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ORIGINAL RESEARCH

Two SNPs in the promoter region of Toll-like receptor 4 gene are not associated with smoking in Saudi Arabia

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Abstract: Defects in the innate immune system, particularly in Toll-like receptors (TLRs), have been reported in several cigarette smoke-promoted diseases. The aim of this study was to examine the impact of tobacco smoke on allelic frequencies of TLR4 single-nucleotide polymorphisms (SNPs) and to compare the genotypic distribution of these SNPs in a Saudi Arabian population with that in previously studied populations. DNA was extracted from 303 saliva samples collected from smokers and nonsmokers. Two transitional SNPs in the promoter region of TLR4 were selected, rs2770150 (T/C) and rs10759931 (G/A). Genotype frequencies were determined using quantitative polymerase chain reaction. Our results showed a slight effect of smoking on the distribution of rs2770150 and rs10759931. However, the differences were not significant. Thus, we conclude that the SNPs selected for this study were independent of smoking and may not be related to smoking-induced diseases.

Keywords: Toll-like receptor 4, polymorphism, genetic variation, smoking

Introduction

Innate immunity, which is considered to be the first line of defense against diseases, is well studied, and its role has been clarified through study of mutations in innate immunity genes as well as of patients with various diseases such as cystic fibrosis.[1] In addition to induction of autoimmune diseases,[2] defects in the innate immune system have been reported in several other diseases such as types of cancer,[3] asthma,[4] psoriasis,[5] and Alzheimer's and other neurodegenerative diseases.[6,7] The innate immune system initially recognizes microorganisms through pattern recognition receptors, in particular Toll-like receptors (TLRs).[8] TLRs are a family of at least 13 transmembrane receptors that are expressed on immune cells as well as on gingival epithelial cells and are involved in the initiation of inflammatory processes.[9–11] Upon induction by certain ligands, TLRs activate intracellular signaling pathways that promote the production of multiple immune mediators that contribute to host defense.[12,13] Several polymorphisms have been reported at different positions in TLR genes[14,15] and were found to be associated with inflammatory diseases.[16,17] Single-nucleotide polymorphisms (SNPs) are a class of polymorphisms involving single-base substitutions.[18] SNPs are thought to constitute the majority of sequence variants in human beings,[19] and they occur approximately once every 300 bases.[20] Several reports have demonstrated the role of TLR SNPs in the development of cancer.[21,22] *TLR4*, which is located on chromosome 9, encodes a protein that plays a critical role in the immune system through recognition of lipopolysaccharides found in Gram-negative bacteria.[23,24] *TLR4* polymorphisms have been reported to be

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involved in different infectious and noninfectious diseases.[25,26] In particular, the *TLR4* SNPs rs2770150 and rs10759931 have been detected in association with many health complications. We previously demonstrated that *TLR4* polymorphisms, specifically the SNPs rs2770150 and rs10759931, are associated with colon cancer.[27] Additionally, variations in the rs2770150 SNP were found to affect antibody response to whole-cell pertussis vaccination.[28] Furthermore, these variations alter the level of susceptibility to pollution-induced asthma.[29] The rs10759931 SNP was demonstrated to be associated with latent tuberculosis infection and subsequent pulmonary tuberculosis.[30] Many of these diseases have been shown to be caused by tobacco smoke, indicating the importance of examining the effects of smoking on these SNPs.[31–33] The huge number of health problems and risks associated with tobacco smoke are widely known, including COPD,[34] different types of cancer,[35,36] and periodontal diseases.[37] Smoking was found to induce epigenetic and genetic alterations that, in turn, may lead to the initiation of different diseases, including those described earlier.[38] Smoking may cause either transition or transversion mutations.[39] However, transitions are reported to generate more radical amino acid changes than transversions.[40] The aim of this study was to examine the impact of tobacco smoke on allelic frequencies of TLR4 rs2770150 and rs10759931 transitional SNPs and to compare the genotypic distribution of these SNPs in a Saudi Arabian population with that in other previously studied populations.

Materials and methods

Saliva collection

Saliva samples were collected from a total of 126 nonsmokers and 177 smokers. Samples were collected from male students and staff at King Saud University (KSU) between January and April 2015. From each participant, 2 mL of saliva was collected in a 15-mL falcon tube. The clinical data for these samples are listed in Table 1. This study was reviewed by the College of Applied Medical Sciences' research ethics committee at KSU and was granted the approval number CAMS 13/3536. Each participant provided informed consent and completed a written survey. Data included in the survey comprised age, number of cigarettes smoked per day, years of smoking, and body mass index (BMI).

DNA extraction

Each saliva sample was diluted with two volumes of phosphate-buffered saline immediately after collection. DNA extraction was performed using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). A NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the DNA concentration and quality. Then, the DNA samples were stored at −20°C for later application.

Table 1 Clinical characteristics of study subjects

Variable	Nonsmokers (n=126)	Smokers (n=177)
Age (years), median ± average	20±21	24±27
BMI		
Obese (≥30 kg/m²)	20/100 (20%)	27/163 (17%)
Nonobese (<30 kg/m²)	80/100 (80%)	136/163 (83%)
Years of smoking		
>5	–	104/165 (63%)
≤5	–	61/165 (37%)
Cigarettes per day		
≥20	–	99/159 (62.3%)
<20	–	60/159 (37.7%)

Abbreviation: BMI, body mass index.

Genotyping

Each DNA sample was diluted to 10 ng/µL before use in the genotyping experiments. Two transitional *TLR4* SNPs were selected: rs2770150 (T/C) and rs10759931 (G/A). Few data are available regarding the association of the selected SNPs with different diseases. However, these SNPs were selected because they occur in the promoter region (Table 2) and thus regulate *TLR4* expression. Reactions were performed using 20 ng of DNA mixed with 5.6 µL of TaqMan® Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA) and 0.2 µL of 40× TaqMan® SNP Genotyping assay (Applied Biosystems), using a QuantStudio™ 7 Flex Real-Time PCR System thermal cycler (Applied Biosystems). The amplification protocol included 40 cycles as follows: a pre-read stage for 30 sec at 60°C, a hold for 10 min at 95°C, amplification for 15 sec at 95°C and 1 min at 60°C, and a post-read stage for 30 sec at 60°C.

Statistical analysis

Genotypic and allelic frequencies were calculated and checked for deviation from Hardy–Weinberg equilibrium, as described in our previous work.[41] Case–control and other

Table 2 Description of the selected SNPs

Gene	SNP ID	SNP location	SNP type	Ancestral allele
TLR4	rs2770150	NC_000009.11:g.120463139	Promoter	T>C
	rs10759931	NC_000009.11:g.120464147	Promoter	G>A

Abbreviations: SNP, single-nucleotide polymorphism; TLR, Toll-like receptor.

genetic comparisons were performed using the chi-square test and allelic odds ratios (ORs), and 95% confidence intervals (CIs) were calculated with Fisher's exact test (two-tailed). Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) 22.0 software (SPSS, Chicago, IL, USA). *P*-values ≤0.05 were considered significant.

Results

Characteristics of the study population

As shown in Table 1, the smokers and nonsmokers did not differ significantly in BMI, because the selection criteria for this study were independent of patient weight. In addition, approximately two-thirds of the samples were collected from students rather than staff, and the smoker and nonsmoker groups did not differ significantly in age. Thus, no genotyping analysis was conducted regarding these parameters. Given that the average age at which participants started smoking was 18.3 years and that half of the smokers (85/176) were less than 24 years of age, we separated the smoker participants into the following two groups: those who had smoked for >5 years and those who had smoked for ≤5 years. Approximately half of the smokers (71/159) consume 20 cigarettes (ie, one pack) per day, and we classified the smokers into the following two categories: those who consume ≥20 cigarettes per day and those who daily consume <20 cigarettes. The characteristics of the subjects are summarized in Table 1.

Genotypic patterns of *TLR4* SNPs among smokers and nonsmokers

A total of 303 saliva samples, 177 from smokers and 126 from nonsmokers, were included in this study to investigate the effects of tobacco smoke on the genotypic distribution of *TLR4* rs2770150 and rs10759931 SNPs. The homozygous ancestral alleles, TT in rs2770150 and AA in rs10759931, were used as references for the genotyping analysis. The allelic frequencies in nonsmokers and smokers, ORs, 95% CIs, chi-square results, and *P*-values are listed in Table 3. Neither SNP was significantly associated with smoking behavior. The genotypic distribution of rs2770150 was 49% TT, 38% TC, and 13% CC in nonsmokers compared to 54% TT, 35% TC, and 11% CC in smokers. The allele frequencies for rs10759931 were 12% AA, 32% AG, and 56% GG in nonsmokers and 8% AA, 37% AG, and 55% GG in smokers.

Long- and short-term smoking and their impacts on *TLR4* polymorphisms

The smokers were divided into two groups based on years of smoking: group A (>5 years) and group B (≤5 years). Table 4 lists the genotypic frequencies and subsequent analysis of the SNPs for each group compared to nonsmokers. No correlation was observed between the SNPs and either long-term or short-term smokers. The genotypic allocation of the rs2770150 SNP in group A was 49% TT, 38% TC, and 13% CC in nonsmokers and 57% TT, 34% TC, and 9% CC in smokers. In group B, however, 53% TT, 33% TC, and 15% CC were observed in smokers compared to 49% TT, 38% TC, and 13% CC in nonsmokers. For the rs10759931 SNP, the genotypic frequencies in group A were 12% AA, 32% AG, and 56% GG in nonsmokers and 8% AA, 36% AG, and 56% GG in smokers. Allele frequencies for this SNP in group B were 8% AA, 32% AG, and 59% GG in smokers and 12% AA, 32% AG, and 56% GG in nonsmokers.

Table 3 Genotype frequencies of *TLR4* gene polymorphism in smoker and control patients

Gene	SNP	Allele	Nonsmokers, n (%)	Smokers, n (%)	OR	95% CI	χ^2	*P*-value
TLR4	rs2770150	Total	112	156				
		TT	55 (0.49)	85 (0.54)	Ref			
		TC	43 (0.38)	54 (0.35)	0.81	0.4807–1.3735	0.6012	0.4381
		CC	14 (0.13)	17 (0.11)	0.79	0.3586–1.7217	0.3640	0.5463
		TC + CC	57 (0.51)	71 (0.46)	0.81	0.4956–1.3108	0.7563	0.3845
		T	153 (0.68)	224 (0.72)	Ref			
		C	71 (0.32)	88 (0.28)	0.85	0.5823–1.2308	0.7617	0.3828
	rs10759931	Total	118	168				
		AA	14 (0.12)	13 (0.08)	Ref			
		AG	38 (0.32)	62 (0.37)	1.76	0.7464–4.1362	1.6870	0.1940
		GG	66 (0.56)	93 (0.55)	1.52	0.6696–3.4392	1.0072	0.3156
		AG + GG	104 (0.88)	155 (0.92)	1.61	0.7250–3.5534	1.3804	0.2400
		A	66 (0.28)	88 (0.26)	Ref			
		G	170 (0.72)	248 (0.74)	1.09	0.7527–1.5905	0.2222	0.6374

Abbreviations: TLR, Toll-like receptor; SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table 4 Comparison of genotype frequencies of *TLR4* gene SNPs with overall controls depending on smoking duration

Gene	SNP	Allele	Nonsmokers, n (%)	Smokers, n (%)	OR	95% CI	χ^2	*P*-value
TLR4	rs2770150	**Patients smoking for >5 years**						
		Total	112	91				
		TT	55 (0.49)	52 (0.57)	Ref			
		TC	43 (0.38)	31 (0.34)	0.76	0.4195–1.3859	0.7924	0.3734
		CC	14 (0.13)	8 (0.09)	0.60	0.2343–1.5594	1.0979	0.2947
		TC + CC	57 (0.51)	39 (0.43)	0.72	0.4149–1.2624	1.3006	0.2541
		T	153 (0.68)	135 (0.74)	Ref			
		C	71 (0.32)	47 (0.26)	0.75	0.4855–1.1593	1.6795	0.1950
	rs2770150	**Patients smoking for ≤5 years**						
		Total	112	55				
		TT	55 (0.49)	29 (0.53)	Ref			
		TC	43 (0.38)	18 (0.33)	0.79	0.3901–1.6159	0.4058	0.5241
		CC	14 (0.13)	8 (0.15)	1.08	0.4075–2.8825	0.0260	0.8720
		TC + CC	57 (0.51)	26 (0.47)	0.87	0.4534–1.6507	0.1934	0.6601
		T	153 (0.68)	76 (0.69)	Ref			
		A	170 (0.72)	146 (0.74)	1.09	0.7123–1.6680	0.1578	0.6912
TLR4	rs10759931	**Patients smoking for >5 years**						
		Total	118	99				
		AA	14 (0.12)	8 (0.08)	Ref			
		AG	38 (0.32)	36 (0.36)	1.66	0.6216–4.4219	1.0309	0.3099
		GG	66 (0.56)	55 (0.56)	1.46	0.5700–3.7314	0.6242	0.4295
		AG + GG	104 (0.88)	91 (0.92)	1.53	0.6145–3.8159	0.8459	0.3577
		A	66 (0.28)	52 (0.26)	Ref			
		G	170 (0.72)	146 (0.74)	1.09	0.7123–1.6680	0.1578	0.6912
	rs10759931	**Patients smoking for ≤5 years**						
		Total	118	59				
		AA	14 (0.12)	5 (0.08)	Ref			
		AG	38 (0.32)	19 (0.32)	1.40	0.4388–4.4667	0.3248	0.5687
		GG	66 (0.56)	35 (0.59)	1.48	0.4941–4.4621	0.5003	0.4794
		AG + GG	104 (0.88)	54 (0.92)	1.45	0.4973–4.2502	0.4717	0.4922
		A	66 (0.28)	29 (0.25)	Ref			
		G	170 (0.72)	89 (0.75)	1.19	0.7181–1.9770	0.4604	0.4974

Abbreviations: TLR, Toll-like receptor; SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Association between individual SNPs and the intensity of smoking

Allele frequency data for the smokers were also analyzed with regard to the quantity of cigarettes consumed per day. Here, we identified two categories: category A (≥20 cigarettes/day) and category B (<20 cigarettes/day). The SNP genotypes of both categories of smokers were compared to those of nonsmokers (Table 5); no significant differences were observed. For the rs2770150 SNP, the genotypes of smokers in category A were 61% TT, 29% TC, and 10% CC and the genotypes of smokers in category B were 45% TT, 42% TC, and 13% CC. Both categories were compared to nonsmokers, in whom the allele frequencies were 49% TT, 38% TC, and 13% CC. For the rs10759931 SNP, nonsmokers showed allele frequencies of 12% AA, 32% AG, and 56% GG. The genotypic distribution of category A smokers for this SNP was 9% AA, 37% AG, and 54% GG. In category B smokers for the same SNP, the allele frequencies were 7% AA, 33% AG, and 60% GG.

Differentiation between the Saudi Arabian population and others and linkage studies

The genotyping results for nonsmokers were used to compare the Riyadh region population in Saudi Arabia (CRS), from which we collected our samples, with other previously studied populations (Table 6). Of 126 samples, we determined the rs2770150 SNP genotypes for 112 samples and the rs10759931 SNP genotypes for 118 samples. The frequency of the various alleles of rs2770150 differed significantly between Chinese (Han Chinese in Beijing), Japanese (Japanese in Tokyo), Nigerian (Yoruba in Ibadan), Kenyan (Maasai in Kinyawa), and Italian (Toscans) populations, and the Saudi population ($P<0.005$ for most). For TLR4 rs10759931, the CRS population differed significantly from African Americans (D-0) and North Americans

Table 5 Genotype frequencies of *TLR4* gene SNPs with overall controls according to the daily quantity of cigarettes

Gene	SNP	Allele	Nonsmokers, n (%)	Smokers, n (%)	OR	95% CI	χ^2	*P*-value
TLR4	rs2770150	**Patients smoking ≥20 cigarettes/day**						
		Total	112	87				
		TT	55 (0.49)	53 (0.61)	Ref			
		TC	43 (0.38)	25 (0.29)	0.60	0.3243–1.1224	2.5621	0.1095
		CC	14 (0.13)	9 (0.10)	0.67	0.2663–1.6714	0.7521	0.3858
		TC + CC	57 (0.51)	34 (0.39)	0.62	0.3507–1.0925	2.7530	0.0971
		T	153 (0.68)	131 (0.75)	Ref			
		C	71 (0.32)	43 (0.25)	0.71	0.4534–1.1036	2.3369	0.1263
	rs2770150	**Patients smoking <20 cigarettes/day**						
		Total	112	53				
		TT	55 (0.49)	24 (0.45)	Ref			
		TC	43 (0.38)	22 (0.42)	1.17	0.5806–2.3676	0.1971	0.6571
		CC	14 (0.13)	7 (0.13)	1.15	0.4106–3.1974	0.0677	0.7948
		TC + CC	57 (0.51)	29 (0.55)	1.17	0.6053–2.2459	0.2108	0.6461
		T	153 (0.68)	70 (0.66)	Ref			
		C	71 (0.32)	36 (0.34)	1.11	0.6785–1.8103	0.1686	0.6814
TLR4	rs10759931	**Patients smoking ≥20 cigarettes/day**						
		Total	118	95				
		AA	14 (0.12)	9 (0.09)	Ref			
		AG	38 (0.32)	35 (0.37)	1.43	0.5513–3.7234	0.5474	0.4594
		GG	66 (0.56)	51 (0.54)	1.20	0.4821–2.9972	0.1561	0.6928
		AG + GG	104 (0.88)	86 (0.91)	1.29	0.5310–3.1161	0.3123	0.5763
		A	66 (0.28)	53 (0.28)	Ref			
		G	170 (0.72)	137 (0.72)	1.00	0.6556–1.5363	0.0003	0.9870
	rs10759931	**Patients smoking <20 cigarettes/day**						
		Total	118	57				
		AA	14 (0.12)	4 (0.07)	Ref			
		AG	38 (0.32)	19 (0.33)	1.75	0.5063–6.0485	0.7943	0.3728
		GG	66 (0.56)	34 (0.60)	1.80	0.5509–5.9016	0.9692	0.3249
		AG + GG	104 (0.88)	53 (0.93)	1.78	0.5595–5.6864	0.9785	0.3226
		A	66 (0.28)	27 (0.24)	Ref			
		G	170 (0.72)	87 (0.76)	1.25	0.7460–2.0978	0.7223	0.3954

Abbreviations: TLR, Toll-like receptor; SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table 6 Allele and genotype frequencies of *TLR4* gene polymorphisms in the Riyadh region compared to other populations

Population	Genotype frequency (n)			Allele frequency		χ^2	*P*-value
	TT	TC	CC	T	C		
(A) TLR4 rs2770150							
CRS (n=112)	0.491 (55)	0.384 (43)	0.125 (14)	0.683	0.317	–	–
CEU (n=226)	0.522 (118)	0.381 (86)	0.097 (22)	0.712	0.288	0.3006	0.5835
HCB (n=86)	0.977 (84)	0.023 (2)	–	0.988	0.012	30.0691	<0.005
JPT (n=88)	1.000 (88)	–	–	1.000	–	33.9123	<0.005
YRI (n=226)	0.796 (180)	0.204 (46)	–	0.898	0.102	24.1620	<0.005
MEX (n=100)	0.640 (64)	0.260 (26)	0.100 (10)	0.770	0.230	1.9997	0.1573
MKK (n=286)	0.720 (206)	0.231 (66)	0.049 (14)	0.836	0.164	11.4701	<0.005
TSI (n=176)	0.625 (110)	0.330 (58)	0.045 (8)	0.790	0.210	4.1589	0.0414
	AA	**AG**	**GG**	**A**	**G**	χ^2	***P*-value**
(B) TLR4 rs10759931							
CRS (n=118)	0.119 (14)	0.322 (38)	0.559 (66)	0.280	0.720	–	–
D-0 (n=48)	–	0.167 (8)	0.833 (40)	0.083	0.917	7.6210	0.0058
E-0 (n=38)	0.105 (4)	0.526 (20)	0.368 (14)	0.368	0.632	1.0657	0.3019
CABG (n=2,156)	0.141 (304)	0.458 (987)	0.401 (865)	0.370	0.630	3.9379	0.0472

Abbreviations: TLR, Toll-like receptor; CABG, coronary artery bypass graft; CEU, Utah residents with Northern and Western European ancestry from the CEPH collection; CEPH, Centre d"Etude du Polymorphisme Humain; HCB, Han Chinese in Beijing, China; JPT, Japanese in Tokyo, Japan; YRI, Yoruba in Ibadan, Nigeria; MEX, Mexican ancestry in Los Angeles, CA, USA; MKK, Maasai in Kinyawa, Kenya; TSI, Toscans in Italy; CRS, Saudi population residing in the Riyadh region of central Saudi Arabia; D-0, from Coriell Human Variation Panel – African American; E-0, from Coriell CEPH/Utah Pedigree – Caucasian; CABG, North American.

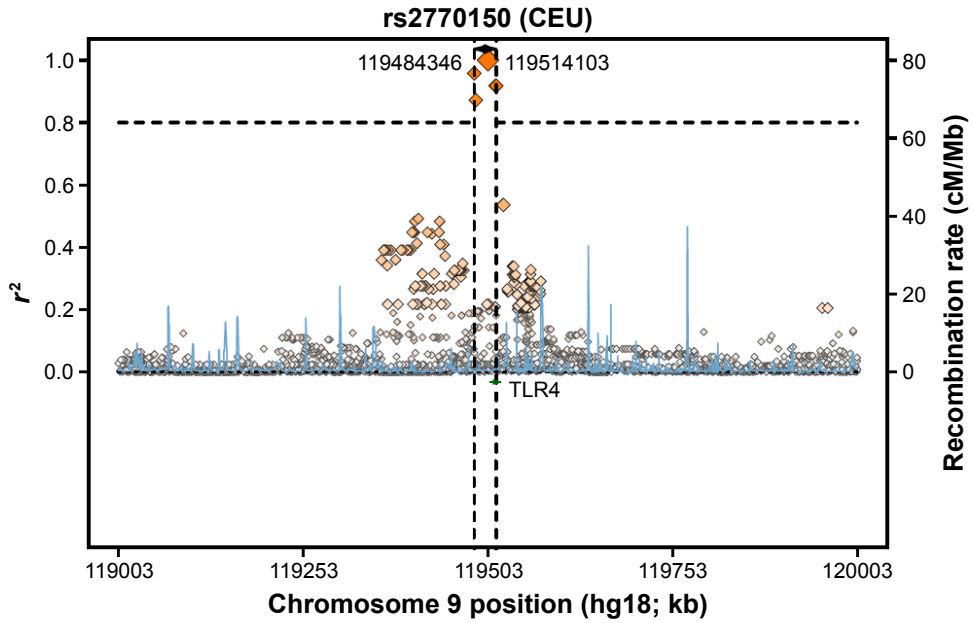

Figure 1 Regional LD plot for the TLR4 rs2770150 SNP.
Abbreviations: LD, linkage disequilibrium; TLR, Toll-like receptor; SNP, single-nucleotide polymorphism; CEU, Utah residents with Northern and Western European ancestry from the CEPH collection; CEPH, Centre d"Etude du Polymorphisme Humain.

(coronary artery bypass graft; $P<0.05$). Additionally, we constructed linkage disequilibrium plots for both the TLR4 rs2770150 and rs10759931 SNPs using SNP Annotation and Proxy Search (SNAP; http://www.broadinstitute.org/mpg/snap/ldplot.php; Figures 1 and 2). The maximum r^2 values for rs2770150 and rs10759931 were 0.958 and 0.965, respectively.

Discussion

Several studies have assessed the genetic changes following cigarette smoke exposure, typically identifying changes in innate immunity genes. Others have evaluated changes in gene expression of gingival epithelial cells in response to cigarette smoke.[42] Previous studies showed a clear link between smoking cigarettes and the pathogenesis of many

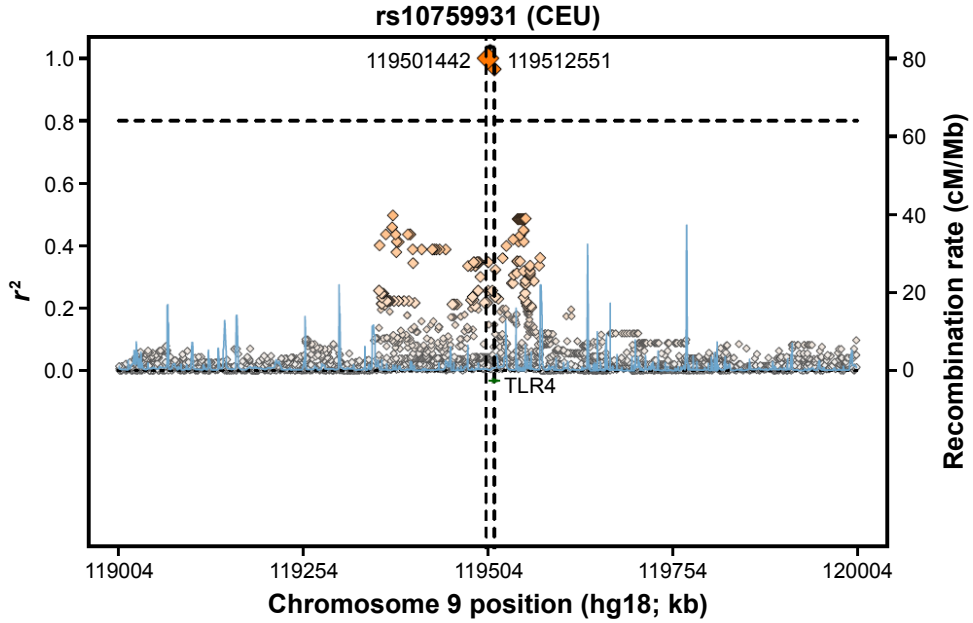

Figure 2 Regional LD plot for rs10759931 SNP in TLR4.
Abbreviations: LD, linkage disequilibrium; SNP, single-nucleotide polymorphism; TLR, Toll-like receptor; CEU, Utah residents with Northern and Western European ancestry from the CEPH collection; CEPH, Centre d"Etude du Polymorphisme Humain.

diseases, in particular, COPD, oral cancer, and periodontal disease. Additionally, we have previously shown that smoking tobacco affects *TLR4* expression via different pathways.[42] Impairment of TLR4 signaling becomes evident through the presence of SNPs that are associated with cancer susceptibility, and we have recently described an association between TLR4 polymorphism and colon cancer development.[27] Although they could have either positive or negative effects, polymorphisms in *TLR4* have been reported in various diseases.[25,26,43,44] These diseases and others have been found to be caused by tobacco smoke.[31–33]

In the present study, we showed that smoking has a slight effect on the rs2770150 and rs10759931 SNPs of TLR4. However, no significant association was observed between cigarette smoking and the genetic distribution of the SNPs investigated. Our results are contradictory to those previously published that show that the TLR4 rs2770150 and rs10759931 SNPs are associated with different diseases.[27–30] The lack of significant results may be explained by the lack of association between smoking and genetic variation in TLR4 rs2770150 and rs10759931 SNPs. Other SNPs in *TLR4*, especially those in regulatory regions or exons, may be associated with various diseases related to smoking. Thus, although these SNPs may not be related to smoking-induced diseases, we recommend performing other studies on SNPs located in the exons of *TLR4*.

Comparison of the data for the TLR4 rs2770150 SNP between the Riyadh population and other populations showed a pattern similar to that reported for SNPs located in other genes, such as the Thr241Met SNP in X-ray repair cross-complementing group 3 (*XRCC3*),[45] which reinforces the historical hypothesis of early human migration out of Africa.[46] The imbalance between the protective/affective effects of polymorphism is a key factor in the development of smoking-related diseases in human beings. Further investigations in larger populations of the same or mixed ethnicity could help to define the effects of smoking on different genes involved in the human innate immune system. Further insight into the genetic factors affected by smoking could lead to new approaches for cessation or prevention of smoking and treatment of many diseases caused by tobacco.

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Disclosure

The authors report no conflicts of interest in this work.

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