

ORIGINAL ARTICLE

CUTLL1, a novel human T-cell lymphoma cell line with t(7;9) rearrangement, aberrant NOTCH1 activation and high sensitivity to γ -secretase inhibitors

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Activating mutations in NOTCH1 are present in over 50% of human T-cell lymphoblastic leukemia (T-ALL) samples and inhibition of NOTCH1 signaling with γ -secretase inhibitors (GSI) has emerged as a potential therapeutic strategy for the treatment of this disease. Here, we report a new human T-cell lymphoma line CUTLL1, which expresses high levels of activated NOTCH1 and is extremely sensitive to γ -secretase inhibitors treatment. CUTLL1 cells harbor a t(7;9)(q34;q34) translocation which induces the expression of a *TCRB-NOTCH1* fusion transcript encoding a membrane-bound truncated form of the NOTCH1 receptor. GSI treatment of CUTLL1 cells blocked NOTCH1 processing and caused rapid clearance of activated intracellular NOTCH1. Loss of NOTCH1 activity induced a gene expression signature characterized by the downregulation of NOTCH1 target genes such as *HES1* and *NOTCH3*. In contrast with most human T-ALL cell lines with activating mutations in *NOTCH1*, CUTLL1 cells showed a robust cellular phenotype upon GSI treatment characterized by G1 cell cycle arrest and increased apoptosis. These results show that the CUTLL1 cell line has a strong dependence on NOTCH1 signaling for proliferation and survival and supports that T-ALL patients whose tumors harbor t(7;9) should be included in clinical trials testing the therapeutic efficacy NOTCH1 inhibition with GSIs.

Leukemia (2006) 20, 1279–1287. doi:10.1038/sj.leu.2404258; published online 11 May 2006

Keywords: T-cell lymphoblastic leukemia; NOTCH1; cell line

Introduction

T-cell lymphoblastic leukemia (T-ALL) is an aggressive hematologic tumor characterized by a high prevalence of activating mutations in the NOTCH1 gene.¹ The NOTCH signaling pathway plays a critical role in the hematopoietic system by maintaining stem cell homeostasis and participating in multiple stages of T-cell development.² During early hematopoiesis, NOTCH signaling is required for the commitment of multipotent hematopoietic progenitors to the T-cell lineage.^{3–6} Thus, immunodeficient mice reconstituted with bone marrow progenitors expressing a constitutively active form of *Notch1* show ectopic T-cell development in the bone marrow and fail to produce B lymphocytes.⁵ Conversely, mice harboring a conditional deletion of NOTCH1 in hematopoietic progenitors fail to develop T-cells, and show ectopic B-cell development in the

thymus.⁶ In addition to this early role in T-lineage commitment, NOTCH signaling participates in essential processes at multiple stages of thymocyte development.⁷ Thus, NOTCH1 activity (i) is required for progression through the early DN1, DN2 and DN3 stages of thymocyte development;⁸ (ii) participates in the regulation of *TCRB* gene rearrangement;⁹ (iii) regulates lineage decisions between $\alpha\beta$ and $\gamma\delta$ lineages;¹⁰ and (iv) at least in some systems, between CD4 and CD8 T-cell lineages.^{11–14}

The NOTCH1 receptor is a class I heterodimeric transmembrane protein comprised of an extracellular and a transmembrane subunit which in the resting receptor are noncovalently associated through a dimerization domain. Activation of the NOTCH1 receptor occurs when NOTCH ligands of the Jagged and Delta-like family bind to the extracellular subunit of NOTCH1. Ligand binding induces dissociation of the two subunits of the receptor and initiates the proteolytic cleavage of the transmembrane subunit, first by an ADAM metalloprotease and subsequently by the γ -secretase complex, which releases the cytoplasmic portion of the receptor from the membrane. Cleaved intracellular NOTCH (ICN) translocates to the nucleus and binds to CSL, a DNA-binding factor, to form a transcriptional complex which activates the expression of target genes. Transcriptional activation by the ICN is coupled with phosphorylation of its carboxy-terminus PEST domain and proteasomal degradation of the protein.¹⁵

The first evidence linking aberrant NOTCH1 signaling to the pathogenesis of T-ALL came from the characterization of the t(7;9)(q34;q34.3) chromosomal translocation, a rare recurrent rearrangement identified in the SUPT1 cell line and present in <1% of human T-ALL cases.¹⁶ This translocation juxtaposes a truncated *NOTCH1* gene next to the *TCRB* locus, leading to the aberrant expression of an intracellular constitutively active form of NOTCH1.¹⁶ In contrast with the rare occurrence of the t(7;9) translocation, activating mutations in the NOTCH1 gene are found in over 50% of patients with T-cell lymphoblastic leukemias and lymphomas, making it the most prevalent oncogene in the pathogenesis of this disease.¹ Activating mutations in NOTCH1 typically involve the dimerization and PEST domains of the receptor. Heterodimerization domain (HD) mutations disrupt the interaction between the extracellular and transmembrane subunits of NOTCH1 and are predicted to induce ligand independent activation of the receptor. Mutations in the PEST domain of NOTCH1 generate carboxy-terminus truncated forms of the receptor which, upon ligand interaction, are predicted to generate ICN forms resistant to proteasomal degradation.¹

Although most of the existent T-ALL cell lines harbor activating mutations in *NOTCH1*, most of them fail to show clear cellular responses to the inhibition of NOTCH signaling.¹ This is in contrast with mouse cell lines established from

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Supported by the Wolf Trust, the Pardee Foundation and Children's Leukemia Research Association (AAF).

Received 9 March 2006; accepted 24 March 2006; published online 11 May 2006

lymphomas harboring NOTCH1 mutations, which respond with rapid cell cycle arrest and apoptosis to inhibition of NOTCH1 with γ -secretase inhibitors (GSI).¹⁷ As part of our effort to develop better tools for understanding the role of NOTCH1 in T-cell malignancies, we have established a new cell line that we named Columbia University T-cell Lymphoblastic Lymphoma 1 (CUTLL1) which has unique features for the characterization of oncogenic NOTCH1. Columbia University T-cell Lymphoblastic Lymphoma 1 cells show an immature T-cell phenotype and harbor a t(7;9)(q34;q34) translocation resulting in juxtaposition of the TCRB and NOTCH1 loci. In contrast with the original t(7;9)(q34;q34) present in SUPT1 cells, this rearrangement generates a fusion transcript encoding a truncated but membrane-bound form of NOTCH1. In addition, CUTLL1 cells are highly sensitive to inhibition of the NOTCH signaling pathway with GSIs. These results show that T-cell tumors harboring a t(7;9)(q34;q34) may be amenable to treatment with GSIs and highlight the value of CUTLL1 cells for the functional characterization of the NOTCH signaling pathway in T-ALL.

Material and methods

Patient material

Lymphoma cells from a pleural effusion in a pediatric patient with T-cell lymphoblastic disease at relapse were obtained from Morgan Stanley Children's Hospital of New York-Presbyterian/Columbia University Medical Center. Informed consent and approval by the institutional review board were obtained according to general guidelines.

Establishment and maintenance of the CUTLL1 cell line

Pleural effusion cells (10^5) were plated in three wells of a 24-well plate in RPMI 1640 supplemented with 20% fetal calf serum (FCS), penicillin, streptomycin, and L-glutamine at 37°C in a 5% CO₂ humidified environment. At 1 week after plating, cultures showed fibroblast looking cells adherent to plastic with lymphoid cells attached to them. Half of the media was replaced with fresh media once a week for 5 weeks with slow but continuous growth of lymphoid cells. After 6 weeks of culture cells were expanded by weekly 1:2 dilutions. At 2 months after initial plating robust lymphoid cell growth was observed and the cells were transferred to six-well plates and subsequently to culture flasks. The cell line established in this way has been named CUTLL1 and has been maintained in continuous culture in RPMI1640 plus 20% FCS for more than 8 months. Columbia University T-cell Lymphoblastic Lymphoma 1 can be frozen in RPMI media containing 20% FCS and 10% dimethyl sulfoxide and thawed back in culture which is most successful if seeded in their own conditioned media.

Karyotype and Fluorescence in situ hybridization analysis

Metaphase chromosome preparations made from CUTLL1 cell line were subjected to G banding and karyotype analysis following standard procedures. Two PAC clones RP11-370H5 and CTD-3242B16, which span the NOTCH1 gene were obtained from Invitrogen. DNA was labeled by nick-translation using spectrum orange dUTP fluorochrome (Vysis, Downers Grove, IL, USA). A spectrum green-labeled alpha-satellite chromosome 9 centromeric probe was obtained from Vysis (Downers Grove, IL, USA). Fluorescence *in situ* hybridization was performed by standard methods on cells used for

cytogenetic analysis. Hybridization signals were scored on at least 20 metaphase spreads on diamino-phenylindole-stained slides using the Cytovision Imaging system attached to a Nikon Eclipse 600 microscope (Applied Imaging, Santa Clara, CA, USA).

Immunophenotypic analysis

Flow cytometry analysis of CUTLL1 cells was performed after 6 months of *in vitro* culture. Cells were stained with four-color antibody cocktails that included anti-CD1a, -CD2, -CD3, -CD4, -CD5, -CD7, -CD8, -CD10, -CD13, -CD16/56, -CD19, -CD33, -CD34, -CD45, -CD117, -TCR $\alpha\beta$ and -TCR $\gamma\delta$ (BD BioSciences, San Jose, CA, USA). Cytoplasmic staining with anti-TdT (Beckman Coulter, Miami, FA, USA) antibody was performed using Fix-and-Perm reagents, according to the manufacturer's instructions (Caltag Laboratories, Burlingame, CA, USA).

DNA fingerprinting

The genetic identity of the derived cell line with the original primary lymphoma cells from the patient was confirmed by analysing 14 tetranucleotide short tandem repeat loci, one pentanucleotide repeat locus (Penta, Germany) and the Amelogenin locus using the PowerPlex^R 16 System (Promega, Madison, WI, USA). Genomic DNA isolated from CUTLL1 cells and from the corresponding primary T-cell lymphoblastic lymphoma sample was amplified following the manufacturer's instructions. The fluorescent products were analysed by capillary electrophoresis in a ABI PRISM310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The results were analysed using GeneMapper v3.0 analysis software.

5' RACE amplification of aberrant NOTCH1 transcripts

Messenger RNA (mRNA) was extracted from CUTLL1 cells using the Nucleotrap mRNA extraction kit from Clontech, and 5'RACE was performed with the SMART RACE kit (Clontech, Mountain View, CA, USA) using an oligonucleotide primer complementary to the sequence of exon 29 of the NOTCH1 gene (5'-TCGTC CATGAGGGCACCGTCTGAAG-3').

PCR amplification and sequence analysis the TCRB-NOTCH1 rearrangement

Total genomic DNA from primary T-cell lymphoblastic lymphoma cells and from the CUTLL1 cell line was extracted using the PuregeneTM Cell and Tissue kit (Gentra, Minneapolis, MN, USA). PCR amplification and sequence analysis of genomic sequences spanning the TCRB-NOTCH1 rearrangement was performed using TCRBJ254 (5'-GGACCCGGCTCTCAGTGCT-3') and NOTCH1 Exon 28 (5'-TCCCCGCCCTCCAAAATAAGG-3') primers using the High Fidelity PCR System (Roche, Indianapolis, IN, USA) following the manufacturer's instructions.

Proliferation, apoptosis and cell cycle analysis

Duplicate cultures of CUTLL1 cells were seeded at 5×10^4 cells/ml and treated with 500 nM Compound E or vehicle (DMSO). Cell growth was analysed every 48 h using a colorimetric MTT assay (MTT Cell Proliferation Kit I, Roche). Cell cycle distribution was performed by assessment of DNA content using PI staining as previously described.¹⁸ Apoptosis was quantified by flow cytometry by AnnexinV/PI staining using the Annexin V: fluorescein isothiocyanate Apoptosis Detection Kit I (BD-Pharmingen, San Diego, CA, USA).

Gene expression profiling

Microarray hybridization of triplicate CUTLL1 cultures treated with GSI (500 nM Compound E) or vehicle (DMSO) for 24 h was performed using Affymetrix U133plus2 arrays following standard procedures. Interarray intensity differences were normalized with Dchip (<http://biosun1.harvard.edu/complab/dchip/>). Unsupervised hierarchical clustering was performed with Dchip. Supervised Nearest-neighbor analysis of genes associated with GSI treatment versus control vehicle (DMSO) treatment were identified by signal-to-noise nearest-neighbor analysis performed with Genecluster 2.0 (<http://www.broad.mit.edu/cancer/software/genecluster2/gc2.html>) on genes that showed at least two fold differences between maximum and minimum values and an absolute difference of at least 100 U.

Results

Establishment and culture of CUTLL1, a new immature T-cell line

A new cell line was established by *in vitro* culture of lymphoblast cells obtained from a pleural effusion sample of a 14-year-old male diagnosed with T-cell lymphoblastic lymphoma at relapse. This cell line was named CUTLL1 for Columbia University T-cell Lymphoblastic Lymphoma 1 and has been maintained in continuous culture for more than 8 months. CUTLL1 cells show robust growth *in vitro* with a doubling time

of 48–72 h and can be frozen and thawed successfully using their own conditioned media. Microsatellite fingerprinting results from CUTLL1 cells and the primary lymphoma sample used to establish this line showed that they share a unique DNA fingerprint. Fingerprint results and allele sizes for CUTLL1 cells and the primary lymphoma cells are provided in the supplemental material (Table S1 and Supplementary Figure 1). This result demonstrates that CUTLL1 is in fact a new T-ALL cell line established from this patient sample and rules out the possibility that it was the result of accidental cross contamination of this culture with a different leukemia cell line.

Immunophenotypic analysis of CUTLL1 cells demonstrated coexpression of CD4 and CD8 as well as CD2, CD5, CD7, surface CD3 and TCR $\alpha\beta$ (Figure 1). Markers associated to B (CD19), NK (CD16/56) or myelomonocytic (CD13, CD33 and CD63) lineage were negative. CUTLL1 cells showed partial expression of CD10 (31%) and CD1a (10%) markers. They expressed TdT and were negative for the early hematopoietic marker CD34.

CUTLL1 is a new T-cell line with the t(7;9) translocation involving the NOTCH1 locus

Cytogenetic analysis of CUTLL1 cells showed a hyperdiploid karyotype [50, XY, t(7;9), +16, +18, +20, +21] with a characteristic t(7;9)(q34;q34) translocation (Figure 2a and b). This recurrent chromosomal rearrangement typically involves the

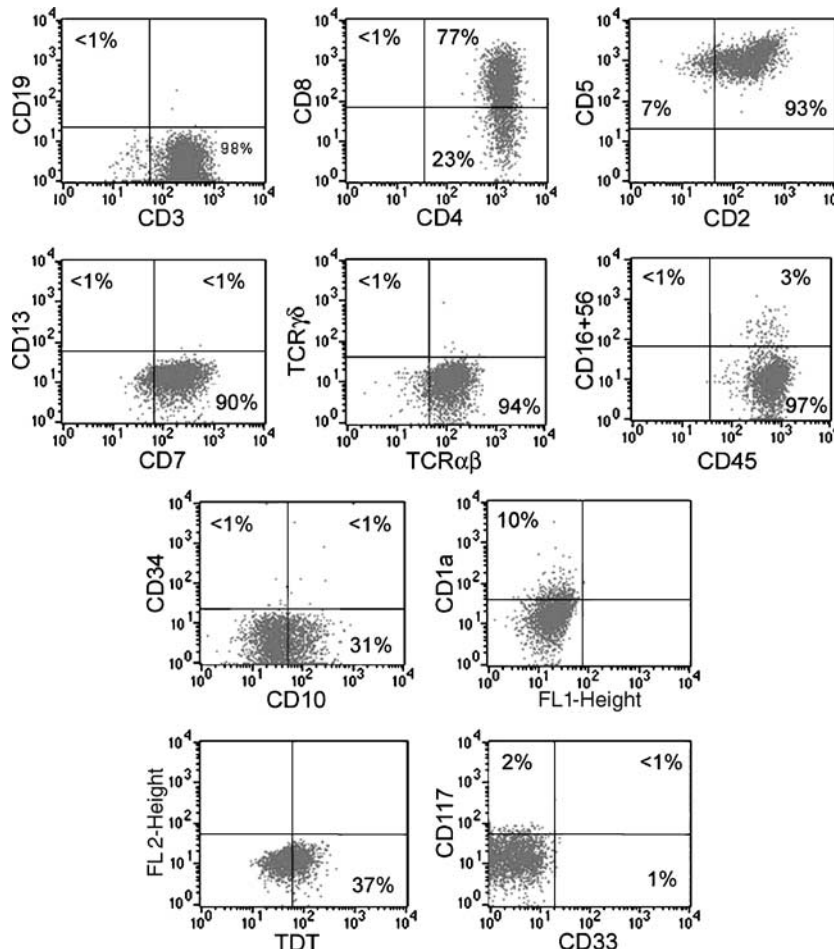


Figure 1 Immunophenotypic characterization of CUTLL1 cell line. CUTLL1 cells were stained with the indicated antibodies and analysed by flow cytometry. High expression of CD3, CD4, CD8 and TCR $\alpha\beta$ indicate cell arrest at the immature cortical thymocyte stage of development.

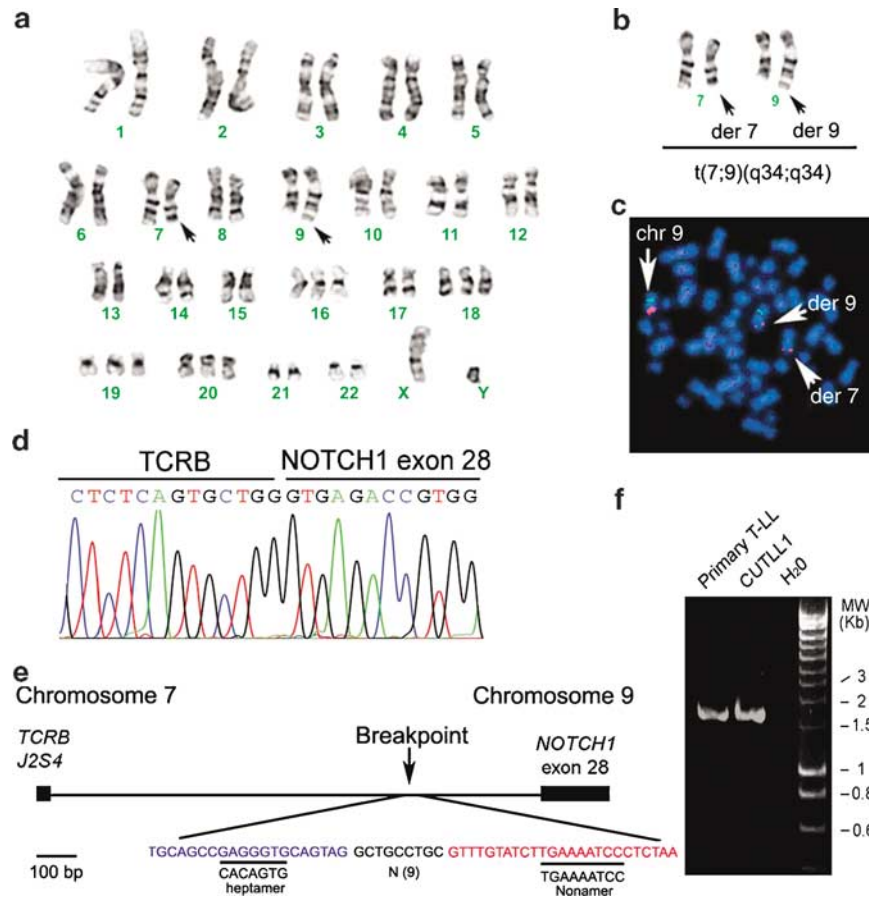


Figure 2 Cytogenetic and molecular characterization of the CUTLL1 cell line. **(a, b)** Karyotype analysis of CUTLL1 cells demonstrated the presence of a $t(7;9)(q34;q34)$. **(c)** Double color FISH with a centromeric chromosome 9 probe (green) and a PAC probe containing the *NOTCH1* locus (red) shows a split signal demonstrating the involvement of *NOTCH1* in the rearrangement. **(d)** Sequence of 5' RACE amplification of *NOTCH1* demonstrating the presence of a *TCRB-NOTCH1* fusion transcript. **(e)** Schematic representation of the *TCRB-NOTCH1* genomic rearrangement in CUTLL1 cells. Sequences from the *TCRB* locus in chromosome 7 (blue) are joined to sequences from the *NOTCH1* locus in chromosome 9 (red) by a stretch of nine bases introduced during the rearrangement. Sequences flanking the breakpoint with homology to heptamer and nonamer rag recombination sequences are underlined. **(f)** PCR analysis of genomic DNA from CUTLL1 cells and the corresponding primary lymphoma cells demonstrates the existence of the *TCRB-NOTCH1* rearrangement in the cell line and primary patient material.

TCRB locus in chromosome 7q34 and the *NOTCH1* gene in 9q34, and is found in less than 1% of T-cell lymphoblastic tumors. Fluorescence *in situ* hybridization analysis with 9q34 PAC clones spanning the *NOTCH1* gene showed a split signal which was translocated to chromosome 7q confirming the involvement of the *NOTCH1* locus in the $t(7;9)$ translocation (Figure 2c). This result strongly suggested that CUTLL1 cells may express a truncated form of NOTCH1. To test this hypothesis, we performed a 5'RACE amplification of the *NOTCH1* gene in CUTLL1 cells using a primer located in exon 29 of *NOTCH1*, which encodes the first intracellular region of the receptor. This experiment amplified a single PCR band corresponding to a truncated *NOTCH1* transcript which spanned *NOTCH1* exons 29 and 28 followed by the *J2S4* sequence of the *TCRB* gene (Figure 2d and e). This chimeric transcript encodes a truncated form of NOTCH1 which retains the transmembrane region of the receptor followed by an extracellular stub encoded by exon 28 and a short peptide derived from the *TCRB* sequence. Importantly, truncated forms of NOTCH1 lacking most of the extracellular fraction of the receptor but retaining the transmembrane domain are constitutively cleaved by the γ -secretase complex resulting in aberrant NOTCH1 signaling.¹⁹

PCR amplification of genomic DNA with primers located in the *J2S4* sequence of the *TCRB* gene and in exon 28 of *NOTCH1*

amplified a 1.6 kb band both in CUTLL1 cells and in the primary lymphoma sample used to establish the cell line (Figure 2f), demonstrating that the *TCRB-NOTCH1* rearrangement was in fact originally present in the primary cells from the patient and is not a secondary event acquired during the *in vitro* immortalization of the CUTLL1 cells. Sequence analysis of the rearranged genomic fragment confirmed the presence of a chromosomal breakpoint in intron 27 of *NOTCH1* joined to *TCRB* sequences from chromosome 7 by a stretch of nine extra bases. The predicted breakpoint positions were flanked by heptamer and nonamer sequences with homology to RAG consensus recombination sequences separated from the breakpoint by 16 bases (Figure 2e). These features are characteristic of rearrangements mediated by the RAG recombinase during thymocyte development and strongly suggest that a recombination error during aberrant *TCRB* rearrangement may have been responsible for the generation of the $t(7;9)$.

CUTLL1 cells have a characteristic gene expression signature

Gene expression profiling with oligonucleotide microarrays showed that CUTLL1 cells have a unique gene expression signature when compared with seven other well characterized

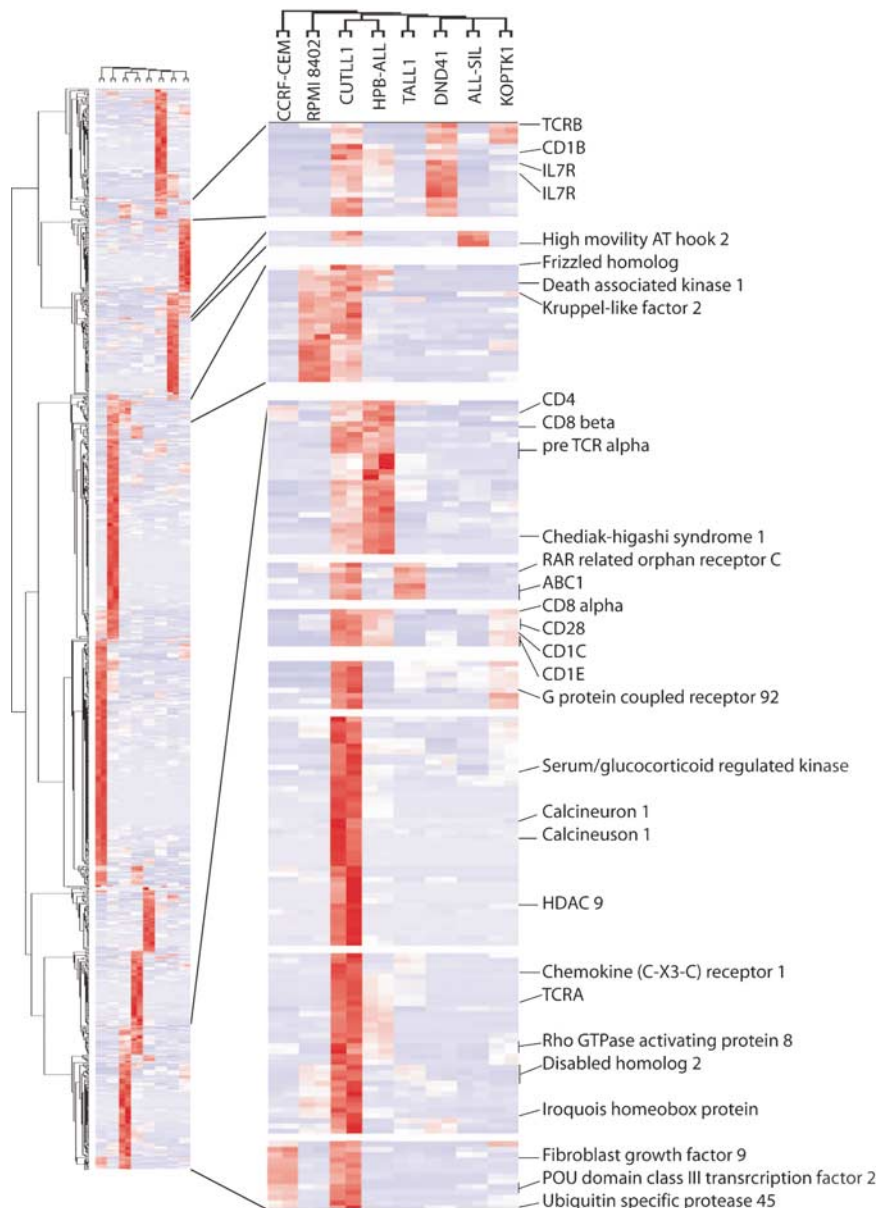


Figure 3 Gene expression profiling of CUTLL1 and T-ALL cell lines. Hierarchical clustering of microarray gene expression data from duplicate samples from eight T-ALL cell lines. Clusters of genes upregulated in CUTLL1 cells are shown in the right. Relative gene expression levels are color coded with red indicating higher levels of expression and blue lower levels of gene expression.

T-cell lymphoblastic cell lines. Unsupervised hierarchical clustering demonstrated that CUTLL1 cells are more related to the HPB-ALL cell line (Figure 3) and to other T-ALL cell lines such as ALL-SIL, DND41, KOPTK1 and TALL1 that show decreased proliferation and G1 cell cycle arrest when treated with GSI than to cell lines which fail to show a growth phenotype upon GSI treatment such as CCRF-CEM and RPMI8402.

CUTLL1 cells have high levels of NOTCH1 activity which is blocked by treatment with a GSI

A dual mechanism of activation of NOTCH1, is generated by the presence of the t(7,9) in T-cell progenitors. First, strong enhancer elements in the vicinity of the TCR locus are predicted to induce high levels of NOTCH1 expression; and second,

truncated *NOTCH1* transcripts generate constitutively active forms of the receptor that signal in the absence of ligand. Consistent with this model, CUTLL1 cells harbor high levels of intracellular activated NOTCH1 protein (NOTCH1IC) measured by Western blot with an antibody (Val1774, Cell Signaling, Danvers, MA, USA) that specifically recognizes the γ -secretase cleaved active form of NOTCH1 (Figure 4a and b).

The original t(7;9) translocation identified in SUPT1 cells generates a cytoplasmic form of NOTCH1 which lacks the transmembrane domain and therefore is not blocked by GSI. However, the retention of the sequences encoding the transmembrane domain of NOTCH1 in the rearranged transcript expressed in CUTLL1 cells prompted us to test the effects of GSI in this cell line. Treatment of CUTLL1 cells with the GSI Compound E for 30 min induced a rapid reduction in the levels of NOTCH1IC with complete clearance of activated NOTCH1

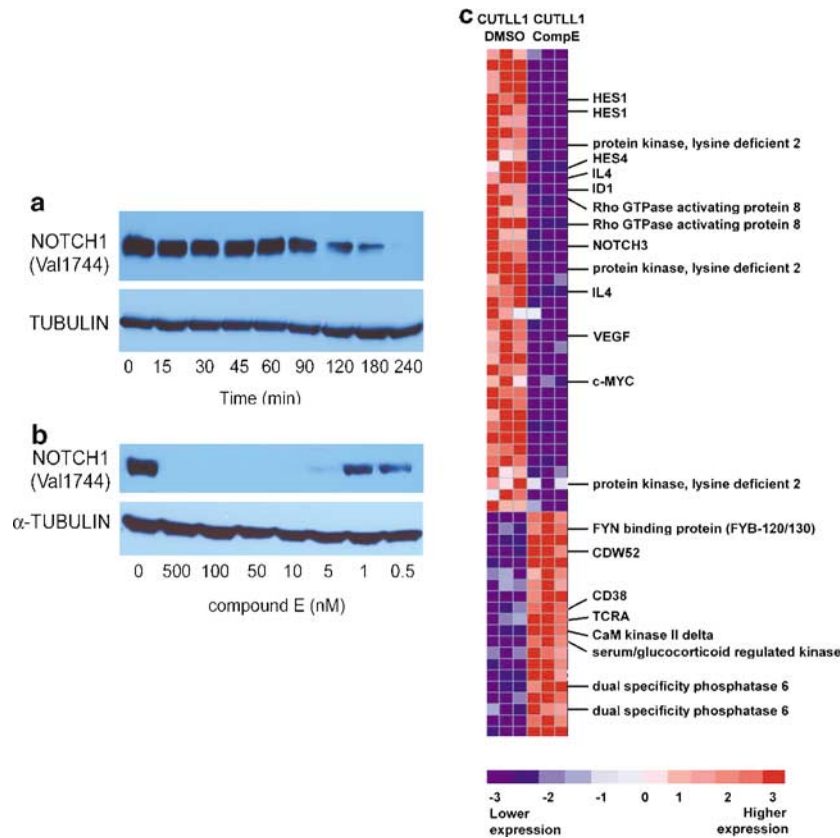


Figure 4 GSI treatment blocks activation of NOTCH1 in CUTLL1 cell lines. The effect of GSI in the levels of activated NOTCH1 in CUTLL1 cells was analysed by Western blot using the NOTCH1 Val1744 antibody (Cell Signaling Inc.) which recognizes only the activated form of NOTCH1 after cleavage by γ -secretase complex. **(a)** Time course Western blot analysis of activated NOTCH1 in CUTLL1 cells treated with GSI (CompE 500 nM). **(b)** Dose-dependent inactivation of NOTCH1 signaling in CUTLL1 cells treated with serial dilutions of CompE for 24 h. **(c)** Nearest-neighbor analysis of microarray gene expression data in CUTLL1 cells treated for 24 h with CompE or vehicle only (DMSO). Relative gene expression levels are color coded with red indicating higher levels of expression and blue lower levels of gene expression. Top genes upregulated and downregulated upon GSI treatment are shown.

by Western blot after 4 h (Figure 4a). NOTCH1 inhibition was sustained for the duration of the treatment with Compound E at least up to 4 days (data not shown). Inhibition of NOTCH1 signaling in CUTLL1 cells by GSI was dose dependent, with complete clearance of NOTCH1C after 24 h with doses as low as 10 nM of Compound E (Figure 4b). These results demonstrate that the t(7;9) present in CUTLL1 cells generates a constitutively active but membrane bound form of NOTCH1 whose activity requires processing by the γ -secretase complex rendering NOTCH1 signaling in these cells susceptible to inhibition by GSI.

GSI inhibition of NOTCH1 signaling induces cell cycle arrest and apoptosis in CUTLL1 cells

To test if sustained high levels of NOTCH1 signaling were required for the maintenance of the leukemic phenotype in CUTLL1 cells we evaluated the effect of Compound E on cell proliferation and survival in this cell line. Treatment of CUTLL1 cells with Compound E drastically impaired cell proliferation by 4 days of treatment (Figure 5a). This reduction in cell growth resulted from a combination of cell cycle arrest and increased apoptosis. Thus, treatment of CUTLL1 cells with compound E induced reduction in S and G2/M phases of the cell cycle and accumulation of cells in G1 after 3 days of treatment. This effect in cell cycle progression was accompanied by increased cell

death. Apoptosis (Annexin V⁺/PI⁻ cells) increased from 5.6 to 9.4% and cell viability (AnnexinV⁻/PI⁻ cells) decreased from 80 to 66%. Thus, in contrast with most human T-ALL cell lines harboring activating mutations in *NOTCH1*, which show weak proliferative responses and no induction of apoptosis upon GSI treatment, CUTLL1 cells require continuous NOTCH1 signaling for proliferation and survival.

GSI treatment of CUTLL1 cell induces changes in gene expression consistent with NOTCH inactivation

To further characterize the transcriptional response of CUTLL1 cells to inhibition of NOTCH1 signaling, we analysed the gene expression profiles of cells treated with Compound E or vehicle only (DMSO) for 24 h using oligonucleotide microarrays. As shown previously, treatment of CUTLL1 cells with Compound E for 24 h induces complete clearance of intracellular activated NOTCH1, but still precedes the occurrence of cell cycle arrest and the induction of apoptosis. Supervised analysis of microarray data revealed a total of 41 genes that were consistently downregulated and 20 genes which were consistently upregulated over two fold upon GSI treatment (Figure 5c). Importantly, genes downregulated after NOTCH1 inhibition included NOTCH1 target genes such as *HES1* and *NOTCH3* and the *MYC* and *ID1* transcription factor oncogenes. Genes upregulated over two fold in CUTLL1 cells after GSI treatment included

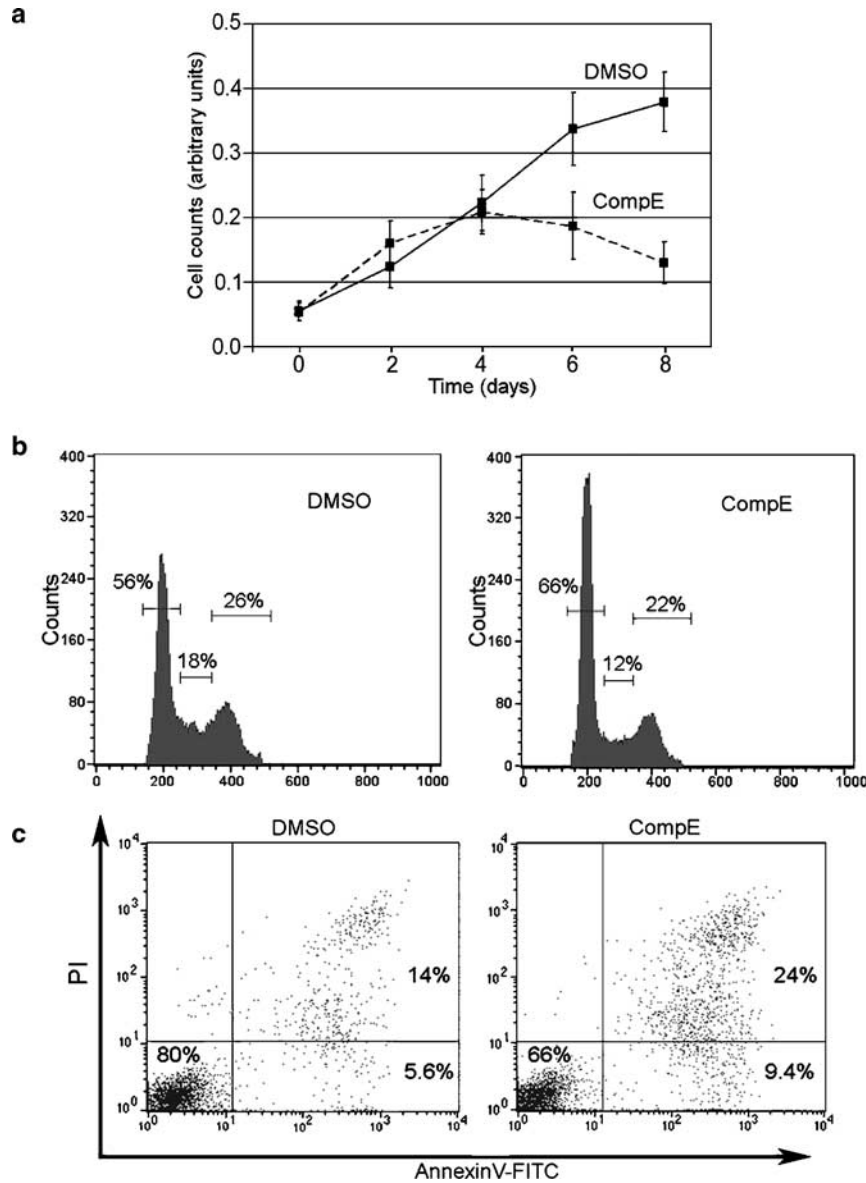


Figure 5 Impaired proliferation cell cycle arrest and apoptosis in CUTLL1 cells treated with a GSI. (a) Analysis of cell growth in CUTLL1 cell treated with GSI (CompE 500 nM) or vehicle (DMSO) using an MTT based assay. (b) Cell cycle analysis of CUTLL1 cells after treatment with GSI (CompE) or vehicle (DMSO). Cell cycle was analysed daily by assessment of propidium iodide incorporation and FACS analysis. (c) Increased apoptosis in CUTLL1 cells treated with Compound E. Cells were treated with compound E 500 nM and apoptosis was quantified by flow cytometry after Annexin V-FITC/PI staining. Figures in the plot show percentage of live, apoptotic and dead cells in control (DMSO) and GSI (CompE) treated cells.

T-cell differentiation markers such as TCRA and important signaling molecules such as FYB-120/130, CaM kinase II delta, the dual specific phosphatase 6 and the serum/glucocorticoid regulated kinase genes.

NOTCH1 cooperative oncogenes and tumor suppressors in CUTLL1 cells

Activation of NOTCH1 in T-ALL is one of several events that contributes to the multistep transformation of T-cell progenitors. Thus, activating mutations in NOTCH1 are found in leukemic cells expressing other transcription factor oncogenes such as *TAL1*, *LYL1*, *HOX11*, *HOX11L2*, *LMO1* or *LMO2*.¹ Quantitative RT-PCR analysis of T-ALL transcription factor oncogenes in CUTLL1 cells showed that this cell line has a very high level of expression of *HES1*, a bHLH factor regulated by NOTCH1 and

lacks significant expression of other T-ALL transcription factor oncogenes such as *HOX11*, *HOX11L2*, *TAL1*, *LMO1* or *LMO2* (data not shown).

Sequence analysis of the *TP53* gene demonstrated the presence of a heterozygous G to A mutation in position 994, which results in the substitution of arginine 248 for glutamine in the DNA-binding domain of this tumor suppressor (Figure 6b). Importantly, analysis of the IARC TP53 mutation database at <http://www-p53.iarc.fr/P53main.html>, revealed that *TP53* mutations in this position are frequent in human cancer and that this exact amino acid change has been reported in 674 human tumors including numerous lung, breast, stomach, esophagus, bladder, pancreas and colon carcinomas, as well as in 12 Li-Fraumeni families. Sequence analysis of DNA from the primary lymphoma cells used to establish the CUTLL1 cell line showed that the 248 arginine to glutamine mutation was originally

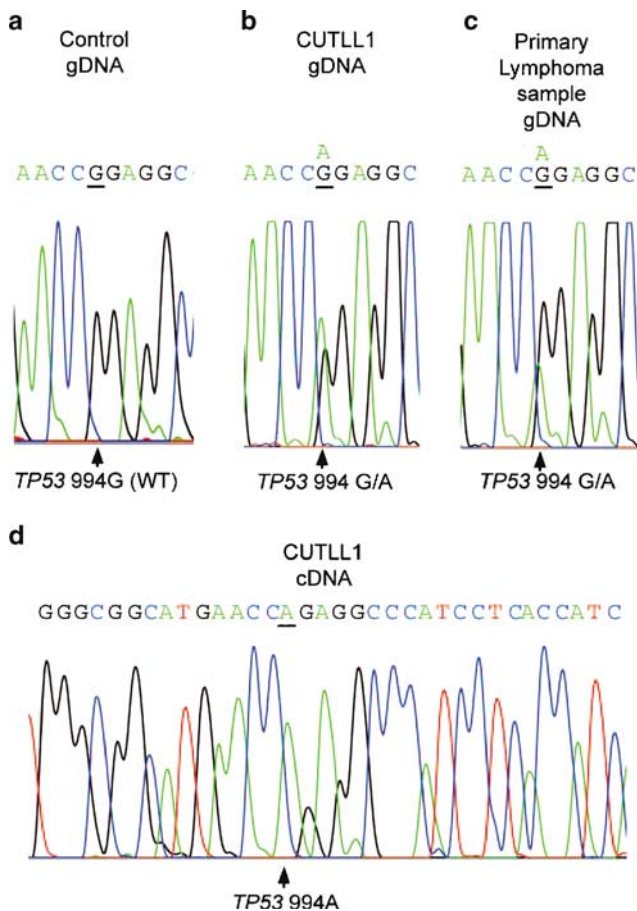


Figure 6 Monoallelic expression of mutant *TP53* in CUTLL1 cells. Sequence analysis of exon 7 of the *TP53* tumor suppressor gene shows the presence of a heterozygous G to A mutation in position 994 present in genomic DNA the CUTLL1 cell line (b), and in the original primary lymphoma sample (c). Sequence analysis of *TP53* cDNA from CUTLL1 cells, shows that the 994A mutant allele is monoallelically expressed in this cell line (d).

present in these cells and is not a secondary event acquired during immortalization of CUTLL1 cells *in vitro* (Figure 6c). In addition, analysis of *TP53* transcripts shows that the 994 A mutant allele is monoallelically expressed in the CUTLL1 cell line (Figure 6d).

Discussion

T-lineage acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic cancer that accounts for 10 to 15% of pediatric and 25% of adult ALL cases.^{20,21} Despite recent progress in the treatment of this disease the prognosis of T-ALL patients with primary resistant or relapsed disease is very poor, underscoring the need to develop more effective antileukemic drugs.^{22–26} The recent identification of activating mutations in NOTCH1 in over 50% of T-ALL patients demonstrates that the NOTCH1 signaling pathway plays a critical role in the pathogenesis of human T-ALL. Thus, inhibition of aberrant NOTCH1 signaling with GSIs to block a critical activation-associated proteolytic cleavage of the NOTCH1 receptor, is emerging as a promising novel strategy for the treatment of T-ALL.

Treatment of human T-ALL cell lines harboring activating mutations in NOTCH1 with GSIs abrogates NOTCH1 signaling

effectively. However, most of human T-ALL cell lines show no apparent cellular phenotype in response to the loss of NOTCH1 signaling. Even in those cases in which inhibition of NOTCH1 signaling induces cell cycle arrest, these responses are modest and are only apparent after relatively long treatments with GSI. In contrast with these results, recent reports have shown that mouse cell lymphoma lines harboring similar activating mutations in *Notch1* are highly sensitive to GSI and show marked growth arrest and apoptosis upon Notch inhibition. This observation suggest that human cell lines which have been in culture for several years may have acquired additional mutations that render NOTCH1 signaling dispensable for growth *in vitro*, while the more recently established mouse lines still require high levels of activated NOTCH1 for maintenance of their tumor phenotype.

Here, we report a new human T-cell lymphoblastic lymphoma cell line, CUTLL1, with robust cell growth arrest and apoptotic responses upon inhibition of NOTCH1 signaling with GSIs. The CUTLL1 cell line has an immature T-cell phenotype and harbors a characteristic t(7;9). Molecular characterization of this chromosomal rearrangement showed the presence of a translocation involving *TCRB* and *NOTCH1* genes that generates a fusion transcript containing *TCRB* sequences followed by *NOTCH1* sequences distal to intron 27. The *TCRB*-*NOTCH1* fusion gene present in CUTLL1 cells encodes a truncated but still membrane bound form of NOTCH1. Similar N-terminus truncated forms of NOTCH1 have been previously found and shown to be constitutively cleaved from the membrane by the γ -secretase complex generating high levels of intracellular activated NOTCH1.

In addition to this *TCRB*-*NOTCH1* rearrangement both CUTLL1 cells and the relapsed patient sample harbor a heterozygous mutation in DNA-binding domain of *TP53*. Interestingly, analysis of *TP53* transcripts showed that this mutant allele is monoallelically expressed in the CUTLL1 cell line. In contrast *TP53* mutations are present in only 5% of T-ALL at diagnosis but have been found to be more frequent after relapse, suggesting that they are selected during chemotherapy and contribute to treatment failure.

Aberrant NOTCH1 signaling in CUTLL1 cells is dependent on γ -secretase processing of the receptor and can be effectively blocked with GSIs. This is in contrast with SUPT1 cells, a previously described t(7;9) leukemia cell line that constitutively expresses truncated cytoplasmic forms of NOTCH1C which do not require γ -secretase activity for signaling.

Inhibition of NOTCH1 signaling with GSIs in CUTLL1 cells impairs cell proliferation inducing G1 cell cycle arrest and increased apoptosis. Importantly these cellular responses occur in the absence of the *TP53* tumor suppressor gene. Cellular responses to GSI treatment are preceded by marked transcriptional changes which include the downregulation of NOTCH1 direct target genes such as *HES1* and *NOTCH3*. Importantly, the gene expression signature induced by NOTCH1 inhibition in CUTLL1 cells includes important genes involved in the regulation of cell growth, proliferation and survival which may mediate the cell cycle arrest and apoptosis induced by GSIs.

Conclusion

In summary, we have established a new T-LL cell line, CUTLL1, harboring a t(7;9) and expressing a constitutively active but membrane-bound form of NOTCH1. These findings support that, in contrast with previous observations, NOTCH1 signaling can be inhibited with GSIs in patients harboring the t(7;9)

translocation and that these patients might benefit from experimental treatments with GSIs. Finally, the CUTLL1 cell line represents a valuable novel tool for the characterization of the NOTCH signaling pathway and for the elucidation of the transcriptional network initiated by oncogenic NOTCH1 in human T-cell precursors.

Acknowledgements

We thank Vladan Miljkovic in the ICG Microarray Facility for assistance with microarray experiments, the Cytogenetics Core Laboratory and Dr Subhadra Nandula for help with karyotype and FISH analysis.

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