

Association between *TLR-9* polymorphisms and colon cancer susceptibility in Saudi Arabian female patients

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Objective: The authors aimed to explore the relationship between the expression/polymorphisms of *TLR-9* and susceptibility to colon cancer development in the Saudi Arabian population.

Methods: In total, blood samples from 115 patients with colon cancer and 102 participants without colon cancer were analyzed in this study. Three single-nucleotide polymorphisms (SNPs) were selected from the *TLR-9* gene, including two sites within the *TLR-9* gene's promoter region (rs352144 and rs187084) and one site in a *TLR-9* intron region (rs5743839). Odds ratios (ORs) and 95% confidence intervals (CIs) were computed from logistic regression models after adjusting for age, gender, and tumor localization. To investigate the differential expression of *TLR-9* in colon cancer, *TLR-9* expression was evaluated using quantitative real-time reverse transcription polymerase chain reaction on 40 matched normal and colon tissues.

Results: The authors found that *TLR-9* expression was decreased in colon cancer tissues as compared with that in normal tissues. Moreover, significant associations between the *TLR-9* rs187084 SNP and colon cancer risk were observed in female patients only. In rs187084, the T allele had a significantly lower frequency (2.8 times) in female cancer patients than in controls (0.27 vs 0.41). The *TLR-9* rs352139 and rs352144 SNPs were significantly associated with colon cancer development when the tumor was located in the rectal area.

Conclusion: The findings support the hypothesis that *TLR-9* has an anticancer role in colon cancer development. Furthermore, genetic variation may influence colon cancer development, and SNPs in *TLR-9* could serve as biomarkers for decision making in the treatment of females with rectal cancer.

Keywords: Innate, immunity, TLR polymorphisms, rs187084, rs352139, rs352144

Introduction

The innate immune system is the first line of defense against pathogens and tissue injury.¹ The human immune system is a well-coordinated network of cells, organs, and glands that protects the body from foreign invaders. Therefore, an optimized immune system is the key to health and longevity, and the immune system plays a crucial role in preventing cancer. Toll-like receptors (TLRs) appear to be involved in the first line of defense against invading pathogens, initiating inflammatory responses and playing a key role in immune cell regulation, survival, and proliferation. Through their role in the immune system, TLRs are potential tools for curing and preventing cancer. It is estimated that between 10 and 13 TLR families (named TLR-1 to TLR-13)²⁻⁵ are activated by various ligands expressed in different types of immune cells. These receptors are located on the cell surface, and TLR-3, TLR-7, TLR-8, and TLR-9 are localized to the endosomal/lysosomal compartment in most mammalian species. Human *TLR-9*,

which has been mapped to chromosome 3p21.3,² spans ~5 kb and contains two exons. The *TLR-9* gene encodes a protein of 1032 amino acids² and is preferentially expressed by B cells and plasmacytoid dendritic cells.⁶ TLR-9 is one of the most important receptors for the initiation of innate immune responses against intracellular pathogens. Unlike other products of the *TLR* gene family, which are membrane-bound pattern recognition receptors, TLR-9 is localized on the endoplasmic reticulum membrane (in the resting state) or on the endosomal/lysosomal membrane (after ligand stimulation and trafficking);^{7,8} however, TLR-9 interacts with unmethylated CpG DNA from bacteria and some viruses.^{9,10} Alternatively, TLR-9 functions through the MyD88-dependent pathway, leading to nuclear factor-kappa-B (NF-κB) activation, cytokine secretion, and inflammatory response.^{11,12} To date, TLR-9 is the only TLR for which a systemically administered specific agonist has shown substantial evidence of anticancer activity in human clinical trials.¹³ Several studies have shown that TLR-9 engagement on cluster of differentiation 4-positive (CD4⁺) T cells can enhance their survival and therefore potentiate anticancer responses by prolonging T-cell activity.¹⁴ Previous studies have indicated that *TLR-9* polymorphisms may be associated with the risk of developing several types of cancers, including bladder cancer,¹⁵ prostate cancer,^{16–18} acute lymphoblastic leukemia,¹⁹ hepatocellular carcinoma,²⁰ gastric cancer,^{21,22} cervical cancer,²³ Hodgkin's lymphoma,²⁴ breast cancer,²⁵ Burkitt's lymphoma,²⁶ non-Hodgkin's lymphoma,²⁷ endometrial cancer,²⁸ esophageal cancer,²³ and lymphoma.^{24,27,29} However, the results are inconsistent and inconclusive. The link between *TLR-9* polymorphisms and cancer was specifically investigated in the context of chronic inflammation, which is thought to increase the risk of cancer.³⁰ Most previous studies have focused on three common single-nucleotide polymorphisms (SNPs), ie, rs352140 (C/T), rs5743836 (T/C), and rs187084 (C/T) (also referred to as 2848C/T, 1237T/C, and 1486C/T, respectively); however, the results of these studies were inconsistent.³⁰

In this study, the authors hypothesized that *TLR* SNPs may affect cancer through inappropriate TLR signaling, causing downstream elevations in proinflammatory cytokine levels, which in turn promotes cancer. In the present study we have investigated the relationship between *TLR-9* expression/polymorphism and susceptibility to colorectal cancer development in the Saudi Arabian population.

Subjects and methods

Study population

A population-based, case-control study that included 115 patients with colon cancer and 102 healthy controls

was conducted in the Kingdom of Saudi Arabia (KSA). The participants were recruited from King Khalid University Hospital in Riyadh, KSA, and the study was approved by the ethics committee at King Khalid University Hospital in Riyadh, KSA (project E-12-596, 12/3352/IRB). All questionnaire data and samples (tissues and blood) were collected during the initial recruitment of both the cases and controls. Informed written consent and a self-administered questionnaire regarding sociodemographic character (eg, age, family history of cancer, etc), lifestyle (eg, smoking habits and alcohol intake), and personal medical history were collected from all the participants. Cases and controls were frequency-matched by age and gender. The clinico-pathological characteristics of the patients, including age, family history, smoking habits, stage of colon cancer, medications, and presence of other diseases, were collected and compared with those of controls. Patients in the study population ranged in age from 45 to 88 years, with mean ages \pm SD of 57.04 ± 14.37 years in patients with colon cancer and 56.51 ± 15.70 years in controls. Among the colon cancer patients, the authors selected 66 males (57.4%) and 49 females (42.6%); among the healthy controls, the authors had 60 males (58.8%) and 42 females (41.2%), as shown in Table 1. Age and gender matched controls were used. All the control subjects were healthy without any health problems. Patients were divided into 2 groups based on the location of the tumor, the controls were chosen so that the tumor tissue and matching control tissue were from the same area, ie, the normal tissues were sampled from a very small area near the tumor. The tissue samples were used for RNA

Table 1 Clinical characteristics of the subjects selected for genotyping

Characteristics	Cancer (n=115)	Control (n=102)
Gender		
Male	66	60
Female	49	42
Age (years)		
Male	55.50 \pm 13.94	53.24 \pm 17.4
Female	56.95 \pm 13.70	50.78 \pm 15.21
Localization		
Colon	76	–
Rectum	39	–
Therapy		
Chemotherapy		
Yes	3	–
No	112	–
Radiotherapy		
Yes	5	–
No	110	–

extraction. Blood samples were stored in ethylenediaminetetraacetic acid (EDTA)-containing tubes at -80°C until genomic DNA extraction.

DNA extraction

Sample collection and storage were performed as previously described.³¹ Genomic DNA was extracted from whole blood using a QIAmp kit (QIAmp DNA Blood Mini Kit; Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Briefly, 200–300 μL blood stored in an EDTA tube at -80°C was equilibrated at room temperature, mixed with protease and lysis buffer, and then incubated at 56°C for 10 min. Next, 100% ethanol was added, and the mixture was spun through the column. The column membrane was washed, and the DNA was eluted with 100 μL elution buffer (AE). The extracted DNA concentration was determined using a NanoDrop8000 spectrophotometer (Thermo Scientific). The DNA purity was evaluated by determining standard A260/A280 and A260/A230 ratios.

Total RNA isolation

Total RNA was extracted from 40 colon cancer tissues and 40 matched normal colon tissues using an AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The isolated RNA concentration, purity, and quality were determined using an Agilent 2100 Bioanalyzer system and Agilent Small RNA analysis kit (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's instructions.

Complementary DNA (cDNA) synthesis

As described by Semlali et al,^{32,33} RNA (1 μg of each sample) was reverse-transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, PA, USA). The preparation conditions of the cDNA templates for polymerase chain reaction (PCR) were as follows: 10 minutes at 25°C , 2 hours at 37°C , and 5 minutes at 85°C . The synthesized cDNA was stored at -20°C or 4°C for immediate use in subsequent PCRs.

Quantitative real-time PCR (qPCR)

qPCR was performed using a SYBR[®] Green PCR Supermix (Applied Biosystems) and specific primers. The primers for *TLR-9* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) control were as follows: *TLR-9*: (forward) 5'-GGACCTCTGGTACTGCTTCCA-3' and (reverse) 5'-AAGCTCGTTGTACACCCAGTCT-3'; *GAPDH*: (forward) 5'-GGTATCGTCAAGGACTCATGAC-3' and (reverse) 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'.

All reactions were performed on a 7500 Real-Time PCR System (Applied Biosystems) with an annealing temperature of 60°C for both *TLR-9* and *GAPDH* genes. Cycle thresholds (Cts) were automatically determined using Applied Biosystems software, with each reaction performed in triplicate. Results were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ (Livak) relative expression method.

Genotyping

TLR-9 SNPs (rs187084, rs352139, and rs352144) were genotyped using a TaqMan allelic discrimination assay, as previously described.³⁴ The additional information of the SNPs is shown in Table S1. From each sample, 10–20 ng DNA was used per reaction with 5.6 μL of 2 \times Universal Master Mix and 200 nM primers. All genotypes were determined by an end-point reading on an ABI 7500 real-time PCR machine. The primer and probe mixtures were purchased from Applied Biosystem's "Assays-on-Demand[™]" service. Five percent of the samples were randomly selected and subjected to repeat analyses as a quality control measure for verifying genotyping procedures.

Statistical analyses

Genotypic and allelic frequencies were computed and checked for deviation with the Hardy–Weinberg equilibrium (<https://ihg.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>). Case–control and other genetic comparisons were performed using the chi-square test with Yates's correction, and allelic odds ratios (ORs) with 95% confidence intervals (CIs) were calculated with Fisher's exact tests (two-tailed). Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) 16.0 software for Windows. The authors considered *P*-values less than 0.05 to be significant. Linkage disequilibrium analysis was conducted using Haploview (v5.0).

Results

Analysis of clinical data parameters

A total of 115 colon cancer cases and 102 healthy controls were included in the study. The clinical characteristics of the patients, including age, gender, nationality, and location of the tumor, were collected and compared between colon cancer patients and controls (Table 1). Non-Saudi Arabian patients or patients with a family history of smoking were excluded from this study. The age of patients in the study population ranged from 45 to 88 years, with mean ages \pm SD of 56.04 ± 14.37 years in patients with colon cancer and 52.84 ± 15.88 years in control patients. There were no significant differences in the ages of participants in the

two groups. The male-to-female ratio was not significantly different between cases and controls (66/49 for patients with colorectal cancer; 60/42 for controls).

TLR-9 expression in colon cancer cells and colon cancer tissues

To determine whether there was a link between *TLR-9* expression and colon cancer, the authors analyzed the expression of *TLR-9* in 10 matched tissues (colon cancer and matched normal tissues) using RT-qPCR to compare *TLR-9* mRNA levels in normal and colon cancer tissues. Analysis of the expression data revealed that *TLR-9* mRNA levels were significantly ($P < 0.0001$) decreased in colon cancer tissues (1.02 ± 0.02 folds in normal colon tissue versus 0.44 ± 0.02 in colon cancer tissue; Figure 1).

TLR-9 polymorphism frequencies and susceptibility to colon cancer development in Saudi Arabian patients

No statistically significant differences were found between colon cancer cases and controls (Table 2). The *TLR-9*

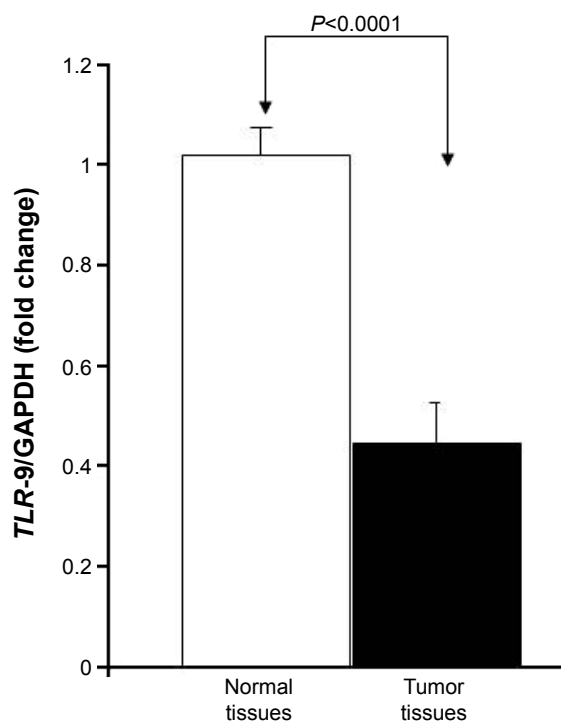


Figure 1 *TLR-9* mRNA expression in colon cancer cells and colon cancer tissues.
Notes: Total RNA of tissues was extracted from matching normal and colon cancer tissues, reverse-transcribed into cDNA, and then used to measure *TLR-9* mRNA expression with specific primers. *TLR-9* expression in colon cancer tissues and matching control tissues is shown as mean \pm SD.
Abbreviations: cDNA, complementary DNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

rs187084 SNP is located in the *TLR-9* gene promoter region. The frequencies of CC, CT, and TT genotypes in the *TLR-9* rs187084 control group were 35% (34/102), 53% (53/102), and 12% (12/102), respectively, whereas those in the case group were 43% (47/115), 43% (47/115), and 14% (15/115), respectively. No significant differences in *TLR-9* rs187084 frequencies were found between the case and control groups ($P > 0.01$). Compared with the CC genotype, carriers of the CT + TT genotype were less susceptible to colon cancer (OR = 0.69, 95% CI: 0.39–1.21; $P = 0.19$; Table 2). The same observations were made for *TLR-9* rs352139 and *TLR-9* rs352144 (Table 2).

Moreover, out of the three SNPs analyzed, all the three SNPs were in linkage disequilibrium in both cases ($r^2 \geq 0.80$) and controls ($r^2 \geq 0.80$) (Figure 2).

Association between the genotype frequencies of *TLR-9* gene polymorphisms and clinical characteristics

Analysis of the *TLR-9* genotype distribution for correlations with age, gender, and tumor localization revealed that there were no significant differences ($P > 0.05$) between cases and controls for both subpopulations of patients aged ≤ 52 and ≥ 52 years for all three *TLR-9* SNPs (rs187084, rs352139, and rs352144; Table 3A and B). The median age of onset in the colon cancer group was 56 years, and the median age of participants in the control group was 52 years. To evaluate the association between colon cancer risk and individual SNPs based on a patient's gender, the genotype distributions according to gender in the colon cancer group were compared with those of the control subjects (Table 4A and B). Interestingly, in the female group (46 patients and 41 controls), the T allele of SNP rs187084 had a significantly lower frequency (2.8 times) in patients than in the controls (0.27 vs 0.41, OR: 0.527, 95% CI: 0.279–0.995; $P = 0.04$). However, no associations were found for the genotype frequencies of *TLR-9* rs352139 and rs352144 in affected and unaffected females ($P > 0.05$; Table 4B). In contrast, all three SNPs from *TLR-9* were not associated with colorectal cancer in males (Table 4A). To investigate the possible association between *TLR-9* genotype polymorphism frequencies and tumor localization, the authors compared *TLR-9* SNP variations in patients with colon cancer when tumors were located in the rectum or in the colon area with variations in control patients. Significant difference was not observed in all three SNPs in patients with tumors located in colon area (Table 5A). However, associations of *TLR-9* rs352139 and

Table 2 Genotype frequencies of *TLR-9* gene polymorphisms in colorectal cancer and controls

Gene	SNP ID	Genotype	Case, n (%)	Control, n (%)	OR	95% CI	χ^2 value	P-value
<i>TLR-9</i>	rs187084	CC	47 (0.43)	34 (0.35)	Ref			
		CT	47 (0.43)	53 (0.53)	0.642	0.355–1.158	2.18	0.13991
		TT	15 (0.14)	12 (0.12)	0.904	0.376–2.176	0.05	0.82221
		CT + TT	62 (0.57)	65 (0.65)	0.690	0.393–1.210	1.68	0.19487
		C	141 (0.65)	121 (0.61)	Ref			
	rs352139	T	77 (0.35)	77 (0.39)	0.858	0.576–1.278	0.57	0.45166
		AA	33 (0.29)	31 (0.30)	Ref			
		AG	55 (0.49)	55 (0.54)	0.939	0.507–1.740	0.04	0.84243
		GG	26 (0.23)	16 (0.16)	1.527	0.691–3.372	1.10	0.29448
		AG + GG	81 (0.71)	71 (0.70)	1.072	0.597–1.923	0.05	0.81642
	rs352144	A	121 (0.54)	117 (0.57)	Ref			
		G	107 (0.48)	87 (0.43)	1.189	0.813–1.740	0.80	0.37163
		AA	2 (0.02)	0 (0)	Ref			
		AC	5 (0.04)	7 (0.07)	0.147	0.006–3.706	2.33	0.12663
		CC	110 (0.94)	94 (0.93)	0.234	0.011–4.932	1.70	0.19294
AC + CC		115 (0.98)	101 (1)	0.228	0.011–4.796	1.74	0.18682	
A		9 (0.04)	7 (0.03)	Ref				
C	225 (0.96)	195 (0.97)	0.897	0.328–2.455	0.04	0.83298		

Abbreviations: CI, confidence interval; OR, odds ratio; Ref, reference; SNP, single-nucleotide polymorphism.

rs352144, but not *TLR-9* rs187084, were found in tumors in the rectal area (Table 5B). In patients with tumors in the rectal area, no significant differences in the frequencies of *TLR-9* rs187084 were found between the case and control groups ($P > 0.05$). The frequencies of the AA, AG, and GG genotypes in *TLR-9* rs352139 in the control group were 30% (31/102), 54% (55/102), and 16% (16/102), respectively, whereas those in the case group were 18% (6/34), 50% (17/34), and 32% (11/34), respectively (Table 5B). The frequencies of the AA, AC, and CC genotypes in *TLR-9* rs352144 in the control group were 0% (0/102), 7% (7/102), and 93% (94/102),

respectively, whereas those in the case group were 3% (1/36), 3% (1/36), and 94% (33/36), respectively (Table 5B).

Discussion

In this study, the authors demonstrated that *TLR-9* mRNA expression was decreased in colon cancer tissues compared with that in normal tissues, suggesting that TLR-9 may have an anticancer role in colon cancer development. These data support the development of *TLR-9* agonists (synthetic oligodeoxynucleotides [ODNs]) for cancer treatment. Baines and Celis³⁵ used a murine cervical carcinoma model, and mice with established subcutaneous tumors that were treated with CpG ODNs injected at a distant site exhibited significant regression. Moreover, CpG ODN-treated mice exhibited significantly improved survival ($P < 0.001$) compared with control mice.³⁵ Tuomela et al³⁶ discovered that low tumor *TLR-9* expression is associated with significantly shortened disease-specific survival in patients with triple-negative, but not with estrogen receptor-positive (ER+), breast cancers.

The authors also demonstrated a significant association between the *TLR-9* rs187084 SNP and colon cancer risk in females, which is closely linked with the female sex hormones. Previous studies have described the role of estrogen and progesterone in protecting against colon cancer in females.^{37–40} There is evidence that gender influences the clinical course of colorectal cancer and can also affect disease severity; indeed, females tend to exhibit a benign course more frequently than males. Moreover, males with colorectal cancer tend to have

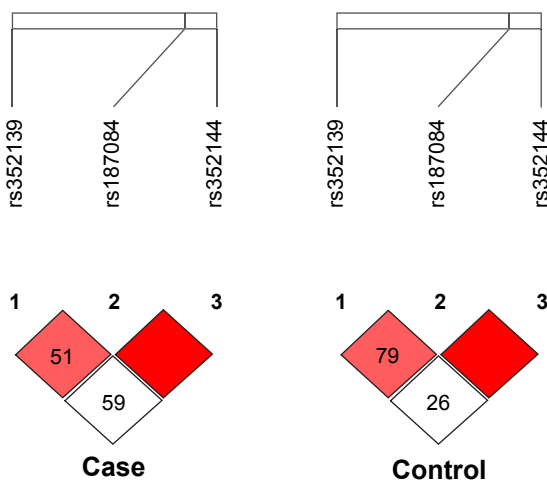


Figure 2 LD of the studied *TLR-9* loci rs187084, rs352139, and rs352144 in colorectal cancer case and control groups.

Abbreviation: LD, linkage disequilibrium.

Table 3A Genotype frequencies of *TLR-9* gene polymorphism in patients younger than 57 years with colorectal cancer

Gene	SNP ID	Genotype	Case, n (Frequency in %)	Control, n (Frequency in %)	OR	95% CI	χ^2 value	P-value
<i>TLR-9</i>	rs187084	CC	22 (0.42)	16 (0.27)	Ref			
		CT	23 (0.43)	35 (0.58)	0.478	0.208–1.098	3.07	0.07989
		TT	8 (0.15)	9 (0.15)	0.646	0.205–2.041	0.56	0.45578
		CT + TT	31 (0.58)	44 (0.85)	0.512	0.232–1.130	2.78	0.09558
	rs352139	C	67 (0.63)	67 (0.56)	Ref			
		T	39 (0.37)	53 (0.44)	0.736	0.431–1.256	1.27	0.26013
		AA	20 (0.36)	24 (0.39)	Ref			
		AG	27 (0.48)	28 (0.45)	1.157	0.523–2.562	0.13	0.71882
		GG	9 (0.16)	10 (0.16)	1.080	0.367–3.175	0.02	0.88876
	rs352144	AG + GG	36 (0.64)	38 (0.61)	1.137	0.538–2.403	0.11	0.73687
		A	67 (0.60)	76 (0.61)	Ref			
		G	45 (0.40)	48 (0.39)	1.063	0.630–1.794	0.05	0.81763
		AA	2 (0.03)	0 (0)	Ref			
		AC	4 (0.07)	4 (0.06)	0.200	0.007–5.453	1.67	0.19671
		CC	53 (0.90)	58 (0.94)	0.183	0.009–3.897	2.15	0.14284
	AC + CC	57 (0.97)	62 (1)	0.184	0.009–3.914	2.14	0.14378	
	A	8 (0.07)	4 (0.03)	Ref				
	C	110 (0.93)	120 (0.97)	0.458	0.134–1.565	1.62	0.20304	

Abbreviations: CI, confidence interval; OR, odds ratio; Ref, reference; SNP, single-nucleotide polymorphism.

a poorer prognosis. The exact mechanisms through which *TLR-9* polymorphisms contribute to cancer pathogenesis are unknown. Based on information from the National Center for Biotechnology Information SNP database, the *TLR-9* rs187084 polymorphism in the *TLR-9* gene is intronic and is most likely not a true disease-causing variant but could reflect the effects of a functional variant located elsewhere in the *TLR-9* gene. Previous studies have shown that C genotype frequencies are closely associated with decreased *TLR-9*

transcription activity when compared with the T genotype. Some introns may contain regulatory sequences or even encode proteins or RNAs with regulatory functions. Additionally, the authors found significant associations between *TLR-9* rs352139/rs352144 SNPs and rectal area localization, but not colon area localization. The *TLR-9* rs352139 and rs352144 SNPs are located in the promoter region and most likely affect *TLR-9* expression. It is possible that the intron and upstream variant of the *TLR-9* gene play an important role in regulating

Table 3B Genotype frequencies of *TLR-9* gene polymorphism in patients older than 57 years with colorectal cancer

Gene	SNP ID	Genotype	Case, n (Frequency in %)	Control, n (Frequency in %)	OR	95% CI	χ^2 value	P-value
<i>TLR-9</i>	rs187084	CC	25 (0.45)	18 (0.46)	Ref			
		CT	24 (0.43)	18 (0.46)	0.960	0.406–2.270	0.01	0.92592
		TT	7 (0.12)	3 (0.08)	1.680	0.382–7.395	0.48	0.48975
		CT + TT	31 (0.55)	21 (0.54)	1.063	0.468–2.415	0.02	0.88428
		C	74 (0.66)	54 (0.69)	Ref			
	rs352139	T	38 (0.34)	24 (0.31)	1.155	0.622–2.147	0.21	0.64774
		AA	13 (0.22)	7 (0.17)	Ref			
		AG	28 (0.48)	27 (0.68)	0.558	0.193–1.612	1.18	0.27836
		GG	17 (0.30)	6 (0.15)	1.526	0.413–5.642	0.40	0.52559
		AG + GG	45 (0.78)	33 (0.83)	0.734	0.264–2.042	0.35	0.55304
	rs352144	A	54 (0.47)	41 (0.51)	Ref			
		G	62 (0.53)	39 (0.49)	1.207	0.682–2.135	0.42	0.51772
		AA	0 (0)	0 (0)	Ref			
		AC	1 (0.02)	3 (0.08)	0.429	0.005–33.596	–	1.000
		CC	57 (0.98)	36 (0.92)	1.575	0.031–81.149	–	1.000
	AC + CC	58 (1)	39 (1)	1.481	0.029–76.201	–	1.000	
	A	1 (0.01)	3 (0.04)	Ref				
	C	115 (0.99)	75 (0.96)	4.600	0.470–45.053	2.06	0.30785	

Abbreviations: CI, confidence interval; OR, odds ratio; Ref, reference; SNP, single-nucleotide polymorphism.

Table 4A Genotype frequencies of *TLR-9* gene polymorphism in male patients with colorectal cancer

Gene	SNP ID	Genotype	Case, n (Frequency in %)	Control, n (Frequency in %)	OR	95% CI	χ^2 value	P-value
TLR-9	rs187084	CC	22 (0.35)	19 (0.33)	Ref			
		CT	30 (0.48)	35 (0.62)	0.740	0.338–1.621	0.57	0.45162
		TT	11 (0.17)	3 (0.05)	3.167	0.768–13.05	2.70	0.10042
		CT + TT	41 (0.65)	38 (0.67)	0.932	0.438–1.985	0.03	0.85473
		C	74 (0.59)	73 (0.64)	Ref			
	rs352139	T	52 (0.41)	41 (0.36)	1.251	0.743–2.108	0.71	0.39956
		AA	19 (0.30)	15 (0.25)	Ref			
		AG	31 (0.48)	34 (0.58)	0.720	0.313–1.657	0.60	0.43896
		GG	14 (0.22)	10 (0.17)	1.105	0.384–3.180	0.03	0.85273
		AG + GG	45 (0.70)	44 (0.75)	0.807	0.365–1.787	0.28	0.59734
	rs352144	A	69 (0.54)	64 (0.54)	Ref			
		G	59 (0.46)	54 (0.46)	1.013	0.613–1.674	0	0.95849
		AA	2 (0.03)	0 (0)	Ref			
		AC	1 (0.01)	3 (0.05)	0.086	0.002–3.101	3.00	0.08326
		CC	64 (0.96)	55 (0.95)	0.232	0.011–4.945	1.69	0.19299
		AC + CC	65 (0.97)	58 (1)	0.224	0.011–4.760	1.76	0.18469
		A	5 (0.04)	3 (0.03)	Ref			
C	129 (0.96)	113 (0.97)	0.685	0.160–2.930	0.26	0.72813		

Abbreviations: CI, confidence interval; OR, odds ratio; Ref, reference; SNP, single-nucleotide polymorphism.

the TLR-mediated immunologic response. However, it is not clear how this intronic SNP (rs352144) reduces *TLR-9* expression. It is possible that this SNP influences signaling by creating an alternative splicing site, thus affecting the mRNA transcription and the final protein product. Mutations in the promoter region are predicted to affect the expression/stability of the regulatory regions (ie, the 5'- and 3'-untranslated regions [UTRs]), explaining why the expression levels of these TLR-9 proteins were decreased in colon cancer tissues

compared with those in normal tissues. The 3'-UTR contains several types of regulatory elements, including binding sites for microRNAs (miRNAs) and AU-rich elements, which are known to regulate gene expression. Moreover, miRNAs can bind to mRNAs and control protein abundance by suppressing translation or marking mRNAs for degradation.⁴¹ Li et al⁴² reported that rs5743305 is located in the promoter region, 1 kb upstream of exon 1 in the *TLR-3* gene, and may influence the transcriptional activity of *TLR-3*.

Table 4B Genotype frequencies of *TLR-9* gene polymorphism in female patients with colorectal cancer

Gene	SNP ID	Genotype	Case, n (Frequency in %)	Control, n (Frequency in %)	OR	95% CI	χ^2 value	P-value
TLR-9	rs187084	CC	25 (0.54)	15 (0.37)	Ref			
		CT	17 (0.37)	18 (0.44)	0.567	0.225–1.424	1.47	0.22539
		TT	4 (0.09)	8 (0.19)	0.300	0.077–1.169	3.18	0.07439
		CT + TT	21 (0.46)	26 (0.63)	0.485	0.205–1.146	2.75	0.09703
		C	67 (0.73)	48 (0.59)	Ref			
	rs352139	T	25 (0.27)	34 (0.41)	0.527	0.279–0.995	3.95	0.04686
		AA	14 (0.28)	15 (0.36)	Ref			
		AG	24 (0.48)	21 (0.50)	1.224	0.481–3.118	0.18	0.67089
		GG	12 (0.24)	6 (0.14)	2.143	0.632–7.266	1.52	0.21765
		AG + GG	36 (0.72)	27 (0.64)	1.429	0.591–3.454	0.63	0.42761
	rs352144	A	52 (0.52)	51 (0.61)	Ref			
		G	48 (0.48)	33 (0.39)	1.427	0.792–2.568	1.41	0.23559
		AA	0 (0)	0 (0)	Ref			
		AC	4 (0.08)	4 (0.1)	1.000	0.016–62.3	–	1.000
		CC	46 (0.92)	38 (0.9)	1.208	0.023–62.29	–	1.000
		AC + CC	50 (1)	42 (1)	1.188	0.023–61.16	–	1.000
		A	4 (0.04)	4 (0.05)	Ref			
C	96 (0.96)	80 (0.95)	1.200	0.291–4.951	0.06	1.000		

Note: Bold values are statistically significantly different.

Abbreviations: CI, confidence interval; OR, odds ratio; Ref, reference; SNP, single-nucleotide polymorphism.

Table 5A Genotype frequencies of *TLR-9* gene polymorphisms in colorectal tumors located in the colon area

Gene	SNP ID	Genotype	Case, n (Frequency in %)	Control, n (Frequency in %)	OR	95% CI	χ^2 value	P-value
<i>TLR-9</i>	rs187084	CC	30 (0.45)	34 (0.35)	Ref			
		CT	27 (0.41)	53 (0.53)	0.577	0.294–1.134	2.56	0.10952
		TT	9 (0.14)	12 (0.12)	0.850	0.315–2.297	0.10	0.74850
		CT + TT	36 (0.55)	65 (0.65)	0.628	0.332–1.188	2.06	0.15130
		C	87 (0.66)	121 (0.61)	Ref			
	rs352139	T	45 (0.34)	77 (0.39)	0.813	0.513–1.287	0.78	0.37640
		AA	23 (0.34)	31 (0.30)	Ref			
		AG	32 (0.47)	55 (0.54)	0.784	0.392–1.569	0.47	0.49166
		GG	13 (0.19)	16 (0.16)	1.095	0.441–2.718	0.04	0.84470
		AG + GG	45 (0.66)	71 (0.70)	0.854	0.443–1.646	0.22	0.63780
	rs352144	A	78 (0.57)	117 (0.57)	Ref			
		G	58 (0.43)	87 (0.43)	1.000	0.645–1.551	0	1.000
		AA	0 (0)	0 (0)	Ref			
		AC	4 (0.06)	7 (0.07)	0.600	0.01–35.86	0.001	1.000
		CC	65 (0.94)	94 (0.93)	0.693	0.014–35.37	0.001	1.000
		AC + CC	69 (1)	101 (1)	0.685	0.013–34.92	0	1.000
		A	4 (0.03)	7 (0.03)	Ref			
		C	134 (0.97)	195 (0.97)	1.203	0.345–4.189	0.08	1.000

Abbreviations: CI, confidence interval; OR, odds ratio; Ref, reference; SNP, single-nucleotide polymorphism.

Genetic variations play crucial roles in phenotypic variation and disease susceptibility by affecting gene expression. Recent studies by the group⁴³ have integrated a number of resources and technologies to assess several aspects of genome variation affecting gene expression. Additionally, recent findings have suggested that protein divergence between species strongly correlates with expression divergence between species.^{44,45}

Results of this study showed that the *TLR-9* rs187084, rs352139, and rs352144 genotypes in the *TLR-9* gene were associated with colon cancer risk, suggesting that *TLR-9* genetic polymorphisms could serve as biomarkers for decision making in the treatment of females with rectal cancer. Further studies are necessary to investigate the potential mechanisms through which *TLR* SNPs may mediate

Table 5B Genotype frequencies of *TLR-9* gene polymorphisms in colorectal cancer tumors located in rectal area

Gene	SNP ID	Genotype	Case, n (Frequency in %)	Control, n (Frequency in %)	OR	95% CI	χ^2 value	P-value
<i>TLR-9</i>	rs187084	CC	14 (0.42)	34 (0.35)	Ref			
		CT	15 (0.46)	53 (0.53)	0.687	0.295–1.602	0.76	0.38390
		TT	4 (0.12)	12 (0.12)	0.810	0.223–2.945	0.10	0.74819
		CT + TT	19 (0.58)	65 (0.65)	0.710	0.317–1.588	0.70	0.40332
		C	43 (0.65)	121 (0.61)	Ref			
	rs352139	T	23 (0.35)	77 (0.39)	0.841	0.470–1.503	0.34	0.55786
		AA	6 (0.18)	31 (0.30)	Ref			
		AG	17 (0.50)	55 (0.54)	1.597	0.570–4.471	0.80	0.37026
		GG	11 (0.32)	16 (0.16)	3.552	1.11–11.37	4.81	0.02825
		AG + GG	28 (0.82)	71 (0.70)	2.038	0.767–5.415	2.09	0.14812
	rs352144	A	29 (0.43)	117 (0.57)	Ref			
		G	39 (0.57)	87 (0.43)	1.809	1.038–3.150	4.44	0.03519
		AA	1 (0.03)	0 (0)	Ref			
		AC	1 (0.03)	7 (0.07)	0.067	0.002–2.563	3.94	0.04722
		CC	33 (0.94)	94 (0.93)	0.118	0.005–2.972	2.79	0.09506
		AC + CC	34 (0.97)	101 (1)	0.113	0.005–2.847	2.91	0.08819
		A	3 (0.04)	7 (0.03)	Ref			
		C	67 (0.96)	195 (0.97)	0.802	0.202–3.189	0.10	0.73581

Note: Bold values are statistically significantly different.

Abbreviations: CI, confidence interval; OR, odds ratio; Ref, reference; SNP, single-nucleotide polymorphism.

pathogenesis and the functional aspects of these *TLR-9* SNP polymorphisms in colon cancer.

Abbreviations

SNP, single-nucleotide polymorphism; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; OR, odds ratio; CI, confidence interval; Ct, cycle threshold; EDTA, ethylenediaminetetraacetic acid.

Acknowledgment

This project was supported by the Research Group Program (number RGP-VPP-260) of the Kingdom of Saudi Arabia.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Characteristics of selected polymorphisms in *TLR-9*

SNP ID	Chromosome/ position	Nucleotide change	Position	Region	Minor allele frequency (%)	
					Cases	Controls
rs187084	3/52227015	C>T	4149	Promoter	77 (0.35)	77 (0.39)
rs352139	3/52216841	A>G	6808	Intron	107 (0.48)	87 (0.43)
rs352144	3/52219937	A>C	3712	Upstream variant	225 (0.96)	195 (0.97)

Abbreviation: SNP, single-nucleotide polymorphism.

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