Reverse association between MTHFR polymorphism (C677T) with sporadic colorectal cancer

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ABSTRACT

Aim: To investigate association between MTHFR gene polymorphism with colorectal cancer.

Background: The enzyme 5,10-methylene-tetrahydrofolate reductase (MTHFR) is linked to DNA methylation, synthesis and repair. One of the most important polymorphisms that has been identified in the MTHFR gene is C677T. The single nucleotide polymorphism C677T has been found to be associated with decreased enzyme activity and decreased plasma folate. Thus it might play an important role in the etiology of colorectal neoplasia.

Patients and methods: Using pyrosequencing, we analyzed the MTHFR genotypes in 234 colorectal cancer patients and 257 normal matched controls.

Results: Whereas the CC, CT and TT genotypes of MTHFR among the colorectal cancer patients were 50%, 29% and 21% respectively, we found 36.6% of 677CC, 31.1% of 677CT and 32.3% of 677TT in the normal controls. We observed a decreased risk of colon cancer when folate intake was high for participants with wild type genotype. This association was stronger at higher levels of folate intake.

Conclusion: Our study corroborates previous findings of an inverse association of the MTHFR 677TT genotype with colorectal cancer, especially at high levels of folate.

Keywords: sporadic colorectal cancer, MTHFR, Pyrosequencing, Polymorphism.

INTRODUCTION

Colon cancer is a complex disease influenced by multiple genetic and environmental factors. It is a prevalent cancer in the United States and other developed countries. Among environmental factors, diet has received a great deal of attention. (1).

Risk of development of colorectal cancer has been linked to diets that are low in the methyl donors, folate and methionine and high in alcohol, a methyl group antagonist (1). Dietary methyl group availability may influence cancer risk by altering DNA methylation or by influencing the rate of DNA mutation. Selective growth and transformation of cells can result from DNA hypomethylation of protooncogenes (2) or hypermethylation of tumor suppressor genes (3) in their promoter regions. In contrast to these mechanisms, in which aberrant DNA methylation influences gene expression, the mutation mediated hypothesis proposes that the oncogenic process is affected by a disproportionately high rate of CpG to TpG transitions, such as those frequently observed in the p53 gene in colorectal tumors (4-6), potentially due to deamination of
5-methylcytosine. Finally, methyl deficient diets may cause imbalances in the pools of nucleotide precursors leading to DNA strand breakages and mutations (7, 8).

Some colon cancers exhibit aberrant DNA methylation or CpG island methylator phenotype, which involves inactivation or silencing of genes by hypermethylation of promoter cytosine-guanosine (CpG) residues. Evidence from epidemiologic studies suggests that adequate folate intake is associated with decreased risk of colorectal cancer (9-11), although not all studies support this association (12, 13).

The 5,10-methylenetetrahydrofolate reductase (MTHFR) enzyme plays an important role in folate metabolism and determines the balance between the different forms of folate for DNA synthesis and DNA methylation (14). MTHFR is a critical enzyme regulating the metabolism of folate and methionine, both of which are important factors in DNA methylation and synthesis. MTHFR irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the primary methyl donor for the remethylation of homocysteine to methionine. A common C677T (Ala → Val) mutation of the gene was found to enhance malfunction of the enzyme (14, 15).

Altered folate metabolism leads to shifts in the balance between availability of 5-methyltetrahydrofolate for methylation reactions and 5,10-methylenetetrahydrofolate for DNA synthesis and repair (16, 17), which may influence colorectal cancer risk. As a matter of fact, polymorphisms in genes related to folate metabolism, specifically MTHFR, are thought to play an important role in carcinogenesis of the large bowel. One polymorphism in the MTHFR gene that affects the efficiency of folate metabolism has been described (18-20). The MTHFR 677 C>T transition in exon 4 is associated with reduced enzyme activity resulting in slower folate metabolism. Individuals with the variant MTHFR 677TT genotype show about 30% of the enzyme activity found among those with the wild-type (CC) enzyme (21). Subjects who are heterozygous for the mutation (CT) have about 65% of wild-type enzyme activity (21).

Among 677TT (val/val) individuals, the MTHFR enzyme is less efficient in converting 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, thus potentially preventing depletion of 5,10-methylenetetrahydrofolate, a cofactor for de novo DNA synthesis, especially dTMP. As a result, cells may be less prone to "dNTP stress" which has been shown to promote cancer-associated genetic alterations due to alterations in the pool of nucleotide precursors available for DNA synthesis. Alteration of these precursor pools induced by methyl (folate) deficiency significantly increases the uracil content and the frequency of chromosome breakages in human leukocyte DNA (22-23).

Individuals with the TT genotype, particularly if combined with a low folate diet, have elevated plasma homocysteine levels, which illustrates the physiological importance of this genotype (23–25).

In this study, we investigated the association of MTHFR polymorphism, codon 677 C>T, among colon cancer cases. We hypothesized that MTHFR polymorphisms are linked with reduced MTHFR enzyme activity, and that the association would be modified by folate status.

**PATIENTS and METHODS**

Blood samples were collected from 234 unrelated Iranian patients with non familial colon cancer and 257 healthy individuals as controls. All subjects were genetically-unrelated ethnic Iranian patients with histopathologically confirmed incident sporadic colorectal cancers and were recruited between September 2003 and December 2007 at the Research Institute for Gastroenterology and Liver Disease (RIGLD). Cancer-free controls were randomly selected from individuals referred to Taleghani hospital during the same time when...
the cases were being recruited. These control subjects had no history of cancer and were frequency-matched to the cases by age within five years and sex.

Questionnaires included information on dietary intake, smoking habits, medical information, and family history of polyps and cancer (especially history of colon, breast, endometrial, or ovarian cancers). In case that data were incomplete, study staff would follow up participants.

Briefly, fasting blood of 234 cases and 257 controls was sampled in the morning. RBC (Red Blood Cells), plasma, and whole blood folate were measured in both groups. Plasma folate was measured by an automated chemiluminescence method (Chiron Diagnostics, East Walpole, MA). For the RBC folate assay, RBCs were isolated by centrifugation at 36,000 rpm for 15 min within 1 h of collection, washed with 9 g/L of sodium chloride solution, and immediately stored at -70°C, until folate analysis. RBC folate was analyzed by affinity chromatography followed by reversed-phase chromatography with electrochemical detection, as previously described (26).

Genomic DNA was isolated from peripheral-blood lymphocytes according to standard procedures (27). All samples (patients and controls) were analyzed for C677T SNP by PCR/pyrosequencing technique and our results were confirmed by direct sequencing of 12 randomly selected samples.

Pyrosequencing is based on the detection of released pyrophosphate (PPi) during DNA synthesis. In a cascade of enzymatic reactions, visible light proportional to the number of incorporated nucleotides is generated.

We designed three primers (forward: 5'-GAGGCTGACCTGAAGCACTTGA-3', reverse: 5'-ATGCCTTCACAAAGCGGAAGA-3' and sequencing: 5'-CGTGATGATGAAATCG-3') of which either forward or reverse was biotinylated. Forward and reverse primers were used for PCR and sequencing primer for running pyrosequencing (Assay Design software v1.0.6). The reaction conditions were as follows: 1µl of genomic DNA solution (10 ng/µl), 20µM of each primer, 0.2 mmol/l of each dNTP, and 2.0 mmol/l of MgCl2 and 0.02 U/µl of Taq DNA polymerase (Fermentase, Germany) in 50µl total volume. Thermal cycling was performed as follows: 94°C for 5 min followed by 35 cycles of 95°C for 30s, annealing temperature for 45s and 72°C for 40s, followed by 72°C for 10 min. The biotinylated products of the single PCR were immobilized on streptavidin-coated paramagnetic beads (Magnetic Biosolutions), and the strands were separated using 0.10 mol/l NaOH. This ssDNA were genotyped in polymorphism locus by sequencing primer and pyrosequencer (PSQ 96MA, Uppsala, Sweden).

Direct sequencing was done for 12 randomly selected subjects to confirm our data. Sequencing results were analyzed by DNASIS MAX software v2.6 (Hitachi Software Engineering Co.).

Standard techniques of matched case-control studies were used. Odds ratios (OD) and 95% confidence intervals were estimated by logistic regression analysis. Exposure was defined as homozygosity for the Valine substitution (TT). Homozygous wild-type individuals (CC) were combined with heterozygotes (CT) as a single “unexposed” group, to increase statistical power in stratified analyses. RBC and plasma folate and other stratification variables were categorized into quartiles. Category boundaries were determined from the exposure distribution of the entire sample.

The association between MTHFR genotype and colorectal cancer was estimated in the entire studied population. We used t- tests to compare mean plasma and RBC folate between levels of genotype and Pearson correlation coefficients were used to determine correlations between the different measures of folate status (using the computer software SPSS for Windows v14.0). All P- values were two-sided; P-values <0.05 were considered statistically significant.
RESULTS

During the accrual period, we identified 263 cases and 268 controls that were potentially eligible; of these, 29 cases and 11 controls refused to interview, thus we analyzed 234 cases and 257 controls by pyrosequencing (Fig. 1). Table 1 represents characteristics of cases and controls in studied population.

<table>
<thead>
<tr>
<th>Table 1. Characteristics of cases and controls in studied population</th>
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<tr>
<td><strong>Risk factor</strong></td>
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<tr>
<td>Gender</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Folates</td>
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<tr>
<td>Total (µg/day)</td>
</tr>
<tr>
<td>RBC (ng/ml)</td>
</tr>
<tr>
<td>Plasma (ng/ml)</td>
</tr>
<tr>
<td>Smoking status</td>
</tr>
<tr>
<td>Current</td>
</tr>
<tr>
<td>Former</td>
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<tr>
<td>Never</td>
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Forty-nine cases and 83 controls were homozygous for the TT genotype. Allele frequencies in cases were T=35.5% and C=64.5%, while among the controls were T= 48% and C= 52%. Table 2 presents the inverse association between TT genotype and colorectal cancer in this population. The frequencies of 677TT (val/val), C677T (ala/val), and CC677 (ala/ala) genotypes among the cases were 21, 29 and 50%, respectively (Table 2). The frequency of val/val genotype among the cases was lower than controls.

<table>
<thead>
<tr>
<th>Table 2. MTHFR C677T genotype prevalence and main effects in Iranian patients with colorectal cancer</th>
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<tr>
<td><strong>Genotype</strong></td>
</tr>
<tr>
<td>TT</td>
</tr>
<tr>
<td>CT</td>
</tr>
<tr>
<td>CC</td>
</tr>
<tr>
<td>TT/CT + CC</td>
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<td>* Odds Ratio</td>
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Fig 1. Pyrosequencing analysis of DNA from colorectal cancer patients containing MTHFR C677T sequence
A: CC wild type genotype; B: TC heterozygote genotype; C: TT homozygote genotype

Table 3 shows the joint effect of MTHFR genotype and folate on adenoma risk. For those with RBC folate levels in the lowest quartile (<165 ng/ml), subjects with the TT genotype had approximately twice the adenoma risk of those with at least one wild-type allele. At the highest folate levels, adenoma risk was <1.0 for both TT homozygotes and those with a wild-type allele. We observed that the association with the TT genotype was limited to advanced colon tumors. Our results bring additional evidence for an inverse
Table 3. ORs and 95% CI for colon cancer in relation to MTHFR C677T genotypes, total folate intake, and supplement use in Iranian population

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Intake*</th>
<th>Food folate</th>
<th>Total folate</th>
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<tbody>
<tr>
<td></td>
<td>Case/control</td>
<td>OR (95% CI)</td>
<td>Case/control</td>
</tr>
<tr>
<td>CC+CT</td>
<td>≤Median</td>
<td>96/98</td>
<td>1.00</td>
</tr>
<tr>
<td>CC+CT</td>
<td>&gt;Median</td>
<td>89/76</td>
<td>1.2(0.77-1.85)</td>
</tr>
<tr>
<td>TT</td>
<td>≤Median</td>
<td>22/47</td>
<td>0.48(0.26-0.89)</td>
</tr>
<tr>
<td>TT</td>
<td>&gt;Median</td>
<td>27/36</td>
<td>0.77(0.41-1.41)</td>
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*The median intake was 320µg/d for folate from foods, 450µg/d for total folate

association between the MTHFR 677TT genotype and colorectal cancer.

**DISCUSSION**

We investigated the association of MTHFR genotypes and colon cancer in a population-based case-control study of Iranian patients. The effect of MTHFR codon 677 was evaluated in relation to total folate intake.

This association was similar in both sexes, stronger at high levels of folate intake. There was a statistically significant trend toward the protective effect of food folate ($P$= 0.01) among those with the TT genotype, compared with the total plasma folate.

Our results revealed an inverse association between the MTHFR 677TT genotype and colorectal cancer. This association was first reported in two male Harvard cohorts [28] and was reproduced in five of eight case-control studies to date (29). Four of five past studies suggested interactions between folate and the TT genotype, with the inverse association being greatest among persons with the highest intake or plasma levels of folate (28, 30-32). Our results are remarkably consistent with those findings.

Colon cancer is difficult to cure when the disease has spread outside the large intestine. Moreover, if the sub-site specificity of the association can be replicated, it will be interesting to see whether the folic acid fortification of the diet initiated in the United States in 1998 will result in a greater decrease in rates, if any, for colon as compared to rectal cancer (33-35). Interestingly, the lower frequency of the T allele in African Americans and Native Hawaiians and the finding of a specificity of its protective effect against advanced colorectal cancer are consistent with the late-stage presentation and poorer survival observed for the disease in these ethnic groups (33-35).

There are several limitations to our study. First, we were unable to distinguish between intake of vitamin B-6, B-12, folate and methyonin in the form of supplements, thus our findings for supplement use could be attributable to folate, vitamin B6, vitamin B12, or other compounds found in dietary supplements. Because dietary intake of folate and B vitamins are often highly correlated (36), some or all of the effect of total folate intake in our study could be attributable to vitamin B-6 or B-12. Second, since alcohol consumption is forbidden in our country, we could not collect clear data from our patients and control subjects. Moreover, since alcohol consumption is not recommended in our culture thereby, we could not gather correct data in this field in order to examine the effect of it on cancer as well.

In conclusion, these data corroborate previous findings of fine inverse association of the MTHFR 677TT genotype with colorectal cancer, especially at high levels of folate. It is also suggested that this effect may be specific to advanced colon cancer.
Acknowledgments

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REFERENCES


