Flow Cytometric Analysis of Ovarian Cancer Ascites: Response of Mesothelial Cells and Macrophages to Cancer

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Abstract. *Aim: To evaluate the response of mesothelial cells and macrophages in the peritoneal fluid of epithelial ovarian malignant tumors using flow cytometry immunophenotyping. Materials and Methods: Thirteen peritoneal fluid samples collected from surgery or scentesis of epithelial ovarian malignant tumor patients were assayed using flow cytometry. Cytological and pathological diagnosis was performed on the same ascites and resected tumor specimens. Samples were treated with antibodies against established markers of mesothelial cells (podoplanin), macrophages (CD14) and the hyaluronan receptor (CD44). Results: A significant association was observed between the results of cytology and expression of podoplanin, CD44 and CD14 ($p<0.05$) in peritoneal macrophages. No significant association was observed between the results of cytology and expression of podoplanin, CD44 and CD14 in mesothelial cells in ascites. Conclusion: Expression of surface molecules, such as podoplanin, CD44 and CD14 was increased in the peritoneal macrophages of epithelial ovarian cancer patients, suggesting that the cell-cell or cell-matrix interaction was enhanced during cancer dissemination in the peritoneum. Analysis of the peritoneal fluid using flow cytometry immunophenotyping may be useful for evaluating the diagnosis and pathophysiology of ovarian cancer dissemination.*

Ovarian cancer has the highest mortality rate of all gynecological cancers. Since early disease is asymptomatic, ovarian cancer is rarely diagnosed until late stages, when the cancer has already spread beyond the primary tumor site (1). This is reflected by the fact that stage I patients have 5- and 10-year survival rates of over 90%, whereas patients with stage III disease have a 5-year survival probability of only about 30% (2). The biological behavior of ovarian cancer is unique, differing markedly from the classic and well-studied pattern of hematogenous metastasis found in other cancers. Epithelial ovarian cancer cells are believed to spread by detaching from the surface of the ovary and, then, carried thorough the body by the physiological movement of the peritoneal fluid, attaching to and invading the peritoneum that lines the organs of the abdominal cavity (3, 4). A combination of factors can contribute to ascites formation in ovarian cancer. Cancer cells can obstruct subperitoneal lymphatic channels and prevent the absorption of the physiologically produced peritoneal fluid (1 l/day). Moreover, secretion of vascular endothelial growth factor by ovarian cancer cells increases the vascular permeability and promotes ascites formation (5). Extensive seeding of the peritoneal cavity by ovarian cancer cells is often associated with the formation of ascites, particularly in advanced, high-grade serous carcinomas. Podoplanin is a 43-kDa mucin-type transmembrane glycoprotein, that is expressed on the peritoneal mesothelial cells, lymphatic endothelial cells, osteocytes, ependymal cells, alveolar type I cells and granulosa cells in normal ovarian follicles (6). Podoplanin has a wide variety of functions, including regulation of organ development, cell motility, tumorigenesis and metastasis (7). Much of the mechanistic insight into podoplanin biology has been gleaned from studies of tumor cells; tumor cells often up-regulate podoplanin as they undergo epithelial mesenchymal transition, with this up-regulation being correlated with increased motility and metastasis. These changes occur through interaction of podoplanin and ezrin, which are radixin and moesin family proteins, respectively, and subsequent modulation of the Rho proteins and actin skeleton (8). As podoplanin lacks any obvious enzymatic motif within its structure, all these activities have to be mediated by protein-protein interactions. Hence, there is a need to identify its binding partner in order to understand its mechanism of action, especially with respect to cancer development. CD44 is a type I transmembrane glycoprotein expressed by multiple hematopoietic and non-hematopoietic

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Table I. *Clinical findings of patients with epithelial malignant ovarian tumors included in this study.*

Patient number	Age	Sample category	Pathological diagnosis	Cytology class	Stage
1	48	Operation	Mucinous borderline	Negative	Ia
2	36	Operation	Mucinous borderline	Negative	Ia
3	49	Operation	Serous borderline	Negative	Ib
4	51	Operation	Serous adenocarcinoma	Suspicious	III
5	50	Operation	Serous adenocarcinoma	Suspicious	III
6	71	Operation	Serous adenocarcinoma	Positive	III
7	59	Operation	Serous adenocarcinoma	Positive	III
8	80	Scentesis	Serous adenocarcinoma	Positive	III
9	63	Operation	Serous adenocarcinoma	Positive	III
10	70	Operation	Mucinous adenocarcinoma	Positive	III
11	71	Scentesis	Clear cell carcinoma	Positive	III
12	44	Operation	Clear cell carcinoma	Positive	III
13	73	Operation	Peritoneal carcinoma	Positive	III

cells. It functions as the major hyaluronan receptor and mediates cell adhesion and migration in a variety of pathophysiological processes, including tumor metastasis, wound healing and inflammation (9). The CD44-podoplanin interaction is important for driving directional cell migration in epithelial and tumor cells (10). In addition, it has been demonstrated that the formation of a stabilized hyaluronan/ versican pericellular matrix surrounding ovarian cancer cells increases motility and protects ovarian cancer cells against the mechanical force in the peritoneal cavity to enable strong adherence to CD44 expressed on peritoneal cells (11, 12).

Thus, the evidence accumulated to date suggests that peritoneal carcinomatosis might rely on the interaction between cancer cells and mesothelial cells and/or macrophages in the local peritoneum and ascites; however, details of such interactions have not been clarified. In this study, we sought to elucidate the response of mesothelial cells and macrophages to cancer cells by conducting a quantitative analysis of podoplanin and CD44 expression in the peritoneal fluid obtained from patients with epithelial ovarian cancer using flow cytometry immunophenotyping.

Materials and Methods

Patients and samples. Thirteen peritoneal fluid samples were used in this study, which were obtained from surgery or scentesis of patients with epithelial malignant ovarian tumor and peritoneal cancer. Cytological and pathological diagnosis was also performed on the fluids and tumor specimens. All patients provided informed consent for inclusion of their samples in this study. This study was approved by the Institutional Review Board of Tokyo Women's Medical University.

Flow cytometry assay. The cell fractions were collected by centrifugation of the 20-100 ml peritoneal fluid samples. After lysis of red blood cells, the numbers of nucleated cells were counted with an automated cell counter (Scepter™; Merck Millipore, Darmstadt, Germany). The cells were stained with labeled antibodies for cell surface molecules, including Alex Fluor 488-conjugated anti-human podoplanin (NC-08; BioLegend, San Diego, CA, USA), Alexa Fluor 647-conjugated anti-human CD14 (M5E2; BioLegend), phycoerythrin (PE)-conjugated anti-human CD44 (IM7; BioLegend), PE-conjugated anti-human CD324 (E-cadherin, 67A4; BioLegend) and PE-conjugated anti-human CD54 (intercellular adhesion molecule 1 (ICAM-1); HA58; eBioscience, San Diego, CA, USA) and were analyzed by flow cytometry (FACS Calibur™; Becton-Dickinson Bioscience, San Jose, CA, USA). The resulting data were analyzed using Cellquest Pro Quick Reference version1.1 software (Becton-Dickinson Bioscience). The mesothelial cell fraction was gated according to the positivity of podoplanin, whereas the macrophage fraction was gated according to the positivity of CD14. The expression rate (%) and fluorescence intensity (FI) of these molecules in both fractions were analyzed in association with the clinical findings of the patients. The increment ratio of FI was evaluated as the ratio of the mean fluorescence intensity (MFI) of the cells of interest compared to that of the negatively stained cell fraction.

Cell block immunohistochemistry. A cell block was prepared from the sediments of ascites. After centrifugation of the ascites, the sediment was fixed with 10% neutral formalin and embedded in paraffin according to routine protocols. The sections were cut at a 4-μm thickness and immunohistochemistry staining was performed with the following primary antibodies: mouse monoclonal antibody against human podoplanin (#11-003; AngioBio, San Diego, CA, USA), rat monoclonal antibody against human CD44 (clone IM7; eBioscience), mouse monoclonal antibody against human CD68 (clone PG-M1; DAKO, Glostrup, Denmark), mouse monoclonal antibody against human EMA (endomysial antibody; clone E29; DAKO) and mouse monoclonal antibody against human E-cadherin (clone NCH-38; DAKO). The slides were stained using Envision+ System (DAKO) and diaminobenzene tetrahydrochloride (0.02%). Cell nuclei were counterstained with hematoxylin.

Statistical analysis. Data are expressed as mean±standard deviation. The statistical significance of the difference between the results of independent experiments was analyzed using the Student's *t*-test. A *p*-value of <0.05 was considered statistically significant.

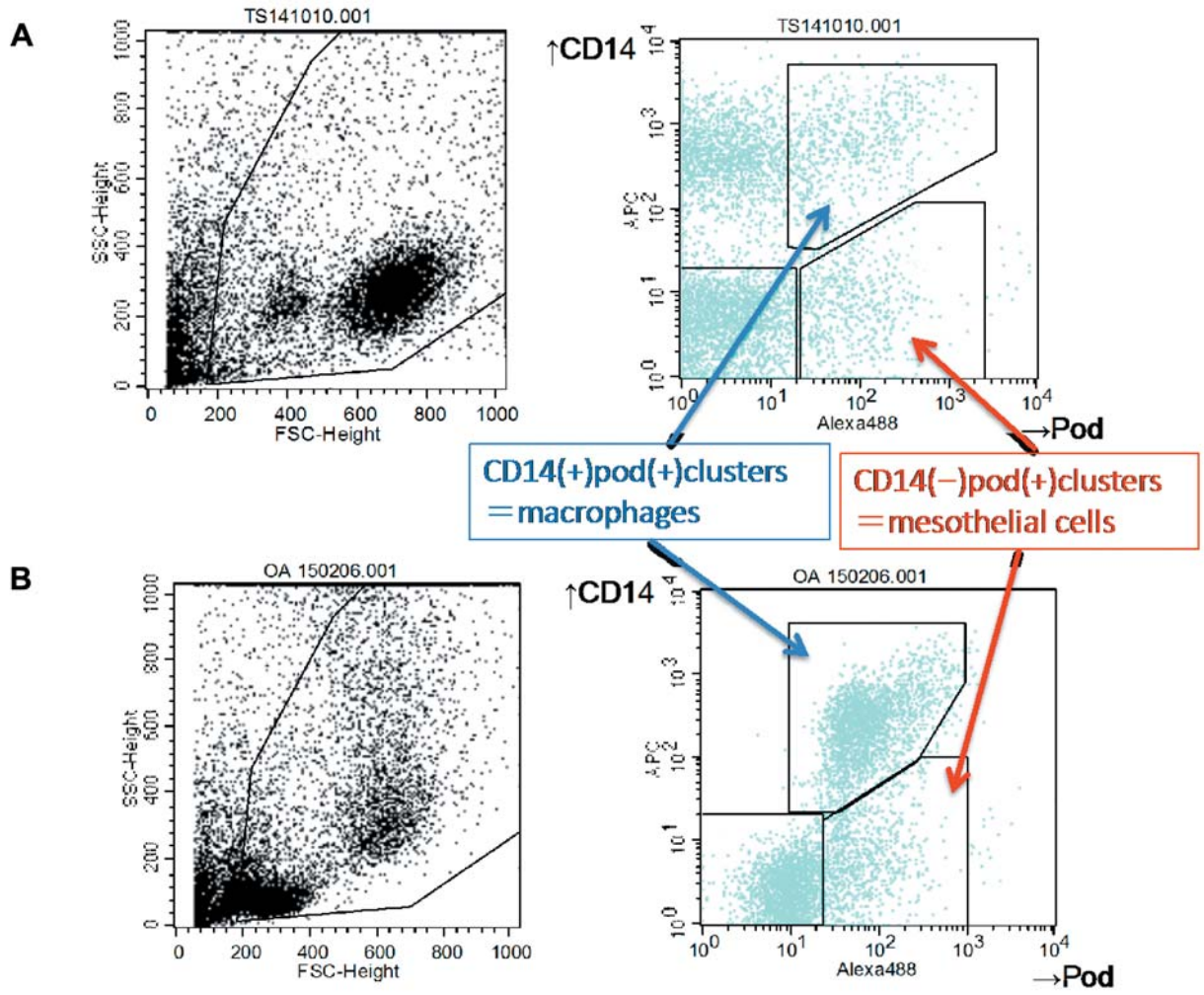

Figure 1. *The cell scattergram of flow cytometry for patient number 8 (A: cytology positive) and number 3 (B: cytology negative).*

Results

Table I shows the clinical findings of the epithelial malignant ovarian tumor patients in the study. Pathological diagnosis showed 7 cases of serous adenocarcinoma, 1 case of serous borderline tumor, 1 case of mucinous adenocarcinoma, 2 cases of mucinous borderline tumor and 2 cases of clear cell adenocarcinoma. Cytological diagnoses of the peritoneal fluid showed 8 positive cases, 2 suspicious cases and 3 negative cases for cancer cells.

Figure 1 shows a representative cell scattergram from flow cytometry for patient number 8 (Figure 1A) and number 3 (Figure 1B). The macrophages were gated according to expression of $CD14^+$/podoplanin+cluster. The mesothelial cells were gated according to expression of $CD14^-$/$podoplanin^+$ cluster.

Figure 2 shows the histograms of the MFI values of the macrophage cluster for patient number 8 (Figure 2A) and number 3 (Figure 2B). The MFI of podoplanin, CD44 and CD14 on the macrophage cluster for patient number 8 (ascites cytology: positive) was higher compared with that of patient number 3 (ascites cytology: negative).

Figure 3 shows the expression of surface markers on $CD14^+$ cells (macrophages). Podoplanin, CD44 and CD14 expression levels on $CD14^+$ cells of ascites-positive patients were higher compared with those of ascites-negative and suspicious patients ($p<0.05$). The expression levels of podoplanin and CD44 on $podoplanin^+$ cells (mesothelial cells) of the ascites-positive patients were higher compared to those of ascites-negative and suspicious patients, although the difference was not statistically significant (data not shown). The E-cadherin expression on $podoplanin^+$ cells of

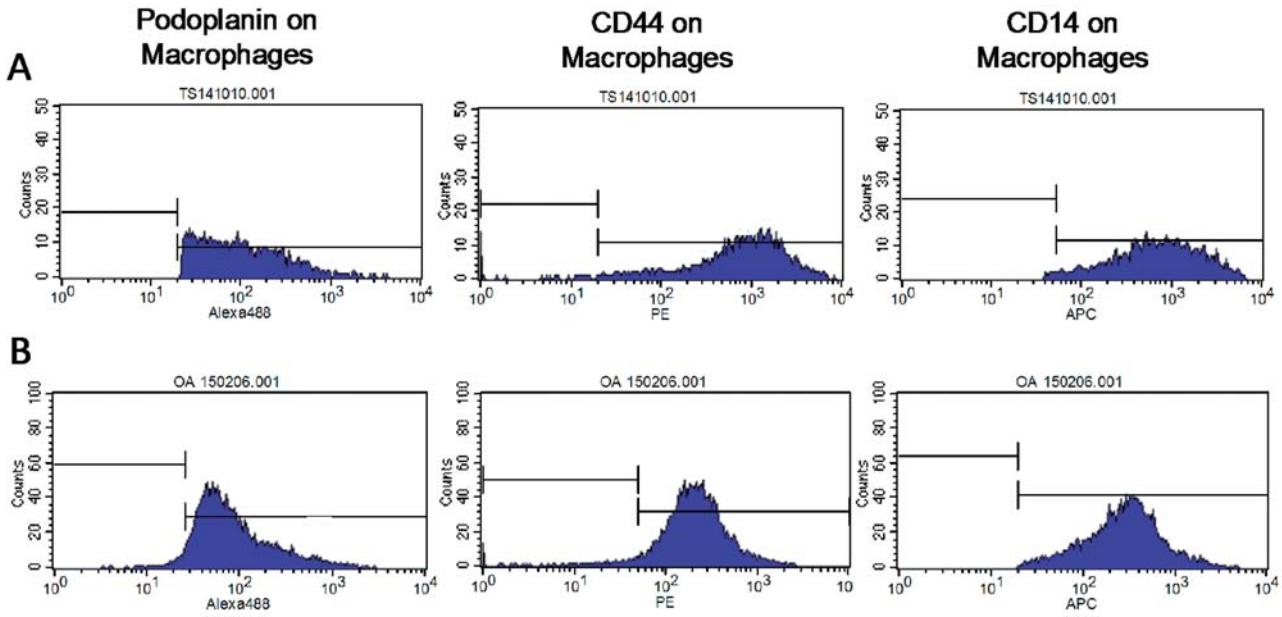

Figure 2. *Histograms of the mean fluorescence intensity of the macrophage cluster in patient number 8 (A) and 3 (B).*

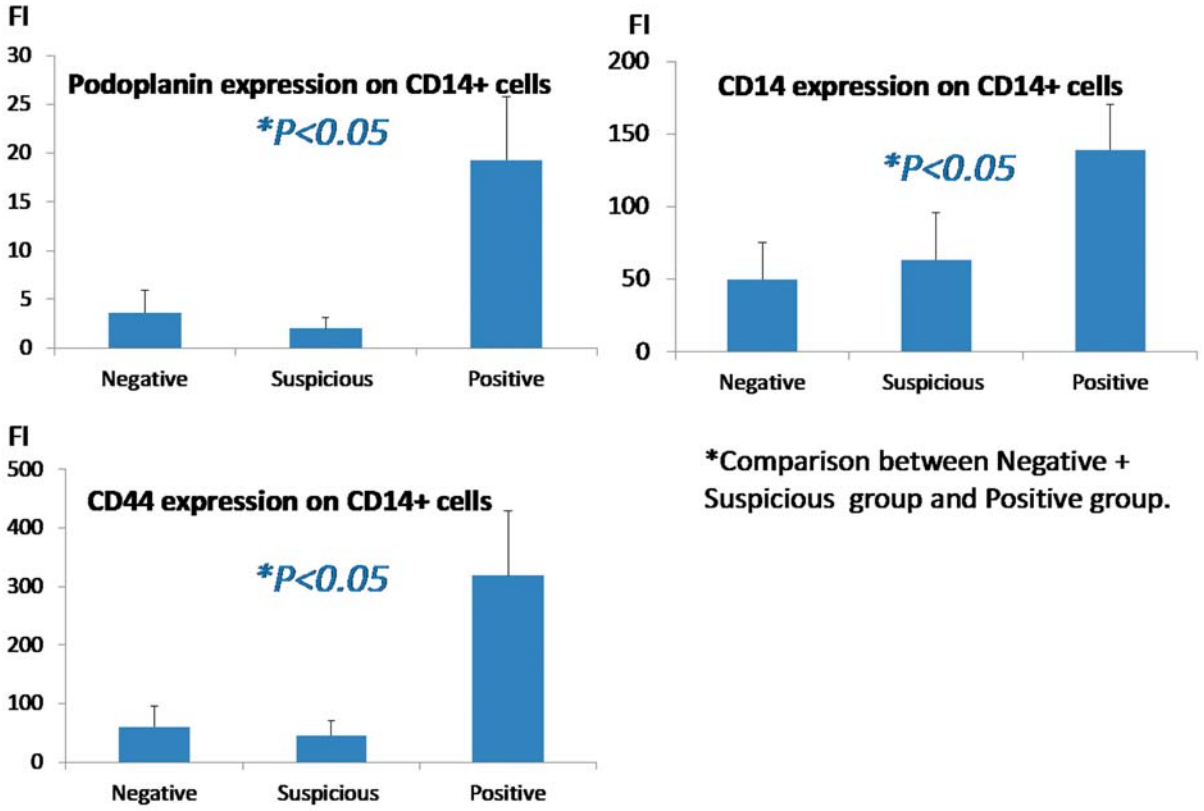

Figure 3. *The expression of surface cell markers on CD14+ cells (macrophages). *p<0.05; positive group vs. negative+suspicious groups.*

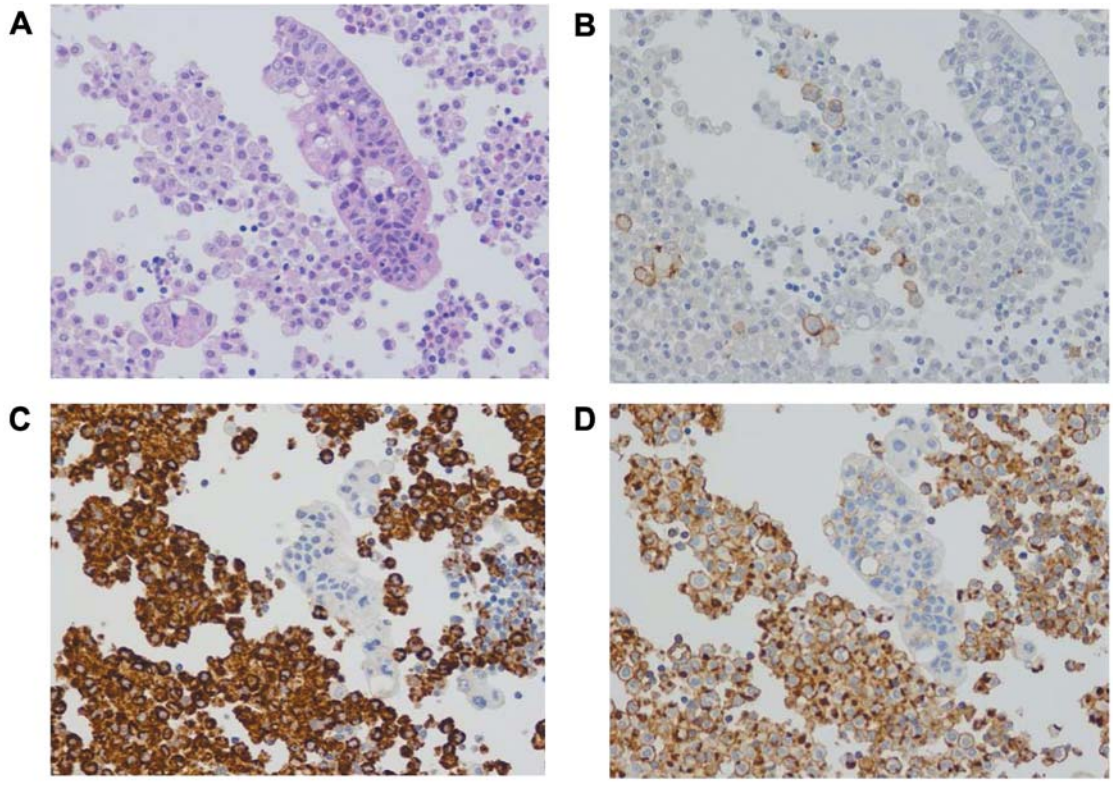

Figure 4. *Cell block immunohistochemistry for patient number 10. (A) Hematoxylin and eosin stain, (B) podoplanin, (C) CD68, (D) CD44.*

ascites-positive patients was higher compared with that from ascites-negative and suspicious patients; however, the difference was not statistically significant (data not shown). The ICAM-1 expression on $CD14^+$ cells of the ascites-positive patients was higher than that of the ascites-negative and suspicious patients but the difference was not statistically significant (data not shown).

Figure 4 shows representative results of the cell block immunohistochemistry for patient number 10. The cell block immunohistochemistry confirmed the enhanced expression of podoplanin and CD44 in the macrophages (CD68-stained cells).

Discussion

In the present study, we found that the expression of podoplanin and CD44, that are associated with cell migration and invasiveness, was up-regulated in macrophages and mesothelial cells of epithelial ovarian cancer ascites and quantitatively detected by a flow cytometry assay. The expression of surface molecules in the macrophages present in the peritoneal fluid of epithelial ovarian cancer patient was enhanced, while the expression patterns in the mesothelial cells showed the same tendency without, however, statistical significance.

All organs within the peritoneal cavity are lined with a continuous monolayer of mesothelial cells (13, 14). Electron micrograph studies of ovarian cancer nodules attached to peritoneal cavity organs have revealed that mesothelial cells are absent from the region underneath the attached tumor mass (15, 16), suggesting that mesothelial cells may act as a protective barrier against ovarian cancer metastasis and, thus, might be excluded during processes leading to successful tumor cell implantation on the peritoneal tissue. To test this hypothesis, we compared the expression of surface molecules in detached mesothelial cells from the peritoneal fluid and the lining mesothelial cells from patients with epithelial ovarian cancer.

The enhanced expression of surface molecules in macrophages may reflect the augmented migration and invasiveness of these cells in the disseminated portion of cancer cells. Tumor-associated macrophages (TAMs) derived from circulating monocytes have been identified as the main components of the tumor microenvironment and shown to stimulate tumor growth and metastasis (17). An increase in TAMs around the tumor microenvironment has been closely associated with a poor prognosis in cancer patients (18-19), whereas M2 TAMs have been shown to promote tumor growth and metastasis by stimulating angiogenesis and

lymphangiogenesis in tumors (20). However, further investigation is needed to clarify whether or not the macrophages in the ovarian cancer ascites are M2 TAMs and how depletion of podoplanin and CD44 in the macrophages of ovarian cancer ascites would affect cancer progression. Recently, the antitumor and antimetastatic activity of synthetic hydroxystilbenes was reported, with the mechanism being attributed to inhibition of lymphangiogenesis and M2 macrophage differentiation of tumor-associated macrophages (21).

In conclusion, the expression of surface molecules, such as podoplanin, CD44 and CD14, related to the cell-cell or cell-matrix interaction was found to be enhanced in the macrophages present in the peritoneal fluid of epithelial ovarian cancer patients. The analysis of the peritoneal fluid using flow cytometry immunophenotyping may be useful for evaluating the diagnosis and discovering the pathophysiological mechanisms underlying the peritoneal dissemination of epithelial ovarian malignant tumors.

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