Dynamics of *BCR-ABL* mutated clones prior to hematologic or cytogenetic resistance to imatinib

Thomas Ernst, Philipp Erben, Martin C. Müller, Peter Paschka, Thomas Schenk, Jana Hoffmann, Sebastian Kreil, Paul La Rosée, Rüdiger Hehlmann, and Andreas Hochhaus

III. Medizinische Klinik, Medizinische Fakultät Mannheim der Universität Heidelberg, Mannheim, Germany

ABSTRACT

Background
Mutations of the *BCR-ABL* tyrosine kinase domain constitute a major cause of resistance to tyrosine kinase inhibitors in patients with chronic myeloid leukemia. We sought to improve the diagnostic armamentarium by screening and to analyze the dynamics of mutated clones in chronic myeloid leukemia patients who experienced hematologic or cytogenetic relapse.

Design and Methods
Ninety-five patients who relapsed during imatinib therapy were screened for *BCR-ABL* kinase domain mutations using sensitive denaturing high-performance liquid chromatography (D-HPLC) and direct sequencing. To investigate the dynamics of mutated clones D-HPLC was applied to 453 cDNA samples tracking back from relapse towards the start of imatinib therapy.

Results
Twenty-two different point mutations affecting 18 amino acids were detectable in 46/79 (58%) and in 7/16 patients (44%) with hematologic or cytogenetic relapse, respectively. A deletion of 81 nucleotides (del248-274) of *ABL* exon 4 was observed in two patients. Three patients had exclusively single nucleotide polymorphisms (K247R, T315T, E499E, n=1 each) within the *BCR-ABL* kinase domain. In patients harboring mutations, hematologic relapse occurred after a median of 12.9 months (range, 0.9-44.2), and *BCR-ABL* mutations first became detectable at a median of 5.8 months (range, 0-30.5) after starting imatinib therapy ($p<0.0001$). Nine patients showed evidence of *BCR-ABL* mutations prior to imatinib therapy (T315I, n=4; M351T, n=3; M244V and Y253H, n=1 each).

Conclusions
We conclude that: (i) D-HPLC is a sensitive method for screening for *BCR-ABL* mutations before and during therapy with tyrosine kinase inhibitors; (ii) the occurrence of *BCR-ABL* mutations during imatinib therapy is predictive of relapse; (iii) mutations may be detectable several months before relapse, and (iv) the sensitive detection of small numbers of mutated clones could provide clinical benefit by triggering early therapeutic interventions.

Key words: chronic myeloid leukemia, imatinib resistance, *BCR-ABL* mutation, D-HPLC

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Correspondence:
Andreas Hochhaus,
III. Medizinische Klinik, Medizinische Fakultät Mannheim der Universität Heidelberg, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany.
E-mail: hochhaus@uni-hd.de

Introduction

Mutations of the *BCR-ABL* tyrosine kinase domain constitute a major cause of resistance to treatment in patients with chronic myeloid leukemia (CML) treated with selective tyrosine kinase inhibitors.[1] To date, more than 50 different point mutations encoding for more than 40 different amino acid substitutions in the BCR-ABL kinase domain have been described in CML patients after relapse due to resistance to imatinib. These mutations affect amino acids involved in imatinib binding or in regulatory regions of the BCR-ABL kinase domain and result in decreased sensitivity to imatinib.[2]

Early detection of *BCR-ABL* mutants may identify patients who are likely to become resistant to imatinib therapy, which in turn would allow optimization of treatment of these high-risk patients with, for example, dose escalation, combination therapy or administration of novel second generation tyrosine kinase inhibitors.[3] However, there is currently no consensus on which technique should be used for mutation analysis screening of the *BCR-ABL* kinase domain of imatinib-resistant CML patients. Mutations can be reliably and sensitively detected by selection and expansion of specific clones followed by DNA sequencing.[4,5] This procedure is, however, cumbersome and not suitable for routine clinical analysis. Alternatively, 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.[6,7] A major drawback of direct sequencing is that its sensitivity for detecting mutations is only 10-20%. Improved sensitivities of 1-5% could be obtained by pyrosequencing,[8] double-gradient denaturing electrophoresis[9] or mass array genotyping.[10] More sensitive methods include peptide nucleic acid based PCR clamping[11] and allele-specific oligonucleotide (ASO) PCR.[12-14] These techniques are, however, specific and cannot be applied for screening for unknown mutations.

Denaturing high-performance liquid chromatography (D-HPLC) has been described as a highly sensitive screening method for the detection of *BCR-ABL* kinase domain mutations, even when the site of the mutation is unknown.[15-17] We sought to employ D-HPLC as a diagnostic method for mutation screening covering the complete *BCR-ABL* kinase domain and to analyze, retrospectively, the dynamics of mutated clones in CML patients who experienced hematologic or cytogenetic relapse.

Design and Methods

Patients' samples

In total, 453 peripheral blood samples from 95 BCR-ABL-positive CML patients (chronic phase, n=47; accelerated phase, n=27; myeloid blast crisis, n=19; lymphoid blast crisis, n=2), who experienced relapse on imatinib therapy (hematologic relapse, n=79; cytogenetic relapse, n=16) were analyzed retrospectively (Table 1). All patients were treated within prospective multicenter trials and received between 400 and 600 mg imatinib/day. The clinical trials were conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000, and were approved by national or international ethics committees. After written informed consent, samples were collected monthly during the first 6 months of imatinib therapy and at 3-monthly intervals thereafter. In addition, 25 patients with CML in chronic phase (Hasford risk score:[18] low risk, n=8; intermediate risk, n=11; high risk, n=6) with a continuous complete cytogenetic remission for more than 2 years after imatinib monotherapy were investigated for low level clones harboring BCR-ABL mutations prior to imatinib therapy.

Table 1. Patients' characteristics.

		N (%)
Gender	Male	51 (54)
	Female	44 (46)
Median age at starting [imatinib (years)	60.4 (range, 19.6-79.6)	
Phase of disease [at imatinib resistance	Chronic phase	47 (50)
	Accelerated phase	27 (28)
	Myeloid BC	19 (20)
	Lymphoid BC	2 (2)
Type of *BCR-ABL* transcript	b3a2	57 (60)
	b2a2	30 (32)
	b3a2/b2a2	4 (4)
	b2a3	1 (1)
	e1a2	2 (2)
	e19a2	1 (1)
Best response to imatinib	CCR (0% Ph^+)	16 (17)
	MCR (1-35% Ph^+)	15 (16)
	Minor CR (36-66% Ph^+)	3 (3)
	CHR	28 (29)
	Return to chronic phase	17 (18)
	No response	14 (15)
	n.a.	2 (2)
Type of relapse	Hematologic	79 (83)
	Cytogenetic	16 (17)

BC: blast crisis; CCR: complete cytogenetic response; MCR: major cytogenetic response; minor CR: minor cytogenetic response; CHR: complete hematologic response; n.a.: not available.

RNA extraction and cDNA synthesis

Total RNA was extracted after hypotonic red cell lysis from at least 20 mL of peripheral blood using commercially available kits (RNeasy™ Mini Kit, RNeasy™ Midi Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized using random hexamer primers and MMLV reverse transcriptase (Invitrogen™, Karlsruhe, Germany) as described elsewhere.[19]

D-HPLC analysis

The entire *ABL* kinase domain (exons 4 to 10) of the rearranged *BCR-ABL* allele was amplified using a nested PCR. The first round of amplification (ABL-A) was performed using a forward primer mapping to *BCR* exon b2 (5'-ACAGCATTCCGCTGACCATCAATAAG-3') or e1 (5'-ACCGCATGTTCCGGGACAAAAG-3'). The common reverse primer (5'-ATGGTCCAGAGGATCGCTCTCT-3') annealed at the junction of *ABL* exons 10 and 11. The size of the ABL-A fragment depends on the respective *BCR-ABL* transcript of the patient (range 1643-1814 bp). In the second round of amplification the coding sequence of the *ABL* kinase domain was divided into three partially overlapping fragments, ABL-B (codons 207-324, 401 bp), ABL-C (codons 279-414, 457 bp) and ABL-D (codons 382-517, 453 bp). The following primers were used: ABL-B-for: 5'-TGGTTCATCATCATTCAACGGTGG-3'; ABL-B-rev: 5'-GTTGCACTCCCTCAGGTAGTC-3'; ABL-C-for: 5'-AAGACCTTGAAGGAGGACACCAT-3'; ABL-C-rev: 5'-AGACGTCGGACTTGATGGAGAACT-3'; ABL-D-for: 5'-ACCACTTGGTGAAGGTAGCTG-3'; ABL-D-rev: 5'-CCTGCAGCAAGGTACTCACA-3'. All PCR reactions were performed in a final volume of 50 µL containing 1 µL of cDNA sample, 1x reaction buffer, 1.5 mM $MgSO_4$, 200 µM dNTP, 0.4 µM each of forward and reverse primers and 1.25 U of Optimase™ polymerase (Transgenomic™, Omaha, NE, USA). Conditions for the first step were as follows: 2 min of denaturation at 95°C followed by a touchdown protocol of 14 cycles of 30 sec at 95°C, 30 sec at 67°C (decrease of 0.5°C per cycle), 3 min at 72°C, and 19 cycles of 30 sec at 95°C, 30 sec at 60°C, and 3 min at 72°C with a final extension for 5 min at 72°C. The second step was performed under the same PCR conditions except for a shorter extension time of 1 min at 72°C and amplification of the first-step PCR product.

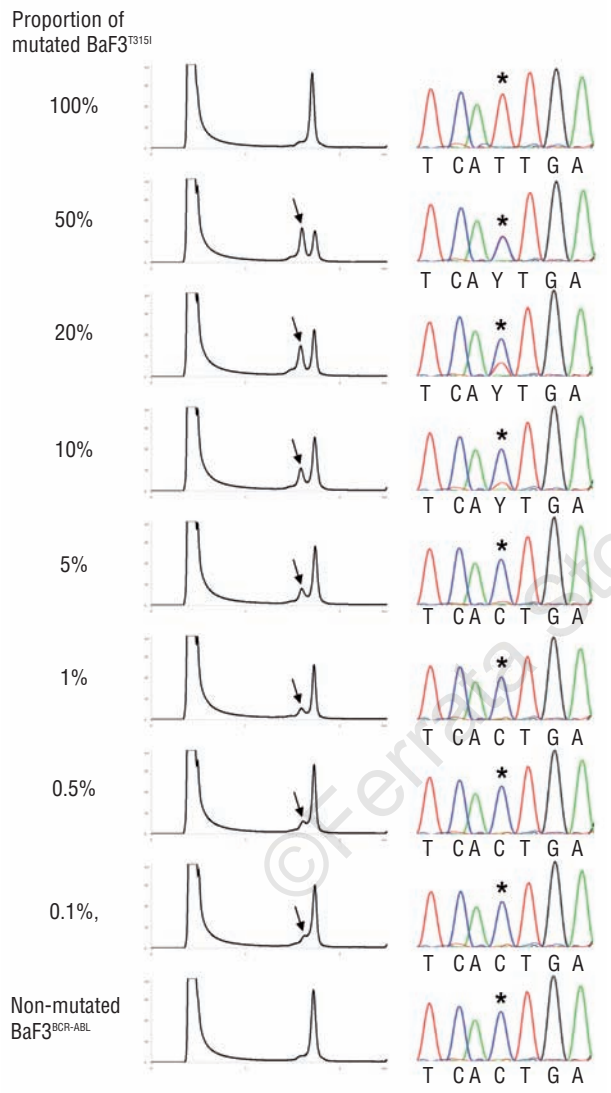

Figure 1. Sensitivity of the D-HPLC assay compared to that of direct sequencing. T315I mutant BaF3^{T315I} and non-mutated BaF3$^{BCR-ABL}$ cell lines were mixed together to contain from 100% to 0% mutant cells and analyzed by D-HPLC (left panel) and direct sequencing (right panel). D-HPLC was optimized to detect 0.1% BaF3^{T315I} in a background of normal BaF3$^{BCR-ABL}$ cells. The degree of difference (marked by arrows) varied from an obvious heteroduplex peak to a slight shouldering of the homoduplex peak depending on the amount of mutant clones. Since mutation detection by D-HPLC depends upon the formation of heteroduplexes between wild-type and mutant sequence strands, no mutation was apparent when the sample comprised 100% mutant alleles. By direct sequencing the mutation was detectable only when the sample comprised ~10% mutant cells. The nucleotide change of interest is marked by asterisks.

PCR products were analyzed by D-HPLC on a Transgenomic Wave™ System Model 3500HT (Transgenomic™, Omaha, NE, USA). Optimal conditions for the resolution of heteroduplexes were calculated using Navigator™ software, version 1.6.0 (Transgenomic™, Omaha, NE, USA). Growth factor-independent BaF3 cell populations[20] expressing full-length non-mutated *BCR-ABL* or full-length *BCR-ABL* with the most clinically common *BCR-ABL* kinase domain mutations, Y253F, E255K, T315I and M351T, were used to optimize the D-HPLC assay and to estimate the sensitivity. Using serial dilutions the detection limit for the T315I and M351T mutations was estimated to be 0.1%, whereas that for the Y253F and E255K mutations was 0.5% and 1%, respectively (Figure 1). The detection limit by direct sequencing was ~10%. We also compared the D-HPLC sensitivity of our nested PCR method for *BCR-ABL* kinase domain amplifications with a single-step PCR method (in which ABL-A was not amplified). D-HPLC analysis of single-step PCR products showed a detection limit when samples contained less than 30% mutated cells. Highly sensitive detection of *BCR-ABL* kinase mutations by D-HPLC requires a nested PCR approach in which the translocated *ABL* allele is specifically analyzed.

Direct sequencing

Direct sequencing was performed for all samples at the time of hematologic or cytogenetic relapse. By hemi-nested PCR, 675 bp products encoding amino acids 207-414 were generated as described previously[7] and sequenced in both directions. For analysis of amino acids 415-517 the ABL-D fragment was sequenced. Sequences were com-

pared with the wild-type *ABL* sequence (GenBank accession # U07563).

Statistical analysis

For statistical analyses, Wilcoxon's matched pairs test was performed using a two-tailed distribution and confidence intervals of 95% (GraphPad Prism™ Version 5.0 software, San Diego, CA, USA).

Results

Mutation analysis of patients who relapsed after imatinib therapy

Twenty-two different point mutations affecting 18 amino acids were detected in 46/79 patients (58%) after hematologic relapse and in 7/16 patients (44%) after cytogenetic relapse (Table 2). Mutations were located in the P-loop (amino acids 248-255, n=17), at T315I (n=9), at M351T (n=7), in the A-loop (amio acids 379-396, n=8), or other sites (n=17). A deletion of 81 nucleotides (del248-274) of *ABL* exon 4 encoding in part for the P-loop region of the kinase domain was observed in two patients. This deletion was caused by the introduction of an alternative splice site due to the L248V mutation.[21,22] Single nucleotide polymorphisms within the *BCR-ABL* kinase domain were detected exclusively in three patients (K247R, T315T, E499E, n=1 each) and additionally to another mutation in four patients (all E499E). Polymorphisms were confirmed by selective sequencing of the normal *ABL* allele.[23]

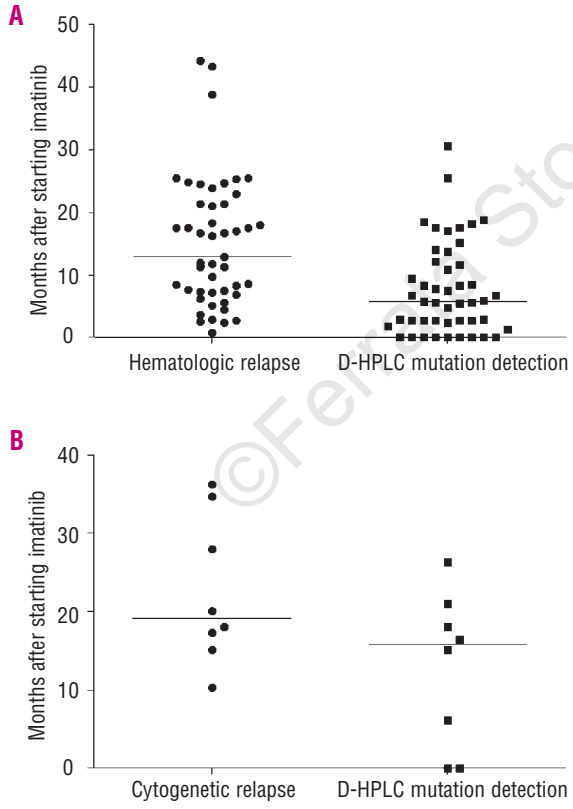

Figure 2. (A) Median time to hematologic relapse after the commencement of imatinib treatment compared with median time to mutation detection by D-HPLC. D-HPLC enables detection of mutations significantly (p<0.0001) earlier than hematologic relapse. Nine patients showed evidence of *BCR-ABL* mutations even prior to imatinib therapy. (B) The median time to cytogenetic relapse after the commencement of imatinib treatment compared with median time to mutation detection by D-HPLC. D-HPLC also enables detection of mutations prior to cytogenetic relapse (p=0.031). In two patients *BCR-ABL* mutations were observed before imatinib therapy was started.

Serial analysis of 55 samples from CML patients harboring BCR-ABL kinase domain mutations

For the 55 CML patients with mutations at hematologic or cytogenetic relapse the mutated clone was tracked back retrospectively from relapse towards the start of imatinib treatment by D-HPLC. *ABL* polymorphisms were not taken into consideration. In the majority of resistant patients, mutations were detectable at various intervals prior to hematologic relapse (Figure 2A). Hematologic relapse occurred at a median of 12.9 months (range, 0.9-44.2) after the start of imatinib therapy. However, *BCR-ABL* mutations first became detectable at a median of 5.8 months (range, 0-30.5) after commencing imatinib. The difference between time to hematologic relapse and earliest detection of a mutation was highly significant (p<0.0001). Nine patients (19%, chronic phase, n=4; accelerated phase, n=3; myeloid and lymphoid blast crisis, n=1 each) showed evidence of *BCR-ABL* mutations prior to imatinib therapy (T315I, n=4; M351T, n=3; M244V and Y253H, n=1 each). In seven patients (15%), the mutation was not detectable before hematologic relapse. Six of these patients (blast crisis, n=4; chronic phase, n=2) had P-loop mutations, one patient in accelerated phase had an A-loop mutation. Cytogenetic relapses occurred at a median of 19.2 months (range, 10.3-36.3) after the start of imatinib therapy (Figure 2B). *BCR-ABL* mutations first became detectable at a median of 15.8 months (range, 0-26.4) after commencing imatinib (p=0.031). In two patients (25%) *BCR-ABL* mutations (M244V in a patient in accelerated phase, L324Q in a patient in chronic phase) were observed in a small clone prior to starting imatinib therapy.

For subgroup analysis, samples of patients with hematologic relapse were divided according to (i) the phase of disease before imatinib treatment and (ii) the site of the mutation. The median intervals between detection of the mutation and hematologic relapse were 5.6 months (range, 0-19.9) for chronic phase patients, 8.1 months (range, 0-41.4) for accelerated phase patients, and 2.4 months (range, 0-14.5) for blast crisis patients (Figure 3A). Mutations can be grouped into five distinguishable clusters: P-loop, T315I, M351T, activation loop (A-loop), and other locations. P-loop mutations were detectable 2.8 months (range, 0-14.5), T315I mutations 6.3 months (range, 2.3-33.7), M351T mutations 10.8 months (range, 7.8-25.5), A-loop mutations 2.9 months (range, 0-6.9), and mutations at other residues 8.7 months (range, 1-41.4) prior to hematologic relapse (Figure 3B and Table 3).

Detection of multiple mutated clones

In nine patients two different nucleotide changes were observed concurrently at the time of hematologic relapse: four patients harbored the E499E polymorphism and an additional mutation, five patients had two mutations associated with imatinib resistance. Mutation analysis of serial blood samples showed that the second mutation appeared a median of 6.4 months (range, 0-8.7) after the first mutation or 2.2 months (range, 1-17) prior to hematologic relapse.

Mutation analysis of patients with continuous complete cytogenetic remission

Samples from 25 CML patients with continuous complete cytogenetic remission for more than 2 years after imatinib monotherapy were screened for baseline *BCR-ABL* mutations. Three samples showed a heterozygous D-HPLC elution profile and subsequent direct sequencing confirmed the nucleotide change 70846 c-t (GenBank accession # U07563), which leads to the silent Y413Y mutation (n=1), and 74901 a-g, which leads to the E499E polymorphism (n=2) as confirmed by selective sequencing of the normal *ABL* allele. No mutations associated with imatinib resistance were found.

Table 2. Mutations and polymorphisms of the *BCR-ABL* kinase domain detected at the time of relapse.

	*Nucleotide change**	*Amino acid change#*	*IC_{50} (nM)§*	*N*
P-loop	58768 a-g	M244V	2,000	6
	58780 c-g	L248V	n.a.	2
	58794 g-c	Q252H	1,325	1
	58795 t-c	Y253H	>6,400	5
	58796 a-t	Y253F	3,475	2
	58801 g-a	E255K	5,200	5
	58802 a-t	E255V	>6,400	2
	67958 a-g	D267G	n.a.	1
	67960 a-g	T277A	n.a.	1
	68710 c-a	F311L	480	1
	68721 c-t	T315I	>6,400	9
	68728 c-g	F317L	1,050	1
	68748 t-a	L324Q	n.a.	1
	68829 t-c	M351T	880	7
A-loop	68841 a-g	E355G	2,300	2
	68853 t-g	F359C	n.a.	1
	70766 t-a	L387M	1,000	2
	70768 g-c	L387F	n.a.	1
	70794 a-g	H396R	1,750	5
	73252 g-a	E450K	n.a.	1
	73253 a-g	E450G	n.a.	1
	73279 g-a	E459K	n.a.	1
Deletions	58780 c-g	del248-274	n.a.	2
Polymorphisms	58778 a-g	K247R	n.a.	1
	68722 t-g	T315T	n.a.	1
	74901 a-g	E499E	n.a.	5

**Nucleotide positions according to GenBank accession number U07563. #Amino acid positions are denoted with the single letter code and correspond to ABL type 1a. §Cellular IC_{50} values as reviewed by O'Hare et al.[2] In nine patients two different mutations were observed in parallel. n.a. - not available.*

Discussion

In this study, we systematically analyzed the dynamics of mutant clones before hematologic or cytogenetic relapse. Mutant clones were detectable several months prior to relapse in patients in all phases of CML. The growth potential of mutated clones may depend on the specific type of the mutation and the selection pressure caused by the tyrosine kinase inhibitor. The longest period between detection of a mutation and subsequent relapse was observed in patients harboring M351T mutations with a relatively low increase of IC_{50} to imatinib (3.4 fold). These patients could benefit from an early increase of the dose of imatinib, as suggested by preclinical data.[24] In contrast, patients harboring mutations with a large increase of IC_{50}, such as T315I (>25 fold) or P-loop mutations (5.1 fold to >25 fold), which were detectable 6.3 months or 2.8 months prior to hematologic relapse, respectively, could benefit from immediate withdrawal of the tyrosine kinase inhibitor.

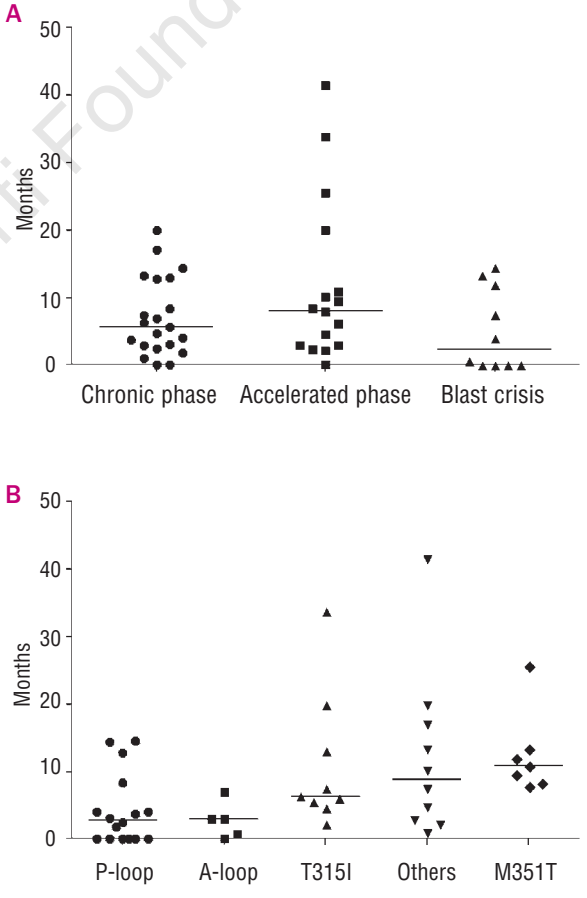

Figure 3. Intervals between D-HPLC positivity and subsequent hematologic relapse. (A) Grouped according to the phase of disease: D-HPLC analysis enables mutation detection several months prior to hematologic relapse in CML patients in all phases of disease. (B) Grouped according to the site of the mutation: Time from first detection of mutation to hematologic relapse was longer in patients harboring mutations associated with minor changes of the IC_{50} to imatinib (e.g., M351T; 3.4-fold increase of IC_{50}, median interval of 10.8 months).

Table 3. Results grouped according to phase of disease and mutation location.

		CP (n=21)	*AP (n=16)*	*BC (n=10)*				
					*Hematologic relapse**	*D-HPLC#*	*p§*	*D-HPLC prior to relapse+*
Mutation location	P-loop (n=16)	9	1	6	8.6	6.9	0.0024	2.8
	T315I (n=9)	4	4	1	7.2	2.7	0.0039	6.3
	M351T (n=7)	1	4	2	18.4	3.0	0.016	10.8
	A-loop (n=5)	1	3	1	17.6	17.0	0.13	2.9
	others (n=10)	6	4	0	19.0	6.8	0.0020	8.8
Hematologic relapse*		16.8	17.2	6.3				
D-HPLC#		6.8	5.4	3.0				
p§		0.0001	<0.0001	0.031				
D-HPLC prior relapse+		5.6	8.1	2.4				

*CP: chronic phase; AP: accelerated phase; BC: blast crisis. *Median time to hematologic relapse after starting treatment with imatinib (months). #Median time to mutation detection by D-HPLC after starting treatment with imatinib (months). §p value for the comparison of median time to hematologic relapse vs. D-HPLC mutation detection as calculated by the Wilcoxon's matched pairs test. +Median interval between D-HPLC positivity and subsequent hematologic relapse (months). Two patients harboring polymorphisms (K247R, E499E) were not taken into consideration.*

There is currently no accepted consensus on when patients should be screened and which technique should be used for analysis of *BCR-ABL* kinase domain mutations. Expert recommendations suggest that mutations should be identified as early as possible because they may indicate the need to reconsider the therapeutic strategy.[25] However, the prognostic impact of early detection of an imminently resistant clone remains to be determined. Since low levels of mutant clones may not necessarily have the same clinical significance as clones that are detected in the context of rising disease burden,[8,14] more sensitive methods, e.g. allele-specific PCR, are probably too sensitive. It is not yet possible to predict imminent disease progression accurately. A more than 2-fold increase of *BCR-ABL* transcripts was suggested to be an indicator for predicting mutations,[26] but other investigators found that this was only a poor predictor.[27] Irrespective of that, since the level of transcripts may rise relatively late in the emergence of a mutant clone, reliance on rising transcript levels may delay mutation detection in comparison to that possible by using sensitive mutation screening techniques.[27] With regard to our results, we suggest that D-HPLC-based screening is a suitable method for routine analysis of *BCR-ABL* mutations. Three-monthly, sensitive mutation analysis seems to be sufficient for predicting disease progression due to mutations several months prior to relapse in patients with suboptimal response according to the current guidelines.[1]

The significance of harboring multiple mutated clones has not been established so far. In this cohort, the second mutation appeared a median of 6.4 months after the first mutation. Since patients relapsed at a median of 2.2 months after detection of the second mutation, the finding of multiple resistant clones could be important. In our small cohort of patients with multiple mutations (n=5), the secondary mutation had (except for one patient initially harboring T315I and later, additionally, M244V) a higher IC_{50} than the primary mutation, indicating that the selective pressure from imatinib may facilitate outgrowth of more resistant clones subsequent to less resistant clones. In order to determine the predictive value of minor clones harboring mutations for consecutive relapses, we screened baseline samples from chronic phase CML patients in complete cytogenetic remission for more than 2 years under ongoing imatinib therapy. No *BCR-ABL* mutations associated with imatinib resistance were found. Thus, the observation of mutations during imatinib therapy seems to be predictive of imminent relapse. In contrast, Chu *et al.* detected *BCR-ABL* kinase mutations in purified $CD34^+$ cells from five of 13 CML patients (38%) in complete cytogenetic remission.[28] Although most of the mutations seen have not been reported in previous clinical studies, two of five patients harboring mutations have relapsed. Others found that mutations in patients in stable complete cytogenetic remission are infrequent, and their detection does not consistently predict relapse.[29]

Another aim of our study was to optimize the D-HPLC method as a diagnostic tool for highly sensitive mutation screening of the entire *BCR-ABL* kinase domain. Using nested PCR for the exclusive amplification of the rearranged *ABL* allele, D-HPLC allowed the detection of minor clones harboring *BCR-ABL* mutations at a level of 0.1-1%. Our D-HPLC assay, which consists of three overlapping *ABL*-fragments, allows an estimation of the site of the mutation, even without sequencing. PCR products harboring the mutation can be enriched by a fragment collector allowing consecutive sequencing (not performed in this study). However, high-sensitivity mutation screening of CML patients prior to therapy is not, to date, recommended for routine monitoring.[25]

In conclusion, sensitive mutation analysis allowed detection of small mutated clones several months prior to hematologic or cytogenetic relapse. We suggest this optimized D-HPLC method as a suitable tool for screening analysis of *BCR-ABL* mutations in CML patients prior to, or during therapy with tyrosine kinase inhibitors. The appearance of *BCR-ABL* mutations during, imatinib therapy seems to indicate imminent relapse. Detection of *BCR-ABL* mutations as soon as possible could provide clinical benefit for CML patients by leading too early reconsideration of therapeutic strategies.

Authorship and Disclosures

TE: performed the experiments, analyzed the data and wrote the manuscript; PE, MCM, PP, TS, JH, SK, PLR: contributed to the design of the study, to the work and to the interpretation of results; RH: supervised the study, revised the manuscript; AH: designed the study, wrote the manuscript. The authors reported no potential conflicts of interest.

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