Int J Clin Exp Pathol 2016;9(10):9853-9866
www.ijcep.com /ISSN:1936-2625/IJCEP0032255

Original Article

Serum amyloid A expression is associated with breast cancer survival

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Received May 16, 2016; Accepted August 1, 2016; Epub October 1, 2016; Published October 15, 2016

Abstract: Serum amyloid A (SAA) is an acute-phase inflammatory protein. A growing body of evidence supports the role of SAA in carcinogenesis and metastasis. Thus, SAA has been suggested as a biomarker for tumor progression. In this study, we detected the expression of SAA in MCF-7 human breast cancer cell lines by immunofluorescence to investigate the effect of human recombinant apo-SAA (rSAA) on MCF-7 cell lines. The expression of SAA in benign and malignant breast tissues was identified by immunochemical staining, and the association of SAA expression in breast cancer with clinicopathologic features and survival was studied. Based on the resulting data, high levels of SAA expression were detected in both the MCF-7 cell line and breast cancer tissues. *In vitro*, SAA induced proliferation, migration, and invasion of MCF-7 cells, and stimulated the expression of matrix metalloproteinase (MMP)-2 and MMP-9 in a dose- and time-dependent manner. Clinically, higher SAA expression in breast cancer tissues was positively associated with tumor T stages ($P<0.001$), lymph node metastasis ($P<0.001$), body mass index ($P<0.001$) and menopausal status ($P=0.016$). After a median 49-month follow-up of 215 breast cancer patients, multivariate Cox's regression analysis confirmed that SAA expression was an independent prognostic factor of breast cancer disease-free survival (hazard ratio =4.950; 95% confidence interval: 1.078-22.731). These results indicated a prognostic value of local SAA expression for breast cancer progression and risk assessment.

Keywords: Serum amyloid A, breast cancer, survival

Introduction

Breast cancer is the most common malignancy among women and one of the leading causes of cancer-related deaths [1], accounting for 14% of all cancer deaths worldwide in 2012 [2]. Identification of novel biomarkers and therapy targets has been proven to be an effective strategy to improve cancer prognosis.

Inflammation has been implicated in the etiology of cancer [3], and increasing evidence has shown that a series of inflammatory molecules is associated with the onset and progression of breast cancer [4-6]. Serum amyloid A (SAA), one of such inflammatory molecules; it is a non-specific acute-phase protein with its primary production ascribed to hepatocytes, as well as adipose tissue. The SAA level may be induced up to 1000-fold following inflammatory stimuli by cytokines, such as IL-8 [7-10]. Biologically, SAA is an important chemoattractant involved in migration, adhesion, proliferation, and recruitment of monocytes and polymorphonuclear leukocytes [11].

Recently, the direct effect of SAA on the invasiveness of tumor cells was also discovered [12]. Further, it has been extensively reported that SAA levels increase in patients afflicted with renal cancer, prostate cancer, lung carcinoma, colorectal cancer [12-15], and breast cancer [4]. Moreover, breast cancer patients with more advanced tumors [4] or with metastasis [16] presented higher SAA level, and such elevated SAA levels were significantly associated with reduced survival [17]. Thus, SAA is considered a potential biomarker and prognostic factor of breast cancer.

Additionally, SAA has been shown to predominately localize to the epithelium of various tissues [18]. Recent data showed that certain cancer cells, including endometrial cancer,

colon carcinoma [19-22], lung carcinoma [23] and glioma [24], could also synthesize and secrete SAA, indicating possible autocrine and intracrine actions of SAA on cancer tissue [24]. The fact that the increased SAA expression in cancer tissue was correlated with poor survival in the case of renal cell cancer [12] implied a possible role of local SAA expression on cancer progression. However, as far as we know, no previous studies have studied the association of local SAA expression and breast cancer progression. Further, the mechanism by which SAA can induce cancer progression is not fully understood.

In this study, we identified the expression of SAA in breast cancer tissue by immunochemical staining and investigated its association with clinicopathologic features and long-term survival. The in-vitro effect of SAA on breast cancer MCF-7 cell lines was also studied. Our findings verified the high expression of SAA in breast cancer tissue and revealed that higher expression of SAA was associated with more advanced disease, lymph node metastasis, and poorer prognosis. Cell culture data indicated that SAA may promote the invasion of breast cancer cells via the matrix metalloproteinase (MMP)-2 or MMP-9 pathway.

Materials and methods

Cell culture

The MCF-7 cells (human breast cancer cell line) were obtained from American Type Culture Collection (Manassas, VA, USA, 2013, Lot Number 61235352), and were cultured in Dulbeco's minimum essential media (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in a 5% CO_2 humidified incubator at 37°C. The MCF-7 cells were passaged every 3 to 4 days.

Cell viability and proliferation assay

Cell viability and proliferation activity were assessed through MTT colorimetric assay. The MCF-7 cells (5,000 cells per well) were seeded onto 96-well plates (Corning Costar, Corning, NY, USA), and cultured in serum free culture medium with the absence or presence of 0.1, 0.5, 1, 5 and 10 μg/ml human recombinant apo-SAA1 (rSAA1; PeproTech, Rocky Hill, NJ, USA) for 12 h, or in the absence or presence of 1 μg/ml, 10 μg/ml rSAA1 for 0, 30 min, 1 h, 3 h, 6 h and 12 h. Then, 20 μl of MTT (5 mg/ml; Sigma-Aldrich, St Louis, MO, USA) were added into each well. After incubation for 4 h, 80 μl of dimethyl sulfoxide (Sigma-Aldrich) were added into each well for another 15 min to fully solubilize formazan (the metabolic product of MTT) and liberate the purple product for its detection using a microplate luminometer at 490 nm.

Scratch wound healing assay (migration assay)

The migration ability of the MCF-7 cells was evaluated by a scratch wound healing assay. Briefly, the MCF-7 cells (1×10^6 cells/well) were seeded onto six-well plates and cultured overnight. After 90% confluence, straight scratches of the same width were made in the monolayer of the MCF-7 cells with a pipette tip. To test the effects of SAA on the migration of the MCF-7 cells, various concentrations of SAA (0, 1, or 10 μg/ml) were added into the plates. At different time points (0, 12 and 24 h), photo images were taken to measure wound healing under a microscope. The migrated area was quantified by AxioVision software (Release 4.8 Carl Zeiss AB, Stockholm, Sweden).

Transwell assay (invasion assay)

The effect of SAA on the invasive ability of the MCF-7 cells was measured using modified Boyden chambers with 8-μm-pore filter inserts in 24-well plates (Corning Costar). The MCF-7 cells (1×10^5 cells/well) were washed with phosphate buffered saline (PBS, pH 7.4) twice, resuspended in the DMEM plus 1% FBS with different concentrations of rSAA1 (0, 1 or 10 μg/ml) seeded into the Matrigel-coated upper chamber, while the lower chamber was filled with DMEM containing 10% FBS and the same concentration as upper chamber. The system was incubated at 37°C and 5% CO_2 for 0, 30 min, 1 h, 3 h, 6 h, and 12 h. After incubation, cells on the upper surface of the inserts were wiped away gently with a cotton wool swab, while the cells invading into the lower surface were fixed with 4% paraformaldehyde for 30 min and stained with hexamethylpararosaniline. The average number of invasive cells was counted in six random high-power fields (×200).

Western blot

The MCF-7 cells were collected and lysed on ice with a lysis buffer containing protein inhibitors (Beyotime Biotecnology, Jiangsu, China) for 30 min. The extracted protein concentration was measured by BCA protein assay kit (Beyotime Biotecnology). Equal amounts of proteins were separated via 10% SDS-polyacrylamide gel in a running buffer, and transferred onto nitrocellulose membranes. The membranes were then blocked and incubated with rabbit anti-MMP-2 antibody, rabbit anti-MMP-9 antibody (Cambridge, UK) or rabbit anti-glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (R&D Systems, Minneapolis, MN, USA) at 4°C overnight. After incubation with suitable secondary antibodies for 2 h at room temperature, the antigen-antibody complexes were visualized with an electrochemiluminescence detection system (Millipore, USA).

Immunofluorescence analysis

Cells were cultured on glass cover slips for immunofluorescent staining. After washing with PBS quickly, cells were fixed in 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.2% Triton X-100 for 10 min ×3 times, blocked with PBS containing 0.3% donkey serum and 0.2% Triton X-100 for 30 min and then incubated at 4°C with goat anti-SAA1 (1:500; R&D Systems) overnight. After washing with PBS, cells were incubated for 90 min at room temperature with secondary antibody Rhodamine (TRITC)-conjugated AffiniPure Donkey Anti-Goat IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), coverslipped with VectaShield Mounting Medium with DAPI (Vector Laboratories, Inc, Burlingame, CA, USA), and sealed with clear nail polish.

Study population and tissue collection

From January 2008 to December 2010, a total of 267 patients with histologically-confirmed breast disease (215 malignant and 50 benign) from Beijing Tiantan Hospital, Capital Medical University, were enrolled in this study. Most of the clinical data were extracted from the medical records, including age at diagnosis, body mass index (BMI), tumor size, histological grade, nodal status, hormone receptor status and human epidermal growth factor receptor-2 (HER2)-status. Each patient's BMI (kg/m^2) was categorized according to the classification of the World Health Organization [25]: normal, 18.5-24.99 kg/m^2; overweight, 25.0-29.99 kg/m^2; and obese, ≥30 kg/m^2. The follow-up data included breast cancer-related recurrence, metastasis, or death. The primary endpoint was disease-free survival (DFS), which was defined as the length of time from diagnosis of breast cancer to the first evidence of invasive relapse at any site, recurrence or death. The secondary endpoint was overall survival (OS). Patients without events were censored at their last follow-up. All the formalin-fixed, paraffin-embedded tissue blocks were obtained from the hospital's department of pathology. Clinical follow-up was carried out by face-to-face consultation at the hospital or by telephone. All patients or their next of kin provided written informed consent for the collection of samples and subsequent analysis. The study was approved by the Institutional Review Board of the Medical Collage of Shandong University.

Immunohistochemistry

All of the tissue blocks were serially cut into 4-μm sections. They were dewaxed in xylene and rehydrated through graded ethanol before being boiled in a pressure cooker with 1.0 mM EDTA (pH 8.0) for 14 min for antigen retrieval. After sections cooled down to room temperature, these were washed twice with PBS; endogenous peroxidase activity and non-specific binding of the second antibody were blocked by 0.3% hydrogen peroxide and 5% bovine serum albumin (Sigma-Aldrich), respectively. After that, sections were incubated with goat anti-SAA1 (1:50; R&D Systems) in PBS containing 1% bovine serum albumin at 4°C overnight and then incubated with the rabbit anti-goat second antibody (1:50; Beyotime Biotecnology). Subsequently diaminobenzidine substrate (Beyotime Biotecnology) was used as a chromogen to visualize antibody-target protein binding in tissue. A hematoxylin solution was used for counterstaining.

Interpretation of immunohistochemical staining

Immunostaining for SAA was regarded as positive when tumor cells evidenced cytoplasmic immunostaining of the lesion, with a minimal background. Staining was categorized into

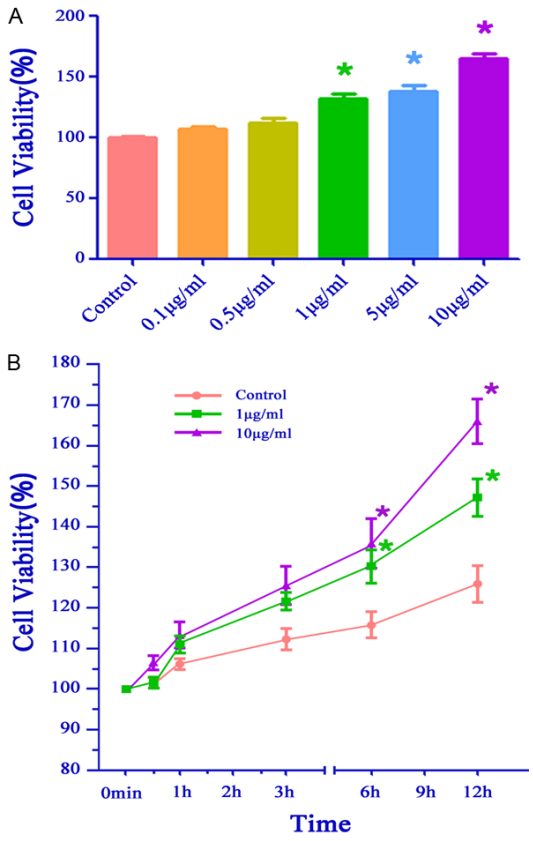

Figure 1. SAA induced proliferation of MCF-7 cells. A. MCF-7 cells were stimulated with SAA of different concentration gradients (0, 0.1, 0.5, 1, 5, 10 μg/ml) for 12 h, and MTT analysis was employed to detect the viability of MCF-7. B. MCF-7 cells were stimulated with PBS, 1 or 10 μg/ml SAA for different time periods (0, 30 min, 1 h, 3 h, 6 h, and 12 h), and MTT analysis was used to detect the viability of MCF-7. *P<0.05 versus control group. Data shown are means ± SEM from three independent experiments in duplicate. SAA, serum amyloid A; SEM, standard error of the mean.

eight grades according to previously described immunohistochemical scores [26-28]. The proportion and intensity of the immunoreactive tumor cells in each species were recorded and required for statistical analysis. Briefly, the proportion score was assigned as the proportion of positive tumor cells (0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; and 5, >2/3). The intensity score was assigned as the average intensity of the positive tumor cells (0, none; 1, weak; 2, intermediate; and 3, strong). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8, except 1. According to the median score of SAA, all patients were divided into low-expression (the score of SAA ≤4) and over-expression groups (the score of SAA ≥5). All of the slides were evaluated independently by two experienced pathologists who were both blinded to the information of sections during microscopic examination and evaluation. If different results were reported by these pathologists, a third pathologist was employed to judge the results.

Statistical analyses

All statistical analyses were performed with SPSS statistical software (version 13.0; Chicago, IL, USA). Chi-square test and one-way ANOVA were used to analyze normally distributed data and nonparametric variables as well as to compare characteristics of the SAA low-expression and over-expression patients. Kaplan-Meier survival curves were compared to estimate DFS and OS, and the differences were tested by the log-rank test. The adjusted hazard ratio (HR) and 95% confidence interval (CI) were calculated by using the multivariable Cox's regression analysis. P-values <0.05 were considered statistically significant.

Results

SAA induced MCF-7 cell proliferation

The effects of SAA on the proliferation of MCF-7 cells were assessed through the MTT method. The MCF-7 cells were stimulated with various concentration of SAA (0, 0.1, 0.5, 1, 5, 10 μg/ml) for 12 h. As shown in **Figure 1A**, SAA increased cell viability of the MCF-7 cells in a dose-dependent manner and reached statistical variance at the concentration of 1 μg/ml (P<0.05). Then, fixed concentrations of SAA (1 and 10 μg/ml) were selected to stimulate MCF-7 for different time periods (30 min, 1 h, 3 h, 6 h and 12 h). As shown in **Figure 1B**, SAA promoted cell viability of the MCF-7 cells in a time-dependent manner and significant increase in proliferation was observed from 6 h in both 1 and 10 μg/ml treatment groups compared with PBS groups (P<0.05).

SAA induced MCF-7 cell migration

The effect of SAA on the migration of the MCF-7 cells was assessed through wound healing assay. The MCF-7 cells were treated with SAA at the concentration of 0, 1, or 10 μg/ml for 12 h or 24 h. As shown in **Figure 2A**, the healing over

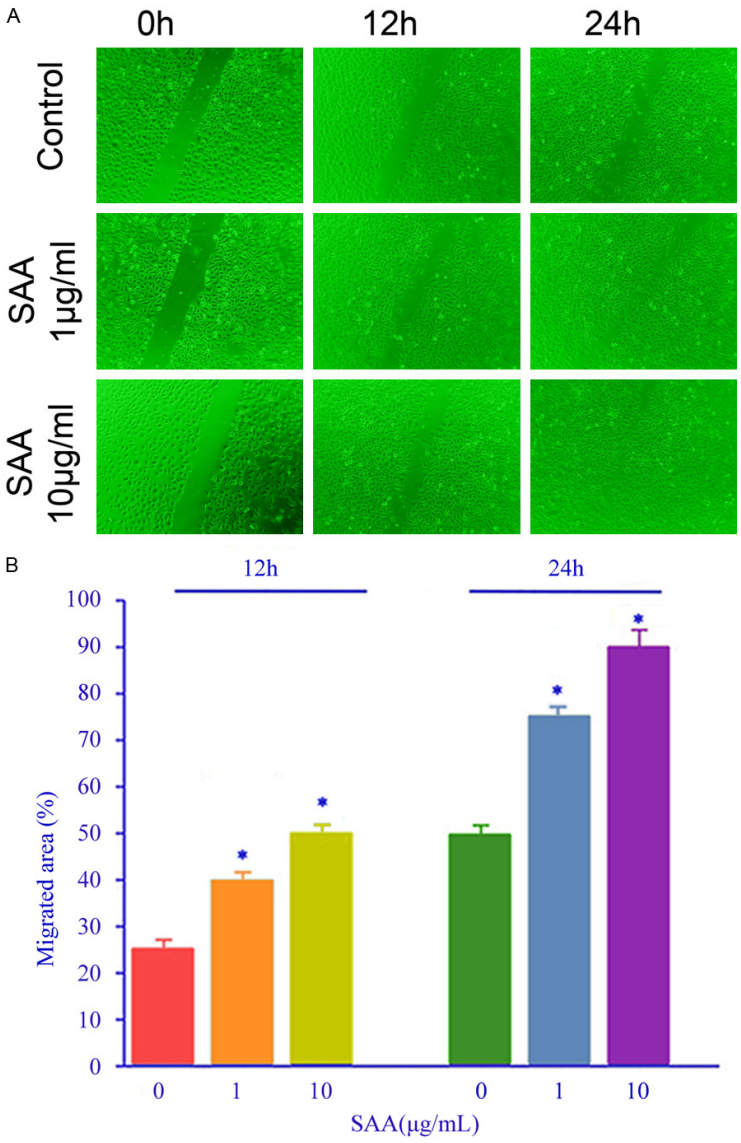

Figure 2. SAA induced the migration of MCF-7 cells. The effect of SAA on the migration of MCF-7 cells was assessed by wound healing assay. A. MCF-7 cells were treated with SAA at the concentration of 0, 1 or 10 μg/ml for 12 or 24 h. Healing over the scratch was photographed under a microscope at different time points (12 and 24 h). B. The percentage of the migrated area of the scratch region. *P<0.05 compared with control group. Data shown are means ± SEM of the three independent experiments. SAA, serum amyloid A; SEM, standard error of the mean.

the scratch increased gradually along with the increased concentrations of SAA. **Figure 2B** showed that SAA of 1 μg/ml had a significant effect on the migration of the MCF-7 cell compared with the control group (P<0.05), while the concentration of SAA of 10 μg/ml displayed a more significant effect (P<0.05), indicating that SAA induced the migration of the MCF-7 cells in a dose-dependent manner.

SAA induced the invasion of the MCF-7 cells

The effect of SAA on the invasion of the MCF-7 cells was assessed by Transwell assays. MCF-7 cells were treated with SAA (1 or 10 μg/ml) for 12 h. As shown in **Figure 3A** and **3C**. Both 1 and 10 μg/ml of SAA significantly promoted the invasive activity of the MCF-7 compared with the control group (P<0.05). Then, fixed concentrations of SAA (1 and 10 μg/ml) and PBS (Control Group) were selected to stimulate MCF-7 for different time periods (30 min, 1 h, 3 h, 6 h, and 12 h). As shown in **Figure 3B**, both 1 μg/ml and 10 μg/ml of SAA strongly promoted the invasive activity of MCF-7 at 6 h compared with the PBS groups (P<0.05). The invasion of the MCF-7 cells increased in a time-dependent manner.

SAA induced the expression of MMP-2 and MMP-9 on MCF-7 cells

MMP-2 and MMP-9 play key roles in the invasion of MCF-7 cells and have been identified as invasion-related protein in breast cancer [29]. To investigate whether SAA could regulate the expression of MMP-2 and MMP-9, MCF-7 cells were treated with SAA of various concentrations for 12 h and the changes of MMP-2 and MMP-9 protein expression was measured with western blot. As shown in **Figure 4A** and **4C**, SAA (1 and 10 μg/ml) significantly enhanced the expression of both MMP-2 and MMP-9 compared with the control groups (P<0.05). In order to investigate the relation between MMP expression and stimulating time, MCF-7 cells were treated with 10 μg/ml SAA for different time periods (0 h, 3 h, 6 h, 12 h, and 24 h). In **Figure 4B** and **4D**, the expression of both MMP-2 and MMP-9 was evidently increased at

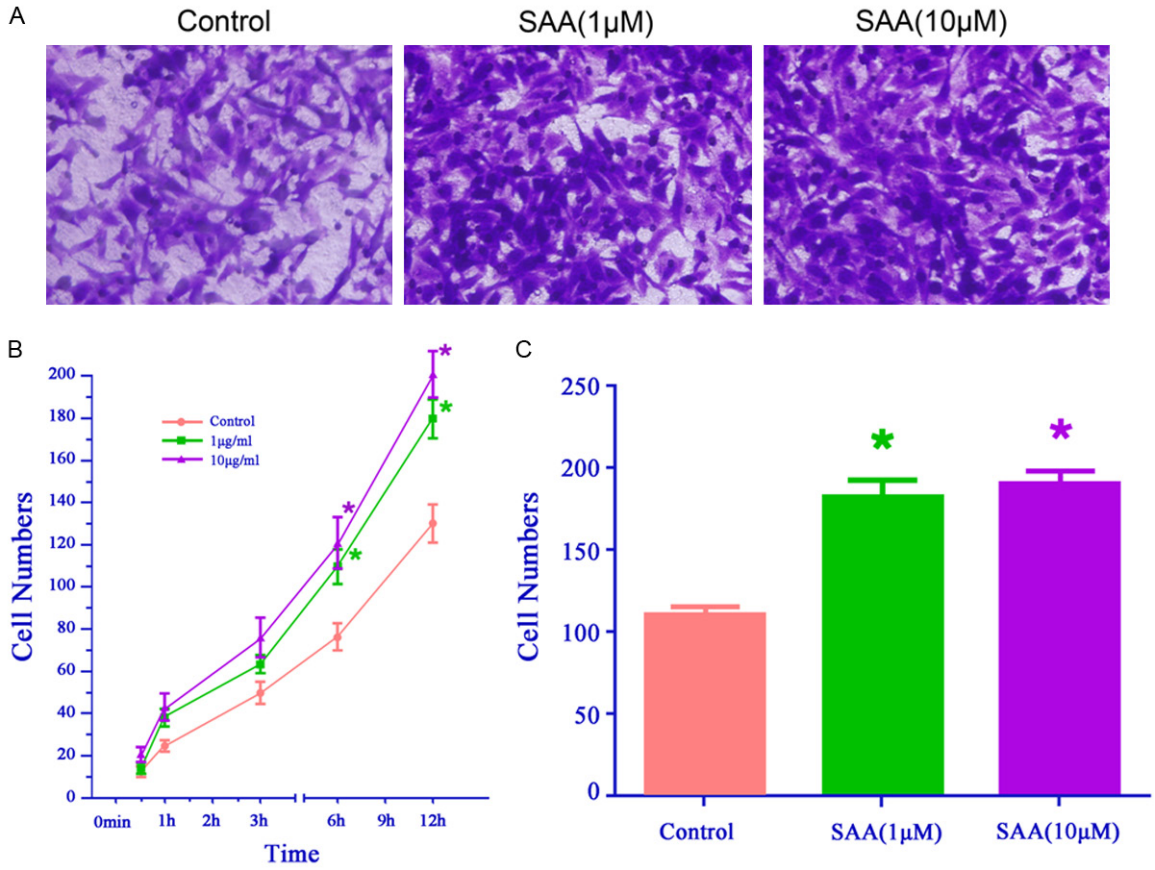

Figure 3. SAA induced the invasion of MCF-7 cells. A. MCF-7 cells were treated with SAA at the concentration of 0, 1 and 10 μg/ml for 12 h. Cells penetrating to the underside surface of Transwell filter were fixed and stained with hexamethylpararosaniline. B. Statistical analysis of invasive cell numbers at different times (30 min, 1 h, 3 h, 6 h and 12 h) after MCF-7 cells were stimulated with PBS, 1 or 10 μg/ml SAA. C. Statistical analysis of invasive cell numbers when MCF-7 cells were treated with SAA at the concentration of 0, 1 or 10 μg/ml for 12 h. *P<0.05 compared with control group. Data shown are means ± SEM from three independent experiments in duplicate.

12 h, and it reached the maximum at 24 h. SAA induced MMP-2 and MMP-9 expression in dose- and time-dependent manner.

SAA expression in the MCF-7 cell line and in benign and malignant breast tissue specimens

The expression of SAA in the MCF-7 cell line was analyzed by immunofluorescence staining. As shown in **Figure 5A-C**, SAA was expressed in the cytoplasm of the MCF-7 cells. A total of 215 breast cancer specimens and 50 benign breast tissue specimens were analyzed through immunohistochemical staining for SAA in this study. We found that the score of SAA in breast cancer was much higher than that in benign breast tissue (3.93 ± 2.403 VS. 0.60 ± 1.125, P<0.001) (**Table 1**). **Figure 5D-I** show the varying degrees of SAA expression in benign and malignant breast tissue specimens.

Patient characteristics

The clinicopathologic characteristics of breast cancer patients in the SAA low-expression or over-expression groups are shown in **Table 2**. The median patient age was 47 (range 27-71) years in the SAA low-expression group, and 51.5 (range 28-74) years in the SAA over-expression group (P=0.014). Patients in the SAA over-expression group had a much higher BMI than those in the low-expression group (P<0.001). Thirty-five (32.7%) patients in the SAA over-expression group and 50 (49.0%) in low-expression group were post-menopausal (P=0.016). Patients in the SAA over-expression group had significantly larger tumors and experienced lymph node metastasis more frequently than patients in the low-expression group (P<0.001). There was no difference between

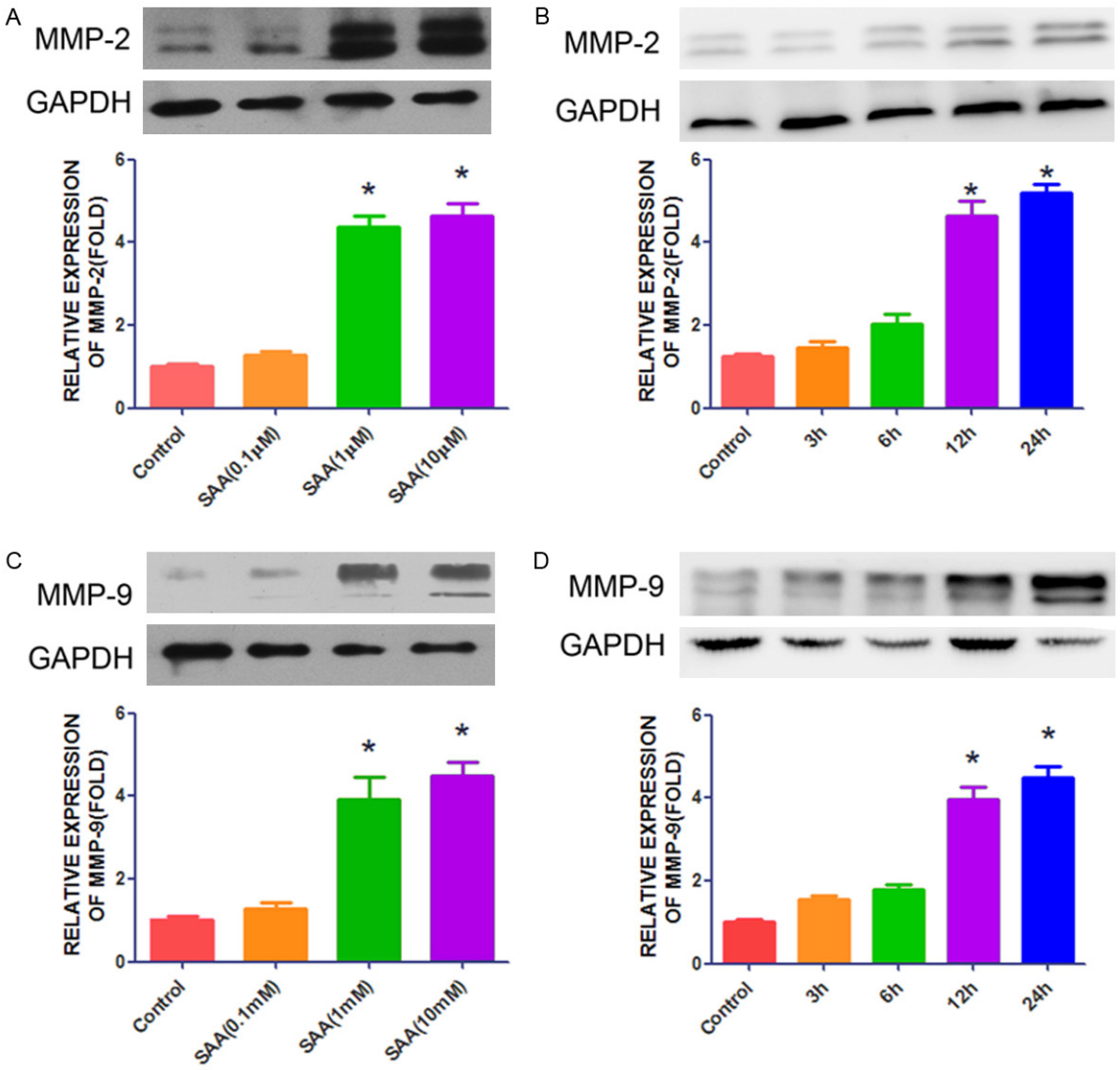

Figure 4. SAA induced the expression of MMP-2 and MMP-9 on MCF-7 cells. A, C. MCF-7 cells were treated with SAA at various concentrations (0, 0.1, 1 or 10 μg/ml) for 12 h and western blot assay was utilized to assess the changes of MMP-2 and MMP-9 protein expression. B, D. MCF-7 cells were treated with SAA at the concentration of 10 μg/ml for various time periods (0, 3, 6, 12 and 24 h) and western blot assay was utilized to assess the expression of MMP-2 and MMP-9 protein. *P<0.05 compared with control group. Data shown are means ± SEM from three independent experiments in duplicate. SAA, serum amyloid A; SEM, standard error of the mean; MMP, matrix metalloproteinase.

the two groups in histological grade, ER, PR, or HER2 (**Table 3**).

Survival analysis

During a median follow-up of 49 months, 31 breast cancer DFS events were noted, and the over-expression of SAA was associated with an increased risk of breast cancer DFS events (28 in SAA over-expression group and 3 in low-expression group; HR=4.950, 95% CI: 1.078-22.731). We noted 14 breast cancer deaths in the SAA over-expression group, and 2 breast cancer deaths in the SAA low-expression group. Given the few deaths at the end of this follow-up, the associations of variables and OS were not performed. Kaplan-Meier curves showed that DFS and OS were lower in the the SAA over-expression group, with 3-year DFS of 96.4% for the SAA low-expression group vs. 81.0% for the over-expression group (log-rank P<0.001) (**Figure 6A**). The 3-year OS was 98.8% for the SAA low-expression group vs. 90.1% for the

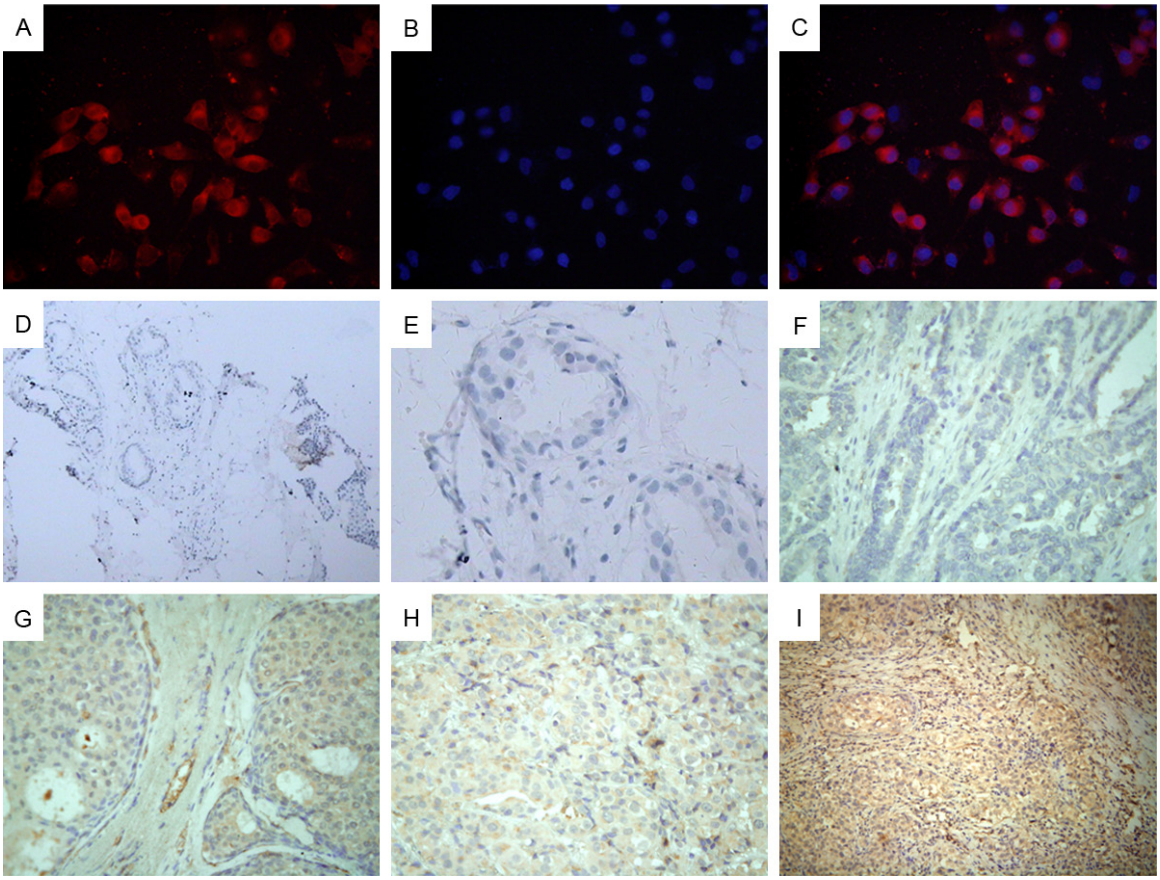

Figure 5. SAA expressed in MCF-7 cell line and breast tissue. (A-C) SAA was positively expressed in the cytoplasm of MCF-7 cells. The localization of SAA (red) was photographed by laser scanning confocal microscopy (A SAA, B DAPI, C Merge). SAA protein was expressed in human breast tissue, and the brown staining represents positive SAA signal. SAA was negatively expressed in the benign breast tissue (D×10, E×40) and invasive breast cancer (F×40). (G) Intraductal carcinoma, with low expression of SAA (×40). (H) Invasive breast cancer, with low expression of SAA (×40). (I) Invasive breast cancer, with overexpression of SAA (×20). SAA, serum amyloid.

Table 1. The score of SAA expression in benign and malignant breast tissue

Characteristics	N	SAA Mean ± SD	F	*P*
Benign	50	0.60 ± 1.125	91.161	<0.001
Malignant	215	3.93 ± 2.403		

SAA, serum amyloid A; SD, standard deviation.

over-expression group (log-rank *P*=0.005) (**Figure 6B**).

Discussion

SAA is an acute-phase inflammatory protein that has been established as a potential serum marker for breast cancer [16] and has been shown to predominately localize to the epithelium of various tissues, including breast tissue [18]. Although many studies have already shown SAA tissue expression associated with cancer progression [19, 30], the relationship between SAA expression in breast tissue and the prognosis of breast cancer patients remains unclear. Furthermore, the effects of SAA on breast cancer cell lines have not been elucidated fully. In this study, we showed the high expression of SAA in breast cancer MCF-7 cell lines by immunofluorescence. Additionally, we showed that the elevated SAA induced the proliferation, migration, and invasiveness of MCF-7 cells in a dose-dependent manner. These findings are similar to those studies on lung carcinoma [23] and glioma cell lines [24]. Apart from the general hepatic synthesis, SAA is also expressed in a series of epithelial cells, including the normal breast lobular epithelium [21]. It is widely accepted that chronic inflammation provides a favorable con-

Table 2. Patient and tumor characteristics by SAA stage

Characteristics		Low-expression (n=112)	Over-expression (n=103)	*P*
Median age at diagnosis (range)		47.0 (27-71)	51.5 (28-74)	0.014
Mean value of Follow-up	± SD	52.66 ± 9.677	45.72 ± 13.890	<0.001
BMI	<25	75 (75%)	30 (30.3%)	<0.001
	25-29.99	18 (18.0%)	52 (52.5%)	
	≥30	7 (7%)	17 (17.2%)	
Menopausal status	Pre	72 (67.3%)	52 (51.0%)	0.016
	Post	35 (32.7%)	50 (49.0%)	
Histological grade	I	7 (7.9%)	4 (4.2%)	0.149
	II	74 (83.1%)	75 (78.1%)	
	III	8 (9.0%)	17 (17.7%)	
Chemotherapy	Yes	90 (96.8%)	81 (97.6%)	1.000
	No	3 (3.2%)	2 (2.4%)	
T stage	1	40 (39.2%)	7 (7.4%)	<0.001
	2	56 (54.9%)	62 (66.0%)	
	3	3 (2.9%)	17 (18.1%)	
	4	3 (2.9%)	8 (8.5%)	
N stage	N0	68 (61.3%)	24 (24.0%)	<0.001
	N1	36 (32.4%)	13 (13.0%)	
	N2	3 (2.7%)	48 (48.0%)	
	N3	4 (3.6%)	15 (15.0%)	
ER	Negative	50 (44.6%)	53 (52.0%)	0.285
	Positive	62 (55.4%)	49 (48.0%)	
PR	Negative	50 (42.9%)	53 (52.0%)	0.183
	Positive	62 (57.1%)	49 (48.0%)	
HER2	Low-expression	104 (92.9%)	92 (92.0%)	0.814
	Over-expression	8 (7.1%)	8 (8.0%)	

Data were presented as median (range), mean ± SD or number (%). SAA, serum amyloid A; SD, standard deviation; T stage, pathologic staging of tumor size; N stage, pathologic staging of lymphatic metastasis; ER, Estrogen receptor; PR, Progesterone receptor; HER2, human epidermal growth factor receptor type 2.

Table 3. Multivariate Cox regression analysis of disease-free survival

Characteristics		No. of events	No. of participants	HR (95% CI)	*P*
T stage	T1	4 (14.8%)	47 (24.0%)	1.0 (Reference)	
	T2	14 (51.9%)	118 (60.2%)	0.668 (0.200-2.227)	0.511
	T3	3 (11.1%)	20 (10.2)	0.582 (0.118-2.880)	0.507
	T4	6 (22.2%)	11 (5.6%)	2.986 (0.790-11.287)	0.107
N stage	N0	2 (6.7%)	92 (43.6%)	1.0 (Reference)	
	N1	5 (16.7%)	49 (23.2%)	4.260 (0.754-24.060)	0.101
	N2	17 (56.7%)	51 (24.2)	5.360 (1.099-26.142)	0.038
	N3	6 (20.0%)	19 (9.0%)	8.325 (1.553-44.641)	0.013
SAA stage	Low-expression	3 (9.7%)	112 (52.1%)	1.0 (Reference)	
	Over-expression	28 (90.3%)	103 (47.9%)	4.950 (1.078-22.731)	0.040

All of the characteristics in **Table 2** were assessed for univariate analysis, and only the significant variables (T stage, N stage, SAA stage; $P<0.05$, data not shown) were assessed in the multivariable Cox's regression analysis. SAA, serum amyloid A; T stage, pathologic staging of tumor size; N stage, pathologic staging of lymphatic metastasis; HR, hazard ratio; CI, confidence interval.

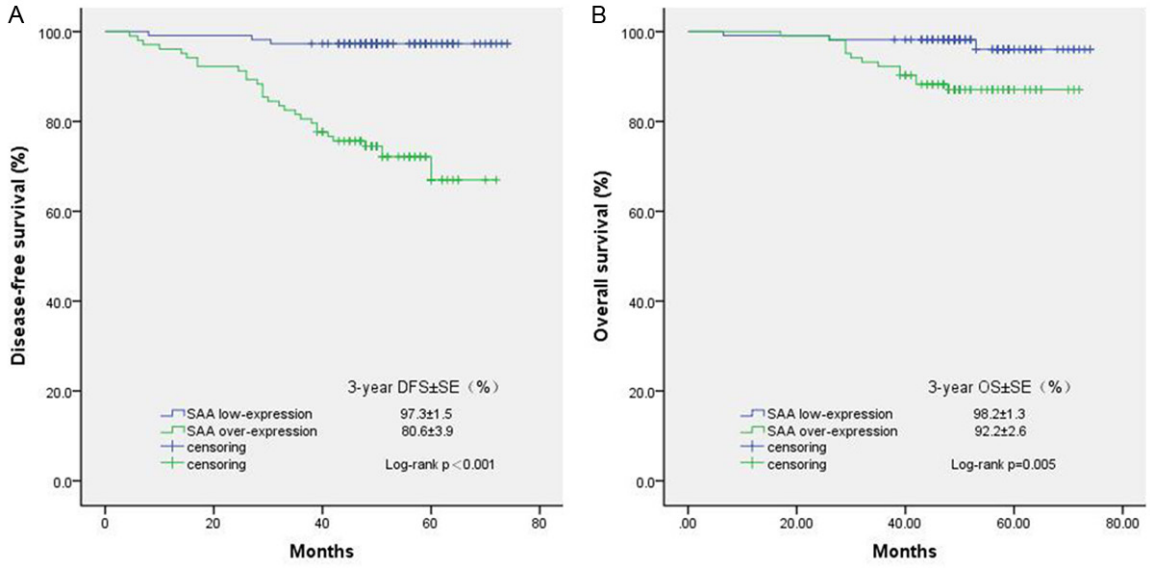

Figure 6. Analysis of DFS and OS by SAA stage. DFS, disease-free survival; OS, overall survival; SE, standard error.

dition for cancer development. Tumor microenvironment is mostly orchestrated by inflammatory cells, which facilitates cancer cell proliferation, survival, and migration [3, 31]. Thus, it was hypothesized that SAA expression in cancer tissue may act in an autocrine or intracrine manner [24]. Our study findings are supportive of this hypothesis.

However, the mechanism by which SAA promotes cancer progression and metastasis has not been well established. It was predicted that SAA might be involved in tumor invasion through extracellular matrix (ECM) alterations by stimulating the matrix MMP production [11]. The MMPs are a class of zinc-dependent proteolytic enzymes that degrade the ECM and basement membrane. Biologically, MMPs are important factors in tissue remodeling and angiogenesis [32]. MMPs are also involved in cancer progression, invasion, and metastasis [33, 34]. Based on their structure and substrate specificity, MMPs are categorized into five groups [35], among which MMP-2 and MMP-9 are two of the most widely studied in cancer. It has been reported that the expression of MMP-2 and MMP-9 were upregulated in breast cancer [36], and the increased MMP expression is commonly correlated with poor prognosis [37, 38]. Several reports suggested a possible link between SAA expression and MMPs. In patients with rheumatoid arthritis, SAA stimulated the MMP-2 and MMP-3 expression in a dose-dependent manner [39], and recombinant SAA (rSAA) induced MMP-1 on human microvascular endothelial cells (HMECs) [36] and MMP-9 in THP-1 cells [40], respectively. However, the interaction between SAA and MMPs in cancer has not been extensively studied. In our study, expression of MMP-2 and MMP-9 was significantly enhanced by SAA in the MCF-7 cells, in a dose- and time-dependent manner. Such inducing effects were also reported in lung cancer [23] and glioma cell lines [24]. Taken together, it is reasonable to hypothesize that SAA promotes breast cancer progression and metastasis through the activation of MMP-2 and MMP-9 pathways, but the detailed interaction pathways require further study.

Consistent with previous cell culture data, the analysis based on clinicopathologic features and follow-up showed a similar effect of SAA on breast cancer. In the present study, expression of SAA in breast cancer tissue was significantly stronger than that in benign breast diseases. Moreover, in breast cancer, SAA expression was also positively correlated with tumor T stages and lymph node metastasis. Because of the limited studies on SAA expression in tumor tissue, we compared our results with studies focused on SAA. Studies by O'hanlo [4] and Zhang [16] showed that breast cancer patients with more advanced tumors (T4 and T2/3/4

respectively) presented higher SAA levels, but there were no differences between T1 tumors and benign breast diseases in both studies. On the contrary, Santana [41] showed no association between SAA and tumor stages in postmenopausal breast cancer patients. The above results may indicate a poor value of SAA in breast cancer detection or differentiation. Regarding metastasic disease, similar to Zhang [16], breast cancer patients with lymph node metastasis or distant metastasis were found to have significantly higher SAA concentration. In our study, only 31 patients (14.4%) experienced distant metastasis or local recurrence in the median 49-month follow-up. Considering the apparently unbalanced data, the association between SAA expression with distant metastasis or local recurrence was not analyzed.

In this study, SAA expression was also positively associated with BMI. The correlation between SAA levels and obesity has been discussed extensively [42]. The meta-analysis by Zhao [43] confirmed that SAA levels are positively associated with BMI levels while weight loss is associated with decreased SAA levels. However, the interaction between SAA and obesity is not well understood, and some hypotheses have been proposed. Inflammation and secretion of SAA and other inflammatory proteins are stimulated, and consequently, adipocytes are led to a chronic inflammatory state, resulting in obesity [44, 45]. Conversely, SAA itself is an important inflammatory adipokine produced by adipocytes [9, 46]. Thus, a two-way interaction and vicious circle between SAA and obesity may exist. With respect to breast cancer, similarly to this study, Santana [41] showed that SAA concentration was higher in overweight or obese postmenopausal breast cancer patients and this condition was dependent on obesity. Considering the fact that obesity is one of the most well-known risk factors for breast cancer and that SAA could promote cancer development, it is reasonable to postulate that obesity may contribute to breast cancer progression partly via the interaction with SAA. From this perspective, the increased SAA expression may be more a result of circulation stimulation rather than the primary production by the tumor itself.

What is interesting is that compared to premenopausal patients, postmenopausal breast cancer patients showed significantly higher local SAA expression, which is not easy to explain. Lee and his colleague [47] reported that in healthy subjects, an increasing SAA level was observed during the menopausal transition as a result of increased central adiposity. This conclusion is based on the evidence that SAA expression is higher in subcutaneous white adipose tissue than in the visceral adipose tissue [48]. Additionally, aging was also considered to be an inflammatory trigger. Dysregulation of cytokine response owing to a lifetime antigen exposure or a decrease in the production of sex hormones may also contribute to the different levels of expression of SAA and other inflammatory molecules [49].

No relationship between SAA expression and other clinicopathologic features, including ER, PR, and Her-2 status, was observed, which may indicate that SAA is more involved in cancer progression rather than carcinogenesis. It should be noted that this result is not in agreement with the results of Pierce [17] and Santana [41], both of which showed higher SAA levels in ER negative subtypes. Because of the relatively limited studies, the association between ER/PR and SAA should be verified in larger samples, or different levels between serum and tissues.

The most important and valuable result of the present study is that increased local SAA expression is associated with reduced DFS. Further, multivariate Cox regression analysis showed that this association was independent of T stage, lymph node metastasis, and BMI level. The findings imply a prognostic value of local SAA expression in breast cancer progression. In fact, Paret [12] also reported previously that expression of SAA1 protein in tumor cells correlated with poor course-specific survival in conventional renal cell carcinomas. Regarding breast cancer, Pierce [17] showed that elevated concentrations of SAA were significantly associated with reduced OS and DFS and were independent of race, tumor stage, and BMI. However, in that study, both the collection and measurement of SAA were carried out approximately 31 months after the diagnosis, which may fail to represent the real status at diagnosis. In another study by Santana [41], a possible role for SAA in the development and prognosis of postmenopausal obesity-related breast cancer was also been suggested, but the follow-up is still in course. Thus, this is the first

study to correlate local SAA expression status at diagnosis with breast cancer survival. However, there is a weaker relationship between SAA expression and OS. This may be attributed to the few deaths at the end of follow-up in our study, and further follow-up data are required.

In conclusion, our studies confirmed the increased SAA expression in both MCF-7 cell lines and breast cancer tissue. The strong association between SAA expression and DFS indicates a prognostic value of local SAA expression for breast cancer progression and risk assessment, which may result from its potential effects of inducing invasion. Further long-term studies of the relationship between SAA, local SAA, and prognosis of breast cancer are needed. Additionally, more *in vivo* mechanistic studies in both serum and tissue are needed to finally identify the complex interaction.

Acknowledgements

We thank Profs. Lei and Yao as well as Drs. Bo and Li for their critical comments on the draft of this report. We also thank the pathologist Xiaoqing Yang for performing the pathologic diagnoses. This research has been sponsored by the Youth Foundation of Beijing Tiantan Hospital, Capital Medical University, China (Grant No. 2015-YQN-09).

Disclosure of conflict of interest

None.

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