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Screening and monitoring of the *BTK*^{C481S} mutation in a real-world cohort of patients with relapsed/refractory chronic lymphocytic leukaemia during ibrutinib therapy

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Summary

The Bruton's tyrosine kinase (BTK) inhibitor ibrutinib has revolutionised the therapeutic landscape of chronic lymphocytic leukaemia (CLL). Acquired mutations emerging at position C481 in the *BTK* tyrosine kinase domain are the predominant genetic alterations associated with secondary ibrutinib resistance. To assess the correlation between disease progression, and the emergence and temporal dynamics of the most common resistance mutation *BTK*^{C481S}, sensitive (10^{-4}) time-resolved screening was performed in 83 relapsed/refractory CLL patients during single-agent ibrutinib treatment. With a median follow-up time of 40 months, *BTK*^{C481S} was detected in 48·2% (40/83) of the patients, with 80·0% (32/40) of them showing disease progression during the examined period. In these 32 cases, representing 72·7% (32/44) of all patients experiencing relapse, emergence of the *BTK*^{C481S} mutation preceded the symptoms of clinical relapse with a median of nine months. Subsequent Bcl-2 inhibition therapy applied in 28/32 patients harbouring *BTK*^{C481S} and progressing on ibrutinib conferred clinical and molecular remission across the patients. Our study demonstrates the clinical value of sensitive *BTK*^{C481S} monitoring with the largest longitudinally analysed real-world patient cohort reported to date and validates the feasibility of an early prediction of relapse in the majority of ibrutinib-treated relapsed/refractory CLL patients experiencing disease progression.

Keywords: chronic lymphocytic leukemia, CLL, ibrutinib, treatment resistance, molecular monitoring.

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Introduction

Chronic lymphocytic leukaemia (CLL) is characterised by significant clinical heterogeneity coupled with a diverse genomic, epigenomic and transcriptomic background.[1] Recurrent molecular and cytogenetic abnormalities have been identified as underlying mechanisms for adverse disease course leading to relapsed/treatment refractory (R/R) CLL. High-risk genetic features such as complex karyotype, deletions of chromosomal regions 11q (*ATM*) and 17p (*TP53*), *TP53* gene mutations as well as unmutated immunoglobulin heavy chain variable (*IGHV*) gene status are commonly associated with refractoriness to standard chemo-immunotherapies, early relapse and inferior survival.[2–4]

Clinical management of patients with R/R CLL or with treatment-naive CLL harbouring high-risk genetic features has been revolutionized by the irreversible Bruton's tyrosine kinase (BTK) inhibitor ibrutinib which confers remarkable response rates both in first-line and in previously treated patient cohorts.[5–9] Although clinical trials and the analyses of real-world patient cohorts have shown a survival advantage with ibrutinib over standard therapies,[6,7,10–13] durable remission is eventually followed by either Richter's transformation or progressive CLL in a subset of the patients. While Richter's transformation tends to occur during the first or second year of ibrutinib treatment and its cumulative incidence shows a plateau after the third year, CLL progression emerges later, typically after a 12–15-month period of BTK inhibition with events regularly occurring during the third, fourth and fifth years after therapy initiation.[13–16] Since ibrutinib failure confers poor survival, early detection of resistance could provide clinically relevant information, potentially optimising the transition of affected patients to alternative treatment strategies with ideal timing during the disease course.

CLL progression on ibrutinib is strongly associated with acquired mutations emerging in *BTK* at the binding site of ibrutinib and/or in the phospholipase Cγ2 (*PLCG2*) gene encoding the protein directly downstream of BTK in the BCR signalling pathway.[15–19] Indeed, while less than half of the patients undergoing Richter's transformation harbour detectable *BTK* and *PLCG2* mutations, these aberrations can be observed in the vast majority (>80%) of patients experiencing progressive CLL.[15,20,21] BTK^{C481S} is by far the most common resistance-associated mutation which, by disrupting the covalent binding between BTK and ibrutinib, renders ibrutinib a reversible inhibitor with decreased BTK binding affinity.[17,22] Although in the presence of the BTK^{C481S} mutation, ibrutinib still allows for some level of disease control via its residual competitive binding, alternative single-agent

or combined targeted therapy options should be considered for patients harbouring this alteration in order to prevent or overcome a potential relapse.[23]

In this study, we assessed the feasibility and clinical value of sensitive longitudinal screening for BTK^{C481S} mutation in a real-world cohort of R/R CLL patients receiving single-agent ibrutinib treatment. Our results provide an insight into the temporal dynamics of this resistance mutation and will expectedly have implications for the molecular monitoring of ibrutinib therapy which may allow for the early identification of patients who could benefit from a switch to alternative treatment modalities.

Materials and methods

Patients and samples

Peripheral blood samples were collected from 83 R/R CLL patients (49 males and 34 females; median age at diagnosis: 57 years, range: 26–85 years) treated with ibrutinib in 13 Hungarian oncohaematological centres. This cohort comprised a subset of 126 CLL patients who had been investigated in the framework of the Hungarian Ibrutinib Resistance Analysis Initiative to date. Selected patients received ibrutinib in a daily dose of 420 mg as a single-agent therapy for a minimum of 13 months (median 36 months, range: 13–68 months) and represented a pretreated cohort with a median of two lines (range: 1–6) of prior therapy. Clinical characteristics are summarised in Table SI. The median follow-up time was 40 months (range: 13–69 months) with <10% of the samples collected retrospectively and over 90% prospectively during the study period. Considering patients progressing on ibrutinib, specimens obtained during ibrutinib treatment prior to the first clinical signs of relapse were available in 56·8% (25/44) of the cases, while the first follow-up sample of 19 patients was received at the time of CLL progression. Follow-up samples of patients harbouring BTK^{C481S} and/or progressing on ibrutinib were received with a median interval of four months (range: 1–11 months) allowing for a real-life time-resolved monitoring of the resistance mutation. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation and the leukaemic cell purity was assessed by flow cytometry using CD5/CD19/CD45 immunophenotypic markers. After DNA extraction, *IGHV* mutation status and *TP53* mutations were analysed according to the most recent European Research Initiative on CLL (ERIC) recommendations.[24,25] Chromosomal abnormalities, including deletions of 11q, 13q and 17p as well as trisomy of chromosome 12, were screened by interphase fluorescence *in situ* hybridisation (FISH) using dual-colour Vysis probe sets (Abbott Molecular, IL, USA). PBMCs from five healthy volunteers were used as negative controls. Written informed consent was obtained from all participants, the study was approved by the Hungarian Medical Research Council (ID: 45371-2/2016/EKU) and it was conducted in accordance with the Declaration of Helsinki.

Droplet digital PCR

Screening and quantitative assessment of the BTK^{C481S} resistance mutation was performed by droplet digital polymerase chain reaction (ddPCR; Bio-Rad Laboratories, CA, USA) using a custom assay that we previously designed for the discriminative analysis of mutant and wild-type alleles.[26] All reactions were carried out according to the manufacturer's recommendations using 100 ng of input DNA. The allelic burden of the mutation was defined as the 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: $FA = a/(a + b)$. *FA* values were normalised to the CLL cell fraction measured by flow cytometry. Quantitative reliability of the assay was determined with linearity measurements using dilution series and the sensitivity of the ddPCR analysis was assessed for each sample. The lower limit of the quantitative range could ubiquitously be determined as 0·01% *FA*.

Ultra-deep next-generation sequencing

Targeted next-generation sequencing (NGS) was performed on follow-up samples of patients harbouring the wild-type BTK^{C481} allele and progressing on ibrutinib using a QIAseq Targeted DNA Custom Panel (Qiagen, Hilden, Germany) covering three genes (*BTK*, *PLCG2* and *TP53*) relevant to ibrutinib therapy and resistance. Libraries were prepared according to the manufacturer's recommendations and sequenced on a MiSeq platform (Illumina, San Diego, CA, USA) with 150 bp paired-end configuration. Data processing and analysis were performed with the smCounter2 workflow utilising unique molecular identifier-based variant calling which facilitates the highly accurate detection of low-frequency variants.[27] Variants were annotated using the dbSNP, COSMIC, ClinVar, SnpSift and SnpEff databases as well as the most recent versions of the *TP53*-specific IARC/Seshat databases.[28,29] Reported variant allele frequencies (VAF) were normalised by considering the ratio of CLL cells in the sample.

Statistics

GraphPad Prism 9.1.0 (GraphPad Software, San Diego, CA, USA) was used for calculating median values and confidence intervals, as well as for analysing the cumulative incidence of disease progression in patients harbouring the BTK^{C481S} resistance mutation. The Mantel–Byar estimate of progression-free survival in patients with or without detectable BTK^{C481S} mutation was calculated by R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria).[30]

Results

Molecular and cytogenetic features considered as independent prognostic markers in CLL were screened as part of the

diagnostic characterisation (Table SI). *TP53* mutation status revealed by ultra-deep NGS and del(17p) status analysed by FISH were available in 75/83 and 81/83 cases, respectively. Patients with del(17p) and/or *TP53* mutation(s) represented 50·6% (42/83) of the study cohort. Further cytogenetic abnormalities including del(11q), del(13q) and trisomy 12 were identified with a frequency of 15·7%, 26·5% and 12·0%, respectively. *IGHV* mutation status was available for 79 patients with 87·3% of them carrying an unmutated *IGHV* gene configuration (IGHV-U). High-risk genetic features associated with adverse prognosis were observed in 89·2% (74/83) of the patients, which together with failures on previous treatment lines is an indicator that our study group adequately represented a real-world R/R CLL cohort with patients typically selected for ibrutinib therapy.

Ultra-sensitive screening for the BTK^{C481S} *resistance mutation*

Retrospective and prospective screening for the ibrutinib resistance-associated *BTK*^{C481S} mutation was performed on a total of 305 samples using a highly sensitive (10^{-4}), locus-specific ddPCR assay. With a median follow-up time of 40 months (range: 13–69 months), *BTK*^{C481S} was detected in 48·2% (40/83) of the patients (Table SII) with 80·0% (32/40) of them experiencing relapse or disease progression during the examined period. These 32 cases represented 72·7% (32/44) of all patients showing signs of clinical disease progression. The median *FA* value at the time of CLL progression was 10·66% (range: 0·01–90·00%; 95% CI: 3·0–23·0%). Richter's transformation was not observed among the 40 *BTK*^{C481S}-positive patients.

Time-resolved monitoring of the BTK^{C481S} *mutation*

The 32 patients harbouring detectable *BTK*^{C481S} mutation and experiencing secondary ibrutinib resistance underwent disease progression after a median of 38 months (range: 13–65 months; 95% CI: 32·0–44·0 months) of ibrutinib therapy. In 19 patients with samples obtained prior to the progression, emergence of the resistance mutation preceded the ibrutinib failure with a median of nine months (range: 0–28 months; 95% CI: 3·0–12·0 months) as shown in Figs 1 and 2. Cumulative incidence of disease progression in patients harbouring the *BTK*^{C481S} resistance mutation and having preprogression samples available for monitoring is shown in Fig 3A. Median *FA* values of *BTK*^{C481S} at the time of the first detection *versus* at the time of disease progression were 0·385% vs 10·66%, demonstrating a median 28-fold increase in the allelic burden of the resistance mutation (Fig 3B). The Mantle–Byar test was used for analysing CLL progression on ibrutinib with all patients starting in the *BTK*^{C481S}-negative group and being transferred to the *BTK*^{C481S}-positive group upon first detection of the mutation. A significantly inferior progression-free survival rate was observed among patients acquiring detectable *BTK*^{C481S} mutation ($P < 0{\cdot}00001$; Figure S1).

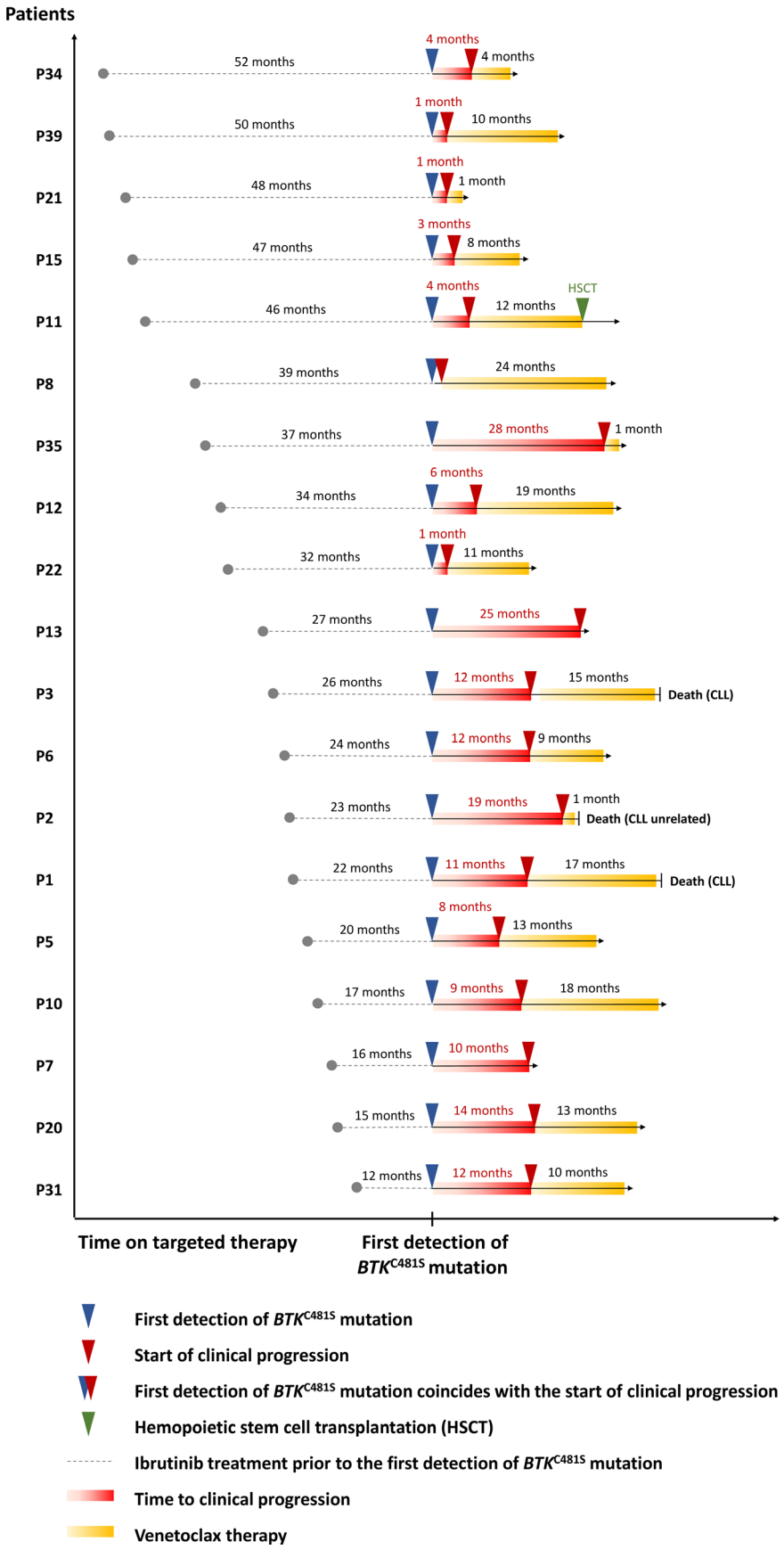
Management of secondary ibrutinib resistance in patients with BTK^{C481S}

Due to secondary ibrutinib resistance and disease progression, 87·5% (28/32) of the patients harbouring *BTK*^{C481S} have discontinued ibrutinib treatment. Subsequent BCL2 inhibitor venetoclax therapy administered to all these patients resulted in clinical remission and elimination of the *BTK*^{C481S} mutant CLL subclones within a median of three months (range: 2–7 months) across the cases. Despite the remarkable and durable decrease in the abundance of the mutations (Fig 4A), eight patients experienced disease progression and developed secondary venetoclax resistance during the follow-up period (Fig 4B). Six out of these eight patients have succumbed to their disease without receiving any further treatment, while two patients are still alive to date with one of them undergoing haematopoietic stem cell transplantation and the other patient receiving salvage chemo-immunotherapy.

Patients harbouring the BTK^{C481S} *mutation with no signs of disease progression*

The *BTK*^{C481S} mutation was detected in eight patients with no clinical evidence of relapse or disease progression during the examined period (Figure S2). These patients, representing 20·0% of all patients harbouring *BTK*^{C481S} (8/40), have continued ibrutinib therapy and were regularly monitored by ddPCR. At the latest follow-up timepoints, the median *FA* value of the resistance mutation was 0·69% (range: 0–20%) after a median of 43 months (range: 23–68 months) of ibrutinib therapy. Interestingly, the emergence of *BTK*^{C481S} was later followed by the complete elimination of the acquired mutation in three cases. Quantitative ddPCR analysis of sequential samples obtained from the remaining five patients revealed an increasing *FA* of the *BTK*^{C481S} resistance mutation, highlighting the significance of monitoring these patients for signs of an impending relapse. Two patients

Fig 1. Treatment timeline of 19 patients with relapsed/treatment refractory (R/R) chronic lymphocytic leukaemia (CLL) harbouring the *BTK*^{C481S} ibrutinib resistance mutation and experiencing relapse or disease progression during the study period. Blue wedges denote the timepoint of the first detection of *BTK*^{C481S} with red wedges indicating the first clinical observation of disease progression. Emergence of the resistance mutation was detectable with a median of nine months (range: 0–28 months) prior to the first clinical signs of CLL progression as indicated by the red bars. Venetoclax therapy was administered in 89·5% (17/19) of the cases as represented by the yellow bars on each timeline.



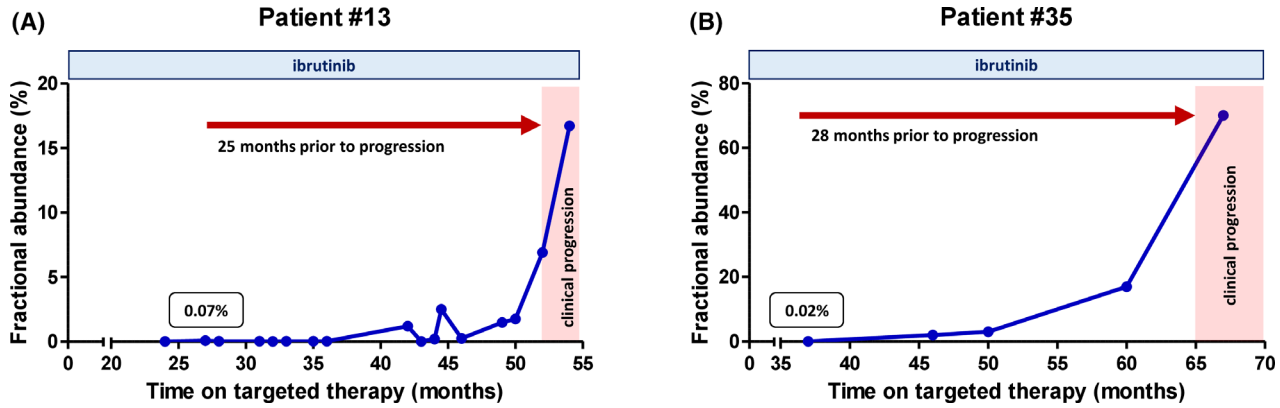


Fig 2. Temporal dynamics of the BTK^{C481S} resistance mutation in two relapsed/treatment refractory (R/R) chronic lymphocytic leukaemia (CLL) patients treated with ibrutinib. (A) In Patient #13, BTK^{C481S} was first detected after 27 months of ibrutinib therapy with a fractional abundance (*FA*) value of 0·07%. Emergence of the resistance mutation predated the first clinical signs of disease progression by 25 months. In the last sample obtained two months after the onset of disease progression, BTK^{C481S} was detected with an *FA* of 16·7%, representing a 239-fold temporal increase in the allelic burden of the mutation. (B) Patient #35 experienced disease progression after 65 months of ibrutinib therapy. The BTK^{C481S} mutation was first detected with a *FA* of 0·02% after 37 months of ibrutinib therapy, hence its emergence preceded the first clinical signs of progression by 28 months. The resistance mutation with gradually increasing allelic burden was detectable in all samples received during the study period. In the latest sample obtained two months after the first signs of clinical disease progression, BTK^{C481S} was observed with a *FA* of 70%, demonstrating a 3 500-fold increase in the allelic burden as compared to the first emergence of the resistance mutation.

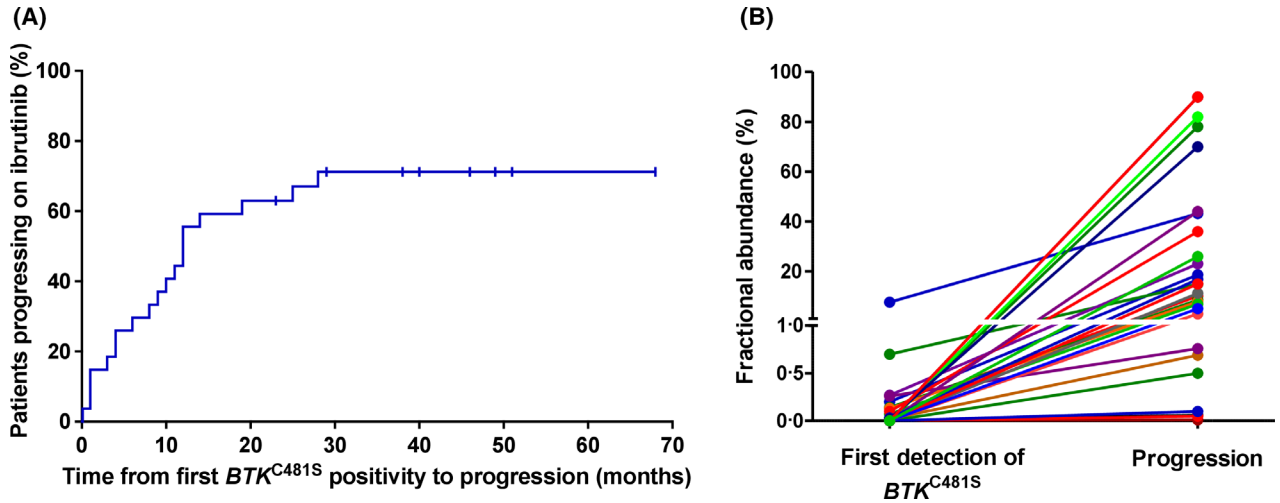


Fig 3. Disease progression and fractional abundance of the mutation in patients harbouring BTK^{C481S}. (A) Cumulative incidence of chronic lymphocytic leukaemia (CLL) progression after the first detection of BTK^{C481S}. Emergence of the resistance mutation preceded the first clinical signs of progression with a median of nine months (range: 0–28 months) in patients experiencing relapse. (B) Fractional abundance (*FA*) of BTK^{C481S} in individual patients at the first detection of the mutation *versus* at the time of disease progression. *FA* values of BTK^{C481S} showed an increasing allelic burden ubiquitously across the patients, including 11 males and eight females experiencing CLL progression. Patients with *FA* values of BTK^{C481S} higher than 50% at the time of progression were all males.

harbouring the resistance mutation but not experiencing disease progression died from reasons not related to CLL during the follow-up period, with last measured BTK^{C481S} *FA* values of 0·08% and 19·1%, respectively (Figure S2).

Secondary ibrutinib resistance in patients with wild-type BTK^{C481}

Twelve patients progressing on ibrutinib carried wild-type BTK^{C481} as assessed by ddPCR. This group, representing 27·3% of the patients experiencing relapse (12/44), showed the first clinical signs of progression after a median of 26 months (range: 13–56 months) of ibrutinib therapy. As an attempt to unveil alternative genomic alterations leading to ibrutinib resistance, targeted ultra-deep sequencing covering all coding regions of the *BTK*, *PLCG2* and *TP53* genes with a median depth of 4 133× (range: 3 390–13 856×) was performed on these patients' samples obtained within a median of 1·5 months (range: 0–7 months) of the first clinical signs of disease progression. Ultra-deep NGS uncovered a novel *PLCG2* mutation in the TIM (X-box) domain of the PLCG2 protein in Patient #75, with no further resistance

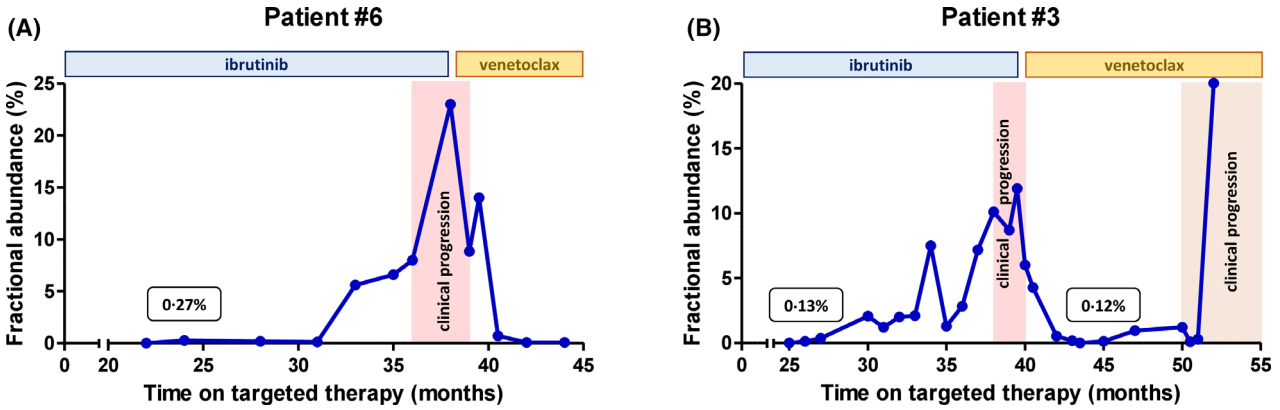


Fig 4. Temporal dynamics of the BTK^{C481S} mutation in two patients with relapsed/treatment refractory (R/R) chronic lymphocytic leukaemia (CLL) receiving ibrutinib and subsequent BCL2 inhibitor venetoclax therapies. (A) In the serial samples of Patient #6, emergence of the BTK^{C481S} was detectable after 24 months of ibrutinib therapy with a *FA* of 0·27%, predating the first clinical signs of disease progression with 12 months. In samples obtained during the ramping-up period of subsequent venetoclax treatment, a temporary increase of the BTK^{C481S} allelic burden was observed which was followed by a sharp decrease in the *FA* values of the mutation. CLL cells carrying the BTK^{C481S} were eliminated by the BCL2 inhibitor therapy and the mutation was undetectable in the sample obtained during the sixth month of venetoclax treatment. The patient was still alive at the latest follow-up timepoint and showed no clinical signs of disease progression. (B) Emergence of BTK^{C481S} in Patient #3 was detected after 26 months of ibrutinib treatment predating the first clinical signs of disease progression by 12 months. After ibrutinib failure, venetoclax therapy was administered which resulted in a sharp decline in the allelic burden of the mutation. Despite the abrupt reduction of the *FA* values, recurrence of BTK^{C481S} was observed after five months of venetoclax treatment predating a second progression by five months. The patient developed venetoclax resistance with an increasing allelic burden of BTK^{C481S} and succumbed to her disease five months after the first clinical signs of venetoclax failure.

mutations affecting the *BTK* and/or *PLCG2* genes in the analysed patients. The identified *PLCG2* p.D334G (c.1001A>G) variant is a previously unreported finding in ibrutinib-resistant R/R CLL; however, the aspartic acid to glycine change at the D334 residue was already found to be associated with Richter's transformation by Maddocks *et al.*[14] Somatic variants detected by ultra-deep NGS across the patients having wild-type BTK^{C481} and progressing on ibrutinib are listed in Table SIII.

Discussion

Ibrutinib has changed the therapeutic landscape of R/R CLL with high response rates and durable progression-free survival.[8,9,11,13,31,32] Long-term outcomes have predominantly been reported in the context of clinical trials while real-world cohort results have started to emerge only recently.[21,26,33–35] Despite the induction of an efficient disease control in the vast majority of R/R CLL patients, ibrutinib tends to lose efficacy in a subset of the cases, eventually leading to disease progression and relapse. Indeed, the number of cases acquiring secondary ibrutinib resistance is gradually accumulating over time with CLL progression emerging regularly in all patient cohorts from the second year of treatment.[15,17,36] Since patients with ibrutinib failure have poor outcome without additional/alternative therapeutic interventions, there is a high clinical interest in the early prediction of CLL progression which could potentially be achieved by screening for resistance mutations in longitudinally collected samples.

In the framework of the nationwide Hungarian Ibrutinib Resistance Analysis Initiative, we investigated the feasibility and value of screening for the most common resistance mutations in ibrutinib-treated R/R CLL patients using an ultra-sensitive ddPCR method. Our patients represent the largest real-world cohort reported to date in which BTK^{C481S} has been monitored longitudinally. The mutation was detected in nearly half of the patients with 80% of them showing disease progression during the examined period. Although, we only analysed a single aberration, this approach identified the underlying resistance mutation and provided informative genetic data for molecular monitoring in nearly 73% of the patients undergoing clinical disease progression. In patients with samples obtained prior to progression, emergence of the resistance mutation predated the first clinical signs of ibrutinib failure with a median of nine months and in 79% (15/19) of these cases, BTK^{C481S} was detectable at least three months before relapse. Consequently, in the majority (73% × 79% = 57%) of our patients with informative follow-up, presence and increasing abundance of the BTK^{C481S} mutation proved to be an indicator of the impending ibrutinib failure well before the first clinical signs of disease progression.

These findings are in line with results of previous studies performed by our group and others. For example, Woyach *et al.* investigated 46 patients experiencing CLL progression in four clinical trials and identified *BTK* and/or *PLCG2* mutations in 87% of the relapse samples. Retrospective analysis of matching serial samples available for 20 of these patients revealed that emergence of the detectable mutations preceded the time

of relapse with an estimated median of 9·3 months.[15] Ahn *et al.* analysed CLL patients harbouring *TP53* defects and receiving ibrutinib in a phase 2 clinical trial and detected BTK^{C481} and/or *PLCG2* mutations in 8/10 patients showing CLL progression. Six out of the eight patients had detectable mutations predating the clinical progression by eight months.[16] In a real-world cohort of CLL patients still being on ibrutinib after at least three years of treatment, Quinquenel *et al.* performed a 'snapshot' screening to determine the prevalence of resistance mutations and found that the presence of the *BTK* mutation was significantly associated with subsequent CLL progression.[21] Serial samples were not investigated in that study. In our previous preliminary project, *BTK* and *PLCG2* mutations were backtracked in five patients, and were detectable on average 10·5 months before the occurrence of clinical relapse.[26] These studies together provide a growing body of evidence that *BTK* and *PLCG2* mutations, with BTK^{C481S} being the predominant alteration, are drivers of ibrutinib resistance and can be important biomarker candidates for molecular disease monitoring in CLL patients receiving ibrutinib.

Nevertheless, the role of testing for mutations before and during ibrutinib treatment is still an intensively studied research area and our limited understanding of the background of resistance poses a number of significant biological and technical challenges. (*i*) Not all mutation-positive patients experience disease progression, although the proportion of patients with stable disease will probably be decreasing with longer follow-up times available in the future. (*ii*) CLL cell populations can be heterogeneous, comprising multiple subclones with different *BTK* and *PLCG2* mutations. The interaction between these subclones and their relative contribution to the clinical relapse is poorly understood.[16,37] (*iii*) At least 40% of the patients progressing on ibrutinib harbour resistance mutations in minor subclones with a cumulative VAF of <30% or even <10%,[37] and the abundance of mutations in the peripheral blood does not always correlate with disease progression. One possible aetiology of this phenomenon is a compartment effect, i.e. the site-specific (e.g. lymph node) presence/dominance of individual mutations conferring underrepresentation of the resistance-associated markers in CLL cells circulating in the peripheral blood.[15,38] Previously, we and others demonstrated that analysis of circulating cell-free DNA in blood plasma could offer a more sensitive minimally invasive screening approach in such cases.[38,39] Another plausible explanation for the low frequency of *BTK/PLCG2* mutations at the time of clinical relapse is the ability of CLL cells harbouring resistance mutations to promote resistance in non-mutated malignant cells. Chen *et al.* performed co-culture experiments with *MYD88*-mutated Waldenström macroglobulinaemia and ABC diffuse large B-cell lymphoma cell lines and observed that BTK^{C481} mutation can confer a protective effect against ibrutinib on neighbouring *BTK* wild-type cells through a paracrine mechanism.[40] (*iv*) Finally, 20% of the patients showing CLL progression on ibrutinib carry no detectable *BTK* or *PLCG2* resistance mutations.[37] Several alternative genetic aberrations and non-genetic mechanisms such as transcriptional and epigenetic changes as well as microenvironmental effects have been described as potential contributors of resistance;[8,14,18,20,41,42] however, further functional and clinical studies are needed to clarify their causative role and the extent of their contribution. Careful consideration of the circumstances mentioned above will be essential to design an efficient disease-monitoring strategy which allows for the reliable prediction of an impending relapse in clinical diagnostics.

Significance of detecting ibrutinib resistance at an early stage has been increasing with the growing number of alternative treatment options emerging on the horizon. Patients with ibrutinib failure can be treated with agents targeting alternative oncogenic pathways, such as PI3K-mTOR and BCL2. In clinical trial and real-world settings, remarkable efficacy has been achieved with venetoclax in patients progressing on ibrutinib and this BCL2 antagonist has also conferred massive reduction of CLL cells resistant to BTK inhibition in our cohort.[43,44] Ongoing clinical trials are evaluating the efficacy of venetoclax in combination with ibrutinib to overcome or prevent ibrutinib resistance.[45–47] Further promising approaches to tackle resistance to current irreversible BTK inhibition strategies include the application of SYK inhibitors, CAR-T cells, BTK degraders, as well as reversible BTK inhibitors which seem to be effective against CLL cells regardless of their C481 mutation status.[48–51] Several studies have been investigating the optimal sequence, combination and duration of the various targeted therapies that have become available recently, and the results will expectedly influence the required structure and organisation of future molecular monitoring strategies.

In summary, our study demonstrates the potential of ultrasensitive monitoring to robustly detect BTK^{C481S} during ibrutinib treatment under real-world circumstances, with the vast majority of mutation-positive patients undergoing CLL progression. Based on the presented data, screening even for this single aberration only can greatly facilitate the early detection of resistance in at least ~60% of the patients experiencing relapse. The predictive value of the test will most likely be increasing with longer follow-up times available in the future. In this regard, establishment of a standardised international monitoring framework with optimal sampling intervals, sample processing steps and assay design/selection will also be essential. Simultaneous interrogation of additional genomic loci recurrently affected during clonal evolution will certainly be unavoidable, especially with the introduction of alternative and combined treatment strategies mentioned above. NGS analysis with a focused gene panel seems to be a plausible method for the comprehensive screening of resistance mutations but affordability, short turn-around time, wide accessibility and high sensitivity fulfilled by our current approach should also be considered as requirements for subsequent disease monitoring. Technical and organisational standardisation coupled with our gradually expanding knowledge of resistance

mechanisms will hopefully further improve the clinical management of R/R CLL patients receiving novel targeted therapies.

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Author contributions

CsB and DA designed the study; GM, SF, PF, AB, TM, JD, JW, HA, BK, ZK, RSz, LG, TGP, AS, BK, ME, MP, LR, LSz, PI, PT, PP, DL TS and ZM provided patient samples and annotations; LK, FT, GB, RK and LLH performed experiments; CsB, LK, TL, FT, GB, RK, AS, PCs, AM and DA performed data analysis; CsB, LK and DA wrote the paper. All authors have read and critically reviewed the final version of the manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Time-resolved BTK^{C481S} monitoring and clinical outcome in ibrutinib-treated CLL patients. Mantel–Byar estimate of progression-free survival from the first detection of BTK^{C481S}. Patients experiencing disease progression with samples obtained prior to relapse and cases with no clinical signs of progression were included in the analysis.

Fig S2. Treatment timeline of eight patients harbouring the BTK^{C481S} resistance mutation with no clinical evidence of disease progression during the study period. In this cohort, representing 20% (8/40) of the cases with BTK^{C481S}, emergence of the resistance mutation was detected after a median of 28 months (range: 19–44 months) of ibrutinib therapy as denoted by blue wedges. Blue *FA* values represent the allelic burden of BTK^{C481S} at the first detection of the resistance mutation while black *FA* values denote the allelic burden at the time of the last patient follow-up.

Table SI. Patient characteristics.

Table SII. Fractional abundance (*FA*) of the BTK^{C481S} mutation assessed by droplet digital polymerase chain reaction (ddPCR) in ibrutinib-treated relapsed/treatment refractory (R/R)) chronic lymphocytic leukaemia (CLL) patients.

Table SIII. Variants detected by ultra-deep next-generation sequencing of the *BTK*, *PLCG2* and *TP53* genes in the samples of patients progressing on ibrutinib but not harbouring the BTK^{C481S} resistance mutation.

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