Core 2 β-1, 6-*N*-acetylglucosaminyltransferase-1 expression in prostate biopsy specimen is an indicator of prostate cancer aggressiveness
(前立腺生検標本における GCNT1 の発現は悪性度の指標になり得る)

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ABSTRACT

Introduction: To avoid over-treatment of early stage prostate cancer (PCa), predictive biomarkers for PCa aggressiveness which can be obtained during pre-treatment evaluation are essential. Core 2 β-1, 6-*N*-acetylglucosaminyl-transferase-1 (GCNT1) is a key enzyme that forms core 2 branched *O*-glycans, the expression of which is associated with aggressive potential of prostate cancer. We examined whether GCNT1 expression in prostate biopsy specimen can predict cancer recurrence after radical prostatectomy for the patients with with PCa. We then investigated molecular background for aggressive malignant potential mediated by GCNT1 expression.

Methods: Paraffin-embedded PCa biopsy specimens were immunohisto-chemically tested for GCNT1 expression using an anti-GCNT1 monoclonal antibody. We also examined the role of GCNT1 in PCa progression using cell lines which express high or low levels of GCNT1.

Results: GCNT1 expression correlated with D'Amico's recurrence risk classification. The GCNT1-positive rate in organ confined PCa was significantly lower than that in PCa with extra-prostatic extension. GCNT1-negative tumors were associated with significantly better prostate-specific antigen (PSA)-free survival compared with GCNT1-positive tumors. Multivariate analysis revealed that GCNT1 expression status was an independent risk factor for PSA recurrence after radical prostatectomy. Subsequent basic study revealed that GCNT1-over-expressing cells produced a significantly larger amount of growth factors when co-cultured with prostate stromal cells compared with GCNT1-knocked down cells and formed larger tumors.

Conclusions: GCNT1 expression in prostate biopsy specimen is a significant and independent predictor of recurrence after radical prostatectomy, which can be used in pre-treatment decision making for the patient. Further validation study is necessary to establish clinical implication of GCNT1 in management of PCa.

INTRODUCTION

In the western countries, prostate cancer (PCa) is the most common malignancy in men and the second-leading cause of cancer-related death [1,2]. Its incidence is rapidly increasing in the Asia–Pacific region [3]. One of the most critical issues related to PCa in clinical practice is over-diagnosis and over-treatment [4]. Over-treatment of indolent PCa with a low malignant potential is a major issue because aggressive treatment of PCa is sometimes associated with adverse events. A promising alternate modality to prevent overtreatment may be active surveillance [5]; however, the identification of suitable patients for aggressive treatment is associated with difficulties. An appropriate tool for patient selection for active surveillance is still lacking. Therefore, development of novel biomarkers of PCa aggressiveness is of vital importance for the prevention of PCa over-treatment.

Preoperative serum PSA levels and biopsy Gleason score are conventional and powerful predictors of biological outcomes after radical prostatectomy [6,7]. To improve the risk stratification for PCa recurrence after primary treatment in patients with localized PCa, many investigators have sought biomarkers that reflect the aggressive potential of PCa [8]. However, the majority of reported biomarkers have not been validated for providing information that is more useful than that provided by conventional clinicopathological parameters. With a novel biomarker representing the malignant potential of PCa, more accurate prediction of PSA recurrence and appropriate treatment selection may be possible.

Core 2 β-1, 6-*N*-acetylglucosaminyltransferase-1 (GCNT1) [9,10] is a key

enzyme that synthesizes core 2 branched *O*-glycans by catalyzing the transfer of *N*-acetylglucosamine (GlcNAc) from uridine diphosphate (UDP)-GlcNAc with a β1, 6-linkage to α-GalNAc of a core 1 *O*-glycan (Fig. 1A). Previously, it was shown that the expression of core 2 branched *O*-glycans is closely correlated with the malignant potential of colorectal cancer [11] and pulmonary adenocarcinoma [12]. Using a polyclonal antibody[10], we already have demonstrated that immunohistochemical testing of GCNT1 was closely related with the aggressive potential of PCa [13], testicular cancer [14], and bladder cancer [15]. However, polyclonal antibody has a weakness in specificity and reproducibility as a clinically useful biomarker. We then established a monoclonal antibody against GCNT1 by peptide immunization [16]. The monoclonal anti-GCNT1 successfully detected GCNT1 expression in paraffin-embedded specimen obtained by radical prostatectomy and post-digital rectal examination urine samples. Although the GCNT1 status were closely related with the aggressive potential of PCa in clinical samples [16], the mechanism of tumor progression still remained unclear. Furthermore, to develop a novel indicator of cancer aggressiveness it is essential to determine the malignant potential before the initiation of treatment for PCa. Therefore, it is very important to test whether GCNT-1 expression in prostate biopsy specimen can predict recurrence after radical prostatectomy.

In this study, we attempted to demonstrate that GCNT1 expression in prostate biopsy specimens was a significant and independent predictor for PSA recurrence after radical prostatectomy. Furthermore, we investigated possible roles of GCNT1 in PCa progression using cell lines which express high or low levels of GCNT1 with *in vitro* and *in vivo* experimental systems.

Materials and methods

Cells

The human androgen-dependent PCa cell line LNCaP and human androgen-independent cell lines DU145 and PC-3 were purchased from the American Type Culture Collection (Rockville, MD, USA). Human prostate stromal cells (PrSCs) were purchased from the Cell Scientific Laboratory (Tokyo, Japan.

Stable transfection

LNCaP cells and DU145 cells were maintained in RPMI1640 medium supplemented with 10% FBS. The cells were plated in a 35-mm cell culture dish 24 h before transfection. The cells were transfected using the X-treme HP DNA transfection reagent (Roche Diagnostics, Basal, Schweiz) with 1 μg of the GCNT1 construct or an empty vector (Mock). The cell clones were selected in the RPMI 1640 medium containing 10% FBS and 100 μg/mL of G418 sulfate. GCNT1 knockdown cells (PC-3 cell line) were as previously described [17].

Immunohistochemical analysis of PCa specimens

Between 2005 and 2011, 687 patients with PCa were treated with radical prostatectomy with or without neoadjuvant therapy at the Department of Urology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan. Of these, 122 patients who did not receive radiation therapy was enrolled in the present study. Staging, grading of the tumors and patient follow-up were previously described described [16,18]. In brief, post-operative PSA levels were considered to be increased (PSA recurrence) if they were ≥ 0.2 ng/mL during two

consecutive visits in a 1-month interval. Time zero was defined as the day of surgery. Patients with constantly detectable PSA levels (< 0.001 ng/mL) after surgery were recorded as recurrences at time zero. Follow-up intervals were calculated from the date of surgery to the last recorded follow-up [16]. Information on PCa patients and tumor characteristics were obtained from medical charts (Table 1). Their biopsy PCa specimens were formalin-fixed and embedded in paraffin. Deparaffinized specimens were incubated with the mouse anti-human GCNT1 mAb (Clone: HU127 [16]), followed by incubation with HRP-conjugated goat anti-mouse IgG antibody (H+L; Millipore). Based on the staining status of Golgi apparatus, specimens with 10% or more positive cancer cells were judged as GCNT1 positive. Informed consents were obtained from all the patients. The ethical committee of Hirosaki University approved the protocol of this study. The study was performed in accordance with the ethical standards of the Declaration of Helsinki.

The orthotopic inoculation system

GCNT1-overexpressing PCa cells (PC3NC and DU145-GCNT1) and cells with low GCNT1 expression (PC3G1KD and DU145-Mock; 10^6 cells/mouse) were orthotopically injected into the prostate of BALB/c-nu/nu mice. Five weeks after injection, the mice were euthanized and the prostate was removed. The collected tissue was measured for size and weight. The PCa tissue specimens were formalin-fixed and embedded in paraffin. Four-μm tissue sections were stained with either hematoxylin–eosin or mouse anti-human GCNT1 mAb.

siRNA transfection in LNCaP prostate cancer cells

LNCaP cells were seeded onto 6-well plates at 4×10^5 cells/well in RPMI1640

containing 10% FBS. After 24 h, siRNAs (60 pmol/well) were transfected using X-treme siRNA transfection reagent (Roche). The siRNA oligonucleotides for GCNT1 (MISSION siRNA, Hs_GCNT1) and control (MISSION siRNA Universal Negative Control, SIC-001) were obtained from Sigma Aldrich (St. Louis, MO, USA). After 48 h, target gene expression was determined using quantitative polymeric chain reaction (qPCR). GCNT1 expression levels were normalized to those of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Identification of prostate stromal cells expressing galectins.

Three μg of proteins from stromal whole-cell lysate or 3 μL of stromal cell supernatants were applied to an SDS-PAGE gel. After electrophoresis, these proteins were transferred to a PVDF membrane, which was blocked with 5% BSA in TBST. Next, galectins were detected using sequential incubation with anti-galectin 1 (Abcam, Cambridge, MA, USA) or anti-galectin 3 antibody (Chemicon) and HRP-conjugated secondary antibodies (1:2000) diluted in 5% BSA-TBST. The signals representing galectins were enzymatically detected using the Novex® ECL Chemiluminescent Substrate Reagent Kit (Life Technologies, Carlsbad, CA, USA).

Determination of cytokine expression in PCa cells with or without galectin-3 and in the PCa cell–stromal cell coculture

To determine the role of GCNT1 in PCa cell–stromal cell interaction, cytokine expression was measured using ELISA. Stromal cells were harvested in 24-well cell culture plates at 50% confluency, and the medium was changed to Opti-MEM I. LNCaP-Mock and LNCaP-GCNT1 cells were seeded at 10^5 cells/well on the stromal cells. Twenty-four hours after co-culture, the culture

medium was collected into microtubes and centrifuged at 20600 × *g* for 5 min. The supernatant was collected in a new tube and cytokine expression was analyzed using the ELISA-based assay (SRL, Tokyo, Japan). For the measurement of galectin-induced cytokine expression, LNCaP-Mock or LNCaP-GCNT1 cells (2×10^4 cells/well) were cultured with or without 2 μg/mL of galectin-1 or galectin-3. Twenty-four hours after co-culture, the culture medium was collected, and we conducted multiplex cytokine analysis using the Procarta Immunoassay kit (Panomics/Affymetrix, CA, USA).

Statistical analysis

The chi-squared test was used to analyze the association of GCNT1 status with clinical and histopathological parameters. PSA-free survival after radical prostatectomy was evaluated using Kaplan–Meier curves, and differences between groups were assessed using the log-rank test. We used the SPSS 21.0 software package (SPSS, Chicago, IL, USA) for all statistical analyses. Multivariate analysis using Cox proportional hazards regression analysis was performed to detect significant and independent parameters with which PSA recurrence after radical prostatectomy can be predicted. Promising parameters included GCNT1 status, patient age, initial PSA, clinical stage and biopsy Gleason score (GS).

Results

GCNT1 expression in biopsy specimen positively correlated with extra-prostatic capsule extension and PSA recurrence

To evaluate the role of GCNT1 in PCa agressivenes, PCa biopsy specimens were

immunohistochemically analyzed using the anti-human GCNT1 mAb. The results demonstrated that GCNT1 was barely expressed in normal prostate gland, whereas PCa cells often expressed significant levels of GCNT1 (Fig. 2A). The GCNT1-positive rate significantly elevated according to the D' Amico's recurrence risk classification [19]. In the pathological parameters, extra-prostatic extension (pT3/4) and lymph node metastasis were positively correlated GCNT1 expression status (Table 2). Moreover, GCNT1-positive patients were at significantly higher risk of PSA recurrence after radical prostatectomy (Fig. 2B). According to multivariate analysis, initial PSA and GCNT1 expression status in biopsy specimen were independent risk factors for PSA recurrence (Table 3). These results indicated that GCNT1 expression in biopsy specimens was a good predictor of PCa aggressiveness.

GCNT1-overexpressing cells formed larger tumors on orthotopic inoculation into the mouse prostate

To determine the role of GCNT1 in PCa growth in nude mice, GCNT1-over-expressing (DU145-GCNT1 and PC3NC) or -under-expressing cells (DU145-Mock and PC3G1KD) were inoculated into the mouse prostate (Fig. 3A). Five weeks after inoculation, the mice were euthanized and the prostates were collected. GCNT1-over-expressing cells were found to form larger tumors compared with GCNT1-underexpressing cells ($P < 0.05$; Figs. 3B and 3C). GCNT1 expressions were stronger in DU145-GCNT1 specimens than that in DU145-Mock (Fig. 3D)

PCa cell and prostate stromal cell interaction regulated cytokine expression in vitro

Cell–cell interactions are important for PCa progression [20]. We next examined the role of GCNT1 expression in the interaction between PCa cells and

prostate stromal cells (PrSCs). When LNCaP cell were co-incubated with PrSCs, LNCaP-GCNT1 cells produced a significantly larger amount of hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), fibroblast growth factor, and keratinocyte growth factor ($P < 0.05$; Fig. 4A).

Stromal cell-derived galectin-3 promoted cytokine production in PCa cells

In our previous study, GCNT1 expressing PCa cells formed poly-*N*-acetyllactosamine on cell surface [17]. The poly-*N*-acetyllactosamine is a ligand for galectins [21,22]. To determine the molecular mechanism of cytokine expression in LNCaP-GCNT1 cells, we examined galectins originating from stromal cell using Western blotting. PrSCs expressed galectin-1 and galectin-3. In the cell culture supernatant, galectin-3 was detected in the stromal cell supernatant, whereas galectin-1 was not detected (Fig. 4B). Galectin-3 stimulated HGF and VEGFA expression in LNCaP cells depending on GCNT1 status ($P < 0.05$; Fig. 4C). GCNT1 expression levels were determined by qPCR methods (Fig. S1).

Discussion

Aberrant glycosylation of cell surface glycoproteins plays an important role in cancer initiation, proliferation, invasion, and metastasis [23,24,25]. Biosynthesis of oligosaccharides on glycoproteins is performed in concert by several glycosyltransferases; GCNT1 is one of the glycosyltransferases that forms the core 2 *O*-glycans on the surface of lymphocytes and various cancer cells [13,14,26,27]. It is noteworthy that the present study clearly demonstrated that immunohistochemical status of GCNT1 expression on prostate biopsy specimen

closely related to extra-prostatic extension, lymph node metastasis of PCa (Table 2). Most importantly, patients with GCNT1-positive PCa exhibit worse PSA-free survival compared to those with a GCNT1-negative tumor after radical prostatectomy (Fig. 2). These results suggests that pre-treatment information on GCNT1 expression provided by immunohistochemical staining of biopsy specimen can precisely predict malignant potential of PCa. Using this information, appropriate patient selection for good candidate of active surveillance could be possible resulting in avoiding over-treatment for early stage prostate cancer.

In this study, we also demonstrated that GCNT1-over-expressing cells produced significantly larger tumors compared with GCNT1-under-expressing cells in nude mice orthotopic model (Fig. 3). We previously showed GCNT1 expression in PCa cells strongly adherent to PrSCs [13]. Therefore, we hypothesized that GCNT1-positive PCa cells had stronger interactions with PrSCs compared with GCNT1-negative PCa cells. In the current co-culture experimental system with PrSCs and PCa cells, significantly large amount of growth factors were produced in combination with GCNT1-over-expressing cancer cells than with GCNT1-under-expressing cells (Fig. 4). These results suggest that GCNT1-expressing PCa cells stimulate cytokine expression mediated by interaction with the stromal cells promoting rapid PCa progression.

Previously, it was reported that GCNT1 expression was associated with the metastatic potential of colorectal [11], lung [12], and testicular cancer [14]. Our research group has already reported that GCNT1-expressing cancer cells can escape from host immune defense system [15,17]. In this immunological evading

mechanism, galectin-3 binding to core 2 branching *O*-glycans expressed on cancer cell surface is an critical step to interfere contact of host natural killer cells with cancer cells [15,17]. Endogenous galectin-3 expression has been found to correlate with the malignant potential of tumors [28,29,30] and PCa progression [31]. Moreover, it was reported that exogenous galectin-3 expression enhances breast cancer invasiveness [32]; however, the effects of exogenous galectin-3 on PCa remain unknown. Therefore, we focused on the effects of exogenous galectin-3 secreted by PrSCs, which may have pivotal roles in PCa progression. LNCaP cells which originally do not express galectin-3 [33], were stimulated to express cytokines by exogenous galectin-3 in the present study. Then, we found that cytokines were produced according to the expression level of GCNT1 (Fig. 4). We provided an evidence that core 2 *O*-glycans can be recognized by galectin-3, thereby stimulating cytokine expression in PCa cells.

Although the GCNT1-driven regulatory mechanism of cancer progression is still poorly understood, our study demonstrates that GCNT1 can be a promising predictor of the malignant potential of PCa. Further clinical trial is necessary to determine the practical implication of GCNT1 as a biomarker of PCa.

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Figure legends

Figure 1

Biosynthetic pathways for core2 *O*-glycan

Starting from *N*-acetylgalactosamine (GalNAc) on serine or threonine residues in a peptide, core1 synthase transfers galactose (Gal) to make the core1 structure (Core1). The core1 structure is converted to branched core2 structure (Core2) by GCNT1, GCNT3, and GCNT4.

Figure 2

Core 2 β-1, 6-*N*-acetylglucosaminyltransferase-1 (GCNT1) expression correlates with prostate cancer (PCa) progression

(A) PCa biopsy specimens were stained with an anti-GCNT1 monoclonal antibody (mAb), followed by staining with a horseradish peroxidase (HRP)-conjugated secondary antibody. Counterstaining was performed using hematoxylin. GCNT1-positive cancer cells are brown. **(B)** Prostate-specific antigen (PSA)-free survival periods were compared between GCNT1-positive and -negative specimens, and survival analysis was conducted using Kaplan–Meier curves. Scale bars: 100 μm

Figure 3

Analysis of tumor formation in nude mice

(A) Core 2 β-1, 6-*N*-acetylglucosaminyltransferase-1 (GCNT1) expression levels were quantified using Western blotting in PC3NC, PC3G1KD (upper panels), DU145-Mock, and DU145-GCNT1 cells (lower panels). Prostate cancer (PCa) cell

lines were injected into the prostates of nude mice. After 5 weeks, the mice were euthanized and their prostates were collected. PC3NC-, PC3G1KD- (**B**), DU145-Mock-, and DU145-GCNT1-derived (**C**) tumor specimens were photographed, and tumor size was measured. (**D**) DU145-Mock- and DU145-GCNT1-derived paraffin-embedded specimens were sliced into 4-μm tissue sections, and the slices were stained with hematoxylin and eosin (upper panels) or an anti-GCNT1 monoclonal antibody (lower panels). Scale bars: 50 μm

Figure 4

Co-culture with prostate stromal cells (PrSCs) and galectin-3 enhanced cytokine expression of core 2 β-1, 6-*N*-acetylglucosaminyltransferase-1 (GCNT1)-expressing PCa cells

(**A**) LNCaP-mock and LNCaP-GCNT1 cells cocultured with PrSCs

After 24 h, the culture supernatants were collected and cytokine expression levels measured using ELISA. (B) Galectin-1 and galectin-3 expression levels in whole-cell lysates and cell culture supernatants of PrSCs were analyzed using Western blotting. (**C**) LNCaP cells were cultured with or without galectins. After 24 h, the cell culture supernatants were collected and hepatocyte growth factor (HGF) and vascular endothelial growth factor A (VEGF-A) expression was measured using a multiplex cytokine assay. *: $p < 0.05$

Supporting information

Fig. S1 Messenger RNA expression of Core 2 β-1,

6-*N*-acetylglucosaminyltransferase-1 (GCNT1) in LNCaP cells

Table 1 Patient characteristics

Age (years, median, IQR)	68 (64-72)
Initial PSA (ng/mL, median, IQR)	7.63 (5.51-13.54)
Clinical T, N (%)	
T1c	58 (47.5)
T2a	30 (24.6)
T2b	9 (7.4)
T2c	4 (3.3)
T3	21 (17.2)
Biopsy Gleason score, N (%)	
6	11 (9.0)
7	82 (67.2)
8≤	29 (23.8)
D'Amico risk classification, N (%)	
Low	6 (4.9)
Intermediate	73 (59.8)
High	43 (35.2)
Follow-up period (months,median, IQR)	73.7 (55.6-94.1)

IQR; interquartile range, PSA; prostate specific antigen

Table 2 Patient characteristics according to GCNT1 status

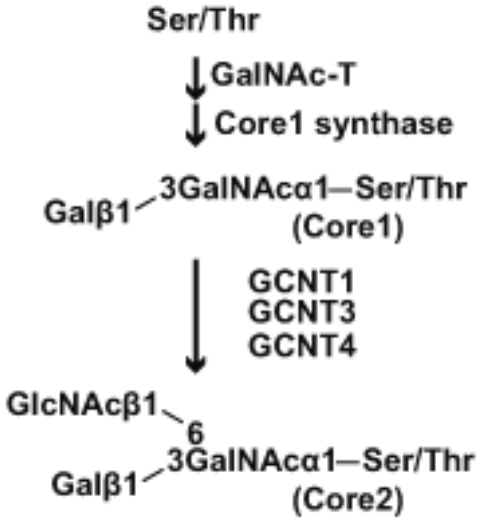
	GCNT1-negative	GCNT1-positive	p-value
Number	31	91	
Age (years, median)	67	68	0.212
Initial PSA (ng/mL, median)	6.08	9.2	0.363
Clinical T, N (%)			
T1	14 (24.1)	44 (75.9)	0.512
T2	11 (25.6)	32 (74.4)	
T3	6 (28.6)	15 (71.4)	
Biopsy Gleason score, N (%)			
6	3 (27.3)	8 (72.7)	0.248
7	22 (26.8)	60 (73.2)	
8≤	6 (20.7)	23 (79.3)	
D'Amico risk classification, N (%)			
Low	2 (33.3)	4 (66.7)	0.037
Intermediate	20 (27.4)	53 (72.6)	
High	9 (20.9)	34 (79.1)	
Extracapsular extension, N (%)	7 (17.5)	33 (82.5)	0.044
Positive surgical margin, N (%)	7 (23.3)	23 (76.7)	0.621
Lymph node involvement, N (%)	0 (0)	1 (100)	< 0.001
Prostatectomy Gleason score, N (%)			
≤6	1 (25.0)	3 (75.0)	0.652
7	18 (27.7)	47 (72.3)	
8≤	12 (22.6)	41 (77.4)	

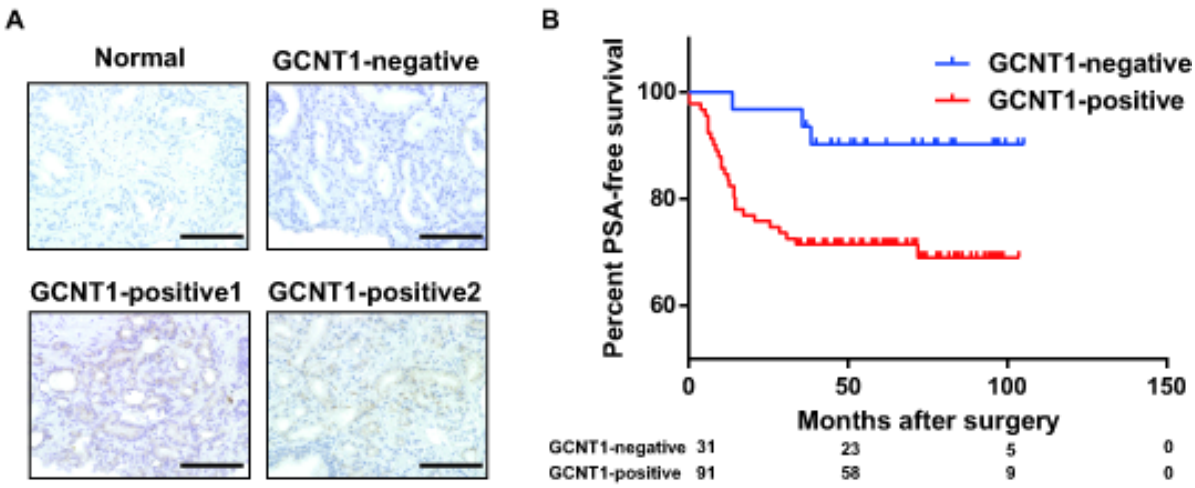
GCNT1; core2 β1,6-*N*-acethylglucosaminyltransferase-1, PSA; prostate specific antigen

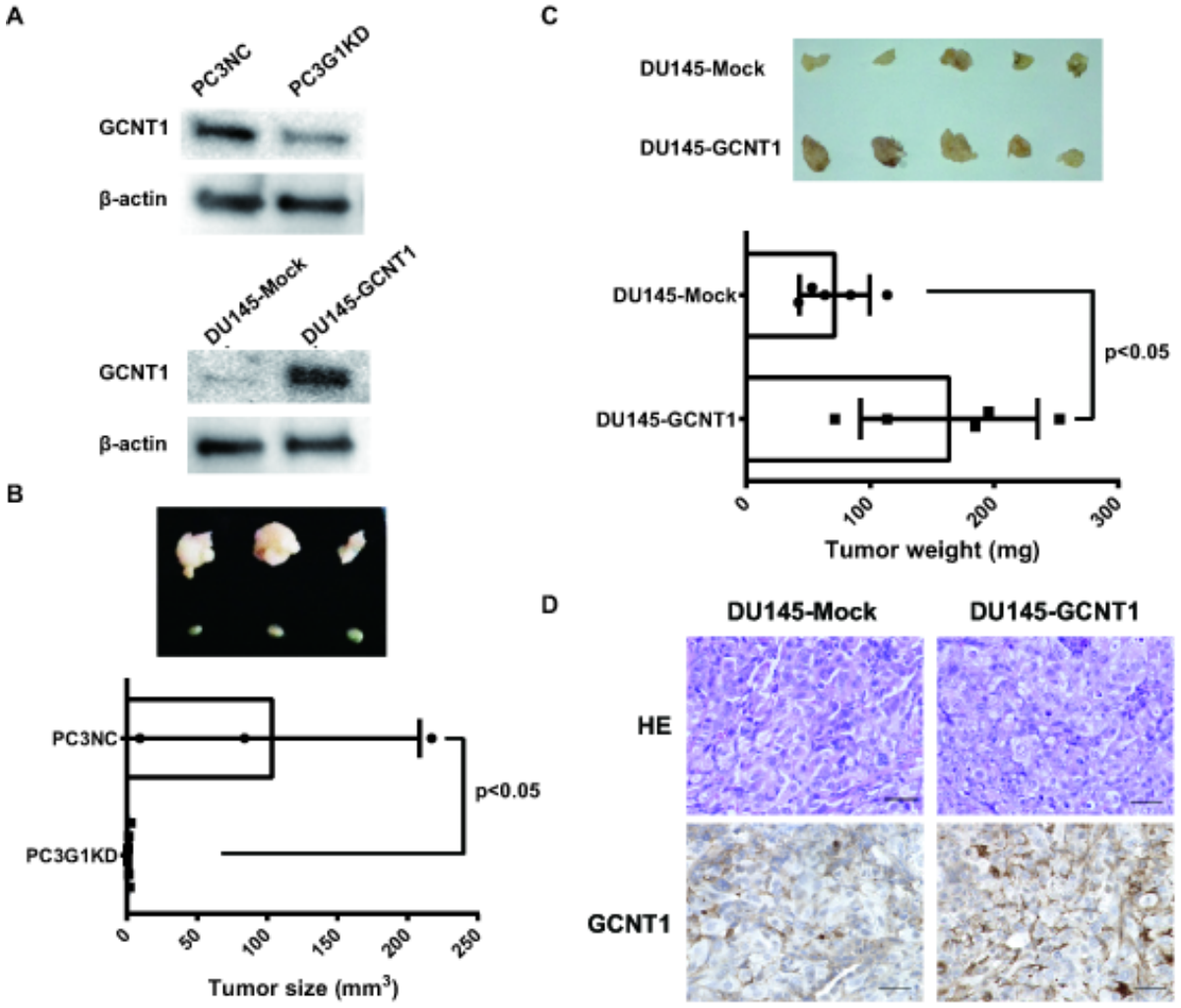
Table 3 Univariate and multivariate analysis for prostate specific antigen recurrence-free survival

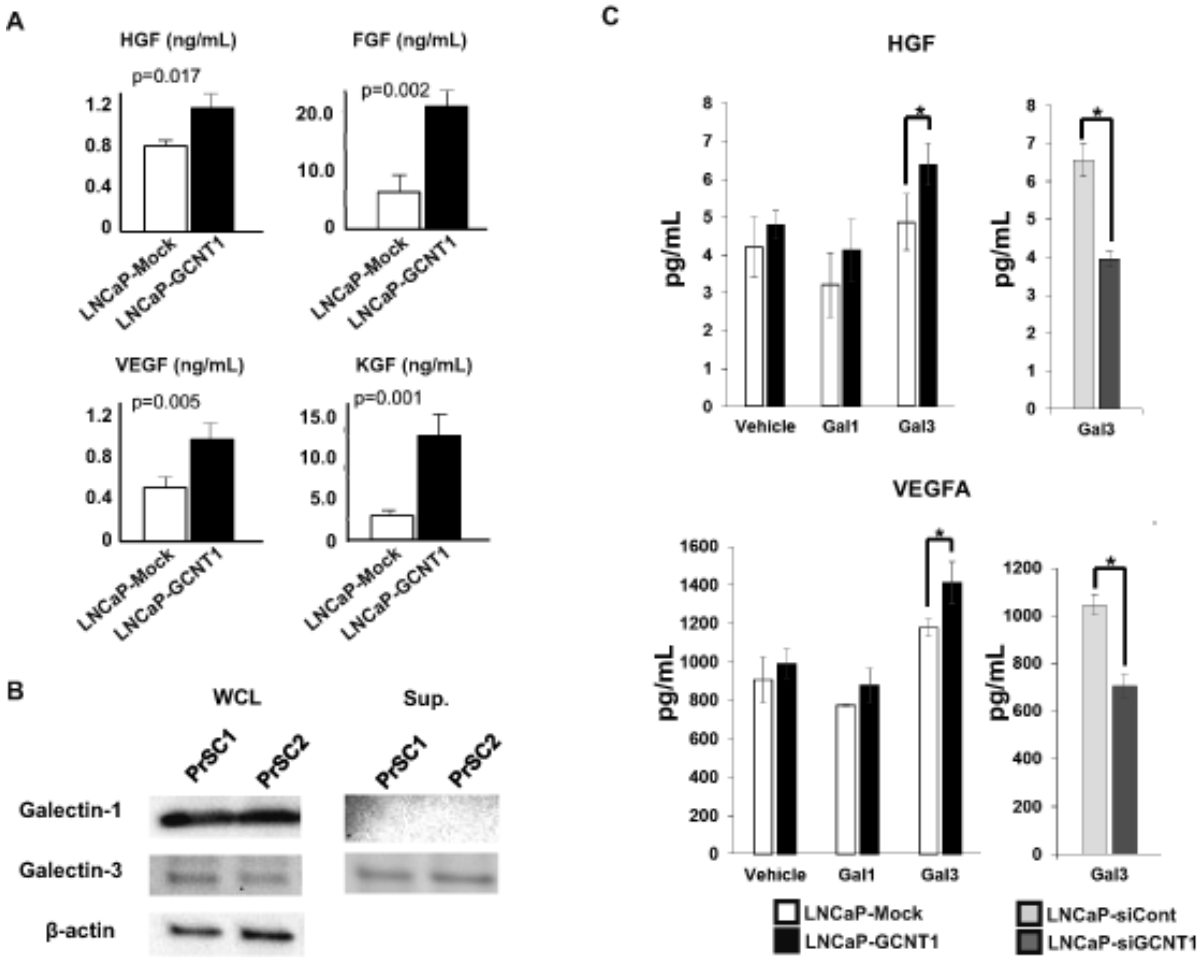
	Univariate	Multivariate	
	p-value	HR (95% CI)	p-value
Age	0.053	1.014 (0.947-1.085)	0.693
iPSA	0.000	1.026 (1.010-1.042)	0.001
cT2c≤	0.173	0.730 (0.236-2.264)	0.586
Biopsy Gleason score 8≤	0.048	1.490 (0.659-3.369)	0.339
GCNT1 status	0.024	3.806 (1.143-12.671)	0.029

iPSA; initial prostate specific antigen, GCNT1; core2 β1,6-*N*-acethylglucosaminyltransferase-1


Ser/Thr
GalNAc-T
Core1 synthase
Galβ1
3GalNAcα1—Ser/Thr
(Core1)
GCNT1
GCNT3
GCNT4
GlcNAcβ1
6
Galβ1
3GalNAcα1—Ser/Thr
(Core2)


A
Normal
GCNT1-negative
GCNT1-positive1
GCNT1-positive2
B
Percent PSA-free survival
100
80
60
0
50
100
150
Months after surgery
GCNT1-negative
GCNT1-positive
GCNT1-negative 31 23 5 0
GCNT1-positive 91 58 9 0


A
PC3NC
PC3G1KD
GCNT1
β-actin
DU145-Mock
DU145-GCNT1
GCNT1
β-actin
B
PC3NC
PC3G1KD
p<0.05
0
50
100
150
200
250
Tumor size (mm3)
C
DU145-Mock
DU145-GCNT1
DU145-Mock
DU145-GCNT1
p<0.05
0
100
200
300
Tumor weight (mg)
D
DU145-Mock
DU145-GCNT1
HE
GCNT1


A
HGF (ng/mL)
p=0.017
FGF (ng/mL)
p=0.002
VEGF (ng/mL)
p=0.005
KGF (ng/mL)
p=0.001
LNCaP-Mock
LNCaP-GCNT1
B
WCL
Sup.
PrSC1
PrSC2
Galectin-1
Galectin-3
β-actin
C
HGF
VEGFA
pg/mL
Vehicle
Gal1
Gal3
LNCaP-Mock
LNCaP-GCNT1
LNCaP-siCont
LNCaP-siGCNT1