The significance of *1793G>A* polymorphism in *MTHFR* gene in women with first trimester recurrent miscarriages

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Abstract

BACKGROUND: Recurrent miscarriages (RM) are significant social and clinical problem. One of suggested reason of RM is hyperhomocysteinemia. Polymorphic genes involved in homocysteine and folate metabolism, including 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene, are considered as an important risk factors for homocysteine accumulation and modulator of RM susceptibility. Therefore the aim of this study was to evaluate the frequency of *MTHFR* polymorphisms (*677C>T*, *1298A>C*, and *1793G>A*) in women with recurrent miscarriages.

MATERIAL AND METHODS: We have analyzed 104 Polish women with a history of 3 or more unexplained recurrent miscarriages in the first pregnancy trimester (6–13 gestation week). The control group consisted of 169 women without obstetrical complication, any history of miscarriage and with at least one live birth in anamnesis. The investigated polymorphisms were determined by PCR/RFLP methods.

RESULTS: For *MTHFR 1793G>A* polymorphism we have observed significant overrepresentation of heterozygotic GA genotypes in RM group (15.38% vs. 4.14% in the controls, OR=4.21, p=0.003). For *677C>T* and *1298A>C* we have shown lack of significant association with RM. Nevertheless, such significant association was observed if more than one mutated *MTHFR* variant was present in one patient.

CONCLUSIONS: Our research indicate the possible role of *MTHFR 1793G>A* polymorphism in pathogenesis of RM. The noticed tendency to more frequent occurrence of haplotypes of *MTHFR* gene including two or three mutated alleles showed the possibility of summarized amplification of these variants effect influencing RM susceptibility.

ORIGINAL ARTICLE

INTRODUCTION

Recurrent miscarriages (RM), appearing with frequency of 1–3% of all pregnancies, are an important medical, social and psychological problem in perinatal medicine. According to WHO criteria three or more consecutive intrauterine deaths and pregnancy losses before 22 week of gestation should be taken as recurrent miscarriages. After exclusion of chromosomal abnormalities, abnormal uterus anatomy, hormonal imbalance, autoimmune and environmental factors, the improper blood clotting tendencies should be considered as etiological explanation of recurrent miscarriages (Rai & Regan 2006; Abbate *et al.* 2002; Martinelli *et al.* 2000).

In recent years hyperhomocysteinemia has been postulated to induce oxidative stress, endothelial cell dysfunction, abnormalities of coagulation, and smooth muscle cell proliferation (Rai 2003). Thus, homocysteinemia could be of importance in recurrent miscarriages etiology. Many researches indicated the possible role of high level of homocysteine and endothelium damage in preeclampsia, intrauterine growth restriction, placental abruption, recurrent miscarriages or late fetal death (Also-Rallo *et al.* 2005; Infante-Rivard *et al.* 2005; Jaaskelainen *et al.* 2006; Nelen *et al.* 2000). Homocysteine is a sulfur amino acid produced in demethylation process of methionine. The 5,10-methylenetetrahydrofolate reductase (MTHFR), a key factor in the remetylation cycle of homocysteine, is cytoplasmic enzyme and catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. This last substance is a methyl donor in the remethylation of homocysteine to methionine and major circulating form of folate (Kang *et al.* 1998; Kim 2005).

Human gene coding for *MTHFR* is located in chromosomal region 1p36.3 (2200 base pair long, consists of 11 exons) (Goyette *et al.* 1994; Homberger *et al.* 2000; Kim 2005) and its polymorphic variants were shown to have important clinical consequences. Thermolabile form of MTHFR with lower activity was firstly described by Kang *et al.* in 1988 (Kang *et al.* 1998). A common alternation in the *MTHFR* gene was reported in 1995 by Frosst *et al.* (1995) as a *677C>T* single nucleotide polymorphism resulting in amino acid exchange in the *MTHFR* polypeptide (Ala222Val) (Frosst *et al.* 1995). Nowadays it is good documented that common *677C>T* polymorphism in the MTHFR gene is associated with increased thermolability, reduced specific activity of the MTHFR enzyme and is responsible for increased plasma concentration of homocysteine (Chango *et al.* 2000; Yamada *et al.* 2001). Several additional variants have been described in the *MTHFR* gene: *1298A>C* (amino acid substitution Glu429Ala) (van der Put *et al.* 1998) and *1793G>A* (Arg594Glu) (Rady *et al.* 2002) which were also suggested to be involved in folate metabolism and cause of lower MTHFR activity (Weisberg *et al.* 1998; Friso *et al.* 2002; Friedman *et al.* 1999; Melo *et al.* 2006).

In numerous studies relationship between two polymorphisms (*677C>T* and *1298A>C*) of *MTHFR* gene and RM appearance have been investigated with conflicting results (Grandone *et al.* 1998; Lissak *et al.* 1999; Mtiraoui *et al.* 2006; Zetterberg *et al.* 2002; Carp *et al.* 2002). Only few studies are related to *1793G>A* genetic variant and pregnancy outcome (Altmae *et al.* 2009; Kordas *et al.* 2009). Thus, the purpose of our study was to evaluate the frequency of genotypes and alleles of *677C>T*, *1298A>C*, *1793G>A* polymorphisms of *MTHFR* gene in the group of Polish women with recurrent miscarriages.

METHODS

Patients

The study was performed from February 2006 to October 2008 in the group of women hospitalized in the Division of Perinatology and Women's Diseases, Department of Perinatology and Gynecology of University of Medical Sciences in Poznan, Poland.

104 women (mean age 30.15±4.07 years, range 23–40 years, median 30 years) with 3 or more unexplained consecutive recurrent miscarriages in the first trimester of pregnancy (6–13 week of gestation) were enrolled in the study. Women with other complications connected to coagulation disturbances, antiphospholipid syndrome, and with other causes of miscarriages (chromosomal abnormalities, hormonal disturbances, uterus abnormality, internal diseases) have been excluded from the study. We have also excluded the change of partners of investigated women during the time period that miscarriages happened.

The control group consisted of 169 women (mean age 29.40±3.56 years, range 22–41 years, median 29 years) without obstetrical complication, any history of miscarriage and with at least one live births in anamnesis. All controls were singleton pregnancies. In each case gestational age was confirmed according to the data of last menstruation and by ultrasound investigation. Each woman was asked about folate and vitamins B intake. Women who did not receive folic acid and vitamins B supplementation in their pregnancy were excluded from the study.

All patients (cases and controls) were Caucasian of Polish origin. For investigation the permission of local Ethics Committee has been given. All women were informed about the goal of the study and given their written consent.

MTHFR genotyping procedure

From each women venous blood samples were collected and stored at minus 20°C until DNA extraction. Genomic DNA was isolated from nucleated blood cells using protocols and reagent supplied in commercial kit QIAamp DNA Blood Mini Kit (Qiagen Inc., Germany). All investigated polymorphisms were determined by polymerase chain reaction/restriction fragment length

polymorphism (PCR/RFLP) methods with primers previously published by Frosst *et al.* for *C677T* polymorphism, van der Put *et al.* for *1298A>C* polymorphism and Rady *et al.* for *1793G>A MTHFR* polymorphism (Frosst *et al.* 1995; van der Put *et al.* 1998; Rady *et al.* 2002).

Statistical methods

Data were analyzed using, the Statistical Package for Social Science v. 17.0 (SPSS Inc., Chicago, Illinois, USA). As a statistically significant we have considered *p*-value lower than 0.05. Frequencies of genotypes were compared by chi-square test; mean values for clinical parameters were compared by one-way ANOVA. The evaluation of haplotype frequency polymorphisms of *MTHFR* gene was performed by PHASE program. Expected genotype frequencies were calculated from allele frequencies applying Hardy-Weinberg equation.

RESULTS

Detailed frequencies of *MTHFR* genotypes are shown in Table 1. For the *677C>T* and *1298A>C* polymorphisms only slight overrepresentation of mutated genotypes and mutated alleles in RM group have been found.

The most interesting results were connected with *MTHFR 1793G>A* polymorphism. We have observed significant overrepresentation of heterozygotic *GA* genotypes in RM group (15.38% *vs*. 4.14% in the controls, OR=4.21, *p*=0.003). The distribution of wild-type *GG* genotype was 84.62% in RM group *vs*. 95.86% in the controls. Mutated homozygous *AA* genotype was absent in both investigated groups. The presence of mutated *A* allele was also statistically significant overrepresented in the RM group (7.69% *vs*. 2.07%, OR=3.94, *p*=0.004; Table 1). The frequency of observed genotypes for all

Tab. 1. The *MTHFR* gene polymorphisms – genotypes and alleles distribution.

	RM (n=104)		Controls (n=169)		OR	95%CI	*p*-value
	Observed n (%)	Expected n (%)	Observed n (%)	Expected n (%)			
MTHFR 677C>T							
Genotypes							
CC	44 (42.31)	43.49	89 (52.66)	52.55	0.66	0.39–1.11	0.06
CT	49 (47.11)	44.96	67 (39.65)	39.88	1.36	0.81–2.28	0.14
TT	11 (10.58)	11.65	13 (7.69)	7.57	1.42	0.55–3.59	0.27
Alleles							
C	137 (65.87)	–	245 (72.49)	–	0.73	0.49–1.08	0.06
T	71 (34.13)	–	93 (27.51)	–	1.36	0.92–2.01	0.06
MTHFR 1298A>C							
Genotypes							
AA	40 (38.46)	39.67	78 (46.15)	46.31	0.73	0.43–1.23	0.13
AC	51 (49.04)	46.63	74 (43.79)	43.48	1.23	0.73–2.07	0.23
CC	13 (12.50)	13.70	17 (10.06)	10.21	1.27	0.54–2.93	0.33
Alleles							
A	131 (62.98)	–	230 (68.05)	–	0.79	0.54–1.17	0.13
C	77 (37.02)	–	108 (31.95)	–	1.25	0.85–1.83	0.13
MTHFR 1793G>A							
Genotypes							
GG	88 (84.62)	85.21	162 (95.86)	95.91	0.24	0.08–0.64	0.003
GA	16 (15.38)	14.20	7 (4.14)	4.05	4.21	1.56–12.50	0.003
AA	0 (0,00)	0.59	0 (0.00)	0.04	–	–	–
Alleles							
G	192 (92.31)	–	331 (97.93)	–	0.25	0.09–0.67	0.004
A	16 (7.69)	–	7 (2.07)	–	3.94	1.50–11.51	0.004

investigated *MTHFR* genotypes were in agreement with Hardy-Weinberg equilibrium.

Further, the interaction of particular genotypes of studied *MTHFR* polymorphisms (*677C>T*, *1298A>C,* and *1793G>A*) has been analysed. The statistically significant prevalence of paired *CT*/*AC* heterozygotes (*677*/*1298 MTHFR* polymorphisms) in the group of women with miscarriages (28.85 *vs.* 15.98%, OR=2.33; 95%CL 1.03–5.34; p=0.02) was shown (Table 2). Moreover, statistically significant prevalence of heterozygote genotypes *AC*/*GA* (*1298*/*1793 MTHFR* polymorphisms) appearance (12.50 *vs.* 3.55%; OR=3.87; 95%CL 1.24–13.35; p=0.008) has been indicated. The most intriguing observation was the coexisting of heterozygote genotypes *CT*/*GA* (*677*/*1793 MTHFR* polymorphisms) in the group of women with miscarriages (9.61%). These genotypes were not observed in the control group (p=0.02) (Table 2).

Table 2. Frequency of paired *MTHFR* genotypes (*677C>T*, *1298A>C,* and *1793G>A*) polymorphisms in *MTHFR* gene in investigated and control groups.

			MTHFR 677C>T **n (%)**			**Total**
			CC	**CT**	**TT**	
MTHFR 1298A>C **n (%)**	**Miscarriages**	AA	10 (9.61)	19 (18.27)	11 (10.58)	40 (38.46)
		AC	21 (20.19)	30 (28.85)*	0 (0.00)	51 (49.04)
		CC	13 (12.50)	0 (0.00)	0 (0.00)	13 (12.50)
		Total	44 (42.31)	49 (47.11)	11 (10.58)	104 (100.00)
	Controls	AA	25 (14.79)	40 (23.67)	13 (7.69)	78 (46.15)
		AC	47 (27.81)	27 (15.98)*	0 (0.00)	74 (43.79)
		CC	17 (10.06)	0 (0.00)	0 (0.00)	17 (10.06)
		Total	89 (52.66)	67 (39.65)	13 (7.69)	169 (100.00)

			MTHFR 1298A>C **n (%)**			Total
			AA	AC	CC	
MTHFR 1793G>A **n (%)**	**Miscarriages**	GG	40 (38.46)	38 (36.54)	10 (9.62)	88 (84.62)
		GA	0 (0.00)	13 (12.50)*	3 (2.88)	16 (15.38)
		AA	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		Total	40 (38.46)	51 (49.04)	13 (12.50)	104 (100.00)
	Controls	GG	78 (46.15)	68 (40.24)	16 (9.47)	162 (95.86)
		GA	0 (0.00)	6 (3.55)*	1 (0.59)	7 (4.14)
		AA	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		Total	78 (46.15)	74 (43.79)	17 (10.06)	169 (100.00)

			MTHFR 677C>T **n (%)**			**Total**
			CC	**CT**	**TT**	
MTHFR 1793G>A **n (%)**	**Miscarriages**	GG	38 (36.54)	39 (37.50)	11 (10.58)	88 (84.62)
		GA	6 (5.77)	10 (9.61)*	0 (0.00)	16 (15.38)
		AA	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		Total	44 (42.31)	49 (47.11)	11 (10.58)	104 (100.00)
	Controls	GG	82 (48.52)	67 (39.65)	13 (7.69)	162 (95.86)
		GA	7 (4.14)	0 (0.00)*	0 (0.00)	7 (4.14)
		AA	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		Total	89 (52.66)	67 (39.65)	13 (7.69)	169 (100.00)

*p<0.05

We have also statistically calculated frequency of haplotypes for all three polymorphisms of *MTHFR* gene. In both groups of patients (with 3 or more pregnancy loss and controls) we have found seven haplotypes (*TAG*, *CAG*, *CCG*, *CCA*, *TCG*, *TCA*, and *CAA* for *MTHFR* gene positions 677, 1298, and 1793, respectively). The tendency to more frequent occurrence of *CCA* (p=0.01), *TCG* (ns), *TCA* (ns) haplotypes (including two or three mutated variants) in women with miscarriages has been noted. In the control group more frequent haplotypes were *CAG* (p=0.04), *CCG* (ns), and *CAA* (ns), which contained only one mutated variant or wild-type variants. In the group of women with miscarriages the occurrence of *TCA* haplotype (including three mutated variants) was noted (not found in the controls) (Table 3).

Tab. 3. Evaluation of haplotypes frequency of investigated polymorphism in the *MTHFR* gene.

MTHFR* haplotype	**RM**		**Controls**	
	Frequency	**SD**	**Frequency**	**SD**
TAG	0.3152	0.000456	0.2745	0.000343
CAG	0.3123**	0.000455	0.4033**	0.000378
CCG	0.2781	0.000440	0.3008	0.000353
CCA	0.0663**	0.000244	0.0181**	0.000103
TCG	0.0174	0.000128	0.0006	0.000019
TCA	0.0084	0.000090	0.0000	0.000000
CAA	0.0019	0.000043	0.0026	0.000039

*Nucleotides position at haplotypes are 677, 1298, and 1793 of *MTHFR* gene, respectively. **p<0.05

DISCUSSION

Negative effects of folate deficiency and homocysteine accumulation on female reproductive function are widely discussed. Nowadays it is suggested that hyperhomocysteinemia is an independent risk factor of recurrent miscarriages. Genes involved in folate metabolism coding for *MTHFR*, cystathionine beta-synthase, methionine synthase, folate receptor α (*FOLR1*) and their polymorphism cold be considered as a important risk factors for hyperhomocyseinemia (Borjel *et al.* 2006; Huemer *et al.* 2006).

In recent years many investigations focused on the role of *677C>T MTHFR* polymorphism in perinatal complications (Raziel *et al.* 2001; Jaaskelainen *et al.* 2006; Infante-River *et al.* 2005; Ren *et al.* 2006; Wiwanitkit 2005; Nelen *et al.* 2000; Foka *et al.* 2000). Several studies also showed the important role and positive association between *677C>T* genetic variant, hyperhomocysteinemia, and recurrent miscarriages (Nelen *et al.* 2000; Ren *et al.* 2006; Wiwanitkit 2005). The possible suggested mechanism of pathologic changes is induction of coagulation cascade in placental vessels (Khong & Hague 1999). This is a cause of thrombosis in spiral arteries and fibrin deposited in intravillous space, endothelium dysfunction, infarctions of placental villous followed by utero-placental insufficiency (Kupferminc 2005; Arias *et al.* 1998; Jivraj *et al.* 2006).

The presence of *677C>T* variant of *MTHFR* gene is correlated firstly with miscarriages in the I trimester of gestation. In analysis made by Nelen *et al.* (2000) it was indicated the correlation between early miscarriages (before 17th week of pregnancy) and the presence of *677TT* genotype of MTHFR (2–3-fold risk) (Nelen *et al.* 2000). But some authors did not shown the correlation of *677TT* genotype with the higher risk of frequency of recurrent miscarriages (Rey *et al.* 2003; Foka *et al.* 2000; Kutteh *et al.* 1999). One of the largest summary of significance of thrombophilia in etiology of miscarriages is meta-analysis (3000 women) made by Rey *et al.* (2003) These authors indicated that early recurrent miscarriages in the I trimester are in close correlation with frequency of factor Leiden (FV), resistance of APC and the presence of *G20210A* mutation of prothrombin gene. In the recurrent miscarriages in II trimester they have suggested the highest role of FV mutation. This analysis does not shown the significant role of *677T* variant of *MTHFR* in the group of women with miscarriages (Rey *et al.* 2003).

Negligible number of observations were connected with the both others *1298A>C* and *1793G>A* genetic variants of *MTHFR* gene investigated in this study. Positive association of *1298A>C* polymorphism and RM was shown in several investigations. In 2009 Rodriguez-Guillén and co-workers indicated the possible role of *677C>T* and *1298A>C* polymorphisms in spontaneous abortion susceptibility (Rodriguez-Guillen *et al.* 2009). For *1793G>A* there is a single investigation showing significant connection of susceptibility to unexplained infertility with *1793G* variant (Altmäe *et al.* 2009).

For two typically investigated *MTHFR* variant (*677C>T*, *1298A>C*) with clearly defined functional consequences in impaired MTHFR activity we have shown in our study lack of significant association with RM. Nevertheless, such significant association was observed if both mutated variants were present in one patient. Moreover, we have shown, at least in investigated group of Polish women with RM, the significant impact of *MTHFR 1793G>A* polymorphism (7.69% *vs.* 2.07%, OR=3.94, p=0.004). However, the functional significance of *1793A* variant is currently unknown, but it is suggested to be connected with lower enzyme activity and connected with homocysteinemia (Altmae *et al.* 2009). Moreover, contrary to results published by Altmae *et al.* (2009) we have also found the high prevalence of wild-type haplotype *CAG* in the control group. In RM group the haplotypes including two or three mutated variants were more frequent. The noticed tendency to more frequent occurrence of haplotypes of *MTHFR* gene including two or three mutated allele

showed the possibility of summarized effect of these variants influencing recurrent miscarriage occurrence possibility (Altmae *et al.* 2009).

Nutrition factors (folate and cobalamin administration) could influence the homocysteine level and its metabolism. Polymorphisms of *MTHFR* gene should be also considered in the context of folate deficiency (Yilmaz *et al.* 2004) since folate intake may modulate the risk of RM and modify the effect of *MTHFR* polymorphisms on the risk of RM appearance. To omit possible nutrition influence on results in our investigation each woman from RM and control group administered 0.4 mg of folic acid per day.

Concluding, we have found that the frequency of *1793G>A* polymorphism of *MTHFR* gene in women with recurrent miscarriage indicated this polymorphism as risk factor for recurrent miscarriages in first trimester of pregnancy, particularly in the early stage of first trimester regardless of folate and vitamins B intake. Furthermore, analysis of genotypes correlation of all three *MTHFR* polymorphisms (*677C>T, 1298A>C, 1793G>A*) showed the overrepresentation of heterozygous genotypes combination (including one mutated allele) that indicated possible summarized amplification of heterozygous genotypes effect in etiology of recurrent miscarriages.

In this investigation it was shown that genetic polymorphisms connected with inherited thrombophilias are very important factors inducing recurrent miscarriages. It suggest possible target to underwent antithrombotic prevention of recurrent miscarriages according to observed individual *MTHFR* genotype/haplotype of woman.

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