Overexpression of *O*-GlcNAc by prostate cancer cells is significantly associated with poor prognosis of patients

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ABSTRACT

BACKGROUND: *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) is a glycan essential for fundamental cellular processes such as transcription/translation, nuclear transport, protein stability, and protein-protein interactions. However, the role of *O*-GlcNAc in prostate cancer progression of patients remains poorly unknown. Here we investigated the clinicopathological significance of *O*-GlcNAc expression level in prostate cancer.

METHODS: *O*-GlcNAc expression level in prostate cancer cells was determined by immunohistochemistry of prostate biopsy specimens obtained from 56 patients later treated with hormone deprivation therapy comparing with adjacent normal prostate glands in the same sections. Overall survival was determined by the Kaplan-Meier and Cox proportional hazards methods with univariate and multivariate models. The effects of reduced *O*-GlcNAc expression level on proliferation and invasion of prostate cancer LNCaP cells were examined using small interfering RNA targeting *O*-GlcNAc transferase (OGT), the enzyme responsible for *O*-GlcNAc biosynthesis.

RESULTS: Defining cancer cells showing stronger cytoplasmic staining than normal prostate glands as overexpression of *O*-GlcNAc, 39% of prostate cancer patients were categorized as overexpression. Kaplan-Meier and Cox proportional hazards methods with univariate model analysis revealed that *O*-GlcNAc overexpression was associated with overall survival ($P = 0.0012$ for Kaplan-Meier and $P = 0.0021$ for Cox univariate hazard model analysis). Furthermore *O*-GlcNAc was the only item in which a significant difference was observed at overall survival by multivariate analysis ($P = 0.0475$). Finally, siRNA-mediated OGT knockdown in LNCaP cells resulted in decreased expression of *O*-GlcNAc and promoted decreased proliferation and tumor cell invasion compared with control siRNA-transfected LNCaP cells.

CONCLUSION: These results indicate that *O*-GlcNAc expression level in prostate cancer cells is associated with poor prognosis of prostate cancer patients and likely enhances tumor cell proliferation and invasion.

Key Words: carcinoma; glycosylation; prognosis; prostate; RNA interference

INTRODUCTION

O-linked β-*N*-acetylglucosamine (*O*-GlcNAc) is a sugar attached to serine or threonine hydroxyl moieties of nuclear and cytoplasmic proteins that are associated with fundamental cellular processes such as cell signalling, metabolism, transcriptional regulation, cell cycle control, protein trafficking, and the regulation of cellular structure.[1] Cycling of *O*-GlcNAc on serine or threonine residues of both is regulated by two distinct enzymes, *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase. *O*-GlcNAc cycles at rates similar to that of *O*-phosphate in response to various cellular stimuli.[2] Because *O*-GlcNAcylation has an important role in fundamental cellular processes, its dysregulation also contributes to the etiology of human diseases, such as diabetes, neurological disorders, and cancer.[3] Recent studies at *in vitro* level indicate that *O*-GlcNAcylation is closely associated with progression of malignant tumors including prostate cancer cells,[4-6] but the clinicopathological significance of *O*-GlcNAc expression level determined by using prostate cancer biopsies has not been characterized to date.

In the present study, we carried out immunohistochemistry to evaluate the expression level of *O*-GlcNAc in prostate cancer cells using prostate biopsy specimens. We then analyzed the relationship between *O*-GlcNAc expression levels and overall survival of the patients. We also examined the effects of *O*-GlcNAc in proliferation and invasion of prostate cancer LNCaP cells *in vitro* using RNA interference targeting the *OGT* gene.

MATERIALS AND METHODS

Patient samples

Tissue blocks representing prostate cancer biopsy specimens from 56 patients dating from 1996 to 2002 at Nagano Municipal Hospital, Nagano, Japan, were examined. These patients did not receive hormonal therapy prior to biopsy, and their survival was followed for 5 years after sextant prostate biopsy. All were subsequently treated with hormonal therapy because of high stage or advanced age. Clinicopathological findings of the patients are summarized in Table 1.

Tumor staging was performed according to the American Joint Committee on Cancer (AJCC) classification system. All tissue specimens were fixed for 48 hr in 20% buffered formalin (pH7.4), embedded in paraffin, and sectioned serially at 3-μm thickness. The Gleason score of each specimen was re-evaluated and confirmed by two certified pathologists (HS and JN). This study was approved by the Ethical Committee of Shinshu University School of Medicine.

Immunohistochemistry

Primary antibodies used were mouse monoclonal anti-*O*-GlcNAc antibody (CTD110.6; Covance Research Products, Denver, PA, USA) and anti-human p63 antibody (4A4; DakoCytomation, Denmark A/S, Glostrup, Denmark). Immunohistochemistry was performed by the indirect immunoperoxidase method. Before immunostaining for both *O*-GlcNAc and p63, antigen retrieval was carried out by microwaving samples for 30 min in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. EnVision™+ System-HRP (DakoCytomation) was used as a secondary antibody, and peroxidase activity was visualized using

diaminobenzidine-hydrogen peroxide (DAB/H_20_2) solution. As negative control for immunohistochemistry, tissue samples incubated with the secondary antibody alone were immersed in DAB/H_20_2 solution, and no specific staining was noted. *O*-GlcNAc expression levels in cancer cells were evaluated by comparison with adjacent normal prostate glands in the same sections. Cancer cells showing stronger cytoplasmic staining than normal prostate glands were defined as overexpression of *O*-GlcNAc, and those that showed comparable or lower cytoplasmic expression to adjacent normal prostate glands were defined as reduced expression of *O*-GlcNAc. Two authors (TK and JN) independently scored the *O*-GlcNAc expression level without knowing information of clinical parameters such as PSA and outcome, and these results gave good agreement.

Cell culture

Human prostate cancer LNCaP cells obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) at 37°C in a humidified incubator containing 5% CO_2.

Cells in log-phase growth were harvested by trypsinization and used for experiments.

RNA interference

Predesigned siRNA targeting OGT (ID no. s16093, Ambion, Austin, TX, USA) or negative control #1 siRNA (Ambion) were electroporated into 1.0 x 10^6 of LNCaP cells using a Nucleofector™ (Amaxa Biosystems, Gaithersburg, MD, USA) using cell line kit R (Amaxa Biosystems) with program T-009. After 24 h of transfection, total RNA was isolated with RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, and 1 μg of each DNase-treated RNA sample was reverse-transcribed with Superscript™ III reverse transcriptase using random primers (Promega, Madison, WI, USA). Then 1/10 aliquots of the cDNA reaction were analyzed by quantitative polymerase chain reaction (PCR) on an ABI PRISM® 7900HT (Applied Biosystems, Foster City, CA, USA) as described.[7] Primers and probes for OGT and control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from

Applied Biosystems™ (OGT, Hs00257895_m1; GAPDH, Hs99999905_m1). Relative expression levels of OGT mRNA in OGT siRNA- or control siRNA-transfected LNCaP cells were evaluated using the comparative threshold cycle method, in which OGT levels are normalized to GAPDH expression.

Western blot analysis

1.0 x 10^6 of LNCaP cells transfected with siRNA targeting OGT (ID no. s16093, Ambion) or negative control #1 siRNA (Ambion) were also subjected to western blot analylsis. After 72 h of transfection, cells were dissolved in 0.1 ml lysis buffer Complete Lysis-M containing a protease inhibitor (Roche, Mannheim, Germany), according to the manufacturer's protocol. Protein concentration was measured using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA), and 55 μg of protein treated with reducing agent were subjected to SDS-PAGE analysis using 3-8% NuPAGE Tris-Acetate mini gels (Life Technologies, Carlsbad, CA, USA). Blotted membrane was immunostained with anti-*O*-GlcNAc antibody (CTD110.6) as described elsewhere.[8] Secondary antibody used was

HRP-conjugated F(ab')$_2$ fragment goat anti-mouse IgM, μ chain specific (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Immunoreactions were detected using the ECL plus Western blotting detection system (GE Healthcare).

Cell proliferation assay

The effect of OGT knockdown in LNCaP cells was assessed using the CellTiter™ 96 AQueous (Promega, Madison, WI, USA) assay in 96-well plates, according to the manufacturer's instructions. OGT siRNA-transfected or control siRNA-transfected LNCaP cells were seeded in 96-well plates with 5,000 cells/well. After 24h incubation at 37°C in 5% CO_2, 20μl of MTS solution was added to a well, and color density was measured as the optical density at 490 nm (OD 490 nm) using an ELISA plate reader. This assay was carried out daily for 5 days in triplicate, and the means ± SD were calculated.

Invasion assay

An invasion assay was performed by a Boyden chamber method using BD Bio-Coat™ Matrigel™ invasion chambers (Becton Dickinson Labware, Bedford, MA, USA) as described.[9] RPMI 1640 was added to the upper cell culture inserts and bottom chambers, and then the inserts were allowed to hydrate for 2 h at 37°C. As a chemoattractant, 10% FBS was added to the bottom chamber. At 24 h after transfection with OGT or control siRNAs, 5×10^4 cells were placed in the insert. After 22 h of incubation at 37°C, the upper face of insert membrane were scrubbed with a cotton bud to remove non-invading cancer cells. Invading cells on the lower face of the insert membrane were fixed and stained using a Diff Quick staining kit (Sysmex, Kobe, Japan) and then counted under a light microscope. The percentage of cells that passed through the Matrigel chamber relative to that seen with control cells was calculated as the percent invasion. The experiment was repeated nine times, and means ± SD were calculated.

Immunofluorescence staining

The siRNA-transfected LNCaP cells were grown on coverslips in RPMI 1640

containing 10% FBS, and then fixed with 20% formaldehyde in phosphate buffered saline (PBS) for 20 min at room temperature. After washing with PBS, these cells were permeabilized with 0.1% saponin, and then incubated with anti-E-cadherin antibody (clone 36; BD Biosciences, Franklin Lakes, NJ, USA). After rinsing with PBS, these cells were incubated with fluorescein isothiocyanate-labeled goat F(ab')2 fragment anti-mouse IgG (H+L) (Beckman Coulter, Marseille, France). After washed with PBS, coverslips were mounted with Vectashield (Vector Laboratories; Burlingame, CA, USA). As negative control, the primary antibody was omitted from the staining procedure, and no specific staining was noted.

Statistics

Student's *t*-test served to determine significant differences between 2-sample data. The overall survival rate was examined using Kaplan-Meier method, and the significance was analyzed by log-rank test. To compare *O*-GlcNAc expression levels in prostate cancer cells with clinicopathological variables such

as age, stage, PSA in serum, and Gleason score at biopsy, results were analyzed using Cox hazard univariate and multivariate tests, and a stepwise model was used to select independent prognostic factors for multivariate analysis. Statistical analysis was performed using JMP8® software (SAS Institute, Cary, NC, USA). $P < 0.05$ was judged significant.

RESULTS

Expression of *O*-GlcNAc in prostate cancer cells

To evaluate *O*-GlcNAc expression level in prostate cancer cells, we immunostained 56 biopsy specimens and compared them to adjacent normal prostate gland. Specimens were also stained with anti-p63 antibody as a marker of basal cells to distinguish normal prostate glands from adenocarcinoma. In normal prostatic glands, *O*-GlcNAc was detected in the cytoplasm and/or nucleus of luminal rather than basal cells, but the staining intensity of *O*-GlcNAc in luminal cells was weak (Fig. 1a). By contrast, expression of the glycan in prostate cancer cells was mostly cytoplasmic rather than in nucleus of prostate

cancer cells from all patients. In particular, 22 (39.3%) of 56 patients were defined as showing *O*-GlcNAc overexpression (Table 1) based on the criteria that prostate cancer cells showed stronger cytoplasmic *O*-GlcNAc staining than did normal adjacent prostate glands (Fig. 1a). On the other hand, 34 (60.7%) of 56 patients were defined as reduced expression of *O*-GlcNAc in the cytoplasm relative to adjacent normal prostate glands (Fig. 1a). When we examined Gleason scores, we found that they were positively correlated with *O*-GlcNAc expression levels (Fig. 1b).

_O_-GlcNAc expression levels in prostate cancer cells is associated with prognosis of patients

To determine whether *O*-GlcNAc expression levels in prostate cancer cells are associated with patient survival, we undertook statistical analysis. Kaplan–Meier analysis for 5-year overall survival in 56 patients showed that survival was inversely correlated with *O*-GlcNAc overexpression with significant difference ($P = 0.0012$) (Fig. 2). Similarly, Cox hazard univariate analysis revealed that

O-GlcNAc overexpression by prostate cancer cells was significantly associated with poor patient prognosis (P = 0.0021) (Table 2). Strikingly, multivariate analysis showed significant differences (P = 0.0475) between *O*-GlcNAc expression level and overall survival (Table 3).

OGT knockdown in LNCaP cells promotes decreased proliferation and tumor cell invasion

To test the effect of OGT in LNCaP cells, we used RNA interference to knock down *OGT* gene. Quantitative PCR analysis of OGT expression in knockdown cells revealed that OGT mRNA expression was significantly reduced by 12 ± 2% compared with control siRNA-transfected LNCaP cells 24h after transfection (Fig. 3a). Western blot analysis demonstrated that two distinct *O*-GlcNAcylated proteins with 39 kDa and 35 kDa in control siRNA-transfected LNCaP cells were decreased in knockdown LNCaP cells transfected by OGT siRNA, confirming the reduced *O*-GlcNAcylation in the knockdown cells (Fig. 3b). Proliferation of LNCaP knockdown cells, as assessed over 5 days using an MTS assay, was

reduced compared with control siRNA-transfected cells from day 2 to 5 ($P < 0.05$) (Fig. 3c). In addition, to evaluate effects of OGT knockdown on LNCaP cell invasion, we performed a Matrigel invasion assay using siRNA-transfected cells. The percent invasion of OGT siRNA-transfected cells was significantly decreased compared with control siRNA-transfected cells (Fig. 3d). Lastly, we compered the immunocytochemical expression of E-cadherin in OGT-knockdown LNCaP cells with control siRNA-transfected LNCaP cells. Although the expression of E-cadherin was weak in both siRNA-transfected LNCaP cells, the results showed that no significant difference was found between them (Fig. 3e).

DISCUSSION

Close association between *O*-GlcNAcylation and tumor progression in several cancers has been shown at *in vitro* level,[4-6] the clinicopathological significance of altered *O*-GlcNAc signalling to onset, progression, and metastasis of cancer is largely unknown.[10] In the present study, our immunohistochemical analysis

showed that overexpression of *O*-GlcNAc in prostate cancer cells compared with adjacent normal prostate glands was significantly associated with decreased overall survival of the patients based on multivariate analysis (see Table 3). This result was also supported by the *in vitro* experiment to examine the proliferation and invasion of LNCaP cells using siRNA-mediated OGT knockdown (see Fig. 3), demonstrating the close association of *O*-GlcNAc expression level and prostate cancer progression. Recently, Lynch et al. demonstrated that reduced *O*-GlcNAcylation in prostate cancer PC3-ML cells resulted in inhibition of invasion and angiogenesis by increased degradation of FoxM1 protein [6]. In other malignancies than prostate cancer, Caldwell et al. demonstrated the inhibition of tumor growth of breast cancer cells following RNA interference knockdown of OGT.[11] Furthermore Mi et al. demonstrated that *O*-GlcNAcylation significantly enhances anchorage-independent growth of lung and colon cancer cells.[4] Yu et al. showed that in breast cancer cells *O*-GlcNAcylation enhances cell migration and metastasis mainly through decreased cell surface expression of E-cadherin.[5] In the present study, we could not obtain significant association

between E-cadherin expression and OGT knockdown in LNCaP cells. However, we found that two distinct *O*-GlcNAcylated proteins with 39 kDa and 35 KDa were decreased in OGT-knockdown LNCaP cells. Although these proteins have not been characterized, it will be of great significance to determine the mechanism why increased *O*-GlcNAc expression level resulted in enhancement of proliferation and cell migration of the prostate cancer cells.

One physiological hallmark of tumors is use of aerobic glycolysis rather than oxidative phosphorylation to produce ATP (also known as the Warburg effect).[12] Aerobic glycolysis is a normal function of rapidly proliferating cells used to fill bioenergetic and biosynthetic needs.[13-14] For cancer cell proliferation, they require carbon- and nitrogen-rich nutrients to biosynthesize metabolites to proliferate rapidly.[15] In addition, cancer cells are addicted to glutamine: the rate of glutamine consumption in tumors is 10-fold higher than in normal cells.[16-17] Two – five % of glucose, which enters the cell, is used to produce uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) through the hexosamine

biosynthetic pathway (HBP).[18] Increased glucose is funneled into the HBP, thus promoting HBP flux and a consequent increase in *O*-GlcNAcylation.[15] Elevated UDP-GlcNAc and subsequent GlcNAcylation could explain why the Warburg effect constitutes an advantage for tumor progression in breast cancer.[5] Our results may imply that the increased *O*-GlcNAcylation in prostatic cancer cells resulted in progression of tumor cells via the Warburg effect. Future study will be of great importance to determine this problem.

In conclusion, this study indicates for the first time that *O*-GlcNAc overexpression in the cytoplasm of prostate cancer cells relative to corresponding benign prostate glands in prostate biopsies is significantly associated with decreased overall survival of patients. Additionally *O*-GlcNAcylation in prostate cancer cells may function in tumor cell invasiveness and proliferation. Understanding *O*-GlcNAcylation regulation in prostate cancer cells could lead to development of novel therapies against this disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. (a) Expression of *O*-GlcNAc and p63 in prostate adenocarcinoma. (Left) Representative cases showing overexpression (upper panels) and reduced expression (lower panels) of *O*-GlcNAc in cancer cells are shown. *O*-GlcNAc staining intensity was evaluated by comparison with adjacent non-neoplastic prostate glands (shown in insets). (Right) Immunostaining with p63 indicates basal cells in non-neoplastic glands (shown in insets). Bar = 50 μm. **(b)** Association between *O*-GlcNAc expression level and Gleason score of prostate adenocarcinoma. *O*-GlcNAc overexpression is significantly correlated with higher Gleason score (***P* < 0.01). Data represent the mean ± SD.

Figure 2. Kaplan-Meier analysis of overall survival based on *O*-GlcNAc expression level (***P* < 0.01).

Figure 3. Effects of OGT knockdown on proliferation and invasion of prostate cancer LNCaP cells. **(a)** Expression levels of OGT mRNA in LNCaP cells

transfected with OGT siRNA is significantly reduced compared with control siRNA (**$P < 0.01$). Fold expression of OGT is normalized to GAPDH expression. Data represent the mean ± SD. **(b)** *O*-GlcNAcylation levels in control siRNA-transfected LNCaP cells is decreased compared with OGT siRNA-transfected LNCaP. Molecular sizes of major *O*-GlcNAcylated proteins seen in control siRNA-transfected LNCaP cells are shown, and star (*) indicates the decreased *O*-GlcNAcylated proteins in OGT siRNA-knockdown LNCaP cells. Western blot analaysis using anti-*O*-GlcNAc antibody CTD110.6. **(c)** Cell growth of OGT knockdown LNCaP cells is significantly decreased compared with cells transfected by control siRNA (*$P < 0.05$). Vertical bars indicate SD. Shown are MTS assays done in triplicate. **(d)** Percent (%) invasion as determined by a Matrigel assay. The percent invasion of knockdown LNCaP cells is significantly decreased compared with cells transfected with control siRNA (**$P < 0.01$). Data represent the mean ± SD. **(e)** Expression of E-cadherin is not altered in knockdown LNCaP cells and control LNCaP cells. Normarski photographs of the same fields are also shown. Bar = 100 · m.

Table 1. Clinicopathological variables and *O*-GlcNAc levels in prostate cancer patients

Characteristics	*Median (range) or N (%)*
Age (years)	76 (60 - 91)
PSA	81 (4 - 11,800)
Gleason score	
7 or Less	22 (39.3%)
8	18 (32.1%)
9 or Greater	16 (28.6%)
Stage	
2	18 (32.1%)
3	10 (17.9%)
4	28 (50.0%)
O-GlcNAc expression	
Reduced expression	34 (60.7%)
Overexpression	22 (39.3%)

Table 2. Cox univariate hazard analysis of overall survival

	Overall survival		
	Hazard ratio	*95% CI*	*P value*
Age (years)			
69 or Less	1		
70 or Greater	0.43	0.17 – 1.36	0.14
PSA			
29.9 or Less	1		
30 or Greater	5.10	1.47 – 32.10	<0.01
Stage			
2 & 3	1		
4	3.10	1.21 – 8.55	0.018
Gleason score			
7 or Less	1		
8 or Greater	2.40	0.93 – 7.41	0.07
O-GlcNAc expression			

Reduced expression	1		
Overexpression	4.09	1.67- 10.96	<0.01

Table 3. Cox multivariate hazard analysis of overall survival

	Overall survival		
	Hazard ratio	*95%CI*	*P value*
PSA			
29.9 or Less vs 30 or Greater	3.06	0.76 – 20.73	0.12
Stage			
2 & 3 vs 4	1.27	0.44 – 4.17	0.66
Gleason score			
7 or Less vs 8 or Greater	1.19	0.40 – 3.69	0.84
O-GlcNAc expression			
Reduced expression vs Overexpression	2.75	1.01 – 8.27	0.0475

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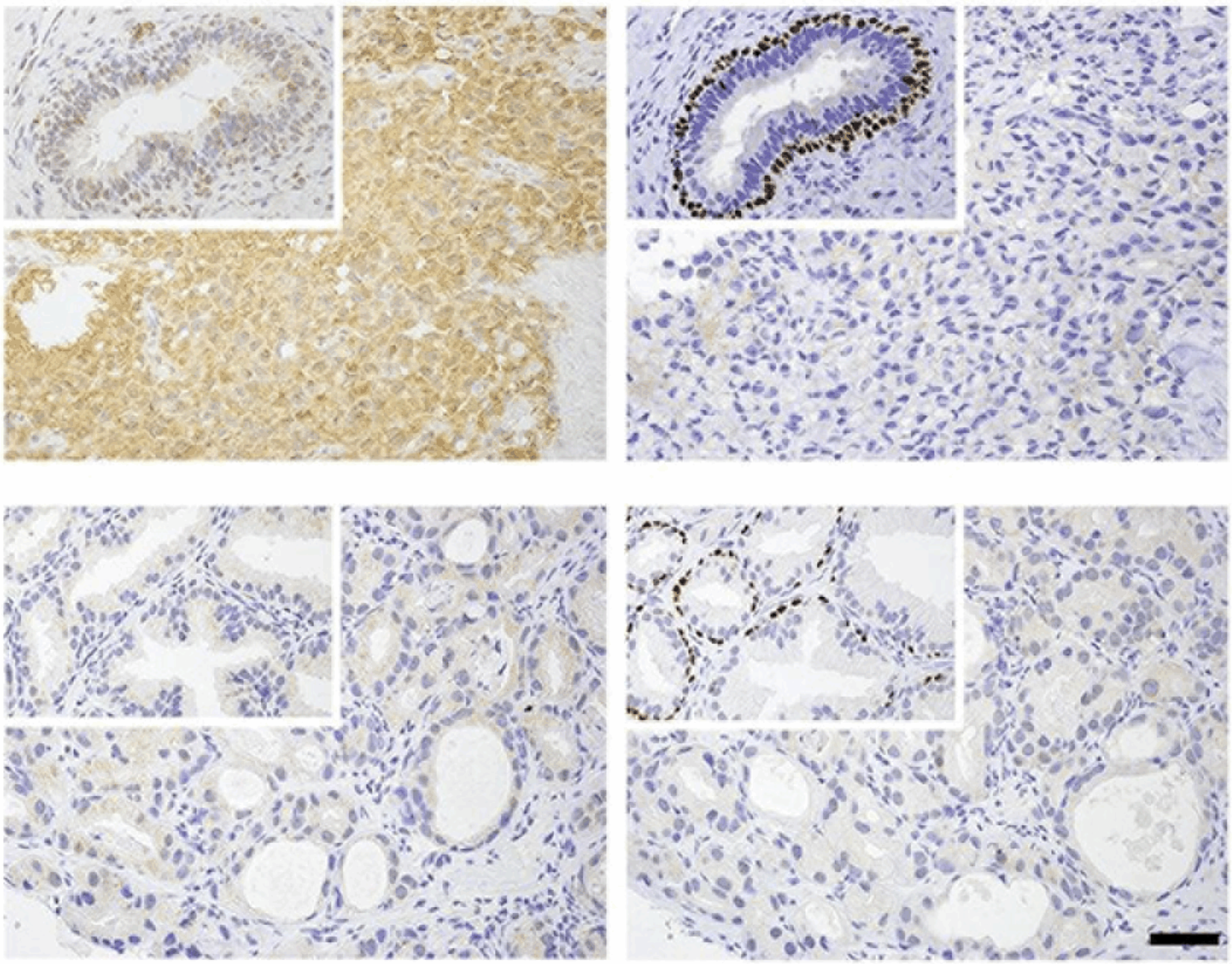
O-GlcNAc
p63

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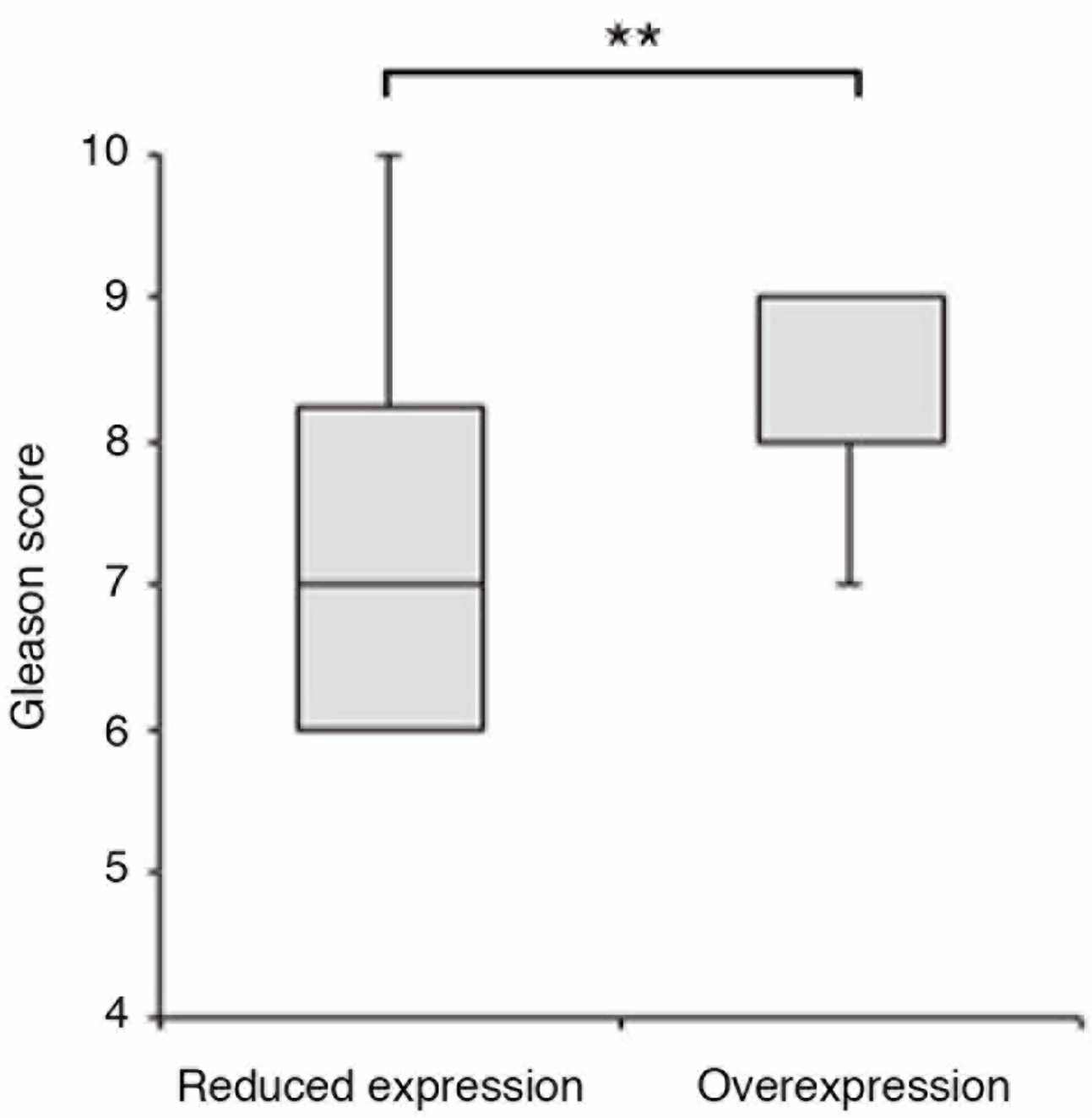
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10
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Gleason score
Reduced expression
Overexpression

Figure 1

x100 (%)

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Overall survival

Reduced expression (n = 34)

Overexpression (n = 22)

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0

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months

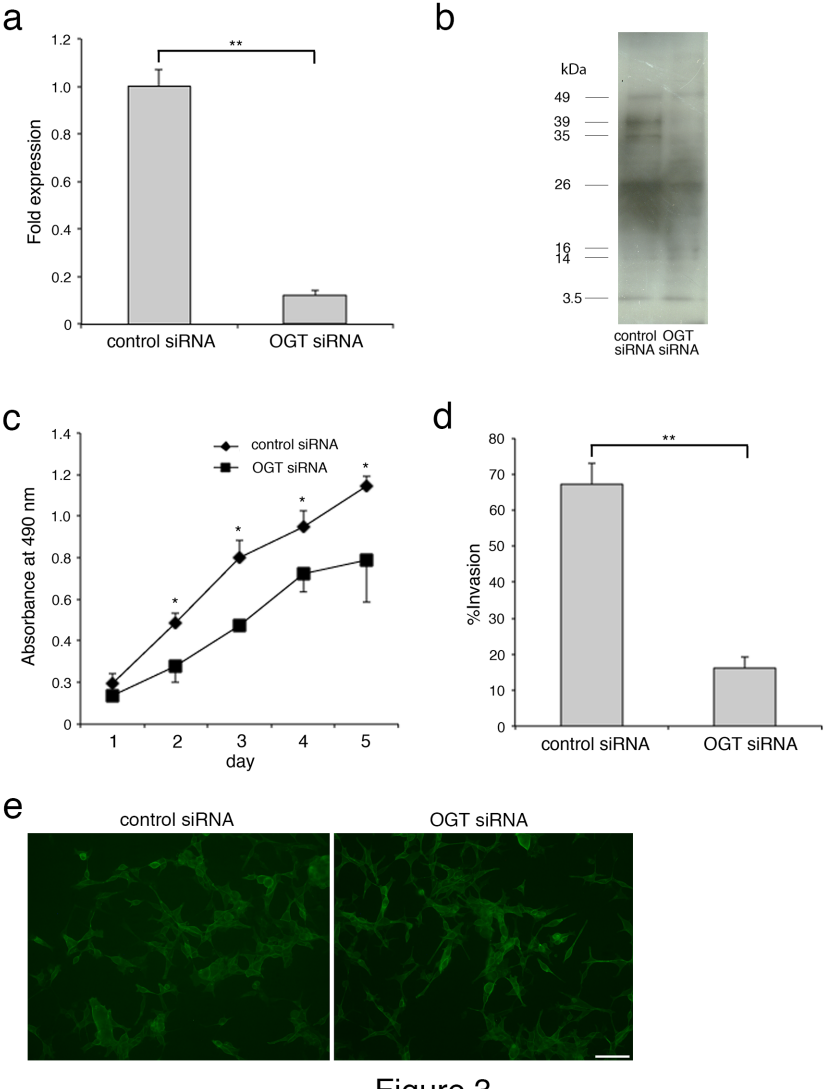
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Figure 3