NOTCH1 mutations in +12 chronic lymphocytic leukemia (CLL) confer an unfavorable prognosis, induce a distinctive transcriptional profiling and refine the intermediate prognosis of +12 CLL

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ABSTRACT

Trisomy 12, the third most frequent chromosomal aberration in chronic lymphocytic leukemia (CLL), confers an intermediate prognosis. In our cohort of 104 untreated patients carrying +12, *NOTCH1* mutations occurred in 24% of cases and were associated to unmutated *IGHV* genes (P=0.003) and +12 as a sole cytogenetic abnormality (P=0.008). *NOTCH1* mutations in +12 CLL associated with an approximately 2.4 fold increase in the risk of death, a significant shortening of survival (P<0.01) and proved to be an independent predictor of survival in multivariate analysis. Analogous to +12 CLL with *TP53* disruption or del(11q), *NOTCH1* mutations in +12 CLL conferred a significantly worse survival compared to that of +12 CLL with del(13q) or +12 only. The overrepresentation of cell cycle/proliferation related genes of +12 CLL with *NOTCH1* mutations suggests the biological contribution of *NOTCH1* mutations to determine a poor outcome. *NOTCH1* mutations refine the intermediate prognosis of +12 CLL.

Key words: chronic lymphocytic leukemia, *NOTCH1* mutations, trisomy 12, prognosis, gene expression profile.

Citation: Del Giudice I, Rossi D, Chiaretti S, Marinelli M, Tavolaro S, Gabrielli S, Laurenti L, Marasca R, Rasi S, Fangazio M, Guarini A, Gaidano G, and Foà R. NOTCH1 *mutations in +12 chronic lymphocytic leukemia (CLL) confer an unfavorable prognosis, induce a distinctive transcriptional profiling and refine the intermediate prognosis of +12 CLL. Haematologica 2012;97(3):437-441. doi:10.3324/haematol.2011.060129*

Introduction

Trisomy 12 represents the third most frequent chromosomal aberration in chronic lymphocytic leukemia (CLL) (15-20% of cases) and often (approx. 60% of cases) occurs as the sole cytogenetic lesion.[1,2] Within the hierarchical model of genetic subgroups commonly used in clinical practice, +12 as single aberration confers an intermediate prognostic risk, with a median time to progression of 33 months and a median overall survival (OS) of 114 months.[1]

The *NOTCH1* gene has been shown to have an essential biological role in hematopoiesis.[3] Following the pivotal study that identified *NOTCH1* mutations in CLL and provided initial evidence on the unfavorable clinical outcome associated with *NOTCH1* alterations,[4] two independent studies of the CLL coding genome have recently identified activating mutations of the *NOTCH1* gene in approximately 10% of CLL at diagnosis.[5,6] The prevalence of *NOTCH1* mutations increases with disease aggressiveness.[5] At diagnosis, *NOTCH1* mutations show an adverse impact on outcome, confirmed in at least four series,[4-7] and act independently of other clinico-biological features, including *TP53* disruption.[7] Among CLL cytogenetic subgroups, *NOTCH1* mutations are distributed in a mutually exclusive fashion with *TP53* disruption and are enriched in CLL carrying +12, where they recur in approximately 25% of patients.[7]

Based on the emerging association between *NOTCH1* alterations and +12, we investigated *NOTCH1* mutations in a series of untreated +12 CLL. We observed that in these patients, *NOTCH1* mutations: i) cluster within cases with no additional cytogenetic abnormalities; ii) induce a particular transcriptional profile; and iii) refine outcome prediction.

The online version of this article has a Supplementary Appendix.
**IDG, DR and SC contributed equally to this study. §GG and RF contributed equally to this study.*
Acknowledgments: this work was supported by AIRC (to RF) and AIRC, Special Program Molecular Clinical Oncology, 5 x 1000, n. 10007, Milan, Italy (to GG and to RF), Compagnia di San Paolo, Torino, Italy, Fondazione per le Biotecnologie, Torino, Italy, Fondazione Buzzati-Traverso, Pavia, Italy, Progetto FIRB-Programa "Futuro in Ricerca" 2008 (to DR), PRIN 2008 (to GG) and 2009 (to DR), MIUR, Rome, Italy, Progetto Giovani Ricercatori 2008 (to DR), Ministero della Salute, Rome, Italy, and Novara-AIL Onlus (to GG), Novara, Italy.
Manuscript received on December 12, 2011. Manuscript accepted on December 28, 2011.
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Design and Methods

Patients

This multicenter study evaluated 104 patients carrying +12: 54 were males and 50 females, with a median age of 65 years (interquartile range 56-72). All cases satisfied the IWCLL diagnostic criteria for CLL[8] and were selected on the basis of: i) untreated disease; ii) availability of biological material; and iii) presence of +12, independent of additional chromosomal abnormalities.

Patients gave their informed consent to blood collection and biological analyses, in agreement with the Declaration of Helsinki. The study was approved by the Ethical Committee of Policlinico Umberto I, "La Sapienza" University of Rome (n. 2182/16.06.2011) and of the Ospedale Maggiore della Carità, Novara, northern Italy, associated with the Amedeo Avogadro University of Eastern Piedmont (protocol code 59/CE; study n. CE 8/11).

Lymphocyte morphology, immunophenotype, FISH analysis, *IGHV* and *TP53* sequencing were performed as previously described.[9]

Mutation analysis of NOTCH1

The *NOTCH1* (exon 34; RefSeq NM_017617.2) mutation hotspot previously identified in CLL[7] was analyzed by direct sequencing of genomic DNA extracted from blood mononuclear cells. Purified amplicons were subjected to conventional DNA Sanger sequencing using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, USA). The presence of *NOTCH1* c.7544_7545delCT alleles was also investigated by ARMS PCR. Further details are reported in the *Online Supplementary Design and Methods.*

Statistical analysis

Overall survival (OS) was measured from the date of initial presentation to the date of death (event) or last follow up (censoring). Survival analysis was performed by the Kaplan-Meier method. Further details are reported in the *Online Supplementary Design and Methods.*

Gene expression profile analysis

For oligonucleotide array analysis, the HGU133 Plus 2.0 gene chips (Affymetrix, Santa Clara, CA, USA) were used. Sample preparation and microarray processing were performed as previously described.[10] Further details are reported in the *Online Supplementary Design and Methods*.

Results and Discussion

Frequency and distribution of NOTCH1 mutations in +12 CLL

NOTCH1 mutations occurred in 25 of the 104 untreated

Table 1A. Characteristics of CLL patients harboring +12 according to the *NOTCH1* mutation status.

Characteristics[a]	All (n=104)		*NOTCH1* wild type (n=79)		*NOTCH1* mutated (n=25)		
	n[b]	%	n[b]	%	n	%	*P*
Age >65 years	52	50	40	51	12	48	0.819
Male	54	52	43	54	11	44	0.363
Binet B-C	37	36	26	33	11	44	0.313
IGHV identity ≥98%	60	58	39	50	21	84	**0.003**
13q14 deletion	19	18	19	24	0	0	**0.006**
Trisomy 12 only	73	70	51	65	23	92	**0.008**
11q22-q23 deletion	8	8	6	8	2	8	1.000
17p13 deletion	5	5	4	5	1	4	1.000
TP53 mutations	4	4	2	3	2	8	0.243
TP53 disruption	8	8	5	6	3	12	0.395

[a]IGHV: *immunoglobulin heavy variable gene;* [b]IGHV: *rearrangement was not available for one* NOTCH1 *wild-type patient.*

Table 1B. Univariate and multivariate analysis for overall survival in CLL patients harboring +12[a].

Characteristics	Events	Total	OS (years) Median	LCI	UCI	Univariate analysis HR	LCI	UCI	*P*	Multivariate analysis HR	LCI	UCI	*P*
Age ≤65 years	15	51	15.6	10.4	20.7	-	-	-		-	-	-	
Age >65 years	21	52	6.7	3.6	9.9	2.92	1.44	5.92	0.003	6.03	2.60	13.95	<0.001
Binet A	19	69	15.6	9.7	21.4	-	-	-		-	-	-	
Binet B-C	17	36	7.8	5.5	10.0	2.47	1.26	4.82	0.008	2.40	1.17	4.89	0.016
IGHV identity <98%	12	43	15.6	9.2	21.9	-	-	-		-	-	-	
IGHV identity ≥98%	23	59	11.1	7.6	14.6	2.35	1.07	5.12	0.032	2.14	0.90	5.11	0.085
NOTCH1 wild-type	21	78	13.0	8.9	17.1	-	-	-		-	-	-	
NOTCH1 mutations	15	25	7.8	1.2	14.3	2.46	1.26	4.81	0.008	3.04	1.39	6.64	0.005
TP53 wild-type	30	95	13.0	10.5	15.5	-	-	-		-	-	-	
TP53 disruption	6	8	5.6	3.0	8.3	3.18	1.29	7.80	0.011	3.46	1.38	8.70	0.008

[a]*OS: overall survival; HR: hazard ratio; LCI: 95% lower confidence interval; UCI: 95% upper confidence interval;* IGHV: *immunoglobulin heavy variable gene.*

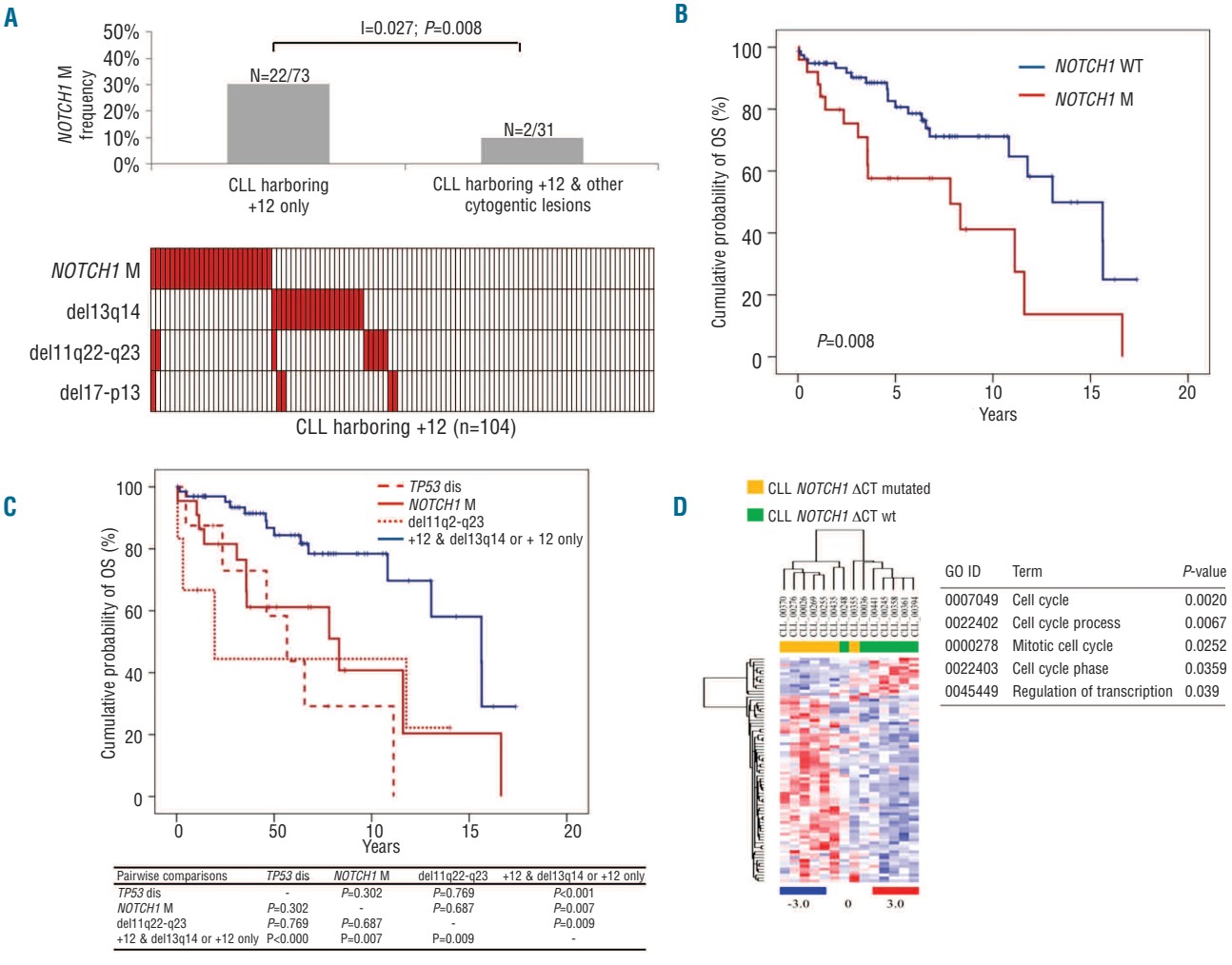

Figure 1. (A) **Distribution of *NOTCH1* mutations among genetic subgroups of +12 CLL.** (B) **Overall survival according to *NOTCH1* mutation status in +12 CLL.** (C) **Hierarchical stratification of overall survival according to genetic lesions in +12 CLL.** (D) **Comparison between *NOTCH1* mutated and *NOTCH1* wt CLL samples. Relative levels of gene expression are depicted with a color scale: red represents the highest level of expression and blue represents the lowest level. The table reports the functional annotation analysis, performed using the DAVID database, of differentially expressed genes between *NOTCH1* mutated and *NOTCH1* wt CLL. The biological processes reported are ordered according to their *P* value.**

CLL with +12 investigated (24%) (Table 1A), were represented in all cases by frameshift deletions, including the c.7544_7545delCT in 22 of 25 (88%) cases, and preferentially associated with use of unmutated *IGHV* genes (84%, *P*=0.003). *NOTCH1* mutations occurred independent of gender, thus suggesting that *NOTCH1* mutations might be an important marker of unfavorable prognosis in both male and female CLL patients.

Trisomy 12 occurred as an isolated chromosomal abnormality in 73 of 104 (70%) cases, while it was associated to other cytogenetic abnormalities in 31 of 104 (30%) cases (Figure 1A). Mutual information analysis revealed a clustering of *NOTCH1* mutations among CLL harboring +12 as a sole abnormality (22 of 73, 30%) compared to patients harboring +12 in addition to other cytogenetic lesions (2 of 31, 6%) (I=0.027; *P*=0.008) (Figure 1A). Consistently, +12 CLL harboring *NOTCH1* mutations carried deletion 13q14 only exceptionally (0 of 19). Consistent with pivotal observations,[5,7] also in +12 CLL, *NOTCH1* mutations distributed in a mutually exclusive fashion with deletions of 17p13 and 11q22-q23 (Figure 1A).

This extended cohort corroborates the high prevalence of *NOTCH1* mutations in +12 CLL, where the overall frequency of *NOTCH1* mutations in +12 patients consistently ranges from 24.5 to 28.6%.[7,11] Furthermore, +12 patients harboring *NOTCH1* mutations prevalently belong to aggressive cases, i.e. cases with an unmutated *IGHV* gene status, in line with recent findings,[7,11] and expression of CD38 (*NOTCH1* mutated/CD38 positive, n=20 of 25 (80.0%) *vs.* *NOTCH1* wild-type/CD38 positive, n=39 of 77 (50.6%) (*P*=0.010).

At variance, no difference emerged in the distribution of ZAP-70 positivity (*NOTCH1* mutated/ZAP-70 positive, n=15 of 24 (62.5%) *vs.* *NOTCH1* wild-type/ ZAP-70 positive, n=34 of 77 (44.2%) (P=0.116).

Finally, the analysis of this specific cohort of patients showed an enrichment of *NOTCH1* mutations in CLL harboring+12 as sole cytogenetic abnormality.

NOTCH1 *mutations and overall survival in +12 CLL*

After a median follow up of seven years, 36 of 103 evaluable patients had died, for a median OS of 11.6 years (95% CI: 10.4-12.7). By univariate analysis, the crude impact of *NOTCH1* mutations on survival in +12 CLL was an approximately 2.4 fold increase in the hazard of death (HR: 2.46; 95% CI: 1.26-4.81) and a significant shortening of OS ($P<0.01$) (Table 1B; Figure 1B). Other variables associated with shorter OS were age, Binet stage, *IGHV* mutation status and *TP53* disruption.

Multivariate analysis selected *NOTCH1* mutations as an independent risk factor of OS (HR: 3.04; 95% CI: 1.39-6.64; $P<0.01$), after adjusting for age (> *vs*. ≤65 years), Binet stage (B-C *vs*. A), *IGHV* mutation status and *TP53* disruption by mutation and/or deletion. *NOTCH1* mutations, 13q14 deletion, 11q22-q23 deletion and *TP53* status were used to build a hierarchical model of genetic subgroups to predict OS in CLL with +12. The outcome of +12 CLL with *NOTCH1* mutations was poor, similar to cases with +12 and *TP53* disruption or 11q22-q23 deletion and significantly worse than patients with +12 as an isolated abnormality or plus 13q14 deletion (Figure 1C).

NOTCH1 mutations represent, therefore, an independent adverse prognostic factor of OS among +12 CLL, providing a new genetic prognostic stratification of patients with this intermediate risk marker.

Gene expression profiling of +12 CLL with NOTCH1 *mutations*

To understand whether *NOTCH1* mutations induced a distinctive transcriptional profile in +12 CLL, we compared 7 *NOTCH1* mutated *vs.* 7 *NOTCH1* wild-type cases in a cohort of patients carrying +12 (*Online Supplementary Table S1*).

This analysis showed that *NOTCH1* mutated cases formed a tight clustering (Figure 1D), with 2 patients incorrectly placed. Of these, one later developed a *TP53* mutation and a myelodysplastic syndrome (CLL_00248, *Online Supplementary Table S1)*.

Sixty-five differentially expressed genes (*Online Supplementary Table S2*) were selected, the majority being upmodulated in *NOTCH1* mutated samples. DAVID functional annotation analysis highlighted an overrepresentation of cell cycle related genes, indicating that *NOTCH1* mutations induce a proliferative advantage that might explain the clinically aggressive behavior (Figure 1D). We also observed significantly higher levels of IgM expression in *NOTCH1* mutated cases. It is known that IgM expression is higher in cells with increased ability to respond to external stimuli,[12,13] indicating that the *NOTCH1* mutated clone might survive and expand also thanks to these interactions. Intriguingly, approximately 30% of the upregulated transcripts were located on chromosome 12. This might be due to the fact that +12 was present as a single alteration in all *NOTCH1* mutated cases, whereas in the *NOTCH1* wild-type subgroup the scenario was more complex, with 3 cases displaying only +12 and 4 cases one or more additional chromosomal aberrations.

Among the transcripts previously reported to be associated with a +12 signature, we confirmed the upregulation of *ANAPC5*, *GLIPR1*, *TIMELESS* and *SLC2A6*.[14,15]

In summary, this study highlights that *NOTCH1* mutations in +12 CLL: i) preferentially cluster with cases harboring +12 as sole genetic abnormality; ii) account for 24% of cases and are more frequently detected in cases with unfavorable biological markers; iii) associate with a particular gene expression profile; iv) predict a poor outcome, stratifying +12 CLL in two distinct subgroups; and v) are not associated with *TP53* disruption or 11q deletion, thus making them even more useful in a genetic hierarchical prognostic model, given their mutual exclusivity. This observation sheds light on the heterogeneous clinical course of +12 CLL patients and allows us to refine the intermediate prognostic risk of this chromosomal lesion. The transcriptional profile characterized by an overrepresentation of genes involved in cell cycle and proliferation suggests the potential biological contribution of *NOTCH1* mutations in determining the aggressive behavior of the disease.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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