Notch-1 Mutations Are Secondary Events in Some Patients with T-Cell Acute Lymphoblastic Leukemia

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Abstract

Purpose: Activating Notch-1 mutations are frequent in T-cell acute lymphoblastic leukemia (T-ALL), occurring in >50% of patients. In murine models of T-ALL, Notch-1 activation can both directly initiate leukemia and cooperate secondarily to other primary events. Whether acquisition of Notch-1 mutations is an early initiating event or a secondary event in the pathogenesis of human T-ALL is unclear.

Experimental Design: We used denaturing high-performance liquid chromatography, sequencing, and fragment analysis to analyze Notch-1 mutational status and mutant level in 62 patients at presentation as well as 16 matched presentation-relapse samples.

Results: We detected Notch-1 mutations in 47 patients (76%). Seven of these were low-level mutations (quantified at ≤10%), despite high blast counts, suggesting that they were acquired as a secondary event in a subclone. Of 16 matched presentation-relapse samples studied, 7 were wild-type at both presentation and relapse. Five of nine mutant-positive patients at presentation relapsed with the same mutation(s) at the same high level. Four patients had evidence of a change in mutant at relapse. One lost a PEST mutation and became wild-type. Two others lost mutations at relapse but acquired different mutations, despite unchanged T-cell receptor rearrangements, suggesting that the latter event predated the acquisition of the Notch-1 mutation. One relapsed with a secondary T-cell leukemia and different Notch mutation.

Conclusions: These results suggest that Notch-1 mutations can sometimes be acquired as secondary events in leukemogenesis and must be used cautiously as solitary minimal residual disease markers.

Notch-1 is thought to play an important role in normal hematopoiesis, where it has been implicated in maintenance of the hematopoietic stem cell niche (1), hematopoietic stem cell self-renewal (2, 3), and determination of lymphoid progenitor cell fate (4). It is a transmembrane receptor and ligand binding to the extracellular domain causes a conformational change in the heterodimerization (HD) domain, exposing sites to cleavage first by a disintegrin and metalloproteinase and then by γ-secretase at the transmembrane domain. This liberates intracellular Notch-1 (ICN) to translocate to the nucleus and induce downstream signaling events (5–7).

The first evidence implicating aberrant Notch signaling in leukemogenesis came from identification of the rare t(7;9) translocation in three pediatric patients with T-cell acute lymphoblastic leukemia (T-ALL), which juxtaposed the T-cell receptor (TCR) β promoter with ICN and led to constitutive Notch-1 activation (8). Subsequently, activating *Notch-1* mutations have been found as a frequent occurrence in both pediatric and adult T-ALL, being detected in >50% of patients at disease presentation (9–11). Mutations tend to cluster in two hotspots: the HD, where they are associated with ligand-independent cleavage of ICN (12), and the PEST domain, where they lead to a COOH-terminally truncated ICN. The latter results in loss of the negative regulatory domain, escape from FBXW7-mediated degradation and prolongation of the half-life of ICN (9, 13, 14). Approximately 20% of patients have mutations in both domains, usually occurring in *cis*, which results in synergistic Notch activation (9). Increased ICN levels have recently been shown to activate important oncogenes, including c-Myc and mammalian target of rapamycin (15–17).

The role of Notch as a primary event in leukemogenesis has been recapitulated in murine models, where animals transplanted with cells transduced with either ICN or the Notch ligand Delta-like 4 developed a lethal T-cell leukemia (18–20). Interestingly, *Notch-1* mutations have also been found in the majority of mice with TAL-1–induced T-ALL as well as in mice deficient in various combinations of *TP53*, *HAXA2*, and *Rag2*

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Table 1. Notch-1 primer sequences and annealing temperatures

Domain/exon	Primer	Sequence	Annealing temperature (°C)
HD-N/exon 26	26-F	5′-GGAAGGCGGCCTGAGCGTGTC-3′	67
	26-R	5′-ATTGACCGTGGGCGCCGGGTC-3′	
HD-C/exon 27	27-F	5′-GCCTCAGTGTCCTGCGGC-3′	Touch down
	27-R	5′-GCACAAACAGCCAGCGTGTC-3′	
TAD/exon 34	TAD-F	5′-GCTGGCCTTTGAGACTGGC-3′	62
	TAD-R	5′-GCTGAGCTCACGCCAAGGT-3′	
PEST/exon 34	PEST-F	5′-CAGATGCAGCAGCAGAACCTG-3′	64
	PEST-R	5′-AAAGGAAGCCGGGGTCTCGT-3′	

genes, suggesting that *Notch-1* mutations can occur as secondary events and play a collaborative role in T-ALL pathogenesis (21, 22).

In human T-ALL while *Notch-1* mutations occur in all cytogenetic and molecular subgroups, it is unclear whether they are primary initiating or secondary cooperating events. To address this issue, we have assessed the relative level of *Notch-1* mutated alleles in a cohort of human patients at presentation with T-ALL and examined stability of these mutations at disease relapse.

Materials and Methods

Patients and samples. Ethical approval for tissue collection for research was obtained from the Multi-Centre Research Ethics Committee for patients entered into clinical trials or from local research ethics committees as appropriate, and informed consent was obtained according to the Declaration of Helsinki. DNA was extracted from bone marrow samples or aspirate slides from 62 patients at presentation with T-ALL (46 adults and 16 children); results on 24 adults were reported previously (10). Samples were available from 16 patients at first relapse; 3 also had samples at second relapse.

Notch-1 mutational analysis. *Notch-1* mutation screening and identification were carried out using heteroduplex analysis and sequencing as previously reported (10). The HD-N, HD-C, transactivation (TAD), and PEST domains were amplified by 35 cycles of the PCR using the primer sequences shown in Table 1. The proofreading enzyme Optimase (Transgenomic) was used according to the manufacturer's specifications for the TAD and HD-C domain with the inclusion of 1 mol/L betaine due to the high GC content of these fragments. However, products of adequate quality could not be obtained using Optimase for the HD-N and PEST domains, and these fragments were amplified using BioTAQ DNA polymerase (Bioline). PCR products were analyzed by denaturing high-performance liquid chromatography (WAVE DNA Fragment Analysis System, Transgenomic). Abnormal chromatograms were confirmed by repeat analysis and samples were sequenced using the Dye Terminator Cycle Sequencing Quick Start kit and analyzed on the CEQ8000 Genetic Analysis System (Beckman Coulter).

For samples where the heteroduplex peaks were much smaller than the homoduplex peaks, the former were purified using the fragment collector facility of the WAVE and sequenced. In samples with insertions or deletions, relative mutant levels were quantified using the methods described above except one primer was fluorescently labeled and only 30 cycles of amplification were done. Products were size separated using fragment analysis on the CEQ8000 Genetic Analysis System. For one sample, a mutant-specific oligonucleotide was designed corresponding to the mutation, and quantification was done using the LightCycler rapid thermal cycler system (Roche Diagnostics Ltd.).

TCR rearrangements. TCR rearrangements were determined at the TCR γ locus using heteroduplex analysis and fluorescently labeled primers as previously reported (23–25). For those patients where no suitable marker was detected at the γ locus, the δ locus was analyzed.

Same patient identity. Same patient identity was confirmed in the paired presentation-relapse samples using size analysis at four highly informative polymorphic short tandem repeat loci (*VWA*, *FES*, *D11S-554*, and *F13I*; ref. 26). PCRs were carried out using BioTAQ DNA polymerase, a fluorescently labeled forward primer, and 30 cycles of amplification with annealing temperatures of 54°C for *F131*, *FES*, and *VWA* and 61°C for D11S-554. Products were sized by fragment analysis.

Results

Notch-1 mutation level. Of the 38 new cases in this cohort of adult and pediatric patients, 30 had one or more *Notch-1* mutations, giving an overall incidence of 47 mutant-positive (76%) patients in the total cohort of 62. This is similar to the incidence observed (71%) in our earlier study of 24 adult patients (10). Most of the 60 mutations detected had heteroduplex patterns that were suggestive of the presence of the mutation in the majority of cells, assuming heterozygosity (Fig. 1). Allele quantification using size analysis was feasible for 32 of the mutations that were either insertions or deletions. The median mutant level was 40% of total Notch alleles (range,

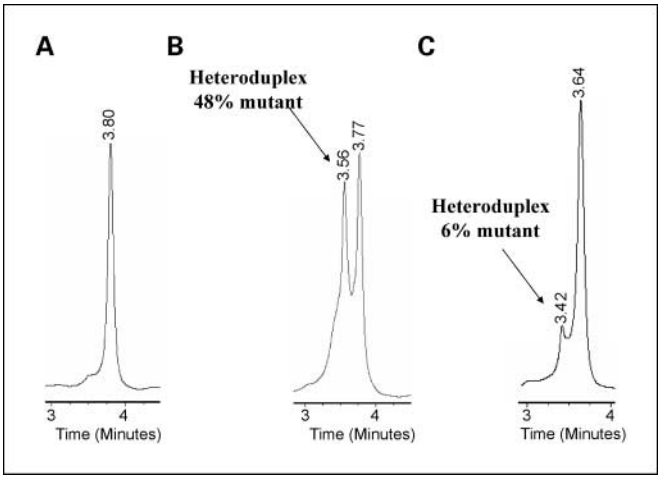

Fig. 1. WAVE chromatograms of the HD-N according to mutant level. *A*, WT chromatogram of the HD-N seen as a single peak. *B*, a high-level heteroduplex pattern of the HD-N (insV1579/1580; quantified at 48% by fragment analysis) of case 54. Similar high-level patterns were seen in the majority of mutant-positive patients. *C*, a low-level heteroduplex pattern (F1593L;1594InsSP) in the HD-N (case 22; quantified at 6% by fragment analysis).

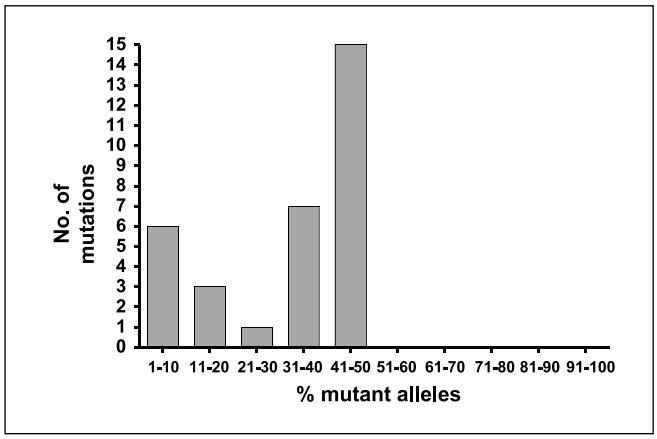

Fig. 2. Distribution of mutant level for 32 insertions or deletions. Mutation level as quantified by labeled primer and fragment analysis.

5-49%; Fig. 2). For 22 of the mutations, the mutant level was >30% of total alleles and therefore consistent with the presence of a heterozygous mutation in the majority of cells (≥60%). However, for 10 mutations, the mutant level was <30% and in six of these it was ≤10% (Fig. 2), indicating that ≤20% of cells carried the mutation (Table 2). Two of these low-level mutations were in the same patient (#50; Table 2). The remaining 27 mutations were nucleotide substitutions, which were not amenable to quantification. Of these, only one case (#40) had an apparent low-level mutation by visual estimation of the WAVE chromatogram, and all others were high level. The low-level mutants were not due to the presence of normal cells, as in each patient morphologic examination revealed at least 75% leukemic blasts. Furthermore, three of these patients had a high-level mutation in another domain. This suggested that the low-level mutations were acquired in a subclone as a later event. None of the patients with low-level mutations had evidence of ploidy or loss of genomic material of chromosome 9q by G-banding cytogenetics.

Paired presentation-relapse samples. To further examine whether the low-level mutations might have been acquired as secondary events, we studied paired presentation-relapse samples from 16 patients. Short tandem repeat analysis of four loci confirmed that all paired samples were from the same individual. Seven cases were wild-type (WT) at both presentation and relapse (Fig. 3A). Of nine mutant-positive patients at presentation, five relapsed with the same mutation(s) at approximately the same or increased mutant level (#8-11; Table 3). In one patient (#10), the same mutation was also present at second relapse. In two patients (#11 and #12), high-level mutations were present in two different domains, both of which recurred at relapse, suggesting they were in the same cell. In these five patients, it is likely that the Notch mutation occurred as an early event. However, in four mutant-positive patients, there were changes at relapse.

At presentation, one patient (#13) had only a PEST domain mutation (insertion of stop codon at amino acid 2468), which was not detected at relapse (Fig. 3B). The same TCR Vγ1 clone was identified at both presentation and relapse. Case 15 had a low-level HD-C mutation at presentation (delV1677; 9% mutant) that was undetectable at relapse. However, the patient relapsed with a new high-level HD-N mutation (H1592Q; F1593T; Fig. 3C). To determine whether the HD-N mutation was present at diagnosis but below the level of detection of the WAVE analysis, mutant-specific quantitative PCR was done on the presentation sample. The HD-N mutation could not be detected at a sensitivity of 1×10^{-4}. Furthermore, the same TCR Vγ1 clone was seen at both presentation and relapse. Another patient (#14) had an 11-bp insertion in the PEST domain at presentation, which was not detected at first or second relapse (22 and 29 months, respectively). However, a 39-bp insertion in the HD-C domain was present at a low level (6%) at diagnosis and progressively increased at each relapse (21% and 33%, respectively; Fig. 3D-F). Of note, at each time point, the patient had the same TCR Vγ11 clone; no new clone was detectable. In these three cases, the results indicate that the Notch-1 mutations occurred after the TCR rearrangement.

Case 16 presented at 5 months of age and relapsed 20 months later. Two high-level mutations (H1545P and S2514F), which were detected at presentation, were lost at relapse, but a new high-level (34% mutant) 2-bp deletion in the PEST domain was acquired. TCR analysis at the γ locus showed loss of the original clone and acquisition of a new unrelated clone (Vγ4 to Vγ2 clone, respectively), with different CDR3 sequence. Therefore, in this patient, the results probably reflect development of a secondary leukemia, similar to cases described previously (25).

Discussion

As increasing numbers of genetic abnormalities are being described in T-ALL (27, 28), it has become feasible to examine

Table 2. Mutant level and features of patients with low-level Notch-1 mutations

Patient no.	WCC × 10^9/L	Blast (%)	Mutation	Mutant level (%)	Cytogenetics
14	2.2	>90	insVNSLNIPYKIEAV1711/1712	6	51XY,+8,+10,+11,+13,+19
15	39.4	90	delV1677	9	Normal
20	2.4	74	insA1594/1595	10	Normal
22	68.2	86	F1593L;insSP1594/1595	6	Failed
40	91.8	84	R1599P	Low*	+8, t(11;19)(q23p13.3)
50	212.7	95	F1607K;insGP1608/1609	6	t(7;11)(q35p13)
			del1675S;del1676I;insTGSHA1675/1676 †	5	

Abbreviation: WCC, white cell count.
*Low level by visual estimation of the WAVE chromatogram.
† Both mutations detected at presentation.

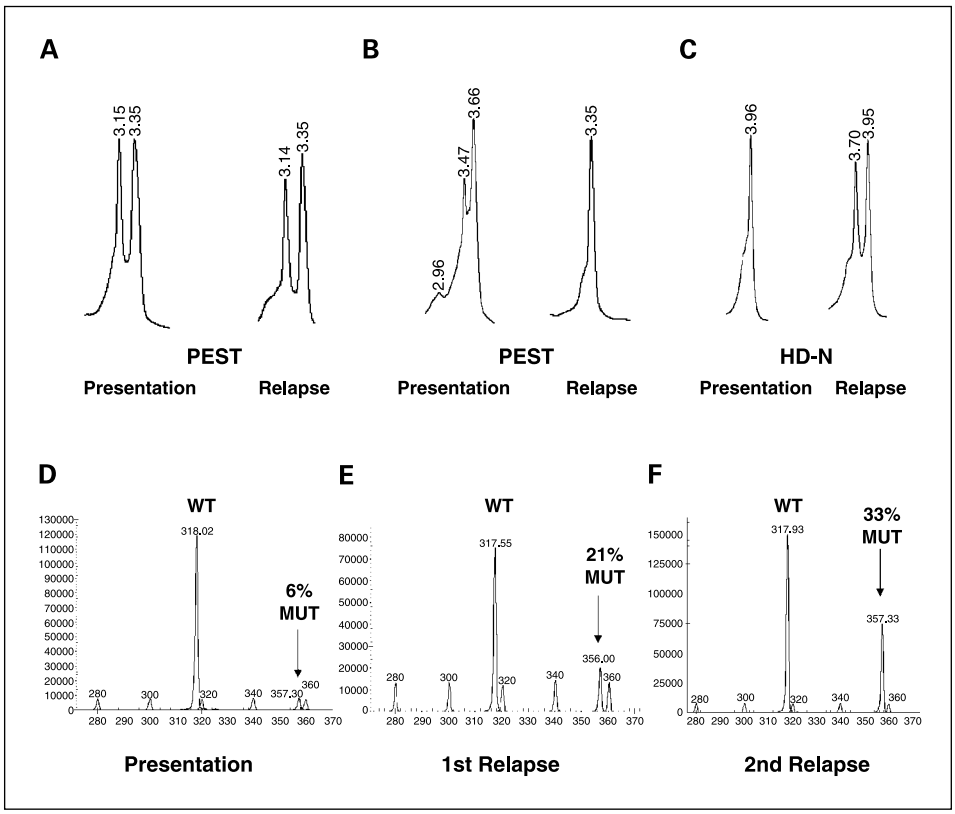

Fig. 3. WAVE chromatograms and fragment analysis for paired presentation-relapse cases. *A,* a high-level PEST domain mutation (11-bp insertion leading to frameshift at amino acid 2444) recurred at relapse (case 10). *B,* a high-level (39% mutant) PEST domain mutation (insertion of stop codon at amino acid 2468) was lost at relapse (case 13). *C,* case 15 developed a new high-level HD-N mutation (H1592Q;F1593T) at relapse, which was undetectable in the presentation sample by quantitative PCR. *D* to *F,* progressive increase of a 39-bp insertion in the HD-C domain (case 14).

the chronology in which these molecular events are acquired. Such information could help to elucidate the mechanisms by which oncogenic pathways interact, further characterize the leukemic stem cell responsible for relapse, and possibly rationalize the molecular pathways that could be targeted for drug development.

In murine models of T-ALL, Notch activation can both directly induce leukemia and collaborate with other initiating

Table 3. Notch-1 mutational status at presentation and relapse in 16 patients with T-ALL

Patient no.	Presentation /1st relapse/2nd relapse			
	HD-N	HD-C	PEST	Overall (% mutant)*
1	-/-	-/-	-/-	WT/WT
2	-/-	-/-	-/-	WT/WT
3	-/-/-	-/-/-	-/-/-	WT/WT/WT †
4	-/-	-/-	-/-	WT/WT
5	-/-	-/-	-/-	WT/WT
6	-/-	-/-	-/-	WT/WT †
7	-/-	-/-	-/-	WT/WT
8	+/+ (L1601P)	-/-	-/-	MUT/MUT (H/H)
9	-/-	-/-	+/+ ‡ (fs@2467)	MUT/MUT (22/45)
10	-/-/-	-/-/-	+/+/+ (fs@2444)	MUT/MUT/MUT † (46/42/47)
11	-/-	+/+ (insG1675)	+/+ (fs@2444)	MUT/MUT (47/46; 44/37)
12	+/+ (L1586P)	-/-	+/+ (2442NGAX)	MUT/MUT (H/H; 47/42)
13	-/-	-/-	+/- (2468X)	MUT /WT (39/-)
14	-/-/-	+/+/+ ‡ (13aa ins)	+/-/- (fs@2444)	MUT/MUT/MUT † (6/21/33; 15/-)
15	-/+ (H1592Q;F1593T)	+/- (delV1677)	-/-	MUT/MUT§ (-/H; 9/-)
16	+/- (H1545P)‖	-/-	+/+§ (S2514F/fs@2515)	MUT/MUT§ (H/-; -/34)

*Percentage mutant level as quantified by fragment analysis. H indicates high mutant level by visual estimation of WAVE chromatogram; fs indicates frameshift, all leading to premature stop codon; MUT indicates Notch-1 mutation present; WT indicates wild-type Notch-1 status; HD indicates heterodimerization NH_2-terminal domain and COOH-terminal domain; PEST indicates proline-glutamine-serine-threonine rich domain; and X indicates stop codon.

† Postallograft but donor cells undetectable.

‡ Increasing mutant level.

§ Different mutation at relapse.

‖ H1545P mutation is in exon 26 but lies in the third Lin12/Notch repeat domain. No mutations were detected in the TAD transactivation domain.

genetic events to perpetuate leukemic growth (18–22). Mice transgenic for *SCL/LMO1* usually acquire a Notch-1 mutation by the time of overt transformation to T-ALL (22). Oligoclonality predates acquisition of the mutation, but it is not until after the latter event that these thymocytes can form transplantable tumors. Whether *Notch-1* mutations are an initiating or secondary collaborating event in human T-ALL is unclear. Our results suggest that both events may occur.

Of the 59 mutations detected in the total cohort of 62 patients, 49 were present at a high level, which was consistent with the presence of a heterozygous mutation in the majority of cells. Furthermore, in five of nine mutant-positive patients in our paired presentation-relapse cohort, the same mutation was present at relapse. In these patients, it is likely that the mutation had occurred early in disease pathogenesis. Two of these patients had mutations in both the HD and PEST domains at presentation and relapse, suggesting they are in the same cell. *In vitro* studies have shown that double mutations of this nature cause synergistic Notch activation when in *cis* (9). It is also noteworthy that two patients (#14 and #9) had evidence of increasing mutant level and one (#15) acquired a new high-level mutation at relapse, suggesting that Notch activation may offer a selection advantage to these cells. The two mutations identified in patient 16 deserve particular mention. The H1545P mutation in exon 26 lies upstream of the HD-N in the third Lin12/Notch repeat (LNR-C); this domain has been shown to play an important role in preventing Notch activation in invertebrates and *in vitro* (29, 30). This mutation was undetectable in the remission sample and thus a true disease-associated mutation rather than constitutional polymorphism and is the first mutation to be described in this domain in T-ALL. Additionally, the S2514F mutation in the PEST domain of this patient involves a highly conserved serine residue, mutation of which prevents cyclin-dependent kinase 8–mediated Notch-1 hyperphosphorylation and leads to stabilization of ICN (31). Whereas most PEST mutations introduce frameshifts or stop codons upstream of S2514, point mutations of this residue have not been reported and provide a potentially novel mechanism of Notch activation.

Results in some of our patients suggested that the *Notch-1* mutations occurred not as a primary event but later in the pathogenesis of the disease. Seven were present at a particularly low level. This was not due to large numbers of normal hematopoietic cells, as review of the morphology showed that all cases had blast counts of $\geq$74%. A low mutant level could occur if the majority of leukemic cells either lost genomic material, including a mutated Notch-1 allele, or gained copies of the WT allele. However, genomic loss of 9q34 has not been described by either genome-wide analysis or fluorescent *in situ* hybridization in two substantial cohorts of T-ALL patients (32–34). Furthermore, the finding that T-ALL tumors frequently acquire a second synergistically activating *Notch-1* mutation suggests that leukemic cells favor increased, rather than decreased, Notch signal strength, making genomic loss of mutated *Notch* alleles unlikely. Although homozygosity of the *Notch-1* mutated allele has been found in some T-ALL lines as a result of loss of heterozygosity (22), the fact that no patient in our cohort had a mutant level of >50% supports the finding of others that this is not a common phenomenon in human T-ALL (32–34). *Notch-1* gene duplication has been described in T-ALL, occurring in a small population of cells as a subclonal phenomenon (35), supporting the hypothesis that Notch activation can be a late secondary event. However, the low mutant levels observed in our cases could only be explained by duplication of the WT gene in the majority of cells with the mutant-carrying clone in the minority. Our results therefore suggest that the low-level mutants described here are due to the gain of a mutation in a subclone, which, in the presence of high blast counts, must be a secondary event. These results were supported by the demonstration that three mutations were lost at relapse, whereas the TCR rearrangements remained unchanged. This suggests the *Notch-1* mutation was acquired after the TCR rearrangement in these three patients, a situation analogous to the secondarily acquired *Notch-1* mutations in murine models of T-ALL.

The acquisition of *Notch-1* mutations at different stages of disease may influence the role of Notch in disease pathogenesis. Early development of a mutation may lead to expansion of the T-cell population before acquisition of other pathogenic events, whereas with later development its effect on activation of other oncogenic pathways may be more important (15–17). Irrespective of the underlying mechanisms, however, results of the current study have implications for Notch as a target both for minimal residual disease analysis and for therapy. The high incidence of *Notch-1* mutations in both pediatric and adult patients makes it an attractive target for minimal residual disease analysis, although its application would also be challenging due to the complexity of some of the mutations. Nevertheless, the lack of mutation stability at relapse and the evident acquisition after the TCR rearrangement observed in a proportion of our patients means that caution should be exercised in using them as solitary markers for minimal residual disease analysis. In addition, there may be implications for Notch pathway inhibitors that are currently in clinical trials, which, even if effective against constitutively activated mutant-positive cells, may select out remaining WT leukemic cells.

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