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ORIGINAL ARTICLE

NOTCH1 mutations identify a genetic subgroup of chronic lymphocytic leukemia patients with high risk of transformation and poor outcome

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NOTCH1 has been found recurrently mutated in a subset of patients with chronic lymphocytic leukemia (CLL). To analyze biological features and clinical impact of *NOTCH1* mutations in CLL, we sequenced this gene in 565 patients. *NOTCH1* mutations, found in 63 patients (11%), were associated with unmutated *IGHV*, high expression of CD38 and ZAP-70, trisomy 12, advanced stage and elevated lactate dehydrogenase. Sequential analysis in 200 patients demonstrated acquisition of mutation in one case (0.5%) and disappearance after treatment in two. Binet A and B patients with *NOTCH1*-mutated had a shorter time to treatment. *NOTCH1*-mutated patients were more frequently refractory to therapy and showed shorter progression-free and overall survival after complete remission. Overall survival was shorter in *NOTCH1*-mutated patients, although not independently from *IGHV*. *NOTCH1* mutation increased the risk of transformation to diffuse large B-cell lymphoma independently from *IGHV*, with this being validated in resampling tests of replicability. In summary, *NOTCH1* mutational status, that was rarely acquired during the course of the disease, identify a genetic subgroup with high risk of transformation and poor outcome. This recently identified genetic subgroup of CLL patients deserves prospective studies to define their best management.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by the proliferation and progressive accumulation of mature clonal B lymphocytes in bone marrow, blood and lymphoid tissues. The clinical course of the disease is highly heterogeneous, with patients requiring early treatment for disease progression and others who have an indolent course that does not affect their life expectancy.[1,2] Several characteristics of the disease including the *IGHV* mutational status, cytogenetics, the expression of several proteins in the leukemic lymphocytes and the response to treatment have been related to the outcome of patients.[3]

Whole-genome and exome sequencing have started to reveal the complex landscape of somatic mutations in CLL with the identification up to now of around 80 recurrently mutated genes with predicted functional impact.[4–6] The distribution of these mutations in different clinical and biological subgroups of patients suggests that they may be relevant in determining the heterogeneous behavior of the disease. However, the clinical impact of these mutations and their stability during the course of the disease is not well known. Activating mutations of *NOTCH1* have emerged as one of the most frequent somatic aberrations in CLL affecting up to 10–15% of patients.[4,6–9] Virtually all these mutations generate a truncated protein lacking the C-terminal domain, that is more stable and activates the *NOTCH1* signaling pathway.[4] The presence of *NOTCH1* mutations in CLL cells seems to be associated with adverse prognosis features and to confer an adverse prognosis.[4,6,8,9]

In this study, we have investigated the presence of *NOTCH1* mutations in a large series of CLL cases to better define their stability along the evolution of the disease, as well as their relationship with other clinical and biological features of the disease and their clinical impact, particularly their influence in the requirement of and response to therapy and the transformation to diffuse large B-cell lymphoma (DLBCL).

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METHODS

Patients

A total of 565 patients diagnosed with CLL according to the World Health Organization criteria[10] with available DNA from samples before treatment, containing more than 30% of tumor cells, were included in the study. Clinical and biological data at diagnosis, treatment and follow-up were recorded and analyzed. Patients were predominantly males (59%) with a median age at diagnosis of 61 years and most of them in Binet stage A (81%). The distribution of prognostic factors with adverse impact on the evolution of patients was: unmutated *IGHV* in 46% of the patients analyzed, adverse cytogenetics (del(11)(q22.3) and del(17)(p13.1)) in 14%, high expression of CD38 or ZAP-70 in leukemic lymphocytes in 33% and 34% of patients, respectively. After a median follow-up of 6.2 years (range 0.2–27) for surviving patients, 259 patients remained untreated. The remainder received different therapies along the years, including chlorambucil ($n=105$), monotherapy with purine analogs ($n=36$), fludarabine-based polychemotherapy without rituximab ($n=51$), fludarabine-based polychemotherapy with rituximab ($n=68$), CHOP-like regimens ($n=26$) and other therapies ($n=20$). Actuarial median time to treatment (TTT) was 5.3 years. Response to treatment was evaluated according to the International Workshop on Chronic Lymphocytic Leukemia criteria.[11] In patients achieving a complete response (CR), minimal residual disease (MRD) was evaluated by sensitive multiparametric flow cytometry (0.01%),[12,13] according to the International Workshop on Chronic Lymphocytic Leukemia recommendations.[11] CR patients in whom no study of MRD was performed were considered as CR MRD-positive in all the analyses. Transformation to DLBCL was diagnosed by cytology in 2 cases and histology in 34 cases. In all, 204 patients died during the follow-up, with a median overall survival (OS) of 12 years.

Informed consent to participate in the study was obtained according to the guidelines of the local Ethic Committees.

Gene amplification and sequencing

DNA and RNA were extracted from mononuclear cells containing more than 30% of tumor cells. The median percent of tumor cells in the samples was 90% (range: 30–100%), with only 24 samples (4%) having less than 50% of CLL cells. PCR for *IGHV* was carried out according to ERIC guidelines.[14,15] *IGHV* sequences were aligned using Immunoglobulin database (http://www.imgt.org).

Exon 34 of *NOTCH1* was amplified with forward: 5′-ATGGCTACCTGTCA GACGTG-3′/ reverse: 5′-TCTCCTGGGGCAGAATAGTG-3′ and forward: 5′-G AGCTTCCTGAGTGGAGAGC-3′/ reverse: 5′-CCTGGCTCTCAGAACTTGCT-3′ primers. These amplifications cover the whole PEST domain and most of the TADD domain and include 97% of *NOTCH1* mutations previously described in CLL. The sensitivity of the Sanger technique employed for assessment of the allelic representation of *NOTCH1* mutational status was 10% (equivalent to 20% tumor cells carrying the mutations in heterozygosis in all tumor cells), as assessed by DNA titration experiments ($n=2$) by diluting genomic DNA from a heterozygous mutated sample with 99% of tumor cells into normal DNA simulating 50–10% of tumor cells. In these two cases, the allelic representation of *NOTCH1* mutations was around 50% as assessed by next-generation sequencing technologies. A clonospecific PCR with a sensitivity of 3% of allelic representation was performed in cases that showed changes in *NOTCH1* mutational status (see Supplementary Material and Supplementary Table 1). Exon 5 of *MYD88* and exons 14, 15, 16 and 18 of *SF3B1* were sequenced as previously described.[4,5] Exons 4 – 9 of *TP53* were amplified as previously described by International Agency for Research on Cancer (IARC) Consortium (http://www-p53.iarc.fr). PCR products were purified and sequenced as previously described.[5]

Statistical analysis

Fisher's test or non-parametric tests were employed to correlate clinical and biological variables according to *NOTCH1* mutational status. The main endpoints were OS, relative survival (adjusted for expected survival in the general population), TTT, progression-free survival (PFS) from CR achievement (considering need of more treatment, transformation to DLBCL or death, whichever occurred first, as events), and incidence of transformation to DLBCL. Survival curves were plotted by the Kaplan and Meier method and compared by the log-rank test. The independent value of *NOTCH1* mutations to predict the various time-to-event outcomes was assessed by multivariate Cox regression analysis. Relative survival was calculated by the cohort method described by Dickman *et al.*[16] Estimates of expected survival were calculated by the Ederer II method[17] from Spanish life tables stratified by age, sex and calendar year that were obtained from the Human Mortality Database (http://www.mortality.org).

The impact of *NOTCH1* on the risk of transformation to DLBCL was evaluated by two different methods. First, a nested case–control study in which 2–4 randomly selected control patients were matched to each case patient by the *IGHV* mutational status and the duration of follow-up, to guarantee that controls have had the same opportunity as cases with transformation to DLBCL. The association between the *NOTCH1* status and the risk of transformation to DLBCL was then assessed by logistic regression, conditional on the set of matched cases and controls. Second, we analyzed the influence of *NOTCH1* on the cumulative incidence of transformation to DLBCL by taking death as a competing risk. The multivariate adjustment for other factors predicting the transformation to DLBCL was performed within the framework of competing risks by the method of Fine and Gray.[18] The replicability of the nested case–control study and the competing-risks model were assessed by bootstrap resampling. For this purpose, control case selection for nested case–control analysis of DLBCL transformation was done by constraining the matching criteria of dates of diagnosis and last follow-up of the controls for a given case that anteceded the diagnosis of CLL and postdated DLBCL transformation, respectively, of that given case. The replicability of the nested case–control study and the competing-risks model analyzing the effect of *NOTCH1* on the risk of DLBCL transformation were assessed by bootstrap resampling. A total of 1000 samples, the same size as the original series, were built through random extraction with reposition, so that in each sample a given patient may either not be represented at all or represented once, twice or more times. The parameters assessed by resampling were the *P*-values of either the subhazard ratios of the Fine and Gray's regression or the odds ratios of the conditional logistic regression. Bootstrap resampling allows verifying that the predictive value of *NOTCH1* status was not critically dependent on the particular composition of the present series.

All statistical tests were two-sided and the level of statistical significance was 0.05. All the analyses were conducted with the use of the Stata 11 software (http://www.stata.com) and the SPSS 19 software (http://www.ibm.com). For relative survival analysis, the Stata routines developed by Paul Dickman (Karolinska Institutet, Stockholm, Sweden; available at http://www.pauldickman.com) were used.

RESULTS

Frequency of NOTCH1 mutations and sequential analysis

Of the 565 patients, 63 (11%) carried somatic mutations of *NOTCH1*. Of them, 54 (86%) had the dinucleotide deletion p.P2514Rfs*4, 3 patients had a p.L2482Ffs*2, 2 patients had a p.Q2394* and 1 patient each p.Q2444*, p.Q2404*, p.Q2503* and p.P2437fs*36.

To determine the stability of the *NOTCH1* mutational status during the clinical evolution of the CLL, we assessed *NOTCH1* mutations in two sequential samples of 200 patients with a median interval of 3.5 years (0.2–21.6 years). The disease status at the time of both samples, interval between samples and changes in *NOTCH1* are described in Table 1. A change in *NOTCH1* was observed in 3 of 200 patients (1.5%). Two patients with p.P2514fs*4 at diagnosis had a wild-type *NOTCH1* 4 and 7 years later after having received two lines of treatment (chlorambucil and fludarabine-containing therapy in one, CHOP-like chemotherapy and an autologous stem cell transplantation in the other). Another patient with unmutated *NOTCH1* at diagnosis acquired a *NOTCH1* mutation (p.Q2501fs*6) after 9.5 years of stable disease. In summary, only 0.5% of patients acquired a mutation in *NOTCH1* during the follow-up, suggesting that in most patients the status of *NOTCH1* is stable throughout the course of the disease. To better assess the changes in *NOTCH1* mutational status, a more sensitive clonospecific PCR was used. Cells carrying *NOTCH1* mutation were detected in samples from these three patients that were negative by Sanger.

NOTCH1 mutations are associated with adverse biological and clinical features

The main biological features of the patients according to the *NOTCH1* mutational status are listed in Table 2. *NOTCH1*-mutated

CLL patients showed more frequently unmutated *IGHV*, as well as elevated expression of CD38 and ZAP-70. In addition, they had more frequently trisomy 12, and less frequently del(13q)(q14.3) than cases with *NOTCH1*-unmutated. No case carried simultaneous mutation of *NOTCH1* and *MYD88*.

NOTCH1-mutated patients had more frequently advanced Binet and Rai stages, elevated serum lactate dehydrogenase and elevated beta-2-microglobulin than unmutated patients (Table 3).

TTT, response and outcome according to NOTCH1 mutational status

A total of 259 patients have not required therapy during the follow-up. This proportion was lower in patients with *NOTCH1*-mutated than in *NOTCH1*-unmutated cases (Table 3). Patients in Binet stage A and B with *NOTCH1*-mutated showed shorter TTT than *NOTCH1*-unmutated patients (median TTT 1.8 vs 6.0 years; $P<0.001$) (Figure 1). Of note, 17 of 63 (27%) *NOTCH1*-mutated patients have never received treatment after a median follow-up of 2.5 years (range: 0.6–16.5 years). The multivariate analysis of TTT, including Binet stage (A vs B), *NOTCH1* and *IGHV* mutational status, showed that only Binet stage and *IGHV* mutational status were independent in predicting need of treatment.

The status of *NOTCH1* was similar among groups of patients receiving different types of treatment. The response to therapy is listed in Table 3. Refractoriness to treatment was significantly more frequent among *NOTCH1*-mutated than in unmutated patients. Moreover, MRD-negative CR rates were lower in *NOTCH1*-mutated patients.

In all, 54 of the 115 patients achieving a CR eventually required further treatment, and 6 died without further therapy. PFS from CR achievement was shorter in *NOTCH1*-mutated than in unmutated patients (Table 3, Figure 2a). Unmutated *IGHV* and MRD-positive CR also predicted shorter PFS from CR. In a multivariate analysis, the three variables, namely *NOTCH1*-mutated ($P=0.02$; hazard ratio (HR)= 2.4), *IGHV* unmutated ($P=0.001$, HR = 4.0) and MRD-positive CR ($P=0.003$, HR= 2.6) maintained independent value to predict failure, in the Cox model with 105 patients. In addition, the survival from CR achievement was significantly shorter in *NOTCH1*-mutated patients (median survival from CR: 4.9 vs 8.7 years, respectively; $P=0.003$; Figure 2b). The prognostic value for OS from CR was not independent from *IGHV* and MRD status. Similar results were observed when the analysis was restricted to patients treated with fludarabine-containing regimens (data not shown).

After a median follow-up of 6.2 years, 204 patients have died. The main variables associated with poor OS were advanced clinical stage, unmutated *IGHV*, high expression of CD38 and ZAP-70, adverse cytogenetics, elevated serum lactate dehydrogenase, high beta-2-microglobulin and short lymphocyte-doubling time ($P<0.001$ in all comparisons). Patients with *NOTCH1*-mutated CLL showed shorter OS when compared with unmutated patients (10-year OS: 35% vs 64%; $P<0.001$) (Table 3, Figure 3). *NOTCH1* p2514fs*4 mutation and the other *NOTCH1* mutations had similar impact on OS (Supplementary Figure 1). Multivariate analysis identified, in a model with 404 cases, the following unfavorable variables to predict OS: age (HR = 1.04; $P<0.001$), advanced Binet stage (HR = 1.7; $P=0.002$), high beta-2-microglobulin (HR = 2.2; $P<0.001$) and unmutated *IGHV* (HR = 4.2; $P<0.001$). *NOTCH1* mutations did not reach independent prognostic value for OS.

To analyze the impact of *NOTCH1* mutations on the life expectancy of CLL patients, relative OS adjusted by general population mortality rates was calculated. As shown in Figure 4, the life expectancy of patients with *NOTCH1*-mutated CLL was significantly lower than that of the general population being around 50% after 10 years.

Table 1. Sequential analysis of *NOTCH1* mutations in patients with CLL

First sample	*Second sample*	N	NOTCH1 *mut/unmut*	*Interval*[a] *(years)*	*Changes*	*Type of change*
	Stable	91	7/84	3.4 (0.5–19)	1	Unmut-->mut
Diagnosis/stable	Progression	44	6/38	2.0 (0.7–22)	0	
	Post-treatment	42	12/30	5.2 (0.6–17)	2	Mut-->unmut Mut-->unmut
Progression	Post-treatment	6	0/6	4.6 (2.5–9)		
Post-treatment[b]	Post-treatment	17	3/14	4.2 (0.2–13)	0	
Total		200	28/172	3.5 (0.2–21.6)	3/200 (1.5%)	

Abbreviations: CLL, chronic lymphocytic leukemia; mut, mutated; unmut, unmutated. The percent of CLL cells (mean and s.d.) in the first and second sample were 83 ± 15% and 92 ± 12%, respectively. [a]Interval expressed as median and range in parenthesis. [b]The 17 patients with both samples after treatment were only collected for the sequential analysis of *NOTCH1* configuration.

Table 2. Main biological features of 565 patients with CLL according to the *NOTCH1* mutational status

Parameter	*Category*	NOTCH1 *unmutated* (n = *502)*	NOTCH1 *mutated* (n = *63)*	P
IGHV	Unmutated	173/416 (42%)	44/53 (83%)	<0.001
CD38	High	127/441 (29%)	37/52 (71%)	<0.001
ZAP-70	High	125/414 (30%)	34/48 (71%)	<0.001
	Del(13q)(q14.3)	179/385 (46%)	14/47 (30%)	0.03
Genetics	Del(11q)(q22.3)	36/339 (11%)	6/43 (14%)	n.s.
	Trisomy 12	50/344 (14%)	15/44 (34%)	0.002
	Del(17p)(p13.1)	11/340 (3%)	2/43 (5%)	n.s.
SF3B1	Mutated	32/332 (10%)	5/39 (13%)	n.s.
MYD88	Mutated	11/367 (3%)	0/48 (0%)	n.s.

Abbreviations: CLL, chronic lymphocytic leukemia; n.s., not significant. CD38 high: >30% of positive CLL cells; ZAP-70 high: ⩾20% of positive CLL cells; *IGHV* unmutated: ⩾98% homology with germline.

Table 3. Main clinical characteristics and outcome of the 565 patients with CLL according to the *NOTCH1* mutation

Parameter	*Category*	NOTCH1 *unmutated* (n = *502*)	NOTCH1 *mutated* (n = *63*)	P
Gender	Male (%)	299 (60%)	37 (59%)	n.s.
Age (years), median (range)		61 (24–93)	63 (43–94)	n.s.
Binet stage	A	414 (83%)	40 (65%)	
	B	65 (13%)	19 (31%)	0.001
	C	22 (4%)	3 (5%)	
	0	285 (57%)	27 (44%)	
Rai stage	I–II	189 (38%)	27 (44%)	0.03
	III–IV	27 (6%)	8 (13%)	
Lymphocytes ($\times 10^9$/l), median (range)[a]		12.7 (1.1–233)	11.4 (1.7–339)	n.s.
Hemoglobin (g/l), median (range)		139 (45–175)	136 (63–177)	n.s.
Platelets (x10^9/l), median (range)		195 (19–470)	196 (92–433)	n.s.
LDH	> UNL	44/468 (9%)	12/59 (19%)	0.02
Beta-2-microglobulin	> UNL	170/421 (40%)	32/56 (57%)	0.02
LDT	< 1 year	65/361 (18%)	11/41 (27%)	n.s.
Never-treated patients		242/502 (48%)	17/63 (27%)	0.002
Response to first-line treatment[b]	MRDneg[c]	51/225 (23%)	4/45 (9%)	0.04
	CR	59/225 (22%)	11/45 (24%)	
	PR	98/225 (44%)	19/45 (42%)	
	Failure	27/225 (12%)	11/45 (24%)	0.03
10-year TTT (95% CI)	Binet stage A and B	59% (54–64)	88% (77–99)	<0.001
Median PFS from CR (years)	CR	4.8	2.0	<0.001
10-year DLBCL (95% CI)	All	6% (3–9)	41% (21–61)	<0.001
10-year OS (95% CI)	All	64% (59–69)	35% (20–50)	<0.001

Abbreviations: CLL, chronic lymphocytic leukemia; CR, complete response MRD positive or MRD not assessed; DLBCL: transformation to diffuse large B-cell lymphoma; LDH, lactate dehydrogenase; LDT, lymphocyte-doubling time; MRDneg, complete response with negative minimal residual disease (MRD) status; n.s., not significant; OS overall survival; PFS, progression-free survival; PR, partial response; TTT, time to treatment; UNL, upper normal level; 95% CI, 95% interval of confidence [a]Of the 565 patients, 70 (12.4%) were diagnosed as small lymphocytic lymphoma. [b]In 36 patients, response was not assessable (35 *NOTCH1* unmutated, 1 *NOTCH1*-mutated. [c]Patients in whom MRD was not available were considered as CR.

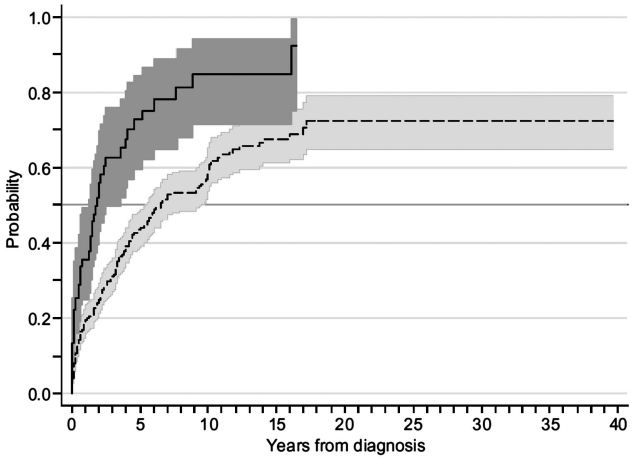

Figure 1. TTT in Binet stage A and B CLL patients according to *NOTCH1*-mutated (solid line) and *NOTCH1*-unmutated (dashed line) ($P < 0 \cdot 001$). The 95% confidence interval for each group of patients is depicted.

NOTCH1 mutations increase the risk of transformation to DLBCL

A total of 36 patients developed transformation to DLBCL. At 10 years from diagnosis, when 143 patients were still at risk, the cumulative incidences of transformation or death without transformation were 8% and 33%, respectively. The influence of *NOTCH1* mutations on the incidence of transformation to DLBCL was investigated after adjustment for other variables associated with transformation, including high expression of CD38, trisomy 12, absence of del(13q), previous exposure to purine nucleoside analogs or anthracyclines and unmutated *IGHV*. Only *NOTCH1*-mutated (HR = 5.2; $P < 0.001$) and *IGHV*-unmutated (HR = 3.6; $P = 0.006$) were independently associated with a higher risk of DLBCL ($n = 469$). At the resampling test of replicability, *NOTCH1*-mutated was selected as an independent predictor of transformation to DLBCL in 63% of the 1000 bootstrap samples, whereas unmutated *IGHV* was selected in 13%. Figure 5 shows the cumulative incidence of transformation to DLBCL according to *NOTCH1* status. At 10 years from diagnosis, the cumulative incidence of transformation was 6% and 31% for *NOTCH1*-unmutated and *NOTCH1*-mutated patients, respectively.

The case–control study included the 36 case patients who evolved into DLBCL and 168 who did not and were matched to the cases by the *IGHV* mutational status and length of follow-up. Median follow-up to diagnosis of DLBCL in case patients or to death or last follow-up in control patients was 4.5 years (range, 0.02–23) and 9.8 years (range, 0.4-39), respectively. At the conditional logistic regression, harboring *NOTCH1* mutations was strongly associated with progression to DLBCL (HR: 8.0, % CI: 3.2–20, $P < 0.001$). At the resampling test of replicability, the association between mutated *NOTCH1* and progression to DLBCL was statistically significant in 79% of the 1000 bootstrap samples.

In 15 patients, *NOTCH1* and *TP53* were analyzed at transformation: 6 cases had mutation in *NOTCH1* (40%), 2 in *TP53*, 5 in both genes and 2 had no mutations. In 8 cases, the status of *NOTCH1* was available in samples before transformation and it was identical than at transformation (5 unmutated and 3 mutated). Regarding *TP53*, two patients of eight analyzed acquired the mutation at transformation (both being *NOTCH1*-mutated). In addition, at time of transformation simultaneous samples of DLBCL and non-transformed peripheral blood CLL were available in six patients. In all cases, *NOTCH1* configuration was identical in both samples (four *NOTCH1*-mutated and two *NOTCH1*-unmutated).

DISCUSSION

The use of whole-genome and exome sequencing has revealed the presence of recurrent somatic mutations in CLL with specific

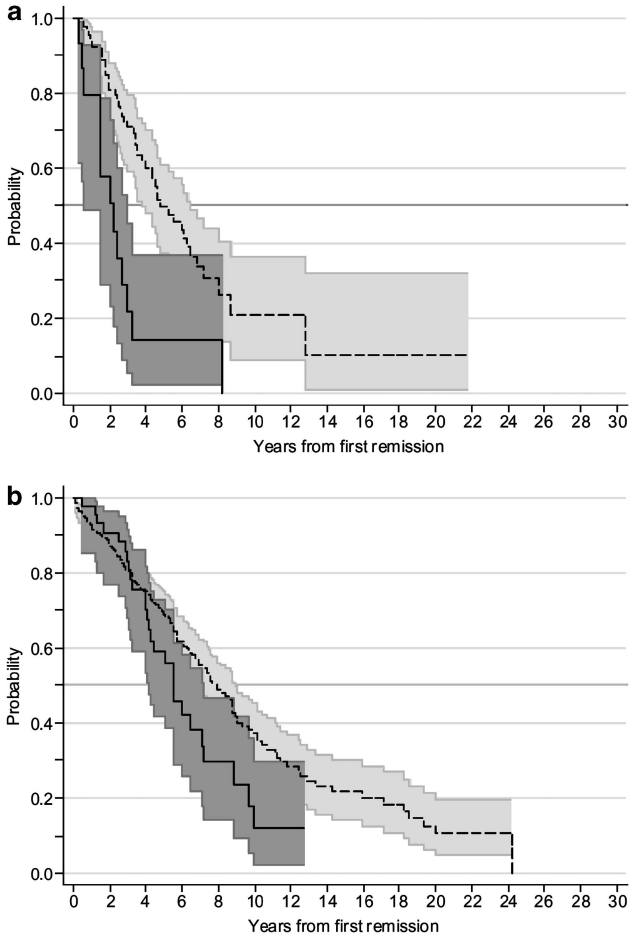

Figure 2. Outcome from CR achievement. (**a**) PFS from CR achievement according to *NOTCH1*-mutated (solid line) and *NOTCH1*-unmutated (dashed line) ($P<0\cdot001$). (**b**) Survival from CR of *NOTCH1*-mutated CLL patients (solid line) and *NOTCH1*-unmutated CLL patients (dashed line) ($P=0\cdot003$).

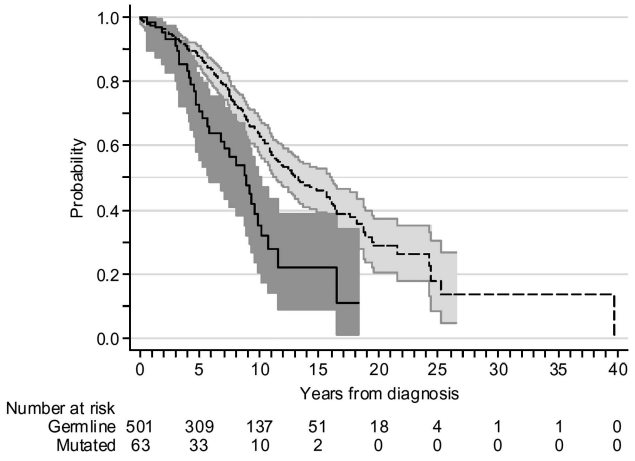

Figure 3. OS in CLL patients according to *NOTCH1*-mutated (solid line) and *NOTCH1*-unmutated (dashed line) ($P<0\cdot001$). The 95% confidence interval for each group of patients is depicted.

gene mutations clustering in one of the two major subgroups of CLL according to the *IGHV* mutational status.[4,5,19,20] Among the most frequently mutated genes, we and others have found

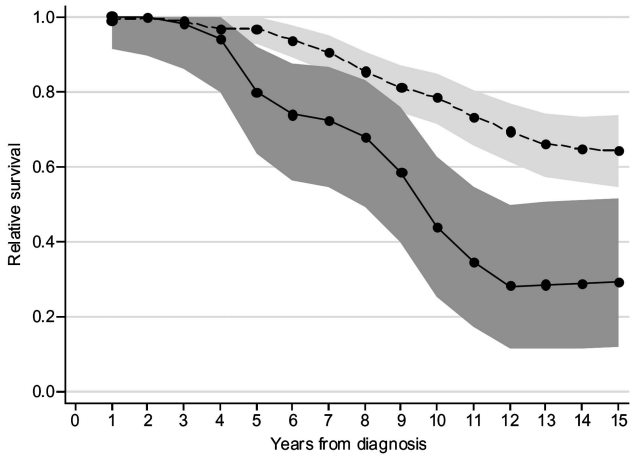

Figure 4. Relative survival of CLL patients with *NOTCH1*-mutated (solid line) and *NOTCH1*-unmutated (dashed line) CLL. The 95% interval of confidence for each cohort is plotted.

NOTCH1 and *SF3B1* mutations in up to 10–15% of CLL samples. Preliminary data suggested an unfavorable prognostic impact of *NOTCH1* mutation.[4,6,8,9,20] Thus, to gain insight into the impact of *NOTCH1* mutations in CLL, we have extended our initial series and we have analyzed in depth the role of *NOTCH1* mutation in the outcome of CLL patients with particular emphasis in the evaluation of the response to treatment and transformation to DLBCL.

Whether *NOTCH1* mutational status remains stable or, on the contrary, it changes over time in the evolution of the disease is an important issue not well established at present. There is evidence that patients at progression and relapse have more frequently *NOTCH1* mutations,[6,8,20] as we also observed in our series (Table 1). This finding is not unexpected since *NOTCH1*-mutated patients have a higher risk of progression. Fabbri *et al.*[6] observed that in 5 of 16 patients with Richter syndrome harboring *NOTCH1* mutation, this alteration was not present in the CLL at diagnosis. However, the mutational status of *NOTCH1* over time in the evolution of CLL before transformation has not been investigated. Herein we report that, in a large series of 200 patients analyzed by Sanger changes in *NOTCH1* status were observed only in 3 patients, and more importantly, only 1 patient acquired the mutation with no evidence of CLL progression. The disappearance of *NOTCH1* mutation after treatment has been previously reported in one patient.[21] The use of the more sensitive clonospecific PCR demonstrated low levels of cells with *NOTCH1* mutation in the three cases. The identification of these small subclones carrying the mutation may reflect the complex fluctuation of different tumor subclones in the evolution of the disease. A recent study using next-generation sequencing in three CLL patients[22] has highlighted the heterogeneous patterns of subclonal evolution of the disease with many subclones present at very low frequencies evolving over the years. The clinical impact of these subclones carrying mutation at low levels will require further specific studies. Moreover, no differences in *NOTCH1* status were found when comparing samples at CLL and at transformation with DLBCL. These results suggest that acquisition of *NOTCH1* mutation during the evolution of the disease, although possible, is an uncommon phenomenon. Further studies should clarify the relevance of the modulation of clones carrying *NOTCH1* mutations and whether clones acquiring such mutation may emerge during the follow-up.

In our non-selected CLL series, we have confirmed that *NOTCH1* mutations are present in 11% of cases. This proportion is similar to that found in a recent study of a similar large series of patients.[6,8,20] *NOTCH1* mutations were associated with

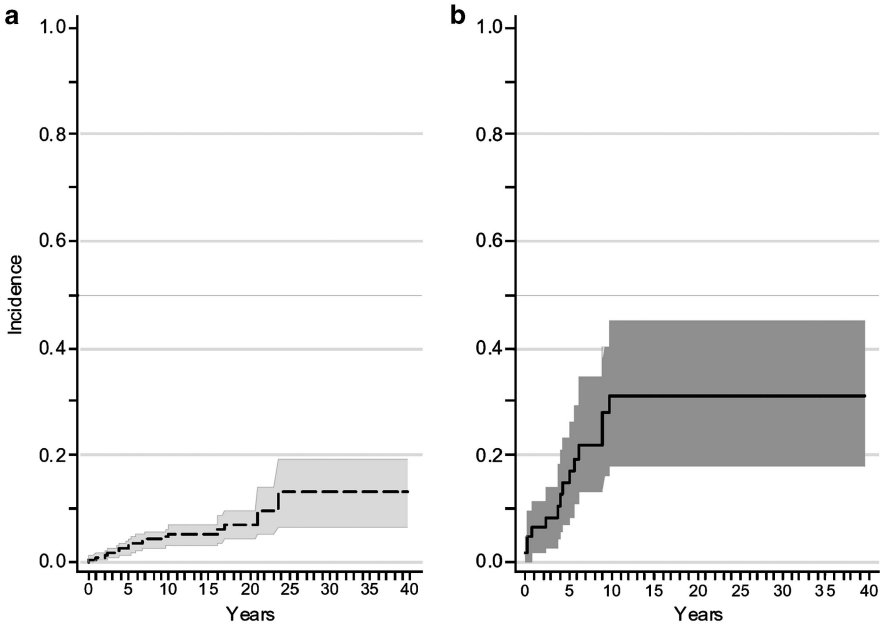

Figure 5. Cumulative incidence of transformation to DLBCL in *NOTCH1*-unmutated CLL patients (**a**) and *NOTCH1*-mutated CLL patients (**b**) ($P < 0 \cdot 001$).

unmutated *IGHV*, and high expression of CD38 and ZAP-70, as well as more frequently trisomy 12.[8,19,23,24] In our study, we have closely analyzed the impact of *NOTCH1* mutations on the requirement and response to treatment. Interestingly, patients with *NOTCH1* mutations required therapy more frequently and earlier than patients with unmutated *NOTCH1*. Moreover, patients with *NOTCH1* mutation showed poorer response to treatment, and shorter PFS and OS from CR achievement. Of note, *NOTCH1*-mutated patients who achieved CR after front-line therapy had poor outcome with 50% of them requiring further therapy within 2 years. These patients, particularly if young and fit, would be candidates to intensive or investigational treatments. However, more information is warranted from prospective clinical trials to define the real impact of *NOTCH1* mutations in CLL patients.

Overall, patients with *NOTCH1*-mutated CLL had poor outcome in terms of OS, which is in agreement with previous reports.[4,8] Rossi *et al*.[8] have recently observed that the impact of *NOTCH1* mutations in OS is independent from *IGHV*. However, we could not confirm this finding in our study, in accordance with a previous smaller series.[20] The small group of patients with *NOTCH1*-mutated *IGHV*-mutated in our series behaved as low-risk CLL, in sharp contrast with the poor prognosis observed by Rossi *et al*.[8]

Transformation to DLBCL is an evolving event of CLL that occurs in 0.5–1% of the patients per year. The development of DLBCL, that confers an ominous prognosis to patients, has been associated with unmutated and stereotyped immunoglobulin genes, trisomy 12, del(11)(q22.3), mutations of *TP53* and *CDKN2A*, among others.[25–28] Expanding our initial previous observation,[4] the results of the current study demonstrate that *NOTCH1* mutation is one of the most important predictors of DLBCL development, with more than 30% of those patients having developed DLBCL at 10 years of diagnosis. Rossi *et al*.[8,29] have reported a similar risk of transformation in *NOTCH1*-mutated patients. In addition, we have observed that the higher risk of DLBCL conferred by *NOTCH1* mutation is independent from the *IGHV* status. Acquisition of *NOTCH1* mutation has been observed in some patients at transformation to DLBCL.[6] None of our patients with sequential or simultaneous sample from non-transformed and transformed tissue had differences in *NOTCH1*. The presence of subclones with *NOTCH1* mutation[6] together with technical reasons could account for the discrepancies.

The molecular mechanisms by which *NOTCH1* mutation confers higher risk of transformation and bad response to treatment are unknown. In our previous study, we showed that the truncated NOTCH1 protein encoded by mutated *NOTCH1* is more stable, accumulates in the cell and activates the downstream *NOTCH1* signaling pathway.[4] NOTCH1 activation induces several cellular functions, including the activation of PI3K/Akt, MYC and NFkB signaling pathways, that promote cell proliferation, survival and angiogenesis, which may be important for the aggressive behavior and frequent transformation of CLL cells.[30–35] The relevance of *NOTCH1* mutation in the CLL biology opens the possibility of designing specific new therapeutic strategies for these patients.[36–38]

In summary, we have shown that *NOTCH1* mutation is a genetic marker that defines a high-risk group of CLL patients characterized by high risk of transformation and poor outcome. Although this newly identified subgroup of patients only represents a 10% of the whole CLL population, their increase risk in developing DLCBL and dismal prognosis deserves specific investigation in prospective clinical trials.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTIONS

LC, MC, AN, MP, VQ, XSP and CL-O performed sequencing analysis. MR, NV, MG-D, JMH, BN, E Colado and E Campo reviewed the pathological data and confirmed the diagnosis. DC, CL, SB carried out genetic and biological studies. AM-T, TB, JD, EG, PA, CR, ARP, MJT, FB and AL-G reviewed clinical data. MA prepared and supervised the bioethics requirements. AP contributed to and critically reviewed statistical analysis. E Campo and AL-G directed the research. NV, E Campo and AL-G wrote the manuscript, which all the authors approved.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)