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Ultra-deep sequencing leads to earlier and more sensitive detection of the tyrosine kinase inhibitor resistance mutation T315I in chronic myeloid leukemia

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ABSTRACT

Chronic myeloid leukemia cells acquire resistance to tyrosine kinase inhibitors through mutations in the *ABL1* kinase domain. The T315I mutation mediates resistance to imatinib, dasatinib, nilotinib and bosutinib, whereas sensitivity to ponatinib remains. Mutation detection by conventional Sanger sequencing requires 10%-20% expansion of the mutated subclone. We studied the T315I mutation development by ultra-deep sequencing on the 454 XL+ platform (Roche) in comparison to Sanger sequencing. By ultra-deep sequencing, mutations were detected at loads of 1%-2%. We selected 40 patients who had failed first-line to third-line treatment (imatinib, dasatinib, nilotinib) and had high loads of the T315I mutation detected by Sanger sequencing. We confirmed T315I mutations by ultra-deep sequencing and investigated the mutation dynamics by backtracking earlier samples. In 20 of 40 patients, we identified the T315I three months (median) before Sanger sequencing detection limits were reached. To exclude sporadic low percentage mutation development without subsequent mutation outgrowth, we selected 42 patients without resistance mutations detected by Sanger sequencing but loss of major molecular response. Here, no mutation was detected by ultra-deep sequencing. Additional non-T315I resistance mutations were found in 20 of 40 patients. Only 15% had two mutations per cell; the other cases showed multiple independently mutated clones and the T315I clone demonstrated a rapid outgrowth. In conclusion, T315I mutations could be detected earlier by ultra-deep sequencing compared to Sanger sequencing in a selected group of cases. Earlier mutation detection by ultra-deep sequencing might allow treatment to be changed before clonal increase of cells with the T315I mutation.

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Introduction

The use of *BCR-ABL1* tyrosine kinase inhibitors (TKI) has revolutionized the treatment of chronic myeloid leukemia (CML) by achieving long remission periods.[1] However, lack of efficacy, progression or adverse events can require treatment discontinuation. For imatinib, the discontinuation rate for first-line treatment was 34% within the first six years.[2] Therapy with the 2nd-generation inhibitors dasatinib or nilotinib for newly diagnosed CML patients had lower discontinuation rates, but still some patients progressed or failed therapy.[3-5]

Mutations in the *ABL1* tyrosine kinase domain (TKD) are the best studied mechanism of acquired TKI resistance.[6-8] Two-thirds of patients who fail imatinib treatment have acquired at least one mutation in the TKD, and at least one-third of resistant patients on dasatinib or nilotinib have developed mutations.[9] Over 100 point mutations leading to amino acid exchanges were described in different TKI resistant CML patients.[10,11] Well characterized resistance profiles of each TKI have been described and this allows the TKIs to be changed and offer the possibility of overcoming resistance.[8] Only the threonine-to-isoleucine exchange at amino acid position 315 (T315I) caused by the single base substitution act>att results in resist-

ance to most TKIs, and sensitivity remains only to ponatinib.[12] Although the mutation itself does not increase the kinase activity,[13] the resistance to imatinib, dasatinib, nilotinib and bosutinib causes patients with T315I to show a rapid increase in malignant cell burden and to progress to blast crisis.[14]

An early detection of the T315I mutation may be advantageous and allow treatment intervention before disease progression. Low-level mutations (≤1%) are selected on treatment in patients receiving a TKI for which the mutation causes resistance, and these lead to lower response rates.[15] However, the sensitivity of conventional Sanger sequencing to detect mutations is generally 10%-20%. Consequently, reliable detection of T315I mutated CMLs by Sanger sequencing requires strong expansion of the mutated clone or the entire CML cell mass to bear the mutation. In contrast, ultra-deep sequencing (UDS) can overcome the sensitivity limits of conventional sequencing studies.[16,17] In comparison to highly sensitive but mutation-specific PCRs (e.g. digital PCR assays[18] or allele specific PCRs), UDS assays can be designed to identify all mutations in the TKD. This is essential in order to discover novel mutations and to diagnose patients who have gained more than one resistance mutation. The evolution of two or more mutations in the same clone (compound mutations) can occur rapidly under treatment with non-sensitive inhibitors. It changes the TKI resistance profile within months[16] and results in an extremely resistant CML.[19] Therefore, these cases need to be distinguished from those with multiple individual clones carrying one resistance mutation each. In previous studies, individual PCR products were cloned before Sanger sequencing to determine the clonal architecture.[20] These elaborate cloning steps can be avoided with UDS because individual sequencing reads are the equivalent to cloned PCR products. CML cells with the T315I in combination with other key position mutations (e.g. G250E, Y253H or E255V) are resistant *in vitro* to currently available TKIs, including ponatinib.[19] For those patients, therapy options without TKIs, such as allogeneic stem cell transplantation (alloSCT), urgently need to be considered.

Here we used UDS in comparison to conventional Sanger sequencing[8] to study the appearance and evolution of the T315I. By backtracking CML patients with a T315I mutation level, which was already detectable by Sanger sequencing, we describe the early dynamics and the progression of the mutational landscape.

Methods

CML patient cohort

Samples were received at our laboratory between June 2006 and October 2014 for routine diagnostic assessment. Patients gave written informed consent in accordance with the Declaration of Helsinki. The study was approved by the Munich Leukemia Laboratory Institutional Review Board. Cohort 1 included 40 CML patients (2-5 time points *per* patient) with a known T315I mutation detected by Sanger sequencing. At the end of the monitoring period, patients had a median age of 61 years (19-85 years) and had received treatment by 1-3 TKIs and alloSCT. We included patients and time points from chronic phase to blast crisis *(Online Supplementary Table S1)*. Cohort 2 consisted of 42 patients who had achieved but subsequently lost major molecular response (MMR). In these patients, no resistance mutations were identified by Sanger sequencing for routine diagnostic purposes. The samples sequenced in this part of the study were selected after MMR loss. Patients had a median age of 58 years (26-79 years). Cytogenetic aberrations in addition to the Philadelphia chromosome are given in *Online Supplementary Table S2*.

RNA isolation, cDNA synthesis

RNA was isolated after red blood cell lysis from peripheral blood or mononucleated bone marrow cells. For isolation and cDNA synthesis, routine protocols were used.[21]

BCR-ABL1 fusion transcript quantification

The *BCR-ABL1/ABL1* ratio was determined according to previously published protocols[22,23] and normalized to %IS (international scale) by the conversion factor 0.989.[24]

BCR-ABL1 TKD sequencing

Sanger sequencing was performed as previously described.[25] For UDS, PCRs were performed with FastStart High Fidelity PCR System (Roche Applied Science, Penzberg, Germany). *BCR-ABL1* fusion transcript was amplified (first PCR), sequencing amplicons of the TKD were generated (second PCR) with Assay-on-Demand (AoD) oligonucleotide primer plates including multiplex identifiers (MIDs) 1-26. AoD plates were part of the IRON-III study (Roche Diagnostics, Penzberg, Germany). Primer sequences and PCR conditions are given in the *Online Supplementary Tables S3 and S4*. PCR products were tested on the Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Products were purified by Agencourt AMPureXP beads (Beckman Coulter, Krefeld, Germany) and pooled (1st/2nd amplicon ratio: 4/5). Pools were quantified using Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA).

Sequencing with the XL+ kit for extended read length on GS Junior (Roche/454, Branford, CT, USA) was performed according to the manufacturer's instructions.

Cytogenetic and molecular genetic analyses

Cytomorphology and chromosome banding analysis combined with interphase fluorescence *in situ* hybridization were performed as previously described.[26]

Data analysis

Sequencing raw data was processed with the long amplicon pipeline 1 and analyzed by GS Amplicon Variant Analyzer 3.0 (Roche/454) and Sequencing Pilot Module SeqNext 4.1.1 (JSI Medical Systems, Kippenheim, Germany). Sanger sequencing was analyzed by Mutation Surveyor 4.0 (SoftGenetics, State College, PA, USA) or Sequencing Pilot Module SeqPatient 4.1.2 (JSI). We calculated the percentage of mutated *BCR-ABL1* transcripts relative to all *BCR-ABL1* transcripts.[16,17] To distinguish compound from multiclonal mutations, individual reads were investigated for the presence of more than one mutation using the GS Amplicon Variant Analyzer 3.0. In cases with ambiguous read distribution, the divergent progression of mutations during disease course was used to identify multiclonal disease development.

Results

Sensitivity of ultra-deep XL+ sequencing

We sequenced the TKD of *ABL1* with high sensitivity on the 454 XL+ platform. Extended read length of the XL+ assay allowed covering the TKD (exons 4-10) by two sequencing amplicons (555 bp, 575 bp). To sequence only *ABL1* from the *BCR-ABL1* fusion gene, we partially amplified the fusion gene from cDNA (first PCR) serving as tem-

plate for the sequencing amplicons in a second (nested) PCR (Figure 1). The percentage of mutated *BCR-ABL1* relative to all *BCR-ABL1* transcripts (in %) was calculated. Equal distribution of forward and reverse reads and a median coverage of 2257X (range 1012-4558X) *per* base were reached.

Dilution tests were performed to prove quantitative and sensitive mutation detection. cDNAs from 2 samples with high and well defined mutation loads (identified by 454 XL+ sequencing) were mixed in different ratios to simulate specific or low percentage mutation loads (*Online Supplementary Table S5*). We obtained a high correlation of the calculated mutation load and the sequenced result for each single nucleotide variant including 1% and 2% mutation loads (Figure 2A; correlation coefficient calculated by linear regression: R^2=0.998). Next, we performed independent technical replicates (n=5) of patient samples with mutations at levels below the Sanger sequencing detection limit. Replicates were performed from one cDNA with independent PCR amplifications and independent sequencing reactions. Different MIDs were used. Mutated reads were detectable for each mutation and in each individual test (Figure 2B), confirming the sensitivity of 1%-2%, which is required for early mutation detection.

T315I mutation detection by Sanger sequencing and UDS

We selected 40 CML patients (cohort 1), who had received treatment with at least one TKI (Table 1), and in whom we had previously identified the T315I mutation by conventional Sanger sequencing for routine diagnostic purposes: 36 of 40 had a *BCR-ABL1/ABL1* (%IS) ≥1 (median 46.139; range 1.572-89.727) and prior TKI treatment of six months or longer (*Online Supplementary Table S1*). Two patients were in the ELN warning categories[1] [*BCR-ABL1/ABL1* (%IS) of 40.200 and treatment with imatinib for four months; *BCR-ABL1/ABL1* (%IS) of 0.812 after 19 months of second-line dasatinib treatment] and 2 patients were sequenced after only two months of TKI treatment due to the cytomorphological detection of a blast crises and *BCR-ABL1/ABL1* (%IS) of more than 10.

Early T315I mutation detection

First, we performed UDS in comparison to Sanger sequencing and obtained a high degree of correlation of the mutation loads (R^2=0.948) (Figure 2C). Next, we sequenced earlier samples of all 40 patients with sensitive UDS. Resistance mutations with less than 5% mutation load were confirmed in an independent sequencing run if they occurred only once *per* patient during disease course. Sequencing analysis of samples with *BCR-ABL1/ABL1* (%IS) 1 or less than 1 were confirmed in independent runs. If possible, we continued the backtracking until we reached a sample without any detectable T315I mutation with 454 XL+ sensitivity (n=37). For 3 patients, we completed the backtracking until we reached a sample with a T315I mutation load below the Sanger sequencing detection limit. We used cDNA from routine *BCR-ABL1/ABL1* ratio analysis with a median interval between the sampling time points of three months. For patients who carried a T315I above the Sanger sequencing limit (15%) in more than one sample, we concluded that also by conventional Sanger sequencing the mutation would have been detected but was not because no Sanger sequencing was performed. For those samples, the earliest time point with a mutation load of more than 15% was defined as the end point of our monitoring interval (*Online Supplementary Figure S1*). The resulting monitoring intervals contained 2-5 samples *per* patient and covered a median period of five months (2-27 months) (Table 1 and *Online Supplementary Table S1*). At the beginning of our monitoring interval, patients had a median *BCR-ABL1/ABL1* (%IS) of 29.739 (interval 0.046-74.007) and 61% (14 of 23) of patients were already in blast phase. During our monitoring interval, 13% (3 of 23) progressed to blast phase in parallel with the T315I mutation outgrowth. An increase in *BCR-ABL1/ABL1* ratio in parallel with the T315I increase was observed for 15% of patients (6 of 39). Only 26% (6 of 23) stayed in complete hematologic response or chronic phase during the entire monitoring interval (*Online Supplementary Table S1*).

In 20 of 40 patients, backtracking by UDS uncovered an early sample with a T315I mutation level below 15% (Patients #1 to #20). In the other 20 of 40 patients (Patients #21 to #40), no low percentage T315I mutation was detected by backtracking. In these patients, the earliest measureable T315I load was 27% or more (range 27%-100%, median 60%), and was, therefore, detectable by both Sanger sequencing and UDS (Figure 3A). Differences between the samples with and without a low level T315I cannot be attributed to different sampling intervals (medi-

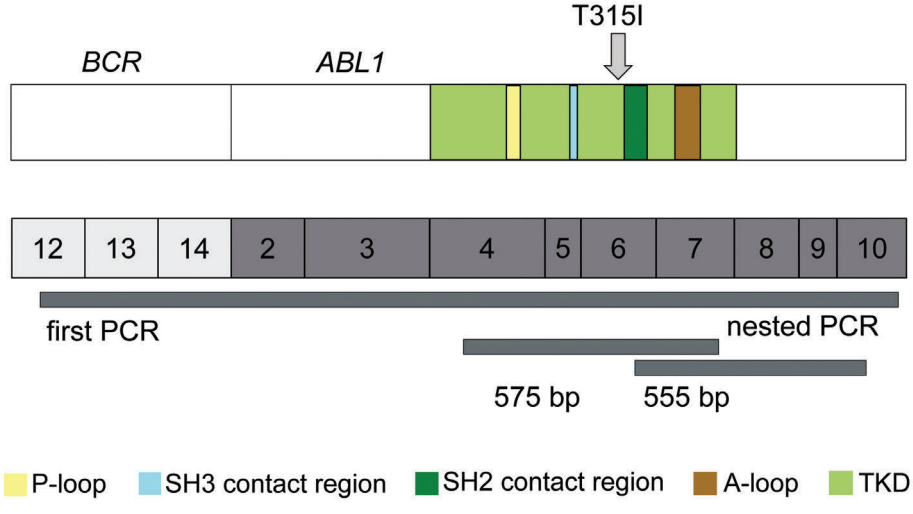

Figure 1. Amplicon design. The fusion transcript was amplified from cDNA (first PCR). The first PCR served as template for amplification of two amplicons for 454 XL+ sequencing (nested PCR). The position of the T315I is marked by an arrow. Key motive structures of the *ABL1* tyrosine kinase domain (TKD, light green) are shown: P-loop (phosphate binding loop, yellow), SH2 contact region (dark green), SH3 contact region (blue), A-loop (activating loop, brown). The b3a2 (e14a2) fusion transcript with the exons of the *BCR* gene in light and the exons of the *ABL1* gene is shown in dark gray.

an interval 3 months for both subsets; range 1-13 months for Patients #1 to #20 *vs.* 1-14 months for Patients #21 to #40). In addition, there was no difference in the time from initial CML diagnosis to T315I onset between the patient subsets with and without a low percentage T315I mutation. The T315I mutation occurred in 21 of 40 patients before or within the first year of disease (11 patients with and 10 patients without detectable low level T315I) (*Online Supplementary Table S1*). T315I could develop up to 14 years after CML was diagnosed. Patients with late T315I development were mainly on second- or third-line TKI therapy (*Online Supplementary Table S1*). For 21 patients, molecular and/or cytogenetic response data was available from initial diagnosis to T315I development. Only 2 patients (#15, #37) had achieved major treatment milestones before mutation development (CCyR/MMR) and lost the response status in parallel with T315I mutation development (*Online Supplementary Table S1*). The other 19 patients did not reach molecular or complete cytogenetic response before T315I mutation development [median period between initial diagnosis and T315I detection: 7 months (interval 2-60 months)]. Importantly, 5 of 19 patients were under TKI treatment for 12 months or longer, a time point at which MMR achievement would be required to define optimal response according to ELN guidelines.[1]

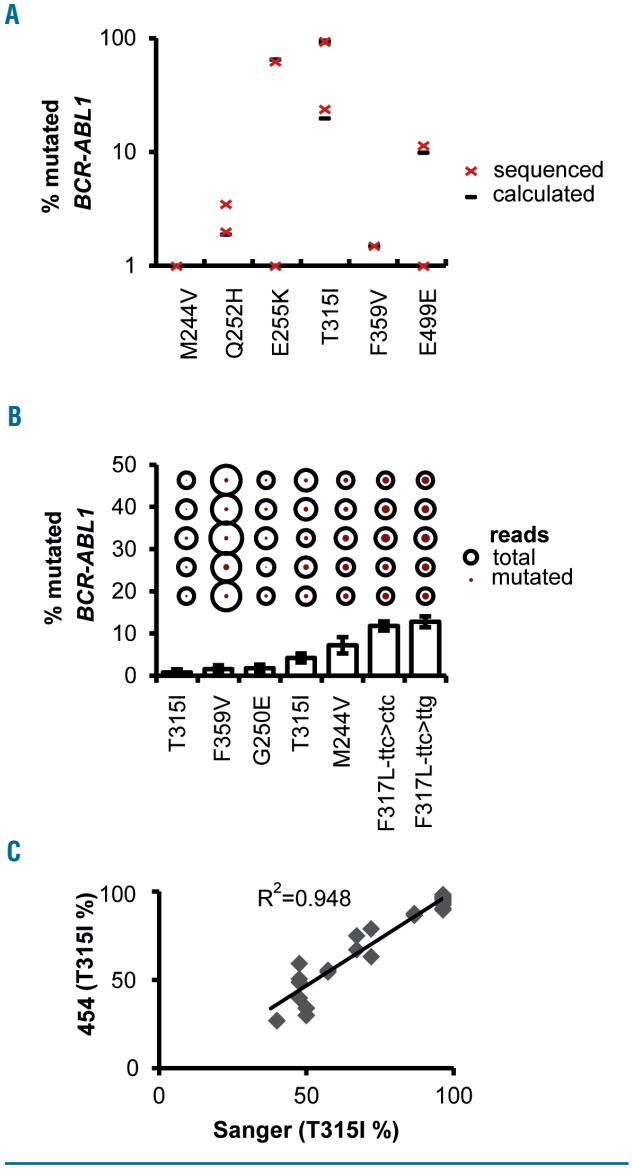

Figure 2. Quality controls for 454 XL+ sequencing of the *BCR-ABL1* kinase domain. (A) Mutation loads (%) are shown as obtained through calculation of the mixing ratio of mutated *BCR-ABL1* from different patients (black line) and as sequenced by the 454 XL+ protocol (red crosses). (B) Sequencing was performed in replicates (n=5) for known resistance mutations. Bar charts show mean (+/-standard deviation). Bubbles display individual sequencing analysis, areas represent read numbers (white: all; red: mutated; example bubbles on the left side: 1000 total and 50 mutated reads). (C) The T315I mutation load is shown as obtained by Sanger sequencing and by 454 XL+ sequencing. Correlation coefficient R^2 was calculated by linear regression.

T315I mutation evolution

Next, we used the patients with low level mutations to uncover the dynamics and time frames until the T315I mutation loads reached 15% or higher. It required a median period of three months (range 1-27 months) (Figure 3B) for the T315I to expand to more than 15%. Patient #17 (indicated by * in Figure 3B) did not show the rapid clonal expansion observed in the other patients. This patient received hydroxyurea alternating with imatinib every four weeks during the period analyzed. For the other samples, usually at the next monitoring time point, the T315I mutation load had greatly expanded.

No low percentage T315I mutation was observed that disappeared during our monitoring period. However, our initial cohort included only patients with at least one Sanger sequencing positive sample (≥15%). According to this study design, we cannot exclude the possibility that low level mutations may arise but disappear before being

Table 1. Patients' characteristics.

	Cohort 1: longitudinal T315I monitoring	Cohort 2: control
Total cohort	n=40	n=42
Sex (male/female)	27/13	23/19
Median monitoring interval, months (range)	5 (2-27)	-
Median number of samples/patient (range)	2 (2-5)	1
	End of monitoring period	
Median age, years (range)	61 (19-85)	58 (26-79)
Peripheral blood counts[1]		
Median WBC, x10⁹/L (range)	12 (3-379)	6.5 (3-53)
Median platelets, x 10⁹/L (range)	164 (20-352)	219 (107-1192)
Median hemoglobin, g/dL (range)	11 (8-15)	13 (9-16)
Therapy[2]		
Imatinib only	12	32
Dasatinib only	2	0
Nilotinib only	1	1
Two TKIs	22	7
Three TKIs	3	1
AlloSCT in addition to TKI	4	1

WBC: white blood cells; alloSCT: allogeneic hematopoietic stem cell transplantation; TKI: tyrosine kinase inhibitors. [1]Available for 28 patients of cohort 1 and 25 of cohort 2. [2]Available for all patients of cohort 1 and 41 of cohort 2; 13 patients of cohort 1 and 10 patients of cohort 2 received antineoplastic agents in addition to TKI: hydroxyurea, interferon alpha, cytarabine, FLAG-Ida [idarubicin, fludarabine, cytarabine, granulocyte colony-stimulating factor (G-CSF)].

detected by Sanger sequencing in a context of routine diagnostics. Therefore, we selected a second control cohort of 42 CML patients (cohort 2) (Table 1). Patients of cohort 2 had lost the MMR status while treated with TKIs, but were negative for *BCR-ABL1* resistance mutations by Sanger sequencing. We performed UDS for all patients, but could neither identify a low percentage T315I mutation nor any other resistance mutation (*data not shown*).

Additional TKI resistance mutations

In cohort 1, sequencing the entire *BCR-ABL1* TKD allowed identifying 20 cases without and 20 with resistance mutations in addition to T315I. For reasons of comparability, we limited our analysis of non-T315I mutations to aberrations detectable in the sample of the first T315I detection. This excluded aberrations, which were completely eradicated by previous therapeutic interventions before the T315I developed. Patients had acquired up to seven different non-T315I mutations. In total, 18 different mutation types were identified to occur with the T315I. The total number of additional mutations was 52. All mutations resulted in amino acid exchanges with known effects on TKI resistance.[8,16] In our T315I mutated cohort, additional mutations were most frequently found at E255 (E255K: n=8 patients; E255V: n=7 patients). Importantly, only 18 of 52 non-T315I mutations had a mutation load above the Sanger sequencing detection limit and 34 of 52 were detectable by UDS only (Figure 4).

Next, we determined the clonal architecture of the CMLs with more than one mutation. Of all additional mutations, 49 of 52 (94%) were located in the first sequencing amplicon together with the T315I mutation. Sequencing reads from multiclonal CMLs should carry only one mutation *per* read, whereas for compound mutated CMLs the aberrations should be seen on the same sequencing read. However PCR-mediated recombination artifacts may cause sequence variants from different CML clones to be seen on the same read.[27] CMLs can, therefore, be falsely classified as compound mutated.[28] We also observed cases with a read distribution, which could be derived from either a multiclonal or a compound mutated case. To determine the extent of false compound mutations in our sequencing assay, we mixed cDNA from patients with only one mutation (Y253H+E255K; Y253H+T315I; E255K+T315I). The recombination rate (calculated as previously described by Deininger *et al.*[29]) was on average 17% per 100 bp (*data not shown*).

In critical cases, we used the longitudinal design of our study as additional information to eliminate misclassification. Patients could be classified as multiclonal, despite reads with a chimeric mutation pattern, if one mutation decreased or disappeared, while the other increased or stayed unaltered during the course of the disease. By applying this additional criterion, we could classify 12 of the 20 patients with additional mutations as multiclonal and 3 as compound mutated (Figure 4). Five patients could not be unequivocally categorized. This was due to technical limitations for 4 patients (mutations in two amplicons or potentially strong PCR-mediated recombination). For Patient #21, the clonal architecture could not be resolved because of an acquired Philadelphia chromosome duplication (*Online Supplementary Table S2*). Here, mutations from different reads could be derived from either different clones or different Philadelphia chromosomes.

Figure 5 shows the detailed clonal development of 4 patients with additional non-T315I resistance mutations. We extended the backtracking to the diagnosis sample. In the multiclonal Patients #15 and #16, the T315I arose and overgrew all otherwise mutated clones within less than one year (Figure 5A and B). In Patient #37, the E255V clone gained a T315I mutation under dasatinib treatment and expanded to 100% within five months (Figure 5C). In Patient #33, the Y253H mutated clone further diversified into three compound mutated subclones, of which the Y253H+T315I clone arose to 76% within 79 days (Figure 5D and *Online Supplementary Figure S2*).

Discussion

By backtracking patients with a high T315I mutation load detected by Sanger sequencing (cohort 1), we identified UDS as a sensitive approach for earlier mutation identification. We excluded the possibility that low level TKD mutations arise sporadically and frequently without subsequent expansion in a second cohort. Cohort 2 was selected to contain CML patients from the European LeukemiaNet (ELN) warning or failure category, in whom sequencing is usually performed. In contrast to cohort 1, UDS in cohort 2 did not reveal any low percentage mutation.

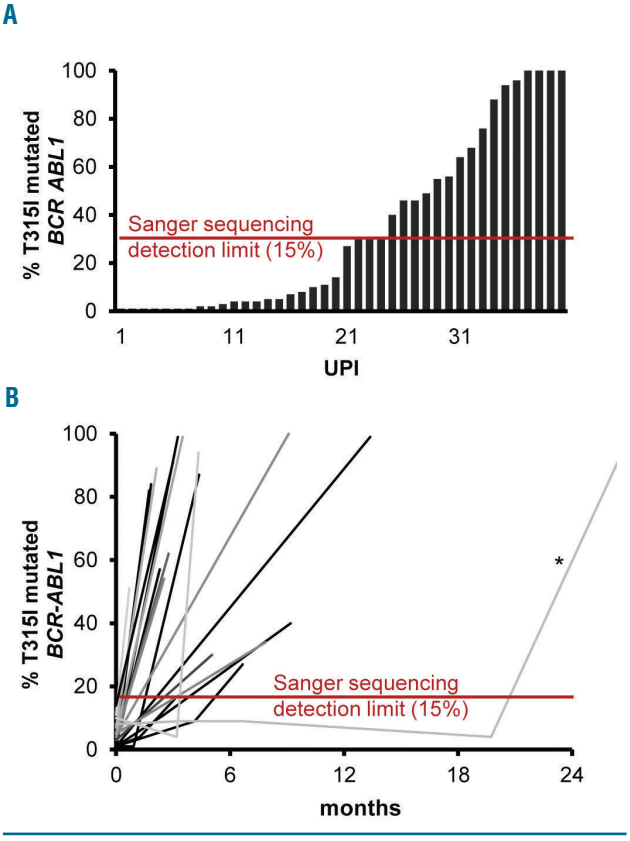

Figure 3. Detection of the T315I by ultra-deep sequencing and development. The percentage of T315I mutated *BCR-ABL1* of all *BCR-ABL1* transcripts is shown as detected by 454 XL+ sequencing. Sanger sequencing detection limit is defined as 15% (red line). (A) The T315I load (%) at the earliest detectable time point is given (UPI: unique patient identifier). (B) In patients for whom a low T315I mutation level (<15%) was detected, subsequent time points are shown. Patient #17 (*) was treated with hydroxyurea or imatinib changing every four weeks for 31 weeks.

In the T315I positive cohort 1, we sequenced two to five longitudinal time points from patients treated with imatinib, dasatinib and/or nilotinib, which allowed a retrospective description of the mutation evolution. We found a rapid and ever increasing nature of the T315I mutation. For the majority of patients under ineffective TKIs, a 1%-2% mutation load expanded to Sanger sequencing detection limits in between two routine analysis time points corresponding to three months. As a consequence of the rapid T315I subclonal outgrowths, each sequenced sample is only a snapshot of the mutational landscape at one time point and despite narrow monitoring intervals, the low percentage mutation onset can be missed. In fact, we only identified the early onset of the T315I mutation in every second patient. For patients without any detectable low percentage T315I mutation, we had sequenced at least one earlier time point and could not detect any mutation with at least a 1% load. The rapid T315I outgrowths demonstrated in our study supports the quest for introduction of UDS in routine clinical procedures[16,30] and the relevance of mutation monitoring requested by ELN guidelines[1] for patients with suboptimal response ("failure category" and "warning category"). After the first year, patients with *BCR-ABL1/ABL1* (%IS) below 1 fall into the "warning category." Therefore, absolute *BCR-ABL1* transcript numbers are low, despite good RNA quality. Mutations in these patients can only be detected if the mutation load is high. Mutations detected at those time points are very informative; however, the number of false negative sequencing results is probably considerable, and negative results should be interpreted with caution. A one-time negative sequencing result cannot exclude the contribution of a mutation in the course of resistance. Patients with rapid clonal evolution might benefit from *BCR-ABL1* sequencing several times during disease evolution.

Although all low percentage (<15%) T315I mutations were detectable by Sanger sequencing at subsequent routine sampling time points, results from UDS would have allowed an earlier therapy intervention and earlier consideration of alloSCT, as recommended by the current ELN guidelines for patients with T315I mutations. This would increase the time frame needed to prepare an alloSCT, e.g. initiation of HLA typing of patient's siblings or the search for unrelated stem cell donors.[1]

Given the retrospective nature of our study, we can only speculate as to the benefit of changing TKI earlier. Firstly, for some patients, the T315I mutation outgrowths was parallel by an increase in the *BCR-ABL1/ABL1* ratio and progress to blast crisis. Support for a change in TKI before progression comes from the phase II trial on ponatinib in Philadelphia chromosome-positive leukemias (PACE). Among chronic phase patients, 66% achieved complete cytogenetic response (CCyR) under ponatinib; however, among patients with accelerated phase and blast phase CML, only 33% and 21% reached CCyR, respectively.[31] Secondly, early eradication of mutated cells could prevent the malignant clone from further diversification, e.g. the gain of cytogenetic aberrations or additional resistance mutations. We observed 50% of patients with other TKD mutations in addition to the T315I mutation and found that more than half of them were also present only at low levels. This showed that not only for T315I, but also for all other resistance mutations, conventional Sanger sequencing would have identified only a small subset of mutations. Importantly, other highly sensitive assays for T315I (e.g. digital PCR or mass spectrometry) can detect the mutation even below 1% (e.g. 0.05%[15] or less than 10 mutated copies[18]), but are limited to the detection of the T315I mutation. These assays do not reveal any of the additional mutations. The high number of additional mutations could explain why we did not see a correlation between the increase in the T315I mutation load and the overall malignant cell burden (*BCR-ABL1/ABL1* ratio) in every patient. The dynamics of total CML cell expansion are likely to be the sum of all resistance mechanisms and depend on the administered TKIs.[32] In line with other previously published *BCR-ABL1* sequencing studies, we also observed that the variety of detected mutations reflected the selective pressure of previously administered TKIs.[16,20,33] In our study, a strong dominant outgrowth of the T315I clone over all other mutated clones was observed. This demonstrated the strong resistance of the mutated cells against first- and second-line TKIs and the extraordinary importance of considering a T315I mutation before changing TKI.

In patients with the T315I mutation and other mutations in one clone, the compound mutated clone strongly expanded and the detected mutation loads of both mutations remained high during the monitoring period. Treatment for patients with compound mutations is chal-

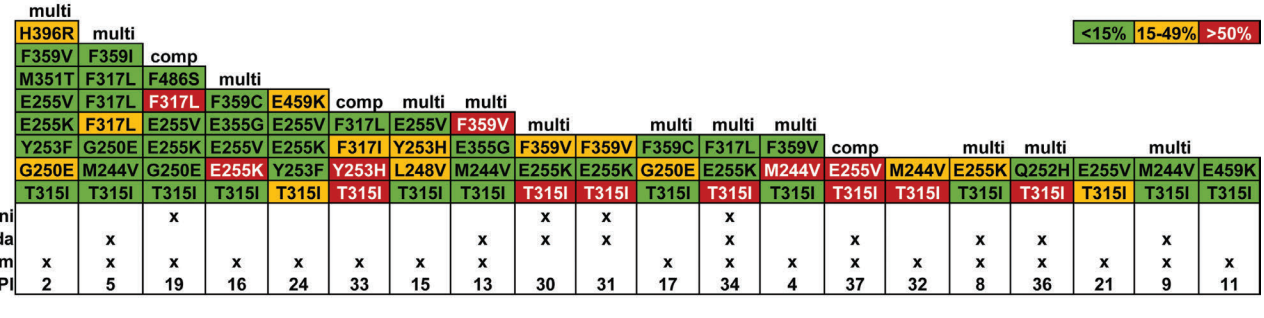

Figure 4. Additional tyrosine kinase inhibitor resistance mutations. The number and type of additional mutations *per* patient is shown. Only mutations present at the time point of initial T315I detection are included. Mutation load is color-coded (green: <15%; orange: 15-49%; dark red: >50%). Cases can have multiclonal (multi) or compound (comp) mutations and 5 cases could not be definitely defined. Tyrosine kinase inhibitor treatment up to the time point of initial T315I mutation detection is displayed (im: imatinib; da: dasatinib; ni: nilotinib). Patient #5 had three mutations resulting in a F317L amino acid change: ttc>ctc, 12%; ttc>tta, 27%; ttc>ttg>12%.

lenging. *In vitro* data suggested that many combinations of mutations (including those with T315I) render cells resistant to available TKIs including ponatinib,[19,34] However, recent data from the PACE trial encourages the use of ponatinib in heavily pre-treated patients with compound mutations.[29] Compound mutated CML cases can be distinguished from cases with multiple independent clones by their read distribution using the longer amplicons design of 454 XL+ sequencing. However, a study by Parker *et al.* suggested that technical artifacts cause a false classification of multiclonal cases as compound mutated.[28] PCR mediated recombination generates one PCR product, which is derived from two original molecules, but presents as one double mutated read.[27] To clarify the clonal

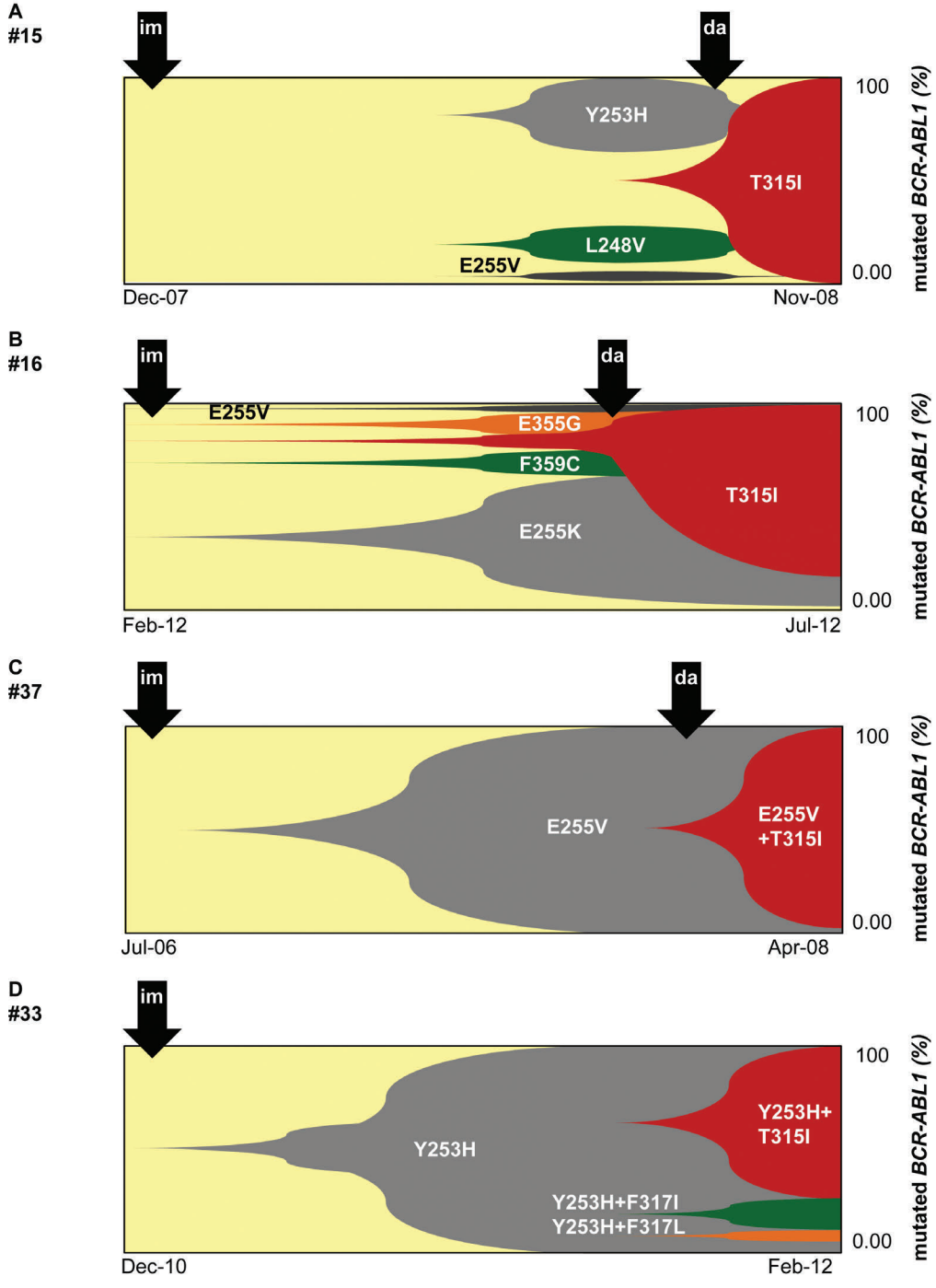

Figure 5. Examples of clonal evolution. Patient #15 and #16 are examples of multiclonal chronic myeloid leukemia (CML) patients (A and B) and patient #37 and #33 (C and D) have compound mutated clones. We extended the backtracking analysis to the initial CML diagnosis sample. Sampling dates are shown on the x-axis and the mutation load on the y-axis. Tyrosine kinase inhibitor treatment by imatinib (im) and dasatinib (da) is indicated by an arrow. (A and B) Each CML subclone is shown as individual area. The T315I is displayed in red; the other resistance mutations are displayed in gray, green and orange. (C) The E255V mutated clone (gray) gained a T315I+E255V double mutated subfraction (red). (D) Of the Y253H mutated *BCR-ABL1* transcripts (gray), 98% gained one additional mutation: 7% the T317L (orange), 15% the T317I (green) and 76% the T315I (red) mutation (*Online Supplementary Figure S2*).

architecture of cases with mixed reads, we used the longitudinal design of our study. Mutations derived from the same clone cannot diverge during disease course. However, we cannot exclude the possibility that, in rare cases, a nucleotide exchange at one position would occur independently several times in one patient but in independent clones. In future studies, the generation of chimeric reads could be reduced by modified sequencing strategies (single molecule consensus sequencing)[28] or could be mathematically corrected.[29]

However, the aforementioned advances are not sufficient to resolve the clonal evolution of cases with cytogenetic aberrations in addition to the initial Philadelphia chromosome, e.g. duplication of the Philadelphia chromosome.[35] We observed additional cytogenetic aberrations in one-third of our cohort with the T315I mutation (cohort 1). We were unable to identify the clonal development of some patients because mutated reads could be assigned to either of the two Philadelphia chromosomes or to different CML clones; only single cell sequencing would clarify their clonal architecture.[36] Importantly, additional cytogenetic aberrations not only add another layer of complexity to clonal heterogeneity, but are also a mechanism of acquired TKI resistance. Therefore, the integrative analysis of all TKD mutations, cytogenetic aberrations, but also of acquired mutations in genes beyond *ABL1*[37,38] or alternative resistance mechanisms (e.g. drug efflux and import deregulation[39]) will help to understand the relative resistance potential of each clone under TKI therapy. The evolution of small clones with the T315I mutation can only be unraveled through prospective studies.

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