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Clinical impact of small subclones harboring *NOTCH1*, *SF3B1* or *BIRC3* mutations in chronic lymphocytic leukemia

Large-scale sequencing approaches have shown that chronic lymphocytic leukemia (CLL) is composed of a mosaic of leukemic subpopulations, each defined by distinct genetic lesions.[1-4] The application of ultra-deep-next generation sequencing (NGS) to track *TP53* mutated subclones in CLL has provided a proof-of-concept that small tumor cell populations of very low clonal abundance can drive the overall disease course, and may represent informative and highly sensitive biomarkers of chemorefractoriness.[5]

In addition to *TP53*, other cancer genes known to be recurrently mutated in CLL and significantly associated with poor survival include *NOTCH1, SF3B1*, and *BIRC3*.[6] The clinical impact of mutations in the *NOTCH1*, *SF3B1*, and *BIRC3* genes has so far only been documented in CLL series investigated by conventional sequencing that has a sensitivity limit of 10%-15%. Here we assessed the frequency, prognostic impact and evolution during CLL course of small subclones mutated in the *NOTCH1*, *SF3B1*, and *BIRC3* genes.

The study population was a consecutive series of 304 newly diagnosed CLL patients (*Online Supplementary Table 1S*) who were prospectively registered in the Amedeo Avogadro University CLL database.[5] CLL diagnosis had been made according to IWCLL-NCI criteria. Median follow up of patients alive at the time of the study was seven years. Patients provided informed consent in accordance with local IRB requirements and the 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 Number CE 8/11).

We applied an ultra-deep-NGS strategy coupled with a robust bioinformatic algorithm for highly sensitive detection of small mutated subclones in the *NOTCH1*, *SF3B1*, and *BIRC3* genes. *NOTCH1*, *SF3B1*, and *BIRC3* mutation hotspots (*Online Supplementary Table 2S*) were investigated on genomic DNA extracted from peripheral blood mononuclear cell samples collected at CLL diagnosis, progression requiring treatment, relapse, and last follow up. In all cases, the fraction of tumor cells corresponded to 70%-98% as assessed by flow cytometry. Ultra-deep-NGS was performed using the 454 chemistry, was based on amplicon libraries, and was tailored at an approximately 200-fold coverage per amplicon (average coverage

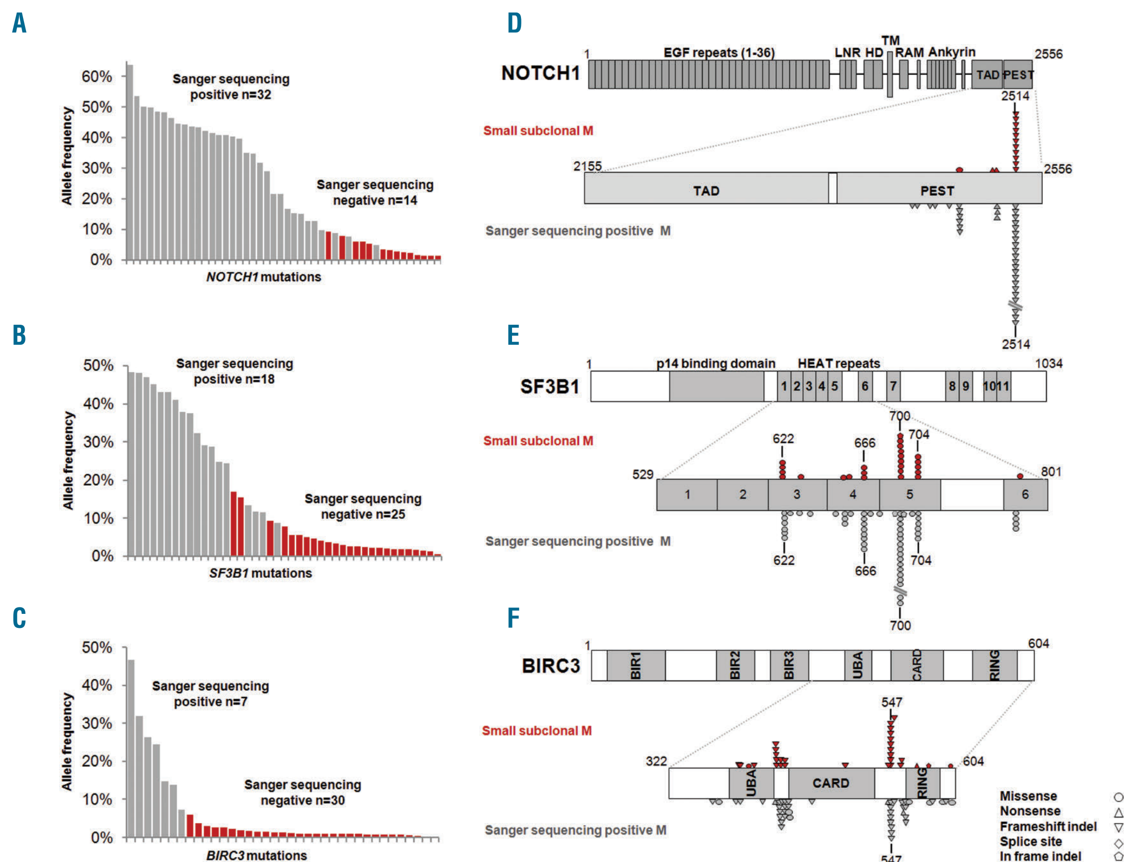

Figure 1. Molecular profile of subclonal *NOTCH1, SF3B1*, and *BIRC3* mutations. Allele frequency of *NOTCH1* (A), *SF3B1* (B), and *BIRC3* (C) mutations identified by ultra-deep-next generation sequencing. Mutations are ordered according to their allelic abundance. Mutations that tested positive (gray bars) and negative (red bars) by Sanger sequencing are indicated. Schematic diagram of the NOTCH1 (D), SF3B1 (E), and BIRC3 (F) proteins with their conserved functional domains. Color-coded shapes indicate the position of small subclonal mutations from the CLL study cohort (red shapes) and mutations detectable by Sanger sequencing in CLL from our internal dataset (gray shapes). Hot spot codons recurrently affected by both subclonal and clonal mutations are highlighted.

2437x; range 1003x-8584x). A previously developed bioinformatic algorithm was applied to call non-silent variants out of background NGS noise.[5] The sensitivity of the ultra-deep-NGS approach allowed mutant allele fractions down to 0.3% to be detected (3 mutant alleles in a background of ~1000 wild type alleles) with a 95% confidence interval of 0.2%-0.5%. Variant calling by the algorithm was validated by Sanger sequencing or, if the variant was below the sensitivity threshold of Sanger sequencing, by both duplicate ultra-deep-NGS and allele specific PCR (AS-PCR) (*Online Supplementary Figure 1S*). To account for tumor representation, the frequency of the mutant alleles provided by ultra-deep-NGS was corrected for the proportion of $CD19^{+}/CD5^{+}$ cells in each sample. Overall survival (OS) from diagnosis was the primary end point and this was measured from date of initial presentation to date of death from any cause (event) or last follow up (censoring). Survival analysis was performed using the Kaplan-Meier method.[7] To identify the optimal cut off of the variant allele frequency to predict CLL OS, we used the outcome-driven maximally selected rank statistics implemented in the Maxstat package of R.[8] Continuous variables from related samples were compared using the Wilcoxon test. Further details are available in the *Online Supplementary Appendix*.

Ultra-deep-NGS identified 46 *NOTCH1* mutations (median allele frequency 24%; range 1.4%-64%) in 14% (43 of 304) of CLL patients, 43 *SF3B1* mutations (median allele frequency 16%; range 0.5%-48%) in 11% (35 of 304), and 37 *BIRC3* mutations (median allele frequency 5.6%; range 0.2%-47%) in 8% (26 of 304). All mutations that had been detected by Sanger sequencing (i.e. Sanger sequencing positive mutations) were also identified by ultra-deep-NGS (Figure 1). Ultra-deep-NGS identified additional small subclonal mutations that, because of their very low abundance, were missed by Sanger sequencing (*NOTCH1* n=14; median allele frequency 3.9%; range 1.4%-9%; *SF3B1* n=25; median allele frequency 4.3%; range 0.5%-17%; *BIRC3* n=30; median allele frequency 1.4%; range 0.2%-6%) (Figure 1A-C and *Online Supplementary Tables S3-S5*). *NOTCH1* mutations were more represented in the leukemic cell population (*Online Supplementary Figure S2*) and, therefore, more frequently detectable by Sanger sequencing than *SF3B1* and *BIRC3* variants. This is consistent with the view that *NOTCH1* mutations are an early event, while *SF3B1* and *BIRC3* lesions are late events in CLL evolution.[2,9,10] These data indicate that a fraction of *SF3B1*, *BIRC3*, and to a lesser extent *NOTCH1* mutations may be restricted to small subclones not detectable by conventional sequencing at the time of CLL presentation.

The molecular spectrum (i.e. type and hot spot distribution) of small subclonal mutations of *NOTCH1*, *SF3B1*, and *BIRC3* was highly consistent with that of variants that were detectable by Sanger sequencing in CLL (Figure 1D-F). These data indicate that small subclonal mutations of *NOTCH1*, *SF3B1*, and *BIRC3* do not represent biologically irrelevant random passenger events, but instead are predicted to have a similar impact on the proteins as that of clonal variants.

There was no statistically significant difference in OS between patients harboring small subclonal mutations of *NOTCH1*, *SF3B1*, or *BIRC3* and wild-type patients (Figure 2A-C), even after adjusting for confounding factors (*Online Supplementary Table S6*). The study, however, was not fully powered to support non-inferiority. Conversely, patients harboring small subclonal mutations of *NOTCH1*, *SF3B1*, or *BIRC3* showed a trend towards a longer OS than patients having a mutation that was detectable by Sanger sequencing (Figure 2A-C). Consistently, outcome-driven approaches documented that *NOTCH1*, *SF3B1*, and *BIRC3* mutations should be represented in at least 25%, 35% and 1% of the alleles, respectively, to have the maximum impact on CLL OS (*Online Supplementary Figure S3*). Application of these approaches to *TP53* mutations failed to identify a clear

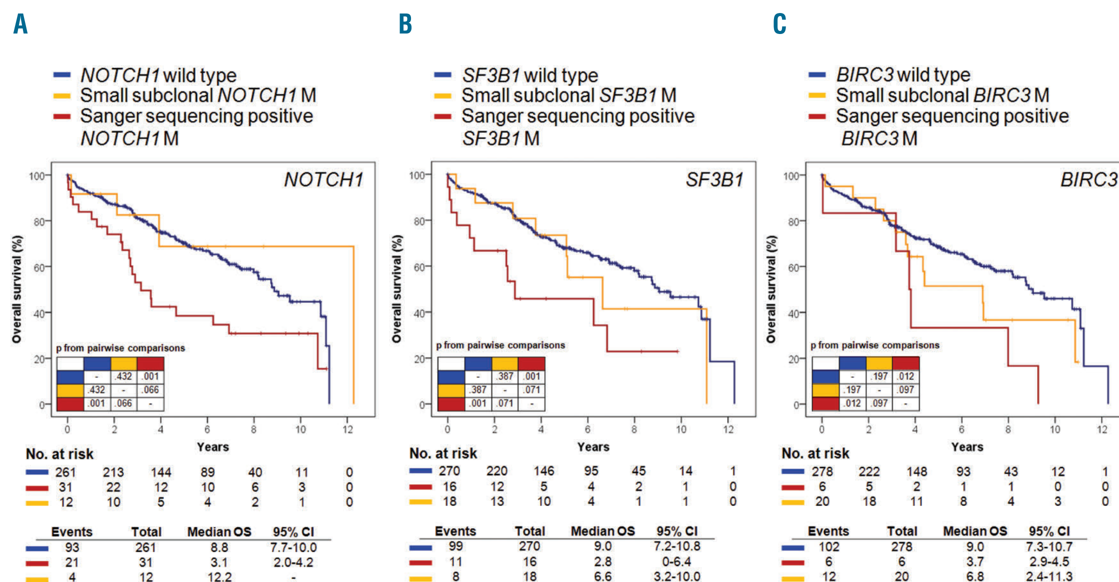

Figure 2. Estimates of overall survival (OS) of patients harboring small mutated subclones. Comparison of OS from chronic lymphocytic leukemia (CLL) diagnosis between patients harboring small subclonal mutations not detectable by Sanger sequencing (yellow line), cases harboring Sanger sequencing positive mutations (red line), and cases harboring an unmutated gene (blue line) of the *NOTCH1* (A), *SF3B1* (B), and *BIRC3* (C) genes. *P*: *P* values by log rank test; M: mutation.

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cut off, suggesting that, in contrast to *NOTCH1*, *SF3B1*, and *BIRC3* mutations, *TP53* lesions are relevant to the outcome of CLL independent of their abundance (*Online Supplementary Figure S4*).[5]

Longitudinal ultra-deep-NGS of sequential samples was carried out in: i) 7 cases harboring small subclonal *NOTCH1* mutations (including 2 patients who required therapy and 5 who were managed with a "watch and wait" approach); ii) in 12 cases harboring small subclonal *SF3B1* mutations (including 4 patients who required therapy and 8 who were managed with a "watch and wait" approach); and iii) in 6 cases harboring small subclonal *BIRC3* mutations (including 4 patients who required therapy and 2 who were managed with a "watch and wait" approach). By longitudinal analysis, the abundance of small subclonal mutations of *NOTCH1*, *SF3B1*, and *BIRC3* did not significantly change in those patients who were managed with a "watch and wait" approach, with only scattered mutations displaying an outgrowth (Figure 3 and *Online Supplementary Figure S5*). Upon treatment, small subclones harboring *NOTCH1* or *BIRC3* mutations either shrank below the sensitivity threshold of our ultra-deep-NGS (Figure 3 and *Online Supplementary Figure S5*) or persisted at low abundance, suggesting that they maintained sensitivity to chemotherapy and did not gain a competitive advantage over the wild-type clones. This observation suggests that the enrichment of *BIRC3* mutations that had previously been documented in advanced relapsed/refractory CLL[11] might be related to other factors that contribute to the fitness of the clones harboring *BIRC3* lesions, rather than a differential sensitivity to therapy. Small subclonal *SF3B1* mutations were more frequently selected than counter-selected (Figure 3 and *Online Supplementary Figure S5*).

The clinical impact of small subclones harboring *NOTCH1*, *SF3B1*, or *BIRC3* mutations appears to be less pronounced than that of small *TP53* mutated subclones. Also, upon treatment, *NOTCH1*, *SF3B1*, and *BIRC3* mutations to do not provide small subclones with the same strength of fitness advantage as *TP53* mutations. However, many homogeneously treated CLL patients and large-scale genotyping of sequential samples collected in a timely fashion are required to quantify precisely the specific fitness of small *NOTCH1*, *SF3B1*, and *BIRC3* mutated subclones in the context of therapy.

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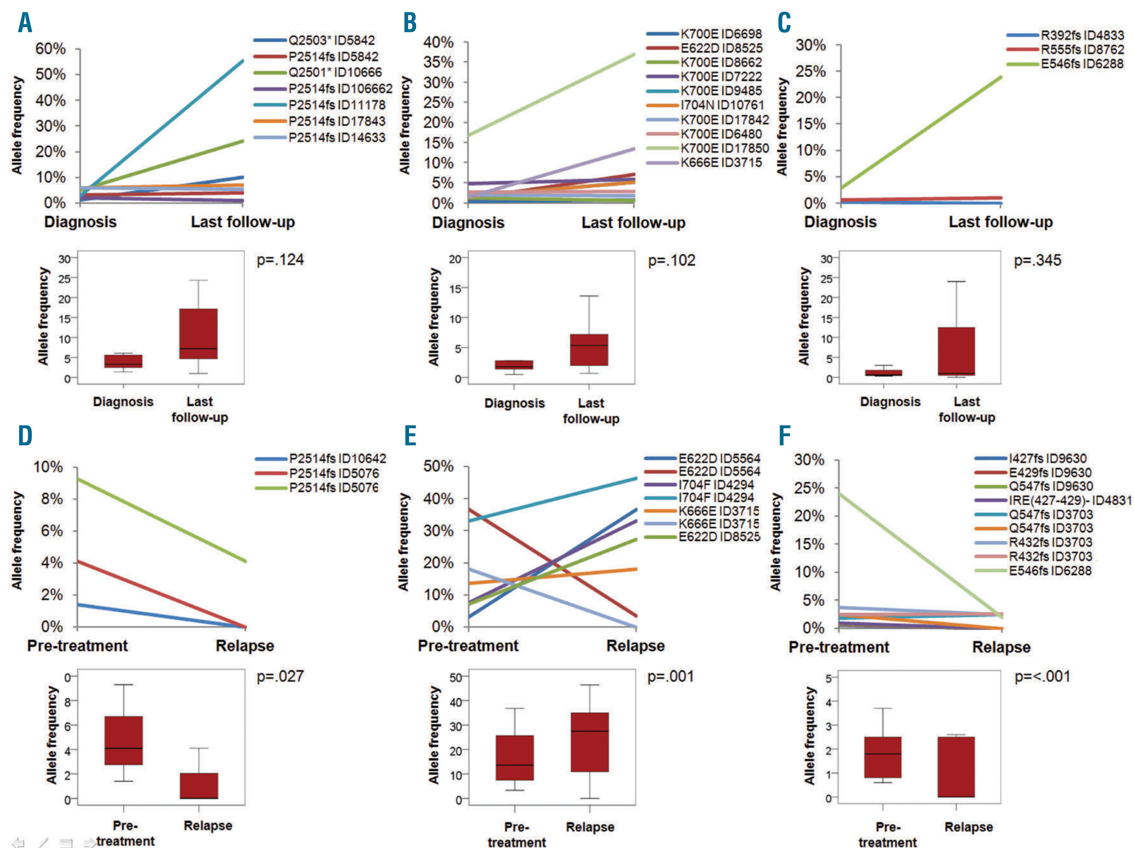

Figure 3. Longitudinal analysis of patients harboring small mutated subclones. Changes of the allele frequency of the *NOTCH1* (A), *SF3B1* (B), and *BIRC3* (C) mutated subclones upon "watch and wait". The upper line graph represents the allele frequency modification of each individual mutation. The lower box plot graph shows in aggregate the mean, interquartile, and extreme values of the allele frequency at diagnosis and last follow up. *P* values by Wilcoxon rank test. Changes of the allele frequency of the *NOTCH1* (D), *SF3B1* (E), and *BIRC3* (F) mutated subclones upon treatment. The upper line graph represents the allele frequency modification of each individual mutation after each individual treatment. The lower box plot graph shows in aggregate the mean, interquartile, and extreme values of the allele frequency before treatment and at relapse. *P*: *P* values by Wilcoxon rank test.

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The online version of this letter has a Supplementary Appendix.

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