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Prognostic impact of genetic abnormalities in 536 first-line chronic lymphocytic leukaemia patients without 17p deletion treated with chemoimmunotherapy in two prospective trials: Focus on IGHV-mutated subgroups (a FILO study)

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► **To cite this version:**

Florence Nguyen-Khac, Marine Baron, Romain Guièze, Pierre Feugier, Alexandra Fayault, et al.. Prognostic impact of genetic abnormalities in 536 first-line chronic lymphocytic leukaemia patients without 17p deletion treated with chemoimmunotherapy in two prospective trials: Focus on IGHV-mutated subgroups (a FILO study). *British Journal of Haematology*, 2024, 205 (2), pp.495-502. <10.1111/bjh.19459>. <hal-04796762>

HAL Id: hal-04796762

<https://hal.science/hal-04796762v1>

Submitted on 22 Nov 2024

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HAL Authorization

1 **Prognostic impact of genetic abnormalities in 536 first-line chronic lymphocytic leukaemia patients**
2 **without 17p deletion treated with chemoimmunotherapy in two prospective trials: focus on IGHV**
3 **mutated subgroups (a FILO study)**

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32
33 **Running title:** Genetic impact according to IGHV status in CLL

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35 **Key words:** CLL, prognosis, IGHV, cytogenetic abnormalities, mutations
36

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45

1 Abstract word count: 200
2 Text word count: 2207
3 Figures: 2
4 Tables: 2
5 Supplementary tables: 8
6 Supplementary figures: 1
7 References: 30
8
9
10
11
12
13
14
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1 **Abstract**

2 The potential prognostic influence of genetic aberrations in chronic lymphocytic leukaemia (CLL) can
3 vary based on various factors, such as the immunoglobulin heavy variable (IGHV) status. We
4 conducted an integrative analysis on genetic abnormalities identified through cytogenetics and
5 targeted NGS in 536 CLL patients receiving first-line chemo(immuno)therapies (CIT) as part of two
6 prospective trials. We evaluated the prognostic implications of main abnormalities, with specific
7 attention to their relative impact according to IGHV status. In the entire cohort, unmutated (UM)-
8 IGHV, complex karyotype, del(11q), and *ATM* mutations correlated significantly with shorter PFS.
9 Focusing on the subset of M-IGHV patients, univariate analysis showed that complex karyotype,
10 del(11q), *SF3B1*, and *SAMHD1* mutations were associated with significant lower PFS. The prognostic
11 influence varied based on the patient's IGHV status, as these abnormalities did not affect outcomes
12 in the UM-IGHV subgroup. *TP53* mutations had no significant impact on outcomes in M-IGHV
13 subgroup. Our findings highlight the diverse prognostic influence of genetic aberrations depending
14 on the IGHV status in symptomatic CLL patients receiving first-line CIT. The prognosis of gene
15 mutations and cytogenetic abnormalities needs to be investigated with a compartmentalized
16 methodology, taking into account the IGVH status of patients receiving first-line BTK and/or BCL2
17 inhibitors.

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1 Introduction

2 Chronic lymphocytic leukaemia (CLL) is a genetically and clinically heterogeneous disease.
3 Cytogenetic abnormalities, IGHV mutational status and more recently gene mutations have led to a
4 better understanding of CLL heterogeneity and a better characterization of patient
5 prognosis(Lazarian *et al*, 2017). Mutations of specific genes including *TP53*, *ATM*, *BIRC3*, *SF3B1*,
6 *NOTCH1*, *EGR2*, *BRAF* or *KRAS* have been associated with poorer outcome, either shorter time to first
7 treatment (TFT), progression-free survival (PFS) and/or overall survival (OS)(Nadeu *et al*, 2016;
8 Blakemore *et al*, 2020; Knisbacher *et al*, 2022; Mansouri *et al*, 2023). However, the frequency and
9 clinical impact of these mutations differed among the analyzed cohorts. Several factors, such as age,
10 variant allele frequency (VAF), co-existing driver mutations and/or chromosomal alterations, could
11 influence the clinical significance of a driver mutation in an individual patient. Therefore, integrative
12 analyses of cytogenetics, IGHV status, and gene mutations are essential to accurately evaluate
13 patient prognosis.

14 Recent years have seen a paradigm change in CLL therapies with the advent of BTK inhibitors (BTKi),
15 the BCL2 inhibitor (BCL2i) venetoclax and the novel anti-CD20 monoclonal antibody obinutuzumab
16 which have been transformative in CLL management(Shadman, 2023). These targeted therapies have
17 demonstrated improved PFS and OS over chemoimmunotherapy (CIT) regimens, mainly
18 bendamustine-rituximab (BR) and chlorambucil-obinutuzumab (CO) in randomized phase III studies
19 in relapsed/refractory or first-line (1L) CLL. However, some questions remain, including whether CIT
20 may still an acceptable option in certain CLL subpopulations, i.e IGHV mutated patients receiving 1L
21 fludarabine-cyclophosphamide-rituximab (FCR). Recently, the fixed-duration combination of BTKi and
22 CIT for 1L treatment of CLL patients was shown to achieve a high rate of undetectable minimal
23 residual disease (uMRD) and sustained long-term remission even after discontinuing BTKi(Davids *et*
24 *al*, 2019; Jain *et al*, 2021; Michallet *et al*, 2023).

25 The aim of this study was to evaluate, in prospective trials using 1L CIT, frequencies of gene
26 mutations, their association with IGHV status and cytogenetic abnormalities and their impact on
27 outcome. To address these questions, we undertook an integrative study including karyotype,
28 fluorescence in situ hybridization (FISH), IGHV status and targeted next generation sequencing (NGS)
29 in two French prospective clinical trials using 1L CIT.

30

31 Material and methods

32 We studied 536 CLL patient samples taken at randomization of two prospective phase 3 trials using
33 1L fludarabine-based CIT (CLL 2007 FMP [NCT00564512] and CLL 2007 SA [NCT00645606]) (Lepretre
34 *et al*, 2012; Dartigeas *et al*, 2018). The CLL FMP 2007 was a phase 3 multicenter trial that involved
35 untreated CLL patients, who were randomly assigned to receive six courses of oral fludarabine and
36 cyclophosphamide (FC) in combination with either rituximab (FCR) or alemtuzumab (FCCam). The CLL
37 FA 2007 was a randomised, multicentre phase 3 trial that included treatment-naïve and fit CLL
38 patients aged 65 years or older. Patients received four cycles of FCR and were randomly assigned, in
39 case of response, to either receive rituximab maintenance every 8 weeks for up to 2 years or
40 undergo observation. Patients were diagnosed using the iwCLL guidelines (Hallek *et al*, 2018), with
41 informed consent obtained in accordance with the declaration of Helsinki. All CLL patients were
42 symptomatic Binet stage B or C and devoid of 17p deletion. Demographic, clinical and biological
43 characteristics of both cohorts are summarized in **Supplementary Table S1**. Our study included
44 patients from these two prospective trials whom DNA material was available for NGS and who
45 received fludarabine-based regimens. These patients (n=536) did not significantly differ from those

1 who were excluded due to insufficient DNA or not receiving protocol treatment (n=154)
2 **(Supplementary Table S1)**. We also did not observe significant differences when excluding patients
3 that received alemtuzumab (n=70) in terms of prognostic factors associated or not with PFS and OS
4 (data not shown). The average lymphocyte percentage of the total white cell count in pre-treatment
5 blood samples was 85% (range, 60-100%; interquartile [IQR]25-75, 82-89%). Baseline assessments
6 included conventional karyotype (K) and FISH analysis for del(13q), trisomy 12 (tri12), del(11q),
7 del(17p) and IGHV mutational status. A karyotype was defined as complex (CK) if ≥ 3 clonal
8 aberrations were present in chromosome banding analysis (CBA) (International Standing Committee
9 on Human Cytogenetic Nomenclature, 2020). Highly CK (high-CK) and non-high-CK were defined if ≥ 5
10 and 3 or 4 clonal aberrations were identified respectively.

11 We investigated by targeted deep sequencing 33 genes and 2 non-coding regions including *TP53*,
12 *SF3B1*, *NOTCH1*, *ATM*, *BIRC3*, *NFKBIE*, *EGR2*, *MYD88* and *POT1* (see **Supplementary Tables S2**).
13 Briefly, DNA was extracted from total blood mononuclear cells. Targeted regions were PCR-amplified
14 using an AmpliSeq (Life technologies) approach and PCR products sequenced in a MiSeq (Illumina)
15 instrument with a mean depth of 1363X (range, 500-22460; IQR25-75, 700-1424) and a detection
16 level of 1%. Targeted sequencing was analyzed according to previously described algorithms with
17 minor modifications (Damm *et al*, 2014). Mutations were considered as clonal if the VAF was $\geq 40\%$
18 and subclonal (high/low) if VAF was between 12.5 and 40% / $\leq 12.5\%$, respectively. This latter cutoff
19 value represents the common detection threshold of mutations by Sanger sequencing (Rossi *et al*,
20 2014). Driver mutations were assigned to signaling pathways (DNA damage, chromatin modification,
21 RNA processing, MAP kinase signaling, NOTCH signaling, NFKB signaling, BCR signaling;
22 **Supplementary Table S3**). A pathway was considered altered if at least one gene in the pathway was
23 mutated.

24 Main clinical and biological characteristics were collected and included in analysis of prognostic
25 factors (age, gender, Binet stage, response to therapy including minimal residual disease [MRD]
26 assessment, FISH, chromosomal abnormalities and mutations). Only subgroups of five or more
27 patients were included for prognostic analyses. Quantitative variables are presented as
28 mean/median and range/IQR according to their distribution. Categorical variables are presented as
29 numbers and related percentage. Analyses were performed as intent to treat. PFS was defined as the
30 time between randomization and the first documented disease progression, death from any cause,
31 or last follow up for surviving patients. PFS and OS were estimated by the non-parametric Kaplan-
32 Meier method and then compared between groups by the log-rank test. The significance level of $p <$
33 0.05 was applied and statistical analyses were performed using the software SAS 9.3 (SAS Inc, Cary,
34 NC) and R v3.4.4 (<http://www.R-project.org>).

35

36 **Results**

37 Main clinical and biological characteristics of CLL patients included in both prospective trials are
38 summarized in **Supplementary Table S1**. Both cohorts harbored similar characteristics particularly
39 regarding IGHV status, cytogenetic abnormalities and gene mutations, with the exception of older
40 age at inclusion, related to specific inclusion criteria, and higher frequencies of *NOTCH1* and *KMT2D*
41 mutations in the 2007 SA cohort. Considering the whole study population (n=536, **Table 1**),
42 unmutated (UM) IGHV status was observed in 293/522 (56%) cases. The median number of
43 cytogenetic abnormalities per sample was one (range, 0-11). Chromosomal abnormalities by K were
44 observed in 319/470 (68%) of evaluable samples, including 17% CK, 4% high-CK, and 30% carrying

1 translocations. FISH analyses revealed 296/503 (59%) del(13q), 88/522 (17%) tri(12) and 114/534
2 (21%) del(11q). Due to specific inclusion criteria, no patient harbored del(17p). For a total of 1121
3 mutations, the median number of gene mutations was two per patient (range, 0-7) (see
4 **Supplementary Table S4** for the complete list of variants). At least one mutation was observed in
5 443/536 (83%) patients, including 117 (22%) harboring one mutation, 127 (24%) two mutations and
6 199 (37%) three or more mutations. The most frequent mutations were identified in *SF3B1* (24%),
7 *NOTCH1* (24%), *ATM* (18%), *NFKBIE* (10%), *KMT2D* (8%), *XPO1* (7%), *POT1* (7%), *EGR2* (7%), *PAX5*
8 enhancer region (7%), *TP53* (6%), *BRAF* (6%), *IRF4* (4%), *FBXW7* (4%) and *BIRC3* (4%) (**Table 1** and
9 **Figure 1A**). *MYD88* and *SAMHD1* mutations were mostly observed as clonal events whereas *BIRC3*,
10 *KRAS* and *NRAS* mutations as subclonal ones (**Supplementary Table S4**). According to pathway
11 assignment, one, two, three and more than three pathways were altered in 177 (33%), 109 (20%), 62
12 (12%), and 37 (7%) patients, respectively. The most frequently altered pathways were NOTCH
13 signaling (26%), DNA damage (23%), and NFKB signaling (16%).
14 Significant associations between cytogenetic and molecular characteristics are detailed in
15 **Supplementary Table S5**. Selected significant associations are presented in **Figure 1B**. In particular,
16 *NOTCH1* mutations were associated with UM-IGHV ($P<0.0001$) and tri(12) ($P=0.002$); *FBXW7*
17 mutations with tri(12) ($P=0.001$); *XPO1* and *NFKBIE* mutations with UM-IGHV ($P=0.0007$ and $P=0.003$
18 respectively); *ATM* mutations with del(11q) ($P=0.0005$); *TP53* with high-CK ($P=0.02$); and *MYD88* and
19 *PAX5* enhancer mutations with M-IGHV ($P=0.0002$ and $P=0.01$ respectively). The frequencies of main
20 cytogenetic and molecular characteristics were thus different in M- and UM-IGHV subpopulations, as
21 represented in **Table 1** and **Figure 1C**.

22 The median follow-up for the whole study population was 61 months (IQR25-75, 40-70). Five-year
23 PFS and OS were 54% (CI95%, 48-60) and 80% (CI95%, 75-83), respectively. Univariate analyses
24 associated with PFS and OS for the whole study population are represented in **Supplementary Table**
25 **S6** and **Supplementary Figure S1**. Prognostic factors associated with shorter PFS included UM-IGHV
26 ($P<0.0001$), CK ($P=0.003$), del(11q) ($P=0.001$), *ATM* mutations ($P=0.01$), DNA damage pathway
27 ($P<0.0001$), the number of mutations ≥ 3 ($P=0.01$), while del(13q) was associated with longer PFS
28 ($P=0.002$). A trend toward shorter PFS was observed in *TP53*-mutated patients ($P=0.06$). OS was
29 pejoratively impacted by UM-IGHV ($P=0.01$), *ATM* ($P=0.01$), *TP53* ($P=0.02$), *FBXW7* ($P=0.002$) and *IRF4*
30 ($P=0.005$) mutations. Del(13q) was associated with longer OS ($P=0.03$). By multivariate analyses, UM-
31 IGHV retained prognostic significance for shorter PFS and OS (**Supplementary Table S6**).

32 Looking specifically to M-IGHV subgroup (n=229), 44% harbored normal karyotype, 60% isolated
33 del(13q) and 9% del(11q) by FISH, which significantly differ from UM-IGHV subgroup (n=293; 24%,
34 28% and 32%, respectively; $P<0.0001$ for each comparison) (**Table 1** and **Figure 1C**). The distribution
35 and frequencies of mutations and altered pathways were also different between M- and UM-IGHV.
36 In the M-IGHV subgroup, the most frequent molecular abnormalities were *SF3B1* (24%), *ATM* (16%)
37 and *NOTCH1* (11%) mutations along with *PAX5* enhancer (10%) variants, while mutations of *NFKBIE*
38 (5%), *EGR2* (4%) and *XPO1* (3%) were uncommon. The mean number of mutations was lower in M-
39 IGHV subgroup and the proportion of patients without any mutation significantly higher (22% vs.
40 13%, $P=0.01$). In this subgroup of M-IGHV patients receiving first-line fludarabine-based regimens,
41 univariate analysis (**Table 2**, **Figure 2**) showed that CK, del(11q), *SF3B1* and *SAMHD1* mutations, and
42 DNA damage pathway were associated with shorter PFS. The shortest PFS were observed in patients
43 harboring CK and del(11q) with respective median PFS of 53.7 (95%CI, 46.6-not reached) and 52.0
44 (95%CI, 43.3-70) months. Nonetheless, no molecular or cytogenetic abnormalities had a significant

1 impact on OS, with the exception of *XPO1* mutations; although it should be taken with caution
2 regarding the low number of *XPO1*-mutated patients (n=6). *TP53* mutations (n=12) did not impact
3 significantly outcomes in M-IGHV patients. Too few samples (n=4) were mutated for *IRF4* and *FBXW7*
4 in M-IGHV subgroup to assess reliably their specific prognostic impact. By multivariate analyses, only
5 del(11q) retained prognostic significance for shorter PFS ($P=0.005$) (**Supplementary Table S7**).
6 Cytogenetic and molecular abnormalities that impact prognosis differed in the UM-IGHV subgroup
7 (**Figure 2J** and **Supplementary Table S8**). Notably, CK, del(11q), *SF3B1* and *SAMHD1* mutations, which
8 were associated with shorter PFS in M-IGHV, had no impact in the UM-IGHV subgroup. On the other
9 hand, *ATM* mutations resulted in significantly shorter PFS/OS, while del(13q) resulted in longer
10 PFS/OS in UM-IGHV, which was not observed in M-IGHV.

11 **Discussion**

12 Adverse genetic features in the context of CIT have been largely described and include, among the
13 most significant, UM-IGHV, del(11q), CK/high-CK, *NOTCH1/SF3B1/BIRC3* mutations and *TP53*
14 abnormalities (Rossi *et al*, 2011, 2013; Baliakas *et al*, 2015). However, clinical impact of genetic
15 abnormalities varied between analyzed cohorts, depending in part on the type of CIT used (CO, BR or
16 FCR) (Knisbacher *et al*, 2022; Rossi *et al*, 2011; Tausch *et al*, 2020a; Spunarova *et al*, 2019), the stage
17 of the disease and associated genetic landscape.

18 Recent studies have indeed demonstrated that mutations may affect outcomes differently in UM-
19 and M-IGHV, in Binet stage A (Mansouri *et al*, 2023; Baliakas *et al*, 2019) and in global CLL cohorts
20 (including both asymptomatic and symptomatic patients, receiving either CIT or BTKi) (Knisbacher *et al*,
21 2022). Comprehensive multiomic analyzes have also recently strongly suggested that UM- and M-
22 IGHV harbored unique and distinct mechanisms of leukemogenesis (Knisbacher *et al*, 2022).

23 Our work focused on a large CLL cohort of 536 patients treated with 1L fludarabine-based regimens
24 as part of two prospective trials. We undertook an integrative analysis of both cytogenetic and
25 molecular abnormalities identified through FISH, CBA, and targeted NGS on 33 genes and 2 non-
26 coding regions, to evaluate the prognostic implications of principal abnormalities, with a particular
27 focus on their relative impact in UM- and M-IGHV. Our study has some limitations. Specifically, the
28 population received CIT, which is currently restricted in CLL. Additionally, del(17p) patients were
29 excluded, potentially influencing the genetic landscape that has been identified; finally, data on
30 stereotyped subsets, particularly subset #2, which has been linked to a worse prognosis in M-IGHV,
31 were not available.

32 The main genetic features identified in our study were in line with those reported previously in
33 symptomatic CLL cohorts, as were the noteworthy links between certain mutations and IGHV status,
34 in particular *NOTCH1/NFKBIE/XPO1* mutations with UM-IGHV and *MYD88/PAX5* enhancer mutations
35 with M-IGHV (Jeromin *et al*, 2014; Puente *et al*, 2015). The significantly lower number of mutations in
36 M-IGHV was also consistent with previous large-scale genomic studies (Knisbacher *et al*, 2022). In the
37 present study, we provide insights into the distinct genetic landscape and varying prognostic impact
38 of genetic abnormalities based on IGHV status of symptomatic CLL patients. We also focused on the
39 specific aspects of the M-IGHV subgroup, in this cohort of 1L CLL patients without del(17p) treated
40 prospectively by fludarabine-based regimens.

41 In M-IGHV patients, we have shown in particular that CK, del(11q), *SF3B1* and *SAMHD1* mutations
42 pejoratively impacted PFS, while *NOTCH1* and *TP53* mutations had no significant impact. These
43 results regarding del(11q), *SF3B1* and *NOTCH1* mutations were concordant with those reported
44 recently in M-IGHV by Knisbacher *et al* (Knisbacher *et al*, 2022) using large-scale genomic sequencing.

1 The specific prognostic value of *SF3B1* mutations could be clarified by taking into account subset #2,
2 which is known to have a negative prognostic impact in M-IGHV and is associated with the presence
3 of *SF3B1* mutations(Jaramillo *et al*, 2020). Regarding the lack of impact of *TP53* mutations in the M-
4 IGHV group, the limited number of mutated patients (n=12) and the exclusion of del(17p) must be
5 taken into account, as numerous studies have shown that prognosis can vary according to the
6 presence of one or more *TP53* abnormalities (mutation and/or deletion)(Malcikova *et al*, 2009;
7 Griffin *et al*, 2023). However, these data are consistent with the lack of impact of *TP53* mutations
8 observed in M-IGHV patients with stage A CLL(Mansouri *et al*, 2023).

9 Whilst CIT is increasingly restricted in CLL, recent findings have revealed that frontline fixed-duration
10 combinations with fludarabine-based CIT and BTKi could lead to remarkable response rates and
11 PFS(Davids *et al*, 2019; Jain *et al*, 2021; Michallet *et al*, 2023). The potential impact of genetic
12 abnormalities regarding IGHV status may be examined in this context, should such combinations be
13 developed in the future. Unlike CIT, durable remissions are seen with 1L venetoclax combination and
14 BTKi, regardless of IGHV status, del(11q) or mutations in *ATM*, *SF3B1*, *BIRC3*, and *NOTCH1*(Tausch *et al*,
15 2020b; Barr *et al*, 2022). Adverse prognostic markers still include del(17p), *TP53* mutations and/or
16 high-CK with fixed-duration BCL2i, continuous BTKi, or fixed-duration combinations(Tausch *et al*,
17 2020b; Brieghel *et al*, 2021; Fürstenau *et al*, 2023). Further research is necessary to investigate the
18 precise prognosis of gene mutations and cytogenetic abnormalities in the context of frontline BTKi
19 and BCL2i therapies, which may be conducted with a compartmentalized approach based on IGVH
20 status.

21
22 **Acknowledgements:** We thank M'Boypa Diop for her help in biostatistical analyses.

23
24 **Data availability statement:** The data that support the findings of this study are available from the
25 corresponding author upon reasonable request.

26
27 **Funding sources:** This study was supported by grants from INCA-DGOS-Inserm_12560 (SiRIC
28 CURAMUS is financially supported by the French National Cancer Institute, the French Ministry of
29 Solidarity and Health and Inserm with financial support from ITMO Cancer AVIESAN).

30
31 **Conflict-of-interest disclosure:** No relevant COI

32
33 **Ethics approval statement:** n/a

34
35 **Patient consent statement:** n/a

36
37 **Permission to reproduce material from other sources:** n/a

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39 **Clinical trial registration:** NCT00564512, NCT00645606

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41 **Author contributions:** FNK, OAB and DRW designed the research. FNK and DRW wrote the
42 manuscript. FNK, LS, RG, SR, ND, FD and DRW performed experiments. FNK, MB, RG, XT, VL, CD,
43 EVdN, SL, PF and DRW recruited patients. FNK, MB, RG, AF, SR, SS, VL, CD, EVdN, SL, PF, OAB and
44 DRW analyzed data. All authors reviewed and approved the manuscript.

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Figures legends

Figure 1. Panorama of mutational and cytogenetic abnormalities in a prospective cohort of 536 CLL patients receiving first-line chemo(immuno)therapy regimens. (A) Recurrent gene mutations, non-coding regions variants and cytogenetic abnormalities according to IGHV status (mutated [M-IGHV], blue; unmutated [UM-IGHV], white). Each column represents a patient sample and each row a mutated gene (italic) or cytogenetic abnormality. Colored box: presence; white box: absence; light grey box: not available. **(B)** Co-occurrence and mutual exclusion of selected mutations and cytogenetic abnormalities. **(C)** Percentages of recurrent gene mutations and cytogenetic abnormalities according to IGHV status (mutated [M-IGHV], blue bar; unmutated [UM-IGHV], white bar). CK, complex karyotype; enh, enhancer. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Figure 2. Somatic alterations and prognosis according to IGHV status.

(A-I). Kaplan-Meier curves for progression-free survival (PFS) according to the presence of recurrent mutations and cytogenetic abnormalities in the IGHV mutated (M-IGHV) subgroup. PFS according to the presence (red) or absence (blue) of the following recurrent molecular/cytogenetic abnormalities: CK **(A)**, del(11q) **(B)**, del(13q) **(C)**, *SF3B1* mutations **(D)**, *NOTCH1* mutations **(E)**, *SAMHD1* mutations **(F)**, *TP53* mutations **(G)**, *ATM* mutations **(H)** and DNA damage pathway **(I)**. Dashed lines represent 95% confidence interval. P values are indicated at the left bottom of each survival plot. CK, complex karyotype; enh, enhancer.

(J). Somatic alterations associated with progression-free survival (PFS, left panel) and overall survival (OS, right panel) in M-IGHV (left boxes) and UM-IGHV (right boxes) in univariate analysis. Heatmap denotes hazard ratios (HRs) and stars P values as indicated in the right part of the figure.