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RESEARCH ARTICLE

Clinical significance of side population in ovarian cancer cells

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Abstract Recently, accumulating evidence has suggested that tumors, including ovarian cancer, are composed of a heterogeneous cell population with a small subset of cancer stem cells (CSCs) that sustain tumor formation and growth. The emergence of drug resistance is one of the most difficult problems in the treatment of ovarian cancer, which has been explained recently by the potential of CSCs to have superior resistance against anti-cancer drugs than conventional cancer cells. In this study, we expanded this line of study to examine whether this phenomenon is also observed in clinical specimens of ovarian cancer cells. In total we could analyze 28 samples out of 60 obtained from ovarian cancer patients. The clinical samples were subjected to testing of the expression of side population (SP) as a CSC marker, and according to the presence of SP (SP+) or absence of SP (SP−), clinicopathological significances were analyzed. Although there was no statistical significance, there were more SP+s in recurrent cases as well as in ascitic and peritoneal dissemination than in primary tumor of the ovary. There was no correlation between SP status and FIGO staging. In 19 cases of those who could be followed more than 6 months from initial therapy, there were 8 cases of recurrence or death from disease, and all of these were SP+. On the other hand, in 11 cases of disease-free survivors, 6 were SP+. There was a significant difference in prognosis between SP+ and SP− ($p = 0.017$). Although this study was limited, it revealed that SP could be contained more in recurrent or metastatic tumors than in primary tumors, and also that the presence of SP could be a risk factor of recurrence in ovarian cancer. Therefore, a novel therapeutic strategy targeting SP could improve the prognosis of ovarian cancer.

Keywords Ovarian cancer · Side population (SP) · Cancer stem cells (CSCs) · Prognosis

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Introduction

Ovarian cancer is the most lethal gynecological malignancy. The number of ovarian cancer patients is gradually increasing in Japan, as well as in the United States and European countries. In 2008, 4,599 patients died of ovarian cancer, making it the tenth most fatal malignancy in Japan. Usually ovarian cancer patients lack symptoms because the ovaries reside within the pelvic cavity, and effective screening methods have not yet been developed. As a result, more than half of the ovarian cancer patients are stage III or IV at the time of diagnosis. Although taxanes improve 5-year survival in advanced ovarian cancer [1], many patients experience recurrence because of resistance to anti-cancer drugs. Therefore, novel therapeutic strategies are needed.

Recently, accumulating evidence has suggested that tumors, including ovarian cancer, are composed of a heterogeneous cell population with a small subset of cancer

stem cells (CSCs) that sustain tumor formation and growth. The emergence of drug resistance is one of the most difficult problems in the treatment of ovarian cancer, but has recently been explained by the discovery that CSCs have resistance against anti-cancer drugs that is superior to that of conventional cancer cells. Therefore, clarifying the mechanism of CSC drug resistance could result in the development of novel therapeutic strategies.

Several reports have shown that CSCs are enriched in a side population (SP) that is recognized by fluorescence-activated cell sorting (FACS) [2–4]. To date, SP has been detected in various solid tumor cells, such as breast [5], colon [6, 7] and pancreatic cancer [8], as well as ovarian cancer [9–11]. We also found that SP is significantly increased in chemoresistant ovarian cancer cell lines (in submission). However, the significance of SP in clinical samples, such as cancer tissue or cancer cells in ascitic fluid, still remains unclear [12, 13]. Therefore, we conducted a clinical study to clarify the significance of SP in clinical samples.

Materials and methods

Clinical samples

Clinical samples, such as cancer tissue or cancer cells in ascitic fluid, were obtained from patients with ovarian, peritoneal or tubal cancer from January 2009 to July 2010 at St. Marianna University Hospital. Informed consent was obtained from all patients, and this study was approved by the internal review board of our institution. Tissues or ascites harvested during laparotomy or by ultrasound-guided puncture were centrifuged to isolate the cellular component and then resuspended in HAM-12 containing antibiotics.

Analysis of the side population in human ovarian cancer tissues and ascites by flow cytometry

Human ovarian cancer tissues were washed with phosphate-buffered saline (PBS) and then cut into 1-mm-diameter pieces. These pieces were then transferred into PBS containing 2.4 U/ml of DISPASE® (GIBCO) and incubated for 60 min at 37°C. After passing through a Cell Strainer™ with 70-μm pore size (BD Falcon), red blood cells were lysed using PharmLyse™ (BD Bioscience), and the resulting single-cell suspension was diluted to 1.0×10^6 cells/ml. For the staining of the SP, cells were pre-incubated for 10 min at 37°C and then incubated for an additional 80 min at 37°C with 5 mg/ml Hoechst 33342 (Sigma-Aldrich, Japan). SP cells were determined by the exclusion of Hoechst 33342, which was inhibited by verapamil (Sigma-Aldrich, Japan). Verapamil was added after pre-incubation at the start of the Hoechst 33342 staining at a final concentration of 50 μM. Live cells were gated based on forward and side scatter as well as lack of propidium iodide uptake. Flow cytometry was performed using a J-SAN® (Bay Bioscience, Japan) instrument and analyzed by FlowJo® software (TOMY Digital Biology Co. Ltd., Japan). Tissues/cells that satisfied the following conditions were defined as tissues/cells containing SP: (1) the percentage of cells in the SP fraction was >0.15%, and (2) after verapamil administration, more than half of the cells were diminished from the SP fraction. Mann-Whitney *U* test or Fisher's exact probability test were used to determine statistical significance, and $p < 0.05$ was considered significant.

Results

Clinical samples were obtained from a total of 60 patients. Six cases were not primary ovarian cancer and were excluded from this study. Among the remaining 54 samples, 26 could not be analyzed because they contained too much debris, were contaminated with normal tissues or were too small to be analyzed. In the 28 samples that were evaluable for the study, the patient age ranged from 20 to 73 years old. Patient characteristics are shown in Table 1.

Of 28 samples, 18 contained an SP (SP+) and 10 did not (SP−). Figure 1 shows the FACS analysis for a SP+ sample. The mean ages of SP+ and SP− patients were 52.6 and 49.2 years old, respectively; however, they were not significantly different. Of 24 patients with samples obtained at primary surgery or neoadjuvant chemotherapy (NAC), 15 were SP+ (62.5%). Of four recurrent cases, three were SP+ (75%). Although the difference between primary and recurrent cases was not significant ($p = 0.55$), more recurrent cases were SP+.

Of 18 samples obtained directly from ovarian tumors, 10 were SP+ (55.6%). However, of eight ascitic fluid and two peritoneal dissemination samples, six (75%) and two (100%) were SP+, respectively. Although the difference was not significant, there were more SP+ samples in ascitic fluid and peritoneal dissemination than in primary ovarian tumors.

Focusing on the histology, there were eight cases of endometrioid carcinoma (SP+: 5 vs. SP−: 3), seven cases of serous carcinoma (4 vs. 3) and five cases of clear cell carcinoma (3 vs. 2), with no correlation between SP+ and histology. According to FIGO staging, there were 11 cases of stage I/II and 17 cases of stage III/IV. Of the stage I/II cases, 5 were SP+ (45.4%), while 13 of the stage III/IV cases were SP+ (76.5%). There was no correlation between SP+ and FIGO staging ($p = 0.22$).

In the 19 patients that could be followed more than 6 months from the initial therapy, there were 8 cases of recurrent or fatal disease, and all 8 were SP+. However, in

Table 1 Patient characteristics that could be analyzed by FACS

Age	Background	Pathology	FIGO	Sample	Prognosis
SP+ group					
37	0	Endometrioid	Ic	T	[a]
38	0	Endometrioid	IIIa	T	DFS
38	0	LPM, serous	Ic	T	[a]
41	0	Endometrioid	IIIc	A	[a]
42	0	Serous	IIIc	T	DOD
43	0	Clear	IIIc	T	DOD
43	1	Clear	IIIc	A	DOD
48	0	Serous	IIIc	T	[a]
48	0	Endometrioid	Ic	T	DFS
56	0	Mucinous	IIIc	T	AWD
56	0	Clear	IIIc	T	DFS
60	0	Mucinous	IIIc	A	DOD
61	1	Serous	IIIc	P	AWD
62	0	Adenocarcinoma[b]	Ia	A	DFS
64	0	Serous	IIIc	P	DOD
67	1	SSPC	IIIc	A	DOD
69	0	Adenocarcinoma[c]	IIIc	A	[a]
73	0	Endometrioid	Ic	T	DFS
SP− group					
20	0	Dysgerminoma	Ia	T	[a]
20	0	LPM, mucinous	Ic	A	[a]
40	1	Endometrioid	IV	T	DFS
41	0	Endometrioid	IIIc	T	[a]
46	0	Endometrioid	Ic	T	DFS
60	0	Clear	Ic	T	DFS
61	0	Serous	IIIc	T	DFS
67	0	Serous	Ic	T	DFS
68	0	Serous	IIIc	A	[a]
69	0	Clear	IIc	T	DFS

Background: 0: obtained from primary surgery; 1: recurrent case

Sample: *T* ovarian tumor, *A* ascites, *P* peritoneal dissemination

DFS disease-free survival, *AWD* alive with disease, *DOD* died of disease

[a] Follow-up period <6 months

[b] Histological diagnosis could not be made due to chemotherapeutic effect

[c] Laparotomy was not done, and histological diagnosis was made only by cytology of ascitic fluid

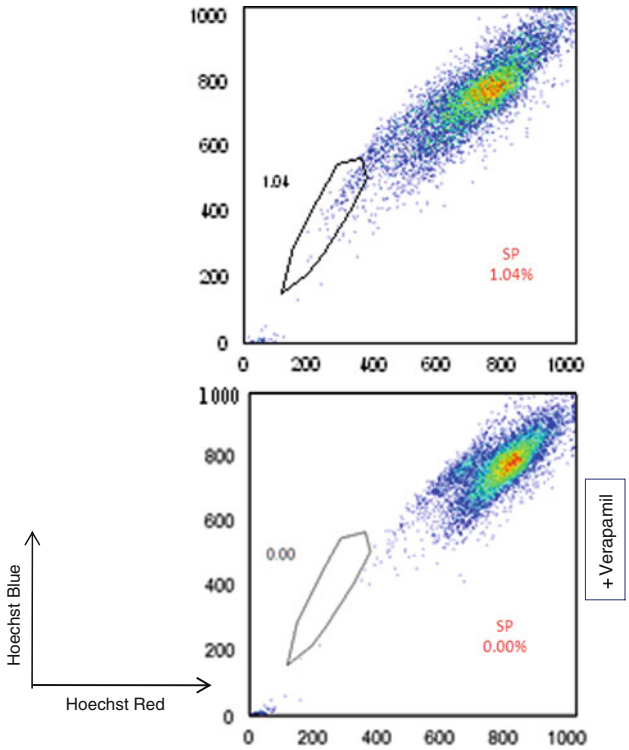

Fig. 1 An example of a patient determined as SP+ by flow cytometric analysis. After administration of verapamil, cells of the SP fraction were diminished. *Vertical axis* intensity of Hoechst blue, *horizontal axis* intensity of Hoechst red

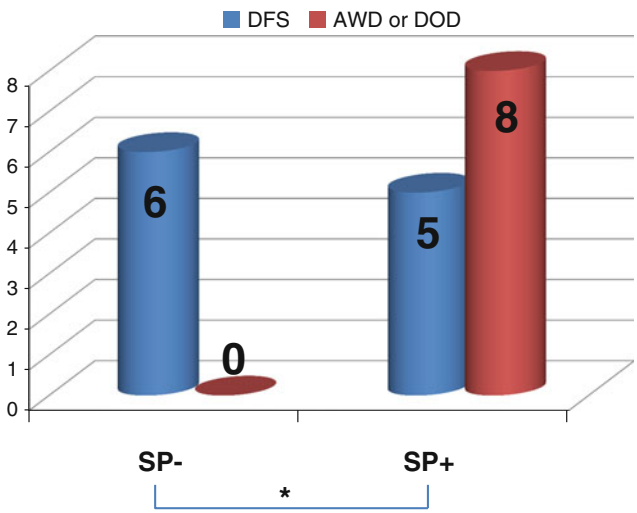

Fig. 2 Prognosis of the patients according to the presence or absence of SP. *There was statistical significance in the prognosis between SP+ and SP− ($p = 0.017$). *Vertical axis* number of cases. *DFS* disease-free survival, *AWD* alive with disease, *DOD* died of disease

11 cases of disease-free survivors, 6 were SP+, and 5 were SP−. SP+ was significantly correlated with a worse prognosis than SP− ($p = 0.017$, Fig. 2). Of the SP+ cases that were histologically designated as serous and mucinous carcinoma, all were recurrent or the patient died of disease. Of the SP+ clear cell carcinomas, two out of three cases recurred or the patient died of disease. However, there was no recurrent case in three SP+ endometrioid carcinoma cases.

Discussion

Several groups have reported about SP in ovarian cancer. Szotek et al. detected SP in human ovarian cancer cell lines

as well as in primary ascites cancer cells. Moreover, SP cells in ovarian cancer were reported to have CSCs characteristics, suggesting that CSCs are also enriched in SP in ovarian cancer [9, 14]. In our clinical study, the presence of SP was assessed in clinical tissues/cells in ovarian, peritoneal and tubal carcinoma patients. A total of 54 cases were analyzed, but the percentage of evaluable cases was only 51.9% (28/54). The evaluable rate was relatively low because cancer tissues in vivo could contain some amount of normal cells, mucous and cell debris. Moreover, cancer tissues could contain various polyclonal cancer cells. These factors might have interfered with analysis by FACS in our study. For this reason, novel procedures that could extract only cancer cells from clinical samples should be developed.

Once ovarian cancer develops, the tumor surface ruptures, and tumor cells are disseminated into the peritoneal cavity. Most ovarian cancers will spread by this process. CSCs were reported to be related to metastasis [15–17], which, if true, suggests that peritoneal dissemination could be promoted by CSCs. In the present study, although it was not statistically significant, SP+ cases were detected more frequently in ascitic fluid or peritoneal dissemination than in primary tumors (75, 100 vs. 55.6%). This result suggests that peritoneal dissemination in ovarian cancer is correlated with SP, which is supported by the fact that SP+ cases were detected more frequently in FIGO stage III/IV patients than in stage I/II (45.5 vs. 76.5%). Stage III/IV patients already have peritoneal dissemination and/or distant metastasis, so it might be reasonable that SP could be detected in tissues from stage III/IV patients.

Although the histological difference was not correlated to the presence or absence of SP, in serous, mucinous and clear cell carcinoma patients that were SP+, most experienced recurrence or died of disease. However, SP+ endometrioid carcinoma patients did not experience recurrence. Although the follow-up term was too short and the number of patients too small to reach a conclusion, in endometrioid carcinoma, the prognosis might not be affected by the presence of SP.

In the present study, all eight SP+ patients experienced recurrence or died of disease within 2 years from initial therapy, suggesting that SP might be correlated with the risk of recurrence or death from disease. Some of these patients were resistant to the initial chemotherapy, which may indicate that SP containing rich CSCs is correlated to chemoresistance. Therefore, although the number of the studied cases was small, it indicates that detecting SP could predict the chemoresistance or prognosis of ovarian cancer.

In conclusion, our study suggests that SP might be correlated to peritoneal dissemination, and might also predict chemoresistance or prognosis in ovarian cancer. Because our study consisted of a small number of patients, a larger study should be conducted in order to define the clinical significance of SP in ovarian cancer. The technical difficulties of treating clinical samples for detecting SP/CSCs should also be overcome.

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