Chronic lymphocytic leukemia cells in lymph nodes show frequent NOTCH1 activation

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the Western world. Pathogenic mechanisms involve multiple external events (such as microenvironmental and antigenic stimuli) and internal events (genetic and epigenetic alterations) that are associated with the transformation, progression and evolution of CLL. CLL is characterized by an accumulation of mature B cells in peripheral blood, bone marrow and lymphoid tissues. Extracellular stimuli play an important role in the development and maintenance of neoplastic cells. B-CLL cells proliferate and activate pathogenic signaling pathways in anatomical structures known as proliferation centers, which are usually more conspicuous in involved lymph nodes.[1] Its clinical course is quite heterogeneous, whereby some patients progress rapidly and have short survival, whereas others have a more stable clinical course that may not need treatment for years.

Several clinical and biological prognostic factors for CLL have been identified, such as the Rai and Binet clinical staging systems, specific cytogenetic alterations, mutational status of immunoglobulin (IgHv) genes, TP53 mutations and the expression levels of CD38 and ZAP70. More recently, massive sequencing data have revealed new genetic alterations, some of which have a significant impact on the clinical course. Of these, mutations in the *NOTCH1* and *SF3B1* genes, which are associated with an adverse clinical outcome, are emerging as the most frequent mutations in CLL.[2]

The *NOTCH1* gene codes for a trans-membrane receptor that regulates critical cellular functions, such as proliferation, apoptosis and differentiation, and is associated with tumorigenesis. NOTCH1 signaling is initiated when a ligand from the Jagged or Delta families binds the receptor and induces a cascade of proteolytic processes that results in the release and nuclear translocation of the NOTCH1 intracellular domain (NCID). This, in conjunction with the transcription factor CBF1/RBP-Jk, leads to the activation or repression of its target genes.[3,4]

Although a considerable number of studies have addressed the consequences of *NOTCH1* and *SF3B1* mutations in CLL,[2] few have so far analyzed the frequency and biological impact of these mutations in lymph nodes (LN) affected by CLL (lnCLL). There is a growing interest in therapeutic targeting of NOTCH1 activation signaling in tumors with agonists such as γ-secretase inhibitors.[5] It has also been proposed that mutation-independent NOTCH1 - activation contributes to tumor cell growth and survival.[6,7]

Therefore, we have analyzed the frequency of *NOTCH1* and *SF3B1* mutations and the functional status of the NOTCH1 pathway through the expression analysis of NOTCH1-induced targets in a series of samples of lnCLL.

The first step was the analysis of *NOTCH1* and *SF3B1* mutations in the LN infiltrated with CLL. The series included 155 LN samples from 147 patients, who were biopsied at diagnosis or during the course of the disease (*Online Supplementary Appendix*). Patients' characteristics are summarized in Table 1. *NOTCH1* and *SF3B1* mutations were successfully analyzed in 140 and 142 samples by qPCR, respectively; 32 of 140 (22.8%) were positive for the p.P2515fs*4 *NOTCH1* mutation and 14 of 142 (9.8%) for the p.K700E *SF3B1* mutation. As this *SF3B1* mutation constitutes only around 50%-60% of all *SF3B1* mutations identified in CLL, we also studied exons 14 and 16 in which most of the other mutations reside. Although capillary

Table 1. Summary of the main clinical and biological characteristics of patients with samples at diagnosis.

Lymph node samples obtained at diagnosis	
Median age at diagnosis, years (range)	69.8 (39-89)
Male, n (%)	65/100 (65.0%)
Binet B-C, n (%)	36/72 (82.0%)
Unmutated IgH, n (%)	40/65 (61.5%)
13q del, n (%)	6/38 (15.8%)
Trisomy 12, n (%)	9/39 (23.1%)
11q del, n (%)	6/60 (10.0%)
17p del, n (%)	4/63 (6.3%)
Lymphocytes x10⁹/L, median (range)	7 (1-130)
CD38-positive, n (%)	43/65 (66.2%)
ZAP70-positive, n (%)	22/45 (49.0%)
Treatment, n (%)	67/134 (50.0%)
Median follow up, months (range)	28 (0-152)
Death from CLL	47/100 (47.0%)

Table 2. Summary of samples with mutations in NOTCH1 and/or SF3B1.

	DX (%)	PR (%)	Total (%)
NOTCH1 mut	28/114 (24.5)	4/22 (18.2)	32/140 (22.8)
SF3B1 mut	14/119 (11.7)	3/19 (15.8)	18/142 (12.7)
Any mutation	39/112 (34.8)	8/19 (42.1)	44/133 (33.1)

Mut: mutated; DX: at diagnosis; PR: progression.

sequencing is a less sensitive method than qPCR, we were still able to identify four additional *SF3B1* mutations in exon 14 and none in exon 16. Therefore, 18 of 142 (12.7%) *SF3B1* gene mutations were detected. Three out of 132 samples showed a mutation in both genes (Table 2). When comparing the rate of mutations in both genes in samples taken at the time of diagnosis or progression, no significant differences were found (Table 2). Samples at diagnosis and during progression were available for 8 patients. *NOTCH1* status did not change in any of the patients (2 mutated and 6 non-mutated), while one acquired an *SF3B1* mutation during progression.

We found that, although the percentage of cases with mutated SF3B1 at the time of diagnosis (11.7%) was similar to that of other series analyzed in peripheral blood (PB) lymphocytes,[2] the *NOTCH1* mutation rate we found in lnCLL (24.5% at diagnosis) seems to be higher than in other studies performed in PB samples, in which the percentages of mutations range from 8% to 31%[8,9] depending on the series and its characteristics. The lowest values (<15%) were obtained from the analysis of samples taken at diagnosis and the highest were found in patients transformed to Richter syndrome[8] or who showed refractoriness to treatment.[10] It is also higher than the frequency found in a different series of CLL PB taken at the time of diagnosis analyzed in our laboratory using the same method in which we found 12% of cases with p.P2515fs*4 *NOTCH1* mutation (*data not shown*).

We only analyzed the p.P2515fs*4 mutation, which accounts for around 80% of all *NOTCH1* mutations reported in CLL (66%-98%)[11] and, therefore, the real percentage of mutated cases in our lnCLL might be even higher. This

could be due to the greater aggressiveness of lnCLL and/or to the high sensitivity of the qPCR technique used for mutation detection.

We next analyzed the association of *NOTCH1* and *SF3B1* mutations in samples taken at diagnosis with patient, clinical, biological and cytogenetic characteristics. We found no significant associations between the presence of *NOTCH1* or *SF3B1* mutations and any biological and clinical characteristics, or with overall survival and time to treatment (*Online Supplementary Table S1*).

The expression of NOTCH1 itself together with *NOTCH1* targets, including NFATc1, p52, p50, C-MYC, MUM1, XBP1s, LEF and HES1,[3,4,12-14] and Ki67, were analyzed by immunohistochemistry in the 155 CLL paraffin samples and in 6 samples of reactive lymphoid tissue included in the tissue micro arrays (TMA) (*Online*

Supplementary Appendix).

Lymphocytes in reactive LN exhibited mainly cytoplasmic expression of NOTCH1, NFAT, p52 and p50. MUM1 expression was restricted to plasma cells and scattered cells within the germinal centers, XBP1 only recognized plasma cells, while MYC, LEF1 and HES1 expression were negative in normal B cells.

NOTCH1 nuclear accumulation was analyzed with a rabbit-mAb recognizing the NICD1 epitope,[15] a surrogate of NOTCH1 activation, and was detected in both 23 of 29 (79.3%) of *NOTCH1*-mutated cases and in 82 of 91 (90%) of *NOTCH1*-wild-type cases. This means that in a large proportion of samples (105 of 120, 87.5%), NOTCH1 could be detected in the nuclear compartment of the tumoral B cells (mainly in the proliferation centers) (*Online Supplementary Table S2*), indicating that the WNT-
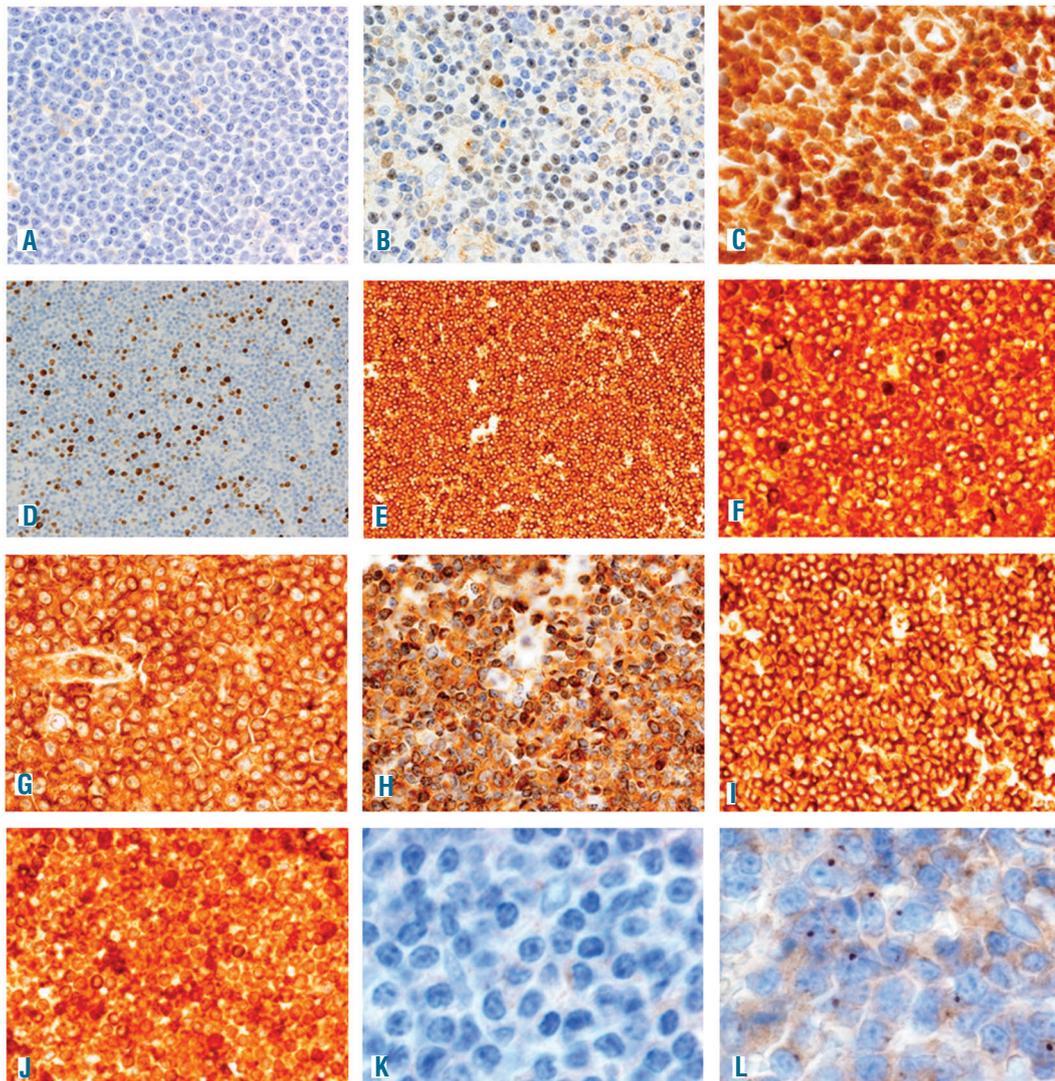
Figure 1. (A) Negative NOTCH1 expression in a wild-type case. (B) Mild NOTCH1 expression in a mutated case. (C) Intense NOTCH1 expression in a wild-type case. (D) NFAT cytoplasmic expression in a negative case. (E) NFAT nuclear expression in a positive case. (F) p50 nuclear expression in a negative case. (G) p50 nuclear expression in a positive case. (H) p52 cytoplasmic expression in a negative case. (I) p52 nuclear expression in a positive case. (J) myc expression in the proliferation center of a wild-type case. (K) JAG-negative stain in a mutated case (L). JAG expression in dots in the Golgi apparatus of a wild-type case.

NOCTH1 pathway is frequently activated in lnCLL, irrespective of the presence of the *NOTCH1* mutation, as has been previously reported by others.[15]

The *NOTCH1* mutation was associated with nuclear expression of NFAT (χ^2=8.081, *P*=0.005), NF-κB p52 subunit (χ^2=5.841, *P*=0.016) and CMYC (χ^2=4.077, *P*=0.043), which was selectively expressed in the proliferation center B cells (Figure 1 and *Online Supplementary Table S2*). The intensity and selectiveness of the expression of C-MYC by neoplastic B cells within proliferation centers were unexpected, being similar to those found in tumoral cells in Burkitt lymphoma samples, although they were restricted to the proliferation centers in our study (Figure 1). Other NOTCH1 downstream targets (such as HES1, LEF1 and XBP1) were also diffusely expressed by neoplastic B cells, and were detected in mutated and non-mutated samples, further supporting the hypothesis that the WNT-NOCTH1 pathway is frequently activated in lnCLL, irrespective of the presence of the *NOTCH1* mutation.

However, NOTCH1 nuclear expression was not significantly associated with the detection of any of the markers analyzed (*Online Supplementary Table S3*), their expression being found in samples with or without nuclear NOTCH1 expression.

The NOTCH1 pathway is activated when ligands of the JAG or Delta families bind to their receptors, leading to the release and nuclear translocation of NOTCH1. These ligands are known to be constitutively expressed in B-CLL cells.[7] Therefore we decided to analyze JAG1 expression in these samples. We found that 31 of 94 (31.9%) expressed the protein and that its detection was associated with the expression of some NOTCH1 targets such as LEF1 (χ^2=7.095, *P*=0.008), MYC (χ^2=5.158, *P*=0.023) and MUM1 (χ^2 =5.369, *P*=0.021), although not with NOTCH1 expression itself (*Online Supplementary Table S4*) as NOTCH1 could be activated by mutation independently of JAG1 presence. JAG1 was expressed in the B-CLL cells in the proliferative centers of wild-type and mutated cases in dots in the Golgi apparatus.

These results reflect the complexity of the NOTCH1 pathway in CLL as it could be constitutively activated by different mechanisms including gene mutation, ligand activation or even by the participation of other members such as NOTCH2, as has also been previously reported.[7]

In conclusion, we have found that the mutational frequency of *NOTCH1*, but not of *SF3B1*, is higher in LN with CLL cell involvement than in other series analyzing CLL PB samples. Another major finding of this study is that NOTCH1 pathway is frequently activated in lnCLL, independently of *NOTCH1* gene mutational status, thus suggesting the relevance of NOTCH1 pathway in the survival of CLL cells, and the existence of previously reported additional mechanisms of NOTCH1 activation.[7] These include the stronger expression of NOTCH1 ligands, which suggests the existence of autocrine/paracrine mechanisms that could lead to NOTCH1 signaling activation in addition to *NOTCH1* gene mutation.

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