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Relationship of ovarian cancer tumour markers' concentration between local fluid and serum: comparison of malignant to benign condition

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Summary

Objective: The aim of the study was to evaluate the relationship of ovarian tumour markers osteopontin (sOPN) and splice variant 6 of sCD44 (sCD44-v6) between serum and local environment, represented by ascites or peritoneal fluid (PF) / peritoneal washing (PW), in patients with malignant and non-malignant conditions, respectively. *Materials and Methods:* The study included 33 women with advanced ovarian malignancy and 33 women with benign gynaecological conditions. Tumour markers concentrations were determined by flow cytometry. *Results:* Mean concentrations in ascites for both tumour markers were significantly higher than in PF or PW. Serum sOPN concentrations did not correlate with local fluid levels in either group and showed a tendency for retention in local fluid which was potentiated in malignant condition. Serum sCD44-v6 concentrations positively correlated with concentrations in ascites, PF, and PW. However, the magnitude of slope of the regression line was lower in malignant situation. *Conclusion:* Although concentrations of both potential biomarkers significantly increase in ascites produced by metastatic ovarian cancer, serum values are not a simple reflection of local environment changes.

Key words: Ovarian cancer; Tumour markers; sOPN; sCD44-v6; Serum; Peritoneal fluid.

Introduction

Ovarian cancer has an excellent prognosis, with a five-year survival rate exceeding 90% if diagnosed at an early stage. However, the vast majority are diagnosed in an advanced stage, when the survival rate is approximately 30% [1]. This is mostly due to the lack of highly sensitive and specific screening tools for detection of early-stage disease. The current annual screening modalities of bimanual examination with serum tumour marker cancer antigen 125 (CA125) and transvaginal ultrasonography together allow the detection of only 30-40% of woman with early stage ovarian cancer [2]. It has been suggested that combining multiple serum markers may help to achieve better sensitivity and the specificity required to detect ovarian cancer early, before any symptoms appear.

It has recently been found that osteopontin (OPN) is among ten genes differentially overexpressed in ovarian cancer cells, more than ten-fold compared to primary human ovarian surface epithelial cells [3]. A soluble form of OPN (sOPN) was included in a list of top promising blood tumour markers for early detection of disease [4]. Cells may bind OPN, among others, via splice variant 6 of CD44 (CD44-v6), thereby initiating cytoskeletal reorganization, cell survival, cell proliferation and migration, and enhancing metastatic behaviour in a tumour [5-7]. Since the binding of OPN with αvβ3 integrin increases cancer cell membrane expression of CD44-v6, there is a need to elucidate whether sOPN concentrations are related to sCD44-v6 concentrations.

Blood assay for detecting tumour markers is an important non-invasive method for establishing a cancer diagnosis [8]. However, it is questionable whether sufficient tumour product can reach the peripheral blood (a range of 0.1−20% of secreted or shed protein is assumed) for early disease detection with diagnostic tests, taking into account the sensitivity of the blood assay [9]. An additional problem in setting the tumour marker cut-off level for distinction between health and disease is posed by background secretion by non-malignant cells, especially by other cells of the body in response to certain benign tumours [9, 10]. Investigation of the relationship between sOPN and sCD44-v6 concentrations in serum and local fluid in patients with benign and malignant gynaecological diseases may help to elucidate whether concentration changes of sOPN and sCD44-v6 in the local environment can be detected with a blood test. To the best of the present authors' knowledge,

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this relationship has not yet been examined in neither benign nor malignant conditions. As local fluid, we could use ascites, peritoneal fluid (PF) or/and peritoneal washing (PW) [11, 12]. Ascites might be an important source of circulating sOPN, since it was reported that ovarian cancer patients with ascites had higher serum sOPN levels than those without ascites [13]. However its concentration in ascites of ovarian cancer patients has not been previously measured. PW is already included in the International Federation of Gynaecology and Obstetrics (FIGO) staging classification for ovarian cancer [14]. In a previous study, the present authors already established a standardized protocol for sampling PF and performing washing during laparoscopy and demonstrated that concentrations of sOPN and sCD44-v6 in PF correlate with their concentrations in PW, so they could use PW as local fluid when PF was absent [15].

In the present study, the authors first compared the concentrations of sOPN and sCD44-v6, in serum and local fluid, between patients with advanced ovarian cancer and patients with benign gynaecological conditions. They then evaluated in both groups whether serum concentrations of sOPN and sCD44-v6 correlate with their concentrations in ascites, PF, and PW. Finally, they investigated the relationship between concentrations of sOPN and sCD44-v6.

Materials and Methods

The study included 33 patients with advanced ovarian cancer [FIGO III-IV] and 33 patients with benign pathology of reproductive organs as a control group, who were operated between December 2011 and December 2013 at the Department of Gynaecology, University Medical Centre Ljubljana. Family, general, gynaecological and obstetric history, indication for surgery, other relevant diseases, and current therapy were collected from medical records. Early-stage (FIGO I, II) and absence of ascites were exclusion criteria for enrolling patients with ovarian malignancy. The control group enrolled patients with common non-malignant gynaecological indications for surgery (e.g., benign ovarian cyst, uterine myoma, chronic pelvic pain). Patients with malignancies and elevated standard tumour marker CA125 were excluded from the control group. The purpose of the study was explained to all patients and written informed consent was obtained prior to enrolment. The study was approved by the Commission of the Republic of Slovenia for Medical Ethics (No. 82/01711) and in accordance with the Declaration of Helsinki.

Venous blood samples for determination of sOPN and sCD44-v6 concentrations were obtained prior to surgery while the patients were hospitalized for preoperative preparation. Four ml of peripheral blood was collected into a vacutainer, without anticoagulant or other additives. Serum was separated by centrifugation at 2000 x *g* for 15 minutes at 4°C. Blood for a haemogram, inflammation parameters (C-reactive protein (CRP), total white blood cells (WBC)) and standard tumour marker CA125, were obtained for analysis at the same time as sOPN and sCD44-v6.

Samples of ascites from patients with ovarian cancer were aspirated immediately after entry to the abdominal cavity using a 50-ml syringe. In controls, samples of PF and PW were collected during laparoscopy using a standard sampling protocol (SSP) as previously described [15]. PW was performed after the aspiration of the whole amount of PF or immediately after entering the abdominal cavity if PF was not present. Samples were transferred into a tube, which was kept on ice until centrifugation at 1000 x *g* for ten minutes at 4°C within 30 minutes. Sera and supernatants of ascites, PF, PW were stored in aliquots at -80°C.

Concentrations of sOPN and sCD44-v6 were measured separately using a FlowCytomix Simplex Kit. The kits consisted of fluorescent microspheres with an emission wavelength of 700 nm. Microspheres were coated with specific antibodies raised against each of the analytes. They also contained a biotin-conjugated second antibody and straptavidine-phycoerythrin emitting at 575 nm. Samples were run on a Cell Lab Quanta SC-MPL. Samples were acquired by Cell Lab Quanta SC-MPL software and analysed using Flowcytomix Pro 3.0 software.

All data are presented as mean ± standard error of mean (SEM). Pearson's and Spearman's correlation coefficients were used to calculate the direction and strength of the relationship between variables as required in terms of the normality of variables. Data were compared by the Student's unpaired *t*-test. Since sOPN serum concentrations were positively associated with age, measured sOPN concentrations in serum were analysed using ANOVA with age as covariate. A p-value of < 0.05 was considered significant. Statistical analysis was performed using software statistical package SPSS, version 19.

Results

The clinical characteristics of the investigated sixty-six patients are summarized in Table 1. The mean concentrations of sOPN in ascites and serum were both significantly higher in ovarian cancer patients than in PF/PW and serum of patients in the control group ($p < 0.001$; Table 2). Whereas the mean concentration of sCD44-v6 was significantly higher in ascites of patients with ovarian cancer compared to PF/PW of controls ($p = 0.001$), the serum mean sCD44-v6 concentration was significantly lower in women suffering from cancer than in patients of the control group ($p = 0.01$; Table 2).

In the group of patients with ovarian cancer, the mean concentration of sOPN in serum was 38-fold lower than in ascites (88.57 ±7.85 *vs.* 3355.67 ± 459.41 ng/ml; $p < 0.001$), while sCD44-v6 serum mean concentration was 1.5-fold higher compared to ascites (111.84±7.18 *vs.* 76.69 ±7.10 ng/ml, $p < 0.001$) (Table 2). In the control group, the mean concentration of sOPN in serum was five-fold lower than that in PF (26.79 ± 2.14 *vs.* 132.14 ± 22.09 ng/ml, $p < 0.001$), and 2.2-times higher than in PW (26.79 ± 2.14 *vs.* 12.37 ± 2.51 ng/ml, $p < 0.001$) (Table 2). The mean concentration of sCD44-v6 in serum was three-fold higher than the mean concentration in PF (146.44 ± 10.84 *vs.* 45.32 ± 3.49 ng/ml, $p < 0.001$) and even 32-fold higher than that in PW (146.44 ± 10.84 *vs.* 4.56 ± 0.56 ng/ml, $p < 0.001$) (Table 2).

In ovarian cancer patients, concentrations of sOPN in serum were not correlated to ascites (Pearson: $r = 0.157$, $p > 0.5$) (Figure 1). In the control group, serum sOPN concentrations were not correlated with levels in PW and in PF (Spearman: $r = 0.289$, $p = 0.108$ and $r = 0.302$, $p = 0.142$,

Table 1. — *Patient characteristics.*

Parameters	Data	
	Control group[1]	**Ovarian cancer group**
Number of patients	33	33
Age (years, value ± SEM)	43 ± 1.82	60.03 ± 2.13
Age range	21−69	28−83
Elevated CRP		
n (%)	4 (12%)	31 (93.9%)
Value (mean ± SEM)	19.25 ± 4.44 mg/L	61.64 ± 10.5 mg/L
Elevated WBC		
n (%)	1 (3%)	8 (24.2%)
Value (mean ± SEM)	18×10^6/L	$13.8 \pm 1.16\times10^6$/L
Elevated CA125		
n (%)	0	33 (100%)
Value (mean ± SEM)	NA	3745.1 ± 1266.2 U/ml
Histological type, n (%)		
Serous	NA	29 (88%)
Endometrioid	NA	3 (9%)
Serous + clear cell	NA	1(3%)
Benign diagnosis, n (%)		
Benign ovarian cyst	10 (30%)	NA
Myoma of the uterus	15 (46%)	NA
Pelvic pain, sterilisation	6 (18%)	NA
Preventive adnexectomy	2 (6%)	NA
FIGO stage, n (%)		
III B	NA	1 (3%)
III C	NA	22 (67%)
IV	NA	10 (30%)
Histological grade, n (%)		
G1	NA	4 (12%)
G2	NA	12 (36%)
G3	NA	17 (52%)
Local fluid, *n* (%)		
Ascites	NA	33 (100%)
Peritoneal fluid	26 (79%)	NA
Peritoneal washing[2]	33 (100%)	NA

[1] The control group was formed by patients with benign gynaecological conditions. [2] Peritoneal washing was performed after the aspiration of PF or immediately after entering the abdominal cavity if PF was not present.

respectively) (Figure 1). In contrast to sOPN, serum concentrations of sCD44-v6 positively correlated with concentrations in all three local fluids: ascites (Pearson: $r = 0.876$, $p < 0.001$), PF (Pearson: $r = 0.949$, $p < 0.001$), and PW (Pearson: $r = 0.496$; $p = 0.002$) (Figure 2). However, the magnitude of slope of linear regression was lower in malignant conditions (Figure 3).

When concentrations of sOPN were compared with concentrations of sCD44-v6 in serum and in all three local fluids of both groups of patients, the authors found only a significant positive correlation between sOPN and sCD44-v6 levels in ascites of patients with ovarian cancer (Pearson: $r = 0.561$; $p < 0.001$) (data not shown).

Discussion

Interpretation of studies measuring soluble forms of OPN and CD44-v6 in blood is clouded by lack of knowledge of which factors determine the steady-state levels of these two molecules in serum. The present authors therefore evaluated the relationship of sOPN and sCD44-v6 between two different environments: local fluid represented by ascites and PF/PW, to which tumour markers are shed or secreted, and serum, in which other influences that determine the steady-state level of tumour markers could be expected. Since the particular interest in this study was to elucidate whether a malignant situation could change this relationship, the authors evaluated the association of both tumour markers between serum and local fluid in patients with advanced ovarian cancer (which are typical representatives

Table 2. — *Concentrations of sOPN and sCD44-v6 (average ± SEM) in different body fluids*

	Ovarian cancer group	Control group[1]	*p*-value[2]
Tumour marker	**sOPN**		
Sample (n)	[ng/ml]	[ng/ml]	
Serum (33 per group)	88.57 ± 7.85	26.79 ± 2.14	< 0.0015[5]
Ascites (33) or PF[3] (26)	3355.67 ± 459.41	132.14 ± 2.51	< 0.001
PW[4] (33)	NA	12.37 ± 12.27	NA
Concentration ratio			
Serum: ascites or PT	0.026	0.2	
Serum: PW	NA	2.16	
Tumour marker	**sCD44-v6**		
Sample (n)	[ng/ml]	[ng/ml]	
Serum (33 per group)	111.84 ± 7.18	146.44 ± 10.84	0.010
Ascites (33) or PF (26)	76.69 ± 7.10	45.32 ± 3.49	0.001
PW (33)	NA	4.56 ± 0.56	NA
Concentration ratio			
Serum: ascites or PT	1.5	3	
Serum: PW	NA	32	

[1] The control group was formed by patients with benign gynaecological conditions. [2] Student's unpaired *t*-test. [3] PF = peritoneal fluid was present in women of control group. [4] PW = peritoneal washing was performed after the aspiration of PF or immediately after entering the abdominal cavity if PF was not present. [5]Age adjusted ANOVA.

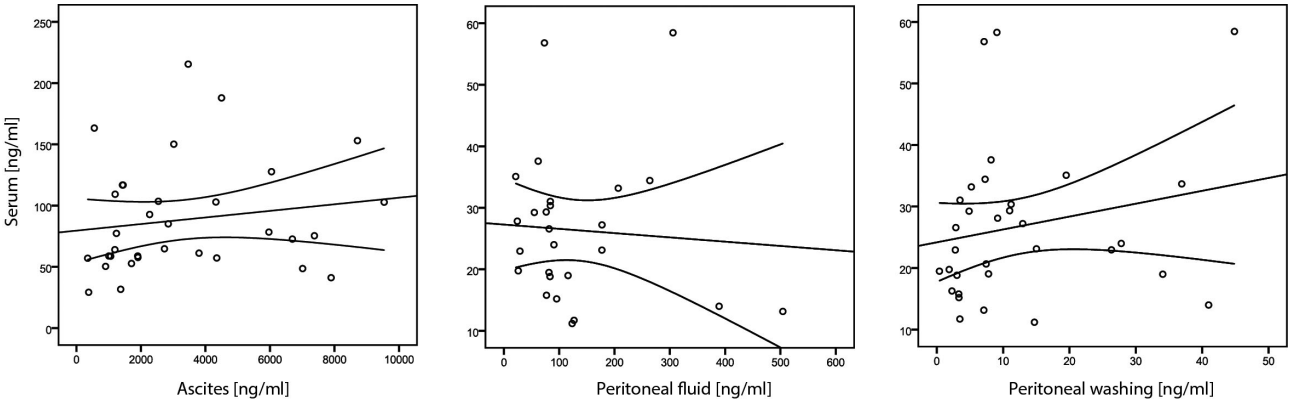

Figure 1. — Correlation of osteopontin (sOPN) concentrations between serum and different local fluids. In ovarian cancer patients, local fluid was represented by ascites, whereas in control patients, local fluid was represented by peritoneal fluid and peritoneal washing. The control group enrolled patients with common non-malignant gynaecological indications for surgery.

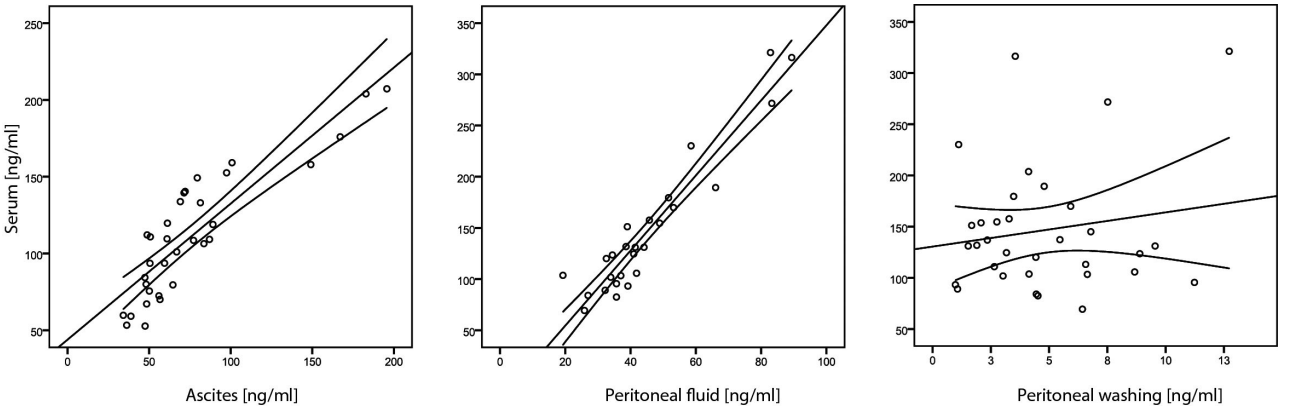

Figure 2. — Correlation of splice variant 6 of sCD44 (sCD44-v6) concentrations between serum and different local fluids. In ovarian cancer patients, local fluid was represented by ascites, whereas in control patients, local fluid was represented by peritoneal fluid and peritoneal washing. The control group enrolled patients with common non-malignant gynaecological indications for surgery.

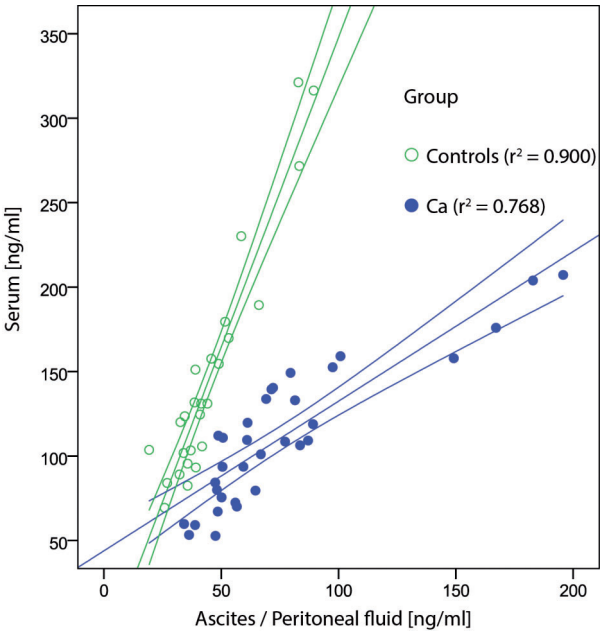

Figure 3. — Comparison of linear regression of sCD44 (sCD44-v6) concentrations between serum and ascites in ovarian cancer patients (group Ca; blue colour, solid dots) and between serum and peritoneal fluid in controls (green colour, empty dots). The control group enrolled patients with common non-malignant gynaecological indications for surgery.

of cancer cell-positive peritoneal fluid) and in patients with benign gynaecological conditions. The present results showed that serum sOPN concentrations were not related to local fluid concentrations in either study group. Although in malignancy the association of sCD44-v6 concentrations between serum and local fluid remain strong, the magnitude of slope of the regression line was lower than with controls, which may indicate that that fraction of sCD44-6 able to reach the serum from ascites in ovarian cancer is lower than in benign disease. In the malignant condition, both tumour markers, but sOPN in particular, in spite of their different kinetics, showed a tendency for retention in ascites.

An important feature of secreted or shed proteins that may serve as blood tumour markers is a low baseline concentration in the serum of the control group. The present authors demonstrated a five-fold lower basal level of serum sOPN compared to PF in controls, which can be explained by considerable local production of sOPN in the peritoneal cavity and/or accumulation of sOPN in local fluid. PF under physiological conditions is mainly formed by exudation of the ovarian capillaries. Fluid secreted by the fallopian tube and follicular fluid drained at ovulation also contribute to the formation of PF, although in small amounts [16, 17]. OPN is synthesized by the oviductal epithelium and was detected in oviductal fluid, where has been observed to bind to oocytes [18, 19]. In addition, OPN expression in the female reproductive tract has been demonstrated in surface mesothelial-like cells of the ovary, the mucinose epithelium of the endocervix, and the secretory endometrium [18, 20]. A high sOPN concentration in local fluid might also be explained by sOPN accumulation due to 'ion trapping'. Ionisation affects the steady-state distribution of molecules between aqueous compartments if a pH difference exists between them [21]. As an acidic protein, sOPN would be more ionised in PF, where the pH is higher than in plasma [22, 23]. Because ionised molecules cannot cross the membrane of mesothelial cells, sOPN accumulates in PF. Another explanation for the high local fluid-to-serum ratio might be that high renal and/or liver clearance of sOPN reduces the amount of circulating sOPN. It has been shown that intact sOPN and COOH-terminal fragments of sOPN are present in the urine of healthy women, as well as in the urine of women with benign ovarian conditions and patients with ovarian cancer [24].

The results of the current study showed that retention of sOPN in local fluid was greater in ovarian cancer than in benign conditions. The authors also found significantly increased mean sOPN concentrations in local fluid and serum of patients with advanced ovarian cancer compared to controls, which is in agreement with previous studies reporting elevated serum sOPN levels in patients with ovarian cancer [25-28]. sOPN is strongly associated with ovarian cancer development and progression. This glycoprotein promotes ovarian cancer growth and increases the survival of ovarian cancer cells under certain stress conditions through activation of the PI3K/Akt/HIF-1α signalling pathway [29]. An increase in HIF-1α synthesis may facilitate angiogenesis in tumours. It has also been demonstrated that plasma OPN concentration varies by FIGO Stage and is correlated to the volume of ascites, the grade of disease, and tumour recurrence, thus suggesting a prognostic value of the marker [30]. In addition to the sOPN retention tendency in local fluid, the present authors found that sOPN concentrations in serum were not associated with concentrations in ascites, PF or PW, suggesting that it would be reasonable to set separate control values of this marker in the blood and in the local fluid. Determination of sOPN concentrations in local fluid may be useful in combination with cytology in order to obtain more accurate results, especially in classification of early stage disease. Knowledge of the tumour microenvironment is also very important in patients with advanced carcinoma, since it might be an important step in the pursuit of new approaches to cancer therapy, with the intraperitoneal method of treatment gaining increasing support. Why the level of sOPN in the serum of patients with ovarian cancer is elevated in spite of the absence of association of sOPN concentrations between serum and ascites is difficult to explain. One hypothesis might be that the fraction of sOPN in ascites able to reach the serum is not in proportion to its concentration in ascites. sOPN could also exit from the primary tumour and peritoneal metasta-

sis through new vessels functionally connected to the systemic circulation.

In contrast to sOPN, the present data of detected sCD44-v6 concentrations in PF and PW showed low basal production in the local environment. In the normal physiology of ovaries, sCD44-v6 mediates apoptosis inhibition, which is important in preventing oocytes from succumbing to atresia during follicular maturation [31]. An explanation for a low local fluid-to-serum ratio of sCD44-v6 could be its high molecular weight (170 kDa), which is almost six times higher than that of the OPN standard form (32 kDa). Although follicular fluid may minimally contribute to PF formation following follicular rupture, the origin of PF is mainly the result of exudation from the ovarian capillaries, so the content of a protein with a high molecular weight in PF would be low because diffusion through the capillary wall is more difficult [16, 17]. A low sCD44-v6 baseline level in PF has already been shown to be useful in differential diagnosis of non-malignant and malignant ascites. The present results of the sCD44-v6 concentration in PF were comparable to previously published results of the sCD44-v6 concentration in non-malignant ascites represented by cirrhotic and tuberculous ascites [32].

Among CD44v isoforms, CD44-v6 appears to be a key functional tumour marker critically involved in the main features of cancer progression [33-36]. In patients with advanced ovarian cancer, the expression of CD44-v6 has been associated with peritoneal metastatic dissemination, tumour resistance to chemotherapy, and shortened overall survival [37]. The present authors found that the mean concentration of sCD44-v6 was significantly higher in ascites than in PF, while the serum mean sCD44-v6 concentration was significantly lower in patients with ovarian cancer than in patients with benign gynaecological conditions. The difference of sCD44-v6 concentrations between serum and ascites was therefore lower (1.5-fold) than between serum and PF (three-fold). Increased sCD44-v6 concentration in ascites compared to PF may reflect mainly the secretion of peritoneal metastasis, since CD44-v6 expression is increased in tumour tissue at the peritoneal metastasis sites compared with those at the corresponding primary tumour [37]. The present result showing a decreased serum concentration in the malignant group compared to controls, which is in accordance with a previously published study by Stickler *et al.* [38], is difficult to explain in view of a published study on increased expression of sCD44-v6 in ovarian cancer [37,39]; the present authors therefore attempted to elucidate the relationship between two different environments, local fluid, and serum. They found a positive correlation between sCD44-v6 concentrations in serum and ascites, as well as between serum and PF; however, the slope of the regression line was much steeper in the case of PF than that of ascites as independent variable. This finding indicates that serum sCD44-v6 concentrations in ovarian cancer patients are less dependent on concentrations in ascites than those in PF. The decreased serum concentration may therefore be because the fraction of sCD44-6 that reaches the serum from ascites in a malignant condition is lower than the fraction of PT in non-malignant conditions, and an elevation of tumour marker level in ascites was not therefore detected in serum.

OPN is a potent inducer of CD44-v6 and thereby provides metastatic activity to tumorigenic cells. OPN binding with CD44 variants/β1-integrins promotes cell spreading, motility, and chemotactic behaviour [5-7]. A recently published study showed a correlation between sOPN and the presence of CD-44v6 in tumour specimens from colorectal cancer patients. sOPN secreted from cancer-associated fibroblasts was observed to increase CD44-v6 in colorectal cancer stem cells but was unable to induce the expression of CD44-v6 on more-differentiated colorectal cancer cells. CD44-v6 expression thus appears to be part of a more complex system that confers self-renewal and metastatic properties to cancer stem cells [40]. The results of the present study showed a weak positive correlation between sOPN and sCD44-v6 ascites concentrations, which might indicate the presence of a high metastatic local environment in patients with advanced ovarian cancer, especially since no correlation between these two tumour markers was found in local fluid of patients with benign gynaecological conditions. Completely different kinetics of sOPN and sCD44-v6 might be an explanation why no correlation was found when the present authors evaluated the association between sOPN and sCD44-v6 concentrations in serum.

Conclusions

In summary, the present authors found that in a malignant situation, both tumour markers, but particularly sOPN, in spite of their different kinetic properties accumulate in ascites. The retention of sOPN in local fluid already exists in the non-malignant situation but it is potentiated in malignant disease. Serum sOPN levels were not related to concentrations in local fluids in either group, so it would be reasonable to set separate control values of sOPN in the blood, ascites, PF, and PW. In contrast to sOPN, serum sCD44-v6 concentrations were positively correlated to those in local fluids in both malignant and non-malignant conditions, although they seem less dependent on the concentration in ascites than in PF. A low concentration of sCD44-v6 in PF/PW shows low baseline production of this tumour marker in the local environment and therefore the sensitivity of sCD44-v6 in local fluid. Finally, sOPN concentrations correlated with sCD44-v6 levels in ascites, which indicates the presence of a high metastatic local environment in patients with advanced ovarian cancer. Further studies are needed to establish the relationship between sCD44-v6 in serum and local fluid of patients with early ovarian cancer.

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