Association of MTHFR polymorphism and periodontitis’ severity in Indonesian males

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Abstract. Periodontitis is an oral disease with a complex etiology and pathogenesis, but with a suspected contribution by genetic factors. This study aimed to assess the association of polymorphism in MTHFR (methylene tetrahydrofolate reductase, C677T) gene and the severity of periodontitis in Indonesian males. Severity of periodontitis was classified as mild, moderate or severe for 100 consenting, 25 to 60 years old male Indonesians. Using PCR amplification for DNA extracted from blood serum samples, the variation at the SNP polymorphism of the MTHFR (C677T) gene was evaluated by using RFLP, cutting by the restriction enzyme Hinf I and subjecting the fragments to electrophoresis on agarose gel. Chi-square testing was mainly used for statistical assessment of the results. The CC genotype (wild type) of the tested polymorphism was the most common variant (78%) and TT (mutant) genotype relatively rare (2%), so that C-allele appeared in 88% of the cases and T-allele in 12% of the cases. The results suggest that there is no significant association between MTHFR C677T polymorphism and the severity of periodontitis in the tested Indonesian males.

Keywords: periodontitis, MTHFR, polymorphism

1. Introduction
Periodontal disease is one of the most common and widely spread human diseases. Periodontal disease has been associated with chronic systemic disorders [1], such as diabetes mellitus [2], osteoporosis [3], cardiovascular disease [4], and stroke [5]. As a result, men and women aged 25-74 years with periodontitis appear to have an increased risk of death from systemic disease [4]. Almost all adults have suffered from gingivitis, periodontitis, or both [6].

A study of Albander and Rams (2002) suggested that Asian populations have highest prevalence rates of periodontal disease cases in the world [7]. The results of a national survey (SKRT 2004) suggest that 39% of Indonesian population is suffering from dental and oral disease [6].

Periodontitis is a disease with unknown exact etiology, but the etiology is believed to be multifactorial. In addition to bacterial pathogens and other environmental factors (poor habits,
hormonal, stress) involved in the pathogenesis of periodontitis, genetic factors are also suspected to be involved. In principle, genetic polymorphism can be used to test this assumption.

Increased homocysteine levels in gingival crevicular fluid (GCF) has been reported for patients with periodontitis [8, 9]. Increased homocysteine level in blood, or hyperhomocysteinemias, can be a risk factor for the onset of cardiovascular disease [10]. Increased homocysteine level may result from decreased levels of folate that is essential in the conversion of homocysteine into methionine [11]. Decreased level of folate can be due to reduced activity of the enzyme methylene tetrahydrofolate reductase (MTHFR) as catalyst to conversion of 5-methyltetrahydrofolate (MTHF), the active form of folate [12]. MTHFR enzyme is encoded by the MTHFR gene, and polymorphism in this gene can have an effect on the activity of MTHFR enzyme [13].

A variety of negative effects have been associated with MTHFR polymorphism. A study conducted by Mills et al (1999) in Ireland showed that homozygous polymorphic T-allele was related to an increased risk of cleft lip [12]. The results of the work by Kalita et al (2006) suggest an association with stroke [15], and those by Deeparani et al (2009) suggest an association with heart diseases [16].

Another study in America by James et al (1999) concluded that MTHFR polymorphism is related with the occurrence of Down's syndrome in children. A study of Gudnason et al (1998) showed that the polymorphic T-allele has an effect on the action of MTHFR enzyme [18]. One of the clinical manifestations appeared was an elevated level of homocysteine on the blood in T-allele individuals [12, 18, 19]. The study from Canada by de Koning et al (2003) has reported an association between the MTHFR polymorphism with atherosclerosis [19]. The results from such studies on systemic disease have prompted the hypothesis that MTHFR gene polymorphism may also be associated with periodontitis.

The lowest prevalence of MTHFR gene polymorphisms is in Africa (6.3%), and the highest has been reported in Europe (Italy, 43.8%) [12]. A study by Schneider et al (1998) has also reported a low rate of polymorphism of 4.5% in Sri Lanka [14]. Asian prevalence levels of MTHFR polymorphisms have been reported for populations in Yemen, Turkey, and Japan [12], and in Hong Kong [14], India [15, 16], and Jordan [17]. However, no study has been conducted on the MTHFR gene polymorphism in Indonesian males. The present study aimed to investigate the association of a selected polymorphism of MTHFR gene (C677T) with the severity of periodontitis in Indonesian men.

2. Materials and methods

2.1. Sampling and ethical approval

In total, 100 consenting Indonesian men aged 25-60 years were included in the study. Their oral health was screened with conventional dental procedures and the classified to subjects with mild, modest or severe periodontitis. Stored (at -20°C) blood serum of the subjects was extracted from the Laboratory of Biology, Faculty of Medicine at the University of Indonesia. The study and applied methods were approved by the ethical committee of the Faculty of Dentistry, University of Indonesia.

2.2. Isolation of DNA

For DNA isolation, thawed serum samples were inserted into Falcon tubes containing RBC anticoagulant (1:3), and the tube was repeatedly inverted for 10 minutes. The tubes were then centrifuged for 15 min at 1500 rpm at room temperature, supernatant was discarded, and this was repeated 3 times. After that CLS 2 mL was added to the pellet and the solution in the up-down with the transfer pipette, and incubated in a water bath at 37°C for 60 min. PP solution of 1.3 mL was added into the tube, vortexed and centrifuged at 40°C/3000 rpm for 5 min. The supernatant was poured into a new tube containing 2.3 mL of cold isopropanol, and the tube was inverted repeatedly until DNA in it appeared white. Cold 70% ethanol was added (1.3 mL) and tube was repeatedly inverted, and washing was repeated twice before drying the tube at tilted position. Then 0.3 mL of TE (adjusted by the amount of DNA) was added, the mixture was incubated in a water bath at 37°C for 2 hours, and finally transferred into Eppendorf tubes (300 μL) and stored at -20°C.
2.3. PCR and RFLP
To assess the status of the samples regarding the selected polymorphism in the MTHFR gene, PCR amplification was performed using an appropriate primer pair (forward) 5'-TGA AGG AGA AGG TGT CTG CGG GA-3’ and (reverse) 5’-AGG ACG GTG CGG TGA GAG TG-3’. PCR amplification was conducted in Elmer GeneAmp PCR System 9700. The 10 μL volume of biomix consisted of 1 μL of genomic DNA, buffer solution containing 0.2 μM of dNTP, 2 μL of each primer, 0.7U Tag polymerase (Promega), and 4 μL ddH$_2$O. The PCR amplification included 35 cycles with an initial denaturation at 95°C for 5 min, then the first cycle of denaturation at 95°C for 30 s, annealing at 64°C for 30 s and elongation at 72°C for 30 s. The final extension was done at 72°C for 7 min. The amplified fragments were separated by electrophoresis on 1.5% agarose gel, to show the amplified target fragment of 198 bp in size. Restriction fragment length polymorphism (RFLP) analysis was performed by adding the restriction enzyme Hinf I (0.1 μL) into a tube containing the amplified DNA fragments, 2.5 μL buffer solution and 12.4 μL ddH$_2$O. The mixture was incubated in a water bath at 37°C for 4 h, then inactivated by incubating at 65°C for 20 min. DNA fragments were subjected to electrophoresis on 3% agarose gel. Cutting with the restriction enzyme resulted in either one single DNA band of 198 bp (genotype CC), three bands (198, 175 bp and 23 bp; genotype CT), or two bands (175 and 23 bp, genotype TT). The genotype coding is based on the nucleotide bases of the cutting site, involving cytosine (C) in the wild type and thymine (T) in the mutant type.

2.4. Statistical analysis
To compare the test groups with mild, moderate and severe periodontitis according to genotypes and allotypes of the tested polymorphism, chi-square testing and logarithmic regression were mainly used for statistical analysis and assessment of the association of polymorphism and risk to periodontitis. Statistical significance was assumed at p < 0.05.

3. Results
PCR amplified DNA samples of the targeted MTHFR gene were shown to provide in electrophoresis the expected single band of 198 bp in size (Fig. 1a). After using the cutting enzyme Hinf I for polymorphism status, the resulting variants of genotypes CC (wild type), CT (heterozygous) and TT (mutant) were observed (Fig. 1b).

![Figure 1. Results of PCR-RFLP: a) PCR amplified target fragment; M = ladder marker; wells 1-6 with samples of 198 bp PCR product (the band below is from remaining primers); b) fragments indicating genotypes after Hinf I enzyme cutting](image-url)

The observed frequencies of the genotypes and allotypes of MTHFR polymorphism for the test subjects grouped according to the severity of periodontitis are shown in Table 1. The genotype CC was by far the most frequent (78%), and TT relatively rare (2%). The C allele was correspondingly common (88%), leaving 12% frequency for the allele T. There is a slight apparent trend of the
genotype CC (and allele C) becoming more common towards increasing severity of periodontitis, and corresponding parallel decrease in the frequency of genotype CT and allele T (Table 1). There is however no statistically significant association to the severity of periodontitis ($p > 0.05$ in Chi-square testing).

**Table 1.** Frequency (%) of genotypes and allotypes for the test subjects grouped according to severity of periodontitis (note that N equals % for genotypes)

| Severity | Genotype | Allotype |
|----------|----------|----------|
|          | CC       | CT       | TT       | C        | T        |
| Mild     | 7 (70.0) | 3 (30.0) | 0 (0.0)  | 17 (85.0)| 3 (15.0) |
| Moderate | 38 (77.6)| 10 (20.4)| 1 (2.0)  | 86 (87.8)| 12 (12.2)|
| Severe   | 33 (80.5)| 7 (17.1) | 1 (2.4)  | 73 (89.0)| 9 (11.0) |
| Total    | 78 (78.0)| 20 (20.0)| 2 (2.0)  | 176 (88.0)| 24 (12.0)|

In Fig. 1a, the rows 1-6 show the PCR product (198-bp) of the MTHFR target. In Fig. 1b, the rows 1-3 and 7 show the undigested products by the enzyme, only forming a single band of 198-bp corresponding to CC, the homozygous wild-type genotype. The rows 4-5 show in principle three bands that are the 198-bp band (undigested) and 175-bp and 23-bp bands (digested, the latter one invisible), corresponding to CT or the heterozygous polymorphic genotype. The row 6 shows the fully digested products of 175-bp and 23-bp bands (again the latter invisible) of the homozygous polymorphic genotype, TT.

4. Discussion
Only 10% of the test population was included in the group of mild periodontitis. Partly this may be related to some bias in sampling, but is also taken to reflect the common status of dental and oral health in the adult Indonesian population [6].

The results appear to be in general agreement with those by Kalita et al (2006) [15], Deeparani et al (2009) [16], and Khabour et al (2009) [17]. The CC genotype of the C667T polymorphism of MTHFR dominated, and only 2% of the subjects were of the TT genotype. Accordingly, C-allele was in the present work overwhelmingly more common than T-allele that appeared in 12% of the cases. This is less than in most studies from Asian populations, although in agreement with the general finding that the T-allele is the minor counterpart in the C677T polymorphism of MTHFR [12, 14].

The results from numerous studies have suggested associations between MTHFR gene polymorphism and systemic disease [12-19], although in some cases such claims have also been refuted and may remain controversial [20- 21]. The reported associations together with the possible relation between periodontitis and systemic disease have prompted the hypothesis that the MTHFR polymorphism may also be associated with periodontitis.

However, the results of the present work show no significant association ($p > 0.05$) between the tested MTHFR polymorphism (C677T) and the severity of periodontitis in adult Indonesian males. In this case the sample size was modest ($n = 100$), but as the rejection was nowhere near bordering significance, any remaining association from a larger sample is unlikely to be strong.

5. Conclusion
In the tested population of adult male Indonesians for the polymorphism (C677T) of the MTHFR gene, the CC genotype (wild type) was the most common variant (78%) and TT (mutant) genotype relatively rare (2%), so that C-allele appeared in 88% of the cases and T-allele in 12% of the cases. The results suggest that there is no significant association between MTHFR polymorphism (C677T) and the severity of periodontitis in the tested Indonesian males.
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