

Immunohistochemical Level of Unsulfated Chondroitin Disaccharides in the Cancer Stroma Is an Independent Predictor of Prostate Cancer Relapse

Andrew J. Sakko,^{1,2} Miriam S. Butler,² Sharon Byers,^{3,4} Betty J. Reinboth,⁵ Jürgen Stahl,⁶ James G. Kench,^{7,8} Lisa G. Horvath,^{7,9} Robert L. Sutherland,⁹ Phillip D. Stricker,^{7,10} Susan M. Henshall,⁷ Willis R. Marshall,¹¹ Wayne D. Tilley,² David J. Horsfall,² and Carmela Ricciardelli^{1,2}

¹Discipline of Obstetrics and Gynecology, Research Centre for Reproductive Health, University of Adelaide; ²Dame Roma Mitchell Cancer Research Laboratories, Hanson Institute, University of Adelaide; ³Matrix Biology Unit, Department of Genetic Medicine, Children, Youth and Women's Health Service; ⁴Paediatrics Department, University of Adelaide; ⁵Department of Pathology, University of Adelaide; ⁶Adelaide Pathology Partners, Mile End, South Australia, Australia; ⁷Cancer Research Program, Garvan Institute of Medical Research, Sydney, New South Wales, Australia; ⁸Department of Tissue Pathology, Institute of Clinical Pathology and Medical Research, Westmead Hospital, Westmead, New South Wales, Australia; ⁹Sydney Cancer Centre, Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia; ¹⁰Department of Urology, St Vincent's Hospital, Darlinghurst, New South Wales, Australia; and ¹¹Surgical and Specialty Services, Royal Adelaide Hospital, Adelaide, South Australia, Australia

Abstract

The glycosaminoglycan chondroitin sulfate is significantly increased in the peritumoral stroma of prostate tumors compared with normal stroma and is an independent predictor of prostate-specific antigen (PSA) relapse following radical prostatectomy. In this study, we determined whether specific alterations in the sulfation pattern of glycosaminoglycan chains in clinically organ-confined prostate cancer are associated with PSA relapse. Immunoreactivity to distinct glycosaminoglycan disaccharide epitopes was assessed by manually scoring the staining intensity in prostate tissues from patients with benign prostatic hyperplasia ($n = 19$), early-stage cancer (cohort 1, $n = 55$ and cohort 2, $n = 275$), and advanced-stage cancer ($n = 20$). Alterations to glycosaminoglycans in benign and malignant prostate tissues were determined by cellulose acetate chromatography and high-pressure liquid chromatography. Glycosaminoglycan disaccharide epitopes were localized to the peritumoral stroma of clinically local-

ized prostate cancer. The level of immunostaining for unsulfated disaccharides (C0S) in the peritumoral stroma, but not for 4-sulfated (C4S) or 6-sulfated disaccharides (C6S), was significantly associated with the rate of PSA relapse following radical prostatectomy. High levels of C0S immunostaining were determined to be an independent predictor of PSA relapse (1.6-fold, $P = 0.020$). Advanced-stage prostate cancer tissues exhibited reduced electrophoretic mobility for chondroitin sulfate and increased unsulfated disaccharides when compared with benign prostatic hyperplasia tissues, whereas the sulfated disaccharide levels were unaffected. The level of C0S immunostaining in the peritumoral stroma is an independent determinant of PSA failure in clinically localized prostate cancer. Specific alterations to chondroitin sulfate side chains occurring during tumor development may be a crucial step for disease progression in prostate cancer. (Cancer Epidemiol Biomarkers Prev 2008;17(9):2488–97)

Introduction

Prostate cancer is the most frequently diagnosed invasive solid cancer in men in the Western world. With the advent of better diagnostic tools [e.g., serum prostate specific antigen (PSA) measurement and transrectal ultrasound guided core biopsy], the majority of men are now diagnosed with early-stage disease, which is potentially curable by surgery or radiotherapy (1). However, up to 20% to 30% of men with presumed organ-confined prostate cancer who undergo curative treatments ultimately relapse with disseminated disease

(2). Furthermore, the mortality rate for prostate cancer has only shown a slight decline during this period (3). Despite recent advances with chemotherapy treatment, the main treatment option for men with metastatic prostate cancer remains androgen ablation therapy (4). Clinical outlook following androgen ablation therapy is poor, with reemergence of tumor after an initial stabilization of disease in a form that is no longer responsive to this treatment (5). Given the poor treatment outcomes for both early and advanced prostate cancer, it is critical that new approaches to understanding prostate cancer progression and metastasis be developed.

Proteoglycans are structural components of cell membranes and extracellular matrix and are important modifiers of cellular proliferation and differentiation, playing leading roles in tissue growth and development. Proteoglycans are involved in both normal and neoplastic growth by participating in cell-cell and cell-matrix adhesion and cell migration and proliferation (6). Many

Received 3/7/08; revised 5/30/08; accepted 6/30/08.

Grant support: Cancer Council South Australia (W Bruce Hall Fellowship, A.J. Sakko), University of Adelaide Faculty of Health Sciences (Hilda Farmer Research Fellowship, C. Ricciardelli) and the National Health and Medical Research Council grant 349457, Cancer Institute NSW, and RT Hall Trust.

Requests for reprints: Carmela Ricciardelli, Discipline of Obstetrics and Gynaecology, University of Adelaide, Frome Road, Adelaide, SA 5005, Australia. Phone: 61-8-8303-8255; Fax: 61-8-8303-4099. E-mail: carmela.ricciardelli@adelaide.edu.au

Copyright © 2008 American Association for Cancer Research.

doi:10.1158/1055-9965.EPI-08-0204

of the functions of proteoglycans are associated with their attached glycosaminoglycan side chains. Glycosaminoglycans are unbranched polysaccharide chains composed of repeating disaccharide units that are linked to the proteoglycan protein core, and are highly negatively charged due to the presence of sulfate and/or carboxyl groups. The composition of these disaccharide units and their sulfation patterns determines distinct glycosaminoglycan types. Chondroitin sulfate is a glycosaminoglycan side chain component of several distinct proteoglycans (e.g., versican) and has a linear polymer structure that possesses repetitive, sulfated disaccharide units containing glucuronic acid and *N*-acetylgalactosamine (GalNAc) joined by $\beta(1-4)$ and $\beta(1-3)$ linkages with sulfate residues at either the C-4 or C-6 position of the disaccharide moiety.

The concentration of chondroitin sulfate is greatly increased over normal tissue levels in several different malignancies (7-10), including prostate cancer (11-13). Our previous studies illustrated that patients treated by radical prostatectomy for early stage (cT₁-cT₂) cancer who showed high chondroitin sulfate concentration in the peritumoral stroma of the prostate had a significantly higher incidence of PSA failure than patients with low chondroitin sulfate concentration (14). Chondroitin sulfate levels in advanced (cT₄) prostate cancer tissues are very similar to the levels present in those early-stage prostate tumors that ultimately progressed,¹² suggesting that metastasis could be associated with increased chondroitin sulfate levels. Although chondroitin sulfate proteoglycans are widely distributed both on the surfaces of most cells and in the extracellular matrix, the structure and function of chondroitin sulfate side chains in malignant tissues have not been thoroughly investigated. Immunologic studies using monoclonal antibodies indicated that the sulfation profile of chondroitin sulfate chains varies according to specific spatiotemporal patterns in embryologic tissues, suggesting that chondroitin sulfate isoforms differing in sulfation position and degree perform distinct functions in embryologic development (15). Variance in chondroitin sulfate structure is largely determined by the specificities of the glycosyltransferases and sulfotransferases involved in chondroitin sulfate synthesis (16). Studies by Kitagawa et al. (17) showed that the ratio of C4S to C6S chondroitin sulfate chains in the embryonic chick brain changes with development and that the levels of specific sulfotransferase activities are closely coordinated with changing levels of individual sulfated disaccharides. Alterations in chondroitin sulfate structure have been observed in many different cancer types (7-10, 18, 19). Importantly, Dietrich et al. (8) showed an increased amount of unsulfated disaccharides in the urine of patients with different malignancies, including prostate cancer.

The aim of this study was to investigate the sulfation pattern of glycosaminoglycan side chains in clinically localized and advanced prostate cancer and to determine whether specific alterations in disaccharide epitopes are associated with PSA relapse.

Materials and Methods

Patient Cohorts. Samples of prostate tissue were collected from patients undergoing retropubic radical prostatectomy for clinically organ confined prostate cancer (Early Stage Pilot Cohort 1, $n = 55$ and Early Stage Cohort 2, $n = 275$) and from men undergoing transurethral resection of the prostate for voiding dysfunction [$n = 39$; 19 from men with advanced metastatic disease, i.e., cT₄N_xM₁ and 20 from men with benign prostatic hyperplasia (BPH)]. All tissue samples were surplus to diagnostic requirements and were obtained either through the Repatriation General Hospital Tissue Bank or the Garvan Institute of Medical Research with approval from the Flinders Medical Centre, Repatriation General Hospital, University of Adelaide, and the St. Vincent's Hospital (Sydney) Human Ethics Committees. Tumors were staged using the International Union against Cancer system (20). The presence of micrometastatic disease at the time of surgery for patients was determined by a PSA failure, that is, a return to measurable serum PSA levels on two sequential measurements subsequent to a postoperative level below the sensitivity threshold of the assay (<0.2 ng/mL). Early Stage Cohort 1 (Repatriation General Hospital cohort), Advanced Cancer Cohort, and BPH Cohort consisted of whole sections of tissues mounted on microscope slides, whereas Early Stage Cohort 2 (St. Vincent's Hospital Campus Prostate Cancer Group tissue microarray cohort) consisted of sections of arrayed prostate tissue cores mounted on microscope slides. In statistical analyses, the Early Stage Pilot Cohort 1 was used as a training set and the Early Stage Cohort 2 was used as a validation set. The cohort profiles are detailed in Table 1.

Immunodetection of Glycosaminoglycan Epitopes. Sections of paraffin-embedded prostate tissue (4 μ m) from the Early Stage Cohort 1 (pilot cohort) were immunostained with a panel of commercially available monoclonal antibodies to the specific glycosaminoglycan disaccharide epitopes that remain after digestion with chondroitinase enzymes (21), that is, 2B6 monoclonal antibody detects 4-sulfated disaccharide (C4S, 1/1000, Seikagaku Corporation), 3B3 monoclonal antibody detects 6-sulfated disaccharide (C6S 1/200, Seikagaku Corporation), and 1B5 monoclonal antibody detects unsulfated disaccharide (C0S, 1/400, Seikagaku Corporation) in chondroitin sulfate and dermatan sulfate glycosaminoglycan side chains. Positive C0S immunoreactivity in cancer and nonmalignant prostate tissues was detected following digestion with chondroitinase ABC [0.1 unit/mL in 100 mmol/L Tris acetate buffer (pH 7.5) containing 0.1% bovine serum albumin, 1 h at 37°C, Sigma Chemical Co.] and with chondroitinase AC [0.1 units/mL in 50 mmol/L sodium acetate buffer (pH 6.0) containing 0.1% bovine serum albumin, 1 h at 37°C, Sigma Chemical]. No immunoreactivity for C0S was detected using chondroitinase B [0.1 unit/mL in 100 mmol/L Tris acetate buffer (pH 8.0) containing 0.1% bovine serum albumin, 1 h at 37°C, Sigma Chemical]. These findings indicate that the C0S disaccharides are

¹² C. Ricciardelli, unpublished observation.

Table 1. Summary of clinical and pathology data for the four cohorts used in this study

| Early Stage Pilot Cohort 1* | |
|---------------------------------------|-----------------------------------------------|
| Patients (n) | 55 |
| Median age at diagnosis (y) | 64 (range 51-73) |
| Median preoperative serum PSA (ng/mL) | 9.0 (range 0.3-47.7, n = 50) |
| Median follow-up (mo) | 79.0 (range 36.0-154.0) |
| Gleason score (n) | |
| 2-4 | 14 |
| 5-6 | 29 |
| ≥7 | 12 |
| PSA failure rate (n) † | 22/55 (40%) |
| Early Stage Cohort 2‡ | |
| Patients (n) | 275 |
| Median age at diagnosis (y) | 63 (range 44-76) |
| Median preoperative serum PSA (ng/mL) | 9.2 (range 0.2-191.0, n = 260) |
| Median follow-up (mo) | 108.5 (range 5.6-260.4) |
| Gleason score (n) | |
| 2-4 | 23 |
| 5-6 | 134 |
| ≥7 | 115 |
| Unknown | 3 |
| PSA failure rate (n) | 124/275 (45%) |
| Advanced Cancer Cohort | |
| Patients (n) | 20 |
| Median age at diagnosis (y) | 73 (range 60-92) |
| Stage | cT ₄ N ₁ M ₁ |
| Gleason score (n) | |
| 2-4 | 5 |
| 5-6 | 7 |
| ≥7 | 8 |
| BPH Cohort | |
| Patients (n) | 19 |
| Median age at diagnosis (y) | 70 (range 54-87) |

*Repatriation General Hospital cohort.

† Gleason score determined by a pathologist (JS or JK).

‡ The presence of micrometastatic disease at the time of surgery for patients was determined by a PSA-failure, i.e. a return to measurable serum PSA levels on two sequential measurements subsequent to a postoperative level below the sensitivity threshold of the assay (<0.2 ng/ml).

§ St. Vincent's Hospital Campus Prostate Cancer Group (SVCPCG) TMA cohort.

present in chondroitin sulfate but not in dermatan sulfate chains. In addition, the predominance of 2B6 staining in tissues treated with chondroitinase B indicated that dermatan sulfate regions in prostate tissues are mostly 4-sulfated (data not shown). The levels of COS disaccharides were also assessed in tissue sections from an independent cohort of patients treated by radical prostatectomy (Early Stage validation Cohort 2), following digestion with chondroitinase ABC. Visualization of the glycosaminoglycan disaccharide epitopes for both cohorts 1 and 2 was achieved with a standard streptavidin immunoperoxidase reaction using biotinylated secondary antibody (Dako Corp.) and diaminobenzidine tetrahydrochloride (Sigma Chemical) to yield an insoluble brown deposit. Negative controls included no pretreatment with chondroitinase enzyme or replacement of the primary antibody with PBS. The immunostaining of each disaccharide epitope was scored manually by two independent observers blinded to clinical outcome in areas identified by urologic pathologists (J.S. and J.K.). The immunostaining intensity in the

stroma was scored as strong (3+), moderate (2+), weak (1+), or negative.

Glycosaminoglycan Isolation and Cellulose Acetate Electrophoresis. The limited amount of malignant tissue that is surplus to diagnostic requirements at radical prostatectomy and available for research studies prevented extraction of glycosaminoglycan from early-stage prostate cancer tissues. Glycosaminoglycan was therefore isolated only from archived frozen tissue obtained from men undergoing transurethral prostatic resection for voiding dysfunction (20 from men with advanced cT₄N₁M₁ disease and 19 from men with BPH). Confirmation of tissue pathology for each patient was determined (J.S.) in the adjacent tissue of a portion of resected tissue fragments, which were divided longitudinally following thawing and then formalin fixed and paraffin embedded. For glycosaminoglycan isolation, resected prostate tissues (100-500 mg) were thawed and digested in 20 volumes of 1 mg/mL papain in 0.5 mol/L sodium acetate buffer (pH 5.8) containing 25 mmol/L EDTA and 10 mmol/L cysteine hydrochloride at 60°C for 48 h. Cold (4°C) trichloroacetic acid was then added to a final concentration of 10% and after 1 h the precipitated protein was removed by centrifugation (2,000 × g, 4°C, 15 min). The supernatant was dialyzed against several changes of water for 48 h. The retentate was concentrated by lyophilization and reconstituted in 50 µL water. Glycosaminoglycans were isolated by precipitation with 4 volumes of cold (4°C) ethanol containing 1% potassium acetate. After 24 h, the glycosaminoglycans were retrieved by centrifugation (2,000 × g, 4°C, 15 min) and reconstituted in water.

Glycosaminoglycans were electrophoretically separated on cellulose acetate membrane (Sepraphore II, Pall Corp.) in 0.2 mol/L calcium acetate buffer (pH 7.2) at a constant voltage of 80 V for 105 min. Individual glycosaminoglycans were visualized by staining with Alcian blue [0.2% in 25 mmol/L sodium acetate buffer (pH 5.8), containing 50 mmol/L magnesium chloride and 50% ethanol] for 30 min (22). The separated bands were quantified by laser densitometry. Glycosaminoglycans extracted from prostate tissue were identified by comparison of band migration distances with standard glycosaminoglycans (22). Further identification of individual glycosaminoglycans was facilitated by enzymatic digestion with chondroitinase ABC or hyaluronidase and by nitrous acid hydrolysis.

Characterization of Sulfation Pattern of Glycosaminoglycan Chains. The total glycosaminoglycan extract was batch absorbed to Q-Sepharose and eluted with 2.5 mol/L NaCl to concentrate the glycosaminoglycan preparation. The inherent disaccharide patterns of the chondroitin sulfate and dermatan sulfate components were determined by digestion of 10 to 20 µg glycosaminoglycan with chondroitinase ABC (0.02 units) in buffer containing 50 mmol/L Tris-HCl (pH 8.0) overnight at 37°C (23). Following digestion, four volumes of absolute ethanol was added, and the mixture was placed at -20°C for 4 h before centrifugation at 15,000 × g. The disaccharides remaining in the supernatant (40 µL) were

separated by high-pressure liquid chromatography on a Partisil-5PAC (5 μ m, 25 cm \times 4.6 mm id) column, and eluted at a flow rate of 1.0 ml/min, in a mobile phase of 52% acetonitrile, 12% methanol, and 36% aqueous buffer [0.5 mol/L Tris HCL, 0.1 mol/L boric acid (pH 8.0); ref. 24]. This system allows the separation and quantification of disaccharides from chondroitin sulfate and dermatan sulfate. Standard disaccharide preparations of Δ Di-4S, Δ Di-6S, Δ Di-0S, Δ Di-4,6S, Δ Di-HA, and *N*-acetylgalactosamine, *N*-acetylgalactosamine-4-sulfate, and *N*-acetylgalactosamine-6-sulfate were used as controls to characterize the elution profile.

Statistical Analysis. Statistical Package for the Social Sciences version 13.0 (SPSS) was used. The Spearman's correlation and χ^2 tests were used to determine correlation between chondroitin sulfate epitope expression and clinicopathologic features. The χ^2 test was used to determine association between the presence of the modified chondroitin sulfate and malignant disease. The Mann-Whitney *U* test was used to assess differences between the proportion of disaccharides in nonmalignant and cancer tissues. Relapse-free survival was used as the end point in Cox regression and Kaplan-Meier analyses to determine whether the levels of glycosaminoglycan disaccharide epitopes were related to risk and rate of relapse, respectively, in Early Stage Cohort 2. Relapse-free survival was calculated from the date of diagnosis to the date of progression or the date of last follow-up if progression-free. Four patients who died from other causes were censored on the date of death. Statistical significance was set at $P < 0.05$.

Results

Glycosaminoglycan Epitopes in Prostatic Tissues. C4S, C6S, and C0S disaccharide moieties were localized to the peritumoral stroma of clinically organ confined prostate cancer tissues (Fig. 1A). Strong immunostaining for C4S disaccharide was present in 50.9% (28 of 55) of tumors whereas strong staining for C6S disaccharide was present in 20.0% (11 of 55) of the cancer tissues. Strong immunostaining for C0S disaccharide was present in 32.7% (18 of 55) of the tumors. C4S disaccharide was observed throughout the stroma adjacent to nonmalignant tissue, whereas C6S disaccharide and unsulfated disaccharide were localized to the periglandular stroma of nonmalignant glands (Fig. 1A). Strong immunostaining for C0S disaccharide was observed in 80% (8 of 10) of advanced cancers, whereas 78% (7 of 9) of the BPH tissues examined exhibited weak or moderate staining levels for C0S (Fig. 1B).

Association of Unsulfated Chondroitin Sulfate Level with PSA Relapse. Kaplan-Meier analyses of the rate of PSA relapse with respect to immunostaining in prostate tissue sections of glycosaminoglycan disaccharide epitope for patients in the Early Stage Cohort 1 are shown in Fig. 2. The levels of C4S disaccharide (Fig. 2A; log-rank statistic = 0.69, $P = 0.709$) and C6S disaccharide (Fig. 2B; log-rank statistic = 2.11, $P = 0.349$) were not associated with the rate of PSA relapse. In contrast, the level of C0S disaccharide was associated with the rate of PSA relapse (log-rank statistic = 11.12, $P = 0.004$; Fig. 2C). Patients with moderate or high levels of C0S disaccharide had a significantly higher rate of relapse compared with

patients with low levels of C0S disaccharide. The level of C0S disaccharide was also significantly associated with the rate of PSA relapse in the independent Early Stage Cohort 2 (log-rank statistic = 6.617, $P = 0.037$; Fig. 3A). Because there were only 13 patients with low levels of C0S disaccharide in this cohort, these patients were grouped with patients with moderate C0S disaccharide levels. Patients with high levels of C0S disaccharide (45.7%, 58 of 129) again experienced significantly more relapses than patients with low or moderate levels of C0S disaccharide (33.6%, 48 of 146, log-rank statistic = 6.25, $P = 0.012$; Fig. 3B).

High levels of C0S were significantly associated with clinical stage cT₂ ($P = 0.021$), pathologic stage pT₃ ($P = 0.006$), the presence of seminal vesicle invasion ($P = 0.007$), and extracapsular extension ($P = 0.011$) by χ^2 analysis, whereas no significant relationship was observed between C0S levels and Gleason score ($P = 0.393$), presence of positive margins ($P = 0.399$), nor preoperative PSA levels ($P = 0.802$). High levels of C0S disaccharide were significantly associated with a 1.60-fold increased risk of relapse following radical prostatectomy (Cox univariate analysis; Table 2A). Comparison with the other statistically significant clinical and pathologic variables by multivariate analysis indicated that the level of unsulfated disaccharide predicted PSA relapse (1.59-fold increased risk) independent of pathologic stage, Gleason score, preoperative serum PSA, surgical margins, seminal vesicle involvement, and extracapsular extension (Cox multivariate analysis; Table 2B).

Chondroitin Sulfate Exhibits Altered Mobility in Advanced Prostate Cancer. Cellulose acetate chromatography indicated that a physicochemical modification to the structure of chondroitin sulfate is present in the majority of advanced prostate cancer tissues compared with the form isolated from BPH tissues (16 of 20 advanced cancers compared with 5 of 19 BPH tissues, $P = 0.0001$, χ^2 test; Fig. 4A). The relative mobility coefficient of chondroitin sulfate for advanced-stage cancers was 0.58 compared with 0.69 for BPH tissues. The relative mobility coefficient of dermatan sulfate was unaltered in BPH and cancer tissues (range, 0.48-0.52).

Characterization of Glycosaminoglycan Sulfation Pattern in Advanced Prostate Cancer. The lower migration rate on cellulose acetate chromatography of chondroitin sulfate from advanced prostate cancer tissues suggested a differential sulfation pattern compared with nonmalignant prostate tissues. To investigate this further, isolated glycosaminoglycan chains were digested with chondroitinase ABC and analyzed by high-pressure liquid chromatography (Fig. 4B). The level of Δ Di-0S disaccharides was significantly increased ($P = 0.016$) in prostate cancer tissues (mean, 7.5%; range, 4.3-16.7%) compared with BPH tissues (mean, 3.7%; range, 3.4-4.2%; Fig. 4C). The proportion of Δ Di-4S disaccharides was reduced in prostate cancer tissue compared with BPH tissues; however, this difference was not statistically significant. The Δ Di-6S disaccharide levels were not altered in prostate cancer compared with BPH tissues (Fig. 4C). Interestingly, an additional unknown peak, which eluted earlier than the Δ Di-0S disaccharide, was observed in all prostate

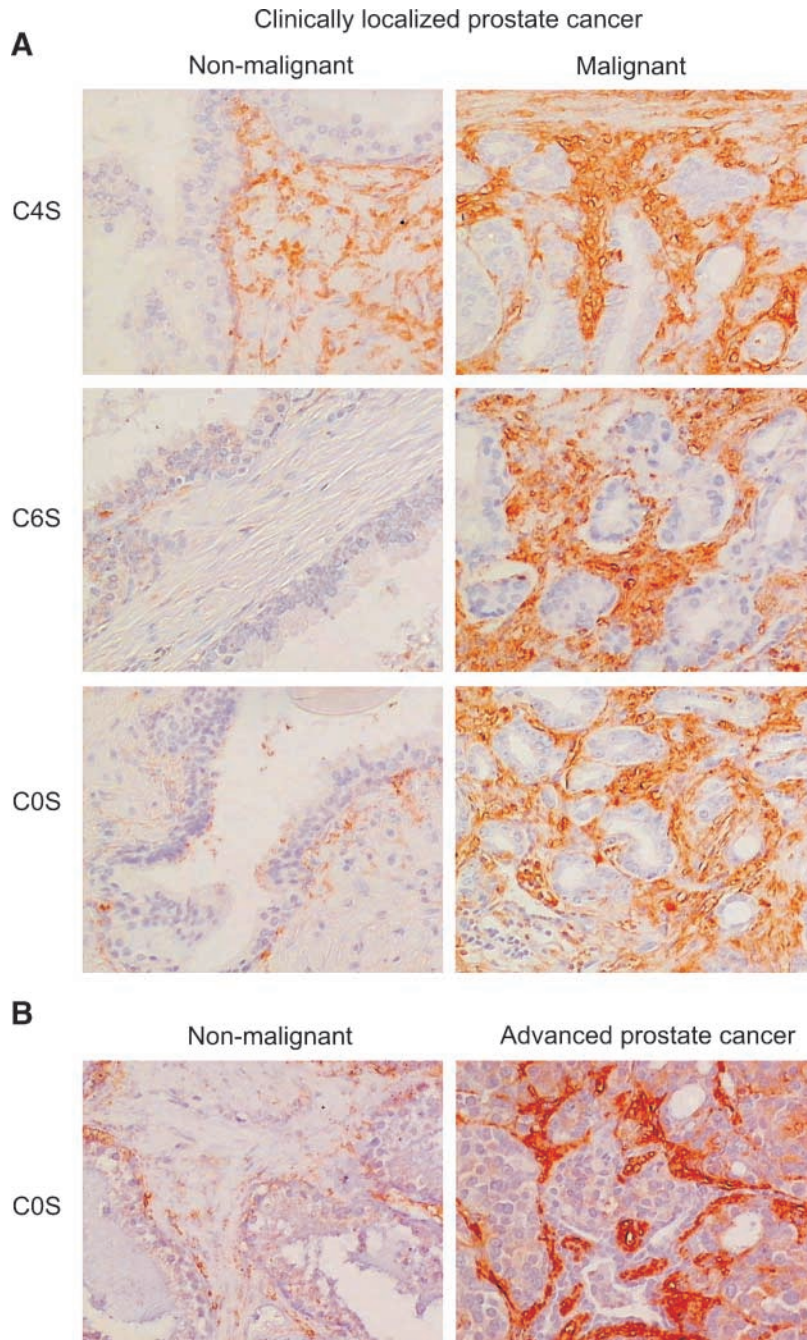


Figure 1. Glycosaminoglycan epitopes in prostatic tissues. Paraffin sections were stained using monoclonal antibodies to C4S (2B6), C6S (3B3), or unsulfated disaccharide (C0S, 1B5) in nonmalignant and malignant tissue areas of clinically localized prostate cancer (**A**) and BPH and advanced prostate cancer (**B**). Magnification, $\times 200$.

cancer tissues examined (Fig. 4B), but was absent in BPH. The identity of this peak is unknown as it did not correspond to the elution profile of any of the following additional glycosaminoglycan structures examined, Δ Di-4,6S, Δ Di-HA, GalNAc-4-S, GalNAc-6-S, and GalNAc (data not shown).

Discussion

The mechanisms underlying the progression of prostate cancer from organ confined to locally invasive and ultimately metastatic disease are poorly understood. Previous studies from this laboratory have shown

increased levels of chondroitin sulfate and the chondroitin sulfate proteoglycan versican in the peritumoral stromal matrix of prostate cancer patients who relapse after surgical treatment for presumed organ-confined disease (13, 14, 25). Those studies showed that measurement of chondroitin sulfate level may assist in predicting patient outcome following surgery, especially when combined with other clinical and pathologic features such as preoperative serum PSA levels. The present study investigated distinct glycosaminoglycan epitopes and showed that C0S disaccharide levels, but not C4S or C6S disaccharide levels, in the peritumoral stroma are significantly associated with rate of PSA failure in

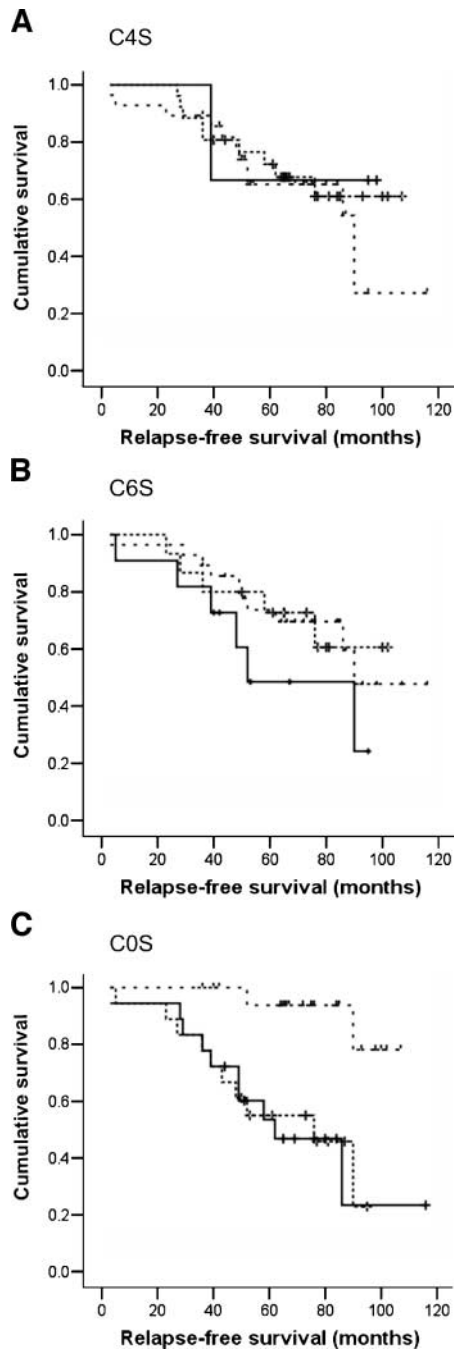


Figure 2. Kaplan-Meier product limit plots illustrating the relationship between glycosaminoglycan epitope levels and PSA relapse in clinically localized prostate cancer (Early Stage Cohort 1). **A.** (----) low C4S (0 or 1+ score), $n = 3$; (.....) moderate C4S (2+ score), $n = 26$; or (—) strong C4S disaccharide immunostaining (3+ score), $n = 26$, log-rank statistic = 0.69, $P = 0.709$. **B.** (----) low C6S (0 or 1+ score), $n = 27$; (.....) moderate C6S (2+ score), $n = 15$ or (—) strong C6S disaccharide immunostaining (3+ score), $n = 29$; log-rank statistic = 2.11; $P = 0.349$. **C.** (----) low unsulfated (0 or 1+ score), $n = 19$; (.....) moderate unsulfated (2+ score), $n = 18$; or (—) strong unsulfated disaccharide immunostaining (3+ score), $n = 18$; log-rank statistic = 11.12; $P = 0.004$.

clinically localized prostate cancer. The level of immunostaining for C0S disaccharide was found to be a predictor of PSA relapse, independent of the standard clinical and pathologic predictors. In addition, we showed that chondroitin sulfate but not dermatan sulfate glycosaminoglycan chains had a reduced electrophoretic mobility in advanced-stage prostate cancer tissues, probably due to a reduced charge density. These findings suggest that specific alterations to chondroitin sulfate side chains, in particular a decrease in overall sulfation attributed to an increased proportion of unsulfated chondroitin side chains, may be a crucial step for promoting disease progression in prostate cancer.

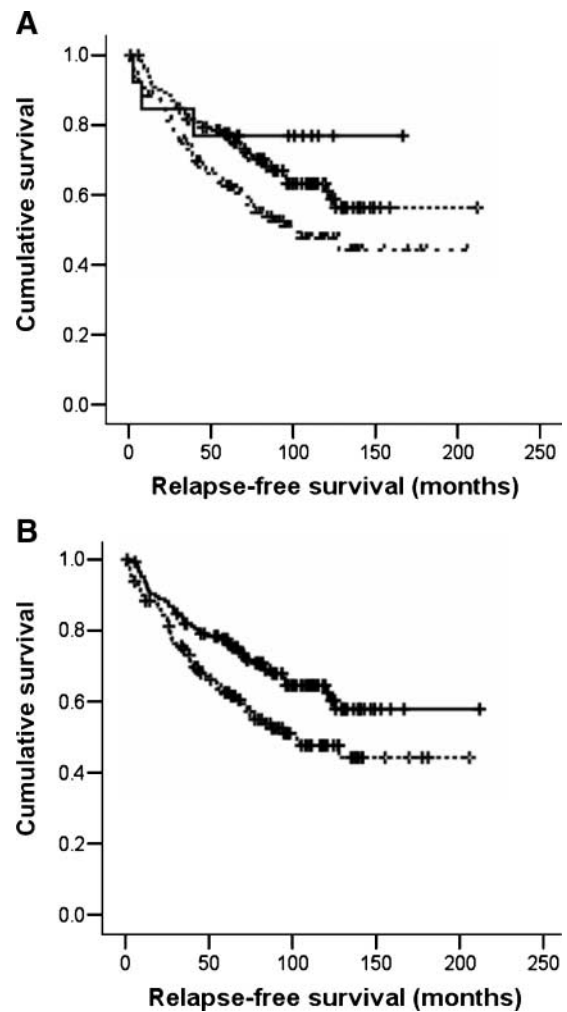


Figure 3. Kaplan-Meier product limit plots illustrating the relationship between chondroitin sulfate epitope levels and PSA progression in clinically localized prostate cancer in an independent cohort derived from an independent institution (Early Stage Cohort 2). **A.** (—) low unsulfated (0 or 1+ score), $n = 12$; (.....) moderate unsulfated (2+ score), $n = 129$ or (----) strong unsulfated disaccharide immunostaining (3+ score), $n = 119$; log-rank statistic = 6.617; $P = 0.037$. **B.** (—) Low or moderate unsulfated (0, 1+, or 2+ score), $n = 146$ or (----) strong unsulfated disaccharide immunostaining (3+ score), $n = 129$; log-rank statistic 6.625; $P = 0.012$.

Table 2. Statistical analysis of specific alterations in the sulfation pattern of GAG chains

| A. Univariate Cox Regression analysis of relapse-free survival in patients treated by radical prostatectomy (Early Stage Cohort 2) | | | |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|-------------------------|-------------------|
| Variable | Relative risk | 95% confidence interval | P |
| Age at diagnosis (<i>n</i> = 275) | 1.00 | 0.98-2.26 | 0.72 |
| Clinical stage* (<i>n</i> = 274) | 1.49 | 0.97-1.02 | 0.061 |
| Pathologic stage [†] (<i>n</i> = 275) | 2.59 | 1.75-3.84 | <0.0001 |
| Preoperative PSA [‡] (<i>n</i> = 260) | 1.78 | 1.22-2.62 | 0.003 |
| Gleason score [§] (<i>n</i> = 272) | 2.58 | 1.76-3.80 | <0.0001 |
| Margins (<i>n</i> = 275) | 1.82 | 1.24-2.68 | 0.002 |
| Seminal vesicle involvement (<i>n</i> = 272) | 2.24 | 1.52-3.30 | <0.0001 |
| Extracapsular extension (<i>n</i> = 275) | 2.30 | 1.56-3.39 | <0.0001 |
| C0S (<i>n</i> = 275) | 1.62 | 1.10-2.36 | 0.013 |
| B. Multivariate Cox regression analysis of relapse-free survival in patients treated by radical prostatectomy (Early Stage Cohort 2). All variables are significant in univariate analysis (<i>n</i> = 255) | | | |
| Pathologic stage | 4.30 | 1.36-13.56 | 0.013 |
| PSA | 1.46 | 0.97-2.19 | 0.073 |
| Gleason score | 2.03 | 1.26-3.27 | 0.002 |
| Seminal vesicle involvement | 0.46 | 0.17-1.27 | 0.133 |
| Margins | 1.20 | 0.39-3.72 | 0.447 |
| Extracapsular extension | 0.79 | 0.31-1.99 | 0.612 |
| C0S | 1.65 | 1.10-2.48 | 0.016 |
| C. Stepwise elimination of nonsignificant variables (<i>n</i> = 272) | | | |
| Pathologic stage | 1.97 | 1.29-3.01 | 0.002 |
| Gleason score | 2.31 | 1.54-3.46 | <0.0001 |
| C0S | 1.59 | 1.07-2.36 | 0.020 |

NOTE: P values highlighted in bold indicate $P < 0.05$

* Clinical stage cT1 vs cT2 and 6 cases that were stage cT3.

[†] Pathological stage pT2 vs pT3.

[‡] Preoperative serum PSA level (prostate specific antigen, ng/mL) dichotomized by cut point <10.0 versus ≥ 10.0 .

[§] Gleason score <7 vs ≥ 7 .

^{||} Immunostaining for unsulfated chondroitin disaccharide (C0S) in peritumoral stroma dichotomized by low or moderate levels vs high level.

Increased proportions of Δ Di-0S disaccharides have been observed in laryngeal (26), rectal (19), colon (7), liver (27), pancreatic (10), and head and neck tumor tissues (9) compared with normal tissue. Δ Di-0S disaccharide is the predominant disaccharide found in pancreatic carcinoma (10) and is increased 4-fold in gastric carcinoma compared with normal tissue (28). Furthermore, gastric carcinomas exhibit increased Δ Di-0S and Δ Di-6S disaccharides with a parallel decrease in Δ Di-4S disaccharides. The current study suggests that increased levels of Δ Di-0S disaccharides also occur in prostate cancer. Thus, a common feature of several cancer types seems to be the presence of chondroitin sulfate harboring specific alterations in disaccharide sulfation.

As sulfation is a nonrandom, biosynthetically regulated process, differences between the sulfation pattern of chondroitin sulfate attached to proteoglycans within normal and neoplastic tissues suggest that tumor factors may affect the synthesis and metabolism of chondroitin sulfate by the cells within the peritumoral stroma. Several growth factors that are secreted by prostate cancer cells at increased levels compared with normal prostate epithelial cells, such as transforming growth factor β 1 and platelet-derived growth factor, have been shown to induce structural modification to chondroitin sulfate chains, while simultaneously modulating proteoglycan core protein expression in arterial smooth muscle cells (29). These structural modifications include increases in chain length and changes in

sulfation resulting in altered charge density. In addition, insulin-like growth factor-1 is known to be increased in prostate cancer cells compared with normal prostate epithelial cells and to stimulate synthesis of undersulfated proteoglycans by the peritubular cells of the testis (30).

Chondroitin chains are synthesized by chondroitin synthase and chondroitin *N*-acetylgalactosaminyl transferase. Chain elongation requires GalNAc transferase II and D-glucuronic acid transferase II activities, whereas chain initiation requires GalNAc transferase I activity. Although little is known as to whether these enzymes are differentially expressed in cancerous tissues, α 1-3 GalNAc transferase has been shown to be activated during induced pancreatic carcinogenesis in the hamster (31). Several mechanisms may thus be used by prostate cancer cells to regulate both the levels and pattern of sulfation of chondroitin sulfate proteoglycans by stromal cells in the prostate and it is unlikely that observed changes are serendipitous. These mechanisms may comprise down-regulation of enzymes involved in chondroitin sulfate sulfation, such as chondroitin 4-*O*-sulfotransferase, chondroitin 6-*O*-sulfotransferase, and chondroitin 4-sulfate-6-*O*-sulfotransferase by either cancer cells or peritumoral stromal cells under the direction of cancer cells, and result in increased amounts of GalNAc residues that are unsulfated (16).

The current study shows that physicochemical alterations to the structure of chondroitin sulfate in the

prostatic stroma occur during prostate cancer progression and may aid prostate cancer metastasis. Changes in chondroitin sulfate structure may contribute to the efficiency with which chondroitin sulfate proteoglycans, such as versican, destabilize tumor cell focal adhesion and promote cancer invasion (32). Other studies have shown that a lowering of the sulfation level altered the ability of chondroitin sulfate to modulate embryonic cell migration on extracellular matrix *in vitro* (33). Alterations in sulfation pattern of chondroitin sulfate may well reflect modified biological roles of chondroitin sulfate proteoglycans between normal and neoplastic tissues (34). Although the biological and developmental significance of the different chondroitin sulfate epitopes is not well understood, studies have found that the sulfation profile of chondroitin sulfate chains changes with specific spatiotemporal patterns in various tissues during embryologic development, suggesting that

chondroitin sulfate isoforms differing in sulfation position and degree perform distinct functions (35, 36). In particular, the ratio of C4S to C6S varies during normal embryonic development (17) and a change in ratio in favor of C6S seems to be associated with cell proliferation (29). Furthermore, knockdown of the chondroitin synthase gene (by RNA interference) revealed that C0S (the only chondroitin synthesized by *Caenorhabditis elegans*) is required for embryonic morphogenesis and cell division (37). Interestingly, differential sulfation of heparan sulfate proteoglycans directs retinal axons through the chiasm during optic innervation (38). In addition, heparan sulfate and chondroitin sulfate have been shown to interact in a sulfation-dependent manner with various axon guidance proteins, including slit2, netrin1, ephrinA1, ephrinA5, and semaphorin5B (39). These studies lend support to the notions that glycosaminoglycans are sulfated in complex and changing

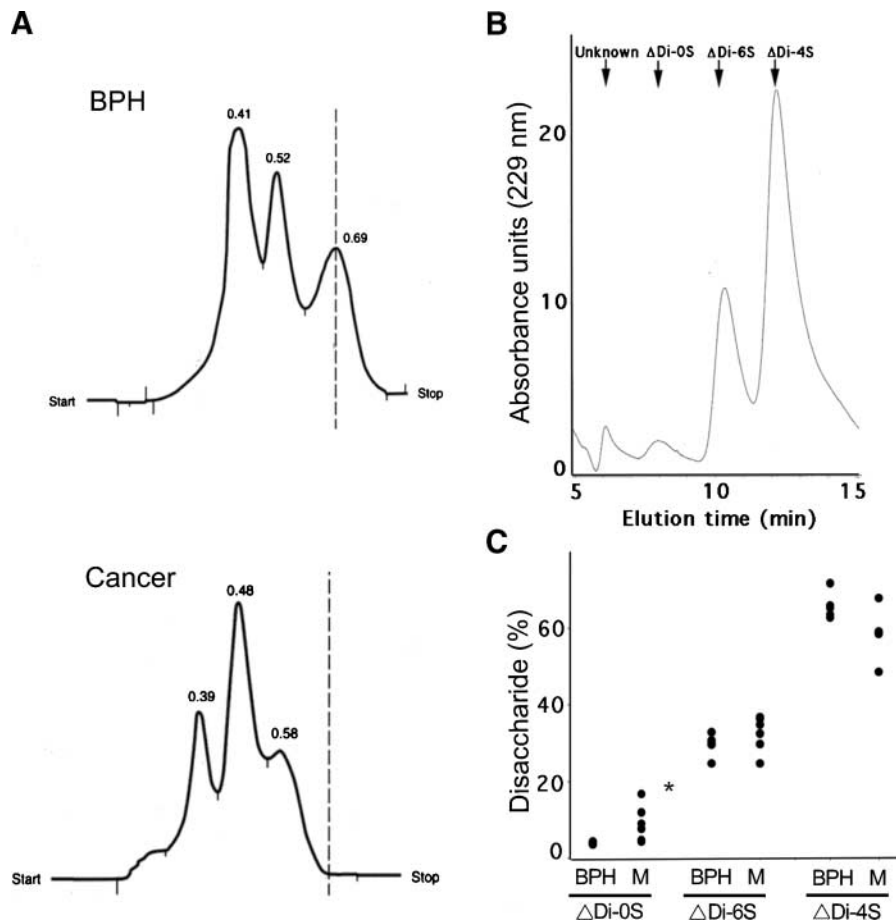


Figure 4. Cellulose acetate electrophoresis of papain-digested glycosaminoglycans from human prostate tissue (A). Profile produced by BPH and advanced prostate cancer tissue. The leading peak (relative mobility, RM 0.69 in BPH and RM 0.58 in cancer) was composed of chondroitin sulfate, the middle peak (RM 0.48-0.52) was composed of dermatan sulfate, and the trailing peak (RM 0.39-0.41) was composed of heparan sulfate and hyaluronan (A). High-pressure liquid chromatography elution profile of disaccharides from human prostate cancer tissue following chondroitinase ABC digestion (B). Absorbance was measured at 229 nm. Glycosaminoglycan chains were digested with chondroitinase ABC and analyzed by high-pressure liquid chromatography. Proportion of Δ Di-4S, Δ Di-6S, and Δ Di-0S disaccharides from chondroitin sulfate and dermatan sulfate isolated from nonmalignant, that is, BPH, and malignant (M) human prostate tissues (C). *, the level of Δ Di-0S disaccharides were significantly increased in prostate cancer tissues compared with BPH tissues ($P = 0.016$, Mann Whitney U test).

patterns and that undersulfation of chondroitin sulfate may be involved in fundamental biological processes involving cell migration.

A reduction in the electrophoretic migration of chondroitin sulfate has been observed in the urine of patients with different malignancies, including prostate cancer, compared with patients without cancer (8). The lower electrophoretic migration of chondroitin sulfate from cancer patients was found to be due to an increased amount of Δ Di-OS disaccharide. A study by Dietrich et al. (8) showed a direct correlation between the relative amounts of the Δ Di-OS disaccharide present in chondroitin sulfate and the stage of malignancy of the cancer. The structural change to chondroitin sulfate occurred early in the development of the tumor and, following surgery and chemotherapy, the structure of chondroitin sulfate in the urine of treated patients was found to revert back to a form similar to that secreted by normal subjects. It is therefore possible that measurement of COS disaccharides in biological fluids may be useful in the diagnosis and follow-up of prostate cancer.

In conclusion, this study supports the tenet that specific glycosaminoglycan alterations occur during tumor development and consequent changes in proteoglycan composition may be a critical step for prostate cancer invasion. Although the physiologic significance of the modifications induced by various growth factors for proteoglycan biosynthesis and particularly undersulfation are not understood, we postulate that changes to chondroitin sulfate structure in the peritumoral stroma of prostate cancer may facilitate cancer invasion by destabilizing focal cell adhesions between prostate cancer cells and the surrounding stroma. Further study of the regulation and biological role of chondroitin sulfate sulfation in prostate cancer will improve our knowledge of both the prostate tumor cell microenvironment and epithelial-stromal cell interactions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Keiko Mayne for performing the cellulose acetate electrophoresis and glycosaminoglycan isolation, Marie Pickering for immunohistochemical expertise, Virginia Papangelis for providing tissue sections from the Repatriation General Hospital cohort, and Anne-Maree Haynes and Ruth PeBenito for their assistance with the management of the patient database and tissue bank in relation to the St. Vincent's Hospital Campus Prostate Cancer Group cohort.

References

- Hoedemaeker RF, Vis AN, Van Der Kwast TH. Staging prostate cancer. *Microsc Res Tech* 2000;51:423–9.
- Ayala G, Tuxhorn JA, Wheeler TM, et al. Reactive stroma as a predictor of biochemical-free recurrence in prostate cancer. *Clin Cancer Res* 2003;9:4792–801.
- Levi F, Lucchini F, Negri E, Boyle P, La Vecchia C. Leveling of prostate cancer mortality in Western Europe. *Prostate* 2004;60:46–52.
- Kumar S, Shelley M, Harrison C, Coles B, Wilt TJ, Mason MD. Neo-adjuvant and adjuvant hormone therapy for localised and locally advanced prostate cancer. *Cochrane Database Syst Rev* 2006:CD006019.
- Walczak JR, Carducci MA. Prostate cancer: a practical approach to current management of recurrent disease. *Mayo Clin Proc* 2007;82:243–9.
- Yip GW, Smollich M, Gotte M. Therapeutic value of glycosaminoglycans in cancer. *Mol Cancer Ther* 2006;5:2139–48.
- Adany R, Heimer R, Caterson B, Sorrell JM, Iozzo RV. Altered expression of chondroitin sulfate proteoglycan in the stroma of human colon carcinoma. Hypomethylation of PG-40 gene correlates with increased PG-40 content and mRNA levels. *J Biol Chem* 1990;265:11389–96.
- Dietrich CP, Martins JR, Sampaio LO, Nader HB. Anomalous structure of urinary chondroitin sulfate from cancer patients. A potential new marker for diagnosis of neoplasias. *Lab Invest* 1993;68:439–45.
- Martins RC, Lima FR, Werneck CC, Neto VM, Silva LC. Patterns of synthesis and secretion of sulfated glycosaminoglycans in primary cortical and cerebellar astrocytes *in vitro*. *Biol Cell* 2000;92:421–7.
- Theocharis AD, Tsara ME, Papageorgakopoulou N, Karavias DD, Theocharis DA. Pancreatic carcinoma is characterized by elevated content of hyaluronan and chondroitin sulfate with altered disaccharide composition. *Biochim Biophys Acta* 2000;1502:201–6.
- De Klerk DP, Lee DV, Human HJ. Glycosaminoglycans of human prostatic cancer. *J Urol* 1984;131:1008–12.
- Iida S, Suzuki K, Matsuoka K, et al. Analysis of glycosaminoglycans in human prostate by high-performance liquid chromatography. *Br J Urol* 1997;79:763–9.
- Ricciardelli C, Mayne K, Sykes PJ, et al. Elevated stromal chondroitin sulfate glycosaminoglycan predicts progression in early-stage prostate cancer. *Clin Cancer Res* 1997;3:983–92.
- Ricciardelli C, Quinn DJ, Raymond WA, et al. Elevated levels of peritumoral chondroitin sulfate are predictive of poor prognosis in patients treated by radical prostatectomy for early-stage prostate cancer. *Cancer Res* 1999;59:2324–8.
- Mark MP, Baker JR, Kimata K, Ruch JV. Regulated changes in chondroitin sulfation during embryogenesis: an immunohistochemical approach. *Int J Dev Biol* 1990;34:191–204.
- Silbert JE, Sugumar G. Biosynthesis of chondroitin/dermatan sulfate. *IUBMB Life* 2002;54:177–86.
- Kitagawa H, Tsutsumi K, Tone Y, Sugahara K. Developmental regulation of the sulfation profile of chondroitin sulfate chains in the chicken embryo brain. *J Biol Chem* 1997;272:31377–81.
- Kovalszky JTA. Differential expression of proteoglycans on the surface of malignant cells and in the tumor stroma. In: Adany R, editor. *Tumor matrix biology*. Boca Raton (FL): CRC Press; 1995. p. 23–53.
- Tsara ME, Papageorgakopoulou N, Karavias DD, Theocharis DA. Distribution and changes of glycosaminoglycans in neoplasias of rectum. *Anticancer Res* 1995;15:2107–12.
- Hermanek P. 1992 tumor classification/developments. *Langenbecks Arch Chir Suppl Kongressbd* 1992;40–45.
- Couchman JR, Caterson B, Christner JE, Baker JR. Mapping by monoclonal antibody detection of glycosaminoglycans in connective tissues. *Nature* 1984;307:650–2.
- Horsfall DJ, Mayne K, Skinner JM, Saccone GT, Marshall VR, Tilley WD. Glycosaminoglycans of guinea pig prostate fibromuscular stroma: influence of estrogen and androgen on levels and location of chondroitin sulfate. *Prostate* 1994;25:320–32.
- Reinhold BJ, Finnis ML, Gibson MA, Sandberg LB, Cleary EG. Developmental expression of dermatan sulfate proteoglycans in the elastic bovine nuchal ligament. *Matrix Biol* 2000;19:149–62.
- Zebrower M, Kieras FJ, Heaney-Kieras J. High pressure liquid chromatographic identification of hyaluronic acid and chondroitin sulphate disaccharides. *Glycobiology* 1991;1:271–6.
- Ricciardelli C, Mayne K, Sykes PJ, et al. Elevated levels of versican but not decorin predict disease progression in early-stage prostate cancer. *Clin Cancer Res* 1998;4:963–71.
- Skandalis SS, Stylianou M, Vynios DH, Papageorgakopoulou N, Theocharis DA. The structural and compositional changes of glycosaminoglycans are closely associated with tissue type in human laryngeal cancer. *Biochimie* 2007;89:1573–80.
- Kovalszky I, Pogany G, Molnar G, et al. Altered glycosaminoglycan composition in reactive and neoplastic human liver. *Biochem Biophys Res Commun* 1990;167:883–90.
- Theocharis AD, Vynios DH, Papageorgakopoulou N, Skandalis SS, Theocharis DA. Altered content composition and structure of glycosaminoglycans and proteoglycans in gastric carcinoma. *Int J Biochem Cell Biol* 2003;35:376–90.
- Schonherr E, Jarvelainen HT, Sandell LJ, Wight TN. Effects of platelet-derived growth factor and transforming growth factor- β 1

- on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells. *J Biol Chem* 1991;266:17640–7.
30. Thiebot B, Bichoual L, Langris M, et al. IGF-1 stimulates synthesis of undersulfated proteoglycans and of hyaluronic acid by peritubular cells from immature rat testis. *Biochim Biophys Acta* 1997;1358:127–41.
 31. Hirota M, Egami H, Ogawa M. Augmentation of UDP-GalNAc: Fuc α 1–2Gal α 1–3 *N*-acetylgalactosaminyl transferase activity in nitrosamine-induced hamster pancreatic cancers. *J Exp Clin Cancer Res* 2000;19:235–9.
 32. Sakko AJ, Ricciardelli C, Mayne K, et al. Modulation of prostate cancer cell attachment to matrix by versican. *Cancer Res* 2003;63:4786–91.
 33. Newgreen DF, Gibbins IL, Sauter J, Wallenfels B, Wutz R. Ultrastructural and tissue-culture studies on the role of fibronectin, collagen and glycosaminoglycans in the migration of neural crest cells in the fowl embryo. *Cell Tissue Res* 1982;221:521–49.
 34. Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. *FASEB J* 1992;6:861–70.
 35. Sorrell JM, Carrino DA, Baber MA, Caplan AI. Versican in human fetal skin development. *Anat Embryol (Berl)* 1999;199:45–56.
 36. Shimazaki Y, Nagata I, Ishii M, et al. Developmental change and function of chondroitin sulfate deposited around cerebellar Purkinje cells. *J Neurosci Res* 2005;82:172–83.
 37. Mizuguchi S, Uyama T, Kitagawa H, et al. Chondroitin proteoglycans are involved in cell division of *Caenorhabditis elegans*. *Nature* 2003;423:443–8.
 38. Pratt T, Conway CD, Tian NM, Price DJ, Mason JO. Heparan sulphation patterns generated by specific heparan sulfotransferase enzymes direct distinct aspects of retinal axon guidance at the optic chiasm. *J Neurosci* 2006;26:6911–23.
 39. Shipp EL, Hsieh-Wilson LC. Profiling the sulfation specificities of glycosaminoglycan interactions with growth factors and chemotactic proteins using microarrays. *Chem Biol* 2007;14:195–208.