Distribution and Clinical Significance of Heparan Sulfate Proteoglycans in Ovarian Cancer

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ABSTRACT

Purpose: **Heparan sulfate proteoglycans have been implicated in cancer cell growth, invasion, metastasis, and angiogenesis. This study was designed to compare their expression in normal ovary and ovarian tumors and then to examine their prognostic significance in ovarian cancer.**

Experimental Design: **The expression of syndecan-1, -2, -3, and -4, glypican-1, and perlecan was assessed by immunohistochemistry in 147 biopsies that included normal ovary and benign, borderline, and malignant ovarian tumors. Clinical data, including tumor stage, performance status, treatment, and survival, were collected. Univariate and multivariate analyses were performed to evaluate prognostic significance.**

Results: **The expression patterns of syndecan-1 and perlecan were altered in ovarian tumors compared with normal ovary. Syndecan-1 was not detected in normal ovary but was present in the epithelial and stromal cells of benign and borderline tumors and in ovarian adenocarcinomas. Perlecan expression was decreased in basement membranes that were disrupted by cancer cells but maintained in the basement membranes of blood vessels. Syndecan-2, -3, and -4, and glypican-1 were expressed in normal ovary and benign and malignant ovarian tumors. Stromal expression of syndecan-1 and glypican-1 were poor prognostic factors for survival in univariate analysis.**

Conclusion: **We report for the first time distinct patterns of expression of cell surface and extracellular matrix heparan sulfate proteoglycans in normal ovary compared with ovarian tumors. These data reinforce the role of the tumor stroma in ovarian adenocarcinoma and suggest that stromal induction of syndecan-1 contributes to the pathogenesis of this malignancy.**

INTRODUCTION

The heparan sulfate proteoglycans (HSPGs) play diverse roles in tumor biology by mediating adhesion and migration and cellular responses to mitogenic and angiogenic growth factors (1). They have been proposed as critical regulators of tumor invasion and metastasis (2). The major HSPG families at the cell surface are the four transmembrane syndecans (syndecan-1 through -4) and the 5 glypican family members (glypican-1 through -5) that are linked to the cell membrane via a phosphatidyl-inositol anchor (1). Perlecan is a large modular protein that is abundant in the extracellular matrix and basement membranes of normal tissues and blood vessels (3, 4).

These distinctly different proteins are designated HSPGs because they are modified after translation by the covalent attachment of two or more chains of the linear polysaccharide heparan sulfate. The heparan sulfate chains are responsible for much of the biological role of the HSPGs because they encode the recognition sites for numerous heparin-binding proteins, such as acidic and basic fibroblast growth factor (bFGF; Ref. 5), vascular endothelial growth factor (6), and hepatocyte growth factor/scatter factor (7, 8), which have well-documented roles in tumor biology. These ligands use HSPGs as co-receptors to mediate their binding to specific tyrosine kinase receptors and promote signal transduction (9). HSPGs also participate in cell-cell and cell-matrix adhesion complexes by binding to structural proteins of the extracellular matrix, including collagens, laminin, and fibronectin. Notably, syndecan-4 cooperates with integrins and regulates the distribution and activity of protein kinase C in focal adhesions (10). Phosphorylation of the cytoplasmic domain of syndecan-2 has also been noted to occur independently of integrin signaling and plays a role in the assembly of fibronectin and laminin (11).

Syndecans, glypicans, and perlecan are expressed in cell-, tissue- and development-specific patterns (2). Several studies have demonstrated altered expression of HSPGs in cancer (1), but with respect to ovarian cancer the only HSPG that has been studied in detail is the phosphatidyl-inositol-linked HSPG, glypican-3. Various mutations in the glypican-3 gene cause Simpson–Golabi–Behmel syndrome, which is associated with an increased risk of Wilm's tumor and neuroblastoma (12, 13). Mutations of glypican-3 and loss of expression due to hypermethylation have been demonstrated in a significant proportion

Received 8/27/03; revised 3/29/04; accepted 5/19/04.
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of sporadic ovarian cancers, suggesting that glypican-3 may act as a tumor suppressor in this and other malignancies (14–17). However, in a study of ovarian cancer cell lines, transmembrane HSPGs, in addition to phosphatidylinositol-linked families, mediated adhesion to collagen I and III and fibronectin, suggesting that other HSPGs in addition to glypican-3 play a role *in vivo* (18). We therefore performed this study to define the expression patterns of syndecan-1, -2, -3 and -4; glypican-1; and perlecan in normal ovary and benign, borderline, and malignant ovarian tumors. We then compared the prognostic importance of HSPG expression with that of standard prognostic factors in ovarian adenocarcinoma.

MATERIALS AND METHODS

Patients and Samples. Samples of frozen ovarian tissue were taken from archived cases held in the tumor bank at the Christie Hospital and Paterson Institute for Cancer Research, Manchester. These had been obtained during surgery, cut into 5-mm-diameter cubes, frozen by immersion in liquid nitrogen within 1 h of resection, and stored at −80°C. In addition, formalin-fixed, paraffin-embedded samples were obtained from histopathology departments where the original surgery had taken place for patients who were referred to our center after initial surgery for chemotherapy. Formalin-fixed, paraffin-embedded samples of age-matched normal ovary were obtained from archive material held in the Histopathology Department of the Christie Hospital NHS Trust. The South and Central Manchester Ethical Committees approved this study.

All histological samples were reviewed by a histopathologist who specializes in gynecological oncology (J. H. S.). Samples that were included in the study had a diagnosis of normal ovary, benign epithelial ovarian tumor, epithelial tumor of borderline malignancy, or primary ovarian adenocarcinoma. Histological grade was assessed as moderately or poorly differentiated. Clinical data were collected on age, clinical stage, and performance status. Information on International Federation of Gynecologists and Obstetricians (FIGO) stage was derived from surgical, histological, and radiological disease assessments. Bulk disease was defined as ≥2 cm in diameter. Karnowsky performance was assessed at the time of referral to our center before chemotherapy and after recovery from initial surgery. Response to chemotherapy was assessed by comparing the size of the largest measurable disease site by computed tomography scanning before and after treatment by standard two-dimensional response assessment criteria.

Immunohistochemistry. Mouse antihuman HSPG antibodies 2E9 (4 μg/ml; syndecan-1 and -3 cytoplasmic domains), 10H4 (1 μg/ml; syndecan-2), 1C7 (2 μg/ml; syndecan-3 ectodomain), 8G3 (10 μg/ml; syndecan-4), syndecan-1 (6 μg/ml; glypican-1), and perlecan mixture (1 μg/ml; perlecan core protein) were kindly provided by Professor G. David (University of Leuven, Leuven, Belgium). Mouse antihuman syndecan-1 ectodomain antibody CD138 (clone B-B4) was obtained from Serotec (Oxford, United Kingdom). Monoclonal mouse IgG1 antibody, unconjugated rabbit antimouse immunoglobulin, and horseradish peroxidase-conjugated rabbit antimouse immunoglobulin were obtained from DAKO Ltd. (Ely, Cambridgeshire, United Kingdom).

Microscope slides, precoated with 2% 3-aminopropyl-triethoxysilate–acetone for 5 min, were prepared. Embedded tissue samples were cut in 7-μm sections, air-dried, and stored at −20°C. The slides were placed in a staining rack at room temperature, immersed in 100% acetone for 10 min, air dried for 1 min, and then immersed in PBS. Formalin-fixed, paraffin-embedded ovarian tissues were cut at a thickness of 3 μm on 3-aminopropyl-triethoxysilate-coated slides, dewaxed by immersion in xylene for 10 min, rehydrated in a graded series of ethanol baths, and finally transferred to a PBS bath for immunostaining. One of the serial sections was stained with hematoxylin and eosin

Conventional immunohistochemical techniques were used. Briefly, after endogenous peroxidase was blocked with 0.3% hydrogen peroxide in PBS–methanol, the slide was placed in a bath of PBS containing 0.05% Tween 20 for 5 min. The sections were then blocked with 20% normal rabbit serum in PBS and incubated with primary monoclonal antibodies, at the concentrations shown above, in 10% normal rabbit serum overnight at 4°C. After washing, horseradish peroxidase-conjugated rabbit antimouse secondary antibody (diluted 1:200 in 10% normal rabbit serum) was added for 30 min at room temperature. The sections were then placed in 0.05% diaminobenzidine solution (DAKO, Ely, United Kingdom) for 5 min. After incubation with diaminobenzidine the slides were washed, counterstained in hematoxylin for 1 min, and washed in running water at 37°C for 1 min. They were then placed in alkaline water, dehydrated, cleared, and mounted. The final dilution of each antibody was determined after preliminary serial dilution studies. These were chosen to yield an intermediate level of positive staining in control tissues. Deglycanation to remove heparan sulfate chains was investigated but did not improve staining and was not required for the present study. Microwave retrieval was also investigated but not required for paraffin-embedded slides. Staining patterns of B-B4 antibody were compared in fresh-frozen, formalin-fixed sections from the same tumor in a pilot study of 10 cases, and no differences in the distribution or intensity of the stain was detected (data not shown). Therefore, data from fresh-frozen, formalin-fixed material were pooled for studies of syndecan-1. Fresh-frozen material was used for the studies of syndecan-2 and -4, glypican-1, and perlecan.

Analysis. Two independent observers who were not aware of the clinicopathological information inspected one slide per patient. Staining patterns were scored by use of a semiquantitative scoring system. A score of 0–3 was given according to the intensity of the staining, with 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining in the relevant cell types. Where there were differences between the findings of the two independent observers, a third observer was asked to review the findings, and the most frequent assessment was accepted.

The progression-free interval was from the date of initial diagnosis to the date of either radiological or clinical disease progression or recurrence. Survival was from the date of diagnosis to the date of most recent follow-up or death. Cases where death was not cancer related were excluded. Univariate graphs for progression-free and overall survival probabilities were calculated using the Kaplan–Meier method. Correlations between the clinical variables and the biological factors were examined

by the Kruskal–Wallis and Mann–Whitney tests and the Spearman correlation coefficient. The log-rank test was used to perform multivariate analysis and establish the independent prognostic value of the biological factors that showed significance in univariate analysis.

RESULTS

Patients and Samples. We studied the expression of syndecan-1 in 12 normal ovaries, 10 benign epithelial ovarian tumors, 10 borderline ovarian tumors, and 115 samples of ovarian carcinoma from patients who presented between 1985 and 1995. The expression of syndecan-2 through -4, glypican-1, and perlecan was assessed in 4 normal ovaries, 10 benign epithelial ovarian tumors, 10 borderline ovarian tumors, and in 65 of the 115 samples of ovarian carcinoma.

The clinicopathological characteristics of the patients are shown in Table 1. The majority of patients with primary ovarian adenocarcinoma ($n = 115$) had poorly differentiated tumors (57 of 115), FIGO stage III (65 of 115), bulk disease (69 of 115), and a good performance status (Karnowsky performance ≥70%; 74 of 115). These clinical characteristics were similar for the subset of 65 patients in whom the other HSPGs were examined (Table 1). The treatment regimens, response rates, median progression-free survivals, and median overall survivals were also comparable. Chemotherapy was given to 94% of patients in both groups. The majority of patients (87% in the syndecan-1 study and 92% in the other HSPG studies) received a platinum-based combination chemotherapy regimen. The proportion of patients who received a platinum and taxol combination was 22% in the syndecan-1 study and 36% in the other HSPG studies. The overall response rates (complete and partial) were 72.3% and 77%, in line with predicted outcomes (19). The median follow-up was 23 months (range, 6–125 months), the median progression-free interval was 12 months (range, 1–75 months), and the median survival for the patients in the syndecan-1 study group was 30 months compared with 29 months for the other group.

Table 1 Clinicopathological characteristics of study cases

Clinicopathological characteristic	S1 study sections	S2, S3, S4, G1, and perlecan studies
Median (range) age (yrs)		
Normal ovary	56 (55–79)	56 (55–79)
Benign ovarian tumors	56 (37–73)	57 (37–73)
Borderline ovarian tumors	62 (20–77)	62 (20–77)
Epithelial ovarian carcinoma	56 (23–80)	59 (23–80)
Histological type (*n*)		
Normal ovary	12	4
Benign epithelial ovarian tumor	10	10
Endometrioid	1	1
Mucinous	5	5
Serous	4	4
Borderline ovarian tumor	10	10
Mucinous	4	4
Serous	6	6
Epithelial ovarian carcinoma	115	65
Adenocarcinoma	27	10
Clear cell	8	2
Endometrioid	23	18
Mucinous	7	7
Serous	50	28
Tumor grade (*n*)		
Well differentiated	18	13
Moderately differentiated	29	15
Poorly differentiated	57	35
Not known	11	2
FIGO stage (*n*)		
I	9	6
II	10	6
III	65	36
IV	31	17
Residual disease (*n*)		
None	1	0
Minimal residual disease (<2 cm)	45	29
Bulk disease	69	36
KP (*n*)		
>70%	74	41
30–70%	36	23
Unknown	5	1
Median	80	80

Abbreviations: S1 through -4, syndecan-1 through -4; G1, glypican-1; FIGO, International Federation of Gynecologists and Obstetricians; KP, Karnowsky performance.

Distribution of HSPGs. The results for expression of each of the HSPGs studied are summarized in Table 2. Syndecan-1 expression was assessed in 115 cases with the B-B4 monoclonal antibody, which binds to the extracellular domain of this transmembrane HSPG. There was no detectable staining of syndecan-1 in the surface mesothelial cell layer or the stromal cells in any sections of normal ovary examined (Fig. 1*A*). In contrast, staining for syndecan-1 was present in the epithelial cells, cancer cells, and stromal cells of a significant proportion of benign, borderline, and malignant ovarian tumor sections (Fig. 1*B*). Cancer cell staining was frequently concentrated in the most infiltrating area at the advancing invasive tumor front (Fig. 1*C*). Similarly, stromal cell staining for syndecan-1 was of highest intensity in samples with invasive adenocarcinoma, and in these sections expression of syndecan-1 in stromal fibroblasts was confined to cells adjacent to invasive cancer cells (Fig. 1*D*). Because B-B4 detects the syndecan-1 ectodomain that can be cleaved and shed from cell surfaces by proteolysis (20, 21), we tested whether stromal expression was due to shedding of syndecan-1 produced by the cancer cells. Cases with negative expression of syndecan-1 in cancer cells and positive syndecan-1 ectodomain in the stromal cells were examined for expression of the 2E9 antibody, which recognizes the syndecan-1 (and syndecan-3) cytoplasmic domain. The 1C7 antibody, which recognizes syndecan-3 ectodomain, was also used as a control for cross-reactivity. The staining patterns obtained were most consistent with stromal synthesis of syndecan-1. In cases in which the adenocarcinoma cells were negative but the stromal cells were positive for syndecan-1 ectodomain, positive staining for the syndecan-1 cytoplasmic domain with 2E9 antibody was observed in stromal cells rather than cancer cells.

In contrast to the data for syndecan-1, the expression of syndecan-2, determined with the 10H4 monoclonal antibody, was confined to the stromal cells. No staining was detected in the epithelial or cancer cells of any of the ovarian sections studied, whereas stromal cell staining was present in all of the normal and benign ovaries and in the majority of borderline and malignant tumor sections (Table 2; Fig. 2*A*). Similarly, there

Table 2 Cellular distribution of heparan sulfate proteoglycans in normal ovaries and ovarian tumors

		Staining intensity	Normal ovary	Benign tumor	Borderline tumor	Carcinoma
Syndecan-1	Epithelial cells	Grade 0	11 (100)	4 (30)	4 (40)	68 (59)
		Grade 1	1 (0)	3 (40)	4 (40)	24 (20)
		Grade 2	0 (0)	1 (10)	1 (10)	15 (13)
		Grade 3	0 (0)	2 (20)	1 (10)	5 [3 NK] (7)
	Stromal cells	Grade 0	11 (100)	8 (70)	5 (50)	64 (56)
		Grade 1	0 (0)	2 (30)	2 (20)	18 (15)
		Grade 2	0 (0)	0 (0)	3 (30)	24 (20)
		Grade 3	1 (0)	0 (0)	0 (0)	7 [2 NK] (8)
Syndecan-2	Epithelial cells	Grade 0	3 (100)	10 (100)	10 (100)	64 (100)
	Stromal cells	Grade 0	1 (0)	0 (0)	2 (20)	6 (7)
		Grade 1	1 (50)	5 (50)	4 (30)	43 (75)
		Grade 2	0 (25)	3 (40)	3 (50)	13 (15)
		Grade 3	1 (25)	2 (10)	1 (0)	3 (3)
Syndecan-3	Epithelial cells	Grade 0	3 (100)	10 (100)	10 (100)	65 (100)
	Stromal cells	Grade 0	0 (0)	0 (0)	0 (0)	2 (0)
		Grade 1	1 (50)	3 (40)	1 (10)	13 (25)
		Grade 2	1 (50)	6 (60)	9 (90)	34 (55)
		Grade 3	1 (0)	1 (0)	0 (0)	16 (20)
	Blood vessels	Grade 0	0 (0)	0 (0)	0 (0)	9 (4)
		Grade 1	1 (25)	1 (0)	2 (20)	9 (15)
		Grade 2	2 (50)	7 (70)	7 (70)	24 (53)
		Grade 3	1 (25)	1 [1 NK] (10)	1 (10)	18 [5 NK] (28)
Syndecan-4	Epithelial cells	Grade 0	1 (25)	3 (30)	2 (20)	9 (11)
		Grade 1	32 (75)	76 (70)	7 (70)	38 (65)
		Grade 2	0 (0)	1 (0)	1 (10)	17 (22)
		Grade 3	0 (0)	0 (0)	0 (0)	1 (2)
	Stromal cells	Grade 0	3 (100)	10 (100)	10 (100)	64 (100); 1 grade 1
Glypican 1	Epithelial cells	Grade 0	3 (100)	10 (100)	10 (100)	65 (100)
	Stromal cells	Grade 0	3 (50)	3 (40)	7 (70)	30 (45)
		Grade 1	0 (25)	7 (60)	1 (10)	31 (48)
		Grade 2	0 (25)	0 (0)	2 (20)	3 (5)
		Grade 3	0 (0)	0 (0)	0 (0)	1 (2)
	Blood vessels	Grade 0	2 [1 NK] (100)	6 [4 NK] (100)	7 [3 NK] (100)	45 [16 NK] (100); 4 grade 1
Perlecan	Basement membrane	Grade 0	0 (0)	0 (0)	3 (20)	44 (67)
		Grade 1	1 (25)	0 (0)	3 (40)	2 (3)
		Grade 2	1 (75)	5 (50)	2 (20)	11 (16)
		Grade 3	1 (0)	5 (50)	2 (20)	8 (14)
	Stroma	Grade 0	0 (0)	0 (0)	0 (0)	0 (0)
		Grade 1	1 (50)	3 (30)	4 (40)	11 (17)
		Grade 2	1 (25)	5 (50)	3 (30)	33 (52)
		Grade 3	1 (25)	2 (20)	3 (30)	21 (31)
	Blood vessel	Grade 0	0 (0)	0 (0)	0 (0)	1 (2)
		Grade 1	1 (25)	1 (10)	2 (10)	3 (4)
		Grade 2	1 (0)	5 (60)	4 (50)	24 (44)
		Grade 3	1 (75)	4 (30)	4 (40)	36 [1 NK] (50)

NOTE. For syndecan-1, the numbers of patients in each group were as follows: normal ovary ($n = 12$), benign tumor ($n = 10$); borderline tumor ($n = 10$), and carcinoma ($n = 115$). For the other heparan sulfate proteoglycans, the numbers of patients in each group were as follows: normal ovary ($n = 3$), benign tumor ($n = 10$); borderline tumor ($n = 10$), and carcinoma ($n = 65$). Values in parentheses indicate percentage of patients.

Abbreviations: NK, not known.

was no epithelial cell staining, but there was positive stromal cell staining for syndecan-3 and glypican-1, detected by the 1C7 and syndecan-1 antibodies, respectively. We also observed that syndecan-3 localized to blood vessels of both benign and malignant tissues (Fig. 2*B*). In contrast, syndecan-4, detected by the 8G3 antibody, was not expressed in the stromal cells of any of the tissue sections studied but was present at the cell membrane and in the cytoplasm of normal epithelial and cancer cells (Table 2; Fig. 2*C*). The intensity of epithelial staining for syndecan-4 was low in the majority of cases. There was no significant relationship between the expression of syndecan-2 through -4 or glypican-1 (Table 2; Fig. 2*D*) and histological subtype or tumor grade.

Perlecan is a 400-kDa modular HSPG that is commonly found in basement membranes and in the extracellular matrix. There was moderate to strong expression of perlecan in all of the

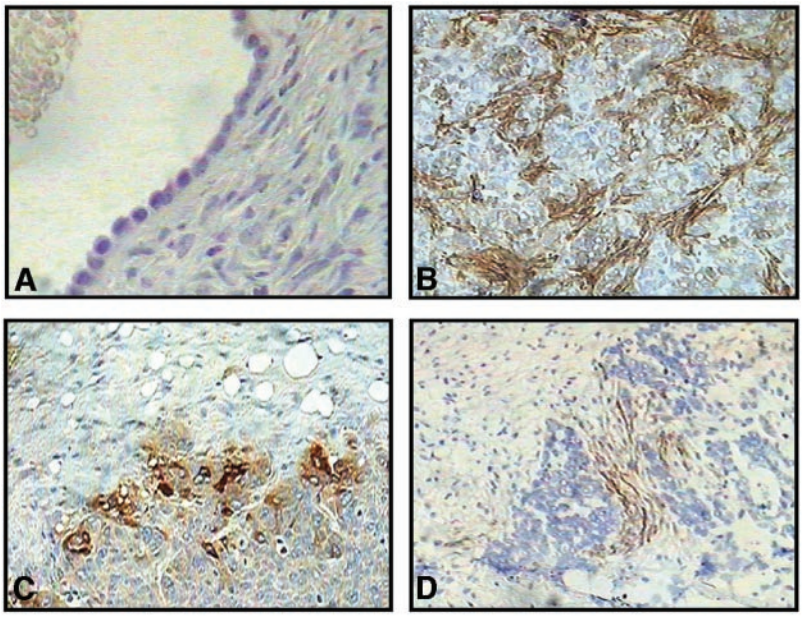

Fig. 1 Syndecan-1 expression. *A*, normal human ovary showing negative staining of mesothelium and stroma with B-B4. *B*, weak (grade 1) focal epithelial cell staining and strong (grade 3) diffuse stromal cell staining in a frozen section of a metastatic lesion of a International Federation of Gynecologists and Obstetricians (FIGO) stage IV poorly differentiated serous ovarian adenocarcinoma. *C*, metastatic tumor from a stage IV poorly differentiated adenocarcinoma in which isolated epithelial cells on the periphery of the invasive tumor show strong (grade 3) staining and the stroma is negative. *D*, FIGO stage III moderately differentiated serous ovarian adenocarcinoma showing moderate (grade 2) focal stromal cell staining (10%) in a region near small areas of invasive carcinoma. The epithelial cells are negative for B-B4.

ovarian tissue sections examined. Perlecan was distributed in basement membranes, the internal elastic lamina of blood vessels, in stroma, and in the submesothelial layer of normal ovary that separates the surface mesothelial layer from the underlying stroma (Fig. 2*E*). In contrast to the homogeneous staining observed for perlecan in the basement membranes of the majority of the benign and borderline tumors, the basement membranes of 67% of ovarian adenocarcinoma sections did not express detectable perlecan, and in many cases loss of perlecan occurred in areas of invasive cancer (Fig. 2*F*). Interestingly, with only one exception, there was no corresponding decrease in perlecan staining of basement membranes surrounding blood vessels or in the stroma in these sections. There was a positive correlation between stromal perlecan staining and perlecan expression surrounding blood vessels ($P = 0.001$) and an inverse relationship with the expression of perlecan in the basement membrane ($P = 0.002$, Kruskal–Wallis and Mann–Whitney tests).

Prognostic Factor Analysis. The clinical prognostic factors for progression-free and overall survival, respectively, were FIGO stage ($P < 0.0005$ for both), residual disease after primary debulking surgery ($P = 0.0001$ and $P < 0.0005$), and Karnowsky performance status ($P = 0.001$ and $P = 0.0003$), consistent with previous reports (Ref. 19; Fig. 3). Treatment regimen was not found to be prognostic in this study (*i.e.*, platinum/Taxol *versus* other platinum combination). Two of the HSPGs examined were of prognostic significance in univariate analysis. Stromal staining of syndecan-1 was associated with a shorter progression-free interval ($P = 0.0025$) and survival ($P = 0.027$) with median progression-free survival and overall survival of 19 and 33 months, respectively, for negatively staining tumors compared with 14 and 29 months for grade 1, 4 and 16 months for grade 2, and 13 and 15 months for grade 3 tumors (Fig. 3). Increased intensity of glypican-1 staining in the stroma was also significantly associated with decreased overall survival ($P = 0.04$) but not progression-free survival ($P = 0.31$). In multivariate analysis, stromal syndecan-1 expression remained significant when adjusted for Karnowsky performance ($P = 0.04$), was of borderline significance when adjusted for FIGO stage ($P = 0.06$), and was not significant when adjusted for residual/bulk disease, Karnowsky performance, and stage ($P = 0.24$).

DISCUSSION

This is the first report that has assessed the expression and prognostic significance of syndecan-1 through -4, glypican-1, and perlecan in normal ovaries and ovarian tumors. These molecules have been implicated in several processes related to malignancy, and novel anticancer therapies designed to modulate their function have been proposed (22, 23). However, clinical data on the role of HSPGs in ovarian cancer are lacking. We show that with the exception of syndecan-1, all of these HSPGs are expressed in normal ovary. In contrast, syndecan-1 is expressed in ovarian adenocarcinoma, and perlecan expression is lost from basement membranes that are breached by invasive cancer.

Syndecan-1 is the best-studied HSPG, and its expression is frequently altered in cancer. It is a pleiotropic molecule that has been demonstrated to have tumor suppressor function and tumor promoter function depending on the model system or tissue examined (1). Initial reports demonstrated that expression of syndecan-1 *in vitro* was associated with maintenance of epithelial morphology, anchorage-dependent growth, and inhibition of invasiveness (24). This “tumor suppressor” function was subsequently supported by immunohistochemical studies that correlated loss of syndecan-1 with reduced survival in patients with malignant mesothelioma (25), squamous cell carcinoma of the head and neck (26), and laryngeal cancer (27). However, in a landmark study, Alexander *et al.* (28) demonstrated that syndecan-1 expression is essential for wnt-1-induced mammary tu-

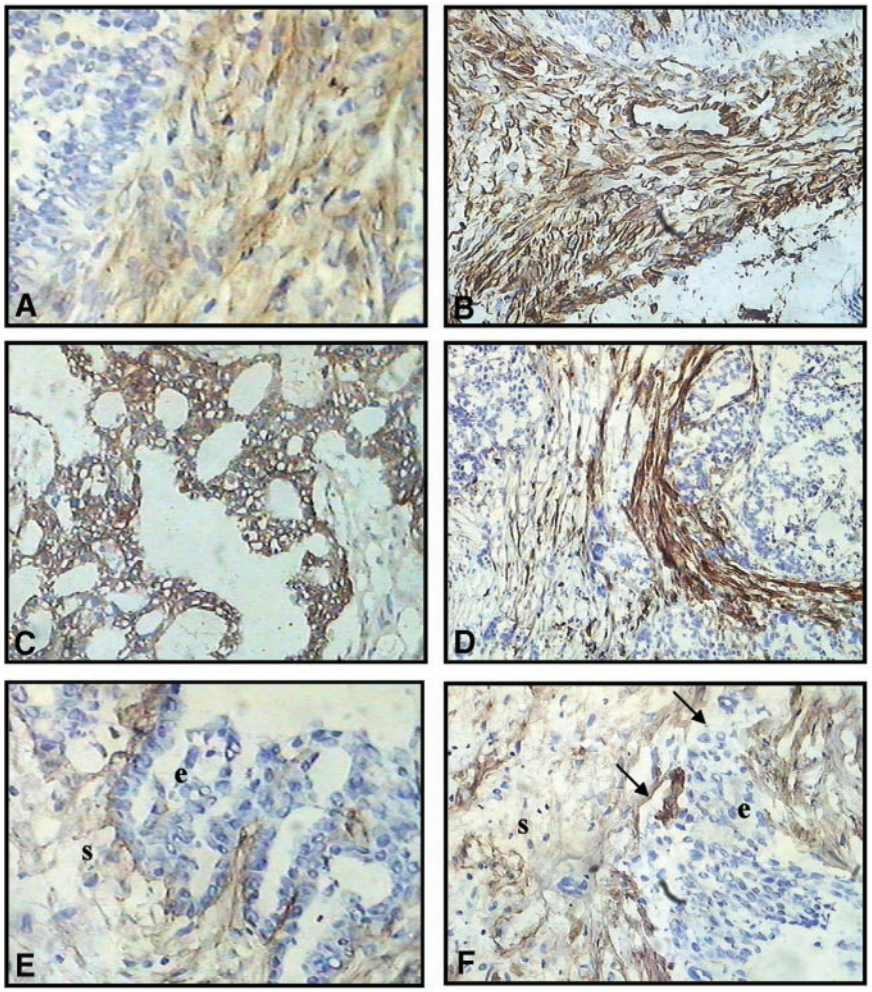

Fig. 2 Syndecans 2-through -4, glypican, and perlecan. *A*, syndecan-2 (10H4) in a International Federation of Gynecologists and Obstetricians (FIGO) stage I, mucinous well-differentiated adenocarcinoma showing moderate (grade 2) diffuse stromal cell staining. *B*, syndecan-3. Strong (grade 3) diffuse stromal cell and blood vessel staining in a FIGO stage I well-differentiated mucinous adenocarcinoma. *C*, syndecan-4. Strong focal cell membrane and cytoplasmic staining of a FIGO stage I clear cell adenocarcinoma. *D*, glypican-1. Strong (grade 3) diffuse stromal cell staining in a FIGO stage IV, moderately differentiated endometrioid adenocarcinoma. *E* and *F*, perlecan. Two areas within a FIGO stage III, moderately differentiated serous adenocarcinoma. *E*, noninvasive area showing moderate (grade 2) basement membrane staining between the epithelial cells (*e*) and the stroma (*s*). *F*, different area of the same tissue section in which there is loss of basement membrane where the epithelial carcinoma cells are invading into the stroma (*arrows*).

morigenesis in mice and demonstrated that syndecan-1 is an essential receptor for an oncogenic growth factor. In addition, studies in breast and gastric cancer have demonstrated an association between increased stromal syndecan-1 expression, loss of cancer cell syndecan-1 expression, and an adverse clinical outcome (29–31). Syndecan-1 ectodomains undergo physiological shedding as a result of proteolytic cleavage (1), and in the latter studies, stromal syndecan-1 expression may have been due to shedding of syndecan-1 ectodomains from the cancer cells (29). In the present study, stromal syndecan-1 expression was associated with decreased survival in univariate analysis. In contrast to the abovementioned studies, stromal syndecan-1 expression was not associated with concomitant loss of syndecan-1 from cancer cells. In addition, an antibody to the syndecan-1 cytoplasmic domain, which was not used in the previously reported studies (29–31), localized to the stromal cells. Thus, the pleiotropic role of syndecan-1 may be determined by its cellular origin, with the tumor promoter role occurring when it is expressed in stromal cells and the tumor suppressor role occurring when it is expressed in epithelial cells.

There is increasing evidence that stromal cells are essential for the progression of cancer (32, 33). In experimental models of ovarian cancer, normal ovarian stromal cells actually inhibit tumor growth, suggesting that recruitment of host stromal cells is essential for tumor progression (32). We frequently observed stromal syndecan-1 expression in the most invasive areas of cancer, and stromal glypican-1 expression also correlated with decreased survival on univariate analysis. During development, the expression patterns of HSPGs are believed to mirror those of ligands that require HSPGs to elicit their cellular responses. For example, glypican-1 expression is up-regulated in pancreatic cancer cells and surrounding fibroblasts and the mitogenic response of pancreatic cells to bFGF and heparin-bonding-epidermal growth factor is abrogated by antisense attenuation of this HSPG (34).

In theory, heparan sulfate-dependent proteins such as bFGF could induce both syndecan-1 and glypican-1 in tumor stroma (35, 36). A number of fibroblast growth factor family members have been implicated in the pathogenesis of ovarian cancer, and high levels of cytoplasmic bFGF correlate with an increased proportion of stromal cells in primary ovarian tumors (37).

In addition to a role in growth factor activation, HSPGs may also modulate interactions with the extracellular matrix. Ovarian carcinoma can disseminate by nonhematogenous mech-

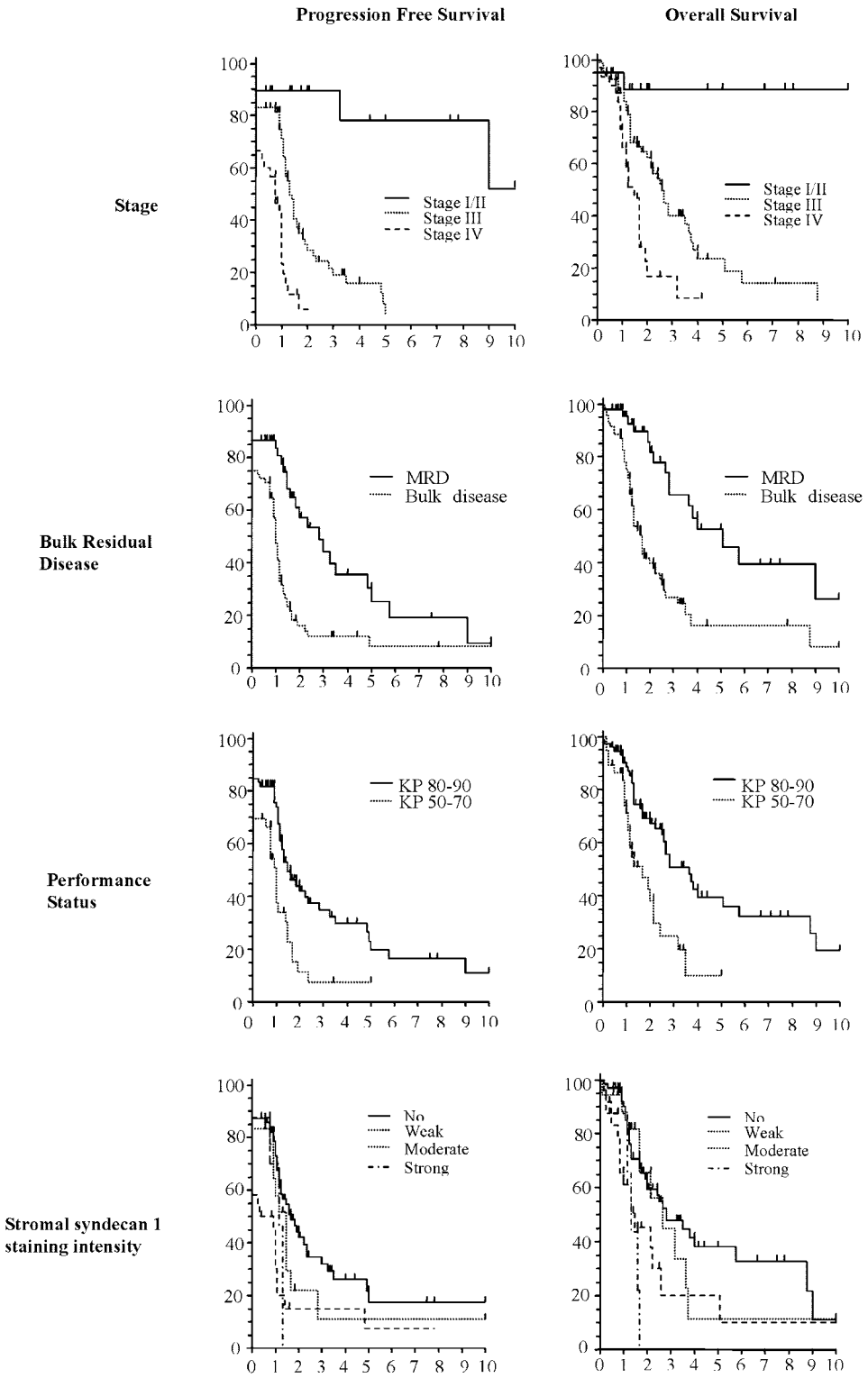

Fig. 3 Progression-free and overall survival. The graphs show the progression free and overall survival of patients according to the International Federation of Gynecologists and Obstetricians stage, bulk residual disease, performance status, and intensity of stromal syndecan-1 staining. The *X axes* represent years of follow up, and the *Y axes* represent the percentage survival. The *P* values for each variable are as follows: Stage: progression-free survival, $P < 0.00005$; overall survival, $P < 0.00005$. Residual disease: progression-free survival, $P = 0.0001$; overall survival, $P < 0.0005$. Performance status: progression-free survival, $P = 0.0006$; overall survival, $P = 0.0003$. Stromal syndecan-1 staining: progression-free survival, $P = 0.005$; overall survival, $P = 0.027$. *KP*, Karnowsky performance; *MRD*, minimal residual disease.

anisms via intraperitoneal adhesion, and invasion of the submesothelial matrix is facilitated by adhesion between the cell-surface receptors type I and III collagen and fibronectin (38). Candidate receptors in ovarian cancer cells include the β1 integrins, transmembrane and phosphatidylinositol-linked HSPGs (18). Although not significant for prognosis, syndecan-1 and -4 were both expressed in cancer cells in a proportion of tumors. Stromal cell syndecan-1, -2, and -3 and glypican-1 may

also play a role in mediating migration and invasion of host stromal cells required to support and maintain tumor growth.

Perlecan expression did not correlate with clinical outcome, but staining for perlecan was notably absent in areas of invasive cancer. Degradation of the basement membrane by proteases such as the matrix metalloproteinases is believed to be a critical process for the invasion of cancer cells. However, we observed that perlecan in the basement membranes of blood vessels was invariably intact despite loss from sites of tumor invasion remote from blood vessels. Perlecan is up-regulated at sites of active angiogenesis, and the angiogenic effects of bFGF are suppressed by experimental down-regulation of perlecan in colon cancer (3). Our data reveal that perlecan expression may be markedly heterogeneous within a given tumor and raise the possibility that cancer cells can either suppress or promote perlecan expression, depending on their location.

In adult tissues, syndecan-3 is found mainly in the central nervous system and peripheral nerves (39), but before the present study there was one report of syndecan-3 in the endothelial cells and walls of hepatic portal blood vessels and stromal mesenchymal cells in hepatocellular carcinoma (40), consistent with our observation of syndecan-3 in blood vessels. In the present study, it was not possible to determine with accuracy whether syndecan-3 was present in the endothelial cells or in smooth muscle pericytes that are in close proximity to the luminal endothelial cells. However, microvessel endothelial cells stain positively for syndecan-3 *in vitro*.[8] We also noted syndecan-3 in the blood vessels of nonmalignant ovarian tissues, although the intensity of staining was weaker than for the blood vessels in ovarian cancer sections. Therefore, syndecan-3 is not specific for tumor vasculature, but this does not preclude a role for syndecan-3 in the regulation of heparin-binding angiogenic cytokines such as bFGF, vascular endothelial growth factor, and hepatocyte growth factor.

Our data demonstrate that with the exception of syndecan-1, the HSPGs examined in this study are normally expressed in the ovary. Stromal syndecan-1 and glypican-1 were poor prognostic factors in univariate analysis but not in multivariate analysis. The other HSPGs were not significant for clinical outcome. It should be noted that the clinical samples were obtained over a time period when taxane and platinum combination chemotherapy was not standard; therefore, the heterogeneity of treatment regimens given may have confounded in our analysis of prognostic significance. We also did not find age and tumor grade to be significant prognostic factors. However, the known adverse prognostic factors of FIGO stage ($P < 0.0005$), residual disease after primary debulking surgery ($P < 0.0005$), and Karnowsky performance status ($P = 0.0003$) were all highly significant for survival, demonstrating that our cohort is representative of ovarian cancer in general.

An important concept when considering the function of HSPGs in any cancer is that their biological effects are influenced by the structure of the heparan sulfate chains that are displayed on the ectodomains of the core proteins (1). Thus, protein expression and turnover may remain stable but HSPG function may be altered by structural changes in the heparan sulfate chains. This has been observed in an experimental model of colon cancer, where transition from adenoma to carcinoma is accompanied by altered heparan sulfate structure that in turn changes reactivity to fibroblast growth factors (41–43). Similarly, heparan sulfate is subject to degradation by proteolytic enzymes called heparanases, which contribute to tumor cell invasion and metastasis by liberating fragments of heparan sulfate that can activate growth factors (20). Thus, the HSPGs have pleiotropic effects depending on their cellular context, the sequences of their heparan sulfate chains, and processing by heparanases that are released by cancer cells into the tumor microenvironment.

In summary, we present novel data on the expression of HSPGs in normal ovary and benign, borderline, and malignant ovarian tumors. The findings implicate stromal induction of syndecan-1 in the pathogenesis of ovarian cancer and reinforce the contribution of stromal cell factors and host-tumor interactions in this malignancy.

[8] Our unpublished observation.

REFERENCES

1. Blackhall FH, Merry CL, Davies EJ, Jayson GC. Heparan sulfate proteoglycans and cancer. Br J Cancer 2001;85:1094–8.

2. Sanderson RD. Heparan sulfate proteoglycans in invasion and metastasis. Semin Cell Dev Biol 2001;12:89–98.

3. Sharma B, Handler M, Eichstetter I, Whitelock JM, Nugent MA, Iozzo RV. Antisense targeting of perlecan blocks tumor growth and angiogenesis in vivo. J Clin Investig 1998;102:1599–608.

4. Iozzo RV, San Antonio JD. Heparan sulfate proteoglycans: heavy hitters in the angiogenesis arena. J Clin Investig 2001;108:349–55.

5. Ostrovsky O, Berman B, Gallagher J, et al. Differential effects of heparin saccharides on the formation of specific fibroblast growth factor (FGF) and FGF receptor complexes. J Biol Chem 2002;277:2444–53.

6. Robinson CJ, Stringer SE. The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J Cell Sci 2001;114:853–65.

7. Lyon M, Deakin JA, Mizuno K, Nakamura T, Gallagher JT. Interaction of hepatocyte growth factor with heparan sulfate. Elucidation of the major heparan sulfate structural determinants. J Biol Chem 1994; 269:11216–23.

8. Lyon M, Deakin JA, Rahmoune H, Fernig DG, Nakamura T, Gallagher JT. Hepatocyte growth factor/scatter factor binds with high affinity to dermatan sulfate. J Biol Chem 1998;273:271–8.

9. Pellegrini L. Role of heparan sulfate in fibroblast growth factor signalling: a structural view. Curr Opin Struct Biol 2001;11:629–634.

10. Oh ES, Woods A, Lim ST, Theibert AW, Couchman JR. Syndecan-4 proteoglycan cytoplasmic domain and phosphatidyl 4,5-bisphosphate coordinately regulate protein kinase C activity. J Biol Chem 1998;273:10624–9.

11. Klass CM, Couchman JR, Woods A. Control of extracellular matrix assembly by syndecan-2 proteoglycan. J Cell Sci 2000;113:493–506.

12. Gonzalez AD, Kaya M, Shi W, et al. OCI-5/GPC3, a glypican encoded by a gene that is mutated in the Simpson-Golabi-Behmel overgrowth syndrome, induces apoptosis in a cell line- specific manner. J Cell Biol 1998;141:1407–14.

13. Filmus J. Glypicans in growth control and cancer. Glycobiology 2001;11:19R–23R.

14. Lin H, Huber R, Schlessinger D, Morin PJ. Frequent silencing of the GPC3 gene in ovarian cancer cell lines. Cancer Res 1999;59:807–10.

15. Midorikawa Y, Ishikawa S, Iwanari H, et al. Glypican-3, overexpressed in hepatocellular carcinoma, modulates FGF2 and BMP-7 signalling. Int J Cancer 2003;103:455–65.

16. Powell CA, Xu G, Filmus J, Busch S, Brody JS, Rothman PB. Oligonucleotide microarray analysis of lung adenocarcinoma in smokers

and nonsmokers identifies GPC3 as a potential lung tumor suppressor. Chest 2002;12:6S–7S.

17. Xiang YY, Ladeda V, Filmus J. Glypican-3 expression is silenced in human breast cancer. Oncogene 2001;20:7408–12.

18. Kokenyesi R. Ovarian carcinoma cells synthesize both chondroitin sulfate and heparan sulfate cell surface proteoglycans that mediate cell adhesion to interstitial matrix. J Cell Biochem 2001;83:259–70.

19. International Collaborative Ovarian Neoplasm Group. Paclitaxel plus carboplatin versus standard chemotherapy with either single-agent carboplatin or cyclophosphamide, doxorubicin, and cisplatin in women with ovarian cancer: the ICON3 randomised trial [Erratum in: Lancet 2003;361:706]. Lancet 2002;360:505–15.

20. Kato M, Wang H, Kainulainen V, et al. Physiological degradation converts the soluble syndecan-1 ectodomain from an inhibitor to a potent activator of FGF-2. Nat Med 1998;4:691–7.

21. Fitzgerald ML, Wang Z, Park PW, Murphy G, Bernfield M. Shedding of syndecan-1 and -4 ectodomains is regulated by multiple signaling pathways and mediated by a TIMP-3-sensitive metalloproteinase. J Cell Biol 2000;148:811–24.

22. Presta M, Leali D, Stabile H, et al. Heparin derivatives as angiogenesis inhibitors. Curr Pharm Des 2003;9:553–66.

23. Jayson GC, Gallagher JT. Heparin oligosaccharides: inhibitors of the biological activity of bFGF on Caco-2 cells. Br J Cancer 1997;75:9–16.

24. Inki P, Jalkanen M. The role of syndecan-1 in malignancies. Ann Med 1996;28:63–7.

25. Kumar-Singh S, Jacobs W, Dhaene K, et al. Syndecan-1 expression in malignant mesothelioma: correlation with cell differentiation, WT1 expression, and clinical outcome. J Pathol 1998;186:300–5.

26. Anttonen A, Kajanti M, Heikkila P, Jalkanen M, Joensuu H. Syndecan-1 expression has prognostic significance in head and neck carcinoma. Br J Cancer 1999;79:558–64.

27. Pulkkinen JO, Penttinen M, Jalkanen M, Klemi P, Grenman R. Syndecan-1: a new prognostic marker in laryngeal cancer. Acta Otolaryngol 1997;117:312–5.

28. Alexander CM, Reichsman F, Hinkes MT, et al. Syndecan-1 is required for Wnt-1-induced mammary tumorigenesis in mice. Nat Genet 2000;25:329–32.

29. Stanley MJ, Stanley MW, Sanderson RD, Zera R. Syndecan-1 expression is induced in the stroma of infiltrating breast carcinoma. Am J Clin Pathol 1999;112:377–83.

30. Barbareschi M, Maisonneuve P, Aldovini D, et al. High syndecan-1 expression in breast carcinoma is related to an aggressive phenotype and to poorer prognosis. Cancer (Phila) 2003;98:474–83.

31. Wiksten JP, Lundin J, Nordling S, et al. Epithelial and stromal syndecan-1 expression as predictor of outcome in patients with gastric cancer. Int J Cancer 2001;95:1–6.

32. Parrott JA, Nilsson E, Mosher R, et al. Stromal-epithelial interactions in the progression of ovarian cancer: influence and source of tumor stromal cells. Mol Cell Endocrinol 2001;175:29–39.

33. Pupa SM, Menard S, Forti S, Tagliabue E. New insights into the role of extracellular matrix during tumor onset and progression. J Cell Physiol 2002;192:259–67.

34. Kleeff J, Wildi S, Kumbasar A, Friess H, Lander AD, Korc M. Stable transfection of a glypican-1 antisense construct decreases tumorigenicity in PANC-1 pancreatic carcinoma cells. Pancreas 1999;19:281–8.

35. Jaakkola P, Maatta A, Jalkanen M. The activation and composition of FiRE (an FGF-inducible response element) differ in a cell type- and growth factor-specific manner. Oncogene 1998;17:1279–86.

36. Jaakkola P, Jalkanen M. Transcriptional regulation of Syndecan-1 expression by growth factors. Prog Nucleic Acid Res Mol Biol 1999;63:109–38.

37. Obermair A, Speiser P, Reisenberger K, et al. Influence of intratumoral basic fibroblast growth factor concentration on survival in ovarian cancer patients. Cancer Lett 1998;130:69–76.

38. Cannistra S. Cancer of the ovary. N Engl J Med 1993;329:1550–9.

39. Carey DJ, Conner K, Asundi VK, et al. cDNA cloning, genomic organization, and in vivo expression of rat N-syndecan. J Biol Chem 1997;272:2873–9.

40. Roskams T, De Vos R, David G, Van Damme B, Desmet V. Heparan sulphate proteoglycan expression in human primary liver tumours. J Pathol 1998;185:290–7.

41. Jayson GC, Vives C, Paraskeva C, et al. Coordinated modulation of the fibroblast growth factor dual receptor mechanism during transformation from human colon adenoma to carcinoma. Int J Cancer 1999;82:298–304.

42. Jayson GC, Lyon M, Paraskeva C, Turnbull JE, Deakin JA, Gallagher JT. Heparan sulfate undergoes specific structural changes during the progression from human colon adenoma to carcinoma in vitro. J Biol Chem 1998;273:51–7.

43. Molist A, Romaris M, Lindahl U, Villena J, Touab M, Bassols A. Changes in glycosaminoglycan structure and composition of the main heparan sulphate proteoglycan from human colon carcinoma cells (perlecan) during cell differentiation. Eur J Biochem 1998;254:371–7.

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Clin Cancer Res 2004;10:5178-5186.