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-α* -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-α* -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-α* -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-α* -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-α* -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

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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 A variant of TNF- α -308G>A (HGVS nomenclature: NG_007462.1:g.4682G>A) is associated with higher levels of TNF expression (Yapikakis *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 (USM/KK/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

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 (Tecan, 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.

Primer	Sequence	Length (bp)	Tm (°C)	GC (%)	References
677 F	5'-TGAAGGAGAAGGATGTCTGGGGGA-3'	23	61.6	56.5	(Wilson <i>et al.</i> , 1992)
677 R	5'-AGGACGGTGCGGTGAGAGTG-3'	20	61.8	65	
308 F	5'-AGGCAATAGGTTTGTGAGGGCCAT-3'	23	59.1	47.8	(Frosst <i>et al.</i> , 1995)
308 R	5'-TCCTCCCTGCTCCGATTCGG-3'	20	61.3	65	

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.

	<i>MTHFR</i> 677C>T		<i>TNF-α</i> -308G>A	
	Required concentration	Volume for 20 µl reaction (µl)	Required concentration	Volume for 20 µl reaction (µl)
Double distilled water	-	7.74	-	7.74
Buffer	1.00 X	2.00	1.00 X	2.00
dNTP	0.20 mM	0.16	0.20 mM	0.16
MgCl ₂	-	-	-	-
Q solution	1.00 X	4.00	1.00 X	4.00
Forward primer	0.50 µM	2.00	0.50 µM	2.00
Reverse primer	0.50 µM	2.00	0.50 µM	2.00
<i>Taq</i> polymerase	1.00 U	0.20	1.00 U	0.20
DNA	50.00 ng	2.00	50.00 ng	2.00

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 μ l of double distilled water, 5.0 μ l of the PCR product, 2.0 U of restriction enzyme and 5.0 μ l 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 *Hinf*I, and for the *TNF- α* -308G>A variant, *Nco*I 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 μ l) was mixed with 0.7 μ l of DNA ladder or 4 μ l 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).

Figure 1. Direct sequencing results for the *MTHFR* 677C>T genetic polymorphism (a) an example of a sample trace with *MTHFR* 677C>T wild type sequence, and (b) an example of a sample trace from a subject having *MTHFR* 677C>T heterozygous sequence.

Figure 2. Direct sequencing results for the *TNF- α* -308G>A genetic polymorphism. Only an example of a sample trace with *TNF- α* -308G>A wild type sequence is included since there was no subject having the *TNF- α* -308G>A heterozygous sequence.

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 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%. For *TNF-α* -308, 125 subjects exhibited the GG genotype, and four had the GA genotype. None of the subjects were identified with the AA genotype. The frequencies of the G and A alleles were 98.45% and 1.55%, respectively.

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

SNP	Band size (bp)	Restriction enzyme	Restriction enzyme recognition sequence
<i>MTHFR</i> 677 C>T		<i>HinfI</i>	5'-GANTC-3'
CC-Wild type	198		
CT-Heterozygote	198, 175		
TT-Homozygote	175		
<i>TNF-α</i>-308 G>A		<i>NcoI</i>	5'-CCATGG-3'
GG-Wild type	87, 20		
GA-Heterozygote	107, 87, 20		
AA-Homozygote	107		

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 wild-type homozygous (GG) genotype of *TNF- α* -308G>A, and the band at 198 bp represents 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; Frosst *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 *HinfI* and *NcoI* 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

simultaneous detection method. In a newer study, *MTHFR C677T* has also been associated with more and more diseases including Sjogren's syndrome and lymphoma development (Fragkioudaki *et al.*, 2017) due to defective DNA methylation, as well as genomic instability, which indicates the importance of investigating the gene.

CONCLUSION

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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