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Research Article

Frequency of TLR 2, 4, and 9 Gene Polymorphisms in Chinese Population and Their Susceptibility to Type 2 Diabetes and Coronary Artery Disease

Fengjing Liu,[1,2] Weixin Lu,[1] Qiaohui Qian,[1] Weigang Qi,[3] Jifan Hu,[2] and Bo Feng[1]

[1] *Department of Endocrinology, Tongji University, Affiliated East Hospital Tongji University, Shanghai 200120, China*
[2] *Department of Medicine, Palo Alto Health Care System, Veterans Affairs, School of Medicine, Stanford University, Palo Alto, CA 94304, USA*
[3] *Department of Cardiology, Affiliated East HospitalTongji University, Tongji University, Shanghai 200120, China*

Correspondence should be addressed to Jifan Hu, jifan@stanford.edu and Bo Feng, fengbo@medmail.com.cn

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Toll-like receptors (TLRs) are pivotal components of the innate immune response. Activation of the innate immune system and subsequent chronic low-grade inflammation are thought to be involved in the pathogenesis of atherosclerosis and type 2 diabetes. In the study, we genotyped TLRs gene polymorphisms, including TLR2 Arg677Trp and Arg753Gln, TLR4 Asp299Gly and Thr399Ile, TLR9-1486T/C and -1237T/C. The frequencies of TT, TC and CC genotype of TLR9-1486T/C mutation were 39.6%, 45.8% and 14.6%, respectively; the frequencies of T allele and C allele were 62.5% and 37.5%. However, neither of these parameters was statistically significant among study groups. In addition, we were surprised to find that the commonly reported TLR SNPs in the Western countries, like TLR2 Arg677Trp or Arg753Gln, TLR4 Asp299Gly or Thr399Ile and TLR9-1237T/C, were not polymorphic at all in all study subjects. In conclusion, our data suggests that TLR2 Arg677Trp or Arg753Gln, TLR4 Asp299Gly or Thr399Ile and TLR9-1237T/C polymorphisms have low frequency and TLR9-1486T/C polymorphism may not be a suitable marker in predicting the susceptibility to type 2 diabetes or coronary artery disease in the Chinese Han population.

1. Introduction

Coronary artery disease (CAD) is the leading cause of death in men and women worldwide, particularly in the developed countries [1]. Type 2 diabetes mellitus, a major cause of cardiovascular morbidity and mortality in developed countries, has increased significantly in East Asia, like Chinese Han population, as the local economy improves significantly in the last decade. Although the primary cause for this increment remains unclear, the activation of innate immunity system and chronic low-grade inflammation may be plausibly involved [2]. Recent studies have suggested the role of certain genetic variants of innate immunity system in the predisposition to the development of the disease.

Toll-like receptors (TLRs) are the family of genetically conserved transmembrane receptors involved in the innate immunity and pathogen recognition. Recognition of pathogen-associated molecular patterns by TLRs activates signaling events that induce the expression of effector molecules, such as cytokines and chemokines, controlling the adaptive immune responses [3, 4].

Genetic variations within genes encoding these receptors have an important influence on the pathogenesis of inflammatory diseases [5]. Variations within genes of the family of innate immune receptors may account, in part, for the inherited differences in the susceptibility to autoimmune diseases or inflammatory disease. The ability of certain individuals to respond properly to TLR ligands may be impaired by single nucleotide polymorphisms (SNPs) within TLR genes, resulting in an altered susceptibility to the disease.

Considering the potential role of TLRs pathway in the overall immune reconstitution, we examined whether the altered immune response caused by TLRs gene variation was associated with type 2 diabetes and/or its macrovascular complications. We were particularly interested in comparing

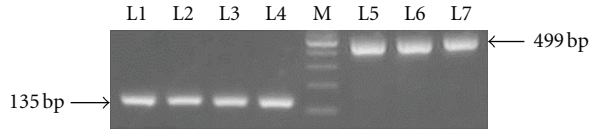

Figure 1: Electrophoretic result of TLR9 gene PCR products.100 bp DNA Ladder Marker is shown in lane M. TLR9 gene PCR products (TLR9-1237T/C = 135 bp, TLR9-1486T/C = 499 bp) are shown respectively in lanes 1–4 and lanes 5–7.

the role of TLR variations in subgroups of CAD patients with and without type 2 diabetes.

2. Materials and Methods

2.1. Study Subjects. A total of 432 subjects who underwent successfully percutaneous coronary intervention were consecutively recruited from the inpatient of Department of Cardiology at Tongji University Affiliated East Hospital between July 2008 and December 2009. Because the study was performed as a monocentric study, care was taken to avoid that any carriers of the polymorphisms studied were related to each other. All subjects were divided into two groups according to the presence ($n = 333$) or absence ($n = 99$) of CAD. And then patients with CAD were divided into two groups according to the presence ($n = 160$) or absence ($n = 173$) of T2DM; the patients without CAD were also divided into two groups according to the presence ($n = 33$) or absence ($n = 66$) of T2DM. The diagnosis of diabetes mellitus was based on the definition and diagnostic criteria for diabetes mellitus of WHO (World Health Organization) in 1999. CAD was defined as the result of coronary angiography, which was performed via the radial or femoral artery. All the angiographies were interpreted with the consensus from two independent operators blind to the results of the genetic analysis. The diagnosis of CAD was based on ≥50% of luminal stenosis in at least one major coronary vessel. Clinical data (Table 1) consist of age, gender, body mass index (BMI), obesity (BMI ≥ 25 kg/m^2), hypertension (≥140/90 mmHg or any antihypertensive treatment), dyslipidaemia (according to NCEP-ATP III criteria or any antilipemic therapy), and smoking status ("ever" or "never," "ever" defined as having smoked more than three cigarettes a day for at least 1 year). Individuals were excluded if they had evidence of normal or nearly normal coronary arteries, congestive heart failure, valvular heart disease, cardiomyopathy, inflammatory or neoplastic condition, and hepatic or renal dysfunction.

2.2. Ethical Approval. All subjects gave written informed consent for the intervention and the genetic analyses, which was reviewed and approved by the Ethics Committees of Tongji University. This study was in accordance with the principles of the Helsinki Declaration (1964).

2.3. Genotyping. Blood samples from all subjects were collected in EDTA vials and stored at −80°C until required. Genomic DNA was isolated from frozen whole blood using the EZ-10 Spin Column Whole Blood Genomic DNA MiniPreps Kit (Sangon, Canada) according to the manufacturer's instructions. The alleles of TLR gene polymorphisms were detected using polymerase chain restriction-restriction fragment length polymorphism (PCR-RFLP) procedure.

PCR reactions were set up in a 25 μL reaction volume containing 1.5μL 10 μM forward primer, 1.5 μL 10 μM reverse primer, 1.5 μL DNA extract, 12.5 μL GoTaq Green Master Mix (Promega, USA), and 8.0 μL sterilized ddH_2O. Reactions were run on a PTC-200 DNA Engine PCR (BIO-RAD, USA) using the following conditions: initial denaturation at 95°C for 5 min followed by 36 cycles of denaturation at 94°C for 40 seconds, annealing at X°C for 40 seconds, and extention at 72°C for 1 minute. A final extension step was at 72°C for 10 minute. The PCR products were held at 4°C until analysis. The primers and PCR conditions used to detect TLR variants were listed in Table 2. PCR products were electrophoresed in an agarose gel and visualized by ethidium bromide staining. TLR9 gene PCR products were shown in Figure 1.

The PCR products (10 μL) were digested overnight or for 12–14 h at 37°C with 5U of restriction endonucleases: AciI for TLR2 Arg677Trp and Arg753Gln polymorphisms, NcoI for TLR4 Arg299Gly and HinfI for TLR4 Arg399Ile polymorphisms, and BstNI for TLR9-1237T/C and AflII for TLR9-1486T/C polymorphisms. Digested PCR products were separated by electrophoresis through 1%–2.5% agarose gel. After digestion, the wild-type allele sizes of TLR2 were 55 bp, 147 bp, and 228 bp; the wild-type allele sizes of TLR4 did not change and they were 249 bp for the Asp299 (A allele) and 406 bp for the Thr399 (C allele); the wild-type allele sizes of TLR9-1237T/C polymorphism were 108 bp and 27 bp; the wild-type allele sizes of TLR9-1486T/C polymorphism were 172 bp and 327 bp. The sizes for polymorphic alleles were 283 bp and 147 bp for TLR2 Arg677Trp; 375 bp and 55 bp for TLR2 Arg753Gln; 223 bp and 26 bp for TLR4 299Gly (G allele) and 377 bp for TLR4 399Ile (T allele); 60 bp, 48 bp, and 27 bp for TLR9-1237T/C and 499 bp for TLR9-1486T/C. Electrophoretic separation of TLR9 gene -1486T/C polymorphism was shown in Figure 2.

2.4. Statistical Analysis. All the statistical analyses were performed using the SPSS software for Windows version 16.0. Chi-square analysis was used to assess deviation from Hardy-Weinberg equilibrium (HWE) and to compare the genotype and allele frequency between groups. Analysis of variance was used to investigate associations with clinical or metabolic parameters. Multivariant analysis was done by ANOVA. A P value < 0.05 was considered statistically significant.

3. Results

Compared with non-CAD group, patients with CAD had higher incidence of DM (33/99 versus 160/333, $P < 0.05$). Also, compared with non-DM group, patients with DM had higher incidence of CAD (173/239 versus160/193, $P < 0.05$).

Table 1: Clinical characteristics among groups in study population.

	Control	CAD	DM	DM + CAD	P
n, male/female	43/23	113/60	20/13	95/65	$P = 0.811$*
Age, years	60.3 ± 8.0	62.9 ± 9.6	61.6 ± 8.7	65.3 ± 8.7	$P = 0.165$†
Smoker, n (%)	20 (30.0%)	76 (43.9%)	9 (27.3%)	58 (36.2%)	$P = 0.108$*
Obesity, n (%)	11 (16.7%)	21 (12.1%)	6 (18.2%)	26 (16.2%)	$P = 0.633$*
Hypertention, n (%)	29 (43.9%)	107 (61.8%)	17 (51.5%)	120 (75.0%)	$P < 0.005$*
Dyslipidaemia, n (%)	26 (39.4%)	93 (53.8%)	18 (54.5%)	97 (60.4%)	$P = 0.037$*

Data were means ± SD or percentages (%). *χ^2 test. †One-way ANOVA.

Table 2: Primers, PCR conditions, and restriction enzymes used for genotyping TLRs gene.

SNPs		Primer sequence 5′-3′	AT	PCR product	RE	Expected product
TLR2	Arg677Trp	F: TAT GGT CCA GGA GCT GGA GA	60°C	430 bp	AciI	Wt: 228 bp
	Arg753Gln	R: TGA CAT AAA GAT CCC AAC TAG ACA A				Arg677Trp: 283 bp Arg677Trp: 375 bp
TLR4	Arg299Gly	F: GAT TAG CAT ACT TAG ACT ACT ACC TCC ATG R: GAT CAA CTT CTG AAA AAG CAT TCC CAC	55°C	249 bp	NcoI	Wt: 249 bp Mt: 223 bp
	Thr399Ile	F: GGT TGC TGT TCT CAA AGT GAT TTT GGG AGA A R:A CCT GAA GAC TGG AGA GTG AGT TAA ATG CT	60°C	406 bp	HinfI	Wt: 406 bp Mt: 377 bp
TLR9	T-1237C	F: ATG GGA GCA GAG ACA TAA TGG A R: CTG CTT GCA GTT GAC TGT GT	62°C	135 bp	BstNI	Wt: 108 bp Mt: 60 bp 48 bp
	T-1486C	F: TCC CAG CAG CAA CAA TTC ATT A R: CTG CTT GCA GTT GAC TGT GT	62°C	499 bp	AflII	Wt: 327 bp Mt: 499 bp

F: forward primer; R: reverse primer; AT: annealing temperature; RE: restriction enzyme.

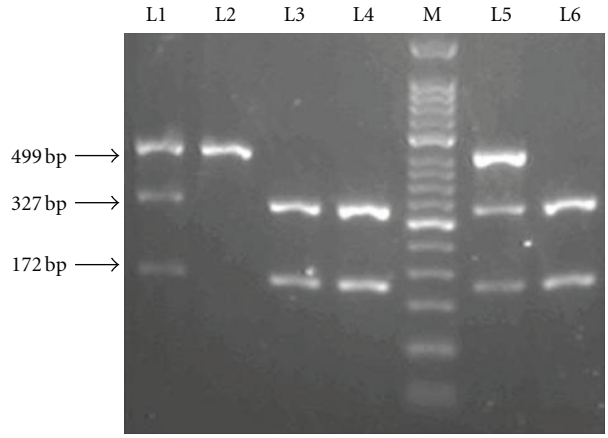

Figure 2: Electrophoretic separation of TLR9 gene PCR fragments after restriction endonuclease cleavage on a 2% agarose gel. Two heterozygous carriers are shown in lane 1 and 5. A homozygous mutation sample is shown in lane 2. Three wild-type samples are shown in lanes 3, 4, and 6. 50 bp DNA Ladder Marker is shown in lane M.

In T2DM group, 14 of the 33 patients were heterozygotes for TLR9-1486T/C polymorphism and 6 were mutant homozygotes. In CAD group, 76 of the 173 patients were heterozygous for TLR9-1486T/C polymorphism and 28 were mutant homozygous. In T2DM and CAD group, 71 of the 160 patients were heterozygous for TLR9-1486T/C polymorphism and 23 were mutant homozygous. In control group, 37 of the 66 patients were heterozygotes for TLR9-1486T/C polymorphism and 6 were mutant homozygotes. The frequencies of TLR-1486T/C genotypes and alleles were not significantly different between groups, respectively ($P = 0.642$, $P = 0.962$, Table 3).

We also examined several other TLR family gene variants that have been commonly reported in Western countries, including TLR9 promoter-1237T/C polymorphism, TLR2 Arg677Trp and Arg753Gln polymorphisms, and TLR4 Asp299Gly and Thr399Ile polymorphisms in all subjects. To our surprise, we did not detect the presence of any variant for these SNPs in our subjects (data not shown).

4. Discussion

It is well known that diabetes mellitus is not only a chronic inflammation disease, but also a disease of innate immune system. Long-term innate immune system activation, resulting in chronic inflammation, elicits disease in individuals who develop T2DM [6, 7]. Schmidt et al. found that markers of inflammation, including total sialic acid (SA), orosomucoid, haptoglobin, and α1-antitrypsin, predict CAD and are increased in patients with T2DM [8]. Another study reported that markers of inflammation, such

Table 3: Genotype distribution of TLR9-1486T/C promoter polymorphism.

SNP	Genotypes			Total	Allele frequency	
	TT	TC	CC		C	T
Non-CAD	46	41	12	99	65 (32.8%)	133 (67.2%)
CAD	135	147	51	333	249 (37.4%)	417 (62.6%)
P		$\chi^2 = 1.304$ $P = 0.521$			$\chi^2 = 1.371$ $P = 0.242$	
Non-DM	91	103	34	239	171 (37.5%)	285 (62.5%)
DM	79	85	29	193	143 (37%)	243 (63%)
P		$\chi^2 = 0.058$ $P = 0.971$			$\chi^2 = 0.018$ $P = 0.892$	
Control	23	37	6	66	49 (37.1%)	83 (62.9%)
CAD	69	76	28	173	132 (38.2%)	214 (61.8%)
DM	13	14	6	33	26 (39.4%)	40 (60.6%)
DM + CAD	66	71	23	160	117 (36.6%)	203 (63.4)
P		$\chi^2 = 4.258$ $P = 0.642$			$\chi^2 = 0.292$ $P = 0.962$	

Control: control group; CHD: CAD group; DM: T2DM group; DM + CAD = type 2 diabetes mellitus plus coronary artery disease group.

Table 4: Comparison of TLR9-1486T/C promoter polymorphism in Asian population.

Nationality	Genotype			P
	TT	TC	CC	
Hong Kong	335 (43.6%)	350 (45.5%)	84 (10.9%)	0.256
Japanese	51 (25.8%)	108 (54.5%)	39 (19.7%)	0.093
This study	23 (34.8%)	37 (56.1%)	6 (9.1%)	

as C-reactive protein (CRP) levels, white blood cell, and fibrinogen, were associated with the development of diabetes in the elderly [9]. In addition, the circulating inflammatory markers interleukin 6, acute-phase reactants, and especially CRP have been shown to predict the development of T2DM [10–12].

The concept of atherosclerosis being a chronic inflammation disease has been reviewed by Ross [13]. So far, many studies have demonstrated that atherosclerosis is an active, inflammatory process, rather than simply a passive infiltration of lipids [14–19]. T2DM is a common and potent risk factor for coronary artery disease. The presence of T2DM significantly increases the risk of atherosclerosis [2, 20–22] as our present study results corroborate. In a word, T2DM and atherosclerotic cardiovascular disease have common antecedents.

A homologous family of toll-like receptors (TLRs) was discovered in 1997 [23]. TLRs are differentially expressed among immune cells and serve as pattern-recognition receptors in mammals. They recognize and bind to conserved pathogen-associated molecular patterns shared by large groups of microorganisms and trigger the activation of signal transduction pathways, which in turn induce dendritic cell maturation and cytokine production [24]. These receptors play a central role in the activation of innate immunity [25]. A growing body of data supports a role of specific single nucleotide polymorphisms (SNPs) in several TLR genes in modulating the risk of bacterial and viral infections.

The relationship between inflammation, innate immunity, and diabetes was not fully understood. To prove whether inflammation contributes to the onset or progression of diabetes, we examined whether a constitutive defect in the innate immune response as occurs in the form of TLRs polymorphism was associated with reduced diabetes risk and its chronic macrovascular complication [26].

First, Our findings showed that there were three genotypes in TLR9 promoter-1486T/C position in Chinese Han population. The frequency of the C allele was 37.5%. The genotype frequencies of the TLR9-1486T/C in Chinese Han population were similar to those of Koreans [27]. But exploratory analyses did not support the association between TLR9-1486T/C polymorphism and the prevalence of T2DM or angiographic coronary artery disease. Although no significant association was found, the CC genotype of TLR9-1486T/C tended to be overrepresented in patients with T2DM compared with controls (18.2% versus 9.1%, $P = 0.055$). However, the frequency of C allele in controls is not in line with the finding from Japanese population ($P = 0.093$, Table 4) [28].

It was reported that Korea population had low frequency of TLR9-1237T/C (<0.3%) [27]. No TLR9-1237T/C polymorphism was present in 183 patients with SLE and 198 controls in a Japanese population [28]. But high frequency of the variant allele was reported in the Caucasian population (11–16%) [29]. The genetic variation at position-1237 is associated with an increased risk of asthma in European American populations [30]. However, we did not detect any SNP at position-1237 in our study, and Korean and Japanese populations and other Chinese population also have much less variations at position-1237 [27, 28, 31]. It would be very interesting to examine whether distinct TLR9-1237T/C genotype variations worldwide may be related to the disease patterns in these countries, especially considering the relatively low incident rate of heart diseases in the Oriental populations.

Many studies in Caucasians suggested that TLR4 polymorphisms were associated with innate immunity-related diseases, such as chronic inflammatory disease and atherosclerosis [32–34]. One study showed that TLR4 Asp299Gly polymorphism was associated with reductions

in vascular inflammation, angiographic coronary artery disease, and clinical diabetes in Caucasian populations [26]. A strong association between the TLR4 Asp299Gly/Thr399Ile polymorphism and diabetic neuropathy was showed in Rudofsky's study [35]. In contrast, TLR4 Asp299Gly and Thr399Ile polymorphisms were reported to be very rare in several studies of Asian ethic groups. Hang et al. failed to detect any homozygous or heterozygous variant genotypes of TLR4 Asp299Gly and Thr399Ile polymorphisms in 491 Han Chinese subjects, consisting of cotton and silk textile workers who was exposed to endotoxins [36]. In addition, almost no Asp299Gly were detected in ethnic Chinese patients in a study that analyzed the association of ischemic stroke with the TLR4 gene polymorphism [37].

In our study subjects, TLR2 Arg677Trp and Arg753Gln polymorphisms, TLR4 Asp299Gly and Thr399Ile polymorphisms, and TLR9-1237T/C mutation were all absent, in a complete agreement with studies in Japanese [38] and Korean population [39]. It is not clear whether the very low frequency of these TLR variants, as reported in this study, would be related to the low incident rates of diseases in the Orientals, such as diabetes and coronary artery disease compared with the western population. A large international study covering both developed countries and Asian populations that have varied TLR variations should be investigated to address this hypothesis.

Our study may have some limitations. Because it is a retrospective case-control study, a selection bias cannot be completely excluded. The study population was comprised entirely of patients. To limit this possibility we included consecutive patients and tried to adjust for known confounding risk factors. On the other hand, the strength of our study is that all subjects are of the same ethnic origin. Furthermore, all subjects were examined in a standardized manner, with well-defined diagnostic criteria. All genotyping was performed blind with respect to case-control status.

It should be noted that the occurrence of diabetes and/or its complications depends on the interaction among multiple risk factors, like the presence of different risk alleles, environmental factors, and the lifestyle. The contribution of any single-gene polymorphism is rather small and the interactive effect of several factors may lead to an underestimation or an overestimation of the role of a given polymorphism in determining the phenotype. Therefore, the results might not apply to groups with different genetic or environmental backgrounds. Despite the limitations of the study, the association between TLRs gene variant and susceptibility to T2DM and/or its complication will strengthen our understanding of the link between innate immunity, T2DM, and atherosclerosis.

In conclusion, the present study suggests that TLR9-1486T/C polymorphism may not be appropriate to predict the susceptibility to T2DM or CAD in Chinese Shanghai Han population. Like other Asian populations, Shanghai Han population has very low occurrence of other TLR SNPs, including TLR2 Arg677Trp and Arg753Gln polymorphisms, TLR4 Asp299Gly and Thr399Ile polymorphisms, and TLR9 promoter-1237T/C mutation, which is unlike western countries. Whether these unusually rare TLR variations are also related to distinct disease patterns in different racial population awaits further investigation.

Abbreviation

TLR:	Toll-like receptor
PCR-RFLP:	Polymerase chain reaction-restriction fragment length polymorphism
CAD:	Coronary artery disease
SNPs:	Single-nucleotide polymorphisms
WHO:	World Health Organization
T2DM:	Type 2 diabetes mellitus
SA:	Sialic acid
F:	Forward primer
R:	Reverse primer
AT:	Annealing temperature
RE:	Restriction enzyme
CRP:	C-reactive protein.

Conflict of Interests

The authors declare they have no Conflict of interests.

Authors' Contribution

Each author contributed to the subject recruitment, data collection, laboratory work, statistical analysis, and paper writing. In addition, F. Liu designed and processed the study and wrote the first draft of the paper. J. Hu helped in the statistical analysis of the data and final paper writing. B. Feng conceived and supervised the study and critically evaluated the study and paper. All authors contributed to and approved the final paper. J. Hu and B. Feng contributes equally to this study.

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