The Frequency of the 677C>T and 1298A>C Polymorphisms in the Methyleneetetrahydrofolate Reductase (MTHFR) Gene in the Population

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ABSTRACT

Background: The gene for 5,10-methylenetetrahydrofolate reductase (NAD(P)H) or MTHFR gene encodes protein methylenetetrahydrofolate reductase (MTHFR), an enzyme important in folate metabolism. Aim: The aim of this study was to determine the frequencies of 677C>T and 1298A>C polymorphisms in the MTHFR gene of healthy subjects from the population.

Material and methods: The blood samples were collected from 164 unrelated and healthy donors from population consisted of 98 females and 66 males. Both the MTHFR 677C>T and 1298A>C single nucleotide polymorphisms (SNPs) were analyzed by Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Linkage disequilibrium (LD) between pair of SNPs was calculated through Haploview analysis.

Results: The frequency of MTHFR 677T allele in the population (32.62%) was in agreement with the frequency of this allele in most other populations, however, the frequency of MTHFR 1298C allele (38.41%) was higher than that reported for most other populations in the world. Haploview analysis showed a relatively strong LD between 677C>T and 1298A>C SNPs with D' values of 0.87.

Conclusion: Regarding the two MTHFR polymorphisms, three of the nine combined genotypes were present in 87.2% of the population. 33.54% subjects were complex heterozygous (677CT/1298CC genotype). The subjects with 677CT/1298AA genotype had a relatively strong LD between 677C>T and 1298A>C SNPs with D' values of 0.87.

Keywords: MTHFR gene, SNPs, 677C>T, 1298A>C, polymorphisms, PCR-RFLP.

1. INTRODUCTION

The gene for 5,10-methylenetetrahydrofolate reductase (NAD(P)H) or MTHFR gene (OMIM: 607093) is located on the short (p) arm of chromosome 1 (cytogenetic location: 1p36.22). It ranges from 11,785,729 base pairs (bp) to 11,806,102 bp (GRCh38, NCBI) and its total length is 20,374 bp. It consists of 11 exons. MTHFR gene encodes an enzyme methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) made from 656 amino acids and a molecular weight is 74,597 Da. Methylenetetrahydrofolate reductase is important for a chemical reaction involving forms of the vitamin folate (vitamin B9). Methylenetetrahydrofolate reductase catalyzes the conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MTHF). This reaction is required for the re-methylation of amino acid homocysteine (Hcy) to methionine. Although a number of mutations were described, 677C>T (rs1801133) and 1298A>C (rs1801131) SNPs (single nucleotide polymorphisms) are the two most common mutations in the MTHFR gene. Many of the MTHFR gene polymorphisms alter or decrease the activity of methylenetetrahydrofolate reductase, leading to an increase of homocysteine in the blood. Decreased folate and increased plasma Hcy levels are associated with a variety of common conditions such as cardiovascular disease, neural tube defects, cleft lip/palate, hypertension, preeclampsia, thrombosis, osteoporosis, dementia, Alzheimer’s disease, Down syndrome, certain types of cancer, glaucoma, pregnancy complications, migraine, epilepsy, depression and schizophrenia.

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Polymorphism 677C>T (OMIM: 607093.0003) in the coding region of the MTHFR gene (position: 11,796,321) was described by Frosst (1). The replacement of cytosine with thymine is a point mutation of the transition type in exon 4 of this gene (c.788C>T) which alanine changes to valine (p.Ala222Val or A222V) in the N-terminal catalytic domain of MTHFR protein and is responsible for the synthesis of a thermolabile form of MTHFR with reduced enzymatic activity. 677C>T polymorphism is relatively common and has been studied for a long time.

Another mutation that often occurs in the MTHFR gene 1298A>C (OMIM: 607093.0004) is the first time described by van der Put (2). 1298AC transversion is located in exon 7 (position: 11,794,419) that leads to the replacement of adenine with cytosine (c.1286A>C) resulting in transfer glutamic acid to alanine (p.Glu429Ala or E429A) within the C-terminal regulatory domain of the protein. The 1298AC mutation is associated with decreased MTHFR activity that is more pronounced in the homozygous than heterozygous state.

The frequency of the MTHFR 677C>T and 1298A>C polymorphisms varies in different geographical regions of the world and among different ethnic groups. Although the frequency of MTHFR 677C>T polymorphism has been established (3, 4), MTHFR 1298A>C polymorphism has not been investigated in Bosnia and Herzegovina so far. The aim of this study was to determine the frequency of 677C>T and 1298A>C polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene in peripheral blood samples of healthy subjects from the population using the polymerase chain reaction (PCR) and the restriction fragment length polymorphism (RFLP) method. Subsequently, we compared the obtained frequencies of minor alleles (MAF) with the heterozygous state. The frequency of the MTHFR 677C>T polymorphism is relatively common and has been studied for a long time.

2. MATERIALS AND METHODS

Samples Collection

The blood samples were collected from 164 unrelated and healthy donors from population consisted of 98 females and 66 males between 17 and 79 years of age (Table 1). Blood samples (3 ml) were taken and collected into tubes with EDTA. All individuals who participated in this study belonged to Caucasians from different regions of Bosnia and Herzegovina between 2011 and 2015. All the subjects included in this analysis gave written informed consent to participate in the study. The study was approved by the Ethics Committee of the Faculty of Science on University of Sarajevo (01/01-556/2-2018).

DNA isolation and detection of MTHFR gene mutations

After genomic DNA was extracted from whole peripheral blood according to a previously described method proposed by Miller et al. (5) with several small modifications, the MTHFR 677C>T and 1298A>C polymorphisms were analyzed using the PCR-RFLP method. A fragment of 198 bp containing the polymorphic site in the exon 4 of MTHFR gene was amplified by PCR on a thermal cycler (Eppendorf M Mastercycler gradient, Hamburg, Germany) in 0.2-ml thin-walled tubes using previously described couples of primers for 677C>T: 5’-TGAAGAGAAGGTGCTCAGCAGG -3’ as the forward and 5’-AGGACGGTGCCGCTAGAGT -3’ as the reverse primer (1) while a fragment of 163 bp containing the polymorphic site in the exon 7 of MTHFR gene was amplified using 5’- CTGGGGGAGCTGAAGACACTAC -3’ as the forward and 5’- CACTTTGTGACCATTCGCTTTT -3’ as the reverse primer (2).

PCR amplifications were performed in 25 μl reaction mixture contained 50–100 ng of template DNA, 1x PCR buffer (10x PCR Buffer without MgCl2; Sigma-Aldrich, USA), 1.5 mM of magnesium chloride solution (25 mM MgCl2; Sigma-Aldrich, USA), 0.2 mM of deoxynucleotide (dTTP) solution mix (equimolar solution of 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP; New England BioLabs, USA), 0.2 μM of each primer (Sigma-Aldrich, USA) and 1.25 U of Taq DNA Polymerase (Tag DNA Polymerase; Sigma-Aldrich, USA). An initial denaturation step of 5 minutes at 95 °C was followed by 37 cycles for 30 seconds at 95 °C, 30 seconds at 61 °C and 30 seconds at 72 °C, and a 10-minute elongation step at 72°C at the end of the cycles. The amplification products were electrophoresed in ethidium bromide-stained 2% agarose gels.

After gel electrophoresis, each remaining PCR product was digested with the restriction endonucleases Hinf I and MboII (New England BioLabs, USA), separately. The digestion reactions contained: 10 μl of PCR product (0.1–0.5μg of DNA), 2.5 μl of buffer (10x NEBuffer Cut Smart, supplied with enzyme) 0.5 μl of restriction enzyme (concentration: 10U/μl) and 12 μl of nuclease-free water. Restriction analysis was performed for 4 hours at 37 °C. The digestion products were separated on ethidium bromide-stained 3% agarose gels at 70 V and visualized under ultraviolet light.

Comparisons of SNP frequencies between males and females. Linkage disequilibrium (LD) analysis was performed using the Haploview software version 4.2 (Daly Lab at the Broad Institute, Cambridge, USA).
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quencies between population and other ethnic popula-
tions were done using a chi-square test with significance
level of 0.05 and 95% confidence intervals (95% CI) using
the MedCalc statistical software package version 12.5.0.0
(Ostend, Belgium).

3. RESULTS

Table 1 gives frequencies of genotypes and alleles of the
MTHFR 677C>T polymorphism. The frequencies of 677CC, CT and TT
genotypes among subjects from the population were 40.85%, 53.05% and 6.1%,
respectively resulting in a T allele frequency of 32.62%.
The genotype distribution for males and females was in

Table 2. Distribution of genotypes and allele frequencies of 677C>T MTHFR gene observed in this study compared with those found in other
populations. *Statistically significant.

| Study | Country | Sample size (n) | Distribution of 677C>T MTHFR genotype (n) | HWE Frequency of 677C>T MTHFR alleles | T, n (%) | 95% CI | χ² P |
|-------|---------|----------------|------------------------------------------|--------------------------------------|----------|-------|------|
| Present study | | 164 | 67 87 10 | 0.008* | 107 (32.62) |
| Biseli et al, 2008 (6) | Brazil | 194 | 100 77 17 | 0.69 | 111 (28.61) |
| Boduroglu et al, 2004 (7) | Turkey | 91 | 58 30 3 | 0.71 | 36 (19.78) |
| Balta et al, 2003 (8) | Turkey | 185 | 90 87 8 | 0.02* | 103 (27.84) |
| Basol et al, 2016 (9) | Turkey | 126 | 86 35 5 | 0.55 | 45 (17.86) |
| Thirumaran et al, 2005 (10) | Germany | 1448 | 600 681 167 | 0.21 | 1015 (35.05) |
| Kurzweili, 2010 (11) | Germany | 212 | 96 96 20 | 0.57 | 136 (32.08) |
| Lightfoot et al, 2005 (12) | UK | 755 | 356 316 83 | 0.31 | 482 (31.92) |
| Chango et al, 2005 (13) | France | 119 | 49 58 12 | 0.39 | 39 (34.45) |
| Coppede et al, 2009 (14) | Italy | 113 | 40 55 18 | 0.90 | 91 (40.26) |
| Kokotas et al, 2009 (15) | Denmark | 1084 | 545 449 90 | 0.85 | 629 (29.01) |
| Martinez-Friaz, 2008 (16) | Spain | 188 | 76 85 27 | 0.68 | 139 (36.97) |
| Meguid et al, 2008 (17) | Egypt | 48 | 33 12 3 | 0.21 | 18 (18.75) |
| D’Leary et al, 2002 (18) | Ireland | 192 | 90 84 18 | 0.80 | 120 (31.25) |
| Wang et al, 2008 (19) | China | 70 | 36 29 5 | 0.79 | 39 (27.86) |
| Muthuswamy et al, 2016 (20) | India | 110 | 80 30 0 | 0.09 | 30 (13.64) |
| Jusić-Karić et al, 2016 (21) | BiH | 207 | 91 92 24 | 0.92 | 140 (33.82) |
| Mahmutbegović et al, 2017 (22) | BiH | 154 | 71 74 9 | 0.07 | 92 (29.87) |
| Damnjanovic et al, 2010 (23) | Serbia | 412 | 163 190 59 | 0.76 | 308 (37.38) |
| Alfrevic et al, 2010 (24) | Croatia | 104 | 37 59 8 | 0.02* | 75 (36.06) |
| Petra et al, 2007 (25) | Slovenia | 258 | 112 110 36 | 0.29 | 182 (35.27) |
| Li et al, 2013 (26) | USA | 564 | 236 246 82 | 0.17 | 410 (36.35) |
| Linz et al, 2003 (27) | Australia | 299 | 145 133 21 | 0.19 | 175 (29.26) |

Table 3. Results

Table 1 gives frequencies of genotypes and alleles of the MTHFR 677C>T and 1298A>C polymorphisms. The frequencies of 677CC, CT and TT genotypes among subjects from the population were 40.85%, 53.05% and 6.1%, respectively resulting in a T allele frequency of 32.62%. The genotype distribution for males and females was in
the Hardy-Weinberg equilibrium. The frequencies of 1298AA homozygotes, AC heterozygotes and CC homozygote were 26.83%, 69.51% and 3.66%, respectively. The 1298A>C genotype distribution deviated from the expected Hardy-Weinberg distribution. The overall C allele frequency was 38.41%.

It was found that there was no statistically significant difference in the distribution of genotypes for 677C>T polymorphism between males and females (P = 0.4030). The frequency of 677TT allele was higher in females (34.18%) than in males (30.3%) but this difference was not statistically significant according to chi-square test (P = 0.5385). Similarly, there were no significant differences in the distribution of genotypes (P = 0.3325) and allele frequencies (P = 0.9617) for 1298C allele in the population (32.62%) observed in this study is consistent with results of previous studies (3, 4) as well as with the frequency of allele in most other populations, however, the frequency of MTHFR 1298C allele (38.41%) was much higher (except for one population from Turkey: 43.96%) than that reported for other populations in the world (Brazil, Turkey, France, Italy, Spain, Egypt, India, Sweden, South Korea, Germany, Russia, USA, Australia and UK). These differences were statistically significant (P < 0.05) except for population from Turkey (one sample), Italy (one sample), Egypt, Russia and Australia (Table 3).

### 4. DISCUSSION

MTHFR 677T and 1298C allele frequencies differ between populations. The frequency of MTHFR 677T allele in the population (32.62%) observed in this study is consistent with results of previous studies (3, 4) as well as with the frequency of allele in most other populations, however, the frequency of MTHFR 1298C allele (38.41%) was much higher than frequency of this allele in the most other populations in the world. These differences were statistically significant (P < 0.05). We found that there were no statistically significant differences in the distribution of genotypes and allele frequencies for MTHFR 677C>T and 1298A>C polymorphisms between males and females.

After analysis of combined genotypes for these two polymorphisms we observed that the most frequent subjects (34.15%) were homozygous for 677C allele and heterozygous of the 1298 locus (677CC/1298AC genotype). But, those who were complex heterozygous for 677 and 1298 locus (677CT/1298AC genotype) were 33.54%. Subjects who were heterozygous for the 677 locus and homozygous for 1298C allele (677CT/1298AA genotype) were 19.51%. The subjects with 677TT genotype had a 1298AA (4.27%) or 1298AC genotype (1.83%) while sub-

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**Table 3. Distribution of genotypes and allele frequencies of 1298A>C MTHFR gene observed in this study compared with those found in other populations.** *Statistically significant.*

| Study                  | Country     | Sample size (n) | Distribution of 1298A>C MTHFR genotype (n) | HWE | Frequency of 1298A>C MTHFR alleles |
|------------------------|-------------|-----------------|---------------------------------------------|-----|-----------------------------------|
|                        |             | AA  | AC  | CC  | P    | C, n (%) | 95% CI       | χ²  | P     |
| Present study          |             | 164 | 44  | 114 | 6.00* | 126 (38.41) |               |     |       |
| Biselli et al, 2008    | Brazil      | 194 | 108 | 74  | 12   | 0.89    | 98 (25.26)  | 6.119% | 10.202% | 13.692 | P = 0.0002* |
| Boduroglu et al, 2004  | Turkey      | 91  | 21  | 60  | 10   | 0.00*   | 80 (43.96)  | -3.618% | -14.756% | 1.276  | P = 0.2587   |
| Baso et al, 2016       | Turkey      | 126 | 74  | 48  | 4    | 0.25    | 56 (22.22)  | 8.455%  | 23.609% | 16.605 | P = 0.0001* |
| Nicot et al, 2006      | France      | 198 | 102 | 81  | 15   | 0.84    | 111 (32.83) | 3.301%  | 17.407% | 8.314  | P = 0.0039* |
| Gemmati et al, 2004    | Italy       | 257 | 126 | 110 | 21   | 0.66    | 152 (29.57) | 2.115%  | 15.593% | 6.681  | P = 0.0097* |
| De Re et al, 2010      | Italy       | 96  | 33  | 54  | 9    | 0.05*   | 72 (37.50)  | -0.072% | 9.709%  | 0.0127 | P = 0.9103   |
| Martinez-Friaz, 2008   | Spain       | 188 | 91  | 78  | 19   | 0.70    | 116 (30.85) | 0.322%  | 14.754% | 4.110  | P = 0.0426* |
| Meguid et al, 2008     | Egypt       | 48  | 18  | 29  | 1    | 0.00*   | 31 (32.29)  | -5.472% | 16.829% | 0.945  | P = 0.3310   |
| Muthuswamy et al, 2016 | India       | 110 | 53  | 50  | 7    | 0.28    | 64 (29.09)  | 0.952%  | 17.394% | 4.647  | P = 0.0311* |
| Berglund et al, 2009   | Sweden      | 449 | 214 | 196 | 39   | 0.53    | 274 (30.51) | 1.754%  | 14.166% | 6.467  | P = 0.0110* |
| Kim et al, 2005        | South Korea | 445 | 308 | 129 | 8    | 0.19    | 145 (16.29) | 16.228% | -28.109% | 66.523 | P = 0.0039* |
| Kurzwelly, 2010        | Germany     | 212 | 106 | 89  | 17   | 0.78    | 123 (16.29) | 2.407%  | 16.369% | 6.960  | P = 0.0083* |
| Weiner, 2011           | Russia      | 503 | 232 | 215 | 56   | 0.56    | 327 (32.50) | -0.168% | -12.124% | 3.594  | P = 0.0580   |
| Li et al, 2013         | USA         | 574 | 265 | 250 | 59   | 0.99    | 368 (32.06) | 0.371%  | -12.476% | 4.339  | P = 0.0373* |
| Lincz et al, 2003      | Australia   | 294 | 124 | 139 | 31   | 0.38    | 201 (34.18) | -2.397% | -10.934% | 1.462  | P = 0.2266   |
| Lightfoot et al, 2005  | UK          | 755 | 347 | 331 | 77   | 0.88    | 485 (32.12) | 0.472%  | -12.273% | 4.525  | P = 0.0334* |
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jects with 1298CC genotype had only 677CC genotype (3.66%). These genotypes represent 12.8%. Just three subjects had a triple 677TT/1298AC genotype, probably as result of recombination between the two ancestor genotypes (most likely 677CT/1298AC genotype). These results suggest that 677T allele is linked with the A allele of 1298 locus and 1298C allele is associated with C allele of locus 677. Only 3.05% subjects were heterozygous for both MTHFR wild-type alleles (677CC/1298AA genotype). The triple 677CT/1298CC and quadruple 677TT/1298CC mutation has not been found suggesting decreased viability of embryos with increased numbers of mutant alleles.

Combined genotypes which contain three or four mutant alleles were not detected or detected several implying linkage disequilibrium between two polymorphisms. The pattern of LD in the MTHFR gene showed a relatively strong LD between 677C>T and 1298A>C SNPs with \( D' \) values of 0.87 and the correlation \( r^2 \) of 0.23. The examples of LD observed in natural populations are the result of a complex interaction between genetic factors and the demographic history of the population. Particularly, recombination shows a significant role in determining the patterns of LD in a population.

5. CONCLUSIONS

The frequency of MTHFR 677T allele in the population (32.62%) was in agreement with the frequency of this allele in most other populations, however, the frequency of MTHFR 1298C allele (38.41%) was higher than frequency of this allele in the most other populations of the world.

- Conflict of interest statement: The authors declare no conflict of interest.

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