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Association between Decreased Plasma Folate Levels and MTHFR *C677T*, and *MTRR A66G* Gene Polymorphisms as Determinants for Elevated Total Homocysteine Concentrations in Pregnant Women

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Abstract

Aims: The aims of this paper were to examine the association between gene polymorphisms of methylenetetrahydrofolate reductase (MTHFR) *C677T* and methionine synthase reductase (*MTRR*) *A66G* with levels of plasma folate and tHcy.

Methods: Peripheral venous blood samples were obtained from 143 pregnant women, who had been fasted for more than 10 hours. Plasma tHcy and folate concentrations were measured in plasma specimens. The genotypes for polymorphisms were identified by the PCR-restriction fragment length polymorphism (RFLP).

Results: Our results demonstrated that MTHFR 677T allele was the predictors of plasma folate levels in pregnant women. While MTHFR 677TT genotype and *MTRR* 66G allele were the predictors of plasma tHcy concentrations. Plasma folate levels were negatively correlated with tHcy concentrations (Pearson's correlation coefficient r=-0.358, p=0.000012). With the increase of gestational weeks, the levels of plasma folate was first risen and then fall, whereas, there was a downtrend between gestational weeks and tHcy levels.

Conclusion: Plasma folate status and total homocysteine level associated with MTHFR C677T and MTRR A66G gene polymorphisms in pregnant women. According to our study, the pregnant women of homozygous mutation of MTHFR 677TT and/or MTRR 66GG genes generally have lower levels of folate. Therefore, we recommend that pregnant women with 677TT and 66GG genotypes should supplement the appropriate dosage of folic acid based on their genotypes.

Keywords: Folic acid; Neural tube defects; Methylenetetrahydrofolate reductase (MTHFR); Methionine synthase reductase (*MTRR*); Genotype; Homocysteine; Gene polymorphism

Introduction

Folic acid, which known as vitamin B9 or folate is an essential nutrient, is required for a series of biochemical pathways included DNA replication and enzymatic reactions involved in the amino acid synthesis [1-3]. Folate demand in pregnant women extremely increases because it is necessary for fetuses to grow and develop [4]. Folic acid cannot be synthesized by the human body and must be obtained from diet and supplements [5], and folic acid deficiency may result in neural tube defects (NTDs) [6,7]. NTDs are birth defects of the spine, spinal cord, or brain that originate during embryonic development. The most common NTDs are spinal cord and spina bifida and anencephaly [8]. Folic acid supplementations for pregnant women are an effective primary care-relevant preventive intervention [9]. America and many other countries issued the regulations to require amending the standards of several grain products and vegetable by folic acid addition [7,10,11]. As recommended, all women of childbearing age should take 400 to 800 micrograms folic acid per day during the periconceptional and pregnancy [9,12-16]. Women who had a fetus diagnosed as affected by a neural tube defect or have given birth to a baby with a neural tube defect have an increased risk and should be administered high-dose supplementation (5 mg folic acid daily) [17].

As inactive form, folic acid must be converted by MTHFR to tetrahydrofolic acid and 5-methyltetrahydrofolate (5-MTHF) for regulating the metabolism of folate and homocysteine [5,18-20]. It is well known that MTHFR *C677T* (rs1801133) genetic variation leads to enzyme activity reducing, and finally, influences susceptibility to occlusive vascular diseases, NTDs, colon cancer and acute leukemia [21-24]. Mutant homozygote (TT genotype) which significantly elevated plasma homocysteine level is a crucial hereditary risk factor in the development of some diseases, and constitutes approximately 20% in northern China [25,26]. Methionine synthase reductase (*MTRR*) which keeps sufficient levels of methylcob(III)alamin, the activated cofactor for methionine synthase, catalyzes homocysteine remethylation to methionine [27,28]. One major allelic variant of *MTRR A66G* (rs1801394) act as a predisposing factor contributing hyperhomocysteinemia [29]. According to Walker (1999), the mean

homocysteine concentration was 5.6 µmol/L (95% confidence interval 3.9-7.3) at 8-16 weeks' gestation [30]. Elevated serum homocysteine of the first trimester is correlated with history of pregnancy losses, vascular-related complications of pregnancy, and preterm birth [31]. Notably, the 66GG genotype increased the risk of NTDs pregnancy outcomes in women with low vitamin B12 levels or when the MTHFR *C677T* mutation is also present [27,28,32]. In addition, the risk of bearing Down's syndrome babies increased among the women who carry both the MTHFR 677T and *MTRR* 66G mutations genes [33,34]. Our objective was to examine the association between gene polymorphisms of MTHFR *C677T* and *MTRR A66G* with levels of plasma folate and tHcy.

Materials and Methods

Study subjects

143 eligible pregnant women (the mean age is 29.7 ± 11.3 years, range: 18-43 years) were recruited. All of them were Han people and had the same or the similar dose of folic acid supplementation (0.4 mg per day). Women with Alcohol consumption, smoking habits, and those with metabolic disorders, renal insufficiency, hemolytic disorders, and malignancies et al. which may lead to decreasing serum folate level were excluded. All subjects were recruited from the department of Obstetrical in First People's Hospital of Kunming during the period from July 1, 2016 to February 28, 2017. Ethical approval for the study was obtained by the Ethics Committee of the Kunming first people hospital Research Center at May 18, 2016 and the version number is 201605182. Informed consents were acquired from all participants.

Genotype analysis

Blood samples were collected in tubes containing 200 µL of 2% EDTA and stored at -80°C or colder until further analysis. Genotyping for MTHFR *C677T* and *MTRR A66G* single nucleotide polymorphisms (SNP) gene mutations were performed by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) and agarose gel electrophoresis. Data were analyzed using Lasergene. The MTHFR *C677T* polymorphism was detected using sequences (forward: 5'-CCCTATTGGCAGGTTACCCC -3'and reverse: 5'-TCTGGGAAGAACTCAGCGAAC -3') and HaeIII endonuclease in PCR-RFLP assays. *MTRR A66G* was genotyped using primers (forward: 5'-CGGATCATTTGGGGAGCTTG -3' and reverse: 5'-TGTAACGGCTCTAACCTTATCGG -3') and NdeI restriction enzyme.

Plasma folate and total homocysteine concentrations measurements

Plasma folate was determined by Semi-quantitative chemiluminescent microparticle immunoassay (ARCHITECT i1000SR immunoassay analyzer, Abbott Laboratories, Abbott Park, IL, USA) using ARCHITECT Folate Reagent Kit and ARCHITECT Homocysteine Reagent Kit (Abbott Laboratories, Abbott Park, IL, USA), respectively [35,36]. Serum tHcy concentration was measured by high-performance liquid chromatography and fluorometric detection following reduction, deproteinization, and derivatization with the fluorophore SBDF (ammonium 7-fluorobenzo-2- oxa-1,3-diazole-4-sullfonate).

Statistical methods

Data analysis was performed by IBM SPSS Statistics 22.0 (SPSS Inc., IBM Corporation, Chicago, IL, USA) and GraphPad Prism 7.03 software (GraphPad Software Inc., La Jolla, CA, USA). Genotype distributions and allele frequencies of MTHFR *C677T* and *MTRR A66G* of participants was determined, and their 95% confidence intervals (CI) were calculated. Hardy-Weinberg equilibrium was determined for all of the genotypes using $\chi2$ testing. Continuous variables including age, blood parameters (folate and tHcy) and gestational weeks were calculated as mean ± S.D., and analyzed using one-way ANOVA, Pearson's correlation coefficient, and Tukey's test. The significance threshold was set at 0.05. Because the distribution of plasma values of tHcy had a positive skew, analyses were carried out after logarithmic transformation, and back-transformed results are shown.

Results

The genotype distributions of the MTHFR *C677T* polymorphisms was in Hardy-Weinberg equilibrium (P=0.668773). Genotype distributions of the MTHFR *C677T* and *MTRR A66G* polymorphism of the 143 Han women of Yunnan Province were analyzed: 50 (35%) subjects were CC, 67 (47%) were CT, and 26 (18%) subjects were TT; 63 (44%) subjects were AA, 51 (36%) subjects were AG, and 29 (20%) subjects were GG. The allele frequencies of MTHFR 677T and *MTRR* 66G were 41.6% and 38%, respectively (Table 1). As shown in Figure 1a, there was a significant effect of *C677T* polymorphism on plasma folate levels, with homozygous wild-type objects having higher folate levels than either of the other two genotype groups (P=0.000).

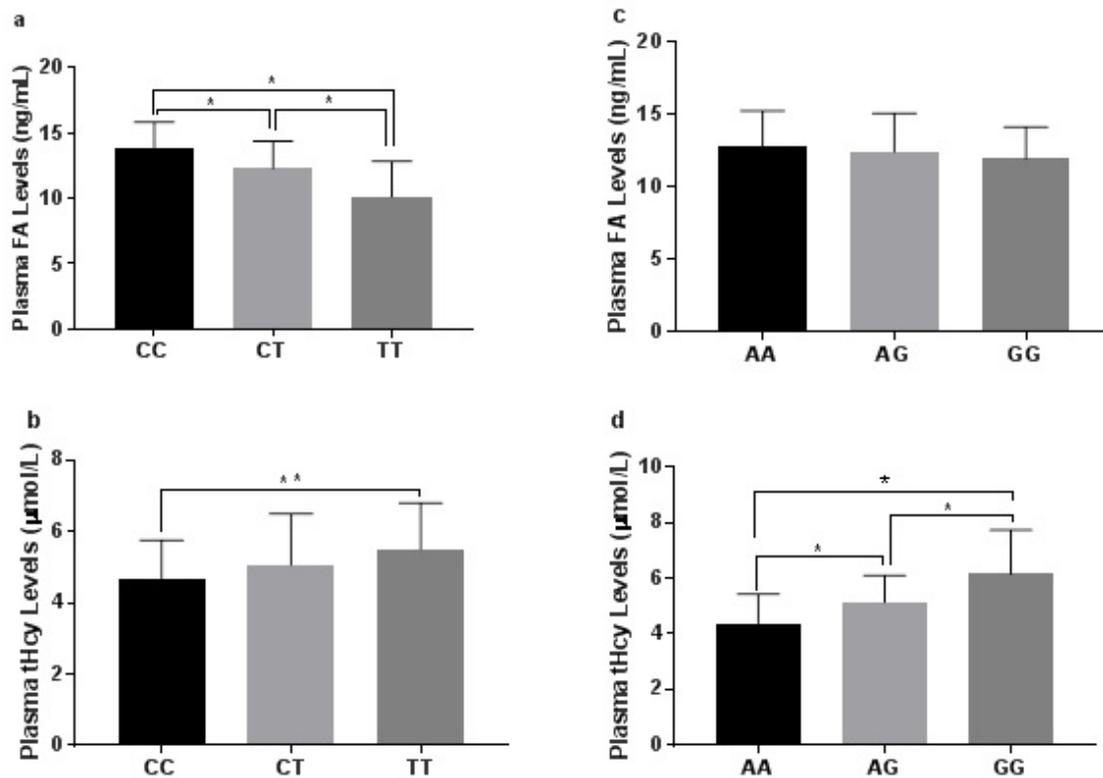| Genotype	n	Genotype Distributions (%)	Allele frequency (%)	95% CI*
MTHFR C677T	-		T of C677T	-
CC	50	35%		
CT	67	47%	42	P=0.668773
TT	26	18%		
MTRR A66G	-		G of A66G	-
AA	63	44%		
AG	51	36%	38	P=0.003528
GG	29	20%		

N: The Number of Subjects in Each Group. * Chi-squared test.

Table 1: Genotype Distributions, and allele frequencies of the MTHFR C677T and MTRR A66G polymorphisms.
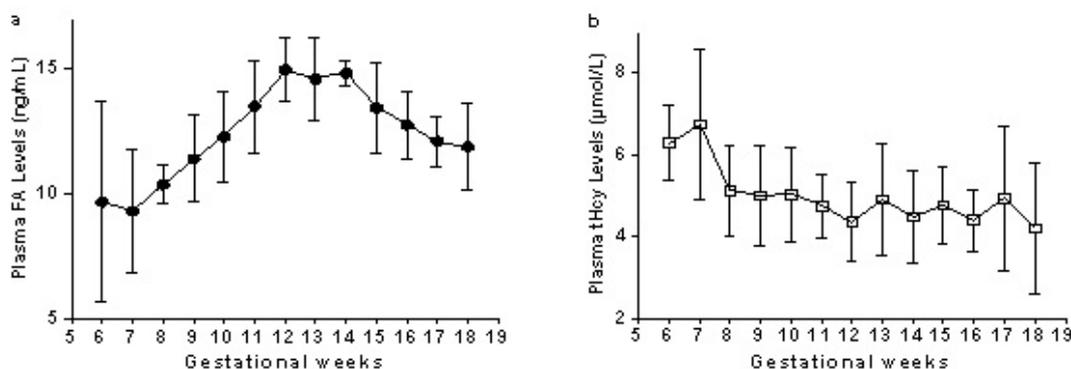
According to the relationship between genotypes and tHcy levels, the significant difference of tHcy levels between CC and TT (P=0.041) was observed (Figure 1b). No statistical significance was found between *MTRR A66G* gene polymorphism and plasma folate concentrations (P=0.506, 0.167 and 0.424, respectively) (Figure 1c). In contrast, tHcy levels significant differences were observed between AA vs. AG and GG (Figure 1d). Plasma folate concentrations were negatively correlated with tHcy levels (Pearson's correlation coefficient r=-0.358, P=0.000012).

With the increase of gestational weeks, the levels of plasma folate rose in the first trimester and then fell in the second (Figure 2a). While there was a downtrend between gestational weeks and tHcy levels (Figure 2b).

Figure 1: Plasma folate and tHcy levels varieties in different genotypes (n=143, x̄ ±s). a, b: Association of plasma folate and tHcy concentrations with MTHFR *C677T* polymorphism; c, d: Association of plasma folate and tHcy concentrations with *MTRR A66G* polymorphism. * $p < 0.01$, ** $p < 0.05$.

Figure 2: Mean Plasma folate and tHcy level trends changed along with increasing of gestational weeks. (a) Plasma FA concentrations rose markedly from the 7th week and reached a peak during the 12th week. Subsequently, a steady downward trend from the 14th to 18th week was observed. (a) Plasma tHcy levels experienced a downward trend along with gestational weeks.

Plasma tHcy concentrations gradually dropped from the 7th to 12th week along with the progress of pregnancy, and then there were an alternating upward and downward variations at successive weeks (Figure 2b).

The effects of the combination of genotypes for MTHFR *C677T* and *MTRR A66G* polymorphisms on folate and tHcy concentrations are shown in Table 2.

MTHFR C677T/ MTRR A66G	Plasma Folate Levels* (ng/mL)	tHcy$^\psi$ (mmol/L)
CC/AA (n=25)	13.65 ± 2.01^a	4.33 ± 0.81^a
CC/AG (n=16)	14.20 ± 1.94^b	4.63 ± 1.07^b
CC/GG (n=9)	14.01 ± 1.76^c	5.82 ± 1.04^{a,c}
CT/AA (n=28)	13.09 ± 1.82^d	4.16 ± 0.82^{c,d}
CT/AG (n=23)	12.18 ± 2.25^e	5.35 ± 0.90^{a,d}
CT/GG (n=16)	11.10 ± 1.69^{a,b,c}	6.27 ± 1.86^{a,b,d}
TT/AA (n=10)	9.45 ± 2.88^{a,b,c,d,e}	5.20 ± 1.66
TT/AG (n=12)	10.49 ± 2.89^{a,b,c,d}	5.47 ± 0.65^{a,d}
TT/GG (n=4)	10.73 ± 1.65	6.43 ± 1.62^{a,d}
tHcy: Total Plasma Homocysteine Concentration. N: The Number of Subjects in Each Group.		
*One-way ANOVA performed. F (8,134) = 8.329, p<0.0005; ψ One-way ANOVA performed on the log-transformed variables. F (8,134) = 7.920, p<0.0005;		
Values in a row with different superscript letters are significantly different, p<0.05 (Tukey's test).		

Table 2: Association analysis of plasma folate and tHcy concentrations according to the combination of genotypes for MTHFR *C677T* and *MTRR A66G* gene polymorphisms.

Age (years)	Samples (n, %)	Plasma Folate Levels ($\bar{x}$ ±s, ng/mL)	tHcy Levels ($\bar{x}$±s, μmol/L)
<25	13 (9%)	11.43 ± 3.20	5.03 ± 1.41
25~	67 (47%)	12.63 ± 2.47	5.19 ± 1.51
30~	38 (27%)	12.51 ± 2.48	4.63 ± 0.82
35~	22 (15%)	12.33 ± 2.32	5.04 ± 1.20
40~	3 (2%)	13.70 ± 2.07	5.68 ± 2.05
tHcy: Total Plasma Homocysteine Concentration. N: The Number of Subjects in Each Group.			

Table 3: Plasma folate and total homocysteine levels varieties in different age women (n=143, $\bar{x}$ ± S.D.).

The one-way ANOVA indicated that pregnant women with the MTHFR 677TT/*MTRR* 66AA haplotype had lower serum folate concentrations than those with MTHFR 677CC/*MTRR* 66AA, MTHFR 677CC/*MTRR* 66AG, MTHFR 677CC/*MTRR* 66GG, MTHFR 677CT/*MTRR* 66AA, MTHFR 677CT/*MTRR* 66AG, and MTHFR 677TT/*MTRR* 66AG. While the MTHFR 677TT/*MTRR* 66GG haplotype had higher tHcy concentrations than MTHFR

677CC/*MTRR* 66AA, MTHFR 677CC/*MTRR* 66GG, MTHFR 677CT/*MTRR* 66AA, MTHFR 677CT/*MTRR* 66AG, MTHFR 677CT/*MTRR* 66GG and MTHFR 677TT/*MTRR* 66AG. No significant difference was observed between age and maternal plasma folate and homocysteine levels (Table 3).

Discussion

Epidemiological evidence has revealed that variety of factors contributes to the risk of adverse pregnancy outcomes. These factors include genetic variation, maternal nutrition and immunological responses, periconceptional intake of folic acid antagonists (trimethoprim, carbamazepine, phenobarbital, phenytoin, et al.) [37-39]. Folate is fundamental for nucleic acid synthesis and needed for homocysteine metabolism and methylation reactions [40]. Maternal plasma folate deficiency is associated with an increased the total plasma homocysteine which is strongly related to adverse pregnancy outcomes and other diseases [24,41,42].

In this paper, we provide evidence those polymorphisms of MTHFR *C677T* and *MTRR A66G* are associated with plasma folate and tHcy levels. The results showed that the *MTRR A66G* polymorphism alone was not related to plasma folate concentrations but significantly related to circulating tHcy levels, and this finding was similar to the pioneering studies of Barbosa et al. 40 and Gaughan et al. 27, respectively. However, exactly the reverse, our study indicated that 66GG, not 66AA significantly elevating tHcy. This may be due to sex and race varieties. 43 There was a significant difference in tHcy levels among 66AA, 66AG, and 66GG (4.39 ± 1.04 μmol/L, 5.15 ± 0.96 μmol/L, 6.15 ± 1.58 μmol/L, AA vs.AG, P=0.001; AA vs.GG, P=0.000; AG vs.GG, P=0.000, respectively). Our results showed that the *MTRR* 66GG genotype confers the relative risks of elevated tHcy. It seemed that *MTRR A66G* gene mutation contributed to lower plasma folate levels (12.73 ± 2.52 ng/mL, 12.42 ± 2.67 ng/mL, 11.95 ± 2.17 ng/mL), although no significant differences were observed.

A possible explanation for this might be that the sample size is too small. In our study, the T allele frequency was 41.5%and individuals with variant allele (T) significantly decreased plasma folate concentration (CC: 13.85 ± 1.93 ng/mL, CT: 12.34 ± 2.10 ng/mL, TT: 10.16 ± 2.65 ng/mL, respectively, P=0.000). In addition, the subjects with TT genotype had significantly higher tHcy levels than those CC genotype (5.44 ± 1.32 μmol/L vs. 4.67 ± 1.07 μmol/L, respectively; P=0.010). The results were consistent with the previous studies [24,43,44]. Because the prevalence (19%) of 677TT genotype is high in China, the risk of exposure to plasma folate deficiency, resultant hyperhomocysteinemia, and adverse pregnancy outcome may elevate [45-47].

The most interesting finding was that plasma folate concentrations rose markedly from the 7th week and reached a peak during the 12th week. Subsequently, a steady downward trend from the 14th to 18th week was observed (Figure 2a). Meanwhile, our data indicated that the plasma tHcy concentrations gradually dropped from the 7th to 12th week along with the progress of pregnancy, and then there were alternating upward and downward variations at successive weeks (Figure 2b).

Although both plasma folate and tHcy levels are significantly different in pregnant women than non-pregnancy women [48], no statistical significances were observed between first trimester and second trimester with plasma folate and tHcy levels. No previous study has investigated the relationship between plasma folate levels tendency

and gestational weeks. This is an important issue for future research. Moreover, our study indicated that there was a significant negative correlation between plasma tHcy and folate levels (r=-0.358, p=0.000012), independently of MTHFR *C677T* and *MTRR A66G* gene polymorphisms.

According to Finer and his colleagues' report, up to 42% of American women and girls got pregnant without intentions during 2011, and the neural tube closes completely by 28 days from conception [37,49,50]. This means that almost half of women and girls may not use sufficient folic acid supplements before and early pregnancy [51]. The prevalence of NTDs will not decrease whether or not folic acid administration after 7 or more weeks of gestation [52].

Previous intervention study recommended the reference interval of plasma/serum folate for adults is 2.5-20 ng/mL [53]. Although WHO has not given an appropriate cutoff for gestation, a consensus suggested a concentration for defining folate deficiency (4 ng/mL) [41]. Besides, Chinese government recommends all of the childbearing age women consuming 0.4 mg folic acid daily, and the rate of NTDs has shown a declining trend [54-56]. However, compared with US women's plasma folate level, our data indicated that the plasma folate level was lower than the geometric mean 37.3 nmol/L [46,57]. In conclusion, as many pregnancies are unplanned, and taking the MTHFR *C677T* and *MTRR A66G* polymorphisms and environmental factors into account, we recommend that health providers supply appropriate folic acid with or without multiple vitamin supplements to all childbearing age women based on their genotypes and vitamin status. Furthermore, additional investigation into the optimal dose is strongly recommended.

Conclusion

According to our study, The MTHFR *C677T*, *MTRR A66G* polymorphism and folate deficiency were significantly associated with elevated serum tHcy levels. The pregnancy of homozygous mutation of MTHFR 677TT and *MTRR* 66GG genes could induce the decrease of folate. Therefore, our research recommended that pregnant women with homozygous mutation of 677TT and 66GG genes should supply the appropriate dosage of folic acid based on their genotypes.

Conflict of Interest

The authors do not have any conflicts of interest concerning disclosure.

Acknowledgements

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