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Relationship of Toll-Like Receptors 2 and 4 Gene Polymorphisms with Essential Hypertension in Chinese Han Population

Huabei Wu[1], Shijie Yin[2]

[1]School of General Medical, Guangxi Medical University, Nanning, China
[2]The First Affiliated Hospital of Guangxi University of Traditional Chinese Medicine, Nanning, China
Email: 280131686@qq.com, 786106740@qq.com

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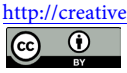

Abstract

Objective: There are numerous studies suggesting that genetic polymorphisms of inflammation factors Toll-like receptors 2 and 4 (*TLR*2, *TLR*4) might play a role in the pathophysiological process of hypertension. In this study, we evaluated the association in a sample of members of the Chinese Han population. **Method:** We selected four single nucleotide polymorphisms (SNP) of *TLR*2 (rs3804099, rs3804100, rs7656411) and *TLR*4 (rs1927906) genes, and measured the distributions of genotypic and allelic frequencies in 1063 participants, including 391 essential hypertension patients and 672 controls. **Result:** No significant differences in the genotypic and allelic frequencies of the four SNPs were detected between cases and controls. However, three haplotypes, CCG, TTG and TTT of *TLR*2, were significantly associated with a decrease in the risk of essential hypertension (OR: 0.512, 95% CI: 0.397 - 0.660, $P < 0.000$; OR: 0.701, 95% CI: 0.550 - 0.892, $P = 0.0038$; OR: 0.797, 95% CI: 0.667 - 0.952, $P = 0.0122$, respectively). Inversely, the risk of essential hypertension increased significantly in patients with the CTG, TCG or TCT haplotypes (OR: 2.924, 95% CI: 2.157 - 3.963, $P < 0.0000$; OR: 3.955, 95% CI: 2.042 - 7.660, $P < 0.0000$; OR: 6.998, 95% CI: 4.137 - 11.838, $P < 0.0000$, respectively). **Conclusion:** Our study suggested that haplotypes (CCG, TTG, TTT, CTG, TCG and TCT) of *TLR*2 might have profound effects on the development of essential hypertension in the Chinese Han population.

Keywords

Toll-Like Receptor 2, Toll-Like Receptor 4, Single-Nucleotide Polymorphisms, Essential Hypertension, Inflammation

1. Introduction

Essential hypertension (EH) is the most prevalent risk factor for cardiovascular morbidity and mortality worldwide [1]. Many studies have concentrated on the genetic and environmental factors that may lead to EH, attempting to find potential therapeutic targets and form prevention strategies. However, the aetiology of essential hypertension (EH) is still not completely known. Recently, there have been many studies that demonstrated how EH may be a low-degree inflammatory disease [2] [3]. A significant increase of a series of circulating inflammatory markers was observed in advanced EH. These markers included C-reactive protein (CRP), Interleukin-6 (IL-6), and so on. Both cross-sectional and prospective studies have shown that CRP is higher in hypertensive patients [4], while IL-6 was correlated with blood pressure measures, as Chae [5] demonstrated. Despite an increase in clinical studies that support the link between inflammation and EH, the mechanisms underlying the process are still unclear.

Toll-like receptors (*TLR*s), which are part of the mediated innate immune transmembrane signalling receptor family, play an important role in signal transduction for the activation of inflammatory cells. They also form a bridge between innate immunity and acquired immunity, characterised by an extracellular leucine-rich repeat domain and an intracellular Toll/IL-1 receptor-like (TIR) domain [6]. *TLR*s have ubiquitously expressed pattern recognition receptors central to the inflammatory response in a broad array of species. In vertebrates, *TLR*s expression was originally described in immune system cells, such as macrophages and neutrophils, but it is now becoming apparent that they are widely expressed throughout the body in cells as diverse as hepatocytes, vascular smooth muscle cells, and neurons. *TLR*2 and *TLR*4 are important members of the Toll-like receptor family. *TLR*2 recognises various lipoproteins from bacteria, mycoplasma and fungi by forming a heterodimer with either *TLR*1 (*TLR*1/*TLR*2 to recognise triacyl lipoproteins) or *TLR*6 (*TLR*2/*TLR*6 to sense diacyl lipoproteins) [7]. *TLR*4, produced by monocytes and endothelial cells [8], can ligate with lipopolysaccharide (LPS) and be activated by cellular fibronectin in response to tissue injury [9] and heat shock protein 60 [10]. At present, many studies have reported close relationships between *TLR*2 or *TLR*4 and cardiovascular diseases. Kuwahata *et al.* [11] reported that high *TLR*2 expression levels in monocytes might be an independent risk factor for atherogenesis. Dzumhur *et al.* [12] indicated that single nucleotide polymorphism (SNP) 1350T/C in *TLR*2 might play a protective role against acute myocardial infarction (AMI) and arterial hypertension. Even though *TLR*4 and *TLR*2 are reported to be associated with other inflammatory diseases, studies regarding the association between with EH have thus far been poor. *TLR*4 expression was reported to increase in hypertensive rats [13]. On the other hand, *TLR*4-deficient mice showed less susceptibly to developing pulmonary hypertension [14]. Moreover, Sollinger [15] suggested that cell damage-associated *TLR*4 signalling might act as a direct mediator for the vascular contractility linking inflammation to hypertension. For *TLR*2, increased expression was observed in

pregnant women with hypertension, compared with controls [16]. These studies indicated that the *TLR*4 and *TLR*2 genes might have a role in EH, as well as in other inflammatory diseases.

So far, 290 SNPs have been identified in the human *TLR*4 gene (http://www.ncbi.nlm.nih.gov, as of the last access on 23 October 2011). Rs1927906 of *TLR*4 is located in the 3'-UTR of gene, which has been documented to differ significantly in pulmonary tuberculosis in the Sudanese [17]. However, to the best of our knowledge, our study is the first to focus on its association with EH. For the *TLR*2 gene, a total of 342 SNPs have been identified in humans to date (http://www.ncbi.nlm.nih.gov, as of the last access on 23 October 2011). Rs3804099 and rs3804100 of *TLR*2 are synonymous SNPs, and rs7656411 of *TLR*2 are located in the 3'-UTR of gene. Although the former SNPs did not lead to a change in the primary polypeptide sequence, many studies have confirmed that synonymous mutations have an impact on gene function and have been implicated in diseases [18] [19] [20]. In addition, some mutations located in the 3'-UTR of the gene have a close contact with EH [21] [22] [23]. In order to study the relationships between these two genes and EH, we investigated the relationship of the SNPs of *TLR*2 and *TLR*4 with EH in members of the Chinese Han population.

2. Methods

2.1. Ethics Statement

The Guangxi Medical Ethics Committee in China approved this study. All of the patients provided written informed consent.

2.2. Subjects

There were 391 EH patients and 672 normotensive controls that were recruited to participate in our study. The participants underwent a physical examination in the Guangxi hospital and two health care centres in the Guangxi province in Nangning between April 2021 and August 2021. The participants self-reported that they were of Han Chinese ethnicity. Hypertension was defined as currently receiving treatment with an antihypertensive drug or having a diastolic blood pressure (DBP) of a minimum of 90 mmHg and/or a systolic blood pressure (SBP) of a minimum of 140 mmHg [24]. The control subjects all had SBP < 140 mmHg and DBP < 90 mmHg [24]. Inclusion criteria: the case group was Han patients with essential hypertension who has been diagnosed, while the control group was Han healthy people with non-essential hypertension, and had no family genetic history of hypertension. Patients with diabetes, tumor, secondary hypertension, coronary heart disease, valvular heart disease, myocarditis and other inflammatory diseases were excluded from both groups. Data on demographic characteristics (age, gender, occupation, etc.), lifestyle (smoking, and alcohol consumption), health status, and medical history were collected using a standardised questionnaire. A family history of hypertension was considered positive if

the participant's parents or siblings had a history of hypertension. Blood pressure was measured three times by trained nurses with a mercury sphygmomanometer in a comfortable sitting position after five minutes of rest, and the mean values of the three trials were obtained for analysis. Participants were asked to avoid vigorous exercise, drinking, and smoking for at least 30 minutes before the measurements were taken. All participants were recruited from Nanning, Guangxi, and provided written informed consent. The Ethics Committee of Guangxi Medical University approved the study.

2.3. DNA Extraction and Genotyping of the *TLR*4 and *TLR*2 Polymorphisms

The SNPs were selected from the National Center for Biotechnology Information db SNP, with minor allele frequencies (MAF) of more than 5%. The MAF was chosen based on the number of SNPs to be genotyped, the available sample size and the desire to ensure a reasonable study power (at least 80%).

Two millilitres of fasting venous blood was collected from the participants from 8 to 11 am in the morning, using ethylene-diamine-tetra-acetic acid (EDTA) as an anticoagulant. Genomic DNA was extracted according to the phenol-chloroform method. The primers for amplifying the *TLR*4 gene (rs1927906) and *TLR*2 gene (rs3804099, re3804100, rs7656411) were designed based on the National Center for Biotechnology Information gene database (Primer sequence of each SNP site is shown in Table 1). Amplification included 1.0 uL DNA, mixing with H_2O 1.8 uL, 10× Taq polymerase chain reaction (PCR) Master-mix 0.5 μL, $MgCl_2$ 0.4 uL, dNTP 0.1 uL, PCR enzyme 0.2uL, DNA 1 uL, and 1 uL of each primer. Cycle conditions involved an initial denaturation for 30 s at 94˚C, followed by 40 cycles of denaturing at 95˚C for 5 s, annealing at 52˚C for 5 s, primer extension at 80˚C for 5 s, 52˚C for 5 s, 80˚C for 1 min, followed by 5 cycles and a final extension at 72˚C for 3 min. The PCR products were purified using a SAP enzyme before being preserved at 4˚C. Genotyping of all SNPs was performed using Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) according to the manufacturer's instructions, with an accuracy of more than 99%.

Table 1. Primer sequence of each SNP site.

SNP_ID	2nd-PCRP	1st-PCRP	UEP_SEQ
1927906-C	ACGTTGGATGTCCTTC CTATCAGTTCCCTC	ACGTTGGATGTGCTT GTCCACCTCACCTG	GTTCCCTCTCCCA GA
3804099-B	ACGTTGGATGCTGCTT CATATGAAGGATCAG	ACGTTGGATGGATCT ACAGAGCTATGAGCC	ggTGAAGGATCAG ATGACTTAC
7656411-A	ACGTTGGATGCCTTTA AATTACTGTGTATC	ACGTTGGATGGTACA TGTGAGCTAAATAG	ggTTTTTGAGTCA TTATGAGGAA
3804100-B	ACGTTGGATGTTCCAG TGTCTTGGGAATGC	ACGTTGGATGGTCAG TGGCCAGAAAAGATG	cccacCTTGGGAAT GCAGCCTGTTAC

2.4. Statistical Analysis

Data was expressed as proportions, means and standard deviations (Mean ± SD). Clinical characteristics were compared between cases and controls using a Student t-test or χ^2 test. The Hardy-Weinberg equilibrium was tested for each SNP among the controls. Differences in allelic and genotypic frequencies between the cases and controls were assessed using the χ^2 test. A logistic regression analysis was used to test the relationships between genotypes, alleles and EH after adjustments were made to the potential confounders, including gender, age, smoking and alcohol consumption. Linkage disequilibrium and haplotypes were analysed using the SHEsis software (http://analysis2.bio-x.cn/myAnalysis.php). The statistical tests were 2-sides, and a P value < 0.05 was considered statistically significant.

3. Results

The clinical characteristics of the case and control subjects are summarised in Table 2. Significant differences were observed in gender, age, smoking and alcohol consumption between the two groups. None of the four SNPs reported in the present study showed significant deviations from the Hardy-Weinberg equilibrium in control subjects ($P > 0.05$).

The distributions of genotypic and allelic frequencies of the four SNPs in each group are shown in Table 3. No statistically significant differences were detected in each SNP between the cases and controls (all P values > 0.05). Multiple logistic regression analyses revealed no significant associations (all P values > 0.05) between these four SNPs with EH, even after adjusting for all confounding factors (As shown in Table 3).

The linkage disequilibrium (LD) analysis results assessed by the SHEsis programme demonstrated that three SNPs (rs3804099, rs3804100, rs7656411) of the *TLR*2 gene were in LD, and their pair-wise LD (D') values are shown in Figure 1. Then, three SNPs (rs3804099, rs3804100, rs7656411) of the *TLR*2 gene were included in the haplotype analysis. In addition, six haplotypes with frequencies ≥ 0.03 were obtained (As shown in Table 4). The CCG, TTG and TTT haplotypes were significantly associated with a decrease in the risk of EH (OR: 0.512, 95% CI: 0.397 - 0.660, $P < 0.000$; OR: 0.701, 95% CI: 0.550 - 0.892, $P = 0.0038$; OR: 0.797, 95% CI: 0.667 - 0.952, $P = 0.0122$, respectively). Inversely, the risk of EH

Table 2. Characteristics of study participants.

Confounding factors	Case (n = 391)	Control (n = 672)	P^* value
Gender (male/female)	153/238	309/363	0.03
Age (year)	56.98 ± 9.48	47.08 ± 10.21	0.00
Smoking (%)	15.9	21.1	0.04
Alcohol consumption (%)	43.2	65.8	0.00

*$P < 0.05$ was defined as a statistical significance.

Table 3. The distributions of genotypic and allelic frequencies in cases and controls and their association with EH.

Polymorphisms		Control	Case	*P** value	Adjusted OR (95% CI)**
Rs3804099 genotypes	C/C	57 (0.085)	30 (0.077)	0.886	1
	C/T	269 (0.400)	156 (0.399)		0.922 (0.538 - 1.578)
	T/T	346 (0.515)	205 (0.524)		1.050 (0.620 - 1.778)
Allelic	C	383 (0.285)	216 (0.276)	0.665	1
	T	961 (0.715)	566 (0.724)		1.072 (0.860 - 1.337)
Rs3804100 genotypes	T/T	389 (0.579)	220 (0.563)	0.715	1
	T/C	237 (0.353)	147 (0.376)		1.056 (0.785 - 1.422)
	C/C	46 (0.068)	24 (0.061)		0.867 (0.484 - 1.555)
Allelic	T	1015 (0.755)	587 (0.751)	0.814	1
	C	329 (0.245)	195 (0.249)		0.989 (0.787 - 1.244)
Rs7656411 genotypes	G/G	151 (0.225)	73 (0.187)	0.181	1
	G/T	319 (0.475)	207 (0.529)		1.296 (0.896 - 1.875)
	T/T	202 (0.301)	111 (0.284)		1.137 (0.755 - 1.713)
Allelic	G	621 (0.462)	353 (0.451)	0.635	1
	T	723 (0.538)	429 (0.549)		1.042 (0.853 - 1.272)
Rs1927906 genotypes	A/A	620 (0.923)	370 (0.946)	0.283	1
	A/G	51 (0.076)	21 (0.292)		0.855 (0.475 - 1.538)
	G/G	1 (0.010)	0 (0)		
Allelic	A	1291 (0.961)	761 (0.973)	0.127	1
	G	53 (0.039)	21 (0.027)		0.836 (0.471 - 1.483)

*$P < 0.05$ was defined as statistically significant. **Adjusted OR (95% CI) were odds ratios and 95% confidence interval calculated by multiple logistic regression analyses after being adjusted for gender, age, smoking and alcohol consumption.

Table 4. Estimated haplotype frequencies and association with the risk of EH in cases and controls.

Haplotypes*	Frequency in cases	Frequency in controls	*P* value**	OR 95% CI
CCG	0.119	0.206	0.000	0.512 (0.397 - 0.660)
TTG	0.144	0.190	0.004	0.701 (0.131 - 0.490)
TTT	0.454	0.502	0.012	0.797 (0.667 - 0.952)
CTG	0.150	0.056	0.000	2.924 (2.157 - 3.963)
TCG	0.038	0.010	0.000	3.955 (2.042 - 7.660)
TCT	0.088	0.013	0.000	6.998 (4.137 - 11.838)

*Haplotypes with frequency less than 0.03 in controls and cases were excluded. **$P < 0.05$ was defined as statistically significant.

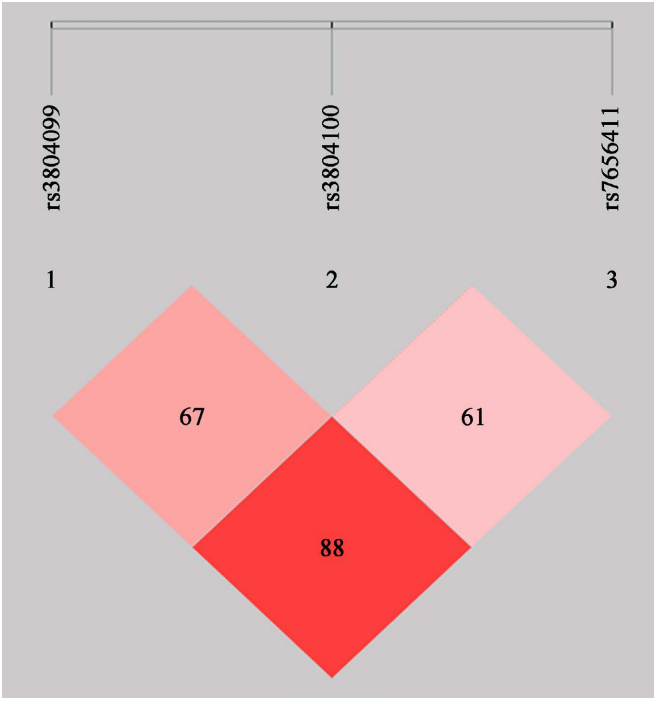

Figure 1. The pair-wise LD (D') values of three SNPs (rs3804099, rs3804100, rs7656411) of the *TLR*2 gene.

increased significantly in patients with the CTG, TCG or TCT haplotypes (OR: 2.924, 95% CI: 2.157 - 3.963, $P < 0.000$; OR: 3.955, 95% CI: 2.042 - 7.660, $P < 0.000$; OR: 6.998, 95% CI: 4.137 - 11.838, $P < 0.000$, respectively).

4. Discussion

Our study demonstrated no significant differences in any of the individual distributions of genotypic and allelic frequencies between the cases and controls for the four SNPs. Multiple logistic regression analyses suggested that these SNPs were not associated with EH. However, the LD analysis revealed that three SNPs of *TLR*2 were in LD, allowing for the construction of haplotype blocks. In addition, three protective haplotypes and three risk haplotypes of EH were found in the haplotype analysis. Therefore, we provided evidence for an association of *TLR*2 gene polymorphisms with the risk of EH, suggesting that the *TLR*2 gene might be involved in the pathogenesis of EH in the Han Chinese population.

As a major risk factor for atherosclerosis, hypertension is considered a low-degree inflammatory disease [25]. Some researchers reported that there was a close link between vascular inflammation caused by endothelial injury and the occurrence and development of hypertension [26] [27]. *TLR*2 can affect inflammatory cytokines and endothelial function. Sabroe *et al.* [28] confirmed that *TLR*2 agonists regulated important neutrophil functions, including adhesion, the generation of reactive oxygen species, and the release of chemokines, in addition to activating major proinflammatory signalling pathways, including the nuclear factor—*κ*B pathway. Jiang [29] and colleagues demonstrated that fragmented hya-

luronan (HA) generated by tissue injury required both *TLR*2 and *TLR*4 to stimulate mouse macrophages and produce inflammatory chemokines and cytokines. Mullick *et al*. [30] reported that *TLR*2 deficiency could reduce hyperlipidemic-induced changes in the morphology of the endothelium. Tzima *et al*. [31] found that the extent of endothelial cell disruption is proportional to the extent of endothelial cell *TLR*2 expression. Therefore *TLR*2 is closely associated with inflammation and vascular endothelial function. Considering the role of *TLR*2 in promoting vascular inflammation, we speculated that a reduced or increased of *TLR*2 expression, or a change in its protein structure, led to functional changes, and consequently, affected the regulation of inflammatory cytokines and endothelial function, resulting in vascular inflammation. This, thus, influenced the susceptibility towards EH. The six haplotypes that were found in our study may enhance or weaken the function of *TLR*2 via changing the protein structure or its expression, so as to affect susceptibility towards EH.

Our study has several limitations. First, we did not study other meaningful SNPs of *TLR*2 and *TLR*4, and therefore we cannot exclude the possibility that they may have linked inheritance with the SNPs in our study. Second, we failed to demonstrate whether the SNPs were related to functional alterations of *TLR*2 or *TLR*4. Third, our study did not clarify the mechanism of the six haplotypes regarding susceptibility towards EH. Thus, more extensive studies, including whole-genome analyses, are required to confirm our results.

In conclusion, there were no genotypic and allelic associations between the four SNPs in the *TLR*2 or *TLR*4 genes and the EH cases. However, the haplotypes, which are based on rs3804099, rs3804100 and rs7656411 of *TLR*2, CCG, TTG and TTT were protective factors regarding EH. Finally, CTG, TCG and TCT were risk factors for EH. These suggested the involvement of *TLR*2 in EH pathogenesis.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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