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CD44 variant 6 is correlated with peritoneal dissemination and poor prognosis in patients with advanced epithelial ovarian cancer

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Key words

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Cancer stem cells (CSCs) drive tumor initiation and metastasis in several types of human cancer. However, the contribution of ovarian CSCs to peritoneal metastasis remains unresolved. The cell adhesion molecule CD44 has been identified as a major marker for CSCs in solid tumors, including epithelial ovarian cancer. CD44 exists as a standard form (CD44s) and also as numerous variant isoforms (CD44v) generated by alternative mRNA splicing. Here we show that disseminated ovarian tumors in the pelvic peritoneum contain highly enriched CD44v6-positive cancer cells, which drive tumor metastasis and are responsible for tumor resistance to chemotherapy. Clinically, an increased number of CD44v6-positive cancer cells in primary tumors was associated with a shortened overall survival in stage III–IV ovarian cancer patients. Furthermore, a subpopulation of CD44v6-positive cancer cells manifested the ability to initiate tumor metastasis in the pelvic peritoneum in an *in vivo* mouse model, suggesting that CD44v6-positive cells show the potential to serve as metastasis-initiating cells. Thus, the peritoneal disseminated metastasis of epithelial ovarian cancer is initiated by the CD44v6-positive subpopulation, and CD44v6 expression is a biomarker for the clinical outcome of advanced ovarian cancer patients. Given that a distinct subpopulation of CD44v6-positive cancer cells plays a critical role in peritoneal metastasis, definitive treatment should target this subpopulation of CD44v6-positive cells in epithelial ovarian cancer.

Epithelial ovarian cancer is the leading cause of death from gynecological malignancies.[1] Because most patients with ovarian malignancies are generally asymptomatic until the cancer has progressed and metastasized, more than two-thirds of tumors are diagnosed at an advanced stage with multiple disseminated tumors in the pelvic peritoneum.[2] The clinical outcomes for women diagnosed with advanced epithelial ovarian cancer are poor even after treatment with extirpative surgery and proper chemotherapy. Although the cancer may respond to primary therapy, chemoresistant residual cancer cells can persist in a dormant state for many months in the pelvic peritoneum, leading to relapse.[3,4] Therefore, elucidating the molecular events that control peritoneal metastasis may provide potential molecular targets for the treatment of advanced epithelial ovarian cancer with multiple peritoneal disseminated tumors.

Cell adhesion molecule CD44 is a polymorphic integral membrane glycoprotein that binds hyaluronic acid and contributes to tumor growth, invasion, and metastasis.[5–7] CD44 exists as a standard form (CD44s) and also as numerous variant isoforms (CD44v) generated by alternative mRNA splicing of up to 10 variant exons that encode parts of the extracellular domain.[6–10] Among CD44v isoforms, CD44v6 was initially found to promote the metastatic potential of a rat pancreatic adenocarcinoma cell line.[11] Furthermore, several previous studies supported the premise that CD44v6 plays a key role in cancer proliferation, migration, and invasion in a variety of human cancers, such as colorectal, breast, lung, and ovarian cancer.[12–15] In epithelial ovarian cancer, it is known that CD44v6 promotes tumor metastasis by binding hyaluronic acid on peritoneal mesothelial cells.[16]

In recent history, the cancer stem cell (CSC) theory has proposed that the bulk of tumor cells are generated by a rare population of tumor-initiating cells, conceptually termed CSCs.[17–19] CSCs possess the ability to self-renew and differentiate into a heterogeneous lineage of cancer cells and inherently drive the metastatic process.[18,20] CD44 has been identified as one of the major cell surface markers associated with CSCs in several types of epithelial tumors, including ovarian cancer.[6,21–23] Intriguingly, recent studies indicated that a subpopulation of CD44v6-positive cells shows a characteristic phenotype of CSCs in colorectal cancer, bladder cancer, and brain tumor.[24–26] These findings led us to hypothesize that CD44v6-positive ovarian cancer cells may possess CSC traits and play a key role in tumor initiation and disseminated metastasis.

Uncovering the molecular mechanisms underlying peritoneal metastasis is the final frontier in ovarian cancer biology. Even

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though ovarian CSCs have not been fully elucidated, these cells are thought to play a crucial role in disseminated metastasis and relapse at peritoneal metastatic sites.[27] The present study was designated to evaluate the role of CD44v6 in peritoneal disseminated metastasis and the potential relevance of CD44v6 to the clinical outcome of patients with advanced epithelial ovarian cancer with long-term follow-up.

Materials and Methods

Patients and tissue preparation. From January 2002 to December 2012, the clinical records of stage III–IV epithelial ovarian cancer patients were reviewed retrospectively, and 59 patients with peritoneal disseminated tumors who underwent primary standard surgery followed by proper chemotherapy at Kumamoto University Hospital were included in this study. Patients were excluded when they had borderline tumors, multiple primary cancers, or non-epithelial tumors. The eligible patients were followed-up until December 2014. Written informed consent was obtained from all patients before treatment, in accordance with the institutional guidelines of our hospital.

Tumor tissues obtained surgically were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 4-μm thickness for histological diagnosis. Sections were stained with H&E, and histologic typing was carried out according to the WHO classification of surface epithelial–stromal ovarian tumors.[28] All tumors were staged according to the International Federation of Gynecology and Obstetrics criteria.[29]

Evaluation of immunohistochemical staining. Immunohistochemical analysis was carried out as described previously.[30] Briefly, the sections were washed with PBS, subjected to antigen retrieval by heating in a microwave in 0.01 M sodium citrate buffer (pH 6.0) for 15 min, and exposed to 3% H_2O_2 in methanol before staining with the primary antibody. Immune complexes were detected with use of the avidin–biotin–peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine substrate (Vector Laboratories), and the sections were counterstained with hematoxylin. CD44v6 was detected with the mouse mAb CD44v6 (2F10; R&D Systems, Minneapolis, MN, USA). The expression level of CD44v6 was quantified as a percentage of the total number of stained cells. The primary ovarian tumors that contained at least 10% CD44v6-positive cancer cells were categorized as the "CD44v6-high" group, whereas the tumors that contained less than 10% CD44v6-positive cells were categorized as the "CD44v6-low" group. The percentage of CD44v6-positive cancer cells in primary tumors was evaluated by counting cells in at least three microscopic fields per slide.

Mice. BALB/c nude mice were obtained from CLEA (Tokyo, Japan) and maintained according to institutional guidelines. All animal experiments were carried out in accordance with protocols approved by the animal ethics committee of Kumamoto University.

Cell line. A human ovarian cancer cell line, ES-2, was obtained from ATCC (Manassas, VA, USA). ES-2 cells were maintained in RPMI-1640 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% FBS at 37°C in a 5% CO_2-containing atmosphere.

Flow cytometry and transplantation assay. Cell sorting and flow cytometric analysis were carried out with the use of a FACS Aria II (BD Biosciences, San Jose, CA, USA). Cells were incubated with allophycocyanin-conjugated mouse mAb CD44v6 (2F10; R&D Systems) and phycoerythrin-conjugated rat mAb CD44 (IM7; BioLegend, San Diego, CA, USA) for 30 min. The FACS-sorted CD44v6-positive or -negative cancer cells were suspended in RPMI-1640 medium and injected i.p. into 7-week-old female BALB/c nude mice. Tumor-initiating frequencies were assessed with the use of ELDA software for limiting dilution analysis.[31]

Immunoblot analysis. Immunoblot analysis was carried out as previously described.[27] In brief, equal amounts of cell lysate protein were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to anti-CD44v6 antibody (VFF-18; Abcam, Cambridge, UK), anti-E-cadherin (36/E-cadherin; BD Biosciences), anti-N-cadherin (32/N-cadherin; BD Biosciences), anti-fibronectin (10/fibronectin; BD Biosciences), anti-vimentin (V9; DakoCytomation, Glostrup, Denmark), and anti-β-actin (13E5; Cell Signaling Technology, Beverly, MA, USA). Immune complexes were visualized by chemiluminescence detection (Pierce Biotechnology, Rockford, IL, USA).

Proliferation and chemosensitivity assay. Cell viability was assessed with MTS assay according to the manufacturer's protocol (CellTiter 96 Aqueous One Solution Cell Proliferation assay; Promega, Madison, WI, USA). Briefly, cells (3×10^3/100 μL per well) were plated in 96-well flat bottom plates and serum starved overnight. Ovarian cancer cells were treated with paclitaxel or cisplatin at the indicated concentrations. At 12 h post-drug treatment, 20 μL MTS assay solution was added to each well for 2 h. Absorbance was recorded at 490 nm on an SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Experiments were carried out in triplicate and repeated three times and the percentage of cell survival was defined as the relative absorbance of untreated versus treated cells.

Statistical analysis. The prognosis of patients was determined according to the cumulative survival rate after treatment. Survival rates were calculated using the Kaplan–Meier method, and differences between curves were assessed with the log–rank test. Correlations between variables were evaluated with the χ^2-test, Fisher's exact test, Mann–Whitney *U*-test, or Wilcoxon test. Data are presented as mean ± SD and were analyzed with the Student's *t*-test. Univariate and multivariate Cox proportional hazard model analyses were carried out to calculate hazard ratios (HRs) using SPSS version 21.0 (IBM, Armonk, NY, USA). In all analyses, a *P*-value of <0.05 was considered statistically significant.

Results

Correlation between CD44v6 expression pattern and clinicopathological features in patients with stage III–IV epithelial ovarian cancer. The association between CD44v6 expression and the clinicopathological characteristics of the 59 patients is shown in Table 1. Thirteen (22.0%) cases belonged in the CD44v6-high group, and 46 (78.0%) cases to the CD44v6-low group. The median age of all patients at diagnosis was 57 years (range, 37–82 years). There were no significant differences in the median age between the CD44v6-high and CD44v6-low groups. In addition, no significant correlation was observed between the immunohistochemical (IHC) expression of CD44v6 and clinicopathological characteristics, such as tumor histological type, tumor marker CA125, and tumor size. Adjuvant systematic chemotherapy was given as clinically indicated in accordance with standard practices, and almost all patients (57/59, 96.6%) received paclitaxel–carboplatin as first-line adjuvant chemotherapy. No significant differences were recorded in the distribution of the number of cycles of

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Table 1. Association between CD44 variant 6 (CD44v6) expression pattern and clinicopathological characteristics in patients with stage III–IV ovarian cancer

	All cases, *n* (%)	CD44v6-high group, *n* (%)	CD44v6-low group, *n* (%)	*P*-value
All cases	59	13	46	
Median age, years (range)	57 (37–82)	59 (43–82)	56 (37–77)	0.84
Age, years				
<50	18 (30.5)	3 (23.1)	15 (32.6)	0.51
≥50	41 (69.5)	10 (76.9)	31 (67.4)	
Histological type				
Serous	42 (71.2)	7 (53.8)	35 (76.1)	0.12
Clear	3 (5.1)	2 (15.4)	1 (2.2)	
Endometrioid	5 (8.5)	1 (7.7)	4 (8.7)	
Mucinous	1 (1.7)	1 (7.7)	0 (0.0)	
Mixed	7 (11.8)	1 (7.7)	6 (13.0)	
Undifferentiated	1 (1.7)	1 (7.7)	0 (0.0)	
CA125, U/mL				
<500	18 (30.5)	6 (46.2)	12 (26.1)	0.16
≥500	41 (69.5)	7 (53.8)	34 (73.9)	
Tumor size, cm				
<10	40 (67.8)	7 (53.8)	33 (71.7)	0.22
≥10	19 (32.2)	6 (46.2)	13 (28.3)	
First-line chemotherapy regmen				
Paclitaxel/ carboplatin	57 (96.6)	12 (92.3)	45 (97.8)	0.33
Other	2 (3.4)	1 (7.7)	1 (2.2)	
No. of cycles of chemotherapy				
<2	41 (69.5)	8 (61.5)	33 (71.7)	0.48
≥3	18 (30.5)	5 (38.5)	13 (28.3)	

chemotherapy between CD44v6-high and CD44v6-low groups (Table 1).

Highly enriched CD44v6-positive ovarian cancer cells in peritoneal disseminated tumors. To investigate whether CD44v6-positive cancer cells are associated with peritoneal metastasis, we compared the average number of CD44v6-positive cells among the 59 samples of primary ovarian tumors to that in samples of peritoneal disseminated tumors taken from the same patients. Representative IHC staining patterns for CD44v6 in primary and disseminated tumors are shown in Figure 1(a,b). Immunohistochemical analysis revealed a significantly higher percentage of CD44v6-positive cells detected in peritoneal disseminated tumors than in corresponding primary ovarian tumors ($P < 0.01$; Fig. 1c). These findings indicated that CD44v6-positive cells are correlated with peritoneal dissemination, and the pelvic peritoneum may have the potential to form a part of the niche microenvironment involved in tumor initiation and metastasis.

Prognostic impact of CD44v6 expression in advanced epithelial ovarian cancer patients. Given that a subpopulation of CD44-positive cancer cells in hierarchically organized ovarian cancer manifests CSC properties,[21] we hypothesized that CD44v6 expression would correlate with aspects of ovarian cancer survival. To address this issue, we used Kaplan–Meier analyses of overall survival (OS) and progression-free survival (PFS) between the CD44v6-high and CD44-low groups. Representative IHC staining patterns for CD44v6 in CD44-high and CD44-low groups are shown in Figure 2(a, b). In the evaluation of the sites of primary lesions, the 5-year OS rates were 18.0% (95% confidence interval [CI], 0.0–40.2) in the CD44-high group and 59.6% (95% CI, 44.3–74.8) in the CD44-low group. Significant differences

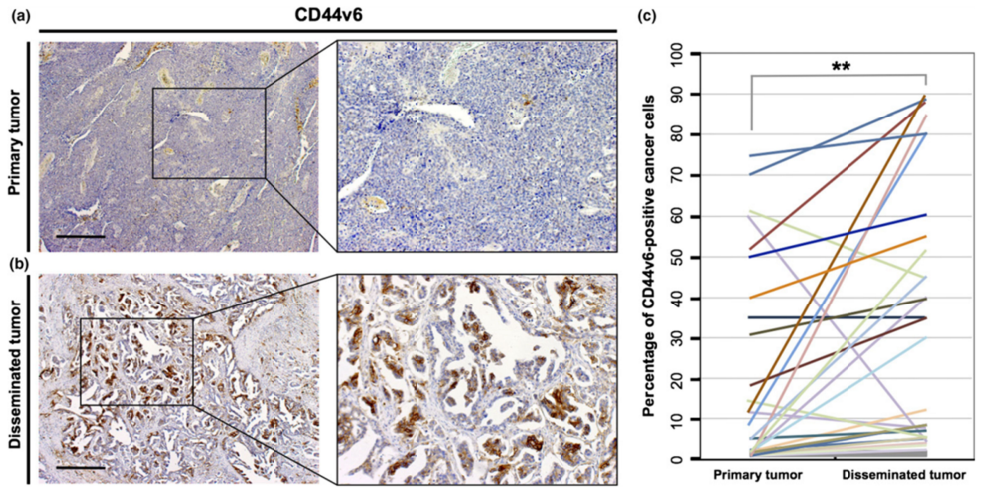

Fig. 1. Disseminated ovarian tumors in the pelvic peritoneum contain highly enriched CD44 variant 6 (CD44v6)-positive cancer cells. (a) Immunohistochemical analysis with an anti-CD44v6 antibody in primary epithelial ovarian tumors. Scale bar = 500 μm. (b) Immunohistochemical staining with an anti-CD44v6 antibody in peritoneal disseminated tumors. Scale bar = 500 μm. (c) The percentage of CD44v6-positive cancer cells in primary and disseminated tumors. Peritoneal disseminated tumors contained significantly higher percentages of CD44v6-positive cells than primary tumors (Mann–Whitney *U*-test, $^{**}P < 0.01$).

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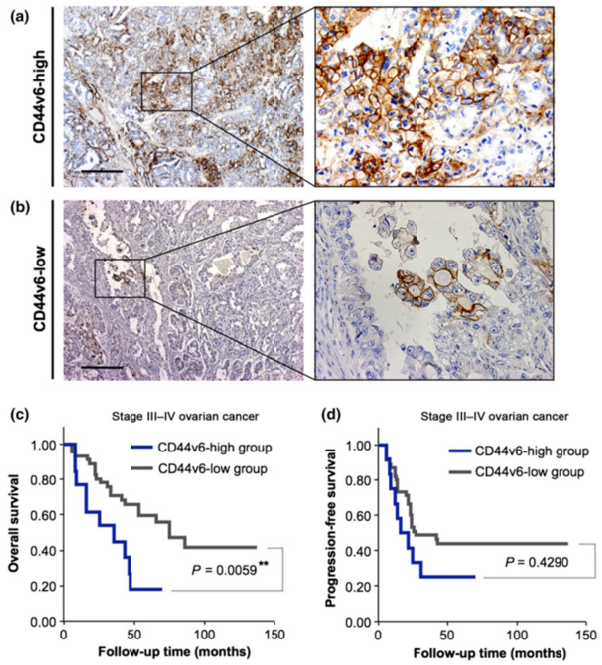

Fig. 2. CD44 variant 6 (CD44v6) expression predicts epithelial ovarian cancer survival. (a) Immunohistochemical analysis with an anti-CD44v6 antibody in primary epithelial ovarian tumors. The tumors that contained at least 10% CD44v6-positive cancer cells were categorized as the CD44v6-high group. Scale bar = 500 μm. (b) Immunohistochemical staining with an anti-CD44v6 antibody in primary tumors. The tumors that contained less than 10% CD44v6-positive cancer cells were categorized as the CD44v6-low group. Scale bar = 500 μm. (c) Kaplan–Meier analysis of overall survival in patients with stage III–IV ovarian cancer according to the expression of CD44v6. There were significant differences in overall survival between the CD44v6-high and CD44v6-low groups (**P = 0.0059). (d) Kaplan–Meier analysis of progression-free survival in patients with stage III–IV ovarian cancer according to the expression of CD44v6. Progression-free survival was not significantly different between the CD44v6-high and CD44v6-low groups (P = 0.4290).

Table 2. Hazard ratios (HRs) using univariate and multivariate Cox proportional hazard model

Prognostic factor	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age, years						
<50						
≥50	1.286	0.574–2.879	0.542			
CA125, U/mL						
<500						
≥500	1.060	0.487–2.306	0.884			
Tumor size, cm						
<10						
≥10	1.063	0.498–2.267	0.874			
First-line chemotherapy						
Paclitaxel/carboplatin						
Other	0.905	0.122–6.727	0.923			
Surgical debulking status						
Optimal surgery (Residual tumor size <1 cm)						
Suboptimal surgery (Residual tumor size ≥1 cm)	2.568	1.247–5.288	0.011	2.283	1.091–4.775	0.028
CD44v6 expression						
Low						
High	2.930	1.334–6.436	0.007	2.568	1.149–5.738	0.022

CD44v6, CD44 variant 6; CI, confidence interval; High,; Low,.

were observed in OS between the CD44v6-high and CD44v6-low groups for patients with stage III–IV ovarian cancer (P = 0.0059; Fig. 2c). In contrast, no significant differences were observed in PFS between the CD44v6-high and CD44v6-low groups (P = 0.4290; Fig. 2d). These findings suggested that CD44v6-positive cancer cells in primary

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