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*CYP2E1 Rsa*I and 96-bp insertion genetic polymorphisms associated with risk for colorectal cancer

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ABSTRACT. We investigated a possible association between alcoholism, cigarette smoking, obesity and *CYP2E1 Rsa*I and 96-bp insertion genetic polymorphisms with risk for colorectal cancer (CRC). Patients with CRC (70 women and 61 men) were matched for gender and age to 206 healthy controls. The mean age of the two groups was 62 years. Meat intake, cigarette smoking and alcohol drinking were assessed using a specific frequency questionnaire. The body mass index was also calculated. DNA was extracted from peripheral blood; *Rsa*I polymorphism genotypes were evaluated by PCR-RFLP and 96-bp insertion genetic polymorphisms were evaluated by specific primers. The distributions of *CYP2E1 Rsa*I c1/c1, c1/c2 and c2/c2 genotypes were 90.2, 9.2 and 0.6%, respectively, in controls and 83.9, 13.7 and 2.4% in CRC cases. Allele c2 was associated with increased risk for CRC [odds ratio (OR) = 1.88, 95% confidence interval (95%CI) = 1.02-3.45]. The *CYP2E1 Rsa*I c2/c2 genotype was associated with an increased risk for rectal cancer (OR = 3.23, 95%CI = 1.26-9.03). The 96-bp insertion was slightly more frequent in the CRC group (9.3 *vs* 11.4%, P = 0.19), especially in females (6.4 *vs* 11.5%, P = 0.34). Smoking, alcohol drinking or high intake of red meat and *CYP2E1* polymorphisms were

not associated with increased risk for CRC. The 96-bp insertion was marginally more frequent (P = 0.07) in undernourished CRC subjects. We concluded that the risk for CRC is higher among individuals with allele c2. The *CYP2E1 Rsa*I c2/c2 genotype was associated with an increased risk for rectal cancer.

Key words: *CYP2E1*; Polymorphism; Colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers in the world. The Brazilian Institute of Cancer (INCA) estimated that 13,310 men and 14,800 women had been newly diagnosed with CRC in Brazil during 2010. These values correspond to an estimated risk of 14 and 15 new cases per 100,000 men and women, respectively (INCA, 2011).

Epidemiological studies have identified dietary factors, such as consumption of meat, especially red meat, and cigarette smoking as possible risk factors for the development of CRC (Le-Marchand et al., 2002; Küry et al., 2007). Obesity and low physical activity have also been associated with a higher incidence of this cancer (Reszka et al., 2006). Dietary carcinogens, radiation, and nicotine tend to promote chromosome instability, while dietary fiber, minerals, and vitamins tend to normalize the cell cycle (Ferreira and Rocha, 2004).

Several carcinogens require metabolic activation by enzymes to exert their genotoxic effects, and inherited variations in genes associated with the metabolization of carcinogens may result in altered enzyme activity and, subsequently, carcinogen activation or deactivation. Phase I enzymes, including cytochrome P450 (CYP), activate several compounds to form genotoxic electrophilic intermediates (Boccia et al., 2007; Neafsey et al., 2009).

CYP2E1, a member of the cytochrome P450 superfamily, is a naturally occurring ethanol- and obesity-inducible enzyme that is primarily responsible for the metabolic activation of many low molecular weight carcinogens, such as *N*-nitrosamines, aniline, vinyl chloride, urethane, and alcohol (Knodell et al., 1991). *N*-nitrosamines present in tobacco and diet are well-recognized carcinogens involved in the development of tumors at various sites. Functional *CYP2E1* gene polymorphisms might, therefore, impact on susceptibility to cancer development (Gao et al., 2007).

Among the known genetic polymorphisms in the *CYP2E1* gene, the *Rsa*I variant corresponding to a C-1054T substitution (rs2031920) and the 96-bp insertion in the 5'-flanking region have drawn much interest because of their potential functionality. According to the conventional nomenclature, the *Rsa*I wild-type allele (commonly called c1 allele) and the variant c2 allele correspond to *CYP2E1**5A and *CYP2E1**5B, respectively. The insertion allele in the 96-bp region is named *CYP2E1**1D, whereas the noninsertion allele is *CYP2E1**1C (Morita et al., 2009).

Few studies have focused on the role of *Rsa*I polymorphism in the pathogenesis of cancers of the colon and rectum, lung, head and neck, bladder, nasopharynx, and stomach, and their results have been inconsistent. The reasons for this discrepancy are not clear, but one problem is the lack of sufficient investigation of gene environmental interactions, including links with dietary and smoking habits and environmental factors that may alter the enzyme activity of *CYP2E1* and thereby modify cancer susceptibility due to *CYP2E1* polymorphisms

(Gao et al., 2007; Masuda et al., 2007; Morita et al., 2009; Jia et al., 2009; Wang et al., 2010; Cantor et al., 2010; Lu et al., 2011).

The aim of the present study was to investigate *CYP2E1* polymorphisms in Brazilian patients from São Paulo.

MATERIAL AND METHODS

A case-control study involving 131 patients with CRC and 206 healthy subjects was carried out between March 2008 and December 2010. We recruited CRC cases that were managed by the Oncology Group, Gastroenterology Division of the Universidade Federal de São Paulo. All the recruited patients were histopathologically diagnosed with colorectal adenocarcinoma. Controls were selected from cancer-free individuals selected during a routine check-up by the Central Laboratory of the Universidade Federal de São Paulo. Eligibility criteria for the controls were as follows: no prior diagnosis of colorectal cancer; age between 18 and 74 years at the time of selection, and history of partial or total removal of the CRC, familial adenomatous polyposis, or inflammatory bowel disease. The study was approved by the local Ethics Committee, and all patients provided written informed consent.

The patients answered a structured questionnaire regarding dietary habits and frequency of red meat intake, whether they were current or former cigarette smokers, and pattern of alcohol consumption. Smokers were divided into 2 groups - never and ever smokers (current and former). Drinkers also were divided into 3 groups (≥3 times/week, <3 times/week, and no drinking) according to drinking frequency. Subjects were also divided into 3 groups according to the frequency of red meat intake (≥3 times/week, <3 times/week, and no red meat intake). Anthropometric questions inquired about height (cm) and body weight (kg) in the current year. Body mass index (BMI; kg/m^2) was analyzed for subjects in each case group. According to the BMI, the subjects were classified into 3 groups: undernourished, well-nourished, and overweight.

Peripheral blood was collected for genomic DNA extraction. *CYP2E1* gene polymorphisms were investigated by the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) genotyping technique.

DNA extraction

Leukocyte DNA was extracted from peripheral venous blood collected in EDTA tubes by using the Invisorb® Spin Blood Mini kit.

Analysis of *CYP2E1* genetic polymorphisms

The genotypes of the *CYP2E1* polymorphism were analyzed as described previously (Morita et al., 2009). Genotyping for the *CYP2E1 Rsa*I polymorphism was determined by the PCR-RFLP using the primers 5'-CCAGTCGAGTCTACATTGTCA-3' (forward) and 5'-TTCATTCTGTCTTCTAACTGG-3' (reverse). The PCR product of 413 bp in length was digested by treatment with 5 U *Rsa*I for 1 h at 37°C. The digestion resulted in fragments of 352 and 61 bp for the c1 allele. The digested fragments were electrophoresed on a 3% agarose LE (Uniscience) gel and visualized using ethidium bromide.

The 96-bp insertion genotype was identified by the PCR method, as described previously, by using the primers 5'-GTGATGGAAGCCTGAAGAACA-3' (forward) and 5'-TTTGG TGGGGTGAGAACAG-3' (reverse). The fragments were electrophoresed on a 3% agarose LE (Uniscience) gel and visualized using ethidium bromide. The PCR products of the 96-bp insertion allele and non-insertion allele were 729 and 633 bp in length, respectively. Genotyping was routinely repeated if there was any uncertainty in determining the genotype.

Statistical analysis

The Student *t*-test was used for the comparison of age between the groups. Differences in the polymorphisms between the groups were determined by the chi-square test. This test was also used to compare the clinical variables between the *CYP2E1* genotypes and alleles in the cancer patients. The association between the risk of developing cancer and these variables was assessed by calculating the odds ratio (OR) and 95% confidence interval (95%CI). A P value of <0.05 was considered to be statistically significant, and a P value of 0.05 to 0.10 was considered to be marginally significant.

RESULTS

A case-control study was conducted on 131 CRC patients and 206 healthy controls to determine whether the *CYP2E1* genetic polymorphisms *Rsa*I and 96-bp insertion are associated with the development of this disease. The characteristics of the cancer patients and controls are shown in Table 1. No difference in age or gender was observed between the groups. Among the 131 cancer patients, 83 (63.3%) had colon cancer and 48 (36.7%) had rectal cancer. According to the TNM classification, most patients were at stage II (43.5%) or III (25.1%) of cancer.

Table 1. Characteristics of the patients.

	Patients [N (%)]	Control [N (%)]	P
Parameters			
Age, years (±SD)	62.4 (13.6)	61.7 (13.3)	0.67[a]
≤50	25 (19.0)	31 (15.1)	
>50	106 (81.0)	175 (84.9)	
Gender			
Male	61 (46.5)	82 (39.8)	0.267[b]
Female	70 (53.5)	124 (60.2)	
Tumor site			
Colon	83 (63.3)		
Rectum	48 (36.7)		
Stage			
I	21 (16.0)		
II	57 (43.5)		
III	33 (25.1)		
IV	20 (15.4)		

[a]*t*-test; [b]χ^2 test.

The distributions of the c1/c1, c1/c2, and c2/c2 genotypes of *CYP2E1 Rsa*I were 90.2, 9.2, and 0.6%, respectively, in the controls, and 83.9, 13.7, and 2.4% in the CRC patients (Table 2). Among the healthy control subjects, the observed genotype frequencies of the *CYP2E1*

polymorphisms were consistent with the frequency expected under the Hardy-Weinberg equilibrium (P = 0.50 for *Rsa*I and P = 0.48 for 96-bp insertion), which suggested that the distribution of the *CYP2E1* genotypes is adequate in the cancer-free population.

Table 2. *CYP2E1* polymorphisms *Rsa*I and 96-bp insertion in all the individuals and according to genders.

Genotype	All individuals		P	Female		P	Male		P
	Control [N (%)]	Case [N (%)]		Control [N (%)]	Cancer [N (%)]		Control [N (%)]	Cancer [N (%)]	
*Rsa*I									
c1/c1	186 (90.2)	110 (83.9)	0.13	112 (90.3)	59 (84.2)	0.35	74 (90.2)	51 (83.6)	0.32
c1/c2	19 (9.2)	18 (13.7)		11 (8.8)	9 (12.8)		8 (9.8)	9 (14.7)	
c2/c2	1 (0.6)	3 (2.4)		1 (0.9)	2 (3.0)		0	1 (1.7)	
96-bp isertion									
0	187 (90.7)	116 (88.6)	0.19	116 (93.6)	62 (88.5)	0.34	71 (86.6)	54 (88.5)	0.16
1	19 (9.3)	13 (9.9)		8 (6.4)	8 (11.5)		11 (13.4)	5 (8.1)	
2	0	2 (1.5)		0	0		0	2 (3.1)	
1 or *2*	19 (9.3)	15 (11.4)		8 (6.4)	8 (11.5)		11 (13.4)	7 (11.4)	

According to the site, the distributions of the genotypes c1/c1, c1/c2, and c2/c2 were 89.1, 8.4, and 2.5% respectively, in colon cancer cases, and 75.0, 22.9 and 2.1% in rectal cancer patients (Table 3). The allele c2 was more frequent in the CRC patients, which indicates that its presence is associated with an increased risk of CRC (P = 0.05; OR = 1.88, 95%CI = 1.02-3.45; Table 4). The c2/c2 genotype of *CYP2E1 Rsa*I was associated with a high risk of rectal cancer (P = 0.06; OR = 3.23, 95%CI = 1.26-9.03), but not colon cancer (Table 4).

Table 3. *CYP2E1 Rsa*I polymorphisms according to the site.

Genotype	All individuals		P	Female		P	Male		P
	Colon [N (%)]	Rectum [N (%)]		Colon [N (%)]	Rectum [N (%)]		Colon [N (%)]	Rectum [N (%)]	
c1/c1	74 (89.1)	36 (75.0)	0.06	39 (86.6)	20 (80.0)	0.25	35 (92.1)	16 (69.5)	0.05
c1/c2	7 (8.4)	11 (22.9)		4 (8.8)	5 (20.0)		3 (7.9)	6 (26.0)	
c2/c2	2 (2.5)	1 (2.1)		2 (4.6)	0		0	1 (4.5)	

Table 4. Allele frequency *Rsa*I polymorphism in both groups and in colon or rectal cancer.

Allele	Control [N (%)]	Cancer [N (%)]	P	OR	95%CI
c1	391 (94.9)	238 (90.8)	0.05	1.88	1.02-3.45
c2	21 (5.1)	24 (9.2)			
CYP2E1 genotype	Colon [N (%)]	Rectum [N (%)]			
c1/c1	74 (89.1)	36 (75.0)			
c1/c2	7 (8.4)	11 (22.9)	0.06	3.23	1.16-9.03
c2/c2	2 (2.5)	1 (2.1)			

OR = odds ratio; 95%CI = confidence interval at 95%.

With regard to the 96-bp insertion polymorphism of *CYP2E1*, non-insertion of 96 bp was more frequent in both groups, with the frequency being 90.7 in the colon cancer cases and 88.6 in the controls. The genotype frequencies of the 96-bp insertion polymorphism did not differ between the cases and controls, but it was slightly more frequent in the CRC group (11.5

vs 6.4%, P = 0.34), especially among female subjects (Table 2).

The polymorphisms studied were not associated with cigarette smoking, alcohol drinking, or red meat intake and risk of colorectal cancer for *Rsa*I polymorphism [OR = 2.11, 95%CI = 0.74-6.03; OR = 1.39, 95%CI = 0.61-3.15, OR = 1.58, 95%CI = 0.69-3.65, respectively], and for *CYP2E1* 96-bp insertion polymorphism [OR = 1.23, 95%CI = 0.36-4.23; OR = 1.11, 95%CI = 0.47-2.61, OR = 0.93, 95%CI = 0.38-2.28].

Undernourished subjects had a marginally higher frequency of the 96-bp insertion (P = 0.07). In *CYP2E1 Rsa*I polymorphism, no difference was found among individuals and BMI (P = 0.44; Table 5).

Table 5. Genotypes of individuals in the colorectal cancer group and body mass index.

Genotype	Under and well nourished [N (%)]	Overweight [N (%)]	P	Overweight [N (%)]	Under nourished [N (%)]	P
96-bp insertion	61	67		67	13	
0	53 (86.8)	60 (89.5)	0.32	60 (89.5)	11 (84.6)	0.07
1	6 (9.8)	7 (10.5)		7 (10.5)	1 (7.7)	
2	2 (3.4)	0		0	1 (7.7)	
1 or *2*	8 (13.2)	7 (10.5)		7 (10.5)	2 (15.4)	
*Rsa*I genotype	61	67		67	13	
c1/c1	51 (83.6)	56 (83.5)	0.2	56 (83.5)	10 (76.9)	0.44
c1/c2	10 (16.4)	8 (11.9)		8 (11.9)	3 (23.1)	
c2/c2	0	3 (4.6)		3 (4.6)	0	

DISCUSSION

CRC is the third and the second most common cancer worldwide in men and women, respectively. Almost 60% of the cases occur in developed regions. About 608,000 deaths from CRC are estimated worldwide, accounting for 8% of all cancer deaths, making it the fourth most common cause of death from cancer (Ferlay et al., 2010).

Among the patients studied, most of them were women (53.5%), and the mean age was 62.4 years. These findings agree with the data published by the Brazilian National Cancer Institute (INCA, 2011).

Variations in the frequency of the *CYP2E1* genotypes among different populations and ethnic groups have been reported in several studies carried out in different regions of the world. These variations may be attributable to differences in race and lifestyle of the subjects and also to the differences in the techniques adopted by the studies.

A meta-analysis performed by Zhou et al. (2010), which examined 10 other case-control studies based on Caucasian and Asian populations, indicated a higher risk for CRC in individuals homozygous c2/c2 genotype of *CYP2E1 Rsa*I. Another study in a Hungarian population also found an association between the c2 allele and increased susceptibility to CRC (Kiss et al., 2000). The c2 allele has been associated with increased cancer risk in the case of head and neck tumors (Tang et al., 2010), but has been found to serve as a protective factor in the case of lung cancer (Zhan et al., 2010).

In this study, the c2 allele polymorphism of *CYP2E1* was found to be more frequent in CRC patients, suggesting a statistically significant increase in the risk of developing CRC (P = 0.05, OR = 1.88, 95%CI = 1.02-3.45). The genotypes c2/c2 and c1/c2 were more common in patients with tumors of the rectum (OR = 3.23, 95%CI = 1.16-9.03).

Hayashi et al. (1991) found a 10-fold higher transcriptional activity of the c2/c2 allele of *CYP2E1* than the c1/c1 allele in the HepG2 cell line, suggesting that the transcriptional activity of c2 allele must be larger that of c1. This overexpression may be partially mediated by the c2 allele rendering the individual more susceptible to CRC.

Morita et al. (2009) reported a higher risk of CRC in patients with the genetic polymorphisms of *CYP2E1* in the 96-bp insertion allele. In this study, the absence of the 96-bp insertion allele was more frequent in both case and control groups, and although not statistically significant, the 96-bp insertion allele was more frequent in the CRC patients, especially those of the female gender, suggesting a possible association with an increased risk of developing cancer if a larger number of participants were studied.

Studies have shown that the activity of *CYP2E1* may be modulated by alcohol drinking, smoking, diet, and obesity (Ginsberg et al., 2009). However, many studies have failed to find associations between genetic polymorphisms, lifestyle habits, diet, and the risk of developing cancer. A possible reason for this lack of significance is the lack of association between external factors, such as diet, alcohol consumption, smoking, and obesity, and genetic factors.

This study examined alcohol consumption, smoking, and red meat intake, and we did not find any association between these aspects, the *CYP2E1 Rsa*I polymorphism or 96-bp insertion allele, and risk of cancer.

BMI-based classification of the subjects revealed that the 96-bp insertion allele was marginally more frequent in overweight individuals ($P = 0.07$). This is a very interesting finding, since the 96-bp insertion allele was slightly more frequent in the case group, and the activity of *CYP2E1* is known to be modulated by obesity. Larger studies are needed to confirm whether the presence of the 96-bp insertion increases the susceptibility of obese subjects to CRC. No association was noted between the *CYP2E1 Rsa*I polymorphism and BMI.

Carcinogenesis is a slow and gradual process, which involves several genetic and environmental factors. Biometabolism enzymes, both of phase I and phase II, are key to the metabolic processes of our body, and are therefore of fundamental importance. Effective detoxification and/or activation of xenobiotics depend on the correct functioning of these enzymes. Genetic polymorphisms in biometabolism enzymes, such as *CYP2E1*, render individuals more susceptible to CRC due to a malfunction in enzymatic processes.

In summary, the risk of CRC was higher among individuals with the allele c2. The *CYP2E1 Rsa*I c2/c2 genotype was associated with an increased risk of rectal cancer. The 96-bp insertion did not increase the risk of colorectal cancer.

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