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## Title page

### Genetic characterization of B-cell prolymphocytic leukemia: a prognostic model involving *MYC* and *TP53*

#### Running Title: Genetic characterization of B-PLL

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## Key Points

B-PLL is tightly linked to *MYC* aberrations (translocation or gain) and 17p (*TP53*) deletion.

Cases of B-PLL with *MYC* aberration and 17p (*TP53*) deletion have the worst prognosis.

## Abstract

B-cell prolymphocytic leukemia (B-PLL) is a rare hematological disorder whose underlying oncogenic mechanisms are poorly understood. Our cytogenetic and molecular assessment of 34 patients with B-PLL revealed several disease-specific features and potential therapeutic targets. The karyotype was complex ( $\geq 3$  abnormalities) in 73% of the patients and highly complex ( $\geq 5$  abnormalities) in 45%. The most frequent chromosomal aberrations were translocations involving *MYC* [t(*MYC*)] (62%), deletion (del)17p (38%), trisomy (tri)18 (30%), del13q (29%), tri3 (24%), tri12 (24%), and del8p (23%). Twenty-six of the 34 patients (76%) exhibit *MYC* aberration, resulting from mutually exclusive translocations or gains. Whole-exome sequencing revealed frequent mutations in *TP53*, *MYD88*, *BCOR*, *MYC*, *SF3B1*, *SETD2*, *CHD2*, *CXCR4*, and *BCLAF1*. The majority of B-PLL used the *IGHV3* or *IGHV4* subgroups (89%), and displayed significantly mutated *IGHV* genes (79%). We identified three distinct cytogenetic risk groups: low-risk (no *MYC* aberration), intermediate-risk (*MYC* aberration but no del17p), and high-risk (*MYC* aberration and del17p) ( $p=.0006$ ). *In vitro* drug response profiling revealed that the combination of a B-cell receptor or BCL2 inhibitor with OTX015 (a bromodomain and extra-terminal motif (BET) inhibitor targeting *MYC*) was associated with significantly lower viability of B-PLL cells harboring a t(*MYC*). We conclude that cytogenetic analysis is a useful diagnostic and prognostic tool in B-PLL. Targeting *MYC* may be a useful treatment option in this disease.

## Introduction

B-cell prolymphocytic leukemia (B-PLL) is a very rare disease that accounts for less than 1% of all cases of chronic B-cell leukemia and generally occurs in elderly people. According to the World Health Organization (WHO) criteria, B-PLL can be diagnosed when prolymphocytes comprise more than 55% of the lymphoid cells in peripheral blood (PB). Diagnosis on the basis of clinical and morphological data can be difficult because B-PLL shares a number of features with other B-cell malignancies, including splenic marginal zone lymphoma (MZL), mantle-cell lymphoma (MCL) or chronic lymphocytic leukemia (CLL). For example, an unusual leukemic form of MCL mimicking B-PLL is distinguished by the presence of the t(11;14)(q13;q32) translocation<sup>1,2</sup>. B-PLL does not have a specific immunophenotype, and has low Matutes scores<sup>3,4</sup>, as observed for other B lymphoproliferative disorders.

Little is known about the oncogenic events leading to the development and clinical heterogeneity of B-PLL. Given the disease's rarity, its cytogenetic features have only been described in case reports and small series<sup>5,6</sup>, and large-scale genomic sequencing has not yet been performed for this disease.

Rituximab-based chemo-immunotherapy is often used to treat patients with B-PLL but no *TP53* aberrations. In patients with *TP53* aberrations, alemtuzumab-based regimens have been implemented. Lasting treatment responses are rare, and the prognosis is generally poor<sup>7</sup>. Recent data from case reports and small series suggest that two agents targeting B-cell signaling (ibrutinib and idelalisib) are efficacious<sup>8-10</sup>. Hence, allogeneic hematopoietic stem cell transplant is still the only curative treatment for B-PLL - albeit in younger patients only<sup>7</sup>.

In the present study, we performed cytogenetic and molecular assessments in one of the largest reported series of patients with a validated diagnosis of B-PLL. Furthermore, we sought correlations between the cytogenetic and molecular findings and the patients' clinical outcomes. Lastly, we studied the primary B-PLL cells' *in vitro* response to novel targeted therapeutics. Overall, the present study is the first to have provided an extensive genetic portrait of B-PLL and to have offered insights into prognostic markers and treatment options.

## Patients, materials and methods

### *Patient selection*

Databases from 28 French healthcare establishments were retrospectively screened for cases of *de novo* B-PLL. Patients with a history of another B-cell malignancy (CLL or MZL) were excluded. A total

of 87 patients with an available blood smear at diagnosis were first identified locally, based on cell morphologic criteria. All 87 blood smears were blind-reviewed by three independent expert cytologists (LB, KM, and CS), and ten representative smears were subsequently reviewed further by a fourth cytologist (J-FL) (Supplementary information and Supplemental Table S1). Only 34 of the 87 cases met the WHO criteria for B-PLL (i.e. prolymphocytes accounted for more than 55% of the lymphoid cells in PB). A diagnosis of MCL was ruled out by karyotyping (K) and FISH assays: no *CCND1* rearrangements or other infrequent translocations involving *CCND2* and *CCND3* were observed<sup>11,12</sup>. Four other patients carrying a translocation t(11;14)(q13;q32) with a *CCND1* rearrangement were diagnosed as having MCL and were excluded. Cytogenetic and molecular analyses were performed on the available material: at diagnosis for 21 patients, during follow-up and before treatment for 10 (median time between diagnosis and sampling: 44 months), and at relapse for 3. The study was performed in accordance with the Declaration of Helsinki, and was approved by the local investigational review board (*CPP-Ile-de-France VI*, Paris, France) on May 21<sup>th</sup>, 2014.

#### *Karyotyping and FISH analysis*

Standard chromosome banding analyses were used to obtain R-or G-banded chromosomes from PB (n=28), bone marrow (n=4) or spleen (n=1). The samples were cultured for 48–72 hours with 12-O-tetradecanoylphorbol-13-acetate (n=6) or CpG-oligonucleotides and interleukin 2 (n=27). All the K results were reviewed by the members of the *Groupe Francophone de Cytogénétique Hématologique* and classified according to the International System for Human Cytogenetic Nomenclature (ISCN 2016). Complex Ks (CKs) and highly complex Ks (HCKs) were defined as the presence of at least three or five numerical or structural chromosomal abnormalities, respectively. Standard FISH was performed in all cases. The specific probes and procedures used are detailed in the supplementary information.

#### *Whole-exome sequencing*

DNA extracted from flow-sorted CD19+ tumor cells (and CD5+, IGL or IGK when appropriate) and non-tumor (CD3+) cells (considered to be germinal controls) was used for exome capture according to standard procedures. Paired-end sequencing was performed using HiSeq2000 sequencing instruments (Illumina, San Diego, CA). More detailed information can be found in the supplementary information.

#### *Targeted deep resequencing*

Mutation validation using targeted deep resequencing was performed as described previously<sup>13</sup>. Details are available in the supplementary information.

### *IGHV analysis*

*IGH* rearrangements sequencing was performed on DNA extracted from sorted tumor cells or from whole blood, as described previously<sup>14</sup>. Depending on the % of identity of the *IGHV* genes with their germline counterparts, sequences were considered as unmutated (100% identity), minimally/borderline mutated (99.9%–97%) or significantly mutated (< 97% identity), following criteria proposed by Hadzidimitriou et al.<sup>15</sup> for MCL and Bikos et al.<sup>16</sup> for MZL.

### *RNA Sequencing*

RNA sequencing was performed on high-quality RNA from 12 B-PLL as described previously<sup>17</sup>. More detailed information can be found in the supplementary information.

### *In vitro cell viability and programmed cell death (PCD) assays*

The viability of primary B-PLL cells was assessed with the ATP-based CellTiter-Glo 2.0 assay (Promega, Madison, WI). Briefly, after pretreatment (or not) with the BET proteins inhibitors (iBET) OTX015 or JQ1 (500 nM), B-PLL cells were exposed for 48h to increasing concentrations of fludarabine, ibrutinib, idelalisib or venetoclax. Viability was determined by normalizing luminescence units against a DMSO control. Alternatively, PCD was measured by flow cytometry using annexin-V and propidium iodide co-labeling, after B-PLL cells treatment for 48h with ibrutinib, idelalisib, venetoclax, or OTX015. Details are provided in the supplemental information. All drugs were purchased from Selleckchem (Houston, TX).

### *Statistical analysis*

Overall survival (OS) was defined as the time interval between diagnosis and death or last follow-up. Categorical variables were compared using a chi-squared test or Fisher's exact test, whereas continuous variables were compared using the Mann-Whitney test. Survival analyses were performed using the Kaplan-Meier method. The log-rank test was used for intergroup comparisons of OS curves. A 2-sided  $P < 0.05$  was considered statistically significant. All statistical analyses were performed using MedCalc software (version 17.8.6, Ostend, Belgium).

### **Accession Numbers**

The WES and RNA-Seq data have been deposited in the European Genome-phenome Archive (EGA) database (accession number EGAD00001002323, EGAD00001002438 and EGAD00001002476).

Additional methods are described in supplementary information.

### **Results**

### *Characteristics of the study population*

The study included 34 patients diagnosed with *de novo* B-PLL between 1992 and 2017 (Table 1). There was a predominance of males (59%), and the median (range) age at diagnosis was 72 (46.2-87.9). The median lymphocyte count was 36 G/L (4.6-244) and the median proportion of prolymphocytes was 79.5 (60-100). Nineteen of the 34 patients (56%) were positive for CD5 (Supplemental Table S2). The median (range) follow-up time after diagnosis was 47 months (0.2-141). Most of the patients had been treated (29/33, 88%) with a median (range) time after diagnosis of 3.2 months (0-106).

### *Chromosomal aberrations: MYC is the most frequently altered gene in B-PLL*

Karyotype was performed in 33 patients (Table 1 and Supplemental Table S3). A CK was found in 73% of the patients, and HCK in 45%. By combining K and FISH data, we observed that the most frequent abnormality, found in 21 of the 34 patients (62%), was a translocation involving the *MYC* gene [t(*MYC*)]. This translocation involved the *IGH* locus in 12 cases, *IGL* in 6 and *IGK* in 1. The *MYC* gene partner was not identified in 2 cases. Five of the 34 patients (15%) had gained copies of the *MYC* gene: three had 3 copies, one had 4 copies and one had *MYC* amplification. Thus the majority of the cases (26 out of 34, 76%) had a *MYC* abnormality, *MYC* translocation and *MYC* gain being mutually exclusive. The other frequent chromosomal aberrations were: del17p including the *TP53* gene (13/34, 38%), trisomy (tri) 18/18q (10/33, 30%), del13q (10/34, 29%), tri3 (8/33, 24%), tri12 (8/34, 24%) and del8p (7/31, 23%) (Figure 1).

An analysis of copy number values (calculated from the WES coverage in 16 patients) confirmed the majority of recurrent losses and gains observed by K and/or FISH analyses, and highlighted other novel recurrent copy number variations: losses of 5q22-23, 9q34, 14q24 and 19p13, and gains of 17q24, 1q31-42 and 4q27-35 (Supplemental Table S4).

### *IGHV analyses: preferential usage of VH3 and VH4 genes, and frequently mutated IGHV genes*

We obtained clonal *IGH* sequences in 19 B-PLL patients. In 17 of the 19 B-PLL cases (89%), a member of the *IGHV3* (n=11, including 4 *IGHV3-23*) or *IGHV4* (n=6) subgroups was involved, while the *IGHV1* subgroup gene (*IGHV1-2*) was used in the two remaining cases. Furthermore, the vast majority of B-PLL sequences (15/19, 79%) displayed significantly mutated *IGHV* genes (Supplemental Table S5).

### *The spectrum of somatic mutations*

WES of paired tumor-control DNA was performed in 16 patients and mutations were validated by targeted deep-resequencing and/or RNA-Seq (Supplemental Figure S1 and Supplemental Table S6). We identified 10 genes that were mutated in at least two patients (Table 2). The most frequently



mutated gene was *TP53*, with a total of 7 mutations observed in 6 of the 16 patients (38%). These *TP53* mutations were predicted to affect the protein's DNA binding domain (Supplemental Figure S2). In all cases, these mutations were associated with a del17p. Mutations in *MYD88* were found in four patients. Interestingly, the *MYD88* mutation was associated with tri3 in two cases, with a VAF of 66.5% (BPLL\_6) and 67.4% (BPLL\_32) indicating that the mutated copy was duplicated. Mutations in genes that have a role in chromatin modifications were recurrent, including the X-linked *BCOR* gene encoding a BCL6 corepressor (n=4), *CHD2* (n=2), *SETD2* (n=2) and other genes mutated in one patient each: *CREBBP*, *EZH2*, *KMT2D*, *NCOR1*, *SETD1B* and *TET2*. The *MYC* gene had missense mutations in 3 patients, all of whom harbored a t(*MYC*). Two mutations were just downstream of the transactivation domain, and one was in the bHLH domain (Supplemental Figure S2). The products of other relevant mutated genes were found to be involved in RNA metabolism (*SF3B1*, *BCLAF1*, *ZRSR2* and *WBP4*), cell migration (*CXCR4*), cell cycle (*CDKN3*, *RB1*), apoptosis (*TP73*) and NOTCH signaling (*NOTCH2* and *FBXW7*) (Figure 2).

#### *IGL/PVT1 fusions*

Using RNA-Seq, we detected *IG* fusion transcripts in 3 of 7 patients with t(*MYC*). In case #27 harboring a t(8;14)(q24;q32), *IGHG1* was fused with intron 1 of *MYC*. In cases BPLL\_4 and BPLL\_30 with a t(8;22)(q24;q11), the *PVT1* gene was fused to *IGLL5* or *IGLV4-69\*01*, respectively. The two latter fusions were confirmed by an RT-PCR assay. No other recurrent fusion transcripts were found in the 12 patients with RNA-Seq data (Supplemental Table S7).

#### *Clonal organization*

With a view to identifying early and late oncogenic events, we evaluated the clonal or subclonal nature of chromosomal abnormalities and recurrent somatic mutations. We observed that t(*MYC*), del17p, tri12 and mutations in the *TP53*, *BCOR*, *MYC*, *SF3B1* and *MYD88* genes were mostly clonal, and should be considered as early events in B-PLL. In contrast, *MYC* gain, del8p and del13q were frequently subclonal indicating that they are acquired (Supplemental Figure S3).

#### *Correlations between cytogenetic aberrations, mutations and gene expression patterns*

We identified three cytogenetic subgroups (Figure 1 and Table 3). The main subgroup of patients (21/34, 62%) had a t(*MYC*). Relative to the patients without t(*MYC*), this subgroup had a higher percentage of prolymphocytes (p=.03), a higher proportion of CD38+ cells (p<.0001), a lower level of cytogenetic complexity (p=.0005), a lower proportion of cases with HCK (p=.0004), fewer unbalanced translocations (p=.04), and fewer cases with del17p (p=.0006). Mutations in *MYC* and in genes involved in RNA metabolism, chromatin remodeling and transcription were almost exclusively

observed in the t(*MYC*) subgroup, whereas mutations in *TP53* were less frequent (Figure 2A). A principal component analysis (PCA) of gene expression data from the 12 cases analyzed by RNA-Seq showed that the 7 patients with t(*MYC*) clustered together (Figure 2B). Taken as whole, these results suggest that patients with t(*MYC*) form a homogeneous subgroup of B-PLL. A second subgroup of B-PLL with *MYC* gain (5/34, 15%) was associated with HCK (p=.01) and tri3 (p=.008). The remaining 8 patients corresponded to a third subgroup lacking *MYC* aberration. Compared with patients with *MYC* aberration, this third subgroup had a lower percentage of prolymphocytes (p=.03), no CD38+ expression (p<.001), a higher number of chromosomal aberrations (p=.02) and a higher frequency of del17p (p=.03) (Table 3).

#### *Survival analyses: the prognostic values of MYC aberrations and del17p*

The median OS for the entire study cohort was 125.7 months. Patients with t(*MYC*) had a median [95% CI] OS of 57.5 months [25.7-132.1], which did not differ significantly from the value observed for patients with *MYC* gain (median: 66.5 months) (Figure 3A). Hence, we pooled patients with *MYC* aberration for subsequent OS comparisons, and found that they had a significantly shorter OS than patients without *MYC* aberrations (p=.03) (Figure 3B).

With regard to the combination of *MYC* and del17p aberrations, we identified three distinct prognostic groups with significant differences in OS (p=.0006) (Figure 3C). The patients without *MYC* aberration (n=8) had the lower risk (median not reached). Patients with *MYC* aberration (translocation or gain) but no del17p (n=18) had an intermediate risk (125.7 months). The high-risk group corresponded to patients with both *MYC* and del17p (n=7, 11.1 months).

Other chromosomal abnormalities and karyotype complexity did not have a significant impact on OS. Patients with del17p tended to have a shorter OS than those without (Supplemental Figure S4).

#### *In vitro* drug response assays

To evaluate the B-PLL cell's sensitivity to drugs used in the treatment of B-cell malignancies, we first used a cell viability assay based on the quantification of cellular ATP levels (an indicator of metabolic activity<sup>18</sup>) on primary B-PLL cells after 48h of exposure to ibrutinib, idelalisib (both B-cell receptor (BCR) inhibitors), venetoclax (a highly specific inhibitor of the anti-apoptotic protein BCL2), and fludarabine. The drugs were tested alone or in combination with OTX015 (an iBET known to suppress *MYC* expression in leukemia and lymphoma cell lines)<sup>19-21</sup>. Treatment with increasing doses of single drugs resulted in reduced cell viability in the 3 cases with t(*MYC*) (BPLL\_8, BPLL\_13 and BPLL\_18), but varied from one drug to another and from one patient to another. Relative to treatments with single drugs, OTX015 co-treatment with low doses of fludarabine, ibrutinib or venetoclax was associated

with a much lower level of viability (<30%) in patients with t(*MYC*) (Figure 4A). In contrast, the patient harboring a del17p but not a *MYC* aberration (BPLL\_34) was significantly more resistant to single drugs and OTX015 co-treatments than patients with t(*MYC*) (Figure 4B). To confirm these results, we applied a complementary cytofluorometric approach. In BPLL\_8 with t(*MYC*), treatments with drugs alone killed between 13 and 27% of the cells. An additive effect of OTX015 was observed; between 48 and 63% of the cells were killed (Figure 4C). Cells from patient BPLL\_34 (del17p but no *MYC* aberration) were less sensitive to all drugs tested in both the presence and absence of OTX015 (Figure 4D). Thus, the ATP assay and flow cytometry assay results highlighted the efficacy of combined drug + iBET treatment of cells from patients with t(*MYC*). The enhancing effect of the iBETs on the PCD induced by fludarabine, ibrutinib, and venetoclax was fully corroborated by the results of co-treatment with JQ1, another well-known iBET (Supplemental Figure S5B-C). It should be noted that we did not test any cases with *MYC* gain.

## Discussion

In the present study, we genetically characterized 34 patients with B-PLL. Given that the diagnosis of B-PLL is challenging, a thorough morphological review of blood smears was performed by three independent cytologists. Although we excluded patients with a history of MZL, the absence of a biopsy analysis means that we cannot fully rule out the possible presence of MZL with a prolymphocytoid transformation- an extremely rare situation<sup>22</sup>.

To the best of our knowledge, the present series is the largest yet to have undergone a comprehensive genomic characterization of B-PLL. We found that the most frequent aberrations are related to *MYC* alterations (in 77% of cases) highlighting the *MYC*'s major role in the pathogenesis of B-PLL. Strikingly, *MYC* translocations and *MYC* gain were found to be mutually exclusive. Translocations in which the *MYC* gene is repositioned under the control of an enhancer (usually immunoglobulin genes enhancers) result in the overexpression of *MYC*, as does the gain of one or more copies of *MYC*<sup>23</sup>. Although *MYC* translocations and mutations are classically associated with Burkitt lymphoma (BL), aberrations in *MYC* have also been linked to almost all B-cell neoplasms. With regard to B-PLL, *MYC* translocations and *MYC* gain have been reported in a few cases<sup>24-29</sup>. In the present series, t(*MYC*) was present in the major clone but *MYC* gain was mainly subclonal and/or overlooked as part of a CK. Thus, performing FISH with a specific probe is essential to identify *MYC* gain or amplification. We found *MYC* gene mutations in 3 cases, all of which also had t(*MYC*). These mutations were not located in the transactivation domain (the hot spot for *MYC* mutations in BL). However, it is likely that *MYC* mutations have a role in the physiopathology of B-PLL (as they do in BL)

by enhancing the oncogenicity of Myc<sup>30</sup>. We found a fusion transcript between *PVT1* and an *IGL* gene in two patients with a variant *MYC* translocation t(8;22). This fusion subsequently disrupted the *PVT1* gene, a long intergenic noncoding RNA located 55 kb downstream of *MYC*, and which has been identified as a recurrent breakpoint in BL harboring variant translocations t(2;8) or t(8;22)<sup>31,32</sup>. In a recent study, the *PVT1* promoter was found to behave like a tumor-suppressor DNA element: silencing the *PVT1* promoter increased *MYC* transcription and cell proliferation<sup>33</sup>. As with *MYC* mutations, disruption of the *PVT1* gene in some B-PLL patients might cooperate functionally with translocations to increase *MYC*-mediated oncogenesis.

Our present results confirmed previous case reports in which a CK was frequent in B-PLL<sup>24,27,34</sup>. The frequency of a CK in B-PLL (73% in the present study) is higher than that reported in BL (34%<sup>35</sup>), MCL (40-60%<sup>36,37</sup>), MZL (53%<sup>38</sup>), and CLL at diagnosis (15%<sup>39-42</sup>), and is similar to that observed in high-risk CLL<sup>43</sup>. Whereas t(*MYC*) is the main abnormality in B-PLL (62%), it is rare in other chronic B-cell disorders (i.e. below 5% in MCL, MZL and CLL<sup>28,38,44</sup>). The frequency of del17p in B-PLL (38%) observed here is higher than for MCL and MZL (both around 20%)<sup>37,38</sup>, or for CLL (5 to 7% at diagnosis)<sup>39,45</sup>. In addition to *MYC* and *TP53* abnormalities, we found several other recurrent chromosomal aberrations in B-PLL, including trisomies 3, 12 and 18, del13q, and del8p. None of the chromosomal abnormalities observed in B-PLL are specific and so can be observed in other mature B-cell malignancies, albeit at different frequencies. Similarly, all the driver genes recurrently mutated in B-PLL are also involved in other chronic B-cell disorders, but not with the same prevalence. In line with previous studies<sup>5,6</sup>, we found a high frequency of *TP53* mutations (37.5%) in B-PLL; this frequency is higher than that observed for other B-cell malignancies, such as MCL (18%)<sup>46</sup>, LZM (14%)<sup>47</sup>, CLL (10%)<sup>48</sup> and BL (24%)<sup>49</sup>.

We identified a high frequency of clonal *BCOR* mutations, which were only present in t(*MYC*) patients. *BCOR* encodes a Bcl6 co-repressor that silences *BCL6* targets by epigenetic modifications<sup>50</sup>. Inactivating mutations of *BCOR* have been found in myeloid disorders<sup>51,52</sup> but are very unusual in B-cell diseases, except in the rare splenic diffuse red pulp lymphoma<sup>53</sup>. The association of *BCOR* mutations and t(*MYC*) might be a specific feature of B-PLL. Indeed, a recent study of E $\mu$ -*Myc* lymphomas in a mouse model revealed a high frequency of *Bcor* mutations and showed that *Bcor* was a cooperative tumor suppressor gene, the disruption of which can accelerate *Myc*-driven lymphomagenesis<sup>54</sup>.

Our data showed that patients with B-PLL and t(*MYC*) form a homogeneous subgroup, with a less karyotype complexity, a higher proportion of prolymphocytes and a higher frequency of CD38 expression. Interestingly, clonal mutations in the *MYC*, *BCOR* and *SF3B1* genes clustered together in

some patients with t(*MYC*), suggesting oncogenic cooperation in the development of B-PLL. *MYC* aberration is a feature shared with BL, however, in contrast to BL, we did not find any mutation in *TCF3*, *ID3* or *CCND3* genes in B-PLL<sup>49</sup>.

Van der Verlen et al. have suggested that B-PLL is a specific subgroup of t(11;14)-negative MCL, based on similarities in the immunophenotype, immunogenotype and gene expression profile<sup>55</sup>. We did not find any *CCND1* or *ATM* mutations (the most frequent mutations in MCL<sup>56</sup>), in our patients with B-PLL. The vast majority of B-PLL expressed significantly mutated *IGHV* genes belonging to the *IGHV3* and *IGHV4* subgroups, in keeping with previous reports<sup>57,58</sup>. This contrasts with MCL, that exhibit predominantly no or minimal somatic hypermutations<sup>15</sup>.

Taken as a whole, our data demonstrate that the genomic profile of B-PLL shares similarities with that of chronic B-cell malignancies but also displays unique combinations of alterations, thus confirming that B-PLL is a distinct entity.

The median OS time in our cohort was 125.7 months - longer than the times reported in two earlier studies<sup>6,59</sup>. This difference might be due to our patients' treatments with rituximab, alemtuzumab or fludarabine – all of which are reportedly efficacious in B-PLL<sup>60,61</sup>. In light of our results, we propose a hierarchical prognostic model that depends on *MYC* aberration (translocation or gain) and del17p. Patients without *MYC* aberration have indolent disease (regardless of their 17p status), whereas patients with *MYC* aberration have a significantly shorter OS time. A combination of *MYC* aberration and del17p confers the worse outcome, with a median OS of less than one year. By analogy with the “double-hit” form of CLL involving del17p and *MYC* gain<sup>43</sup>, these patients may be considered as having a very high-risk form of B-PLL. Due to the rarity of this disease, our data were obtained with a relatively small number of patients and so need to be confirmed in future studies.

Our *in vitro* data showed that a combination of ibrutinib, idelalisib or venetoclax with OTX015 is more cytotoxic than the drugs used alone - especially in patients with *MYC* translocation. This is concordant with the known effects of OTX015, an inhibitor of the bromodomain-containing proteins BRD2, BRD3 and BRD4. These proteins act as epigenetic readers and have an important role in the regulation of transcription and the modulation of oncogene expression. Like other iBETs, OTX015 strongly downregulates the transcription of genes regulated by superenhancers, such as *MYC*<sup>20</sup>. Current approaches to the treatment of B-PLL (like CLL) are based on del17p. However, our results strongly suggest that *MYC* should be taken into account, and could be targeted. OTX015 is already in Phase I clinical studies with a relatively good safety profile<sup>62</sup>; when combined with targeted drugs, it might constitute an epigenetic treatment option in patients with B-PLL and *MYC* translocation. In any case,

further investigations with a larger number of patients (especially those with *MYC* gain) are required to make progress in this field.

In conclusion, we found that patients with B-PLL have CKs and HCKs, a high frequency of *MYC* aberration (by translocation or gain), frequent del17p, and frequent mutations in the *TP53*, *BCOR*, *MYD88* and *MYC* genes. We identified three prognostic subgroups, depending on *MYC* and 17p status. Patients with both *MYC* aberration and del17p had the shortest OS, and should be considered as a high-risk subgroup. Our results showed that cytogenetic analysis is a useful diagnostic and prognostic tool in B-PLL. We recommend K and FISH (for *MYC* and *TP53*) analyses whenever B-PLL is suspected. Moreover, our *in vitro* data strongly suggest that drugs targeting the BCR and BCL2 in combination with an iBET might be a treatment option for patients with B-PLL.

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### **Authorship Contributions**

F.NK, OA.B, SA.S and E.C designed the study. E.C, E.P, K.D, D.RW, C.D, C.G, M.Y, C.L, F.D, M.LT, N.D, P.D, S.B, LW, SS performed experiments and analyzed data. K.M, C.S, L.B, and J-F.L performed the morphological review. C.A, V.L, J.G, V.E, B.G, E.CB, M.M, C.L, N.N, A.I, S.S, MA.CR, B.Q, S.FF, N.A and I.RW provided samples and clinical data. E.C, F.D, T.T, S.S, OA.B and F.NK wrote the manuscript.

### **Disclosure of Conflicts of Interest**

The authors declare no competing financial interests.

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## Figures legends

### Tables

**Table 1. Characteristics of the patients with B-PLL**

**Table 2. Recurrently mutated genes identified using WES in 16 patients with B-PLL**

**Table 3. Comparison of the three cytogenetic subgroups (S): significant results**

### Figures

**Figure 1. Distribution of chromosomal abnormalities detected in 34 patients with B-PLL: three cytogenetic subgroups of patients: *MYC* translocation, *MYC* gain, and no *MYC* aberration.** Each column represents one patient, and each row one particular genetic or laboratory parameter. Color code: black or blue: presence; grey: absence; white: not available. CK: complex karyotype (three or more chromosomal abnormalities), HCK: highly complex karyotype (five or more chromosomal abnormalities), del: deletion, tri: trisomy. The percentages of polymphocytes (indicated in black boxes) correspond to above-median values, relative to the cohort as a whole.

**Figure 2. Whole-exome sequencing and RNA-seq.** A. Distribution of the chromosomal aberrations and mutations in the three cytogenetic subgroups (*MYC* translocation, *MYC* gain, and no *MYC* aberration) in the 16 cases analyzed using WES. Chromosomal abnormalities: grey: absence; color: presence. Mutations: grey: WT; color: mutated. B. Principal component analysis of differential gene expression patterns for 12 samples with various cytogenetic abnormalities.

**Figure 3. Overall survival in patients with B-PLL.** (A) and (B) as a function of *MYC* aberrations (translocation (t(*MYC*) or gain) and the presence or absence of *MYC* aberration (ab); the t(*MYC*) and *MYC* gain data were pooled. (A) t(*MYC*) median [95%CI]: 57.5 months [25.7-132.1] vs *MYC* gain median [95%CI]: 66.5 months [4.7-undetermined]. (B) *MYC* aberration (t(*MYC*) + *MYC* gain) median [95%CI]: 57.5 [25.7-132.1]. (C) *MYC* and del17p status. *MYC* aberration without del17p median [95%CI]: 125.7 months [52.2-132.1]; *MYC* aberration with del17p median [95%CI]: 11.1 months [4.7-66.5]

**Figure 4. The combination of drugs frequently used to treat B-cell malignancies with BET protein inhibitors enhances the killing of primary B-PLL cells with t(*MYC*).** (A) B-PLL cells from 3 patients with t(*MYC*) were treated with the indicated concentrations of ibrutinib, idelalisib, venetoclax or fludarabine in the presence or absence of OTX015 (500 nM). Viability was assessed with an ATP-based CellTiter-Glo® 2.0 kit. (B) B-PLL cell viability was measured and analyzed as in (A) in a patient with del17p but no *MYC* aberration. (C) Cell death was quantified in primary B-PLL cells from patient BPLL\_8 (with t(*MYC*)) with or without pretreatment with OTX015 (500 nM) and exposure for 48h to

ibrutinib (7.5  $\mu$ M), idelalisib (50  $\mu$ M) or venetoclax (10 nM). The percentages refer to annexin-V-positive or annexin-V/PI-positive cells. (D) Primary B-PLL cells from BPLL\_34 (with del17p but no *MYC* aberration) were treated with drugs and analyzed as in (C). Bars represent the mean  $\pm$  SEM.

	<b>Whole cohort</b>
<b>Parameters</b>	<b>n=34</b>
<b>Age at diagnosis, years (median, range)</b>	72 [46.2-87.9]
<b>Sex (M/F)</b>	20/14
<b>Splenomegaly</b>	18/31 (58%)
<b>Lymphadenopathy</b>	6/31 (19%)
<b>Lymphocytosis G/L (median, range)</b>	36 [4.6-244]
<b>% of prolymphocytes (median, range)</b>	79.5 [60-100]
<b>Treated patients</b>	29/33 (88%)
<b>Death</b>	14/33 (42%)
<b>Follow-up (median, range)</b>	47 m [0,2-141]
<b>OS, median (95%CI)</b>	125.7 m (52.3-132.1)
<b>TTT, median (95%CI)</b>	5.8 m (1.5-27.5)
<b>CD38+</b>	20/33 (61%)
<b>Matutes' score</b>	
<b>0</b>	8/33 (25%)
<b>1</b>	11/33 (33%)
<b>2</b>	9 (27%)
<b>3</b>	5 (15%)
<b>CD5+</b>	19/34 (56%)
<b>CD23+</b>	11/34 (32%)
<b>CK <math>\geq</math>3 abnormalities*</b>	24/33 (73%)
<b>HCK <math>\geq</math>5 abnormalities*</b>	15/33 (45%)
<b>Number of karyotypic aberrations (median, range)*</b>	4 [1-21]
<b>Unbalanced translocations*</b>	23/33 (70%)
<b>t(MYC)</b>	21/34 (62%)

<b>del17p (<i>TP53</i>)</b>	13/34 (38%)
<b>Tri12</b>	8/34 (24%)
<b>Tri3</b>	8/33 (24%)
<b>Tri18</b>	10/33 (30%)
<b><i>MYC</i> gain</b>	5/34 (15%)
<b>del13q</b>	10/34 (29%)
<b>del8p (<i>TNFRSF10</i>)</b>	7/31 (23%)
<b><i>IGHV</i> mutated</b>	16/19 (84%)
<b><i>TP53</i> mutated</b>	6/16 (38%)
<b><i>MYD88</i> mutated</b>	4/16 (25%)
<b><i>BCOR</i> mutated</b>	4/16 (25%)
<b><i>MYC</i> mutated</b>	3/16 (19%)
<b><i>SF3B1</i> mutated</b>	3/16 (19%)

**Table 1. Characteristics of the patients with B-PLL**

m: months; OS: overall survival; TTT: time to first treatment; t(*MYC*): translocation involving *MYC*; CK: complex karyotype; HCK: highly complex karyotype; del: deletion; tri: trisomy.

\*The frequency of chromosomal abnormalities did not differ significantly when comparing patients at diagnosis vs. patients during follow up; and treated patients vs. untreated patients.

Gene	Number of cases	Number of mutations identified	Mutations	Biological process
<b>TP53</b> (NM_000546)	6	7	p.A129Pfs*41 + p.V132L, T205C, V236G, A248D, L275Q, R282P	Cell cycle, apoptosis
<b>MYD88</b> (NM_002468)	4	4	L265Px2, M232T, S219C	NF-kB pathway
<b>BCOR</b> (NM_001123385)	4	4	F924C, N1459Sx2, P1648Lfs*4	Chromatin remodeling
<b>MYC</b> (NM_002467)	3	3	V160L, S161L, F389L	Cell cycle
<b>SF3B1</b> (NM_012433)	3	3	K700Ex3	Spliceosome
<b>SETD2</b> (NM_014159)	2	2	2 p.Gly878Glnfs*14, L1577P	Chromatin remodeling (histone H3K36 methyltransferase)
<b>CHD2</b> (NM_001271)	2	2	P1387Rfs*13, V1163Gfs*3	Chromatin remodeling
<b>CXCR4</b> (NM_003467)	2	2	S338X, S325Qfs*22	Cell migration
<b>BCLAF1</b> (NM_001077440)	2	2	R820C, W211X	RNA processing factor
<b>NFASC</b> (NM_001005388)	2	2	T946I, V644M	Neuronal development

**Table 2. Recurrently mutated genes identified using WES in 16 patients with B-PLL.**

Parameters	S1 t(MYC) n=21 (62%)	S2 MYC gain n=5 (15%)	S3 Without MYC n=8 (23%)	Comparison	p-value
Prolymphocytes % (range)	86% (61-100)	79 (66-91)	76% (60-80)	S1 vs S2+S3 S3 vs S1+S2	.03 .03
CD38+	18/20 (90%)	2/5 (40%)	0/8 (0%)	S1 vs S2+S3 S3 vs S1+S2	<.0001 <.001
Number of chromosomal abnormalities (range)	3 (1-9)	6 (5-21)	10 (1-15)	S1 vs S2+S3 S3 vs S1+S2	.0005 .02
HCK $\geq$ 5	4/20 (20%)	5/5 (100%)	6/8 (75%)	S1 vs S2+S3 S2 vs S1+S3	.0004 .01
Unbalanced translocations	11/20 (55%)	5/5 (100%)	7/8 (88%)	S1 vs S2+S3	.04
del17p	3/21 (14%)	4/5 (80%)	6/8 (75%)	S1 vs S2+S3 S3 vs S1+S2	.0006 .03
Tri3	4/20 (20%)	4/5 (80%)	0/8 (0%)	S2 vs S1+S3	.008

**Table 3. Comparison of the three cytogenetic subgroups (S) (MYC translocation, MYC gain, without MYC activation): significant results. HCK: highly complex karyotype; del: deletion; tri: trisomy**