ORIGINAL ARTICLE

Arylsulfatase B (N-acetylgalactosamine-4-sulfatase): potential role as a biomarker in prostate cancer

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BACKGROUND: The enzyme arylsulfatase B (ARSB; N-acetylgalactosamine-4-sulfatase) degrades chondroitin-4-sulfate (C4S) and is reduced in malignant colonic and mammary tissues but has not previously been evaluated in prostate cancer.

METHODS: ARSB immunostaining was performed on two tissue microarrays (TMAs) and analyzed by digital image analysis, generating ARSB H-scores for prevalence and intensity of epithelial, stromal and combined epithelial and stromal immunostaining. Also, paired malignant and normal prostate tissues were analyzed for ARSB activity, C4S, total sulfated glycosaminoglycans and versican content. The quantities of C4S and of the epidermal growth factor receptor (EGFR) that co-immunoprecipitated with versican were determined in the normal and malignant paired prostate tissues.

RESULTS: Forty-four cases of prostate cancer were paired by age (± 5 years), race, Gleason score (in order) and pathological TNM (tumor, node, metastasis) score. The pairs differed by recurrence vs non-recurrence of elevated PSA at ≥ 4 years. When TMA cores were analyzed for ARSB H-score, 18 of the 22 pairs had lower ARSB H-scores in the recurrent member of the pair, whereas higher initial PSA values were associated with recurrence in only 65% of the paired cases. In a second TMA, Gleason scores 6 and 7 were associated with higher ARSB H-scores than Gleason scores 8 and 9 for stroma, epithelium and stroma and epithelium combined (P = 0.052, P = 0.015, P < 0.0001, respectively) and were inversely correlated (r = −0.98, −0.97 and −0.99, respectively). In other paired normal and malignant prostate tissues, ARSB activity was significantly higher in the normal tissues, and C4S and versican values were lower (P < 0.0001). C4S that co-immunoprecipitated with versican was greater in the malignant than in the normal tissue, whereas total EGFR that co-immunoprecipitated with versican was reduced.

CONCLUSIONS: Study findings suggest that ARSB may be useful as a prognostic biomarker in prostate cancer and that the biological action of ARSB on chondroitin sulfate may impact upon versican’s effects in the tumor microenvironment.

INTRODUCTION

Arylsulfatase B (ARSB; N-acetylgalactosamine-4-sulfatase) is the lysosomal enzyme that removes the 4-sulfate group of N-acetylgalactosamine-4-sulfate at the non-reducing end of chondroitin-4-sulfate (C4S) and dermatan sulfate and thereby regulates their degradation. Recent studies demonstrated extralysosomal localization of ARSB in epithelial and endothelial membranes in human cells. Decline in ARSB activity was shown in malignant mammary and colonic epithelial tissues and in metastatic colonic epithelial cells, and the intensity and localization of ARSB immunostaining was reduced in higher grade colonic adenocarcinomas. The current studies were undertaken to determine if the previously identified reductions in ARSB in malignant mammary and colonic tissues were also evident in malignant prostate tissue.

Previously, chondroitin sulfate and versican, an extracellular matrix proteoglycan with chondroitin sulfate attachments, were reported to predict progression in early-stage prostate cancer and considered as potential biomarkers of prostate cancer. Versican is an important extracellular matrix proteoglycan composed of three domains: the G1 domain has hyaluronic acid repeats and a carbohydrate recognition domain. These domains enable versican to interact with multiple binding partners, including type 1 collagen, tenascin-R, fibronectin, P- and L-selectins, β1-integrins, EGF receptor (EGFR) and P-selectin glycoprotein ligand-1. Versican is regarded as a critical factor affecting the attachment of prostate cancer cells to fibronectin in the stroma, thereby mediating motility and invasiveness. As decline in ARSB activity leads directly to increase in chondroitin sulfation and transcriptionally to increase in versican expression, the associations among versican, chondroitin sulfate and ARSB are of interest and were addressed in the studies in this report.

Although PSA has been widely used as a biomarker of prostate cancer, the benefits of screening by PSA remain controversial and a better prognostic marker of prostate cancer has been the subject of considerable investigation. In this report, the potential role of ARSB as a biomarker of prostatic malignancy was considered. Measurements of the intensity of ARSB immunostaining by digitized image analysis (H-scores) were calculated for prostate cancers in two small tissue microarrays (TMAs), and the associations of H-scores with recurrence vs non-recurrence and Gleason score were determined. In addition, ARSB domain at the C-terminus has epidermal growth factor (EGF)-like repeats and a carbohydrate recognition domain. These domains enable versican to interact with multiple binding partners, including type 1 collagen, tenascin-R, fibronectin, P- and L-selectins, β1-integrins, EGF receptor (EGFR) and P-selectin glycoprotein ligand-1. Versican is regarded as a critical factor affecting the attachment of prostate cancer cells to fibronectin in the stroma, thereby mediating motility and invasiveness. As decline in ARSB activity leads directly to increase in chondroitin sulfation and transcriptionally to increase in versican expression, the associations among versican, chondroitin sulfate and ARSB are of interest and were addressed in the studies in this report.

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enzyme activity, C4S and versican were compared between normal and malignant regions from radical prostatectomies performed for prostate cancer. The study data that follow suggest that further evaluation of ARSB as a biomarker and possible tumor suppressor in prostate cancer is warranted.

MATERIALS AND METHODS
Cancer tissue arrays and tissue samples
Prostate cancer tissues from three sources were analyzed. These included a cancer TMA obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA, USA), which included 30 cores with Gleason scores from 6 to 9. A second cancer TMA from the Cooperative Prostate Cancer Tissue Resource (CPCTR, from AK-B) included 22 cases of pairs that varied by biochemical recurrence (increased PSA) vs non-recurrence after ≥4 years and were matched by age ≤5 years, race, Gleason score matched by sequence and score, treatment (radical prostatectomy) and pathological TNM (tumor, node, metastasis) stage.19 Also, fresh frozen tissues from nine prostatectomies for prostate cancer were obtained from the University of Illinois at Chicago (UIC) Tissue Bank under a protocol approved by the Institutional Review Board and the Cancer Center of UIC. Frozen sections were made from paraffin blocks. Histological sections consisting of epithelium and stroma, were identified by two observers (GG and LF), dissected out and frozen for subsequent analysis, as described.

ARSB immunostaining and digitized image analysis
TMA slides were hydrated using xylen and an alcohol gradient and rinsed in distilled water. Antigen unmasking was performed with a 10× concentrated retrieval solution by Dako (DakoCyton, Carpinteria, CA, USA), according to the manufacturer’s instructions, then slides were rinsed in phosphate-buffered saline for 5 min. Endogenous peroxidase activity was blocked by H2O2 blocking reagent for 10 min at room temperature, then the TMA slides were treated with a protein blocking solution for 10 min at room temperature, rinsed and incubated with ARSB polyclonal rabbit antiserum (Open Biosystems, ThermoFisher Scientific, Huntsville, AL, USA; 1:100) or negative immunoglobulin G (IgG) control for 30 min at room temperature. Slides were rinsed and then treated with EnVision Plus labeled polymer (DakoCyton) for 30 min at room temperature. DAB Plus (DakoCyton) was used for 10 min to detect ARSB, and slides were rinsed in distilled water, counterstained with hematoxylin, dehydrated through an alcohol gradient and mounted with Permount. The TMA slides were digitally scanned at ×20 magnification on an Aperio ScanScope C5 (Aperio Technologies, Vista, CA, USA) using the Aperio ImageScope program (v10.0.35.1800) and loaded into Spectrum version 11.1. Other prostate cancer and normal tissue sections from frozen tissue were immunostained with ARSB polyclonal antibody. Negative IgG controls were also prepared and imaged.

The TMA Lab software module was used to segment the TMA into individual cores, while the Genie module was used to map distinct epithelial and stromal regions within each core. Genie is a machine learning program that classifies each pixel in an image according to a set of hand-drawn, pre-classified training images provided by a skilled human operator. For this study, we created three classes: epithelial, stromal and no-tissue, using 16, 7 and 1 training images, respectively. The resulting classifier algorithm, which was determined to be highly accurate in classifying pixels within the training set of images, was then applied to the entire TMA set. Once epithelial and stromal regions were mapped, it was possible to score staining solely within epithelial or stromal compartments or in combination. The Positive Pixel Count (Aperio) algorithm was used within the epithelial and stromal compartments to measure brown enzyme staining in each relevant pixel at four ordinal intensity levels, from 0 to 3. The H-score, an index that combines stain prevalence and intensity, was determined based on the proportion of weakly, moderately and strongly stained pixels in each core using the formula: (% weak × 1 + % moderate × 2 + % strong × 3)/100. The H-scores were calculated independently for the NDRI and CPCTR cores. Mean H-score ± s.d. for Gleason scores 6–9 in the NDRI TMA was calculated. The H-scores for the recurrent vs non-recurrent member of the paired samples in the CPCTR were compared. When multiple cores from the same surgery were present on an array, the H-scores for the cores were averaged, and the average value used in subsequent analysis. Each TMA core was reviewed visually before scoring to exclude artifacts or missing tissue and again after scoring. No gross discrepancies with the automated scoring were identified.

ARSB activity assay and western blot
Tissue homogenates were prepared from the normal and malignant foci isolated from the prostatectomies performed at UIC. ARSB activity was determined using a fluorometric assay and the exogenous substrate 4-methylumbelliferyl sulfate, as previously described. Briefly, 20 µl of tissue homogenate and 80 µl of assay buffer (0.05 N Na acetate buffer, pH 5.6) were combined with 100 µl of substrate (5 mM 4-MUS in assay buffer) in wells of a microplate. After incubation for 30 min at 37 °C, the reaction was stopped by 150 µl of stop buffer (Glycine-Carbonate buffer) at pH 10.7, and fluorescence was measured at 360 nm (excitation) and 465 nm (emission) in a microplate reader (FLUOstar, BMG, Cary, NC, USA). ARSB activity was expressed as nmol per mg protein per h, based on a standard curve for ARSB activity prepared with known quantities of 4-methylumbiliferyl at pH 5.6. Protein content of the tissue homogenate was determined by total protein assay kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA).

Western blot for ARSB was performed using the paired normal and malignant prostate tissue samples from three of the UIC cases. Tissue lysates were prepared from prostate tissue with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) and protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Scientific, Pittsburgh, PA, USA). Western blot of ARSB was performed on 10% sodium dodecyl sulfate gel with ARSB antibody, as above, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). An ARSB inhibitory peptide, composed of the sequence which was used to generate the antibody, was added to three of the wells to show the specificity of the ARSB band. The sequence of the inhibitory peptide used in the western blot was: RLQFYHKHSVPVYFPAQDPR (NP_15848.1; AA: 501-520). Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham, GE Healthcare, Piscataway, NJ, USA). Density of the ARSB was compared with β-actin in the malignant and normal samples.

Measurement of sulfated glycosaminoglycans (GAGs)
Total sulfated GAG content in the normal and malignant prostate tissues was measured using the substrate 1,9-dimethylmethylene blue (Blucolor, Biocontrol, Newtownabbey, Northern Ireland), which detects C4S, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate, heparan sulfate and heparin, but does not detect unsulfated GAGs or disaccharides. The substrate 1,9-dimethylmethylene blue combines with the sulfate groups of the sulfated GAG and detects the polysaccharide component of proteoglycans and the protein-free sulfated GAG chains. Tissue lysates were prepared using radio-immunoprecipitation assay buffer (50 mmol/l Tris-HCl containing 150 mmol/l NaCl, 1% Nonidet P40, 0.5% deoxycholic acid and 0.1% sodium dodecyl sulfate, pH 7.4). Absorbance maximum of 1,9-dimethylmethylene blue was detected at 656 nm (FLUOstar), and sulfated GAG concentration expressed as µg per mg of protein of tissue lysate.

Immunoprecipitation of tissue lysates by C4S antibody
Tissue lysates were prepared using radio-immunoprecipitation assay buffer, as above. Antibody specific to native C4S (4D1, Abnova, Littleton, CO, USA) was previously tested by the recovery of pure C4S following immunoprecipitation with the C4S antibody (1 µg) and shown to be 93.3 ± 2.7%. Cross-reactivity of the antibody with CS-E or C6S was excluded by similar tests. The C4S antibody (1 µg per mg of cell lysate protein) was added to the prostate cell lysates in tubes, and tubes were rotated overnight in a shaker at 4 °C. Next, 100 µl of pre-washed protein L-agarose (Santa Cruz Biotechnology) was added to each tube, and tubes were incubated overnight at 4 °C. Subsequently, the beads were washed three times with phosphate-buffered saline containing protease inhibitor mixture, and the precipitate was eluted with dye-free elution buffer and subjected to C4S antibody measurement by Blucolor assay, as described above.

Determination of versican by competitive enzyme-linked immunosorbent assay (ELISA)
Human versican was measured by a competitive ELISA (My BioSource, San Diego, CA, USA), in which color development was inversely proportional to the versican content in the test samples. Standards, ranging from 1 to 25 µg/l, tissue samples and versican-horseradish peroxidase
The samples were extrapolated from a standard curve and normalized. The concentration of versican in the samples was determined using Instat (GraphPad Software, San Diego, CA, USA) by paired test, two-tailed. Lower arylsulfatase B H-scores predict recurrence in paired prostate cancer cases. (a) Mean ARSB H-scores were calculated for stroma, epithelium and combined stroma and epithelium for 22 pairs of prostate cancer cases that differed by recurrence vs non-recurrence at > 4 years of follow-up. Recurrences had lower mean ARSB H-scores for stroma (0.10 ± 0.08 vs 0.19 ± 0.09), epithelium (0.48 ± 0.32 vs 0.86 ± 0.24) and combined stroma and epithelium (0.28 ± 0.26 vs 0.62 ± 0.28), and differences were highly significant (P = 0.0021, P = 0.0003, P = 0.0006, respectively, paired t-test, two-tailed). (b) Scattergram shows the ARSB H-scores in the 22 pairs of prostate cancer cases for epithelium and stroma combined. H-scores < 0.25 accurately predicted recurrence, and H-scores > 0.70 were associated with non-recurrence in 8 of 9 cases. Squares indicate non-recurrence, and diamonds indicate recurrence.

Measurement of total EGFR by ELISA
Total human EGFR (phosphorylated and unphosphorylated EGFR and ErbB1) was measured in the tissue extract using a standardized ELISA (R&D, Minneapolis, MN, USA). Total EGFR in the samples was captured in the wells of microtiter plates that were pre-coated with specific capture antibody. The immobilized total EGFR was detected by a biotinylated second EGFR, and streptavidin-hydrogen peroxidase was added. The bound enzyme activity was determined by chromogenic substrate (hydrogen peroxide/tetramethylbenzidine), and color development due to hydrogen peroxidase activity was stopped by 2N sulfuric acid. Intensity of color was measured at 450 nm in a plate reader (BMG). The concentration of versican in the samples was extrapolated from the standard curve and expressed per mg of total tissue protein, measured by protein assay (Pierce).

Measurement of C4S immunoprecipitated with versican
Versican was immunoprecipitated from tissue lysates using versican antibody (V0 isoform; Santa Cruz Biotechnology) covalently bound to Dynabeads (Life Technologies, Carlsbad, CA, USA). Total versican concentration in the immunoprecipitate was determined by competitive ELISA as described above. Prostate cancer samples were diluted by 50% in diluent to bring versican to approximately the same concentration as in the normal tissue immunoprecipitates. Blyscan assay for C4S was conducted as described above to detect the C4S that co-immunoprecipitated with versican.

Immunohistochemistry of C4S
Tissue sections were prepared from the frozen normal and malignant prostate tissues and immunostained with C4S mouse monoclonal antibody (4D1 clone, Santa Cruz Biotechnology; 1:100). Sections were incubated with primary antibody or IgG negative control overnight at 4 °C, washed, and then incubated with secondary antibody, which was conjugated with hydrogen peroxidase for 1 h at room temperature. Color was developed following wash with 3,3-diaminobenzidine and counterstained with hematoxylin. Digitized images were obtained with QCapture software (QImaging, Surrey, BC, Canada) at ×20 magnification. Background color was modified with GIMP Portable software (Portable Apps, New York, NY, USA).

Table 1. Lower arylsulfatase B H-scores and higher PSA values predict recurrence in paired prostate cancer cases

| Pair number | H-score combined—recurrence | H-score combined—no recurrence | PSA—recurrence | PSA—no recurrence |
|-------------|-----------------------------|--------------------------------|----------------|------------------|
| 1           | 0.26                        | 0.46                           | 2.1            | 8.5              |
| 2           | 0.48                        | 0.36                           | 7.6            | 6.1              |
| 3           | 0.052                       | 0.53                           | 39             | 14.3             |
| 4           | 0.034                       | 1.07                           | 32             | 8.4              |
| 5           | 0.077                       | 0.41                           | 10             | NA               |
| 6           | 0.11                        | 0.56                           | 8.6            | 9.3              |
| 7           | 0.22                        | 1.03                           | 4.9            | 10.7             |
| 8           | 0.32                        | 0.51                           | 29.2           | 5.9              |
| 9           | 0.17                        | 0.78                           | 13.4           | 1.9              |
| 10          | 0.13                        | 0.30                           | 14.6           | 4.6              |
| 11          | 0.37                        | 0.53                           | 11.6           | 3.6              |
| 12          | 0.28                        | 0.92                           | 20             | 5.6              |
| 13          | 0.38                        | 0.75                           | 6.8            | 4.7              |
| 14          | 0.64                        | 0.80                           | 12             | 6                |
| 15          | 0.66                        | 0.25                           | 4.3            | 7.6              |
| 16          | 0.040                       | 0.34                           | 8.6            | 30.1             |
| 17          | 0.60                        | 0.48                           | 13.8           | 11.5             |
| 18          | 0.12                        | 0.28                           | 8.2            | 10               |
| 19          | 0.13                        | 0.54                           | 10.8           | 7.3              |
| 20          | 0.075                       | 1.29                           | 10             | NA               |
| 21          | 0.041                       | 0.51                           | 3              | 17               |
| 22          | 1.045                       | 0.85                           | 7.6            | 5.1              |

Abbreviation: NA, not available.
Bold numbers indicate lower H-score predictive of recurrence. Italicized numbers indicate higher PSA predictive of recurrence.

Figure 1. Lower arylsulfatase B (ARSB) H-scores predict recurrence in paired prostate cancer cases. (a) Mean ARSB H-scores were calculated for stroma, epithelium and combined stroma and epithelium for 22 pairs of prostate cancer cases that differed by recurrence vs non-recurrence at > 4 years of follow-up. Recurrences had lower mean ARSB H-scores for stroma (0.10 ± 0.08 vs 0.19 ± 0.09), epithelium (0.48 ± 0.32 vs 0.86 ± 0.24) and combined stroma and epithelium (0.28 ± 0.26 vs 0.62 ± 0.28), and differences were highly significant (P = 0.0021, P = 0.0003, P = 0.0006, respectively, paired t-test, two-tailed). (b) Scattergram shows the ARSB H-scores in the 22 pairs of prostate cancer cases for epithelium and stroma combined. H-scores < 0.25 accurately predicted recurrence, and H-scores > 0.70 were associated with non-recurrence in 8 of 9 cases. Squares indicate non-recurrence, and diamonds indicate recurrence.

(4D1 clone, Santa Cruz Biotechnology; 1:100). Sections were incubated with primary antibody or IgG negative control overnight at 4 °C, washed, and then incubated with secondary antibody, which was conjugated with hydrogen peroxidase for 1 h at room temperature. Color was developed following wash with 3,3-diaminobenzidine and counterstained with hematoxylin. Digitized images were obtained with QCapture software (QImaging, Surrey, BC, Canada) at ×20 magnification. Background color was modified with GIMP Portable software (Portable Apps, New York, NY, USA).

Statistics
Results are expressed as mean ± s.d. Statistical significance of differences in H-scores between paired samples that varied by recurrence vs non-recurrence and association between H-scores and Gleason scores was determined using Instat (GraphPad Software, San Diego, CA, USA) by paired or unpaired t-tests, two-tailed or by one-way analysis of variance, followed by the Tukey–Kramer post-test to correct for multiple comparisons. Paired t-tests were performed with six pairs of normal and malignant biological samples using averages of technical duplicates of each measurement. P-value of < 0.05 was considered statistically significant.

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RESULTS

Lower ARSB H-score predicts recurrence in paired cancer cases

Prostate cancer cases in the CPCTR array were paired for age ± 5 years, race, treatment intervention, Gleason scores (in same sequence) and pathological TNM stage and were differentiated only by biochemical (elevated PSA) recurrence vs non-recurrence after ≥4 years of follow-up. ARSB (N-acetylgalactosamine-4-sulfatase) immunostaining of the TMA containing 22 pairs of cores from prostatectomies was performed. ARSB H-scores for epithelium, stroma and combined epithelium and stroma were determined by digitized image analysis for each case and were compared between the recurrent and non-recurrent members of the pairs. In all, 82% (18/22) of the pairs had higher H-scores for the non-recurrence than for recurrence using the combined stroma and epithelium ARSB H-score (Table 1). In contrast, initial PSA values were lower in only 65% (13/20) of the recurrences, making the PSA in this sample less effective as a predictor of recurrence than the ARSB H-score. The combination of higher ARSB H-score and lower PSA value predicted 95% (21/22) of the recurrences.

Mean ARSB H-score for recurrence for combined stroma and epithelium was 0.28 ± 0.26 and for non-recurrence was 0.62 ± 0.28. By paired t-test, the difference in ARSB H-scores was highly significant for combined (P = 0.0006, paired t-test, two-tailed), for epithelium (P = 0.0003) and for stroma (P = 0.0021) (Figure 1a). ARSB H-scores < 0.25 for combined predicted recurrence with 100% specificity, and H-scores > 0.70 for combined were highly predictive of non-recurrence (Figure 1b). Overlap between the scores for recurrence and non-recurrence was evident between 0.25 and 0.70, and > 50% (23/44) of the cases were in this range.

Inverse association between ARSB immunostaining and Gleason score

Mean ARSB H-scores for cores on the NDRI array were calculated and associated with the corresponding Gleason scores. Eleven cores were designated Gleason 6, six were Gleason 7, eight were Gleason 8, and five were Gleason 9. Mean ARSB H-scores were compared between Gleason scores 6 ± 7 and 8 ± 9.
for stroma, epithelium and combined epithelium and stroma and found to be significant \((P = 0.052, P = 0.015\) and \(P < 0.0001\), respectively, unpaired t-test, two-tailed; Figure 2a). Representative images demonstrate greater intensity of ARSB immunostaining for Gleason scores 6 and 7 compared with scores 8 and 9 (Figures 2b–e). The ARSB-positive epithelial cell membrane becomes increasingly less continuous and more punctate with increasing Gleason score. Overall intensity of stromal and epithelial staining declined with increasing Gleason score. Representative TMA cores demonstrate positive staining for ARSB (Figure 2f) and negative staining with IgG control (Figure 2g).

Linear regression analysis demonstrated inverse correlations for ARSB H-scores and Gleason scores (7–9) for combined epithelium and stroma, epithelium and stroma (Figure 2h). The \(r\)-values were \(-0.99\), \(-0.97\) and \(-0.98\), respectively.

Reduced ARSB associated with increased sulfated GAGs and C4S in the malignant prostatic tissue compared with the normal. Western blot performed using normal and malignant areas from prostate tissue obtained at the time of prostatectomy shows higher density bands in the normal tissues than in the malignant. The specificity of the antibody is confirmed by the use of the inhibitory peptide that is composed of the amino-acid sequence used to generate the antibody. \(\beta\)-Actin bands confirm equal loading. N, normal; CA, cancer. (b) Densitometry confirms the visual impression that ARSB intensity is reduced in the malignant tissue, compared with the normal \((P = 0.003)\). Addition of the peptide, which was the epitope for the ARSB antibody, inhibits the band formation \((P < 0.0001)\). ARSB activity is significantly reduced in the malignant tissue from the prostatectomy samples obtained from the University of Illinois at Chicago Tissue Bank, compared with the normal tissue. Mean ARSB activity in normal tissue was over 63 ng per mg protein per h greater \((**P < 0.0001, \text{paired } t\text{-test, two-tailed}; n = 6 \text{ pairs})\). (c) Corresponding to the decline in ARSB activity, the total sGAGs and C4S both increased significantly in the malignant prostate tissue, compared with the normal tissue \((**P < 0.0001, \text{paired } t\text{-test, two-tailed}; n = 6 \text{ pairs})\). (e–g). C4S antibody was used for immunostaining in normal and malignant prostate tissue. Increased intensity of C4S is apparent in the malignant tissue (f), particularly in the stroma and in the epithelial cell nuclei. Negative control shows no staining for C4S (original magnification \(\times 10\)).
the malignant tissue, an increase of 2.6 times the baseline ± biomarker of prostate cancer. Measurements of versican showed Versican, an extracellular matrix proteoglycan with chondroitin Versican increased in malignant prostate tissue control staining is negative (Figure 3g).

Immunohistochemistry of C4S also demonstrates less intense staining of C4S in the normal (Figure 3e), compared with the normal tissue. ARSB activity in the normal paired samples was almost 6 mg per mg protein in the normal tissue to 68.8 ± 7.1 ng per mg protein in the malignant tissue, an increase of 2.6 times the baseline (P < 0.0001). sGAG, sulfated glycosaminoglycan.

ranged from 6 to 9. In these samples, the ARSB activity in the malignant prostate tissue was ~50% of the value in the normal tissue. Mean ARSB activity in the normal tissue was 139.3 ± 13.4 nmol per mg protein per h, compared with 76.1 ± 7.1 ng per mg protein per h in the malignant tissue (P < 0.0001, paired t-test, two-tailed; Figure 3c).

As decline in ARSB activity leads to reduced degradation of C4S, the content of C4S and total sulfated GAGs were measured in the normal and malignant prostate samples. C4S was significantly increased in the malignant prostate tissue, compared with the normal tissue (P < 0.0001, paired t-test, two-tailed) (Figure 3d). The mean difference in C4S content between the malignant and normal paired samples was almost 6 ng per mg total protein and accounted for 81% of the increase in the total sulfated GAGs in the malignant tissue compared with the normal tissue. Immunohistochemistry of C4S also demonstrates less intense staining of C4S in the normal (Figure 3e), compared with the malignant prostate tissue (Figure 3f), consistent with the decline in ARSB in the malignant tissue and the resultant increase in C4S. IgG control staining is negative (Figure 3g).

Versican increased in malignant prostate tissue
Versican, an extracellular matrix proteoglycan with chondroitin sulfate attachments, has previously been considered as a biomarker of prostate cancer. Measurements of versican showed significant increases in versican in the malignant prostate tissues,

Decline in total EGFR that co-immunoprecipitated with versican in malignant prostate tissue
Specific versican isoforms and overexpression of specific domains of versican have been reported to impact on cell proliferation and EGFR-EGFR receptor (EGFR) signaling. Particular attention has been focused on the two EGF-like motifs in the G3 domain at the C-terminus. To detect whether there were changes in the EGFR in the malignant vs the normal prostate tissue, the EGFR was quantified by ELISA. In contrast to the increase in C4S that co-immunoprecipitated with versican was measured. In the malignant tissue, C4S increased to 2.6 times the level in the normal prostate tissue (Figure 4b). This increase is consistent with the marked decline in ARSB activity and the overall increase in C4S content in the malignant tissue. Levels of versican and C4S directly correlated in both the normal and malignant prostate tissues, with lower values for the normal tissue and higher values for the malignant tissue (r = 0.94).

To the best of our knowledge, this is the first report of decline in ARSB in prostatic malignancy. The study findings are consistent
with a role for ARSB in the determination of the composition of the tumor microenvironment and suggest that decline in ARSB activity contributes to the malignant phenotype, as we have previously reported in other epithelial tissues.18-20 Study data suggest that ARSB may be useful as a biomarker of prostate cancer. In 82% of paired cases, the biochemical recurrences had lower ARSB immunostaining at the time of prostatectomy. This contrasts with the results for PSA, as PSA values were higher in only 65%. ARSB immunostaining, determined by digitized analysis, was inversely associated with Gleason scores for epithelial and stromal compartments separately and in combination. Also, when ARSB activity was determined in normal and malignant regions of prostatectomies, ARSB activity was significantly less in the malignant compared with the normal tissue. In association with reduced ARSB activity, total sulfated GAGs and C4S content were increased in the malignant prostatic tissue, and the C4S containing matrix proteoglycan versican was also increased. The C4S that co-immunoprecipitated with versican (V0 isoform) was increased in the malignant prostate tissue, whereas the total EGFR that co-immunoprecipitated with versican declined.

Versican was previously reported as a biomarker of prostate malignancy, and increases in versican and in chondroitin sulfate have been identified as predictors of disease progression by other investigators.11,12 This report suggests that the decline in ARSB activity and the associated increase in C4S may impact on versican-associated processes in the stroma and on the stromal–epithelial interactions. By presenting and recruiting molecules to the epithelial cell surface receptors and can modulate signaling pathways, including the EGFR-EGFR pathway, as two EGF-like motifs are located at the C-terminus of the G3 domain of versican.13-14,22-27 The interaction of the versican EGF-like repeats with the epithelial cell EGFR has been reported to affect EGFR signaling and to influence malignant growth and invasiveness.

Other work has shown that exogenous EGF influenced prostate cancer behavior, including the migration of malignant cells to metastatic sites, cell-cycle activation through Cyclin D1 and invasiveness through the urokinase-type plasminogen activator pathway.28-30 Study findings suggest that increased chondroitin sulfate may inhibit the interaction of the versican EGF-like repeats with the endogenous EGFR and may have consequences for the development of the malignant phenotype and/or invasiveness.

Further studies are required to determine the usefulness of ARSB as an effective biomarker of prostate cancer aggressiveness, and the combination of ARSB and PSA may be more informative than either test alone. Analysis of larger databases with outcome data will enable clarification if measurements of ARSB activity or ARSB immunohistochemical scores can help to predict recurrence and severity of disease. Specific cutoffs for ARSB H-scores or for ARSB activity may become associated with recurrence or non-recurrence. Standardization of H-scores may be difficult, as variation in the range of H-scores was present in the two small arrays analyzed in this report. Potentially, microgram quantities of tumor tissue from biopsy samples can be studied to determine ARSB activity and correlate activity with clinical data. As ARSB treatment is used safely and effectively for replacement in Mucopolysaccharidosis VI,31 a therapeutic role for ARSB in prostate cancer may emerge in the future.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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