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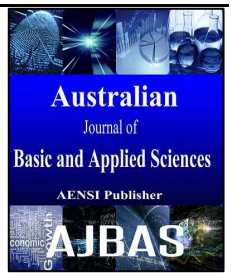

Development of High Resolution Melting (HRM) Methods to Detect Dopamine Transporter (DAT) Gene (SLC6A3) (rs27072) Polymorphisms in Human

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ABSTRACT

Background: Polymorphism of dopamine transporter (DAT) gene (SLC6A3) caused by variation occurring in variable number of tandem repeat (VNTR) is known to be associated with many genetic-diseases. rs27072 is one of the polymorphisms occurring in dopamine transporter (DAT) gene (SLC6A3) that is believed to be associated with nicotine dependence. PCR-based and PCR-RFLP analysis is a common method for genotyping the dopamine transporter gene (SLC6A3) polymorphisms in human. Although these methods are available but it still not considered as cost-effective and time-consuming. In this study, we have developed an alternative HRM-based technology without any post PCR to provide rapid and efficient investigation of dopamine transporter (SLC6A3) (rs 27072) gene polymorphism. Using the HRM analysis, optimization was done by first varying the annealing temperature and further optimized by varying the primer concentration and number of cycle. We found non-specific amplification presented in melting behaviour of the target amplicon was caused by the high concentration of primer used in the study. Additionally, we also discovered that insufficient number of cycles can lead to the late amplification of target amplicon. For future plan, other parameters that is known to influence optimization of HRM analysis such as DNA concentration, primer size, GC content and annealing/extension time will be tested and examined until good HRM analysis was obtained.

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INTRODUCTION

Dopamine, a major neurotransmitter of the central nervous system is thought to be involved in the mesolimbic reward pathway (Wise *et al*., 1989; Koob *et al*., 1992). Thus, dopamine plays a major role in activating reward-motivated behaviour, one which affects the dopamine level in the brain. Many addictive drugs are believed to enhance dopaminergic neurotransmission by increasing the level of dopamine released (DA) in midbrain (Venton *et al*., 2006). One study done by Pontieri *et al*., (1996) showed the presence and stimulation of nicotinic receptors on dopaminergic cell bodies resulted in an increased release of dopamine in nucleus accumbens of the mesolimbic system.

Dopamine transporter (DAT) is a membrane-spanning protein that regulates the temporal and spatial activity of released dopamine (DA) through rapid uptake of dopamine neurotransmitter into presynaptic terminal. The dopamine transporter gene (SLC6A3) is an obligatory target for several addictive psychostimulants including cocaine, and amphetamine (Giros *et al*., 1996; Kilty *et al*., 1991; Chang *et al*., 2001). Dopamine transporter gene (SLC6A3) contains variable number of tandem repeat (VNTR) in its 3'-untranslated region which is known to be associated with a variety numbers of disease-associated genes. Therefore, dopamine transporter (SLC6A3) gene polymorphism is raised from variability occurring in variable number of tandem repeat (VNTR). The variation of variable number of tandem repeat (VNTR) of SLC6A3 in terms of length and sequence was shown to affect the amount of DAT protein expressed in the brain (Miller and Madras, 2002: Fuke *et al*., 2001).

Several genotyping technologies are available for the determination of dopamine transporter SLC6A3 gene polymorphism, including PCR, and PCR-RFLP. In the presented study, we have developed an alternative new HRM-based technology without any post PCR to determine the dopamine transporter SLC6A3 gene polymorphism. HRM analysis is a mutation scanning method that

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employs dsDNA intercalating dye and its analysis is based on the form of thermodynamics difference between DNA fragments. HRM methods also are rapid, simple and efficient technique since the PCR amplification and HRM analysis is performed in one run. The objective of this study was to develop a high resolution melting methods for further use in the investigation of dopamine transporter gene (SLC6A3) (rs27072) polymorphisms. The successful optimization high resolution melting methods would result in good analysis of DAT gene polymorphisms.

MATERIALS AND METHODS

DNA Sample:

DNA samples were obtained from human adult. Prior to that, all subjects were given informed consent form to participate in the study. This study was approved by the Ethics Committee of School of Medical Sciences, Universiti Sains Malaysia.

DNA Extraction:

Genomic DNA was extracted from 200ul blood per sample using G-spin Total DNA Extraction Mini kit (iNtRON, Korea) and the genotyping from each sample was determined by the high resolution melting (HRM) analysis. Concentration of DNA was estimated using Qubit® dsDNA HS Assay kit (Invitrogen™, USA), while the DNA purity was assessed by spectrophotometer, Biophotometer Uvette (Eppendorf, Germany), at 280 nm absorbance.

Primer Design:

The rs27072 sequence of SLC6A3 is available on the National Centre for Biotechnology Information (NCBI) database (dbSNP cluster ID: rs27072). Primer3 programme (Rozen and Skaletsky, 2000) was used to design the primers. PCR primer was designed and had predicted length 114 bp. Then, the primers were optimized for annealing temperature. The specificity of primers were confirm with Primer BLAST.

PCR and HRM analysis:

The PikoReal 96 Real-Time PCR system (Thermo Scientific, USA) was used for PCR amplification and high resolution melting (HRM) analysis. PCR amplifications were performed in 10 µl reaction volumes consisting of 2ul of genomic DNA template, 5ul of Luminaris Color HRM Master Mix (2x), and 0.5ul of forward and reverse primers (final concentration: 0.5 uM) and 2ul of nuclease free water. For HRM analysis, first step was to optimize and determine the optimum annealing temperature. Optimization was done by varying the annealing temperature using 3-step PCR cycling protocol as follows: an initial denaturation step for 10 min at 95 C, followed by 40 cycles of 10 sec at 95 C, 30 sec at 60°C,58°C and 56°C of annealing, and 30 sec final extension at 72°C. HRM analysis was performed automatically after the PCR, and programmed to ramp temperature from 60 to 95°C, raised by 0.2 degree/step after the final extension step. Melt curves were analysis using the PikoReal software version 2.1(Thermo Scientific, USA). According to Luminaris Color HRM Master Mix protocol, the DNA template concentration should not exceed 20 ng in the final volume. Both PCR amplification and high resolution melting (HRM) analysis was performed using the Luminaris Color Master Mix purchase from Research Instrument (Thermo Scientific, USA).

DNA Sequencing:

To validate the HRM methods, PCR purified products were performed using ABI 3130x1 Genetic Analyzer (Applied Biosystems, Foster City, CA) for sequencing.

RESULT AND DISCUSSION

High resolution melting analysis is a recently rapid, simple and efficient technique that intensively used in identifying sequence variation in human, plant, and microorganisms (Reed and Wittwer, 2004; Krypuy *et al*., 2007; Liu *et al*., 2014) In previous study, gene polymorphisms of dopamine transporter (SLC6A3) (rs 27072) were investigated by using PCR-RFLP methods (Ling *et al*., 2003). Therefore, in this study we have developed an alternative technique HRM analysis to provide better detection of dopamine transporter (DAT) gene (SLC6A3) (rs27072) polymorphisms. This highly sensitive method also has ability to detect accurately small differences in melting profile of interest amplicons (Liew *et al*., 2004). Additionally, with an access to HRM devices, PCR amplification and HRM analysis can be performed in one run thus making it a convenient and time-consuming application for genotyping. Good HRM assay is very critical for HRM analysis. Thus, most attention is given during the optimization of HRM assay development.

Our study showed that primer concentration and number of cycle highly influenced the determination of annealing temperature (Ta) for HRM analysis. We tested the annealing temperature with nine samples of 20 ng of purified DNA at 60°C (as recommended by protocol) and later Ta was reduced from the original protocol to 58°C and 56°C (Figure 1). During optimization of annealing temperature, 0.5µM of primer concentration and 40 numbers of cycles were used. We obtained suboptimal annealing temperature for all tested annealing temperature since we found the presence of non-specific peaks in the melt curve graph, indicating the presence of non-specific amplification of the DNA (Figure 2). Additionally, the late amplification of target amplicon also was demonstrated in all tested Ta (Cq value > 30) (Figure 4). However, annealing at 58°C

has potential to be the optimal Ta for genotyping the SLC6A3. Then, to observe the effect of varying the primer concentration and number of cycles on annealing temperature, we tested three samples of 20 ng of purified DNA using 0.2 µM (Figure 3) and 45 numbers of cycles (Figure 5).

The non-specific peak resolved when 0.2 µM of primer concentration was applied to the per PCR reaction (Figure 3). After using 45 number of cycle, the overall Cq value for all tested Ta was declined. Annealing at 58 °C (Cq value=22) showed the lowest Cq value compared to annealing at 56°C (Cq value=24) and 60°C (Cq value=26) (Figure 4). Hence, we obtained optimal primer concentration and number of cycle with 0.2 µM per PCR reaction tube and 45 cycles. We concluded that using excessive concentration of primers can increase the chance of primers binding nonspecifically to undesired sites on the template. Increasing the number of amplification cycles can lead to the higher initial number of DNA molecules in the sample. Thus, the fluorescence will be detected in earlier cycles.

The non-specific amplicon and late amplification performed in this study would greatly affect the overall final analysis of HRM in determining the variants of polymorphisms studied. Although annealing at 58 °C (Figure 6) showed different melting profiles of target amplicon, the analysis of different genotype group (homozygous SNP&heterozygotes) results still difficult to be distinguished. For future plan, primer size, GC content and annealing/extension time will be optimized to maximise good HRM analysis.

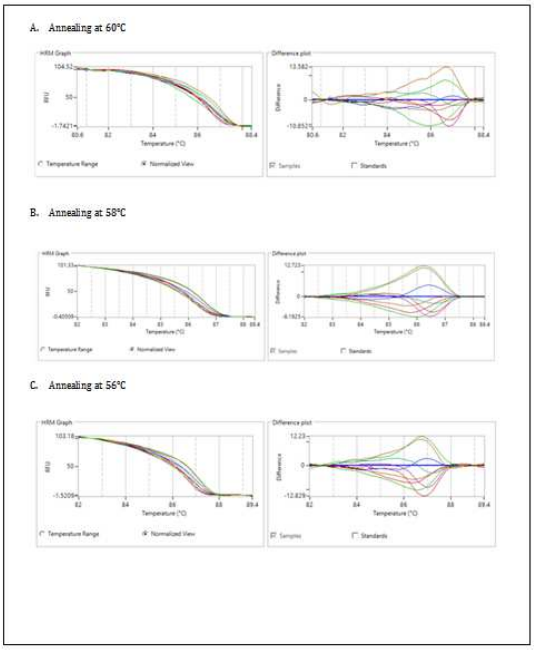

Fig. 1: HRM of a 114 bp product containing SNP rs27072 (C/T): this SNP is part of the haplotypes involved in determining dopamine transporter polymorphisms. This target was amplified using 30 sec annealing at 60°C, 58°C and 56°C. annealing temperature variation. Normalized melting curves and difference plots for the genotyping of rs 27072 from the HRM analysis program (40 cycles) are shown. 60°C, 58°C and 56°C of annealing temperature were tested.

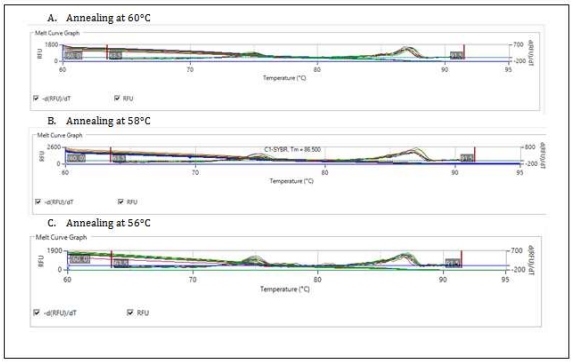

Fig. 2: The melt curve graph of 0.5µM concentration of primer for annealing at 60°C, 58°C and 56°C.

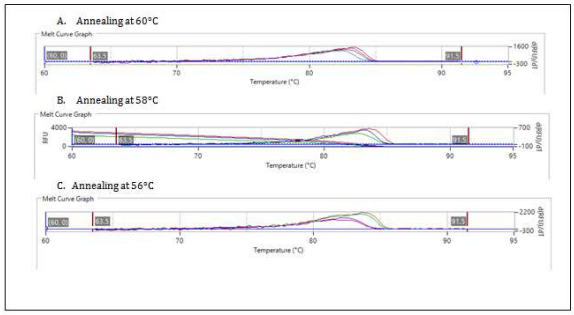

Fig. 3: The melt curve graph of 0.2µM concentration of primer for annealing at 60°C, 58°C and 56°C.

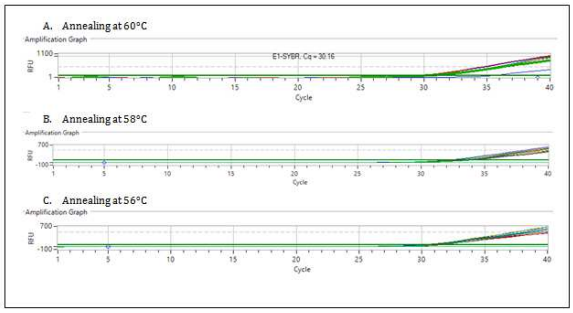

Fig. 4: The Cq-value of 40 number of cycles for annealing at 60°C,58°C and 56°C .

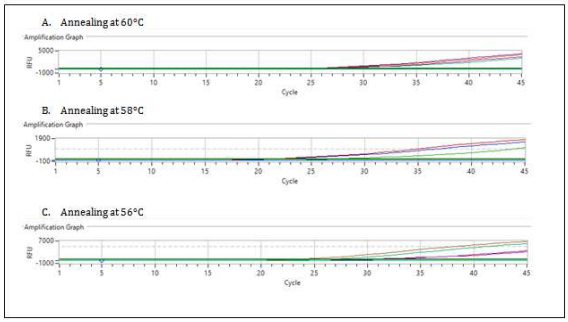

Fig. 5: The Cq-value of 45 number of cycles for annealing at 60°C,58°C and 56°C .

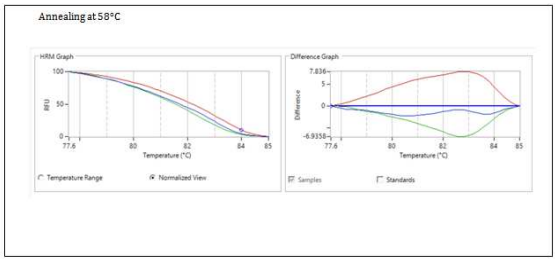

Fig. 6: Normalized melting curves and difference plots for the genotyping of rs 27072 from the HRM analysis program, 0.2µM of primer concentration and 45 numbers of cycles are shown at 58°C of annealing temperature.

Conclusion:

As a conclusion, successful HRM assay development will help in dopamine transporter (SLC6A3) (rs 27072) gene polymorphisms investigations due to its efficiency in accurately detecting the heterozygous and homozygous nucleotide substitution (Lince *et al*., 2009). HRM analysis also is not laborious methods since no post-PCR was conducted after the PCR amplification. However, the HRM genotyping methods development in the present study will be further optimized prior to the polymorphisms detection.

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