

Relationship of the methylenetetrahydrofolate reductase C677T polymorphism with microsatellite instability and promoter hypermethylation in sporadic colorectal cancer

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ABSTRACT. The methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism is associated with the expression of a thermolabile enzyme with decreased activity that influences the pool of methyl-donor molecules. Several studies have reported an association between C677T polymorphism and susceptibility to colorectal cancer (CRC). Considering that methylation abnormalities appear to be important for the pathogenesis of CRC, we examined the correlation between the genotype of the MTHFR C677T polymorphism, hypermethylation of the promoter region of five relevant genes (*DAPK*, *MGMT*, *hMLH1*, *p16^{INK4a}*, and *p14^{ARF}*), and microsatellite instability, in 106 patients with primary CRCs in Brazil. We did not find significant differences in the genotypic fre-

quencies of the MTHFR C677T polymorphism when one or more loci were hypermethylated. However, we did find a significant excess of 677TT individuals among patients with CRC who had microsatellite instability. This strong association was independent of the methylation status of *hMLH1* and of the biogeographical genomic ancestry of the patients. Although the mechanism responsible for the link between the C677T polymorphism and microsatellite instability was not apparent, this finding may provide a clue towards a better understanding of the pathogenesis of microsatellite instability in human colorectal cancer.

Key words: Methylenetetrahydrofolate reductase, MTHFR, C677T polymorphism, Microsatellite instability, Hypermethylation, Colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is one of the most common human malignancies, being responsible for more than 100,000 cases annually in the United States (Jemal et al., 2004). There is thus an urgent need to identify possible predisposing conditions, to help identify susceptible individuals for clinical surveillance.

One of the possible risk factors recently proposed for CRC is the genotype of the methylenetetrahydrofolate reductase enzyme (MTHFR) C677T polymorphism (reviewed in Sharp and Little, 2004). Such a link is of special interest, because altered patterns of DNA methylation are very common in this malignancy (Herman and Baylin, 2003; Esteller, 2005). For instance, we recently found that at least 58% of human CRCs show hypermethylation of the promoter region of at least one human gene (Anacleto et al., 2005a,b).

In the C677T polymorphism of the MTHFR gene, there is a change from an evolutionary conserved alanine to a valine, resulting in a thermolabile enzyme with low activity (Frosst et al., 1995). Individuals with the 677TT and 677CT genotypes show 30 and 65% activity levels of MTHFR, respectively, compared with the 677CC homozygote (Sharp and Little, 2004). The 677T allele shows geographical variation in frequency, being least frequent in sub-Saharan Africa and more uniformly common in other continents (Botto and Yang, 2000). The 677T allele occurs in the same haplotype background in European, Asian and African individuals, suggesting that it only occurred once in human evolution, probably in Africa, and that it spread due to some yet unknown selective advantage (Rosenberg et al., 2002).

Numerous studies have been done on the association between the C677T polymorphism and susceptibility to CRC, many of them pointing to a lower prevalence of CRC among individuals with the 677TT genotype (Sharp and Little, 2004; Kono and Chen, 2005). There seems to be a dependency on adequate folate status; when the dietary intake is low, since the protective effect of the 677TT genotype is not evident in heavy alcohol drinkers (Bailey, 2003; Sharp and Little, 2004). Nevertheless, several other studies, including some with a large number of patients (Keku et al., 2002), have failed to show any such association.

Two different major pathogenetic mechanisms have been proposed for the development of CRC (Ionov et al., 1993). The first, the so-called “classic pathway”, seems to be the most common and depends on multiple additive mutational events (germline and/or somatic) in tumor suppressor genes and oncogenes, frequently involving chromosomal deletions in key genomic regions (Rajagopalan et al., 2003). On the other hand, the “mutator pathway”, operationally recognizable by the presence of microsatellite instability, depends on early mutational loss of the mismatch repair system (germline and/or somatic), leading to accelerated accumulation of gene mutations in critical target genes and progression to malignancy (Lynch and de la Chapelle, 2003). The distinction between these pathways seems to be more than academic, since there is evidence that the tumors emerging from the mutator pathway have a specific “mutator phenotype”, which includes preferential localization in the right colon, undifferentiated histology, lymphocyte infiltration, and a better prognosis (Aaltonen et al., 1993; Thibodeau et al., 1993). Recently, it has also been discovered that in both of these pathogenetic pathways, loss of activity of key genes can occur through hypermethylation of their promoter region, rather than by genetic means (Herman and Baylin, 2003).

Recently, we examined microsatellite instability (MSI), and hypermethylation of the promoter region of five genes (*DAPK*, *MGMT*, *hMLH1*, *p16^{INK4a}*, and *p14^{ARF}*) in 106 patients with primary colorectal cancers in Brazil (Anacleto et al., 2005a,b). We have now made genetic typing studies of the MTHFR C677T polymorphism in these patients and in a control population.

MATERIAL AND METHODS

Patients

Primary tumor samples from 106 patients diagnosed with CRC were collected at the A.C. Camargo Cancer Hospital in São Paulo, Brazil. Informed consent was obtained from all patients, and this research was approved by the Research Ethics Committee of the A.C. Camargo Hospital and the Ludwig Institute for Cancer Research, São Paulo Branch. We also obtained matching normal colon tissue from 30 of the patients. To avoid selection bias, the samples were collected from sequential surgical cases of CRC. We also obtained medical information on the nature of the cancer, sex, age, tumor location, histological features, and clinical evolution for each patient. This is an extension of our previous study, which should be consulted for detailed information (Anacleto et al., 2005b). As a control group, we studied 150 healthy individuals from the southeast region of Brazil.

MTHFR genotyping

DNA from CRC cases (normal and tumor tissue) and from control subjects were genotyped for MTHFR C677T by examining polymerase chain reaction-restriction fragment length polymorphisms produced with the *HinfI* restriction enzyme, as described elsewhere (Frosst et al., 1995). The 677T allele creates a *HinfI* restriction site, producing 175- and 23-bp fragments, while there is no cleavage for the 677C allele (which is then identified by a 198-bp fragment). All the samples were scored independently and blindly by two researchers at our laboratory, in order to guarantee the quality of genotyping.

Methylation and microsatellite instability

All samples were studied for methylation of the promoter region of the following genes: *DAPK*, *MGMT*, *hMLH1*, *p16^{INK4a}*, and *p14^{ARF}*, as described previously (Anacleto et al., 2005b). We also studied MSI in all samples using the Bethesda consensus panel (Boland et al., 1998), composed of two mononucleotide repeat microsatellites (*BAT25* and *BAT26*) and three dinucleotide repeat microsatellites (*D2S123*, *D5S346*, and *D17S250*), also as described elsewhere (Anacleto et al., 2005b).

Genomic ancestry analysis

All cancer samples were submitted to an analysis of biogeographical genomic ancestry. Each sample was independently typed for 40 biallelic short insertion/deletion polymorphisms (indels) as described previously (Bastos-Rodrigues et al., 2006). Amplicons were then sized using a MegaBACE 1000 DNA sequencer (Amersham) and analyzed using the Genetic Profiler (version 2.2) and Fragment Profiler (version 1.2) programs (Amersham).

We used the program ADMIX.PAS (Long, 1991) to estimate the proportion of European, African and Amerindian biogeographical ancestry of each group (MSI+ and MSI-). We used the European, African and Amerindian individuals of the HGPD-CEPH Human Genome Diversity Cell Line Panel (Boland et al., 1998; Cann et al., 2002) as parental populations.

Statistical analysis

Hardy-Weinberg equilibrium was tested by the exact method of Guo and Thompson (1992), with 10,000 Markov steps. Data in a 2 x 2 format were analyzed using the Fisher exact test available in the Epi-Info software, version 6.0. Data on 2 x N tables were analyzed using the CLUMP software (Sham and Curtis, 1995), which was especially designed for use in genetic case-control association studies. In all tests, statistical significance was assumed at $P < 0.05$.

RESULTS

We genotyped the MTHFR C677T polymorphism in each of 106 dissected primary CRCs. In 32 patients for whom we had both cancer tissue and adjacent normal tissue, the typing results were identical in the two samples. We also typed 150 healthy controls from the southeast region of Brazil (Table 1). These two samples showed no significant departures from Hardy-Weinberg equilibrium, and there were no significant differences in the three genotype classes between cancer patients and healthy controls (Table 1).

To decrease the degrees of freedom and increase the power of the subsequent statistical tests, we pooled the 677CC and 677CT individuals, who had ample MTHFR activity, and contrasted them with the 677TT group. The clinicopathological information (patient sex and age, tumor location, histological features, Duke's stage (Williams and Beart, 1992), and recurrence) of 91 patients and the data for the 677CC + CT and the 677TT groups are shown in Table 2. No significant associations emerged. We also found no significant association between MTHFR polymorphism and promoter hypermethylation of the five tumor-associated genes

Table 1. Genotype frequencies of MTHFR C677T polymorphism in patients with colorectal cancer and in controls.

	CC + CT (%)	TT (%)	OR	95% CI	P
Controls	137 (91)	13 (9)	-	-	-
Cases	91 (86)	15 (14)	1.74	0.47-4.10	0.17

OR = odds ratio; 95% CI = 95% confidence interval for the odds ratio.

Table 2. The MTHFR C677T polymorphism and clinicopathological features of patients with colorectal cancer.

	CC + CT (%)	TT (%)	OR	95% CI	P
Men (N = 41)	36 (88)	5 (12)	-	-	-
Women (N = 50)	43 (86)	7 (14)	1.17	0.30-4.72	0.80
Age >70 (N = 36)	29 (81)	7 (19)	-	-	-
Age < 70 (N = 55)	50 (91)	5 (9)	2.41	0.61-9.82	0.20
Duke A (N = 15)	13 (87)	2 (13)	1.00	-	-
Duke B (N = 38)	32 (84)	6 (16)	1.22	0.18-10.10	0.99
Duke C (N = 37)	32 (86)	5 (14)	1.02	0.14-8.74	0.99

OR = odds ratio; 95% CI = 95% confidence interval for the odds ratio.

For Duke's stages (A, B, C), see Williams and Beart, 1992.

(*DAPK*, *MGMT*, *hMLH1*, *p16^{INK4a}*, and *p14^{ARF}*) in the 106 CRC samples (Table 3). Among the 106 tumors, 14 (13.3%) exhibited a high degree of MSI (MSI+). Fifty percent of the patients with MSI+ tumors (7/14) were TT genotype, in contrast with only 9% of the MSI- group (Table 4). This difference was very highly significant (P = 0.00057), indicating a strong association between MSI+ tumors and the 677TT genotype (odds ratio: 10.4; 95% confidence limits: 2.5-45.4).

Table 3. The MTHFR C677T polymorphisms and aberrant DNA methylation of five tumor-associated genes (*DAPK*, *MGMT*, *hMLH1*, *p16^{INK4a}*, and *p14^{ARF}*).

	CC + CT	TT	OR	95% CI	P
Not methylated	41	8	1	-	-
<i>hMLH1</i> methylated	15	4	1.37	0.29-6.12	0.72
<i>DAPK</i> methylated	20	1	0.26	0.01-2.30	0.26
<i>p16^{INK4a}</i> methylated	14	3	1.10	0.20-5.57	0.99
<i>p14^{ARF}</i> methylated	10	4	2.05	0.41-9.87	0.43
<i>MGMT</i> methylated	25	5	1.02	0.26-4.00	0.99
>3 loci methylated (10)	7	3	2.20	0.36-12.84	0.37
>2 loci methylated (26)	21	5	1.22	0.30-4.85	0.75
>1 locus methylated (57)	50	7	0.72	0.72-2.42	0.55

OR = odds ratio; 95% CI = 95% confidence interval for the odds ratio.

Table 4. Association between the MTHFR C677T polymorphism and microsatellite instability in colorectal cancer.

	CC + CT (%)	TT (%)	OR	95% CI	Pp
MSI- (91)	83 (91)	8 (9)	-	-	-
MSI+ (14)	7 (50)	7 (50)	10.4	2.5 - 45.4	0.00057

OR = odds ratio; 95% CI = 95% confidence interval for the odds ratio.
MSI- = no microsatellite instability; MSI+ = microsatellite instability.

Brazilians form a very heterogeneous population, which is the result of five centuries of interethnic crosses between peoples from three continents: the European colonizers, mainly represented by the Portuguese, African slaves, and the autochthonous Amerindians. These three groups have admixed to a point in which there is very little correlation between skin color and ancestry in Brazil (Parra et al., 2003). Since both the frequency of the 677T allele (Botto and Yang, 2000) and the incidence of MSI in CRC (Ashktorab et al., 2003) seem to differ between Africans and Europeans, we had to rule out the possibility that the significant association between MSI+ tumors and the 677TT genotype was due to ethnic stratification in our sample. We genotyped all cancer samples for 40 polymorphic indel loci, which form a powerful ancestry informative test battery (Bastos-Rodrigues et al., 2006). For the MSI+ group, the proportions of Europeans, Africans and Amerindians were 0.851 ± 0.046 (mean \pm SE), 0.0935 ± 0.029 and 0.055 ± 0.032 , respectively, while for the MSI- group, the results were 0.868 ± 0.092 , 0.115 ± 0.057 and 0.017 ± 0.063 , respectively. The differences in the proportions of genomic ancestry between the two groups were not significant.

DISCUSSION

We did not observe an association between the MTHFR C677T genotype and CRC. Other studies also found no such correlation (Keku et al., 2002). We had no information about the folate status of our patients, though there is evidence that the association of MTHFR C677T and CRC is only evident when folate intake is adequate (Bailey, 2003; Sharp and Little, 2004). We also did not find any correlation of the MTHFR genotype with sex, age, tumor location, histological features, Duke's stage, recurrence, or promoter methylation of five tumor-associated genes (*hMLH1*, *DAPK*, *p16^{INK4a}*, *p14^{ARF}*, and *MGMT*).

However, we did find a very highly significant positive association between the 677TT genotype and MSI (Table 4). Such an association had been seen in some previous studies (Shannon et al., 2002), but not in others (Toffoli et al., 2003; Eaton et al., 2005). We tested for two possibilities that could explain the correlation that we observed. The first, ethnic stratification, was ruled out by genomic ancestry analysis. The second was the methylation status of the promoter region of the mismatch repair gene *hMLH1*. In fact, hypermethylation of *hMLH1* is the single most common recognizable form of MSI in sporadic colorectal tumors (Kane et al., 1997). However, as we (Anacleto, 2005b) and others (Yamashita et al., 2003) previously found, MSI is not always associated with hypermethylation of *hMLH1*. Indeed, when we analyzed the proportion of *hMLH1* hypermethylation among MSI tumors, there was no correlation with the 677TT genotype.

In conclusion, we found a strong correlation between the MTHFR C677TT genotype and MSI in CRC. This strong association appears independent of the biogeographical ancestry of the patients or of the methylation status of *hMLH1*. Although the mechanism responsible for the link between the C677T polymorphism and MSI was not apparent, this finding may provide a clue for a better understanding of the pathogenesis of MSI in human CRC.

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