Clinical significance of a single nucleotide polymorphism and allelic imbalance of matrix metalloproteinase-1 promoter region in prostate cancer

NORIHICO TSUCHIYA1, SHINTARO NARITA1, TERUAKI KUMAZAWA1, TAKAMITSU INOUE1, ZHIYONG MA1, HIROSHI TSURUTA1, MITSURU SAITO1, YOHEI HORIKAWA1, TAKESHI YUASA1, SHIGERU SATOH1, OSAMU OGAWA2 and TOMONORI HABUCHI1

1Department of Urology, Akita University School of Medicine, 1-1-1 Hondo Akita 010-8543; 2Department of Urology, Graduate School of Medicine, Kyoto University, 54 Shogoin, Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

Received April 16, 2009; Accepted June 5, 2009

DOI: 10.3892/or_00000462

Abstract. Matrix metalloproteinase-1 (MMP-1) is associated with cancer invasion and metastasis. The 2G allele of the polymorphic site in the MMP-1 promoter was demonstrated to have a higher transcription activity than the 1G allele. Allelic imbalance (AI) at 11q22 harboring the MMP-1 is frequently observed in various cancers and may be associated with an advanced disease. We conducted a case-control study to determine the association of the MMP-1 genotype with susceptibility to prostate cancer involving 283 prostate cancer patients and 251 controls. Furthermore, AI, retention allele of the MMP-1 promoter, and MMP-1 protein expression were analyzed in 77 prostate cancer specimens. The MMP-1 promoter polymorphism was associated with neither susceptibility nor progression of prostate cancer. Tumors with 1G/2G or 2G/2G genotypes had a significantly higher MMP-1 expression level compared to those with 1G/1G genotype (P=0.006 and 0.013, respectively). AI at 11q22 was significantly associated with the Gleason score (P=0.003) and pathological stage (P=0.022). In addition, AI was significantly associated with the Gleason score (P=0.003) and pathological stage (P=0.022). In addition, retention of the 2G allele showed a significant association with the pathological stage (P=0.026). AI at 11q22 region, retention of the 2G allele, specifically appeared to be involved in the progression of prostate cancer. However, the presence of the 2G allele of the MMP-1 promoter polymorphism itself seems to influence neither the susceptibility nor the progression of prostate cancer.

Introduction

Tumor invasion or metastasis is one of the most crucial events for determining the outcome of cancer patients. In the first step of these events, several proteinases such as serine proteinase urokinase plasminogen activator and matrix metalloproteinases (MMPs) degrade the extra-cellular matrix (ECM), so that the tumor cells can migrate to the stromal tissue through the basement membrane (1). MMP-1, a member of MMP family, is abundantly expressed in many types of cancer cells and adjacent stromal fibroblasts (2-5), and digests various elements of the ECM including collagen types I, II, II, VII, VIII, X, and XI (6). The overexpression of MMP-1 has been shown to be associated with tumor progression and poor outcomes for cancers of the digestive system and melanoma (3,5,7,8).

The expression of MMP-1 is mainly regulated by activated protein-1 (AP-1) transcription factor that mediates signal transduction from cytokines and growth factors such as interferons, interleukins, epidermal growth factor, and fibroblast growth factor (9). In the promoter region of the MMP-1, three AP-1 binding sites are located at -72, -186, and -1062 bp from the transcription start site. Insertion of an extra guanine residue adjacent to the AP-1 binding site at -1062 bp creates a new binding site for ETS transcription factor at -1602 bp (5'-AAGGAT-3'; 2G), and both the AP-1 and ETS cooperatively enhance the expression of MMP-1 (10). AP-1 binding to the -1062 bp acts as a repressor of transcription when the polymorphic site has only one guanine residue (5'-AAGGAT-3'; 1G) (11).

Recent epidemiological studies have demonstrated almost consistent results regarding the biological function of the MMP-1 promoter polymorphism and its association with the susceptibility or progression in several cancer types (4,12-23). The 2G allele of the polymorphism has been reported to be related with increased risk of colorectal, lung, endometrial, ovary, kidney, and bladder cancers (4,12-20), while the 2G allele was associated with tumor progression or patient survival but not with susceptibility of uterus, stomach, and colorectal.
cancers (21-23). However, the results of most studies are consistent with each other regarding the risk associated with the 2G allele. Meanwhile, 11q22 harboring the MMP-1 gene exhibits amplification in esophageal, colorectal, cervical, and prostate cancers (24-27). Determining which of the polymorphic alleles is amplified and how the amplified allele affects the expression of MMP-1 or phenotypes of cancers is also of interest. In prostate cancer, immunohistochemical and in situ hybridization analysis have detected MMP-1 in cancer cells as well as in normal adjacent and prostatic intraepithelial cells (28). However, a previous study that evaluated the effect of the polymorphism on prostate cancer found no association between MMP-1 and susceptibility or metastatic status of prostate cancer (29). There have been few studies focusing on the clinical implication of MMP-1 expression, promoter polymorphism, and allelic imbalance (AI) of 11q22.

We hypothesized that the 1G/2G polymorphism or AI of the MMP-1 promoter region affects the development and/or progression of prostate cancer. To validate this hypothesis, we first conducted a case-controlled study to examine the effect of the polymorphism on the risk and progression of prostate cancer, and then investigated how the genotype and AI of the region affected the malignant potential, invasiveness of the tumors, and protein expression of MMP-1.

Materials and methods

Subjects. We studied a series of 524 registered subjects, including 283 patients with prostate cancer and 251 control males at the Akita University Medical Center and related community hospitals in Akita prefecture, who agreed to participate in this study and provided blood specimens. Prostate cancer patients were selected from April 1997 to December 2003 and control subjects were selected from March 1998 to September 2001.

For all patients with prostate cancer, histological evaluation was performed on specimen obtained by transrectal needle biopsy or transurethral resection of the prostate for voiding symptoms. The clinical or pathological stage of prostate cancer at the time of diagnosis was determined by reviewing the medical records based on the Tumor-Node-Metastasis system. Prostate cancer was classified into stage A (T1a-bN0M0), stage B (T1c-2N0M0), stage C (T3-4N0M0), or stage D (T1-4N1M0-1 or T1-4N0-1M1) by the modified Whitmore-Jewett system. In patients who underwent radical prostatectomy, the final pathological stage was applied and in patients who did not undergo radical prostatectomy, clinical stage was applied. Pathological grading of PCa was determined according to the General Rule for Clinical and Pathological Studies on Prostate Cancer by the Japanese Urological Association and the Japanese Society of Pathology, which is mostly based on the WHO criteria and the Gleason score. All pathological grading was based on needle biopsy specimens in stages B-D patients and surgical specimens in stage A patients. Well, moderately, and poorly differentiated carcinomas generally correspond to Gleason scores of 2-4, 5-7, and 8-10, respectively. In the present study, because the two grading systems were individually used by local pathologists, the tumor grading system was newly categorized as follows: low grade cancer included well-differentiated or Gleason 2-4 carcinomas, intermediate grade cancer included moderately differentiated or Gleason 5-7 carcinomas, and high grade cancer included poorly differentiated or Gleason 8-10 carcinomas. No definitive pathological grade could be determined in 24 patients due to inadequate information or inappropriate classification by local pathologists.

The male controls comprised 251 volunteers without any apparent voiding symptoms. They were selected randomly from the native Japanese population attending community-based medical check-ups. They were all tested for total serum PSA levels (the Tandem-R assay), and those with abnormal total PSA levels (4.0 ng/ml or more) were omitted from the study. Written informed consent was obtained from all the subjects. The present study was approved by the Institutional Review Board of the Akita University School of Medicine, Akita, Japan.

Genotyping of the MMP-1 promoter polymorphism. DNA was extracted from the collected blood samples of each subject using a QIAamp Blood Kit (Qiagen, Hilden, Germany). The PCR was performed in a final volume of 15 μl containing 20 ng genomic DNA, 5 pmol forward primer (5'-GGT ATG CCA CTT AGA TGA GG-3'), 5 pmol reverse primer (5'-CTT GGA TTG ATT TGA GAT AAG-3'), 0.2 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 1.0 mM MgCl2, and 0.5 unit of Ampli-Taq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ). Initial denaturation at 94°C for 30 sec, annealing at 55°C, and extension at 72°C for 7 min. The forward primer was labeled with a fluorescent dye, HEX, to examine the size of PCR products with an autosequencer (ABI 310, Applied Biosystems, Foster, CA) as described previously (30). The size of the PCR products was determined in comparison with an internal ROX 400-size standard (Applied Biosystems) and analyzed using GENESCAN software v3.1 (Applied Biosystems) (Fig. 1A). The 72-bp and 73-bp fragments of amplified DNA are equivalent to 1G allele and 2G allele, respectively. The validity of the analysis was confirmed by direct sequencing of several PCR samples using ABI Prism 310 DNA sequencer (Applied Biosystems).

Analysis of allelic imbalance (AI). AI of the polymorphic site of MMP-1 promoter was analyzed using DNA pairs obtained from the tumor and peripheral blood samples in a subgroup of 77 patients who underwent radical prostatectomy. Pathological diagnosis was confirmed by microscopic examination of hematoxylin and eosin (H&E)-stained sections. Regions of >80% tumor density were marked on H&E-stained slides to be used as guidelines for microdissection. The target foci were microdissected using a 20-gauge needle, comparing the slide with the H&E staining in the same position. DNA was extracted using a DEXPAT kit (Takara Biomedical Inc., Shiga, Japan), precipitated in cold ethanol with sodium acetate and Pellet Paint NF, Co-Precipitant (Novagen, Madison, WI), and resuspended in Tris-EDTA buffer. Peripheral blood and corresponding prostate tissue DNA samples were simultaneously amplified and both the PCR products were analyzed using ABI PRISM 310 DNA sequencer as described above. AI was determined by measuring the signal imbalance between the opposing alleles. Presence of AI was detected when one
of the signal peaks in a PCR product from prostate tumor DNA was <70% of that from the corresponding peripheral blood (leukocyte) DNA. Representative results for AI analysis, no AI, retention of the 1G allele, and retention of the 2G allele (from the left to the right), are shown (B). Arrows indicate retention alleles.

Immunohistochemical analysis of MMP-1 expression.
Seventy-seven prostate specimens obtained at radical prostatectomy were subject to immunohistochemical analysis. The specimens were fixed in 10% buffered formalin and embedded in paraffin. Individual paraffin blocks containing cancer lesions with representative Gleason scores were selected from each specimen. Tissue sections (5 μm) were de-paraffinized in xylene and rehydrated through a graded ethanol series. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 15 min. The sections were boiled in 0.01 M citric acid (pH 6.0) to retrieve the antigen and non-specific binding was blocked with 5% goat serum for 10 min. After washing, mouse monoclonal anti-human MMP1 antibody (F-67; Daiichi Fine Chemical Co., Ltd., Toyota, Japan) at 1:1000 was applied and incubated at 4˚C overnight. After washing in PBS, secondary antibody conjugated with horseradish peroxidase (EnVision system; Dako, Japan Inc., Tokyo, Japan) was applied, followed by incubation at room temperature for 30 min. After washing in PBS again, tissue sections were developed with diaminobenzidine (DAB; Nichirei Biosciences Inc., Tokyo, Japan) and counter-stained with hematoxylin.

The expression of MMP-1 was classified into four categories according to the staining intensity (i.e., negative, weak, intermediate, and strong) (Fig. 2). Assessment was performed by two independent observers unaware of clinical information. In the statistical analysis, patients with Gleason score 8 or more were compared with those with a Gleason score of 7 or less, because patients with a Gleason score of 8 or more had a greater chance of recurrence after radical prostatectomy than those with a Gleason score of 7 or less (31). Similarly, pathological categorization of tumor stage was dichotomized as T3a or less versus T3b or more, because previous studies have demonstrated that patients with pT3b or more tumor stage had a significantly greater chance of recurrence than those having pT3a or less tumor stage (31).

Statistical analysis. The data were analyzed by SSPS version 16.0J software (SSPS Inc., Chicago, IL). Hardy-Weinberg equilibrium analyses were performed to compare the observed and expected genotype frequencies using the χ² test. The age-adjusted odds ratio (aOR) and 95% confidence interval (CI) for the relative risk of prostate cancer in each genotype were determined by multiple logistic regression analysis with the inclusion of age. The relationships between tumor stage and AI status, and between the genotype and MMP-1 expression level were analyzed by the χ² test. A probability of <0.05 was required for statistical significance.

Results
Association of the MMP-1 promoter polymorphism with a risk and progression of prostate cancer. The demographics of the study subjects are shown in Table I. The mean age of male controls and prostate cancer patients was 70.0±7.6, 72.0±8.3, respectively. The numbers of 1G/1G, 1G/2G, and
Table II. Genotype distribution and logistic regression analysis of the MMP-1 promoter polymorphism.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control (%)</th>
<th>Prostate cancer (%)</th>
<th>Tumor stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>33 (13.1)</td>
<td>218 (86.9)</td>
<td>10 (14.4)</td>
</tr>
<tr>
<td>1G/1G</td>
<td>1G/2G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aOR</td>
<td>(95% CI)</td>
<td>P-value</td>
<td></td>
</tr>
<tr>
<td>1.005</td>
<td>(0.622-1.739)</td>
<td>0.880</td>
<td></td>
</tr>
<tr>
<td>0.386</td>
<td>(0.196-0.763)</td>
<td>0.202</td>
<td></td>
</tr>
<tr>
<td>1.170</td>
<td>(0.983-1.404)</td>
<td>0.094</td>
<td></td>
</tr>
<tr>
<td>0.774</td>
<td>(0.598-1.000)</td>
<td>0.049</td>
<td></td>
</tr>
<tr>
<td>0.952</td>
<td>(0.752-1.209)</td>
<td>0.746</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>(0.510-2.000)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>0.885</td>
<td>(0.506-1.559)</td>
<td>0.697</td>
<td></td>
</tr>
<tr>
<td>0.369</td>
<td>(0.203-0.666)</td>
<td>0.282</td>
<td></td>
</tr>
</tbody>
</table>

Association of AI of the MMP-1 promoter region with pathological stage or tumor grade of prostate cancer. Association of AI with pathological T status or tumor grade is summarized in Table III. Of the 77 prostate cancer specimens, an AI was evaluated in 32 informative cases (41.6% of all the cases), of which 13 (40.6% of informative cases) had an AI. Two (13.3%) of the 15 cases with a Gleason score of 7 or less had an AI, whereas 11 (64.7%) of the 17 cases with a Gleason score 8 or more had an AI. Similarly, 7 (29.2%) of 24 cases with pT3a or less disease had an AI, whereas 10 (52.6%) of 19 cases with pT3b or more disease had an AI. Among 13 cases with an AI, retention of 1G allele was observed in 4 (30.8%) and 9 (69.2%) cases, respectively. The association of the AI status was significant in association with a higher Gleason score (P=0.003) and a higher pathological tumor stage (P=0.022). Although a retention allele showed no statistically significant association with a Gleason score (P=0.522), the frequency of the 2G allele retention significantly increased with tumor invasiveness (P=0.026) (Table III).

Association of MMP-1 expression with the MMP-1 promoter polymorphism and AI of the MMP-1 promoter region. In 77 patients who underwent radical prostatectomy, we evaluated the relationship between the MMP-1 promoter polymorphism and MMP-1 expression levels (Table IV). In immunohistochemical analysis of MMP-1, negative, low, moderate, and high expression was observed in 3 (3.9%), 14 (18.2%), 39 (50.6%), and 21 (27.3%) cases, respectively. Tumors with 1G/2G and 2G/2G genotypes had a significantly higher MMP-1 expression level compared to those with 1G/1G genotype (P=0.006 and 0.013, respectively), whereas there was no significant difference in the expression level between the 1G/2G and 2G/2G genotypes (P=0.581). AI status of...
tumors was not significantly associated with the MMP-1 expression level (P=0.156) (Table IV).

**Discussion**

The association of the 2G allele of the polymorphism with a higher MMP-1 expression has been reported in several cancer types (4,12,21,32). Immunohistochemical or gene expression analyses of surgical specimens showed that ovarian, cervical, and endometrial cancers with the 2G allele had a higher expression of MMP-1 than those with the 1G/1G genotype (4,12,21), and tongue cancers with the 2G/2G genotype showed a higher MMP-1 expression than those with the 1G allele (32). Our study demonstrated that prostate cancer with the 2G allele also had a significantly higher MMP-1 expression level than that with 1G/1G genotype, the observation being consistent with previous studies. The increased expression of MMP-1 under the existence of the 2G allele was confirmed by the luciferase assay using constructs with either 1G or 2G at the promoter region (-1602 bp) in fibroblast, melanoma, and breast cancer cell lines (10,11). In these cells, the 2G construct resulted in a

![Figure 2. Representative immunohistochemical stainings demonstrating weak (A) and strong (B) expression of MMP-1.](image)

<table>
<thead>
<tr>
<th>Allelic imbalance</th>
<th>Retention allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1G</td>
</tr>
<tr>
<td>+</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>T status</th>
<th>Allelic imbalance</th>
<th>Retention allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤pT3a</td>
<td>45</td>
<td>18</td>
</tr>
<tr>
<td>≥pT3b</td>
<td>22</td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IHCa</th>
<th>Genotype</th>
<th>Allelic imbalance</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>1G/1G</td>
<td>1G/2G</td>
<td>2G/2G</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1 (11.0)</td>
<td>1 (3.1)</td>
<td>1 (2.8)</td>
</tr>
<tr>
<td>Weak</td>
<td>4 (44.5)</td>
<td>4 (12.5)</td>
<td>6 (16.7)</td>
</tr>
<tr>
<td>Moderate</td>
<td>4 (44.5)</td>
<td>16 (50.0)</td>
<td>19 (52.8)</td>
</tr>
<tr>
<td>Strong</td>
<td>0 (0.0)</td>
<td>11 (34.4)</td>
<td>10 (27.8)</td>
</tr>
<tr>
<td></td>
<td>0.006b</td>
<td>0.013b</td>
<td>0.581c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.006b</td>
<td></td>
</tr>
</tbody>
</table>

*IHC, immunohistochemistry; *vs. 1G/1G; *vs. 1G/2G.
2- to 29-fold increase in reporter transcription compared with the 1G construct. In the present study, despite the increased expression of MMP-1 in specimens with the 2G allele, progression of the disease did not seem to be affected by the existence of the 2G allele alone. Since the 11q22 region contains a cluster of MMP genes including MMP-1, 3, 7, 8, 10, 12, 13, 20, and 27 (33), the amplification of 11q22 region may induce co-overexpression of other MMPs concurrent with MMP-1 and cooperatively facilitates tumor invasion.

In this study, the tumor progression was significantly associated with an AI, especially retention of the MMP-1 promoter region, whereas our case-control study showed that the presence of the 2G allele alone did not seem to have an effect on the tumor progression. Noll et al also demonstrated that 83% of metastatic melanoma with an AI of the region had retention of the 2G allele (34). Our study, however, did not reveal whether each AI case had a loss or gain of either allele. A previous study using comparative genomic hybridization (CGH) analyses showed that 11q22 was one of the most frequently amplified regions in localized prostate cancer (27). In the study, 5 (22.7%) of 22 cases with pT2 disease and 6 (75.0%) of 8 with pT3 disease had a gain in 11q22 region, an observation consistent with our findings. The finding that advanced tumors have a disproportionate representation of the 2G allele implies that the retention and probably amplification of the 2G allele has a selective advantage for tumor cells to acquire metastatic or invasive potential. Further studies combined with fluorescence in situ hybridization or CGH analysis are warranted to determine the gene dosage effect of the 2G allele on prostate cancer progression. In renal cell cancer, although AI of the 11q22 region was not detected, the 2G/2G genotype showed a significant association with cancer susceptibility (17). The contribution of the 2G allele dosage to carcinogenesis or tumor progression varies among different cancer types (17).

There is only one small-scale study regarding prostate cancer, which showed that the MMP-1 promoter polymorphism was not associated with susceptibility to the disease (29). Our relatively large-scale study also did not find any association with susceptibility to prostate cancer. Some potential explanations for the lack of association include the fact that MMP-1 polymorphism is not involved in carcinogenesis of prostate cancer, but contributes to tumor invasion or progression. Another reason could be that the sample size in this case-control study did not have sufficient statistical power to detect minimal differences in genotype frequency between the control and patient groups. A larger-scale study is therefore needed to validate our results.

It is unclear whether the polymorphism possesses direct effects on the development of malignant tumors or is only a genetic marker predicting susceptibility to cancers. The MMP-1 promoter polymorphism is known to be in linkage disequilibrium with the 5A/6A polymorphism in the promoter region of the MMP-3, (35,36) and it is known that spontaneous breast cancer develops in MMP-3 transgenic mice (37). Although a previous study indicated that the 2G allele, which creates ETS binding site, induced higher ERK-mediated MMP-1 expression (11), there has been no apparent evidence that higher expression of MMP-1 itself promotes carcinogenesis. Moreover, the amplified 11q22 region harbors several important genes involved in carcinogenesis and/or cancer progression such as other MMPs, BIRC2, and BIRC3. Thus, when we evaluate the effect of the MMP-1 polymorphism or AI on the cancer susceptibility or progression, consideration should be given to the relative contribution of linkage disequilibrium between polymorphisms and other genes located in the co-amplified region.

In conclusion, the presence of the 2G allele was associated with higher expression of MMP-1 in prostate cancer tissue and AI of the MMP-1 promoter region, specifically in retention of the 2G allele, was suggested to be involved in the progression of prostate cancer. However, the MMP-1 polymorphism itself did not influence susceptibility nor progression of the prostate cancer.

Acknowledgements

This study was supported by Japan Society for Promotion of Science (19390411, 19591832, 19591833, 19659406). We are grateful to Ms. Y. Mitobe and Ms. T. Shibata (Department of Urology, Akita University School of Medicine, Japan) for their excellent technical assistance.

References


