Role of serum-derived hyaluronan-associated protein-hyaluronan complex in ovarian cancer

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Abstract. The objective of this study was to determine if the level of serum hyaluronan (HA), serum-derived HA-associated protein (SHAP)-HA complex, and urinary trypsin inhibitor (UTI) correlate with the clinical outcome of ovarian cancer patients. The relationship of metalloproteinase and its inhibitor with HA and the SHAP-HA complex was also examined. Serum and urine samples were obtained from 45 patients with ovarian cancer, 22 patients with benign ovarian tumors and 50 healthy women. Concentrations of serum HA and UTI were measured by an inhibitory sandwich enzyme-linked immunosorbent assay, and concentrations of the serum SHAP-HA complex were measured by a sandwich enzyme-linked immunosorbent assay. Concentrations of MMP-2, MMP-9 and TIMP-1 were measured by a one-step enzyme immunoassay. The levels of HA, SHAP-HA complex, MMP-9 and TIMP-1 were higher in the ovarian cancer group than in the benign ovarian tumor group. In ovarian cancer patients, the levels of HA, SHAP-HA complex and MMP-9 were higher in the stage III/IV group than in the stage I/II group, and the levels of SHAP-HA complex, MMP-9 and TIMP-1 were higher in the non-responder group than in the responder group. The serum concentration of SHAP-HA complex had a significant correlation with HA, MMP-9 and TIMP-1 in ovarian cancer patients. The patients with elevated SHAP-HA complex had a shorter disease-free survival compared with those with normal levels of SHAP-HA complex. The multiple regression analysis revealed that SHAP-HA complex is the significant independent variable for progression-free survival. The elevated level of SHAP-HA complex may indicate the prognosis of recurrence and reflect the tumor metastasis associated with MMP-9 in ovarian cancer patients.

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Introduction

Epithelial ovarian cancer is the prime cause of cancer death among women with gynecological malignancies. Late stage at diagnosis is responsible for the high mortality rate for this cancer, as more than 70% of patients are diagnosed at an advanced stage. The main route of metastatic dissemination of epithelial ovarian cancer is by exfoliation of the tumor cells, which migrate, implant and invade throughout the peritoneal cavity. The molecular mechanisms underlying this process are not well characterized; but, it is likely that the interaction between the ovarian cancer cells and peritoneal mesothelium is mediated by specific adhesion molecules (1).

Hyaluronan (HA) is an extracellular polysaccharide typically present in the extracellular matrix of some epithelial and neural tissues. HA is particularly abundant in connective tissues. HA controls cell migration, differentiation and proliferation (2), thereby influencing tissue morphogenesis, wound healing and tumor growth (3,4). HA levels correlate with the invasiveness and metastatic capacity of tumor cells (5,6). Increased HA concentrations may help invasion by providing a less dense matrix for cancer cells (7), stimulating cancer cell motility, and forming an immunoprotective coat for cancer cells (8).

Associations with various HA-binding proteins (HABPs), including proteoglycans, result in tremendously diverse physiological functions for HA. Extracellular matrix containing HA as a major component, called HA-rich matrix, plays important roles in regulating cellular behavior in a variety of physiological and pathological processes via cell-surface HA receptors, such as CD44 and receptor for hyaluronan-mediated motility (RHAMM) (9-12).

Serum-derived HA-associated proteins (SHAPs) correspond to the heavy chains of plasma inter-α-trypsin inhibitor (ITI) family molecules and are bound to HA via a unique ester bond (13-15). ITI family molecules are synthesized by hepatocytes and secreted into the blood at high concentrations (16). The heavy chains of these molecules are derived from 3 different genes, and either 1 or 2 of the chains are covalently bound to the light chain, bikunin, to form ITI family members such as ITI, pre-α-trypsin inhibitor, and inter-α-trypsin-like inhibitor (17). During the formation of SHAP-HA

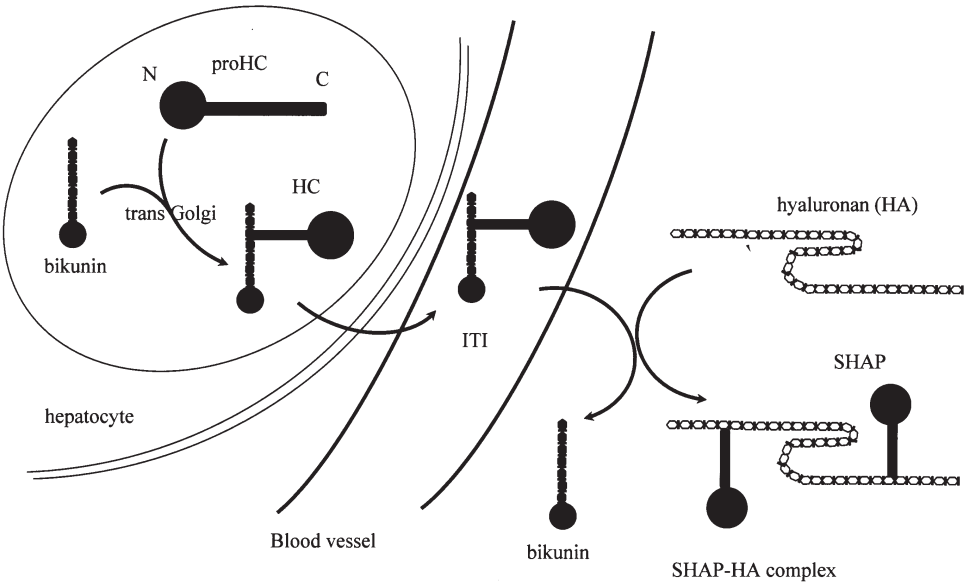

Figure 1. Schematic representation of the synthesis of the SHAP-HA complex. The biosynthesis of inter-α-trypsin inhibitor (ITI) occurs in hepatocytes. The heavy chains (HC) are first synthesized in their precursor forms. Then, in the trans Golgi, they are coupled to the chondroitin sulfate chain of bikunin, accompanied by the removal of the C-terminal extensions. The HC-bikunin complexes are circulating in the blood at high concentrations. When necessary, they are recruited to local sites and the heavy chain moieties were transferred to hyaluronan (HA) to form the SHAP-HA complex. The released bikunin is rapidly excreted into the urine, where it is also widely known as the urinary trypsin inhibitor.

complexes, HA is substituted for the chondroitin sulfate chain of bikunin, accompanied by the release of bikunin (Fig. 1) (14,18). Released bikunin is excreted in urine as urinary trypsin inhibitor (UTI). The SHAP-HA complex was originally discovered in HA-rich matrix from cultured mouse dermal fibroblasts, and SHAP was found to be derived from serum supplemented to culture media (13,15).

Matrix metalloproteases (MMPs) play a crucial role in tissue remodeling in a variety of physiological and pathological processes, similar to the roles of HA. In ovarian cancer, high levels of HA and MMP-9 in tumor stroma predict poor survival, and HA may possibly influence the expression of MMP-9 as well as the conversion of the inactive pro-forms to active forms (19,20).

The goal of the present study was to determine if the levels of serum HA, the SHAP-HA complex and UTI correlate with the clinical outcome of ovarian cancer patients. The relationship of metalloproteinase and its inhibitor, tissue inhibitor of metalloproteinases 1 (TIMP1), with HA and the SHAP-HA complex was also examined.

Materials and methods

Serum and urine were obtained from 45 patients with ovarian cancer, 22 patients with benign ovarian tumors and 50 healthy women. All patients attended the gynecology clinic at Aichi Medical University Hospital from June 1995 to December 2004. A laparotomy and pathological diagnosis were performed in all cases of ovarian cancer and benign ovarian tumors. The study was approved by the regional ethics committee of Aichi Medical University School of Medicine. Written informed consent was obtained from all participants prior to study enrollment. Of the 45 ovarian cancer patients, 19 had stage I disease, 6 had stage II disease, 16 had stage III disease, and 4 had stage IV disease. Nineteen patients had serous adenocarcinoma, 1 had mucinous adenocarcinoma, 6 had endometrioid adenocarcinoma, 16 had clear cell adenocarcinoma, 1 had carcinosarcoma, 1 had squamous cell carcinoma and 1 had undifferentiated adenocarcinoma (Table I). After laparotomy, 25 patients received 4 cycles of adjuvant chemotherapy with paclitaxel and carboplatin, and 20 patients who had residual tumors received 6 cycles of remission-induction chemotherapy using carboplatin and paclitaxel or irinotecan. The observed progression-free survival time was longer than 24 months.

Concentrations of HA were measured using an inhibitory enzyme-linked immunosorbent assay (ELISA) (21). HA-bovine serum albumin (BSA) plates (Seikagaku Corp., Tokyo, Japan) were washed 3 times with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T). Then, 50 μl each of sample (diluted 1:5-10 with PBS-T), and 50 μl each of biotinylated HABP (0.5 μg/ml in 1% BSA/PBS-T) was applied to each well, and plates were incubated at 37˚C for 1 h. After washing with PBS-T, 50 μl of horseradish peroxidase-streptavidin (1:500) was added to each well, and plates were further incubated at 37˚C for 1 h. Color development was achieved by incubating with 50 μl of tetramethylbenzidine (TMB) solution at 37˚C for 10 min; then, the reaction was stopped using 50 μl of 1 M HCl. Absorbance at 450/630 nm was measured with an immuno Mini NI-2300 spectrophotometer. Assays were performed in triplicate.

Concentrations of SHAP-HA complex in serum were determined by measuring the amount of SHAPs using a sandwich ELISA (21). Microtiter plates were coated with HABP (4 μg/ml in 0.1 M sodium carbonate buffer; pH 9.5) at 4˚C for 15 h. Wells were washed twice with 200 μl of PBS, followed by blocking with 200 μl of 3% BSA in PBS-T at room temperature for 1 h. After washing the wells 3 times

Table I. Characteristics of 45 patients with ovarian cancer.

Age	57.9±11.7 (31-84)
Stage	
Ia	1
Ib	7
Ic	11
IIa	1
IIb	1
IIc	4
IIIa	2
IIIb	1
IIIc	13
IV	4
Histology	
Serous	19
Mucinous	1
Endometrioid	6
Clear cell	16
Others	3
Response	
CR	8
PR	6
NC	1
PD	5

CR, complete response; PR, partial response; NC, no change; PD, progressive disease.

with 200 μl of PBS-T, 50 μl each of sample (serum diluted 1:5-10 with PBS) in 1% BSA/PBS-T was added to each well. Then, plates were incubated at 37˚C for 1 h. After washing, 25 μl of rabbit anti-human ITI antibody (diluted 1:3000 with 1% BSA/PBS-T) and 25 μl of HRP-conjugated goat anti-rabbit immunoglobulins antibody (diluted 1:3000 with 1% BSA/PBS-T) was added to each well and incubated at 37˚C for 1 h. Wells were washed 3 times, then they were incubated with 50 μl each of TMB solution at 37˚C for 10 min. The reaction was stopped by the addition of 50 μl of 1 M HCl, and absorbance at 450/650 nm was measured. Assays were performed in triplicate.

Concentrations of UTI were measured using an inhibitory ELISA. Microtiter plates were coated with UTI (2 μg/ml in 0.1 M sodium carbonate buffer; pH 9.5) at 4˚C overnight and then blocked with 3% BSA as above. The plates were washed 3 times with PBS-T, then 50 μl of sample (urine diluted 1:5-10 with PBS) and 50 μl of anti-UTI antibodies were applied to each well and incubated at 37˚C for 1 h. Wells were washed, and 100 μl of HRP-conjugated goat anti-rabbit antibody was added to each well. Plates were then further incubated at 37˚C for 1 h. After washing with PBS-T, color development was achieved by incubation with 50 μl of TMB solution at 37˚C for 10 min, then the reaction was stopped using 50 μl of 1 M HCl. Absorbance at 450/650 nm was measured. Assays were performed in triplicate.

Concentrations of serum MMP-2, MMP-9 and TIPM-1 were measured with a one-step sandwich EIS system by using monoclonal antibodies against human MMP-2, MMP-9 and TIMP-1 (Fuji Chemical Co., Toyama, Japan) as reported previously (22-24). A specimen (10 μl) was mixed with 100 μl of 50 μg/l each antibody conjugated with HRP in 10 mM sodium phosphate buffer (pH 7.0) containing 10 g/l BSA, 10 nM EDTA and 0.1 M NaCl. A 100 μl aliquot of the mixture was transferred to each microplate well previously coated with each antibody. The plate was incubated for 60 min at room temperature and then washed 3 times with PBS. Color development was achieved by incubation with 100 μl of citric acid-sodium phosphate buffer containing phenylenediamine and hydrogen peroxide at room temperature for 20 min. The reaction was stopped with 100 μl of 1 M HCl, and the absorbance at 492 nm was measured. Assays were performed in triplicate.

The upper limits of normal levels were defined as the mean value plus 2 times the standard deviation of the values from the control group (50 healthy women). These upper limits of normal values were: 100 ng/ml for HA, 5 U/ml for SHAP-HA complex, 5 μg/ml for UTI, 1000 ng/ml for MMP-2, 500 ng/ml for MMP-9 and 200 ng/ml for TIMP-1.

Differences between categories were analyzed with the Student's t-test. Correlation was analyzed by using Pearson's correlation coefficient, and multivariate analysis was performed by using the multiple regression method. Progression-free survival was analyzed by the Kaplan-Meier method and log-rank test. P-values <0.05 were considered statistically significant.

Results

The levels of HA, SHAP-HA complex, MMP-9 and TIMP-1 were higher in the ovarian cancer group than in the benign ovarian tumor group (Table II). Among the 45 ovarian cancer patients, the levels of HA, SHAP-HA complex and MMP-9 were higher in the stage III/IV group than in the stage I/II group (Table III). However, no differences in the levels of HA, SHAP-HA complex, UTI, MMP-2, MMP-9 or TIMP-1 were found among the histological types (Table IV). In the 20 ovarian cancer patients treated with remission-inducing chemotherapy after tumor-reduction surgery, the levels of SHAP-HA complex, MMP-9 and TIMP-1 were higher in the non-responder group than in the responder group (Table V). The serum concentration of the SHAP-HA complex was significantly correlated with the levels of MMP-9, and TIMP-1 in the 45 ovarian cancer patients (Fig. 2). The patients with elevated levels of the SHAP-HA complex had a shorter disease-free survival compared with patients with normal levels of the SHAP-HA complex (Fig. 3). For HA, UTI, MMP-9 and TIMP-1, the positive group had also a shorter disease-free survival compared with the negative group, whereas no difference was found for MMP-2. Multiple regression analysis revealed that the level of the SHAP-HA complex is a significant independent variable that predicts a shorter progression-free survival (Table VI).

Table II. Serum HA, SHAP-HA complex, MMP-2, MMP-9, TIMP-1 and UTI in ovarian cancer patients and benign ovarian tumor patients.

Diagnosis	No.	HA (ng/ml)	SHAP-HA (U/ml)	UTI (μg/ml)	MMP-2 (ng/ml)	MMP-9 (ng/ml)	TIMP-1 (ng/ml)
Ovarian cancer	45	488.8±902.0	7.01±10.93	7.70±11.37	769.7±302.0	632.6±355.5	262.5±113.4
Benign ovarian tumor	22	44.6±28.6	2.19±2.29	3.65±5.77	818.1±164.4	419.1±248.3	156.6±25.0
P-value		0.0246	0.0456	ns	ns	0.0140	<0.0001

ns, non-significant.

Table III. Serum HA, SHAP-HA complex, MMP-2, MMP-9, TIMP-1 and UTI in stages I/II and III/IV ovarian cancer patients.

Stage	No.	HA (ng/ml)	SHAP-HA (U/ml)	UTI (μg/ml)	MMP-2 (ng/ml)	MMP-9 (ng/ml)	TIMP-1 (ng/ml)
I/II	25	218.8±251.9	4.10±4.51	4.81±5.83	792.9±343.3	487.9±330.9	249.4±131.6
III/IV	20	826.4±1260.2	10.63±15.02	11.30±15.22	740.6±246.4	813.4±303.9	278.9±86.2
P-value		0.0229	0.045	ns	ns	0.0015	ns

ns, non-significant.

Table IV. Serum HA, SHAP-HA complex, MMP-2, MMP-9, TIMP-1 and UTI in different histological types of ovarian cancer.

Histology	No.	HA (ng/ml)	SHAP-HA (U/ml)	UTI (μg/ml)	MMP-2 (ng/ml)	MMP-9 (ng/ml)	TIMP-1 (ng/ml)
Serous	19	357.9±387.9	4.85±7.28	6.92±10.02	768.3±292.8	598.3±288.1	261.5±111.8
Mucinous	1	106.7	1.26	2.3	356	390	198
Endometrioid	6	806.3±1065.5	11.74±23.22	3.90±1.20	732.5±279.6	613.3±352.9	262.7±96.3
Clear cell	16	265.8±289.1	7.02±6.38	2.88±10.32	808.5±314.9	663.6±449.9	269.9±138.4
Others	3	2000.2±2952.9	12.99±17.73	23.10±26.76	783.7±440.1	803.3±331.7	250.3±31.9

Table V. Serum HA, SHAP-HA complex, MMP-2, MMP-9, TIMP-1 and UTI in the responder group and non-responder group in ovarian cancer patients.

Response	No.	HA (ng/ml)	SHAP-HA (U/ml)	UTI (μg/ml)	MMP-2 (ng/ml)	MMP-9 (ng/ml)	TIMP-1 (ng/ml)
CR/PR	14	700.6±1387.2	5.78±8.45	11.42±15.64	765.6±270.6	703.4±265.3	251.4±63.3
NC/PD	6	1119.8±940.6	21.96±21.24	11.03±15.62	682.2±185.7	1070.0±234.7	343.0±103.6
P-value		ns	0.0227	ns	ns	0.0091	0.0248

[a]These patients were treated with chemotherapy after reduction surgery. CR, complete response; PR, partial response; NC, no change; PD, progressive disease; ns, non-significant.

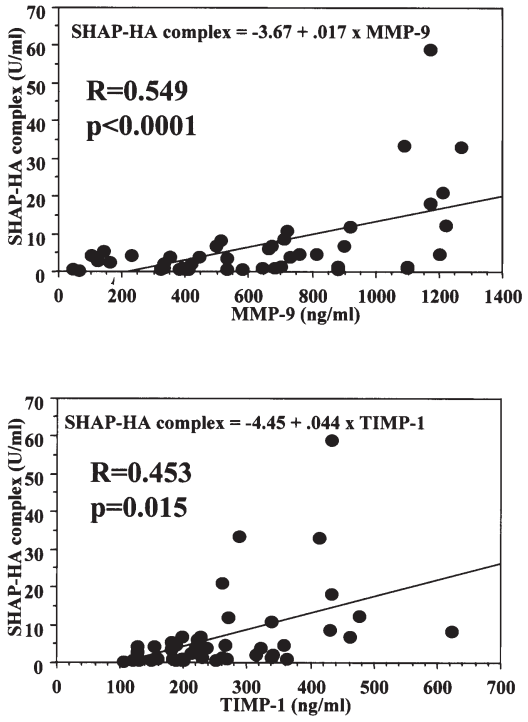

Figure 2. Correlations of serum SHAP-HA complex with MMP-9 and TIMP-1 in patients with ovarian cancer.

Table VI. Multiple regression analysis for progression-free survival in ovarian cancer patients.

Variables	Regression coefficient	Standard error	T-value	P-value
Intercept	79.34	13.02	6.093	<0.0001
Age	-0.251	0.19	-1.895	0.066
Stage	-0.15	2.19	-1.109	0.274
HA	0.114	0	0.667	0.508
SHAP-HA complex	-0.384	0.29	-2.094	0.043
UTI	-0.173	0.2	-1.305	0.2
MMP-2	-0.248	0	-1.79	0.081
MMP-9	-0.188	0	-1.117	0.271
TIMP-1	0.016	0.02	0.098	0.922

Discussion

The multistage process of tumor invasion and metastasis depends on several mechanisms, including stimulation of cell growth by growth factors, destruction of the extracellular matrix by proteolytic enzymes (25), neovascularization due to the presence of angiogenic factors (26,27), and cell-to-cell or stromal adhesion regulated by cell adhesion molecules.

Significantly increased levels of HA are often associated with certain types of human tumors, and the levels of HA in the serum of some cancer patients are significantly greater than those of normal individuals (7,28-30). Although increased HA synthesis is not a universal characteristic of tumors, there seems to be an overall tendency for transformed cells to exhibit higher levels of HA production (31-33). In addition, a close relationship has been demonstrated between HA production and malignant phenotype, such as invasiveness (34). Our and another group found that highly metastatic cell lines release more HA into culture medium than less metastatic variants (5,6). Furthermore, Zhang *et al* (4) reported that HA on the surface of tumor cells is correlated with metastatic behavior.

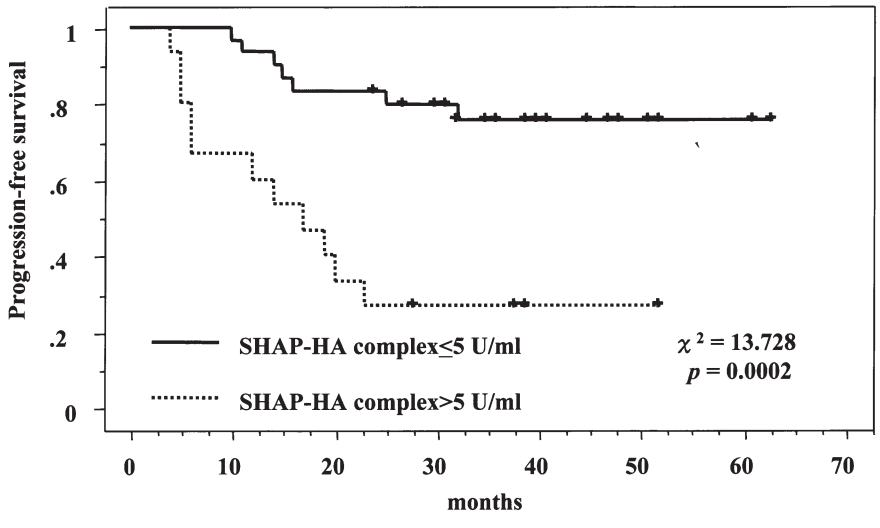

Figure 3. Progression-free survival in ovarian cancer patients was compared between 15 cases with positive SHAP-HA complex levels and 30 cases with negative SHAP-HA complex levels.

HA has either directly or indirectly been implicated in cell adhesion, motility, growth and differentiation (35). HA-binding proteins regulate cellular behavior by interacting with HA and forming the HA pericellular matrix (36). Increased matrix deposition of HA may favor tumor growth and invasion by increasing tissue hydration and providing a suitable environment for cell migration analogous to embryonic cell movement. Additional mechanisms may also help the HA matrix in favoring tumor growth. For example, the HA pericellular coat may reduce the access of immune cells to tumor cells (8). Tumor cells are surrounded by a thick pericellular coat that is sensitive to hyaluronidase. Removal of this coat may allow lymphocytes to exert their cytolytic effect on tumor cells. Additionally, partially degraded HA fragments promote angiogenesis, an important host contribution to tumor cell viability (37).

Our previous study (38) demonstrates that increased expression of HA synthase 1 (HAS1) in tumor cells is a significant prognostic parameter, independent of conventional factors related to tumor spreading at diagnosis and residual tumor size. Furthermore, HAS1 expression correlated with the microvessel density in the tumor, but it was unrelated to CD44 expression. Yamada *et al* (39) reported that elevated transcription levels of the HAS1 gene correlated with poor prognosis of human colon cancer. Anttila *et al* (40) reported that elevated levels of stromal HA indicate that the tumor is aggressive and predict poor disease outcome in ovarian cancer patients, especially those with serous tumors. Kayastha *et al* (41) reported that CD44 expression is associated with the spread of ovarian cancer and is an independent predictor of survival. HA contributes to tumor growth, invasion and metastasis through cell proliferation, movement, adhesion and angiogenesis. It is possible that these functions of HA depend on the overexpression of the HAS genes. The three subtypes of HAS may synthesize HAs with different biological functions. Our data suggest that HA synthesized by HAS1 is associated with tumor neovascularization and predicts tumor aggressiveness and patient survival in ovarian cancer (38).

In the present study, the levels of serum HA and the SHAP-HA complex were greater in the ovarian cancer group than in the benign ovarian tumor group. This finding shows that the synthesis of HA in ovarian cancer tissue is increased and that this HA reacts with ITI, resulting in increased levels of the SHAP-HA complex. In the ovarian cancer patients, the serum levels of HA and the SHAP-HA complex were greater in the stage III/IV group than in the stage I/II group, indicating that the increased levels of HA and the SHAP-HA complex are related to tumor invasion and metastasis. In the same way, the serum levels of MMP-9 and TIMP-1 were significantly greater in the ovarian cancer group than in the benign ovarian tumor group, and serum MMP-9 was elevated according to tumor progression. Thus, MMP-9 is associated with tumor invasion and metastasis in ovarian cancer. Moreover, the ovarian cancer patients with elevated levels of SHAP-HA complex had a shorter disease-free survival time compared to those patients with normal levels of SHAP-HA complex, and the multiple regression analysis revealed that the SHAP-HA complex is a significant independent variable predicting a shorter progression-free survival. These results suggest that the SHAP-HA complex is a useful marker to predict disease recurrence. Because the levels of the SHAP-HA complex showed a significant positive correlation with the levels of MMP-9 and TIMP-1, the SHAP-HA complex may promote the synthesis and activation of MMP-9 and TIMP-1 in ovarian cancer progression.

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