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The Frequency of the 677C>T and 1298A>C Polymorphisms in the Methylenetetrahydrofolate 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/1298AC* genotype), 34.15% subjects had *677CC/1298AC* and 19.51% of *677CT/1298AA* genotype. The subjects with *677TT* genotype had a *1298AA* or *1298AC* genotype while subjects with *1298CC* genotype had only *677CC* genotype. The subjects with *677CC/1298AA* genotype were only 3.05%. We were not found triple *677CT/1298CC* and quadruple *677TT/1298CC* mutation suggesting decreased viability of embryos with increased numbers of mutant alleles.

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.

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 methylenethetrahydrofolate 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 frequencies of these polymorphisms in some other populations of the world.

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 Mastercycler gradient, Hamburg, Germany) in 0.2-ml thin-walled tubes using previously described couples of primers for *677C>T*: 5'-TGAAGGAGAAGGTGTCTGCGGGA -3' as the forward and 5'-AGGACGGTGCGGTGAGAGTG -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'- CTTTGGGGAGCTGAAGGACTACTAC -3' as the forward and 5'- CACTTTGTGACCATTCCGGTTTG -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 $MgCl_2$; Sigma-Aldrich, USA), 1.5 mM of magnesium chloride solution (25 mM $MgCl_2$; Sigma-Aldrich, USA), 0.2 mM of deoxynucleotide (dNTP) solution mix (equimolar solution of 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM TTP; New England BioLabs, USA), 0.2 µM of each primer (Sigma-Aldrich, USA) and 1.25 U of *Taq* DNA Polymerase (*Taq* 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.

Regarding the *677C>T* SNP, since mutation creates a *Hinf I* restriction site (5'- G|ANTC -3'), the amplicon of the wild-type allele (198 bp) is not cut, while the mutant one is cut into two fragments of 175 and 23 bp respectively. In agarose gel electrophoresis it is possible to see the 198 and 175 bp fragments, the 23-bp fragment is not retained in the gel. Nevertheless, concerning the *1298A>C* SNP, since mutation abolishes a restriction site for *MboII* enzyme (5'- GAAGAN8 -3'), the amplicon (163 bp) of the mutated allele will be cut into four (84, 31, 30 and 18 pb) instead of five fragments (56, 31, 30, 28 and 18 bp) as a wild type allele. In agarose gel electrophoresis it is possible to see the 84 and 56 bp fragments and fragments of 30, 31 and 28 bp as one band.

Statistical Analysis

Allelic and genotypic frequencies were calculated by the gene counting method. The chi-square (χ^2) test was used to evaluate the Hardy-Weinberg equilibrium for the distribution of genotypes of subjects and to determine differences in the distribution of genotypes and allele 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). Comparisons of SNP fre-

		MTHFR 677C>T polymorphism					MTHFR 1298A>C polymorphism			
	Genotype/ Allele	Total n (%)	Male n (%)	Female n (%)	χ2 test	Genotype/ Allele	Total n (%)	Male n (%)	Female n (%)	χ2 test
Sample size		164 (100)	66 (100)	98 (100)			164 (100)	66 (100)	98 (100)	
Age (mean ± SD)		34.85±14.58	34.59±15.10	35.03±14.30			34.85±14.58	34.59±15.10	35.03±14.30	
Genotype frequency	CC	67 (40.85)	28 (42.42)	39 (39.8)	χ2 = 1.817 P = 0.4030	AA	44 (26.83)	19 (28.79)	25 (25.51)	χ2 = 2.202 P = 0.3325
	CT	87 (53.05)	36 (54.55)	51 (52.04)		AC	114 (69.51)	43 (65.15)	71 (72.45)	
	TT	10 (6.1)	2 (3.03)	8 (8.16)		CC	6 (3.66)	4 (6.06)	2 (2.04)	
HWE	χ2	7.01022	0.08635	2.40155		χ2	36.0918	9.23188	27.88707	
	P value	0.00810*	0.76885	0.12121		P value	0.00000*	0.00237*	0.00000*	
Allele frequency	C	221 (67.38)	92 (69.7)	129 (65.82)	χ2 = 0.378 P = 0.5385	A	202 (61.59)	81 (61.36)	121 (61.73)	χ2 = 0.00230 P = 0.9617
	T	107 (32.62)	40 (30.3)	67 (34.18)		C	126 (38.41)	51 (38.64)	75 (38.27)	
	Total	328 (100)	132 (100)	196 (100)		Total	328 (100)	132 (100)	196 (100)	

Table 1. Genotype and allele frequencies of MTHFR gene polymorphisms in the population. *Statistically significant.

		Sample size (n)	Distribution of 677C>T MTHFR genotype (n)			HWE	Frequency of 677C>T MTHFR alleles			
Study	Country		CC	CT	TT	P	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)	-2.941% - 10.98%	1.167	P = 0.2800
Boduroglu et al, 2004 (7)	Turkey	91	58	30	3	0.71	36 (19.78)	4.58% - 20.542%	8.939	P = 0.0028*
Balta et al, 2003 (8)	Turkey	185	90	87	8	0.02*	103 (27.84)	-2.233% - 11.789%	1.668	P = 0.1965
Basol et al, 2016 (9)	Turkey	126	86	35	5	0.55	45 (17.86)	7.442% - 21.779%	15.300	P = 0.0001*
Thirumaran et al, 2005 (10)	Germany	1448	600	681	167	0.21	1015 (35.05)	-3.211% - 7.78%	0.663	P = 0.4154
Kurzwelly, 2010 (11)	Germany	212	96	96	20	0.57	136 (32.08)	-6.341% - 7.494%	0.00615	P = 0.9375
Lightfoot et al, 2005 (12)	UK	755	356	316	83	0.31	482 (31.92)	-4.898% - 6.557%	0.0327	P = 0.8564
Chango et al, 2005 (13)	France	119	49	58	12	0.39	82 (34.45)	-6.234% - 9.991%	0.134	P = 0.7148
Coppede et al, 2009 (14)	Italy	113	40	55	18	0.90	91 (40.26)	-0.75% - 16.036%	3.076	P = 0.0794
Kokotas et al, 2009 (15)	Denmark	1084	545	449	90	0.85	629 (29.01)	-1.806% - 9.304%	1.616	P = 0.2036
Martinez-Friaz, 2008 (16)	Spain	188	76	85	27	0.68	139 (36.97)	-2.912% - 11.526%	1.273	P = 0.2592
Meguid et al, 2008 (17)	Egypt	48	33	12	3	0.21	18 (18.75)	3.328% - 22.884%	6.221	P = 0.0126*
O'Leary et al, 2002 (18)	Ireland	192	90	84	18	0.80	120 (31.25)	-5.664% - 8.442%	0.0963	P = 0.7563
Wang et al, 2008 (19)	China	70	36	29	5	0.79	39 (27.86)	-4.871% - 13.771%	0.826	P = 0.3635
Muthuswamy et al, 2016 (20)	India	110	80	30	0	0.09	30 (13.64)	11.693% - 25.821%	24.297	P < 0.0001*
Jusić-Karić et al, 2016 (3)	BiH	207	91	92	24	0.92	140 (33.82)	-5.834% - 8.153%	0.0708	P = 0.7902
Mahmutbegović et al, 2017 (4)	BiH	154	71	74	9	0.07	92 (29.87)	-4.679% - 10.124%	0.438	P = 0.5080
Damnjanovic et al, 2010 (21)	Serbia	412	163	190	59	0.76	308 (37.38)	-1.547% - 10.849%	2.105	P = 0.1469
Alfirevic et al. 2010 (22)	Croatia	104	37	59	8	0.02*	75 (36.06)	-5.008% - 12.013%	0.527	P = 0.4679
Petra et al, 2007 (23)	Slovenia	258	112	110	36	0.29	182 (35.27)	-4.118% - 9.278%	0.513	P = 0.4737
Li et al, 2013 (24)	USA	564	236	246	82	0.17	410 (36.35)	-2.329% - 9.544%	1.385	P = 0.2392
Lincz et al, 2003 (25)	Australia	299	145	133	21	0.19	175 (29.26)	-2.975% - 9.833%	0.976	P = 0.3232

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.

quencies between population and other ethnic populations 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* 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

		Sample size (n)	Distribution of 1298A>C MTHFR genotype (n)			HWE	Frequency of 1298A>C MTHFR alleles			
Study	Country		AA	AC	CC	P	C, n (%)	95% CI	χ2	P
Present study		164	44	114	6	0.00*	126 (38.41)			
Biseli et al, 2008 (6)	Brazil	194	108	74	12	0.89	98 (25.26)	6.119% - 20.102%	13.692	P = 0.0002*
Boduroglu et al, 2004 (7)	Turkey	91	21	60	10	0.00*	80 (43.96)	-3.618% - 14.756%	1.276	P = 0.2587
Basol et al, 2016 (9)	Turkey	126	74	48	4	0.25	56 (22.22)	8.455% - 23.609%	16.605	P < 0.0001*
Niclot et al, 2006 (26)	France	198	102	81	15	0.84	111 (28.03)	3.301% - 17.407%	8.314	P = 0.0039*
Gemmati et al, 2004 (27)	Italy	257	126	110	21	0.66	152 (29.57)	2.115% - 15.593%	6.681	P = 0.0097*
De Re et al, 2010 (28)	Italy	96	33	54	9	0.05*	72 (37.50)	-8.072% - 9.709%	0.0127	P = 0.9103
Martinez-Friaz, 2008 (16)	Spain	188	91	78	19	0.70	116 (30.85)	0.322% - 14.754%	4.110	P = 0.0426*
Meguid et al, 2008 (17)	Egypt	48	18	29	1	0.00*	31 (32.29)	-5.472% - 16.829%	0.945	P = 0.3310
Muthuswamy et al, 2016 (20)	India	110	53	50	7	0.28	64 (29.09)	0.952% - 17.394%	4.647	P = 0.0311*
Berglund et al, 2009 (29)	Sweden	449	214	196	39	0.53	274 (30.51)	1.754% - 14.166%	6.467	P = 0.0110*
Kim et al, 2005 (30)	South Korea	445	308	129	8	0.19	145 (16.29)	16.228% - 28.109%	66.523	P < 0.0001*
Kurzwelly, 2010 (11)	German y	212	106	89	17	0.78	123 (29.01)	2.407% - 16.369%	6.960	P = 0.0083*
Weiner, 2011 (31)	Russia	503	232	215	56	0.56	327 (32.50)	-0.168% - 12.124%	3.594	P = 0.0580
Li et al, 2013 (24)	USA	574	265	250	59	0.99	368 (32.06)	0.371% - 12.476%	4.339	P = 0.0373*
Lincz et al, 2003 (25)	Australia	294	124	139	31	0.38	201 (34.18)	-2.397% - 10.934%	1.462	P = 0.2266
Lightfoot et al, 2005 (12)	UK	755	347	331	77	0.88	485 (32.12)	0.472% - 12.273%	4.525	P = 0.0334*

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.

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 *677T* 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 *1298A>C* polymorphism between males and females (Table 1).

Regarding the two common *MTHFR* polymorphisms, three of the nine combined genotypes were present in 87.2% of the population. Fifty five subjects (33.54%) were heterozygous for both *MTHFR* polymorphisms (*677CT/1298AC* genotype). We detected 56 individuals (34.15%) with the *677CC/1298AC* genotype and 32 (19.51%) with the *677CT/1298AA* genotype. The combined genotypes *677TT/1298AA* (4.27%), *677CC/1298CC* (3.66%) and *677CC/1298AA* (3.05%) as well as the triple mutation *677TT/1298AC* (1.83%) represent total 12.8%. The subjects with triple *677CT/1298CC* and quadruple *677TT/1298CC* mutations were not found.

Haploview analysis showed a relatively strong LD between *MTHFR 677C>T* and *1298A>C* SNPs with *D'* values of 0.87 and the correlation r^2 of 0.23.

The frequency of *MTHFR 677T* allele in the population (32.62%) was in agreement with the frequency of this allele in most other populations in the world (Brazil, Turkey, Germany, UK, France, Italy, Denmark, Spain, Ireland, China, Bosnia and Herzegovina, Serbia, Croatia, Slovenia, USA and Australia) but significantly different from the frequency of *677T* allele in the population of Turkey for two samples (P = 0.0028 and P = 0.0001), the samples from Egypt (P = 0.0126) and India (P <0.0001) (Table 2). However, the frequency of *MTHFR 1298C* allele in the population (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 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-

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 suggestion 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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