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Rarity of TLR4 Asp299Gly and Thr399Ile Polymorphisms in the Korean Population

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Purpose: Activation of the innate immune system and chronic low-grade inflammation are thought to be involved in the pathogenesis of atherosclerosis and also thought to be associated with type 2 diabetes and its complications. As a receptor for bacterial lipopolysaccharide and heat-shock proteins, Toll-like receptor 4 (TLR4) is one of the central regulators of the immune response. Recent studies have reported an association between TLR4 polymorphisms and diabetes and its complications in Caucasian populations. **Materials and Methods:** In this study, we analyzed the association between TLR4 gene polymorphisms in patients with features of type 2 diabetes and healthy controls in Korea. Two polymorphisms of the TLR4 gene (Asp299Gly and Thr399Ile) were examined in 225 diabetic patients and 153 healthy controls using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and single-strand conformation polymorphism (SSCP). **Results:** No Asp299Gly or Thr399Ile mutations were detected in any of the 378 subjects. Seven subjects from each group who had slightly different SSCP patterns were selected for sequencing, but we found no TLR4 polymorphisms on Exon3. The Asp299Gly and Thr399Ile TLR4 gene polymorphisms were absent in both groups, which was similar to the results for Japanese and Chinese Han subjects. **Conclusion:** Our data and other Asian data suggest that a racial difference can be found in the frequency of the TLR4 polymorphism.

Key Words: Type 2 diabetes, TLR4, polymorphism, inflammation, innate immunity

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INTRODUCTION

A homologous family of toll receptors, the toll-like receptors (TLRs), was discovered in 1997.[1] TLRs serve as pattern-recognition receptors in mammals and play a critical role in the recognition of microbial components, such as lipopolysaccharides (LPSs), which initiate the innate immune response.[1] TLR4, a member of the TLR family, is expressed on cardiomyocytes, macrophages, airway epithelium, and endothelial and smooth muscle cells.[2,3] TLR4 interacts with endogenous ligands, including oxidized low-density lipoprotein, heat-shock proteins 60 and 70, fibrinogen, and fibronectin, which are elevated in diabetic patients,[4-8] as well as with exogenous ligands, such as LPS.[2,9-12] Twenty-nine single nucleotide polymorphisms (SNPs) have been identified in the human TLR gene.[13] Of these, the Asp299Gly and 399 Thr399Ile polymorphisms have been shown to cause hyporesponsiveness to LPS in human alveolar macrophages and airway epithelial cells.[14] Individuals carrying the Asp299Gly TLR4 allele have lower levels of proinflammatory cytokines, acute-phase reactants, and soluble adhesion molecules, such as interleukin 6 and fibrinogen.[15] Countering the increased risk of severe bacterial infection, they have a lower risk of carotid atherosclerosis and smaller intima-media thickness in the common carotid artery.[15]

Many studies have suggested that activation of the innate immune system is closely associated with type 2 diabetes. The circulating inflammatory markers interleukin 6, acute-phase reactants, and especially C-reactive protein, have been shown to predict the development of type 2

diabetes.[16-20]

Studies have analyzed the association between TLR4 polymorphisms and the features of type 2 diabetes. Although one study reported a poor relationship,[21] a recent study of 776 Caucasians, including 246 with type 1 diabetes and 530 with type 2 diabetes, indicated that 68 of those with type 2 diabetes were heterozygous for the Asp299Gly polymorphism (carrier rate (CR) 12.8%, allelic frequency (AF) 6.4%), 67 of them were heterozygous for the TLR4 Thr399Ile polymorphism (CR 12.6%, AF 6.3%), and the type 2 diabetic subjects showed a strong association between the Asp299Gly/TLR4 Thr399Ile polymorphism and diabetic neuropathy.[22] Since only one other previous study has examined the TLR4 polymorphism in ethnic Korean subjects,[23] we analyzed the association between the TLR4 polymorphism and features of type 2 diabetes in Koreans.

MATERIALS AND METHODS

The study groups consisted of 378 Korean subjects: 225 had type 2 diabetes (mean age, 54.5 ± 10.0 years; 114 women and 111 men) and 153 were controls (mean age 46.1 ± 12.9 years; 77 women and 76 men). The diabetic patients were recruited from the outpatient clinic of the Department of Endocrinology, Gachon University of Medicine and Science, Gil Medical Center, Incheon, Korea. To be representative of the controls in Korea, we selected people who were recruited from the health care center during the same study period and whose age was between 20 - 70 years old to cover all age groups. Physical examinations and laboratory testing was performed on all patients (Table 1).

PCR

DNA from both patients and controls was extracted from peripheral white blood cells using the standard method. The PCR primers for Asp299Gly and TLR4 Thr399Ile had the following sequences:

TLR4 Asp299Gly: forward 5'GATTAGCATAC TTAGACTACTACCTCCATG3'

reverse 5'GATCAACTTCTGAAAAAGCATTC CCAC3',

TLR4 Thr399Ile: forward 5'GGTTGCTGTTCTCA

Table 1. Basal Characteristics of Patients with type 2 Diabetes and Normal Controls

Variables	Type 2 diabetes	Normal controls
No. of patients	225	153
Sex (F/M)	80 : 73	140 : 85
Age (yrs)	55.9 ± 10.8	42.3 ± 9.2
BMI* (kg/㎡)	23.6 ± 3.8	22.9 ± 3.0
Duration of diabetes	8.3 ± 6.4	-
SBP† (mmHg)	124.2 ± 15.6	113.3 ± 12.3
DBP‡ (mmHg)	72.6 ± 10.5	78.0 ± 46.6
FBS§ (mmol/L)	159.4 ± 53.8	83.2 ± 4.0
HbA1c (%)	8.3 ± 1.9	5.1 ± 0.3
Cholesterol (mmol/L)	186.9 ± 38.0	181.3 ± 30.0
Triglycerides (mmol/L)	164.8 ± 114.7	100.4 ± 71.3
HDL-Chol.‖ (mmol/L)	47.9 ± 15.2	55.4 ± 13.2

Data are means ± SD or %.
*Body mass index.
†Systolic blood pressure.
‡Diastolic blood pressure.
§Fasting blood glucose.
‖High density lipoprotein-cholesterol.

AAGTGATTTTGGGAGAA3'

reverse 5'ACCTGAAGACTGGAGAGTGAGAG TTAAATGCT3'.

In total, 50 ng DNA were amplified in a 20-μL volume containing 0.4 μL primer, 1 μL DNA extract, 0.4 μL Taq polymerase (Takara Bio, Otsu, Japan), 2 μL 10 × PCR buffer, and 2 μL dNTPs. Amplification consisted of two initial cycles at 94℃ for 30 s, 52℃ for 1 min, and 72℃ for 1 min, followed by 30 cycles at 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s, followed by 5 min at 72℃, and ending at 10℃.

Restriction enzymes

To screen for the TLR4 Asp299Gly and Thr 399Ile polymorphisms, the sequence was cleaved using NcoI and HinfI restriction endonucleases, respectively. Eight microliters of PCR product were treated with 0.5 μL restriction endonuclease, and a drop of mineral oil was added. The mixture was incubated at 37℃ for 24 h, and then electrophoresed on 2.5% NuSieve® GTG (GeneFrontier, Tokyo, Japan) and Seakem® LE agarose gels (GeneFrontier) at 100 V for 30 min.

SSCP (Single Strand Conformation Polymorphism)

To locate the TLR4 Asp299Gly polymorphism, the PCR product was subjected to an SSCP study. A mixture of 8 μL of SSCP solution and 2 μL of PCR product was heated at 94°C for 5 min and then iced for 2 min. The product was run on a 10% acrylamide gel at 150 V for 1.5 h and stained using a Silverstar® Staining Kit (Bioneer, Daejeon, Korea).

DNA sequencing

Samples with different SSCP patterns were selected, and the PCR products were sequenced with the TLR4 Asp299Gly forward primer using a commercial service (Macrogen®, Seoul, Korea).

RESULTS

PCR DNA from type 2 diabetic patients using the TLR4 Asp299Gly and TLR4 Thr399Ile primers was not cut by the NcoI or HinfI restriction endonucleases. No TLR4 Asp299Gly or Thr399Ile polymorphisms were detected in the type 2 diabetics.

We examined the SSCP band patterns from the DNA of 225 type 2 diabetics and 153 controls. Seven SSCP bands from the 225 diabetic patients and 7 of the 153 controls differed slightly from the others. Therefore, we sequenced these 14 DNA samples but detected no Asp299Gly polymorphisms at the TLR4 Exon 3 (Fig. 1).

DISCUSSION

Many studies in Caucasians suggest that the Asp299Gly polymorphism is associated with innate immunity-related diseases, such as chronic inflammatory disease and atherosclerosis.[15,24,25] Therefore, association with the TLR4 polymorphism has been analyzed in type 2 diabetics with features of mild systemic inflammation. Rudosky et al. reported that the Asp 299Gly and Thr399Ille genotypes of the TLR4 gene are associated with a reduced prevalence of diabetic neuropathy in type

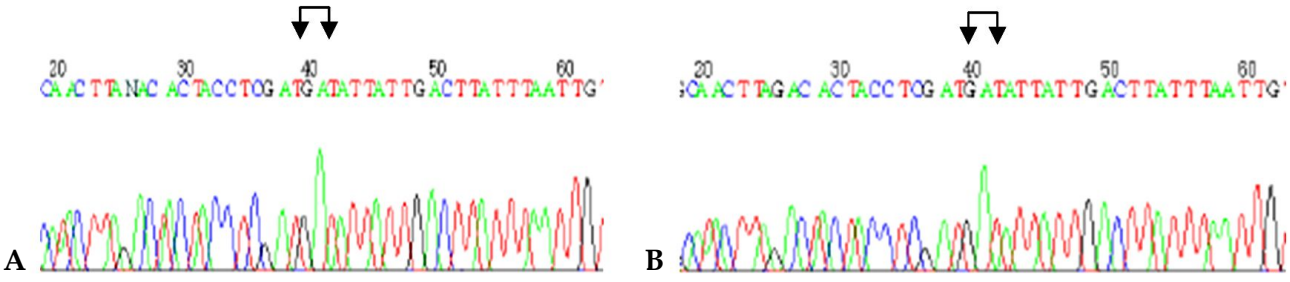

Fig. 1. The sequencing results for the samples in diabetic patients (A) and normal controls (B). The sequencing results in all lanes were homozygous for adenine at position 896. No GAT(Asp)→GGT(Gly) substitution was observed.

2 diabetes.[21] In contrast, the Asp299Gly polymorphism was reported to be very rare in several studies of Asian ethic groups.[26-29] Therefore, we examined the ethnic differences in the TLR4 polymorphism and its association with type 2 diabetes, but were unable to find Asp299Gly and Thr399Ile polymorphisms in 378 Korean subjects (225 type 2 diabetics, and 153 controls).

Hang et al. failed to detect any homozygous or heterozygous variant genotypes of the Asp299Gly and Thr399Ile polymorphisms in 491 Han Chinese subjects, consisting of cotton and silk textile workers who were exposed to endotoxins.[26] In addition, no Asp299Gly polymorphisms were detected in ethnic Chinese patients in a study that analyzed the association of ischemic stroke with the TLR4 gene polymorphism[27] (although one TLR4 gene C119A was found). A study on polymorphisms of the TLR4 and CD 14 genes in ulcerative colitis patients revealed no TLR4 Asp299Gly mutation in any Chinese patients or healthy controls but detected mutations in 10% of the Caucasian Dutch subjects.[28] Of 411 Japanese subjects, including 197 critically ill patients and 214 healthy controls, no TLR4 Asp299Gly or Thr399Ile polymorphisms were detected.[29]

Even among Caucasian subjects, some studies have suggested that the TLR4 Asp299Gly polymorphism is not related to conditions such as rheumatoid arthritis and systemic lupus erythematosus[30] or even the incidence of myocardial infarction (MI) and stroke in a large prospective study of US men.[31] Yang et al. reported that the TLR4 polymorphism was not related to the severity of atopy in asthmatics[33] or that of coronary artery stenosis in 695 patients with atherosclerotic MI.[33] Moreover, the TLR4 Asp299Gly variant had no influence on LPS responsiveness or the susceptibility to pulmonary tuberculosis in a study in Gambia.[34]

In conclusion, Asp299Gly and Thr399Ile TLR4 gene polymorphisms were not found in diabetic patients and healthy controls in a Korean population, findings that are similar to those for Japanese and Chinese Han subjects. Therefore, our data and other Asian data suggest that a racial difference can be found in the frequency of the TLR4 polymorphism.

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