



## A wide role for NOTCH1 signaling in acute leukemia

Raffaella Chiaramonte<sup>a,\*</sup>, Andrea Basile<sup>a</sup>, Elena Tassi<sup>a</sup>, Elisabetta Calzavara<sup>a</sup>,  
Valentina Cecchinato<sup>a</sup>, Vincenzo Rossi<sup>b</sup>, Andrea Biondi<sup>b</sup>, Paola Comi<sup>a</sup>

<sup>a</sup>Department of Biomedical Science and Technology, University of Milano, LITA via Fratelli Cervi 93, 20090 Segrate, Milano, Italy

<sup>b</sup>Centro M. Tettamanti, Clinica Pediatrica, University of Milano-Bicocca, S. Gerardo Hospital, Monza, Italy

Received 1 June 2004; received in revised form 18 July 2004; accepted 20 July 2004

### Abstract

NOTCH1 is involved in the pathogenesis of T-acute lymphoblastic leukemia (T-ALL) carrying the very rare translocation t(7;9)(q34;q34.3).

We analyzed the expression of genes belonging to NOTCH pathway, in acute leukemia primary samples and lymphoblastoid cell lines. NOTCH1 pathway activation represents a common feature of T-ALL when compared to acute myelogenous leukemia (AML) and B-cell precursor acute lymphoblastic leukemia. The contemporary expression of *NOTCH1* and its ligands on cell surface contributes to high levels of pathway activity. AML primary samples show high levels of *JAGGED1* expression despite the low NOTCH1 pathway activation, consistent with an autonomous *JAGGED1* signaling in myeloid leukemogenesis. © 2004 Elsevier Ireland Ltd. All rights reserved.

**Keywords:** NOTCH1; JAGGED1; T-ALL; BCP-ALL; AML

### 1. Introduction

*NOTCH* gene family encodes single pass transmembrane receptors able to transduce intercellular signals involved in cell-fate determination [1]. NOTCH1 signal is triggered by the Delta/Serrate/Lag2 (*DSL*) ligands [1]. The *DSL* family of genes include the vertebrate homologues *JAGGED1*, *JAGGED2*, *DLL-1* [1], *DLL-3* and *DLL-4* [2].

NOTCH pathway plays a role in haematopoiesis affecting both stem cells and committed progenitors [3]. The expression of NOTCH1 and its ligands in thymic stromal cells and in developing thymocytes is consistent with NOTCH function. NOTCH1 is turned on before T-cell commitment along the lymphoid development; a variety of model systems indicates that NOTCH1 signaling is required for the earliest stages of T-cell commitment in the thymus and that, in its absence, cells develop toward the B-lineage [4]. NOTCH1 regulates myeloid cells differentiation and safeguards them from apoptosis [5]. In myeloid environment, *JAGGED1* appears to play a critical role as its enforced expression causes the loss of cell

\* Corresponding author. Tel.: +39-025 033 0404/7/9; fax: +39-025 033 0411.

E-mail address: [raffaella.chiaramonte@unimi.it](mailto:raffaella.chiaramonte@unimi.it) (R. Chiaramonte).

ability to differentiate following the stimulation with GM-CSF [6].

The fundamental role played by the NOTCH pathway in T-lineage proliferation and differentiation has a counterpart in the involvement of NOTCH signaling alterations in the pathogenesis of T-cell acute lymphoblastic leukemia (T-ALL). The t(7;9)(q34;q34.3) translocation, which occurs specifically in T-ALL, places the 3' half of *NOTCH1* gene sequence downstream *TCR*- $\beta$  regulative sequence [7] and leads to the overexpression of a constitutively active intracellular portion of NOTCH1 (NICD) [8]. This form has been proved to be neoplastic in vivo and, if over expressed in mouse bone marrow, to induce selectively T-ALL [8] suggesting that *NOTCH1* behaves as a gate keeping gene in the T-lineage. Yan et al. [9] demonstrated that the induction of the *DLL-4* gene expression in bone marrow cells gives rise to T-cell leukemia. Moreover, it has been proposed that the ligand JAGGED1 may be responsible of NOTCH activation found in T-cell-derived anaplastic large cell lymphoma by inducing proliferation and inhibiting apoptosis [10]. Regarding NOTCH signaling in myeloid neoplastic counterpart, acute myelogenous leukemia is characterized by abnormalities in the NOTCH1–JAGGED1 system which would give rise to excessive self-renewal and block of differentiation [11].

We have previously demonstrated that, independently from the translocation t(7;9)(q34;q34.3), the NOTCH pathway is activated in T-ALL cell lines when compared to B-cell lymphomas (EBV negative) or to normal B and T lymphocytes from healthy donors [12].

In the present study we further extend these observations by analysing the expression of a large panel of genes involved in NOTCH pathway activity, in a series of primary leukemia cells isolated from bone marrow of pediatric patients with T-ALL, B cell precursor ALL (BCP–ALL) and AML. Our findings reinforce the notion that NOTCH pathway over-signaling represents a hallmark of the T-ALL since AML and BCP–ALL showed significantly lower levels of expression. Of note T ALL cell lines and primary samples are both characterized by the contemporary expression of NOTCH receptor and its ligands supporting the hypothesis that NOTCH pathway activation in T-ALL can be due, at least in part, to an auto-activation of this pathway. By contrast

in AML we found high levels of *JAGGED1* expression despite of the low expression of NOTCH1 pathway genes, consistent with a potential role of autonomous JAGGED1 signaling in myeloid leukemogenesis.

## 2. Materials and methods

### 2.1. Cell cultures and blood samples

Under informed consent by the parents or the guardians, 32 children with T-ALL, 25 with B-cell precursor ALL and 20 with AML were included in the study. All patients have been referred to a single Institution (Clinica Pediatrica of Monza, Italy) and treated according to the protocols of the 'Associazione Italiana Ematologia ed Oncologia Pediatrica' (AIEOP). Diagnoses have been made according to standard morphological, immunological and molecular genetics criteria. Peripheral blood mononuclear cells (PBMC) have been separated on Ficoll-Hypaque gradient, frozen as viable cells and kept at  $-80^{\circ}\text{C}$ . Mononuclear cells isolated from peripheral blood of healthy volunteers were used as controls. The following T-ALL cell lines, Jurkat, SupT1, MOLT4, CEM, HuT78, RPMI-8402, FRO and KE37, and B-cell derived leukemia/lymphoma cell lines, BC2, Raji, AS283, VAL, Daudy, BL135, BL41, EW36, Ly8, were used. Cell lines were cultured as previously reported [12] in 5%  $\text{CO}_2$  atmosphere in complete RPMI-1640 medium supplemented with 10% foetal calf serum.

### 2.2. Retrotranscription and amplification

Total RNA was extracted according to Chomczynski and Sacchi [13]. RNA samples were retrotranscribed by M-MLV Reverse Transcriptase (Gibco BRL-Life Technologies) in the conditions suggested. PCR was performed in a 20  $\mu\text{l}$  reaction mixture containing 2  $\mu\text{l}$  of cDNA, 1  $\times$  PCR Buffer II, 1.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  dNTPs, 0.25 units of AmpliTaq DNA Polymerase (Perkin–Elmer Corporation). Amplifications were performed for the number of cycles specified in Table 1 according to the following regime: denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $57^{\circ}\text{C}$  for 30 s and extension at  $72^{\circ}\text{C}$  for 30 s.

Table 1  
PCR primers and number of cycles performed for each amplification

<i>NOTCH1</i> (22 cycles)	Fwd	CCCACTCATTCTGGTTGTCC	<i>HES5</i>	Fwd	AGAAAAACCGACTGCGGAAG
	Rev	CGCCTTTGTGCTTCTGTCT	(26 cycles)	Rev	GACAGCCATCTCCAGGATGT
<i>JAGGED1</i> (25 cycles)	Fwd	GTCGGTCTCCAGTCTCCAG	<i>IFI-16</i>	Fwd	CCAGCTTTTCTGCTTTCGAC
	Rev	TGATTTCTTGATCGGGTTC	(24 cycles)	Rev	GTTGGTGGCATCTGAGGAGT
<i>JAGGED2</i> (25 cycles)	Fwd	AATGGTGGCATTCTGTGTTGA	<i>MNDA</i>	Fwd	CAAGCAAGCATCTGGAACAA
	Rev	GCGATACCCGTTGATCTCAT	(25 cycles)	Rev	AGCTTGCGGTCAACTGTTCT
<i>DLL-1</i> (26 cycles)	Fwd	TATCCGCTATCCAGGCTGTC	<i>MELTRIN</i> $\beta$	Fwd	TGCAGGAACACCTCCTTCTT
	Rev	GGTGGGCAGGTACAGGAGTA	(26 cycles)	Rev	CATAGGCCCACTGTCGATAC
<i>DLL-3</i> (23 cycles)	Fwd	TCCCAGAATTCAAACCCAA	<i>Id3</i>	Fwd	ACTCAGCTT GCCAGGTGGA
	Rev	TCACCTCCAATCTGGTCTCC	(23 cycles)	Rev	AAGTCCTTTTGTCTGTTGGA
<i>DLL-4</i> (25 cycles)	Fwd	ACTACTGCACCCACCACTCC	<i>HERP2</i>	Fwd	AACTGTTGGTGGCCTGAATC
	Rev	CCTGTCCACTTCTTCTCGC	(26 cycles)	Rev	GCTGGTAAATGCAGGCGTAT
<i>HES1</i> (23 cycles)	Fwd	ACGACACCGGATAAACCAAA	<i>DELTEX</i>	Fwd	CTTCCCTGATACCCAGACCA
	Rev	CGGAGGTGCTTCACTGTGAT	(24 cycles)	Rev	CGTGCCGATAGTGAAGATGA
<i>pT<math>\alpha</math></i> (23 cycles)	Fwd	CTGGATGCCTTACCTATGG	<i>GAPDH</i>	Fwd	CCATGGAGAAGGCTGGGG
	Rev	CAGGTCCTGGCTGTAGAAGC	(20 cycles)	Rev	CAAAGTTGTCATGGATGACC

Amplified cDNAs were separated by agarose gel electrophoresis in the presence of ethidium bromide (100 ng/ml). Results of electrophoresis were acquired by the image acquisition system EDAS 2400 (Kodak) and following analysed by Kodak 1D image analysis system.

### 2.3. Statistical analysis

All values resulting from image analysis of PCR products were normalized against the corresponding values obtained from *GAPDH* amplification. The resulting values were then statistically analysed by ANOVA (analysis of variance) test to reveal if there is any statistical difference in gene expression associated with the different leukemia cell samples, T-ALL, BCP-ALL and AML. Analysis was independently performed for each gene. The tested hypothesis was:

$H_0$  = gene expression values are equal across the different cell types; versus

$H_1$  = at least two cell types gene expression values are different.

The rejection of the null hypothesis ( $H_0$ ) ( $P$ -value < 0.01) drives to the conclusion that the analysed gene expression levels are different in, at least, two among the analysed populations. A following multiple comparison test (Tukey test) was performed to determine which particular population differs from the others: the difference between values distributions of two samples groups is significant when  $P < 0.05$ ; is not significant when  $P > 0.05$ .

## 3. Results

### 3.1. Identification of NOTCH pathway target genes

We have previously reported the concordance between *HES1* and *NOTCH1* genes expression both in T-cell ALL and B-cell lymphoma cell lines [12]. To identify further NOTCH pathway target

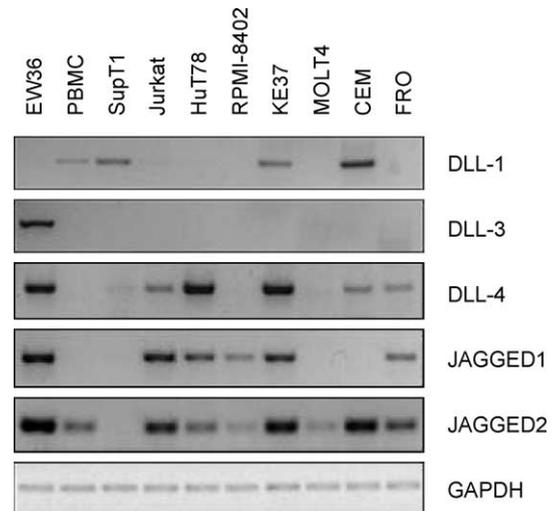


Fig. 1. RT-PCR analysis of DSL ligands expression in T-ALL cell lines; EW36 B cell line and PBMC have been used as control for *JAGGED1*, *JAGGED2*, *DLL-1*, *DLL-3*, *DLL-4*. To verify the specificity of the reactions, parallel amplifications have been performed on genomic DNA resulting in amplified DNA fragments of different size (not shown). The house keeping gene *GAPDH* has been used for normalization.

genes we have expanded the analysis to the genes reported to be modulated by NOTCH pathway: *pTα* (the gene for preTCR- $\alpha$ ), *DELTEX*, *HERP2*, *HES5*, *MELTRIN $\beta$*  (an ADAM family metallo-protease), *IFI-16* and *MNDA* [14,15]. Only the expression of *pTα* gene correlates with *NOTCH1* and *HES1* expression (data not shown). Accordingly, the subsequent analyses on primary leukemic cells were limited to *NOTCH1*, *HES1* and *pTα* as marker of NOTCH1 signaling.

### 3.2. NOTCH1 and ligands expression in T-ALL cell lines

To assess a possible contribution of the ligands in the NOTCH pathway activation we performed RT-PCR analyses on T-ALL leukemia cell lines of transcripts for all the DSL ligands. The following T-ALL cell lines were analysed: SupT1 carrying t(7;9)(q34;q34.3), Jurkat, HuT78, RPMI-8402, KE37, MOLT4, CEM, FRO. PBMC isolated from normal

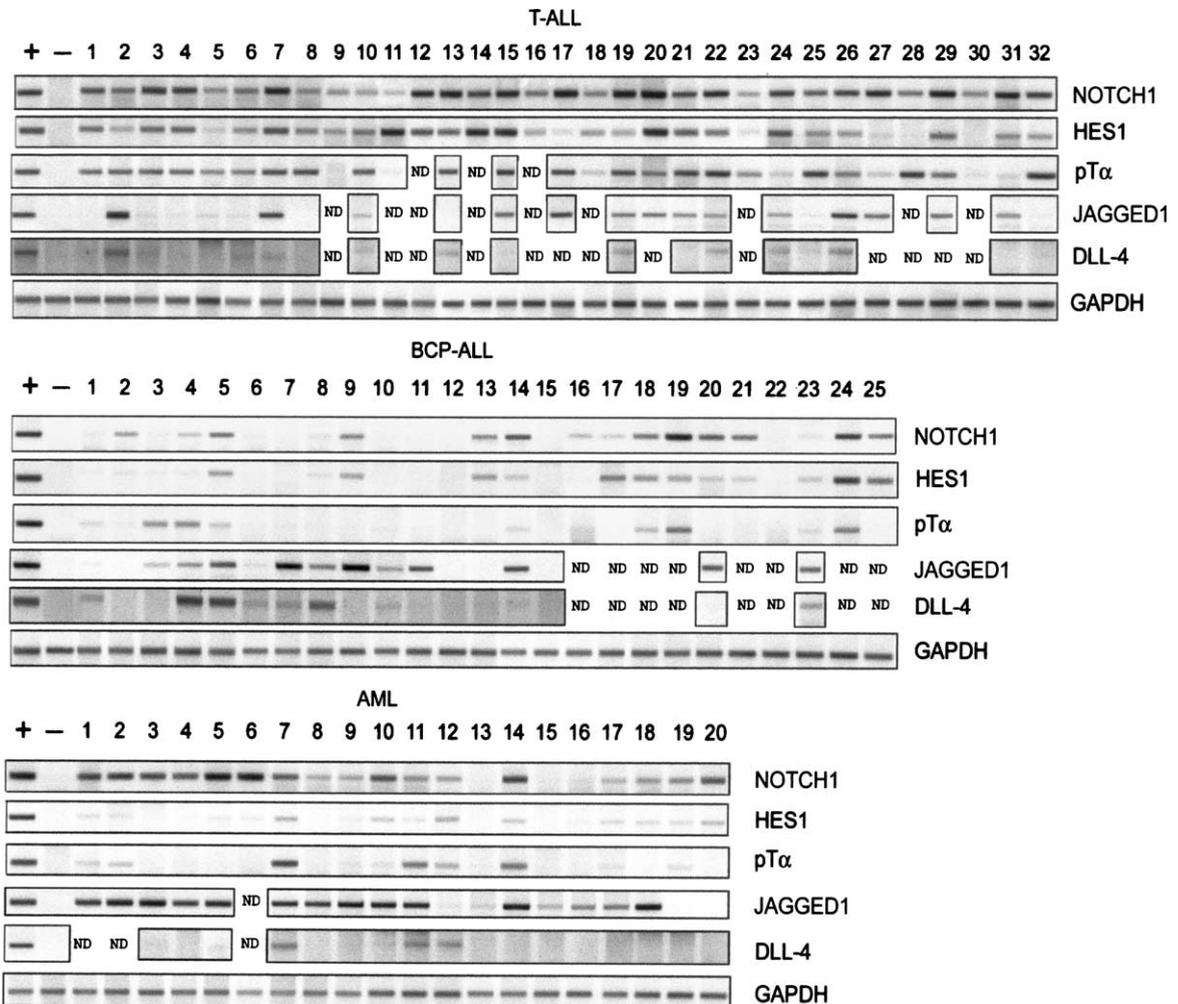


Fig. 2. RT-PCR analysis of Notch pathway-related genes, *NOTCH1*, its target genes, *HES1* and *pTα*, and the ligands *JAGGED1* and *DLL-4*, in blood samples from patients with acute leukemia T-ALL, BCP-ALL and AML. To verify the specificity of the reactions, positive and negative controls were inserted: for the amplification of *NOTCH1*, *HES1* and *pTα* positive and negative controls are, respectively, MOLT4 and BL41 cell lines, for *JAGGED1* and *DLL-4* amplifications EW36 and MOLT4 cell lines. The house keeping gene *GAPDH* has been used for normalization. Results of one representative experiment are reported.

donors and the EW36 B lymphoma cell line were used as controls.

As shown in Fig. 1 all T-ALL cell lines, that we have previously assessed to be characterized by NOTCH1 pathway activation [12], expressed one or more NOTCH ligands encoding genes.

### 3.3. Expression analysis of NOTCH pathway related genes on primary acute leukemia samples

Fig. 2 shows the representative results of RT-PCR analysis of *NOTCH1*, *HES1*, *pTα*, *JAGGED1* and *DLL-4* genes expression in primary ALL and AML samples and PBMC from normal donors. RT-PCR analysis was performed three independent times and showed reproducible results (data not shown).

Results of all experiments performed in 32 T-ALL, 25 BCP-ALL and 20 AML patient samples are shown in Fig. 3. The box-plots used for presenting data show

the median value (dark line) within a box containing 50% of the samples (25–75th percentile). Statistical analyses were performed by using ANOVA and Tukey tests. T-ALL samples showed *NOTCH1* levels of expression equivalent to AML ( $P > 0.05$ ), whereas they are higher if compared to BCP-ALL ( $P < 0.01$ ). With respect to NOTCH1 pathway signaling, as measured by *HES1* and *pTα* genes expression, T-ALL samples showed the highest levels of expression as compared to AML ( $P < 0.01$ ) and furthermore in BCP-ALL samples ( $P < 0.01$ ).

Finally, the analysis of the DSL ligands expression was performed for *JAGGED1* and *DLL-4*. Primary samples from both B and T-cell leukemia showed comparable levels of mRNA expression. AML samples displayed the highest levels of *JAGGED1* gene expression if compared to T-ALL ( $P < 0.01$ ) and BCP-ALL ( $P < 0.05$ ). No differences were detected in the three different leukemia subgroups with respect to *DLL-4* gene expression (data not shown).

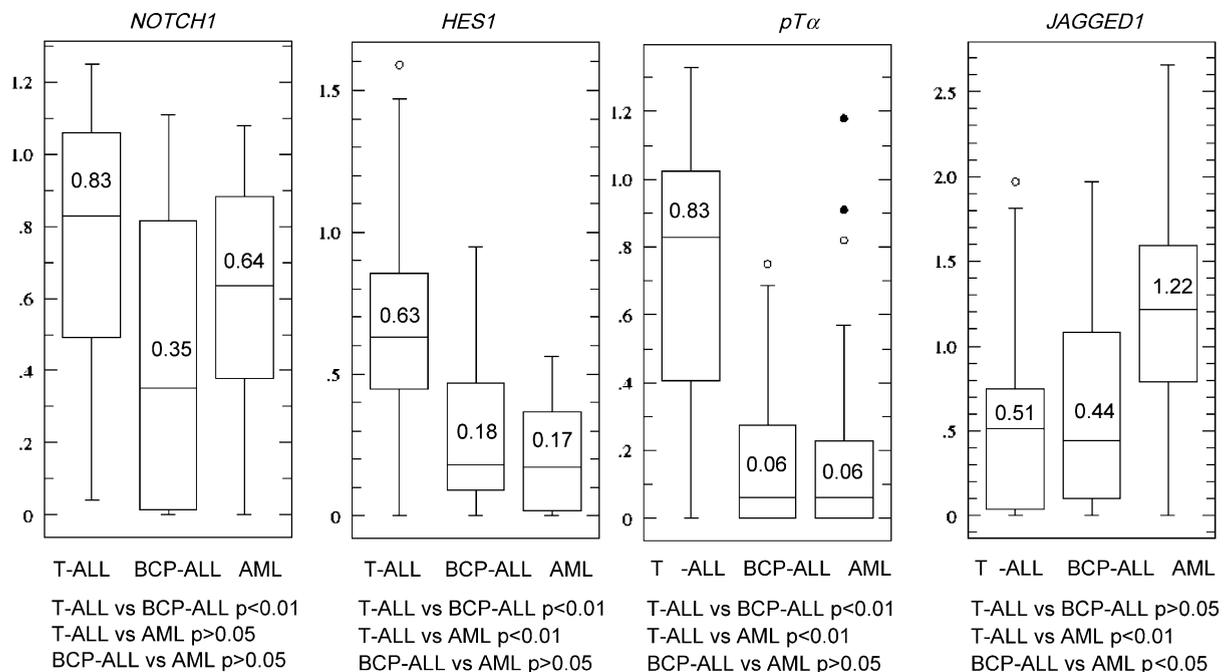


Fig. 3. Median and distribution of the expression values of NOTCH related genes (*NOTCH1*, *HES1*, *pTα* and *JAGGED1*) measured in blood samples from patients with T-ALL, BCP-ALL and AML. Normalization has been performed against *GAPDH* gene expression. Box-plots have been used to represent data and the median value (dark line) is reported within a box containing 50% of the samples (25–75th percentile). Results of Tukey test (multiple comparison) are reported: the difference between values distributions of two samples groups is significant when  $P < 0.05$ ; is not significant when  $P > 0.05$ .

#### 4. Discussion

During T-cell lineage development *NOTCH1* expression is finely regulated [16]: *NOTCH1* drives cell fate decision (the choice between  $\text{TCR}\gamma/\delta$  or  $\alpha/\beta$  and between  $\text{CD4}^+$  or  $\text{CD8}^+$ ) by inductive interactions from thymic stromal cells [17]; its expression is physiologically down-regulated to permit successful maturation by interfering with TCR signal strength [18]. *NOTCH1* seems to play a role in differentiation also through its ability to affect cell proliferation by modulation of cell cycle [19] with the consequent accelerated progression through  $G_1$  phase: by maintaining cells in a proliferative state *NOTCH1* signaling may interfere with differentiation. Furthermore, during T-cell lineage development, *NOTCH1* plays a role in rescuing cells from apoptosis [20]. The present study confirms and further extends the role of the signaling driven by *NOTCH1* and its ligands in

the pathogenesis of different types of acute leukemia: T-ALL, BCP-ALL and AML. We show that high levels of *NOTCH1* gene expression represent a common feature of T-ALL independently from the presence of the  $t(7;9)(q34;q34.3)$  translocation observed in less than 1% patients.

It has been suggested that the oncogenic ability of *NOTCH1* truncated form may be due to the observed failure of *NOTCH1* pathway down-regulation at the DP stage of T lineage development leading to a differentiation block at intermediate stages and malignant transformation proper of T-ALL [18]. The widespread over-signaling of *NOTCH1* pathway relieved in T-ALL suggests that *NOTCH* pathway deregulation may represent a common way by which different genetic alterations lead to the deregulation of thymocytes development and to leukemogenesis. Recent evidences further support the hypothesis that several

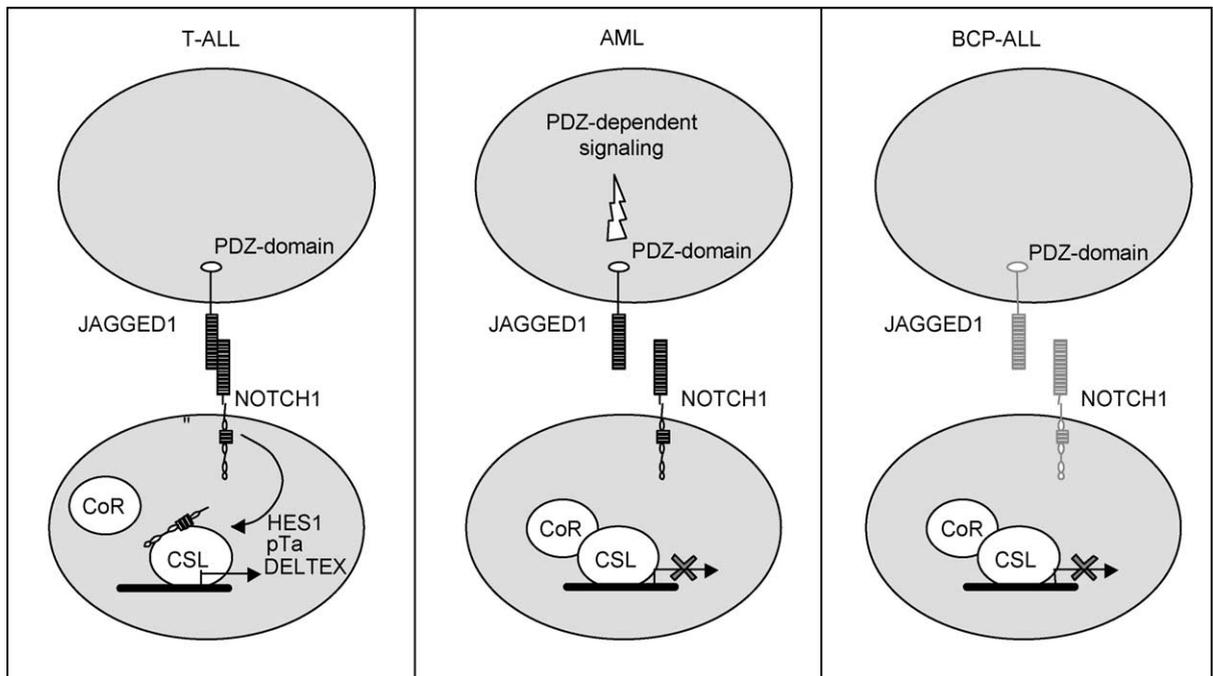


Fig. 4. A scheme of the possible bidirectional signaling of Notch1/DSL ligands. In T-ALL Jagged1, or other DSL ligands, activate *NOTCH1* signaling and the transcription of target genes; on the contrary the high levels of *JAGGED1* in AML do not produce the activation of *NOTCH1* signaling, even if *NOTCH1* is expressed on these cells, possibly because of a lack of sensibility of *NOTCH1* to *JAGGED1*-induction due to a decreased binding affinity; on the other side in AML cells the signaling starting from the PDZ domain of *JAGGED1*, reported from other work, might contribute to leukemogenesis. In BCP-ALL low levels of both *NOTCH1* and *JAGGED1* suggest they do not play any role in this neoplasia. The interaction between *NOTCH1* and the DSL ligands, here represented on different cells, might also take place inside the same cell driving to an autocrine signaling.

oncogenes, as *IKAROS* dominant negative form, *BMP2*, the bHLH transcriptional regulators, *TAL1*, *TAL2*, *LYL1*, *BHLHB1* and *LMO* may play their well-assessed role in T-ALL genesis by acting upstream NOTCH1 pathway [21–24].

A possible role for NOTCH ligands in inducing high level of pathway activity was assessed through the analysis of *JAGGED1* and *DLL-4* transcription levels in acute leukemia blood samples. Both ligands were selected since *DLL-4* overexpression in mouse bone marrow results in T Cell leukemia [9]. Moreover, *JAGGED1* expression in stromal cells of bone marrow and thymus seems to drive haematopoietic differentiation by interacting with NOTCH1 in haematopoietic progenitors [6]. Finally, a possible role for *JAGGED1* has been suggested for T-cell-derived anaplastic large cell lymphoma [10] and acute myelogenous leukemia [11].

We have found that *JAGGED1* is highly expressed only in AML samples, thus confirming a selective role of this ligand in AML [11]. By contrast, *DLL-4* generally showed low levels of expression without any evident differences among the different leukemia subgroups.

The high level of *JAGGED1* expression suggests that this ligand might be important per se and not necessarily in relation to NOTCH pathway activation, according to the data recently reported by Ascano et al. [25] who demonstrated the existence of a NOTCH-independent pathway driven directly by *JAGGED1*: a scheme of NOTCH1/DSL ligands signaling in acute leukemia is reported in Fig. 4.

In conclusion the expression analysis of genes involved in NOTCH1 signaling suggests that NOTCH1 pathway exerts a wide role in acute leukemia. More specifically in T-ALL, its function is not restricted to those cases carrying the t(7;9)(q34;q34.3) translocation, due to its central role in driving cell development. In AML our findings suggest a possible autonomous role of *JAGGED1* in supporting AML growth.

## Acknowledgements

This work was supported by grants from MIUR to PC and to AB, from University of Milano (FIRST) to

RC, to PC and to AB, from Associazione Italiana per la Ricerca sul Cancro (AIRC) to AB.

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