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Relationship between Arg753Gln Toll-like receptor 2 and Asp299Gly Toll-like receptor 4 genetic variations and susceptibility to colorectal cancer in southern Iran

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Abstract: Variations in Toll-like receptor 2 (*TLR2*) and Toll-like receptor 4 (*TLR*4) encoding genes have been associated with tumorigenesis through the disruption of immune and inflammatory responses. The aims of this study were to evaluate the two single nucleotide polymorphisms (SNPs) of the genes Arg753Gln *TLR2* (rs5743708) and Asp299Gly *TLR4* (rs4986790) in colorectal cancer patients in southern Iran. Colorectal cancer patients and healthy controls were included in this study (150 Persian subjects in each group). Blood samples were used for genotyping by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The association between these SNPs and colorectal cancer and clinicopathological factors, including age, gender, tumor stage and differentiation were also investigated. A significant association was found between Arg753Gln *TLR2* SNP and colorectal cancer. This SNP was significantly more frequent in male patients. However, there was no association between Asp299Gly *TLR4* and colorectal cancer. Therefore, Arg753Gln *TLR2* SNP can be considered as a risk factor for colorectal cancer incidence in southern Iran, especially in men. Further investigations in other populations are recommended in order to assess the association of this SNP with colorectal cancer.

Key words: colorectal cancer; polymorphism; *TLR*2; *TLR4;* SNP

INTRODUCTION

Colorectal carcinoma is a major cause of cancer-associated morbidity and mortality worldwide [1]. Despite the lower incidence of colorectal cancer in Asian countries in the past, recent studies point to its growing incidence in countries such as Iran [2-4]. According to annual reports, this type of cancer is the fifth and the third most common cancer in Iranian men and women, respectively [5,6]. It seems that the accumulation of genetic mutations and environmental factors along with lifestyle changes are the main causes of the elevated colorectal cancer rate [2,7]. A massive population of microorganisms (~10^{14}) inhabits the human large intestine and evidence indicates that chronic inflammation is an important factor in the development of tumors in the large intestine [8]. Toll like receptors (*TLRs*) belong to the family of pattern-recognition receptors. They are expressed on immune cells, and they have a critical role in the identification of microbial components. These receptors maintain the mucosal immune system and intestinal microbial balanced through regulation of the gut barrier function and inflammation [9]. *TLR2* and *TLR4* are two key receptors from this family. Pathogen-associated molecular patterns (PAMP) from Gram-positive bacteria and lipopolysaccharide (LPS) from Gram-negative bacteria are recognized by *TLR2* and *TLR4*, respectively [10]. Evidence suggests that *TLR2* and *TLR4* gene polymorphisms lead to dysregulation of *TLR2*- and *TLR4*-dependent signaling pathways. These disturbed pathways result in the disruption of the immune and inflammatory responses and consequently provide a microenvironment favoring tumorigenesis [10-12]. Arg753Gln *TLR2* (rs5743708) and Asp299Gly *TLR4* single nucleotide polymorphisms (SNPs) of *TLR* genes have been linked to chronic inflammation-related cancers such as gastric and

prostate cancers [13-17]. A meta-analytical study confirmed the association between *TLR4* Asp299Gly SNP and an increased risk of gastric cancer and decreased risk of prostate cancer [18].

The association between Arg753Gln *TLR2* (rs5743708) and Asp299Gly *TLR4* (rs4986790) SNPs and the risk of colorectal cancer have been investigated in different populations. While some studies found associations between these two SNPs and colorectal cancer, [10,19,20] others found no such associations [8,21,22]. Due to the limited and contradictory results in different populations, it seems that more studies are needed to examine the possible association of Arg753Gln *TLR2* and Asp299Gly *TLR4* SNPs with colorectal cancer. As far as we know, no study has been conducted to evaluate the association of these SNPs and colorectal cancer in the Iranian population. Therefore, the aim of this study was to investigate the Arg753Gln *TLR2* and Asp299Gly *TLR4* SNPs in colorectal cancer patients and normal population in southern Iran. The potential association of these SNPs and the risk of colorectal cancer and some clinicopathological factors such as age, gender, tumor stage and tumor differentiation were evaluated, as well.

MATERIALS AND METHODS

Study population and blood samples

The ethical approval of the Ethics Committee of Shiraz University of Medical Sciences was obtained prior to conducting this study. All study participants signed informed consents. Colorectal cancer patients at the university hospital in Shiraz (Shahid Faghihi Hospital) and age- and gender-matched healthy controls from the same geographic area were included in the study (150 Persian subjects in each group). Both groups comprised 81 males (54%) and 69 females (46%) (p=1). Subjects were more likely to be younger than 60 years in both groups (61.7% of patients and 70% of controls), and the remaining were 60 years or older (38.3% of patients and 30% of controls) (p=0.14). Available demographic and clinical characteristics of healthy controls and colorectal cancer patients, including age, gender, tumor stage (I, II, III, IV) and tumor differentiation (well, moderate, poor) were obtained from the subjects' files (Table 3).

Blood sampling, DNA extraction and genotyping

Blood samples were collected in EDTA-anticoagulant tubes. Standard proteinase K salting out was applied for the extraction of total genomic DNA. After DNA quantification using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA), the DNA samples were stored at -20°C. *TLR2* Arg753Gln (rs5743708) and *TLR4* Asp299Gly (rs4986790) genotyping was carried out by PCR-RFLP. Details about the sequences of the primers (SinaClon Co., Iran), enzymes (MBI Fermentas, Vilnius, Lithuania) and PCR products are summarized in Table 1. Digested products were stained with GelRed (Biotium, Belgium) and electrophoresed. Visualization was performed under UV illumination.

Statistical analysis

SPSS software package (version 18; Chicago, IL, USA) was used for data analysis. Given that the variables were qualitative, a Chi-square test was applied to determine possible differences related to *TLR2* and *TLR4* SNPs and demographic and clinical characteristics in the case and control groups. The statistical significance was considered when two-tailed p was less than 0.05.

RESULTS

Two missense single nucleotide polymorphisms of *TLR2* Arg753Gln (rs5743708) and *TLR4* Asp299Gly (rs4986790) were investigated in 150 colorectal cancer patients and 150 healthy controls. Genotype and allele frequencies in the case and control groups are summarized in Table 2. The subjects were evaluated using the Hardy-Weinberg exact test, with both case and control groups in agreement with the exact test (p>0.05). In the case-control comparisons, the distribution of *TLR2* Arg753Gln genotype was significantly different (p=0.03) (Table 2). However, *TLR4* Asp299Gly variation was not detected in any patients or controls. The potential association between *TLR2* Arg753Gln and some demographic and clinical characteristics including age, gender, tumor stage and tumor differentiation were evaluated in colorectal cancer patients. As described in Table 3, no significant differences were

Table 1. Primers and enzymes used for PCR-RFLP and their products

Enzyme recognition site	PCR product	Primer sequences	Digestion product
PstI 5′C TGCAG↓3′ 3′ G↑ACGT C5′	337 bp	***TLR2* Arg753Gln (rs5743708)** Forward: 5′- AACTGTCTTTGTGCTTTCTG-3′ Reverse: 5′- TGTCTGAATGAACTTAACTATAACTA-3′	Wild type allele G: One fragment (337 bp) Mutant allele A: Two fragments (165 and 172 bp)
NcoI 5′C↓CATG G3′ 3′G GTAC↑C5′	229 bp	***TLR4* Asp299Gly (rs4986790)** Forward: 5′- AGCATACTTAGACTACTACCTC**C**ATG-3′* Reverse: 5′- AGCATTCCCACCTTTGTTG-3′	Wild type allele A: One fragment (229 bp) Mutant allele G: Two fragments (207 and 22 bp)

* The fourth nucleotide from the 3' side of the TLR4 Asp299Gly forward primer was modified from G to C in order to create the *NcoI* enzyme recognition site.

Table 2. *TLR2* and *TLR4* genotypes and allele frequencies in colorectal cancer patients and healthy controls.

Groups	*TLR2* Arg753Gln						*TLR4* Asp299Gly					
	Genotype				Allele		Genotype				Allele	
	GG	AG	AA	p	G	A	AA	AG	GG	p	A	G
patients N=150, n (%)	143 (95.3)	1 (0.7)	6 (4)	0.03	287 (95.7)	13 (4.3)	150 (100)	0 (0)	0 (0)	*	150 (100)	0 (0)
Controls N=150, n (%)	150 (100)	0 (0)	0 (0)		150 (100)	0 (0)	150 (100)	0 (0)	0 (0)		150 (100)	0 (0)

* The genotype of all cases and controls was wild-type homozygote (AA)

Table 3. *TLR2* Arg753Gln SNP and demographic and clinical characteristics of colorectal cancer patients.

Parameters	Arg753Gln			
	GG	AG	AA	p
Gender				**0.03**
Patients				
Male	74 (91.4)	1 (1.2)	6 (7.4)	
Female	69 (100)	0 (0)	0 (0)	
Controls				
Male	81(100)	0 (0)	0 (0)	-
Female	69(100)	0 (0)	0 (0)	
Age				
Patients				
<60	87 (94.6)	1 (1.1)	4 (4.3)	1.0
≥60	55 (96.5)	0 (0)	2 (3.5)	
Controls				
<60	105 (100)	0 (0)	0 (0)	-
≥60	45 (100)	0 (0)	0 (0)	
Tumor stage				
I	37 (100)	0 (0)	0 (0)	
II	55 (96.4)	1 (1.8)	1 (1.8)	0.11
III	36 (92.3)	0 (0)	3 (7.7)	
IV	15 (88.2)	0 (0)	2 (11.8)	
Tumor Differentiation				
Well	93 (95.9)	0 (0)	4 (4.1)	
Moderate	37 (94.9)	1 (2.6)	1 (2.6)	0.37
Poor	13 (92.9)	0 (0)	1 (7.1)	

Data are given as *n* (%)

found in the frequencies of *TLR2* Arg753Gln based on age, tumor stage and tumor differentiation. However, the distribution of this genetic variation was significantly different based on gender (p=0.03). The homozygote wild-type genotype (GG) was detected in all the female patients: genotypes with at least one mutant allele (AG/AA) were found in 7 male patients.

DISCUSSION

Available reports in different populations pointed to the association of *TLR* genetic variations with tumor initiation or progression. Due to the lack of prior studies in the Iranian population, we evaluated two *TLR* SNPs (*TLR2* Arg753Gln, *TLR4* Asp299Gly) in colorectal cancer patients and healthy controls in southern Iran. Our results showed a significant association between *TLR2* Arg753Gln and colorectal cancer. No difference was found between patients and controls based on *TLR4* Asp299Gly SNP. Similarly, there was no association between *TLR4* Asp299Gly SNP and colorectal cancer in Malaysia [8], Saudi Arabia [21], Spain [22], China [23], India [24], and Tunisia [25]. In

contrast, Pedro Pimentel-Nunes et al. [10] in Portugal, described a 3-fold elevated risk of colorectal cancer in a Caucasian population who were carriers of mutant homozygote *TLR4* 299Gly. They also found no significant association between *TLR2* Arg753Gln SNP and risk of colorectal cancer. Jelavić et al. [19] presented similar results to Pedro Pimentel-Nunes et al. in a Croatian population. Kutikhin et al. [20] also found a higher risk of rectal cancer in a Russian population with *TLR4* Asp299Gly SNP. Our results are consistent with Guo's [23] study, in which no *TLR4* Asp299Gly variation in any colorectal cancer patients and normal controls in the Chinese Han population was detected. It appears that the frequency of *TLR4* Asp299Gly SNP is very low in the Iranian and Chinese populations. The functional consequence of *TLR2* Arg753Gln SNP seems to be colorectal tumorigenesis as a result of intestinal microbial imbalance and dysfunction of the immune and inflammatory responses. Nihon-Yanagi et al. [26] showed that *TLR2* mRNA expression was significantly higher in cancerous colorectal tissue than in normal tissue; however, *TLR4* mRNA expression was not different. In our study, all of the patients with at least one mutant allele (AG/AA) were male. It appears that this SNP can be considered as a potential risk factor for colorectal cancer incidence in southern Iran, especially in males. Available evidence indicates that there are also differences in the expression of *TLR2* in men and women. The evaluation of *TLR2* and *TLR4* expression in gastric cancer patients revealed significantly higher expression of *TLR2* but not of *TLR4* in women than in men before surgery [27]. In another study, Li et al. [28] showed that *TLR2* knockout only in male mice decreased post-ischemic myocardial inflammatory response resulting in improved cardiac function. Roberts et al. [29] also showed that *TLR2*- and *TLR4*-signaling pathways suppress T regulatory cells in male but not female mice, which was associated with elevated susceptibility to myocarditis in males.

CONCLUSION

TLR2 Arg753Gln SNP but not *TLR4* Asp299Gly SNP was significantly associated with colorectal cancer in southern Iran. Further studies will be required to consider this genetic variation as a biomarker for risk assessment of colorectal cancer in other populations.

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