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Research paper

Impact of gene mutations and chromosomal aberrations on progression-free survival in chronic lymphocytic leukemia patients treated with front-line chemoimmunotherapy: Clinical practice experience

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ABSTRACT

The impact of genetic aberrations on rituximab-based therapeutic regimens has been intensely studied in chronic lymphocytic leukemia (CLL). According to the current consensus chemoimmunotherapy consisting of rituximab and DNA-damaging drugs is not suitable for patients with *TP53* defects. In our study, we focused on CLL patients with an intact *TP53* gene and investigated four recurrently mutated genes in CLL, genomic aberrations by FISH, and IGHV status with the aim of analyzing their impact on progression-free survival (PFS) after front-line therapy with FCR (fludarabine, cyclophosphamide, rituximab) or BR (bendamustine, rituximab) regimens. Using next-generation sequencing, we analyzed 120 patients treated with FCR and 57 patients treated with BR at a university hospital. We used a 10% cut-off for variant allele frequency and recorded the following mutation frequencies in the pre-therapy samples: *ATM* 23%, *SF3B1* 20%, *NOTCH1* 19% and *BIRC3* 11%. The data on cytogenetic aberrations (11q22, 13q14, trisomy 12) and *IGHV* mutation status were also considered in PFS analyses. In univariate analyses, we observed a negative impact of *BIRC3* mutations and 11q22 deletion in both regimens, while the unmutated *IGHV* status was associated with a significantly shorter PFS only in the FCR-treated cohort. In a multivariate analysis, only deletion 11q22 in both regimens, and the unmutated *IGHV* in the FCR cohort maintained an independent association with the reduced PFS. Notably, sole 11q22 deletion, without an *ATM* mutation on the other allele, manifested the shortest PFS of all analyzed markers. Deletion 11q22 and *IGHV* status predict PFS in previously untreated CLL patients.

1. Introduction

Therapy of chronic lymphocytic leukemia (CLL) is currently undergoing a revolution with the introduction of B-cell receptor signaling inhibitors [1–3]. Nevertheless, fludarabine with cyclophosphamide and rituximab (FCR) still remain a therapeutic option for young and physically fit CLL patients with wild-type *TP53* [4–6]. The FCR is, however, associated with hematologic toxicity, in particular myelosuppression and infectious complications [6–8]. A combination of bendamustine with rituximab (BR) represents an option for physically less-fit patients or those ineligible to receive fludarabine-based treatment [9]. For the FCR regimen, some well-established durable remission markers have been reported, namely mutated *IGHV* status and lack of unfavorable cytogenetic aberrations such as deletions 17p13 and 11q22 [10,11]. For the BR regimen, a simple biological score based on *IGHV* status and the presence of deletions 11q22 and 17p13 has also been reported [12].

In addition, the impact of some recurrently mutated genes has also been investigated in FCR treated patients. Thus, mutations in *NOTCH1* have been associated with the lack of benefit from rituximab added to FC, while *SF3B1* mutations predicted short time to progression after the frontline FCR therapy [13]. Mutations in *NOTCH1* also predicted shortened time to treatment failure after FCR, similar to *SF3B1* mutations or unmutated *IGHV* status [14].

It remains unclear which of these factors are essential to make a real life therapeutic decision in patients routinely treated by chemoimmunotherapy (patients with wild-type *TP53*). Additionally, some recurrently mutated genes have never been, according to our best knowledge, analyzed in relation to progression after FCR or BR treatment. The

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lack of data on prognostic relevance concerns especially mutations in the *ATM*, a gene encoding a kinase with a critical role in p53 pathway activation [15] and representing one of the most frequently mutated genes in CLL [16,17]. Despite indisputable progress, *ATM* mutation identification in CLL remains challenging, due to a large gene size (62 coding exons) and difficult interpretation of some variants, resulting from only vague information about their functional consequences. In addition to *ATM*, we also analyzed mutations in the following genes: *SF3B1*, a splicing factor involved in ribosomal processing of RNA transcripts [18]; *NOTCH1*, a transcription factor aberrantly activated in CLL cells [19,20]; and *BIRC3*, a negative regulator of the NF-κB pathway vital for CLL cell survival [21]. Furthermore, we also considered classic cytogenetic aberrations detected in CLL patients (FISH panel) and the *IGHV* mutation status.

Here we demonstrate that deletion 11q22 in both regimens, and the *IGHV* status in FCR treated patients, have a prognostic power in the previously untreated patients.

2. Materials and methods

2.1. Patient cohort and treatment

The study was performed on purified (see below) peripheral blood samples from 177 CLL patients with no *TP53* defect (*TP53* mutation and/or 17p deletion) treated with a front-line therapy between 2008 and 2016 (FCR) or 2013 and 2016 (BR) at the Department of Internal Medicine, Hematology and Oncology, University Hospital Brno. From this cohort, 68 patients were also investigated in the first relapse. Written informed consent was provided in accordance with the Declaration of Helsinki. Clinical and biological characteristics of the patients are summarized in Table 1. In total, 120 patients were treated with either FCR (n = 89; 74%) or FCR-Lite (n = 31; 26%; reduced doses of the drugs) [22] and 57 patients with BR. Altogether, 171 patients experienced partial or complete remission and were included in the PFS analyses. Six patients were excluded from PFS analyses since they exhibited stable disease or progression during therapy; these patients did not harbor a specific genetic trait. The median age at therapy administration was 61 years in the FCR cohort and 70 years in the BR cohort. The standard FCR treatment consisted of rituximab (375 mg/m^2 in the first cycle and 500 mg/m^2 in the subsequent cycles) followed by fludarabine (25 mg/m^2) and cyclophosphamide (250 mg/m^2). The reduced doses usually involved 12 mg/m^2 of fludarabine and 150 mg/m^2 of cyclophosphamide. For BR the treatment consisted of bendamustine at a dose of 70–90 mg/m^2 and rituximab (375 mg/m^2 in the first cycle and 500 mg/m^2 in the subsequent cycles). The number of cycles provided to a particular patient was at the discretion of the physician, depending on the response and toxicity reflecting a routine clinical approach. The drug dosages were individually modified according to age, renal function, and anticipated or documented myelosuppression. The PFS was defined as time from therapy initiation to clinically observable progression.

Table 1
Clinical and biological characteristics of CLL patients before treatment initiation.

(n = 177)	FCR (n = 120)*		BR (n = 57)	
	No. of patients	*%*	*No. of patients*	*%*
Gender				
Male	75	63	38	67
Female	45	37	19	33
Clinical Stage				
Rai 0/I	33	28	13	23
Rai II	24	20	10	17
Rai III	33	27	14	25
Rai IV	30	25	20	35
IGHV status				
unmutated	90	75	43	75
mutated	30	25	14	25
FISH				
17p13 deletion	0	0	0	0
11q22 deletion	46	38	18	32
Trisomy 12	10	8	9	16
13q14 deletion	60	50	33	58
Normal	27	23	11	19
Mutations				
TP53	0	0	0	0
ATM	26	22	15	26
SF3B1	25	21	10	18
NOTCH1	19	16	15	26
BIRC3	13	11	6	11
no mutation	52	43	23	40

* 74% FCR, 26% FCR Lite

2.2. Mutations analysis by next-generation sequencing

Next-generation sequencing (NGS) analysis was performed on MiSeq (Illumina, San Diego, CA, USA) using gDNA from cryopreserved separated CD19+ B-lymphocytes or mononuclear cells; the percentage of CLL cells (CD5+CD19+) exceeded 80% in all cases (median 96.6%). We did sequencing using three separate panels: (i) *TP53* (exons 2–11), (ii) *SF3B1* (exons 13–16) / *NOTCH1* (hot spot mutation c.7541_7542delCT and part of 3´UTR) / *BIRC3* (exons 7–10) and (iii) *ATM* (exons 2–63) using an in-house multiplex PCR protocol (University Hospital Brno). In all samples, 20 ng of patient DNA was amplified with highly accurate proof-reading Q5 Polymerase (New England Biolabs, Ipswich, MA, USA) using exon-specific primers (Supplemental Table S1). The experimental design and reaction conditions followed the manufacturer's recommendations. Briefly, PCR products were pooled, purified with Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA), and quantified using Qubit dsDNA HS Assay Kit (Life Technologies, Waltham, MA, USA). The purified amplicon pools were diluted to a total amount of 1 ng. The indexed paired-end library was prepared with Nextera XT DNA Sample Preparation Kit (Illumina) and sequenced using MiSeq Reagent Kit v2 300 cycles (Illumina). To avoid cross-contamination, samples obtained from the same patient in different time periods were sequenced in separate runs. PCR amplicons and libraries for each run were prepared separately. The median coverage per base achieved was 31 900 reads for the *TP53* gene, 12 000 reads for *SF3B1/NOTCH1/BIRC3* genes and 6 300 reads for the *ATM* gene. The *ATM* variants' functional impact was verified by on-line tools SIFT and PolyPhen. Reads' quality trimming, merging and mapping onto the reference genome (GRCh37) as well as variant calling, was done using CLC Genomic Workbench [version 7.5]. A cut-off for the variant allele frequency (VAF) was set at 1%. In the PFS clinical analyses, the cumulative cut-off for mutation presence was set at 10% to consider only well selected clones. Twenty mutations (five for each gene) had been repeatedly analyzed in an independent NGS run, and were confirmed in all cases including the similar VAF frequency.

2.3. Other molecular characterization of the cohort

Cytogenetic aberrations were detected by interphase FISH using probes from MetaSystems (Altlussheim, Germany). PCR and direct sequencing were used to analyze the *IGHV* rearrangement and mutation status as described previously [23].

2.4. Immunoblot analysis

Cells were lysed in ice-cold RIPA buffer with protease and phosphatase inhibitor cocktail (Merck). Protein concentrations were determined by BCA Protein Assay Kit (Merck). For *NOTCH1* and *BIRC3* analyses, 50 µg of lysates were separated on 10% sodium dodecyl

sulfate polyacrylamide gel (SDS-PAGE) and transferred to a nitrocellulose membrane. For ATM analysis, 100 µg of lysates were run on NuPAGE Novex 3–8 % Tris-Acetate Gel (Invitrogen) and transferred to a nitrocellulose membrane. The membranes were incubated overnight at 4 °C with the following primary antibodies from Cell Signaling Technology (Danvers, Massachusetts, USA): anti-ATM (clone D2E2), anti-cleaved-NOTCH1 (clone D3B8), anti-BIRC3 (clone 58C7) and anti-β-actin (clone 13E5). The proteins were visualized using Lumi-Light Western Blotting Substrate (Roche) or Clarity Western ECL Substrate (Bio-Rad) and analyzed by Alliance 4.7 software (Uvitec, Cambridge, UK).

2.5. Statistical analyses

The PFS analyses were performed by log-rank (Mantel-Cox) test using the interval from therapy initiation to clinical progression (according to the iwCLL recommendations) [24] or the last follow up. Survival analysis was calculated using the Kaplan–Meier survival estimator. Median survival time and differences between the curves were evaluated by the log-rank test using the GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). The effect of multiple factors on PFS was tested by multivariate Cox proportional hazard regression analyses using Statistica 13.2 (TIBCO Software Inc.). The differences between categorical variables were evaluated by Fisher exact test.

3. Results

3.1. Frequency of mutations and their impact on protein levels

In our study, the only exclusion criterion in the sample selection was the presence of a *TP53* abnormality, since such patients are not indicated for therapy with FCR/BR. We therefore analyzed 177 CLL patients with no *TP53* pre-therapy defect who were treated with at least two cycles (median 4, range 2–6) of front-line FCR or BR therapy. Despite having no *TP53* defects, our cohort had a prognostically unfavorable profile with 75% of the patients harboring the unmutated *IGHV*. The aberrations detected in individual patients are summarized in Fig. 1. Using the NGS cut-off 10% VAF, we observed that only one patient harbored three mutated genes, while 25 patients exhibited two mutated genes, 76 patients had one mutated gene, and 75 patients did not exhibit a single mutated gene. In 38 patients, more than one mutation (VAF > 1%) in a given affected gene was noted.The identified aberrations and biological and clinical characteristics of the patients are summarized in Supplemental Tables S2a and S2b. Concerning the tested genes, we observed the highest mutation frequency for *ATM* (23%), followed by *SF3B1* (20%), *NOTCH1* (19%), and *BIRC3* (11%) genes. We noted the expected co-occurrence of *ATM* mutations with 11q22 deletion ($P < 0.001$) as well as *BIRC3* mutations with 11q22 deletion ($P = 0.012$). We also recorded mutual exclusivity of *NOTCH1* mutations with *SF3B1* mutations ($P = 0.011$) and trisomy 12 with the other two cytogenetic aberrations, *i.e.* deletions 11q22 and 13q14 (Fig. 1).

We also verified the effect of mutations in *ATM*, *NOTCH1* and *BIRC3* genes on the protein level using immunoblotting (Supplemental Figs. S1–S3). This analysis showed that *ATM* mutations resulted in a null or reduced ATM protein level. On the other hand, mutations in *NOTCH1* led to a strong accumulation of the intracellular portion of the protein (ICN1) in some mutated samples. The *BIRC3* mutations led to shortened protein variants, reflecting mostly the presence of frame-shift mutations. Hence, mutations in all three genes were associated with the expected phenotype on the protein level.

3.2. Deletion 11q22 and IGHV status are robust independent markers of shorter PFS

To test for the posibility of merging the FCR and BR treated patients in some analyses we initially compared the PFS in these cohorts. The FCR and BR regimens had a nearly identical median PFS of 27 months and 31 months, respectively (Supplemental Fig. S4). In the merged cohort, we did not observe an impact on the number of administered therapeutic cycles (Supplemental Fig. S5). The subsequent analyses were done separately for both regimens and included all studied genes, cytogenetic aberrations and *IGHV* status. In the case of FCR, we observed significantly shortened PFS for unmutated *IGHV* status (median 22 vs. 52 months in patients with mutated *IGHV*; $P < 0.001$), deletion 11q22 (median 20 vs. 38 months in the other patients; $P < 0.001$) and *BIRC3* mutations (median 20 vs. 30 months in *BIRC3*-wt patients; $P = 0.015$) (Fig. 2 and Supplemental Table S3). Other aberrations did not have an impact on the PFS in the univariate analysis; there was, however, a trend towards a longer PFS for patients with trisomy 12

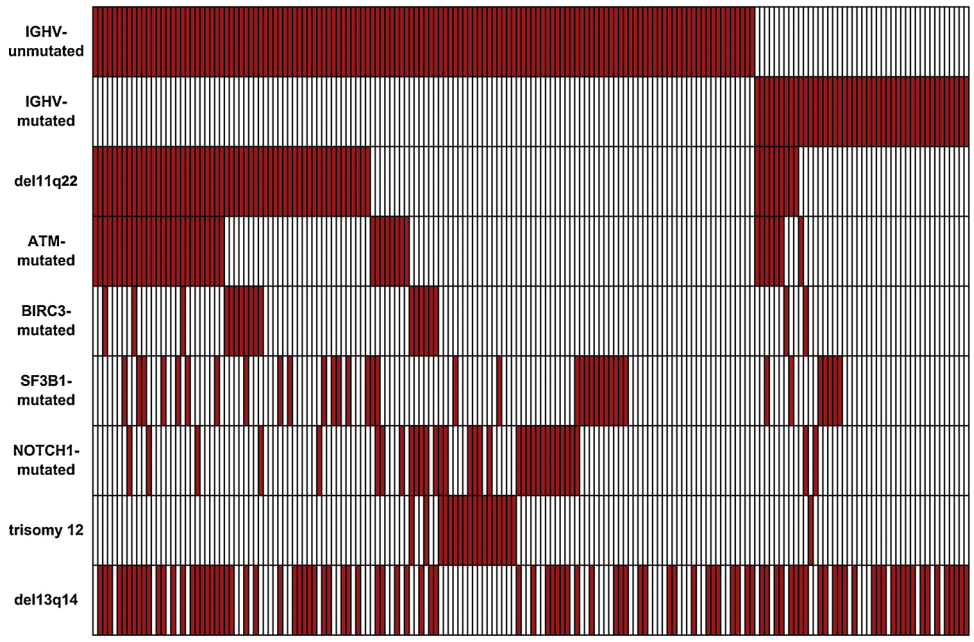

Fig. 1. Distribution of aberrations detected in individual CLL patients before FCR or BR therapy.

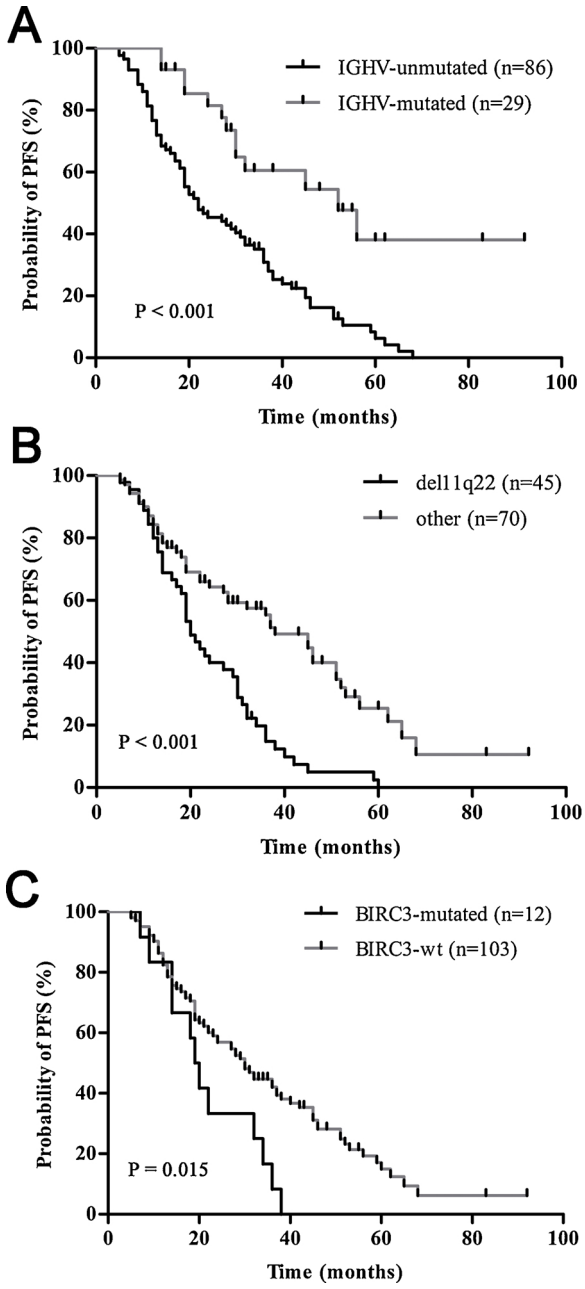

Fig. 2. PFS in 115 CLL patients treated with front-line FCR therapy. (A) *IGHV* status. Median PFS in months: *IGHV*-unmutated: 22; *IGHV*-mutated: 52. (B) The samples harboring del11q22. Median PFS in months: del11q22: 20; other: 38. (C) *BIRC3* mutations. Median PFS in months: *BIRC3*-mutated: 20; *BIRC3*-wt: 30.

(median 51 vs. 28 months in the other patients; P = 0.056) (Supplemental Fig. S6 and Supplemental Table S3). In a multivariate analysis, only unmutated *IGHV* (P = 0.003; HR: 2.5; 95% CI: 1.4–4.7) and deletion 11q22 (P = 0.002; HR: 2.0; 95% CI: 1.3 – -3.2) remained an independent association with the shortened PFS in FCR treated patients (Supplemental Table S4).

In the BR regimen, we observed in the univariant analyses, significantly reduced PFS for deletion 11q22 (median 25 vs. 38 months in the other patients; P = 0.011) and mutations in *BIRC3* gene (median 23 vs. 36 months in the other patients; P = 0.044; Fig. 3 and Supplemental Table S5). All other aberrations had no significant impact on the PFS (Supplemental Fig. S7 and Supplemental Table S5). In the multivariant analysis, only deletion 11q22 remained an independent marker of

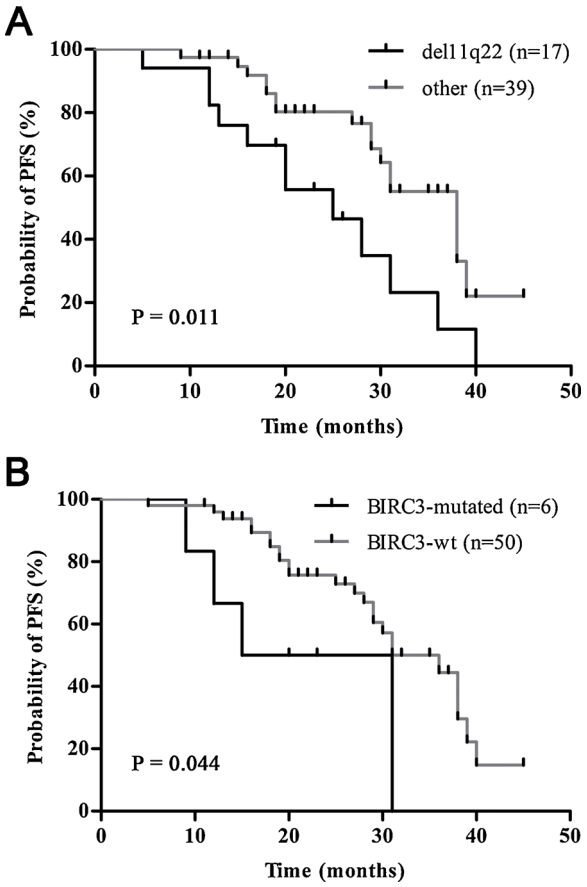

Fig. 3. PFS in 56 CLL patients treated with front-line BR therapy. (A) The samples harboring del11q22. Median PFS in months: del11q22: 25; other: 38. (B) *BIRC3* mutations. Median PFS in months: *BIRC3*-mutated: 23; *BIRC3*-wt: 36.

reduced PFS (P = 0.002; HR = 2.5; 95% CI: 1.2–5.4; Supplemental Table S6).

These data suggest 11q22 deletion's crucial importance on CLL prognosis. Since *ATM* is located in a minimally deleted region, we subsequently compared *ATM* mutations with the sole 11q22 deletion (Fig. 4). In the merged cohort of FCR and BR treated patients, the sole 11q22 deletion, with no *ATM* mutation on the other allele, manifested the worst outcome with the median PFS reaching only 16 months; by

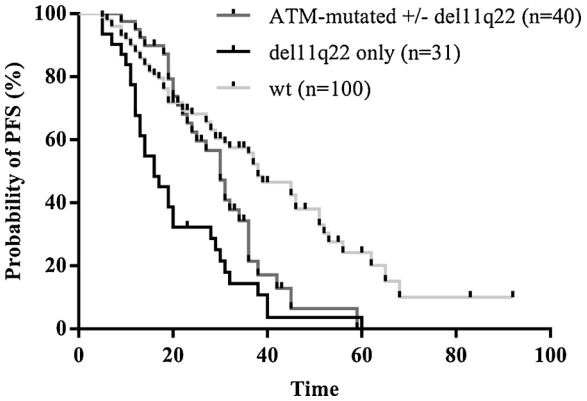

Fig. 4. PFS according to the allelic status of *ATM* gene in 171 patients. Median PFS in months: ATM-mutated +/- del11q22: 31; del11q22 only: 16; wt: 38. Statistical significance: ATM-mutated +/- del11q22 vs. del11q22 only P = 0.009; HR 0.47; 95% CI: 0.27 – 0.83; ATM-mutated +/- del11q22 vs. wt P = 0.014; HR 1.9; 95% CI: 1.1–3.2; del11q22 only vs. wt P < 0.001; HR 4.7; 95% CI: 2.6 – 8.5.

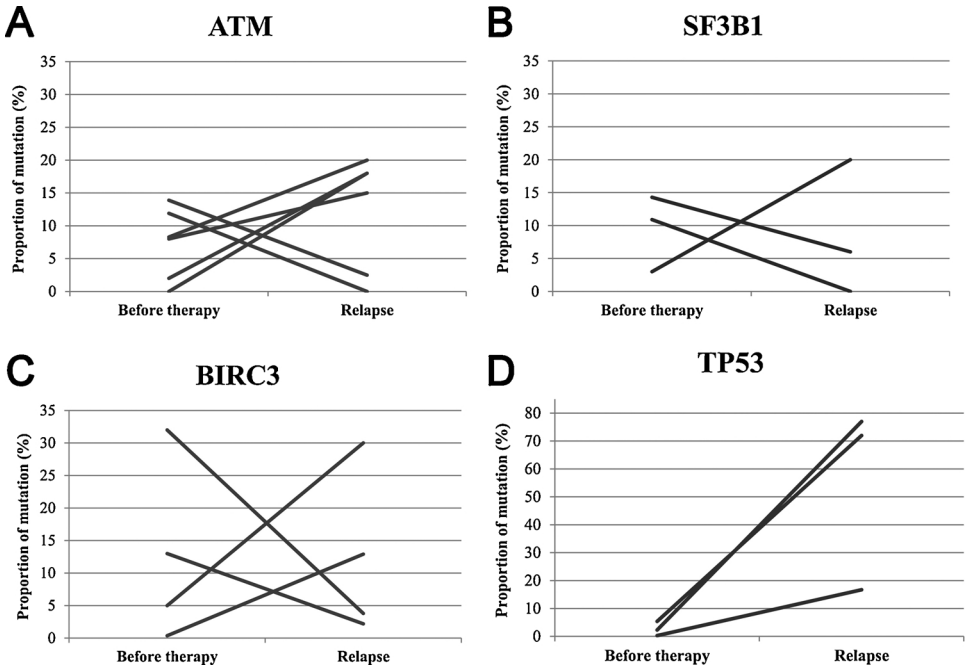

Fig. 5. Clonal evolution of mutations. A) *ATM* gene, B) *SF3B1* gene, C) *BIRC3* gene and D) *TP53* gene. The samples with the change of mutation status (VAF 10%) are shown. Clonal evolution of *NOTCH1* gene was not detected. Clonal evolution of deletion 11q22 is shown in Supplemental Figure S8.

contrast, *ATM* mutations, either alone (n = 9 samples) or accompanying 11q22 deletion (n = 31 samples) were associated with a significantly longer PFS (median 31 months; P = 0.009).

3.3. Clonal evolution of mutations at relapse

We and others have shown that clonal evolution is a frequent phenomenon in CLL patients undergoing therapy [16,17,25]. We therefore analyzed the potential evolution of the four studied genes and also of *TP53* in 136 paired samples obtained from 68 patients before therapy vs. at first relapse (median time between the samplings: 32 months; range 8–87 months). Using again the cut-off 10% VAF, four out of the five genes exhibited a shift in mutation status in a proportion of the samples (Fig. 5). We observed four gains and two losses of *ATM* mutations, one gain and two losses of SF3B1 mutations and two gains and two losses of *BIRC3* mutations. Further, we recorded an expansion of three *TP53* mutations, which is in line with the known *TP53* defects' selection under therapy presure. There was no change in *NOTCH1* mutation status in any sample despite the fact that subclonal *NOTCH1* mutations (VAF between 1–10%) were relatively frequent (n = 8 patients [12%]). Altogether, there was no clear trend towards clonal evolution for any of the four analyzed mutated genes, but notably, the FCR therapy completely eliminated one *ATM* mutated clone in a patient.

4. Discussion

In our study, we analyzed four recurrently mutated genes, three chromosomal abberations and *IGHV* status that might be used as markers of time to progression in CLL patients with an intact *TP53* gene treated with the frontline FCR or BR regimens in a routine clinical setting. We have analyzed a cohort of 177 CLL patients with a high mutation rate (despite our conservative VAF cut-off) and a predominance of *IGHV* unmutated status, reflecting the general spectrum of more agressive CLL cases treated in a large university hospital. In line with previous reports [11,26,27], we observed a clear association between the unmutated *IGHV* status and an inferior PFS in patients treated with FCR. Notably, we did not observe such a difference in the patients treated with BR. In this regimen, the association between the unmutated *IGHV* status and reduced PFS was recorded in some [12,28], but not all studies [9]. We admit, however, that our data must be interpreted with caution due to the relatively low number of patients investigated.

In univariant analyses of the mutations' impact on PFS, we observed very similar effect in both regimens: no negative impact for *ATM*, *SF3B1* and *NOTCH1* mutations on the PFS, but a significantly shortened time to progression in patients with *BIRC3* mutations. BIRC3 is a negative regulator of the NF-κB pathway [21,29] which is central to CLL cell survival [30,31]. A negative *BIRC3* mutations' impact on PFS after the front-line therapies (other than FCR/BR) has been shown previously in CLL [32]. The *BIRC3* mutations have also been associated with fludarabine chemo-refractoriness resembling *TP53*-defective CLL [33]. Thus, our data support this gene's important role in CLL progression after chemoimmunotherapy. However, because of the BIRC3 mutations' association with 11q22 deletion, the prognostic relevance in a multivariant analysis is diminished.

In the study by Stilgenbauer et al. [13] *SF3B1* and *NOTCH1* mutations were predictive of shorter PFS in a multivariant analysis in FCR treated patients albeit with a relatively small hazard ratios. These gene mutations are clearly relevant for malignant B cell biology, but have a less clear impact on the PFS in a real life population of CLL patients that tend to have shorter PFS then a clinical trial population. Additionally, in our FCR cohort the total number of *SF3B1* or *NOTCH1* mutated cases was relatively low (n = 25 and n = 19, respectively), which might contribute to the absence of a predictive value. However, this is well illustrating a real life scenario with these mutations being relatively infrequent, and often associated with other factors that have a clear negative impact on PFS, namely *IGHV* status and presence of del11q22.

We also demonstrate a strong negative impact of deletion 11q22 on the PFS in both studied regimens, which is in line with previous studies [8,10–12]. In addition, we present an analysis concerning the *ATM* defects, in which the sole 11q22 deletion leads to a significantly worse outcome than inactivating *ATM* alleles involving mutation. A mutation on the residual *ATM* allele has been shown to negatively affect the response of 11q22-deleted CLL cells to DNA-damaging chemotherapy [34]. Hence, a significantly better outcome concerning the PFS in our

ATM-mutated patients could be accounted for by the presence of rituximab. In fact, we reported previously that *ATM*-mutated CLL cells manifest a higher CD20 level (target of rituximab) than the *ATM*-wt samples, including those with sole 11q22 deletion [35]. The higher CD20 level was associated with *ATM*-mutated CLL cells' better *in vitro* response to rituximab and ofatumumab [35]. We have also shown that intraclonal CLL cells' subpopulations with the higher CD20 level are preferentially eliminated by rituximab *in vivo* [36]. Thus, our current clinical data seem to be in line with previous preclinical studies, although it is clear that other factors apart from hypothetically CD20 level also influence the clinical outcome. Interestingly, another factor also previously associated with the higher cell-surface CD20 level, *i.e.* trisomy 12 [37], showed a trend towards a longer PFS in our study.

Complete *ATM*-mutated clones' eradication by FCR regimen has been reported in CLL patients [16,38] and we also observed such a case. Therefore, it seems that *ATM* mutation presence *per se* does not preclude successful CLL patients' therapy with rituximab-based regimens. Nevertheless, among our studied genes, *ATM* mutations were the most frequently expanded in relapse, which indicates that in some cases these mutations likely represent a relapse-driving event.

A sole 11q22 deletion, *i.e.* without an accompanying *ATM* mutation on the other allele, was associated in our study with the shortest PFS of all markers, with the median reaching only 16 months. This adverse phenotype can probably be partially accounted for by the *BIRC3* mutations, which co-occur in a proportion of these patients. However, also the remaining cases, *i.e.* those with the sole 11q22 deletion and no *BIRC3* mutation manifested very short PFS (identical median of 16 months; data not shown). We noticed that some of these patients experienced complete remission and some had only partial remission, suggesting that both more rapid cell proliferation and drug resistance can be responsible for the observed unfavorable outcome.

In summary, we confirm that unmutated IGHV status is a marker of adverse prognosis in *TP53*-wt CLL patients treated by frontline FCR therapy. In both FCR and BR regimens, deletion 11q22 can set aside CLL patients at high-risk of early progression after front-line chemoimmunotherapy. Based on our data, special attention should be given to CLL patients harboring a sole 11q22 deletion, with no *ATM* mutation on the other allele, who manifest particularly short PFS.

Conflict of interests

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2019.04.015.

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