

C1GALT1 Enhances Proliferation of Hepatocellular Carcinoma Cells via Modulating MET Glycosylation and Dimerization

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Abstract

Altered glycosylation is a hallmark of cancer. The core 1 β 1,3-galactosyltransferase (C1GALT1) controls the formation of mucin-type O-glycans, far overlooked and underestimated in cancer. Here, we report that C1GALT1 mRNA and protein are frequently overexpressed in hepatocellular carcinoma tumors compared with nontumor liver tissues, where it correlates with advanced tumor stage, metastasis, and poor survival. Enforced expression of C1GALT1 was sufficient to enhance cell proliferation, whereas RNA interference-mediated silencing of C1GALT1 was sufficient to suppress cell proliferation *in vitro* and *in vivo*. Notably, C1GALT1 attenuation also suppressed hepatocyte growth factor (HGF)-mediated phosphorylation of the MET kinase in hepatocellular carcinoma cells, whereas enforced expression of C1GALT1 enhanced MET phosphorylation. MET blockade with PHA665752 inhibited C1GALT1-enhanced cell viability. In support of these results, we found that the expression level of phospho-MET and C1GALT1 were associated in primary hepatocellular carcinoma tissues. Mechanistic investigations showed that MET was decorated with O-glycans, as revealed by binding to *Vicia villosa* agglutinin and peanut agglutinin. Moreover, C1GALT1 modified the O-glycosylation of MET, enhancing its HGF-induced dimerization and activation. Together, our results indicate that C1GALT1 overexpression in hepatocellular carcinoma activates HGF signaling via modulation of MET O-glycosylation and dimerization, providing new insights into how O-glycosylation drives hepatocellular carcinoma pathogenesis. *Cancer Res*; 73(17); 5580–90. ©2013 AACR.

Introduction

Hepatocellular carcinoma is the fifth most common solid tumor and the third leading cause of cancer-related deaths worldwide (1). Because of late-stage diagnosis and limited therapeutic options, the prognosis of patients with hepatocellular carcinoma after medical treatments remains disappointing (2). Diverse posttranslational modifications control various properties of proteins and correlate with many diseases, including cancer (3). Although comprehensive genomic and proteomic analyses have identified many key drivers of hepatocellular carcinoma, the posttranslational

modifications remain poorly understood (4, 5). Thus, elucidation of the precise molecular mechanisms underlying hepatocellular carcinoma progression is of great importance for developing new reagents to treat this aggressive disease.

Glycosylation is the most common posttranslational modification of proteins, and aberrant glycosylation is often observed in cancers (6, 7). Accumulated evidence indicates that alterations in N-linked glycosylation are a hallmark of various liver diseases, including hepatocellular carcinoma (5, 8). For instance, expression of N-acetylglucosaminyltransferase-III and -V is increased in hepatocellular carcinoma (9, 10). An N-glycan profiling study identified novel N-glycan structures in serum as prognostic markers of hepatocellular carcinoma (11). In addition, α -1,6-fucosyltransferase can generate fucosylated α -fetoprotein (AFP), which provided a more accurate diagnosis of hepatocellular carcinoma from chronic liver diseases (12, 13). However, changes in O-linked glycosylation have been overlooked in the past. The O-glycosylation of proteins is difficult to explore, as consensus amino acid sequences of O-glycosylation remain unclear and effective releasing enzymes for O-glycans are not available (14). Recently, a systematic analysis of mucin-type O-linked glycosylation revealed that mucin type O-glycans are decorated not only on mucins but on various unexpected proteins, and functions of the O-glycosylation are largely unknown (15). Several lines of evidence indicate that O-glycosylation of proteins plays critical

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-13-0869

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roles in cancer. O-glycans on major histocompatibility complex class I-related chain A (MICA) enhance bladder tumor metastasis (16), and O-glycosylation of death receptor controls apoptotic signaling in several types of cancer (17). In addition, our previous study showed that GALNT2, an O-glycosyltransferase, regulates EGF receptor activity and cancer behaviors in hepatocellular carcinoma cells (18). Therefore, understanding the roles of O-glycosylation in hepatocellular carcinoma may provide novel insights into the pathogenesis of hepatocellular carcinoma.

Core 1 β 1,3-galactosyltransferase (C1GALT1) is a critical mucin-type O-glycosyltransferase that is localized in the Golgi apparatus (19, 20). C1GALT1 transfers galactose (Gal) to N-acetylgalactosamine (GalNAc) to a serine (Ser) or threonine (Thr) residue (Tn antigen) to form the Gal β 1-3GalNAc α 1-Ser/Thr structure (T antigen or core 1 structure; ref. 21). The core 1 structure is the precursor for subsequent extension and maturation of mucin-type O-glycans (22). C1GALT1 has been shown to regulate angiogenesis, thrombopoiesis, and kidney development (23, 24). Although mucin-type O-glycosylation and C1GALT1 have been shown to play crucial roles in a variety of biologic functions, the expression and role of C1GALT1 in hepatocellular carcinoma remain unclear. Here, we found that C1GALT1 is frequently overexpressed in hepatocellular carcinoma and its expression is associated with poor survival of hepatocellular carcinoma patients. We therefore hypothesized that C1GALT1 can regulate the malignant growth of hepatocellular carcinoma cells and contribute to the pathogenesis of hepatocellular carcinoma.

Materials and Methods

Human tissue samples

Postsurgery frozen hepatocellular carcinoma tissues for RNA extraction and Western blotting and paraffin-embedded tissue sections were obtained from the National Taiwan University Hospital (Taipei, Taiwan). This study was approved by the Ethical Committees of National Taiwan University Hospital, and all patients gave informed consent to have their tissues before collection.

Cell culture

Human liver cancer cell lines, Huh7, PLC5, Sk-Hep1, and HepG2, were purchased from Bioresource Collection and Research Center in the year 2008. HA22T, SNU387, and HCC36 cells were kindly provided by Prof. Shiou-Hwei Yeh (National Taiwan University) in the year 2010. All cell lines were authenticated by the provider based on morphology, antigen expression, growth, DNA profile, and cytogenetics. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS in 5% CO₂ at 37°C. To analyze growth factor-induced cell signaling, cells were starved in serum-free DMEM for 5 hours and then treated with 25 ng/mL of hepatocyte growth factor (HGF) or insulin-like growth factor (IGF) at 37°C for 30 minutes.

Reagents and antibodies

Vicia villosa agglutinin (VVA)- and peanut agglutinin (PNA) lectin-conjugated agarose beads, fluorescein isothio-

cyanate (FITC)-, and biotinylated VVA were purchased from Vector Laboratories. Recombinant EGF, HGF, IGF-I, and protein deglycosylation kits were purchased from Sigma. PHA665752 was purchased from Tocris Bioscience. Antibodies against C1GALT1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and IGF-IR (receptor) were purchased from Santa Cruz Biotechnology, Inc.. Antibodies against MET pY 1234/5, IGF-IR pY1135/1136, p-AKT, p-ERK1/2, and ERK1/2 were purchased from Cell Signaling Technology, Inc. Antibodies against total MET and AKT were purchased from GeneTex, Inc.

Tissue array and immunohistochemistry

Paraffin-embedded human hepatocellular carcinoma tissue microarrays were purchased from SuperBioChips and BioMax. Arrays were incubated with anti-C1GALT1 monoclonal antibody (1:200) in 5% bovine serum albumin/PBS and 0.1% Triton X-100 (Sigma) for 16 hours at 4°C. After rinsing twice with PBS, SuperSensitive Link-Label IHC Detection System (BioGenex) was used and the specific immunostaining was visualized with 3,3-diaminobenzidine liquid substrate system (Sigma). All sections were counterstained with hematoxylin (Sigma).

cdNA synthesis and quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of total RNA was used in reverse transcription reaction using the Superscript III First-Strand cDNA Synthesis Kit (Invitrogen). The cDNA was subjected to real-time PCR. Primers for C1GALT1 were 5'-TGGGAGAAAAGGTTGACACC-3' and 5'-CTTTGACGTGTTTGGCCTTT-3'. Primers for GAPDH were 5'-GACAAGCTTCCCGTTCTCAG-3' and 5'-ACAGTCAGCCG-CATCTTCTT-3'. Quantitative real-time PCRs were carried out as described previously (18).

Transfection

To overexpress C1GALT1, cells were transfected with pcDNA3.1/C1GALT1/mycHis plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Empty pcDNA3.1/mycHis plasmid was used as mock transfectant. The transfected cells were selected with 600 μ g/mL of G418 for 14 days and then pooled for further studies.

RNA interference

Two siRNA oligonucleotides against C1GALT1 (5'-UUA-GUAUACGUUCAGGUAA GGUAGG-3' and 5'-UUA UGU UGG CUA GAA UCU GCA UUG A-3') and a negative control siRNA of medium GC were synthesized by Invitrogen. For knockdown of C1GALT1, cells were transfected with 20 nmol of siRNA using Lipofectamine RNAiMAX (Invitrogen) for 48 hours. The pLKO/C1GALT1-shRNA plasmid and nontargeting pLKO plasmids were purchased from National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). The short hairpin RNA (shRNA) plasmids were transfected with Lipofectamine 2000 and selected with 500 ng/mL of puromycin for 10 days. Knockdown of C1GALT1 in single colonies was confirmed by Western blotting.

Western blotting

Western blotting was conducted as reported previously (18).

Phospho-receptor tyrosine kinase array

A human phospho-receptor tyrosine kinase (p-RTK) array kit was purchased from R&D systems. Hepatocellular carcinoma cells were serum starved for 5 hours and then treated with 20% FBS for 30 minutes. Cells were lysed and 500 μ g of proteins were subjected to Western blotting according to the manufacturer's protocol.

Cell viability, proliferation, and cell cycle

Cells (4×10^4) were seeded in each well of 6-well plates with DMEM containing 10% FBS. Viable cells were analyzed by Trypan blue exclusion assay at 0, 24, 48, and 72 hours. Cell proliferation was evaluated by immunostaining with anti-Ki67 antibody (1:1000; Vector Laboratories). For cell-cycle analysis, 1×10^6 cells were stained with propidium iodide (Sigma) for 30 minutes. The percentages of cells in G₁, S, and G₂-M phases were analyzed by flow cytometry (Becton Dickinson).

Deglycosylation and lectin pull down

Protein deglycosylation was carried out using an Enzymatic Protein Deglycosylation Kit (Sigma). Briefly, cell lysates were treated with neuraminidase or PNGase F at 37°C for 1 hour. For lectin blotting, 20 μ g of cell lysate was separated by an 8% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Millipore), and blotted with biotinylated VVA (1:10,000). For lectin pull-down assay, cell lysates (0.3 mg) were incubated with or without deglycosylation enzymes and then applied to VVA- or PNA-conjugated agarose beads at 4°C for 16 hours. The pulled down proteins were analyzed by Western blotting.

Tumor growth in immunodeficient mice

For tumor growth analysis, 7×10^6 of hepatocellular carcinoma cells were subcutaneously injected into severe combined immunodeficient (SCID) mice ($n = 4$ for each group). Tumor volumes were monitored for 36 or 56 days. Excised tumors were weighed and lysed for Western blotting and immunohistochemistry.

Dimerization of MET

To analyze dimerization of MET, hepatocellular carcinoma cells were incubated with or without 25 ng/mL of human HGF in DMEM on ice for 5 minutes. Cross-linker Bis(sulfosuccinimidyl) suberate (BS³, 0.25 mmol/L, Thermo Scientific) was added to cells and reacted at 37°C for 5 minutes. Cells were then transferred on ice for 10 minutes. Reactions were blocked by adding 50 mmol/L of Tris-HCl (pH 7.4). Cell lysates were separated by 6% SDS-PAGE and immunoblotted with anti-MET antibody.

Statistical analysis

Student *t* test was used for statistical analyses. Paired *t* test was used for the analyses of paired hepatocellular carcinoma tissues. Mann-Whitney U test was used to compare unpaired

nontumor liver tissue and hepatocellular carcinoma tissues. Two-sided Fisher exact test was used for comparisons between C1GALT1 expression and clinicopathologic features. Kaplan-Meier analysis and the log-rank test were used to estimate overall survival. Pearson correlation test was used to assess C1GALT1 and phospho (p)-MET expression. Data are presented as means \pm SD. $P < 0.05$ is considered statistically significant.

Results

Expression of C1GALT1 is upregulated in hepatocellular carcinoma and correlates with advanced tumor stage, metastasis, and poor survival

We first investigated expression of *C1GALT1* in hepatocellular carcinoma tissues. Paired hepatocellular carcinoma and adjacent nontumor liver tissues ($n = 16$) were analyzed by real-time reverse transcriptase PCR (RT-PCR). Results showed that *C1GALT1* mRNA was significantly upregulated in hepatocellular carcinoma tissues compared with adjacent nontumor liver tissues (paired *t* test, $P < 0.05$; Fig. 1A). Consistently, Western blotting showed that C1GALT1 protein was overexpressed in hepatocellular carcinoma tissues of paired specimens (Fig. 1B). We further conducted immunohistochemical analysis for 72 primary hepatocellular carcinoma tissues and 32 nontumor livers to investigate the expression of C1GALT1. The immunohistochemistry showed dot-like precipitates of C1GALT1 in the cytoplasm of hepatocellular carcinoma (Fig. 1C), which is similar to the intracellular localization of the Golgi apparatus in hepatocytes (25). We did not observe expression of C1GALT1 in surrounding stromal cells under our experimental conditions. The intensity of staining was scored according to the percentage of C1GALT1-positive cells in each sample (0, negative; +1, < 20%; +2, 20%–50%; +3, > 50%). Our data revealed that C1GALT1 was highly expressed (+2 and +3) in 54% of hepatocellular carcinoma tumors, whereas only 19% of nontumor liver tissues expressed high levels of C1GALT1 (Mann-Whitney *U* Test, $P = 0.002$; Fig. 1C and D). Consistently, results from tissue microarrays also showed increased expression of C1GALT1 in hepatocellular carcinomas compared with normal liver tissues (Supplementary Fig. S1). These results indicated that C1GALT1 expression was significantly higher in hepatocellular carcinoma than that in nontumor liver tissues.

We next investigated the relationship between C1GALT1 expression and clinicopathologic features in patients with hepatocellular carcinoma. We found that high expression of C1GALT1 correlated with advanced tumor stage (Fisher exact test, $P < 0.05$) and metastasis (Fisher exact test, $P < 0.01$) of hepatocellular carcinoma tumors (Table 1 and Supplementary Table S1). A Kaplan-Meier survival analysis showed that the survival rate of patients with hepatocellular carcinoma with high C1GALT1 expression was significantly lower than those with low C1GALT1 expression. (log-rank test, $P = 0.001$; Fig. 1E). Collectively, these data suggest that C1GALT1 is frequently upregulated in hepatocellular carcinoma and its expression is associated with advanced tumor stage, metastasis, and poor survival in hepatocellular carcinoma.

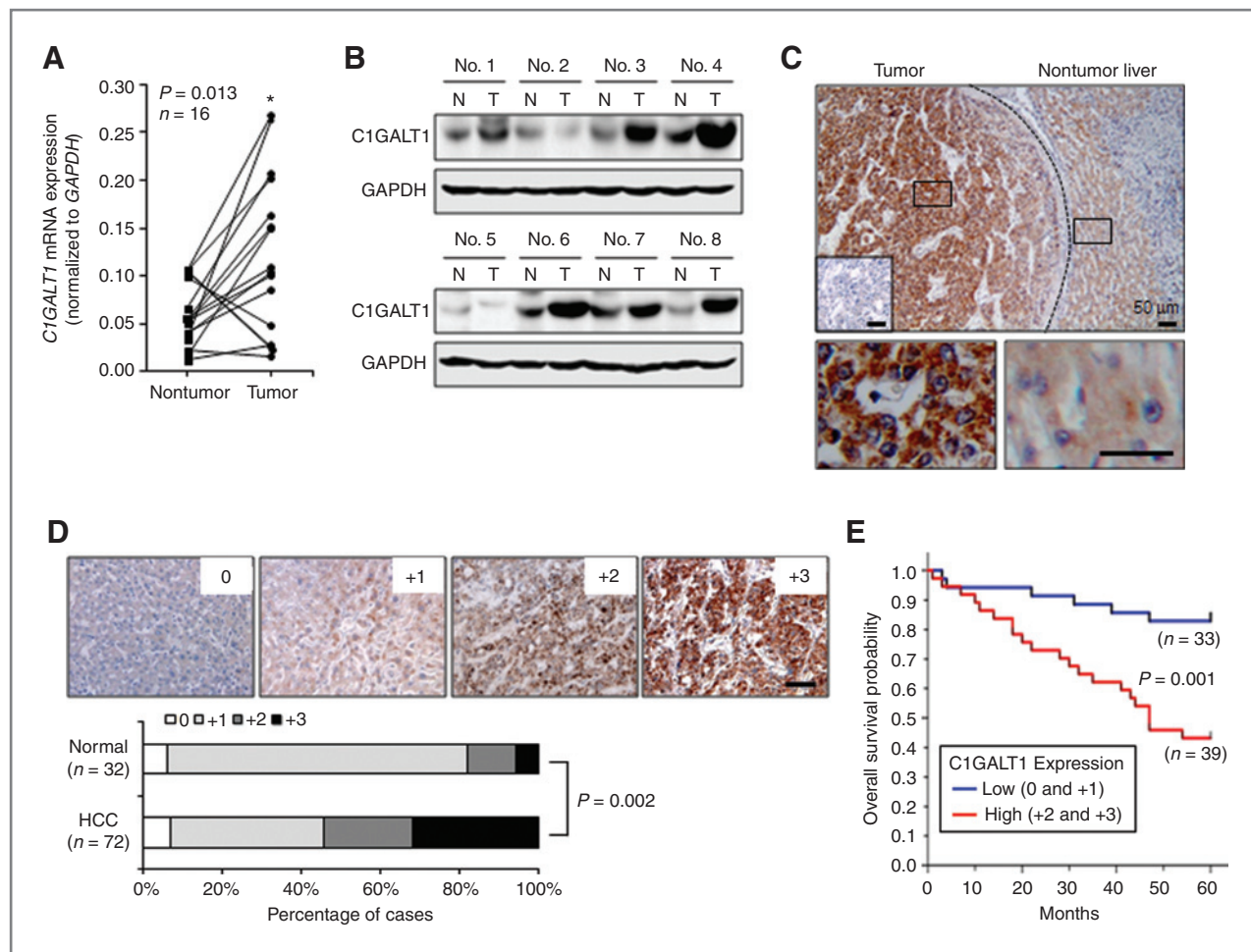


Figure 1. Expression of C1GALT1 in human hepatocellular carcinoma. **A**, expression of C1GALT1 mRNA in 16 paired hepatocellular carcinoma tissues. The mRNA levels of C1GALT1 were analyzed by real-time RT-PCR and normalized to GAPDH. Paired *t* test, *, $P = 0.013$. **B**, Western blot analyses showing C1GALT1 expression in paired hepatocellular carcinoma tissues from **A**. Representative images are shown. N, nontumor liver tissue; T, tumor tissue. **C**, immunohistochemistry of C1GALT1 in paired primary hepatocellular carcinoma tissues. The brown stained cells in the tumor part are hepatocellular carcinoma cells, and those in the nontumor part are hepatocytes. The staining was visualized with a 3,3'-diaminobenzidine liquid substrate system, and all sections were counterstained with hematoxylin. Representative images of tumor (left) and nontumor liver tissue (right) are shown. The negative control does not show any specific signals (bottom left of the top panel). Amplified images are shown at the bottom. Scale bars, 50 μm . **D**, statistical analysis of immunohistochemistry in hepatocellular carcinoma tissues. The intensity of C1GALT1 staining in tissues is shown at the top. Scale bars, 50 μm . Results are shown at the bottom. Mann-Whitney *U* Test, $P = 0.002$. **E**, Kaplan-Meier analysis of overall survival for patients with hepatocellular carcinoma. The analyses were conducted according to the immunohistochemistry of C1GALT1. Probability of overall survival was analyzed after the initial tumor resection. Log-rank test, $P = 0.001$.

C1GALT1 modifies mucin-type O-glycans on hepatocellular carcinoma cells

To investigate functions of C1GALT1 in hepatocellular carcinoma, knockdown or overexpression of C1GALT1 was conducted in multiple hepatocellular carcinoma cell lines. Western blotting showed that the average expression level of C1GALT1 was significantly higher in hepatocellular carcinoma cell lines compared with that of nontumor liver tissues (Fig. 2A). We conducted knockdown of C1GALT1 with two different C1GALT1-specific siRNAs in HA22T and PLC5 cells, which express high levels of C1GALT1 and overexpression of C1GALT1 in Sk-Hep1 and HCC36 cells as they express low levels of C1GALT1 (Fig. 2B). Immunofluorescence microscopy further confirmed the knockdown and overexpression of

C1GALT1 in hepatocellular carcinoma cells and its subcellular localization in the Golgi apparatus (Supplementary Fig. S2). Furthermore, we showed that knockdown of C1GALT1 enhanced binding of VVA to glycoproteins, whereas overexpression of C1GALT1 decreased the VVA binding (Fig. 2B), indicating that C1GALT1 catalyzes the formation of Tn to T antigen. Seven proteins had evident changes in VVA binding, including p50, p60, p80, p90, p110, p140, and p260, as shown in Fig. 2B. Among them, p140 showed changes in all four tested cell lines. Consistently, flow cytometry showed that C1GALT1 altered VVA binding to the surface of hepatocellular carcinoma cells (Fig. 2C). These results indicate that C1GALT1 modulates the expression of mucin-type O-glycans on hepatocellular carcinoma cells.

Table 1. Correlation of C1GALT1 expression with clinicopathologic features.

| Factor | | C1GALT1 expression | | P | Method |
|------------------|---------|--------------------|---------------|--------------------|-----------------------------|
| | | Low (n = 33) | High (n = 39) | | |
| Sex | Male | 27 | 26 | 0.185 | Two-sided Fisher exact test |
| | Female | 6 | 13 | | |
| Age, y | <55 | 11 | 9 | 0.430 | |
| | ≥55 | 22 | 30 | | |
| Histology grade | 1 + 2 | 18 | 28 | 0.147 | |
| | 3 + 4 | 15 | 11 | | |
| Tumor stage | T1 + T2 | 27 | 20 | 0.025 ^a | |
| | T3 + T4 | 6 | 19 | | |
| Metastasis | No | 33 | 30 | 0.003 ^a | |
| | Yes | 0 | 9 | | |
| Overall survival | | | | 0.001 ^a | Log rank |

^aP < 0.05 is considered statistically significant.

C1GALT1 regulates hepatocellular carcinoma cell proliferation *in vitro* and *in vivo*

Effects of C1GALT1 on cell viability and proliferation of hepatocellular carcinoma cells were analyzed by Trypan blue exclusion assays and fluorescent staining of Ki67, respectively. Knockdown of C1GALT1 significantly suppressed cell viability, whereas overexpression of C1GALT1 enhanced cell viability (Fig. 3A). Furthermore, Ki67 staining showed that C1GALT1 modulated cell proliferation (Fig. 3B). We also found that knockdown of C1GALT1 led to G₁-phase arrest in HA22T and PLC5 cells, whereas overexpression of C1GALT1 increased the number of cells in S phase in Sk-Hep1 cells (Supplementary Fig. S3).

To analyze the effect of C1GALT1 on tumor growth *in vivo*, we stably knocked down C1GALT1 with C1GALT1-specific shRNA in HA22T and PLC5 cells (Supplementary Fig. S4). Clone number 8 of HA22T cells and clone number 10 of PLC5 cells were subcutaneously xenografted in SCID mice. The results showed that knockdown of C1GALT1 significantly suppressed the volume and weight of hepatocellular carcinoma tumors. Immunohistochemistry of excised tumors for Ki67 expression revealed that knockdown of C1GALT1 suppressed tumor cell proliferation *in vivo* (Fig. 3C). Knockdown of the C1GALT1 in the tumors was confirmed by Western blotting (Supplementary Fig. S5). These data provide evidence that C1GALT1 can modulate hepatocellular carcinoma cell proliferation *in vitro* and *in vivo*.

C1GALT1 regulates phosphorylation of MET

Because RTKs are crucial for hepatocellular carcinoma proliferation (4, 26) and their activities have been found to be regulated by O-glycosylation (18, 27), we investigated whether C1GALT1 could affect RTK signaling pathways in hepatocellular carcinoma cells. A human p-RTK array was used to detect the tyrosine phosphorylation level of 42 different RTKs. Our data indicated that knockdown of C1GALT1 in HA22T cells decreased phosphorylation of

ERBB3, MET, and EPHA2, whereas phosphorylation of VEGFR1 was increased (Fig. 4A). MET plays crucial roles in multiple functions in hepatocellular carcinoma, including cell proliferation (28), hepatocarcinogenesis (29), and metastasis (28). In addition, NetOGlyc 3.1 predicts one potential O-glycosylation site in the extracellular domain of MET (data not shown). Therefore, we further investigated the role of C1GALT1 in glycosylation and activation of MET in hepatocellular carcinoma cells. Our results showed that knockdown of C1GALT1 inhibited HGF-induced phosphorylation of MET at Y1234/5 and suppressed phosphorylation of AKT in HA22T and PLC5 cells (Fig. 4B, top). In contrast, overexpression of C1GALT1 enhanced HGF-induced activation of MET and increased p-AKT levels in Sk-Hep1 and HCC36 cells. In addition, C1GALT1 expression did not significantly alter IGF-I-induced signaling in all tested hepatocellular carcinoma cell lines (Fig. 4B, bottom). These results suggest that C1GALT1 selectively activates the HGF/MET signaling pathway.

To investigate the role of the MET signaling pathway in C1GALT1-enhanced cell viability, we treated hepatocellular carcinoma cells with PHA665757, a specific inhibitor of MET phosphorylation (30). Trypan blue exclusion assays showed that C1GALT1-enhanced cell viability was significantly inhibited by the blockade of MET activity (Fig. 4C). In addition, we observed that knockdown of C1GALT1 decreased HGF-induced cell migration and invasion, whereas overexpression of C1GALT1 enhanced HGF-induced cell migration and invasion (Supplementary Fig. S6).

We next analyzed whether C1GALT1 expression correlated with MET activation in primary hepatocellular carcinoma tissues. Western blotting (Fig. 4D) and Pearson test (Fig. 4E) from 20 hepatocellular carcinoma tumors showed a significant correlation ($R^2 = 0.73$, $P < 0.0001$) between expression levels of C1GALT1 and phospho-MET. These results suggest that C1GALT1 could regulate MET activation in hepatocellular carcinoma.

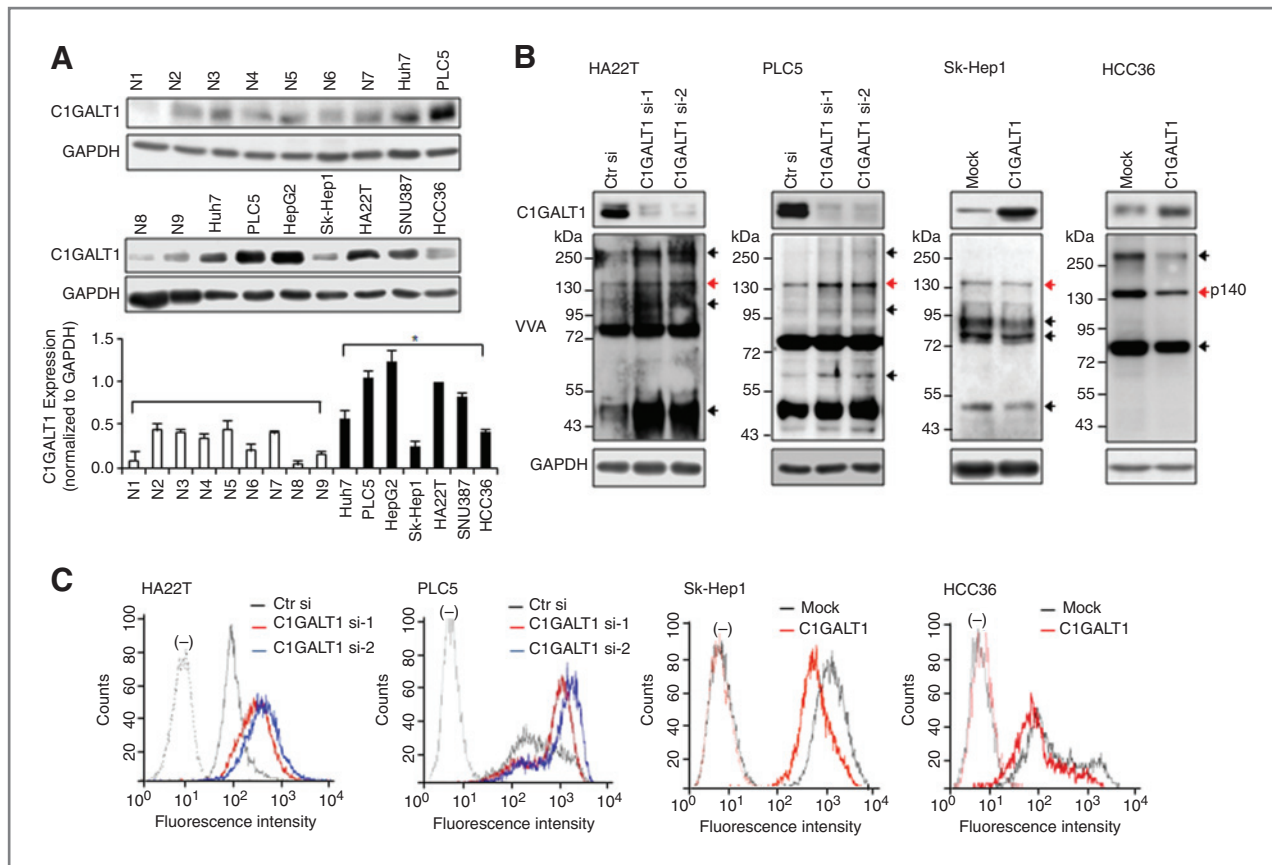


Figure 2. C1GALT1 regulates O-glycosylation in hepatocellular carcinoma cells. A, expression of C1GALT1 in hepatocellular carcinoma cell lines. C1GALT1 expression in seven hepatocellular carcinoma cell lines and nine nontumor (N) liver tissues was analyzed by Western blotting. GAPDH was used as an internal control. Signals were quantified by ImageQuant5.1. *, $P < 0.05$. B, effects of C1GALT1 on binding of VVA to glycoproteins. Western blotting shows C1GALT1 expression after knockdown with two C1GALT1 siRNAs compared with control (Ctr) siRNA in HA22T cells and PLC5 cells. Overexpression of C1GALT1 in Sk-Hep1 and HCC36 cells transfected with C1GALT1/pcDNA3.1 plasmid (C1GALT1) was compared with pcDNA3.1 empty plasmid (mock). The changes in O-glycans on glycoproteins were revealed by Western blotting with biotinylated VVA. Proteins with evident changes in VVA binding are indicated by arrows. p140 changes in all tested cell lines are indicated by red arrows. C, effects of C1GALT1 on O-glycans of hepatocellular carcinoma cell surfaces. Surface O-glycans were analyzed by flow cytometry with FITC-VVA. Negative (-) refers to cells without addition of VVA-FITC.

C1GALT1 modifies O-glycans on MET and regulates HGF-induced dimerization of MET

To investigate the mechanisms by which C1GALT1 regulates HGF/MET signaling, we analyzed the effects of C1GALT1 on glycosylation and dimerization of MET in hepatocellular carcinoma cells. Because C1GALT1 is an O-glycosyltransferase, we first analyzed whether MET is O-glycosylated using VVA and PNA lectins, which recognize tumor-associated Tn and T antigen, respectively. Lectin pull-down assays with VVA or PNA agarose beads showed that endogenous MET expressed Tn and T antigens in all seven hepatocellular carcinoma cell lines tested (Supplementary Fig. S7). Moreover, we found that VVA binding to MET in HA22T cells was further increased after removal of N-glycans on MET with PNGaseF (Fig. 5A), indicating the specificity of VVA binding to O-glycans on MET. We also observed that removal of sialic acids by neuraminidase enhanced VVA binding, suggesting that MET expresses sialyl Tn in addition to Tn. These findings strongly suggest that MET expresses short mucin-type O-glycans in hepatocellular carcinoma cells.

To investigate whether C1GALT1 can modify O-glycans on MET, VVA binding to MET was analyzed in hepatocellular carcinoma cells with C1GALT1 knockdown or overexpression. We found that knockdown of C1GALT1 increased VVA binding to MET in both HA22T and PLC5 cells (Fig. 5B). Conversely, overexpression of C1GALT1 decreased VVA binding to MET in Sk-Hep1 and HCC36 cells. Consistently, we observed that removal of sialic acids enhanced VVA binding to MET in these cell lines. These findings indicate that C1GALT1 can modify O-glycans on MET in hepatocellular carcinoma cells.

We next explored the effects of altered O-glycosylation on MET properties. Our results showed that C1GALT1 expression did not significantly alter the protein level of MET analyzed by Western blotting (Fig. 5B) and flow cytometry (data not shown). Because HGF-induced dimerization of MET is an initial and crucial step for the activation of MET signaling (31), we analyzed whether C1GALT1 could affect MET dimerization. Our data showed that knockdown of C1GALT1 suppressed HGF-induced dimerization of MET in both HA22T and PLC5 cells. In contrast, overexpression of C1GALT1 enhanced

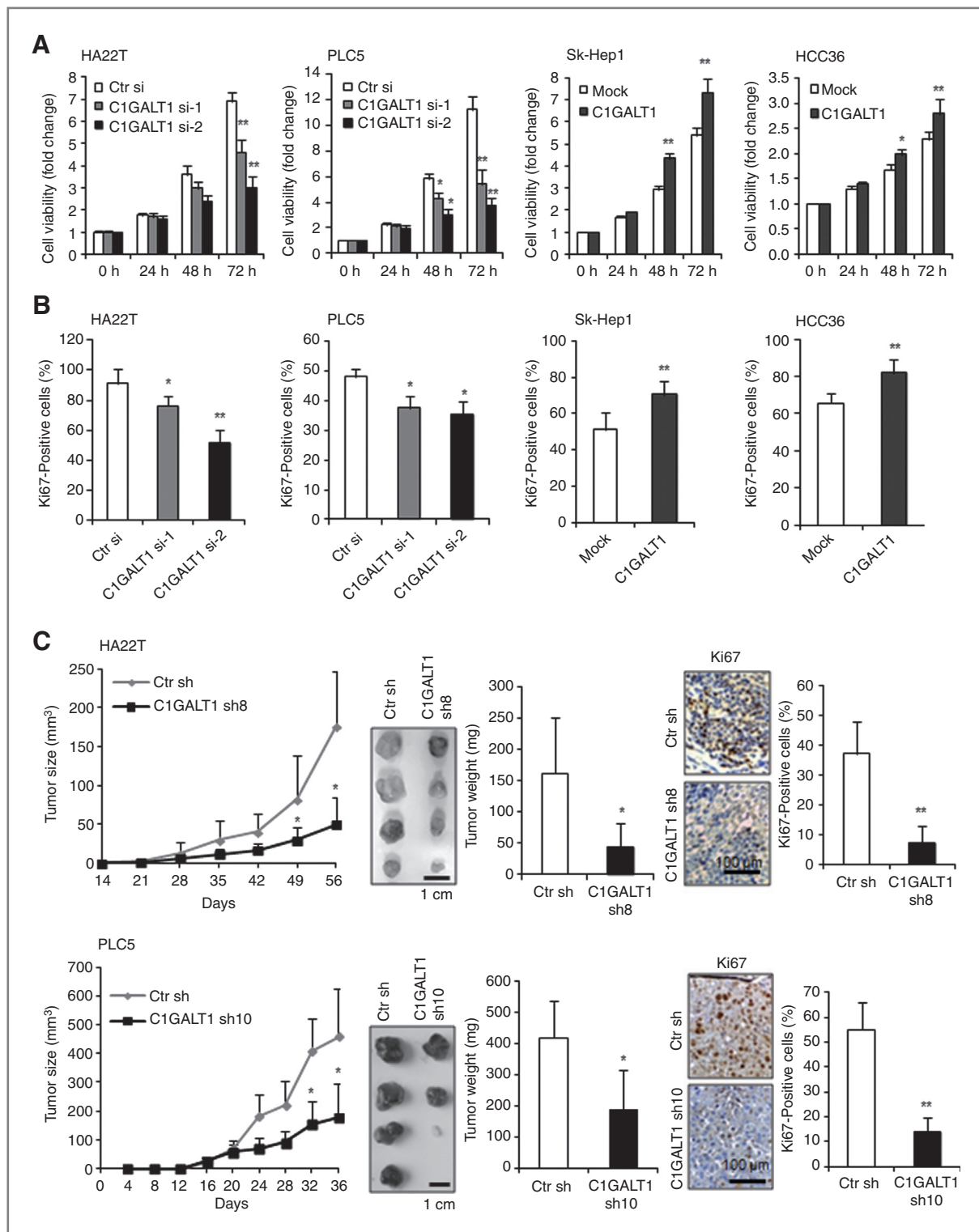


Figure 3. C1GALT1 regulates hepatocellular carcinoma cell proliferation *in vitro* and *in vivo*. **A**, C1GALT1 modulated cell viability *in vitro*. Cell viability of HA22T, PLC5, Sk-Hep1, and HCC36 cells was analyzed by Trypan blue exclusion assays at different time points for 72 hours. The results are standardized by the cell number at 0 hour. Data are represented as means \pm SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$. **B**, effects of C1GALT1 on cell proliferation. Cells were immunofluorescently stained for Ki67 and Ki67-positive cells were counted under a microscope. Results are presented as means \pm SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$. **C**, effects of C1GALT1 on hepatocellular carcinoma tumor growth and proliferation in SCID mouse model. HA22T (top) and PLC5 (bottom) cells were subcutaneously injected into SCID mice. Four mice were used for each group. The volume of tumors was measured at different time points, as indicated (left). Mice were sacrificed at day 56 for HA22T cells and day 36 for PLC5 cells. Tumors were excised and weighted (middle). Cell proliferation of tumor cells was evaluated by immunohistochemical staining for Ki67, and representative images are shown (right). Results are presented as the mean \pm SD from 4 mice for each group. *, $P < 0.05$.

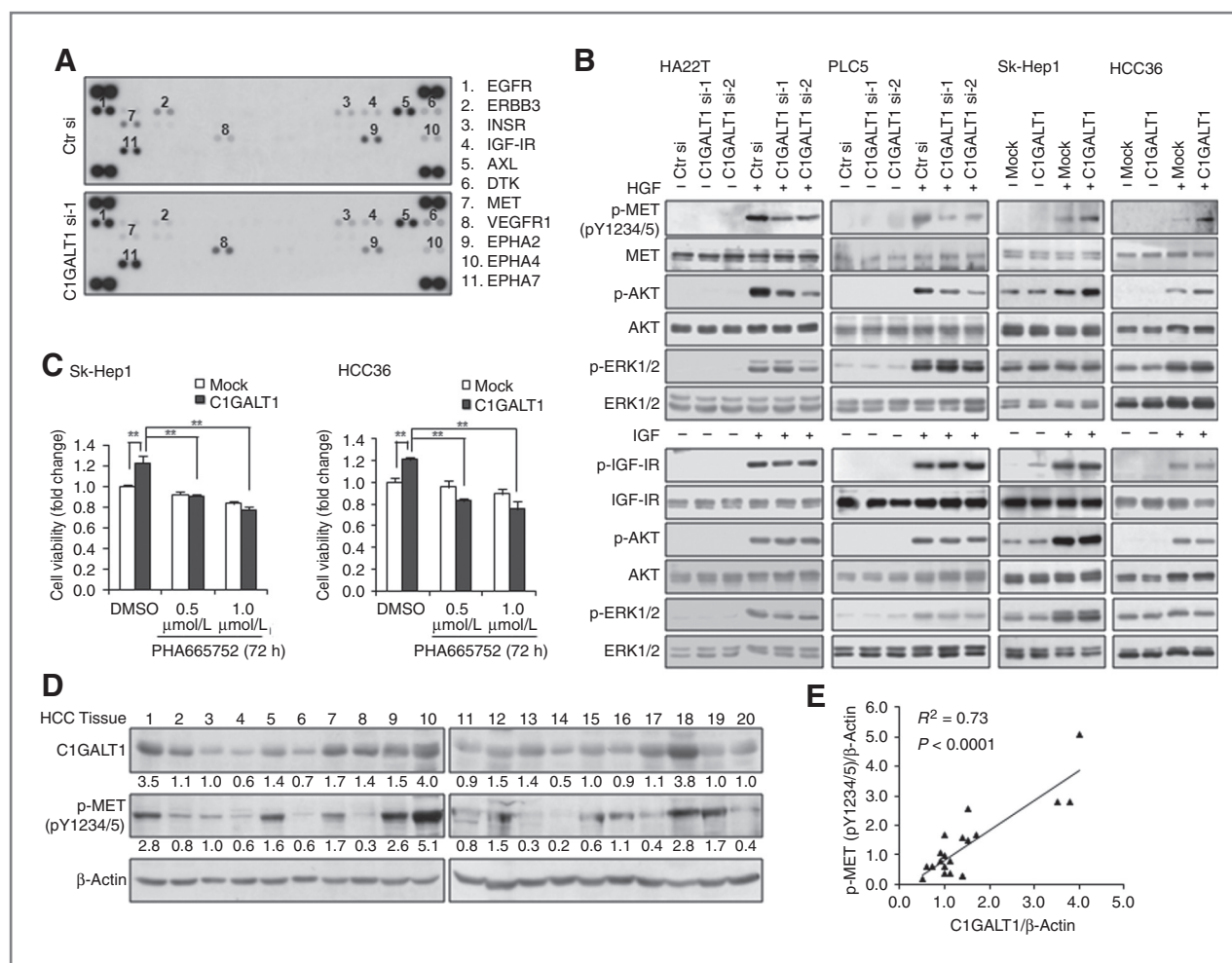


Figure 4. C1GALT1 regulates activity of MET in hepatocellular carcinoma cells. **A**, human p-RTK array showing the effect of C1GALT1 on the phosphorylation of RTKs. Cell lysates of control and C1GALT1 knockdown HA22T cells were applied to p-RTK array including 42 RTKs. **B**, C1GALT1 modulates HGF-induced signaling in hepatocellular carcinoma cells. HA22T, PLC5, Sk-Hep1, and HCC36 cells were starved for 5 hours and then treated with (+)/without (–) HGF (25 ng/mL) or IGF (25 ng/mL) for 30 minutes. Cell lysates (20 μg) were analyzed by Western blotting with various antibodies, as indicated. **C**, effects of MET inhibitor, PHA665752, on C1GALT1-enhanced cell viability. Sk-Hep1 and HCC36 cells were treated with PHA665752 at the indicated concentration and then analyzed by Trypan blue exclusion assays at 72 hours. Data are represented as means \pm SD from three independent experiments. **, $P < 0.01$. **D**, expression of C1GALT1 and p-MET in hepatocellular carcinoma tissues. Tissue lysates (20 μg for each tumor) were analyzed by Western blotting. Signals of Western blotting were quantified by ImageQuant5.1. β-actin was a loading control. **E**, correlation of C1GALT1 and p-MET expression in 20 hepatocellular carcinoma tumors. Pearson test was used to analyze the statistical correlation of C1GALT1 and p-MET expression in **D**.

dimerization of MET in Sk-Hep1 and HCC36 cells (Fig. 5C). These results suggest that C1GALT1 modifies O-glycans on MET and regulates HGF-induced dimerization of MET in hepatocellular carcinoma cells.

Discussion

This study showed that overexpression of C1GALT1 in hepatocellular carcinoma tissues was associated with advanced tumor stage, metastasis, and poor prognosis. C1GALT1 expression regulated hepatocellular carcinoma cell viability and proliferation *in vitro* and *in vivo*. The C1GALT1-enhanced cell viability was inhibited by MET inhibitor. MET carried O-glycans, and these structures were modified by C1GALT1. Furthermore, C1GALT1 could regulate HGF-induced dimerization and activity of MET in hepatocellular

carcinoma cells. Taken together, this study is the first to show that C1GALT1 was able to regulate hepatocellular carcinoma cell proliferation *in vitro* and *in vivo*, and modulation of O-glycosylation and activity of MET may be involved in this process. Our findings provide novel insights not only into the role of O-glycosylation in the pathogenesis of hepatocellular carcinoma but also in the development of reagents for hepatocellular carcinoma treatment.

In colon and breast cancer, an increase in the expression of short O-glycans, such as Tn, sialyl Tn, T, and sialyl T, often alters the function of glycoproteins and their antigenic property, as well as the potential of cancer cells to invade and metastasize (32). Short O-glycans have been developed as carbohydrate vaccines for cancer treatment (33). The expression of short O-glycans in human hepatocellular

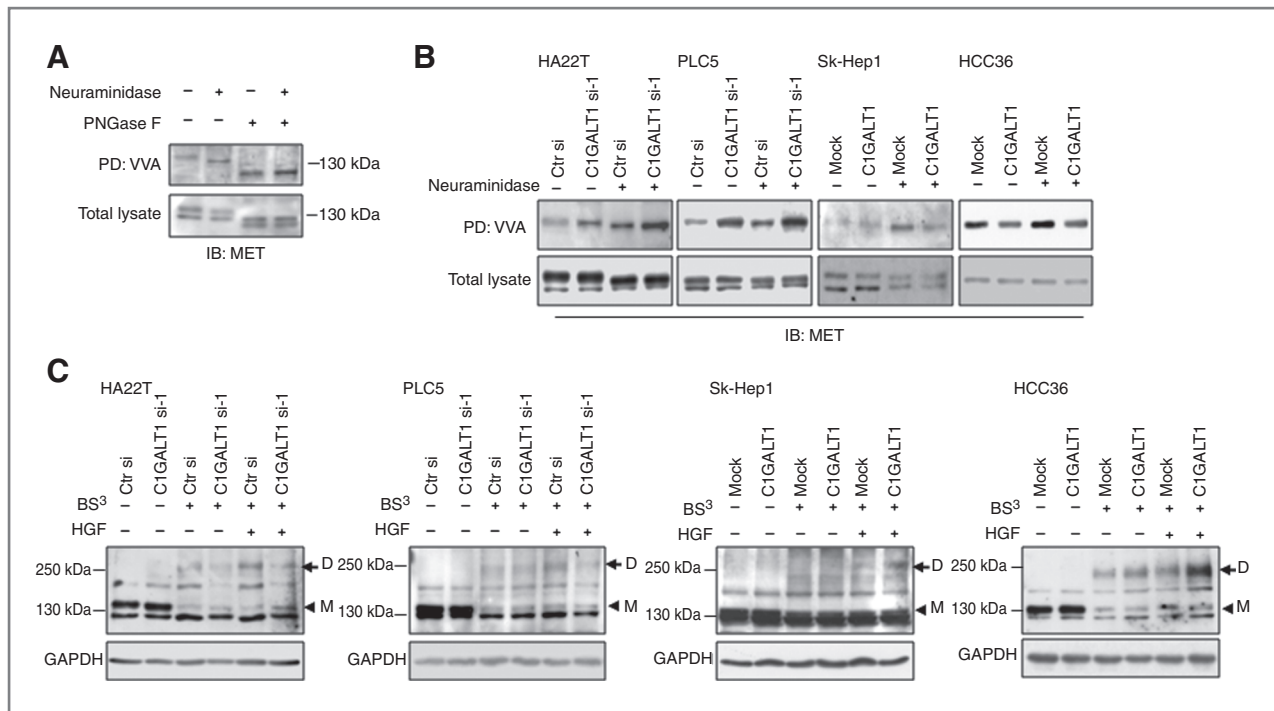


Figure 5. C1GALT1 regulates glycosylation and dimerization of MET. **A**, MET is decorated with short O-glycans. Lysates of HA22T cells were treated with neuraminidase and/or PNGaseF and then pulled down (PD) by VVA agarose beads. The pulled down proteins were analyzed by immunoblotting (IB) with anti-MET antibody. The molecular mass is shown on the right. **B**, C1GALT1 modifies O-glycosylation of MET in hepatocellular carcinoma cells. Cell lysates were treated with (+) or without (-) neuraminidase and then pulled down by VVA agarose beads. The pulled down glycoproteins were immunoblotted (IB) with anti-MET antibody. **C**, C1GALT1 regulates dimerization of MET in hepatocellular carcinoma cells. Hepatocellular carcinoma cells were starved for 5 hours and then treated with (+) or without (-) 25 ng/mL of HGF. Cell lysates were cross-linked by BS³ and then analyzed by Western blotting with anti-MET antibody. The arrows indicate the dimer (D) of MET, and the arrowheads indicate the monomer (M). Markers of molecular weight are shown on the left. GAPDH is an internal control.

carcinoma has been reported (34, 35). However, glycogenes responsible for these O-glycans and their functions in hepatocellular carcinoma remain largely unknown. Previously, we reported that GALNT1 and GALNT2 are the major GalNAc transferases in liver tissues and that GALNT2 modulates the sialyl Tn expression on EGF receptor in hepatocellular carcinoma cells and suppresses their malignant properties (18). Here, we further report that C1GALT1 expression is dysregulated in hepatocellular carcinoma and C1GALT1 modulates the expression of mucin-type O-glycans on hepatocellular carcinoma cell surfaces.

We found that O-glycans can be decorated on MET, an important protooncogene in a variety of human cancers. Our data showed that MET from all tested seven hepatocellular carcinoma cell lines could be pulled down by VVA and PNA lectins, suggesting the presence of Tn and T antigens on MET. Removal of sialic acids by neuraminidase enhanced VVA binding to MET, indicating that some of the Tns are sialylated in hepatocellular carcinoma cells. Removal of N-glycans by PNGaseF further enhanced VVA binding to MET. Moreover, prediction of O-glycosylation sites using NetOGlyc 3.1 indicates that there is one potential O-glycosylation site in the extracellular domain of MET. These results strongly suggest the presence of O-glycans on MET. Glycosylation has long been proposed to control various protein properties, including

dimerization, enzymatic activity, secretion, subcellular distribution, and stability of RTKs (14, 36). However, most studies focused on effects of N-glycans on RTKs. Importantly, we found that C1GALT1 can modulate the O-glycans on MET and enhance dimerization and phosphorylation of MET. Because receptor dimerization is a key regulatory step in RTK signaling (37), it is highly possible that C1GALT1 modulates MET activity via the enhancement of its dimerization. To our knowledge, we are the first to report that MET expresses O-glycans and changes in these carbohydrates regulate the activity of MET. It will be of great interest to further investigate the exact structures and sites of O-glycans on MET to understand how O-glycosylation modulates the structure and function of RTKs.

Recent studies have shown that aberrant activation of MET signaling correlates with the increased cell proliferation, poor prognosis, and poor outcome of human hepatocellular carcinoma (38–40). HGF/MET signaling has been shown to promote invasion and metastasis of hepatocellular carcinoma cells (41, 42). Our data show that C1GALT1 can increase dimerization and phosphorylation of MET. Consistent with previous findings, we also found that C1GALT1 can enhance HGF-induced migration and invasion. Targeting MET is considered to be an attractive strategy for treating many human cancers, including hepatocellular carcinoma (43). Thus, a complete understanding of the mechanisms by which the structure and

function of MET signaling are regulated is crucial to improve the effect of MET-targeted therapies in human cancers. This study provides novel insights into the role of O-glycosylation in modulating MET activity. It will be important to further investigate whether changes in O-glycans on MET can affect hepatocellular carcinoma cell sensitivity toward targeted therapeutic drugs, including small-molecule inhibitors and therapeutic antibodies. We found that C1GALT1 expression modulates HGF-, but not IGF-mediated signaling, suggesting the selectivity of C1GALT1 activity toward certain RTKs. However, we observed that C1GALT1 expression changes binding patterns of VVA to several glycoproteins and knockdown of C1GALT1 in hepatocellular carcinoma cells also modulates phosphorylation of ERBB3, VEGFR1, and EPHA2, suggesting that there are other acceptor substrates, in addition to MET. Therefore, it remains possible that several signaling pathways may be involved in mediating the biologic functions of C1GALT1 in hepatocellular carcinoma cells. Thus, targeting C1GALT1 could have effects similar to those from targeting multiple RTKs. This study opens up avenues for treating cancers by targeting not only the receptors themselves but also their O-glycosylation regulators.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y.-M. Wu, C.-H. Liu, M.-C. Huang

Development of methodology: Y.-M. Wu, C.-H. Liu

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-M. Wu, C.-H. Liu, P.-H. Lee, R.-H. Hu

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-M. Wu, C.-H. Liu, M.-C. Huang

Writing, review, and/or revision of the manuscript: Y.-M. Wu, M.-J. Huang, M.-C. Huang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-M. Wu, H.-S. Lai, P.-H. Lee, R.-H. Hu

Study supervision: Y.-M. Wu, H.-S. Lai, M.-C. Huang

Grant Support

This study was supported by grants from the National Taiwan University 101R7808 (M.-C. Huang), the National Science Council NSC NSC 99-3111-B-002-006 (Y.-M. Wu), NSC 101-2314-B-002-053-MY2 (R.-H. Hu), and NSC 101-2320-B-002-007-MY3 (M.-C. Huang).

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Received March 24, 2013; revised June 9, 2013; accepted June 24, 2013; published OnlineFirst July 5, 2013.

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C1GALT1 Enhances Proliferation of Hepatocellular Carcinoma Cells via Modulating MET Glycosylation and Dimerization

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Cancer Res 2013;73:5580-5590. Published OnlineFirst July 5, 2013.

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