A co-operative evaluation of different methods of detecting BCR-ABL kinase domain mutations in patients with chronic myeloid leukemia on second-line dasatinib or nilotinib therapy after failure of imatinib

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The online version of this article contains a supplementary appendix.

ABSTRACT

Background

Various techniques have been employed to detect BCR-ABL kinase domain mutations in patients with chronic myeloid leukemia who are resistant to imatinib. This has led to different reported frequencies of mutations and the finding of a heterogeneous pattern of individual mutations.

Design and Methods

We compared direct sequencing alone and in combination with denaturing high-performance liquid chromatography and two high-sensitivity allele-specific oligonucleotide polymerase chain reaction approaches for analysis of BCR-ABL mutations in 200 blinded cDNA samples prior to and during second-line dasatinib or nilotinib therapy in patients with chronic myeloid leukemia in whom imatinib treatment had failed.

Results

One hundred and fourteen mutations were detected by both direct sequencing alone or in combination with high performance liquid chromatography and 13 mutations were additionally detected by the combined technique. Eighty of 83 mutations (96%) within a selected panel of 11 key mutations were confirmed by both allele-specific oligonucleotide polymerase chain reaction techniques and 62 mutations were identified in addition to those detected by combined liquid chromatography and direct sequencing, indicating the presence and a high prevalence of low-level mutations in this cohort of patients. Furthermore, 125 mutations were detected by only one allele-specific oligonucleotide polymerase chain reaction technique. Pre-existing mutations were traceable 4.5 months longer and emerging clones were detectable 3.0 months earlier by allele-specific oligonucleotide polymerase chain reaction than by direct sequencing together with liquid chromatography.

Conclusions

Our results suggest that denaturing high performance liquid chromatography combined with direct sequencing is a reliable screening technique for the detection of BCR-ABL kinase domain mutations. Allele-specific oligonucleotide polymerase chain reaction further increases the number of detected mutations and indicates a high prevalence of mutations at a low level. The clinical impact of such low-level mutations remains uncertain and requires further investigation. Allele-specific oligonucleotide polymerase chain reaction allows detection of defined mutations at a lower level than does denaturing high performance liquid chromatography combined with direct sequencing and may, therefore, provide clinical benefit by permitting early reconsideration of therapeutic strategies.

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).[1] After the 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%.[2] 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 sensitivity to imatinib over time and thereby experience relapse.[3] 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.[4] 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.[5]

Various techniques have been employed to detect BCR-ABL kinase domain mutations, resulting in different reported frequencies of mutations and the finding of 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.[6,7] However, this procedure is cumbersome and not suitable for routine clinical analysis. As an alternative, sequencing of nested polymerase chain reaction (PCR)-amplified BCR-ABL products has been widely used to search for known and unknown BCR-ABL kinase domain mutations.[8,9] A potential drawback of direct sequencing (DS) is its detection sensitivity of only 10-20%. In comparison, sensitivities of 1-5% can be obtained using denaturing high-performance liquid chromatography (D-HPLC),[10-13] double-gradient denaturing electrophoresis,[14] pyrosequencing,[15] high-resolution melting,[16] or array-based assays.[17,18] More sensitive methods include peptide nucleic acid-based PCR clamping[19] and allele-specific oligonucleotide (ASO) PCR.[20-23] 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 – such 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 from patients on second-generation tyrosine kinase inhibitor (TKI) therapy were simultaneously analyzed using two screening methods (DS and D-HPLC in combination with DS) and two high-sensitivity ASO PCR techniques: an amplification refractory mutation system (ARMS) PCR and ligation-PCR (L-PCR). In particular, we sought to evaluate (i) the reliability of D-HPLC/DS results compared to DS and (ii) whether ASO PCR-based genotyping approaches – despite their obvious drawback of detecting known mutations only – confer a clinically advantageous level of sensitivity of mutation detection which eventually could justify implementation of these techniques in clinical monitoring of CML patients.

Design and Methods

Patients' samples

In total, 200 blood samples from 40 BCR-ABL-positive CML patients with treatment failure or suboptimal response to imatinib according to the European LeukemiaNet recommendations[24] were investigated. All patients gave written informed consent, had previously received between 400 and 800 mg imatinib/day and were included in the clinical trials NCT00109707, NCT00384228, CA180013, CA180005 and CA180006 (*www.clinicaltrials.gov*). These trials were conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000, and approved by national and/or international ethics committees. The patients' characteristics are given in Table 1. Mutation analysis was performed prior to start of second-line TKI therapy with dasatinib 140 mg/day (n=20) or nilotinib 800 mg/day (n=20) and after 3, 6, 9, and 12 months on therapy. Patients were randomly selected and samples were blinded in the Mannheim (Germany) laboratory. Two hundred cDNA aliquots were shipped to each of the other two participating laboratories in Leipzig (Germany), and Tromsø (Norway).

RNA extraction and cDNA synthesis

Total RNA was extracted after hypotonic red cell lysis from at least 20 mL of peripheral blood using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) or TRIzol® reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturers' instructions. Complementary DNA synthesis was performed using random hexamer primers and MMLV reverse transcriptase (Invitrogen) as described elsewhere.[25]

Table 1. Patients' characteristics prior to the start of second-line TKI therapy.

Gender	Male	24
	Female	16
Age	Median [years]	64
	Range	39-78
Phase of disease	Chronic phase	31
	Accelerated phase	7
	Myeloid blast crisis	2
Mutation status	Mutation	29
	No mutation	11
Second-line TKI therapy	Dasatinib	20
	Nilotinib	20

TKI: tyrosine kinase inhibitor.

Quantitative reverse-transcriptase polymerase chain reaction

BCR-ABL, total ABL and β-glucuronidase (GUS) transcripts were amplified in Mannheim using the LightCycler™ technology (Roche Diagnostics, Mannheim, Germany) and detected via specific hybridization probes as described previously.[26,27] The control genes were selected based on recommendations of the Europe Against Cancer (EAC) protocol.[28] Two microliters of cDNA were used as the template for the quantitative reverse transcriptase-PCR reactions.

Direct sequencing

DS was performed in Tromsø as described previously.[9] Hemi-nested PCR products of 675 bp encoding amino acids 207-414 were amplified and sequenced in both directions using an ABI PRISM® 3130 Genetic Analyzer and the BigDye Terminator kit version 3.1 (Applied Biosystems, Foster City, CA, USA). Sequences were compared with the wild-type ABL sequence (GenBank accession no. U07563). In our hands this approach allows the detection of mutations if the mutated transcripts represent at least 20% of the entire BCR-ABL transcriptome using mutant BaF/3 cell line dilution series.

Denaturing high-performance liquid chromatography in combination with direct sequencing

D-HPLC was performed in Mannheim on a Transgenomic Wave™ System Model 3500 HT (Transgenomic, Omaha, NE, USA) according to previous descriptions.[13] 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 Y253F, E255K, T315I or M351T were used to optimize the D-HPLC assay and to estimate its sensitivity. Using serial dilutions the detection limit for T315I and M351T mutations was estimated as 0.1%, whereas the limits for the Y253F and E255K mutations were 0.5% and 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 from this study.[29]

Amplification refractory mutation system polymerase chain reaction

ARMS PCR was performed in Tromsø according to previous descriptions.[21] Complementary DNA corresponding to 80 ng RNA was subjected to a single step of PCR amplification (95°C 10 for min, 50 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 min) 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, Mx3000P real time platform (Stratagene, La Jolla, CA, USA)]. Briefly, primers were chosen to complement the mutated allele at the 3'-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 *Hind*III). For normalization, copy numbers were finally related to total BCR-ABL and expressed as BCR-ABL^{mutant}/BCR-ABL^{total}. This single step approach has previously been shown to detect mutated transcripts if they contribute at least 0.1% of the total BCR-ABL and ABL transcript count.[21]

Ligation polymerase chain reaction

L-PCR was performed in Leipzig as described previously.[23] 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 S1*). 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 detection 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 values 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 patients' 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: $\%\ mutant\ allele = 2^{-(ct\ BCR\text{-}ABL_{mutant} - ct\ BCR\text{-}ABL_{total})} \times 100$. 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 are 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 level of statistical significance 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 at 3-monthly intervals during 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.

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 direct sequencing alone and in combination with denaturing high-performance liquid chromatography

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. One hundred and fourteen mutations affecting 16 different amino acids were detected by both techniques in 100 of 174 samples. DS did not detect any 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 13 mutations, nine (69%) were minor clones of compound mutations with a low proportion of mutant alleles. Differences between DS and D-HPLC/DS were not statistically different (Fisher's exact test).

Comparison of denaturing high-performance liquid chromatography in combination with direct sequencing and allele-specific oligonucleotide polymerase chain reaction

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 techniques. The number of mutations detected by the different 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), Y253F (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 to as *high-level* mutations with a median proportion of mutant alleles of 49% (range, 0.79%-100%) BCR-ABL^{mutant}/BCR-ABL^{total} (Figure 2A). One F317L mutation was missed by both ASO PCR methods and one F317L by ARMS and one G250E by L-PCR. 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 amplification refractory mutation system- and ligation- polymerase chain reaction

The 187 mutations not found by DS and/or D-HPLC/DS are here referred to as *low-level* mutations (Figure 2B). Sixty-two of these 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-ABL^{total}. In addition 125 of the 187 mutations (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 125 mutations, 48 (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). The other 77 (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).

Table 2. Number of mutations at baseline and during 12 months of second-line TKI therapy detected by different mutation analysis methods.

	Baseline (month 0)			Dasatinib (months 3-6-9-12)			Nilotinib (months 3-6-9-12)		
	D-HPLC/DS	ARMS	L-PCR	D-HPLC/DS	ARMS	L-PCR	D-HPLC/DS	ARMS	L-PCR
M244V	2	--	--	0	--	--	0	--	--
G250E	3	5	4	6	9	7	6	11	7
Q252H	1	1	1	0	1	2	0	1	0
Y253H	2	4	5	1	8	5	0	7	9
Y253F	1	1	1	0	0	0	4	4	4
E255K	1	3	4	0	9	8	6	14	14
E255V	1	2	4	4	4	4	4	4	4
D276G	1	--	--	0	--	--	1	--	--
T277A	0	--	--	3	--	--	0	--	--
V299L	0	0	0	0	5	4	0	3	0
F311L	1	--	--	0	--	--	2	--	--
F311I	0	--	--	0	--	--	3	--	--
T315I	2	2	4	9	18	35	4	13	28
F317L	1	6	7	13	18	18	0	6	5
L324Q	1	--	--	3	--	--	0	--	--
L341L	0	--	--	0	--	--	1	--	--
M351T	4	8	6	0	2	1	0	4	4
E355A	0	--	--	0	--	--	2	--	--
E355G	2	--	--	0	--	--	0	--	--
F359V	2	4	4	0	2	1	8	12	20
L387F	1	--	--	2	--	--	0	--	--
M388L	1	--	--	2	--	--	0	--	--
H396R	4	--	--	0	--	--	7	--	--
H396P	1	--	--	4	--	--	0	--	--
E453K	1	--	--	0	--	--	0	--	--
E459K	3	--	--	1	--	--	1	--	--
E466X	0	--	--	0	--	--	1	--	--
C475Y	0	--	--	1	--	--	1	--	--
F486S	1	--	--	3	--	--	2	--	--

–: not investigated by the respective method; D-HPLC/DS: D-HPLC in combination with direct sequencing; ARMS: amplification refractory mutation system PCR; L-PCR: ligation-PCR.

Table 3. Comparison of the four different mutation analysis methods for 11 key mutations.

	DS	D-HPLC/DS	ARMS	L-PCR
Total number of detected mutations	79	83	191	220
Additionally detected mutations to DS	–	4	112	142
Additionally detected mutations to D-HPLC/DS	0	–	110	139
Additionally detected mutations to ARMS	0	0	–	77
Additionally detected mutations to L-PCR	0	0	48	–

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

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

We examined whether or not the greater sensitivity of ASO PCR could be exploited for clinically relevant purposes. Two categories of mutant clones were investigated: (i) clones harboring baseline mutations which disappeared according to D-HPLC/DS during second-line TKI treatment (n=10); and (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). ARMS allowed a median 6 months (range, 0-9 months) longer follow-up of pre-existing clones than did D-HPLC/DS. By L-PCR, pre-existing mutant clones were traceable for a median of 3 months (range, 0-9 months) longer. Regarding clones emerging during 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 for a median of 4.5 months longer and emerging clones were detectable 3.0 months earlier by ASO PCR than by 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 study time points (months 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 had been identified by D-HPLC/DS at baseline but was only detectable by ARMS and L-PCR at 3 months and became undetectable thereafter during 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 are needed for a satisfactory investigation of the influence of an interaction between treatment and progression on the development of a F317L mutation.

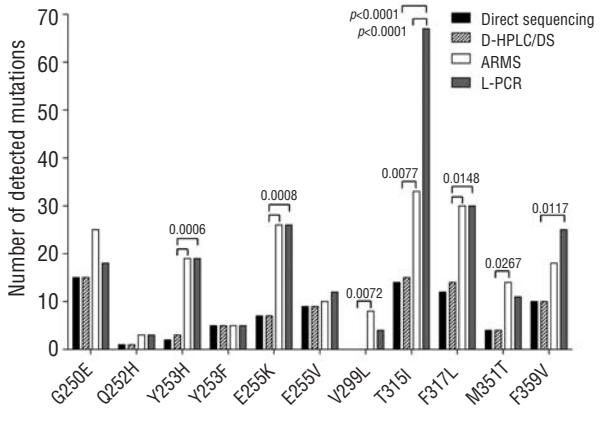

Figure 1. Comparison of the number of mutations detected by the four different mutation analysis methods (for a panel of 11 key mutations). In total, 200 blinded cDNA samples prior to and during second-line TKI therapy were simultaneously analyzed by all techniques. Differences between direct sequencing and D-HPLC/DS were not statistically significant. For Y253H, E255K, T315I, and F317L, statistically significant differences were observed between D-HPLC/DS and both ASO methods. Numbers over the bars indicate p values in cases of statistically significant differences, as obtained by Fisher's exact test.

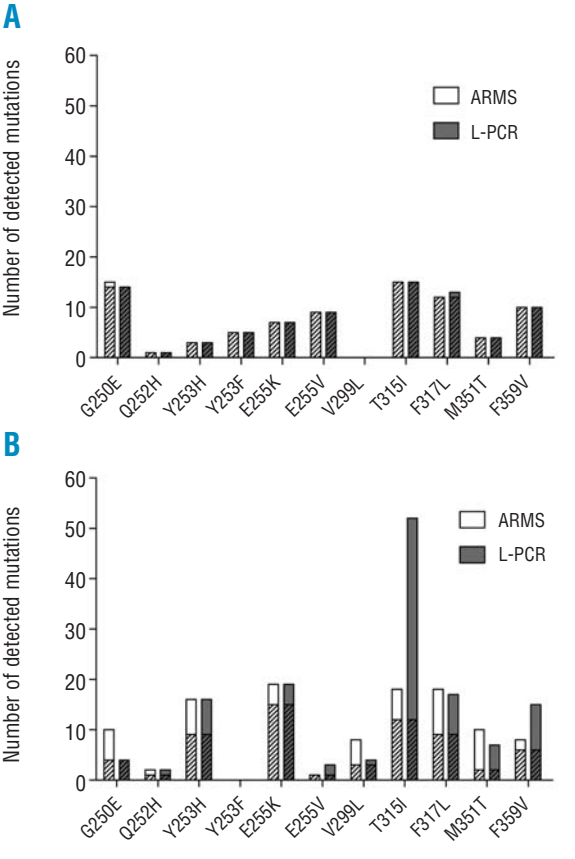

Figure 2. (A) Comparison of both ASO PCR methods for high-level mutations (i.e. mutations which were also detected by D-HPLC/DS). Eighty of 83 high-level mutations (96%) were independently detected by both ASO methods. Hatched areas correspond to mutations which were detected by both ASO methods in the same sample. ARMS detected one G250E mutation which was not detected by L-PCR and L-PCR revealed one F317L mutation which was not detected by ARMS. (B) Comparison of both ASO PCR methods for low-level mutations (i.e. mutations which were not detected by D-HPLC/DS). Sixty-two of 187 low-level mutations (33%) were independently detected by both ASO techniques (hatched areas). Forty-eight low-level mutations were detected by ARMS only and 77 by L-PCR only. L-PCR detected statistically significantly more T315I low-level mutations than did ARMS (p<0.0001).

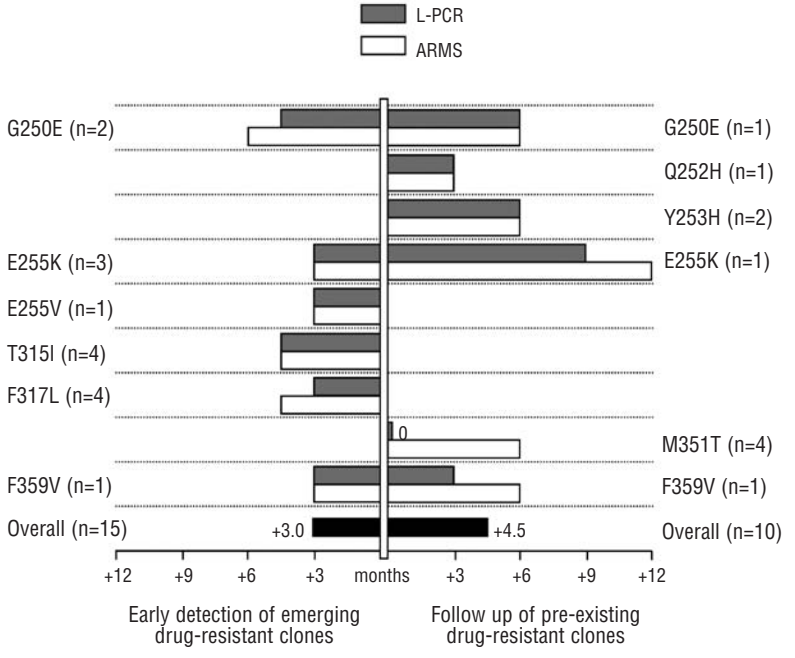

Figure 3. Evaluation of the diagnostic window for emerging drug-resistant clones and follow-up of pre-existing clones as obtained by ASO PCR versus D-HPLC/DS. A total of 15 mutant clones emerged and 10 pre-existing clones disappeared as detected by D-HPLC/DS over a 12-month period of second-line TKI treatment. Detection of these clones by ASO PCR was compared to that by D-HPLC/DS in serial samples. For individual mutations the respective median differences (months) compared to D-HPLC/DS are illustrated by bars. ASO PCR detected emerging mutations a median of 3.0 months earlier and traced pre-existing mutations 4.5 months longer than D-HPLC/DS.

Discussion

Since the introduction of imatinib and the discovery of BCR-ABL kinase domain mutations as the main mechanism of resistance, mutation detection in the case of treatment 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*[4,5] and *in vivo*[30,31] 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 DS and DS 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 the 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 cases of resistance or suboptimal response to TKI treatment in CML. Since there are other 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.[32]

Another aim of our study was to perform a blinded comparison of D-HPLC/DS *vs.* two 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)[21] or to provide accurate quantification 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-ABL^{mutant}/BCR-ABL^{total}, 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 for six (ARMS) and five (L-PCR) of eleven mutations tested. Among low-level mutations identified by ASO PCR, 33% were confirmed independently by both techniques. These clones were present at a higher level (median 1.7% BCR-ABL^{mutant}/BCR-ABL^{total}) than the unconfirmed mutations (median 0.73% BCR-ABL^{mutant}/BCR-ABL^{total}), suggesting that the discrepancies between the results of the two ASO PCR techniques usually occur below a level of 1% mutant clones. Only ASO PCR results with Ct values below the cross-reactivity of every specific run were 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 considered: (i) whether we could identify associations with clinical characteristics, and (ii) whether we were able to increase the diagnostic window for detection of emerging or pre-existing clones prior to progression.

Due to the fact that this study was set up with the primary aim of comparing different techniques, only a limited number of patients and clinical characteristics were available. However, we observed more emerging F317L mutant clones on dasatinib therapy (n=4) than on nilotinib therapy (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.[33] The low number of patients with F317L did not enable a test for independency of these two characteristics. Therefore, more data are 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.

As reported for the specific D-HPLC/DS technique used in this study, BCR-ABL mutations were detectable a median of 7.1 months prior to hematologic relapse.[13] The ASO PCR techniques might further increase the diagnostic window from 7.1 to 10.1 months, by detecting the G250E, E255K/V, T315I, F317L and F359V mutatios 3 months earlier than D-HPLC/DS can do. Almost all of these mutations have been shown to arise on treatment or have lower response rates to dasatinib (IC_{50}>3 nM; Q252H, E255K/V, V299L, F317L, T315I), or nilotinib (IC_{50}>150 nM; Y253H, E255K/V, F359V/C, T315I).[30,31] Hence, 3-monthly serial monitoring by D-HPLC/DS for patients on second-line TKI treatment and an unsatisfactory response is likely to be useful. A further increase of the diagnostic window by using 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 those mutations at risk on the specific treatment (i.e. IC_{50}>3 nM for dasatinib and IC_{50}>150 nM for nilotinib). It remains to be seen whether threshold levels of mutant clones or the kinetics of increase over time would 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 an 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. All authors contributed to the final version of the article.

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