ORIGINAL ARTICLE

Development and Validation of High Resolution Melting Assays for High-Throughput Screening of *BDNF* rs6265 and *DAT1* rs40184

Asraa Faris[1], Hadri Hadi Bin Md Yusof[1], Shahidee Zainal Abidin[1], Omar Habib[1], Pike-See Cheah[2], Johnson Stanslas[3], Normala Ibrahim[4], Munn Sann Lye[5], Abhi Veerakumarasivam[6], Rozita Rosli[1], King Hwa Ling[1]

[1] Department of Biomedical Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2] Department of Human Anatomy, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[3] Department of Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[4] Department of Psychiatry, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[5] Department of Community Health, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[6] Department of Biological Sciences, School of Science and Technology, Sunway University, 47500 Subang Jaya, Selangor, Malaysia

ABSTRACT

Introduction: One of the commonly used techniques for mutation screening is High Resolution Melting (HRM) analysis. HRM is a post PCR method that relies on the detection of the fluorescent signals acquired due to the release of DNA intercalated dyes upon the melting of dsDNA to ssDNA. The method is simple, inexpensive and does not require post PCR-handling, making it suitable for high throughput screening. **Methods:** This study aimed to develop and validate HRM technique for the screening of two disease-associated single nucleotide polymorphisms (SNPs) namely *BDNF* rs6265 and *DAT1* rs40184 using a total of 30 gDNA samples. The obtained results were confirmed and validated by sequencing. **Results:** HRM analysis showed that the predicted genotypes of *BDNF* rs6265 and *DAT1* rs40184 among all the gDNA samples were in 100% concordance with the sequencing results, making it an accurate and sensitive method for the detection of SNPs. **Conclusions:** The application of HRM can accurately determine the genotype of *BDNF* rs6265 and *DAT1* rs40184 SNPs, making it a promising tool for rapid and high-throughput screening of targeted SNPs in a large population study.

Keywords: BDNF, DAT1, HRM, PCR

Corresponding Author:
King Hwa Ling, PhD
Email: lkh@upm.edu.my
Tel: +603-8947 2564

INTRODUCTION

The brain derived neurotrophic factor (BDNF) is a growth factor and is the most expressed neurotrophin in the adult brain (1). Upon the binding of BDNF to the receptor tyrosine kinase B (TrkB), pathways such as the phosphatidylinositol-3-kinase (PI3K) pathway, the phospholipase C (PLCγ) pathway, and the mitogen-activated protein kinase (MAPK) pathway are activated (2,3). The activation of the above pathways results in neuronal growth, differentiation, and synaptic plasticity and was shown to be crucial to the mechanisms related to memory solidation (4,5). BDNF has crucial roles in neuronal development and maintenance of both central and peripheral nervous system. A single nucleotide polymorphism (SNP) in the *BDNF* gene known as rs6265 was found to be associated with the development of several mental illnesses such as bipolar disorder, Alzheimer's disease, schizophrenia and depression (6–9). The rs6265 results in a substitution of the amino acid valine with methionine at codon 66, leading to alteration in the secretion of the mature BDNF protein (10,11).

Dopamine is a neurotransmitter that regulates several body functions such as movement, reward, cognition and mood (12). The availability of dopamine is regulated by the dopamine transporter (DAT1). DAT1 is a plasma membrane protein that regulates the reuptake of dopamine in the presynaptic neurons (13). Deficiency of dopamine has been associated with several disorders such as bipolar disorder, Parkinson's disease, schizophrenia and depression implicating a role of DAT1 in the aetiology of these disorders (14–17). A single nucleotide polymorphism in the 14th intron of the dopamine transporter gene, rs40184, has been shown to be associated with the development of neuropsychiatric disorders such as depression (18), bipolar disorder(19) and attention deficit hyperactivity disorder (ADHD) (20).

Despite the association to several disorders, to date, there is no clear mechanism that explains the role and function of this SNP in the development of the disorders in humans (21). Studies on mice models, however, found that polymorphisms on the *DAT1* gene affect the normal functionality of the gene, leading to hyperactivity and diminished dopaminergic tone (22,23).

One of the most employed techniques for the screening of *BDNF* rs6265 and *DAT1* rs40184 is polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (17,24–26). PCR-RFLP is a technique that relies on the use of restriction enzymes to identify the sequence of specific regions or motifs of DNA. PCR-RFLP approach involves the amplification of the targeted DNA fragment bearing the variation via PCR and subsequently treating the amplicon with an appropriate restriction enzyme that results in the formation of restricted fragments of variable sizes. These fragments are electrophoresed using agarose or polyacrylamide gel before being stained for fluorescence visualization upon excitation by UV (27). Among the major disadvantages of this technique is the requirement for the usage of specific endonucleases for the detection of SNPs, which limits the range of detectable SNPs. Other disadvantages include being laborious, time-consuming and unusable for high-throughput screening (28), thus indirectly escalating the cost per man-hour production.

An alternative technique to PCR-RFLP is High Resolution Melting (HRM). HRM is a post-PCR method used for the detection of several genetic modifications such as mutations and single nucleotide polymorphisms in the PCR amplified DNA products. The detection is achieved through analysing the melting behaviour of the nucleic acids via the detection of the fluorescence signal of the double-stranded DNA intercalating dye (29,30). The analysis of the melting curve is carried out by using computer-based software to track the melting transition of the PCR product through the detection of the change in fluorescence signal as the temperature gradually increases and releases the saturating dye binding to the double-stranded DNA (31). The differentiation of the genotypes is based on monitoring changes in the temperature and shape of the melting curve. Homozygous (wild-type and mutant) genotypes can be discriminated by a slight change in the melting temperature, whereas the discrimination of heterozygous genotypes is displayed by a change in the shape of the melting curve (32). As compared to other real-time amplification methods such as TaqMan and fluorescence resonance energy transfer, HRM uses low-cost fluorescent dyes that require minimum optimization (33,34).

The purpose of this study was to develop and validate HRM assays for the detection of *BDNF* rs6265 and *DAT1* rs40184 single nucleotide polymorphisms. The assays were optimized for future, rapid and low-cost high-throughput screening of samples in 96- or 384-well formats in suitable real-time PCR machines.

METHODS

Ethical approval and sample acquisition

Ethical clearance and approval was obtained from the Malaysian National Institute of Health (NIH) and Medical Research and Ethics Committee (MREC) (ID number: NMRR-14-688-19696). Written consent was obtained from all the subjects who participated in the study. A total of 30 genomic DNA (gDNA) samples were obtained for this study.

Genomic DNA preparation

Genomic DNA samples were extracted from the buffy coat using QIAamp® DNA Mini Kit (QIAGEN, Germany) following the manufacturer's instructions. The quality of the DNA was measured using NanoVue Plus UV spectrophotometer (GE Healthcare, USA). Only samples with absorbance 260/280 ratio of 1.7-1.9 were considered pure. The extracted gDNA were then electrophoresed using 1% (w/v) agarose gel for 40 minutes at 100V to measure the integrity. The gel was stained with ethidium bromide (Bio-Rad, USA) for 10 minutes followed by destaining with distilled water. The bands were then viewed by G:BOX BioImaging System (Syngene, USA). The generation of the gel image was obtained by GeneSnap software (Syngene, USA). All the DNA samples were stored in -20°C until use.

Primer design and gradient PCR

Primers for the detection of *BDNF* rs6265 and *DAT1* rs40184 were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and were synthesized by Integrated DNA Technologies, Singapore (Table I). To detect the optimal annealing temperature, a gradient PCR was performed with the temperature ranges between 55°C-65°C using MasterCycler Gradient Thermal Cycler machine (Eppendorf, Germany). The product was then electrophoresed using a 2% (w/v) agarose gel for 60 minutes at 80V to determine the best annealing temperature.

PCR efficiency, specificity, and HRM analysis

Serially diluted PCR amplicons were used to test on PCR efficiency and reproducibility using HRM. Each 10µl HRM-reaction mixture consisted of 1X LightCycler® 480 High Resolution Melting Master (Roche, Germany), 15ng of genomic DNA, 0.35 µmol forward and reverse primers, 2mM MgCl2 and PCR-grade water. The reaction was performed in triplication on a LightCycler® 480 Multiwell Plate 96 from Roche, Germany. The amplification of the SNP was carried out using LightCycler® 480 System (Roche,Germany) using the following cycle condition: initial denaturation at 95°C was done for 10 min followed by 45 cycles of

Table I: The properties of the designed primers for high resolution melting assays.

SNP	Primer sequence (5′→3′)		GC% / Tm (°C)	Amplicon size (bp)
BDNF rs6265	Forward	CTTGACATCATTGGCTGACACT	45.5/60.2	146
	Reverse	GCTCCAAAGGCACTTGACTACT	50.0/ 60.0	146
DAT1 rs40184	Forward	CACAGTCTCGCGGCTTTT	55.6/ 60.1	100
	Reverse	TGGACCAACACACCCTTGA	52.6 / 61.0	100

amplification at 95°C for 10s, annealing at 60°C (*BDNF* rs6265) and 61°C (*DAT1* rs40184) for 15s, and a final extension at 72°C for 10s. Subsequently, amplicons were subjected to HRM analysis followed by melting analysis in the same machine using a temperature range of 65°C to 95°C with 25 acquisitions per every 1°C increment. To obtain the best discrimination, validated samples of each genotype were included in each run to serve as references.

Following HRM, the Light Cycler® released 1.5.1.62SP3 software was employed to view the real-time amplification process and analyse the melting profiles of all samples. The normalized melting curves and the temperature shifted differential plots were obtained from the gene scanning module of the software to determine the genotype for each sample based on the profiles of validated samples.

Sequencing

The HRM product of each genotype was purified using High Pure PCR purification kit (Roche, Germany) following the manufacturer's instructions and was sent for Sanger sequencing using services provided by First BASE Laboratories Sdn. Bhd., Malaysia. The assembly of the sequences and the generation of the contigs were performed by using SNAPGENE software (GSL Biotech; available at snapgene.com). A Phred score of >20 was used as a cut-off for good quality sequences.

RESULTS

Sample preparation and PCR optimization

The quality of DNA is an important criterion for the success of HRM. The DNA samples used for this study displayed an A260/280 absorbance of 1.7-1.9 indicating that the samples were pure. The samples were then electrophoresed using 1% (w/v) agarose gel to check the integrity of the DNA (Fig.1A). All the bands appeared intact above the 10kb marker without smearing indicating that the samples were in good gDNA integrity and not degraded.

In this study, primers were designed to amplify the targeted sequence of the *BDNF* and *DAT1* genes including both rs6265 and rs40184 SNPs, respectively. The best annealing temperature was determined by a clear and bright gel electrophoresis band with a specific product. Based on the gel electrophoresis result, the best annealing temperature for *BDNF* rs6265 and for *DAT1* rs40184 was determined at 60°C (Fig.1B) and 61°C, respectively (Fig.1C). A standard curve was then obtained to evaluate the efficiency of the PCR for both primers (Fig.1D and Fig.1E). Based on the equation derived from the standard curve, the PCR efficiency for *BDNF* rs6265 was 99.71% and for *DAT1* rs40184 was 92.37%. R-squared values for both PCR assays were greater than 0.98 indicating that the assays were highly reproducible.

Validation of HRM specificity

Distinct peaks were obtained for the three genotypes (wild-type, heterozygous, and recessive) of *BDNF* rs6265 (Fig.2A and Fig.2B) and *DAT1* rs40184 (Fig.2C and Fig.2D) using high resolution melting. The distinction was based on the shape of the melting curve and on the melting temperature differences. The difference in the plot observed in Fig.2C occured because of the GC rich nature of the *DAT1* amplicon. Due to their stable properties, the GC rich region only melt when a sufficient high temperature is reached, resulting in a shoulder peak. To evaluate the accuracy of the HRM method, the HRM results were compared to the results obtained from Sanger sequencing for *BDNF* rs6265 (Fig.2E) and *DAT1* rs40184 (Fig.2F). The electropherogram displayed a single peak for the wild-type genotype (GG) and the mutant genotype (AA). A distinct overlapping double peaks of different colours corresponding to two different bases indicated the heterozygous genotype (GA). All the 30 samples for each SNP were sequenced and matched 100% with the genotypes predicted by HRM analysis for both *BDNF* rs6265 and *DAT1* rs40184.

DISCUSSION

One of the several approaches for the detection of single nucleotide polymorphism is the High Resolution Melting (HRM) analysis. It is a cheap and rapid SNP detection technique that is easy to setup and requires shorter time and minimum optimization of the saturating double-stranded DNA binding dyes (34). A major advantage of this technique is that the method uses the normal PCR reagents with double-stranded DNA intercalating dye and needs no post PCR handling which is very convenient for daily diagnostic and screening settings (35,36). The HRM technique undergoes the normal step of PCR amplification of a DNA template intercalated to a binding dye with an additional melting step. Following the amplification, the dyes fluoresce at a high level which slowly reduces as the DNA dissociates due to melting.

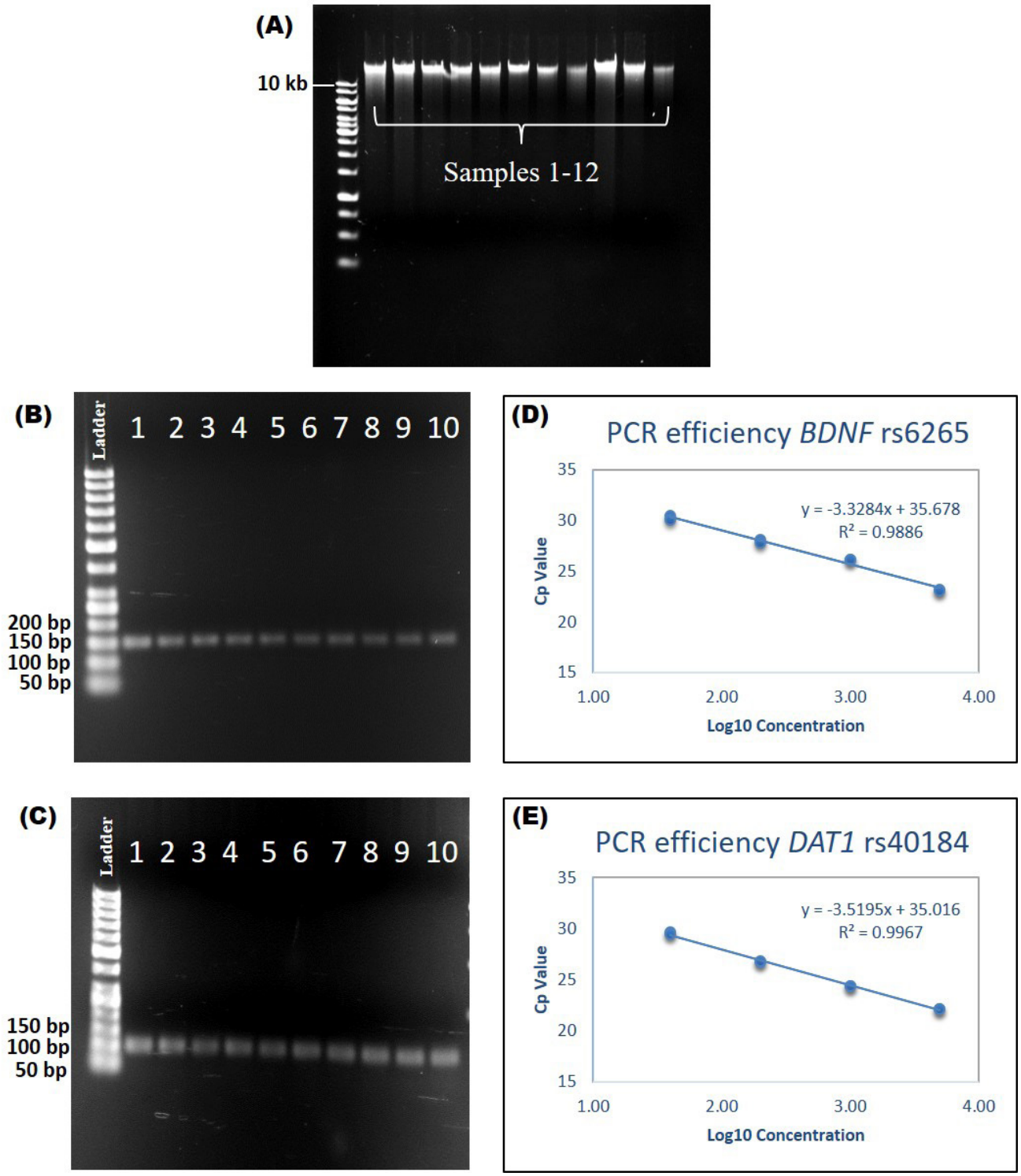

Figure 1: (A) A representative image of agarose gel electrophoresis to determine DNA integrity of 12 samples. **(B)** The optimization of the PCR annealing temperature for *BDNF* rs6265 primers. **(C)** The optimization of the PCR annealing temperature for *DAT1* rs40184. The gradient PCR for both primers was performed with the annealing temperature ranges between 55-66°C (1=55.0°C, 2=55.7°C, 3=56.6°C, 4=57.8°C, 5=59.1°C, 6=60.5°C, 7=61.8°C, 8=63.1°C, 9=64.2°C, 10=65.0°C) **(D)** The standard curve demonstrating the PCR efficiency of *BDNF* rs6265 at 99.7% **(E)** The standard curve demonstrating the PCR efficiency of *DAT1* rs40184 at 92.37%.

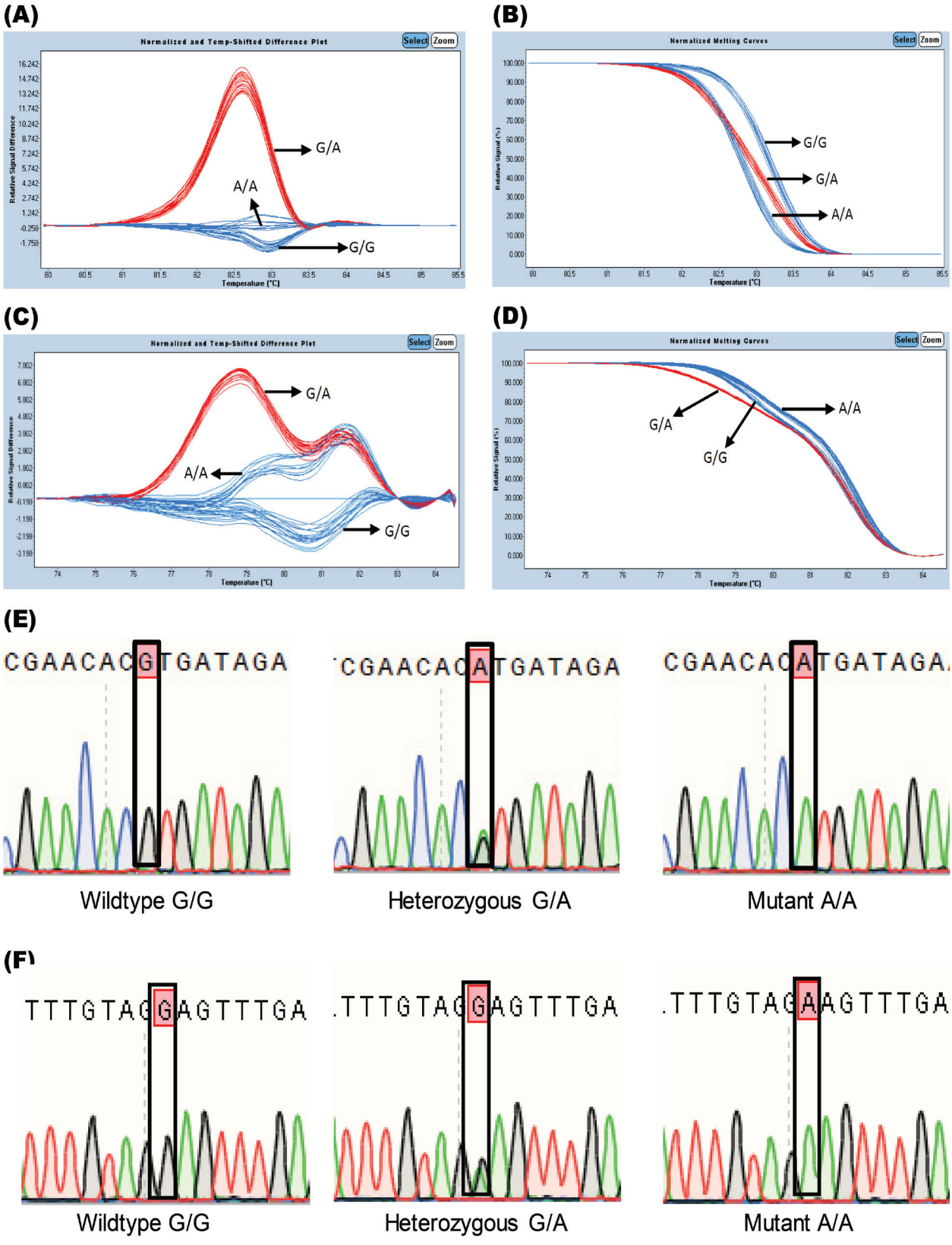

Figure 2: **(A)** A representative result of the temperature shifted difference plot and **(B)** the normalized melting curves of *BDNF* rs6265. **(C)** A representative result of the temperature shifted difference plot and **(D)** the normalized melting curves of *DAT1* rs40184. **(E)** A representative result for the three genotypes obtained from the sequencing of *BDNF* rs6265 and **(F)** *DAT1* rs40184

Product length, sequence, and GC content are some of the factors that play role in the melting behavior. One of the criteria to increase the sensitivity of the technique is through the usage of a shorter amplicon to broaden the variation among the melting profiles (32). As the amplicon length decreases, the difference in the melting temperature among the different genotypes increases resulting in a better profile differentiation. In addition, smaller amplicons would possibly reduce the cycling times as the denaturation occurs at a lower temperature (30).

In this paper, HRM assays were developed and validated for the screening of *BDNF* rs6265 and *DAT1* rs40184 using genomic DNA samples. As shown in Figure 2 A-D, three genotypes existed for both *BDNF* rs6265 and *DAT1* rs40184 that demonstrated distinct profiles making them distinguishable from each other. The wild-type and the mutant genotypes were distinguishable from each other by analysing the difference in the melting temperature, which was predominantly determined by the energy needed to break up all the hydrogen bonds to completely separate the double-stranded DNA into single-stranded. The G-C base pairing (three hydrogen bonds) had a higher melting temperature as compared to A-T (two hydrogen bonds) base pairing, whereas heterozygous samples were differentiated by a curve with a distinct shape due to the occurrence of mismatching or formation of heteroduplexes during the DNA denaturation-reannealing process (35).

The validation of the HRM results was conducted by Sanger sequencing. All the results obtained from the HRM agreed with the sequencing results indicating that the HRM method is 100% accurate and specific in detecting different genotypes for *BDNF* rs6265 and *DAT1* rs40184. Apart from Sanger sequencing, other methods that can be used for measuring the accuracy of the HRM include pyrosequencing, microarrays and the usage of restriction enzymes and hybridization labelled probe, which despite being costly are still equally advisable to be used for the determination of the accuracy (37,38).

CONCLUSION

High resolution melting assays were successfully developed and validated for the detection of two SNPs namely *BDNF* rs6265 and *DAT1* rs40184. Comparing the HRM result to the result obtained from sequencing shows that this technique is reliable and accurate for high-throughput screening approach. As compared to other methods, this method has the advantage of being easy, efficient and rapid. The use of HRM for the screening of SNP enables the elimination of additional sequencing analysis that in most cases is costly, involves multi-steps, and is time-consuming.

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Author Contributions

Conceived and designed the experiment: AF, PSC, KHL. Analyzed the data: AF, HHBMY, SZA, OH. Wrote the first draft of the manuscript: AF, KHL. Contributed to the writing of the manuscript: PSC, JS, MSL, NI, RR, AV. Jointly developed the structure and arguments for the paper: AF, PSC, JS, MSL, NI. Made critical revisions and approved final revision: PSC, KHL. All authors agree with manuscript result and conclusion. All authors reviewed and approved the final manuscript.

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