

Analysis of Toll-like receptor 2, 4, 6 and 9 genome DNA mutations in patients with tractable and intractable gastric mucosal diseases

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Abstract. The possible involvement of Toll-like receptor (TLR) genome DNA in the prolongation and relapse of inflammatory intestinal diseases and alcoholic hepatic diseases has been reported. In this study, we examined the relationship of mutations of the TLR 2, 4, 6 and 9 genomic DNA to recurrent or intractable gastritis or gastric ulcers. The subjects were 32 patients, including 6 with *H. pylori* (Hp)-positive gastritis, 4 with Hp-negative gastritis, 10 with Hp-positive tractable gastric ulcer, 5 with Hp-positive recurrent gastric ulcer after Hp eradication, and 7 with Hp-negative easily recurrent gastric ulcer after Hp eradication. Gastric mucosal tissue and peripheral blood specimens were collected from each of the patients. DNA was extracted from the tissue and blood specimens and subjected to electrophoresis by the PCR method, using the oligonucleotide primers of TLR 2, 4, 6 and 9. The gastric mucosal tissue specimens were collected endoscopically from the sites of the lesions. Subsequently, the presence or absence of genomic DNA mutations in the blood and tissue specimens was examined using a DNA sequencer. TLR 2, 4, 6 or 9 DNA mutations were not observed in any of the gastric mucosal or peripheral blood specimens obtained from patients with tractable gastritis or gastric ulcer, or from those with intractable gastric ulcer who were Hp-positive or Hp-negative or had become Hp-negative after eradication therapy. These data suggest that mutations of the

TLR 2, 4, 6 and 9 genome DNA may not be involved in the recurrence, delayed healing or intractability of gastritis and gastric ulcers.

Introduction

The Toll-like receptors (TLRs) are expressed mainly in macrophages and dendritic cells which are involved in spontaneous immunity. These receptors have been reported to recognize a structure common to various pathogenic microorganisms and thereby to protect the host from these organisms (1-3). TLRs have been reported to play important roles in not only spontaneous immunity, but also acquired immunity (4,5). Their expression is not limited to spontaneous immunocompetent cells, such as macrophages and dendritic cells, but is also observed in mast cells and epithelial cells (6,7). TLR is a type I transmembrane receptor, with the extracellular domain consisting of leucine-rich repeats and the intracellular portion composed of the Toll/IL-1 receptor (TIR) domain, which is homologous to the IL-1 receptor (4). Ten types of TLRs have been discovered in humans, and their ligands have also been revealed sequentially (5). Analyses of various pathological conditions that are associated with the appearance of TLRs have been conducted. In diseases of the upper digestive tract, chronic atrophic gastritis has been reported to be possibly associated with the appearance of TLR 4, irrespective of the presence/absence of Hp infection (8,9). The possible involvement of DNA mutations, such as SNPs, has been reported in the onset/delayed healing of various diseases (10-13). While prevention of recurrence of gastritis and peptic ulcer by Hp eradication has been reported in numerous cases, recurrent disease despite Hp eradication has also been reported in some cases (14,15). There have been no reports of the onset of intractability of these lesions being associated with mutations of the TLR genome. In the present study, we determined whether or not mutations of the TLR 2, 4, 6 and 9 genomic DNA might be involved in the recurrence, delayed healing or intractability of the lesions in cases of gastritis and gastric ulcer.

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Patients and methods

The subjects were 6 patients with Hp-positive gastritis, 4 with Hp-negative gastritis, 10 with Hp-positive tractable gastric ulcer, 5 with Hp-positive recurrent gastric ulcer after Hp eradication, and 7 with Hp-negative easily recurrent gastric ulcer after Hp eradication. Gastric mucosal tissue specimens from the lesion sites and peripheral blood specimens were collected from these patients in order to obtain DNA samples. The gastric mucosal tissue specimens were collected endoscopically. Taking into consideration the potential confounding of the results by DNA damage caused by external stimuli, such as food and drugs, DNA was prepared from both the specimens obtained from the lesion sites and peripheral blood cells. Gastric mucosal specimens were collected from the site of redness or atrophic mucosa in patients with gastritis, and from the scarred regions or marginal lesions in gastric ulcer patients. None of our study subjects had a history of non-steroidal anti-inflammatory drug (NSAID) use. Hp infection was diagnosed based on the results of Hp culture and the urea breath test (UBT), and subjects who tested negative on both were judged to be free of Hp infection.

DNA isolation. A GENOMIX kit (TALET, Italy) was used for extracting the DNA from the peripheral blood cells. After addition of the buffy coat, the specimens were heated for 15 min at 65°C. Then, 3 ml of chloroform was added, followed by further mixing. The specimens were then centrifuged at 3000 rpm for 10 min. The upper layers were collected and centrifuged again at 3000 rpm for 10 min. The supernatants were decanted, and the precipitates were completely dissolved using a vortex mixer. Then, 3 ml of 100% ethanol was added to precipitate the DNA. The phenol chloroform method was used for extraction of the DNA from the gastric mucosal tissue specimens. The samples were cut into small pieces, and TE buffer, 10% SDS and proteinase K were added. The final concentrations were adjusted to 2% SDS and 1 mg/ml proteinase K. Subsequently, the samples were heated at 65°C for 2 h, and then further heated at 56°C overnight. Next, phenol chloroform isoamyl was added and the supernatants were collected after 10-min centrifugation at 3000 rpm. Chloroform isoamyl was added followed by further mixing, and centrifugation was continued at 3000 rpm. Ethanol was added to the supernatants which were then centrifuged again. The supernatants were decanted, followed by the addition of 2 ml of 70% ethanol to the precipitate, and vortex mixing and centrifugation at 3000 rpm for 10 min. The DNA thus obtained was dissolved in TE buffer and the DNA concentrations were determined by spectrophotometry.

PCR method. 10X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl) was added to 50 mM MgCl₂ to achieve a final concentration of 1 mM, 10 mM dNTPs to a final concentration of 200 μM, and 50 μM forward primers and reverse primers to obtain final concentrations of 0.5 μM each. Plasmid DNA was added as the template for the amplification reaction, to achieve approximately 0.01 fM, and then 2.5 units of KOD-plus-DNA polymerase (Toyobo) was added. The Gene Amp PCR System 2400 (Perkin Elmer) was used for the PCR amplification. The PCR product was subjected to 0.8% agarose

Table I. PCR oligonucleotide primers.

TLRs	Forward	Reverse
TLR 2	tatgacgcaatggtatctg	ggcttgaaccaggaagacga
TLR 4	ggagcctttctggactatc	atgtagaacccgcaagtctg
TLR 6	gtgggtccattacgaactct	caaataaagtccactgcgtca
TLR 9	tgccctgcgcttctattca	caatttccgccacatcgag

gel electrophoresis, and amplification of DNA fragments to the target length was confirmed. The PCR oligonucleotide primers of TLR 2, 4, 6 and 9 used for the present study are listed in Table I.

Base sequence determination. The base sequence was determined by means of the chain elongation reaction termination method using dideoxynucleotide. An ABI PRISM and a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) were used, using plasmid DNA (0.2-0.5 μg) as the template. For this reaction, the reaction mixture with a total volume of 20 μl, consisting of 8.0 μl of the Terminator Ready Reaction Mix, 0.2-0.5 μg of the template, 3.2 pmol of the primer, and ddH₂O q.s., was prepared using the Gene Amp PCR System 2400 (Perkin Elmer). The reaction conditions were as follows: 25 cycles at 96°C for 10 sec, at 50°C for 5 sec, and at 60°C for 4 min. Subsequently, the system was incubated at 4°C. After completion of the reaction, the reaction liquid was precipitated with ethanol. After elimination of the supernatant, the system was washed with 70% ethanol and dried under reduced pressure. The precipitate was dissolved in 50 μl of TSR, degenerated for 2 min at 95°C, and allowed to stand on ice. The resultant DNA was analyzed using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems) sequencer.

Results

The results of gel electrophoresis of TLR 2, TLR 4, TLR 6, and TLR 9 with oligonucleotide PCR primers are shown in Fig. 1. Numbers 1, 2, 3, 4, 5, 6 and 7 represent cases of easily recurrent and tractable gastritis. Numbers 8, 9 and 10 represent cases of tractable gastric ulcers. Number 11 represent a case of an easily recurrent gastric ulcer after *H. pylori* eradication therapy. All samples in TLR 2, TLR 4, TLR 6 and TLR 9 were expressed uniformly by PCR gel electrophoresis. The oligonucleotide PCR primers of TLR 2, TLR 4, TLR 6 and TLR 9 were made up of 277 bases, 229 bases, 168 bases and 294 bases, respectively. Fig. 2a, b, c and d show the results of the analysis of the DNA sequences of TLR 2, TLR 4, TLR 6 and TLR 9, respectively. No mutations were observed in the TLR 2, 4, 6 or 9 DNAs prepared from any of the specimens obtained from either Hp-positive or Hp-negative patients with gastritis, Hp-positive patients with tractable gastric ulcers, or Hp-positive patients with recurrent or Hp-negative patients with easily recurrent gastric ulcers after Hp eradication. The analyzed data for the TLR 2, 4, 6, 9 DNA sequences in cases numbers 9 and 11 are given as representative data.

Expressions of TLR 2, TLR 4, TLR 6, and TLR 9 analyzed by Gene Amp PCR System 9700

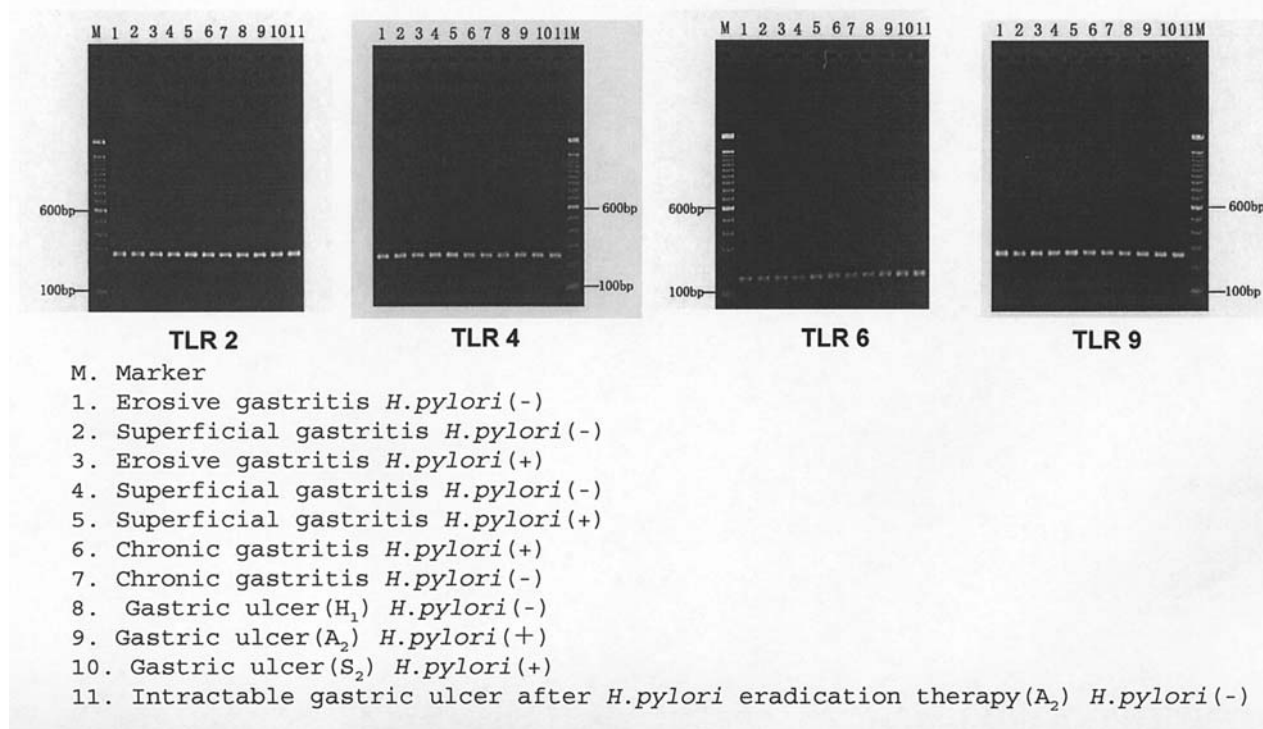


Figure 1. Numbers 1 and 3 represent cases of easily recurrent erosive gastritis where the patients were treated with NSAID. Numbers 2, 4, and 5 represent cases of tractable gastritis. Numbers 6 and 7 represent cases of chronic atrophic gastritis, following while case no. 11 represents an intractable gastric ulcer in a patient after Hp eradication therapy, and nos. 8, 9, and 10 represent cases of tractable gastric ulcers both Hp-positive and -negative patients.

Discussion

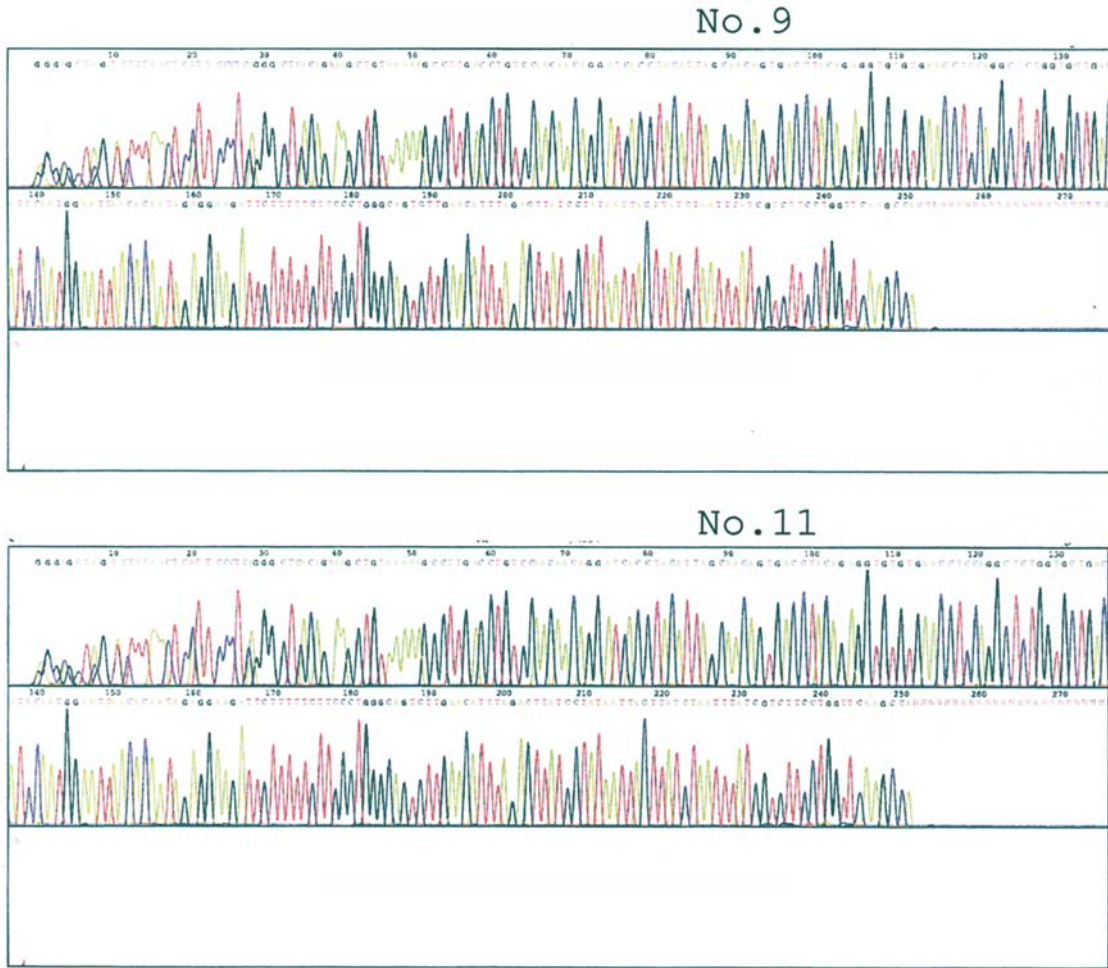
Expressions of pathogenic genes can trigger the onset of various diseases, and various molecules have been recognized to mediate this process, thereby controlling the pathological process in a complex manner. The relationships of various diseases to the expressions of genes have been analyzed comprehensively using such systems as gene chips. Recently, there have been a number of reports on the involvement of gene mutations in the onset, intractability and delayed healing of diseases (16-18). TLR mutations in relation to gastrointestinal diseases have been reported in cases of ulcerative colitis, Crohn's disease and alcoholic liver disease (19-22). Related studies have raised the possibility that mutations of the TLR 4 genomic DNA in ulcerative colitis, of the TLR 9 genomic DNA in Crohn's disease and of the TLR 2 genomic DNA in alcoholic liver disease may be associated with prolongation of the clinical course and intractability of the inflammation. In addition to these conditions, association of TLR mutations with prolongation of the clinical course and intractability of inflammation has also been reported for many other inflammatory diseases (23-25).

The TLRs were originally regarded as molecules involved in innate immunity and they were recognized to have physiological functions. However, more recently, these molecules were revealed to be involved in a broad spectrum of inflammatory and immune reactions, ranging from innate

immune reactions to acquired immune reactions. TLR 2, TLR 4, TLR 6 and TLR 9 have been shown to recognize peptidoglycan, LPS, lipoprotein, and CpG DNA as pathogen-associated molecular patterns. The expressions of TLRs have been seen in not only mucosal epithelia, but also in macrophages, dendritic cells and mast cells. As TLR ligands, fibronectin, hyaluronic acid and heparan sulfate fragments, as well as HSP60 and HSP70, have been identified, in addition to bacteria and viruses (26-30). From these reports, it can be readily understood that various pathological conditions processes, including those involved in delayed healing, are related to genomic mutations of the TLRs in the host.

A wide variety of factors, such as Hp infection and the use of NSAIDs, have been reported to be involved in the recurrence and intractability of gastritis and peptic ulcer (31-34), however, their possible association with TLR mutations has not been reported until now. TLR mutational analysis is designed to identify the molecular patterns of clinically important pathogenic factors, because DNA mutations of the TLR genome impact various functions, extending from innate to acquired immunity, potentially leading to changes in and even destruction of intra-mucosal signaling and defense systems. We identified no mutations of the TLR 2, 4, 6 or 9 genome in any of our current patients with tractable and intractable gastritis and peptic ulcer. These results constitute an important new finding, and future studies of TLR mutations in various diseases, especially in patients with malignancies

a



b

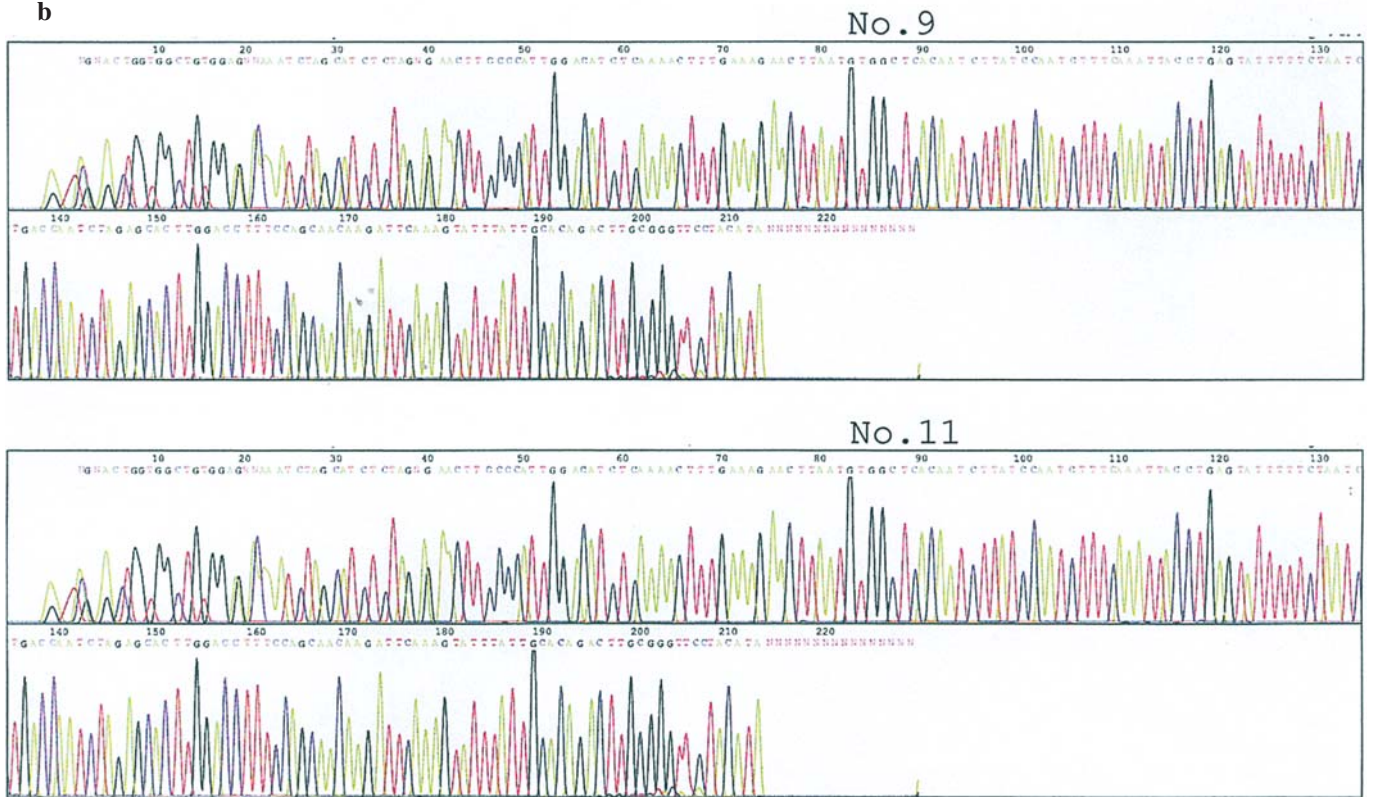


Figure 2. The chromatographs in (a) and (b) represent the DNA sequences of TLR 2 and 4, respectively.

such as gastric cancer, would be of great interest. In addition to TLR mutations, it is also essential to simultaneously study TLR ligands.

From our present results, we conclude that mutations of TLR 2, 4, 6 and 9 genomic DNA may not be involved in recurrence, delayed healing or intractability of gastritis and ulcer.

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