Original

**Association of MTHFR C677T, MTHFR A1298C and MTRR A66G Polymorphisms with Birth Defects in Southern China**

Minmin Jiang¹, Shengwen Huang¹, Jun Yuan², Xingwei Ma¹, Xiaoli Wu¹, Zhaozhen Zhuo¹, Lingyan Ren¹ and Qian Jin¹

¹ Prenatal Diagnosis Center, Guizhou Provincial People's Hospital, Guiyang, China
² Clinical Laboratory, Guiyang Second People's Hospital, Guiyang, China

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Abstract: To investigate the association of MTHFR C677T, MTHFR A1298C and MTRR A66G polymorphisms with birth defects in southern Chinese population. Genotyping was performed by Fluorescence Quantitative Analyzer using the Sequencing Reaction Universal Kit. Association analysis method was used to explore the relationship between genetic polymorphisms in MTHFR, MTRR gene and birth defects. Our results showed that serum folic acid level of genotype TT in MTHFR C677T was significantly lower than other genotypes, while homocysteine level significantly higher compared with CC and CT (P < 0.05). In addition, genotype GG in MTRR A66G might also promote homocysteine accumulation (P < 0.05). Results of logistic regression represented that MTHFR C677T, MTHFR A1298C, and MTRR A66G polymorphisms were not important or independent risk factors for predicting birth defects. Besides, genotype distribution of MTHFR C677T was significantly different in normal and abnormal pregnancy population, and genotype TT might affect folic acid metabolism and promote homocysteine accumulation. However, MTHFR C677T, MTHFR A1298C, and MTRR A66G polymorphisms were not critical or independent risk factors for predicting birth defects in this study.

Key words: Birth defect, MTHFR, MTRR, Folic acid, Homocysteine, Genetic polymorphism

**Introduction**

Birth defects are inborn errors of development, and it is also one of the most frequent maternal risk factors. In a broad sense, birth defects contain any structural or functional anomaly with measurable effects on physical, intellectual, and social welfare. Previous studies have reported that approximately one in thirty-three babies born in the United States each year was affected by a birth defect. In Dalian, China, the perinatal prevalence of birth defects from 2006 to 2010 was 101.14 per 10,000 live births. In addition, birth defects have an adverse effect on population health worldwide, which brings a huge economic burden. According to statistics in 2013, hospitalization costs related to birth defects in the United States were approximately $22.9 billion. Such economic burden is accompanied by great stress and disruption of family life. Therefore, reducing the incidence of birth defects is a project that all countries in the world need to pay attention to and conquer.

Folic acid deficiency or abnormal metabolism is one of the main factors leading to birth defects. In the period of fetal development, with the widespread distribution and constant division of cells, the growing embryo undergoing rapid changes. As an essential coenzyme in DNA synthesis, folic acid has an important impact on the methylation of the universal methyl donor S-adenosylmethionine (SAM). And, it also plays an important role in methionine metabolic pathway, protein synthesis and metabolism, cell proliferation, and tissue growth. To our knowledge, folic acid deficiency or abnormal metabolism is related to altered chromatin structure and epigenomic changes. In previous studies, researchers were most concerned about the fact that folic acid supplementation prior to conception and during pregnancy could reduce the prevalence of neural tube defects (NTD). Up to now, the exact etiology of NTD remains unclearly. Given the importance of folic acid in normal fetal development, health authorities in some countries recommend that women capable of becoming pregnant supplement a certain amount of folic acid in their daily diet.

The effect of folic acid on embryonic development, and its correlation with birth defects is not only the applicable dose of folic acid, but individual genotype also plays a crucial role in folic acid metabolism. Meanwhile, it was estimated that 65% to 75% of birth defects are due to unknown causes for suspected polygenic and multifactorial etiologies. Hence, folic acid metabolism-related gene polymorphisms have become an important research point. Methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) play central roles in the regulation of folic acid metabolism and homocysteine synthesis. Kaouther Nasri et al. investigated the associations of MTHFR C677T, MTHFR A1298C, and MTRR A66G polymorphisms with NTD in a Tunisian population. Their results showed that MTHFR (C677T, A1298C) and MTRR A66G polymorphisms in the mother group were protector factors for NTD fetuses, while MTHFR C677T in father group was a risk factor for NTD. Jiang Su et al. reported the distributions of the MTRR A66G were significant differences between ventricular septal defects group and control. Besides, another study on population of northern China has reported MTHFR C677T and MTRR A66G polymorphisms might be related to non-syndromic cleft lip with or without cleft palate, and the interactions were detected between the methionine synthase (MTR) A2756G, MTRR A66G, and MTHFR C677T polymorphisms with MTHFR A1298C.
Reference to current researches, the *MTHFR C677T, MTHFR A1298C* and *MTRR A66G* polymorphisms were closely related to birth defects. However, the climate and environment are quite different from northern to southern China, so the impact of the environment on the population must be considered. Nevertheless, there is still no research report on these three polymorphisms in the population of southern China. Thus, in this current study, we aimed to investigate the association of *MTHFR C677T, MTHFR A1298C* and *MTRR A66G* polymorphisms with birth defects in southern Chinese population, and to provide medication guidance of folic acid.

**Materials and Methods**

**Patients and samples**

A total of 297 women were included in the case-control study. All of them received pre-pregnancy check-ups, pre-natal check-ups and hospitalized delivery at the Guizhou Provincial People’s Hospital (December, 2016, to December, 2018). The study was approved by the Ethics Committee of Guizhou Provincial People’s Hospital (Approval No. 2019-21). The purpose and procedures of the study were carefully explained to all participants, and written informed consent was obtained from each participant.

All the participants were divided into three groups: abnormal pregnancy group (abnormal pregnancy with birth defects, 31 cases; In detail, there were 2 cases for anencephaly, 10 cases for open spina bifida, 6 cases for meningocele, 7 cases for cleft lip and 6 cases for congenital heart disease), normal pregnancy group (normal pregnancy with non-birth defects, 122 cases), and healthy control group (healthy women of childbearing age, 144 cases). Peripheral blood of the participants was collected, and the serum and plasma were immediately separated and then kept into different sterile tubes.

**Inclusion criteria and exclusion criteria**

The health data of pregnant women and fetus were monitored throughout this study. For the rigor of the experiment, we have set up inclusion and exclusion criteria, respectively. The inclusion criteria were: i) the abnormal pregnancy group was diagnosed by color Doppler ultrasound and found that fetus with edema, anencephaly, open spina bifida, meningocele, cleft lip or congenital heart disease; ii) the normal pregnancy group was no fetal abnormalities were found by color Doppler ultrasound during pregnancy, or no fetal abnormalities after birth; iii) the healthy control group is women of childbearing age who were identified as healthy before pregnancy. For another, the exclusion criteria were: i) the abnormal pregnancy group, excluding fetal malformations identified or diagnosed malformations after birth; ii) in the abnormal pregnancy group, we excluded the patients who showed abnormal soft indicators (NT thickening, ventricular bright spot, choroidal cyst, etc.) by color Doppler ultrasound but the fetus was not diagnosed as malformation after birth; iii) in the healthy control group, women of 15–49 years of healthy childbearing age but no menstrual cramps and menopausal were excluded.

**Genotyping and gene polymorphism analysis**

In our study, three gene polymorphisms were selected, including *MTHFR C677T* site, *MTHFR A1298C* site, and *MTRR A66G* site. The target genes were extracted from the whole blood samples of 297 patients using Sequencing Reaction Universal Kit SNP-U1 (Tianlong Science&Technology Co., Ltd; Xi’an; China) according to the manufacturer’s instruction. Briefly, all samples were genotyped and quality-controlled using Fascan48E Multichannel Fluorescence Quantitative Analyzer (Tianlong Science&Technology Co., Ltd; Xi’an; China) according to the protocol of the manufacturer.

**Detection of folic acid and homocysteine in vivo**

The folic acid levels were measured in serum by chemiluminescence instrument (Architect-i2000SP, America), and the homocysteine levels were measured in plasma by automatic biochemical analyzer (Hitachi-7180, Japan) with enzymatic cycling method. Then, we analyzed the correlation between *MTHFR* polymorphisms and folic acid, homocysteine levels in this study.

**Statistical analysis**

Statistical analysis was performed with SPSS software (IBM Inc. Armonk, NY, USA). Quantitative variables were subjected to mean comparisons by Student’s t test or one-way analysis of variance (ANOVA). Differences in the distribution of genotype and allele between groups were assessed by Pearson’s χ² test. But when the expectation of a single genotype is less than 5, Fisher’s exact test was applied instead. Utilized binary logistic regression models to test how the association of each polymorphism with birth defects is independent of potential confounding factors. The association between these genotypes and the risk of birth defects was estimated by the odds ratio (OR). Results were considered statistically significant when the P value was less than 0.05.

**Results**

**Age of cases**

The average age of the normal pregnancy group, abnormal pregnancy group and healthy control were 32.56 ± 5.71 years, 34.50 ± 5.81 years, 29.48 ± 4.76 years, respectively. There was no significant difference between normal pregnancy and abnormal pregnancy group (P > 0.05). The age of the healthy control group was significantly younger than pregnancy groups (P < 0.05). However, the differences in age did not have any significant impact on the conclusions of this study.

**Genotype distribution**

The information of genotype frequencies, χ² (for *MTHFR C677T*) and *fisher* value (for *MTHFR A1298C* and *MTRR A66G* ) at different locus were presented in Table 1. It was observed that the genotype frequency distributions were in accordance with the Hardy-Weinberg equilibrium. For locus *MTHFR C677T*, frequencies of genotype CC, CT, and TT among normal pregnancy group, abnormal pregnancy group and healthy controls were statistically different (P < 0.05). In detail, genotype CC accounted for the highest proportion (48.36%) in normal pregnancy group, whereas genotype CT accounted for the highest proportion in abnormal pregnancy and healthy control groups (58.06% and 50.00%, respectively). And the proportion of genotype TT in the normal pregnancy group was the lowest (17.21%). This phenomenon probably indicates allele C at *MTHFR C677T* is a common situation in a normal pregnancy.

Meanwhile, for *MTHFR A1298C*, genotype CC accounted for the lowest proportion in three groups, whereas genotype AA accounted for the highest proportion followed by genotype AC. However, there was no statistical difference between three groups (P > 0.05). Additionally, for *MTRR A66G* locus, frequencies of genotype AA, AG, and GG in three groups had no statistically significant difference (P > 0.05). It might indicate that *MTHFR C677T* locus is more closely related to pregnancy and birth defects than *MTHFR A1298C* and *MTRR A66G*.
Table 1. Genotype distribution frequency of MTHFR C677T, MTHFR A1298C, and MTRR A66G

| Groups                  | MTHFR C677T | MTHFR A1298C | MTRR A66G |
|-------------------------|-------------|-------------|-----------|
|                         | n (%)       | Fisher P    | n (%)     | Fisher P   | n (%)     | Fisher P   |
| Normal pregnancy        |             |             |           |            |           |            |
| CC                      | 59 (48.36)  |             | 73 (59.84) |             | 78 (63.93) |             |
| CT                      | 42 (34.43)  |             | 39 (31.97) |             | 36 (29.51) |             |
| TT                      | 21 (17.21)  |             | 10 (8.20)  |             | 8 (6.56)   |             |
| Abnormal pregnancy      |             |             | 23 (18.13) |             | 8 (13)     |             |
| CC                      | 8 (25.81)   |             | 11.622     | 0.020       | 16 (51.61) |             |
| CT                      | 18 (58.06)  |             | 74.19      | 0.000       | 13 (41.94) |             |
| TT                      | 5 (16.13)   |             | 25.81      | 0.000       | 2 (6.45)   |             |
| Healthy control         |             |             | 23 (8)     |             | 103 (36.39)|             |
|                         | 45 (31.25)  |             | 78 (25.81) |             | 92 (30.56) |             |
|                         | 72 (50.00)  |             | 36 (12.75) |             | 44 (14.19) |             |
|                         | 27 (18.75)  |             | 8 (2.63)   |             | 8 (2.63)   |             |

Note: Fisher’s exact test was used in MTHFR A1298C and MTRR A66G, while Pearson’s $\chi^2$ test was used in MTHFR C677T. P: Hardy–Weinberg P-value.

Table 2. Serum folic acid levels of different genotypes

| Genotypes | Cases, n | Serum folic acid (Mean±SD) |
|-----------|----------|---------------------------|
| CC        | 68       | 14.82±2.18                |
| CT        | 77       | 15.65±3.99                |
| TT        | 33       | 13.34±3.41                |
| F         | 1.542    |                           |
| P-value   | 0.023    |                           |

Table 3. Homocysteine levels of different genotypes

| Genotypes | Cases, n | Homocysteine (Mean±SD) |
|-----------|----------|------------------------|
| CC        | 67       | 7.52±1.47              |
| CT        | 80       | 7.84±1.53              |
| TT        | 33       | 8.13±1.98              |
| F         | 2.143    |                        |
| P-value   | 0.001    |                        |

Table 4. Variables in the Equation of Logistic Regression

| End event | Polymorphisms | Genotypes | B$^a$ | S.E.$^b$ | Wald | P   | Exp(B)$^c$ | 95%CI for Exp(B) |
|-----------|----------------|-----------|-------|---------|------|-----|-----------|-------------------|
| With or without birth defects | MTHFR C677T | CC | -0.479 | 0.652 | 0.54 | 0.462 | 0.619 | 0.172 | 2.223 |
|                       | MTHFR C677T | CT | 0.682 | 0.601 | 1.29 | 0.256 | 1.979 | 0.61 | 6.422 |
|                       | MTHFR C677T | TT | 5.674 | 0.059 | 0.08 | 0.843 | 0.837 | 0.144 | 4.857 |
|                       | MTHFR A1298C | AA | -0.178 | 0.897 | 0.039 | 0.843 | 0.837 | 0.144 | 4.857 |
|                       | MTHFR A1298C | AC | -0.365 | 0.938 | 0.152 | 0.697 | 0.694 | 0.11 | 4.361 |
|                       | MTHFR A1298C | CC | 0.223 | 0.894 | 0.00 | 0.983 | 0.983 | 0.984 | 1.96 |
|                       | MTRR A66G | AA | -0.863 | 0.784 | 1.213 | 0.271 | 0.422 | 0.091 | 1.96 |
|                       | MTRR A66G | AG | -1.214 | 0.84 | 2.087 | 0.149 | 0.297 | 0.057 | 1.542 |
|                       | MTRR A66G | GG | 2.107 | 0.349 | 0.00 | 0.999 | 0.999 | 0.999 | 1.96 |

$^a$B is regression coefficients in model; $^b$S.E. is standard error; $^c$Exp(B) is equivalent to odds ratio (OR).
Serum folic acid levels and homocysteine levels

As presented in Table 2, serum folic acid levels of different genotypes were significant differences in MTHFR C677T polymorphisms (P < 0.05). Compared with genotype CC and CT, serum folic acid level in genotype TT of MTHFR C677T was significantly lower. However, there was no significant difference of serum folic acid levels in MTHFR A1298C and MTRR A66G polymorphisms (P > 0.05).

As showed in Table 3, homocysteine levels were significant differences in all genotypes of MTHFR C677T, A1298C and MTRR A66G polymorphisms (P < 0.05). It is worth mentioning that homocysteine level of genotype TT (8.13±1.98) in MTHFR C677T polymorphisms was significantly higher than that of genotype CC and CT (7.52±1.47 and 7.84±1.53, respectively; P < 0.05). Considering the relatively low amount of serum folic acid level in genotype TT of MTHFR C677T polymorphisms, allele T probably is one of the negative factors of folic acid metabolism.

MTHFR C677T, A1298C and MTRR A66G were not independent factors in predicting birth defects

To test how the association with each gene polymorphism with birth defects was independent of potential confounding factors, we used binary logistic regression models to make predictions. The results were listed in Table 4. Unexpectedly, we found that all the three polymorphisms (MTHFR C677T, A1298C and MTRR A66G) were not important or independent factors in the construction of the birth defect prediction model (P > 0.05).

Discussion

Folic acid plays a very important role in cell division, proliferation, damage repair and so on13. In previous studies, there has been widespread controversy over the dosage of folic acid14. In this study, we explored the relationship between the key enzyme genes MTHFR and MTRR of folic acid metabolism and birth defects. Our results showed the genotype distribution of MTHFR C677T, MTHFR A1298C and MTRR A66G polymorphisms was significantly different between normal and abnormal pregnancy populations in southern China. Genotype TT in MTHFR C677T might not be conducive to folic acid metabolism and normal pregnancy, while promoting homocysteine accumulation. However, the diverse genotypes of MTHFR C677T, A1298C and MTRR A66G polymorphisms were not independent risk factors for predicting birth defects.

MTHFR, as a hot spot gene in the study of folic acid gene polymorphisms and birth defects, plays an important role in folic acid metabolism in the one carbon metabolism and methylation pathways. Meanwhile, MTHFR is also involving in the homocysteine metabolism. Until now, the reports of MTHFR are often seen in studies of congenital heart disease (CHD). A review, which was consisted of 58 child and parent research groups with 12,347 cases and 18,106 controls worldwide, showed that both MTHFR C677T and A1298C were risk factors for CHD in children with transregenental effects from their parents16,17. And, another one research found that children with CHD and their mothers had a significantly different frequencies of genotype TT in MTHFR C677T from non-CHD control group18. In addition, many studies have discussed the relationship between gametes and birth defects in recent years. Wen-Jie Huang et al.17 focused on the effect of MTHFR gene on male infertility after folic acid supplementation, and then found that folic acid significantly improved semen parameters, as well as reduced sperm malondialdehyde and sperm DNA fragmentation index of patients with MTHFR 677 TT genotype. The clinical outcomes of spontaneous pregnancy rate and live birth rate of these male patients were significantly higher than patients in placebo group. However, folic acid treatment did not exhibit any advantage in MTHFR 677 CT, 1298 AC, 1298 CC, 1793 GA, or combined 677 CT/1298 AC genotype17. Moreover, a recent study, which was on maternal gene polymorphisms of folic acid metabolism-related enzymes and risk of Down’s syndrome in offspring, showed no significant differences in the frequencies of MTHFR C677T and A1298C, MTR A2755G, and MTRR A66G. But in infants with Down syndrome, the homocysteine level of MTHFR 677 TT genotype was significantly higher than that of CC18. And, a total of 37 articles related to maternal MTHFR C677T polymorphism and Down syndrome were summarized into a meta-analysis by Mandeep Kaur et al.19. Their results suggested a highly significant association between homozygous mutant TT and birth of Down’s syndrome child. Likewise, the genetic models they built suggested that allele T possesses high risk for Down’s syndrome, whether present in dominant, codominant or recessive form15. Although the number of research reports in recent years is limited, the results and conclusions tended to be similar. All of these have suggested that genotype TT in MTHFR 677 is the main risk factor for reproductive gametes and genetic birth defects. In the present study, it was also found allele T was not dominant in normal pregnancy group because the genotype TT in MTHFR C677T was only 17.21%. This phenomenon appears to be consistent with previous studies to some extent. Instead, allele C was prevailing in normal pregnancy group. The proportion of CC genotypes was 48.36%, and the sum of the proportions of CC and CT genotypes was 82.79% in normal pregnancy group. This probably indicated the allele C related to normal pregnancy, while allele T had a negative effect. Notably, we found that genotype TT in MTHFR C677T accounted for the lowest proportion in this study, regardless of the pregnancy status. It is possible that in the evolution of the survival of the fittest, genes beneficial to individual organisms will be retained, such as allele C, while the proportion of relatively weak genotypes will gradually shrink.

In this study, further analysis was applied to discover the effects of different genotypes on folic acid and homocysteine metabolism. As well known, one of the important functions of folic acid metabolism is the conversion of homocysteine to methionine by the action of 5-methyltetrahydrofolate (5-methyl-THF)14. MTHFR is an essential enzyme in folic acid metabolism. The mutation of the MTHFR gene at position C677T, which converts alanine to valine, resulting in a decrease in MTHFR enzyme activity. A reduction of 65% enzymatic activity in homozygous TT as well as 30% in heterozygous CT has been associated with elevated homocysteine levels, DNA hypomethylation, and genomic instability14. This probably explains why MTHFR C677T genotype is closely related to abnormal reproductive gametes, birth defects of CHD, and high homocysteine levels in Down’s syndrome babies16,17. Hyperhomocysteinemia (greater than 15 μmol/l in blood) or high plasma homocysteine level has been reported as a risk factor for multiple diseases, such as congestive heart failure, chronic renal failure, neural tube defects and so on20. Folic acid and homocysteine are also related to malignant tumors. When folic acid is deficient, cytosine methylation in DNA will be reduced, which might result in proto-oncogene expression and potential malignancy transformation14. In view of the vital role of folic acid involved in homocysteine metabolism, epigenetic modification, cell division, DNA damage repair, and other in vivo biochemical processes, it is recommended that women of childbearing age be supplemented with at least 400 μg folic acid per day, and high-risk women up to 5 mg per day21,22. Consistent with previous researches, we found folic acid levels of genotype TT in MTHFR C677T were significantly lower than.
that of CC and CT in this study. On the contrary, homocysteine levels of TT genotype in MTHFR C677T were significantly higher than that of CC and CT. It is speculated that genotype TT in MTHFR C677T reduced the efficiency of folic acid metabolism and promoted the accumulation of homocysteine. Perhaps this phenomenon can also explain why the genotype TT in MTHFR C677T was rare in normal pregnant population. However, there were no significant different in serum folic acid levels of polymorphisms of MTHFR A1298C and MTRR A66G. It might suggest that these two loci have little effect on folic acid metabolism. On the contrary, homocysteine levels were significant different in the three polymorphisms of MTHFR C677T and A1298C and MTRR A66G. In addition to the genotype TT of MTHFR C677T, genotype GG of MTRR A66G may also play a role in promoting the accumulation of homocysteine. Unfortunately, limited by the amount of genotype data used in this study, the conclusions need to be further verified.

According to the distribution of MTHFR C677T, A1298C and MTRR A66G polymorphisms, and the differences in serum folic acid and homocysteine levels, we further constructed a logistic regression model to explore the specific relationship between the three gene polymorphisms and birth defects. However, the logistic regression model showed that the polymorphisms of MTHFR C677T, MTHFR A1298C, and MTRR A66G were not critical or independent factors for predicting birth defects. Based on the differences in the distribution of three loci polymorphisms among groups, as well as the significant differences in folic acid and homocysteine levels among the genotypes, it was inferred that different genotypes, especially the genotype TT in MTHFR C677T, were related to folic acid levels, homocysteine levels, and birth defects. However, these polymorphisms were not independent risk factors for birth defects. The exact causes of most birth defects are unclear, thus these potential pathogenic gene loci cannot be ignored. For health authorities, if genotype TT of MTHFR C677T is detected in the prenatal genetic test, it is necessary to pay more attention to the condition of the pregnant woman and fetus in the routine prenatal examination and color Doppler ultrasound examination, especially focus on fetal malformation. In addition, folic acid supplementation may help combat homocysteine accumulation. Thus, women during pregnancy should pay attention to dietary balance, and supplement the appropriate amount of folic acid.

To be honest, the present study has some limitations. First, the sample size included in this experiment was limited, especially in cases of birth defects with genetic testing and folic acid and homocysteine levels testing. Second, this study only contains data from one hospital. Although the hospital is a major hospital in southern China and the population is representative, the statistics of multi-centers are more reliable. Third, due to the limited number of cases, the birth defects group cannot be further divided into subgroups, which may bring some bias to the final data analysis.

In conclusion, our present study found that MTHFR C677T, MTHFR A1298C, and MTRR A66G polymorphisms have different distribution in people with normal pregnancy or not. Genotype TT in MTHFR C677T may have a certain inefficient effect on folic acid metabolism. It can promote the accumulation of homocysteine, which is not conducive to normal pregnancy. In addition, MTHFR C677T, MTHFR A1298C, and MTRR A66G polymorphisms are not independent risk factors for predicting birth defects in southern China population. It is still necessary to further explore whether these loci combined with other factors to cause birth defects or become protective factors. Even so, appropriate folic acid supplementation is still helpful for pregnancy and prevention of birth defects.

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Competing Interest
The authors declare that they have no competing interests.

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Minmin Jiang et al.: Gene Polymorphisms and Birth Defects
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