Original Research

DEVELOPMENT OF A METHOD FOR GENOTYPING THE *MTHFR* RS1801133 POLYMORPHISM IN VIETNAMESE, USING THE POLYMERASE CHAIN REACTION WITH CONFRONTING TWO-PAIR PRIMERS

Tran Quang Thuyen[1], Bui Thi Thuy Nga[2], Nguyen Anh Ngoc[2], Tran Quang Binh[2,✉]

[1] *Military Institute of Preventive Medicine, Viet Nam*
[2] *National Institute of Nutrition, Viet Nam*

ABSTRACT

Aims: To develop a method using polymerase chain reaction with confronting two-pair primers (PCR-CTPP) to genotype *MTHFR* rs1801133 polymorphism in Vietnamese people.

Methods: Fifteen DNA samples from a group of Vietnamese people were used to evaluate this assay. The Sanger sequencing method was used to identify the standard genotypes of the rs1801133 polymorphism. Some bioinformatic softwares were used to design four a multiplex PCR-CTPP. Experimental procedures were conducted to verify the primers and components of the PCR method.

Results: The protocol of the PCR-CTPP was successful developed with optimal inner/outer primers ratio, the melting temperature of primers, the components and the thermal cycling to identify *MTHFR* rs1801133 polymorphism.

Conclusions: The PCR-CTPP protocol should be applied to genotyping *MTHFR* rs1801133 polymorphism in large cohorts to investigate the association between the polymorphism and metabolic syndrome in Vietnamese population.

Keywords: *rs18021133, MTHFR gene, PCR-confronting two-pair primers*

I. INTRODUCTION

Metabolic syndrome is a collection of metabolic disorders that include abdominal obesity, dysglycemia, high blood sugar, and high blood pressure [1]. Metabolic syndrome is a risk factor for several diseases that are leading causes of death such as cardiovascular disease, diabetes, and cancer. On the other hand, metabolic syndrome is formed through the interaction of many genes and environmental factors. The consequences of this interaction can be different in each population or individual. Research on genetic factors is considered a screening tool to determine the risk of developing metabolic syndrome in each individual. However, the influence of these factors on metabolic syndrome is formed through the interaction between genetic factors and environmental factors. In Vietnam, there have been publications on lifestyle, and socio-economic factors related to metabolic syndrome [2, 3], while there is a lack of studies on genetic factors with metabolic syndrome.

✉ Corresponding author: Tran Quang Binh
Email: tranquangbinh@dinhduong.org.vn
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Therefore, it is necessary to have more studies on genes related to this syndrome to have an early and effective prevention of metabolic syndrome for the community and for each individual.

The *MTHFR* gene is located at position 36.22 on the short arm of human chromosome 1. This gene encodes for methylenetetrahydrofolate reductase (MTHFR). That plays a role in the synthesis of the amino acid methionine. In particular, MTHFR takes part in converting folate from 5,10-methylenetetrahydrofolate to 5-methyl-tetrahydrofolate. The rs1801133 polymorphism located on the intron of the *MTHFR* gene changes the C allele to the T allele, leading to the conversion of the amino acid Alanine to Valin. This modification leads to a decrease in the activity of methylenetetrahydrofolate reductase, then causes an increase in homocysteine levels in the blood [4]. Some previous studies demonstrated the rise in homocysteine levels leads to an increased risk of hypertension, hyperglycemia, and decreasing high-density lipoprotein-cholesterol [5, 6, 7].

To determine the genotype of rs1801133, some previous studies used AS-PCR (allele-specific polymerase chain reaction) [8], and other studies used RFLP-PCR (restriction fragment length polymorphism- polymerase chain reaction) [9]. However, when carrying out the methods, it is ineffective and costly due to having to perform two PCR reactions (AS-PCR) or needing a couple of electrophoreses and using restriction enzymes (RFLP-PCR). This study aims to develop a multi-primer PCR procedure to determine the genotype of rs1801133 on the *MTHFR* gene by performing only a PCR reaction and one of electrophoresis.

II. METHODS

2.1. Research design

Our study aimed to optimize the multi-primer PCR reaction to detect the genotype of rs1801133 polymorphism of the *MTHFR* gene. This study is part of the project "5-year cohort study of diabetes and metabolic syndrome in Vietnamese: Role of lifestyle factors and genetics". The topic was approved by the Medical Ethics Committee of the Central Institute of Hygiene and Epidemiology with decision No. IRB-VN01057-34/2016.

2.2. Research subjects

The subjects of our study were 15 DNA samples of 15 Vietnamese individuals participating in the study for reference. After extraction, DNA samples were stored at -20^{o}C for later use.

2.3. Experimental methods

DNA preparation

The DNA samples were extracted from whole blood using the Wizard® Genomic DNA Purification Kit (Promega, USA) and concentration was estimated by measuring the absorbance at 260 nm in the ND2000 machine (Thermo Scientific, USA). The DNA samples used for the PCR reaction were diluted at a concentration of 16-20 ng/µl. All the DNA samples had an A_{260}/A_{280} ratio of 1.8–2.0.

Primer's design

This *MTHFR* gene sequence was obtained from the US Center for Biotechnology Information (NCBI) database. Primers used to detect the genotype of rs1801133 were designed according to the principles of Hamajima N. et al. [10]. In this study, we designed four primers in which two outer primers (F and R) will synthesize a DNA fragment (321bp) containing the rs1801133 polymorphism; two inner primers (Fc and Rt) were designed to genotype the rs1801133 polymorphism. When 2 pairs of primers were combined, pair Fc and R detect the C allele (119 bp) and pair F and Rt detect the T allele (224 bp). In addition, to increase the specificity of the inner primers, we designed a mismatch at the position adjacent to the first nucleotide at the 3' end of the two inner primers (Fc and Rt) [11]. The primers were checked for specificity and properties by using Primer-BLAST software, OligoAnalyzer, and then synthesized at IDT company (USA). The sequence of the primers: F: 5'-GAAGAACTCAGCGAACTCAG-3'; R: 5'-ACTGTCATCCCTATTGGCAG-3'; Fc: 5'-GCTGCGTGATGATGAAATCTG-3'; Rt: 5'-AGAAGGTGTCTGCGGGAAT-3'.

Gene sequencing to identify positive controls

In this study, the outer primers (F and R) were used to amplify the DNA fragment around the rs1801133 polymorphism. The total volume of the PCR reaction was 15.5 µl including 4 µl of purified water (UltraPure Distilled Water, Invitrogen); 7.5µl GoTaq Green Master Mix 2x (Promega, USA); 1 µl for each primer and 2 µl of sample DNA. Amplification was achieved by a series of three steps: denaturation phase at 94^{o}C for 30 seconds, followed by annealing temperature set at 57^{o}C for 30 seconds and extension at 72^{o}C for 30 seconds. These steps were repeated 30 times to exponentially produce exact copies of the target DNA. After that, the period lasted at 72^{o}C for 8 minutes. Amplified products were sent for sequencing at the Genlab company – in Vietnam. Select 3 DNA samples with 3 different rs1801133 genotypes to optimize multiplex primer PCR reaction.

PCR reaction optimization

PCR reactions were performed by a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA). This process determined the ratio of the primers and PCR conditions to produce DNA fragments of different sizes corresponding to different genotypes.

The ratio of the primers F, R, Fc, and Rt was tested in different ratios. The ratio was considered appropriate when the PCR reaction showed the bands corresponding to the genotypes. In addition, the annealing temperature of the PCR reaction was optimized with the same PCR components at different annealing temperatures at 53, 55, 57 and 59^{o}C. The annealing time was set for 30 and 40 s with the above thermal cycling to optimize the coupling time of the reaction.

PCR products were evaluated by electrophoresis with 2.0% agarose, 0.5× TBE buffer at 100 volts for 30 min. Electrophoresis results were read on a GelDocXR+ gel imaging system (Bio-rad- USA).

III. RESULTS

3.1. Genotyping by sequencing method

The research samples were numbered from 1 to 15. Below is the image of the sequencing results by Sanger for rs11801133 of 3 DNA samples with 3 different genotypes. The above DNA samples were used as standard samples to optimize the multiplex PCR reaction to determine the genotype of rs1801133.

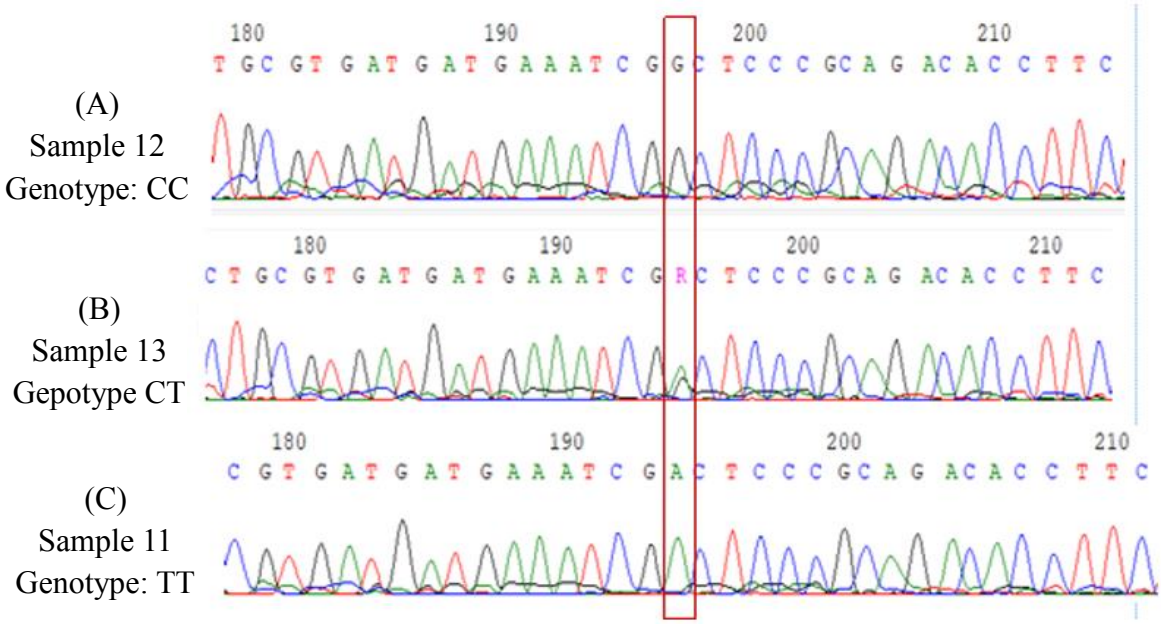

Figure 1. *The sequence data generated were analyzed for MTHFR rs1801133.*
(A) Sequencing results of genotype CC, (B) Sequencing results of genotype CT, (C) Sequencing results of genotype TT (the red square indicates the variants, respectively)

3.2. Optimization results of the multi-primer PCR reaction

Table 1. *The components for the multi-primer PCR reaction*

Components	Volume (µl)
Nuclease-Free Water	3.75 µl
PCR Master mix	4.50 µl
Primer F	0.50 µl
Primer R	0.25 µl
Primer Fc	0.25 µl
Primer Rt	0.75 µl
DNA	2.00 µl
Total volume	12.0 µl

Table 1 describes the volume and components of the multiplex PCR reaction. With the setting in PCR programmer for 35 thermal cycles: activation enzyme at 94^{o}C for 3 minutes, denaturation phase at 94^{o}C for 30 seconds; hybrid at 53^{o}C for 40 seconds; elongation at 72^{o}C for 40 seconds, elongation at 72^{o}C for 8 minutes, then finish phase at 25^{o}C forever. These components and thermal cycling were used to determine 15 unknown samples, including three known genotype samples as positive controls.

3.3. The results determine the genotype of rs1801133

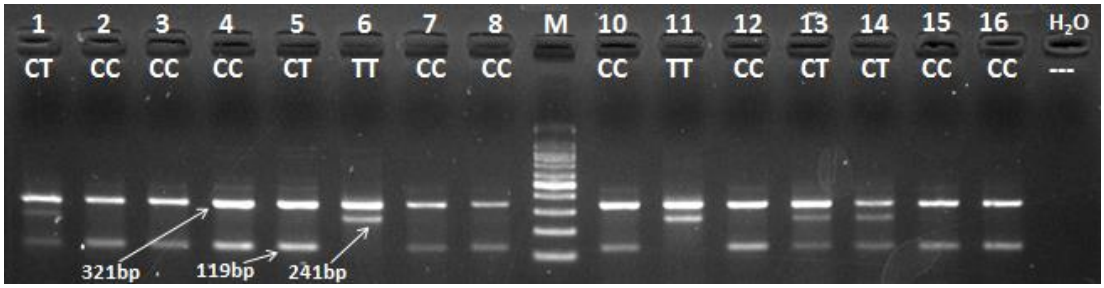

Figure 2. *Representative PCR products of the MTHFR rs1801133 genotype were detected using a gel electrophoresis process in 90 minutes with the multiplex polymerase chain reaction.*

From the left side to the right side, which was 15 samples in respective 15 lanes, M :ladder, 11 : positive control for the TT genotype, 12: was the CC genotype, 13: was the CT genotype, and 17: was blank. This result showed that PCR products were loaded on gel electrophoresis, as can easily be seen in Figure 2 showed bands with the expected length for each lane, indicating that our DNA molecule was succeeded by PCR reactions with primer F-R (321bp) to amplify target DNA outside, primer Fc-R (119bp) to indicate allele C and primer F-Rt (224bp) to indicate allele T. Therefore, the CC genotype got 2 bands (321bp and 119bp); the CT genotype got 3 bands (321bp,241bp and 119bp) and the TT genotype got 2 bands (321bp and 241bp) respectively.

IV. DISCUSSION

The *MTHFR* gene is located on the short arm of chromosome 1, which encodes the methylenetetrahydrofolate reductase, an enzyme that plays an important role in the converting homocysteine to methionine [12]. The rs1801133 is associated with decreased activity of methylenetetrahydrofolate reductase, which in turn causes elevated homocysteine levels in the blood [4]. The study by Platt D.E.et al. in Lebanon showed that carriers of the T allele had a 1.48-fold increased risk of having a blood homocysteine concentration ≥15 mmol/ml [13]. Elevated homocysteine levels cause an increased risk of many pathological disorders related to metabolic syndromes such as cardiovascular disease, and diabetes [14]. Therefore, rs1801133 is considered an important marker in the study of the interaction relationship between genetic factors and the environment for metabolic syndrome formation.

In recent years, there are many technical methods for genotyping SNPs such as AS-PCR, RFLP-PCR, TaqMan method, gene chip and sequencing. Each method has advantages and disadvantages. While RFLP-PCR has a simple design method, this technique requires restriction-specific enzymes to detect genotypes with time for results depending on the characteristics of each enzyme. The AS-PCR method is a method for accurate and fast results, but this technique still needs to perform 2 PCR reactions in parallel, thus doubling the cost. The CTPP multiplex polymerase chain reaction (PCR) method does not require complex equipment, so it can be deployed at many molecular biology laboratories. In addition, this technique requires only needs to use one PCR reaction and one-time electrophoresis similar to conventional PCR techniques, which reduces the time and cost of implementation.

In the PCR-CTPP method, the design of the annealing temperature (Tm) of the primers is a more important role than that of the PCR-RFLP method. When the Tm values of the primers are similar between the four primers, it leads to competition for the formation of amplification products is equal in the multiplex PCR reaction [15]. In this study, to design primers with similar Tm, we also experiment to determine the optimal Tm (53^0C). On the other hand, to minimize competition and increase specific amplification, this study designed nucleotides a mismatch at the position adjacent to the first nucleotide at the 3' end of the inner primers (Fc and Rt) and simultaneously changed the concentration of primers in the reaction to find the most suitable concentration volume.

V. CONCLUSION

This study successfully designed the multiplex PCR procedure to determine the genotype of the rs1801133 polymorphism on the *MTHFR* gene. The procedure is simple and does not require expensive equipment, giving fast and accurate results. The results of this study can be applied to larger sample size to analyze genotypes and evaluate the relationship between rs1801133 and metabolic syndromes.

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