Extravasated platelet aggregation contributes to tumor progression via the accumulation of myeloid-derived suppressor cells in gastric cancer with peritoneal metastasis

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Abstract. Extravasated platelet aggregation (EPA) serves an important role in the cancer microenvironment during cancer progression, and has been demonstrated to interact with tumor cells in several types of cancer. EPA induces epithelial-mesenchymal transition (EMT) via transforming growth factor-β, and also recruits immunosuppressive cells, including regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSCs). However, the role of EPA in gastric cancer with peritoneal metastasis remains unknown. The present study analyzed the association between EPA and prognosis in patients with gastric cancer with peritoneal metastasis. The present study evaluated 62 patients diagnosed with advanced gastric cancer with peritoneal metastasis between 2001 and 2016. EPA, EMT, Treg cells and MDSCs in peritoneal metastatic lesions were detected by immunohistochemical evaluation of CD42b, SNAIL, FOXP3 and CD33, respectively. CD42b expression was observed in 56.5% (35/62) of peritoneal metastatic lesions. CD42b expression in peritoneal metastatic lesions was associated with poor overall survival compared with lower frequencies (hazard ratio, 2.03; 95% confidence interval, 1.12-3.69; P=0.018). SNAIL, FOXP3 and CD33 expression were not associated with overall survival, but CD33 expression was markedly higher in CD42b-positive patients (P=0.022). These results indicated that EPA affects immunosuppression by recruiting MDSCs in the tumor microenvironment via the secretion of soluble factors, resulting in tumor progression. EPA may be a novel therapeutic target for gastric cancer with peritoneal metastasis.

Introduction

Gastric cancer is one of the most common types of cancer worldwide, and a leading cause of cancer-related deaths (1). Peritoneal metastasis is a frequent recurrent pattern in gastric cancer, and is related to the poor prognosis. Although various treatments including systemic or intraperitoneal chemotherapy for peritoneal metastasis improved, satisfactory outcomes have not been achieved (2,3). There is thus a need for novel treatments in addition to conventional surgery and chemotherapy.

Interactions between cancer cells and tumor stromal cells have a key role in tumor progression, invasion and metastasis. The tumor microenvironment consists of several kinds of cells, such as endothelial cells, cancer-associated fibroblasts (CAFs), and immune cells. We previously reported that activated peritoneal mesothelial cells by transforming growth factor beta 1 (TGF-β1) caused to tumor invasion and progression (4). Moreover, the intraperitoneal cavity contains a lot of M2 anti-inflammatory phenotype of macrophages, which caused to the development of peritoneal metastasis in gastric cancer (5).

Platelets are the discoid anucleate hematopoietic cells that are responsible for maintaining hemostasis. On the other hand, they have been recognized as key regulators for tumor development and metastasis in several tumors (6-8). Platelet aggregation in the blood vessels protects cancer cells from several stress and immunocompetent cells through the platelet coating around tumor cells. Platelets further promote cancer cell attachment to intravascular endothelial cells, leading to extravasation and the colonization of secondary tumors in new microenvironments (9). However, few studies have examined the role of platelets in primary tumors. We previously found a correlation between extravasated platelet aggregation (EPA) and epithelial-mesenchymal transition (EMT) in breast cancer, and showed that patients with EPA were less responsive to

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neo-adjuvant chemotherapy (10). Furthermore, EPA in primary gastric cancer biopsy specimens was inversely correlated with pathological response to preoperative chemotherapy, and was identified as an independent prognostic factor (11).

Platelets contain a lot of TGF-β, which they secrete following platelet activation (12,13). TGF-β promotes the invasion ability and chemoresistance of tumor cells via the induction of EMT, and also facilitates the induction of immunosuppression by regulatory T (Treg) cells accumulation into the tumor microenvironment (14). TGF-β-induced forkhead box (FOX)P3-positive Treg cells have been shown to participate in the maintenance of immunosuppression, and to play critical roles in chemoresistance (15,16). In addition to Treg cells, several studies have demonstrated the importance of myeloid-derived suppressor cells (MDSCs) in tumor-associated immune suppression (17,18). MDSCs may promote the Treg cells infiltration into tumor stroma through the secretion of TGF-β. Collectively, these findings suggest that EMT, MDSCs, and Treg cell infiltration induced by EPA are key regulators of cancer progression.

In the present study, we investigated the relationship between EPA and prognosis in patients with gastric cancer with peritoneal metastasis by analyzing the expression of CD42b, SNAIL, FOXP3, and CD33 using immunohistochemistry.

Materials and methods

Patient samples. Sixty-two patients diagnosed with advanced gastric cancer with peritoneal metastasis between 2001 and 2016 were evaluated retrospectively. As inclusion criteria, all patients had collected the peritoneal nodule by surgery included the staging laparoscopy and diagnosed pathologically. Specimens from peritoneal metastatic lesions were collected before chemo/radiotherapy. Patients were excluded for the following reason: Poor general condition or refuse treatment and are unable to treat the gastric cancer.

All procedures were carried out in accordance with the ethical standards of the responsible committees on human experimentation and with the Helsinki Declaration of 1964 and later versions. This study was approved by the Institutional Review Board of Kanazawa University Graduate School of Medical Sciences (study no. 2789). Written informed consent was obtained from all patients.

Evaluation for clinical status. Primary and metastatic lesions were evaluated by gastrointestinal endoscopy and contrast-enhanced computed tomography scan. Peritoneal metastasis was diagnosed by laparoscopy examination or open surgery before chemotherapy, and classified into three categories according to the 15th edition of the General Rules for Gastric Cancer Study of the Japanese Research Society for Gastric Cancer: P1a (greater omentum, lesser omentum, anterior lobe of the transverse colonic membrane, or membrane of the pancreatic surface or spleen), P1b (a few scattered metastases to upper abdominal peritoneum, namely, the parietal peritoneum close to the umbilical side and the visceral peritoneum close to the cranial transverse colon), and P1c (many metastases to middle or lower peritoneum). The ascites level was evaluated by CT and classified into four groups: None, mild (limited the pelvic cavity), moderate (over the pelvic cavity), and severe (all over the abdominal cavity). Univariate analyses of prognostic factors for overall survival (OS) were performed. Patient-related factors included age, sex, and European Cooperative Oncology Group (ECOG) performance status was retrospectively examined. Tumor-related factors were categorized according to the WHO Classification of tumours, 5th edition (19).

Immunohistochemistry. EPA was investigated by immunostaining for CD42b. CD42b (GPIbα) is platelet activation specific marker involved in the process of coagulation (20). All specimens were fixed in 10% formalin, embedded in paraffin, and cut into the 3-μm tissue sections. The specimens were deparaffinized through a graded series of xylene and ethanol. For antigen retrieval, sections were pretreated in 1 mM citrate buffer (pH 6.0), and autoclaved for 10 min at 120°C. Endogenous peroxidase block was used by peroxidase block solution provided with the EnVision kit for 20 min. After blocking endogenous peroxidase, sections were incubated with 5% normal goat serum for 20 min to block nonspecific staining. Sections were subsequently incubated for 2 h at room temperature with anti-platelet antibody (1:100 dilution, anti-CD42b rabbit monoclonal; Abcam), anti-SNAIL antibody (1:50 dilution, anti-SNAIL rabbit polyclonal antibody, ab180714; Abcam), anti-FOXP3 antibody (1:50 dilution, anti-FOXP3 mouse monoclonal, 236A/E7; Abcam), or anti-CD33 antibody (1:100 dilution, anti-CD33 mouse monoclonal antibody, NCL-L-CD33; Leica Biosystems). After the sections were washed in Phosphate-buffered saline: PBS, immunoreactivity was visualized by EnVision reagent (Dako Co.), and the slides were developed with diaminobenzidine and counterstained with hematoxylin.

Evaluation of immunostaining. Immunostaining sections were evaluated in tumor sites containing cancer cells. To evaluate CD42b expression, immunostained cells were observed in five non-overlapping intratumoral fields at 400x magnification. Cancer cells and immunostained cells in the field were counted, and ≥10% of cancer cells were stained were defined as positive and <10% were as negative. For SNAIL evaluation, an immunoreactive score was used by multiplying the staining intensity (0-3) and the stained cell ratio (0-4). Specimens with a score 0 were classified as negative and samples with a score 1-12 were classified as positive (21). FOXP3 cells were evaluated by counting intratumoral fields under high power (x400) and the number of FOXP3 positive cells was defined as the mean number per field. The average number of FOXP3 positive cells was calculated, and ≥5.5 was defined as high infiltration and <5.5 as low infiltration (22). CD33-positive cell infiltration was evaluated by counting intratumoral fields under high power (x400). The average number of CD33-positive cells was evaluated: ≥11 was defined as high infiltration and <11 as low infiltration (23).

Statistical analysis. Differences in CD42b expression and categorical variables were analyzed using a χ^2 test. Overall survival rates were calculated by the Kaplan-Meier method, and the log-rank test was used to compare results between survival times and between subgroups. P<0.05 was taken to indicate statistical significance. All statistical analysis was performed using SPSS v23 (SPSS).

Results

Patient and clinicopathological characteristics. The clinicopathological characteristics of the 62 patients at the time of diagnosis of peritoneal metastasis are shown in Table I. The median age was 63 (range, 28-83) years, and 26 patients were men and 36 patients were women. 12 patients had a performance status (PS) ≥1, and the remaining 50 patients had a PS of 0. 49 patients had initial and 13 patients had recurrent peritoneal metastasis. Primary gastric cancer was intestinal-type adenocarcinoma in 13 patients and diffuse-type adenocarcinoma in the remaining 49. 27 patients had a macroscopic classification of Borrmann type 4. The P statuses according to the Japanese Classification of Gastric Carcinoma 15th edition were P1a in 8 cases, P1b in 5 cases, and P1c in 49 cases. The levels of ascites were none in 23, mild in 17, moderate in 8, and severe in 14. Ten patients had other distant metastases, including liver, lung, or lymph node metastasis.

CD42b expression in peritoneal metastasis. We investigated CD42b expression as a marker of EPA in 62 patients with peritoneal metastasis. All peritoneal metastasis specimens were collected before chemotherapy. CD42b expression was observed in 56.5% (35/62) of peritoneal metastatic lesions (Fig. 1A and B). EPA was observed around tumor cells and around CAFs (Fig. 1C).

Relationship between CD42b expression and clinicopathological features. The relationships between CD42b expression and clinicopathological features, including age, sex, PS, Borrmann type, microscopic type, T stage, N stage, P status, ascites volume, and other distant metastasis are shown in Table II. CD42b expression was clearly related to sex ($P<0.025$) and microscopic type ($P<0.038$), but not to age, performance status, T stage, N stage, P status, ascites volume, or other distant metastasis.

SNAIL, FOXP3, and CD33 expression. SNAIL expression was mainly confirmed in the nuclei of cancer cells. Positive SNAIL expression was observed in 67.7% (42/62) of cases (Fig. 1D). There was no relationship between SNAIL and CD42b expression ($P=0.271$, Table II). Furthermore, there was no association between SNAIL expression and OS ($P=0.601$, Table III). The Treg cell marker FOXP3 was also confirmed in the nuclei of T cells. High infiltration of FOXP3-positive cells was detected in 16.1% (23/62) of cases (Fig. 1E). There was no relationship between FOXP3 and CD42b expression ($P=0.564$, Table II) or OS ($P=0.823$, Table III). High infiltration of CD33-positive cells was detected in 59.6% (37/62) of cases (Fig. 1F), and was clearly correlated with CD42b expression ($P=0.022$, Table II), but not correlated with OS ($P=0.111$).

Relationship between patient characteristics and overall survival. The relationships between clinicopathological features and OS were evaluated by log-rank tests (Table III). OS was clearly lower in patients with P1c (compared to P1a/P1b status), with severe ascites (compared with no or moderate ascites), and in patients with other distant metastasis (Table III). OS was not significantly related to age, sex, initial

Table I. Clinical and pathological data of 62 patients with gastric cancer with peritoneal metastasis.

Characteristics	Value
Age, years (median, range)	63 (28-83)
Sex, n	
Male	26
Female	36
Initial or recurrence, n	
Initial	49
Recurrence	13
ECOG performance status, n	
≥1	12
0	50
Borrmann macroscopic type, n	
1	1
2	3
3	26
4	27
5	5
Differentiation (Lauren classification), n	
Intestinal	13
Diffuse	49
Clinical T stage, n	
T1	0
T2	0
T3	11
T4	51
Clinical N stage, n	
N0	15
N1	12
N2	7
N3	28
P status, n	
P1a	8
P1b	5
P1c	49
Ascites, n	
None	23
Mild	17
Moderate	8
Severe	14
Other distant metastasis, n	
Negative	52
Positive	10

P1a, greater omentum, lesser omentum anterior lobe of the transverse colonic membrane, or membrane of the pancreatic surface or spleen; P1b, a few scattered metastases to upper abdominal peritoneum, namely, the parietal peritoneum close to the umbilical side and the visceral peritoneum close to the cranial transverse colon; P1c, many metastases to middle or lower peritoneum; Ascites mild, limited to the pelvic cavity; Ascites moderate, over the pelvic cavity; Ascites severe, all over the abdominal cavity; ECOG, European Cooperative Oncology Group.
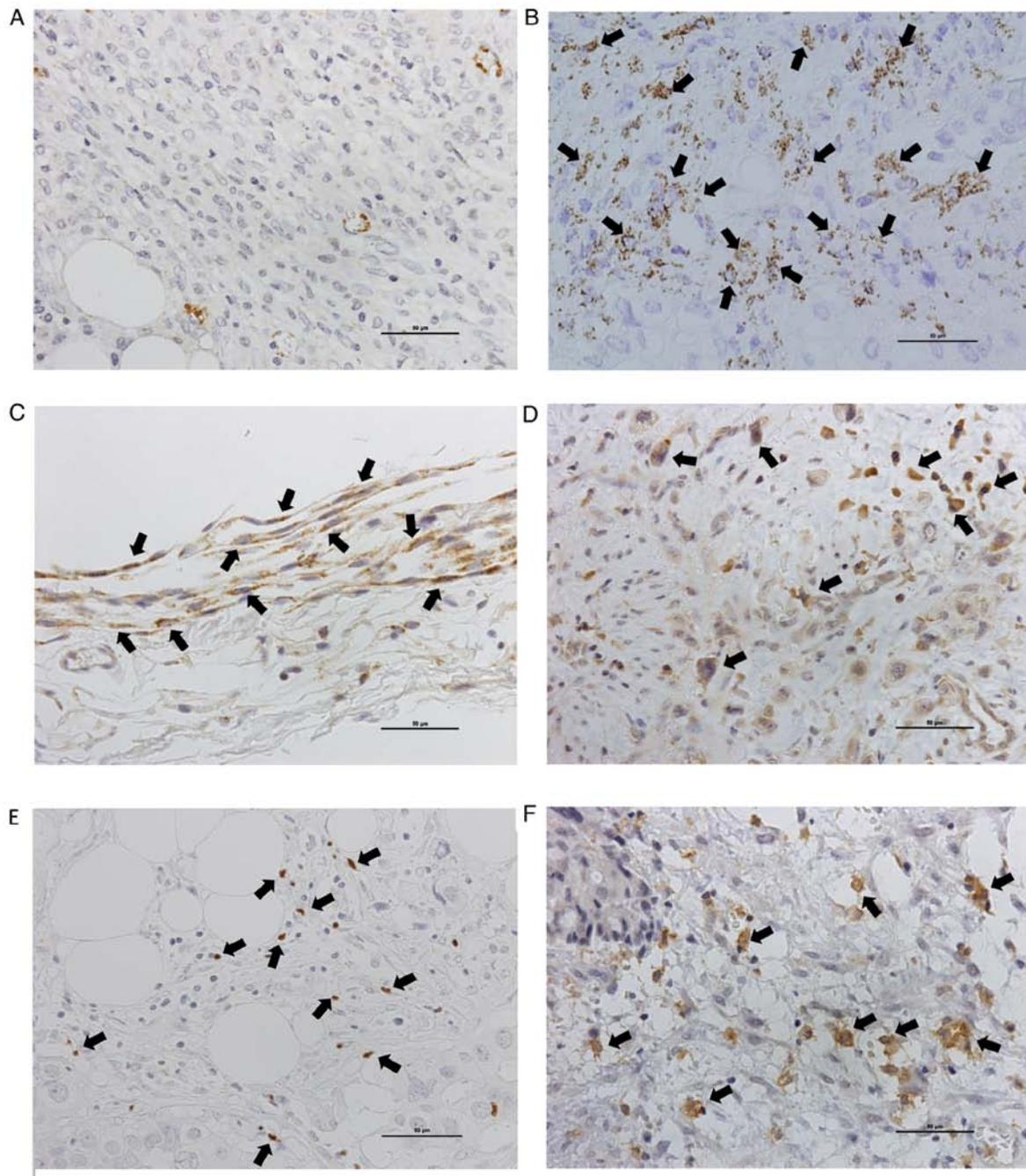
Figure 1. Representative immunostaining images of peritoneal metastasis. (A) Cancer cells and immunostained cells in the field were counted. Positive, ≥10% of cancer cells were stained; negative, <10% of cancer cells were stained. Negative expression of CD42b. (B) CD42b-positive platelets (black arrows) were detected around tumor cells (>10%). (C) Platelet aggregation (CD42b expression) was observed around the CAFs (black arrows). (D) SNAIL expression was mainly confirmed in the nuclei of cancer cells (black arrows). (E) The regulatory T cell marker forkhead box P3 was also identified in the nuclei of T cells (black arrow). (F) CD33 expression was observed in cancer stroma (black arrows). Scale bar, 50 μm. CAFs, cancer-associated fibroblasts.

or recurrent peritoneal metastasis, ECOG PS, Borrmann type, or microscopic type.

Survival curves according to CD42b, SNAIL, FOXP3, and CD33 expression. OS curves for gastric cancer with peritoneal metastasis are shown in Fig. 2. Median OS for CD42b-positive patients were 13.6 months compared with 28.4 for CD42b-negative patients (hazard ratio 2.03, 95% confidence interval 1.12-3.69, P=0.018). In contrast, SNAIL, FOXP3, and CD33 expression in peritoneal metastatic lesions were not significantly related to OS (Fig. 2B-D).

Discussion

We detected CD42b expression as a marker of EPA in 56.4% of peritoneal metastases for patients with gastric cancer in the current study, and clarified that it was a poor prognosis factor. All patients were diagnosed as Stage IV gastric cancer with peritoneal metastasis. In our previous study, there were no significant association between CD42b expression and clinical stage (11). Generally, tumor stroma contains fibroblasts which express the p-selectin, and tend to aggregate the platelets (Fig. 1C). In this study, we evaluated the gastric cancer cells and

Table II. Association between CD42b expression and the clinicopathological characteristics of patients with gastric cancer with peritoneal metastasis.

Variables	CD42(-), n (n=27)	CD42(+), n (n=35)	P-value
Age, years			0.639
≥70	7	10	
<70	20	25	
Sex			0.025
Male	7	19	
Female	20	16	
Initial or recurrence			0.697
Initial	21	28	
Recurrence	6	7	
ECOG performance status			0.425
0	24	26	
≥1	3	9	
Borrmann macroscopic type			0.373
Type 4	10	17	
Not	17	18	
Microscopic			0.038
Intestinal	9	4	
Diffuse	18	31	
Clinical T stage			0.678
2-3	5	6	
4	22	29	
Clinical N stage			0.405
0-2	16	18	
3	11	17	
P status			0.228
1a,1b	8	5	
1c	19	30	
Ascites			0.084
None-Moderate	23	25	
Severe	4	10	
Other distant metastasis			0.296
Negative	24	28	
Positive	3	7	
SNAIL expression			0.271
Negative	11	9	
Positive	16	26	
FOXP3 infiltration			0.564
Low	18	21	
High	9	14	
CD33 infiltration			0.022
Low	17	8	
High	10	27	

ECOG, European Cooperative Oncology Group; FOXP3, forkhead box P3.

platelet aggregation (Fig. 1B), and did not evaluate the platelets around the fibroblasts. Platelets play an important role in the tumor microenvironment during cancer development, and have been shown to interact with tumor cells. Mikami *et al* (24) investigated that platelets facilitated the gastric cancer cells growth and that this growth was disturbed by antiplatelet

Table III. Univariate analyses of clinicopathological parameters associated with overall survival in patients with gastric cancer with peritoneal metastasis.

Variables	Odds ratio	95% CI	No.	P-value
Age, years				0.287
≥70	1.519	0.700-3.293	17	
<70			45	
Sex				0.522
Male	1.211	0.673-2.180	26	
Female			36	
Initial or recurrence				0.286
Initial	1.466	0.723-2.972	49	
Recurrence			13	
ECOG performance status				0.331
0	1.556	0.838-2.888	50	
≥1			12	
Borrmann macroscopic type				0.736
Type4	0.905	0.506-1.619	27	
Not			35	
Microscopic				0.535
Intestinal	0.811	0.418-1.575	13	
Diffuse			49	
P status				0.022
1a,1b	2.242	1.070-4.698	13	
1c			49	
Ascites				0.009
None-Moderate	2.555	1.325-4.928	48	
Severe			14	
Other distant metastasis				0.043
Negative	2.231	1.006-4.948	10	
Positive			52	
CD42b expression				0.018
Negative	2.029	1.115-3.690	27	
Positive			35	
SNAIL expression				0.606
Negative	0.85	0.459-1.576	20	
Positive			42	
FOXP3 infiltration				0.823
Low	1.073	0.580-1.983	39	
High			23	
CD33 infiltration				0.111
Low	1.712	0.878-3.341	25	
High			37	

P-values were obtained using a log-rank test. ECOG, European Cooperative Oncology Group; FOXP3, forkhead box P3.

drugs *in vitro* and *in vivo*. Platelets contain a large amount of growth factors, such as TGF-β, platelet-derived growth factor, epidermal growth factor, vascular endothelial growth factor (VEGF), sphingosine 1-phospate, and basic fibroblastic growth factor within the α-granules that are secreted following platelet activation (25). Han *et al* (26) reported that platelet pellet (10^6 platelets) from breast cancer patients contained higher TGF-β1 level (median 15.3 ng/ml) than control group (median 4.3 ng/ml). These growth factors affect the tumor progression, angiogenesis, invasion, EMT, and metastasis, not only in the blood vessels but also in the tumor stroma. Previous reports have suggested a correlation between EPA
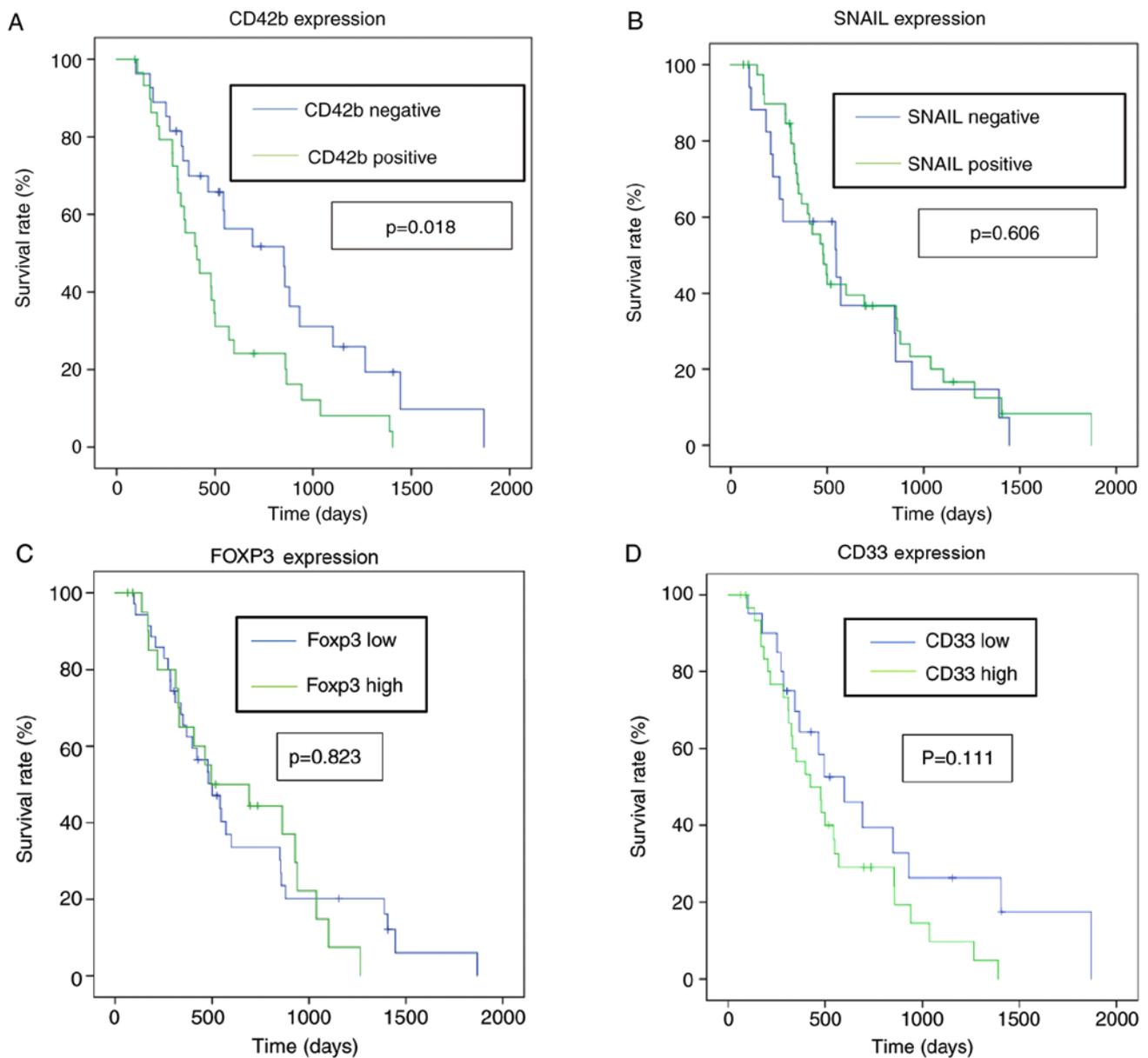
Figure 2. OS curves. (A) Median OS for CD42b-positive patients was 13.6 months compared with 28.4 months for CD42b-negative patients (hazard ratio, 2.03; 95% confidence interval, 1.12-3.69; P=0.018) (B) OS according to SNAIL expression (P=0.606; log-rank test). (C) OS according to FOXP3 expression. (D) OS according to CD33 expression. SNAIL (P=0.606), FOXP3 (P=0.823) and CD33 (P=0.111) expression in peritoneal metastatic lesions were not significantly associated with OS. FOXP3, forkhead box P3; OS, overall survival.

and EMT (10); however our data found no association between expression of the EMT marker SNAIL and EPA. This apparent discrepancy could be explained by differences in the tumor microenvironment between primary lesions and metastatic lesions, given that various stromal cells, including CAFs, human peritoneal mesothelial cells, mesenchymal stem cells, and M2 macrophages are present in the peritoneal metastasis environment and affect the EMT in tumor cells (5,27-29).

CD33 and CD11b are considered as basic markers of MDSCs. Yu *et al* (30), also reported that most CD33-positive cells in primary solid tumors were MDSCs. The current results found a close relationship between CD42b expression and CD33 infiltration in peritoneal metastasis. MDSCs play a pivotal role in tumor-related immunosuppression, and are recruited by several factors, including TGF-β, VEGF, and

matrix metalloproteinase 9, which are also secreted by platelets (31-33).

MDSCs promote tumor growth by shaping immuno-suppressive responses towards tumor tolerance, and also by supporting several processes necessary for neoplastic progression, such as tumor angiogenesis, cancer stemness, and metastasis dissemination. Our findings thus showed that the presence of EPA in the tumor microenvironment may induce the recruitment of MDSCs, resulting in tumor progression.

A large volume of ascites fluid was associated with a worse prognosis in this current study. However there was no significant correlation between platelet aggregation and ascitic fluid volume, there was a tendency for the volume of ascitic fluid to be higher in CD42b-positive cases. We previously reported

a close relationship between ascites volume and VEGF levels in the peritoneal cavity, with high levels of VEGF being correlated with a poor prognosis (34). Expression for VEGF was also detected in >70% of peritoneal metastases. These results indicate that VEGF secretion by cancer cells and platelets promote tumor development by inducing the angiogenesis in the peritoneal cavity.

VEGF is important for inducing an immunosuppressive microenvironment in several tumors via MDSCs (35). Horikawa *et al* (36) reported that VEGF in ovarian cancer with peritoneal metastatic lesions inhibited immune functions through MDSCs. Intratumoral MDSCs have also been shown to express VEGF receptor 2, and VEGF/VEGF receptor 2 signaling directly promoted MDSC differentiation and tumor infiltration (37,38). Collectively, these data suggest that MDSCs induced by tumor- and platelet-derived VEGF signaling play important roles in tumor immune evasion.

The present study had several limitations. First, regarding possible heterogeneity of tumor characteristics; the pathology of gastric cancer with peritoneal metastasis is complicated, and it is difficult to prove if a small biopsy sample is characteristic of metastatic lesion. Second, it is not sufficient to identify the MDSC by the CD33 staining, though the MDSC marker is lack of defined it. Furthermore, the accumulation of MDSCs by platelets is consideration in this study, it is necessary to study *in vitro* and *in vivo* experiments. However, there were few reports on the immune environment in gastric cancer with peritoneal metastasis, our results are important. Third, this investigation was conducted at a single institution, retrospective study, with a relatively small sample size. However, it is difficult to collect peritoneal metastatic tissue because gastric cancer patients with peritoneal metastasis generally do not undergo surgery. These factors should therefore be taken into account, and further, prospective, multi-center studies are needed to confirm the results before they can be generalized to daily clinical work.

In conclusion, the results of the present study suggest that EPA is associated with a poor prognosis in gastric cancer patients with peritoneal metastasis. EPA may not only increase tumor malignancy by secreting soluble factors such as platelet-derived growth factor, basic fibroblastic growth factor, and VEGF, but may also affect immunosuppression through the infiltration of MDSCs into the tumor microenvironment. These data indicate that EPA may represent a novel therapeutic target in gastric cancer with peritoneal metastasis.

Acknowledgements

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

TY and SF conceived the study. TY performed the experiments, analyzed data and wrote the manuscript. SF analyzed and interpreted the data. JK, MO, SI, YO, ST, KO, SN and IM were involved in conception and design. KN, TM, HTaj, HTak, IN and TO were involved in analysis and interpretation of data. SF contributed to manuscript writing and revision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were carried out in accordance with the ethical standards of the responsible committees on human experimentation and with the Helsinki Declaration of 1964 and later versions. This study was approved by the Institutional Review Board of Kanazawa University Graduate School of Medical Sciences (study number 2789). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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