Matrix metalloproteinase 3 is a stromal marker for chicken ovarian cancer

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Abstract. Matrix metalloproteinases (MMPs) are involved in the degradation of the extracellular matrix and basement membranes. Due to this, MMPs have been thought to promote invasion and metastasis of cancer cells and angiogenesis in tumors. Even though the chicken is a useful animal model for studying human ovarian cancer, no reports exist of the MMP expression pattern in chicken ovarian cancer. Therefore, we investigated the expression pattern of MMPs in chicken ovarian cancer. Results of RT-PCR and quantitative RT-PCR analyses showed *MMP3* to be over-expressed in cancerous hen ovaries. *In situ* hybridization analysis of cancerous chicken ovaries showed that *MMP3* mRNA was predominantly localized in the stroma, which is similar to *MMP3* expression in human cancers. The results suggest that the expression pattern of *MMP3* mRNA in chicken ovarian cancer is similar to that in various types of human cancer. Moreover, *MMP3* potentially plays a significant role in developing ovarian cancer in chickens. The cell type-specific expression of *MMP3* makes this gene a unique marker for ovarian cancer in chickens.

Introduction

Of all the gynecologic cancers, ovarian cancer has the highest mortality rate. Even though the survival rate following early detection of the disease is relatively high, a diagnosis of ovarian cancer often occurs at a late stage in the disease. The mechanisms responsible for ovarian cancer are not completely known, and research is minimal due to a lack of suitable animal models (1).

The laying hen is a suitable animal model for human ovarian cancer due to the similarities between human and chicken ovarian cancers. Most human ovarian cancers arise spontaneously in cells derived from the ovarian surface epithelium (2,3). This is consistent with chicken ovarian cancer, which originates from ovarian epithelial cells (4). Support for the hypothesis regarding incessant ovulation that explains ovarian cancer in humans (2,3) is the fact that hens ovulate almost daily, resulting in genomic damage to the ovarian surface epithelium and increasing the likelihood of mutations that lead to the development of spontaneous ovarian adenocarcinoma (5). Moreover, anti-tumor antibodies common to human and chicken cancer carcinomas include cancer antigen 125, cytokeratin, pan cytokeratin, proliferating cell nuclear antigen (PCNA), carcinoembryonic antigen, cytokeratin AE1/AE3, epidermal growth factor receptor, ERBB2, Lewis Y, selenium-binding protein 1 and tumor-associated glycoprotein 72 (6-8). Despite these similarities, further characterization of chicken ovarian cancer is crucial for a comparative study of ovarian cancers in humans and chickens.

Proteases are involved in controlling multiple biological processes and multiple diseases, including cancer (9). It was recently reported that cysteine proteases, known as cathepsins, were involved in chicken ovarian cancer (10). One of the protease groups, matrix metalloproteinases (MMPs), is involved in the degradation of the extracellular matrix and basement membranes. Due to their function, MMPs have long been considered to play an essential role in cancer progression by promoting tumor cell invasion, angiogenesis and metastasis of cancer cells (11-13). In human ovarian cancer, certain MMPs are abundantly expressed in epithelial ovarian cancer cells (14,15). However, the expression of MMPs in cancerous chicken ovaries has yet to be investigated. We therefore examined the expression patterns of MMPs in cancerous and normal chicken ovaries, with a particular emphasis on MMP3.

Materials and methods

Animals. The care and experimental use of White Leghorn (WL) hens (*Gallus gallus domesticus*) was approved by the Institute of Laboratory Animal Resources, the Seoul National University (SNU-070823-5), Korea. The hens were maintained

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Table I. Primer sequences used for RT-PCR and cloning.

Gene	Sequence (5'-3'): forward and reverse	Gene bank accession no.	Product size (bp)
MMP1	CTAATGGGCTGCTGGCTCA GACCTCTCAGGATGTTTGCG	XM_417176	406
MMP2	GTGGCAATGGTGATGGACAG TCCTGAGAAAGGCGGAAGTT	NM_204420	460
MMP3	CACTGGGATAGGAGGGGATG TCTGTGGGTGCCATTTCTGT	XM_417175.2	348
MMP7	CCTCCTACTTTGTGCTGCCA ATGATGTCTGCCTGTCCCG	NM_001006278.1	453
MMP9	CGGCTTAGAGGTGAAGACCC GGAAGGTGAAGGGGAAGACA	NM_204667.1	416
MMP11	CCAGCCAGACCTTGAAACAA CAATCTCCTGTGGGACACCA	XM_001232776	491
MMP13	CGGGTGCTGTGGAAGAAATA TTGGTGTAGTTGGGGCAGAC	XM_001235204	407
MMP15	GACGCTGGAAAACACGGAC ACCACTTGCCCTTGAACACA	XM_413995	422
MMP16	CAACTGACCCCAGAATGTCG AAAAATCCTCCCTCCCCATC	NM_205197	454
MMP17	TTTGGGTATCTGCCTCCTCC CCTGCTGTGTGATGGTCTCC	XM_415092	482
MMP23B	CGTAGTGGCTTTGCTGGCTA CAAGTTCCCCTGTTGTTCCA	XM_417569	425
MMP24	CGACTCTTCCTGTTCGCAGA TCGCTCTCTTGTCCTCGTTG	XM_417326	498
MMP27	CAGGAAAACCAGACACCGAG GAGCAGCAACCAGGAACAAA	NM_205000	423
MMP28	CAGCACCTACTACTGCCACTCC AATAGCGGTCATCCCGAAAG	XM_415771	500
GAPDH	CACAGCCACACAGAAGACGG CCATCAAGTCCACAACACGG	NM_204305	443

in a standard management program at the University Animal Farm at Seoul National University. The procedures used for animal management, reproduction and embryo manipulation followed the standard operating protocols of our laboratory.

Tissue samples. Cancerous (n=5) ovaries were obtained from 2- and 3-year-old WL hens with spontaneously developed ovarian cancer. Normal (n=3) ovaries were obtained from 2- and 3-year-old healthy WL hens without any histological changes in the ovaries. Sections of these ovaries were frozen or embedded in paraffin for further analysis. For diagnosis, paraffin-embedded tissues were sectioned at 5 μm and stained with hematoxylin and eosin.

RT-PCR analysis. Total RNA was extracted from frozen tissues by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using AccuPower® RT PreMix (Bioneer, Daejeon, Korea). Specific primer sets were used for RT-PCR (Table I). PCR amplification was performed as follows: 95˚C for 3 min; followed by 30 cycles of 95˚C for 20 sec, 60˚C for 40 sec and 72˚C for 1 min; and a final extension of 72˚C for 5 min. PCR products were analyzed on a 1% agarose gel stained with ethidium bromide.

Quantitative RT-PCR analysis. Quantitative RT-PCR was performed using SYBR-Green (Sigma, St. Louis, MO, USA) and a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). Relative quantification of gene expression was calculated using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{GAPDH})_{\text{cancerous tissue}} - (Ct_{\text{target gene}} - Ct_{GAPDH})_{\text{normal tissue.}}$ The information for the primer sets is shown in Table II.

In situ hybridization. *In situ* hybridization was conducted as previously described (16). For hybridization probes, PCR

Table II. Primer sequences used for quantitative RT-PCR.

Gene	Sequence (5'-3'): forward and reverse	Gene bank accession no.	Product size (bp)
MMP3	ACCTGGGCTTTCCCAGAAGT CTGAAGGGCAGCATCAACGA	XM_417175.2	194
GAPDH	ACACAGAAGACGGTGGATGG GGCAGGTCAGGTCAACAACA	NM_204305	193

products were generated from ovarian cancer cDNA with the primers used in RT-PCR analysis. Products were then gel-extracted and cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). Following the verification of sequences, a DIG-labeled RNA probe was prepared using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN, USA). Frozen sections (10 μm) were mounted on slides pretreated with 3-aminopropyltriethoxysilane (Sigma), dried on a 50˚C slide warmer, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), treated with 1% Triton X-100 in PBS for 20 min, washed three times in PBS and incubated with a prehybridization mixture [50% formamide and 5X standard saline citrate (SSC)] for 15 min at room temperature. Following prehybridization, sections were incubated in a hybridization mixture (50% formamide, 5X SSC, 10% dextran sulfate sodium salt, 0.02% bovine serum albumin, 250 μg/ml yeast tRNA and denatured DIG-labeled cRNA probes) for 18 h at 55˚C in a humidified chamber. Sections were then washed for stringency in a series of solutions containing formamide and SSC. Following blocking with 1% blocking reagent (Roche), sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche). Following incubation, a visualization solution (0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.4 mM nitroblue tetrazolium, and 2 mM levamisole; Sigma) was used. Sections were counterstained with 1% (w/v) methyl green (Sigma). Images were captured with a Zeiss Axiophot light microscope equipped with an AxioCam HRc camera (Carl Zeiss, Inc., NY, USA).

Statistical analysis. Statistical analysis was performed using the Student's t-test using the SAS program (SAS Institute, Cary, NC, USA). $P<0.05$ was considered to be statistically significant.

Results

Pathological characteristics of chicken ovarian cancer. Normal ovaries had typically developing follicles surrounded by stroma (Fig. 1A). The morphology of cancerous ovaries was entirely different, showing gland-like growth of cancer cells invading stromal tissues (Fig. 1B). The difference between normal and cancerous ovaries was similar to that reported previously (17,18).

Increased expression of MMP3 in cancerous ovaries. We initially performed RT-PCR analysis to determine the expression of MMPs that have been identified in chickens. *MMP3* mRNA was markedly expressed in cancerous ovaries but almost undetectable in normal ovaries (Fig. 2A), whereas the expression of the remaining MMPs were weak or undetectable in cancerous and normal ovaries (data not shown). Therefore, further study was focused on *MMP3*.

To measure relative mRNA levels between normal and cancerous ovaries, we performed quantitative RT-PCR. As expected, *MMP3* mRNA expression in cancerous ovaries was approximately 16-fold higher than that in normal ovaries ($P<0.05$, Fig. 2B).

Localization of MMP3 mRNA in normal and cancerous ovaries. The expression pattern for *MMP3* was further analyzed by the localization of *MMP3* mRNA by *in situ* hybridization. The results indicated an absence of the cell-specific expression of *MMP3* mRNA in normal ovaries (Fig. 3A and D), whereas abundant *MMP3* mRNA was observed in the stroma between the gland-like areas in the cancerous ovaries (Fig. 3B, C, E and F).

Discussion

MMP3 (also known as stromelysin-1) plays significant roles in regulating extracellular matrix remodeling and in activating other MMPs (19). Over-expression of *MMP3* has also been demonstrated in various types of cancer (20). In human cancer, MMPs are also commonly expressed in the stromal cells rather than in the tumor cells: expression of MMPs in stromal cells has been reported in breast, colorectal, lung, prostate, and pancreatic cancers (20). Our results also show that *MMP3* mRNA is localized in stromal cells in chicken ovarian cancer suggesting that the roles of MMP3 in cancer are conserved between chickens and humans, and the expression of *MMP3* may be regulated by similar mechanisms in the two species. In humans, cancer cells induce *MMP1, 2* and *3* expression by secreting signaling molecules known as extracellular matrix metalloproteinase inducers (21,22). However, further studies are necessary to determine the detailed mechanism(s) of tumorigenesis in chicken ovaries.

Although the role of MMP3 in cancer has not been fully elucidated, certain studies have revealed its tumorigenic functions. Sternlicht *et al* showed that MMP3 over-expression in transgenic mice leads to enhanced mammary carcinogenesis (23), and Witty *et al* showed that MMP3 targets relevant substrates to promote apoptosis in neighboring epithelial cells, which is relevant for cancer (24). In addition to their ability to degrade major protein components of the extracellular matrix or the basement membrane, MMPs play a role in

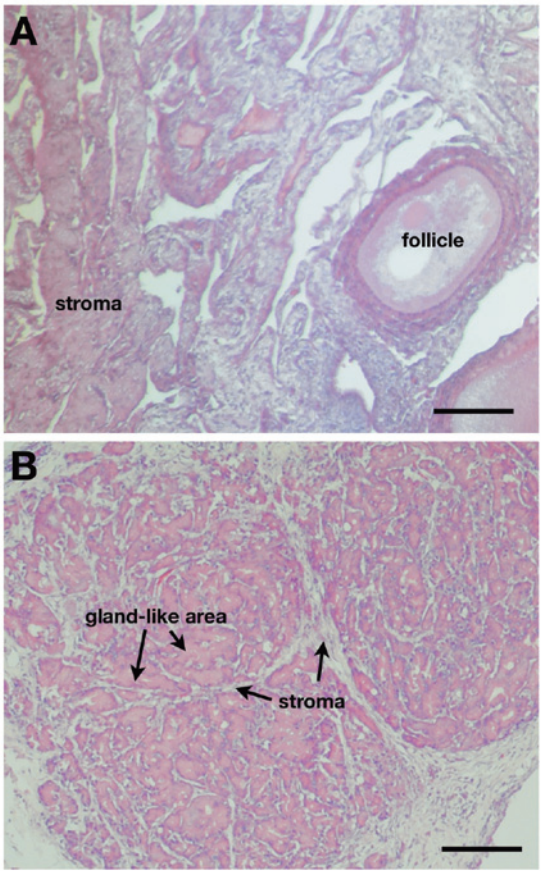

Figure 1. Hematoxylin and eosin staining of (A) normal and (B) cancerous hen ovary. Scale bar, 100 μm.

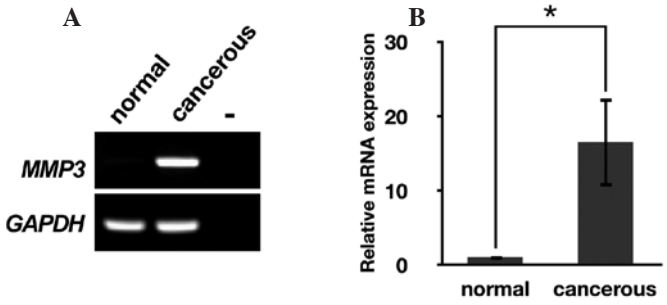

Figure 2. Increased expression of *MMP3* mRNA in normal and cancerous chicken ovaries. (A) RT-PCR analysis of ovaries. (B) Relative mRNA expression of *MMP3* between normal and cancerous ovaries from chickens indicated that *MMP3* mRNA was greater in cancerous ovaries (mean ± SEM, P<0.05).

the early stages of tumorigenesis by stimulating cell proliferation and modulation of angiogenesis (25). A focus on the early stages of ovarian cancer in chickens may reveal new roles for MMPs in the early developmental stages of cancer and thus improve the ability of medical professionals to make an early diagnosis.

In conclusion, this study has demonstrated the overexpression of *MMP3* in chicken ovarian cancer. The expression pattern of *MMP3* is relatively similar to that of MMPs in human cancer. The cell type-specific expression of the *MMP3* gene renders this gene a unique marker for epithelial chicken ovarian cancer and suggests the crucial role MMP3 plays in its development.

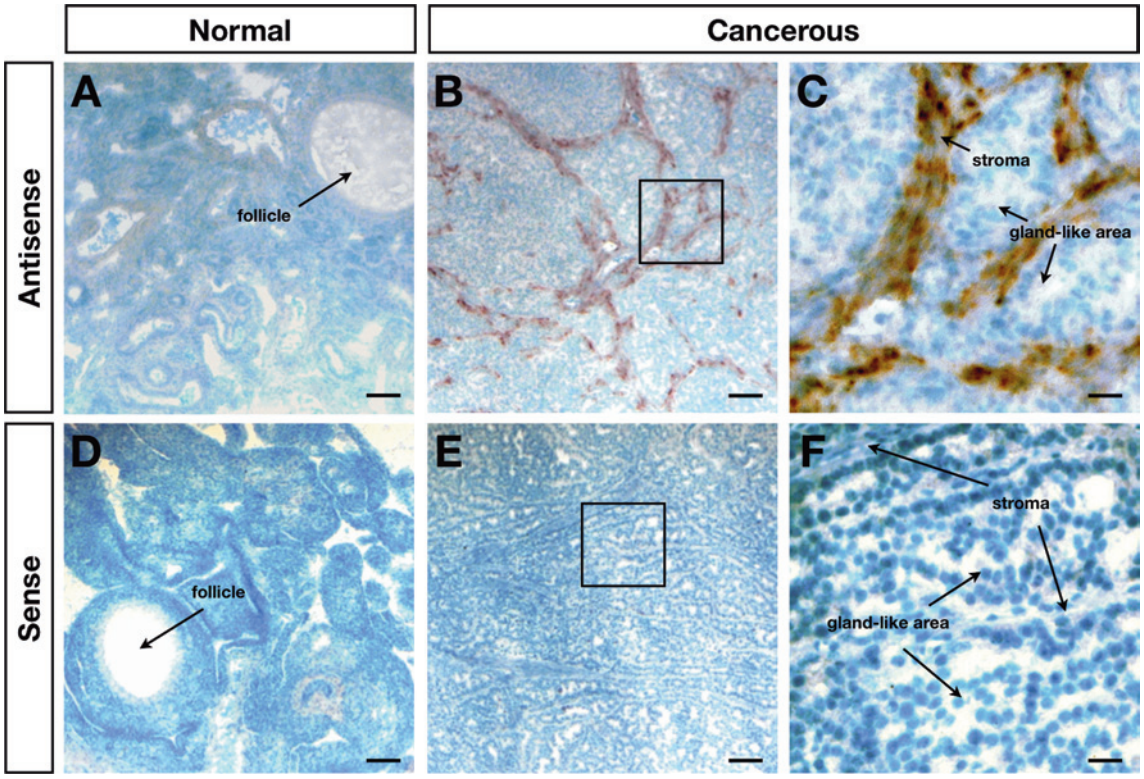

Figure 3. *In situ* hybridization analysis of *MMP3* mRNA in normal and cancerous chicken ovaries. (A) No cell-specific localization of *MMP3* mRNA was observed in normal ovaries. (B and C) *MMP3* mRNA was markedly expressed in the stroma of cancerous ovaries. (D-F) Negative controls with the sense probe. Scale bar, 100 μm (A, B, D and E) and 25 μm (C and F).

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