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**BCR-ABL kinase domain mutations on second line dasatinib or nilotinib therapy in chronic myeloid leukemia patients after imatinib failure: a cooperative evaluation of different detection methods**

Thomas Ernst,1 Franz X. Gruber,2 Oliver Pelz-Ackermann,2 Jacqueline Maier,3 Markus Pfirrmann,4 Martin C. Müller,1 Ingvild Mikkola,5 Kimmo Porkka,2 Dieter G. Niederwieser,3 Andreas Hochhaus,3 and Thoralf Lange2

1III. Medizinische Klinik, Universitätsmedizin Mannheim, Universität Heidelberg, Mannheim, Germany; 2Institute of Pharmacy, University of Tromsø, Tromsø, Norway; 3Abteilung für Hämatologie, Onkologie und Hämostaseologie, Universitätsklinikum Leipzig, Germany; 4Institut für Medizinische Informationsverarbeitung, Biometrie und Epidemiologie, Ludwig-Maximilians-Universität, München, Germany, and 5University Hospital of Helsinki, Helsinki, Finland

**ABSTRACT**

**Background**

Various techniques to detect BCR-ABL kinase domain mutations in imatinib-resistant patients with chronic myeloid leukemia (CML) have been employed, resulting in different frequencies of mutations and a heterogeneous pattern of individual mutations.

**Design and Methods**

We sought to compare direct sequencing (DS), denaturing high-performance liquid chromatography in combination with DS (D-HPLC/DS) and two high-sensitivity allele-specific oligonucleotide (ASO) PCR approaches for analysis of BCR-ABL mutations in 200 blinded cDNA samples prior to and during second line dasatinib or nilotinib therapy in CML patients after imatinib failure.

**Results**

Comparing DS and D-HPLC/DS, 114 mutations were detected by both approaches and 13 mutations were additionally detected by D-HPLC/DS. Eighty of 83 mutations (96%) within a selected panel of 11 key mutations were confirmed by both ASO PCR techniques and 62 mutations were detected by one ASO PCR technique only. Pre-existing mutations were traceable 4.5 months longer and emerging clones were detectable 3.0 months earlier by ASO PCR than D-HPLC/DS.

**Conclusions**

Comparing DS and D-HPLC/DS, 114 mutations were detected by both approaches and 13 mutations were additionally detected by D-HPLC/DS. Eighty of 83 mutations (96%) within a selected panel of 11 key mutations were confirmed by both ASO PCR techniques and 62 mutations were identified additionally to D-HPLC/DS indicating the presence and a high prevalence of low-level mutations in this cohort of patients. Furthermore, 125 mutations were detected by one ASO PCR technique only. Pre-existing mutations were traceable 4.5 months longer and emerging clones were detectable 3.0 months earlier by ASO PCR than D-HPLC/DS.

Key words: BCR-ABL mutation, DHPLC, ARMS, ligation PCR, imatinib resistance, nilotinib, dasatinib, chronic myeloid leukemia.


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Introduction

The advent of imatinib mesylate has revolutionized the treatment of patients with chronic myeloid leukemia (CML). After 7-year follow-up of the IRIS study, the estimated overall survival of patients who received imatinib as initial therapy was 86% and freedom from progression to accelerated phase or blast crisis was 93%. However, a minority of patients in chronic phase and a substantial proportion of patients in advanced phases of CML are either initially refractory to imatinib treatment or lose imatinib sensitivity over time and thereby experience relapse. The most frequently identified mechanism of acquired imatinib resistance is the emergence of point mutations within the BCR-ABL kinase domain impairing imatinib binding either by interference with the imatinib binding site or by stabilizing a BCR-ABL conformation with reduced binding affinity for imatinib. To date, more than 70 different BCR-ABL kinase domain mutations encoding for more than 50 different amino acid substitutions have been described in imatinib-resistant CML patients.

Various techniques have been employed to detect BCR-ABL kinase domain mutations, resulting in different frequencies of mutations and a heterogeneous pattern of individual mutations. There is currently no consensus concerning the technique that should be used for routine monitoring of CML patients and there are still difficulties in clinical interpretation of specific mutations. One particularly reliable and sensitive approach is the selection and expansion of specific clones followed by DNA sequencing. However, this procedure is cumbersome and not eligible for clinical routine analysis. As an alternative, sequencing of nested PCR-amplified BCR-ABL products has been widely used to search for known and unknown BCR-ABL kinase domain mutations. A potential drawback of direct sequencing is the sensitivity of only 10-20%. In comparison, sensitivities of 1-5% could be obtained by denaturing high-performance liquid chromatography (D-HPLC), double-gradient denaturing electrophoresis, pyrosequencing, high-resolution melting, or array based assays. More sensitive methods include peptide nucleic acid based PCR clamping and allele-specific oligonucleotide (ASO) PCR. However, these techniques are specific for known mutations and cannot be applied for screening of unknown mutations. To date, these methods have been used for scientific purposes - as quantification of low-level drug resistance - rather than clinically relevant monitoring of drug-resistant clones. A comparative assessment of technical features characterizing individual detection techniques has not been performed to date.

In this study we sought to compare different techniques for the detection of BCR-ABL kinase domain mutations in CML patients after imatinib failure. Serial blinded samples on second generation tyrosine kinase inhibitor (TKI) therapy were simultaneously analyzed using two screening methods (direct sequencing (DS) and D-HPLC in combination with DS) and two high-sensitivity ASO PCR techniques, amplification refracto-
**Table 1.** Patients’ characteristics prior to start of second line TKI therapy.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>Female</td>
</tr>
<tr>
<td>Age, median [years]</td>
<td>64</td>
</tr>
<tr>
<td>Phase of disease</td>
<td>Chronic phase</td>
</tr>
<tr>
<td></td>
<td>Accelerated phase</td>
</tr>
<tr>
<td></td>
<td>Myeloid blast crisis</td>
</tr>
<tr>
<td>Mutation status</td>
<td>Mutation</td>
</tr>
<tr>
<td></td>
<td>No mutation</td>
</tr>
<tr>
<td>Second line TKI therapy</td>
<td>Dasatinib</td>
</tr>
<tr>
<td></td>
<td>Nilotinib</td>
</tr>
</tbody>
</table>

TKI: tyrosine kinase inhibitor.

were compared with the wild-type ABL sequence (GenBank accession no. U07563). In our hands this approach allows to detect mutations if the mutated transcripts represent at least 20% of the entire BCR-ABL transcriptome using mutant Ba/F3 cell line dilution series.

**D-HPLC in combination with direct sequencing (D-HPLC/DS)**

Denaturing high-performance liquid chromatography (D-HPLC) was performed in Mannheim on a Transgenic Wave™ System Model 3500 HT (Transgenic, Omaha, NE, USA) according to previous descriptions. This technique is based on heteroduplex formation by PCR products amplified from wild-type and mutant alleles and allows high throughput screening applications. Briefly, three overlapping fragments covering the entire BCR-ABL kinase domain (amino acids 207 through 517) were generated by nested PCR and analyzed for the presence of sequence variations by D-HPLC. PCR products with an abnormal D-HPLC profile were sequenced in both directions to characterize the precise nucleotide substitution(s) and compared to the ABL wild-type sequence (GenBank accession no. U07563). Growth factor-independent Ba/F3 cell populations expressing full-length non-mutated BCR-ABL or full-length BCR-ABL with the clinically most common BCR-ABL kinase domain mutations Y255F, E255K, T315I or M351T were used to optimize the D-HPLC assay and to estimate the sensitivity. Using serial dilutions the detection limit for T315I and M351T mutations was estimated as 0.1%, for the Y255F or E255K mutations as 0.5% or 1%, respectively. Selected mutant and non-mutant Ba/F3 cell lines were used as internal controls for each D-HPLC run. ABL single nucleotide polymorphisms were identified as described previously and excluded for this study.

**Amplification refractory mutation system (ARMS) PCR**

ARMS PCR was performed in Tromsø according to previous descriptions. Complementary DNA corresponding to 80 ng RNA was subjected to a single step of PCR amplification (95 °C 10 minutes, 50 cycles of 95 °C 30 seconds, 60 °C 30 seconds, 72 °C 1 minute) using assays which were optimized for selective amplification of mutations known to confer TKI resistance (total volume 25 µL, 2x master mix (Eurogentec, Brussels), 1 µM Taqman probe, 2 µM primers, Mxs300F primers real time platform (Stratagene, La Jolla, CA, USA)). Briefly, primers were chosen to complement the mutated allele at the 5’-end. For the purpose of increased affinity the primers contained additional mismatches allowing a preferential amplification of the mutated template over an almost 4-log range. Samples giving rise to amplification curves crossing a threshold significantly earlier compared to curves derived from non-mutant Ba/F3 cells were classified as positive. The resulting Ct values were subsequently translated into copy numbers after comparison to standard curves derived from amplification of a 10-fold diluted plasmid containing the respective mutation (pCR 2.1 vector, linearized with HindIII). For normalization, copy numbers were finally related to total BCR-ABL and expressed in BCR-ABL mutant/BCR-ABL total. This single step approach has earlier been shown to detect mutated transcripts if they contribute at least 0.1% of the total BCR-ABL and ABL transcript count.

**Ligation PCR (L-PCR)**

L-PCR was performed in Leipzig as published previously. In addition to the already described hybridization, ligation, and quantification procedures to detect BCR-ABL T315I and BCR-ABL E255K, additional hybridization probes were used for the remaining mutations (Online Supplementary Table 1). In serial 4-fold dilutions of Ba/F3 cells expressing BCR-ABL T315I or BCR-ABL E255K mutants in Ba/F3 cells expressing non-mutated BCR-ABL, mutation specific L-PCR assays achieved sensitivities of 0.1% to 0.05% BCR-ABL mutant/BCR-ABL total. Similar sensitivities were achieved for the other mutations tested with the exception of BCR-ABL M351T, which was detectable only down to 5%. Ba/F3 cells expressing non-mutated BCR-ABL were used as negative controls in order to reveal any cross-reactivity with the mutation-specific oligonucleotides. Samples were scored positive only if the Ct value of the sample did not exceed those of 0.05% BCR-ABL mutant/BCR-ABL total and those of the negative control in two independent reactions. The dynamic range of the L-PCR approach, which is particularly important for the successful monitoring of a mutant clone, typically covers 100% to <0.1% or 0.05% mutant (3–3.5 log). Ba/F3 cell lines containing the respective BCR-ABL mutant or patient samples with a known ratio of BCR-ABL mutant/BCR-ABL total were used as positive controls in all assays. The comparative Ct method was used to calculate the relative percentage of mutated cells in the positive samples directly according to the equation:

\[
\% \text{ mutant allele} = \frac{2^{-(Ct \text{ BCR-ABL mutant} - Ct \text{ BCR-ABL total})}}{100}
\]

The robustness of the PCR conditions contributes to a very low inter-assay variation in the calculated reproducibility of mutants of around 20% (0.2 log) over the entire detection range (data not shown).
Statistical analysis

Since DS and D-HPLC/DS led to dichotomous results (mutation detection yes or no), the scaling of both parameters was always categorical. In general, BCR-ABL/ABL and BCR-ABL/GUS ratios as well as the various mutation levels measured by ARMS or L-PCR were given as continuous data. If frequencies of mutation detection were compared between the methods, results of ARMS- and L-PCR were dichotomized (mutation detection yes or no) as well. To assess the relation between two categorical variables, Fisher's exact test was applied. Groups with continuous data were compared by the Mann-Whitney U test. The association between two continuous parameters was described by the Spearman's rank correlation coefficient. Due to the explorative nature of the analyses, the significance level was 0.05 for all tests. Only two-sided \( p \)-values were recorded. All calculations were performed using SAS/STAT software, Version 9.1.3 for PC.

Results

BCR-ABL/ABL and BCR-ABL/GUS ratios

BCR-ABL fusion mRNA was quantified and related to the expression of two reference genes prior to and in 3-monthly intervals of second line dasatinib (n=20) or nilotinib (n=20) therapy. Median BCR-ABL/total ABL and BCR-ABL/GUS ratios were 90% (range 5.5%-260%) and 21% (range 0.56%-128%) in patients prior to dasatinib therapy. After 12 months of dasatinib therapy, BCR-ABL/total ABL and BCR-ABL/GUS ratios were reduced to 4.5% (range 0%-71%) and 2.1% (range 0%-34%), respectively. Median BCR-ABL/total ABL and BCR-ABL/GUS ratios were 56% (range 11%-100%) and 17% (range 4.8%-109%) in patients prior to nilotinib therapy. After 12 months of nilotinib therapy, BCR-ABL/total ABL and BCR-ABL/GUS ratios were reduced to 8.2% (range 0%-81%) and 2.9% (range 0%-65%), respectively.
**Table 3. Comparison of the four different mutation analysis methods for 11 key mutations.**

<table>
<thead>
<tr>
<th>Method</th>
<th>DS</th>
<th>D-HPLC/DS</th>
<th>ARMS</th>
<th>L-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of detected mutations</td>
<td>79</td>
<td>83</td>
<td>191</td>
<td>220</td>
</tr>
<tr>
<td>Additionally detected mutations to DS</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Additionally detected mutations to D-HPLC/DS</td>
<td>0</td>
<td>-</td>
<td>110</td>
<td>139</td>
</tr>
<tr>
<td>Additionally detected mutations to ARMS</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>77</td>
</tr>
<tr>
<td>Additionally detected mutations to L-PCR</td>
<td>0</td>
<td>0</td>
<td>48</td>
<td>-</td>
</tr>
</tbody>
</table>

**DS:** Direct sequencing; D-HPLC/DS: D-HPLC in combination with direct sequencing; ARMS: amplification refractory mutation system PCR; L-PCR: ligation-PCR.

**Analyzed samples and number of detected mutations**

In total, 174 of 200 samples (87%) were comparable between the different mutation detection approaches. The remaining 26 samples had a BCR-ABL/total ABL ratio <0.1% on second line TKI therapy and the amplification of BCR-ABL failed in at least one laboratory. Table 2 gives an overview of all mutations detected by the four different methods in regard to the underlying second line TKI therapy. In total, 667 mutations were identified (DS, n=114; D-HPLC/DS, n=142; ARMS, n=191; L-PCR, n=220).

**Comparison of DS vs. D-HPLC/DS**

To analyze the reliability of D-HPLC as a screening method for routine use we compared DS of all samples to the results obtained by D-HPLC in combination with sequencing of suspect D-HPLC products (D-HPLC/DS). Analyzed sequences of DS and D-HPLC/DS overlapped at ABL type 1a amino acids 207 to 414. 114 mutations affecting 16 different amino acids were detected by both techniques in 100 of 174 samples. DS found no mutations which were not identified by D-HPLC/DS. In contrast, D-HPLC/DS detected 13 additional mutations which were not found by DS resulting in a total of 127 mutations affecting 19 amino acids in 104 of 174 samples. Of these, 9 of 13 mutations (69%) were minor clones of compound mutations with a low proportion of mutant alleles. Differences between DS and D-HPLC/DS were statistically not different (Fisher’s exact test).

**Comparison of D-HPLC/DS vs. ARMS**

ASO PCR was performed for a panel of 11 clinically relevant mutations (G250E, Q252H, Y253H/F, E255K/V, V299L, T315I, F317L, M351T, F359V) according to the respective ARMS and L-PCR technique. The number of detected mutations as obtained by the different detection methods are shown for individual mutations in Figure 1 and summarized in Table 3. Eighty of 83 mutations (96%) detected by D-HPLC/DS within the ASO PCR panel were confirmed by both PCR techniques (G250E (n=14), Q252H (n=1), Y253H (n=5), Y253H (n=3), E255K (n=7), E255V (n=9), T315I (n=15), F317L (n=12), M351T (n=4), F359V (n=10)) and referred as high-level mutations with a median proportion of mutant alleles of 49% (range 0.79%-100%) BCR-ABL mutant/BCR-ABLtotal (Figure 2A). One F317L mutation was missed by both ASO PCR methods and one F317L or G250E was missed by ARMS or L-PCR, respectively. D-HPLC/DS detected 59 additional mutations outside the mutation panel of the ASO PCR assays. A total of 187 mutations [G250E (n=10), Q252H (n=3), Y253H (n=23), E255K (n=23), E255V (n=3), V299L (n=9), T315I (n=58), F317L (n=26), M351T (n=15), F359V (n=17)] in 120 of 174 samples were additionally detected by both or only one of the specific ASO PCR techniques and were not found by D-HPLC/DS. Significant differences (Fisher’s exact test) in the number of detected mutations between D-HPLC/DS and both ASO PCR methods were identified for the following mutations: Y253H (p=0.0006), E255K (p=0.0008), T315I (p=0.0077) [D-HPLC/DS vs. ARMS], p<0.0001 [D-HPLC/DS vs. L-PCR], and F317L (p=0.0148). In addition, differences were significant for D-HPLC/DS vs. ARMS of V299L (p=0.0072) and M351T (p=0.0267) and for D-HPLC/DS vs. L-PCR of F359V (p=0.0117).

**Comparison of ARMS vs. L-PCR**

The 187 mutations not found by DS and/or D-HPLC/DS are further referred to as low-level mutations (Figure 2B). Sixty-two of 187 mutations (33%) were independently detected by ARMS and L-PCR [G250E (n=4), Q252H (n=1), Y253H (n=9), E255K (n=15), E255V (n=1), V299L (n=3), T315I (n=12), F317L (n=9), M351T (n=2), F359V (n=6)] with a median proportion of mutant alleles of 1.7% (range 0.04%-100%) BCR-ABL mutant/BCR-ABLtotal. In addition 125 of 187 muta-
tions (67%) not found by D-HPLC/DS were detected by one ASO PCR technique only, with a lower median proportion of mutant alleles of 0.73% (range 0.01%-100%) BCR-ABL mutant/BCR-ABL total. Of these, 48 of 125 mutations (38%) were detected by ARMS only: G250E (n=6), Q252H (n=1), Y253H (n=7), E255K (n=4), V299L (n=5), T315I (n=6), F317L (n=9), M351T (n=8), F359V (n=2). Seventy-seven of 125 mutations (62%) were detected by L-PCR only: Q252H (n=1), Y253H (n=7), E255K (n=4), E255V (n=2), V299L (n=1), T315I (n=40), F317L (n=8), M351T (n=5), F359V (n=9). Differences in the number of detected mutations between ARMS and L-PCR were significant for the T315I mutation only ($p<0.0001$, Fisher’s exact test).

Evaluation of the diagnostic window for emerging drug resistant clones and follow-up of pre-existing clones

We examined whether or not the higher sensitivity level of ASO PCR can be exploited for clinically relevant purposes. Two categories of mutant clones were investigated: (i) Clones harboring baseline mutations which disappeared by D-HPLC/DS during second line TKI treatment (n=10). (ii) Clones emerging by D-HPLC/DS upon second line TKI treatment (n=15). ASO PCR detection of these clones was compared to D-HPLC/DS detection in serial samples (Figure 3). In median, ARMS allowed a 6 months (range 0-9 months) longer follow-up of pre-existing clones than D-HPLC/DS. By L-PCR, pre-existing mutant clones were traceable in median 3 months (range 0-9 months) longer. Regarding clones emerging upon treatment, both ASO PCR techniques detected mutant clones a median of 3 months (range 0-12 months) earlier than did D-HPLC/DS. Overall, pre-existing mutations were traceable in median 4.5 months longer and emerging clones were detectable 3.0 months earlier by ASO PCR than D-HPLC/DS.

Association of detected mutations and clinical characteristics

All mutational findings of DS, D-HPLC/DS, ARMS and L-PCR at the four respective time points (month 3, 6, 9, and 12) were correlated with the clinical characteristics disease phase, second line TKI treatment (dasatinib or nilotinib), and a BCR-ABL/total ABL level ≤ 0.1% at 12 months. Only F317L findings showed associations with clinical characteristics. One F317L mutation has been identified by D-HPLC/DS at baseline but was only detectable by ARMS and L-PCR at 3 months and became undetectable thereafter on nilotinib therapy by all techniques. In contrast, four patients on dasatinib (chronic phase, n=1; accelerated phase, n=2; myeloid blast crisis, n=1) but no patient on nilotinib showed a F317L mutation after 12 months by D-HPLC/DS ($p=0.0392$, Fisher’s exact test). Each of these mutations was already detectable by at least one of the ASO PCR techniques at a low level at baseline and became detectable by D-HPLC/DS after 3 (n=2), 6 and 9 months, respectively. However, more data is needed to satisfactorily investigate the influence of an interaction between treatment and progression on the development of a F317L mutation.

Discussion

Since the introduction of imatinib and the discovery of BCR-ABL kinase domain mutations as the main mechanism of resistance, mutation detection in case of failure or suboptimal response has become an increasingly important issue in the management of CML patients.24 With the availability of second generation TKI with different in vitro and in vivo activity against mutant clones, the presence of mutations provides information relevant to the selection of the optimal second or third line treatment. However, a range of different screening and mutation detection techniques has been employed. Here, we present a blinded comparison of D-HPLC screening followed by direct sequencing and direct sequencing alone initiated by investigators of the European LeukemiaNet. Consistent with the higher sensitivities expected from the smaller PCR fragments amplified in D-HPLC/DS, we detected 13 additional mutations (+11.4%) by this technique. However, only...
four of these samples were diagnosed as wild-type by DS alone. Thus, in our study the higher sensitivity of D-HPLC/DS resulted mainly in the detection of second or third mutated clones (n=9). However, D-HPLC/DS has some additional advantages: First, the initial screening for mutations by D-HPLC has proven to be more rapid and cost effective in our hands than the full procedure of DS. Second, the three overlapping fragments effectively cover the entire BCR-ABL kinase domain (amino acids 207-517) compared with two fragments (amino acids 207-414) analyzed by DS. Taken together, in our study both techniques were suitable and closely comparable for the detection of major resistant clones in case of resistance or suboptimal response to TKI treatment in CML. Since there are further techniques available with different laboratory-specific modifications and applications, this study provides a basis for further comparisons and standardization efforts comparable with the introduction of the international scale for quantification of BCR-ABL transcripts.22

A further aim of our study was to perform a blinded comparison of D-HPLC/DS vs. two allele-specific oligonucleotide (ASO) PCR techniques which are currently experimental but potentially more sensitive and quantitative. So far, these techniques have been employed not for mutation screening, but rather to follow known mutated clones over time (ARMS)23 or to provide accurate quantitation of specific mutant clones (L-PCR).23 Taking the mutations detected by D-HPLC/DS as a high level reference, both ASO PCR techniques showed a low false negative rate, missing only two of 83 mutations (2.4%). Among the mutations detected by both ASO PCR and D-HPLC/DS, the lowest proportion quantified by ASO PCR was 0.79% BCR-ABLmutant/BCR-ABLtotal, confirming the reported sensitivity of the D-HPLC/DS technique. The higher sensitivities of the ASO PCR techniques resulted in the detection of 187 additional mutations referred to here as low level mutations. The difference in the incidence of detected mutations compared to D-HPLC/DS was significant in six (ARMS) or five (L-PCR) of eleven mutations tested. Among low-level mutations identified by ASO PCR, 55% were confirmed independently by both techniques. These clones were present at a higher level (median 1.7% BCR-ABLmutant/BCR-ABLtotal) than the unconfirmed mutations (median 0.75% BCR-ABLmutant/BCR-ABLtotal), suggesting that the discrepancies between the results of the two ASO PCR techniques usually occur below 1% mutant clones. Only ASO PCR results with Ct values below the cross-reactivity of every specific run have been scored as positive, so we can largely exclude false positive results for both ASO PCR techniques.

Given that the implications for high sensitivity and quantitative mutation testing in the specific management of CML patients are unknown, we asked (i) if we can identify associations with clinical characteristics and (ii) if we were able to increase the diagnostic window for detection of emerging or pre-existing clones prior to progression. (i) Due to the fact that this study has been set up with the primary aim of comparing different techniques, only a limited number of patients and their clinical characteristics were available. However, we observed more emerging F317L mutant clones on dasatinib (n=4) compared to nilotinib (n=0) within 12 months. All of these F317L clones were already detectable at a very low level by the ASO PCR techniques prior to initiation of dasatinib. Furthermore, we observed an association of F317L mutations and advanced disease phase as previously reported.24 The low number of F317L patients did not support a test for independency of these two characteristics. Therefore, more data is needed to investigate the interaction between advanced disease phase, dasatinib treatment and the occurrence of a F317L mutation as well as the additional role of high sensitivity mutation testing. (ii) As reported for the specific D-HPLC/DS technique used in this study, BCR-ABL mutations were detectable in median 7.1 months prior to hematological relapse.19 The ASO PCR techniques might give a further increase of the diagnostic window from 7.1 to 10.1 months, by detecting the mutations G250E, E255K/V, T315I, F317L and F359V three months earlier than D-HPLC/DS. Almost all of these mutations have been shown to arise on treatment or have lower response rates on dasatinib (IC50>5 nM; Q252H, E255K/V, V299L, F317L, T315I), or nilotinib (IC50>150 nM; Y253H, E255K/V, F359V/C, T315I).20,21 Hence, 3-monthly serial monitoring by D-HPLC/DS for patients on second line TKI treatment and unsatisfactory response is likely useful. A further increase of the diagnostic window by the more sensitive techniques provides an opportunity for earlier optimization of the TKI therapy and increases the time available for identification of a stem cell donor in those cases in which allogeneic transplantation is an option.
Based on the current study, this goal should be achievable by serial monitoring of these mutations at risk for the specific treatment (i.e., IC50<3 nM for dasatinib and IC50>150 nM for nilotinib). It remains to be seen whether threshold levels of mutant clones or the kinetics of increase over time will be most informative in this respect. Based on this study and considering the narrowing spectrum of mutations causing resistance on second or third line TKI treatment, we foresee potential advantages for highly sensitive and quantitative monitoring of mutant clones in the future management of CML patients with unsatisfactory response.

**Authorship and Disclosures**

TE, FXG, AH and TL were involved in the conception and design of the experiments, analyzed the data and wrote the manuscript. MCM, OPA, JM, IM, FXG, TE and TL analyzed samples, MP was responsible for statistics, AH, KP and DN were involved in data interpretation and finalized the manuscript while all authors contributed to the article version.

The authors reported no potential conflicts of interest.


