Mucin glycosylating enzyme GALNT2 promotes the malignant character of hepatocellular carcinoma by modifying the EGF receptor

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Abbreviations: HCC, hepatocellular carcinoma; GALNT, Polypeptide N-acetylgalactosaminyltransferase; EGF, epidermal growth factor; HGF, hepatocyte growth factor; VVA, Vicia villosa agglutinin; GalNAc, PNA, peanut agglutinin; N-acetylgalactosamine; GnT, N-acetylglucosaminyltransferase; PNGaseF: Peptide: N-Glycosidase F

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Abstract

Extracellular glycosylation is a critical determinant of malignant character. Here we report that N-acetylgalactosaminytransferase 2 (GALNT2), the enzyme that mediates the initial step of mucin type-O glycosylation, is a critical mediator of malignant character in hepatocellular carcinoma (HCC) that acts by modifying the activity of the EGF receptor (EGFR). GALNT2 mRNA and protein were downregulated frequently in HCC tumors where these events were associated with vascular invasion and recurrence. Restoring GALNT2 expression in HCC cells suppressed EGF-induced cell growth, migration, and invasion in vitro and in vivo. Mechanistic investigations revealed that the status of the O-glycans attached to the EGFR was altered by GALNT2, changing EGFR responses after EGF binding. Inhibiting EGFR activity with erlotinib decreased the malignant characters caused by siRNA-mediated knockdown of GALNT2 in HCC cells, establishing the critical role of EGFR in mediating the effects of GALNT2 expression. Taken together, our results suggest that GALNT2 dysregulation contributes to the malignant behavior of HCC cells, and they provide novel insights into the significance of O-glycosylation in EGFR activity and HCC pathogenesis.
Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third leading cause of cancer-related death worldwide (1). The primary curative treatment for HCC is hepatic resection. Although clinical treatment of HCC is continuously evolving, the prognosis of HCC patients remains poor. To improve the survival of HCC patients, further understanding of HCC pathogenesis and novel treatment agents are needed.

Glycosylation is the most common post-translational modification of proteins. Aberrant glycosylation is a hallmark of most human cancers and affects many cellular properties, including cell proliferation, apoptosis, differentiation, transformation, migration, invasion, and immune responses (2). Tumor-associated carbohydrate antigens have drawn global attention to develop diagnostic reagents and vaccines for cancer therapy (3). However, the glycogenes responsible for the expression of these antigens and their pathophysiological roles in human cancers are still largely unknown.

Two major types of protein glycosylation in mammalian cells exist: $N$-linked and $O$-linked. The most frequently occurring O-glycosylation is the mucin-type, initiated by the transfer of UDP-$N$-acetylglactosamine (UDP-GalNAc) to the hydroxyl group of serine (S) or threonine (T) residue forming Tn antigen (GalNAcα-S/T) (4). This
reaction is catalyzed by a large family of polypeptide GalNAc transferases (GALNTs), consisting of at least 20 members in humans, namely GALNT1 to 14 and GALNTL1 to L6 (5, 6).

Studies have shown that O-glycans and GALNT genes play critical roles in a variety of biological functions and human disease development. For instance, loss of GALNT1 activity in mice results in bleeding disorder (7). Risk of epithelial ovarian cancer (8) and coronary artery disease (9) have been associated with single nucleotide polymorphisms of GALNT1 and GALNT2, respectively. GALNT3 expression is a potential diagnostic marker for lung (10) and pancreatic (11) cancers. GALNT6 modifies mucin 1 glycosylation and regulates proliferation of breast cancer cells (12).

The epidermal growth factor receptor (EGFR) is a promising therapeutic target as its overexpression is associated with various cancers and plays a crucial role in tumor malignancy (13). Overexpression of EGFR in HCC (14) and up-regulation of EGF in advanced HCC compared with the control liver tissue and early HCC (15) suggest the potential role of EGFR-ligand interaction in HCC progression. Phase 2 clinical trials of erlotinib, an EGFR inhibitor, showed 9% partial response and 25% partial response combined with bevacizumab (antibody for VEGF) for advanced HCC.
(16). The phase 3 clinical trial of erlotinib for advanced HCC is still ongoing.

**However, the clinical efficacy is still unsatisfactory.** Thus, to improve the effect of EGFR-targeted therapies, molecular mechanisms by which EGFR regulates HCC properties should be further investigated.

In HCC, the expression pattern and function of GALNT family have never been reported, although O-glycosylation can regulate multiple cellular properties. Here, we report that GALNT2 is frequently downregulated in HCC. Moreover, GALNT2 modifies EGFR O-glycosylation and activity, and plays a critical role in the malignant phenotype of HCC cells *in vitro* and *in vivo*.  

**Materials and methods**

**Tissue samples**

Post surgery fresh tissue samples were collected from patients receiving treatment at the National Taiwan University Hospital (Supplementary Table S1). The tumor samples were taken from the central part of the resected tumor and the paired non-tumor samples were taken 2 cm away from the tumor. For immunohistochemistry, specimens were fixed in 4% (w/v) paraformaldehyde/phosphate buffered saline (PBS). For RNA extraction, specimens were soaked in RNALater™ (Qiagen Corp, Tokyo,
Japan) at 4°C overnight and then stored at -20°C. Samples used for western blotting were stored at -80°C. Ethics approval was obtained from the local hospital ethic committees and a written consent was obtained from each patient before sample collection.

**Cell line and cell culture**

Human liver cancer cell lines Huh7, PLC5, and HepG2 were purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan) in 2008. HA22T, SUN387, and HCC36 cells were kindly provided by Shiou-Hwei Yeh (National Taiwan University, Taiwan) in 2010. All cell lines were authenticated by the provider based on morphology, antigen expression, growth, DNA profile, and cytogenetics. Cells were maintained with Dulbecco's modified Eagle's medium (DMEM) (Biowest, Miami, FL) containing 10% fetal bovine serum (FBS) (PAA Laboratories, Linz, Austria), 100 IU/ml penicillin and 100 μg/ml streptomycin (Biowest) in tissue culture incubator at 37°C, 5% CO₂. All cell culture experiments were conducted with cells at less than 30 passages after receipt. Cells were tested to be *Mycoplasma* free prior to experiments.

**cDNA synthesis and real time RT-PCR**

The total RNA was isolated using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. In real-time PCRs, quantitative PCR System Mx3000P (Stratagene, La Jolla, CA) was used. Primers were
designed by Primer 3 (v.0.4.0) (Supplementary Table S2). Relative quantity of gene expression normalized to GAPDH was analyzed with MxPro Software (Stratagene).

**Immunohistochemistry**

Paraffin-embedded tissue sections were incubated with anti-GALNT2 polyclonal antibody (1:75, Sigma, St Louis, MO) diluted with 5% non-fat milk/PBS for 16 h at 4°C. After rinsing twice with PBS, Super Sensitive™ Link-Label immunohistochemistry Detection System (BioGenex, San Ramon, CA) was used and the specific immunostaining was visualized with 3,3-diaminobenzidine liquid substrate system (Sigma). All sections were counterstained with hematoxylin. Negative controls were performed by replacing primary antibody with control IgG. Tumor cell proliferation was assessed by Ki67 immunoreactivity. Anti-Ki67 rabbit polyclonal antibody (Vector Laboratories, Burlingame, CA) was applied to the slides at 1:500 dilution. Cells with positively stained nuclei were counted in five random fields.

**Plasmid construction**

RT-PCR was performed for cloning of full-length human GALNT2 (Accession No. NM_004481) from nontumorous liver total RNA (BD Biosciences, Palo Alto, CA). The sense primer was 5′-ATGCGGCGGCCTCGCGGAT-3′, and the anti-sense primer was 5′-CTGCTGCAGGTTGAGCGTGAT-3′. The RT-PCR products were cloned into pcDNA3.1/myc-His (Invitrogen Life Technologies) to generate the
GALNT2/myc-His fusion gene. The insert was confirmed by DNA sequencing.

Transfection

Overexpression of GALNT2 gene was achieved by transfecting cells with pcDNA3.1/GALNT2/mycHis plasmids using Lipofectamine 2000 (Invitrogen, Life Technologies) according to the manufacturer’s protocol. The transfected cells were selected with 600 μg/ml of G418 for 14 days and then pooled for further studies.

siRNA knockdown of GALNT2 expression

SMARTpool siRNA oligonucleotides against GALNT2 and siCONTROL Non-targeting siRNA were synthesized by Dharmacon Research (Thermoscientific, Lafayette, CO). For knockdown of GALNT2, cells were transfected with siRNA using DharmaFECT 4 (Thermoscientific) with a final concentration of 100 nmol siRNA for 48 h.

Western blot analysis

GALNT2 proteins were detected with rabbit anti-GALNT2 polyclonal antibody (Sigma). For detection of EGFR and its downstream signaling molecules, anti-phospho-tyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, NY) and antibodies against total EGFR, EGFR pY 845, EGFR pY1068, p-Src, Src, p-Shc, Shc, p-AKT, AKT, p-ERK1/2, and ERK1/2 (Cell Signaling Technology, Inc., Beverly, MA)
were used. Detection of glycoproteins decorated with Tn antigens was achieved by using biotinylated *Vicia villosa* agglutinin (VVA, Vector Laboratories) with or without neuraminidase (Sigma) treatment. GAPDH was detected with anti-GAPDH monoclonal antibody (BD Pharmingen, San Diego, CA).

**Lectin pull down and immunoprecipitation**

To detect the Tn, T, and T/sialyl T on glycoproteins, VVA, peanut agglutinin (PNA), and Jacalin agarose beads (Vector Laboratories) were used, respectively. Briefly, cell lysates (0.5 mg) were incubated with VVA, PNA, or Jacalin agarose beads for 4 h. Peptide: N-Glycosidase F (PNGaseF, Sigma) was used to remove N-glycans. Protein G sepharose beads (Amersham Pharmacia, Piscataway, NJ) conjugated with 1 μg of anti-EGFR antibody were used in immunoprecipitation. The precipitated proteins were then subjected to western blotting.

**Matrigel invasion assay**

Cell invasion assays were performed in BioCoat Matrigel Invasion Chambers (Becton Dickinson, Bedford, MA) according to the manufacturer's protocol, as previously described (17). Briefly, 500 μl DMEM with or without chemoattractants was loaded in the lower part of the chamber and 3×10^4 of transfected cells in 500 μl serum-free DMEM were seeded onto the upper part. Chemoattractants were 10% FBS (PAA on April 20, 2017. © 2011 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from
Laboratories), 100 ng/ml EGF (Sigma), or 50 ng HGF (Sigma). Cells were allowed to invade the matrigel for 24 h. In some experiments, 70 μM erlotinib (Santa Cruz Biotechnology, Santa Cruz, CA) or dimethyl sulfoxide (DMSO) was included in the upper-chamber medium. The invading cells were fixed and stained with 0.5% (wt/vol) crystal violet (Sigma). The numbers in each well were counted and values are presented as mean ± SD.

**Transwell migration assay**

The transfected cells (3×10^4) were resuspended in serum-free DMEM and added to the top well of each migration chamber with an 8-μm pore size membrane (Corning). Cell migration was induced by 10% FBS (PAA Laboratories), 100 ng/ml EGF (Sigma), or 50 ng HGF (Sigma) in the bottom chambers and analyzed after 24 h. To assess the effect of erlotinib on cell migration, erlotinib (70 μM) or DMSO was included in the upper-chamber medium.

**Cell growth analysis**

Cells (4×10^4) were seeded in 6-well plates with serum-free DMEM or DMEM containing 10% FBS (PAA Laboratories), 100 ng/ml EGF (Sigma), or 50 ng HGF (Sigma). Viable cells were determined at 24 h intervals for 72 h using hemocytometer with trypan blue exclusion. Erlotinib (70 μM) and DMSO control were used to assess
the effect of EGFR inhibitor.

**Bromodeoxyuridine (BrdU) incorporation and immunofluorescence microscopy**

Cells were plated in chamber slides and subjected to serum starvation for 16 h, and treated with 10% FBS (PAA Laboratories), 100 ng/ml EGF (Sigma), or 50 ng HGF (Sigma) for 2 h and then bromodeoxyuridine (BrdU; 10 µM) for 0.5 h. The cells were fixed and incubated with anti-BrdU antibody (Sigma). BrdU staining was completed with Cy3-conjugated goat anti-mouse IgG (Jackson Immunoresearch), and counterstained by DAPI. For GALNT2 staining, cells cultured in complete DMEM were stained with anti-GALNT2 antibody (Sigma) and Cy3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch).

**Internalization of EGFR**

Cells were starved for 4 h and then treated with EGF (100 ng/ml) (Sigma) for 10 min at 37°C. The cells were washed with ice cold PBS, fixed, and then immunostained by anti-EGFR (Cell Signaling) and anti-EEA1 (early endosome antigen 1) antibody (Santa Cruz) and counterstained by DAPI (Sigma).

**Tumor growth in nude mice**

For tumor growth analysis, 6-week-old female BALB/c nude mice (National Laboratory Animal Center, Taiwan) were injected subcutaneously with $5 \times 10^6$ of
Mock cells \((n=6)\) or \(GALNT2\) transfectants \((n=6)\). At day 28 after injection, tumors in each group were excised for analyses. Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University College of Medicine.

Statistical analysis

Student's \(t\)-test was used for statistical analyses. Data are presented as means ± SDs. We performed paired \(t\)-tests for the analysis of paired HCC tissues. Where appropriate, a two-way ANOVA followed by a Bonferroni post hoc test for significance was applied, as indicated in figure legends. \(P < 0.05\) was considered statistically significant.

Results

Expression of \(GALNT2\) is frequently downregulated in human hepatocellular carcinoma

To investigate the potential role of \(GALNT\) gene family in HCC, we first analyzed \(GALNT1-14\) and \(GALNTL1-L6\) expression in primary liver tissues by real-time RT-PCR. Among them, only \(GALNT1\) and \(GALNT2\) were found to be highly expressed in the non-tumorous liver tissues \((n = 8)\) (Fig. 1A). We therefore further analyzed \(GALNT1\) and \(GALNT2\) expression in paired HCC tissues \((n = 6)\). Paired \(t\)-tests showed that \(GALNT2\), but not \(GALNT1\), exhibited significant downregulation in HCC tissues.
compared with their non-cancerous parts (Fig. 1B, upper and middle panel). Moreover, real-time RT-PCR revealed downregulation of *GALNT2* expression in 72.1% (31/43) of HCC tissues (Fig. 1B, lower panel). Interestingly, *GALNT2* downregulation was found in 90.9% (10/11) and 83.3% (10/12) of HCC patients with vascular invasion and recurrence, respectively. Consistent findings of lower expression levels of GALNT2 protein in HCC tissues were observed by western blotting and immunohistochemistry (Fig. 1C and 1D). These results suggest that GALNT2 expression is frequently downregulated and associated with invasive properties of HCC.

**Stable transfection of Huh7 cells with GALNT2**

To investigate the role of GALNT2 in HCC, we first analyzed GALNT2 expression in six HCC cell lines by western blotting. We found that Huh7 expressed lower levels of GALNT2, whereas PLC5 expressed higher levels of GALNT2 in these cell lines (Fig. 2A). We therefore chose Huh7 cells and PLC5 cells to overexpress and knockdown the GALNT2 expression, respectively. For establishing stable transfectants, G418-resistant clones of transfected Huh7 cells were pooled. Mock and GALNT2 stable transfectants were obtained from the pooled colonies of Huh7 cells transfected with pcDNA3.1 and GALNT2/pcDNA3.1 plasmids, respectively. The overexpression of GALNT2 was confirmed by Western blot analysis and immunofluorescence microscopy (Fig. 2B). An
increased binding of VVA lectin to glycoproteins in GALNT2-overexpressing lysates was observed, reflecting enhanced O-glycosylation (Fig. 2C).

**GALNT2 suppresses malignant phenotypes in Huh7 cells**

To investigate effects of GALNT2 on malignant phenotypes in Huh7 cells, cell growth, migration, and invasion were analyzed. We found that GALNT2 suppressed FBS- and EGF-induced cell growth (Fig. 3A). Our data further showed that GALNT2 was able to inhibit FBS- and EGF-induced cell proliferation (Fig. 3B), but has no significant effect on apoptosis and cell cycle (data not shown). Furthermore, re-expression of GALNT2 significantly inhibited FBS- and EGF-induced migration and invasion revealed by transwell migration and matrigel invasion assays, respectively (Fig. 3C and 3D). In contrast, we did not observe significant changes in cell growth, proliferation, migration, and invasion when cells treated with HGF or under serum free conditions. To further confirm the effects of GALNT2 overexpression on Huh7 cells, GALNT2 overexpression was knocked down by siRNA. Our data showed that the suppressive effects of GALNT2 overexpression were significantly blocked by GALNT2 siRNA but not control siRNA (Supplementary Fig. S1). To know whether EGF plays a role in FBS-induced malignant phenotypes, EGFR inhibitor erlotinib was used to treat mock and GALNT2 stable transfectants. Our data showed that cell growth, migration, and
invasion were significantly suppressed by erlotinib (Supplementary Fig. S2). These results suggest that GALNT2 can suppress the malignant behavior of Huh7 cells and the EGF-mediated pathway may be involved in this process.

GALNT2 inhibits tumor growth in nude mice

To investigate the effect of GALNT2 on tumor growth in vivo, Mock and GALNT2 transfected Huh7 cells were subcutaneously xenografted in nude mice. We observed that GALNT2 significantly suppressed tumor volume (Fig. 4A) and tumor weight (Fig. 4B) after 28 days. Immunohistochemical analysis showed a significant decrease in the percentage of Ki67-positive cells in GALNT2 tumors compared with Mock tumors (Fig. 4C and 4D). These results suggest that GALNT2 inhibits HCC tumor cell growth and proliferation in vivo.

GALNT2 modifies glycosylation and activity of EGFR in Huh7 cells

Since we found that GALNT2 can suppress EGF-induced malignant phenotypes, we analyzed whether EGFR glycosylation and activity were modulated by GALNT2 expression. We observed that endogenous EGFRs in HCC cells could not be pulled down by VVA (data not shown). Interestingly, EGFR could be easily pulled down by VVA after neuraminidase treatment (Fig. 5A, upper panel). In addition, more EGFR
molecules were pulled down after the removal of N-glycans by PNGaseF than those without treatment. We also showed that EGFRs could be precipitated by PNA after neuraminidase treatment and pulled down by jacalin without neuraminidase treatment. To further confirm the presence of O-glycans, EGFR was immunoprecipitated, followed by neuraminidase treatment, and then immunoblotted with VV A. The results obtained consistently show the expression of sialyl Tn structure on EGFRs (Fig. 5A, lower panel), suggesting that short O-glycans, preferentially sialyl Tn, were decorated on EGFRs. Notably, forced expression of GALNT2 enhanced the expression of sialyl Tn on EGFR (Fig. 5B). To know the effects of glycosylation on EGFR molecules, we analyzed the cell surface expression of EGFR, EGF-induced endocytosis, and EGF-induced dimerization of EGFR. Our data showed that neither the surface expression (Fig. 5C) nor EGF-induced dimerization (data not shown) of EGFR was affected by GALNT2. Interestingly, fluorescence microscopy showed that GALNT2 inhibited co-localization of EGFR with EEA1 when cells were treated with EGF (Fig. 5C). In addition, endocytosis triggered by Alexa488-EGF was suppressed by GALNT2 (Supplementary Fig. S3A). Overexpression of GALNT2 significantly inhibited the elimination of biotinylated EGFR from the cell surface (Supplementary Fig. S3B). These results suggest that GALNT2 overexpression inhibits EGF-triggered endocytosis of EGFR.
We next examined the effect of GALNT2 on tyrosine phosphorylation of cellular proteins. We found that GALNT2 mainly suppressed tyrosine phosphorylation of proteins located at 175 kDa and 140 kDa (Fig. 5D). We then investigated whether phosphorylation and activity of EGFR (175 kDa) were modulated by GALNT2. Our data showed that GALNT2 expression inhibited the EGF-induced phosphorylation of EGFR at Y845 and Y1068, as well as the total tyrosine phosphorylation levels revealed by 4G10 mAb (Fig. 5E). Furthermore, phosphorylation levels of EGFR downstream signaling molecules, including p-Src, p-Shc, pAkt, and p-ERK1/2, also diminished (Fig. 5E). These results suggest that GALNT2 expression down-regulates EGF-induced phosphorylation of EGFR and, which in turn, suppresses the downstream signaling pathways of EGFR.

**Effects of GALNT2 knockdown on HCC cells**

To verify the effect of GALNT2 on HCC cells, endogenous GALNT2 expression was knocked down with siRNA. Our data showed that GALNT2 pooled siRNA efficiently inhibited GALNT2 protein expression in PLC5 cells and suppressed the expression of short O-glycans recognized by VVA lectins compared with the control siRNA (Fig. 6A). In addition, the knockdown of GALNT2 diminished binding of VVA to neuraminidase-treated EGFR, indicating that less sialyl Tn were present on the EGFR.
in the GALNT2-knockdowned cells (Fig. 6B). In these cells, the EGF-induced phosphorylation of EGFR at Y1068 and Y845 was increased (Fig. 6B), suggesting that EGFRs decorated with less sialyl Tn exhibit higher activity. Moreover, our results showed that knockdown of GALNT2 enhanced EGF-induced cell growth (Fig. 6C), migration (Fig. 6D), and invasion (Fig. 6E) in PLC5 cells, whereas no significant changes were observed for cells treated with HGF or under serum free conditions.

**Surprisingly, PLC5 cells invaded in higher numbers than migrated.** In addition, we observed that FBS-induced cell growth, but not migration and invasion, was enhanced by GALNT2 siRNA. Notably, the increase in the EGF-triggered malignant properties induced by GALNT2 knockdown was significantly suppressed by erlotinib, suggesting that EGFR plays a critical role in the phenotypic changes mediated by GALNT2.

To further confirm the effect of GALNT2 on HCC cells, we knocked down GALNT2 expression in another HCC cell line HA22T. Our data showed that knockdown of GALNT2 with siRNA increased EGFR activation but decreased VVA binding to EGFR (Supplementary Fig. S4A). In addition, EGF-induced cell growth, migration, and invasion were significantly increased by GALNT2 siRNA (Supplementary Fig. S4B-D). These results further demonstrate a role of GALNT2 in regulating the malignant behavior of HCC cells.
Discussion

We showed that GALNT1 and GALNT2 are the major GALNT enzymes in human liver. Downregulation of GALNT2 was frequently found in primary HCC tissues and associated with vascular invasion and recurrence. Interestingly, GALNT2 modulates the structure of short O-glycans on EGFR. Moreover, GALNT2 could regulate the malignant phenotype and phosphorylation levels of EGFR and its downstream signaling molecules. Here, we show for the first time that GALNT2 can modulate the malignant behavior of HCC cells, and that O-glycosyltransferase is a novel regulator of EGFR function.

Expression of short O-glycans, such as Tn, sialyl Tn, T, and sialyl T, are found in many types of cancer and exploited to develop cancer vaccines (18). Changes in these structures often alter the function of the cell and its antigenic property, as well as its potential to invade and metastasize (18). The T antigen expression is associated with lower survival probability and is an independent prognostic factor in colorectal cancer (19). Sialyl Tn expression is associated with poor clinical outcome in endometrial and colorectal cancer patients (20, 21). In contrast, the presence of sialyl Tn in keratoacanthoma is associated with tumor regression (22). Several short O-glycans have also been detected in human HCC, but not in the normal liver, by monoclonal
antibodies or lectins (23, 24). However, the role of short O-glycans in HCC progression and prognosis remains unknown. This study reveals that GALNT1 and GALNT2 are the major GalNAc transferases in liver tissues, and that GALNT2 can modulate the sialyl Tn expression in HCC cells and suppress their malignant properties. Thus, it is of great interest to further investigate the significance of the short O-glycans and the GALNT family in HCC malignancy.

Since there is no consensus sequence for GalNAc addition by GALNTs and O-glycans have never been reported on EGFR, it has long been thought that EGFR carries only N-glycans. This study also showed that neuraminidase-treated EGFR can be pulled down by VVA and PNA. Binding of VVA to the neuraminidase-treated EGFR was enhanced after removal of N-glycans, and GALNT2 enhanced the VVA binding to neuraminidase-treated EGFR. Although the exact sites of O-glycosylation on EGFR require further investigation, our data strongly suggest that EGFR carries short O-glycans. Interestingly, we found that EGF-induced endocytosis of EGFR was suppressed by GALNT2 overexpression, suggesting that changes in O-glycosylation on EGFR could modulate EGFR internalization and thereby regulate its downstream signaling. Indeed, it has been reported that clathrin-mediated internalization is essential for sustained EGFR signaling (25). To our knowledge, this study is for the first time to
show that EGFR may express short O-glycans.

We found that GALNT2 can modulate EGF-induced phenotypes in all tested HCC cell lines. In contrast, there is no significant effect for GALNT2 on HGF-triggered phenotypes. These findings suggest that the effect of GALNT2 exhibits selectivity to EGF-, but not HGF-induced phenotypes. We also observed that GALNT2 can significantly modulate 10%FBS-triggered cell growth in HCC cells. Since many substrates for GALNT2 are present in HCC cells, it remains possible that GALNT2 mediates its effects through other receptors in addition to EGFR. EGFR inhibitors prevent the development of HCC in animal models (26) and erlotinib has shown some activity in the treatment of human HCC (16, 27). Sorafenib, a multikinase inhibitor, has been demonstrated to provide a significant survival benefit for patients with advanced HCC (28). However, the efficacy of these agents still remains to be improved. So far, no single target is identified to play the major role in HCC progression, suggesting that multiple pathways should be targeted for HCC treatment. There are currently many clinical trials evaluating TKIs for HCC, including those tested in combination with erlotinib as a first-line therapy (29). A better understanding of the molecular mechanism that regulates the activity and signaling of RTKs is important for developing novel targeted treatments. This study has identified a novel mechanism by
which the activity and downstream signaling of RTKs can be modified by O-glycosyltransferase, which may offer novel insights into the development of new therapeutic agents for HCC.

In conclusion, the results obtained in this study suggest that GALNT2 could modify EGFR glycosylation and activity, and thereby regulate the malignant behavior of HCC cells. This study not only demonstrates a pathophysiologic role of GALNT2 in HCC cells but also contributes to shed light on the significance of abnormal O-glycosylation in HCC tumor progression. Understanding effects and mechanisms of O-glycosylation on the activity of EGFR or other receptor tyrosine kinases by GALNT family genes may offer a novel strategy for the development of HCC therapeutic agents. These include anti-microRNAs, siRNAs, carbohydrate mimetics, or small molecule compounds that can modulate GALNT gene expression or enzyme activity.

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Figure legends

Figure 1. Expression of GALNT2 in human hepatocellular carcinoma. A,

Expression of the GALNT family genes in primary liver tissues. The expression of GALNT1-14 and GALNTL1-L6 mRNA, as indicated, in pooled non-tumorous parts of HCC patients (n = 8) was analyzed by real-time RT-PCR. The relative level of mRNA was normalized to GAPDH and obtained from three separate experiments. Error bars, ±SD. B, GALNT1 and GALNT2 mRNA expression in HCC tissues. GALNT1 (upper panel) and GALNT2 (middle panel) expression in 6 paired HCC tissues (from No. 1 to 6) and GALNT2 expression in 43 paired HCC tissues (n = 43) were analyzed (lower panel). T: Tumor tissues; N: paired non-tumorous liver tissues. Paired t-tests were performed. *P < 0.05, T/N ratio < 0.5 is considered as T < N. C, Expression of GALNT2 in paired HCC tissues (n = 6) by western blotting. Representative images are shown. The signals were quantified from three separate experiments. Paired t-tests also showed significant decrease of GALNT2 expression in HCC tumors (not shown). D,

Immunohistochemistry of GALNT2 in paired HCC tissues. Representative images (patient No. 3) are shown. The subcellular localization of GALNT2 was shown in the Golgi apparatus of normal hepatocytes. Amplified images are shown in the lower right. The negative control did not show any specific signals (data not shown). Scale bars = 50 μm.
Figure 2. Stable transfection of Huh7 cells with GALNT2. A, Expression of GALNT2 in HCC cell lines. The GALNT2 protein expression was analyzed by western blotting, and the relative intensity of signals is presented as the mean ± SD. n = 3. B, Re-expression of GALNT2 in Huh7 cells. GALNT2 overexpression was confirmed by western blotting. Immunofluorescence microscopy showed overexpression of GALNT2 (red) in > 90% of GALNT2 stable transfectants. Nuclei were counterstained with DAPI (blue). The changes in carbohydrates on cellular proteins were detected by Vicia villosa agglutinin (VVA), specific for GalNAc-O-Ser/Thr.

Figure 3. Effects of GALNT2 on malignant phenotypes in Huh7 cells. A, Effects of GALNT2 on cell growth analyzed by trypan blue exclusion assays. Cells were grown in serum free DMEM or DMEM containing 10% FBS, 100 ng/ml EGF, or 50 ng/ml HGF; and the number of live cells at different time points were counted. The results were graphed after standardization by Mock (0 h) to 1.0. Results were analyzed by two-way ANOVA and are represented as the mean ± SD from three independent experiments. *P < 0.05; **P < 0.01. B, Effects of GALNT2 on cell proliferation. Proliferative cells were analyzed by BrdU incorporation assays. **P < 0.01; n = 3; Error bars, mean ± SD. C, Effects of GALNT2 on cell migration by transwell migration assays. Data are represented as mean ± SD from three independent experiments. *P < 0.05; **P < 0.01.
**D**, Effects of GALNT2 on invasion by matrigel invasion assays. Data are represented as mean ± SD from three independent experiments. **P < 0.01.

**Figure 4. GALNT2 inhibits tumor growth in nude mice.** **A**, the effect of GALNT2 on tumor volumes observed for 28 days. Data are shown as mean± SD. **P < 0.01, n = 6 for each group. B**, GALNT2 inhibited tumor weights. After implantation for 28 days, tumors were excised and their weights were shown as mean± SD. *P < 0.05, n = 6. C, Immunohistochemistry of xenografts. Paraffin-embedded sections were immunostained with GALNT2, Ki67, or control IgG. Scale bars = 100 μm. **D**, GALNT2 inhibited tumor cell proliferation *in vivo*. The number of Ki67-positive cells was counted from randomly selected microscopic fields. Data are shown as mean ± SD from six tumors and five fields for each tumor were counted. **P < 0.01.

**Figure 5. GALNT2 modifies glycosylation and activity of EGFR in Huh7 cells.** **A**, EGFR was decorated with short O-glycans. In the upper panel, cell lysates of Huh7 cells was treated with/without neuraminidase, and then pulled down (PD) by lectins. PNGaseF was used to remove N-glycans in cell lysates before lectin pulldown. The pulled down molecules were immunoblotted (IB) with anti-EGFR antibody. In the lower panel, cell lysates were immunoprecipitated with anti-EGFR antibody, and then
immunoblotted with VVA or anti-EGFR antibody. B, GALNT2 increased VVA binding to the neuraminidase-treated EGFR. The cell lysates treated with neuraminidase were pulled down with VVA and then immunoblotted with anti-EGFR antibody. C, GALNT2 suppressed EGF-induced endocytosis. Upper panel, GALNT2 did not change the surface expression of EGFR by flow cytometry. Lower panel, representative images of EGF-induced endocytosis. Endocytosis was triggered by 100 ng/ml EGF for 10 min. EGFR (red) and early endosome antigen 1 (EEA1) (green) were immunostained with anti-EGFR and anti-EEA1 antibody, respectively. Nuclei were stained with DAPI (blue). Scale bars indicate 5 μm. A significant decrease in the percentage of co-localization between EGFR and EEA1 was observed in GALNT2 stable transfectants compared with mock transfectants. Data are represented as mean ± SD from three independent experiments. Five fields for each image were quantified. D, GALNT2 inhibited EGF-induced tyrosine phosphorylation. Phosphorylated tyrosines in cell lysates were immunoblotted with 4G10 antibody. E, GALNT2 suppressed EGF-induced phosphorylation of EGFR and its downstream signaling molecules. Mock or GALNT2 stable transfectants were starved for 4 h and then treated with (+) or without (-) EGF (100 ng/ml) for 10 min. Cell lysates were immunoprecipitated with anti-EGFR polyclonal antibody and then immunoblotted with antibodies mentioned. Alternatively, cell lysates were directly immunoblotted with various antibodies, as
indicated. Signals of western blotting were quantified by ImageQuant5.1 (Amersham Biosciences, UK) and presented as means ± SD from three independent experiments.

**Figure 6. Effects of GALNT2 knockdown on PLC5 cells.**

A, Knockdown of GALNT2 with siRNA in PLC5 cells. GALNT2 expression was knocked down by GALNT2 siRNA compared with the control (Ctr) siRNA by western blotting. Binding of VVA to cellular proteins was decreased in GALNT2-knockdowned cells. B, Knockdown of GALNT2 inhibited O-glycosylation but increased EGF-induced phosphorylation of EGFR. The neuraminidase-treated cell lysate of PLC5 cells knockdowned with the control (Ctr) siRNA or GALNT2 siRNA was pulled down (PD) by VVA and then immunoblotted (IB) with anti-EGFR polyclonal antibody. The cells transfected with the control (Ctr) siRNA or GALNT2 siRNA were starved for 4 h and then treated with (+) or without (-) EGF (100 ng/ml) for 10 min. The phosphorylation of EGFR was analyzed. C-E, Effects of GALNT2 knockdown and EGFR inhibitor erlotinib on EGF-induced malignant phenotypes. Effects GALNT2 knockdown on malignant properties including cell growth (C), cell migration (D), and cell invasion (E) were analyzed. Cells were treated with 10% FBS, 100 ng/ml EGF, or 50 ng/ml HGF. Some were treated with DMSO control (-) or 70 μM of erlotinib, as indicated. Data are shown as mean ± SD from three independent experiments. A two-way ANOVA followed by a Bonferroni post hoc test for significance was applied,*P < 0.05; **P <
0.01.
**Fig. 1**

**A**

Relative mRNA expression (normalized to GAPDH) for GALNT1.

**B**

Relative mRNA expression (normalized to GAPDH) for GALNT1.

**C**

Western blot analysis of GALNT2 and GAPDH in Normal and Tumor samples. kDa markers are shown.

**D**

H&E stains of Normal and Tumor samples with scale bars of 50 μm.

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<th>T ≥ N</th>
<th>T &lt; N</th>
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<td>HCC case (n = 43)</td>
<td>27.9% (12/43)</td>
<td>72.1% (31/43)</td>
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<td>Vascular invasion (n = 11)</td>
<td>9.1% (1/11)</td>
<td>90.9% (10/11)</td>
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<td>Recurrence (n = 12)</td>
<td>16.7% (2/12)</td>
<td>83.3% (10/12)</td>
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Fig. 2
Fig. 3
Fig. 4
Fig. 5
Mucin glycosylating enzyme GALNT2 promotes the malignant character of hepatocellular carcinoma by modifying the EGF receptor

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