MOLECULAR BACKGROUND OF RESISTANCE TO TARGETED THERAPIES IN CHRONIC LYMPHOCYTIC LEUKEMIA

PhD THESIS

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I. INTRODUCTION

Chronic lymphocytic leukemia (CLL), a malignant tumor of mature B cells, is one of the most common types of oncohematological diseases in the developed countries with an age-adjusted incidence of 4-5 per 100,000 people. CLL is characterized by the monoclonal expansion and proliferation of typically CD5 expressing and mature B cells in the peripheral blood, bone marrow and other lymphoid organs, including lymph nodes and the spleen. CLL is most common in the elderly, with a median age of 72 years at the diagnosis.

CLL has highly variable and heterogeneous clinical manifestations, albeit the most common presentation of the disease is the incidental discovery of asymptomatic lymphocytosis during complete blood count analysis obtained for reasons unrelated to CLL. Lymphadenopathy is the second most common manifestation, whereas the B symptoms, such as fever, weight loss, night sweats, and organomegaly present less frequently. Single- or multi-lineage cytopenias due to lymphocytic infiltration of the bone marrow may also be associated with the disease in some cases.

Substantial heterogeneity of the initial presentation and genetic background of CLL is complemented by a highly variable clinical course. Some patients survive for relatively long periods without requiring therapeutic intervention, whereas others succumb to an aggressive and rapidly progressing illness that is often refractory to standard chemoimmunotherapies. Recent advances in molecular biology and understanding of the genetic changes

driving leukemogenesis have led to the identification of markers associated with high-risk disease and refractoriness to standard chemoimmunotherapies conferring poor prognosis. Genetic changes defining high-risk CLL include the deletion of chromosomal region 17p, *TP53* mutations and unmutated heavy chain of the B cell receptor immunoglobulin status.

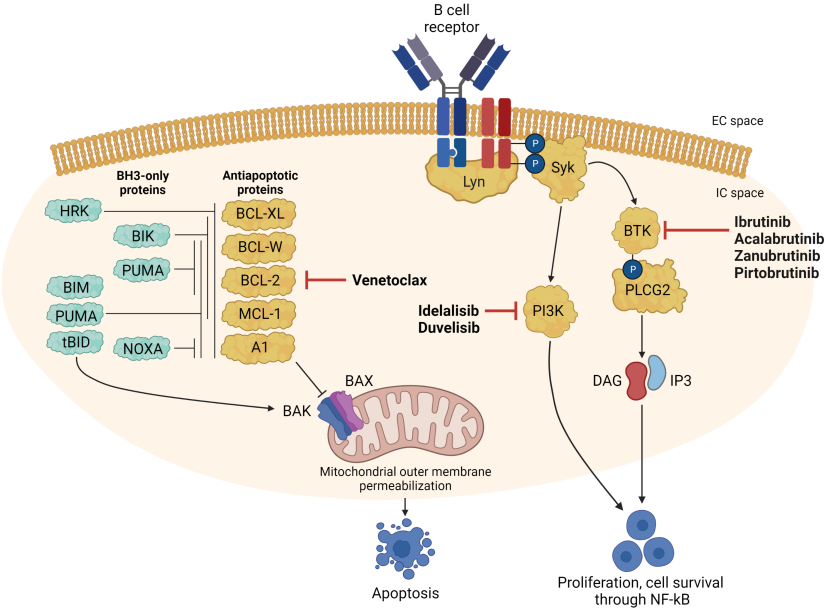

Figure 1. Targeted agents and their respective intracellular targets in CLL. Recently, the irreversible Bruton's tyrosine kinase (BTK) inhibitors ibrutinib, acalabrutinib and zanubrutinib together with the Bcl-2 inhibitor venetoclax have entered clinical practice achieving remarkable response rates across heterogeneous patient populations.

Clinical management of cases with relapsed or therapy refractory (R/R) CLL or treatment-naïve disease with high-risk genetic

features has been revolutionized by the introduction of novel targeted agents. Over the last decade, a handful of potent targeted drugs with various targets have been introduced to clinical practice (Figure 1).

Among the numerous targeted drugs recently introduced in patient care, two highly potent agents, the first-in-class small molecules Btk inhibitor ibrutinib and Bcl-2 inhibitor venetoclax have emerged and essentially changed the treatment of patients with high risk CLL by targeting the B cell receptor and Bcl-2 antiapoptotic pathways, respectively.

Despite the high response rates and prolonged progression-free survivals in both ibrutinib- and venetoclax-treated patient populations, eventual loss of efficacy and subsequent Richter's transformation or acquired therapy resistance occur in some cases, alike with other targeted therapies in hematologic and solid tumors. While Richter's transformation is recognized to occur within the first two years of targeted therapy and its cumulative incidence plateaus after the third year, secondary or acquired resistance and subsequent progressive CLL emerges after longer periods of targeted treatment. Progressive CLL typically occurs two to five years after therapy initiation and is the leading cause of treatment failure. Since secondary ibrutinib and venetoclax resistance confers dismal outcomes, early detection of loss of efficacy could potentially allow for the introduction of alternative treatment strategies with ideal timing over the disease course and the identification of patients who may benefit from change of therapy.

II. OBJECTIVES

With a series of studies, we aimed to unveil distinct genetic changes underlying resistance to targeted therapies in CLL and to scrutinize the correlation between the emergence of selected gene mutations and relapse or disease progression. We collected and analysed specimens from CLL patients treated with ibrutinib or venetoclax with special emphasis on pre-progression and progression samples to assess the clinical value and feasibility of the sensitive screening for the most frequent resistance mutations. Our data may have implications for the sensitive monitoring of targeted therapies which may allow for the early detection of an impending relapse and the identification of patients who could potentially benefit from change of therapy.

Specific objectives:

- To develop a droplet digital polymerase chain reaction (ddPCR)-based method for the detection of the most frequent resistance mutations conferring secondary resistance to ibrutinib or venetoclax therapy

- To dissect the temporal heterogeneity observed in R/R CLL in the context of ibrutinib therapy by the molecular monitoring of the most common *BTK* p.Cys481Ser mutation

- To unveil clonal evolution during venetoclax therapy by the molecular screening for the most frequent variants *BCL2* p.Gly101Val and p.Asp103Tyr, as well as other pathogenic *BCL2* mutations by targeted next-generation sequencing (NGS)
- To assess the correlation between acquired ibrutinib resistance and the emergence of *BTK* p.Cys481Ser

- To assess the correlation between acquired venetoclax resistance and selected *BCL2* mutations

- To aid the standard-of-care monitoring of the efficacy of ibrutinib and venetoclax therapies in CLL

III. MATERIALS

III.1. Patient samples

Serial peripheral blood (PB) samples were collected from 83 R/R CLL patients treated with ibrutinib. This cohort comprised a subset of 126 patients investigated within the framework of the Hungarian Ibrutinib Resistance Analysis Initiative. Selected patients received single agent ibrutinib in a daily dose of 420 mg for a minimum of 13 months (median 36 months, range: 13-68 months).

Peripheral blood samples were collected from 67 patients with R/R CLL treated with venetoclax monotherapy or venetoclax in combination with rituximab. Fixed duration venetoclax *plus* rituximab combination was applied in 23.9% (16/67) of the patients, with time-limited and continuous venetoclax monotherapy administered in 7.5% (5/67) and 68.6% (46/67) of the cases, respectively. Following the ramp-up period, selected patients received venetoclax in a daily dose of 400 mg for a median 20 months (1-83 months) complemented with rituximab during the first 6 months of therapy in cases with fixed duration treatment setup.

Progressive disease and relapse were defined as the emergence of at least one novel symptom based on the iwCLL criteria for active disease and change of therapy was issued at the physician's discretion.

III.2. Molecular, cytogenetic and flow cytometry analyses

Peripheral blood mononuclear cells were separated by density gradient centrifugation from the PB specimens following the assessment of leukemic cell purity by flow cytometry using CD5/CD19/CD45 immunophenotypic markers. DNA isolation was performed using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Basel, Switzerland) following the manufacturer's instructions. After DNA extraction, *TP53* mutations and the IGHV mutation status were analysed according to the most recent ERIC guidelines. Chromosomal abnormalities, including del(11q), del(13q) and del(17p), as well as the trisomy of chromosome 12, were investigated by FISH using dual-colour Vysis probe sets (Abbott Molecular, Illinois, USA).

III.3. Droplet digital PCR

Screening and quantitative assessment of the *BTK* p.Cys481Ser and selected *BCL2* resistance mutations (p.Gly101Val, p.Asp103Tyr, p.Asp103Glu, p.Ala113Gly, p.Val156Asp) were screened by ddPCR using assays designed for the discriminative analysis of mutant and wild-type alleles. Reactions were carried out on the QX200 Droplet Digital PCR platform (Bio-Rad Laboratories, California, USA) according to the manufacturer's instructions. Allelic burden of each mutation was defined as fractional abundance (FA) calculated from the ratio of the number of mutant DNA molecules (a) and the total number of mutant (a) plus wild-type (b) DNA molecules detected: $FA = \frac{a}{a+b}$.

III.4. Ultra-sensitive next-generation sequencing

Targeted ultra-deep NGS was performed on follow-up samples of ibrutinib-treated patients harbouring the wild-type *BTK* Cys481 allele and progressing on ibrutinib, as well as samples of venetoclax-treated patients with progressive CLL obtained at the time of relapse or disease progression. NGS was carried out using a QIAseq Targeted DNA Custom Panel (Qiagen, Hilden, Germany) covering four genes (*BTK*, *PLCG2*, *BCL2* and *TP53*) relevant to ibrutinib or venetoclax therapy and resistance.

III.5. Statistics

GraphPad Prism 9.1.0 (GraphPad Software, California, USA) was utilised for calculating median values and confidence intervals (CI), as well as for analysing the cumulative incidence of disease progression in patients harbouring the *BTK* p.Cys481Ser resistance mutation, and for performing the Mantel–Cox test to compare PFS and OS between patients harbouring *BCL2* resistance and cases with wild-type *BCL2* Gly101 and Asp103. The Mantel–Byar estimate of PFS in patients with or without detectable *BTK* p.Cys481Ser resistance mutation was calculated by R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

IV. RESULTS

IV.1. Molecular monitoring of ibrutinib resistance

IV.1.1. Ultra-sensitive screening for the BTK p.Cys481Ser resistance mutation

With a median follow up of 40 months (range: 13-69 months), *BTK* p.Cys481Ser was detected in 48.2% (40/83) of the patients with 80.0% (32/40) of them experiencing relapse or disease progression during the follow up period. These 32 patients represented 72.7% (32/44) of all cases with progressive CLL or relapse. Median FA value at the time of relapse or disease progression was 10.66% (range: 0.01-90.00%).

IV.1.2. Time-resolved monitoring of the BTK p.Cys481Ser mutation

The 32 patients carrying detectable *BTK* p.Cys481Ser and experiencing acquired ibrutinib resistance underwent disease progression after median 38 months (range: 13-65 months) of ibrutinib therapy. In 19 patients with samples obtained prior to disease progression, emergence of the resistance mutation preceded the first clinical signs of ibrutinib failure with median 9 months (range: 0-28 months) (Figure 2). Median FA values of *BTK* p.Cys481Ser at the time of first detection versus at the time of relapse or disease progression were 0.385% versus 10.66%, demonstrating a median 28-fold increase in the allelic burden of the resistance mutation.

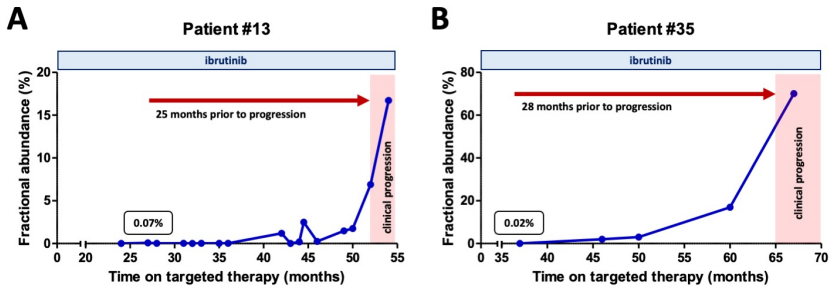

Figure 2. Temporal dynamics of the *BTK* p.Cys481Ser resistance mutation in two patients with relapsed or therapy refractory chronic lymphocytic leukemia treated with ibrutinib.

IV.1.3. Management of secondary ibrutinib resistance in patients with BTK p.Cys481Ser

Due to ibrutinib failure and subsequent disease progression, 87.5% (28/32) of the patients carrying *BTK* p.Cys481Ser have discontinued ibrutinib treatment. Successive Bcl-2 inhibition by venetoclax was applied in all these cases resulting in clinical remission and elimination of the *BTK* p.Cys481Ser mutant CLL subclones within median 3 months (range: 2-7 months) across the patients. Despite the remarkable and durable response rates and decrease in the allelic burden of *BTK* p.Cys481Ser, 8 patients experienced relapse or disease progression, and developed secondary venetoclax resistance during the follow-up period. Six out of these 8 patients have succumbed to their disease without receiving any subsequent therapy, while two patients are still alive to date with one of them undergoing hematopoietic stem cell transplantation and the other patient receiving salvage chemoimmunotherapy.

IV.2. Molecular monitoring of venetoclax resistance

IV.2.1. Sensitive screening for BCL2 p.Gly101Val and p.Asp103Tyr

Screening for *BCL2* p.Gly101Val and p.Asp103Tyr was performed on samples obtained from 67 R/R CLL patients using locus-specific ddPCR assays designed and optimized for the sensitive (10^{-4}) interrogation of these two mutational hotspots. *BCL2* p.Gly101Val and p.Asp103Tyr were detected in 10.4% (7/67) and 11.9% (8/67) of the cases, respectively, with 4 patients harbouring both mutations. The two resistance mutations were first observed after median 15 months (range: 5-48 months) of venetoclax therapy.

Ten out of eleven (90.9%) patients carrying p.Gly101Val and/or p.Asp103Tyr experienced relapse or disease progression, representing 43.5% (10/23) of all cases developing secondary venetoclax resistance during the follow-up period. Intriguingly, all *BCL2* p.Gly101Val and p.Asp103Tyr variants were detected in patients receiving venetoclax as a continuous single-agent treatment, while the two resistance mutations were not observed during or after fixed duration venetoclax therapy. In the analysed patient population, significantly inferior PFS as defined by the iwCLL 2018 criteria was observed among patients carrying *BCL2* p.Gly101Val and/or p.Asp103Tyr mutations as compared to cases with wild-type *BCL2* Gly101 and Asp103 loci (Mantel–Cox test, p value = 0.0192).

IV.2.2. Time-resolved monitoring of co-occurring BCL2 resistance mutations

Four patients harbouring multiple *BCL2* mutations relapsed or experienced disease progression after median 29 months (range: 18-48 months) of continuous single agent venetoclax therapy. Quantitative ddPCR using locus-specific and custom-designed assays was performed on longitudinally collected samples of all four cases allowing for the retrospective temporal dissection of the additional *BCL2* variants p.Asp103Glu, p.Ala113Gly and p.Val156Asp detected by NGS. In samples obtained at the time of relapse or disease progression, median FA values of *BCL2* p.Gly101Val, p.Asp103Tyr, p.Asp103Glu, p.Ala113Gly and p.Val156Asp were 0.50% (range: 0.16-2.20%), 1.15% (range: 0.05-3.40%), 1.16% (range: 0.37-1.95%), 0.37% (range: 0.05-5.76%), and 2.20%, respectively.

In all cases with samples obtained prior to progression, emergence of *BCL2* p.Gly101Val and p.Asp.103Tyr, as well as co-occurring *BCL2* mutations preceded the first clinical signs of disease progression with median 11 months (range: 5-16 months) (Figure 3A-C, Patient #43 harbouring exclusively p.Gly101Val mutation is not shown). Allelic burdens of *BCL2* p.Gly101Val, p.Asp103Tyr, p.Asp103Glu, p.Ala113Gly and p.Val156Asp were increased at the time of relapse as compared to samples collected prior to disease progression, demonstrating a median of 6.61-fold (range: 1.2-18.75) increase in the FA values of each mutation over time.

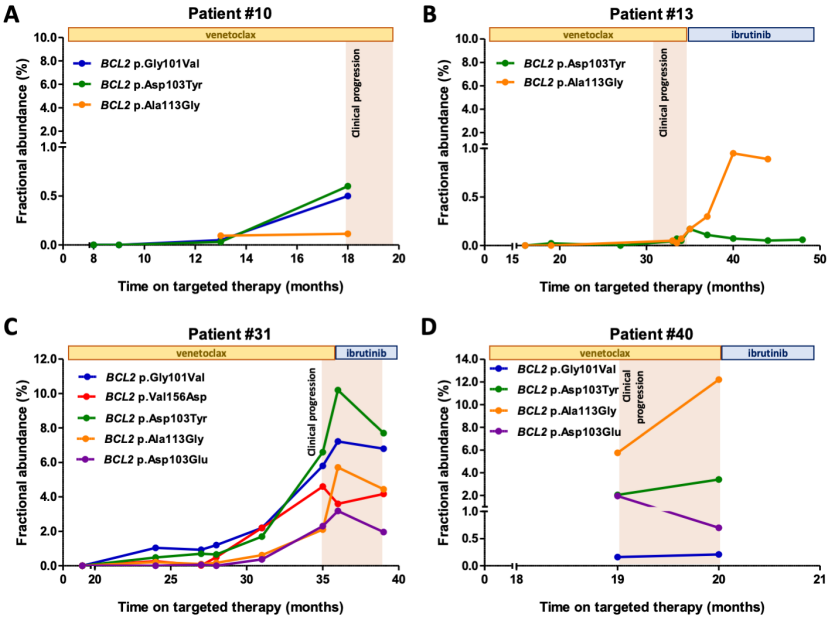

Figure 3. Temporal dynamics of *BCL2* mutations in 4 patients harbouring multiple variants associated with venetoclax resistance.

IV.2.3. Secondary venetoclax resistance in cases with wild-type BCL2

Thirteen patients progressing on venetoclax harboured wild-type *BCL2* Gly101 and Asp103 as assessed by ddPCR. Interestingly, all patients experiencing relapse or disease progression received venetoclax as a continuous single agent therapy. Interestingly, no further resistance mutations affecting the *BCL2* gene were identified in any of the analysed cases suggesting the presence of alternative genomic lesions localized outside the coding region of *BCL2*, or the emergence of epigenomic/transcriptomic changes eventually leading to manifest venetoclax resistance.

V. DISCUSSION

In the framework of a nationwide research initiative, we investigated the feasibility and clinical value of screening for the most common resistance mutations underlying acquired ibrutinib and venetoclax resistance in R/R CLL patient populations using an ultra-sensitive digital droplet polymerase chain reaction (ddPCR)-based method. In our study we performed ddPCR and targeted next-generation sequencing for the sensitive detection of the most common resistance mutations underlying ibrutinib and venetoclax failure in 83 and 67 R/R CLL patients treated with ibrutinib and venetoclax, respectively.

With the ultra-sensitive ddPCR-based analyses of one mutation hotspot associated with ibrutinib resistance and two hotspots linked to venetoclax failure, we successfully identified the underlying molecular mechanisms in two-thirds (32/44 and 10/23 of all ibrutinib- and venetoclax-resistant cases, respectively) of the patients undergoing relapse or disease progression during targeted treatments. Our results imply the selected gene mutations as promising potential molecular biomarkers for use in the standard-of-care diagnostic work-up of targeted therapy resistance in CLL.

Additionally, in the ibrutinib-treated cohort, emergence of the resistance mutation predated the first clinical observation of relapse or disease progression with median 9 months. Consequently, the presence and increasing abundance of *BTK* p.Cys481Ser proved to be a good indicator of an impending relapse well before the first clinical signs of relapse or disease

progression in the majority (79% × 73%) of our patients with informative follow up.

In the venetoclax-treated patients, retrospective analysis and temporal dissection of the uncovered co-occurring *BCL2* variants in longitudinally collected samples of patients receiving venetoclax revealed heterogeneous and dynamically changing subclonal FA values of mutations during venetoclax therapy. This suggests a complex underlying architecture of resistant CLL cell compartments, coupled with subsequent progressive CLL in all four cases analysed longitudinally.

In summary, our series of studies demonstrate the feasibility and clinical value of robustly detecting the most frequent *BTK* p.Cys481Ser mutation underlying acquired ibrutinib resistance and the two most common *BCL2* mutations p.Gly101Val and p.Asp103Tyr associated with secondary resistance under real-world circumstances. To our knowledge, our patient cohorts were the largest real-world R/R CLL patient populations at the time of publishing, in which resistance mutations have been molecularly monitored during Btk or Bcl-2 inhibition.

VI. CONCLUSIONS

Novel findings of my thesis are:

- Sensitive ddPCR-based methods are optimal for the early detection of an impending relapse as well as for the identification of underlying molecular mechanisms in up to two-thirds of R/R CLL patients treated with targeted agents experiencing relapse or disease progression.
- In R/R CLL patients progressing on ibrutinib with PB specimens obtained prior to ibrutinib failure, emergence of the underlying resistance mutation *BTK* p.Cys481Ser predated the first clinical observation of acquired ibrutinib resistance with median 9 months. In the majority of the cases using sensitive ddPCR, the mutation was detectable at least 3 months prior to relapse or disease progression.
- We unveiled genetic alterations conferring resistance to the Bcl-2 inhibitor venetoclax by the molecular screening for the most frequent underlying variants *BCL2* p.Gly101Val and p.Asp103Tyr in nearly half of the patients with venetoclax failure and subsequent disease progression.
- We dissected the temporal heterogeneity observed in R/R CLL in the context of venetoclax therapy by the molecular monitoring of pathogenic *BCL2* variants using ddPCR and ultra-deep targeted NGS. Convergent evolution of distinct mutation-bearing CLL subclones was observed in the longitudinally collected samples of four patients.

VII. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

Publications related to the PhD thesis:

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