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ORIGINAL ARTICLE: RESEARCH

Early detection and quantification of mutations in the tyrosine kinase domain of chimerical *BCR–ABL1* gene combining high-resolution melting analysis and mutant-allele specific quantitative polymerase chain reaction

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Abstract

***BCR–ABL1* point mutations are the most common cause of resistance in patients with chronic myeloid leukemia (CML) who fail or lose response to tyrosine kinase inhibitors. We have developed a rapid method to screen *BCR–ABL1* mutations by high resolution melting (HRM). We designed a strategy based on amplification refractory mutational system-quantitative polymerase chain reaction (ARMS-qPCR) to identify and quantify several clinically relevant mutations. From 856 patients with CML studied during 2 years in our laboratory, we selected 32 who showed persistent levels of *BCR–ABL1* transcripts (≥0.1%) in at least two consecutive studies. Using our strategy, we identified mutations in 11/32 cases (34.4%), while only two of them (6.2%) were detectable by sequencing. Furthermore, we were able to estimate the timing and dynamics of mutated clones, evaluating retrospective samples from the same patient. In cases with lack or loss of molecular response this analysis might be useful for designing early therapeutic strategies.**

Keywords: CML, HRM, ARMS, screening, mutations

Introduction

The treatment of chronic myeloid leukemia (CML) represents a model for targeted cancer therapy. Adenosine triphosphate (ATP)-competitive kinase inhibitors that block *BCR–ABL1* kinase activity, particularly imatinib mesylate (Gleevec), can induce durable responses in the vast majority of patients [1]. However, the emergence of resistant leukemia clones bearing mutations in the *BCR–ABL1* kinase domain (KD) represents a major mechanism of disease recurrence that can be overcome by using another tyrosine kinase inhibitor (TKI) [2]. However, the absence of a *BCR–ABL1* KD mutation does not exclude drug resistance [3], since other mechanisms of resistance can be acquired, including *BCR–ABL1* gene amplification, transcript overexpression, alterations in drug-efflux kinetics, up-regulation of other kinase pathways, and rare *BCR–ABL1* mutations outside of the KD [4]. One or more *BCR–ABL1* KD mutations have been detected by direct DNA sequencing in 30% and 50% of patients with chronic phase CML who develop resistance to imatinib [5,6]. Mutation frequency is higher in patients with the accelerated or blast phase of the disease, especially the lymphoid blast phase [7]. Importantly, in those patients with imatinib resistance due to KD mutations, the use of more potent kinase inhibitors, including dasatinib, nilotinib and bosutinib, can often help to overcome resistance [8]. Particular methods used to detect *BCR–ABL1* KD mutations will obviously have a great influence on detection frequency and clinical decision. A variety of mutation detection methods exist, showing a wide range of analytical sensitivities, from the least sensitive direct Sanger sequencing method, detecting a mutation in approximately 1 in 5 *BCR–ABL1* transcripts, to the highly sensitive mutation-allele specific quantitative polymerase chain reaction (PCR) methods, which can reliably detect a mutant transcript down to 1 in 10 000 *BCR–ABL1* transcripts. Other reported screening methods for *BCR–ABL1* KD mutations include denaturing high performance liquid chromatography (DHPLC), targeted microarrays and high resolution melting (HRM) [9]. Pyrosequencing and the amplification refractory mutation system (ARMS) have been adopted as quantitative mutation detection methods to track the level or proportion of a mutated clone after therapy switch [10,11].

This study was undertaken with the dual purpose of carrying out early screening of mutations by HRM and quantifying them by mutation-specific quantitative PCR in a series of patients with CML who never achieved or lost the major molecular response (MMR) in a course of targeted therapy. Molecular monitoring and detection of mutations

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in retrospective samples allowed us to track the dynamics of mutated clones.

Materials and methods

Patients' characteristics

The study was carried out according to an institutional review board-approved laboratory protocol. Informed consent was obtained from all subjects. Bone marrow aspirate or peripheral blood specimens from 65 patients with clinically resistant CML with imatinib failure [12] had previously been studied by direct sequencing (50 unmutated [UMut] cases and 15 harboring mutation [Mut] in the *BCR–ABL1* KD). Furthermore, we selected 32 patients who showed a suboptimal molecular response to TKI [13] (persistent levels of *BCR–ABL1* transcripts >0.1% in at least two consecutive studies) from 856 patients with CML studied during 2 years in our laboratory. Also, 15 healthy donors were analyzed.

Sample preparation

Total RNA was extracted from leukocyte TRIzol lysates (Invitrogen). The extraction procedure was followed according to the manufacturer's manual; cDNA synthesis was performed using random hexamer primers and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). A 1313 bp large PCR fragment containing *BCR* and *ABL1* sequences was amplified using primers BCR13 and ABL7 (Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.718767). This PCR product was used as a universal template for HRM, ARMS and single-strand conformation polymorphism (SSCP) and for second-round PCR for direct sequencing analysis.

BCR–ABL1 transcript quantification

Quantitative real time (qRT)-PCR assay was performed on total RNA extracted from peripheral blood using the Rotor-Gene PCR Cycler (Qiagen). The *BCR–ABL1*/*ABL1* ratio was determined using the *BCR–ABL1* qRT-PCR Kit (MolecularMD), according to the manufacturer's instructions. The methodology that we employed to quantify *BCR–ABL1* expression was cross-validated with the Institute of Medical and Veterinary Science (Adelaide, South Australia) [14].

Direct sequencing

BCR–ABL1 rearrangement was first amplified (1313 bp, 1000× diluted when visible under ultraviolet [UV] light after agarose electrophoresis) followed by a nested PCR that amplifies the KD of *ABL1* (exons 4–7: 586 bp, covering amino acids 228–423) of rearranged *BCR–ABL1* (Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.718767). The 586 bp PCR products were purified using GFX columns (GE Healthcare). The DNA sequencing reaction for both strands was carried out at the Macrogen Inc. facility (Seoul, Korea). Sequences were evaluated using the Mutation Surveyor program (SoftGenetics, State College, PA).

Plasmid control preparation

A TOPO TA Cloning Kit (Invitrogen) was used to obtain positive controls for genetic variants of interest: positive plasmid control (PPC) and negative plasmid control (NPC). The 1313 bp large PCR fragments containing *BCR* and *ABL1* sequences were amplified using cDNA from patients harboring each KD mutation of interest; column purified PCR product was cloned into a TOPO TA vector. In each case, the presence of the specific mutation was further confirmed by direct sequencing. Plasmids were linearized and evaluated for DNA mass by spectrophotometry at 260 nm, and finally mass values were converted to copy number values.

HRM study design and analysis

For HRM analysis using the fragment of 1313 bp, we generated three amplicons designated as HRM-P, -A and -C of 170, 131 and 100 bp, corresponding to nucleotides (nt) 957–1126, 1150–1280 and 1323–1422 (NM_005157.4), respectively (Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.718767). For HRM, a PCR reaction was performed in 25 μL containing 2 μL of 1/1000 diluted template generated as described above, 12.5 μL of Mezcla Real 2× (Biodynamics, Argentina) and 500 pmol of each primer. The quality of HRM results is highly dependent on the quality of real-time amplification; for this reason, only when a strong fluorescent signal was generated ($C_t < 30$) was the sample considered for post-PCR analysis by HRM. HRM melting curve data were obtained by slowly increasing the temperature from 75 to 95°C at a rate of 0.1°C/s. The melting status and changes in T_m value were analyzed using Rotor-Gene software (Rotor-Gene Q Series software 1.7). For HRM scoring, one of the reference triplicate NPCs was set up as a UMut genotype. Results were automatically analyzed by the software and confirmed by viewing normalized melt curves and difference graphs.

Single-strand conformation polymorphisms

The same HRM-PCR products (HRM-P, HRM-A and HRM-C) were also analyzed by SSCP. Polyacrylamide gel running conditions were optimized in order to discriminate Mut PCR products from UMut products using controls with previously identified mutations. The SSCP analysis was carried out as follows: 3 μL of PCR product was added to 2 μL of denaturating solution (0.05% of xylene-cyanol, 0.05% of bromophenol blue dye and 20 mM ethylenediaminetetraacetic acid [EDTA] in formamide 95%). After heat denaturation (95°C for 5 min), samples were immediately chilled on ice and then run (3 h and 30 min at 200 V, 4°C) on 17.5% acrylamide:bisacrylamide gels (37.5:1) in 1× Tris-borate-EDTA buffer. The run was performed in a Mini-Protean III system (7.3 × 10.2 × 0.1 cm) (BioRad, Hercules, CA). The nucleotide change was confirmed by direct sequencing of the PCR product.

Amplification refractory mutational system-quantitative PCR study design

We designed a set of primers, some of them previously published [11], to detect 15 nucleotide changes corresponding to 12 clinically relevant mutations by ARMS-qPCR (Table I,

Table I. Primer specificity evaluation* used for ARMS-qPCR.

	PPC		NPC				Genetic variants		
Assay name	*Ct* ND	*Ct* D	*Ct* ND	*Ct* D	$\Delta Ct^{(ND-D)}$	Bgd	Codon number	Amino acid change	Nucleotide change
V299La	15.74	16.24	16.92	30.80	13.88	6.6×10^{-3}	299	Val > Leu	G > T
V299Lb			16.92	35.63	18.71	2.3×10^{-4}	299	Val > Leu	G > C
F311I	16.48	16.47	16.42	28.46	12.04	2.3×10^{-2}	311	Phe > Ile	T > A
T315I	17.55	17.92	18.00	28.77	10.77	5.7×10^{-2}	315	Thr > Ile	C > T
F317La	15.83	15.92	16.45	25.52	9.07	1.8×10^{-1}	317	Phe > Leu	T > C
F317Lb			16.45	39.43	22.85	1.3×10^{-5}	317	Phe > Leu	C > G
F317Lc			16.45	32.81	16.41	1.1×10^{-3}	317	Phe > Leu	C > A
E255K	16.33	16.77	17.36	29.13	11.77	2.8×10^{-2}	255	Glu > Lys	G > A
E255V	17.65	17.62	18.65	33.04	14.39	4.6×10^{-3}	255	Glu > Val	A > T
M351T	15.12	15.45	16.52	33.47	16.95	7.8×10^{-4}	351	Met > Thr	T > C
E355G	15.69	15.63	17.14	30.24	13.10	1.1×10^{-2}	355	Glu > Gly	A > G
M244V			17.24	28.77	11.53	3.3×10^{-2}	244	Met > Val	A > G
L248V			14.83	25.00	10.70	6.0×10^{-2}	248	Leu > Val	C > G
G250E			19.26	30.06	10.80	5.6×10^{-2}	250	Gly > Glu	G > A
F359V			15.95	32.76	16.81	8.7×10^{-4}	359	Phe > Val	T > G

*C_t value for perfect match (PPC amplified with D) and mismatch (NPC amplified with D) templates. Blank cell indicates that the corresponding mutated plasmid was not available. V299La, GTG > CTG; V299Lb, GTG > TTG; F317La, TTC > CTC; F317Lb, TTC > TTG; F317Lc, TTC > TTA.
ARMS-qPCR, amplification refractory mutational system-quantitative polymerase chain reaction; PPC, positive plasmid control; NPC, negative plasmid control; C_t, cycle threshold; ND, non-discriminative primer; D, discriminative primer; Bgd, background signal.

Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.718767). The assay is based on the detection of mutated clones in RNA templates transcribed to cDNA. Clones were detected and quantified by real-time PCR using SYBR-Green chemistry. PPCs for eight mutations were obtained (V299L, F311I, F317L, T315I, E255K, E255V, M351T and E355G), with the aim of determining sensitivity and specificity thresholds. ARMS-qPCR reactions were prepared in a final volume of 25 µL using the SYBR-Green Universal PCR Master Mix 2× (Roche). Thermal cycling conditions consisted of an initial incubation at 50°C for 2 min, DNA polymerase activation at 95°C for 5 min, 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s and extension at 72°C for 10 s. Gene-specific reverse primers complementary to sequences immediately downstream of the mutation were used in combination with a common forward primer. Templates were either a 1000-fold dilution of the previously described PCR amplification of patient samples or serial 10-fold dilutions of the matched (MAT; PPC amplified with mutation specific primer) and mismatched (MIS; NPC amplified with mutation specific primer) plasmid. Samples, PPCs, NPC and distilled water, as a negative control, were analyzed in duplicate with both primer sets: non-discriminative (ND) and discriminative (D) within the same run. All real-time PCR analyses were performed on the Rotor-Gene real-time PCR platform (Qiagen). To estimate primer efficiency in each case, serial 10-fold dilutions of a MAT plasmid were used to construct standard curves (five points). The resulting C_t values were plotted against plasmid copy number.

The background of each assay was estimated as follows:

$$\text{Bgd} = \frac{100}{(1+E_{\text{MUT}})^{\Delta C_t^{(\text{MIS}-\text{MAT})}}}$$

where Bgd = background of the assay, E_{MUT} = discriminative MUT primer efficiency, MIS = mismatch template, MAT = match template.

Percentage of tumor burden was calculated taking into account primer efficiencies and background as follows:

$$\text{Fgd} = \left[\frac{(1+E_{\text{ND}})^{C_t^{(\text{ND})}}}{(1+E_D)^{C_t^{(\text{D})}}} - \text{Bgd}\right]\times 100$$

where Fgd = foreground of the assay, C_t = cycle threshold, E_{ND} = non-discriminative primer efficiency, E_{D} = discriminative primer efficiency.

By calculating the difference between the ratios of ARMS-qPCR and qRT-PCR at different time points we could estimate the dynamics of the mutation and evaluate the accumulation rate of the mutation:

$$\Delta\text{MUT}^{\text{AR}} = \frac{\left[\frac{(\text{ARMS}-\text{qPCR})}{(\text{qRT}-\text{PCR})}\right]_{T_{\text{L}}} - \left[\frac{(\text{ARMS}-\text{qPCR})}{(\text{qRT}-\text{PCR})}\right]_{T_{\text{F}}}}{\Delta\text{Time}}$$

where $\Delta\text{MUT}^{\text{AR}}$ = accumulation rate of mutation, T_{L} = last study time point, T_{F} = first study time point, ΔTime = difference in months between T_{L} and T_{F}.

Results

Serial-dilution experiments with plasmid DNA for HRM analysis

We examined three regions of *ABL1* KD: a 170 bp amplified region containing amino acids (aa) 221–278 (HRM-P), a second region (131 bp) containing aa 286–329 (HRM-A) and a third region (100 bp) from aa 343 to 376 (HRM-C) (Supplementary Figure 1 available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.718767). Specific primer pairs that generated specific PCR products with no evidence of primer dimer formation were controlled on a derivative plot using standard melt analysis and electrophoresis on 2.5% agarose gel. First, using 10^5 copies each of different plasmid samples with mutations (PPCs), HRM analysis in duplicate was performed, generating constant positive melting curves in terms of both shape and peak height with a range of

melting temperatures (T_m) from 82 to 86°C. On the other hand, NPC constantly produced the UMut scanning profile. After PCR amplification and subsequent HRM analyses of serial dilutions of eight PPCs (V299L, E255K, E255V, F311I, T315I, F317L, M351T, E355G) into NPC (PPC/NPC; 100%, 50%, 25%, 10%, 5%, 2.5%, 1.5%), we obtained fluorescence curve plots relative to UMut plasmid, as shown (Figure 1). We always ran two replicates for each mutation

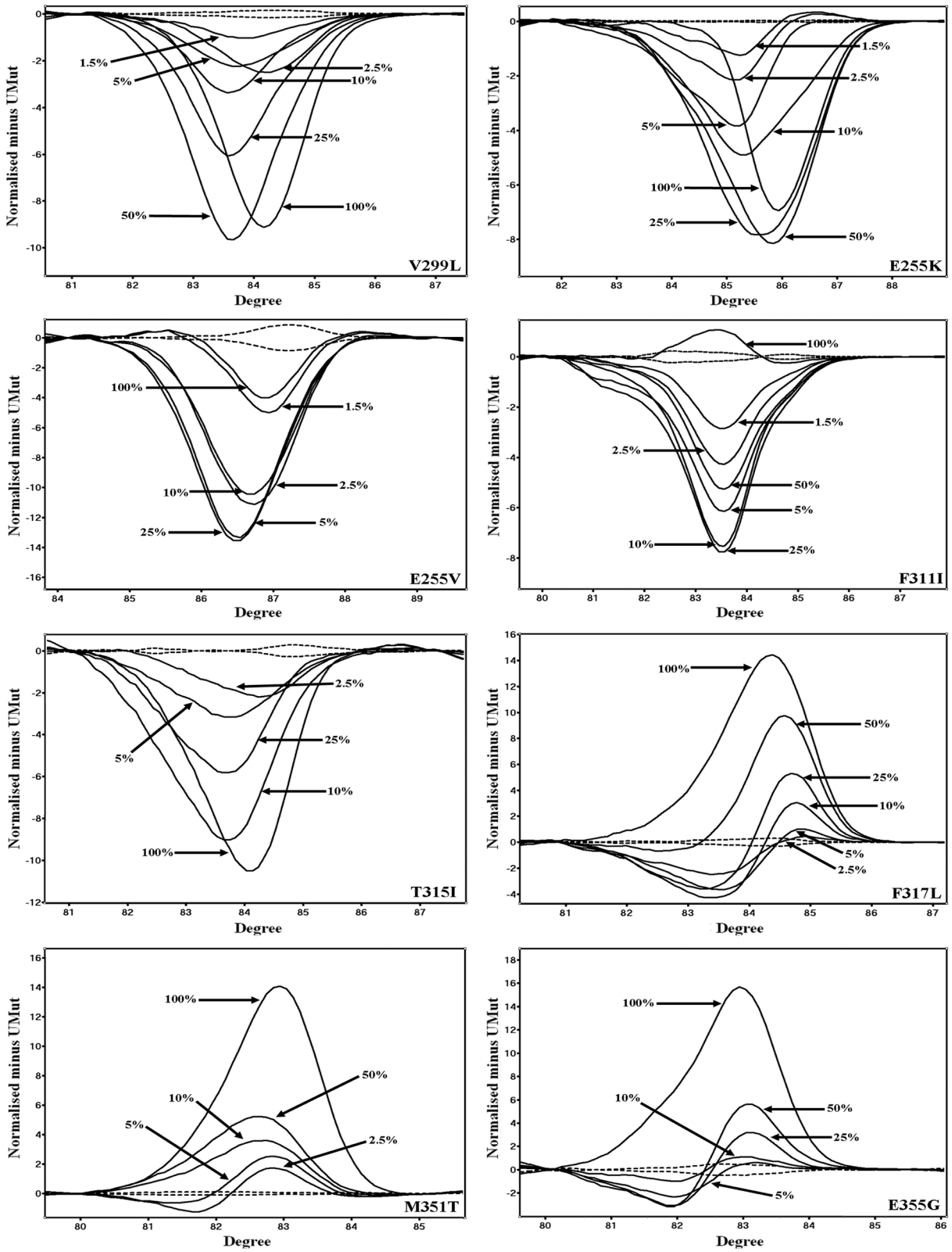

Figure 1. Normalized melting curve graphs from HRM analysis. Eight different PPCs were serially diluted in NPC (100%, 50%, 25%, 10%, 5%, 2.5%, and 1.5%) with the aim of estimating selectivity of the HRM method. Changes in peak heights and T_m values revealed a difference that was detectable in the ratio of Mut and UMut plasmids. HRM assay revealed signals that were not directly proportional to mutation burden, indicating that it is not suitable as a quantitative method. PPC (continuous line), positive plasmid control; NPC (dashed line), negative plasmid control.

and four replicates of UMut samples. Apart from E355G mutation, whose selectivity was between 5 and 10%, the selectivity value (considering all mutations) was on average close to 2.5% (range 1.5–5%). Our experiments with the three amplicons produced comparable results and indicated, approximately, 10-fold of detection selectivity improvement by HRM compared with conventional sequencing.

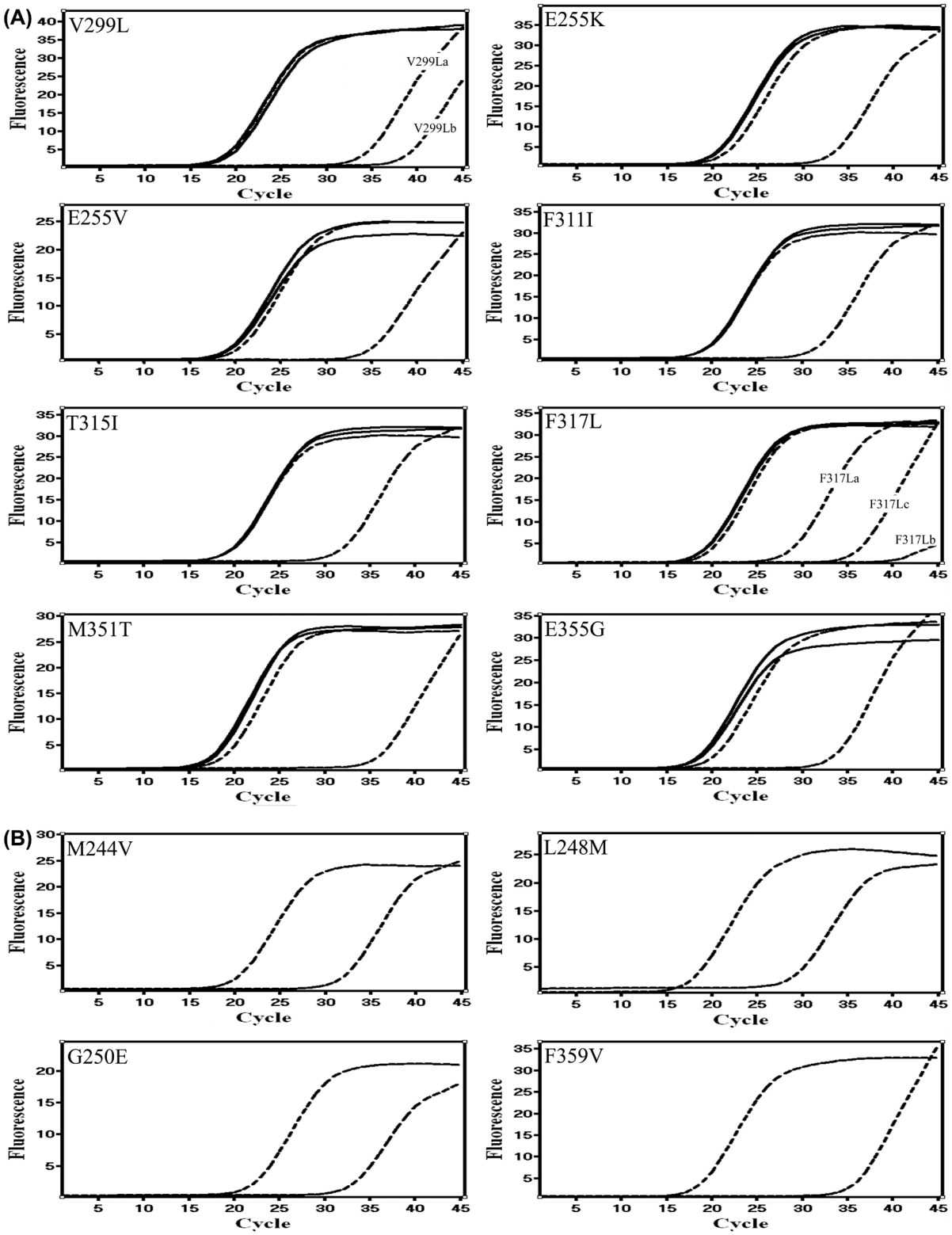

Figure 2. Amplification curves from ARMS-qPCR analysis. (A) Specificity of assays for eight different mutations was determined by calculating ΔCt values between Ct^{MAT} (PPC with D primers) and Ct^{MIS} (NPC with D primers). (B) ARMS-qPCR assays for which PPCs were not available. Dashed line with earlier amplification corresponds to negative plasmid control with non-discriminative primer, and later curve is due to unspecific amplification. This unspecific amplification was considered in all cases for calculation of the mutant clone. ND, non-discriminative primer; D, discriminative primer; F317L Da/Db/Dc, corresponding to nucleotide change TTC > CTC; TTC > TTG; TTC > TTA, respectively. Dashed lines: NPC with D and ND primers. Continuous lines: PPC with D and ND primers.

Serial-dilution experiments with plasmid DNA for ARMS-qPCR analysis

In Table I are described 15 relevant nucleotide changes that represent 12 amino acid substitutions associated with resistance to TKIs. Similarly to HRM analysis, eight PPCs and one NPC were used as templates for mutation targeted assays (ARMS-qPCR). Standard curves were constructed to determine whether PCR was efficient. The sensitivity was further improved by a nested approach. A series of 10-fold dilutions from 1×10^6 to 1×10^1 copies/μL of plasmid controls were used in the real-time PCR assay with discriminative and non-discriminative primers. Standard curves showed a range of slope from −3.2 to −3.5, indicating that PCR efficiency was close to 100%. A system based on plasmids containing UMut or Mut sequences and C_t^{MAT}, C_t^{MIS} and ΔC_t (see "Materials and methods" section) was used to evaluate the specificity of the real-time PCR approach for detection of *BCR–ABL1* mutations. The ΔC_t values were determined using 10^5 copies of PPC and NPC controls. The goal was to maximize the difference in amplification of the matching versus the mismatching template $[\Delta C_t^{(MIS-MAT)}]$ (Figure 2; Table I). In Table I it can be appreciated that background (Bgd) values were very low, and in all cases the selectivity of the assays was 0.1% or lower.

Comparison of qualitative detection of *ABL1* KD mutations by HRM with direct sequencing

To determine whether the validated HRM methodology using plasmids could also be applied to patient samples, a total of 65 (15 Mut and 50 UMut) previously sequenced samples from patients with chronic phase CML and 15 healthy donors were analyzed. HRM analysis showed a concordance of 100% regarding mutations detected in the 15 patients with Mut CML (Table II). No mutated profile was found in peripheral blood samples derived from 15 healthy individuals. The 50 imatinib-resistant UMut patients were subsequently analyzed by HRM. A total of 13/50 patients showed an HRM profile compatible with the presence of a mutation: three mutated profiles were detected in HRM-P, seven in HRM-A and three in HRM-C fragments (Table III).

Confirmation and quantification of mutated HRM profiles by ARMS-qPCR

The 13/50 patients positive by HRM were further genotyped by ARMS-qPCR. We confirmed the presence of mutations in eight of them (Table III). The median mutated burden measured by ARMS-qPCR of those cases that were sequencing-negative and HRM-positive was markedly lower than 20% (median 8.8%, range 1.2–23%), indicating higher selectivity of HRM with respect to direct sequencing. Five HRM-positive cases could not be confirmed by ARMS-qPCR, probably because we sought only some clinically relevant mutations. However, the SSCP method, which is less sensitive (5–10%) than HRM, was useful in two of five cases (Table III).

Mutation detection and monitoring in patients with CML with suboptimal response

Among 856 CML patient-samples sent to our laboratory over a period of 2 years for *BCR–ABL1* mRNA quantification, we selected 32 from patients who showed characteristics of suboptimal response (less than major molecular response after 18 months of treatment). These samples were examined for the existence of TKI resistance-associated mutations using HRM for initial screening, detecting an abnormal HRM pattern in 13 out of 32. ARMS-qPCR confirmed the presence of mutations in 11 cases (11/32, 34.4%). The remaining two HRM-positive cases could not be confirmed by ARMS-qPCR, because one case presented the L298L polymorphism, and the other, probably, harbored a rare mutation (Table IV). Moreover, using ARMS-qPCR, we were able to track mutation back over time (Figure 3) in all of them, thereby permitting the monitoring of mutated clones from a low concentration (0.1%). In four cases harboring four different mutations (G250E, T315I, V299L and F317L), we calculated the ratio between relative mutation quantification using specific ARMS-qPCR and relative quantification of *BCR–ABL1* transcripts obtained by qRT-PCR. As shown in

Table II. Patients with mutations detected by direct sequencing and confirmed by HRM.

Sample	Sequencing	HRM-P	HRM-A	HRM-C
Mut 1	T315I		Mut	
Mut 2	T315I		Mut	
Mut 3	T315I		Mut	
Mut 4	T315I		Mut	
Mut 5	T315I		Mut	
Mut 6	F317L		Mut	
Mut 7	F317L/E255Q/V299L	Mut	Mut	
Mut 8	V299L/E355G		Mut	Mut
Mut 9	E255K	Mut		
Mut 10	E255V	Mut		
Mut 11	E255V	Mut		
Mut 12	G250E	Mut		
Mut 13	Y253H	Mut		
Mut 14	F359I			Mut
Mut 15	M351T			Mut

HRM, high resolution melting; Mut, mutated.

Table III. Sequence-negative patients analyzed by HRM, SSCP and ARMS-qPCR.

Sample	Sequencing	HRM	*BCR–ABL1*/*ABL1* (%)	ARMS-qPCR (%)	SSCP
HRM-P					
1	UMut	Mut	15	UMut*	Mut
2	UMut	Mut	9	UMut*	Mut
3	UMut	Mut	25	G250E (4.24)	UMut
HRM-A					
4	UMut	Mut	40	T315I (4)	UMut
5	UMut	Mut	35	T315I (9.6)	UMut
6	UMut	Mut	37	T315I (22)	Mut
7	UMut	Mut	24	V299L (10)	Mut
8	UMut	Mut	15	T315I (8)	Mut
9	UMut	Mut	0.75	UMut*	UMut
10	UMut	Mut	23	UMut*	UMut
HRM-C					
11	UMut	Mut	12	UMut*	NA
12	UMut	Mut	22	F359V (1.17)	UMut
13	UMut	Mut	0.97	F359V (23)	Mut

*The sequence was unmutated for mutations studied in the P-loop (M244V, L248V, G250E, E255V/K), ATP-binding (V299L, F311I, T315I, F317L) and catalytic domain (M351T, F359V, E355G).
HRM, high resolution melting; SSCP, single-strand conformation polymorphism; ARMS-qPCR, amplification refractory mutational system-quantitative polymerase chain reaction; UMut, unmutated, Mut, mutated; NA, non-applicable.

Table IV. Mutational analysis of patients with CML with suboptimal response to TKIs.

Case	Age	Sex	Time of treatment (months)	Previous treatment			Treatment in course			Sequencing	*BCR–ABL1*/*ABL1* (%)	HRM	ARMS-qPCR (%)	ΔMUT^{AR}
				TKI	mg	Months	TKI	mg	Months					
1	48	M	24				Imatinib	400	24	T315I	14.8	Mut	T315I (21)	53.2
2	45	M	24	Imatinib	400	12	Imatinib	600	12	UMut	0.20	Mut	T315I (5.40)	
3	55	M	47				Imatinib	400	47	UMut	1.00	Mut	T315I (2.35)	
4	43	M	61	Imatinib	400	36	Dasatinib	100	25	F317L	5.05	Mut	F317L (36)	0.1
5	50	M	23	Imatinib	400/800	8/5	Dasatinib	100	10	UMut	0.13	Mut	F317L (8)	
6	20	M	38	Imatinib	400	25	Imatinib	800	13	UMut	0.28	Mut	F317L (3.85)	
7	41	F	33	Imatinib	600	15	Nilotinib	800	18	UMut	0.24	Mut	V299L (8.5)	0.4
8	78	F	24				Imatinib	600	24	UMut	0.11	Mut	V299L (2.85)	
9	36	M	37	Imatinib	400	24	Nilotinib	400/800	12/1	UMut	25.0	Mut	V299L (2)	
10	68	F	108	Imatinib	400/600	84/12	Nilotinib	200	12	UMut	16.0	Mut	V299L (1)	
11	54	M	23				Imatinib	400	23	UMut	3.33	Mut	G250E (11.5)	0.3
12	69	M	42				Imatinib	600	42	L298L*	0.28	Mut	UMut	
13	51	F	55				Imatinib	600	55	UMut	0.87	Mut	UMut†	

*Polymorphism.
†Sequence was unmutated from 12 mutations studied by ARMS-qPCR.
CML, chronic myeloid leukemia; TKI, tyrosine kinase inhibitor; ΔMUT^{AR}, accumulation rate of mutation (see "Materials and methods"); ARMS-qPCR, amplification refractory mutational system-quantitative polymerase chain reaction; UMut, unmutated, Mut, mutated.

Table IV, these ratios are indicative of the accumulation rate for each mutation (ΔMUT^{AR}). T315I mutation showed a significantly higher accumulation rate ($\Delta MUT^{AR} = 53.2$, case 1) than other mutations such as F317L ($\Delta MUT^{AR} = 0.1$, case 4), V299L ($\Delta MUT^{AR} = 0.4$, case 7) or G250E ($\Delta MUT^{AR} = 0.3$, case 11) (see "Materials and methods" section).

Discussion

BCR–ABL1 point mutations are the most common mechanism of resistance in patients with CML who fail or lose response to TKI treatment [15,16]. These mutations have the ability to impair the drug–protein interaction while preserving kinase activity. To date, more than 100 different mutations have been described, affecting more than 70 amino acids of the *ABL1* kinase domain with varying degrees of clinical relevance [17]. Tyrosine kinase domain (TKD) mutation precedes or accompanies disease relapse and progression to an advanced phase, and hence its importance for early detection, in order to choose the most appropriate treatment. Several methods have been developed for mutation detection in *ABL1* KD with different sensitivities and selectivities. The most commonly used is direct sequencing, with a sensitivity of about 20% [18]. Other methodologies such as DHPLC and pyrosequencing have been reported, and showed an increased sensitivity [19], although none of them, up to now, are routinely used in the practical clinical setting, maybe due to their high cost and the necessity of specific equipment. Recently, HRM, performed with the same real-time PCR thermocycler that is routinely used to monitor molecular response in patients with CML, was reported to be a useful method for analyzing genetic variations in patients with CML [20,21]. This method is simple to perform, cheap and fast, and could be used for initial screening.

In this study, we carried out the screening of *ABL1* variants using HRM and subsequent identification and quantification of mutations by ARMS-qPCR. Both methods were tested with plasmids containing corresponding mutant or unmutated sequences. This procedure enabled us to evaluate the proportion of a mutated clone by calculating the ratio of mutant *BCR–ABL1* transcripts to total *BCR–ABL1* as measured by real-time PCR. This ratio also allowed us to evaluate the dynamics of mutated clones by retrospective analysis of samples from the same patient.

The specificity of the HRM methodology was evaluated by analyzing blood from healthy controls or from patients with CML harboring different TKD mutations previously detected by direct sequencing. The melting curves and difference graphs showed no false-positive or false-negative results, confirming the high specificity of the HRM method. In our hands, exhaustive analysis of plasmid mixtures containing variable concentrations of mutated plasmids confirmed a reproducible selectivity between 1.5 and 5% depending on the investigated mutation.

Taking into account the high power of detection of HRM, we decided to carry out screening of mutations in a population of 50 clinically resistant patients with imatinib failure [12], in whom direct sequencing was negative. HRM methodology detected 13 out of 50 cases with a profile consistent with some nucleotide change. Performing ARMS-qPCR analysis in HRM-positive cases, we identified and quantified mutations in eight of them, increasing the detection of mutations by 16% (8/50). In all cases the size of the mutated clone was below or near the detection limit for the direct sequencing method. Since ARMS-qPCR was performed to identify only some clinically relevant point mutations, we speculate that the remaining HRM-positive patients ($n = 5$) could be carriers of rare mutations. These rare mutations could be further characterized by cloning and sequencing of an appropriate number of clones. Current recommendations suggest that mutations should be identified as early as possible, since clinical evidence indicates that some mutations are less sensitive to nilotinib (Y253H, E255K/V and F359V/C), dasatinib (F317L and V299L) or to all available TKIs (T315I). Therefore, it is very important to identify mutations early and investigate the dynamics of mutant clones, before hematologic, cytogenetic or molecular manifestations would permit definition of early therapeutic strategies [22].

Taking into account that high levels of *BCR–ABL1* transcripts can predict the occurrence of mutations [23,24], we performed the screening of mutations by applying

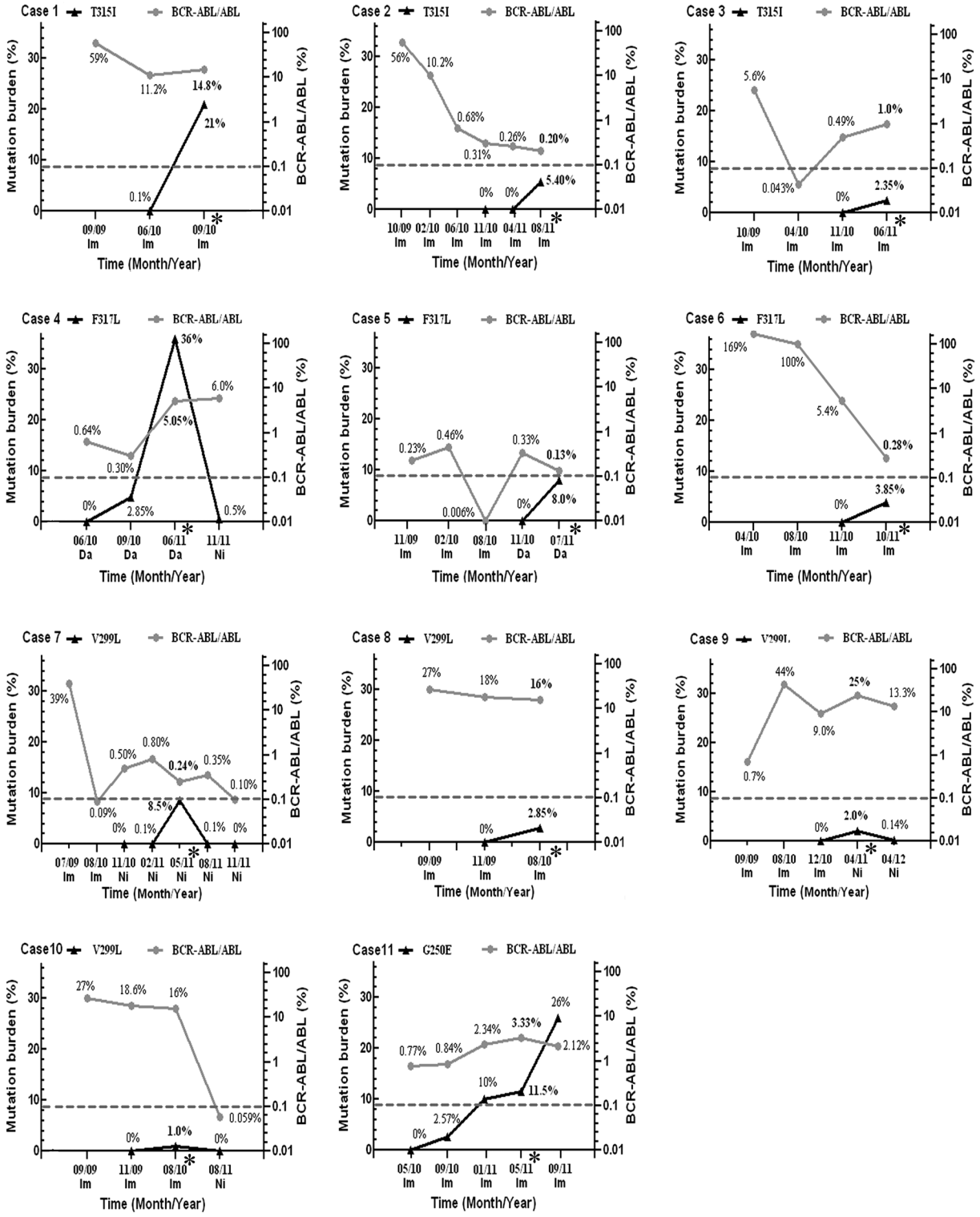

Figure 3. Serial and parallel measurement of *BCR–ABL1* transcript levels and *ABL1* TKD mutation levels. These graphs show the dynamics of *BCR–ABL1* (ARMS-qPCR) mutated clones and *BCR–ABL1* transcripts (qRT-PCR) for 11 patients with a suboptimal response to TKIs. Only in cases 4, 7, 9, 10 and 11 could we track the mutated clone before and after detection of the mutation. In cases 4, 7, 9 and 10, for which treatment was rotated to nilotinib, a significant decrease in the mutant clone burden was observed. In contrast, in case 11, for which treatment was not modified, the percentage of mutated clone increased. *First study of mutation detection.

HRM/ARMS-qPCR in 32 patients with persistently high levels of *BCR–ABL1* transcripts (over 0.1%) in at least two consecutive studies, as proposed by Wang *et al.* [25]. We identified mutations in 11 out of 32 cases (34.4%), while only two cases (6.2%) were positive by sequencing. The results demonstrated that HRM/ARMS-qPCR is more sensitive than the sequencing strategy, and the assay is capable of detecting mutated clones earlier. These findings suggest the necessity to use more sensitive methods, in particular during the early phase of resistance.

ARMS-qPCR assay also allowed tracking of mutated clones. We could estimate the accumulative rate of four mutations during follow-up (ΔMUT^{AR}) as a measure of mutation aggressiveness. The V299L, F317L and G250E mutations showed a significantly lower rate of accumulation (Table IV), likely indicating that in these cases the progression time would be slower. However, T315I displayed a faster accumulation, suggesting increased oncogenic fitness for this mutation under TKI treatment pressure [26]. Additionally, mutant transcripts seemed to exhibit a lower decrease in patients harboring the T315I after cessation of TKI [27].

Although the mutation V299L was detected at levels below 10% in cases 7, 9 and 10 under nilotinib treatment, the mutant clone decreased almost to undetectable levels, indicating the effectiveness of this TKI over this mutation (Figure 3).

In summary, HRM/ARMS-qPCR gives important information that can be useful for monitoring patients with CML under TKI therapy. In particular, the quantitative aspect of the assay is highly relevant for patients exhibiting treatment resistance or suboptimal response, in order to optimize treatment.

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Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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Supplementary material available online

Table listing primers and sequences and Figure showing details of study design.