

# CD44 Expression Indicates Favorable Prognosis in Epithelial Ovarian Cancer

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## ABSTRACT

**Purpose:** The purpose of this study was to investigate the expression and prognostic significance of CD44 in epithelial ovarian cancer.

**Experimental Design:** We analyzed the expression of CD44 by immunohistochemistry in 307 epithelial ovarian cancers and evaluated its relation to hyaluronan, clinicopathological factors, and prognosis.

**Results:** Fifty-one percent of the tumors had a high proportion of CD44-positive cells (*i.e.*,  $\geq 10\%$ ), and this high CD44 expression was significantly associated with cancer cell-associated hyaluronan, well-differentiated tumor, mucinous histological type, and early stage of the tumor. High CD44 expression predicted better 5-year overall survival (50% versus 22%) and recurrence-free survival (70% versus 34%) in the univariate analyses ( $P < 0.00005$  for both). In the Cox multivariate analyses, the independent predictors of overall survival at 5 years were primary residual tumor ( $P < 0.0005$ ), International Federation of Gynecologists and Obstetricians (FIGO) stage ( $P = 0.001$ ), histological grade ( $P = 0.014$ ), adjuvant chemotherapy ( $P = 0.004$ ), and stromal hyaluronan level ( $P < 0.0005$ ), but not CD44. However, the expression of CD44 ( $P = 0.04$ ) and stromal hyaluronan ( $P = 0.005$ ) were both independent predictors of recurrence-free survival at 5 years, together with the size of the primary

residual tumor ( $P < 0.0005$ ) and histological type ( $P = 0.043$ ).

**Conclusions:** The relatively frequent ectopic expression of CD44 on ovarian cancer cells is thus related to well-differentiated, early-stage tumor and long survival of the patients. Thus, whereas CD44-expressing cancer cells may adhere and implant to the hyaluronan-positive mesothelium, at least in model systems, high expression of CD44 in the tumor does not bring about an unfavorable prognosis.

## INTRODUCTION

Ovarian cancer is the sixth most common cause of death from cancer in women (1). The prognosis for ovarian cancer patients is poor because metastasis is frequently present at the time of diagnosis (2). In epithelial ovarian cancer, metastasis occurs mainly by exfoliation of the tumor cells from the primary tumor, followed by migration, implantation, and invasion throughout the peritoneal cavity (3). For this to happen, tumor cells must alter their adhesive properties.

CD44 is a transmembrane protein widely distributed in both epithelial and nonepithelial normal tissues (4). CD44 is encoded by a single gene located on human chromosome 11. However, alternative splicing of mRNA produces several larger CD44 isoforms in addition to the standard isoform, CD44s (5). CD44 is involved in many cell-cell and cell-matrix interactions, including cell adhesion, migration, and lymphocyte functions (6). CD44 is considered the principal cell surface receptor for hyaluronan (7), an extracellular polysaccharide involved in a variety of physiological and pathological processes (8), including the progression of malignancies such as ovarian cancer (9–11).

Induction of CD44 expression has been noted during the development of ovarian carcinoma, but the issues of whether high CD44 expression represents a relatively favorable prognosis (12) or an aggressive behavior of the tumor and unfavorable prognosis (13) and whether CD44 has any prognostic significance (14–16) have remained controversial. To clarify the role of CD44 expression in epithelial ovarian cancer, we studied a large series of samples by immunohistochemistry and analyzed the prognostic value of CD44 by univariate and multivariate analyses.

## PATIENTS AND METHODS

**Patients.** The material of the present study was selected from a consecutive series of 445 women diagnosed and treated for ovarian malignancy at Kuopio University Hospital and Jyväskylä Central Hospital, Finland between 1976 and 1992. The relevant clinical data were collected by retrospective review of the patients' files. Patients with the nonepithelial type of neoplasia ( $n = 36$ ) and all patients treated before operation ( $n = 33$ ) or patients who were not treated with operation ( $n = 35$ ) were excluded. Depending on the availability of representative tumor

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samples, a total of 307 patients were included in the analysis. Forty-four of the patients had metastatic lesions available that were also analyzed. Borderline tumors were excluded from the study.

Tumor staging was based on the standards of the FIGO<sup>2</sup> (17). In addition to postoperative adjuvant chemotherapy, 8 patients received postoperative radiotherapy, and 38 patients received both of these adjuvant therapies. Tumor recurrence was observed in 72 patients (23%), no recurrence was observed in 94 patients (31%), and tumor was present or progressing in 141 patients (46%) during the follow-up. The median follow-up time was 27 months (range, 0.3–237 months) for all patients ( $n = 307$ ) and 106 months (range, 21–237 months) for patients still alive ( $n = 77$ ). Patients who died because of any postoperative complications were excluded from the survival analyses ( $n = 9$ ). The clinicopathological characteristics of the patients are summarized in Table 1.

**Histology.** Of all tumors, 5- $\mu$ m-thick paraffin-embedded tissue sections were stained with H&E. Histological typing and grading were re-evaluated for this study according to the WHO classification (18).

**CD44 Immunohistochemistry.** Deparaffinized and re-hydrated sections were heated in a microwave oven in 0.01 M citrate buffer (pH 6.0) for  $3 \times 5$  min, incubated in the citrate buffer for 18 min, and washed for  $2 \times 5$  min with PBS. Endogenous peroxidase activity was blocked by 5% hydrogen peroxide for 5 min, followed by a wash for  $2 \times 5$  min with PBS. The sections were incubated with 1% BSA in PBS for 30 min at 37°C. The primary antibody (mouse monoclonal antihuman CD44, clone 2C5; R&D Systems, Abingdon, United Kingdom), which recognizes all forms of CD44 (19), was diluted with 1% BSA to 1:1200 and incubated on the slides overnight at 4°C. The slides were incubated with the secondary antibody for 1 h and washed with PBS for  $2 \times 5$  min. The slides were then incubated for 1 h in preformed avidin-biotin peroxidase complex and washed twice for 5 min with PBS, developed with 3,3'-diaminobenzidine, counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted in DePex. An adjacent section, processed without the primary antibody, was used as a negative control. The intensely CD44-positive inflammatory cells were used as internal positive controls, and lung samples showing strong staining for CD44 were used as external positive controls.

**Histochemistry of Hyaluronan.** The biotinylated complex of hyaluronan binding region of aggrecan and link protein (bHABC) was prepared from bovine articular cartilage and tested for purity, as described previously (20, 21). The staining procedure has been described in detail previously (20).

**Evaluation of the Stainings.** All samples were analyzed by two observers (M. A. A. and V-M. K.) who were unaware of the clinical data. Disagreement in the assessment of staining was found in <10% of the slides examined, and consensus was reached on further review. About 20% of the samples were

Table 1 Clinicopathological characteristics of the patients ( $n = 307$ )

Variable	<i>n</i>	%
FIGO stage		
I	84	27
II	47	15
III	144	47
IV	32	10
Histological type		
Serous	109	36
Mucinous	31	10
Endometrioid	82	27
Clear cell	32	10
Other epithelial <sup>a</sup>	53	17
Histological grade		
1	43	14
2	104	34
3	160	52
Primary residual tumor		
None	122	40
≤2 cm	51	17
>2 cm	108	35
No data	26	8
Adjuvant chemotherapy		
Platinum-containing	163	53
No platinum	97	32
None	44	14
No data	3	1
Chemotherapy response <sup>b</sup>		
CR	139	45
PR	39	13
SD	22	7
PD	56	18
No data	51	17
CD44 expression		
Low	152	49
High	155	51
Stromal hyaluronan level		
Low	94	31
Moderate	116	37
High	97	32
Cell-associated hyaluronan staining		
Negative	47	15
Positive	260	85

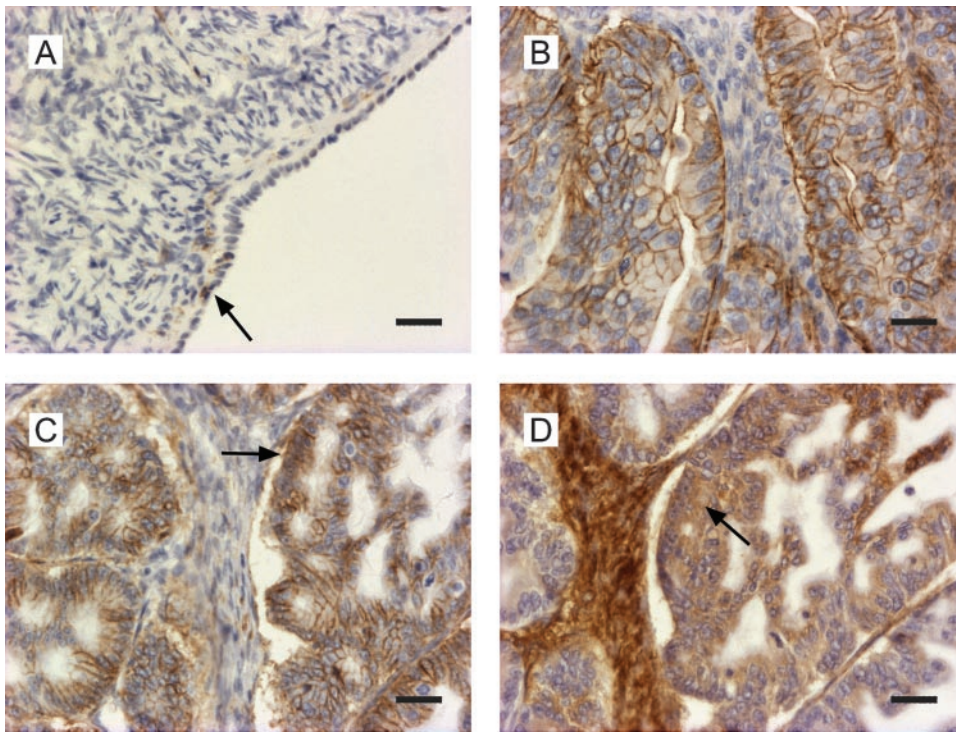
<sup>a</sup> Includes 20 mixed epithelial, 1 Brenner, 32 unclassified epithelial cancers.

<sup>b</sup> CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

stained with another CD44 antibody (monoclonal mouse anti-human CD44, clone DF 1485; Dako Cytomation, Glostrup, Denmark). Two investigators (S. S. and V-M. K.) evaluated the stainings. The expression of CD44 was scored as a fraction of positive cancer cells in the whole tumor area. The samples were considered CD44 positive if any positive staining was detected, unless there were only rare, isolated single cells. The intensity of CD44 was categorized into three grades: 0, negative; 1, weak to moderate; or 2, strong. For further statistical analysis, CD44 expression was categorized into two groups according to the median percentage of the expression: low expression, ≤10% of the tumor cells expressed CD44; and high expression, >10% of the tumor cells expressed CD44.

The percentage of stroma with the strongest hyaluronan intensity of the total peri- and intratumoral stromal area was graded into three categories (low, <35%; moderate, 35–75%; or

<sup>2</sup> The abbreviations used are: FIGO, International Federation of Gynecologists and Obstetricians; RFS, recurrence-free survival; OS, overall survival.



**Fig. 1** Immunohistochemical staining of normal and ovarian cancer tissue sections using monoclonal antihuman CD44. **A**, normal ovary with a few epithelial cells (arrow) positive for CD44. **B**, serous ovarian carcinoma showing high expression of CD44 on the cancer cell membrane. **C**, mucinous ovarian carcinoma showing high cancer cell-associated expression of CD44 (arrow). **D**, the same tumor as in **C** with hyaluronan (arrow). Bars, 30  $\mu$ m.

high, >75%), according to the 33rd and 66th percentiles in a frequency distribution. The percentage of hyaluronic acid-positive tumor cells of all neoplastic cells in the section was also estimated, but for statistical analysis the tumors were eventually categorized in two groups, hyaluronan positive or hyaluronan negative. The evaluation of hyaluronan has been described in detail previously (11).

**Statistical Analyses.** Statistical analyses were performed using the SPSS computer program package. Spearman correlation coefficients and Wilcoxon tests were used to evaluate the relationships between continuous variables. Frequency tables were analyzed using a  $\chi^2$  test. Univariate survival analyses were based on Kaplan-Meier method (22). The differences between curves were analyzed using the log-rank test. OS was defined as the time interval between the date of surgery and the date of death due to ovarian cancer. RFS was defined by the time interval between the date of surgery and the date of recurrence. Multivariate survival analysis was calculated by means of Cox's proportional hazards model in a forward stepwise manner with the log-likelihood ratio significance test (23). The assumption of proportional hazards was tested by logminlogplots. Probability values of <0.05 were regarded as significant.

## RESULTS

**CD44 Protein Expression.** Only a few epithelial cells of the normal ovary expressed CD44, in contrast to the tumor samples with frequent cell surface staining for CD44 (Fig. 1, **A** and **B**). The mean percentage of CD44-positive cells was 20% in the primary tumors ( $n = 307$ ) and 14% in the metastatic lesions ( $n = 44$ ; Fig. 2). About 20% of the samples stained with another CD44 antibody were analyzed by two investigators. No statis-

tically significant difference was noted between the antibodies used ( $Z = -1.88$ ,  $P = 0.06$ , Wilcoxon test). In addition, there was no difference in CD44 expression (clone 2C5) in older samples compared with more recent samples ( $P = 0.68$ , Kruskal-Wallis test). Lymphocytes infiltrating the stromal areas were always CD44 positive. The level of CD44 expression in the primary tumors correlated with that of the metastatic lesions ( $r = 0.61$ ,  $P < 0.0005$ ).

**Correlation between CD44 and Hyaluronan.** In 85% of the cases, at least some hyaluronan-positive cancer cells were found in the tumors. A positive correlation was found between the levels of CD44 and hyaluronan-positive cancer cells in the primary tumors ( $r = 0.2$ ,  $P < 0.0003$ ;  $n = 298$ ; Fig. 1, **C** and **D**), a finding in line with the hyaluronan receptor function of CD44. In the metastases ( $n = 44$ ), however, CD44 expression level was not correlated with cell-associated hyaluronan. Stromal hyaluronan level was scored low, moderate, and high in 31%, 37%, and 32% of the cases, respectively. No correlation existed between CD44 and stromal hyaluronan in the primary tumors, whereas the metastases showed a modest inverse relationship between CD44 and stromal hyaluronan ( $r = -0.3$ ,  $P = 0.03$ ).

**CD44 Expression and Clinicopathological Factors.** Well-differentiated ( $P < 0.0005$ ) and mucinous ( $P < 0.0005$ ) tumors showed high CD44 expression (Table 2). The tumors with high CD44 expression also responded better to adjuvant chemotherapy ( $P = 0.008$ ). The advanced-stage tumors had low CD44 expression ( $P < 0.0005$ ). Low CD44 expression was also significantly associated with a large primary residual tumor (>2 cm), tumor recurrence, and death of the patient (Table 2).

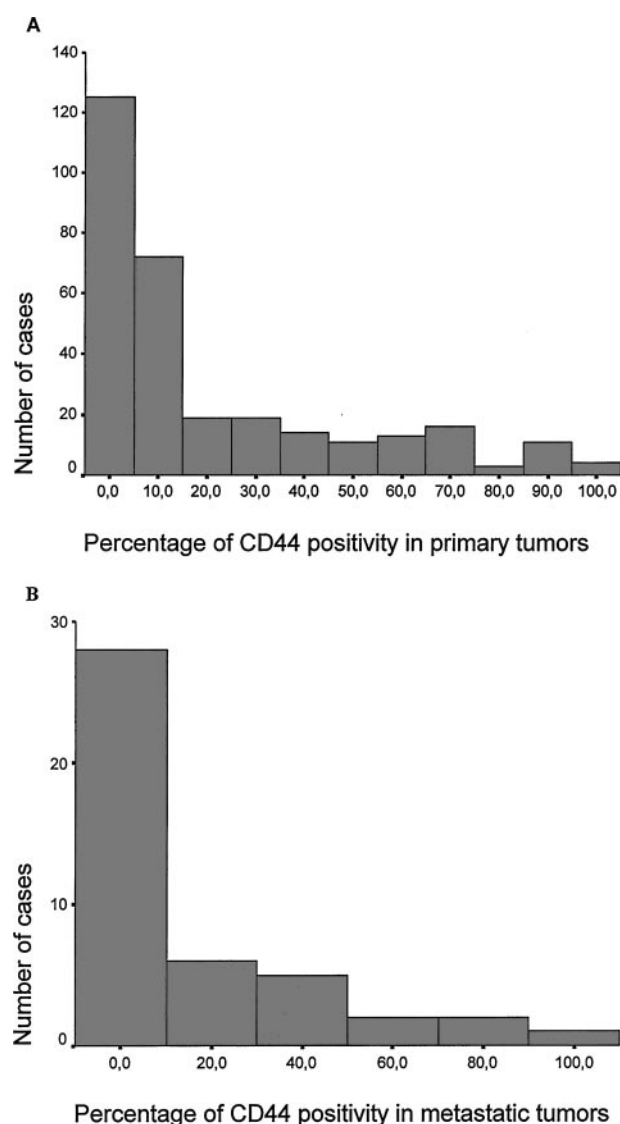


Fig. 2 Histograms. Distribution of CD44 in (A) primary ovarian tumors (mean percentage of CD44-positive cells = 20%) and (B) metastases (mean percentage of CD44-positive cells = 14%). Columns represent the numbers of cases in the groups determined by the percentage of CD44 positivity detected in cancer cells.

**Survival.** The 5-year prognosis for patients with high CD44 expression compared with that for patients with low CD44 expression was better in both OS (50% versus 22%,  $P < 0.00005$ ) and RFS (70% versus 34%,  $P < 0.00005$ ; Fig. 3). Other factors in the univariate analysis significantly indicating better OS were well-differentiated tumor, early FIGO stage, no primary residual tumor ( $P < 0.00005$  for all) and younger than median age at diagnosis ( $P = 0.05$ ). Longer RFS was predicted by the mucinous histological type ( $P = 0.025$ ), well-differentiated cancer ( $P = 0.049$ ), early FIGO stage ( $P = 0.0001$ ), and no primary residual tumor ( $P < 0.00005$ ).

CD44, stromal hyaluronan, histological type and grade, FIGO stage, primary residual tumor, age at diagnosis, and adjuvant chemotherapy were entered in Cox's multivariate anal-

Table 2 Distribution (%) of clinicopathological variables within CD44 categories

Variable	CD44 expression		Total
	Low <sup>a</sup>	High <sup>b</sup>	
FIGO stage			
I	32	68	100
II	32	68	100
III	62	38	100
IV	66	34	100
Histological type			
Serous	60	40	100
Mucinous	13	87	100
Endometrioid	51	49	100
Clear cell	41	59	100
Other epithelial	53	47	100
Histological grade			
1	21	79	100
2	42	58	100
3	62	38	100
Primary residual tumor			
None	31	69	100
≤2 cm	53	47	100
>2 cm	63	37	100
Chemotherapy response <sup>c</sup>			
CR	42	58	100
PR	59	41	100
SD	77	23	100
PD	54	46	100
Age at diagnosis (yrs)			
<62	46	54	100
≥62	54	46	100
Recurrence			
Yes	60	40	100
No	28	72	100
End state			
Dead	56	44	100
Alive	31	69	100

<sup>a</sup> <10%.

<sup>b</sup> ≥10%.

<sup>c</sup> CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

yses (Table 3). CD44 did not retain its statistical significance in predicting OS, as did stromal hyaluronan and the clinicopathological factors shown in Table 3. In contrast, both CD44 and stromal hyaluronan had independent prognostic value in RFS, together with the size of the primary residual tumor and histological type (Table 3).

## DISCUSSION

The set of splice variants and overall expression level of CD44 change during the development of several malignant tumors (24). These changes can lead to altered adhesion between tumor cells and extracellular matrix, facilitate invasion (24, 25), and enhance growth (4). We show here that decreased expression of CD44 is associated with advanced stages of epithelial ovarian tumors and acts as an independent factor predicting short RFS.

The general prognostic role of CD44 in human cancers is complicated. Decreased expression of CD44 and its variants has been associated with poor outcome in melanoma (26) and prostate (27–29) and colorectal cancer (30). On the other hand, in

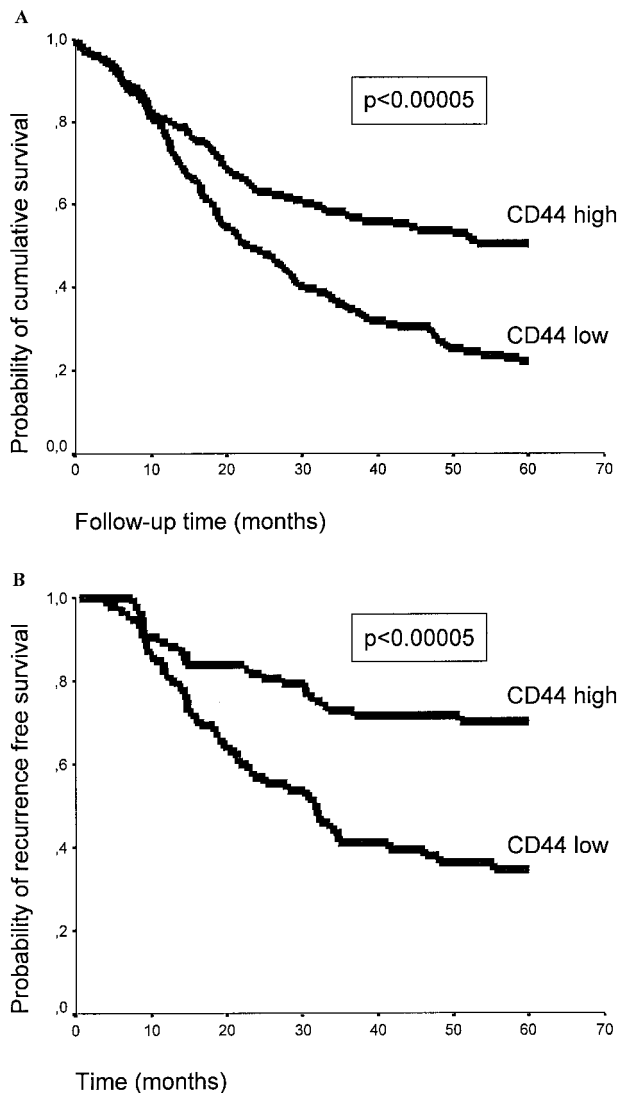


Fig. 3 A, Kaplan-Meier curves for OS of epithelial ovarian cancer patients according to the percentage of low ( $\leq 10\%$ ) and high ( $> 10\%$ ) cancer cell-associated CD44 staining (log-rank analysis). B, Kaplan-Meier RFS curves of patients according to the percentage of low ( $\leq 10\%$ ) and high ( $> 10\%$ ) cancer cell-associated CD44 staining (log-rank analysis).

renal cell carcinoma (31, 32), non-small cell lung carcinomas (33), stage II and III breast cancer (34), and cervical cancer FIGO stage Ib (35), increased expression of CD44 predicts poor survival. Furthermore, no relationship between CD44 expression and prognosis has been found in gastric cancer (36). Obviously, the role of CD44 in the later progress of the disease is related to tumor type.

However, inconsistent results on the prognostic role of CD44 have also been found within a certain tumor type, with epithelial ovarian cancer being one example (Table 4). Thus, using relatively fewer patient materials, one previous study has agreed with the present study that high CD44 expression is associated with relatively good prognosis in ovarian carcinoma (12), three studies have detected no association at all

(14–16), and one study suggested poor prognosis with high CD44 (13). We think that the defined and large body of patient material in the present study has eliminated potential problems related to the samples, but there are other issues [such as the different antibodies used (Table 4)] that may lay behind the controversies.

Epithelial ovarian cancer spreads by implantation of tumor cells through the mesothelial lining of the peritoneal cavity. *In vitro* studies have suggested that CD44 on the surface of ovarian cancer cells binds to the hyaluronan coat on mesothelial cells and may contribute to peritoneal metastasis (37, 38). Monoclonal antibodies against CD44 significantly inhibit ovarian cancer cell adhesion to mesothelial cells and peritoneal implantation in mice (39, 40). The present study, which is based on a large body of clinical material as well as data by others (12), does not concur with the idea of tumor cell CD44 supporting human ovarian cancer spreading. On the contrary, a high proportion of CD44-positive cancer cells occurred in well-differentiated tumors and less aggressive histological subtypes in the present material and was associated with a relatively favorable prognosis in both OS and RFS. Other molecular mechanisms, such as integrins (41–43) and proteoglycans (44), may have to be considered for the implantation stage of ovarian cancer cells.

No change in CD44 expression was observed during the metastatic process, whereas others have observed down-regulation of CD44 during tumor progression in mice (45) and in human ascitic tumor cells (12). Naturally, changes in CD44 other than the total expression level may contribute to its role in malignant growth (46). One such property is the regulated ability of this receptor to bind hyaluronan (6). In the present material, a positive correlation noted in the primary tumors between cell-associated hyaluronan and CD44 was lost in the metastases, perhaps reflecting changes in the hyaluronan receptor function of CD44.

The inverse correlation between CD44 expression and stromal hyaluronan in the metastases is not surprising, given that both parameters are indicators of advanced disease and poor prognosis. However, the present data do not give any hints of a molecular or metabolic connection between stromal hyaluronan accumulation and CD44 expression. The fact that stromal hyaluronan accumulation and CD44 depletion in the primary tumors were both independent prognostic factors in the Cox analysis of RFS rather suggests that they act separately.

Also, when comparing the expression of CD44 and hyaluronan in the tumor cells, it must be acknowledged that the affinity of CD44 to hyaluronan is regulated rather than constitutive, and there is no way of detecting the fraction of total CD44 capable of binding hyaluronan in each case. Therefore, the expression of CD44 in itself does not guarantee cell adherence to hyaluronan, and the level of total CD44 may not correlate with hyaluronan affinity.

In conclusion, 51% of epithelial ovarian cancers express CD44 in  $> 10\%$  of tumor cells, and this high expression rate is associated with better survival of these patients and with other favorable prognostic factors. Accordingly, the high level of ectopic CD44 expression that often occurs in human epithelial ovarian cancer is not a negative prognostic indicator, despite the

Table 3 The independent prognostic factors in Cox's multivariate analysis for OS ( $n = 269$ ) and RFS ( $n = 151$ )

Factor	Category	Relative risk	95% CI <sup>a</sup>	P <sup>b</sup>
OS				
Adjuvant chemotherapy	Platinum-containing therapy	<sup>c</sup>		0.004
	Nonplatinum therapy	1.469	1.020–2.116	0.039
	None	2.648	1.438–4.877	0.002
FIGO stage	I–II vs. III–IV	2.399	1.451–3.968	0.001
Primary residual tumor	Negative vs. positive	3.928	2.268–6.803	<0.0005
Histological grade	Grade 1–2 vs. 3	1.518	1.089–2.117	0.014
Stromal hyaluronan level	Low vs. high	1.379	1.167–1.630	<0.0005
RFS				
Primary residual tumor	Negative vs. positive	2.990	1.735–5.151	<0.0005
Histological type	Serous vs. other	1.704	1.018–2.854	0.043
CD44 expression	Low vs. high	2.062	1.025–3.067	0.040
Stromal hyaluronan level	Low vs. high	1.456	1.120–1.891	0.005

<sup>a</sup> CI, confidence interval.

<sup>b</sup> Log-likelihood ratio significance test.

<sup>c</sup> Reference category.

Table 4 Studies on CD44 and its prognostic significance in ovarian cancer

Author (ref no.)	CD44s antibody	Manufacturer	n	CD44 positivity	Prognostic significance
Cannistra <i>et al.</i> (16)	Clone 25.32	R&D Systems	31	82%	No significance
Kayastha <i>et al.</i> (13)	Anti-CD44s monoclonal antibody	Bender MedSystems	56	39%	Positivity → poor prognosis in UV <sup>a</sup> and MV analyses
Saegusa <i>et al.</i> (15)	Anti-h phagoc glycop-1 mouse monoclonal antibody	Dako	115	Score	No significance
Berner <i>et al.</i> (14)	Anti-CD44s monoclonal antibody	Bender MedSystems	67	64%	No significance
Ross <i>et al.</i> (12)	A3D8 mouse monoclonal antibody	Sigma Immunochemicals	64	23%	Low expression → poor prognosis in UV, not in MV analyses
Sillanpää <i>et al.</i> , this work	Clone 2C5	R&D Systems	307	50% (high)	Low expression → poor prognosis in UV and MV analyses

<sup>a</sup> UV, univariate; MV, multivariate; anti-h, antihuman; phagoc, phagocytic; glycop-1, glycoprotein-1.

fact that in some models and under some conditions, CD44 may aid the mesothelial adhesion of cancer cells.

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