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Review Paper

Clinical Relevance of Notch1 in T-Cell Acute Lymphoblastic Leukaemia

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Abstract

Notch1 signalling is an essential event in the thymopoiesis. Activating Notch1 mutations are frequently seen in T-Acute Lymphoblastic Leukaemia (T-ALL). Notch1 is mainly involved in the T cell proliferation, differentiation and apoptosis. The mutations in the Notch1 have been reported in more than 50% of T-ALL cases. The aberrant Notch1 signalling has a prominent role in the biology of T-ALL. Activating mutations of Notch1 were clinically significant in the molecular pathogenesis of T-ALL. The Crucial role of Notch1 in T cell lineage commitment and in the making of T-ALL paves the way to study Notch1 as a therapeutic target in the T-ALL therapy. In this review, the role of Notch1 in T-ALL, its signalling mechanism, Notch1 mutations, its impact on the disease prognosis and its therapeutic significance has been discussed to reiterate Notch1 as a molecular marker in T-ALL.

Keywords: T-ALL, Notch1, mutations, T- cell differentiation, FBXW7, ICN1, GSI.

Introduction

T-cell acute lymphoblastic leukaemia (T-ALL) is an aggressive haematological malignancy of thymocytes[1], accounting for 25-30% of paediatric and 25% of adult ALL cases registered in Cancer Institute (WIA), India. Although the treatment outcome in T-ALL has been improved tremendously with the current treatment regimens, but challenges remain such as understanding the factors that contribute to the resistance to chemotherapeutic agents and dismal outcome of relapse in T-ALL. Notch1 is an oncogenic transcription factor initially identified from chromosomal translocation and its aberrant expression seen in T-ALL. The identification of mutations in Notch1 in the 50% of T-ALLs has generated the major interest to study Notch1 signalling, its significance in the prognosis and in developing the Notch1 specific molecular therapies in T-ALL[1].

Notch1 is a 300 KDa single pass transmembrane receptor cum transcription factor. Binding of Delta/Serrate/lag-2 ligands to Notch1 receptor, it undergoes two proteolytic cleavages that releases the intracellular part of the receptor from membrane into nucleus where it induces transcription of target genes such as c-myc, pTα, HES1, HEY1, mTOR, CDK25, CDK2 and cyclin D1 and D3 to promote S-phase entry in the cell proliferation. In late 1980, Jeff Sklar's group first discovered the human Notch1 as a gene activated due to the chromosomal translocation t (7; 9), that fuses the ICN1 form of the Notch1 to the TCR β promoter/enhancer in the lymphoblasts of pre T-ALL patient[2]. Notch1 is required for the commitment of hematopoietic progenitor cells to T cell development and differentiation. Aberrant activation of Notch1 signalling due to the gain of function mutations contributes to the deregulation of differentiation, self renewal and proliferation of hematopoietic stem cells which leads to T-ALL in children[2].

Notch1 and Its Structure

Notch gene was discovered in drosophila almost 100 years ago and it encodes highly conserved cell surface receptor. The Notch gene has four isoforms which encode a family of highly conserved type I transmembrane receptors that are normally activated by five canonical ligands of the Delta/Serrate/lag-2 family [Jag1, Jag2, Dll1, Dll3, and Dll4]. The variety of receptor-ligand combinations could generate distinct cellular responses. The N terminal large extra cellular domain of Notch1 contains 36 tandem epidermal growth factor (EGF) like repeats and 3 cysteine rich Lin-12 Notch1 (LNR) repeats and the heterodimerisation domain (HD). The RAM domain, ankyrin repeats, transcriptional activational domain (TAD) and PEST domain are found within the C terminal intracellular part of Notch1 as shown in figure 1. The C-terminal PEST degron is responsible for the ICN1 turn over in association with the F-box WD7 proteins through ubiquitination. Notch1 activation can influence many specific developmental events especially in the T-cell fate determination from pluripotent stem cells.

Role of Notch1 in T-Cell Development

Notch1 is essential for T cell differentiation and its activity found to be triphasic during normal T cell development[1]. Notch1 expression levels are highest in the earlier CD4-CD8- double negative (DN) cells, low in CD4+CD8+ double positive (DP) stage and intermediate in the both CD4 and CD8 single positive (SP) cells. The level of Notch1 expression during thymopoiesis is tightly regulated by pre TCR signalling. During

β selection, Pre TCR signalling leads to Id 3 expression which decreases the Notch1 transcription rate, thereby reduces the Notch1 protein level[3]. Notch1 signalling is required not only for generation of the early T-cell precursor population but also for transitions of those precursors to DP and SP suggesting that Notch1 activation is important to promote survival and proliferation during the early T-cell development. The constitutive activation of Notch1 causes the maturational arrest of T lymphoblasts at the CD 4 and CD 8 double positive stage and the event may lead to the clonal proliferation of immature T- lymphoblasts in the T-ALL[4].

Notch1 Isoforms

Mammalian Notch1 gene has four isoforms comprised of multiple structural motifs. The intracellular portion of ICN1-4 has RAM domains containing high binding affinity to CSL, and highly conserved Ankyrin domain. Between the Ankyrin and the PEST domains lies the most varied portion of ICN1-4 as shown in Figure 2. These structural variations affect the stepwise assembly of the CSL/ICN1/MAML transient transcriptional activation complex of ICN 1-4. A study on the variations among the isoforms was done by J.C Aster et al 2011[5]. The study states that the high binding affinity of the RAM to CSL is essential for the transcriptional activation and subsequent downstream functions.

ICN1-3 has relatively high binding affinity to the SPS (sequence- paired- site) of the CSL and readily forms the dimeric CSL/ICN/MAML transcription complex; thereby transactivate c- Myc, which an important player in the making of T-ALL. ICN 4 fails to form dimeric CSL/ICN/MAML transcriptional complex due to the varied ankyrin domain and fails to bind the SPS which governs c-Myc expression. The leukemeogenecity of ICN1-4 forms have been correlated with their ability to turn on the c-Myc expression. The c-Myc provides leukemogenic signals which has been implicated in the pathogenesis of T-ALL. To date in T-ALL, translocations/ mutations have been identified in Notch1but not in Notch 2 and Notch 3. This rationale could be due to the differences in the intrinsic transforming potential of Notch1 and its ability to activate the CSL dependant promoter elements in T-ALL cells.

Notch1 Signalling

Notch1 signals have highly pleiotropic effects in regulating the cell fate specification, proliferation, self renewal, survival and apoptosis of the developing animals[6]. Notch1 core signalling pathway has simple molecular design and affects the cell differentiation decision not only across the species but also across different cell types in an organism. Unlikely to most other pathways, Notch1 core signalling lacks integrated signal amplification steps such as phosphorylation of proteins involved in the pathway. Each activated Notch1 receptor is utilised to yield one activated ICN1 thereby exhibits a stoichiometry between the signalling input and output. The signalling strength of Notch1 is indeed for its action[7].

The signalling strength depends on the post translational modification of the EGF repeats of Notch1 receptors. The extracellular epidermal factor (EGF) repeats of newly synthesized Notch1 are glycosylated in the endoplasmic reticulum. The glycosylation starts with the addition of O-fucose to the EGF repeats by O- fucosyltransferase (Ofut), followed by the addition of N-Acetyl glucosamine (GlcNAc) to the attached fucose moiety by fringe proteins in the Golgi apparatus. The fringe mediated glycosylation of Notch1 receptors confers the ligand specific effects especially favouring the Delta like ligand interactions. Fringe mediated glycosylation is essential for maintaining the Notch1 receptor input to the membrane to strengthen the Notch1 signalling and the expression level of its downstream target genes. After the glycosylation by fringe, the Notch1 receptor will be repeatedly cleaved at the S1 site by a furin like convertases in the Trans Golgi apparatus for its maturation and to find its way to the cell surface.

The matured Notch1 receptor contains three major sub units such as extracellular domain, heterodimerisation domain and intracellular domain. Upon ligand activation, the Notch1 receptor has been pulled by the ligand and the negative regulatory region (NRR) of the receptor exposes the ADAM cleavage site (S2) and it is proteolytically cleaved by ADAM protease. The cleavage by ADAM protease is the key regulatory step in the ligand dependant activation of Notch1. The protelytic cleavage causes the release of a short lived membrane tethered Notch1 and it is further subjected to the intramembrane proteolysis at the S3 site by an enzyme called γ secretase. The γ secretase cleavage releases ICN1 the active form of Notch1. The freed ICN1 have either N-terminal Valine or N-terminal Serine/leucine which determines the half life of the ICN. The released ICN1 locates into the nucleus, and forms a dimeric transient transcription complex (TTC) with the binding factor such as CSL (also known as RBJ-k) and cofactors of the MAML family[8]. Finally the TTC recruits additional activators like p300 to turn on the CSL dependant genes expression such as c-myc, pTα, HES1, HEY1, mTOR and CDK25, CDK2 and cyclin D1 and D3 to promote the cell proliferation. The Notch1 signalling has been pictorially described in figure 3.

Notch1 ICN turn over can be maintained by the negative regulations such as ubiquitination, phosphorylation and hydroxylation. The serine residues in the PEST domain are hyperephosphorylated by CDK8 and then further ubiquitylated by FBXW7, an E3 ubiquitin ligase. Ubiquitination controls the availability of ICN1 in the nucleus to govern the Notch1 mediated gene expression[9]. The data of Zheng et al., 2010 suggests that asparagines residues at 1945 and 2012 of ICN would be hydroxylated by F1H1 (the asparagine hydroxylase factor – inhibiting H1F1α) and the hydroxylation negatively regulates the Notch1 signalling[10].

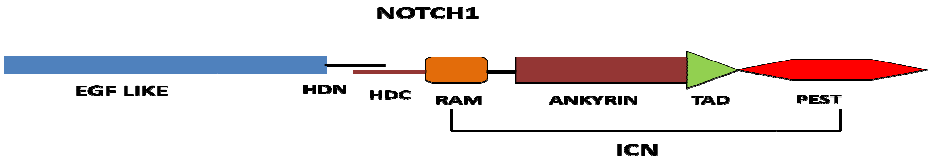

Figure-1
Structure of Notch1

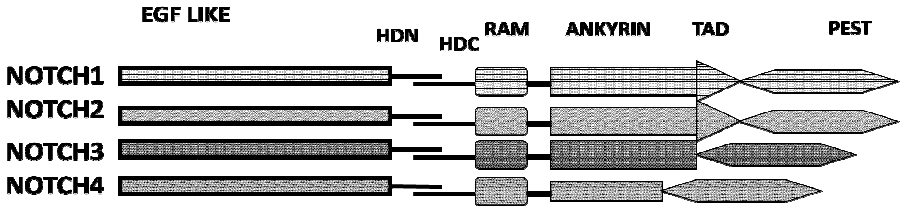

Figure-2
Four Isoforms of Notch

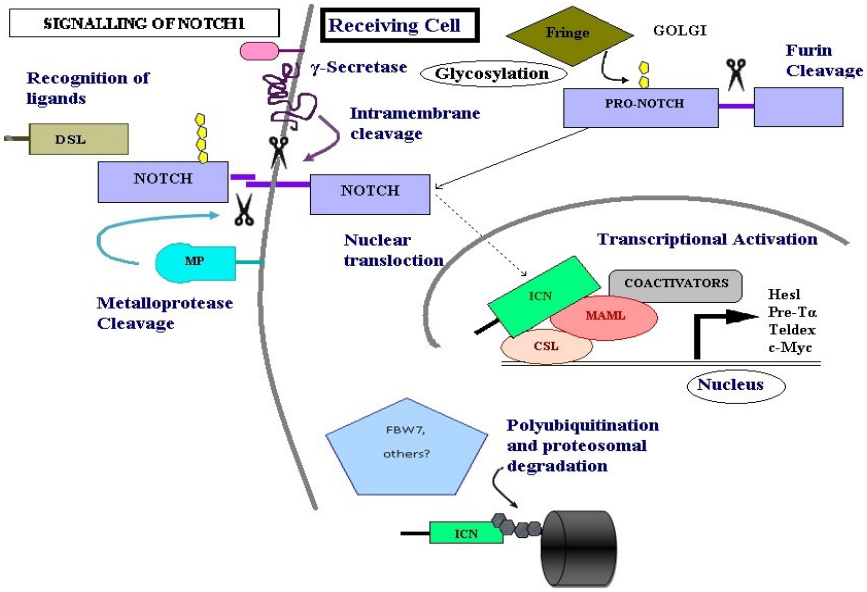

Figure-3
Notch1 Signalling Mechanism

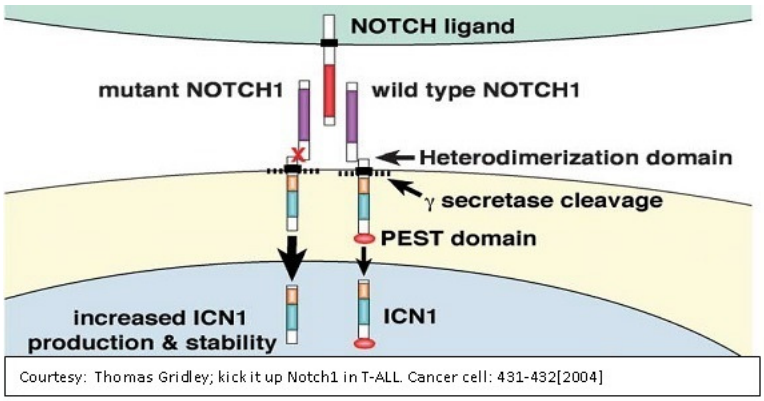

Figure-4
Effects of Activated Mutations of Notch1 on its Signalling

Notch1 Targeted Leukemogenic Genes

C-myc is the direct target of Notch1 in human T-ALL. Notch1 upregulates the c-Myc expression which favours the expression of genes involved in the anabolic proliferation of leukemic cells. Role of C-myc expression has its own foot prints in leukaemia in association with Notch1. C-myc is also a key target of Notch1 at the TCR β signalling in T- cell development by rearranging the TCR β locus to produce a pre TCR[11]. HES 1 is yet another important highly conserved target of Notch1. The expression of HES1 is directly proportional to the Notch1 signalling and it potentially triggers the T-cell malignancy in the transgenic mice[12]. HES1 inactivates PTEN thereby PTEN loses its regulatory control on the PI3K-AKT signalling leads to m-TOR expression[13]. Thus Notch1 induced over expression of HES1 could hyper activate the mTOR expression via affecting the AKT signalling. HES1 has inhibitory effect on CYLD, a deubiquitinase whose inhibition could prolong the half life of NF-kB. Thus Notch1 appears to favour NF-kB activity via the activation of HES1. Notch3 is the direct target of activated Notch1, which in association with IKK kinase complex turns on NF-kB expression. The active ICN1 of Notch1 can activate the NF-kB activation through the activation of TRAF genes[14]. The ICN1 could directly activate cyclin D1 transcription through CDK2 activity which involved in cell cycle progression from G1 to S phase[15]. Notch1 also regulate the CDK complex and activate CDK4 and CDC25A via C-myc activation and retains cyclin D1 in the nucleus to progress the cell cycle to S phase.

Aberrant Notch1 Signalling In T-ALL

Aberrant activation of Notch1 signalling plays a prominent role in the molecular pathogenesis of T-ALL through the activation of protooncogenes involved in the cell cycle progression. Mutations in the LNR, Heterodimerisation (HD) and juxta membrane domain are typically single aminoacid substitutions and small in-frame deletions/insertions. These mutations no longer will regulate the S2 cleavage and allow the ligand independent cleavage which facilitates the constitutive release of ICN1. Mutations in the PEST domain lead to the truncated ICN proteins which devoid of phosphodegron sites (PEST). The truncated ICN1 is the resultant of the premature stop codons occurred by the nonsense mutations. The loss of phosphodegron site causes the impaired degradation of activated Notch1 via proteasome mediated ubiquitination, leading to increased levels of ICN1 as shown in the figure 4. Inactivating mutations of FBXW7 also fails to degrade the activated ICN1 causes the prolonged survival of ICN1 in the nucleus. Inactivating mutations of FBXW7 seen in 10-14% of human T-ALL. Thus HD and PEST domain mutations of Notch1 prolong the half-life of ICN1 and thereby increase the Notch1 dependant target genes expression.

Notch1 mutations are generally heterozygous, in which malignant clones continue to express on the nonmutated Notch1 allele. When both, mutations are found in Cis in the same Notch1 allele, it produces potential increase in Notch1 signalling than trans mutations[8]. Mutations can either exhibits amino acid substitutions at conserved residues or short insertion/deletion in the LNR, HD, juxtamembrane region which causes destabilisation of short-lived NTM* and creating an open conformation that exposes the S2 site. Thus it favours the ligand independent release of intracellular Notch1 (ICN1) by γ secretase and thereby increases the intracellular ICN1 level. Mutations in the PEST domain will not let PEST to bind with FBXW7-E3 ligase, thus ubiquitination mediated degradation of ICN1 will not be favoured. Thus the mutations occurs in these hot spot regions contributes to the constitutive Notch1 signalling and the over expression of Notch1 targeted genes. The higher frequency of multiple mutations on the same Notch1 allele highlights that the acquired mutations aberrantly activate the Notch1 pathway.

Notch1 in the Prognosis of T-ALL

Notch1 mutations have a significant role in the prognosis of T-ALL. Many studies reported the prognostic significance of Notch1 in the T-ALL. Several groups have reported that the paediatric T-ALL patients who harboured mutations in Notch1 or Notch1/FBXW7 showed good clinical outcome than the Adult T-ALL. A few studies have reported a poor prognosis in Notch1/FBXW7 mutated patients. The outcome is influenced by the treatment protocols. Though each protocol has the same principal drugs, they differ in dosage, tolerance of the individual against the toxicity. Neutropenia and thrombocytopenia are the major side effects caused during the treatment and obstructs the further treatment proceedings to next stages. The delay in the treatment completion due to the side effects acquired could also be a notable remark in the concept of prognosis. The risk stratification in T-ALL patients is highly effective to design the appropriate treatment regimens with reduced adverse side effects. The development of candidate prognostic marker will be helpful for the risk stratification and good clinical outcome in T-ALLs. The prognoses of T-ALLs with Notch1mutations in different cohorts are tabulated in table 1. From the tabulation, it is clear that Notch1 mutations reports good clinical outcome in the paediatric T-ALL than adults. Notch1 could be an ideal prognostic marker for risk stratification in T-ALLs.

Targeting Notch1 against T-ALL

Targeted therapies are necessary for the cancer cells which addicted to the oncogenic signal driving tumour cell growth proliferation and its immortal survival. New therapeutic strategies are needed for T-ALL patients with poor prognosis and to increase the event free survival in the patients who show good prognosis. In principle, drugs that selectively target specific molecular lesions in T-ALL such as Notch1 inhibition would appear to be ideal therapeutic target[8]. Notch1 inhibition leads to the cell cycle arrest in human T-ALL cell lines and apoptosis in murine T-ALL cell lines[12]. Small molecule γ secretase inhibitors (GSI) block the intracellular Notch1 generation at the S3 cleavage level and the ICN1 turn over in

the nucleus. GSI inhibits the activated Notch1 release and the transcriptional down regulation of Notch1 target genes. These findings led to the phase I clinical trial of Mrk-0752 in seven relapsed T-ALL patients[17]. The major limitation of the trial was the gastrointestinal toxicity induced as the side effect due to the GSI therapy.

K.D Keersmaecker et al 2008 studied the combination of Dexamethasone with GSI in T-ALL cell lines. The study showed the effective inhibition of Notch1 and the results emphasized that T-ALL cells becomes more susceptible to the Dexamethasone when it is pre-treated with GSI[18]. Mutations leading to loss of PTEN seen in 8% T-ALL which causes the constitutive activation of PI3K-AKT-mTOR pathway and the mutations of FBXW7 seen in 10% of the T-ALL which causes the prolonged ICN1 survival are the two major contributing factors towards the resistance of GSI treatment. The CK2 inhibitors restore the PTEN function and the CK2 inhibitors treated T-ALL cell lines became more susceptible to the GSI compounds[19]. The in-vitro study reveals that the efficacy of GSI therapy was good in combination with the CK2 inhibitors when the mutated PTEN detected in T-ALL. A small molecule CDK4 inhibitor makes the T-ALL cells to be susceptible to GSI treatment. CDK4-6RB pathway is an important downstream mechanism through which GSI inhibits the proliferation of T-ALL cells, thus CDK4 inhibitors also could enhance the efficacy and specificity of GSI therapy without generating adverse side effects. Due to Notch1 inhibition by GSI, the expression of downstream targets gens such as c-MYC, HES1 was impaired. The loss of proliferation signal due to the impaired expression of c-MYC, HES1 causes the G1 arrested cells to exit the cell cycle via the inactivation of CDKN2D and activation of CDKN1B[15].

AntiNotch1 immuno therapy in T-ALL has gathered much attention as advancement over the conventional GSI therapy. The approach is more specific and thereby reduces risk of side effects induced by the GSI treatment on the global inhibition of Notch1 signalling. In T-ALL, the AntiNotch1 antibodies could antagonise only the Notch1 without blocking the other isoforms. The efficacy is the major limitation of AntiNotch1 antibodies and it is not so in GSI which has potent cell penetrating ability. The anti-Notch1 monoclonal antibody OMP-59R5 is still under the Phase I clinical trials[16]. More focussed research in AntiNotch1 immuno therapy will pave the way to develop highly efficient and specific treatments in T-ALL.

Table-1
Comparison of Prognostic Significance of the Notch1 and FBXW7 Mutations in T-ALL

Group	Size of the Study	Population	Protocol	Notch1 Mutated (%)	FBXW7 Mutated (%)	Prognosis	Author
Children	55	Japan	JACLS	40	14.5	Favourable only if both NOTCH1/ FBXW7 mutated	Myoung-Ja Park 2008[24]
Children	157	Germany	ALL BFM 2000	52.2	-	Favourable outcome	Breit 2009[29]
Children	77	China	Customised protocol	37.7	-	Poor prognosis	Zhu 2006[23]
Children	162	Europe	MRC-UKALL 2003	38	26	Favourable only if both NOTCH1/ FBXW7 mutated	S.Jenkinson 2012[28]
Adult	126	Multicenter USA	GMALL 05/93	56	11	No significant prognosis	C.D.Baldus 2009[30]
Adult	88	London	UKALL XII/ECOG E2993	71	18	No significant prognosis	Mansour 2009[22]
Adult	141	France	LALA 94	60	-	Favourable prognosis	Asnafi 2008[26]
Children/young adults	50	India	ALL BFM 97	40	10	Too early to predict	Cancer institute W.I.A (current study)

Stapled α-helical peptides derived from MAML1 (SAHM) are cell permeable stabilised peptides. SAHM is the structural analogue of MAML1 at its interface with ICN1 and CSL which prevents assembling the Notch1 transcriptional complex by disrupting the Notch1 dimerisation. Similar to GSI, SAHM also globally repress the Notch1 signalling but did not induce the adverse gastrointestinal toxicity[12]. Notch1 inhibition found to be insufficient with GSI treatment due to its resistance on T-ALL cells. The Combinatorial therapy has turned out to be more potential on Notch1 inhibition with reduced side effects. Nutlin 3 is a small molecule inhibitor of MDM2 and P53 association thereby it increases the expression of Notch1 through the prolonged half life of active P53 [20]. Nutlin 3 molecule is a non cytotoxic compound which inhibits the apoptosis and suppresses the osteocalcigenesis which the common induced side effects by the GSI therapy. The combinatorial approach of Nutlin3 with GSI could improve the efficacy and reduce the toxicity in T-ALL patients. In addition the combination of m-TOR inhibitors with GSI also found to be very effective on the Notch1 inhibition in T-ALL[15]. Since Notch1 signalling associated with many oncogenic signals, the combinatorial therapy has the promising role on the effective inhibition of Notch1 in T-ALL.

Conclusion

A major goal of T-ALL studies is the risk adapted stratification and the individualised treatment. An ideal prognostic factor is essential to save the high risk patients from eventual relapse and reduce treatment intensity to the good responders. Though many clinically significant genes and its expression were studied in T-ALL, Notch1 turns out to be more specific due to its mutational frequency in T-ALLs but not in B-ALLs [21] and its crucial role in the T-cell lineage differentiation as discussed in the introduction. Many studies have correlated the prognostic significance of Notch1 with different treatment regimens and supported Notch1 as a promising prognostic marker in T-ALL. Advancement of Notch1 targeted therapies such as small peptide inhibitors and anti Notch1 immunotherapy in T-ALL are the much appreciated upcoming therapeutic approaches. Many advanced research techniques such as Next generation Sequencing, array CGH and Micro RNA expression help the researchers to elucidate the critical role of Notch1 and its associated genes in the making of T-ALL. The deeper research will facilitate to explore the finer details of how Notch1 signalling involved in the biology of T-ALL.

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