PROCEEDINGS ARTICLE

The genome of chemorefractory chronic lymphocytic leukemia reveals frequent mutations of *NOTCH1* and *SF3B1*D Rossi¹, S Rasi¹, V Spina¹, A Bruscaggin¹, S Monti¹, S Cresta¹, R Famà¹, C Deambrogi¹, M Greco¹, M Fangazio¹, C Ciardullo¹, D Piranda¹, GM Casaluci¹, M Messina², ID Giudice², S Chiaretti², M Marinelli², A Guarini², R Foà^{2,3} and G Gaidano¹

Next-generation whole-exome sequencing has revealed two novel genes, namely *NOTCH1* and *SF3B1*, whose mutations predict poor outcome and preferentially associate with chemorefractory chronic lymphocytic leukemia (CLL). Analysis of 539 CLL cases documents that *NOTCH1* mutations i) represent one of the most frequent cancer gene mutations involved at presentation; ii) cluster with cases harboring trisomy 12 and tend to be mutually exclusive with *TP53* disruption among genetic subgroups; iii) identify high-risk patients showing poor survival similar to that associated with *TP53* abnormalities; and iv) exert a prognostic role independent of widely accepted clinical and genetic risk factors. Mutations of *SF3B1*, a splicing factor that is a critical component of the spliceosome, recurrently associate with fludarabine-refractory CLL, occur at a low rate at CLL presentation and have a minor role in Richter transformation, corroborating the notion that CLL histological shift is molecularly distinct from chemorefractory progression without the Richter transformation.

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Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults.^{1–3} The clinical course of CLL ranges from very indolent, with a nearly normal life expectancy,^{1–3} to rapidly progressive, leading to death, and occasionally undergoing transformation to aggressive lymphoma, known as Richter syndrome (RS).^{1–5}

At presentation, several clinical and biological features may help predict, at least in part, the clinical course of CLL.^{6–8} Of the biological prognosticators that have been developed, current guidelines for clinical practice recommend screening only for *TP53* disruption by mutation and/or deletion of the locus, which identifies a fraction of high-risk CLL patients destined to experience a very short survival.^{8–15} High-risk CLL, however, cannot be fully recapitulated by *TP53* disruption, as 40–50% high-risk CLL patients are devoid of *TP53* abnormalities.¹⁶ Thus, it is conceivable that other genetic lesions may drive CLL aggressiveness and/or may cause the chemorefractory phenotype of the disease.

Genome-wide methods, including whole-exome sequencing (WES), allow the characterization of the entire spectrum of mutations present in a given disease, as documented by the example of other B-cell malignancies.^{17,18} On these grounds, investigating the CLL genome may be useful to provide further insights into CLL pathogenesis, and might contribute to elucidate the molecular basis of CLL clinical evolution, including RS transformation and development of chemorefractoriness. We have exploited an integrated approach based on next-generation WES to investigate the CLL-coding genome. This approach has revealed two novel genes, namely *NOTCH1* and *SF3B1*, whose mutations predict poor outcome and preferentially associate with chemorefractory CLLs.^{19–21}

MUTATIONS OF *NOTCH1* IN HIGH-RISK CLL

We have assessed the prognostic role of *NOTCH1* mutations in CLL.²⁰ Two series of previously untreated CLLs were used as

training ($n=309$, median follow-up 6 years) and validation ($n=230$, median follow-up 7 years) cohorts. *NOTCH1* mutations were analyzed by DNA Sanger sequencing in blind with respect to clinical data. In the training series, *NOTCH1* mutations occurred in 34/309 (11.0%) patients, being mostly represented (26/34, 76.5%) by a recurrent 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 ($n=1$). All mutations were predicted to disrupt the *NOTCH1* PEST domain. CLL with *NOTCH1* mutations preferentially carried unmutated *IGHV* genes (76.5%, $P<0.001$). Other characteristics at presentation associated with *NOTCH1* mutations were advanced Rai stage (26.5%, $P=0.006$) and trisomy 12 (44.1%, $P<0.001$). By univariate analysis, *NOTCH1* mutations were associated with an increase in the hazard of death (hazards ratio: 3.77; 95% confidence interval: 2.14–6.66) and a significant shortening of overall survival (OS) ($P<0.001$). Multivariate analysis selected *NOTCH1* mutations as an independent risk factor of OS (hazards ratio: 4.22; 95% confidence interval: 2.15–8.28; $P<0.001$), after adjusting for age ($P<0.001$), Rai stage ($P=0.005$), *IGHV* mutation status ($P=0.465$), 11q22-q23 deletion ($P=0.128$), trisomy 12 ($P=0.183$) and *TP53* disruption ($P<0.001$). The poor prognosis conferred by *NOTCH1* mutations was attributable, at least in part, to a shorter time to progression requiring treatment ($P<0.001$), and a higher cumulative probability of RS development ($P=0.026$). Although *NOTCH1*-mutated patients were devoid of *TP53* disruption in 31/34 (91.2%) cases, the OS predicted by *NOTCH1* mutations was similar to that of *TP53*-mutated/-deleted CLL. Analysis of the validation series confirmed the following: i) the prevalence of *NOTCH1* mutations at CLL presentation (26/230, 11.3%); ii) the spectrum of *NOTCH1* mutations at CLL presentation (c.7544_7545delCT: 21/26, 80.7%; other mutations: 5/26, 19.3%); iii) the adverse prognostic impact of *NOTCH1* mutations in CLL both by univariate analysis and

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multivariate analysis (hazards ratio: 2.08; 95% confidence interval: 1.10–3.93; $P=0.023$); iv) the preferential mutually exclusive distribution of *NOTCH1* mutations with respect to *TP53* disruption (25/26, 96.2%); and v) the similar poor OS of *NOTCH1*-mutated and *TP53*-disrupted CLL.

The current study on 539 CLL cases documents that *NOTCH1* mutations i) represent one of the most frequent cancer gene mutations known to be involved at CLL presentation; ii) cluster with cases harboring trisomy 12 and tend to be mutually exclusive with *TP53* disruption among CLL genetic subgroups; iii) identify a high-risk subgroup of patients showing poor survival similar to that associated with *TP53* abnormalities; and iv) 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 biological predictors of CLL identified to date, *TP53* disruption is the sole risk factor consistently associated with high-risk patients^{6–13}. The genetics of high-risk CLL, however, is not fully recapitulated by *TP53* disruption, as 40–50% high-risk CLL patients are devoid of *TP53* abnormalities.¹⁶ Conceivably, other genetic lesions may drive CLL aggressiveness. This study is consistent with a role of *NOTCH1* mutations in contributing to CLL clinical aggressiveness, as 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.²⁰ 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* disruption in 50% of the patients. 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, histological 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. However, these studies were based on small CLL series, used treatment-free survival as surrogate clinical end point and lacked a formal demonstration that the clinical effect of *NOTCH1* mutations is reproducible and independent of confounders.²⁰ 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.

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 treatment-free survival of ~2 years for mutated cases versus ~9 years for *NOTCH1* germline 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.¹⁹ 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,^{19,20} and that *NOTCH1* activation *in vitro*

confers resistance to apoptosis through nuclear factor- κ B pathway activation.

The external validation approach exploited in our study documents that *NOTCH1* mutations are an independent prognostic factor retaining its predictive value in CLL patients followed up at different institutions. This observation suggests that the prognostic value of *NOTCH1* mutations, although detected retrospectively, is independent of a potential bias due to patient referral or patient management at a single center. 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. 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, which in normal conditions is required to limit the intensity and duration of *NOTCH1* activation.^{19,20} Removal of the PEST domain results in *NOTCH1*-impaired degradation and accumulation of an active *NOTCH1* isoform sustaining deregulated signaling. 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 for 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, and that prompt future studies of molecular therapy for *NOTCH1*-mutated CLL patients.

MUTATIONS OF *SF3B1* IN CLL

Our WES analysis of fludarabine-refractory CLL has led to the identification of recurrent mutations of *SF3B1*, a critical component of the cell spliceosome, prompting further investigations of these alterations in a large CLL panel.²¹ The study population comprised three clinical cohorts representative of the following: i) fludarabine-refractory CLL ($n=59$), including cases ($n=11$) subjected to WES; ii) newly diagnosed and previously untreated CLL ($n=301$); and iii) clonally related RS ($n=33$). Tumor samples were obtained for the following: i) fludarabine-refractory CLL, immediately before starting the treatment to which the patient eventually failed to respond, and ii) for newly diagnosed and previously untreated CLL, at disease presentation. All RS studies were conducted on RS diagnostic biopsies. Mutation analysis of *SF3B1* was performed on genomic DNA by a combination of Sanger sequencing and targeted next-generation sequencing. *SF3B1* was altered in 10/59 (17%) fludarabine-refractory CLL by missense mutations ($n=9$) or in-frame deletions ($n=1$) clustering in the HEAT3, HEAT4 and HEAT5 repeats of the *SF3B1* protein.²¹ Two sites that are highly conserved among species (codon 662 and codon 700) were recurrently mutated in three and five cases, respectively. *SF3B1* mutations were monoallelic and were predicted to be functionally significant according to the PolyPhen-2 algorithm. Mutations occurred irrespective of *IGHV* mutation status, CD38 expression and ZAP70 expression.²¹ At the time of fludarabine refractoriness, *SF3B1* mutations were enriched in cases harboring a normal fluorescence *in situ* hybridization karyotype ($P=0.008$) and distributed in a mutually exclusive manner with *TP53* disruption (mutual information $I=0.0609$; $P=0.046$). By

combining *SF3B1* mutations with other genetic lesions enriched in chemorefractory cases (*TP53* disruption, *NOTCH1* mutations, *ATM* deletion), fludarabine-refractory CLL appeared to be characterized by multiple molecular alterations that, to some extent, are mutually exclusive.²¹ We then compared the prevalence of mutations observed at the time of fludarabine refractoriness with that observed in other disease phases. At diagnosis, *SF3B1* mutations were rare (17/301; 5%) and showed a crude association with short treatment-free survival ($P < 0.001$) and OS ($P = 0.011$).²¹ Remarkably, 5/17 (29%) *SF3B1* mutated CLL were primary fludarabine-refractory patients. In CLLs investigated at diagnosis, the hotspot distribution and molecular spectrum of *SF3B1* mutations, as well as their mutual relationship with other genetic lesions, were similar to those observed in fludarabine-refractory CLL. *SF3B1* mutations were restricted to 2/33 (6.0%) clonally related RS. Across the different disease phases investigated, mutations were somatically acquired in all cases ($n = 18$) for which germline DNA was available. These data document that mutations of *SF3B1*, a splicing factor that is a critical component of the spliceosome, recurrently associate with fludarabine-refractory CLL, occur at a low rate at CLL presentation and have a minor role in RS transformation, corroborating the notion that CLL histological shift is molecularly distinct from chemorefractory progression without RS transformation.²¹ The identification of *SF3B1* mutations points to the involvement of splicing regulation as a novel pathogenetic mechanism in CLL. The pathogenicity of *SF3B1* mutations in CLL is strongly supported by clustering of these mutations in evolutionarily conserved hotspots localized within HEAT domains, which are tandemly arranged curlicue-like structures serving as flexible scaffolding on which other components can assemble.

PERSPECTIVES

To date, sequencing analysis of the CLL coding genome has revealed at least two novel genetic alterations, namely mutations of *NOTCH1* and *SF3B1*, that help identify high-risk patients.^{19,21} 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 in clinical trials, indirect evidence for this hypothesis comes from the observation that *NOTCH1* mutations are enriched among chemorefractory CLL patients, and that *NOTCH1* activation *in vitro* confers resistance to apoptosis through nuclear factor- κ B pathway activation. Given the availability of *NOTCH1* inhibitors that are under clinical development, mutations of *NOTCH1* in CLL may also provide a novel molecular therapeutic target. The observation that *SF3B1* regulates the alternative splicing program of genes controlling cell cycle progression and apoptosis points to a potential contribution of *SF3B1* mutations in modulating tumor cell proliferation and survival. In addition to pathogenetic implications, *SF3B1* mutations might also provide a therapeutic target for *SF3B1* inhibitors, which are currently under preclinical development as anticancer drugs. The number of novel molecular markers of chemorefractoriness in CLL is rapidly increasing, and genes of the nuclear factor- κ B pathway appear to be also involved in determining treatment failure, as documented by our recent identification of an association between *BIRC3* disruption by mutation and/or deletion and refractoriness to fludarabine in *TP53* wild-type CLL.²²

CONFLICT OF INTEREST

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