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Molecular pathogenesis and targeted therapies for NOTCH1-induced T-cell acute lymphoblastic leukemia

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SUMMARY

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic tumor resulting from the malignant transformation of immature T-cell progenitors. Originally associated with a dismal prognosis, the outcome of T-ALL patients has improved remarkably over the last two decades as a result of the introduction of intensified chemotherapy protocols. However, these treatments are associated with significant acute and long-term toxicities, and the treatment of patients presenting with primary resistant disease or those relapsing after a transient response remains challenging.

T-ALL is a genetically heterogeneous disease in which numerous chromosomal and genetic alterations cooperate to promote the aberrant proliferation and survival of leukemic lymphoblasts. However, the identification of activating mutations in the *NOTCH1* gene in over 50% of T-ALL cases has come to define aberrant NOTCH signaling as a central player in this disease. Therefore, the NOTCH pathway represents an important potential therapeutic target. In this review, we will update our current understanding of the molecular basis of T-ALL, with a particular focus on the role of the NOTCH1 oncogene and the development of anti-NOTCH1 targeted therapies for the treatment of this disease.

Keywords

T-ALL; NOTCH; gamma-secretase inhibitors; targeted therapy; prognosis; leukemia

Introduction

Acute lymphoblastic leukemias (ALL) are characterized by the uncontrolled clonal proliferation of immature lymphoid cells which infiltrate the bone marrow. In T-cell acute lymphoblastic leukemias (T-ALL) the malignant clone is derived from T-cell progenitor cells and expresses immature immunophenotypic markers characteristic of the T-cell lineage. T-ALL represents about 15% of pediatric and 25% of adult ALLs and is typically associated with very high white cell counts, mediastinal masses with pleural effusions, and increased risk of leptomeningeal infiltration at diagnosis [1]. Although initially associated

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with a particularly bad prognosis, the introduction of intensified treatment protocols has improved the outcome of this disease and current therapies achieve five-year relapse-free survival rates of about 75% in children and 50% in adults [2–8].

T-cell transformation is a multistep oncogenic process in which multiple lesions involving different oncogenes and tumor suppressor genes cooperate to disrupt the normal circuitry that controls cell proliferation, differentiation and survival during T-cell development [9–12]. Most of what we know about the molecular basis of T-ALL has been learned from the study of recurrent cytogenetic alterations [9]. The most common genetic alteration in T-ALL is the presence of deletions in the *CDKN2A* tumor suppressor locus containing the *P16/INK4A* and the *P14/ARF* tumor suppressor genes, which control cell cycle progression and p53 mediated apoptosis, respectively [13]. In addition, over 50% of T-ALLs harbor activating mutations in the NOTCH signaling pathway making of NOTCH1 the most prominent T-ALL specific oncogene [14] and defining T-ALL as a disease primarily characterized by aberrant NOTCH1 activation [15,16]. However, T-ALL is a heterogeneous disease in which different molecular groups, primarily defined by the expression of T-ALL transcription factor oncogenes, are associated with specific patterns of gene expression, a specific block in cell differentiation and distinct clinical characteristics [10–12]. Thus, T-ALL-associated chromosomal translocations typically result in the juxtaposition of a selective group of oncogenic transcription factors next to strong regulatory elements located in the vicinity of the T-cell receptor β (TCRB) gene in chromosome 7q34 or the T-cell receptor α - δ (*TCRAD*) locus in chromosome 14q11 [9,17]. These T-ALL-specific transcription factor oncogenes include basic helix-loop-helix (bHLH) family members such as *TAL1* [18–21], *TAL2* [22], *LYL1* [23] and *BHLHB1* [24]; LIM-only domain (LMO) factors such as *LMO1* and *LMO2* [25–29]; *TLX1/HOX11* [30–33], *TLX3/HOX11L2* [34], *NKX2.5* [35,36] and *HOXA* homeobox genes [11,37]; *MYC* [38–42] and *MYB* [43] oncogenes; and *TANI*, a truncated and constitutively activated form of the NOTCH1 receptor [44,45]. In some cases, these factors can also be activated in the context of other non-TCR-associated chromosomal abnormalities. This is the case for small deletions activating *TAL1* [46] and *LMO2* [47]; duplications of the *MYB* oncogene [48,49]; and the t(5;14)(q32;q11) translocation which activates the *TLX3* oncogene in chromosome 5 by relocating it to the vicinity of the *BCL11B* locus in chromosome 14.

Additional molecular alterations present in T-ALL include transcription factor fusion oncogenes such as *PICALM/MLLT10/CALM-AF10* [50–52], *MLL-MLLT1/MLL-ENL* [53,54], *SET/NUP214* [55], *NUP98-RAP1GDS1* [56,57]; activation of signaling factors driving proliferation such as *LCK* [58], *CCND2* [59,60], *JAK1* [61], *NUP214-ABL1* [62], *EML1-ABL1* [17], and *NRAS* [63,64]; and the loss of tumor suppressor genes in the RAS (*NF1* [65]) and PI3K (*PTEN* [66]) signaling pathways. However, the catalog of genetic alterations involved in the pathogenesis of T-ALL is not yet complete as shown by the recent identification of loss-of-function mutations in *WT1* [67], the *PTPN2* phosphatase [68] and the *PHF6* tumor suppressor gene [69].

NOTCH1 signaling pathway

The NOTCH1 receptor is a class I transmembrane protein that functions as a ligand-activated transcription factor (Figure 1) [15]. Thus, NOTCH1 directly transduces information from extracellular signals into changes in gene expression in the nucleus. The main components of NOTCH1 signaling include: the Delta/Serrate/LAG-2 (DSL) family of ligands (Delta-like 1, 3, and 4 as well as Jagged 1 and 2); the NOTCH1 receptor (NOTCH1); the RBPJ/CSL (CBF1/Su(H)/LAG-1) DNA-binding protein; and the mastermind-like family of coactivators. In resting conditions, NOTCH1 sits in the membrane as a heterodimeric complex composed of an N-terminal extracellular subunit

(N_{EC}) and a C-terminal transmembrane and intracellular subunit (N_{TM}). The N_{EC} subunit interacts with Delta-like and Jagged ligands through 36 epidermal growth factor (EGF)-like repeat domains. In addition, it contains a negative regulatory region (NRR) composed of three Lin12/NOTCH repeats (LNRs). These LNR domains fold over and stabilize the heterodimerization domain (HD), which consists of the C-terminus of N_{EC} and the N-terminus of N_{TM} in close interaction, to prevent the spontaneous activation of the receptor in the absence of ligand. The N_{TM} subunit contains a transmembrane sequence followed by a series of cytoplasmic domains, including a RAM domain, a series of ankyrin repeats, a transactivator domain, and several nuclear localization signals, which collectively function as a ligand activated transcription factor. The N_{TM} also contains a C-terminal PEST (proline (P), glutamic acid (E), serine (S), and threonine (T) rich) domain, which is responsible for the proteosomal degradation of activated NOTCH1 in the nucleus [15].

Under physiologic conditions the NOTCH1 receptor is activated via interaction with a Jagged or Delta-like ligand molecule. This ligand-receptor interaction induces a conformational change in the NRR regulatory region and triggers the cleavage of the HD domain by the ADAM10 and ADAM17 metalloproteases at the cell surface [70–74]. This first activation-associated cleavage, also known as S2, is then followed by a second proteolytic cleavage (S3) catalyzed by the γ -secretase complex in the transmembrane region of the receptor [70–72]. Thus, the γ -secretase complex releases the intracellular domain of NOTCH1 (ICN1) into the cytosol and allows its translocation into the nucleus, where it associates with the RBPJ/CSL DNA-binding protein, recruits members of the mastermind (MAML) family of coactivators and p300, and through these interactions, activates gene expression [15]. Finally, recruitment of the RNA polymerase II holoenzyme to the ICN1-RBPJ/CSL-MAML transcriptional complex triggers the phosphorylation of the PEST domain of NOTCH1 by cyclin-dependent kinase 8 and recruits the FBXW7-SCF ubiquitin ligase complex, which ultimately mediates the polyubiquitination and proteasomal degradation of the activated receptor in the nucleus [15].

NOTCH1 in T-cell development

The NOTCH signaling pathway is responsible for cell fate specification and tissue patterning in multiple cellular and tissue contexts during development. In the lymphoid system NOTCH signals provided by the thymic microenvironment are essential for the specification and development of T-cell progenitors [75,76]. Consistent with this model, conditional inactivation of NOTCH1 results in a complete ablation of T-cell lymphopoiesis and differentiation accompanied by ectopic B-cell development in the thymus [77,78]. Likewise, overexpression of an active, intracellular form of NOTCH1 in bone marrow progenitors results in ectopic pre-T cell development in the bone marrow [79].

Upon T-cell specification, thymocytes differentiate into $\alpha\beta$ or $\gamma\delta$ T-cell lineages, and while the development of $\gamma\delta$ T-cells seems to be independent of NOTCH [80,81], $\alpha\beta$ T-cells require continuous NOTCH1 activation for their maturation to the DN3 stage of development [81]. During this process, several important factors required for T-cell development are transcriptionally controlled by NOTCH1, including the pre-T-cell receptor alpha (*PTCRA*) [82], the IL7 receptor alpha (*IL7RA*) [83] and *MYC* [84]. Both preTCR signaling and NOTCH activation are needed for growth and survival at the so called β -selection checkpoint [80], at which point NOTCH1 signaling is critically required to sustain cell metabolism via activation of the PI3K-AKT cascade [85].

Aberrant NOTCH1 activation in T-ALL

The first evidence of the role of *NOTCH1* in the pathogenesis of T-ALL resulted from the cloning of *TAN1*, a truncated and constitutively active form of NOTCH1, at the breakpoint

of the t(7;9)(q34;q34.3) chromosomal translocation present in about 1% of T-ALL cases [44]. In this translocation, the *NOTCH1* locus in 9q34 is broken so that the derivative chromosome 9 retains the N-terminal domains of NOTCH1, including the NRR region, while the transmembrane and intracellular domains of the receptor are translocated to the derivative chromosome 7 where they are aberrantly expressed under the control of the *TCRB* regulatory sequences. Ultimately this rearrangement results in constitutive activation of NOTCH1 signaling due to the expression of high levels of N_{TM} and/or ICN1 in T-cell precursors (Figure 1) [44,45]. The pathogenic role of activated NOTCH1 in T-ALL was fully demonstrated when irradiated mice reconstituted with bone marrow progenitors expressing activated forms of NOTCH1 developed clonal hematopoietic tumors characterized as T-ALL [86]. In addition, T-cell tumors generated by insertional mutagenesis showed a high incidence of retroviral integrations resulting in constitutive activation of NOTCH1 [87]. However, it was only after the identification of activating mutations in *NOTCH1* in over 50% of human T-ALL cases (Figure 1) [14] that the central role of NOTCH1 in the pathogenesis of this disease was fully appreciated.

Activating mutations in *NOTCH1* typically result in the disruption of molecular locks responsible for preventing the spontaneous activation of the receptor at the membrane or mediating the termination of NOTCH1 signaling in the nucleus [14]. Thus, most mutations in the HD domain (exon 26 and exon 27), which are present in approximately 40% of human T-ALLs, destabilize the interaction between the N-terminal and C-terminal HD subunits and result in ligand-independent activation or ligand hypersensitivity (HD class 1 mutations) (Figure 1) [88]. A second mutational hotspot is located at the 3' end of the gene, which encodes the C-terminal PEST domain [14]. PEST domain mutations are present in about 15% of T-ALL samples and are typically truncating and nonsense mutations, which result in deletion of the recognition sequence for proteasomal degradation of ICN1 by the FBXW7/SCF complex in the nucleus (Figure 1). In rare cases, NOTCH1 is activated as the result of in-frame insertions in the distal part of the HD domain (HD class 2 mutations) that result in the displacement and constitutive processing at the ADAM protease cleavage site [88]. Alternative mechanisms of NOTCH1 activation include juxtamembrane expansion NOTCH1 mutations (JME alleles), which consist of extracellular in frame insertions that displace the HD domain away from the membrane (Figure 1) [89] and the *NOTCH1* H1545P mutation –located in the NOTCH1 LNR-C repeat– that disrupts the activity of the NRR and facilitates S2 processing of the HD domain (Figure 1) [90]. In addition to these mutations in the *NOTCH1* gene, about 15% of T-ALLs harbor mutations in *FBXW7*, which typically involve key arginine residues responsible for the recognition of phosphorylation sites in the PEST domain of NOTCH1 [91–93]. These *FBXW7* mutations impair the substrate recognition function of the FBXW7/SCF complex and impair the degradation of activated NOTCH1 (Figure 1) [91,92]. In addition, the oncogenic effects of *FBXW7* mutations may extend beyond the NOTCH1 signaling pathway as this ubiquitin ligase also mediates the proteasomal degradation of additional oncoproteins such as MYC, JUN, Cyclin E, Aurora-A and m-TOR [94–96]. Finally, about 20% of T-ALL patients harbor either dual mutations in the HD and PEST domains of NOTCH1 or both a *NOTCH1* HD allele and a *FBXW7* mutation. The combined effect of these mutations results in exceedingly high levels of NOTCH signaling as a result of NOTCH ligand-independent activation at the membrane plus impaired ICN1 degradation in the nucleus [14,91].

An important point worth emphasizing here is that not all NOTCH mutations are functionally equivalent. Indeed each of the different types of NOTCH1 alleles described above has very different effects in its mechanism of action and its level of activation. *NOTCH1* PEST mutations, when present alone, are typically weak alleles and are predicted to be functional only in the presence of NOTCH ligands [14]. *NOTCH1* HD alleles result in variable levels of spontaneous NOTCH1 activation and, although some may induce ligand-

independent activation of the receptor, others probably only confer ligand hypersensitivity [88]. In contrast, truncated *NOTCH1* alleles resulting from the t(7;9) translocation, *NOTCH1* insertion mutations (class 2 HD mutations and JME alleles), and double mutant alleles (*NOTCH1* HD plus PEST or *NOTCH1* HD plus *FBXW7* mutations) result in remarkably high levels of NOTCH1 activation [14]. Consistently, each of these alleles and allele combinations has shown very different effects in its capacity to induce leukemia when expressed in mouse hematopoietic progenitor cells [97]. Specifically, weak *NOTCH1* alleles failed to induce T-ALL by themselves although they accelerate T-cell transformation in hematopoietic progenitors expressing the *k-ras* oncogene [97]. Overall, strong *NOTCH1* mutants may work as major drivers of the tumor phenotype, acting potentially probably as initiating events in T-ALL, while weaker alleles may function as secondary events that contribute to tumor progression.

Genes and pathways controlled by NOTCH1 in T-cell transformation

The identification of genes and pathways controlled by NOTCH in T-ALL has been the focus of extensive research over the last years. These studies have defined a prominent role for NOTCH1 as a central regulator, promoting leukemia cell growth by multiple direct and indirect mechanisms. Gene expression profiling of T-ALL cell lines and ChIP-on-chip analysis of NOTCH1 in T-ALL cells revealed a prominent role of oncogenic NOTCH1 as a direct transcriptional activator of multiple genes involved in anabolic cell growth and metabolism [98]. In addition, this study also identified the *MYC* oncogene as a prominent direct target gene regulated by NOTCH1 in human leukemias [98]. Notably, most of the genes controlled by NOTCH1 that regulate cell growth, proliferation and metabolism are also targets of *MYC* [98,99]. The resulting NOTCH1-MYC feed-forward-loop transcriptional regulatory network reinforces the expression of genes implicated in anabolic pathways, ribosome biosynthesis, protein translation and nucleotide and amino acid metabolism downstream of NOTCH1 [84,98]. Consistent with these observations, analysis of mouse tumor cells also revealed *c-Myc* as a prominent NOTCH1 target gene in T-cell transformation [84,100].

In addition to its direct effect on anabolic genes and facilitating cell growth via upregulation of *MYC*, NOTCH1 facilitates the activation of the PI3K-AKT-mTOR signaling pathway, a critical regulator of cell growth and metabolism [66,85]. The first indication of the key interaction between NOTCH and the PI3K pathway was provided in a seminal manuscript by Ciofani and coworkers who demonstrated that NOTCH signals regulate cell size, glucose uptake and glycolysis via activation of the PI3K-AKT signaling pathway during T-cell development [85]. More recently, phosphoproteomic analysis demonstrated a marked suppression of mTOR signaling in T-ALL cells upon inhibition of NOTCH signaling [101]. Overall, NOTCH1 seems to facilitate the activation of the PI3K-AKT-mTOR pathway at multiple levels. In T-cell progenitors and T-ALL lymphoblasts, a transcriptional repressor directly downstream of NOTCH1 signaling, *HES1*, can downregulate the expression of *PTEN*, a critical negative regulator of the PI3K pathway [66]. In addition NOTCH1 can activate AKT via the LCK tyrosine kinase in T-cells [102] and MYC can rescue the inhibitory effects of blocking NOTCH1 on the m-TOR pathway [101]. Finally, numerous signaling molecules upstream of PI3K, including the interleukin 7 receptor alpha chain (*IL7RA*) [83] and the pre-T-cell receptor alpha (*PTCRA*) [82], are upregulated upon activation of NOTCH1 signaling in T-cell progenitors and in T-ALL lymphoblasts.

The transcriptional program activated by oncogenic NOTCH1 also has a direct effect on cell cycle progression. For instance, oncogenic NOTCH1 signaling promotes G1/S cell cycle progression in T-ALL [14,45,103,104] [105]. These effects are mediated in part by transcriptional upregulation of *CCND3*, *CDK4*, and *CDK6* [103]. Notably, *CCND3* is a

direct NOTCH1 target gene in T-ALL and is strictly required for NOTCH1-induced transformation [106]. Moreover, inhibition of NOTCH signaling in T-ALL is associated with upregulation of the cyclin-dependent kinase inhibitors *CDKN2D* (p19/*INK4d*) and *CDKN1B* (p27/*Kip1*) [98,105]. Finally, in hematopoietic progenitors, NOTCH1 can induce the transcription of the S phase kinase-associated protein 2 (SKP2), which mediates the proteasomal degradation of CDKN1B (p27/*Kip1*) and CDKN1A (p21/*Cip1*) [104].

Finally, NOTCH1 signaling can also regulate the survival of T-ALL cells via interaction with the NFκB. Specifically, activation of NOTCH signaling upregulates NFκB activity by increasing expression of IκB-kinase [107] and upregulating both the expression and the nuclear localization of NFκB [108]. The critical role of this interaction is demonstrated by the antileukemic effects of NFκB inhibition in T-ALL and the strict requirement of NFκB signaling for NOTCH-induced transformation [109].

NOTCH1 mutations and clinical prognosis in T-ALL

Since the identification of activating mutations in *NOTCH1*, a number of studies have addressed the prognostic significance of these alterations in T-ALL. Initially, a study reporting results from a cohort of 157 pediatric T-ALL patients treated with the ALL-BFM 2000 protocol found that *NOTCH1* mutations were associated with increased prednisone sensitivity, lower levels of minimal residual disease and favorable long-term outcomes [110]. Similarly, analysis of 55 pediatric T-ALL and 14 T-cell lymphoblastic lymphoma patients treated in the Japan Association of Childhood Leukemia Study (JACLS) protocols ALL-97 and NHL-98 showed an improved outcome in patients harboring *NOTCH1* and/or *FBXW7* mutations [111]. In adult T-ALL, analysis of patients treated in the LALA-94 or GRAALL-2003 studies also identified NOTCH1 and/or *FBXW7* mutations as favorable prognostic markers [112]. However, these results have not been fully validated in other series. Analysis of 72 pediatric T-ALL patients treated with the ALL-7, ALL-8 or ALL-9 protocols by the Dutch Childhood Oncology Group [113] and a study analyzing a cohort of 88 adult T-ALL patients treated according to the MRC UKALLXII/ECOG2993 protocol [114] failed to detect a significant association between *NOTCH1* and *FBXW7* mutations and clinical outcome.

Notably, three timely reports have recently readdressed the association of NOTCH activation with outcome, clarifying some of the uncertainties raised by earlier studies. First, a retrospective study on the relevance of *NOTCH1*/*FBXW7* mutations in pediatric T-ALL analyzed patients enrolled on Dutch DCOG ALL7/8 or ALL9 or the German COALL-97 protocols and combined mutation analysis of *NOTCH1* and *FBXW7* with direct measurement of activated NOTCH1 protein using reverse-phase protein microarrays [115]. This analysis confirmed that *NOTCH1* and *FBXW7* mutations are associated with increased intracellular NOTCH1 levels in clinical samples [115]. In this series, the presence of *NOTCH1*/*FBXW7* mutations was associated with a good initial *in vivo* prednisone response. However, this improved response to therapy did not translate into a superior outcome [115]. Similarly, analysis of *NOTCH1* and *FBXW7* mutations in 134 children with T-ALL enrolled in EORTC-CLG trials showed that mutation-positive patients have a better response to prephase therapy and lower levels of minimal residual disease at the end of induction [116]. However, this improved therapeutic response once again, did not result in improved outcome [116]. Finally, and in contrast with the results of these reports, an extended analysis of the effects of *NOTCH1* and *FBXW7* mutations in patients treated on ALL-BFM protocols confirmed the overall favorable effect of activating NOTCH1 mutations in prognosis originally observed in the BFM2000 study [117]. This series included 151 cases from the original report of the ALL-BFM 2000 protocol [110] and extended this series by including 150 new cases. *NOTCH1* and *FBXW7* mutations in this cohort were associated with rapid

early treatment response both in terms of prednisone sensitivity and as measured by minimal residual disease [117]. Notably, this improved therapeutic response resulted in improved outcome and decreased risk of relapse [117]. Overall the results of these studies show that activation of NOTCH pathway is associated with improved therapeutic response and high sensitivity to glucocorticoid therapy in T-ALL. However, the ultimate effect of these mutations in terms of clinical outcome seems to be therapy-dependent.

Targeted inhibition of NOTCH1 for the treatment of T-ALL

Perhaps the most exciting opportunity derived from the identification of *NOTCH1* mutations in T-ALL is the possibility of developing anti-NOTCH1 targeted therapies in this disease. The γ -secretase complex, responsible for the proteolytic processing and activation of NOTCH signaling can be inhibited with small molecule inhibitors (GSIs) and has been the focus of extensive research by pharmaceutical companies because of its role in the pathogenesis of Alzheimer's disease [118,119]. These GSIs function as pan-NOTCH inhibitors blocking the activity of all 4 NOTCH receptors. Early studies on the activity of GSIs as an anti NOTCH-therapy for T-ALL showed that treatment of T-ALL cell lines with these drugs resulted in rapid clearance of activated NOTCH1 protein and effective downregulation of NOTCH1 target genes [14,45,84,98,101]. Most notably, NOTCH inhibition reduced growth and proliferation by inducing G1 cell cycle arrest and decreasing cell size [14,45,66,101]. Following these encouraging results, the Dana-Farber Cancer Institute performed a phase I clinical trial testing MK-0752, an oral GSI developed by Merck for the treatment of Alzheimer's disease, in T-ALL patients [120]. Six adults and two children with leukemia (seven with T-ALL and one with AML) were enrolled in this study and four of the seven T-ALL patients showed activating mutations in NOTCH1. Treatment duration ranged from 2 to 56 days, and one patient with T-ALL and a NOTCH1 mutation achieved a 45% reduction in a mediastinal mass after 28 days. However, this patient subsequently progressed, and no patient achieved an objective response before discontinuation because of disease progression or drug-related toxicity [120]. The most common dose-limiting toxicity was grade 3/4 diarrhea, revealing an unfavorable toxicity profile most probably related to inhibition of NOTCH signaling in the gut. The development of gastrointestinal toxicity in the context of GSI therapy was not completely unanticipated and has emerged as a significant obstacle for the clinical development of these drugs. NOTCH1 and NOTCH2 play an important role in the intestinal epithelium, where they are involved in the control of cell proliferation and differentiation, and as noted above, GSIs are pan-NOTCH inhibitors that cause a systemic block of all 4 NOTCH receptors. Genetic inhibition of NOTCH signaling in the gut using animal models via deletion of the *Rbpjk* gene [121] or in the context of double *Notch1/Notch2* conditional knockouts [122] induces cell cycle arrest and differentiation to secretory cell lineages at the expense of the absorptive epithelium; a phenotype that is recapitulated upon pharmacologic inhibition of the Notch pathway with GSIs [121,123]. Overall, these results strongly suggest that alternative strategies with an improved therapeutic window may be needed for the successful implementation of GSIs as anti-NOTCH therapies in T-ALL. In this regard, a recent report from Merck has shown that three days of >70% Notch inhibition with a GSI is sufficient to induce effective antileukemic responses in T-ALL xenograft models and is well-tolerated [124]. A similar intermittent dosing approach has shown to reduce the toxicity associated with PF-03084014, a GSI developed by Pfizer [125]. These results illustrate that secretory metaplasia induced by GSIs is time- and dose-dependent and can be avoided using intermittent dosing schemes. An alternative approach to improve the safety and efficacy of anti-NOTCH therapies in T-ALL may result from the combined use of GSIs with chemotherapy or other molecularly targeted drugs. The idea is to use GSIs at high doses for short periods of time to avoid the development of gastrointestinal toxicity while using drug combinations that increase their antileukemic efficacy. Combination therapies of GSIs with

CDK inhibitors [105], drugs targeting NF κ B signaling [91], or small molecule inhibitors of CK2 [125] and the PI3K-AKT-mTOR pathway [66] [101,126] have been shown to increase the antileukemic effects of these pan-NOTCH inhibitors. In addition, prolonged exposure to GSIs may increase the response to glucocorticoid treatment [127], and inhibition of NOTCH signaling with a GSI can sensitize glucocorticoid-resistant T-ALL cell lines to glucocorticoid-induced apoptosis [128]. Importantly, *in vivo* testing of GSIs and glucocorticoids in combination in a mouse model of glucocorticoid resistant T-ALL showed that glucocorticoid treatment has a direct protective effect against GSI-induced intestinal toxicity in mice [128,129]. These results have now been confirmed and extended in a report showing that glucocorticoids abrogate the gastrointestinal toxicity induced by the GSI PF-03084014 and that delayed administration of glucocorticoids does not impair their protective effect against GSI-induced gut toxicity [130]. Overall, these results strongly suggest that glucocorticoid treatment may enhance the antileukemic effects of GSIs, while at the same time ameliorating the intestinal toxicity typically associated with systemic inhibition of NOTCH signaling [128].

Modulators of clinical response to GSI in T-ALL

Despite the prominent role of NOTCH1 in the pathogenesis of T-ALL, inhibition of NOTCH1 signaling seems to have only modest antileukemic effects against human T-ALL cell lines. Thus, inhibition of NOTCH signaling with GSIs is effective only in a fraction of these tumors and induces primarily a cytostatic effect [14,45,98], although it can also result in the induction of apoptosis in some instances [45,124,130]. In contrast, *Notch*-induced mouse T-ALLs seem to be more sensitive to inhibition of NOTCH signaling [97] [126]. Comparative analysis of GSI-sensitive and GSI-resistant T-ALL cell lines showed that GSI treatment can effectively decrease the level of active NOTCH1 protein and the expression of NOTCH1 target genes in both sensitive and resistant tumors [66,98]. These results demonstrate that GSI resistance in T-ALL cell lines is not mediated by defects in drug uptake or impaired inhibition of the γ -secretase complex and suggests that human T-cell leukemia cell lines may have accumulated additional mutations that sustain leukemic cell growth and bypass the effects of NOTCH1 inhibition. Detailed molecular analysis of GSI-sensitive and GSI-resistant T-ALL cell lines showed a striking correlation between *PTEN* mutational status and GSI sensitivity, as all GSI-sensitive tumors were *PTEN* wild type while each of the GSI-resistant lines analyzed showed mutational loss of this tumor suppressor gene [66]. However, analyses of mouse models of NOTCH1-induced leukemias and primary T-ALL samples in culture suggest that additional mutations may be required to confer full resistance to GSI therapy [131]. Notably, *FBXW7* mutations, which upregulate the expression of MYC, JUN and Cyclin E in addition to contributing to increased ICN1 stability, are also more prevalent in GSI-resistant T-ALL cell lines [91,92]. As clinical trials testing the safety and efficacy of GSI therapy in T-ALL progress, it will be important to analyze the effect of these genetic alterations in the response to anti-NOTCH1 therapies.

New and emerging anti-NOTCH therapies

The limitations of GSIs in the clinic suggest that alternative strategies may be needed for the therapeutic targeting of NOTCH1 in T-ALL. One possibility resides in the use of synthetic peptides to block the NOTCH transcriptional complex directly in the cell nucleus. This approach would confer direct NOTCH inhibition and may have a more rapid inhibitory effect on NOTCH signaling in the cell than GSIs. Following this approach, Moellering and coworkers have recently shown that SAHM1, a cell-permeable, stabilized alpha-helical peptide targeting the protein-protein interface responsible for the recruitment of MAML1 into the NOTCH-CSL transactivation complex, can effectively block NOTCH signaling and

has potent, NOTCH-specific antileukemic effects both in human T-ALL cell lines and in a mouse model of NOTCH1-induced T-ALL [132].

Finally, given that NOTCH proteins are surface molecules, specific antibodies could provide selective blocking of NOTCH1, specifically, while preserving the activity of the other three NOTCH family members. An elegant study by Wu and coworkers at Genentech has demonstrated that highly specialized antibodies can block NOTCH1 signaling by binding to and stabilizing the LNR-HD complex [133], locking the receptor in an “off” conformation. Notably, this anti-NOTCH1 antibody blocked leukemic cell growth in pre-clinical models and inhibited angiogenesis [133]. Moreover, the anti-Notch1 antibody did not affect the activity of Notch2, which precluded the development of overt gastrointestinal toxicity [133]. In a related study, Aste-Amézaga and coworkers showed that anti-NOTCH1 antibodies can block ligand-independent signaling driven by Notch1 receptors with diverse class I HD point mutations, the most common type of mutation found in T-ALL [134].

Conclusions and future directions

Aberrant activation of the NOTCH signaling pathway plays a central role in T-ALL, a tumor in which multiple oncogenic and tumor suppressor pathways coordinately disrupt the regulatory programs controlling cell proliferation, differentiation and survival in normal developing thymocytes. The introduction of anti-NOTCH therapies in clinical trials may result in urgently needed improvements in therapy, particularly for patients with primary refractory and relapsed disease. Detailed correlative studies analyzing the genetic background of the tumors treated as well as elucidation of the mechanisms that mediate response to therapy and the genetic events implicated in resistance and relapse will be instrumental in defining the next steps towards achieving novel, more effective targeted treatments in this disease.

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Figure 1. NOTCH1 mutations in T-ALL

Schematic representation of the NOTCH1 receptor structure and types of NOTCH1 mutations found in T-ALL. EGF-like, EGF-like repeats involved in ligand recognition. NRR, negative regulatory region. LNR, Lin12-NOTCH repeats. HD, heterodimerization domain. RAM, RAM domain. ANK, ankyrin repeats. TAD, transactivation domain. PEST, proline, glutamic acid, serine and threonine rich domain. ICN1, intracellular NOTCH1.