High-Resolution Melting Analysis for Detection of Internal Tandem Duplications

Cecily P. Vaughn* and
Kojo S. J. Elenitoba-Johnson*†

From the Associated Regional and University Pathologists
Institute for Clinical and Experimental Pathology* and the
Department of Pathology,[†] University of Utah School of Medicine,
Salt Lake City, Utah

High-resolution melting analysis (HRMA) is a recently introduced closed-tube fluorescence-based method for rapid mutation screening and detection. However, all of the targets by which this technique has been validated thus far have had single-base substitutions, deletions, or similarly small mutational deviations from the wild-type sequence. In the current study, we sought to determine the feasibility of utilization of HRMA for the detection of larger sequence aberrations, using internal tandem duplications (ITD) in the juxtamembrane domain of the *FLT3* gene as a model system. This gene is important in the growth and differentiation of hematopoietic progenitors and ITDs in this gene have been identified in a subset of poor-prognosis acute myelogenous leukemias (AML). DNA extracted from 62 AML samples was analyzed on a prototype high-resolution melting instrument. The samples interrogated for the *FLT3* ITDs were subjected to post-amplification denaturation with frequent and regular fluorescence acquisition. The fluorescence *versus* temperature melting graphs generated were analyzed for deviation from the profiles reproducibly obtained for the wild-type samples. Results by HRMA were compared to results obtained using capillary electrophoresis-based fragment analysis, temperature gradient capillary electrophoresis detection, and sequencing of ITDs. *FLT3* ITDs were detected in 13 of 62 AML samples with 100% concordance between the detection methods. This study demonstrates the utility of HRMA to rapidly and accurately screen samples for the presence of large sequence aberrations including *FLT3* ITDs. (*J Mol Diagn* 2004, 6:211–216)

The detection of sequence variations in DNA is important in the diagnosis of both genetically and somatically acquired diseases.¹ Multiple methods have been developed to scan DNA for polymorphisms and mutations without the need for direct sequencing.^{2–6} Many of these methods such as single-strand conformation polymorphism (SSCP),⁴ denaturing high performance liquid chro-

matography (dHPLC), denaturing gradient gel electrophoresis (DGGE),⁵ and temperature gradient capillary electrophoresis (TGCE)⁷ require PCR products to be analyzed by gel or column separation.

Fluorescence monitoring of PCR product melting profiles permits the detection of DNA mutations in solution, without the need for gel or column separation.⁸ Fluorescently labeled probe-based methods such as dual hybridization,⁹ exonuclease (TaqMan),¹⁰ or hairpin (Molecular Beacon)¹¹ probes may be used for mutation detection, but only for the bases covered by the probe; thus, they are not amenable to mutation scanning. Mutational scanning requires methods that can detect aberrations over larger regions. A number of methods have been described which use SYBR Green I and melting curve analysis.^{6,12,13} However, these methods required either primer modification with GC-rich sequences and/or extensive post-amplification manipulation before melting analysis. Additional homogeneous methods for scanning by melting analysis include the use of labeled primers¹⁴ or multiple overlapping probes to cover the length of the amplicon.¹⁵ Recently, a mutation scanning method using high-resolution melting analysis (HRMA) has been described.¹⁶ HRMA can detect mutations over the length of an amplicon without the need for gel or column separation. This closed-tube method employs the dsDNA binding dye LCGreen I and obviates the need for fluorescently labeled primers or probes. To date, the utility of this method has only been investigated for the detection of point mutations, single nucleotide polymorphisms, or similarly subtle variations in nucleic acid sequences.

In this study, we examine the feasibility of using HRMA for the detection of large sequence aberrations, using internal tandem duplications (ITD) in the juxtamembrane domain of the *FLT3* gene as a model system. ITD mutations in *FLT3* have been identified in approximately 20% of acute myelogenous leukemias (AML).¹⁷ These mutations occur in exon 14 and intron 14 of the *FLT3* gene but the exact location and length of the mutations varies between patients. We screened DNA from 62 AML samples by HRMA for the presence of ITD mutations. Samples were also analyzed by capillary electrophoresis

Supported by the ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT.

Accepted for publication April 16, 2004.

Address reprint requests to Kojo S. J. Elenitoba-Johnson, M.D., Department of Pathology, University of Utah Health Sciences Center, 50 North Medical Drive, Salt Lake City, UT 84132. E-mail: kojo.elenitobaj@path.utah.edu.

(CE)-based fragment analysis and TGCE. All ITD mutations were further confirmed by sequencing. Our studies show a 100% correlation between HRMA and the other methods, thereby illustrating the utility of HRMA for the detection of large sequence aberrations.

Materials and Methods

DNA Samples

For genotypic *FLT3*, DNA was extracted from bone marrow or peripheral blood samples using the MagNA Pure LC (Roche Molecular Biochemicals, Indianapolis, IN). All samples were obtained from the archived inventories of ARUP Laboratories (Salt Lake City, UT) with institutional review board approval. We analyzed DNA from 62 AML samples for ITD mutations using HRMA and CE-based fragment analysis. Thirty-six of the samples were also analyzed by TGCE. In addition to the patient samples, DNA from the MV4-11 cell line, obtained from DSMZ (Braunschweig, Germany), was analyzed by HRMA, CE, and TGCE.

Polymerase Chain Reaction

DNA samples were amplified for the *FLT3* locus using the previously described primers 11F, 5'-GCAATTTAGGTAT-GAAAGCCAGC-3', and 12R, 5'-CTTCAGCATTGACG-GCAACC-3'.¹⁸ Analysis by high-resolution melting and TGCE required product that was free of polymerase-dependent errors, so high fidelity amplification was used. One hundred nanograms of DNA was amplified in a 50 μ l reaction containing 0.3 μ mol/L each of each primer and 1X High Fidelity Master Mix (Roche Molecular Biochemicals) (containing *Taq* and *Tgo* DNA polymerases, 0.2 mmol/L each dNTP, and 1.5 mmol/L $MgCl_2$). The amplification was performed on the GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA) and entailed an initial denaturation of 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 5 minutes. DNA samples for *FLT3* mutation analysis by capillary electrophoresis were amplified using a modified version of the above protocol with the 11F primer 5'-labeled with 6-FAM.

Amplicon Detection

High-Resolution Melting Analysis

All *FLT3* amplicons were subjected to HRMA. LCGreen I dye (Idaho Technology, Salt Lake City, UT) was added to each amplicon at a final concentration of 10 μ mol/L. To facilitate heteroduplex formation, 10 μ l of each sample was heated in a glass capillary on the LightCycler (Roche Molecular Biochemicals) at 95°C for 1 minute and then cooled to 40°C for 2 minutes with a temperature transition of 20°C/second. Samples were then heated at 0.2°C/second from 60 to 90°C on a prototype of the Idaho Technology HR-1

instrument. This glass capillary-based single-sample high-resolution melting instrument collects fluorescence data at a rate of approximately 50 data points per 1°C.

Results were analyzed as fluorescence (F) *versus* temperature (T) graphs. The data were also analyzed using normalization, temperature shifting, difference plots, and derivative melting curves, as previously described.¹⁶ Briefly, for normalization, temperature ranges on each side of the melting transition were chosen and the data points for a given sample were scaled between 0 and 100% fluorescence. For temperature shifting, the melting curves of the samples were superimposed over the same temperature range. This aided in the differentiation between wild-type and mutant samples by emphasizing curve shape in conjunction with melting temperature. Difference plots were generated by choosing a wild-type control as the baseline and subtracting the fluorescence of each sample relative to this baseline. Derivative plots were visualized as the negative derivative of the fluorescence relative to the temperature (-dF/dT) *versus* temperature.

Capillary Electrophoresis-Based Fragment Analysis

FLT3 PCR products were run on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using a 50-cm array with Performance Optimized Polymer-6 (POP-6). One μ l of PCR product was mixed with 8.5 μ l formamide and 0.5 μ l ROX500 internal size standard. One μ l of this mixture was electrokinetically injected at 10 volts for 1 second. Electrophoresis was performed at 1500 volts at 55°C with a data delay time of 1120 seconds and a run time of 4000 seconds. Data were analyzed with the ABI Filter Set D using the 3100 Data Collection software, version 1.1 (Applied Biosystems).

Temperature Gradient Capillary Electrophoresis

Temperature gradient capillary electrophoresis was performed post-amplification for 36 of the AML patient samples and for the MV4-11 cell line. Following amplification, samples were heated to 94°C for 5 minutes and then cooled to 4°C for a minimum of 5 minutes for heteroduplex formation. Samples were injected at 3 kV for 20 seconds and assayed on the SpectruMedix Reveal System (State College, PA) with a temperature range of 45 to 60°C and a ramp time of 23 minutes. Results were analyzed using the SpectruMedix Revelation software. Samples were genotyped by comparison of the electropherograms to those of the wild-type control. Samples were scored as positive for an ITD mutation if they exhibited peaks in addition to the pattern of the wild-type control.

Analytical Sensitivity

DNA from the MV4-11 cell line was serially diluted into placental DNA. Dilutions of 1X, 1:2, 1:5, 1:10, and 1:20 were

Figure 1. Detection of *FLT3* ITD by high-resolution melting analysis. **A:** Fluorescence (F) versus temperature (T) melting curves using raw fluorescence data. Four wild-type (black, brown, green, and purple line), and four ITD mutant (red, orange, blue, and light blue line) patient samples are shown. The insertions within the ITD mutant samples ranged from 6 to 102 bp. **B:** Melting curves after fluorescence-normalization, for the samples depicted in A. The wild-type samples have narrow melting transitions, whereas the ITD mutant samples have broad or multiple melting transitions. **C:** Temperature shifted melting curves. ITD mutant samples are easily distinguished by the low temperature decrease in fluorescence. **D:** Fluorescence difference plots of the normalized data. A wild-type sample is used for the baseline and the curves for the remaining wild-type samples cluster around the baseline. The curves for the mutant samples are depicted in the positive scale with a greater fluorescence difference. **E:** Derivative (-dF/dT versus T) melting curves of the normalized fluorescence data. The wild-type sample has a single peak, whereas the ITD mutant samples have either a low temperature shoulder or two peaks.

amplified using the *FLT3* primer set and assessed using both high-resolution melting and capillary electrophoresis.

Sequencing

PCR amplicons from samples with ITD mutations were sequenced to confirm the presence of and to characterize the internal duplication. The amplicons were separated by electrophoresis on 2% or 4% agarose gels and the ITD bands were purified using the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA). Automated DNA sequencing of PCR products was performed using dideoxynucleotide termination chemistry and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Results

High-Resolution Melting Analysis

HRMA for *FLT3* is illustrated in Figure 1. The raw fluorescence data are shown in Figure 1A. Absolute fluorescence varied from sample to sample. Wild-type samples were characterized by narrow melting transitions, while samples containing ITD mutations showed either a broad melting transition or multiple melting domains. Samples showing multiple melting domains were easily distinguished using the raw data. However, in this format, samples with broad melting transitions were more difficult to visualize and distinguish from wild-type samples.

Figure 2. Detection of *FLT3* internal tandem duplications by capillary electrophoresis-based fragment analysis. **A:** Electropherogram of a wild-type clinical sample showing a single peak at 329 bp (**blue peak**). Size standards at 300, 340, 350, and 400 bp are also shown (**red peaks**). **B:** Electropherogram of a mutant clinical sample showing a peak at 359 bp in addition to the wild-type peak at 329 bp.

Results were better visualized using fluorescence-normalized melting curves (Figure 1B) or temperature-shifted melting curves (Figure 1C). Although the T_m of each wild-type sample differed slightly, ITD samples were easily distinguished from wild-type samples at the low temperature end of the melting transition where the difference in curve shape was greatest. The low end of the melting transition represents the melting of heteroduplexes present in the ITD samples.

Results were also visualized as difference plots and derivative plots ($-dF/dT$ versus T graphs) of the melting curve data (Figure 1, D and E). Using the fluorescence difference plots, the curves for the wild-type samples were clustered around the baseline while the curves for the mutant samples were always seen in the positive scale regardless of which wild-type sample was used as the control for the baseline. The derivative plots of wild-type samples showed a single peak. Samples with ITD mutations exhibited either a single peak with a shoulder or two peaks.

The MV4-11 cell line and five of the samples containing ITD mutations exhibited multiple melting domains. The five AML samples all had significant populations of the ITD allele relative to the wild-type allele as determined by the peak heights in the CE electropherograms. All other ITD samples resulted in broad melting transitions.

Capillary Electrophoresis-Based Fragment Analysis

Wild-type samples yielded a single peak at 329 bases and ITD mutant samples yielded one or two peaks greater than 329 bases (see Figure 2). The wild-type peak was present in all clinical samples. However, the relative intensity of the ITD allele to the wild-type allele varied greatly. The MV4-11 cell line exhibited a single peak at 359 bases.

Figure 3. Detection of *FLT3* internal tandem duplications by temperature gradient capillary electrophoresis (TGCE). **A:** Electropherogram of a wild-type clinical sample showing a single dominant peak at approximately 1560 frames. **B:** Electropherogram of a mutant clinical sample showing multiple peaks, representing heteroduplexes, in addition to the wild-type peak.

Temperature Gradient Capillary Electrophoresis

Wild-type samples exhibited a single dominant peak at 1613 ± 63 frames (Figure 3A). Patient samples containing ITD alleles exhibited multiple peaks, representing heteroduplexes, in addition to the wild-type peak (Figure 3B). The MV4-11 cell line resulted in a single peak at 1750 frames.

Correlation of FLT3 Results

FLT3 ITD mutations were detected by HRMA and CE in 13 of 62 (21%) AML samples. ITD mutations were detected in 9 of the 36 samples tested by TGCE. Results between the three methods were 100% concordant.

Analytical Sensitivity

The ITD mutation was detectable at a dilution of 1:5 using HRMA and at a dilution of 1:10 using CE-based fragment analysis.

Sequencing Analysis

ITD mutations were confirmed by DNA sequencing of PCR products. Internal tandem duplication PCR products ranged in size from 335 to 431 bases in length, representing duplications of 6 to 102 bases. One sample contained two ITD products with lengths of 389 and 431 bases. Although the length and exact location of the insertions varied between samples, all ITD mutations resulted in in-frame shifts. An example of the sequence

Figure 4. Sequencing results for sample 26. The sequence shown corresponds to bases 1764 to 1803 of GenBank Accession Number 406322. The 24 base insertion occurs after position 1793.

associated with an internal tandem duplication is illustrated in Figure 4.

Discussion

In this report, we have established the utility of an approach combining the dsDNA binding dye LCGreen I and HRMA in the rapid interrogation of DNA samples containing heterozygous, and/or homozygous somatically acquired genetic mutations. In addition, we show that this approach is useful for screening for the presence of large sequence aberrations. Specifically, our current study shows that this approach for the detection of ITDs in the *FLT3* gene (which ranged in size from 6 to 102 bp) achieved 100% accuracy when compared with detection by CE, TGCE, and sequencing.

The employment of solution-based or real-time fluorescence methods for the detection of structural variations in DNA sequences has entailed the utilization of sequence-specific fluorescently labeled probes or non-specific DNA binding dyes.^{19–22} The sequence-specific DNA probe-based methods are generally limited by their inability to detect sequence alterations that occur outside the coverage area of the “reporter” oligonucleotide probe. By comparison, the non-specific dsDNA binding dye-based techniques for mutational scanning require somewhat extensive post-amplification manipulation for the reliable detection of variations between two DNA sequences. In this regard, we previously demonstrated the feasibility of detection of single-base substitutions in amplicons > 200 bp in length, by incorporating GC-clamps into one of the PCR primers used for the amplification of the DNA segment of interest, and performing a post-amplification melting protocol which involved the incorporation of urea into the reaction mixture.⁶ This procedure is labor intensive, and subject to the achievement of variable results due to the multiple post-amplification steps required for scanning of DNA for sequence alterations. Discrimination of mutated sequences has also been achieved using 5'-fluorescently labeled primers in PCR.¹⁴ With this approach, genotyping of amplicons of up to 300 bp in size was successfully performed, but was limited by the requirement that the sequence alteration interrogated be within a melting domain that included the fluorescently labeled primer.¹⁴ Hence, the development of simple closed-tube fluorescence-based methods that discriminate between related sequences would be a desirable improvement in molecular diagnostics.

Post-amplification analysis of amplicons by gradual melting of the DNA sequences in combination with acquisition of multiple fluorescence data points (50 per second) permits detailed evaluation of the status of homo- and heteroduplexes, and strand dissociation states during dsDNA strand denaturation.¹⁶ The HRMA instrument collects more fluorescence data per degree Celsius than other instrumentation such as the LightCycler or ABI PRISM 7900HT. This approach and the utilization of a dsDNA binding dye such as LCGreen I, which does not significantly alter the relationship between DNA duplexes in solution, constitute the underlying principles inherent to

the feasibility of high-resolution melting in nucleic acid genotyping. Notwithstanding the promising results demonstrated in the genotypic discrimination of sequences with germ-line alterations comprising single-base substitutions, the utility of identification of larger sequence aberrations and somatically acquired mutations has not been previously evaluated.

In this study, we have demonstrated the utility of high-resolution melting in conjunction with the generic dsDNA binding dye LCGreen I in the identification of sequence alterations larger than point mutations. While the majority of our experiments incorporated LCGreen I post-amplification, we have demonstrated that this dye is compatible with PCR and its inclusion at the beginning of PCR does not adversely affect the ability to perform HRMA. In addition to being PCR-compatible, the use of LCGreen I is advantageous because unlike SYBR Green I, LCGreen I does not appear to undergo significant repartitioning within the double-stranded DNA strand during post-amplification melting.¹⁶ This permits finer resolution of melting profiles and thus improves the ability to detect nucleic acid sequence variations. Using internal tandem duplications of the *FLT3* gene as a model target, we have demonstrated that high-resolution melting using LCGreen I can reliably detect DNA insertions ranging from 6 to 102 bases within amplicons ranging from 335 to 431 bp in length. Each ITD was detected as a reproducible variation in the shape of the *F* versus *T* curves of the samples containing the ITDs when compared to the curves of the samples with the wild-type sequence. The difference in the curve-shape is attributable to heteroduplex formation between the mutated sequence contributed by the abnormal alleles in the tumor DNA and wild-type sequence contributed by a normal allele in the tumor DNA, or from DNA from contaminating normal cells.

Interestingly, we also reproducibly detected the presence of a homozygous *FLT3* ITD in an acute myelogenous leukemia-derived tumor cell line (MV4–11) within which no wild-type sequence was demonstrable by CE, TGCE, or DNA sequencing. The ITD in this sample was composed of a 30-bp sequence insertion, and yielded an interesting *F* versus *T* profile with two distinct melting transitions. Analysis of the sequence within the ITD revealed a likelihood for the formation of misannealed heteroduplexes in configurations following re-annealing of the DNA strands in the homozygous 30-bp ITD present in the MV4–11 DNA.

The detection of *FLT3* ITD mutations by HRMA was 100% concordant with the results obtained by CE, TGCE, and sequencing for the same samples. HRMA was advantageous because samples could be screened within 2 minutes post-amplification. HRMA was effective at detecting mutations across the length of the amplicon since the position of the mutation varied between samples. Theoretically, sequence aberrations such as deletions and random insertions would be similarly detectable in heterogeneous samples with wild-type and mutated species. Although only sequencing can provide exact characterization of each mutation, HRMA was able to both rapidly and accurately determine which samples contained mutations. We found the primary drawback of the HRMA method to be its inability to detect small quantities

of mutant DNA species in an overwhelming background of wild-type DNA species. This is particularly important in the identification of somatically acquired mutations, wherein the tumor cells account for only a small proportion of the total cell population. With the dilutional series study, we found that ITD alleles were only detected in samples when comprising up to 20% of the entire target DNA content analyzed. Capillary electrophoresis could distinguish ITD populations present at 10%. Both systems are limited by the preferential amplification of the dominant species.

In conclusion, we successfully identified somatic mutations of the *FLT3* gene in 13 of 62 (21%) AML patient samples assessed using HRMA. These results correlated 100% with CE, TGCE, and sequencing results. Our studies show that HRMA has the advantage of screening each sample for mutations within 2 minutes post-amplification. We found the main limitation of HRMA to be its inability to identify mutations when the relative abundance of mutated cells was less than 20% of the total cell population. Future developments to increase the frequency of fluorescence acquisition may increase sensitivity and allow the system to discriminate sequence variations in longer DNA segments. Nevertheless, our studies illustrate that HRMA in its current design can be used to quickly and accurately screen DNA samples for large sequence aberrations, including *FLT3* ITDs.

Acknowledgments

We thank Leah Hartung and Pam Wilfahrt of ARUP Laboratories for the provision of clinical samples and Mohamed Jama, Sam Page, and Lan-Szu Chou, also of ARUP Laboratories, for their assistance with CE, DNA sequencing, and TGCE, respectively.

References

1. Botstein D, White RL, Skolnick M, Davis RW: Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am J Hum Genet* 1980, 32:314–331
2. Flavell RA, Kooter JM, De Boer E, Little PF, Williamson R: Analysis of the β - δ -globin gene loci in normal and Hb Lepore DNA: direct determination of gene linkage and intergene distance. *Cell* 1978, 15:25–41
3. Wyman AR, White R: A highly polymorphic locus in human DNA. *Proc Natl Acad Sci USA* 1980, 77:6754–6758
4. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T: Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci USA* 1989, 86:2766–2770
5. Fischer SG, Lerman LS: DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. *Proc Natl Acad Sci USA* 1983, 80:1579–1583
6. Elenitoba-Johnson KS, Bohling SD: Solution-based scanning for single-base alterations using a double-stranded DNA binding dye and fluorescence-melting profiles. *Am J Pathol* 2001, 159:845–853
7. Gelfi C, Righetti PG, Cremonesi L, Ferrari M: Detection of point mutations by capillary electrophoresis in liquid polymers in temporal thermal gradients. *Electrophoresis* 1994, 15:1506–1511
8. Wittwer CT, Herrmann MG, Gundry CN, Elenitoba-Johnson KS: Real-time multiplex PCR assays. *Methods* 2001, 25:430–442
9. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP: Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* 1997, 22:130–131, 134–138
10. Heid CA, Stevens J, Livak KJ, Williams PM: Real-time quantitative PCR. *Genome Res* 1996, 6:986–994
11. Tyagi S, Kramer FR: Molecular beacons: probes that fluoresce upon hybridization. *Nature Biotechnol* 1996, 14:303–308
12. Germer S, Higuchi R: Single-tube genotyping without oligonucleotide probes. *Genome Res* 1999, 9:72–78
13. Lipsky RH, Mazzanti CM, Rudolph JG, Xu K, Vyas G, Bozak D, Radel MQ, Goldman D: DNA melting analysis for detection of single nucleotide polymorphisms. *Clin Chem* 2001, 47:635–644
14. Gundry CN, Vandersteen JG, Reed GH, Pryor RJ, Chen J, Wittwer CT: Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. *Clin Chem* 2003, 49:396–406
15. Millward H, Samowitz W, Wittwer CT, Bernard PS: Homogeneous amplification and mutation scanning of the p53 gene using fluorescent melting curves. *Clin Chem* 2002, 48:1321–1328
16. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ: High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin Chem* 2003, 49:853–860
17. Kiyoi H, Towatari M, Yokota S, Hamaguchi M, Ohno R, Saito H, Naoe T: Internal tandem duplication of the *FLT3* gene is a novel modality of elongation mutation which causes constitutive activation of the product. *Leukemia* 1998, 12:1333–1337
18. Kiyoi H, Naoe T, Yokota S, Nakao M, Minami S, Kuriyama K, Takeshita A, Saito K, Hasegawa S, Shimodaira S, Tamura J, Shimazaki C, Matsue K, Kobayashi H, Arima N, Suzuki R, Morishita H, Saito H, Ueda R, Ohno R: Internal tandem duplication of *FLT3* associated with leukocytosis in acute promyelocytic leukemia: Leukemia Study Group of the Ministry of Health and Welfare (Kohseisho). *Leukemia* 1997, 11:1447–1452
19. Elenitoba-Johnson KS, Bohling SD, Wittwer CT, King TC: Multiplex PCR by multicolor fluorimetry and fluorescence melting curve analysis. *Nat Med* 2001, 7:249–253
20. Vaughn CP, Elenitoba-Johnson KS: Hybridization-induced de-quenching of fluorescein-labeled oligonucleotides: a novel strategy for PCR detection and genotyping. *Am J Pathol* 2003, 163:29–35
21. Bohling SD, Wittwer CT, King TC, Elenitoba-Johnson KS: Fluorescence melting curve analysis for the detection of the *bcl-1/JH* translocation in mantle cell lymphoma. *Lab Invest* 1999, 79:337–345
22. Bohling SD, King TC, Wittwer CT, Elenitoba-Johnson KS: Rapid simultaneous amplification and detection of the *MBR/JH* chromosomal translocation by fluorescence melting curve analysis. *Am J Pathol* 1999, 154:97–103