

## ***MTHFR* Gene Polymorphism and Its Relationship with Plasma Homocysteine and Folate in a North Indian Population**

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**Abstract** There is a paucity of data on the prevalence of elevated homocysteine and its relation with plasma folate and the methylenetetrahydrofolate reductase (*MTHFR*) gene in the population of North India. This study evaluates *MTHFR* gene polymorphism and its relationship with plasma homocysteine and folate levels in a healthy North Indian population. The age of the 200 subjects included in this study was in the range 18–73 (mean 39.4) years. The plasma homocysteine level was elevated in 56.5%, and the plasma folate level was low in 49.5% of the subjects. Heterozygous *MTHFR* gene polymorphism (CT) was present in 15.5%, and homozygous (TT) in 3.5% of the subjects. Age, diet, and *MTHFR* gene polymorphisms were related to homocysteine level. All the subjects with the TT and 79% with the CT genotype had a high level of plasma homocysteine, whereas 51% with the CC genotype had a high homocysteine level. After adjustment for the effect of covariates, however, homocysteine was not related to *MTHFR* gene polymorphism.

**Keywords** *MTHFR* gene polymorphism · Homocysteine · Folate · Dietary habit · Healthy population · Indian population

### **Introduction**

Folate is one of the most important determinants of plasma homocysteine concentration (Evans et al. 2003; Brattstrom and Wilcken 2000). In addition to

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dietary intake of substrate and vitamin cofactors of homocysteine metabolism, the methylenetetrahydrofolate reductase (*MTHFR*) gene regulates folate-dependent remethylation of homocysteine to methionine. The 677C → T substitution polymorphism within this gene causes thermolability and reduces the activity of the methylenetetrahydrofolate reductase enzyme. It has been suggested that polymorphism of this gene accelerates coronary artery disease in patients with familial hypercholesterolemia or a previous myocardial infarction (Kawashiri et al. 2000).

In Southeast Asia, including India, premature atherosclerosis has been attributed to metabolic syndrome, comprising hyperglycemia, obesity, and elevated triglycerides. This has been suggested to result in a high incidence of premature coronary artery disease and may be a risk factor of stroke in the young (Hughes et al. 1989; Balian et al. 1999). The Indian diet usually contains cereals, pulses, and vegetables that have adequate folic acid; however, in a vegetarian diet, the vitamin B<sub>12</sub> level is usually low, and it requires at least 1 liter of milk or milk products to meet the daily requirement of vitamin B<sub>12</sub> (Gopalan et al. 1996). There is a paucity of studies from Northern India correlating folic acid, homocysteine level, and *MTHFR* gene polymorphism in normal subjects. A few studies have examined *MTHFR* gene polymorphism in normal individuals and have reported the occurrence of a homozygous gene polymorphism in 0–3.5% and a heterozygous gene polymorphism in 18.1–40.8% (Singh et al. 2005; Alam et al. 2008; Kalita et al. 2006; Panigrahi et al. 2006). In these studies, however, a detailed correlation of various demographic data, dietary intake of folate and vitamin B<sub>12</sub>, smoking, body mass index, life style, and *MTHFR* gene polymorphism with homocysteine was not evaluated. In a study from Pune, India, on 63 normal individuals, homocysteine was high in 81%, serum B<sub>12</sub> was low in 46%, serum folate was low in 7.9%, and none had a homozygous *MTHFR* gene polymorphism (Refsum 2001). In this study, we report the relation among plasma folic acid, homocysteine, and *MTHFR* gene polymorphism in apparently healthy normal individuals and evaluate the predictors of high homocysteine level.

## Materials and Methods

This study included 200 consecutive healthy volunteers and the relatives of the patients attending the neurology department of Sanjay Gandhi Postgraduate Institute of Medical Science, Lucknow, India. This study was approved by the Institute Ethics Committee, and informed consent was taken from the subjects. There was no history of neurological or medical diseases, and their clinical examination was normal. All the subjects underwent a detailed dietary history to determine the daily intake of vitamin B<sub>12</sub> and folic acid, using a food frequency questionnaire that has been validated in an Indian population (Kaur and Sangha 2006). The subjects were shown a standard 150 ml cup and 5 ml spoon and asked to compare their consumption of folate and vitamin B<sub>12</sub> sources such as cereals, pulses, vegetables, legumes, milk, milk products, fish, meat, and so on. A weekly food frequency was formulated, and the frequency of consumption of these food items was determined. The average daily consumption of folate and vitamin B<sub>12</sub>

was calculated by comparing to per 100 g versus milliliter values as per the Indian Council of Medical Research guidelines (Gopalan et al. 1996). The height and weight of each subject were measured, and body mass index was calculated as weight in kilograms divided by height in  $m^2$ .

There were 200 subjects with an age range of 18–73 (mean 39.4) years. All the individuals were from Uttar Pradesh; 83 subjects were from a rural area, 124 were males, 32 were Muslim, and 168 were Hindu. Among the Hindu subjects, 78 were Brahmin, 48 Kshatriya, 12 Vaishya, and 35 belonged to other castes. Twenty-seven (13.5%) subjects were sedentary, 36 (18%) smoked, and 30 (15%) consumed alcohol. The body mass index was above 25 in 36 (18%) subjects. The mean systolic blood pressure was  $129 \pm 5$  mm of Hg, and the diastolic blood pressure was  $81 \pm 4$  mm of Hg. Of the 200 subjects, 111 were nonvegetarians and 99 were lacto-vegetarians.

Subjects were requested to fast overnight, and 10 ml venous blood was collected by venepuncture in an EDTA vial between 8 and 9 AM. Blood was immediately kept on ice. A 5 ml blood sample was centrifuged at 10,000 rpm for 10 min at 4°C, and plasma was separated. Plasma and the remaining 5 ml blood were preserved in a –40°C refrigerator until analyzed.

#### Plasma Homocysteine, Vitamin B<sub>12</sub>, and Folate Estimation

The plasma homocysteine level was estimated by enzyme-linked immunosorbent assay (ELISA) using the Axis Shield diagnostic kit. Plasma folate and B<sub>12</sub> were estimated by radioimmunoassay (DPC, USA). The plasma homocysteine (>13.9  $\mu\text{mol/l}$ ), B<sub>12</sub> (<211 pg/ml), and folate (<5.38 ng/ml) levels in our subjects were considered to be abnormal (Kalita et al. 2007).

#### *MTHFR* Gene Analysis

Deoxyribonucleic acid (DNA) was extracted from blood by the salting out method using phenol–chloroform as described by Comey et al. (1994). The *MTHFR* 677 C-T substitution was identified with the use of restriction enzyme digestion of the polymerase chain reaction (PCR) amplified products (Alam et al. 2008). Exonic 5'-TGAA GGAGAA GGTGTCTGCG GGA-3' and intronic 5'-AGG ACGG TCG GGTG AGAGTG-3' primers (Fermentas, USA) were used. This generates a 198-bp fragment. The C-T substitution creates a *HinfI* recognition sequence, and digestion of the PCR product results in fragments of 175 and 23 bp. The fragment size was determined by gel electrophoresis (Frosst et al. 1995).

#### Statistical Analysis

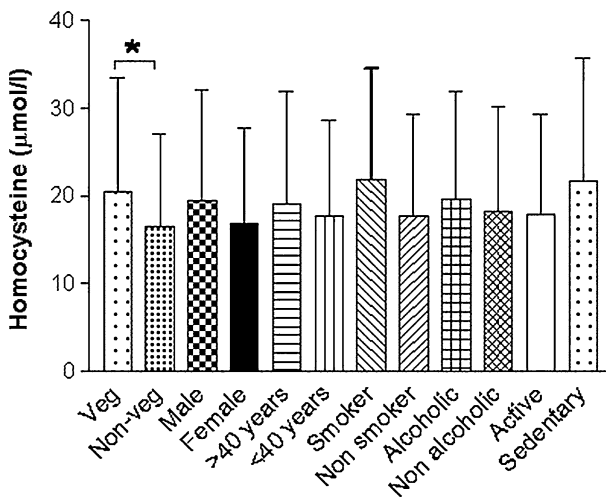
The effect of gender, age, life style, body mass index, smoking, diet, plasma folate and B<sub>12</sub> level and *MTHFR* polymorphism on homocysteine was evaluated using linear regression analysis. The residual values of homocysteine, after regressing out for significant covariates, were analyzed using one-way analysis of variance to test

the significance among the significant covariates. Statistical analysis was done using the Statistical Package for Social Science version 10 (SPSS Inc., Chicago).

## Results

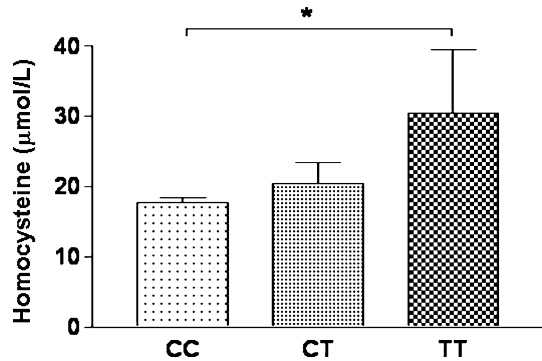
The daily folate intake was normal in all the subjects (median 249.3  $\mu\text{g}$ ). The median homocysteine level was 14.7 (1.3–65.8)  $\mu\text{mol/l}$ . The mean homocysteine level was above 13.9  $\mu\text{mol/l}$  in 113 (56.5%) subjects. The elevation of homocysteine was mild (14–39  $\mu\text{mol/l}$ ) in 91 and moderate (40–100  $\mu\text{mol/l}$ ) in 22 subjects. None of the subjects had severe hyperhomocysteinemia exceeding 100  $\mu\text{mol/l}$ .

The median plasma folate level was 3.1 (0.1–22.2)  $\text{ng/ml}$ . The plasma folate level was low (<5.38  $\text{ng/ml}$ ) in 99 (49.5%) subjects. The median plasma  $\text{B}_{12}$  level was 210.5 (28.9–1,795.9)  $\text{pg/ml}$  and was low (<211  $\text{pg/ml}$ ) in 99 subjects. The *MTHFR* gene polymorphism study revealed TT (homozygous) in 7 (3.5%) and CT (heterozygous) in 24 (12%) subjects. On linear regression analysis, homocysteine was significantly related to age ( $P = 0.01$ ), dietary habit ( $P = 0.01$ ), and *MTHFR* gene polymorphism ( $P = 0.01$ ), whereas smoking ( $P = 0.33$ ), alcohol consumption ( $P = 0.11$ ), body mass index ( $P = 0.22$ ), life style ( $P = 0.44$ ), plasma folate ( $P = 0.76$ ), and  $\text{B}_{12}$  ( $P = 0.72$ ) were not related to homocysteine (Fig. 1). After regressing out the effect of significant covariates, however, homocysteine was not significantly related to *MTHFR* gene polymorphism ( $P = 0.41$ ). All the subjects with the TT (homozygous) polymorphism and 19 (79%) with the CT (heterozygous) polymorphism had hyperhomocysteinemia, whereas 58 (51%) without polymorphism had hyperhomocysteinemia. Of the subjects with low folate, 55% had hyperhomocysteinemia; 50% with normal folate also had hyperhomocysteinemia



**Fig. 1** Plasma homocysteine level ( $\mu\text{mol/l}$ ) in various clinical parameters (\*  $P < 0.05$ )

**Fig. 2** Relationship between plasma homocysteine and *MTHFR* gene polymorphism (\*  $P < 0.05$ )



(Fig. 2). Elevated homocysteine level was more common in vegetarians (63%) than in nonvegetarians (50%).

## Discussion

In our study, 56.5% of the apparently healthy subjects had mild to moderate elevation of plasma homocysteine levels which were not related to *MTHFR* gene polymorphism. The homocysteine level in Indian subjects is reported to be higher than in western populations (Anand et al. 2000; Chambers et al. 2000; Misra et al. 2002; Kumar et al. 2005). In Southeast Asians and Orientals, serum homocysteine levels are high when compared with Americans and Europeans (Hughes et al. 1989; Balian et al. 1999). In healthy well-nourished individuals, homocysteine metabolism is well-regulated, and the range of total levels is 5–13.9 µmol/l (Welch and Loscalzo 1998). More than half of our normal volunteers had total homocysteine levels above the defined normal range. Deficiency of enzymes or cofactors that are involved in the metabolism of homocysteine can result in aberrant intracellular processing, leading to elevated homocysteine. In the metabolism of homocysteine, vitamin B<sub>12</sub>, folate in the remethylation pathway, and vitamin B<sub>6</sub> in the trans-sulfuration pathway are crucial. A deficiency of these vitamins can result in varying degrees of hyperhomocysteinemia (Welch and Loscalzo 1998).

We have studied the *MTHFR* C677T polymorphism. Among the genes involved in homocysteine metabolism, *MTHFR* plays a key role. *MTHFR* converts methylenetetrahydrofolate to methyltetrahydrofolate, which is a primary methyl donor in the transmethylation reaction by which homocysteine is converted to methionine. Therefore, defects in *MTHFR* genes may result in elevation of homocysteine. In our study, *MTHFR* gene polymorphism is significantly related to a raised homocysteine level, but after adjustment of the other covariate, it did not achieve significance. All the 3.5% homozygous and 79% heterozygous subjects had hyperhomocysteinemia, whereas only 51.5% without the *MTHFR* gene polymorphism had elevated homocysteine levels.

In a study on distribution of the *MTHFR* C677T gene polymorphism in Indians, 3% of the subjects were homozygous and 18% were heterozygous (Kaur and Sangha

2006). In another study, the frequency of *MTHFR* C677T reveals homozygous alleles in 3.5% and heterozygous alleles in 40.8% of the subjects (Reddy and Jamil 2006). Kumar et al. (2005) did not find an association of allele level with the *MTHFR* C677T polymorphism, but they did find a relationship with the *MTHFR* A1298C polymorphism. The *MTHFR* A1298C polymorphism in the homozygous condition has been reported in 18% of the population and the heterozygous condition in 71%. That study, however, did not include subjects from the state of Uttar Pradesh (the source of the subjects in this study). It has been reported that the C677T polymorphism results in reduction of *MTHFR* activity by about 30% in heterozygous and by 60% in homozygous individuals (Kalita et al. 2006). In the A1298C polymorphism, *MTHFR* activity was reduced by 10% in heterozygotes and 35–45% in homozygotes, which is much lower than the C677T polymorphism. We, therefore, have evaluated the C677T polymorphism.

In our study, homocysteine was related to the vegetarian diet on a linear regression analysis, highlighting the inadequacy of vitamin B<sub>12</sub> in a vegetarian diet in the absence of adequate milk and milk product consumption. Dietary folate consumption was within the normal range in our subjects, but plasma folate was low in half the subjects. This may be due to typical cooking practices in India, as folate is lost during prolonged cooking at high temperatures. Low plasma folate in our subjects may also be due to common prevalence of gastrointestinal infections and infestations; however, the presence of these infections and infestations was not confirmed.

From this study, it can be concluded that about half the apparently healthy individuals from North India have high plasma homocysteine levels which are not associated with *MTHFR* C677T polymorphism.

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