

## Serum Amyloid A Promotes Invasion of Feline Mammary Carcinoma Cells

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**ABSTRACT.** The serum amyloid A (SAA) concentration is higher in mammary tumors with metastases in both humans and animals. In the present study, the direct effects of recombinant feline SAA (rfSAA) protein on invasiveness of feline mammary carcinoma cells were evaluated. As an indicator of invasiveness, matrix metalloproteinase-9 (MMP-9) expression was investigated in 4 feline mammary carcinoma cell lines of different origins. In 3 of 4 cell lines, MMP-9 expression was significantly increased by rfSAA stimulation. The invasive capacities of feline mammary carcinoma cells were also stimulated by rfSAA. The findings of this study have identified a novel role for SAA in mammary tumorigenesis and suggest that therapeutic strategies targeting SAA may provide new alternatives in treating tumor invasion and metastasis.

**KEY WORDS:** feline, invasiveness, mammary carcinoma, matrix metalloproteinase, serum amyloid A

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Serum amyloid A (SAA) is one of the major acute phase proteins (APPs) in mammals, including humans and cats [6, 27]. Acute phase reaction is a systemic response to inflammatory stimulations, such as infection or trauma, and APPs including SAA are synthesized mainly in the liver as part of the reaction [7]. Gene expression and protein synthesis of SAA are stimulated by several inflammatory cytokines, such as IL-1 $\beta$ , IL-6 and TNF- $\alpha$  [5]. The synthesized SAA protein is released into circulation, and the secretion of SAA into blood results in a marked increase in SAA concentration in serum. SAA concentration occasionally increases up to 1,000-fold above the basal level [10].

Because of the dramatic increase in serum SAA concentration during inflammation, serum SAA concentration is used as an inflammatory marker in humans [13] and cats [24]. Elevated SAA concentration has been described in various inflammatory and infectious diseases [3, 25]. Increased SAA concentration has also been demonstrated in humans with neoplastic diseases [1, 30]. Furthermore, the relationship between serum SAA concentration and clinical stage of the tumor has also been shown in earlier studies [15]. Patients with tumor metastasis have a higher serum SAA concentration than do patients without metastasis. Thus, SAA is also considered as a useful biomarker in monitoring the progression of neoplastic diseases.

Besides the potential of SAA as a cancer biomarker, the role of SAA in the progression of neoplastic diseases is of current interest. An acute immune response may cause an increased risk of peripheral metastases [4]. On the other hand,

cytokine-like functions of SAA protein were demonstrated in both humans [19] and cats [23]. SAA protein stimulates the production of various cytokines by macrophages and can play an important role in acute immune response. Therefore, elevated SAA concentration can stimulate tumor metastasis indirectly. The SAA protein also stimulates matrix metalloproteinase-9 (MMP-9) production by macrophages [11]. Matrix metalloproteinases (MMPs) are a family of extracellular matrix degrading proteases that are zinc-dependent and associated with invasion and metastasis during tumor progression, because of their ability to degrade extracellular matrix and basement membrane [20]. Type IV collagen is one of the integral components of the basement membrane, and its collagenase, MMP-9, is believed to play a key role in tumor invasion and metastasis [17]. Although these indirect effects of SAA protein in tumor progression have been relatively well-evaluated, there is limited information about the direct activities of SAA protein on the invasiveness of tumor cells [8].

Mammary tumors are common neoplasms in cats, and feline mammary carcinoma shows an age-dependent incidence, histopathological features and metastasis pattern similar to human breast cancer [12]. Moreover, human breast cancer and feline mammary carcinoma share the similar biological features at the molecular level [2]. Thus, feline mammary carcinoma could be a suitable animal model for human breast cancer. In human patients with breast cancer, elevated serum SAA concentration has been described, and the SAA concentration has also been correlated with the stage of the cancer [33]. Therefore, it is suggested that SAA affects the progression and metastasis of breast cancer in a direct or an indirect way.

In the present study, the direct effects of recombinant feline SAA (rfSAA) protein on the invasiveness of feline mammary carcinoma cells were evaluated. As the indicator of invasiveness, MMP-9 mRNA expression and protein synthesis were investigated in 4 feline mammary carcinoma cell lines of different origins. The effect of SAA on the actual

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invasive capacity of these cells was also estimated.

To determine the partial sequence of feline MMP-9, feline peripheral macrophages were prepared according to a previously described method [23]. These procedures were conducted in accordance with the guidelines of the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo. Total cellular RNA was extracted by using a commercial kit (Illustra RNeasy Mini RNA Isolation Kit; GE Healthcare, Buckinghamshire, U.K.). Obtained RNA was treated with DNase. Reverse transcription was performed using a commercially available reverse transcriptase (ReverTra Ace qPCR RT Master Mix; TOYOBO, Tokyo, Japan), according to the manufacturer's instructions. Template cDNA was amplified by PCR using Taq polymerase (AmpliTaq Gold 360 Master Mix; Applied Biosystems, Foster City, CA, U.S.A.) with the following primer pair: 5'-AAC CAC CAC CACACC TGA AT-3' (forward) and 5'-CAA AGG TCA CGT AGC CCA CT-3' (reverse). These primers were designed based on the predicted sequences of feline MMP-9 (GenBank accession number, XM\_003983412). The PCR cycles were as follows: pre-denaturing (95°C for 10 min); 30 cycles of denaturation (95°C for 30 sec), annealing (57°C for 30 sec) and extension (72°C for 45 sec); and final extension (72°C for 7 min). The PCR products were electrophoresed through a 2% agarose gel and purified from the gel by using a commercially available kit (Wizard SV Gel and PCR Clean-Up System; Promega Corp., Madison, WI, U.S.A.). The purified PCR products were directly sequenced by the dideoxy chain termination method using an ABI prism BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Gene specific primers described above were used for sequencing. The analysis was performed in triplicate to avoid errors in sequence analysis. The sequences matched in all 3 analyses were adopted.

The synthesis of rfSAA was carried out using a previously described method [23]. The synthesized rfSAA was desalted by using a commercial desalting column (PD-10 Desalting Columns; GE Healthcare) according to the manufacturer's instructions. The purity of rfSAA was analyzed by SDS-PAGE (12.5% SDS-polyacrylamide gel) and was determined to be greater than 95% by using a software package (Image Lab Software; Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Feline mammary tumor cell lines (FKN-p, FNN-m, FON-p and FON-m) were kindly provided by the Laboratory of Veterinary Surgery, the University of Tokyo. Cell lines, FKN-p and FON-p, were established from primary lesions, and FNN-m and FON-m were established from metastatic lesions in feline patients bearing spontaneous mammary tumors. FON-p and FON-m were established from the same patient. Details of each of the cell lines were described previously [29]. All cell lines were grown in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaille, France), 100 µg/ml streptomycin and 100 IU/ml penicillin (Nacalai Tesque, Kyoto, Japan). Cells were maintained at 37°C in 5% CO<sub>2</sub>.

Subconfluent (70–80% confluent) cells were detached and resuspended at  $1 \times 10^6$  cells/ml in serum-free media (RPMI

1640 containing 1% bovine serum albumin; BSA) and added in 100 µl aliquots to a 96-well plate. The cells were incubated for 24 hr at 37°C in 5% CO<sub>2</sub>. After incubation, culture media were replaced, and cells were stimulated with rfSAA (1, 5 and 25 µg/ml) for 24 hr. The rfSAA concentration added to cell culture was determined on the basis of previous studies [8, 23]. Cells and cell-free supernatants of culture media were collected separately by centrifugation and stored at –80°C until analysis. Each experiment was performed in duplicate and repeated 3 times in separate experiments.

Total cellular RNA was extracted from the cells, and reverse transcription was performed as described above. For quantitative RT-PCR, the cDNA was amplified with SYBR green (THUNDERBIRD SYBR qPCR Mix; TOYOBO) by using gene-specific primers and a thermal cycler (Thermal Cycler Dice Real Time System; Takara Bio, Otsu, Japan) with the following program: a 10 min preincubation at 95°C, 50 cycles of PCR (5 sec at 95°C and 30 sec at 60°C) and dissociation (95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec). The reactions were performed with primers specific to feline MMP-9 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank accession number, NM\_001009307). Sequences of primers are shown in Table 1. Data were normalized relative to GAPDH as an endogenous control. Quantification of mRNA transcription was performed using the comparative cycle threshold (Ct) method. Each sample was assessed in duplicate.

MMP-9 levels in culture media were examined by gelatin zymography. Samples were diluted in sample buffer (125 mM Tris-HCl pH 6.8, 25% glycerol, 5% SDS and 0.2% bromophenol blue), and a dilution containing an equal volume of culture media was subjected to electrophoresis on a 10% SDS-PAGE gel co-polymerized with 0.1% gelatin. Following electrophoresis, the gels were rinsed in 2.5% Triton X-100 for 2 hr at room temperature and incubated in enzymatic activation buffer (50 mM Tris-HCl, 200 mM NaCl and 5 mM CaCl<sub>2</sub>, pH 7.6) for 24 hr at 37°C with gentle shaking. The gels were stained with 0.25% Coomassie Brilliant Blue R-250, 50% methanol and 5% acetic acid for 30 min and then de-stained in 5% methanol and 7% acetic acid for 1 hr. A commercially available zymography marker (Gelatin Zymo MMP Marker; Life Laboratory, Yamagata, Japan) was run on each gel as a positive control. MMP-9 levels were assessed on the basis of gelatinolytic activity, indicated as clear bands against the dark blue background. All gels were analyzed with an imaging analyzer system (Cool Saver version 1.0; ATTO, Tokyo, Japan) and software (CS Analyzer version 2.0; ATTO). To obtain a semi-quantitative value for each sample, the imaging assessment value of each unknown band was compared with the value of a MMP-9 standard band. The ratio of unknown to standard was calculated, and an arbitrary unit (a.u.) value was assigned to each sample. Each sample was assessed in duplicate.

Invasion of cell lines was assessed by the matrigel transwell assay [8]. For the analysis, 24-well chambers with 8-µm pore filters (Chemotaxicell; Kurabo, Osaka, Japan) were coated on the upper surface with matrigel (BD Matrigel matrix; Becton–Dickinson, Franklin Lakes, NJ, U.S.A.).

Table 1. Sequences of oligonucleotide primers used for quantitative RT-PCR

Primer set	Primer sequence (5'-3')	Position of the primers	Product size	GenBank accession number
<b>MMP-9</b>				
Forward	GCCCCTACAGTGTCTTTGGA			
Reverse	TCCCATCCTTGAAGAAATGC	123–225	103bp	AB858226
<b>GAPDH</b>				
Forward	GCTGCCCAAGAACATCATCC			
Reverse	GTCAGATCCACGACGGACAC	598–731	134bp	NM_001009307

MMP-9: Matrix Metalloproteinase-9, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Matrigel serves as a reconstituted basement membrane *in vitro*. By coating the upper surface of membrane, matrigel occludes the pores of the membrane and blocks non-invasive cells from migrating through the membrane. In contrast, invasive cells secrete proteases that degrade matrigel and enable invasion through the membrane pores. Cells ( $7 \times 10^4$ /well) in 200  $\mu$ l of serum-free medium (RPMI 1640 containing 1% BSA) were added to the upper chamber with rfSAA (1, 5 and 25  $\mu$ g/ml). The lower wells were filled with 600  $\mu$ l of media (RPMI 1640 containing 10% FBS). After 24 hr, the cells that invaded the matrigel and reached the lower surface of the filter were fixed in methanol and stained with Wright-Giemsa solution. The upper surfaces of the filters were scraped twice with cotton swabs to remove non-invading cells. The number of invading cells was counted in 5 high-power (200 $\times$  magnification) microscope fields per filter, and the ratio of rfSAA stimulated cells to control was calculated. Each experiment was performed in duplicate and repeated 3 times in separate experiments.

Statistical analyses were performed using a statistical software package (JMP version 5.0.1J; SAS Institute, Cary, NC, U.S.A.). Student's *t*-test was used to compare individual treatment with their respective control values. In all cases,  $P < 0.05$  was considered to indicate significance. All data are expressed as mean  $\pm$  SEM.

The partial sequence of feline MMP-9 cDNA was determined (GenBank accession number, AB858226). The amino acid sequence of the feline MMP-9 deduced from the partial cDNA sequence determined in this study was shown to have 77%, 66% and 87% identity with those of human, mouse and dog counterparts, respectively (data not shown).

The basal level of MMP-9 mRNA expression was significantly higher in FON-p than those in other 3 cell lines (Fig. 1). MMP-9 mRNA expression in FON-p was at least 10 times greater than that of the other 3 cell lines. In FKN-p and FNN-m cell lines, MMP-9 mRNA expression after rfSAA stimulation increased significantly in a dose-dependent manner (Fig. 1). In FON-p, MMP-9 expression was significantly decreased with 1  $\mu$ g/ml and 5  $\mu$ g/ml treatment with rfSAA. However, FON-p constantly expressed more MMP-9 mRNA than other 3 cell lines (Fig. 1). Although dose-dependent increase of MMP-9 expression was observed in FON-m cells, statistical significance was not reached (Fig. 1).

MMP-9 levels in culture media were examined by gelatin zymography, and a semi-quantitative value of MMP-9 levels for each sample was calculated. Relatively high level

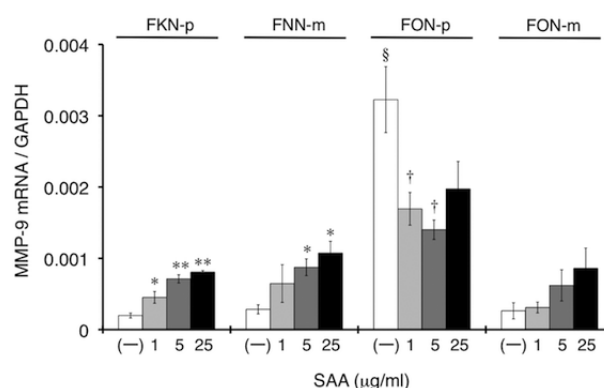


Fig. 1. MMP-9 mRNA expression with rfSAA stimulation. FKN-p, FNN-m, FON-p and FON-m cells were grown in the absence and presence of 1, 5 or 25  $\mu$ g/ml rfSAA for 24 hr. Total cellular RNA was extracted, and the expression of MMP-9 mRNA was examined by quantitative RT-PCR. The expression levels were normalized with that of GAPDH. Each experiment was performed in duplicate and repeated 3 times in separate experiments. Data represent mean  $\pm$  SEM of 3 experiments. \*  $P < 0.05$  and \*\*  $P < 0.01$  increased versus control. †  $P < 0.05$  decreased versus control. §  $P < 0.01$  increased compared with other cells.

of MMP-9 was observed in the FON-p cell line with no stimulation (Fig. 2A), and there was significant difference between FON-p and the other 3 cell lines in basal MMP-9 activity (Fig. 2B). In all 4 cell lines, clear bands of MMP-9 were detected by gelatin zymography with 1–25  $\mu$ g/ml of rfSAA stimulation (Fig. 2A). In FKN-p, FNN-m and FON-p cell lines, MMP-9 levels increased significantly in a dose-dependent manner (Fig. 2B). Although a dose-dependent increase in MMP-9 level was observed in FON-m cells, statistical significance was not reached (Fig. 2B).

The invasiveness of tumor cells was assessed by the matrigel transwell assay. FNN-m cells showed relatively higher invasiveness than the other 3 cell lines with no stimulation (Fig. 3A). The number of invading cells increased with rfSAA stimulation in FNN-m, FON-p and FON-m cell lines (Fig. 3A). In FKN-p cells, however, no difference was observed with or without rfSAA stimulation (Fig. 3A). Moreover, invasion of cells decreased significantly with 5  $\mu$ g/ml of rfSAA in FKN-p (Fig. 3B). In FNN-m and FON-m cells, cell invasion increased significantly with  $\geq 1$   $\mu$ g/ml of rfSAA stimulation. Invasions of both cells after 24 hr in-

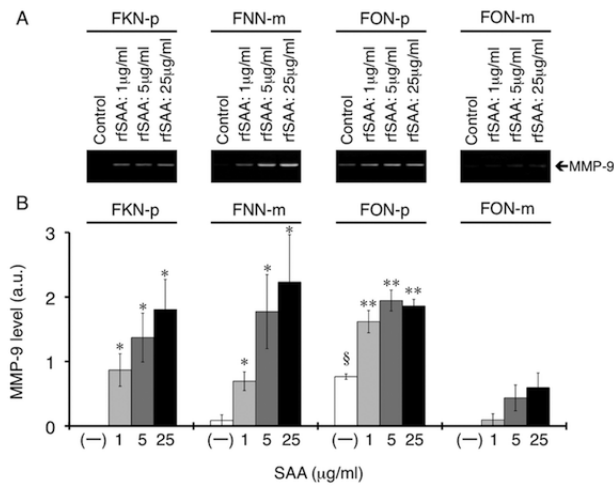


Fig. 2. MMP-9 levels with rfSAA stimulation examined by gelatin zymography. (A) FKN-p, FNN-m, FON-p and FON-m cells were grown in the absence and presence of 1, 5 or 25  $\mu\text{g/ml}$  rfSAA for 24 hr. Cell-free culture media were collected, and MMP-9 levels were examined by gelatin zymography. MMP-9 levels were assessed on the basis of gelatinolytic activity, indicated as clear bands against the dark blue background. Representative data are shown. (B) To obtain a semi-quantitative value for each sample, the expression levels were normalized with that of a commercially available standard. Each experiment was performed in duplicate and repeated 3 times in separate experiments. Data represent mean  $\pm$  SEM of 3 experiments. \*  $P < 0.05$  and \*\*  $P < 0.01$  increased versus control.  $\S$   $P < 0.01$  increased compared with other cells.

creased more than twice with rfSAA stimulation. Although a dose-dependent increase in invasion was observed, statistical significance was not reached in FON-p cells (Fig. 3B).

In the present study, the direct effects of rfSAA on tumor cells were demonstrated. Feline mammary carcinoma cell lines expressed and produced MMP-9 with rfSAA stimulation in a dose-dependent manner. rfSAA also stimulated the actual invasion of the cells through the extracellular matrix component. These findings show that SAA may have a role in tumor progression and metastasis. The association between SAA and tumor progression was indicated by several previous clinical studies [1, 15, 30] and *in vitro* studies [11]. SAA also stimulates inflammatory responses [19], and inflammation is considered as one of the important factors for tumor progression [4]. However, these previous studies focused on the indirect effects of SAA on tumor progression, while the direct effects remain unexplored.

Although direct effects of SAA, such as MMP-9 production and tumor cell invasion, were previously reported in 2 human glioma cell lines [8], contradictory effects of SAA were shown in the study. SAA stimulated tumor cell invasion in one cell line; however, tumor cell invasion was significantly suppressed by SAA in the other cell line. In the present study, increased MMP-9 production or increased tumor cell invasion with rfSAA stimulation was observed in 4 feline mammary carcinoma cell lines. The suppressive effect of rfSAA in the cells was almost imperceptible.

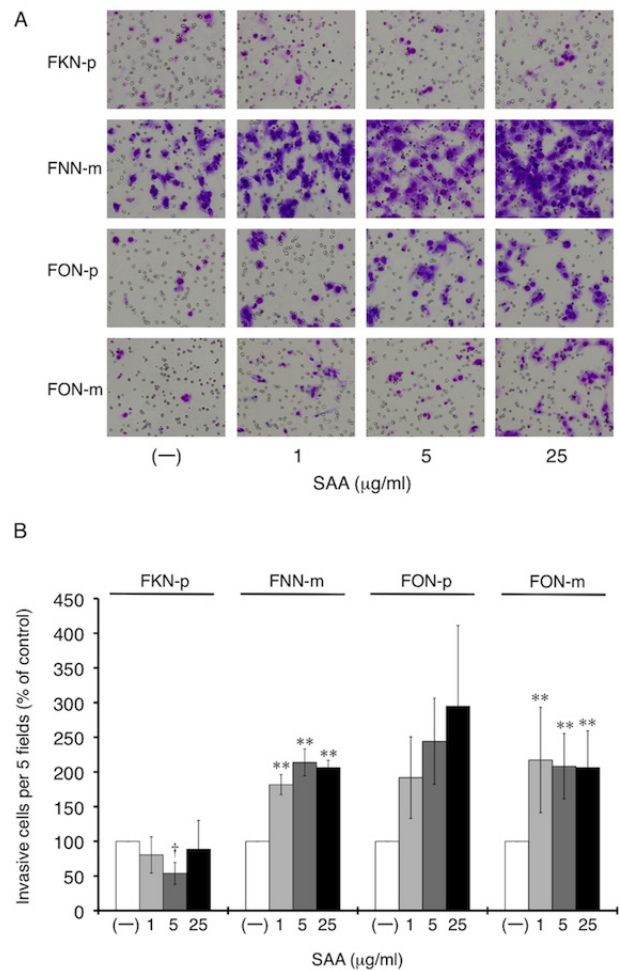


Fig. 3. Tumor cell invasion with rfSAA stimulation. (A) Invasiveness of tumor cells was assessed by using the matrigel transwell assay. FKN-p, FNN-m, FON-p and FON-m cells were grown in the absence and presence of 1, 5 or 25  $\mu\text{g/ml}$  rfSAA on upper surface of the chamber for 24 hr. After incubation, cells that reached the lower surface of the filter were fixed and stained with Wright-Giemsa solution. Representative data are shown. (B) The number of invading cells was counted in 5 high-power (200 $\times$  magnification) microscope fields per filter, and the ratio of rfSAA stimulated cells to the control was calculated. Each experiment was performed in duplicate and repeated 3 times in separate experiments. Data represent mean  $\pm$  SEM of 3 experiments. \*\*  $P < 0.01$  increased versus control.  $\dagger$   $P < 0.05$  decreased versus control.

In humans and animals with mammary tumors, it has been observed that serum SAA concentration is higher in patients with metastasis [26, 33]. Furthermore, both SAA and MMP-9 were described as prognostic markers in human patients with breast cancer [14, 21]. Thus, the findings that rfSAA stimulated, not suppressed, MMP-9 production and facilitated invasion of mammary tumor cells are considered as reasonable outcomes.

MMP-9 mRNA expression and protein synthesis were increased in FKN-p cells with rfSAA stimulation. However,

rfSAA could not stimulate tumor cell invasion in these cells. Many other proteinases and their inhibitors may be involved in tumor cell invasion, and the expression of the inhibitor, such as tissue inhibitor of metalloproteinases (TIMPs), might suppress cell invasion [31]. Although TIMPs were also described as important factors in the prognosis of breast cancer [16, 32], the expression of TIMPs was not examined in this study.

FNN-m cells were established from cells in the pleural fluid of a feline patient with thoracic metastasis [29]. The cells showed relatively higher invasiveness than the other 3 cell lines with or without rfSAA, and MMP-9 mRNA expression and protein synthesis increased significantly in the presence of rfSAA. Cell lines that had originally high invasive or metastatic capacity might be more susceptible to SAA.

FON-p cells showed unique features compared with the other 3 cells. MMP-9 mRNA expression and protein synthesis in these cells without rfSAA stimulation were significantly higher than in the other cells. Although MMP-9 mRNA expression was suppressed with 1 or 5 mg/ml rfSAA, MMP-9 protein synthesis was stimulated significantly with rfSAA. As MMP-9 mRNA was expressed highly without stimulation, there is a possibility that MMP-9 mRNA expression was increased more rapidly with rfSAA stimulation and decreased over time. Alternatively, rfSAA might stimulate only protein translation and down-regulate mRNA transcription under a certain condition.

FON-m cells were established from the same patient as FON-p [29]; however, these cells showed different responses to SAA. Although a dose-dependent increase in MMP-9 production was observed in FON-m, the changes were smaller than those observed for FON-p and statistical significance was not reached. The colonized cells after metastasis might be less susceptible to SAA.

SAA protein is mainly synthesized in the liver and released into the blood stream. In humans, it was described that SAA was expressed in various tumor tissues, such as squamous cell carcinoma [18] and ovarian epithelial tumor [28]. Moreover, tumor cells themselves can produce SAA protein [9]. Therefore, in addition to the systemic increase in SAA concentration, the local expression of SAA might affect the tumor cells and their invasiveness through autocrine or paracrine effects. It has also been described that lung cancer cells with forcibly expressed SAA gene showed higher metastatic capacity [22]. However, SAA expression could not be detected in feline mammary carcinoma cell lines used in this study (data not shown). To the best of our knowledge, local expression of SAA has not been examined in feline tumors.

In conclusion, SAA stimulates MMP-9 production and tumor cell invasion in feline mammary tumor cells. Thus, a novel role for SAA in mammary tumorigenesis is suggested. The suppression of effects of SAA may provide new therapeutic strategies for tumor invasion and metastasis. Further investigation is needed to determine the receptors and the signaling pathways of feline SAA in tumor cells.

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