

## Original Article

# Relationship of folate metabolism related enzymes MTHFR and MTRR gene polymorphisms with unexplained recurrent spontaneous abortion

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**Abstract:** The role of methylenetetrahydrofolate reductase (MTHFR) C677T, A1298C, methioninesynthase reductase (MTRR) A66G was unclear in repeated spontaneous abortion. To provide experimental basis for etiological diagnosis and treatment of recurrent spontaneous abortion, we selected 197 patients with recurrent spontaneous abortion and 116 normal women, extracting the oral mucosal epithelial cells and detect to the MTHFR gene C677T, A1298C and MTRR gene loci of A66G single nucleotide polymorphisms. The result showed that compared with the control group, the distribution of C677T MTHFR allele in URSA group was statistically significant ( $P < 0.05$ ). The frequency of TT allele was higher than that of the control group, and the frequency of CT allele was lower than that of the control group. MTHFR A1298C and MTRR mutation frequency between the URSA group and the control group no significant difference ( $P > 0.05$ ); URSA group the activity of MTHFR, red cell folate and plasma folate levels lower than control group ( $P < 0.05$ ) and homocysteine levels higher than control group ( $P < 0.05$ ); URSA patients in different ages of human serum folate and red cell folate, homocysteine homocysteine levels does not exist significant difference ( $P > 0.05$ ). We considered that the mutation of C677T gene in MTHFR gene is associated with the risk of URSA, which suggests that TT allele may be a susceptibility gene of URSA. URSA group in age and plasma folate and red cell folate and homocysteine homocysteine levels is not relevant, which for pregnant and maternal folic acid supplement guidance and monitoring, so as to further reduce recurrent spontaneous abortion rate has important significance.

**Keywords:** Unexplained recurrent spontaneous abortion, 5,10-methylenetetrahydrofolate reductase, methionine-synthase reductase, hyperhomocysteine, single nucleotide polymorphism

## Introduction

Recurrent spontaneous abortion (RSA) refers to the abortion that occurs for 2 or more consecutive times. Its incidence accounts for 1%-2% of total pregnancy and 15%-20% of natural abortion [1]. The pathogeneses of RSA are very complex, including chromosomal abnormalities, endocrine abnormalities, anatomical factors, blood group incompatibility, immune factors, infection and even mental factors. At present, there are still 40%-80% of patients with unknown pathogeneses, namely unexplained recurrent spontaneous abortion (URSA) [2] is the difficult miscellaneous disease in Obstetrics and Gynecology. Currently, many scholars have focused on the relationship between polymorphism of thrombophilic gene,

Methylenetetrahydrofolate (MTHFR) and Methionine synthase reductase (MTRR) genes and URSA [3]. By studying the polymorphism of MTHFR C677T genetic locus, A1298C genetic locus and MTRR A66G genetic locus related to folic acid metabolism, this paper explores their relationships with erythrocyte folic acid, plasma folic acid, HCY and URSA, which is of important significance for guiding and monitoring folic acid supplementation before and during pregnancy, and thus further reducing the rate of RSA.

## Materials and methods

### Research objects

**URSA Group:** 197 non-pregnant patients who saw doctors in the Eugenics Genetic Counseling

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**Table 1.** Taqman-MGB primer (refer to kit)

MTHFR C677T-F	MTHFR C677T-R
GAAAAGCTGCGTGATGATG	TTGAAGGAGAAGGTGTC
MTHFR A1298C-F	MTHFR A1298C-R
AAGAACGAAGACTTCAAA	TGGGGGGAGGAGCTGAC
MTRR A66G-F	MTRR A66G-R
AGGCAAAGGCCATCGCA	ATCCATGTACCACAGCTT

**Table 2.** Taqman-MGB probe gene sequence (refer to kit)

MTHFR C677T-V2	MTHFR C677T-M2
AATCGGCTCCCGC	AATCGACTCCCGC
MTHFR A1298C-V2	MTHFR A1298C-M2
ACACTTGCTTCACT	ACACTTTCTTCACT
MTRR A66G-V2	MTRR A66G-M2
AAGAAATATGTGAG	AAGAAATGTGTGAG

**Table 3.** Age distribution of URSA group and control group

Age	URSA Group Cases (%)	Control Group Cases (%)
< 5	21 (10.64)	17 (4.65)
25~	59 (29.79)	31 (26.73)
30~	54 (27.66)	27 (23.28)
35~	46 (23.4)	30 (25.86)
40~	17 (8.5)	11 (9.48)
Total	197 (100)	116 (100)

Clinic of General Hospital of Tianjin Medical University and Screening Department of Tianjin Women and Children Health Center from January 1 2013 to December 31 2014 were selected; they were at the age of 20-41 and at the average age of 30.17. They met the following conditions: (1) spontaneous abortion  $\geq$  2 times, with or without a history of normal childbirth; (2) normal PB karyotype analysis and no history of familial genetic disease; (3) no genital anatomy deformity through Doppler ultrasound, hysterosalpingography and other diagnosis; (4) normal endocrine through examination, including normal menstrual cycle, diphasic nasal body temperature, ovulation detection, normal thyroid function, no history of diabetes; (5) elimination of mycoplasma, chlamydia, toxoplasma gondii, cytomegalovirus, syphilis, simple herpes viruses and other pathogens; (6) negative anti-nuclear antibodies, antiphospholipid antibodies, anti-sperm antibodies and other immune-

related antibodies; (7) no history of venous thrombosis, no liver and kidney acute and chronic diseases; (8) no vitamin supplementary therapy within recent three months.

*Control group:* 116 female workers and volunteers of Tianjin Women and Children Health Center at the age of 21-43 and the average age of 31.43 were selected, with healthy non-pregnancy or at least one successful pregnancy, no history of natural abortion, embryo damage, fetal growth restriction, pregnancy-induced hypertension and intrauterine fetal death as well as thrombotic disease.

### *DNA extraction and genotyping*

The sterile cotton swab was used to repeatedly scrap epithelial cells of oral mucosa (scrap up and down at one side in the month with the strength of slight bump), and then the sample DNA was extracted with the column extraction kit.

The PCR amplification primers and probes of MTHFR C677T and A1298C genetic loci and MTRR A66G genetic locus are shown in **Tables 1 and 2**. Related instruments and reagents are the products of US Applied Biosystems (ABI). The experimental method was based on the kit instructions. The fluorogenic quantitative PCR was respectively conducted for three loci. PCR system (10  $\mu$ L), included DNA template (20 ng/ $\mu$ L) 1  $\mu$ L, Taqman Universal Master Mix 5  $\mu$ L, Taqman-MGB probe 0.5  $\mu$ L, deionized water 3.5  $\mu$ L, PCR reaction condition (two-step method): 95°C initial denaturation for 10 min, 92°C denaturation 15 s, 60°C annealing extension 1 min, 20 cycles; 89°C denaturation 15 s, 60°C annealing extension 90 s, 30 cycles. After reaction, read the ending fluorescence of sample hole on ABI7900 fluorescence quantitative PCR, and analyzed the genotyping results of each sample with software.

### *Detection of homocysteine, erythrocyte folic acid and plasma folic acid*

Five ml of fasting venous blood was taken with EDTA-K2 anticoagulant vacuum blood collection tube and heparin anticoagulant vacuum blood collection tube, respectively. And then fully mixed, and centrifuged within 40 min of blood collection at 3500 rpm/min for 15 min. After centrifugation, immediately plasma was

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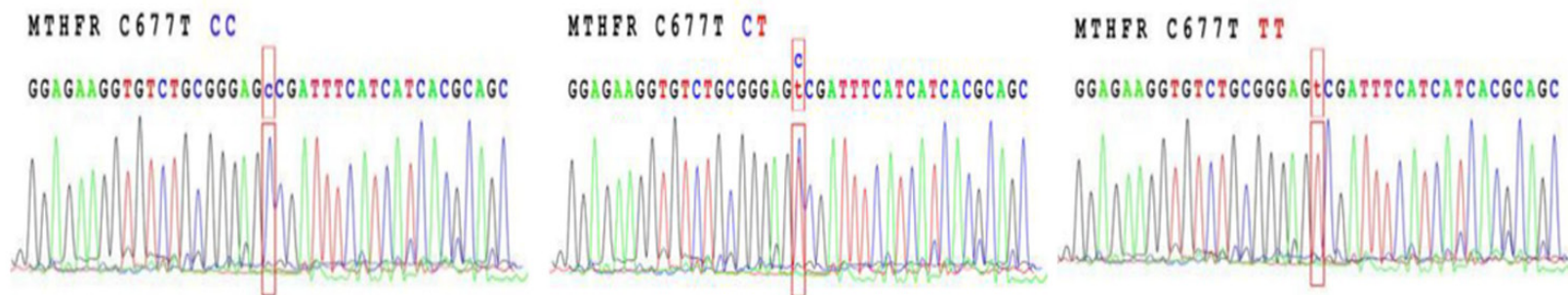


Figure 1. CC, CT and TT, three gene types of MTHFR C677T.

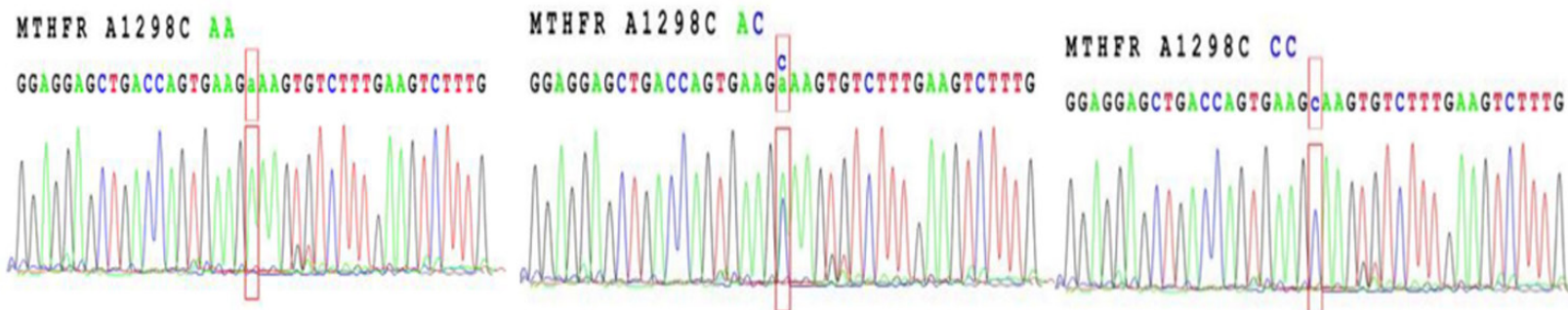


Figure 2. AA, AC and CC, three gene types of MTHFR A1298C.

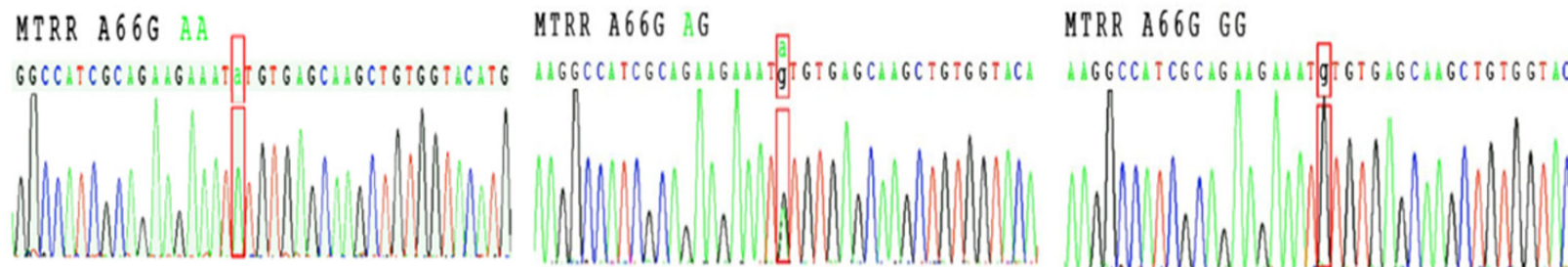


Figure 3. AA, AG and GG, three gene types of MTRR A66G.

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**Table 4.** MTHFR C677T proportion (%) in URSA group and control group

Group	Cases	MTHFR C677T gene		
		CC	CT	TT
URSA group	197 (100)	54 (27.5)	74 (37.5)	69 (35)
Control group	116 (100)	23 (19.8)	62 (53.4)	31 (26.7)
		7.521		
<i>P</i>		0.023		

separated and all specimens were stored at -20°C to avoid repeated freezing and thawing.

The microbial culture method was used to detect plasma folic acid and erythrocyte folic acid. The used detection microorganism was *Lactobacillus*. The Homocysteine was detected by Toshiba TBA-120FR fully-automatic biochemical analyser.

### Statistical analysis

The data was processed with SPSS19.0 software, the measurement data was expressed by mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ), and group comparison was analyzed by variance. The count data was expressed by rate or proportion, and chi square test was used for group comparison. Chi square test was adopted for the genotype and gene frequency between two groups, and  $P < 0.05$  was considered as the indication of great statistic significance.

## Results

### Age distribution of URSA group and control group

There were totally 313 study cases, including 197 cases in the URSA group and 116 cases in the control group. In URSA group, the age was 20-41, and the average age was 30.17. In control group, the age was 21-43 and the average age was 31.43. The difference in age distribution between two groups was of no statistical significance ( $\chi^2 = 1.95$ ,  $P = 0.74$ ,  $P > 0.05$ ), as shown in **Table 3**.

### MTHFR C677T, A1298C and MTRR A66G polymorphism

MTHFR C677T gene includes three gene types, namely CC, CT and TT, as shown in **Figure 1**; MTHFR A1298C gene includes three gene types, namely AA, AC and CC, as shown in

**Figure 2**; MTRR A66G gene includes three gene types, namely AA, AG and GG, as shown in **Figure 3**.

### Distribution frequency of MTHFR C677T, A1298C and MTRR A66G genes in URSA group and control group

The difference in gene type distribution of MTHFR C677T between URSA group and control group was of statistical significance,  $\chi^2 = 7.521$ ,  $P$  (C677T) = 0.023 < 0.05. The TT gene type in URSA group accounted for 35.0%, higher than 26.7% in control group; while CT gene type in URSA group accounted for 37.5%, lower than 53.4% in control group, as shown in **Table 4**.

There was no significant difference between the MTHFR A1298C and MTRR A66G distribution frequencies of URSA group and control group  $P$  (A1298C) = 0.196 > 0.05,  $P$  (A66G) = 0.669 > 0.05, as shown in **Table 5**.

### Comparison of MTHFR activity, plasma homocysteine, erythrocyte folic acid and plasma folic acid concentration between URSA group and control group

Compared URSA group with control group, and established the single-factor variance analysis model. Under the 95% of confidence interval,  $P$  (MTHFR activity) = 0.026,  $P$  (plasma folic acid) = 0.000,  $P$  (erythrocyte folic acid) = 0.000,  $P$  (homocysteine) = 0.000, as shown in **Table 6**. Results showed that there was a significant difference in MTHFR activity, plasma homocysteine, erythrocyte folic acid and plasma folic acid ( $P < 0.05$ ).

### Relationships between age and plasma folic acid, erythrocyte folic acid and homocysteine in URSA group

In URSA group, there was no significant difference in plasma folic acid, erythrocyte folic acid and homocysteine between people at different ages in URSA group ( $P > 0.05$ ). Under the 95% of confidence interval,  $P$  (plasma folic acid) = 0.274,  $P$  (erythrocyte folic acid) = 0.578,  $P$  (homocysteine) = 0.331, as shown in **Table 7**.

## Discussion

Due to the reduction of enzymatic activity caused by gene mutation of folic acid metabolism

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**Table 5.** MTHFR A1298C and MTRR A66G proportion (%)

Group	Cases	MTHFR A1298C gene			MTRR A66G		
		AA	AC	CC	AA	AG	GG
URSA group	197 (100)	125 (63.5)	67 (34)	5 (2.5)	115 (58.4)	70 (35.5)	12 (6.1)
Control group	116 (100)	82 (70.7)	29 (25)	5 (4.3)	64 (55.2)	42 (36.2)	10 (8.6)
$\chi^2$			3.257			0.805	
P			0.196			0.669	

**Table 6.** Comparison of mthfr activity, plasma homocysteine, erythrocyte folic acid and plasma folic acid concentration between URSA group and control group

	MTHFR activity %	Plasma homocysteine ( $\mu\text{mol/L}$ )	Erythrocyte folic acid ( $\mu\text{g/L}$ )	Plasma folic acid ( $\mu\text{g/L}$ )
URSA group	44.46 $\pm$ 22.54	11.91 $\pm$ 4.9	330.21 $\pm$ 106.55	8.90 $\pm$ 4.99
Control group	54 $\pm$ 25	8.73 $\pm$ 5.07	516.12 $\pm$ 165.37	16.19 $\pm$ 6.94
F	5.041	13.751	72.594	56.329
P	0.026	0.000	0.000	0.000

**Table 7.** Relationships between age and plasma folic acid, erythrocyte folic acid and homocysteine in URSA group

	Plasma homocysteine ( $\mu\text{mol/L}$ )	Erythrocyte folic acid ( $\mu\text{g/L}$ )	Plasma folic acid ( $\mu\text{g/L}$ )
< 35 years old	10.08 $\pm$ 5.45	445.1 $\pm$ 188.3	13.52 $\pm$ 7.16
$\geq$ 35 years old	10.19 $\pm$ 3.98	410.1 $\pm$ 141.18	10.19 $\pm$ 6.7
F	1.187	0.893	1.275
P	0.331	0.578	0.274

enzyme MTHFR, the methylation of DNA and protein necessary for embryonic development is insufficient, fetal malformation or URSA occurs, the concentration of plasma homocysteine increases, the vascular endothelial cell damage and toxic effect may be caused, and meanwhile, the adhesion and aggregation of blood platelet is activated, resulting in placental vascular thrombotic disease. Therefore, MTHFR gene mutation is the risk factor of neural tube defect and URSA [4, 5].

In this study, the difference in genotype distribution of MTHFR C677T between two groups is of statistical significance ( $P < 0.05$ ). The TT gene type in URSA group accounts for 35.0%, higher than 26.7% in control group; while CT gene type in URSA group accounts for 37.5%, lower than 53.4% in control group, indicating that MTHFR C677T mutation is related to the risk of URSA, and TT genotype may be a genetic susceptibility gene of URSA, and pregnant women with TT genotype may have a larger risk

of URSA. There is no significant difference in gene distribution frequency of MTHFR A1298C and MTRR A66G ( $P = 0.196 > 0.05$ ,  $P = 0.669 > 0.05$ ), indicating that MTHFR A1298C and MTRR A66G gene mutation is not correlated to the risk of URSA. Meanwhile, there is a significant difference in MTHFR activity and concentrations of erythrocyte folic acid, plasma folic acid and HCY between two groups ( $P < 0.05$ ). MTHFR activity and concentrations of erythrocyte folic acid

and plasma folic acid in URSA group are lower than those in control group ( $P < 0.05$ ), while the concentration of HCY in URSA group is higher than that in control group ( $P < 0.05$ ). This verifies that the mutation of these genes is closely related to the levels of erythrocyte folic acid, plasma folic acid and HCY [6], suggesting that the gene mutation reduces the enzymatic activity and levels of erythrocyte folic acid and plasma folic acid, increases the level of plasma HCY, and increases the risk of URSA.

The concentration of serum folic acid is easily affected by the assimilation status of pregnant women as well as recent dieting, and it will obviously drop with the increase of blood volume during pregnancy. The microbial culture method is used to detect the relatively stable erythrocyte folic acid, which can better reflect the reserve of folic acid in body, and especially significant for pregnant women [7]. In this study, the level of erythrocyte folic acid in the control group is 516.12 $\pm$ 165.37 nmol/L, and 330.21 $\pm$

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106.55 nmol/L in URSA group, which are lower than the average level of  $891.6 \pm 102$  nmol/L of Chinese female population [8]. Suggest that although the living standard in China has been improving and dietary structure is changing, the shortage of folic acid is on the rise, which shall be paid attention to. Meanwhile, the result in this study shows that there is no significant difference in plasma folic acid, erythrocyte folic acid and HCY between people at different ages in URSA group ( $P > 0.05$ ), indicating that in URSA group, age is not correlated to levels of plasma folic acid, erythrocyte folic acid and HCY, suggesting that people at different ages shall promptly supplement folic acid.

There were studies suggesting that 0.4-0.8 mg/d of low-dose folic acid could be added during early pregnancy [9], or decavitamin verified by evidence-based medicine, containing folic acid [10]. Pregnant women who once suffered neural tube defect (NTD) shall daily supplement 4 mg of folic acid [11]. The folic acid metabolic disorder and hyperhomocysteinemia caused by the genetic factors of MTHFR will lead to abnormal clotting mechanism [12] and increase the risk of URSA. The content of blood coagulation factor relied by VitK can be reduced by supplementing folic acid, thus reducing the risk of placental vascular thrombotic disease during pregnancy [13], and playing a protective role. Hao Ling et al [14] considered the folic acid ADI of normal pregnant women was significantly positively correlated to the level of 5-methyl tetrahydrofolate, and they shall take in more folic acid. For patients with TT genotype as well as a history of URSA, the supplement of normal dose of folic acid cannot meet the need during pregnancy, and it is particularly important to increase the dose of folic acid supplemented. However, there shall be deeper research on the amount of folic acid supplemented, medication time as well as adverse impacts of large dose of folic acid on fetus and pregnant woman. This will open a new way to study the pathogen of URSA and find the effective treatment measure.

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### Disclosure of conflict of interest

None.

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