Platelets surrounding primary tumor cells are related to chemoresistance

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Abstract. Platelets are crucial components of the tumor microenvironment that function to promote tumor progression and metastasis. In the circulation, the interaction between tumor cells and platelets increases invasiveness, protects tumor cells from shear stress and immune surveillance, and facilitates tumor cell extravasation to distant sites. However, the role and presence of platelets in the primary tumor have not been fully determined. Here, we investigated the presence of platelets around breast cancer primary tumor cells and the associations between these cells. We further investigated the associations among platelets, tumor cells, chemoresistance, and epithelial-mesenchymal transition (EMT). We retrospectively analyzed data from 74 patients with human epidermal growth factor receptor 2 (HER2)-negative breast cancer who underwent biopsies before treatment and subsequent neo-adjuvant chemotherapy. In biopsy specimens, we evaluated the expression of platelet-specific markers and EMT markers using immunohistochemistry. The associations among the expression of platelet-specific markers in biopsy specimens, EMT, response to neo-adjuvant chemotherapy, and survival were analyzed. The presence of platelets was observed in 44 out of 74 (59%) primary breast cancer biopsy specimens. Platelet-positive tumor cells showed EMT-like morphological changes and EMT marker expression. Primary tumor cells associated with platelets were less responsive to neo-adjuvant chemotherapy (pCR rate: 10 vs. 50%, respectively; p=0.0001). Platelets were an independent predictor of the response to chemotherapy upon multivariable analysis (p<0.0001). In conclusion, there was a significant association between platelets surrounding primary tumor cells in the biopsy specimens and the chemotherapeutic response in breast cancer. Platelets surrounding primary tumor cells may represent novel predictors of chemotherapeutic responses.

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Introduction

Cancer is the leading cause of death worldwide, accounting for 8.2 billion deaths in 2012 alone (1). Multimodal treatment, including chemotherapy, surgery, and radiotherapy, has dramatically reduced cancer mortality and improved the quality of life of individuals with cancer (1-3). However, not all patients respond positively to currently available therapies, and relapse is common in patients who initially respond to chemotherapy. The epithelial-mesenchymal transition (EMT) is an essential mechanism involved in tumor progression and metastasis, and the tumor microenvironment, including the extracellular matrix and numerous stromal cell types, has been shown to induce EMT (4,5). Therefore, the development of novel treatments targeting the EMT process may provide effective therapies for patients who do not respond to current treatments or who experience chemoresistant relapse.

Platelets, the smallest anucleate hematopoietic cells, are now recognized as key regulators of tumor progression and metastasis (6-8). In the circulation, platelet aggregation protects cancer cells from shear stress and immune surveillance through the formation of a platelet cloak. Platelets also facilitate cancer cell adherence to vascular endothelial cells, which leads to extravasation into the stroma and the formation of secondary tumors (9). However, the presence and role of platelets in primary tumors are not well understood.

Platelets contain numerous platelet-derived growth mediators and cytokines related to EMT, such as transforming growth factor-β (TGF-β), vascular endothelial growth factor-A (VEGF-A), and plasminogen activator inhibitor-1 (PAI-1). Labelle *et al* reported that direct signaling between platelets and breast cancer cells in the vasculature induces the latter to undergo EMT (10). Furthermore, investigations have demonstrated that tumors undergoing EMT show increased resistance to chemotherapy (11,12). Moreover, in a

study targeting chemoresistant breast cancer cells following neo-adjuvant chemotherapy, we found that some patients achieved pathological complete response (pCR; defined as no residual invasive cancer in the breast and lymph nodes), which would be expected to be associated with a more favorable prognosis than that in patients who did not achieve pCR (13). Chemoresistance involves numerous complex mechanisms, including gene pathways associated with apoptosis/senescence and DNA repair, which are often influenced by communication between host and tumor cells (14). Furthermore, EMT, anti-apoptotic mechanisms, and stemness induced by the cancer microenvironment have been shown to play important roles in chemoresistance (15).

Therefore, we hypothesized that platelets surrounding tumor cells could also be detected in primary sites and could be associated with EMT and chemoresistance. The aims of this study were as follows: i) to confirm the presence of platelets surrounding primary tumor cells in breast cancer; ii) to explore the associations between tumor cells associated with platelets and EMT; and iii) to evaluate the association between the presence of platelets surrounding tumor cells and chemoresistance, and survival.

Materials and methods

Patients and clinical specimens. We retrospectively analyzed data from 74 patients with human epidermal growth factor receptor 2 (HER2)-negative breast cancer who had undergone neo-adjuvant chemotherapy at Kanazawa University Hospital between 2006 and 2013. Patients were selected according to the following inclusion criteria: women, histologically confirmed invasive ductal carcinoma of the breast with no evidence of metastatic disease and defined as clinical stage I to IIIC (any T, N3, M0) with the same neo-adjuvant chemotherapy regimen [four cycles of docetaxel (Taxotere) 75 mg/m^2 followed by four cycles of fluorouracil, epirubicin 100 mg/m^2, and cyclophosphamide (FEC-100)]. Additionally, patients were excluded from the analysis if they met any of the following criteria: i) invasive lobular carcinoma; ii) ductal or lobular carcinoma *in situ*; and iii) HER2-positive breast cancer, defined as immunohistochemistry (IHC) 3+ or fluorescence *in situ* hybridization (FISH)/dual *in situ* hybridization (DISH) positive. All study procedures were approved by the Ethics Committee of the Kanazawa University Hospital. Written informed consent was obtained from each patient enrolled in the study.

The tumors were staged according to the International Union against Cancer tumor-node-metastasis (TNM) classification 7th edition (16). Histological subtype and grade were classified on the basis of the World Health Organization (WHO) guidelines for the Pathology and Genetics of Tumors of the Breast and Female Genital Organs (17). ER status, progesterone receptor (PR) status, Ki-67 index, histology, and nuclear grade were evaluated in biopsy specimens analyzed prior to neo-adjuvant chemotherapy. Biopsies were performed by taking 3-5 extra cores in a needle biopsy with a 14-gauge needle or by vacuum-assisted biopsy with an 11-gauge needle. Biopsy samples were obtained uniformly from various regions of the entire tumor.

The pathological response to neo-adjuvant chemotherapy, including anthracycline and/or taxanes, was evaluated in surgical specimens after therapy. pCR was defined as the complete eradication of all invasive cancer in both the breast and axillary nodes. Any other response was considered to be non-pCR.

Table I. Patient characteristics.

Characteristics	Data
Age (years), median (range)	52 (27-73)
Menopause, n (%)	
Premenopause	31 (48)
Postmenopause	33 (52)
Stage, n (%)	
I	22 (30)
IIA	18 (24)
IIB	14 (19)
IIIA	7 (9)
IIIB	4 (6)
IIIC	9 (12)
ER status, n (%)	
Positive	48 (65)
Negative	26 (35)
Ki-67 index, % (range)	40 (0-90)
Histology, n (%)	
Scirrhous carcinoma	55 (74)
Papillotubular carcinoma	8 (11)
Solid-tubular carcinoma	9 (12)
Unknown	2 (3)
Nuclear grade, n (%)	
1	14 (19)
2	11 (15)
3	34 (46)
Unknown	15 (20)

Associations between clinicopathological parameters, including CD42b expression and pCR, were investigated with univariable/multivariable logistic regression. Odds ratios (ORs) and 95% confidence intervals (CIs) with two-sided p-values were used. $p<0.05$ was considered statistically significant. Overall survival (OS) was defined as the time between the first day of chemotherapy and the date of breast cancer-related death; patients still alive were censored at the last date of follow-up. Recurrence-free survival (RFS) was defined as the interval between the first day of chemotherapy and the date of disease relapse or death from related causes; patients still alive were censored at the last date of follow-up.

Immunohistochemical examination. The Dako Envision system, with dextran polymers conjugated to horseradish peroxidase (Dako, Carpinteria, CA, USA), was used for immunohistochemical staining to avoid any endogenous biotin contamination. Formalin-fixed, paraffin-embedded tissues were cut into sections (4 μm thick). The sections were deparaffinized with xylene and rehydrated in increasing dilutions of ethanol. Endogenous peroxidase was blocked by immersing

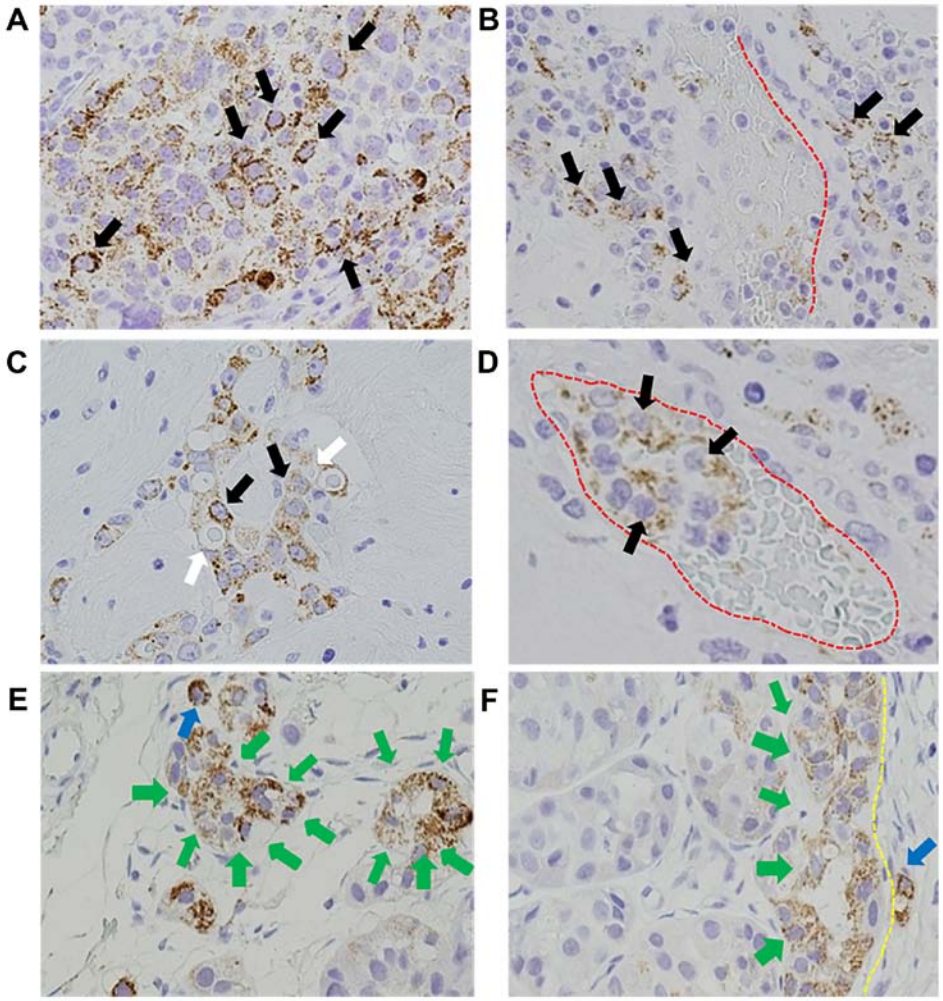

Figure 1. Immunohistochemical analysis of CD42b expression in biopsy specimens from patients with primary breast cancer. (A) Expression of CD42b (black arrow) was detected around primary tumor cells in the biopsy specimens, but not within the membrane or cytoplasm. Tumor cells with CD42b expression (black arrow) were observed in (B) perivascular tissue (red dotted line shows the blood vessels), (C) around the capillaries (white arrow), and (D) in the blood vessels (red dotted line). (E) Tumor cells with CD42b expression showed EMT-like morphological changes [i.e., loss of apical-basal polarity (green arrow) and tumor cell migration (blue arrow)]. (F) These were detected at the invasive front (yellow dotted line). Magnification, x400.

sections in 3% H_2O_2 and 100% methanol for 20 min at room temperature. Antigen retrieval was achieved by microwaving sections at 95˚C for 10 min in 0.001 M citrate buffer (pH 6.7). After blocking the endogenous peroxidase, the sections were incubated with Protein Block Serum-Free (Dako) at room temperature for 10 min to prevent non-specific staining. Sections were then incubated with primary antibodies [anti-glycoprotein Ib (CD42b; Abcam, Cambridge, UK) at a 1:100 dilution for platelet identification; anti-E-cadherin (clone 4A2C7; Zymed) at a 1:50 dilution; anti-vimentin (ab92547; Abcam) at a 1:250 dilution; and anti-β-catenin (ab16051; Abcam) at a 1:1,000 dilution as a marker for EMT] followed by quenching of the endogenous peroxidase activity. Peroxidase activity was detected using the enzyme substrate 3-amino-9-ethylcarbazole. Sections were incubated in Tris-buffered saline without the primary antibodies as negative controls, counterstained with Mayer's hematoxylin, and mounted with mounting medium.

All biopsy specimens were fixed with 10% formalin and embedded in paraffin. The percentage of stained cells was recorded in at least five fields at x400 magnification in randomly selected areas. Cases in which >10% of cancer cells were stained were defined as positive. To eliminate sampling bias, we confirmed that there was no difference between available resected specimens and biopsy specimen for this evaluation method. Two observers who were unaware of the clinical data independently reviewed all the pathological slides.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software following the guidelines described by Bremer and Doerge (18) and the GraphPad Prism User Guide. Differences in categorical variables were tested for significance using χ^2 tests. Results were considered significant when $p<0.05$. OS and RFS rates were estimated using Kaplan-Meier method and compared using the log-rank test.

Results

Patient and clinicopathological characteristics. Patient characteristics, including age, menopausal status, and tumor stage are summarized in Table I. The median patient age was 52 years (range, 27-73 years), and 31 patients were premeno-

Table II. Relationship between CD42b expression and the clinicopathological characteristics of the primary breast cancer cases.

Clinicopathological parameters	CD42b expression		t or χ^2 test (p-value)
	Positive (≥10%) n (%)	Negative (<10%) n (%)	
Patients	44 (59)	30 (41)	
Stage			0.2280
I	11 (25)	11 (36)	
II	18 (41)	14 (46)	
III	15 (34)	5 (18)	
Nuclear grade			0.1539
G1	6 (14)	8 (26)	
G2	6 (14)	5 (18)	
G3	25 (56)	10 (33)	
Unknown	7 (16)	7 (23)	
Histology			0.4172
Scirrhous carcinoma	33 (75)	22 (73)	
Papillotubular carcinoma	3 (7)	5 (16)	
Solid-tubular carcinoma	6 (13)	3 (11)	
Unknown	2 (5)	0 (0)	
ER status			0.6115
Positive	29 (66)	22 (73)	
Negative	15 (34)	8 (27)	
Chemotherapy response			0.0001
pCR	4 (9)	15 (50)	
Non-pCR	40 (91)	15 (50)	

pausal. The tumor stages were as follows: stage I, n=22; stage II, n=32; and stage III, n=20. ER status was positive in 48 tumors and negative in 26 tumors. The median Ki-67 index was 40 (range, 0-90). With respect to histological subtype, 72 tumors were invasive ductal carcinoma, and two were of an unknown subtype. The nuclear grades of the tumors were as follows: 1 in 14 cases, 2 in 11 cases, 3 in 34 cases, and unknown in 15 cases. The median follow-up time was 69 months (range, 28-117 months). There were 5 deaths (4 deaths in CD42b-positive and 1 death in CD42b-negative groups) and 9 recurrences (7 recurrences in the CD42b-positive and 2 recurrences in the CD42b-negative group). The median RFS and OS values were not reached. The 5-year RFS and OS rates were 93.2 and 98.6%, respectively.

Platelets surrounding primary tumor cells. All tumors were evaluated for CD42b expression, a platelet-specific marker. CD42b expression was observed in 44 of 74 (59%) primary breast tumors (Fig. 1A), with particularly strong staining at the invasive front, which was observed in 37 of 74 (84%) specimens (Fig. 1F), and migratory tumor cells in the perivascular tissue, which was observed in 30 of 44 (68%) specimens (Fig. 1B-D).

Relationship between platelets surrounding primary tumor cells and clinicopathological features. The relationships between CD42b expression and clinicopathological features, including stage, nuclear grade, histology, ER status, and pathological responce are summarized in Table II. A statistically significant association was noted between CD42b expression and pathological response ($p<0.0001$). There were no significant associations between CD42b expression and stage, nuclear grade, histology, or ER status.

Expression of EMT markers in primary tumor cells associated with platelets. Tumor cells associated with CD42b immunoreactivity showed EMT-like morphological changes, including loss of apical-basal polarity and detachment from the basement membrane at the invasive front (Fig. 1E and F). In order to investigate the expression of EMT markers in CD42b-positive tumor cells, immunohistochemistry was performed on biopsy specimens. All tumors were evaluated for E-cadherin, vimentin, and β-catenin expression. We found nuclear staining of β-catenin in CD42b-positive tumor cells (Fig. 2A and C), while CD42b-negative tumor cells showed a membranous pattern of β-catenin staining (Fig. 2B and D). CD42b-positive tumor cells also showed loss of E-cadherin expression and gain of vimentin expression (Fig. 2E and G). In contrast, CD42b-negative tumor cells showed membranous expression of E-cadherin and β-catenin, but loss of vimentin expression (Fig. 2F and H).

Relationship between platelets surrounding primary tumor cells in biopsy specimens and pathological response to

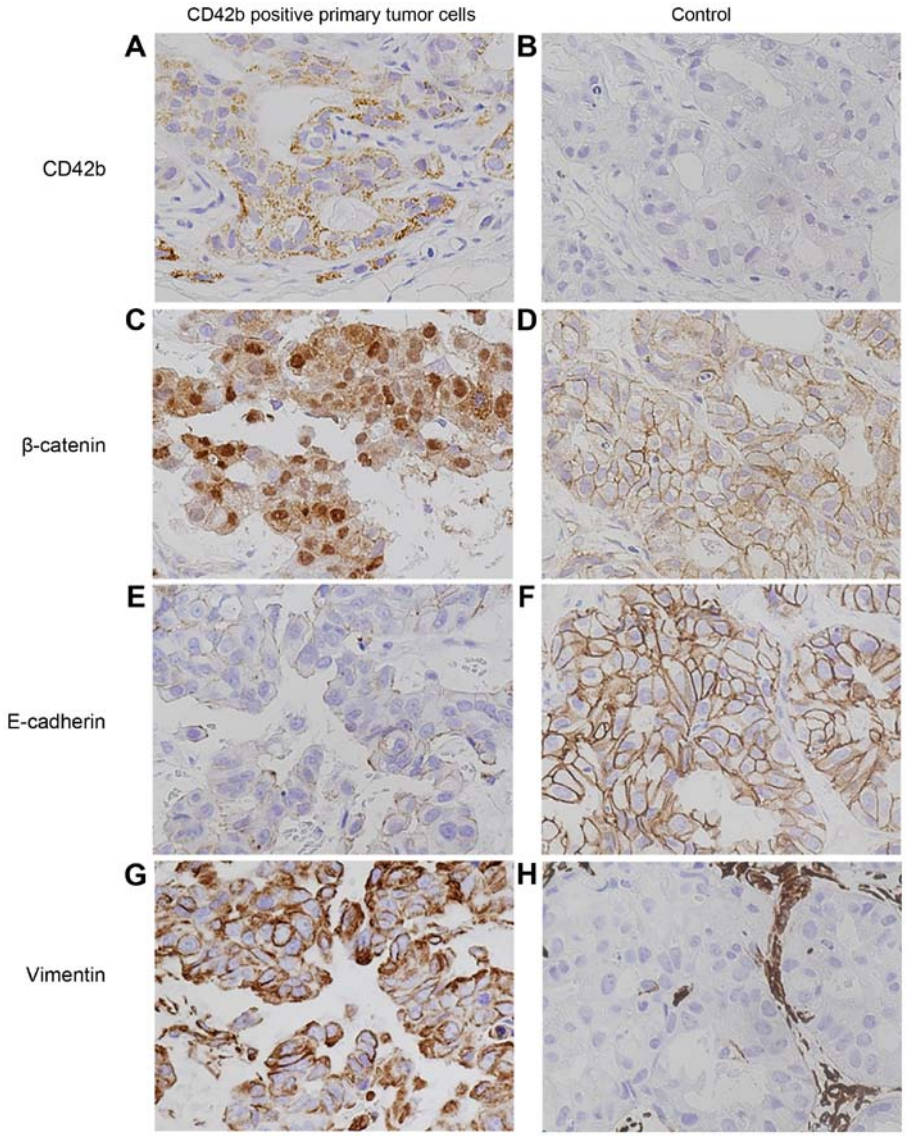

Figure 2. Expression of epithelial-mesenchymal transition markers in CD42b-positive tumor cells in serial sections. (A) CD42b expression around primary tumor cells. (B) Absence of CD42b expression around primary tumor cells. (C) Nuclear staining of β-catenin in CD42b-positive primary tumor cells. (D) Membranous staining pattern of β-catenin in CD42b-negative primary tumor cells in serial sections from a sample from a single patient. (E) CD42b-positive primary tumor cells showing loss of E-cadherin expression. (F) CD42b-negative primary tumor cells showing membranous staining of E-cadherin in serial sections from a sample from a single patient. (G) CD42b-positive primary tumor cells showing membranous staining of vimentin. (H) CD42b-negative primary tumor cells showing loss of vimentin expression in serial sections from a sample from a single patient. Magnification, x400.

neo-adjuvant chemotherapy. Analysis of the relationship between CD42b expression and pathological response to neo-adjuvant chemotherapy showed that pCR differed significantly with respect to CD42b expression. When compared to patients with CD42b-positive tumors, those with CD42b-negative tumors achieved a pCR far more frequently (10 vs. 50%, respectively; p=0.0001) (Table II).

Univariate analysis of clinicopathological parameters showed that CD42b expression (p<0.0001) was significantly associated with pCR rate. Multivariate analysis identified CD42b expression (p<0.0001), ER status (p=0.03), and nuclear grade (p=0.02) as independent predictors of pCR rate (Table III).

Relationship between CD42b expression and survival outcomes. RFS and OS between tumors with CD42b expression and those without CD42b expression are shown in Fig. 3. RFS and OS displayed no significant differences, regardless of CD42b expression (p=0.18 and 0.24) (Fig. 3).

Discussion

In cancer progression and metastasis, platelets play an essential role in the host tumor microenvironment and have been shown to interact with cancer cells. In this study, we demonstrated that platelets aggregated around primary tumor cells in 59% of the breast cancer specimens. Moreover, we showed that primary tumor cells surrounded by platelets were found in sites in which EMT was occurring based on molecular and morphological changes. Finally, we also found that primary tumor cells associated with platelets exhibited chemoresistance to common anticancer drugs (including anthracycline and taxanes). Therefore, our data provide important insights into the mechanisms of breast cancer progression.

Table III. Univariable and multivariable analysis of clinicopathological parameters including CD42b expression for prediction of pCR.

	Univariable analysis			Multivariable analysis		
Clinicopathological parameters	OR	95% CI	P-value	OR	95% CI	P-value
CD42b expression (≥10 vs. <10%)	0.1	0.02-0.34	<0.0001	0.03	0.003-0.15	<0.0001
ER status (positive vs. negative)	0.49	0.17-1.44	NS	0.21	0.04-0.9	0.03
Clinical stage (III vs. I-II)	0.45	0.11-1.78	NS	1.08	0.18-5.76	NS
Nuclear grade (G3 vs. G1-2)	1.77	0.61-5.1	NS	5.31	1.27-29.4	0.02

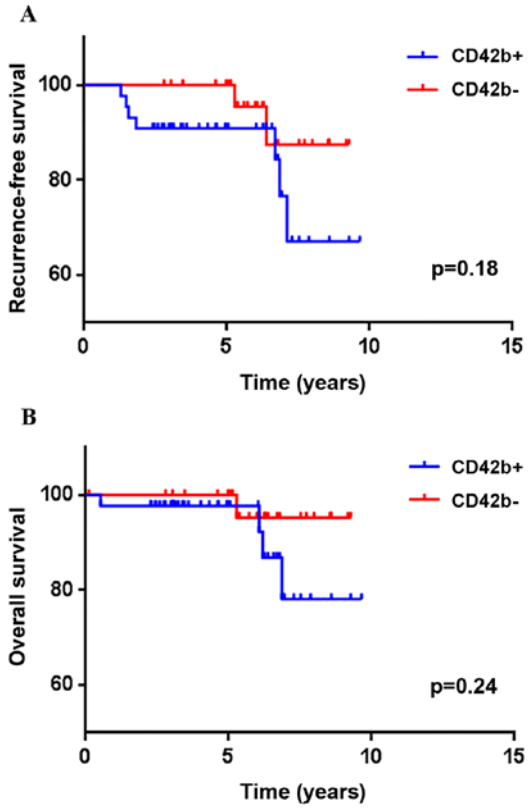

Figure 3. (A) Recurrence-free survival and (B) overall survival according to CD42b expression status.

In patients with platelets surrounding primary tumor cells, wherein platelets were present at numbers >10% of the total number of tumor cells, significant residual invasive carcinoma cells were observed in surgical specimens resected after chemotherapy. These results indicated that platelet aggregation around primary tumor cells may play a crucial role in inducing EMT and chemoresistance. Thus, platelet aggregation around primary tumor cells may be an effective predictor of chemoresistance and a novel therapeutic target for overcoming chemoresistance, one of the major complications of cancer therapies.

Platelets, the smallest anucleate hematopoietic cells, cannot be detected by traditional hematoxylin and eosin staining. Therefore, the presence of platelet aggregation around primary tumor cells is difficult to recognize. Cancer cells were shown to have the ability to interact with platelets *in vitro* several decades ago (19-22). Furthermore, the interaction between circulating tumor cells (CTCs) and circulating platelets is now recognized as a hallmark of the metastatic potential of cancer (6,8). Recent studies have demonstrated that the presence of platelet aggregation around tumor cells can be detected both in the circulation and in primary tumor cells in patients with pancreatic cancer (23). Here, we used immunohistochemistry to demonstrate that platelets aggregated around primary tumors in about half of the breast cancer patients. Therefore, these data further support that the metastatic potential of platelets in primary sites, in addition to those in circulation, should also be analyzed.

Platelets were detected in HER2-negative breast cancer, regardless of stage, nuclear grade, ER status, and Ki-67 index. Other studies have reported the interaction between intrinsic subtype and tumor cell-platelet interactions. Luminal-type breast cancer cells have been reported to induce greater aggregation of platelets than other types of breast cancer cells *in vitro* (19,24). Moreover, in addition to facilitating tumor invasiveness, migration, tumor growth, cell survival, and angiogenesis, circulating platelets also play a crucial role in inducing EMT in malignancy (10). Circulating platelets are supported by chemical mediators, such as TGF-β, VEGF-A, and PDGF, released from activated platelets (10,25). We demonstrated that primary tumor cells associated with platelet aggregation showed morphological and molecular characteristics of EMT in breast cancer. In particular, we observed nuclear translocation of β-catenin, which reflects the down-regulation of E-cadherin and may lead to activation of the Wnt pathway, thus inducing transcriptional enhancement of c-Myc and cyclin-D (26). These events could promote tumor cell migration from the primary site. Miyashita *et al* reported that primary tumor cells surrounded by platelets exhibited characteristics of EMT in pancreatic cancer (23). Thus, these findings support our hypothesis that induction of EMT by platelet aggregation may occur during early processes of metastasis, even at primary tumor sites.

In this study, we also showed that primary tumor cells associated with platelet aggregation were less responsive to chemotherapy. Patients whose pre-treatment biopsy specimens contained platelets surrounding tumor cells at a rate >10% of the total number of tumor cells showed significant residual cancer cells in surgical specimens following chemotherapy. Recent reports have demonstrated that human platelets increase cancer cell survival, proliferation, and chemoresistance to 5-fluorouracil and paclitaxel in colon and ovarian

cancer *in vivo* (27). Moreover, chemoresistance could be induced by platelets throughout EMT (28), PAI-1-mediated anti-apoptotic pathways, direct protection, or immunosuppression mediated by downregulation of NKG2-D (29,30). These results indicate that platelet aggregation surrounding primary tumor cells may be a predictive factor for chemotherapeutic success. Additionally, if platelet-mediated EMT and chemoresistance could be modulated, we may be able to achieve enhanced chemotherapeutic efficacy. As such, it is imperative to elucidate the mechanisms of tumor cell-platelet interactions in primary tumor sites.

In the present study, we were unable to demonstrate a significant relationship between CD42b expression and survival outcomes. The prognostic impact of pathological complete response (pCR) varies dependent on the intrinsic subtype of breast cancer. pCR is a suitable surrogate end point for luminal B/HER2-negative, HER2-positive, and triple-negative disease, but not in luminal breast cancer. Because our study consisted of these different subtypes, there is necessity to evaluate a greater number of samples. Moreover, duration of follow-up limited the ability to evaluate the recurrence and death of breast cancer; increased follow-up time is required.

This study had several limitations, including its retrospective nature, small sample size, potential selection bias, and heterogeneity of tumor characteristics. With respect to the heterogeneity of tumor characteristics, we performed preliminary experiments on the expression of CD42b in available resected specimens and biopsies as consistently as possible to reduce the effects of tumor heterogeneity. We confirmed that there was no difference between available resected specimens and biopsy specimen for this evaluation method.

We concluded that platelets may have tremendous potential to induce tumor progression and metastasis, even when found within the primary tumor site. This phenomenon may represent a novel predictive factor for chemoresistance, and our results may provide important insights into new therapeutic targets in breast cancer. To discover and validate novel therapeutic targets, we are now conducting research to elucidate the mechanisms of the chemoresistance caused by platelets in breast cancer cells.

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