SHOULD MTHFR 1298 A>C BE TESTED TOGETHER WITH MTHFR 677 C>T POLYMORPHISM IN WOMEN WITH REPRODUCTIVE CHALLENGES?

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Methylenetetrahydrofolate reductase (MTHFR) plays a critical role in the folate metabolism. The polymorphism 677C>T of the MTHFR gene, producing thermolabile enzyme with decreased function, is widely studied and associated with many conditions. Additionally, it has been shown that another polymorphism, 1298A>C, also reduces the activity of this enzyme, although to a lesser extent. The aim of this study is to evaluate the clinical informativeness of testing both MTHFR polymorphisms. Genomic DNA, were extracted from peripheral blood of 180 female patients with pregnancy complications and 183 healthy female controls, and genotyped for MTHFR 677C>T and 1298A>C loci, using TaqMan assays. Our study found similar frequency of alleles and genotypes between two groups. Based on MTHFR 677C>T genotype, 11.7% of patients homozygous for this mutation were under the possible risk. When the position 1298 was included in the testing, 22.8% of the patients were heterozygous for both polymorphisms. Additionally, 8.9% of the patients were homozygous only for the MTHFR 1298 mutation. Although, there was no differences compared to healthy control (p>0.05), 43% of patients were found to have elevated risk which is about four times higher than results with only MTHFR 677C>T genotyping. After obtaining information for the 677 position, testing for the second polymorphism (1298A>C) should be...
considered, since we have shown that it dramatically increases the rate of detection of patients who are potentially at risk for MTHFR associated conditions.

Keywords: MTHFR polymorphisms, 677C>T and 1298A>C, testing together, pregnancy

INTRODUCTION

Methylenetetrahydrofolate reductase (MTHFR) plays a critical role in the folate dependent remethylation of homocysteine. In 1988, Kang et al. (KANG et al. 1988) described a thermolabile variant of MTHFR, associated with decreased enzyme activity and mildly elevated plasma homocysteine levels. The responsible mutation in the MTHFR gene, 677C>T, was identified by Frost et al. in 1995 (FROSST et al. 1995). High prevalence of 677C>T polymorphism in whites and Japanese (but not in Africans), precipitated the need for more intense studies of biochemical implications of this MTHFR gene variant (ROSENBERG et al. 2002). It is also has been observed the north-to-south increase of the prevalence of 677T allele in Europe (BOTTO and YAN, 2000). This kind of distribution may be influenced by the higher folic acid content in the food of Mediterranean populations, compared with northern European populations. The low frequency among the Africans could also be related to nutritional folate deficiency. MTHFR enzyme activity in individuals heterozygous or homozygous for this mutation was 72% or 39% of the homozygous wild type form, respectively (CHRISTENSEN et al. 1997). In the meta-analysis of data, performed by Brattstrom et al. (BRATTSTROM et al. 1998), it has been shown that MTHFR 677TT genotype had plasma homocysteine concentrations 25% higher than those with 677CC genotype, and could cause the mild hyperhomocysteinemia.

A second common polymorphism in MTHFR gene, 1298A>C, results in the replacement of the glutamate with the alanine at the position 429. An in vitro study (WEISBERG et al. 2001) revealed that this polymorphism codes for the enzyme form with the decreased activity but, in contrast to the 677C>T, not rendering its thermolability. The variant enzymes, expressed in bacteria from constructs made from cDNAs with 677T or 1298C mutation, retained only 45% and 68%, respectively, of the normal enzyme activity. Moreover, enzymes with both mutations (677T and 1298C) were even less active (41% from base activity). These in vitro results were similar to the earlier published measurements from lymphocyte extracts (WEISBERG et al. 1998, VAN DER PUT et al. 1998). However, this haploid system cannot fully mimic the in vivo condition, as it was shown that, in its functional form, this enzyme is a homodimer (OGINO and WILSON, 2003, ULVIK et al. 2007). Different combinations of peptides with single mutated amino acids could possibly render different enzyme activities.

The focus of many studies since 1995 has been the association between the 677 C>T polymorphism, plasma homocysteine concentrations, and various clinical conditions: cardiovascular diseases-CVD (FROSST et al. 1995, CHRISTENSEN et al. 1997, SAFFARI et al. 2013, HANSON et al. 2001), vein thrombosis (DJORDJEVIC et al. 2011), and neural tube closure defects (VAN DER PUT et al. 1998, BEAUDIN and STOVER, 2009). More recently, the findings that individuals homozygous for 677TT exhibit lower levels of genomic DNA methylation under low folate status (FRISO et al. 2012) and that global DNA hypomethylation is observed in early tumorogenesis (JONES 2005), suggested that the 677 C>T mutation could also be connected with the development of cancer (ARAUJO et al. 2015, AL-MOTASSEM et al. 2015).

Recognized as an important predictive genetic parameter in women with different complications during pregnancy (fetal loss, intrauterine growth restriction, infertility,
unsuccessful IVF procedure), the polymorphism 677 C>T in *MTHFR* gene is widely tested in maternity clinics (KOVAC et al. 2010, SAFDARIAN et al. 2014, CORIU et al. 2014, TIWARI et al. 2015). However, the second important locus, 1298A>C, is still not routinely tested in many laboratories. The aim of this study was to estimate the percentage of the patients which could be considered as being at increased risk for adverse effects of possibly disturbed folate pathway, when both loci are tested together.

**MATERIALS AND METHODS**

This study included 180 female patients (mean age 38.5±5.7 years) with various pregnancy complications (infertility, fetal loss, and unsuccessful *in vitro* fertilizations) and 183 healthy fertile controls. The peripheral blood was collected in EDTA tube and DNA was isolated with commercial kit for DNA isolation (Qiagen, GmbH, Hilden, Germany).

To analyze *MTHFR* polymorphisms, 677 C>T (rs1801133) and 1298 A>C (rs1801131), we used Applied Biosystems’ validated TaqMan SNP Genotyping Assays in ViiA7 Real Time PCR system (ABI, Foster City, CA, USA).

Allelic frequencies were calculated by the gene counting method. In order to test for Hardy-Weinberg equilibrium, expected frequencies were compared to the observed ones, using the Pearson Chi-square test. To compare the genotypes and allele frequencies between patient and control group we used SPSS software (version 17; SPSS Inc., Chicago, IL). The Pearson Chi-square test was used to determine the statistically significant differences in frequency distribution of *MTHFR* alleles and genotypes between cases and controls and results with p values less than 0.05 were accepted as statistically significant. Logistic regression were used to calculate the OR and 95%CI interval among estimated genotypes, where 677CC/1298AA genotype was considered as reference.

**RESULTS**

Genotypic and allelic frequencies of each polymorphism in *MTHFR* gene in tested subjects (Table 1) were in accord with the Hardy-Weinberg expectations for genetic equilibrium (p>0.05).

<table>
<thead>
<tr>
<th>MTHFR SNP</th>
<th>Genotype</th>
<th>n (%) of participants</th>
<th>Allele</th>
<th>Allele frequency n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>677 C&gt;T</td>
<td>CC</td>
<td>73 (40.5)</td>
<td>80 (43.7)</td>
<td>C 232 (64) T 243 (66)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>86 (47.8)</td>
<td>83 (45.4)</td>
<td>T 128 (36)</td>
</tr>
<tr>
<td></td>
<td>TT*</td>
<td>21 (11.7)</td>
<td>20 (10.9)</td>
<td></td>
</tr>
<tr>
<td>1298 A&gt;C</td>
<td>AA</td>
<td>82 (45.5)</td>
<td>83 (45.4)</td>
<td>A 246 (68)</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>82 (45.5)</td>
<td>72 (39.3)</td>
<td>T 114 (32)</td>
</tr>
<tr>
<td></td>
<td>CC*</td>
<td>16 (8.9)</td>
<td>28 (15.3)</td>
<td></td>
</tr>
</tbody>
</table>

*high risk genotype*
When *MTHFR* 677C>T polymorphism was genotyped, 73 patients (40.5%) had only wild type variant, 86 (47.8%) of them were heterozygous, and remaining 21 (11.7%) patients were homozygous for high risk allele. The frequency of the high risk 677T allele was 36%, as shown in Table 1. For the control group we found 80 samples (43.7%) as wild type, 83 (45.4) of them as heterozygous and 20 (10.9%) as homozygous for the mutation. The frequency of high risk 677T allele was 34% as shown in Table 1. We found a higher frequency of high risk allele in patients compared to controls, but it’s not reach the statistically significance (p>0.05).

For the *MTHFR* 1298A>C polymorphism, 82 (45.5%) patients had two wild type alleles, 82 (45.5%) were heterozygous and 16 (8.9%) homozygous for high risk allele. The frequency of high risk allele for the patients group was 32% (Table 1). For the control group, the genotype distribution was: 83 (45.4%) wild type, 72 (39.3) heterozygous and 28 (15.3) homozygous mutation as it is shown in Table 1. The allele frequency was 35% for the high risk 1298C allele, which was not statistically different compared to study group (p>0.05).

Table 2 shows the frequency of the nine combinations of genotypes for two tested positions (677/1298). The most common genotypes in patients group were CT/AA (25%), CC/AC (22.8%) and CT/AC (22.8%). The genotype arising from two copies of the most common allele CC/AA was less frequent (8.9%), as well as CC/CC (8.9%) and TT/AA (11.7%) genotype. For the control group, the both loci heterozygous genotype CT/AC (23.5%) was similar as in patients group, as well as CT/AA (21.9%) and TT/AA (10.9%). The genotype with all wild type allele was more frequent than in patients group CC/AA (12.6%), as well as CC/CC (15.3%), while the CC/AC (15.8%) genotype was less frequent than in patients group, but not statistically different (p>0.05). No tested subjects in both group had 3 or 4 high risk alleles (CT/CC, TT/AC or TT/CC combination), suggesting that two high risk alleles cannot coexist on the same gene copy (i.e., cannot be in the *cis* position). The binary logistic regression analysis was performed and 677CC/1298AA genotype was taken as referent for calculating odds ratio (OR) and 95% confidence interval (CI). No one of the genotype showed increased or decreased risk for pregnancy complication (p>0.05) (Table 2).

Table 2 Different genotypes of *MTHFR* 677C>T/1298A>C in female patients with pregnancy complication and healthy control

<table>
<thead>
<tr>
<th>MTHFR 677C&gt;T/1298A&gt;C</th>
<th>Patients n (%)</th>
<th>Controls n (%)</th>
<th>OR</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC/AA</td>
<td>16 (8.9)</td>
<td>23 (12.6)</td>
<td>1</td>
<td>/</td>
<td>Ref.</td>
</tr>
<tr>
<td>CC/AC</td>
<td>41 (22.9)</td>
<td>29 (15.8)</td>
<td>2.032</td>
<td>0.917-4.5041</td>
<td>0.081</td>
</tr>
<tr>
<td>CC/CC*</td>
<td>16 (8.9)</td>
<td>28 (15.3)</td>
<td>0.821</td>
<td>0.339-1.992</td>
<td>0.663</td>
</tr>
<tr>
<td>CT/AA</td>
<td>45 (25)</td>
<td>40 (21.9)</td>
<td>1.617</td>
<td>0.751-3.483</td>
<td>0.219</td>
</tr>
<tr>
<td>CT/AC*</td>
<td>41 (22.8)</td>
<td>43 (23.5)</td>
<td>1.371</td>
<td>0.636-2.955</td>
<td>0.421</td>
</tr>
<tr>
<td>CT/CC</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>/</td>
<td>NC</td>
<td>/</td>
</tr>
<tr>
<td>TT/AA*</td>
<td>21 (11.7)</td>
<td>20 (10.9)</td>
<td>1.509</td>
<td>0.623-3.655</td>
<td>0.362</td>
</tr>
<tr>
<td>TT/AC</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>/</td>
<td>NC</td>
<td>/</td>
</tr>
<tr>
<td>TT/CC</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>/</td>
<td>NC</td>
<td>/</td>
</tr>
</tbody>
</table>

*high risk genotype; Ref-reference genotype for logistic regression analysis; NC-not calculated
DISCUSSION

The MTHFR 677 C>T polymorphism is widely studied for possible association with various clinical conditions. Some studies have shown an association between MTHFR 677TT genotype and various CVD (Klerk et al. 2002), supporting the hypothesis that moderate hyperhomocysteinemia is a causal factor for CVD. However, other reports failed to confirm such an association neither with CVD nor specifically with coronary artery disease, in spite of the higher plasma homocysteine level in patients with 677T high risk alleles (Saffari et al. 2013). Earlier studies in Serbia didn’t find positive association between MTHFR 677 locus and vein thrombosis, fetal loss or chronic myeloid leukemia (Djordjevic et al. 2011, Kovac et al. 2010, Jakovljevic et al. 2012).

Prevalence of inherited thrombophilia has also been studied in women with different complications during pregnancy and patients with neural tube defect (NTD). Homozygous MTHFR 677TT mutations and Factor V Leiden mutation were significantly more prevalent in women with unsuccessful IVF procedure, compared to control women (Safdarian et al. 2014). It is also has been shown that MTHFR 677 mutation is a risk factor for preterm delivery, fetal death and low birth weight (Tiwari et al. 2015). Some studies that have tested both loci together find that MTHFR 677 and 1298 polymorphisms are associated with recurrent pregnancy loss and miscarriage (Parveen et al. 2013, Yang et al. 2016). However, it is still unclear if women homozygous for 677 mutation should be offered the anticoagulant therapy during pregnancy, or not, although it has been shown that, in these individuals, folate supplementation has resulted in lower homocysteine levels. Pregnant women with inherited thrombophilia require an individualized risk assessment, considering particular thrombophilic defects, together with acquired and circumstantial risk factors such as age, antiphospholipid antibodies, cancer, immobility, surgery, obesity and travel (Varga and Kujoovich, 2012).

In 1998, van der Put et al., (Van der Put et al. 1998) have been studied the joint effect of two common mutations in MTHFR gene and showed that the simultaneous presence of 677 C>T and 1298 A>C in MTHFR gene leads to an even more reduced MTHFR specific activity, higher plasma homocysteine levels, and decreased plasma folate levels, compared to a single mutation carriers, making the 1298 C variant an additional genetic risk factor for NTD. Moreover, thrombophilia inherited through combined heterozygosity of two polymorphic loci in MTHFR, may cause intrauterine growth restriction (Coriu et al. 2014). The pregnancy outcome therefore depends not only on dietary folate supplementation, but also on the combination of maternal MTHFR activity, affecting the quantity and form of folate delivered to the fetus, and fetal MTHFR activity, which subsequently affects the utilization and distribution of the supplied folate (Donnelly and Isotalo, 2001).

Recent studies show that the 677 and 1298 polymorphisms could be a risk factor for different type of cancer (Araujo et al. 2015, Al-Motassem et al. 2015), and could also influence the radiotherapy treatments in patients with head and neck squamous cell carcinoma, presumably through the role of MTHFR in DNA methylation (Friso et al. 2012, Jones 2015, Anders et al. 2015).

It was postulated (Ogino and Wilson, 2003, Ulvik et al. 2007) that the two polymorphisms are usually in trans configuration, and because of the small distance that separates them on the chromosome, crossover rarely occurs. Indeed, many studies, cited in Ogino and Wilson (Ogino and Wilson, 2003), failed to find any individual with more than two functional mutations at MTHFR loci.
On the other hand, 677CT/1298CC and 677TT/1298CC genotypes (i.e., genotypes with three or four mutations) were observed in aborted fetal material, although no such genotypes were found in the neonatal group (ISOTALO et al. 2000). This could demonstrate the potential role of these genotypes in compromised fetal viability. However, the same study identified carriers of the 677TT/1298AC genotype, although at a very low frequency, in both neonatal and aborted fetal group. This finding suggested that MTHFR high risk variants could occur in cis configurations. Also, Weisberg et al. (WEISBERG et al. 1998) identified one child with an NTD who had an MTHFR 677TT/1298AC genotype, supporting the possibility of cis configurations of MTHFR high risk variants. Ogino and Wilson (OGINO and WILSON, 2003) in their meta-analysis suggested that extremely rare findings of individuals with CT/CC, TT/AC or TT/CC genotype in all tested populations could even be explained as a rare misgenotyping due to allele dropout error (BLAIS et al. 2015). Van der Put et al. (VAN DER PUT et al. 1998) speculated that, if cis configuration of MTHFR mutations occurs, either through de novo mutation, or through recombination, it would result in a selective disadvantage because of the expression of severe phenotypes, NTDs and spontaneous abortions. Polymorphisms at 677 and 1298 loci give three common haplotypes (C-A, C-C and T-A) and one rare (T-C) haplotype. Ulvik et al. (ULVIK et al. 2007) describe the enzyme as a homodimer that mainly exist in six different configurations arising from the three common alleles. The rare genotypes 677TT/1298CC, 677TC/1298CC and 677TT/1298AC could produce the deleterious or lethal enzyme dimer configuration.

Similar to other investigators (VAN DER PUT et al. 1998, HANSON et al. 2001) our study also found no individual homozygous at both loci for high risk allele (677TT/1298CC), or any genotype with the combination of three or more high risk alleles. Moreover, in more than 400 additional subjects from Serbia, tested so far in our laboratory (data not shown), such genotypes were not found.

Although the association between the homozygous 677TT mutation and consequently decreased enzyme activity and increased homocysteine level has been quite well established, the variability in the clinical effect of this mutation, and the lack of association between two groups could be the extrinsic and intrinsic factors (folate level being the most important). Despite the controversial data in the literature about association of MTHFR polymorphism with various conditions, these tests nevertheless entered in daily clinical and diagnostic practice, providing important information to physicians about the possible disturbance of the folate dependent pathways. Namely, when only MTHFR 677 polymorphism was typed, around 11.7% of patients under the possible higher risk for thrombosis and folate depended malformation during pregnancy could be identified. However, when we included the second polymorphism of MTHFR, 1298A>C, the percentage of patients with possible higher risk was increased dramatically to 43.4%. Although statistical significance is absent in frequency of these genotypes in relation to control, contribution of these genotypes to hyperhomocysteinemia in every patient should be verify and examine by adequate biochemical test. On the basis of this results, we suggest that 1298 A>C polymorphism should be tested after obtaining the results for 677 C>T genotype. If the MTHFR 677 genotype is homozygous for the mutation, there is no need for testing the other locus because of very rare cis configuration and no additional clinical informativeness. On the other hand, if the 677 genotype is wild type or heterozygous, testing the 1298 locus can be very important to detect the patients under the possible risk for MTHFR associated conditions.
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DA LI MTHFR 1298 A>C POLIMORFIZAM TREBA TESTIRATI ZAJEDNO SA 677 C>T KOD ŽENA SA REPRODUKTIVNIM IZAZOVOM

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Izvod

Methylenetetrahydrofolat reduktaza (MTHFR) ima ključnu ulogu u folatnom metabolizmu. Polimorfizam 677 C>T u MTHFR genu, koji dovodi do stvaranja termolabilnog enzima sa smanjenom aktivnošću se danas ispituje u vezi sa mnogim fiziološkim stanjima. Pored toga, pokazano je da drugi polimorfizam u MTHFR genu, 1298 A>C, takođe redukuje aktivnost enzima. Cilj ove studije je bio da ispiša kliničku informativnost rezultata testiranja oba polimorfizma zajedno kod svake osobe. Na uzorcima genomsk eDNK izolovane iz periferne krvi 180 pacijentkinja sa komplikacijama tokom trudnoće i 183 zdrave osobe ženskog pola kao kontrole, utvrđen je genotip MTHFR gena na lokusima 677 i 1298, korišćenjem komercijalnih TaqMan eseja. Naši rezultati pokazuju da nema statistički značajne razlike u učestalosti alela i genotipova između ispitivanih grupa (p>0.05). MTHFR 677C>T genotip je utvrđen kod 11.7% pacijenata sa homozigotnom mutacijom na ovom lokusu, što ukazuje na povećan rizik zbog smanjene enzimske aktivnosti. Kada je pozicija 1298 uključena u testiranje, za 22.8% pacijentkinja se pokazalo da su heterozigotni nosioci na oba lokusa, dok je 8.9% pacijentkinja bilo sa homozigotnom mutacijom na 1298 lokusu. Ukupno 43% pacijentkinja je detektovano sa MTHFR genotipom koji daje enzim smanjene aktivnosti što je približno četiri puta više u odnosu na rezultate koji se dobijaju kada se testira samo MTHFR 677 C>T polimorfizam. Testiranje i drugog lokusa (1298 A>C), nakon dobija informacije o genotipu na 677 poziciji, dramatično povećava stopu detektovanih pacijenata koji mogu biti pod rizikom za stanja asocirana sa smanjenom aktivnošću MTHFR enzima što ukazuje da je neophodno susksesivno testiranje oba polimorfna lokusa.