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XPO1 MUTATIONS IDENTIFY A SUBGROUP OF EARLY STAGE CLL PATIENTS CHARACTERIZED BY SHORTER TIME TO FIRST TREATMENT CONCEIVABLY DUE TO ENHANCED B CELL RECEPTOR SIGNALING

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Abstract

Most early-stage chronic lymphocytic leukemia (CLL) patients do not require immediate treatment and are actively managed with watch & wait strategy. While few clinical and molecular factors predict treatment requirement, the impact of gene mutations in unknown. In this study, we examined a total of 1092 early-stage CLL cases, subdivided into a training group (N=295) and two validation cohorts (N=402 and N=395). Through NGS analysis, we identified *XPO1* mutations as predictor of shorter time to first treatment (TTFT). Importantly, *XPO1* mutations retained their prognostic significance independently of IGHV status and variables incorporated into the prognostic models that predict TTFT in early-stage CLL (IPS-E and Rai 0 Model). To elucidate the mechanisms underlying the higher proliferation rate of *XPO1* mutated cells, we conducted RNA and ATAC-seq on 8 *XPO1*-mutated CLL samples and 15 *XPO1* wild-type CLL cases, matched for IGHV status, FISH karyotype, and *TP53* status. The analysis revealed that chromatin regions with increased accessibility in *XPO1*-mutated CLL were enriched with binding sites for transcription factors regulated by BCR-related pathways, including NF-kB signaling, p38-JNK, and RAS-RAF-MEK-ERK. Pathway enrichment analysis of upregulated genes from RNA-seq data indicated that XPO1 mutant CLL exhibited transcriptomic features associated with BCR and cytokine signaling. Combining epigenomic and transcriptomic data, we found that *MIR155HG* (the host gene of miR-155) and *MYB* (the transcription factor regulating *MIR155HG*) were upregulated, and their promoters were more accessible in *XPO1*-mutated CLL. Further confirmation through RT-qPCR demonstrated higher expression of miR-155 in *XPO1*-mutated cells. In conclusion, *XPO1* mutations, conceivably by increasing miR-155 levels, enhance BCR signaling, resulting in a higher proliferation rate and a shorter TTFT in early-stage CLL. The integration of *XPO1* mutations into existing scoring systems may aid in identifying patients who would derive the greatest benefit from early intervention clinical trials.

Introduction

Chronic lymphocytic leukemia (CLL) is one of the most prevalent B cell malignancies and the most common form of leukemia in Western countries, boasting an annual incidence of 5.1 per 100,000 individuals [1]. The nature of CLL is notably diverse; some patients may never necessitate treatment, while others experience early relapse following therapy [2]. The extensive array of molecular investigations in CLL has significantly enhanced our comprehension of disease pathogenesis and led to the identification of molecular biomarkers, aiding clinicians in the precise management of individual patients [2-7]. The discovery of molecular predictors, combined with the introduction of innovative and highly effective drugs, empowers the optimization of treatment strategies for CLL on an individualized basis. The application of precision medicine to neoplastic disorders involves tailoring the management and treatment of the disease according to the specific tumor genes, alongside the host's characteristics. Over the past few years, a precision medicine approach has emerged for CLL, propelled by the expanding biological and genomic insights into this form of leukemia. In this study, we will provide a translational perspective of the precision management of the various phases of CLL, including asymptomatic patients, patients requiring first line therapy, relapsed/refractory (R/R) disease, and Richter syndrome (RS).

Genomic Landscape and Biological Mechanisms of CLL

Numerous molecular investigations have extensively analysed the molecular profile of CLL, revealing a diverse range of molecular abnormalities driving disease pathogenesis, progression, and transformation, rather than a singular, unifying genetic lesion. CLL is associated with various genetic lesions that deregulate different biological pathways contributing to its pathogenesis [2-7].

In the case of all B cell malignancies, the distinctive rearrangement of the immunoglobulin heavy variable (IGHV) gene serves as the distinguishing feature for each CLL clone. Pivotal studies have demonstrated that the *IGHV* gene repertoire in CLL exhibits an imbalance, suggesting the involvement of antigen selection in the development of the disease [8,9]. Another significant characteristic of *IGHV* genes employed by CLL is the extent of similarity between the IGHV rearrangement and its normal counterpart. In about 60% of CLL cases, the *IGHV* genes utilized by the leukemic clone exhibit a homology to the normal counterpart of less than 98%. Referred to as IGHV mutated CLL (M-CLL), these cases are theorized to stem from B cells that have undergone somatic hypermutation of immunoglobulin genes - a physiological process occurring as B cells transit through the germinal center. In contrast, 40% of CLL cases exhibit *IGHV* genes with a homology to the normal counterpart equal to or exceeding 98%. These cases are termed as IGHV unmutated CLL (U-CLL) and are postulated to derive from naïve B cells that have undergone maturation independent of the germinal center reaction (Figure 1).

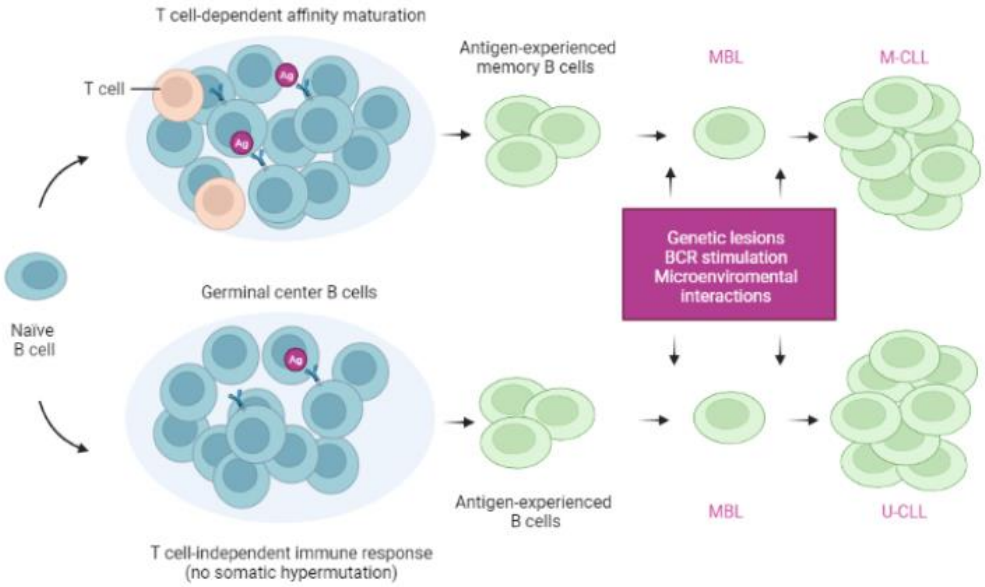

Figure 1. Cell-of-origin of CLL

The mutational status of *IGHV* genes identifies CLL subgroups that exhibit significant molecular and clinical distinctions. IGHV U-CLL is associated with adverse prognostic genomic aberrations, increased B cell receptor signaling (BCR) capacity, a shorter time to progression, and inferior survival when compared to IGHV mutated patients [10-12]. Beyond its prognostic value, the mutational status of *IGHV* genes also represents a predictive biomarker. CLL patients with mutated *IGHV* genes, devoid of *TP53* abnormalities, may still derive benefits from chemoimmunotherapy (CIT), a treatment typically considered suboptimal for those with IGHV unmutated CLL [13-15].

The apoptosis pathway is commonly compromised, and the predominant genetic abnormality in CLL, the deletion of 13q14 (*del13q14*), plays a crucial role in the dysregulation of apoptosis in many CLL patients, though not all. *Del13q14* is detected in 50% to 60% of cases, often manifesting as a monoallelic lesion [16]. This genetic alteration is an early occurrence in the pathogenesis of CLL and may be detected as early as the stage of monoclonal B-lymphocytosis (MBL), which often precedes the diagnosis of CLL [17]. The minimal deleted region on 13q14 includes two microRNAs (miR), namely miR-15 and miR-16, which naturally inhibit the function of the anti-apoptotic protein BCL2 [18]. The absence of miR-15/16 removes a suppressor of BCL2 expression, consequently fostering the continuous survival of tumor B cells *in vitro* and inducing CLL development in mouse models [19,20]. Individuals with *del13q14* exhibit a favorable prognosis, provided they do not concurrently harbor other genetic abnormalities (such as 17p deletion, 11q deletion) that are linked to a less favorable outcome [16]. While the identification of *del13q14* has elucidated the significance of the apoptotic pathway in the disease, and guidelines recommend its assessment through FISH [21], this genetic alteration is not presently employed as a biomarker for precision medicine. This is because CLL responds to BCL2 inhibitors irrespective of the *del13q14* status.

A critical pathway in CLL pathogenesis, significantly influencing treatment response, is the DNA damage response pathway [2]. Molecular alterations affecting genes within this pathway, notably *TP53* and *ATM*, are the most prevalent lesions [22]. The *TP53* gene encodes a central regulator of the DNA damage response pathway and becomes the target of the genotoxic impact of chemotherapy. Chemotherapy operates by inducing DNA damage, activating the TP53 pathway, ultimately leading to apoptosis of CLL cells. Conversely, when *TP53* is compromised by mutation and/or deletion, chemotherapy loses its ability to induce apoptosis in CLL cells. Consequently, these cells may proliferate continuously, accumulating multiple additional genetic abnormalities that facilitate progression and clonal evolution [23]. In line with this, CLL patients experiencing *TP53* disruption exhibit a markedly unfavorable response to chemoimmunotherapy (CIT) and are recommended for treatment with novel therapeutic agents [13,21,24]. *ATM*, functioning as a tumor suppressor gene pivotal for the DNA damage response, is situated in the 11q22-23 region, which is deleted in around 15-20% of newly diagnosed CLL cases. Patients with *del11q* or *ATM* mutations are associated with an intermediate prognosis [22].

The nuclear factor-κB (NF-κB) signaling pathway plays a pivotal role in CLL pathogenesis, comprising two pathways known as canonical and non-canonical [25]. The BCR signaling enhances the canonical pathway, while the non-canonical pathway is typically activated by cytokines or other microenvironmental interactions [26]. In CLL, the disruption of *BIRC3*, a negative regulator of non-canonical NF-κB, is a common occurrence, resulting in abnormal and sustained activation of this biological pathway, fostering proliferation and survival [2]. *BIRC3* mutations are not present in MBL, occur infrequently at the time of CLL diagnosis (3-4%), but become detectable in around 25% of patients resistant to fludarabine [27]. A recent study has shown that *BIRC3* mutations play a role in conveying resistance to fludarabine, cyclophosphamide, and rituximab (FCR), both *in vitro* and *in vivo* [28].

Consistently, patients with *BIRC3* mutations undergoing FCR treatment exhibit a similar unfavorable outcome as those with *TP53* disruption, which stands out as the most robust predictor of chemorefractoriness in CLL [28]. The potential predictive value of *BIRC3* mutations for failure after CIT is supported by findings from the CLL14 phase 3 clinical trial, which compared chlorambucil-obinutuzumab with venetoclax-obinutuzumab in previously untreated CLL patients [29]. Indeed, *BIRC3* mutations were linked to a shorter progression-free survival (PFS) in the chlorambucil-obinutuzumab group, underscoring the role of *BIRC3* mutations as a biomarker for chemorefractoriness [29].

Additional genetic abnormalities contributing to CLL pathogenesis involve the deregulation of the *NOTCH* signaling pathway, often linked to mutations in *NOTCH1* or *FBXW7* genes [22,30]. The *NOTCH1* gene encodes a transmembrane receptor that, upon ligand binding and subsequent migration of the NOTCH1 intracellular domain to the nucleus, triggers the transcription of pro-survival and anti-apoptotic genes [31]. *NOTCH1* mutations typically manifest within the PEST domain, which contains the amino acid sequences recognized by the ubiquitin ligase F-box and WD repeat-containing protein 7 (*FBXW7*). Physiologically, the FBXW7 protein recognizes the PEST domain of the NOTCH1 protein, and, upon ubiquitination, induces its degradation through the proteasomal pathway. Consistently, in case of *NOCTH1* mutations that disrupt the recognition sequence in the PEST domain, the NOTCH1 protein does not undergo proteasomal degradation, and rather retains its function as a positive transcription factor for NOTCH1 target genes [22]. Additionally, NOTCH1 signaling can be enhanced by mutations in *FBXW7* that impede the ubiquitination of the NOTCH1 protein [32,33].

Mutations in the *NOTCH1* gene are identified in about 10% of CLL patients at the time of diagnosis and show an increase in patients with relapsed/refractory (R/R) disease [34]. Despite *NOTCH1*-mutated patients experiencing a shorter survival compared to those with

wild-type *NOTCH1*, the inclusion of *NOTCH1* mutational screening in clinical practice has not yet occurred due to the absence of conclusive evidence establishing *NOTCH1* mutations as a reliable predictor for treatment decisions [34]. The evaluation of *NOTCH1* mutations in the CLL8 trial, which compared FCR to FC in first line CLL therapy, has indicated that patients with *NOTCH1* mutations may no benefit from the addition rituximab into the FC regimen [24]. Correspondingly, CLL cells from patients with *NOTCH1* mutations exhibit lower CD20 expression and diminished cell lysis when exposed to anti-CD20 *in vitro* compared to patients with wild-type *NOTCH1*. Additionally, the blockade of NOTCH1 signaling by γ-secretase inhibitors or NOTCH1-specific small interfering RNAs upregulates CD20 expression on CLL cells. These biological findings potentially signify a disrupted epigenetic loop linked to impaired histone deacetylase (HDAC) function induced by *NOTCH1* mutations, which is partially restored by treatment with HDAC inhibitors [35]. The novel anti-CD20 antibody obinutuzumab, demonstrating higher efficacy compared to rituximab, has proven effective in overcoming refractoriness to anti-CD20 therapy in CLL patients with *NOTCH1* mutations [36].

Another frequently disrupted molecular process in CLL is splicing [2,22]. The most common gene mutations affecting this pathway target the *SF3B1* gene [37]. This gene encodes a critical component of the U2 snRNP, crucial for the initial stages of RNA splicing. The consequences of *SF3B1* mutations are not fully understood but appear to result in abnormal splicing of genes encoding proteins involved in various biological pathways, including the DNA damage response [22]. *SF3B1* is mutated in approximately 10% of newly diagnosed CLL patients and is associated with a worse outcome than wild-type cases [37].

Recently, somatic mutations in the U1 spliceosomal RNA gene have been identified in various cancer types, including CLL [38]. Biologically, this mutation generates new splice junctions and disrupts the splicing pattern of multiple genes. Present in about 3-4% of CLL

cases at diagnosis, it is linked to a shorter TTFT [38]. However, mutations in spliceosome genes do not currently influence the management or treatment decisions in CLL.

Biological markers for prognostic evaluation and predictive insights

A prognosticator refers to a clinical or biological characteristic that provides insights into the natural course and prognosis of a disease, regardless of the treatment administered [39]. In contrast, a predictor is a biomarker that furnishes information regarding the potential advantages of a particular treatment [39]. Within the spectrum of CLL genetic aberrations, *TP53* abnormalities and IGHV mutational status presently meet the criteria as predictive biomarkers, with their usage being endorsed by clinical guidelines for the management of CLL [21,39]. As previously stated, patients with CLL experiencing *TP53* disruption, either through deletion or mutation, exhibit resistance to conventional CIT regimens [2]. The advent of biological agents targeting the BCR pathway or inhibiting BCL2 has partially mitigated the adverse effects associated with *TP53* disruption, although complete elimination of the negative impact has not been achieved. Consequently, patients with *TP53* disruption are treated upfront with biological agents, whose mechanisms of action are independent of the DNA damage response [21].

Conversely, patients with mutated *IGHV* genes, without *TP53* disruption, may still find benefit in chemoimmunotherapy (CIT). As of now, phase 3 clinical trials comparing CIT to biological drugs have not demonstrated the superiority of biological drugs over CIT in this specific subgroup of patients [40-42].

According to guidelines, the assessment of *TP53* status must be conducted through both FISH and mutational analysis prior to initiating treatment, and this evaluation should be repeated at each subsequent relapse [21]. Similarly, the mutational status of *IGHV* genes should be examined before commencing treatment.

Nevertheless, since the pattern of *IGHV* gene mutations remains stable over time, there is no need for retesting at the time of relapse [21]. In the absence of treatment indications at the time of diagnosis, testing for *TP53* abnormalities or *IGHV* mutational status should not be conducted in clinical practice; such testing is reserved for research purposes. It is crucial to note that the decision to initiate treatment is not contingent on the results of these tests but solely on the patient's clinical stage and symptoms [21].

As treatment decisions are influenced by *TP53* and *IGHV* mutational status, it is essential to standardize the analysis of these molecular predictors across laboratories, employing validated methodologies and guidelines [43,44]. The European Research Initiative on CLL (ERIC) has formulated guidelines for the analysis of both *TP53* and *IGHV* mutational status, and it offers a global accreditation process to centers [43,44].

Peripheral blood is a suitable material for *TP53* mutation analysis when the lymphocyte count is $>10 \times 10^9$/L [43]. The sequenced region of the *TP53* gene must encompass exons 4-10, including both the DNA-binding domain and oligomerization domain. Ideally, analysis should extend to exons 2, 3, and 11 to cover the entire coding region [43]. Sanger sequencing or Next Generation Sequencing (NGS) methods can be employed for *TP53* gene sequencing, with a variant allele frequency (VAF) cutoff of 10% for variant calling. This approach is employed because the clinical significance of small subclones of *TP53* is not yet fully comprehended [43]. A few studies have indicated that also *TP53* mutations with a VAF below the conventional 10% threshold are associated with a worse outcome in patients undergoing CIT [45,46]. Nonetheless, these initial investigations are retrospective, and it is imperative to gather additional evidence from prospective trials before reevaluating the clinical significance of small TP53-mutated subclones in making treatment decisions.

Concerning *IGHV* genes, peripheral blood is a suitable material for the test, and B cell purification is typically unnecessary unless the patient exhibits a low fraction of leukemic cells [44]. According to the ERIC guidelines, leader primers are preferred as they enable the amplification of the entire sequence of the rearranged IGHV gene. This approach allows for the comprehensive determination of the somatic hypermutation level in IGHV genes utilized by the CLL clone [44]. Following sequencing, specific bioinformatic tools facilitate the analysis of the IGHV rearrangement [44]. The ERIC network has generated extensive datasets, enabling the analysis of thousands of molecular IGHV sequencing data [47,48]. A subset of unrelated CLL patients carries quasi-similar, if not identical, IGHV sequences known as stereotyped BCR [47,48]. Various groups of stereotyped BCR sequences have been identified, some of which exhibit unique molecular and clinical features [47,48]. For instance, the stereotyped BCR subset #2 identifies a subgroup of CLL patients who, despite carrying mutated IGHV genes, have a very poor outcome and may warrant novel therapeutic strategies [49]. While the incorporation of stereotyped BCR subsets among predictive biomarkers holds promise for a precision medicine approach to CLL in the future, current guidelines do not include them among the biomarkers utilized for treatment decisions in clinical practice.

Management of asymptomatic CLL patients

In most cases, CLL is an incidental diagnosis, often discovered during a complete blood count conducted for unrelated reasons [50]. Additionally, 70% of newly diagnosed CLL patients present at an early stage, as per the Binet and Rai staging systems. These individuals may never necessitate treatment and may experience a life expectancy comparable to the general population [51,52]. Despite the generally indolent nature of CLL, certain patients exhibit a CLL clone with a high proliferation rate, potentially leading to an early need for

treatment due to progressive lymphocytosis, enlarged lymph nodes, cytopenia, and systemic symptoms [21].

Currently, asymptomatic early-stage CLL patients follow a watch-and-wait strategy, with treatment initiated only in the presence of symptomatic disease, as outlined in the latest iwCLL guidelines [21]. Two clinical trials that compared chlorambucil and fludarabine versus placebo in asymptomatic CLL patients failed to show a survival advantage for early treatment compared to observation [53,54]. Preliminary findings from the CLL12 clinical trial, a phase 3 trial comparing ibrutinib versus observation in asymptomatic CLL, have indicated a higher PFS in the ibrutinib arm. However, the results are not considered mature enough to establish a definitive advantage of ibrutinib over observation in terms of OS [55]. Consequently, initiating early intervention in CLL without clinical indications for treatment is not currently justified, and guidelines recommend a watch-and-wait strategy for these patients [21]. The molecular analysis of the CLL12 trial may unveil whether patients with specific genetic lesions could potentially benefit from early treatment, paving the way for future clinical trials designed to evaluate the value of intervention for early-stage CLL patients with high-risk molecular features.

Recent research has aimed to identify the clinical and molecular characteristics of early-stage CLL patients undergoing a watch-and-wait approach, particularly those who may require treatment soon after diagnosis [6, 56-59]. The investigation into the tumor growth pattern of untreated CLL involved the analysis of serial longitudinal samples collected from diagnosis until the onset of treatment requirements [6]. Two distinct growth patterns have been discerned. The exponential growth pattern is characterized by rapid proliferation of the CLL clone, whereas the logistic growth pattern exhibits a slower rate of progression [6]. These growth patterns are associated with specific molecular features.

Cases with exponential growth are primarily associated with IGHV unmutated CLL and demonstrate a higher frequency of clonal and subclonal somatic genetic lesions compared to patients with a logistic growth pattern [6]. The validation of these different growth patterns and the connection between exponential growth, unmutated IGHV genes, and additional genetic lesions have been confirmed in an independent cohort of CLL patients [6].

Other studies have aimed to pinpoint the clinical and molecular characteristics that can identify early-stage CLL patients at risk of progressing soon after diagnosis. Identifying such patients in advance could potentially allow them to benefit from clinical trials comparing early intervention to observation. In this context, a combination of straightforward clinical features and molecular biomarkers—specifically, a lymphocyte count > 15,000/μL, palpable lymph nodes, and unmutated IGHV genes—distinguishes three distinct subgroups of Binet A and treatment-naive CLL patients with a high likelihood of requiring early treatment [56]. This risk model, known as IPS-E (International Prognostic Score-Early), has been validated in multiple independent series and serves as a robust tool to provide information at the time of diagnosis regarding the likelihood that a given CLL patient in the early stage will progress and require treatment [56].

Furthermore, leveraging the genetic diversity within CLL, mutations in genes integral to CLL pathogenesis have been investigated as biomarkers for identifying early-stage CLL patients at an elevated risk of progression and subsequent treatment necessity. These investigations highlight mutations in *SF3B1*, *NOTCH1*, *ATM*, *U1*, and *XPO1* as molecular indicators linked to a shorter time to first treatment (TTFT) [38,57,58]. Interestingly, disruption of *TP53* does not correlate with a shorter TTFT. This aligns with the idea that *TP53* disruption interacts with the treatment involving chemotherapeutic agents, as opposed to a watch-and-wait strategy that does not subject CLL cells with *TP53* disruption to the selective pressure exerted by ineffective chemotherapy [56,57].

The precise contribution of gene mutations in identifying asymptomatic CLL patients at an impending risk of requiring treatment is yet to be fully elucidated and is currently under investigation.

Management of first line therapy in CLL

The choice of first line treatment for CLL is determined by the molecular characteristics of the disease, in addition to patient attributes and the availability of novel drugs in various geographic regions. The latest guidelines advocate for conducting IGHV mutational analysis, FISH cytogenetics (including assessments for 13q, 11q, and 17p deletion, and trisomy12), and TP53 mutational status assessment before initiating treatment. Individuals with TP53 abnormalities, encompassing 17p deletion and/or TP53 mutation, are recommended to undergo treatment with biological drugs, steering clear of CIT [21].

Recent findings from multiple phase 3 clinical trials that compared CIT with chemo-free regimens have highlighted the superiority of chemo-free approaches in the initial treatment of CLL. Nonetheless, when conducting subgroup analyses based on IGHV mutation status, substantial distinctions have emerged between patients with IGHV mutations and those without. This emphasizes the importance of biomarkers in adopting a precision medicine approach for CLL patients in need of first-line therapy [40-42,60].

Patients harboring unmutated IGHV genes exhibited a less favorable outcome when subjected to CIT across the mentioned trials, necessitating the use of biological agents in this specific molecular subgroup [40-42,60]. In contrast, individuals with mutated IGHV genes experienced positive outcomes when treated with CIT, irrespective of age and comorbidities. In the E1912 trial, which aimed to compare ibrutinib-rituximab versus FCR in treatment-naive, young, and fit patients, the outcomes for patients with mutated IGHV genes were comparable in both treatment arms [40].

Similar outcomes were observed in the phase 3 trial that compared first-line bendamustine-rituximab (BR), ibrutinib, or ibrutinib-rituximab (IR) in patients aged >65 years, as well as in patients participating in the CLL14 trial, which compared obinutuzumab-venetoclax to obinutuzumab-chlorambucil in elderly patients or those with comorbidities [41,42]. In summary, these findings establish that IGHV-mutated CLL without TP53 disruption may derive benefits from both CIT and biological drugs without significant statistical differences. However, in the Illuminate trial, where patients were randomized to receive ibrutinib-obinutuzumab or chlorambucil-obinutuzumab, the chemo-free arm demonstrated superiority even in the subset of patients with mutated IGHV [60].

As expected, patients with *TP53* abnormalities undergoing treatment in the CIT arms experienced early failures. However, the impact of *TP53* abnormalities was not evident in patients treated with biological agents, except for the CLL14 trial, where *TP53*-mutated patients exhibited a poor outcome even in the obinutuzumab-venetoclax arm [29].

Numerous ongoing clinical trials are currently comparing various chemo-free regimens as first-line treatments, aiming to eliminate the CLL clone through a defined duration therapy protocol. In a phase 2 trial involving high-risk patients (those with >1 of the following features: 17p deletion, TP53 mutation, 11q deletion, unmutated IGHV), a combination of ibrutinib and venetoclax was administered for 24 cycles [61]. If minimal residual disease (MRD) negativity was achieved, the therapy was discontinued. In this high-risk population, following 12 cycles of the combined therapy with ibrutinib and venetoclax, 88% of patients attained complete remission, and 61% achieved remission with undetectable cytofluorimetric MRD [61].

These findings underscore the synergistic effect of ibrutinib and venetoclax, encouraging the formulation of clinical trials with the objective of determining the optimal combination for potentially eradicating the CLL clone in individual patients.

The *XPO1* pathway

Ensuring proper nucleocytoplasmic partitioning of proteins is crucial for cellular homeostasis. Among the key human shuttle proteins regulating macromolecular traffic between the nucleus and cytoplasm, Exportin-1 (XPO1) holds significant importance. XPO1 interacts with a diverse range of proteins via their canonical leucine-rich nuclear export signal (NES) domain, serving as a consensus sequence for nuclear export (Figure 2) [62].

The majority of *XPO1* mutations identified in human cancer involve the E571K substitution, conferring a gain-of-function. In cells with *XPO1*^{E571K} mutations, a negatively charged glutamic acid at position E571 is replaced by a positively charged lysine, promoting enhanced interaction of XPO1 with proteins possessing a negatively charged NES domain. This heightened interaction leads to increased export of negatively charged cargo proteins [63]. *XPO1* is often overexpressed in cancer patients, including those with pancreatic, gastric, prostate, and colorectal cancers, and such overexpression is linked to disease progression, treatment resistance, and poorer survival [64]. The biological implications of *XPO1* mutations in CLL have yet to be characterized.

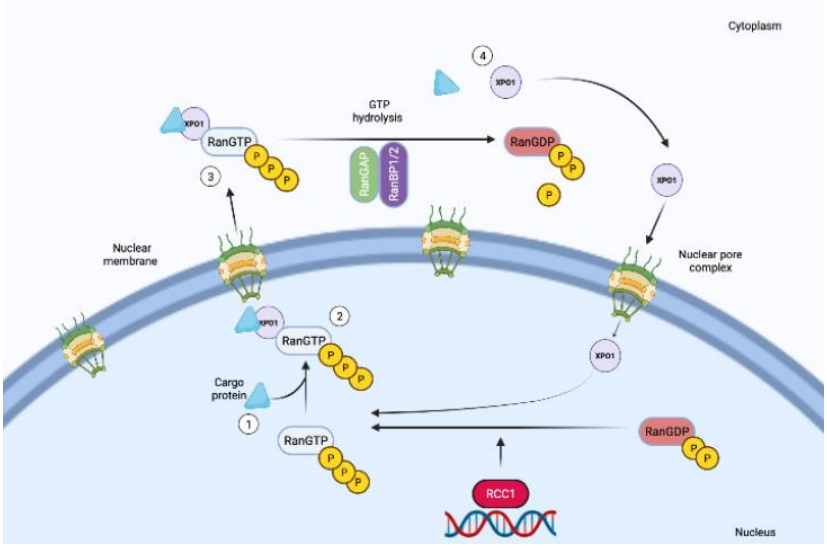

Figure 2. Schematic representation of XPO1-mediated nuclear export

Objective of the study

The objective of this current study is to assess the prevalence of *XPO1* mutations across various stages of the CLL clinical trajectory, to characterize the transcriptomic and epigenomic profiles of CLL with *XPO1* mutations in comparison to *XPO1* wild type, and to investigate whether *XPO1* mutations may predispose individuals to disease progression and an early need for treatment.

Materials and methods

Patients

A cohort of 295 consecutive newly diagnosed Binet A CLL patients referring to our institution was included in this study (training cohort). The 1st validation cohort was of a multicenter cohort of 402 Binet A CLL. The 2nd validation cohort was of a multicenter cohort of 395 Rai 0 CLL previously described elsewhere [59]. The study was approved by the local ethical committee (study number CE 120/19).

Mutational analysis

In the training cohort, tumor genomic DNA (gDNA) was analyzed in the coding exons plus splice sites of the most frequently mutated genes in CLL with a Next Generation Sequencing (NGS) approach using a variant allele frequency (VAF) threshold of 5%. In the validation series, the *XPO1* gene (exons 15 and 16) was analyzed by NGS or by Sanger sequencing. A robust and previously validated bioinformatics pipeline was used for variant calling [28].

RNA-seq and ATAC-seq on primary CLL cells

Peripheral blood mononuclear cells from 8 patients with *XPO1* mutations were sorted to purify the CLL CD19+/CD5+ tumoral cells and were subjected to RNA-seq and ATAC-seq. Fifteen *XPO1* wild type cases, matched for IGHV status, *TP53* status and for FISH karyotype, were analyzed for comparative purposes.

For RNA-seq, prior to library construction, 10-100 ng total RNA was treated with the NEBNext® Poly(A) mRNA Magnetic Isolation Module. Library construction was performed with the NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina®, according to the manufacturer's protocol. RNA concentration and integrity were assessed using Bioanalyzer

2100 (Agilent Technologies, Santa Clara, CA) and all samples had an RNA integrity number equivalent (RIN) value of 9 or more). Raw counts were obtained by mapping the reads to the genome using STAR Counts tool. Genes were considered non expressed and filtered out when less than 10 samples had a cpm value above 0.5. This resulted in keeping only 15162 genes. Counts were normalized using the trimmed mean of M values (TMM) method in the edgeR package in R. Log2-cpm values were used for downstream analysis and visualization. Pairwise differential expression was performed using the limma package. Differentially expressed genes were defined by a p-value < 0.01. Gene set enrichment was performed using the EnrichR online tool with KEGG and Wikipathways human specific databases and with published online databases (https://lymphochip.nih.gov/signaturedb/). Statistical significance was determined by adjusted p-value (q) < 0.05.

For ATAC-seq, the Nextflow-based pipeline "ATAC-seq" was used (https://nf-co.re/ATAC-seq). In the pipeline reads were trimmed using TrimGalore! tool and aligned to the GRCh37/hg19 assembly of the human genome using BWA. Duplicate reads were marked using Picard and removed. Reads mapping to mithocondrial DNA and to ENCODE blacklisted regions were also removed. Genome browser tracks were created with the genomeCoverageBed command in BEDTools and normalized such that each value represents the read count per base pair per million mapped. Finally, the UCSC Genome Browser's bedGraphToBigWig tool was used to produce a bigWig file. Peak calling was performed using MACS2 using the '-nomodel' parameter and reads counted for each sample in each consensus peak using featureCounts to create a matrix of count for each sample and each peak region. The list of consensus peaks was obtained by merging all peaks in all samples by bedtools merge. Consensus peaks were mapped to genomic location, including gene and distance from nearest Transcription Start Site (TSS) using the ChIPpeakAnno and the BiomaRt packages in R. Read counts were normalized across samples using the quantiles normalization function

normalize.quantiles from the preprocessCore package in R. The combat function was used to remove inter-patient effects for visualization purposes such as Principal Component Analysis (PCA). Supervised differential accessibility was performed using DeSeq2 package in R, using patient IDs as an independent variable in the linear model to remove batch effects to due inter-patient variability. To map peak regions to gene promoters and enhancers, a comprehensive map of CLL promoters and enhancers was obtained from the literature. Active promoters and strong enhancer regions were merged from 7 publicly available CLL chromatin states. For enrichment of differentially accessible peaks, regions were tested for enrichment of TF targets from ChiP-seq studies available on ENCODE project in the cell line GM12878 using a hypergeometric test.

Assessment of miR-155-5p expression

Cellular RNA was isolated by Trizol (Invitrogen) from 8 *XPO1* mutated CLL and from 8 *XPO1* wild type CLL matched for IGHV status, *TP53* status and for FISH karyotype. The cDNA templates were prepared using TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems). RT-qPCR reactions were performed using TaqMan Fast Advanced Master Mix (Applied Biosystems) with miR-155-5p TaqMan Advanced MicroRNA Assay. The miR-186-5p was used as housekeeping genes. The experiment was carried out in duplicate. Quantitative miR expression data were acquired using StepOnePlus Real-Time PCR system. The relative miR-155-5p expression levels were determined using the comparative Ct ($2^{-\Delta\Delta Ct}$) method and the relative expression between the two groups was compared by the Wilcoxon rank sum exact test.

Statistical methods

The study end point was time to first treatment (TTFT), defined as the time between presentation and start of first treatment of CLL because of progression to symptomatic disease according to the National Cancer Institute-Working Group/International Workshop on Chronic Lymphocytic Leukemia guidelines (patients without a documented event were censored at the date of last observation or death) [21]. Survival analysis was performed by Kaplan-Meier method and compared between strata using the Log-rank test. The adjusted effects of *XPO1* mutations and the IPS-E [56] and Rai 0 prognostic model [59] variables on TTFT were estimated by Cox regression. The analysis was performed with the Statistical Package for the Social Sciences (SPSS) software v.24.0 (Chicago, IL).

Results

Incidence of *XPO1* mutations across different phases of CLL

Prevalence of *XPO1* mutations was assessed across different phases of CLL, including MBL (n=95), early stage CLL (n=997), relapsed CLL after chemoimmunotherapy or pathway inhibitors (n=81), and CLL transformed into an aggressive lymphoma (Richter syndrome) (n=55). *XPO1* mutations occurred in 3.2% (n=3/97) of MBL, 2.7% (n=28/997) of early stage CLL, 4.9% (n=4/81) of relapsed CLL after chemoimmunotherapy or pathway inhibitors, and 12.7% (7/55) of CLL transformed into diffuse large B cell lymphoma (Richter syndrome).

The *XPO1* gene exhibited two hotspots. The first hotspot, located at p.E571, constituted 93.0% of all mutations and impacted a conserved position throughout phylogenesis. The glutamic acid at position 571 was frequently mutated to lysine, and less commonly to glycine, alanine, or valine. These mutations were specifically chosen to convert the negatively charged glutamic acid at position 571 into a positively charged amino acid. While variations affecting the p.E571 position have been observed in various cancer types, the p.D624 mutation is uniquely confined to CLL.

We investigated whether specific patient or disease characteristics were linked to *XPO1* mutations in untreated CLL. After adjusting for multiple comparisons, we found a significant correlation between *XPO1* mutations and unmutated IGHV genes, as well as with the presence of a lymphocyte doubling time before the initiation of treatment.

We examined the potential longitudinal clonal evolution of *XPO1* mutations in three groups: patients with asymptomatic CLL progressing to a treatment-requiring stage (N=61), patients with active CLL on ibrutinib treatment (N=33), and patients with CLL transformed into Richter syndrome (N=29). In the first scenario, involving five patients with asymptomatic CLL carrying *XPO1* mutations at the time of diagnosis, the median VAF of *XPO1* mutations remained consistent at the onset of treatment requirement (27.0% at treatment requirement vs.

19.0% at the time of diagnosis, p=0.206). In asymptomatic CLL cases without *XPO1* mutations, the watch-and-wait strategy did not lead to the emergence of *XPO1* mutations. All 56 cases analyzed at the time of diagnosis remained confirmed as wild type when treatment was required. Among the 33 cases under ibrutinib treatment, none of the patients followed for at least 96 weeks acquired an *XPO1* mutation. Regarding Richter syndrome, 29 patients were examined both in the CLL phase and in the lymph nodes affected by Richter syndrome. Notably, among the 4 cases with *XPO1* mutations in the lymph node biopsy, 3 cases (all clonally related) did not have the *XPO1* mutant clone in the CLL phase but acquired it at the time of Richter transformation.

Chromatin state is more accessible in *XPO1* mutated CLL cells

To better clarify whether *XPO1* mutated cells show a distinct proliferative course, we looked for cellular programs associated with *XPO1* mutations in CLL. CLL cells were purified through flow-sorting from 8 patients harboring *XPO1* mutations (VAF from 25% to 50%) and from 15 patients lacking *XPO1* mutations which represented the controls. *XPO1* mutated and *XPO1* wild type CLL were matched for the main biological CLL characteristics (IGHV, *TP53* and FISH karyotype) to mitigate biases related to the genetic background of the disease. Samples were profiled for both chromatin accessibility and transcriptome.

By principal component analysis (PCA), *XPO1* mutated CLL clearly separated and showed a distinct chromatin rearrangement compared to wild type cases (Figure 3A). Overall, *XPO1* mutated CLL had a chromatin landscape that was more accessible than that of *XPO1* wild type CLL. Differentially accessible chromatin regions were decorated with the CLL-specific map of enhancers and active promoters, and with the mature B cell specific map of 96 transcription factor-biding sites (Figure 3B). Chromatin regions that were more accessible in *XPO1* mutated CLL were enriched of binding sites for transcription factors that are regulated

by the pathways that emanate from B cell receptor (BCR), including NF-kB signaling (NF-kB, BCL3, OCT2), p38-JNK (JUNB, JUND, MEF2A, MEF2C, ATF2), RAS-RAF-MEK-ERK (CEBPB, SP1) and calcium-calmodulin (NFATC1). Regions of chromatin that exhibited reduced accessibility in *XPO1*-mutated CLL were enriched with binding sites for FOXO1, whose nuclear localization is hindered by active BCR signaling. Conversely, chromatin regions with increased accessibility in *XPO1*-mutated CLL were also enriched with binding sites for transcription factors regulated by cytokine/inflammation signaling, such as STAT3 and STAT5 (Figure 3C).

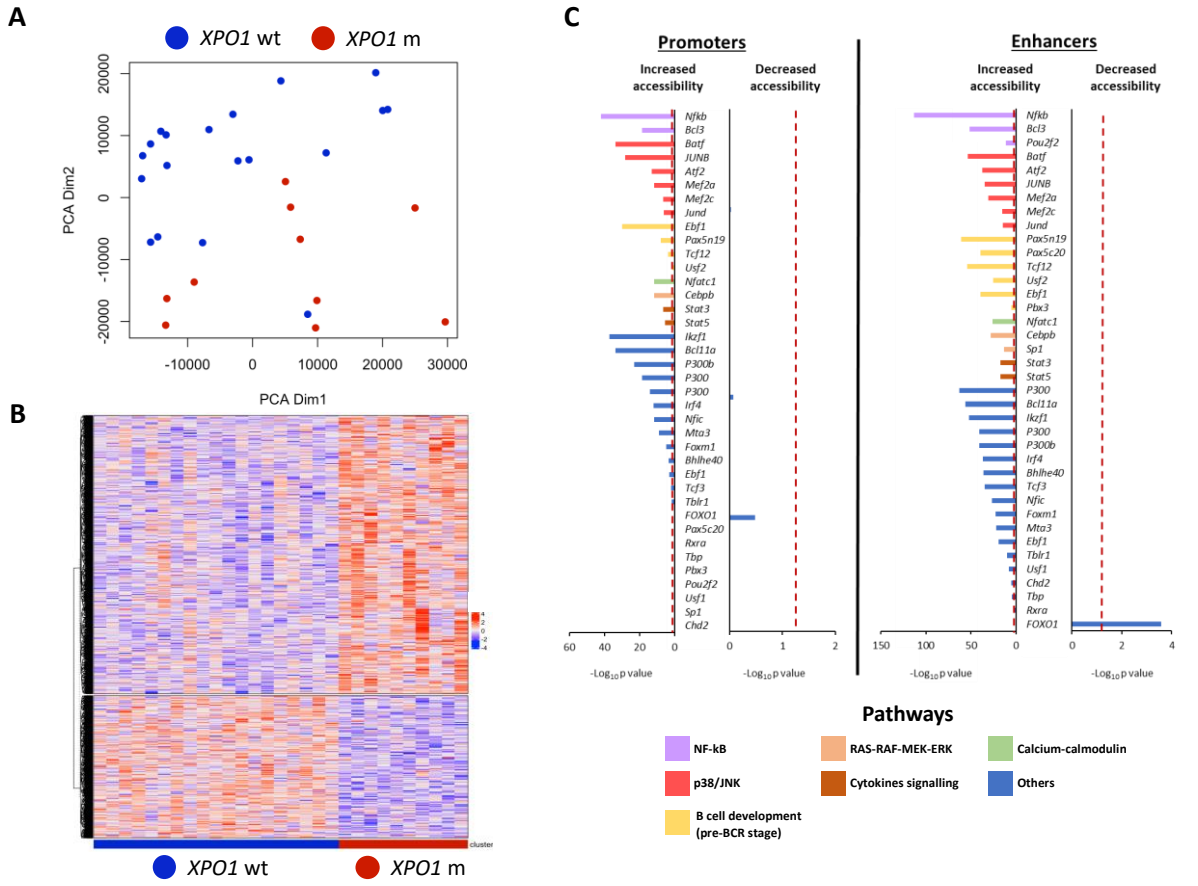

Figure 3. Different chromatin accessibility in XPO1 mutated compared to wild type CLL. **(A)** Multidimensional scaling (MDS) plot of the diverse chromatin accessibility between *XPO1* mutant (in red) and *XPO1* wild type cases (in blue) demonstrates distinct clustering between mutated and non-mutated CLL samples along MDS dimension. **(B)** Heatmap of signal intensity characterized showing the different chromatin accessibility in *XPO1* mutant and in *XPO1* wild type CLL. **(C)** Bar plots showing the genes that present in their promoter and/or enhancer region a significantly different chromatin accessibility in *XPO1* mutated CLL. The dot red lines denote statistical significance. The bars are color-coded according to signaling pathways reported in the legend below the graph.

The transcriptome of *XPO1* mutated CLL cells is enriched in MAPK and inflammation signaling genes

Overall, 236 genes were upregulated, and 296 genes were downregulated in *XPO1* mutant CLL compared to *XPO1* wild type CLL and by PCA *XPO1* mutated cases showed a distinct transcriptomic profile (Figure 4A-B). Consistent with the chromatin accessibility changes in *XPO1* mutated CLL, they also showed several transcriptomic features associated with BCR and cytokine signaling. Pathway enrichment analysis of the upregulated genes showed that *XPO1* mutant CLL were enriched of immediate early response to BCR activation, TGFβ signaling, EGF-EGFR signaling, FOSB gene expression, regulation of MAPK through DUSP (Figure 4C).

Among the genes frequently involved in pathways that are overexpressed in *XPO1* mutated cases, DUSP1, a nuclear phosphatase that inhibits the JUN/FOS signaling, represents a potential candidate for the interaction with the XPO1 protein. Using the Wregex prediction tool [65] we identified 2 different NESs in the DUSP1 protein, both with a negative charge in the amino-acid C-terminal residues suggesting that DUSP1 could interact with XPO1. *XPO1* mutations are known to enhance the nuclear export of proteins with a negative charge in the NES. Therefore, DUSP1 could be exported out of the nucleus with a greater extent in cases of *XPO1* mutations thus leading to reduced phosphorylation and therefore a more active JUN/FOS signaling.

The *miR-155/MYB* pathway is upregulated in *XPO1* mutant CLL

By combining transcriptomic and epigenomic data we identified 15 genes that were upregulated by RNA-seq and whose promoters were more accessible by ATAC-seq (Figure 4D). Among these genes *MIR155HG*, the host gene that generates miR-155, and *MYB*, the transcription factor that positively regulates the expression of miR-155 were further

investigated since their role in the BCR signaling. As expected, higher levels of *MIR155HG* correlate with higher levels of *MYB* (Figure 4E). miR-155 expression favors BCR signaling though several mechanisms including the downmodulation of SHIP1, a phosphatase that inhibits the BCR cascade. As expected, an inverse correlation between *MIR155HG* and *MYB* with *SHIP1* was observed (Figure 4F- G). To confirm that the higher expression of *MIR155HG* correlates with higher miR-155 levels, we performed a RT-qPCR in 8 *XPO1* mutated cases and in 8 *XPO1* wild type cases matched for IGHV status and FISH karyotype. Quantitative analysis confirmed the higher expression of miR-155 in *XPO1* mutated CLL (p=0.065) (Figure 4H). Another finding that validates the enhanced activation of the miR-155 pathway in *XPO1* mutated cells is represented by the downregulation of the *SPI1/PU.1* gene, a transcription factor that is significantly downregulated in *XPO1* mutated cases (p=0.0029).

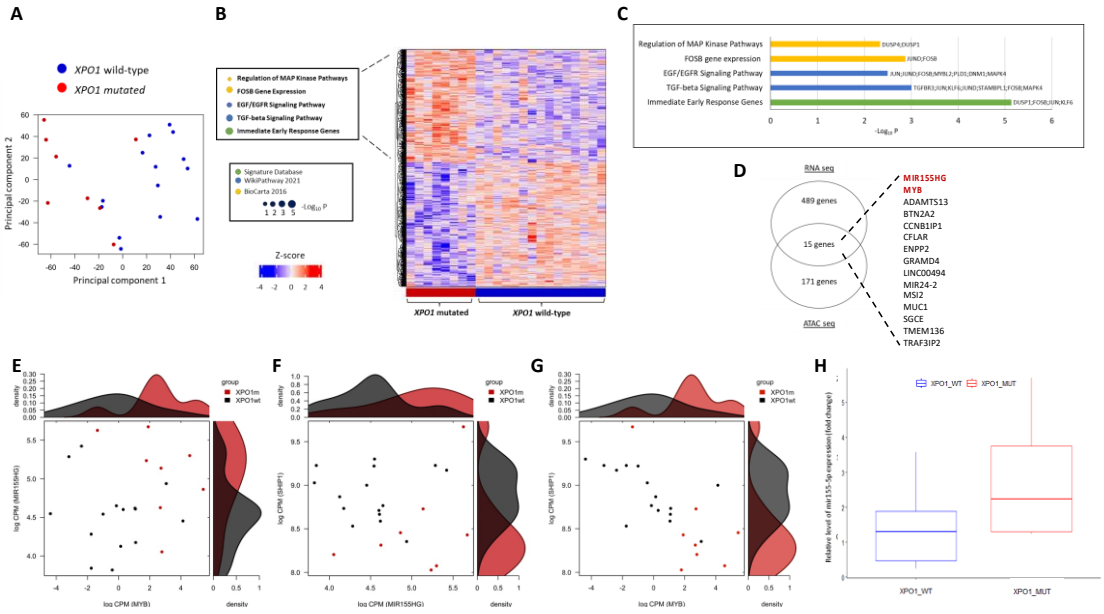

Figure 4. Different gene expression profile in XPO1 mutated compared to wild type CLL. **(A)** Multidimensional scaling plot of the diverse chromatin accessibility between *XPO1* mutant (in red) and XPO1 wild type cases (in blue) demonstrates distinct clustering between mutated and non-mutated CLL samples along MDS dimension. **(B)** Heatmap demonstrating differential expression of genes between *XPO1* mutant (in red) and *XPO1* wild type (in blue) CLL. On the right side of the heatmap is represented the gene ontology analysis for biological processes significantly enriched in *XPO1* mutated CLL. **(C)** The histogram represents the pathway enrichment analysis of the upregulated genes showed that *XPO1* mutant CLL. **(D)** Venn diagrams representing the number of genes that are upregulated by RNA-seq and whose promoters are more accessible by ATAC-seq in *XPO1* mutated CLL. The intersection between the diagrams represents the 15 genes that are upregulated and whose promoter is more accessible. The density plots represented in panel **E**, **F** and **G** denote the association of *MIR155HG, MYB* and *SHIP1* in *XPO1* mutated (in red) and in *XPO1* wild type (in grey) CLL cells. **(H)** The box plot denotes the relative expression of target miR-155-5p compared to endogenous control (miR-186-5p) in *XPO1* mutated (in red) and *XPO1* wild type cells (in blue).

Association between *XPO1* mutations and time to first treatment

As *XPO1* mutated CLL showed an enrichment in cellular programs that activate the BCR signaling cascade and cell proliferation, we tested whether early stage CLL patients are characterized by a shorter time to first treatment using a training/validation approach. *XPO1* was mutated in 2.7% of patients (8/295) of the training cohort (all Binet A patients), 3.7% (15/402) patients in the 1st validation cohort (all Binet A patients), and in 2.0% (8/395) patients 2nd validation cohort (all Rai 0 patients). The complete clinical and biological profile of the cohorts is reported in Table 1. As expected for patients with early stage CLL, high risk molecular features were rare.

After a median follow-up of 10.4 years, *XPO1* mutations significantly associated with a shorter TTFT in the training cohort, with a median TTFT of 3.3 years compared to 21.0 years in wild type cases (Figure 5A). In multivariate analysis including the IPS-E variables, which are validated for anticipating TTFT and include unmutated IGHV genes, palpable lymph nodes and lymphocyte count >15,000/μL, *XPO1* mutations maintained an independent association with a shorter TTFT (HR 2.47, 95% C.I. 1.05-5.80, p=0.039) (Table 2). *XPO1* mutations maintained an independent association with a shorter TTFT (HR 13.19, 95% C.I. 4.07-42.81, p<0.001) also after adjusting for variables of the Rai 0 prognostic score (Table 3).

To confirm the observation that *XPO1* mutated patients experience a more progressive disease, we evaluated the TTFT stratified based on *XPO1* mutation status in the 1st and 2nd validation cohorts. In the 1st validation cohort, after a median follow-up of 5.8 years, patients with *XPO1* mutations had a significantly shorter median TTFT of 5.0 years compared to 11.5 years in patients with wild type *XPO1* (Figure 5B). In the 2nd validation cohort, after a median follow-up of 6.7 years, patients with *XPO1* mutations had a significantly shorter median TTFT of 3.3 years compared to 14.1 years in patients with wild type *XPO1* (Figure 5C).

By combining the training and the validation cohorts (N=1092 patients), patients carrying either *XPO1* E571 (N=28) or D624 (N=3) mutations showed superimposable outcome in terms of TTFT (p=0.345) (Figure 5D). In addition, the prognostic value of *XPO1* mutations in terms of shorter TTFT was maintained in patients with both mutated and unmutated IGHV genes (Figure 5E-F).

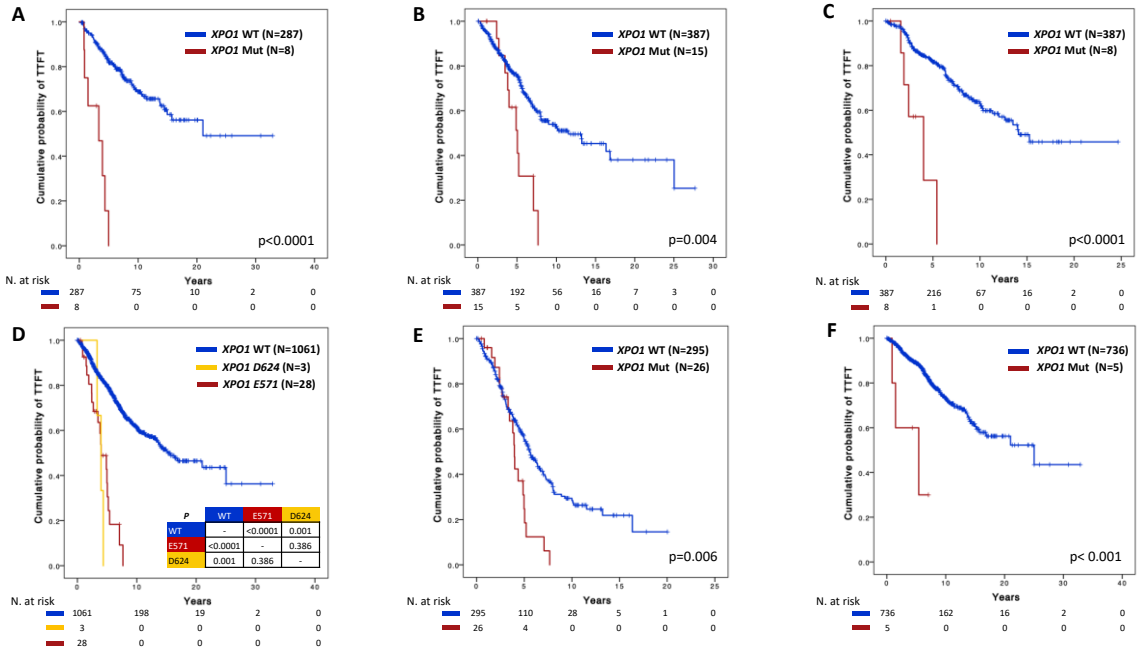

Figure 5. Prognostic impact of XPO1 mutations. Kaplan-Meier estimates of TTFT in the training cohort of 295 Binet A CLL patients **(A)**, in the 1st validation cohort of 402 Binet A CLL patients **(B)**, and in the 2nd validation cohort of 395 Rai 0 CLL patients **(C).** Cases harboring *XPO1* mutations are represented by the red line and wild type cases are represented by the blue line. Panel D denotes Kaplan-Meier estimates of TTFT according *XPO1* mutation type combining the training and the validation cohorts (N=1092). Patients with *XPO1* mutation in codon E571 are represented by the red line, in codon D624 by the yellow line and wild type cases by the blue line. Panel **E** and **F** represent the clinical impact of *XPO1* mutations in IGHV unmutated and IGHV mutated patients, respectively. Cases harboring *XPO1* mutations are represented by the red line and wild type cases are represented by the blue line. The Log-rank statistics p values are indicated adjacent to the curves.

XPO1 mutations as a novel predictor of early treatment requirement

The IPS-E and Rai 0 models provide a benchmark of the expected TTFT of patients with high-risk early stage CLL. We sought to understand whether early stage CLL with *XPO1* mutations have a similarly poor TTFT as early stage CLL nominated as high-risk by the IPS-

E or Rai 0 model group. The TTFT of early-stage patients harboring *XPO1* mutations, irrespective of their actual IPS-E score, was short as the TTFT of early-stage patients belonging to the high-risk class according to IPS-E (Figure 6A). Similarly, the TTFT of early-stage patients harboring *XPO1* mutations, irrespective of their actual Rai 0 score, was short as the TTFT of early-stage patients belonging to the high-risk class according to the Rai 0 model (Figure 6B).

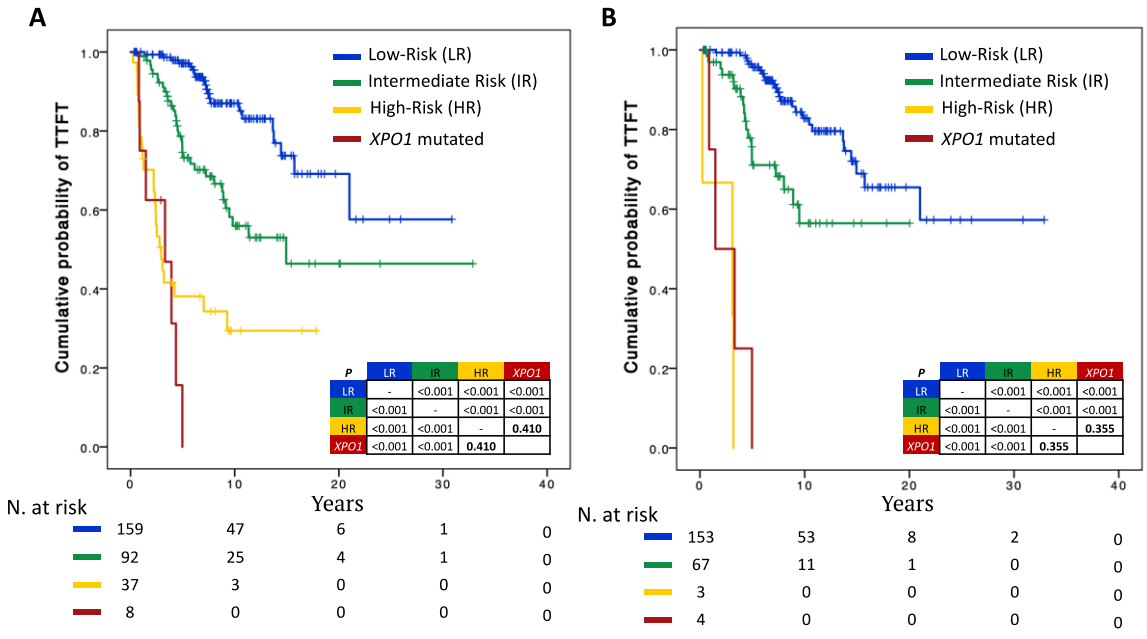

Figure 6. Prognostic impact of XPO1 mutations within prognostic scores for early stage CLL patients. **(A)** Kaplan-Meier estimates of TTFT in 295 Binet A CLL according the IPS-E and *XPO1* mutations. Low-risk patients are represented by the blue line, intermediate-risk patients by the green line, high risk patients by the yellow line and *XPO1* mutated patients by the red line. **(B)** Kaplan-Meier estimates of TTFT in 227 Rai 0 CLL according to the Rai 0 prognostic score and *XPO1* mutations. Low-risk patients are represented by the blue line, intermediate-risk patients by the green line, high risk patients by the yellow line and *XPO1* mutated patients by the red line. The pairwise log-rank statistics p values are indicated in the tables adjacent to the curves.

Discussion

Most CLL patients are diagnosed in an asymptomatic stage and are managed with a watch & wait strategy [21]. However, their clinical outcome is heterogenous, with certain patients progressing and necessitating therapy at distinct time points. In the present study, employing a training-validation approach, *XPO1* mutations emerged as an independent predictor of shorter time to first treatment that is able to capture a subset of patients requiring treatment shortly after diagnosis. Transcriptomic and epigenomic data also indicate that *XPO1* mutant cells exhibit increased BCR signaling activity, potentially predisposing them to heightened proliferative behavior.

The *XPO1* gene encodes a nuclear exportin crucial for regulating nuclear-cytoplasmic partitioning, a process essential for cell homeostasis [62,64,66]. *XPO1* mutations have been identified in various tumor types, including pancreatic, gastric, prostate, and colorectal cancers [22-24]. In hematological malignancies, *XPO1* is commonly mutated in primary mediastinal and Hodgkin lymphoma, and it also plays a role in CLL, albeit with a lower frequency of mutations [67-69]. In most instances, *XPO1* mutations affect the glutamic acid at position 571 of the protein. Beyond mutations at position 571, this study, along with others, has identified mutations at position 624 to be exclusive to CLL [69]. Our findings further confirm that *XPO1* is mutated in approximately 5% of CLL cases, with associations to unmutated IGHV genes and a lymphocyte doubling time indicative of an exponential growth pattern commonly observed in CLL [6].

Given the accessibility of tumoral sampling, CLL is an ideal disease for evaluating clonal evolution. Through the examination of longitudinal samples, it was observed that neither the watch-and-wait strategy nor therapies (including chemoimmunotherapy and ibrutinib) led to the expansion of the mutant clone in patients with *XPO1* mutations. Likewise, the watch-and-wait strategy did not trigger the emergence of *XPO1* mutations in patients who were wild

type at the time of diagnosis. These findings highlight the notion that *XPO1* mutations represent an initial event in CLL pathogenesis and therefore remain stable over the disease course of CLL [3]. Conversely, the mechanisms that lead to Richter transformation differ from mechanisms leading to CLL progression. Interestingly, in 3 out of 4 cases of clonally related Richter syndrome patients with *XPO1* mutation detected in the lymph node biopsy, the *XPO1* mutation was absent in the CLL phase pointed to *XPO1* mutations as a novel mechanism that may lead to Richter transformation.

Prediction of time to treatment requirement is essential in the management of CLL patients since most of them do not require therapy at the time of diagnosis. Prediction of TTFT is essential to allocate resources in CLL since it may identify high risk patients that are suitable to early intervention clinical trials and, on the other hand, may reassure patients with a very indolent disease that may be followed with a less intense schedule [56,59]. In this study *XPO1* mutations sorted out as a novel predictor of shorter TTFT in 1092 early stage CLL using a training-validation approach. Superimposable outcome has been obtained in Binet A (training cohort and 1st validation cohort) and in Rai 0 CLL patients (2nd validation cohort). Previous reports did not identify *XPO1* mutations as a marker for shorter TTFT conceivably because CLL patients at different clinical stages were not included and not initially homogenously managed with a watch & wait strategy [69,70]. Importantly, the prognostic role of *XPO1* mutations in anticipating TTFT in early stage CLL, is independent by IGHV status and by the variables enclosed in the two prognostic scores dedicated to early stage CLL patients [56,59]. Since *XPO1* mutations affect two unique hotspots, the aspartic acid in position 624 and the glutamic acid in position 571, polymerase chain reaction-based methods may be used to identify *XPO1* mutations in a simple and time effective manner.

To gain some insights into the mechanisms that may drive the higher proliferation rate of *XPO1* mutated CLL, we evaluated the transcriptomic and the epigenomic profile of *XPO1*

mutated CLL compared to wild type cases matched for IGHV status, FISH karyotype and *TP53* status. Previous reports showed that *XPO1* mutated patients present an enrichment in pathways that regulate lymphocytes activation, NFAT signaling and NF-κB activation [69]. In the present study we further refine this notion and the BCR signaling sorted out as a potential candidate that might explain the higher proliferation rate of *XPO1* mutated CLL. Transcriptomic and epigenomics data suggest that the BCR signaling is more active in *XPO1* mutated cases conceivably due to the higher expression of miR-155 that, by inhibiting SHIP1, stimulates the BCR pathway [71]. In addition, enhanced BCR signaling actively promotes the expression and transcription of *MYB*, that leads to *MIR115HG* transcription further enhancing the transcription of miR-155 and therefore the BCR signaling [72].

What remains to be explained is the mechanism by which *XPO1* mutations enhance the miR-155 pathway. The first hypothesis is related to DUSP1. Utilizing its negatively charged nuclear export signal (NES), DUSP1 could be exported more extensively from the nucleus in XPO1 mutant cells. Although we did not conduct *in vitro* nuclear-cytoplasmic partitioning assays for the DUSP1 protein, the higher expression of DUSP1 observed in our samples through RNA-seq may indicate an effort by XPO1 mutant cells to compensate for the reduced nuclear presence of DUSP1. Consequently, the JUN/FOS signaling in the nucleus, no longer inhibited by DUSP1, becomes more active. Interestingly, the promoter region of the *MIR155HG* gene harbors a conserved binding site region for JUN/FOS [73]. Therefore, in *XPO1* mutated cells the active JUN/FOS signaling, caused by the lower nuclear activity of DUSP1, could promote the transcription of *MIR155HG* that through miR-155 promotes the BCR signaling. The second hypothesis that may lead to the enhanced miR-155 expression is related to its potential higher exportation out of the nucleus in cases harboring *XPO1* mutations. The 2,2,7-trimethyl-guanosine (TMG)-cap, a recognized signal on micro-RNA for binding to XPO1 [74], possesses a negative charge that can be recognized and consequently exported

more extensively from the nucleus by mutant XPO1. As a result, elevated levels of miR-155 in the cytoplasm may enhance BCR signaling.

In conclusion, we have identified *XPO1* mutations as a new independent predictor of a shorter TTFT in early-stage CLL patients. The increased proliferation rate observed in *XPO1*-mutated CLL cells may be attributed to the heightened activity of the BCR signaling pathway, specifically through the MYB/miR-155 axis. The integration of *XPO1* mutations into existing predictive scores could enhance the ability to anticipate treatment requirements in early-stage CLL patients, facilitating the management of the majority of CLL cases and aiding in the selection of patients who may derive the greatest benefit from participation in clinical trials exploring early intervention in CLL.

The present study has been published in 2023 [75].

Tables

Table 1. Patient characteristics

	Training cohort	1st validation cohort	2nd validation cohort
Characteristics	**Values**	**Values**	**Values**
Median Age	70.8 (62.2-76.9)	67.0 (56.0-73.0)	>65 years 55.0%
Median lymphocytes/μL	8500 (5700-13000)	4151 (900-10556)	NA
B2M mg/L	2.1 (1.7-2.7)	NA	NA
Gender			
Male	159 (53.9%)	125 (57.8%)	NA
Female	136 (46.1%)	91 (42.1%)	NA
13q deletion			
Yes	141 (48.5%)	NA	NA
No	150 (51.5%)	NA	NA
Trisomy 12			
Yes	44 (15.1%)	44 (12.8%)	56 (14.2%)
No	247 (84.9%)	299 (87.2%)	339 (85.8%)
11q deletion			
Yes	15 (5.2%)	NA	34 (8.6%)
No	276 (94.8%)	NA	361 (91.4%)
17p deletion			
Yes	12 (4.1%)	19 (5.5%)	25 (6.3%)
No	279 (95.9%)	326 (94.5%)	370 (93.7%)
IGHV mutational status			
Mutated	214 (75.1%)	236 (61.5%)	291 (74.0%)
Unmutated	71 (24.9%)	148 (38.5%)	102 (26.0%)

B2M, beta-2-microglobulin; IGHV, immunoglobulin heavy chain variable

Table 2. Multivariate analysis for *XPO1* mutations and IPS-E variables

Variable	HR	95% C.I.	p value
Unmutated IGHV	3.78	2.36-6.08	<0.0001
Palpable lymph nodes	2.67	1.66-4.32	<0.001
Lymphocyte >15,000/μL	2.21	1.36-3.59	0.001
XPO1 mutations	2.47	1.05-5.80	0.039

HR, Hazard Ratio; CI, Confidence Interval; IGHV, immunoglobulin heavy chain variable

Table 3. Multivariate analysis for *XPO1* mutations and Rai 0 prognostic score variables

Variable	HR	95% C.I.	p value
WBC > 32,000/μL	4.99	1.49-16.78	0.009
Unmutated IGHV	3.35	1.69-6.61	<0.0001
Del 17p	2.08	0.28-15.50	0.474
Tris 12	2.71	1.26-5.83	0.011
Del 11q	0.55	0.13-2.39	0.427
XPO1 mutations	13.19	4.07-42.81	<0.001

HR, Hazard Ratio; CI, Confidence Interval; WBC, white blood cells; IGHV, immunoglobulin heavy chain variable

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