Association of methylenetetrahydrofolate reductase gene polymorphisms & colorectal cancer in India


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Background & objectives: Methylenetetrahydrofolate reductase (MTHFR) is a critical enzyme in folate metabolism and involved in DNA synthesis, DNA repair and DNA methylation. The two common functional polymorphisms of MTHFR, 677 C→T and 1298 A→C have shown to impact several diseases including cancer. This case-control study was undertaken to analyse the association of the MTHFR gene polymorphisms 677 C→T and 1298 A→C and risk of colorectal cancer (CRC).

Methods: One hundred patients with a confirmed histopathologic diagnosis of CRC and 86 age and gender matched controls with no history of cancer were taken for this study. DNA was isolated from peripheral blood samples and the genotypes were determined by PCR-RFLP. The risk association was estimated by compounding odds ratio (OR) with 95 per cent confidence interval (CI).

Results: Genotype frequency of MTHFR 677 CC, CT and TT were 76.7, 22.1 and 1.16 per cent in controls, and 74, 25 and 1.0 per cent among patients. The ‘T’ allele frequency was 12.21 and 13.5 per cent in controls and patients respectively. The genotype frequency of MTHFR 1298 AA, AC, and CC were 25.6, 58.1 and 16.3 per cent for controls and 22, 70 and 8 per cent for patients respectively. The ‘C’ allele frequency for 1298 A→C was 43.0 and 45.3 per cent respectively for controls and patients. The OR for 677 CT was 1.18 (95% CI 0.59-2.32, *P = 0.642), OR for 1298 AC was 1.68 (95% CI 0.92-3.08, *P = 0.092) and OR for 1298 CC was 0.45 (95% CI 0.18-1.12, *P = 0.081). The OR for the combined heterozygous state (677 CT and 1298 AC) was 1.18 (95% CI 0.52-2.64, *P =0.697).

Interpretation & conclusion: The frequency of the MTHFR 677 TT genotype is rare as compared to 1298 CC genotype in the population studied. There was no association between 677 C→T and 1298 A→C polymorphisms and risk of CRC either individually or in combination. The homozygous state for 1298 A→C polymorphism appears to slightly lower risk of CRC. This needs to be confirmed with a larger sample size.

Key words Colorectal cancer - MTHFR gene polymorphisms

Folate deficiency resulting from low consumption of vegetables and fruits is associated with increased risk of several cancers, including colorectal cancer. An important biological function of folate is to provide methyl groups required for intracellular methylation reactions and de novo DNA synthesis. Folate deficiency is thought to be carcinogenic through disruption of DNA methylation, synthesis and impaired
DNA repair. However, folate requires metabolic transformation catalysed by several enzymes including methylenetetrahydrofolate reductase.

MTHFR has a major impact on the regulation of the folic acid pathway due to the conversion of 5, 10 methylenetetrahydrofolate (methylene-THF) to 5-methyl-THF. The two common polymorphisms of the MTHFR gene are 677 C→T and 1298 A→C which are known to reduce the enzyme activity leading to a decreased pool of methyl-THF and associated with hyperhomocysteinemia, particularly in folate deficiency. The 677 C→T transition (exon 4) causes an amino acid substitution from alanine to valine at codon 222 within the catalytic region of the enzyme. The 1298 A→C substitution is within the proposed regulatory region of the methylenetetrahydrofolate reductase enzyme and may influence overall enzyme function, particularly in combination with the 677 C→T polymorphism.

MTHFR variants are known to influence disease processes and several studies have been reported on reduced enzyme activity and the susceptibility to different disorders, including vascular diseases, neural tube defects and cancer. Homozygous for MTHFR 677 C→T and 1298 A→C polymorphisms have been shown to reduce the risk of CRC and acute lymphoblastic leukaemia. Considerable ethnic variations have been reported in the frequency of the 677 C→T and 1298 A→C polymorphisms.

This case control study looks at the association of the 677 C→T and 1298 A→C genetic polymorphisms in the MTHFR gene and risk of CRC in a predominantly South Indian population.

Material & Methods

A total of 100 patients have been randomly selected from among the patients attending the Surgical Oncology outpatient department of the Kidwai Memorial Institute of Oncology, Bangalore. Patients initially selected for the study were those clinically diagnosed as having CRC and were definitely included in the study after histopathological confirmation.

Eighty six age and gender matched healthy individuals without gastrointestinal symptoms or family history of cancer, belonging to the same socio-economic group formed the control group from among our hospital attenders and attenders of patient’s other than cases enrolled into this study. The study was carried out between February 2006 and February 2008 and included a predominantly South Indian population. The study protocol was approved by the Institutional Ethical Committee and informed consent was obtained from the subjects under study.

DNA extraction: Five ml of blood was collected from all subjects in tubes containing dipotassium EDTA as anti coagulant. Genomic DNA was isolated from peripheral leukocytes following the standard phenol- chloroform extraction method. DNA thus obtained was stored at -80° C till analysis. The quantity and purity of DNA was checked by spectrophotometric analysis and gel electrophoresis.

Genotyping of the MTHFR polymorphisms: Custom synthesised primers and other PCR reagents namely Taq polymerase, 10x PCR buffer, dNTPs, MgCl2, and Hinfl were purchased from Bangalore Genie (India). MboII was procured from Fermentas (USA). Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) was employed to study the two common MTHFR polymorphisms namely 677 C→T and 1298 A→C.

677 C→T polymorphism: The method described by Frosted et al was used for detection of 677 C→T polymorphism A length of 198 base pairs on exon 4 of MTHFR gene was amplified using 5 TGA AGG AGAAGG TGT CTG CGG GA 3 primer and 5 AGG ACG GTG CGG TGA GAG TG 3′ as reverse primer. The C to T polymorphism at codon 677 introduces a restriction site for enzyme Hinfl. The PCR was carried out in a total volume of 25 μl containing about 200 ng/ml genomic DNA, 2 μl of 2.5 mM of each of the dNTP’s, 0.5 μl of 25 mM MgCl2, 10 pmol of both primers and 0.5 U Taq polymerase. PCR cycling conditions were an initial denaturation step at 94° C for 5 min, and 30 cycles of the following: 94°C for 1 min, 57°C for 1 min and 72°C for 15 sec. This was followed by a 10 min extension at 72°C.

Restriction digestion with Hinfl was carried out on 1μl buffer, 1 μl (10 U) of Hinfl, 4 μl water and 4 μl of PCR amplicons and incubated at 37°C for 4 to 6 h. The digested product was sized by electrophoresis on a 10 per cent non denaturating polyacrylamide gel and genotype was determined by examination of 0.2 per cent ethidium bromide stained gels under a UV transilluminator. Wild type (677CC) shows a single band of 198 bp. The presence of the ‘T’ allele introduces a cut among heterozygous (677 CT) and 3 bands of 198, 175 and 23 bp were seen. The homozygous (677 TT) have two bands of 175 bp and 23 bp (Fig. 1).
1298A→C polymorphism: 1298 A→C mutation was analysed by the method described by Ramadevi et al. PCR amplification of exon 7 of MTHFR gene using 5’CTT TGG GGA GCT GAA GGA CTA CTA C-3’ as forward primer and 5’CAC TTT GTG ACC ATT CCG GTT TG-3’ as reverse primer results in a 163-bp product. The PCR product was digested with MboII restriction enzyme. 1298 A→C mutation abolishes one restriction site of MboII, resulting in the merger of the 56 and 28 bp bands into 84 bp. The PCR conditions were similar to MTHFR 677 C→T polymorphism except that the primer concentrations were 30 pmol.

Restriction digestion with MboII was carried out on 1μl buffer, 0.5 U of MboII, 4.5 μl water and 4 μl of PCR amplicon and incubated at 37°C for 10 to 12 h. The MboII digested product was sized on a 20 per cent polyacrylamide gel. The gel was stained with 0.2 per cent ethidium bromide and viewed under a UV transilluminator. Wild type (1298 AA) produced fragments of 56 bp, 30/31 bp, 28 bp and 18 bp. Heterozygous (1298 AC) produced fragments of 84, 56, 30/31, 28 and 18 bp, whereas homozygous for 1298 polymorphism (1298 CC) produced 84, 30/31 and 18 bp (Fig. 2).

The PCR and RFLP were carried out in batches of 15-20. In each batch apart from positive control for homozygous and heterozygous, a few samples whose genotype was known from pervious batches were run to check repeatability. Some samples were randomly genotyped by two different individuals to check sample replicability and found to be repeatable. In all about 20 per cent of study samples were analysed in duplicate, with 100 per cent concordance in genotype calling.

Statistical analysis: Data were analysed using SPSS 16.0 and STATA 9.0. The frequency of genotype was determined by direct counting. OR and 95 per cent CI were estimated as a measure of association between genotype and CRC. Conformity to Hardy-Weinberg equilibrium was determined by Chi square (χ²) test/ Fisher’s exact test (two tailed). A P value of < 0.05 was considered statistically significant.

Results

Patients and controls were matched for age, sex and socioeconomic status. Of the 100 patients, 60 were males and 40 females (age 17-75 yr; mean 58 yr) and mean age was 57 yr for controls.

Frequency of MTHFR 677 CC, CT and TT were 76.7, 22.1 and 1.16 per cent in controls, and 74.0, 25.0 and 1.0 per cent among patients (Table I). The ‘T’ allele frequency was 12.21 and 13.5 per cent in controls and cases respectively. The frequency of MTHFR 1298 AA, AC, and CC were 25.6, 58.1 and 16.3 per cent for controls and 22.0, 70.0 and 8.0 per cent for patients respectively. The ‘C’ allele frequency for 1298 A→C was 43.0 and 45.34 per cent (Table I). The genotype distribution pattern for 677 C→T followed the Hardy-Weinberg equilibrium with a P value of 0.667 and 0.481 for controls and cases respectively. The genotype distribution for 1298 A→C polymorphism followed the Hardy-Weinberg equilibrium in controls (P= 0.109) which was significantly different from the patient groups (P = 0.001).

Table II shows the combined genotype distribution for both polymorphisms. The heterozygous/ homozygous state for both polymorphisms showed a 1.48 fold increased risk of CRC, with a 95 per cent confidence limit of 0.83-2.65 and a P value of 0.182 which was not significant.
Discussion

Variant forms of MTHFR are associated with increased risk to several diseases like neural tube defects, Down’s syndrome, vascular disease, stroke, male infertility and cancer\textsuperscript{10,11}. MTHFR polymorphisms have been hypothesized to be protective against certain types of cancer as the reduced enzyme activity shunts more of the folate substrate, methylene-THF towards thymidylate synthesis and away from DNA methylation. However, when the folate intake is deficient, both DNA methylation and thymidylate synthesis may be impaired in patients with MTHFR polymorphisms leading to carcinogenesis. Among cancer, the polymorphic form of 677C→T is known to increase the risk of cervical, esophageal, endometrial and breast cancers while decreasing the risk of ALL and colorectal cancer\textsuperscript{12-15,6,5}.

Several epidemiological studies have focused on associations between MTHFR polymorphisms and colon cancer. Homozygous (TT) individuals have reduced incidence of colorectal cancer, and the incidence could be further modified by age, dietary habits and life style\textsuperscript{6,16}.

In our study we have found only one homozygous (677 TT) genotype in each group. A number of published studies have also reported low prevalence of homozygous genotype (TT) for MTHFR 677 both in their control group and among cases\textsuperscript{5,9,10}. Le Marchand \textit{et al}\textsuperscript{17} in a multiethnic cohort study reported that the frequency of the ‘T’ allele was relatively low in African Americans and Native Hawaiians and they have also reported an inverse association between colorectal cancer risk and the TT genotype. This association was similar in both sexes, stronger at high levels of folate intake and more specific to advanced colon cancer\textsuperscript{17}. In some populations like African Blacks from Brazil, and among Thai patients with venous thrombosis, homozygosity was completely absent\textsuperscript{10}.

Two other studies revealed weak inverse association between MTHFR 677 TT genotype and colon cancer which is independent of intake of folate or alcohol, while one study found no association.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cases (n=100)</th>
<th>Controls (n=86)</th>
<th>OR</th>
<th>95% CI</th>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>74 (74)</td>
<td>66 (76.7)</td>
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<tr>
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<td>19 (22.1)</td>
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<td>1.0 (1.16)</td>
<td>0.86</td>
<td>0.05 - 13.94</td>
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<tr>
<td>‘T’ allele frequency</td>
<td>13.5%</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>22 (22)</td>
<td>22 (25.6)</td>
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<td>AC</td>
<td>70 (70)</td>
<td>50 (58.1)</td>
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<td>CC</td>
<td>8 (8)</td>
<td>14 (16.3)</td>
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<td>45.34%</td>
<td>43.0%</td>
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Figures in parentheses are percentage values

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<th>MTHFR 677 C→T</th>
<th>MTHFR 1298 A→C</th>
<th>No: Patients (100)</th>
<th>No: Controls (86)</th>
<th>OR</th>
<th>95% CI</th>
<th>P value</th>
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between the low activity MTHFR 677 TT genotype and colon cancer\(^1\,8,19\). A case-control study from Japan showed that individuals with the MTHFR 677 TT genotype have a decreased risk of colorectal cancer when folate is adequate, suggesting a protective role of folate by ensuring a sufficient thymidylate pool for DNA synthesis\(^20\). Kono et al\(^21\) have reviewed 16 studies which have addressed the association between MTHFR 677 C→T polymorphism and colorectal cancer in 10 countries. Decreased risk of colorectal cancer associated with the 677 TT genotype has been fairly consistently observed with few exceptions and this decrease was observed in people with both high and low folate.

Lima et al\(^22\) in a recent study have reported higher frequencies of the MTHFR 677 TT and CT genotypes in patients less than 50 yr compared with those of an older age. Our data show that MTHFR 677 C→T polymorphism does not affect the susceptibility to colorectal cancer, a finding similar to that reported by Wang et al\(^5\) in colorectal cancer patients in a predominantly South Indian population.

Prevalence of the variant allele (677 ‘T’) varies among different ethnic groups around the world. The frequency distribution of MTHFR homozygous genotype (TT) showed a marked variation ranging from 10 to 40 per cent\(^23\). High prevalence of MTHFR 677 TT genotype has been reported from Canadian (15.3\%)\(^6\) Italian (23.8\%)\(^24\) French (18.2\%)\(^25\) Chinese (14.3\%)\(^26\) and Japanese (10.2\%)\(^27\) populations while low frequency has been reported from Turkish (6\%) and Norwegian (8\%)\(^28\) populations. Among Indians prevalence of homozygous for MTHFR 677 C→T polymorphism is approximately 3.0 per cent and the frequency of ‘T’ allele is low ranging from 9.6 to 13.5 per cent\(^8\,10\). The ‘T’ allele frequency in our study was 12.21 and 13.55 per cent in controls and cases respectively.

The prevalence of MTHFR 1298 A→C polymorphism and its association with CRC has not been extensively studied. A case-control study of Japanese population observed no association between 1298 A→C polymorphism and risk of colorectal cancer\(^29\). Only one published report is available from India on the association of CRC and MTHFR 1298 polymorphism. Wang et al\(^2\) in a case-control study in colorectal cancer patients in an Indian population have reported that the MTHFR 1298 ‘C’ allele is common among Indians (41\%) and the MTHFR 1298 CC genotype was significantly associated with decreased colon and rectal cancer risk. In the present study, we have found that 1298 CC had an OR of 0.45 with a non significant \(P\) value suggesting that there may be slight reduction in CRC risk.

A study among Tamil population from India, reported 41 per cent ‘C’ allele frequency for MTHFR 1298 polymorphism\(^30\). Ramadevi et al\(^3\) have also reported a similar finding among South Indian population for 1298 ‘C’ allele. Similar results were seen in our study also. The ‘C’ allele frequency was 43 per cent among controls and 45.35 per cent among cases.

In conclusion, this case-control study exhibited that the frequency of MTHFR 677 ‘T’ allele is rare, while the MTHFR 1298 ‘C’ is common among Indians. There was no association between 677 C→T and 1298 A→C polymorphisms and risk of CRC either individually or in combination. However, the homozygous state for 1298 (CC) had an OR of 0.45, suggests that 1298 polymorphism may be more important than 677 polymorphism for CRC among Indians. These results need to be confirmed in larger studies.

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