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ORIGINAL ARTICLE

Distinct patterns of novel gene mutations in poor-prognostic stereotyped subsets of chronic lymphocytic leukemia: the case of *SF3B1* and subset #2

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Recent studies have revealed recurrent mutations of the *NOTCH1*, *SF3B1* and *BIRC3* genes in chronic lymphocytic leukemia (CLL), especially among aggressive, chemorefractory cases. Nevertheless, it is currently unknown whether their presence may differ in subsets of patients carrying stereotyped B-cell receptors and also exhibiting distinct prognoses. Here, we analyzed the mutation status of *NOTCH1*, *SF3B1* and *BIRC3* in three subsets with particularly poor prognosis, that is, subset #1, #2 and #8, aiming to explore links between genetic aberrations and immune signaling. A remarkably higher frequency of *SF3B1* mutations was revealed in subset #2 (44%) versus subset #1 and #8 (4.6% and 0%, respectively; $P<0.001$). In contrast, the frequency of *NOTCH1* mutations in subset #2 was only 8%, lower than the frequency observed in either subset #1 or #8 (19% and 14%, respectively; $P=0.04$ for subset #1 versus #2). No associations were found for *BIRC3* mutations that overall were rare. The apparent non-random association of certain mutations with stereotyped CLL subsets alludes to subset-biased acquisition of genomic aberrations, perhaps consistent with particular antigen/antibody interactions. These novel findings assist in unraveling specific mechanisms underlying clinical aggressiveness in poor-prognostic stereotyped subsets, with far-reaching implications for understanding their clonal evolution and implementing biologically oriented therapy.

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Keywords: chronic lymphocytic leukemia; immunoglobulin genes; stereotyped B-cell receptors; *NOTCH1* mutations; *SF3B1* mutations; prognosis

INTRODUCTION

Different lines of research have established a role for (auto)antigenic stimulation in the ontogeny and evolution of chronic lymphocytic leukemia (CLL). In particular, CLL exhibits a highly skewed immunoglobulin (IG) gene repertoire,[1] while patient survival is associated with the somatic hypermutation status of the clonotypic IG heavy variable (*IGHV*) *genes*.[2,3] A remarkable phenomenon in CLL is that subsets of cases carry quasi-identical or 'stereotyped' B-cell receptors (BcR), in up to 30% of patients.[4–11] BcR stereotypy strongly implies that CLL ontogeny is not stochastic but instead driven by interactions between the clonogenic cells and a restricted set of antigenic elements.[12,13]

CLL subsets expressing certain stereotyped BcRs share both clinical and biological features. For instance, CLL patients assigned to subset #1 (*IGHV1/5/7/IGKV1(D)-39*, unmutated CLL) or subset #2 (*IGHV3-21/IGLV3-21*, variable mutational status, however primarily mutated CLL), comprising the largest stereotyped subsets overall, collectively account for 6% of all CLL patients[11] and display a very poor clinical outcome.[7,9,14,15] Furthermore, patients belonging to subset #8 (*IGHV4-39/IGKV1(D)-39*, unmutated CLL) appear to have a particularly poor outcome, even when compared with other clinically aggressive subsets, and exhibit the highest risk of Richter transformation among all CLL cases.[16,17]

Of note, emerging evidence suggests that these distinct clinical profiles may be linked to a precise functional and genetic make-up. In particular, certain subsets, including #1, #2 and #8, have been reported to exhibit subset-biased immune signaling, gene expression, DNA methylation profiles and genetic aberrations, for example, (i) high frequency of del(11q) in subset #2 and (ii) high frequency of trisomy 12 and t(14;19)(q32;q13) in subset #8.[18–26] Altogether, these findings imply that distinctive modes of

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microenvironmental interactions mediated by certain stereotyped BcRs may be associated with selection or occurrence of particular genetic aberrations, with the combined effect determining both clinical evolution and eventual outcome.

Very recently, whole-genome and/or exome sequencing have identified a number of novel recurrent genetic lesions in CLL, including *NOTCH1*, the splicing factor *SF3B1* and the antiapoptotic gene *BIRC3*.[27–35] In most published studies, *NOTCH1* and *SF3B1* mutations were detected at frequencies ranging from 5 to 10% of patients at diagnosis and, in contrast, sharply increased in frequency (17–24%) among patients with progressive, refractory disease and poor outcome.[28–30,36–39] In addition, *NOTCH1* mutations appeared to be associated with increased risk of Richter transformation and were reported to be more frequent among patients harboring trisomy 12.[34,38] Similarly, *BIRC3* mutations were associated with clinical aggressiveness, being detected in up to 24% of fludarabine-resistant CLL.[35]

Considering the distinct clinical outcomes of various stereotyped subsets, where antigen involvement is almost undisputable, and the observation that while novel gene mutations are present in only a minor proportion of patients they are associated with distinct clinical outcome, it became intriguing to explore whether these mutations were associated with subsets expressing specific stereotyped BcRs, and thus, by extension, whether such mutations could correlate with particular immune signaling profiles orchestrated by their stereotyped BcRs. To this end, we evaluated the mutation status of the *NOTCH1*, *SF3B1* and *BIRC3* genes, all shown to be associated with clinical aggressiveness, in poor-prognostic CLL subsets #1, #2 and #8 identified from our large-well annotated CLL cohort.[11] This approach enabled us to uncover striking differences in the genetic make-up of various stereotyped subsets, pointing to distinct paths by which CLL clones expressing different BcRs acquire genomic aberrations.

MATERIALS AND METHODS

Patients and subsets

Patients with CLL assigned to stereotyped subsets #1, #2 and #8 ($n = 170$) following the criteria recently described by our group[8,10,11] were included in this study. All CLL samples are part of a multicenter cohort with cases from the Czech Republic, Denmark, France, Greece, Italy, Sweden and the UK (including patients enrolled onto the LRF UK CLL4 trial[40]). All cases were diagnosed according to the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) established criteria, showing a typical CLL immunophenotype.[41] Informed consent was collected according to the Declaration of Helsinki and ethical approval was granted by local ethical review committees.

Detection techniques

Polymerase chain reaction amplification and Sanger sequencing of exon 34 of the *NOTCH1* gene; exons 14–16 of the *SF3B1* gene; and exons 7–10 of the *BIRC3* gene were performed using standard protocols (protocols and primers available on demand).

Statistics

Differences in frequencies were evaluated using descriptive statistics. Overall survival was measured from the date of diagnosis until last follow-up or death. Time to treatment was evaluated from the diagnostic date until date of initial treatment. Survival curves were constructed with the Kaplan–Meier method, and the log-rank test was used to determine differences between survival proportions. All statistical analyses were performed using the Statistica Software 10.0 (Stat Soft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

All previous studies describing novel recurrent gene mutations in CLL included more general CLL cohorts,[27–39] thus offering only a generic overview of their incidence and potential clinical impact. Considering the underlying heterogeneity of this disease, this inevitably leads to ambiguity, leaving open many questions as to their precise relevance in particular subgroups of CLL. To obtain a more accurate and detailed view, we here for the first time profiled more homogeneous stereotyped subsets following a conceptual approach that places the BcR at the center of patient stratification.[11] This strategy overcomes the shortcomings of other existing stratification schema (for example, clinical staging, fluorescence *in situ* hybridization-based groupings), which can only recognize more 'crude' and conspicuously less homogeneous categories.

In particular, we evaluated the mutation status of the *NOTCH1*, *SF3B1* and *BIRC3* genes, by sequence analysis of exons with known hot-spot regions (*NOTCH1*, exon 34; *SF3B1*, exons 14–16; *BIRC3*, exons 7–10), in well-characterized, aggressive stereotyped CLL subsets identified from our large CLL cohort.[11] Overall, out of 170 CLL patients from stereotyped subsets #1 ($n = 82$), #2 ($n = 66$) and #8 ($n = 22$), we detected 23/160 (14.4%) *NOTCH1* mutations, 24/119 (20%) *SF3B1* mutations and 2/31 (6.5%) *BIRC3* mutations. In the case of *NOTCH1* and *SF3B1* mutations, the frequencies reported here are in line with those reported in the literature for advanced, refractory CLL (~17–24%),[34,36,38,39] as one might expect because of case selection since subsets #1, #2 and #8 represent very aggressive subgroups of CLL. On the other hand, few mutations were detected in the *BIRC3* gene, in keeping with previous studies in more general cohorts, indicating that this genetic event is uncommon at diagnosis of CLL and instead tends to accumulate among refractory CLL.[35] This is also supported by recent findings in our population-based CLL cohort,[37] where *BIRC3* mutations were extremely rare and detected in very few patients (R Rosenquist, unpublished data).

Notably, when analyzing separately the frequency of *NOTCH1* and *SF3B1* mutations in subsets #1, #2 and #8, significant differences emerged (Figure 1). The most striking difference concerned the very high frequency of *SF3B1* mutation in subset #2 patients (44%) compared with the other poor-prognostic subsets analyzed (subset #1, 4.7%; subset #8, 0%; $P < 0.001$; Table 1) as well as to the literature, where even among refractory CLL or cases with Richter transformation the frequency was distinctly lower (17%).[37,39] This finding, herein reported for the first time, strongly points to a subset-biased genetic event of critical importance in the pathobiology of subset #2. That said, the biological reason as to why subset #2 patients exhibit such a remarkable propensity to associate with *SF3B1* mutations remains elusive and requires further investigation.

Another unresolved issue concerns the precise clinical significance of these aberrations, in particular since we found no

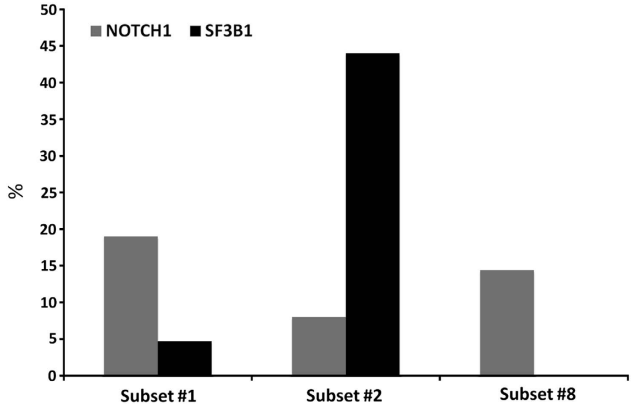

Figure 1. Distinct mutation patterns in different stereotyped subsets. Striking differences ($P < 0.001$) were identified between subsets #1, #2 and #8 with regard to the frequency of *SF3B1* mutations. The frequency of the *NOTCH1* exon 34 mutation was also different between the subsets and in the case of subset #1 versus #2 also reached statistical significance ($P = 0.04$).

differences in time-to-first-treatment ($P = 0.67$) when comparing subset #2 cases with/without *SF3B1* mutations (Figure 2). Therefore, *per se*, *SF3B1* dysregulation, although remarkably enriched in subset #2, does not seem to explain the particularly aggressive phenotype of this subset. Admittedly, the possibility exists that additional genetic or epigenetic mechanisms may be responsible for impairing spliceosomal activity among subset #2 cases negative for the tested *SF3B1* mutations. Hence, it remains to be elucidated how the specific antigenic stimulation through this distinctive BcR may be implicated in this process of genetic dysregulation, opening new possibilities for guided research into the pathogenesis and, in particular, the progression of subset #2.

Table 1. *SF3B1* mutation profile in stereotyped subsets of CLL

Subset # 1, n = 3	*Subset # 2, n = 21*	*Exon*	*Codon*	*Amino-acid change*	*Mutation type*
0	14	15	700	Lys–Glu	Missense
1	3	16	742	Gly–Asp	Missense
0	1	14	626	Asn–Ile	Missense
1	0	15	704	Ile–Asn	Missense
1	0	14	626	Asn–Ser	Missense
0	1	14	622	Glu–Asp	Missense
0	1	16	784–785	—	Deletion*
0	1	16	745	Ala–Pro	Missense
0	1	14	622	Glu–Val	Missense

Analysis of exons 14, 15 and 16 of the *SF3B1* gene in 119 cases assigned to subsets #1, #2 or #8 revealed 25 mutations. No mutations were observed in subset #8 cases, three mutations were observed in subset #1, while the remaining 22 mutations were found in subset #2 Notably, the recurrently targeted hotspot (codon 700) was only found mutated in subset #2 (14/25). One subset #2 case carried a mutation at both codon 700 and codon 742.
*c.2352_2354delGAA

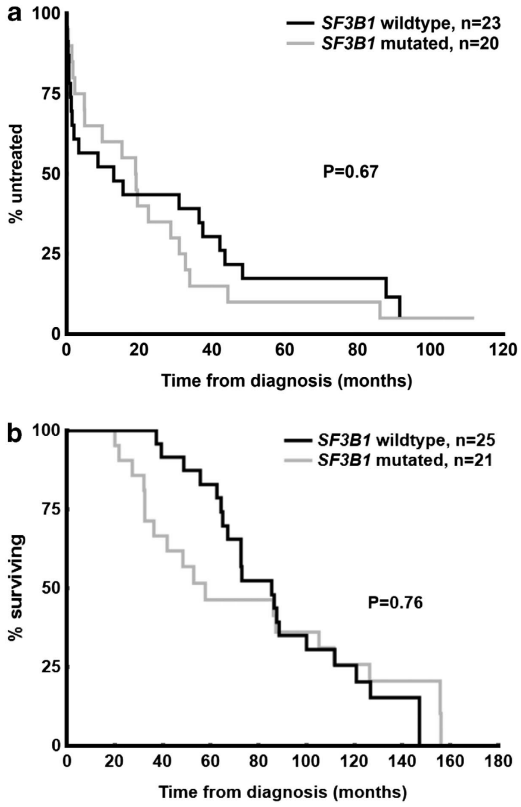

Figure 2. Survival curves for subset #2. No differences were identified with regard to time-to-first-treatment (**a**) or overall survival (**b**) in subset #2 patients based on the presence or absence of *SF3B1* mutations.

The notion that BcR-mediated selection may be intimately linked to the acquisition of certain genetic aberrations is also underscored by the profiling of subsets #1, #2 and #8 for the 2-bp deletion in exon 34 of the *NOTCH1* gene, which accounts for more than 90% of all *NOTCH1* gene mutations.[28] In contrast to mutations in *SF3B1*, this aberration is relatively rare in subset #2, being present in only 8% (5/60) analyzed cases, similar to published reports on more general cohorts.[37,39] In our study, *NOTCH1* hotspot mutation was more frequent in both subset #1 (19%) (15/78) and subset #8 (14%) (3/22), similar to the frequency reported for advanced, refractory CLL[36,38] of note, the difference between subsets #1 and #2 was statistically significant ($P = 0.04$). This result suggests that *NOTCH1* aberrations either do not occur or are selected against in subset #2, and hence are less relevant clinically as compared with other poor-prognostic CLL cases.[36,38] However, their precise significance, especially in subset #8, requires further analysis in a larger group of patients, although this might prove logistically difficult if only for the fact that subset #8 accounts for just 0.2–0.3% of all CLL. Notwithstanding, this analysis would elucidate the links, if any, between *NOTCH1* defects with trisomy 12 and Richter's transformation, both reported as distinctive features of subset #8.

Overall, these novel findings are particularly intriguing as they raise a number of puzzling questions. Any given CLL is 'born' with a distinct BcR, while the novel mutations linked to aggressive disease are thought to accumulate later in the natural history of the disease, due either to the occurrence of *de novo* mutations or to the expansion of initially minor subclones bearing mutations. If this holds true, the strong association between the subset #2 BcR and *SF3B1* mutations observed herein suggests a potential direct or indirect causative relationship between particular antigenic stimulation/immune signaling and the occurrence of genetic lesions in selected pathways. As antigen stimulation is thought to occur in many if not all CLL cases, the finding that specific gene mutations, on the contrary, cluster mainly within specific antigen-defined subsets indicates that BcR stimulation *per se* can be important for the occurrence of the genetic lesions, at least for certain subsets of cases. Along this reasoning, one might postulate that the qualitative or quantitative aspects of each specific BcR stimulation endowed by/linked to certain distinctive BcR must be relevant, being different in each subset depending on the type of antigenic elements and the type of antigen/antibody interactions that are distinct for different subsets.

In summary, for the first time, we unveiled distinct patterns of mutations in the *NOTCH1* and *SF3B1* genes among different major, poor-prognostic stereotyped subsets, pointing to subset-biased acquisition of genetic events along with distinct antigen stimulation during disease evolution. This new concept, whereby specific antigenic stimulation and occurrence of genetic lesions in key cellular pathways are linked, offers a compartmentalized view of the biology of CLL with implications for individualized therapies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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