

Association of matrix metalloproteinase-9 and decorin expression with the infiltration of cervical cancer

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Abstract. Matrix metalloproteinase-9 (MMP9) has been recognized to be an important factor in cancer invasion and metastasis. In contrast, decorin has been revealed to inhibit primary tumor development. The aim of the present study was to investigate the function of MMP9 and decorin in cervical cancer. Three experiments were performed to analyze the function of MMP9 and decorin in the invasion of cervical cancer by: i) Analyzing the expression of MMP9 and decorin by immunohistochemistry in 100 cervical specimens; ii) determining the concentration of decorin by an enzyme-linked immunosorbent assay (ELISA) using the human squamous cervical cancer cell line CaSki and human endometrial stromal cell line CRL4003 and iii) evaluating the invasion ability of CaSki cells in a cervical invasion model by an invasion assay. Immunohistochemistry revealed that MMP9 was overexpressed in microinvasive carcinoma (100.0%) but was less strongly expressed in normal or pre-malignant squamous epithelium (0-41.9%). In contrast, the activity of decorin in stroma adjacent to neoplastic cells was lower in microinvasive carcinoma (9.1%) compared with in normal or pre-malignant lesions (74.2-100.0%). An ELISA revealed that MMP9 released from CaSki cells resolved the decorin released from CRL4003 cells. An invasion assay demonstrated that the invasive ability of CaSki cells was suppressed by an MMP inhibitor, and decorin was released from CRL4003 cells. These data suggested that decorin prevented the invasion of malignant cells in uterine cervical cancer; however, MMP9 promotes cell invasion by destroying decorin.

Introduction

Matrix metalloproteinases (MMPs) are a family of zinc- and carboxym-dependent proteolytic enzymes (1). These enzymes are normally involved in the breakdown of the extracellular matrix within the context of physiological tissue remodeling and angiogenesis (2). MMP9 is normally associated with bone remodeling (2) and dysregulated states, such as rheumatoid arthritis and osteosarcoma (3). It also has a strong influence on many phases of cancer progression, including angiogenesis, invasiveness and metastasis. An excessive production of MMP9 has been recognized to be an important factor in cancer invasion and metastasis (4).

Decorin is a member of the extracellular matrix small leucine-rich proteoglycan (SLRP) family of proteins that exists and functions mainly in the stroma and epithelial cells (5). It also has been found to play an important role in tumor development and progression, angiogenesis and metastasis (6). Several studies have shown that decorin inhibits primary tumor development and progression by antagonistically targeting multiple tyrosine kinase receptors, such as epidermal growth factor receptor (EGFR) (7).

The data in the pertinent literature show that members of the MMP family are able to cleave some SLRPs (8-11) including decorin (9). We hypothesize that cervical cancer cells display enhanced MMP9 expression, which results in infiltration to the extracellular matrix and uterine stroma destroying the basement membranes. The resolution of decorin by MMP9 from cancer cells promotes further infiltration, which results in further destruction of the extracellular matrix and stroma, where decorin is produced. The aim of the present study was to investigate the role of MMP9 and decorin in cervical cancer.

Materials and methods

Participants. The present study included 100 randomly selected patients who underwent cervical conization for squamous cell neoplasia at Osaka Medical College between 2010 and 2012. The Institutional Review Board approved this study (Ethics Committee of Osaka Medical College, 0277), and informed consent was obtained from all patients for the use of their tissue samples.

Expression of MMP9 and decorin by immunohistochemistry. The specimens were fixed in 10% formalin and embedded

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Abbreviations: MMP9, matrix metalloproteinase-9; ELISA, enzyme-linked immunosorbent assay; MMPs, matrix metalloproteinases; SLRP, small leucine-rich proteoglycan; EGFR, epidermal growth factor receptor

Key words: cervical cancer, matrix metalloproteinase-9, decorin, invasion, metastasis

in paraffin. Serial sections cut out from paraffin-embedded blocks were used for routine histopathology. A 4- μ m section was cut from a paraffin embedded block and immunohistochemically analyzed for the expression of MMP9 and decorin. Deparaffinized and rehydrated sections (4 μ m) were autoclaved in 0.01 mol/l citrate buffer Ph 6.0 for 15 min at 121°C for antigen retrieval. Endogenous peroxidase activity was blocked with 0.3% solution hydrogen peroxide in methanol for 30 min. Tumor sections were incubated at 4°C for 12 h with anti-MMP9 rabbit polyclonal antibodies (AB13458; 1:50 dilution; EMD Millipore, Billerica, MA, USA) and anti-decorin rabbit polyclonal antibodies (ab151988, 1:100 dilution; Abcam, Cambridge, MA, USA). The sections were washed with 1X phosphate-buffered saline (PBS) and incubated with Histofine simple stain MAX PO (multi; Nichirei, Tokyo, Japan) for 30 min at room temperature. Finally, the sections were washed with 1X PBS, signals and then visualized by incubation with H₂O₂/diaminobenzidine substrate solution for 5 min. The sections were counterstained with hematoxylin prior to dehydration and mounting.

The evaluation of the immunohistochemical data was performed by two independent observers who were blinded to the clinicopathological data. The expression of MMP9 for each sample was defined as detectable immunoreactions in cancer cells, as described previously (12). The evaluation of MMP9 expression was performed for stroma under the basement membrane or epithelial cells with no invasive neoplasia. In cases of invasive carcinoma, malignant cells or the stroma adjacent to malignant cells were evaluated. Briefly, the MMP9 expression was considered to be negative (no more than 10% of cells positive), low (more than 10% and up to 50% of cells positive) or high (more than 50% of cells positive). Based on these data, two groups were established: Negative MMP9 (no/low expression) and positive MMP9 expression (high expression).

The evaluation of the stromal decorin expression was also performed under the basement membrane with no invasive neoplasia. In cases of invasive carcinoma, the stroma adjacent to the malignant cells was evaluated. The expression of decorin was assessed using a semiquantitative system. Briefly, the decorin expression was scored as 0 (no stain), 1+ (weak immunoreactivity in more than 10% of stromal cells), 2+ (moderate immunoreactivity in more than 10% of stromal cells), or 3+ (strong immunoreactivity in more than 10% of stromal cells). Based on these data, two groups were established: Including a low decorin expression group (0 and 1+) and a high decorin expression group (2+ and 3+).

Cell lines. We used the human cervical cancer CaSki and the CRL-4003 immortalized human endometrial stromal cells, both purchased from the American Type Cultured Collection (Manassas, VA, USA). We also performed an STR polymorphism profiling analysis (Wakennyaku Co., Ibaraki, Japan) to confirm the cell line identity. These cells were cultured in growth media [DMEM/F12 10% FBS, 1% BD Insulin, Transferrin, Selenous (ITS) +Premix Universal Culture Supplement (catalog no: 354352; BD Biosciences, Franklin Lakes, NJ, USA)], in a humidified atmosphere of 5% CO₂ with 95% air at 37°C.

Measurement of the concentration of decorin with an enzyme-linked immunosorbent (ELISA). We used the cervical

cancer cell line CaSki and endometrial stromal cell line CRL4003. DMEM was used as cell culture medium. A total of 10⁶ CaSki cells and CRL4003 cells each were cultured at 37°C in a humidified, 5% CO₂ atmosphere in DMEM for 96 h. CaSki culture medium was then obtained as medium containing MMP9. CRL4003 culture medium was also obtained as medium containing decorin. The concentration of decorin in the CRL4003 culture medium with or without CaSki culture medium or MMP inhibitor (MMP-9 inhibitor, ab142180; Abcam) was investigated by an ELISA. The experiment was carried out five times, and the concentration of decorin was expressed as the mean \pm standard deviation (SD).

Influence of MMP9 and decorin on the invasion ability using an invasion assay. The invasive potential was assessed using an invasion assay. We examined the effects of MMP9 and decorin on the invasive potential of CaSki cells with or without CRL4003 cells and MMP inhibitor using this assay. A total of 5 \times 10⁵ CaSki cells were cultured at 37°C in a humidified, 5% CO₂ atmosphere in serum-free DMEM for 24 h. These cells with or without MMP inhibitor were then seeded into upper wells coated with a thin layer of Matrigel. The lower chamber contained 600 μ l of DMEM with or without 5 \times 10⁵ CRL4003 cells; we established four groups: CaSki, CaSki with CRL4003, CaSki with MMP inhibitor, CaSki with CRL4003 and MMP inhibitor. Following 24-h incubation at 37°C, noninvading cells on the surface of the Matrigel-coated membrane were removed by scraping with a cotton swab. Cells that migrated through the Matrigel were stained with hematoxylin. Following several washes with PBS, the stained cells were manually counted for two independent experiments. Each point represents the mean \pm SD of four replicates.

Statistical analyses. All statistical analyses were performed using the JMP software package (v11.1.1; SAS Institute Inc, Tokyo, Japan). Continuous variables are expressed as the mean \pm SD. The Mann-Whitney U-test was used to compare continuous variables between the two groups. When making multiple comparisons in datasets with continuous variables containing more than two groups, the Tukey honestly significant difference (HSD) was used. Fisher's exact test with Bonferroni's correction was used to compare frequencies between the three groups. Pearson's correlation coefficient was calculated to determine the correlation between two variables. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of MMP9 and decorin in surgical specimens. The immunochemical staining of MMP9 and decorin is shown in Fig. 1. In patients with cervical intraepithelial neoplasia (CIN) I (Fig. 1A), the expression of MMP9 was not seen under the basement membrane (Fig. 1B); however, decorin was expressed abundantly in the stroma (Fig. 1C). In contrast, in patients with microinvasive carcinoma (Fig. 1D), the expression of MMP9 was abundant (Fig. 1E); however, decorin was not seen in the stroma adjacent to malignant cells (Fig. 1F). The results of the immunohistochemical analyses are shown in Table I. The patients were categorized into three groups: those with normal

Table I. Expression of MMP9 and decorin in uterine cervical neoplasm.

Variables	Low-grade neoplasia (n=33)	Premalignant (n=31)	Malignant (n=36)	P-value
Age ^a , years	37.2±10.0	40.9±11.2	38.6±10.4	N.S.
Positive for MMP9 (%)	0/33 (0)	13/31 (41.9)	36/36 (100)	P<0.01
High decorin expression (%)	33/33 (100)	23/31 (74.2)	3/36 (9.1)	P<0.01

MMP9, Matrix metalloproteinase-9; N.S., not significant. ^aBased on an analysis of variance (mean ± standard deviation).

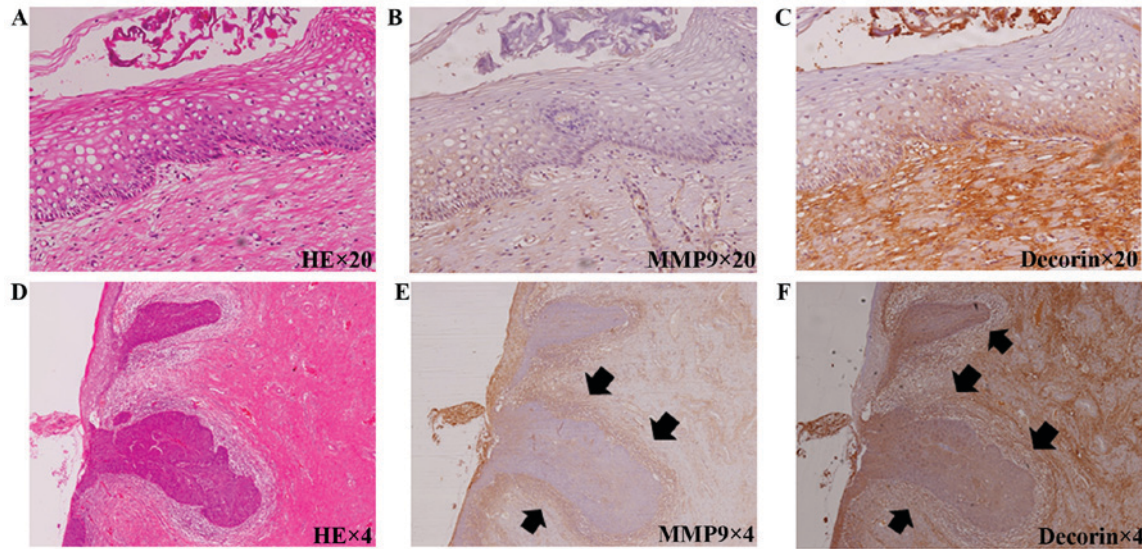


Figure 1. Immunohistochemical staining of MMP9 and decorin in specimens from normal cervical epithelium (A-C) and microinvasive carcinoma (D-F). (A) Hematoxylin and eosin staining of normal epithelium. (B) MMP9 was not stained in the normal epithelium or stroma under the basement membrane. (C) Decorin was overexpressed in the stroma under the basement membrane. (D) Hematoxylin and eosin staining of microinvasive squamous cell carcinoma. (E) MMP9 was overexpressed in the stroma adjacent to cancer cells (arrows). (F) Decorin expression was weaker in the stroma adjacent to cancer cells (arrows). MMP9, matrix metalloproteinase-9.

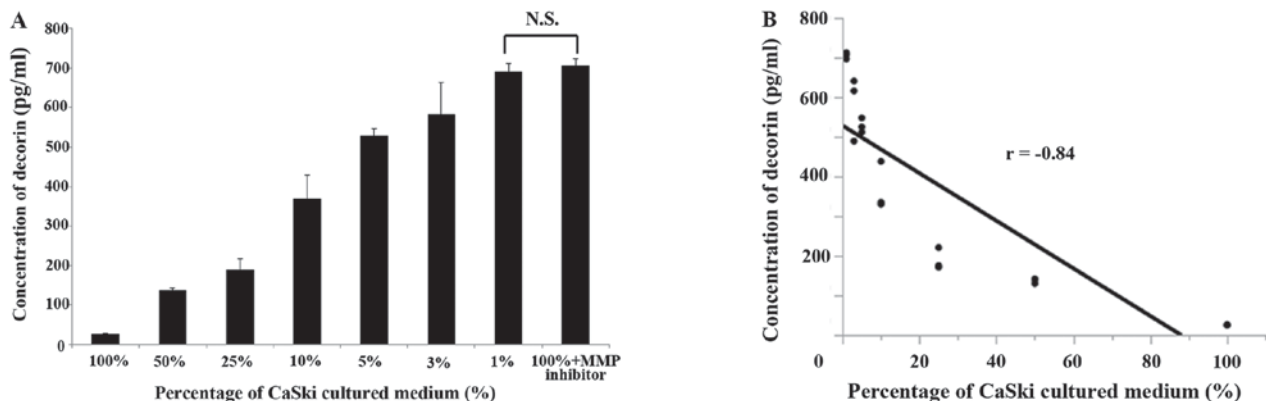


Figure 2. (A) Concentration of decorin in CRL4003 cell culture medium by enzyme-linked immunosorbent assay (ELISA). The concentration of decorin decreased when CaSki culture medium was added but not when MMP inhibitor was added. (B) Pearson's correlation coefficient showed a strong correlation between the concentration of decorin and the amount of CaSki cultured medium ($r = -0.84$, $P < 0.05$). This finding indicates that MMP9 released from CaSki cells destroys decorin released from CRL4003 cells. MMP, matrix metalloproteinase.

to CIN2 were categorized as having low-grade neoplasia, those with CIN3 were categorized as being premalignant, and those with microinvasive carcinoma (FIGO stage IA1 squamous cervical cancer) were categorized as being malignant. Five patients had no neoplasia, 14 had CIN 1 and 14 had CIN 2; a

total of 33 patients had normal to CIN2 (low-grade neoplasia group), 31 had CIN3 (pre-malignant group), and 36 had microinvasive carcinoma (malignant group). The mean ± SD age in the low-grade neoplasia, pre-malignant and malignant groups was 37.2±10.0, 40.9±11.2 and 38.6±10.4 years old, respectively. No

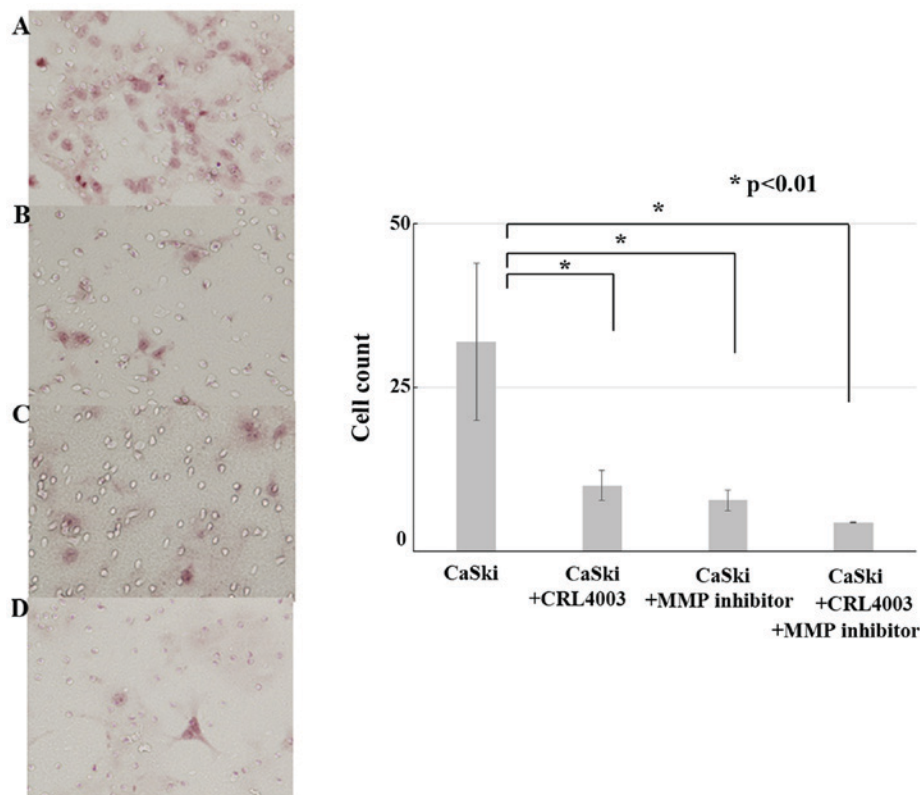


Figure 3. Migration and invasion assays in a cervical cancer model. (A) CaSki cells without CRL4003 or matrix metalloproteinase (MMP) inhibitor. (B) CaSki cells with CRL4003. (C) CaSki cells with MMP inhibitor. (D) CaSki cells with CRL4003 and MMP inhibitor. CRL4003 and MMP inhibitor suppressed the invasive ability of CaSki cells.

significant difference was found between the groups. The rate of MMP9 positivity was 0% in the low-grade neoplasia group, 41.9% in the premalignant group and 100% in the malignant group. There were significant differences between each group ($P<0.01$). In contrast, the rate of high decorin expression was 100% in the low-grade neoplasia group, 74.2% in the premalignant group and 9.1% in the malignant group. There were significant differences between each group ($P<0.01$).

Relationship between MMP9 and decorin. The concentration of decorin in CRL4003 culture medium with varying amounts of CaSki culture medium by an ELISA (Fig. 2A). When the ratio of CaSki culture medium to CRL4003 culture medium was 100, 50, 25, 10, 5, 3 and 1%, the concentration of decorin was 26.4 ± 1.2 , 138 ± 6.4 , 189 ± 28 , 368 ± 61 , 529 ± 18 , 582 ± 81 , 690 ± 21 and 705 ± 19 pg/ml, respectively. However, the concentration of decorin in CRL4003 culture medium with the same volume of CaSki culture medium and MMP inhibitor was 705 ± 19 pg/ml. This value was not significantly different in comparison to the concentration of decorin in the CRL4003 cultured medium with 1% CaSki cultured medium ($P=0.82$). Pearson's correlation coefficient showed a strong correlation between the concentration of decorin and the amount of CaSki cultured medium ($r=-0.84$, $P<0.05$) (Fig. 2B). These findings indicate that the decorin released from CRL4003 cells was resolved by MMP9 released from CaSki cells.

Migration and invasion in the cervical invasion model. The results of migration and invasion assays is show in Fig. 3. The

cell counts of CaSki, CaSki with CRL4003, CaSki with MMP inhibitor and CaSki with CRL4003 and MMP inhibitor were 32 ± 12 , 10 ± 2.3 , 7.8 ± 1.6 and 4.4 ± 1.1 , respectively. CRL4003 culture medium and MMP inhibitor decreased the CaSki cell migration and invasion ($P<0.01$). CRL4003 culture medium with MMP inhibitor suppressed the CaSki cell migration and invasion the most effectively ($P<0.01$).

Discussion

In the present study, immunohistochemical analyses in patients with cervical cancer showed an increased MMP9 expression and a decreased decorin expression in the stroma adjacent to malignant cells. *In vitro*, the endometrial stromal cell line CRL4003 produced decorin, which suppressed cell invasion. Furthermore, the cervical cancer cell line CaSki produced MMP9, which promoted cell invasion and decomposed decorin.

MMPs are a family of metalloendopeptidases that break down the protein components of the extracellular matrix; they play an important role in tissue remodeling and degradation (1). Angiogenesis is one of the most important process of tumor growth and requires the degeneration and remodeling of the extracellular matrix, cell migration and proliferation, and tube formation (13); the role of MMP in tumor growth and progression is associated with angiogenesis. MMP9, which is over-expressed in cancer cells, is a potent gelatinase that has been shown to be associated with the process of tumor cell invasion and metastasis (14,15). The

overexpression of MMP9 has been observed in pre-cancer and cancer lesion of the uterine cervix. The published literature states that MMP9 is overexpressed in more than 90% of squamous cell carcinomas and 83-100% of high-grade squamous intraepithelial lesions (HSILs) but is less strongly expressed in low-grade squamous intraepithelial lesions (LSILs) and normal squamous epithelium (13%) (16-28). Most reports have shown that the activity of MMP9 tends to increase from normal cervix to HSIL and SCC, being more strongly expressed in more advanced stages (27,28). MMP9 is expressed in stromal cells and inflammatory cells around tumors (29). These findings suggest the importance of MMP9 in the pathogenesis of uterine cervical cancer; MMP9 affects various inflammatory cells infiltrating the tumor area, the extracellular matrix and stromal cells and proteins released from those cells.

Decorin, which is a member of the extracellular matrix SLPR, has been thought to act exclusively as a structural component (5). It is expressed in a wide range of connective tissues, including skin, bone, cartilaginous tissue and stroma and is secreted into the extracellular matrix (30,31). Fibrillar collagen, which is the main structural constituent of tendons, ligaments, skin and other connective tissues, is bound and cross-linked by decorin (32-36). Decorin knockout mice show skin fragility and abnormal collagen fibril morphology (37), and a lack of decorin in these mice causes a significant delay in the healing of the excisional and incisional dermal wound compared to decorin wild-type mice (38). Furthermore, crossing decorin-null mice with P53-null mice caused early lethality of double-mutant animals because of massive organ infiltration by T-cell lymphoma (39). These findings indicate that decorin thus plays a role in tissue development.

As mentioned below, decorin has biological functions as a degenerator of endocytosis and inhibitor of tumor cell growth, migration, angiogenesis, endothelial cell proliferation and motility. Decorin affects signal transduction via erbB family receptors characterized by the stimulation of mitogen-activated protein kinases, mobilization of intracellular calcium and up-regulation of P21WAF1/CIP, a potent inhibitor of cyclin-dependent kinases, ultimately leading to growth suppression (31,40-43). These signal transductions are associated with modulating cell proliferation, cell cycle progression and apoptosis. Decorin is known to be a promoter of matrix organization (44), which constitutes a physical barrier against the migration/motility of cancerous cells; decorin-collagen interactions prevent metastasis (45). Decorin is also known to be a negative regulator of signal transduction by interfering with growth factor binding including TGF- β (46,47); the synthesis of matrix molecules and the growth factor-dependent modulation of cell proliferation and migration is inhibited by decorin. For these reasons, decorin has recently emerged as a potential natural anticancer agent produced by normal host cells against cancer cells. There have been a number of reports about the anti-tumor effects of decorin on various malignant tumors, including uterine cervical carcinoma cells (48), ovarian cancer cells (49), breast cancer cells (50), colon carcinoma cells (51) and pancreatic cancer cells (52).

The published literature shows that MMPs digest decorin into fragments by cleaving within the leucine-rich region

at multiple site (9). Those previous authors examined the susceptibility of decorin to five different MMPs. MMP2, MMP3 and MMP7 exhibited digestive activity for decorin, with MMP1 and MMP9 showing lower activity. In the present study, concentration of decorin in CRL4003 cultured medium was increased by MMP9 inhibitor. These data suggest that MMP9 not only directly digests decorin but also suppresses the production of decorin by impairing the tissue.

In conclusion, MMP9 was found to be overexpressed in uterine cervical cancer, but it was less strongly expressed in normal or pre-malignant squamous epithelium. In contrast, the activity of decorin in stroma adjacent to neoplastic cells was lower in microinvasive carcinoma than in normal or pre-malignant lesions. Decorin prevents the invasion of malignant cells, but MMP9 promotes cell invasion by destroying decorin, the extracellular matrix and stroma.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contribution

TT and MO designed the study and performed the experimental data collection and analysis. TT and YT provided the diagnosis based on immunohistochemical analyses, and TT and MO wrote the paper.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Osaka Medical College, and written informed consent to participate in the study was obtained from all patients involved.

Patient consent for publication

No identifying patient information is included in the published manuscript.

Competing interests

The authors declare that they have no competing interests.

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