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Promoter Polymorphism of Toll-Like Receptor 4 is Associated with a Decreased Risk of Coronary Artery Disease: A Case-Control Study in the Chinese Han Population

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Background: Coronary artery disease (CAD) is considered a chronic inflammatory disease of the blood vessels. Toll-like receptor 4 (TLR4) is a transmembrane receptor involved in inflammatory reactions. The aim of this study was to determine the association between polymorphisms in the promoter region and 3'-untranslated region (3'-UTR) of TLR4, and the associated CAD risk.





Material/Methods: This study enrolled 424 participants with CAD and 424 controls without CAD. The polymorphisms in the promoter region and 3'-UTR of TLR4 were identified from the HapMap database, including rs10116253, rs10983755, and rs11536889. Genomic DNA was extracted from peripheral blood. Polymerase chain reaction-restriction fragment length polymorphism was performed to identify genotype polymorphisms. Relative luciferase activity was measured using the dual-luciferase reporter assay system.

Results: TLR4 rs10116253 in the promoter region was associated with CAD risk. The variant (CC+TC) genotypes of rs10116253 were associated with a decreased CAD risk (OR 95% CI 0.73 (0.54–0.98), $p=0.034$). In the stratification analyses, the variant (CC+TC) genotypes of rs10116253 were observed to have a relationship with decreased CAD risk in the male subgroup (OR: 95% CI 0.68 (0.48–0.98), $p=0.041$). Moreover, the variant CC and (CC+TC) genotypes of rs10116253 were correlated with a decreased CAD risk in participants younger than 60-year-old (TC: OR (95% CI 0.62 (0.39–0.98), $p=0.042$; TC+CC: OR 95% CI 0.63 (0.41–0.98), $p=0.039$). Regarding rs10116253, the luciferase activity of the mutant C allele construct was lower than that of the wild T allele construct (5.215 ± 0.009 vs. 5.304 ± 0.041 ; $p=0.087$).

Conclusions: The results provided evidence of an association between the TLR4 rs10116253 in the promoter region and a reduced risk of CAD.

MeSH Keywords: **Coronary Artery Disease • Polymorphism, Single Nucleotide • Promoter Regions, Genetic • Toll-Like Receptor 4**

Full-text PDF: <http://www.medscimonit.com/abstract/index/idArt/899587>

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Background

Coronary artery disease (CAD) is a major life-threatening complication of atherosclerosis worldwide, which has resulted in an increasing number of deaths in low- and middle-income countries, especially in China [1]. There are many factors involved in the pathogenesis of atherosclerosis. It has become evident that inflammatory reaction and immune response play an important role in the initiation and progression of atherosclerosis, from the formation of fatty streak to the destabilization of atherosclerotic plaque [2]. In general, the immune system responds effectively to harmful agents by two closely related pathways: the innate and the adaptive immune recognition systems [3]. As the gate of innate immune system and the trigger of adaptive immune system, toll-like receptors (TLR) can affect atherosclerosis in many ways [4].

So far, 13 TLRs have been identified in mammalian species, including 10 in humans [5]. Toll-like receptor 4 (TLR4) is regarded as the first human homologue of the *Drosophila* Toll, which has been extensively studied in the field of atherosclerosis [6]. It has been shown that TLR4 can not only recognize lipopolysaccharide (LPS), a major cell wall component of Gram negative bacteria, but also several endogenous ligands, such as minimally modified low density lipoprotein (mmLDL), the extra domain A (EDA) in fibronectin, heat shock protein (HSP). It has been confirmed that the endogenous ligands aforementioned are closely related to atherogenesis [7,8].

It is well known that genetic factors play important roles in the pathogenesis and development of atherosclerosis; and genes in several pathways that have been implicated in the pathogenesis and development of atherosclerosis have been considered. Some studies have focused on genes involved in lipid metabolism while others addressed endothelial, inflammatory response and mechanisms involved in the pathogenesis of hypertension [9]. Turner et al. revealed that a novel single nucleotide polymorphism (SNP) at the *SMAD3* locus correlated with lower *SMAD3* expression in blood and human plaque, which conferred protection from CAD [10]. Waart et al. reported that smokers with glutathione transferase M1 null genotype had a greater carotid intima-media thickness (IMT) as well as a higher 2-year progression rate of IMT [11]. It was reported that genetic variations in TLR4 could alter the expression of TLR4, thus influencing the pathogenesis of some inflammation-related diseases, including atherosclerosis and its complications [12]. Recently, two genetic variants (rs4986790 and rs4986791) of TLR4 gene in the coding region have been shown to be related to reduced CRP levels and, in parallel, a decreased risk of CAD [13]. However, a number of studies conducted in China, Japan, and South Korea have revealed that the frequencies of rs4986790 and rs4986791 variant genotype are zero [14].

In addition to the known rs4986790 and rs4986791 polymorphisms, studies have identified some polymorphisms in the promoter region and 3'-untranslated region (3'-UTR) of TLR4. Some researchers reported that the polymorphisms in the promoter region of TLR4 might be associated with decreased risks of prostate cancer and gastric cancer [15,16]. Castaño-Rodríguez et al. found that polymorphism in 3'-UTR of TLR4 was related to an increased risk of gastric cancer [17]. However, no study has reported on the relationship between polymorphisms in the promoter region and 3'-UTR of TLR4, and the risk of CAD. Thus, the aim of this study was to investigate the association between three tag-SNPs (rs10116253, rs10983755, and rs11536889), which capture the essential genetic information about the promoter region, and the 3'-UTR of TLR4 gene, and the risk of CAD in the Chinese Han population of Northern China.

Material and Methods

Study population

We enrolled 848 Chinese participants who underwent coronary angiography at The First Affiliated Hospital of Chinese Medical University from October 2012 to January 2016. Written informed consent was obtained from each participant, which was approved by the Ethics Committee of The First Affiliated Hospital of Chinese Medical University.

According to the presence or absence of CAD, the cohort participants were classified into a CAD group and a control group. CAD was defined as $\geq 50\%$ of luminal stenosis in at least one major coronary vessel based on the result of coronary angiography, which was determined by agreement of two independent operators. The exclusion criteria were: participants with cardiomyopathy, auto-immunologic disease, severe kidney or liver disease, or malignant disease [18].

Demographic data included sex, age, smoking status (smokers were defined as having smoked at least one cigarette per day for more than one year), alcohol consumption (drinkers were defined as having consumed at least one alcoholic drink a day for a minimal period of six months), blood pressure (hypertension was defined as $\geq 140/90$ mm Hg, or any antihypertensive treatment), blood glucose (diabetes mellitus (DM) was defined as fasting plasma glucose ≥ 7.0 mmol/L, or 2-hour plasma glucose ≥ 11.1 mmol/L, or any hypoglycemic therapy), and blood lipid (hyperlipidemia was defined as plasma cholesterol concentration ≥ 5.17 mmol/L, or plasma triglyceride concentration ≥ 1.70 mmol/L, or plasma low-density lipoprotein cholesterol concentration ≥ 2.58 mmol/L, or any anti-lipidemic therapy), were retrospectively extracted from registered documents or questionnaires.

SNP selection

Genotype data were downloaded from online database (Phase I+II+III, Release 27, <http://www.HapMap.org>). The selection criteria of tag-SNPs were as follows [19]: 1) tag-SNPs were in Chinese Han Beijing population; 2) tag-SNPs were within extended gene regions of TLR4 (10 kb upstream of the transcription initiation site and 10 kb downstream of the termination site); 3) tag-SNPs were in strong pairwise linkage disequilibrium ($r^2 > 0.8$); and 5) the minor allele frequency of tag-SNPs was larger than 1%. Accordingly, seven tag-SNPs were listed (rs10759930, rs2737191, rs10116253, rs10983755, rs11536889, rs7873784, and rs11536898). Then, using the FuncPred software (<http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm>), three tag-SNPs were selected for further analyses. Among them, rs10116253 and rs10983755 were predicted to be putative transcription factor binding sites, and rs11536889 might be a functional site mapped to the 3'-UTR.

Genotyping

The genotyping method was described previously [20]. Genomic DNA of each participant was extracted from blood clots using the standard phenol-chloroform method. TLR4 polymorphisms were detected using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) procedure. A 25 μ L reaction volume was mixed with 0.5 μ L of 10 μ M forward primer, 0.5 μ L of 10 μ M reverse primer, 0.5 μ L of DNA extract, 0.25 μ L of rTaq DNA polymerase, 2 μ L of 2.5 mM dNTPs mixture, 2.5 μ L of 10 \times PCR buffer, and 18.75 μ L of purified ddH₂O. Reaction conditions were 95°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, X °C for 30 seconds (X=63°C for rs10116253, 60°C for rs10983755, and 63°C for rs11536889), and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. The primer sequences are listed in Supplementary Table 1. The PCR products were digested overnight with restriction endonucleases (Fermentas, USA): BsmAI for rs10116253 at 37°C, Tail for rs10983755 at 65°C, and Taal for rs11536889 at 65°C, respectively. Eventually, the genotypes were discriminated on 3.0% agarose gel and visualized by staining with GeneFinder (Biov, China). The wild-type T allele of rs10116253 produced two fragments of 470 bp and 131 bp, and the polymorphic C allele produced a single 601 bp fragment. The wild-type G allele of rs10983755 produced two fragments of 342 bp and 121 bp, and the variant A allele produced a single 463 bp fragment. The wild-type G allele of rs11536889 produced a single 391 bp fragment, and the polymorphic C allele produced two fragments of 292 bp and 99 bp. Electrophoretic separations of TLR4 polymorphisms are shown in Figures 1–3.

Transient transfection and dual-luciferase assay

The fragment of TLR4 promoter region (–2700 to –246) was amplified from genomic DNA, which was subsequently cloned

into the luciferase expression vector pGL3 (Promega, Madison, Wisconsin, USA). Plasmids pGL3-basic-T and pGL3-basic-C (pGL3-basic; Promega) were constructed through site-directed mutagenesis. All plasmids used in this study were verified by sequencing to be fully consistent with the target sequence.

Human embryonic kidney (HEK) 293T cells were cultured in DMEM with 10% fetal bovine serum (Hyclone, USA). Then plasmids pGL3-basic, pGL3-basic-G, and pGL3-basic-A were transfected with the HEK293T cells using Lipofectamine 2000 (Invitrogen, USA). The empty vector (pGL3-basic) was used as a negative control, and pGL3-control vector (Promega) was used as a positive control in the transfection experiments. After 30 hours of incubation, the cells were collected, and the luciferase activities were analyzed using the Dual-Glo Luciferase Assay System (Promega) according to manufacturer's protocol. Each experiment was performed in triplicates. The relative luciferase activity in each well was calculated as the ratio of firefly luciferase activity to Renilla luciferase activity.

Statistical analyses

All statistical analyses were performed using the SPSS 16.0 statistical software package (SPSS, Chicago, USA). Continuous variables were presented as mean \pm SD and compared by ANOVA test. Discrete variables were represented as frequencies and percentages and evaluated by χ^2 test. The Hardy-Weinberg equilibrium (HWE) in the control group was evaluated for each SNP by χ^2 test. To assess the association between each SNP and CAD risk, the odds ratio (OR) with 95% confidence intervals (CI) was determined using multiple logistic regression analyses after adjustment for potentially confounding factors (age, sex, hypertension, DM, and hyperlipidemia). Differential luciferase expression between the wild type and mutant type was analyzed by Student's t-test. Results were considered to be statistically significant if *p* values were less than 0.05.

Results

Characteristics of the participants

The study cohort included 424 participants with CAD and 424 controls without CAD. The clinical characteristics are summarized in Table 1. No significant differences existed in baseline parameters of age and sex ($p > 0.05$). Compared with the control group, a higher prevalence of hypertension, DM, and hyperlipidemia was found in the CAD group ($p < 0.05$).

Association of TLR4 SNPs with CAD in total population

Genotype distributions of the three tag-SNPs in the control group followed HWE ($p > 0.05$). Compared with the common

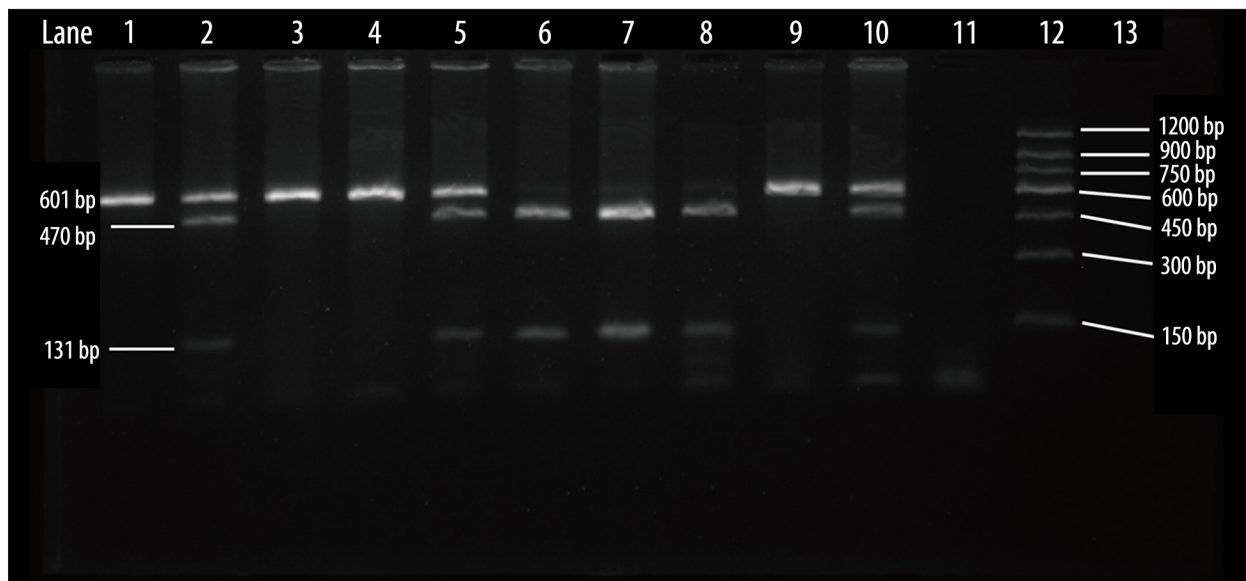


Figure 1. Polymerase chain reaction–restriction fragment length polymorphism assay for analyzing TLR4 rs10116253 polymorphism. Lane 12 shows 150 bp DNA marker; Lane 11 shows purified ddH₂O; Lane 13 shows blank; Lanes 2, 5, and 10 show rs10116253 TC genotype; Lane 6, 7, and 8 show rs10116253 TT genotype; Lanes 1, 3, 4, and 9 show rs10116253 CC genotype.

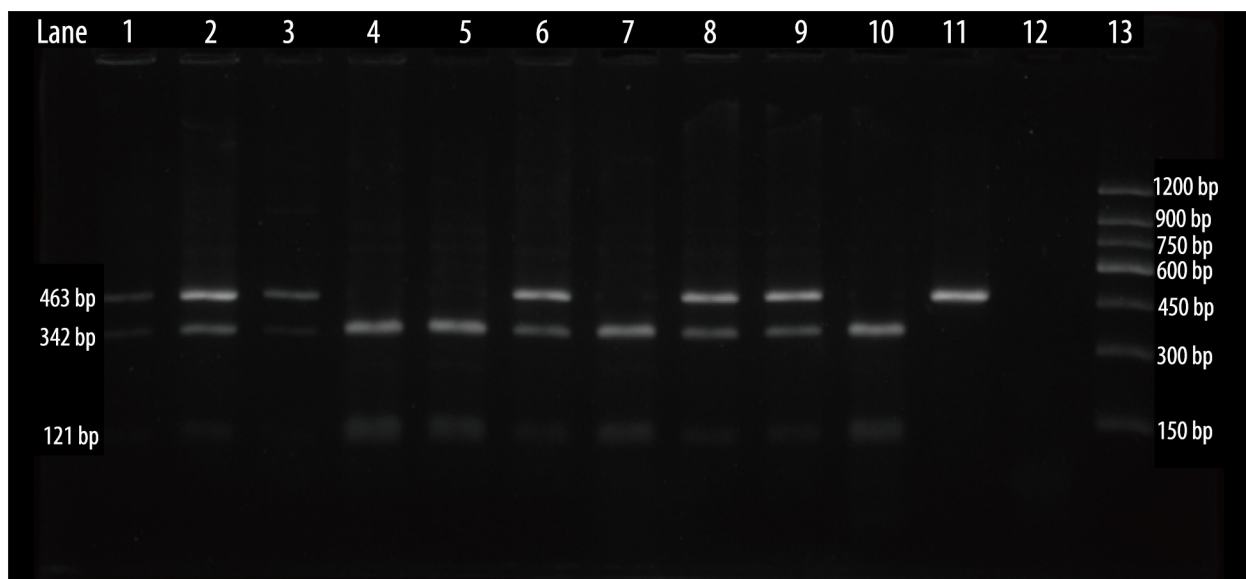


Figure 2. Polymerase chain reaction–restriction fragment length polymorphism assay for analyzing TLR4 rs10983755 polymorphism. Lane 13 shows 150 bp DNA marker; Lane 12 shows purified ddH₂O; Lane 1, 2, 3, 6, 8, and 9 show rs10983755 GA genotype; Lanes 4, 5, 7, and 10 show rs10983755 GG genotype; Lane 11 shows rs10983755 AA genotype.

homozygous genotype, we found that the (CC+TC) variant genotypes of rs10116253 were associated with a decreased risk of CAD in the total population, with corresponding OR of 0.73 (95% CI: 0.54–0.98, $p=0.034$) (Table 2). However, rs10983755 and rs11536889 were not statistically associated with CAD risk in the total population ($p>0.05$) (Table 2).

Association of TLR4 SNPs with CAD in subpopulation

In the stratification analyses, it was revealed that the (CC+TC) variant genotypes of rs10116253 were associated with reduced CAD risk in the male group (OR: 95% CI 0.68 (0.48–0.98), $p=0.0410$). In addition, the TC and TC+CC variant genotypes of rs10116253 was correlated with a decreased risk of CAD in participants younger than 60-year-old (TC, OR: 95% CI

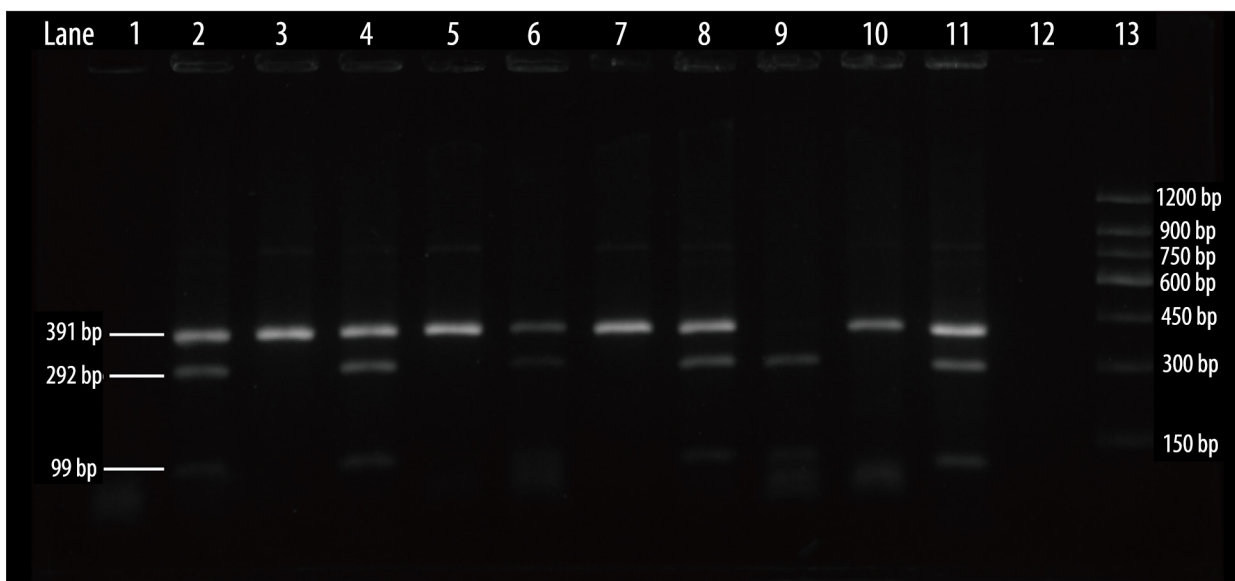


Figure 3. Polymerase chain reaction–restriction fragment length polymorphism assay for analyzing TLR4 rs11536889 polymorphism. Lane 13 shows 150 bp DNA marker; Lane 1 shows purified ddH₂O; Lane 12 shows blank; Lane 2, 4, 6, 8, and 11 show rs11536889 GC genotype; Lanes 3, 5, 7, and 10 show rs11536889 GG genotype; Lane 9 shows rs11536889 CC genotype.

Table 1. Baseline characteristics of the study subjects.

Index	Cases		Controls		P
Total	N=424		N=424		
Age	59.47±10.81		59.27±10.89		0.790
Sex					
Male	269	(63.4)	258	(60.8)	0.436
Female	155	(36.6)	166	(39.2)	
Smoking					
Yes	166	(39.2)	124	(29.2)	0.090
No	257	(60.6)	247	(66.3)	
Missing	1	(0.2)	53	(12.5)	
Alcohol consumption					
Yes	63	(14.9)	67	(15.8)	0.230
No	360	(84.9)	304	(71.7)	
Missing	1	(0.2)	53	(12.5)	
Hypertension					
Yes	291	(68.6)	244	(57.5)	0.001
No	133	(31.4)	180	(42.5)	
DM					
Yes	165	(38.9)	101	(23.8)	0.000
No	259	(61.1)	323	(76.2)	
Hyperlipidemia					
Yes	227	(53.5)	186	(43.9)	0.005
No	197	(46.5)	238	(56.1)	

DM – diabetes mellitus.

Table 2. Association of TLR4 polymorphisms with the risk of coronary artery disease.

Genotype	Crude		Adjust		
	OR (95% CI)	P	OR (95% CI)*	P	
TLR4 rs10116253	TC vs. TT	0.72 (0.53–0.97)	0.032	0.74 (0.54–1.01)	0.061
	CC vs. TT	0.69 (0.46–1.02)	0.063	0.68 (0.45–1.03)	0.066
	TC+CC vs. TT	0.71 (0.53–0.95)	0.019	0.73 (0.54–0.98)	0.034
TLR4 rs10983755	GA vs. GG	0.75 (0.56–0.99)	0.044	0.77 (0.58–1.03)	0.077
	AA vs. GG	0.82 (0.50–1.35)	0.436	0.77 (0.46–1.28)	0.311
	GA+AA vs. GG	0.76 (0.58–1.00)	0.046	0.77 (0.58–1.01)	0.059
TLR4 rs11536889	GC vs. GG	1.15 (0.86–1.53)	0.358	1.16 (0.86–1.56)	0.343
	CC vs. GG	1.26 (0.69–2.31)	0.453	1.31 (0.70–2.42)	0.397
	GC+CC vs. GG	1.16 (0.88–1.53)	0.289	1.18 (0.89–1.56)	0.263

OR – odds ratio; CI – confidence intervals; * ORs and 95% CI and corresponding P values were calculated by logistic regression.

Table 3. Stratification analyses of association between TLR4 polymorphisms and the risk of coronary artery disease.

Variable	TLR4 rs10116253			TLR4 rs10983755			TLR4 rs11536889		
	Genotype	OR (95% CI)	P	Genotype	OR (95% CI)	P	Genotype	OR (95% CI)	P
Sex*	TC vs. TT	0.70 (0.47–1.03)	0.067	GA vs. GG	0.76 (0.53–1.10)	0.141	GC vs. GG	1.17 (0.81–1.70)	0.405
	CC vs. TT	0.66 (0.40–1.10)	0.113	AA vs. GG	0.65 (0.33–1.27)	0.209	CC vs. GG	1.12 (0.51–2.44)	0.777
	TC+CC vs. TT	0.68 (0.48–0.98)	0.041	GA+AA vs. GG	0.73 (0.52–1.04)	0.081	GC+CC vs. GG	1.17 (0.82–1.67)	0.400
Female	TC vs. TT	0.85 (0.50–1.47)	0.571	GA vs. GG	0.77 (0.48–1.25)	0.289	GC vs. GG	1.10 (0.66–1.82)	0.717
	CC vs. TT	0.67 (0.33–1.37)	0.267	AA vs. GG	0.92 (0.40–2.11)	0.848	CC vs. GG	1.87 (0.67–5.22)	0.233
	TC+CC vs. TT	0.82 (0.49–1.39)	0.467	GA+AA vs. GG	0.80 (0.50–1.27)	0.342	GC+CC vs. GG	1.20 (0.74–1.92)	0.463
Age#	TC vs. TT	0.86 (0.56–1.33)	0.502	GA vs. GG	0.88 (0.58–1.32)	0.535	GC vs. GG	1.35 (0.90–2.04)	0.150
	CC vs. TT	0.71 (0.40–1.24)	0.227	AA vs. GG	0.64 (0.31–1.32)	0.223	CC vs. GG	0.92 (0.39–2.16)	0.854
	TC+CC vs. TT	0.81 (0.54–1.22)	0.310	GA+AA vs. GG	0.82 (0.56–1.21)	0.316	GC+CC vs. GG	1.28 (0.87–1.90)	0.209
	TC vs. TT	0.62 (0.39–0.98)	0.042	GA vs. GG	0.66 (0.43–1.01)	0.052	GC vs. GG	1.00 (0.64–1.56)	0.994
<60	CC vs. TT	0.63 (0.34–1.16)	0.134	AA vs. GG	0.86 (0.41–1.82)	0.692	CC vs. GG	2.08 (0.80–5.42)	0.134
	TC+CC vs. TT	0.63 (0.41–0.98)	0.039	GA+AA vs. GG	0.70 (0.46–1.04)	0.077	GC+CC vs. GG	1.12 (0.74–1.71)	0.591

OR – odds ratio; CI – confidence intervals; * ORs and 95% CI and corresponding P values were calculated by logistic regression analyses adjusted by age, hypertension, diabetes mellitus and hyperlipidemia; # ORs and 95% CI and corresponding P values were calculated by logistic regression analyses adjusted by sex, hypertension, diabetes mellitus and hyperlipidemia.

0.62 (0.39–0.98), $p=0.042$); TC+CC, OR: 95% CI 0.63 (0.41–0.98), $p=0.039$) (Table 3). There was no relationship between TLR4 rs10983755, TLR4 rs11536889, and CAD risk in the stratification analyses ($p>0.05$) (Table 3).

Effects of TLR4 polymorphism on gene promoter activity

Regarding TLR4 rs10116253, the wild T allele and mutant C allele constructs were tested for their effects on transcriptional activity in HEK293T cells along with the control vector (pGL3-basic). Compare to the wild T allele construct, the mutant C allele construct showed a trend toward decreased luciferase

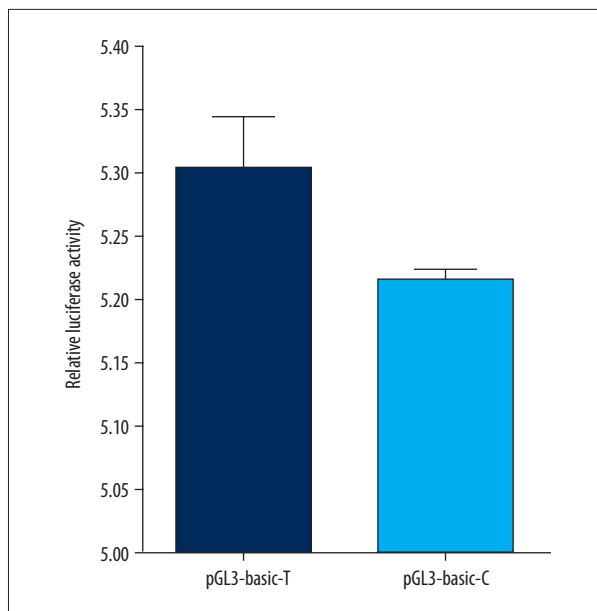


Figure 4. Effect of rs10116253 on the transcriptional activity of TLR4. The transcriptional activity is expressed as relative luciferase activity (the ratio of Firefly to Renilla activity). pGL3-basic T: the promoter construct containing wild T allele; pGL3-basic C, the promoter construct containing variant C allele.

activities (5.215 ± 0.009 vs. 5.304 ± 0.041), although it did not reach statistical significance ($p=0.087$) (Figure 4).

Transcription factor prediction

The promoter of genes can interact with transcriptional factors, which have various versatilities in regulating the expression of genes [21]. The promoter sequence variation can result in changeable transcriptional activities, and thus alter the gene's transcription and thereby modulating disease risks. Driven by such an assumption, the partial promoter sequences containing the rs10116253 locus were applied to predict potential transcription factors using the JASPAR online tools (<http://jaspar.genereg.net/>). Interestingly, rs10116253 wild T allele was predicted as a transcription factor binding site of NFATC2, but the variant C allele was not supposed to have such ability.

Discussion

In this study, we found that TLR4 rs10116253 variant genotypes were related to decreased risk of CAD in the Chinese Han population, especially in males and participants younger than 60-year-old. The mutant C allele construct showed a trend toward decreased luciferase activities in comparison with the wild T allele construct, which might partly explain the protection effect of TLR4 rs10116253 on CAD risk.

TLR4, one of best characterized TLRs, is a transmembrane receptor involved in inducing inflammatory responses [22]. As a chronic inflammatory vessel disease, CAD is one of the leading causes of death worldwide [23]. Molecular biology and epidemiology studies have been accumulating suggesting that TLR4 is related to the occurrence and development of CAD [24]. Recently, the association between TLR4 polymorphism and CAD risk has generated increased interest. Clinical studies have found the missense polymorphisms rs4986790 and rs4986791 in the coding region of TLR4 were associated with blunted receptor activity and subsequently diminished inflammatory response [25]. These two genetic polymorphisms are closely related to the onset of acute coronary events independent of other traditional risk factors [26]. The variant genotypes of rs4986790 and rs4986791 are almost never found in Asian populations [27]. We have known that the sequence variants in the promoter region may change the binding capacity of certain transcription factor, which holds great promise in altering the gene's transcription [28]. So far, no study has explored the relationship of polymorphism in the promoter region of TLR4 with CAD risk. This is the first study to evaluate the genetic effects of TLR4 promoter polymorphisms on the risk of CAD.

In the present case-control study, we have reported that TLR4 rs10116253 was related to decreased risk of CAD in the Chinese Han population, which may be attributed to the important role TLR4 played in the process of atherogenesis. There could be a number of explanations for this. As the gate of host defense, TLR4 not only could be recognized by some exogenous ligands (for example, LPS of Gram-negative bacteria, lipoteichoic acids of Gram-positive bacteria and chlamydial HSP60), but also it could be activated by a few endogenous ligands (HSP60, EDA in fibronectin, hyaluronan, and mmLDL), all of which have been shown to played an important role in the initiation of atherosclerosis [29–31]. In addition, with activation of the arterial wall cells (e.g., endothelial cells, vascular smooth muscle cells, adventitial fibroblasts, dendritic cells, and macrophages), TLR4 expression has been shown to be upregulated [32–34]. Edfeldt et al. showed that TLR4 expression was markedly augmented in the human atherosclerotic vessel. In contrast, TLR4 was expressed at low level in normal artery [35]. Furthermore, the activation of TLR4 could lead to upregulation of NF κ B and IRF3, which further mediated pro-inflammatory cytokines production, adhesion molecules expression, and leukocyte infiltration [36]. It has been reported that the Fas death pathway could be stimulated by TLR4 activation, which could induce the expression of apoptotic molecules, and thus degraded the plaque [37]. All of the aforementioned demonstrates that TLR4 exerted effects on almost every step of atherogenesis.

The 5'-flanking sequences of human TLR4 consist of several important transcriptional regulatory regions that give rise

to alternative transcripts. Some of these regions contain potential transcription factor binding sites (including Ik-2 site, GATA-2 binding site, N-myc site), which are involved in regulating TLR4 transcription [18,38,39]. In the present study, it was found that the rs10116253 polymorphism in the promoter region of TLR4 had a protective effect on CAD risk in the Chinese Han population. To determine the effect of this rs10116253 T/C variant on gene expression, transient transfection studies in HEK293T cells were conducted. We found that the promoter construct containing the mutant C allele had a lower transcriptional activity than that of the wild T allele construct in HEK293T cells, although it did not reach statistical significance. As TLR4 is a transmembrane receptor, we conjecture that the difference in luciferase activity may be more significant if HEK293T cells were stimulated by some ligands. The results of this inquiry indicated that TLR4 rs10116253 affected the occurrence of CAD, possibly by altering gene promoter activity. Furthermore, we identify that rs10116253 wild T allele created a putative transcription factor binding site for NFATC2, while the mutant C allele did not have such capability. Thus, it was deduced that the TLR4 promoter might lose its ability to directly interact with transcription factor NFATC2 when the T allele was replaced by the C allele. However, no evidence has been shown that this predicted transcription factor has an effect on TLR4 levels directly or indirectly. Further investigations are needed to enlighten our understanding of the involved molecular mechanisms.

Stratification analyses demonstrated an association between rs10116253 variant genotypes and decreased risk of CAD in the male subgroup. In general, men are more likely than women to suffer from CAD due to the atheroprotective effects of estrogen [40]. In the most striking contrast, Rettew et al. demonstrated that TLR4 expression was upregulated in macrophages response to estrogen, and thus proinflammatory cytokines

production (e.g., IL-6, IL-1 β , and TNF- α) was increased [41]. The effect of estrogen on TLR4 expression might account for the sex disparity in our results. We also observed that rs10116253 variant genotypes were related to reduced risk of CAD in participants younger than 60-year-old. Hears et al. found that intermediate and nonclassical monocytes from aged individuals produced significantly higher levels of TNF α compared with young individuals, suggesting age was associated with the heightened inflammatory response of monocytes to TLR4 stimulation [42], which partially explains the association of rs10116253 polymorphisms with reduced risk of CAD in those younger than 60-year-old.

There were some limitations to this study. First, the relatively inadequate number of participants made it difficult to perform further analyses, such as haplotype analyses. Second, some information was lost in a small sample of participants, including smoking and drinking status, so that these elements could not be included as environmental factors in our multivariate logistic regression. Third, additional molecular-biology studies are necessary to identify the mechanisms and the biological effects of the promoter polymorphism of TLR4.

Conclusions

In conclusion, this study, for the first time, reported a modest association between rs10116253 polymorphism in the promoter region of TLR4 and the risk of CAD in the Chinese Han population. The TLR4 rs10116253 variant genotype conferred reduced risk of CAD, especially in males and participants younger than 60-year-old. The luciferase activity of the mutant allele construct was lower than that of the wild allele construct, although it did not reach a statistical significance, which might partly explain the effect of TLR4 rs10116253 on CAD.

Supplementary Table

Supplementary Table 1. Primer sequences of TLR4 polymorphisms.

Polymorphisms	Primer sequences
rs10116253	F: GGGTGAAAAGCCAGGTAGAGGAGGT; R: TGGAAAGTAGCAAGTGCAATGTAAGT
rs10983755	F: GCCAGAACATCAAGAACACAGAAAAG; R: TGGAAAGTAGCAAGTGCAATGTAAGT
rs11536889	F: GCAGGAAGGAAGTGGGATGAC; R: TGTTCTGAGGAGGCTGGATG

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