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Simple Genotype Analysis of the Asp299Gly Polymorphism of the *Toll-Like Receptor-4* Gene That is Associated With Lipopolysaccharide Hyporesponsiveness

Naoko Okayama,[1] Kozue Fujimura,[1] Yutaka Suehiro,[2] Yuichiro Hamanaka,[2] Motoki Fujiwara,[3] Tomoyo Matsubara,[3] Tsuyoshi Maekawa,[4] Shoichi Hazama,[5] Masaaki Oka,[5] Hiroaki Nohara,[6] Kozo Kayano,[6] Kiwamu Okita,[6] and Yuji Hinoda[1,2]*

[1]Division of Clinical Laboratory, Yamaguchi University Hospital, Yamaguchi University School of Medicine, Yamaguchi, Japan

[2]Department of Clinical Laboratory Science, Yamaguchi University School of Medicine, Yamaguchi, Japan

[3]Department of Pediatrics, Yamaguchi University School of Medicine, Yamaguchi, Japan

[4]Department of Critical Care and Emergency Medicine, Yamaguchi University School of Medicine, Yamaguchi, Japan

[5]Department of Surgery II, Yamaguchi University School of Medicine, Yamaguchi, Japan

[6]Department of Internal Medicine I, Yamaguchi University School of Medicine, Yamaguchi, Japan

A nonsynonymous single nucleotide polymorphism (Asp299Gly) in the *Toll-like receptor-4* (*TLR-4*) gene affects the responsiveness to lipopolysaccharide in humans. To analyze this important polymorphism more efficiently, we developed a simple polymerase chain reaction (PCR) restriction length fragment polymorphism (RFLP) assay and examined the Asp299Gly allele frequency in a Japanese population. The PCR primer was designed with 1- or 2-bp mismatches, creating the recognition sequence for restriction enzyme *Bsa*BI or *Bst*XI, allowing RFLP analysis of the digested products. Genotyping was carried out with this assay for 275 DNA specimens from 107 healthy volunteers and 168 patients with various diseases, including ulcerative colitis (n = 86). The Asp299Gly allele of the *TLR-4* gene was not detected in any of the specimens, suggesting that it is very rare in Japanese. J. Clin. Lab. Anal. 16:56–58, 2002.

Key words: *TLR-4* gene; PCR-RFLP; polymorphism; LPS; ulcerative colitis

INTRODUCTION

The Toll-like receptor-4 (TLR-4) is a member of the interleukin-1 receptor (IL-1R)/toll-like receptor (TLR) superfamily (1,2). This conserved cytoplasmic region is termed a Toll/IL-1R (TIR) domain, which is derived from the fact that it is commonly found in IL-1R and a receptor-like protein of the *Drosophila* fruit fly called Toll. So far, six superfamily members have been identified, but, with the exception of TLR-2 and -4, little is known about their in vivo functions. Recently, accumulating evidence has suggested that TLR-4 is required for lipopolysaccharide (LPS) responsiveness, and thus is involved in host defense against Gram-negative bacteria (3,4). LPS is known to play a pivotal role in the pathogenesis of a variety of infectious and allergic diseases (5,6). Furthermore, it has recently been shown that LPS may stimulate the growth of human colon carcinoma cell line, suggesting a possible direct effect in the promotion of colon cancer (7). Individual differences in response to LPS could be of importance in determining the susceptibility, severity, and prognosis of these disorders. In this context, a recent report concerning nonsynonymous, cosegregating single-nucleotide polymorphisms (Asp299Gly and Thr399Ile) of the *TLR-4* gene by Arbour et al. (8) should be noted. In addition to several in vitro transfection experiments indicating the relation of Asp299Gly with decreased response to LPS, they found that the airway responsiveness to inhaled LPS was significantly lower in subjects heterozygous or homozygous for the Asp299Gly and Thr399Ile alleles than in those with the wild-type genotype. Although in the subjects tested there were 73

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*Correspondence to: Yuji Hinoda, M.D., Department of Clinical Laboratory Science, Yamaguchi University School of Medicine, 1-1-1, Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan. E-mail: hinoda@po.cc.yamaguchi-u.ac.jp

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TABLE 1. Sequences of primers used in this study

Primers for restriction enzyme *Bas*BI	
F1	5′-ttagaaatgaaggaaacttggaaaag-3′
R1	5′-tttgtcaaacaattaaataagtGaTtaata-3′
R1′	5′-tttgtcaaacaattaaataagtGaTtaata**C**catc-3′
Primers for restriction enzyme *Bst*XI	
F2	5′-agcatacttagactacCacctcgatg-3′
R2	5′-gttgccatccgaaattataagaaaag-3′
F2′	5′-agcatacttagactacCacctcgatg**G**tat-3′

F, forward primer; R, reverse primer. Bold capital characters indicate the polymorphic site. Underlined capital characters indicate mismatched nucleotides.

wild-types, nine heterozygotes, and one homozygote, data on the allele frequency in a given population were not shown. The sequence analysis was performed with polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) and direct sequencing. In this study, we develop a simple assay for Asp299Gly by PCR-restriction fragment length polymorphism (RFLP) using mismatch primers, and examine the allele frequency of Asp299Gly in a Japanese population.

MATERIALS AND METHODS

DNA Specimens

For DNA analysis, 7 ml of peripheral blood was obtained from 107 unrelated healthy volunteers and 168 patients with ulcerative colitis (n = 86), colon cancer (n = 47), viral hepatitis (n = 32), bacterial infection (n = 1), or Kawasaki vasculitis (n = 2), after informed consent was given. The experimental protocol was approved by the institutional ethics committee of the Yamaguchi University School of Medicine, Ube, Yamaguchi, Japan. The tested subjects were all Japanese. DNA was isolated based on a conventional NaI method (9).

PCR-RFLP

The primers designed in this study are listed in Table 1. Two primer sets, F1-R1 and F2-R2, were used for the PCR-RFLP assay for identifying the Arg299Gly allele. PCR was carried out in a total volume of 25 μl consisting of 0.5 μl of 10 μM solution of each primer, 2.5 μl of 10× reaction buffer (100 mM Tris-HCl pH 8.3 at 25°C, 500 mM KCl, 15 mM $MgCl_2$), 4 μl of 1.25 mM solution of 4 dNTPs, 1 μl of AmpliTaq DNA polymerase (Perkin Elmer, Branchburg, NJ), 1 μl of genomic DNA (80 ng/μl), and 15.5 μl H_2O. This was performed with a Robocycler (Gene Amp PCR System 9600; Perkin Elmer) under the conditions of initial denaturation of 2 min at 95°C followed by 38 cycles of 30 sec at 95°C, 30 sec at 51°C, and 30 sec at 72°C. The PCR products were digested with the restriction enzyme *Bsa*BI or *Bst*XI (New England Biolabs, Inc., Beverly, MA) and separated on a 7% horizontal nondenaturing polyacrylamide gel at 100 V for 1.5 hr. The gel was then stained with ethidium bromide.

RESULTS AND DISCUSSION

We tested two kinds of restriction enzymes, *Bsa*BI and *Bst*XI, for the PCR-RFLP assay of the Asp299Gly allele. The reverse primer R1 and the forward primer F2 were designed to anneal to the proximity of the polymorphism with 1- or 2-bp mismatches, as shown by underlines in Table 1, creating recognition sequences, 5′-gatnn/nnatc-3′ and 5′-ccannnnn/ntgg-3′ for restriction enzymes *Bsa*BI and *Bst*XI, respectively. Consequently, the DNA template containing adenine (A) at the polymorphic site (wild-type allele) was sensitive to *Bsa*BI, and that containing guanine (G) (mutated allele) was sensitive to *Bst*XI, allowing RFLP analysis of the digested products. Other restriction enzymes would be applicable to this method. However, it should be noted that the amplification efficiency of PCR was clearly decreased when the primer contained mismatches within 3 bp from the 3′ end (data not shown). Mismatches of primers R1 and F2 were therefore introduced into further nucleotide positions from the 3′ end (Table 1).

Genotyping was performed with these PCR-RFLP assays for 275 DNA specimens from 107 healthy volunteers and 168 patients with ulcerative colitis, colon cancer, viral hepatitis,

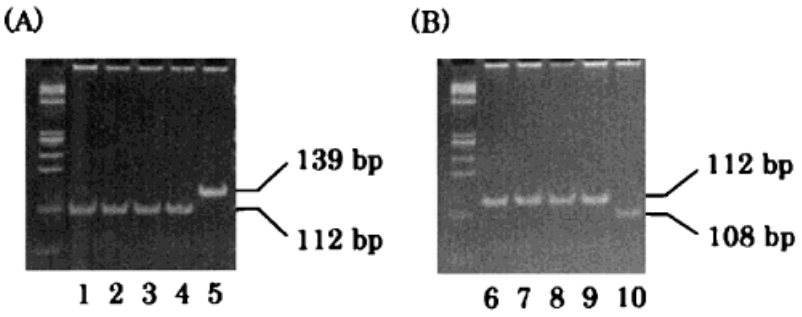

Fig. 1. Electrophoresis patterns for the Asp299Gly allele of the *TLR-4* gene analyzed by PCR-RFLP-based assays. Restriction endonucleases (**A**) *Bsa*BI and (**B**) *Bst*XI were used. Lanes 1–4 and 6–9 showed digestion of amplified DNAs from four healthy volunteers with *Bsa*BI and *Bst*XI, respectively. The four samples were all sensitive to *Bsa*BI and resistant to *Bst*XI, indicating homozygous wild-type. Lanes 5 and 10 showed digestion patterns of positive control DNAs containing guanine (G) at the polymorphic site. In contrast to homozygous wild-type, they were resistant to *Bsa*BI and sensitive to *Bst*XI. Control DNAs for *Bsa*BI and *Bst*XI were amplified with the primer R1′ and F2′ (Table 1), respectively.

bacterial infection, or Kawasaki vasculitis. The Asp299Gly allele of the *TLR-4* gene was not detected in any specimens from the Japanese individuals tested. However, 245 DNA samples were sensitive to *Bsa*BI and resistant to *Bst*XI. Representative results of the assay are shown in Fig. 1. Digestion of the 139 bp fragment of PCR products at the *Bsa*BI site, and of the 131 bp fragment at the *Bst*XI site yielded fragments of 112 bp + 27 bp and 108 bp + 23 bp, respectively. The smaller fragments were not visible under the electrophoresis conditions used in this study. As positive control DNA samples, we prepared the reverse primer R1′ and the forward primer F2′, which were designed to cover the polymorphic site containing G (Table 1). These control PCR products were resistant to *Bsa*BI and sensitive to *Bst*XI, respectively, as demonstrated in Fig. 1. Thus, the Asp299Gly allele was considered to be very rare in a Japanese population.

Cario and Podolsky (10) recently showed that TLR-4 was strongly upregulated in ulcerative colitis and Crohn's disease, which may be caused by an exaggerated host defense reaction of the intestinal epithelium to endogenous luminal bacterial flora. It is intriguing to hypothesize that the Asp299Gly allele of the *TLR-4* gene may be related to such an imbalanced reaction. We examined 86 ulcerative colitis patients for this polymorphism, but all of their alleles were wild-type.

REFERENCES

1. Medzhitov R, Preston-Hurlburt P, Janeway CA. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997;388:394–397.
2. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature 2000;406:782–787.
3. Poltorak A, He X, Smirnova I, Liu MY, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998;282:2085–2088.
4. Qureshi ST, Lariviere L, Leveque G, et al. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (TLR4). J Exp Med 1999;189:615–625.
5. Heumann D, Glauser MP, Calandra T. Molecular basis of host-pathogen interaction in septic shock. Curr Opin Microbiol 1998;1:49–55.
6. Herz U, Lacy P, Renz H, Erb K. The influence of infections on the development and severity of allergic disorders. Curr Opin Immun 2000;12:632–640.
7. Kojima M, Morisaki T, Izuhara K, et al. Lipopolysaccharide increases cyclo-oxygenase-2 expression in a colon carcinoma cell line through nuclear factor-kappa B activation. Oncogene 2000;19:1225–1231.
8. Arbour NC, Lorenz E, Schutte BC, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nature Genet 2000;25:187–191.
9. Wang L, Hirayasu K, Ishizawa M, Kobayashi Y. Purification of genomic DNA from human whole blood by isopropanol-fractionation with concentrated NaI and SDS. Nucleic Acids Res 1994;22:1774–1775.
10. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun 2000;68:7010–7017.