Development of a multiplex PCR-RFLP method for simultaneous detection of the MTHFR 677C>T and TNF-α -308G>A variants in a Malay population

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Abstract. Methylenetetrahydrofolate reductase (MTHFR) and tumor necrosis factor-alpha (TNF-α) play major roles in cardiovascular and inflammatory disorders. This study aimed to develop a new multiplex PCR genotyping method for the simultaneous detection of MTHFR 677C>T and TNF-a -308G>A, which are the two single nucleotide polymorphisms (SNPs) that are widely known to confer susceptibility to major vascular and inflammatory disorders. DNA was amplified using multiplex PCR, which was optimized by evaluations of the annealing temperature, the effects of various magnesium chloride, primer and enzyme concentrations, and the amount of DNA template. Restriction fragment length polymorphism (RFLP) analysis was performed in two separate tubes followed by agarose gel electrophoresis. One hundred twenty-nine healthy volunteers were recruited, and the MTHFR 677C>T and TNF-a -308G>A variants were genotyped using a novel multiplex PCR-RFLP technique. The results were confirmed by DNA sequencing. The allele frequencies of MTHFR 677C>T were 97.29% (C allele) and 2.71% (T allele). For TNF-a -308G>A, the allele frequencies were 98.45% (G allele) and 1.55% (A allele). The PCR-RFLP method developed in this study is simple, cost-effective and time-saving. It can be used to simultaneously genotype subjects for the MTHFR 677C>T and TNF-a -308G>A variants with 100% concordance with DNA sequencing data. This method can be routinely used for rapid investigation of the MTHFR 677C>T and TNF-a -308G>A variants.

Keywords: MTHFR, TNF-α, PCR, PCR-RFLP, homocysteine

INTRODUCTION

The flavoenzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) regulates the flow of folate (vitamin B9) during methionine synthesis (Selhub, 1999) and has major effects on the distribution of intracellular folate (Bagley and Selhub, 1998; Smulders et al., 2007). MTHFR also plays a pivotal role in the pathogenesis of migraines and hyperhomocysteinemia. Hyperhomocysteinemia may occur due to single nucleotide polymorphisms (SNPs) in MTHFR 677C>T (HGVS nomenclature: NG_013351.1:g.14783 C>T), resulting in a missense mutation at position 222, leading to the substitution of alanine with valine. Increased plasma homocysteine concentration is a widely accepted major independent risk factor for several diseases including cardiovascular diseases, neural tube
defects, leukemia, colon cancer and peripheral vascular disease (Frosst et al., 1995).

Tumor necrosis factor (TNF) is a pro-inflammatory molecule and a polypeptide effector of the inflammatory reaction that also appears to play a role in various inflammatory diseases. Findings from in vitro and in vivo experiments have suggested that homocysteine may provoke intestinal mucosal injury by modulating TNF-α-mediated cytotoxicity (Ratter et al., 1999). Indeed, plasma homocysteine has been regarded as a determinant of TNF-α in pathological conditions characterized by low-grade inflammation (Bogdanski et al., 2008). Therefore, the targeting of the TNF pathway can significantly reduce plasma homocysteine levels (Sattar et al., 2007), suggesting a role for this cytokine in homocysteine metabolism. The Δ variant of TNF-α -308G>A (HGVS nomenclature: NG_007462.1:g.4682G>A) is associated with higher levels of TNF expression (Yapajakis et al., 2009), and this SNP has been linked to a wide variety of conditions, including migraine (Chen et al., 2015), cancer (Ma et al., 2014), type 2 diabetes mellitus (Liu et al., 2013), and neurological disorders (Pereira et al., 2007; Schurks et al., 2011), compared with other variants of TNF-α.

PCR methods for the amplification of MTHFR 677C>T (Frosst et al., 1995; Barbaux et al., 2000; Yi et al., 2002) and TNF-α -308G>A (Wilson et al., 1992; Keso et al., 2001) have previously been described. A uniplex PCR method for the amplification of MTHFR 677C>T which is widely used has previously been reported (Frosst et al., 1995). An additional study has reported the multiplex PCR amplification of MTHFR 677C>T and 1298A>C variants (Yi et al., 2002). Nevertheless, although the above mentioned methods are simple and have the potential to be developed further, they were validated without the use of positive controls, and DNA sequencing was not performed (Yi et al., 2002). Another study has reported a multiplex hetero-duplex PCR method for genotyping MTHFR 677C>T and 1298A>C SNPs along with methionine synthase (Barbaux et al., 2000). Although this method has the potential to generate reliable and high-quality data, it is complex and time-consuming due to a cloning step that must be performed before the generation of hetero-duplexes.

Wilson et al. (1992) have reported a uniplex PCR method for genotyping -308G>A SNPs of TNF-α (Wilson et al., 1992). This method is simple but requires overnight digestion, which may be time-consuming. Another study has reported a uniplex PCR method for the genotyping of TNF-α -308G>A (Keso et al., 2001). This method also requires a time-consuming overnight digestion step. Moreover, it utilizes a high-concentration polyacrylamide gel (9%) for the separation of PCR products before visualization, which may pose a health hazard to researchers.

Both the MTHFR 677C>T and TNF-α -308G>A genotypes are associated with various pathological conditions, such as colorectal cancer (Ferroni et al., 2009), breast cancer (Ferroni et al., 2009), hypertension (Bogdanski et al., 2008), rheumatoid arthritis (Ranganathan, 2008), and cardiovascular diseases (Dedoussis et al., 2005). To date, there is no reported multiplex PCR method available for the simultaneous detection of the MTHFR 677C>T and TNF-α -308G>A genotypes. The simultaneous detection of the two genes may help in correlating the genes and the aforementioned diseases. The expense of reagents and the preparation time are decreased in multiplex PCR compared with systems in which several uniplex PCR tubes are used (Edwards and Gibbs, 1994). A multiplex reaction is therefore ideal for conserving costly polymerase and templates that are in short supply as well as for detecting both genes simultaneously. Therefore, this study aimed to develop a simple, rapid and robust multiplex PCR method for simultaneous detection of the two genes for disease association.

**MATERIALS AND METHODS**

**Sample collection.** This study was conducted at Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian, Kelantan, Malaysia, between January and June 2014 and was approved by the local institutional review board at the Universiti Sains Malaysia (USMKK/PPP/JEPeM 231.3.(08)]) in compliance with the Declaration of Helsinki.

**DNA extraction.** Blood (1 ml) was collected from the subjects and stored in

PCR-RFLP for SNPs variants detection in Malay population

ethylenediaminetetraacetic acid (EDTA) tubes (Becton Dickinson, New Jersey, USA). Genomic DNA was extracted from the whole blood (200 µl) using a GeneAll® Blood Kit (GeneAll Biotechnology, Seoul, South Korea). DNA concentration and purity were determined using an Infinite® 200 NanoQuant (Tcan, Switzerland). Good-quality DNA was indicated by a 260/280 nm absorbance ratio between 1.8 and 2.0 (Santella, 2006).

**PCR primers.** The primers used for analyses of MTHFR 677C>T and TNF-α -308G>A by multiplex PCR are described in Table 1. Prior to use, the selected primer pair was validated against the Basic Local Alignment Search Tool (BLAST) database (version 7.0.5.3, Maryland, USA) (Kakavas et al., 2008) to determine primer specificity and eliminate any possibility of repetitive sequences.

**Table 1. Primers for MTHFR 677 C/T and TNF-α -308 G/A.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Length (bp)</th>
<th>Tm (°C)</th>
<th>GC (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>677 F</td>
<td>5’-TGAAGGAGAAGGATGCTGGGGA-3’</td>
<td>23</td>
<td>61.6</td>
<td>56.5</td>
<td></td>
</tr>
<tr>
<td>677 R</td>
<td>5’-AGGACGGGTGAGTGAGGTG-3’</td>
<td>20</td>
<td>61.8</td>
<td>65</td>
<td>(Wilson et al., 1992)</td>
</tr>
<tr>
<td>308 F</td>
<td>5’-AGGCAATAGGTTTTGAGGCCAT-3’</td>
<td>23</td>
<td>59.1</td>
<td>47.8</td>
<td>(Frosst et al., 1995)</td>
</tr>
<tr>
<td>308 R</td>
<td>5’-TCTCTCCTGTGCCATTCCG-3’</td>
<td>20</td>
<td>61.3</td>
<td>65</td>
<td></td>
</tr>
</tbody>
</table>

**Multiplex genotyping.** The multiplex PCR method was developed in accordance with the Qiagen® Multiplex PCR Handbook. The primer specificity, functionality and annealing temperature were tested using uniplex PCR methods that were combined into a single multiplex PCR. A total of 20 µl of PCR mixture was prepared on ice in 200 µl PCR tubes. The mixture consisted of 1X PCR buffer with potassium chloride (KCl), 0.5 µM of each primer, 0.2 mM of each dNTP, 1X Q solution, 1 U Taq DNA polymerase (Qiagen, Venlo, Netherlands) and 50 ng of DNA sample. The final optimized parameters are as that summarized in Table 2.

A negative control containing distilled water, instead of genomic DNA was also prepared. Samples that had been previously sequenced and found to be positive or negative for the SNPs were used as positive controls. The reaction mixture was gently tapped and centrifuged before being placed in a thermocycler. The PCR parameters consisted of an initial denaturation step for 4 min at 94°C followed by 35 cycles of 60 sec at 94°C, 60 sec at 62°C and 60 sec at 72°C with a 5 min final extension at 72°C. A 198-bp fragment was yielded after running the sample on a 1% agarose gel at 80 V for 45 min, and a 107-bp fragment was produced after it was run on a 2% agarose gel at 80 V for 60 min.

**Table 2. Final optimized parameters of PCR reactions for MTHFR 677C>T and TNF-α -308G>A polymorphisms.**

<table>
<thead>
<tr>
<th></th>
<th><strong>MTHFR 677C&gt;T</strong></th>
<th></th>
<th><strong>TNF-α -308G&gt;A</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Required</strong></td>
<td><strong>Volume for 20 µl reaction (µl)</strong></td>
<td><strong>Required</strong></td>
<td><strong>Volume for 20 µl reaction (µl)</strong></td>
</tr>
<tr>
<td>Double distilled water</td>
<td>7.74</td>
<td>Buffer</td>
<td>7.74</td>
</tr>
<tr>
<td>Buffer</td>
<td>1.00 X</td>
<td>1.00 X</td>
<td>2.00</td>
</tr>
<tr>
<td>dNTP</td>
<td>0.20 mM</td>
<td>0.20 mM</td>
<td>0.16</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>-</td>
<td>Q solution</td>
<td>-</td>
</tr>
<tr>
<td>Q solution</td>
<td>1.00 X</td>
<td>4.00</td>
<td>1.00 X</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.50 µM</td>
<td>2.00</td>
<td>1.00 µM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.50 µM</td>
<td>2.00</td>
<td>0.50 µM</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>1.00 U</td>
<td>0.20</td>
<td>1.00 U</td>
</tr>
<tr>
<td>DNA</td>
<td>50.00 ng</td>
<td>2.00</td>
<td>50.00 ng</td>
</tr>
</tbody>
</table>

dNTP: deoxyribonucleotide triphosphate; MgCl₂: magnesium chloride; Q solution: provided by manufacturer (Qiagen, Germany) to remove non-specific bands; Taq polymerase: Thermus aquaticus polymerase enzyme.
Endonuclease restriction assay - RFLP method. The PCR product was separated into two tubes for analysis of SNPs. Each tube consisted of 12.8 µl of double distilled water, 5.0 µl of the PCR product, 2.0 U of restriction enzyme and 5.0 µl buffer (New England BioLabs, Boston, USA). The tubes were gently tapped and briefly centrifuged before they were incubated at 37°C for 2 hours. The restriction enzyme used for the MTHFR 677C>T variant was HinfI, and for the TNF-a -308G>A variant, NcoI was used.

Agarose gel electrophoresis. The PCR products were analyzed using stained agarose gel electrophoresis (2%) with 0.5X Tris borate EDTA (TBE) buffer and ethidium bromide staining. Blue loading dye (New England BioLabs) (1 µl) was mixed with 0.7 µl of DNA ladder or 4 µl of each of the PCR and PCR-RFLP products before they were added into the wells. The system was run at 80 V for 1 hour. The fragments were visualized using an Alpha Innotech® Ultraviolet Transilluminator (Alpha Innotech® USA) before the image was captured.

PCR product purification and DNA sequencing. The PCR products were sent to a commercial laboratory for sequencing after purification using a PCR cleanup kit (GE HealthCare, Germany). Three samples from each different genotypes were randomly selected for sequencing. Sequencing was performed using an Applied Biosystems 3730 XL Genetic Analyzer (Applied Biosystems, Foster City, USA). The SNPs were analyzed by aligning the resulting sequences with reference gene sequences using Bio Edit Sequence Alignment Editor.

RESULTS

Using this novel simultaneous detection PCR-RFLP method, a total of 129 subjects were successfully genotyped. As a confirmatory measure, individuals with known genotypes were genotyped using the uniplex method and subsequently sent for sequencing. The results obtained from this technique showed 100% concordance (Figures 1 and 2).
Amplification of MTHFR 677C>T and HinfI.
The size of the PCR product for the MTHFR 677C>T genotype was 198 bp (Table 3). The HinfI restriction enzyme recognizes the sequence 5’-GANTC-3’ (N = G, A, T, or C). Therefore, the MTHFR 677 TT genotype generates two bands at 175 bp and 23 bp and can easily be differentiated from the wild-type (MTHFR 677CC) genotype. In contrast, the lack of an HinfI recognition site (5’-GANCC-3’) due to the C allele in MTHFR 677C>T produces only one undigested band at 198 bp. A heterozygous individual will have three bands, with two bands derived from the T allele and the other from the C allele (Figure 3).

The size of the PCR product for TNF-α -308G>A was 107 bp (Table 3). The NcoI restriction enzyme recognizes the sequence 5’-CCATGG-3’. Therefore, the TNF-α -308GG genotype generates two bands at 87 bp and 20 bp that can easily be differentiated from the mutant genotype (TNF-α -308AA). In contrast, the lack of an NcoI recognition site (5’-CCATGG) due to the A allele in TNF-α -308G>A produces only one undigested band at 107 bp. A heterozygous individual will have three bands, with two bands derived from the G allele and one from the A allele (Figure 3).

Of the 129 subjects, 122 had the MTHFR 677CC genotype and 7 possessed the MTHFR 677CT genotype, while none had the MTHFR 677TT genotype. The frequency of the C allele was 97.29%, and that of the T allele was 2.71%.

Table 3. Summary of the products and their sizes following digestion with restriction enzymes.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Band size (bp)</th>
<th>Restriction enzyme</th>
<th>Restriction enzyme recognition sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR 677 C&gt;T</td>
<td>198</td>
<td>HinfI</td>
<td>5’-GANTC-3’</td>
</tr>
<tr>
<td>CC-Wild type</td>
<td>198</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT-Heterozygote</td>
<td>198, 175</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT-Homozygote</td>
<td>175</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α -308 G&gt;A</td>
<td></td>
<td>NcoI</td>
<td>5’-CCATGG-3’</td>
</tr>
<tr>
<td>GG-Wild type</td>
<td>87, 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA-Heterozygote</td>
<td>107, 87, 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA-Homozygote</td>
<td>107</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

We have successfully developed a new, simple method for the simultaneous detection of the MTHFR 677C>T and TNF-α -308G>A SNPs. In addition, our study is the first to simultaneously determine the genotype and allele frequencies of the MTHFR 677C>T and TNF-α -308G>A gene polymorphisms in a Malaysian population. This method was successfully applied to genotype 129 healthy Malay volunteers with significantly reduced pre-PCR preparation and reaction times compared with methods involving several single PCRs.

In this study, the concentrations of PCR components, such as the primers, dNTP and Taq DNA polymerase, were optimized so that the lowest concentrations possible were used to produce bands with the greatest intensity. In addition to the PCR components, the conditions were also optimized. Generally, lower annealing temperatures result in higher non-specific products (Rychlik et al., 1990).
Figure 3. Representative gel electrophoresis of PCR-RFLP products for analysis of MTHFR 677C>T and TNF-α-308G>A. Lanes A, C: a single band at 198 bp represents the wild-type homozygous (CC) genotype of MTHFR 677C>T, and the band at 107 bp represents the PCR product of TNF-α-308G>A produced from multiple PCRs; Lanes B, D: the three bands at 198 bp, 175 bp and 23 bp represent the heterozygous variant (CT) of MTHFR 677C>T, and the band at 107 bp represents the PCR product of TNF-α-308G>A produced from multiple PCRs; Lanes E, K: negative control; Lanes G, I: the two bands at 87 bp and 20 bp represent the PCR product of MTHFR 677C>T produced from multiple PCRs; Lanes H, J: the three bands at 107 bp, 87 bp and 20 bp represent the heterozygous variant (GA) of TNF-α-308G>A, and the bands at 198 bp represent the PCR product of MTHFR 677C>T produced from multiple PCRs; Lane F: the positive control for the heterozygous variant of MTHFR 677C>T (three bands at 198, 175 and 23 bp), and band at 107 bp represents the PCR product for TNF-α-308G>A produced from multiple PCRs; Lane L: the positive control for the heterozygous variant of TNF-α-308G>A (three bands at 107, 87 and 20 bp), and the band at 198 bp represents the PCR product of MTHFR 677C>T produced from multiple PCRs; bp: base pair; CC: Cytosine-Cytosine; CT: Cytosine-Thymine; GG: Guanine-Guanine; and GA: Guanine-Adenosine; N/B: Lanes A to F represent PCR-RFLP products for MTHFR 677C>T, and Lanes G to L represent PCR-RFLP products for TNF-α-308G>A.

In this study, the primers were adopted from previously published uniplex methods (Wilson et al., 1992; Frosts et al., 1995). Both the forward and reverse primers were less than 20 base pairs (bp) in length. Primer length is related to primer annealing temperature and hybridization stability. An optimal primer length is between 18 and 30 bases, and the final eight to ten bases at the 3’ end play a major role in its specificity. The melting temperature of a PCR product also plays a major role in the efficiency of the multiplex PCR. The annealing temperature of the PCR must ensure that both sets of forward and reverse primers bind specifically and efficiently to the template DNA. The formula recommended by Rychlik et al. (1990) was used to calculate the annealing temperatures for the MTHFR 677C>T and TNF-α-308G>A genetic polymorphisms.

When optimizing the gradient annealing temperatures, two bands that corresponded to the desired SNPs were observed at 62°C. During the extension step, 1000 bases are typically incorporated into a DNA product every minute (Kakavas et al., 2008). The extension step of the PCR is 60 seconds in duration at 72°C for our
method, which theoretically should allow for the incorporation of 1 kb of nucleotides.

For this method, Q solution® (Qiagen, Germany) was used to remove any non-specific bands. This PCR additive facilitates the amplification of difficult templates by modifying the melting behavior of DNA. This reagent was used to enable or improve suboptimal PCRs. The advantages of the Q solution® can be utilized with any primer/template system, and it is non-toxic.

To identify the MTHFR 677C>T and TNF-α -308G>A SNPs, 2.0 U of the Hinfl and Ncol restriction enzymes were used, respectively, in separate tubes. Restriction enzymes possess the unique ability to recognize a particular DNA sequence. Therefore, a series of SNPs can be simultaneously detected from the same PCR product. The reactions were conducted in separate tubes to avoid cross-digestion, which may result in the incomplete or partial digestion of PCR products, which in turn may lead to the misinterpretation of results. Uniplex reactions have the advantage of producing very specific results and can be distinguished easily because each gel lane represents a particular SNP, whereas in mixed reactions, bands that correspond to various SNPs are observed in a single lane.

Our method was successfully used to genotype 129 healthy subjects from Kelantan. Kelantan is located in the northeast of Peninsular Malaysia. To validate our newly developed multiplex PCR method, DNA samples from variants were selected for sequencing as a confirmatory step. The DNA sequencing and subsequent gel analysis clearly verified the genetic variants detected by chromatogram, confirming the reliability of the developed method.

The frequency of the C allele (97.29%) in healthy Kelantanese Malay for MTHFR 677C>T was higher, while that of the T allele was lower compared with previous studies (Choo et al., 2011; Mejia Mohamed et al., 2011). The frequency of the T allele (2.71%) was lower for the healthy Malay in this study compared with other studies conducted on healthy Malay from other parts of Malaysia (Choo et al., 2011; Mejia Mohamed et al., 2011). A previous study has reported frequencies of 92.9% for the homozygous CC genotype and 7.1% for the CT heterozygous genotype for the MTHFR 677C>T variant among 20 Malaysian healthy subjects and 22 patients with neural tube defects (Hayati et al., 2008). Although this study was conducted using a conventional PCR-RFLP method to genotype MTHFR 677C>T, it was limited by a small sample size. The lower T allele frequency can be attributed to the fact that this study population was composed entirely of females, and all subjects were selected from the HUSM. However, Kelantanese Malay residing in the remote northeastern regions of the Malaysia are believed to have a unique genetic signature (Loo and Gan, 2014). Similarly, the T allele among Malaysian Chinese (14%) (Choo et al., 2011) is much higher compared with the Kelantanese Malay. Therefore, these results indicate that female Malay have a lower T allele frequency compared with other Malaysian ethnicities.

The frequency of the G allele (98.45%) among healthy Kelantanese Malays for the TNF-α -308G>A SNPs was similar to that reported in other studies (Montazeri et al., 2010). Montazeri et al. have reported frequencies of 95.1% for the G allele and 4.9% for the A allele among Malaysians (Montazeri et al., 2010). These authors did not specifically state the frequency according to ethnicities and their study population consisted of 68% ethnic Malays. In Asia, the frequency of the A allele in TNF-α -308G>A genetic polymorphisms varies between ethnicities. For instance, the TNF-α -308A allele is found in 0.8% of the Japanese population (Takashige et al., 1999), 7.7% of Chinese-Hans (Fei et al., 2002), 9.0% of Hong Kong Chinese (Lee et al., 2000) and 9.6% of Koreans (Um and Kim, 2003). A previous study has reported frequencies of 94.0% for the homozygous GG genotype, 6.0% for the heterozygous GA genotype and 2.0% for the homozygous AA genotype for the TNF-α -308G>A variant among 102 healthy Malaysians (Montazeri et al., 2010). These findings, which were based on a multiplex PCR method for the genotyping of TNF-α and TNF-β variants, are similar to those reported in our study.

In another study, 29 genes that are involved in coagulation, homocysteine metabolism, blood pressure regulation cell-cell interactions and inflammatory response, including MTHFR C677T and TNF-α -308G>A, have been associated with higher risks of human limb deficiency anomalies among infants (Carmichael, 2006) indicating the utility of the developed
The newly developed simultaneous detection method for these two SNPs is simple, inexpensive and reproducible and can be routinely applied in most laboratories for the simultaneous identification of the two genes.

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