Association between Genetic Polymorphism of Methylenetetrahydrofolate Reductase with Non Familial Colorectal Cancer in Iranian Population

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Abstract

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme regulating folate metabolism, which affects DNA methylation and synthesis. One of the most important polymorphisms identified in the MTHFR gene is C677T. MTHFR activity is lowered in individuals with 677TT genotype.

Using pyrosequencing, we analyzed the MTHFR genotypes in 118 colorectal cancer patients and 189 normal matched controls. Whereas the CC, CT and TT genotypes of *MTHFR* among the colorectal cancer patients were 51.7%, 28.0 % and 20.3% respectively, we were able to find 47.1% of 677CC, 27.0% of 677CT and 25.9% of 677TT in normal controls. An inverse association was observed between the risk of colorectal cancer and TT genotype with the odds ratios (OR) of 1, 0.94 and 0.71 for CC, CT, and TT genotypes, respectively. This association was similar in both sexes, but in patients with high levels of folate intake. Our study corroborates previous findings of an inverse association between MTHFR 677TT genotype and colorectal cancer, especially at high levels of folate.

Keywords: non familial colorectal cancer, methylenetetrahydrofolate reductase, pyrosequencing, polymorphism

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Introduction

Colorectal cancer is one of the most common cancers in the world, accounting for nearly 10% of new cases of all cancers. The incidence of colorectal cancer varies substantially worldwide, with high rates in Western countries and low rates in African and Asian countries in general. [1]. Risk of developing colorectal cancer has been linked to diets that are low in methyl donors, folate and methionine and high in alcohol, a methyl group antagonist. Dietary methyl group availability may influence cancer risk by altering DNA methylation or by influencing the rate of DNA mutation. [2] Selective growth and transformation of cells can result from DNA hypomethylation of protooncogenes or hypermethylation of tumor suppressor genes in their promoter regions. [3]

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme regulating folate metabolism, which is thought to influence DNA methylation and synthesis. MTHFR irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which provides the methyl group to convert homocysteine to methionine, the precursor of S-adenosylmethionine (SAM). SAM is the universal methyl-group donor for methylation of a wide variety of biological substrates. It has been hypothesized that folate/methyl depletion may result in not only global genomic hypomethylation, but also aberrant methylation of CpG clusters in the promoters of tumor suppressor and DNA repair genes, probably via upregulation of DNA methyltransferase. [4-5] The substrate of MTHFR, 5, 10-methylenetetrahydrofolate, is required for the conversion of deoxyuridylate to thymidylate. Depletion of the thymidylate pool results in uracil misincorporation into DNA, leading to single- and double-strand breaks. [6-8]

The MTHFR 677 C>T transition in exon 4 is associated with reduced enzyme activity resulting in slower folate metabolism. Individuals with MTHFR 677TT genotype variant show 30% of the enzyme activity found among those with the wild-type (CC) enzyme [9]. Subjects who are heterozygous for the mutation (CT) have 65% of wild-type enzyme

activity [9]. Individuals with the TT genotype, particularly if combined with a low folate diet, have elevated plasma homocysteine levels, thus illustrating the physiological importance of this genotype [10-13].

In this study, we evaluated the consistency of reported associations between the MTHFR polymorphisms and colorectal cancer and discussed the implications of the MTHFR polymorphisms with respect to nutritional status in colorectal cancer. We hypothesized that MTHFR polymorphisms are linked with reduced MTHFR enzyme activity and that the association is modified by folate status.

Materials and Methods

Patients and controls

Blood samples were collected from 118 unrelated Iranian patients with non familial colon cancer and 189 healthy individuals as controls. Their characteristics are shown in table 2.

All subjects were genetically-unrelated ethnic Iranian. Patients with histopathologically confirmed incident sporadic colorectal cancer were recruited between September 2003 and December 2007 in the Research Center for Gastroenterology and Liver Diseases (RCGLD) in Taleghani hospital Tehran. Cancer-free controls were randomly selected from individuals referred to Taleghani hospital during the same time when the cases were recruited. These control subjects had no history of cancer and were frequency-matched to the cases by age within five years and sex.

Data Collection

Data was collected by in-person interviews in the hospital or at another convenient location (such as the health department) by trained physician interviewers. Study participants were given a brief explanation of the interview process.

Red Cell and Plasma Folate

Briefly, a fasting blood sample was drawn in the morning from 118 cases and 189 controls. RBCs, plasma, and whole blood folate were determined for the cases and controls. We calculated red cell folate values from whole blood folate concentrations and corrected for hematocrit and plasma folate levels, according to an established formula [14].

C677T Polymorphism analysis

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

Genotyping by pyrosequencing

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. [15, 16]

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

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

Table 1: PCR and Pyrosequencing primers used to genotype MTHFR C677T

PCR and Pyrosequencing primers (5' → 3')				Score: 98 Quality: High	
Primer	**Id**	**Sequence**	**Bp**	**Tm°C**	**GC (%)**
PCR	Forward	GAGGCTGACCTGAAGCACTTGA	22	65.1	54.5
PCR	Reverse	ATGCCTTCACAAAGCGGAAGA	21	65.5	47.6
Sequencing	Sequencing	CGTGATGATGAAATCG	16	51.0	43.8

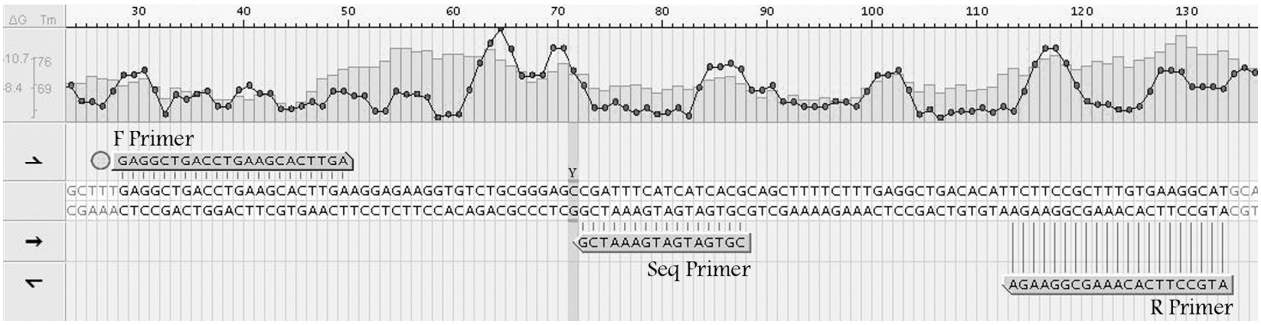

Figure 1. MTHFR pyrosequencing assay design. PCR amplification of C677T polymorphism.
FP: Biotinlated forward primer; RP: reverse primer; Seq: sequencing primer.

Statistical analysis

Standard techniques for matched case-control studies were used. Odds ratios 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 and ANOVA to compare mean plasma and RBC folate between levels of genotype and Pearson product 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 147 cases and 193 controls who were potentially eligible. Of them, 29 cases and 4 controls refused interview, thus we analyzed 118 cases and 189 controls by pyrosequencing [Fig. 2]. Twenty-four cases and 49 controls were homozygous for the TT genotype. Allele frequencies in cases were T=34% and C=66% while in controls T= 39% and C= 61%. Table 3 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 in cases were 20.3, 28.1 and 51.7%, respectively (Table 3). The frequency of val/val genotype in our cases was lower compared to the controls; the age OR for this genotype was 0.96 (95% CI, 0.59-1.68). The ORs were similar in men with and without a family history of colorectal cancer.

Table 4 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 TT genotype had approximately twice the risk of adenoma comparing 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.

In summary, we report that the association with the TT genotype is limited to advanced colon tumors. Our results bring additional evidence for an inverse association between the MTHFR 677TT genotype and colorectal cancer.

Discussion

The association between the MTHFR C677T polymorphism and colorectal adenoma has been examined in eight studies in the U.S.,[17-20] Mexico,[21] Japan,[23,24] and Norway [25].Decreased risk associated with 677TT was not observed in case-control studies in Mexico,[21] the United Kingdom,[26] Germany,[27] Australia,[28] or Japan.[29] Inconsistent findings in small studies can be ascribed to random variations, but such findings in large studies are difficult to interpret. Particularly notable is the lack of association reported in two large case-control studies in the United Kingdom [26] and Australia.[28]

We investigated the association between MTHFR genotypes and colon cancer in a population-based case-control study of Iranian patients. The effects of MTHFR codon 677, as well as the combined effects of genotypes at both loci, were evaluated in relation to total folate intake.

Table 2: Characteristics of cases and controls in studied population

Risk factor	Iranians	
	Cases (*n* = 118) Mean (SD)	Controls (*n* = 189) Mean (SD)
Gender		
Male	73(61.9%)	108(57.1%)
Female	45(38.1%)	81(42.9%)
Age	69.3 (8.1%)	57.1 (6.3%)
Folate		
Total (µg/day)	446.1 (252.1)	448.5 (260.3)
RBC (ng/ml)	258.4 (166.7)	269.5 (143.6)
Plasma (ng/ml)	10.8 (7.6)	14.1 (9.1)
Smoking status		
Current	21 (17.8%)	24 (12.7%)
Former	18 (15.3%)	55 (29.1%)
Never	79 (66.9%)	110 (58.2%)
Location of tumor		
Distal	89 (75.4%)	-
Proximal	29 (24.6%)	-

Table 3: MTHFR C677T genotype prevalence and main effects in Iranian patients with colorectal cancer

Genotype	Cases (%)	Controls (%)	OR	95% CI
TT	24 (20.3)	49 (25.9)	0.71	0.38-1.34
CT	33 (28.0)	51 (27.0)	0.94	0.53-1.69
CC	61 (51.7)	89 (47.1)	1.00	Reference
TT/CT + CC	24/94	49/140	1.04	0.54 -1.95

Table 4: Ors and 95% CI for colon cancer in relation to MTHFR C677T genotypes, total folate intake, and supplement use in Iranian population

Genotype	Intake[a]	Food folate		Total folate	
		n	OR (95% CI)	*n*	OR (95% CI)
CC+*CT*	≤Median	49/79	1.00	53/73	1.00
CC+*CT*	>Median	45/61	0.84 (0.50-1.37)	41/67	0.44 (0.28-1.25)
TT	<Median	11/28	1.02 (0.60-1.77)	15/24	0.98 (0.50-1.37)
TT	>Median	13/21	0.57 (0.37-1.15)	9/25	1.12 (0.64-1.59)
		***P*=0.01**		***P*=0.72**	

[a] The median intake was 320µg/d for folate from foods, 450µg/d for total folate.

In the present study, association was similar in both sexes but stronger at high levels of folate intake. The frequency of the T allele was found to differ markedly across ethnic/racial groups. There was a statistically significant trend toward a protective effect of food folate (P= 0.01) among those with the TT genotype, compared with the total plasma folate.

Our results bring additional evidence for an inverse association between the MTHFR 677T genotype and colorectal cancer .This association was first reported in two male Harvard cohorts [30] and has been reproduced in five of eight case-control studies to date [30]. Four of five previous 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 [31]. Our results are remarkably consistent with those findings.

Low activity of MTHFR or the 677TT genotype is probably advantageous because it ensures an adequate thymidylate pool for DNA synthesis when folate supply is sufficient, as originally proposed by Chen et al [17] In a folate-depleted situation, as suggested by Keku et al [32] high activity of the enzyme or the 677CC genotype may be disadvantageous because 5,10-methylenetetrahydrofolate is converted and the thymidylate pool is depleted. Increased risk for 677TT versus 677CC would be seen in the folatedepleted situation if aberrant DNA methylation is a primary mechanism, but no such increase has been observed.

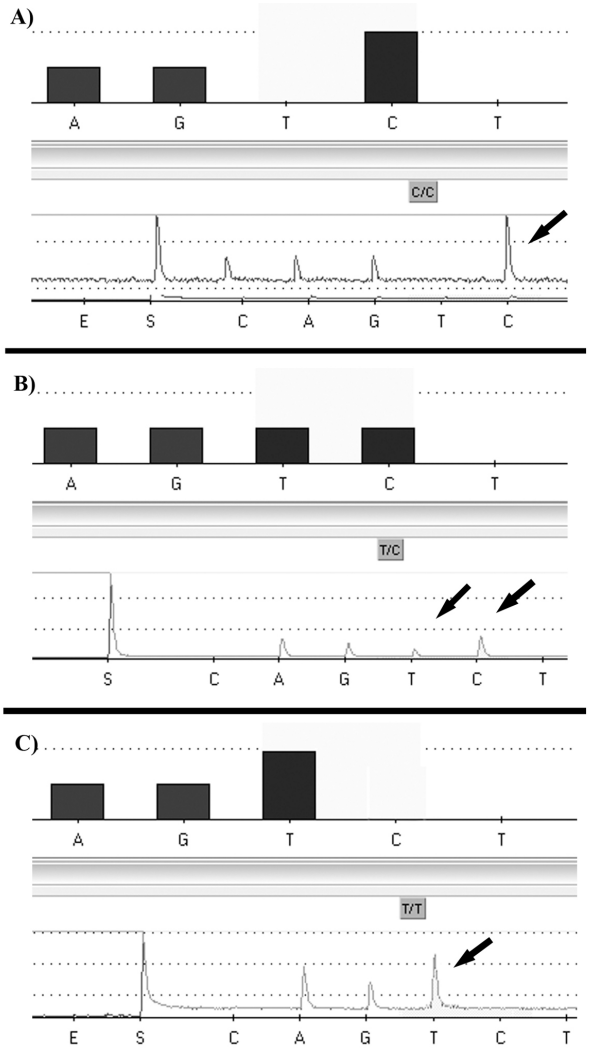

Figure 2. Pyrosequencing analysis of DNA from colorectal cancer patients containing MTHFR C677T sequence. A) CC wild type genotype, B) TC heterozygote genotype and C) TT homozygote genotype.

Decreased risk of colorectal cancer associated with the 677TT genotype has been observed in different populations with few exceptions. This decrease is observable in either high or low folate status. The thymidylate pool associated with MTHFR activity is a probable mechanism underlying the decreased risk for the 677TT versus 677CC genotype. The effect modification of nutritional factors such as folate and alcohol remains possible, even when there is no overall.

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 is often highly correlated [33], some or all of the effect of total folate intake in our study could be attributable to vitamin B-6 or B-12.

In conclusion, these data corroborate previous findings of fine inverse association between MTHFR 677TT genotype with colorectal cancer, especially at high levels of folate.

Acknowledgments

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Electronic databases

Electronic-Websites for data in this article are as follows:

Online Mendelian Inheritance of Man (OMIM), http:// www.ncbi.nlm.nih.gov/omim/

(for Colorectal Cancer [MIM #114500]).

(MTHFR gene accession number: A U09806)

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