HHS Public Access

Author manuscript

Prostate. Author manuscript; available in PMC 2018 March 21.

Published in final edited form as:

Prostate. 2014 July ; 74(10): 1059–1067. doi:10.1002/pros.22826.

Increased Expression of *GCNT1* is Associated With Altered *O*-glycosylation of PSA, PAP, and MUC1 in Human Prostate Cancers

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Abstract

BACKGROUND—Protein glycosylation is a common posttranslational modification and glycan structural changes have been observed in several malignancies including prostate cancer. We hypothesized that altered glycosylation could be related to differences in gene expression levels of glycoprotein synthetic enzymes between normal and malignant prostate tissues.

METHODS—We interrogated prostate cancer gene expression data for reproducible changes in expression of glycoprotein synthetic enzymes. Over-expression of *GCNT1* was validated in prostate samples using RT-PCR. ELISA was used to measure core 2 *O*-linked glycan sialyl Lewis X (sLe^x) of prostate specific antigen (PSA), Mucin1 (MUC1), and prostatic acidic phosphatase (PAP) proteins.

RESULTS—A key glycosyltransferase, *GCNT1*, was consistently over-expressed in several prostate cancer gene expression datasets. RT-PCR confirmed increased transcript levels in cancer samples compared to normal prostate tissue in fresh-frozen prostate tissue samples. ELISA using PSA, PAP, and MUC1 capture antibodies and a specific core 2 *O*-linked sLe^x detection antibody demonstrated elevation of this glycan structure in cancer compared to normal tissues for MUC1 ($P = 0.01$), PSA ($P = 0.03$) and near significant differences in PAP sLe^x levels ($P = 0.06$). MUC1, PSA and PAP protein levels alone were not significantly different between paired normal and malignant prostate samples.

CONCLUSIONS—*GCNT1* is over-expressed in prostate cancer and is associated with higher levels of core 2 *O*-sLe^x in PSA, PAP and MUC1 proteins. Alterations of *O*-linked glycosylation could be important in prostate cancer biology and could provide a new avenue for development of prostate cancer specific glycoprotein biomarkers.

Keywords

prostate cancer; GCNT1; O-glycosylation; sialyl Lewis X

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The authors declare that they have no conflicts of interest.

INTRODUCTION

Most serum proteins are glycoproteins including prostate specific antigen (PSA) [1]. A growing body of evidence suggests that protein glycosylation is altered significantly in a variety of malignancies. Altered glycosylation of MUC1, for example, has been demonstrated to occur in pancreatic neoplasms and is currently being developed as a candidate early detection biomarker for testing in pancreatic cyst fluid [2–5]. Profiling using MALDI-TOF-MS or LC-MS technologies have shown significant differences between the branching structure of PSA glycoproteins in normal seminal plasma, with many sialylated biantennary complex oligosaccharide structures, and the prostate cancer cell line LNCaP, which contains a mixture of neutral biantennary and triantennary complex forms of oligosaccharides [6,7]. Serum PSA from patients with prostate cancer has higher levels of α2,3-linked sialic acid compared to that from seminal fluid or serum from patients with documented benign prostatic hyperplasia (BPH) [8–10]. A recent report demonstrated increased core fucosylation in prostate cancer serum glycans compared to patients with BPH [11].

Continued improvements in mass spectrometry and hybrid antibody lectin microarrays will allow unbiased detection of candidate glycoprotein biomarkers that could be useful for cancer detection in a variety of malignancies. However, limitations in sensitivity of available technologies hampers development of new biomarkers, particularly since cancer-specific glycoproteins would be expected to be found at relatively low levels in early stage malignancies, particularly when diluted in the serum of an average sized patient [12]. Since polysaccharides are synthesized by a wide array of unique enzymes, we reasoned that expression levels of those enzymes in the cancer tissue samples could provide insights into candidate glycoprotein alterations in human prostate cancer. We therefore interrogated existing prostate cancer gene expression datasets for differential expression of glycoprotein synthetic enzymes between normal and malignant prostate tissues. We identified *GCNT1* as significantly up-regulated in prostate cancer. The GCNT1 glycosyltransferase catalyzes the transfer of GlcNAc from its UDP carrier to form the β1,6-linkage (core 2 branch) at GalNAc of core 1 *O*-linked glycans. To demonstrate activity by this core 2 *O*-glycan branching glycosyltransferase, we assessed the expression levels of the core 2 *O*-sLeX. This functional glycan structure is recognized by members of the selectin family of adhesion molecules [13–17], which facilitate leukocyte migration and cancer cell metastasis [18].

MATERIALS AND METHODS

Sample Collection

All prostate tissue samples used in this study were obtained from patients undergoing radical prostatectomy at Stanford University Hospital (Table I). These patients were consented for the use of their tissues under an Institutional Review Board-approved protocol. Frozen sections of these prostate samples were evaluated by a genitourinary pathologist. The tumor and adjacent normal areas were marked and contaminating tissues were trimmed away from the block as described previously [19]. Tumor samples in which at least 90% of the

epithelial cells were cancerous and adjacent normal samples having no observable tumor epithelium were selected for extraction of total protein, DNA, and RNA.

Reagents

Monoclonal antibodies against PSA, PAP, and MUC1 were purchased from Meridian (Saco, ME), Cosmo Bio Co (Tokyo, Japan) and Abnova (Taipei City, Taiwan) respectively. For detection of the core 2 *O*-linked glycan sialyl Lewis X (sLe^x), we used the CHO131-monoclonal antibody which has been characterized previously [17]. Tissue lysis buffer and Halt Protease Inhibitor Cocktail were purchased from Thermo Scientific (Rockford, IL). Antibody coating buffer was purchased from Immunochemistry Tech (Bloomington, MN). The PSA standard used in this study was prepared previously in the Department of Urology at Stanford University [20]. The PAP standard and MUC1 recombinant protein were purchased from Mybiosource (San Diego, CA) and Abnova (Taipei City, Taiwan) respectively. HRP-streptavidin and peroxidase substrates were purchased from R&D (Minneapolis, MN).

Gene Expression Datasets and Analysis

Gene expression data was analyzed using a dataset containing 81 prostate cancer samples and 52 normal prostate tissue samples harvested from radical prostatectomy specimens [19]. Transcript levels were normalized across the microarrays and extracted for the following glycosylation genes: O-Linked Glycosylation: *A4GNT*, *B3GALTL*, *B3GNT8*, *B4GALT5*, *C1GALT1*, *C1GALT1C1*, *GALNT1*, *GALNT10*, *GALNT11*, *GALNT12*, *GALNT13*, *GALNT14*, *GALNT2*, *GALNT3*, *GALNT4*, *GALNT6*, *GALNT7*, *GALNT8*, *GALNT9*, *GALNTL1*, *GALNTL5*, *GALNTL6*, *GCNT1*, *GCNT3*, *GCNT4*, *OGT*, *POFUT1*, *POFUT2*, *POMGNT1*, *POMT1*, *POMT2*, *ST3GAL1*, *ST3GAL2*, *ST6GALNAC1*, *ST8SIA3*, *ST8SIA6*. N-Linked Glycosylation: *AGA*, *B3GNT2*, *B3GNT3*, *B3GNT8*, *B4GALT1*, *B4GALT2*, *B4GALT3*, *EDEM1*, *EDEM2*, *EDEM3*, *FUCA1*, *FUCA2*, *FUT11*, *FUT8*, *GANAB*, *GLB1*, *GNPTAB*, *GNPTG*, *HEXA*, *HEXB*, *MAN1A1*, *MAN1A2*, *MAN1B1*, *MAN1C1*, *MAN2A1*, *MAN2A2*, *MAN2B1*, *MANBA*, *MGAT1*, *MGAT2*, *MGAT3*, *MGAT4A*, *MGAT4B*, *MGAT4C*, *MGAT5*, *MGAT5B*, *MOGS*, *NAGPA*, *NEU1*, *NEU2*, *NEU3*, *NEU4*, *PRKCSH*, *ST6GAL1*, *ST8SIA2*, *ST8SIA3*, *ST8SIA4*, *ST8SIA6*, *UGGT1*, *UGGT2*. Relative expression levels between the prostate cancer and normal tissues were compared using the two-class significance analysis of microarray (SAM) test [21].

For the datasets from LaPointe et al. [22] and Gulzar et al. [19], data from each microarray were mean centered across the array prior to data extraction. To look at relative levels of expression between adjacent normal and malignant tissues, expression levels were mean centered across experiments. For experiments from Stamey et al. [23], Singh et al. [24], Taylor et al. [25], and Glinsky et al. [26] and Sboner et al. [27], raw expression levels were normalized to the median array intensity. For *GCNT1*, absolute expression levels were plotted and expression levels compared using the Student's *t*-test.

TaqMan Gene Expression Assay

RNA expression levels of *GCNT1* were measured in four benign adjacent and 15 prostate tumor samples using the TaqMan Gene Expression Assay. We used Applied Biosystems

inventoried assays with FAM/MGD labeled probes (Hs01922706_s1) and the Human *B2M* (beta-2-microglobulin) as an endogenous control. Total RNA (1 μg) was reversed transcribed to cDNA and assayed in triplicate using the Stratagene Mx3005P QPCR System in accord with manufacturer's protocols. Using MxPro qPCR software, the average CT and delta-CT were calculated for *GCNT1* and normalized between samples by integrating the average CT value from the *B2M* to obtain the delta-delta-CT.

Protein Extraction and ELISA Assay

Prostate tissues (approximately 100 mg each) were homogenized in 400 μl of lysis buffer containing protease inhibitors at 4°C for 2 min. The tissue lysates were centrifuged at 10,000 × g in 4°C for 5 min and supernatants containing total proteins were collected, aliquoted and stored at −80°C until use. Antibodies (100 μl) against PSA (M66276M, Meridian), PAP (Hyb-7412 Cosmo Bio) and MUC1 (H00004582-AP41, Abnova) were immobilized on ELISA plates at 4°C overnight. The ELISA plates were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBST) and then blocked with 1% BSA in PBS buffer, pH 7.4 for 90 min. Total protein extract from paired normal and prostate tumor tissues were added to the appropriate wells and incubated at room temperature for 60 min. The plates were washed three times with PBST followed by addition of 100 μl (2 μg/ml) biotinylated anti-sLe^x detection antibody. In parallel experiments, total PSA, PAP, and MUC1 proteins were also measured with 100 μl (0.5 μg/ml) detection antibodies against PSA (Meridian *M86506M-biotin*), PAP (Cosmo Bio *Hyb-7432-biotin*), and MUC1 (Abnova H00004582-AP41). These proteins were detected by streptavidin-HRP based detection method according to manufacturer's instructions (R&D Biosystems-USA). The levels of sLe^x glycoprotein signals were normalized to tissue PSA, PAP, and MUC1 protein levels. The normalized data for normal and malignant tissues was compared using a paired *t*-test.

RESULTS

We interrogated a gene expression dataset of 81 tumors and 52 adjacent normal prostate tissue samples harvested at radical prostatectomy for transcript levels of 84 glycosylation linked enzymes [19]. We used a two class SAM to assess for differences in levels of gene expression between normal and tumor samples. Of the 84 transcripts, 25 were significantly up-regulated and 7 were significantly down-regulated. However, only 7 of these transcripts showed expression differences of 1.5-fold or more: *GCNT1* (2.5-fold), *GALNT7* (2.1-fold), *ST6GALNAC1* (1.8-fold), *GALNT3* (1.7-fold), *B4GALT3* (1.6-fold), *MOGS* (1.6-fold), and *EDEM3* (1.5-fold). Notably, *GCNT1*, *GALNT7*, *GALNT3*, and *GALNT1*, all of which catalyze synthesis of *O*-linked glycoproteins, were expressed at significantly higher levels in malignant compared to normal prostate tissue samples ($P < 0.001$) (Fig. 1A). Of these transcripts, *GCNT1* was the most differentially expressed between normal and malignant prostate tissues and we focused our analysis on this transcript. GCNT1 catalyzes the transfer of GlcNAc from its UDP carrier to form the β1,6-linkage to the α-GalNAc of core 1 *O*-linked glycan that is bound to Ser/Thr residues of polypeptides to form the *O*-glycan core 2 branch (Fig. 1B).

We validated the gene expression data with quantitative PCR for *GCNT1* on a set of normal and malignant prostate cancer samples. In agreement with the microarray data, *GCNT1* transcript levels were increased in the cancer samples compared to those in normal prostate tissues (Fig. 2). Together these data strongly suggest that *GCNT1* is over-expressed in prostate cancer compared to normal prostate tissues.

We further investigated expression levels of *GCNT1* in other publicly available datasets that had data from normal and malignant prostate tissues. *GCNT1* transcript levels were significantly elevated ($P < 0.001$) in malignant tissues compared to normal prostate tissues datasets from LaPointe et al. [22] and Singh et al. [24] (Fig. 3). To better understand how altered levels of *GCNT1* varied across the spectrum of normal prostate tissue, prostate dysplasia, low grade cancer and high grade cancer, we interrogated gene expression levels in a third independent set of carefully dissected prostate tissues samples generated by Stamey et al. [23]. *GCNT1* levels were higher in prostate cancer samples and dysplasia compared to benign prostatic hyperplasia (Fig. 4). *GCNT1* transcript levels in cancers with pure Gleason pattern 3 did not differ significantly from those with pure Gleason pattern 4 (Fig. 4). However, there appeared to be a slight trend toward higher expression in pattern 4 compared to pattern 3 cancer as has been reported previously [28].

Given this trend, we assessed whether *GCNT1* transcript levels predicted clinical outcome in available gene expression datasets with associated clinical follow-up. *CGNT1* expression levels appeared normally distributed across tumor samples, therefore samples were divided at the median into high and low expressing tumors. Tumors expressing higher *GCNT1* transcript levels were associated with a significantly increased risk of biochemical recurrence in the Glinsky et al. [26] dataset ($P < 0.001$, Log rank test) while *GCNT1* gene expression levels were not associated with biochemical recurrence in data from Gulzar et al. [19] ($P = 0.11$) and Taylor et al. [25] ($P = 0.22$), nor were expression levels associated with prostate cancer death in Sboner et al. [27] ($P = 0.08$). Although *GCNT1* transcript levels did not reach significance, it is notable that lower *GCNT1* transcript levels trended with increased post-surgical biochemical recurrence in the Gulzar dataset [19] and with prostate cancer specific mortality in the Sboner dataset [27]. This is in contrast to the Glinsky dataset and previous reports [28], where higher transcript levels were associated with biochemical recurrence and potentially calls into question the association between *GCNT1* levels and prostate cancer aggressiveness.

Since GCNT1 and other *O*-glycoprotein synthetic enzymes were significantly over-expressed in cancer, we next examined the glycosyltransferase's functional activity to determine whether this occurred at higher levels in malignant compared to normal prostate tissues. We selected three proteins to interrogate for glycosylation changes in prostate tissues: two proteins specifically expressed in the prostate: prostate specific antigen (PSA) and prostatic acid phosphatase (PAP), as well as Mucin1 (MUC1), a glycoprotein we have identified as over-expressed in aggressive prostate cancers and with documented glycosylation changes observed in other cancer types [2–5,22].

Total protein was extracted from 10 paired normal and malignant prostate tissue samples harvested fresh from radical prostatectomy specimens (Table I). We developed sandwich

ELISA immunoassays for total PSA, PAP, and MUC1 protein levels, and a parallel assay to measure levels of the *O*-glycan core 2 branch sLe^x structure on PSA, PAP and MUC1 using a detection antibody (CHO-131) specific for the capping structure sLe^x on core 2 branched *O*-glycans. CHO-131 does not recognize the structurally similar sLe^a glycan branch structure [17] Total PSA, PAP, and MUC1 protein levels did not differ significantly between normal and malignant prostate tissues ($P = 0.14$, $P = 0.15$, $P = 0.86$, respectively) (Fig. 5A–C). However, levels of core 2 *O*-linked sLe^x modified PSA and MUC1 (normalized by total PSA or MUC1 levels in each sample) were significantly higher in the cancer tissues compared to the paired normal prostate tissues (Fig. 5D and E; PSA $P = 0.03$; MUC1 $P = 0.01$). Similarly, total PAP levels did not differ between normal and malignant prostate tissues, while normalized PAP core 2 *O*-sLe^x levels were found at higher levels in the cancer tissues compared to the paired normal prostate tissues that approached statistical significance ($P = 0.06$) (Fig. 5F).

DISCUSSION

By interrogating a set of transcripts encoding for glycoprotein synthetic enzymes, we were able to take advantage of large existing prostate cancer gene expression data sets to identify *GCNT1*, as well as other enzymes involved in O-linked glycosylation, as being transcriptionally up-regulated in malignant compared to normal tissues. This finding was consistent across several datasets encompassing several hundred specimens, showing that up-regulation of *GCNT1* is a common feature in prostate cancer. Increased expression occurred in pre-cancerous dysplasia, low grade and high-grade prostate cancer, implying it is an early change in prostate carcinogenesis. In addition, using sandwich ELISA assays, we were able to document increased *O*-linked glycosylation with sialyl Lewis X in PSA, MUC1, and PAP proteins in malignant prostate tissues compared to adjacent normal prostate tissues. The finding of altered *O*-linked glycosylation in all three proteins investigated suggests that this change could be pervasive across many glycoproteins in prostate cancer.

Altered protein glycosylation has been studied in target proteins in prostate cancer previously, although most work has centered on *N*-linked glycosylation changes. PSA is one of the best-studied proteins and has been used as a standard for comparison of different methods of assessing *N*-linked glycoprotein structure [29]. PSA derived from cancer appears to have a greater number of *N*-linked branch structures compared to PSA from benign prostate tissues [6,7] However, far less has been reported regarding *O*-linked glycoproteins in prostate cancer. GCNT1 protein levels assessed by immunohistochemistry have been reported previously to be up-regulated in 45 of 69 prostate cancer cases, and higher expression was associated with more aggressive disease [28]. Stable expression of GCNT1 in LNCaP cells resulted in larger tumor growth in an orthotopic mouse model as well as increased adherence of the cells to collagen IV, although GCNT1 expression did not alter growth kinetics in vitro [28]. We did not find consistent association of *GCNT1* transcript levels with clinical outcome in four large datasets, although it is possible that protein levels do not correlate directly with transcript levels. Regardless, the finding of increased GCNT1 protein expression in human prostate cancers agrees with our finding of up-regulation of transcript levels of *GCNT1* and other *O*-linked core 2 synthetic enzymes, as well as our finding of increased core 2 *O*-sLe^x in PSA, MUC1, and PAP in malignant compared to

normal prostate tissues. Furthermore, our findings are consistent with previous reports of increased levels of α2-3-linked sialic acid on serum glycoproteins in patients with prostate cancer compared to men with BPH [11].

Cancer-associated alterations of *O*-linked glycosylation, particularly of core 2 *O*-sLe^x, have been observed in a variety of contexts and could influence prostate carcinogenesis through several different mechanisms. For example, core 2 *O*-linked glycosylation of MUC1 on the surface of prostate cancer cells allows them to evade immune destruction by NK cells [30]. Similarly immune cloaking has been observed in other cancer types and core 2 *O*-linked glycosylation of MUC1 also has been shown to block antigen presentation to CD8+ T cells [31]. Aberrant *O*-linked glycosylation of the extracellular domain of MUC1 also has been shown to mediate up regulation of b-catenin and ERK1/2 cell signaling pathways through the MUC1 cytoplasmic tail region [32,33]). In addition, *O*-glycosylation of oncofetal fibronectin has been reported to drive the epithelial to mesenchymal transition (EMT) induced by transforming growth factor-β (TGF-β) in prostate cancer cells [34], and abundant evidence implicates the TGF-β signaling pathway in prostate cancer genesis and progression [35]. Furthermore, a growing body of evidence suggests that core 2 *O*-sLe^x expression on the cell surface of circulating tumor cells (CTCs) can facilitate the interaction of circulating tumor cells and endothelial selectins and integrins, which could facilitate CTC tethering, rolling motion and eventual extravasation of cancer cells [26,36]. Since we observed core 2 *O*-sLe^x modifications on all three proteins we tested, it is plausible that other proteins in prostate cancer cells show similar changes, meaning that alterations in *O*-glycosylation could act through several mechanisms to facilitate prostate cancer cell growth.

Altered *O*-glycosylation is being explored broadly in cancer detection and treatment, and could be applied to prostate cancer management. For example, several platforms have been developed using lectins or core 2 *O*-sLe^x specific antibodies to detect *O*-glycosylation changes in tissues, serum and plasma samples of patients in pancreatic and colon neoplasms that could be developed as detection biomarkers [37]. In pancreatic cancer, knock-down of the *O*-glycosylation synthetic enzyme GalNAc-T3 has been shown to attenuate growth and induce apoptosis, suggesting that this enzyme activity could be targeted therapeutically [38]. In addition, *O*-linked glycosylation changes in cancers have been reported to be the targets of immunotherapy and to influence immune activity [31]. Since we have found evidence for pervasive changes in *O*-glycosylation in prostate cancer, it is possible that these strategies could be used to improve specificity of prostate cancer detection or provide new therapeutic avenues including improved immunotherapy.

CONCLUSIONS

By leveraging gene expression data in human prostate samples, we have identified increased expression of *GCNT1* and other *O*-glycoprotein synthetic enzymes as a relatively common event in prostate cancer. In addition, we document that these changes in expression appear to have functional consequences based on our finding of increased levels of core 2 *O*-sLe^x modifications in PSA, MUC1 and PAP in human prostate cancers compared to paired normal prostate tissues. *O*-linked glycosylation changes might be found in additional

proteins in prostate cancer and should be explored for the biological effects and therapeutic potential, and as a possible source of cancer-specific biomarkers.

Acknowledgments

Grant sponsor: Department of Defense; Grant number: W81XWH-11-1-0380; Grant sponsor: NIH, Early Detection Research Network; Grant number: 1U01CA152737-01.

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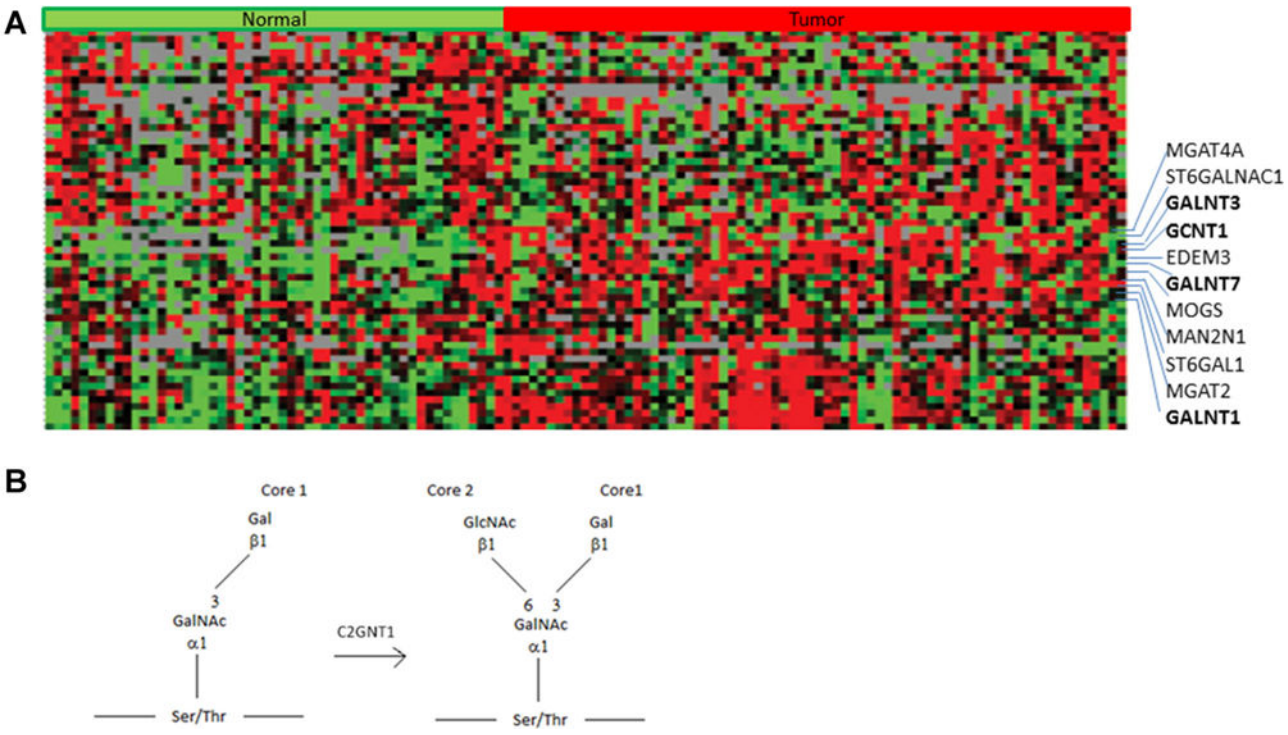

Fig. 1.

A:Two-class SAM analysis comparing 56 benign adjacent normal and 81 prostate cancer samples. Listed are transcripts differentially expressed between the two groups with a False Detection Rate (FDR) < 0.05. Each tumor sample is represented in a column and individual transcripts are displayed in rows. Red indicates relative increased expression level of transcripts relative to the median level across the samples, whereas green represents relative decrease in expression levels, and the degree of color saturation corresponds to the degree of change. **B**:*GCNT1* geneproductb-1,6-N-acetylglucosaminyltransferase-1 (C2GNT1) catalyzes formation of the core 2 branch in O-linked glycans.

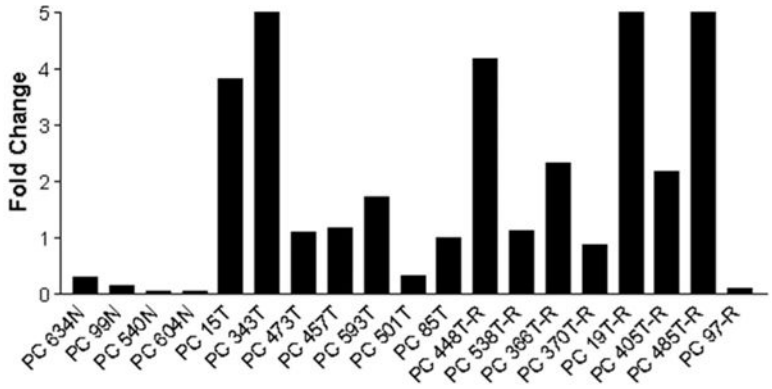

Fig. 2.

Normalized *GCNT1* expression levels obtained by TaqMan RT-PCR gene expression assay (N, normal; T, Tumor; R, biochemical recurrence following surgery).

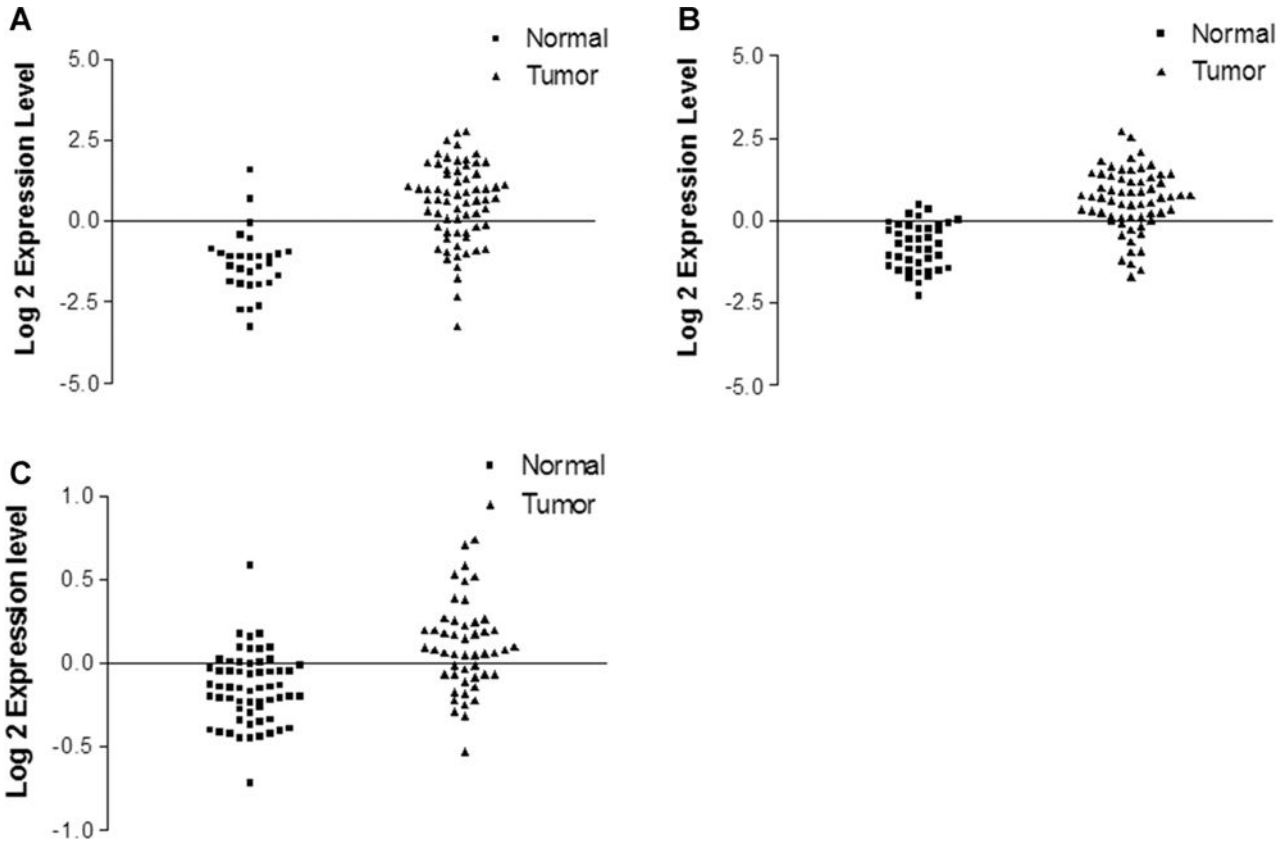

Fig. 3.

Transcript levels of *GCNT1* in normal and malignant tissues from human prostate tissue gene expression datasets: A) Gulzar et al. (2012), B) LaPointe et al. (2004),C) Singh et al. (2001). Mean-centered normalized log2 fluorescence ratios for *GCNT1* from normal and prostate cancer samples are shown.

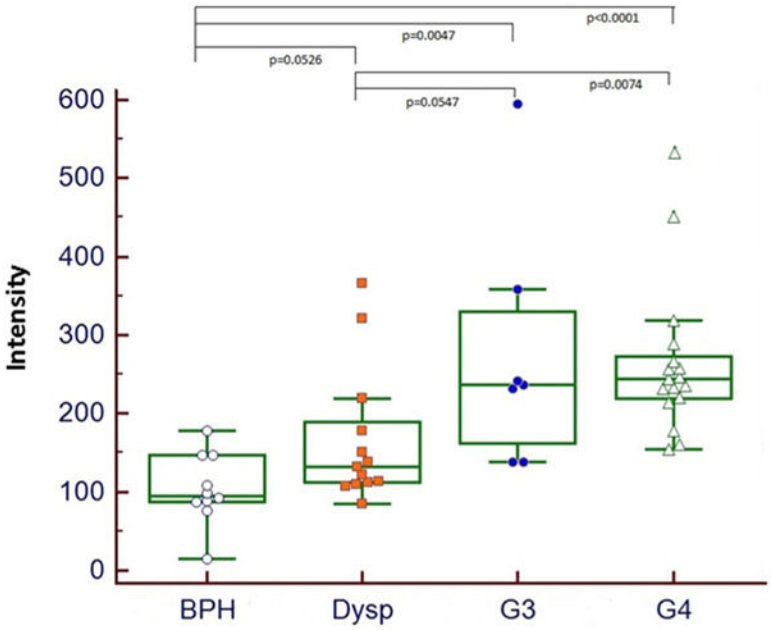

Fig. 4.

Comparison of *GCNT1* transcripts levels in microdissected tissues from human BPH (n = 10), prostatic dysplasia (n = 13), pure Gleason pattern 3 prostate cancer (n = 7) and pure Gleason pattern 4/5 prostate cancer (n = 17). Normalized log2 intensity ratios from the Affymetrix U133A gene chip set are shown.

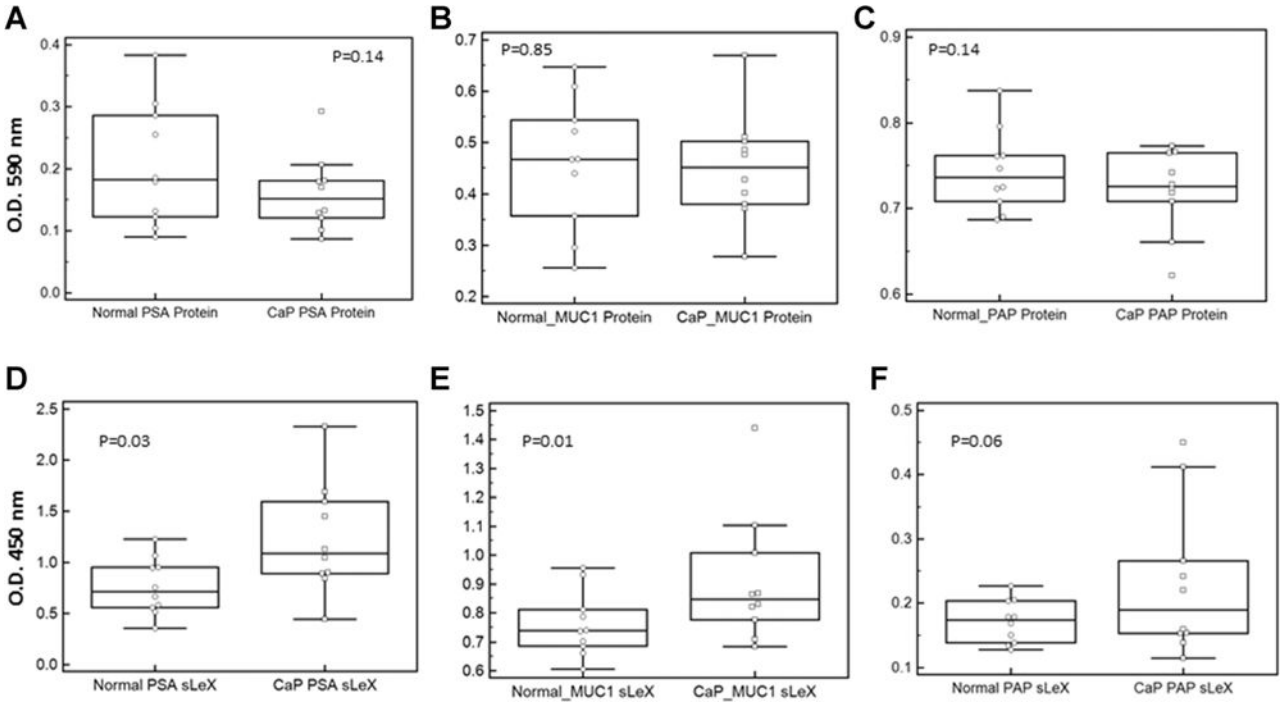

Fig. 5.

Total PSA, MUC1, and PAP protein levels and parallel measurements of core 2 *O*-sLe^x modified PSA, MUC1, and PAP glycoprotein levels. Upper panels show total protein values (A590): **A**:PSA, (**B**) MUC1 and (**C**) PAP. All were normalized to total protein levels in each sample. Bottom panels: core 2 *O*-sLex values (A450/A590) normalized to cognate protein levels: **D**: PSA core 2 *O*-sLex, **E**: MUC1 core 2 *O*-sLex and (**F**) PAP core 2 *O*-sLex.

TABLE I

Clinical Annotations for the Patient Samples Used

S.No.	Age	Gleason score	Stage	Pre-Op-PSA
1	74	3 + 4	pT2c	4.5
2	56	3 + 4	pT1c	18.5
3	74	3 + 3	pT3b	15.9
4	69	4 + 5	pT2c	3.4
5	69	3 + 3	pT1c	10.3
6	58	3 + 3	pT2c	12
7	56	3 + 4	pT2c	4.1
8	51	4 + 3	pT2a	12.7
9	56	3 + 4	pT3b	38.48
10	72	3 + 4	pT3a	5.3