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Raised Serum Chondroitin Sulfate Epitope Level in Ovarian Epithelial Cancer

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Objective: To determine the value of serum chondroitin sulfate epitope WF6 and hyaluronan (HA) levels as a biomarker for early detection of ovarian epithelial cancer and other gynecological disorders. Method: Serum WF6 CS epitope and HA were measured in 91 patients with ovarian epithelial cancer, 39 patients with non-cancer gynecological disorders and 30 healthy women. Serum chondroitin sulfate (CS) WF6 epitope was determined by a competitive immunoassay with the monoclonal antibodies WF6, which specifically recognizes an epitope in native CS chains. In addition, serum HA concentration was measured by an ELISA-based assay with a biotinylated affinity HA-binding proteins. Results: The serum concentration of CS (WF6) epitope was highly increased in epithelial types of ovarian cancer and at all stages of development ($p < 0.005$). Serum HA in ovarian cancer patients was significantly higher than normal controls ($p < 0.05$). Conclusion: These results reflect changes in ECM metabolism in progressive ovarian cancer, which cause an increase in serum CS epitopes and HA. Therefore, serum CS epitopes may provide useful biomarkers for cancers and other disorders of the ovary. Measurement of serum HA provided complementary information, which may be useful as a discriminator between benign ovarian disorders and malignant ovarian diseases.

Key words: chondroitin sulfate epitopes, hyaluronan, ovarian epithelial cancer, serum biomarker.

Ovarian cancer is a major health threat to women over age of 35 and represents the fifth most common type of women's cancer and the second most common gynecological cancer (*1*, *2*). The high mortality rate of ovarian cancer is due to the clinical symptoms of this disease, which are often mild and nonspecific during its early stage: typically it goes undetected and untreated until it reaches an advanced stage (*3*). Initial clinical evaluation of the symptoms is limited by a lack of easily available laboratory tests or imaging modalities. Therefore simple, specific and sensitive methods are needed for early diagnosis and monitoring of this disease. Early detection will enable better clinical intervention and it would be a major help in improving patient's quality of life, morbidity and mortality.

Currently, a number of biomarkers involved in tissue remodeling have been identified as potentially useful in detecting ovarian cancers. Most of these consist of high-molecular weight glycoproteins such as CA125, CA15-3, carcinoembryonic antigen (CEA), prostasin, the kallikrein family (including hK13, hK10, hK 5, and hK6), osteopontin, lysophosphatidic acid (LPA), YKL-40 (glycoprotein in the chitinase protein family) (*4*, *5*). CA-125 was the first biomarker identified for ovarian cancer and it is approved for monitoring recurrence of disease. The serum prostasin, a serine protease, has been shown to be significantly higher in patients with ovarian cancer and could be useful in the detection of early stage disease. Similar to prostasin, the human kallikreins, which are secreted serine proteases (*6*) have been proposed to be involved in the progression and metastasis of human cancer (*7*–*9*). However, there are the conflicting reports in the success of detecting significant difference in serum levels of LPA in patients with ovarian cancer compared with matched controls (*10*). YKL-40 is reported to be expressed in pathological condition involving extracellular matrix (ECM) degradation (*11*) and recent studies have detected YKL-40 protein in serum in early stage (*12*) and in recurrent ovarian cancer (*13*).

Structurally, CSPGs consist of a core protein to which is attached one or more chondroitin sulfate (CS) chains, which are linear, sulfated glycosaminoglycans (GAGs). They have multiple roles in the normal physiology of animal connective tissues, such as regulating cell migration, cell recognition, growth factor binding and tissue morphogenesis. HA is a high molecular weight linear

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polysaccharide, which is a ubiquitous component of extracellular matrix and cell associated matrices in most body tissues. It is essential for growth and motility of both normal and transformed cells. There is a family of large CSPGs, which bind to HA forming large supramolecular assemblies and contribute to ECM properties. Animal studies have shown that HA is synthesized and turned over in the body at the rate of about 15g/day (*14*).

It has been reported that CSPGs and HA are overexpressed in pathological conditions involving the accumulation and turnover of extracellular matrix. Using various methods they have been detected a serum and shown to vary in rheumatoid arthritis (*15–17*), knee osteoarthritis (*18*), hepatic fibrosis (*19*, *20*) and cirrhosis (*21*). Many studies have also reported the elevation of CSPGs levels in tumors, compared with the non-malignant tissue in which the tumor originated. Some relationship has been proposed between tumor glycosaminoglycans and tumor-cell properties (*22–25*). Currently, there is evidence to show that CSPGs are synthesized by ovarian carcinoma cells and are involved in cell-matrix adhesion to interstitial matrix (*26*). In addition, several types of malignant ovarian tumor are reported to accumulate HA, which may be related to its role in the invasiveness of ovarian tumor cells (*27*). These observations have led us to speculate that increased metabolism and clearance of these matrix proteoglycans and HA in the circulation may provide potential markers for these types of cancers.

There is a previous study reporting an elevated level of HA in urine and serum correlated with advanced breast cancer (*28*). There have also been investigations of serum CSPG epitopes and HA in ovarian cancer. HA concentrations in body fluids were measured by He *et al.* (*29*), who reported an elevated level of HA in serum and urine in malignant ovarian tumors compared with benign ovarian tumors. There is also evidence that there are not only changes in CS expression in pathology, but also changes in the pattern of sulfation within chondroitin sulfate chains and these changes have been detected by antibodies recognizing specific epitope sequences in CS (*30*). In this study, we have therefore brought together sensitive analyses of serum HA and a CS epitope in patients with ovarian cancer and with non-cancer gynecological disorders. Assays were established for the investigation of HA by an ELISA-based assay with a biotinylated affinity probe specific for HA and CS epitopes were investigated with a novel monoclonal antibody (WF6), which recognizes a native epitope, which is variably expressed in chondroitin 6-sulfate chains (*31*).

MATERIALS AND METHODS

Cell culture and Antibodies Reagents—Hybridoma cells synthesizing mAb WF6, a mouse monoclonal antibody (IgM) raised against shark aggrecan (a CS-PG), were grown using a standard hybridoma technique as previously described (*31*, *32*).

Serum Specimens—All subjects gave their informed consent before participation. Blood samples from healthy women were collected at the Gynecology clinic, Maharaj Nakorn, Chiang Mai Hospital, (Department of Obstetrics and Gynecology, Faculty of Medicine, Chiang Mai University, Thailand). Patients were selected based on a diagnosis of ovarian cancer and were operated on between March 2003 and April 2004. Serum samples were collected from 30 healthy women with an average age of 46.8 years (range 19–70 years). Since CSPG epitopes and HA have been detected at increased levels in serum in arthritis and in liver diseases, healthy donors and patients provided a medical history and were given a physical examination to verify that they were free of articular, bone, liver, endocrine, or other chronic disorders.

We selected serum from 91 women with preoperative ovarian epithelial cancer and 39 women having benign gynecological disorders with an average age of 51 years (range 32–67 years) and 45 years (range 23–70 years), respectively. This cross sectional study included glynecological examination of 91 consecutive women with International Federation of Gynecology and Obstetrics stage 1 ($n = 46$), II ($n = 7$), III ($n = 23$), or IV ($n = 3$) of preoperative ovarian epithelial cancer. Histologically, 39 tumors were serous adenocarcinoma, 23 as clear cell carcinomas, 14 as endometrioid , 10 as mucinous adenocarcinoma, and 5 as mixed type carcinomas. All patients underwent the standard cytoreductive surgery and FIGO staging. Thirty-nine individuals were identified with benign gynecological conditions based on transvaginal sonogram and pathology reports were identified from the ovarian screening check-up program. Diagnoses included benign cystadenoma (10 patients), dermoid cyst (8 patients), Leiomyoma (12 patients), endometriosis (4 patients) and complex ovarian cysts (5 patients). These patients remained disease-free at 12 months' follow up. The study was approved by the Ethics Committee of Faculty of Medicine, Chiang Mai University.

Preparation of Shark Aggrecan and HA-Binding Proteins—Shark cartilage was dissected and the proteoglycan extracted with buffer containing 0.2 M Tris-HCl (pH 8.0), 4 M guanidine HCl, 10 mM EDTA, 10 mM 6-aminohexanoic acid, 10 mM *N*-ethylmaleimide, 2 mM PMSF and then dialyzed against distilled water. The high buoyant density (A_1) fraction was prepared by CsCl isopycnic centrifugation (*33*). After stirring overnight at 4°C, the solution was brought to a density of 1.7 g/ml with solid CsCl. A density gradient was established by centrifugation at 48,000 rpm at 4°C for 48 h (the first centrifugation). The gradient was partitioned into 5 fractions. The bottom fraction (A_1 fraction) contained most of the aggregated aggrecan and was pooled and subjected to a second centrifugation in the presence of 4M guanidine HCl, with an initial density of 1.6 g/ml. After being partitioned into 5 fractions, the disaggregated aggrecan in the bottom fraction (A_1D_1) was dialysed against water and freeze dried.

HA-binding proteins, HABPs comprising cartilage link protein and aggrecan G1 domain (*33*), were released from bovine articular cartilage by trypsin digestion and purified by HA-sepharose affinity chromatography (*34*). Proteins bound to HA-sepharose were eluted with 4 M GuHCl. HABPs were biotinylated as described by Rappuoli (1981) (*35*). HABPs were dissolved in 0.1 M $NaHCO_3$ buffer pH 9.6 and mixed with 5 ml of HA-Sepharose and left overnight at 4°C. At this stage HABPs were re-associated with HA-sepharose in order to protect the functional binding sites. The HABP was mixed with *N*-hydroxysucinimido–biotin at ratio 3:1 (w/w) and incubated overnight with agitation at room temperature. The unbound biotin

was removed from the B-HABP gel filtration. The biotinylated HABPs (B-HABPs) was stored frozen (–20°C) in aliquots.

Competitive Immunoassay Using Monoclonal Antibody WF6—A mouse monoclonal antibody WF6 was raised against a shark cartilage aggrecan preparation (*31*) and a quantitative ELISA for the epitope recognized by monoclonal antibody WF6 was modified from a previous study (*32*). The antibody was specific for intact chondroitin sulfate chains and showed no interaction with other sulfated glycosaminoglycans, hyaluronan or other polyanions, such as DNA, RNA or dextran sulfate (*31*). The standard used in the assay was shark cartilage aggrecan (A_1D_1 fraction) at concentrations 19–10,000 ng/ml in 6% BSA in TE buffer (0.1 M Tris HCl, pH 7.4 containing 0.15 M sodium chloride, 0.1% Tween 20 and 0.1% BSA). Diluted human serum samples (1:5 in 6% BSA-TE) were added to 1.5 ml plastic tubes containing an equal volume of WF6 (cell culture supernatant, 1:200 dilution in TE buffer). They were incubated at 37°C for 1 h, and then added to the microtitre plate, which was pre- coated with shark aggrecan (A_1 fraction). Non-specific protein binding was blocked with BSA. The plates were incubated at 37°C for 1 h., and the wells were then washed and peroxidase-conjugated anti–mouse IgM antibody (1:2,000) was added (100 μl/well; in TE buffer). The bound conjugate was detected by adding *o*-PD (*ortho*-phenylenediamine) substrate (100 μl/well in 0.05 M citrate buffer, pH 5.0). The reaction was stopped after 10 minutes with 50 μl/well of 4 M sulfuric acid and the absorbance was determined using a microplate reader at 492/690 nm. The concentration of WF6 epitope in supernatant samples was calculated by reference to a standard curve.

ELISA-Based Assay for HA Using Biotinylated HA-Binding Proteins—Human serum samples or standard HA (Healon^R) at various concentrations (19–10,000 ng/ml in 6% BSA-PBS pH 7.4), were added to 1.5 ml plastic tubes containing biotinylated HABPs prepared as described above (1:200 in 0.05 M Tris-HCl buffer, pH 8.6). The tubes were incubated at room temperature for 1 h., and then samples were added to the microplate, which was precoated with umbilical cord HA (100 μl/well of 10 μg/ml) and blocked with 1% BSA (150 μl/well). The plate was then incubated at room temperature for 1 h. The wells were then washed and peroxidase-conjugated anti-biotin antibody (1:2,000 dilution), 100 μl/well in PBS was added. The plate was incubated at room temperature for another hour. The detection of conjugated antibody was with *o*-PD substrate and plate reading was carried out as described above. The concentration of HA in samples was calculated from the standard curve.

CA 125 Serum Analysis—CA125 serum testing was performed in the clinical chemistry laboratory of Maharaj Nakorn Chiang Mai Hospital, on an Immuno 1 analyzer from Bayer Diagnostics. For data analysis, the upper limit of normal for CA 125 (*36*) was defined as 35 U/ml.

Statistical Analysis—Concentration of analytes was determined by reference to a standard curve, using genesis software using the absorbance values falling between 40–50% inhibition of the standard curve. The ELISA data were analyzed using the statistical program SPSS.

To analyze the data, we divided patients into different groups according to clinical and pathologic parameters. The Mann-Whitney U-test was used for comparison of different serum components. In all cases, p values less than 0.05 were considered significant. All results compared are from assays performed at the same time and carried out at least in triplicate. A receiver-operating characteristic (ROC) curve was constructed to establish the diagnostic cut-off level of serum HA and WF6 epitope in order to discriminate ovarian cancer group from other groups (*37*). Sensitivity, specificity and accuracy were calculated in accordance with standard methods (*38*).The statistic significance of the relative accuracy of WF6 epitope versus that of either HA or CA125 in detecting cancer among subgroups of patients was based on cases where two tests gave discrepant results using Wilcoxon Sign Rank Test.

RESULTS

Serum CS Epitope in Ovarian Epithelial Cancer and Gynecological Disorder—We investigated native CS epitope (WF6) in serum samples from 30 healthy women (normal), 91 women with preoperative ovarian epithelial cancer and from a further group of similar age with non-cancerous gynecological disorders. As depicted in Table 2, the range of WF6 epitope levels in the normal patients was 14.5 to 527.8 ng/ml. The mean and median WF6 epitope values was 106.1 ng/ml and 63.4 ng/ml, respectively. The upper limit of normal for WF6 epitope in this group of normal individuals was defined as 351 ng/ml, base on the mean value plus two standard deviations (95% CI). Thus, an abnormal WF6 epitope serum level was determined to be $\geqslant$351 ng/ml. Two of 30 individuals had WF6 value $\geqslant$351 ng/ml; These values were 361 and 527.78 ng/ml.

The serum WF6 epitope in healthy women was 106 ± 123 ng/ml. In contrast in patients with ovarian cancer and benign gynecological disorders, the serum levels of WF6 were much higher, 4,969 ± 3,204 ng/ml and 1,675 ± 673 ng/ml, respectively (Table 1). As illustrated in Fig. 1, preoperative WF6 epitope levels were significantly higher in ovarian epithelial cancer patients ($p < 0.001$) relative to both normal controls and individual with benign gynecological disorders. Patients with stage I tumors had preoperative serum WF6 epitope levels approximately 41 times higher than normal controls.

The optimal cutoff values to maximize the discriminating power of WF6 epitope, between patients with ovarian cancer and the other groups (benign gynecological disorders and healthy women) were determined using a ROC analysis (*37*). For WF6 the cut off value was 851.74 ng/ml, the sensitivity was 75.8%, and the specificity 59.4%. The area under the curve for WF6 was 0.736 [95% confidence interval (CI) 0.657–0.816]. The analysis of serum CA 125 in all normal women and non-cancerous gynecological disorders patients was less than 35 U/ml and as expected, it was elevated in ovarian epithelial cancer patients (402 ± 453 U/ml).

These results indicate that WF6 epitope was markedly increased in serum in women with ovarian cancer and with benign gynecological disorders. In each case the difference was highly significant compared to sera of healthy controls.

Serum HA Level in Ovarian Epithelial Cancer Patients—Serum HA was determined using a biotinylated

affinity probe specific for HA in an ELISA-based assay. As depicted in Table 2, the range of HA levels in the normal patients was 20.6 to 360.8 ng/ml. The mean and median HA values was 118.5 ng/ml and 94.3 ng/ml, respectively. The upper limit of normal for WF6 epitope in this group of normal individuals was defined as 297 ng/ml, base on the mean value plus two standard deviations (95% CI). Thus, an abnormal WF6 epitope serum level was determined to be ⩾297 ng/ml. Three of 30 individuals had HA value ⩾297 ng/ml; These values were 298.1, 341.93 and 360.78 ng/ml.

Results showed that serum HA of ovarian epithelial cancer patients (242 ± 52 ng/ml) was markedly elevated (~2-fold, $p = 0.05$) above normal (119 ± 89 ng/ml), and non-cancerous gynecological disorders the serum HA (104 ± 138 ng/ml) was much lower than in women with ovarian cancer, ($p < 0.05$) and was not significantly different from normal (Fig. 1).

In order to discriminate ovarian cancer from other groups with an optimal accuracy, an analysis of ROC curve was performed (Fig. 2). The area under the curve of serum HA of ovarian cancer and the other 2 groups (non-cancerous gynecological disorders and control) were 0.611 [95% confidence interval (CI) 0.525–0.698]. This result indicated that 77% of randomly selected patients with ovarian cancer would have a higher HA value than a patient randomly selected from the other groups. Base on the ROC curve analysis, the cut-off point for the serum HA concentration to achieve the highest accuracy for the diagnosis of ovarian cancer was 76.4 ng/ml. At this concentration, the sensitivity and specificity for differentiating between ovarian cancer and other groups in serum HA were 57.1% and 55.1% respectively.

Serum CS Epitope Levels in Patients with Early Stage Ovarian Epithelial Cancer—In a retrospective study of 91 patients, the mean and median of serum WF6 epitope levels in stage I ovarian epithelial cancer were 3,698 ng/ml and 1,841 ng/ml, respectively (range, 23 to 18,200 ng/ml). Preoperative serum levels were elevated in 32 of 46 (70%) for WF6 epitope, 12 of 46 (25%) for HA and 13 of 46 (28%) for CA 125, in stage I ovarian epithelial cancer patients. The analysis of the CS epitope, WF6 epitope, therefore showed a strong potential to detect early stage ovarian epithelial cancer.

Serum WF6 Epitope Levels in Patients with Recurrent Ovarian Epithelial Cancer—The mean and median WF6 epitope levels in 26 advanced stage ovarian epithelial cancer patient were 3,616 ng/ml and 895 ng/ml (range 18 to 12,100 ng/ml), respectively. In 30 recurrent patients the mean and median WF6 epitope were 6,212 and 6,092 ng/ml (range, 56 to 16,200 ng/ml), respectively. Applying the cut off value of mean ± 2SD, the serum levels of WF6 epitope were elevated in patients 17 of 26 (68%) patients with advanced stage and in 27 of 30 (89%) patients with recurrent ovarian epithelial cancer. Serum HA levels

Table 1. **Serum WF6 epitope, hyaluronan and CA125 for normal, benign gynecological disorders and ovarian cancer patients.**

	Normal	Benign gynecological disease	Ovarian epithelial cancer
Number of patient	30	39	91
WF6 epitope (ng/ml)			
Mean	106.1	1,674.6**	4,969.1**,†
Median	63.4	1,468.2	3,845.9
Range	14.5–527.8	292.8–7,530.7	27.4–11,200
One SD	122.5	673.4	3,204.4
Mean + 2SDs	351	3,021	11,378
Hyaluronan (ng/ml)			
Mean	118.5	104.1	241.5*
Median	94.3	31.08	730.1
Range	20.6–360.8	13.2–507.3	19.7–5,087.9
One SD	89.1	137.9	51.6
Mean + 2SDs	297	379	345
CA125 (U/ml)			
Mean	16	NP	401.5
Median	15.5	NP	25
Range	5–35	NP	5–4,227

Abbreviations: SD, standard deviation; NP, not performed. *, ** P <0.05 , <0.001 for ovarian epithelial cancer or benign gynecologic disease versus normal. † $P < 0.001$ for ovarian epithelial cancer versus benign gynecologic disease.

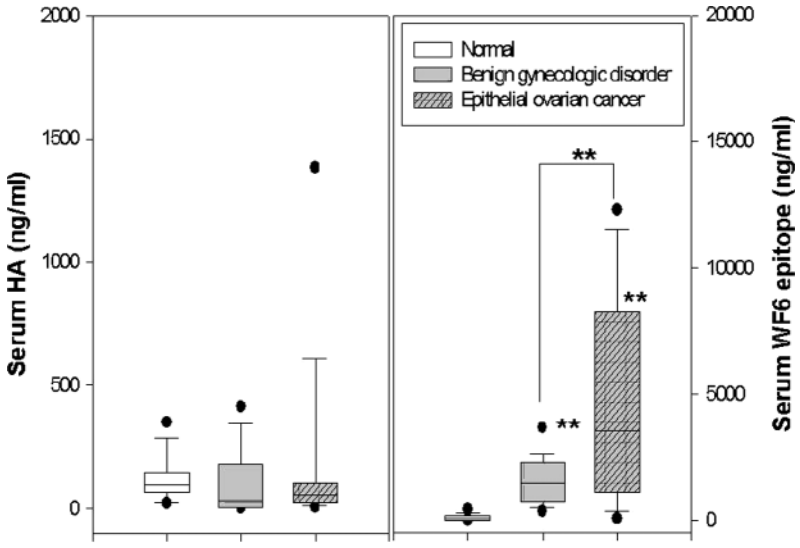

Fig. 1. **Serum concentration of the biomarkers WF6 epitope and hyaluronan in ovarian disease.** Comparison of healthy women ($n = 30$), women with ovarian epithelial cancer ($n = 91$) and women with gynecological disorder with non-ovarian cancer ($n = 39$). Boxes represent median and the interquartile range, between 5th and 95th quartile with error bars. Statistically significant difference ($p < 0.05$ and $p < 0.001$ shown with an asterisk and double asterisks, respectively) relative to the median of the healthy women.

were elevated in 11 of 26 (38%) patients with advanced and recurrent ovarian epithelial cancer and in 12 of 30 (48%) patients with stage III/IV and recurrent tumors. Serum level of CA 125 were elevated in 11 of 26 (43%) patients with advance and recurrent ovarian cancer and 22 of 30 (73%) patients with stage III/IV and recurrent tumors.

Frequency of Serum WF6 Epitope Values in Ovarian Epithelial Cancer Patients as a Function of Tumor Stage and Histology—Analysis in Table 3 shows the frequency of elevation of preoperative serum level of WF6 epitope, HA and CA125 in patient who were subsequently diagnosed as having primary ovarian, fallopian tube, or peritoneal cancer on surgical pathologic review. This table further delineates the frequency of elevation of these three serum markers, taking into consideration primary diseases site, stage and histology. The number of patients with elevated serum WF6 epitope values was higher than that for HA and CA125 in all groups, regardless of these variables.

The serum levels of WF6 epitope were elevated across all stages of tumor (stage I v II v III/IV by Mann-Whitney U-test; $p < 0.005$) (Fig. 3). In addition, patients with recurrent tumors had higher values of serum WF6 epitope than patients with any stage of newly diagnosed tumors.

Table 2. **Ovarian epithelial cancer patient demographics.**

	No. of patients	%
Age, year		
Median	46.8	
Range	19–70	
Primary disease site		
Ovarian	91	100
Stage		
I	40	44
II	7	8
III	23	25
IV	3	3
Recurrent	18	20
Histological diagnosis		
Serous	39	44
Clear cell	23	25
Endometrioid	14	15
Mucinous	10	11
Mixed type	5	5
Patient status		
NED	70	65
Recurred	37	34
Living	99	91
Death	1	1
Unknown	2	2

DISCUSSION

Ovarian cancer is a leading cause of death from gynecological malignancies. In general, the mortality is made worse by late diagnosis. This is because the disease is clinically silent in the early stages and symptoms appear only late in the disease process. There is therefore an important need for improved methods for early diagnosis, for monitoring subsequent therapy, and for predicting outcomes. Ovarian cancer is classified as a solid neoplasm, which depends on an extracellular matrix for physical coherence and biochemical support; it also requires vascular channels (*39*). Dynamic turnover of the extracellular matrix is undoubtedly a major factor in the processes of tissue invasion and metastasis. This may be accompanied by altered patterns of CS sulfation, which have been identified in other examples of pathology involving extracellular matrix remodelling (*40*) and these frequently also include increased turnover of HA (*14–17*, *20*, *21*, *28*, *29*). The changes in GAG detected in serum are therefore likely to reflect essential aspects of tumor biology and tissue invasion (*41*). These ECM components are not specific to ovarian tissues and the circulating levels in serum are likely to be affected by other disease processes with active tissue turnover and with disease which compromised liver or kidney function (*42*). We have detected high CS epitope WF6 in osteoarthritis and rheumatoid arthritis (*32*), although the levels were not as elevated as in the ovarian cancers reported here. The application of these analyses

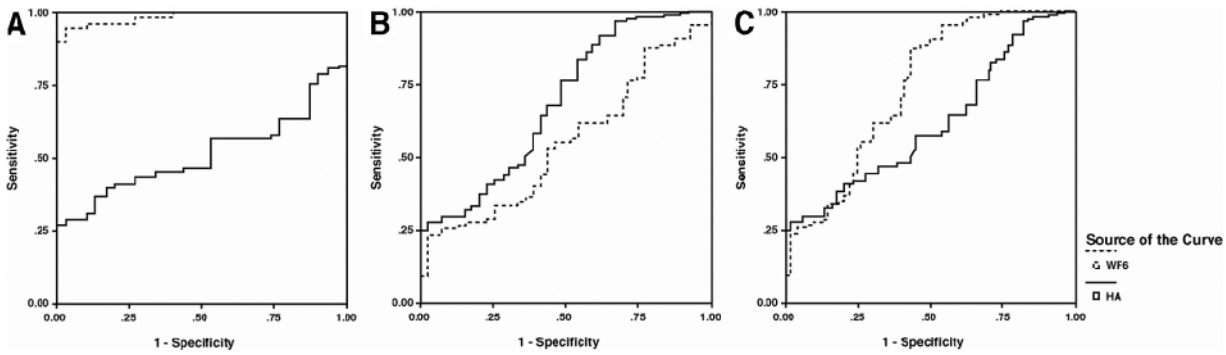

Fig. 2. **The ROC curve of serum biomarkers distinguishing between: (A) the healthy group and the ovarian cancer group; (B) the gynecological disorder with the non-ovarian cancer group; (C) the combined ovarian cancer plus the gynecological disorder groups with the non-ovarian cancer group.** For serum HA, the area under the curve (Curve C, solid line) is 0.611 (0.525–698), $p = 0.016$. A cut-off point considered as the highest accuracy for diagnosing ovarian cancer is 76.4 ng/ml, with the sensitivity and specificity of 57.1% and 55.1%, respectively. For serum WF6 epitope, the area under the curve (Curve C, dash line) is 0.739 (0.657–0.816) A cut-off point considered as the highest accuracy for diagnosing ovarian cancer is 851.74 ng/ml, with the sensitivity and specificity of 57.8% and 59.4%, respectively.

Table 3. **Preoperative serum WF6 epitope, HA and CA 125 values for ovarian epithelial cancer patients.**

	Elevated WF6 epitope (≥351 ng/ml)		Elevated HA (≥297 ng/ml)		Elevated CA 125 (>35 U/ml)		*P* value*		
	No. of patients	%	No. of patients	%	No. of patients	%	WF6 epitope *vs.* CA125	HA *vs.* CA125	WF6 epitope *vs.* HA
Total	81/91	88	26/91	28	39/91	43	0.000	0.000	0.000
Stage									
I	34/40	85	12/40	30	13/40	33	0.000	0.000	0.000
II	2/8	25	1/8	13	1/8	13	0.018	0.043	0.000
III/IV	22/26	85	12/26	46	11/26	42	0.000	0.000	0.000
Recurrent	13/18	72	4/18	22	12/18	66	0.000	0.000	0.000
Histological diagnosis									
Serous	32/39	82	5/39	13	6/39	15	0.000	0.001	0.000
Clear cell	20/23	87	2/23	9	13/23	57	0.000	0.407	0.000
	13/14	93	3/14	21	8/14	57	0.001	0.470	0.001
Endometrioid									
Mucinous	10/10	100	5/10	50	3/10	30	0.001	0.015	0.078
Mixed type†	5/5	100	4/5	80	2/5	40	0.043	0.080	0.50

*Based on Wilcoxon Signed Ranks test. †Mixed type: Mixed histology (*eg.* Clear cell/papillary serous, adenocarcinoma, poorly differentiation, papillary serous/endometrioid).

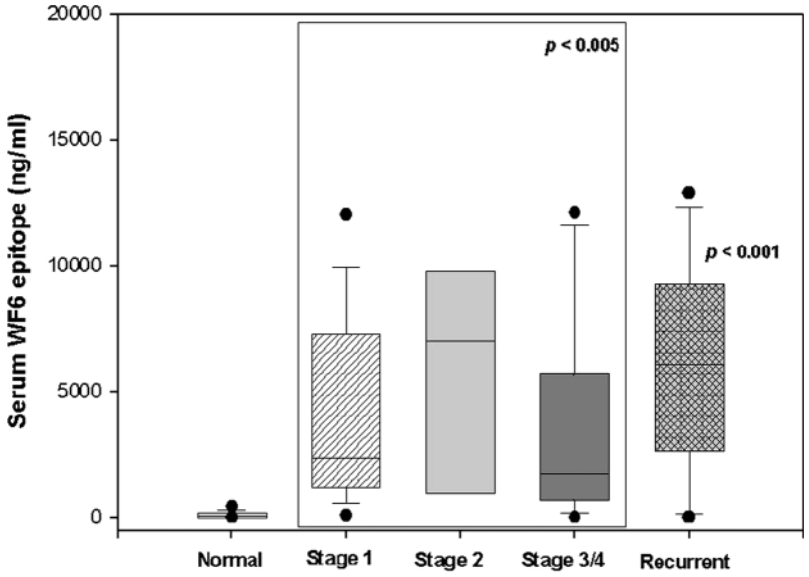

Fig. 3. **Serum concentration of the biomarkers in patients with early and late stage ovarian epithelial cancers.** Comparison of the value obtained from healthy women (n = 30), stage I (n = 46), stage II (n = 7), late stage; stage III-IV (n = 26) and recurrent (n = 30). Boxes represent median and the interquartile range, between 5th and 95th quartile with error bars.

would therefore be most appropriate in those patients, who are clear of other active disease.

Many previous studies have described ECM markers in ovarian cancer tissue with synthesis and accumulation of CSPGs (*26*) and HA (*43*), which mediate cell adhesion, cell motility and metastasis. In contrast, there are few reports describing these components in the circulation (or body fluids) for possible use as biomarkers (*28*, *29*). In the present study we investigated a specific serum CS epitope WF6 as a biomarker in patients with ovarian epithelial cancer and non-cancer gynecological disorders. The antibody WF6 is highly specific for intact chondroitin sulfate and shows no interaction with other sulfated glycosaminoglycans (*31*). It recognizes an epitope composed of a pattern of sulfation found in CS chains and the expression of this epitope varies with tissue source and in pathology (*31*). The appearance of WF6 on CS in serum will depend on factors affecting its expression in tissues and its clearance into the circulation. The initial results of serum analysis in this study showed there were interesting changes in its concentration that were associated with pathology. Analysis of serum WF6 epitope reliably distinguished normal individuals from patients with benign gynecologic disorder and ovarian epithelial cancer (both, $p < 0.001$). Interestingly, this marker also distinguished patients with benign gynecological disorder from those with ovarian epithelial cancer ($p < 0.001$). Strikingly, preoperative serum WF6 epitope levels were elevated in 88% of all patients with ovarian epithelial cancer. By contrast, 28% and 43% of patients had elevated serum HA and CA125 values.

In this study, elevated serum WF6 epitope levels were detected in stage I of ovarian epithelial cancer patients 85% of the time, compare with 30% and 33% for HA and CA125, respectively. In addition, WF6 epitope also reliably predicted advanced-stage and recurrent ovarian epithelial cancer. Preoperative serum WF6 epitope levels increased in stage III/V ($p < 0.005$) and recurrent ($p < 0.001$) ovarian epithelial cancer patients. Importantly, WF6 epitope

predicted the presence of ovarian cancer regardless of the histological subtype as it was reliably elevated in serous, mucinous, endometrioid and clear cell tumors.

The accumulation of CS-PG especially chondroitin 6-sulfate PGs, which are likely to be rich in WF6 epitope, may be synthesized by the tumor cells themselves and may be correlated with previous immunohistochemical localization with mAb 3B3 (anti-C6S epitope) of the ECM of ovarian cancer tumors (*23*). CSPGs have been identified on tumor cell surfaces; it has been suggested that they promote tumor invasion by reducing interaction of cells with the interstitial extracellular matrices (*44*), whilst permitting attachment to basement membrane. In other reports, the degradation of GAGs on the ovarian carcinoma cell showed that CS chains were components of cell surface PGs involved in mediating cell adhesion (*26*), cell motility and invasion (*41*). Ovarian cancer cells are thus likely to be significant producers of CSPGs and may contribute directly to elevated level of CS epitopes in serum. However, it will require further study to understand the cellular source and turnover of WF6 epitope and how its serum concentration varies with the different pathological processes associated with ovarian cancer.

In conclusion this study is the first report of serum CS epitopes and HA in patients with ovarian epithelial cancers and non-cancer ovarian diseases. We have identified WF6 epitope as a potential serum marker for the early detection of ovarian epithelial cancer. In addition, the results suggest that altered and/or elevated serum CS-epitope and HA levels may provide non-invasive methods for the screening and monitoring of disease in patients with ovarian epithelial cancer.

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