Regular article

LYMPHÖID NEOPLASIA

Presence of multiple recurrent mutations revealed by targeted NGS confers poor trial outcome of relapsed/refractory CLL

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Short Title: targeted mutational landscape of R/R CLL

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Key point

- Targeted NGS of relapsed/refractory CLL reveals high incidence of concurrent mutations that mostly affect the **TP53**, **ATM** and **SF3B1** genes.
- Concurrent mutations of the **TP53**, **ATM** and/or **SF3B1** genes confer short survival in patients with relapsed/refractory CLL.
Abstract

While TP53, NOTCH1 and SF3B1 mutations may impair prognosis of patients with CLL receiving frontline therapy, the impact of these mutations or any other, alone or in combination, remains unclear at relapse. The genome of 114 relapsed/refractory patients included in prospective trials was screened using targeted NGS of TP53, SF3B1, ATM, NOTCH1, XPO1, SAMHD1, MED12, BIRC3 and MYD88 genes. We performed clustering according to both number and combinations of recurrent gene mutations. The number of genes affected by mutation was ≥ 2, 1 and 0 in 43 (38%), 49 (43%) and 22 (19%) respectively. Recurrent combinations of ≥ 2 mutations of the following genes: TP53, SF3B1 and ATM were found in 22 (19%) patients. This 'multiple-hit' profile was associated with a median PFS of 12 months compared to 22.5 months in the remaining patients (P = .003). Concurrent gene mutations are frequent in patients with relapsed/refractory CLL and are associated with worse outcome.
Introduction

Chronic lymphocytic leukemia (CLL) is characterized by its unique immunophenotype of CD19^+CD5^+CD23^+sig^dim expressing clonal mature B cells but also by a highly variable clinical course. With the emergence of new classes of drugs such as the inhibitor of PI3-kinase idelalisib^1 and the irreversible inhibitor of Bruton's tyrosine kinase ibrutinib^2, available treatment options have increased significantly and allow us to begin to develop models of treatment stratification. Over the past 3 years, the CLL genome has been thoroughly characterized by Next-Generation Sequencing (NGS).^3 Pioneer reports using this approach unraveled somatic mutations recurrently targeting multiple genes among which TP53, SF3B1, NOTCH1, MYD88 and ATM were the most frequent.^4^-^9 Large retrospective studies in untreated patients from historical cohorts have recently shown the adverse prognostic impact of the TP53, NOTCH1 and SF3B1 mutations on time to treatment and overall survival (OS).^10^-^12 In addition, mutations in these genes may also be associated with poor progression-free survival (PFS) in frontline patients treated in prospective trials.^13,14 Conversely, the pejorative impact of TP53, SF3B1, and NOTCH1 mutations on clinical outcome of patients with relapsed/refractory (R/R) CLL is controversial.^15^-^17 Compared to untreated CLL, relapse, as advanced disease, may be associated with a high level of genomic diversification.^9,18 This process might result in accumulation and co-occurrence of these or other genomic events leading to interactions that could be of prognostic relevance. Here, we therefore investigated relapsed/refractory CLL genomes within a clinical trial setting using a comprehensive NGS gene panel.

Methods

Patients and treatment

The present study reports on 114 patients with R/R CLL treated by conventional immunochemotherapy in the setting of prospective clinical trials (flow-chart in Supplementary Figure S1). Fifty-five patients were enrolled into the ICLLO1 trial by the French CLL Intergroup GCCLL/MW-
GOELAMS prior to a planned interim analysis (phase II salvage treatment with bendamustine, ofatumumab and methylprednisolone in relapsed CLL). The remaining patients were treated in two phase II trials from the United Kingdom National Cancer Research Network and selected on the basis of the availability of DNA samples; 37 were enrolled in the CLL201 trial (a randomized phase II trial of fludarabine, cyclophosphamide and mitoxantrone with or without rituximab in previously treated CLL) and 26 in the CLL202 trial (a phase II study of subcutaneous alemtuzumab plus fludarabine in patients with fludarabine refractory CLL). Studies were approved by all relevant institutional ethical committees and regulatory review bodies, and were conducted in accordance with the Declaration of Helsinki. Patient characteristics are shown in Supplementary Table 1. Fludarabine-refractoriness was defined by a response less than a partial remission to a fludarabine-based regimen or a remission lasting < 6 months on discontinuation of treatment with a fludarabine-based regimen. Median follow-up was 23.6 months. The study has been approved by the IRB (CPP Sud-Est 6) of Clermont-Ferrand University hospital in France and the French CLL Intergroup committee (samples from ICCL01 trial), and the UK NCRI CLL Trials biobanks (samples from CLL201 and CCLL202 trials).

Genetic characterization

Cytogenetic aberrations and IGHV status were respectively analyzed by FISH for 17p and 11q regions and Sanger sequencing. For prognostic analyses, patients were distributed in 3 groups according to their cytogenetic features by FISH in a hierarchical model: i) 17p deletion, ii) 11q deletion without 17p deletion and iii) no 17p or 11q deletion. Copy number variations were generated for 59 patients using HumanOmni1-Quad BeadChip (Illumina) and following the manufacturer’s instructions.

Somatic gene mutational status was determined by targeted NGS. A TruSeq Custom amplicon panel of 9 recurrently mutated genes in CLL (i.e. TP53, SF3B1, ATM, NOTCH1, XPO1, SAMHD1, MED12, BIRC3 and MYD88) was designed using Design Studio (http://designstudio.illumina.com/) (supplementary Methods). Sequencing libraries were prepared according to the manufacturer’s
instructions and run on the Illumina MiSeq instrument (Illumina Inc.). According to Illumina RTA (v.1.18.42) a mean of 14.1 M reads were obtained per run of which 96.8% were identified reflecting an acceptable signal to noise ratio. The yield was 4.1 Gb and 95.9% of reads were above Q30 across 6 MiSeq runs. The data were analyzed using in-house bioinformatics pipeline consisting of a combination of two analyses. After demultiplexing and generation of FASTQ files the data were processed in MiSeq Reporter (v.2.3.32.0) using a banded Smith-Waterman algorithm to align the sequences to the reference genome and Illumina Somatic Variant caller (variant caller suitable for the detection of somatic variants in cancer samples). A second alignment followed by variant calling using the packages Stampy (v. 1.0.22)24 and Platypus (v.0.5.1)25 was used to specifically identify additional insertions and deletions (InDels) not detected with MiSeq Reporter. The VCF files were annotated using Annovar26 and filtered according to Depth and Variant allele frequency (VAF), position and function of the variant in the coding sequence (hg19 refGene), common polymorphism databases (dbSNP v.132 & 137, 1000 genomes Apr2012), the cancer specific database COSMIC (v.67), our in-house CLL specific database, and the prediction score SIFT (from ljb2). All alignments and annotations were performed using the hg19 reference genome (Supplementary Figure S2). Median coverage of the genes was 2266X. 97.1% and 92.1% of patients had a mean coverage across all genes greater than 100X and 500X, respectively. (Supplementary Figure S3). Only NOTCH1 had a less uniform coverage across its targets. However the amplicon covering the hotspot 7541_7542delCT performed as well as the other genes with 95.6% of patients with at least 100X and 87.9% with at least 500X.

Sub-clonal distribution

We inferred the likely sub-clonal distribution using the VAF from the sequencing data and corrected for copy number with information obtained from 17p and 11q FISH data and from the B-allele frequency distributions derived from whole genome array where available. We then accounted for
some degree of germ-line contamination and assessing the tumor purity with an error rate of +/- 5% for the VAFs. If the VAF was <55% we supposed it was a heterozygous mutation, rather then a subclonal homozygous mutation. If two mutations in the same patient showed a VAF difference of less than 5% we took the average to calculate the percentage of cell carrying the mutations assuming they occurred in the same clone; if the VAF difference was greater than 5% we presumed that the two mutations were independent (the mutation with the highest VAF occurred prior the second lower VAF mutation).

Statistical analyses

Overall response rate (ORR) and complete remission rate (CRR) were defined using the International Workshop on CLL (IWCLL) Criteria. Categorical factors between groups of patients were compared using Pearson Chi-squared or Fisher’s exact test. Logistic regression analysis was used to examine the influence of various factors on ORR. Survival time data were calculated using the Kaplan-Meier method for OS and PFS and were compared by logrank testing. Proportional hazards models (Cox regression) were fitted to investigate effects of prognostic factors for PFS and OS. All variables with \( P < 0.1 \) were included. Multivariate Cox models were established after backward selection. Interactions were tested and the proportional hazard hypothesis verified by visual display of the Schönfeld residuals. The models were validated by a bootstrapping process. In each step, 1,000 bootstrap samples with replacements were created from the training set. The bootstrap estimates of each covariate coefficient and SE was averaged from those replicates. The analysis was performed with SPSS software v22 (IBM).

Results

Frequency of gene mutations and correlation to other CLL features
We identified a total of 191 mutations (average of 1.68 per sample) in 92 (81%) patients: 135 missense mutations, 40 substitutions/InDels, 12 nonsense and 4 splicing mutations distributed as shown in Figure 1A (list of all mutations in Supplementary Table S2).

*TP53*, *NOTCH1* and *SF3B1* mutations occurred in 26 (22.8%), 17 (14.9%) and 32 (28.1%) patients, respectively (Figure 1A). The usual distribution of mutations in these 3 genes was found. *TP53* mutations were mostly missense (62%) and located in the DNA-binding domain. *NOTCH1* mutations were virtually confined (88%) to the 2-bp deletion (c.7541_7542delCT) in exon 34. *SF3B1* mutations were all missense, occurring most frequently as p.K700E/c.A2098G (46%). Thirty (26.3%) patients harbored *ATM* mutations. Consistent with previous observations, *ATM* mutations were distributed across the whole gene; 9 mutations clustered in the 3’ terminal region of the gene encoding FAT and PI3kinase functional domains. Mutations in the other genes sequenced were distributed as follows: *XPO1* mutations in 17 (14.9%), *SAMHD1* mutations in 11 (9.6%), *MED12* mutations in 10 (8.8%), *BIRC3* mutations in 6 (5.3%) and *MYD88* mutations in 3 (2.6%) patients (Figure 1A).

A correlation analysis of gene mutations with other CLL features revealed few significant relations (Figure 1B). As expected, significant overrepresentation of mut-*TP53* was noted in the 17p deletion subset (65.4% vs 9.4%, \( P < .001 \)) and in *IGHV* unmutated cases (26.9% vs 0%, \( P = .01 \)). Of note, 55% of 11q deleted patients had either mut-*SF3B1* (n=9) and/or mut-*ATM* (n=7) without reaching statistical significance. All patients with mut-*MYD88* had mutated *IGHV* (n=3) and all mut-*BIRC3* occurred in *IGHV* unmutated cases (n=6).

**Distribution of gene mutations**

To analyze the relative distribution of gene mutations we performed clustering according to i) the number of mutated genes (0 in cluster #1, 1 in cluster #2, ≥2 in cluster #3&4) and ii) the recurrence of combinations of 2 gene mutations (≥5% of cases in cluster #3 vs <5% of cases in cluster #4) (Figure 2).
Twenty-two (19.3%) patients did not have any mutations (cluster #1) and 49 (43%) patients had 1 gene mutated only (cluster #2). The remaining 43 (37.7%) patients had ≥2 genes mutated (clusters #3 & #4). Recurrent combinations of mutations (affecting >5% of patients) were found in a group of 22 (19.3%) patients. These combinations of mutations comprised ≥2 of the following genes: TP53, SF3B1 and ATM (cluster #3, so called multiple-hit (MH) profile). Remarkably, these 3 gene mutations were found more frequently associated with each other than alone.

Relative frequencies of each cluster were significantly affected by neither clinical features nor IGHV status and were well balanced amongst trials (Supplementary Table S3). Conversely, in line with the well-known link between TP53 mutation and 17p deletion, MH patients were more frequent in the 17p deleted subset (42.3%) compared to 11q deleted subset (8.3%) and other cytogenetics (13.3%) (P = .002) (Figure 2).

In an attempt to infer the likely sub-clonal distribution of the mutations carried by the patients with MH-profile from a combination of their respective VAFs and the results from FISH and copy number variations analyses (Supplementary Table S2), we established at least that in 8 patients (80%) with a TP53 mutation, this mutation occurred as a second hit. ATM mutations, although generally accepted as a secondary event in CLL leukemogenesis, arose before TP53 or SF3B1 mutations in 15 patients (83%), whereas SF3B1 came first in just over half of patients with this mutation (n=11; 65%). We discovered 3 recurrent patterns of clonal evolution: ATM and SF3B1 mutations arose in the same clone (pattern 1 in 5 patients); ATM was mutated first, then TP53 was mutated second (pattern 2 in 5 patients); ATM mutation came first, then SF3B1 mutation originated later (pattern 3 in 4 patients) (Figure 3).
Predictive markers of response to salvage therapy

To investigate the clinical significance of gene mutations and their distribution, we evaluated their impact on the prospectively recorded therapeutic response. Univariate analysis on response is reported in Supplementary Table S4. In the entire cohort, ORR and CR rate were 73.9% and 20.7% respectively. Poor ORR was strongly predicted only by fludarabine-refractoriness (55.9% vs 81.8%, \( P = .004 \)) and \( TP53 \) disruption (57.1% vs 81.3%, \( P = .007 \)). If considered individually, no other gene mutation influenced ORR. Conversely, MH-profile was associated with poorer ORR (42.9%) compared to other genomic clusters (86.4%, 83.3%, 71.4% in cluster #1, #2, #4 respectively) \( (P = .002) \). Logistic regression analysis revealed that ORR was significantly and independently influenced by MH-profile (OR = 5.16 [95%CI = 1.62-16.45], \( P = .006 \)) and fludarabine-refractoriness (OR = 4.89 [95%CI = 1.77-13.55], \( P = .002 \)) but not by \( TP53 \) disruption (OR = 2.52 [95%CI = 0.89-7.11], \( P = .081 \)). In terms of CRR, a significantly lower response rate was noted in the \( TP53 \) disrupted (8.6% vs 26.7%, \( P = 0.03 \)) and \( ATM \) mutated (3.4% vs 26.8%, \( P = 0.008 \)) patients. While unexpected, \( NOTCH1 \) mutations conferred a better CRR (41.2% vs 17%, \( P = 0.045 \)). Furthermore, none of the patients with MH-profile achieved complete remission compared to 24% for the remaining patients \( (P = .006) \).

Survival analyses

To address the question of the prognostic relevance of gene mutations we first performed univariate analysis on PFS (Supplementary Table S4). Overall, median PFS was 19.1 months but shorter in fludarabine-refractory cases (12.3 vs 22.5 months, \( P = .001 \)). No significant influence of either 17p or 11q deletion was found. Furthermore, when considered individually, \( TP53 \) mutations were the only gene mutations that adversely impacted PFS (12 vs 20.7 months, \( P = .004 \)). Patients harboring the MH-profile displayed a significant shorter median PFS of 12 months compared to 22.5 months in the other patients \( (P = .003) \) (19.1 months in cluster #1, 23 months in cluster #2 and 17.5 months in cluster #4) (Figure 4A and Supplementary Table S4). Multivariate analysis for PFS confirmed the
independent and adverse prognostic value of the MH-profile (HR=2.24 [95%CI=1.24-4.03], P = .007) as well as those of fludarabine-refractoriness (HR = 3.36 [95%CI=1.61-7.058], P = .001) while TP53 mutation was not significant anymore after the bootstrapping process (Table 1). Interestingly, among the TP53-disrupted patients, the MH-profile retained its prognostic impact with a median PFS of 11.2 months vs 22.4 months for the TP53 disrupted patients with wt-SF3B1 and wt-ATM (P = .03) (Figure 4B).

In terms of OS, advanced Binet staging and fludarabine-refractoriness adversely impacted the outcome while no gene mutation alone appeared as discriminant (Supplementary Table S4). The patients with more than one gene mutation (i.e. cluster #3 & #4) had however a significantly poorer outcome than others (i.e. cluster #1 & #2) (median OS of 28.2 and 27.1 months vs not reached and 46.6 months respectively, P = .019) (Figure 5 and Supplementary Table S4). Multivariate analysis confirmed the independent adverse impact of either more than one gene mutated (model A, HR=2.91 [95%CI=1.52-5.60], P = .001) or the MH profile only (model B, HR=2.85 [95%CI=1.37-5.94], P = .005) (Table 2). Interestingly, besides the retained impact of fludarabine-refractoriness, multivariate analysis uncovered an adverse and independent impact of BIRC3 mutation although it did not retain significance after bootstrapping.

Discussion

Here, we present the largest NGS study of patients with R/R CLL recruited prospectively into clinical trials to date. We reveal a high incidence of concurrent mutations that mostly affect the TP53, ATM and SF3B1 genes. This MH-profile is associated with a significantly poorer response to salvage conventional immunochemo therapy and with a significant and independent shorter survival than those of patients of similar clinical phenotype with single or no mutation.
So far, the incidence of gene mutations has largely been reported in NGS studies of heterogeneous and mostly treatment-naïve patients or focusing on the TP53, SF3B1 and NOTCH1 genes only using Sanger sequencing. Now that main CLL genomic drivers have been uncovered by WES or WGS studies and targeted NGS has been demonstrated as an accurate and reproducible technique in CLL, it offers an affordable and more sensitive tool for mutational screening of patients with CLL in clinical practice. We focused on previously identified CLL drivers and used stringent quality filters and visual inspection to remove all possible false positive calls. Next, we annotated all remaining variants with the latest versions of public and cancer-specific databases, in silico prediction scores, as well as the CLL literature to systematically identify all variants of potential pathogenicity. This approach is however limited by the quality of existing databases.

Our targeted NGS analysis confirms the increased incidence of the TP53, NOTCH1 and SF3B1 mutations in relapsing patients matching the range of what was previously reported in Sanger sequencing studies including R/R CLLs. Indeed, the frequency of NOTCH1 mutation remains comparable at around 12-14.9% among both previous studies and ours. Statistically significant differences in the incidence of TP53 mutations were found between our study (22.8% of cases) and the ERIC study (13%) and the CLL2H (39%) but not CLL3X trials (30%). The frequency of SF3B1 mutations was also statistically different between our study (28.1%) and the ERIC study (16%), but not the CLL2H (17.5%) or CLL3X (26%). These differences in the mutation incidence might be explained by different inclusion criteria such as definitions of relapse and refractoriness. In addition, although our study is not reporting on gene mutations occurring only in very minor sub-clones (i.e. VAF < 8%), targeting NGS is more sensitive than Sanger Sequencing and exon-wide as opposed to focused on mutation hotspots.

Furthermore, our study provides new insights in the spectrum of gene mutations of R/R CLL, which, overall, present with a high frequency of other gene mutations. While poorly documented so far, notably at relapse, frequency of ATM mutations is markedly increased as compared with series of
patients in need of first treatment.\textsuperscript{32,33} We also show that SAMHD1 mutations are more common compared to untreated patients\textsuperscript{34} in addition to mutations in XPO1 (14.9\% vs 3.4\%).\textsuperscript{11} The incidence of BIRC3 mutations is 2-fold higher than in untreated patients.\textsuperscript{10,12} Finally, MED12 mutations are more frequent in our cohort than in those recently reported (5.2\%).\textsuperscript{35} By contrast, MYD88 is rarely affected at relapse, which is consistent with its association with mutated IGHV status.

To investigate the prognostic significance of gene mutations at relapse, we chose to focus our analysis on 114 patients coming from prospective clinical trials to avoid potential bias in terms of response evaluation or survival indicators. While the patients come from 3 trials, response and survival do not significantly differ between them in univariate analysis and we included trial in the multivariate analysis to avoid a potential trend of treatment effect.

Other recent prognostic studies of R/R CLL focused on the TP53, SF3B1 and NOTCH1 genes but not ATM.\textsuperscript{15-17} We confirm that neither SF3B1 nor NOTCH1 mutations adversely influence the outcome when evaluated at relapse. The presence of NOTCH1 mutation is even associated with a significantly better PFS, which is supported by the previous analysis of the CLL2H trial.\textsuperscript{15} The present study also shows that, if considered individually, no other gene mutations except for TP53 or BIRC3 impact on prognosis in the present analysis. In line with such observations, Rossi et al. have proposed a hierarchical model in which these two genomic events were considered as conferring the worst outcome in CLL.\textsuperscript{12}

Importantly, our work challenges conventional single gene analyses aimed at establishing a specific prognosis for each driver. Indeed, we clearly demonstrate that contrary to previous studies\textsuperscript{36} most R/R CLL cases have more than one recurrently mutated gene and that these are not mutually exclusive. Therefore an accurate prognostic dissection of R/R CLL requires the integration of such mutational complexity.

Complex karyotype is defined by multiple numerical/structural cytogenetic changes and discriminates also poor outcome in patients with CLL undergoing salvage treatment.\textsuperscript{37,38} The number
of gene mutation did not seem to correlate with number of cytogenetic abnormalities revealed by metaphase cytogenetics in the 46 documented patients of our series (data not shown). However, there is evidence from previous whole genome array studies that there is a strong correlation between genomic complexity and the presence of TP53 disruption.\textsuperscript{39,40} Therefore, large patient cohorts will be required to unravel potential independence of these phenomena.

Evolutionary principles in CLL as in other cancers supposes that genetic diversification may fuel clonal evolution.\textsuperscript{3,9,18,39} Using our targeted NGS approach on bulk leukemia cells, the likely sub-clonal distribution of the MH-profile could only be inferred. Longitudinal genome-wide studies including multiple time-points or single cell approaches are required to precisely reconstruct the tumor phylogeny.\textsuperscript{18} However, within the limitations of the single time-point targeted approach presented here, our results are intriguing and suggest different patterns of clonal evolution in which TP53 mutation appears as a late event, which is in line with recently reported mathematical modeling.\textsuperscript{41} Furthermore, our data are consistent with the idea that genomic complexity, intra-tumor heterogeneity and cooperation between certain mutations may drive CLL evolution. Indeed, the OS of patients with more than one mutated gene was significantly shorter in our study. In line with our results, genomic intra-tumor heterogeneity by itself was recently linked to poor outcome since the presence of subclonal drivers were shown to adversely influence survival.\textsuperscript{9,18,42} Conversely, regarding response to therapy and PFS, it seems that the combinations of two of the TP53, ATM and SF3B1 gene mutations are the most powerful combination to provide therapeutic resistance. Interestingly, these three genes are all known to be involved in DNA damage response.\textsuperscript{43} Therefore, multiple hits of the TP53, ATM and SF3B1 genes might specifically cooperate to drive tumor resistance in addition to being a surrogate for genomic complexity.

Clearly, our results demonstrate that the heterogeneity in survival after cytotoxic therapy of patients with R/R CLL is at least in part due to a specific acquired mutational architecture. It remains to be seen whether this will continue to be the case in the BCR inhibitors era. Our results argue for the
systematic evaluation of mutational profiles including ATM status of patients with R/R CLL to assess the accurate prognosis within future clinical trials to establish whether those with ultra-high risk disease are patients who will benefit most from novel non-chemotherapy based targeted multi-modality regimen such as novel CD20-targeting antibodies, BCL2 or BCR inhibitors combinations.
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Authorship

Contribution: R.G., P.R., O.T, A.S. designed the research, analyzed the data and wrote the paper. B.P. performed statistical analyses. F.N.K. performed FISH analyses. F.D. performed IGHV genes sequencing. P.R., R.C., A.T., M.C., A.B., R.A. and A.H. designed, performed and analyzed NGS experiments. J.M., S.J.L.K. and J.T. supervised NGS work. S.D.G. and O.T. designed the ICLL01 trial and enrolled patients. M.S.D., L.Y. and V.L enrolled patients in the ICLL01 trial. P.H., A.P., L.V., P.C., H.M.B and M.L.G.T provided samples. All the authors approved the final version of this manuscript.
Conflict-of-interest disclosure: V.L. has received honoraria, consultancy, and advisory board fees from Roche, Janssen, GSK and Gilead. The remaining authors declare no competing financial interest.

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References


Table 1.

**Multivariate analysis for progression-free survival (treatment adjusted)**

<table>
<thead>
<tr>
<th>Prognostic factors</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
<th>P*</th>
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<td>Fludarabine-refractoriness</td>
<td>3.36</td>
<td>1.65-6.86</td>
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<td>TP53 mutation</td>
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<td>Multiple-hit profile</td>
<td>2.24</td>
<td>1.24-4.03</td>
<td>0.007</td>
<td>0.015</td>
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Including variables with $P < 0.1$ in univariate analysis (i.e. fludarabine-refractoriness, TP53 mutation, ATM mutation, Multiple-hit profile and trial)

*After bootstrapping process (1000 samples)
Table 2.

Multivariate analyses for overall survival (treatment adjusted)

<table>
<thead>
<tr>
<th>Multivariate models</th>
<th>Prognostic factors</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
<th>P*</th>
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</thead>
<tbody>
<tr>
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<td></td>
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</tbody>
</table>

Both models include variables with \( P < 0.1 \) in univariate analysis (i.e. Binet/RAI staging, fludarabine-refractoriness, BIRC3 mutation, trial). In addition, model #1 includes cluster #3 & #4 vs #1 & #2 and model #2 includes cluster #3 (multiple-hit profile) vs #1, #2 & #4.

*After bootstrapping process (1000 samples)
**Figure Legends**

**Figure 1.** Incidence of gene mutations and correlation with genomic features. A) Number and type of mutation (left) and number of affected patients (right) of each sequenced gene. (B) Circos diagrams illustrating pairwise co-occurrence of gene mutations with cytogenetics (left, n = 110 patients) and IGHV status (right, n = 96 patients). The length of the arc depicts the frequency of the marker and the width of the ribbon corresponds to the proportion of co-occurrence with the second marker. Co-occurrences among genes mutations or among cytogenetics are not shown. 17p, 17p deletion; 11q, 11q deletion; IGHV M, mutated IGHV; IGHV UM, unmutated IGHV; *, $P < .05$.

**Figure 2.** Clustering diagram of gene mutations distribution. Rows correspond to cytogenetics (11q or 17p deletion) or sequenced gene and columns represent individual patients. Patients are clustered according to i) number of genes mutations (0 in cluster #1, 1 in cluster #2, ≥2 in cluster #3 & #4) and ii) recurrence of combinations (≥5% of cases in cluster #3 or <5% of cases in cluster #4). Color-coded is based on the marker status (white: no 11q deletion, no 17p deletion or no gene mutation, black: 17p deletion or 11q deletion, red: gene mutated, gray: missing data).

**Figure 3.** Recurrent patterns of clonal evolution in the multiple-hit profile cluster. Only the locus for TP53, ATM and SF3B1 are represented. The figures in percentage (%) represent the proportion of cells carrying the mutations. The VAFs represent the frequency of alleles affected by the mutation. The top cell represents the closest common ancestor. The bottom cells represent the cell population when the sample was collected (pre-treatment).

**Figure 4.** Impact of the multiple-hit profile on progression-free survival in univariate analysis. (A) Kaplan-Meier curves of progression-free survival according to the presence of the multiple-hit profile (combinations of mutations comprising ≥2 of the following genes: TP53, SF3B1 and ATM) in the whole population (A) and in the TP53 disruption subgroup (B).
Figure 5. Impact of the multiple-hit profile on overall survival in univariate analysis. (A) Kaplan-Meier curves of overall survival according to the presence of the multiple-hit profile (combinations of mutations comprising ≥2 of the following genes: TP53, SF3B1, and ATM), the cluster #4 and the clusters #1 & #2.
Figure 1.
Incidence of gene mutations and correlation with genomic features in 114 patients with relapsed/refractory CLL.
Figure 2.
Clustering diagram of gene mutations distribution in the 114 patients with relapsed/refractory CLL.
Figure 3.
Recurrent patterns of clonal evolution in the multiple-hit profile cluster.

**Example of pattern 1**
(patient 201-2635)

VAFs

- ATM 0.486
- ATM 0.665
- SF3B1 0.484

**Example of pattern 2**
(patient 202-2316)

VAFs

- ATM 0.512
- TP53 0.253

**Example of pattern 3**
(patient 201-2545)

VAFs

- ATM 0.875
- SF3B1 0.0806

- ATM 0.875
- SF3B1 0.0806

- ATM 11q 0.27
- ATM 11q 0.79
Figure 4. Impact of the multiple-hit profile on progression-free survival.

A

\[ P = 0.003 \]

Progression-free survival

No multiple-hit profile
\( n = 92 \)

Multiple-hit profile
\( n = 22 \)

Time (Months)

B

\[ P = 0.03 \]

Progression-free survival

TP53-disruption without multiple-hit profile
\( n = 21 \)

TP53-disruption with multiple-hit profile
\( n = 14 \)

Time (Months)
Figure 5.
Impact of the multiple-hit profile on overall survival.

\[ P = .008 \]

- Cluster \#1 and \#2
  - \( n = 71 \)

- Multiple-hit profile
  - \( n = 22 \)

- Cluster \#4
  - \( n = 21 \)
Presence of multiple recurrent mutations revealed by targeted NGS confers poor trial outcome of relapsed/refractory CLL

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