Expression of the Hyaluronan Receptor, CD44S, in Epithelial Ovarian Cancer Is an Independent Predictor of Survival

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ABSTRACT

Most ovarian carcinomas present at advanced stage, principally as the result of dissemination to peritoneal sites. Standard CD44 (CD44S) is the principal receptor for hyaluronic acid, and *in vitro* and animal studies have suggested that the attachment of ovarian carcinoma cells to the peritoneal mesothelium involves the interaction between CD44S on ovarian carcinoma cells and hyaluronic acid on mesothelial surfaces. We, therefore, analyzed a series of ovarian carcinomas for the expression of CD44S by immunohistochemistry to see whether expression of this receptor by tumor cells correlated with clinicopathological factors and measures of patient outcome. Fifty-six fixed, paraffin-embedded primary epithelial ovarian tumors were immunostained with antibody to CD44S. Membrane staining was considered positive, and results were correlated with stage, grade, age, histology, and survival. Twenty-two (39%) tumors were positive for CD44S. There was no correlation between CD44 expression and histological type, grade, age, or stage. However, CD44 expression was significantly associated with survival in both univariate ($P = 0.003$) and multivariate ($P = 0.006$) analyses. These results support a role for CD44S expression in the spread of ovarian epithelial cancer and suggest that expression of this molecule is a significant independent predictor of survival in women with this disease.

INTRODUCTION

Ovarian epithelial cancer was responsible for an estimated 26,800 cases and 14,200 deaths in 1997 (1). Late stage at diagnosis is responsible for the high mortality rate for this cancer, as more than 70% of patients are diagnosed at an advanced stage (2). Spread of ovarian cancer almost always occurs within the abdominal cavity, with death resulting from symptoms related to the intra-abdominal tumor mass. Although lymphatic spread occurs with some frequency, hematogenous dissemination of tumor is comparatively rare.

CD44 is a cell adhesion molecule that is expressed on the cell membrane in either the standard form (CD44S) or as a higher-molecular-weight form generated from mRNAs that include additional exons not present in CD44S mRNA (variant CD44s). CD44S is the principal matrix adhesion molecule expressed by normal ovarian epithelium and ovarian carcinoma cell lines, and *in vitro* binding of ovarian carcinoma cells to mesothelium is inhibited by antibodies to CD44S but not by antibodies to other adhesion molecules (3). Although metastatic behavior in some other cancers has been correlated with expression of CD44 variants, i.p. spread of ovarian carcinoma cells seems to be related exclusively to the expression of the standard CD44 (4).

Further evidence for the role of CD44S in the metastatic spread of ovarian cancer comes from animal studies, in which i.p. inoculation of human ovarian carcinoma cells into athymic nude mice results in disseminated tumor nodules that are dramatically decreased in number if tumor cells are preincubated with antibodies to CD44 (5).

Correlations of expression of CD44S by tumor cells with the extent of disease and survival in ovarian carcinoma patients would be a significant step toward confirming the importance CD44S expression in the spread of human ovarian cancer. Thus far, no such studies have been reported. We, therefore, evaluated a series of fixed ovarian carcinoma specimens for expression of CD44S and compared expression with clinical and pathological factors as well as with outcome measures.

MATERIALS AND METHODS

Patient Population. Tissue blocks from fixed, paraffin-embedded malignant ovarian epithelial tumors were retrieved from the surgical pathology files of the RPCI.[2] Patients with primary epithelial ovarian cancer who were treated at RPCI and who had completed an epidemiological questionnaire provided to the initial roster of eligible patients. Patients with recurrent disease or those who had received adjuvant chemotherapy were specifically excluded from the analysis. Fifty-six patients had tumor blocks available in the surgical pathology files, and follow-up information was available on all of these patients from the Tumor Registry. Patients were not selected on the basis

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[2] The abbreviations used are: RPCI, Roswell Park Cancer Institute; SEER, Surveillance, Epidemiology and End Results Program; FIGO, International Federation of Gynecologists and Obstetricians; RHAMM, receptor for hyaluronic acid mediated motility.

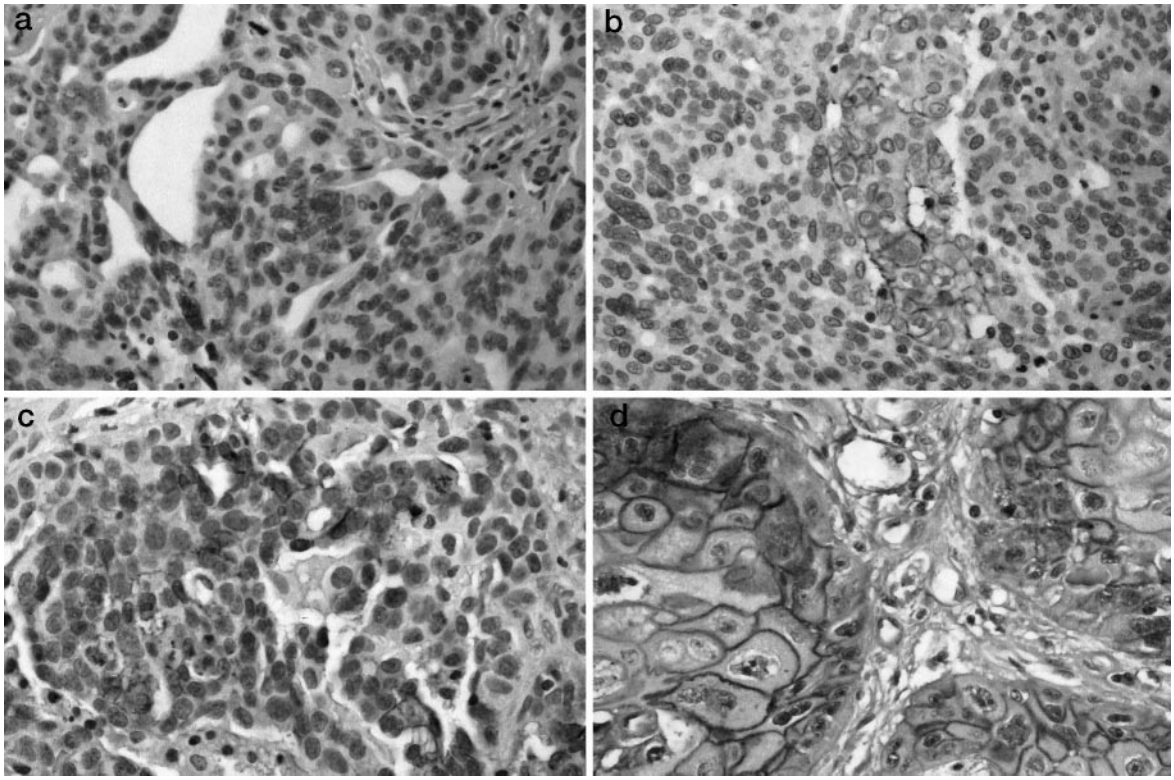

Fig. 1 Selected tumors exhibiting varying degrees of CD44S membrane staining. *a-c*, poorly differentiated serous carcinomas. *a*, rare foci of small groups of positive cells; *b*, tumor with scattered larger nests of positive cells in a background of negative tumor cells (most common pattern); *c*, tumor with most cells showing some membrane staining in all of the tumor nests; *d*, squamous cell carcinoma with staining of most of the cells in all of the areas. ×200.

of any other criteria. Stage was recorded by the Tumor Registry using SEER (6) rather than FIGO (7) staging criteria. The SEER system is divided into local, regional, and distant disease. Although SEER local disease corresponds to FIGO stage I, SEER regional disease includes some FIGO stages II and IIIC (ipsilateral pelvic peritoneal spread and retroperitoneal lymph nodes, respectively), and SEER distant disease includes other FIGO stages II and IIIC (contralateral pelvic disease and inguinal lymph node metastasis, respectively) as well as distant hematogenous dissemination (FIGO stage IV). All of the research was carried out under a protocol approved by the RPCI Institutional Review Board.

Immunohistochemical Analysis. H&E-stained slides were reviewed to confirm that the blocks contained tumor. Tumor type and grade were determined by one of the authors (B. A. W.) based on modified WHO criteria (8). Sections (4-μM) were cut, deparaffinized, and stained with a monoclonal antibody to standard CD44 molecule (CD44S, Bender MedSystems, Vienna, Austria) at a dilution of 1:50. Sections were subjected to heat-induced antigen retrieval for 15 min at 100°C in 10 mM citrate buffer before the addition of the primary antibody. All of the staining was carried out using the avidin-biotin method on a Ventana 320 Automated System (Ventana Medical System, Tucson, AZ). Di-amino-benzidine was used as the chromogen. Negative controls consisted of substituting an isotypic mouse IgG for the primary antibody. Positive control consisted of normal skin, which normally expresses CD44S. Any membrane staining of tumor cells was considered positive, and the percentage of positive cells as well as the intensity of staining was recorded. Any positive staining except for rare, isolated single cells was considered positive.

Outcome Measures. All of the patients were followed until the time of analysis or until death. No patient was lost to follow-up. Outcome measures were overall survival and disease-free survival. These were correlated with clinical and pathological factors and with results of immunohistochemistry for CD44S, p53, and $p21^{waf1/cip1}$. Associations between CD44S expression and p53, p21, stage, differentiation, histology, and age at diagnosis were assessed using Fisher's exact and χ^2 tests. Associations were considered statistically significant if two-tailed *P*s were less than 0.05. Kaplan-Maier curves were used to estimate survival probability as a function of time, and survival differences were analyzed by the log-rank test. A proportional-hazards model was used to estimate and test the influence of CD44S expression, stage, differentiation, and histology on survival.

RESULTS

The majority of tumors were serous (38 of 56; 67.8%), poorly differentiated (43 of 56; 76.7%) and high grade (49 of 56; 87.5%). All of the positive tumor cells were characterized by

Table 1 CD44 positivity by histology, grade, and stage

	No. of cases	Positivity, *n* (%)	*P*
Histology			
Serous	38	16 (42)	
PD NOS[a]	6	3 (50)	
Mucinous	5	0 (0)	
Clear cell	3	1 (33)	
MMMT	2	1 (50)	
Endometrioid	2	1 (50)	
Total	56	22 (39)	0.566
Grade			
Well	5	1 (20)	
Moderate	8	4 (50)	
Poor	43	17 (40)	
Total	56	22 (39)	0.558
Stage			
Distant	49	21 (43)	
Nondistant	7	1 (14)	0.226

[a] PD, poorly differentiated; NOS, not otherwise specified; MMMT, malignant mixed Müllerian tumor.

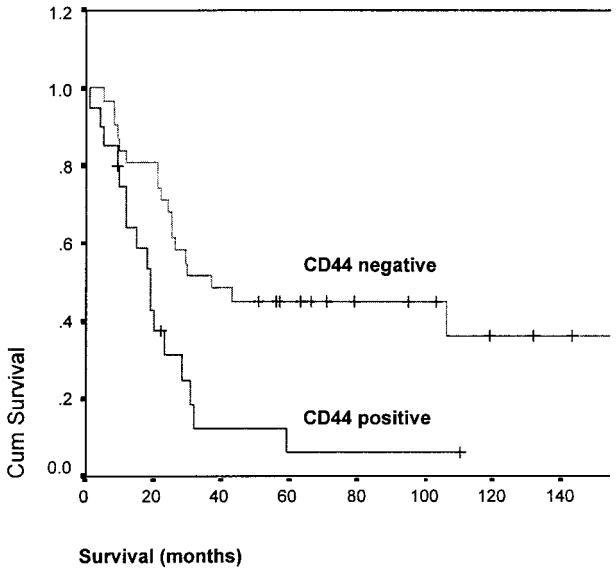

Fig. 2 Kaplan-Meier survival plots for women whose tumors expressed CD44S and for women whose tumors were negative for CD44S.

intense membrane staining. Most of the positive tumors had single or multiple small foci of tumor cells exhibiting intense membrane reactivity, with the majority of tumor cells negative. Only rare positive tumors expressed CD44S in the majority of cells. Fig. 1 shows several tumors differing in the extent of membrane immunoreactivity for CD44S. Twenty-two (39%) of 56 primary tumors expressed CD44. Table 1 lists CD44S immunohistochemical expression by histological type, grade, and stage. There was no significant association between histological type and CD44S expression, although 0 of 5 mucinous tumors expressed CD44S compared with 16 (43%) of 38 serous tumors. Higher grade tumors (grades II and III) and those from patients with distant disease were more likely to be positive for CD44S (41.1 *versus* 20% and 43 *versus* 14%, respectively), but this did not reach statistical significance.

CD44S expression was significantly associated with poorer disease-free survival in univariate analysis ($P = 0.003$). Fig. 2 shows the Kaplan Meier survival curves for disease-free survival. Fifty-eight % of the patients whose tumors did not express CD44S died in the follow-up period compared with 85% of patients whose tumors expressed CD44S. Patients whose tumors were CD44-positive had a mean survival of 25 months (SD ± 24 months) compared with patients with CD44-negative tumors whose mean survival was 52 months (SD ± 42 months). In multivariate Cox regression analysis, CD44 was independently related to survival, even after adjusting for age, histology, stage, and differentiation ($P = 0.006$).

DISCUSSION

Most women dying from epithelial ovarian cancer succumb to the effects of i.p. spread and growth of tumor, rather than from vascular or lymphatic dissemination. Ovarian cancer cells attach to peritoneal mesothelial cells using adhesion molecules on the cell membrane. Several independent lines of evidence have suggested that CD44S is the principal matrix adhesion molecule expressed by normal ovarian epithelium and ovarian carcinoma cell lines. Cannistra and colleagues (3) demonstrated that, of the three classes of adhesion molecules (integrins, selectins and proteoglycans, the latter of which includes CD44S), only β1 integrins and CD44S were expressed by normal ovarian epithelial cells and ovarian carcinoma cells. Antibodies to CD44S, but not to β1 integrins, inhibited binding of carcinoma cells to mesothelial cells *in vitro*. The importance of CD44S for adhesion was supported by their finding that malignant cells from solid tumor, but not from ascites, both expressed CD44S and bound to mesothelium *in vitro*. Mediation of binding by hyaluronic acid was confirmed by demonstrating that a significant fraction of binding could be prevented by pretreating mesothelial cells with hyaluronidase. Subsequent studies have shown that the strength of adhesion of ovarian carcinoma cells to mesothelial cells is correlated with the degree of CD44S expression, and that transfection of CD44S-expressing cDNA to weak binders restored adhesion to peritoneal mesothelium (4).

Animal studies have also provided support for the importance of CD44S expression in peritoneal adhesion by ovarian carcinoma cells. Inoculation of the peritoneal cavity of syngeneic mice with a mouse ovarian carcinoma cell line was associated with increased levels of hyaluronic acid in the ascites and increased hyaluronic acid deposition in the stroma of the mesentery at the sites of tumor cell attachment (9). Although only 10% of ascites cells were positive for CD44, this is the population that attached to these sites. In another study, human ovarian carcinoma cells were inoculated in the peritoneal cavity of athymic mice, and the ability of antibodies to CD44S to inhibit tumor formation was evaluated. The number but not the size of tumor nodules was decreased, which is consistent with the notion that the inhibition of CD44S decreases the opportunity for attachment but once attached does not interfere with the growth of the nodule (5).

Hyaluronic acid has been implicated in the invasion and metastasis of tumor cells in animal models (10, 11) and is found

at high levels in certain tumor types (12) and in the serum of some cancer patients (13, 14). A number of hyaluronic acid-binding proteins have been identified (15), but only two, RHAMM and CD44, have been shown to mediate cell-stroma interactions in tumors. RHAMM (receptor for hyaluronic acid mediated motility) was identified as a cell-surface protein responsible for the hyaluronic acid binding seen as a result of H-*ras*-induced cell motility (16). RHAMM expression has been identified on human cancer cells (17, 18) and has been implicated in tumor progression (18) RHAMM also seems to play a direct role in cell transformation (19). The most well-studied hyaluronan receptor in human tumors, however, has been CD44. Expression of many different variants of the CD44 molecule (CD44v) is possible by inclusion of a variety of additional exons not present in the standard (CD44S) transcript (20), some of which also bind to hyaluronan. Expression of these variants has been correlated with adverse prognostic features in some tumor types (21–23), but these findings have not been consistent (24). Two previous studies (4, 25) of CD44 expression and prognosis in epithelial ovarian cancer measured expression of variant rather than standard CD44. One study demonstrated a correlation of CD44v expression with survival, (25), and the other did not (4). The current study is the first study to report the influence of standard CD44 (CD44S) expression on survival in patients with epithelial ovarian cancer.

In summary, we found that immunohistochemical expression of CD44S on ovarian epithelial cancers is a significant independent prognostic factor in this disease. This finding supports previous *in vitro* and animal studies showing the importance of CD44S expression on ovarian tumor cells in peritoneal dissemination.

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