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[Continued on next page]

(54) Title: METHODS FOR DETECTING PROSTATE CANCER

(57) Abstract: Disclosed are methods of for predicting the severity or likelihood of recurrence of prostate cancer in a subject with prostate cancer by measuring the amount or activity of N-acetylgalactosamine-4-sulfatase in prostate cancer tissue and comparing the amount or activity to that of a control sample, a reduced amount or activity being indicative of the severity or likelihood of recurrence of prostate cancer in the subject.

Lower Arylsulfatase B H-scores predict recurrence in paired prostate cancer cases.

FIG. 1A

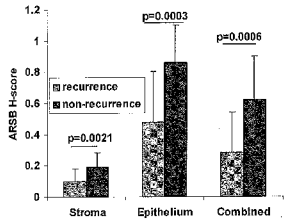
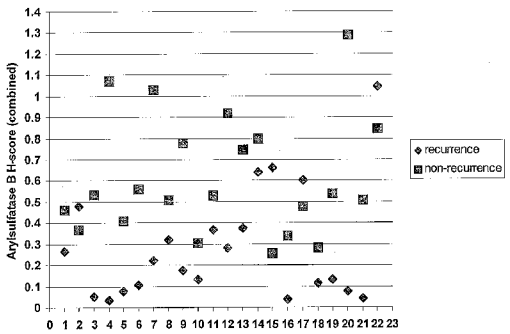


FIG. 1B



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METHODS FOR DETECTING PROSTATE CANCER

CROSS-REFERENCE TO RELATED PATENTS

This application claims the benefit of U.S. Provisional Application No. 61/834,044 filed June 12, 2013, which is incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

The ability to accurately stage cancers, including prostate cancer, is an important factor in the treatment and prognosis of cancer in patients. Cancer staging is used to assess the severity of a cancer, to predict the likelihood of recurrence, and to select treatment.

Currently, staging of prostate cancers is based primarily on prostate biopsy results (including Gleason scores), prostate-specific antigen (PSA) levels, the extent of the tumor, and the presence or absence of lymph node involvement or metastasis.

The predictive value of a diagnostic test is a function of its sensitivity and specificity. Although PSA has been widely used as a biomarker of prostate cancer, the use of PSA to screen for or stage prostate cancer remains controversial because PSA levels are of questionable predictive value.

There is a demand for improved methods of detecting prostate cancer. The present invention satisfies this demand.

SUMMARY OF THE INVENTION

The role of arylsulfatase B (ARSB) as a biomarker of prostatic malignancy was investigated. Measurements of the intensity of ARSB immunostaining by digitized image analysis (H-scores) were calculated for prostate cancers in two small tissue microarrays (TMA) and the associations of H-scores with recurrence vs. non-recurrence and Gleason score were determined. In addition, ARSB enzyme activity, chondroitin-4-sulfate (C4S), and versican were compared between normal and malignant regions from radical prostatectomies performed for prostate cancer. The study data that follow show the usefulness of ARSB as a biomarker and possible tumor suppressor in prostate cancer.

Accordingly, in an embodiment, this invention is a method of predicting the severity or likelihood of recurrence of prostate cancer in a subject comprising i)

measuring the amount of ARSB in a sample of prostate tissue from the subject; and ii) comparing the amount of ARSB in the tissue sample to the amount of ARSB in a control sample, wherein a decrease in the amount of ARSB in the tissue sample relative to the amount of ARSB in the control is indicative of the severity or likelihood of recurrence of prostate cancer in the subject.

In another embodiment, this invention is a method of predicting the severity or recurrence of prostate cancer in a subject comprising i) measuring the ARSB activity in a sample of prostate tissue from the subject; and ii) comparing the ARSB activity in the tissue sample to the ARSB activity in a control sample, wherein a decrease in the ARSB activity in the tissue sample relative to the ARSB activity in the control is indicative of the severity or recurrence of prostate cancer in the subject.

In another embodiment, the amount of ARSB in the prostate tissue samples is determined by ARSB immunostaining.

In another embodiment, the amount of ARSB in the prostate tissue is determined spectrophotometrically.

In another embodiment, H-scores are calculated from the prevalence and intensity of the immunostaining.

In another embodiment, the H-scores are used to predict the severity or likelihood of recurrence of prostate cancer in the subject.

In other embodiments, the prostate tissue from the subject is malignant prostate tissue.

In other embodiments, the prostate tissue from the subject comprises a mixture of normal and malignant prostate tissue.

In other embodiments, the control comprises samples of normal prostate tissues obtained from existing tissue sources, samples of normal prostate tissues obtained from the subject and/or samples of prostate tissue obtained from the subject at an earlier timepoint.

In other embodiments, the control obtained as described above comprises a mixture of normal and malignant prostate tissues.

In other embodiments, the method further comprises measuring the subject's PSA values, wherein a decrease in the amount of ARSB in the subject's prostate tissues and/or the ARSB activity in the subject's prostate tissues relative to the control amount in combination with PSA values is indicative of the severity or likelihood of recurrence of prostate cancer in the subject.

The present invention and its attributes and advantages will be further understood and appreciated with reference to the detailed description below of presently contemplated embodiments, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The preferred embodiments of the invention will be described in conjunction with the appended drawings provided to illustrate and not to the limit the invention.

Fig. 1A is a graph showing mean ARSB H-scores for stroma, epithelium, and combined stroma and epithelium for 22 pairs (recurrent vs. non-recurrent) of prostate cancer cases.

Fig. 1B. is a scattergram showing the ARSB H-scores in the 22 pairs of prostate cancer cases for epithelium and stroma combined.

Fig. 2A is a graph comparing mean ARSB H-scores to Gleason scores 6+7 (n=17) and Gleason 8+9 (n=13) for stroma, epithelium and combined stroma and epithelium for the prostate cancer cores in a National Disease Research Interchange (NDRI) array.

Fig. 2B-E are images of representative prostate cancer cores immunostained for ARSB, with Gleason scores of 6 (Fig. 2B), 7 (Fig. 2C), 8 (Fig. 2D) or 9 (Fig. 2E).

Fig. 2F and Fig. 2G. are images of representative TMA cores stained with ARSB polyclonal rabbit antibody or with control IgG.

Fig. 2H is a plot of ARSB H-scores vs. Gleason scores (7-9) for the NDRI tissue cores.

Fig. 3A is a Western blot using normal (N) and malignant (C) prostate tissue.

Fig. 3B is a densitometric scan of the Western blot of Fig. 3B.

Fig. 3C is a graph showing ARSB activity in normal and malignant prostate tissue.

Fig. 3D is a graph of total sulfated glycosaminoglycans (GAGs) and the chondroitin-4-sulfate (C4S) in normal and malignant prostate tissue.

Fig. 3E and Fig. 3F are images of normal and malignant prostate tissue immunostained using C4S antibody. Fig. 3G is a negative control.

Fig. 4A is a graph of versican protein levels in normal and malignant prostate tissue.

Fig. 4B is a graph showing levels of chondroitin-4-sulfate immunoprecipitated with versican in normal and malignant prostate tissue.

Fig. 5A shows EGFR that co-immunoprecipitated with versican as a function of total EGFR (Fig. 5B) in normal and malignant prostate tissue.

DETAILED DESCRIPTION

Arylsulfatase B (ARSB) or N-acetylgalactosamine-4-sulfatase has been described as a lysosomal enzyme that catalyzes the removal of the 4-sulfate group of N-acetylgalactosamine-4-sulfate at the non-reducing end of C4S, and dermatan sulfate (DS), thereby regulating degradation of C4S and DS. Extra-lysosomal localization of ARSB in epithelial and endothelial membranes has been demonstrated in human cells. Reduced ARSB activity has been found in malignant mammary and colonic epithelial tissues and in metastatic colonic epithelial cells, and the intensity and localization of ARSB immunostaining is reduced in higher grade colonic adenocarcinomas.

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 extracellular matrix proteoglycan with three domains: the G1 domain has hyaluronan attachments that interact with the CD44 cell surface protein; the G2 domain has chondroitin sulfate attachments; and the G3 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, and P-selectin glycoprotein ligand-1 (PSGL-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.

The studies described herein below provide the first evidence that ARSB is reduced in malignant prostate tissue, and that ARSB may serve as a marker for prostate cancer.

Materials and Methods

Cancer tissue arrays and tissue samples

Prostate cancer tissues from three sources were analyzed, including: a cancer tissue microarray (TMA) obtained from the National Disease Research

Interchange (NDRI, Philadelphia, PA), which includes 30 cores with Gleason scores from 6-9; a cancer tissue array from the Cooperative Prostate Cancer Tissue Resource (CPCTR), which includes 22 pairs of cases that vary by biochemical recurrence (increased PSA) vs. non-recurrence after four or more years, matched by age ± 5 y, race, Gleason score matched by sequence and score, treatment (radical prostatectomy), and pathologic TNM stage; and fresh frozen tissues from nine prostatectomies for prostate cancer obtained from the University of Illinois Hospital and Health Sciences System (UIHHSS) Biorepository Core Facility under a protocol approved by the Institutional Review Board and the Cancer Center of UIHHSS. Frozen sections were performed and benign and malignant foci, consisting of epithelium and stroma, were identified by two observers, isolated, dissected out, and frozen for subsequent analysis, as described below.

Arylsulfatase B immunostaining and digitized image analysis

Tissue microarray slides were hydrated using xylene and an alcohol gradient, and rinsed in distilled water. Antigen unmasking was performed with a 10X concentrated retrieval solution by Dako (DakoCytomation, Carpenteria, CA), according to the manufacturer's instructions, then slides were rinsed in phosphate-buffered saline (PBS) for 5 minutes. Endogenous peroxidase activity was blocked by H_2O_2 blocking reagent for 10 minutes at room temperature, then the TMA slides were treated with a protein blocking solution for 10 minutes at room temperature, rinsed and incubated with arylsulfatase B polyclonal rabbit antiserum (Open Biosystems, ThermoFisher Scientific, Huntsville, AL; 1:100) or negative IgG control for 30 minutes at room temperature. Slides were rinsed and then treated with EnVision Plus labeled polymer (DakoCytomation) for 30 minutes at room temperature. DAB Plus (DakoCytomation) was used for 10 minutes 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 20x magnification on an Aperio ScanScope® CS (Aperio Technologies, Inc., Vista, CA) 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 slides were digitally scanned at 20x magnification on an Aperio ScanScope® CS (Aperio Technologies, Inc., Vista, CA) using the Aperio

ImageScope program (v10.0.35.1800) and loaded into Spectrum version 11.1. The TMA Lab® software module was used to segment the TMAs 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, three classes were created: 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 TMAs. 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, Inc.) algorithm was used within the epithelial and stromal compartments to measure brown chromogen 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 x 1 + % moderate x 2 + % strong x 3) / 100.

The H-scores were calculated independently for the NDRI and CPCTR cores. Mean H-score ± standard deviation (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.

Arylsulfatase B activity assay and Western blot

Tissue homogenates were prepared from the normal and malignant foci isolated from the prostatectomies performed at UIC. Arylsulfatase B (ARSB) activity was determined using a fluorometric assay and the exogenous substrate 4-methylumbeliiferyl sulfate, as previously detailed (Bhattacharyya et al., J Steroid Biochem Mol Biol 2007; 103: 20-34, which is incorporated by reference in its entirety). Briefly, 20 µl of tissue homogenate and 80 µl of assay buffer (0.05 M Na acetate buffer, pH 5.6) were combined with 100 µl of substrate (5mM 4-MUS in assay buffer) in wells of a microplate. After incubation for 30 minutes 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). ARSB activity was expressed as nmol/mg protein/hour, based on a standard curve for ARSB activity prepared with known quantities of 4-methylumbilleferyl at pH 5.6. Protein content of the tissue homogenate was determined by total protein assay kit (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL).

Western blot for ARSB was performed using 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, Inc., Danvers, MA) and protease and phosphatase inhibitors (Halt™ Protease and Phosphatase Inhibitor Cocktail, Thermo Scientific, Pittsburgh, PA). Western blot of ARSB was performed on 10% SDS gel with ARSB antibody, as above, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). An ARSB inhibitory peptide with the sequence used to generate the anti-ARSB antibody, was added to three of the wells to verify the specificity of the ARSB band. The sequence of the inhibitory peptide used in the Western blot is RLQFYHKHSVPVYFPAQDPR (SEQ ID NO:1) (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 to β-actin in the malignant and normal samples.

Arylsulfatase B spectrophotometric analysis

ARSB measurements were performed following a standard protocol with the synthetic substrate, *p*-nitrocatechol sulfate. The reaction utilized the substrate 0.1 M *p*-nitrocatechol sulfate (NCS) in H_2O and the buffer 1.0 M Na acetate at pH 6.0 with 20 mM barium acetate at 37°C. Cells were sonicated in 1.00 ml ddH_2O twice for 10 seconds. Assays were run in duplicate on at least three samples, and the difference in measurements of optical density at 30 minutes and 90 minutes was used to calculate enzymatic activity. The linearity of the reaction was verified by performing determinations of activity at multiple time points. Controls from patients with MLS were used as negative controls for some of the experiments. 75 µl of buffer, 75 µl of substrate, 20 µl of cell extract and 130 µl of de-ionized water were combined, and

the reaction was stopped by addition of 0.7 ml of 0.064 N NaOH. Blanks were prepared by adding the substrate after the reaction had been terminated by addition of NaOH. The extent of the reaction was measured by optical density (OD) readings at 515 nm in the Beckman DU65 spectrophotometer. Enzymatic activity was calculated using the constant 0.0112 to solve for activity expressed as nmol/min/mg protein. Protein content of the cell lysate was measured using BCA™ Protein Assay Kit (Pierce).

Measurement of sulfated glycosaminoglycans

Total sulfated glycosaminoglycan (GAG) content in normal and malignant prostate tissues was measured using the substrate 1,9-dimethylmethylene blue (Blyscan™, Biocolor Ltd., Newtownabbey, Northern Ireland), which detects chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin, but does not detect unsulfated glycosaminoglycans or disaccharides. The substrate 1,9-dimethylmethylene blue combines with the sulfate groups of the sulfated GAG and detects the sulfated polysaccharide component of proteoglycans and the protein-free sulfated GAG chains. Tissue lysates were prepared using RIPA buffer (50 mmol/L Tris-HCl containing 0.15 mol/L NaCl, 1% Nonidet P40, 0.5% deoxycholic acid and 0.1% SDS, pH 7.4). Absorbance maximum of 1,9-dimethylmethylene blue was detected at 656 nm (FLUOstar), and sulfated GAG concentration expressed as µg/mg of protein of tissue lysate.

Immunoprecipitation of tissue lysates by chondroitin-4-sulfate antibody

Tissue lysates were prepared using RIPA buffer, as above. Antibody specific to native chondroitin-4-sulfate (C4S; 4D1, Abnova, Littleton, CO) 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/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, Santa Cruz, CA) 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 Blyscan assay, as described above.

Determination of versican by competitive ELISA

Human versican was measured by a competitive ELISA (My BioSource, San Diego, CA), in which color development was inversely proportional to the versican content in the test samples. Standards, ranging from 1 to 25 ng/ml (µg/L), tissue samples, and versican-horseradish peroxidase conjugate were added to wells pre-coated with versican antibody, incubated for 1 hour at 37°C, and washed three times. Color was developed by adding hydrogen peroxide/ tetramethylbenzidine (TMB) substrate. The reaction was stopped by 2N sulfuric acid, and the color was read 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 total EGFR by ELISA

Total human epidermal growth factor receptor (phosphorylated and unphosphorylated EGFR and ErbB1) was measured in the tissue extract using a standardized ELISA (R&D, Minneapolis, MN). 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 (HRP) was added. The bound enzyme activity was determined by chromogenic substrate [(hydrogen peroxide/tetramethylbenzidine (TMB)], and color development due to HRP activity was stopped by 2N sulfuric acid. Intensity of color was measured at 450 nm in a plate reader (BMG), and the values of the samples were extrapolated from a standard curve and normalized using the total tissue protein concentration as measured by protein assay (Pierce).

Measurement of C4S immunoprecipitated with versican

Versican was immunoprecipitated from tissue lysates using versican antibody (V0 isoform; SCBT, Santa Cruz, CA) covalently bound to Dynabeads (Life Technologies, Carlsbad, CA). 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 chondroitin-4-sulfate

Tissue sections were prepared from the frozen normal and malignant prostate tissues and immunostained with chondroitin-4-sulfate (C4S) mouse monoclonal antibody (4D1 clone, SCBT; 1:100). Sections were incubated with primary antibody or IgG negative control overnight at 4°C, then washed, then incubated with secondary antibody which was conjugated with HRP 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 20X magnification. Background color was modified with GIMP Portable software (Portable Apps, New York, NY).

Statistics

Results are expressed as mean ± standard deviation. 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) by either 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 6 pair of normal and malignant biological samples using averages of technical duplicates of each measurement. P-value of less than 0.05 was considered statistically significant. One asterisk represents $p \leq 0.05$, ** represents $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$.

Results

Lower Arylsulfatase B 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 or more years of followup. Arylsulfatase B (ARSB; N-acetylgalactosamine-4-sulfatase) immunostaining of the tissue microarray 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. 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, indicating that PSA is 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.

With reference to Fig. 1A, mean ARSB H-scores were calculated for stroma, epithelium, and combined stroma and epithelium for the 22 pairs of prostate cancer cases that differed by recurrence vs. non-recurrence at 4 or more 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). Fig. 1B shows the ARSB H-scores in the 22 pairs of prostate cancer cases for epithelium and stroma combined. H-scores of less than 0.25 accurately predicted recurrence, and H-scores of greater than 0.70 were associated with non-recurrence in 8 of 9 cases.

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	*7.8*	*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*

NA = not available;
bold = non-recurrence with higher H-score; italics = non-recurrence with lower PSA;
* = neither lower ARSB H-score nor higher PSA predicted recurrence

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) (Fig. 1A). ARSB H-scores less than 0.25 for combined predicted recurrence with 100% specificity, and H-scores greater than 0.70 for combined were highly predictive of non-recurrence (Fig. 1B). Overlap between the scores for

recurrence and non-recurrence was evident between 0.25 and 0.70, and over 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. 11 cores were designated Gleason 6, 6 were Gleason 7, 8 were Gleason 8, and 5 were Gleason 9. Mean ARSB H-scores were compared between Gleason scores 6+7 and Gleason scores 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) (Fig. 2A). Representative images demonstrate greater intensity of ARSB immunostaining for Gleason scores 6 and 7 compared to scores 8 and 9 (Fig. 2B-2E). 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 (Fig. 2F) and negative staining with IgG control (Fig. 2G).

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

Reduced ARSB associated with increased sulfated glycosaminoglycans and chondroitin-4-sulfate in malignant prostatic tissue compared to normal

Western blot demonstrated greater ARSB in the normal than in the malignant prostate tissue (Fig. 3A). The ARSB inhibitory peptide, composed of the epitope to which the antibody was developed, completely blocked the appearance of the ARSB band, demonstrating the specificity of the antibody. B-actin band demonstrated equal loading. Densitometry confirms the visual impression (Fig. 3B) and indicates significant differences between intensity of bands between normal and malignant tissue and inhibition by ARSB inhibitory peptide.

In the paired normal and malignant fresh frozen tissue samples obtained from the UIC Tissue Bank from radical prostatectomies performed as initial treatment for localized prostate cancer, PSA at diagnosis ranged from 4.3 to 25.4 ng/ml (µg/L), and Gleason scores ranged from 6-9. 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/mg protein/h, compared to 76.1 ±

7.1 ng/mg protein/h in the malignant tissue ($p<0.0001$, paired t-test, two-tailed) (Fig. 3C).

Because a decline in ARSB activity leads to reduced degradation of chondroitin 4-sulfate (C4S), the content of C4S and total sulfated glycosaminoglycans (GAGs) were measured in normal and malignant prostate samples. C4S was significantly increased in the malignant prostate tissue, compared to the normal tissue ($p<0.0001$, paired t-test, two-tailed) (Fig. 3D). The mean difference in C4S content between the malignant and normal paired samples was almost 6 µg/mg total protein, and accounted for 81% of the increase in the total sulfated GAGs in the malignant tissue compared to the normal tissue. Immunohistochemistry of C4S also demonstrates less intense staining of C4S staining in the normal (Fig. 3E), compared to the malignant prostate tissue (Fig. 3F), consistent with the decline in ARSB in the malignant tissue and the resultant increase in C4S. IgG control staining is negative (Fig. 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, compared to levels in the normal tissues. The mean value of the increase between the malignant and the normal paired tissues was 119.4 ng/mg protein, an increase of more than 76% over the baseline ($p<0.0001$, paired t-test, two-tailed) (Fig. 4A).

The G2 domain of versican has sites where chondroitin sulfate attaches, and versican isoforms with differences in these attachment sites have been associated with changes in cell proliferation and apoptosis. To determine if the amount of C4S associated with versican differed between the normal and malignant prostate tissue, the 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 (Fig. 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 chondroitin-4-sulfate directly correlated in both the normal and malignant prostate tissues, with lower values for the normal tissue and higher values for the malignant tissue ($r = 0.94$).

Decline in total EGFR that co-immunoprecipitates 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 EGF-EGF receptor (EGFR) signaling. Particular attention has been focused on the two epidermal growth factor (EGF)-like motifs in the G3 domain at the C-terminus. To detect if 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 in the malignant compared to the normal prostate tissue (above), the EGFR that co-immunoprecipitated with versican in the malignant tissue declined to 17.6 ± 1.0% of the value in the normal tissue (Fig. 5A). In contrast, the total EGFR in the malignant tissue increased to over three times the baseline value (Fig. 5B). These results suggest that the increased C4S bound with versican in malignant prostate tissue may impede the binding of the stromal versican EGF-like repeats with epithelial EGFR. The overall increase of EGFR in the malignant tissue suggests that more EGFR may be available for interaction with endogenous EGF, leading to effects on cell proliferation.

Discussion

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. 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, since 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 to normal tissue. In association with reduced ARSB activity, total sulfated glycosaminoglycans and chondroitin-4-sulfate content were increased in the malignant prostatic tissue, and the chondroitin-4-sulfate containing matrix proteoglycan versican was also increased. The chondroitin-4-sulfate 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. This report suggests that the decline in ARSB activity and the associated increase in C4S may impact versican-associated processes in the stroma and on the stromal-epithelial interactions. By presenting and recruiting molecules to the epithelial cell surface, stromal versican can interact with epithelial cell surface receptors and can modulate signaling pathways, including the EGF-EGFR pathway, since two epidermal growth factor (EGF)-like motifs are located at the C-terminus of the G3 domain of versican. 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 prostatic 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 (uPA) pathway. 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 development of the malignant phenotype and/or invasiveness.

It is envisioned that the combination of ARSB and PSA may be more informative than either test alone. Analysis of larger databases with outcome data will provide confirmation that ARSB activity and/or ARSB immunohistochemical scores can help to predict recurrence and severity of disease. Using the teachings disclosed herein, it is well within the ability of one of ordinary skill in the art to identify specific cutoffs for ARSB H-scores or for ARSB activity for recurrence or non-recurrence. Standardization of H-scores may be difficult, since 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 to correlate activity with outcome data. Because ARSB treatment has been used in the treatment of people with mucopolysaccharidosis VI, a condition caused by a deficiency of ARSB, it is envisioned that ARSB may be administered in therapeutic amounts to treat prostate cancer.

While the disclosure is susceptible to various modifications and alternative forms, specific exemplary embodiments of the present invention have been shown

by way of example in the drawings and have been described in detail. It should be understood, however, that there is no intent to limit the disclosure to the particular embodiments disclosed, but on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the scope of the disclosure as defined by the appended claims.

CLAIMS

What is claimed is:

1. A method for predicting the severity or likelihood of recurrence of prostate cancer in a subject comprising

measuring the amount of ARSB in a sample of prostate tissue from the subject; and

comparing the amount of ARSB in the tissue sample to the amount of ARSB in a control sample,

wherein a decrease in the amount of ARSB in the tissue sample relative to the amount of ARSB in the control is indicative of the severity or likelihood of recurrence of prostate cancer in the subject.

2. The method of claim 1 wherein the control comprises samples of prostate tissues obtained from existing tissue sources.

3. The method of claim 1 wherein the control comprises samples of prostate tissues obtained from the subject.

4. The method of claim 1 wherein the control comprises samples of prostate tissue obtained from the subject at an earlier timepoint.

5. The method of claim 1 wherein the sample of prostate tissue from the subject comprises malignant prostate tissue.

6. The method of claim 1 further comprising measuring the subject's PSA values,

wherein a decrease in the amount of ARSB in the subject's prostate tissues relative to the control amount in combination with PSA values is indicative of the severity or lilelihood of recurrence of prostate cancer in the subject.

7. The method of claim 1 wherein the amount of ARSB in the prostate tissue samples is determined by ARSB immunostaining.

8. The method of claim 7 wherein H-scores are calculated from the prevalence and intensity of the immunostaining.

9. The method of claim 8 wherein the H-scores are used to predict the severity or likelihood of recurrence of prostate cancer in the subject.

10. A method of predicting the severity or recurrence of prostate cancer in a subject
comprising
measuring the ARSB activity in a sample of prostate tissue from the subject; and
comparing the ARSB activity in the tissue sample to the ARSB activity in a control sample,
wherein a decrease in the ARSB activity in the tissue sample relative to the ARSB activity in the control is indicative of the severity or likelihood of recurrence of prostate cancer in the subject.

11. The method of claim 10 wherein the control comprises samples of prostate tissues obtained from existing tissue sources.

12. The method of claim 10 wherein the control comprises samples of prostate tissues obtained from the subject.

13. The method of claim 10 wherein the control comprises samples of prostate tissue obtained from the subject at an earlier timepoint.

14. The method of claim 10 wherein the sample of prostate tissue from the subject comprises malignant prostate tissue.

15. The method of claim 10 further comprising measuring the subject's PSA values,
wherein a decrease in ARSB activity in the subject's prostate tissues relative to the control amount in combination with PSA values is indicative of the severity or recurrence of prostate cancer in the subject.

16. The method of claim 10, initiating prostate cancer treatment when ARSB activity is decreased relative to the control, indicating recurrence of prostate cancer.

17. A method of treating a subject with prostate cancer comprising administering to the subject an effective amount of ARSB.

18. A prostate tissue sample immunostained with an ARSB-specific antibody.

19. An image of the sample of claim 18.

20. A digitized image analysis of the sample of claim 18 or the image of claim 19.

Lower Arylsulfatase B H-scores predict recurrence in paired prostate cancer cases.

FIG. 1A

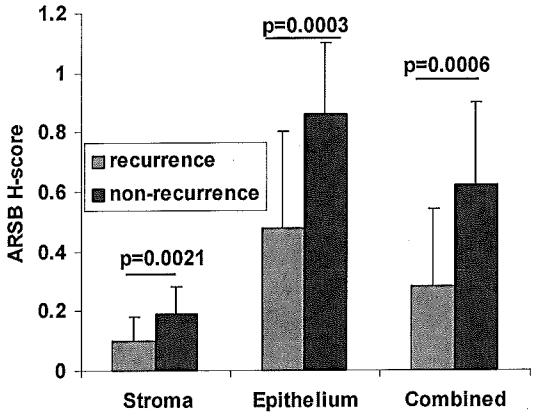
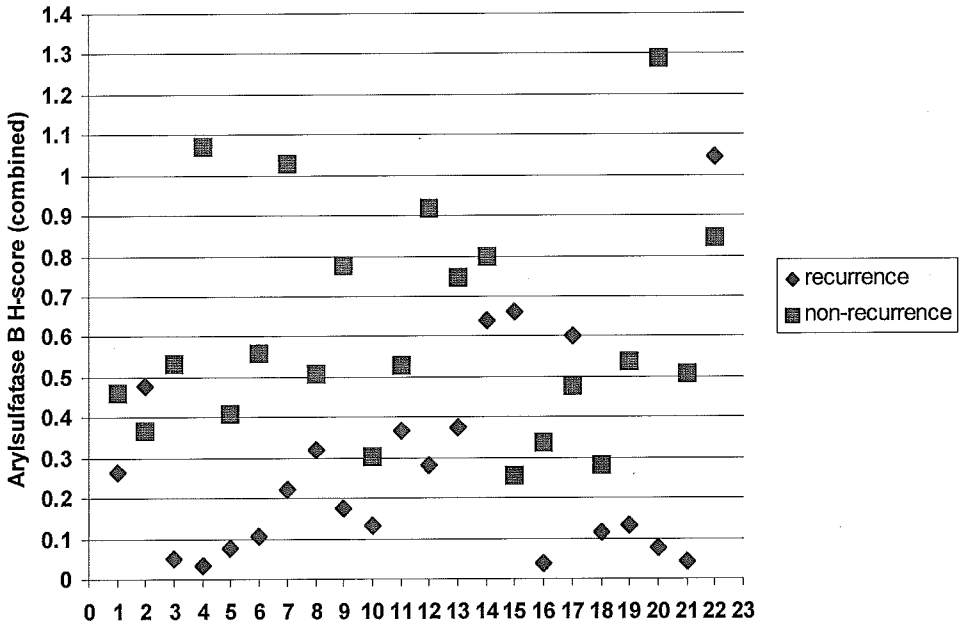


FIG. 1B



Inverse Correlation between ARSB immunostaining and Gleason score

FIG. 2A

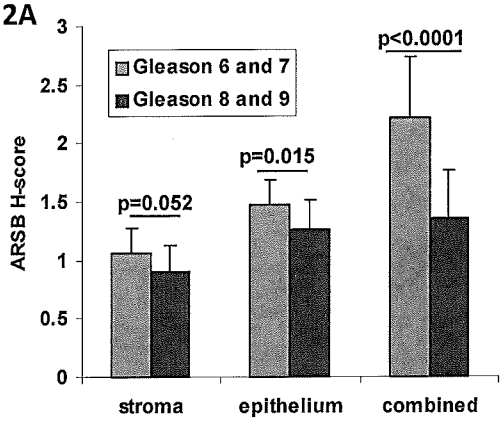


FIG. 2B-E

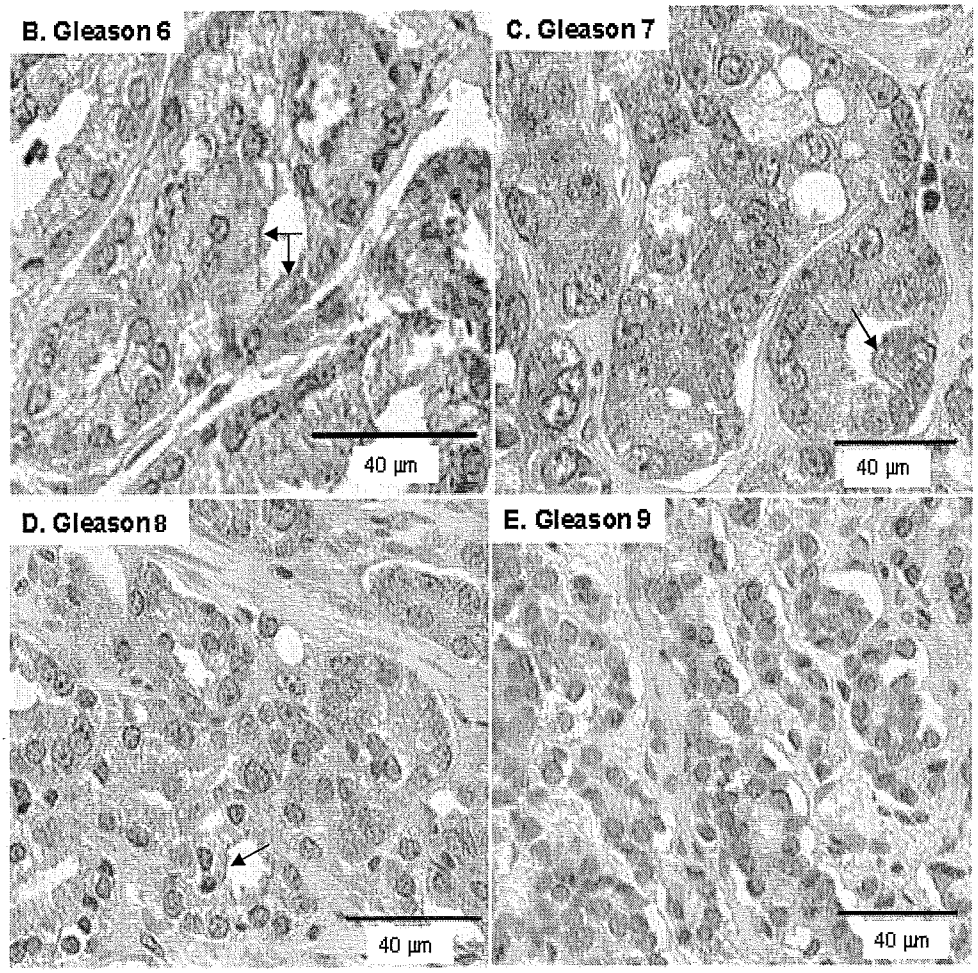


FIG. 2F Positive ARSB control

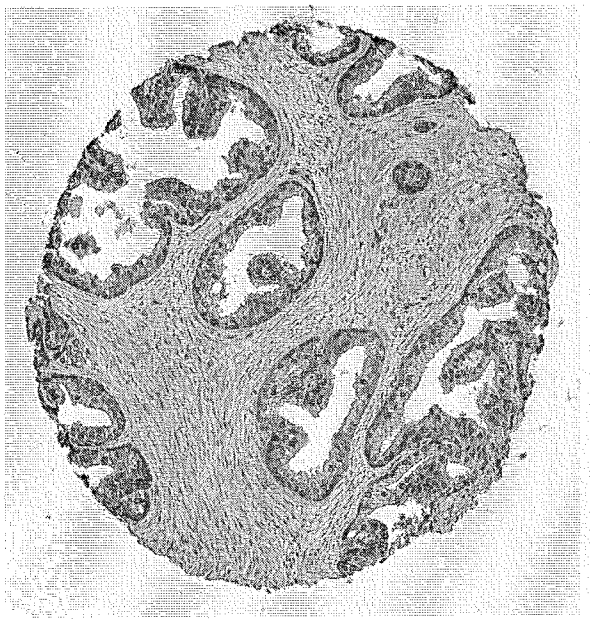
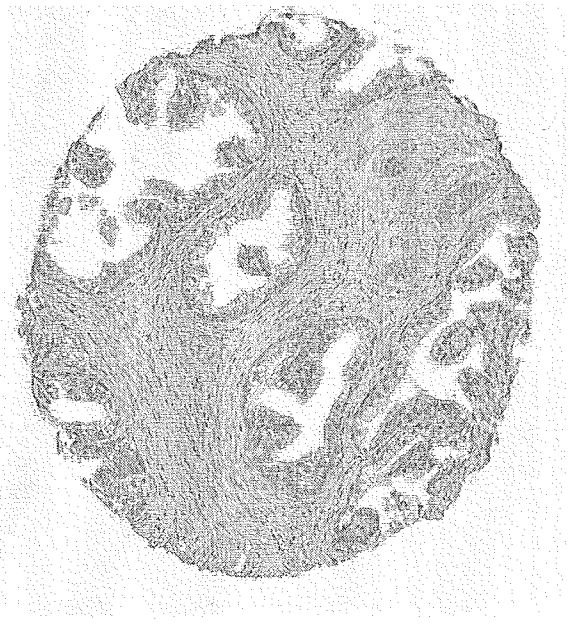


FIG. 2G Negative ARSB control



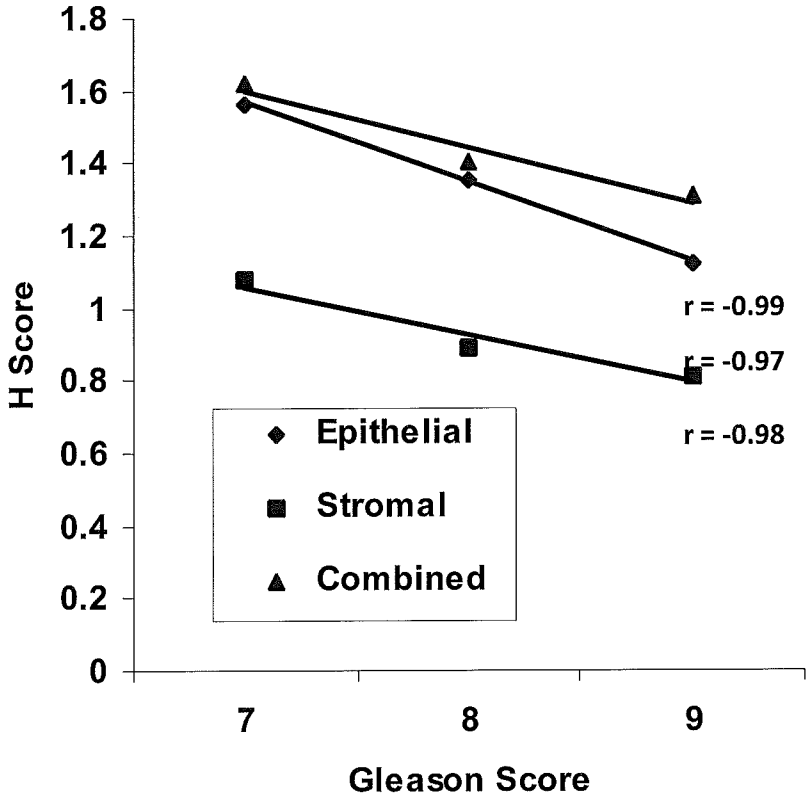


FIG. 2H

Reduced ARSB associated with increased sulfated glycosaminoglycans and chondroitin-4-sulfate in malignant prostatic tissue compared to normal

FIG. 3A

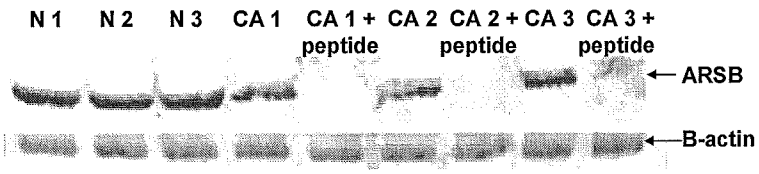


FIG. 3B

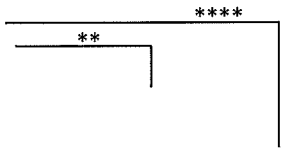


FIG. 3C

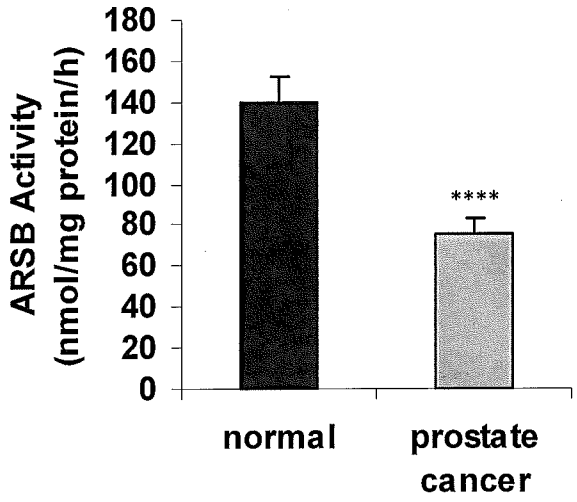
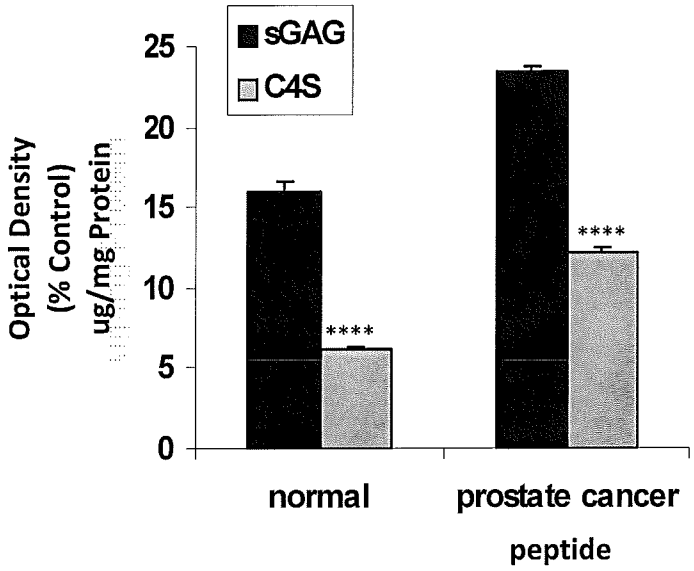


FIG. 3D


sGAG
C4S
25
20
15
10
5
0
Optical Density
(% Control)
ug/mg Protein
****
****
normal
prostate cancer
peptide

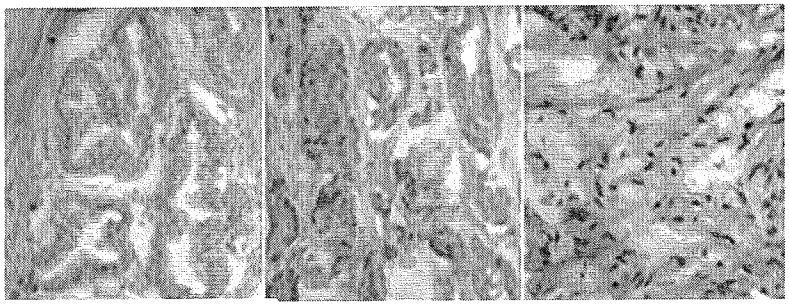


FIG. 3E FIG. 3F FIG. 3G

Normal Prostate Cancer Negative control

Increase in versican and in C4S immunoprecipitated with versican in malignant prostatic tissue.

FIG. 4A

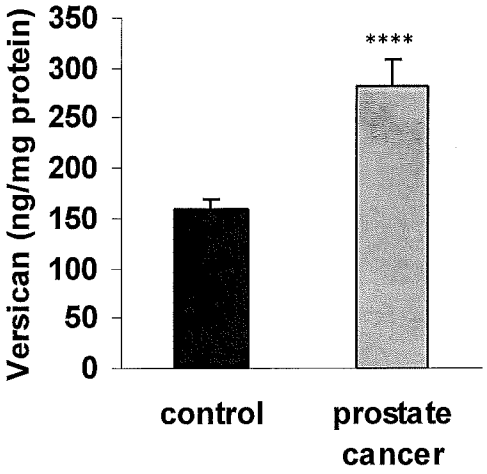
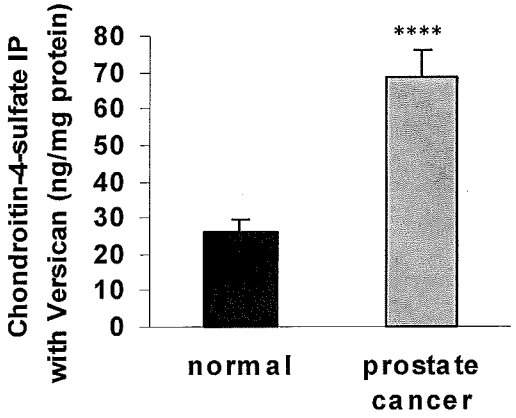


FIG. 4B



Decline in total EGFR that co-immunoprecipitates with versican in malignant prostate tissue.

FIG. 5A

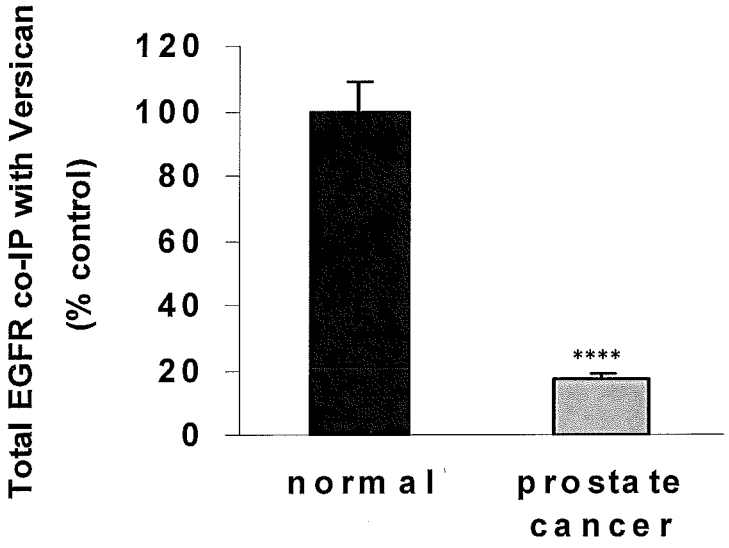
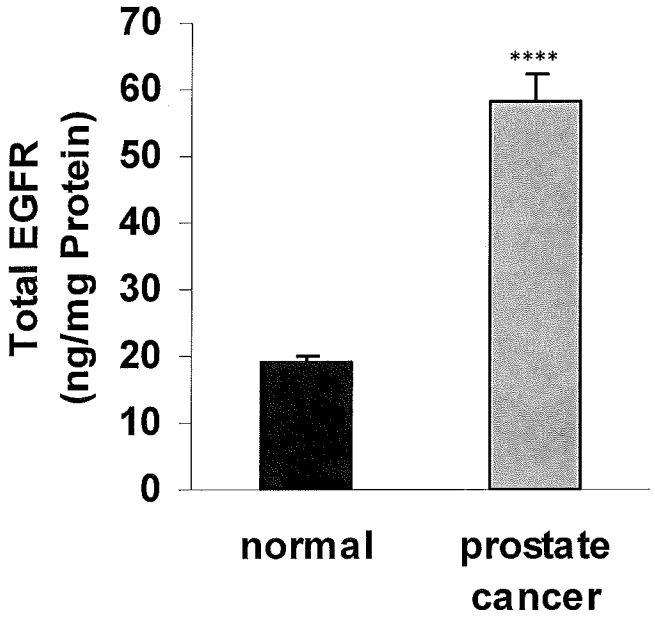


FIG. 5B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/42074

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 9/16; A61K 38/46, 48/00; C07K 16/18, 16/40 (2014.01)
CPC - C12N 9/16; A61K 38/46; C07K 16/18, 16/40
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12Q 1/34, 1/44, 1/68; C12N 9/00, 9/16; A61K 38/00, 38/46, 48/00, 39/395; C07K 16/00, 16/18, 16/40 (2014.01)
CPC: C12Q 1/6883, 1/6886, 1/6827; C12N 9/00, 9/16, 9/93; A61K 38/00, 38/46, 39/00; USPC: 435/7.19, 7.4, 183, 195, 196

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Espacenet; Google; Google Scholar; PubMed; 'arysulfatase B,' 'ARSB,' 'N-acetylgalactosamine-4-sulfatase,' prostate, treat, administer, 'patient/subject,' chondroitinase, chondroitinsulfatase, antibody, stain

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	FEFERMAN, L et al. Reduced Arylsulfatase B Enzyme Activity And Immunostaining In Malignant Prostate Tissue. University of Illinois Cancer Center 2012 Cancer Research Forum. 06 March 2012; Chicago, IL; Carcinogenesis and Chemoprevention: abstract nr 11; page 7, column 3, paragraph 4; page 7, paragraph 5; Materials and Methods, Results and Conclusion.	1-5, 7-14, 16, 18-20 ----- 6, 15
Y	RICCIARDELLI, C et al. Elevated Stromal Chondroitin Sulfate Glycosaminoglycan Predicts Progression In Early-Stage Prostate Cancer. Clin. Cancer Res. June 1997, Vol. 3, No. 6: pages 983-992; abstract; page 983, column 2, paragraph 3; page 984, column 2, paragraph 2; Table 1; page 986, column 1, paragraph 3; figure 3; page 987, column 2, paragraph 2; page 990, column 2, paragraph 3.	6, 15
Y	US 2006/0078959 A1 (PRABHAKAR, V et al.) April 13, 2006; abstract; paragraphs [0003], [0042], [0175], [0176], [0183], [0185]	17
Y	US 2012/0189605 A1 (KOPPAKA, V et al.) July 26, 2012; paragraphs [0015], [0029], [0039], [0041], [0042]	17

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 September 2014 (30.09.2014)	07 NOV 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774