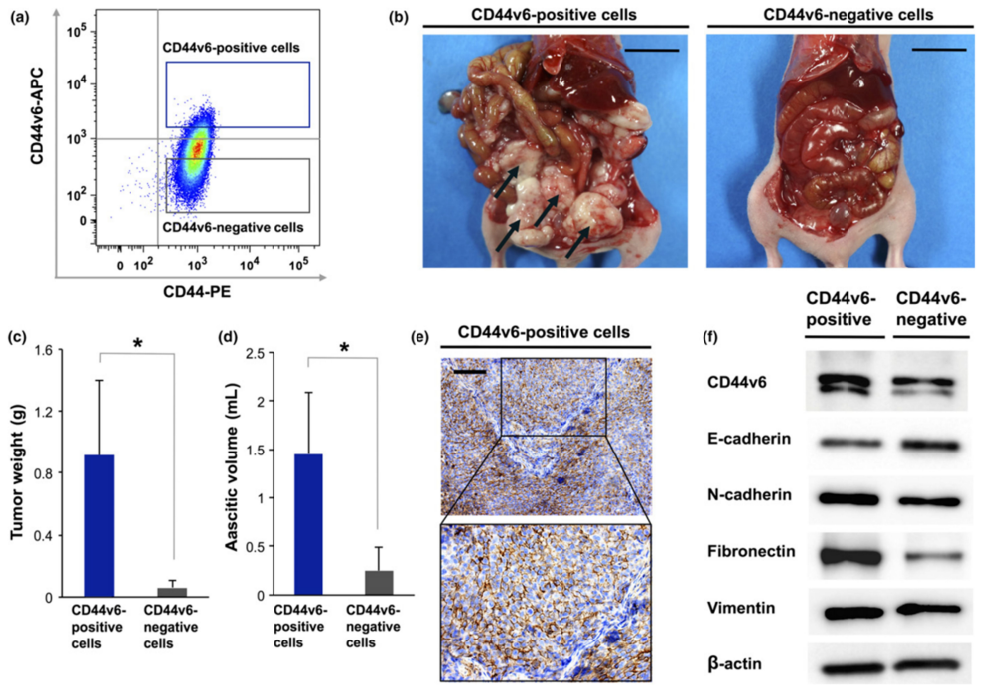

Fig. 3. Subpopulation of CD44 variant 6 (CD44v6)-positive ovarian cancer cells possesses a high peritoneal metastatic ability. (a) Flow cytometric analysis of CD44v6 expression in ES-2 ovarian cancer cells. (b) Macroscopic appearance of disseminated tumors at 35 days after cell transplantation. CD44v6-positive cells generated more extensive disseminated tumors in the peritoneal cavity than CD44v6-negative cells. Scale bar = 2 cm. (c) Total weight of peritoneal disseminated tumors determined at 35 days after cell injection. Quantitative data are presented as mean ± SD for five mice. *$P < 0.05$. (d) Ascitic volume determined at 35 days after transplantation. Quantitative data are presented as mean ± SD for five mice. *$P < 0.05$. (e) Immunohistochemical analysis with antibody to CD44v6 in peritoneal disseminated tumors in a mouse model. Paraffin-embedded sections of disseminated tumors generated by CD44v6-positive cancer cells were subjected to immunohistochemical staining with an anti-CD44v6 antibody. Scale bar = 200 μm. (f) Western blot analysis of CD44v6 and epithelial–mesenchymal transition regulatory proteins, including E-cadherin, N-cadherin, fibronectin, and vimentin in FACS-sorted CD44v6-poisitive cells *versus* FACS-sorted CD44v6-negative cells.

tumors play an important role in the survival of advanced ovarian cancer patients.

Univariate and multivariate analysis of various clinicopathological factors in relation to OS are shown in Table 2. Immunohistochemical expression of CD44v6 proved to be a highly predictive factor based on the univariate Cox proportional hazards model ($P = 0.007$; HR, 2.930; 95% CI, 1.334–6.436) and the multivariate Cox proportional hazards model ($P = 0.022$; HR, 2.568; 95% CI, 1.149–5.738). In addition, surgical debulking status also significantly correlated with OS based on the univariate Cox proportional hazards model ($P = 0.011$; HR, 2.568; 95% CI, 1.247–5.288) and the multivariate Cox proportional hazards model ($P = 0.028$; HR, 2.283; 95% CI, 1.091–4.775).

High metastatic ability in a subpopulation of CD44v6-positive ovarian cancer cells. Given that CD44v6-positive cancer cells showed high metastatic potential in patients with advanced ovarian cancer, we next examined the relevance of peritoneal metastasis in a subpopulation of CD44v6-positive cells in an *in vivo* mouse model. To compare the peritoneal metastatic abilities of CD44v6-positive and CD44v6-negative cancer cells, we sorted CD44v6-positive and CD44v6-negative cells from the ES-2 ovarian cancer cell line (Fig. 3a) and serially transplanted them intraperitoneally into nude mice. Limiting dilution assay revealed that CD44v6-positive cells had a greater tumor initiating ability than CD44v6-negative cells, suggesting that a subpopulation of CD44v6-positive cells is highly efficient at metastatic dissemination (Table 3). The CD44v6-positive cells generated extensive disseminated tumors, resulting in massive abdominal distension by hemorrhagic ascites, within 5 weeks of inoculation, whereas CD44v6-negative cells showed little ability to form disseminated tumors in the peritoneal cavity (Fig. 3b). The total weight of peritoneal disseminated tumors formed by CD44v6-positive cells was significantly greater than that of those formed by CD44v6-negative cells ($P < 0.05$; Fig. 3c). In addition, transplantation of CD44v6-positive cells caused a significant increase in the ascitic volume in comparison with that resulting from transplantation of CD44v6-negative cells ($P < 0.05$; Fig. 3d). A representative IHC staining pattern for CD44v6 in peritoneal disseminated tumors generated by CD44v6-positive cancer cells is shown in Fig-

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Table 3. ***In vivo*** **tumorigenicity of CD44 variant 6 (CD44v6)-positive and CD44v6-negative cells**

	No. of transplanted cells			Frequency of metastasis-initiating cells (95% CI)
	10, 000	1000	100	
CD44v6-positive cells	6/6	6/6	4/5	62.6** (21.4–185.0)
CD44v6-negative cells	3/12	1/12	0/12	29, 211.2 (10, 813.7–78, 910.0)

CD44v6-positive and -negative cancer cells were separated by FACS, and the indicated numbers of cells were transplanted intraperitoneally into nude mice. The incidence of tumor formation within 8 weeks was scored. Data represent the number of tumors per number of injections. Tumorigenic cell frequencies were estimated with the use of ELDA software for limiting dilution analysis. $^{**}P < 0.01$.

ure 3(e). These results suggested that CD44v6-positive cells play a crucial role in the formation of disseminated tumors in the pelvic peritoneum and have the potential to contain specialized metastasis-initiating cells.

Epithelial–mesenchymal transition (EMT) is an important step in invasion and metastasis of cancer.[32] When the ovarian cancer cells detach and start their metastatic journey, it is believed that they frequently undergo EMT.[3] We therefore hypothesized that CD44v6 has an important role in the EMT phenomenon of ovarian cancer. To investigate the relationship between CD44v6 expression and EMT, we evaluated the expression of EMT regulatory proteins, such as E-cadherin, N-cadherin, fibronectin, and vimentin in FACS-sorted CD44v6-poisitive cells *versus* FACS-sorted CD44v6-negative cells by Western blot analysis. In consequence, E-cadherin expression was downregulated in FACS-sorted CD44v6-positive cells in comparison with FACS-sorted CD44v6-negative cells and concomitant upregulation of N-cadherin, fibronectin, and vimentin was observed in CD44v6 - positive cells (Fig. 3f). These findings suggested that a subpopulation of CD44v6 regulates the metastatic ability of ovarian cancer cells, which is relevant to the process of EMT.

Chemoresistance in a subpopulation of CD44v6-positive ovarian cancer cells. CSCs are inherently responsible for tumor resistance to conventional chemotherapy.[17,18] Given that the primary ovarian tumors containing at least 10% CD44v6-positive cancer cells showed significantly poorer prognosis, we next evaluated the relevance of chemoresistance in CD44v6-positive cells as a potential cause of the poor prognosis. To investigate whether the subpopulation of CD44v6-positive cells correlates with resistance to chemotherapy, ES-2 ovarian cancer cells were exposed to paclitaxel or cisplatin *in vitro*. Flow cytometric analysis showed that treatment with paclitaxel or cisplatin results in enhanced expression of CD44v6 in residual cancer cells as compared to untreated cells (Fig. 4a,b). Furthermore, FACS-sorted CD44v6-positive ovarian cancer cells showed significantly higher viability compared to FACS-sorted CD44v6-negative cells in MTS assay (Fig. 4c,d), indicating that a subpopulation of CD44v6-positive cells is associated with tumor resistance to chemotherapy.

Discussion

We have identified that disseminated tumors in the pelvic peritoneum are highly enriched in CD44v6-positive cancer cells, which prominently contributes to peritoneal metastasis of advanced epithelial ovarian cancer. Of particular interest in this study was that an increased number of CD44v6-positive cancer cells were associated with a shortened OS in the evaluation of the sites of primary tumors. Furthermore, we showed that a subpopulation of CD44v6-positive ovarian cancer cells possesses a strong ability to initiate tumor metastasis in the pelvic peritoneum in an *in vivo* mouse model, indicating that CD44v6-positive cells have the potential to serve as metastasis-initiating cells.

Epithelial ovarian cancer is a highly lethal malignancy that represents a great clinical challenge in gynecologic oncology.[4] Given that peritoneal dissemination and metastasis is responsible for most cancer-related deaths in patients with advanced ovarian cancer, the elucidation of molecular mechanisms underlying the peritoneal metastasis and the characteristics of ovarian CSCs is essential to combat this fatal disease. Although CD44v6 plays an important role in the tumor growth and metastasis of several types of tumors,[24,25] the functions of CD44v6 have not been completely characterized in ovarian cancer metastasis. In the current study, we showed that CD44v6 expression is increased in tumor tissues at the peritoneal metastasis sites compared with those at the corresponding primary tumors, indicating that CD44v6 is clinically associated with the induction of metastasis in the pelvic peritoneum.

Although previous studies have focused on the potential correlation of CD44v with ovarian cancer survival to address the diagnostic and prognostic values of CD44v, there is no unified view on this issue.[33] Some authors suggested that the expression of the CD44v6 is not correlated with tumor development and prognosis of epithelial ovarian cancer,[12,34] whereas others showed that CD44v6 expression levels are involved in ovarian cancer progression, metastasis, and relapse.[35] Taken together, several questions regarding the relationship between CD44v6 expression and prognosis remain to be resolved. In the light of these unanswered questions, we evaluated the association between CD44v6 expression and OS and PFS in the sites of primary lesions. As a result, the tumors containing at least 10% CD44v6-positive cancer cells showed significantly poorer prognosis in terms of OS than those containing less than 10% CD44v6-positive cells in the evaluation of the sites of primary tumors. Furthermore, the multivariate Cox proportional hazards model showed that the expression of CD44v6 is an independent prognostic factor for the OS of patients with advanced ovarian cancer.

In recent years, emerging evidence has provided support for the existence of CSCs in various cancers, including epithelial ovarian cancer.[17,20] Even though previous studies indicated that a CD44v6-positive cell population possesses CSC properties in several types of tumors,[24,25] the correlation between CD44v6-positive cells and ovarian CSCs remained unclear. To investigate whether a subpopulation of CD44v6-positive cancer cells manifest highly metastatic activity, we compared the tumorigenic and peritoneal metastatic potential of CD44v6-positive and CD44v6-negative cells in an *in vivo* mouse model. Consistent with our clinical observations, we found that a subpopulation of CD44v6-positive cells is prominently involved in peritoneal metastasis in a mouse model. In a set of experiments, we also showed that CD44v6 expression demarcates a highly tumorigenic ovarian CSC population with peritoneal metastatic potential and CD44v6-positive cells possess the potential to serve as metastasis-initiating cells. Recent evidence indicates the existence of a "CSC niche," a specialized

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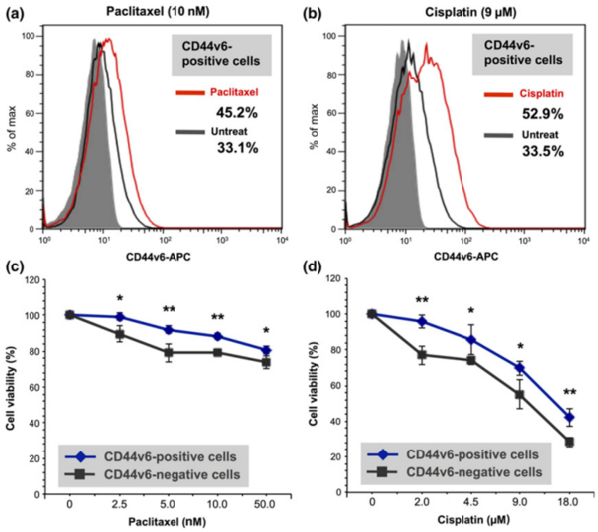

Fig. 4. CD44 variant 6 (CD44v6)-positive ovarian cancer cells are associated with chemoresistance. (a) Flow cytometric analysis of CD44v6 expression in ES-2 ovarian cancer cells treated with paclitaxel and untreated ES-2 cells. (b) Flow cytometric analysis of CD44v6 expression in ES-2 ovarian cancer cells treated with cisplatin and untreated ES-2 cells. (c) Chemosensitivity assay in FACS-sorted CD44v6-positive and FACS-sorted CD44v6-negative cells. Cells were subjected to MTS assay to evaluate viability in the presence of paclitaxel. $*P < 0.05$, $**P < 0.01$. (d) Chemosensitivity assay. FACS-sorted CD44v6-positive and FACS-sorted CD44v6-negative cells were subjected to MTS assay to assess the viability in the presence of cisplatin. $*P < 0.05$, $**P < 0.01$.

microenvironment that regulates CSC properties and contributes to tumor initiation, growth, and metastasis.[36,37] The present study revealed the close relationship between CD44v6 expression and the pelvic peritoneum and thereby, raises the possibility that the microenvironment of the pelvic peritoneum forms a possible CSC niche for epithelial ovarian cancer.[38]

Recent evidence suggested that CD44v manifests enhanced protection against species (ROS), rendering them resistant to chemotherapy in several types of solid tumors.[22,23] In the current study, we showed that a subpopulation of CD44v6-positive cancer cells is correlated with tumor resistance to chemotherapy. In view of this, the results of our present study raise the possibility that CD44v6 potentiates the ability of ovarian cancer cells to defend themselves against chemotherapy-induced ROS.

In conclusion, the biological and molecular heterogeneity of ovarian CSCs represents a highly promising area of research that may provide new insights that could lead to prognostic and therapeutic breakthroughs for advanced epithelial ovarian cancer. CD44v6-positive cancer cells may be a potential molecular therapeutic target for eliminating ovarian CSCs and metastasis-initiating cells. The finding that a distinct subpopulation of CD44v6-positive CSCs plays a central role in peritoneal metastasis suggests that definitive treatment should target the CD44v6-positive cell population in epithelial ovarian cancer.

Acknowledgments

We thank A. Aoki for technical assistance and Y. Hisako, K. Kawaguchi, S. Miyaji, and R. Tanaka for help in the preparation of the manuscript.

Disclosure Statement

The authors have no conflict of interest.

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