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Polymorphisms of Genes Involved in the Folate Metabolic Pathway Impact the Occurrence of Unexplained Recurrent Pregnancy Loss

Li Luo, MS[1], Yueming Chen, MS[2], Li Wang, BS[2], Guangchao Zhuo, MS[2], Chunning Qiu, BS[2], Qiaofeng Tu, BS[2], Jin Mei, MS[3], Wen Zhang, MS[3], Xia Qian, MS[3], and Xianjun Wang, MS[1,2]

Abstract

Low levels of folate combined with high levels of homocysteine may cause unexplained recurrent pregnancy loss (URPL). However, the relationships between polymorphisms in genes of the folate metabolic pathway and URPL remain controversial. We conducted a case-control study to explore polymorphisms of the major folate pathway genes, including methylenetetrahydrofolate reductase (MTHFR) 677C>T, MTHFR 1298A>C, methionine synthase (MTR) 2756A>G, methionine synthase reductase (MTRR) 66A>G and reduced folate carrier 1 (RFC-1) 80A>G, and their associations with URPL. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine the distributions of MTHFR, MTR and RFC-1 polymorphisms, and the results were validated using direct sequencing. The polymorphisms in MTRR were determined using direct sequencing. Haplotypes were analyzed using SHEsis, an online tool for biological analysis. We found that the MTHFR 677T allele and the 677T/1298A/2756A/66A/80G haplotype were risk factors for URPL, while the MTR 2756G allele and the 677C/1298A/2756A/66A/80A haplotype exhibited protective effects on susceptibility to URPL in a Chinese Han population from the Hangzhou area.

Keywords

folate metabolism, unexplained recurrent pregnancy loss, gene polymorphism, haplotype

Introduction

Recurrent pregnancy loss (RPL) refers to 2 or more consecutive spontaneous abortions, which occur in 1% to 2% of women of childbearing age.[1,2] There are many causes of RPL, including chromosomal abnormalities, anatomic malformations, endocrine and metabolic imbalances, antiphospholipid antibody syndrome, hypothyroidism, autoimmune diseases, coagulant function abnormalities, exposure to poisons, reproductive tract infections, environmental factors, and so on.[3,4] Recurrent pregnancy loss with no clear cause is often called unexplained recurrent pregnancy loss (URPL).

Regarding the etiologies of URPL, a number of studies have shown that a low level of folate and a high level of homocysteine in the folate metabolic pathway were significantly associated with URPL.[5-8] The maintenance of normal levels of folate is dependent on food supply. Consumption of foods that lack folate, absorption disorders, and mutations in genes involved in metabolism could contribute to low levels of folate in some women. Some mutations in genes involved in the folate metabolic pathway have been associated with a low level of folate and a high level of homocysteine, such as polymorphisms of methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), methionine synthase reductase (MTRR), and reduced folate carrier 1 (RFC-1). Hence, it has been suggested that some genetic polymorphisms in genes of the folate metabolic pathway might impact the occurrence of URPL.

Compared with the MTHFR 677CC genotype, carriers of the MTHFR 677TT genotype showed approximately 30% of

[1] School of Laboratory Medicine & Life Science, Wenzhou Medical University, Wenzhou, China
[2] Department of Laboratory Medicine, Hangzhou First People's Hospital, Hangzhou, China
[3] Department of Obstetrics/Gynaecology and Genetics, Hangzhou First People's Hospital, Hangzhou, China

Corresponding Author:
Xianjun Wang, School of Laboratory Medicine & Life Science, Wenzhou Medical University, Wenzhou 325035, China; Department of Laboratory Medicine, Hangzhou First People's Hospital, Hangzhou, 310001, China.
Email: wangxj0525@126.com

Table 1. The Etiologies of Recurrent Pregnancy Loss (RPL) and the Number of Previous Pregnancy Losses.

Recurrent Pregnancy Loss (RPL)	n	Number of Previous Pregnancy Losses, n	
		2	≥3
Explained recurrent pregnancy loss	11	5	6
Abnormal chromosomal carrier	3	0	3
Uterine anomalies (arcuate or septate)	3	1	2
Antiphospholipid antibody syndrome	1	0	1
Polycystic ovary syndrome	2	2	0
Vesicular mole	2	2	0
Unexplained recurrent pregnancy loss (URPL)	125	88	37

enzyme activity, while carriers of the MTHFR 677CT genotype displayed almost 65% of normal enzyme activity.[9] Reduced enzymatic activity could lead to dysfunction in the conversion of 5′10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in vivo. Methionine synthase, which is a cobalamin (VB12)-dependent enzyme, combined with MTRR, which is required for the maintenance of MTR activity, plays an important role in the conversion of homocysteine into methionine. The RFC-1 is the primary carrier that transports 5-methyltetrahydrofolate into the cell, and an A80G mutation results in low levels of 5-methyltetrahydrofolate in cells. Dufficy et al showed that the amount of folate transported into cells was negatively correlated with the RFC-1 80G allele.[10] Low levels of 5-methyltetrahydrofolate could inhibit the conversion of homocysteine into methionine, which in turn could cause elevated levels of homocysteine in vivo. Thrombophilia, vascular endothelial cell injury, and toxic effects on the embryo could result from a high level of homocysteine and could result in pregnancy loss.[11-13] At the same time, decreased levels of intracellular 5-methyltetrahydrofolate could result in low levels of *S*-adenosylmethionine, a direct methyl donor, which could in turn affect the methylation processes of DNA, proteins, and lipids.

However, many studies of gene variants in the folate metabolic pathway and URPL have mostly been concerned with the polymorphisms of MTHFR 677C>T and/or MTHFR 1298A>C; these studies have ignored the other relevant genes in the folate metabolic pathway.[5,14,15] In addition, the association between the polymorphism of MTHFR 677C>T with URPL has remained controversial.[14,15] With regard to the different distributions of the polymorphisms of genes involved in folate metabolism in people from different regions,[5,14-16] we performed a case–control study to ascertain the associations between polymorphisms in these genes with URPL in a Chinese Han population from the Hangzhou area.

Materials and Methods

Participants

A total of 136 women who had 2 or more consecutive spontaneous abortions, including first, second, or third trimester losses, were recruited for this study from the outpatient Department of Obstetrics/Gynaecology and Genetics at Hangzhou First People's Hospital. All participants were enrolled prior to 12 weeks of gestation.

The patients underwent standardized clinical and laboratory tests, which included evaluations of the following: parental karyotype analysis, hysteroscopy (prior to the current pregnancy), protein C, protein S, coagulation, sex hormones, thyroid function, human chorionic gonadotropin, genital tract secretions (eg, *Ureaplasma urealyticum*, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae*), HIV antibodies, syphilis antibodies, TORCH, antinuclear antibodies, antineutrophil antibodies, and hepatitis B virus. In addition, semen examinations of the participant's husbands were normal. Eleven patients who showed 1 or more anomalies during the investigation were excluded. The etiologies of RPL and the numbers of previous spontaneous abortions are shown in Table 1. The remaining 125 women with URPL without any explainable etiology were included in the present study. The control participants were 135 women who met the following criteria: more than 1 normal-term delivery of a single live birth at the Hangzhou First People's Hospital, no history of spontaneous abortion, no gestational hypertension, no diabetes, no ectopic pregnancy, no history of preterm birth, no thromboembolic disease, and no family history of genetic disease. Informed consent was provided by the participants prior to their participation in the study, which was approved by Medical Ethics Committee of Hangzhou First People's Hospital.

The average ages of the cases and controls were 30.89 $\pm$ 4.3 years (mean $\pm$ standard deviation; range, 23-43 years) and 29.4 $\pm$ 3.3 years (mean $\pm$ standard deviation; range, 21-41 years), respectively. No significant difference in age was found between the 2 groups ($P > .05$).

Genotyping

Genomic DNA was extracted from 200 μL of whole blood using a DNA Extractor Kit (Axygen, California). The purified DNA was dissolved in 150 μL of Tris-EDTA buffer and was stored at −20°C until the assay was performed. The 4 polymorphisms, MTHFR 677C>T, MTHFR 1298A>C, MTR 2756A>G, and RFC-1 80A>G, were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis. Polymerase chain reactions were

performed in a final volume of 50 μL with a reaction mixture containing 5 μL 10× buffer (Mg^{2+} free), 800 μmol/L deoxynucleotide triphosphates, 1.5 mmol/L $MgCl_2$, 200 pmol each primer, 5 U Taq polymerase, and 5 μL (400 ng) of template DNA; deionized water was added to reach a final volume (Takara, Otsu, Shiga, Japan). Table 2 lists the amplification reaction programs for each polymorphism. Finally, a restriction endonuclease reaction was used to analyze the genotypes. The enzyme reaction mixture contained 17 μL of the PCR products, 2 μL of 10× buffer, and 1 μL of restriction endonuclease (Thermo Scientific, Massachusetts; 10 U/μL) in a total volume of 20 μL; the reactions were incubated for 16 hours at 37°C. The RFLP products were run on 3% agarose gel at 70 V for 2 hours; then, the digested bands were observed under a ultraviolet gel imager (BioRad, California). The PCR reaction conditions used to amplify MTRR 66A>G were the same as those used for MTHFR 677C>T; however, the amplification protocol differed, as shown in Table 2. The MTRR genotypes were analyzed by direct sequencing with the same primers that were used for PCR (Invitrogen, California).

Validation by Sequencing

We randomly selected 40 samples from each group for direct sequencing (Invitrogen) with the PCR amplification primers to validate the MTHFR 677C>T, MTHFR 1298A>C, MTR 2756A>G, and RFC-1 80A>G genotypes that were obtained by the RFLP analysis. The sequences were aligned and analyzed with Lasergene sequencing analysis software (DNAStar, Madison, Wisconsin).

Statistical Analyses

Hardy-Weinberg equilibrium analyses were performed to compare the observed and expected genotype frequencies using the chi-square (χ^2) test. The distributions of the alleles and genotypes among the groups were evaluated using the χ^2 test. Odds ratios (ORs) were calculated and are presented within the 95% confidence intervals (95% CIs). Haplotypes were analyzed using SHEsis, an online software tool for biological analysis (http://analysis.bio-x.cn/myAnalysis.php). P values <.05 were considered statistically significant. Statistical analyses were conducted using the SPSS software package, version 17.0 (IBM, New York).

Results

Allele and Genotype Frequencies of the 5 Loci

We recruited 125 patients with URPL for this study, including women who experienced spontaneous abortion (n = 24), inevitable abortion (n = 83), missed abortions (n = 7), cessation of intrauterine growth and development (n = 9), and stillbirths (n = 2); biochemical pregnancies, however, were excluded.

The results of the PCR-RFLP analysis exactly matched those of the sequencing analysis, indicating that the genotyping results in this study were reliable. All of the allele and genotype frequencies of the 5 loci were in Hardy-Weinberg equilibrium (P > .05). Table 3 shows the frequencies of the 5 polymorphic alleles and genotypes in the cases and in the controls. The distributions of the C and T alleles of MTHFR 677 were significantly different between the 2 groups; the T allele frequency distribution in the case group was significantly higher than that in the control group (OR = 1.451, 95% CI = 1.012-2.081, P = .043). In addition, the prevalence of the CT+TT genotype in the case group was significantly higher than in the control group (OR = 1.700, 95% CI = 1.024-2.821, P = .039). However, we found no differences when we performed pairwise comparisons of the CC and CT, CC and TT, and CT and TT genotypes for the MTHFR 677C>T polymorphism. In addition, the frequencies of the A and G alleles of MTR 2756 showed a statistically significant difference between the 2 groups; the G allele frequency distribution in the test case group was significantly lower than that in the control group (OR = 0.509, 95% CI = 0.272-0.952, P = .032).

No differences were found in the MTHFR 1298A>C, MTRR 66A>G, or RFC-1 80A>G polymorphisms between the 2 groups (P > .05).

Haplotype Analysis of the Cross-Effects of 5 Loci

We used SHEsis, an online software program for haplotype analyses, to compare the cross-effects between the 2 groups. Table 4 shows that the 677C/1298A/2756A/66A/80A haplotype was a protective factor against URPL (OR = 0.237, 95% CI = 0.156-0.361, P < .001) and that the 677T/1298A/2756A/66A/80G haplotype was a risk factor for URPL (OR = 5.749, 95% CI = 2.997-11.029, P < .001).

Discussion

In the present study, we found that MTHFR 677T was associated with URPL and that the T allele increased the risk of URPL (OR = 1.451, 95% CI = 1.012-2.081, P = .043). Additionally, the frequency of the CT+TT genotype in the case group was significantly higher (OR = 1.700, 95% CI = 1.024-2.821, P = .039) than that in the control group. This result was consistent with the meta-analyses of Ren and Wang[17] and Cao et al,[18] who showed that MTHFR 677T was a genetic risk factor for URPL in a Chinese population. Rohini study noted that MTHFR 677C>T might be an early genetic screening indicator for URPL in a northern Indian population.[5] In addition, Baumann reported that MTHFR 677C>T could play a role in the pathophysiological mechanism of URPL.[7]

Different results regarding the relationship between MTHFR 677C>T and URPL have been reported. A case–control study conducted in Egyptian women showed that, although the frequency of the TT genotype was higher in the case group compared with the control group, the difference was not statistically significant.[15] Another study conducted in Japanese women also showed no association of the MTHFR 677C>T polymorphism with URPL.[14] These controversial results might have resulted from the diverse ethnicities of the women

Table 2. PCR-RFLP/Direct Sequencing Parameters for the Detection of 5 Candidate Loci.

Locus	Forward Primer	Reverse Primer	PCR Program	Restriction Endonuclease/ Direct Sequencing
MTHFR 677C>T	5′-TGAAGGAGAAGGTGTCTGCGGGA-3′	5′-AGGACGGTGCGGTGAGAGTG-3′	95°C 5 min, 40× (95°C 30 s, 59°C 30 s, 72°C 30 s), 72°C 5 min	H*inf*I
MTHFR 1298A>C	5′-AAGGAGGAGCTGCTGAAGATG-3′	5′-CTTTGCCATGTCCACAGCATG-3′	95°C 5 min, 40× (95°C 30 s, 51°C 30 s, 72°C 30 s), 72°C 5 min	M*bo*II
MTR 2756A>G	5′-TGTTCCCAGCTGTTAGATGAAAATC-3′	5′-GATCCAAAGCCTTTTACACTCCTC-3′	95°C 5 min, 40× (95°C 30 s, 52°C 30 s, 72°C 30 s), 72°C 5 min	H*ae*III
MTRR 66A>G	5′-CAGGCAAAGGCCATCGCAGAAGA-3′	5′-CACTTCCCAACCAAAATTCTTCAAAG-3′	95°C 5 min, 40× (95°C 30 s, 52°C 30 s, 72°C 30 s), 72°C 5 min	Direct sequencing
RFC-1 80G>A	5′-AGTGTCACCTTCGTCCCCTC-3′	5′-CTCCCGCGTGAAGTT CTT-3′	95°C 5 min, 40× (95°C 30 s, 60°C 30 s, 72°C 30 s), 72°C 7 min	H*ha*I

Abbreviations: MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; RFC-1, reduced folate carrier 1; PCR-RFLP, polymerase chain reaction–restriction fragment length polymorphism; min, minutes; s, seconds.

Table 3. Genotype Frequencies and Allele Frequencies of 5 Single-Nucleotide Polymorphisms (SNPs) Between the Test Cases and Control Groups.

Locus	Case (n = 125), n (%)	Control (n = 135), n (%)	OR (95% CI)	*P* Value
MTHFR 677C>T				
C	150 (60.0)	185 (68.5)	1	
T	100 (40.0)	85 (31.5)	1.451 (1.012-2.081)	0.043
CC	40 (32.0)	60 (44.5)	1	
CT	70 (56.0)	65 (48.1)	1.615 (0.957-2.727)	0.072
TT	15 (12.0)	10 (7.4)	2.250 (0.920-5.504)	0.072
CT+TT	85 (68.0)	75 (55.5)	1.700 (1.024-2.821)	0.039
MTHFR 1298A>C				
A	204 (81.6)	210 (77.8)	1	
C	46 (18.4)	60 (22.2)	0.789 (0.514-1.213)	0.28
AA	82 (65.6)	78 (57.8)	1	
AC	40 (32.0)	54 (40.0)	0.705 (0.422-1.177)	0.18
CC	3 (2.4)	3 (2.2)	0.951 (0.186-4.855)	0.952
MTR 2756A>G				
A	234 (93.6)	238 (88.1)	1	
G	16 (6.4)	32 (11.9)	0.509 (0.272-0.952)	0.032
AA	109 (87.2)	106 (78.5)	1	
AG	16 (12.8)	26 (19.3)	0.598 (0.304-1.179)	0.135
GG	0 (0.0)	3 (2.2)	–[a]	–[a]
AG+GG	16 (12.8)	29 (21.5)	0.537 (0.276-1.045)	0.064
MTRR 66A>G				
A	193 (77.2)	190 (70.4)	1	
G	57 (22.8)	80 (29.6)	0.701 (0.473-1.041)	0.077
AA	77 (61.6)	68 (50.4)	1	
AG	39 (31.2)	54 (40.0)	0.638 (0.377-1.079)	0.093
GG	9 (7.2)	13 (9.6)	0.611 (0.246-1.519)	0.286
RFC-1 80A>G				
A	114 (45.6)	130 (48.1)	1	
G	136 (54.4)	140 (51.9)	1.108 (0.785-1.564)	0.561
AA	22 (17.6)	26 (19.3)	1	
AG	70 (56.0)	78 (57.8)	1.061 (0.552-2.038)	0.86
GG	33 (26.4)	31 (22.9)	1.258 (0.594-2.664)	0.548

Abbreviations: CI, confidence interval; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; RFC-1, reduced folate carrier 1; OR, odds ratio.

[a]Statistical analyses were not performed because of limited data.

Table 4. Haplotype Analysis of the 5 Loci.

Haplotype	Case (n = 250), n (%)	Control (n = 270), n (%)	*P* Value	OR (95% CI)
677C/1298A/2756A/66A/80A	40 (16.0)	123 (45.6)	<.001	0.237 (0.156-0.361)
677C/1298A/2756A/66A/80G	45 (18.0)	56 (20.7)	.596	0.888 (0.571-1.379)
677T/1298A/2756A/66A/80G	51 (20.4)	12 (4.4)	<.001	5.749 (2.997-11.029)

Abbreviations: CI, confidence interval; OR, odds ratio.

involved, as well as the different geographical regions that were represented in these studies.

Methionine synthase reductase helps to maintain the activity of MTR in the folate metabolic pathway, and both enzymes play important roles in the maintenance of homocysteine levels.[19] Our study showed that MTR 2756G was a protective factor against URPL in women of childbearing age (OR = 0.509, 95% CI = 0.272-0.952, $P = .032$). Currently, this result has rarely been reported in the literature. However, a number of studies have suggested that MTR 2756G was a risk factor for malignancy. The mechanism involves reduced synthesis of methionine, a low level of *S*-adenosylmethionine, and DNA hypomethylation, all of which result in DNA damage and chromosomal instability.[20-22] However, the protective mechanism of MTR 2756G against URPL requires further clarification.

A haplotype analysis of 5 polymorphic loci showed that the 677T/1298A/2756A/66A/80G haplotype could increase the risk of URPL (OR = 5.749, 95% CI = 2.997-11.029, $P < .001$), while the 677C/1298A/2756A/66A/80A haplotype could reduce the risk of URPL (OR = 0.237, 95% CI = 0.156-0.361,

$P < .001$). The results of the haplotype analysis suggested that gene–gene interactions could significantly impact the occurrence of URPL. However, the relationship between haplotype and URPL must be further elucidated to obtain a more accurate assessment of the risk of URPL.

Additionally, serum homocysteine levels have been shown to be involved in URPL and are associated with the MTHFR 677C>T polymorphism.[9,23,24] In general, the evidence shows that individuals with the MTHFR 677TT genotype have almost double the homocysteine levels as individuals carrying the MTHFR 677CT or 677CC genotypes.[9] Therefore, we did not explore the relationship between serum homocysteine levels and URPL in this study.

In summary, this study showed that individuals, specifically women of Chinese Han nationality in Hangzhou, who carry the MTHFR 677T allele and the 677T/1298A/2756A/66A/80G haplotype could be at an increased risk of URPL. In contrast, women who carry the MTR 2756G allele and the 677C/1298A/2756A/66A/80A haplotype might be protected from URPL. We suggested that MTHFR 677C>T was an early genetic screening indicator for URPL and that individuals with a CT or TT genotype should increase their intake of folic acid supplements to prevent miscarriage.

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Authors' Note

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Declaration of Conflicting Interests

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