Single nucleotide polymorphism typing of the human toll-like receptor 4 gene at the 2-kb upstream region of the 5' untranslated region: New enclosure strategy for the risk grouping of poorly-differentiated gastric adenocarcinoma patients

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Abstract. To date, no enclosure method for risk grouping patients with poorly-differentiated gastric adenocarcinoma has been identified. We examined the relationship between mutations in toll-like receptor 4 (TLR4) and patients with poorly-differentiated gastric adenocarcinoma. Genomic DNA was extracted from the peripheral blood of 38 patients, 20 with well-differentiated and 18 with poorly-differentiated gastric cancer, from 25 patients with colorectal cancer and from 10 healthy volunteers. The polymorphism of TLR4 up to the 2-kb upstream region of the 5' untranslated region (UTR) was analyzed. The results revealed the presence of single nucleotide polymorphisms (SNPs) only among patients with poorly-differentiated gastric adenocarcinoma. SNPs were found at 3 sites: -2081, -2026 and -1601 in 12, 15 and 15 of the 18 cases of poorly-differentiated gastric adenocarcinoma, respectively. The results of the determination of a consensus among the base sequences of the core promoter, basal promoter and upstream promoter elements reveal that the variant sites were present in the TLR4 mRNA promoter region, suggesting that they were biologically significant variations. Polymorphism analysis of the upstream region of the 5' UTR of TLR4 may be a useful new enclosure strategy for the risk grouping of poorly-differentiated gastric adenocarcinoma patients.

Introduction

With the recent development of new techniques, such as polymorphism analysis of the genome and genes, screening methods for gastrointestinal tract cancer are changing (1-3). Significant enclosure methods for risk grouping patients with well-differentiated gastric adenocarcinoma have been developed, as represented by the *Helicobacter pylori* (*H. pylori*) and pepsinogen methods (4-6). However, to date no enclosure method for risk grouping patients with poorly-differentiated gastric adenocarcinoma at the time of diagnosis of gastric cancer has been established. Structurally, human genes consist of exons and introns, or the so-called gene-coding region and untranslated region (UTR). Upstream of the 5' UTR terminal side is the promoter region, which is the binding site of RNA polymerase to the UTR of DNA. The binding of RNA polymerase to this promoter initiates transcription into RNA. Thus, the upstream region of the 5' UTR terminal side is a biologically important region involved in gene expression and regulation.

Toll-like receptors (TLRs) are signal transduction molecules involved in the conversion of natural immunity to acquired immunity (7,8). Among them, toll-like receptor 4 (TLR4) recognizes lipopolysaccharides such as *H. pylori* as pathogen-associated molecular patterns, and is closely involved in the formation of mucosal lesions.

In this study, in order to examine the genetic background of patients with gastrointestinal tract carcinoma and particularly those with poorly-differentiated gastric adenocarcinoma, polymorphism analysis of the TRL4 gene at the 2-kb upstream region of the 5' UTR was performed in patients with gastric cancer or colorectal cancer and in healthy volunteers. Differences in the sites of single nucleotide polymorphisms (SNPs) and the relationship between these sites and the promoter region were examined.

Patients and methods

A total of 73 cases were included in the study. The subjects included 38 patients with gastric cancer (mean age, 66.8 years). Twenty of these had well-differentiated adenocarcinomas (15 *H. pylori*-positive and 5 *H. pylori*-negative) and 18 had poorly-differentiated adenocarcinomas (8 with signet-ring cell

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Table I. PCR amplification conditions and names and sequences of the primers used.[a]

Primer	Sequence (5'→3')	Thermal conditions
Genomic PCR primer		
TLR-U1	AGCACCTACTCTGTGTCAGG	(96˚C for 20 sec, 60˚C for 30 sec and 68˚C for 3 min) 30 cycles.
TLR-L1	GCGAGGCAGACATCATCC	
2nd PCR primer		
TLR-U1.1	ATAATCTCAATACTCAGAGC	
TLR-L1.1	CATCATCCTGGCATCATCC	

[a]PCR was conducted using the Gene Amp™ PCR System 9700 (Applied Biosystems) and enzyme KOD-Plus (Toyobo).

Table II. Conditions for base sequence analysis, and names and sequences of the primers used.[a]

Primer	Sequence (5'→3')
TLR-U1.1	ATAATCTCAATACTCAGAGC
TLR-U2	GACGTTTTACGTAAGTTAGC
TLR-U3	TCCAAAAGAATTGAAAACAG
TLR-U4	TTCCACTTCTAAGAGCTGCC
TLR-U5	TTCTTCACAAGAAGGGGC
TLR-U6	AGAACCTGATTGTTTTCC
TLR-L1.1	CATCATCCTGGCATCATCC
TLR-L2	GCTTTTATCCCAATTAGTC
TLR-L3	TAGAGGGGTTAGGACTTC
TLR-L4	TTTTGTACAATCATCACCAC
TLR-L5	CCATTTGTCTGTTCATGGAC
TLR-L6	AACGAAGATAATCGCATGTG

[a]Base sequence analysis was performed using the ABI PRISM™ 3700 DNA Analyzer and BigDye® Terminator Cycle Sequencing Kit (both from Applied Biosystems).

carcinomas; 9 *H. pylori*-positive and 9 *H. pylori*-negative). The cases also included 25 patients with colorectal cancer (mean age, 62.7 years) and 10 healthy volunteers (mean age, 62.9 years) with no family history of malignant disease (6 *H. pylori*-positive and 4 *H. pylori*-negative). Informed consent was obtained from all the patients and volunteers prior to the study. Genomic DNA was extracted from the peripheral blood of the study subjects and applied in the following examinations.

DNA isolation. A Genomix Kit (Tale, Italy) was used to extract the DNA from the peripheral blood cells. After the addition of a buffy coat, the specimens were heated for 15 min at 65˚C, then 3 ml of chloroform was added followed by further mixing for 5 min. The specimens were then centrifuged at 3000 rpm for 10 min and the upper layers were collected and again centrifuged at 3000 rpm for 10 min. The supernatants were decanted and the precipitates completely dissolved using a vortex mixer. One hundred percent ethanol (3 ml) was then added to precipitate the DNA. The obtained DNA was dissolved in TE buffer, and DNA concentrations were determined by spectrophotometry.

Polymorphism analysis of the human toll-like receptor 4 genes. Analysis consisted of three parts: i) using the genomic DNA sample (20 ng) extracted from each case as the template, a 2277-bp region including the TRL4 gene at the 2-kb upstream region of the 5' UTR was amplified by PCR; ii) using the PCR amplification product as the template, a 2nd PCR was conducted which yielded a 2223-bp product; iii) the 2nd PCR product obtained in (ii) was purified and subjected to base sequence analysis, then the base sequence of the entire length of the double strand was determined. PCR amplification conditions, the names and sequences of the primers used for the PCR amplification, the conditions for the base sequence analysis and the names and sequences of the primers used for the base sequence analysis are shown in Tables I and II. The base sequences obtained were compared between the samples using the 5' UTR upstream sequence in the Ensembl Exon Report (database version 38, Ensembl transcript ID ENST 00000354834) as a reference, and SNP typing was conducted in the analyzed region. Sequencher™ Software (gene codes) was used for base-sequence analysis and SNP typing.

Examination of the RNA polymerase II promoter region of the hTLR4 gene. In prokaryotes such as *E. coli*, several types of RNA are synthesized by one type of RNA polymerase, while in eukaryotes, the structure of the promoter is more complex. There are 3 types of RNA polymerase in eukaryotes: type I is mainly involved in rRNA synthesis, type II in mRNA synthesis, and type III in tRNA synthesis. Of these, mRNA is the molecule that plays a key role in the transmission of genetic information from transcription to the protein-synthesizing system. A promoter consists of one or multiple promoter elements upstream of the core promoter in addition to the core and the basal promoters (9-11). Each RNA polymerase recognizes a specific promoter sequence (12,13). Therefore, in order to locate the RNA polymerase II promoter region which is the key for hTLR4 genetic information in the upstream region of the 5' UTR, the region up to 2-kb upstream of the 5'UTR was examined. The consensus of these base sequences has already been clarified. In this study, the narrowing down of the promoter region was performed by determination of the consensus of the base sequences of the core promoter [TATA box, initiator sequence (Inr sequence)] and basal promoter

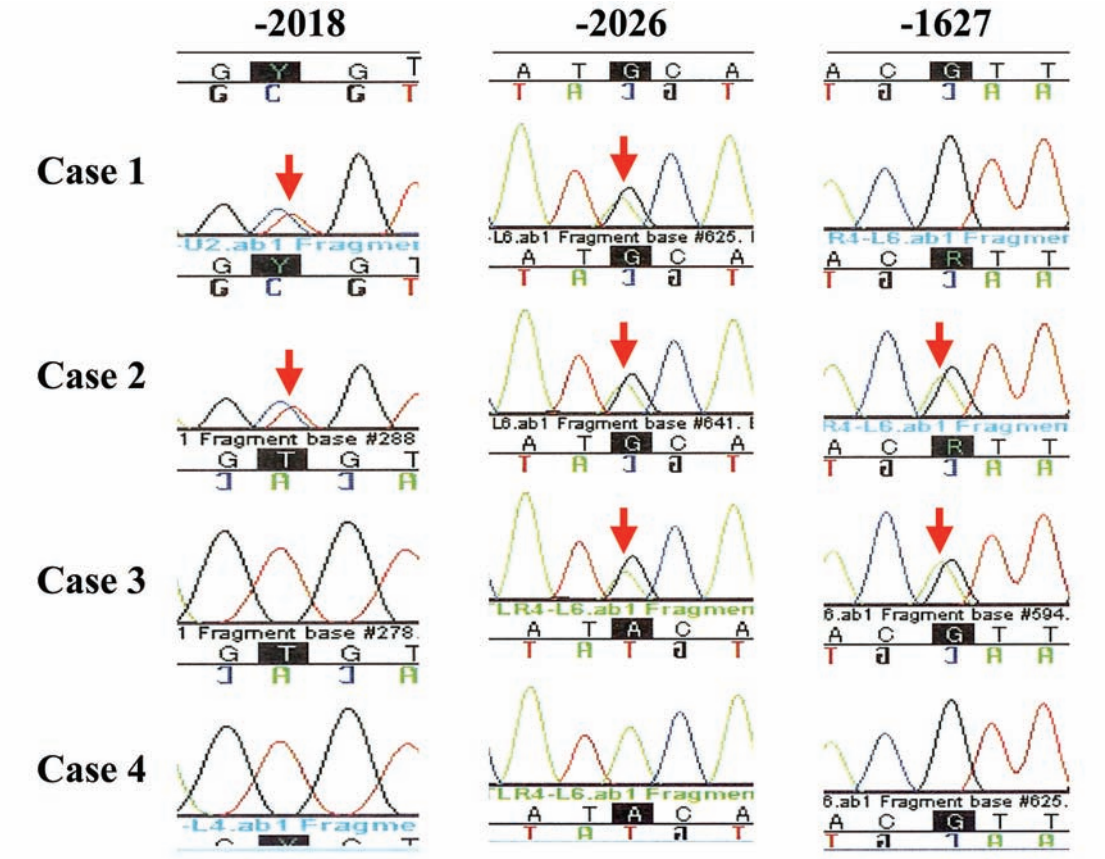

Figure 1. SNPs of the hTLR4 DNA sequence in the 2-kb upstream region of the 5' UTR examined by chromatographs. Cases 1, 2, 3 and 4 indicate poorly-differentiated gastric adenocarcinoma (Signet-ring cell carcinoma), poorly-differentiated gastric adenocarcinoma (poorly-differentiated), poorly-differentiated gastric adenocarcinoma (Signet-ring cell carcinoma) and well-differentiated gastric adenocarcinoma, respectively. Red arrows indicate heterozygous points.

(CAAT box, GC box). TATA box, Inr sequence, CAAT box and GC box are 5'-TATAWAW-3' (W: A or T), 5'-YYCARR-3' (Y: C or T; R: A or G), 5'-CCAAT-3' and 5'-ATGCAAAT-3', respectively (14-17).

Results

The study of the 2-kb upstream region of the 5' UTR of the hTRL4 gene in patients with gastric cancer and colorectal cancer located SNPs at three sites: -2081, -2026 and -1601. All SNPs were found only in cases of poorly-differentiated gastric adenocarcinoma. The SNPs were found in 12 (-2081), 15 (-2021) and 15 (-1601) of 18 cases, irrespective of the presence or absence of *H. pylori* infection (Table III, Fig. 1). Possible occurrence of the RNA polymerase II (mRNA synthesis) promoter region in the hTLR4 gene in the 2-kb upstream region of the 5' UTR terminal side was suggested by the consensus sequence of the base sequences of the core and basal promoters (Fig. 2).

Discussion

Upon diagnosis of gastric cancer, the risk grouping of patients is important. For the risk grouping of patients with well-differentiated gastric adenocarcinoma (at onset), the presence or absence of *H. pylori* infection (5,6), smoking history (18,19), history of alcohol consumption (19) and serum pepsinogen levels (4,6) have been reported as relevant. However, these factors have not been demonstrated to be useful for risk grouping poorly-differentiated gastric adenocarcinoma patients. Meanwhile, E-cadherin inactivation, abnormal amplification of K-*sam* and c-*met*, excessive expression of growth factors such as TGFß, IGF-II and bFGF and methylation of the promoter of the retinoic acid receptor *RAR*ß have been reported as gene abnormalities in poorly-differentiated gastric adenocarcinoma, but have yet to be applied clinically (20-28). Therefore, there are no reliable enclosure methods for risk grouping poorly-differentiated gastric adenocarcinoma patients at the time of diagnosis. The SNP-ID sites found in the 2-kb upstream region of the 5' UTR terminal side that have been published to date are TRL4, -2196, -2081, -2026, -1633, -1607, -1601, -1459, -1395, -1376, -728, -533, -69, -57, -55, -23 and -20; a total of 16 sites. In the present study, the sites at which SNPs were found in cases of poorly-differentiated gastric adenocar-cinoma were -2081, -2026 and -1601; a total of 3 sites. These sites are among those listed in the previously published SNP-ID. To have biological significance, a variant site must be in the promoter region of the mRNA. Variations in other regions do not affect gene expression or regulation, or protein synthesis, and have no biological significance. Therefore, it is very important to examine whether or not a variant site is in the mRNA promoter region. However, the mRNA promoter region of TLR4 has not as yet been clarified. It has been reported that in most of the mRNA promoter regions in human and mouse genes clarified to date, the mRNA promoter

Table III. SNPs of the hTLR4 gene at the 2-kb upstream region of 5 'UTR.

	H. pylori infection	-2081	-2026	-1601
Well-differentiated gastric adenocarcinoma (n=20)	+	0/15	0/15	0/15
	-	0/5	0/5	0/5
Poorly-differentiated gastric adenocarcinoma (n=18)				
Poorly-differentiated	+	3/5[a]	4/5[a]	4/5[a]
	-	3/5[a]	4/5[a]	4/5[a]
Signet-ring cell carcinoma	+	3/4[a]	3/4[a]	3/4[a]
	-	3/4[a]	4/4[a]	4/4[a]
Colorectal cancer (n=25)		0/25	0/25	0/25
Healthy volunteers (n=10)	+	0/5	0/5	0/5
	-	0/5	0/5	0/5

Denominators of fractions indicate the number of cases of genomic mutation. [a]Heterozygosity.

Figure 2. The base sequence of the hTLR4 gene at the 2-kb upstream region of the 5 'UTR upstream. The green-colored region indicates the first exon; the pink region the 5'UTR. Characters in red indicate the upper genomic PCR primer; those in blue, the lower genomic PCR primer. Boxes indicate heterozygous points (SNPs) in the hTLR4 gene at the 2-kb upstream region of the 5' UTR included in a total of sixteen published SNP-ID sites. The red-underlined regions indicate the Inr sequence and TATA box, respectively. The blue-underlined regions indicate the CAAT and GC boxes, respectively. The yellow-underlined boxes indicate the SNP points of this study. The red broken line indicates the speculated region of the mRNA promoter of hTLR4.

region is present at the -100 to -200 upstream region of the Inr sequence (16,17). This suggests that the aforementioned 3 SNP sites of may have biological significance. We are currently accumulating a larger population sample and further narrowing down the mRNA promoter region of TLR4.

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