

# Suppression of Chemokine Gene Expression and Production in LPS-Stimulated Macrophages by a 130 kDa Glycoprotein from Plerocercoids of *Spirometra erinaceieuropaei*

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Previous studies have shown that excretory/secretory (ES) products from plerocercoids of *Spirometra erinaceieuropaei* have immunosuppressive activities. We report here that a 130 kDa glycoprotein (ES130) purified from ES products as a suppressive factor of nitric oxide synthesis in LPS-stimulated RAW 264.7 cells inhibited the gene expression of 3 chemokines, regulated on activation normal T cell expressed and secreted (CCL5/RANTES), macrophage inflammatory protein 2 (CXCL2/MIP-2), interferon-inducible protein 10 kDa (CXCL10/IP-10) in RAW 264.7 cells and mouse peritoneal macrophages stimulated with LPS for 3 h. These chemokines are important factors for recruitment of inflammatory leukocytes. RANTES acts on monocytes, basophils, lymphocytes, natural killer cells and eosinophils. MIP-2 is a potent chemoattractant for neutrophils, while IP-10 binds to Th1 cells. Nearly 80% of MIP-2 gene expression and 50% of IP-10 gene expression in peritoneal macrophages stimulated with LPS for 8 h was suppressed as well as these chemokine production by the preincubation with 100 ng/mL of ES130 or 5000 ng/mL crude ES products for 24 h. On the other hand the mRNA expression of RANTES in macrophages stimulated with LPS for 8 h or 24 h was not inhibited by ES130 or crude ES products, while the RANTES chemokine levels in the incubation medium were significantly suppressed. These results suggest that ES130 may attenuate inflammation around the plerocercoids by inhibiting these chemokine production.

**Key words:** macrophage; chemokine; excretory/secretory products; lipopolysaccharide; *Spirometra erinaceieuropaei*

The larval plerocercoids of *Spirometra erinaceieuropaei* are known to cause “sparganosis” in various tissues in the human (Kudesia et al., 1998). When the plerocercoids are taken orally in many mammals including humans or rodents, the head portion of the larva migrated into the peritoneal cavity from the intestines. These plerocercoids

may have various bacteria on their surface and they bring these bacteria into the peritoneal cavity which, in turn, activates the peritoneal macrophages by the bacterial lipopolysaccharide (LPS). LPS is a ligand of Toll-like receptor 4, and has been known to be a potent activator of the immune system that induces local inflammation and

Abbreviations: ELISA, enzyme-linked immunosorbent assay; ES, excretory/secretory; FBS, fetal bovine serum; IFN, interferon; IL-1, interleukin-1; ISRE, IFN-stimulated response element; IP-10, IFN-inducible protein 10 kDa; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; RT, reverse transcriptase; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor

septic shock (Rietschel and Brade, 1992; Guha and Mackman, 2001). It causes multiple effects on macrophages, including the secretion of chemokines for recruitment of inflammatory leukocytes and the inflammatory mediators such as interleukin-1 (IL-1) and tumor necrosis factor (TNF)- $\alpha$  (Hsi and Remick, 1995).

The chemokines are divided into four families on the basis of the arrangement of the first 2 of 4 conserved cysteins (Ben-Baruch et al., 1995; Rottman, 1999). Many of the CXC chemokines are potent chemoattractants for neutrophils, but not monocytes, while CC chemokines act on monocytes, basophils, lymphocytes, natural killer cells and eosinophils (Wang et al., 1998; Rottman, 1999). In the mouse, the CXC family includes macrophage inflammatory protein 2 (CXCL2/MIP-2), interferon-inducible protein 10 kDa (CXCL10/IP-10) and KC (CXCL1/Gro- $\alpha$ ). MIP-2 and KC bind to CXCR1 and/or CXCR2 expressed on neutrophils, while IP-10 binds to CXCR3 expressed on Th1 cells. The CC family includes JE/monocyte chemoattractant protein (CCL2/MCP-1) and regulated on activation normal T cell expressed and secreted (CCL5/RANTES). RANTES is a ligand for CCR1/3/5 expressed on monocytes, macrophages, T cells, dendritic cells and eosinophils (Wang et al., 1998; Rottman, 1999).

The experimentally infected plerocercoids do not induce the strong inflammatory responses around the parasites and can survive for long periods in the tissues of mice, hence we hypothesized that the larval plerocercoids of *S. erinaceieuropaei* secreted an immunosuppressive factor(s). We previously showed that the excretory/secretory (ES) products from the plerocercoids suppress the LPS-induced expressions of chemokines KC and JE (Fukumoto et al., 1997), TNF- $\alpha$  (Miura et al., 2001; Dirgahayu et al., 2002), and IL-1 $\beta$  (Dirgahayu et al., 2004) in murine macrophages.

In the present study, we purified an immunosuppressive factor (ES130) of a 130 kDa glycoprotein from ES products of plerocercoids and examined the effect on the chemokine gene expression.

## Materials and Methods

### **Preparation of ES products from plerocercoids of *Spirometra erinaceieuropaei***

Plerocercoids of *Spirometra erinaceieuropaei* were collected from 2 species of snakes (*Elaphe quadrivirgata* and *Rhabdophis tigrinus*) in the southern part of Ehime Prefecture, Japan and stored for over 6 to 10 months in the subcutaneous tissue of golden hamsters, which were housed and maintained according to the guidelines for proper treatment of animals at the Research Center for Bioscience and Technology, Tottori University, Japan. ES products were obtained as described previously (Miura et al., 2001). To obtain ES products, 25 plerocercoids aseptically removed from hamsters were incubated for 24 h in 25 mL of Dulbecco's modified Eagle's medium (DMEM: Invitrogen, Carlsbad, CA) in a 10 cm dish. The medium was centrifuged at 10,000  $\times$  g for 30 min at 4°C to remove insoluble debris, then dialyzed against 25 mM Tris-HCl (pH 7.4) and concentrated with Amicon Ultra-15 Centrifugal Filter Units (Nihon Millipore, Tokyo, Japan), and subsequently sterilized with a 0.22  $\mu$ m filter (Nihon Millipore). The ES products after sterilization are referred to as crude ES products hereafter. The protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), and adjusted to 500  $\mu$ g/mL for culture or 250  $\mu$ g/mL for purification.

### **Purification of an immunosuppressive factor from ES products**

The crude ES products were loaded onto a new MonoQ HR5/5 anion-exchange column (Amersham Bio Sciences, Piscataway, NJ) equilibrated in 25 mM Tris-HCl (pH 7.4), and the column was washed with 10 column volumes of same buffer. The bound materials were eluted with a step gradient consisting of 10 column volumes each of 25 mM Tris-HCl (pH 7.4) containing 0.1, 0.2, 0.3, 0.4,

0.5 M NaCl. These samples were determined for their inhibitory effect on the nitrite production in RAW 264.7 macrophages (RCB00535) that were stimulated with LPS (*Escherichia coli* serotype 005:B5) (Difco Laboratories, Detroit, MI). The strongest inhibition was obtained in the fraction eluted with Tris-HCl containing 0.3 M NaCl. For further purification, the fractions eluted with 0.3 M NaCl were applied on *Ricinus communis* agglutinin (RCA120)- and wheat germ agglutinin (WGA)-agarose column (Seikagaku, Tokyo) sequentially according to the manufacture's instructions. To measure the protein concentration, the purified proteins and bovine serum albumin (Sigma Aldrich, St. Louis, MI), as a standard protein, were applied to 5% to 20% linear gradient polyacrylamide gel (SPG-R520L; ATTO, Osaka, Japan) for SDS-PAGE. After that, the polyacrylamide gel was stained with SYPRO Ruby gel staining (Invitrogen), and the concentration of the purified proteins was quantified by densitometry (ATTO Densitograph 4.0; ATTO).

### Preparation and culture of macrophage

RAW 264.7 cells were cultured according to Dirgahayu et al. (2004). Male C57/BL6 mice (8-week-old) were purchased from Japan SLC (Shizuoka, Japan). These mice were housed and maintained at the Research Center for Bioscience and Technology, Tottori University. Peritoneal macrophages were harvested with 10 mL of ice-cold phosphate-buffered saline on the 3rd day af-

ter intraperitoneal injection of 2 mL thioglycolate (Sigma-Aldrich). The macrophages in DMEM containing penicillin G (Banyu Pharmaceutical, Tokyo), streptomycin (Meiji Seika, Tokyo) and 10% heat-inactivated fetal bovine serum (FBS; Invitrogen,) were plated in 60 mm tissue culture dish (Greiner, Bayern, Germany). Then the cells were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> and were left for more than 14 h before each experiment.

The culture medium was replaced with a fresh DMEM including penicillin G, streptomycin and 10% FBS, and either with crude ES products (0.5–5.0 µg/mL) or purified ES products (10–200 ng/mL). The cells were incubated for 24 h, and were stimulated with 100 ng/mL LPS. After 3 h, 8 h or 24 h of incubation, the cells were collected for isolation of RNA, and the supernatant fluids of these dishes were collected for the measurement of chemokines by enzyme-linked immunosorbent assay (ELISA).

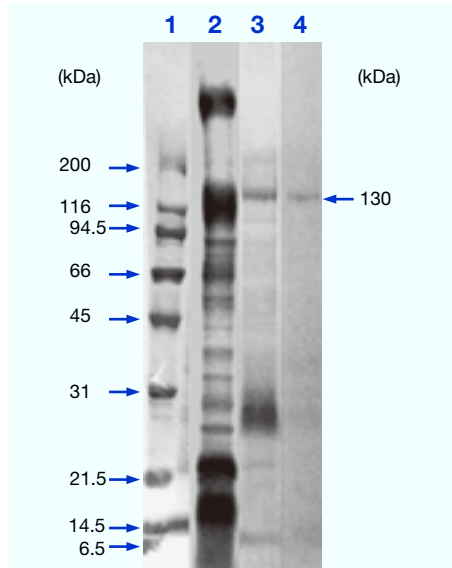
### RNA isolation and semi-quantitative reverse transcription-PCR

Total RNA was prepared using an ISOGEN Kit (Nippon Gene, Tokyo) according to the manufacture's protocol. Total RNA (1 µg) was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Promega, Tokyo) and random primers. The cDNA was subjected to PCR amplification with *Taq* DNA polymerase (Gene *Taq*) (Nippon Gene). The primers for IL-1β and TNF-

**Table 1. Chemokine primers and cycle number of PCR**

Gene	Primer	Product size (bp)	Cycle
CCL5/RANTES	5'-CCT CAC CAT CAT CCT CAC TGC A-3'	(forward)	334
	5'-CAT CCC CAA GCT GGC TAG GAC T-3'	(reverse)	
CXCL2/MIP-2	5'-AGT TTG CCT TGA CCC TGA AGC C-3'	(forward)	466
	5'-TGG GTG GGA TGT AGC TAG TTC C-3'	(reverse)	
CXCL10/IP-10	5'-CCT ATC CTG CCC ACG TGT TGA G-3'	(forward)	431
	5'-CGC ACC TCC ACA TAG CTT ACA G-3'	(reverse)	
β-actin	5'-TGG AAT CCT GTG GCA TCC ATG AAA-3'	(forward)	349
	5'-TAA ACC GCA GCT CAG TAA CAG TCC-3'	(reverse)	

IP-10, interferon-inducible protein 10 kDa; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted.



**Fig. 1.** An immunosuppressive factor, ES130, purified with ion-exchange chromatography and lectin chromatography was analyzed by SDS-PAGE with SYPRO Ruby gel staining. Lane 1, molecular marker; lane 2, crude ES products; lane 3, the fraction eluted from the MonoQ column with Tris-HCl containing 0.3 M NaCl; lane 4, the fraction eluted from the RCA120- and WGA-agarose columns sequentially. ES, excretory/secretory; RCA, *Ricinus communis* agglutinin; WGA, wheat germ agglutinin.

$\alpha$  were used according to Kina et al. (2005), and those for cyclooxygenase-2 (COX-2) were based on Fukumoto et al. (2006). The primers that we used for RANTES (Heeger et al., 1992), MIP-2 (Su et al., 1996), IP-10 (Baker et al., 2003) and  $\beta$ -actin (Alonso et al., 1986) are listed in Table 1. The PCR cycles consisted of denaturation at 94°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 1 min. The number of cycles for semi-quantitative reverse transcriptase-PCR (RT-PCR) of each gene was also listed in Table 1. The PCR products were separated by electrophoresis on 1.5 % agarose gels, and stained with ethidium bromide and photographed under UV light with a digital camera. For quantification, electrophoretic gels were analyzed using the software Image J version 1.33 (National Institutes of Health Bethesda, MD). The amount of mRNAs was normalized to that of  $\beta$ -actin.

## ELISA analysis

The culture medium of  $4.0 \times 10^6$  macrophages in 60 mm dishes was harvested after incubation with LPS for 8 h or 24 h. The collected supernatants were stored at  $-80^\circ\text{C}$  before measurement. The concentrations of RANTES, MIP-2 and IP-10 were assayed using ELISA kit (R & D Systems, Minneapolis, MN), following the manufacturer's protocol.

Statistical analysis was performed by non-repeated measures analysis of variance. If there was significant difference between the positive control and treatment groups, Dunnett's test was subsequently performed. Data were analyzed with statistical package software SPSS 12.0 for Windows.

## Results

### Purification of an immunosuppressive factor (ES130)

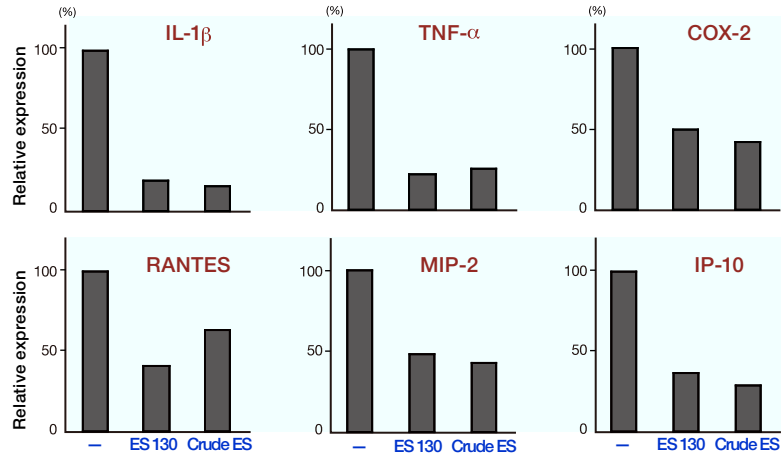
A suppressive effect on NO synthesis in LPS-stimulated RAW 264.7 cells was found in 0.3 M NaCl fraction of crude ES products by an anion exchange chromatography. Then we purified a glycoprotein of 130 kDa, which suppressed NO synthesis in RAW 264.7 cells, using 2 lectin columns, RCA-agarose- and WGA-agarose-columns sequentially (Fig. 1).

### Suppression of chemokine mRNA expression in RAW 264.7 cells by ES130

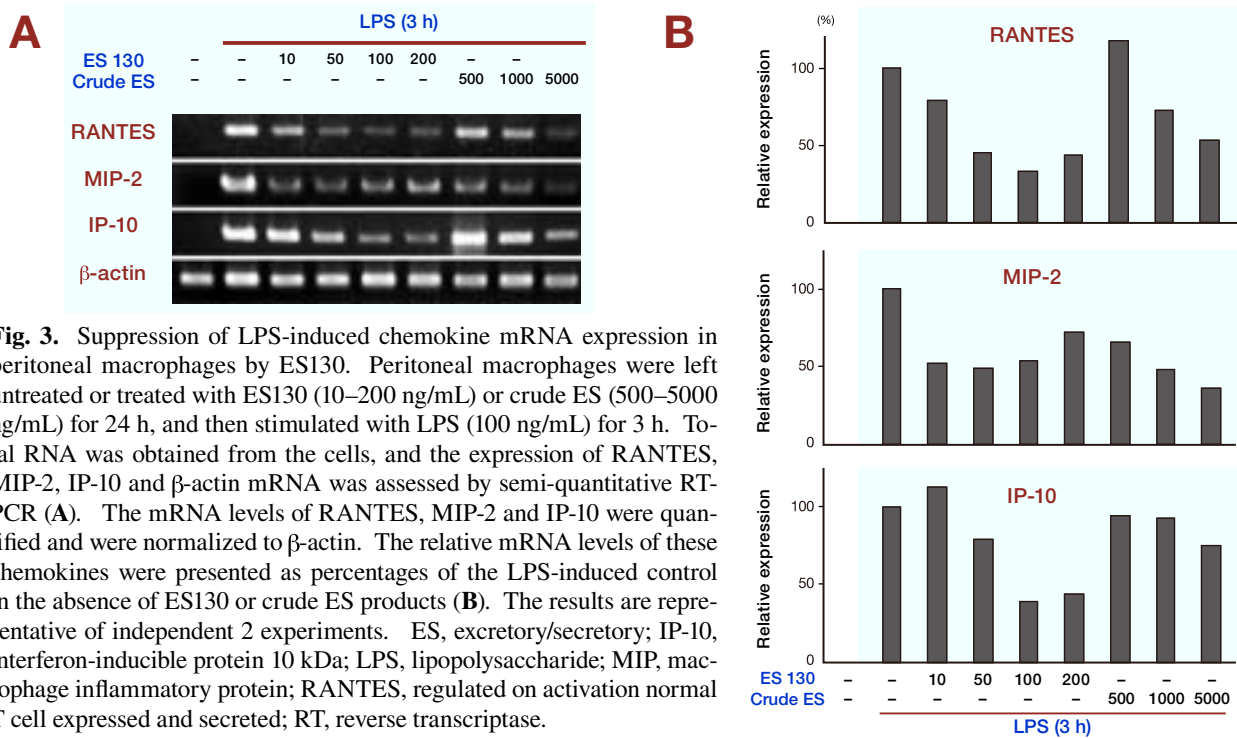
Preincubation with 100 ng/mL of ES130 for 24 h suppressed the mRNA expression of 3 proinflammatory genes, IL-1 $\beta$ , TNF- $\alpha$  and COX-2, and 3 chemokine genes, MIP-2, RANTES and IP-10 as well as 5  $\mu\text{g}/\text{mL}$  of crude ES in RAW 264.7 cells stimulated with LPS for 3 h (Fig. 2).

### Suppression of chemokine mRNA expression in peritoneal macrophages by ES130

No appreciable expression of chemokines was observed in control macrophages without LPS



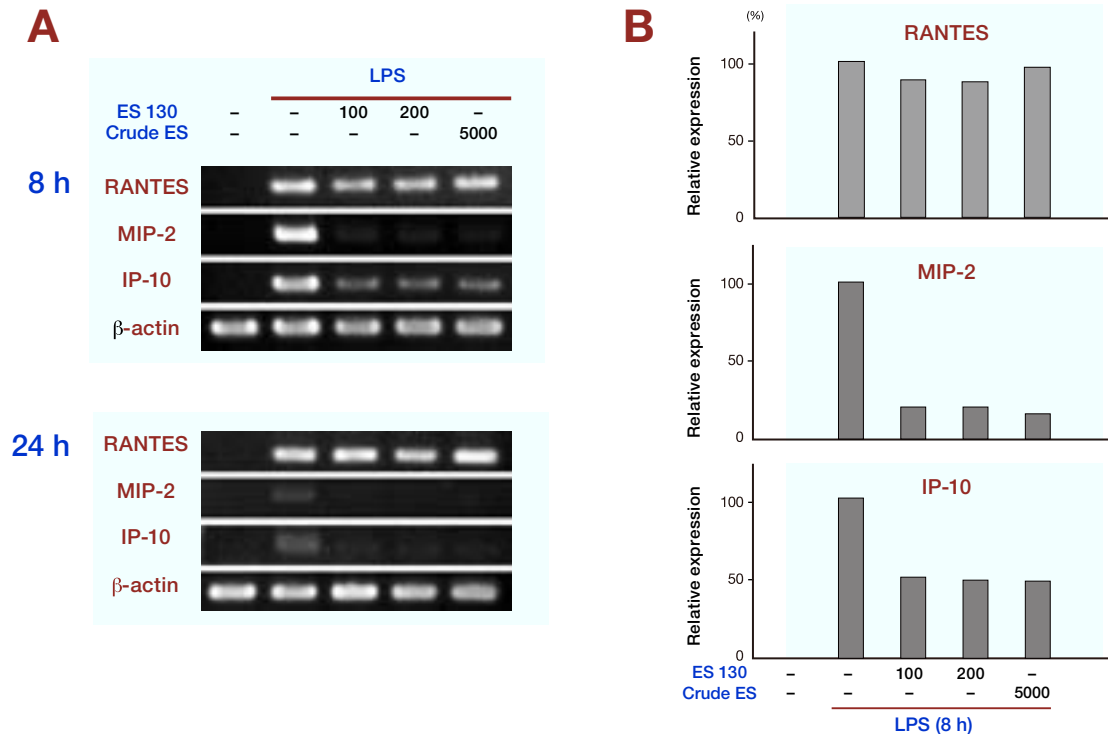
**Fig. 2.** ES130 inhibited the mRNA expression of proinflammatory genes including 3 chemokines in LPS-stimulated RAW 264.7 macrophages. RAW 264.7 cells were left untreated or incubated with ES130 (100 ng/mL) or crude ES products (5  $\mu$ g/mL) for 24 h, then stimulated with LPS (100 ng/mL) for 3 h. Total RNA was obtained and the mRNA expression of IL-1 $\beta$ , TNF- $\alpha$ , COX-2, MIP-2, RANTES, IP-10 and  $\beta$ -actin was analyzed by semi-quantitative RT-PCR. The mRNA levels of proinflammatory genes were quantified and were normalized to  $\beta$ -actin. Relative mRNA levels of these genes were presented as percentages of the LPS-induced control in the absence of ES130 or crude ES products. ES, excretory/secretory; IL-1, interleukin-1; IP-10, interferon-inducible protein 10 kDa; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; RT, reverse transcriptase.



**Fig. 3.** Suppression of LPS-induced chemokine mRNA expression in peritoneal macrophages by ES130. Peritoneal macrophages were left untreated or treated with ES130 (10–200 ng/mL) or crude ES (500–5000 ng/mL) for 24 h, and then stimulated with LPS (100 ng/mL) for 3 h. Total RNA was obtained from the cells, and the expression of RANTES, MIP-2, IP-10 and  $\beta$ -actin mRNA was assessed by semi-quantitative RT-PCR (A). The mRNA levels of RANTES, MIP-2 and IP-10 were quantified and were normalized to  $\beta$ -actin. The relative mRNA levels of these chemokines were presented as percentages of the LPS-induced control in the absence of ES130 or crude ES products (B). The results are representative of independent 2 experiments. ES, excretory/secretory; IP-10, interferon-inducible protein 10 kDa; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; RT, reverse transcriptase.

stimulation. Mouse peritoneal macrophages were either left untreated or preincubated with various doses of ES130 or crude ES products for 24 h. The cells were stimulated with 100 ng/mL LPS for 3

h. Crude ES products suppressed 3 chemokine gene expression in a dose-dependent manner. Ten to 100 ng/mL of ES130 also suppressed RANTES and IP-10 in dose-dependent manner, while about



**Fig. 4.** Suppression of LPS-induced chemokine mRNA expression in murine peritoneal macrophages by ES130. Peritoneal macrophages were left untreated or treated with ES130 (100 or 200 ng/mL) or crude ES (5000 ng/mL) for 24 h, and then stimulated with LPS (100 ng/mL) for 8 h or 24 h. Total RNA was obtained from the cells, and the mRNA expression of RANTES, MIP-2, IP-10 and  $\beta$ -actin was assessed by semi-quantitative RT-PCR (A). The mRNA levels of RANTES, MIP-2 and IP-10 in macrophages stimulated with LPS for 8 h were quantified and were normalized to  $\beta$ -actin. The relative mRNA levels of these chemokines were presented as percentages of the LPS-induced control in the absence of ES130 or crude ES products (B). The results are representative of 2 independent experiments. ES, excretory/secretory; IP-10, interferon-inducible protein 10 kDa; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted; RT, reverse transcriptase.

half of the MIP-2 gene expression was inhibited by 10 to 100 ng/mL of ES130 (Fig. 3). However, 200 ng/mL of ES130 did not inhibit the gene expression of 3 chemokines more than the dose of 100 ng/mL.

Nearly 80% of MIP-2 gene expression and 50% of IP-10 gene expression in peritoneal macrophages stimulated with LPS for 8 h were suppressed by the preincubation with 100 or 200 ng/mL of ES130 or 5000 ng/mL crude ES products for 24 h. The suppressive effect on the gene expression of MIP-2 and IP-10 continued in macrophages after stimulation with LPS for 24 h. On the other hand, the mRNA expression of RANTES in macrophages stimulated with LPS for 8 h or 24 h was not inhibited by ES130 or crude ES products (Fig. 4).

### ***Suppressive effect of ES130 on chemokine production in the medium of macrophages***

The effects of ES130 and crude ES products on 3 chemokine levels in the incubation medium of peritoneal macrophages stimulated with LPS for 8 h and 24 h were examined by ELISA.

MIP-2 concentrations in the medium of LPS-stimulated macrophages for 8 h and 24 h were deeply suppressed by ES130 or crude ES products (Fig. 5) as well as the suppression of MIP-2 mRNA levels (Fig. 4). The IP-10 concentration in the medium of macrophages stimulated for 8 h or 24 h was also suppressed by ES130 or crude ES products significantly (Fig. 5).

# Functional Polymorphisms in the Promoter Regions of Matrix Metalloproteinase-2, -3, -7, -9 and TNF-alpha Genes, and the Risk of Colorectal Neoplasm in Japanese

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Colorectal carcinogenesis involves environmental factors and genetic predispositions. Recent studies have suggested the associations between colorectal neoplasm and functional polymorphism of matrix metalloproteinases (MMPs) and cytokine genes. In this study, we analyzed polymorphisms of MMPs and tumor necrosis factor (TNF)-alpha genes, focusing on the susceptibility to colorectal neoplasm and the tumor progression. The subjects were 186 patients (95 men and 91 women) who underwent total colonoscopy, and were classified into cancer, adenoma and non-neoplasm (control) groups of 47, 72 and 67 patients, respectively. The polymorphisms at the MMP-2 –1306C/T, MMP-3 –1171 5A/6A, MMP-7 –181A/G, MMP-9 –1562C/T and TNF-alpha –308G/A loci were analyzed. Regarding background factors, significant differences were found in the age, sex ratio and alcohol-drinking and cigarette-smoking histories in the adenoma and cancer groups, compared to those in the control group. On these factors-adjusted logistic regression analysis of polymorphisms and disease susceptibility, no significant difference was noted in the frequency of any polymorphism in the adenoma and cancer groups, compared to those in the control group. The analysis of the involvement of polymorphisms in tumor progression in the adenoma and cancer groups revealed that the odds ratio for the MMP-3 5A allele was significantly higher in the cancer group (2.74; 95% confidence interval = 1.11–6.74,  $P = 0.02$ ). The polymorphisms of MMP genes and TNF-alpha genes were not associated with the susceptibility to colorectal neoplasm, but the involvement of the MMP-3 5A allele in the progression of adenoma to cancer was suggested.

**Key words:** colorectal neoplasm; gene analysis; matrix metalloproteinase; polymorphism; tumor necrosis factor-alpha

Recently, the rate of colorectal cancer has been increasing rapidly in Japan, and it is now the main cause of death from malignant disease, as in many other countries (Yoshimi and Sobue, 2004). In fact, age-standardized rates are similar to those in Caucasian populations of the United States (Yiu et al., 2004). The reason has generally been ascribed to the Westernized diet, characterized by a high intake of fat and meat, popular after World

War II (Kono, 2004), whereas the relevance of genetic predispositions has not been sufficiently analyzed in Japanese. Colorectal neoplasm is known to be a multifactorial disease, with dietary factors, lifestyle habits and genetic predispositions contributing to its development.

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Abbreviations: MMP, matrix metalloproteinase; RFLP, restriction fragment length polymorphism; TNF, tumor necrosis factor

Matrix metalloproteinases (MMPs) are proteolytic enzymes that play key roles not only in extracellular matrix degradation but also in all stages of cancer initiation, invasion and metastasis (Curran and Murray, 1999; Zhu et al., 2001; Behrens et al., 2003). Furthermore, recent studies have suggested that MMPs are involved in tumor initiation and development, including the regulation of cell proliferation, apoptosis, angiogenesis, loss of cell adhesion and immune responses to cancer (Egeblad and Werb, 2002). In fact, the over-expression of MMPs has been demonstrated in various cancers (Adachi et al., 1999; Ohashi et al., 2000; Aglund et al., 2004; Jordan et al., 2004). Polymorphisms of MMPs in the promoter region, naturally occurring sequence variations, may result in the differential expression of MMPs in individuals (Ye, 2000). To date, the promoters of MMP-2, -3, -7 and -9 genes have been reported to contain polymorphisms, exhibit allele-specific effects on the regulation of MMP gene transcription, and have been associated with changes in the susceptibility to or development of some cancers (Liang et al., 2002; Ghilardi et al., 2002, 2003; Matsumura et al., 2005).

On the other hand, previous studies have shown that tumor necrosis factor (TNF)-alpha expression may act as a high-risk factor or a poor prognostic factor in some cancers (Warzocha et al., 1997; El-Omar et al., 2003; Machado et al., 2003; Sharma et al., 2008). However, a few previous reports have suggested that there is no signifi-

cant association between the TNF-alpha -308A/G polymorphism allele and colorectal cancer development (Park et al., 1998; Landi et al., 2003).

To explore the possible association between these polymorphisms and the risk of colorectal neoplasms, we analyzed promoter genes in MMP-2, -3, -7 and -9 and TNF-alpha in a Japanese sample of colorectal neoplasm patients and controls who had no finding of colonoscopy.

## Materials and Methods

### Subjects

A total of 186 Japanese subjects (91 women and 95 men, with a mean age of  $64 \pm 13$  years) were studied between August 2003 and March 2007. All subjects had undergone total colonoscopy just prior to enrollment. One hundred nineteen patients with histologically confirmed colorectal neoplasm (47 cancers and 72 adenomas) and 67 non-neoplasm subjects (controls) were interviewed regarding their medical history, family history of colorectal cancer, anamnesis of diabetes, and habits such as alcohol intake and cigarette smoking. Before enrollment and blood sampling, written informed consent was obtained from all recruited subjects. This study was approved by the Ethics Committee of Tottori University Faculty of Medicine.

**Table 1. Sequences of PCR primers**

Gene	Polymorphism	Forward primer and reverse primer (5'-3')	T <sub>m</sub> (°C)	Restriction enzyme
MMP-2	-1306 C/T	<i>CTT CCT AGG CTG GTC CTT ACT GA</i> <i>CTG AGA CCT GAA GAG CTA AAG AGC T</i>	60	<i>Xsp I</i>
MMP-3	-1171 5A/6A	<i>CTT CCT GGA ATT CAC ATC ACT GCC ACC ACT</i> <i>GGT TCT CCA TTC CTT TGA TGG GGG GAA AGA</i>	65	<i>Tth111 I</i>
MMP-7	-181 A/G	<i>TGG TAC CAT AAT GTC CTG AAT GAT ACC TAT G</i> <i>TCG TTA TTG GCA GGA AGC ACA CAA TGA ATT</i>	65	<i>EcoR I</i>
MMP-9	-1562 C/T	<i>GCC TGG CAC ATA GTA GGC CC</i> <i>CTT CCT AGC CAG CCG GCA TC</i>	60	<i>Sph I</i>
TNF-alpha	-308 G/A	<i>GAG GCA ATA GGT TTT GAG GGC CAT</i> <i>GGG ACA CAC AAG CAT CAA G</i>	55	<i>Nco I</i>

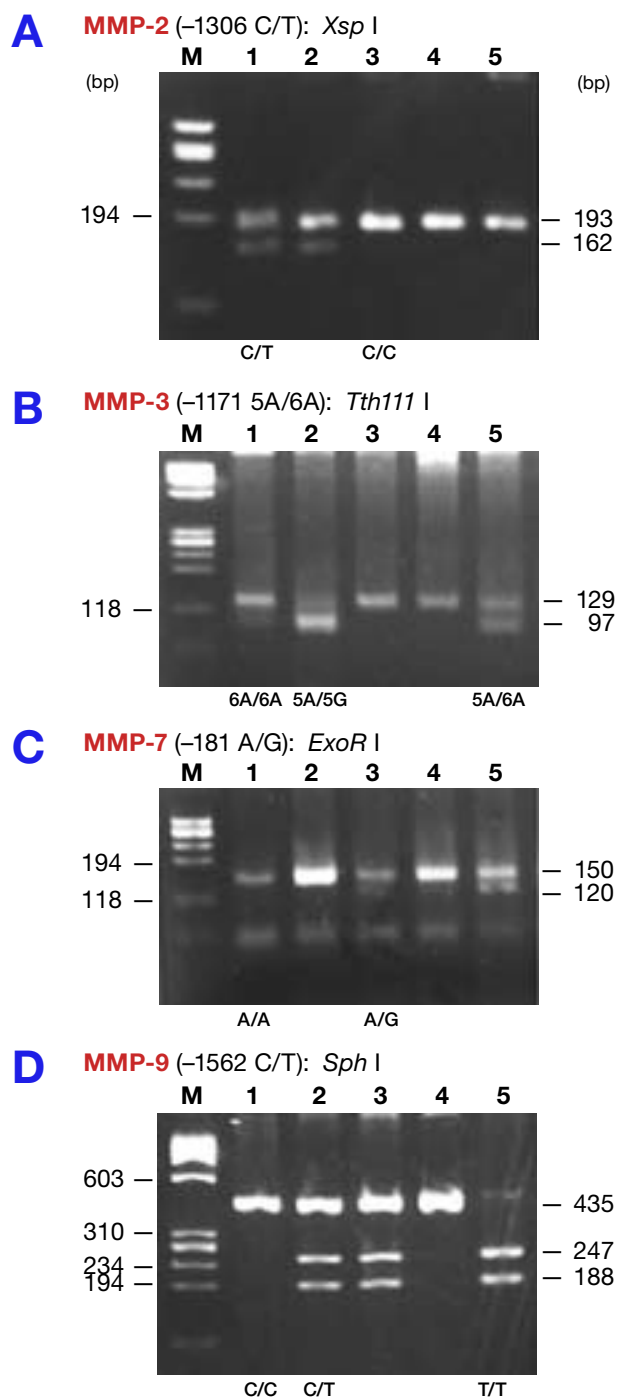


## DNA extraction

Genomic DNA samples were extracted from peripheral white blood cells using a DNA extracting kit (DnaQuickII: Dainipponseiyaku, Osaka, Japan) according to the manufacturer's instructions.

## Determination of genotypes

Using the extracted DNA as a template, the promoter region of each MMP was amplified by PCR using commercially available kits (HotStarTaq: QIAGEN, GmbH, Germany), following the manufacturer's instructions. The sequences of primers are summarized in Table 1. The methods used to type the MMP-2 -1306 C/T, MMP-3 -1171 5A/6A, MMP-7 -181A/G, MMP-9 -1562 C/T and TNF-alpha -308 G/A polymorphisms have been described previously (Jormsjö et al., 2001; Vasku et al., 2004; Zhang et al., 2004; Perri et al., 2005; Tuet al., 2007). Briefly, reactions were carried out under the following conditions: 5 min at 95°C, and then amplification for 35 cycles consisting of 30 s at 95°C, 30 s at an appropriate temperature and 30 s at 72°C. A final extension step at 72°C for 5 to 10 min was added to terminate the amplification. Subsequently, the PCR products were digested with appropriate restriction enzymes that cleave from 1 to 2 fragments. The digests were then electrophoresed on a 1 to 2% agarose gel using TBE buffer (45 mM Tris-borate, pH 8.3, and 2 mM EDTA) to confirm cleavage using molecular size marker IV (Nippon Gene, Tokyo, Japan). Gels were stained with ethidium bromide and visualized under UV light. As examples for gel documentation, the results of MMPs genotyping were shown in Fig. 1.



**Fig. 1.** Genotyping of MMP-2 C/T, MMP-3 5A/6A, MMP-7 A/G and MMP-9 C/T by PCR-restriction fragment length polymorphism.

**A:** MMP-2 C/T. The PCR products were digested with *Xsp*I restriction enzyme and subjected to electrophoresis on a 2.5% agarose gel. M, molecular marker; 1 and 2, C/T genotype; 3, 4 and 5, C/C genotype.

**B:** MMP-3 5A/6A. The PCR products were digested with *Tth111*I restriction enzyme and subjected to electrophoresis on a 2.5% agarose gel. M, molecular marker; 1, 3 and 4, 6A/6A genotype; 2, 5A/5A genotype; 5, 5A/6A genotype.

**C:** MMP-7 A/G. The PCR products were digested with *EcoR*I restriction enzyme and subjected to electrophoresis on a 2.5% agarose gel. M, molecular marker; 1, 2 and 4, A/A genotype; 3 and 5, A/G genotype.

**D:** MMP-9 C/T. The PCR products were digested with *Sph*I restriction enzyme and subjected to electrophoresis on a 2.5% agarose gel. M, molecular marker; 1 and 4, C/C genotype; 2 and 3, C/T genotype; 5, C/T genotype.

**Table 2. Characteristics of subjects**

		Control [67] Ratio	Patient with				
			Adenoma [72]		Cancer [47]		
			Ratio	<i>P</i> <sup>†</sup>	Ratio	<i>P</i> <sup>†</sup>	<i>P</i> <sup>‡</sup>
Age (year)		61.3 ± 2.0	66.3 ± 1.1	0.04	67.3 ± 1.7	0.04	NS
Sex	Male/female	25/42	42/30	0.02	28/19	0.03	NS
Family history <sup>§</sup>	Yes/no	8/59	3/69	NS	10/37	NS	0.008
Smoking status	Smoker/non-smoker	19/48	31/41	NS	26/21	0.006	NS
Alcohol status	Drinker/non-drinker	21/46	36/36	0.03	25/22	0.03	NS
Diabetes	Yes/no	5/62	5/67	NS	9/38	NS	NS

[ ], number of subjects.

Ratio, except age (mean ± SD).

NS, not significant.

<sup>†</sup> Compared with the control subjects.

<sup>‡</sup> Compared with patients with adenoma.

<sup>§</sup> Patients with family history of colorectal cancer in the 1st degree relatives.

### Statistical analysis

The significance of differences in means or proportions was measured using analysis of variance or the chi-square test. Comparison of the genotypes of MMP-2, -3, -7 and -9 and TNF-alpha and the allelotype distribution in the study groups was performed by means of a 2-sided contingency table using the chi-square test. Relationships between the genotypes and clinicopathological characteristics of the patients were evaluated by Fisher's exact test. To evaluate the increased risk of colorectal neoplasm associated with the presence of polymorphic alleles, odds ratios and 95% confidence intervals were computed, adjusted by logistic regression for several covariates potentially associated with the colorectal neoplastic risk such as age, sex, family history of colorectal cancer, alcohol intake, smoking status and diabetes mellitus. All statistical analyses were performed using the software package SPSS II for Windows (version 11.0 J, SPSS Japan, Tokyo)

## Results

### Characteristics of subjects

Subjects who had undergone colonoscopy were divided into a control group with no finding and patient groups with adenoma or cancer of the

colon. The backgrounds of groups are shown in Table 2. The mean age was 67.3 ± 1.7 years (range: 27–90) in the colorectal cancer group and 66.3 ± 1.1 years (range: 36–88) in the adenoma group. Compared to the control group, significant differences were noted in the age, sex ratio and alcohol-drinking history in the adenoma and cancer groups. No significant difference was noted in the cigarette-smoking history between the adenoma and control groups (*P* = 0.10), but one was present between the cancer and control groups (*P* = 0.01). There were no significant differences in the familial medical history of colorectal cancer or anamnesis of diabetes, compared to the control group.

On comparison of the adenoma and cancer groups, no significant differences were present in the age, sex ratio, or alcohol-drinking or cigarette-smoking history, but the frequency of familial medical history of colorectal cancer was significantly higher in the cancer group (*P* = 0.007). The frequency of anamnesis of diabetes was slightly higher in the cancer group, but the difference was not significant (*P* = 0.08).

### Genotype distributions and the susceptibility for adenoma and cancer

The frequencies and odds ratios of polymorphisms of the MMP-2, -3, -7 and -9 and TNF-

**Table 3. Genotype and allele frequencies of polymorphisms of the MMP-2, -3, -7 and -9 and TNF-alpha gene in controls and patients**

Genotype and allele	Control [67]	Patient with adenoma [72]			Patient with cancer [47]		
	Number (%)	Number (%)	Odds ratio† (95% CI)	P	Number (%)	Odds ratio† (95% CI)	P
<b>MMP-2 -1306 C/T</b>							
CC	64 (95)	66 (92)	1		41 (87)	1	
CT	3 (5)	6 (8)			5 (11)		
TT	0 (0)	0 (0)			1 (2)		
CT + TT	3 (5)	6 (8)	2.39 (0.51–11.7)	0.26	6 (13)	2.98 (0.62–14.29)	0.17
T	0.02	0.04			0.07		
<b>MMP-3 -1171 5A/6A</b>							
6A6A	50 (75)	58 (81)	1		31 (66)	1	
6A5A	17 (25)	13 (18)			14 (30)		
5A5A	0 (0)	1 (1)			2 (4)		
6A5A + 5A5A	17 (25)	14 (19)	0.79 (0.34–1.84)	0.59	16 (34)	1.56 (0.64–3.81)	0.32
5A	0.13	0.1			0.19		
<b>MMP-7 -181 A/G</b>							
AA	55 (82)	65 (90)	1		45 (96)	1	
AG	12 (18)	7 (10)			2 (4)		
GG	0 (0)	0 (0)			0 (0)		
AG + GG	12 (18)	7 (10)	0.61 (0.21–1.75)	0.35	2 (4)	0.21 (0.40–1.11)	0.06
G	0.09	0.05			0.02		
<b>MMP-9 -1562 C/T</b>							
CC	47 (70)	54 (75)	1		30 (64)	1	
CT	19 (28)	17 (24)			16 (34)		
TT	1 (2)	1 (1)			1 (2)		
CT + TT	20 (30)	18 (25)	0.78 (0.35–1.71)	0.53	17 (36)	1.41 (0.61–3.28)	0.41
T	0.16	0.19			0.13		
<b>TNF-alpha -308 G/A 1</b>							
GG	65 (97)	71 (99)	1		46 (98)	1	
GA	2 (3)	1 (1)			1 (2)		
AA	0 (0)	0 (0)			0 (0)		
GA + AA	2 (3)	1 (1)	0.62 (0.04–8.02)	0.71	1 (2)	0.77 (0.06–9.31)	0.83
A	0.01	0.01			0.01		

[ ], number of subjects.

CI, confidence interval.

† Odds ratio adjusted on age, sex, smoking and alcohol status.

alpha gene are shown in Table 3. Regarding the distribution of alleles in our study, most patients had C/C alleles in MMP-2, 6A/6A in MMP-3, A/A in MMP-7, C/C in MMP-9 and G/G in TNF-alpha, and, in reverse, none had G/G alleles in MMP-7, nor A/A alleles in TNF-alpha.

Logistic regression analysis adjusted for age, sex ratio, and alcohol-drinking and cigarette-smoking histories, which were background factors

with significant differences, was performed in the control, adenoma and cancer groups.

There were no significant differences of these genotypes between controls and adenoma patients, and between controls and cancer patients, suggesting that colorectal neoplasm susceptibility was not found in any polymorphism of the MMP-2, -3, -7 and -9 or TNF-alpha gene.

### Association with tumor progression

To investigate the association with tumor progression from adenoma to cancer, the frequencies and odds ratios of polymorphisms of the MMP-2, -3, -7 and -9 and TNF-alpha gene in the adenoma and cancer groups were calculated (Table 4). Logistic regression analysis adjusted for a familial history of colorectal cancer and anamnesis of diabetes was performed. We found that patients with the MMP-3 5A allele had significantly higher odds ratio (2.74) of cancer ( $P = 0.02$ ). However, other genotypes showed no significant difference between the adenoma and cancer groups.

### Discussion

It has been reported that MMPs play an important role in various cancer metastases and invasion including colorectal cancer (Asano et al., 2007), but no consistent finding has been obtained with regard to the association of any MMP gene polymorphism with colorectal cancer progression. Similarly, no association between TNF-alpha and colorectal cancer has been identified. We investigated polymorphisms of the MMP-2, -3, -7 and -9 and TNF-alpha gene and the susceptibility to colorectal neoplasm. In addition, considering that most colorectal cancer cases develop via the adenoma-carcinoma sequence (Fearon and Vogelstein, 1990), we studied the association of tumor progression with each gene polymorphism. MMP-2 expression in colorectal cancer tissue has been reported to be correlated with the disease stage and prognosis, and considered to play an important role in colorectal cancer invasion and metastasis (Turpeenniemi-Hujanen, 2005). In the MMP-2 gene, C/T polymorphism is present at -1306, and the transcription activity is higher in the C than in the T allele (Price et al., 2001). In studies reported by Heittaratchi et al. (2007) and Elander et al. (2006), -1306 C/T was not associated with the sensitivity of colorectal cancer, 5-year survival rate or tumor characteristics, whereas Xu

**Table 4. Genotype and allele frequencies of polymorphisms of the MMP-2, -3, -7 and -9 and TNF-alpha gene in patients with adenoma and cancer**

Genotype and allele	Patient with			<i>P</i>
	Adenoma	Cancer		
	[72] Number (%)	[47] Number (%)	Odds ratio† (95% CI)	
<b>MMP-2 -1306 C/T</b>				
CC	66 (92)	41 (87)	1	0.49 (0.43–5.488)
CT + TT	6 (8)	6 (13)	1.54	
<b>MMP-3 -1171 5A/6A</b>				
6A6A	58 (81)	31 (66)	1	0.02 (1.11–6.74)
6A5A + 5A5A	14 (19)	16 (34)	2.74	
<b>MMP-7 -181 A/G</b>				
AA	65 (90)	45 (96)	1	0.52 (0.11–3.00)
AG + GG	7 (10)	2 (4)	0.58	
<b>MMP-9 -1562 C/T</b>				
CC	54 (75)	30 (64)	1	0.41 (0.61–3.37)
CT + TT	18 (25)	17 (36)	1.43	
<b>TNF-alpha -308 G/A</b>				
GG	71 (99)	46 (98)	1	0.58 (0.13–36.13)
GA + AA	1 (1)	1 (2)	0.58	

CI, confidence interval.

†Odds ratio adjusted on family history and diabetes.

et al. (2004) reported that the risk of colorectal cancer development was higher in CC than in CT and TT types, showing that no consistent findings have been obtained with regard to the association of MMP-2 gene polymorphisms with colorectal cancer. In our study, no association of MMP-2 gene polymorphisms with the susceptibility to colorectal neoplasm or tumor progression was noted.

In colorectal cancer tissues, MMP-3 is mainly expressed in stromal cells (Newell et al., 1994). In the MMP-3 gene, 5A/6A polymorphism is present at -1171, and the transcription activity is higher in the 5A than in the 6A allele (Ye et al., 1996). Hinoda et al. (2002) reported that the 6A allele with low transcription activity indirectly contributed to colorectal cancer development, but no other study has supported this finding (Biondi et al., 2000; Ghilardi et al., 2001; Xu et al., 2006;

Hettiaratchi et al., 2007). The clinicopathological significance of the MMP-3 expression in colorectal cancer remains unclear. No association with colorectal neoplasm susceptibility was noted in the present study, but the frequency of the 5A allele with high transcription activity during the progression of adenoma to cancer was high in the cancer group. Sternlicht et al. (1999) investigated the MMP-3 in the breast cancer, and reported that MMP-3 promoted spontaneous premalignant changes and malignant conversion in the mammary glands of transgenic mice. Furthermore, regarding breast cancer, Nelson et al. (2008) reported that the overproduction of MMP-3 in mammary gland tissue triggered surrounding cells to increasingly produce reactive oxygen species, and induced DNA injury and genetic instability, finally leading to malignant conversion. Colorectal cancer may develop via a similar course.

While MMP-7 has a broad substrate specificity, it also exhibits “shedase” activity mediating cell surface protein release, and its influence on the growth and progression of colorectal, esophageal, stomach, and lung cancers has been investigated (Leeman et al., 2003; Zhang et al., 2005). A strong correlation of MMP-7 with colorectal cancer malignancy has been reported, in which the expression frequency of MMP-7 was particularly high in cancer tissues, and the MMP-7 expression level was higher in metastatic lesions than in the primary lesion (Yoshimoto et al., 1993). In the MMP-7 gene, A/G polymorphism is present at -181 of the promoter lesion, and the transcription activity is higher in the G than in the A allele (Ghilardi et al., 2003). Ghilardi et al. (2003) reported that the MMP-7 -181 G/G genotype was involved in colorectal cancer and tumor progression in Italians, but we detected no association of MMP-7 gene polymorphisms with colorectal neoplasm susceptibility or tumor progression. One reason may have been the biased distribution of MMP-7 polymorphisms. The frequency of MMP-7 G/G was about 0.5% in healthy subjects in a study performed in China (Lu et al., 2006), but about 20% in another Asian country,

India (Singh et al., 2008), suggesting regional variation. Actually, none of the subjects analyzed showed MMP-7 G/G in our study.

Regarding MMP-9, Zucker and Varcroca (2004) reported that the MMP-9 level rose in an early stage after the progression of adenoma to colon cancer, and Roeb et al. (2001) reported that MMP-9 transcription activity was 5 times higher in colorectal cancer than in the normal mucosa. In the MMP-9 gene, T/C polymorphism is present at -1562, and the transcription activity is higher in the C than in the T allele (Zhang et al., 1999). Matsumura et al. (2005) reported a relationship between stomach cancer invasion and the MMP-9 T allele position, and Grieu et al. (2004) reported that C homo improved the prognosis of breast cancer. Regarding colorectal cancer, Elander et al. (2006) investigated the relationship between C/T polymorphism at -1562 and colorectal cancer, and found no correlation, as observed in our study.

In the TNF-alpha gene, G/A polymorphism is present at -308, and the transcription activity is higher in the A than in the G allele (Abraham and Kroeger, 1999). Previous reports, however, support that there is no significant association between the TNF-alpha -308 A/G allele and colorectal cancer susceptibility (Park et al., 1998; Landi et al., 2003). The findings of our study are similar to those reports.

The present study clarified that the susceptibility to colorectal cancer is more strongly affected by age, gender, and cigarette-smoking and alcohol-drinking histories in Japanese, as reported by Otani et al. (2003), than gene polymorphisms studied. Colorectal cancer is a multifactorial disease involving environmental factors as well as a genetic predisposition, and properly cannot be explained by genetic predisposition alone. However, detailed clarification of the involvement of a genetic predisposition may contribute to the prediction and early discovery of cancer development as well as elucidation of the molecular mechanism involved in the carcinogenic factors and early stage of carcinogenesis.

In conclusion, no association with the susceptibility to colorectal neoplasm was noted in any polymorphism of the MMP-2, -3, -7 and -9 and TNF-alpha genes, but the involvement of the MMP-3 5A allele in tumor progression was suggested.

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