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Prognostic and predictive role of gene mutations in chronic lymphocytic leukemia: results from the pivotal phase III study COMPLEMENT1

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ABSTRACT

Next generation sequencing studies in chronic lymphocytic leukemia (CLL) have revealed novel genetic variants that have been associated with disease characteristics and outcome. The aim of this study was to evaluate the prognostic value of recurrent molecular abnormalities in patients with CLL. Therefore, we assessed their incidences and associations with other clinical and genetic markers in the prospective multicenter COMPLEMENT1 trial [treatment naive patients not eligible for intensive treatment randomized to chlorambucil (CHL) *vs.* ofatumumab-CHL (O-CHL)]. Baseline samples were available from 383 patients (85.6%) representative of the total trial cohort. Mutations were analyzed by amplicon-based targeted next generation sequencing (tNGS). In 52.2% of patients we found at least one mutation; the incidence was highest in *NOTCH1* (17.0%), followed by *SF3B1* (14.1%), *ATM* (11.7%), *TP53* (10.2%), *POT1* (7.0%), *RPS15* (4.4%), *FBXW7* (3.4%), *MYD88* (2.6%), and *BIRC3* (2.3%). While most mutations lacked prognostic significance, *TP53* (HR2.02, $P<0.01$), *SF3B1* (HR1.66, $P=0.01$), and *NOTCH1* (HR1.39, $P=0.03$) were associated with inferior progression-free survival (PFS) in univariate analysis. Multivariate analysis confirmed the independent prognostic role of *TP53* for PFS (HR1.71, $P=0.04$) and overall survival (OS) (HR2.78, $P=0.02$), and of *SF3B1* for PFS only (HR1.52, $P=0.02$). Notably, NOTCH1 mutation status separates patients with a strong from those with a weak benefit from addition of ofatumumab to CHL (*NOTCH1*^wt: HR0.50, $P<0.01$; *NOTCH1*^mut: HR0.81, $P=0.45$). In summary, *TP53* and *SF3B1* were confirmed as independent prognostic factors and *NOTCH1* as a predictive factor for reduced ofatumumab efficacy in a randomized chemo/immunotherapy CLL trial. These results validate NGS-based mutation analysis in a multicenter trial and provide a basis for expanding molecular testing in the prognostic workup of patients with CLL. (*Trial registered at clinicaltrials.gov identifier: NCT00748189.*)

Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease. Many prognostic factors have been identified in the last decades, but only a few have found their way into clinical practice.[1] In a variety of different CLL trials, genomic aberrations, particularly 17p deletion and mutation status for *TP53*[2-7] had the strongest relation to clinical outcome. Their assessment before institution of therapy has therefore been recommended for every CLL patient.[1] The availability of new CLL treatment modalities have raised the question of the influence of these genomic aberrations as predictive factors for treatment response.

Recent insights from unbiased next generation sequencing (NGS) approaches describe more than 40 recurrently mutated cancer driver genes in CLL, the clinical significance of which often remains undefined.[8-11] The clinical value of single mutations was mostly studied in heterogeneous patient cohorts and assessed outside of the context of clinical and biologic features.[12-16] Therefore, there is a need to perform mutation screening in large clinical trials, as they provide a homogeneous cohort of patients, standardized assessment of clinical and laboratory parameters, and valid outcome data. The UK CLL4 trial and the GCLLSG CLL8 trials served to determine the prognostic impact of recurrent mutations among the minority of young/fit patients. Both cohorts were screened for mutations in *TP53, NOTCH1* and *SF3B1*, as mutations in these genes have a high incidence (10-15%) and typically occur in hotspots, which allows the use of Sanger sequencing for mutation screening. While mutated *TP53* (*TP53*mut) was validated as an independent prognostic factor for progression-free survival (PFS) and overall survival (OS) in both studies, results for the other genes were less clear: mutated *SF3B1* (*SF3B1*mut) was associated with decreased PFS in CLL8 and decreased OS in UK CLL4 but not vice versa.[12,13,17] Notably, and in contrast to previous reports, mutated *NOTCH1* (*NOTCH1*mut) was not an independent prognostic factor for PFS in both trials, but was identified as predicting the lack of efficacy of the addition of CD20 antibody (rituximab) in the CLL8 trial;[13] this provocative finding needs confirmation in additional data sets.

The COMPLEMENT1 trial evaluated chlorambucil (CHL), a less intense chemotherapy backbone for which the majority of CLL patients are eligible, with or without the addition of the CD20 antibody ofatumumab.[15,16] Ofatumumab binds to a different epitope of CD20 compared to rituximab and is more efficacious in triggering complement-dependent cytotoxicity (CDC) *in vitro*. The major result of COMPLEMENT1 was a significantly prolonged PFS by the addition of ofatumumab (22.4 months; 95%CI: 19.0-25.2) to chlorambucil[18,19] (13.1 months; 95%CI: 10.6-13.8) in a population who cannot tolerate more intensive therapy. The well characterized patient cohort and mature follow up of COMPLEMENT1 provided an ideal background to study incidence, associations, and prognostic as well as the predictive value of gene mutations in a typical, elderly, front-line CLL patient population.

Methods

Patients

The GlaxoSmithKline-sponsored phase III trial COMPLE-MENT1 (OMB110911) enrolled 447 untreated patients ineligible for fludarabine-based therapy and in need of treatment. Subjects were randomly assigned to receive CHL or O-CHL with a ratio of 1:1. The primary study end point was PFS as determined by an independent review committee. Biomarker analysis was implemented in the study protocol and was approved by the institutional review board or ethics committee of each participating institution. Each patient provided written informed consent before enrolment. For 383 of 447 patients (85.7%), a baseline sample with sufficient DNA and informed consent for research purposes were available. This subset was representative of the intent-to-treat population with regard to clinical, laboratory and genetic baseline characteristics (*Online Supplementary Table S1*).

Sequencing

We designed a customized Illumina™ Truseq amplicon panel for all coding regions of *TP53, ATM, BIRC3, MYD88, FBXW7, POT1*, and for the most commonly affected exons 14, 15, 16 and 18 in *SF3B1* and exon 34 in *NOTCH1*. The cumulative target size was 24,161 basepairs (bp) covered by 221 amplicons with a length of up to 250 bp each. Adjacent 10 intron bp were included to cover splice site mutations. Input of 250 ng DNA from peripheral blood mononuclear cells isolated by Ficoll gradient centrifugation were sufficient for libraries according to the Illumina TruSeq Custom Amplicon (TSCA) protocol. Sequencing was performed on an Illumina MiSeq™ with the 500-cycle MiSeq Reagent Kit v2. Mutations in the 3'UTR of *NOTCH1* and in *RPS15* exon 4 were analyzed *via* Sanger sequencing.

Statistical analysis

Statistical analyses were performed on an intent-to-treat basis including all patients with samples available. Analysis was performed using R studio 1.1.447 with survival package (RStudio Inc., Boston, MA, USA). Categorical variables were compared using the Fisher Exact test, and continuous variables were compared using non-parametric rank-sum tests. Statistical tests were two-sided; $P<0.05$ was considered statistically significant. There were no adjustments for multiple testing, so that all reported *P*-values have an exploratory character for all analysis except for *Online Supplementary Figure S2* for which we used false discovery rate (FDR) as an adjustment.

Time to event was analyzed by Kaplan-Meier estimates and log-rank. To identify independent prognostic factors, we included treatment arm, del11q, del17p and IGHV mutation status as factors associated with PFS in univariate analysis in addition to all genetic subgroups defined by gene mutations. These 13 parameters were tested in 383 patients without forward or backward selection procedures to explore the independent prognostic character. In addition, we performed a multivariate treatment-gene mutation interaction analysis to explore treatment dependent and therefore predictive value. We used R studio 1.1.447 (RStudio Inc., Boston, MA, USA) with survival package for all statistical analyses.

Results

Spectrum and associations of mutations

Sequencing of 383 patient samples resulted in a mean coverage of 2,852 reads and 98.2% of target reads above 100x. We identified a total of 304 mutations in the selected gene set. Mutation incidences were *NOTCH1* (17.0%), *SF3B1* (14.1%), *ATM* (11.7%), *TP53* (10.2%), *POT1* (7.0%), *RPS15* (4.4%), *FBXW7* (3.4%), *MYD88* (2.6%), and *BIRC3* (2.3%) (Figure 1A). When looking at the muta-
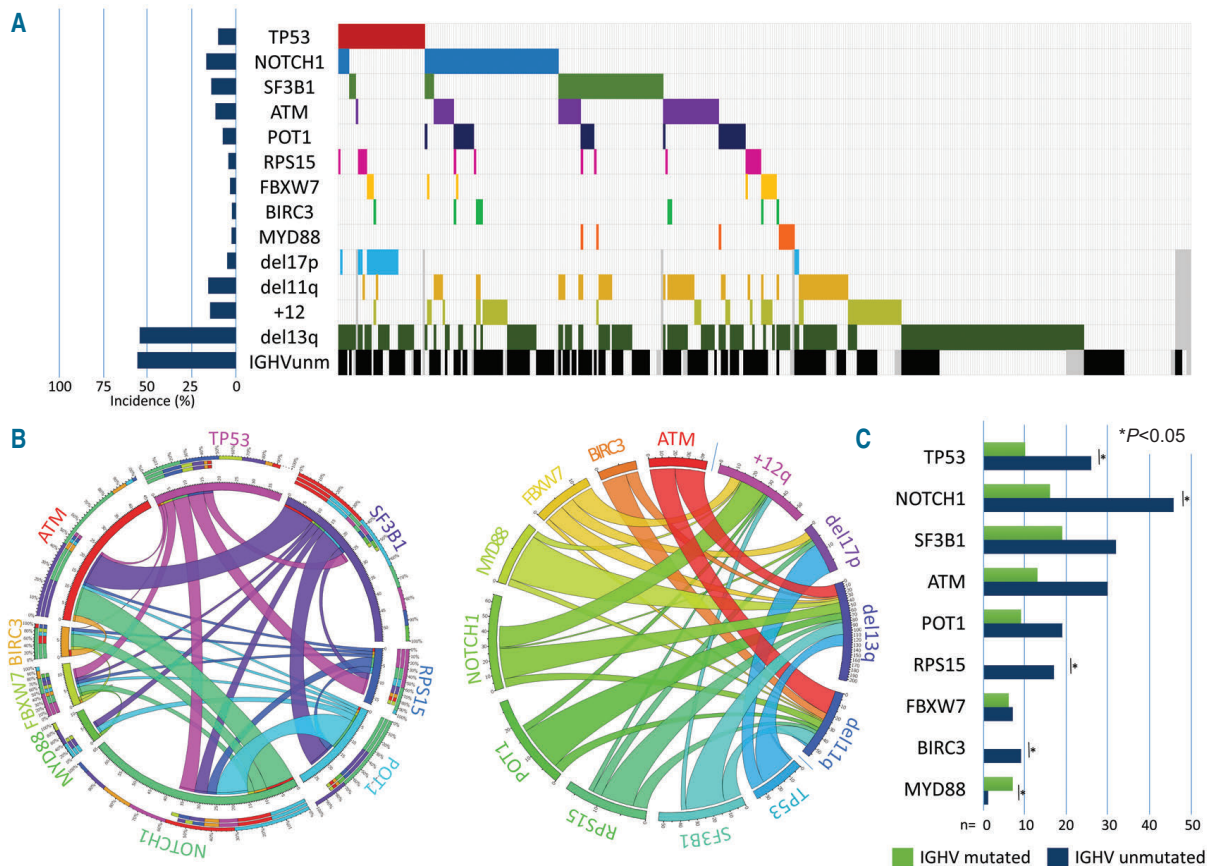
Figure 1. Incidence and distribution of genetic parameters (gene mutations, genomic aberrations and IGHV status). (A) Cluster diagram of patients (columns) with data for all genetic parameters (rows) (right) and overall incidence (left). Distribution of markers is ordered by rows. (B) Circos plots of the co-occurrence of gene mutations with each other (left) and pairwise with chromosomal aberrations (right). Lengths of arcs correspond to total incidences of respective markers while the width of each ribbon corresponds to the proportion of co-occurrence with a respective second marker. (C) Distribution of gene mutations in the IGHV mutation status subgroups.

tion type, we observed 195 missense mutations, 3 insertions, 56 deletions, 27 nonsense mutations, 11 splice site and 12 3'UTR mutations. Of all variants, 122 were selected for validation *via* Sanger sequencing and all of them were confirmed, so that further validation procedures were omitted (*Online Supplementary Table S2*). While the number of mutations was slightly higher in CHL *versus* O-CHL (178 *vs.* 126, not significant), the number of patients with at least one mutation was similar in both arms: 55% for CHL and 53% for O-CHL (see *Online Supplementary Figure S1* for a detailed overview). In the total cohort, only 11.5% of patients had neither a mutation nor a chromosomal aberration in the analyzed targets. As previously reported, mutations in *SF3B1* and *MYD88* were exclusively single nucleotide variants (SNV). Of 66 NOTCH1 mutations, 52 caused stop codons *via* frameshift or nonsense mutation and 12 affected the 3'UTR (*Online Supplementary Table S2*). *TP53* and *BIRC3* showed various patterns of mutations including insertions, deletions and exonic as well as splice site SNV.

Regarding associations, all but one case with *MYD88* mutation had mutated IGHV, while *TP53, RPS15* and *BIRC3* mutations were found predominantly in patients with unmutated IGHV (Figure 1). As to cytogenetics, we found deletion of 11q associated with mutations of ATM and *BIRC3* and trisomy 12q associated with mutations in

NOTCH1, BIRC3 and *FBXW7*. Interestingly, of the nine *BIRC3* mutated cases, six had an aberration in 11q and four in 12q. As expected, high concordance was found between 17p deletion and mutation in *TP53*, but also with *FBXW7*^mut. These associations hold true when considering correction for multiple testing with false discovery rate (*Online Supplementary Figure S2*).

Analyzing mutations in nine different genes and correlation with genomic aberrations and IGHV mutation status allowed us to derive an interaction network based on significant correlations considering incidence, co-occurrence, or mutual exclusivity (Figure 2). Interestingly, two dichotomies emerge from the complex network. Firstly, there is a mutual exclusivity between del13q and +12q. While del13q does not significantly associate with any of the gene mutations, cases with +12q cluster with mutated *NOTCH1, BIRC3* and *FBXW7*. The second dichotomy is found in mutation of *TP53* and del17p on one side and a cluster including del11q, +12q and *ATM* on the other side. Interestingly, mutations of *SF3B1* are not associated with either group, suggesting that these mutations represent an independent pathogenic mechanism. Concerning clinical associations, *SF3B1* mutations were more common in male patients (*Online Supplementary Table S3*) and associated with high absolute lymphocyte count (ALC) and CD19^+CD5^+ fraction by flow cytometry (*P*<0.01 and

Table 1. Prognostic associations of gene mutations and progression-free survival (PFS) / overall survival (OS) in the full trial cohort (both treatment arms combined) in univariate analysis (log rank test).

Gene mutation	PFS			OS			N (events PFS/OS)
	HR	95%CI	P	HR	95%CI	P	
TP53	2.02	1.18-3.45	**<0.01**	4.25	1.65-10.92	**<0.01**	39 (27/16)
NOTCH1	1.39	1.04-1.86	**0.03**	0.84	0.49-1.44	0.62	65 (50/10)
SF3B1	1.66	1.12-2.47	**<0.01**	1.39	0.67-2.87	0.32	54 (43/11)
ATM	1.16	0.84-1.60	0.42	0.89	0.48-1.68	0.78	45 (34/7)
POT1	1.25	0.73-1.92	0.33	1.15	0.50-2.58	0.77	29 (21/5)
RPS15	1.08	0.62-1.87	0.81	2.06	0.72-5.93	0.11	17 (10/5)
FBXW7	1.51	0.67-3.39	0.22	2.19	0.52-9.33	0.12	13 (9/4)
MYD88	0.59	0.31-1.11	0.19	NA	NA	NA	10 (6/0)
BIRC3	1.63	0.69-3.87	0.23	1.29	0.34-4.92	0.72	9 (6/2)

NA: not applicable due to no events in mutated patients. CI: Confidence Interval; N: number. Statistical significance in bold.

P=0.03), whereas ALC in *MYD88* mutated patients was low. WBC >50x10⁹/L were found more often in patients with mutated *TP53, RPS15* or *ATM* (P=0.05, P=0.02 and P=0.03, respectively).

Clinical outcome and prognostic impact of gene mutations in the full cohort

A significantly lower overall response rate was found only for *TP53* mutated cases (HR 5.20, P<0.01). In univariate analysis, significantly decreased PFS was found for patients with mutations in *TP53* (HR 2.02, P<0.01), *SF3B1* (HR 1.66, P<0.01), and *NOTCH1* (HR 1.39, P=0.03), but not for patients with mutations in ATM (HR 1.16, P=0.42) or BIRC3 (HR 1.63, P=0.23) (Table 1 with 95%CI, Figure 3, and *Online Supplementary Figure S3*). OS was significantly shorter in patients with mutations of *TP53*, and this was observed both in the total cohort (HR 4.25, P<0.01) as well as in patients without deletion of 17p (HR 2.56, P=0.03) (*data not shown*). Remarkably, no death event was observed among *MYD88* mutated patients. The number of mutated genes correlated with PFS, and patients harboring two or more mutated genes had shorter PFS (*Online Supplementary Figure S4*). Mutation in at least one of the target genes associated with significantly shorter OS, while the number of mutated genes did not play a significant role.

Taking into account that a disruption of both alleles of *BIRC3* or *ATM* could be required to observe any effect as described in the UK CLL4[20] trial, we considered the del11q status for the impact of mutations in both genes. However, in our patient cohort, mutation of ATM did not add significant prognostic value either in the 11q deleted subgroup or in the 11q disomic subgroup (*Online Supplementary Figure S5*). As *BIRC3* was mutated in only nine patients, the subgroups were too small to address this question.

Mutations in *TP53* and *ATM* showed high variant allelic fractions (VAF) of the mutant allele, mainly explained by a high co-occurrence with deletion of the other allele (*Online Supplementary Figure S6A*). Also mutations with a variant allele fraction (VAF) ≤10%, usually undetectable by Sanger sequencing, were rare in *TP53* (2 of 38) and absent in *MYD88* in this trial, but present in a significant proportion of cases with mutation of *SF3B1, NOTCH1* or *FBXW7*. As the role of mutations with minor allelic fraction is still unclear, we performed survival analyses including patients with wild-type (WT) *SF3B1/NOTCH1* and

with major and minor (< 10% VAF) mutated fractions (only NGS data considered). Interestingly, patients affected by only minor *SF3B1* mutated clones showed significantly shorter PFS in comparison to WT (HR 3.09, 95%CI: 0.67-14.30, P=0.01), while for *NOTCH1*, minor mutations had no significant impact on the full cohort, mainly due to low numbers (HR 1.54, 95%CI: 0.71-3.34, P=0.26) (*Online Supplementary Figure S6B and C*).

We performed multivariable analyses including treatment arm, IGHV mutation status, del17p, del11q and gene mutations to examine the independent prognostic value of these parameters. Besides ofatumumab+chlorambucil, IGHV status, del17p and del11q, only mutations of *TP53* (HR 1.71, P=0.04) and *SF3B1* (HR 1.52, P=0.02) were identified as independent factors associated with decreased PFS. For OS, only presence of del17p and *TP53* mutation (HR 2.78, P=0.02) retained a significant independent role (Table 2 with 95%CI).

Predictive value for the efficacy of ofatumumab addition

Randomization of patients into treatment arms with and without ofatumumab allowed the evaluation of differential CD20 antibody efficacy in subgroups defined by mutations. In the total cohort of 383 patients, treatment with ofatumumab was beneficial with regard to PFS (HR 0.53, P<0.01) as published for the whole trial population.[19] Analyses of both treatment arms separately generally reiterated the results obtained for the total cohort, with mutations in *SF3B1* and *TP53* being associated with shorter PFS. However, for *NOTCH1*, we observed an impact on PFS in the O-CHL treatment arm (HR 1.94, 95%CI: 1.25-3.92, P<0.01) but not with CHL alone (HR 1.01, 95%CI: 0.69-1.47, P=0.98) (Figure 4). Conversely, the addition of ofatumumab to chlorambucil in patients with *NOTCH1*ᵂᵗ status was strongly beneficial (mPFS 23.8 *vs.* 13.3 months, HR 0.50, 95%CI: 0.39-0.63, P<0.01), while the benefit in the *NOTCH1*ᵐᵘᵗ group was not significant (17.2 *vs.* 13.1 months, HR 0.81, 95%CI: 0.50-1.31, P=0.45). Notably, the same analysis confirmed the addition of ofatumumab to be beneficial in *SF3B1* and *TP53* mutated subgroups (for SF3B1: mPFS 17.3 *vs.* 10.8 months, HR 0.53, 95%CI: 0.29-0.97, P=0.03; for TP53: mPFS 12.8 *vs.* 3.7 months, HR 0.49, 95%CI: 0.23-1.05, P=0.05). This impact was strongest with *NOTCH1* mutations at a mutant allele fraction >40% and weaker with smaller *NOTCH1* variant fraction (*Online Supplementary Figure S7*). To investigate the rela-
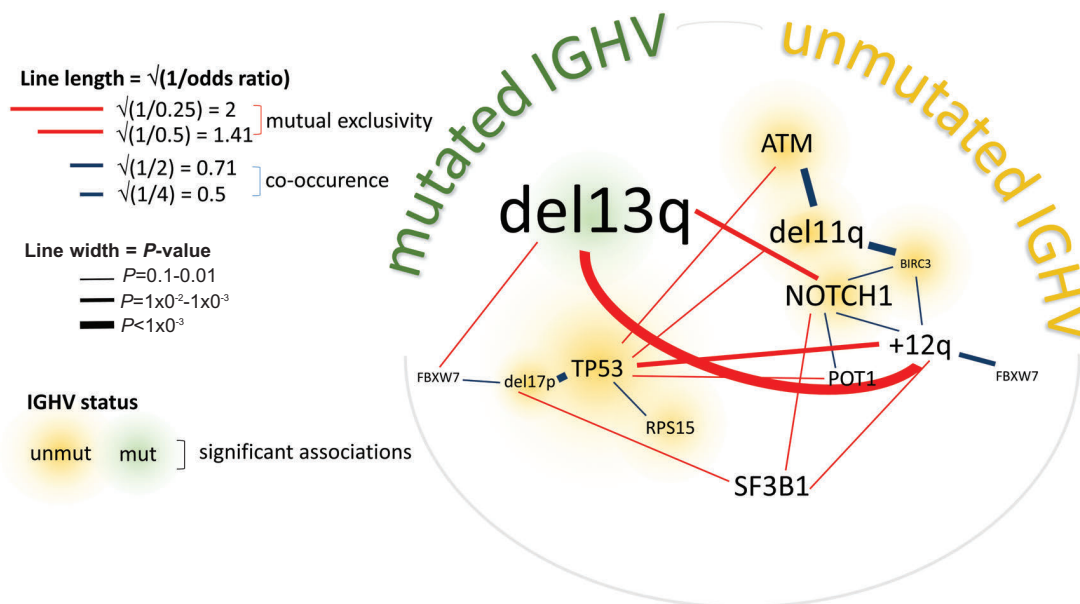
Figure 2. Visualization of co-occurrence of gene mutations and genomic aberrations based on pairwise Fisher exact test. Line length corresponds to $\sqrt{(1/\text{odds ratio})}$. Therefore, lines with a length >1 show mutual exclusivity (red) and lines with a length <1 co-occurrence (blue). Line width corresponds to stated *P*-value of pairwise comparison; when *P*>0.1, no line is depicted. Font size characterizes incidence of mutation/aberration; green indicates association with mutated IGHV, and yellow indicates association with unmutated IGHV (*P*<0.1 each). FBXW7 is depicted twice.

tion between treatment and *NOTCH1* mutational status, we performed an interaction-focused multivariate test. This analysis attributed a predictive impact to *NOTCH1* (*P*=0.05), while for all other parameters no interaction was observed (*Online Supplementary Table S4*). There were no differences in CD20 surface levels measured with flow cytometry between subgroups defined by mutation of *NOTCH1* suggesting that differential CD20 expression is not the cause of the lower CD20 antibody efficacy (*data not shown*).

Discussion

Over recent years, in an increasing number of laboratories, tNGS has replaced Sanger sequencing as a tool for mutation analysis. The current report represents a combined analysis of recurrent gene mutations studied by tNGS with a comprehensive dataset in a large multicenter phase III trial in CLL. In contrast to prior analyses, we used amplicon-based tNGS which allowed sequencing of 119 exons in eight different genes with a detection limit of 5% VAF. Although some tNGS approaches allow detection of subclones with only 1% VAF and below,[21] the aim of this project was to evaluate the prognostic value of molecular abnormalities for a better refinement of risk stratification. Development of such a robust, reproducible and affordable assay, which can be easily adopted into a diagnostic setup, could help in the clinical management of CLL patients. In favor of a uniform coverage of all targets, we trimmed sensitivity to achieve reliable results with high specificity. Mutations with minor allelic fractions are not detectable *via* Sanger sequencing and there is little evidence of their clinical impact, apart from in *TP53*.[21,22] Therefore, not surprisingly, councils like the European Initiative for Research in CLL (ERIC) caution against deriving clinical conclusions from small subclonal mutations.[23]

The low number of detected minor mutations in *TP53* and other genes in COMPLEMENT1 does not allow any conclusions to be drawn about their prognostic significance. However, for minor *SF3B1* mutations, we found an impact similar to mutations with VAF>10%. Further studies on clinical trials focusing on minor mutations are necessary to explore the impact of such variants with a comprehensive validation setup.

Our data support prior observations characterizing *SF3B1, NOTCH1, ATM* and *TP53* as the most frequently mutated genes in decreasing order of frequency, while mutations in *BIRC3* and *MYD88* remain very rare events in untreated CLL.[10,11] However, putting associations of these mutations and chromosomal aberrations in a correlation network uncovers two substantial associations. First, the mutual exclusivity between del13q and +12q and associated mutations, which is supported by prior clonality and lineage analysis showing that both aberrations are clonal events in CLL associating with tumor pathogenesis, but independently of each other.[10] Second, intermediate events, namely del17p and *TP53* on one side and different genomic abnormalities, mainly connected to 11q and *ATM* on the other side. As these abnormalities associate with growth advantage and accumulation in pretreated CLL, one can envisage that either mutation of *TP53* or mutation of one of the other genes is sufficient to cause a clonal survival advantage and therefore make the respective other event redundant.

Besides genetic interactions, analysis of the impact on clinical parameters, and especially on outcome after therapy, was the major aim of this study. In the current COMPLEMENT1 data set, *TP53* was the only gene mutation associated with a decrease in PFS and OS independent of other prognostic factors in multivariable analyses. Notably, this correlation was observed despite the high degree of collinearity with 17p deletion, confirming the need of *TP53* mutation testing in addition to 17p deletion
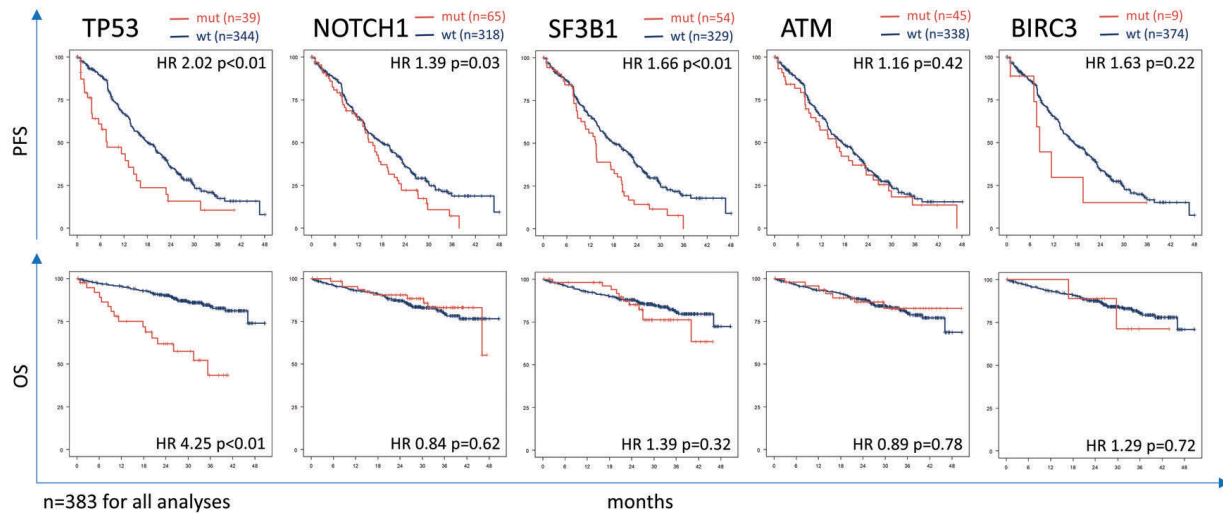
Figure 3. Kaplan-Meier estimates of progression-free survival (PFS) (left) and overall survival (OS) (right) according to the status of selected gene mutations for the total patient cohort. Red lines: mutated (mut) subgroups; blue lines: wild-type (wt). Denoted *P*-values were calculated by log-rank test (mut *vs.* unmutated subgroup).

Table 2. Multivariate analysis based on Cox-regression for progression-free survival (PFS) / overall survival (OS).

Parameter present	PFS			OS			N (events PFS/OS)
	HR	95%CI	*P*	HR	95%CI	*P*	
O-CHL arm	0.47	0.35-0.61	**<0.01**	0.76	0.44-1.29	0.31	183 (116/28)
TP53	1.71	1.04-2.81	**0.04**	2.78	1.17-6.62	**0.02**	39 (27/16)
NOTCH1	1.32	0.95-1.84	0.10	0.88	0.43-1.81	0.74	65 (50/10)
SF3B1	1.52	1.06-2.17	**0.02**	1.31	0.62-2.8	0.48	54 (43/11)
ATM	1.02	0.67-1.56	0.93	1.01	0.43-2.38	0.98	45 (34/7)
POT1	1.28	0.79-2.1	0.32	1.65	0.62-4.4	0.31	29 (21/5)
RPS15	0.82	0.42-1.59	0.56	1.16	0.44-3.06	0.76	17 (10/5)
FBXW7	1.15	0.58-2.29	0.69	1.64	0.56-4.83	0.37	13 (9/4)
MYD88	0.72	0.29-1.78	0.47	NA	NA	NA	10 (6/0)
BIRC3	1.12	0.47-2.66	0.79	0.63	0.15-2.69	0.53	9 (6/2)
IGHV^unmut	1.46	1.09-1.95	**0.01**	1.72	0.95-3.13	0.08	199 (141/44)
del17p	2.90	1.49-5.67	**<0.01**	3.22	1.18-8.76	**0.02**	19 (12/10)
del11q	1.72	1.19-2.51	**<0.01**	1.94	0.98-3.83	0.06	58 (46/14)

NA: not applicable due to no events in mutated patients. CI: Confidence Interval; N: number. Statistical significance in bold.

diagnostics in routine practice, as recommended by current ERIC and International Workshop on CLL (iwCLL) guidelines.[1] Accordingly, as only about half of the *TP53* mutated cases harbor an additional 17p deletion, mutation status for *TP53* should be assessed in addition to del17p to allocate patients to treatment with novel agents. However, this subgroup accounts for just over 10% (here 11.7%) of front-line CLL cases, and does not identify all patients with poor response and short survival times. Therefore, the major question was whether other genetic factors characterize aggressive CLL to the same degree. In previous studies, this had been shown for disrupted *BIRC3*, that affected the response to therapy and PFS in an extent comparable to mutation of *TP53*.[24] In COMPLEMENT1, only nine patients were mutated in *BIRC3*, having an overall response rate (ORR) of 75% in contrast to 47% responders among *TP53* mutated cases. While *TP53* significantly shortened PFS (HR 2.02, *P*<0.01) and OS (HR

4.25, *P*<0.01), *BIRC3* did not associate with either (HR 1.63 for PFS and 1.29 for OS, both not significant). The same applies to mutations in ATM, which together with mutations in *BIRC*3 associate with del11q, but do not add any additional prognostic value to the impact of 11q on PFS and OS in this trial. This is in contrast to data published within the UK CLL4 trial,[20] which may be explained by a different treatment and the lack of a CD20 antibody in UK CLL4, a different filtering approach for *ATM* variants, or just smaller patient numbers. However, also other groups did not find an adverse impact of a biallelic inactivation in *ATM*,[25,26] and therefore a more comprehensive setup with a bigger patient cohort with matched germline samples for validation of the somatic origin of these variants is required to definitively answer this question.

In contrast, for mutations of *SF3B1*, we observed a higher risk for early progression independently from other prognostic factors, but this was less pronounced as com-
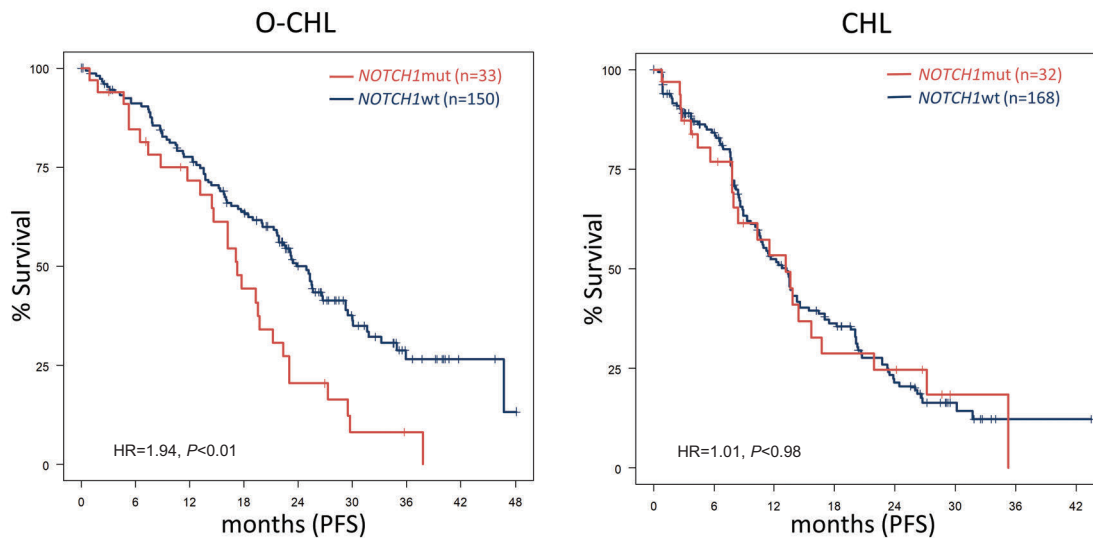
Figure 4. Kaplan-Meier estimates of progression-free survival (PFS) in the chlorambucil + ofatumumab (left) and chlorambucil alone (right) treatment arms according to *NOTCH1* mutation status. Mutated subgroups are depicted by red lines, wild type by blue lines. Denoted *P*-values were calculated by log-rank test (mutated *vs.* unmutated subgroup).

pared to *TP53* or del17p. These results conform to a number of previous observations.[8,13,27,28] Some approaches integrated *SF3B1* and additional novel mutations into the hierarchical classification for prognostication established by Döhner in 2000.[3] This is challenging as coincidences like mutations of *NOTCH1* and +12q or *ATM* and del11q are not easy to resolve in these models and currently available data are not powered to address correlations of small subgroups with clinical parameters. In a recent analysis, del11q and mutations of *SF3B1* and *NOTCH1* were categorized as intermediate risk.[29] Although del11q and *SF3B1* mutation showed similar hazard ratios for PFS (1.72 and 1.52) in the multivariate analysis of COMPLEMENT1, the former shows a trend to inferior OS (HR 1.94, *P*=0.06) while *SF3B1*[mut] did not (HR 1.31, *P*=0.48). In addition, the prognostic impact of *NOTCH1* appeared to strongly depend on the type of therapy (see below and Stilgenbauer *et al.*[13]), indicating that its use as a prognostic marker must be approached with caution.

As in previous genomic studies, we observed an adverse outcome of *NOTCH1*[mut] patients on the total trial cohort, which is more pronounced in cases with coding mutations. However, when analyzing both treatment arms separately, this prognostic impact turned out to be due to differential treatment effect, i.e. it identified *NOTCH1* mutation as a predictive factor. This finding reiterates the result obtained with rituximab, confirming a lesser efficacy of type 1 CD20 antibodies in *NOTCH1* mutated CLL[13] compared to WT. The impact on PFS is strongest in patients with a high mutant allele burden in *NOTCH1* and less pronounced in patients with minor mutations, but can only be observed with O-CHL and not with CHL treatment. This predictive value remains after adding 3'UTR mutations in *NOTCH1* and persisted in a treatment interaction analysis in contrast to all other markers. This may explain the discrepancies in previously published data: mutation of *NOTCH1* was an independent prognostic factor for PFS in heterogeneous cohorts of patients mainly treated with CD20 antibodies as the current standard of therapy but was not in the UK LRF CLL4

trial that did not contain CD20 antibody treatment.[12,14,15,30,31] Interestingly, in contrast to the previously published relation between *NOTCH1* mutation and CD20 expression,[32] *NOTCH1*[mut] cases showed no difference in CD20 surface expression as analyzed by flow cytometry. This is in line with the results from the CLL8 trial but differs from recently published data.[13,32] Even though the underlying molecular interrelation of *NOTCH1* mutations with CD20 remains unclear, the predictive impact of *NOTCH1* mutation is now confirmed in two independent clinical phase III trial cohorts. Although O-CHL shows slightly longer mPFS than CHL in *NOTCH1*[mut] patients, it is noteworthy that both mutation subgroups, *NOTCH1*[mut] and *TP53*[mut], have a similar initial impact in the setting of chemo-immunotherapy with CD20-targeted therapy outcome (HR on PFS 1.88 for *TP53*[mut] and 1.94 for *NOTCH1*[mut]). Moreover, the proportion of patients affected by mutated *NOTCH1* in the front-line setting is bigger than the group defined by *TP53* mutation. Based on current treatment guidelines, chemo-immunotherapy is still a valuable option for a number of patients.[33] Our findings raise a note of caution on the use of such therapy in a significant subset of CLL patients with mutated *NOTCH1*. Furthermore, CD20 antibodies remain important elements in combination with novel compounds e.g. with venetoclax[34] or ibrutinib,[35] and it is still unclear whether specific subgroups have a particular benefit of such combinations in comparison to single agent. The major implication from our observation is the need to understand and circumvent the resistance against CD20 antibodies as they remain an important element in the treatment of CLL. Furthermore, our results underline the role of recurrent mutations also in trials with novel treatment principles such as BTK-, PI3K- and BCL2 inhibition.

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