DEVELOPMENT OF PCR-BASED GENOTYPING METHOD FOR FAT MASS OBESITY-ASSOCIATED (FTO) GENE POLYMORPHISM RS9939609

Yusof H[1], Mohd Rodzi NA[1], Shakir NS[1], Mohd Ali AB[2], Kareem ZJ[3,4], and Mohd Hatta FH[5].
[1]Faculty of Health Sciences, UiTM Selangor Branch, Puncak Alam Campus, 42300 Puncak Alam, Selangor
[2]Apical Scientific Sdn Bhd, No. 7-1 to 7-4, Jln S/P2/7 Taman Serdang Perdana, Seksyen 2 Seri Kembangan, 43300 Seri Kembangan, Selangor, Malaysia
[3]Faculty of Health Science, Qaiwan International University (QIU), Slemani Heights, Sulaymaniyah, Iraq
[4]Kurdistan Institution for Strategic Studies and Scientific Research (KISSR), Kurdistan Region, Iraq
[5]Department of Pharmaceutical Pharmacology and Chemistry, Faculty of Pharmacy Universiti Teknologi MARA Selangor Branch, Puncak Alam Campus, 42300 Puncak Alam, Selangor

Correspondence:
Fazleen Haslinda Mohd Hatta,
Department of Pharmaceutical Pharmacology and Chemistry,
Faculty of Pharmacy,
Universiti Teknologi MARA Selangor Branch,
Puncak Alam Campus,
42300 Puncak Alam, Selangor
Email: fazleen@uitm.edu.my

Abstract

Malaysia has the highest obesity rate in Southeast Asia and is growing. Environmental and genetic factors influence weight increase and obesity, leading to numerous diseases and mortality. This study aimed to develop a genotyping method to detect FTO gene variant rs9939609, known for its association with body mass index (BMI) and obesity. In-house primers detected rs9939609. Two common primers flanked the variation region, and two more primers identified the wild type and alternative allele. Capillary sequencing verified results. We developed FTO gene variant rs9939609 genotyping. This method can be used to genotype FTO variant rs9939609 in any research.

Keywords: Obesity, Body Mass Index (BMI), FTO Gene Variant rs9939609, Capillary Sequencing

Introduction

Obesity is a medical disorder defined as excess weight in the form of fat that, as it builds up, may trigger serious medical issues (1). It is also classified as an epidemic chronic, complex, and multifactorial metabolic disease, with prevalence reaching 20% of the global population (2). Obesity, as stated by World Health Organization (WHO), has almost tripled internationally since 1975, with 13% of the adult population in 2016 being obese. Nauru has the greatest obesity rate in the world, with a national average BMI of 32.5 (3). The number of overweight children under five has increased by about 24% across Africa since 2000. In 2019, Asia accounted for almost half the obesity percentage among children under five (1). Obesity is on the rise in both developed and developing countries. It is linked to many chronic illnesses, including metabolic syndrome, type 2 diabetes, and cardiovascular disease, which should be worrisome (4). Several single nucleotide polymorphisms (SNPs), including rs9939609, rs8050136, rs17817449, rs1421085, and rs1121980, were discovered to have a substantial risk of obesity (5). However, the rs9939609 polymorphism (A/T) mutation in intron one was found to have the largest impact on obesity (6).

Numerous studies have confirmed the rs9939609 gene to have a substantial link to obesity in adults, children, and adolescents, with most of the evidence relevantly focused on adults (7). Shifts in dietary and physical activity habits typically come from societal and environmental changes, influencing a person's susceptibility to obesity and overweight (1). This FTO rs9939609 gene polymorphism (A/T substitution) expressed in humans aids in additional weight gain due to changes in hypothalamus FTO gene expression with the A allele recognised as a causal factor to obesity vulnerability (8). This study aims to develop a method to detect FTO alleles corresponding to rs9939609.

Materials and Methods

Subjects and DNA

This study comprised 51 undergraduates from the Faculty of Health Sciences with ages ranging from 18 to 30 years old, recruited by convenience sampling. This research protocol was approved by the Research Ethics Committee UiTM; REC/662/19). We excluded subjects from other faculties, subjects whose blood failed to be extracted, and subjects with low DNA quality. The selected subjects were willing to comprehend the research instructions, free from chronic and blood-borne diseases, and ready to sign informed consent forms. Subjects' refusal to participate at any point in the research timeline was accepted willingly. After obtaining informed consent, the students were led through the blood-drawing procedure. A 5ml of fresh blood from each individual was obtained in EDTA, maintained at -80°C during collection, and appropriately thawed before DNA extraction.

A commercial DNA extraction kit (Bio Basic Inc., USA) extracted the DNA from the whole blood samples. The final pure genomic DNA aliquots were kept at -20°C for long-term use. The amount of DNA was measured using UV absorbance at A260 (1.0 OD until its equivalent of 50 ug), and the purity of genomic DNA was examined using an analytical 0.7 percent agarose gel.

Polymerase Chain Reaction (PCR)

The PCR primers used to amplify the FTO variant rs9939609 were designed using the online software Primer3Plus. The list of primers and their properties were as listed in Table 1. The designed primers were aligned with genomic sequence NC 000016.10 from the NCBI Reference Sequence (Homo sapiens chromosome 16, GRCh38.p14 Primary Assembly - Nucleotide - NCBI (nih.gov)).

Table 1: Primer's sequence and melting temperature. The black bold coloured base was the wild type and alternative base pairing to the template DNA

Primer	Sequence	Melting temperature (°C)	Amplicon size (base pairs)
FTO Common Forward	5'- GGA TTC AGA AGA GAT GAT CTC AAA	60	195
FTO Common Reverse	5'- CAC TCC ATT TCT GAC TGT TAC C	60	
FTO Wild-Type Reverse	5'- GAG ACT ATC CAA GTG CAT CAC **A**	58.4	152
FTO Forward alternative	5'-CTT GCG ACT GCT GTG AAT TT**A**	58.4	85

About 10 pmol of primers were used in the PCR mix for each PCR reaction. Optimisation for annealing temperature was done by gradient PCR, where the annealing temperature was set at a gradient of 50-65°C. The component of the assay is shown in Table 2.

Table 2: PCR mixture

Constituent	Volume (µL)
exTEN 2X PCR master mix (1st BASE, Singapore) (PCR buffer, 3mM $MgCl_2$, 0.08U/µL DNA polymerase, 400µM dNTP mix)	12.5
Nuclease-free water	9
LEP common forward primer (IDT, US)	1
LEP common reverse primer (IDT, US)	1
LEP wild-type/mutant primer (IDT, US)	0.5
DNA template	1

Result validation

Six samples that were identified as homozygous wild types (2 samples), heterozygous (2 samples), and homozygous alternatives (2 samples) were sent to a sequencing service provider along with the FTO common forward and reverse primers to validate whether the primers adhered to the specific region on the genetic materials. For capillary sequencing, these samples were amplified without the wild type and alternative allele primers. Each of the samples that gave out their genotype results were randomly picked to be sequenced. The six samples' PCR amplification mixes were first placed through a PCR Clean-up for PCR product purification before sequencing. Upon receiving the chromatography result, Chromas version 2.6.6 (Technelysium Pty Ltd, Australia) was used to interpret the data.

Results

Among 51 subjects recruited, 10 subjects were male (19.6%), while 41 were female (80.4%). Native (*Bumiputra*) ethnics represented this data with a mean age of 23.20 (± 1.56) years old and a mean body mass index (BMI) of 22.74 (± 4.91) kg/m2.

PCR Optimisation

Figure 1 shows the annealing temperature mapping for detecting FTO variant rs9939609.

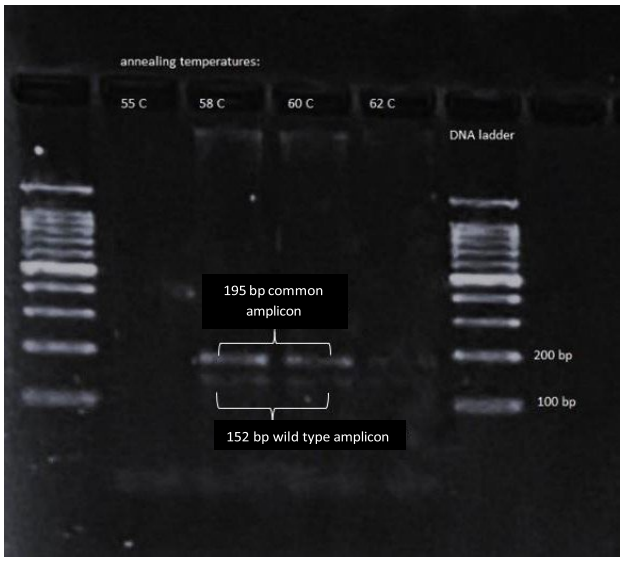

Figure 1: *Agarose gel under UV showing results for an annealing temperature gradient. Lanes loaded with amplicon from annealing temperatures 58°C and 60°C show successful amplifications. A 100 bp ladder was used for this gel.*

Figure 2 shows the six samples that were selected for validation: two with the wild-type genotype (samples A & B), two with the alternative genotype (samples C & D), and two with the heterozygous-type genotype (samples E & F).

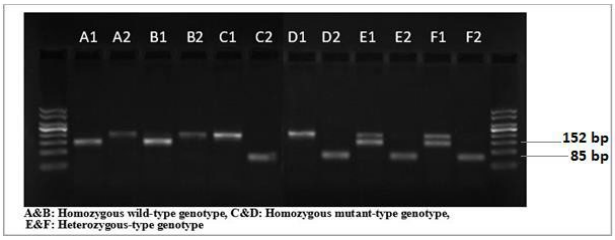

Figure 2: *PCR crossmatch result for the FTO gene visualized in 2% agarose gel electrophoresis.* Based on agarose gel band images, each DNA sample was loaded side by side, with A1, B1, C1, D1, E1, and F1 lanes having wild-type primers and A2, B2, C2, D2, E2, and F2 lanes having mutant primers. Wild and mutant genotypes were identified at 150bp and 80bp ladders, whereas heterozygous genotypes were identified at both base pairs. A 50 bp ladder was used in this gel.

Discussion

The optimised PCR reaction began with 95°C initial denaturation for 5 minutes. Then 30 cycles of thermal cycling comprising a 95°C denaturation for 30 seconds, a 58°C annealing phase for 30 seconds, and a 72°C extension phase for 30 seconds. The final extension was at 72°C for 1 minute, followed by a cooling-off step of 15°C for 5 minutes. For the most specific DNA amplification, the annealing temperature must be optimal for the primer to anneal. All PCR processes were carried out using

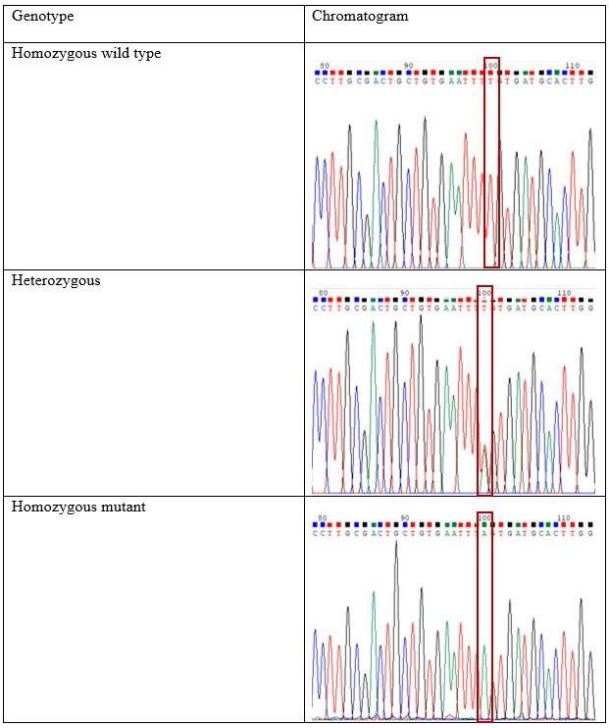

Figure 3: Capillary sequencing result. The target area of the DNA single base was substituted for the A-allele. The heterozygous genotype has shown two chromatography peaks at once. Therefore, the sequencing result is successful and can be used as validation for the FTO gene through PCR.

MyCyclerTM Thermal Cycler (Bio-Rad, US).

The resultant amplicons were identified based on their sizes. The common primers produce 190 bp amplicons. The internal positive control may not appear if there is a wild type of alternative band, but this amplicon serves as an internal positive control when the wild type or alternative allele is absent in the gel. The wild-type amplicon will appear at the size of 130 bp, and the alternative allele will appear at 60 bp (Figure 1). As there are other genotyping methods described in other publications (9), this method will be used as an in-house genotyping method and can be used as a validation for other methods of genotyping the FTO variant rs9939609.

The amplified PCR products were then resolved by electrophoresis on a 2% agarose gel. In this experiment, Tris-borate-EDTA buffer (1X TBE), agarose gel electrophoresis for Molecular Biology (Vivantis, USA), and nuclear acid staining were used (SYBRTM Safe DNA Gel Stain, Thermo Fisher Scientific, USA). The first and last wells were loaded with 1.5 µL of VC 50 base pair DNA ladder (Vivantis, USA), while the remaining wells were loaded with 10 µL of PCR products. The gel was electrophoresed for 30 minutes at 90 V power. Following gel electrophoresis, the agarose gel electrophoresis material was viewed using an ultraviolet

(UV) transilluminator, ImageQuant LAS 500 (GE Healthcare Bio-Sciences AB, Sweden).

Referring to Figure 2, samples A and B are representations of a wild-type genotype. There is an amplification at 130 bp (A1 and B1) and no amplification at 60 bp (A2 and B2). Instead, the common amplicon appears as an indication that there is DNA available to produce amplification. Samples C and D are the opposite of the first two samples, where they lack amplification at 130 bp (C1 and D1), but amplification happens at lanes C2 and D2 (60 bp). Heterozygous genotypes were detected for samples E and F, where 130 bp and 60 bp amplicons were detected. Due to competitive binding between the common primers and the allele-specific primers, sometimes the common amplicon bands fade significantly if there is binding between the allele-specific primers to the DNA templates.

The capillary sequencing result has succeeded and validated the FTO genotyping through PCR (Figure 3). The method was repeated with a different technician following the same protocol and successfully replicated the protocol and 100% accuracy.

Conclusion

In this study, we have successfully developed a PCR screening method for FTO allele rs9939609. This study may assist in further understanding the correlation between the A19G polymorphism with BMI. The findings may also present data regarding the frequency of occurrence of the gene variant rs9939609 alleles in the FTO gene in the targeted group and suggest that those prone to unhealthy eating behaviour change their lifestyle to adopt a healthier diet intake. In the bargain, the data obtained may help provide knowledge and baseline data for future research.

Acknowledgement

This work is supported by the Research Management Centre (RMC), UiTM, under the *Geran Khas Penyelidikan* (GPK) (600-RMC/GPK 5/3 (026/2020)).

Competing interests

The authors declare that they have no competing interests.

Ethical Clearance

This research protocol was approved by the Research Ethics Committee UiTM; REC/662/19).

Financial support

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

References

1. World Health Organization. Obesity and overweight. 2021. Available at: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight. Accessed 24 May 2022.
2. Gómez JC, Ena J, Lorido JA, Ripoll JS, Carrasco-Sánchez FJ, Gómez-Huelgas R, *et al*. Obesity is a chronic disease. Positioning statement of the Diabetes, Obesity and Nutrition Workgroup of the Spanish Society of Internal Medicine (SEMI) for an approach centred on individuals with obesity. Revista Clínica Española (English Edition) 2021;221(9):509-516.
3. Hanson D. The 20 Most Obese Countries in the World in 2019. Money Inc. 2019. Available at: https://moneyinc.com/most-obese-countries-in-the-world/. Accessed 1 Jun 2022.
4. Kaakinen M, Laara E, Pouta A, Hartikainen AL, Laitinen J, Tammelin TH, *et al*. Life-Course Analysis of a Fat Mass and Obesity-Associated (FTO) Gene Variant and Body Mass Index in the Northern Finland Birth Cohort 1966 Using Structural Equation Modeling. American Journal of Epidemiology 2010;172(6):653-665.
5. Peng S, Zhu Y, Xu F, Ren X, Li X, Lai M. FTO gene polymorphisms and obesity risk: a meta-analysis. BMC Medicine 2011;9(1).
6. Mehrdad M, Fardaei M, Fararouei M, Eftekhari MH. The association between FTO rs9939609 gene polymorphism and anthropometric indices in adults. Journal of Physiological Anthropology 2020;39(1).
7. Jiang Y, Mei H, Lin Q, Wang J, Liu S, Wang G, Jiang F. Interaction effects of FTO rs9939609 polymorphism and lifestyle factors on obesity indices in early adolescence. Obesity Research & Clinical Practice 2019;13(4):352-357.
8. Ali AHA, Shkurat TP, Abbas AH. Association analysis of FTO gene polymorphisms rs9939609 and obesity risk among the adults: A systematic review and meta-analysis. Meta Gene 2021;27:100832.
9. Mozafarizadeh, M., Mohammadi, M., Sadeghi, S., Hadizadeh, M., Talebzade, T., & Houshmand, M. (2019). Evaluation of FTO rs9939609 and MC4R rs17782313 Polymorphisms as Prognostic Biomarkers of Obesity: A Population-based Cross-sectional Study. Oman Medical Journal, 34(1), 56–62.