Prevalence of C677T Single Nucleotide Polymorphism of Methylenetetrahydrofolate Reductase Gene and its Relationship With Serum Levels of Homocysteine, Vitamin B12, Folate, and Cholesterol in Alzheimer’s Patients

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Abstract
Objectives: Many single nucleotide polymorphisms affect the incidence of Alzheimer’s disease (AD). This investigation aimed to consider the frequency of the C677T single nucleotide polymorphism of the methylenetetrahydrofolate reductase (MTHFR) gene in Alzheimer’s patients.

Materials and Methods: This study was conducted in two groups of control (n = 80) and patient (n = 80) with a ratio of 1:1 male to female. Amplification-refractory mutation system-Polymerase Chain Reaction (ARMS-PCR) method was used to study mutations and ELISA was used to measure homocysteine and the chemiluminescence method was used to measure cholesterol, vitamin B12 and folate.

Results: Based on the results of the PCR test of the MTHFR gene, the incidence rate of mutation in the healthy allele was 44.6% and in the mutant allele was 27.9% of the total study population.

Conclusion: In this study, it was discovered that an increase in cholesterol levels is related with an increased risk of developing the disease, but more studies are needed to confirm this. It should also be noted that this increase is not related to the MTHFR gene polymorphism at the C677T position.

Keywords: Alzheimer’s, SNP, Homocysteine, Vitamin B12, Folate, Cholesterol, C677T, MTHF

Introduction
One of the most common types of dementia is Alzheimer’s disease (AD), which is a kind of brain dysfunction in which the patient’s mental abilities gradually decline. The most noticeable kind of dementia is memory impairment. Memory impairment usually develops gradually and progresses. Symptoms include near-memory impairment and impaired consciousness at the time of place, and late symptoms include psychosis, paranoia, and delusions (1-3). One of the mechanisms in the development of AD is vascular disorders, so the factors that cause vascular disease can increase the risk of AD (4). One of these factors is hyperhomocysteine or increased plasma concentration of homocysteine, which was seen in 25% of patients with vascular disease. Therefore, the relationship among homocysteine levels and AD was considered and most studies on the relationship between serum homocysteine levels and AD confirmed the presence of hyperhomocysteine in these patients (5). The molecular mechanism of AD is associated with the extracellular accumulation of amyloid-β (Aβ) peptides produced by the cleavage of the amyloid precursor protein, strings of hyperphosphorylated Tau proteins collecting inside neurons known as neurofibrillary tangles. Generally, the function of Aβ is regarded as an important trigger in AD pathobiology that brings about neurofibrillary tangles, neuronal dysfunction, and dementia. In this way, the stages ending in AD, tau, and Aβ first misfold and shape collections in one brain area, from where they scatter to the interconnected region of the brain thereby inducing its gradual functional deterioration. Homocysteine is a non-protein amino acid derived from methionine. In this case, methionine loses its methyl agent in biochemical reactions and converts itself to hyperhomocysteine or increased plasma concentration of homocysteine, which was seen in 25% of patients with vascular disease.
mutations in the MTHFR gene (5-7).

The MTHFR gene is located on chromosome 1 at position 3p36. This gene encodes the methyl-hydro folate reductase enzyme and its function is to assist control of homocysteine levels in the body. C677T and A1298C are the most important polymorphisms identified in this gene (8,9). In MTHFR C677T polymorphism, cytosine is replaced by thymine (T> C), which converts the amino acid alanine to valine (10). MTHFR gene mutation has been related to an increased chance of having children with neural tube defects (8-10). The MTHFR gene is required to uptake folic acid and other conditions of folate by cells. Patients with a polymorphism in the MTHFR gene called C677T are incapable of producing the most effective form of folate, methyl folate (40% to 60%). Methyl folate is a nutritious, important, and practical substance for the production of carriers of the nervous system and cardiovascular system. Methyl folate indirectly affects the level of hormones and detoxification of the body (9-11).

The MTHFR gene produces a vital compound called S-adenosine methionine, commonly referred to as SAME. SAME is essential for the production of CoQ10, carnitine, and creatine. Homocysteine is an additional product after the production of SAME. Methyl folate, along with methylcobalamin, helps convert homocysteine to methionine. This cycle will continue as long as the MTHFR gene produces methyl folate during its function (11,12).

In addition, studies have shown that cholesterol metabolism and homeostasis in the brain can contribute to this disease, and considering the association between cholesterol biosynthesis and AD, the regulation of cholesterol homeostasis is of great importance (12-14). So far, many researches have been performed to investigate the role of polymorphism of different MTHFR genes and different results have been obtained (15,16). Remarkably, the findings of genetic risk factors are critical to better explain the pathophysiological functions in the progression of AD. However, such factors are not feasible for any intervention until now. According to this fact, changeable risk factors including hypertension, diabetes, dyslipidemia, and other parameters declared before should be closely investigated to prevent complications favoring cognitive strategy. Importantly, this study aimed to investigate the frequency of single nucleotide polymorphism of the C677T MTHFR gene and its relationship with serum levels of homocysteine, vitamin B12, folate, and cholesterol in Alzheimer’s patients.

Materials and Methods

Study Population

Eighty patients (F/M = 1) with Alzheimer’s and eighty healthy subjects (F/M = 1) participated in this case-control study. The age range of both groups was 40-70 years. A written consent form was taken from all subjects. The protocol of this study was confirmed by the Ethic committee of the Shahrood branch of Islamic Azad University.

Determination of Plasma Homocysteine Levels and Serum Level of Vitamins B12, Folate, and Cholesterol Analysis

A fasting blood sample was taken from all subjects at 8 mL in vials containing EDTA and plasma was prepared for homocysteine assay; all other factors were measured in the serum sample. The plasma and serum samples were immediately separated and kept at -20°C until further analysis. Plasma level of homocysteine was determined via Axis Homocysteine-EIA Kit (Axis-Shield Diagnostic Company, UK). Serum level of VitB12 and folate was assessed by a Chemiluminescence kit (Abbott Company, Diagnostic Division, Ireland). Also, cholesterol was measured by an enzymatic kit.

DNA Extraction and ARMS-PCR

Polymerase chain reaction (PCR) technique was done using a thermocycler PCR set. Relatively, the PCR cycling program was followed. Considerably, after the PCR technique, the PCR product was controlled qualitatively, by using electrophoresis on agarose gel 1%. A High-Pure PCR Template Preparation Kit was used to extract DNA samples (Roche). DNA band analysis was performed by electrophoresis on 1.5% agarose gel. To determine the MTHFR genotype, ARMS-PCR (Allele-specific method) was used. The starting sequence was as follows in Table 1. The PCR program was started at 94°C for 1 minute and then performed with 35 cycles at 93°C for 10 seconds, 64°C for 10 seconds, and 72°C for 20 seconds. Finally, the final expansion was performed at 72°C for 1 minute. PCR products were then separated by 3% agarose gel electrophoresis (W/V). After this step, the Amplification-refractory mutation system (ARMS)-PCR method was used to study the mutation and to perform a homocysteine test, ELISA kit (Axis-Shield Diagnostic Company, UK).

Table 1. PCR Primer Sequence Used in Genotyping

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’ → 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR 677T (F)</td>
<td>GAA GGA GAA GGT TGC GGT AGC</td>
</tr>
<tr>
<td>MTHFR 677T (R)</td>
<td>GAA GGA GAA GGT TGC GGA AGT</td>
</tr>
<tr>
<td>MTHFR 677C (F)</td>
<td>GGA AGC GTG CGT GGA AGT</td>
</tr>
<tr>
<td>MTHFR 677C (R)</td>
<td>CA AAG ACA CTT TCT TCACT</td>
</tr>
</tbody>
</table>
was used to determine cholesterol, vitamin B12 and folate levels, abbot kit (Abbott Company, Diagnostic Division, Ireland) was used by Chemiluminescence method. All steps were performed according to the kit protocol. Also, cholesterol was determined by the enzymatic Kit.

Statistical Analysis
Statistical analysis was performed by Statistical Package for Social Sciences (SPSS) software version 21.0. Kruskal-Wallis test and Mann-Whitney-U test were used for the analysis of clinical and laboratory parameters. The P value of less than 0.05 was considered statistically significant.

Results
Amplification-Refractory Mutation System-Polymerase Chain Reaction
In this study, during the polymerase chain reaction, in addition to replicating the putative fragment, special shear sites at both ends of the direct and reverse primers were added and propagated to the end of the desired fragment in the third reaction cycle. To determine the optimal connection temperature of the primers, a polymerase chain reaction was performed on the temperature slope of 72-55°C.

According to the obtained results, the temperature of 58.1°C is known as the optimal binding temperature of the primers during the polymerase chain reaction. Then, using the above conditions, the main reaction was done to amplify the fragment and the results were analyzed using electrophoresis.

Determination of the Frequency of C677T Single Nucleotide Polymorphism of MTHFR Gene in Alzheimer’s Patients and Control Group, and Their Comparison
In this study, the incidence of C677T mutation in both healthy individuals and patients was investigated. Based on the conclusion of PCR reaction of the MTHFR gene, the incidence rate of mutation in allele I (healthy) is 44.6%, and in allele 2 is 27.9% of all subjects. As shown in Figure 1, the frequency of mutations at the C677T site is higher in patients than in healthy individuals. In addition, the frequency of mutations in healthy alleles is higher in sick women than in healthy individuals and sick men. Also, the frequency of mutation in healthy alleles is higher than that of allele II (mutant) in all subjects.

On the other hand, according to Figure 2, the frequency of heterozygous genotypes in healthy and sick individuals is more than in other genotypes. In addition, the frequency of CT heterozygous genotype was higher in patients than in healthy individuals, but this difference was not statistically significant. The frequency of TT homozygous genotype is higher in healthy individuals than in patients.

Determination of Polymorphism of C677T Mutation Site in MTHFR Gene With Serum Cholesterol Level in Alzheimer’s Patients
Mean serum cholesterol levels in different genotypes of the C677T mutation site in the MTHFR gene were compared separately in men and women. The results showed that the level of cholesterol in individuals with the CC genotype was higher compared to other genotypes in both groups (Table 2).

Determining the Association Between C677T Mutation Polymorphism in the MTHFR Gene and Mean Serum Vitamin B12 Level in Alzheimer’s Patients and Control Group
In general, vitamin B12 levels were lower in patients than in healthy people. In addition, serum levels of vitamin B12 were higher in sick women compared to sick men. Also, serum levels of vitamin B12 in people with CT (heterozygous) genotype were higher than in other genotypes in patients (Table 3).

Determination of Polymorphism of C677T Mutation Site in MTHFR Gene in Comparison With Mean Serum Folate Level in Alzheimer’s Patients and Control Group
Based on the comparison of the mean serum folate level in each genotype, the results show that the folate level in all genotypes of patients, both male and female, did
not change much compared to controls and with 95% confidence, the changes were not significant. However, serum folate levels in the TT genotype of patients showed a decrease of almost 50% compared to the control (Table 4).

Determination of Polymorphism of C677T Mutation Site in MTHFR Gene With Mean Serum Homocysteine Level in Alzheimer’s Patients and Control Group

Based on the results, the level of homocysteine in patients showed a higher rate compared to healthy individuals. Also, in patients with heterozygous genotype (CT), the level of homocysteine is higher than in other genotypes. Based on these results, it can be pointed out that there is a direct relationship between the level of homocysteine and the heterozygous genotype of this mutation site. According to analysis, serum level of heterozygous genotype is significant with 95% confidence compared to healthy individuals (Table 5).

Discussion

The MTHFR gene is found on chromosome 1 at position p36.3, which encodes the enzyme methyl-hydro folate reductase, which functions to help level homocysteine in the human body. C677T polymorphism is one of the most important known polymorphisms in this gene. In C677T, thymine is replaced by cytosine, which converts the amino acid alanine to valine. The MTHFR gene plays a vital role in the uptake of folic acid or any other form of folate by cells. People with a polymorphism in MTHFR called C677T are 40% to 60% incapable of producing methyl folate, a form of vitamin B9 that reduces the risk of AD (17).

In the present study, the PCR technique was used to investigate the polymorphism at site 677. In these studies, it was observed that the frequency of mutation at the C677T site is higher in patients than in healthy individuals, and also that the mutation rate in allele I is higher than that of II (mutant). Mutation rates were higher in sick women than in sick men, also, the frequency of CT genotype was higher in patients than in healthy people. Because the MTHFR gene produces an enzyme that plays a key role in homocysteine metabolism, the conversion of C→T to position 677 causes valine to replace alanine, a highly conserved amino acid. Increasing the amount and types of polymorphisms at this site reduces the function of this enzyme, so a deficiency in this enzyme can be associated with increased levels of homocysteine as well as decreased levels of folic acid and vitamin B12 (18).

Polymorphism has also been demonstrated to be related to increased T-Hcy levels in Alzheimer’s patients (18).

### Table 2. Comparison of Mean Cholesterol Levels With Different Types of C677T Genotypes in Alzheimer’s Patients and Healthy Individuals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Genotype Type</th>
<th>Mean Cholesterol (mg/dl) ± SD in Healthy Individuals</th>
<th>Mean Cholesterol (mg/dL) ± SD in Patients</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women</td>
<td>CC</td>
<td>125.5±17.67</td>
<td>173.14±34.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>152±37.7</td>
<td>163.56±34.46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>140.23±10.82</td>
<td>166.12±26.5</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>CC</td>
<td>163.67±29.26</td>
<td>181.89±23.65</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>141.55±32.82</td>
<td>168.14±37.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>134.42±35.73</td>
<td>138.8±9.85</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>142.89±27.33</td>
<td>165.6±27.78</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

### Table 3. Mean Serum Level of Vitamin B12 in Each of the Genotypes at the C677T Locus of the MTHFR Gene in Alzheimer’s Patients and Healthy Individuals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Genotype Type</th>
<th>Mean B12 (pmol/L) ± SD in Healthy Individuals</th>
<th>Mean B12 (pmol/L) ± SD in Patients</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women</td>
<td>CC</td>
<td>248±93.33</td>
<td>213.57±95.83</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>343.48±89.85</td>
<td>294.36±89.55</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>332.08±41.92</td>
<td>223.62±94.5</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>CC</td>
<td>173±45.8</td>
<td>182.44±78.51</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>242.77±88.78</td>
<td>207.42±67.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>411±46.65</td>
<td>173±79.85</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>291.72±67.72</td>
<td>215.74±84.25</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

### Table 4. Comparison of Mean Folate Levels With C677T Genotypes in Alzheimer’s Patients and Healthy Individuals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Genotype Type</th>
<th>Mean folate (nmol/L) ± SD in Healthy Individuals</th>
<th>Mean folate (nmol/L) ± SD in Patients</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women</td>
<td>CC</td>
<td>16.4±3.81</td>
<td>8.14±5.83</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>10.54±3.95</td>
<td>9.28±6.78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>11.08±5.74</td>
<td>4.75±0.88</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>CC</td>
<td>10.13±2.33</td>
<td>9.22±1.31</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>8.64±4.05</td>
<td>8.56±1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>8.35±5.37</td>
<td>3.6±0.55</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>10.85±25.25</td>
<td>7.26±2.76</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Table 5. Comparison of Mean Homocysteine Levels with Genotypes at the C677T Locus in Alzheimer's Patients and Healthy Individuals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Genotype Type</th>
<th>Mean Homocysteine (µmol/L) ± SD in Healthy Individuals</th>
<th>Mean Homocysteine (µmol/L) ± SD in Patients</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women</td>
<td>CC</td>
<td>10.35±1.9</td>
<td>19.34±6.19</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>12.86±6.04</td>
<td>26.54±9.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>14.59±5.7</td>
<td>21.36±7.88</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>CC</td>
<td>10.56±1.07</td>
<td>24.55±2.31</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>15.34±1.97</td>
<td>23.88±2.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>15.55±4.37</td>
<td>13.26±0.49</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>13.2±3.5</td>
<td>21.4±4.09</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

In addition, the MTHFR gene in the region at 1p36 is a region prone to AD. Thus, in combination with APOE 4, the MTHFR C677T polymorphism may alter Alzheimer's susceptibility in the elderly at elevated T-Hcy levels. Accordingly, the results of the present research, it can be said that polymorphism in position 677 can increase the risk of disease. Although the present study showed that this polymorphism was higher in women with AD, this difference was not statistically significant. The results of previous reports have shown that CT polymorphism has increased in patients compared to healthy individuals and TT homozygosity has been seen more in healthy individuals (19) which is in line with the results of the present study.

Based on the results obtained in the present study and comparing the mean serum cholesterol level in each genotype of the C677T locus mutation in the MTHFR gene, Serum cholesterol levels in patients with CC genotype are higher than other genotypes in patients as well as healthy individuals. Since the study of the relationship between serum cholesterol levels and CC genotype in Alzheimer's patients has not been performed. In a study, Ford et al reported that the risk of cognitive diseases, including Alzheimer's, in the homozygous TT genotype was higher than in the homozygous CC genotype, which did not have a meaningful association between this type of genotype and cognitive diseases such as Alzheimer's (20).

A study that was done by Brunelli et al did not confirm that mutations in MTHFR and gene polymorphism in C677T could be associated with Alzheimer's and increased folate levels because they did not observe significant differences in genotype distribution and allele abundance between Alzheimer's patients and the group (21). A study by Rai showed that there is a direct association between heterozygous CT and homozygous TT mutations compared with wild-type CC alleles with an increased risk of AD (22).

Recent studies, unlike previous studies, seem to emphasize the role of polymorphism in increasing the risk of AD, which is due to changes in the catalytic activity of the enzyme involved in folate metabolism. The results of the present experiments also confirm the results of those studies, because it shows that the serum folate level in patients has decreased compared to the control group, which can indicate a defect in the function of the enzyme due to mutations causing polymorphism. In addition, the results obtained in the study of Liew and Gupta showed that polymorphism of the MTHFR gene at the C677T position decreases serum levels of vitamin B12 and folic acid, and increases serum levels of homocysteine by decreasing these two vitamins; also, they recommended the use of vitamin B12 and folic acid to reduce serum homocysteine levels for the prevention and treatment of many diseases, which could confirm the present results (23).

Examination of MTHFR gene polymorphism in the C677T position showed that the risk of AD increased in individuals with heterozygous CT genotype and TT homozygous genotype compared to CC homozygous genotype. In addition, in individuals with TT genotype, an increase in serum homocysteine levels and a decrease in folate and vitamin B12 were observed simultaneously. These results can be explained by examining the function of the enzyme methylene-tetrahydrofolate reductase and the function of this enzyme in the homozygous TT and heterozygous CT genotypes may be defective. This enzyme cannot convert homocysteine to folate and vitamin B12 well and this defect can, under certain mechanisms, destroy nerve cells and dementia diseases, including Alzheimer's.

However, more extensive studies to evaluate the function of this enzyme and the results of enzyme dysfunction in this disease are needed to confirm these results.

It is suggested that the more specific role of this enzyme in dementia and ADs is further determined by studying the mechanism of action of this enzyme in neurons and studying the role of homocysteine concentration in the formation of amyloid plaques and increased degradation in neurons. Vitamin B12 and folate can be used to treat or prevent the disease by lowering the serum level of homocysteine or increasing its concentration in the diet of patients or people at risk for this disease. Conspicuously, the quality and quantity of extracted nucleic acids of are great importance (24).

Conclusions

In this study, no significant difference was observed between cholesterol levels in men and women, which indicates that the concentration of cholesterol was independent of AD. It was also found that an increase in cholesterol concentration is associated with an increased risk of this disease, but further studies are needed to...
confirm this, and it was also found that this increase is not related to the MTHFR gene polymorphism at the C677T position. It was also shown that the risk of AD increased in individuals with heterozygous CT genotype and homozygous TT genotype compared to homozygous CC genotype by examining the polymorphism of the MTHFR gene at the C677T position; also, in individuals with TT genotype, a decrease in folate and vitamin B12 was observed simultaneously with an increase in serum homocysteine levels.

**Authors’ Contribution**

**Conceptualization:** Ebrahim Mirzajani.

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**Validation:** Ebrahim Mirzajani, Mohammad Taghi Goodarzi, Amir Reza Ghayeghran.

**Formal analysis:** Sanaz Amani.

**Investigation:** Sanaz Amani.

**Resources:** Sanaz Amani.

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**Visualization:** Ebrahim Mirzajani, Mohammad Taghi Goodarzi, Amir Reza Ghayeghran.

**Supervision:** Ebrahim Mirzajani.

**Project administration:** Ebrahim Mirzajani.

**Conflict of Interests**

None declared.

**Ethical Issues**

This study was approved by Islamic Azad University, Shahrood Branch (No. IR.IAU.SHAHROOD.REC.1399.054).

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