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CLINICAL RESEARCH

Methylenetetrahydrofolate reductase C677T genotype affects promoter methylation of tumor-specific genes in sporadic colorectal cancer through an interaction with folate/vitamin B_{12} status

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Abstract

AIM: To evaluate joint effects of *Methylentetrahydrofolate reductase* (*MTHFR*) *C677T* genotypes, and serum folate/vitamin B_{12} concentrations on promoter methylation of tumor-associated genes among Iranian colorectal cancer patients.

METHODS: We examined the associations between *MTHFR C677T* genotype, and promoter methylation of *P16*, *hMLH1*, and *hMSH2* tumor-related genes among 151 sporadic colorectal cancer patients. The promoter methylation of tumor-related genes was determined by methylation-specific PCR. Eighty six patients from whom fresh tumor samples were obtained and 81 controls were also examined for serum folate and vitamin B_{12} concentrations by a commercial radioimmunoassay kit.

RESULTS: We found 29.1% of cases had tumors with at least one methylated gene promoter. In case-case comparison, we did not find a significant association between methylation in tumors and any single genotype. However, in comparison to controls with the *CC* genotype, an increased risk of tumor methylation was associated with the *CT* genotype (OR = 2.5; 95% CI, 1.1-5.6). In case-case comparisons, folate/vitamin B_{12} levels were positively associated with tumor methylation. Adjusted odds ratios for tumor methylation in cases with high (above median) *versus* low (below median) serum folate/vitamin B_{12} levels were 4.9 (95% CI, 1.4-17.7), and 3.9 (95% CI, 1.1-13.9), respectively. The frequency of methylated tumors was significantly higher in high methyl donor than low methyl donor group, especially in those with *MTHFR CT* (P = 0.01), and *CT/TT* (P = 0.002) genotypes, but not in those with the *CC* genotype (P = 1.0).

CONCLUSION: We conclude that high concentrations of serum folate/vitamin B_{12} levels are associated with the risk of promoter methylation in tumor-specific genes, and this relationship is modified by *MTHFR C677T* genotypes.

Key words: *Methylentetrahydrofolate reductase*; Folate; Vitamin B_{12}; Methylation; Colorectal cancer

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INTRODUCTION

Colon cancer (CLC) is one of the most common cancers in the world, with high rates in Western countries[1]. A significant increase in CLC incidence with the predominant localization in the left colon has also been reported in Iran over the last decade[2,3]. However, little is known about the molecular mechanism of CLC in this region.

One of the pathways by which that CLC can progress involves transcriptional silencing by hypermethylation of CpG islands referred as methylator phenotype ($CIMP^+$)[4]. The $CIMP^+$ in CLC is characterized by frequent hypermethyaltion of specific CpG sites, including those present in the promoter regions of tumor suppressor genes such as the cell cycle regulator, *p16* and genes involved in DNA mismatch repair like *hMLH1*[4]. The *hMLH1* promoter region is methylated in about 90% of microsatellite unstable (MSI-positive) colon cancers that leads to the silencing of *hMLH1* expression[5]. The $CIMP^+$ phenotype may be the result of more widespread aberration in methyl-group metabolism in cancer cells.

Interaction of the epigenome with the environment, including nutrition, can alter patterns of gene expression. It has been proposed that polymorphisms in folate-metabolizing enzymes and genes involved in DNA methylation are associated with colon cancer. MTHFR is a key enzyme regulating folate metabolism, which affects DNA methylation and synthesis. MTHFR converts 5, 10-methylentetrahydrofolate to 5-methyl tetrahydrofolate, which is required for homocysteine methylation to methionine. Methionine is then activated to S-adenosylmethionine, a universal methyl donor in numerous transmethylation reactions, including methylation of DNA, RNA, proteins, and other molecules[6]. The *MTHFR* gene is polymorphic with single nucleotide variants within codon 677 in exon 4 (C to T, Ala to Val). This variant encodes a thermolabile enzyme with reduced activity that leads to a reduced plasma folate level[7].

Several case-control studies have shown a reduced risk of CLC for homozygous *MTHFR TT* individuals. The protective effect appears to depend on an adequate level of dietary folate intake, gender, age, and location of the tumor in the proximal or distal colon[8,9]. In some circumstances, the *MTHFR-TT* genotype seems to increase the risk of CLC[10,11]. It has been suggested that deficient activity of MTHFR affects DNA methylation status through an interaction with folate status[12]. Several data provide evidence that individuals with the common *C677T* mutation in the *MTHFR* gene and with low levels of folate had a diminished level of DNA methylation compared with those with the *C/C* wild type. Folate deficiency may be involved in carcinogenesis through impaired synthesis and repair of DNA, or by causing global hypomethylation of DNA, a possible early event in carcinogenesis[13]. Although a protective role against cancer was suggested for the high dietary folate intake, epidemiological evidence has not consistently shown a protective effect of high folate intake against CLC[14,15]. There are few studies addressing joint effects of *MTHFR C677T* genotypes, and methyl donor coenzymes status on promoter methylation of tumor-associated genes in CLC[16,17]. In the current study we investigated the role of *MTHFR C677T* genotype, and serum folate/vitamin B_{12} concentrations on methylation of CpG islands at *p16*, *hMLH1*, and *hMSH2* tumor-associated genes among Iranian sporadic CLC patients.

MATERIALS AND METHODS

Study population, and samples

A total of 151 sporadic primary CLC tumor samples (86 fresh and 65 formalin fixed and paraffin embedded) as well as corresponding normal mucosa were collected from surgical patients at 3 hospitals of the Shiraz University of Medical Sciences in Shiraz, Southern Iran from July, 2003 to September, 2005. Institutional review board approval was granted for this study. The fresh samples were snap frozen in liquid nitrogen immediately after resection and stored at -70℃ until processing. All samples were evaluated and subjected to histological diagnosis by an expert pathologist, who also selected representative tissue sections for DNA extraction, and further molecular analyses. The splenic flexure was used as the anatomical boundary to define proximal and distal CLC. Sociodemographic characteristics such as age and gender were obtained by completion of a detailed questionnaire.

Extraction of DNA and MTHFR genotyping

Genomic DNA was extracted from micro-dissected formalin-fixed, paraffin embedded tumor samples and adjacent normal tissues using the pinpoint slide DNA isolation kit (ZYMORESEARCH, CA, USA). We used the standard phenol/chloroform method for DNA extraction from fresh tumor samples. Genotyping of *MTHFR* at codon 677 of DNA from control and CLC cases was performed using a modification of the mutagenically separated PCR (MS-PCR) method described by Hill and FitzPatrick[18]. Genotyping for *MTHFR* involved analysis of PCR product size by electrophoresis on 3% agarose gels. PCR reactions were carried out in a volume of 50 μL containing 50 ng DNA, 1 × polymerization buffer (MBI Fermentas, Lithuania), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, and 1.5 U Taq polymerase. The primers and concentrations used for PCR reactions were as follows: forward mutant (29 bases) 0.35 μmol/L 5´-CACTTGAAGGAGAAGGTGTCTGCGGGACT-3´, forward normal (49 bases) 0.19 μmol/L 5'-GCTTTGAGGCTGACCTGAAGA-CCTTGAAGGAGAAG GTGTCTGCGGCAGC-3'

and the reverse primer (20 bases) 0.23 μmol/L 5'-TCACCTGGATGGGAAAGATC-3'. The two forward primers are complementary to the normal (677C) and mutant (677T) alleles and differed in length by 20 bases at their 5' ends. The cycling parameters were 5 min at 95℃ followed by 35 cycles of 45 s at 95℃, 1 min at 55℃, and 45 s at 72℃ followed by a single 10-min extension at 72℃. Twenty μL of each reaction mixture was separated on agarose gel and stained with ethidium bromide and visualized under UV illumination.

Serum folate and vitamin B_{12} measurement and methylation specific PCR (MSP)

Folate and vitamin B_{12} measurements were limited to sera from 86 patients with freshly studied tumors and 81 age and sex matched normal controls, selected among healthy volunteers from the general population with no history of any cancer. Blood samples were drawn from patients before operation and serum was prepared within two hours of blood collection. Sera were frozen immediately at -70℃ until used. The concentrations of folate and vitamin B_{12} in each specimen were measured in duplicate by a commercial radioimmunoassay kit (SimulTRAC-SNB RIA, DRG International Inc. USA) using a gamma counter (Contron, Switzerland). We determined the *p16*, *hMLH1*, and *hMSH2* promoter methylation status by chemical treatment with sodium bisulfite and subsequent MSP as described[19]. In brief, this technique uses bisulfite modification to convert unmethylated, but not methylated, cytosine to uracil. MSP utilizes this difference to amplify specifically either methylated or unmethylated DNA. The sequences of primers used for amplification of the promoter region of each of the 3 genes were as follows: *p16* methylated, sense 5'-TTATTAGAGGGTGGGGC-GGATCGC-3' and antisense 5'-GACCCCGAACCGCGACCGTAA-3', which produce a 150 bp fragment; *p16* unmethylated: sense 5'-TTATTAGAGGGTGGGGTGGATTGT-3' and antisense 5'- CAACCCCAAACCACAACCATAA-3', which produce a 151 bp fragment; *hMLH1* methylated, sense 5'-ACGTAGACG-TTTTATTAGGGTCGC-3' and antisense 5'-CCTCATCGTAACTACCCGCG-3', which produce a 112 bp fragment; *hMLH1* unmethylated, sense 5'-TTTTGATGTAGATGTTTTATTAGGGTTGT-3' and antisense 5'-ACCACCTCATCATAACTACCCACA-3',which produce a 124 bp fragment; *hMSH2* methylated, sense 5'-TCGTGGTCGGACGTCGTTC-3' and antisense 5'-CAACGTCTCCTTCGACTACACCGG-3', which produce a 133 bp fragment; *hMSH2* unmethylated, sense 5´-GGTTGTTGTGGTTGGATGTTGTTT-3' and antisense 5'-CAACTACAACATCTCCTTCAAC TACACCA-3', which produce a 144 bp fragment. The hot-started PCR reactions were performed in a 50 μL reaction volume containing 25 pmol of each of sense and antisense primer, 0.2 mmol/L dNTPs, and 80 ng bisulfite-modified DNA in 1× PCR buffer provided by Taq enzyme supplier. The reaction mixture was denatured at 95℃ for 5 min, after which 1.5 U Taq polymerase was added; then amplified by 40 cycles, each consisting of 30 s denaturation at 95℃, 45 s annealing at 58℃, and 30 s polymerization at 72℃, followed by a single 10-min extension at 72℃.

Table 1 Frequency distributions of selected characteristics in CLC patients and control subjects

Variables	Cases			Control (n = 81)	P^1
	Proximal	Distal	Total (n = 151)		
Gender					0.8
Male (%)	28 (31.1)	62 (68.9)	90 (59.6)	50 (61.7)	
Female (%)	32 (52.5)	29 (47.5)	61 (40.4)	31 (38.3)	
Smoking status					0.1
Smokers[2] (%)	22 (34.4)	42 (65.6)	64 (42.4)	25 (30.9)	
Non-smokers	38 (43.7)	49 (56.3)	87 (57.6)	56 (69.1)	
Age groups (yr)					0.4
< 60 (%)	24 (36.4)	42 (63.6)	66 (43.7)	40 (49.4)	
≥60 (%)	36 (42.4)	49 (57.6)	85 (56.3)	41 (50.6)	
Median (range)	61 (40-87)	60 (28-90)	60 (28-90)	60 (28-89)	
Mean (SD)	62.22 (10.97)	59.24 (12.7)	60.42 (12)	58.98 (15.66)	

[1]Fisher's exact test; [2]Current and former smokers.

Statistical analysis

Statistical analysis was performed using the SPSS version 11.5 software package (Chicago, IL). Associations between methylation of loci and clinical, biological and genotypic features were evaluated using Chi square and Fisher's exact test as appropriate. Logistic regression was used to calculate odds ratio (OR) and 95% confidence intervals (95% CI). We adjusted for covariates, specifically including age, gender, and smoking status. Comparing serum folate and vitamin B_{12} levels in cases and controls was performed using two-sided *t*-test, Mann-Whitney test, and Kruskal-Wallis test appropriately.

RESULTS

Distribution of selected characteristics of cases and controls

Selected characteristics of the study population are presented in Table 1. One hundred and fifty one patients and 81 controls entered the study. The distribution was similar in cases and controls by virtue of the study design. Sixty percent (91) of patients had distal CLC and 40% (60) had proximal CLC. Cases were more likely to be males and to be non-smokers. No statistically significant differences were found between cases and controls or between proximal and distal cancer cases with respect to distributions of age, and smoking status. The frequency of distal CLC in males and females was 68.9% and 47.5% (Table 1), respectively, indicative of a significantly higher left CLC incidence in males than females (OR = 2.65; 95% CI, 1.3-5.2).

MTHFR genotypes and the methylation status of tumor-associated genes promoter

Illustrative examples of genotyping of *MTHFR* gene are shown in Figure 1. In 42 patients for whom we performed genotyping in both the cancer tissue and adjacent normal tissue, the typing results were identical in the two samples. CpG island promoter

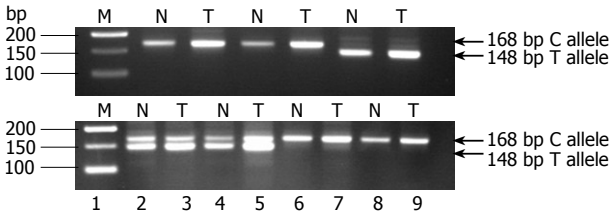

Figure 1 Representative example of MS-PCR assay for genotyping of codon 677 of *MTHFR* gene. For PCR-primers and reaction conditions see methods. In case of the *MTHFR 677 C* allele, a product with 168 base pairs (bp) in length was generated, whereas the *MTHFR 677 T* allele yielded a 148-bp product. The differently sized allele-specific PCR products were separated by agarose gel electrophoresis. In 42 patients genotyping was performed in both cancer tissue (T) and adjacent normal tissue (N). Lanes 2-5 in the lower panel show heterozygote (*CT*) samples. M: DNA size marker.

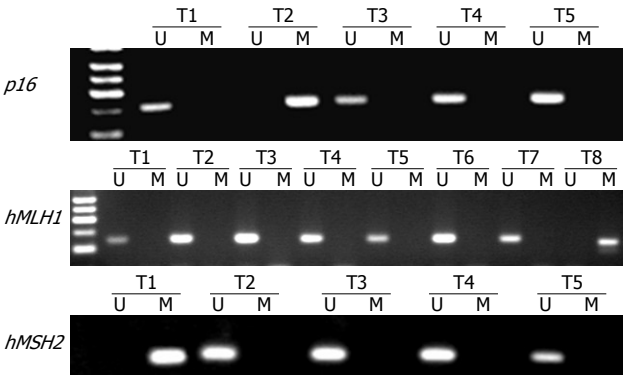

Figure 2 Representative examples of MSP reactions for promoter methylation analysis of *p16*, *hMLH1*, and *hMSH2* genes in primary CLC tumors. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated genes; the presence of a product in those lanes marked M indicates the presence of methylated genes. Lane 1 indicates the 50 bp DNA size marker.

hypermethylation was analyzed in the primary tumors by methylation specific PCR as described in "MATERIALS AND METHODS". Illustrative examples are shown in Figure 2. Table 2 summarizes the association of promoter methylation of genes and *MTHFR* genotype, and other clinical-biological characteristics of CLC patients. Several studies have reported age-dependent variation in the frequency of the *MTHFR* genotypes[20,21]. Therefore, we divided both CLC and control groups into $\geqslant$ 60 and < 60-year old groups. The median age of CLC patients (60 years) was chosen for this division. The most frequently methylated locus was *p16* (19.9 %; 30 of 151), followed by *hMLH1* (13.2 %; 20 of 151), and *hMSH2* (2.6 %; 4 of 151). Eight of 151 (5.3 %) of tumors had both P16 and *hMLH1* CpG island hypermethylation while 2 of 151(1.3%) had both *hMLH1* and *hMSH2* promoter hypermethylation. None of the tumors had simultaneous CpG island hypermethylation of all three genes. There were no significant differences in association of methylation of any individual gene investigated by age or sex of patients. The frequency of tumor methylation (tumors with at least one gene methylated) was 44/151 (29.1%). The latter group of tumors is collectively referred to as "methylated tumors". A significantly higher risk of tumor methylation was found in females (OR = 2.3; 95% CI, 1.1-5.04) (Table 2). Gene promoter methylation was also strongly associated with tumor site, the highest frequency (more than 97%) of methylation occurring in the proximal tumors.

We confronted *MTHFR* genotype with the methylation of tumors using the *CC* genotype as the reference group. Results from case-case comparison, showed no statistically significant genotype dependent differences in the frequency of any specific gene promoter methylation (Table 2). In comparison to cases with the *CC* genotype, we did not find any significant association between tumor methylation or "methylated tumors" , defined above and any single genotype in the entire group of patients, but cases with the *CT* genotype were slightly more likely to have methylated tumors (OR = 1.9; 95% CI, 0.9-4.2). Results from the case-control comparison, showed that the *CT* genotype was significantly associated with tumor methylation in the entire group of patients (OR = 2.5; 95% CI, 1.1-5.6; Table 3). More than five-fold increased risk of tumor methylation was also observed for the *CT* genotype, in male CLC cases compared with age-matched male controls. The *CT* genotype also presented significantly increased tumor methylation in the proximal and in the older age group (OR = 2.7, 95% CI, 1.2-6.2; OR = 3.8, 95% CI, 1.2-12, respectively). The same trend was also observed for CT + TT genotypes. These results suggest that the *C677T* genotype of *MTHFR* can predispose some of CLC patients to the methylation of genes promoter.

Serum folate/vitamin B_{12} status and genes promoter methylation

Due to the previously observed interaction between folate and the *MTHFR* genotype in CLC[22], we investigated the influence of serum folate/vitamin B_{12} levels on tumor methylation in 86 fresh tissue samples in which their corresponding blood samples were also available. We observed no significant differences in serum folate/vitamin B_{12} levels between cases and controls (Table 4). There were also no differences in association of serum folate/vitamin B_{12} levels by sex, and tumor location. Comparing two age groups of patients, a trend for higher serum folate/vitamin B_{12} levels was observed in older age group, with the older cases presenting 15% higher serum folate ($P = 0.04$).

In case-control comparisons, we found no significant difference in serum folate/vitamin B_{12} levels by *MTHFR* genotypes (data not shown). However, in case-case comparisons, serum folate levels appear to be associated with *MTHFR* genotypes (Table 5). Patients with the homozygous *TT* genotype had significantly lower concentrations of folate in their blood than those with *CT* or *CC* genotypes (Mann-Whitney test, $P = 0.007$ and $P = 0.03$, respectively). We found no significant difference in serum vitamin B_{12} concentrations between subjects with the CC and *TT* genotypes.

To test the association between serum folate/vitamin B_{12} levels and methylation of genes promoter and tumors methylation, we stratified serum folate/

Table 2 Stratification analysis of tumors and genes promoter methylation frequencies

Variables	Methylation positive (%)						Methylated tumors[2] *n* (%)	OR (95% CI, *P*)[3]
	p16	*P*[1]	*hMLH1*	*P*[1]	*hMSH2*	*P*[1]		
Sex								
Male (90)	13 (14.4)	0.06	10 (11.1)	NS	4 (4.4)	NS	21 (23.3)	1
Female (61)	17 (27.9)		10 (16.4)		0 (0)		23 (37.7)	2.3 (1.1–5.04, 0.03)
Total (151)	30 (19.9)		20 (13.2)		4 (2.6)		44 (29.1)	
Age (yr)								
< 60 (66)	15 (22.7)	NS	7 (10.6)	NS	0 (0)	NS	19 (28.8)	1
⩾ 60 (85)	15 (17.6)		13 (15.3)		4 (4.7)		25 (29.4)	1.1 (0.6–2.4, 0.7)
Tumor site								
Proximal (60)	29 (48.3)	0.00	20 (33.3)	0.00	4 (6.7)	0.02	43 (71.7)	1
Distal (91)	1 (1.1)		0 (0)		0 (0)		1 (1.1)	0.002 (0.00–0.023, 0.00)
Serum folate (86)								
Low (38)	2 (5.3)	0.04	4 (10.5)	NS	0 (0)	NS	4 (10.5)	1
High (48)	10 (20.8)		10 (20.8)		3 (6.3)		16 (33.3)	4.9 (1.4 -17.7, 0.01)
Serum vitamin B_{12} (86)								
Low (42)	2 (4.8)	0.02	5 (11.9)	NS	0 (0)	NS	5 (11.9)	1
High (44)	10 (22.7)		9 (20.5)		3 (6.8)		15 (34.1)	3.9 (1.1-13.9, 0.03)
MTHFR 677 genotype[4]								
CC (64)	13 (20.3)	NS	7 (10.9)	NS	0 (0)	NS	15 (23.4)	1
CT (80)	17 (21.3)	NS	12 (15)	NS	3 (3.8)	NS	28 (35)	1.92 (0.9-4.2, 0.09)
TT (7)	0 (0)	NS	1 (14.3)	NS	1 (14.3)	NS	1 (14.3)	0.7 (0.07-6.1, 0.7)
CT + *TT* (87)	17 (19.5)	NS	13 (14.9)	NS	4 (4.6)	NS	29 (33.3)	1.8 (0.8-3.9, 0.125)

[1]Fisher's exact test; [2]tumors with at least one methylated gene promoter; [3]the first category was taken as reference. Odds ratio adjusted for age, sex, and smoking status; [4]For genotype comparison CC used as the reference category. NS: Not significant.

Table 3 Association between MTHFR genotypes and tumor methylation in relation to clinical-biological features of CLC patients (case-control comparison, *n*)

Genotype (%)	Controls	Cases	Methylated	OR (95% CI, *P*)[1,2]	Unmethylated	OR (95% CI, *P*)[1,2]
Total	81	151	44		107	
CC	40 (49.4)	64 (42.4)	15 (34.1)	1	49 (45.8)	1
CT	31 (38.3)	80 (53)	28 (63.6)	2.5 (1.1-5.6, 0.02)	52 (48.6)	1.4 (0.7-2.6, 0.3)
CT or TT	41 (50.6)	87 (57.6)	29 (65.9)	2.1 (0.9-4.5, 0.07)	58 (54.2)	1.1 (0.7-2.1, 0.6)
Sex						
Male	50	90	21		69	
CC	28 (56)	33 (36.7)	5 (23.8)	1	28 (40.6)	1
CT	17 (34)	51 (56.7)	15 (71.4)	5.3 (1.6-18.2, 0.008)	36 (52.2)	1.9 (0.9-4.3, 0.09)
CT or TT	22 (44)	57 (63.3)	16 (76.2)	4.3 (1.3-14.3, 0.01)	41 (59.4)	1.7 (0.8-3.6, 0.1)
Female	31	61	23		38	
CC	12 (38.7)	31 (50.8)	10 (43.5)	1	21 (55.3)	1
CT	14 (45.2)	29 (47.5)	13 (56.5)	1.1 (0.3-3.6, 0.9)	16 (42.1)	0.7 (0.2-1.9, 0.5)
CT or TT	19 (61.3)	30 (49.2)	13 (56.5)	0.9 (0.3-2.8, 0.8)	17 (44.7)	0.5 (0.2-1.4, 0.2)
Age						
< 60	40	66	19		47	
CC	18 (45)	31 (47)	6 (31.6)	1	25 (53.2)	1
CT	18 (45)	34 (51.5)	13 (68.4)	1.4 (0.4-5.1, 0.6)	21 (44.7)	0.8 (0.3-1.9, 0.6)
CT or TT	22 (55)	35 (53)	13 (68.4)	1.1 (0.3-3.8, 0.9)	22 (46.8)	0.7 (0.3-1.6, 0.3)
⩾ 60	41	85	25		60	
CC	22 (53.7)	33 (38.8)	9 (36)	1	24 (40)	1
CT	13 (31.7)	46 (54.1)	15 (60)	3.8 (1.2-12.0, 0.02)	31 (51.7)	2.6 (0.9-6.6, 0.09)
CT or TT	19 (46.3)	52 (61.2)	16 (64)	2.7 (0.9-8.2, 0.08)	36 (60)	1.9 (0.8-4.2, 0.1)
Site						
Proximal		60	43		17	
CC		22 (36.7)	15 (34.9)		7 (41.2)	
CT		37 (61.7)	27 (62.8)	2.7 (1.2-6.2, 0.02)	10 (58.8)	1.4 (0.5-4.8, 0.4)
CT or TT		38 (63.3)	28 (65.1)	2.1 (1.03-4.99, 0.05)	10 (58.8)	1.2 (0.4-3.7, 0.7)
Distal		91	1		90	
CC		42 (46.2)	0 (0)		42 (46.7)	
CT		43 (47.3)	1 (100)	ND	42 (46.7)	1.3 (0.7-2.5, 0.4)
CT or TT		49 (53.8)	1 (100)	ND	48 (53.3)	0.9 (0.5-1.6, 0.7)

[1,2]For odds ratio and 95% CI calculations, controls with CC genotype was used as reference category, odds ratio adjusted for age, sex and smoking status. ND: Not determined.

vitamin B_{12} levels in two groups of low (below median), and high (above median) levels. The prevalence of

Table 4 Associations between serum folate/vitamin B_{12} concentrations and tumor methylation in relation to the clinical-biological characteristics of patients

Variables	*n* (%)	Folate (ng/mL) mean (range)	*P*[1]	High folate/Low folate[2] *n* (%)	*P*[3]	Vit.B_{12} (pg/mL) mean (range)	*P*[1]	High B_{12}/Low B_{12}[2] *n* (%)	*P*[3]
Controls (81)		6.3 (3.8-12)	0.1[4]	49 (60.5)/32(39.5)	0.6[4]	312.8 (108-995)	0.1[4]	40 (49.4)/41 (50.6)	0.9[4]
Total cases (86)		5.9 (2.1-12)		48 (55.8)/38(44.2)		269 (50-681)		44 (51.2)/42 (48.8)	
Unmethylated	66 (76.7)	5.7 (2.4-12)		32 (48.5)/34 (51.5)		255.2 (79-681)		29 (43.9)/37 (56.1)	
Methyalated	20 (23.3)	6.4 (2.1-10)	0.06	16 (80)/4 (20)	0.02	314.7 (50-571)	0.1	15 (75)/5 (25)	0.02
Sex									
Male (55)		5.9 (2.4-12)		29 (52.7)/26 (47.3)		274.2 (67-681)		28 (50.9)/27(49.1)	
Unmethylated	43 (78.2)	5.8 (2.4-12)		19 (44.2)/24 (55.8)		325.5 (67-571)		18 (41.9)/25 (58.1)	
Methylated	12 (21.8)	6.5 (3.9-9.5)	0.1	10 (83.3)/2 (16.7)	0.02	254.4 (83-681)	0.04	10 (83.3)/2 (16.7)	0.02
Female (31)		5.7 (2.1-10)	0.6[5]	19 (61.3)/12 (38.7)	0.5[5]	259.7 (50-523)	0.7[5]	16(51.6)/15 (48.4)	1.00[5]
Unmethylated	23 (74.2)	5.6 (3-8)		13 (56.5)/10 (43.5)		260. 3 (79-521)		11 (47.8)/12 (52.2)	
Methylated	8 (25.8)	6.2 (2.1-10)	0.4	6 (75)/2 (25)	0.4	257.8 (50-523)	0.9	5 (62.5)/3 (37.5)	0.7
Age									
< 60 yr (36)		5.4 (2.1-8)		18 (50)/18 (50)		238.1(50-571)		14 (38.9)/22(61.1)	
Unmethylated	30 (83.3)	5.4 (3-8)		14 (46.7)/16 (53.3)		229.8 (79-465)		11 (36.7)/19 (63.3)	
Methylated	6 (16.7)	5.5 (2.1-7.9)	0.5	4 (66.6)/2 (33.3)	0.7	279.5 (50-571)	0.8	3 (50)/3 (50)	0.7
⩾ 60 yr (50)		6.3 (2.4-12)	0.04[5]	30 (60)/20 (40)	0.4[5]	291.3 (67-681)	0.1[5]	30 (60)/20(40)	0.1[5]
Unmethylated	36 (72)	6.1 (2.4-12)		18 (50)/18 (50)		276.3 (83-681)		18 (50)/18 (50)	
Methylated	14 (28)	6.8 (3.9-10)	0.1	12 (85.7)/2 (14.3)	0.03	329.7 (67-543)	0.1	12 (85.7)/2 (14.3)	0.03
Site									
Distal (62)		5.8 (2.4-12)		32 (51.6)/30 (48.4)		260.6 (79-681)		28 (45.2)/34 (54.8)	
Unmethylated	61 (98.4)	5.8 (2.4-12)		31 (50.8)/30 (49.2)		255.5 (79-681)		27 (44.3)/34 (55.7)	
Methylated	1 (1.6)	7.9	0.3	1 (100)/0 (0)	ND	271 (271)	0.1	1 (100)/0 (0)	ND
Proximal (24)		6.1 (2.1-10)	0.4[5]	16 (66.7)/8 (33.3)	0.2[5]	290.7 (50-543)	0.5[5]	16 (66.7)/8 (33.3)	0.1[5]
Unmethylated	5 (20.8)	5.3 (4.5-6.4)		1 (20)/4 (80)		251 (109-483)		2 (40)/3 (60)	
Methylated	19 (79.2)	6.3 (2.1-10)	0.06	15 (78.9)/4 (21.1)	0.03	301.2 (50-543)	0.5	14 (73.7)/5 (26.3)	0.3

[1]*P*-value from Mann-Whitney and *t*-test where appropriate; [2]Median was taken as cut off point for low and high categorical value for serum folate/vitamin B_{12} levels (5.5 ng/mL, and 240 pg/mL, respectively); [3]*P* value from Fisher's exact test; [4]Case/control comparison; [5]Subgroups comparison, cases only. ND: Not determined.

Table 5 Combined effects of *MTHFR 677* genotype and serum folate/vitamin B_{12} concentrations on risk of tumor methylation (case-case comparison)

MTHFR 677 genotype	*n* (%)	Folate (ng/mL) mean (range)	*P*[1]	High folate/Low folate[2] *n* (%)	*P*[3]	Vit. B_{12} (pg/mL) mean (range)	*P*[1]	High B_{12}/Low B_{12}[2] *n* (%)	*P*[3]
Cases (86)									
CC (42)		5.8 (2.1-8.8)		25 (59.5)/17 (40.5)		254.6 (50-673)		20 (47.6)/22 (52.4)	
Unmethylated	34 (81)	5.9 (3.4-8.8)		20 (58.8)/14 (41.2)		267.3 (107-673)		16 (47.5)/18 (53.9)	
Methylated	8 (19)	5.4 (2.1-7.8)	0.6	5 (62.5)/3 (37.5)	1.00	200.9 (50-429)	0.2	4 (50)/4 (50)	1.00
CT (38)		6.3 (3-12)	0.5[5]	22 (57.9)/16 (42.1)	1.00	296 (79-681)	0.4[5]	21 (55.3)/17 (44.7)	0.5
Unmethylated	27 (71.1)	5.9 (3-12)		12 (44.4)/15 (55.6)		254.1 (79-681)		11 (40.7)/16 (53.9)	
Methylated	11 (28.9)	7.2 (5.3-10)	0.06	10 (90.9)/1 (9.1)	0.01	398.7 (150-571)	0.007	10 (90.9)/1 (9.1)	0.01
TT (6)[4]		4.2 (2.4-5.9)	0.03[5]	1 (16.7)/5 (83.3)	0.08	198.5 (83-300)	0.4[5]	3 (50)/3(50)	1.00
Unmethylated	5 (83.3)	3.8 (2.4-5.1)		0/5 (100)		178.2 (83-258)		2 (40)/3 (60)	
Methylated	1 (16.7)	5.9		1 (100)/0		300		1 (100)/0	
CT + *TT* (44)		5.9 (2.4-12)	0.9[5]	23 (52.3)/21 (47.7)	0.5	282.7 (79-681)	0.5[5]	24 (54.5)/20 (45.5)	0.7
Unmethylated	32 (72.7)	5.6 (2.4-12)		12 (37.5)/20 (62.5)		242.3 (79-681)		13 (40.6)/19 (59.4)	
Methylated	12 (27.3)	7.1 (5.3-10)	0.002	11(91.7)/1 (8.3)	0.002	390.5 (150-571)	0.002	11 (91.7)/1 (8.3)	0.003

[1]*P*-values for methylated and unmethylated cases, and genotype comparison, Mann-Whitney, *t*-test and Kruskal Wallis where appropriate; [2]Median was taken as cut off point for low and high categorical value for serum folate/vitamin B_{12} levels (5.5 ng/mL, and 240 pg/mL, respectively); [3]*P*-value from Fisher's exact test; [4]Since we did not have enough *TT* cases, the association of serum methyl donors and tumor methylation was not determined in these individuals; [5]Comparison with the *CC* genotype, cases only.

hypermethylation within the promoter of *p16* gene, but not in either *hMLH1* or *hMSH2* genes, was higher in CLCs derived from patients with high serum folate ($P = 0.04$) and vitamin B_{12} ($P = 0.02$) when compared with CLCs from patients with low serum folate/vitamin B_{12} levels status (Table 2).

The association between tumor methylation and serum folate/vitamin B_{12} levels is shown in Table 4. A small trend for higher levels of serum folate was found in the entire group of patients with methylated tumors compared to those with unmethylated tumors ($P = 0.06$). The percentage of methylated tumors in patients with high serum methyl donors was also higher than those with low serum methyl donors (80% in high folate group, and 75% in high B_{12} group; $P = 0.02$), whereas no such difference was found for unmethylated tumor group (Table 4). The OR for tumor methylation was 4.9 (95% CI, 1.4-17.7) for patients with high serum folate

versus low serum folate (Table 2). The risk of tumor methylation was also positively associated with serum vitamin B_{12} status (OR = 3.9, 95% CI, 1.1-13.9). The high serum folate/vitamin B_{12} levels were particularly associated with tumor methylation in males (P = 0.02), but not in females (P = 0.4, Table 4). The association was also age and site dependent, being significant for older cases and those with proximal tumors (Table 4). Since we did not have enough distal methylated tumors, we could not examine the association of tumor methylation with serum folate status in such tumors.

Joint effects of serum methyl donors, and MTHFR C677T genotypes on promoter methylation of tumor-associated genes

To investigate further whether the relationship between serum folate/vitamin B_{12} status and DNA methylation is modified by the *MTHFR* genotype, we evaluated the joint effects of *MTHFR* codon 677 genotypes and serum folate/vitamin B_{12} levels on tumor methylation. The combined effects of serum folate/vitamin B_{12} levels and *MTHFR* polymorphism on tumor methylation are presented in Table 5. While the *CC* genotype showed no association with serum folate/vitamin B_{12} levels with respect to tumors methylation, the *CT* and *CT/TT* genotypes of *MTHFR* exhibited a significant association of tumor methylation with high serum methyl donors. Insufficient *TT* cases eliminated the ability to examine their association with serum methyl donors and tumor methylation in these individuals. The frequency of methylated tumors was significantly different between cases with high and low serum methyl donors only in those with the *CT* and *CT/TT* genotype, but not with the *CC* genotype. More than 90% of methylated tumors in cases with *CT* and *CT/TT* genotypes had high serum methyl donors (Table 5). Among *CC* individuals no significant differences in mean serum folate/vitamin B_{12} levels between cases with methylated and unmethylated tumors or an association between tumor methylation and folate/vitamin B_{12} levels was observed. Therefore, the *677T* allele seems to increase the risk of methylation associated with high serum folate/vitamin B_{12}. We conclude that for the heterozygous or homozygous *C677T* genotypes, increased concentrations of folate and vitamin B_{12} are associated with increased risks of tumor methylation. Our data suggest that the *MTHFR C677T* genotype might be a genetic modifier of the effect of the folate/vitamin B_{12} status on the risk of methylation of genes promoter.

DISCUSSION

It is well established that loss of proper gene expression in human cancer can occur through epigenetic mechanisms. The effect of a common polymorphism in the *MTHFR* gene (*C677T*) on colorectal cancer risk in relation to folate status is controversial. Both global DNA hypomethylation and gene promoter hypermethylation associated with the *MTHFR TT* genotype under low folate intake have been reported[12]. In the present study, we investigated the association between the *MTHFR C677T* genotype and methylation of three putative tumor-associated genes, *p16*, *hMLH1*, and *hMSH2*, in 151 unselected series of sporadic CLC.

In our study (Table 2) the number of CLCs with at least one gene methylated was higher in females than males, and in those with proximal tumor location than those with distal tumors. Proximal tumor location, higher frequency in female subjects, and older age are characteristics that were previously associated with CIMP+ CLC[4,10].

In comparison to controls, *MTHFR C677T* allele was associated with the elevation of tumor methylation in the entire group of cases, as well as in males and older patients (Table 3). Therefore, our finding is consistent with those reports in which increased genes promoter methylation was associated with the *MTHFR C677T* genotype in CLC[10,23]. We found no apparent association between methylation of any of the individual gene examined and the *MTHFR* genotypes. Therefore, DNA methylation at specific loci appears to be random. Consistent with a previous report that there are more frequent CIPM+ proximal tumors in subjects with alleles conferring low MTHFR enzyme activity[23], the majority of methylated tumors with 677T variants in our study were also located in the proximal colon (Table 3).

Vitamin B_{12} and folate are two important cofactors of methyl-group metabolism. We noted a trend for association between serum folate/vitamin B_{12} levels and gene promoter methylation (Table 2). Higher serum folate and vitamin B_{12} levels were strongly associated with promoter methylation of the key tumor suppressor gene *p16* (P = 0.04, and P = 0.02, respectively). There was also a trend, although not statistically significant, in the association between the serum folate/vitamin B_{12} levels with promoter methylation of *hMLH1* and *hMSH2* genes.

Given the interaction between folate and the *MTHFR* genotype for CLC risk, we stratified the analyses of tumor methylation based on *MTHFR* genotypes and serum folate/vitamin B_{12} status (Tables 4 and 5). In case-case comparisons, we found no significant difference in methyl donor status by age, sex, or tumor location. However, the serum folate level was significantly lower in cases homozygous for the *C677T* variant compared to those with the *CT* and *CC* genotypes (P = 0.04, Table 5). We found the *TT* variant of *MTHFR* associated with lower levels of folate in patient's sera. Although the blood folate level is mainly determined by dietary intake, the *MTHFR C677T* polymorphism might modify its metabolism and serum concentration[24].

In our study, we found significant differences in the serum folate and vitamin B_{12} levels in patients with methylated and unmethylated tumors (Table 4). None of the patients in our study used vitamin supplementation. Therefore, the increased tumor methylation observed in our study was associated with the high serum methyl donor status in physiological range. We noted also the same trend in the association between serum vitamin B_{12} levels and tumor methylation (Table 4). Therefore, our data are consistent with those reports where a

positive association of dietary folate intake with DNA methylation and CLC risk was observed[25,26].

Both folate deficiency and the *MTHFR C677T* polymorphism have been previously linked to global DNA hypomethylation in lymphocytes and colon tissue[27,28]. However, few studies have addressed the joint effects of methyl donors in blood and *MTHFR* genotypes on promoter-specific DNA methylation in malignancies[12,29]. Here, we assessed the association between methylation of genes promoter, the circulating levels of folate/vitamin B_{12} and the influence of the *MTHFR* 677 genotypes in CLC patients. While no significant difference in serum folate/vitamin B_{12} status was observed between those with methylated and unmethylated tumors in *CC* individuals, the *CT*, and *CT*/*TT* genotypes of *MTHFR* exhibited a significant positive correlation with elevated folate/vitamin B_{12} levels for promoter methylation silencing (Table 5). Previously, an interaction between dietary folic acid and vitamin B_{12} supplementation with promoter methylation in colorectal adenomas has been suggested, especially for subjects with *MTHFR TT* genotype[16,30]. Because we did not have enough *TT* cases, we could not evaluate correlation of serum folate levels with tumor methylation in these individuals. Among our study group there was only one *TT* individual with a methylated tumor who also had higher serum folate/vitamin B_{12} level than *TT* cases with unmethylated tumors (Table 5). Genomic DNA methylation in leukocytes and in transformed human lymphoblasts was shown to be positively correlated to folate status in those with the *TT* genotype, but not with wild-type *MTHFR CC* genotype[12,27,31]. An inverse trend of serum and erythrocyte folate with DNA hypomethylation was also reported in normal colonic mucosa[28].

In some studies, no interaction was reported between either *MTHFR* genotype and folate intake in association with CIMP + colon tumors[17,32]. Further investigation is needed focusing on ethnic variations in the relationships between the *MTHFR* polymorphism, folate intake, and tumors methylation in CLC. The majority of previous case-control studies have assessed dietary folate or vitamin B intake from questionnaires rather than their blood measurements, a procedure which is prone to some degree of miscalculation. Moreover, other factors like alcohol intake and iron status may be related to folate availability and biological activity[33]. In the present work, the direct measurement of serum folate/vitamin B_{12} was correlated with CLC and tumor methylation. It has been previously reported that the colonic mucosal folate concentration correlates directly with serum folate concentration in the physiological range at each time point[34]. Therefore, the serum folate measurement could be an accurate reflection of the folate status in the colonic mucosa. High intracellular concentrations of folate intermediates are associated with aberrant methylation within promoter regions of cancer – associated genes in colorectal tumors[35].

There is evidence that the epigenetic mechanism of gene silencing by methylation may play a differential role in proximal versus distal colon carcinogenesis. A different role for the *MTHFR 677 TT* genotype in the tumorigenesis of proximal and distal CLC has been also suggested[8]. Our finding of an increased risk of tumor methylation associated with high serum folate/vitamin B_{12} levels in those with proximal tumors, and in older patients (Table 4) might be related to the previous observation of a high concentration of folate in tumors from older patients and proximal CLC[35].

Our results indicate that a high serum folate/vitamin B_{12}, in combination with a heterozygous or homozygous *C677T MTHFR* genotype, predisposes tumor-specific genes to promoter hypermethylation. Conversely, folate could be protective or have no effect in developing CLC in subjects with the wild type *MTHFR 677* CC genotype. The *MTHFR C677T* mutation reduces MTHFR activity, which leads to lower levels of 5-methylTHF in individuals with a marginal folate status. However, in the presence of high folate levels, the negative effect of *MTHFR TT* on the efficiency of the methylation process might be masked possibly by maximizing the catalytic activity of MTHFR[36]. Indeed, under conditions of high folate status no differences in the *K*m or *V*max values were detected between the wild type and mutant enzymes[37]. Therefore, hypermethylation of CpG islands could occur in individuals with the *MTHFR 677T* allele under high folate status.

A major draw-back of the present study is the fact that the serum data collection occurred after the onset of tumor formation. Although, more studies are needed to determine whether *MTHFR C677T* genotypes, together with high serum folate/vitamin B_{12} levels, could serve as risk factors for the CIMP + CLC subgroup, the findings of this study are in agreement with other recent reports which together provide additional evidence for caution in the mandatory fortification of cereals with folic acid.

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COMMENTS

Backgrounds

Transcriptional silencing of tumor suppressor genes by hypermethylation of capillary blood gases (CpG) islands located in the promoter region is very common in human colorectal cancer. *P16, hMLH1, hMSH2* are key tumor suppressor genes frequently silenced by promoter methylation in sporadic colon cancer (CLC). Methylentetrahydrofolate reductase (*MTHFR) C677T* genotype has been associated with reduced enzyme activity and altered cellular folate composition. In this study, we investigated the association between serum folate/vitamin B_{12}, *MTHFR C677T* genotype, and promoter methylation of three tumor-associated genes in solid tumors among sporadic CLC patients.

Research frontiers

Dietary folate/vitamin B_{12} intake and *MTHFR C677T* genotype was suggested to protect against colorectal cancer. However, only a few studies have addressed the joint effects of circulating levels of folate/vitamin B_{12} and the *MTHFR C677T* genotype on the risk of epigenetic inactivation of specific tumor suppressor genes in CLC patients.

Innovations and breakthroughs

Our data indicate that serum folate/vitamin B_{12} levels are directly associated with the DNA hypermethylation of CpG island within promoter of the tumor specific genes and to the *C677T* genotype of *MTHFR*. We identified that

the *T* allele of *MTHFR* has strong influence on the risk of tumor methylation associated with high serum folate/vitamin B_{12} levels.

Applications

The results from the study support other recent reports that high folate and vitamin B_{12} status might serve as risk factors for CLC. This study provides additional evidence for caution in terms of CLC risk because of the mandatory fortification of cereals with folic acid in certain countries.

Peer review

This is a population-based, case-controlled, molecular epidemiological study on the interaction of *MTHFR C677T* genotype and circulating folate/vitamin B_{12} with the CpG island hypermethylation of tumor-associated genes in sporadic colorectal cancer. This result indicated that for the *MTHFR C677T* genotypes, increased concentrations of folate and vitamin B_{12} are associated with increased risks of tumor methylation. This demonstration might give a suggestion to protect against colorectal cancer, at least, in Iranian sporadic CLC population.

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