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Association of the 677C→T Polymorphism in the *MTHFR* Gene with Colorectal Cancer in Mexican Patients

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Abstract. *The 5,10-methyl-tetrahydrofolate reductase (MTHFR) enzyme plays a critical role in folate and homocysteine metabolism, and its gene, MTHFR, displays common genetic polymorphisms that influence its activity. Clinical implications of MTHFR polymorphisms have been reported for several diseases, including a variety of solid tumors such as colorectal cancer (CRC). Here, the role of the 677C→T polymorphism of MTHFR was evaluated by genotyping 369 patients and 170 healthy controls from the Mexican population. The observed genotype frequencies for the controls and patients, respectively, were: 18.8% and 32% for 677TT; 34.7% and 34% for 677CC; 46.4% and 34% for 677CT. The odds ratio (OR) was 2.0 (95% confidence intervals CI; 1.3-3.3) (p<0.05). The data suggested that the 677C→T polymorphism in MTHFR contributes significantly to the risk of CRC susceptibility in the Mexican population*

Colorectal cancer (CRC) is a one of the leading causes of death in American countries (1). CRC invariably shows a progression through several clinical and histopathological steps that are the outcome of genetic changes involving the activation of oncogenes or inactivation of tumor-suppressor genes (2). To date, little is known about the etiology of CRC. Nonetheless, genetic susceptibility and environmental risk factors have recently been described (2). Folates are involved in a large number of biochemical processes and genetic or dietary deficiencies of these vitamins lead to hyperhomocysteinemia. This leads to the impairment of several cellular functions, such as cytokine expression, the bioability of nitric oxide, the induction of oxidative stress, apoptosis activation, DNA methylation and nucleotide synthesis (3, 4). A relationship between plasma folate levels, content of uracil, and DNA damage in dividing cells renders *MTHFR* a suitable candidate for studies of CRC susceptibility (5-7). Specifically, 5,10-methyl-tetrahydrofolate reductase (MTHFR) plays an important role in cellular DNA methylation, catalyzing the conversion of 5, 10-methyl-THF to 5-methyl-THF. It also has an important regulatory function in folate metabolism, where it directs the folate pool to remethylate homocysteine at the expense of DNA and RNA synthesis (8, 9). The single nucleotide polymorphism g. 677C→T (p. Ala222Val) of the *MTHFR* gene encodes a thermolabile variant of the protein that reduces its global activity by 50% (10).

Evidence of an association between the g.677C→T and g.1298A→C *MTHFR* polymorphisms and an increased risk of developing malignancies (including CRC) has recently been reported (11-14). Osian *et al*. reported that individuals with the g.677TT and g.1298CC polymorphisms in *MTHFR* had an increased risk of CRC (15). Rayn *et al*. found a high frequency of the T allele in CRC patients (16), and Urlich *et al*. found a novel mechanism by which MTHFR polymorphisms can affect the risk of CRC (17). More recently, studies aiming to establish correlation between C677T polymorphism and CRC yield not conclusive data, because some of them suggest this allele increase risk for CRC (18, 19), but other authors conclude C677T does not affect this risk (20).

It has also been observed that the frequency of the 677C→T polymorphism varies among diverse ethnic groups. For example, the frequencies of 677TT in the Dutch and Canadian populations are 8.5% and 12%, respectively (21, 22). Several reports studying the general Mexican population have estimated it, as one of the highest homozygote

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Table I. *Characteristics of the study groups.*

	Controls (n=170)		CRC patients (n=369)		*p*-value
Age (years)					
Mean (SD)	49.3	(15.7)	59.26	(13.25)	$p<0.05^2$
<50 years (%)	72	(42)	86	(23)	
≥50 years (%)	98	(58)	283	(77)	
Gender (%)					
Female	93	(54.7)	170	(46)	$p>0.05^1$
Male	77	(45.3)	199	(54)	
Family History* (%)					
No	156	(92)	310	(84)	$p<0.05^1$
Yes	14	(8)	59	(16)	
Smoking status, (%)					
Current smokers	67	(39)	181	(49)	$p<0.05^1$
Smoking in the past	24	(14)	54	(14)	$p>0.05^1$
Non-smokers	79	(47)	134	(37)	$p<0.05^1$
Tumor localization, (%)	-				
Right colon			32	(7)	
Left colon			207	(56)	
Rectum			130	(37)	
Clinical stage (%)	-				
I-II (Dukes A-B)			100	(27)	
III-IV (Dukes C-D)			269	(73)	

[1]Fisher's exact test, [2]Student's *t*-test. SD, standard deviation. *Positive familial history of cancer and leukemia in first and second degree relatives of patients.

prevalences of the 677TT polymorphism (23-26). However, there are no descriptions of an association of the *MTHFR* 677C→T polymorphism and adult Mexican individuals affected with CRC. Here a possible role for the 677C→T polymorphism of *MTHFR*, as a risk factor for the development of CRC in the Mexican population, is reported.

Materials and Methods

DNA was extracted by standard protocols (27) from peripheral blood lymphocytes collected from 170 healthy individuals and 369 patients with CRC from the metropolitan area of Guadalajara and its surroundings. The patients were recruited from January 2006 to July 2008; healthy individuals were recruited from volunteer blood donors. All the samples were taken after obtaining appropriate written informed consent. Efforts were taken to ensure that siblings of those already sampled were excluded. Clinical and demographical data were obtained by questionnaire; all the patients were interviewed to investigate occupational exposure and chemotherapy agent use was recorded. *MTHFR* gene amplification was performed by PCR using the following primers: 5'-CCTTGAACAGGTGGAGGCC-3' and 5'-CAAAGAAAAGCTGCGTGATGAT-3'. The reactions were performed in a total PCR volume of 15 μL containing 200 μM dinucleotide triphosphates (dNTPs) (Invitrogen, Los Angeles, CA, USA), 2.5 pmol of primers, 3.0 mM $MgCl_2$ and 1.5 U Taq polymerase (Invitrogen, Los Angeles, CA, USA). The PCR conditions were 94˚C (4 min), followed by 30 cycles of 94˚C (1 min), 57˚C (1 min), and 72˚C, (1 min). By this procedure a fragment of 158 bp was obtained.

Table II. *Genotype distribution of the 677C→T MTHFR polymorphism in CRC patients and healthy controls.*

	Genotype			Alleles	
	677CC * n (%)	*677CT* * n (%)	*677TT* n (%)	*677C* 2n (%)	*677T* 2n (%)
Controls	59 (34.7)	79 (46.5)	32 (18.8)	197 (58)	143 (42)
CRC patients	124 (34)	126 (34)	119 (32)	374 (51)	364 (49)
Controls *vs.* CRC patients					
OR	2.05	1.34			
95% CI (low-high)	(1.3-3.3)	(1.03-1.75)			
p-value	0.001	0.03			

*This reference category combined the two genotypes, because the odds ratio (OR) showed an equal risk for each group. CI: confidence interval.

For allele discrimination, the amplified product was subjected to restriction enzyme analysis with *Hinf*I (New England Biolabs, Beverly, MA, USA) according to the manufacturer's instructions. The samples were separated using 6% polyacrylamide electrophoresis gels (29:1), followed by silver staining (28). This revealed the 158 bp fragment for the wild type, three fragments of 158, 130, and 28 bp for the heterozygous, and two fragments of 130 and 28 bp for the homozygous variants.

The allele frequencies were obtained by direct counting. The Hardy-Weinberg equilibrium was tested by a goodness-of-fit Chisquare test to compare the observed genotype frequencies with the expected frequencies among the control subjects. Odds ratios (OR) and 95% confidence intervals (CI) were also calculated. A two-sided $p<0.05$ was assumed to be statistically significant. All the statistical analyses were performed with the Statistical Analysis System software SSPS 13.0 (Chicago, IL, USA).

Results

Table I shows the comparative epidemiological data for the CRC patients and controls. In the CRC group, the observed average age was 59.26 years (range: 18 to 91 years). Sixteen (59/369) of these patients had a positive family history for cancer.

Table II shows the genotypes and allele frequencies of the *MTHFR* polymorphism. The .677TT genotype was observed in 18.8% (32/170) of the controls and 32% (119/369) of the CRC patients; this difference was statistically significant (p=0.001, OR 2.0 [95% CI1.3-3.3]). All of the genotype distributions were under Hardy-Weinberg equilibrium.

Table III shows the frequency distributions of the *MTHFR* polymorphisms when compared for variables such as gender, age, smoking habits and positive family history for cancer. The interaction of the genotype g.677C→T between the patients and controls showed statistically significant differences with male gender (OR 2.5 [CI 95% 1.3-5.2]; $p<0.005$), age (the stratified group ≥50 years old, based on the average of CRC symptom onset from the literature) (OR 2.1 [CI 95% 1.2-4.0];

Table III. *Genotype distribution of the 677C→T MTHFR polymorphism in CRC patients with respect to gender, age, smoking status and family history.*

	Patients (n=369)			Controls (n=170)			(CI 95% Low-high)	*p*-value
Gender								
	Female	Male		Female	Male			
	n	n		n	n			
677TT	48	69		18	14		2.5 (1.3-5.2)[1]	0.005
**677CT +677CC*	122	130		75	63			
Age								
	<50 years	≥50 years		<50 years	≥50 years			
	n	n		n	n			
677TT	23	94		13	19		2.1 (1.2-4.0)[2]	0.01
**677CT +677CC*	63	189		59	79			
Smoking status								
	non-smoking	smoking	ex-smoking	non-smoking	smoking	ex-smoking		
	n	n	n	n	n	n		
677TT	41	57	19	18	9	5	3.0 (1.4-7.4)[3]	0.005
**677CT +677CC*	93	124	35	61	58	19		
Family history								
	yes	no		yes	no			
	n	n		n	n			
677TT	22	97		3	29		2.0 (1.2-3.3)[4]	0.005
**677CT +677CC*	37	213		11	127			

*This reference category combined the two genotypes, because the odds ratio (OR) showed an equal risk for each group. CI: confidence interval, [1]Male patients *vs*. male controls, [2]≥50 years patients *vs*. ≥50 years controls, [3]smoking patients *vs*. smoking controls, [4]Non-familial antecedents of cancer patients *vs*. No familial antecedents of cancer controls.

Table IV. *Genotype distribution of the 677C→T MTHFR polymorphism in CRC patients with respect to gender, age, smoking status, and familial history of cancer combined.*

	Patients	Controls	OR (CI 95% Low-high)	*p*-value
677TT, male, ≥50 years, smoker, non familial history of cancer	18	7	10.4 (3.59-32.2)	<0.001
677CC, 677CT, female, <50 years, non-smokers, family history of cancer	101	25		

Table V. *Multivariate logistic regression analysis of the study groups.*

							95% CI	
Variable	Beta	SE	Wald	df	Significance	OR	Lower	Upper
677TT/677TT	1.2	0.265	22.41	1	0.000	3.5	2.1	5.9
Age stratified	1.5	0.246	38.85	1	0.000	4.6	2.8	7.5
Smoking	1.5	0.263	33.86	1	0.000	4.6	2.7	7.7
Family history of cancer	0.95	0.35	7.4	1	0.007	2.6	1.3	5.1

$p<0.01$), tobacco consumption (OR 3.0 [CI 95% 1.4-7.4]; $p<0.005$), and positive family history of cancer (OR 2.0 [CI95% 1.2-3.3]; $p<0.005$). In the homozygous g.677TT group, an increased risk of developing CRC was detected. Table IV reveals the increased risk of CRC for patients with the g.677TT genotype along with concomitant variables (gender, age, smoker status, and no familial antecedent of cancer) (OR 10.4 [CI 95% 3.59-32.2] $p<0.001$).

A multivariate logistic regression analysis, in which the groups were considered to be the dependent variable, was

carried out with the following independent variables: g.677TT genotype, stratified age, tobacco consumption and family history of cancer (Table V).

Discussion

In Mexico, as well as many other countries around the world, CRC incidence has increased over the last 50 years. In the present study, 77% of the patients were ≥50 years, consistent with internationally described reports in which the incidence of colon cancer is higher in people over the age of 50 years (29, 30). This may be due to changes in the lifestyle in the Mexican population, such as diet, constant exposure to toxic substances, additives and contaminants. These lifestyle changes, combined with longevity, contribute to CRC incidence in this country.

A slight predominance of men (54%) compared with women (46%) was observed in the CRC group. This was again consistent with findings described in the literature that report an expected men:women ratio of 1.2:1 (31, 32). Recent studies have suggested that the high incidence in men is due to lower participation in the diagnosis of CRC in comparison to women. With regard to the location, gender differences with regard to the proportion of patients with cancer of the distal colon and rectal cancer were observed; these were lower in women than men (33-35). Overall, left CRC was most frequently observed (56%), followed by a rectosigmoidal (37%) and right (7%) localization. Some reports have suggested that the anatomical distribution of CRC is biased in terms of proximal to distal localization. This is probably the result of preventive measures and new techniques and approaches that permit better detection of CRC now being used in developed countries with a high incidence of this disease (36).With regard to family history of cancer, 16% of the patients had a positive history had cancer (3% of these had grade I CRC, whereas the rest had other types of cancer, data no showed). The frequency was consistent with the overall rates reported in the literature for cancer in control and CRC families (37). Tobacco consumption has been associated with the development of CRC in different studies (38-42), and the present results were consistent with this association. A significant difference was observed in cigarette smokers (49%) compared to the controls (39%) and it has been postulated that tobacco consumption causes chromosomal instability (43).

Little attention has been paid to the role of genetic susceptibility and environmental exposure in the etiology of CRC. In particular, people with an altered ability to activate pro-carcinogens and detoxify carcinogens may have an increased risk of developing cancer (44). Several reports have suggested that reduced activity of MTHFR may decrease the methylation of homocysteine to methionine and in turn the level of S-adenosyl methionine (SAM), resulting in DNA hypomethylation (17, 45). On the other hand, the reduced level of the MTHFR substrate 5,10-methylene-THF, which is required for thymidylate synthesis, could lead to uracil misincorporation into DNA, diminished DNA repair, and an increased frequency of chromosomal breaks and damage (46).

In the present study, a significant difference in the distribution of the 677TT genotype between the CRC patients and the healthy controls was observed (OR 2.0 CI 95% 1.3-3.3; $p<0.05$). This association was most evident in the patients with a genotype of 677TT/677TT who were male, ≥50 years old, tobacco consumers and not positive for a family history of cancer (OR 2.5 95% CI 1.3-5.2; 2.1 95% CI 1.2-4.0; 3.0 95% CI 1.4-7.4 and 2.0 95% CI 1.2-3.3, respectively; $p<0.05$). Moreover, analyzing these variables together in patients with the genotype 677TT revealed a strong association with an OR of 10.39 (95% CI 3.59-32.19, $p<0.001$).

The association between the 677C→T polymorphism and CRC is a contradictory issue; some studies have described a reduction in CRC risk in individuals with the 677TT genotype in comparison to the 677CT and 677CC genotypes. However, it has been shown that this association is influenced by adequate dietary folate intake (44, 45). Other studies suggested an increased risk of CRC in male patients with the 677TT genotype who exhibit high alcohol consumption or poor dietary folate intake (46, 47), suggesting that the risk of CRC conferred by the 677TT polymorphism in *MTHFR* is related to individual differences in age, gender, diet, alcohol and smoking habits. In this context, it is possible that *MTHFR* polymorphisms may mediate CRC risk with respect to low folate intake. Unfortunately, the folate status and dietary intake of the individuals were not analyzed in this study. Nonetheless, an emphasis on appropriate dietary folate intake for reducing CRC risk in older men and smokers with the 677TT genotype might be advisable in our populations.

In conclusion, that the 677TT genotype of the MTHFR gene shows significant differences between controls and CRC patients. These differences suggest that the polymorphism may serve as a good marker for men with CRC.

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