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ORIGINAL ARTICLE

Basic Study

TLR2 and *TLR4* polymorphisms influence mRNA and protein expression in colorectal cancer

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Author contributions: Proença MA planned and conducted the study, collected and interpreted the data, drafted and wrote the manuscript; de Oliveira JG collected data on genotyping of *TLR2* and *TLR4* polymorphisms in the control group; Cadamuro ACT collected data on immunohistochemistry of both TLR2 and TLR4 proteins; Succi M collected data on CRC samples; Netinho JG collected data on CRC samples; Goloni-Bertolo EM and Pavarino ÉC planned the study; Silva AE conceived and planned the study and critically revised the manuscript.

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Abstract

AIM: To evaluate the effect of promoter region polymorphisms of toll-like receptor (*TLR*)*2-196* to *-174del* and *TLR4-1607T/C* (rs10759932) on mRNA and protein expression in tumor tissue and of *TLR4+896A/G* (rs4986790) on colorectal cancer (CRC) risk.

METHODS: The *TLR2-196* to *-174del* polymorphism was investigated using allele-specific polymerase chain reaction (PCR) and the *TLR4-1607T/C* and *TLR4+896A/G* by PCR-restriction fragment length polymorphism (RFLP). We genotyped 434 DNA samples from 194 CRC patients and 240 healthy individuals. The mRNA relative quantification (RQ) was performed in 40 tumor tissue samples by quantitative PCR TaqMan assay, using specific probes for *TLR2* and *TLR4* genes, and *ACTB* and *GAPDH* reference genes

were used as endogenous controls. Protein expression was analyzed by immunohistochemistry with specific primary antibodies.

RESULTS: No association was found for *TLR4-1607T/C* and *TLR4+896A/G* by three statistical models (log-additive, dominant and recessive). However, based on dominant and log-additive models, the polymorphic variant *TLR2-196* to *-174del* was associated with increased CRC risk [dominant: odds ratio (OR) = 1.72, 95%CI: 1.03-2.89; *P* = 0.038 and log-additive: OR =1.59, 95%CI: 1.02-2.48; *P* = 0.039]. *TLR2* mRNA expression was increased in tumor tissue (RQ = 2.36) when compared to adjacent normal tissue (RQ = 1; *P* < 0.0001), whereas the *TLR4* mRNA showed a basal expression (RQ = 0.74 *vs* RQ = 1, *P* = 0.452). Immunohistochemistry analysis of TLR2 and TLR4 protein expression was concordant with the findings of mRNA expression. In addition, the *TLR2-196* to *-174del* variant carriers showed mRNA relative expression 2.19 times higher than wild-genotype carriers. The TLR2 protein expression was also higher for the *TLR2-196* to *-174del* variant carriers [117 ± 10 arbitrary unit (a.u.) *vs* 95 ± 4 a.u., *P* = 0.03]. However, for the *TLR4 -1607T/C* polymorphism no significant difference was found for both mRNA (*P* = 0.56) and protein expression (*P* = 0.26).

CONCLUSION: Our findings suggest that *TLR2-196* to *-174del* polymorphism increases *TLR2* mRNA expression and is associated with higher CRC risk, indicating an important role in CRC genetic susceptibility.

Key words: Toll-like receptor 2; Toll-like receptor 4; Colorectal cancer; Protein expression; Gene expression; Genetic polymorphisms

Core tip: This study investigated the influence of the toll-like receptor (*TLR*)*2* and *TLR4* functional polymorphisms on mRNA and protein expression levels in colorectal cancer samples and the association of these polymorphisms with the risk of developing this neoplasm. Increased expression of TLR2 (mRNA and protein) in tumor tissue was observed compared with adjacent normal tissue. Moreover, for the first time, the polymorphism *TLR2-196* to *-174del* was associated with a higher risk of developing this type of cancer, and *TLR2-196* to *-174del* allele carriers showed mRNA relative expression approximately two times higher than wild-genotype carriers. Thus, functional polymorphism in *TLR2* may change gene expression levels, accentuating inflammation and aggravating the development of colorectal cancer.

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INTRODUCTION

Chronic inflammation has emerged as one of the main risk factors and features of cancer. It can affect any stage of tumorigenesis, generating a microenvironment conducive to tumor development and progression, and promoting the survival, proliferation and migration of cancer cells[1-3]. Thus, many cancers can arise from local irritation, inflammation and chronic infection[4,5]. The inflammatory process occurs through a network of chemical signals that initiate and maintain a host immune response in order to heal the affected tissue[4]. The activation of innate and adaptive immune responses is the main mechanism involved in the homeostasis alteration caused by tumors in adjacent tissues[6,7].

Colorectal cancer (CRC) is one of the main examples of the inflammation-cancer association[4]. Moreover, experimental models have provided evidence that innate immune system chemical mediators and bacterial toxins play key roles in CRC development[8,9]. CRC reports show a yearly worldwide incidence of approximately 1 million cases and a mortality rate of over 500000, representing the second or third leading cause of cancer-related death in many countries[10,11]. In 2012, the estimated incidence of CRC in Brazil was 16368 cases in men and 17581 cases in women, and 8549 men and 9058 women died of the disease[12]. Compared to the hereditary form, sporadic CRC (SCRC) is the most common type, accounting for more than 90% of cases.

As the intestine is under a constant inflammatory process due to the presence of microorganisms and their pathogen-associated molecular patterns (PAMPs)[13], changes in proteins or receptors involved in the inflammatory and immune responses may contribute to an increased risk of developing cancer[14]. In this respect, the toll-like receptor (*TLR*) family that encodes type I transmembrane proteins plays an essential role in pathogen recognition by the extracellular matrix[15], leading to activation of innate and adaptive immune responses and to a process of controlled inflammation[10,16]. The first step in the interaction of commensal bacteria with the intestinal epithelium is their recognition by TLR2 and TLR4 receptors, which recognize lipoproteins and PAMPs, and lipopolysaccharides (LPS) of Gram-negative bacteria, respectively[17,18]. TLRs activate the nuclear factor kappa B (NF-κB) pathway, the main regulatory inflammation signaling pathway, and this activation is involved in the pathogenesis of CRC[19,20]. Certain TLRs have been reported to play a role in bowel diseases, and it is believed that one of these roles is to induce

Table 1 Characteristics of patients with colorectal cancer and controls *n* (%)

Variables	CRC	Controls	OR (95%CI)	*P* value
Individuals, *n*	194	240		
Age (yr)			0.4513 (0.3063-0.6649)	
< 60	72 (37.1)	136 (56.7)		< 0.0001
⩾ 60	122 (62.9)	104 (43.3)		
mean ± SD	62 ± 12	56 ± 18		
Variation	24 to 88	20 to 93		
Gender			0.8619 (0.5898-1.259)	0.4988
Female	89 (45.9)	119 (49.6)		
Male	105 (54.1)	121 (50.4)		
Smoking habit			1.910 (1.299-2.808)	0.0013
Non-smokers	101 (52.1)	87 (36.3)		
Smokers	93 (47.9)	153 (63.7)		
Alcoholic habit			0.4963 (0.3305-0.7454)	0.0010
Non-alcoholic	114 (58.8)	178 (74.1)		
Alcoholics	80 (41.2)	62 (25.9)		

CRC: Colorectal cancer; OR: Odds ratio.

cell death by apoptosis of neoplastic cells[10].

Genes *TLR2* (4q32) and *TLR4* (9q33.1) are highly polymorphic, which may cause changes in protein expression or function[21], resulting in a differentiated inflammatory response that in turn can influence the progression of several cancer types, such as CRC[22-26].

Among the *TLR2* polymorphisms, a 22 bp deletion at position -196 to -174 of the promoter region appears to produce a reduction in gene transcription activity[21]. Variations in the *TLR2* gene have been associated with susceptibility to various infectious and inflammatory diseases[27] and some types of cancer associated with inflammation[26,28]. Similarly, the *TLR4* gene also shows polymorphisms located in the promoter region, such as *-1607T/C* (rs10759932), still poorly studied in association with cancer[29-31] and no reported studies on CRC. A *TLR4* polymorphism that has been largely studied and has received special attention consists of the substitution of an aspartic acid residue for glycine in amino acid 299 ($Asp^{299}Gly$) corresponding to *TLR4 +896A/G* polymorphism (rs4986790), but the results regarding its association with infectious diseases and cancer are still controversial[26,32-35]. However, alterations in *TLR4* expression levels have been reported to influence the innate immune response and to be potentially related to variation in the promoter sequence, with susceptibility to human diseases such as cancer[35].

Therefore, we aimed to evaluate whether common gene variants involved in the inflammatory response, such as *TLR2-196 to -174del* and *TLR4 -1607T/C* occurring in the promoter region influence gene expression in tumor tissue and whether these functional polymorphisms together with the *TLR4 +896A/G* were associated with colorectal cancer risk.

MATERIALS AND METHODS

Approval and consent

This study was approved by the Ethics in Research Committee IBILCE/UNESP, nº027/11 (protocol: 0009.0.229.000-11). All participants gave written informed consent, and the epidemiological data on the study population were collected using a standard interviewer-administered questionnaire, with questions on current and past occupation, smoking habits, alcohol intake and family history of cancer or adenomatous polyps and lesions.

Study populations

This case-control study comprised 434 individuals (Table 1). The case group (CRC) consisted of 194 samples from patients with a confirmed diagnosis of sporadic CRC by clinical histopathological parameters, 160 of which were studied based on samples of peripheral blood and 40 on samples of biopsies or surgical fragments (6 patients had both biopsy and blood samples) and normal adjacent mucosa (105 men and 89 women; mean age: 62 ± 12 years). All CRC samples were collected between December 2010 and August 2012 at the Cancer Institute (ICA) and the Proctology Service of the Base Hospital in São José do Rio Preto, SP, Brazil and all required information on clinical histopathological parameters was obtained from the patients' medical records. The inclusion criterion was as follows: patients with sporadic cancer and the exclusion criterion was patients with hereditary cancer. The control group (C) consisted of 240 healthy blood donors (121 men and 119 women; mean age: 56 ± 18 years), according to the criteria described in a previous study[26], whose DNA samples from leukocytes were stored in our laboratory.

With regard to risk factors, statistically significant differences were found between the group aged younger than 60 years and the group greater than or equal to 60 years, and between non-smokers and smokers and non-alcoholics and alcoholics. Gender was not significantly different between the groups, according to Fisher's exact test (Table 1).

Nucleic acid extraction

DNA was extracted from peripheral blood leukocytes of the CRC group according to the technique of Miller *et al*[36], with modifications (using Ficoll-Paque™ PLUS to separate the blood phases), and stored a -20 ℃ for subsequent genotyping. Simultaneous extraction of total RNA and DNA from tissue samples was performed, using the Trizol® reagent protocol.

Polymorphism genotyping

To investigate the *TLR2-196 to -174del* polymorphism,

Table 2 Polymerase chain reaction-restriction fragment length polymorphism conditions, primers sequences, restriction enzyme and fragment sizes

Polymorphisms	Primers (5'-3')	Enzymes T°/time	Fragment (bp)	Ref.
TLR2-196 to *-174del*	F: CACGGAGGCAGCGAGAAA	-	*ins*: 286	[59]
	R: CTGGGCCGTGCAAAGAAG		*del*: 264	
TLR4 +896A/G	F: AGCATACTTAGACTACCACCTCGATG	*Bst*XI	A: 131	[60]
(rs4986790)	R: GTTGCCATCCGAAATTATAAGAAAAG	37 ℃/3 h	G: 108; 23	
TLR4 -1607T/C	F: TTTGTATAATTTGACTACCATTGCGT	*Hha*I 37 ℃/3 h	T: 139	[30]
(rs10759932)	R: CATTTTTTCACATCTTCACCAGC		C: 117; 22	

ins: Wild-type allele (insertion); *del*: Polymorphic allele (deletion); bp: Base pairs; T°: Temperature; F: Forward; R: Reverse.

allele-specific PCR was performed, and polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) was used to assess the *TLR4* +896A/G and *TLR4* -1607T/C polymorphisms (Table 2). For both PCR techniques, the reaction solution contained the following: 1X buffer, 0.10 μmol/L of dNTPs, 0.5 μmol/L of MgCl2, 0.5 μmol/L of each primer, 1 U of Taq DNA polymerase, 13.3 μL of ultrapure Milli-Q H_2O, and 200 ng of genomic DNA. The material was processed in an automatic thermocycler and for *TLR4 +896A/G* and *TLR4 -1607T/C* the material was also subjected to enzymatic digestion. The amplification products of polymorphism *TLR2-196 to -174del* and the digestion products of *TLR4* polymorphisms *+896A/G* and *-1607T/C* were visualized on 3% agarose 1000 gel (Invitrogen®) stained with ethidium bromide in the presence of a 100 bp molecular marker. To ensure greater genotyping reliability, a positive control was included in all reactions, consisting of a sample that was heterozygous for the polymorphism under evaluation.

mRNA relative quantification by quantitative PCR

A reverse transcriptase reaction was performed using a High Capacity cDNA kit (Applied Biosystems). The cDNA was validated by PCR amplification of a 613 bp fragment of the *ACTB* gene (β-actin). A quantitative PCR (qPCR) reaction was performed by means of a TaqMan® gene expression assay (Applied Biosystems), using specific probes for genes *TLR2* (Hs_00610101m1 inventoried) and *TLR4* (Hs_01060206m1 inventoried). Both reference genes *ACTB* (Catalog#: 4352935E) and *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) (Catalog#: 4352934E) (Applied Biosystems) were used as endogenous controls in all analyses.

The reactions were performed in triplicate, using 25 ng of cDNA in StepOnePlus™ Real-Time PCR equipment (Applied Biosystems), and in all experiments a negative control was used to determine contamination. Relative quantification (RQ) was calculated using the $2^{-\Delta\Delta}$Ct method[37] compared to both reference genes, using four pools of normal adjacent tissue samples as a calibrator, grouped relating to polymorphism *TLR2-196 to -174del* and *TLR4 -1607T/C* genotypes, located in the promoter region (pool1 and pool4: ins/ins + T/T; pool2: T/C + C/C; pool3: ins/del).

RQ was also calculated for the samples stratified by polymorphism genotypes in the promoter region (*TLR2-196 to -174del* and *TLR4 -1607T/C*). They were grouped according to genotypes for each polymorphism separately (at least one polymorphic allele *vs* wild homozygote). The tumor tissue samples with wild genotype were used as a calibrator in comparison with those with at least one polymorphic allele.

Protein expression by immunohistochemistry

Immunohistochemical analysis was performed using a total of 20 tumor and normal adjacent samples. Consecutive 4 μm-thick sections were cut from each trimmed paraffin block. Deparaffinized tissue slides were submitted to antigen retrieval, using a high-temperature antigen-unmasking technique. The sections were incubated with specific primary antibodies: rabbit polyclonal antibody anti-TLR2 (06-1119, 1:50 dilution; Millipore) and mouse monoclonal anti-TLR4 (76B357.1, 1:200 dilution; Abcam). Next, the slides were incubated with biotinylated secondary antibody (Picture Max Polymer Detection Kit, Invitrogen) for 30 min, following the manufacturer's protocol. Immunostaining was carried out with 3,3'diaminobenzidine tetrahydrochloride containing 0.005% H_2O_2 and hematoxylin counterstain. Placental mucosa and appendix tissue were used as positive controls for proteins TLR2 and TLR4, respectively. Immunostaining was evaluated in the epithelial cytoplasm by densitometric analysis according to an arbitrary scale from 0 to 255 arbitrary unit (a.u.), performed with AxioVision software under a Zeiss-Axioskop Ⅱ light microscope. A total of 60 points equally distributed in each of the regions were scored, and values were expressed as mean ± SE.

Statistical analysis

SNPStats software was used to perform multiple logistic regression to evaluate the association of polymorphisms with CRC risk, including a log-additive model (major allele homozygotes *vs* heterozygotes *vs* minor allele homozygotes), a dominant model (major allele homozygotes *vs* heterozygotes + minor allele homozygotes), and a recessive model (major

Table 3 Allele and genotype frequencies of *TLR2* and *TLR4* polymorphisms and multiple logistic regression analysis between case and control groups *n* (%)

Polymorphisms	Statistical Models	Genotypes /alleles	C	CRC	*P* value
TLR2-196 to *-174del*			*n* = 240	*n* = 188	
		ins/ins	200 (83.0)	144 (77.0)	
		ins/del	36 (15.0)	39 (21.0)	
		del/del	4 (2.0)	5 (3.0)	
		ins	436 (0.9)	327 (0.9)	
		del	44 (0.1)	49 (0.1)	
	Dominant	*ins/ins*	200 (83.3)	144 (76.9)	0.038
		ins/del + del/del	40 (16.7)	44 (23.1)	
	OR (95%CI)		1.72(1.03-2.89)		
	Recessive	*ins/ins + ins/del*	236 (98.3)	183 (97.3)	0.360
		del/del	4 (1.7)	5 (2.7)	
	OR (95%CI)		1.90 (0.48-7.58)		
	Log-additive	*ins/ins*	200 (83.0)	144 (77.0)	0.039
		ins/del	36 (15.0)	39 (21.0)	
		del/del	4 (2.0)	5 (3.0)	
	OR (95%CI)		1.59 (1.02-2.48)		
TLR4 -1607T/C			*n* = 208	*n* = 190	
		T/T	166 (79.0)	154 (81.0)	
		T/C	39 (19.0)	33 (17.0)	
		C/C	3 (2.0)	3 (2.0)	
		T	371 (0.9)	341 (0.9)	
		C	45 (0.1)	39 (0.1)	
	Dominant	*T/T*	166 (79.0)	154 (81.0)	0.860
		T/C + C/C	42 (21.0)	36 (19.0)	
	OR (95%CI)		0.95 (0.56-1.63)		
	Recessive	*T/T + T/C*	205 (98.0)	187 (98.0)	0.940
		C/C	3 (2.0)	3 (2.0)	
	OR (95%CI)		0.93 (0.14-5.95)		
	Log-additive	*T/T*	166 (79.0)	154 (81.0)	0.860
		T/C	39 (19.0)	33 (17.0)	
		C/C	3 (2.0)	3 (2.0)	
	OR (95%CI)		0.96 (0.59-1.55)		
TLR4 +896A/G			*n* = 240	*n* = 190	
	Dominant	*A/A*	224 (93.3)	172 (90.5)	0.520
		A/G	16 (6.7)	18 (9.5)	
	OR (95%CI)		1.28 (0.60-2.73)		
		A	464 (0.97)	349 (0.95)	
		G	16 (0.03)	17 (0.05)	

The data are adjusted for age, gender, smoking and drinking status. CRC: Colorectal cancer; C: Controls; OR: Odds ratio.

allele homozygotes + heterozygotes *vs* minor allele homozygotes)[38]. Age, gender, smoking and drinking as covariates were adjusted to obtain statistical significance between groups for all polymorphisms evaluated. The GraphPad InStat (version 3.00) software was used to perform Fisher's exact test for assessing the effect of combined genotypes, and the HaploView software (version 4.0) was used to analyze the distribution of haplotype frequencies.

TLR2 and *TLR4* mRNA and protein expression analysis was performed using the GraphPad Prism software (version 6.01), and the gene expression analysis results were validated and confirmed by ExpressionSuite software (Life Technologies, version 1.0). RQ values were used for statistical analysis. For protein expression, the means of densitometry analysis of tumor and normal adjacent samples were compared. Continuous data distribution was evaluated using D'Agostino and Pearson's omnibus normality test. Student's *t*-test or correspondent nonparametric tests, such as the Mann-Whitney and Wilcoxon's signed rank test, were used for comparisons between groups. The Benjamini-Hochberg correction[39] was applied to the analysis. The probability level of $P \leqslant 0.05$ was considered statistically significant in all analyses.

RESULTS

TLR2 and TLR4 polymorphisms

The allele and genotype frequency distributions of polymorphisms *TLR2* and *TLR4* (Table 3) were consistent with the Hardy-Weinberg equilibrium in the control (C) group (data not shown).

TLR2-196 to -174del was associated with increased CRC risk by both the dominant [odds ratio (OR) = 1.72, 95%CI: 1.03-2.89; $P = 0.038$] and the log-additive models (OR = 1.59, 95%CI: 1.02-2.48; $P = 0.039$), while *TLR4 -1607T/C* and *TLR4 +896A/G* were not (Table 3). With regard to polymorphism *TLR4 +896A/G*, it was not detected in individuals with a homozygous

Table 4 Combined effect of polymorphisms *TLR2* -196 to -174 del, *TLR4* +896 A/G and *TLR4* -1607 T/C on the risk of colorectal carcinoma

Risk genotypes	C	CRC	OR (95%CI)	*P* value
	n = 240	*n* = 188		
None	104	136	1.00 (reference)	
TLR2 *ins/del* or *del/del*	4	3	0.98 (0.21-4.48)	1.000
TLR4 +896 *A/G*				
TLR2 *ins/del* or *del/del*	11	11	1.31 (0.54-3.13)	0.650
TLR4 -1607 *T/C* or *C/C*				
TLR4 +896 *A/G/*	2	2	1.31 (0.18-9.44)	1.000
TLR4 -1607 *T/C* or *C/C*				

CRC: Colorectal cancer; C: Controls; OR: Odds ratio.

Table 5 Haplotype frequency distribution of variants *-1607 T/C* and *+896 A/G* of *TLR4* gene in the case and control groups

Haplotypes[1]	C	CRC	χ^2	*P* value
***TLR4* -1607/+896**				
T-A	0.861	0.849	0.207	0.649
C-A	0.108	0.103	0.052	0.819
T-G	0.031	0.048	1.414	0.234

[1]C-G haplotype was not found. CRC: Colorectal cancer; C: Controls.

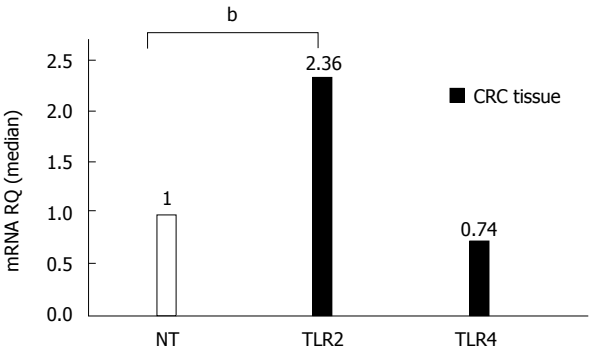

Figure 1 Distribution of the median mRNA relative quantification values of genes *TLR2* and *TLR4*. Using the Wilcoxon's signed rank test, a statistically significant difference was found for *TLR2* ([b]*P* < 0.0001), but not for *TLR4* (*P* = 0.452), when comparing the relative quantification (RQ) in colorectal cancer tissue (CRC) with adjacent normal tissue pools (NT). The reference genes *ACTB* and *GAPDH* were used as endogenous controls.

polymorphic genotype G/G in either group, precluding analysis by the three statistical models.

We intended to evaluate the combined effect of the three polymorphisms (*TLR2-196 to -174del*, *TLR4 +896A/G* and *TLR4 -1607T/C*) on the risk of CRC. However, no individuals with the combination of these three variant alleles were observed in our study subjects, and the combinations of two variant alleles showed no statistically significant difference between the control and the CRC group (Table 4). To further investigate the polymorphisms in gene *TLR4*, we also performed a haplotype analysis of polymorphisms *-1607T/C* and *+896A/G* (Table 5). Haplotype CG was not found, and the other haplotypes showed no statistical difference in the frequencies of allele combinations between the CRC and C groups.

Table 6 *TLR2* and *TLR4* mRNA relative quantification values in colorectal carcinoma, stratified according to wild and polymorphic genotype

	TLR2 -196 to -174 del		*TLR4 -1607 T/C*	
	ins/ins	*ins/del*[2]	*T/T*	*T/C + C/C*
n (%)[1]	27 (73.0)	10 (27.0)	31 (83.8)	6 (16.2)
Median	3.57	6.95	0.75	0.86
Range	0.49-13.59	0.62-14.69	0.20-23.89	0.47-2.27
P value[3]	0.035		1.000	

[1]3 tumor tissue samples which were not genotyped were excluded from the analysis; [2]No individuals with a del/del genotype in tumor tissue samples were found; [3]Results of the nonparametric Mann-Whitney *U* test. *ins*: Wild-type allele (insertion); *del*: Polymorphic allele (deletion).

mRNA and protein expression

We observed significantly increased *TLR2* relative gene expression in tumor tissue (RQ = 2.36) compared to adjacent normal tissue (RQ = 1; *P* <0.0001) (Figure 1). With regard to the *TLR4* gene, however, we did not find statistically significant differences between the relative expression in normal and tumor tissue that showed basal relative gene expression (RQ = 0.74, *P* =0.452) (Figure 1).

In the immunohistochemical analyses of TLR2 and TLR4 protein expression, we considered only the epithelial cytoplasm. In normal adjacent tissues, this analysis showed weak or moderate cytoplasm staining in the epithelium for both proteins (Figure 2A and D). In tumor tissue, although a strong cytoplasm immunostaining pattern in the epithelium was found for the TLR2 protein (Figure 2B), the TLR4 protein showed weak or moderate expression (Figure 2E). The mean optical densitometry values for TLR2 in tumor tissue (154 ± 5 a.u.) were statistically higher than those in normal adjacent tissues (109 ± 6 a.u., *P* < 0.0001; Figure 2C), but no difference was found for TLR4 (CRC = 123 ± 4 a.u, NT=111 ± 6 a.u, *P* = 0.117; Figure 2F). Thus, these results are concordant with the findings of mRNA expression.

Stratification of functional polymorphisms and influence on gene and protein expression

In order to evaluate the influence of the functional polymorphisms *TLR2-196* to *-174del* and *TLR4 -1607T/C* on mRNA and protein expression, the samples were grouped according to genotypes, considering the presence of at least one polymorphic allele compared to wild genotype samples (Table 6). For the mRNA expression analysis, the wild genotype group was used as a calibrator (RQ = 1) to calculate the relative gene expression. Individuals with at least one polymorphic allele *TLR2-196 to -174del* had more than two times higher *TLR2* expression in tumor tissue (RQ = 2.19; *P* = 0.03) compared to those with the wild

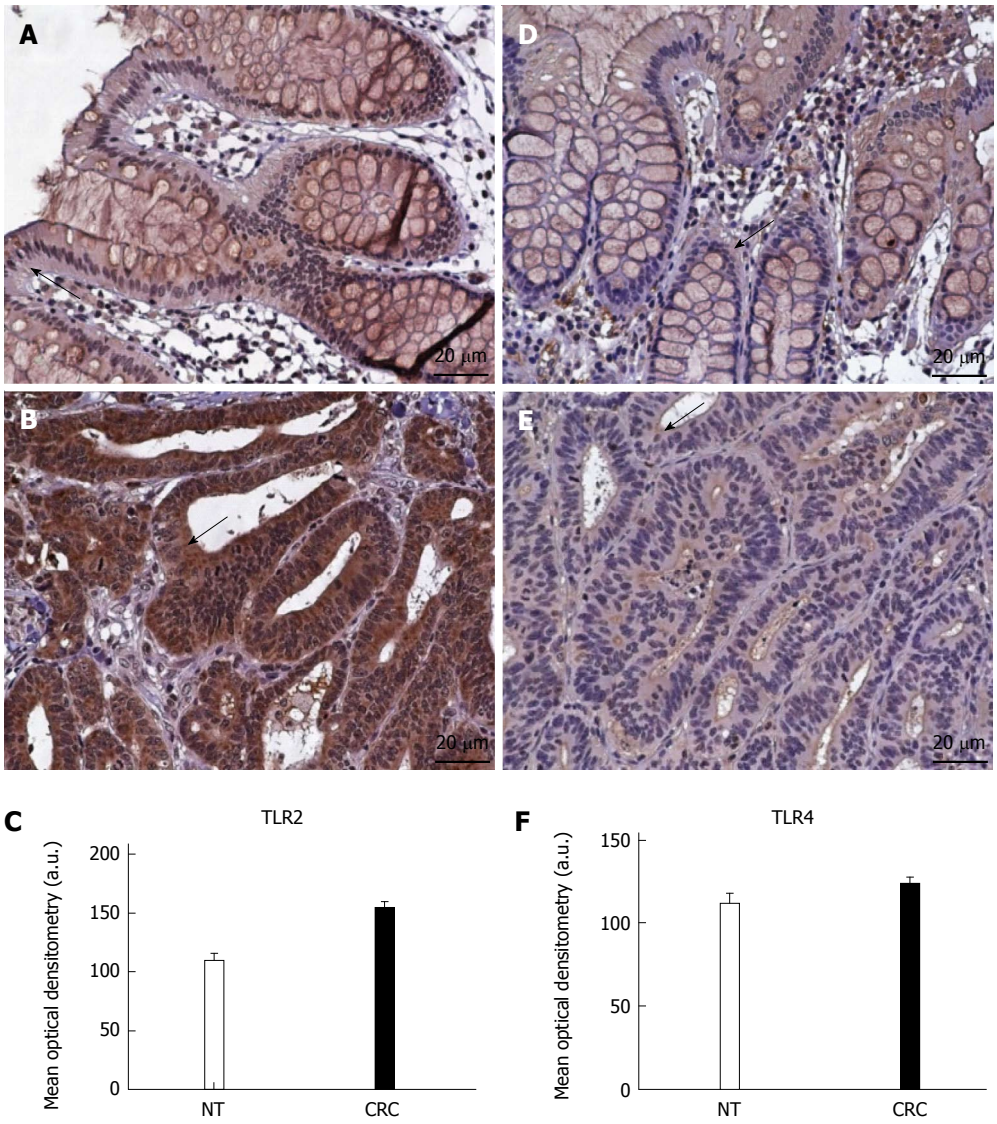

Figure 2 Toll-like receptor 2 and toll-like receptor 4 protein expression in intestinal mucosa (cytoplasm staining). Moderate expression of TLR2 in normal adjacent mucosa (NT), predominantly in epithelial cells (arrowhead) (A); compared with intense immunostaining in colorectal cancer (B); Low expression of TLR4 in normal adjacent mucosa, predominantly in the epithelium (arrowhead) (D); Moderate TLR4 immunostaining in the epithelial cells (arrowhead) of colorectal cancer (E); Harris' Hematoxylin counterstain. Bar: 20 μm. Densitometry analyses (mean ± SE) (C and F); $P < 0.001$. All images are from the same patient. a.u.: Arbitrary unit; CRC: Colorectal cancer.

genotype (Figure 3A). However, for the *TLR4 -1607T/C* polymorphism, this analysis showed no statistically significant difference in gene expression between individuals with the polymorphic allele (RQ = 0.98; $P = 0.56$) and those with the wild allele in homozygosis (Figure 3B).

A similar result was observed when protein expression was compared according to genotypes. Individuals carrying the polymorphic genotype had higher protein expression (117 ± 10 a.u. *vs* 95 ± 4 a.u., $P = 0.03$) than those carrying the wild homozygous genotype for the *TLR2-196 to -174del* polymorphism (Figure 4). However, for the *TLR4 -1607T/C* polymorphism no significant difference was found (139 ± 10 a.u. *vs* 128 ± 4 a.u., $P = 0.26$) (Figure 4).

DISCUSSION

To the best of our knowledge, this is the first report of an evaluation of Toll-like receptor polymorphisms *TLR2-196* to *-174del*, *TLR4 -1607T/C* and *TLR4 +896A/G* in a group of patients with sporadic CRC, which showed that the presence of polymorphic allele *TLR2-196 to -174del* is associated with increased risk of developing this type of cancer, and influences mRNA and protein expression in tumor tissue of patients with CRC compared to those with wild genotype. However, no change in *TLR4* mRNA and protein expression or an association with polymorphisms *TLR4 -1607T/C* and *+896A/G* were found.

The association between CRC risk and the polymor-

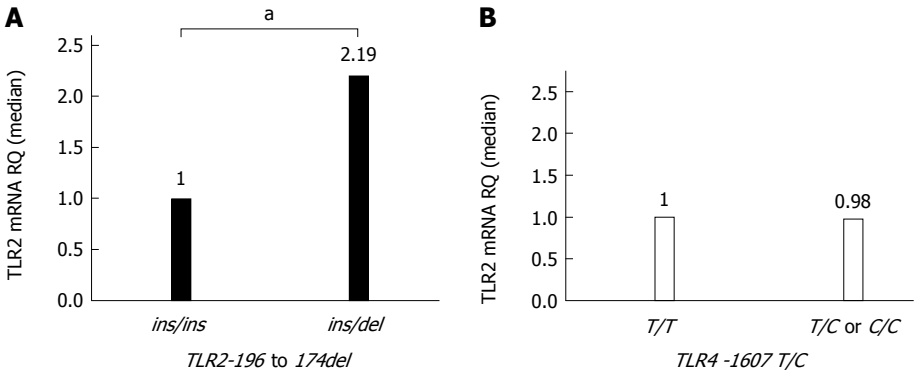

Figure 3 Toll-like receptor 2 (A) and toll-like receptor 4 (B) mRNA relative quantification, using tumor tissue samples from a wild-genotype colorectal cancer group as a calibrator, compared to those with at least one polymorphic allele. Using Wilcoxon's signed rank test, a statistically significant difference was found for the gene expression of *TLR2* (^{a}P = 0.037), but not for *TLR4* (P = 1.000). The reference genes *ACTB* and *GAPDH* were used as endogenous controls. CRC: Colorectal cancer.

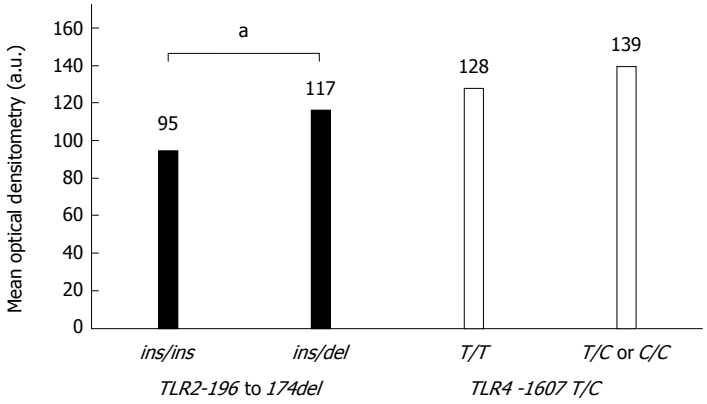

Figure 4 Densitometry values of immunohistochemistry analysis for toll-like receptor 2 and toll-like receptor 4 proteins, stratified according to polymorphic and wild genotypes. Carriers of the polymorphic genotype del/del for *TLR2-196* to *-174del* polymorphism showed higher protein expression than those with homozygous wild genotype ins/ins (^{a}P = 0.03). No statistical difference was found for the values of TLR4 protein. *ins:* Wild-type allele (insertion); *del:* Polymorphic allele (deletion).

phic variant *TLR2-196 to -174del* was demonstrated by both the dominant and the log-additive statistical models. Other studies showed an association between the *TLR2-196* to *-174del* variant and breast cancer in the Greek population[40], with gastric cancer in the Brazilian[26] and the Chinese[41] populations, and with bladder[42], prostate[43] and cervical cancer[44] in a northern Indian population. However, no association between gastric cancer was found in the Japanese population[45], nor did we find any studies reporting the presence of this polymorphism in CRC.

In the present study, polymorphisms *TLR4 +896A/G* and *TLR4 -1607T/C* were not associated with susceptibility to CRC. Studies on the functional polymorphism *TLR4-1607T/C* and cancer are scarce. This polymorphism has been described as relatively common, with a frequency of over 5% for the polymorphic allele C[30], as found in the present study (10% in both groups). This polymorphism was associated with a protective effect for gastric[30] and prostate[31] cancer. In contrast, a positive association was found for prostate cancer risk in a North American population[29].

The *TLR4 +896A/G* variant is located in a coding region and causes a substitution of the amino acid 299 glycine for asparagine (Asp^{299}Gly). Some studies have indicated that this variant is associated with a change in the extracellular domain structure of the TLR4 receptor and suggest that the polymorphic allele G is associated with an attenuated immune response to LPS and lower secretion levels of pro-inflammatory cytokines[46-48], justifying, to a certain extent, the lack of association between this polymorphism and the risk of CRC.

Our study found a low frequency of the *TLR4 +896 G* allele and no homozygous G/G subjects were detected in either the CRC or the control group. The rarity of this genotype has also been observed in other populations, such as those of Croatia[22], northern India[44] and Greece[40]. In line with our results, other studies have also reported absence of the polymorphic homozygous G/G genotype, such as one conducted in Spain[45] and a previous study from our laboratory in the Brazilian population[26].

A lack of association with the *TLR4 +896A/G* polymorphism has also been reported in CRC by some studies conducted in Spanish[49] and Chinese[50] populations, in addition to other types of cancer,

such as cervical in northern India[44] and prostate in Sweden[51]. This lack of association may be due to this polymorphism having no effect, or only a reduced effect, on the biological development of CRC, therefore unnoticeable in the analyses of these samples[49]. Thus, due to the low frequency of the G variant and the rarity of G/G homozygotes, much larger samples may be needed to allow a more robust conclusion on the association of this polymorphism with the development of cancer, although other authors did find an association between this variant and CRC in populations of Croatia[22] and Europe[52]. In another, recently completed study by our research group, although no homozygous G/G subjects were detected, an association between *TLR4 +896A/G* and the risk of gastric cancer and chronic gastritis was observed[26].

An evaluation of the combined effect of the three polymorphisms (*TLR2-196* to *-174del*, *TLR4 +896A/G* and *TLR4 -1607T/C*) on CRC risk showed that none of the combinations of two variant alleles produced any significant differences between the CRC and control groups, which suggests that these combinations do not affect the risk of developing CRC. However, the above-mentioned recent study conducted by our research group showed that the combination of genotypes *TLR2-196 to -174 ins/del* and *del/del* with *TLR4 +896A/G* leads to a higher risk of developing gastric cancer[26].

In addition, haplotype analysis of *TLR4* polymorphisms *-1607T/C* and *+896A/G* showed no statistically significant differences in the distribution of allele combination frequencies between the CRC and control groups, suggesting that the possible formation of haplotypes with these gene polymorphisms does not affect CRC risk. In line with our findings, other studies also failed to find an association between *TLR4* gene haplotypes and either CRC risk[53], or chronic gastritis and intestinal metaplasia[54]. However, an association between other *TLR4* gene haplotypes with some types of cancer, such as *TLR4 G-C* (^{299}Gly-^{399}Thr) with increased gastric cancer risk[26], and Asp^{299}-Ileu^{399} with increased gastritis and precancerous lesions risk[34] were reported.

The mRNA relative expression analysis of genes *TLR2* and *TLR4* in tumor and adjacent normal tissues from patients with CRC showed a 2.36-fold increased gene expression of *TLR2* in tumor tissue, unlike *TLR4* which showed basal relative gene expression. Furthermore, in line with this result, the immunohistochemistry assay showed that only the TLR2 protein was overexpressed in tumor tissue, demonstrating a change in the expression of this receptor in CRC.

The TLR2 receptor operates in the recognition of microorganisms in the intestinal mucosa, leading to their activation and triggering an inflammatory process in the organ microenvironment. Therefore, TLR2 overexpression may cause a more accentuated inflammatory response, recruiting MyD88 for the TLR/TIR domain and thereby inducing pro-inflammatory cytokine production by a classical signaling pathway. The IKK protein is activated in a process that involves IRAK-1 and TRAF6. The IKK complex catalyzes IkB phosphorylation and degradation by the proteasome, allowing NF-κB displacement to the nucleus. In the nucleus, NF-κB regulates pro-inflammatory cytokine expression and molecule adhesion[55], thus facilitating tumor progression.

High *TLR2* expression in tumor tissue of patients with CRC has been reported, while no difference was found regarding *TLR4* expression[10,16]. In gastric cancer, increased expression of *TLR2,* but not of *TLR4* mRNA[56] was also reported, and was also associated with *Helicobacter pylori* infection[57].

When the mRNA and protein expression of TLR2 and TLR4 from CRC patients were stratified according to genotypes (wild and carrying at least one variant allele), it became clear that in the subgroup of patients with at least one polymorphic allele of *TLR2-196* to *-174del*, the expression of TLR2 mRNA and protein was significantly higher than that of wild genotype carriers.

In contrast, another study conducted in a Japanese population, found that the polymorphic genotype *del/del* decreases the transactivation of responsive promoters, causing a decrease in gene transcription and thus a decrease in gene expression[21]. However, considering the important role of this gene in the induction of inflammatory processes and the association between polymorphism *TLR2-196* to *-174del* and several types of cancer, taken together these data suggest that this deletion must increase the level of gene transcription in tumor tissue, enhancing the inflammatory process and favoring cancer progression. Therefore, these are novel findings, as they indicate for the first time the influence of the *TLR2-196* to *-174del* polymorphism on increased expression of *TLR2* gene in CRC. Another study has reported the importance of polymorphisms on gene expression levels in ulcerative colitis[58].

Unlike *TLR4 -1607T/C* polymorphism, we did not find any influence of the polymorphic allele *C* on relative gene expression in CRC tumor tissue. This polymorphism was shown by a functional luciferase expression assay not to influence gene transcription[35]. This result confirms the lack of association between this functional polymorphism and CRC risk, indicating that its presence does not cause significant changes in gene transcription.

Considering that in most cases CRC has a good prognosis and is treatable when diagnosed at an early stage, it is of the utmost importance to establish molecular markers capable of identifying risk groups and providing early diagnosis in individuals with increased risk of developing this neoplasm. Overall, our results indicate that the *TLR2* gene plays an important role in colorectal carcinogenesis, highlighting the importance of the *TLR2-196* to *-174del* polymorphism in increasing gene expression and possibly triggering a stronger inflammatory response, which in turn enhances the risk of tumor progression.

In conclusion, *TLR2* mRNA and protein expression are increased in CRC tissue. In addition, the functional polymorphism *TLR2-196* to *-174del* influences mRNA and protein expression in tumor tissue of patients with CRC promoting its increase in relation to wild-type genotype carriers, thus emphasizing their important role in colorectal carcinogenesis. Furthermore, the polymorphic variant *TLR2-196* to *-174del* is associated with increased CRC risk and may contribute to the identification of CRC risk groups. However, there is no evidence of an association between CRC and *TLR4 -1607T/C* and *TLR4 +896A/G* polymorphisms or of these polymorphisms on *TLR4* gene expression. In future studies, these genes should be evaluated in other intestinal diseases, such as in precancerous lesions, as well in other populations to better understand their importance and function in colorectal carcinogenesis.

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COMMENTS

Background

Colorectal cancer is one of the main inflammation-cancer association models, therefore it is interesting to evaluate polymorphisms in genes related to the inflammatory process which are associated with the development of some cancers, such as toll-like receptors (TLRs). *TLR2* and *TLR4* genes have promoter region polymorphisms which may change the levels of gene transcription. However, only a few studies have investigated the influence of functional polymorphisms on changes in mRNA or protein expression levels in colorectal cancer and their role in carcinogenesis.

Research frontiers

Epidemiological studies on the association between polymorphisms and susceptibility to disease such as cancer frequently present conflicting results. However, together with other analyses such as gene and protein expression this may help clarify their effects on gene regulation at the transcription level.

Innovations and breakthroughs

This study showed, for the first time, that the presence of polymorphic allele *TLR2-196* to *-174del* is associated with an increased risk of developing colorectal cancer, and this polymorphism enhances mRNA and protein expression in tumor tissue from patients with colorectal cancer compared to those with the wild genotype.

Applications

The data show that carriers of the *TLR2-196* to *-174del* polymorphism constitute a risk group for the development of colorectal cancer. Thus, considering the high incidence of this cancer in the Brazilian population, an understanding of the mechanisms involved in activation of the immune and inflammatory response mediated by these receptors is important in the development of preventive and therapeutic strategies for this neoplasm.

Terminology

TLR2: toll-like receptor 2, lipoprotein bacterial receptor, gene located on chromosome 4; TLR4: toll-like receptor 4, lipopolysaccharide receptor, gene mapped on chromosome 9.

Peer-review

The study explores the role of TRL2 and TRL4 polymorphism and colorectal cancer risk. The article is meaningful and well written.

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