Original Article

Cyclooxygenase-2 gene polymorphisms and susceptibility to colorectal cancer in a Brazilian population

Michele Tatiana Pereira Tomitão[1,2], Sergio Carlos Nahas[1,3], Marcia Saldanha Kubrusly[4], Tatiane Katsue Furuya[2,5], Marcio Augusto Diniz[6], Suely Kazue Nagahashi Marie[7], Adriana Vaz Safatle-Ribeiro[1,3], José Eluf-Neto[8], Ivan Cecconello[1], Ulysses Ribeiro Junior[1,3]

[1]Departamento de Gastroenterologia, Faculdade de Medicina da Universidade de São Paulo, São Paulo/SP, Brasil; [2]Centro de Investigação Translacional em Oncologia, Instituto do Câncer do Estado de São Paulo, São Paulo/SP, Brasil; [3]Instituto do Câncer do Estado de São Paulo, São Paulo/SP, Brasil; [4]Divisão de Transplante de Órgãos Digestivos LIM 37, [5]Departamento de Radiologia e Oncologia LIM 24, Faculdade de Medicina da Universidade de São Paulo, São Paulo/SP, Brasil; [6]Cedars-Sinai Samuel Oschin Comprehensive Cancer Institute, Los Angeles, CA, USA; [7]Departamento de Neurologia LIM 15, Faculdade de Medicina da Universidade de São Paulo, São Paulo/SP, Brasil; [8]Departamento de Medicina Preventiva, Faculdade de Medicina da Universidade de São Paulo, São Paulo/SP, Brasil

Contributions: (I) Conception and design: U Ribeiro-Junior, MTP Tomitão, AV Safatle-Ribeiro, MS Kubrusly; (II) Administrative support: MTP Tomitão; (III) Provision of study materials or patients: SC Nahas, J Eluf-Neto, SKN Marie, I Cecconello; (IV) Collection and assembly of data: MTP Tomitão, MS Kubrusly; (V) Data analysis and interpretation: TK Furuya, MA Diniz, MTP Tomitão; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Ulysses Ribeiro Junior. Departamento de Gastroenterologia, Faculdade de Medicina da Universidade de São Paulo, Brasil. Email: ulysses.ribeiro@fm.usp.br.

Background: Multi-ethnicity of Brazilian population displays high levels of genomic diversity. Polymorphism may detect people at higher risk of developing cancer, distinctive response to treatment, and prognosis. Cyclooxygenase-2 (COX-2) is induced in response to growth factors and cytokines, and is expressed in inflammatory diseases, precancerous lesions and colorectal cancer (CRC). The aim of this study was to evaluate the influence of *COX-2* –1195A > G and 8473T > C polymorphisms as a risk factor of developing CRC.

Methods: We evaluated *COX-2* Single Nucleotide Polymorphism (SNP) of 230 CRC patients and 196 healthy controls by Real-Time Polymerase Chain Reaction.

Results: Populations were in Hardy-Weinberg equilibrium (HWE), except for control group of 8473T > C SNP. The frequencies were similar in both groups for genotypes and haplotypes. There was no association between studied polymorphisms and risk of CRC.

Conclusions: The gene polymorphisms studied do not participate in the genetic susceptibility to CRC in a Brazilian population.

Keywords: Polymorphism; genes; single nucleotide polymorphism (SNP); cyclooxygenase-2 (COX-2); colorectal cancer (CRC); risk; susceptibility

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Introduction

Colorectal cancer (CRC) is considered a public health problem worldwide (1). In Brazil, it was registered 15,067 death for this malignancy in 2013 (2) and the incidence is estimated at 34,280 people in 2016 (3).

The multi-ethnicity of Brazilian population represents a challenge in terms of genetic variability characterization, and the intra-individual genetic variation represented by genetic polymorphisms can be a relevant factor in susceptibility to developing cancer, distinct response to

chemotherapy and radiotherapy, and prognosis (4). SNPs are the most common intra-species variants, exceeding 150 million cataloged in humans according to NCBI SNP database. SNPs may vary according to ethnic differences, explaining some discrepancies between the results found in several studies on this topic (5,6).

Cyclooxygenases are key enzymes of the inflammatory process, by converting arachidonic acid to prostaglandin H_2, precursor of prostaglandins, prostacyclins and thromboxanes (7). The Cyclooxygenase-2 (COX-2), undetectable under normal conditions, is readily induced in response to growth and inflammatory factors, and it is expressed in inflammatory diseases, pre-malignant lesions and colorectal tumors (8). The enzyme may be detected in adenomas and adenocarcinomas (9). Recent studies have found COX-2 overexpression in up to 72% of CRC (10,11).

The promoter of the *COX-2* gene is rich in recognition sites for nuclear proteins, which is critical in gene transcription induction (12). The *COX-2*-1195G > A (rs689466) SNP creates a recognition sequence for c-MYB transcription factor that results in increased gene transcription (12). This SNP has been associated to an increased risk of gastrointestinal tumors, including CRC (6,13).

The *COX-2* + 8473T > C polymorphism (rs5275), can affect the affinity of transcription factor binding sites, influence the stability and/or translational efficiency of mRNA, and may modulate cancer susceptibility (14,15).

Thus, the aim of the present study was to evaluate the influence of *COX-2* –1195A > G and 8473T > C polymorphisms as a risk factor of developing CRC.

Methods

Patients and controls

We evaluated 230 patients with CRC, who underwent surgical resection between September 2001 and November 2006, at the Hospital das Clinicas, University of São Paulo. The control group included 196 patients operated for benign disease at the same hospital, matched for sex and age, and no individual or familial history of cancer. All participants signed an informed consent form, approved by the Ethics Committee for Research Projects Analysis of the University of São Paulo, School of Medicine (protocol nº0803/11).

DNA isolation

Genomic DNA was isolated from the buffy coat using the extraction and purification Kit PureLink™ Genomic DNA Mini Kit (Invitrogen- Thermo Fisher Scientific, Carlsbad, USA) according to manufacturer's instructions. The concentration and the purity of the DNA samples were determined in spectrophotometer NanoDrop™ ND-1000 (NanoDrop Technologies, Inc. Wilmington, USA). The integrity was checked by electrophoresis in 1% agarose gel.

Genotyping

Determination of genotype of *COX-2* –1195A > G and 8473T > C SNPs was performed using TaqMan Kits (Thermo Fisher Scientific, Foster City, CA; Assay-on-demand, products: C_1647381_10 e C_16198794_10, respectively), according to manufacturer's instructions. The selection of SNPs was based on association to CRC and functional effects described in the literature, available in the dbSNP database (NCBI; www.ncbi.nlm.nih.gov). Twenty percent of the samples were randomly selected and re-genotyped. The results showed 100% of similarity.

Statistical analysis

The genotype frequencies were determined by direct counting of the alleles. The Hardy-Weinberg equilibrium (HWE) was evaluated through χ^2 test. The haplotype frequencies were calculated using the Expectation-Maximization (EM) algorithm. Linkage disequilibrium (LD) between polymorphisms was verified using the Haploview software (version 4.2). We evaluated the association of genotypes and alleles of the *COX-2* gene between case and control groups using the χ^2 test or Fisher's exact test. The odds ratio and 95% confidence intervals were calculated for genotypes and estimated haplotypes. The logistic regression model adjusted for age, sex, ethnicity, level educational, alcohol drinking and smoking status were calculated in case and control groups. The genotype, dominant and recessive models were used in all analyzes, performed with R software, version 3.1.2. Data were considered significant when $P<0.05$.

Results

Demographics characteristics of the cases and controls are summarized in *Table 1*. There was no statistical difference between the groups regarding age (P=0.84), sex (P=1.00), ethnicity (P=0.58) and smoking status (P=1.00). Education level and alcohol consumption showed statistical difference

Table 1 Description of the participants

Variable	Cases (n=230) n (%)	Controls (n=196) n (%)	OR (95% IC)	P	Total (n=426) n (%)
Age (years)[a]				0.8410[c]	
Mean (SD)	61.96±14.01	62.03±14.81	1.00 (0.99–1.01)	0.9600	61.99±14.37
Median	64	63			64
Sex				1.0000[d]	
Female	106 (46.09)	90 (45.92)	1.00	0.6560	196 (46.01)
Male	124 (53.91)	106 (54.08)	1.00 (0.68–1.47)	0.9890	230 (53.99)
Ethnicity[b]				0.5820[d]	
Caucasian	197 (85.65)	158 (80.61)	1.00		355(83.33)
Afrodescendent	23 (10.00)	31 (15.82)	0.60 (0.33–1.06)	0.0790	54 (12.68)
Asian	10 (04.35)	7(03.57)	1.15 (0.43–3.08)	0.7870	17 (03.99)
Education level (years)				0.0001[d]	
0–5	128 (55.65)	86 (43.88)	1.00		214 (50.23)
6–9	19 (08.26)	5 (02.55)	2.55 (0.92–7.10)	0.0720	24 (05.63)
10–12	55 (23.91)	53 (27.04)	0.70 (0.44–1.11)	0.1290	108 (25.35)
>12	28 (12.17)	52 (26.53)	0.36 (0.21–0.62)	0.0001	80 (18.78)
Alcohol drinking				0.0001[d]	
Non-consumers	139 (60.43)	154 (78.57)	1.00		293 (68.78)
Ex-consumers	33 (14.35)	16 (08.16)	2.28 (1.21–4.33)	0.0110	49 (11.50)
Alcoholics	58 (25.22)	26 (13.27)	2.47 (1.48–4.14)	0.0010	84 (19.72)
Smoking status				1.0000[d]	
Non-smokers	129 (56.09)	118 (60.20)	1.00		247(57.98)
Exsmokers	71 (30.87)	50 (25.51)	1.30 (0.84–2.02)	0.2440	121 (28.40)
Current smokers	30 (13.04)	28 (14.29)	0.98 (0.55–1.74)	0.9450	58 (13.62)

[a], Age of CRC diagnosis for patients and age of informed consent form application day for controls; [b], self-reported ethnicity; [c], Mann-Whitney Test; [d], Fisher's exact test; [e], logistic regression analysis. SD, standard deviation.

Table 2 Polymorphisms, chromosome position, HWE value, minor Alelle frequency and alleles studied for each polymorphism

SNP	Chromosome position	HWpval	HWpval cases	HWpval controls	MAF	Alele
−1195A > G	186650751	0.52	0.43	0.80	0.19	A:G
+8473T > C	186643058	0.14	1.00	0.02	0.34	T:C

SNP, single nucleotide polymorphism; HWpval, Hardy-Weinberg value; MAF, Minor Alelle Frequency.

(P<0.001).

The position of the *COX-2* polymorphisms on chromosome, information about HWE and minor allele frequency are shown in *Table 2*. The genotype frequencies of the *COX-2* SNPs were consistent with HWE with P-value ≥0.05, except for control group of the 8473T > C SNP (P=0.02).

We also evaluated the allelic segregation of the

polymorphisms, in order to verify the LD. There is a high LD between both polymorphisms, which are distant from each other in 7 kilobase (D' =97). The two SNPs were grouped into a single block and formed three haplotypes, as shown in *Figure 1*.

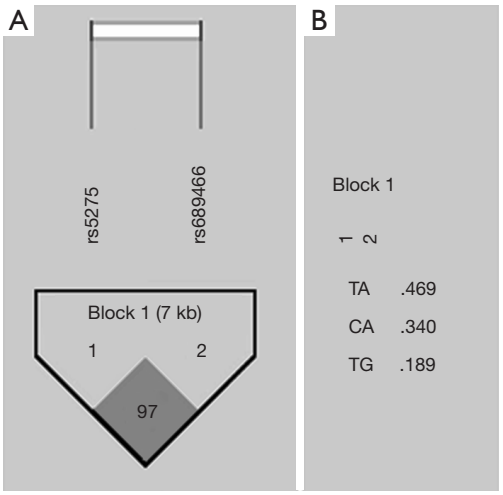

Figure 1 Schematic representation of the linkage disequilibrium structure between COX-2 gene polymorphisms. (A) Pattern of LD of two SNPs in the COX-2 gene; (B) haplotype blocks and haplotype estimated frequencies. LD, linkage disequilibrium; SNP, single nucleotide polymorphisms.

We determined the genotype frequencies of the CRC patients and control subjects. The results are summarized in *Table 3*. They were similar in both logistic regression models (P>0.05). The unadjusted model showed a trend to lower risk, but without statistical significance, for the 8473TT genotype (P=0.05).

We evaluated the association between alleles of the *COX-2* SNPs in cases and controls. No statistically significant differences were detected among groups (P>0.05). The same results were obtained with the haplotypes in both simple and multiple logistic regression models, as shown in *Table 4*.

Discussion

This is the first report of the impact of *COX-2* –1195A > G and 8473T > C polymorphisms in CRC risk among Brazilian population.

In the present study, the frequencies of the *COX-2* SNPs were consistent with HWE in the general population, except for the SNP 8473T > C in control group (P=0.02). Deviation of HWE may be caused by interbreeding, population stratification, natural selection or unknown errors of the genotype determination among others (16). The Brazilian population may have poor compatibility for genetic ancestry among study groups because it is

Table 3 Genotype distribution, frequencies and association test of the COX-2 SNPs in cases and controls

Genotype	Cases (n=230) n (%)	Controls (n=196) n (%)	OR (95% IC)[a]	P	OR (95% IC)[b]	P	Total n (%)
–1195A > G							
AA	146 (63.48)	135 (68.88)	1.00		1.00		281 (65.96)
AG	72 (31.30)	55 (28.06)	1.21 (0.79–1.84)	0.37	1.26 (0.80–1.99)	0.31	127 (29.81)
GG	12 (5.22)	6 (3.06)	1.85 (0.68–5.07)	0.23	2.23 (0.70–7.14)	0.18	18 (4.23)
AA/AG×GG	218 (94.78)	190 (96.94)	1.74 (0.64–4.74)	0.27	2.04 (0.65–6.43)	0.22	408 (95.77)
AA×AG/GG	84 (36.52)	61 (31.12)	1.27 (0.85–1.91)	0.24	1.34 (0.86–2.07)	0.17	145 (4.04)
+8437T > C							
TT	104 (45.22)	88 (44.90)	1.00		1.00		192 (45.07)
TC	102 (44.35)	75 (38.27)	1.15 (0.76–1.74)	0.50	1.26 (0.81–1.97)	0.31	177 (41.55)
CC	24 (10.43)	33 (16.84)	0.62 (0.34–1.12)	0.11	0.71 (0.37–1.36)	0.29	57 (13.38)
TT/TC×CC	206 (89.57)	163 (83.16)	0.58 (0.33–1.01)	0.05	0.63 (0.34–1.16)	0.14	369 (86.62)
TT×TC/CC	126 (54.78)	108 (55.10)	0.99 (0.67–1.45)	0.95	1.10 (0.73–1.67)	0.65	234 (54.93)

[a], Unadjusted logistic regression model; [b], multiple logistic regression model, adjusted for sex, age, ethnicity, education level, alcohol drinking and smoking habits. COX-2, Cyclooxygenase-2; OR, odds ratio; CI, confidence interval.

Table 4 Haplotype frequencies and association test of the *COX-2* SNPs between cases and controls

Haplotype		Cases (n=230) (%)	Controls (n=196) (%)	OR (95% CI)[a]	P	OR (95% CI)[b]	P	Total (%)
A	T	32.23	35.97	1.00		1.00		46.90
A	C	46.90	46.94	1.04 (0.70–1.57)	0.834	1.19 (0.77–1.84)	0.440	34.00
G	T	20.49	17.09	1.27 (0.83–1.94)	0.281	1.38 (0.87–2.19)	0.177	18.90
*	*	0.38	0.00	–	–	–	–	0.20
Total		100.00	100.00					100.00

[a], Unadjusted logistic regression model; [b], multiple logistic regression model, adjusted for sex, age, ethnicity, education level, alcohol drinking and smoking habits. COX-2, Cyclooxygenase-2; OR, odds ratio; CI, confidence interval.

originated from a combination of many ethnic groups (17). Nonetheless, there are no standard guidelines for rejecting SNPs that deviate from HWE (17).

We evaluated the association between alleles of the *COX-2* SNPs in cases and controls, as well as genotypes and haplotypes, and there was no statistically significant association to CRC risk. Functional studies have demonstrated the effect of the SNPs in major *loci* in the *COX-2* gene, and these may be related to increased levels of mRNA in CRC (18). The SNP –1195A > G lies in a region rich in recognition sites for nuclear transcription factors, that are critical in the transcription activation (12).

Our results are similar to others findings identified in the literature. Hoff *et al.* (19) found no significant association of the *COX-2* –1195A > G SNP with the risk of CRC in the Dutch population. Similar results were found in the Spanish and Danish population (20,21) and in two systematic reviews (22,23). In contrast, some researches detected significant association, but the results are conflicting (5,6). A case-control study in Jordan associated protective effect on the development of polyps and CRC in the presence of wild-type allele –1195A (24). Pereira *et al.* associated the G allele to predisposition to CRC increased in 1.73 fold (25). These results corroborate the findings of a functional study that identified overexpression of *COX-2* gene in cell lines carrying the G allele (26). However, Tan *et al.* (13) associated the AA and GA genotypes with increased predisposition of CRC (OR =1.77 and 1.24, respectively), and the same association was detected in a systematic review (6). Vogel *et al.*, showed that carriers of the G allele had a reduced risk of CRC (18). The allele creates a site for the transcription factor c-MYB, which would cause the increase of transcripts according to Zhang *et al.*(12).

The 8473T > C SNP may modify the binding affinity for regulatory factors and influence the mRNA stability and/or translational efficiency (14). This polymorphism was not associated to susceptibility to CRC in this study. Cox *et al.* (27) evaluated *COX-2* SNPs, including the 8473T > C, and did not detect association. The same results were observed in four European studies (18,20,21,25) and in two systematic reviews (22,23).

The divergent results in different population and tumor types suggest that the SNPs play a role in CRC, but are influenced by other molecular factors including functional polymorphisms not yet described, which can increase or neutralize the function effect of the SNPs *COX-2* –1195A > G and 8473T > C (12). Similarly there may be a cell-tissue-specific modulation, which could explain the different findings related to mechanism of action of the variants in gene and protein expression (28). Another possible explanation may be associated to interference of environmental factors and ethnic composition of the population, demonstrated by studies results in different countries and ethnic groups (29).

This study has some limitations. The number of cases was relatively small for this study design, which may result in decreased statistical power. SNPs were selected according to their frequency in Caucasian populations with more than 10% of polymorphic frequency to minimize the small sample size. Moreover, ethnicity was self-reported by the patient. The most appropriate method to determine this variable is through the characterization of informative autosomal markers of ancestry, which brings more specific information, especially in populations with a high degree of admixture, as in Brazil.

Therefore, under the conditions of this research, we concluded that the variants *COX-2* –1195A > G and 8473T > C do not participate in the genetic susceptibility to CRC in the Brazilian population.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: All participants signed an informed consent form, approved by the Ethics Committee for Research Projects Analysis of the University of São Paulo, School of Medicine (protocol nº0803/11).

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