

## RESEARCH COMMUNICATION

# Interactions Between MTHFR C677T - A1298C Variants and Folic Acid Deficiency Affect Breast Cancer Risk in a Chinese Population

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### Abstract

**Background:** Our objective was to evaluate the MTHFR C677T-A1298C polymorphisms in patients with breast cancer and in individuals with no history of cancer, to compare the levels of genetic damage and apoptosis under folic acid (FA) deficiency between patients and controls, and to assess associations with breast cancer. **Methods:** Genetic damage was marked by micronucleated binucleated cells (MNBN) and apoptosis was estimated by cytokinesis-block micronucleus assay (CBMN). PCR-RFLP molecular analysis was carried out. **Results:** The results showed significant associations between the MTHFR 677TT or the combined MTHFR C677T-A1298C and breast cancer risk (OR = 2.51, CI = 0.85 to 7.37,  $p = 0.08$ ; OR = 4.11, CI = 0.78 to 21.8,  $p < 0.001$ ). The MNBN from the combined MTHFR C677T-A1298C was higher and the apoptosis was lower than that of the single variants ( $p < 0.05$ ). At 15 to 60 nmol/L FA, the MNBN in cases with the TTAC genotype was higher than controls ( $p < 0.05$ ), whereas no significant difference in apoptosis was found between the cases and controls after excluding the genetic background. **Conclusions:** Associations between the combined MTHFR C677T-A1298C polymorphism and breast cancer are possible from this study. A dose of 120 nmol/L FA could enhance apoptosis in cases with MTHFR C677T-A1298C. Breast cancer individuals with the TTAC genotype may be more sensitive to the genotoxic effects of FA deficiency than controls.

**Keywords:** Folic acid - combined MTHFR C677T-A1298C - breast cancer - genetic damage - apoptosis

*Asian Pacific J Cancer Prev*, 13, 2199-2206

### Introduction

Folate is an important micronutrient found in green vegetables, fruits and beans. The synthetic form that is added to foods and found in supplements is known as folic acid (FA). FA plays an essential role in several complex metabolic pathways including DNA synthesis and pathways that are involved in the methionine metabolic pathway, which is crucial for DNA methylation (Fenech et al., 2001; Eichholzer et al., 2006; Mazz et al., 2010; Bouckaert et al., 2011).

FA levels are determined by both dietary FA intake and FA metabolism. FA deficiency induces deficient methylation of deoxyuridylic acid (dUMP) to deoxythymidylic acid (dTMP) leading to uracil misincorporation (Fenech, 2001; Mazz et al., 2010) and DNA global hypomethylation (Rampersaud et al., 2000); this not only leads to point mutations, the generation of single- and double-stranded DNA breaks, chromosome breakage, micronucleus formation and abnormal apoptosis (Everson et al., 1988; Fenech et al., 2001; Fenech et al., 2002; Eichholzer et al., 2006; Yang et al., 2008; Mazz et al., 2010; Bouckaert et al., 2011), but also may

also be associated with aneuploidies of the increment chromosomes 17, 21 and 8 (Wang et al., 2004; Ruosaari et al., 2008; Thomas et al., 2008; Ni et al., 2010). Low dietary FA intake also results in an interruption of the DNA repair capability (Wei et al., 2003). The majority of prospective and case-controlled studies showed that low levels of serum FA, red cell FA concentration and low dietary FA intake are inversely correlated with colon cancer, breast cancer, adenoma and other cancers at several sites (Kim et al., 2001; Wang et al., 2006; Lin et al., 2010; Johnson et al., 2011).

Disturbances in the FA metabolic pathway are associated with a wide range of conditions, and variants from genes involved in this pathway may play a role in the genesis of these conditions. At least 30 different enzymes are involved in this complex pathway including methylenetetrahydrofolate reductase (MTHFR), methionine synthase, methionine synthase reductase, and thymidylate synthase (Kim et al., 1999; Suleeporn et al., 2010). Defects or polymorphic enzymes may alter the bioavailability of FA and influence cancer susceptibility (Maruti et al., 2009). MTHFR is responsible for the availability of methyl groups for biological

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methylation reactions and catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is a co-substrate for homocysteine remethylation to methionine (Dhillon et al., 2009); mutations of MTHFR will alter the activity of the enzyme. The best-characterized polymorphism of MTHFR is the C677T transition. This mutation causes an alanine to valine substitution and reduces the enzyme's catalytic activity (Thomas et al., 2008; Rodrigues et al., 2010). An additional polymorphism, MTHFR A1298C, results in the change from a glutamic acid to an alanine residue and decreases the enzyme's activity (Weisberg et al., 1998; Promthet et al., 2010). Reduced MTHFR activity increases the 5, 10-methylenetetrahydrofolate concentration, whereas it decreases the 5-methyltetrahydrofolate concentration. Such a situation is expected to favor the synthesis of dTMP over methylation of CpG, and to minimize both the uracil incorporation into DNA and also the chromosomal breaks caused by uracil. Two common polymorphisms have been reported that are linked to an increased risk of a wide range of adverse health conditions (such as birth defects, slow growth, anemia, weight loss, digestive disorders, some behavioral issues, cardiovascular disease and several cancers) (Weisberg et al., 1998; Huang et al., 2008; Thomas et al., 2008; Dhillon et al., 2009; Promthet et al., 2010; Rodrigues et al., 2010).

Epidemiological studies indicate that the 677TT genotype is associated with a lower risk of acute lymphocytic leukemia (Skibola et al., 1999) and colon carcinoma (Promthet et al., 2010), especially in low-risk subjects who consume a diet high in folate (Crott et al., 2001). In contrast, under FA deficiency, the 677TT variant genotype has been associated with an increased risk of cancer at various sites (breast, gastric, cervical and prostate) (Beilby et al., 2004; Zoodma et al., 2005; Bistulfi et al., 2010). Previous studies showed that a variation of the FA concentration within the physiological range (15 to 240 nmol/L) significantly influenced the genomic instability and cytotoxicity in cultured human lymphocytes with the C677T polymorphism (Wu et al., 2009). However, the extent of the genetic damage and the levels of apoptosis under FA deficiency between breast cancer cases and noncancer control cases with the combined MTHFR polymorphisms of C677T and A1298C have not been elucidated. In the present work, we investigated and compared the effects of FA deficiency between breast cancer cases and non-cancer controls on the levels of genetic damage and apoptosis of human lymphocytes with combined MTHFR C677T-A1298C polymorphisms.

## Materials and Methods

### Study design

Approval for the present study was obtained from the National Natural Sciences Foundation of China (NSFC) and the Yunnan Scientific and Technological Committee. The peripheral blood from 75 female breast cancer patients and 75 control female volunteers who did not receive any vitamin B supplements was utilized for the study after obtaining informed consent.

All of the cases were pathologically diagnosed females in the Second Affiliated Hospital of Kunming Medical College, Yunnan, China. Eligible cases were newly diagnosed breast-cancer females that had not yet received chemotherapy and did not have any previous history of any cancer.

### Genotype assays

Peripheral blood samples were collected from the cancer and control groups, and genomic DNA was extracted from whole blood samples by the hydroxybenzene and chloroform method (Song et al., 1999). DNA fragments containing the MTHFR nucleotides 677 and 1298 were amplified according to the conditions described by P. Yi et al. (2002). The PCR primers to amplify the 677-fragment are 5'CCTTGAACAGGTGGAGGCC3' and 5'CAAAGAAAAGCTGCGTGATGAT3', and the primers for the 1298-containing segment are 5'GCAAGTCCCCAAGGAGG3' and 5'GGTCCCCACTTCCAGCATC3'. The PCR conditions were one cycle of 94 °C, 4 min; 40 circles of 94 °C, 45 s, 59 °C, 45 s, and 72 °C, 45 s, and a final cycle of 72 °C, 10 min. PCR products with nucleotides 677 and 1298 were subjected to HinfI and MboII digestion, respectively, followed by electrophoresis using a 2% agarose gel.

The PCR products were validated by sequencing analysis. Mutagenesis was performed to generate all nine possible combinations of the polymorphic variants: CCAA (wild-type), CCAC (heterozygous mutant at 1298), CCCC (homozygous mutant at 1298), CTAA (heterozygous mutant at 677), CTAC (heterozygous mutant at both 677 and 1298), CTCC (heterozygous mutant at 677 and homozygous mutant at 1298), TTAA (homozygous mutant at 677), TTAC (homozygous mutant at 677 and heterozygous mutant at 1298) and TTCC (homozygous mutant at both 677 and 1298).

### Lymphocyte culture, viability and cell growth assays

Each individual donated 5 ml of peripheral blood by venipuncture after an overnight fast. The donated blood was collected in heparinized tubes. The lymphocytes were isolated using lymphocyte separation medium (Shanghai Huajing Biotech Company, China). The lymphocyte cultures were prepared at a concentration of  $0.5 \times 10^6$  cells / ml in 1 ml of RPMI 1640 medium containing varying concentrations of FA (15, 30, 60, 120 and 240 nmol/L). All of the other constituents of the RPMI 1640 medium were as previously described, and all were purchased from Gibco® Media, American. Five percent dialyzed fetal calf serum (FCS, PAA the cell culture company, American), 10 kU/L interleukin 2 (Shenzhen Xinjier Pharmaceutical, China), 2 mmol/L L-glutamine (Sigma), 100 U/ml penicillin G and 100 mg/ml streptomycin (Beijing Xiasi Biotechnology, China) were added to the medium. The lymphocytes were cultured at a concentration of  $0.5 \times 10^6$  cells / ml in 5 ml volumes after stimulation with phytohemagglutinin (45 mg/ml) (PHA; Beijing Biotech Company, China), and cultures were incubated at 37 °C and 5% CO<sub>2</sub> in a humidified incubator. After 3 days, the cell number and viability were determined using a hemocytometer and trypan blue exclusion, respectively.

The cultures were continued in 0.7 ml fresh medium and 0.3 ml "conditioned" medium from the previous 3-day culture at a concentration of  $0.5 \times 10^6$  viable cells/ml. The components of the fresh medium were the same as above but without PHA. The medium change was repeated at 6 days post-PHA treatment, and a final viable cell count was measured on day 9.

#### Cytokinesis-block micronucleus (CBMN) assays

Eight days after PHA treatment, cytochalasin B (4.5 µg/ml; Sigma) was added to each tube, and cells were harvested 28 h later onto microscope slides using a cytocentrifuge (Shandon Southern Products, Cheshire, UK). The slides were then air-dried, fixed with 3 ml each methanol:acetic acid and cold methanol for 10 min and stained with 5% Giemsa for 5 min. All of the slides were coded prior to scoring. Scoring was performed by a single individual using an Olympus light microscope at 1000× magnification under oil immersion. Coded slides were scored for the frequency of micronucleated binucleated (MNBN) cells, micronucleated mononucleated (MNed mono) cells, nucleoplasmic bridges (NPBs) cells and nuclear buds (NBUDs) cells, apoptotic cells and necrotic cells. The scoring criteria followed the cytokinesis-block micronucleus (CBMN) assay established by Fenech (Fenech, 2006). In this study, the genetic damage was measured by the frequency MNBN.

#### Statistical analysis

Genotypic frequencies were assessed by the  $X^2$  test. The frequencies of MNBN and apoptosis for the various cultures were compared using a one-way ANOVA. The mean values of the frequencies of MNBN and apoptosis for the control and breast cancer groups with different genotypes under varying FA conditions were compared using the Newman-Keuls ANOVA post-test (two-tailed). The differences in sensitivity to FA deficiency between breast cancer and control groups were determined using a difference of difference analysis by subtracting the pooled observed frequencies of relative biomarkers at 240 nmol/L FA from those observed at 15 to 120 nmol/L FA for each individual (Andrew et al., 2001).

The differences for these results were then compared between the groups using a two-tailed Student's *t*-test. The analyses were conducted using SPSS (Statistical Package for the Social Sciences) (Version 12.0) and Prism 4.0 software (GraphPad, San Diego, CA).

## Results

#### Frequencies of combined MTHFR C677T-A1298C genotypes

The distributions of the MTHFR polymorphisms in case and control groups did not deviate significantly from the Hardy-Weinberg equilibrium. The frequencies of 677C and 677T alleles were 0.63 and 0.37 in case group and 0.71 and 0.29 in control group, respectively, while the frequencies of 1298A and 1298C alleles were 0.71 and 0.29 in case group and 0.74 and 0.26 in control group, respectively. The differences in frequencies of C677T and A1298C genotype between the case group and

**Table 1. Distribution of MTHFR C677T and A1298C Polymorphisms**

Genotype	Cases (%)		Controls (%)		Allelic frequency: $\chi^2$ P	
	N = 75	N = 75	Cases (%)	Controls (%)	T of 677, C of 1298	
MTHFR677	75(100.0)	75(100.0)	37.3	29.3	1.447	>0.05
CC	32(42.7)	37(49.3)			0.671	>0.05
CT	30(40)	32(42.7)			0.11	>0.05
TT	13(17.3)	6(8.0)			2.953	>0.05
MTHFR1298	75(100.0)	75(100.0)	29.3	26	0.226	>0.05
AA	37(49.3)	42(56.0)			0.669	>0.05
AC	32(42.7)	28(37.3)			0.444	>0.05
CC	6(8.0)	5(6.7)			0.098	>0.05

Genotypic frequencies were assessed by the  $X^2$  test

**Table 2. The Frequency of Combined MTHFR C677T-A1298C Polymorphisms**

MTHFR 677	MTHFR 1298	Cases (%)	Controls (%)	$\chi^2$	P
CC	AA	23 (30.6)	27 (36.0)	0.48	> 0.05
CC	AC	8 (10.7)	7 (9.3)	0.074	> 0.05
CC	CC	1 (1.33)	3 (4.0)	1.027	> 0.05
CT	AA	10 (13.3)	11 (14.7)	0.055	> 0.05
CT	AC	17 (22.7)	19 (25.3)	0.146	> 0.05
CT	CC	3 (4.0)	2 (2.7)	0.207	> 0.05
TT	AA	4 (5.3)	4 (5.3)	0	> 0.05
TT	AC	7 (9.3)	2 (2.7)	2.955	> 0.05
TT	CC	2 (2.7)	0 (0.0)	-	-

Genotypic frequencies were assessed by the  $X^2$  test

**Table 3. Odd Ratios (ORs) and 95% CI for MTHFR Polymorphisms with Risk of Breast Cancer**

Genotype	No. cases (%)	No. controls (%)	OR(95% CI)*	p
677CC	32(42.7)	37(49.3)	1.0 (Reference)	
677CT	30(40)	32(42.7)	1.08(0.55 to 2.16)	0.84
677TT	13(17.3)	6(8.0)	2.51(0.85 to 7.35)	0.08
1298AA	37(49.3)	42(56.0)	1.0 (Reference)	
1298AC	32(42.7)	28(37.3)	1.10(0.66 to 2.54)	0.64
1298CC	6(8.0)	5(6.7)	1.16(0.38 to 4.83)	0.53
Combined genotype				
677CC-1298AA	23(30.6)	27(36.0)	1.0 (Reference)	
677CC-1298AC	8(10.7)	7(9.3)	1.04(0.42 to 4.27)	0.96
677CC-1298CC	1(1.33)	3(4.0)	1.09(0.38 to 4.02)	0.91
677CT-1298AA	10(13.3)	11(14.7)	1.07(0.38 to 2.96)	0.92
677CT-1298AC	17(22.7)	19(25.3)	1.05(0.45 to 2.48)	0.96
677CT-1298CC	3(4.0)	2(2.7)	1.76(0.27 to 11.47)	0.17
677TT-1298AA	4(5.3)	4(5.3)	1.17(0.26 to 5.23)	0.51
677TT-1298AC	7(9.3)	2(2.7)	4.11(0.78 to 21.8)	0.001
677TT-1298CC	2(2.7)	0(0.0)	-	-

\*OR, odds ratio; CI, confidence interval status, ORs were further adjusted for age at enrollment

control group were insignificant (Table 1-2). No TTCC genotype was detected in the control group. The genotype CC, CT and CC frequencies of the C677T polymorphism were respectively 44%, 43% and 13% for patients, and 46%, 40% and 14% in controls. There were no significant differences between groups ( $p=0.92$ ).

Multiple logistic regression tests were applied to evaluate the effect of variables on disease progression (genotypes) (Table 3). MTHFR 677TT (OR = 2.51; CI= 0.85 to 7.37;  $p=0.08$ ) and MTHFR 677TT-1298AC (OR= 4.11; CI=0.78 to 21.8;  $p<0.001$ ) were predictors of breast cancer.

Genotype	FA (nmol/L)					ANOVA p
	15	30	60	120	240	
Apoptosis /500 cells						
CCAA	4.07±0.99 <sup>a</sup>	5.63±1.04 <sup>a</sup>	5.82±2.04 <sup>a</sup>	3.39±1.17 <sup>a</sup>	2.94±1.37 <sup>a</sup>	3.23±0.93 <sup>a</sup>
CCAC	3.85±1.17 <sup>a</sup>	5.23±1.39 <sup>a</sup>	5.58±1.63 <sup>a</sup>	3.07±0.46 <sup>a</sup>	3.30±0.64 <sup>a</sup>	3.23±0.64 <sup>a</sup>
CCCC	3.86±1.47 <sup>a</sup>	5.13±1.04 <sup>a</sup>	6.08±0.69 <sup>a</sup>	3.41±0.53 <sup>a</sup>	3.06±0.25 <sup>a</sup>	3.32±0.70 <sup>a</sup>
CTAA	4.07±0.92 <sup>a</sup>	5.49±0.83 <sup>a</sup>	6.16±0.90 <sup>a</sup>	3.22±1.23 <sup>a</sup>	2.91±0.79 <sup>a</sup>	3.36±0.99 <sup>a</sup>
CTAC	2.62±0.83 <sup>a</sup>	3.84±0.68 <sup>a</sup>	4.29±1.01 <sup>a</sup>	2.40±0.65 <sup>a</sup>	2.28±0.39 <sup>a</sup>	2.41±0.67 <sup>a</sup>
CTCC	3.02±0.09 <sup>a</sup>	4.06±1.04 <sup>a</sup>	4.64±0.32 <sup>a</sup>	2.79±0.17 <sup>a</sup>	2.50±0.57 <sup>a</sup>	2.60±0.35 <sup>a</sup>
TTAA	3.23±1.19 <sup>a</sup>	4.51±0.94 <sup>a</sup>	5.29±0.80 <sup>a</sup>	2.97±0.37 <sup>a</sup>	2.58±0.81 <sup>a</sup>	2.37±0.88 <sup>a</sup>
TTAC	2.80±0.65 <sup>a</sup>	3.60±1.0 <sup>a</sup>	4.57±1.48 <sup>a</sup>	1.95±0.62 <sup>a</sup>	1.74±0.32 <sup>a</sup>	1.78±0.47 <sup>a</sup>
TTCC	1.35±0.25 <sup>a</sup>	2.47±0.50 <sup>a</sup>	2.54±0.70 <sup>a</sup>	1.31±0.39 <sup>a</sup>	1.30±0.01 <sup>a</sup>	1.28±0.21 <sup>a</sup>
CCA	3.53±1.13 <sup>a</sup>	4.92±1.26 <sup>a</sup>	5.37±1.45 <sup>a</sup>	2.97±0.89 <sup>a</sup>	2.80±0.88 <sup>a</sup>	2.93±0.21 <sup>a</sup>
CCCA						
CCCC						
CTAA	21.20±2.71 <sup>a</sup>	16.90±1.77 <sup>a</sup>	11.05±3.70 <sup>a</sup>	8.69±2.73 <sup>a</sup>	8.79±1.64 <sup>a</sup>	8.87±1.31 <sup>a</sup>
CTAC	19.56±1.64 <sup>a</sup>	17.02±1.82 <sup>a</sup>	11.12±0.76 <sup>a</sup>	8.31±0.78 <sup>a</sup>	8.34±0.92 <sup>a</sup>	8.33±1.64 <sup>a</sup>
CTCC	18.82±1.36 <sup>a</sup>	15.51±1.28 <sup>a</sup>	11.12±0.75 <sup>a</sup>	8.49±1.02 <sup>a</sup>	8.31±1.12 <sup>a</sup>	8.56±1.33 <sup>a</sup>
TTAA	20.73±2.86 <sup>a</sup>	16.91±1.97 <sup>a</sup>	11.26±2.19 <sup>a</sup>	8.84±0.70 <sup>a</sup>	8.84±2.26 <sup>a</sup>	8.08±1.13 <sup>a</sup>
TTAC	22.62±2.34 <sup>a</sup>	17.07±1.17 <sup>a</sup>	12.58±1.29 <sup>a</sup>	8.73±0.80 <sup>a</sup>	8.73±0.77 <sup>a</sup>	8.70±2.28 <sup>a</sup>
TTCC	20.99±0.08 <sup>a</sup>	17.58±0.66 <sup>a</sup>	12.16±0.18 <sup>a</sup>	9.05±0.81 <sup>a</sup>	9.24±0.68 <sup>a</sup>	9.40±2.28 <sup>a</sup>
Total	20.30±1.32 <sup>a</sup>	16.24±2.20 <sup>a</sup>	12.29±1.61 <sup>a</sup>	8.86±1.22 <sup>a</sup>	8.04±1.13 <sup>a</sup>	8.57±1.84 <sup>a</sup>
CCA	24.33±1.84 <sup>a</sup>	21.74±2.09 <sup>a</sup>	14.54±0.91 <sup>a</sup>	10.38±1.19 <sup>a</sup>	10.34±0.76 <sup>a</sup>	10.33±1.08 <sup>a</sup>
CCA	25.78±0.93 <sup>a</sup>	22.26±1.18 <sup>a</sup>	15.97±1.37 <sup>a</sup>	12.78±0.34 <sup>a</sup>	12.57±0.62 <sup>a</sup>	12.35±0.20 <sup>a</sup>
CCA	21.32±2.74 <sup>a</sup>	17.40±2.29 <sup>a</sup>	12.23±2.41 <sup>a</sup>	9.15±1.69 <sup>a</sup>	8.76±1.63 <sup>a</sup>	9.16±1.76 <sup>a</sup>

**Table 4. The Frequencies of Apoptosis and MNBN in Case Group under Various FA Concentrations.** Values are means ± SD; n = 23 for CCAA; n = 8 for CCAC; n = 1 for CCCC; n = 10 for CTAA; n = 17 for CTAC; n = 3 for CTCC; n = 4 for TTAA; n = 7 for TTAC; n = 2 for TTCC. Bars marked with different letters differ from each other in the same type in different medium; Repeated measures one-way ANOVA of data

Genotype	FA (nmol/L)					ANOVA p
	15	30	60	120	240	
Apoptosis /500 cells						
CCAA	4.70±0.82 <sup>a</sup>	8.21±1.22 <sup>a</sup>	8.56±1.32 <sup>a</sup>	3.72±0.72 <sup>a</sup>	3.75±0.99 <sup>a</sup>	3.68±0.98 <sup>a</sup>
CCAC	4.96±0.90 <sup>a</sup>	8.24±1.55 <sup>a</sup>	8.81±1.62 <sup>a</sup>	4.05±0.82 <sup>a</sup>	4.30±1.07 <sup>a</sup>	4.17±0.66 <sup>a</sup>
CTAA	4.69±1.40 <sup>a</sup>	8.20±1.09 <sup>a</sup>	8.81±1.62 <sup>a</sup>	3.14±0.79 <sup>a</sup>	3.20±0.28 <sup>a</sup>	3.50±0.32 <sup>a</sup>
CTAC	4.35±0.65 <sup>a</sup>	7.17±1.26 <sup>a</sup>	7.63±0.98 <sup>a</sup>	3.26±0.85 <sup>a</sup>	3.40±0.87 <sup>a</sup>	3.62±0.88 <sup>a</sup>
CTCC	2.84±0.90 <sup>a</sup>	6.14±1.14 <sup>a</sup>	6.64±1.26 <sup>a</sup>	2.57±0.81 <sup>a</sup>	2.75±1.04 <sup>a</sup>	2.77±1.11 <sup>a</sup>
TTAA	3.21±0.37 <sup>a</sup>	6.99±0.12 <sup>a</sup>	6.83±0.19 <sup>a</sup>	3.16±0.23 <sup>a</sup>	3.08±1.29 <sup>a</sup>	3.48±0.78 <sup>a</sup>
TTAC	3.61±0.63 <sup>a</sup>	5.36±1.04 <sup>a</sup>	6.54±1.33 <sup>a</sup>	3.27±0.82 <sup>a</sup>	3.40±0.71 <sup>a</sup>	3.61±0.84 <sup>a</sup>
Total	3.03±0.07 <sup>a</sup>	3.94±0.23 <sup>a</sup>	4.54±0.70 <sup>a</sup>	2.31±0.39 <sup>a</sup>	2.59±0.66 <sup>a</sup>	2.70±1.23 <sup>a</sup>
CCA	4.21±1.11 <sup>a</sup>	7.73±1.62 <sup>a</sup>	8.00±1.63 <sup>a</sup>	3.52±0.93 <sup>a</sup>	3.54±1.06 <sup>a</sup>	3.64±1.03 <sup>a</sup>
CCCA						
CCCC						
CTAA	21.20±2.71 <sup>a</sup>	16.90±1.77 <sup>a</sup>	11.05±3.70 <sup>a</sup>	8.69±2.73 <sup>a</sup>	8.79±1.64 <sup>a</sup>	8.87±1.31 <sup>a</sup>
CTAC	19.56±1.64 <sup>a</sup>	17.02±1.82 <sup>a</sup>	11.12±0.76 <sup>a</sup>	8.31±0.78 <sup>a</sup>	8.34±0.92 <sup>a</sup>	8.33±1.64 <sup>a</sup>
CTCC	18.82±1.36 <sup>a</sup>	15.51±1.28 <sup>a</sup>	11.12±0.75 <sup>a</sup>	8.49±1.02 <sup>a</sup>	8.31±1.12 <sup>a</sup>	8.56±1.33 <sup>a</sup>
TTAA	20.73±2.86 <sup>a</sup>	16.91±1.97 <sup>a</sup>	11.26±2.19 <sup>a</sup>	8.84±0.70 <sup>a</sup>	8.84±2.26 <sup>a</sup>	8.08±1.13 <sup>a</sup>
TTAC	22.62±2.34 <sup>a</sup>	17.07±1.17 <sup>a</sup>	12.58±1.29 <sup>a</sup>	8.73±0.80 <sup>a</sup>	8.73±0.77 <sup>a</sup>	8.70±2.28 <sup>a</sup>
TTCC	20.99±0.08 <sup>a</sup>	17.58±0.66 <sup>a</sup>	12.16±0.18 <sup>a</sup>	9.05±0.81 <sup>a</sup>	9.24±0.68 <sup>a</sup>	9.40±2.28 <sup>a</sup>
Total	20.30±1.32 <sup>a</sup>	16.24±2.20 <sup>a</sup>	12.29±1.61 <sup>a</sup>	8.86±1.22 <sup>a</sup>	8.04±1.13 <sup>a</sup>	8.57±1.84 <sup>a</sup>
CCA	24.33±1.84 <sup>a</sup>	21.74±2.09 <sup>a</sup>	14.54±0.91 <sup>a</sup>	10.38±1.19 <sup>a</sup>	10.34±0.76 <sup>a</sup>	10.33±1.08 <sup>a</sup>
CCA	25.78±0.93 <sup>a</sup>	22.26±1.18 <sup>a</sup>	15.97±1.37 <sup>a</sup>	12.78±0.34 <sup>a</sup>	12.57±0.62 <sup>a</sup>	12.35±0.20 <sup>a</sup>
CCA	21.32±2.74 <sup>a</sup>	17.40±2.29 <sup>a</sup>	12.23±2.41 <sup>a</sup>	9.15±1.69 <sup>a</sup>	8.76±1.63 <sup>a</sup>	9.16±1.76 <sup>a</sup>

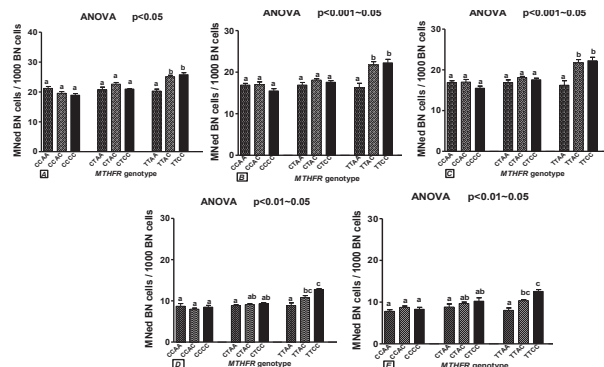
**Table 5. The Frequencies of Apoptosis and MNBN in Control Group under Various FA Concentrations.** Values are means ± SD; n = 27 for CCAA; n = 7 for CCAC; n = 3 for CCCC; n = 11 for CTAA; n = 19 for 677CT/1298AC; n = 2 for CTCC; n = 4 for TTAA; n = 2 for TTAC; n = 0 for TTCC. Bars marked with different letters differ from each other in the same type in different medium; Repeated measures one-way ANOVA of data

**MNBN and apoptosis in different FA concentration**

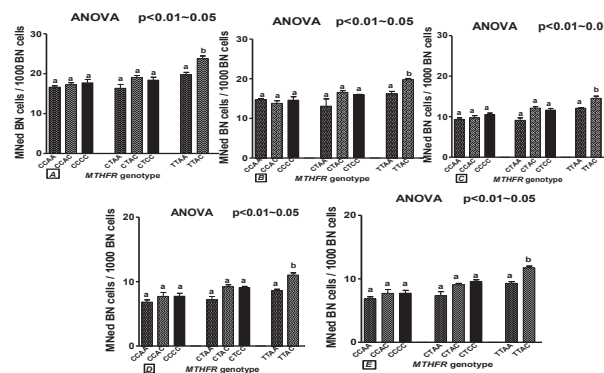
The frequency of MNBN significantly decreased with increasing FA concentration from 15 to 120 or 240 nmol/L FA (p < 0.05 to 0.001), and the frequency of apoptosis increased with increasing FA concentration from 15 to 30 or 60 nmol/L. The frequency of apoptosis was likewise reduced when the FA concentration increased from 60 to 120 or 240 nmol/L in all tested groups (p < 0.05 to 0.001). There was no significant difference between the detected biomarkers between 120 and 240 nmol/L FA in all of the groups (Table 4, 5).

**Frequencies of MNBN in MTHFR C677T-A1298C combined genotypes in cancer cases**

The combined genotypes of 677CC, 677CT with any 1298 alleles and of 677TT with 1298AA showed the lowest frequencies of MNBN under any concentration of FA, and no significant difference was detected. The genotype of 677TT with either 1298AC or CC (TTAC and TTCC) showed significant increases in the frequencies of MNBN regardless of the FA concentration relative to the frequencies of MNBN from the genotypes 677CC with any 1298 alleles and to CTAA or TTAA (p < 0.05 to 0.001). The difference of the frequencies of MNBN



**Figure 1. The Frequencies of MNBN in the Case and Control Group with the Combined MTHFR C677T-A1298C Genotypes.** (A) 15 nM FA (B) 30 nM FA (C) 60 nM FA (D) 120 nM FA (E) 240 nM FA



**Figure 2. The Frequencies of MNBN in the Control Group with the Combined MTHFR C677T-A1298C Genotypes.** (A) 15 nM FA (B) 30 nM FA (C) 60 nM FA (D) 120 nM FA (E) 240 nM FA

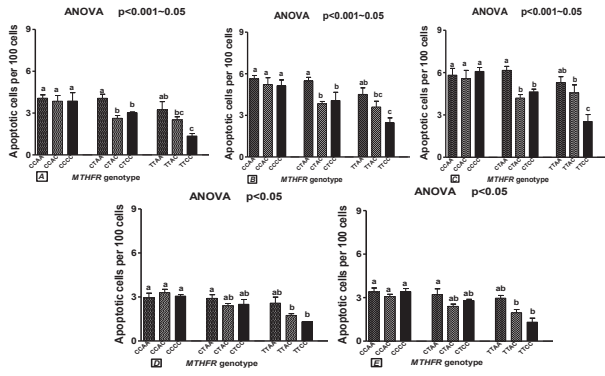
in lymphocytes with the 677CT, 677TT genotype with 1298AC, 1298CC (CTAC, CTCC, TTAC and TTCC) was not significant at concentrations of 120 to 240 nmol/L FA, but CTAC or CTCC was significantly lower than the TTAC or TTCC genotypes at 15 to 60 nmol/L in the cancer group (p < 0.05 to 0.01) (Figure 1).

**Frequencies of MNBN among MTHFR C677T-A1298C genotypes in controls**

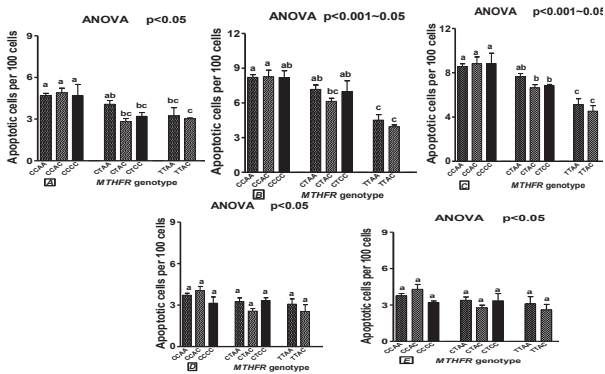
The 677CC, 677CT with any 1298 alleles and TTAA genotypes had the lowest frequencies of MNBN, and there was no significant difference between them. No TTCC individuals were found in the control group. The MNBN levels from the TTAC genotype were significantly increased relative to the MNBN levels from the 677CC and 677CT genotypes with any 1298 alleles (p < 0.05 to 0.01), regardless of FA concentration change (Figure 2).

**Frequencies of apoptosis among MTHFR C677T-A1298C genotypes in cancer cases**

The 677CC with any 1298 alleles and 677CT with 1298AA genotypes showed higher frequencies of apoptosis in lymphocytes than other genotypes (p < 0.05 to 0.01), and there were no significant differences in the levels of apoptosis between them at any concentration of FA. The 677TT with 1298 AC or CC (TTCC and TTAC) genotypes had lower frequencies of apoptosis than the 677CC with any 1298 alleles and CTAA genotypes at any FA concentration (p < 0.05 to 0.01). The frequencies of



**Figure 3. The Frequency of Apoptosis in the Case Group with the Combined MTHFR C677T-A1298C Genotypes.** (A) 15 nM FA (B) 30 nM FA (C) 60 nM FA (D) 120 nM FA (E) 240 nM FA



**Figure 4. The Frequencies of Apoptosis in the Control Group with the Combined MTHFR C677T-A1298C Genotypes.** (A) 15 nM FA (B) 30 nM FA (C) 60 nM FA (D) 120 nM FA (E) 240 nM FA

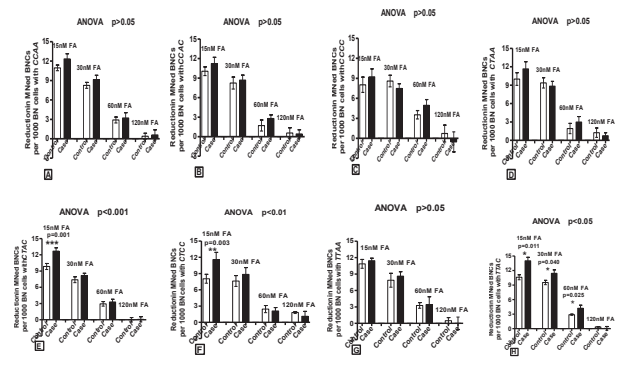
apoptosis of 677CT heterozygote with either the 1298AC or CC (CTAC and CTCC) genotypes were significantly lower than from the 677CC with any 1298 alleles and the CTAA genotypes in all of the FA concentration groups ( $p < 0.01$ ); however, the frequency of apoptosis was increased when FA was increased to 120 nmol/L ( $p < 0.05$ ). The 677TT or 677 CT with 1298 AA (TTAA and TCAA) and 677CC with 1298AC or CC (CCAC and CCCC) genotypes did not show significant differences in the levels of apoptosis relative to CCAA in the cancer case group (Figure 3).

*Apoptosis among MTHFR C677T-A1298C genotypes in controls*

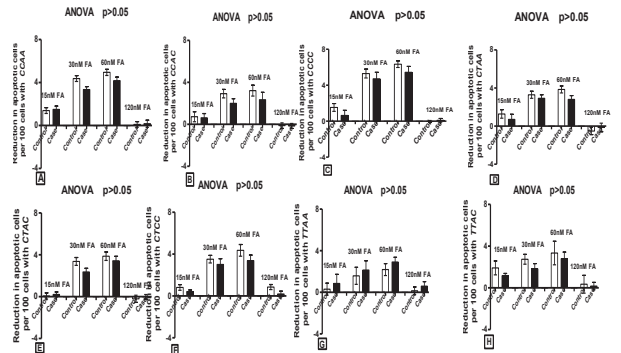
The genotype 677CC with any 1298 alleles (CCAA, CCAC and CCCC) had higher levels of apoptosis than other genotypes ( $p < 0.01$ ) and did not have significant differences in the level of apoptosis relative to each other. The combination of the 677TT and 677CT with any 1298 alleles (TTAA, TTAC, CTAA and CTAC) showed a significant decrease in the levels of apoptosis relative to the 677CC with any 1298 allelegenotypes at 15 to 60 nmol/L of FA ( $p < 0.05$  to 0.001), and increased significantly as FA was increased to over 120 nmol / L ( $p < 0.05$ ) (Figure 4).

*Comparison of MNBN/apoptosis frequency between cancer cases and controls*

A difference of differences analysis showed that



**Figure 5. Comparison of the MNBN Frequencies Between the Case and Control Groups with the Combined MTHFR C677T-A1298C Genotypes after the Difference of Differences Analysis under FA Deficiency.** The results are shown as column and bar of results for each individual. (A) CCAA genotype (B) CCAC genotype (C) CCCC genotype (D) CTAA genotype (E) CTAC genotype (F) CTCC genotype (G) TTAA genotype (H) TTAC genotype



**Figure 6. Comparison of the Apoptosis Frequencies Between the Case and Control Groups with the Combined MTHFR C677T-A1298C Genotypes after the Difference of Differences Analysis under FA Deficiency.** The results are shown as column and bar of results for each individual. (A) CCAA genotype (B) CCAC genotype (C) CCCC genotype (D) CTAA genotype (E) CTAC genotype (F) CTCC genotype (G) TTAA genotype (H) TTAC genotype

MNBN levels in individuals with the 677CT with a mutated 1298 alleles from the cancer group were significantly higher than those in the respective control group at 15 nmol/L FA ( $p < 0.001$  to 0.01), whereas the MNBN levels in the cancer case group sharing the 677TT with 1298AC genotype were significantly higher at 15 to 60 nmol/L FA ( $p < 0.05$ ) than in the controls (Figure 5).

There were no significant differences in the occurrence of apoptosis among individuals with any genotype between the cancer case and control groups as determined using a difference of differences analysis by subtracting the pooled values of the biomarker at 240 nmol/L FA from those observed at 15 to 120 nmol/L FA for each individual (Figure 6).

**Discussion**

Reports studying the association of folate metabolism and changes in MTHFR activity with breast cancer are conflicting at all time (Campbell et al., 2002; Rodrigues et al., 2010). Therefore, the present report

studied the relationship between combined MTHFR polymorphisms and genetic damage and apoptosis under folate deficiency, and compared the differences in response to folate deficiency between breast cancer and noncancer populations with a combined MTHFR genotype. However, the results must be considered with some caution because the culture conditions may not predict precisely what happens in vivo.

In this study, we found that superfluous supplementation of FA (more than 60 or 120 nmol/L) decreased the frequency of apoptosis. In contrast, MNBN exhibited a strong negative correlation with folic acid concentration but plateaued at 120 nmol/L (120 versus 240 nmol/L;  $p > 0.05$ ). Taken together, these results suggest that FA deficiency may increase the risk of cancer by inducing an imbalance in DNA precursors, leading to modified DNA synthesis and repair, and a concentration of 120 nmol/L FA is required to minimize cytotoxicity and genetic damage.

Changes of MTHFR activity may tilt the balance of one carbon metabolism in favor of DNA synthesis at the expense of methyl supply for methylation reactions; a suboptimal methyl supply can lead to aberrant DNA methylation, which has been associated with breast cancer etiology. The C677T polymorphism is in exon 4, which is within the N-terminal catalytic domain of the enzyme, and the A1298C polymorphism is in exon 7, which is within the C-terminal regulatory domain. The more dynamic effect of C677T is due to its location within the catalytic region. The A1298C polymorphism may affect enzyme regulation possibly by influencing S-adenosylmethionine, which is an allosteric inhibitor of MTHFR and is known to bind in the C-terminal region (Yi et al., 2002; Promthet et al., 2010). Consequently, effects on MTHFR activity may become significant when an individual carries both mutations. The study found that the activity for the 1298A→C polymorphism was approximately 65% of that of the controls and the activity for the 677C→T mutation was approximately 40% of that of the controls. However, individuals that were heterozygous for both the 1298A→C and 677C→T mutation had somewhat lower activities in lymphocytes (50% to 60% of controls) than single 677C→T heterozygotes (Weisberg et al., 1998). Low enzyme activity may reduce the capacity of DNA methylation and possibly reduce uracil misincorporation into DNA (Weisberg et al., 1998; Skibola et al., 1999; Crott et al., 2001; Huang et al., 2008; Thomas et al., 2008; Dhillon et al., 2009; Promthet et al., 2010; Rodrigues et al., 2010). One puzzling result of the present study is that there was no trend toward a reduction of MNBN levels in single mutant (TTAA) cells compared with wild-type (CCAA) cells. Additionally, there was an increased frequency of MNBN in the homozygous mutant at both 677 and 1298 (TTCC) compared with homozygous mutant at 677 (TTAA) cells within the cancer case group and in homozygous mutant at 677 and heterozygous mutant at 1298 (TTAC) compared with homozygous mutant at 677 (TTAA) cells within both groups. This is counterintuitive because uracil in DNA is positively correlated with MNBN in other studies using this system (Crott et al., 2001). However, MNBN may originate not only from chromosome breakage caused by uracil in

DNA, but also from chromosomal loss events that may be caused by hypomethylation of DNA (Friso et al., 2002). One study reported that the 677TT with a mutated 1298 locus genotype increases DNA hypomethylation in lymphocytes in vivo (Campbell et al., 2002), and DNA hypomethylation in lymphocytes in vitro or in vivo increases the loss of chromosomes 1, 9 and 16, which are then included in MNBN (Guttenbach et al., 1994; Xu et al., 1999). Therefore, the marginal increase in MNBN levels in 677TT with mutated 1298 locus cells may be due to increased chromosomal loss events; thus, combined mutations could enhance the instability of the genome in the Chinese population.

In accordance with the results discussed above, apoptosis levels were lower in the 677TT with 1298CC than the 677TT with 1298AA genotype and lower in the 677CT with 1298AC genotype than the 677CT with 1298AA genotype at 15 to 60 nmol / L FA in cancer cases, but the lower apoptosis level in the 677TT with 1298AC genotype relative to the 677TT with 1298AA genotype and in 677CT with 1298AC genotype relative to the 677CT with 1298AA genotype was not significant in the control group. To the best of our knowledge, this is the first report showing that spontaneous apoptosis in breast cancer patients is associated with or may even depend on MTHFR genotypes. It also found that the spontaneous apoptosis rates after 24 h were significantly increased in chronic lymphocytic leukemia patients with the MTHFR 677CC genotype versus MTHFR 677CT and MTHFR 677TT genotypes (41, 35 and 25%, respectively,  $p = 0.045$ ). Additionally, the spontaneous apoptosis rate was significantly decreased in the 1298AA genotype vs. 1298AC genotype (29 and 40%, respectively,  $p = 0.015$ ) (Bistulfi et al., 2010). It reported on the diversion of one-carbon units toward nucleotide synthesis at the expense of DNA methylation in young women with the 677TT genotype after 7 weeks of folate restriction (Quinlivan et al., 2005), which suggested that the decrease in apoptosis was related to DNA synthesis. Meanwhile, the study found that apoptosis was observably lower in the 677TT with 1298CC genotype than 677TT with 1298AA genotype and lower in the 677CT with 1298AC genotype than the 677CT with 1298AA genotype at 15 to 60 nmol/L FA in cancer cases, which suggested that the combined mutation of MTHFR may be more likely to influence apoptosis processes in the cancer cases group with FA deficiencies. Our data provide some evidence suggesting that the hypomethylation of DNA might decrease apoptosis processes in some cancer patients; this result was in agreement with those of Celtikci et al. (2009) and Xiao et al. (2006). In that of my study, apoptosis did not appear to be related to the combined MTHFR polymorphisms in the control group. Although apoptosis was lower in 677CT with 1298AC cells than in 677CT with 1298AA cells in the control group, the magnitude of this difference was small, and it is unclear if this is a physiologically relevant difference. A possible weakness of our study is that we did not measure DNA methylation directly.

It is known that malignancies derived from rapidly proliferating tissues are expected to have an increased requirement for DNA synthesis, and patients with such

malignancies could, therefore, be even more endangered by folate deficiency and the resulting DNA damage. By the difference of differences analysis, MNBN from the TTAC genotype at 15 to 60 nmol/L FA and the 677CT with mutated 1298 locus genotype at 15 nmol/L FA in cancer cases is significantly higher than in controls, and in individuals receiving adequate FA, there were no significant differences in sensitivities to the genomic damage between the cancer cases and controls in all of the genotypes. This finding seems to suggest that the breast cancer patients with the combined mutation of 677TT with 1298AC genotype may be more sensitive to FA deficiency than controls, and the effect of the resulting genomic damage in the cancer case groups by MTHFR polymorphisms seems to be dependent on folate status. Additionally, for the occurrence of apoptosis among individuals with any genotype, our data showed no significant differences between cancer cases and controls, which suggests that women with the combined mutation of MTHFR C677T-A1298C genotypes have significantly increased odds of developing breast cancer.

In summary, our study has shown that the combined mutants of MTHFR C677T-A1298C have a significant effect on increasing genetic damage and decreasing apoptosis levels compared with single variants in breast cancer patients. Breast cancer patients with the combined mutation of MTHFR C677T-A1298C may be more sensitive than controls to FA deficiency that can cause the induction of genomic instability. An association was found between MTHFR C677T and combined MTHFR C677T-A1298C polymorphism and breast cancer in our series. Unfortunately, there was no 677TT with 1298CC genotype control in the study, so we cannot compare the difference on genetic damage and apoptosis in breast cancer cases and non-cancer controls with the 677TT with 1298CC genotype.

## Acknowledgements

We are grateful to the volunteers for their participation in the study. This work is supported by the National Natural Science Foundation of China (No. 30760098, 30960166) and the Natural Science Foundation of Yunnan Province (No.2008C008Z, 2008CD108). The author(s) declare that they have no competing interests.

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