Mutations of *NOTCH1* are an independent predictor of survival in chronic lymphocytic leukemia

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Analysis of the chronic lymphocytic leukemia (CLL) coding genome has recently disclosed that the *NOTCH1* proto-oncogene is recurrently mutated at CLL presentation. Here, we assessed the prognostic role of *NOTCH1* mutations in CLL. Two series of newly diagnosed CLL were used as training (n = 309) and validation (n = 230) cohorts. *NOTCH1* mutations occurred in 11.0% and 11.3% CLL of the training and validation series, respectively. In the training series, *NOTCH1* mutations led to a 3.77-fold increase in the hazard of death and to shorter overall survival (OS; *P* < .001). Multivariate analysis selected *NOTCH1* mutations as an independent predictor of OS after controlling for confounding clinical and biologic variables. The independent prognostic value of *NOTCH1* mutations was externally confirmed in the validation series. The poor prognosis conferred by *NOTCH1* mutations was attributable, at least in part, to shorter treatment-free survival and higher risk of Richter transformation. Although *NOTCH1* mutated patients were devoid of *TP53* disruption in more than 90% cases in both training and validation series, the OS predicted by *NOTCH1* mutations was similar to that of *TP53* mutated/deleted CLL. *NOTCH1* mutations are an independent predictor of CLL OS, tend to be mutually exclusive with *TP53* abnormalities, and identify cases with a dismal prognosis. (*Blood*. 2012;119(2): 521-529)

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults.[1-4] The clinical course of CLL ranges from very indolent, with a nearly normal life expectancy,[5-9] to rapidly progressive leading to death and occasionally undergoing transformation to aggressive lymphoma, known as Richter syndrome (RS).[10-18]

At presentation, several clinical and biologic features may help to predict, at least in part, the clinical course of CLL.[19-21] Of the biologic prognosticators that have been developed, current guidelines for clinical practice recommend screening only for *TP53* disruption by mutation, deletion, or both of the locus, that identifies a fraction of high-risk CLL destined to experience a very short survival.[2,21-28] High-risk CLL, however, cannot be fully recapitulated by *TP53* disruption, and other lesions of cancer genes may be implicated in this aggressive phenotype.[29]

Recently, two independent investigations of the CLL coding genome have revealed that activating mutations of the *NOTCH1* proto-oncogene are recurrently associated with CLL.[30,31] Based on current knowledge, *NOTCH1* mutations occur in ~ 10% CLL at diagnosis and their frequency increases in advanced disease phases, as exemplified by the case of RS.[30,31] The relevance of *NOTCH1* mutations in CLL is reinforced by knowledge of activation of the *NOTCH1* pathway in this leukemia,[32] and by the possibility of targeting NOTCH1 with drugs currently under development in other clinical contexts.[33] Although not designed to fully assess clinical correlates, the pivotal studies that have identified *NOTCH1* mutations in CLL have provided initial evidence suggesting that *NOTCH1* alterations might be associated with an unfavorable clinical outcome.[30,31,34] However, several aspects of the clinical implications of *NOTCH1* mutations in CLL still remain to be elucidated, including: (1) their distribution among well established CLL genetic subgroups, including those defined by FISH abnormalities and *TP53* status; and (2) their independent prognostic role, given the tight association between *NOTCH1* mutations and unmutated immunoglobulin heavy variable (*IGHV*) genes, one of the most widely accepted prognosticators in CLL.

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By using a training-validation approach, we hereby report that *NOTCH1* mutations: (1) cluster with CLL harboring trisomy 12, suggesting that aberrant NOTCH signaling plays an important role in this genetic subgroup; (2) tend to be mutually exclusive with *TP53* disruption in the same patient; and (3) are an independent predictor of CLL overall survival (OS) because they identify a subset of high-risk patients with dismal prognosis similar to that associated with *TP53* abnormalities.

Patients and methods

Patients

The study used a training-validation design. The training cohort was a consecutive series of 309 previously untreated CLL who presented for initial evaluation at a single center. The training series was provided with prospectively collected biologic samples drawn at presentation and with a prospectively maintained clinical database updated in May 2010. Median follow-up of alive patients was 6 years. No patient was lost at follow-up. The validation cohort was represented by a retrospective series of 230 previously untreated CLL from 3 institutions participating to the same national CLL network. Inclusion criteria for the validation series were availability of: (1) biologic samples collected at presentation, and (2) clinical follow-up. Median follow-up of alive patients for the validation series was 7 years.

For sample size definition, we assumed a prevalence of *NOTCH1* mutations at presentation of at least 10% and a 5-year OS of 80% for the entire population. Based on these assumptions, the sample size would allow detection of at least 15% and 19% difference in 5-year OS for the training series and the validation series, respectively (power = 80%; $\alpha = .05$).

CLL diagnosis was based on International Working Group on CLL–National Cancer Institute criteria.[1,2] RS diagnosis was histologically proven and was represented by diffuse large B-cell lymphoma (clonally related to the CLL phase).[1,35]

The Reporting Recommendations for Tumor Marker Prognostic Studies criteria were followed throughout this study.[36] Patients provided informed consent in accordance with local institutional review board requirements and Declaration of Helsinki. The study was approved by the Ethical Committee of the Ospedale Maggiore della Carità di Novara associated with the Amedeo Avogadro University of Eastern Piedmont (protocol code 59/CE; study CE 8/11).

Molecular studies

NOTCH1, *TP53*, and *IGHV* mutations were analyzed by DNA Sanger sequencing.[17,26,31,37,38] *NOTCH1* c.7544_7545delCT mutation also was investigated by amplification refractory mutation system (ARMS) PCR. Probes (Abbott) used for FISH analysis were LSI13 and LSID13S319, CEP12, LSIp53, and LSIATM.[18,23] Molecular studies were performed on tumor samples collected from peripheral blood: (1) at CLL presentation for both the training (n = 309) and validation (n = 230) CLL series; and (2) at the time of first progression requiring treatment for progressive CLL that were treated with fludarabine-based regimens (n = 113). Details of molecular methods are in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Flow cytometry

CD38 and ZAP-70 expression was analyzed by flow cytometry on PBMCs collected at presentation. Cut-off points of 30% and 20% were used to define positivity for CD38 and ZAP-70, respectively. Details are reported in supplemental Methods.

Serum β2-microglobulin quantification

β2-microglobulin levels were quantified by nephelometry (Siemens Healthcare Diagnostics; reference range, 1.8-2.5 mg/L) on serum samples collected at presentation.

Statistical analysis

OS was measured from date of initial presentation to date of death (event) or last follow-up (censoring). Treatment-free survival (TFS) was measured from date of initial presentation to date of progressive and symptomatic disease requiring treatment according to International Working Group on CLL–National Cancer Institute guidelines (event), death, or last follow-up (censoring).[2] Time to RS transformation was measured from date of initial presentation to date of the biopsy documenting occurrence of RS transformation (event), death, or last follow-up (censoring).[17] OS from first line treatment was assessed among cases treated with fludarabine-based regimens (n = 113) and was measured from date of treatment start to date of death (event) or last follow-up (censoring). Survival was estimated by Kaplan–Meier method.[39] The crude association between exposure variables and outcome was estimated by univariate Cox regression analysis.[40] The independence of *NOTCH1* mutations as a predictor of CLL OS was estimated after controlling for confounding variables by multivariate Cox regression analysis.[40-42]

Covariates included in the multivariate analysis along with *NOTCH1* mutation status were selected according to the following criteria: (1) wide acceptance as clinical or biologic prognosticators in CLL, (2) availability of the information in both the training and validation series, and (3) limitation of the number of predictors to no more than ~ 1/10 uncensored events to avoid overfitting.[41,42] Based on these criteria, the following variables were included in multivariate analysis: *NOTCH1* mutations (present vs absent), age (continuous variable), sex (male vs female), Rai stage (III-IV vs 0-II), *IGHV* identity ≥ 98% (present vs absent), trisomy 12 (present vs absent), 11q22-q23 deletion (present vs absent), and *TP53* disruption by mutation, deletion, or both (present vs absent). None of the covariates violated the proportional hazard assumption as documented by plotting the smoothed Schoenfeld residuals and by performing a correlation test between time and residuals.[41-43] The assumption of effect additivity of predictors was not violated, as documented by a global test of additivity including interactions between *NOTCH1* mutations and other covariates.[41,42] None of the covariates showed colinearity.[41,42] Age was treated as a continuous variable and did not violate the linearity assumption as assessed by plotting the smoothed martingale residuals.[41,42,44]

The prediction accuracy of the multivariate model was verified by assessing model discrimination and calibration (see supplemental Methods for details).[41,42,45] The stability and predictive performance of *NOTCH1* mutations as an independent predictor of CLL OS was validated both internally in the training series and externally in an independent validation series. Internal validation was performed using a bootstrapping resampling procedure (see supplemental Methods for details).[41,42,46] The more general validity of *NOTCH1* mutations as an independent predictor of CLL OS was tested using an external validation approach. In this step, Cox regression was applied to an independent validation cohort that included *NOTCH1* mutations and the confounding variables also tested in the training series.

Recursive-partitioning analysis for censored survival data was performed to hierarchically classify CLL patients into risk categories based on *NOTCH1* and *TP53* status.[47] Categorical variables were compared by χ^2 test and Fisher exact test when appropriate. Continuous variables were compared by Mann-Whitney test. All statistical tests were 2-sided. Statistical significance was defined as P value $< .05$. The analysis was performed with the Statistical Package for the Social Sciences Version 18.0 software (SPSS) and with R statistical package 2.13.0 (http://www.r-project.org).

Results

Frequency and distribution of *NOTCH1* mutations in the training series

The training series (n = 309) was representative of the main clinical and biologic characteristics of CLL (Table 1). *NOTCH1* mutations (n = 34, all heterozygous) occurred in 34/309 (11.0%) patients, being mostly represented (26/34, 76.5%) by a recurrent

Table 1. Characteristics of the CLL training series according to *NOTCH1* mutation status

	All (n = 309)		*NOTCH1* wild type (n = 275)		*NOTCH1* mutated (n = 34)		
Characteristic	**n***	**%**	**n***	**%**	**n***	**%**	***P***
Age, y (range)	69 (60-76)		69 (60-76)		69 (60-76)		.615
Male	170	55.0	152	55.3	18	52.9	.797
Rai stage III-IV	34	11.0	25	9.1	9	26.5	.006
IGHV identity ≥ 98%	103	33.3	77	28.0	26	76.5	< .001
13q14 deletion	160	51.8	151	54.9	9	26.5	.002
Normal FISH	92	29.8	81	29.5	11	32.4	.727
Trisomy 12	61	19.7	46	16.7	15	44.1	< .001
11q22-q23 deletion	25	8.1	24	8.7	1	2.9	.334
17p13 deletion	25	8.1	24	8.7	1	2.9	.334
TP53 mutations	23	7.4	21	7.6	2	5.9	1.000
TP53 disruption	33	10.7	30	10.9	3	8.8	1.000

*Median and 25th-75th percentiles are reported for continuous variables.

2-bp frameshift deletion (c.7544_7545delCT). The remaining *NOTCH1* mutations (8/34, 23.5%) were frameshift deletions other than c.7544_7545delCT (n = 7) and frameshift insertions (n = 1; supplemental Table 1). All mutations were predicted to disrupt the NOTCH1 PEST domain.

The clinical and biologic features of *NOTCH1* mutated CLL are summarized in Table 1. CLL with *NOTCH1* mutations preferentially carried unmutated *IGHV* genes (76.5%; $P < .001$). Other characteristics at presentation associated with *NOTCH1* mutations were advanced Rai stage and trisomy 12 (Table 1). Consistent with the mutually exclusive distribution of trisomy 12 and 13q14 deletion,[23] *NOTCH1* mutated CLL were less frequently deleted on 13q14 (Table 1).

NOTCH1 mutations are an independent prognosticator of OS in the training series

After a median follow-up of 6 years, 135/309 patients from the training series had received treatment, 19/309 had developed RS and 78/309 had died, accounting for a median TFS of 7.1 year (95% confidence interval [CI], 4.5-9.8), a 5-year risk of RS of 7.9% (95% CI, 4.4-11.4), and a median OS of 13.0 years (95% CI, 10.2-15.9).

By univariate analysis, the crude impact of *NOTCH1* mutations on survival was an ~ 3.8-fold increase in the hazard ratio (HR, 3.77; 95% CI, 2.14-6.66) and a significant OS shortening ($P < .001$; Table 2; Figure 1A) that occurred irrespective of the *NOTCH1* mutation type (c.7544_7545delCT, $P < .001$; other mutations, $P = .009$; supplemental Figure 1). Other variables associated with shorter OS were age, Rai stage, *IGHV* mutation status, trisomy 12, 11q22-q23 deletion, and *TP53* disruption (Table 2; supplemental Figure 2).

The adjusted impact of *NOTCH1* mutations on OS was estimated after controlling for confounding variables by multivariate Cox regression analysis. Along with *NOTCH1* mutations, other variables included in the analysis were: (1) those known a priori to be widely accepted clinical (age and Rai stage)[3-14] and genetic (*IGHV* mutation status, 11q22-q23 deletion, and *TP53* disruption)[20-28,37,38] risk factors affecting CLL OS; (2) trisomy 12, given its double association with *NOTCH1* mutations and OS in this series (Tables 1-2); and (3) sex.

Table 2. Univariate and multivariate analysis for overall survival in the CLL training series

																Internal bootstrapping validation	
			OS, y			Univariate analysis				Multivariate analysis*†‡				Bootstrap parameter, mean			Bootstrap selection, %
Characteristics	**Event**	**Total**	**Median**	**LCI**	**UCI**	**HR**	**LCI**	**UCI**	***P***	**HR**	**LCI**	**UCI**	***P***	**HR**	**LCI**	**UCI**	
Age§						1.06	1.03	1.08	< .001	1.07	1.04	1.10	< .001	1.07	1.05	1.10	100
Female	29	139	13.9	10.8	17.0												
Male	49	170	12.2	8.0	16.5	1.53	.096	2.43	.069	1.96	1.20	3.20	.007	2.17	1.28	3.68	91.1
NOTCH1 germline	62	275	13.9	10.5	17.3												
NOTCH1 mutations	16	34	3.5	0	7.3	3.77	2.14	6.66	< .001	3.99	2.05	7.76	< .001	4.55	2.23	9.31	98.0
Rai stage 0-II	55	275	15.6	13.1	18.7												
Rai stage III-IV	23	34	6.0	2.5	9.6	3.99	2.44	6.50	< .001	2.33	1.25	3.99	.007	2.57	1.38	4.82	90.0
IGHV identity < 98%	42	206	NR														
IGHV identity ≥ 98%	36	103	11.7	6.3	17.2	2.23	1.42	3.52	.001	1.44	0.83	2.50	.191	1.53	0.86	2.71	50.4
No trisomy 12	52	248	NR														
Trisomy 12	26	61	10.8	5.5	16.0	1.93	1.21	3.01	.006	1.50	0.91	2.64	.108	1.62	0.96	2.74	55.4
No 11q22-q23 deletion	65	284	15.6	13.3	17.9												
11q22-q23 deletion	13	25	6.8	0	14.1	2.25	1.23	4.11	.008	1.57	0.76	3.27	.220	1.77	0.81	3.90	47.4
TP53 germline	61	276	13.9	10.5	17.3												
TP53 disruption	17	33	4.6	1.8	7.3	3.74	2.16	6.49	< .001	3.27	1.80	5.95	< .001	3.38	1.78	6.43	96.5

LCI indicates 95% lower confidence interval; UCI, 95% upper confidence interval; and NR, not reached.

*Shrinkage coefficient, 0.91.

†Discrimination: c-index of the original model, 0.792; bias-corrected c-index, 0.015; and optimism, 0.777.

‡Calibration: calibration slope of the original model, 1.000; bias-corrected calibration slope, 0.910; and optimism, 0.090.

§Age was treated as a continuous variable.

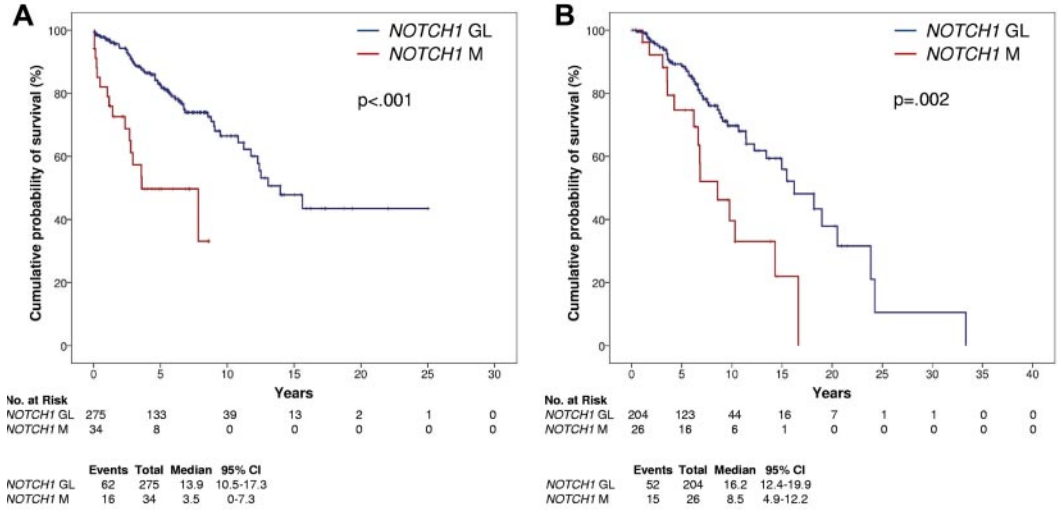

Figure 1. Kaplan-Meier estimates of OS according to *NOTCH1* mutation status. Overall survival according to *NOTCH1* mutation status in the CLL training series (n = 309; A) and in the CLL validation series (n = 230; B). *NOTCH1* germ line cases (*NOTCH1* GL) are represented by the blue line. *NOTCH1* mutated cases (*NOTCH1* M) are represented by the red line.

Multivariate analysis selected *NOTCH1* mutations as an independent risk factor of OS (HR, 4.22; 95% CI, 2.15-8.28; $P < .001$; Table 2). The inclusion of *NOTCH1* mutations in addition to age, sex, Rai stage, *IGHV* mutation status, trisomy 12, 11q22-q23 deletion, and *TP53* disruption significantly improved the fit (-2LL of the model without *NOTCH1* mutations, 692 vs -2LL of the model with *NOTCH1* mutations, 677; likelihood ratio statistics, 14.5; $P < .001$) and the predictive accuracy (c-index of the model without *NOTCH1* mutations, 0.768 vs c-index of the model with *NOTCH1* mutations, 0.792; $P < .001$) of the model. The *IGHV* mutation status that was significant in the model without *NOTCH1* mutations (supplemental Table 2) was no longer retained as an independent prognosticator of OS after inclusion of *NOTCH1* mutations. By bivariate analysis, the *IGHV* mutation status maintained its prognostic relevance in CLL devoid of *NOTCH1* mutations (supplemental Figure 3).

The stability and predictive performance of *NOTCH1* mutations as an independent prognostic factor of CLL OS was internally validated in the training series using a bootstrapping resampling procedure. The first step of the internal validation showed that *NOTCH1* mutations were selected at high frequency (> 98.3%) as an independent prognosticator of CLL OS in each of the 1000 bootstrap samples that were generated (Table 2). This step validated *NOTCH1* mutations as one of the most important variables affecting OS in the training series. The second step of the internal validation demonstrated that the hazard ratios produced from the original series were very close to those produced from the 1000 bootstrap samples (Table 2).

An exploratory analysis applied only to the training series demonstrated that *NOTCH1* mutations maintained their independent prognostic role also after adjusting for β2-microglobulin levels, ZAP-70 expression, and CD38 expression (supplemental Table 3).

NOTCH1 mutations predict an increased risk of CLL progression, RS transformation, and short survival after treatment

At CLL diagnosis, *NOTCH1* mutations identified CLL patients with rapidly progressive disease and patients at risk of RS development. In fact, patients from the training series carrying *NOTCH1* mutations displayed a shorter time to progression requiring treatment compared with patients without *NOTCH1* mutations ($P < .001$; Figure 2A). In addition, *NOTCH1* mutated patients from the training series displayed a higher cumulative probability of RS compared with patients without *NOTCH1* mutations ($P = .026$; Figure 2B), that occurred irrespective of the *NOTCH1* mutation type.

In CLL patients treated with fludarabine-based regimens at first progression requiring treatment, *NOTCH1* mutations occurred in 23/113 (20.4%) cases (supplemental Table 4) and associated with an OS from treatment similar to that marked by *TP53* disruption ($P = .600$) and significantly shorter compared with that of patients lacking both *NOTCH1* and *TP53* lesions ($P = .041$; Figure 3).

NOTCH1 mutations are an independent prognosticator of OS in the validation series

The prognostic value of *NOTCH1* mutations as a risk factor was externally validated in an independent CLL series (n = 230; supplemental Table 5). *NOTCH1* mutations (n = 26, all heterozygous) occurred in 26/230 (11.3%) patients and affected in all cases the PEST domain, with a mutational spectrum similar to that of the training series (c.7544_7545delCT, 21/26 [80.7%]; other mutations, 5/26 [19.3%]; supplemental Table 1). Survival analysis in the validation series confirmed that *NOTCH1* mutations represent an adverse prognostic factor in CLL. By univariate analysis, *NOTCH1* mutated patients were confirmed to display a significantly shorter OS compared with *NOTCH1* germ line patients ($P = .002$; Figure 1B). Similarly to the training series, also in the validation series *NOTCH1* mutations predicted poor OS irrespective of mutation type (supplemental Figure 1). By multivariate analysis, *NOTCH1* mutations were selected as an independent risk factor of OS also in the validation series (HR, 2.15; 95% CI, 1.13-4.11; $P = .019$), after controlling for the same covariates applied to the training series.

CLL with *NOTCH1* mutations have a poor prognosis similar to CLL with *TP53* disruption

NOTCH1 mutations and *TP53* disruption tended to distribute in a mutually exclusive fashion in both the training and validation

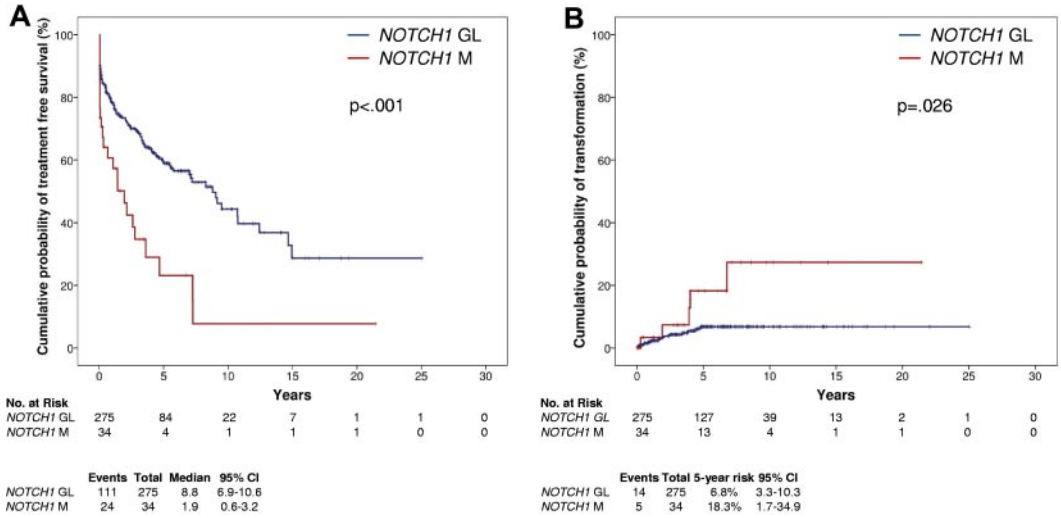

Figure 2. Kaplan-Meier estimates of TFS and cumulative probability of transformation to RS according to *NOTCH1* mutation status. TFS according to *NOTCH1* mutation status (A) and cumulative probability of transformation to RS (B) in the CLL training series (n = 309). *NOTCH1* germ line cases (*NOTCH1* GL) are represented by the blue line. *NOTCH1* mutated cases (*NOTCH1* M) are represented by the red line.

series (Figure 4A-B). Patients harboring *NOTCH1* mutations were devoid of *TP53* disruption in 31/34 (91.2%) and in 25/26 (96.2%) cases of the training and validation series, respectively.

Because *TP53* disruption identifies patients with the shortest survival in CLL,[22-28] the outcome of *NOTCH1* mutated cases was compared with that of patients with *TP53* disruption. Cases harboring both *NOTCH1* and *TP53* lesions were compiled to cases harboring only *TP53* disruption, because of the low number (3 in the training series and 1 in the validation series) of double mutated cases, and based on a recursive partitioning analysis for risk of death. In both the training and validation series, CLL harboring *NOTCH1* mutations displayed an OS similar to that of CLL harboring *TP53* disruption (Figure 4C-D). The results were superimposable also when the few double mutated cases were analyzed as a separate subgroup (supplemental Figure 4).

ARMS is a useful tool for *NOTCH1* mutation screening

A PCR-based test was designed to detect the c.7544_7545delCT mutation that accounts for ~ 80% of *NOTCH1* mutations in CLL. ARMS was calibrated to detect a mutation present in great than or equal to 10% alleles, approximating the sensitivity of DNA Sanger sequencing, and was applied in blind to the training series. Under these conditions, ARMS showed a 100% sensitivity and specificity in detecting *NOTCH1* c.7544_7545delCT ($\kappa = 1$). In fact, all 26 CLL from the training series harboring c.7544_7545delCT by DNA Sanger sequencing scored positive by ARMS, whereas all 283 CLL lacking c.7544_7545delCT by DNA Sanger sequencing scored negative by ARMS (Figure 5). By survival analysis, CLL that scored positive by ARMS for c.7544_7545delCT showed a shorter OS compared with negative patients ($P < .001$; Figure 5). These results confirm that ARMS is a useful tool for *NOTCH1* c.7544_7545delCT mutation screening.

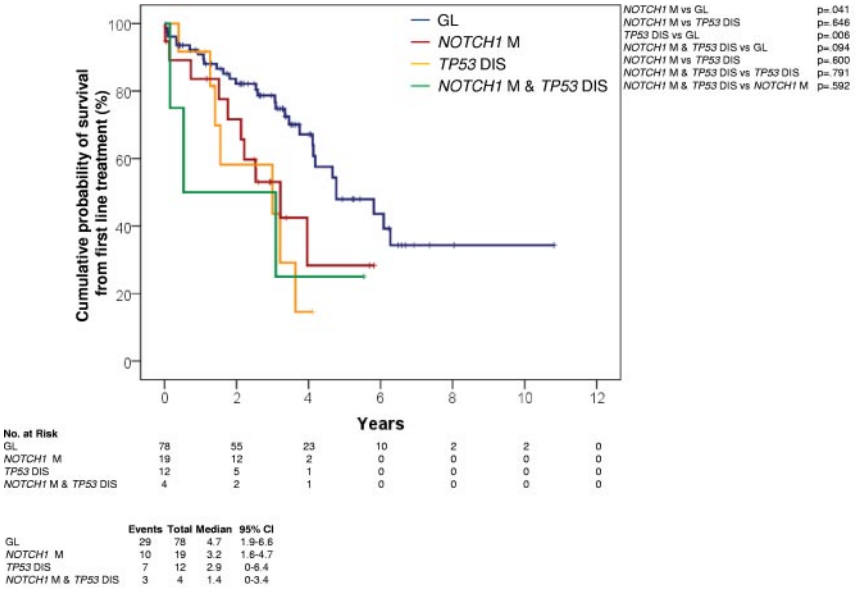

Figure 3. Kaplan-Meier estimates of OS from first-line treatment. OS from first line treatment according to *NOTCH1* mutation status in CLL treated with fludarabine-based regimens series (n = 113). Cases with germ line *NOTCH1* and *TP53* genes (GL) are represented by the blue curve. Cases harboring *NOTCH1* mutations without *TP53* disruption (*NOTCH1* M) are represented by the red curve. Cases harboring *TP53* disruption without *NOTCH1* mutations (*TP53* DIS) are represented by the yellow curve. Cases harboring both *NOTCH1* mutations and *TP53* disruption (*NOTCH1* M and *TP53* DIS) are represented by the green curve.

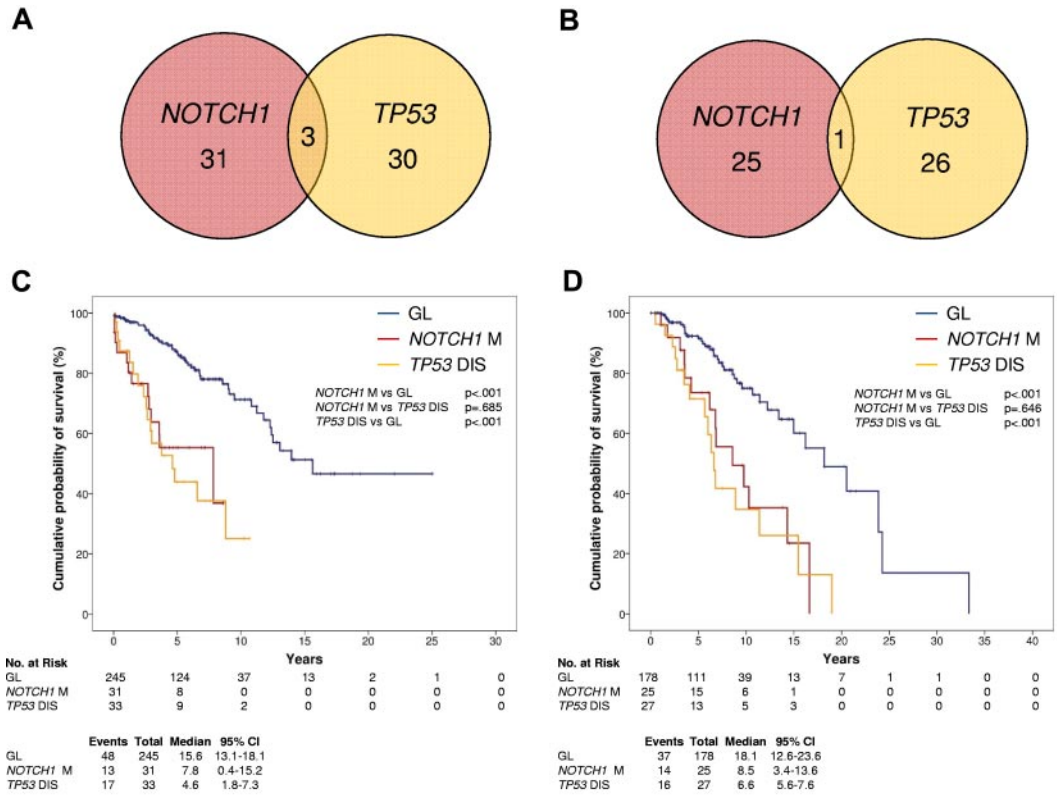

Figure 4. Comparative analysis of *NOTCH1* mutations and *TP53* disruption. (A-B) Venn diagram illustrating the overlap between *NOTCH1* mutations and *TP53* disruption by mutations, deletion, or both in the CLL training series (A) and in the CLL validation series (B). Numbers within the red and yellow circles indicate the number of cases harboring *NOTCH1* mutations without *TP53* disruption (red circle) and cases with *TP53* disruption without *NOTCH1* mutations (yellow circle). Numbers within the overlaps between circles indicate the number of cases harboring both *NOTCH1* mutations and *TP53* disruption. (C-D) Kaplan–Meier estimates of OS according to *NOTCH1* mutation and *TP53* disruption in the CLL training series (n = 309; C) and in the CLL validation series (n = 230; D). Cases with germ line *NOTCH1* and *TP53* genes (GL) are represented by the blue line. Cases harboring *NOTCH1* mutations without *TP53* disruption (*NOTCH1* M) are represented by the red line. Cases harboring *TP53* disruption (*TP53* DIS) are represented by the yellow line. Cases harboring both *NOTCH1* and *TP53* disruption were compiled to cases harboring only *TP53* disruption, because of the low number (3 in the training series and 1 in the validation series) of double-mutated cases, and based on a recursive partitioning analysis for risk of death.

Timing of *NOTCH1* mutations and relationship with *TP53* disruption in high-risk CLL

Paired sequential samples were tested in selected cases of high-risk CLL. *NOTCH1* mutations were acquired at chemorefractoriness in 1/4 cases and at RS transformation in 4/11 (supplemental Table 6).

The relationship between *NOTCH1* mutations and *TP53* disruption was investigated in high-risk CLL, including fludarabine-refractory CLL (n = 59) and RS (n = 26; Figure 6). At the time of fludarabine-refractoriness, *NOTCH1* mutations and *TP53* disruption overlapped in 11.8% (7/59) of cases (Figure 6B). Consistent with the whole CLL series, *NOTCH1* mutations and *TP53* disruption distributed in a mutually exclusive manner also in CLL that subsequently transformed to RS (Figure 6A). However, on RS transformation, *NOTCH1* mutations and *TP53* disruption occurred simultaneously in a fraction of patients as documented by: (1) acquisition of *TP53* disruption in 2 RS that already harbored *NOTCH1* mutations in the CLL phase; and (2) acquisition of both *NOTCH1* mutations and *TP53* disruption in 3 RS devoid of these alterations in the CLL phase (Figure 6A).

Discussion

The current study on 539 CLL documents that *NOTCH1* mutations: (1) represent one of the most frequent cancer gene mutations known to be involved at CLL presentation; (2) among CLL genetic subgroups, cluster with cases harboring trisomy 12 and tend to be mutually exclusive with *TP53* disruption; (3) identify a high-risk subgroup of patients showing poor survival similar to that associated with *TP53* abnormalities; and (4) exert a prognostic role independent of widely accepted clinical and genetic risk factors, and in series from different institutions, as documented by the training-validation approach chosen for the design of this study.

Of the biologic predictors of CLL identified to date,[19-21] *TP53* disruption is the sole risk factor consistently associated with high-risk patients.[22-29] The genetics of high-risk CLL, however, is not fully recapitulated by *TP53* disruption, because 40% to 50% high-risk CLL are devoid of *TP53* abnormalities.[29] Conceivably, other genetic lesions may drive CLL aggressiveness. This study is consistent with a role of *NOTCH1* mutations in contributing to CLL clinical aggressiveness, because these genetic alterations identify patients whose survival is similar to that associated with *TP53* disruption.

The role of *NOTCH1* mutations in determining CLL aggressiveness is independent of the effect exerted by *TP53* disruption. In fact, at presentation, *NOTCH1* mutations in both the training and validation series tend to distribute in a mutually exclusive manner with *TP53* disruption. Consistently, the impact of *NOTCH1* mutations on CLL survival is independent of *TP53* disruption by multivariate analysis. The scenario observed in CLL differs from that of RS, in which mutations of *NOTCH1* associate with *TP53*

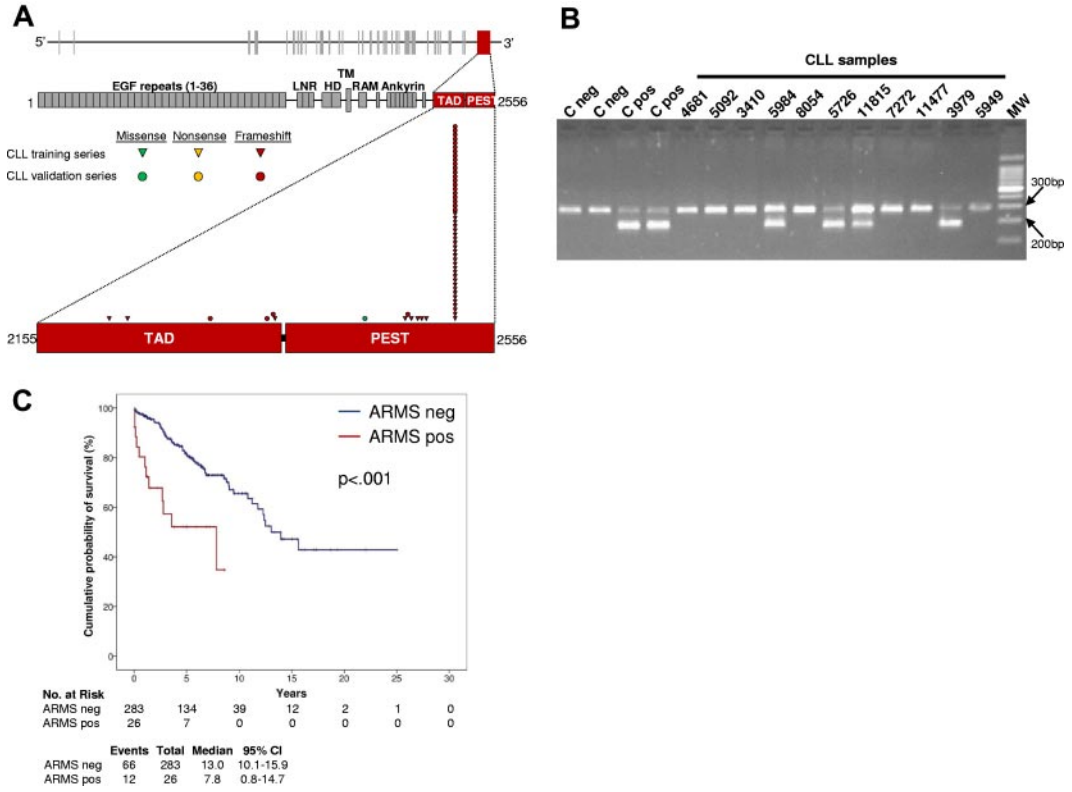

Figure 5. ARMS to detect *NOTCH1* c.7544_7545delCT mutation. (A) Schematic diagram of the *NOTCH1* gene (top) and protein (bottom), with its conserved functional domains (EGF-like repeats: LNR, LIN-12/NOTCH repeats; HD, heterodimerization; TM, transmembrane; Ankyrin repeats: TAD, transactivation domain; PEST, proline, glutamic acid, serine, threonine sequence). The TAD domain and the PEST sequence, both coded by exon 34, are magnified. Color-coded shapes indicate the position of the mutations found in the CLL training series (n = 34) and in the CLL validation series (n = 26). (B) Representative results of the ARMS assay showing 4 CLL samples that scored positive for the c.7544_7545delCT mutation (codes 5984, 5726, 11815, 3979) and 7 CLL samples that scored negative for the c.7544_7545delCT mutation (codes 4681, 5092, 3410, 8054, 7272, 11477, 5949). Negative samples show a normal band of 284 bp. Positive samples show an additional mutant band of 183 bp. Negative (C neg) and positive (C pos) controls also are included. Molecular weight (MW) is the 100-bp DNA ladder. Camera: Gel Doc 1000, BioRad; image acquisition software: Quantity One 4.5.0, BioRad. (C) Kaplan-Meier estimates of overall survival according to the results of the ARMS assay in the CLL training series (n = 309). Cases that scored negative by ARMS for the *NOTCH1* c.7544_7545delCT mutation (ARMS neg) are represented by the blue line. Cases that scored positive by ARMS for the *NOTCH1* c.7544_7545delCT mutation (ARMS pos) are represented by the red line.

disruption in 50% of the patients (Fabbri et al[31]; this study). A likely explanation is that the concomitant occurrence of *NOTCH1* mutations and *TP53* disruption in the same CLL clone causes further clinical aggressiveness and, potentially, histologic transformation to aggressive lymphoma.

The pivotal studies on *NOTCH1* mutations in CLL have provided initial evidence that *NOTCH1* alterations might be associated with an unfavorable clinical outcome.[30,31,34] However, these studies were based on small CLL series,[34] used TFS as surrogate clinical end point,[34] and lacked a formal demonstration that the clinical effect of *NOTCH1* mutations is reproducible and independent of confounders.[30,31] Our results add to the current knowledge on the clinical aspects of *NOTCH1* mutations in CLL by demonstrating the robustness and reproducibility of these genetic alterations as a risk factor, thus providing a new tool for the early identification of high-risk patients.

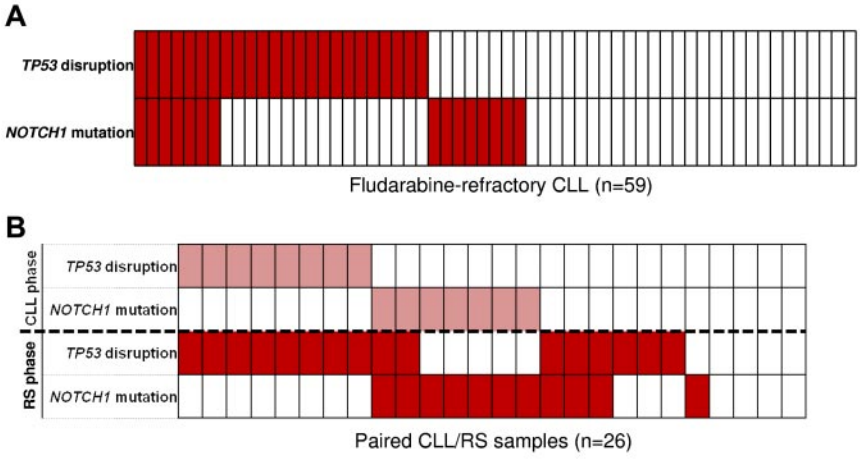

Figure 6. Timing of *NOTCH1* mutations and relationship with *TP53* disruption in high-risk CLL. (A) *NOTCH1* mutations and *TP53* disruption in fludarabine-refractory CLL. In the heatmap, rows correspond to the *NOTCH1* and *TP53* genes, and columns represent individual patients color-coded based on the gene status (white, wild type; red, mutations of *NOTCH1* and disruption of *TP53*). (B) *NOTCH1* mutations and *TP53* disruption in sequential CLL/RS samples. In the heatmap, rows correspond to the *NOTCH1* and *TP53* genes. Columns represent individual patients color-coded based on the gene status (white, wild type; pink, mutations of *NOTCH1* and disruption of *TP53* in the CLL phase; red, mutations of *NOTCH1* and disruption of *TP53* at RS transformation).

Different mechanisms might explain, at least in part, the poor prognosis associated with *NOTCH1* mutations in CLL. First, *NOTCH1* mutations lead to the acquisition of a progressive clinical phenotype that mandates treatment shortly after initial presentation, as documented by a median TFS of ~ 2 years for mutated cases versus ~ 9 years for *NOTCH1* germ line patients. Second, our actuarial analysis indicates that *NOTCH1* mutated patients display a higher risk of developing RS, a condition that is frequently lethal and recurrently harbors *NOTCH1* mutations that, importantly, are present already at the time of CLL presentation in a significant fraction of RS patients.[15-18,31] A potential association of *NOTCH1* mutations with chemorefractoriness may further explain the poor outcome associated with *NOTCH1* alterations. Although the relationship between *NOTCH1* mutations and response to treatment needs to be formally tested within clinical trials, indirect evidence for this hypothesis comes from the observation that *NOTCH1* mutations are enriched among chemorefractory CLL patients[31] and that *NOTCH1* activation in vitro confers resistance to apoptosis through NF-κB pathway activation.[32]

The external validation approach exploited in the current study documents that *NOTCH1* mutations are an independent prognostic factor retaining its predictive value in CLL followed at different institutions. This observation suggests that the prognostic value of *NOTCH1* mutations, though detected retrospectively, is independent of a potential bias because of patient referral or patient management at a single center.[42] The general validity of *NOTCH1* mutations as a prognosticator in CLL is further supported by the consistent association of *NOTCH1* mutations with poor outcome in all series tested to date by independent investigators, although previous studies were not designed for a comprehensive survival analysis, or included a limited number of patients.[30,31,34] Confirmation within the frame of prospective clinical trials will be helpful to fully assess the generalization of *NOTCH1* mutations as a prognostic marker in CLL.

Consistent with the mutational spectrum of *NOTCH1* in CLL, all mutations disrupted the C-terminal PEST domain that in normal conditions is required to limit the intensity and duration of *NOTCH1* activation.[33,48,49] Removal of the PEST domain results in NOTCH1 impaired degradation and accumulation of an active NOTCH1 isoform sustaining deregulated signaling.[30] A practically important feature of the *NOTCH1* mutational spectrum is that one single recurrent mutation, c.7544_7545delCT, accounts for ~ 80% of all *NOTCH1* mutations detectable in CLL. The high recurrence of c.7544_7545delCT in CLL has prompted the design of a simple PCR-based strategy for its rapid detection. This assay allows the reliable detection of all cases harboring the c.7544_7545delCT mutation, translates into prognostically meaningful results, and might provide a potentially helpful approach for a first-level screening of *NOTCH1* alterations avoiding the need of DNA sequencing procedures. In addition to prognostic implications, *NOTCH1* mutations might also provide a therapeutic target for *NOTCH1* inhibitors that are currently under development in other clinical contexts[33,49] and that prompt future studies of molecular therapy for *NOTCH1*-mutated CLL patients.

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Authorship

Contribution: D.R., R.R., L.P., R.D.-F., R. Foà, and G.G. designed the study, interpreted data, and wrote the manuscript; D.R., M.F., and P.B. performed statistical analysis; S.R., V.S., A.B., M.C., S.C., and R. Famà performed molecular analysis; S.M. performed FISH analysis; L.D.P, F.F., L.L., and R.M. provided well characterized biological samples and clinical data; G.F., V.G., A.G., and S.D. contributed to interpretation of biological data; and D.C. was in charge of the ARMS assay.

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