

High-Resolution Melting Analysis for a reliable and two-step Scanning of Mutations in the Tyrosine Kinase Domain of the Chimerical *bcr-abl* gene

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Running title: HRM Scanning for chimerical *bcr-abl* TKD

Abstract

For relevant Imatinib therapy against Philadelphia (Ph)-positive leukemias, it is essential to monitor mutations in the chimerical *bcr-abl* tyrosine kinase domain (TKD). However, there is no universally acceptable consensus on how to efficiently identify mutations in the target TKD. Recently, high-resolution melting (HRM) technology was developed, which allows gene scanning using an inexpensive generic heteroduplex-detecting dsDNA binding dye. This study aimed to validate HRM's introduction in a practical clinical setting for screening of mutations in sporadic sites of the chimerical *bcr-abl* TDK. All chimerical and wild-type *abl* TKD regions selectively amplified were used for HRM assays and direct sequencing. The HRM test had approximately 2.5 to 5% detection sensitivity for mutations. In contrast to mixture samples with mutant and wild-type cells, all mutant cell samples had indeterminate melting curves equivalent to those of the wild-type due to formation of only a homoduplex. This issue was improved by the addition of exogenous wild-type DNA after PCR. Subsequently, HRM results gave a high accordance rate of 98.7% (44/45 samples) compared to the sequencing data. The discordant results in the one appear to be due to unsuccessful amplification. Thus, HRM is thought to be suitable for reliable scanning for mutations in the chimerical *abl* TDK in a clinical setting.

Key words: Ph, *bcr-abl*, mutation, melting analysis, HRM

Introduction

The small chemical agent of Imatinib has a high therapeutic response rate for diseases carrying the chimerical *bcr-abl* gene. For example, the first Imatinib treatment was reported to give approximately 80% and 60% complete molecular response rates in chronic myelogenous leukemia (CML) and Philadelphia (Ph)-positive acute lymphoblastic leukemia (ALL), respectively^{1,2}. However, in many cases mutations in the chimerical *abl* Tyrosine kinase domain (TKD) were revealed to precede resistance to Imatinib, resulting in disease relapse and progression to advanced disease. At present, although it is known that there are several causative factors in resistance, such as expression of a rapid drug efflux protein and non *bcr-abl*-dependent transformation involving the *src* family, TDK mutations in the chimerical gene are thought to play a major role in resistance acquisition³. Therefore, detection of mutations becomes essential in cases treated with Imatinib. Moreover, mutations associated with Imatinib therapy emerge as a Ph-positive subclone from minimal residual leukemia (MRD) even in the hematological remission period^{4,5}. This indicates the need for highly sensitive tests to detect only Ph-positive leukemic clones. To date, several methods to analyze mutations including direct and subcloning sequencing have been employed, but respective methods have merits and demerits, and are not always sensitive⁶. Recently, to analyze genetic variations (SNPs, Mutations, and methylations), a novel melting analysis called high-resolution melting (HRM) with an automated instrument and real-time PCR apparatus has been used^{7, 8}. HRM is used to characterize samples according to their dissociation profile as they transit from double-strand DNA (dsDNA) to single-strand (ssDNA). Therefore,

mixture samples with mutant and wild-type cells are easily identified by differences in melting curve shapes. mutant sequence variants produce a T_m shift compared with the wild-type⁹⁾. In addition, it is a reliable- and closed-tube system without high-cost fluorescence probes^{10, 11)}. Thus, to introduce HRM assays in clinical settings to detect Ph-positive subclones with *bcr-abl* kinase domain mutations, the relevance and validation of the assay prior to direct sequencing was studied.

Materials and Methods

Samples and processing of cDNA

A total of 19 Ph-positive samples were used, consisting of 10 unlinked and already mutation-known specimens, 8 fresh practical samples from 6 patients with CML, 2 patients with ALL and one sample from a Ph-positive K562 cell line. All patients with Ph-positive leukemias were being treated with Imatinib at 400-800mg per day and had hematological remission, but were positive for *bcr-abl* real-time RT PCR. As controls, 16 peripheral blood samples from normal volunteers and 10 cell lines consisting of HTLV-1-associated cell lines (Hut102, KK1, KOB, OMT, MT2, SO4, ST1), T-cell lines of Jurkat and MOLT4, and the monocytic line U937 were used.

The total RNA was extracted from total leukocyte guanidinium thiocyanate lysates using an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using oligo-dT primers and Superscript III reverse transcriptase (Invitrogen, Carlsland, CA, USA). Practical and stocked samples used in this study were applied under the approval (15040708) of the Ethics Committee and

the condition of the criteria of the Japanese Association of Laboratory Medicine.

Study Design for HRM assay in the chimerical *abl*-TDK region:

Our study for detection of mutation was designed as the two step manner: firstly, genetic alteration screening by HRM analysis (Figure 1), using the LightCycler 480 (Roche Molecular System, Alameda, CA, USA) for HRM and real-time thermal cycling, secondly, only samples positively screened by HRM were directly sequenced. First of all, each sample was examined for *bcr-abl* chimerical status by the conventional method¹²⁾. If positive for Major or minor chimerical types, these *bcr-abl* kinase domains were selectively amplified, generating a fragment of 1504 bp for b2/a2 and 1579 bp for b3/a2, using primers previously reported¹³⁾.

For HRM analysis, PCR products of 50-250bp length are recommended for best discrimination. Therefore, we applied a modified method previously reported by Polakova et al¹⁰⁾, generating 4 amplicons, designated as HRM1, 2, 3, and 4 of 220 bp, 225 bp, 239 bp and 241 bp corresponding to nt 629-848, 830-1054, 1030-12689, and 1266-1506 (NM_005157), respectively. For HRM, a PCR reaction was performed in 20 µl reaction volumes containing 1µl of 1/200 diluted template generated as described above, Master Mix, Taq DNA Polymerase, dNTP Mix, HRM Dye, 3mM MgCl₂, primers¹⁰⁾, and 1M GC Melt, according to the instructions of Roche Applied Science (Manheim, Germany). The PCR was monitored by real-time cycling and a strong fluorescent signal was generated only when bound to dsDNA, that is the touch down PCR cycling and HRM conditions¹⁷⁾. HRM melting curve data were obtained by slowly increasing the

temperature, from 60 to 90°C at a rate of 100 acquisitions per 1°C. The melting status and changes in T_m value were analyzed using the Roche HRM algorithm (Gene Scanning Software, Roche Supplied Science, Mannheim, Germany), depicting graphs of fluorescence-normalized and temperature-shifted melting curves and difference plots. The cell line K562 was used as a wild-type reference sample.

Sequencing:

In this study, to compare to accuracy of HRM analysis, regardless of the first step negative samples, all of the samples used were confirmed by sequencing the regions of the selectively amplified chimerical *abl* TKD, as well as *abl* TKD from Ph-negative controls using a Big Dye terminator kit Ver 3.1 (Applied Biosystems, Carlsland, CA, USA) and the ABI Prism 3130 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Results

HRM assay validation:

For HRM scoring, Ph(+) K562 was set up as a wild-type genotype and the 2 or 3 normal blood samples were monitored as negative controls. First of all, using three different samples with mutations, HRM analysis in duplicate was performed, generating constant positive melting curves both in terms of shape and peak height with a range of melting temperatures (T_m) from 84-86°C (Fig 2). On the other hand, 16 samples without the Ph-chromosome constantly produced the wild-type scanning profiles according to the Roche HRM program, as shown

in Fig. 3 (Normalized and Temp-shifted melting curves and difference plots).

Interestingly, as shown in Fig 2 panel-(B), no correlation was observed between fluorescence heights and the ratio of the mutant and wild-type: the peak was higher in mixture samples with mutant and wild-type cells than samples with only mutant clones. To address this strange relation, variable mixture-samples with mutant and wild-type cells diluted by exogenous control DNA from wild-type cells were subjected to the HRM assay. Fig 4 shows that samples containing only mutant cells (bottom graph) produced indeterminate signals with low peaks, but the mixture samples containing variable wild-type cell burden (5-90%) displayed apparently higher peaks, indicating the existence of mutation. This shows that 100% mutant samples may become false negative, with indistinguishable from only wild-type patterns. To form only homoduplex dsDNA in either mutant or wild-type DNA probably accounted for the indeterminate evaluation in all mutant or wild-type samples.

Accordingly, to avoid false-negatives in samples with all mutant cells, we organized screen mutations using two-divided samples; one was an original and the other mixed by exogenous wild-type cells, as shown in Fig 5. Using this strategy, the test performance of HRM was examined in this study.

HRM assay results

The HRM assay was blindly examined in duplicate by a single researcher and then compared to sequencing data. As summarized in Table 1, the HRM test was positive for 13 (72.2%) of 19 Ph-positive leukemias, including a Ph-positive K562 cell line. Using the same amplicons as above, direct sequencing identified

14 missense mutations (73.6%) out of 19 Ph(+) leukemias. The positive and negative accordance rate of both tests was 94.7% among 19 Ph(+or-) samples. The discrepancy in sample No 5, negative in the HRM and positive in the sequencing, was expected before HRM analysis, because the PCR efficiency was not so good. As expected, the direct sequencing disclosed a problematic issue for PCR in that a mutation (nt 838) existed within the annealing sequence (nt830-849) of the primer.

Next, of 26 Ph-negative samples consisting of 10 cell lines and 16 normal blood controls, HRM assays produced negative findings in all but one. The positive one for HRM was U937 derived from myelomonocytoid leukemic cells. The sequencing revealed a mutation of E308V, which was expected to be somatic as it is one of the oncogenes.

Conclusively, the accordance rate of the two methods was 98.7% in all 45 cases of Ph-positive/-negative leukemias and controls.

Discussion

Most patients with Ph-positive leukemias, especially chronic CML, who receive Imatinib as first-line therapy achieve good cytogenetic and molecular responses. However, long-term molecular studies suggest that around 25-30% of patients seem not to achieve successful responses and undergo disease progression. Major causes of Imatinib resistance include the emergence of leukemic clones with mutations in the tyrosine kinase domain of *bcr-abl*. This indicates that it is necessary to screen for mutations in early phase of the emergence of mutation clones. Unfortunately, there is generally no acceptable

consensus when and by which technology the TKD mutations should be screened. At present, direct sequencing, denaturing high-performance liquid chromatography (D-HPLC), denaturing gradient gel electrophoresis (DGDE), allele specific oligonucleotide-polymerase chain reaction (ASO-PCR) and pyro-sequencing are available, but the respective methods have merits and demerits for practical clinical settings¹⁴⁾. A novel technology of HRM with development of instruments and saturating intercalating dyes is emerging for detection of nucleic acid sequence variations and is now applied in practical diagnostic settings^{15,16)}. the two step method allows to avoid the direct sequencing for the entire region of all samples.

In this study, the HRM in our system was shown to efficiently and simply differentiate mutations in the chimerical *bcr-abl* TDK region by using LightCycler technology and a software algorithm. In particular, it is noteworthy that mixture samples with mutant and wild-type cells was easily and high sensitively (approximately 5%) detectable. On the other hand, samples with all mutant cells often presents with indeterminate low peaks in difference plots, causing confusing interpretations. This is probably the main defect in this HRM technology resulting from a dependency on heteroduplex formation due to the mixture ratio of the mutant and wild-type. Therefore, to avoid false-negatives with samples containing all mutant cells, it was shown that the addition of exogenous control DNA was useful (Fig 4 and 5). Actually, since the ratio of mutant cells in the samples is unknown in practical samples, we adopted an HRM assay system which measures using a double feature; an original one and mixtures of the mutant and exogenous wild-type DNA with the ratio of 1:1.

Practical examples are shown in Fig 5, indicating that the mixed sample (left panel) makes it easy to discriminate, whereas the change in the positive peak pattern was tolerable if a 40%mutant sample was diluted up-to 50%.

Finally, our HRM results were accordant in all but one out of 45 samples with an accordance rate of 98.7% compared with sequencing data. The discrepancy in the results in one sample was expected due to an accidental relation between the primer and mutation sites, as described above. The quality of HRM is thought to be highly dependent on real-time amplification, so that we are now revising part of the primer set and appropriate sequence length for HRM.

Clearly, this is a rapid, simple, accurate screening method using HRM technology for chimerical *bcr-abl* TDK mutations involved in resistance to Imatinib. Since resistant Ph-subclones emerge from MRD and increase step by step in parallel with the long Imatinib therapy duration, the HRM assay system is a suitable and useful method to better manage Ph-positive leukemias, for example to decide in dose escalation or cessation of Imatinib, alternation of new drugs or different therapies with Dasatinib and bone marrow transplantation. Actually, we are applying this method in a routine clinical setting prior to sequencing to select only mutation-positive samples.

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Figures

Figure 1. Study design of High-Resolution Melting (HRM) and the structure of the tyrosine kinase domain (TKD). Firstly, a part of chimerical *bcr-abl* TKD is selectively amplified by nested PCR. Using amplified chimerical *bcr-abl* products as a template, the second PCR for the HRM assay is performed, generating four intercalating dye amplicons, HRM-1, -2, -3, and -4. Then, HRM analyses were done by using the LightCycler Gene Scanning Application.

Figure 2. Validation of the HRM assay using samples with 100% mutant cells and samples with variable % mutant cells. Duplicate assays gave rise to the similar results, indicating the good reproducibility in both Normalized and Tem-shifted melting curves and difference plots. Ph-positive K562 cells were used as a wild-type (Wt) reference.

Figure 3. Validation of HRM test performance using negative controls with wild-type TKD demonstrated by direct sequencing in peripheral blood from 16 healthy persons and 9 hematopoietic cell lines, excluding the U937 cell line. All 4 HRM analyses were evaluated to be wild-type by both normalized-Tem shifted melting curves according to the Gene Scanning Application Algorithm. Normal peripheral blood from volunteers was used as a wild-type reference.

Figure 4. Changes in heights of peaks and T_m values depending upon the difference in the mixture ratio of mutant and wild-type cells. HRM assay

revealed indeterminate signals in samples with only mutant cells (around 100%mutant cell samples). On the other hand, the mixture samples diluted up-to 5% mutant cells produced typical positive scanning patterns, indicating that the best mixture ratio is 50% vs 50%.

Figure 5. Representative cases (panels A and B) of HRM and sequencing analyses. The left panel showed that the addition of wild-type DNA into the 100% mutant (760T>C) sample made discrimination easy. On the other hand, the right panel showed the change in the melting curves between the original (947C>T) and the mix was tolerable for discrimination.

Table

Table 1. Summary of the results on mutations examined by both HRM and direct sequencing analyses. Mut;mutation, Wt:wild-type, NE: not evaluated

Figure 1

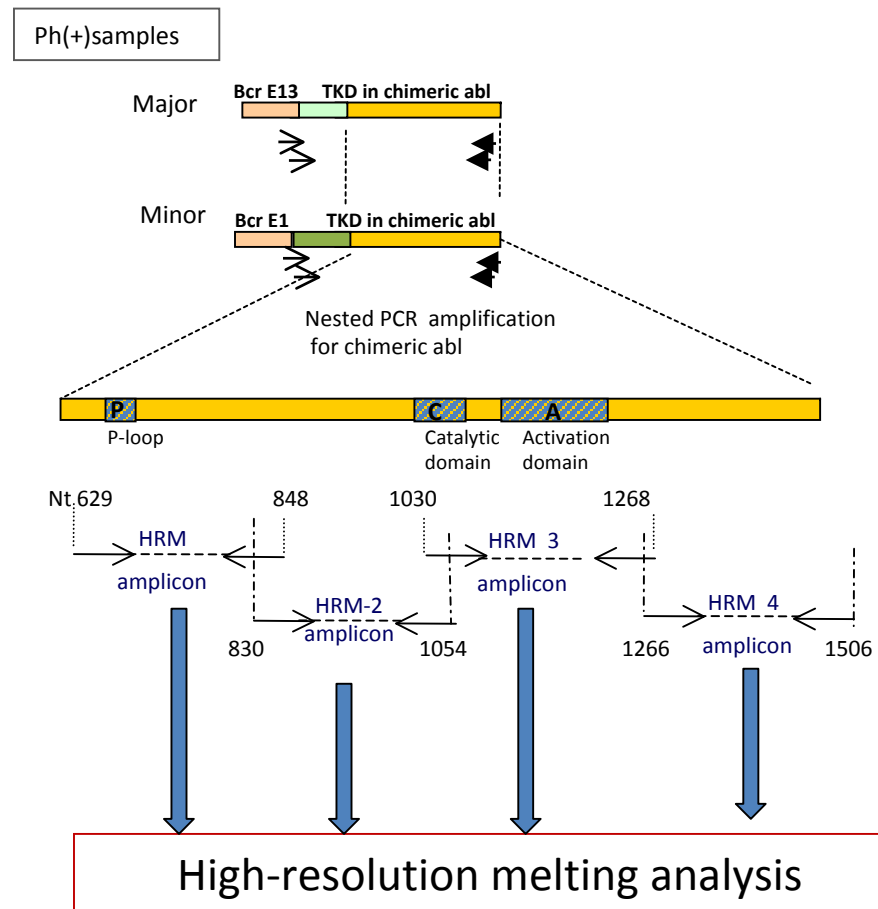
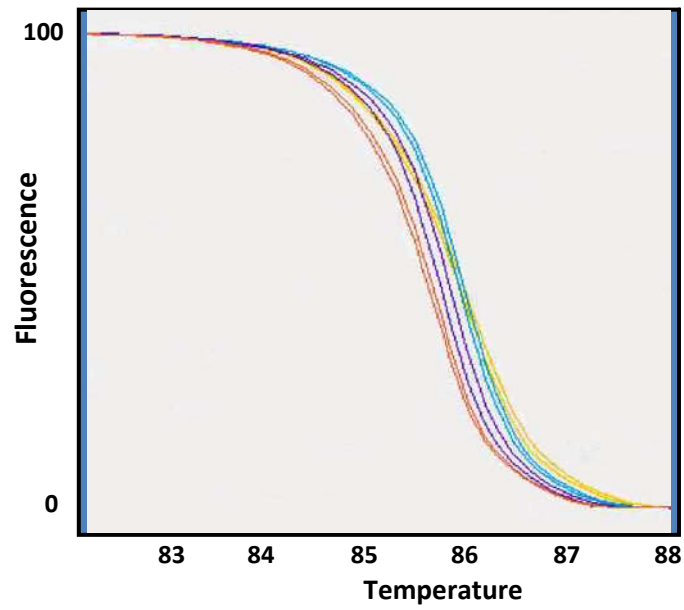


Figure 2.

Normalized and Temp-shifted melting curves



Difference plot

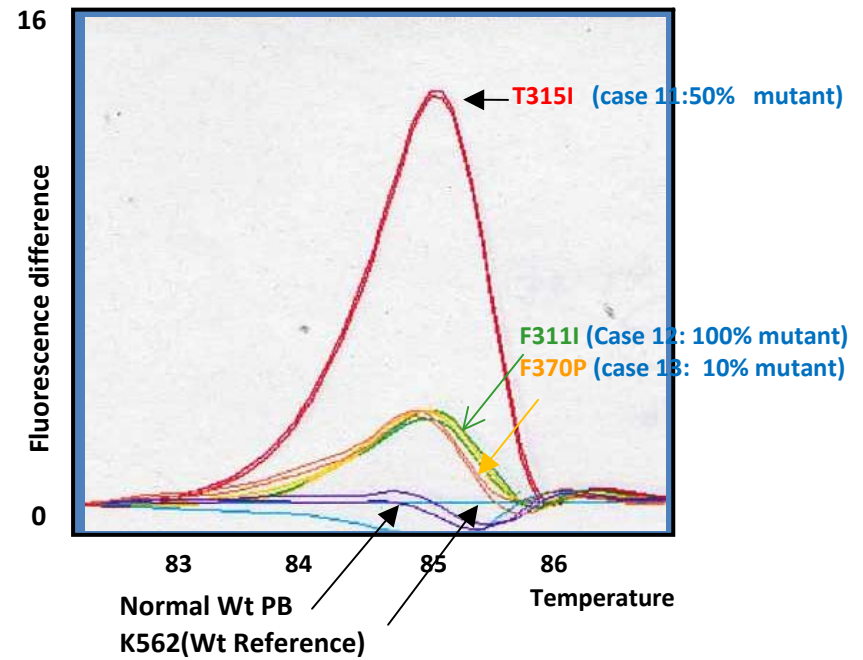


Figure 3

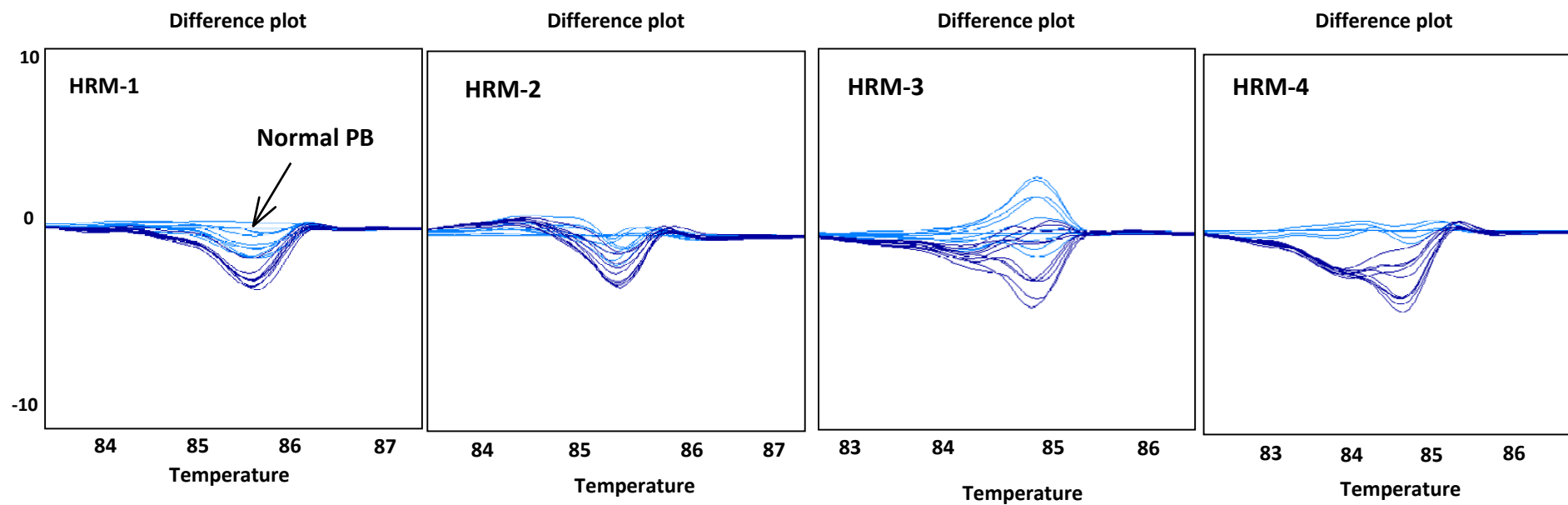


Figure 4

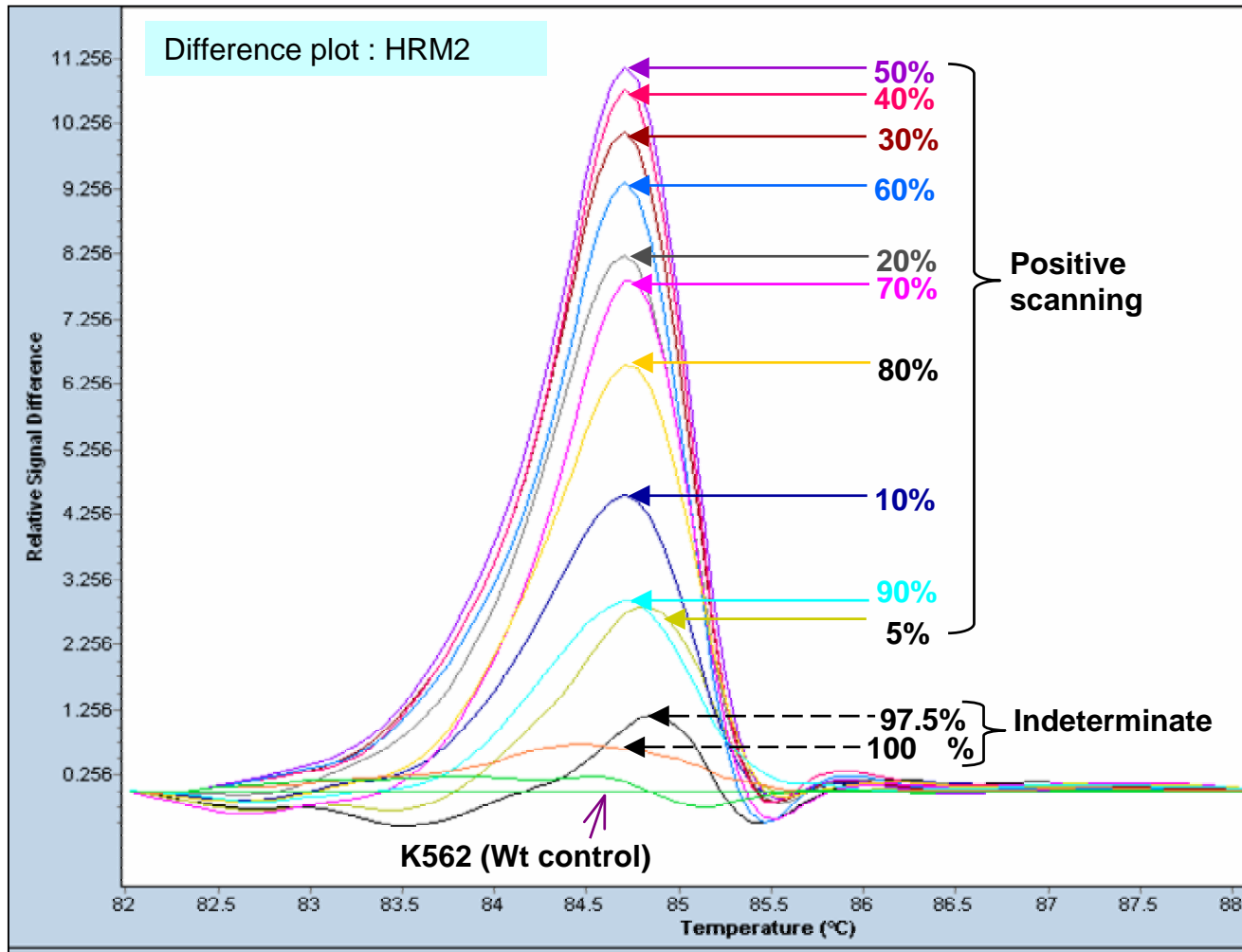


Fig 5.

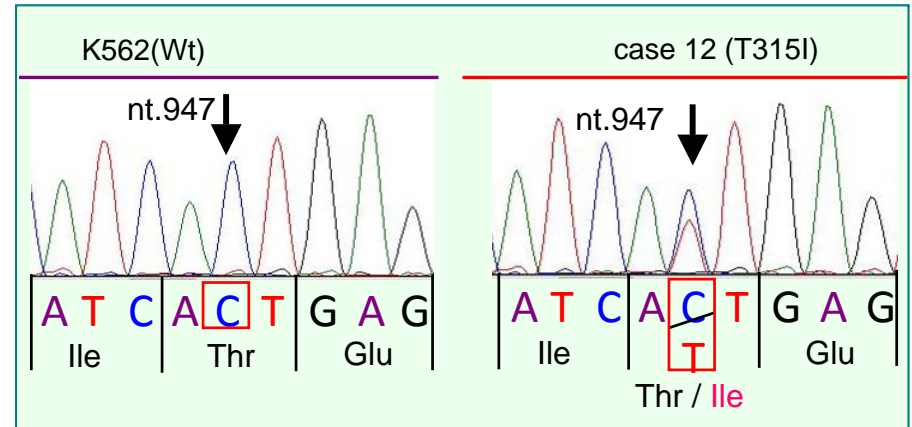
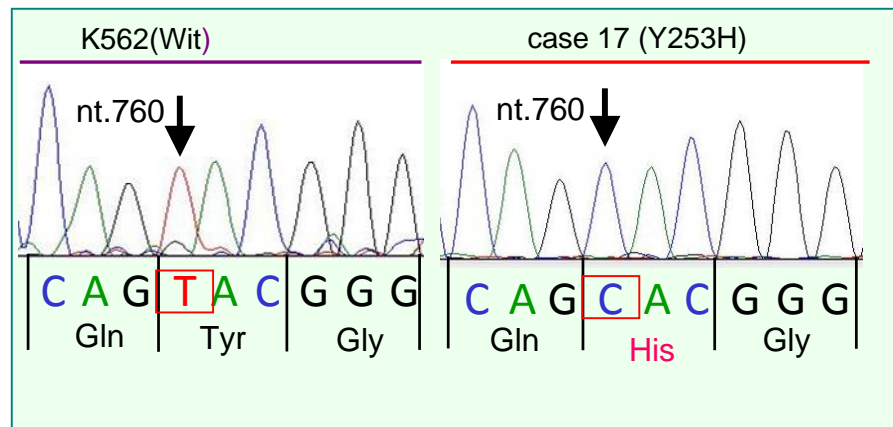
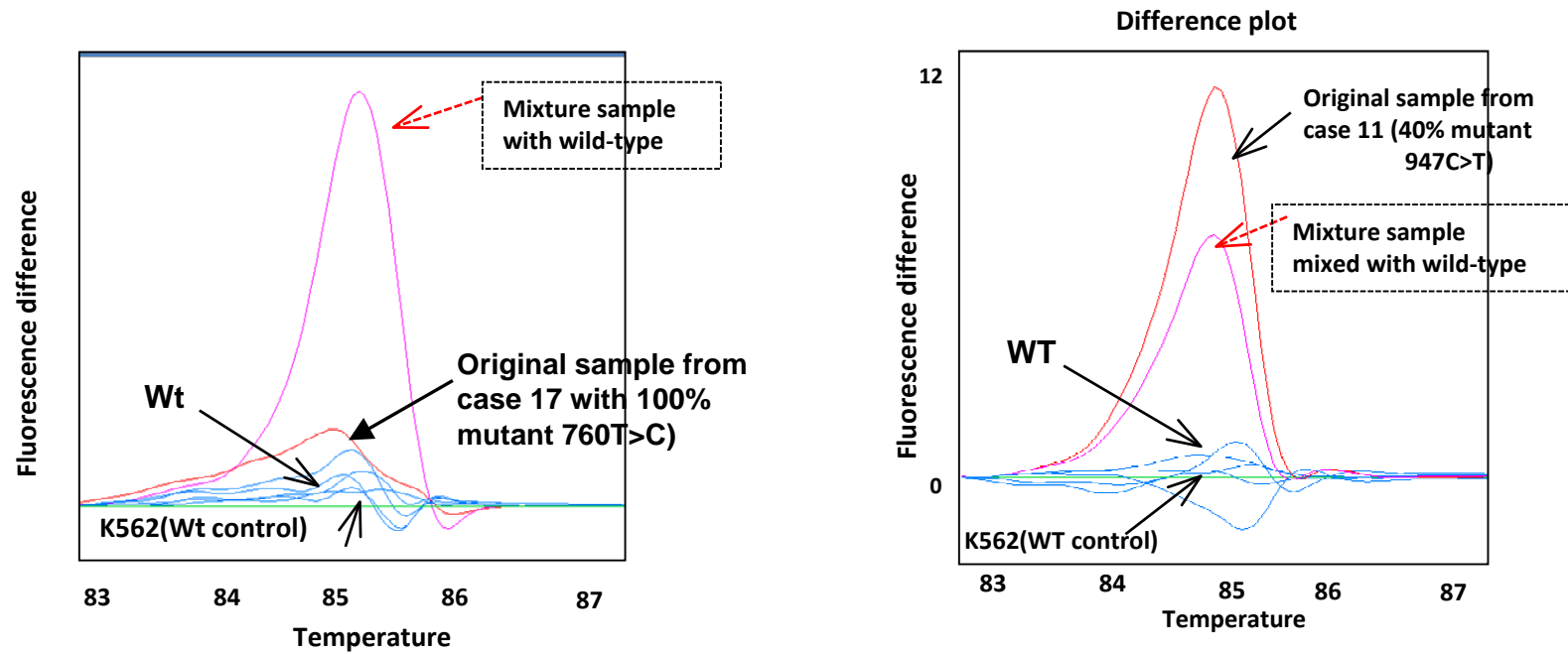


Table 1

sample	Clinical dx	bcr-abl	Results	TKD	nt substitute\	exon	
Unlikned	1	CML	Major	Mut	Y253F	761 A>T	4
	2	CML	Major	Mut	Y253H	760T>C	4
	3	CML	Major	Mut	E255E	767A>T	4
	4	CML	Major	Mut	E255K	766G>A	4
	5	CML	Major	NE	E279K	838G.>A	5
	6	CML	major	Mut	T315I	947C>T	6
	7	CML	major	Mut	F317L	954C>A	6
	8	CML	major	Mut	M351I	1055T>CX	6
	9	CML	major	Mut	H396R	1188A>G	7
	10	CML	major	Mut	F486S	1461T<C	9
fresh	11	CML	major	Mut	T315I		6
	12	CML	major	Mut	F311I		6
	13	CML	major	Mut	I370R		7
	14	CML	major	Wt	Wt		
15	CML	major	Wt	Wt			
16	CML	major	Wt	Wt			
17	ALL	minor	Mut	Y253H	760T>C	4	
18	ALL	minor	Mt	Wt			
Cell lines	19	K562	minor	Wt	Wt		
	20	U937	(-)	Mut	E308V	926A>T	5
	21	KOB	(-)	Wt	Wt		
	22	KK1	(-)	Wt	Wt		
	23	ST1	(-)	Wt	Wt		
	24	SO4	(-)	Wt	Wt		
	25	OMT	(-)	Wt	Wt		
	26	MT2	(-)	Wt	Wt		
	27	Hut102	(-)	Wt	Wt		
	28	Jurkat	(-)	Wt	Wt		
	29	MOLT4	(-)	Wt	Wt		
Normal PB	30-45		(-)	Wt	Wt		