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***MTHFR* C677T and A1298C variant genotypes and the risk of microsatellite instability among Iranian colorectal cancer patients**

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Abstract

Methylenetetrahydrofolate reductase (*MTHFR*) is a key enzyme in the folate metabolic pathway. We aimed to test the hypothesis that *C677T* and *A1298C* variants of *MTHFR* predispose to microsatellite instable (MSI) colorectal cancer. We determined *MTHFR* genotypes in 175 sporadic colorectal cancer patients and a total of 231 normal controls in Shiraz, Southern Iran. Among the genotypes found in our samples, *MTHFR CT* and *CT + TT* were associated with increased risk for CRC incidence [odds ratio (OR) = 2.4, 95% confidence interval (95%CI) = 1.8–4.4; OR = 2.4, 95%CI = 1.6–3.6, respectively]. Double heterozygotes *677CT/1298AC* and double homozygote *677TT/1298AA* and *677CC/1298CC* genotypes also showed a significantly increased risk of developing CRC compared with the wild-type *677CC/1298AA* genotypes of the controls. Among the 151 tumors tested, 36 (23.8%) were MSI+. MSI was more common in proximal tumors (OR = 10.4; 95%CI = 3.9–27.8) and in smokers (OR = 2.9; 95%CI = 1.3–6.7). In a case–control comparison, the *MTHFR 677CT + TT* genotype was strongly associated with MSI (OR = 2.6; 95%CI = 1.3–5.3). Hypermethylation of mismatch repair genes was positively related with MSI incidence in these tumor series ($P = 0.00$). Our data suggest that the *MTHFR 677CT + TT* variant genotype may be a risk factor for MSI+ cancer.

1. Introduction

The incidence of colorectal cancer (CRC) has rapidly increased in Iran during the last decade [1]. The increase has generally been ascribed to smoking and a Westernized diet, which is characterized by a high intake of fat and meat, but there have been a limited number of studies done on the molecular pathology of CRC in this region.

Sporadic CRC is a multifactorial disease caused by numerous interacting genetic, epigenetic, and environmental risk factors. There is increasing evidence suggesting that colorectal cancers develop through different molecular pathways [2]. Based on genetic and epigenetic profiles, Shen et al. [3] proposed three distinct subclasses of primary colon cancers, each with homogenous molecular features.

The enzyme methylene tetrahydrofolate reductase (*MTHFR*) functions at a critical juncture between DNA synthesis and methylation of DNA, proteins, and lipids [4]. Two common variant genotypes of the *MTHFR* gene, *C677T* in exon 4 (Ala222Val) and *A1298C* in exon 7 (Glu429Ala), are associated with reduced enzyme activity [5,6]. It has been suggested that mutations in *MTHFR*, which alter both DNA methylation and nucleotide synthesis, are related to CRC incidence [7,8].

The results of previous CRC studies with regard to its relationship with *MTHFR* polymorphism were controversial [9,10]. Several case-control studies have shown a reduction in CRC risk for individuals with the *TT* genotype compared with those with *CC* or *CT* genotypes, but the protective effect appeared to depend on an adequate dietary folate intake. Other studies did not find a reduced risk of CRC or rectal cancer among those with a variant genotype of the *MTHFR* polymorphism; rather, they observed an increased risk of colon cancer, suggesting that the effects of the *MTHFR* genotype may differ in populations with different levels of folate intake [11,12].

Microsatellites are short tandem sequence repeats in DNA, and their length changes are termed microsatellite instability (MSI), which have been found to be associated with about 15–20% of sporadic colorectal cancers [13]. MSI-positive colon cancers comprise a distinct group that also shows widespread gene promoter methylation, a feature referred to as the CpG island methylator phenotype. MSI occurs as a result of a deficiency in a group of nuclear proteins collectively known as the mismatch repair (MMR) system. Mutations in genes coding for enzymes involved in folate metabolism might play a role in altered gene promoter methylation and thus predispose to MSI CRC. On the basis of the functional effects of *MTHFR* genotypes and their role in methylation, variants may be expected to be associated with the risk of MSI in colorectal cancer. Little is known about the role of *MTHFR* variant genotypes in MSI-related colon carcinogenesis. A few studies have investigated the role of *MTHFR* variant genotypes in MSI-related colon carcinogenesis, but the findings were not consistent. While several investigators reported that the *MTHFR* homozygote *TT* genotype was associated with an increased risk of MSI-positive CRC [14–17], some studies found either no association or a reduced risk of MSI tumors associated with *MTHFR* variant genotypes [18,19].

As far as we know, there is currently no information in the available literature concerning the possible association of two *MTHFR* variants with CRC in Iranians. We recently examined the association between the *MTHFR C677T* genotype and hypermethylation of the promoter region of three tumor-associated genes (*P16*, *hMLH1*, and *hMSH2*) among 151 sporadic CRC patients in Iran [20]. We have now made MSI analysis and genetic typing studies of the *MTHFR A1298C* in these series of patients. Therefore, this should be considered as an extension of our previous study, which could be consulted for detailed information.

2. Materials and methods

2.1. Study population and DNA preparation

A total of 175 sporadic primary tumor samples (90 fresh and 85 paraffin-embedded samples) and their corresponding adjacent normal colon mucosa were obtained from surgical patients

at university hospitals in Shiraz, Southern Iran. The Institutional Ethics Committee approved the study protocol. Fresh samples were snap-frozen immediately after surgery and stored at -70°C . All samples were subjected to re-evaluation of the original histologic diagnosis made by an expert pathologist, who also selected representative tissue section areas for DNA extraction and further molecular analyses. Genomic DNA was extracted from tumor and normal tissues, as described previously [20].

2.2. MTHFR genotyping

DNA extracted from peripheral blood lymphocytes of healthy volunteers was used as normal controls for genotype analysis. Genotyping of *MTHFR* at codon 677 and 1298 of DNA from control and CRC cases was performed using the mutagenically separated polymerase chain reaction (MS-PCR) method, as described [20,21].

2.3. MSI analysis

DNA from 151 tumor samples were tested for MSI at the Bethesda Consensus recommended loci (*BAT25*, *BAT26*, *D2S123*, *D5S346*, and *D17S250*). Target DNA sequences were amplified by PCR in a 50- μL reaction volume containing 100 ng genomic DNA. The PCR products were analyzed under denaturing conditions on a 6.7% polyacrylamide gel containing 50% urea. After electrophoresis, the polyacrylamide gel was fixed and the PCR products were visualized by silver staining. MSI analysis was based on the interpretation of the mobility shift of tumor DNA in comparison to normal DNA. MSI was defined by the presence of a ladder-like expansion or contraction of the microsatellite repeat unit. MSI analysis results for 53 samples and their normal matched tissues were also verified by an automatic DNA sequencer (ABI Prism 3130 Genetic Analyzer; Applied Biosystems, Foster City, CA). Tumors were classified as having microsatellite instability (MSI) if two or more microsatellite markers showed changes compared with matched normal tissue. Tumors with only one MSI-positive marker defined as MSI-low (MSI-L) and were grouped together with no MSI+ specimens as microsatellite stable (MSS).

2.4. Statistical analysis

Depending upon the sample size, associations between clinical, biologic, and genotypic features were evaluated using either the chi-square test or the Fisher's exact test. The level of significance was assessed at $P < 0.05$. Logistic regression was used to calculate odds ratio (OR) and 95% confidence intervals (95%CI). We also adjusted for potential confounding variables such as age, gender, tumor site, and cigarette smoking. All statistical computations were performed by SPSS software (version 11.5; SPSS Inc., Chicago, IL).

3. Results

In this study, we investigated the association of two common functional polymorphisms of *MTHFR* with reduced enzyme activity (*C677T* and *A1298C*), as well as CRC incidence among Iranian sporadic colon cancer patients. Sixty percent of patients had distal and 40% had proximal tumors. Cases were more likely to be males older than 60 years.

3.1. Distribution of genotypes

Genotype frequencies and odds ratios for *MTHFR* genotypes and colon cancer are presented in Table 1. Prevalence of the *MTHFR* 677T allele did not differ significantly between controls (20.3%) and cases (31.1%). The distribution of *MTHFR* 677 genotypes among controls (*CC*, 64.9%; *CT*, 29.4%; *TT*, 5.6%) agreed with that expected from the Hardy-Weinberg equilibrium ($\chi^2 = 1.22$, $P = 0.3$). However, significant departures from Hardy-Weinberg equilibrium were observed for *MTHFR* 677 genotypes among cases ($P = 0.004$).

The genotype distributions of CRC patients (*CC*, 42.4%; *CT*, 53%; *TT*, 4.6%) were significantly different from controls ($P = 0.00$). To increase precision, we combined heterozygous and homozygous variant genotypes. The *MTHFR* 677*CT* + *TT* genotype in controls was less frequent than in CRC cases ($P = 0.00$; Table 1). Similar frequencies of the *A1298C* genotypes were seen in patients and controls. Both patients and controls were in Hardy-Weinberg equilibrium for the distribution of the *A1298C* polymorphism ($\chi^2 = 0.77$, $P = 0.4$; $\chi^2 = 0.01$, $P = 9$, respectively).

We examined the relationship between two *MTHFR* polymorphisms and the risk of CRC. According to the logistic regression model, in the entire group of patients, *MTHFR CT* and *CT* + *TT* genotypes were strongly associated with a higher risk of cancer (Table 1). The adjusted OR for *CT* and *CT* + *TT* genotypes were 2.8 (95% CI = 1.8 – 4.4) and 2.4 (95% CI = 1.6 – 3.6), respectively. No CRC risk associated with *MTHFR A1298C* genotypes was found when we compared patients with controls and considered the *AA* genotype as the referent (Table 1). For *MTHFR 677* and *MTHFR 1298* haplotypes, double heterozygotes (677*CT*/1298*AC*) had 3.6-fold (95% CI 1.5 – 8.6) increased risk compared with the wild-type (677*CC*/1298*AA*) genotypes of controls. The homozygous 677*TT*/1298*AA* genotype was also associated with an increased risk of developing CRC in comparison to controls (OR = 3.9, 95% CI = 1.0 – 14.4; Table 1). Due to the small number of cases in the current study, it was not possible to perform analyses of CRC risk associated with other combined *MTHFR* genotypes.

3.2. Correlation between MTHFR genotypes and patients' characteristics

Patients' clinicopathologic characteristics in relation to *MTHFR* genotypes are summarized in Tables 2 and 3. No differences in frequencies of the *MTHFR A1298C* genotypes were found in patients stratified by age, sex, smoking status, and tumor differentiation stage (Table 2). Grouping the patients according to their tumor localization, significant differences in *MTHFR 1298* genotype distribution were observed between patients with proximal colon tumor (*AA*, 20.7%; *AC*, 55.2%; *CC*, 1.7%) and patients with distal CRC (*AA*, 43.8%; *AC*, 49.3%; *CC*, 6.8%), as verified by the chi-square test ($P = 0.02$). The frequency of the *MTHFR 1298AC* + *CC* genotype in patients with proximal cancer was significantly higher than distal CRC cases ($P = 0.02$). We did not also find a CRC risk associated with the *AC* + *CC* genotype for any of the sex and age groups, when cases were compared with controls and the *AA* genotype was used as a reference category (Table 2). However, the *AC* + *CC* genotype was associated with a threefold-increased CRC risk in proximal patients compared with controls (OR = 3; 95% CI = 1.1–7.6).

In case–case comparisons, we observed no differences in frequencies of *MTHFR C677T* genotypes in patients stratified by the clinicopathologic variables (Table 3). In case–control comparisons, however, we found an increased cancer risk associated with the *CT* + *TT* genotypes in females (OR = 2.5; 95% CI 1.3 – 4.6), in older patients (OR = 2.7; 95% CI 1.5 – 4.8), and in both proximal (OR = 3.4; 95% CI 1.8 – 5.9) and distal (OR = 1.8; 95% CI 1.1 – 3.2) tumors (Table 3). Compared with controls, the *CT* + *TT* genotype was strongly associated with CRC in smokers (OR = 4.4; 95% CI 1.6 – 12.5), but not in nonsmokers. This genotype also showed a significant association with both well/moderately differentiated and poorly differentiated CRC (Table 3).

3.3. Relationship between MTHFR genotypes and MSI

We examined the relationship between *MTHFR* polymorphisms and MSI in CRC from 151 patients. PCR amplification of the extracted DNA was performed for the five microsatellite markers recommended by the National Cancer Institute (i.e. BAT25, BAT26,

D17S250, D2S123, and D5S346 [22]. Illustrative examples of MSI analysis of the five microsatellite markers in CRC tumors are shown in Fig. 1.

Table 4 provides the OR with 95%CI for the association between MSI and clinicopathologic characteristics among colon cancer cases. Among the 151 tumors, 36 (23.8%) exhibited a high degree of MSI (MSI+). Proximal location of the tumor was significantly associated with the likelihood of having MSI ($P = 0.00$). A total of 26/60 (43.3%) patients with proximal CRC showed MSI tumors, and only 10/91 (11%) patients with distal CRC showed MSI. Patients with MSI CRC were smokers and more likely to have poorly differentiated tumors. A small trend for a higher risk of MSI was observed in younger patients compared with the old ones ($P = 0.06$; Table 4).

The associations between MSI and the genetic features of tumors are shown in Table 5. The frequency of *MTHFR* 677 and *MTHFR* 1298 genotypes were not statistically different in MSI tumors compared with MSS tumors. In case-control comparisons, however, a significant positive association was observed between the *MTHFR* CT+ TT genotype and MSI status (OR = 2.6, 95%CI 1.3 – 5.3; $P = 0.01$).

The majority of CRC exhibiting the MSI phenotype result from hypermethylation of the *hMLH1* gene promoter [23]. The CRC series investigated in this study for MSI analysis (151 tumors) had been characterized previously for hypermethylation of the promoter region of *hMLH1* and *hMSH2* genes [20]. Therefore, this allowed us to examine the association between MSI status and hypermethylation of the two MMR genes in the present study. Ninety-five percent of the patients with methylated *hMLH1* (19/20) and 75% of those with methylated *hMSH2* (3/4) exhibited a high degree of MSI, indicating a strong association between hypermethylation of MMR genes and MSI incidence in CRC tumors ($P = 0.00$; data not shown).

4. Discussion

The current case-control study aimed to explore the role of two common functional polymorphisms of *MTHFR* gene, *C677T* and *A1298C*, in altering the susceptibility to cancer and MSI in sporadic CRC in a Southern Iranian population. These variants may be involved in the development of CRC by aberrant methylation of genes promoter and thus predispose to microsatellite instability.

The frequencies of *MTHFR* 1298 genotypes in patients corresponded to those of the controls (Table 1). The distribution of *MTHFR* 677 genotypes among patients, however, was significantly different from that of the controls ($P = 0.00$). The TT genotype frequency observed in our control group (5.6%) was lower than the 12% generally reported for Caucasian and Asian populations [24].

Previous studies on the association between the *C677T* polymorphism and susceptibility to CRC showed no consistent results. Some studies have reported a significantly reduced risk of developing CRC in TT individuals with an adequate dietary folate intake [25,26], which suggests a protective effect of this genotype. Several other studies, however, have failed to show a protective effect of the *MTHFR* 677TT genotype against CRC, even when folate intake was high [11,27,28].

Because of the relatively small numbers, we combined individuals with *MTHFR* 677TT and CT genotypes. In agreement with the previous findings of an increased CRC risk associated with the *MTHFR*-TT genotype, we found more than a twofold-increased risk of CRC incidence associated with the CT+ TT genotype in the entire group of patients compared with age-matched controls (Table 3). The proportion of CT+ TT individuals was not

significantly elevated in male patients ($P = 0.06$). Compared with controls, however, the *MTHFR CT + TT* genotype showed a higher risk of cancer in females ($OR = 2.5$; $95\%CI = 1.3 - 4.6$), but not in males (Table 3). We observed no significant difference in the distribution of the *CT + TT* genotype between patients with proximal and distal tumors. In case-control comparisons, the *CT + TT* genotype was positively associated with cancer incidence in older cases. Shannnon et al. [14] suggested that the increased risk of CRC associated with the *MTHFR TT* genotype in older populations is possibly due to age-related disturbances in folate metabolism. In our previous study, we examined the association among the *MTHFR C677T* genotype, serum folate status, and hypermethylation of the promoter region of three tumor-related genes (*p16*, *hMLH1*, and *hMHS2*) in CRC patients [20]. In that study, we found that levels of serum folate and vitamin B12 were positively associated with tumor methylation, especially for those with the *MTHFR CT + TT* genotype.

We found no significant difference in the risk associated with the *MTHFR AC + CC* genotype when all the CRC patients were compared with controls and the wild-type *AA* genotype was used as a reference category (Table 2). A significant risk difference was observed between homozygous *AA* and the *AC + CC* genotypes when proximal cases were compared with controls and the wild-type *AA* was used as the reference category ($OR = 3$, $95\% = CI 1.1-7.6$). Such a relationship was not observed when cases with distal tumors or any sex and age group of CRC patients were compared with controls.

While the genotype of *MTHFR 1298* was not associated with the overall risk of CRC in our study, a significantly increased cancer risk was observed in individuals with double heterozygotes (*677CT/1298AC*), as well as homozygous *677TT/1298AA* and *677CC/1298CC* genotypes (Table 1). The lack of risk associated with the *MTHFR A1298C* alone could be due to no significant alteration in the activity of the enzyme caused by this polymorphism [5,29]. However, the coincidence of the *1298C* polymorphism with the *677T* allele, which adversely affects the *MTHFR* activity [6], could increase the risk of cancer development.

We had previously reported that the *MTHFR TT* genotype, combined with a high serum folate status, may be a risk factor for tumor-specific gene promoter hypermethylation among Iranian CRC patients [20]. We have now conducted MSI analysis in these patients. We aimed to test the hypothesis that polymorphisms in the *MTHFR* enzyme that are involved in folate metabolism may play a role in altered promoter-specific hypermethylation and, thus, predispose to MSI CRC.

We scored MSI status by examining a combination of three dinucleotide markers (D2S123, D5S346, and D17S250) and two mononucleotide markers (BAT25 and BAT26) in sporadic CRC based on National Cancer Institute (NCI) workshop criteria [22]. MSI was scored if two or more markers showed changes. Some investigators have defined tumors as microsatellite instable if only mononucleotide markers show instability [30]. In our study, all tumors showing instability in dinucleotide makers were also MSI-positive at least in one mononucleotide maker.

In agreement with previous reports [15,31], MSI CRC cases in our study group were more likely to be proximally located and poorly differentiated (Table 4). Our result is consistent with the findings of other studies showing a positive association between cigarette smoking and microsatellite instability in CRC [32,33].

Several studies have addressed the relationship among *MTHFR* polymorphisms and MSI in colon cancer. The findings of some previously published studies suggest that individuals with one or two *MTHFR 677T* alleles were at increased MSI CRC risk [15,17]. Compared

with age-matched controls, we observed that the *MTHFR* 677CT + TT genotype is also associated with MSI cancer (OR = 2.6, 95%CI = 1.3 – 5.3; Table 5). Such a relationship was not observed in case–case comparisons. Clarizia et al. [16] observed no association between the *C677T* polymorphism and microsatellite instability in the Brazilian population. Our results suggest that the *MTHFR* *C677T* variant genotype might be associated with an increased risk of MSI tumors. In case–case comparisons, we found a small trend of higher MSI risk in cases with the *MTHFR* 1298AC + CC variant genotype (OR = 2.9, 95%CI = 0.8 – 9.7; *P* = 0.07; Table 5).

MSI occurs as a result of a deficiency of the MMR genes [34]. This deficiency is identified in 15 – 20% of all CRC [35]. Our finding is in general agreement with previous studies showing that silencing of MMR gene expression by hypermethylation is associated with MSI-CRC [36]. A strong association was observed between genes promoter methylation and MSI incidence in these tumors (data not shown). Ninety-one percent of 22 tumors, which had at least one methylated *MMR* gene, were MSI+. The balance of DNA synthesis and DNA methylation determined by *MTHFR* genotypes may play an important role in the regulation of gene expression influencing cancer risk.

In conclusion, we found the *MTHFR* 677T allele to be strongly associated with CRC and MSI. Our data suggest that this risk may be mediated, at least in part, by inactivation of *MMR* genes caused by their promoter hypermethylation. The major limitation of this study is its sample size, and it is also necessary to investigate gene–nutrient interactions to better predict risk factors.

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Figure 1. MSI analysis in colorectal cancer using five microsatellite markers (Bat-25, Bat-26, D2S123, D17S250, and D5S346) by PCR-SSCP. The gels were stained by the silver nitrate method. N, normal DNA pattern; T, tumor specimens.

Table 1

The frequencies of the *C677T* and *A1298C* genotypes of the *MTHFR* gene in sporadic CRC patients and controls

<i>MTHFR</i> genotypes	Cases <i>n</i> (%)	Controls <i>n</i> (%)	<i>P</i> *	OR (95%CI) **	<i>P</i> **
<i>C677T</i>					
<i>CC</i> ref	64 (42.4)	150 (64.9)		1	
<i>CT</i>	80 (53)	68 (29.4)		2.8 (1.8–4.4)	0.000
<i>TT</i>	7 (4.6)	13 (5.6)	0.000	0.7 (0.3–1.9)	0.6
<i>CT + TT</i>	87 (57.6)	81 (35.1)	0.000	2.4 (1.6–3.6)	0.000
Total	151	231			
<i>A1298C</i>					
<i>AA</i> ref	38 (37.3)	79 (42.5)		1	
<i>AC</i>	52 (51)	85 (45.7)		1.3 (0.8–2.2)	0.3
<i>CC</i>	12 (11.8)	22 (11.8)	0.6	1.2 (0.5–2.7)	0.7
<i>AC + CC</i>	64 (62.7)	107 (57.5)	0.5	1.2 (0.8–2.1)	0.4
Total	102	186			
Combined genotype					
<i>CC/AA</i> ref	12 (14)	46 (24.7)	0.03	1	
<i>CC/AC</i>	20 (23.3)	57 (30.6)		1.4 (0.6–3.1)	0.5
<i>CC/CC</i>	10 (11.6)	14 (7.5)		2.6 (0.9–7.5)	0.07
<i>CT/AA</i>	14 (16.3)	27 (14.5)		2.0 (0.8–4.9)	0.1
<i>CT/AC</i>	23 (26.7)	25 (13.4)		3.6 (1.5–8.6)	0.004
<i>CT/CC</i>	1 (1.2)	5 (2.7)		ND	
<i>TT/AA</i>	6 (7)	6 (3.2)		3.9 (1.0–14.4)	0.04
<i>TT/AC</i>	0 (0)	3 (1.6)		ND	
<i>TT/CC</i>	0 (0)	3 (1.6)		ND	

OR was adjusted for age, sex, and smoking status.

Abbreviation: ND, not determined.

* Statistical test for case–control comparisons, chi-square test.

** For OR and 95%CI calculations, controls with the wild-type *CC* and *AA* *MTHFR* genotype were used as reference category.

Table 2
Characteristics of patients and controls according to *MTHFR* 1298A/C genotypes

	Cases				OR (95%CI)*	Controls				OR (95%CI)**
	Number	AA n (%)	AC n (%)	AC + CC n (%)		Number	AA n (%)	AC n (%)	AC + CC n (%)	
Total	102	38 (37.3)	52 (51)	64 (62.7)		186	79 (42.5)	85 (45.7)	107 (57.5)	1.3 (0.8–2.1)
Age										
< 60	40	12 (30)	24 (60)	28 (70)	1	86	36 (41.9)	45 (52.3)	50 (58.1)	1.7 (0.7–3.9)
60	62	26 (41.9)	28 (45.2)	36 (58.1)	0.6 (0.3–1.4)	100	43 (43)	40 (40)	57 (57)	1.01 (0.5–1.9)
Sex						186				
Male	68	26 (38.2)	41 (60.3)	42 (61.8)	1	117	48 (41)	60 (51.3)	69 (59)	1.2 (0.6–2.2)
Female	34	12 (35.3)	11 (32.4)	22 (64.7)	1.1 (0.4–2.6)	69	31 (44.9)	25 (36.2)	38 (55.1)	1.4 (0.6–3.4)
Smoking						74				
Never	51	17 (33.3)	29 (56.9)	34 (66.7)	1	50	17 (34)	23 (46)	33 (66)	0.9 (0.4–2.3)
Current/past	51	21 (41.2)	23 (45.1)	30 (58.8)	0.8 (0.3–1.8)	24	9 (37.5)	12 (50)	15 (62.5)	0.8 (0.3–2.4)
Location										
Proximal	29	6 (20.7)	16 (55.2)	23 (79.3)	1	–	–	–	–	3 (1.1–7.6)
Distal	73	32 (43.8)	36 (49.3)	41 (56.2)	0.3 (0.1–0.8)	–	–	–	–	0.9 (0.6–1.7)
Differentiation	94									
Well/moderate	89	34 (38.2)	43 (48.2)	55 (61.8)	1	–	–	–	–	1.2 (0.8–2.0)
Poor	5	0(0)	5 (100)	5 (100)	ND					N.D

OR was adjusted for age, sex, and smoking status.

Abbreviation: ND, not determined.

* OR and 95%CI for frequency of the AC or CC genotype in CRC patients; the first category was taken as reference.

** OR and 95%CI for frequency of the AC or CC genotype in CRC patients compared with the control group; controls with the wild-type AA genotype were used as reference category.

Table 3

Characteristics of patients and controls according to *MTHFR 677C/T* genotypes

	Cases					Controls				
	Number	CC n (%)	CT n (%)	CT + TT n (%)	I _{OR} (95% CI)	Number	CC n (%)	CT n (%)	CT + TT n (%)	² OR (95% CI)
Total	151	64 (42.4)	80 (53)	87 (57.6)		231	150 (64.9)	68 (29.4)	81 (35.1)	2.4 (1.6–3.6)
Age										
< 60	66	31 (47)	34 (51.5)	35 (53)	1	100	60 (60)	35 (35)	40 (40)	1.3 (0.7–2.7)
60	85	33 (38.8)	46 (54.1)	52 (61.2)	1.3 (0.7–2.5)	131	90 (68.7)	33 (25.2)	41 (31.3)	2.7 (1.5–4.8)
Sex										
Male	90	33 (36.7)	51 (56.7)	57 (63.3)	1	140	87 (62.1)	46 (32.9)	53 (37.9)	1.6 (0.8–3.2)
Female	61	31 (50.8)	29 (47.5)	30 (49.2)	0.6 (0.3–1.2)	91	63 (69.2)	22 (24.2)	28 (30.8)	2.5 (1.3–4.6)
Smoking						^{*81}				
Never	87	40 (46)	44 (50.6)	47 (54)	1	56	23 (41.1)	24 (42.9)	33 (58.9)	0.8 (0.4–1.6)
Current/Past	64	24 (37.5)	36 (56.3)	40 (62.5)	1.2 (0.6–2.4)	25	17 (68)	7 (28)	8 (32)	4.4 (1.6–12.5)
Location										
Proximal	60	22 (36.7)	37 (61.7)	38 (63.3)	1	–	–	–	–	3.4 (1.8–5.9)
Distal	91	42 (46.2)	43 (47.3)	49 (53.8)	0.6 (0.3–1.2)	–	–	–	–	1.8 (1.1–3.2)
Differentiation										
Well/Moderate	131	58 (44.3)	66 (50.4)	73 (55.7)	1	–	–	–	–	2.3 (1.5–3.6)
Poor	20	6 (30)	14 (70)	14 (70)	2.1 (0.7–6.1)	–	–	–	–	4.5 (1.7–12.3)

^{1/1} Odds ratio and 95% confidence interval for frequency of the *CT* or *TT* genotype in CRC patients taking the first category as reference;

² Odds ratio and 95% confidence interval for frequency of the *CT* or *TT* genotype in CRC patients compared with the control group; controls with the wild type *CC* genotype were used as reference category.

^{1,2} Adjusted for age, sex, and smoking status.

ND: Not Determined.

* Missing information on variable of smoking status explains why n for controls does not equal 231.

Table 4

Associations between MSI status and clinicopathological features of CRC

	Number	MSI n (%)	MSS n (%)	OR (95% CI)	p Value
Total	151	36 (23.8)	115 (76.2)		
Age					
< 60	66	20 (30.3)	46 (69.7)	1	
60	85	16 (18.8)	69 (81.2)	0.5 (0.5–1.0)	0.06
Sex					
Male	90	23 (25.6)	67 (74.4)	1	
Female	61	13 (21.3)	48 (78.7)	0.97 (0.4–2.3)	0.9
Smoking					
Never	87	14 (16.1)	73 (83.9)	1	
Current/past	64	22 (34.4)	42 (65.6)	2.9 (1.3–6.7)	0.01
Location					
Distal	91	10 (11.0)	81 (89)	1	
Proximal	60	26 (43.3)	34 (56.7)	10.4 (3.9–27.8)	0.00
Differentiation					
Well/Moderate	131	27 (20.6)	104 (79.4)	1	
Poor	20	9(45)	11 (55)	3.5 (1.2–9.9)	0.02

Table 5

Association between MTHFR genotypes and MSI and MSS CRC

Genotype	MSI cancers n (%)	MSS cancers n (%)	¹ OR (95% CI)	² OR (95% CI)
<i>MTHFR C677T</i>				
CC	15 (41.7)	49 (42.6)	Ref	2.3 (0.5–9)
CT	18 (50)	62 (53.9)	0.9 (0.4–2.1)	2.6 (1.3–5.6)
CT + TT	21 (58.3)	66 (57.4)	1.1 (0.4–2.2)	2.6 (1.3–5.3)
<i>MTHFR A1298C</i>				
AA	11 (64.7)	21 (30.4)	Ref	N.D
AC	6 (35.3)	37 (53.6)	2.7 (0.8–9.3)	N.D
AC + CC	6 (35.3)	48 (69.6)	2.9 (0.8–9.7)	0.4 (0.14–1.1)

¹OR and 95% CI for MSI-cases versus MSS-cases, OR adjusted for age, sex, and smoking status

²OR and 95% CI for MSI-cases versus controls, controls with the wild type genotype were used as reference category

OR adjusted for age, sex, and smoking status

ND: Not determined