The Number of Signaling Pathways Altered by Driver Mutations in Chronic Lymphocytic Leukemia Impacts Disease Outcome

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Running title: Clinical Impact of Pathways Altered by CLL Driver Mutations

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Statement of Translational Relevance

Assessment of the international prognostic index in chronic lymphocytic leukemia, CLL-IPI, is recommended prior to treatment of patients with chronic lymphocytic leukemia (CLL). An increasing proportion of patients are diagnosed with CLL-IPI low risk, which challenges prognostication and allocation of highly specialized hematology resources. By adding the prognostic value of recurrent mutations at the time of CLL diagnosis, time to first treatment and overall survival can be predicted with improved accuracy.

For prognostication, including the number of signaling pathways altered by driver mutations as a biomarker – rather than the number of driver mutations per se – significantly impacted time to first treatment independent of CLL-IPI. Thus, operationalization of genetic phenotyping can improve risk stratification and guide individualized management of newly diagnosed CLL patients.

Abstract

Purpose: Investigation of signaling pathways altered by recurrent gene mutations and their clinical impact in a consecutive cohort of patients with newly diagnosed chronic lymphocytic leukemia (CLL). The heterogeneous clinical course and genetic complexity of CLL warrant improved molecular prognostication. However, the prognostic value of recurrent mutations at time of diagnosis remains unclear.

Experimental Design: We sequenced samples from 314 consecutive, newly diagnosed patients with CLL to investigate the clinical impact of 56 recurrently mutated genes assessed by next-generation sequencing.

Results: Mutations were identified in 70% of patients with enrichment among IGHV unmutated cases. With 6.5 years of follow-up, 15 mutated genes investigated at time of diagnosis demonstrated significant impact on time to first treatment (TTFT). Carrying driver mutations was associated with shorter TTFT and poor overall survival. For outcome from CLL diagnosis, the number of signaling pathways altered by driver mutations stratified patients better than the number of driver mutations. Moreover, we demonstrated gradual impact on TTFT with increasing number of altered pathways independent of CLL-IPI risk. Thus, a 25-gene, pathway-based biomarker assessing recurrent mutations refine prognostication in CLL; in particular for CLL-IPI low and intermediate risk patients. External validation emphasized that a broad gene panel including low burden mutations was key for the biomarker based on altered pathways.

Conclusions: We propose to include the number of pathways altered by driver mutations as a biomarker together with CLL-IPI in prospective studies of CLL from time of diagnosis for incorporation into clinical care and personalized follow-up and treatment.
Introduction

Chronic lymphocytic leukemia (CLL) is a common B-cell malignancy characterized by a heterogeneous clinical course and complex genetics (1). With 30-50% of patients requiring CLL treatment throughout disease course, prognostication is essential (2, 3). According to the validated prognostic index, CLL-IPI, the 10-year overall survival (OS) is 80% for patients with low risk disease, whereas the median overall survival (OS) is less than 4 years for patients with very high risk CLL among cohorts treated with chemoimmunotherapy (4, 5). A highly varying genetic landscape comprising approximately 2,000 recurrently mutated genes have been identified in CLL with just a handful of genes mutated in more than 5% of patients (6-8). Interestingly, only 25 genes have been identified as common mutational drivers in previous work (6, 7, 9). For instance, driver mutations in ATM, BIRC3, MGA, NOTCH1, POT1, SF3B1, and TP53 have been associated with short time to first treatment (TTFT) and poor OS (6, 7, 10).

Apart from clinical features, molecular markers like IGHV mutational status and copy number alterations (CNAs) are used for risk stratification in prognostic indices such as the CLL-IPI (4, 11-13). Certain recurrent mutations and CNAs often cooccur indicating a common pathogenesis such as TP53 mutations with del(17p) and ATM mutations with del(11q) (9, 14, 15). In contrast, isolated del(13q) and trisomy 12 [tri(12)] have shown mutual exclusivity and isolated del(13q) rarely cooccur with recurrent mutations (6, 9). Further assessment of subclonal mutations identifies CLL as a complex disease; approximately half of patients harbor more than one driver mutation, and 20% harbor more than 3 driver mutations—often with subclonal development (9). Moreover, an increasing number of mutational drivers correlate with worse clinical outcome (6, 9).

As mutations affect various signaling pathways, actionable lesions may be targeted by novel therapies in a substantial proportion of cases (6). Nevertheless, the clinical impact of recurrent genetic alterations has not yet translated into personalized treatment; TP53 aberrations (del(17p) and/or TP53 mutations) remain the only biomarker influencing clinical practice (16). BTK inhibitors as well as PI3K and BCL2 inhibitors alone or in combination with rituximab (R) are approved for first line treatment of patients with TP53 aberration and relapsed/refractory (R/R) CLL (16). Even though durable responses are seen, patients with TP53 aberration and R/R CLL are still at increased risk of progression due to early Richter’s transformation and acquired resistance mutations in BTK, PLCG2 and BCL2 (17-20). Combination targeted therapies show promising results in ongoing trials with durable undetectable minimal residual disease (21-23). For now, assessment of recurrent mutations (except TP53) is not advised in routine clinical practice (16).

Upon disease progression and subsequent therapy, patients may acquire new mutations, while mutations already present may either expand or diminish (9, 24). Such clonal evolution is key in disease progression and relapse, and crucial low burden mutations may be detected early on with sensitive techniques (25). Although some recurrent mutations at time of treatment are emerging as predictors of treatment, less is known about the clinical impact of mutations in population-based cohorts at time of diagnosis. To investigate the clinical impact of signaling pathways altered by recurrent mutations, we here present sequencing data in a population-based cohort of 314 consecutive patients with newly diagnosed CLL.
Materials and Methods
Patients and materials

We included all samples from a single center cohort of consecutive patients with newly diagnosed CLL between Jan 2008 and July 2015. The cohort is population-based due to referral of all patients with persistent lymphocytosis (>5x10^9/L) from a well-defined geographical area. Patients were diagnosed according to iwCLL criteria (16). Clinical data was retrieved from the Danish national CLL registry (26). CLL-IPI risk was calculated as previously described (5). The study was approved by the National Committee on Health Research Ethics, the Data Protection Agency, and the Health Authorities. According to Danish legislation, the study did not require written informed consent due to the retrospective biobank usage. Additionally, patients from two independent cohorts with data on CLL-IPI and outcome was included for external validation (9, 27).

High-throughput sequencing

Library preparation was performed using KAPA Library Preparation protocol (Roche NimbleGen, Madison, WI, USA). In brief, DNA extracted from peripheral blood mononuclear cells (PBMC) was fragmented and purified using AMPure XP (Beckman Coulter, Brea, CA, USA). Following end repair and poly(A)-tailing, fragments were indexed using NEXTflex™ DNA Barcodes 96 (Bioo Scientific, Austin, TX). Using a SeqCap EZ Choice (Roche NimbleGen) NGS panel, we targeted 56 genes and 2 miRNAs including 25 genes classified as driver genes in CLL (Supplementary Table S1) (6, 7, 9). Pooled libraries were double captured using SeqCap EZ Kit (Roche NimbleGen) and sequenced as paired-end on a HiSeq2500 using HiSeq® SBS Kit v4 (2x125 base PE; Illumina, San Diego, CA, USA).

Bioinformatic analysis

A workflow for detection of nucleotide variants was developed in CLC Biomedical Genomics Workbench 3.0.1 (CLC BGW, Qiagen, Hilden, Germany). In brief, paired reads were trimmed to remove 1 bp 5’ and 3’, as well as selecting high quality reads (q-score of 0.01). Following alignment (hg19/GRCh37), we called variants with a minimum coverage of 30 reads with at least 3 variant reads and a minimum variant allele frequency (VAF) of 0.5% to enable modeling of stereotypic errors (28). Due to lack of paired germline samples, we used a three-pronged approach to filter single nucleotide polymorphisms (SNPs). 1) Ingenuity® Variant Analysis (Qiagen) excluded SNPs present in the 1,000 Genomes Project and/or ExAC databases with population frequencies of 0.5% or greater. 2) Local Danish germline variants identified in at least two of 670 internal control germline samples were excluded. 3) All missense and in-frame variants with a VAF between 40-60% were manually reviewed in Alamut Visual 2.11 (Interactive Biosoftware, Rouen, France) prioritizing variants described in ClinVar (www.ncbi.nlm.nih.gov/clinvar ) as likely pathogenic, pathogenic or with an align-GDGV score of C35 or greater (29). Subsequently, VAFs were modeled to fit gamma distributions and true mutations were identified as outliers as previously described (modified using P < 0.0001)(30). Next, any variants identified in 16 healthy gender-matched donors younger than 30 years of age were also excluded. With a median coverage of 906 x (99% above 37 x), a dynamic limit of detection (LOD) according to read depth was applied allowing a minimum LOD of 2% VAF with a read depth above 1085x (Supplementary Fig. S1) (31). Genes were considered recurrent if mutated in at least 2 cases. Any mutation in 25 common driver genes described in three seminal papers were defined as drivers, while all other mutations were considered as non-drivers (Supplementary Table S1) (6, 7, 9). Driver mutations were
assigned to signaling pathways according to consensus among the two seminal papers (Table 1) (6, 7), and a pathway was considered altered if at least one gene in the pathway was mutated. Note that genes may be involved in multiple pathways. Thus, a single mutation can count as more than one altered pathway.

**FISH and IGHV mutational status**

Results from IGHV and FISH analyses were retrieved from the Danish national CLL registry (26). Rearrangement of IGHV-IGHD-IGHJ was amplified and sequenced as described elsewhere (32). Cytogenetic aberrations were analyzed for del(13q), tri(12), del(11q), and del(17p) at time of diagnosis using FISH and considered positive if present in at least 10% of 200 interphases. Using only registry data to calculate CLL-IPI, del(17p) was used as the only TP53 aberration as previously described (5), while only TP53 mutations without concomitant del(17p) were included as CLL drivers in multivariable analyses to avoid overfitting Cox models.

**Statistical analyses**

Recurrent mutations present in more than 10 cases, CNAs and IGHV mutational status as well as altered pathways were compared using pairwise Fisher’s exact tests with P-correction using false discovery rate (FDR). Patients were followed from date of diagnosis until CLL specific treatment, death or end of follow-up, whichever came first. Patients receiving CLL specific treatment were further followed from date of treatment initiation until second line treatment, death or end of follow-up for treatment-free survival (TFS). Time to first treatment (TTFT) was estimated as cumulative incidence rates considering death as a competing event. Cause-specific multivariable Cox analyses adjusted for CLL-IPI risk were used for TTFT. Log-rank tests were used for overall survival (OS) and TFS, while K-sample tests were used for TTFT. P-values less than 0.05 were considered significant. P-adjustment using FDR indicated by asterisk (*) for Q-values less than 0.1 throughout the paper. All analyses downstream of CLC BGW were performed with R version 3.4.1 (33). Custom scripts are available upon request.

**Results**

**Patient characteristics**

We sequenced a diagnostic sample from 314 consecutive patients with CLL. Among the 291 patients with complete baseline data, risk according to CLL-IPI was low, intermediate, high and very high in 57%, 30%, 11% and 2% of patients, respectively (Table 2). With a median follow-up of 6.5 years (95% CI: 5.9-7.1), 117 (37%) patients had received treatment and 96 (31%) died; 49 (16%) died without receiving treatment. Median time to first treatment was 14.2 months (IQR: 2.4-35.2) for treated patients. For external validation, two previously published cohorts from Scandinavia and Spain were included (Supplementary Table S2) (9, 27). Both validation cohorts included patients with more advanced clinical stage and higher 3- and 5-year incidence of treatment.

**Mutations and patterns of cooccurrence**

Among the 56 genes (Supplementary Table S1), we identified 515 mutations in 219/314 (70%) patients with a mean of 1.6 mutations (Supplementary Table S3). The most frequently mutated genes were IGLL5,
NOTCH1, ATM, SF3B1, MGA, TP53, CHD2, KHL6, POT1, and FBXW7 (Supplementary Fig. S2). At time of diagnosis, 165 (53%) patients carried driver mutations and 54 (17%) patients carried only non-driver mutations. Having 1, 2, 3, and >3 driver mutations occurred in 97 (31%), 34 (11%), 15 (5%) and 19 (6%) patients, respectively (Fig. 1). Although mutations in FBXW7 (50%), MED12 (67%), NOTCH1 (42%) and TP53 mutations (42%) seemed more common among patients harboring only 1 driver mutation, no mutations were significantly enriched when stratified based on the number of driver mutations (P = 0.16; Supplementary Table S4).

Following FDR correction, patterns of cooccurring genetic alterations revealed enrichment of ATM, NFKBIE, and NOTCH1 mutations as well as del(11q) in patients with unmutated IGHV status (IGHV-U). The mutational load was significantly higher in patients with IGHV-U status compared to those with mutated IGHV status (IGHV-M) (mean of 2.6 and 1.3 mutations per patient, respectively, P < 0.0001, Fig. 1). As expected, cooccurrence was seen for del(17p) and TP53 mutations, del(11q) and ATM mutations, as well as for tri(12) and FBXW7. Del(13q) mutually excluded NOTCH1 as well as tri(12), and IGHV-U status mutually excluded IGLL5 mutations and del(13q) (Fig. 2). Although IGLL5 mutations were highly prevalent and primarily located to exon 1 (73%), only two patients with IGHV-U harbored IGLL5 exon 1 mutations (Supplementary Fig. S2). The 30% of patients without any identified mutations were primarily IGHV-M (87%) with isolated del(13q) or normal FISH (Fig. 1).

Clinical impact of specific recurrent mutations

In univariate analyses, we demonstrated a significant shorter TTFT for patients with ATM, BCOR, BIRC3, BRAF*, DDX3X*, EWSR1*, FAM50A*, FBXW7*, FUBP1, IKZF3*, MGA*, NFKBIE*, NOTCH1*, POT1*, SETD2*, SF3B1*, TP53*, and ZMYM3* mutations compared to patients with wild-type (wt; Fig. 3[*] and Supplementary Fig. S3A). Enriched in IGHV-M patients, carrying IGLL5* mutations was associated with a favorable TTFT. Inferior OS was demonstrated for patients with ASXL1, BIRC3, GNB1, TP53 and ZMYM3* mutations (Supplementary Fig. S3B). In a subgroup analysis stratified on high and low burden mutations (>10% vs 2-10% VAF), low burden BCOR*, DDX3X*, EWSR1*, IKZF3*, MGA*, NOTCH1*, POT1*, SETD2*, SF3B1* and TP53* retained impact for TTFT (Supplementary Fig. S4). Unexpectedly, recurrent mutations in XPO1 and EGR2 did not impact outcome in univariate analyses. Thus, we further investigated whether restricting the analyses to mutations within the hotspot regions of MYD88, NOTCH1, SF3B1, POT1, TP53, NFKBIE, BIRC3, XPO1, and EGR2 as proposed by ERIC (http://www.ericll.org) would change the outcome. This analysis resulted in loss of significance for POT1 on TTFT, while a better distinction between mutated and wt groups for NOTCH1 and SF3B1 were seen. Still, we saw no impact of MYD88, XPO1 or EGR2 mutations on TTFT or on OS (Supplementary Table S5).

IGHV mutational status and recurrent mutations

For patients with IGHV-M, we did not have data to support that concomitant TP53 aberration affected TTFT (P = 0.09), whereas TP53 aberrations had a significant negative impact on TTFT for IGHV-U patients (P = 0.01). This could not be shown for OS (Supplementary Figs. S5A-B). Due to enrichment of driver mutations in patients with IGHV-U (P < 0.0001), we further investigated the impact of driver mutations in relation to IGHV mutational status. Carrying a driver mutation (0 vs ≥1) had negative impact on TTFT for patients with both IGHV-M (P = 0.004) and IGHV-U patients (P = 0.01). This association could not be demonstrated for OS (P > 0.27; Supplementary Figs. SSC-D). Due to the small number of patients belonging to each IGHV
stereotypic subset, we could not assess the distribution of recurrent mutations within the different stereotyped subsets (15).

Clinical impact following treatment

First and second line treatment were administered to 117 and 41 patients, respectively, as detailed in Supplementary Table S6. Survival following first line treatment was inferior for patients with ASXL1*, KRAS, MGA, TP53 and ZMYM3 mutations at time of diagnosis in univariate analyses (Supplementary Fig. S6A). Inferior TFS from first line treatment was demonstrated for patients with CHD2*, NFkBIE, TP53* and ZMYM3 mutations (Supplementary Fig. S6B).

Clinical impact of number of driver or non-driver mutations

We identified mutations in all 25 genes defined as drivers based on the consensus of three prior seminal papers on recurrent mutations in CLL (Fig. 1 and Supplementary Table S3) (6, 7, 9). Significantly shorter TTFT could be demonstrated for patients with at least one driver mutation when compared to patients without identified recurrent mutations, while only inferior TTFT (but not OS) was demonstrated for patients with driver mutations compared to patients with non-driver mutations only (Supplementary Fig. S7A-B).

We next investigated the impact of the number of driver mutations (0 vs 1 vs 2 vs 3 vs >3). Inferior outcome was demonstrated with increasing number of driver mutations for TTFT ($P < 0.0001$) as well as for OS ($P = 0.04$. Fig. 4A and Supplementary Fig. S8A). In a multivariable model of TTFT, the number of driver mutations was further independent of CLL-IPI but showed alternating hazard ratios with increasing number of drivers (Fig. 4C).

Altered pathways and patterns of cooccurrence

To investigate a more biologically meaningful approach, each driver mutation was assigned according to involved signaling pathway(s) based on the 25-gene panel (Table 1). According to this pathway assignment, 1, 2, 3 and >3 pathways were altered in 79, 54, 16, and 16 patients, respectively; 149 patients had no altered pathway as they harbored no driver mutations (Fig. 1). The most frequently altered pathways were DNA damage response (21%), RNA and ribosomal processing (18%), and Notch signaling (16%). For pathways not sharing common driver genes, cooccurrence was only demonstrated between apoptosis and NFκB pathways (odds ratio 3.3 [1.4;7.8]; $Q = 0.02$; data not shown).

Clinical impact of pathways altered by driver mutations

Mutually adjusted for each altered pathway, apoptosis, RNA metabolism, Notch, and DNA damage pathways significantly impacted TTFT (Supplementary Fig. S8C). Next, we demonstrated a negative correlation for the number of pathways altered by driver mutations and TTFT ($P < 0.0001$; Fig. 4B) as well as for OS ($P = 0.02$; Supplementary Fig. S8B). For TTFT, 5-year cumulative incidence rates of 17%, 41%, 53%, 75%, and 81% was demonstrated when stratifying patients into having 0, 1, 2, 3 or >3 altered pathways, respectively. In cause-specific Cox analysis of TTFT, each additional altered pathway was associated with a 1.62 [1.44;1.82] increased hazard of treatment. As CLL-IPI has been validated for both TTFT and OS (also within this cohort; $P < 0.0001$)(34), we next investigated whether the number of altered pathways could add prognostic value to CLL-IPI at time of diagnosis. In a cause-specific Cox model of TTFT, the number of
altered pathways adjusted for CLL-IPI risk demonstrated independent impact ($P < 0.0001$; Fig. 4D). A subsequent univariate subgroup analysis revealed stratification based on number of altered pathways only in patients with CLL-IPI low to intermediate risk (Supplementary Fig. S9). The multivariable model of TTFT showed increased adverse hazard ratios with increasing number of altered pathways – while this was not the case for number of driver mutations (Figs. 4C, D). Thus, when assessing the number of altered pathways in multivariable analysis adjusted for CLL-IPI, the negative correlation with TTFT appeared stronger than for the number of driver mutations.

Exploring restricted gene panels

Without clear consensus on CLL driver genes, we explored whether 1) a 10-driver gene panel with high burden variants (VAF > 10%) in hotspot regions (Supplementary Table S5 including the entire CDS of ATM), 2) a 22-driver gene panel, and 3) a 29-driver gene panel would improve prognostication for TTFT (Supplementary Table S1). Interestingly, both the 22- and 29-driver gene panel showed gradual and clear discriminatory capabilities for the number of altered pathways in univariate analyses and steady increasing hazards in multivariable analysis adjusted for CLL-IPI risk similar to the 25-gene panel, while this was not the case for number of drivers. By contrast, the high burden 10-gene panel was insufficient for prognostication based on number of altered pathways (Supplementary Fig. S10).

Validation of altered pathways as an independent prognostic marker

For validation of the 25-gene panel pathway-based biomarker, we assessed the impact of number of altered pathways for TTFT in a validation cohort of newly diagnosed (sampled within one year of diagnosis), untreated Spanish patients with complete CLL-IPI scores available from the International Cancer Genome Consortium (ICGC)(9). Mutations in the 25-gene panel (Supplementary Table S1) down to 2% VAF were included for an even external validation of the Danish results. In a cause-specific Cox model adjusted for CLL-IPI risk, the number of altered pathways demonstrated negative impact on TTFT. However, the gradual adverse impact was only clear for patients with IGHV-M status (Supplementary Fig. S11).

Finally, we assessed a high burden (VAF > 10%) 10-driver gene panel to test whether a restricted gene panel would be sufficient for a pathway-based biomarker. Neither in the Danish, the Spanish nor in the Scandinavian population-based CLL (SCAN) cohort (Supplementary Table S2) could the 10-gene panel perform as basis for a pathway-based biomarker (Supplementary Figs. S10D and S12).

Discussion

Our study confirms the complexity of recurrent mutations in consecutive, newly diagnosed patients with CLL. Several recurrently mutated genes are each associated with poor OS and short TTFT. However, the clearest discrimination was demonstrated for the number of pathways altered by driver mutations based on a 25-gene panel, which correlated with inferior TTFT independent of CLL-IPI risk also in an independent external validation cohort, in particular for patients with IGHV-M patients and CLL-IPI low to intermediate risk.

With 70% of patients harboring recurrent mutations at time of diagnosis and only 17 genes being mutated in more than 2% of cases, the genetic complexity is apparent already at time of CLL diagnosis. Using a limited 56-gene panel, the mutational load at time of diagnosis is lower than previously reported (6, 7) and
showed lower prevalence of BIRC3 and MYD88 but a higher prevalence of FBXW7, IGLL5, MGA, and NFKBIE mutations compared to untreated samples reported by the ICGC in CLL (6). In contrast to our study, only half of the untreated WES/WGS sequenced samples in the ICGC cohort were obtained within a year from diagnosis (median time to sampling of 1 year), which may explain a lower mutational load yet higher prevalence of individual mutations in our more indolent population-based cohort. Compared to Danish nation-wide data (5), this cohort presents a similar proportion of patients with IGHV-U (32% vs 32%) and CLL-IPI low risk (57% vs 56%) while patients were younger (60% vs 70% above 65 years) with less advanced Binet stage (87% vs. 78% with Binet stage A) and fewer del(17p) (4.7% vs 5.9%). In accordance with previous reports, the 30% of patients without any proven mutations were predominantly IGHV-M (87%) with isolated del(13q) or normal FISH cytogenetics. Cooccurrence of aberrations were similar to previous reports (6, 9).

The previously reported negative impact of carrying a driver mutation could be confirmed. In addition, non-driver genes such as EWSR1, FAM50A, IKZF3, and SETD2 are here reported for the first time to be independently associated with shorter TTFT and should be further investigated as putative drivers (6, 7). While IKZF3 was identified as a common driver by Landau and colleagues as well as by the ICGC, EWSR1 was only recognized as such by the former and SETD2 by the latter authors (6, 7). Notably, all four SETD2 mutated patients in our study progressed within 11-66 days after diagnosis. Despite cooccurring driver mutations and IGHV-U status, our results support both EWSR1 and SETD2 as CLL drivers in line with a previous study (35). To assure robustness of the 25-gene panel pathway-based biomarker, these putative driver genes were not included, thus only allowing driver genes with consensus across the three seminal papers in the gene panel (Supplementary Table S1) (6, 7, 9). As a result of somatic hypermutation, a high prevalence of IGLL5 mutations mainly among patients with IGHV-M status is demonstrated, while the association of IGLL5 mutations with a favorable outcome seems of limited clinical use.

Restricting the target region to hotspot mutations in a proposed 10-gene panel by ERIC (www.ericll.org), indicate that NOTCH1 and SF3B1 hotspots are superior in identifying pathogenic mutations compared to assessing the entire coding sequence (CDS) including the 3’UTR of NOTCH1 (Supplementary Table S5). Without paired germline samples for this study, we can only speculate whether germline variants may have been reported or whether hotspot mutations are more pathogenic. By contrast, impact on TTFT decreased or disappeared for genes such as POT1 and NFKBIE, when restricting the analysis to hotspots, suggesting the need to investigate the entire CDS.

For patients receiving treatment, MGA and TP53 mutations at time of diagnosis correlated with a poor OS from time of treatment. While chemotherapy resistance of TP53 aberrations is well-known, MGA mutations have also predicted poor survival following treatment in CLL (36). Additionally, CHD2, NFKBIE and ZMYM3 mutations also showed inferior TFS following 1st line treatment and may constitute novel predictors of treatment outcome. Longitudinal resequencing was not possible at time of treatment and relapse in this study but might have pointed to alternative mutations predictive of treatment outcome, while the introduction of targeted therapies may have influenced results.

In the ICGC CLL cohort, the number of CLL drivers was demonstrated to independently impact patient outcome when adjusting for clinical stage and IGHV status (6). Further including common driver alterations including CNAs identified by Landau and colleagues (7), the number of CLL driver alterations were reported to be independent of age, Binet stage, IGHV status and del(17p) for impact on patient outcome (9). Even
though we similarly demonstrate gradual adverse TTFT with increasing number of driver mutations, a better discrimination was demonstrated for the number of signaling pathways altered by driver mutations based on the 25-gene consensus panel. Thus, the number of altered pathways – and not only the number of driver mutations per se – negatively impacted TTFT independently of CLL-IPI risk groups. Although an ever-evolving understanding of signaling pathways make pathway assignment somewhat unclear, we here assigned genes to individual pathways based on the consensus of the three seminal papers on recurrent mutations in CLL, thus assuring an operational assignation to pathways (6, 7, 9). In a clinical setting with predominantly low and intermediate risk patients at diagnosis (5, 13), the number of pathways altered by mutations in 25 driver genes had particular strong prognostic impact. This was validated in an independent cohort of untreated patients with IGHV-M status.

In our cohort, the Spanish and the SCAN cohort, using only high burden (VAF > 10%) mutations in the hotspot region of 10 selected genes proposed by ERIC was insufficient to add prognostic value independent of CLL-IPI risk group. As low burden mutations in several genes had impact on TTFT, we further demonstrated that the biomarker based on number of altered pathways is robust, with similar performance when 22 or 29 common driver genes assessed by NGS including low burden mutations (VAF > 2%) form the basis for the biomarker. Contrary to using the number of driver mutations, we demonstrate that the number of altered pathways extend the CLL-IPI and benefit prognostication for newly diagnosed patients. Counting the number of altered pathways is not only powerful and easily applicable, it also markedly simplifies the genetic complexity in CLL in a biologically meaningful way, as seen for other B-cell malignancies (37-39).

In conclusion, the complex genetic landscape and heterogeneous clinical course in CLL make prognostication based on individual recurrent mutations impractical. By investigating common driver mutations in CLL based on altered signaling pathways, a biologically meaningful assessment of clinical impact of driver mutations is achieved. External validation emphasized that CLL-IPI can be improved by including the number of pathways altered by mutations in 25 driver genes among newly diagnosed patients with CLL as a new biomarker. A broad gene panel allowing for low allele burden mutations is needed for molecular prognostication at diagnosis; particularly patients with low to intermediate risk CLL demonstrated improved prognostication by this approach. We propose to include the number of pathways altered by driver mutations together with CLL-IPI in prospective studies of CLL from time of diagnosis for possible incorporation into clinical care and personalized follow-up and treatment.

Authors’ Contributions
Acknowledgments

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References


## Tables

### Table 1. Pathway assignment. Pathways altered by driver mutations according to Landau et al and Puente et al (Nature; 2015). Note that genes may be involved in more than one pathway.

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<td>TRAF3</td>
<td>IRF4</td>
<td>SF3B1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NFKBIE</td>
<td>XPO1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ZNF292</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Patient characteristics for 314 newly diagnosed patients with CLL. CLL-IPI modified as previously described (da Cunha-Bang et al, Blood; 2016).

<table>
<thead>
<tr>
<th>Variable</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>≤65 years</td>
<td>125 (39.8)</td>
</tr>
<tr>
<td>&gt;65 years</td>
<td>189 (60.2)</td>
</tr>
<tr>
<td>Binet stage</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>273 (86.9)</td>
</tr>
<tr>
<td>B/C</td>
<td>41 (13.1)</td>
</tr>
<tr>
<td>β-2-microglobulin</td>
<td></td>
</tr>
<tr>
<td>≤4.0 mg/L</td>
<td>263 (85.9)</td>
</tr>
<tr>
<td>&gt;4.0 mg/L</td>
<td>43 (14.1)</td>
</tr>
<tr>
<td>Unknown</td>
<td>8</td>
</tr>
<tr>
<td>IGHV status</td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>211 (67.4)</td>
</tr>
<tr>
<td>Unmutated</td>
<td>102 (32.6)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>del(17p)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>284 (95.3)</td>
</tr>
<tr>
<td>Yes</td>
<td>14 (4.7)</td>
</tr>
<tr>
<td>Unknown</td>
<td>16</td>
</tr>
<tr>
<td>CLL-IPI risk</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>167 (57.4)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>87 (29.9)</td>
</tr>
<tr>
<td>High</td>
<td>31 (10.7)</td>
</tr>
<tr>
<td>Very high</td>
<td>6 (2.1)</td>
</tr>
<tr>
<td>Unknown</td>
<td>23</td>
</tr>
</tbody>
</table>
**Figure legends**

**Figure 1.** Driver mutations, copy number alterations by FISH (CNAs), IGHV mutational status and CLL-IPI risk in 314 newly diagnosed patients with CLL. The top segment plot indicates the number of (no.) altered pathways, while the bar plot indicates the no. driver mutations. Mutated genes are indicated regardless of the number of mutations in each gene. High burden mutations with a variant allele frequency (VAF) above 10% were predominant compared to low burden mutations with a VAF below 10%. Note that the 47% of patients without driver mutations (blue segment) were predominantly IGHV mutated (IGHV-M) with normal FISH status or isolated del(13q).

**Figure 2.** Patterns of co-occurrence of genetic alterations. A) Copy number alterations, IGHV mutational status and genes mutated in more than 10 cases were investigated using pairwise Fisher’s exact tests. False discovery rate (FDR) was used to adjust for multiple testing, and adjusted $P$-values ($Q$-values) <0.1 were considered significant. Cooccurring alterations were primarily identified in IGHV unmutated (IGHV-U) patients. Significant cooccurrence of del(17p), del(11q) and tri(12) was seen with TP53, ATM and FBXW7 mutations, respectively. Del(13q) and NOTCH1 mutations mutually excluded one another.

**Figure 3.** Recurrently mutated genes with significant impact on time to first treatment (TTFT) following adjustment for multiple testing with false discovery rate (FDR, $Q < 0.1$). $Q$-values indicated.

**Figure 4.** Clinical impact on time to first treatment (TTFT) for A) the number of (no.) driver mutations and B) pathways altered by driver mutations. Pairwise K-sample tests indicated. Multivariable Cox analysis for the clinical impact on TTFT of the C) no. driver mutations adjusted for CLL-IPI and D) no. altered pathways adjusted for CLL-IPI risk. For multivariable analyses, only TP53 mutations without concomitant del(17p) were included as CLL drivers, whereas del(17p) regardless of TP53 mutational status was included as the only TP53 aberration for CLL-IPI.
Clinical Cancer Research

The Number of Signaling Pathways Altered by Driver Mutations in Chronic Lymphocytic Leukemia Impacts Disease Outcome


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