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Polypeptide *N*-acetylgalactosaminyl transferase 3 independently predicts high-grade tumours and poor prognosis in patients with renal cell carcinomas

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Background: The polypeptide *N*-acetylgalactosaminyltransferases (GalNAc-Ts) family of enzymes regulates the initial steps of mucin-type O-glycosylation. *N*-acetylgalactosaminyltransferases might show novel patterns of GalNAc-T glycosylation on tumour-derived proteins, which could influence cancer biology, but its mechanisms are unclear. We investigated the association of GalNAc-T3 and -T6 expressions with clinicopathological features and prognoses of patients with renal cell carcinomas (RCCs).

Methods: Expressions of GalNAc-T3/6 and cell-adhesion molecules were analysed immunohistochemically in 254 paraffin-embedded tumour samples of patients with RCC.

Results: Of 138 GalNAc-T3+ cases, 46 revealed significant co-expression with GalNAc-T6. *N*-acetylgalactosaminyltransferases-3+ expression showed a close relationship to poor clinical performance and large tumour size, or pathologically high Fuhrman's grading, and presence of vascular invasion and necrosis. The GalNAc-T3-positivity potentially suppressed adhesive effects with a significantly low β -catenin expression. Univariate and multivariate analyses showed the GalNAc-T3+ group, but not the GalNAc-T6+ group, to have significantly worse survival rates.

Conclusion: *N*-acetylgalactosaminyltransferases-3 expression independently predicts high-grade tumour and poor prognosis in patients with RCC, and may offer a therapeutic target against RCC.

Renal cell carcinoma (RCC) is the most common primary malignancy of the kidney and one of the most lethal genitourinary malignancies (Siegel *et al*, 2012). Approximately 16 000 new cases of RCC are diagnosed each year; 7500 patients die of it in Japan alone (<http://www.ganjoho.jp/professional/index.html>, 2012), and it recurs within 5 years in up to 20% of RCC patients, even after

curative nephrectomy (Zisman *et al*, 2002). Metastasis of RCC is primarily responsible for its poor prognosis, although immunotherapy and several targeted agents, including tyrosine kinase inhibitors, prolong survival in certain populations (Motzer *et al*, 2009). Predicting which patients are prone to develop recurrence and mortality after surgery is therefore critical. Although

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clinicopathological factors, such as the tumour node metastasis (TNM) stage, Fuhrman's nuclear grading, Eastern Cooperative Oncology Group performance status (ECOG PS), or tumour necrosis, can indicate RCC prognosis (Finley *et al*, 2011), molecular and genetic factors have not notably done so; the clinical significance of biological markers is under evaluation. The clinical picture of RCC seems to be strongly determined by the complex interplay of additional cellular alterations, for example, epigenetic modulation of gene expression (Baldewijns *et al*, 2008).

Malignant transformation and progression in tumours are closely related to alterations in cell-surface carbohydrate antigens (CAs), and with frequent aberrant glycosylation (Brockhausen, 1999). Glycosylation, a major post-translational modification for most secretory and cellular proteins, can alter the physicochemical properties and biological activities of these proteins; in cancer, it can change glycoprotein functions and transform cellular phenotypes.

O-glycosylation encompasses diverse classes of glycoproteins. In mammals, the most common types are mucin-type O-linked glycans, which constitute up to 80% of the total amount of CAs (Brockhausen, 1999; Hollingsworth and Swanson, 2004). Mucin-type O-glycosylation in the Golgi apparatus is initiated by uridine diphosphate (UDP) *N*-acetyl- α -D-galactosamine polypeptide (GalNAc) *N*-acetylgalactosaminyltransferases (GalNAc-Ts), which catalyse the transfer of GalNAc from the sugar donor UDP-GalNAc to serine and threonine residues on the protein-synthesising CAs (Schwientek *et al*, 2002; Ten Hagen *et al*, 2003). O-linked glycans are aberrantly expressed in many epithelial cancers with the presentation of shortened glycan structures and changes in sialylation patterns, which could affect cell differentiation, adhesion, invasion, and metastasis (Brockhausen, 1999; Schwientek *et al*, 2002; Ten Hagen *et al*, 2003). However, they are also involved in other numerous biological functions and processes such as fertilisation, anticoagulation, or inflammatory responses (Dwek, 1996; Rudd *et al*, 2001). To date, 15 distinct members of the GalNAc-T family have been identified and characterised in mammals; as many as 24 human isozymes of it may exist (Schwientek *et al*, 2002; Ten Hagen *et al*, 2003). Interestingly, the GalNAc-Ts show tissue-specific expression and have different (but partly overlapping) kinetic properties and acceptor substrate specificities. In contrast, some of them work in a concerted, hierarchical manner to form O-glycans (Ten Hagen *et al*, 2003). Several studies have indicated that GalNAc-T3 might be useful in the evaluation of various cancer types, including the colorectal (Shibao *et al*, 2002), lung (Dosaka-Akita *et al*, 2002; Gu *et al*, 2004), gastric (Ishikawa *et al*, 2004), gallbladder (Miyahara *et al*, 2004), pancreatic (Yamamoto *et al*, 2004; Li *et al*, 2011), prostatic (Landers *et al*, 2005), and extrahepatic bile duct carcinomas (Inoue *et al*, 2007). Furthermore, it is possible that GalNAc-Ts expression is not only a cancer-specific event but also found in inflamed tissue.

N-acetylgalactosaminyltransferases-6 shows high homology in DNA and amino-acid sequence to GalNAc-T3 throughout the coding region, with identical organisation for nine conserved intron/exon boundaries in the coding regions, and similar kinetic properties that are distinct from other GalNAc-Ts (Schwientek *et al*, 2002; Ten Hagen *et al*, 2003; Li *et al*, 2011). *N*-acetylgalactosaminyltransferases-6 could thus also be a marker for aberrant O-linked glycans in carcinogenesis. However, we have seen no studies of possible associations between GalNAc-T3 and/or -T6 expressions in RCCs and their clinicopathological features, including tumour grade or patients' prognoses.

In the current study, using specific polyclonal antibodies against GalNAc-T3 and -T6, we evaluated expressions of GalNAc-T3/6 in RCC specimens with patients' clinicopathological data, showing that only GalNAc-T3 was expressed in most human RCCs, especially those with worse ECOG PS, or higher Fuhrman's grade

and vascular invasion (VI). Furthermore, GalNAc-T3+ expression has been significantly associated with a poor outcome.

MATERIALS AND METHODS

Patients and tissue specimens. All the intended procedures of the present study, including use of specimens from human subjects, were approved by the Ethics Committee of University of Occupational and Environmental Health (UOEH) in Kitakyushu, Japan. Pathological reports were reviewed to identify patients who underwent radical or partial nephrectomy for RCC between 1999 and December 2011 at the hospital of UOEH. Patients who suffered perioperative deaths, defined as death during the patient's initial hospitalisation or within 30 days of surgery, were excluded. A total of 254 patients with available follow-up data comprised the cohort of this retrospective study, after further excluding those with the following characteristics: (a) other prior or concomitant malignant tumours; (b) coexisting medical problems of sufficient severity to shorten life expectancy; (c) adjuvant therapies after surgery; and (d) non-carcinomas. Three pathologists examined all resected specimens to confirm their histopathological features. The TNM system of the Union for International Cancer Control (UICC) 7th Edition was used for staging (Sobin *et al*, 2009); all RCCs were graded based on the Fuhrman four-tiered nuclear grading system (Fuhrman *et al*, 1982). Clinical information was gathered from patients' records. The survival duration was based on the date of surgery until patient's death or most recent clinical visit. Each patient was assigned an ECOG PS score at the time of diagnosis. Patients were categorised as low, intermediate, or high risk, and were prospectively evaluated every 3 months for the first 2 years after surgery, every 6 months for the next 3 years, and yearly thereafter, using chest X-ray or thoracic CT scan, abdominal ultrasonography or CT scan or MRI, and serum biochemistry. A few patients with metastases but without medical contraindications were given palliative interferon- α - and interleukin-2-based immunotherapy. No targeting agents such as tyrosine kinase inhibitors were used. Formalin-fixed, paraffin-embedded tissue blocks came from our Department of Pathology. Normal human tissue was taken from non-tumour portions of surgically resected specimens, and then stained with haematoxylin and eosin, elastica van Gieson (EVG), or immunohistochemical preparations in sequential sections. Elastica van Gieson staining revealed VI very clearly.

Preparation of antibodies against GalNAc-Ts and other Golgi enzymes. Polyclonal antibody was raised against GalNAc-T3 by multiple immunisations of New Zealand white rabbits with a synthetic peptide, based on the previously published work (sequence: GYYTAAELKPVLDRPPQDS; Nomoto *et al*, 1999). Anti-GalNAc-T6 polyclonal antibody was generated in the same way, as described in the previously published paper (synthetic peptide sequence: GFYTPAELKPFWERPPQDP; Li *et al*, 2011). The specificity of our two antibodies was confirmed by western blotting and immunohistochemistry, with peptide competition against GalNAc-T3 (Nomoto *et al*, 1999; Dosaka-Akita *et al*, 2002) and GalNAc-T6 (Li *et al*, 2011), respectively. For immunohistochemistry of GalNAc-T3, we used well-differentiated tubular colorectal adenocarcinoma cells as positive controls (Shibao *et al*, 2002); well-differentiated tubular pancreatic adenocarcinoma cells were used as positive GalNAc-T6 controls (Li *et al*, 2011).

To analyse staining or expression profiles of other Golgi enzymes or GalNAc-Ts, rabbit polyclonal anti- β -1,2-*N*-acetylglucosaminyltransferase II (GnT-II), goat polyclonal anti-*N*-acetylglucosaminyltransferase III (GnT-III), -*N*-acetylglucosaminyltransferase V (GnT-V), and -Golgi α -mannosidase II (MAN2A1) antibodies (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or

anti-GalNAc-T1, -T2, and -T4 goat monoclonal antibodies (1:50; Santa Cruz Biotechnology) were applied.

Immunohistochemistry of tissue samples. Immunohistochemical staining was performed by the antibody-linked dextran polymer method (EnVision; DAKO Cytomation, Tokyo, Japan). Deparaffinised and rehydrated 5- μ m sections were incubated in 10% H₂O₂ for 5 min to block endogenous peroxidase activity. The sections were thereafter rinsed and incubated with rabbit polyclonal anti-GalNAc-T3 (diluted 1:3000) and -T6 (diluted 1:1000), mouse monoclonal β -catenin (diluted 1:100, BD Biosciences, San Jose, CA, USA), and mouse monoclonal E-cadherin (diluted 1:1000, BD Biosciences) antibodies for 30 min. The second antibody-peroxidase-linked polymers were then applied, and the sections were incubated with a solution consisting of 20 mg of 3,3'-diaminobenzidine tetrahydrochloride, 65 mg of sodium azide, and 20 ml of 30% H₂O₂ in 100 ml of Tris-HCl (50 mM, pH7.6). After counterstaining with Meyer's haematoxylin, sections were observed under a light microscope. For immunohistochemistry of RCC tissues, positive areas comprising <10% of the neoplasms were considered negatively stained. For cytoplasmic GalNAc-Ts expressions and apical membranous E-cadherin and β -catenin expressions, respectively, positive areas that were \geq 10% were considered positively stained and were graded into three categories: weak, positive area of 10–30%; moderate, 30–80%; and strong, >80% positive areas. All histological and immunohistochemical slides were evaluated by two independent observers (certified surgical pathologists in our department) using a blind protocol design (observers were blinded to the clinicopathological data). Agreement between observers was excellent (>0.9) for all antibodies investigated, as measured by interclass correlation coefficient. For the few instances of disagreements, a consensus score was determined by the third board-certified pathologists in our department.

Cell culture. Human RCC cell line KPK1 (a kind gift from the Department of Urology, Faculty of Medicine, Kyushu University, Japan) and human lung adenocarcinoma cell line PC9 (Igarashi *et al*, 2007) were maintained in DMEM and RPMI medium, respectively, containing 10% foetal calf serum (Gibco, Carlsbad, CA, USA) at 37 °C in an atmosphere of 95% air and 5% CO₂ (Naito *et al*, 1982).

Immunofluorescence of RCC tissue samples and cell line KPK1. The frozen sections of human RCC and non-RCC tissue samples were labelled with the above polyclonal rabbit GalNAc-T3 (1:3000) and -T6 (1:1000) antibodies, polyclonal rabbit G α T-II, polyclonal goat G α T-III, -V, and MAN2A1 (1:100; Santa Cruz Biotechnology) antibodies or monoclonal goat GalNAc-T1, -T2, and -T4 (1:50; Santa Cruz Biotechnology) antibodies, incubated with Hoechst 33258 (0.5 mg ml⁻¹; Dojindo, Kumamoto, Japan), and then visualised with goat anti-rabbit or donkey anti-goat IgG antibodies conjugated with Alexa Fluor Dyes (green-stained; Invitrogen, Carlsbad, CA, USA).

The RCC cell line KPK1 was cultured on coverslips, fixed with 95% acetone for 5 min, and allowed to air dry. Cells were then incubated with Hoechst 33258 (0.5 mg ml⁻¹; Dojindo) and anti-GalNAc-T3 or -T6 antibodies for 1 h at room temperature (RT), washed with PBS, and reacted with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG for 1 h at RT. After being washed with PBS, specimens were observed under a Nikon (Tokyo, Japan) Eclipse E600 inverted fluorescence microscope.

GalNAc-T3 and GalNAc-T6 siRNA knockdown. Twenty-five base pair double-stranded RNA oligonucleotides were commercially generated (Invitrogen): 5'-AAACAUGUCCAACAACA GAGCAAGG-3' and 5'-CCUUGCUCUGUUGUUGGACAUG UUU-3' for GalNAc-T3#84; 5'-UUCUGCUGCUGUAUAAU AUCCUUGC-3' and 5'-GCAAGGAUUAUUAACAGCAGCAG AA-3' for GalNAc-T3#85; 5'-UUUCUCUUGGGCCAUCUUUG

CUGCC-3' and 5'-GGCAGCAAAGAUGGCCCAAGAGAAA-3' for GalNAc-T6#36; and 5'-UUCUCAUGUGGAGGAAGUGUU UCCC-3' and 5'-GGGAAACACUCCUCCACAUGAGAA-3' for GalNAc-T6#38. siRNA transfections were performed according to the manufacturer's instructions with modifications (Invitrogen; Igarashi *et al*, 2007; Miyamoto *et al*, 2008). Five microlitres of Lipofectamine 2000 (Invitrogen) was diluted in 250 μ l Opti-MEM medium (Invitrogen) and incubated for 5 min at RT. Next, 250 pmol of GalNAc-T3, GalNAc-T6 and Lipofectamine as control siRNA diluted in 250 μ l Opti-MEM were added gently and incubated for 20 min at RT. Oligomer-lipofectamine complexes and aliquots of 2 \times 10⁵ PC9 cell (human lung adenocarcinoma cell line; Igarashi *et al*, 2007) in 500 μ l culture medium were combined and incubated for 48 h. Whole-cell extracts (50 μ g) were prepared from PC9 after siRNA transfection and western blotting was performed.

Western blotting. The PC9 cells after siRNA transfection were washed and lysed with RIPA buffer. Whole-cell lysates were prepared as previously described (Igarashi *et al*, 2007). Whole-cell lysates (100 μ g) were separated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinylidene difluoride membranes (Bio-Rad Laboratories, K.K., Tokyo, Japan) using a semi-dry blotter. The blotted membranes were treated with 5% (w/v) skimmed milk in 10 mM Tris, 150 mM NaCl, and 0.2% (v/v) Tween 20, and were incubated for 1 h at RT with the primary antibody. The following antibodies and dilution were used: 1:1000 dilution of anti-GalNAc-T3 and anti-GalNAc-T6, and 1:5000 dilution of anti- β -actin (Santa Cruz Biotechnology). The membranes were then incubated for 45 min at RT with a peroxidase-conjugated secondary antibody, visualised using an ECL kit (GE Healthcare Bio-Science, Buckinghamshire, UK).

The bands on the western blots were analysed densitometrically using Scion Image software (version 4.0.2; Scion Corp., Frederick, MD, USA).

Statistical analysis. Significance of correlations was calculated by χ^2 -test to assess relationships between immunohistochemical expressions and clinicopathological variables. Survival curves were plotted using the Kaplan–Meier method and compared with the log-rank test. Hazard ratios and 95% confidence intervals were estimated using univariate or multivariate Cox proportional-hazard models. All statistical tests were two-tailed, with $P < 0.05$ considered significant. All above statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0; Kanda, 2013). More precisely, it is a modified version of R commander (version 1.6-3) designed to add statistical functions frequently used in biostatistics.

RESULTS

Patient characteristics. The cohort included 254 patients (181 men, 73 women) with clinicopathological features representative of RCC (Table 1). The average age at surgery was 63 years. Most patients (191 out of 254) were ECOG 0 patients; the remaining patients (63 out of 254) were \geq 1 PS. The median tumour size was 4.5 cm (range: 1.0–20.0 cm). At diagnosis, 14 patients had lymph node metastases and 34 had distant metastases. Most tumours (218) were classified as clear cell RCC. On the basis of the UICC criteria, 161 patients had stage I disease. Post-operative follow-up was available for all 254 patients (median: 49.0 months; range: 1.0–163.1 months). Median post-operative disease-specific survival (DSS) duration was 36.6 months, with 1- and 5-year survival rates of 96% and 84%, respectively.

Table 1. Detailed patients' clinicopathological characteristics

Characteristic	Patients (n = 254)
Age (years)	
Average	63
Median	64
Range	30-84
> 60	160
≤ 60	94
Sex	
Male	181
Female	73
ECOG PS	
≥ 1	63
0	191
Months after surgery	
Average	49
Median	37
Range	1-163
Tumour size (cm)	
Average	5.2
Median	4.5
Range	1-20
Tumour stage	
I	161
II	21
III	36
IV	36
Regional lymph metastasis	
N0	240
N+	14
Distant metastasis	
M0	220
M+	34
Fuhrman's grade (G)	
G1	41
G2	156
G3	38
G4	19
Histopathological subtype	
Clear cell RCC	218
Papillary RCC	19
Chromophobe RCC	4
Unclassified RCC	13
Abbreviations: ECOG PS = Eastern Cooperative Oncology Group performance status; RCC = renal cell carcinoma.	

GalNAc-Ts or other Golgi enzyme expression in normal tissues and RCC specimens. Specificity of GalNAc-T3/6 polyclonal antibodies was tested using immunohistochemistry and western blotting (Nomoto *et al*, 1999; Dosaka-Akita *et al*, 2002; Li *et al*, 2011). *N*-acetylgalactosaminyltransferases-3 and -6 showed only cytoplasmic immunohistochemical expression (Figure 1).

N-acetylgalactosaminyltransferases-3/6 expressions were not detectable or very weakly detectable in adjacent normal tubular epithelium on paraffin-embedded tissues (Figure 1); whereas GalNAc-T3 expression was occasionally and weakly seen in the proximal tubular epithelium on frozen samples, GalNAc-T6 was not (Supplementary Figure 1). *N*-acetylgalactosaminyltransferases-3 was expressed in 138 of 254 (54%) RCC specimens: 41% weak, 10% moderate, 3% strong. GalNAc-T6 was expressed in 62 of 254 (24%) specimens: 23% weak, 0% moderate, 1% strong. When GalNAc-Ts expression was split into groups of either positive (weak to strong staining) or negative, the GalNAc-T3/6 immunoprofile was 39.4 -/-, 36.2 +/-; 6.3 -/+, and 18.1% +/+. Correspondingly, frozen RCC tissue samples showed strong expression of GalNAc-T3 but weak expression of GalNAc-T6 (Supplementary Figure 1), whereas other GalNAc-Ts (-T1, -T2, and -T4) were expressed neither in RCC nor in adjacent normal tubular epithelium on frozen samples (data not shown).

In contrast with the GalNAc-T3 expression profile, frozen RCC specimens demonstrated no expression of other Golgi enzymes, GnT-II and -III, or very weak expression of GnT-V and MAN2A1, despite the specific and functional expression of GnT-II, -III, and -V and MAN2A1 in non-RCC tissues (Supplementary Figure 1). Hence, these obtained data can support that the staining with GalNAc-T3 reflects results of specifically glycosylated structures, but not of merely increased Golgi glycosylation in hyperproliferating RCC cells.

Analysis of GalNAc-Ts expression in human RCC tissues and cell line. Immunofluorescence staining of the RCC surgical specimens and RCC cell line (KPK1) showed specific expression of cytoplasmic GalNAc-T3 in a perinuclear fashion, but very weak expression of cytoplasmic GalNAc-T6 (Supplementary Figures 1 and 2A).

Analysis of specific GalNAc-T3/6 expressions in human cancer cell lines. Western blotting revealed that both proteins of GalNAc-T3 and GalNAc-T6 were expressed in PC9 cell lines. We performed siRNA transfection of GalNAc-T3 or GalNAc-T6 to the wild-type PC9 cells. Expression of GalNAc-T3 was significantly decreased in PC9 cells of GalNAc-T3 siRNA, but not in PC9 cells of GalNAc-T6 siRNA (Supplementary Figure 2B). On the other hand, expression of GalNAc-T6 was significantly downregulated in PC9 cells of GalNAc-T6 siRNA, whereas GalNAc-T6 protein was expressed very well in PC9 cells of GalNAc-T3 siRNA (Supplementary Figure 2B). These western blotting analyses further confirmed that each antibody of GalNAc-T3 and GalNAc-T6 was specific for each protein of GalNAc-T3 and GalNAc-T6, respectively.

Association of GalNAc-T3/6 expression with clinicopathological variables. To identify association of GalNAc-Ts expression (GalNAc-Ts - vs GalNAc-Ts +) with clinicopathological characteristics of the cohort, the variables were split as shown in Table 2. There were no significant differences between patients with GalNAc-T3/6 - and GalNAc-T3/6 + tumour expressions in terms of age and gender ($P > 0.05$). However, GalNAc-T3 + expression was closely associated with > 1 ECOG PS ($P = 0.02$), high tumour Fuhrman's grade (Figure 1), and advanced disease stage manifesting as larger tumour size and presence of VI and necrosis ($P < 0.001$) in all tumours. GalNAc-T6 stain status also significantly correlated with ECOG PS ($P = 0.03$), tumour Fuhrman's grade (Figure 1), VI ($P < 0.001$), and necrosis ($P = 0.01$). Particularly, both GalNAc-T3 and -T6 expressions were evident in VI of high-grade RCC components, as shown by EVG stains (Supplementary Figure 3). The high-grade ($> G3$) tumour rate in GalNAc-T3 + samples was 49 out of 138 (35.5%), but 7 out of 116 (6.0%) in GalNAc-T3 - samples. In the GalNAc-T3 + group, 97 out of 138 cases (70.2%) were classified as \geq stage Ib by UICC

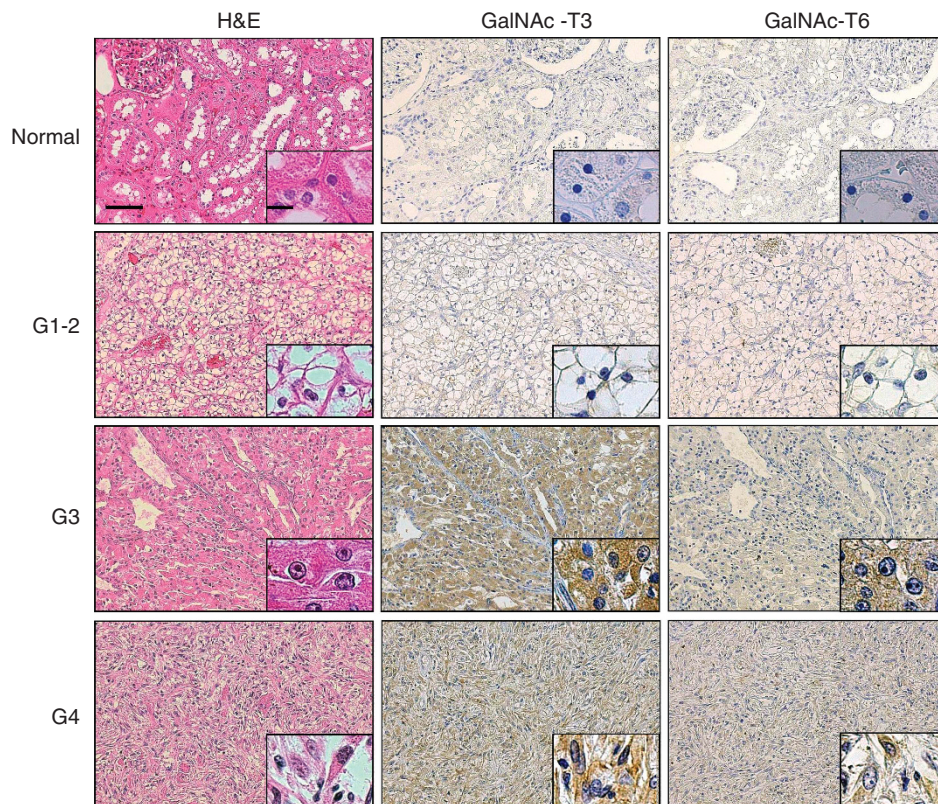


Figure 1. Representative images of immunohistochemical analyses of GalNAc-T3 and GalNAc-T6 in human RCC (GalNAc-T3: G1–G2, negative; G3: strongly positive; G4: strongly positive; GalNAc-T6: G1–G2, negative; G3, weakly positive; G4: weakly positive) and normal tubule specimens (GalNAc-T3: negative; GalNAc-T6: negative) Original magnification: $\times 100$; inset: $\times 400$. Bar = $100\ \mu\text{m}$ ($\times 100$) or $20\ \mu\text{m}$ ($\times 400$). Abbreviations: H&E = haematoxylin and eosin; G = Fuhrman's grade.

TNM classification, compared with 53 out of 116 (45.7%) in the GalNAc-T3 – group.

In a Kaplan–Meier analysis (Figure 2), RCC patients with GalNAc-T3/6+ expression had a significantly shorter post-operative median DSS (-T3: 34.5 months; -T6: 34.5 months) compared with those who had GalNAc-Ts – expressions, (-T3: 40.5 months; -T6: 37.0 months; -T3: $P < 0.001$; -T6: $P = 0.03$, Figure 2A and B). For patients with GalNAc-T3+ RCCs, increasing levels of GalNAc-T3 expression were not associated with worse clinicopathological features or longer survival (data not shown).

GalNAc-T3 represents an independent prognostic indicator for RCC. To assess whether GalNAc-T3/6 expressions were independent predictors of post-operative DSS, a Cox proportional-hazards model was created in a forward fashion including only covariates that had statistically significant correlations with DSS, using an inclusion threshold of $P < 0.05$ (Table 3). Univariate analysis showed that ECOG PS, tumour size, Fuhrman's grade, presence of VI and necrosis, and GalNAc-T3/6+ status were significant predictors of poorer survival ($P < 0.001$, $= 0.002$, < 0.001 , < 0.001 , $= 0.005$, < 0.001 , and 0.03 , respectively). Furthermore, multivariate analysis demonstrated that, after correction for confounding variables, GalNAc-T3+ expression remained an independent prognostic indicator for DSS, as did ECOG PS, Fuhrman's grade, and VI ($P = 0.03$, 0.02 , 0.002 and 0.001 , respectively).

Complementary correlations between GalNAc-T3 and -T6. Besides, this cohort was divided in two ways: (i) double negative vs either GalNAc-T3+ or -T6+ and (ii) GalNAc-T3+ -only vs GalNAc-T6+ -only (Figure 3A–C); the Kaplan–Meier method was used to verify them. The DSS of RCC patients showed

significance and borderline significance (-T3: $P < 0.001$ and -T6: $P = 0.06$, respectively) in the former (i) classification approach, but not in the latter (ii) $P = 0.3$, indicating that there were complementary, but not competitive, correlations between GalNAc-T3 and -T6.

Competitive correlations between GalNAc-T3 and adhesion molecule. Immunohistochemical expressions of β -catenin and E-cadherin were present in 144 (57%) and 65 (26%) of 254 specimens, respectively. When GalNAc-T3 and β -catenin expressions were split into groups of either positive or negative, their immunoprofiles were 10.2 -/–, 33.1, +/–, 35.4 -/+ , and 21.3% +/+ . These +/- and -/+ subgroups, present in 84 and 90 cases, respectively, showed significant correlation with VI ($P < 0.001$). The DSS of RCC patients, similarly divided into two: (i) -/- vs +/- (Figure 4A) or -/+ (Figure 4B), and (ii) +/- vs -/+ (Figure 4C), revealed significant differences ($P < 0.01$; Figure 4C) only in the latter (ii) classification approach, indicating that there were significantly competitive correlations between the GalNAc-T3 and β -catenin (Tables 2 and 3). Actually, VI components of high-grade RCCs showed immunohistochemical, GalNAc-T3+ but β -catenin– expression (Supplementary Figure 3). By contrast, no significant immunoprofile relationships were detected between GalNAc-T3 and E-cadherin (data not shown).

DISCUSSION

In the current large cohort, we showed how GalNAc-T3 and -T6 expressions could be closely related to various clinicopathological characteristics of the 254 patients with RCCs, using antihuman

polyclonal GalNAc-Ts antibodies raised against the distinctive synthetic peptides. Our collected data, including immunofluorescence or western blotting after siRNA knockdown, can confirm

Table 2. Detailed correlation between GalNAc-T3 or -T6 expression and clinicopathological variables

	Total n (%)	GalNAc-T3 positive, n (%)		GalNAc-T6 positive, n (%)	
Variables	n = 254	Positive n = 138	P-value	Positive n = 62	P-value
Age			0.18		0.77
> 60 years	160 (63.0)	92 (66.7)		40 (64.5)	
≤ 60 years	94 (37.0)	46 (33.3)		22 (35.5)	
Sex			0.45		0.55
Male	181 (71.3)	101 (73.2)		46 (74.2)	
Female	73 (28.7)	37 (26.8)		16 (25.8)	
ECOG PS			0.02		0.03
≥ 1	63 (24.8)	42 (30.4)		22 (35.5)	
0	191 (75.2)	96 (69.6)		40 (64.5)	
Tumour size			<0.001		0.19
> 4 cm	150 (59.0)	97 (70.3)		41 (66.1)	
≤ 4 cm	104 (41.0)	41 (29.7)		21 (33.9)	
Fuhrman's grade (G)			<0.001		<0.001
≥ G3	56 (22.0)	49 (35.5)		25 (40.3)	
≤ G2	198 (78.0)	89 (64.5)		37 (59.7)	
VI			<0.001		<0.001
Positive	77 (30.3)	59 (42.8)		27 (43.5)	
Negative	177 (69.7)	79 (57.2)		35 (56.5)	
Necrosis			<0.001		0.01
Positive	126 (49.6)	88 (63.8)		39 (62.9)	
Negative	128 (50.4)	50 (36.2)		23 (37.1)	
GalNAc-T3			—		<0.001
Positive	138 (54.3)	—		46 (74.2)	
Negative	116 (45.7)	—		16 (25.8)	
GalNAc-T6			<0.001		—
Positive	62 (24.4)	46 (33.3)		—	
Negative	192 (75.2)	92 (66.7)		—	
β-catenin			<0.001		0.96
Positive	144 (56.7)	54 (39.1)		35 (56.5)	
Negative	110 (43.3)	84 (60.9)		27 (43.5)	

Abbreviations: ECOG PS = Eastern Cooperative Oncology Group performance status; GalNAc-T = N-acetylgalactosaminyltransferases; VI = vascular invasion.

that those antibodies used in the present studies have not only been very sensitive to GalNAc-T3/6 but also can support that the selective staining with GalNAc-T3, as shown here in RCC samples, reflects significant upregulation of specific glycosyltransferases concomitant with specific and functional effects, but not of merely increased Golgi glycosylation in rapidly growing RCC cells. The present findings indicate, for the first time, that GalNAc-T3 is an independent novel and powerful marker for poor prognosis in patients with RCCs, whereas GalNAc-T6 is not. Our results show GalNAc-T3 + expression in RCC (1) to be significantly closely related with poor ECOG PS, large tumour size, high Fuhrman's grade, and presence of VI and necrosis, manifesting as advanced-stage under the TNM system of UICC, that is, poorly differentiated characteristics or invasive/aggressive behaviours; (2) has significantly high co-expression with, and has potentially complementary relevance to, GalNAc-T6; and (3) apparently ameliorates adhesive effects together with significant low expression of β-catenin, seemingly leading to severe VI. A diagram depicting GalNAc-T3's relationships from this study is summarised in Supplementary Figure 4. Furthermore, we can present a critical RCC-specific molecule, GalNAc-T3, which could potentially be a tumour marker or therapeutic target for RCC.

O-linked glycans are aberrantly expressed in many epithelial cancers with the presentation of shortened glycan structures and changes in sialylation patterns, which could affect differentiation, adhesiveness, and invasion of various cancer cells (Brockhausen, 1999; Schwientek *et al*, 2002; Ten Hagen *et al*, 2003). Our immunohistochemical examination in RCC displayed a subcellular cytoplasmic pattern for GalNAc-Ts, apparently localised to the Golgi apparatus (Grabenhorst and Conrad, 1999). In contrast, no or very weak staining was noted in adjacent normal tubular epithelium, although proximal tubule epithelial cells were rarely stained for GalNAc-T3. This implies its potentially crucial role in acquired renal carcinogenesis through aberrant O-linked glycosylation. GalNAc-T3 could be a specific diagnostic tumour marker for RCC. As GalNAc-Ts appear in external secretions such as the colostrum (Hagen *et al*, 1993; Bennett *et al*, 1996), GalNAc-T3 could be secreted into body fluids as a quantitative soluble marker. Moreover, current data suggest that GalNAc-T3 is an ideal therapeutic target with minimum risk of side effects, a nontrivial consideration, as adverse reactions caused by anticancer agents are a serious issue in clinical management. We reported that positive GalNAc-T3 expression is found in normal pancreatic ducts and bronchial epithelium (Dosaka-Akita *et al*, 2002; Li *et al*, 2011), and many carcinoma types (Shibao *et al*, 2002; Gu *et al*, 2004; Ishikawa *et al*, 2004; Miyahara *et al*, 2004; Yamamoto *et al*, 2004; Landers *et al*, 2005; Inoue *et al*, 2007; Li *et al*, 2011). Obviously, however, the utility of GalNAc-T3 inhibitors as therapeutic modalities requires much further study.

Our obtained results accord with studies of several other carcinomas, including colon (Shibao *et al*, 2002), gastric (Ishikawa *et al*, 2004), gallbladder (Miyahara *et al*, 2004), prostate (Landers

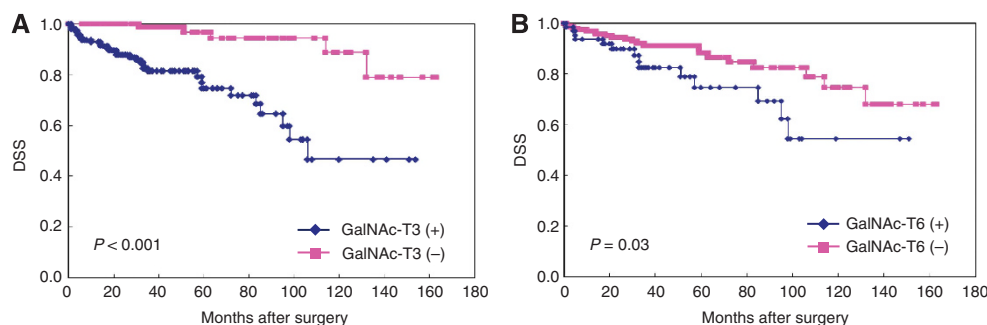


Figure 2. Kaplan–Meier curves of disease-specific survival (DSS) in patients with RCCs after surgery according to GalNAc-T3 (A) and GalNAc-T6 (B).

Table 3. Univariate and multivariate analyses of survival in 254 patients with RCC, according to clinicopathological variables and GalNAc-T3/6 expressions

Risk factors	Univariate			Multivariate		
	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
ECOG PS	5.43	2.78–10.58	<0.001	2.45	1.19–5.05	0.02
Tumour size	4.46	1.73–11.48	0.002	1.24	0.42–3.61	0.70
Fuhrman's grade	10.57	5.07–22.03	<0.001	3.96	1.63–9.64	0.002
VI	9.46	4.27–20.94	<0.001	4.63	1.80–11.92	0.001
Necrosis	2.84	1.37–5.90	0.005	0.71	0.30–1.66	0.42
GalNAc-T3 expression	7.20	2.77–18.73	<0.001	3.43	1.13–10.38	0.03
GalNAc-T6 expression	2.09	1.06–4.09	0.03	0.95	0.44–2.06	0.89
β -catenin expression	0.90	0.46–1.76	0.76	0.74	0.36–1.52	0.41

Abbreviations: CI = confidence intervals; ECOG PS = Eastern Cooperative Oncology Group performance status; VI = vascular invasion. The bold entries indicate specifically independent prognostic indicators for DSS in RCC.

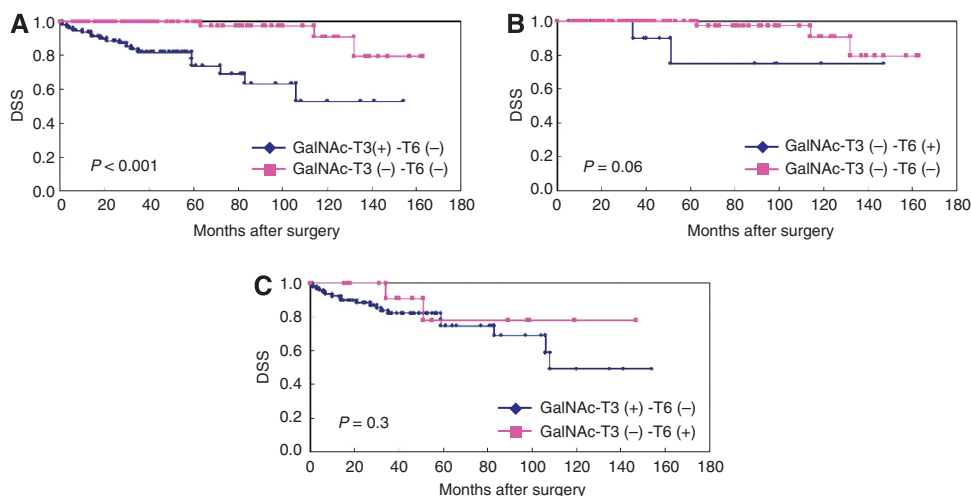


Figure 3. Kaplan–Meier curves of disease-specific survival (DSS) in patients with RCC after surgery according to split approaches to GalNAc-T3/6 immunoprofiles: (i) double negative vs either GalNAc-T3 (A) or -T6 (B) positive; and (ii) GalNAc-T3 + only vs GalNAc-T6 + only (C).

et al, 2005), and extrahepatic bile duct (Inoue *et al*, 2007) epithelial cancers, where upregulated GalNAc-T3 expression was closely associated with more aggressive tumour behaviour and poor outcome. The present cohort study shows GalNAc-T3 as a powerful and independent negative indicator of DSS in patients with RCC and, by extension, GalNAc-T3 may be a novel prognostic marker for an array of different cancer types. Reportedly, the overall 5-year survival rate is 60% in RCC, but <10% in RCC patients with metastases (Cohen and McGovern, 2005). Cytokines or novel targeted therapies also seem to be of limited benefit, even after complete resection of primary RCCs (Escudier *et al*, 2007; Motzer *et al*, 2009). *N*-acetylgalactosaminyltransferases-3 expression patterns in primary RCC should allow improved patient selection for adjuvant systemic therapies soon after surgery and prediction of appropriate post-operative courses. Although relapse occurs in 20–30% of RCC surgical cases (Zisman *et al*, 2002), there have been no reliable predictors of progressive potential to date (Chow *et al*, 1999; Cohen and McGovern, 2005). *N*-acetylgalactosaminyltransferases-3, used as a molecular marker, may be able to refine individual risk stratification and treatment plans, possibly including specific anti-GalNAc-T3 enzymes. This intriguing prospect warrants more experimentation on the potential invasive and metastatic functions of GalNAc-T3 in RCC.

Univariate and multivariate Cox survival analyses confirm that GalNAc-T3 expression is an independent prognostic factor in RCC if the cut-off value is set at 10%, but not at 30 or 80%. There seems to be some danger of data fitting in arbitrary cut-off values; however, the blinded protocol design used should prevent that it would have influenced the final conclusive results. Nevertheless, this discrepancy could be due to the heterogeneity of cancer or the size of cohort, and further experiments are thus needed to address the methodology standardisation for GalNAc-T3 in clinical specimens after collecting and investigating a much larger number of surgical RCC cases.

The *GalNAc-T3* exhibits a high sequence homology to the *GalNAc-T6* gene, located on chromosome 12q13 and containing 10 exons of 1869 base pairs that encode a type-II transmembrane protein (Bennett *et al*, 1999). As GalNAc-T3 and -T6 are thus nearly identical, but distinct, substrate specificities and represent the only two enzymes to form glycosylated oncofoetal fibronectin *in vitro* (Wandall *et al*, 1997; Bennett *et al*, 1998), biosynthesis of the glycosylated form of oncofoetal fibronectin *in vivo* quite possibly results from cooperating expression of an alternative spliced variant of fibronectin containing the glycosylation site, initiated by at least either of two GalNAc-T3 or -T6 (Wandall *et al*, 2007). More recently, our laboratory also found GalNAc-T3

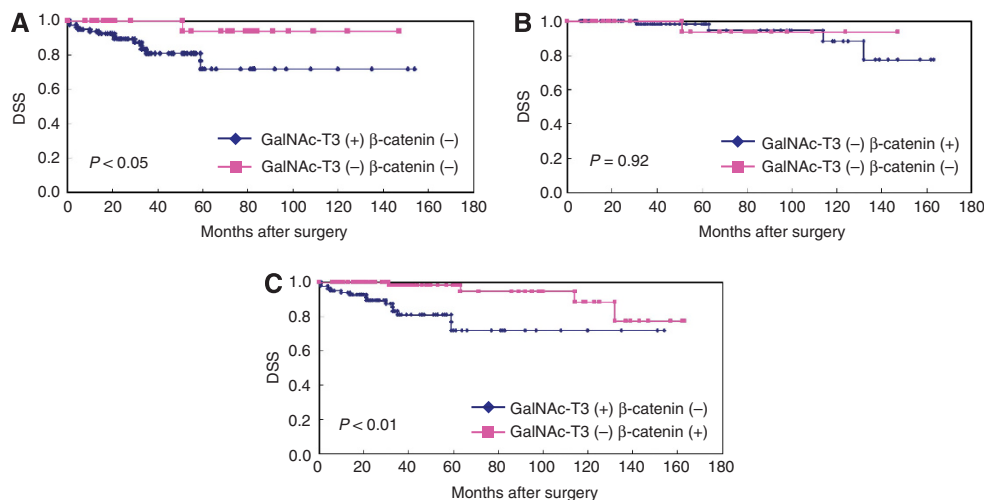


Figure 4. Kaplan–Meier curves of disease-specific survival (DSS) in patients with RCCs after surgery according to split approaches to GalNAc-T3 and β -catenin immunoprofiles: (i) double negative vs either GalNAc-T3 (A) or β -catenin (B) positive; and (ii) GalNAc-T3+ only vs β -catenin+ only (C).

and -T6 to co-express significantly in a cohort of surgical pancreatic cancer cases, although they might function separately (Li *et al*, 2011). Although similar to our obtained data regarding GalNAc-T3, the present findings on GalNAc-T6 are also consistent with our own and others' previous studies in part, showing *de novo* expression of GalNAc-T6 in several other carcinomas, including oral (Wandall *et al*, 2007), breast (Berois *et al*, 2006; Freire *et al*, 2006), gastric (Gomes *et al*, 2009), and pancreas (Li *et al*, 2011) epithelial cancers, where the malignant cells specifically demonstrated GalNAc-T6 expression associated occasionally with more progressive tumour characteristics and bad prognosis. On the basis of these features, we can easily hypothesise that GalNAc-T3 *in vivo* should be concurrently expressed with GalNAc-T6 in RCC as well. Here, we have revealed a significantly close relationship between these two GalNAc-Ts, and survival analyses further show significant differences, especially in a double-negative vs either-positive classification approach, which suggests complementary correlations between GalNAc-T3 and -T6. In this context, upregulation of GalNAc-T3 within the malignant transformation, differentiation, invasion, or progression of RCC cells could partly depend on that of GalNAc-T6 in both synergistic and compensatory ways. However, this relationship remains to be elucidated more in detail; further follow-up cohort and molecular studies are needed to determine the comprehensive mechanisms between GalNAc-T3 and -T6 expressions.

To further clarify GalNAc-T3's function in RCC, we examined expression patterns of two cell-adhesion molecules, β -catenin and E-cadherin, which critically affect cell morphology and carcinogenesis (Yuan *et al*, 2007; Park *et al*, 2010). A study that focused on modulation of cell-adhesion function by GalNAc-Ts suggested that GalNAc-T6 is involved in mammary carcinogenesis through aberrant glycosylation and stabilisation of an oncoprotein and O-glycoprotein mucin 1 (MUC1), accompanied by the down-regulation of E-cadherin and β -catenin (Park *et al*, 2010). Correspondingly, the GalNAc-T3+ expression in RCC was also found to suppress one of the two cell-adhesion molecular markers, β -catenin, probably resulting in anti-adhesive effects and leading to vessel permeation, that is, severe VI and advanced TNM stage, as shown here. Furthermore, our preliminary findings suggest that the protein glycosylated by GalNAc-T3 in RCC is MUC1; it is partly supported by the indication that MUC1 could be a therapeutic agent against RCC (Aubert *et al*, 2009). We could also hypothesise that GalNAc-T3 promotes RCC invasion and

metastasis by affecting cell–stromal interaction and cell–cell adhesion. A recent paper has confirmed that GalNAc-T6 also accelerates epithelial–mesenchymal transition and facilitates metastases in breast cancer (Park *et al*, 2011). Although further in-depth analyses are needed to elucidate this field, our original data both indicate the crucial role of aberrant O-linked glycosylation via GalNAc-T3 in renal carcinogenesis and RCC invasiveness, but also imply the potential of GalNAc-T3 as a target in RCC treatment. It might be suggested that a gene therapy with RNA interference for GalNAc-T3 is very promising.

In conclusion, our findings show that GalNAc-T3+ expression in RCC is significantly related to histopathological phenotypes with poor prognoses, for example, those with VI, high Fuhrman's grade, or advanced TNM stage. Moreover, outcomes of patients whose RCCs had GalNAc-T3+ were markedly worse compared with those with GalNAc-T3– RCCs. These analyses indicate, for the first time, that GalNAc-T3 is a novel and useful independent predictor of histologically high-grade tumour and poor prognosis in patients with RCC.

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