Circulating versus cellular tumor DNA for the detection of BTK resistant CLL clones

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ABSTRACT

Resistance mutations can be detected in 75% of CLL patients progressing under BTK inhibitor therapy. Using semiquantitative wild-type-blocking (WTB) RT-PCR for BTK and Sanger sequencing for PLCG2 mutations, we compared detection sensitivity of cellular versus circulating tumor DNA (ctDNA) in 20 sample pairs of 13 consecutive patients.

With an assay sensitivity of 0.06%, 7 patients had a BTK-C481S and one a PLCG2-G667E mutation. Cellular DNA was positive in 10 but ctDNA only in 6 samples, giving false-negative results in samples with low mutational burden.

In summary, WTB-PCR is cost-effective and routinely applicable but misses low frequency mutations when using ctDNA.

Abbreviations
alloSCT allogeneic stem cell transplantation
BTKi Bruton tyrosine kinase inhibitor
cfDNA cell-free DNA
tctDNA circulating tumor DNA
PB peripheral blood
PLCG2 Phospholipase C Gamma 2
RT Richter transformation
WTB wild-type blocking

1. Introduction

BTK inhibitors (BTKi) like ibrutinib and acalabrutinib have been approved for front- and further-line therapy in CLL and treatment is usually indefinite until progression or unacceptable toxicities occur [1–3]. Up to 25% of patients progressing under BTKi therapy develop a Richter transformation (RT) [4], mostly in the first 15 months after treatment initiation, while at least 75% show CLL progression, often between the second and fourth year of ibrutinib treatment [5]. In the latter cohort, BTK and/or PLCG2 mutations can be detected in about 75% of patients but in only 40% of patients with RT. The BTK C481X mutation leads to a reduced binding affinity of the BTK inhibitors while the PLCG2 mutation activates the pathway downstream of BTK [5]. However, in almost half of clinically progressing patients only a low mutant allele fraction (MAF) below 10% of BTK and/or PLCG2 mutations can be found in peripheral blood (PB) while proliferating mutant cells remain in the lymph nodes. Vice versa, in the presence of CLL proliferation in PB, the MAF is usually higher [6,7].

Recently, detection of circulating tumor DNA (ctDNA) has also been evaluated for hematological malignancies either as initial biomarker, response monitoring or for early detection of relapse [8–11].

We were therefore interested if detection of BTKi resistance mutations by routinely applicable RT-PCR in patients progressing under BTKi or during post-progression therapy achieves a higher sensitivity using ctDNA from PB samples than cellular DNA.
2. Materials and methods

2.1. Patients and study design

From July 2016 until July 2021, PB samples were taken simultaneously in EDTA (9ml) and cfDNA tubes (10ml PAXgene Blood, PreAnalytiX, Suisse) from 13 consecutive CLL patients at the time of progression under BTKi. Patients were eligible if iwCLL criteria for progression were met [12]. From 4 of the 8 patients with proven BTK or PLCG2 mutation, further simultaneous EDTA and cfDNA samples were taken at different time points during subsequent therapy.

Follow-up data were obtained until December 2021 during routine visits. The study was approved by the institutional ethics committee of Hannover Medical School, Hannover, Germany and informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

2.2. Quantitative wild-type blocking RT-PCR

Cellular DNA was isolated from whole blood using a Whole Blood Kit (Promega, Germany) while cfDNA was isolated using a QIAamp Circulating Nucleic Acid Kit including mini silica columns and a vacuum pump, according to the manufacturer (Qiagen, Germany).

Quantification of cfDNA was performed by fluorometry using the Qubit® dsDNA HS Assay Kit (ThermoFischer Scientific, Germany) and plates were read in a Quantus fluorometer (Promega, Germany).

DNA copy numbers were determined by using a modification of a published RT-PCR protocol [13] with the aid of a fluorescence labelled detection probe for the BTK mutation in Exon 14 containing locked nucleic acids as well as sequence specific primers (TIB MOLBIOL, Germany) (Table 1). Copy number quantification was enabled by short (105bp) control fragments for mutated and wildtype DNA of which a known amount (10.000 fragments/5µl) was included in the RT-PCR reaction. Semiquantitative results are therefore given as a ratio of mutated to wildtype fragments (MUT/WT ratio).

In detail, quantification of one sample required analysis of 12 PCR preparations: each contained a master-mix of 10µl reverse primer “D”, 2.0µl FastStart Mastermix (Roche Diagnostics, Germany), 20µl MgCl, 10.7µl sterile water and 50µM detection probe “Y”. 15µl of this mastermix was given into 12 wells of a 96-well plate and 5µl of each of the 3 forward primers “A”, “B” or “C” (10µM) were added to 4 wells, respectively. Finally, each of these wells was filled with 5µl of WT control fragment “1”, BTK mutation fragment “2” (as positive control), sterile water (negative control) and cellular or cell-free DNA.

All PCR reactions were performed in a LightCycler 480 II (Roche Diagnostics, Germany) at following settings: initial denaturation for 10min at 95°C; 40 cycles with denaturation for 10 s at 95°C and primer and detection probe annealing for 30 s at 62°C. Analysis of FAM fluorescence signal was performed by LightCycler Software (Roche).

2.3. BTK and PLCG2 sequencing

CLL samples without the BTK-C481S mutation (Exon 14) were screened for BTK mutations in Exon 11, 15 and 16 as well as PLCG2 mutations in Exon 19, 20 and 24 by Sanger sequencing.

In detail, 10µM forward and reverse primer were mixed with 4µl sterile water and 10µl HotStartTag Mastermix (Qiagen, Germany) as well as 10ng sample DNA to a final volume of 20µl. Sterile water served as negative control. PCR was run in a Biometra T3000 Thermocycler (Analytik Jena, Germany) using the following settings: initial denaturation for 5min at 95°C; 40 cycles with denaturation for 30 s at 95°C, primer annealing for 30 s at 56°C and extension for 60 s at 72°C; final extension for 10min at 72°C. PCR products were cleaned using a ExoSAP-IT PCR Product Cleanup Reagent (ThermoFischer Scientific, Germany) and a capillary electrophoresis was run on an Agilent 2100 (Agilent Technologies, Germany). PCR products were then prepared for sequencing by PCR (25 cycles: 10 s at 96°C, 5 s at 56°C, 2 min at 60°C) including 5µM of primers and 1.5µl BigDye terminator (ThermoFischer Scientific, Germany) and purification using AMPure XP Beads (Beckman Coulter, Germany) and ethanol precipitation according to manufacturer.

Sequencing was carried out on a Biosystems 3130 Genetic Analyzer (ThermoFischer Scientific, Germany), edited using “Sequencing Analysis 5.2” software (Applied Biosystems, USA) and finally analyzed using “Seqman” software (DNASTAR, USA).

2.4. Sensitivity, stability and standard curve quantification for RT-PCR

For determination of detection limit and establishment of standard curve for quantification, dilution series experiments were repeatedly performed by either diluting BTK mutation control fragments (“dilution series A”: 10.000/1.000/100/10 fragments) in 1ng wildtype DNA (controls: 1ng wildtype DNA and sterile water) or by taking 10 BTK mutation control fragments in diluted amounts of wildtype DNA (“dilution series B”: 100/30/10/1ng; controls: 10ng wildtype DNA, 100 fragments BTK mutation control, sterile water), resembling a small BTK mutated clone in a patient with high CLL counts in PB.

Table 1

| Primer/Control Fragments | Mutated DNA Concentration (ul) | Wildtype DNA Concentration (ul) |
|--------------------------|-------------------------------|-------------------------------|
| BTK Exon 11 Forward Primer | 10ng | 10ng |
| BTK Exon 11 Reverse Primer | 10ng | 10ng |
| BTK Exon 15 Forward Primer | 10ng | 10ng |
| BTK Exon 15 Reverse Primer | 10ng | 10ng |
| BTK Exon 16 Forward Primer | 10ng | 10ng |
| BTK Exon 16 Reverse Primer | 10ng | 10ng |
| PLC/2 Exon 19 Forward Primer | 10ng | 10ng |
| PLC/2 Exon 19 Reverse Primer | 10ng | 10ng |
| PLC/2 Exon 20 Forward Primer | 10ng | 10ng |
| PLC/2 Exon 20 Reverse Primer | 10ng | 10ng |
2.5 Statistics

Statistical analysis was carried out using SPSS statistics 28.0 software.

Differences in frequency of detected BTK mutations were compared between cellular and ctDNA using Chi-Square test. Results of MUT/WT ratios and ctDNA concentrations were compared using Mann-Whitney-U test. Correlation of PB lymphocytes and MUT/WT ratio or ctDNA was analyzed using Spearman rank correlation.

Overall survival was assessed from initiation of subsequent therapy. Possible association with patient or treatment parameters as well as BTK mutation were analyzed using Cox regression and median survival times by Kaplan-Meier analysis.

3. Results

3.1 Patient characteristics

13 consecutive patients (3 female, 10 male) with clinical progression under Ibrutinib (n=12) or acalabrutinib (n=1) were screened for BTK and PLCG2 mutations. Median age was 75.0 years (range: 54 – 82 years) and patients had received a median of 2 therapies (0–4) before starting BTKi with 3 patients being treatment-naive. Median time until progression under BTKi was 23.0 months (2 – 40 months). Median time between analysis and subsequent therapy was 7.0 days (4.5 – 35 days) except for patient #6 who remained on Ibrutinib for 172 days despite progression. Other subsequent therapies included venetoclax (n=10) as well as R-CHOP (n=1) or R-miniCHOP (n=1) for the 2 patients with RT. Median number of lymphocytes in PB at time of first analysis was 7470/µl (350-228.000/µl). Median follow-up was 7.1 months (0.1 – 53.2 months). Eight patients progressed under the subsequent therapy, including 3 RTs, and 11 patients died during follow-up, including 3 due to progression, 3 due to RT, 2 due to infection and 3 due to other causes (Table 2).

3.2 BTK and PLCG2 mutations

In 8 out of the 13 patients (61.5%) we were able to detect a mutation causing BTKi resistance: 7 (53.8%) showed a BTK-C481S mutation (including two patients with RT) and one (7.7%) a PLCG2-G667E mutation, which has not been described so far and belongs to the SH2 domain.

3.3 Sensitivity and stability of BTK assay

We were able to repeatedly detect down to 10 BTK fragments in up to 100ng wildtype DNA. This resembles a sensitivity of approximately 0.06% (10 BTK fragments = 10 cells; 100ng = 16.600 cells with 6pg DNA/lymphocyte [14]).

3.4 Comparison of cellular and ctDNA

We were able to compare results from 20 sample pairs of cellular DNA and ctDNA simultaneously taken from PB at first analysis or during subsequent therapy. As patient #7 developed secondary AML, we could also investigate bone marrow (BM) blood.

Altogether 10 of the 20 samples pairs showed a positive result (Table 2). While cellular DNA was positive in all 10 samples, ctDNA gave positive results in only 6 samples which was significantly lower (p=0.025). The 4 “false-negative” ctDNA samples were either obtained after response to subsequent therapy (#1 and #4) or from a slowly progressing patient (#7), all of which showed low MUT/WT ratios in cellular DNA (0.0004 – 0.079). While there was a significant correlation of PB lymphocytes to MUT/WT ratio (r=.611; p=.02), there was no correlation of ctDNA concentration to PB lymphocytes. Furthermore, ctDNA concentration did not differ between BTK positive and negative samples with median ctDNA concentrations of 1.85 and 1.90ng/µl, respectively. Only patient 4, who progressed with RT and was BTK mutated, showed a significantly higher ctDNA concentration (88ng/µl).

For those 4 patients who had further sample pairs taken during subsequent therapy, MUT/WT ratios rapidly decreased: the mean ratio for first analysis of cellular and ctDNA was 7.3 and the mean ratio for the second analysis with a median of 83 days after start of subsequent therapy was 0.002 (including ratios of both cellular and ctDNA).

One BM sample showed a positive result (MUT/WT ratio: 0.47) while cellular and ctDNA from PB were negative.

3.5 Outcome

In univariate Cox regression, presence of a BTK mutation (p = .038; HR = 0.10, 95%-CI: 0.01-0.88) was associated with longer overall survival (OS). Accordingly, Kaplan-Meier analyses gave a median OS of 15.3 months for patients with BTK mutation vs. 2.5 months for patients without evidence of mutation (p = .01).

4. Discussion

In patients progressing under BTKi, BTK C481X and/or PLCG2 mutations can be found in around 80% of cases (with 70% BTK and 10% PLCG2 mutations) [7,15-17] when using highly sensitive methods (NGS, ddPCR). We used wildtype blocking RT-PCR (WTB-PCR), which has also demonstrated high sensitivity [18] and, in fact, the overall mutation detection rate of 62% (54% for BTK C418S and 8% for PLCG2-G667E mutations) in our series of consecutive patients was in a comparable range.

Mutational burden can differ between tissue sites and it has been demonstrated that in almost half of clinically progressing patients resistant CLL cells persist in secondary compartments (e.g. lymph nodes, BM, spleen) while mutational burden in PB remains low [6,7]. In this situation, ctDNA might be shed by CLL cells from these compartments into PB and might allow better detection of mutant clones than cellular DNA.

However, our detection rate for ctDNA was 40% lower compared to cellular DNA and the “false-negative” samples were taken at time points with low mutational burden in cellular DNA. This lack of sensitivity might be related either to the amount of ctDNA or ctDNA. As ctDNA concentration was rather high and did not differ between BTK positive and negative samples, it is unlikely that ctDNA amounts were a limiting factor. In contrary, as detailed by Roschewski et al. [8], ctDNA constitutes only about 5% of ctDNA in lymphoma patients and its concentration correlates to tumor burden. Correspondingly, lymphocyte counts of our cohort correlated to the mutational burden (MUT/WT ratio) which was high at the initial time of progression but rapidly decreased during subsequent therapy. Thus, it appears likely that the amount of mutated ctDNA was below the sensitivity of our assay.

These results might appear contrary to the ones of Albitar et al. [18]: they investigated 9 paired CLL samples of cellular and ctDNA and found 7 vs. 7 BTK and 4 vs. 2 PLCG2 mutations, respectively. However, they did not provide data regarding the corresponding lymphocyte counts and mutational burden in these samples and it is possible that these samples were taken at time of progression with rather high ctDNA. Regarding PLCG2, different mutations can sometimes be found in multiple subclones with low allelic burden within the same patient [5] and, therefore, it is more likely for mutated PLCG2 clones to be present only in a second compartment but not in PB. Consequently, ctDNA might in fact be more sensitive for the detection of PLCG2 mutations than cellular DNA but these subclones are mostly clinically irrelevant [17] as pointed out below.

We identified only one patient with a PLCG2 mutation (by Sanger sequencing), which has not been described so far and belongs to the classical SH2 domain. Mutations within the SH2 domain inactivate its inhibitory function, leading to hyperfunctional PLCG2 and BTK-
Table 2
Patient data, resistance mutations and outcome.

| Age | Sex | Diagnosis at Progression under BTKi | Previous therapies (pre-Ibr) | BTKi therapy until progression (in months) | Number of lymphocytes at first analysis (G/l) | Subsequent therapy | Timepoint (days from therapy start to analysis) | Sample type | BTK mutation (yes/no=1/0) | Ratio MUT/WT | Progress (yes/no=1/0) | PFS (months) | Death (yes/no=1/0) | OS (months) |
|-----|-----|-------------------------------------|-------------------------------|------------------------------------------|---------------------------------------------|-------------------|------------------------------------------|-------------|---------------------------|-------------|-----------------------|-------------|------------------------|-------------|
| 75  | m   | B-CLL | 4 | 29 | 1330 | Venetoclax | -7 | EDTA blood | 1 | 1.97 | 0 | 53.2 | 1 | 53.2 |
| 71  | m   | B-CLL | 3 | 23 | 350 | Venetoclax | 35 | EDTA blood | 1 | 0.065 | 1 | 3.4 | 1 | 3.9 |
| 68  | f   | B-CLL | 1 | 23 | 7470 | Venetoclax | 0 | EDTA blood | 1 | 2.12 | (RT) | 4.0 | 1 | 4.2 |
| 75  | m   | RT from B-CLL | 3 | 31 | 70880 | R-CHOP → Venetoclax | 3 | EDTA blood | 1 | 16.52 | 1 | 13.4 | 1 | 15.3 |
| 74  | f   | B-CLL | 2 | 19 | 1550 | Venetoclax | -45 | EDTA blood | 0 | 1 | 1.9 | 1 | 2.5 |
| 64  | m   | B-PLL | 0 | 16 | 590 | Ibrutinib → Venetoclax → R-idelalisib → alloSCT | -172 | EDTA blood | 1 | 10705 | 1 | 20.2 | 1 | 25.3 |
| 70  | m   | B-CLL | 2 | 33 | 3860 | Venetoclax | -26 | EDTA blood | 0 | 0 | 0 | 450 | 0.47 |
| 78  | f   | B-CLL | 0 | 40 | 8060 | Venetoclax | -21 | EDTA blood | 1 | 0.09 | 1 | 7.1 | 1 | 7.1 |
| 54  | m   | B-CLL | 2 | 36 | 93700 | Venetoclax | 1 | EDTA blood | 1 | 0.02 | 1 | 10705 | 1 | 13.2 | 1 | 16.2 |

(continued on next page)
independent activation of B cell receptor signaling pathway [17,19]. However, it appears that the PLCG2 mutation was rather a bystander effect of other resistance mechanisms [17] as the clinical course of the patient notably showed a slow progression allowing continuation of ibrutinib, but during further treatments the patient did not respond to venetoclax and idelalisib, went on to allogeneic stem cell transplantation and finally died due to alloSCT complications 7 months thereafter.

There are only scarce data regarding the course of BTK mutation burden under subsequent therapies [20,21]. Similar to 5 patients described by Kanagal et al. [20], we observed a rapid decline of BTK resistant clones under both R-CHOP for RT and venetoclax for CLL progression. However, Lucas et al. [21] identified 5 of 8 patients with CLL progression after ibrutinib and venetoclax having a nearly identical variant allele frequency (VAF) of their C418S mutation pre- and post-venetoclax. Furthermore, they detected several novel BTK and PLCG2 mutations that increased throughout venetoclax treatment, demonstrating that the disappearance of ibrutinib resistant clones under venetoclax is mostly transient and that these clones often recur when patients become refractory to venetoclax.

The small patient cohort is a possible limitation of our study. However, Woyach et al. [7] demonstrated that progression due to resistant clones occurs rather late during ibrutinib therapy while RT as well as infection and other toxicities usually occur early. Accordingly, detection of a BTK mutation was associated with longer overall survival even within our cohort, arguing against any selection bias.

Another limitation might be the WTB-PCR itself as it is a single mutation method with a maximum sensitivity of 1:2000 per ml plasma while multiple mutation assays like CAPP-seq NGS or PhasED-seq can reach a sensitivity up to 2.5:100.000 [8,9,22]. However, these NGS methods are costly and not yet available in daily routine.

In conclusion, WTB-PCR is a sensitive, cost-effective and routinely applicable method for the detection of BTK resistant CLL clones under BTK inhibitor therapy but misses low frequency mutations when using ctDNA compared to cellular DNA. For now, ctDNA should only be applied for research use in combination with recently presented deep sequencing NGS methods.

Authorship

AT was the principal investigator, had the initial idea, designed the research and participated in the data analysis and writing of the paper. WS, HH and JC designed the RT-PCR and performed laboratory work as well as laboratory data analysis. JK participated in clinical data collection and analysis and writing of the paper.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Arne Trummer reports a relationship with Janssen-Cilag GmbH that includes: consulting or advisory and speaking and lecture fees. Arne Trummer reports a relationship with AbbVie Inc that includes: consulting or advisory, speaking and lecture fees, and travel reimbursement.

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