

MORPHOLOGIC AND MOLECULAR ANALYSIS OF RICHTER SYNDROME IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS TREATED WITH IBRUTINIB OR VENETOCLAX

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Running title: Richter syndrome in ibrutinib/venetoclax-treated CLL

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Key words: Chronic lymphocytic leukemia, Richter syndrome, ibrutinib, clonal evolution

Declarations of interest: none.

ABSTRACT

Richter syndrome (RS) represents the development of high-grade lymphoma in patients with chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL) and presents a diagnostic and therapeutic challenge with an adverse prognosis. The genetic background and morphology of RS in CLL patients treated with chemoimmunotherapy is extensively characterized, however, our knowledge about RS in patients treated with targeted oral therapies should be extended.

To understand the morphologic and molecular changes leading to RS in CLL patients treated with the Bruton's tyrosine kinase inhibitor, ibrutinib and the BCL2 inhibitor, venetoclax, sequential samples from six CLL/SLL patients undergoing RS were collected in both the CLL and RS phases. A detailed immunophenotypic analysis of formalin-fixed, paraffin-embedded tissue specimens of RS phase was performed, followed by extensive molecular characterisation of CLL and RS samples, including the immunoglobulin heavy chain gene (*IGH*) rearrangement, *TP53* mutations, drug-induced resistance mutations in *BTK* and *BCL2* genes and various copy number changes and point mutations detectable with multiplex ligation-dependent probe amplification (MLPA).

Rare, non-diffuse large B cell lymphoma phenotypes of RS were observed in 3/6 cases, including plasmablastic lymphoma and a transitory entity between diffuse large B cell lymphoma and classical Hodgkin lymphoma. The majority of cases were clonally related and harbored an unmutated variable region of the immunoglobulin heavy chain gene. Abnormalities affecting the *TP53* gene occurred in all patients, and every patient carried at least one genetic abnormality conferring susceptibility to RS. In the background of RS, 2/5 patients treated with ibrutinib showed a *BTK* C481S resistance mutation. One patient developed a *BCL2* G101V mutation leading to venetoclax resistance and RS.

In conclusion, our findings contribute to better understanding of RS pathogenesis in the era of targeted oral therapies. Rare phenotypic variants of RS do occur under the treatment of ibrutinib or venetoclax, and genetic factors leading to RS are similar to those identified in patients treated with chemoimmunotherapy. To our best knowledge, we have reported the first *BCL2* G101V mutation in an RS patient treated with venetoclax.

1. Introduction

Richter syndrome (RS) represents the development of high-grade lymphoma in the setting of an antecedent or concomitant chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL)¹. Histologically, two main types of RS are distinguished, diffuse large B-cell lymphoma (DLBCL) and classical Hodgkin lymphoma (cHL), with the former entity comprising the majority (80-90%) of cases¹. Rarely, transformation to plasmablastic lymphoma (PBL), histiocytic sarcoma, lymphoblastic lymphoma and interdigitating dendritic cell sarcoma may occur². The DLBCL type of RS appears as confluent sheets of large, CD20+ neoplastic B cells with a variable positivity of markers characteristic for the preceding CLL³. The vast majority (90-95%) of DLBCL type RS cases exhibit a non-germinal center phenotype (CD10-, MUM1/IRF4+)³⁻⁵. The cHL variant of RS is characterized by CD30+/CD15+/CD20- phenotype, with Hodgkin and Reed-Sternberg cells located in the cHL-specific inflammatory background of T cells, histiocytes, eosinophil granulocytes and plasma cells. However, in some CLL cases Hodgkin-like cells with CD30+ expression are in the background of small CLL lymphocytes instead of the reactive cellular milieu of cHL, which is not considered as a 'true' transformation^{6,7}.

Clonal relatedness is the most important prognostic factor in RS and represents a true transformation in contrast to clonally unrelated lymphomas developing in patients with CLL. While ~80% of DLBCL-RS is clonally related to the antecedent CLL based on *IGHV-D-J* rearrangement, only 40-50% of the cHL-RS arises in a clonally related manner⁸. RS may develop in patients carrying either mutated or unmutated *IGHV*, however, in most of the cases only the latter represents a clonally related transformation⁹. Although the term 'clonally unrelated RS' is being used widely, these cases are biologically *de novo* lymphomas with higher response rates to therapy and more favorable prognosis compared to their clonally related counterparts³⁻⁵. Patients with clonally unrelated DLBCL-RS have a

median survival of ~5 years, in contrast to the much poorer outcome in clonally related DLBCL-RS (8-16 months)⁵. The outcome of HL-RS patients is more favorable compared to DLBCL-RS, but worse than that of the primary classical HL¹⁰. RS developing in patients treated with novel targeted therapies confers a highly adverse prognosis, with only 13% of patients achieving remission^{11,12}.

Over the past years several molecular alterations connected with the development of DLBCL-RS were identified. Risk factors associated with the high-grade transformation of CLL comprise hereditary single nucleotide polymorphisms (SNP), somatically acquired genetic lesions and clinical features. Hereditary SNPs including *BCL2* rs4987852, *CD38* rs6449182 and *LRP4* rs2306029 predisposing to RS confer a moderate risk via incompletely understood functional mechanisms¹³⁻¹⁵. Cases of CLL carrying an unmutated *IGHV* gene are prone to transform into RS, especially when harboring the *IGHV4-39* gene solely or as a part of the stereotyped B-cell receptor subset #8⁴. CLL patients with *NOTCH1* mutation exhibit a 10-fold higher probability of developing RS compared to their *NOTCH1* wild-type counterparts¹⁶⁻¹⁸. TP53 defects (mutations or 17p deletion) are present in 60% of RS patients, acquired mainly at transformation⁵. Various genetic events affecting *MYC* and *CDKN2A* genes lead to their deregulation and are acquired at the time of transformation^{5,19-21}. Among clinical features, advanced stage (Rai III-IV) and a lymph node size >3 cm are the only factors associated with transformation²²⁻²⁴. Predictive markers of CLL progression (decrease in lymphocyte doubling time, bone marrow involvement, elevated β 2-microglobulin and elevated lactate dehydrogenase (LDH) level) are not associated with RS development, suggesting a biologically distinct way of CLL progression and transformation^{24,25}. Several studies investigated the impact of the therapy on the development of RS. Exposure to fludarabine, a widely used purine analogue was found to be associated with increased risk of RS in some series²⁶, although not in others^{25,27}.

Small molecule inhibitors targeting the B cell receptor (BCR) pathway (especially ibrutinib) and Bcl2 related apoptosis pathway (venetoclax) have recently revolutionized the therapy of CLL, conferring remarkable outcomes in high-risk as well as in relapsed/refractory patients²⁸⁻³¹. In spite of

the remarkable results, a proportion of patients undergo progression or Richter transformation (RT) due to the emergence of resistance mutations or additional genetic abnormalities^{32,33}. However, our knowledge about the genetic changes leading to RT in CLL patients treated with novel targeted therapies is incomplete and should be further elucidated.

In this study, we aimed to scrutinize the histological and molecular background of RS in six ibrutinib and/or venetoclax treated patients who underwent high-grade transformation to gain a better insight into the process leading to the diagnostically and therapeutically challenging phenomenon of RS.

2. Patients and methods

Sequential samples from six CLL/SLL patients undergoing RT were collected in the CLL phase and at RS diagnosis except for Patient 4 (P4) from whom only a single RS sample was available (although basic cytogenetic characterisation had been performed). The patient cohort consisted of one female and five male patients, their median age at CLL diagnosis was 58 years (38-75 years), the median follow-up time was 109.5 months (53-355 months). Four patients were treated with ibrutinib, and one patient with venetoclax and ibrutinib+venetoclax combination, respectively. Median time from the initiation of targeted therapy to RT was 20.0 months (2-52 months), the median survival following RT was 1.5 months (0-29 months). Additional clinical data is presented in Table 1 and Figure 1.

Morphologic and immunophenotypic analysis of RS samples was carried out using formalin-fixed, paraffin-embedded (FFPE) tissue specimens. CD19+ lymphocytes were purified from peripheral blood and bone marrow aspirate with EasySep™ Human CD19 Positive Selection Kit II (StemCell Technologies, Vancouver, Canada) and DNA was isolated using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany). DNA isolation from FFPE tissues was performed with QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). After fluorimetric quantification we performed *IGHV-IGHD-IGHJ*

sequencing, next-generation sequencing (NGS) of the *TP53* gene, multiplex ligation-dependent probe amplification (MLPA) targeting relevant abnormalities in CLL and droplet digital PCR for the detection of resistance mutations against targeted therapies.

The analysis of *IGHV-IGHD-IGHJ* rearrangements was performed as described by Agathangelidis *et al.*³⁴. DNA amplification with leader primers was followed by bidirectional Sanger sequencing and results were obtained by immunoinformatic analysis using IMGT V-QUEST and ARResT/AssignSubsets software in concordance with the updated ERIC recommendations³⁵. The whole coding region of *TP53* gene including the splice sites was analyzed using the SureMASTR TP53 library preparation kit (Agilent, Santa Clara, CA, USA) and MiSeq sequencing with a minimum mean coverage of 10000x. The pathogenicity of *TP53* variants was determined using the Seshat tool (<https://p53.fr/tp53-database/seshat>). Multiplex ligation-dependent probe amplification (MLPA) with SALSA P037 and P038 CLL-specific probemixes (MRC-Holland, Amsterdam, The Netherlands) was performed to detect various copy number changes and mutations of great importance in CLL (including deletions of chromosomes 11q, 13q, 17p, trisomy of chromosome 12, and *NOTCH1* c.7541_7542delCT, *SF3B1* K700E and *MYD88* L265P mutations), following the manufacturer's instructions. Resistance mutations in *BTK* and *BCL2* genes were analyzed using droplet digital PCR (ddPCR) method with mutation specific assays for the detection of the most frequent variants (*BTK* C481S – dHsaMDS802598840, *BCL2* G101V wild-type - dHsaADS52164188, *BCL2* G101V mutant type – dHsaADS26433961, *BCL2* D103Y wild-type – dHsaADS13624642, *BCL2* D103Y mutant type – dHsaADS77894415). Droplet generation and reading was performed using QX200 AutoDG Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA). The mean sensitivity of the method was 0.01%.

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Medical Research Council in Hungary.

3. Results

3.1. Morphologic patterns of transformation

All diagnoses of this study were rendered by two independent expert hematopathologists. Three out of the six Richter syndrome cases (P2, P3 and P4) were diagnosed as DLBCL showing diffuse proliferation of neoplastic large B lymphocytes with a markedly increased Ki67 proliferation activity (Table 1).

RS of P1 presented 5 months after venetoclax initiation as a paravertebral mass causing low back pain. The histological examination of the core biopsy specimen revealed a tumor mass with a diffuse pattern composed of polymorphic large cells. The sample was tested with a wide range of immunohistochemical markers. The tumor cells were positive for LCA/CD45 (partial), MUM1, p53, S100 and CD4 stainings with partial Oct-2 and Fascin-1 positivity and a proliferation activity of 60% by Ki67. The following markers proved to be negative: CD20, PAX5, CD79a, CD21, CD23, Bcl-6, CD10, CD138, CD30, CD3, CD5, CD7, PD-L1, CD68, ALK1, kappa, lambda, CD15 and melanoma markers (MelanA, HMB45 and SOX10). Based on the partial LCA and Oct-2 expression and the strong Ki67 positivity and the lack of more lineage specific markers (B-cell, T-cell markers, lack of CD30), the initial diagnosis of “null phenotype” high-grade lymphoma with a morphology resembling Hodgkin lymphoma or anaplastic large cell lymphoma had been established which was later confirmed to be of B-cell origin with monoclonal IgH gene rearrangement (Figure 3).

P5 experienced a clinically suspicious right inguinal lymph node enlargement two months after initiating ibrutinib, concurrently with lymph node regression in other localizations. Histological examination of the core biopsy specimen revealed a diffuse lymphoid infiltrate and besides small lymphocytes, it consisted mainly of atypical, CD20 and CD30 positive large cells. A proportion of the large cells proved to be multinucleated giant cells. The small cell component showed strong CD5 and

weak CD20 positivity. The neoplastic large cells showed a partial expression of C-MYC, a proliferation activity of 60% by Ki67 and MUM1 expression of 100% while being negative with Bcl-6 staining. Genomic DNA of Epstein-Barr virus (EBV) was not detectable by real-time PCR. In summary, both the morphology and immunophenotype of the large cell component showed mixed features between classical Hodgkin lymphoma and non-germinal center type DLBCL (Figure 4).

After 18 months of ibrutinib treatment, P6 showed multiple gingival proliferations which have been excised for histological evaluation. Wide subepithelial sheets of tumor cells were observed, the proliferation activity was approximately 90% as determined by Ki67 staining. The tumor cells showed a marked LCA, CD79a, CD138 and kappa light chain positivity, while CD20, CD3, CD30, MPO and Pan-CK stainings were negative. Based on this immunophenotype, the diagnosis of plasmablastic lymphoma was established in the patient without previous immunodeficiency that is not associated with CLL or CLL-related therapies (e.g. HIV infection) (Figure 5).

3.2. Genetic background of transformation

3.2.1. Clonal relationship determined by the IGHV-D-J rearrangement

IGHV analysis was successful in 4/6 CLL-RS paired samples. Three out of the four successfully analyzed sample pairs were clonally related (3/6 patients overall), with *IGHV* mutational status and *IGHV-D-J* genes being identical. Further details of *IGHV-D-J* mutation analysis are shown in Table 2.

3.2.2. Chromosome 17p deletions and TP53 mutations

Abnormalities affecting the key cell cycle regulator TP53 occurred in all patients. TP53 abnormalities were present in all CLL samples and 4/6 RS samples. Detailed results of *TP53* analysis are shown in Figure 2 and Table 3.

3.2.3. Other genetic aberrations and clinical correlations

13q deletion proved to be the second most frequent chromosomal aberration with 3/6 affected cases, followed by trisomy 12 (two affected cases). The chromosomal abnormalities occurred in various constellations and were mainly monoallelic (Figure 2). Using the SALSA P038 CLL-2 probemix, we were able to investigate the presence of three mutations frequently occurring in CLL (*SF3B1* c.2098A>G (p.K700E), *NOTCH1* c.7541_7542delCT (p.P2514fs) and *MYD88* c.794T>C (p.L265P)). Although cases carrying trisomy 12 are frequently accompanied by *NOTCH1* mutations, these two aberrations predisposing to RS occurred independently in our cohort. Interestingly, P2 proved to be the sole patient to harbor a 9p21.3 deletion, leading to *CDKN2A* and *CDKN2B* loss in both CLL and RS phases. Further details are shown in Figure 2.

There were remarkable differences among the clinical features of the patients in our cohort, many of which may be linked to certain genetic alterations. P2 carried the largest number of aberrations (six lesions in CLL and ten lesions in RS phase), resulting in a complex karyotype and genomic instability leading to a highly aggressive large cell transformation of CLL resembling acute leukemia. P1 harboring 13q del and *IGHV*-M (germline homology of 96.14%) was observed for 12 years after diagnosis with no need of treatment, and RS proved to be clonally unrelated due to the use of different *IGH* genes, but still being *IGHV*-M (germline homology of 93.51%). Although trisomy 12 confers an intermediate prognosis, the unmutated *IGHV* gene with the PBL phenotype and the presence of stereotyped BCR subset #1 may lead to a particularly dismal outcome as documented in the case of P6. The total follow-up time (53 months) of P6 is the shortest in our cohort, the patient had to be treated immediately after diagnosis and PBL transformation occurred 18 months after ibrutinib initiation (Figure 1). P1 and P6 succumbed to RS after a total follow-up time of 355 and 53 months, respectively (Figure 1).

P5 harbored an adverse *SF3B1* mutation with a VAF of 3% and an *IGHV*-U genotype. Although generally stereotyped BCRs confer a more unfavorable outcome in the *IGHV*-U patients, subset #6 carried by P5 did not have such a negative impact on survival as subset #1 in P6. At transformation, P5 continued ibrutinib therapy with the addition of 6 cycles of rituximab-bendamustine and is still in remission 29 months after developing clonally related RS (follow-up: 70 months).

3.2.4. Ibrutinib/venetoclax resistance mutations

Two out of five RS samples analyzed showed a *BTK* C481S ibrutinib resistance mutation (P4 and P2). One patient carried a *BCL2* G101V venetoclax resistance mutation (P2), the RS sample of the other patient undergoing venetoclax therapy (P1) was not available in the required amount for ddPCR analysis. P4 developed a *BTK* C481S mutation with a variant allele frequency (VAF) of 0.21% that was detectable in the RS phase 22 months after ibrutinib initiation. P2 was treated with ibrutinib until she progressed 40 months after initiating ibrutinib. After progression, venetoclax and rituximab were added to ibrutinib and this combination was administered for 12 months until RT occurred. CLL sample of P2 was collected at progression and showed a *BTK* C481S mutation with a VAF of 11.9%. One year later, at the time of transformation the *BTK* C481S VAF decreased to 0.95% while the *BCL2* G101V mutation emerged with a VAF of 4.6%.

4. Discussion

The BTK inhibitor ibrutinib and BCL2 inhibitor venetoclax have revolutionized the treatment of CLL over the past years, especially in the setting of relapsing/refractory or TP53 mutated disease. However, clonal evolution initiated by conventional chemoimmunotherapy continues under the selective pressure exerted by targeted therapies, and drives progression, relapse and transformation.

RS developing under chemoimmunotherapy was studied excessively, but the genetic and morphologic characterization of ibrutinib- or venetoclax-induced RT needs further elucidations.

Although our knowledge is rapidly growing on the molecular and radiological features of RS, histological examination has an outstanding impact on diagnosis establishment and the morphology correlates frequently with molecular and clinical features. In the era of cytotoxic therapies, CLL transformation to PBL occurred extremely rarely³⁶. However, in a cohort of 4 post-ibrutinib RS cases reported by Chan *et al.*, 50% of the patients showed PBL morphology³⁷, and a further similar case was reported by others³⁸. The molecular mechanism underlying PBL transformation of ibrutinib treated CLL is unclear. Most of the PBL cases are extranodal and arise *de novo* in association with immunodeficiency or EBV positivity. Secondary cases show monoclonal *IGH*³⁹ and *MYC* rearrangement⁴⁰, and cases with trisomy 12 have also been reported⁴¹ similar to our P6. Considering that BTK is downregulated in plasma cells⁴², plasmablastic transformation of ibrutinib treated CLL may be an escape mechanism to overcome BTK inhibition³⁷ without developing a resistance mutation. In line with this theory, P6 in our cohort underwent RT characterized by PBL morphology without the canonical *BTK* C481S resistance mutation, similarly to three, previously published cases^{37,38}.

The development of ibrutinib resistance mutations in *BTK* and *PLCG2* genes is the leading cause of CLL progression^{38,43}. RS during ibrutinib treatment occurs in 5-7.3% of patients within 17-24 months after ibrutinib initiation, as reviewed by Lampson *et al.*⁴⁴. The mean period (20 months) from ibrutinib start to RT in our cohort is in line with these findings. *BTK* or *PLCG2* mutations occur in ~80% of patients with CLL progression, but in only ~40% of RS cases^{45,46}. This difference may be explained by the underestimated mutation frequency because of the limited availability of RS tissues compared to peripheral blood, or more likely by biologically distinct ways of CLL progression and RS. In our cohort, 2/5 patients harbored a *BTK* C481S mutation among those who underwent RT during ibrutinib treatment. This prevalence corresponds to the literature; however, we should note that non-C481S *BTK* mutations or *PLCG2* mutations were not analyzed in this study.

RS during venetoclax therapy occurred in two patients in our cohort 8.5 months after therapy initiation. Interestingly, the high frequency of RS observed in heavily pretreated patients⁴⁷ may not correspond to the frequency of RS in a less heavily pretreated cohort⁴⁸. Refractoriness to fludarabine and complex karyotype are the main risk factors of early progression in venetoclax treated patients¹¹, the latter presumably being responsible for the early RS and aggressive clinical course of P2 in our cohort. Acquired abnormalities in the *BTG1*, *TP53*, *CDKN2A/B*, *SF3B1* and *BRAF* genes have been reported in the background of CLL progression and RS in venetoclax-treated patients⁴⁹. To our best knowledge, *BCL2* mutations have not been reported in RS by now⁵⁰, therefore this study might be the first to reveal a *BCL2* G101V resistance mutation in RS developing during venetoclax therapy. However, as remnant CLL clones were observed besides the DLBCL component in the patient's bone marrow biopsy, a CLL origin of *BCL2* G101V mutation could not be excluded.

Given that some genetic lesions present in CLL phase were absent in RS phase, they could have been replaced by CLL subclones with a higher potential towards transformation. Indeed, 13q deletion observed in CLL phase of P3 has been eliminated, while an *SF3B1* K700E mutation predisposing to RS emerged. Furthermore, subclonal dynamics affecting *TP53* mutations has also been detected. All six patients carried *TP53* defect (17p deletion, *TP53* mutation or both). In line with our previous findings in ibrutinib-treated CLL⁴³, the majority of *TP53* mutated subclones have been eliminated under the selective pressure of ibrutinib. In the CLL phases of P1 and P6, six *TP53* mutations have been identified altogether, and all of them were absent in the RS phase. The four *TP53* mutations of the CLL sample of P3 were replaced with only one mutation detected in the RS phase.

In summary, our findings support the importance of morphologic and molecular analysis in Richter transformation of CLL in the era of oral targeted therapies. Since all patients except for one had received multiple lines of cytotoxic chemotherapy before initiating targeted therapies, the morphologic and genetic changes observed do not necessarily represent the features of cytotoxic therapy-naïve RS patients. Every patient in our cohort carried at least one genetic aberration conferring susceptibility to RS, with the predisposing factors in ibrutinib/venetoclax-treated patients being the

same as identified in patients treated with chemoimmunotherapy (unmutated *IGHV*, stereotyped BCRs, TP53 defect, trisomy 12, *CDKN2A/B* loss, *MYC* aberrations, *NOTCH1* and *SF3B1* mutations). *BTK* or *BCL2* mutations play a less prominent role in RS pathogenesis compared to CLL progression or relapse. To our best knowledge, we have reported the first *BCL2* G101V mutation in an RS patient treated with venetoclax.

TABLES

Table 1. Patient characteristics

Patient ID	Gender	Age at CLL diagnosis (years)	Age at RS (years)	Time from CLL diagnosis to RS (months)	Total follow-up time (months)	Therapy lines prior to RS	RS morphology
P1	M	38	67	355	355	Chl, Flu, R-B, R-B, R-CVP, IBR, VEN-R	null phenotype high-grade lymphoma
P2	F	48	62	178	180	FC, ofa, R-FC, R-CHOP, R-B, IBR, R-IBR-VEN	
P3	M	49	59	123	126	FC, R-CHOP, B, B, Chl, ofa, duve, IBR (only for 3 w), CHOP, COPP/ABV + irradiation (for cHL), IBR	DLBCL
P4	M	67	74	92	93	R-FC, R-B, IBR	DLBCL
P5	M	75	78	41	70	IBR	DLBCL/cHL
P6	M	75	79	52	53	R-CVP, CHOP, R, alem, R-B, IBR	plasmablastic lymphoma

alem: alemtuzumab; B: bendamustine; Chl: chlorambucil; cHL: classical Hodgkin lymphoma; CHOP: cyclophosphamide, doxorubicin, vincristine and prednisolone; CLL: chronic lymphocytic leukemia; COPP/ABV: cyclophosphamide, vincristine, prednisone and procarbazine/ doxorubicin, bleomycin and vinblastine; DLBCL: diffuse large B-cell lymphoma; duve: duvelisib; F: female; FC: fludarabine, cyclophosphamide, FCM: fludarabine, cyclophosphamide, mitoxantrone; Flu: fludarabine; IBR: ibrutinib; M: male; ofa: ofatumumab; R: rituximab; R-B: rituximab, bendamustine; R-Chl: rituximab, chlorambucile; R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone; R-CVP: rituximab, cyclophosphamide, vincristine, prednisolone; R-FC: rituximab, fludarabine, cyclophosphamide; RS: Richter syndrome; VEN: venetoclax; w: weeks

Table 2. Clonality analysis

Patient ID	Immunoglobulin heavy chain (CLL)						Immunoglobulin heavy chain (RS)						RS clonality
	M/U	V	D	J	CDR3	Subset	M/U	V	D	J	CDR3	Subset	
P1	M	IGHV4-34*01 F	IGHD6-13*01 F	IGHJ6*02 F	14	unassigned	M	IGHV1-18*01 F	IGHD2-2*01 F	IGHJ6*02 F	20	unassigned	unrelated
P2	U	IGHV3-74*01 F	IGHD5-12*01 F	IGHJ6*02 F	16	unassigned	U	IGHV3-74*01 F	IGHD5-12*01 F	IGHJ6*02 F	16	unassigned	related
P3	U	IGHV3-33*01 F, IGHV3-33*06 F	IGHD6-13*01 F	IGHJ6*02 F	23	unassigned	U	IGHV3-33*01 F, IGHV3-33*06 F	IGHD6-13*01 F	IGHJ6*02 F	23	unassigned	related
P4			no data				U	IGHV3-23*01 F, IGHV3-23D*01 F	IGHD1-26*01 F	IGHJ4*02 F	22	unassigned	NA
P5	U	IGHV1-69*01 F, IGHV1-69*01D F	IGHD3-16*02 F	IGHJ3*02 F	21	CLL#6	U	IGHV1-69*01 F, IGHV1-69*01D F	IGHD3-16*02 F	IGHJ3*02 F	21	CLL#6	related
P6	U	IGHV1-3*01 F	IGHD6-19*01 F	IGHJ4*02 F	13	CLL#1			no data				NA

CDR3: complementarity determining region 3; CLL: chronic lymphocytic leukemia; D: diversity; J: joining; M: mutated; NA: not applicable; RS: Richter syndrome; U: unmutated; V: variable

Table 3. TP53 mutations identified in the paired samples

Patient ID	TP53 mutation (CLL)					TP53 mutation (RS)				
	cDNA change	Amino acid change	VAF (%)	COSMIC ID	Clinical significance	cDNA change	Amino acid change	VAF (%)	COSMIC ID	Clinical significance
P1	c.533delA	p.H178Pfs*69	1,8%	COSM6960219	deleterious					
	c.533delinsCC	p.H178Pfs*3	1,3%	novel	probably deleterious					
	c.536A>G	p.H179R	3,2%	COSM10889	pathogenic			negative		
	c.713G>A	p.C238Y	1,6%	COSM11059	pathogenic					
	c.814G>A	p.V272M	22,0%	COSM10891	pathogenic					
P2	c.425C>T	p.P142L	*	COSM43583	VUS	c.595G>T	p.G199*	2,9%	COSM44537	deleterious
						c.611A>C	p.E204A	1,1%	COSM46223	VUS
P3	c.472C>G	p.R158G	4,6%	COSM11087	deleterious					
	c.503A>G	p.H168R	2,1%	COSM43545	deleterious					
	c.652_654delGTG	p.V218del	3,7%	COSM6496	VUS	c.517G>T	p.V173L	1,6%	COSM43559	pathogenic
	c.747G>T	p.R249S	12,0%	COSM10817	pathogenic					
P4			no data			c.466C>G	p.R156G	*	COSM45154	probably deleterious
P5			negative			c.584T>C	p.I195T	*	COSM11089	pathogenic
P6	c.771_777delGGAAGAC	p.E258Pfs*85	8,5%	novel	probably deleterious			negative		

VAF: variant allele frequency; VUS: variant of unknown significance; *: samples analyzed by Sanger sequencing outside of the setting of this study. VAF must be over the 20% sensitivity of Sanger sequencing, but its exact value is unknown. COSMIC IDs are given according to COSMIC version v91.

FIGURE LEGENDS

Figure 1. Timeline and main clinical features of CLL patients developing Richter syndrome during ibrutinib or venetoclax treatment. The left part of the graph illustrates the clinical course of patients from diagnosis to ibrutinib initiation with lines of therapies. Numbers above the line indicate time to first treatment (TTFT) while numbers below represent time to ibrutinib initiation in months. Patient 2 received ibrutinib and venetoclax simultaneously for 12 months before transformation. Before long-term ibrutinib therapy, P3 received ibrutinib for 3 weeks, but discontinued due to drug intolerance. P3 developed classical HL simultaneously with CLL and received therefore cHL-directed chemotherapy as well. (PB: peripheral blood, BM: bone marrow, LN: lymph node).

Figure 2. Heat map displaying the *IGHV* mutational status, chromosomal aberrations and mutations identified across the six patients. CLL sample from P4 was not available. “NA” (not applicable) denotes absent data due to sample unavailability, unsuccessful or unreasonable analysis (e.g. *BCL2* analysis in venetoclax-naïve patients).

Figure 3. Richter transformation of CLL into null-phenotype high-grade lymphoma presented as a paravertebral mass (P1). The neoplastic large cells with lobulated nuclei and prominent nucleoli (hematoxylin-eosin, panel A) are of hematopoietic origin (LCA, B) and show negativity of CD20 (D) and CD30 (E). CD5 positive CLL cells are scattered within the population of large cells (C). The proliferation activity is approximately 60% (Ki67, F) (200x magnification).

Figure 4. Richter transformation of CLL into mixed phenotype B-cell lymphoma with features of DLBCL and classical HL in an inguinal lymph node (P5). The diffuse lymphoid infiltrate effaces the original structure of the lymph node (hematoxylin-eosin, panel A). The anaplastic large cell component mixed with the remnant CLL cells (CD5, B) is positive for CD20 (C), CD30 (D) and C-MYC (E). The proliferation activity is approximately 60% (Ki67, F) (200x magnification).

Figure 5. Richter transformation of CLL into plasmablastic lymphoma (PBL) manifested in the oral mucosa (P6). The neoplastic cells show an immunoblastic morphology with several mitoses

(hematoxylin-eosin, panel A). The plasmablastic/plasmacytic differentiation is characterized by CD20 negativity (B) and CD138 positivity (C) among other markers. Monoclonal tumor cells show a kappa light chain positivity (D) and lambda negativity (E). The proliferation activity of PBL cells is 90% (Ki67, F) (200x magnification).

ACKNOWLEDGEMENTS

This work was funded by the KH17_126718, K21_137948, FK20_134253 and K_16 #119950 grants of the Hungarian National Research, Development and Innovation Office (NKFIH), the EU's Horizon 2020 research and innovation program under grant agreement No. 739593 and the János Bolyai Research Scholarship (BO/00320/18/5) of the Hungarian Academy of Sciences, the ÚNKP-19-3-I-SE-52 and ÚNKP-20-5-SE-22 grants of the Ministry of Human Capacities, and the Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary, within the framework of the Molecular Biology thematic programme of the Semmelweis University and the ELIXIR Hungary.

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