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Designing a PCR-Based Genotyping Technique for the LEP A19G Polymorphism: Method Development and Optimization

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ABSTRACT

The LEP gene is a protein-encoding gene for the satiety hormone called leptin, which stimulates appetite, impeding food intake and anorexigenic peptide upregulation. This research aims to develop a genotyping method to detect the LEP gene variant A19G, known for its association with appetite and eating preference. Convenience random sampling was performed by disseminating the subject's recruitment on social media among the students of Faculty of Health Sciences, Universiti Teknologi MARA. A total of 51 undergraduates (mean age, 23.20 (Standard Deviation, SD 1.56) years old; 41 females, 10 males) from the faculty were recruited. Questionnaires have been provided to assess the subjects' meal preferences and food intake frequency. A19G genotyping was performed using polymerase chain reaction (PCR) and subsequent agarose gel electrophoresis (AGE). The analysis confirmed the successful development of the A19G genotyping method. The variant allele frequency (VAF) for A19G was 0.13. This approach provides a reliable genotyping tool for LEP A19G in future investigations despite data suggesting that A19G may not be a marker for appetite differences in this subject group. Further research with a larger, more diverse population is warranted to confirm these results and explore the influence of other LEP gene variants on appetite and eating preferences.

INTRODUCTION

A healthy and balanced food intake would protect individuals from malnutrition and diet-related noncommunicable diseases (NCDs) such as stroke, diabetes, cancer, and heart disease (WHO, 2019). Healthy diet practices recommended by the World Health Organization (WHO) include terminating saturated and industrially manufactured trans fats from the diet, opting for unsaturated fats consumption, balancing calorie intake and output, and limiting salt and sugar consumption. An unhealthy diet is the leading global challenge in the health sector. Even though people living in urban areas have easy access to clean, healthy, and adequate food, a trend of satisfying sweet and fatty food cravings has been incorporated into an urbanised lifestyle. Appetite is an intricate concept. It is regarded as the inner urge to look for, choose, and consume food from a biological standpoint (de Graaf et al., 2004). In the broader context, appetite is an interrelated process of physiological, psychological, and environmental factors that regulate an individual's quotidian eating pattern (Halford & Blundell, 2000). The correlation between appetite and eating patterns is evident, as an intricate interplay of hormonal and neural factors governs appetite. This regulatory mechanism aims to maintain a balanced energy intake that adequately sustains metabolic needs. This system stimulates eating behavior by starvation, hedonic sensations, and food cravings (Das & Roy, 2017). Comprehending food choice preference, meal timing, and frequency of food intake is imperative in tackling the epidemics of cardiovascular diseases, obesity, and diabetes. According to the National Health and Morbidity Survey, diabetes, hypertension, or hypercholesterolemia affects at least one-third of the Malaysian population (NIH, 2020).

Based on Noncommunicable Diseases Country Profiles 2018, 74% of overall deaths in Malaysia were accounted for NCDs, with cardiovascular diseases recorded as high as 35%, where salt intake, diabetes, and obesity are some risk factors for mortality due to NCDs (WHO, 2018). Leptin is a hormone that comprises 167 amino acids and is released by the hypothalamus to regulate food intake (Facey et al., 2017). The leptin (LEP) gene is located at chromosome 7q31.3, the short arm of chromosome 7, and encompasses approximately 20kb length of 3 exons (Isse et al., 1995), which is responsible for encoding for the 16-kDa leptin hormone.

The leptin receptor is a transmembrane protein from the class I cytokine family receptor, and by binding to this receptor, only leptin can carry out its physiological function (Fan & Say, 2014). Variations in genes of digestive neuroendocrine hormones and enzymes such as cholecystokinin (CCK), leptin, fat mass and obesity (FTO), and glutamic acid decarboxylase (GAD) have been observed to increase the risk of obesity by regulating satiety and hunger (Grimm & Steinle, 2011). Variants in CCK and leptin hormone contribute to imprudent meal size and excessive snacking behaviour, respectively (de Krom et al., 2007).

Meanwhile, FTO gene variants are associated with increased caloric intake (Tanofsky-Kraff et al., 2009), and increased carbohydrate intake behaviour is observed in individuals with GAD gene variants (Choquette et al., 2009). Polymorphism of LEP and LEPR genes that encode for leptin hormone is usually assessed for dietary deficiency-related problems such as obesity. A19G is one of the leptin gene alleles typically assessed for its polymorphisms in other research studies. The A19G polymorphism showed no significant association as a potential obesity-predisposing marker in Malaysian populations (Wan Rohani et al., 2018). A study reported that single nucleotide polymorphisms (SNPs) of the leptin gene in Malaysia were associated with ethnicity rather than body mass index (BMI) and gender (Fan & Say, 2014).

In addition, more investigations are needed on the association between A19G polymorphisms and appetite; hence, future research and genotypic data are limited. Therefore, this study aims to develop a method to detect the LEP gene corresponding to the variant A19G. We studied the relationship between A19G polymorphism on the LEP gene and appetite among the Faculty of Health Sciences, Universiti Teknologi MARA students, to understand the association between appetite and A19G polymorphism.

MATERIALS AND METHODS

Subject Recruitment

This study comprised of 51 undergraduate students from the Faculty of Health Sciences, Universiti Teknologi MARA whose ages ranged from 18 to 30 years, recruited by convenience sampling. Recruitment of participants was done using social media. We excluded students from other faculties, participants whose blood failed to be extracted, and participants with low DNA quality. The selected participants were willing to follow the research instructions, free from chronic and blood-borne diseases, and ready to sign informed consent forms. Participants will not be recruited if they refuse to participate.

Data and Blood Sample Collection

The study was approved by the Research Ethics Committee of Universiti Teknologi MARA with reference number REC/662/19, and informed consent was obtained from all participants. Participants were briefed about the research procedure, and their height and weight were measured. Participants' information on demographic data and appetite was collected through written interviews. The assessment questions consisted of the subjects' eating preferences such as whether food was taken during the indicated eating time and where the food was consumed, with a scale of frequency of food consumption. The questionnaire was adapted from validated questionnaires which include the Adult Eating Behaviour Questionnaire (AEBQ) (Hunot, et al., 2016), Self-Regulation of Eating Behaviour Questionnaire (SREBQ) (Kliemann, et al., 2016) and Adolescents' Food Habits Checklist (AFHC) (Johnson, et al., 2002). Participants' venous blood was collected by qualified personnel into a 5ml ethylenediaminetetraacetic acid (EDTA) blood collection tube. The blood was frozen at -80°C during collection and appropriately thawed before DNA extraction.

Genotyping

Blood DNA was extracted utilising the EZ-10 Spin Column DNA Cleanup Minipreps Kit (Bio Basic Inc., USA). The DNA extraction was done according to the kit's manual. The gene was subsequently amplified using two sets of designed primers. The sequence of the leptin A19G nucleotides was determined using the National Center for Biotechnology Information (NCBI) website, which is accessible at https://www.ncbi.nlm.nih.gov. The leptin A19G accession number was NM_000230.3 (Homo sapiens). The primer was created using the Primer-BLAST website, and the NCBI website's FASTA sequence was used as a reference. After the Primer-BLAST generated primer sequences, the primer pair of interest was chosen from the detailed primer reports as depicted in Table 1. Determination of primer specificity was assessed employing the BLAST application (https://blast.ncbi.nlm.nih.gov). The validated primer sequence was procured from a primer synthesis company.

Table 1: Primer sequences and melting temperature.

Primer	Sequence	Melting temperature (°C)
LEP Common Forward	5'-GAG CTG GCG CTA GAA ATG C	59.4
LEP Common Reverse	5'-TGC CAA GAA AGA CCA GCA GA	58.4
LEP 19G (wild-type)	5'-GCG GTT GCA AGG TAA GGC	59
LEP 19A (mutant)	5'-TAG GAA TCG CAG CGC CAA	59

Leptin A19G genotypes were identified through polymerase chain reaction (PCR) in a 25µl amplification mixture. The mixture comprised specific primers for both wild-type and alternative alleles. LEP common forward and reverse primers (0.2µM each) and 0.1µM of LEP 19G wild-type primers were used in the wild-type tube. Meanwhile, 0.2µM each of LEP common forward and reverse primers, along with 0.1µM of LEP 19G mutant primers, were employed in the alternative allele tube. The amplification mixture also included 12.5µl of 2X Taq Master Mix from Vivantis Technologies (Malaysia) and double-distilled water to reach a total volume of 25µl. The amplification process was carried out using the MyCyclerTM Thermal Cycler from Bio-Rad (USA). It is important to note that this is a double-tube reaction, where one tube represents the wild-type allele, and the other represents the alternative allele for each sample. The mixture was made to ensure an internal control of the common PCR band resulting from the amplification of both the common primers pairing was present regardless of the genotype state, indicating that there was DNA and that the PCR was working.

PCR began with 5 min of 95°C denaturation, followed by 32 cycles of 30 sec each of denaturation, annealing, and fragment extension at 95°C, 61°C, and 72°C, respectively. The cycle was terminated with a subsequent elongation for 2 min at 72°C. The PCR was set to retain the temperature at 20°C before collecting the PCR products. Before amplifying the samples, a temperature gradient was performed to determine the optimal primer annealing temperature empirically. In line with the PCR program and the predetermined PCR parameters, five PCR reaction mixtures with identical DNA samples were run at five different temperatures per the PCR program and PCR parameters set. The temperatures mentioned ranged from 55°C to 65°C. Ultimately, 61°C was the best annealing temperature based on the amplicon band thickness and lack of an unspecific band of the observed agarose gel.

PCR amplification products were detected using the agarose gel electrophoresis (AGE) method. The AGE method necessitated agarose powder, Tris-borate-EDTA (1X TBE) buffer, and SafeGreen gel stain. The first and last wells were loaded with a 1.5µL VC 50bp DNA ladder (Vivantis, Malaysia). The remaining wells were loaded with 10µL of PCR products. The gel was electrophoresed with 90V power for 28 min. Subsequently, the AGE product was visualized using ImageQuant LAS 500 (GE Healthcare Bio-Sciences AB, Sweden), an automated gel documentation system. The gel was placed onto the provided DNA tray, and an orange filter was inserted for fluorescence detection. DNA stain imaging was automated, and image analysis output was viewed using the ImageQuant TL v8.1 software.

To evaluate the normality of genotypic distribution in the study, the Hardy-Weinberg Equation was assessed using the formula as follows:

$$p2 + 2pq + q2 = 1 \ldots\ldots (3.3)$$

p = frequency of dominant allele

q = frequency of mutant allele

The Hardy-Weinberg Equilibrium formula was derived from allelic frequencies tabulated in a Punnet square. By plugging in p=43/51 and q=8/51 derived from genetic counting, the Hardy-Weinberg Equilibrium was calculated as follows:

$$(86/102)2 + 2([86/102]\ [16/102]) + (16/102)2 = 1$$

Capillary Sequencing

Samples from each genotype group obtained from the PCR experiment were sent for capillary sequencing. This step was vital to endorse the primers in targeting the correct sequence of the DNA genome, concomitantly verifying the results obtained. Before sending for capillary sequencing, the polymorphic bands were eluted and purified using the EZ-10 Spin

Column DNA Cleanup Minipreps Kit (Bio Basic Inc., USA). The company's capillary sequencing results were viewed using Chromas version 2.6.6 DNA sequencing software (Technelysium Pty Ltd, Australia).

Statistical Analysis

Statistics analyses were executed using the Statistical Package for Social Sciences (SPSS) version 28 (SPSS Inc, Chicago, IL, USA). Numerical data were described as mean (± Standard Deviation, SD), while categorical data were expressed as frequency (%). Genotype and allele frequencies were assessed using gene counting, and the Hardy-Weinberg Equilibrium value was calculated for the genotype distribution of the LEP A19G. The association between A19G polymorphism regarding eating preference and frequency of food consumption was assessed using the Chi-square ($\chi 2$) test. p-value <0.05 was considered statistically significant.

RESULTS

Development of PCR Method for Detection of A19G Polymorphism

The PCR temperature gradient was done using MyCyclerTM Thermal Cycler (Bio-Rad, US) and DNA extracted from a blood sample to establish the optimal annealing temperature for the primers. This step was essential to ensure appropriate primer-to-template integration. As outlined on the specification sheet, the calculated annealing temperature for the common forward and reverse primers was 56.7°C and 56.8°C, respectively. Therefore, the optimal temperature was empirically determined from 48°C to 62°C. Five different temperatures were run for the DNA sample to determine the outcomes of the gel electrophoresis band. As a result, the PCR procedure was run for 32 cycles with an optimized annealing temperature of 61°C, which was higher than the predicted temperature for both primers. The difference in calculated annealing temperature from the actual annealing temperature was probably influenced by magnesium (Mg2+) and potassium (K+) concentrations (Porta & Enners, 2012) in the buffer.

A19G Polymorphism Detection

A wild-type allele was visualized as a 130bp band, while an 80bp band represented a mutant allele. Gene carrying both alleles produced DNA bands at 130bp and 80bp. The A19G polymorphism detection products are shown in Figure 1. The DNA band bases were relatively compared to the 50bp ladder on lanes 1 and 8. The upper and lower columns were electrophoresed PCR products of wild-type and mutant primers, respectively. The combination of wild type and mutant electrophoresed on the upper and lower lanes gave the genotype for each sample. Samples 302, 304, 306, and 307 indicated homozygous wild type. Sample 303 was shown to be heterozygous, carrying both wild-type and mutant alleles, while sample 305 was genotyped as a homozygous mutant.

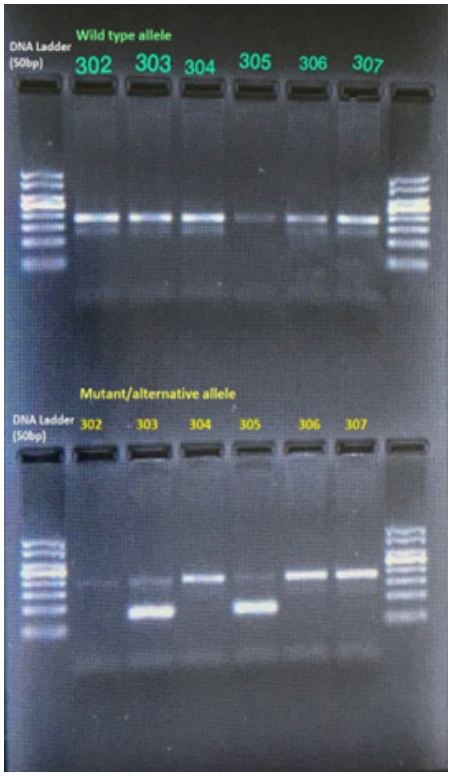


Figure 1: Genotyping result for samples 302-307.

Subjects' Demographics, A19G Genotypic Frequencies and Distribution

Among the 51 participants, there were 10 males (19.6%) and 41 females (80.4%), with a mean age of 23.20 (SD1.56) years old and a mean body mass index (BMI) of 22.74 (SD 4.91) kg/m2. This data was also represented by native (Bumiputra) ethnics, especially Malays, as Universiti Teknologi MARA was established to cater specifically to native ethnics' needs for academic studies and professional development (Universiti Teknologi MARA, 2020). The subjects' mean BMI was 22.74 (± 4.91) kg/m2, categorized as normal weight based on global WHO recommendations (WHO, 2010) The most significant percentage of overall A19G genotype distribution was weighed by homozygous wild-type genotype with over 37 (72.5%), followed by heterozygous mutant genotype with 12 (23.5%) and homozygous s mutant genotype with 2 (3.9%). The wild-type allele was assigned as G, while A was mutant.

Association between A19G Polymorphism and Eating Preference

Further analysis was performed to assess the association between A19G polymorphism and the eating preference of the subjects using the Chi-square ($\chi 2$) test presented in Table 2.

The findings of the Fisher-Freeman-Halton Exact test were interpreted because the predicted frequencies for the study were not fulfilled.

Table 2: The relationship between A19G polymorphism and eating preference at breakfast, lunch, and supper (n=51).

Breakfast	**Skip n (%)**	**At home n (%)**	**At café n (%)**	**X^2**	**p-value[a]**
Wild-type	9(24.3)	14(37.8)	14(37.8)	1.843	0.916
Heterozygous	3(25.0)	4(33.3)	5(41.7)		
Mutant	1(50.0)	1(50.0)	0(0.0)		
Lunch	**At home n (%)**	**Fast food/ cafe/ restaurant n (%)**	**Pace lunch prepared at home n**		
Wild-type	4(10.8)	33(89.2)	0(0.0)	8.227	0.064
Heterozygous	1(8.3)	10(83.3)	1(8.3)		
Mutant	0(0.0)	1(50.0)	1(50.0)		
Supper	**At home n (%)**	**Fast food/ cafe/restaurant n (%)**			
Wild-type	28(75.7)	9(24.3)		0.537	0.822
Heterozygous	10(83.3)	2(16.7)			
Mutant	2(100.0)	0(0.0)			

[a] Fisher-Freeman-Halton Exact test

Validation of A19G Polymorphism Detection Results

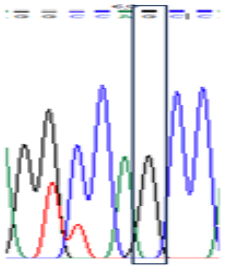
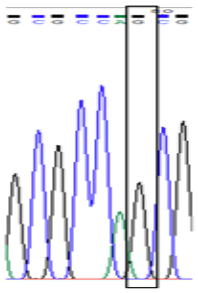
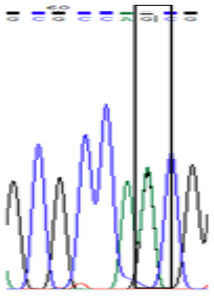
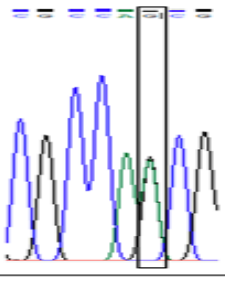
The results of polymorphism detection by the second person were assessed and compared to the results previously obtained by the method. The polymorphism detection was observed in Figure 1. Capillary sequencing results delivered by the sequencing companies were viewed and interpreted. The base composing the gene mutation point was regarded to determine the polymorphism. After the 5'-GCCA-3' sequence in the genome, the guanine

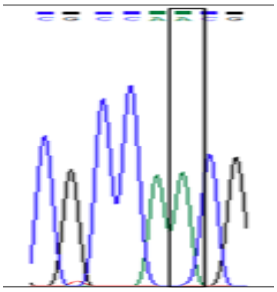
(G) base was dominant, while the adenine (A) base was regarded as recessive. Table 3 outlines the capillary sequencing results. The chromatogram revealed that guanine composed the mutation point for samples 301 and 311, while 305 bases comprised adenines. Peaks of guanine and adenine were observed for samples 127 and 319.

DISCUSSION

Robust detection of minute DNA samples has been established with PCR. Optimizing the protocol is critical in yielding amplicons with high fidelity. Yields' performance relied on parameters such as DNA template, buffer condition, PCR cycle number, and primer

Table 3: Capillary sequencing of polymorphic PCR products.

Sample number	Chromatogram	Base at mutation point	A19G polymorphism
301		Guanine (G)	Homozygous wild-type
311		Guanine (G)	Homozygous wild-type
127		Guanine (G) > Adenine (A	Heterozygous mutan
319		Guanine (G) > Adenine (A)	Heterozygous mutant

305		Adenine (A)	Homozygous mutant

sequences (Porta & Enners, 2012). First and foremost, DNA was extracted from venous blood. The blood sample has a high yield of DNA templates and can be kept for a long in a proper storage condition in case of delayed molecular testing. An efficient buffer condition was ensured with the appropriate concentration of DNA polymerase, magnesium chloride (MgCl2), and deoxynucleoside triphosphate (dNTPs). Also, the PCR cycle number plays a pivotal role in producing excellent yields. Setting the cycle number too high would be time-consuming. Initial PCR was done with 30 cyclical reactions during optimization but failed to visualize any bands on the electrophoresed agarose gel. Optimal 32-cycle PCR succeeded in observing bright and distinct bands.

It took specific complementary primers only to amplify the specified target product. Using the Primer-BLAST software, the right flanking area on the genome sequence could be easily identified. To prevent self-annealing and nonspecific primer binding, a universal primer concentration of 0.2 M was employed (Sachse, 2003). In this study, the primer generated the correct bands at 180, 130, and 80 bp.

The A19G genotypic distribution among the subjects was in Hardy-Weinberg equilibrium to indicate no deviation of the genotypic distribution of the expected results from the previous generation and studies. A19G genotype of the homozygous wild type was predominant, followed by heterozygous mutant and homozygous mutant. The frequency trend was similar to several studies in Malaysia (Fan & Say, 2014; Wan Rohani et al., 2018). However, contradicting genotype frequencies can be observed (Liew et al., 2009), where the heterozygous mutant was predominant, followed by the homozygous mutant and homozygous wild type.

Based on gender stratification, males and females have the highest frequencies of homozygous wild-type genotypes. The frequency of A19G genotypic distribution followed the trend of the Malaysian suburban population in Perak (Fan & Say, 2014). Still, it contradicted Mestizos in western Mexico (Partida-Pérez et al., 2011) and the Italian population (Lucantoni et al., 2000). The variation in trend can be attributed to various ethnicities (Fan & Say, 2014). In this study, A19G polymorphism was higher in female subjects than in males. Some studies disclosed that the polymorphism was unrelated to gender (Fan & Say, 2014; Mizuta et al., 2008). The gender difference in polymorphism distribution might arise due to the small sample number and female subjects predominating the sample. Leptin hormone was also reported to be positively associated with estradiol and negatively associated with testosterone, leading to gender dimorphism of the A19G polymorphism (Lagiou et al., 1999).

Based on the Fisher's Exact test, there was no significant association between A19G polymorphism and breakfast (p=0.916), lunch (p=0.064), and supper (p=0.822) preferences among the subjects. As no correlation was

reported in the literature related to the A19G polymorphism, little was known about the association between the polymorphism and eating preference. Nevertheless, some studies have provided insights into the association between meal intake and leptin hormone encoded by the LEP gene (Dashti et al., 2021; Forester et al., 2018; Paz-Filho et al., 2012; Whincup et al., 2005). Whincup PH indicated a significant association of leptin levels between young pupils consuming home-cooked meals and school dinners (Whincup et al., 2005). Contrary to the current findings, a previous report found that leptin hormone levels tended to be elevated in breakfast-skipper subjects than in breakfast-eater subjects (Forester et al., 2018). Other authors reported that elevated insulin levels (Forester et al., 2018; Paz-Filho et al., 2012) may have caused the high leptin levels since insulin boosted the leptin hormone. Another study also supports this statement, where high morning leptin level was distinguished in subjects who were partial to late dinner eating, suppressing their morning appetite (Dashti et al., 2021). Socioeconomic status and food availability might explain an insignificant association between A19G polymorphism and eating preference. Students with unsatisfied life inclined to skip breakfast and dinner (Schnettler et al., 2015). Low dietary restraint was associated with fat-free mass changes (Finlayson et al., 2012). The intake of convenient takeaway meals was evident in university students being away from family (Papadaki et al., 2007). These findings suggest that restrictions in low socioeconomic status and food variations restricted students' means to choose healthful eating and hindered the consumption of food the students craved.

Research indicates that the A19G mutation may influence leptin mRNA translation, potentially altering serum leptin levels (Zhang et al., 2021). Some studies suggest that individuals with the A19G mutation might exhibit different leptin levels compared to those without the mutation, which could affect their appetite regulation and susceptibility to obesity (Liu et al., 2014). However, the evidence is mixed, with some research finding no significant association between this polymorphism and leptin levels or obesity traits (Fan et al., 2014). This suggests that the relationship between the LEP A19G mutation and leptin levels is complex and may be influenced by other genetic and environmental factors.

Besides genetic factors, different environments provide various sensory cues that can significantly affect appetite. For instance, the sight and smell of food in a restaurant can stimulate hunger and increase food intake, even if one is not particularly hungry. Bright lighting and vibrant colors in fast-food restaurants are designed to encourage quick eating and turnover, often leading to overeating. Conversely, a calm and quiet dining environment, such as a home-cooked meal in a cozy kitchen, can promote slower eating and better recognition of satiety signals. Additionally, social settings, like dining with friends or family, can lead to longer mealtimes and increased consumption due to social interactions and the enjoyment of shared experiences. These sensory cues, whether visual, olfactory, or social, play a crucial role in influencing our eating behaviors and overall appetite. We found that the eating habit of our subjects are mostly the same when dietary intake and eating behaviors are controlled or consistent across subjects, it could affect genetic factors, such as the LEP A19G mutation, on obesity. This consistency minimizes the influence of environmental and behavioral variables, making it easier to observe the direct impact of the mutation on body weight and appetite regulation.

Our research had some limitations. The sample size was small, so future studies should include larger-scale sampling. The convenience sampling method might cause gender dimorphism and mono-ethnic distribution among the subjects. We propose

sampling subjects from all ethnicities and ages with balanced gender stratification. This study relied on self-reports from the subjects, which might result in recall bias as they might incorrectly gauge their actual food intake. Thus, deviation from accurate results is plausible. Furthermore, this study did not consider anthropometric measures or leptin hormone levels, which might give an essential insight into the results.

CONCLUSION

In this study, we have successfully developed a PCR screening method for A19G polymorphism. However, polymorphism was not associated with the frequency of meal preferences and food intake among Faculty of Health Sciences students. This variant allele was lower than the average Malaysian population variant allele frequencies. The A19G polymorphism may not be a marker in different appetite regulation among the subjects. An insignificant association between A19G polymorphism and appetite might be attributed to students' education, socioeconomic status, and lifestyle.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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