C1GALT1 enhances proliferation of hepatocellular carcinoma cells via modulating MET glycosylation and dimerization

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**Abbreviations:** HCC, hepatocellular carcinoma; C1GALT1, core 1 β1,3-galactosyltransferase; RTK, receptor tyrosine kinase; EGF, epidermal growth factor; HGF, hepatocyte growth factor; VVA, *Vicia villosa* agglutinin; PNA, peanut agglutinin; PNGaseF, Peptide: N-Glycosidase F

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ABSTRACT

Altered glycosylation is a hallmark of cancer. The core 1 β1,3-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 (HCC) tumors compared with non-tumor liver tissues, where it correlates with advanced tumor stage, metastasis and poor survival. Enforced expression of C1GALT1 was sufficient to enhance cell proliferation, whereas RNAi-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 HCC 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 HCC tissues. Mechanistic investigations showed that MET was decorated with O-glycans, as revealed by binding to Vicia villosa agglutinin (VVA) and peanut agglutinin (PNA). Moreover, C1GALT1 modified the O-glycosylation of MET, enhancing its HGF-induced dimerization and activation. Together, our results indicate that C1GALT1 overexpression in HCC activates HGF signaling via modulation of MET.
O-glycosylation and dimerization, providing new insights into how O-glycosylation drives HCC pathogenesis.
INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common solid tumor and the third leading cause of cancer-related deaths worldwide (1). Due to late stage diagnosis and limited therapeutic options, the prognosis of HCC patients after medical treatments remains disappointing (2). Diverse posttranslational modifications control various properties of proteins and correlate with many diseases, including cancer (3). While comprehensive genomic and proteomic analysis have identified many key drivers of HCC yet, the posttranslational modifications remain poorly understood (4, 5). Thus, elucidation of the precise molecular mechanisms underlying HCC progression is of great importance for developing new reagents to treat this aggressive disease.

Glycosylation is the most common post-translational modification of proteins and aberrant glycosylation is often observed in cancers (6, 7). Accumulating evidence indicates that alterations in N-linked glycosylation are a hallmark of various liver diseases, including HCC (5, 8). For instance, expression of N-acetylglucosaminyltransferase-III and -V is increased in HCC (9, 10). N-glycan profiling study identified novel N-glycan structures in serum as prognostic markers of HCC (11). In addition, α1,6-fucosyltransferase can generate fucosylated α-fetoprotein (AFP), which provided a more accurate diagnosis of HCC 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 roles in cancer. O-glycans on MICA enhance bladder tumor metastasis (16), and O-glycosylation of death-receptor controls apoptotic signaling in several types of cancers (17). In addition, our previous study reported that GALNT2, an O-glycosyltransferase, regulates epidermal growth factor (EGF) receptor activity and cancer behaviors in HCC cells (18). Therefore, understanding the roles of O-glycosylation in HCC may provide novel insights into the pathogenesis of HCC.

Core 1 β1,3-galactosyltransferase (C1GALT1) is a critical mucin-type O-glycosyltransferase which 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 Galβ1-3GalNAcα1-Ser/Thr structure (T antigen or core 1 structure) (21). The core 1 structure is the precursor for subsequent extension and maturation of mucin-type O-glycans (22). C1GALT1 have
been shown to regulate angiogenesis, thrombopoiesis, and kidney development (23, 24). Although mucin-type O-glycosylation and C1GALT1 have been demonstrated to play crucial roles in a variety of biologic functions, the expression and role of C1GALT1 in HCC remain unclear. Here, we found that the expression of C1GALT1 is frequently overexpressed in HCC and its expression is associated with poor survival of HCC patients. We therefore hypothesized that C1GALT1 can regulate the malignant growth of HCC cells and contribute to the pathogenesis of HCC.

MATERIALS AND METHODS

Human tissue samples

Post surgery frozen HCC tissues for RNA extraction and Western blotting, and paraffin-embedded tissue sections were obtained from the National Taiwan University Hospital. This study was approved by the Ethics Committees of National Taiwan University Hospital and all patients were 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 (Hsinchu, Taiwan) in year 2008. HA22T, SNU387, and HCC36 cells were kindly provided by Prof. Shiou-Hwei Yeh (National Taiwan University, Taiwan) in year 2010. All cell lines were authenticated
by the provider based on morphology, antigen expression, growth, DNA profile, and
cytogenetics. Cells were cultured in DMEM containing 10% fetal bovine serum
(FBS) in 5% CO₂ at 37°C. To analyze growth factor-induced cell signaling, cells
were starved in serum free DMEM for 5 h, and then treated with 25 ng/ml of HGF, or
IGF at 37°C for 30 minutes.

Reagents and antibodies

Vicia villosa agglutinin (VVA) and peanut agglutinin (PNA) lectins conjugated
agarose beads, FITC- and biotinylated VVA were purchased from Vector
Laboratories (Burlingame, CA). Recombinant EGF, hepatocyte growth factor (HGF),
insulin-like growth factor 1 (IGF-1), and protein deglycosylation kit were purchase
from Sigma (St Louis, MO). PHA665752 was purchased from Tocris Bioscience
(Ellisville, MO). Antibodies against C1GALT1, GAPDH and IGF1R were purchased
from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against MET pY
1234/5, IGF1R pY1135/1136, p-AKT, p-ERK1/2, and ERK1/2 were purchased from
Cell Signaling Technology Inc. (Beverly, MA). Antibodies against total MET and
AKT were purchased from GeneTex Inc. (Irvine, CA).

Tissue array and immunohistochemistry
Paraffin-embedded human HCC tissue microarrays were purchased from SUPER BIO CHIPS (Seoul, Korea) and Biomax (Rockville, MD). Arrays were incubated with anti-C1GALT1 monoclonal antibody (1:200) in 5% BSA/PBS and 0.1% Triton X-100 (Sigma) for 16 h at 4°C. After rinsing twice with PBS, Super Sensitive™ Link-Label IHC 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 (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’-ACAGTCAGCCGCATCTTCTT-3’. Quantitative real-time PCRs were performed 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 transfection. The transfected cells were selected with 600 μg/ml of G418 for 14 days and then pooled for further studies.

RNA interference

Two siRNA oligonucleotides against C1GALT1 (5’-UUAGUAUACGUUCAGGUAA 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 h. The pLKO/C1GALT1-shRNA plasmid and non-targeting pLKO plasmids were purchased from National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). The 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 performed as reported previously (18).

Phospho-receptor tyrosine kinase (p-RTK) array

A human p-RTK array kit was purchased from R&D systems (Minneapolis, MN). HCC cells were serum starved for 5 h and then treated with 20% FBS for 30 min. Cells were lysed and 500 μg 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 h. 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, CA).

**De-glycosylation and lectin pull down**

Protein deglycosylation was carried out using Enzymatic Protein Deglycosylation Kit (Sigma). Briefly, cell lysates were treated with neuraminidase or PNGaseF at 37°C for 1 h. For lectin blotting, 20 μg of cell lysate was separated by an 8% SDS-PAGE, transferred to PVDF membrane (Millipore, Billerica, MA), 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 h. The pulled down proteins were analyzed by Western blotting.

**Tumor growth in immunodeficient mice**

For tumor growth analysis, 7×10^6 of HCC cells were subcutaneously injected into
severe combined immnodeficient (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, HCC cells were incubated with or without 25 ng/ml of human HGF in DMEM on ice for 5 min. Cross-linker Bis(sulfosuccinimidyl) suberate (BS³, 0.25 mM, Thermo Scientific, Lafayette, CO) was added to cells and reacted at 37°C for 5 min. Cells were then transferred on ice for 10 minutes. Reactions were blocked by adding 50 mM of Tris-HCl (pH 7.4). Cell lysates were separated by 6% SDS-PAGE and immunoblotted with anti-MET antibody.

**Statistical analysis**

Student's *t*-test was used for statistical analyses. Paired *t*-test was used for the analyses of paired HCC tissues. Mann-Whitney U test was used to compare unpaired non-tumor liver tissue and HCC tissues. Two-sided Fisher's 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’s correlation test was used to assess C1GALT1 and phosphor (p)-MET expression. Data are presented as means ± SD. *P* < 0.05 is considered statistically significant.
RESULTS

Expression of C1GALT1 is up-regulated in HCC and correlates with advanced tumor stage, metastasis, and poor survival

We first investigated expression of C1GALT1 in HCC tissues. Paired HCC and adjacent non-tumor liver tissues (n = 16) were analyzed by real-time RT-PCR. Results showed that C1GALT1 mRNA was significantly up-regulated in HCC tissues compared with adjacent non-tumor liver tissues (paired t test, p < 0.05) (Fig. 1A). Consistently, Western blotting showed that C1GALT1 protein was overexpressed in HCC tissues of paired specimens (Fig. 1B). We further performed immunohistochemistry (IHC) for 72 primary HCC tissues and 32 non-tumor livers to investigate the expression of C1GALT1. The immunohistochemistry showed dot-like precipitates of C1GALT1 in the cytoplasm of HCC (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
HCC tumors, while only 19% of non-tumor liver tissues expressed high levels of C1GALT1 (Mann-Whitney U Test, p = 0.002; Fig. 1C and 1D). Consistently, results from tissue microarrays also showed increased expression of C1GALT1 in HCCs compared with normal liver tissues (Supplementary Fig. S1). These results indicated that C1GALT1 expression was significantly higher in HCC than that in non-tumor liver tissues.

We next investigated the relationship between C1GALT1 expression and clinicopathologic features in HCC patients. We found that high expression of C1GALT1 correlated with advanced tumor stage (Fisher's exact test, p < 0.05) and metastasis (Fisher's exact test, p < 0.01) of HCC tumors (Table 1 and Supplementary Table 1). A Kaplan-Meier survival analysis showed that the survival rate of HCC patients 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 up-regulated in HCC and its expression is associated with advanced tumor stage, metastasis, and poor survival of HCC.

C1GALT1 modifies mucin-type O-glycans on HCC cells

To investigate functions of C1GALT1 in HCC, knockdown or overexpression of
C1GALT1 was performed in multiple HCC cell lines. Western blotting showed that the average expression level of C1GALT1 was significantly higher in HCC cell lines compared with that of non-tumor liver tissues (Fig. 2A). We performed knockdown of C1GALT1 with 2 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 HCC 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. There are 7 proteins with 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 4 tested cell lines. Consistently, flow cytometry showed that C1GALT1 altered VVA binding to the surface of HCC cells (Fig. 2C). These results indicate that C1GALT1 modulates the expression of mucin-type O-glycans on HCC cells.

C1GALT1 regulates HCC cell proliferation in vitro and in vivo
Effects of C1GALT1 on cell viability and proliferation of HCC 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, while 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#8 of HA22T cells and clone#10 of PLC5 cells were subcutaneously xenografted in SCID mice. The results showed that knockdown of C1GALT1 significantly suppressed the volume and weight of HCC 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 HCC cell proliferation in vitro and in vivo.
C1GALT1 regulates phosphorylation of MET

Since RTKs are crucial for HCC 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 HCC cells. A human phospho-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 HCC, 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 HCC 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, upper panels). 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 IGF1-induced signaling in all tested HCC cell lines (Fig. 4B, lower panels). These results suggest that C1GALT1 selectively activates the HGF/MET signaling pathway.
To investigate the role of MET signaling pathway in C1GALT1-enhanced cell viability, we treated HCC 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 also observed that knockdown of C1GALT1 decreased HGF-induced cell migration and invasion, while 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 HCC tissues. Western blotting (Fig. 4D) and Pearson’s test (Fig. 4E) from 20 HCC 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 HCC.

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

To investigate the mechanisms by which C1GALT1 regulate HGF/MET signaling, we analyzed the effects of C1GALT1 on glycosylation and dimerization of MET in HCC cells. Since 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 7 HCC 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 HCC cells.

To investigate whether C1GALT1 can modify O-glycans on MET, VVA binding to MET was analyzed in HCC 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 HCC 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).

Since 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 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 HCC cells.

DISCUSSION

This study showed that overexpression of C1GALT1 in HCC tissues was associated with advanced tumor stage, metastasis, and poor prognosis. C1GALT1 expression regulated HCC cell viability and proliferation \textit{in vitro} and \textit{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 HCC cells. Taken together, this study is the first to show that C1GALT1 was able to regulate HCC cell proliferation \textit{in vitro} and \textit{in vivo} and modulation of
O-glycosylation and activity of MET may be involved in this process. Our findings provide novel insights into not only the role of O-glycosylation in the pathogenesis of HCC but also the development of reagents for HCC 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 HCC has been reported (34, 35). However, glycogenes responsible for these O-glycans and their functions in HCC remain largely unknown. Previously, we reported that GALNT1 and GALNT2 are the major GalNAc transferases in liver tissues, and GALNT2 modulates the sialyl Tn expression on EGF receptor in HCC cells and suppresses their malignant properties (18). Here, we further reported that C1GALT1 expression is dysregulated in HCC and C1GALT1 modulates the expression of mucin-type O-glycans on HCC cell surfaces.

We found that O-glycans can be decorated on MET, an important proto-oncogene in a variety of human cancers. Our data showed that MET from all tested 7 HCC 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 Tn are sialylated in HCC 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. Since receptor dimerization is a key regulatory step in RTK
signaling (37), therefore 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 increased cell proliferation, poor prognosis, and poor outcome of human HCC (38-40). HGF/MET signaling has been demonstrated to promote invasion and metastasis of HCC cells (41, 42). Our data showed 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 as an attractive strategy for treating many human cancers, including HCC (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 HCC 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 HCC 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 HCC cells. Thus, targeting C1GALT1 could have similar effects as targeting multiple RTKs. This study opens avenues for treating cancers by targeting not only the receptors themselves but also their O-glycosylation regulators.
REFERENCES


FIGURE LEGENDS

Figure 1. Expression of C1GALT1 in human HCC. A, expression of C1GALT1 mRNA in 16 paired HCC 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 blots showing C1GALT1 expression in paired HCC tissues from A. Representative images are shown. N, non-tumor liver tissue; T, tumor tissue. C, immunohistochemistry of C1GALT1 in paired primary HCC tissues. The brown stained cells in the tumor part are hepatocellular carcinoma cells and those in the non-tumor part are hepatocytes. The staining was visualized with 3,3-diaminobenzidine liquid substrate system and all sections were counterstained with hematoxylin. Representative images of tumor (left) and non-tumor liver tissue (right) are shown. The negative control does not show any specific signals (lower left of the upper panel). Amplified images are shown at the lower panels. Scale bars, 50 μm. D, statistical analysis of immunohistochemistry in HCC tissues. The intensity of C1GALT1 staining in tissue s is shown in the upper panel. Scale bars, 50 μm. Results
are shown at the lower panel. Mann-Whitney U Test, \(p = 0.002\). E, Kaplan-Meier analysis of overall survival for HCC patients. The analyses were performed according to the immunohistochemistry of C1GALT1. Probability of overall survival is analyzed after the initial tumor resection. Log-rank test, \(p = 0.001\).

**Figure 2. C1GALT1 regulates O-glycosylation in HCC cells.** A, Expression of C1GALT1 in HCC cell lines. C1GALT1 expression in 7 HCC cell lines and 9 non-tumor (N) liver tissues were analyzed by Western blotting. GAPDH is an internal control. Signals are quantified by ImageQuant5.1 and shown. B, effects of C1GALT1 on binding of *Vicia villosa* agglutinin (VVA) to glycoproteins. Western blotting shows that C1GALT1 expression after knockdown with two *CIGALTLI* siRNAs compared with control (Ctr) siRNA in HA22T cells and PLC5 cells. Overexpression of C1GALT1 in Sk-Hep1 and HCC36 cells transfected with *CIGALTLI*/pcDNA3.1 plasmid (C1GALT1) 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 and indicated by red arrows. C, effects of C1GALT1 on O-glycans of HCC cell surfaces. Surface O-glycans were analyzed by flow cytometry with FITC-VVA. Negative (-) shows cells without adding VVA-FITC.
Figure 3. C1GALT1 regulates HCC 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 h. The results are standardized by the cell number at 0 h. Data are represented as means ± SD from three independent experiments. *P < 0.05; **P < 0.01. B, effects of C1GALT1 on cell proliferation. Cells were immunofluorescently stained for Ki-67 and Ki-67 positive cells were counted under microscope. Results are presented as means ± SD from three independent experiments. *P < 0.05; **P < 0.01. C, effects of C1GALT1 on HCC tumor growth and proliferation in SCID mouse model. HA22T (upper) and PLC5 (lower) 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 ± SD from 4 mice for each group. *P < 0.05.

Figure 4. C1GALT1 regulates activity of MET in HCC cells. A, Human phospo-RTK array showing the effect of C1GALT1 on the phosphorylation of RTKs. Cell lysates of control and C1GALT1 knockdown HA22T cells were applied to
phospho-RTK array including 42 RTKs. **B**, C1GALT1 modulates HGF-induced signaling in HCC cells. HA22T, PLC5, Sk-Hep1, and HCC36 cells were starved for 5 h and then treated with (+) / without (-) HGF (25 ng/ml) or IGF (25 ng/ml) for 30 min. 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 h. Data are represented as means ± SD from three independent experiments. **P < 0.01. **D**, expression of C1GALT1 and p-MET in HCC 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 HCC tumors. Pearson’s test was used to analyze the statistical correlation of C1GALT1 and p-MET expression in (D).

**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 HCC 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 HCC cells. HCC cells were starved for 5 h 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 arrow heads indicate the monomer (M). Markers of molecular weight are shown on the left. GAPDH is an internal control.
Fig. 1
Fig. 3
Fig. 5
Table 1. Correlation of C1GALT1 expression with clinicopathologic features.

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*P < 0.05 is considered statistically significant.
C1GALT1 enhances proliferation of hepatocellular carcinoma cells by modulating MET glycosylation and dimerization

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