

ORIGINAL ARTICLE

Proteomics pattern of peritoneal sApo-2L but not CD200 (OX-2) as a possible screening biomarker for metastatic ovarian, endometrial and breast carcinoma

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Summary

Purpose: The purpose of this study was to evaluate the soluble Apo-2L (sApo-2L) levels in the ascitic fluid and to study its potential in detecting malignant ascites and soluble CD200 (sCD200,sOX-2) levels so as to predict its clinical usage for detecting stage 4 metastatic endometrial, ovarian and breast cancer in serum samples.

Methods: Ascitic fluid from 53 and blood from 25 subjects without known malignancy on admission were collected. There were 14 breast cancer (BC), 17 ovarian cancer (OC) and 19 endometrial cancer (EC) patients diagnosed later on. Blood samples for sApo-2L, sCD200, liver function tests and CEA, CA-19.9 and CA-125 were always taken and assayed in the morning.

Results: Significantly low levels of sApo-2L were observed in peritoneal fluid from OC and EC patients compared to benign peritoneal fluid from control individuals. Positive

correlation was observed between sApo-2L and aspartate aminotransferase (AST) in benign peritoneal fluid and sCD200, and creatinine and sCD200 and platelets in OC patients; also, sCD200 and CEA in EC patients and sCD200 and blood urea nitrogen (BUN) in healthy subjects.

Conclusions: Our data indicate that low proteomics pattern of sApo-2L but not sCD200 is a good biochemical marker. Further decline in the level of sApo-2L was seen in EC compared to OC. Since higher levels of sApo-2L were seen with higher levels of AST, the liver might be involved in its metabolism. The positive correlation detected between sCD200 and creatinine, platelets, CEA and BUN needs to be elucidated.

Key words: metastatic breast carcinoma, metastatic endometrial carcinoma, metastatic ovarian carcinoma, sCD200, soluble TRAIL

Introduction

Cancer is the second most frequent cause of death. Among women, the most frequent cancer is BC (26%) and EC is the most frequent gynecological cancer (7%). Although not so common, OC is the most lethal form of gynecological cancer. CA-125 has been found to have great value in initial diagnosis and in the follow-up for early detection of recurrence [1]. Elevated CA-125 levels are found in 82% of patients with OC and in 28% of patients with non-gynecological cancers (including pancreatic, breast, and colon cancer) [2]. Its

sensitivity is 91% and specificity 100% in the detection of OC [3]. Though observed in other malignancies like colon cancer, serum CEA is elevated in approximately 35% of all OC cases [4] and high preoperative concentrations of CEA are associated with poor prognosis in BC [5].

Death receptor ligands such as soluble tumor necrosis factor-related apoptosis inducing ligand (sTRAIL,sApo-2L) is a type II transmembrane glycoprotein. Its extracellular domain is homologous to that of other TNF family members and shows a homotrimeric subunit structure. Like TNF, TRAIL also exists physiologically in a biologically active

soluble homotrimeric form, serum soluble TRAIL (sApo-2L) [6]. Several recent studies have indicated that TRAIL in malignant effusions from OC patients protects against TRAIL-induced apoptosis and is associated with shorter disease-free interval [7,8].

CD200 (OX-2), though not a cytokine, is a cell surface membrane glycoprotein expressed on macrophages and reported to play a key role in the regulation of the immune system, enabling a response to tumor antigens [9]. CD200 was also defined as a p53-target gene [10]. It was found that CD200 is expressed in many solid tumors, including BC and OC [11] and was shown that it plays an important role in the regulation of immune rejection of leukemic tumor cells in mice [12]. A recent study conducted on genetically engineered mice that overexpressed CD200 showed that metastatic growth of breast tumor cells was increased [13]. Further evidence for a role of tumor CD200 expression in BC metastasis conducted on animals showed that the phenotype of mice lacking expression of functional CD200R1 (CD200R1KO) failed to deliver an immune-suppressive signal and led to decreased ability for metastasis. We investigated soluble CD200 that can also be linked to apoptosis and is an immunosuppressive ligand [14].

The purpose of this study was to evaluate sApo-2L level in ascitic fluid and sCD200 level in blood and their predictive values as biomarkers useful in the early diagnosis of patients in whom the initial diagnoses were EC, OC and BC.

Methods

Patient population

The study was approved by the Antalya Training Hospital local ethics committee, and written informed consent was obtained from all patients and healthy volunteers.

Endometrial and ovarian carcinoma

The baseline clinical characteristics of EC and OC patients are summarized in Table 1. All patients underwent preoperative exploratory laparoscopy. No bacterial contamination was detected in all peritoneal fluid samples.

Group 1 (control group) consisted of healthy volunteers who had no history of allergy/atopy, family atopy history, cardiac, liver, renal or pulmonary diseases. These subjects were found to have cytologically benign peritoneal fluid. Group 2 (OC patients) and Group 3 (EC patients) consisted of patients with stage 4 disease, with liver metastasis and cytologically malignant peritoneal fluid at the time of diagnosis. No chemotherapy was administered to these groups. Blood samples for sApo-2L, sCD200, liver function tests and CEA, CA-19.9 and CA-125 measurement were always taken in the morning between 8 and 10 a.m. Medical history and physical examination were performed on the same day.

Breast carcinoma

The baseline clinical characteristics of BC patients are summarized in Table 2.

Group 1 (control group) consisted of healthy volunteers who had no history of allergy/atopy, family atopy, cardiac, liver, renal or pulmonary diseases.

Group 2 consisted of patients with invasive ductal

Table 1. Clinical and laboratory findings of the gynecological patients. Group 1 consisted of individuals who underwent exploratory laparoscopy for suspected malignancy but found to have benign peritoneal fluid. Group 2: ovarian carcinoma and Group 3: endometrial carcinoma

Parameters	Groups		
	Group 1 (N=53)	Group 2 (N=17)	Group 3 (N=19)
Age (years)	45.79 ± 1.67	48.76 ± 3.47	55.47 ± 2.08
CEA (0-5 ng/mL)	1.19 ± 0.07	3.96 ± 1.23	1.08 ± 0.09
CA-19.9 (0-35 U/mL)	11.57 ± 1.23	6.96 ± 1.11	9.94 ± 2.33
CA-125 (0-35 U/mL)	12.39 ± 1.12	58.87 ± 6.78	21.73 ± 3.73
BUN (8-20 mg/dL)	11.70 ± 0.40	8.59 ± 0.76	13.21 ± 0.89
AST (0-50 U/L)	17.52 ± 0.61	19.00 ± 1.52	17.05 ± 1.03
ALT (0-50 U/L)	14.74 ± 0.66	16.59 ± 1.22	15.11 ± 1.30
Hb (11.5-15g/dL)	11.97 ± 0.24	12.04 ± 0.32	13.18 ± 0.36
WBC (4-10 ³ /mm ³)	7626.15 ± 272.58	8064.67 ± 588.23	8862.88 ± 348.62
PLT (150-400 ³ /mm ³)	234540 ± 9437.67	276285.71 ± 16001.86	295277.78 ± 14396.76
sTRAIL (113-145pg/mL)*	128.61 ± 14.17	69.92 ± 15.67	29.01 ± 6.07
sCD200 (28-40.1pg/mL)*	38.47 ± 0.92	38.15 ± 1.56	38.89 ± 0.98

* for peritoneal fluid. Values are given as mean±standard deviation. Hb: hemoglobin, WBC: white blood cells, PLT: platelets

carcinoma diagnosed by histopathology who had metastases at the time of diagnosis. Blood samples for sApo-2L, sCD200, liver function tests and CEA, CA-125 and CA15.3 measurements were always taken in the morning between 8 and 10 A.M. Medical history and physical examination were performed on the same day.

Experimental procedures

Concentrations of sCD200 in the serum samples were assessed using ELISA kit (Sino Biological Inc., Catalog no: SEK10886, China). CEA (Beckman Coulter System, Germany. Catalog no: 33200), CA-19.9 (Beckman Coulter System. Catalog no: 387687), CA-125 (Beckman Coulter System. Catalog no:386357) and CA15.3 (Beckman Coulter System. Catalog no: 387620). Levels were estimated by fluoroenzyme immunoassay. Concentrations of sApo-2L in the serum samples were quantified using ELISA kits (Diaclone, France).

Chemotherapy protocol for metastatic breast cancer

Group 2a: Stage 4. Metastatic breast cancer patients. Pre-treatment period without any treatment.

Group 2b: Stage 4. Metastatic breast cancer patients. Patients were treated with metronomic chemotherapy (cyclophosphamide 50mg daily p.o. and etoposide 50mg twice weekly p.o) for 3 months after which reassessment of the factors in question was performed.

Statistics

Data were expressed as mean \pm standard error of the mean (SEM) of duplicate measurements and were analyzed using the SPSS for Windows (Version 18.0; SPSS Inc, Chicago, Ill, USA). Comparison of parameters between groups was performed using Student's t-test. The correlations between variables were evaluated by linear regression analysis. P values less than 0.05 were considered statistically significant.

Results

Endometrial and ovarian carcinoma

The baseline clinical characteristics and the sApo-2L and sCD200 concentrations among Groups are summarized in Table 1, and Figures 1 and 2.

Although no significant differences were noticed between Groups 1, 2 and 3 for sCD200 levels ($p=0.2$), significant sApo-2L difference was observed between Group 1 and Group 2 ($p=0.008$), between Group 1 and Group 3 ($p=0.000$) and between Group 2 and Group 3 ($p=0.024$; Figure 1). Also, significant difference in age was observed between Group 1 and Group 3 ($p=0.000$). Significant CEA differences were observed between Group 1 and Group 2 ($p=0.043$) and Group 2 and Group 3

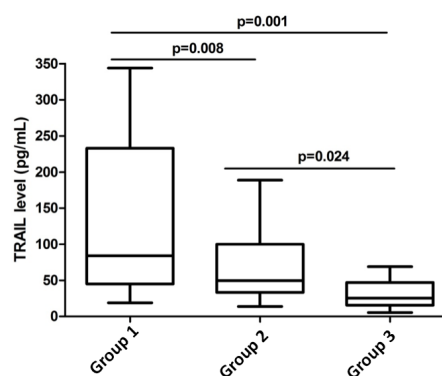


Figure 1. Scatter dot plots of soluble tumor necrosis factor-related apoptosis inducing ligand (sTRAIL,sApo-2L) levels (pg/ml) in healthy controls (Group 1), Group 2 (ovarian carcinoma) and Group 3 (endometrial carcinoma).

($p=0.037$). Significant CA19.9 differences were observed between Group 1 and Group 2 ($p=0.008$). Concerning CA125, significant differences were observed between Group 1 and Group 2 ($p=0.000$), between Group 1 and Group 3 ($p=0.028$) and between Group 2 and Group 3 ($p=0.000$). Significant BUN differences were observed between Group 1 and Group 2 ($p=0.001$) and between Group 2 and Group 3 ($p=0.000$). Significant platelet differences were observed between Group 1 and Group 3 ($p=0.024$).

Multivariate regression analysis showed positive correlations between sApo-2L and AST ($r=0.477$, $p<0.001$); between CA19.9 and CA125 ($r=0.419$, $p<0.05$); between age and BUN ($r=0.413$, $p<0.005$); between CEA and ALT ($r=0.323$, $p<0.05$); between AST and ALT ($r=0.519$, $p<0.001$) for Group 1 patients and between sCD200 and creatinine ($r=0.565$, $p<0.05$); between sCD200 and platelet ($r=0.566$, $p<0.05$) between age and BUN ($r=0.577$, $p<0.05$); between ALT and AST ($r=0.554$, $p<0.05$); between AST and Hb ($r=0.539$, $p<0.05$) for Group 2 patients and between sCD200 and CEA ($r=0.482$, $p<0.05$) for Group 3 patients.

Negative correlations were detected between CA125 and BUN ($r=0.315$, $p<0.05$) and between CA19.9 and Hb ($r=0.390$, $p<0.05$) for Group 1 patients and between sCD200 and age ($r=0.491$, $p<0.05$); between CA125 and AST ($r=0.536$, $p<0.05$); between WBC and BUN ($r=0.516$, $p<0.05$); between AST and WBC ($r=0.512$, $p<0.05$) for Group 3 patients.

Breast carcinoma

The baseline clinical characteristics and the sApo-2L and sCD200 concentrations among

Table 2. Clinical and laboratory findings of breast cancer patients. Group 1: Healthy volunteers, Group 2a: Pre-treatment BC (invasive ductal carcinoma patients). Group 2b: Three months post-BC treatment

Parameters	Group 1 (N=25)	Group 2a (N=14)	Group 2b (N=14)
Age (years)	58.9± 3.65		56.7 ± 1.71
CEA (0-5 ng/mL)	16.9 ±2.84	15.98 ±1.3	13.9 ± 2.2
CA15.3 (0-31.3 U/mL)	78.5± 2.9	64.3± 1.02	16.9 ± 2.1
CA125 (0-35 U/mL)	19.4 ± 1.2	18.9 ± 0.9	10.9 ± 0.1
BUN (8-20 mg/dL)	14.5 ± 1.3	13.9 ± 0.4	9.19 ± 1.4
AST (0-50 U/L)	39.6 ± 1.1	39.6 ± 1.1	13.4 ± 0.2
ALT (0-50 U/L)	36.29 ± 0.9	37.08 ± 1.9	15.5 ± 0.9
Hb (11.5-15g/dL)	10.84 ± 0.3	10.6 ± 0.58	13.7 ± 1.3
WBC (4-10 ⁵ /mm ³)	8986.5 ± 312.8	8195.5 ± 231.7	4516.9 ± 209.7
PLT (150-400 ³ /mm ³)	285327.99 ± 81216.91	279645.71 ± 8032.62	373890.80 ± 9591.00
sTRAIL (1428-1915 pg/mL)*	1701.6 ±215.8	1699.69 ± 198.7	1751.2 ±220.3
sCD200 (38-42.1pg/mL)*	41.09 ± 0.3	41.62 ± 1.5	41.89 ± 0.27

*for serum samples. For abbreviations see footnote of Table 1 and the text

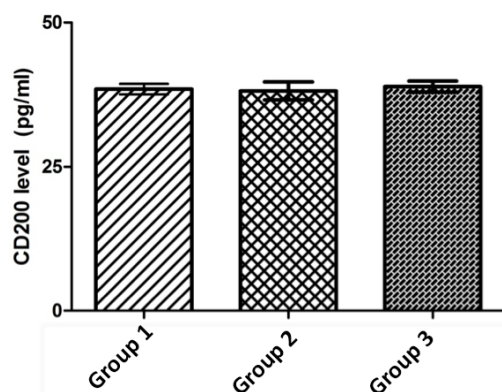


Figure 2. Scatter dot plots of soluble CD200 (sCD200,sOX-2) levels (pg/ml) in healthy controls (Group 1), Group 2 (ovarian carcinoma) and Group 3 (endometrial carcinoma) ($p=0.2$).

Groups are summarized in Table 2.

We compared patients and control group and found that BUN, AST, ALT, CA-15.3, CEA, CA-125 were significantly elevated in Group 2 ($p<0.005$). Hb and platelet levels were significantly decreased in Group 2 ($p<0.005$). We also observed that decreased Hb and platelet levels were significantly elevated after chemotherapy in Group 2b.

Statistical analysis showed positive correlations between BUN and sCD200 levels for Group 1 patients (control patients) ($r=0.452$, $p<0.05$).

Discussion

New tumor markers useful in the early diagnosis and in monitoring treatment and also to help diagnose disease recurrences are still being sought. Evidence implicates a critical role

for sCD200 and sApo-2L in antitumor immune response [15-17]. This study was undertaken to assess whether sApo-2L levels in ascitic fluid obtained from women with EC and OC and sCD200 serum levels obtained from women with BC could help initial diagnosis of these conditions.

When a physician deals with ascites in a patient over 40 years old, malignancy must be ruled out. Although the cytological diagnoses of serous effusions are usually made by routine cytomorphology with certainty, benign reactive mesothelial cells resemble malignant cells, since long-term benign ascites causes mesothelial cell to proliferate. Biochemical markers produced by tumor cells like CEA, CA19.9, CA125 and CA15.3 are also valuable in certain cases and their use is recommended as a surrogate marker of cancer diagnosis and disease evolution. However, in a study [18] only a small proportion of patients had isolated asymptomatic tumor marker progression. It was found that sensitivity to diagnosis of a recurrent BC using CEA is between 17% and 89% [19] and isolated rising in serum CEA was seen only in 38.6% of recurrent BC [20] while isolated rising in serum CA125 was seen only in 48% of recurrent OC [21]. There is no actual marker for detecting EC or its recurrence.

In vitro experimental studies demonstrated that sApo-2L efficiently killed human ovarian cancer cell lines by activating caspases [22]. Despite its anticancer activities, we showed that sApo-2L level in malignant ascites was lower compared to benign peritoneal fluid. In the AJCC 2011 protocol, malignant tumor cells in ascites or peritoneal washings are described as pT2c and stage IIC. Besides its spread to the abdominal cavity, our patient population had advanced disease (stage

IV) and the lower level of sApo-2L in malignant ascites we found in this study supports previous observations. A limited number of studies have shown that malignant ascites prevents sApo-2L to induce apoptosis in a wide variety of tumor types by activating death receptors [7,23]. It was found that sApo-2L induced apoptosis in tumor cell microenvironment, i.e. malignant effusions, could block sApo-2L-induced apoptosis in ovarian cancer cells [8]. This prosurvival activity of ascites against sApo-2L-induced apoptosis was found to be associated with shorter disease-free interval [7], as in our cases. *In vitro* observations indicated that ovarian cancer ascites increased Mcl-1 expression in tumor cells through ERK1/2-Elk-1 signaling to reduce sApo-2L-induced apoptosis [23]. Its level in blood didn't change in BC patients.

The magnitude of sApo-2L-induced cell death was found to vary among ascites of different origins [8]. For instance, we found that sApo-2L levels were decreased more in EC than in OC patients. Embryologically of similar origin both mesothelial lining and OC (especially ovarian serous tumors) but not EC may participate in this context. Mesothelial lining produces fluids under normal conditions. Thus, OC cells grow and proliferate easily in the peritoneal cavity which provides genetically and phenotypically similar environment and produce more ascitic fluid than EC cells, and we speculate that either mesothelial cells or OC cells produce and secrete more sApo-2L compared to EC cells. The precise origin of sApo-2L is debatable. The higher level of sApo-2L seen in patients with higher AST level in our study may reflect its metabolism in the liver.

Soluble CD200 was shown to attenuate TNF α production *in vivo* and was associated with suppression of natural killer cells [24,25]. Overall function of sCD200 is immune evasion of tumor cells [23]. Data from two studies conducted on BC and transplantable thymoma cell lines implied its unequivocal role in tumor growth [12,13]. *In vivo* testing in a murine model of passive cutaneous anaphylaxis revealed that inhibition *in vivo* by CD200R1 cross-linking was much more sensitive than that seen *in vitro*, perhaps reflecting a higher constitutive expression of CD200R1 in mast cells *in vivo* compared with cells maintained in culture, and/or the existence of other cell-cell interactions *in vivo* which could lower the threshold for CD200R1-mediated suppression. The data from these *in vivo* studies were taken to imply a potential clinical utility for CD200:CD200R1 in the regulation of allergic inflammatory disease. In a pre-

vious study [30], we reported a patient who had a pruritic bullous pemphigoid and very high levels of total IgE (5000 kU/L) which was refractory to the aggressive immunosuppressive regimens for bullous pemphigoid but responded rapidly to systemic anti-IgE. The circulating level of sOX-2 was 48.45 pg/mL in serum and 243 pg/mL in blister fluid. Soluble OX-2 levels were higher in blister fluid than in serum. During the second month of follow-up, the patient's sOX-2 level decreased to 26.7 pg/mL. Reduction in serum levels sOX-2 with anti-IgE treatment suggests that sOX-2 could be pro-inflammatory. Soluble OX-2 might also play a role in immune response in the pathogenesis of autoimmune and inflammatory skin disorders [27-31].

CD200R deficient-mice showed reduced tumor growth and metastasis [14] and the levels of sCD200 in human BC patient sera were found to be correlated with more aggressive disease behavior and metastasis in an in-preparation study [9]. In our recent study [32] we reported that the overall survival of newly diagnosed stage IV non-small cell lung cancer was correlated with sCD200. To investigate this finding in our institute, tissues from BC patients were retrieved from the files of the pathology laboratory. sCD200 was planned to be performed on paraffin-embedded BC tissues but we failed to obtain positive results from control slides taken from the appendix and the pancreas. Moreover, sCD200 levels in blood performed on human subjects in our study failed to detect any valuable prognostic information, neither in BC nor in EC and OC patients. Also, neither sApo-2L nor sCD200 levels in BC patients were found to provide significant information on clinical grounds. On the other hand, positive correlation between sCD200 and serum creatinine was noticed, and sCD200 and platelets in peritoneal fluid from OC patients and sCD200 and CEA in peritoneal fluid from EC patients were also noticed. Positive correlation between sCD200 and BUN levels was also observed in healthy volunteers. Since sCD200 was found to be correlated with creatinine levels in OC and BUN levels in healthy volunteers, sCD200 expression in renal cell carcinoma needs to be elucidated.

Conclusion

In conclusion, sApo-2L levels decreased in malignant ascites in EC and OC patients as mentioned above. We should screen metastatic cancer patients' ascites for sApo-2L levels.

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Author's contributions

Conceived and designed the study:ADY, LGS. Analyzed the data:SG, GEG. Pathology:BC

Coordination: ADY, LGS. Wrote the paper, Tables, Figures: BC, ADY. Laboratory: GEG, ADY, SG. Guarantor: ADY. Contributed reagents/materials: ADY.

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