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Advances in Brief

Correlation between Expression of the *Matrix Metalloproteinase-1* Gene in Ovarian Cancers and an Insertion/Deletion Polymorphism in Its Promoter Region[1]

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Abstract

Matrix metalloproteinases (MMPs), a family of closely related enzymes that degrade the extracellular matrix, are likely to be involved in invasion and metastasis of tumor cells. A guanine (G) insertion/deletion polymorphism within the promoter region of *MMP-1* influences the transcription of this gene; *i.e.*, the 2G (insertion-type) promoter possesses greater transcriptional activity than the 1G (deletion-type) promoter. To investigate whether this feature contributes to cancer development and/or progression, we genotyped 163 ovarian cancer patients for the polymorphism and then analyzed levels of expression of the *MMP-1* gene in their tumors. The proportion of patients who were either heterozygotes or homozygotes for the *2G* allele was significantly higher than that observed among 150 individuals without cancer ($P = 0.028$). Moreover, the levels of *MMP-1* expression in cancer tissues among the patients carrying *2G* alleles were elevated significantly in comparison with 1G homozygotes ($P = 0.0038$). By stimulating degradation of extracellular matrix, an excess of MMP-1 production may enhance development and/or rapid progression of ovarian cancers.

Introduction

Ovarian cancer, one of the most common gynecological malignancies, has an aggressive phenotype and a relatively poor prognosis; peritoneal dissemination and/or retroperitoneal lymph node metastases are found in two-thirds of patients at the time of diagnosis (1). To improve prognosis of this disease, it is vital to clarify the molecular mechanisms involved in the invasion and metastasis of ovarian cancers.

Tumors spread by way of a multistep process, in which degradation of extracellular matrix and basement membrane barriers is a key feature (2–4). MMPs[3] comprise a family of at least 16 proteolytic enzymes that degrade extracellular matrix in a substrate-specific manner, and they are thought to have important roles in tumor invasion and metastasis (2, 5–7). Among the MMPs, MMP-1 (interstitial collagenase) degrades fibrillar collagens, the most abundant class of extracellular matrix proteins in interstitial connective tissue. Overexpression of MMP-1 has been demonstrated in tumor tissues and cell lines (8–10), and patients whose tumors express a high level of MMP-1 have particularly poor prognoses (11, 12).

Rutter *et al.* (13) recently investigated the effect of insertion of a guanine (G) nucleotide at −1607 bp in the *MMP-1* promoter sequence on regulation of transcription of the gene. This guanine creates the sequence 5′-GGA-3′, which is a core recognition sequence of the binding site for members of the Ets family of transcription factors (14–16). Promoters containing this 2G sequence display significantly higher transcriptional activity than 1G promoters (13). In tumor cell lines derived from melanomas or breast cancers, 2G homozygotes are found significantly more often than they are in normal population. Such observations have suggested that the *2G* allele might be a susceptibility factor for tumor progression.

To investigate whether the insertion/deletion polymorphism in the *MMP-1* promoter has any correlation with the development or progression of ovarian cancer in particular, we compared the frequency of each allele in DNAs from ovarian cancer patients with control samples from healthy women. We also examined whether the genotype for the polymorphism affected expression of the *MMP-1* gene in ovarian cancer tissue.

Materials and Methods

Samples. Whole blood and tumor specimens were obtained from 163 patients who had undergone surgery for ovarian cancer. All patients gave informed consent prior to collection of samples, according to institutional guidelines. Tumor specimens had been collected at the time of surgery, snap-frozen, and stored at −80°C after being histologically confirmed. Borderline tumors were excluded a priori from our study. Genomic DNA was prepared by digestion with proteinase K and phenol-chloroform extraction. Control samples consisted of DNA extracted from whole blood collected from 150 healthy women.

Radiolabeled-PCR Assay. Analysis of the insertion/deletion polymorphism in the *MMP-1* promoter was performed as described elsewhere (13), with some modifications. Primers for PCR amplification were: sense, 5′-GTTATGCCACTTAGATGAGG-3′; and antisense, 5′-TTCCTCCCCTTATGGATTCC-3′. The antisense primer (500 pmol) was end-labeled with ^{32}P by polynucleotide kinase in a 50-μl reaction mixture containing 2.5 μCi of [α-^{32}P]dATP. PCR amplification was performed in a 10-μl reaction volume containing 20 ng of genomic DNA, 5 pmol of each primer, 1.25 mM dNTPs, 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 6 μM EDTA, 6.7 mM $MgCl_2$, 10% DMSO, and 0.25 units of EX Taq DNA polymerase (TaKaRa, Tokyo, Japan). PCR conditions were as follows: 2 min at 94°C; 25 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C; and then 5 min at 72°C. Following amplification, an equal volume of stop buffer was added, and the products were denatured for 5 min at 80°C. Samples (2.5 μl each) were loaded onto a 7% denaturing polyacrylamide gel. After electrophoresis, the gels were dried and exposed to X-ray films. We invoked a χ^2 test to examine associations between genotypes of the insertion/deletion polymorphism and the presence of cancer (StatView software).

RNA Extraction and Semiquantitative RT-PCR. Total RNA was extracted from the 29 tumor tissue samples, for which genotypes of the insertion/deletion polymorphism in corresponding normal tissues were determined (9 *1G/1G* homozygotes, 10 *1G/2G* heterozygotes, and 10 *2G/2G* homozygotes), using TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's protocol. For RT-PCR, 10 μg of total RNA were reverse-transcribed to

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[3] The abbreviations used are: MMP, matrix metalloproteinase; RT-PCR, reverse transcriptase-PCR; LOH, loss of heterozygosity.

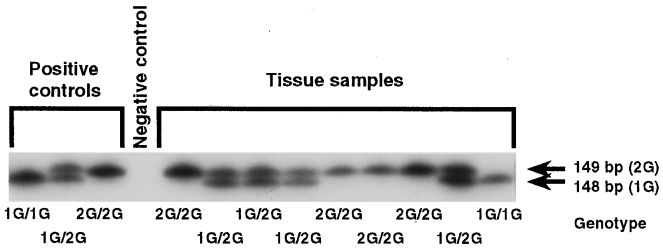

Fig. 1. PCR amplification of genomic DNA sequences that include the polymorphic site in the *MMP-1* promoter region. A 148-bp band corresponds to the *1G* (deletion-type) allele and a 149-bp band corresponds to the *2G* (insertion-type) allele. As positive controls, we used genomic DNAs with genotypes for the insertion/deletion polymorphism that had been determined by direct sequencing (*bottom*).

Table 1 *Frequency of each genotype of the MMP-1 promoter*

	No.	*1G/1G*	*1G/2G*	*2G/2G*
Controls	150	30 (0.20)	56 (0.37)	64 (0.43)
Ovarian cancer[a]	163	18 (0.11)	84 (0.52)[b]	61 (0.37)[b]

[a] Normal tissue samples from ovarian cancer patients. The relative frequency of each genotype is shown in parentheses.

[b] The frequency of *1G/2G* and *2G/2G* genotypes in ovarian cancers (89%) was higher than that in controls (80%; $P = 0.028$).

single-stranded cDNA using oligo$(dT)_{15}$ primer (Boehringer Mannheim) and Superscript II RNaseH$^-$ reverse transcriptase (Life Technologies, Inc.). Primers used to amplify *MMP-1* cDNA were: sense, 5′-GGACTCTCCCATTC TACTGA-3′ (exon 5); and antisense, 5′-CCCCGAATCGTAGTTATAGC-3′ (exon 6). The PCR was carried out in 20-μl reaction volumes under the following conditions: 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and then 5 min at 72°C. Amplified products of 303 bp were electrophoresed in 2% agarose gels, transferred to nylon membranes, hybridized to an internal oligonucleotide (5′-GCCATATATGGACGTTCCCAA-3′, exons 5–6), end-labeled with ^{32}P, and examined by means of a bioimaging analyzer (BAS 1000, Fujifilm) and autoradiography. As a reference, *β-2 microglobulin* cDNA (304 bp) was amplified in 25 cycles (sense primer, 5′-CACCCCCACTGAAAAAGATGA-3′; antisense primer, 5′-TACCTGTG-GAGCAACCTGC-3′; and internal oligonucleotide for hybridization, 5′-ATCTTCAACCTCCATGATG-3′). The intensity of *MMP-1* expression was calculated as a ratio against the intensity of corresponding *β-2 microglobulin* expression, measured by the BAS 1000. A Mann-Whitney *U* test was used to analyze differences in *MMP-1* expression according to the genotypes for the polymorphism.

Results

The insertion/deletion polymorphism in the *MMP-1* promoter yielded PCR products of 148 bp (deletion type) and 149 bp (insertion type; Fig. 1). When we amplified DNAs from blood samples of 163 ovarian cancer patients and 150 control individuals, we found a significant difference in allelic frequencies at this polymorphic site between patients and controls (Table 1). The frequency of heterozygotes and homozygotes for the *2G* allele (*1G/2G* and *2G/2G*) was significantly higher among women with ovarian cancers (89%) than that in individuals without cancer (80%; $\chi^2 = 4.827$, $P = 0.028$), indicating that individuals who carry even one *2G* allele are more susceptible to ovarian cancer than women with a constitutional *1G/1G* genotype.

To further investigate whether this insertion/deletion polymorphism influences expression of the *MMP-1* gene in ovarian cancers, we performed semiquantitative RT-PCR experiments using tumor tissues with genotypes that had been determined (Fig. 2*A*). In tumors carrying *2G* alleles (*1G/2G* heterozygotes or *2G/2G* homozygotes), the median expression levels of *MMP-1* were >7 times the median level of tumors having no *2G* alleles (Fig. 2*B*). The intensities of the *MMP-1* bands calculated as a ratio against intensities of corresponding *β-2 microglobulin* bands were 1.5 *versus* 0.2, respectively (Mann-Whitney *U* test, $P = 0.0038$).

Discussion

The insertion or deletion of a guanine nucleotide in the promoter region of the *MMP-1* gene, which determines the presence or absence of an Ets binding site, affects the transcriptional level of *MMP-1* in normal fibroblasts and in melanoma cells. Furthermore, in tumor cell lines, the frequency of homozygotes for the insertion (*2G*) type was significantly higher than that in normal population (13). On the basis of those findings, Rutter *et al.* (13) hypothesized that the insertion/deletion polymorphism might influence the transcriptional responsiveness of the *MMP-1* promoter in clinical settings because excessive production of MMP-1 is a major contributor to the stromal degradation involved in tumor invasion (11, 12). The high frequency of *2G* homozygotes in tumor cell lines has been taken to imply an increase in invasive behavior due to high levels of *MMP-1* expression.

In the work reported here, we investigated the frequencies of the different insertion/deletion alleles in blood samples from patients with ovarian cancer and examined expression of *MMP-1* in some of their

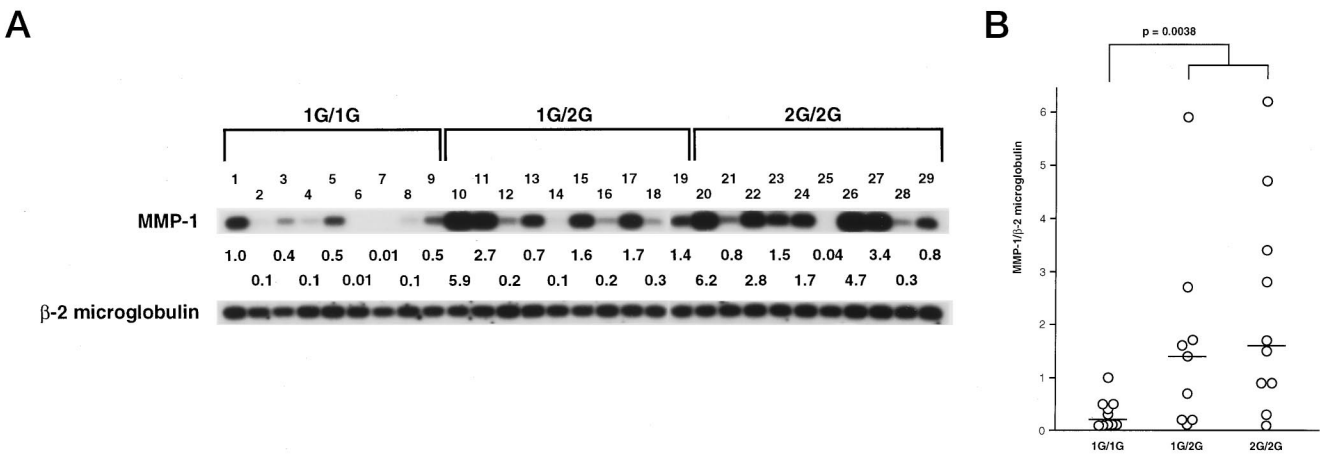

Fig. 2. *A*, expression of the *MMP-1* gene according to genotype for the insertion/deletion polymorphism in ovarian cancers. *Bottom*, ratios of *MMP-1* expression level against corresponding *β-2 microglobulin* expression. *B*, plot of the ratios of *MMP-1* expression against corresponding *β-2 microglobulin* expression for each genotype of the polymorphism. *Horizontal lines*, medians of each group.

tumors. We demonstrated that *1G*/*2G* and *2G*/*2G* genotypes were significantly more frequent in patients with ovarian cancer than they were in normal controls. Although a high frequency of *2G* homozygotes might have been anticipated in view of the earlier report concerning tumor cell lines (13), we also found that heterozygosity for the *2G* allele, not only homozygosity, seemed to increase susceptibility to ovarian cancer.

Subsequent analysis of *MMP-1* expression in ovarian cancers of all three genotypes revealed that expression of this gene was markedly higher in tumors having *1G*/*2G* and *2G*/*2G* genotypes than in tumors with the homozygotic *1G* genotype. We examined the tumors of the 10 constitutional heterozygotes for LOH at the *MMP-1* locus to compare the effect of LOH on expression of *MMP-1* in normal and tumor tissues. Because only one of those cases examined revealed LOH, we were unable to obtain any useful information. However, in the tumor that had lost the *2G* allele (corresponding to *Lane 18* in Fig. 2*A*), the level of *MMP-1* expression was low. Histopathological type of ovarian cancers did not correlate to the level of *MMP-1* expression.

Our overall results imply that the genotype for the insertion/deletion polymorphism in its promoter sequence influences the expression of the *MMP-1* gene in ovarian cancer tissues. Because the 2G type of promoter possesses a binding site for the Ets transcription factor, such binding is likely to lead to higher transcription of *MMP-1*. An excess of MMP-1 production may contribute to enhance degradation of the extracellular matrix and, thereby, promote invasion and metastasis of tumor cells. This scenario could partially explain the generally aggressive character of ovarian tumors. Because the expression of MMP-1 are heterogeneous in each class (Fig. 2), other factors/sequences will be also contribute to this activity.

We have suggested here that a *2G* genotype of the *MMP-1* promoter might represent a risk factor for development and progression of ovarian cancer. A clinical study should be undertaken in larger populations to investigate the correlation between alleles of this polymorphic site and prognosis of individual ovarian cancers.

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