Genotype distribution of methylenetetrahydrofolate reductase A1298C and C677T gene in Indonesian infertile men

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Abstract

Background: Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme of folate and methionin metabolism, making it crucial for DNA synthesis and methylation. Variants of MTHFR C677T and A1298C gene result in reduced plasma folate levels and increase the susceptibility to spermatogenic arrest. This research aims to analyses MTHFR C677T and A1298C gene polymorphism in Indonesian infertile men with azoospermia and oligozoospermia.

Methods: This cross sectional study takes 3 mL of blood from 150 infertile men with oligozoospermia and azoospermia. MTHFR gene is analyzed using polymerase chain reaction technique (PCR) with specific primers. PCR-RFLP analysis of the MTHFR gene using restriction enzymes MboII and HinfI determines allotypes, both of SNP A1298C and C677T in oligozoospermia and azoospermia in Indonesian population.

Results: The results show that the distribution of allotypes of MTHFR gene SNP A1298C and A677T is not significantly different (p>0.05) between patient groups with oligozoospermia and azoospermia. Moreover, distributions of allotypes also show no significant differences (p>0.05) between men with severe oligozoospermia and azoospermia.

Conclusion: MTHFR gene polymorphisms, both of SNP A1298C and C677T are not associated with male infertility in Indonesian men including patients with severe oligozoospermia and azoospermia. (Med J Indones 2012;21:23-7)

Keywords: DNA methylation, MTHFR, spermatogenic arrest

Spermatogenic arrest or interruption of the process of spermatogenesis in the seminiferous tubules is a phenomenon, which causes azoospermia or severe oligozoospermia (sperm count < 2 million/ejaculate). Spermatogenic arrest can be caused by external factors such as diet, high temperatures, chemicals, cigarette smoke, alcohol, and psychological stress and by internal factors such as loss of one of the genes, which control the process of spermatogenesis, e.g., SRY, AZF, or MTHFR genes.1

Methylenetetrahydrofolate reductase (MTHFR) gene is located on the short arm of the distal 36.32 region of chromosome 1 that consists of 11 exons. The cDNA consists of 2.2 kbp and encodes a 70 kDa enzyme involved in folate metabolism as a cofactor for the methylation of homocysteine to methionin.

DNA methylation is a process of adding a methyl group to cytosine bases that will alter the structure of DNA without changing the composition of the nitrogenous bases.2,3

The MTHFR gene is polymorphic in 20 points and two of them exert a very significant effect on enzyme activity, i.e. at positions C677T and A1298C. MTHFR gene polymorphism in position 677 causes a change of alanine to valine and in position 1298 of glutamate
to alanine. These changes cause a decrease in enzyme levels in the blood, which will influence the disregulation of folic acid metabolism that resulted from errors in the methylation of genomic DNA.4,7

Many studies on MTHFR gene polymorphism indicated its association with colon cancer, leukemia, schizophrenia, migraine, Down syndrome and other neurological disorders.8-12 Studies on the association of infertility with MTHFR gene variations have also been conducted in African, American, Hispanic, European, Romanian and Indian populations. They showed that a change to valine reduces homocysteine levels with implications on spermatogenesis in populations in Romania and India, but not in other populations.13,14

In the population of the United States most individuals are found with homozygous dominant genotype: frequency in Mediterranean / Hispanic > Caucasian > African / Afro-American populations. Homozygous recessive genotype was found in the Romanian population at 35.7% among infertile men. The MTHFR gene mutation causes the inactivation of the germ cells and will eventually lead to spermatogenic arrest. Increase in oxidative status in reproductive tissues was also shown as a result of DNA damage in spermatozoa.10

It is known that the polymorphism is influenced by race. Therefore, in the Indonesian population we should know whether the frequency and distribution of MTHFR gene polymorphism and variation of this gene affects spermatogenesis in infertile men. This data will be considered in the management and handling of infertile men who want a child through assisted fertilization program.

METHODS

Subjects

This study was conducted from February until October 2011 and research subjects were male patients diagnosed with infertility. All subjects gave their informed consent before participating in this research. The diagnosis of infertility, which included azoospermia and severe oligozoospermia was determined by an andrologist. Participants were infertile subjects who followed the IVF program at the Yasmin Clinic, Cipto Mangunkusumo Hospital, Jakarta, Indonesia.

Study design

The design of this study was a cross-sectional study with laboratory approach. The sample used in this study was 3 mL of peripheral blood. According to the minimum formula of Sastroasmoro and Ismael,15 the total amount of samples is 138 for oligozoospermia and azoospermia. This study used DNA preparations that we obtained from previous studies entitled correlation microdeletion of AZFc genes with sperm quality of azoospermic and severely oligozoospermic men who followed the ICSI program (Ethical Approval No: 62/PT02.FK/ETIK/2011).

Blood samples were obtained from Yasmin Clinic, Cipto Mangunkusumo Hospital (RSCM) and analyzed for MTHFR gene using PCR technique followed by RFLP technique conducted at the Department of Medical Biology, Faculty of Medicine Universitas Indonesia (FMUI).

Genomic DNA isolation

The DNA isolation method of this study is commonly used in the Department of Medical Biology FMUI.16 An amount of 3 mL of peripheral blood containing EDTA was transferred into a falcon tube (15 mL) containing 9 mL of red blood cell (RBC) lyses solution (1.45 M NH4Cl, 5 mM anhydrous EDTA, and 0.1 M KHCO3), then inverted 2-3 times and incubated at room temperature for 10 minutes. During the process of incubation, the tube was inverted 3-4 times as much. The sample was then centrifuged in an Expender Centrifuge 5702R at a speed of 1,500 rpm for 10 minutes at room temperature. The supernatant was removed by leaving the pellet in the form of mononuclear leucocytes.

The above steps were repeated to obtain a white pellet, which did not contain red blood cells. Then, to the pellet was added 2 mL of Nuclei Lyses Solution (NLS) (10 mM Tris-HCl pH 8, 0.25 mM EDTA, and 0.5% SDS), then pipetted until it became a homogeneous solution. Further samples were incubated in the water bath (NESLAB, RTE-111) at 37°C for 30-60 minutes until the solution was completely homogeneous, marked through the absence of small granules. Then were added 1.3 mL of protein precipitation solution (ammonium acetate 5 M) and vortexed with an Auto Vortex (Stuart Scientific SA 6) for 15-20 seconds. The lysate was centrifuged at 3000 RPM for 15 minutes at 4°C. Centrifugation produced a light-brown precipitate of protein, the supernatant containing DNA. Then, the supernatant was poured into a new falcon tube filled with 2.3 mL of cold isopropanol. The tube was inverted for 20-30 times until it looked like a collection of DNA strands. The samples were incubated at -20°C overnight (16-18 hours).

The next day, samples were centrifuged at 3,000 RPM for 5 minutes at 4°C, so that the DNA-containing
precipitate appeared white. The supernatant was removed and then added 1.3 mL of cold 70% ethanol, the tube was inverted to wash the DNA. DNA solution was centrifuged at 3,000 RPM for 5 minutes at 4°C. The supernatant was discarded and the DNA dried in the open air by reversing the tube for approximately 2 hours. DNA was rehydrated with 200-300 mL of TE solution (Tris-HCl EDTA) and then incubated in a water bath at 37°C for 2 hours. The solution was then transferred into a tube with 1.5 mL of sterile expender. Then, DNA solution was stored at -20°C until further examination.

**MTHFR DNA amplification and PCR-RFLP technique**

Amplification MTHFR gene was performed by using forward primer 5’-CTTGGGGAG CTGAGCACATCT-3’ and reverse primer 5’-CACCTTTGGTGGACCTCTC-3’ for SNP A1298C and forward primer 5’-TGAAGGAGAAGGTGGTCGCGG-3’ and reverse primer 5’-AGACGCTGCGGAGGACTGTG-3’ for SNP C677T. PCR method was performed using PCR GotaqTM Core System kit consisting of 25 mM MgCl2, 5x Green Gotaq, dNTP mix and Gotaq Polymerase. The volume of each reagent was 50 μL consisting of 100-500 ng genomic DNA, 20 ng of each oligonucleotide primer, 1x Green Gotaq, 2.5 mM MgCl2, 200 mM dNTP mix (mixture of dATP, dTTP, dCTP and dGTP) and 1.25 U Tag DNA polymerase. The DNA sample was amplified through 30-35 cycles with initial denaturation at 94°C for 5 minutes, then entered into the cycle consisting of denaturation, annealing and elongation. At the end of the cycle performed lengthening the time extension 72°C for 7 minutes. After the DNA amplification process was completed, the amplicon was stored at 4°C. The results of electrophoresis was band size 340 bp (C677T) and band size 133 bp (A1298T).

Amount of 10 μl amplicon DNA (C677T) results by PCR digested using 1 unit digestion enzyme (HinfI) and the amount of amplicon DNA (A1298T) results by PCR also digested using 1 unit MboII. The DNA incubated at a temperature of 37°C for 3 hours. The results of digested DNA were separated by electrophoresis on 3% agarose gel (Promega) containing 1 μL ethidium bromide (0.5 mg/mL) in 1X TAE buffer solution (0.04 M Tris-acetate, 0.002 M EDTA pH 8, 0). DNA digested in 5 μL mixed with 2 μL tracking dye (0.25% bromophenol blue,0.25%xylene cyanole,25%sucrose), then inserted into the electrophoresis wells. Bands of DNA fragments were then separated by electrophoresis at 80 volts for 60 minutes. As markers a 100 bp DNA ladder was used. Results of electrophoresis bands of DNA fragments were observed by UV illuminator and photographed with a Polaroid camera.

**Statistical analysis**

This research used nonparametric statistical analysis, to evaluate distribution and relations between two populations of genotype and allele of MTHFR gene Chi-square test was used.17

**RESULTS**

DNA amplification to detect MTHFR gene polymorphism

Interpretation of digestion results are shown by different patterns. RFLP analysis for the A1298C on a 163 bp PCR fragment with MboII: Digestion of the 163 bp fragment of 1298 AA genotype gives five fragments of 56, 31, 30, 28 and 18 bp, whereas the 1298CC genotype results in four fragments of 84, 31, 30, and 18 bp. The 18 bp fragment ran off the gel (Figure 1). RFLP analysis for the C677T on a 307 bp PCR fragment with HinfI digestion of the 307 bp fragment of 677CT genotype gives two fragments of 200 and 175 bp, genotype CC gives one fragment of 200, whereas the 677 TT genotype results in four fragments of 175 bp (Figure 2).

![Figure 1. RFLP analysis for the SNP A1298C](image1)

![Figure 2. RFLP analysis for the SNP C677T](image2)
Distribution of MTHFR SNP A1298C in oligozoospermic and azoospermic patients

The distribution of genotypes and allele frequencies of SNPs A1298C MTHFR gene on oligozoospermia and azoospermia is shown in Table 1 and Figure 3. We can see the genotype distribution and allele frequency of MTHFR gene in the groups with oligozoospermia and azoospermia. Table 1 indicates that genotype has distribution of MTHFR gene in oligozoospermia and azoospermia shows a spreading pattern that is not flattened. In the oligozoospermia group, AC genotype has highest proportion (63.45%), AA genotype has lower proportion (35.58%), and CC genotype lowest proportion (0.97%). In the azoospermia group, AC genotype has highest proportion (86.96%), CC genotype has lower proportion (13.04%), and CC genotype has lowest proportion (0.00%).

Table 1. Comparison of genotype distribution and allele frequency of MTHFR gene SNP A1298C between oligozoospermia and azoospermia groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>AA</th>
<th>AC</th>
<th>CC</th>
<th>Allele Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A(%)</td>
<td>C(%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(33.58%)</td>
<td>(63.45%)</td>
<td>(0.97%)</td>
<td></td>
</tr>
<tr>
<td>Oligozoospermia</td>
<td>104</td>
<td>37</td>
<td>66</td>
<td>1</td>
<td>67.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32.70</td>
</tr>
<tr>
<td>Azoospermia</td>
<td>46</td>
<td>6</td>
<td>40</td>
<td>0</td>
<td>56.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>43.47</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>43</td>
<td>106</td>
<td>1</td>
<td>64.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>36.00</td>
</tr>
</tbody>
</table>

Furthermore, for the allele frequency, in the oligozoospermia group, A allele has high frequency (67.30%), whereas C allele has low frequency (32.70%). In the azoospermia group, A allele has high frequency (56.52%), whereas C allele has low frequency (43.47%). As a whole in the population, this indicates that A allele has high frequency (64.00%), whereas C allele has low frequency (36.00%). By chi-square ($\chi^2$) test, allele frequency of the MTHFR gene in the oligozoospermia group was not significantly different from the azoospermia group (p>0.05). This condition indicates that allele frequency of MTHFR gene SNP A1228C is not disposition and MTHFR gene does not contribute to the pathogenesis of male infertility.

Distribution of MTHFR SNP C677T in oligozoospermic and azoospermic patients

The distribution of genotypes and allele frequencies of SNPs C677T MTHFR gene on oligozoospermia and azoospermia are shown in Table 2 and Figure 4. We can see the genotype distribution and allele frequency of MTHFR gene in oligozoospermia and azoospermia groups. Table 2 indicates that genotype distribution of MTHFR gene in oligozoospermia and azoospermia shows a spreading pattern that is not flattened. In the oligozoospermia group, CC genotype has highest proportion (68.26%), CT genotype has lower proportion (30.77%), and TT genotype has lowest proportion (0.97%).

Table 2. Comparison of genotype distribution and allele frequency of MTHFR gene SNP C667T between oligozoospermia and azoospermia groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>Allele Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C(%)</td>
<td>T(%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(68.26%)</td>
<td>(30.77%)</td>
<td>(0.97%)</td>
<td></td>
</tr>
<tr>
<td>Oligozoospermia</td>
<td>104</td>
<td>71</td>
<td>32</td>
<td>1</td>
<td>83.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16.35</td>
</tr>
<tr>
<td>Azoospermia</td>
<td>46</td>
<td>27</td>
<td>18</td>
<td>1</td>
<td>78.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>21.74</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>98</td>
<td>50</td>
<td>2</td>
<td>82.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18.00</td>
</tr>
</tbody>
</table>

In the azoospermia group, CC genotype has highest proportion (58.69%), CT genotype has lower proportion (39.15%), and TT genotype has lowest proportion (2.16%). Furthermore, for the allele frequency, in the oligozoospermia group, C allele has high frequency (83.65%), whereas T allele has low frequency (16.35%). In the azoospermia group, C allele has high frequency (78.26%), whereas T allele has low frequency (21.74%). As a whole, in the population indicates that C allele has high frequency (82.00%), whereas T allele has low frequency (18.00%). By chi-square ($\chi^2$) test, allele frequency MTHFR gene in the oligozoospermia group was not significantly different from the azoospermia group (p>0.05). This condition indicates that allele frequency of MTHFR gene is not disposition and MTHFR gene does not contribute to the pathogenesis of male infertility.

Figure 3. Allele frequency of SNP A1298C MTHFR gene on oligozoospermia and azoospermia groups (chi square, p> 0.05).

Figure 4. Allele frequency of SNP C677T MTHFR gene in oligozoospermia and azoospermia groups (chi square, p> 0.05).
DISCUSSION

Table 1 and Figure 3 indicate that the distribution of genotype MTHFR gene A1298C in oligozoospermia and azoospermia shows a spreading pattern. By chi-square test, allele frequency of MTHFR gene A1298C between oligozoospermia and azoospermia groups was not significantly different (p>0.05). Table 2 and Figure 4 indicate that the distribution of genotype MTHFR gene C677T in oligozoospermia and azoospermia shows a spreading pattern. By chi-square test, allele frequency of MTHFR gene C677T between oligozoospermia and azoospermia groups was not significantly different (p=0.05).

Several studies have shown a significant statistical correlation between MTHFR polymorphisms and male infertility, whereas other researchers did not find any significant association. These discrepancies could be explained by the fact that, in some patients with male infertility, the reduction of MTHFR activity may be linked to other modifications, like, for instance, abnormal methylation of its promoter region. Indeed, epigenetic modifications at several genes, such as methylation of the promoter region and the subsequent down-regulation of gene expression, have been shown to act similarly and in some cases in parallel to genetic mutations in a number of pathologies.

In these studies both mutant genotypes at C677T or A1298C are very little with the frequency of less than 2% in the azoospermia or oligozoospermia groups. This suggests that the spermatogenic arrest in Indonesian men may not be caused by a mutation in the MTHFR gene. Therefore, further research should be conducted to determine the role of other genes as AZF, Boule and DAZL genes that play a role in controlling the mechanism of the process of spermatogenesis.

In conclusion, we found no association of methylenetetrahydrofolate reductase A1298C and C677T gene polymorphism and male infertility in Indonesian men with azoospermia and oligozoospermia. Such studies need to be extended to get a better predictive association of the genotype and environment interaction in spermatogenic arrest.

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