# Analysis of Gene Variation in the Middle Region of Melanocortin 4 Receptor Gene in Obese Thai Individuals

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## ABSTRACT

At present, obesity is a growing health problem both globally and in Thailand, with a remarkable impact on health and economy. Assessment of the risk and the tendency of individual to become obese by analyzing specific gene variations is therefore an important option. In this study, the middle region of the melanocortin 4 receptor (*MC4R*) gene was analyzed in obese Thais to investigate the impact of nucleotide variations on the occurrence of obesity. The obtained data can be used as a database for the prognosis and the prevention of obesity. The *MC4R* gene encodes a receptor protein of the leptin-melanocortin pathway, which is involved in regulating appetite and energy expenditure in the body. Firstly, DNA was extracted from the hair roots of 44 Thai volunteers aged between 18-60 years old with a body mass index (BMI) of more than 25.0. The middle region of *MC4R* gene (position 5,555-6,166) was then amplified by polymerase chain reaction (PCR). PCR products were analyzed by agarose gel electrophoresis and single specific PCR product of 612 base pairs was obtained. Concentrations of PCR product were adequate for the nucleotide sequence analysis by Big Dye Terminator technique. All PCR products were sent to APICAL SCIENTIFIC SDN. BHD. (Malaysia) for nucleotide sequence analysis using the MC4R-F1 primer. In the final step, the nucleotide sequences of the middle region of the *MC4R* gene of Thai volunteers were compared with the reference nucleotide sequence from the National Center for Biotechnology Information (NCBI) to identify single nucleotide polymorphisms (SNPs). DNA analysis of 44 samples identified two heterozygous SNPs at G5726A and T6100C. The odds ratio for G5726A is 13.72, indicating that individuals with this SNP have a 13.72 times greater likelihood of developing obesity compared to those without SNP. The G5726A mutation causes a valine to isoleucine substitution at position 103 (V103I) in the Mc4 receptor. As it is a conservative missense mutation, the chemical properties and the function of the protein is supposedly not significantly affected. In contrast, the T6100C is silent mutation, meaning it does not alter the amino acid sequence or protein function. This finding provides information that could assist a better understanding about the correlation between gene variations and obesity, which may facilitate an obesity risk assessment and prognosis, and help establishing suitable prevention strategies for the Thai population in the future.

## OBJECTIVES

- To investigate the impact of nucleotide variations in the *MC4R* gene on the occurrence of obesity.
- To obtain a database for the prognosis and the prevention of obesity in the Thai population.

## INTRODUCTION

- Obesity is a growing global health problem, including in Thailand, with significant impacts on public health and the economy. It is a chronic disease influenced by factors such as diet, physical activity, genetics, and the environment, and is linked to comorbidities like sleep apnea, cardiovascular diseases, type 2 diabetes, and cancer.
- A key genetic factor in obesity is the *MC4R* gene, which regulates appetite and energy balance through the leptin-melanocortin pathway. This gene encodes a G protein-coupled receptor in the hypothalamus, binding α-MSH to reduce appetite and increase energy expenditure (Figure 1). Mutations in *MC4R* gene can disrupt this pathway, leading to excessive weight gain.
- These mutations can be analyzed through SNPs, which are variations in a single nucleotide at specific positions in the genome that differ among individuals (Figure 2). SNP analysis can help identify genetic variations associated with various diseases.

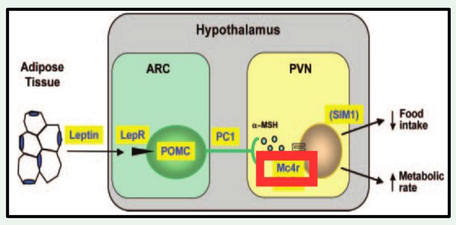


**Figure 1.** The leptin-melanocortin pathway (Modified from Cummings and Schwartz, 2003)

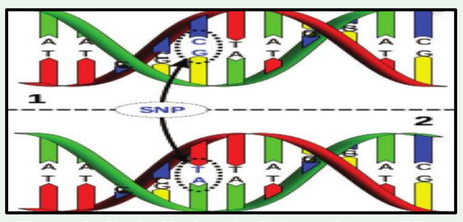


**Figure 2.** Single Nucleotide Polymorphisms (SNPs); adapted from International Society of Genetic Genealogy Wiki, 2017)

## METHODOLOGY

Volunteer recruitment from the Thai population (age 18-60, BMI > 25.0).

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Extraction of genomic DNA from hair roots obtained from volunteers using the PrimeWay Genomic DNA Extraction Kit (1st Base, Malaysia).

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Quality determination of genomic DNA by 1.2% agarose gel electrophoresis.

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Determination of the optimum annealing temperature for *MC4R* gene amplification by gradient PCR, with temperatures ranging between 57.0°C to 77.0°C.

**Table 1.** Annealing temperatures examined by gradient PCR

| Annealing Temperature (°C) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tube No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | 57.0 | 57.6 | 58.9 | 61.0 | 63.4 | 65.8 | 68.2 | 70.6 | 73.0 | 75.1 | 76.4 | 77.0 |

**Table 2.** Nucleotide sequences of primers used for *MC4R* gene amplification

| Primer | Name | Nucleotide sequence from 5'→ 3' | Tm (°C) | G+C (%) |
|---|---|---|---|---|
| Forward primer | MC4R-F1 | GTCTCTCCTGAGGTGTTTGTG | 65.0 | 52 |
| Reverse primer | MC4R-R1 | GATGGTCAAGGTAATCGCTCC | 65.0 | 52 |

**Table 3.** PCR cycle conditions for gradient PCR

| PCR Reaction steps | Temp (°C) | Time | Cycle |
|---|---|---|---|
| Initial denaturation | 95.0 | 10 min | 1 |
| Denaturation | 94.0 | 30 sec | 30 |
| Annealing | 57.0-77.0 | 30 sec | |
| Extension | 68.0 | 1 min | |
| Final extension | 68.0 | 10 min | 1 |
| Hold | 10.0 | ∞ | - |

→

PCR amplification of the middle region of the *MC4R* gene using the optimum annealing temperature.

**Table 4.** Preparation of PCR reaction

| PCR Components | Stock Concentration | Final Concentration | Volume (µL) |
|---|---|---|---|
| DNA | 10 ng/µL | 20 ng/reaction | 3.0 |
| KOD One™ PCR Master Mix | 2X | 1X | 25.0 |
| Forward primer | 10 | 0.6 µM | 3.0 |
| Reverse primer | 10 | 0.6 µM | 3.0 |
| Sterile DI | - | - | 16.0 |
| Total volume | - | - | 50.0 |

**Table 5.** PCR cycle conditions for *MC4R* gene amplification

| PCR Reaction steps | Temp (°C) | Time | Cycle |
|---|---|---|---|
| Initial denaturation | 95.0 | 10 min | 1 |
| Denaturation | 94.0 | 30 sec | 30 |
| Annealing | 65.8 | 30 sec | |
| Extension | 68.0 | 1 min | |
| Final extension | 68.0 | 10 min | 1 |
| Hold | 10.0 | ∞ | - |

↑

**Nucleotide sequence analysis**

- A total of 44 PCR products were sent to APICAL SCIENTIFIC (Malaysia) for nucleotide sequence analysis with the Big Dye Terminator method using MC4R-F1 primer.
- The electropherogram was analyzed with BioEdit 7.7.1, and sequences of the *MC4R* gene were compared to the NCBI reference sequence to identify SNP.

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Amino acid sequence analysis to identify possible amino acid substitution in the Mc4 receptor.

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Assessment of correlation between SNPs and obesity by odds ratio analysis.

## RESULTS AND DISCUSSION

### Optimization of *MC4R* gene amplification by gradient PCR

The optimal annealing temperature for primer-template binding was determined to be 65.8°C as it produced a single PCR product of 612 bp without non-specific bands, analyzed by 1.2% agarose gel electrophoresis.

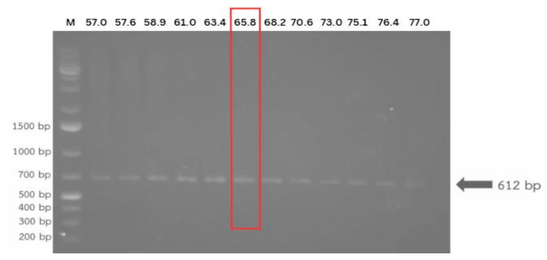


**Figure 3.** Examination of gradient PCR products by 1.2% agarose gel electrophoresis
Lane M : GeneRuler 1 kb Plus DNA ladder
Lane 1-12 : Gradient PCR products obtained from the annealing temperatures of 57.0-77.0, respectively

### PCR amplification of *MC4R* gene

All 44 DNA samples provided a specific 612 bp PCR product with high intensity, non-specific band was not observed. The absence of PCR product in Lane N (negative control ) confirmed there was no DNA contamination in the PCR reaction.

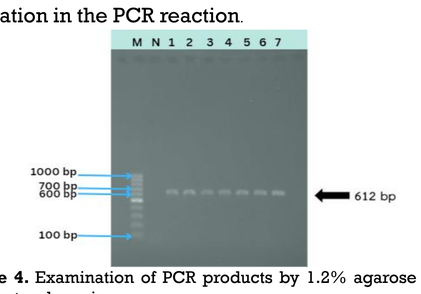


**Figure 4.** Examination of PCR products by 1.2% agarose gel electrophoresis
Lane M : GeneRuler 100 bp Plus DNA ladder
Lane N : negative control
Lane 1 : sample T07 ; Lane 2 : sample T46 ; Lane 3 : sample N19 ; Lane 4 : sample N27 ; Lane 5 : sample N36 ; Lane 6 : sample N37 ; Lane 7 : sample 130

### Nucleotide sequence analysis

- The electropherogram was analyzed to determine the nucleotide sequence and identify SNP positions.

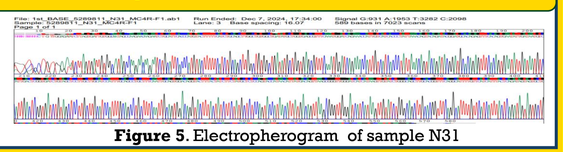

**Figure 5.** Electropherogram of sample N31

- Among 44 samples, two types of heterozygous SNPs were observed. The (A/G) SNP presented in samples T07, N02, 140, and U38 (BMI: 53.15, 27.47, 25.64 and 26.44, respectively), and the (T/C) SNP observed in sample U18 (BMI: 27.53).

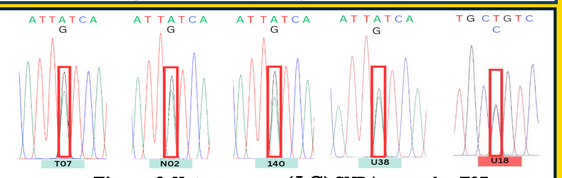


**Figure 6.** Heterozygous (A/G) SNP in samples T07, N02, 140, and U38, and (T/C) SNP in sample U18

- The multiple nucleotide sequence alignment between samples and NCBI reference sequence revealed the G to A substitution at position 5726 (G5726A) in samples T07, N02, 140 and U38 , and U38, and the T to C substitution at position 6100 (T6100C) in sample U18.

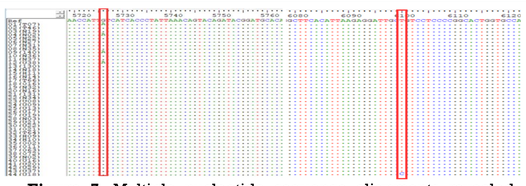

**Figure 7.** Multiple nucleotide sequence alignments revealed nucleotide substitutions in samples T07, N02, 140, U38, and U18

### Amino acid sequence analysis

- The G5726A mutation caused a valine to isoleucine substitution at position 103 (V103I) in the Mc4 receptor, which is a conservative missense mutation.

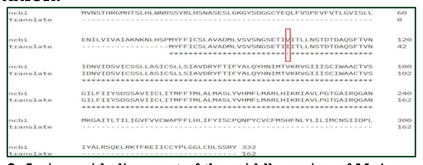

**Figure 8.** Amino acid alignment of the middle region of Mc4 receptor with the full sequence from NCBI, highlighting an amino acid substitution

As both valine and isoleucine are non-polar aliphatic amino acids, structure and function of Mc4 receptor may not be significantly affected.

- In contrast, the T6100C mutation was silent.

### Odds ratio analysis

Odd ratio analysis to assess the correlation between SNP and obesity revealed that the presence of G5726A SNP increased the risk of obesity by 13.72 times.

**Table 6.** Correlation between SNP and obesity (Data were kindly provided by Papada Saipoti and Benyapa Thimai.)

| | Obesity | Normal weight |
|---|---|---|
| EXPOSED (SNP) | 5.5 (A) | 0.5 (B) |
| UNEXPOSED (without SNP) | 48.5 (C) | 60.5 (D) |

Calculation:

$$\text{Odds Ratio (OR)} = \frac{AD}{BC}$$

$$\text{Odds Ratio (OR)} = \frac{5.5 \times 60.5}{0.5 \times 48.5}$$

$$\text{Odds Ratio (OR)} = 13.72$$

## CONCLUSIONS

- The optimum annealing temperature for *MC4R* gene amplification was 65.8°C of which the expected PCR product of 612 bp was obtained with relatively high intensity.
- Two types of heterozygous SNPs were observed. The G5726A presented in samples T07, N02, 140, and U38 of which the BMI values ranging from 25.64-53.15, and the T6100C was observed in sample U18 (BMI: 27.53).
- The G5726A caused a valine to isoleucine substitution (V103I), which supposedly had minimal impact on protein properties and functions.
- The T6100C was silent mutation, therefore, amino acid sequence and protein function were not affected.
- The odds ratio analysis indicated an increased risk of obesity in individuals with the G5726A by 13.72 times.

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- Division of Biochemistry and Biochemical Innovation, Department of Chemistry, Faculty of Science, Chiang Mai University

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