

Comparison of high-resolution melting analysis to denaturing high performance liquid chromatography in the detection of point mutations in *MEFV*, *F5*, and *F2* genes

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Background/aim: Sensitive and cost-effective detection of point mutations is important in genetics research. Denaturing high-performance liquid chromatography (DHPLC) is known to be one of the most sensitive techniques for point mutation detection. A more recent technique, high-resolution melting (HRM), is based on the melting behavior of PCR products. In this study, the efficiency and sensitivity of HRM and DHPLC for the detection of *MEFV*, *F5*, and *F2* gene point mutations were evaluated.

Materials and methods: We studied 15 patients with *MEFV* mutations (E148Q, M680I, M694V, or V726A), 7 patients with the F51691G>A mutation, and 12 patients with the F220210G>A mutation. All mutations were screened by HRM and DHPLC.

Results: All mutations were successfully detected by HRM. However, only 4 (*MEFV* E148Q and M680I, F51691G>A, and F220210G>A) of 6 mutations were successfully detected with DHPLC.

Conclusion: Our study showed that HRM is more sensitive than DHPLC for detection of the studied point mutations.

Key words: Point mutations, high-resolution melting analysis, denaturing high-performance liquid chromatography

1. Introduction

A fast, accurate, and cost-effective mutation screening technique is important, especially for clinical settings. Familial Mediterranean fever (FMF) is an autoinflammatory and autosomal recessive disorder that is common in the eastern Mediterranean region and particularly affects people of Armenian, Turkish, Arab, and non-Ashkenazi Jewish ancestry (1,2). The prevalence of the disease is 1/1000 in Turkey (3). FMF is characterized by recurrent fever attacks and is often caused by mutations in the *MEFV* gene, which encodes a protein called pyrin. (4). To date, around 30 mutations associated with FMF have been reported. Out of these mutations, E148Q, M680I, M694V, and V726A are the most common mutations routinely screened for FMF diagnosis (5–7).

Meanwhile, thrombophilia is a multifactorial disease with significant morbidity and mortality (8,9). Factor V Leiden, a mutation in the *F5* gene at position 1691, and factor II G20210A, a mutation in the 3' untranslated region of the prothrombin gene at position 20210, are the most common and routinely screened mutations in hereditary thrombophilia (10–12).

A sensitive, accurate, and cost-effective mutation screening method is indispensable for routine diagnostic

screening. There are several different methods for the analysis of DNA sequence variations, such as single-strand conformation polymorphism (SSCP) (13), restriction fragment length polymorphism (RFLP) (14), denaturing gradient gel electrophoresis (DGCE) (15), temperature gradient capillary electrophoresis (16), denaturing high-performance liquid chromatography (DHPLC) (17), and DNA sequencing. The sensitivity and specificity of these methods vary.

DHPLC is one of the most reliable techniques in the detection of point mutations. In the presence of heterozygote mutations, it detects changes in the melting profile of amplified DNA fragment (18). Certainly, DNA sequencing is widely accepted as the golden standard in mutation detection. However, these 2 techniques are expensive and time-consuming.

In 2002, high-resolution melting (HRM) was introduced to mutation analysis research. Many applications of HRM analysis have been described, including methylation, small amplicon genotyping, mutation scanning, and unlabeled probe genotyping. This method is based on the characterization of DNA sequence variations through the analysis of the melting profile of a DNA sample (19). The thermal stability and melting behavior of a PCR product

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depend on its base sequence, GC content, and length. HRM does not require post-PCR processing, unlike gel electrophoresis or chromatographic separation. Following PCR, the melting profiles of the amplicons are monitored through fluorescence signal analysis. The melting profile of the amplicon bearing a sequence alteration deviates from the melting profile of the wild-type sample (20,21).

In this study, the efficiency and sensitivity of HRM and DHPLC for the detection of *MEFV* (E148Q, M680I, M694V, and V726A), F51691G>A, and F220210G>A gene mutations were evaluated.

2. Materials and methods

2.1. Samples

Peripheral blood samples were collected from 34 patients and 5 healthy controls genotyped for *MEFV* (E148Q, M680I, M694V, and V726A), F51691G>A, and F220210G>A mutations. The details of the samples are listed in Table 1.

2.2. DNA isolation

Genomic DNA was isolated from peripheral blood samples with phenol/chloroform extraction followed by ethanol precipitation. Following DNA isolation, the quantity and

Table 1. Samples analyzed in the study.

Mutations	Number of samples and genotype
<i>MEFV</i> E148Q	2 (heterozygous)
<i>MEFV</i> M680I	5 (heterozygous)
<i>MEFV</i> M694V	3 (heterozygous) and 1 (homozygous)
<i>MEFV</i> V726A	4 (heterozygous)
F51691G>A	4 (heterozygous) and 3 (homozygous)
F220210G>A	6 (heterozygous) and 6 (homozygous)

the quality of samples were assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

2.3. HRM analysis

Primers were designed using Primer3 software. Primer sequences are listed in Table 2.

HRM analysis consists of 3 steps: PCR reaction, DNA melting, and data analysis. These analyses were performed using the LightCycler 480 High-Resolution Melting Master Kit according to the manufacturer's instructions. PCR amplification and HRM analysis were carried out in a LightCycler 480 real-time PCR system (Roche Diagnostics, Germany) with a 384-well plate.

The PCR conditions for the *MEFV* (exon 10), *F5*, and *F2* genes were: 20 ng of genomic DNA, 10 µL of LightCycler 480 High-Resolution Melting Master (Roche Diagnostics; Taq polymerase, nucleotides, and HRM dye), 0.25 µM of each primer, and 2.0 mM MgCl₂. The final volume of the reaction was 20 µL. The PCR conditions for *MEFV* (exon 2) were: 20 ng of genomic DNA, 10 µL of LightCycler 480 High-Resolution Melting Master (Roche Diagnostics; Taq polymerase, nucleotides, and HRM dye), 0.25 µM of each primer, 1.2 mM MgCl₂, and 5% DMSO. The final volume of the reaction was 20 µL.

PCR amplification started with a preincubation step at 95 °C for 10 min, which included activation of FastStart Taq DNA polymerase and denaturation of DNA, followed by a 45-cycle program (denaturation at 95 °C for 15 s, annealing primer pair-dependent, and elongation at 72 °C for 15 s, with a single acquisition mode) and finally melting with continuous fluorescence measurement from 60 to 90 °C (25 acquisitions/°C) and rapid cooling at 40 °C. LightCycler and HRM data were analyzed with LightCycler software.

2.4. DHPLC analysis

PCR for *MEFV* (exon 10), *F5*, and *F2* genes was performed in 25 µL with the following conditions: 2.5 µL of 10X Taq

Table 2. Primers used for HRM and DHPLC analysis.

Gene	Primer	Amplicon size
<i>MEFV</i> (exon 2)	F: AGCCTGAAGACTCCAGACC R: AGGCCTTCTCTCTGCGTT	148 bp
<i>MEFV</i> (exon 10)	F: TGGGAGCCTGCAAGACATC R: AAAGAGCAGCTGGCGAATG	244 bp
<i>F5</i>	F: CAGAAAATGATGCCAGTGC R: GCCCATTTATTAGCCAGGA	218 bp
<i>F2</i>	F: TTCCGCCTGAAGAAGTGGA R: TCCTGAGCCCAGAGAGCTG	197 bp

buffer $(\text{NH}_4)_2\text{SO}_4$, 1 U of Taq DNA polymerase (5 U/ μL), 25 mM MgCl_2 , 5 pmol of each primer, 10 mM dNTPs, and 100 ng of DNA. *MEFV* (exon 2) PCR was performed in 25 μL with the following conditions: 2.5 μL of 10X Taq buffer $(\text{NH}_4)_2\text{SO}_4$, 2 U of Taq DNA polymerase (5 U/ μL), 25 mM MgCl_2 , 5 pmol of each primer, 10 mM dNTPs, and 100 ng of DNA.

PCR products of the wild-type control and homozygous samples were mixed for heteroduplex formation. The mixed PCR products were denatured at 95 °C for 5 min and cooled to 40 °C to produce heteroduplex formations. DHPLC analysis was performed with a Transgenomic Wave Nucleic Acid Fragment Analysis System (Transgenomic). DHPLC melt software was used for the optimum temperature for separation heteroduplex formations (<http://insertion.stanford.edu/melt.html>). Temperatures were as follows: *F5*, 57.6 and 59.1 °C; *F2*, 58.6 and 59.8 °C; *MEFV* (exon 2), 64.8 and 68.5 °C; and *MEFV* (exon 10), 59.3 and 60.9 °C. In the same running, standards and samples were analyzed. Analysis of samples took about 7–8 min.

3. Results

In this study, *MEFV* (E148Q, M680I, M694V, and V726A), F51691G>A, and F220210G>A mutations were analyzed by HRM and DHPLC. The melting curves and DHPLC traces of samples were compared with wild-type samples to assess the sensitivity and specificity of each method. All of the point mutations were detected by HRM, while only 4 of 6 mutations (*MEFV* E148Q and M680I, F51691G>A, and F220210G>A) were detected by DHPLC. The results of the HRM and DHPLC analysis are listed in Table 3 and shown in Figures 1–7.

4. Discussion

The detection of clinically important mutations for inherited disorder studies is crucial for the diagnosis and prognosis of disease. However, the choice of the method for mutation screening is not always straightforward. To date, several mutation detection techniques, such as RFLP, SSCP, HA, DGCE, DHPLC, and DNA sequencing, have been developed. DHPLC is one of the most reliable techniques for identifying sequence variations and it was used for mutation screening in several studies to date

Table 3. High-resolution melting and denaturing high-performance liquid chromatography analysis of samples.

Mutation	HRM analysis	DHPLC analysis
<i>MEFV</i> gene E148Q	+	+
<i>MEFV</i> gene M680I	+	+
<i>MEFV</i> gene M694V	+	-
<i>MEFV</i> gene V726A	+	-
F51691G>A	+	+
F220210G>A	+	+

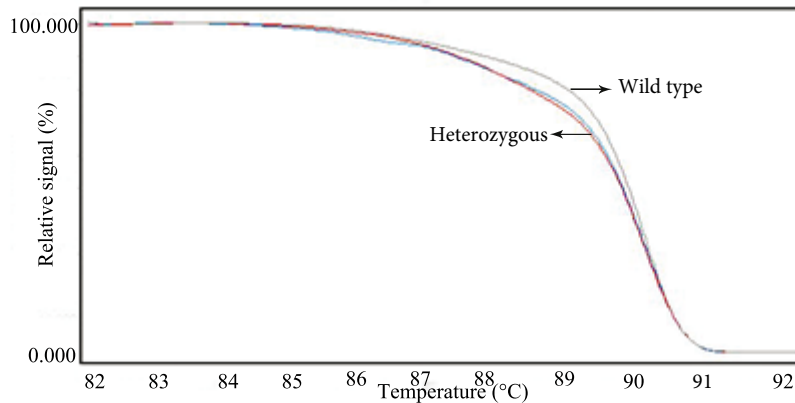


Figure 1. Normalized and temperature-shifted high-resolution melting curves, *MEFV* E148Q.

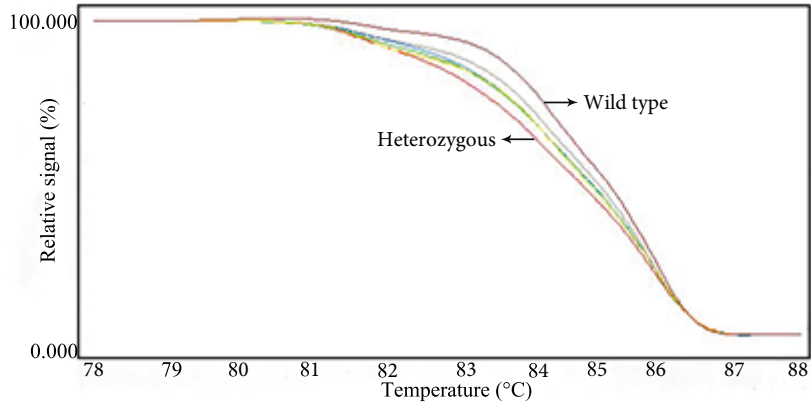


Figure 2. Normalized and temperature-shifted high-resolution melting curves, *MEFV* M680I.

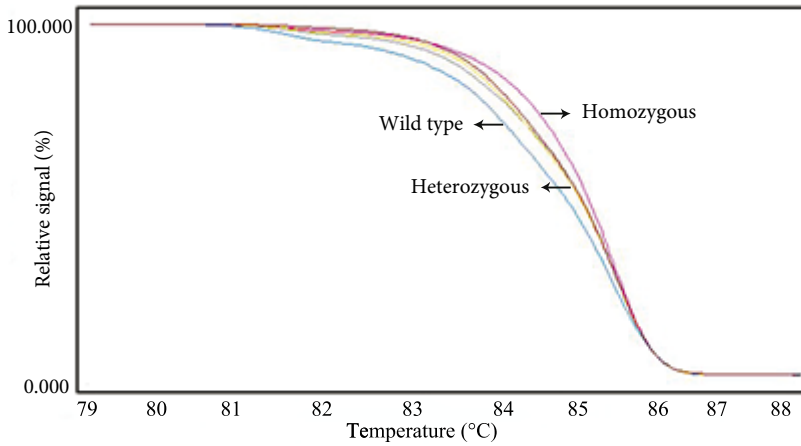


Figure 3. Normalized and temperature-shifted high-resolution melting curves, *MEFV* M694V.

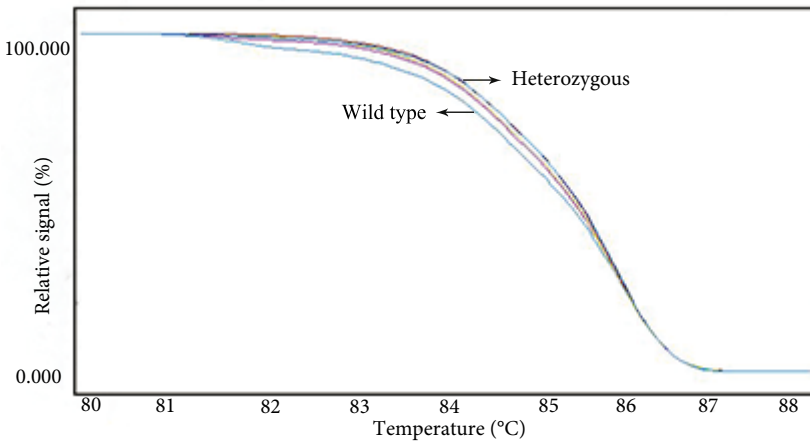


Figure 4. Normalized and temperature-shifted high-resolution melting curves, *MEFV* V726A.

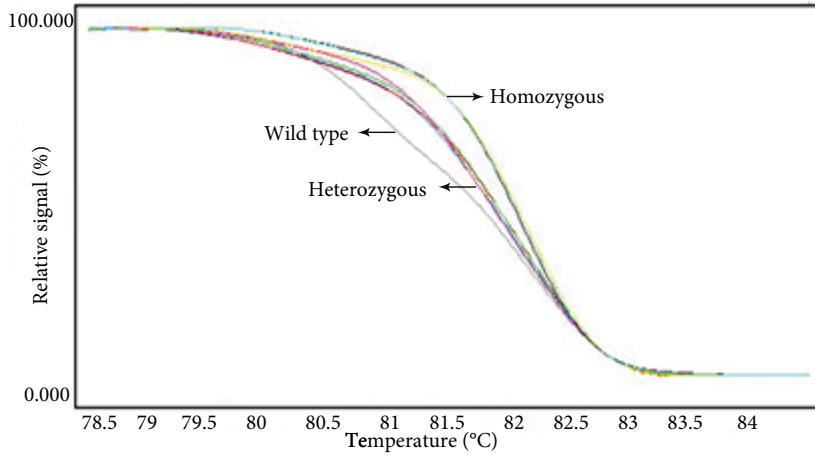


Figure 5. Normalized and temperature-shifted high-resolution melting curves, F51691G>A.

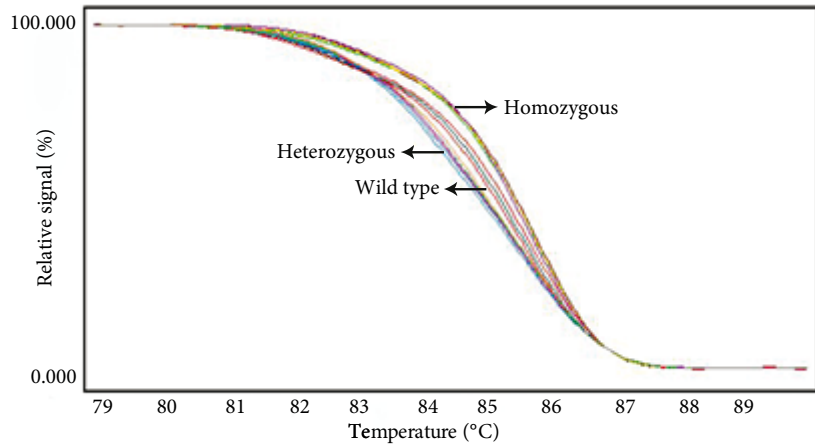


Figure 6. Normalized and temperature-shifted high-resolution melting curves, F220210G>A.

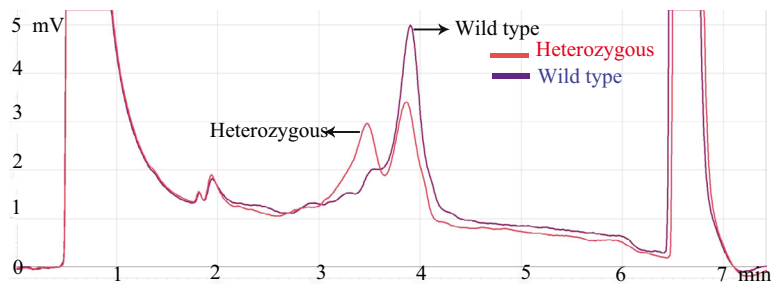


Figure 7. A representative DHPLC profile obtained from heterozygous sample as compared with the wild type.

(22,23). Meanwhile, HRM is another mutation screening method that was introduced in the early 2000s. HRM, although newer compared to DHPLC, is a well-established technique.

To evaluate the use of a mutation screening method in a clinical setting, different mutations should be tested with the method of choice. *BRCA1*, *BRCA2*, *BRAF* (V600E) *c-KIT*, and *PDGFRA* gene mutations were shown to be effectively detected by DHPLC (24–26). For HRM, a closed-tube method with no need for postprocessing has been used in several studies, including detection of the *KRAS* mutations, *MYD88* L265P mutation, *RET* protooncogene, and *CDKL5* gene mutations (27–30).

In this study, we investigated the effectiveness of HRM in the detection of *MEFV* (E148Q, M680I, M694V, and V726A), F51691G>A, and F220210G>A mutations and compared the sensitivity of the technique with DHPLC. All point mutations were successfully detected by HRM analysis and only 4 of the 6 were detected by DHPLC.

There are several studies comparing HRM with DHPLC. The detection of *BRAFV600E* mutations by DHPLC and HRM was evaluated and the results of that study showed that DHPLC and HRM can both be reliably used for the detection of this mutation (31). In another study, all coding exons of *TSC1* and *TSC2* were analyzed using DHPLC and HRM. The sensitivities of HRM and DHPLC

were estimated as 95% and 75% and the specificities were assessed as 91% and 98%, respectively. Based on these results, the authors concluded that their HRM protocol can be used for *TSC* gene mutation screening for clinical applications (32).

To evaluate the efficiency and sensitivity of HRM compared with DHPLC for the detection of genetic variations, different investigations including *LMNA*, *SCN5A*, and *ABCA4* gene mutations were conducted (33–35). In those studies, HRM was found to be faster compared with DHPLC; moreover, HRM showed better sensitivity and specificity than DHPLC.

In the present study, HRM detected all of the point mutations, whereas DHPLC could detect only 4 out of 6 mutations. Our results indicated that HRM is the more sensitive technique in the detection of *MEFV* M694V and V726A mutations.

HRM is an accurate, cost-effective, fast, and high-throughput technique. The technique has considerable potential for the detection of DNA sequence variations for both research and diagnostic applications.

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References

1. Ben-Chetrit E, Levy M. Familial Mediterranean fever. *Lancet* 1998; 351: 659–664.
2. Onen F. Familial Mediterranean fever. *Rheumatol Int* 2006; 26: 489–496.
3. Tunca M, Akar S, Onen F, Ozdogan H, Kasapcopur O, Yalcinkaya F, Tutar E, Ozen S, Topaloglu R, Yilmaz E et al. Familial Mediterranean fever (FMF) in Turkey: results of a nationwide multicenter study. *Medicine (Baltimore)* 2005; 84: 1–11.
4. French FMFC. A candidate gene for familial Mediterranean fever. *Nat Genet* 1997; 17: 25–31.
5. Gershoni-Baruch R, Shinawi M, Leah K, Badarnah K, Brik R. Familial Mediterranean fever: prevalence, penetrance and genetic drift. *Eur J Hum Genet* 2001; 9: 634–637.
6. Akar N, Misiroglu M, Yalcinkaya F, Akar E, Cakar N, Tumer N, Akcakus M, Tastan H, Matzner Y. *MEFV* mutations in Turkish patients suffering from familial Mediterranean fever. *Hum Mutat* 2000; 15: 118–119.
7. Touitou I. The spectrum of familial Mediterranean fever (FMF) mutations. *Eur J Hum Genet* 2001; 9: 473–483.
8. Khan S, Dickerman JD. Hereditary thrombophilia. *Thromb J* 2006; 4: 15.
9. Zoller B, Garcia de Frutos P, Hillarp A, Dahlback B. Thrombophilia as a multigenic disease. *Haematologica* 1999; 84: 59–70.
10. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 1996; 88: 3698–3703.
11. Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; 369: 64–67.
12. Şahin Ş, Benli İ, Aydoğan L. Distribution of prothrombin G20210A, factor V Leiden, and MTHFR C677T mutations in the middle Black Sea area (Tokat) of Turkey. *Turk J Med Sci* 2012; 42: 1093–1097.
13. Nataraj AJ, Olivos-Glander I, Kusukawa N, Highsmith WE Jr. Single-strand conformation polymorphism and heteroduplex analysis for gel-based mutation detection. *Electrophoresis* 1999; 20: 1177–1185.
14. Pourzand C, Cerutti P. Genotypic mutation analysis by RFLP/PCR. *Mutat Res* 1993; 288: 113–121.

15. Abrams ES, Murdaugh SE, Lerman LS. Comprehensive detection of single base changes in human genomic DNA using denaturing gradient gel electrophoresis and a GC clamp. *Genomics* 1990; 7: 463–475.
16. Murphy KM, Berg KD. Mutation and single nucleotide polymorphism detection using temperature gradient capillary electrophoresis. *Expert Rev Mol Diagn* 2003; 3: 811–818.
17. Liu W, Smith DI, Reichtzigel KJ, Thibodeau SN, James CD. Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. *Nucleic Acids Res* 1998; 26: 1396–1400.
18. Xiao W, Oefner PJ. Denaturing high-performance liquid chromatography: a review. *Hum Mutat* 2001; 17: 439–474.
19. 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.
20. Reed GH, Wittwer CT. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clin Chem* 2004; 50: 1748–1754.
21. Montgomery J, Wittwer CT, Palais R, Zhou L. Simultaneous mutation scanning and genotyping by high-resolution DNA melting analysis. *Nat Protoc* 2007; 2: 59–66.
22. Nelken J, Meshkani R, Chahal N, McCrindle B, Adeli K. Detection of familial defective apoB (FDB) mutations in hypercholesterolemic children and adolescents by denaturing high performance liquid chromatography (DHPLC). *Clin Biochem* 2008; 41: 395–399.
23. Wagner T, Stoppa-Lyonnet D, Fleischmann E, Muhr D, Pages S, Sandberg T, Caux V, Moeslinger R, Langbauer G, Borg A et al. Denaturing high-performance liquid chromatography detects reliably *BRCA1* and *BRCA2* mutations. *Genomics* 1999; 62: 369–376.
24. Battochio A, Mohammed S, Winthrop D, Lefresne S, Mulder K, Chu Q, O'Hara C, Lai R. Detection of *c-KIT* and *PDGFRA* gene mutations in gastrointestinal stromal tumors: comparison of DHPLC and DNA sequencing methods using a single population-based cohort. *Am J Clin Pathol* 2010; 133: 149–155.
25. Kim TE, Jung ES, Jung CK, Bae JS, Kim SN, Kim GS, Lee HN, Kang CS, Choi YJ. DHPLC is a highly sensitive and rapid screening method to detect *BRAF(V600E)* mutation in papillary thyroid carcinoma. *Exp Mol Pathol* 2013; 94: 203–209.
26. Gross E, Arnold N, Pfeifer K, Bandick K, Kiechle M. Identification of specific *BRCA1* and *BRCA2* variants by DHPLC. *Hum Mutat* 2000; 16: 345–353.
27. Krypuy M, Newnham GM, Thomas DM, Conron M, Dobrovic A. High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: *KRAS* codon 12 and 13 mutations in non-small cell lung cancer. *BMC Cancer* 2006; 6: 295.
28. Margraf RL, Mao R, Highsmith WE, Holtegaard LM, Wittwer CT. Mutation scanning of the *RET* protooncogene using high-resolution melting analysis. *Clin Chem* 2006; 52: 138–141.
29. Raymond L, Diebold B, Leroux C, Maurey H, Drouin-Garraud V, Delahaye A, Dulac O, Metreau J, Melikishvili G, Toutain A et al. Validation of high-resolution DNA melting analysis for mutation scanning of the *CDKL5* gene: identification of novel mutations. *Gene* 2013; 512: 70–75.
30. Wang CZ, Lin J, Qian J, Shao R, Xue D, Qian W, Xiao GF, Deng ZQ, Yang J, Li Y et al. Development of high-resolution melting analysis for the detection of the *MYD88 L265P* mutation. *Clin Biochem* 2012; 46: 385–387.
31. Carbonell P, Turpin MC, Torres-Moreno D, Molina-Martinez I, Garcia-Solano J, Perez-Guillermo M, Conesa-Zamora P. Comparison of allelic discrimination by dHPLC, HRM, and TaqMan in the detection of *BRAF* mutation V600E. *J Mol Diagn* 2011; 13: 467–473.
32. Tsai TS, Huang MY, Chang YT, Wang CY, Lin JL, Hung PC, Lin SP, Sun CF, Wang WS, Chang CM et al. High-resolution melting analysis is a more effective approach for screening *TSC* genes mutations. *Genet Test Mol Biomarkers* 2011; 15: 415–421.
33. Aguirre-Lamban J, Riveiro-Alvarez R, Garcia-Hoyos M, Cantalapiedra D, Avila-Fernandez A, Villaverde-Montero C, Trujillo-Tiebas MJ, Ramos C, Ayuso C. Comparison of high-resolution melting analysis with denaturing high-performance liquid chromatography for mutation scanning in the *ABCA4* gene. *Invest Ophthalmol Vis Sci* 2010; 51: 2615–2619.
34. Millat G, Chanavat V, Rodriguez-Lafrasse C, Rousson R. Rapid, sensitive and inexpensive detection of *SCN5A* genetic variations by high resolution melting analysis. *Clin Biochem* 2009; 42: 491–499.
35. Millat G, Chanavat V, Julia S, Crehalet H, Bouvagnet P, Rousson R. Validation of high-resolution DNA melting analysis for mutation scanning of the *LMNA* gene. *Clin Biochem* 2009; 42: 892–898.