations are acquired within the malignant clones[125]. These mutations, ranging from simple point mutations to large insertions and deletions that drastically alter the amino acid sequence and can result in early termination, are found mainly in the HD and PEST domains[87, 107, 125-127], but can also be located in the juxtamembrane (JME) region[128]. Mutations in the HD domain affect dimer stability, thus making the receptor more sensitive to γ-secretase[127, 129], even in the absence of ligand. Mutations in the PEST domain increased the receptor's half-life[127], and can prevent its recognition by FBW7 and subsequent proteasomal degradation. Reporter gene assays have demonstrated that these mutations can render the receptor constitutively active[125]. However, to date, the activating effects of NOTCH1 mutations have only been confirmed in a few cases. It appears that not all mutations

effect NOTCH1 signaling in the same manner, as mutational effect have been reported as ranging from non-functional to significantly activating[127, 129].

Recent reports have also described a high frequency of mutations in the FBW7 substrate binding domain (WD40 repeats) in up to 30% of pediatric T-ALLs[91-93] that would alter NOTCH1 signaling. Such mutations prevent the binding of FBW7 to ICN and subsequent ubiquitination, thus leading to prolonged activity of ICN. Likewise, it's believed that mutations in the PEST domain of ICN lying within the CPD will prevent recognition and binding of FBW7 and also result in prolonged NOTCH1 signaling activity. Some of these studies have found patients with mutations in FBW7 and/or NOTCH1 have a more favorable outcome[91].

1.7 The Clinical Significance of NOTCH1

The mechanism in which abnormal NOTCH1 signaling is involved in the deregulation of thymocyte development and subsequent T-ALL leukemogenesis is not well understood. It's widely believed that the oncogenic effect of constitutively active ICN in T-cells is associated with its capability to promote T-cell commitment and thereafter block differentiation at the double positive ($CD4^+CD8^+$) stage. It is likely that aberrant NOTCH1 signaling cooperates with other signaling pathways to promote cell survival in hematopoietic stem cells (HSCs) and T-cell precursors[38, 130]. This uncontrolled proliferation is thought to put these cells at a higher risk of acquiring more genetic abnormalities that further promote transformation[38, 130].

1.7a The Clinical Significance of NOTCH1 in Pediatric T-ALL

Since the landmark study by Weng *et al.*[125] that first describe the presence of NOTCH1 mutations in pediatric T-ALL patients, several groups have tried to determine

Table 1: Summary of Pediatric and Adult T-ALL Studies Assessing the Prognostic Value of NOTCH1 Mutations Alone or in Combination with FBW7 Mutations.

AUTHORS	YEAR	SPECIMEN	MUTATION FREQUENCY	PROTOCOL	FINDINGS
Zhu, Y. *et al.*	2006	53 Pediatric T-ALL 24 Adult T-ALL	NOTCH1 (37.7%)	Ped: VDLP; hd MTX Adult: VDCP; hd MTX	NOTCH1 mutations were more frequent in patients with WBC count $>10x10^9$/L; Poor relapse-free survival and overall survival rate were correlated with NOTCH1 mutation; NOTCH1 mutations were significantly associated with poorer prognosis in Adult T-ALL patients.
Breit, S. *et al.*	2006	157 Pediatric T-ALL	NOTCH1 (52.2%)	ALL-BFM 2000	NOTCH1 mutant patients had an event-free survival of 90% compared with 71% in wild-type patients
Mansour, M.R. *et al.*	2006	24 Adult T-ALL	NOTCH1 (70.8%)	UKALLXII	NOTCH1 mutations may be good MRD markers.
Malyukova, A. *et al.*	2007	26 Pediatric T-ALL	NOTCH1 (30.8%) FBW7 (30.8%) Both (7.7%)	NOPHO	NOTCH1 mutations alone or in combination with FBW7 mutations show a strong association with favorable outcome.
Van Grotel, M. *et al.*	2007	72 Pediatric T-ALL	NOTCH1 (55.6%)	DCOG ALL-7, ALL-8 or ALL-9	EGIL or TCR classification subgroups are not associated with outcome. Presence of NOTCH1 mutations is not associated with disease-free survival.
Mansour, M.R. *et al.*	2009	88 Adult T-ALL	NOTCH1 (60%) FBW7 (18%) Both (21%)	UKALLXII/ ECOG2993	There is a trend towards better EFS in patients with at least 1 mutations in the Notch pathway; this is not significant
Marks, D.I. *et al.*	2009	356 Adult T-ALL	NOTCH1 (61%) FBW7 (18%) Both (3%)	UKALLXII/ ECOG2993	Patients with mutations in NOTCH1 and/or FBW7 have higher event-free survival than wild-type patients (51% vs. 27%), but this is not significant
Asnafi, V. *et al.*	2009	141 Adult T-ALL	NOTCH1 (62%) FBW7 (24%)	LALA-94 GRAALL-2003	NOTCH1/FBW7 status is a major prognostic significance and confer overall good results for T-ALL
Baldus, C.D. *et al.*	2009	126 Adult T-ALL	NOTCH1 (57%) FBW7 (12%)	GMALL 05/93 or 06/99	No significant differences were observed in the complete remission, relapse, or event-free survival rates between NOTCH1/FBW7 wild-type and mutant cases.

the prognostic value of these mutations in both pediatric and adult T-ALLs, and whether or not they could be used to stratify patients for therapy. These studies are summarized in Table 1. In 2006, Zhu *et al.*[131] published a report examining the prognostic value of NOTCH1 mutations in 24 adult and 53 pediatric T-ALL patients, who were treated with very similar protocols. In total, 29 of 77 patients had NOTCH1 mutations. These mutations were located in the HD, PEST, TAD and ANK domains. They found NOTCH1 mutations were more common in patients with presenting WBC over $10x10^9$/L. NOTCH1 mutations were also correlated with poor relapse-free survival and overall survival rates, such that the 3 year relapse free survival and overall survival rates for patients with NOTCH1 mutations were 28.8% and 31.8% respectively, significantly shorter than patients without mutations (59.8% and 71.7%; p=0.0053). However, when the patient cohort was divided by age groups, they found that in the pediatric T-ALL patients (<18 years old), there was no significant difference in survival between those patient who harbored NOTCH1 mutations and those who did not. In contrast, the adult T-ALL patients with NOTCH1 mutations had a far worse overall survival rate than those who were wild-type for NOTCH1 (p=0.0041). At least in this adult cohort, NOTCH1 mutations were associated with a poor prognosis[131].

That same year, Breit *et al.*[132] published a report that suggested NOTCH1 mutations were associated with a more favorable outcome[132]. In this study of 157 pediatric T-ALLs treated with a single protocol (ALL-BFM 2000), 52.2% of the cohort harbored NOTCH1 mutations. Nearly 62% of these mutations were novel (i.e., were different from those reported by Weng *et al.*[125]). For this cohort, treatment response was assessed by MRD measurements at 33 and 78 days after the completion of induction

therapy. At both time points, most of the T-ALL patients with a favorable MRD status ($<10^{-4}$) also harbored NOTCH1 mutations. Not surprisingly then, NOTCH1 mutations were more prevalent in prednisone good-responders than in the poor-responder group ($p=0.001$); therefore patients with NOTCH1 mutations were 3 times less likely to show a poor prednisone response. Patients with NOTCH1 mutations also showed a significantly better relapse-free survival compared with those without mutations ($p=0.004$). In this pediatric cohort, NOTCH1 mutations had favorable effects on treatment response, with a better relapse-free survival[132].

The pediatric T-ALL patients in the Zhu[131] and Breit[132] studies were treated with different treatment protocols. Both protocols used similar chemotherapeutic drugs, but the dosing schedule and amounts varied. In both protocols, induction therapy included the use of vincristine, daunorubicin (or similar analog) and L-asparaginase, followed by treatment with cyclophosphamide, cytarabine and 6-mercaptopurine. The main differences between the 2 protocols was that in the ALL-BFM 2000 protocol used by Breit *et al*. report, induction therapy included a 7-day monotherapy with orally administered prednisone and 1 dose of intrathecal methotrexate, and MRD analysis at days 33 and 78 were used for risk-adapted treatment stratification[133]. This suggests that the prognostic value of NOTCH1 mutations may be treatment-dependent. Further studies are needed to confirm this. It should also be noted, as mentioned previously, adult T-ALL patients typically have many poor prognostic markers associated with their disease. Accordingly, the presence of NOTCH1 mutations may have very little effect on outcome in these patients.

In a 2007 report, Malyukova *et al.*[91] confirmed the major findings of Breit *et al.*[132]. In this study, they analyzed a small cohort of 26 pediatric T-ALLs, who were treated with high-risk protocols prepared by the Nordic Society of Pediatric Hematology and Oncology (NOPHO). This treatment protocol included pulses of high-dose methotrexate alone, or in combination with high-dose cytosine arabinoside, in conjunction with multiple intrathecal injections of methotrexate[134]. Of the 26 patients analyzed, 8 patients (30.8%) harbored NOTCH1 mutations and 8 patients had mutations in the E3 ubiquitin ligase FBW7, which has been reported to result in elevated levels of ICN. Only 2 of the 26 T-ALL patients had mutations in both NOTCH1 and FBW7. They found that NOTCH1 mutations alone, or in combination with FBW7 mutations, were associated with favorable outcome[91].

Adding to the controversy in 2008 was a report published by van Grotel *et al.*[135] in which they found no associations between NOTCH1 mutations and treatment outcome in a sizable cohort of 72 pediatric T-ALL patients[135], all of whom were treated according to Dutch Childhood Oncology Group (DCOG) protocols. These protocols are described as being very similar to the ALL-BFM90 protocols, using high doses of intravenous 6-mercaptopurine with medium risk patients, high doses of L-asparaginase in standard risk patients, and avoidance of cranial irradiation. In this pediatric cohort, 40 of the 72 T-ALL patients (55.6%) had at least 1 mutation in NOTCH1. There was absolutely no association between the presence of NOTCH1 mutations and disease-free survival.

Collectively, the results of these studies of pediatric T-ALLs suggest that the presence of NOTCH1 mutations may not be a reliable prognostic indicator and that its ability to predict prognosis may rely on other factors, including initial response to

therapy, which in and of itself is highly dependent upon the biology of the disease. However, caution must be taken when comparing these reports head-to-head, as the pediatric cohorts were treated with different therapeutic regimens and not all patients shared that same risk for relapse. It is very likely that the chemotherapeutic protocol can influence the prognostic value of NOTCH1 mutations. Future studies are warranted in which NOTCH1 mutations are analyzed in patient cohorts treated with very similar chemotherapeutic protocols. What we do know is that NOTCH1 mutations play both an initiating role in the leukemogenic process, as mutations have been identified in a T-ALL patient at both diagnosis and 7 years before the development of full blown leukemia[136], and a secondary role in disease progression, as mutations have also been identified in subclonal populations[137] that can eventually lead to relapse.

1.7b The Clinical Significance of NOTCH1 in Adult T-ALL

Similar studies focusing on the prognostic value of NOTCH1 mutations were conducted with adult T-ALL cohorts. As noted above, adult T-ALL is considered to be more aggressive and has much more dismal overall survival rate than pediatric T-ALL. In 2006, Mansour *et al.*[138] in a cohort of 24 adult T-ALL patients treated on the MRC UKALLXII trial, identified 17 patients (70.8%) who harbored NOTCH1 mutations. As had been reported in the pediatric T-ALL, the NOTCH1 mutations were not detected in remission. Thus, NOTCH1 mutations may be good markers for MRD detection. In another study, Mansour *et al.*[139] analyzed the presence of NOTCH1 mutations in 88 adult T-ALL patients. While 53 patients (60%) had NOTCH1 mutations, they were evenly distributed between the standard- and high-risk groups. There was a trend towards better event-free survival (EFS) and overall survival (OS) rates in patients with NOTCH1

mutations compared those who were wild-type (51% vs. 27% (EFS) and 54% vs. 41% (OS)), but this trend was not significant (p=0.1 and p=0.3).

In a study by Asnafi *et al.*[140], of the 141 adult (>15 yrs old) T-ALL patients treated with either the Lymphoblastic Acute Leukemia in Adults (LALA)-94 (87 patients) protocol or the pediatric-inspired Group for Research on Adult Acute Lymphoblastic Leukemia (GRAALL)-2003 (54 patients) protocol, NOTCH1 mutations were identified in 88 (62%) cases and FBW7 mutations were present in 34 cases (24%). Overall, 101 cases were classified as NOTCH1 and/or FBW7 mutant (70%). There was a trend for a higher WBC count and more frequent CNS involvement in patients with wild-type NOTCH1. This is likely due to the fact that high risk features, such as age over 35 and WBC >100 g/L, were found much more frequently in the NOTCH1/FBW7 wild-type patients (72.5% vs. 55%; p=0.085). The complete remission rate was similar in patients with NOTCH1 mutations as compared to WT NOTCH1 patients. However, the median EFS was significantly less in the wild-type patients (22 months vs. 36 months; p=0.03). Interestingly, the median overall survival was 38 months for wild-type patients, but had not yet been reached for the mutant NOTCH1/FBW7 patients (p=0.03) at the time this report was published. This study suggests that NOTCH1/FBW7 status has significant prognostic value in modern trials and gives overall good results for adult T-ALL.

In another report, Baldus *et al.*[141] analyzed the prognostic value of NOTCH1 and FBW7 mutations in 126 adult T-ALL patients on the GMALL 05/93 and 06/99 protocols. Both of these protocols included intensive chemotherapy and autologous or allogeneic stem cell transplantation. NOTCH1 mutations were identified in 72 of the 126 (57%) patients, and FBW7 mutations were found in 14 of the 112 patients (12%). It was

observed that the wild-type NOTCH1/FBW7 patients predominantly exhibited an immature double-negative phenotype that was defined by a lack of CD1a, CD4, CD8 and CD3 expression. CD1a expression was highly indicative of presence of NOTCH1 mutations, suggesting that this may be a direct target of NOTCH1 signaling ($p<0.001$). There was no significant difference observed in the complete remission, relapse or EFS rates between wild-type NOTCH/FBW7 and mutant NOTCH1/FBW7 patients, suggesting that there is no prognostic value for NOTCH1/FBW7 mutations.

In a large study by Marks *et al.*[142], the presence of NOTCH1 and FBW7 mutations was analyzed in 356 adult T-ALL specimens. These patients were treated on the UKALLXII/ECOG2993 protocol, the same as the Mansour *et al.*[139] study. NOTCH1 mutations were present in 61% of the cohort and FBW7 mutations were seen in 18% of the patients. Only 3% of the cohort had mutations in both NOTCH1 and FBW7. It was observed that patients with NOTCH1/FBW7 mutations had a higher EFS when compared to wild-type patients (51% vs. 27%), however this was not significant. Nearly 37% of the cohort experienced a relapse that occurred at a median of 12 months, with the majority arising within 2 years of remission. The overall 5-year survival rate for this cohort was 48%. It was observed that the overall survival in CD1a+ patients was 64%, compared 30% in CD1a- patients. This suggests that CD1a status may be a prognostic indicator.

Much like the pediatric studies, the adult T-ALL studies do not give any definitive evidence to the prognostic value of NOTCH1 mutations. Again, this could possibly be due to the fact that in these studies, patients were treated with different chemotherapeutic protocols. With reports from Marks *et al.*[142] and Mansour *et al.*[139] , where the patients were treated with the identical protocol, they could only conclude that there was a trend

towards better survival in patients with NOTCH1/FBW7 mutations. Thus, the prognostic value of NOTCH1 mutations in both pediatric and adult T-ALLs remains extraordinarily controversial.

1.7c The Clinical Significance of NOTCH1 in Solid Tumors

Aberrant NOTCH signaling has been implicated to play a role in the biology of solid tumors as well. However, unlike T-ALLs, abnormal NOTCH signaling is not caused by mutations to the receptor. Instead, it's due to increases in expression for both NOTCH1 ligands and receptors. For example, an upregulation of Jagged1 mRNA has been observed in pancreatic cancer[143] and the over expression of Jagged1 protein has been reported in cancers of the prostate[144], cervix[145] and brain[146, 147]. The upregulation of Jagged2 mRNA has also been observed in cervical cancer[145], along with Jagged2 protein over expression reported in pancreatic cancer[143]. *DLL1* mRNA has been reported as being over-expressed in both cervical[145] and brain cancers, where proteins levels are elevated as well[147]. There is an increase in expression of NOTCH1, at the protein level, in cancers of the cervix[145, 148], colon[148], lung[148], pancreas[143], skin[149] and brain[146, 147]. The role of NOTCH1 signaling has been most extensively studied in breast cancers. In a clinical study of 7 breast cancer specimens, NOTCH1 protein expression was detected to a greater extent in all the tumors examined but not in the normal breast tissue at the margins of the tumors[150]. In another study of 25 breast cancer specimens, the mRNA expression of all 4 NOTCH receptors were detected at varying frequencies[151]. In a subsequent study involving 97 specimens, elevated NOTCH1 protein levels were associated with reduced patient survival[152]. It's estimated that more than half of all human breast tumors express reduced protein levels of Numb, a negative regulator of

NOTCH signaling and that a negative correlation exists between Numb expression and breast tumor grade[153].

1.8: Therapeutic Targeting of NOTCH1

NOTCH1 is believed to be an ideal target for therapy because it's mutated form is generally considered to increase the overall activity of the receptor in >50% of T-ALLs and it can easily be inhibited by small molecule inhibitors, called γ-secretase inhibitors (GSIs), which prevent the cleavage of the intracellular form, an essential requirement for activity. GSIs have been shown to induce cell-cycle arrest at G0/G1, decrease cell viability and cause some apoptosis in a subset of T-cell lines carrying NOTCH1 activating mutations[125, 154]. The problem with GSIs is that they are not specific to NOTCH1, as γ-secretase targets over 30 other transmembrane proteins, one of which is the amyloid precursor protein involved in Alzheimer's disease[155]. Animal studies had shown that systemic inhibition of NOTCH signaling results in gastrointestinal toxicity because of the accumulation of secretory goblet cells in the intestine[156-159]. Initial clinical trials using GSIs have failed miserably[160, 161]. A Phase 1 clinical trial conducted by the Dana-Farber Cancer Institute (04-390) used the GSI MK-0725 for 7 patients with relapsed T-ALL. This trial revealed that GSIs caused severe, dose-limiting, gastrointestinal toxicities[160, 162]. Similar gastrointestinal toxicities have been seen in the GSI clinical trials for Alzheimer's disease, along with skin and immune system abnormalities[157, 159, 163]. The T-ALL clinical trials also suggested that GSIs may be more cytostatic than cytotoxic in humans as they were unable to induce significant apoptosis in T-ALL leukemic blasts in patients[125, 164]. Despite these clinical findings, inhibition of NOTCH1 signaling has been reported to exert a profound effect on the regulation of T-

ALL lymphoblasts[104, 165, 166], suggesting that GSIs may sensitize T-ALL cells to chemotherapy[167]. One characteristic that makes GSIs potentially attractive therapeutic agents is that they can penetrate the blood-brain barrier, as is evident from the Alzheimer's disease studies[167, 168]. Accordingly, if such molecules can be safely and effectively used in combinational chemotherapy, they may aid in the elimination of CNS-sequestered T-ALL.

To further explore the sensitizing of T-ALL to chemotherapy by GSIs, Real *et al.*[169] examined the effects of GSIs in combination with glucocorticoids in T-ALL cells. Using a glucocorticoid resistant T-ALL cell line (CUTLL1) and primary pediatric T-ALL specimens, they found that GSIs could inhibit NOTCH1 signaling, render the cells more sensitive to glucocorticoids, and result in glucocorticoid-induced apoptosis. Thus, it appears that the use of GSIs can reverse glucocorticoid resistance. Interestingly, this effect was specific to glucocorticoids as GSIs did not sensitize T-ALLs to etoposide, methotrexate, vincristine and L-asparaginase. This reversal of glucocorticoid resistance is likely due to the increased expression of the glucocorticoid receptor (NR3C1) and glucocorticoid-regulated genes upon NOTCH1 inhibition[169]. Real *et al.* demonstrated the NOTCH1 target, HES1, can bind to the glucocorticoid receptor promoter and inhibit its expression. Thus, upon NOTCH1 inhibition, there is a decrease in the expression of HES1, thereby releasing the negative transcriptional regulation of the glucocorticoid receptor. *In vivo* studies validated the effectiveness of the combination of GSIs and glucocorticoids. Also surprising was that glucocorticoid treatment seemed to decrease the gastrointestinal toxicities associated with GSI usage.

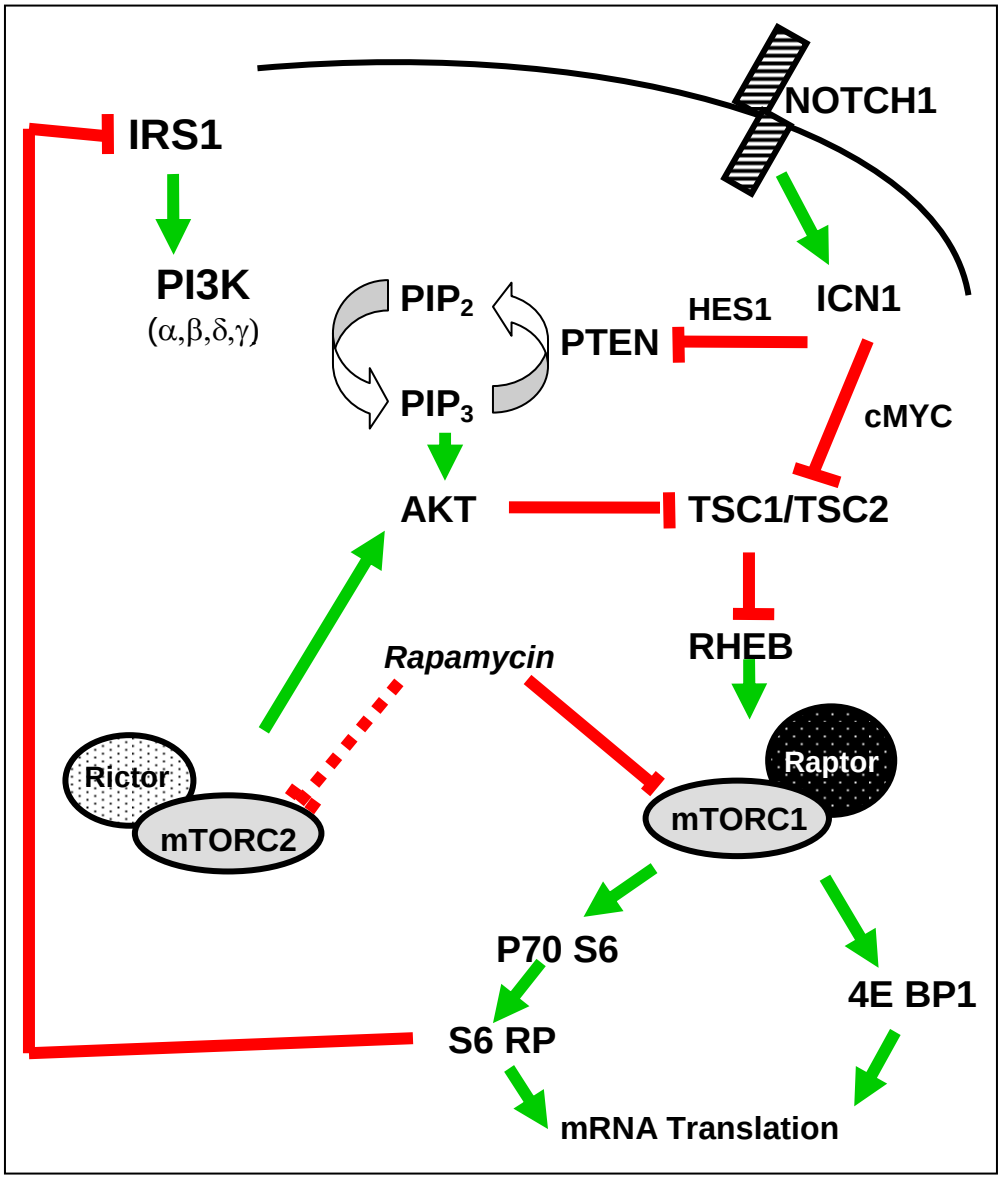
In a study by Cullion *et al.*[170] mice with end-stage T-ALL were treated with the GSI MRK-003, and it appears that this extended the life of these mice but did not cure the disease. Cells isolated from GSI-treated mice exhibited increased mTOR activity, which can promote cell survival (see below for more on mTOR). These results imply that mTOR inhibitors may synergize with GSIs. In fact, the treatment of mouse T-ALL cells with both GSI and the mTOR inhibitor rapamycin resulted in an escalation of apoptosis[170]. The combined treatment of GSIs and rapamycin decreased the proliferation of the leukemic cells and increased overall survival of the mice. This study provides further evidence that NOTCH1 inhibition, possibly through the use of GSIs, is a potential therapeutic option. Obviously, further studies are needed.

1.9 The PI3K-Akt and mTOR Pathways and Their Involvement in T-ALL

It has been suggested that the constitutive expression of oncogenic responder genes (such as MYC) and the activation of other signaling pathways (PI3K/Akt/mTOR) may account for how NOTCH1 drives the pathogenesis of T-ALL[25]. NOTCH1 signaling has been implicated to play a direct role in both the PI3K-Akt and mTOR pathways (Figure 4; discussed in more detail below). These two highly intertwined pathways are linked to cell survival and proliferation. The pathways are stimulated by the activation of the phosphatidylinositol-3-kinases (PI3Ks) by receptor tyrosine kinases. The PI3Ks function as a heterodimer, consisting of a catalytic subunit and a regulatory subunit. There are 2 subclasses of PI3Ks, class 1A and class1B. Class 1A PI3Ks consists of the catalytic subunits PIK3CA (p110α), PIK3CB (p110β) and PIK3CD (p110δ), along the regulatory subunits PIK3R1 (p85α, p55α, p50α), PIK3R2 (p85β) and PI3KR3 (p55γ)[171]. PIK3CA and PIK3CB are expressed ubiquitously, while PIK3CD is expressed mainly in

leukocytes. PIK3R1 (p85a) and PIK3R2 are also widely expressed in most cell types, while the other isoforms have a more limited expression[171]. PIK3CG is the only catalytic member of class IB PI3Ks, and is expressed mainly in leukocytes[171]. The PI3K heterodimers phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). The accumulation of PIP3 on the cell membrane recruits both Akt and phosphoinositide-dependent protein kinase 1 (PDK1), and leads to the phosphorylation of AKT on T308[172]. Once Akt is phosphorylated at S473 by mTOR2, it is fully active[173]. It is the downstream effects of Akt that promote cell survival and activation (cell growth, increased glucose uptake and oxidation, cell cycle progression and cell survival through multiple direct and indirect mechanisms[174-179]). Akt can also phosphorylate tuberous sclerosis 2 (TSC2), disrupting the tuberous sclerosis 1 (TSC1)-TSC2 complex, thus releasing the inhibition of a Ras homologue enriched in brain (RHEB),which can, in turn, activate mammalian target of rapamycin (mTOR1)[180]. cMYC, a downstream target of NOTCH1 has also been shown to inhibit TSC2, leading to the activation of mTOR1[181]. mTOR1 is a complex comprised of the catalytic mTOR subunit, raptor and mLST8[182]. mTOR1 can stimulate the synthesis of proteins needed for cell growth, survival and metabolism by directly phosphorylating and activating ribosomal S6 kinase 1 (S6K1), and eukaryotic translation initiation factor 4E binding proteins (4EBP)[182, 183]. S6K1 has been shown to be involved in a feedback loop for the PI3K-Akt pathway by inhibiting insulin receptor substrate 1 (IRS1), which in turn inhibits the initial activation of AKT[182]. Little is known about the regulation of mTOR2. It too is a complex comprised of the catalytic mTOR subunit and mLST8, but it also

Figure 4: The Role of NOTCH1 Signaling in the PI3K-Akt/mTOR Pathways



Loss of PTEN, either by inhibition of by HES1, mutations, or posttranslational modifications, results in hyperactivated AKT and mTOR signaling. Rapamycin is an inhibitor of mTOR1, and results in the inhibition of cell growth and cap-dependent translation. In certain contexts, mTOR2 may also be inhibited. Repression of TSC2 transcription by cMYC results in increased mTOR1 activity.

contains rictor and mSin1[182]. The mTOR2 complex is directly involved in the PI3K-Akt pathway by its phosphorylation of Akt at S473, leading to its full activation.

The tumor suppressor PTEN (phosphatase and tensin homolog) is a plasma-membrane lipid phosphatase that negatively regulates the activity of the PI3K-Akt pathway[184]. It has been described as an indirect target of NOTCH1 (via HES1 and cMYC), resulting in increased PI3K-Akt signaling[166] (Figure 4). When PTEN is rendered non-functional, whether by deletion, mutation or posttranslational modifications (see below), subsequent inactivation of PI3K targets (mainly Akt) can occur in the absence of stimuli[175]. Numerous tumor types can be linked to alterations in PTEN expression[185], including homozygous and heterozygous somatic mutations[186-188]. Palomero *et al.* found 17% of T-ALL cases at diagnosis harbored complete loss of the PTEN protein[166], with 8% of the T-ALL specimens harboring PTEN mutations. In a small number of paired diagnostic and relapse samples, relapse specimens had loss of PTEN, suggesting that the loss of PTEN is associated with tumor progression in T-ALL[166].

PTEN defects in mouse models recapitulate the broad tumor spectrum often seen in humans, including T-cell malignancies[189, 190]. Several human T-ALL cell lines lack PTEN as a result of deletions or mutations to the gene[191, 192]. As expected, such alterations result in the constitutive activation of the PI3K-Akt pathway. Studies have also shown that the activity of PTEN can be down regulated by post-translational activities such as phosphorylation and oxidation[193-196] and also by the activities of miR19[197]. In a study by Silva *et al.*, it was found that nearly 88% of patients in a cohort of 24 T-ALLs had hyperactivation of the PI3K-Akt pathway[198]. While some specimens

had alterations in their PTEN coding sequence and some had an overall lack of PTEN expression, a vast majority of the specimens had wild-type PTEN protein expression in conjunction with hyperactivation of PI3K-Akt pathway. It was determined that PTEN activity was downregulated by casein kinase 2 (CK2)-mediated phosphorylation and ROS-dependent oxidation of PTEN[198]. This suggests that the potential impact of PTEN in T-ALL and on chemotherapeutic response in this disease is immense. It has also been shown that leukemia cells over-express CK2[198], and that both wild-type and mutant NOTCH1 T-ALL specimens have significantly higher PTEN protein levels than normal human thymocytes. There is experimental evidence that the treatment of T-ALL cell lines with GSI and CK2-specific inhibitors have a mild but consistent cooperative effect in diminishing leukemia proliferation[198, 199]. This suggests that the combination of GSIs with CK2 inhibitors, or even Akt inhibitors may be beneficial in the treatment of T-ALL. There are several other potential mechanisms in which the activity of PI3K-Akt pathway can be inhibited in combination with GSIs. They include pan- and isotype-specific inhibitors of the PI3Ks, as well as Akt inhibitors, some of which have begun clinical trials[172]. One of the major downstream effectors of Akt signaling is mTOR1, which can be effectively inhibited by rapamycin. As mentioned previously, mouse studies have shown that GSIs may synergize with rapamycin to induce apoptosis in T-ALL[170].

In a more detailed study, Silva *et al.*[199] found that in the 9 patients with NOTCH1 mutations (out of a cohort of 19 pediatric T-ALLS), there were significantly elevated PTEN mRNA levels ($p=0.021$) and lower PTEN protein levels. The use of GSIs resulted in an up-regulation of PTEN protein expression. Palomero *et al.* demonstrated that growth arrest induced by GSI treatment of T-ALL cell lines was similar to the growth

defect caused by nutrient deprivation, cytokine withdrawal and inhibition of the PI3K pathway[166]. Both of these studies provide further evidence linking NOTCH1 signaling to the PI3K-Akt pathway. The Palomero report[166] further demonstrated that both HES1 and cMYC can bind to the PTEN promoter in T-ALL cells. HES1 reduces the activity of PTEN promoter, while MYC can cause a moderate increase in PTEN promoter activity. However, it's believed that the MYC induction of PTEN expression is overridden by the activity of HES1. The combined effects of HES1 and MYC downstream of NOTCH1 signaling in T-cell progenitors is thought to increase the activity of the PI3K-Akt signaling pathway in response to extracellular stimuli and to promote cell growth without inducing full oncogenic activation of Akt[166]. However, inactivation of PTEN, either by mutations or posttranslational modifications, uncouples the PI3K-Akt pathway from extracellular signals, bypassing the requirement for NOTCH1 signaling to maintain cell growth[166].

It is believed that the loss of functional PTEN, as the result of mutations or posttranslational modifications, may contribute to the GSI-resistance seen in some human T-ALL cell lines and primary specimens[166]. The overall lack of response following GSI treatment is not due to GSI inactivity, because these treatments can still block γ-secretase activity in GSI-resistance T-ALL cell lines[166]. As mentioned above, these GSI-resistant cells lines typically have decreased expression of PTEN[166] or the functional activity of PTEN is loss due to posttranslational modifications[198], which results in the constitutive activation of AKT and appears to be sufficient to relieve the decrease in cell growth caused by GSI treatments[166]. Further, shRNA knockdown of PTEN in GSI-sensitive/PTEN positive cells could induce GSI resistance. However, this may not be the

only mechanism for GSI resistance. Medyouf *et al.*[200] reported that the association between PTEN loss and GSI-resistance could not be detected in both murine leukemias on PTEN null and wild-type PTEN backgrounds and primary human T-ALL samples. They suggest that the GSI-resistance seen in human T-ALL cell lines may be due to the fact that these cell lines were developed from relapsed T-ALLs which that may have acquired other mechanisms (genetic alterations caused by extensive chemotherapy) to induce resistance[200]. It remains unknown what exactly induces a GSI-resistant phenotype, but it's speculated that this may not occur very frequently in the clinic[200].

1.10 The Role of microRNAs in ALL

Recent studies have highlighted the increasing complexity of transcriptional regulation with the discovery of microRNAs (miRs). miRs are small RNA species (18-22 nucleotides long) that mediate the expression of target genes with complementary sequences in their 3'untranslated regions (UTRs)[201]. miRs are initially transcribed into primary transcripts in the nucleus[202, 203]. These pri-transcripts can be polycistronic in that they encode more than one miR. The pri-microRNA is processed into a 60-70 nucleotide pre-microRNA transcript by Drosha[204]. The pre-miRNA is then transported to the cytoplasm where it is further processed by Dicer into its mature 22 nucleotide form[201, 205]. The mature miR acts mainly through translational repression[206, 207], but may have transcriptional effects, as well. It binds to complementary target sequences in the 3'UTR of mRNAs and prevents/disrupts translation[208]. While a single miR can have several hundred downstream targets, a single gene can also harbor binding sites for multiple miRs[208-210].

Altered expression of a limited number of miRs has been found in some cancers[211-214]. Differential expression of miRs can be used to distinguish mechanisms of transformation or tumors of different developmental origins[215]. In general, tumors and cancer cell lines typically have lower expression of miRs[215]. It is believed that miRs function to regulate and prevent cell division and drive terminal differentiation[215]. For example, miR expression profiling of CLL patients demonstrated that the expression levels of certain miRs could distinguish between cases of CLL with high and low expression of ZAP-70 and from those with different mutational status of IgVH[216]. Nearly 65% of the cases had deletions in hsa-miR-15a and hsa-miR-16-1, both which have been shown to down regulate Bcl-2[208, 217]. A followup study by Calin *et al.* of 94 CLL patients identified a 13-gene miR signature that was prognostically significant[216]. The involvement of miRs in the biology and therapy of T-ALL is poorly understood. It remains unknown what the total impact of miR expression profiling will be on the prognosis and treatment of T-ALL.

1.11 Significance of this Study

The long term survival rate for T-ALL patients typically lags behind BP-ALL patients nearly 20%. This is likely due to the fact that there are few prognostic markers associated with T-ALL on which to base chemotherapy on. NOTCH1 has been implicated as a biomarker with the potential to be a prognostic marker. However, there is still much unknown about the biology of NOTCH1 and its downstream targets, and their roles in T-ALL etiology and therapy. This study aims to help determine the overall prognostic value of NOTCH1 mutations alone, or in combination with mutations in other key T-ALL genes, FBW7 and PTEN. We will also shed light on whether NOTCH1

mutations are truly ‘gain-of-function’, as has been previously suggested by the study of a small number of NOTCH1 mutations. By fully understanding the biology of NOTCH1 signaling in T-ALL, especially its downstream effects, we may begin to identify new targets that could be prognostically and therapeutically important. Studies such as ours can aid in identifying new NOTCH1 therapeutic targets and/or give rise to knowledge for better usage of existing small molecule inhibitors for NOTCH1 in combination with downstream pathways. Lastly, NOTCH1 signaling is not limited to pediatric T-ALL patients. Our studies will certainly be applicable to NOTCH1 signaling in adult T-ALL. NOTCH1 signaling has also been implicated in other cancer types, including both breast and prostrate cancers. Thus, results of our research into NOTCH1 signaling in pediatric T-ALLs may also impact the understanding of the biology and therapy of these cancers as well.

CHAPTER 2

THE PROGNOSTIC VALUE OF NOTCH1, FBW7 AND PTEN MUTATIONS IN PEDIATRIC T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

2.1 Introduction

NOTCH1 signaling is involved in key cellular processes (such as cell fate) by regulating proliferation, survival and differentiation[24], and is crucial for T-cell commitment in early lymphoid progenitors. Studies have shown that constitutive activation of NOTCH1 inhibits B-cell development and is a potent inducer of T-ALL in murine models[24, 79]. The initial oncogenic properties of NOTCH1 were first discovered by the identification of t(7;9)(q34;134.3). This translocation fuses the C-terminal portion of NOTCH1 to the promoter/enhancer region of TCRβ, resulting in constitutively activated NOTCH1 signaling in the absence of any ligand. However, this translocation occurs in less than 1% of all T-ALL cases.

In 2004, Weng *et al.* discovered activating NOTCH1 mutations in more than 50% of pediatric T-ALL patients. Such mutations were confined to 'hot-spot' regions within the HD and PEST domains. These mutations are described as 'gain-of-function' mutations, as they are believed to result in constitutively active ICN and increase the activity of NOTCH1 signaling[125]. Mutations within the HD domain destabilize the heterodimerization of the receptor, making it more susceptible to cleavage by γ-secretase[125, 127, 218], whereas mutations in the PEST domain increase the half-life of ICN and reduce protein turnover[125, 127, 218, 219]. These mutations are unlikely to be all 'gain-of-function' mutations. In the 2004 report[125], single L to P amino acid substitutions in the HD domain at positions 1575, 1594 or 1601 yielded a 3- to 9-fold increase in reporter

activity. A PEST domain deletion at position 2471 yielded a 1.5- to 2-fold increase in reporter activity. Even more interesting was the 20- to 40-fold increase in reporter activity seen when each of the above HD mutations were in *cis* with the PEST domain deletion. Collectively, these results suggest that not all NOTCH1 mutations are activating and in the same degree.

NOTCH1 mutations are considered to be ideal biomarkers because they are acquired in malignant blasts and disappear upon remission[131]. However, the prognostic value of these mutations remains controversial. Some reports claim that NOTCH1 mutations are associated with favorable prognosis, while other reports claim these mutations indicate an unfavorable prognosis[91, 131, 132, 139, 140, 142]. It also remains unclear what role NOTCH1 mutations may play in the development of relapse. Other factors such as FBW7 mutations, which result in prolonged NOTCH1 signaling, can have a significant impact on the role of NOTCH1 signaling in progression of T-ALL and its therapeutic response.

PTEN inactivation, whether by mutations or posttranslational modifications, likely contributes to T-ALL development and progression and influences overall responses to chemotherapy. Inactivation of PTEN, by homozygous deletion or mutations in many tumors, results in constitutive Akt signaling, inhibition of TSC1/TSC2[182], and consequent mTOR1 activation. Results from cancer cell lines and tumor xenografts establish a strong association between the losses of PTEN function and the antiproliferative effects of the macrolide rapamycin, an effective inhibitor of mTOR1[182, 220]. Losses of PTEN are associated with the pathogenesis of T-cell tumors based on PTEN knockout mouse models[190, 221, 222]. Conditional PTEN deletions in mouse hematopoietic stem cells lead to

myeloproliferative disorder, followed by T-ALL[223]. Recent studies indicate that PTEN mutations and losses of protein occur at high frequencies in primary T-ALLs with no associations with T-ALL oncogenic subgroups[166]. In T-ALL cell lines, PTEN mutations were associated with increased Akt phosphorylation and GSI resistance[166]. To date, the prognostic value of PTEN mutations in T-ALL has not been studied extensively.

Relapse is the most common cause of off-therapy events and can account for nearly 90% of treatment failures in ALL[105]. The rate of relapse is dependent upon the immunophenotypic subtype, genetic subtype and/or other risk classifications of ALL[47, 48, 224]. Relapse typically occurs within the first 3-5 years following diagnosis but can also arise 10 or more years post diagnosis[2]. Relapse can arise from the outgrowth of residual leukemic cells that escape initial chemotherapy and are below the limit of detection at the time remission was declared, or very rarely relapse can result from a new secondary leukemia that may or may not be a direct side effect of chemotherapy. Relapse typically occurs in the bone marrow and/or extramedullary tissues, including CNS and testis[2]. Extramedullary relapse is thought to arise from leukemic cells that are 'hidden' from chemotherapy in sanctuary sites[225], whereas bone marrow relapse essentially develops in much the same way as the initial leukemia. The bone marrow is the most common site of relapse and has the worst prognosis[226]. It is generally accepted that relapsed ALL is morphologically and immunophenotypically similar to diagnostic ALL[2], although relapsed disease may also exhibit new genetic abormalities[227]. It is believed that relapse arises from (i) induction of resistance via acquisition of new genetic alterations after diagnosis, (ii) selection and expansion of an already present resistant sub-population at diagnosis, or very rarely as (iii) a secondary, *de novo* ALL[26-28]. A more comprehensive

understanding of the etiology of relapse may lead to better therapeutic strategies that may prevent relapse from occurring.

Studies in this chapter were designed to explore the potential prognostic significance of NOTCH, FBW7 and PTEN mutations, individually and in combination, and what implications these may have on the development of relapse. They also address the functional activity of NOTCH1 and FBW7 mutations (both individually and in combination) *in vitro* to determine if they are indeed activating, and *in situ* to assess the net downstream effects of activated NOTCH1 signaling resulting from these alterations in cells.

2.2 Materials and Methods

2.2a Patient Specimens

2.2a i Patient Specimens for Prognostic Studies

Forty-seven T-cell ALL patient specimens (including 24 patients who did not fail treatment [‘not failed’] and 23 patients who failed treatment [‘failed’]) were obtained from the Children’s Oncology Group (COG) ALL cell bank and used for this study. Patients were treated on Pediatric Oncology Group (POG) protocols including POG 8704 (14 failed, 14 not failed patients), 9086 (4 failed, 3 not failed patients), 9295 (1 failed patient), 9296 (2 failed, 1 not failed patient), 9297 (2 failed, 3 not failed patient) and 9398 (1 failed, 2 not failed patients). Patients in the ‘not failed’ group were children who remained in remission for 4 or more years following diagnosis, and patients in the ‘failed’ group were children who suffered bone marrow relapses within 4 years of diagnosis. Major chemotherapy drugs used were L-asparaginase, doxorubicin, 6-mercaptopurine,

methotrexate, prednisone and vincristine. Patients who died in remission within 4 years of diagnosis were excluded from this study.

2.2a ii Patient Specimens for Relapse Studies

Paired diagnostic and relapsed bone marrow aspirate slides and/or cryopreserved cells from 11 T-ALL pediatric specimens were obtained from Children's Hospital of Michigan. All relapses occurred in the bone marrow. This study included patients who died during relapse.

2.2b Amplifying and Sequencing Mutations

2.2b i Amplifying and Sequencing Mutations in Prognostic Studies

Sample handling and data analysis protocols were approved by the Committee on Investigation Involving Human Specimens at Wayne State University. Leukemic blasts were purified by standard Ficoll-Hypaque density centrifugation. Total RNAs were extracted from primary ALL lymphoblasts using the RNEasy Midiprep Kit (Qiagen; Valencia, CA). cDNAs were prepared from 1μg RNAs using random hexamers and a RT-PCR kit (PerkinElmer; Boston, MA), and purified with the QIAquick PCR Purification kit (Qiagen).

Mutations in NOTCH1 (HD and PEST domain) and PTEN (entire coding sequence) were identified in cDNAs by nested PCR methods. Primer sequences and PCR conditions are summarized in Table 2. FBW7 mutations were identified either in cDNAs or in genomic DNAs by amplifying sequence including exons 11, 12 and 14, previously reported as mutational 'hotspots'[125]. PCR products were separated on 1% agarose gels with ethidium bromide and purified with a gel extraction kit (Marligen Biosciences; Ijamsville, MD). Alternatively, PCR products were directly purified using the QIAquick

Table 2: Primer Sequences and PCR Conditions Used to Identify Mutations in NOTCH1, FBW7 and PTEN in the 47 Pediatric T-ALL Cohort

GENE	EXON/ DOMAIN	PCR TYPE	PRIMER POSITION	PRIMER SEQUENCE	ANNEALING (°C)	CYCLES
NOTCH1	HD	Primary	4430	5'GCGGTGACTGCTCCCTCAACTTCAAT	58°C	35
			5446	5'GGAACTTCTTGGTCTCCAGGTCCTCGTC		
		Nested	4580	5'GCCAGTGCAACCCCCTGTACGACCAGTA	61°C	38
			5402	5'GTCGTCCATGAGGGCACCGTCTGAAG		
	PEST	Primary	6615	5'GTCACCCCATGGCTACCTGTCAGAC	58°C	35
			7926	5'CGTAGGAAAACCCTGGCTCTCAGAACTT		
		Nested	6874	5' GGAGGGGCCCTGAATTTCACTGTG	61°C	38
			7747	5' TGTGTTTTAAAAAGGCTCCTCTGGTCGG		
FBW7	8	Primary	962	5'GATAGAACCCCAGTTTCAACGAGAC	56°C	35
	13	Nested	1674	5' ACTAACAACCCTCCTGCCATCATA		
	12	Primary	1581	5'TCTCGAGATGCCACTCTTAGGGT	56°C	35
	14		2456	5'ACGCCTCTCTTGTCAGTTATGGTTT		
	11	Primary	Intron	5'ATTTTCTGAAGAGCCAAACA	52°C	35
			Intron	5'CTAATTTAAGAGCACACTGTCACTA		
	12	Primary	Intron	5'TCCCAACTTCCCATTCCCTTAT	54°C	35
			Intron	5' CATAGCAAACTTAGAGCCCCAAAG		
	14	Primary	Intron	5'ACCTAGTCACATTGGAGAGTG	54°C	35
			Intron	5'TCTTCTTTTCCTTCTTAGTCTGTAG		
	6	Primary	670	5'ATGGTTCTGAGGTCCGCTCTTTTTC	57°C	35
	13		1841	5'CCCTGTCTCCACATCCCAAACA		
PTEN	5'UTR	Primary	762	5'CGTTCGGAGGATTATTCGTC	54°	35
	3'UTR		2681	5'GAAACCTCTCTTAGCCAACTGC		
	5'UTR	Nested	925	5'CAGCTACCGCCAAGTCCA	56°C	38
	3'UTR		2510	5'ATAAAACGGGAAAGTGCCATCT		
	CDS	Sequencing	1599	5'CCAGTGGCACTGTTGTTTCACA	N/A	N/A
	CDS		1755	5'CAGGTAACGGCTGAGGGAACTC		
	CDS		512	5'AGAGGCGCTATGTGTATTATT		
	CDS		1048	5' TTTGACGGCTCCTCTACTGT		

The position of each primer is based upon the database sequences for NOTCH1 (NM_017617.3), FBW7 (NM_033632.2), and PTEN (NM_000314.4). Abbreviations: CDS, coding sequence; NA, not applicable; UTR, untranslated region

cDNA purification kit (Qiagen) and sequenced in both directions with M13 forward and reverse primers (these primer sites were located on the primers used for PCR) or gene-specific primers at either the Wayne State University Applied Genomics Technology Core or Genewiz, Inc. (South Plainfield, NJ). For a small number of samples, PCR products were subcloned into a TA-cloning vector [pCRII-TOPO (Invitrogen; Carlsbad, CA)] and transformed into One Shot® MACH1T1 competent cells. Plasmids were isolated using the Wizard® Plus Mini Prep DNA purification system (Promega; Madison, WI) for DNA sequencing.

2.2b ii Amplifying and Sequencing Mutations in Relapse Study

Total RNAs and genomic DNAs (gDNAs) were isolated from cyropreserved lymphoblasts using Trizol® (Invitrogen) and the recommended protocol. cDNAs were amplified as described in 2.2b.1. gDNA was isolated from bone marrow aspirate slides with Wright-Giesma staining by first immersing the slide in *p*-xylene for 2-3 days to remove the cover slips. Secondly, the cellular material was scraped off the slides into a microcentrifuge tube. Lastly, gDNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. For those specimens in which cDNAs were amplified, mutational analysis of NOTCH1, FBW7 and PTEN was conducted as described in 2.2b.1. For specimens obtained from bone marrow aspirate slides, gDNAs were PCR amplified with intron primers flanking the HD and PEST domains of NOTCH1, intron primers designed to amplify exons 1-9 of PTEN, and primers amplifying exons 11-14 of FBW7. Primer sequences and PCR conditions are summarized in Table 3. Amplicons were either sequenced directly or subcloned into a T/A cloning vector prior to sequencing (as previously described).

Table 3: Primer Sequences and PCR Conditions Used to Identify NOTCH1, FBW7 and PTEN Mutations in Diagnosis/Relapse Specimens

Gene	Primer Name	Primer Position	Primer Sequence	Annealing (°C)	Cycles
NOTCH1	FHD1	Intron	5' CGAGTGGGACGGGCTGGACTG	65	35
	RHD1		5' AAAGGGTGTGGCTGTGGGGTCA		
	FHD2	Intron	5' TCCCAGCCCCTCTCTGATTGTC	62	35
	RHD2		5' CGGACGGCAACGCTCACAC		
	PDF	Intron	5' GTCTCCGTCCGTGCCCCTCAACCAC	62	35
	PDR		5' GTCGGCCCTGGCATCCACAGAGC		
FBW7	FBW7/F962	962	5' GATAGAACCCCAGTTTCAACGAGAC	56	35
	FBW7/R1674	1674	5' ACTAACAACCCTCCTGCCATCATA		
	FBW7/F1581	1581	5' TCTCGAGATGCCACTCTTAGGGT	56	35
	FBW7/R2456	2456	5' ACGCCTCTCTTGTCAGTTATGGTTT		
	FBW7-EX11F	Intron	5' ATTTTCTGAAGAGCCAAACA	52	35
	FBW7-EX11R		5' CTAATTTAAGAGCACACTGTCACTA		
	FBW7-EX12F	Intron	5' TCCCAACTTCCCATTCCCTTAT	54	35
	FBW7-EX12R		5' CATAGCAAAACTTAGAGCCCCAAAG		
	FBW7-EX14F	Intron	5' ACCTAGTCACATTGGAGAGTG	54	35
	FBW7-EX14R		5' TCTTCTTTTCCTTCTTAGTCTGTAG		
	FBW7/F670	670	5' ATGGTTCTGAGGTCCGCTCTTTTTC	55	35
	FBW7/R1841	1841	5' CCCTGTCTCCACATCCCAAACA		
PTEN	PTEN1F	Intron	5' GCCGTTCGGAGGATTATTCGT	56	35
	PTEN1R		5' AGTTCCGTCTAGCCAAACACACC		
	EX2F/PTEN	Intron	5' TTGTTTTGATTTTTGGTTTTTGAC	51	35
	EX2R/PTEN		5' GTATCCCCCTGAAGTCCATTAG		
	EX3F/PTEN	Intron	5' AGGGGTATTTGTTGGATTATTTATT	51	35
	EX3R/PTEN		5' CCCTAACAGCTTTTTCAGTCAAT		
	EX4F/PTEN	Intron	5' TTTTATTATTATAATATGGGGGTGA	51	35
	EX4R/PTEN		5' CTATCGGGTTTAAGTTATACAACAT		
	EX5F/PTEN	Intron	5' GTATGCAACATTTCTAAAGTTACCT	51	35
	EX5R/PTEN		5' TTGTCAATTACACCTCAATAAAAC		
	EX6F/PTEN	Intron	5' CCCAGTTACCATAGCAATTTAGTGA	51	35
	EX6R/PTEN		5' CTTCTTTAGCCCAATGAGTTGAAC		
	EX7F/PTEN	Intron	5' TTGCAGATACAGAATCCATATTTCG	51	35
	EX7R/PTEN		5' TATAATGTCTCACCAATGCCAGAGT		
	EX8F/PTEN	Intron	5' GAAAATGCAACAGATAACTCAGAT	51	35
	EX8R/PTEN		5' ATCACATACATACAAGTCAACAACC		
	EX9F/PTEN	Intron	5' GATCATGTTTGTTACAGTGCTTA	51	35
	EX9R/PTEN		5' CCATTTTCAGTTTATTCAAGTTTAT		

The position of each primer is based upon the database sequences for NOTCH1 (NM_017617.3), FBW7 (NM_033632.2), and PTEN (NM_000314.4).

2.2c Real-Time RT-PCR Analysis of Gene Expression Profiles

Analyses of gene expression levels were performed in a blinded manner. Transcript levels for 22 chemotherapy-related genes, PTEN, downstream NOTCH1 targets (HES1, DTX1, cMYC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured with a LightCycler real-time PCR machine (Roche; Indianapolis, IN). The primer sequences and PCR conditions for the 22 chemotherapy-related genes are summarized in Table 4. Reactions contained 2 μl of purified cDNA or standard plasmid, 4 mM $MgCl_2$, 0.5 μM each of sense and antisense primers, and 2 μl of FastStart DNA Master SYBR Green I enzyme-SYBR reaction mix (Roche), as described[228]. Specificity of the amplifications was confirmed by melting curve analysis and comparisons to standard templates. For each gene of interest, external standard curves were constructed using serial dilutions of linearized templates, prepared by amplification from cDNA templates, subcloned into a TA-cloning vector and restriction digested with KpnI. Transcript levels for genes of interest were normalized to GAPDH transcripts.

2.2d NOTCH1 and FBW7 Mutagenesis

The wild-type NOTCH1 expression construct in pcDNA3 was a gift from Dr. Spyros Artavanis-Tsakonas (Harvard University; Cambridge, MA). Full-length wild-type FBW7 cDNA (variant 1; NM_033632.2) was amplified from the T-ALL cell line MOLT4 (American Type Culture Collection; Rockville, MD) using primers located in the 5' untranslated region (UTR) (TTCAC**GGTACC**CGAAGGAGGAAGGGAACCAA CC; bold sequence indicates Kpn1 site) and 3'UTR (TTCAC**GAATTC**AGGGGGAAG GGCAGGGAGTA; bold sequence indicates EcoRI site). Following PCR amplification

Table 4: Real-Time PCR Primers and Conditions for 22 Chemotherapy-Related Genes

GENE	PRIMER SEQUENCES	SIZE (BP)	ANNEALING (°C)	GENBANK ACCESSION
ABCC1 (MRP1)	Forward: 5'ACCCTAATCCCTGCCCAGAG Reverse: 5'CGCATTCCTTCTTCCAGTTC	186	60	NM_004996
ABCC2 (MRP2)	Forward: 5'ACGGGCACATCACCATCAAG Reverse: 5'CTCCAGGCAGCATTTCCAAG	171	63	NM_000392
ABCC3 (MRP3)	Forward: 5'CGCCTGTTTTTCTGGTGGTT Reverse: 5'TTGTGTCGTGCCGTCTGCTT	164	63	NM_020038
ABCC4 (MRP4)	Forward: 5'GCGGCTGACGGTTACCCTCTT Reverse: 5'TCTGATGCCTTATCCCAAAAAGCAGT	189	60	NM_005845
ABCC5 (MRP5)	Forward: 5'CCAAGCTGACCCCCAAAATGAAAAA Reverse: 5'TGGATGTGCTTGCCTTCTTCCTCTTC	175	63	NM_005688
ABCG2 (BCRP)	Forward: 5'GGTGGAGGCAAATCTTCGTTATTAGA Reverse: 5'GAGTGCCCATCACAACATCATCTT	154	59	AF098951
ASNS	Forward: 5'TCGGAAGAACACAGATAGCGTGGTGA Reverse: 5'TGCGCGGAGAACATCAAACAAATAGAG	161	60	NM_133436
BCL2	Forward: 5'CTGCACCTGACGCCCTTCACC Reverse: 5'CACATGACCCCACCGAACTCAAAGA	119	61	BC027258
BCL-XL	Forward: 5'GATCCCCATGGCAGCAGTAAAGCAAG-3' Reverse: 5'CCCCATCCCGGAAGAGTTCATTCACT-3'	164	63	Z23115
c-MYC	Forward: 5'AATGAAAAGGCCCCCAAGGTAGTTATCC Reverse: 5'GTCGTTTCCGCAACAAGTCCTCTTC	112	55	NM_002467
DTX-1	Forward: 5'CAGCCGCCTGGGAAGATGGAGTT Reverse: 5'TGGATGCCTGTGGGGATGTCATAGAC	104	60	NM_004416
DHFR	Forward: 5'CATGGTTGGTTCGCTAAACTGC Reverse: 5'GAGGTTGTGGTCATTCTCTGGAAATA	126	60	BC071996
FPGS	Forward: 5'GCTGCAGGTGGAGGACTTGGAC Reverse: 5'CAGGCCATAGCTTCGGAGGATACATT	109	60	NM_004957
GAPDH	Forward: 5'AACGGGAAGCTTGTCATCAATGGAAA Reverse: 5'GCATCAGCAGAGGGGGCAGAG	194	60	NM_002046
GCR	Forward: 5'GCTTGCTCAGGAGAGGGGAGATGT Reverse: 5'CAAAAGTCTTCGCTGCTTGGAGTCTG	133	62	X03225
GGH	Forward: 5'GAGTCTGCAGGTGCGAGAGTTGTA Reverse: 5'TTTGGCCACTTTAGCATAATCTGAGC	144	60	NM_003878
HES1	Forward: 5'CCAAGCTGGAGAAGGCGGACATTC Reverse: 5'ACGTGGACAGGAAGCGGGTCAC	165	61	NM_005524
HPRT	Forward: 5'GCTATAAATTCTTTGCTGACCTGCTG Reverse: 5'AATTACTTTTATGTCCCCTGTTGACTGG	140	62	NM_000194
hRFC	Forward: 5'GTGGAGAAGCAGGTGCCCGTGGAA Reverse: 5'CGTGACCTGCTCCCGCGTGAAGTT	175	64	NM_003056
MAP4	Forward: 5'TGGCCACCAATACTTCTGCTCCTGAT Reverse: 5'GGGCCGGCTGTTTTAGTGACTGC	172	60	NM_002375
MDR1	Forward: 5'CAGGAACCTGTATTGTTTGCCACCAC Reverse: 5'TGCTTCTGCCCACCACTCAACTG	188	60	NM_000927
PTEN	Forward: 5'CCAGTGGCACTGTTGTTTCACA Reverse: 5'CAGGTAACGGCTGAGGGAGCTC	178	60	NM_000314
TPMT	Forward: 5'AGCGGTTGAGATGAAATGGTTTGC Reverse: 5'ACAGTACAATGAAATGTTCCCCGAAGAA	181	62	BC009596
TOP2A	Forward: 5'TGAAGAAGACAGCAGCAAAAAGTCAGT Reverse:5'AAAATTAGAGTCAGAATCATCAGAAGTGG	189	60	NM_001067
TOP2B	Forward:5'ACATCCAAAACAACAAGCAAGAAACCGA Reverse:5'GCAGAGAAGGTGGCTCAGTAGGGAAGTCT	105	62	NM_001068
TUBB1	Forward: 5'TTGGCCAGATCTTTAGACCAGACAAC Reverse: 5'CCGTACCACATCCAGGACAGAATC	122	62	NM_178014
TUBB3	Forward: 5'GCTCAGGGGCCTTTGGACATCTCTT Reverse: 5'TTTTCACACTCCTTCCGCACCACATC	148	63	NM_006086

Abbreviations are: MRP, multidrug resistance-associated protein; ABCG2 (BCRP), breast cancer resistance protein; ASNS, asparagine synthetase; BCL2, B-cell leukemia/lymphoma 2; BCL-XL, B-cell leukemia/lymphoma X long isoform; c-MYC, Myelocytomatosis viral oncogene homolog; DTX, Deltex1; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthase; GAPDH, Glyceraldehye-3-phosphate dehydrogenase; GCR, alpha-glucocorticoid receptor; GGH, gamma-glutamyl hydrolase; HES1, Hairy and enhancer of split; UTR, Untranslated region; HPRT, hypoxanthine phosphoribosyl transferase; hRFC, human reduced folate carrier; MAP4, microtubule-associated protein 4; MDR1, multidrug resistance 1; PTEN, phosphatase and tensin homolog; TPMT, thiopurine s-methyltransferase; TUBB1, tubulin beta; TUBB3, tubulin beta 3.

and subcloning into pCRII-TOPO (Invitrogen), FBW7 constructs were digested with KpnI and EcoRI, and purified with the QIAquick Gel Extraction Kit (Qiagen). Digested FBW7 was subcloned into pcDNA3 and transformed into JM109 competent cells (Promega). FBW7-pcDNA3 constructs were isolated with the Wizard® Plus Midiprep DNA purification system (Promega). Site-directed mutagenesis of the HD and PEST domains of wild-type NOTCH1 and WD40 domains of FBW7 used the QuikChange Lightning site-directed mutagenesis kit (Stratagene; La Jolla, CA), following the manufacturer's protocol with these modifications: (a) 100 ng of wild-type NOTCH1 or FBW7 (both in pcDNA3) were used as template; (b) extension time was 30 sec/kb at 68°C; and (c) DpnI digestions were for 10 minutes. Mutant plasmids were transformed into XL10-Gold ultracompetent cells and (d) LB-ampicillin agar plates were incubated at 37°C for >24 h to prevent recombination. Mutant constructs were transformed into JM109 competent cells to obtain higher copy number plasmids. Plasmids were isolated and the mutants were confirmed by DNA sequencing.

2.2e Generation of HES1 Promoter Reporter Construct and Reporter Gene Assays

A construct with an artificial luciferase reporter gene under the control of a HES1 promoter containing CSL/ICN1 binding sites (HES1-Luc) in pGL3-Basic (Promega) was prepared as follows. The promoter region of the human HES1 gene between positions -942 and -158 (NM_005524) from the translational start site was isolated by PCR from gDNA prepared from CMK16 cells (DSMZ; Braunschweig, Germany) using forward (5'TTCAC**GCTAGC**GTCTAAGGCCCCAAATCCAAACGAG) and reverse

(5'TTCAC**CTCGAG**CAGTAGCGCTGTTCCAGGACCAAG) primers (bold sequences indicate NheI and XhoI restriction sites, respectively). The amplified fragment was digested with NheI and XhoI and subcloned into pGL3-Basic vector (Promega).

Human U20S osteosarcoma cells (American Type Culture Collection) were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone; Logan, UT), 1% penicillin (100 U/mL)/streptomycin (100 μg/ml) (Invitrogen), and 1% L-glutamine (200 mM) under 5% CO_2.

NOTCH1 and FBW7 expression plasmids were transiently transfected into U20S cells with HES1-Luc and pRL-SV40, using Lipofectamine Plus (Invitrogen). Briefly, 3.2 x 10^5 U20S cells were seeded per well (35 mm) of a six well plate, allowed to adhere overnight and then co-transfected with 1 μg of HES1-Luc, 30 ng of pRL-SV40 (Promega), and wither 0.9 μg of wild-type or mutation NOTCH1-pcDNA3 constructs or ICN1-pcDNA3 (provided by Dr. Lucio Miele, Loyola University; Chicago, IL). Total DNA was maintained constant by adding empty pcDNA3 plasmid (Invitrogen). For the FBW7-NOTCH1 co-transfections, wild-type and mutant FBW7-pcDNA3 constructs (0.9 μg) were co-transfected into U20S with wild-type and mutant NOTCH1-pcDNA3 constructs (0.9 μg) with 500 ng of HES1-Luc/30 ng of pRL-SV40. For both series, 48 h post-transfection, the cells were lysed and luciferase activities were assayed using a Dual Luciferase reporter assay system (Promega) on a Turner Designs 20/20 luminometer. Relative luciferase activities of the cell lysates were normalized to *Renilla* luciferase activity (encoded by pRL-SV40). Data are reported as mean values plus/minus SEM from replicate assays.

2.2f Detection of Relapse Clones at Diagnosis

Real time PCR and melting curve analysis with genomic DNAs from paired diagnostic-relapse T-ALL specimens was used to determine whether newly identified relapse T-ALL clones were present at the time of diagnosis as a minor subclonal population. NOTCH1 hybridization probes (TIB MOLBIOL; Berlin, Germany) were designed to detect mutant NOTCH1 sequence in diagnostic specimens. The probe sequences are summarized in Table 5. The genotype analysis was performed on a LightCycler real time PCR machine (Roche) with gene-specific primers and 3'fluorescein-labeled and 5'LC-red640-labeled hybridization probes. Samples were amplified over 35 cycles, after which melting curves for the products were analyzed at 640 nm from 40°C to 80°C at a rate of 0.3°C/s.

2.2g Statistical Methods

Patient statistical analysis was performed by Dr. M. Devidas of the Children's Oncology Group (COG) Statistical Office in Gainesville, FL. Data analyses were performed using the SAS System (SAS Institute Inc. SAS OnlineDoc® 9.1.3. Cary, NC: SAS Institute Inc, 2005) and R (R Foundation for Statistical Computing,;Vienna, Austria. ISBN 3-9000051-07-0, URL http://www.R-project.org. 2005), or GraphPad Prism 4.0. For analyses of overall NOTCH1 signaling, transcript levels for HES1, DTX1 and cMYC were categorized into low and high levels, respectively, corresponding to values below and above the median values. The non-parametric Wilcoxon test was used for comparisons of transcript levels between various subgroups (cases versus controls, NOTCH1 mutant versus non-mutant group, high and low WBC, high versus low HES1/DTX1/cMYC transcripts). The associations between high/low transcript levels of

Table 5: Sequence of the NOTCH1 Hybridization Probes

Probe	Probe Type	Probe Name	Sequence	Annealing (°C)
T5039A	PCR Amplification	Notch1 F1	5’ GGGTAGCTGCTGTCAGACC	57
		Notch1 R1	5’ CCTCGATCTTGTAGGGGATGT	
	Sensor		5’ GCCGGTTGTCAATCTCCAGGTAG	
	Anchor		5’ CGATGGAGCTGGGCGGACAA	
T5153C	PCR Amplification	T5153CF1	5’ TCCTCGCAGTGCTTCCA	57
		T5153CR1	5’ CAAACAGCCAGCGTGTCT	
	Sensor		5’ CCTACAAGACCGAGGCCGTG	
	Anchor		5’ AGAGTAAGTGTGGCCCCATCCCGG	

The position of each NOTCH1 primer is based upon the database sequences for NOTCH1 (NM_017617.3).

a gene and outcome or prognostic factors such as age group or WBC group were tested using Fisher's Exact test. The non-parametric Spearman's correlation coefficient was used to measure the associations between NOTCH1 target genes. The paired t test was used to make comparisons between the luciferase activities associated with the NOTCH1 mutants and wild-type NOTCH1.

2.3 Results

2.3a Identification of NOTCH1 Mutants in Primary T-ALL Specimens

Although significant improvements have been documented in the treatment outcome of T-ALL in children, T-ALL remains an aggressive disease with a substantially poorer prognostic outlook than that for BP-ALL. Following reports of high frequency mutations in the NOTCH1 receptor[125, 131, 132] involving the HD (positions 4710 to 5163) and PEST (positions 6930 to 7665) domains [position numbers based upon NOTCH1 sequence (NM_017617.3)], we became interested in the prognostic significance of mutant NOTCH1 and possible explanations for disparate reports of both good and poor prognoses for T-ALL patients with NOTCH1 mutations[91, 125, 131, 132, 135, 140]. A well-characterized cohort of 47 pediatric T-ALLs with documented treatment outcomes was used to explore this clinically important question. The 47 children included 38 boys and 9 girls diagnosed with T-ALL, 23 of whom relapsed within 4 years of diagnosis (failed) and 24 of whom remained in remission for 4 or more years after diagnosis (not failed). Patient ages ranged from 1.8 to 19.9 years (median = 7.48 years) and WBCs ranged from 8.2 to 999 x 10^9 cells/L (median = 240 x 10^9 cells/L) (Table 6).

RNAs from the 47 T-ALL specimens were reverse transcribed and cDNAs were PCR amplified across the NOTCH1 HD and PEST domains. The amplicons were

Table 6: Characteristics of the 47 Pediatric T-ALL Specimens

GENDER	Age (years)						WBC ($x10^9$ cells per l)					
	N	Minimum	Median	Mean	s.d.	Maximum	N	Minimum	Median	Mean	s.d.	Maximum
Male	38	1.83	7.48	8.66	4.86	19.86	38	8.20	267.50	335.86	262.81	999.90
Female	9	4.13	6.45	6.49	2.26	11.62	9	20.00	171.80	242.77	224.12	680.00
Total	47	1.83	7.15	8.24	4.54	19.86	47	8.20	240.00	318.03	256.25	999.90

Abbreviation: s.d., standard deviation

sequenced in both directions with M13 primers to identify potential HD and PEST mutations. In a few cases, amplicons were subcloned into a T/A cloning vector and individual plasmid clones were isolated for DNA sequencing. Twenty-five samples showed a high frequency polymorphism (C5094T) in the HD domain that was silent (GAC and GAT both encode aspartic acid). NOTCH1 mutations resulting in modified primary sequence were detected in16 patients (9 HD, 4 PEST, 3 HD and PEST) and wild-type NOTCH1 sequences were detected in 31 patients (Table 7). NOTCH1 mutations included single point mutations, deletions and insertions in the HD and PEST domains that variously resulted in amino acid substitutions and premature translation terminations (Table 7). With few exceptions (V1671I, 2514 RVP*Stop, 2459*Stop and 2503*Stop), these mutations are unique from those previously described as 'gain-of-function' in T-ALL[125, 132]. The frequency (34%) of NOTCH1 mutations in our analysis is somewhat lower than that originally reported in pediatric T-ALL[125], and may reflect the unique features of our T-ALL cohort (~50% of patients relapsed). Nonetheless, similar frequencies have been reported in both pediatric and adult T-ALL patients in other studies[125, 131, 132, 138].

For our 47 patient cohort, there were no associations between age or WBC and the presence of NOTCH1 mutations. In contrast to recent reports that the presence of NOTCH1 mutations were good prognostic factors in T-ALL[91, 132], no statistically significant differences were seen in frequencies of mutations between patients who relapsed (9 of 23 patients harbored NOTCH1 mutations) and patients who did not (7 of 24 patients harbored NOTCH1 mutations; p=0.5469, by Fisher's exact test) (Figure 5A). Interestingly, for the 28 patients treated on a single (POG8704) protocol (14 failed, 14 not

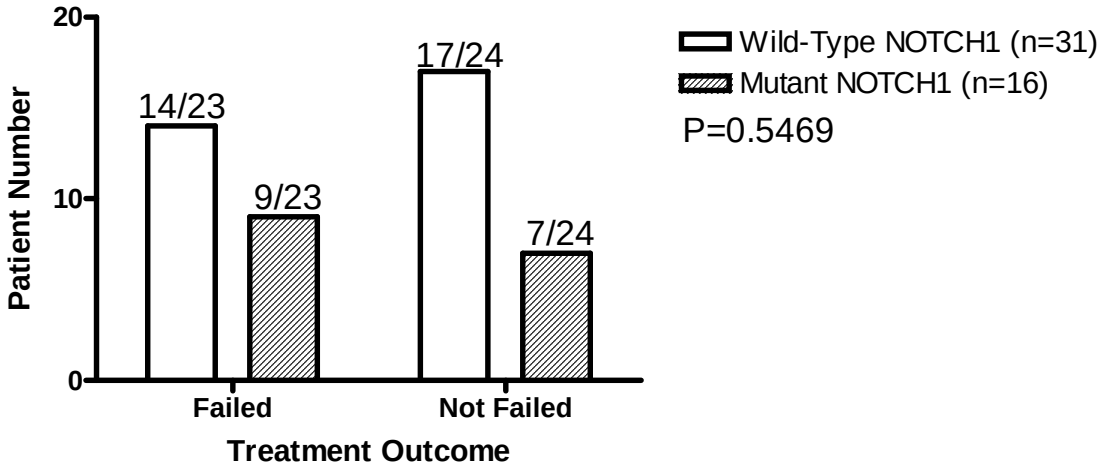
Table 7: Summary of NOTCH1 Mutations in the 47 Pediatric T-ALL Specimens

Sample	*DNA Mutations*		*Amino Acid Changes*
	HD Domain	**PEST Domain**	
1	INS4827(CCCCAACCT); G4828A	Normal	INS1609(PQP); A1610T
2	G4898T; C5094T	Normal	R1633L
3	G4966A; C5094T	A7233G; DEL7541-7542(CT)	G1654S; P2514R; E2515V; S2516P; 2517*
4	G4985T; C5094T	C7507T	R1662L; Q2503*
5	G5011A; C5094T	T7515G	V1671I
6	G4948A	Normal	A1650T
7	Normal	C7375T	Q2459*STOP
8	Normal	G7392A; T7515A; DEL 7518-7537 (GCACCCCTTCCTCACCCCGT); INS7518(TCTCCTACC)	E2506D; H2507L; P2508L; F2509P; L2510P; T2511*
9	G4893T	DEL 7531-7541 (ACCCCGTCCCC)	2511*
10	G5011A	T7515G	V1671I
11	G4976A; C5094T	Normal	G1659D
12	DEL5024-5026(TCG); C5094T	Normal	DEL 1676(V)
13	Normal	C7322T; DEL 7541-7542(CT)	A2441V; P2514R; E2515V; S2516P; P2517*
14	G4900T	Normal	A1634S
15	INS4776(CTGCCGCGCCTTCCCCA) with DUP of 4758-4776 (CAACAGCTCCTTCCACTTC)	C7530T	INS1588(SFHFLPRLPHNS)
16	INS4776(CTGCCGCGCCTTCCCCA) with DUP of 4758-4776 (CAACAGCTCCTTCCACTTC)	INS7313(CT)	INS1588(SFHFLPRLPHNS); S2451*

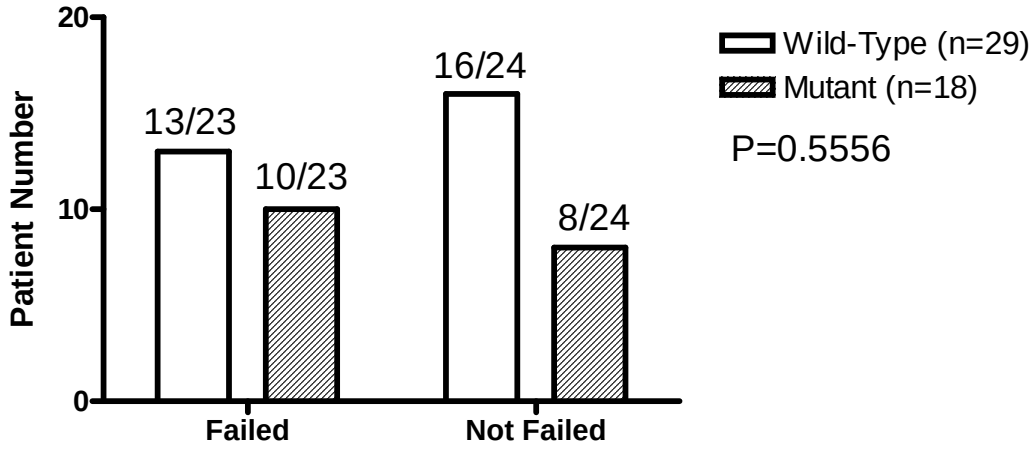
The position of NOTCH1 mutations is based upon the database sequences for NOTCH1 (NM_017617.3). Abbreviations are: HD, Heterodimerization; INS, Insertion; DEL, Deletion; DUP, Duplication; *, Stop codon; nt, Nucleotide; N/A, Not Available; UTR, Untranslated Region; WT, Wild type; Δ, changed sequence.

Figure 5: Association of NOTCH1 Mutations, Alone or in Combination with FBW7 Mutations, with Treatment Outcome

A.



B.



The associations of NOTCH1 mutations, alone and in combination with FBW7 mutations, with treatment outcomes were tested using Fisher's Exact test. (5A) There was no difference in the frequency of NOTCH1 mutations between those patients who failed treatment (9 of 23 patients harbored NOTCH1 mutations) and those who did not fail treatment (7 of 24 patients harbored NOTCH1 mutations; p=0.5469). (5B) Likewise, there is no difference in the frequency of NOTCH1 mutations and FBW7 mutations between those patients who failed treatment (10 of 23 patients had either NOTCH1 and/or FBW7 mutations) and those who did not fail treatment (8 of 24 patients had either NOTCH1 and/or FBW7 mutations; p=0.5556). Patients who failed treatment relapsed with in 4 years of treatment. Patients who did not fail treatment were in remission at least 4 years following treatment.

failed), there was a decrease in the frequency of NOTCH1 mutations in patients who relapsed (3 of 14 patients harbored NOTCH1 mutations) compared to those who responded to treatment (6 of 14 patients harbored NOTCH1 mutations). However, this difference was still not statistically significant (p=0.4197).

We reasoned that our inability to establish statistically significant associations between relapse and mutant NOTCH1 in our 47 patient cohort could be due to (i) various levels of overall signaling, resulting from different activating potencies for the assorted NOTCH1 mutants. Other factors may also be important such as (ii) high frequency mutations in the FBW7 ubiquitin ligase that impact steady state levels of ICN independent of the NOTCH1 mutational status[91, 92], or (iii) decreased expression (due to HES1 and cMYC) and/or inactivating mutations involving the PTEN gene, resulting in increased AKT signaling[166]. Finally, (iv) the T-ALL specimens were from patients treated with different protocols and aberrant NOTCH1 signaling may impact sensitivities to various chemotherapy drugs to different extents.

2.3b Identification of FBW7 Mutations in Primary T-ALL Specimens

Since high frequency mutations in the E3-ubiquitin ligase FBW7 [NM_001013415.1] substrate binding domain were reported in up to 30% of pediatric T-ALL patients[91-93], the 47 T-ALL specimens were analyzed for mutations in the FBW7 gene. cDNAs from 44 specimens were amplified across exons 8-14 for direct sequencing of the amplicons, whereas for one specimen, the product was subcloned (pCRII-TOPO) and multiple plasmid clones were sequenced. For 2 samples, exons 11, 12 and 14 were individually amplified and sequenced from genomic DNAs. FBW7 mutations were detected in exon 11 for 5 patients (11%, Table 8), all of which were heterozygous and

one (R465C) of which was previously documented as inactivating[91-93]. For one sample, there was an additional heterozygous insertion of 49 nucleotides in exon 8 that is predicted to result in early translation termination. As expected, all of the samples with FBW7 mutations were accompanied by wild-type PEST sequence for NOTCH1. Thus, when combined with the 7 T-ALL specimens with PEST domain mutations, 12 of 47 (25%) samples exhibited disruptions of FBW7 function. Two of the samples with FBW7 mutations contained wild-type NOTCH1, whereas the other three of the FBW7 mutants were accompanied by mutations in the HD domain of NOTCH1 (Table 8).

We found no statistically significant difference in the frequencies of NOTCH1 plus FBW7 mutations between the 23 patients who failed treatment (10 of 23) and the 24 patients who did not fail treatment (8 of 24) (p=0.5556) (Figure 5B).

2.3c PTEN Levels and Mutations in Primary T-ALL Specimens

Since NOTCH1 has been reported to directly (activate, via CSL)[229] and indirectly (repress, via HES1 and cMYC)[166] regulate PTEN, we extended our analysis of our T-ALL cohort to include this important gene, given its likely relevance to clinical responses to therapy. We initially performed real-time RT-PCR analysis of PTEN transcript levels for the 47 T-ALLs. PTEN transcript levels spanned an 833-fold range. PTEN transcripts exhibited a slight positive correlation (Spearman's analysis) with transcript levels of both HES1 (r=0.3507; p=0.0157) and cMYC (r=0.3840; p=0.0077).

For 43 samples for which there was sufficient RNA, the entire PTEN coding region was amplified using primers in the 5' and 3' UTRs for direct sequencing with gene-specific primers. With a few samples, amplicons were subcloned into a T/A cloning vector and individual plasmid clones were sequenced. Altogether, PTEN

Table 8: Summary of FBW7 Mutations in the 47 Pediatric T-ALL Specimens

Sample	DNA Changes	Amino Acid Changes	NOTCH1 Status
1	INS1011 (49nt); G1543T	Stop@322; R465L	INS1609(PQP); A1610T
2	C1542T	R465C	R1633L
12	C1662A	R505S	DEL 1676(V)
17	G1543T	R465L	WT
18	C1542T	R465C	WT

The position of FBW7 mutations is based upon the database sequences for FBW7 (NM_033632.2 [isoform 1], NM_018315.4 [isoform 2], NM_001013415.1 [isoform 3]). Abbreviations are: INS, Insertion; DEL, Deletion; DUP, Duplication; *, Stop codon; nt, Nucleotide; N/A, Not Available; UTR, Untranslated Region; WT, Wild type; Δ, changed sequence.

mutations were detected in 25 of 43 specimens, 22 of which would result in truncated proteins (Table 9). Eight of the 25 mutations were homozygous. The higher frequency of both heterozygous and homozygous PTEN mutations in our T-ALL cohort are different from results previously reported[166, 181], but this may reflect inclusion of approximately 50% of patients who relapsed within 4 years in our study. Regardless of the NOTCH1 and FBW7 mutational status, the loss of PTEN in these samples should result in increased AKT signaling and resistance to standard chemotherapy and GSIs[166]. However, constitutively high AKT signaling may also occur independent of PTEN mutations due to inactivating posttranslational modifications of the PTEN protein[198], however, this has not been independently confirmed. Further, NOTCH1 may also activate mTOR independent of the PTEN/PI3K/AKT axis[181].

For the 43 patients whose PTEN status was established, we found no significant difference in the frequencies of PTEN mutations between the 22 patients who failed treatment (15 of 22 PTEN mutants) and 21 patients who did not fail treatment (12 of 21 PTEN mutants) (p=0.5365). With the 43 patients for whom all three genes (PTEN, NOTCH1 and FBW7) were analyzed for mutations, there was no significant difference between the number of children who failed treatment and had any combination of PTEN, NOTCH1 and FBW7 mutations (16 of 22) and those who harbored these mutations and did not relapse (14 of 21 patients) (p=0.7470) (Figure 6).

2.3d Activating Potential of Patient-Derived NOTCH1 Mutations

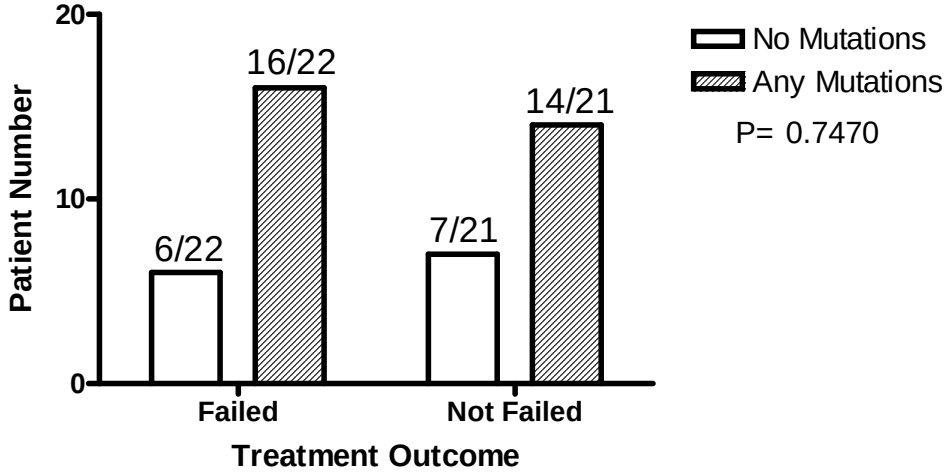
To consider the possibility that the different NOTCH1 mutations identified in 16 primary T-ALL patient specimens may exhibit different activating potentials, we prepared mutant NOTCH1 constructs containing these HD and PEST domain mutations.

Table 9: Summary of PTEN Mutations in the 47 Pediatric T-ALL Specimens

Sample	DNA Changes	Amino Acid Changes
1	N/A	N/A
2	G115T	G39F
3	G509C; C541A	S170T; L181M
4	N/A	N/A
5	INS209 (52nt)	C71W; A72G; E73I; R74*
6	A80G; INS83 (67nt); DEL 84-487; G492T	Δ27-46(CIHFCGCSSLPFCHSLRTWE): N48*
7	C733T; G735C; C737A; G738A; A741C; INS743 (CA); INS745 (TTTCT)	Q245Y; P246Q; L247F; V249M; C250F; G251L; D252V; I253W; K254*
8	DEL697 (C)	Δ233-253 (EEKTSSCTLSSLSRYLCVVIS); V255*
9	WT	WT
10	DEL 165-209; T750G; G752A;T253G; G754A; T756A; A757T	R55S; DEL 56-70 (FLDSKHKNHYKIYNL); C250W; G251E; D252K; I253F
11	DEL 245-1212; INS 34nt from 3'UTR	N82R; C83*
12	WT	WT
13	N/A	N/A
14	INS209 (52nt)	C71W; A72G; E73I; R74*
15	N/A	N/A
16	N/A	N/A
17	WT	WT
18	WT	WT
19	A80G; DEL84-208; INS83 (71nt)	Y27C; Δ30-41 (FCGCSSLPFCHS) 43-46 (RTWE); R47*
20	INS211 (39nt) after 211 G877C; A878G; A879G; T881G; DEL 883-900	INS 71 (SWSYQGTANHTDI); G306A; S307G; L308R; A309*
21	DEL 164-1026	F56*
22	G29T; INS492 (156nt)	S10I; Δ165-170 (ILQEVF) 172-177 (IKALLS); Y178*
23	G698T; A699T; C700A; INS702 (AG)	R233L; Δ235-249 (RKTSSCTLSSLSRYL) 251-252 (VV) 254-255(SK); E256*
24	G738A; INS738 (GAGCCCCT)	L247E; Δ249-257 (LYLCVVISK); F258*
25	INS736 (GG); C737G; T882C; G949A	Δ246-249 (TGYL) 251-252 (VV) 254-255 (SK); E256*
26	G566C; C737A; INS737 (GAATAGGGA)	R189T; P246Q; INS 246 (NRE)
27	DEL 262-979	DEL 88-327
28	INS79 (186nt); DEL80-209; INS209 (52nt); DEL635-1026	Δ27-32 (LYLTRH); I33*
29	A80G; DEL84-164; INS83 (68nt); DEL 165-487; G492T	Y27C; Δ29-41 (HFCGCSSLPFCAS) 43-46 (RTWE); R47*
30	DEL493-634	G165I; V166L; T167S; I168L; P169W; Q171A; R172S; R173*
31	G698A; INS698 (GGTAT); INS753 (GC)	Δ143-228 (QVYGKTSSCTLSSLSRYLCVVRYQSRVLPQTEQDAK) 230-239 (GQNVSLLGKY) 241-248 (LHTRTRGNLRKSRKWKSM); V249*
32	C697G; INS697(A)	Δ293-201 (ETGRQVHVL); T202*
33	DEL493-634	G165I; V166L; T167S; I168L; P169W; Q171A; R172S; R173*
34	INS645 (ACCCTTTT); G766C	Δ216-222 (TLLWSAS); K223*

The position of PTEN mutations is based upon the database sequences for PTEN (NM_000314.4). Abbreviations are: INS, Insertion; DEL, Deletion; DUP, Duplication; *, Stop codon; nt, Nucleotide; N/A, Not Available; UTR, Untranslated Region; WT, Wild type; Δ, changed sequence.

Figure 6: The Prognostic Value of PTEN Mutations in Combination with NOTCH1 and/or FBW7 Mutations



The association between the presence of at least one mutation in NOTCH1, FBW7, or PTEN, or a combination of all three genes, with treatment outcome was tested using Fisher's Exact test. There was no difference in the frequency of mutations (single gene or combination of all 3) between those patients who failed treatment (16 of 22 patients harbored mutations) and those who did not fail treatment (14 of 21 patients harbored mutations; p=0.7470). Patients who failed treatment relapsed with in 4 years of treatment. Patients who did not fail treatment were in remission at least 4 years following treatment.

.

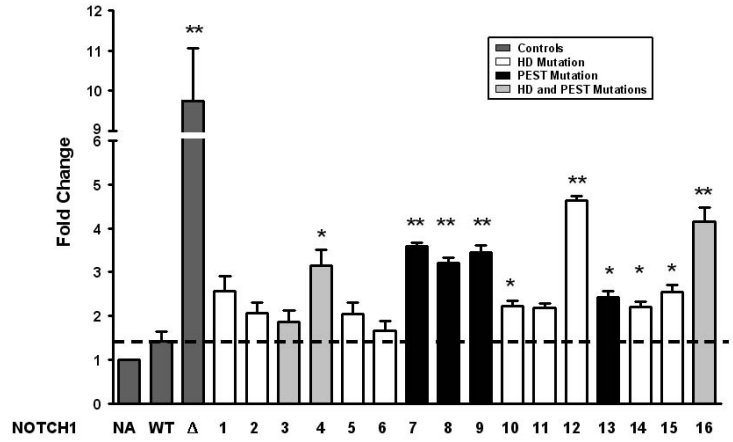
Mutant NOTCH1 constructs in pcDNA3 were transiently transfected into U20S cells with a HES1-Luc reporter construct; firefly luciferase activities (normalized to *Renilla* luciferase) were compared to those for wild-type NOTCH1 and ICN1. The 16 clinically relevant NOTCH1 mutants showed increased transactivating potentials toward HES1-Luc over wild-type NOTCH1 (1.3-3.3-fold), albeit consistently less than by ICN1 (Figure 7A). Interestingly, most NOTCH1 constructs with mutations in either the PEST domain alone, or in combination with HD domain mutations, showed higher levels of reporter gene activation than constructs with mutations in the HD domain alone. This result is somewhat different from that reported by Weng *et al.*[125] based on a much smaller group of clinically relevant NOTCH1 mutants.

2.3e Analysis of Downstream Gene Targets of NOTCH1 as a Measure of Downstream Signaling

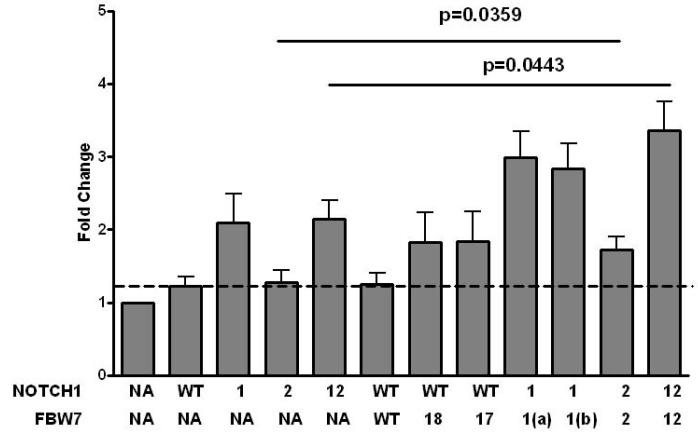
To evaluate overall NOTCH1 signaling resulting from mutations in NOTCH1 and FBW7 genes as measures of possible 'gain-of-function' or constitutive activity, real-time PCR was used to measure transcript levels for HES1, DTX1 and cMYC, all documented NOTCH 1 gene targets[105-108], in the 47 T-ALL specimens. Transcript levels for cMYC and DTX1 significantly correlated with HES1 transcripts [Spearman's correlation coefficient r=0.5219 (cMYC) and 0.6829 (DTX1); p=0.0002 and p<0.0001, respectively] over a 38-300-fold range of expression. Median transcript levels for HES1, DTX1 and cMYC were all increased in the NOTCH1/FBW7 mutant group over specimens expressing wild-type NOTCH1/FBW7 (5.6-, 4.0-and 1.9-fold, respectively); however, transcript levels were remarkably variable and appreciably overlapped between the groups. For HES1 and cMYC, differences between the mutant and wild-type groups

Figure 7: Potencies of Clinically Relevant NOTCH1 and FBW7 Mutations as Measured by Reporter Gene Assays

A:

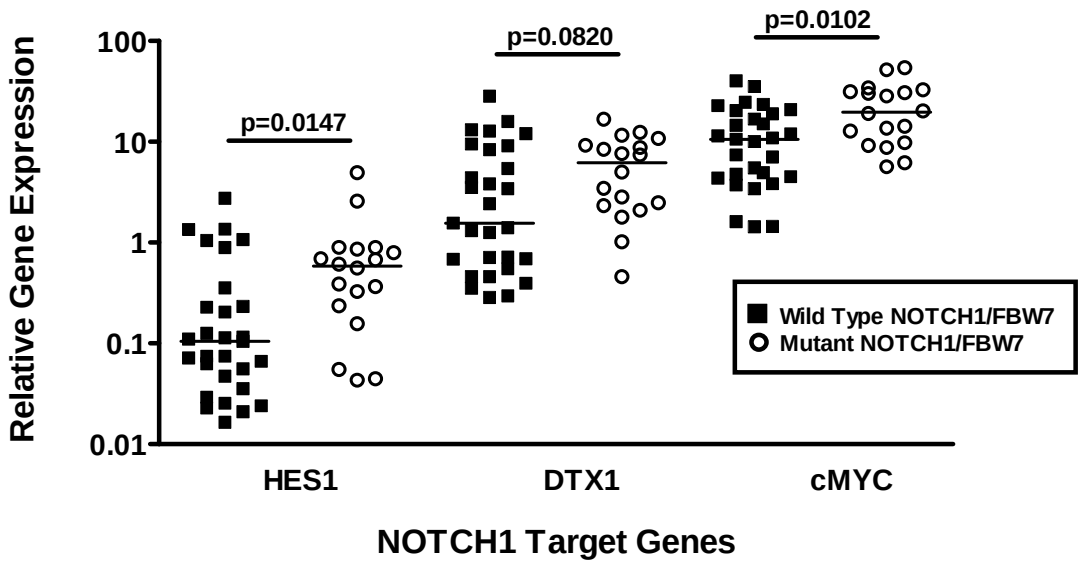


B:



Human U20S cells were transiently co-transfected in 35mm dishes with 0.9 μg of the indicated NOTCH1 expression plasmid alone (A), or with 0.9 μg of both a NOTCH1 expression plasmid and FBW7 expression plasmid (B). For (A), 1 μg of HES1-Luc reporter gene construct and 30 ng of Renilla luciferase (pRL-SV40) internal control were used, whereas for (B), 500 ng HES1-Luc and 30 ng of pRL-SV40 were used. For all transfections, constant plasmid was maintained a 0.9 μg of pcDNA3 plasmid per well. Results represent normalized luciferase activities of whole cell lysates, relative to a control in which HES1-Luc was co-transfected with 0.9 μg pcDNA3 vector in lieu of NOTCH1/FBW7 (assigned a value of 1). Results were presented as mean values ± standard errors [n=6 for (A); n=6 for (B)]. For (A), p-values were calculated using paired t-tests, comparing the luciferase activities of the different NOTCH1 mutations to wild-type NOTCH1 (*, $p \leq 0.05$: **, $p \leq 0.005$). For (B), p-values were calculated using paired t-tests, comparing the clinically relevant NOTCH1 and FBW7 mutants as shown in the figure. For (A), the sample numbers designate the patient samples listed in Table 3. For (B), NOTCH1 and FBW7 forms refer to the sample numbers in Table 4. For sample 1, (a) is the early termination at position 322 and (b) is R465L. Abbreviations are: WT, wild-type; Δ, ICN; NA, no addition.

Figure 8: Expression of HES1, DTX1 and cMYC Transcripts in Patients Harboring NOTCH1 and/or FBW7 Mutations



Transcript levels were measured using real-time RT-PCR and normalized to those for GAPDH. Results are shown for HES1, DTX1 and cMYC transcript levels in T-ALL specimens exhibiting NOTCH1 and/or FBW7 mutations and T-ALL specimens characterized by wild-type NOTCH1 and FBW7. Data were analyzed using the nonparametric Wilcoxon test. Horizontal bars represent median values.

were statistically significant (p=0.0147 and 0.0102 respectively) (Figure 8).

For the 13 specimens with only NOTCH1 mutations, we showed similarly increased levels of HES1 (5.8-fold; p=0.0817), DTX1 (3.2-fold, p=0.2010), and cMYC (2.7-fold; p=0.0083) over the 29 specimens without either NOTCH1 or FBW7 mutations. In contrast to our results for the HES1-luciferase reporter assays (Figure 7A), for samples identified as harboring NOTCH1 mutations, there was no difference in the patterns of expression for downstream target genes between samples with HD mutations alone, versus those with PEST mutations alone, or between samples with only one mutant domain versus those with mutations in both the HD and PEST domain. Further, for individual NOTCH1 mutations, there was no consistent association between reporter activities and overall NOTCH1 signaling (as reflected in HES1/DTX1/cMYC transcripts). These results likely reflect contributions from other factors such as FBW7 in determining levels of overall NOTCH1 signaling, as noted above.

Since three of the samples with FBW7 mutations also contained NOTCH1 HD mutations (Table 8), it was possible to separate the impact of FBW7 mutations from that resulting from NOTCH1 mutations on overall signaling for only a very small number of samples. For the 5 samples with FBW7 mutations (with and without NOTCH1 mutations), the transcript levels of HES1 and DTX1 were increased (4.6- and 4.0-fold respectively) over samples with wild-type FBW7. However, these differences were not significant.

The impact of the clinically relevant FBW7 mutations on transactivation of a HES1 reporter (HES1-Luc) on top of that resulting from the clinically relevant NOTCH1 mutations (in samples 1, 2, 12; Tables 7 and 8) or wild-type NOTCH1 (for samples 17

and 18) was further analyzed by reporter gene experiments (Figure 7b). Mutant FBW7 constructs in pcDNA3 were transiently transfected into U20S cells with the HES1-Luc reporter, together with wild-type or mutant NOTCH1 constructs, as appropriate. Whereas wild-type FBW7 had minimal impact on HES1 transactivation with wild-type NOTCH1, when tested in their clinically relevant contexts (Table 8), the FBW7 mutants augmented transactivation by both wild-type and mutant NOTCH1 (1.4- to 1.6-fold). For FBW7 mutants 2 and 12, these increases were statistically significant ($p=0.0359$ and $p=0.0443$, respectively).

Thus, although levels of HES1/DTX1/cMYC transcript in T-ALLs might be expected to be the most accurate measures of overall NOTCH1 signaling and reflect the impact of both NOTCH1 and FBW7 mutations along with other factors (e.g., NUMB), we found no significant difference in the distribution of HES1/DTX1/cMYC transcript levels (i.e., greater or less than the median value) between the group of patients who failed therapy and patients who did not [$p=0.7683$ (HES1), 0.559 (DTX1), and 0.7683 (cMYC)].

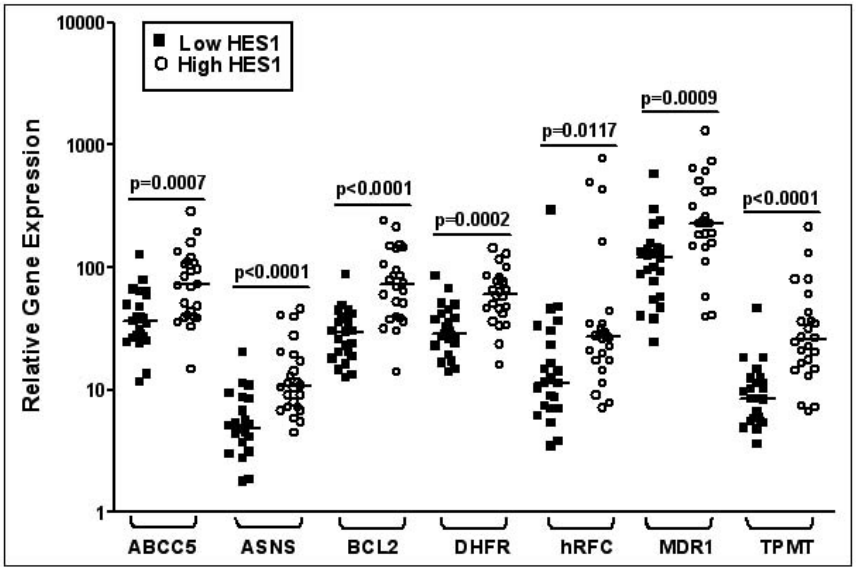
2.3f Expression Analysis of Chemotherapy-Related Genes in Primary T-ALLs and Relationship to NOTCH1 Signaling

We hypothesized that differences in prognostic value of NOTCH1 and FBW7 between studies[91, 125, 131, 132, 135, 138, 140] (including our own) may reflect the inclusion of T-ALL specimens from patients treated with different chemotherapy protocols and the possibility that aberrant NOTCH1 signaling may impact sensitivities to various chemotherapy drugs to different extents. We used real-time RT-PCR with our 47 patient T-ALL cohort to measure transcript levels for 22 gene targets most relevant to major

drugs used to treat T-ALL, including asparaginase, doxorubicin, 6-mercaptopurine, methotrexate, corticosteroids and vincristine. Genes of interest encoded drug transporters, drug metabolizing enzymes, drug targets or apoptosis signaling proteins that included (a) ABCG2, (b) ABCC1, (c) ABCC2, (d) ABCC3, (e) ABCC4, (f) ABCC5, (g) asparagine synthetase, (h) B-cell leukemia/lymphoma 2 (BCL2), (i) B-cell leukemia/lymphoma X long isoform (BCL-XL), (j) dihydrofolate reductase, (k) folypolglutamate synthetase, (l) γ glutamyl hydrolase, (m) glucocorticoid receptor, (n) human reduce folate carrier, (o) hypoxanthine guanine phosphoribosyl transferase, (p) MDR1, (q) microtubule-associated protein 4, (r) thiopurine methyltransferase, (s) topisomerase 2α, (t) topoisomerase 2β, (u) β tubulin class 1, and (v) β tubulin class 3.

For each of these genes, a broad range of transcript levels was detected, from slightly over 569-fold for AGCG2 to 6.7-fold for BCL-XL. When transcript levels for the individual genes were correlated with relative NOTCH1 signaling, as reflected in HES1, DTX1 and cMYC transcript levels, elevated transcript for these established NOTCH1 gene targets were accompanied by consistent and statistically significant increases (1.5-3.0 fold) in transcript levels for MDR1, ABCC5, asparagine synthetase, Bcl-2, human reduced folate carrier, dihydrofolate reductase, and thiopurine methyltransferase (Figure 9; Tables 10-12). Folylpolyglutamate synthetase and hypoxanthine phosphoribosyltransferase were associated with elevated expression for 2 or the 3 established NOTCH1 targets (HES1 and DTX1, and HES1 and cMYC, respectively). For the entire cohort of 47 T-ALL patients, transcript levels for none of these 22 genes were prognostic, in contrast to our previous findings in BP-ALL[228].

Figure 9: Expression of Relevant Chemotherapy Genes in Relation to HES1 Expression



Patients with HES1 transcript levels below the median value were considered to have low HES1 expression, and those with HES1 transcript expression above HES1 median values were considered to have high HES1 expression. Relative transcript levels for 22 chemotherapy-related genes were measured by real-time RT-PCR. Horizontal bars represent median values. Twelve of 22 genes were significantly over-expressed in samples with high HES1 transcripts ($p<0.05$ by non-parametric Wilcoxon test) and of these, the 7 gene targets in the figure also showed a statistically significant association with levels of DELTEX1 and cMYC transcripts. Abbreviations: ABCC5, Multidrug resistance-associated protein 5 (MRP5); ASNS, asparagine synthetase; BCL2, B-cell leukemia/lymphoma 2; DHFR, dihydrofolate reductase; hRFC, human reduced folate carrier; MDR1, multidrug resistance 1; TPMT, thiopurine-S-methyltransferase

Table 10: Expression of NOTCH1 Target Genes and Chemotherapy Relevant Genes in Both Low and High HES1 Expression Patients

SYMBOL	TRANSCRIPTS (RELATIVE UNITS)				*P*
	Low HES1 Expression (n=23)		High HES1 Expression (n=24)		
	Range	Median	Range	Median	
ABCC1	16.09-199.8	46.08	14.64-272.0	48.00	0.9915
ABCC2	0.6033-77.90	5.877	1.718-54.40	9.019	0.3437
ABCC3	**0.2319-12.40**	**2.619**	**0.3660-42.78**	**4.672**	**0.0235**
ABCC4	**5.061-37.22**	**15.14**	**4.159-95.96**	**24.02**	**0.0124**
ABCC5	**11.70-127.8**	**36.86**	**14.92-286.0**	**71.95**	**0.0007**
ABCG2	0.3620-6.856	1.580	0.06943-39.50	1.069	0.2293
ASNS	**1.794-20.40**	**4.829**	**4.466-45.83**	**10.68**	**<0.0001**
BCL2	**12.55-87.73**	**29.75**	**14.10-240.5**	**71.72**	**<0.0001**
BCL-XL	14.15-78.15	28.53	11.63-76.31	30.18	0.6473
DHFR	**13.99-86.02**	**28.70**	**16.14-143.2**	**60.78**	**0.0002**
FPGS	**7.993-43.58**	**17.00**	**12.57-63.21**	**25.37**	**0.0117**
GCR	**24.57-368.8**	**63.53**	**38.67-665.2**	**133.7**	**0.0026**
GGH	0.1907-6.639	1.878	0.2334-14.57	1.604	0.8565
HPRT	***6.223-25.00***	***12.22***	***5.946-54.13***	***18.30***	***0.0037***
hRFC	**3.485-294.4**	**11.50**	**7.127-779.8**	**27.11**	**0.0117**
MAP4	7.153-49.64	14.16	1.686-70.70	16.05	0.6627
MDR1	**24.40-577.1**	**118.9**	**39.56-1301**	**230.2**	**0.0009**
TPMT	**3.612-46.23**	**8.528**	**6.630-213.8**	**25.58**	**<0.0001**
TOP2A	1.007-19.13	4.463	0.1085-26.64	7.014	0.9915
TOP2B	8.263-129.0	22.82	5.078-86.55	34.42	0.1036
TUBB1	204.1-912.5	330.1	105.2-1320	375.9	0.8232
TUBB3	0.08313-2.510	0.2958	0.08392-1.295	0.3261	0.8232

n=number of patients studied. Patients with HES1 transcript expression below the median value were considered to have low HES1 expression, and those with HES1 transcript expression above HES1 median values were considered to have high HES1 expression**.** Relative transcript levels for the target genes were measured by real-time RT-PCR as described in Materials and Methods. Gene abbreviations are summarized in the legend to Table 4. The non-parametric Wilcoxon test was used for comparisons of transcript levels between groups and the *p* values are reported in the table. Bold and italicized entries were statistically significant between the groups.

Table 11: Expression of NOTCH1 Target Genes and Chemotherapy Relevant Genes in Both Low and High DELTEX1 Expression Patients

SYMBOL	TRANSCRIPTS (RELATIVE UNITS)				*P*
	Low DTX1 Expression (n=23)		High DTX1 Expression (n=24)		
	Range	Median	Range	Median	
ABCC1	14.64- 101.8	42.80	19.15- 272.0	48.34	0.2211
ABCC2	**0.6033- 37.03**	**3.910**	**1.718- 77.90**	**10.10**	**0.0325**
ABCC3	0.2319- 42.78	3.385	0.3660- 25.09	4.582	0.4006
ABCC4	9.428- 95.96	16.79	4.159- 59.19	25.90	0.1509
ABCC5	**11.70- 121.2**	**38.89**	**24.00- 286.0**	**70.75**	**0.0059**
ABCG2	0.1010- 6.856	1.598	0.06943- 39.50	1.069	0.4126
ASNS	**1.794- 40.37**	**4.829**	**4.466- 45.83**	**10.67**	**0.0007**
BCL2	**12.55- 215.1**	**29.75**	**18.82- 240.5**	**66.37**	**<0.0001**
BCL-XL	11.63- 78.15	28.39	14.04- 76.31	35.29	0.3021
DHFR	**14.78- 143.2**	**33.13**	**13.99- 128.8**	**57.83**	**0.0132**
FPGS	**7.993- 41.81**	**17.78**	**13.36- 63.21**	**25.08**	**0.0042**
GCR	**24.57- 336.5**	**58.69**	**58.50- 665.2**	**133.7**	**0.0003**
GGH	0.2483- 6.305	1.792	0.1907- 14.57	1.806	0.9406
HPRT	6.223- 31.62	12.87	5.946- 54.13	17.41	0.0722
hRFC	**3.485- 429.8**	**14.23**	**7.088- 779.8**	**27.68**	**0.0277**
MAP4	**4.404- 49.64**	**11.49**	**1.686- 70.70**	**18.23**	**0.0081**
MDR1	**24.40- 1301**	**131.0**	**40.43- 729.9**	**225.5**	**0.0149**
TPMT	**3.612- 213.8**	**9.779**	**5.546- 131.4**	**19.31**	**0.0034**
TOP2A	0.7263- 22.05	5.242	0.1085- 26.64	4.432	0.6321
TOP2B	7.936- 129.0	22.89	5.078- 87.59	29.50	0.1129
TUBB1	134.3- 759.6	391.3	105.2- 1320	346.5	0.9068
TUBB3	0.08313- 1.513	0.2262	0.08392- 2.510	0.3261	0.4892

n=number of patients studied. Patients with DELTEX1 transcript expression below the median value were considered to have low DELTEX1 expression, and those with DELTEX1 transcript expression above DELTEX1 median values were considered to have high DELTEX1 expression**.** Relative transcript levels for the target genes were measured by real-time RT-PCR, as described in Materials and Methods. Gene abbreviations are summarized in the legend to Table 4. The non-parametric Wilcoxon test was used for comparisons of transcript levels between groups and the *p* values are reported in the table. Bold and italicized entries were statistically significant between the groups.

Table 12: Expression of NOTCH1 Target Genes and Chemotherapy Relevant Genes in Both Low and High cMYC Expression Patients

SYMBOL	TRANSCRIPTS (RELATIVE UNITS)				*P*
	Low cMYC Expression (n=24)		High cMYC Expression (n=23)		
	Range	Median	Range	Median	
ABCC1	16.09- 199.8	49.89	14.64- 272.0	43.30	0.3021
ABCC2	0.6033- 48.27	6.053	1.060- 77.90	8.680	0.6021
ABCC3	**0.2319- 8.135**	**2.404**	**0.3660- 42.78**	**5.142**	**0.0034**
ABCC4	**5.061- 42.09**	**14.61**	**4.159- 95.96**	**29.81**	**<0.0001**
ABCC5	**11.70- 111.4**	**36.03**	**31.10- 286.0**	**68.57**	**0.0002**
ABCG2	0.4111- 6.856	1.563	0.06943- 39.50	1.249	0.2549
ASNS	**1.794- 17.13**	**5.540**	**4.466- 45.83**	**10.48**	**0.0020**
BCL2	**12.55- 94.73**	**30.26**	**13.29- 240.5**	**68.92**	**0.0001**
BCL-XL	14.04- 78.15	27.15	11.63- 76.31	31.46	0.4892
DHFR	**13.99- 83.52**	**28.13**	**23.56- 143.2**	**63.01**	**<0.0001**
FPGS	7.993- 44.48	17.65	8.093- 63.21	20.11	0.1082
GCR	32.49- 212.2	70.28	24.57- 665.2	130.6	0.0597
GGH	0.2334- 6.720	2.225	0.1907- 14.57	1.591	0.5027
HPRT	**6.223- 42.61**	**12.34**	**5.946- 54.13**	**18.04**	**0.0308**
hRFC	**3.485- 294.4**	**11.37**	**7.127- 779.8**	**26.62**	**0.0097**
MAP4	7.153- 70.70	14.42	1.686- 59.68	15.03	0.9915
MDR1	**24.40- 424.2**	**128.2**	**40.43- 1301**	**230.8**	**0.0017**
TPMT	**3.612- 36.73**	**10.73**	**5.791- 213.8**	**26.57**	**0.0008**
TOP2A	0.9799- 19.75	4.751	0.1085- 26.64	5.242	0.9406
TOP2B	8.263- 129.0	23.02	5.078- 87.59	30.66	0.1036
TUBB1	198.9- 1320	359.6	105.2- 912.5	362.8	0.9745
TUBB3	0.08313- 1.513	0.2646	0.08392- 2.510	0.3405	0.5874

n=number of patients studied. Patients with cMYC transcript expression below the median value were considered to have low cMYC expression, and those with cMYC transcript expression above cMYC median values were considered to have high cMYC expression**.** Relative transcript levels for the target genes were measured by real-time RT-PCR, as described in Materials and Methods. Gene abbreviations are summarized in the legend to Table 4. The non-parametric Wilcoxon test was used for comparisons of transcript levels between groups and the *p* values are reported in the table. Bold and italicized entries were statistically significant between the groups.

2.3g Identification of NOTCH1, FBW7 and PTEN Mutations at Diagnosis and Relapse

To begin to investigate potential genetic alterations that contribute to relapse in T-ALL, we assessed the frequencies of mutations in NOTCH1 alone, and in combination, with mutations in FBW7 and PTEN at the time of diagnosis and relapse in paired clinical T-ALL specimens. The immediate goal was to evaluate the stability of alterations in these three genes and to determine if any genetic alterations were associated with disease progression and treatment failure. It is reasonable to hypothesize that these mutations may be causal factors in relapsed T-ALL since we have shown that aberrant NOTCH1 signaling is associated with the increased expression of chemotherapy drug resistance/sensitivity genes (above), and we and others have shown that PTEN is frequently inactivated, either by mutations or post-translational modifications, which can lead to increased Akt activity and chemotherapy resistance[198].

For this study, we analyzed paired diagnostic and relapsed samples from 11 T-ALL patients. The patient characteristics are summarized in Table 13. The cohort included eight males with a median diagnostic age of 120 months (10 years) and three females with a median diagnostic age of 161 months (13.4 years). The overall time to relapse (from the date of the initial diagnosis) was 10 months, with the males exhibiting a slightly faster relapse time of 9.5 months.

2.3g i Identification of NOTCH1 and FBW7 Mutations

NOTCH1, FBW7 and PTEN mutations were amplified and identified in the RNAs or gDNAs isolated from cryopreserved lymphoblasts or DNAs isolated from bone marrow aspirate slides. The mutational status of this paired cohort is summarized in

Table 13: Characteristics of the 11 Paired Pediatric T-ALL Specimens

Sample ID	*Sex*	*Race*	*WBC/µl*	*Age at Diagnosis*	*Time to Relapse*
T20184	F	n/a	n/a	84m (7y)	11m
T20319	M	n/a	187,000	120m (10y)	4m
T20320	M	W	n/a	72m (6y)	29m (2y5m)
T20321	M	W	n/a	163m (13y7m)	17m (1y5m)
T20322	F	A	29,337	161m (13y5m)	10m
T20323	M	n/a	160,000	48m (4y)	6m
T20324	M	A	160,000	180m (15y)	3m
T20326	M	n/a	n/a	69m (5y9m)	14m (1y2m)
T20327	M	A	n/a	156m (13y)	5m
T20328	M	H	107,000	120m (10y)	13m (1y1m)
T20329	F	W	n/a	180m (15y)	5m

The overall median age at the time of diagnosis was 120 months (10 years) with a standard deviation of 48.28 months. The median time to progression was 10 months with a standard deviation of 7.632 months. For the 8 males, the median age at diagnosis was 120 months (10 years), while the female had a median age of 161 months (13.4 years). The males had a median time to relapse of 9.5 months, slightly shorter than the female median age to relapse of 10 months. Abbreviations: F, female; M, male; W, White; A, African American; H, Hispanic; n/a, not available; WBC, white blood cell; m, months; y, years.

Table 14. Surprisingly, all eleven patients had wild-type FBW7 at both diagnosis and relapse. Seven patients exhibited wild-type NOTCH1 at both diagnosis and relapse. For these patients, the median age at diagnosis was 156 months (13 years) with a median time to relapse of 6 months. Three of these patients with wild-type NOTCH1 (T20184, T20324 and T20327) had single nucleotide polymorphisms (C5094T or G7083A) that did not change the NOTCH1 amino acid sequence. The exception is patient T20327, who at relapse acquired a SNP in NOTCH1. These data suggest that for this group of patients, mutant NOTCH1 does not appear to be a casual factor of relapse.

Four patients harbored NOTCH1 mutations at diagnosis, relapse, or both. Together, these four mutant NOTCH1 patients had a median diagnostic age of 120 months (10 years) with a median time to progression of 11.5 months. Two patients (T20320 and TT20322) had NOTCH1 mutations at both diagnosis and relapse, but the mutation at relapse differed from that at diagnosis. Interestingly, these patients relapsed 29 months and 10 months later. One patient (T20319) had wild-type NOTCH1 at the time of diagnosis, but acquired a NOTCH1 mutation at relapse, and experienced relapse within 4 months. The last patient (T20328) harbored the same NOTCH1 mutation at both diagnosis and relapse. This patient relapsed 13 months after initial diagnosis. It can be concluded that for this cohort, patients that were wild-type for NOTCH1 were diagnosed with T-ALL at a later age (13 years vs. 10 years), but generally experienced more rapid rate of relapse (6 months vs. 11.5 months), however these differences were not statistically significant.

2.3g ii Identification of PTEN Mutations at Diagnosis and Relapse

Six patients were wild-type for PTEN at both diagnosis and relapse (Table 14), suggesting that relapse was not caused by genetic alterations to PTEN. These patients had a median diagnostic age of 120 months (10 y) and a median time to relapse of 5.5 months. The remaining 5 patients harbored PTEN mutations at diagnosis, relapse or both. These patients had a median diagnostic age of 156 months (13 y) and a median time to relapse of 14 months. Two patients (T20320 and T20327) had detectable PTEN mutations at the time of diagnosis that completely disappeared by relapse. The time to progression to relapse for these two individuals was 29 months and 5 months, respectively. Two patients (T20322 and T20326) had the same PTEN mutation at the time of diagnosis and relapse. The time to relapse progression was 10 months and 14 months, respectively. Only one patient (T20321) had different PTEN mutations at both diagnosis and relapse, and experienced relapse 17 months after diagnosis.

2.3g iii The Impact of the Combination of NOTCH1 and PTEN Mutations at Diagnosis and Relapse

Of the 11 patients, only 4 (36%) were completely wild-type for both NOTCH1 and PTEN. These 4 patients (T20184, T20323, T20324 and T20329) had a median age at diagnosis of 132 months (11 y) with a median time to progression of 5.5 months. From these 4 patients, neither NOTCH1 activation (via presence of NOTCH1 mutations) nor PTEN inactivation (via presence of PTEN mutations) appeared to play an appreciable role in relapse. The remaining 7 patients showed either NOTCH1 mutations, PTEN mutations or both either at diagnosis, relapse or both. These patients had a median age at diagnosis of 120 months (10 y) and a median time to progression of 13 months.

Table 14: Mutational Status of the 11 Paired Diagnosis/Relapse Patients

Patient	Diagnosis or Relapse	NOTCH1		PTEN	
		DNA Mutations	AA Change	DNA Mutations	AA Change
T20184	D	C5094T	WT	WT	WT
	R	C5094T	WT	WT	WT
T20323	D	WT	WT	WT	WT
	R	WT	WT	WT	WT
T20324	D	C5094T	WT	WT	WT
	R	C5094T	WT	WT	WT
T20329	D	WT	WT	WT	WT
	R	WT	WT	WT	WT
T20326	D	WT	WT	INS1732(ACCG), INS1768(CT), G1769A	Δ235-243
	R	WT	WT	INS1732(ACCG), INS1768(CT), G1769A	Δ235-243
T20328	D	T4754T/C	L1585P	WT	WT
	R	T4754C	L1585P	WT	WT
T20321	D	WT	WT	INS1730(A)	Δ234-241, stop@242
	R	WT	WT	Del 1348-2473	Δ106-119, Δ121-125, stop@126
T20320	D	A4808A/G	N1603S	C1768C/G	P246R
	R	G4948A	A1650T	WT	WT
T20327	D	WT	WT	A1111G	Y27C
	R	G7083G/A	WT	WT	WT
T20319	D	C5094C/T	WT	WT	WT
	R	T5039T/A, C5094C/T	I1680N	WT	WT
T20322	D	C5094C/T, C7470C/A	stop@ 2490	T1913T/G	S294R
	R	C5094C/T, T5153T/C	I1718T	T1913T/G	S294R

The position of NOTCH1 mutations is based upon the database sequences for NOTCH1 (NM_017617.3). The position of PTEN mutations is based upon the database sequences for PTEN (NM_000314.4). Abbreviations are: INS, Insertion; DEL, Deletion; WT, Wild type; Δ, changed sequence.

Based on these data in this small cohort of paired specimens, the presence of NOTCH1 and/or PTEN mutations may indeed be associated with a delay in relapse. However, it does not appear that relapse is associated with the presence of these mutations, and that some other underlying factor is most likely causal.

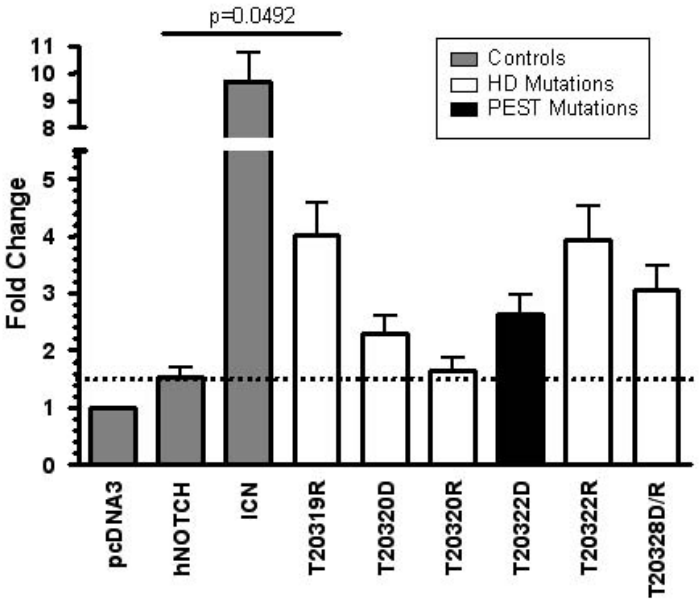
2.3g iv Activating Potential of NOTCH1 Mutations in Diagnostic and Relapse Samples

The transactivating potential of the NOTCH1 mutations was assessed by determining the impact of these mutations on the transactivation of a HES1-Luc reporter (Figure 10). As described above, for these experiments, mutant NOTCH1 constructs in pcDNA3 were transiently transfected into U20S cells with the HES1-Luc reporter. As was seen with the 16 NOTCH1 mutations in the 47 T-ALL cohort, these mutations were activating to different degrees, ranging from 1.5 to 2.6-fold increases in the HES1-Luc reporter activity. Interestingly, only the NOTCH1 mutation in T20319R induced a statistically significant increase in activity compared to wild-type NOTCH1 ($p=0.0492$).

2.3g v Detection of Relapse Clones at Diagnosis

For 5 patients (T20319, T20320, T20321, T20322 and T20327), there is the emergence of a new mutation, either in NOTCH1 and/or PTEN, at the time of relapse, or in the case of T20327R, the loss of a mutation (Table 14). From these data, we can only assume that relapse must have arisen from a new leukemic clone. However, whether this clone is identical to the diagnostic clone, but acquired a new genetic mutation, or if it is a completely different clone that was present as a minor subpopulation at diagnosis is unknown. We made these assumptions based upon the fact that different NOTCH1 and PTEN mutations were detected at the time of diagnosis and relapse (Table 14). We hypothesized that these relapse leukemic clones were already present at the time of

Figure 10: Activity of NOTCH1 Mutations in the Diagnostic/Relapse Patients



Human U2OS cells were transiently co-transfected in 35 mm dishes with 0.9 μg of the indicated NOTCH1 expression plasmid. 1 μg of HES1-Luc reporter gene construct and 30 ng of *Renilla* luciferase (pRL-SV40) internal control were used. For all transfections, constant plasmid was maintained at 0.9 μg of pcDNA3 plasmid per well. Results represent normalized luciferase activities of whole cell lysates, relative to a control in which HES1-luc was co-transfected with 0.9 μg pcDNA3 vector *in lieu* of NOTCH1 (assigned a value of 1). Results are presented as mean values $\pm$ standard errors. p-values were calculated using paired t tests, comparing the luciferase activities of the different NOTCH1 mutations to wild-type NOTCH1.

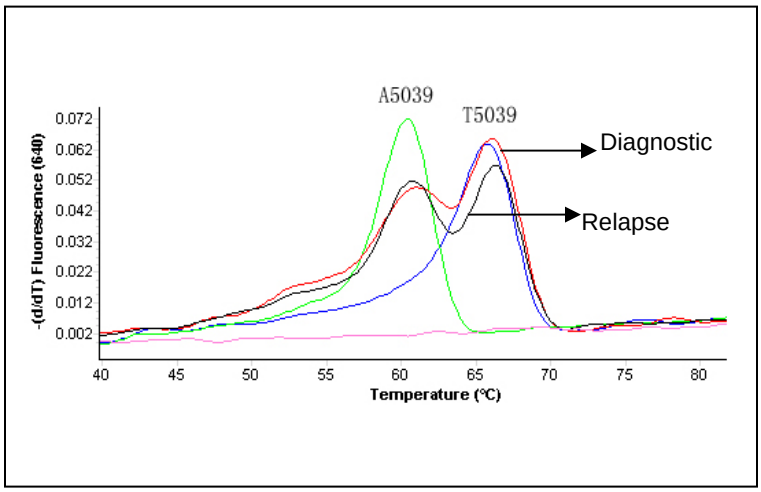
diagnosis, but were just below our limit of detection. To address this possibility, we used real-time PCR to detect whether the newly identified relapse T-ALL clones could be detected in the diagnostic specimens as a minor subclinical population. For this, specific NOTCH1 hybridization probes were designed to detect the relapse NOTCH1 mutation in patient T20319 (T5039A) and patient T20322 (T5153C) in lymphoblasts collected at diagnosis. These methods were validated by plasmid constructs including pure wild-type or mutant NOTCH1 sequence. For T20319, the relapse NOTCH1 mutation (T5039T/A) was detected in the diagnostic sample at a low frequency (Figure 11), suggesting that in this patient, the relapse leukemic clone was indeed present at diagnosis and was able to escape chemotherapy, perhaps because this NOTCH1 mutation rendered it resistant to chemotherapy. For T20322, the relapse NOTCH1 mutation (T5131T/C) was not detected at diagnosis (Figure 11). This suggests that either this leukemic clone arose after chemotherapy or our detection method was not sufficiently sensitive to detect it at diagnosis.

2.4 Conclusions

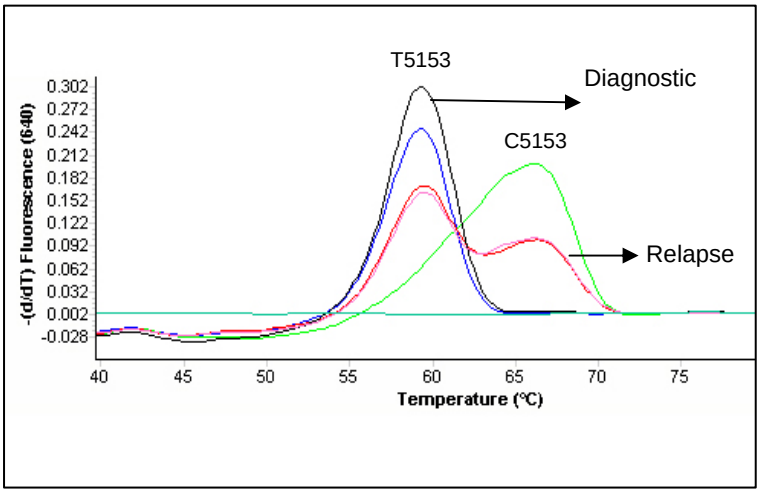
Our results suggest that multiple factors should be considered when attempting to identify molecularly-based prognostic factors for pediatric T-ALL. Our results further established the presence of high frequency mutations in NOTCH1 and FBW7 in pediatric T-ALL. Mutant NOTCH1 was associated with a range of activating potentials, as reflected in activities from HES1 promoter-reporter gene assays that were consistently elevated over wild-type NOTCH1. Although mutant NOTCH1 and mutant FBW7 were associated with increased HES1 promoter-reporter activities and increased transcript levels for the NOTCH1 target genes, HES1, DTX1, and cMYC, the range of transcripts

Figure 11: Detection of Relapse T-ALL Clone as a Subclone at Diagnosis

A. T20319



B. T20322



Genotype analysis of (A) T20319 (NOTCH1 mutation T5039A) and (B) T20322 (NOTCH1 mutation T5153C) was performed using a LightCycler real time PCR with gene-specific primers and 3'fluorescein-labeled and 5'LC-red640-labeled hybridization probes. Samples were amplified over 35 cycles, and melting curves for the products were analyzed at 640nm for 40°-80°C at a rate of 0.3°C/sec. For A, the blue line is pure wild-type NOTCH1 template (TT) and the green line is the pure mutant template (AA). Patient gDNAs are designated by the red line (Diagnostic) and the black line (Relapse). The pink line designates the negative control. For B, the blue line designates pure wild-type NOTCH1 template (TT) and the green line is the pure mutant template (CC). Patient gDNAs are designated by the black line (Diagnostic) and the red line (Relapse). The teal-green line designates the negative control.

was surprisingly broad and there was significant overlap between the mutant and wild-type T-ALL samples. This appears to reflect the different transactivating potencies for the various NOTCH1 mutants and possibly other factors that impact overall NOTCH1signaling [e.g., NUMB[90]]. Collectively, these results imply that, rather than simply scoring the mutant status of NOTCH1 and/or FBW7, overall signaling activity, as reflected in the cumulative transcript levels for these established NOTCH1 target genes, is likely to be far more meaningful to the biology and therapy of T-ALL.

In our analysis, neither the presence of NOTCH1 and/or FBW7 mutations, nor relative HES1/DTX1/cMYC transcript levels, were directly associated with treatment failure in our pediatric cohort. Likewise, neither PTEN mutations alone, nor combinations of mutations in PTEN with NOTCH1 and FBW7, were prognostic.

Interestingly, elevated transcripts for the downstream NOTCH1 gene targets were accompanied by consistent and statistically significant increases in transcript levels for chemotherapy-related genes including MDR1, ABCC5, asparagine synthetase, Bcl-2, human reduced folate carrier, dihydrofolate reductase and thiopurine methyltransferase. While the nature of these associations, including causal mechanisms, is not established, from these results, the net level of chemotherapy drug response would seem to reflect a composite phenotype, including an increased sensitivity to methotrexate due to increased human reduced folate carrier, and increased resistance for assorted chemotherapy agents due to increased Bcl-2 (multiple agents), MDR1 (doxorubicin, vincristine), ABCC5 (6-mercaptopurine, methotrexate), asparagine synthetase (L-asparaginase), and dihydrofolate reductase (methotrexate). Perhaps most importantly, the relative importance of these mechanisms would reflect the combinations of chemotherapy drugs

administered, along with drug doses and schedule. Additional determinants of chemotherapy activity include PTEN levels or the presence of inactivating PTEN mutations and potential downstream effects of NOTCH1 on PI3K-AKT and mTOR signaling pathways.

Additional studies are undoubtedly necessary to establish the mechanisms (i.e., direct or indirect effects of NOTCH1 and downstream signaling) that result in altered expression of these drug resistance/sensitivity genes, along with studies to extend these results to other tumors with aberrant NOTCH1 signaling. Deregulated NOTCH signaling involving NOTCH receptors, ligands and targets has been also observed in solid tumors and high levels of NOTCH1 and Jagged1 ligand were associated with a poor prognosis in breast cancer[230] and metastasis in prostate cancer[144]. Finally, an important implication of our results is that, depending on the NOTCH1 signaling status, modifications in the types or dosing of standard chemotherapy drugs for T-ALL, or combinations of agents capable of targeting NOTCH1 such as GSIs[231] or AKT and mTOR inhibitors, with standard chemotherapy agents may be warranted.

We also extended our studies of NOTCH1, PTEN and FBW7 mutations to their potential roles in the occurrence of relapse, which is the most common cause of treatment failure[25]. The 11 paired specimens were analyzed for the presence of NOTCH1, FBW7 and PTEN mutations at both diagnosis and relapse. Surprisingly, FBW7 mutations were not detected. This implies that these mutations are not causal factors for relapse. It was observed that the seven patients harboring mutations at some stage in their disease had a longer remission period (13 months vs. 5.5 months), and were typically diagnosed at an earlier age (120 months vs. 132 months). In these seven patients, nearly 70% of relapse

appeared to be associated with the emergence of a new leukemic clone, an assumption based on our detection of a new mutation or loss of mutation at relapse. Whether or not these new leukemic clones were the result of an induced acquired genetic alteration or were already present at diagnosis as a subpopulation was analyzed for 2 patients. Using real-time PCR techniques with specific hybridization probes, the relapse clone (as identified by the emergence of a NOTCH1 mutation) for T20319 was detected at diagnosis, at very low levels. This establishes that this clone which may directly or indirectly contribute to relapse was indeed present in the initial diagnostic leukemia specimen. For patient T20322, the relapse clone (again, identified by the presence of a different NOTCH1 mutation) was not detected at diagnosis. Assuming that our method of detection is sufficiently sensitive to detect the clone, it appears that the relapse clone was induced following chemotherapy. This study strongly warrants future studies with a larger patient cohort to systematically identify specific hallmarks of relapse.

It has been documented that MRD levels are good indicators of relapse risk. In a recent study, patients with low levels of MRD had about a 13% chance of relapsing within 5 years, while patients with undetectable MRD following induction therapy had only a 5% chance of relapsing[232]. However, such studies may be difficult to conduct since T-ALL accounts for less than 15% of all ALL cases, and modern aggressive therapies are increasing the cure rates for this disease.

CHAPTER 3

THE DOWNSTREAM EFFECTS OF NOTCH1 SIGNALING: THE DEVELOPMENT OF CELL LINE MODELS

3.1 Introduction

The discovery of 'gain-of-function' NOTCH1 mutations in both children and adults with T-ALL suggests that aberrant NOTCH1 signaling is important in the pathogenesis of T-ALL[91, 125, 131, 132, 138-140]. However, the exact role of NOTCH1 in the etiology and therapy of this disease has not been well established. Inhibiting NOTCH1 signaling with γ-secretase inhibitors (GSIs) is an attractive therapeutic strategy because NOTCH1 is mutated in such a large number of T-ALLs, and GSIs are effective at inhibiting the NOTCH1 signaling process. Previous studies have shown that treatment of T-ALL cell lines with GSIs can induce cell growth arrest and apoptosis[125, 127, 154]. However, Liu *et al.* demonstrated that GSI treatments can have different effects on different cell lines[233]. Treating GSI-sensitive T-ALL cell lines TALL-1 and HSB2 with the GSI Compound E (CompE) for 3 or 4 days resulted in G0/G1 arrest, as indicated by accumulation of cells in G0/G1 and retention of cells in both S-phase and G2/M. GSI treatment also induced apoptosis in these cells nearly 2.3-2.9-fold. However, in the GSI-resistant cell lines, CCRF-CEM (CEM) and Jurkat, little-to-no changes were observed in cell cycle arrest or apoptosis following GSI treatment. Pretreating TALL-1 and HSB2 cells with CompE augmented the apoptotic effect induced by treating with L-asparaginase (L-Asp) or dexamethasone. Conversely, treating CEM and Jurkat cells with CompE prior to chemotherapeutic agents appeared to antagonize their apoptotic effect. Thus, it appears that depending on the cell type, NOTCH1 inhibition either can induce

apoptosis and synergize with chemotherapy, or is ineffective at inducing apoptosis and can antagonize the chemotherapeutic effect. This is entirely consistent with the studies described in Chapter 2.

In order to use NOTCH1 inhibition for targeted therapy, we need to fully understand the downstream functions of NOTCH1 signaling. Only then can we begin to apply our mechanistic insights related to NOTCH1 signaling to improved therapy for T-ALL. This is particularly true in the case of GSIs' cell-specific effects. An important goal of Chapters 3 and 4 is to better understand the downstream effects of NOTCH1 signaling, specifically the complex relationships between NOTCH1 with both the PI3K-Akt and mTOR pathways, and how this maybe exploited for therapy. These pathways are key mediators of cell proliferation and survival and are of particular interest because there are clinically relevant inhibitors available for both pathways. The effects of NOTCH1 activity on these two pathways are likely to have significant impacts in conferring chemotherapy sensitivity or resistance. We suspect that the effect of NOTCH1 targeting in T-ALL therapy may synergize or antagonize the activity of standard chemotherapy agents, depending upon the characteristics of the T-ALL cell. This chapter and the following chapter will focus on the effects of NOTCH1 signaling on novel genes and pathways likely to impact disease progression and chemotherapy sensitivity in clinically relevant T-ALL cell line models with differences in PTEN status and AKT signaling.

3.2 Materials and Methods

3.2a Cell Lines

The human T-ALL cell lines used in these studies, along with their unique characteristics, are summarized in Table 15. The cell lines were grown in RPMI 1640, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/mL) and streptomycin (100 μg/mL) in a humidified atmosphere at 37°C in the presence of 5% CO_2. Transient inhibition of NOTCH1 was performed using 1 μM of Compound E ([(2S)-2-([(3,5-difluorophenyl)acetyl]amino)-*N*-[(3S)-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl]propanamide]; Axxora, San Diego, CA). Control cells were treated with an equal volume of DMSO for the same time period.

3.2b Isolation of RNA

RNAs were isolated using the TRIzol® (Invitrogen) protocol. Briefly, cells were lysed in 1 mL TRIzol®; and phase separation was induced with 200 μL chloroform (Fisher). RNA was then precipitated with isopropyl alcohol (Fisher), pelleted and washed with 75% ethanol. The RNA pellet was allowed to air dry, followed by resuspension in PCR-grade water. cDNAs were prepared using random hexamer primers (see 2.2b) or oligo(dT) from SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen), and purified with the QIAquick PCR Purification kit (Qiagen).

3.2c RT²Profiler™ PCR Array

Total RNAs were purified with the SuperArray RT² qPCR-Grade RNA Isolation Kit (SABiosciences; Fredrick, MD). The PI3K-AKT Signaling Pathway RT²Profiler™ PCR Array (SABiosciences) contained 84 genes deemed most relevant to PI3K-Akt signaling pathway. cDNAs were prepared from 1μg RNA using RT² First Strand Kit

Table 15: Summary of Human T-ALL Cell Lines Used and Their Reported Characteristics

Cell Line	NOTCH1 Status	FBW7 Status	PTEN Status	GSI Sensitivity
HPB-ALL	L1575L/P; heterozygous ins 2442 (EGRGRCSHWAPAAWRCTLFCPRRAPP CPRRCHPRWSHP*STOP	WT	WT	Sensitive
DND-41	L1594L/P; D1610D/V; heterozygous ins 2444 (CCSHWAPAAWRCTLFCPRRAPPCPRR CHPRWSHP*STOP)	WT	WT	Sensitive
ALL-SIL	L1594L/P; 2475 (ASP*STOP)	WT	WT	Sensitive
TALL-1	WT	WT	N/A	Sensitive
Jurkat	WT	R505R/C	2bp deletion and 9bp insertion (exon 7) or 39bp insertion (exon 7)	Resistant
CEM	Heterozygous ins 1595 (PRLPHNSSFHFL)	R465R/H	Deletion exons 2-5	Resistant
RPMI-8402	Heterozygous ins 1584 (PVELMPPE)	R465H	Frameshift sequence at AA236; R159S	Resistant
MOLT4	L1601L/P; heterozygous del 2515 (RVP*STOP)	WT	WT	Resistant

Adapted from: O'Neil, J et al JEM VOL. 204, August 6, 2007; Palomero, T et al Nat Med 2007 Oct; 13(10): 1203-10; Sakai, A et al Blood, Vol 92, No 9 (November 1), 1998: pp3410-3415.

(SABiosciences) and added to RT^2 qPCR Master Mix containing SYBR Green and reference dye. This master mix was aliquoted across the PCR plate. Thermal cycling was performed by LightCycler 480 (Roche) and data were analyzed by software provided by SABiosciences. Each cell line treatment was performed in duplicate, and validated by real-time qPCR, as described in Chapter 2.

3.2d Cell Proliferation Assays

Cells were seeded at 7.5 x 10^4 cells/mL in a total of 10 mL of media. At 24h intervals, cells were counted using Trypan Blue and a hemocytometer. Prism Software v. 4.0 (GraphPad) was used to graph growth and calculate population doubling times.

3.2e Western Blot Analysis

Proteins were isolated from whole cell extracts. Briefly, cells were pelleted and dissolved in protein inhibitor (PI) mix containing 10 mM Tris, 0.5% sodium dodecyl sulfate (SDS) and 1 tablet of Phos-STOP (Roche). Membranes were disrupted by sonication, cell debris was collected by centrifugation and protein-containing supernatant was transferred to new microcentrifuge tube. Proteins were quantitated using a bovine serum albumin (BSA) standard curve.

Proteins were electrophoresed on 10% polyacrylamide gels in the presence of SDS and transferred to polyvinylidene difluoride membranes (Fisher). Proteins were detected using primary antibodies of choice (see below) and a secondary IRDyeTM 800-conjugated Antibody (Rockland). Detection and densitometry were performed with the Odyssey® Imaging System (Licor; Lincoln, NE). The primary antibodies used were:

- Cleaved Notch1 (Val1744) antibody (Cell Signaling Technology [CST]; Danvers, MA) was used in a 1:250 dilution with a tertiary detection method.

Following incubation with Val1744, the blot was washed with PBS+0.1%Tween, then incubated for 1hr with goat-anti-rabbit antibody in a 1:1000 dilution, followed by another wash with PBS+0.1%Tween. Lastly, the antibody was incubated for 1hr with anti-goat 800 in a 1:1000 dilution.

- Phospho-Akt Ser473 antibody (CST) was used in a 1:250 dilution
- total Akt antibody (CST) was used in a 1:1000 dilution
- 4E-BP1 (CST) antibody was used in a 1:1000 dilution
- PI3 Kinase p110α, PI3 Kinase p110γ, PI3 Kinase p110δ (CST) antibodies were used in 1:250 dilutions
- Phospho-S6 Ribosomal Protein (Ser235/236) (CST) antibody was used in 1:1000 dilution
- S6 Ribosomal Protein (CST) antibody was used in 1:1000 dilution
- PTEN (CST) antibody was used in a 1:1000 dilution
- β-Actin (Sigma) antibody was used in 1:2000 dilution.

3.2f Lenti-viral Knockdown of NOTCH1

Jurkat cells were seeded at 2 x 10^5 cells/well in 1mL media in 24-well plate. NOTCH1 shRNA particles[234] and a non-targeted control (ntc, or scrambled) shRNA were pre-packaged by Sigma (St. Louis, MO). The lentiviral particles were added to cells with 4 μg/ul polybrene and allowed to incubate for 24h at 37°C. The viral particles were removed by centrifugation and the cells were transferred to new 24-well plate with 1mL media and 0.25 μg/mL puromycin. The mixed transduced cultures were expanded and plated in soft-agar to allow for the selection and isolation of single clones. Clones were then tested for the knockdown of NOTCH1 by Western blotting and real-time qPCR.

3.2g AnnexinV/PI/Fluorescent Bead *In-Vitro* Cytotoxicity Assay

The NOTCH1 knocked-down stable clones (J.ntc, J.N1KD 2-4 and J.N1KD 2-7) in logarithmic growth phase were resuspended thoroughly and 50μl aliquots of each were processed in triplicate using the AnnexinV-FITC/Propidium Iodide (PI) staining kit (Immunotech; Marseille, France), according to manufacturer's instructions. After incubation, samples were diluted with 400 μl of 1X AnnexinV Buffer containing approximately 5% of FlowCount Fluorospheres (Beckman Coulter; Miami, FL) as an internal monitor for determining relative absolute counts. Tubes were vortexed and analyzed immediately using a Beckman Coulter XL Flow Cytometer equipped with an Argon laser (Beckman Coulter). Cells were gated to include the viable target cell population based on inspection of forward scatter (FS)/ side scatter (SS) characteristics, and absolute relative counts of AnnexinV-/PI- events (i.e.; the viable cell fraction) were determined. Additionally, total AnnexinV+ events were recorded from the ungated cell population to assess overall early and late apoptotic induction. All results were compared to control tubes.

3.2h Cell Cycle Analysis

The NOTCH1 knocked-down stable clones (1×10^6 cells) were washed once in 2 mL cold PBS and resuspended in 150μl cold PBS+0.1% glucose. While vortexing, 1 mL of cold 80% ethanol was added in a drop-wise fashion to each sample. For analysis, cells were resuspended gently and centrifuged for 3min at 500g. The supernatant was removed, leaving approximately 300 μl of residual volume. The cells were again gently vortexed and 2 mL of cold Coulter DNA-Prep reagent (Beckman Coulter) was added. The samples were left at room temperature in the dark for 1-2h with occasional

vortexing, and stored overnight at 4ºC before being analyzed. The samples were analyzed on a Beckman Coulter XL Flow Cytometer (Beckman Coulter) by measuring PI fluorescence on FL3 and histograms were analyzed for G0/G1, S and G2/M phase content using defined parameters.

3.2i Cell Surface Marker Expression Analysis

The NOTCH1 knocked-down clones were resuspended in PBS+30% adult bovine serum at a concentration of approximately 5 x 10^6 cells/mL and aliquoted to tubes containing both FITC and phycoerythrin (PE)-conjugated monoclonal antibodies (Immunotech). Cells were stained in the dark for 20 min at room temperature and washed with 1 mL of cold PBS. The cells were then resuspended in 0.5 mL PBS+Fix (PBS+0.4% formaldehyde). The samples were screened for two-color analysis on a Beckman Coulter XL Flow Cytometer (Beckman Coulter) equipped with an Argon laser. Samples were gated on the viable cell fraction as inferred from FS/SS characteristics. Percent positivity and mean channel fluorescence rations of specific antigens were assessed by comparisons to isotype-matched controls.

3.2j MTT Cytotoxicity Assay

Cells were resuspended in RPMI1640 containing 20% dialyzed fetal bovine serum and 2mM L-glutamine, and seeded at 4000 cells/well in a 96-well plate. Chemotherapeutic agents were diluted in the above medium at the highest concentration needed, and then serially diluted until the smallest concentration needed was achieved. The chemotherapy drug, or vehicle control, was then added to the appropriate wells. The following chemotherapeutic agents were analyzed:

- Methotrexate (MTX)

- Daunorubicin (DNR; Sigma)
- VP16 (Sigma)
- L-Asparginase (Sigma)

Cells were then allowed to incubate with the drug for 4 days at 37°C at 5% CO_2 in a humidified incubator. Next, 10 µl of 2.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) in sterile PBS was added to cells and incubated for 4h at 37ºC. Cells were then lysed by adding 50 µl of 10%SDS in HCl to each well and incubated overnight at dark. Plates were read on visible microplate reader at 595 nm.

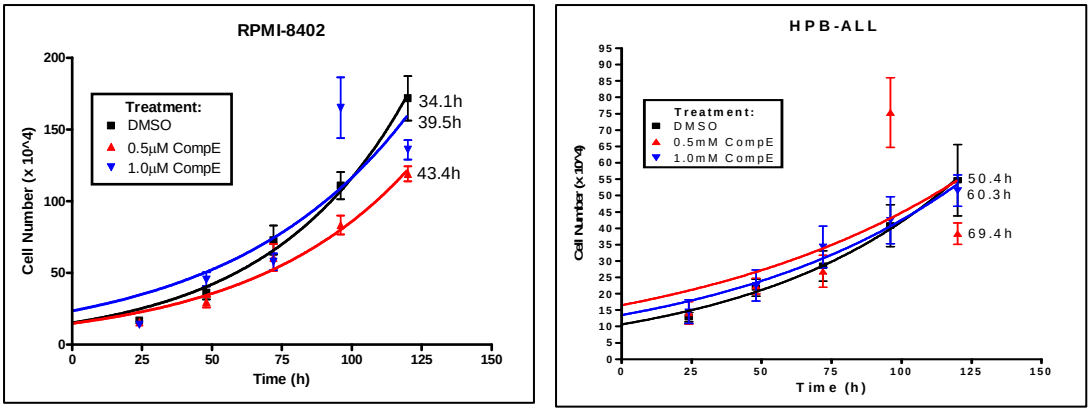
3.3 Results

3.3a Effects of NOTCH1 Inhibition on Cell Proliferation

RPMI-8402 (mutant PTEN) and HPB-ALL (wild-type PTEN) cells were seeded at 7.5 x 10^4 cells/mL and treated with 1.0 µM or 0.5 µM Compound E (CompE) or 10 µl of DMSO as a control. Cells were counted at 24 h intervals for 5 days and the population doubling times were calculated using GraphPad Prism (v. 4.0). There was an increase in doubling times for both RPMI-8402 and HPB-ALL upon CompE treatment (Figures 12A (RPMI-8402) and 12B (HPB-ALL)). For RPMI-8402, there was a 5-9 h increase in population doubling when treated with CompE. Similar results were seen in the HPB-ALLs treated with CompE; however the increase in doubling time was much greater (10-19 h). Thus, CompE inhibits cell growth, although the magnitude of the observed effect may be dependent upon other cellular factors. For example, the RPMI-8402 cells have a mutation in PTEN that likely inactivates the function of this protein. As a result, Akt activity is expected to be high and to promote cell survival despite the presence of

Figure 12: Effects of GSI Treatment on Cell Growth

12A: 12B:



Cells were seeded at 7.5 x 10^4 and treated with either 0.5μM or 1.0μM of CompE or an equal volume of DMSO at time 0h. Cells were counted at 24h intervals with a hemacytometer and Trypan Blue. Growth was graphed and population doubling times were calculated using GraphPad Prism (v. 4.0) software. (12A) RPMI-8402 cells, which harbor a PTEN mutation, exhibited an increase in population doubling time when treated with increasing amounts of CompE. (12B) HPB-ALL cells, which are wild-type for PTEN, also exhibited an increase in population doubling time when treated with CompE.

CompE. It is presumed that these cells, and others that have inactivated PTEN, are more dependent upon Akt for survival than NOTCH1 signaling. Therefore, these cells may be more resistant to GSI treatment, or experience little negative impact resulting from CompE treatment.

3.3b Effects of NOTCH1 Inhibition on Key Regulators of the PI3K-Akt/mTOR Pathway

It has been reported that NOTCH1 can downregulate the expression of PTEN via HES1 resulting in constitutively active PI3K-Akt signaling[166] and activation of mTOR indirectly by cMYC[181], thus promoting cell survival (Figure 4). To further explore the involvement of NOTCH1 in these two critical cell survival pathways at the level of gene expression, we treated both Jurkat (mutant PTEN) and HPB-ALL (wild-type PTEN) cells for 48 h with either 1μM CompE or DMSO, then isolated and reverse transcribed total RNAs. The cDNAs were mixed with 2X SuperArray RT^2 qPCR Master Mix and ddH_2O, and then aliquoted into the appropriate wells of a PI3K-Akt/mTOR SuperArray. SuperArray real-time PCR analysis was performed on the Light Cycler 480 real-time PCR machine. Data were analyzed using the ΔΔCt method. Each cell line was analyzed in duplicate.

The results suggest that the impact of NOTCH1 inhibition on the PI3K-Akt and mTOR pathways is highly cell-type dependent (Figure 13A). For example, in Jurkat cells, NOTCH1 inhibition was associated with significantly decreased expression of PI3KCA and TSC2. In a 'normal' wild-type PTEN cell, a decease in the expression of any PI3K gene could lead to a decrease in Akt activity. However, since PTEN is inactivated in the Jurkat cells, this decrease probably had little impact on Akt activity since it is unable to downregulate this pathway. It is plausible that the various PI3K

isoforms may share redundant functions and could therefore compensate for loss of one activity. However, the decrease in TSC2 would likely increase mTOR activity since it is a negative regulator of mTOR[182]. This increase would likely be much greater in a PTEN-null cell. The inhibition of NOTCH1 in the wild-type PTEN cell line HPB-ALL was associated with significant decreases in expression of EIF4EBP1, PI3KR2 and RPS6KB1 and significant increases in expression of FOXO1, PI3KCG, PI3KR1 and PTEN (Figure 13A). Real-time qPCR validated these changes in expression for PTEN, PI3KCG and EIF4EBP1 in HPB-ALL cells (Figure 13B).

3.3c Long-Term Downstream Effects of NOTCH1 Inhibition

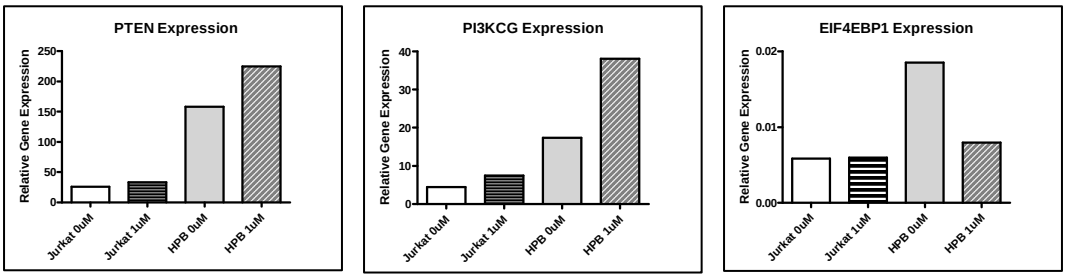
A problem with GSIs is that they are not completely specific to NOTCH1 and can potentially inhibit other targets with transcriptional effects independent of NOTCH1. Further, it is not entirely certain how stable these agents are under standard culture conditions. Accordingly, to better assess the effects of NOTCH1 inhibition on downstream targets, we knocked down NOTCH1 in Jurkat cells, using shRNA lentiviral particles specific for NOTCH1. We chose the Jurkat cell line for our studies since these cells exhibit increased NOTCH1 signaling due to a FBW7 mutation, have inactivated mutant PTEN and therefore represent a large fraction of T-ALL cases, and are easily transduced by lentivirus. Stable clones were selected with puromycin and single clones were isolated following plating in soft agar. RNA and whole cell extracts (WCE) were prepared to identify clones with decreased expression of NOTCH1. We identified 2 clones in which NOTCH1 was significantly decreased (55% knock down for J.N1KD 2-4 and 79% knock down for J.N1KD 2-7) when compared to the non-targeted clones.

Figure 13: The Involvement of NOTCH1 in the PI3K-Akt/mTOR Pathways

13A:

Genes	Jurkat			HPB-ALL		
	1uM CompE	0uM CompE	pValue	1uM CompE	0uM CompE	pValue
AKT1	**4.4E-02**	**5.8E-02**	**0.0230**	5.6E-02	5.7E-02	0.9111
EIF4EBP1	5.5E-02	6.9E-02	0.0988	**3.4E-02**	**1.2E-01**	**0.0002**
FOXO1	1.4E-03	1.3E-03	0.4419	**4.2E-03**	**1.4E-03**	**0.0001**
PI3KCA	**3.3E-02**	**4.1E-02**	**0.0170**	7.6E-02	6.1E-02	0.2438
PI3KCG	5.0E-02	1.1E-02	0.1358	**3.4E-02**	**1.4E-02**	**0.0022**
PI3KR1	7.4E-02	7.1E-02	0.4462	**1.1E-01**	**7.2E-02**	**0.0222**
PI3KR2	1.1E-02	1.2E-02	0.7217	**9.8E-03**	**1.9E-02**	**0.0017**
PTEN	1.1E-01	1.0E-01	0.3840	**2.3E-01**	**1.6E-01**	**0.0115**
RPS6KA1	4.3E-05	8.3E-04	0.1041	7.0E-03	3.8E-02	0.2018
RPS6KB1	3.1E-02	3.1E-02	0.7763	**2.5E-02**	**3.0E-02**	**0.0322**
TSC2	**4.9E-03**	**7.5E-03**	**0.0248**	2.6E-03	3.2E-03	0.4800

13B:



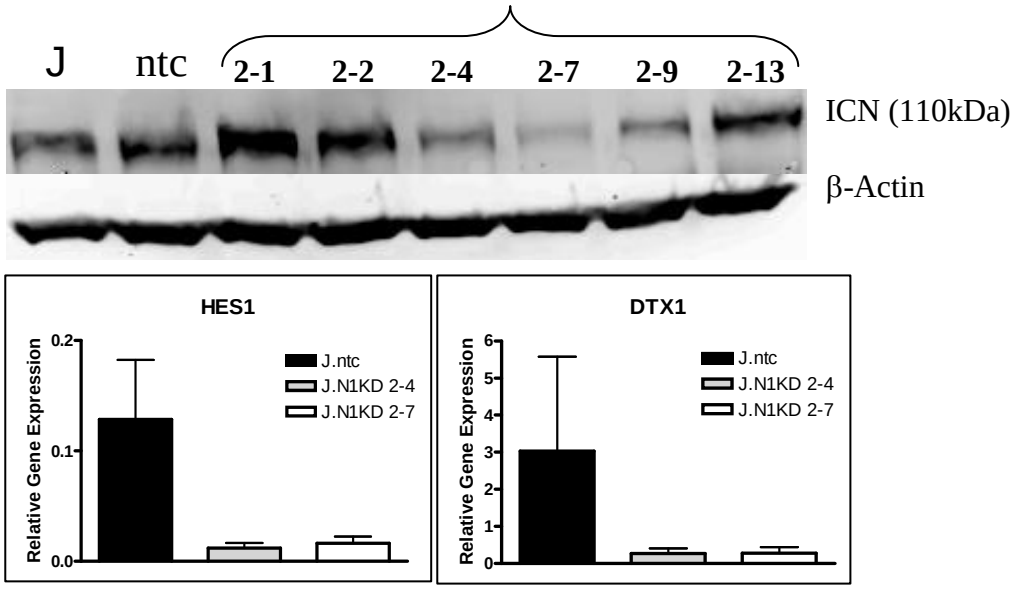
RNAs were isolated from Jurkat and HPB-ALL cells treated with either DMSO (control) or 1uM CompE for 48h. cDNAs were amplified and run on a PI3K-Akt/mTOR SuperArray plate from SA Biosciences that contained primers for 84 genes most relevant to the pathways. The SuperArray was performed on a LightCycler 480 real-time PCR machine. (13A) A summary of genes in which there was a significant ($p \leq 0.05$) change in transcript levels when cells were treated with CompE. Expression levels and p-values in bold depict statistically significant changes. (13B) Real-time PCR was used to validate the expression of some of the genes of interest in both Jurkat and HPB-ALL cells treated with either DMSO or CompE. Transcript levels were normalized to h18S.

Decreased NOTCH1 was verified by real-time qPCR and Western blot analysis (Figure14).

To examine the impact of loss of NOTCH1 on cell proliferation, non-targeted control (J.ntc) and NOTCH1 knock down sublines (J.N1KD 2-4 and J.N1KD 2-7) were seeded at 7.5 x 10^5 cells/mL in 10 mL of RPMI1640, containing 15% FBS and 0.25 mg/mL puromycin. Cells were counted at 24 h intervals using Trypan blue to stain the non-viable cells blue and a hemocytometer. Cell counts were graphed and the exponential population doubling time was calculated. There was a nominal effect on the population doubling times between the J.ntc clone (30.46 h) and J.N1KD 2-4 (33.96 h) and J.N1KD 2-7 (30.68 h) (Figure 15A). Thus, in PTEN deficient Jurkat T-ALL cells, the loss of NOTCH1 has minimal impact on cell proliferation.

The effects of NOTCH1 inhibition on cell cycle progression and baseline apoptosis were analyzed using flow cytometery techniques. There was a slight albeit statistically insignificant increase in the number of cells in the S-phase in J.N1KD 2-4 (23.67%) and J.N1KD 2-7 (22.68%) cells compared to J.ntc (19.8%) cells (Figure 15B). There was also a slight increase in baseline apoptosis in the NOTCH1 knockdown clones (24% and 27% for the J.N1KD 2-4 and J.N1KD 2-7, respectively) (Figure 15C). However, this increase in apoptosis was statistically insignificant. Thus, in the Jurkat T-ALL model, NOTCH1 inhibition appeared to have little effect on either cell cycle progression or spontaneous apoptosis. This is likely a unique characteristic of these PTEN-null T-ALL cells as they are probably more dependent upon Akt for survival as a result of PTEN inactivation than cells with an intact PTEN.

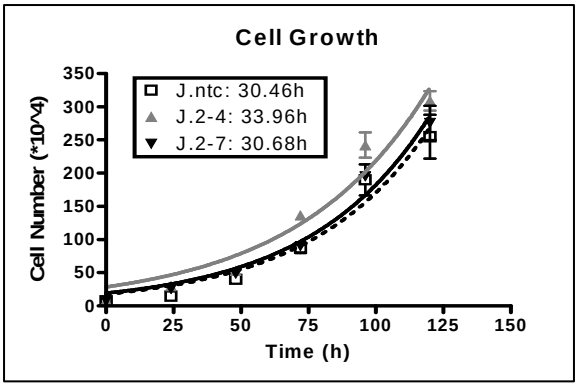
Figure 14: Development of NOTCH1 Knockdown Model



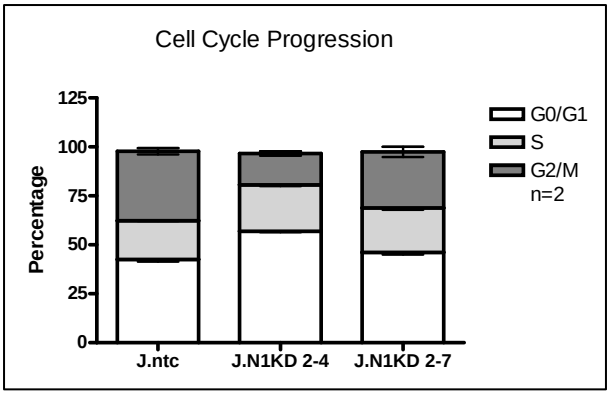
The Jurkat T-ALL cell line, which is PTEN null and has increased NOTCH1 activity as the result of a mutation in FBW7, was transduced with Lenti-viral particles containing shRNA specific for NOTCH1 or a non-targeting sequence (ntc). Transduced cells were subjected to puromycin selection and individual clones were isolated from soft agar. Several clones were analyzed by Western Blot techniques for the knocked-down expression of ICN. J.N1KD clones 2-4 and 2-7 showed the most reduced expression of ICN. The reduced expression of ICN in J.N1KD 2-4 and J.N1KD 2-7 were accompanied by a significant decrease in expression of HES1 and DTX1, both known NOTCH1 target genes. Abbreviations: J, Jurkat; ntc, J.ntc.

Figure 15: Effects of NOTCH1 Inhibition on Cell Growth, Cell Cycle Progression and Apoptosis

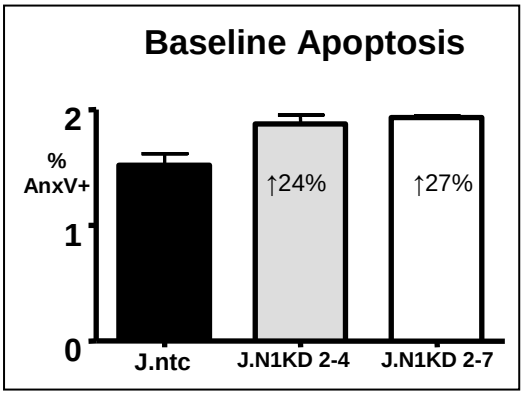
15A:



15B:



15C:



(15A) Cell growth of J.ntc, J.N1KD 2-4 (J.2-4) and J.N1KD 2-7 (J.2-7) was measured with a hemacytometer and Trypan Blue. Viable cells were counted in triplicate for 5 days and the population doubling times were calculated. There was no significant difference between the population doubling times for J.ntc, J.N1KD 2-4 and J.N1KD 2-7. (15B) Inhibition of NOTCH1 has very little effect on cell cycle progression, as is evident by lack of changed in the percentage of cells retained in each phase of the cell cycle when comparing the NOTCH1 knock-down clones to J.ntc. (15C) Inhibition of NOTCH1 has a very modest effect on apoptosis, as evident by the slight increase (24-27%) of AnnexinV+ cells in the NOTCH1 knock-down clones when compared to J.ntc.

3.3d NOTCH1 Inhibition has No Effect on Chemotherapeutic Response

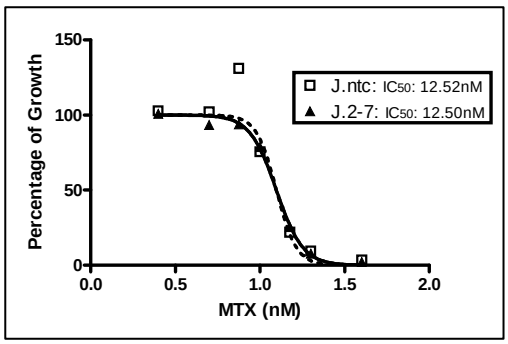
To determine the impact of loss of NOTCH1 on chemotherapy sensitivity or resistance, J.ntc, J.N1KD 2-4 and J.N1KD 2-7 were seeded at 4000 cells/well in 96 well plates and incubated for 4 days with varying concentrations of chemotherapeutic agents, including methotrexate (MTX), daunorubicin (DNR), VP16 and L-asparaginase (L-ASP). The cells were exposed to 2.5 mg/mL MTT substrate for 4 h and lysed with 10% SDS in 10 mM HCl overnight. A visible microplate reader was used to read the plates and the IC_{50}s for each chemotherapeutic agent were calculated graphically using GraphPad Prism software. Loss of NOTCH1 had very little effect on the IC_{50} for each agent (Figure 16). Thus, it appears that in Jurkat cells, loss of NOTCH1 has very little impact on the chemotherapy sensitivity. This lack of an augmented anti-proliferative effect resulting from decreased NOTCH1 is likely the result of Jurkat cells being dependent (or maybe even addicted) to Akt for survival due to the fact that there is an overall lack of PTEN function, as noted above. I would suspect that if the Jurkat cells were treated with an Akt inhibitor in combination with these chemotherapeutic agents, the cells would become more sensitive to the latter, and the IC_{50}s would decrease.

3.3e NOTCH1 Inhibition is Associated with Changes in Cell Surface Marker Expression

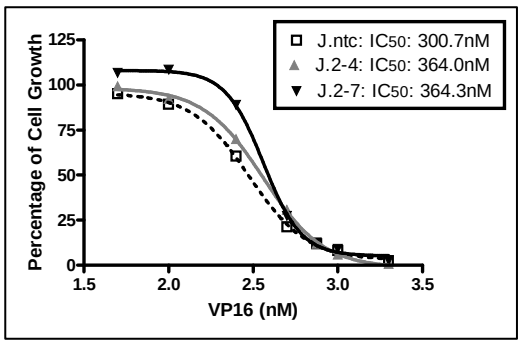
Flow cytometery was used to measure cell surface marker expression as a means to monitor cell differentiation. NOTCH1 inhibition was associated with decreased expression of CD3, CD4, CD5, CD7, CD69, CD95 and CD28 (Figure 17). Decreased expressions of CD1a, CD4, CD7 and CD3 are signs of an “immature” phenotype displayed in adult T-ALLs where NOTCH1/FBW7 are wild-type[141]. Consistent with this,

Figure 16: Effects of NOTCH1 Inhibition on Chemotherapeutic Response

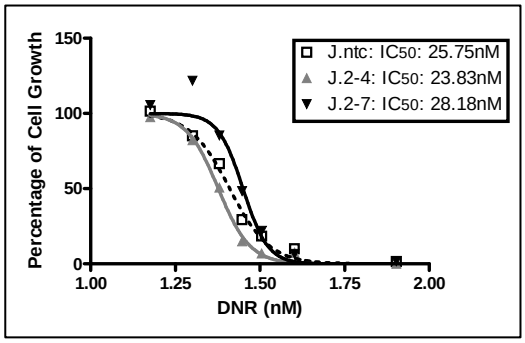
16A:



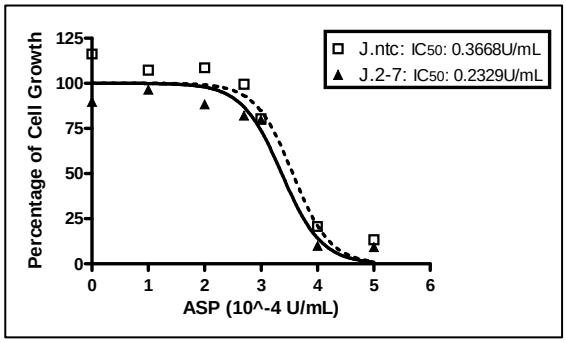
16B:



16C:

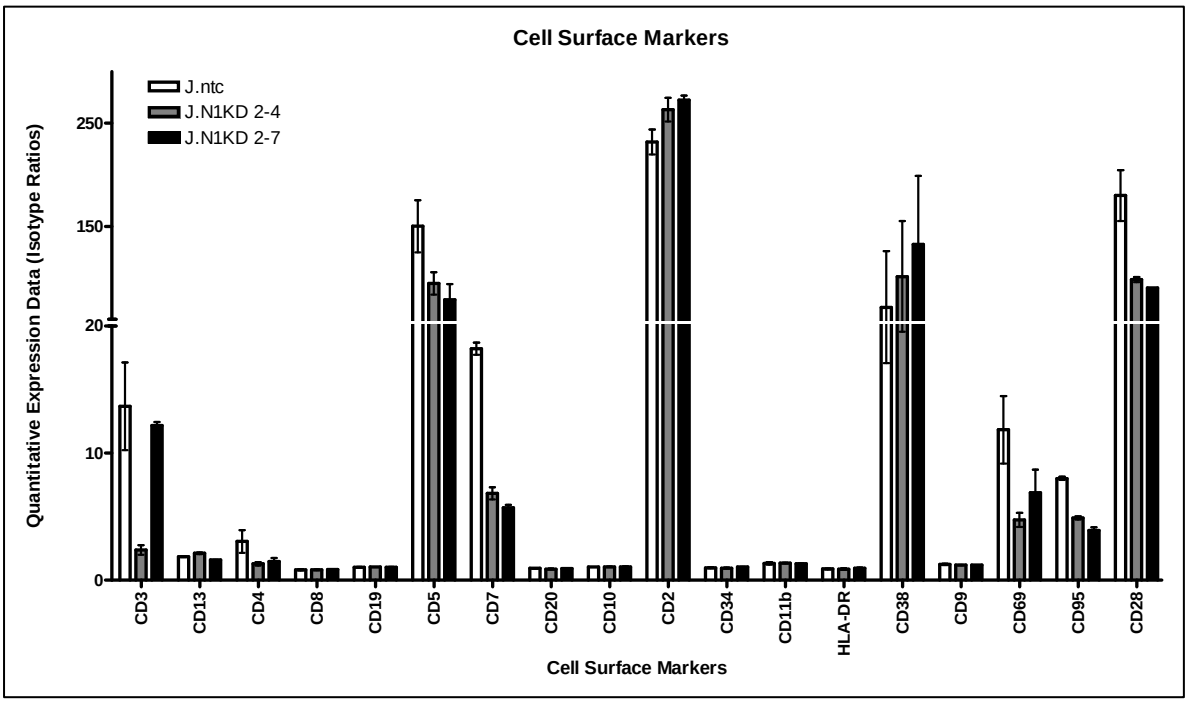


16D:



Using the MTT assay, the impact of NOTCH1 inhibition on the chemotherapeutic response to single agents was evaluated. (16A) Response to methotrexate (MTX). (16B) Response to VP-16. (16C) Response to daunorubicin (DNR). (16D) Response to L-asparaginase (ASP). The IC_{50} for each agent was calculated using GraphPad Prism software (v. 4.0). Abbreviations: J.2-4, J.N1KD 2-4; J.2-7, J.N1KD 2-7.

Figure 17: Effects of NOTCH1 Inhibition on Cell Differentiation



Cell surface markers that are key indicators of cell differentiation were measured by flow cytometry. NOTCH1 knock-down was accompanied by a decrease in expression of CD3, CD4, CD5, CD7, CD69 and CD28.

in the Jurkat cell line model, NOTCH1 expression is indicative of a more “mature” phenotype.

3.4 Conclusions

Our studies hint that NOTCH1 signaling may play a more complicated role in the PI3K-Akt/mTOR pathways than previously considered, and that the impact of NOTCH1 signaling on these pathways may be dependent upon the status of PTEN. Although we saw alterations in expression of other key PI3K-Akt/mTOR pathway genes, including some that would increase Akt activity (and thus promote survival) and others that would downregulate Akt, the composite phenotype would ultimately depend upon the functional status of PTEN. For example, while NOTCH1 inhibition was associated with a decrease in expression of PI3KCA in Jurkat cells, this probably wouldn’t have much of an effect on cell survival since PTEN is non-functional and Akt activity is essentially unregulated. Likewise, in the HPB-ALL cells, the increased expression of PTEN, due to NOTCH1 inhibition, would likely result in decreased Akt activity, while increased expression of PI3KCG could possibly promote increased Akt activity and subsequent cell survival. These effects could impact the response to chemotherapy. For example, an increase in PTEN expression could lead to chemotherapy sensitivity because it will restrict the activation of Akt, thus limiting the signals for cell survival. However, an increase in PI3KCG could lead to chemotherapy resistance because it will increase overall Akt activity, and thus promote cell survival. Thus, the overall effect on the cell proliferation and survival would be reflecting a composite phenotype, which may be ultimately dependent/regulated by the functional status of PTEN.

In this study, it is evident that NOTCH1 inhibition has very little effect on cell survival and chemotherapeutic response in T-ALLs that are PTEN null. This is supported by the fact that NOTCH1 inhibition was not associated with significant changes in population doubling time, changes in apoptosis or cell progression, or even in responses to chemotherapeutic agents. It is likely that our PTEN inactivated model is addicted to Akt signaling for survival, and that NOTCH1 signaling is dispensable. This may explain why initial clinical trials with GSIs showed these drugs were at the very most cytostatic[125, 164]. The T-ALL patients enrolled in these trials could have easily have inactive PTEN, either by mutation or posttranslational modifications, as such events can occur in up to 70% of T-ALLs[166, 198] Accordingly, targeting NOTCH1 for T-ALL therapy would be far more beneficial in T-ALLs in which PTEN is wild-type and active. Such cells would not be nearly as dependent upon Akt for survival because PTEN is able to regulate Akt activation, and thus would be more dependent on aberrant NOTCH1 signaling for survival. These studies suggest that PTEN status need to be taken into consideration when targeting NOTCH1 for therapy, not only its mutational status but its functional status as well.

CHAPTER 4

IDENTIFICATION OF NOVEL NOTCH1 TARGETS

4.1 Introduction

Despite the extensive analysis of NOTCH1 during the past 6 years, including the discovery of NOTCH1 mutations in >50% of T-ALLs, there is surprisingly limited knowledge of the downstream gene targets of NOTCH1 signaling. NOTCH1 functions through ICN, which is cleaved during ligand binding by γ-secretase[78, 79, 85]. ICN translocates to the nucleus, binds to CSL, converting it to a coactivator, and recruits additional coactivators to initiate transcription of target genes[78, 79, 85]. The most well known transcriptional targets of NOTCH1 include HES1 and HES5, HERP family and DTX1[106-108], all basic helix-loop-helix (bHLH) proteins[106-108]. NOTCH1 can also indirectly regulate the transcription of other target genes through the activities of its own direct targets. For example, HES1 and HERP proteins negatively regulate the expression of many downstream target genes, including those involved with apoptosis and proliferation such as PTEN[106, 166]. Other direct or indirect NOTCH1-regulated genes include p21Cip/Waf[235], CD25[236], pre-Tα[237], cyclin D1[238], the proapoptotic receptor NUR[239] and transcription factors of the NF-κB family[35]. cMYC has been identified as a direct NOTCH1 target, as well[103-105]. By identifying the downstream transcriptional targets of NOTCH1 signaling, we can achieve a better understanding of the role of NOTCH1 in T-ALL and how it may be exploited for new therapeutic advantage.

In a recent study by Buonamici *et al.*[240], a murine model was used to demonstrate that the oncogenic expression of NOTCH1 (i.e., ICN) could induce the development of T-ALL and target leukemic cells to the CNS[240]. They discovered that a number of

NOTCH1-targeted genes could be regulators of cell adhesion, migration and metastasis, and could play a significant role in the infiltration of T-ALL cells to the CNS. A gene of particular interest was CCR7, a chemokine receptor that is a known regulator of lymphocyte migration[241]. This gene was significantly upregulated when NOTCH1 was induced. The expression of CCR7 in T-ALL cell lines was enough to target the cells to the CNS. It's unlikely that CCR7 is sufficient alone, but that other factors are also involved in targeting leukemic cells to the CNS.

The study described in this chapter was designed to expand upon these studies and to identify novel NOTCH1 downstream targets that have not been previously reported, in hope that this may better elucidate the relationship between NOTCH1 signaling and disease progression, as well as its relationship to chemotherapy sensitivity.

4.2 Materials and Methods

4.2a Microarray and Validation

RNAs were isolated from Jurkat T-ALL cells that were transduced with shRNA particles to knock down the expression of NOTCH1 using the TRIzol ® protocol (see Chapter 3). A quality check of the total RNA was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies; Palo Alto, CA) to determine if the 18S and 28S ribosomal bands were defined and to ensure no RNA degradation had occurred. Aminoallyl-aRNA was produced with 500 ng of total RNA and TargetAMP1-Round Amnoallyl-aRNA Amplification Kit 101 (Epicentre; Madison, WI). First strand cDNA synthesis used oligo(dT) primers containing a phage T7 RNA polymerase promoter sequence (Invitrogen). Second strand cDNA synthesis and *in vitro* transcription were generated with the UTP nucleotide is partially substituted with an aminoallyl-UTP.

Aminoallyl-aRNA was purified using Qiagen RNeasy mini kit (Qiagen) and the concentration was determined using a NanoDrop ND-1000.

Alexa fluor Reactive Dyes Alexa 555 or Alexa 647 (Molecular Probes; Eugene, OR) was used to label 5 μg of each Aminoallyl-aRNA. The samples were incubated with the dye for 30 min at room temperature, and run through another RNeasy column to remove any unincorporated dye. The samples were quantitated on a NanoDrop spectrophotometer. The Agilent 60-mer microarray (Whole Human Genome Arrays 4x44K p/n G4112F) processing protocol was followed. Briefly, 0.825 μg of Alexa 555-labeled Aminoallyl-aRNA and 0.825μg of Alexa 647-labeled Aminoallyl-aRNA were mixed together and allowed to co-hybridize on the array for 17 h at 65°C. Agilent's SureHyb hybridization chambers were used to allow complete mixing of the hybridization solution in a rotation rack in a hybridization oven. Following hybridization, the slides were washed according to Agilent's protocol.

Slides were immediately scanned with the Agilent dual laser scanner. The photo multiplier tube (PMT) setting with extended dynamic range was at Hi 100% and Lo 10% for the red and green channels. Tiff images were analyzed using Agilent's feature extraction software to obtain fluorescent intensities for each spot on the arrays. Linear and LOWESS normalization was performed on the intensity values.

Changes in gene expression were validated by real-time qPCR. Total RNAs were isolated using the TRIzol® protocol. cDNAs were amplified using random hexamer primers and real-time qPCR was used to quantitate transcript levels. Changes in gene expression levels were calculated using the ΔΔCp method.

4.2b microRNA Array and Validation

Agilent's miRNA arrays were processed using the miRNA Microarray System protocol (Agilent). Each Human miRNA Microarray V2 slide contained 8 miRNA arrays. Each array consisted of human (n=732) and human viral microRNAs from the Sanger miRBASE 10.1. Agilent's "miRNA Microarray System" protocol (v.1.5) was followed during miRNA labeling and array hybridization. Briefly, each total RNA was analyzed with Agilent 2100 Bioanalyzer. Total RNA (100 ng) underwent phosphatase treatment using calf intestine alkaline phosphatase (GE Healthcare Bio-Sciences Corp,; Piscataway, NJ) at 37°C for 30 min. The samples were then denatured and labeled by ligation of one cyanine 3-pCp molecule to the 3' end of the RNA molecule. The samples were incubated at 16°C for 2 h to permit ligation. The labeled miRNAs were cleaned up using Micro Bio-Spin 6 columns (Bio-Rad Laboratories). The samples were dried in a speed-vac and then resuspended in 18μl of nuclease-free water, in which 4.5 μl of 10X GE Blocking and 22.5 μl of 2XHi-RPM Hybridization Buffers (Agilent) were added and incubated at 100°C for 5 min, followed by cooling on ice for 5 min. Samples were immediately added to the array in an Agilent SureHyb Hybridization chamber. The chambers were rotated at 20 rpm in a hybridization oven for 20 h at 55°C.

Following hybridization, the slides were removed from the chamber and washed in GE Wash Buffer 1 for 5 min and pre-warmed 37°C GE Wash Buffer 2 for 5 min (Agilent). The slides were removed from Wash Buffer 2 and allowed to dry, and then scanned using the Agilent dual laser scanner. The PMT settings are set at 100% and 5% for the green channel. Tiff images are analyzed using Agilent's feature extraction software.

The relative expression level of each microRNA (miR) was validated with Applied Biosystems TaqMan® MicroRNA Assays (Applied Biosystems). Total RNAs, including miRs, were isolated using the TRIzol® protocol. Each RNA sample was reversed transcribed with a master mix consisting of 100 mM dNTPs, MultiScribe Reverse Transcriptase (50 U/μL), 10X RT Buffer and RNase Inhibitor. This master mix was aliquoted to the appropriate number of PCR tubes and an RT primer was added. Each primer was specific for a miR of interest. RTs were amplified with a thermocycler. Each miR was quantitated using a Light Cycler 480 (Roche) and each RNA and primer combination was quantitated in triplicate. The Light Cycler master mix consisted of TaqMan 2X Universal PCR Master Mix, without AmpErase and nuclease-free water. This master mix was aliquoted and 20X TaqMan MicroRNA assay mix was added, along with the RT product. This final master mix was aliquoted into 3 wells of a 96-well plate. The fold changes for each miR were calculated by the ΔΔCp method.

4.3 Results

4.3a Microarray Results

In 2009, Chadwick *et al.* prepared RNA from Jurkat cells that were retrovirally transduced with constitutively active forms of NOTCH1 and used Affymetrix microarray analysis to identify novel NOTCH1 gene targets[242]. They identified several genes that appeared to be regulated by NOTCH1, including IGF1R, CD28 and HERP2. In a further attempt to identify novel NOTCH1 target genes, we isolated total RNAs from the J.ntc, J.N1KD 2-4 and J.N1KD 2-7 sublines and analyzed differentially expressed genes with an Agilent Human Whole Genome Oligonucleotide array. Over 1200 genes showed differential expression between the non-targeted control Jurkat cells and the two

NOTCH1 knockeddown Jurkat clones (cutoff was a 1.5-fold change in expression with $p \leq 0.05$). There was a 20- to 40-fold decrease in expression of the well known NOTCH1 target gene HES1, and an 11-fold decrease in expression of DTX1 in the NOTCH1 knocked-down clones (Figure 18A). There was some agreement in the differentially expressed gene targets identified on our microarray and those from the Chadwick study, including EFEMP1, RANBP2, GIMAP5, SHQ1, IGF1R, BMP2K and CD28 (Figure 18A). Other differentially expressed genes of interest from the microarray included IL-7R, a documented NOTCH1 target gene[243], DR4, TGFβ1, PI3KR2, IGF1R and Rictor (Figure 18A). The expression of all of these genes, except for Rictor, was decreased upon knockdown of NOTCH1. Real-time qPCR was able to validate the association of NOTCH1 inhibition with the decreased expression of established NOTCH1 targets including DTX, HES1, and IL7R, along with decreased TGFβ1 and with increased Rictor (Figure 18B).

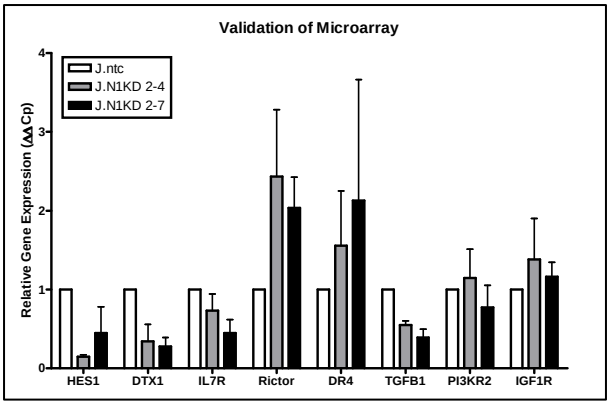
Rictor forms a complex with mTOR2, which can promote cell survival thorough the phosphorylation and complete activation of Akt (phosphorylation occurs on S473)[182]. Concurrent with the real-time data, western blot analysis shows an increase in Rictor protein levels and increased pAKT S473 (Figure 18C). The promoter for Rictor was identified using genomic sequence approximately 2.0kb from the atg translation start site for the Rictor coding sequence and Genomatix software. Genomatix predicted the promoter (632bp) to lie with the 2.0kb genomic sequence. Matinspector software identified both NOTCH1 indirect (via CSL; a.k.a. RBPJκ) and HES1 binding sequences within the Rictor promoter sequence.

Figure 18: Identification of Novel NOTCH1 Downstream Targets

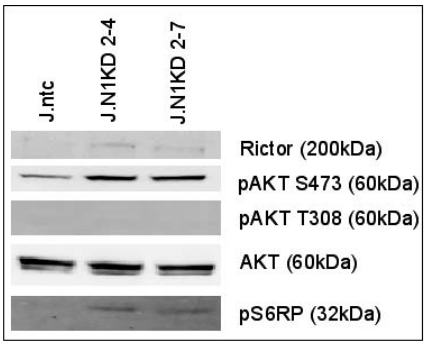
18A:

GENE	FOLD CHANGE
Known NOTCH1 Target Genes:	
HES1	↓ 20-40 fold
DTX1	↓ 11.1 fold
IL7R	↓ 3.2 fold
Genes from Chadwick *et al.*[242]	
EFEMP1	↓ 20 fold
RNABP2	↓ 2.6 fold
GIMAP5	↓ 4.8 fold
SHQ1	↓ 1.7 fold
IGF1R	↓ 2.2 fold
BMP2K	↓ 1.7 fold
CD28	↓ 1.5 fold
Potential Novel NOTCH1 Target Genes	
Rictor	↑1.6 fold
DR4	↓ 5.4 fold
TGFβ1	↓ 2.9 fold
PI3KR2	↓ 1.6 fold
IGF1R	↓ 2.2 fold

18B:

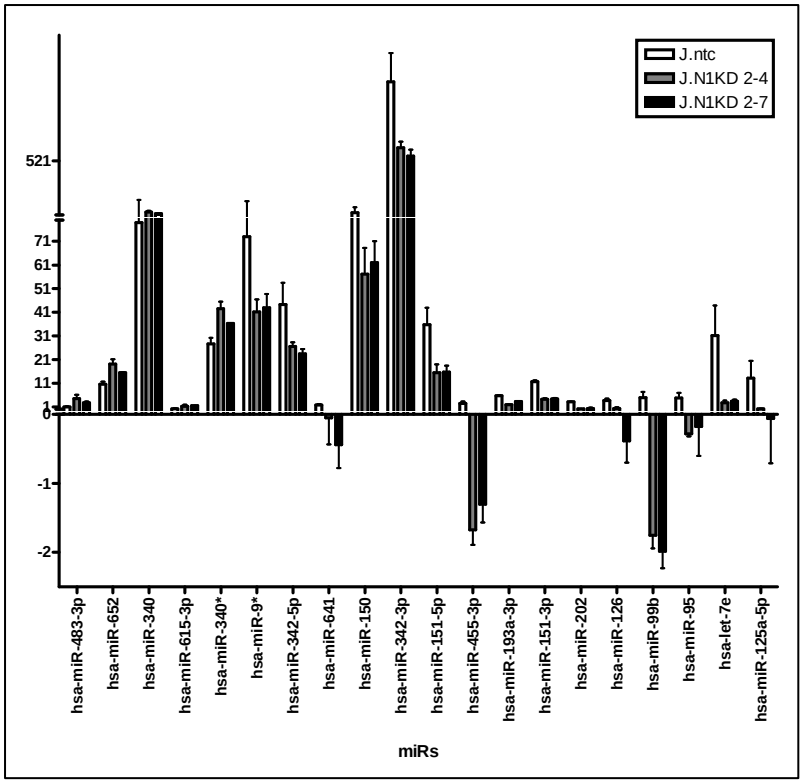


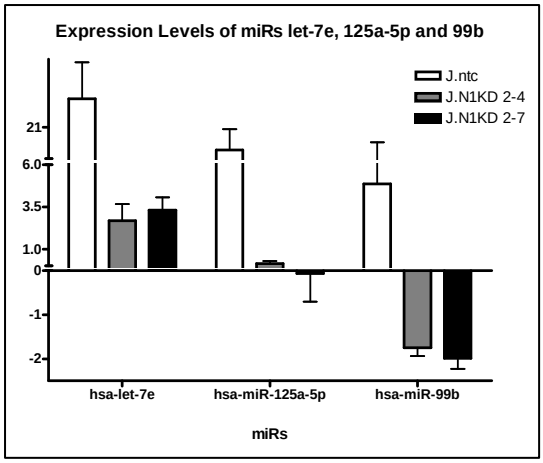
18C:



Total RNA was isolated from J.ntc, J.N1KD 2-4 and J.N1KD 2-7 and differentially expressed genes determined on an Agilent Human Whole Genome Oligonucleotide array. The cutoff limit was at least a 1.5-fold change in expression in conjunction with $p \leq 0.05$. (18A) Summary of the change in expression of known NOTCH1 target genes, potential NOTCH1 target genes previously identified by Chadwick et al and novel NOTCH1 target genes that appear interesting and relevant. (18B) Real-time RT-PCR validation of novel NOTCH1 target genes. (18C) Western blot analysis of Rictor expression in J.ntc, J.N1KD 2-4 and J.N1KD 2-7.

Figure 19: NOTCH1 Inhibition is Associated with the Significant Change in Expression of 20 miRs





Total RNAs were isolated from J.ntc, J.N1KD 2-4 and J.N1KD 2-7 and used determinations of differentially expressed miRNAs on an Agilent Human microRNA Version 2 array. The expression of 732 human miRs were determined. The cutoff limit was 1.5-fold change in expression accompanied by $p \leq 0.05$. A significant change in expression for 20 miRs was observed between J.ntc and the 2 NOTCH1 knock-down clones (Top Panel). Of the 20 miRs, 2 were of particular interest because they are polycistronic and believed to share a common promoter (Bottom Panel).

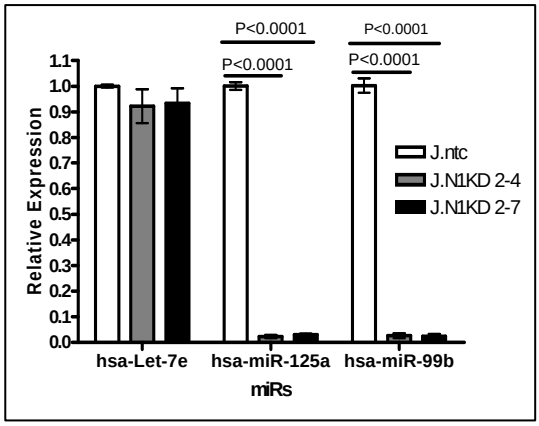
4.3b Potential microRNA Targets of NOTCH1

To identify potential miRNAs regulated by NOTCH1, total RNAs were isolated from J.ntc, J.N1KD 2-4 and J.N1KD 2-7 cells and analyzed on an Agilent Human microRNA V2 array, encompassing 732 human microRNAs (miRs). Knockdown of NOTCH1 was associated with a change in expression of twenty miRs (cutoff was 1.5-fold and $p \leq 0.05$; Figure 19). This suggests that several miRs may be downstream targets of NOTCH1. Of the 20 miRs that showed the differential levels between the non-targeted control and knockdown cell lines, the three most significant changes were for hsa-Let-7e, hsa-miR-99b and hsa-miR-125a-5p. These miRs are believed to be polycistronic, transcribed from the same promoter, and therefore share the same pri-transcript.

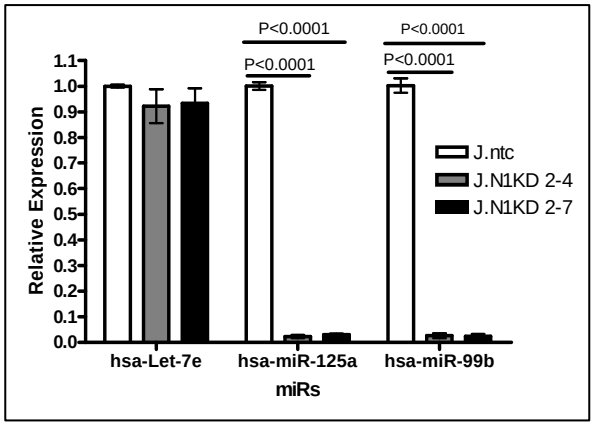
Differential expression of mature hsa-Let-7e, hsa-miR-125a-5p and hsa-miR-99b was further tested using TaqMan microRNA assay probes, with the original RNAs used for the microarray and with additional RNA isolations (Figure 20A (original array RNA) and 20B (additional RNA isolation)). Significantly decreased hsa-miR-125a-5p and hsa-miR-99b was seen in both RNA preparations for the knockdown cell lines compared to the non-targeted control cell line. A decrease in miR expression was also seen in Jurkat cells that were treated with the GSI CompE (Figure 20C). Unexpectedly, hsa-Let-7e expression did not adhere to the same expression pattern as hsa-miR-125a-5p and hsa-miR-99b. Although its expression was significantly decreased when NOTCH1 was inhibited on the original microRNA array, it failed to validate with the TaqMan microRNA assays. If hsa-Let-7e, hsa-miR-125a-5p and hsa-miR-99b are truly polycistronic, we would have expected the expression of all 3 miRs to decrease upon

Figure 20: Validation of miRs Let-7e, 125a-5p and 99b Upon NOTCH1 Inhibition

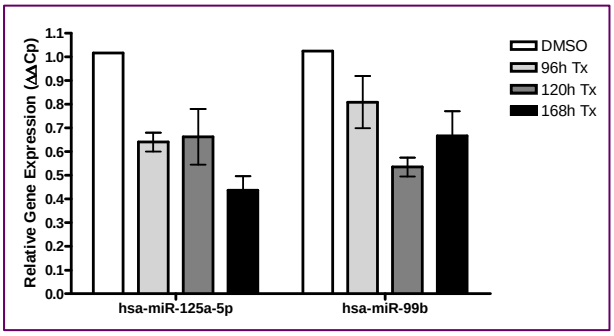
20A:



20B:



20C:



Expression of mature hsa-Let-7e, hsa-miR-125a-5p and hsa-miR-99b were validated using TaqMan microRNA assays from Applied Biosystems. The mature transcript of each miR was quantitated by real-time qPCR using miR-specific probes. We validated miR expression in both the original RNAs used for the initial microRNA array (16A) and subsequent RNA isolations from J.ntc, J.N1KD 2-4 and J.N1KD 2-7 (16B). Jurkat cells were treated with either DMSO or 1uM of CompE for 4-7 days, and miR expression levels were measured to validate the results seen with J.ntc, J.N1KD 2-4 and J.N1KD 2-7 (16C). A time-dependent decrease in the expression of -miR-125a-5p and hsa-miR-99b was seen upon NOTCH1 pharmaceutical inhibition.

NOTCH1 inhibition. We suspect that hsa-Let-7e may undergo additional regulation, independent of hsa-miR-125a-5p and hsa-miR-99b. While these results strongly suggest that levels of hsa-miR-125a-5p and hsa-miR-99b are *bona fide* downstream targets of NOTCH1, the mechanism is unclear; i.e., regulation may be direct or indirect. Future studies will focus on identifying and amplifying the promoter with 5'RACE, and to address NOTCH1 involvement with reporter gene assays. These studies will also identify and validate the downstream targets of these 2 miRs as well, and to role T-ALL progression and chemotherapy response.

4.4 Conclusions

Our studies have identified three potential novel targets of NOTCH1 signaling. The first target is Rictor. It appears that NOTCH1 signaling represses the expression of Rictor because upon NOTCH1 shRNA knockdown there was a significant increase in the expression of this critical gene, at both the transcript and protein levels. This increase in Rictor would likely lead to increased Akt phosphorylation at S473, thus promoting cell survival signals[173]. These findings suggest some caution should be exercised when attempting to inhibit NOTCH1 with GSIs in T-ALL therapy, especially in T-ALLs, for which PTEN is frequently inactivated by mutation or posttranslational modification and activation of Akt is unregulated. Thus, inhibition of NOTCH1 with small molecule GSIs may need to be combined with an Akt inhibitor to disrupt Akt signaling or perhaps with rapamycin to potentially inhibit mTOR2 activity[172]. To date, there are no specific inhibitors of mTOR2. However, rapamycin has been shown to inhibit mTOR1 and in some cases mTOR2 in a cell context-dependent manner[172].

The other two new targets of NOTCH1 signaling identified in our study are hsa-miR-125a-5p and hsa-miR-99b. Our data suggest that NOTCH1 can promote the expression of miRs-125a-5p/99b because when NOTCH1 is inhibited by shRNA knockdown or GSI treatment, there is a significant decrease in expression of these two miRs. The implications for the impact of miRs on chemotherapy have yet to be determined.

CHAPTER 5

CONCLUSIONS

A major confounding problem in the biology and therapy of pediatric T-ALL has been determining the prognostic value of NOTCH1 mutations. In our studies, we attempted shed important new light on this question. We confirmed the presence of high frequency mutations in NOTCH1 and FBW7 in our cohort of 47 T-ALL specimens and we were the first to suggest that PTEN is much more frequently mutated in primary T-ALL specimens than originally believed. However, in our cohort of pediatric T-ALLs, we also found absolutely no association between NOTCH1 mutations, alone or in combination with FBW7 and/or PTEN, and treatment outcome. This result is in agreement with those in a report published by van Grotel *et al.*[135].

Upon further review of our findings, this may not be that surprising after all. Ultimately, the underlying biology of the disease determines how a patient is going to respond to therapy. NOTCH1 mutations can be found in patients generally considered to be both "good responders" to therapy (low to undetectable MRD levels following induction therapy) and "poor responders" (detectable MRD levels following induction therapy). Likewise, NOTCH1 mutations can be found in patients who are classically defined into low, standard and high risk of relapse. Thus, the prognostic merit of NOTCH1 mutations in this disease may be dependent in large part on the chemotherapy regimen that is administered to the patient. Supporting this notion, our studies demonstrated that several chemotherapy relevant genes may be potential direct or indirect downstream targets of NOTCH1 signaling. These genes encoded drug efflux pumps that would likely render cells more chemotherapy resistant (i.e., MDR1, ABCC5) and others

that could render cells more chemotherapy sensitive (i.e., hRFC). The overall net effect of these genes can significantly impact net chemotherapeutic response, depending upon types and doses of drugs used.

We saw evidence of this in our analysis of NOTCH1 mutations. Overall, within our cohort, NOTCH1 mutations were present in 39% of the patients who relapsed and 29% of the patients who responded to therapy, independent of the chemotherapy protocol used. When this analysis was restricted to patients treated with a single chemotherapy regimen, by focusing only on the patients treated with POG8704, we saw a marked decrease in the frequency of mutations in patients who relapsed (21.4%) compared to those who responded to treatment (42.9%). However, this decrease in mutation frequency was not statistically significant.

We contend that it is unlikely that the presence of NOTCH1 mutations alone is enough to predict treatment outcome, as not all NOTCH1 mutations are activating to the same degree. On this basis, it seems more likely than not that the overall level of NOTCH1 signaling, as reflected in levels of NOTCH1 targets such as HES1, could be prognostically important. This is also supported in a recent report by Rao *et al.*[244], in which they used the average expression value of 10 known NOTCH1 target genes (NOTCH10) to predict GSI sensitivity[244]. The use of such a "gene signature" profile, such as NOTCH10, will take into account all factors that regulate the potency of the NOTCH1 signal, including ligand activation and protein turnover regulated by the E3 ubiquitin ligases, such as Numb and FBW7.

To better study the prognostic value of NOTCH1 mutations in T-ALL, the composition of the study population also needs to be carefully controlled. For example,

if the patient cohort contains mostly good responders, NOTCH1 mutations will undoubtedly be associated with favorable outcomes. Likewise, if the cohort contains mainly poor responders, NOTCH1 mutations will be associated with poor outcomes. Clearly, a carefully crafted study design will be important for finally answering this important question.

Although NOTCH1 mutations may not necessarily be prognostic, NOTCH1 remains an attractive and potentially useful therapeutic target for T-ALL. In many cases, mutations in NOTCH1 increase the activity of the receptor, even constitutively in some instances, either by increasing its susceptibility to γ-secretase cleavage in the absence of ligand or by inhibiting the ubiquitination and turnover of ICN[125]. The activation of NOTCH1 can be blocked with the use of GSIs, which prevents the cleavage of ICN. Although GSIs have failed miserably in clinical trials, they should not be completely abandoned[160, 161]. Recent studies have been able to optimize GSI doses without causing much gastrointestinal toxicity[169, 170]. With that being said, GSIs may not be beneficial to every T-ALL patient with NOTCH1 mutations. Indeed, GSIs are likely to be effective in those T-ALLs that are completely dependent upon NOTCH1 for survival. This was evident in our J.N1KD cell line models, for which significantly decreased NOTCH1 levels had little effect on cell survival. We suspect this is due to the fact that Jurkat cells harbor a PTEN mutation that causes the loss of PTEN expression. As a result, these cells (and likely a substantial number of T-ALLs, overall) have lost functional PTEN and are highly dependent upon Akt for survival. Thus, T-ALL patients should be thoroughly screened for mutations other than NOTCH1 that may contribute to the overall cell survival before GSIs are given.

As noted above, our study is the first to report such a high frequency of PTEN mutations in T-ALL (~60% as compared to 17%[166]). Whether this is just a unique feature of our patient cohort or is a more common occurrence in T-ALL still needs to be determined. It is assumed that such mutations would inactivate PTEN, or at the very least diminish its function. Our findings, in combination with those of Silva *et al.* that PTEN is frequently posttranslationally inactivated in up to 70% of T-ALLs[198] suggests that PTEN plays a far more important role in the biology and therapy of T-ALL than previously considered. Indeed, it seems likely that the vast majority of T-ALLs have non-functional PTEN protein and are generally "addicted" to Akt signaling for survival. It is suspected that this addiction can lead to chemotherapy resistance, as unregulated Akt activity promotes cell growth and survival. In these T-ALLs, combinational therapies that employ the use of Akt inhibitors and agents that target the downstream events of Akt may be beneficial. More studies are needed to understand the essential requirements of T-ALL survival and resistance mechanisms so that more targeted therapies can be developed.

Lastly, our study highlights the urgent need for more sophisticated studies that focus on the biology of relapse. Relapse remains the number one cause of treatment failure. By better understanding the mechanism of relapse, and identifying common traits that are unique to relapsed T-ALL clones, we may be able to tailor therapies to prevent chemoresistance in these clones. Likewise, if we can identify genetic alterations that are required for relapse, we may also be able to develop targeted therapies for relapsed disease, as well. Of course, such studies may prove difficult since T-ALL in children is a

comparatively rare disease with too few cases to effectively evaluate therapies and research.

REFERENCES

1. Smith, M.A. et al. Outcomes for children and adolescents with cancer: challenges for the twenty-first century. *J Clin Oncol* **28**, 2625-34 (2010).
2. Onciu, M. Acute lymphoblastic leukemia. *Hematol Oncol Clin North Am* **23**, 655-74 (2009).
3. Spector, L.G., Ross, J.A., Robison, L.L., and Bhatia, S. in Childhood leukemias (ed. Pui, C.H.) (Cambridge University Press, New York, 2006).
4. Greaves, M. Infection, immune responses and the aetiology of childhood leukaemia. *Nat Rev Cancer* **6**, 193-203 (2006).
5. Kinlen, L. Infections and immune factors in cancer: the role of epidemiology. *Oncogene* **23**, 6341-8 (2004).
6. Wartenberg, D., Groves, F.D., and Adelman, A.S. in Acute Leukemias (ed. Estey, E.H., Faderl, S., and Kantarjian, H.) 77-93 (Springer, Berlin, Germany, 2008).
7. de Jonge, R. et al. Polymorphisms in folate-related genes and risk of pediatric acute lymphoblastic leukemia. *Blood* **113**, 2284-9 (2009).
8. Healy, J. et al. Promoter SNPs in G1/S checkpoint regulators and their impact on the susceptibility to childhood leukemia. *Blood* **109**, 683-92 (2007).
9. Pui, C.H., Robison, L.L. & Look, A.T. Acute lymphoblastic leukaemia. *Lancet* **371**, 1030-43 (2008).
10. Caughey, R.W. & Michels, K.B. Birth weight and childhood leukemia: a meta-analysis and review of the current evidence. *Int J Cancer* **124**, 2658-70 (2009).
11. Gilham, C. et al. Day care in infancy and risk of childhood acute lymphoblastic leukaemia: findings from UK case-control study. *BMJ* **330**, 1294 (2005).

12. Kamper-Jorgensen, M. et al. Childcare in the first 2 years of life reduces the risk of childhood acute lymphoblastic leukemia. *Leukemia* **22**, 189-93 (2008).

13. Ma, X. et al. Ethnic difference in daycare attendance, early infections, and risk of childhood acute lymphoblastic leukemia. *Cancer Epidemiol Biomarkers Prev* **14**, 1928-34 (2005).

14. Nachman, J. Clinical characteristics, biologic features and outcome for young adult patients with acute lymphoblastic leukaemia. *Br J Haematol* **130**, 166-73 (2005).

15. Stock, W. et al. What determines the outcomes for adolescents and young adults with acute lymphoblastic leukemia treated on cooperative group protocols? A comparison of Children's Cancer Group and Cancer and Leukemia Group B studies. *Blood* **112**, 1646-54 (2008).

16. Faderl, S. et al. Adult acute lymphoblastic leukemia: concepts and strategies. *Cancer* **116**, 1165-76 (2010).

17. Bleyer, A. Young adult oncology: the patients and their survival challenges. *CA Cancer J Clin* **57**, 242-55 (2007).

18. Armstrong, S.A. & Look, A.T. Molecular genetics of acute lymphoblastic leukemia. *J Clin Oncol* **23**, 6306-15 (2005).

19. Kersey, J.H. Fifty years of studies of the biology and therapy of childhood leukemia. *Blood* **90**, 4243-51 (1997).

20. Pui, C.H. & Evans, W.E. Treatment of acute lymphoblastic leukemia. *N Engl J Med* **354**, 166-78 (2006).

21. Pui, C.H., Relling, M.V. & Downing, J.R. Acute lymphoblastic leukemia. *N Engl J Med* **350**, 1535-48 (2004).

22. Ferrando, A.A. et al. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell* **1**, 75-87 (2002).

23. Ferrando, A.A. & Look, A.T. Clinical implications of recurring chromosomal and associated molecular abnormalities in acute lymphoblastic leukemia. *Semin Hematol* **37**, 381-95 (2000).

24. Uckun, F.M. et al. Biology and treatment of childhood T-lineage acute lymphoblastic leukemia. *Blood* **91**, 735-46 (1998).

25. Pui, C.H. T cell acute lymphoblastic leukemia: NOTCHing the way toward a better treatment outcome. *Cancer Cell* **15**, 85-7 (2009).

26. Henderson, M.J. et al. Mechanism of relapse in pediatric acute lymphoblastic leukemia. *Cell Cycle* **7**, 1315-20 (2008).

27. Mullighan, C.G. et al. Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. *Science* **322**, 1377-80 (2008).

28. Zuna, J. et al. Childhood secondary ALL after ALL treatment. *Leukemia* **21**, 1431-5 (2007).

29. Barrett, A.J. et al. Bone marrow transplants from HLA-identical siblings as compared with chemotherapy for children with acute lymphoblastic leukemia in a second remission. *N Engl J Med* **331**, 1253-8 (1994).

30. Biggs, J.C. et al. Bone marrow transplants may cure patients with acute leukemia never achieving remission with chemotherapy. *Blood* **80**, 1090-3 (1992).

31. Palomero, T., Dominguez, M. & Ferrando, A.A. The role of the PTEN/AKT Pathway in NOTCH1-induced leukemia. *Cell Cycle* **7**, 965-70 (2008).

32. Schroeder, H. et al. Allogeneic bone marrow transplantation in second remission of childhood acute lymphoblastic leukemia: a population-based case control study from the Nordic countries. *Bone Marrow Transplant* **23**, 555-60 (1999).

33. Van Vlierberghe, P., Pieters, R., Beverloo, H.B. & Meijerink, J.P. Molecular-genetic insights in paediatric T-cell acute lymphoblastic leukaemia. *Br J Haematol* **143**, 153-68 (2008).

34. Zhao, W.L. Targeted therapy in T-cell malignancies: dysregulation of the cellular signaling pathways. *Leukemia* **24**, 13-21 (2010).

35. Vilimas, T. et al. Targeting the NF-kappaB signaling pathway in Notch1-induced T-cell leukemia. *Nat Med* **13**, 70-7 (2007).

36. dos Santos, N.R. et al. RelB-dependent stromal cells promote T-cell leukemogenesis. *PLoS One* **3**, e2555 (2008).

37. Medyouf, H. & Ghysdael, J. The calcineurin/NFAT signaling pathway: a novel therapeutic target in leukemia and solid tumors. *Cell Cycle* **7**, 297-303 (2008).

38. Grabher, C., von Boehmer, H. & Look, A.T. Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukaemia. *Nat Rev Cancer* **6**, 347-59 (2006).

39. Chabner, B.A. & Roberts, T.G., Jr. Timeline: Chemotherapy and the war on cancer. *Nat Rev Cancer* **5**, 65-72 (2005).

40. Farber, S. & Diamond, L.K. Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl-glutamic acid. *N Engl J Med* **238**, 787-93 (1948).

41. Hitchings, G.H. & Elion, G.B. The chemistry and biochemistry of purine analogs. *Ann N Y Acad Sci* **60**, 195-9 (1954).

42. Skipper, H.E., Thomson, J.R., Elion, G.B. & Hitchings, G.H. Observations on the anticancer activity of 6-mercaptopurine. *Cancer Res* **14**, 294-8 (1954).

43. Carroll, W.L., Bhojwani, D., Min, D.J., Moskowitz, N. & Raetz, E.A. Childhood acute lymphoblastic leukemia in the age of genomics. *Pediatr Blood Cancer* **46**, 570-8 (2006).

44. Frei, E., 3rd et al. The effectiveness of combinations of antileukemic agents in inducing and maintaining remission in children with acute leukemia. *Blood* **26**, 642-56 (1965).

45. Carroll, W.L. et al. Pediatric acute lymphoblastic leukemia. *Hematology Am Soc Hematol Educ Program*, 102-31 (2003).

46. Gaynon, P.S. Childhood acute lymphoblastic leukaemia and relapse. *Br J Haematol* **131**, 579-87 (2005).

47. Faderl, S., Jeha, S. & Kantarjian, H.M. The biology and therapy of adult acute lymphoblastic leukemia. *Cancer* **98**, 1337-54 (2003).

48. Pui, C.H. in Childhood leukemias (ed. Pui, C.H.) 439-472 (Cambridge University Press, New York, 2006).

49. Dordelmann, M. et al. Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood* **94**, 1209-17 (1999).

50. Schrappe, M. et al. Philadelphia chromosome-positive (Ph+) childhood acute lymphoblastic leukemia: good initial steroid response allows early prediction of a favorable treatment outcome. *Blood* **92**, 2730-41 (1998).

51. Schrappe, M. et al. Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90. German-Austrian-Swiss ALL-BFM Study Group. *Blood* **95**, 3310-22 (2000).

52. Hongo, T., Yajima, S., Sakurai, M., Horikoshi, Y. & Hanada, R. In vitro drug sensitivity testing can predict induction failure and early relapse of childhood acute lymphoblastic leukemia. *Blood* **89**, 2959-65 (1997).

53. Klumper, E. et al. In vitro cellular drug resistance in children with relapsed/refractory acute lymphoblastic leukemia. *Blood* **86**, 3861-8 (1995).

54. Kaspers, G.J. et al. Immunophenotypic cell lineage and in vitro cellular drug resistance in childhood relapsed acute lymphoblastic leukaemia. *Eur J Cancer* **41**, 1300-3 (2005).

55. Campana, D. Status of minimal residual disease testing in childhood haematological malignancies. *Br J Haematol* **143**, 481-9 (2008).

56. Hoelzer, D.a.G., N. in Treatment of Acute Leukemias: New Directions for Clinical Research (ed. Pui, C.H.) (Humana Press Inc., Totowa, 2003).

57. Graux, C., Cools, J., Michaux, L., Vandenberghe, P. & Hagemeijer, A. Cytogenetics and molecular genetics of T-cell acute lymphoblastic leukemia: from thymocyte to lymphoblast. *Leukemia* **20**, 1496-510 (2006).

58. Apperley, J.F. Part I: mechanisms of resistance to imatinib in chronic myeloid leukaemia. *Lancet Oncol* **8**, 1018-29 (2007).

59. Ferrer-Soler, L. et al. An update of the mechanisms of resistance to EGFR-tyrosine kinase inhibitors in breast cancer: Gefitinib (Iressa) -induced changes in the expression and nucleo-cytoplasmic trafficking of HER-ligands (Review). *Int J Mol Med* **20**, 3-10 (2007).

60. Mellor, H.R. & Callaghan, R. Resistance to chemotherapy in cancer: a complex and integrated cellular response. *Pharmacology* **81**, 275-300 (2008).

61. Morgan, T.H. The theory of the gene. *The American Naturalist*, 513-544 (1917).

62. Radtke, F. & Raj, K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat Rev Cancer* **3**, 756-67 (2003).

63. Wharton, K.A., Johansen, K.M., Xu, T. & Artavanis-Tsakonas, S. Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* **43**, 567-81 (1985).

64. Lai, E.C. Notch signaling: control of cell communication and cell fate. *Development* **131**, 965-73 (2004).

65. Radtke, F., Wilson, A., Mancini, S.J. & MacDonald, H.R. Notch regulation of lymphocyte development and function. *Nat Immunol* **5**, 247-53 (2004).

66. Harper, J.A., Yuan, J.S., Tan, J.B., Visan, I. & Guidos, C.J. Notch signaling in development and disease. *Clin Genet* **64**, 461-72 (2003).

67. Weinmaster, G. & Kopan, R. A garden of Notch-ly delights. *Development* **133**, 3277-82 (2006).

68. Bettenhausen, B., Hrabe de Angelis, M., Simon, D., Guenet, J.L. & Gossler, A. Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to Drosophila Delta. *Development* **121**, 2407-18 (1995).

69. Dunwoodie, S.L., Henrique, D., Harrison, S.M. & Beddington, R.S. Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo. *Development* **124**, 3065-76 (1997).

70. Lindsell, C.E., Shawber, C.J., Boulter, J. & Weinmaster, G. Jagged: a mammalian ligand that activates Notch1. *Cell* **80**, 909-17 (1995).

71. Shawber, C., Boulter, J., Lindsell, C.E. & Weinmaster, G. Jagged2: a serrate-like gene expressed during rat embryogenesis. *Dev Biol* **180**, 370-6 (1996).

72. Shutter, J.R. et al. Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev* **14**, 1313-8 (2000).

73. Palomero, T. & Ferrando, A. Therapeutic targeting of NOTCH1 signaling in T-cell acute lymphoblastic leukemia. *Clin Lymphoma Myeloma* **9 Suppl 3**, S205-10 (2009).

74. Weerkamp, F., van Dongen, J.J. & Staal, F.J. Notch and Wnt signaling in T-lymphocyte development and acute lymphoblastic leukemia. *Leukemia* **20**, 1197-205 (2006).

75. Wu, L. T lineage progenitors: the earliest steps en route to T lymphocytes. *Curr Opin Immunol* **18**, 121-6 (2006).

76. Pui, C.H. et al. Improved outcome for children with acute lymphoblastic leukemia: results of Total Therapy Study XIIIB at St Jude Children's Research Hospital. *Blood* **104**, 2690-6 (2004).

77. Radtke, F. et al. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10**, 547-58 (1999).

78. Artavanis-Tsakonas, S., Rand, M.D. & Lake, R.J. Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-6 (1999).

79. Miele, L. Notch signaling. *Clin Cancer Res* **12**, 1074-9 (2006).

80. Lawrence, N., Klein, T., Brennan, K. & Martinez Arias, A. Structural requirements for notch signalling with delta and serrate during the development and patterning of the wing disc of Drosophila. *Development* **127**, 3185-95 (2000).

81. Rebay, I. et al. Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* **67**, 687-99 (1991).

82. Sanchez-Irizarry, C. et al. Notch subunit heterodimerization and prevention of ligand-independent proteolytic activation depend, respectively, on a novel domain and the LNR repeats. *Mol Cell Biol* **24**, 9265-73 (2004).

83. Blaumueller, C.M., Qi, H., Zagouras, P. & Artavanis-Tsakonas, S. Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. *Cell* **90**, 281-91 (1997).

84. Logeat, F. et al. The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proc Natl Acad Sci U S A* **95**, 8108-12 (1998).

85. Brou, C. et al. A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol Cell* **5**, 207-16 (2000).

86. Mumm, J.S. et al. A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. *Mol Cell* **5**, 197-206 (2000).

87. Shah, S. et al. Nicastrin functions as a gamma-secretase-substrate receptor. *Cell* **122**, 435-47 (2005).

88. Lai, E.C. Protein degradation: four E3s for the notch pathway. *Curr Biol* **12**, R74-8 (2002).

89. Qiu, L. et al. Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. *J Biol Chem* **275**, 35734-7 (2000).

90. McGill, M.A. & McGlade, C.J. Mammalian numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain. *J Biol Chem* **278**, 23196-203 (2003).

91. Malyukova, A. et al. The tumor suppressor gene hCDC4 is frequently mutated in human T-cell acute lymphoblastic leukemia with functional consequences for Notch signaling. *Cancer Res* **67**, 5611-6 (2007).

92. O'Neil, J. et al. FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. *J Exp Med* **204**, 1813-24 (2007).

93. Thompson, B.J. et al. The SCFFBW7 ubiquitin ligase complex as a tumor suppressor in T cell leukemia. *J Exp Med* **204**, 1825-35 (2007).

94. Welcker, M. & Clurman, B.E. FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. *Nat Rev Cancer* **8**, 83-93 (2008).

95. Oberg, C. et al. The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. *J Biol Chem* **276**, 35847-53 (2001).

96. Spruck, C.H. et al. hCDC4 gene mutations in endometrial cancer. *Cancer Res* **62**, 4535-9 (2002).

97. Bai, C. et al. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* **86**, 263-74 (1996).

98. Hao, B., Oehlmann, S., Sowa, M.E., Harper, J.W. & Pavletich, N.P. Structure of a Fbw7-Skp1-cyclin E complex: multisite-phosphorylated substrate recognition by SCF ubiquitin ligases. *Mol Cell* **26**, 131-43 (2007).

99. Orlicky, S., Tang, X., Willems, A., Tyers, M. & Sicheri, F. Structural basis for phosphodependent substrate selection and orientation by the SCFCdc4 ubiquitin ligase. *Cell* **112**, 243-56 (2003).

100. Tang, X. et al. Suprafacial orientation of the SCFCdc4 dimer accommodates multiple geometries for substrate ubiquitination. *Cell* **129**, 1165-76 (2007).

101. Welcker, M. & Clurman, B.E. Fbw7/hCDC4 dimerization regulates its substrate interactions. *Cell Div* **2**, 7 (2007).

102. Zhang, W. & Koepp, D.M. Fbw7 isoform interaction contributes to cyclin E proteolysis. *Mol Cancer Res* **4**, 935-43 (2006).

103. Sjolund, J., Manetopoulos, C., Stockhausen, M.T. & Axelson, H. The Notch pathway in cancer: differentiation gone awry. *Eur J Cancer* **41**, 2620-9 (2005).

104. Palomero, T. et al. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc Natl Acad Sci U S A* **103**, 18261-6 (2006).

105. Weng, A.P. et al. c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev* **20**, 2096-109 (2006).

106. Iso, T., Kedes, L. & Hamamori, Y. HES and HERP families: multiple effectors of the Notch signaling pathway. *J Cell Physiol* **194**, 237-55 (2003).

107. Yamamoto, N. et al. Role of Deltex-1 as a transcriptional regulator downstream of the Notch receptor. *J Biol Chem* **276**, 45031-40 (2001).

108. Yun, T.J. & Bevan, M.J. Notch-regulated ankyrin-repeat protein inhibits Notch1 signaling: multiple Notch1 signaling pathways involved in T cell development. *J Immunol* **170**, 5834-41 (2003).

109. Ishibashi, M. et al. Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. *Genes Dev* **9**, 3136-48 (1995).

110. Ohsako, S., Hyer, J., Panganiban, G., Oliver, I. & Caudy, M. Hairy function as a DNA-binding helix-loop-helix repressor of Drosophila sensory organ formation. *Genes Dev* **8**, 2743-55 (1994).

111. Van Doren, M., Bailey, A.M., Esnayra, J., Ede, K. & Posakony, J.W. Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of achaete. *Genes Dev* **8**, 2729-42 (1994).

112. Castella, P., Sawai, S., Nakao, K., Wagner, J.A. & Caudy, M. HES-1 repression of differentiation and proliferation in PC12 cells: role for the helix 3-helix 4 domain in transcription repression. *Mol Cell Biol* **20**, 6170-83 (2000).

113. Chen, H. et al. Conservation of the Drosophila lateral inhibition pathway in human lung cancer: a hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression. *Proc Natl Acad Sci U S A* **94**, 5355-60 (1997).

114. Kim, H.K. & Siu, G. The notch pathway intermediate HES-1 silences CD4 gene expression. *Mol Cell Biol* **18**, 7166-75 (1998).

115. Blacklow, S.C. A new niche for Notch on Deltex? *Structure* **13**, 1579-80 (2005).

116. Matsuno, K., Diederich, R.J., Go, M.J., Blaumueller, C.M. & Artavanis-Tsakonas, S. Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development* **121**, 2633-44 (1995).

117. Xu, T. & Artavanis-Tsakonas, S. deltex, a locus interacting with the neurogenic genes, Notch, Delta and mastermind in Drosophila melanogaster. *Genetics* **126**, 665-77 (1990).

118. Izon, D.J. et al. Deltex1 redirects lymphoid progenitors to the B cell lineage by antagonizing Notch1. *Immunity* **16**, 231-43 (2002).

119. Kiaris, H. et al. Modulation of notch signaling elicits signature tumors and inhibits hras1-induced oncogenesis in the mouse mammary epithelium. *Am J Pathol* **165**, 695-705 (2004).

120. Sestan, N., Artavanis-Tsakonas, S. & Rakic, P. Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. *Science* **286**, 741-6 (1999).

121. Ellisen, L.W. et al. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66**, 649-61 (1991).

122. Allman, D. et al. Separation of Notch1 promoted lineage commitment and expansion/transformation in developing T cells. *J Exp Med* **194**, 99-106 (2001).

123. Aster, J.C. et al. Essential roles for ankyrin repeat and transactivation domains in induction of T-cell leukemia by notch1. *Mol Cell Biol* **20**, 7505-15 (2000).

124. Pear, W.S. et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J Exp Med* **183**, 2283-91 (1996).

125. Weng, A.P. et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* **306**, 269-71 (2004).

126. Lai, E.C. Keeping a good pathway down: transcriptional repression of Notch pathway target genes by CSL proteins. *EMBO Rep* **3**, 840-5 (2002).

127. Weng, A.P. et al. Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. *Mol Cell Biol* **23**, 655-64 (2003).

128. Sulis, M.L. et al. NOTCH1 extracellular juxtamembrane expansion mutations in T-ALL. *Blood* **112**, 733-40 (2008).

129. Malecki, M.J. et al. Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two distinct mechanistic classes. *Mol Cell Biol* **26**, 4642-51 (2006).

130. Sade, H., Krishna, S. & Sarin, A. The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. *J Biol Chem* **279**, 2937-44 (2004).

131. Zhu, Y.M. et al. NOTCH1 mutations in T-cell acute lymphoblastic leukemia: prognostic significance and implication in multifactorial leukemogenesis. *Clin Cancer Res* **12**, 3043-9 (2006).

132. Breit, S. et al. Activating NOTCH1 mutations predict favorable early treatment response and long-term outcome in childhood precursor T-cell lymphoblastic leukemia. *Blood* **108**, 1151-7 (2006).

133. Stanulla, M. et al. Thiopurine methyltransferase (TPMT) genotype and early treatment response to mercaptopurine in childhood acute lymphoblastic leukemia. *JAMA* **293**, 1485-9 (2005).

134. Gustafsson, G. et al. Improving outcome through two decades in childhood ALL in the Nordic countries: the impact of high-dose methotrexate in the reduction of CNS irradiation. Nordic Society of Pediatric Haematology and Oncology (NOPHO). *Leukemia* **14**, 2267-75 (2000).

135. van Grotel, M. et al. Prognostic significance of molecular-cytogenetic abnormalities in pediatric T-ALL is not explained by immunophenotypic differences. *Leukemia* **22**, 124-31 (2008).

136. Eguchi-Ishimae, M., Eguchi, M., Kempski, H. & Greaves, M. NOTCH1 mutation can be an early, prenatal genetic event in T-ALL. *Blood* **111**, 376-8 (2008).

137. Mansour, M.R. et al. Notch-1 mutations are secondary events in some patients with T-cell acute lymphoblastic leukemia. *Clin Cancer Res* **13**, 6964-9 (2007).

138. Mansour, M.R., Linch, D.C., Foroni, L., Goldstone, A.H. & Gale, R.E. High incidence of Notch-1 mutations in adult patients with T-cell acute lymphoblastic leukemia. *Leukemia* **20**, 537-9 (2006).

139. Mansour, M.R. et al. Prognostic implications of NOTCH1 and FBXW7 mutations in adults with T-cell acute lymphoblastic leukemia treated on the MRC UKALLXII/ECOG E2993 protocol. *J Clin Oncol* **27**, 4352-6 (2009).

140. Asnafi, V. et al. NOTCH1/FBXW7 mutation identifies a large subgroup with favorable outcome in adult T-cell acute lymphoblastic leukemia (T-ALL): a Group for Research on Adult Acute Lymphoblastic Leukemia (GRAALL) study. *Blood* **113**, 3918-24 (2009).

141. Baldus, C.D. et al. Prognostic implications of NOTCH1 and FBXW7 mutations in adult acute T-lymphoblastic leukemia. *Haematologica* **94**, 1383-90 (2009).

142. Marks, D.I. et al. T-cell acute lymphoblastic leukemia in adults: clinical features, immunophenotype, cytogenetics, and outcome from the large randomized prospective trial (UKALL XII/ECOG 2993). *Blood* **114**, 5136-45 (2009).

143. Miyamoto, Y. et al. Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. *Cancer Cell* **3**, 565-76 (2003).

144. Santagata, S. et al. JAGGED1 expression is associated with prostate cancer metastasis and recurrence. *Cancer Res* **64**, 6854-7 (2004).

145. Gray, G.E. et al. Human ligands of the Notch receptor. *Am J Pathol* **154**, 785-94 (1999).

146. Cuevas, I.C. et al. Meningioma transcript profiles reveal deregulated Notch signaling pathway. *Cancer Res* **65**, 5070-5 (2005).

147. Purow, B.W. et al. Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. *Cancer Res* **65**, 2353-63 (2005).

148. Zagouras, P., Stifani, S., Blaumueller, C.M., Carcangiu, M.L. & Artavanis-Tsakonas, S. Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proc Natl Acad Sci U S A* **92**, 6414-8 (1995).

149. Nickoloff, B.J., Osborne, B.A. & Miele, L. Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. *Oncogene* **22**, 6598-608 (2003).

150. Weijzen, S. et al. Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. *Nat Med* **8**, 979-86 (2002).

151. Callahan, R. & Egan, S.E. Notch signaling in mammary development and oncogenesis. *J Mammary Gland Biol Neoplasia* **9**, 145-63 (2004).

152. Parr, C., Watkins, G. & Jiang, W.G. The possible correlation of Notch-1 and Notch-2 with clinical outcome and tumour clinicopathological parameters in human breast cancer. *Int J Mol Med* **14**, 779-86 (2004).

153. Pece, S. et al. Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis. *J Cell Biol* **167**, 215-21 (2004).

154. Lewis, H.D. et al. Apoptosis in T cell acute lymphoblastic leukemia cells after cell cycle arrest induced by pharmacological inhibition of notch signaling. *Chem Biol* **14**, 209-19 (2007).

155. Tolia, A. & De Strooper, B. Structure and function of gamma-secretase. *Semin Cell Dev Biol* **20**, 211-8 (2009).

156. Milano, J. et al. Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. *Toxicol Sci* **82**, 341-58 (2004).

157. Searfoss, G.H. et al. Adipsin, a biomarker of gastrointestinal toxicity mediated by a functional gamma-secretase inhibitor. *J Biol Chem* **278**, 46107-16 (2003).

158. van Es, J.H. et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* **435**, 959-63 (2005).

159. Wong, G.T. et al. Chronic treatment with the gamma-secretase inhibitor LY-411,575 inhibits beta-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation. *J Biol Chem* **279**, 12876-82 (2004).

160. DeAngelo, D.J. et al. Phase 1 clinical results with tandutinib (MLN518), a novel FLT3 antagonist, in patients with acute myelogenous leukemia or high-risk myelodysplastic syndrome: safety, pharmacokinetics, and pharmacodynamics. *Blood* **108**, 3674-81 (2006).

161. Ferrando, A.A. The role of NOTCH1 signaling in T-ALL. *Hematology Am Soc Hematol Educ Program*, 353-61 (2009).

162. Staal, F.J. & Langerak, A.W. Signaling pathways involved in the development of T-cell acute lymphoblastic leukemia. *Haematologica* **93**, 493-7 (2008).

163. Li, T. et al. Epidermal growth factor receptor and notch pathways participate in the tumor suppressor function of gamma-secretase. *J Biol Chem* **282**, 32264-73 (2007).

164. Palomero, T. et al. CUTLL1, a novel human T-cell lymphoma cell line with t(7;9) rearrangement, aberrant NOTCH1 activation and high sensitivity to gamma-secretase inhibitors. *Leukemia* **20**, 1279-87 (2006).

165. Margolin, A.A. et al. ChIP-on-chip significance analysis reveals large-scale binding and regulation by human transcription factor oncogenes. *Proc Natl Acad Sci U S A* **106**, 244-9 (2009).

166. Palomero, T. et al. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. *Nat Med* **13**, 1203-10 (2007).

167. De Strooper, B., Vassar, R. & Golde, T. The secretases: enzymes with therapeutic potential in Alzheimer disease. *Nat Rev Neurol* **6**, 99-107 (2010).

168. Wolfe, M.S. Inhibition and modulation of gamma-secretase for Alzheimer's disease. *Neurotherapeutics* **5**, 391-8 (2008).

169. Real, P.J. et al. Gamma-secretase inhibitors reverse glucocorticoid resistance in T cell acute lymphoblastic leukemia. *Nat Med* **15**, 50-8 (2009).

170. Cullion, K. et al. Targeting the Notch1 and mTOR pathways in a mouse T-ALL model. *Blood* **113**, 6172-81 (2009).

171. Amzel, L.M. et al. Structural comparisons of class I phosphoinositide 3-kinases. *Nat Rev Cancer* **8**, 665-9 (2008).

172. Engelman, J.A. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer* **9**, 550-62 (2009).

173. Sarbassov, D.D., Guertin, D.A., Ali, S.M. & Sabatini, D.M. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**, 1098-101 (2005).

174. Bader, A.G., Kang, S., Zhao, L. & Vogt, P.K. Oncogenic PI3K deregulates transcription and translation. *Nat Rev Cancer* **5**, 921-9 (2005).

175. Cully, M., You, H., Levine, A.J. & Mak, T.W. Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat Rev Cancer* **6**, 184-92 (2006).

176. Hay, N. The Akt-mTOR tango and its relevance to cancer. *Cancer Cell* **8**, 179-83 (2005).

177. Katso, R. et al. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu Rev Cell Dev Biol* **17**, 615-75 (2001).

178. Samuels, Y. & Ericson, K. Oncogenic PI3K and its role in cancer. *Curr Opin Oncol* **18**, 77-82 (2006).

179. Sulis, M.L. & Parsons, R. PTEN: from pathology to biology. *Trends Cell Biol* **13**, 478-83 (2003).

180. Manning, B.D. & Cantley, L.C. United at last: the tuberous sclerosis complex gene products connect the phosphoinositide 3-kinase/Akt pathway to mammalian target of rapamycin (mTOR) signalling. *Biochem Soc Trans* **31**, 573-8 (2003).

181. Chan, S.M., Weng, A.P., Tibshirani, R., Aster, J.C. & Utz, P.J. Notch signals positively regulate activity of the mTOR pathway in T-cell acute lymphoblastic leukemia. *Blood* **110**, 278-86 (2007).

182. Sabatini, D.M. mTOR and cancer: insights into a complex relationship. *Nat Rev Cancer* **6**, 729-34 (2006).

183. Brown, V.I., Seif, A.E., Reid, G.S., Teachey, D.T. & Grupp, S.A. Novel molecular and cellular therapeutic targets in acute lymphoblastic leukemia and lymphoproliferative disease. *Immunol Res* **42**, 84-105 (2008).

184. Stambolic, V. et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95**, 29-39 (1998).

185. Eng, C. PTEN: one gene, many syndromes. *Hum Mutat* **22**, 183-98 (2003).

186. Bussaglia, E., del Rio, E., Matias-Guiu, X. & Prat, J. PTEN mutations in endometrial carcinomas: a molecular and clinicopathologic analysis of 38 cases. *Hum Pathol* **31**, 312-7 (2000).

187. Celebi, J.T., Shendrik, I., Silvers, D.N. & Peacocke, M. Identification of PTEN mutations in metastatic melanoma specimens. *J Med Genet* **37**, 653-7 (2000).

188. Wang, S.I., Parsons, R. & Ittmann, M. Homozygous deletion of the PTEN tumor suppressor gene in a subset of prostate adenocarcinomas. *Clin Cancer Res* **4**, 811-5 (1998).

189. Di Cristofano, A., Pesce, B., Cordon-Cardo, C. & Pandolfi, P.P. Pten is essential for embryonic development and tumour suppression. *Nat Genet* **19**, 348-55 (1998).

190. Podsypanina, K. et al. Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. *Proc Natl Acad Sci U S A* **96**, 1563-8 (1999).

191. Sakai, A., Thieblemont, C., Wellmann, A., Jaffe, E.S. & Raffeld, M. PTEN gene alterations in lymphoid neoplasms. *Blood* **92**, 3410-5 (1998).

192. Shan, X. et al. Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. *Mol Cell Biol* **20**, 6945-57 (2000).

193. Lee, S.R. et al. Reversible inactivation of the tumor suppressor PTEN by H2O2. *J Biol Chem* **277**, 20336-42 (2002).

194. Leslie, N.R. et al. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *Embo J* **22**, 5501-10 (2003).

195. Torres, J. & Pulido, R. The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. *J Biol Chem* **276**, 993-8 (2001).

196. Vazquez, F., Ramaswamy, S., Nakamura, N. & Sellers, W.R. Phosphorylation of the PTEN tail regulates protein stability and function. *Mol Cell Biol* **20**, 5010-8 (2000).

197. Mavrakis, K.J. et al. Genome-wide RNA-mediated interference screen identifies miR-19 targets in Notch-induced T-cell acute lymphoblastic leukaemia. *Nat Cell Biol* **12**, 372-9 (2010).

198. Silva, A. et al. PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability. *J Clin Invest* **118**, 3762-74 (2008).

199. Silva, A. et al. Regulation of PTEN by CK2 and Notch1 in primary T-cell acute lymphoblastic leukemia: rationale for combined use of CK2- and gamma-secretase inhibitors. *Haematologica* **95**, 674-8 (2010).

200. Medyouf, H. et al. Acute T-cell leukemias remain dependent on Notch signaling despite PTEN and INK4A/ARF loss. *Blood* **115**, 1175-84 (2010).

201. Gregory, R.I. & Shiekhattar, R. MicroRNA biogenesis and cancer. *Cancer Res* **65**, 3509-12 (2005).

202. Cai, X., Hagedorn, C.H. & Cullen, B.R. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA* **10**, 1957-66 (2004).

203. Lee, Y. et al. MicroRNA genes are transcribed by RNA polymerase II. *Embo J* **23**, 4051-60 (2004).

204. Lee, Y. et al. The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415-9 (2003).

205. Zhang, H., Kolb, F.A., Jaskiewicz, L., Westhof, E. & Filipowicz, W. Single processing center models for human Dicer and bacterial RNase III. *Cell* **118**, 57-68 (2004).

206. Bartel, D.P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281-97 (2004).

207. Carrington, J.C. & Ambros, V. Role of microRNAs in plant and animal development. *Science* **301**, 336-8 (2003).

208. Lawrie, C.H. MicroRNAs and haematology: small molecules, big function. *Br J Haematol* **137**, 503-12 (2007).

209. John, B. et al. Human MicroRNA targets. *PLoS Biol* **2**, e363 (2004).

210. Lewis, B.P., Burge, C.B. & Bartel, D.P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**, 15-20 (2005).

211. Calin, G.A. et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* **99**, 15524-9 (2002).

212. Eis, P.S. et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci U S A* **102**, 3627-32 (2005).

213. Johnson, S.M. et al. RAS is regulated by the let-7 microRNA family. *Cell* **120**, 635-47 (2005).

214. Michael, M.Z., SM, O.C., van Holst Pellekaan, N.G., Young, G.P. & James, R.J. Reduced accumulation of specific microRNAs in colorectal neoplasia. *Mol Cancer Res* **1**, 882-91 (2003).

215. Lu, J. et al. MicroRNA expression profiles classify human cancers. *Nature* **435**, 834-8 (2005).

216. Calin, G.A. et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med* **353**, 1793-801 (2005).

217. Cimmino, A. et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A* **102**, 13944-9 (2005).

218. Gupta-Rossi, N. et al. Monoubiquitination and endocytosis direct gamma-secretase cleavage of activated Notch receptor. *J Cell Biol* **166**, 73-83 (2004).

219. Chiang, M.Y. et al. Identification of a conserved negative regulatory sequence that influences the leukemogenic activity of NOTCH1. *Mol Cell Biol* **26**, 6261-71 (2006).

220. Neshat, M.S. et al. Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. *Proc Natl Acad Sci U S A* **98**, 10314-9 (2001).

221. Suzuki, A. et al. High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. *Curr Biol* **8**, 1169-78 (1998).

222. Suzuki, A. et al. T cell-specific loss of Pten leads to defects in central and peripheral tolerance. *Immunity* **14**, 523-34 (2001).

223. Guo, W. et al. Multi-genetic events collaboratively contribute to Pten-null leukaemia stem-cell formation. *Nature* **453**, 529-33 (2008).

224. Kantarjian, H. et al. Long-term follow-up results of hyperfractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone (Hyper-CVAD), a dose-intensive regimen, in adult acute lymphocytic leukemia. *Cancer* **101**, 2788-801 (2004).

225. Henze, G.a.v.S., A. in Treatment of Acute Leukemias: New Directions for Clinical Research (ed. Pui, C.H.) 119-219 (Humana Press Inc, Totowa, 2003).

226. Gadner, H., Mann, G. and Peters, C. in Treatment of Acute Leukemias: New Directions for Clinical Research (ed. Pui, C.H.) 183-197 (Humana Press Inc., Totowa, 2003).

227. Brunning, R.D.a.M., R.W. in Tumors of the bone marrow 100-136 (Armed Forces Institute of Pathology, Washington, D.C., 1994).

228. Ge, Y. et al. Prognostic role of the reduced folate carrier, the major membrane transporter for methotrexate, in childhood acute lymphoblastic leukemia: a report from the Children's Oncology Group. *Clin Cancer Res* **13**, 451-7 (2007).

229. Whelan, J.T., Forbes, S.L. & Bertrand, F.E. CBF-1 (RBP-J kappa) binds to the PTEN promoter and regulates PTEN gene expression. *Cell Cycle* **6**, 80-4 (2007).

230. Reedijk, M. et al. High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. *Cancer Res* **65**, 8530-7 (2005).

231. Fortini, M.E. Gamma-secretase-mediated proteolysis in cell-surface-receptor signalling. *Nat Rev Mol Cell Biol* **3**, 673-84 (2002).

232. Stow, P. et al. Clinical significance of low levels of minimal residual disease at the end of remission induction therapy in childhood acute lymphoblastic leukemia. *Blood* (2010).

233. Liu, S., Breit, S., Danckwardt, S., Muckenthaler, M.U. & Kulozik, A.E. Downregulation of Notch signaling by gamma-secretase inhibition can abrogate chemotherapy-induced apoptosis in T-ALL cell lines. *Ann Hematol* **88**, 613-21 (2009).

234. Delaney, C., Varnum-Finney, B., Aoyama, K., Brashem-Stein, C. & Bernstein, I.D. Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. *Blood* **106**, 2693-9 (2005).

235. Rangarajan, A. et al. Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *Embo J* **20**, 3427-36 (2001).

236. Deftos, M.L., Huang, E., Ojala, E.W., Forbush, K.A. & Bevan, M.J. Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes. *Immunity* **13**, 73-84 (2000).

237. Reizis, B. & Leder, P. Direct induction of T lymphocyte-specific gene expression by the mammalian Notch signaling pathway. *Genes Dev* **16**, 295-300 (2002).

238. Ronchini, C. & Capobianco, A.J. Induction of cyclin D1 transcription and CDK2 activity by Notch(ic): implication for cell cycle disruption in transformation by Notch(ic). *Mol Cell Biol* **21**, 5925-34 (2001).

239. Jehn, B.M., Bielke, W., Pear, W.S. & Osborne, B.A. Cutting edge: protective effects of notch-1 on TCR-induced apoptosis. *J Immunol* **162**, 635-8 (1999).

240. Buonamici, S. et al. CCR7 signalling as an essential regulator of CNS infiltration in T-cell leukaemia. *Nature* **459**, 1000-4 (2009).

241. Forster, R., Davalos-Misslitz, A.C. & Rot, A. CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol* **8**, 362-71 (2008).

242. Chadwick, N. et al. Identification of novel Notch target genes in T cell leukaemia. *Mol Cancer* **8**, 35 (2009).

243. Gonzalez-Garcia, S. et al. CSL-MAML-dependent Notch1 signaling controls T lineage-specific IL-7R{alpha} gene expression in early human thymopoiesis and leukemia. *J Exp Med* **206**, 779-91 (2009).

244. Rao, S.S. et al. Inhibition of NOTCH signaling by gamma secretase inhibitor engages the RB pathway and elicits cell cycle exit in T-cell acute lymphoblastic leukemia cells. *Cancer Res* **69**, 3060-8 (2009).

ABSTRACT

MOLECULAR AND THERAPEUTIC IMPLICATIONS OF NOTCH1 SIGNALING IN PEDIATRIC T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

by

AMANDA LARSON GEDMAN

August 2010

Advisor: Dr. Larry H. Matherly

Major: Cancer Biology

Degree: Doctor of Philosophy

T-cell acute lymphoblastic leukemia (T-ALL) accounts for 15% of pediatric ALL cases and is associated with early relapse and inferior outcome. The poorer prognosis of T-ALL compared to B-precursor ALL may in part reflect the lack of unique features on which to base therapy. NOTCH1 mutations are of particular interest since these were reported in 37-71% of T-ALLs. The prognostic value of NOTCH1 mutations remains controversial as both favorable and unfavorable associations were reported, whereas in other studies, there were no associations between NOTCH1 mutations and treatment outcome. We explored the impact of mutations in NOTCH1, FBW7 and PTEN on prognosis and downstream signaling in pediatric T-cell acute lymphoblastic leukemia. We identified a high frequency of mutations in NOTCH1 (16 patients), FBW7 (5 patients) and PTEN (26 patients) in a well defined cohort of 47 pediatric T-ALL specimens. NOTCH1 mutations showed a 1.3-3.3-fold increase in activation over wild-type NOTCH1 in reporter assays; mutant FBW7 resulted in further augmentation of NOTCH1 activity. NOTCH1 and FBW7 mutations were accompanied by increases in

median transcripts for NOTCH1 target genes (HES1, DELTEX1 and cMYC). However, none of these mutations were associated with treatment outcome. Increased HES1, DELTEX1 and cMYC transcript levels were associated with significant increases in the transcript levels of several chemotherapy relevant genes, including MDR1, ABCC5, reduced folate carrier, asparagine synthetase, thiopurine methyltransferase, Bcl-2 and dihydrofolate reductase. Our results suggest (1) multiple factors should be considered with attempting to identify molecular-based prognostic factors for pediatric T-ALL and (2) that, depending on the NOTCH1 signaling status, modifications in the types or dosing of standard chemotherapy drugs for T-ALL, or combinations of agents capable of targeting NOTCH1, AKT and/or mTOR with standard chemotherapy agents may be warranted.

Relapse is the most common caused of off-therapy events and is responsible for the majority of ALL treatment failures. Relapse can arise from the (i) the induction of resistance via acquisition of new genetic alterations after diagnosis, (ii) the selection and expansion of an already present resistant-subpopulation at the time of diagnosis, or very rarely as (iii) a secondary, *de novo* ALL. To determine the contribution of genetic alteration to the development of relapse in T-ALL, we assessed the frequency of mutations in NOTCH1 alone or in combination with mutations in FBW7 and PTEN at the time of diagnosis and relapse in 11 paired clinical T-ALL specimens. We observed that the 7 patients harboring mutations in NOTCH1 and/or PTEN at some stage in their disease had a longer remission period (13 months vs. 5.5 months), and were typically diagnosed at an early age (120 months vs. 132 months). In these 7 patients, nearly 70% of relapse appeared to be associated with the emergence of a new leukemic clone, an

assumption made by the presence of a new mutation or loss of a mutation at relapse. Using real-time PCR techniques with specific hybridization probes, we were able to determine that the leukemic clone for one patient was present at the time of diagnosis, but at a very low expression level. This suggests that the clone responsible for relapse was resistant to the initial chemotherapy treatment. For another patient, the relapse clone could not be detected at diagnosis, suggesting that it was induced following chemotherapy. This study strongly warrants future studies with a larger patient cohort to systematically identify specific hallmarks of relapse.

NOTCH1 is a potentially attractive therapeutic target for T-ALL since constitutively activating effects of mutant NOTCH1 can be abolished with γ-secretase inhibitors (GSIs). Because of possible effects of GSIs on other cellular targets in addition to NOTCH1, we explored shRNA knockdown of NOTCH1 to identify novel NOTCH1-regulated genes that may serve as prognostic indicators or therapeutic targets in T-ALL. NOTCH1 expression was knockeddown in Jurkat T-ALL cells using lentivirus expressing shRNAs for NOTCH1 or a non-targeted control (J.ntc) sequence. NOTCH1 knockdown was verified using western blots to measure activated NOTCH1 (ICN1) protein levels, and real-time RT-PCR to measure transcript levels of known NOTCH1 targets (e.g., HES1). Two clonal sublines (J.N1KD 2-4 and J.N1KD 2-7) were identified with significantly decreased expression of NOTCH1 compared to J.ntc. The J.N1KD 2-4 and J.N1KD 2-7 sublines showed minimal changes in cell growth, cell cycle progression and apoptosis. To characterize genotypic changes accompanying NOTCH1 knockdown, we performed microarray analysis with Agilent Whole Genome oligonucleotide microarrays and microRNA (miR) HumanV2 arrays. The microarray identified Rictor, a

key component to in the mTOR2 complex, as a novel downstream target of NOTCH1 signaling. Upon NOTCH1 inhibition, an increase in the expression of Rictor was observed, both at the transcript and protein levels. Initial computational analysis of the Rictor promoter suggests that NOTCH1 may regulate its expression directly (via RBPJκ) or indirectly (via HES1). The miR array identified 20 miRs in J.N1KD 2-4 and J.N1KD 2-7 cells with altered expression compared to J.ntc greater than 1.5-fold ($p<0.05$) and ranging from 3-to10-fold. miRs hsa-Let-7e, hsa-miR-125a-5p and hsa-miR-99b, reportedly derived from a polycistronic transcript, were decreased 10-fold accompanying NOTCH1 knockdown. Using miR qPCR, we confirmed decreased levels of hsa-miR-125a-5p and hsa-miR-99b in the J.N1KD 2-4 and J.N1KD 2-7 sublines. In conclusion, we have developed novel T-ALL cell line models to study the impact of decreased NOTCH1 levels and activity independent of GSI treatment. Our results implicate NOTCH1 in regulating levels of Rictor and hsa-miR-125a-5p, and suggest that caution may be warranted in targeting NOTCH1 with GSIs in the therapy of T-ALL, reflecting the potential promotion of cell survival via the upregulation of Rictor. The downstream effect of regulating hsa-miR-125a-5p has yet to be determined.

AUTOBIOGRAPHICAL STATEMENT

Amanda Larson Gedman

EDUCATION

Wayne State University **Detroit, MI 2005-2010**
Doctor of Philosophy: Cancer Biology
Minor in Pharmacology

University of Michigan-Flint **Flint, MI 1998-2003**
Bachelor of Science: Molecular Biology and Biotechnology
Minor in Chemistry

PUBLICATIONS

Medyouf, H., Gao, X., Armstrong, F., Fusscott, S., Liu, Q., **Larson Gedman, A.**, Matherly, L.H., Schultz, K.R., Pflumio, F., You, M.J., Weng, A.P.: Acute T-cell leukemias remain dependent on Notch signaling despite PTEN loss. Blood. 2009 Feb 11 (115): 1175-84.

Larson Gedman, A., Chen, Q., Kugel Desmoulin, S., Ge, Y., LaFiura, K., Haska, C.L., Devidas, M., Linda, S.B., Taub, J.W., Matherly, L.H.: Associations of NOTCH1 mutations and downstream signaling with prognosis in pediatric T-cell acute lymphoblastic leukemia: A report from the Children's Oncology Group. Leukemia. 2009 Aug;23(8):1417-25.

PUBLISHED ABSTRACTS

Larson Gedman, A., Dombkowski, A., Taub, J.W., Matherly, L.H.: Loss of NOTCH1 is associated with decreased expression of has-miR-125a-5p in T-cell acute lymphoblastic leukemia: Implications for using NOTCH1 inhibition in chemotherapy. Abstracts, American Association for Cancer Research 51: 1058, 2010.

Medyouf, H., Gao, X., Armstrong, F., Fusscott, S., Liu, Q., **Larson Gedman, A.**, Matherly, L.H., Schultz, K.R., Pflumio, F., You, M.J., Weng, A.P.: Acute T-cell leukemias remain dependent on Notch signaling despite PTEN loss. Abstracts, Blood (ASH Annual Meeting Abstracts), 2009.

Larson Gedman, A., Chen, Q., Kugel Desmoulin, S., Cherian, C., Devidas, M., Linda, S.B., Ge, Y., Taub, J.W., Matherly, L.H.: The impact of NOTCH1 on PI3K-AKT signaling and dependence on the PTEN status in T-cell acute lymphoblastic leukemia. Abstracts, American Association for Cancer Research 50: 532, 2009.

Chen, Q., **Larson Gedman, A**., Matherly, L.H., Taub, J.W.: Analysis of clonal T-cell receptor gamma rearrangements, NOTCH1, FBW7 and PTEN mutations in paired pediatric T-cell acute lymphoblastic leukemia samples at diagnosis and relapse reveals evidence of relapse in new leukemic clones. Abstracts, Blood (ASH Annual Meeting Abstracts), Nov 2008;112:1488.

Larson Gedman, A., Ge, Y., LaFiura, K., Devidas, M., Linda, S.B., Taub, J.W., Matherly, L.H.: Association of NOTCH1 mutations in pediatric T-cell acute lymphoblastic leukemia with treatment relapse and expression of genes relevant to chemotherapy response. Abstracts, American Association for Cancer Research 48: 428, 2007.