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High-resolution melt curve analysis: Initial screening for mutations in BCR-ABL kinase domain

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Abstract

Mutations in BCR-ABL kinase domain are associated with resistance to tyrosine kinase inhibitors in some patients with chronic myeloid leukemia. Therefore, mutation detection becomes essential in such patients. We aimed to apply high-resolution melt curve analysis (HRM) for a rapid screening prior to sequencing to select only mutation positive samples. One hundred and one samples with different mutations and mutational ratios were used for HRM testing. HRM results of 100/101 samples were concordant with sequencing data. We found HRM as a suitable and sensitive method for initial rapid screening of BCR-ABL KD mutations to sequence only positive samples.
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1. Introduction

Mutations in BCR-ABL kinase domain (KD) were found to be one of the mechanisms associated with resistance to imatinib [1–3], in patients with chronic myeloid leukemia (CML). In many cases KD mutation precedes or accompanies the disease relapse and progression to advanced-phase disease [4,5]. Therefore, mutation monitoring in CML patients with suboptimal response or resistance to imatinib has become important to indicate the need to reconsider the therapeutic strategy. There is currently no universally accepted consensus when patients should be analyzed for KD mutations in BCR-ABL, which technique should be used, and how the data should be reported [6]. Up to now, several techniques were described in BCR-ABL KD mutation detection. Namely, direct sequencing [5], subcloning and sequencing [2], denaturing high performance liquid chromatography [7], pyrosequencing [8], double-gradient denaturing electrophoresis [9], fluorescence PCR and PNA clamping [10], allele-specific oligonucleotide PCR (ASO-PCR) [11,12] and SEQUENOM Mass Array [13].

A recently developed technique high-resolution melt curve analysis (HRM) has appeared along with the introduction of a new family of LC Green dyes [14,15]. HRM is an extension of previous melting analysis often used as a low-resolution tool for confirming the identity of PCR products on real-time PCR instruments. It is used to characterize DNA samples according to their dissociation behavior as they transit from double stranded DNA to single stranded DNA with increasing temperature. Homozygous sequence changes produce a Tm shift compared with the wild type. In contrast, heterozygous samples are identified by differences in melting curve shape [16].

This study was aimed to test HRM for mutation screening in BCR-ABL kinase domain.

2. Material and methods

2.1. Patients

One hundred and one samples were taken during imatinib treatment of 16 CML patients (Table 1) (9 females and 7 males; 1–17 samples per patient, median 5). Each patient possessed a mutation in BCR-ABL kinase domain; altogether

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Table 1
Patient characteristics and type of mutation detected with sequencing

Patient no.	Sex	Age at diagnosis	Disease phase at IM commencement	Prior therapy	Disease phase at mutation first time detection	Mutation
1	F	50.7	1.CP	HU, HU + IFN, IFN	CP, CHR	M244V, H396R
2	M	33.9	1.CP	HU, IFN	CP, partial CR	E255K
3	F	58	2.CP, hematological relapse	Combined chemotherapy	AP	F311I
4	M	53.3	1.CP	HU, HU + IFN	CP, minimal CR	M244V
5	M	42.9	1.CP, hematological relapse	HU + IFN	CP, CCR	F317L, G250E
6	M	47.3	2.CP, hematological relapse	HU, HU + IFN, IFN	CP, additional chromosomal aberrations	M244V
7	F	58.7	1.CP, hematological relapse	IFN	CP, CHR	M351T
8	F	57.7	1.CP	HU	CP, CHR	F359V
9	F	59.6	1.CP, hematological relapse	HU, HU + IFN	CP	F311I
10	F	30.6	AP, hematological relapse	HU, IFN	CP, CHR, additional chromosomal aberrations	Y253H
11	F	60.8	1.CP, hematological relapse	HU, HU + IFN, IFN	CP, CHR	M244V
12	M	30.8	1.CP	HU	CP, CHR	M351T, F317L
13	M	57.1	AP, hematological relapse	HU, IFN	CP, CHR	Y253F
14	F	31	AP	HU, HU + IFN, IFN	AP	T315I, G250E
15	F	45.1	1.CP	HU, HU + IFN, IFN	AP	E459K, Y253H
16	M	21.8	AP	HU, HU + IFN, IFN, ICT	AP	M351T

AP, accelerated phase; CP, chronic phase; CCR, complete cytogenetic remission; CHR, complete hematological remission; CR, cytogenetic response; HU, hydroxyurea; ICT, intensive chemotherapy; IFN, interferon alpha; IM, imatinib.

12 different mutations with different ratio in sequenced samples were found. The cell line K562 was used as a wild type control (reference sample). Patients' samples were obtained with the consent of the Ethics Committee of the Institute of Hematology and Blood Transfusion, Prague in agreement with the Helsinki Declaration.

2.2. Sample processing

Total RNA was extracted from total leukocyte guanidinium thiocyanate lysates using RNeasy Mini kit (Qiagen, Hilden, Germany). The extraction procedure was followed according to the manufacturer's manual excluding the lysis step. A cDNA synthesis was performed using random hexamer primers and Superscript II reverse transcriptase (Invitrogene, Carlsbad, CA, USA).

2.3. Selective amplification of BCR-ABL kinase region

This step selectively amplified a fragment (1504 bp for b2a2, 1579 bp for b3a2) of BCR-ABL kinase domain region prior to sequencing and HRM. The amplified fragment was generated using published primers [17]. The PCR amplifications were performed in 25 μl reaction volumes containing 1× AccuPrime PCR buffer II (Invitrogene), each primer at 0.2 μM, 1 U of AccuPrime Taq polymerase (Invitrogene) and 2 μl cDNA. The PCR amplification was carried out for 2 min at 94 °C, followed by 35 cycles of 15 s 94 °C, 30 s 60 °C, 2 min 68 °C in thermocycler 9700 (Applied Biosystems, Foster City, CA, USA). The quality of PCR amplification was checked by electrophoresis on 2% agarose gel after staining with ethidium bromide.

2.4. Sequencing

Twelve different mutations were initially detected in 16 patients by sequencing (Table 1). Mutation status was retrospectively analyzed during therapy in 101 samples. The selectively amplified fragment was used as the template (1000× diluted when visible under UV light after agarose electrophoresis) for nested PCR amplifying KD region using primers forward [12] 5′-ACAGCATTCCGCTGACC-ATCAATA-3′ and newly designed reverse 5′-GATACTG-GATTCCTGGAACA-3′. A 914 bp PCR product was purified using QIAquick PCR purification kit (Qiagen). A cycling sequencing reaction was prepared with the same primers using BigDye Terminator kit v. 3.1 (Applied Biosystems) according to the manufacturer's manual. Sequencing products were purified with DyeEx 2.0 Spin kit (Qiagen). The samples were then incubated at 55 °C for 30–60 min to dry the sequencing products, which were further dissolved in 25 μl of formamide (Applied Biosystems) and denatured at 96 °C for 1 min. Sequencing of both strands was carried out in 3130 sequencer (Applied Biosystems). Sequences were evaluated with the Mutation Surveyor program (SoftGenetics, State College, PA, USA). The sensitivity of sequencing with mutation detection using the program was tested with cDNA mixtures containing 75%, 50%, 20%, 15%, 10% and 5% of mutant BCR-ABL diluted with wild type BCR-ABL cDNA, respectively. The quantities of mutant BCR-ABL and wild

type BCR-ABL cDNAs analyzed by real-time RT PCR [17] were equal. After sequencing, the percentage of mutant signal in both strands was estimated by Mutation Surveyor's quantification tool. In these diluted samples as well as in patients' samples, the sensitivity of the assay was approximately 20%. This is in line with other authors [12,18]. However, the program was able to find and quantify as little as 5% of mutant signal when sequences of all samples with the same type of mutation but with different percentage of mutant allele were evaluated in one file. If such sample was tested separately it was detected as negative.

2.5. HRM design and optimization

Generally, it is recommended to use PCR product of up to 250 bp for best discrimination. Therefore, four primer pairs were newly designed using WWW primer tool Primer3 [19]. A sequence with accession number M14752 (NCBI databank) was used as a reference. For better identification we named the primer pairs and appropriate PCR fragments as HRM1–HRM4 (see positions in Fig. 1). (1) HRM1 a 221 bp fragment: forward 5′-CTCATCACCACGCTCCATTA-3′, reverse 5′-TCTTCCACCTCCATGGTGTC-3′, corresponding to nt 989–1209 and covering amino acids P216-E275; (2) HRM2 a 225 bp fragment: forward 5′-ACACCATGGAGGT-GGAAGAG-3′, reverse 5′-TGGCTGACGAGATCTGA-GTG-3′, corresponding to nt 1191–1415 and covering amino acids F283-M343; (3) HRM3 a 239 bp fragment: forward 5′-ATGGCCACTCAGATCTCGTC-3′, reverse 5′-ACGTCGGACTTGATGGAGAA-3′, corresponding to nt 1391–1629 and covering amino acids A350-K415; (4) HRM4 a 241 bp fragment: forward 5′-CGTCTGGGCATTTGGAGTAT-3′, reverse 5′-ACTGGATTCCTGGAACATTG-3′, corresponding to nt 1627–1867 and covering amino acids L429-T495.

Twelve samples with different mutations mostly 100% mutant together with reference wild type (0% mutation) were used to optimize HRM1–HRM4. Reaction conditions were universal for all four primer pairs. The selectively amplified product of BCR-ABL kinase domain was used as a template. The template was 1000× diluted when the amplicon was visible under the UV light after electrophoresis. Each run contained samples in duplicate and wild type control as the reference in triplicate to ensure reproducibility of the melt curves. Real-time PCR followed by HRM was carried out in Rotor Gene 6000 (Corbett Research, Sydney, Australia) for 10 min at 95 °C followed by 40 cycles of 5 s 95 °C, 30 s 58 °C (fluorescence acquisition on HRM channel) and 20 s 72 °C. Cycling phase was followed by hold at 53 °C for 1 min to obtain complete renatured PCR products. The melting phase started at 80 °C, each step rising by 0.1 °C (fluorescence acquisition on the HRM channel), and finished at 93 °C with hold 90 s on the 1st step and 1 s in the next steps. If uncertain results were observed, we repeated HRM phase with 0.02 °C rise for better discrimination. PCR reaction was performed in 10 μl reaction volumes containing 2 μl of template, 1×

PCR buffer (10× Fast Start Taq polymerase buffer, Roche, Basel, Switzerland), 3 mM MgCl$_2$, 1× LCGreen^{TM} I (Idaho Technology, Salt Lake City, Utah, USA), 200 μM dNTPs (Invitrogene), 300 nM primers (Invitrogene) and 0.5 U Fast Start Taq polymerase (Roche). The control without template was used for all four PCR mixes in every run to check contaminations. Further, 44/101 samples were screened in a blind manner with HRM1, 30/101 samples with HRM2, 20/101 with HRM3 and 7/101 with HRM4, respectively (Table 2).

2.6. HRM evaluation

The quality of HRM results is highly dependent on the quality of real-time amplification. Therefore, the quantitative real-time amplification data and data from standard melt analysis were evaluated prior to HRM. This can easily highlight any poorly performing individual assay. Therefore, we assessed Ct values, end point fluorescence level, amplification efficiency and specificity of each sample including reference to identify outliers and filtering them out of subsequent HRM analysis. Ct values reflected the initial amount of template which ideally would be similar for samples and reference. Ct 30 and higher indicated too little starting template amount or sample degradation. Samples with Ct ≥ 30 were repeated with increased template amount. Assays with low end point fluorescence, which could indicate incorrect dye amount, incorrect levels of reaction components, or reaction inhibition, were not scored for HRM. Reactions with amplification efficiency different from reference or with efficiency less than about 1.4 were omitted from evaluation as an outlier and were repeated.

For HRM scoring, one of the reference triplicates was set up as a wild type genotype. The other two were analyzed as controls and scored as wild types. The melt curve regions in raw data window were adjusted to encompass representative baseline data for the pre-melt and post-melt phases. Results were automatically called by the software and confirmed with viewing normalized melt curves and difference graphs (Fig. 2).

2.7. Post-HRM sequencing

HRM1–HRM4 positive amplicons were purified using QIAquick PCR purification kit (Qiagen) before sequencing. Cycling sequencing reaction was prepared with HRM1–HRM4 primers using BigDye Terminator kit v. 3.1. (Applied Biosystems) according to the manufacturer's manual. The subsequent process was the same as described above in *Sequencing*.

3. Results

3.1. HRM1–HRM4 analysis

A total of 101 samples were tested (Table 2). Mutations in BCR-ABL kinase domain were previously found

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Table 2
Characterization of samples used for HRM1–HRM4 testing and results of HRM analyses

HRM1			HRM2			HRM3			HRM4		
Mutation	Mutation ratio* (%)	HRM results	Mutation	Mutation ratio (%)	HRM results	Mutation	Mutation ratio (%)	HRM results	Mutation	Mutation ratio (%)	HRM results
M244V A/G	100	+	T315I	68	+	M351T	100	+	E459K	100	+
	100	+	C/T	52	+	T/C	79	+	G/A	89	+
	100	+		48	+		79	+		30	+
	64	+		40	+		76	+	WT	0	−(4 samples)
	64	+		37	+		57	+			
	30	+		27	+		53	+			
	30	+		14	+		16	+			
	30	+		14	+		16	+			
	25	+	F311I	100	+		8	+			
	25	+	T/A	33	+	H396R	24	+			
	5	+		25	+	A/G	23	+			
	5	+									
E255K G/A	100	+	C/A	91	+		22	+			
	100	+		73	+		19	+			
	60	+		73	+		17	+			
	60	+		23	+	F359V	100	+			
	18	+		23	+	T/G					
	18	+		7	+	WT	0	−(4 samples)			
G250E G/A	68	+	WT	0	−(12 samples)						
	52	+									
	40	+									
	14	+									
	11	+									
Y253H T/C	80	+									
	45	+									
	45	+									
	32	+									
	30	+									
	30	+									
	30	+									
Y253F A/T	78	+									
	22	+									
	5	−									
WT	0	−(11 samples)									

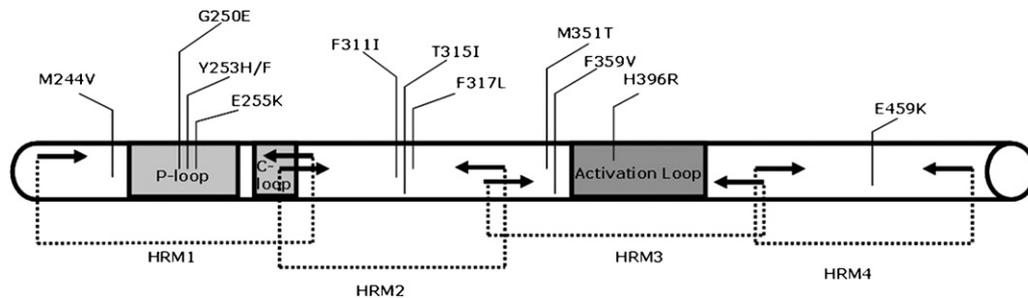WT, wild type; *, mutation ratio in patient's samples analyzed; +, positive; −, negative.

Fig. 1. HRM1–HRM4 regions in BCR-ABL KD. Black arrows indicate positions of each HRM primer pair.

by direct sequencing in 16 CML patients with tyrosine kinase targeted treatment. Altogether 12 different mutations were detected, with double mutations in 5 patients (Table 1) at different times from the beginning of the treatment. The percentage of mutant alleles, determined after sequencing by the DNA quantification tool of Mutation Surveyor program (SoftGenetics), ranged from 0 to 100%.

HRM1–HRM4 primer pairs (Fig. 1) generated specific PCR products with no evidence of primer dimers formation controlled on a derivative plot using the standard melt analysis with software Rotor Gene 6000 Series 1.7 (Corbett Research) and after electrophoresis on 2% agarose gel. Eleven mutations have been detected with the temperature discrimination set to 0.1 °C and in case of M351T to 0.02 °C.
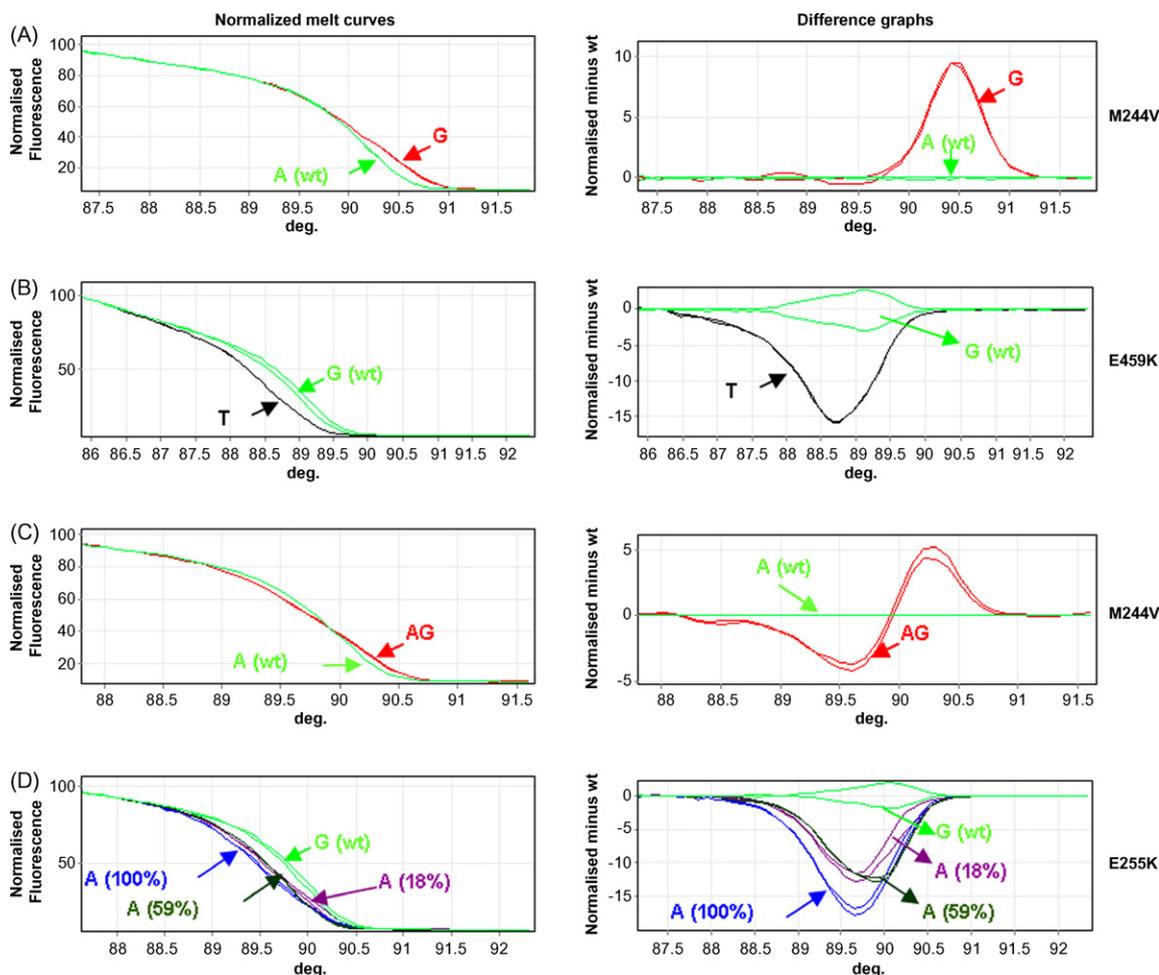
Fig. 2. Examples of high-resolution normalized melt curves. Difference graphs quantitatively determined sample deviation relative to reference (wt). (A) Melt curves of wild BCR-ABL (A) and 100% mutant BCR-ABL (G) of mutation M244V; (B) melt curves of 0% mutant (G) and 100% mutant (A) BCR-ABL of E459K; (C) melt curves of 0% mutant (A) and heteroduplex (AG) formed from 50% of mutant (G) and 50% of wild BCR-ABL (A) of M244V; (D) HRM detection of E255K in samples with different amount of mutant BCR-ABL (A).
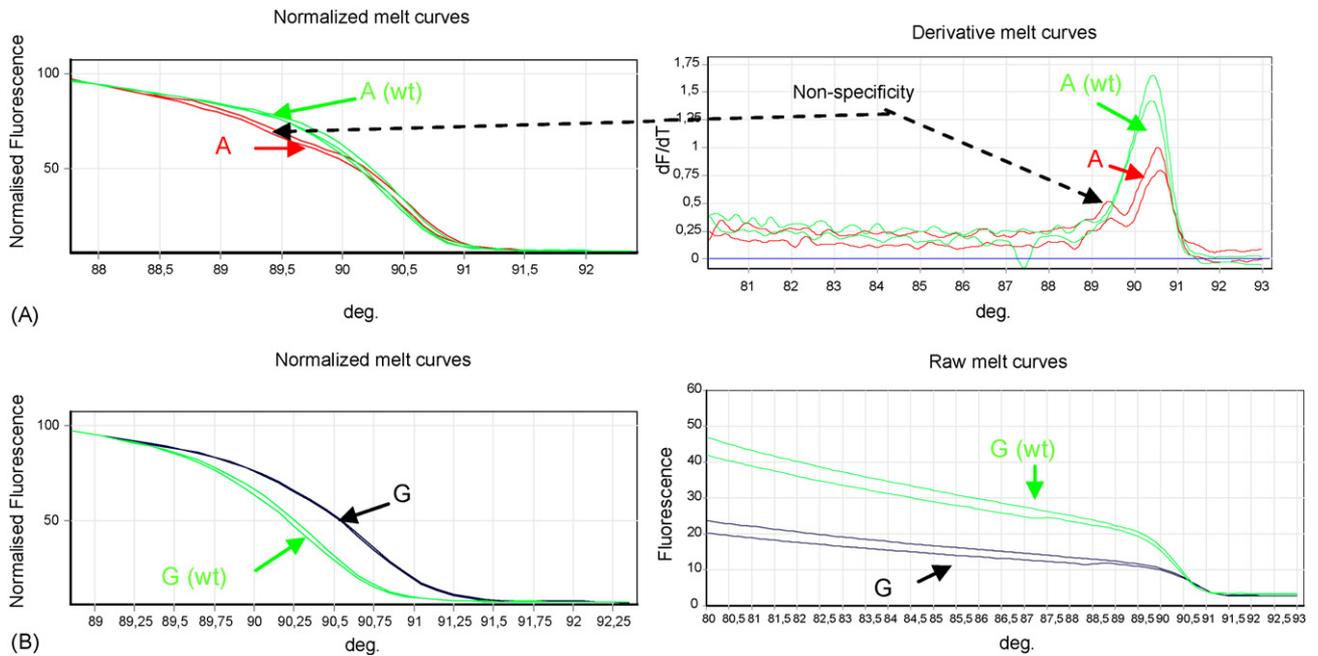
Fig. 3. Examples of HRM false positive results. (A) A false positive sample caused by a non-specific PCR product or a PCR artifact revealed by derivative melt curves of conventional melt curve analysis; (B) A false positive sample caused by low fluoresce at the end point of real-time PCR and at the beginning of melting, respectively.

HRM1 primer pair flanks a region (M1475: 989–1209) with mutations in P-loop. Forty-four samples were processed with these primers. At first, three samples (3/44) were excluded from the HRM evaluation based on real-time PCR and standard melting curve data to avoid false positives (see example in Fig. 3). Assays of these samples were repeated achieving acceptable parameters for HRM. Results of 43/44 samples corresponded to sequencing data. Eleven samples were scored as wild types. Thirty-two samples were positive (12 M244V, 6 E255K, 5 G250E, 7 Y253H, and 2 Y253F). One sample was found to be negative by HRM but contained 5% allele with mutation Y253F (A/T).

HRM2 PCR product (M1475: 1191–1415) encompasses mutations in codons encoding amino acids which directly contact tyrosine kinase inhibitors. Thirty samples were ana-

lyzed. Analysis of one sample (1/30) was repeated with increased template amount due to initial poor amplification (Ct = 30). Results of all 30 samples corresponded to sequencing data. Twelve (12/30) samples were identified as wild types and 18 as mutants (8 T315I, 3 F311I, and 7 F317L).

HRM3 primers amplified a fragment (M1475: 1391–1629) detecting mutations in and around activation loop. Twenty samples were analyzed with these primers. Real-time PCR and HRM were repeated with two samples because of low endpoint fluorescence. Results from HRM3 in several samples were not certain. Therefore, only the HRM step was repeated with 0.02 °C rise. Then the results were scored with certainty. Obtained data were concordant with sequencing; four samples were detected as wild types and 20 as mutants (9 M351T, 6 H396R, and 1 F359V). Retrospectively we found,
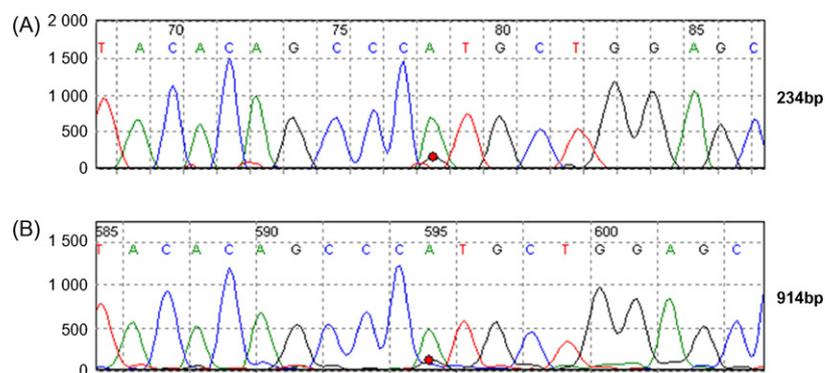

Fig. 4. Comparison of post-HRM sequencing and conventional sequencing. (A) A region of the sequence performed from HRM3 product of a sample with estimated 21% of mutant allele (H396R). The mutant signal is highlighted with a dark dot. (B) A region of the sequence of the same sample performed from a 914 bp PCR product with 24% of mutant signal (dark dot).

that the samples with previous uncertain results contained M351T mutation.

HRM4 (M1475: 1627–1867) was tested with seven samples. In all cases the results of sequencing analyses were confirmed. Four samples were scored correctly as wild types and 3/7 as mutants.

3.2. Post-HRM sequencing

It would be advantageous to directly sequence the PCR product after positive HRM to characterize and quantify the mutation. Therefore, we tested LC Green I interference during sequencing of HRM product. We did not observe any interference as the sequencing product was read in denatured status, so it was improbable the intercalating dye would emit fluorescence. Also the quantity of mutant allele was not influenced by this procedure in comparison to previous sequencing (Fig. 4). This means that we can characterize the mutation by sequencing after positive HRM on the same day.

4. Discussion

For routine practice, sequencing is a laborious and expensive procedure to check, whether the sample is positive on mutation in BCR-ABL KD. Therefore, another method which is simple to perform, cheap and fast, should be used for initial screening. Only positive results would then be sequenced. With the aim of reducing the number of samples that need to be sequenced we tested a new technique- high resolution melting. We screened 101 samples from CML patients with mutation ratios varying from 0 to 100% (Table 2). HRM results of 100/101 samples were concordant with sequencing. Only one sample with 5% of mutant allele (Y253F) was scored by HRM as negative. It was not a real discrepancy, because the value of 5% was estimated after sequencing only under specific assay order (see Section 2). The Y253F mutation is caused by purine/purine single nucleotide substitution (A/T). This probably contributed to the reduced efficiency of discrimination of melting curves. Generally, the best discrimination efficiency in HRM is achieved when purine/pyrimidine and pyrimidine/purine nucleotide substitutions are detected. Other mutations with low ratio in the samples were detected (e.g. M244V, 5%; F317L, 7%, see Table 2). In this study, the sensitivity of HRM detection of mutations tested was higher or comparable to conventional sequencing (see Table 2). Using HRM, it was possible to detect as low as 5% of mutation in the sample.

Different genotypes showed unique transitions that were revealed based on the basis of shape comparison and difference plots of the HRM melting curves (Fig. 2). The shape of melting curves was influenced by the amplification efficacy, different initial template amount, or non-specificity (Fig. 3). We primarily identified 6/101 outliers not involved in HRM evaluation, avoiding thereby false positive/negative results based on real-time PCR data. Thus, the assays were repeated

achieving optimal parameters for HRM evaluation. All mutations tested were detected with significant differences in melting curves of mutant and wild type PCR products. However, for HRM3 there would be necessary to use 0.02 °C rise during melting due to poorer discrimination efficiency using 0.1 °C rise in the M351T detection.

One of the great advantages of HRM was a short time of analysis. HRM analyses of 72 samples on Rotor Gene 6000 took the same time as used for regular PCRs. HRM is unusual among the conventional mutation screening techniques in that homozygous changes can be detected without mixing with wild type [20]. We proved this on all samples with high mutation ratio (Table 2). Of all available mutation screening techniques, HRM is the only method that can be performed in the same container that was used for PCR amplification. Conventional mutation screening techniques (e.g. DHPLC, denaturing gradient electrophoresis, pyrosequencing) require additional steps after PCR and increase the risk of contamination in next reactions due to PCR products exposition to the environment [21].

In ideal case, it would be great in routine practice to perform only one HRM per sample to detect all possible mutations in the whole ABL kinase domain (approximately 1000 bp); however HRM is the most sensitive with short PCR products (up to 250 bp). Therefore, it is necessary to perform four PCRs per each sample. On the other hand this enables us to predict the mutation position in KD before sequencing. Mutation positive samples identified by HRM, DHPLC and double-gradient denaturing electrophoresis have to be sequenced to characterize the type of mutation. This does not hold for pyrosequencing, allele-specific PCR and SEQUENOM Mass Array. On the other hand, multiple individual assays are necessary to perform.

In conclusion, HRM seems to be suitable for initial rapid screening of BCR-ABL KD mutations followed by direct sequencing only positive samples. This approach reduces the number of samples for sequencing. We proved that the HRM dye did not interfere during sequencing. Therefore it was possible to directly sequence the HRM positive products, which accelerated whole assay that could be done within 1 day.

revision and final approval of the article. Jana Moravcova drafting of the article, critical revision and final approval of the article.

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