Nucleotide Sugar Transporter SLC35A2 Is Involved In Promoting Metastatic Potential Of Hepatocellular Carcinoma

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Research Article

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Abstract

Purpose

Recently, aberrant glycosylation has been recognized to be relate to malignant behaviors of cancer and outcomes of patients in various cancers. SLC35A2 serves as a nucleotide sugar transporter and plays an indispensable role on glycosylation. However, effects of SLC35A2 on malignant behaviors of cancer cells and alteration of cancer cells surface glycosylation profile are still not fully understood, particularly in liver cancer. Hence, from an glycosylation prospective, we assessed the effects of SLC35A2 on malignant behaviors including metastatic potential of liver cancer cells.

Methods

SLC35A2 expression in HCC cells was examined by Western blot and quantitative PCR and was regulated by RNA interference or vectors-mediated transfection. Effects of SLC35A2 expression alteration on malignant behaviors and membrane glycan profile of HCC cells by using respectively invasion, migration, cell adhesion assay and lectins microarray. Co-location among proteins in HCC cells was observed by fluorescence microscope and detected by an in vitro co-immunoprecipitation assay.

Results

SLC35A2 was upregulated in HCC, and associated with poor prognosis of patients. SLC35A2 knockdown led to the decrease of invasion, adhesion, metastasis and the alteration of membrane glycan profile as well as the dysregulated expressions or glycosylation of cell adhesion-related molecules in HCC. Mechanistically, delivery of B4GalT1 to Golgi apparatus required the involvement of SLC35A2 in HCC cells and the site G266 was critical in maintaining the intact activity of B4GalT1.

Conclusion

SLC35A2 plays important roles in promoting HCC metastasis by regulating glycosylation modification on HCC cell surface and inducing cell adhesive ability.

Introduction

As an integral feature of almost all biomolecules including nearly all cell surfaces and over 70% of secretory proteins, glycans contribute the most abundant and diverse post-translation modification [1]. At present, glycosylation has gained recognition for its pivotal role in virtually all aspects of our body, from embryogenesis to pathogenesis [2]. Thus, it is not surprising that glycosylation changes are also a universal feature of most major diseases, directly/indirectly associated with a change in the glycosylation pattern of at least one central structure [3, 4].

Page 2/20
Until now, a clear correlation between aberrant glycosylation status of cancers and outcomes of patients has been demonstrated in numerous cancers [5, 6]. However, whether aberrant glycosylation as a result or cause for carcinogenesis is a long-standing debate. In these years, many studies suggested that glycosylation of functionally important proteins, such as E-cadherin and immunoglobulin family receptors (e.g., CD44), altered the strength of cell-cell interaction in cancer, which led to invasion and metastasis of cancers [7, 8]; moreover, aberrant glycosylation of proteins functions as an essential mechanism in defining stage, direction and fate of cancer progression [9, 10]. However, the structural and functional complexity of protein glycosylation make it more difficult to define cancer cell phenotypes than certain proteins/genes [6, 11, 12].

As we know, the glycosylation of glycoconjugates and the biosynthesis of polysaccharides need activated nucleotide-sugars, which serve as the substrates for glycosyltransferases [13]. In these biochemical processes, it is nucleotide sugar transporters (NSTs) that mediate nucleotide sugars transportation from the cytosol into the ER/Golgi lumen [14]. NSTs, classified as the drug metabolite transporter family (solute carrier family 35: SLC35), serve as antiporters of intracellular nucleotide-sugar/nucleotide-monophosphate and nucleotide-sugar/nucleotide-sugar [15–17]. Furthermore, the activity of endogenous NSTs has been implicated in organogenesis, development, mammalian cellular immunity and pathogenicity of human diseases nowadays [15, 18]. For instance, a defect in a human NST have been described to be directly associated with congenital disorders of glycosylation (CDG) [19].

However, far fewer attentions are paid to NSTs, as one of most important functional proteins in glycol chains synthesis, than to various glycosyltransferases in current disease-associated glycobiological studies. This could be mainly because most researchers assume the expression and functions of glycosyltransferases are the major contributor for glycosylation process; in contrast, NSTs are only the subordinated factor, because they just provide substrates for glycosyltransferases [14]. Nevertheless, NSTs can modulate concentration of specific nucleotide-sugar through changing their activity under certain conditions. As a member of NSTs family, SLC35A2 is being found to be closely associated with a variety of cancers [20, 21]. However, effects of SLC35A2 on cancer cells malignant behaviors and alteration of cancer cells surface glycosylation profile is still not well-known, particularly in liver cancer.

In the present study, we examined SLC35A2 expression in various HCC cell lines with different metastatic potentials and tissues and evaluated effects of SLC35A2 on the malignant biological behaviors of HCC cells and glycosylation pattern on HCC cell surface, particularly on carbohydrate structure of key adhesive factor-E-cadherins. Furthermore, it further showed high tumoral SLC35A2 expression can serve as a sensitive ‘readout’ for high-risk HCC metastasis and poor outcome. Altogether, it suggests that SLC35A2 plays significant roles in promoting HCC metastasis, which could rely on altering glycosylation modification on HCC cell surface and inducing cell adhesive ability.

**Materials And Methods**

**Cell Culture**
Five human HCC cell lines including Hep3B, HepG2, MHCC97L, MHCC97H and HCCLM3 (from the Liver Surgery Department at Zhongshan Hospital, Fudan University.) with consecutively increased metastasis potentials were routinely cultured at 37°C in 5% CO₂ in high-glucose MEM or DMEM medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, UT, USA). Briefly, the cells were grown to 90% confluency and harvested by treating with 0.25% trypsin and 0.02% EDTA. After rinsed three times with PBS, cells were centrifuged for further RNA isolation and protein extraction.

**Lentivirus-induced SLC35A2 Deficiency (Lv-shSLC35A2)**

Three different sequences targeted to 3 different sites of SLC35A2 mRNA (NM_0005660) were designed without off-target effects and cloned into expression vector pGCsi-SLC35A2shRNA. After validation of inhibited efficiency in HCC cells, a most efficient target sequence (5’-CAGUAUGUUGCCAUCUCUA-3’) was identified and then modified their structure to form short hairpin RNA (shRNA-2): 5’-CCGGCAGTATGTTGCCATCTTATTCAAGAGATAGAGATGGCAACATACTGTTTTTG-3’. Viral vector generation was obtained by co-transfection of 293T cells by the calcium phosphate precipitation (CPP) method on 10-cm plates with 20 μg of PGC-LV, 15 μg of pHelper1.0EGFP, and 10 μg of pHelper2.0. Infectious lentiviruses were harvested, centrifuged to eliminate cell debris, and then filtered through 0.22-μm cellulose acetate filters. Infectious titer was determined by fluorescence-activated cell sorting analysis of EGFP positive in 293T cells. A multiplicity of infection (MOI) of 20 for HCC cells in serum-free growth medium was used. Transduced cells were selected by 1μg/mL puromycin and used in subsequent assays.

**Construction of pcDNA3.1-based wild-type and mutant vectors**

The coding sequences of SLC35A2 and B4GalT1 were amplified by RT-PCR and cloned respectively into pcDNA3.1(+)-flag and pcDNA3.1(+)-HA eukaryotic expression vectors. Moreover, de novo mutant in SLC35A2 gene (c.797G > T, p.G266V) was constructed by using site-directed mutagenesis system. These cloned sequences were confirmed by carrying out a sequencing analysis. The transfection of these vectors into HCC cells was Lipofactamin 3000-mediated, according to the manufacturer's protocol. Co-localization of SLC35A2 with B4GalT1 in HCC cells through co-transfecting pcDNA3.1-HA-SLC35A2 and pcDNA3.1-Flag-B4GalT1 was observed by fluorescence microscope (Leica, DM500).

**Western blot**

Equivalent protein amounts were separated on a denaturing SDS polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membrane. After blocked with 5% nonfat dry milk in PBS containing 0.05% Tween-20, membranes were incubated with primary antibodies. Visualization used horseradish peroxidase linked anti-rabbit secondary antibodies (Cell Signaling) and ECL-Plus blotting substrate detection kit (Pierce). Quantification of Western blots was analyzed densitometrically by using Quantity One software (Bio-Rad Laboratories).

**Polymerase Chain Response**
RT-PCR assay was carried out according to manufacturer’s instruction. Briefly, 1-2μg RNA was incubated at 70°C for 5 min and then placed on ice. After addition of oligo-(dT)$_{18}$ primer, RT reaction was performed at 42°C for 60 min followed by 97°C for 2 min. For PCR amplification, 1μL cDNA from RT was used in a final volume of 25μL, PCR program for SLC35A2 was performed as 95°C for 5 min, followed by 25 cycles of 95°C for 30s, 60°C for 50s and 72°C for 1 min and a final extension at 72°C for 10min. The products were separated by electrophoresis in a 1.2% agarose gel and visualized after staining with ethidium bromide.

For quantitative RT-PCR (qRT-PCR), Platinum® SYBR® Green qPCR Super Mix kit (Invitrogen, Carlsbad, CA, USA) was used. Reactions were carried out using three independent technical replicates for each sample which was quantified in IQ5 real-time PCR system (Bio-rad). The copy number was adjusted by the geometric mean of two house-keeping genes (GAPDH and ACTB).

**Invasion and migration assay**

In vitro invasion assay was performed using 24-well Transwell unit with polycarbonate filters transwell cell culture plates (8-μm pore size; Costar, Acton, MA, USA). Briefly, 1x10$^5$ cells were seeded onto Matrigel-coated (0.8 μg/μL) upper chambers of the membrane and the lower chambers were filled with 600 μL NIH-3T3 conditioned media as a resource of chemoattractants. After 36 hours, cells were fixed with 0.5% glutaraldehyde and stained with Gimsa. Cotton swabs were used to remove the cells from the upper surface of the membrane, leaving the cells on the underside. For the migration assay, experimental procedures are the same as the in vitro invasion assay described above except that the filter was not coated with Matrigel. The membrane was removed and mounted onto glass slides and scanned.

**Cell adhesion assay**

Cell adhesion assay was performed by using CytoSelectTM cell adhesion assay (Cell Biolabs, Inc.). In brief, under sterile conditions, allow the Fibronectin, Collagen III and Laminin Adhesion Plate or flat-bottom culture plates were coated with E-selectin to warm up at room temperature for 10 minutes. Prepare a cell suspension containing 0.5x10$^6$ cells/ml in serum free media. Add 150 μL of the cell suspension to the inside of each well (BSA-coated wells are provided as a negative control). Incubate for 60 min in a cell culture incubator. Carefully aspirate the media from each well. Gently wash each well 4-5 times with 250 μL PBS. Aspirate the PBS from each well and add 200 μL of Cell Stain Solution. Incubate for 10 minutes at room temperature. Discard the Cell Stain Solution from the wells. Gently wash each well 4-5 times with 500 μL deionized water. Discard the final wash and let the wells air dry. Add 200 μL of Extraction Solution per well, and then incubate 10 minutes on an orbital shaker. Transfer 150 μL from each extracted sample to a 96-well microtiter plate and measure the OD 560nm in a plate reader.

For cell-cell adhesion assay, human endothelial cells (HEC) were seeded in 96-well plates and cultured for 48-72 hr until 100% confluent. Then pretreated or untreated HCC cells with sustained expression of EGFP protein were added to each well as 2.0x10$^4$/well and incubated at 37°C for 60 min. After washed three times, plates were subjected to fluorescence microscopy and remaining HCC cells were counted.
**In vivo metastasis assay**

An orthotopic liver xenograft model was established for tumor growth and metastasis analysis as previously described. In brief, 1×10^7 stably transfected MHCC-97H cells were resuspended in PBS buffer and injected subcutaneously into the capsule of the left hepatic lobe of male BALB/c nude mice. Tumour volume was monitored and calculated as volume (mm^3) = [width^2 (mm^2) × length(mm)]/2. After 7 weeks, mice were sacrificed and the lungs were removed and fixed in formalin, embedded in paraffin. Lung colonization was carefully counted by histological examination under microscope after consecutive tissue sections (5 µm/each). The Animal Experimentation Ethics Committees of our institutes approved the animal study.

**Immunoprecipitation of E-cadherin**

Equal amounts of total protein of each canine cell line (750 µg) were precleared with 50 µl of protein G-sepharose beads for 1.2 h. After centrifugation, the supernatant was incubated overnight with 5 µg of monoclonal antibody (mAb) against E-cadherin. After that, incubation with protein G-sepharose for 2 h was performed. Next, the beads were washed three times with an immunoprecipitation buffer. The immune complexes were released by boiling 5 min at 95°C in Laemmli sampling buffer and the immunoprecipitants were subjected to 7.5% SDS–PAGE and the separated proteins were transferred to a nitrocellulose membrane. The mobility shift was evaluated.

**Cell surface glycosylation pattern analysis**

13 kinds of tumor-associated lectins (Vector laboratory, Burlingame, CA, USA) were dissolved with chip-spotting buffer (CapitalBio, Beijing, China) at a concentration of 1 mg/mL respectively, and spotted on gel-substrate chip using a microarray printing robot Smart Arrayer-48 (CapitalBio, Beijing, China). The diameter of each point is 150µm, and distance between two points is 400µm. Each lectin point has 4 repeats. Then the chip was incubated in a vacuum chamber with humidity greater than 80% at 25°C overnight to immobilize the lectins.

Cells were cultured and reached greater than 80% confluence. Then cells were harvested by cell-scraping and collected by centrifugation at 1200 rpm for 5 min. The resulting pellet was washed 4-5 times with warmed PBS (PH 7.4) and cells were resuspended in 0.5-1 ml cy3 in NaHCO3-NaH2CO3 (PH 9.3) at 10^6 cells/ml. After incubation at room temperature for 30-60 min, stained cells were washed three times in PBS and resuspended in 500 ml PBS. The lectin arrays were blocked by 20% BSA-PBS at room temperature for 1 hr, followed by washing with 0.1% Tween 20-PBS for 5 min, three times. Stained cells were added into sub-array and incubated in the dark for 15-30 min in humidified incubator at 37°C. The binding fluorescence signals of the glycoproteins with lectins were obtained with fluorescence scanner LuxScan 3.0 (CapitalBio, Beijing, China). The net intensity value for each spot was calculated by subtracting background value. Median rectification was used to calculate the dye-bias-corrected ratios. Median corrected data were used to calculate q value by T-statistical analysis with Significance Analysis of Microarrays (SAM, version 2.1, Stanford University, CA, USA) software.
**In vitro co-immunoprecipitation assay**

The cell culture dishes were placed on ice and the cells were washed with ice cold PBS. After the PBS was drained, then the cells were treated with buffer (170 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM NaF, 0.5% NP-40) containing protease inhibitors. After clearing with protein-G-sepharose, supernatants were incubated with protein-G-sepharose conjugated with anti-GP73 monoclonal antibody at 4 °C overnight. Subsequently, protein–beads complexes were washed with PBS for three times and then re-suspended in 20 μL loading buffer. Lastly, the proteins were detected by western blotting.

**Bioinformatics analysis**

The expression data of patients with live hepatocellular carcinoma (LIHC, n=371) was downloaded from TCGA database (https://portal.gdc.cancer.gov) by using the data transfer tool. The differential expression analysis of SLC35A2 among a variety of groups in LIHC was performed through GEPIA2 web server (http://gepia2.cancer-pku.cn). Overall survival probability and progress-free survival probability was plotted by using the Kaplan-Meier analysis. The comparison was performed at the 0.05 level of significance. The gene list positively correlated with SLC35A2 in human hepatocellular carcinoma was downloaded from the public cancer UALCAN databases (http://ualcan.path.uab.edu). Then Metascape database (http://www.metascape.org, v3.5.20220101) was used for pathway enrichment analysis, in which P value less than 0.05 was considered as significant.

**Statistical analysis**

Data were expressed as mean ± standard error (SE) and analyzed using analysis of variance (ANOVA). Student’s t-test was used in two-group comparisons. The association between the various factors was determined using the Pearson correlation. P<0.05 was considered to be statistically significant.

**Results**

**Association of SLC35A2 expression with HCC metastatic potential**

To investigate the association of SLC35A2 expression with HCC metastatic potential, we performed firstly a bioinformatics analysis on SLC35A2 expression feature in HCC by exploring the public cancer databases including GEPIA, UALCAN and Oncomine databases. It was found that SLC35A2 expression was significantly increased in HCC, compared to normal tissues (Fig.1A); the increased expression of SLC35A2 was also associated with tumor grades in HCC (Fig.1B). Of note, it was further observed that SLC35A2 expression was significantly higher in HCC tissues with lymph node invasion or metastasis than those without with lymph node invasion or metastasis (Fig.1C-D). Moreover, overall survival of HCC patients with SLC35A2 high-expression was significantly decreased, compared to those with SLC35A2 low-expression (45.6 vs 70.1 months, P<0.01) (Fig.1E); meanwhile, there was a significant difference of progress-free survival between patients with SLC35A2 high-expression and with SLC35A2 low-expression (13.1 vs 29.7 months, P<0.01) (Fig.1F). In addition, SLC35A2 expression was detected in a variety of HCC
cells (HepG2, Hep3B, MHCC97L, MHCC97H and HCCLM6) with various metastatic potential. It was also shown that SLC35A2 expression in HCC cells (MHCC97L, MHCC97H and HCCLM6) with high metastatic potential was significantly higher than in HCC cells without nearly metastatic potentials (HepG2 and Hep3B) (Fig. 1G-I).

**Impacts of SLC35A2 knockdown on HCC invasion, adhesion and metastasis**

Based on high endogenous levels of SLC35A2 protein in metastatic HCC cells, MHCC97L/H, HCCLM6 with high metastatic potential was used for subsequent knockdown experiments. After transfection with Lv-shSLC35A2, SLC35A2 protein levels were significantly decreased in HepG2, Hep3B and MHCC97H, respectively (Supplementary Fig.S1). Stable integration and expression of lentivirus-mediated SLC35A2-shRNA in MHCC97H cells were monitored for more than 10 passages and did not alter their morphology, which remained epithelial in appearance.

Because carbohydrate determinants are known to involve in mediating cancer cells metastasis and adhesion [22], impacts of SLC35A2 deficiency on HCC cells adhesion, invasion and metastasis were investigated in this study. Before these investigations, effects of SLC35A2 deficiency on HCC cells proliferation and apoptosis were assessed. It was found that proliferation and apoptosis of HCC cells were not significantly different after SLC35A2 knockdown in HCC cells (Supplementary Fig.S1).

Subsequently, the effects of SLC35A2 down-regulation on abilities of HCC cells to invade Matrigel and migration were examined. As shown in Fig.2A, HCC cells across the membranes were significantly decreased after SLC35A2 knockdown, compared to control and mock-transfected HCC cells. Furthermore, adhesive ability of HCC cells with SLC35A2 knockdown to different matrix was tested. It was shown that, although number of HCC cells adhesion to Collagen ⊂, Fibronectin (FN), E-selectin and Matrigel was lower than control group, the decrease extent of HCC cells adhesion to E-selectin was largest, followed by Matrigel, then FN and Collagen ⊂ after SLC35A2 deficiency (Fig.2B). In addition, adhesion of HCC cells with Lv-shSLC35A2 transfection to vascular endothelial cells was also observed. As expected, HCC cells adhered to vascular endothelial cells was significantly after SLC35A2 knockdown (Fig.2C-D).

Furthermore, 7 weeks after orthotopic liver xenograft of SLC35A2 knockdown (Lv-shRNA) HCC cells, tumor weight and volume were calculated and no difference was found between Mock group and Lv-shRNA group (p>0.05) (Fig.2E-G); while there was significant difference in the number of metastatic foci in lung between the two groups (p<0.5) (Fig.2H).

**Effect of SLC35A2 knockdown on membrane glycan profile and carbohydrate determinants of HCC cells**

To elucidate the membrane glycan profile alteration of HCC cells with SLC35A2 knockdown, lectins array experiments were performed through interaction of labeled cells with immobilized lectins on array. Compared to control HCC cells, HCC cells with SLC35A2 knockdown present significant differential binding ability to five classes of lectins, including AAL, DSA, LCA, MAL-II and WGA, SNA (Fig.3A-C). To investigate further whether a change in expression of these specific carbohydrate determinants, such as Thomsen-Friedenreich (TF) antigen, sialyl Lewis A (SLea) and sialyl Lewis X (SLex), after SLC35A2 alteration, HCC cells were transfected with pcDNA3.1-HA-SLC35A2. The result showed that SLC35A2 up-
regulated expression induced TF antigen, SLea and SLex expression in HCC cells, few increases in HepG2, a moderate increase in Hep3B but a distinct increase in MHCC97H (Fig. 3D). Moreover, some cell adhesion-related molecules and glycosyltransferases were further investigated after SLC35A2 knockdown. It was found that the expressions of cell adhesion-related molecules such as ICAM-1, integrin β1, integrinα2 and E-cadherin were down-regulated while the expressions of glycosyltransferases including Gnt3, Fut8 and B4GalT1 were not significantly altered in HCC cells with SLC35A2 knockdown (Fig.4A-D). In addition, to further clarify whether the glycosylation of cell-adhesion molecules can be affected by SLC35A2, glycosylation of E-cadherin was also investigated after SLC35A2 knockdown and the result from the western blotting assay showed that E-cadherin protein from HCC cells with SLC35A2 knockdown exhibited the mobility shifts at some extent (Fig.4E).

**Maintain of SLC35A2 activity was necessary in recruiting B4GalT1 to Golgi apparatus**

To further explore the mechanism of SLC35A2 affecting the intracellular glycosylation, we performed a KEGG pathway enrichment analysis on genes positively correlated with SLC35A2 in HCC by exploring the public cancer UALCAN databases and found that these genes were enriched in histone modification, RNA splicing and Golgi vesicle transport, nucleocytoplasmic/nuclear transport (Fig.4F). As mentioned above, SLC35A2 can transport UDP-galactose, the substrates for galactosyltransferase, from the cytosol into Golgi vesicles, an important organelle for glycosylation modification of proteins. Meanwhile it is well known that among galactosyltransferase, β-1,4-galactosyltransferase (B4GalT1) is unique because it encodes an enzyme that participates both in glycoconjugate and lactose biosynthesis [23]. Herein, the levels of β-1,4-galactosyltransferase (B4GalT1) were detected in a variety of HCC cell lines. It was found that the levels of B4GalT1 were higher in MHCC97L, MHCC97H and HCCLM6 cells with high metastatic potential, compared to Hep3B and HepG2 without metastatic potentials (Fig.5A-B). Furthermore, it was also found that SLC35A2 was significantly co-located with B4GalT1 in HCC cells through co-transfecting pcDNA3.1-HA-SLC35A2 and pcDNA3.1-Flag-B4GalT1 (Fig.5C). It is well-known that Golgi apparatus provides the location for glycosylation catalyzed by various glycosyltransferase in cells. Hence, Golgi marker GP73 was further detected in SLC35A2-B4GalT1 complex. The result showed that in metastatic HCC cells, Golgi marker GP73 was obviously detectable in SLC35A2-B4GalT1 coimmunoprecipitated complex; in contrast, in HCC cells without metastatic potential, GP73 was few detected (Fig.5D-E). To investigate the role of SLC35A2 in B4GalT1 translocation to Golgi apparatus, we detected the binding of GP73 and B4GalT1 in HCC cells with SLC35A2 knockdown and found that SLC35A2-knockdown induced the dislocation of B4GalT1 with Golgi marker GP73 (Fig.5F). In addition, a sequencing analysis revealed a de novo mutation (c.797G>T, p.G266V) in SLC35A2 gene can lead to the loss of normal SLC35A2 activity [24]. Here, it was further found that compared to wild-type SLC35A2, SLC35A2 (G266V) impeded the binding of B4GalT1 to GP73 (Fig.5G).

**Discussion**

In recent years, accumulating information regarding molecular aspects of nucleotide sugar transport (NSTs) have been obtained [25, 26]; however, we still know little about the relationship of their aberrant
expression and function, as well as their association with cancer cell malignant phenotypes. As an important NST, SLC35A2 holds UDP-galactose transporter activity and carbohydrate-proton symporter activity, responsible for transporting mainly UDP-galactose from the cytosol into Golgi vesicles where glycosyl-transferases function and glycans generation. Mutations in SLC35A2 can cause some diseases associated with congenital disorder of glycosylation, such as Congenital Disorder Of Glycosylation, Type Iim and Isolated Focal Cortical Dysplasia Type Ia [27]. However, until now, there is few studies to explore the biological roles of SLC35A2 in cell phenotype, particularly in cancer cells. To our knowledge, this is the first study to elucidate the effects of SLC35A2 on HCC cells malignant behaviors and investigate its clinical significance.

In this study we found a significant increase of SLC35A2 expression in metastatic HCC cells compared to non-metastatic HCC cells and primary lesions. It was also observed that this increase was more frequent in HCC patients with lymph node or distant metastasis than in patients without metastasis; meanwhile high-expressed SLC35A2 indicated the poor outcomes in HCC. These results suggested that up-regulated expression of SLC35A2 was in accordance with enhancement of metastatic capability of HCC cells and poor outcomes of HCC patients.

To determinate the functional consequences of SLC35A2 aberrant expression, we chose HCC cell lines overexpressing endogenous SLC35A2 and efficiently knocked down intercellular SLC35A2 expression. Based on observations to HCC cells biological behaviors changes after SLC35A2 deficiency, we found that the knockdown of SLC35A2 didn't result in alteration of HCC cells proliferation and apoptosis, in spite of some reports in which some NSTs are responsible for a retarded growth in human [17], but significantly decreased their invasion, migration and adhesion. Notably, in adhesion assay, we coated the flat-bottom plate with different extracellular matrix, taking into account of substrate specificity of each nucleotide-sugar transporter, which is responsible for specific glycol-structure [28], and found that after intercellular SLC35A2 deficiency, abilities of HCC cells adhesion to collagen  I, FN, E-selectin and Matrigel was decreased, but the decrease extent of HCC cells adhesion to E-selectin was largest, followed by Matrigel, then FN and collagen I. Mounting evidence suggests that carbohydrates expressed in tumor cells are directly involved in cell-extracellular matrix adhesion [29]. More importantly, the glycosylation changes associated with cancer cell adhesion include the under- and/or overexpression of naturally occurring glycans [30]. Although extra molecular mechanism of SLC35A2 knockdown-induced down-regulated adhesion to extracellular matrix, particularly E-selectin, was not investigated in this study, it was partly explained that down-regulated levels of SLC35A2 could decrease the expression of galactose-containing markers of HCC cells surface such as the TF antigen, SLea and SLeα. These carbohydrate determinants are believed to be linked with the metastatic nature of these cells and the cell adhesion qualities of cancerous cells are altered [31]. They are recognized by selectins or other carbohydrate-binding proteins or by complementary carbohydrates (through carbohydrate-carbohydrate interaction) [32]. Furthermore, in our study, we also found up-regulated expression of SLC35A2 through exogenous SLC35A2 transfection induced TF antigen, SLea and SLeα expression in HCC cells, particularly in metastatic HCC cells. This result indirectly supported the above explanation about different adhesion
ability of HCC cells with SLC35A2 knockdown to various extracellular matrixes, which may contribute to their metastatic potentials.

Recently, it has also been suggested that changes in glycosylation profiles occur during the development of hepatocellular carcinoma (HCC) [33, 34]. However, little is known concerning the effects of some specific glycosyl epitopes on malignant phenotypes of cancer cells, particularly on liver cancer, because the analysis of protein glycosylation processing seems especially relevant to liver pathology, due to this organ majorly influencing on the homeostasis of glycoproteins [35]. From the NST’s point of view, the issue mentioned above might be addressed. To elucidate the effect of SLC35A2 on the glycosylation pattern of HCC cells surface, we performed a lectins microarray analysis on a gel substrate lectins microarray, which has been established in our lab [36], through comparing the surface glycosylation profile of SCL35A2-knockdown HCC cells with parental or mock-transfected HCC cells. Significant differential binding ability to five classes of lectins, including RCA-I, DSA, LCA, MAL-I and WGA, suggesting that intercellular SCL35A2 knockdown affected glycol-structure, such as core fucose (α1–6 linked), α2-3sialic acid, (GlcNAc)n and terminal galactose. These cell surface glycosylation changes have been reported to be associated with tumor development and progress [37]. For example, an increase in sialylation and polysialic acid synthesis is the most frequent tumor-associated aberrant glycosylation, the glycosyl epitopes promotes cancer invasion and metastasis [38].

In addition, because the mechanisms through which specific glycosyl epitopes induce invasive and metastatic phenotypes of tumor cells are extremely complicate [39], at the same time, in present, there is also lack of efficient tool to explore specific glycosyl structure [40]. Despite such dilemma, it has been accepted that an alternative strategy to the analysis of glycosylation is the analysis of specific protein-associated glycans [41], because glycosylation of functionally important membrane proteins may alter tumor cell adhesion or motility in a direction that either promotes or inhibits invasion and metastasis [42, 43]. As a cell surface protein, E-cadherin is glycosylated. Disturbances in E-cadherin-based adhesion contribute to tumor progression [44], including HCC [45]. Characterizing the glycosylation profile of E-cadherin may evaluate the importance of glycosylation modifications in tumor malignant phenotype, such as involved in the intrahepatic metastasis of HCC [46]. Therefore, we analyzed the glycosylation extent alteration of purified E-cadherin from SCL35A2-knockdown HCC cells. Down-regulated glycosylation of E-cadherin suggested SLC35A2 was involved in being of carbohydrate determinant of E-cadherin in HCC cells. This is in accordance with some reports in which E-cadherin displayed high mannose type glycans as well as β1,6-branched oligosaccharides with poly-N-acetyl lactosamine structures and α2,3-linked sialic acid residues [41]. However, the molecular mechanism needs to be conformed in further experiments.

As we known, the glycan portion of glycoproteins, proteoglycans and polysaccharides is synthesized and modified by glycosyltransferases located in the lumen of the endoplasmic reticulum (ER) and Golgi apparatus [47]. During the process, a variety of nucleotide sugars need to be transported by specific transporters from cytosol into the lumen of the ER and Golgi compartment [15]. In our study, we found the level of B4GalT1 in metastatic HCC cells was higher than non-metastatic HCC cells. Furthermore,
interaction between SLC35A2 and B4GalT1 in HCC cells, in accordance with some reports [48], may collaboratively realize specific glycosylation structure. In metastatic HCC cells, Golgi marker GP73 were detectable in SLC35A2-B4GalT1 complex; however, in HCC cells without metastatic potentials, GP73 was few detected. Furthermore, it was also demonstrated that delivery of B4GalT1 to Golgi apparatus requires the involvement of SLC35A2 in HCC cells and the site G266 is critical in maintaining the intact activity of B4GalT1. Altogether, the results indicated that the translocation of SLC35A2-B4GalT1 complex into Golgi apparatus is necessary for malignant glycosylation features and is associated with metastatic feature in HCC.

There are some limitations in the present study, partly due to technical drawback. For example, whether SCL35A2 can generate unique glycol-structure phenotype in HCC cells? What is the extra molecular mechanism of SLC35A2 involved in HCC cells invasion, adhesion and metastasis and so on. Nevertheless, the current data are sufficient to demonstrate that SLC35A2 may function as a regulator of HCC metastasis process, particularly in adhesion, through modulating cell surface carbohydrate determinants. In general, cell social function is predominantly affected by glycosylation [49]. At present, various reagents that block carbohydrate-mediated tumor cell adhesion or block glycosylation processing have been shown to inhibit tumor cell metastasis [50]. This study provides the experimental basis for further development of “anti-adhesion target therapy” through blocking glycosylation signaling.

**Abbreviations**

NSTs (nucleotide sugar transporters)

ER (endoplasmic reticulum)

SLC35 (solute carrier family 35)

CDG (congenital disorders of glycosylation)

SLC35A2 (solute carrier family 35 member A2)

HCC (hepatocellular carcinoma)

MEM (minimum essential medium)

DMEM (dulbecco's modified eagle medium)

EDTA (ethylene diamine tetraacetic acid)

PBS (phosphate buffered saline)

RNA (ribonucleic acid)

mRNA (messenger ribonucleic acid)
shRNA (short hairpin ribonucleic acid)
CPP (calcium phosphate precipitation)
MOI (multiplicity of infection)
B4GalT1 (beta-1,4-galactosyltransferase 1)
RT-PCR (reverse transcription-polymerase chain reaction)
RT (reverse transcription)
SDS (sodium dodecyl sulfate)
PVDF (polyvinylidene difluoride)
PCR (polymerase chain reaction)
cDNA (complementary deoxyribonucleic acid)
qRT-PCR (quantitative reverse transcription-polymerase chain reaction)
GAPDH (lyceraldehyde-3-phosphate dehydrogenase)
ACTB (actin beta)
BSA (bovine serum albumin)
OD (optical density)
HEC (human endothelial cells)
SDS–PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis)
GP73 (golgi protein 73)
LIHC (liver hepatocellular carcinoma)
FN (fibronectin)
AAL (aleuria aurantia lectin)
DSA (datura stramonium)
LCA (lens culinaris agglutinin),
MAL-II (maackia amurensis lectin II)
WGA (wheat germ agglutinin)
SNA (sambucus nigra)
TF (thomsen-friedenreich)
SLe\(^{a}\) (sialyl Lewis A)
SLe\(^{x}\) (sialyl Lewis X)
Gnt3 (n-acetylglucosaminyltransferase-3)
Fut8\(^{\beta}\) fucosyltransferase8\(^{\beta}\)
(GlcNAc)n (n-acetylglucosamine)
OS (overall survival)
mAb (monoclonal antibody)
SAM (Significance Analysis of Microarrays)
ANOVA (analysis of variance)
SE (standard error)

**Declarations**

**Author contribution**

GK and ZJB designed the experiments; CHX, WSK and YKK, LMM generated the data; WSK and YKK performed bioinformatics and statistics analysis; CHX, ZJB and GK drafted the manuscript; all authors read and revised the manuscript; GK and ZJB guided the project.

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**Data availability**

All available material and data are presented in the manuscript.

**Code availability**

Not applicable.
Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflicts of interest/competing interests

The authors declare no conflicts of interest.

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Figures

Figure 1

SLC35A2 expression and prognostic value in HCC. Based on the liver hepatocellular carcinoma (LIHC) data from public cancer databases (TCGA), the difference of SLC35A2 expression between in normal tissues and in primary liver cancer (A), the association between SLC35A2 expression and tumor grade (B),
lymph node invasion status (C) or metastatic status (D) were analyzed by independent t test through GEPIA2 web server. Data are presented as Median (upper quartile-lower quartile). Moreover, Overall survival probability (E) and progress-free survival probability (F) was plotted by using the Kaplan-Meier analysis. The comparison was performed at the 0.05 level of significance. In addition, the levels of SLC36A2 were detected by real-time PCR (A) and western blotting (B) in five HCC cell lines with different metastatic potential. Experiments were carried out three times. Data are presented as Mean ±SE (independent t test).

**Figure 2**

**Effects of SLC35A2 knockdown on malignant behaviors and metastasis of HCC cells.** After SLC35A2 knockdown in HCC cells (MHCC97H), migration and invasion ability of HCC cells were evaluated by a transwell assay using a chamber. The results were reproducible in three independent experiments; data are presented as Mean±SE (**p<0.001) (A). The adhesion of control, Mock and Lv-shRNA-SLC35A2 transfected MHCC97H cells to one of a variety of extracellular matrix proteins including Fibronectin, Collagen IV, Laminin and E-selectin was quantified using colorimetric detection (C). Data are the Mean±SE of three independent experiments. **p<0.01 (D). In addition, in vivo experiment showed, 7 weeks after orthotopic liver xenograft of Mock and SLC35A2 knockdown (Lv-shRNA) MHCC97H (E), tumor weight (F) and volume (G) were respectively 24.5±1.7 g vs 22.2±2.4 g and 40.4±11.0 mm³ vs 39.6±9.9 mm³; no difference was found between Mock group (n=5) and Lv-shRNA group(n=5) (p>0.05). However, there was significant difference in the number of metastatic foci in lung between the two groups:22.0±2.3 vs 14.7±1.9 (p<0.05) (H).

**Figure 3**

**Effect of SLC35A2 on membrane glycan profile and carbohydrate determinants of HCC cells.** A lectins array experiment was performed in control, Mock and Lv-shRNA-SLC35A2 transfected MHCC97H cells through interaction of labeled cells with immobilized lectins on array; Combination of visual inspection of scanned images and fluorescence signal over background was considered as a positive interaction (A). Different binding ability to five classes of lectins between control, Mock and Lv-shRNA-SLC35A2 transfected MHCC97H cells was calculated; data are presented as Mean±SE (*p<0.005) (B). Schematic diagrams of sugar chain structure binding to several differential lectins including AAL, DSA, LCA, MAL-II and WGA, SNA were presented (C). The expression of three specific carbohydrate determinants, such as Thomsen-Friedenreich (TF) antigen, sialyl Lewis A (SLea) and sialyl Lewis X (SLex) was analysis by flow cytometer (D).
Figure 4

Effect of SLC35A2 on cell adhesion-related molecules and glycosyltransferases of HCC cells. The expressions of cell adhesion-related molecules such as ICAM-1, integrin β1, integrinα2, E-cadherin and glycosyltransferases including Gnt3, Fut8 and B4GalT1 in control, Mock and Lv-shRNA-SLC35A2 transfected cells (HepG2, Hep3B and MHCC97H) were detected by western blotting (A); data are presented as Mean ±SE (B-D). Moreover, glycosylation of E-cadherin was investigated after SLC35A2 knockdown by the western blotting assay and the mobility shifts was observed (E). In addition, a KEGG pathway enrichment analysis was performed on genes positively correlated with SLC35A2 in HCC by exploring the public cancer UALCAN databases (http://ualcan.path.uab.edu) (F).

Figure 5

Effect of SLC35A2 on recruiting B4GalT1 to Golgi apparatus. The levels of B4GalT1 were detected by real-time PCR (A) and western blotting (B) in five HCC cell lines with different metastatic potential. Experiments were carried out three times. Data are presented as Mean ±SE (independent t test). Co-localization of SLC35A2 with B4GalT1 in HCC cells through co-transfecting pcDNA3.1-HA-SLC35A2 and pcDNA3.1-Flag-B4GalT1 was observed by fluorescence microscope (Leica, DM500) (C). Moreover, coimmunoprecipitation of SLC35A2 and B4GalT1 in HCC cells was detected by in vitro co-immunoprecipitation assay with anti-flag and anti-HA, respectively (D-E). In addition, the binding of B4GalT1 to GP73 in HCC cells (MHCC97H) with SLC35A2 knockdown (F) and WT or mutant SLC35A2 (G) was evaluated by in vitro co-immunoprecipitation assay with anti-GP73 (G).

Supplementary Files

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- floatimage6.png
- floatimage7.png