

## C677T and A1298C Mutations in the Methylenetetrahydrofolate Reductase Gene in Patients with Recurrent Abortion from the Iranian Azeri Turkish

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### Abstract

**Background:** To assess whether the C677T and A1298C mutations in the methylenetetrahydrofolate reductase (*MTHFR*) gene are associated with recurrent abortion (RA), we determined the frequencies of the T677 and C1298 mutations in patients and controls.

**Materials and Methods:** Mutations were determined by a RFLP-PCR method in 53 patients and 61 matched controls.

**Results:** The frequencies of T alleles were 0.26 in patients and 0.29 in controls. The frequencies of C/C, T/C and T/T genotypes were 34 (55.7%), 22 (36.1%) and 5 (8.2%) in patients, and 27 (50.9%), 21 (39.6%) and 5 (9.43%) in controls. The C allele frequencies were 0.38 in patients and controls. C/C, A/C and A/A genotype distributions were 9 (14.8%), 28 (45.9%) and 24 (39.3%) in patients, and 8 (15.1%), 24 (45.3%) and 21 (39.6%) in controls.

**Conclusion:** There were no significant differences between patients and controls concerning the T677 and C1298 mutations.

**Keywords:** MTHFR, Pregnancy, Recurrent Abortion

### Introduction

The methylenetetrahydrofolate reductase (MTHFR) enzyme plays important roles in metabolism of folates, remethylation of homocysteine to methionine and reduces 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (1). It has been established that MTHFR enzyme activity is associated with mutations within the MTHFR gene. The two most defined mutations of the MTHFR gene are missense mutations that include substitution of cytosine to thymine at nucleotide 677 which results in the conversion of alanine to valine (2, 3). Another mutation is the transversion of adenosine to cytosine at nucleotide 1298 which results in the conversion of glutamate to alanine (2, 3). The influence of these mutations varies in degree from mild to severe regarding the deficiency of MTHFR enzyme activity. Folate, as a universal methyl donor, contributes to the synthesis of nucleic acids, repair and methylation, and gene expression (2, 4, 5). This function implies that gene-nutrient interactions mainly influence the pattern of DNA polymorphisms (6, 7). MTHFR C677T and A1298C SNPs have been associated with human disorders

such as neural tube defects (8-13), cancer (14,15), cardiovascular and cerebrovascular disease (16-18), psychiatric diseases (19, 20), arteriosclerosis (2, 4, 21-23), male infertility (24), hyperhomocysteinemia (2), recurrent pregnancy loss (RPL) and related complications (25-29). Within our population, no studies have addressed distribution of the C677T and A1298C mutations in the MTHFR gene in patients with recurrent abortion (RA) and healthy controls from the Iranian Azeri Turkish. Therefore, we carried out the present study to evaluate whether C677T and A1298C mutations in the MTHFR gene are associated with a predisposition for RA.

### Materials and Methods

The Ethics Committee of Urmia University of Medical Sciences approved the present study. A minimum sample size of 45 patients in the case groups had a statistical power of approximately 90% (two-tailed,  $\alpha=0.05$ ). Totally, 53 cases with unexplained RA and 61 healthy controls voluntarily entered into the present study. Cases had a his-

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tory of at least three consecutive fetal losses before 20 weeks of gestation from the same partner. Cases were diagnosed and sequentially selected among patients referred to the Department of Genetics at Motahari Hospital (Urmia, West Azerbaijan, Iran), from the Obstetrics and Gynecology Department at Urmia University of Medical Sciences and other centers. The control group consisted of fertile females from the general population who had at least one uncomplicated pregnancy and no history of abortion. Controls were randomly selected from the same ethnic group among participants in genetic counseling sessions which occurred in the Genetic Center at Urmia University of Medical Sciences. They were selected with regard to their past medical history and exclusion of any specific disorders such as genetic, congenital diseases and history of pregnancy loss. Patients and controls with vascular disease, obesity, chromosomal, hormonal, immunological and anatomical abnormalities as confounding factors were excluded. All individuals (patients and controls) were matched for age, body mass index (BMI), ethnicity and geographical region. Written informed consent was obtained from patients and controls. DNA was isolated with the standard method from 2-3 ml EDTA-blood of samples (30). MTHFR C677T alleles and genotypes were determined by RFLP-PCR using primers 5'-CAT CCC TAT TGG CAG GTT AC-3' and 5'-GAC GGT GCG GTG AGA GTG-3'. The reaction profile was: denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds for 35 cycles and 72°C for 5 minute (31). MTHFR A1298C alleles and genotypes were determined by RFLP-PCR using primers 5'- ATG TGG GGG GAG GAG CTG AC -3' and 5'- GTC TCC CAA CTT ACC CTT CTC CC-3' and their reaction program was as follows: denaturation at 94°C for 30 seconds, annealing at 51°C for 30 seconds, extension at 72°C for 30 seconds for 35 cycles and 72°C for 5 minute (32). Restriction digestion with *HinfI* (Fermentas Life Sciences, Germany) and *MboII* enzymes (Fermentas Life Sciences, Germany) was used for MTHFR C677T and A1298C genotyping, respectively. Digestion of

the PCR products was performed at 37°C for two hours. Separation of fragments was done by electrophoresis on 3% agarose gel containing ethidium bromide. Presence or absent of different fragments were visualized under UV transilluminator. The presence of T allele at nucleotide 677 of the *MTHFR* gene naturally produces a restriction site for the *HinfI* enzyme. Individuals homozygous for the T allele show two bands of 171 and 94 bp. Individuals homozygous for the C allele show a single un-cut band of 265 bp. Those heterozygous for both the C and T alleles show three bands of 265, 171 and 94 bp (31).

The presence of A allele at nucleotide 1298 of the *MTHFR* gene naturally produces a restriction site for *MboII* enzyme. Individuals homozygous for A allele show two bands of 204 and 37 bp. Individuals homozygous for C allele show a single un-cut band of 241 bp. Individuals heterozygous for C and A alleles show three bands of 241, 204 and 37 bp (32). The frequencies of alleles and genotypes of *MTHFR* gene were determined via direct counting in the studied groups. Cases and healthy controls were tested for their fit to the Hardy-Weinberg equilibrium regarding allelic and genotypic frequencies. For every group, the expected values were calculated and then data were compared to the observed genotype frequencies. All frequencies of the *MTHFR* gene in cases versus healthy controls were compared using either the  $\chi^2$  test or Fisher's exact test. For all statistical analysis, the  $\chi^2$  and p value, odds ratio (OR) and 95% confidence interval (CI) were calculated by SPSS v.16.0 and Microsoft Excel 2003. Two-sided tests were performed and for statistical analysis, a p value less than 0.05 was considered significant.

## Results

The general characteristics of the patients and controls are presented in table 1.

The results of the present study are summarized in tables 2 and 3.

Figure 1 shows the frequency (%) of MTHFR C677T and A1298C genotypes and alleles frequencies in cases and controls.

**Table 1: Characteristics of patients and controls.**

	Patients	Controls
<b>Ethnicity</b>	Native Azeri Turkish	Native Azeri Turkish
<b>No. of abortions (median)</b>	3.2 ± 1.1 (3)	0
<b>No. of successful live-births</b>	0	at least two
<b>Mean age (years)</b>	28.3 ± 5.3	29.6 ± 5.0
<b>BMI* (kg/m<sup>2</sup>)</b>	24.8 ± 3.4	23.3 ± 3.2

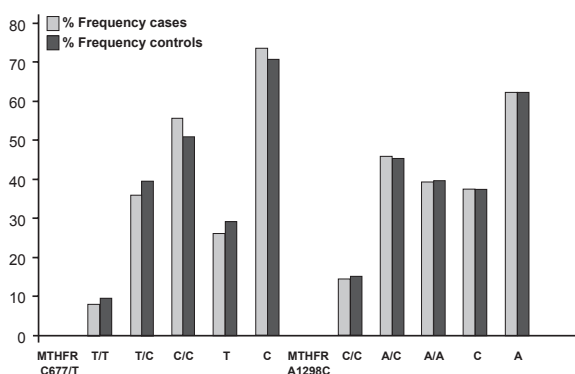
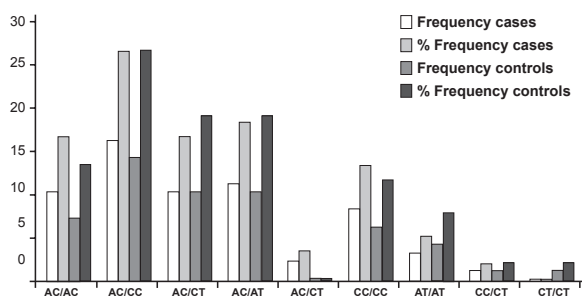
\*  $\chi^2=0.9$ ,  $df=4$ ,  $p=0.3>0.05$

**Table 2: MTHFR C677T and A1298C genotypes and allele frequencies in cases and controls.**

Genotype/allele	F (%F) cases	F (%F) controls	OR (95%CI)	$\chi^2$	P-value
<b>MTHFR C677T</b>					
T/T	5 (8.197)	5 (9.434)	0.86 (0.23-3.14)	0.05425	0.816
T/C	22 (36.07)	21 (39.62)	0.86 (0.4-1.84)	0.15275	0.696
C/C	34 (55.74)	27 (50.94)	1.21 (0.58-2.54)	0.26203	0.609
T	32 (26.23)	31 (29.25)	0.86 (0.48-1.54)	0.25797	0.612
C	90 (73.77)	75 (70.75)	1.16 (0.65-2.08)	0.25797	0.612
<b>MTHFR A1298C</b>					
C/C	9 (14.75)	8 (15.09)	0.97 (0.35-2.73)	0.00259	0.959
A/C	28 (45.9)	24 (45.28)	1.03 (0.49-2.15)	0.00437	0.947
A/A	24 (39.34)	21 (39.62)	0.99 (0.47-2.1)	0.00092	0.976
C	46 (37.7)	40 (37.74)	1 (0.58-1.71)	2.3E-05	0.996
A	76 (62.3)	66 (62.26)	1 (0.59-1.71)	2.3E-05	0.996

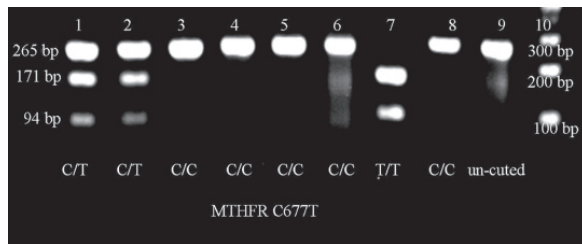
**Table 3: MTHFR C677T/A1298C combined genotype frequencies in cases and controls.**

Combined C677T/A1298C	F (%F) cases	F (%F) controls	OR (95%CI)	$\chi^2$	P-value
AC/AC	10 (16.39)	7 (13.21)	1.289 (0.45 - 3.66)	0.227	0.634
AC/CC	16 (26.23)	14 (26.42)	0.99 (0.43 - 2.28)	5E-04	0.982
AC/CT	10 (16.39)	10 (18.87)	0.843 (0.32 - 2.22)	0.12	0.729
AC/AT	11 (18.03)	10 (18.87)	0.946 (0.37 - 2.44)	0.013	0.909
AT/CT	2 (3.279)	0 (0)	-	1.769	0.184
CC/CC	8 (13.11)	6 (11.32)	1.182 (0.38 - 3.66)	0.085	0.771
AT/AT	3 (4.918)	4 (7.547)	0.634 (0.14 - 2.97)	0.34	0.56
CC/CT	1 (1.639)	1 (1.887)	0.867 (0.05 - 14.2)	0.01	0.92
CT/CT	0 (0)	1 (1.887)	0	1.161	0.281

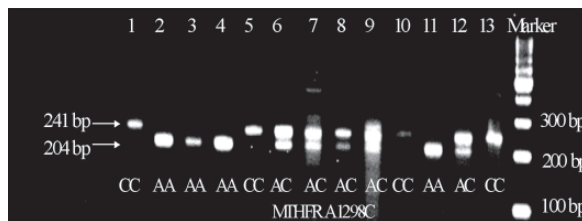
**Fig 1: Frequency of MTHFR C677T and A1298C genotype and allele frequencies in cases and controls.****Fig 2: Frequency and percentage of frequency of MTHFR C677T/A1298C combined genotype in cases and controls.**

The frequencies of C and T alleles were 0.74 and 0.26 in the patient group and 0.71 and 0.29 in healthy controls, respectively. The frequencies of C/C, T/C and T/T genotypes were 34 (55.7%), 22 (36.1%) and 5 (8.2%) in patients, whereas they were 27 (50.9%), 21 (39.6%) and 5 (9.43%) in healthy controls. The C and A allele frequencies were 0.38 and 0.62 in patients, and 0.38 and 0.62 in healthy controls. C/C, A/C and A/A genotypic distributions among patients were 9 (14.8%), 28 (45.9%) and 24 (39.3%), whereas they were 8 (15.1%), 24 (45.3%) and 21 (39.6%) in healthy controls, respectively. Alleles and genotypes were consistent with the Hardy-Weinberg equilibrium in patients (C677T:  $\chi^2=0.28$ ,  $df=2$ ,  $p=0.86$ ; and A1298C:  $\chi^2=0.03$ ,  $df=2$ ,  $p=0.98$ ) and healthy controls (C677T:  $\chi^2=0.09$ ,  $df=2$ ,  $p=0.95$ ; and A1298C:  $\chi^2=0.07$ ,  $df=2$ ,  $p=0.96$ ). Graph 2 shows the frequency and percentage of the MTHFR C677T/A1298C combined genotype among cases and controls. The compound genotype (haplotype) frequencies of the A1298C and C677T mutations in the MTHFR gene in patients versus healthy controls were: AC/AC [10 (16.39%) vs. 7 (13.21%)], AC/CC [16 (26.23%) vs. 14 (26.42%)], AC/CT [10 (16.39%) vs. 10 (18.87%)], AC/AT [11 (18.03%) vs. 10 (18.87%)], AT/CT [2 (3.279%) vs. 0 (0%)],

CC/CC [8 (13.11%) vs. 6 (11.32%)], AT/AT [3 (4.918%) vs. 4 (7.547%)], CC/CT [1 (1.639%) vs. 1 (1.887%)] and CT/CT [0 (0%) vs. 1 (1.887%)], respectively. The comparisons of the alleles, genotypes and haplotype frequencies of MTHFR, C677T and A1298C mutations between the patients and the healthy controls imply that there are no statistically significant differences. Figures 3 and 4 are representative images of the gels.



**Fig 3:** Eight samples were analyzed for MTHFR C677T mutation using *Hinf* I based RFLP-PCR by electrophoresis on a 3% agarose gel. C/C: homozygous for wild allele; C/T: heterozygous for wild allele and mutant allele; T/T: homozygous for mutant allele). (Lane 10: Marker, 100 bp DNA ladder; Lanes 3, 4, 5, 6, 8, 9: C/C genotype-[265-bp un-cut]; Lanes 1, 2: C/T genotype-(265,171,94-bp); Lane 7: T/T genotype-(94-bp).



**Fig 4:** Thirteen samples were analyzed for MTHFR A1298C mutation using *Mbo*II based RFLP-PCR by electrophoresis on a 3% agarose gel. (A/A: homozygous for wild allele; C/A: heterozygous for wild allele and mutant allele; C/C, homozygous for mutant allele). (Lane 14: Marker, 100 bp DNA ladder; Lanes 1, 5, 10, 13: C/C genotype-(241-bp); Lanes 6, 7, 8, 9, 12: C/A genotype-(241,204-bp); Lane 2, 3, 4, 11: A/A genotype-(204-bp).

### Discussion

Low levels of nutrients such as folate have been associated with abnormal epigenetic features and methylation of DNA, which leads to susceptibility and the development of human diseases (33). In the present study, we determined both the allelic and genotypic frequencies of MTHFR, C677T and A1298C polymorphisms in a control group (fertile females) and patients with unexplained RA in the Iranian Azeri Turkish (Urmia, Iran). MTHFR enzyme activity in the homozygote T/T and heterozygote C/T at position 677 MTHFR gene was 30% and 65% of the homozygote CC genotype, respectively. Interestingly, MTHFR enzyme activity in the compound heterozygote genotype for

MTHFR (C677T/A1298C) is less than carriers for 677T or the 1298C alleles in the MTHFR gene (3, 34). It has been documented that the defective form of MTHFR enzyme is a predispositional factor for RA via increased levels of homocysteine (35). Several studies have examined the association between MTHFR SNPs and risk of predisposition to RA. Some have described an association between MTHFR SNPs at positions 677 (T allele) and 1298 (C allele) and unexplained RPL (25-29). Our finding shows that differences of MTHFR C677T and A1298C alleles, genotypes and haplotype frequencies between patients with RA and healthy controls were not statistically significant. These results fail to suggest that the C677T and A1298C mutations in the MTHFR gene play a role in RA predisposition. Our analysis has also shown that carriers for 677T or 1298C alleles and individuals who are compound heterozygous for MTHFR C677T/A1298C genotypes in the MTHFR gene were more frequent in our population. In our studied groups, the frequencies of AT/CT, CC/CT and CT/CT haplotypes in MTHFR gene were fewer than other haplotypes and their distributions equaled each other. In the case of CC/CC and AT/AT haplotype frequencies in the MTHFR gene, no differences were found. Several investigations have reported no association between the C677T and A1298C mutations in the MTHFR gene and unexplained RA (36-45). Behjati et al. (2006), in a study on patients with infertility and recurrent spontaneous abortion reported that the MTHFR 677T mutation frequency was more frequent among recurrent spontaneous abortion patients compared to controls (63.1% vs. 38.7%) and the MTHFR 677T mutation in patients with infertility was not statistically different from those of controls (50.0% vs. 38.7%) (46). Yenicesu et al. analyzed 12 thrombophilic gene mutations including FV Leiden, factor V H1299R, factor II prothrombin G20210A, F XIII V34L, beta-fibrinogen -455G>A, plasminogen activator inhibitor-1, GPIIIa L33P (HPA-1 a/b L33P), MTHFR C677T, MTHFR A1298C, ACE I/D, Apo B R3500Q and Apo E in Turkish couples with RPL and reported that heterozygous mutations of FV Leiden, FXIII V34L, GPIIIa L33P, Apo E4 and prothrombin G20210A, and homozygous mutations of PAI-1 and MTHFR C677T were associated with RPL (47). According to their research, no association with RPL was noted with factor V H1299R, beta-fibrinogen -455G>A, MTHFR A1298C, ACE I/D and Apo B R3500Q (47). The etiology of RA is still unclear (48), therefore controversial findings may be the results of ethnic differences in populations (49). The role of genetic

and environmental factors in susceptibility and predisposition to RA is individual-specific which can be related to genetic polymorphisms. To the best of our knowledge, the present study is the first study on MTHFR SNPs at positions C677T and A1298C as well as allelic, genotypic and haplotypic frequencies in women with normal fertility and those with unexplained RA in Iranian Azeri Turkish.

## Conclusion

Our results imply that MTHFR C677T and A1298C SNPs have not been associated with fetal loss in the tested group.

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## References

- Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, et al. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation*. 1996; 93(1): 7-9.
- Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet*. 1995; 10(1): 111-113.
- van der Put NM, Gabreëls 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): 1044-1051.
- Christensen B, Frosst P, Lussier-Cacan S, Selhub J, Goyette P, Rosenblatt DS, et al. Correlation of a common mutation in the methylenetetrahydrofolate reductase gene with plasma homocysteine in patients with premature coronary artery disease. *Arterioscler Thromb Vasc Biol*. 1997; 17(3): 569-573.
- Friso S, Girelli D, Trabetti E, Olivieri O, Guarini P, Pignatti PF, et al. The MTHFR 1298A>C polymorphism and genomic DNA methylation in human lymphocytes. *Cancer Epidemiol Biomarkers Prev*. 2005; 14(4):938-943.
- Friso S, Choi SW. Gene-nutrient interactions and DNA methylation. *J Nutr*. 2002; 132(8 Suppl): 2382S-2387S.
- 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): 849-853.
- van der Put NM, Steegers-Theunissen RP, Frosst P, Trijbels FJ, Eskes TK, van den Heuvel LP, et al. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. *Lancet*. 1995; 346(8982): 1070-1071.
- Naushad SM, Devi AR. Role of parental folate pathway single nucleotide polymorphisms in altering the susceptibility to neural tube defects in South India. *J Perinat Med*. 2010; 38(1): 63-69.
- van der Put NM, Gabreëls 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): 1044-1051.
- Trembath D, Sherbondy AL, Vandyke DC, Shaw GM, Todoroff K, Lammer EJ, et al. Analysis of select folate pathway genes, PAX3, and human T in a Mid-western neural tube defect population. *Teratology*. 1999; 59(5): 331-341.
- Rampersaud E, Melvin EC, Siegel D, Mehlretter L, Dickerson ME, George TM, et al. Updated investigations of the role of methylenetetrahydrofolate reductase in human neural tube defects. *Clin Genet*. 2003; 63(3): 210-214.
- Volcik KA, Shaw GM, Lammer EJ, Zhu H, Finnell RH. Evaluation of infant methylenetetrahydrofolate reductase genotype, maternal vitamin use, and risk of high versus low level spina bifida defects. *Birth Defects Res A Clin Mol Teratol*. 2003; 67(3): 154-157.
- De Re V, Cannizzaro R, Canzonieri V, Cecchin E, Caggiari L, De Mattia E, et al. MTHFR polymorphisms in gastric cancer and in first-degree relatives of patients with gastric cancer. *Tumour Biol*. 2010; 31(1): 23-32.
- Agodi A, Barchitta M, Cipresso R, Marzagalli R, La Rosa N, Caruso M, et al. Distribution of p53, GST, and MTHFR polymorphisms and risk of cervical intraepithelial lesions in sicily. *Int J Gynecol Cancer*. 2010; 20(1): 141-146.
- Nakata Y, Katsuya T, Takami S, Sato N, Fu Y, Ishikawa K, et al. Methylenetetrahydrofolate reductase gene polymorphism: relation to blood pressure and cerebrovascular disease. *Am J Hypertens*. 1998; 11(8 Pt 1): 1019-1023.
- Verhoef P, Rimm EB, Hunter DJ, Chen J, Willett WC, Kelsey K, et al. A common mutation in the methylenetetrahydrofolate reductase gene and risk of coronary heart disease: results among U.S. men. *J Am Coll Cardiol*. 1998; 32(2): 353-359.
- Kluijtmans LA, Kastelein JJ, Lindemans J, Boers GH, Heil SG, Brusckhe AV, et al. Thermolabile methylenetetrahydrofolate reductase in coronary artery disease. *Circulation*. 1997; 96(8): 2573-2577.
- Bönig H, Däublin G, Schwahn B, Wendel U. Psychotic symptoms in severe MTHFR deficiency and their successful treatment with betaine. *Eur J Pediatr*. 2003; 162(3): 200-201.
- Bjelland I, Tell GS, Vollset SE, Refsum H, Ueland PM. Folate, vitamin B12, homocysteine, and the MTHFR 677C->T polymorphism in anxiety and depression: the Hordaland Homocysteine Study. *Arch Gen Psychiatry*. 2003; 60(6): 618-626.
- Kluijtmans LA, van den Heuvel LP, Boers GH, Frosst P, Stevens EM, van Oost BA, et al. Molecular genetic analysis in mild hyperhomocysteinemia: a

- common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. *Am J Hum Genet.* 1996; 58(1): 35-41.
22. Rozen R. Genetic predisposition to hyperhomocysteinemia: deficiency of methylenetetrahydrofolate reductase (MTHFR). *Thromb Haemost.* 1997; 78(1): 523-526.
23. Gallagher PM, Meleady R, Shields DC, Tan KS, McMaster D, Rozen R, et al. Homocysteine and risk of premature coronary heart disease. Evidence for a common gene mutation. *Circulation.* 1996; 94(9): 2154-2158.
24. Li SS, Li J. MTHFR gene polymorphism and male infertility. *Zhonghua Nan Ke Xue.* 2010; 16(1): 60-64.
25. Nelen WL, Steegers EA, Eskes TK, Blom HJ. Genetic risk factor for unexplained recurrent early pregnancy loss. *Lancet.* 1997; 350(9081): 861.
26. Quere I, Bellet H, Hoffer M, Janbon C, Mares P, Gris JC. A woman with five consecutive fetal deaths: case report and retrospective analysis of hyperhomocysteinemia prevalence in 100 consecutive women with recurrent miscarriages. *Fertil Steril.* 1998; 69(1): 152-154.
27. Sarig G, Younis JS, Hoffman R, Lanir N, Blumenfeld Z, Brenner B. Thrombophilia is common in women with idiopathic pregnancy loss and is associated with late pregnancy wastage. *Fertil Steril.* 2002; 77(2): 342-347.
28. Nelen WL, Blom HJ, Steegers EA, den Heijer M, Eskes TK. Hyperhomocysteinemia and recurrent early pregnancy loss: a meta-analysis. *Fertil Steril.* 2000; 74(6): 1196-1199.
29. Unfried G, Griesmacher A, Weismüller W, Nagele F, Huber JC, Tempfer CB. The C677T polymorphism of the methylenetetrahydrofolate reductase gene and idiopathic recurrent miscarriage. *Obstet Gynecol.* 2002; 99(4): 614-619.
30. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 1988; 16(3): 1215.
31. A ZC, Yang Y, Zhang SZ, Li N, Zhang W. Single nucleotide polymorphism C677T in the methylenetetrahydrofolate reductase gene might be a genetic risk factor for infertility for Chinese men with azoospermia or severe oligozoospermia. *Asian J Androl.* 2007; 9(1): 57-62.
32. Donnelly JG. The 1298(A-->C) mutation of methylenetetrahydrofolate reductase should be designated to the 1289 position of the gene. *Am J Hum Genet.* 2000; 66(2): 744-745.
33. Friso S, Choi SW. Gene-nutrient interactions in one-carbon metabolism. *Curr Drug Metab.* 2005; 6(1): 37-46.
34. Chen M, Xia B, Rodriguez-Gueant RM, Bigard M, Gueant JL. Genotypes 677TT and 677CT+1298AC of methylenetetrahydrofolate reductase are associated with the severity of ulcerative colitis in central China. *Gut.* 2005; 54(5): 733-734.
35. Mtiraoui N, Zammiti W, Ghazouani L, Braham NJ, Saidi S, Finan RR, et al. Methylenetetrahydrofolate reductase C677T and A1298C polymorphism and changes in homocysteine concentrations in women with idiopathic recurrent pregnancy losses. *Reproduction.* 2006; 131(2): 395-401.
36. Vettrisilvi V, Vijayalakshmi K, Paul SF, Venkatachalam P. ACE and MTHFR gene polymorphisms in unexplained recurrent pregnancy loss. *J Obstet Gynaecol Res.* 2008; 34(3): 301-306.
37. Ren A, Wang J. Methylenetetrahydrofolate reductase C677T polymorphism and the risk of unexplained recurrent pregnancy loss: a meta-analysis. *Fertil Steril.* 2006; 86(6): 1716-1722.
38. Grandone E, Margaglione M, Colaizzo D, d'Addetta M, D'Andrea G, Pavone G, et al. Methylenetetrahydrofolate reductase (MTHFR) 677T-->C mutation and unexplained early pregnancy loss. *Thromb Haemost.* 1998; 79(5): 1056-1057.
39. Carp H, Salomon O, Seidman D, Dardik R, Rosenberg N, Inbal A. Prevalence of genetic markers for thrombophilia in recurrent pregnancy loss. *Hum Reprod.* 2002; 17(6): 1633-1637.
40. Foka ZJ, Lambropoulos AF, Saravelos H, Karas GB, Karavida A, Agorastos T, et al. Factor V Leiden and prothrombin G20210A mutations, but not methylenetetrahydrofolate reductase C677T, are associated with recurrent miscarriages. *Hum Reprod.* 2000; 15(2): 458-462.
41. Holmes ZR, Regan L, Chilcott I, Cohen H. The C677T MTHFR gene mutation is not predictive of risk for recurrent fetal loss. *Br J Haematol.* 1999; 105(1): 98-101.
42. Kutteh WH, Park VM, Deitcher SR. Hypercoagulable state mutation analysis in white patients with early first-trimester recurrent pregnancy loss. *Fertil Steril.* 1999; 71(6): 1048-1053.
43. Makino A, Nakanishi T, Sugiura-Ogasawara M, Ozaki Y, Suzumori N, Suzumori K. No association of C677T methylenetetrahydrofolate reductase and an endothelial nitric oxide synthase polymorphism with recurrent pregnancy loss. *Am J Reprod Immunol.* 2004; 52(1): 60-66.
44. Rey E, Kahn SR, David M, Shrier I. Thrombophilic disorders and fetal loss: a meta-analysis. *Lancet.* 2003; 361(9361): 901-908.
45. Robertson L, Wu O, Langhorne P, Twaddle S, Clark P, Lowe GD, et al. Thrombophilia in pregnancy: a systematic review. *Br J Haematol.* 2006; 132(2): 171-196.
46. 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): 268-271.
47. 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): 126-136.
48. Nelen WL, Blom HJ, Thomas CM, Steegers EA, Boers GH, Eskes TK. Methylenetetrahydrofolate reductase polymorphism affects the change in homocysteine and folate concentrations resulting from low dose folic acid supplementation in women with unexplained recurrent miscarriages. *J Nutr.* 1998; 128(8): 1336-1341.
49. Peng F, Labelle LA, Rainey BJ, Tsongalis GJ. Single nucleotide polymorphisms in the methylenetetrahydrofolate reductase gene are common in US Caucasian and Hispanic American populations. *Int J Mol Med.* 2001; 8(5): 509-511.