

Methylenetetrahydrofolate Reductase C677T and A1298C Polymorphisms in Male Partners of Recurrent Miscarriage Couples

Somayeh-Sadat Tara¹, Fatemeh Ghaemimanesh², Saeed Zarei², Fakhreddin Reihani-Sabet³, Zhamak Pahlevanzadeh³, Mohammad Hosein Modarresi⁴, Mahmood Jeddi-Tehrani^{2*}

1- Department of Genetics, Science and Research Branch, Islamic Azad University, Tehran, Iran

2- Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

3- Avicenna Fertility Center, Avicenna Research Institute, ACECR, Tehran, Iran

4- Department of Medical Genetics, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Background: Methylenetetrahydrofolate reductase (MTHFR) single-nucleotide polymorphisms (SNPs) C677T and A1298C have been described as strong risk factors for idiopathic recurrent miscarriage (RM). However, very few studies have investigated the association of paternal MTHFR SNPs with RM. The aim of the present study was to evaluate the prevalence of paternal C677T and A1298C SNPs among Iranian RM couples.

Methods: The study subjects comprised 225 couples with more than three consecutive pregnancy losses, and 100 control couples with no history of pregnancy complications. All females in the case group had MTHFR polymorphisms; and genotype SNPs were analyzed by PCR-RFLP. Groups were statistically compared using Mann Whitney U-test and Chi-square statistical tests. The $p < 0.05$ were considered significant.

Results: Statistically significant difference was detected in the frequency of MTHFR SNPs in male partners of the two groups ($p = 0.019$). Combined heterozygosity of MTHFR polymorphisms was a common phenomenon in the males; 52 (23.1%) and 14 (14%) of males in RM and control groups, respectively. Absence of combined homozygosity for both SNPs in all studied groups/genders was observed.

Conclusion: The MTHFR gene composition of male partners of RM couples may contribute to increased risk of miscarriage.

Keywords: Male partners, Methylenetetrahydrofolate reductase, Polymorphism, Recurrent miscarriage, Thrombophilia.

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Introduction

Pregnancy is a hypercoagulable state due to an addition in coagulation factors, a reduction in anticoagulants and a deficiency in fibrinolysis (1). Recurrent miscarriage (RM) is an important clinical problem with many etiologies such as genetic, anatomic, endocrine, and infectious diseases as well as immune defects. Furthermore, pregnancy complications such as RM and recurrent implantation failure have been found to be related to thrombophilic gene mutations (2-7).

RM is defined as the loss of two or more successive pregnancies before 20th week of gestation and affects ~1% of couples (8).

Methylenetetrahydrofolate reductase (MTHFR) is an essential enzyme to the folic acid pathway. In this regard, insufficiencies of folic acid or defects in MTHFR have demonstrated DNA hypomethylation and abnormal biochemical and/or phenotypic changes in animal models (9, 10), cell culture (11, 12) and humans (13-16). The gene

* Corresponding Author:
Mahmood Jeddi-Tehrani,
Monoclonal Antibody
Research Center,
Avicenna Research
Institute, ACECR,
P.O. Box: 19615-1177,
Tehran, Iran
E-mail:
mahjed@avicenna.ac.ir;
mahjed@yahoo.com

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encoding MTHFR has been mapped to the chromosomal region 1p36.3. MTHFR is involved in the synthesis of 5-methylenetetrahydrofolate, which is a cofactor in the enzymatic formation of methionine from homocysteine; and therefore plays a critical role in early embryonic development.

The MTHFR C677T polymorphism results in an amino acid replacement (alanine to valine), which causes the decrease in the activity of the encoded enzyme at 37°C or more (17). In the MTHFR A1298C allele, a polymorphism in exon 7 results in an amino acid replacement (glutamate to alanine). The activity of the encoded enzyme is decreased, although less than that in C677T allele (18). Most studies show the association of these two polymorphisms with RM in women (6, 19-21). However, in couples suffering from RM, the role of MTHFR gene polymorphisms in the male partner has not been well defined. The prevalence of the MTHFR mutation in combination with other thrombophilia mutations in RM couples in comparison with controls has been reported (22, 23). However, these studies reported no significant difference in the prevalence of inherited thrombophilia between the two groups.

The aim of this study was to analyze the frequencies of MTHFR C677T and A1298C polymorphisms in male partners of RM couples whose female partners suffered from extensive mutations in MTHFR, compared to those in control couples.

Methods

Patients and controls: Two hundred and twenty five couples with at least three successive pregnancy losses below 20th week of gestation, who were referred to Avicenna Fertility Center, Tehran, Iran, were selected as the case group. All females in the case group were carrying either double heterozygotic polymorphisms of MTHFR C677T and MTHFR A1298C or had at least a homozygotic polymorphism at either MTHFR gene loci (these conditions are termed "MTHFR high risk" in this study).

The control group included 100 couples with at least two live births and no history of pregnancy complications such as miscarriage, stillbirth, small for gestational age fetuses or pre-eclampsia. The exclusion criteria for the case group included anatomic disorders, infectious diseases, single heterozygotic or normal genotype of MTHFR gene at positions 677 and 1298.

The mean age of the different gender groups were 32.4±6.0 and 35.2±4.5 years in females and 37.5±8.4 and 39.8±7.9 years in males, in case and control groups, respectively. The average miscarriage rate of the case group was 3.6±1.5. All couples entered in the study after signing informed written consent and the study was also approved by the ethical committee of Avicenna Research Institute.

DNA extraction and PCR-RFLP analysis: Five milliliters of venous blood were collected from each individual (both men and women) in tubes containing EDTA, and DNA was extracted from the samples using salting out method. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) amplifications of the DNA samples for MTHFR C677T and A1298C genes were performed using 5'-TGAAGGAGAA GGTGTCTGCGGA-3' and 5'-CAAGGAGGAG CTGCTGAAGA-3' as forward and 5'-AGGACG GTGCGGTGAGAGTG-3' and 5'-CCACTCCAG CATCACTCACT-3' as reverse specific primers, respectively (24). The amplification products were analyzed on 1.5% ethidium bromide-stained agarose gels and then subjected to enzymatic digestion using Hinf I and Mbo II restriction enzymes for MTHFR C677T and A1298C SNPs, respectively.

Statistical analysis: The genotype distributions of each SNP between the case and the control groups were compared by Mann-Whitney U-test. Heterozygous and homozygous mutations of MTHFR C677T and A1298C and also distribution of them were compared between the case and the control groups using Chi-square test. Statistical analysis software program SPSS-13.0 was used for data analysis. The p<0.05 were considered statistically significant.

Results

MTHFR C677T and A1298C genotype analysis: PCR-RFLP was carried to analyze the MTHFR genotype composition of the male partners of RM couples whose females were suffering from "MTHFR high risk". To achieve this goal, 225 RM women with "MTHFR high risk" SNPs were selected and their male partners were genotyped for MTHFR A1298C and C677T SNPs. The results were compared with genotype frequency of males in 100 control couples. Genotype distributions in the maternal and paternal control groups of MTHFR C677T polymorphism and also mater-

nal control group of A1298C polymorphism were in Hardy-Weinberg equilibrium ($p>0.05$). Paternal control group of A1298C polymorphism was in borderline. The numbers and frequencies of MTHFR C677T and A1298C SNPs in males and females of the case and control groups are shown in table 1. The individuals in both groups were further categorized based on gender, RM/Normal and the type of MTHFR SNPs. Table 1 shows the combinations of the two polymorphisms in both groups. Combined heterozygosity of the two polymorphisms in the males of the two groups is presented as a common phenomenon, 52 (23.1%) and 14 (14%) of males in RM and control groups, respectively. Rare combinations of A1298C/C677T SNPs including homo/hetero-zygote or hetero/homo-zygote variants were observed in males and females of the both groups (Table 1). A major observation of this study was the absence of combined homozygosity for both SNPs in all studied

groups/genders. The pattern of individual grouping in table 1 allowed us to further group them into MTHFR high risk and low risk groups. The data was summarized in table 2 where the males were statistically compared depending on their degree of RM risk. Surprisingly, a statistically significant difference in the frequency of MTHFR high risk group among the male partners in the RM group was detected compared to that in the normal males ($p=0.019$).

Discussion

This study was aimed to survey the possible role for paternal MTHFR SNPs in RM. In this regard, MTHFR C677T and A1298C SNPs in male partners of RM couples whose female partners had MTHFR high risk genotypes were compared to that in normal counterparts. The results suggest that RM male MTHFR genotypes could also be involved in the pregnancy outcome.

Few studies on MTHFR polymorphism variants and RM have been performed in both male and female partners. In this regard, Jivaraj et al. studied factor V Leiden, MTHFR C677T and prothrombin G20210A SNPs in males and females of RM couples and compared their results with those in normal male and female counterparts (22). They concluded that multiple genetic thrombophilic mutations, but not single mutations, in either partner, significantly increased the risk of miscarriage (22). In a similar study, Toth et al. reported no association between paternal thrombophilia and RM (23). In the study performed by Yenicesu et al., 12 thrombophilic gene polymorphisms including MTHFR C677T and A1298C in RM and normal couples were considered (5). They reported significantly higher frequency of MTHFR C677T homozygous mutations in RM males (5).

Role of male MTHFR in RM has also been addressed through the analysis of MTHFR gene promoter hypermethylation, where Rotondo et al. elegantly showed the significant increase in the promoter hypermethylation in RM males (25). This is in line with the fact that hypermethylation of gene promoters can suppress the transcription process leading to silencing of gene expression (26). Lack of MTHFR activity due to presence of its polymorphic variants will reduce the availability of methyl group donors that are needed for methylation of DNA (9) which may adversely affect the global genome methylation and also the genomic imprinting of paternal genes in sperma-

Table 1. Combination of MTHFR A1298C and C677T SNPs in RM and normal groups

Group	A1298C SNP	C677T SNP			Total n (%)
		CC	CT	TT	
RM (n: 225)					
Maternal	AA	0	0	47	47 (20.8)
	AC	0	114	2*	116 (51.6)
	CC	62	0	0	62 (27.6)
Paternal	AA	28	46	14	88 (39.2)
	AC	55	52	2*	109 (48.4)
	CC	27	1*	0	28 (12.4)
Normal (n: 100)					
Maternal	AA	36	19	4	59 (59)
	AC	26	6	0	32 (32)
	CC	8	1*	0	9 (9)
Paternal	AA	25	28	3	56 (56)
	AC	18	14	0	32 (32)
	CC	9	3*	0	12 (12)

* Rare combination of A1298C and C677T SNPs including homo/hetero- or hetero/homo-zygotes; RM: recurrent miscarriage

Table 2. Frequency of individuals with high and low risk MTHFR genotypes in different groups

	Female		Male*	
	High risk	Low risk	High risk	Low risk
RM n (%)	225 (100)	0 (0)	96 (42.7)	129 (57.3)
Normal n (%)	19 (19)	81 (81)	29 (29)	71 (71)

* $P=0.019$, Males in MTHFR high and low risk groups were compared using Chi-square test; High risk: Individuals who carried either combined heterozygous C677T/A1298C MTHFR SNPs or at least one homozygous SNP; Low risk: Individuals who carried no C677T/A1298C MTHFR SNPs or had just one heterozygous SNP; RM: recurrent miscarriage

togenetic cells. On the other hand, genomic imprinting which is involved in silencing of genes at the level of imprinted loci is highly regulated by DNA methylation (27). In this regard, impaired methylation imprints have been shown to lead to generation of poorly developed placenta that carried trophoblasts with proliferation, apoptosis and differentiation abnormalities (28-31). These data suggest that a global hypomethylation of sperm DNA following hypermethylation of MTHFR promoter can cause pregnancy loss through male factor in RM couples (25). Such an effect of MTHFR polymorphism may also account for repeated implantation failure in infertile couples undergoing *in vitro* fertilization treatments.

Considering the nature of our study, significantly increased MTHFR SNPs were found in RM males compared to that in males of normal couples. This will increase the Mendelian risk for the embryo to gain polymorphic MTHFR variants. Interestingly, no combined homozygosity for C677T and A1298C SNPs was found among the individuals in this study and thus our data cannot exclude the possibility that inheritance of such combination might predispose the product of conception to embryonic/fetal death.

It is also well documented that DNA hypermethylation directly affects chromosomal instability (32). It is thus suggested that folate deficiency and DNA hypomethylation following increased frequency of MTHFR polymorphisms may be associated with chromosomal instability (33) which may lead to production of aneuploid gametes and embryos. Chromosomal aneuploidies are well known causes of first trimester miscarriage (34).

Conclusion

Previous reports (6, 19, 35) as well as the data presented in this study are in favor of an increased number of MTHFR polymorphic variants donated by both male and female partners of RM couples to the embryo. The MTHFR gene composition of the embryo may thus be adversely affected by DNA hypomethylation that may increase the risk of embryonic/fetal death and be reflected as recurrent pregnancy loss in the couples. In this regard, MTHFR genotyping of couples with multiple pregnancy losses or repeated implantation failures who remain unresponsive to treatments, followed by selection of IVF embryos with the least frequencies of MTHFR polymorphic variants by preimplantation genetic diagnosis (PGD) may

play a significant role in increasing the survival chance of embryo/fetus.

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Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

References

1. Stirling Y, Woolf L, North WR, Seghatchian MJ, Meade TW. Haemostasis in normal pregnancy. *Thromb Haemost.* 1984;52(2):176-82.
2. Khosravi F, Zarei S, Ahmadvand N, Akbarzadeh-Pasha Z, Savadi E, Zarnani AH, et al. Association between plasminogen activator inhibitor 1 gene mutation and different subgroups of recurrent miscarriage and implantation failure. *J Assist Reprod Genet.* 2014;31(1):121-4.
3. Behjati R, Modarressi MH, Jeddi-Tehrani M, Dokoohaki P, Ghasemi J, Zarnani AH, et al. Thrombophilic mutations in Iranian patients with infertility and recurrent spontaneous abortion. *Ann Hematol.* 2006;85(4):268-71.
4. Jeddi-Tehrani M, Torabi R, Mohammadzadeh A, Arefi S, Keramatipour M, Zeraati H, et al. Investigating Association of Three Polymorphisms of Coagulation Factor XIII and Recurrent Pregnancy Loss. *Am J Reprod Immunol.* 2010;64(3):212-7.
5. Yenicesu GI, Cetin M, Ozdemir O, Cetin A, Ozen F, Yenicesu C, et al. A prospective case-control study analyzes 12 thrombophilic gene mutations in Turkish couples with recurrent pregnancy loss. *Am J Reprod Immunol.* 2010;63(2):126-36.
6. Torabi R, Zarei S, Zeraati H, Zarnani AH, Akhondi MM, Hadavi R, et al. Combination of thrombophilic gene polymorphisms as a cause of increased the risk of recurrent pregnancy loss. *J Reprod Infertil.* 2012; 13(2):89-94.
7. Idali F, Zareii S, Mohammad-Zadeh A, Reihany-Sabet F, Akbarzadeh-Pasha Z, Khorram-Khorshid HR, et al. Plasminogen activator inhibitor 1 and methylenetetrahydrofolate reductase gene mutations in iranian women with polycystic ovary syndrome. *Am J Reprod Immunol.* 2012;68(5):400-7.
8. Rai R, Regan L. Recurrent miscarriage. *Lancet.* 2006;368(9535):601-11.

9. Chen Z, Karaplis AC, Ackerman SL, Pogribny IP, Melnyk S, Lussier-Cacan S, et al. Mice deficient in methylenetetrahydrofolate reductase exhibit hyperhomocysteinemia and decreased methylation capacity, with neuropathology and aortic lipid deposition. *Hum Mol Genet.* 2001;10(5):433-43.
10. Kim YI, Pogribny IP, Basnakian AG, Miller JW, Selhub J, James SJ, et al. Folate deficiency in rats induces DNA strand breaks and hypomethylation within the p53 tumor suppressor gene. *Am J Clin Nutr.* 1997;65(1):46-52.
11. Duthie SJ, Narayanan S, Blum S, Pirie L, Brand GM. Folate deficiency in vitro induces uracil misincorporation and DNA hypomethylation and inhibits DNA excision repair in immortalized normal human colon epithelial cells. *Nutr Cancer.* 2000;37(2):245-51.
12. Kimura M, Umegaki K, Higuchi M, Thomas P, Fenech M. Methylenetetrahydrofolate reductase C677T polymorphism, folic acid and riboflavin are important determinants of genome stability in cultured human lymphocytes. *J Nutr.* 2004;134(1):48-56.
13. Castro R, Rivera I, Ravasco P, Camilo ME, Jakobs C, Blom HJ, et al. 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C-->T and 1298A-->C mutations are associated with DNA hypomethylation. *J Med Genet.* 2004;41(6):454-8.
14. Friso S, Choi SW, Girelli D, Mason JB, Dolnikowski GG, Bagley PJ, et al. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. *Proc Natl Acad Sci USA.* 2002;99(8):5606-11.
15. Jacob RA, Gretz DM, Taylor PC, James SJ, Pogribny IP, Miller BJ, et al. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. *J Nutr.* 1998;128(7):1204-12.
16. Stern LL, Mason JB, Selhub J, Choi SW. Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase gene. *Cancer Epidemiol Biomarkers Prev.* 2000;9(8):849-53.
17. Kang SS, Wong PW, Susmano A, Sora J, Norusis M, Ruggie N. Thermolabile methylenetetrahydrofolate reductase: an inherited risk factor for coronary artery disease. *Am J Hum Genet.* 1991;48(3):536-45.
18. van der Put NM, Gabreels F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *Am J Hum Genet.* 1998;62(5):1044-51.
19. Altmae S, Stavreus-Evers A, Ruiz JR, Laanpere M, Syvanen T, Yngve A, et al. Variations in folate pathway genes are associated with unexplained female infertility. *Fertil Steril.* 2010;94(1):130-7.
20. Govindaiah V, Naushad SM, Prabhakara K, Krishna PC, Radha Rama Devi A. Association of parental hyperhomocysteinemia and C677T Methylenetetrahydrofolate reductase (MTHFR) polymorphism with recurrent pregnancy loss. *Clin Biochem.* 2009;42(4-5):380-6.
21. Jeddi-Tehrani M, Torabi R, Zarnani AH, Mohammadzadeh A, Arefi S, Zeraati H, et al. Analysis of plasminogen activator inhibitor-1, integrin beta3, beta fibrinogen, and methylenetetrahydrofolate reductase polymorphisms in Iranian women with recurrent pregnancy loss. *Am J Reprod Immunol.* 2011;66(2):149-56.
22. Jivraj S, Rai R, Underwood J, Regan L. Genetic thrombophilic mutations among couples with recurrent miscarriage. *Hum Reprod.* 2006;21(5):1161-5.
23. Toth B, Vocke F, Rogenhofer N, Friese K, Thaler CJ, Lohse P. Paternal thrombophilic gene mutations are not associated with recurrent miscarriage. *Am J Reprod Immunol.* 2008;60(4):325-32.
24. Miranda-Vilela AL, Lordelo GS, Akimoto AK, Alves PC, Pereira LC, Klautau-Guimaraes Mde N, et al. Genetic polymorphisms influence runners' responses to the dietary ingestion of antioxidant supplementation based on pequi oil (*Caryocar brasiliense* Camb.): a before-after study. *Genes Nutr.* 2011;6(4):369-95.
25. Rotondo JC, Bosi S, Bazzan E, Di Domenico M, De Mattei M, Selvatici R, et al. Methylenetetrahydrofolate reductase gene promoter hypermethylation in semen samples of infertile couples correlates with recurrent spontaneous abortion. *Hum Reprod.* 2012;27(12):3632-8.
26. Rajender S, Avery K, Agarwal A. Epigenetics, spermatogenesis and male infertility. *Mutat Res.* 2011;727(3):62-71.
27. Piedrahita JA. The role of imprinted genes in fetal growth abnormalities. *Birth Defects Res A Clin Mol Teratol.* 2011;91(8):682-92.
28. Diplas AI, Lambertini L, Lee MJ, Sperling R, Lee YL, Wetmur J, et al. Differential expression of imprinted genes in normal and IUGR human placentas. *Epigenetics.* 2009;4(4):235-40.
29. Georgiades P, Watkins M, Burton GJ, Ferguson-Smith AC. Roles for genomic imprinting and the zygotic genome in placental development. *Proc Natl Acad Sci USA.* 2001;98(8):4522-7.

30. Lim AL, Ferguson-Smith AC. Genomic imprinting effects in a compromised in utero environment: implications for a healthy pregnancy. *Semin Cell Dev Biol.* 2010;21(2):201-8.
31. Serman L, Vlahovic M, Sijan M, Bulic-Jakus F, Serman A, Sincic N, et al. The impact of 5-azacytidine on placental weight, glycoprotein pattern and proliferating cell nuclear antigen expression in rat placenta. *Placenta.* 2007;28(8-9):803-11.
32. Vilain A, Vogt N, Dutrillaux B, Malfoy B. DNA methylation and chromosome instability in breast cancer cell lines. *FEBS Lett.* 1999;460(2):231-4.
33. Hobbs CA, Sherman SL, Yi P, Hopkins SE, Torfs CP, Hine RJ, et al. Polymorphisms in genes involved in folate metabolism as maternal risk factors for Down syndrome. *Am J Hum Genet.* 2000;67(3):623-30.
34. Robberecht C, Pexsters A, Deprest J, Fryns JP, D'Hooghe T, Vermeesch JR. Cytogenetic and morphological analysis of early products of conception following hystero-embryoscopy from couples with recurrent pregnancy loss. *Prenat Diagn.* 2012;32(10):933-42.
35. Stangler Herodez S, Zagradisnik B, Erjavec Skerget A, Zagorac A, Takac I, Vlaisavljevic V, et al. MTHFR C677T and A1298C Genotypes and Haplotypes in Slovenian Couples with Unexplained Infertility Problems and in Embryonic Tissues from Spontaneous Abortions. *Balkan J Med Genet.* 2013;16(1):31-40.