Modification of Sialylation Mediates the Invasive Properties and Chemosensitivity of Human Hepatocellular Carcinoma*§

Yongfu Zhao‡§, Yanping Li‡, Hongye Ma‡, Weijie Dong¶, Huimin Zhou||, Xiaobo Song**, Jianing Zhang¶‡‡, and Li Jia‡§§

Aberrant sialylation is closely associated with malignant phenotypes of tumor cells, including invasiveness and metastasis. This study investigated sialylation with regard to the modification of invasive properties and chemosensitivity in human hepatocellular carcinoma (HCC) cell lines and the association between the sialyltransferase gene family and clinicopathological characteristics in HCC patients. Using mass spectrometry analysis, we found that the composition profiling of sialylated N-glycans differed between MHCC97H and MHCC97L cells with different metastatic potential. The expression profiles of 20 sialyltransferase genes showed differential expression in two cell lines, transitional and tumor tissues, from the same patients. Two genes, ST6GAL1 and ST8SIA2, were detected as overexpressed in MHCC97H and MHCC97L cells. The altered expression levels of ST6GAL1 and ST8SIA2 corresponded to a changed invasive phenotype and chemosensitivity of MHCC97H and MHCC97L cells both in vitro and in vivo. Further data indicated that manipulation of the expression of the two genes led to altered activity of the phosphoinositide-3 kinase (PI3K)/Akt signaling pathway. Targeting the PI3K/Akt pathway by its specific inhibitor wortmannin or by Akt RNA interference resulted in a reduced capacity for invasion and chemoresistance of MHCC97H cells. Our results imply that sialylation may function as an internal factor, regulating the invasion and chemosensitivity of HCC, probably through ST6GAL1 or ST8SIA2 regulation of the activity of the PI3K/Akt pathway. Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.034025, 520–536, 2014.

Metastasis of tumor cells and the development of resistance to antitumor therapies are the major causes of death in cancer patients. Specific changes in the glycosylation patterns of cell surface glycoproteins have been shown to enhance the metastatic efficiency of tumor cells, in particular that of terminal sialylation (1). It is well known that alterations in cell surface sialylated antigens affect many cellular properties—for example, cell–cell adhesion, cell–extracellular matrix adhesion, immune defense, cell metastasis, and invasion abilities (2–5). Sialyltransferases catalyze the transfer of sialic acid from cytidine 5′-monophospho-N-acetylneuraminic acid to terminal positions of glycoprotein and glycolipid carbohydrate groups (6).

The sialyltransferase (ST)1 family is a family of anabolic enzymes consisting of 20 members that are divided into three subfamilies (7). α-2,3-sialyltransferases mediate the transfer of sialic acid with an α-2,3-linkage to it with terminal Gal residues (ST3Gall-VI). α-2,6-sialyltransferases mediate the transfer of sialic acid with an α-2,6-linkage to it with terminal Gal (ST6Gall-II) (8, 9) or GalNac residues (ST6GalNACl-VI). α-2,8-sialyltransferases mediate the transfer of sialic acid with an α-2,8-linkage (ST8Sia-VI). Changes in specific sialyltransferase expression in several tumors have been reported. ST3Galllll modulates pancreatic cancer cell motility and adhesion in vitro and enhances its metastatic potential in vivo (10). The high expression of ST3GallIV is associated with the malignant behavior of gastric cancer cells (11). ST6Gall is up-regulated in colon adenocarcinoma, and its expression is positively correlated with tumor cell invasiveness and metastasis (12–14). ST6GalNAC expression is sufficient to enhance the tumorigenicity of MDA-MB-231 breast cancer cells (15). Overexpression of ST6GalNAcIl has been correlated with

1 The abbreviations used are: ST, sialyltransferase; HCC, hepatocellular carcinoma; MS, mass spectrometry; PI3K, phosphoinositide 3 kinase; PBS, phosphate-buffered saline; 5-FU, 5-fluorouracil; DMSO, dimethyl sulfoxide; MTT, methyl thiazolyl tetrazolium; IHC, immunohistochemical.
poor patient survival (16). ST6GalNAcV has recently been reported to mediate brain metastasis of breast cancer cells (17). ST8Sia I is also overexpressed in neuroectoderm-derived malignant tumors such as melanoma, glioblastoma, and neuroblastoma, as well as in estrogen receptor negative breast cancer, where it plays a role in cell proliferation, migration, adhesion, and angiogenesis (18).

The phosphoinositide 3 kinase (PI3K)/Akt pathway is involved in many cellular processes, including proliferation, differentiation, apoptosis, cell cycle progression, cell motility, tumorigenesis, tumor growth, and angiogenesis (19, 20). In addition, several reports highlight that the PI3K/Akt pathway is responsible for the proliferation, invasion, metastasis, and drug resistance of hepatocellular carcinoma (HCC), and targeting PI3K/Akt inhibits the proliferation and tumorigenesis of HCC cells (21, 22). MicroRNA-7 plays a substantial role in inhibiting the tumorigenesis and reversing the metastasis of HCC through the PI3K/Akt/mTOR signaling pathway in vitro and in vivo (23). The proliferation and invasion of HCC cells are inhibited by lipocalin 2 through the blockade of PI3K/Akt signaling (24). Activation of the PI3K/Akt pathway mediates rapamycin and sorafenib resistance in HCC cells (25, 26). However, little is known about the ST family and its signaling pathway in relation to malignant phenotypes of human HCC.

Therefore, the aims of the present study were to determine sialylated oligosaccharide alteration and expression levels of ST genes among the MHCC97H and MHCC97L cell lines and HCC patient cells by using MS and real-time PCR. In addition, we investigated whether the ST gene family participates in the regulation of tumor invasion and chemosensitivity via the PI3K/Akt pathway and the possible mechanisms.

EXPERIMENTAL PROCEDURES

Cell Culture—Human hepatocarcinoma cell lines MHCC97H and MHCC97L were obtained from the Liver Cancer Institute Zhongshan Hospital, Fudan University (China). Two cell clones of the same genetic background but with different metastatic potential were established from parental HCC cell line MHCC97 (obtained from the Liver Cancer Institute Zhongshan Hospital, Fudan University, China). The parental cell line MHCC97 is a human HCC cell line created in the animal model of human HCC LCI-D20. Relative to MHCC97L, MHCC97H has a high metastasis rate. The two cell lines were cultured in 90% DMEM (Invitrogen) supplemented with antibiotics (1× penicillin/streptomycin, 100 U/ml, Invitrogen) and 10% heat-inactivated fetal bovine serum (Invitrogen). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2. The two cell lines had activated fetal bovine serum (Invitrogen). Cells were incubated at 37 °C in a CO2 incubator to

Samples of Patients—Human primary HCC and transitional tissues (3 cm from the tumor edge) were extracted from 101 patients who underwent surgical resections from July 2010 to May 2012 at the Second Affiliated Hospital of Dalian Medical University (Dalian, China). The patients were 68 men and 33 women, with ages ranging from 19 to 78 years (mean age of 50.3 years). No patients had received chemotherapy or radiation therapy. All samples were confirmed by pathological diagnosis and classified according to the 2012 HCC staging system of the International Union against Cancer. The clinicopathological factors of HCC are summarized in Table I. The investigation project and notices of informed consent have been examined and certified by the Ethics Committee of the Second Affiliated Hospital of Dalian Medical University. All of the patients signed a written informed consent document. The patient tissues were snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

Membrane Protein Extract—A total of 1 × 106 cells were washed with phosphate-buffered saline (PBS) and lysed on a plate with lysis and separation buffer containing a protease inhibitor mixture. Cell membrane proteins were extracted from the cell suspension using a Cellytic MEM Protein Extraction kit (Sigma, St. Louis, MO). The membrane protein concentration was measured with a Micro BCA Protein Assay kit (Pierce, Rockford, IL) and used for further experiments as described below.

Preparation of N-glycans—Three 100-μg aliquots of cell membrane proteins were lyophilized and then resuspended in 20 μl of 50 mM ammonium bicarbonate and 1 μl of 50 mM DTT prior to incubation at 56 °C for 30 min. A 1-μl aliquot of 50 mM iodoacetamide was then added to each sample solution, and samples were allowed to react at room temperature for 1 h in the dark. Three samples were changed via dialysis against 3 l of 50 mM ammonium bicarbonate (pH 8.5) at 4 °C for 24 h. After dialysis, the samples were lyophilized and dissolved in 50 μl of 50 mM ammonium bicarbonate. A 4-μl aliquot of trypsin was then added to each sample solution, and samples were allowed to react at 37 °C for 16 h. A 20-μl aliquot of 1% SDS was added to each sample solution, and samples were then heated at 100 °C for 5 min. Subsequently, 5 μl PNGaseF (Takara Bio Inc., Dalian, China) was added to the solutions, and they were then incubated at 37 °C for 16 h to remove N-glycans from proteins. The released N-glycans were purified using an Oasis HLB cartridge (60 mg/3 ml; Waters, Beijin, China) and then lyophilized.

Permethylation was performed via the solid NaOH technique. Small NaOH pellets (∼50 mg; Fluka, Xian, China) were first mixed with 250 μl of dimethyl sulfoxide (DMSO). The released N-glycans from membrane protein samples were dried in a glass tube, and −50 μl of the NaOH/DMSO slurry was added to the sample, followed by 50 μl of iodoiodamide. The reaction mixture was agitated at room temperature for 30 min. The reaction was terminated by the addition of 1 ml of 10% ice-cold acetic acid. The diluted reaction solution was applied to a Sep-Pak Vac 18 cartridge (50 mg/ml; Waters), and this was followed by the elution of permethylated glycans with 750 μl of acetonitrile and SpeedVac drying. Three aliquots of permethylated glycan samples were finished.

MS Analysis of Permethylated Glycans—Mass spectra were acquired using an Ultraflextreme MALDI-TOF/TOF (Bruker Daltonics, Bremen, Germany). For sample preparation, the dried permethylated matrix solution (10 μg of 2.5-DHB dissolved in 1 ml of 30% ethanol) and 0.5 μl of the diluted analyte solution were spotted on the plate and mixed on the plate. The plate was analyzed via MS, with spectra obtained from Na+ adduct ions.

Real-time PCR Analysis—Real-time PCR was used to analyze the gene expression level of the ST family. Total RNA was isolated from human tissues or HCC cell lines using the RNasy Mini Kit (Qiagen, Valencia, CA), and cDNA was synthesized using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Real-time PCR was carried out on an ABI Prism 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA) using a QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA). The primer pairs for PCR are listed in Table I. The expression levels of target genes were determined relative to GAPDH and calculated as 2−(CtTarget gene−CtGAPDH).
obtain 60% to 80% confluence. The cell cultures were transfected with ST6GAL1 or ST8SIA4 specific shRNA Transfection Reagent Complex, respectively, which was prepared according to the protocol. Scrambled shRNA was used as the negative control. The transfection efficiency was about 80% and cell viability was 90% according to trypan blue dye exclusion assay. Four weeks later, several colonies were picked and expanded for further study. The knockdowns had no effects on the cell morphology (supplementary Fig. S5B).

Overexpression of ST6GAL1 or ST8SIA2—The human ST6GAL1 and ST8SIA2 encoding sequences were obtained from TaKaRa (Dalian, China) and were inserted into the pEGFP-N2 vector (Invitrogen, Carlsbad, CA) using EcoRI and XhoI sites, respectively. Cells were transfected with 5 μg of target gene expression vector or empty vector in 100-mm dishes using PolyFect Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The cell transfection efficiency was 74%, and the survival rate was 89%. After 4 weeks of screening, the cell lines stably expressing ST6GAL1 (MHCC97L/mock, MHCC97H/mock) and ST8SIA2 (MHCC97L/ST6GAL1) or empty vector (MHCC97L/mock, MHCC97H/mock) were established. Then cells were collected for gene expression assay and for further explorations.

Western Blot Analysis—Whole cell proteins were electrophoresed under reducing conditions in 10% polyacrylamide gels. The separated proteins were transferred to a polyvinylidene difluoride membrane. After blocking with 5% skimmed milk in PBS containing 0.1% Tween 20, the membrane was incubated with antibody (1/1000 diluted; Abcam, Cambridge, UK) and then with peroxidase-conjugated anti-rabbit IgG (1/100,000 diluted; GE Healthcare UK Ltd., Little Chalfont, UK). A GAPDH antibody (1/200 diluted; Santa Cruz Biotechnology, Santa Cruz, CA) was used as a control. All bands were detected using an ECL Western blot kit (Amersham Biosciences, UK), and the bands were analyzed with LabWorks (TM ver.4.6, UVP, Biolimaging Systems, NY, USA).

In Vitro Extracellular Matrix Invasion Assay—The cell invasion in vitro was demonstrated by using 24-well transwell units (Corning, NY, USA) with an 8-μm pore polycarbonate filter coated with ECMatrix gel (Chemicon, CA, USA) to form a continuous thin layer. Cells (3 × 10⁴) were harvested in serum-free medium containing 0.1% BSA and added to the upper chamber. The lower chamber contained 500 μl of DMEM. Cells were incubated for 24 h at 37 °C in 5% CO₂. At the end of the incubation, the cells on the upper surface of the filter were completely removed with a cotton swab. Then the filters were fixed in methanol and stained with Wright-Giemsa. Cells that invaded the Matrigel and reached the lower surface of the filter were counted under a light microscope at a magnification of 400×.

In Vivo Drug Sensitivity Assay—Drug sensitivity was measured by using an MTT assay. Cells (1 × 10⁴) were plated in 96-well plates (Costar, Charlotte, NC) and incubated with 5-fluorouracil (5-FU) (Sigma) for 48 h. Then cells were treated with 100 μl of MTT (5 mg/ml; Sigma). After 4 h of incubation at 37 °C in 5% CO₂, 100 μl of DMSO (Invitrogen) was pipetted to solubilize the formazan product for 30 min at room temperature. Spectrometric absorbance was measured at 490 nm by using a microplate reader. Each group contained three wells and was repeated three times. The concentrations required for 50% growth inhibition (IC₅₀ values) were determined by the drug dose that caused 50% cell viability. In Vivo Antitumor Activity—To investigate whether ST6GAL1 or ST8SIA4 is related to the drug sensitivity of tumor cells in vivo, the anitumor activity of 5-FU was examined in nude mice bearing tumor cell xenografts. Five-week-old male athymic nude mice were obtained from the Animal Facility of Dalian Medical University and were provided with sterilized food and water. Approximately 1 × 10⁷ cells were injected subcutaneously into the right flank of each nude mouse. Once bearing palpable tumors (about 1 week after tumor cell inoculation), tumor-bearing mice were randomly divided into control and treatment groups (n = 6 animals per group). The treatment groups received 30 mg/kg 5-FU intraperitoneally three times per week for 3 weeks, and the control groups received physiological saline alone. Mice were sacrificed and their tumors were isolated, weighed, and photographed. The tumor inhibition rate (IR) was calculated according to the following equation: IR (%) = (W₀ - W₁)/W₀ × 100%, where W₀ and W₁ represent the mean tumor weights of the control group and the treatment group, respectively.

| Gene         | Primers                                                                 | Amplicon (bp) |
|--------------|-------------------------------------------------------------------------|---------------|
| ST3GAL1      | 5'-CAGAGATGGAGCTGTGGTCTAG-3'                                           | 197           |
| ST3GAL2      | 5'-GTCGCTCTGAGCTGTGGTCTAG-3'                                           | 191           |
| ST3GAL3      | 5'-TATGCTTCCAGCTGTGGTCTAG-3'                                           | 164           |
| ST3GAL4      | 5'-ATGTTGGCTCTGAGCTGTGGTCTAG-3'                                         | 176           |
| ST3GAL5      | 5'-CAAGAGCTGTTAGTGTTAGTCTAG-3'                                         | 213           |
| ST3GAL6      | 5'-AGTCTTTCCAGCTGTGGTCTAG-3'                                           | 189           |
| ST6GAL1      | 5'-CTTTGTTTTCCAGCTGTGGTCTAG-3'                                         | 166           |
| ST6GAL2      | 5'-ACGTCTCTTCCAGCTGTGGTCTAG-3'                                         | 160           |
| ST6GALNAC1   | 5'-CTGTGTTCTTCCAGCTGTGGTCTAG-3'                                         | 192           |
| ST6GALNAC2   | 5'-CTTTGCTGTTAGTGTTAGTCTAG-3'                                         | 205           |
| ST6GALNAC3   | 5'-GGACACCTGCTACCTGGTTAGTCTAG-3'                                         | 174           |
| ST6GALNAC4   | 5'-ACGCTTCCAGCTGTGGTCTAG-3'                                           | 188           |
| ST6GALNAC5   | 5'-TGACACGCTGCTGTGGTCTAG-3'                                           | 121           |
| ST6GALNAC6   | 5'-ACCTACCCCTCTGCTACCTGGTTAGTCTAG-3'                                     | 179           |
| ST8SIA1      | 5'-TATGCTTCCAGCTGTGGTCTAG-3'                                           | 149           |
| ST8SIA2      | 5'-CTTGTTCTTCCAGCTGTGGTCTAG-3'                                         | 140           |
| ST8SIA3      | 5'-ATTCCTTCACCAGCTACCTGGTTAGTCTAG-3'                                     | 141           |
| ST8SIA4      | 5'-CAAGACCTGAGCTGTTAGTCTAG-3'                                          | 140           |
| ST8SIA5      | 5'-CTCTTTCCAGCTGTGGTCTAG-3'                                           | 152           |
| ST8SIA6      | 5'-CCTTTCCAGCTGTGGTCTAG-3'                                            | 126           |
| GAPDH        | 5'-CTCTTTCCAGCTGTGGTCTAG-3'                                            | 175           |
Inhibition of PI3K/Akt Signaling—Wortmannin (Sigma) was used to suppress the activity of PI3K/Akt signaling in MHCC97H cells. Briefly, HMCC97H cells (1 × 10^4 cells per well) were incubated with DMSO or the PI3K inhibitor wortmannin (1 mM) dissolved in DMSO and collected after 24 h. In addition, Akt expression was also silenced by RNAi. Changes in chemosensitivity and gene expression were measured via MTT assay and Western blot analysis, respectively.

Immunohistochemical Staining Analysis—Immunohistochemistry assays were performed on paraffin-embedded tumor sections. The slides were dried, deparaffinized, rehydrated, and then immersed in 3% hydrogen peroxide for 10 min to quench the endogenous peroxidase. After consecutive washings with PBS, the slides were labeled overnight at 4 °C with antibodies (Abcam, Cambridge, UK) at a dilution of 1:200 or isotype immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, CA) as a negative control. The staining was performed at room temperature for 60 min with secondary streptavidin-HRP-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Finally, the sections were counterstained with hematoxylin and coverslipped. Image-Pro Plus 4.5 Software (Media Cybernetics, Shanghai, China) was used to analyze the expression of proteins.

Statistical Analysis—Each experiment was performed at least in triplicate, and the measurements were performed in three independent experiments. Data are expressed as means ± standard deviation (S.D.). Student’s t test was used to compare the means of two groups. p < 0.05 was considered statistically significant. All analyses were performed using SPSS 13.0 statistical packages (SPSS Inc., Chicago, IL).

RESULTS

MALDI-MS Analysis of N-glycan Composition Profiling from HCC Cell Lines—MALDI-TOF-MS analysis was utilized to evaluate the N-glycan composition profiling of MHCC97H and MHCC97L cell lines. Fig. 1A shows the peaks (1–33) of MS spectrum signals of N-glycans indicating N-glycans released from cell membrane. The assigned N-glycan signals are summarized in Table III. The intensities of the observed MS signals are presented as a histogram in Fig. 1B, labeled with the estimated monosaccharide composition. High-mannose gly-
cans (peaks 4, 5, 7, 10, and 13) were observed in both cell lines (Table III). The N-glycan composition profiles detected in MHCC97H cells were remarkably different from those in MHCC97L cells. Peaks 9 and 27 were exclusively detected only in the high-metastasis MHCC97H cell line. Also, peaks 12, 14, 16, 18, 19, 22, 28, 31, and 33 were detected more frequently in MHCC97H cells (≥2-fold). Peaks 26 and 29 presented more frequently in the MHCC97L cell line (≥2-fold). Surprisingly, all the peaks corresponding to sialylated oligosaccharides were significantly more often observed in MHCC97H cells. The MS profiles of N-glycan composition also showed different sialylation levels between MHCC97H and MHCC97L cell lines, with a higher level of sialylated oligosaccharides in MHCC97H cells (Fig. 1 and Table II). These data indicate that differential N-glycan composition profiling might be associated with metastatic ability in human HCC, especially of sialylated oligosaccharides.

**Differential Expression of ST Gene Family in HCC Cell Lines**—No significant differences were found in the transcription levels of ST3GAL1, ST3GAL2, ST3GAL3, ST3GAL4, ST6GAL2, ST6GALNAC1, and ST8SIA1 mRNA (Figs. 2A–2D).

### TABLE II
Clinicopathological characteristics of HCC patients

| Characteristics                  | n  |
|----------------------------------|----|
| Cancer tissues                   | 101|
| Transitional tissues             | 101|
| Gender                           |    |
| Male                             | 68 |
| Female                           | 33 |
| Age (yr)                         |    |
| ≥50                              | 57 |
| <50                              | 44 |
| Serum AFP (mg/l)                 |    |
| ≥20                              | 71 |
| <20                              | 30 |
| Distant metastasis               |    |
| Yes                              | 32 |
| No                               | 69 |
| Lymph node metastasis            |    |
| Absent                           | 47 |
| Present                          | 54 |
| Clinical stage                   |    |
| I–II                             | 40 |
| III–IV                           | 61 |

**TABLE III**
N-glycans analyzed in MHCC97H and MHCC97L cell lines via MALDI-TOF-MS

| Peak number | Observed m/z | Composition |
|-------------|--------------|-------------|
|             | MHCC97H      | MHCC97L     | Hex | HexNAc | Man | GlcNAc | NeuAc | Deoxyhexose |
| 1           | 1345.80      | 1345.65     | 0   | 0      | 3   | 2      | 0     | 1          |
| 2           | 1375.88      | 1375.63     | 1   | 0      | 3   | 2      | 0     | 0          |
| 3           | 1416.85      | 1416.77     | 0   | 1      | 3   | 2      | 0     | 0          |
| 4           | 1579.88      | 1579.73     | 0   | 0      | 5   | 2      | 0     | 0          |
| 5           | 1783.98      | 1783.83     | 0   | 0      | 6   | 2      | 0     | 0          |
| 6           | 1836.07      | 1835.77     | 0   | 2      | 3   | 2      | 0     | 0          |
| 7           | 1988.07      | 1987.92     | 0   | 0      | 7   | 2      | 0     | 0          |
| 8           | 2156.15      | 2155.95     | 1   | 1      | 3   | 2      | 1     | 1          |
| 9           | 2186.17      | No           | 2   | 1      | 3   | 2      | 1     | 1          |
| 10          | 2192.14      | 2191.95     | 2   | 1      | 3   | 2      | 1     | 1          |
| 11          | 2244.28      | 2243.99     | 2   | 2      | 3   | 2      | 0     | 0          |
| 12          | 2360.17      | 2360.07     | 2   | 2      | 3   | 2      | 0     | 1          |
| 13          | 2396.20      | 2396.00     | 2   | 2      | 3   | 2      | 0     | 1          |
| 14          | 2634.20      | 2634.00     | 3   | 1      | 3   | 2      | 1     | 1          |
| 15          | 2645.27      | 2584.05     | 3   | 1      | 3   | 2      | 1     | 1          |
| 16          | 2693.25      | 2693.03     | 3   | 1      | 3   | 2      | 1     | 1          |
| 17          | 2799.32      | 2799.10     | 3   | 1      | 3   | 2      | 1     | 1          |
| 18          | 2799.33      | 2799.09     | 3   | 1      | 3   | 2      | 1     | 1          |
| 19          | 2880.23      | 2880.07     | 3   | 1      | 3   | 2      | 1     | 1          |
| 20          | 2913.28      | 2913.08     | 3   | 1      | 3   | 2      | 1     | 1          |
| 21          | 2966.34      | 2966.08     | 3   | 1      | 3   | 2      | 1     | 1          |
| 22          | 3054.38      | 3054.15     | 3   | 1      | 3   | 2      | 1     | 1          |
| 23          | 3142.43      | 3142.26     | 4   | 4      | 3   | 2      | 0     | 1          |
| 24          | 3415.52      | 3415.41     | 3   | 3      | 3   | 2      | 1     | 1          |
| 25          | 3503.43      | 3503.03     | 4   | 4      | 3   | 2      | 1     | 1          |
| 26          | 3602.31      | No           | 3   | 3      | 3   | 2      | 3     | 1          |
| 27          | 3776.37      | 3776.20     | 3   | 3      | 3   | 2      | 3     | 1          |
| 28          | 3864.49      | 3864.92     | 4   | 4      | 3   | 2      | 2     | 1          |
| 29          | 3952.45      | 3952.04     | 5   | 5      | 3   | 2      | 1     | 1          |
| 30          | 4225.32      | 4225.85     | 4   | 4      | 3   | 2      | 3     | 1          |
| 31          | 4314.22      | 4314.00     | 5   | 5      | 3   | 2      | 2     | 1          |
| 32          | 4586.98      | 4586.58     | 4   | 4      | 3   | 2      | 4     | 1          |

Notes: The N-glycans were observed as [M+Na]+. Hex, hexose; HexNAc, N-acetyhexosamine; Man, mannose; GlcNAc, N-acetylglicosamine; NeuAc, N-acetyleneuraminic acid.
mRNA (1.85-fold). Relative to MHCC97L cells, MHCC97H cells showed a remarkable increase in the mRNA level of genes ST6GAL1 (4.35-fold), ST3GAL5 (3.84-fold), ST3GAL6 (2.89-fold), and ST6GALNAC6 (2.71-fold) (Fig. 2E), suggesting that MHCC97H cells expressed high levels of α2,3- and α2,6-linked sialylation (core sialylation). In contrast, MHCC97L cells showed high expression levels of ST8SIA2 (5.86-fold), ST6GALNAC2 (2.45±0.52), and ST6GALNAC4 (2.16±0.50), whereas ST8SIA1, ST6GALNAC3, ST8SIA3, ST8SIA5, and ST8SIA6 were undetectable in both cell lines (Fig. 2). These data indicate that differential ST gene expression might be associated with the metastasis of human HCC cell lines.

Effects of Altered Expression of ST6GAL1 on the Invasive Ability and Chemosensitivity of HCC Cells in Vitro and in Vivo— ST6GAL1 mRNA showed the highest expression in MHCC97H cells (Fig. 2E), so ST6GAL1 was silenced by shRNA in order to elucidate its direct effect on the invasive ability and chemosensitivity of MHCC97H cells. As shown in Figs. 3A and 3B, the expression level of ST6GAL1 was significantly reduced in MHCC97H transfectants relative to the control (Figs. 3A and 3B and supplemental Fig. S3A). The α2,6 sialylation level detected by FITC-SNA lectin (SNA, Sambucus nigra) on the cell surface was also reduced in MHCC97H-ST6GAL1 shRNA1 cells (Fig. 3C). However, no changes in the α1,6 fucosylation level were detected by...
FITC-LCA lectin (LCA, Lens culinaris) on the cell surface of MHCC97H-control shRNA or MHCC97H-ST6GAL1 shRNA1 cells (supplemental Fig. S2A).

Transwell assay revealed that knockdown of the ST6GAL1 gene significantly inhibited invasive activity in MHCC97H-ST6GAL1 shRNA1 cells (Fig. 3D). Moreover, MHCC97H-ST6GAL1 shRNA1 cells had an obviously decreased capacity for proliferation relative to MHCC97H and MHCC97H-control shRNA1 cells in the MTT assay (supplemental Fig. S1A). The cell chemoresistance showed the same tendency to decrease (Fig. 3G). The IC_{50} value of 5-FU was significantly less in the MHCC97H-ST6GAL1 shRNA1 groups than in the MHCC97H-control shRNA1 groups, suggesting that cell proliferation was inhibited by the therapeutic drug when MHCC97H cells were treated with ST6GAL1 shRNA1.

Nude mice bearing MHCC97H, MHCC97H-control shRNA, and MHCC97H-ST6GAL1 shRNA1 xenografts were used to analyze the differences in tumor weight when 5-FU was administrated. Fig. 3F shows a significant reduction in mean tumor weight relative to the control. The inhibition rate of 5-FU was 14.38%, 15.60%, and 31.78% in the shRNA-treated groups. IHC staining of ST6GAL1 expression patterns showed that the protein was reduced in the mouse group treated with shRNA1 relative to the untreated group or control group (Fig. 3G).

In order to further investigate the effects of ST6GAL1 on invasive ability and chemosensitivity, an ST6GAL1 expression vector was transfected in MHCC97L cells. Higher expression levels of ST6GAL1 were detected in MHCC97L/ST6GAL1 cells (Figs. 4A, 4B, 4C and 4D; supplemental Fig. S3B), but the fluorescence intensity on FITC-LCA revealed no variation of α-1,6 fucosylation in the three cell lines (supplemental Fig. S2B). The invasive ability, proliferative capability, and chemoresistance were increased after the transfection of the ST6GAL1 expression vector in vitro and in vivo (Figs. 4D–4F; supplemental Fig. S1B). These results evidenced that ST6GAL1 was associated with the invasion and chemosensitivity of HCC cell lines and that altering the expression level of ST6GAL1 might modulate invasion and chemosensitivity in vitro and in vivo.

Altered Expression of ST8SIA2 Gene Influences the Invasive Ability and Chemosensitivity of HCC Cells in Vitro and in Vivo—Because the mRNA and protein levels of ST8SIA2 were found to decrease notably in MHCC97L-ST8SIA2 shRNA1 transfectants (Figs. 5A and 5B), we targeted ST8SIA2 to elucidate the role of ST8SIA2 in the invasion and chemosensitivity of MHCC97L cells. Fluorescence intensity on FITC-Sialyllectin revealed less α-2,8 sialylation in MHCC97L-ST8SIA2 shRNA1 cells than in nontransfection cells (Fig. 5C). However, the α-1,6 fucosylation level detected by FITC-LCA lectin on the cell surface showed no change in MHCC97L-control shRNA and MHCC97L-ST8SIA2 shRNA1 cells (supplemental Fig. S2C).

In vitro ECMMatrix gel analysis showed that knockdown of ST8SIA2 expression significantly increased MHCC97L-ST8SIA2 shRNA1 cells’ invasion ability relative to the MHCC97L-control shRNA cells (Fig. 5D). MTT assay showed that a significant increase in cell number was observed in MHCC97L cells transfected with ST8SIA2-specific shRNA1 in 5-day experiments (supplemental Fig. S1C). Furthermore, the MHCC97L-ST8SIA2 shRNA1 cells also showed decreased sensitivity to 5-FU relative to MHCC97L-control shRNA groups in vitro (Fig. 5E).

The antitumor activity of 5-FU in vivo was assessed by means of intraperitoneal administration using nude mice bearing MHCC97L, MHCC97L-control shRNA, and MHCC97L-ST8SIA2 shRNA1 tumors. A significant increase in mean tumor weight of MHCC97L-ST8SIA2 shRNA1 tumor was observed (Fig. 5F). The inhibition rate of 5-FU was 42.11%, 46.15%, and 22.55% in this shRNA-treated group. We further analyzed the ST8SIA2 expression pattern in tumor by means of IHC staining, and the ST8SIAII protein was reduced in the mouse group with shRNA treatment relative to the untreated group or control group (Fig. 5G).

In order to determine whether overexpression of ST8SIA2 could mediate the invasive ability and chemosensitivity of MHCC97H cells, a higher level of ST8SIA2 was detected in MHCC97H-ST8SIA2 cells (Figs. 6A and 6B). However, fluorescence intensity on FITC-LCA revealed no change of α-1,6 fucosylation in the three cell lines (supplemental Fig. S2D). The invasion, proliferation, and chemosensitivity of MHCC97H cells were altered with the ST8SIA2 expression vector transfection in vitro and in vivo (Figs. 6D–6F and supplemental Fig. S1D). IHC staining and flow cytometry analysis revealed elevated expression of ST8SIAII in MHCC97H-ST8SIA2 cells (Figs. 6C–6E).
FIG. 4. Overexpression of ST6GAL1 enhanced the invasive ability and chemoresistance of MHCC97L cells both in vitro and in vivo. After full-length sequence transfection, ST6GAL1 mRNA (A) and protein (B) were increased notably in MHCC97L cells as shown by real-time PCR and Western blot. C, flow cytometry analysis showed that the α-2,8 sialylation level detected by FITC-SNA on the cell surface was also increased in MHCC97L/ST6GAL1 cells. D, in vitro ECMMatrix gel analysis was performed. The average number of cells that invaded through the filter was counted. MHCC97L/ST6GAL1 cells were significantly more invasive (*p < 0.05) than the MHCC97L and MHCC97L/mock cells. The chemoresistance of MHCC97L cells increased with the ST6GAL1 expression vector transfection in vitro (E) and in vivo (F). G, up-regulation of ST6GAL1 was also shown by IHC staining in xenograft tumors derived from MHCC97L/ST6GAL1 cells (400×). The data are means ± S.D. of three independent assays (*p < 0.05).
Fig. 5. **Silence of ST8SIA2 gene enhances the invasive ability and chemoresistance of MHCC97L cells both in vitro and in vivo.**

A, silencing of ST8SIA2 in MHCC97L cells was analyzed via the RNAi approach. ST8SIA2 transcripts were decreased apparently by shRNA treatment in MHCC97L cells. B, after shRNA transfection, a distinct reduction of ST8SIAII was observed at the protein level in Western blot analysis. C, differential FITC-Siglec7 binding profiles of MHCC97L, MHCC97L-control shRNA, and MHCC97L-ST8SIA2 shRNA1 cell lines using flow cytometry. Histograms of fluorescence intensities of cells with specific carbohydrate expression as determined. D, in vitro ECMatrix gel analysis was
Overexpression of ST8SIA2 gene inhibits the invasive ability and chemoresistance of MHCC97H cells both in vitro and in vivo. After full-length sequence transfection, ST8SIA2 mRNA (A) and ST8SIAII protein (B) were increased notably in MHCC97H cells as shown by real-time PCR and Western blot. C, flow cytometry analysis showed that the α-2,8 sialylation level detected by FITC-Siglec7 on the cell surface was also increased in MHCC97H/ST8SIA2 cells. D, in vitro ECM matrix gel analysis was performed. The average number of cells that invaded through the filter was counted. MHCC97H/ST8SIA2 cells were significantly less invasive (*p < 0.05) than the MHCC97H and MHCC97H/ST8SIA2 cells. The chemosensitivity of MHCC97H cells was increased with ST8SIA2 expression vector transfection in vitro (E) and in vivo (F). G, up-regulation of ST8SIAII was also shown by IHC staining in xenograft tumors derived from MHCC97H/ST8SIA2 cells (400×). The data are means ± S.D. of three independent assays (*p < 0.05).
Sialylation Alters the Invasive Ability and Chemosensitivity of MHCC97H in Vitro—To test directly whether the sialylation influenced MHCC97H cells' invasive ability and chemosensitivity, the regulation of sialylation was investigated. MHCC97H cells were exposed to an exogenous sialidase to remove sialic acid. As illustrated in supplemental Fig. S4A, the SNA level of sialidase-treated MHCC97H cells was much lower than that of MHCC97H cells in the absence of sialidase, suggesting that sialidase effectively inhibited the sialylation process in MHCC97H cells.

To further investigate whether removing sialic acid from MHCC97H cells modulated their invasive ability, we performed an in vitro ECM matrix gel analysis. Under sialidase treatment, the MHCC97H cells showed decreased invasive ability relative to the MHCC97H groups in the absence of sialidase (supplemental Fig. S4B). MTT assay showed a significant reduction in the cell number in MHCC97H cells treated with sialidase in 5-day experiments (supplemental Fig. S4C). Furthermore, the sialidase-treated MHCC97H cells showed increased sensitivity to 5-FU relative to the MHCC97H cells in the absence of sialidase (supplemental Fig. S4D). These results also supported the hypothesis that sialylation is closely related to the activities of invasion, proliferation, and chemosensitivity in HCC cells.

ST6GAL1 or ST8SIA2 gene mediates the activity of the PI3K/Akt signaling pathway in MHCC97H and MHCC97L cell lines. A and C, expression of PI3K/Akt/NF-κB signaling molecules was repressed at the protein level with ST6GAL1 or ST8SIA2 shRNA1 transfection in MHCC97H-ST6GAL1 shRNA1 or MHCC97L-ST8SIA2 shRNA1 cells. B and D, the increased protein levels of PI3K/Akt/NF-κB signaling molecules were determined via Western blot in MHCC97L/ST6GAL1 or MHCC97H/ST8SIA2 cells. The data are means ± S.D. of three independent assays (p < 0.05).
Ser473, Akt Thr308, and mTOR, as illustrated in Fig. 7C. Over-expression of ST8SIA2 in MHCC97H cells showed the reverse tendency (Fig. 7D). These data might indicate a possible mechanism of ST6GAL1 or ST8SIA2 involving the PI3K/Akt pathway that regulates the invasion and chemosensitivity of HCC cell lines.

**Blocking PI3K/Akt Modulates the Invasive Ability and Chemosensitivity of MHCC97H Cells in Vitro and in Vivo**—To further determine the role of the PI3K/Akt signaling pathway, specific inhibitors of PI3K/Akt or Akt shRNA to silence Akt were selected to treat MHCC97H cells. The protein levels of PI3K110α, Akt Ser473, Akt Thr308, Akt, and NF-κB were measured. As shown in Fig. 8A, MHCC97H cells with the inhibitor wortmannin and Akt shRNA treatment exhibited significantly decreased protein levels of the main signal molecules of the PI3K/Akt pathway. The inhibition of the PI3K/Akt pathway made the MHCC97H cells less invasive and susceptible to chemotherapy (Figs. 8B and 8C). Similar results were also observed in in vivo chemosensitivity analysis; reduced tumor weights were measured in mice bearing MHCC97H tumors with impaired PI3K/Akt signaling (Fig. 8D).

**Differential Expression of ST Family in HCC and Transitional Tissues**—To understand whether the ST gene family was involved in hepatocarcinogenesis, we examined the expression of the ST gene family in human primary HCC and transitional tissues. Quantitative real-time PCR confirmed increased mRNA levels of ST3GAL5 (p = 0.0092), ST3GAL6 (p = 0.0092), ST6GAL1 (p = 0.0092), and ST6GALNAC6 (p = 0.0092) in HCC tissues (Table I). However, levels of ST6GALNAC2 (p = 0.0092) and ST8SIA2 (p = 0.0092) were higher in transitional tissues than in HCC samples. No significant difference was shown in the expression levels of the other members of the ST family between the two groups, except for ST6GALNAC3, ST6GALNAC5, ST8SIA3, ST8SIA5, and ST8SIA6. These observations indicated that differential expression of the ST gene family might be linked to tumorigenesis and the progression of HCC.

**DISCUSSION**

In this study, we intensively elucidated the possible effects of sialylation modification on tumor invasion and chemosensitivity in the human HCC cell lines MHCC97H and MHCC97L with high and low metastatic potential, respectively. We further analyzed the differential expression of the ST gene family, which was reported to be correlated with tumorigenesis and progression in HCC patients.

Recent developments in MS technology, thousands of new formerly N-linked glycopeptides from different tissues, cells, and bodily fluids have been identified and quantified (29, 30). Zhang et al. have used MALDI-TOF-MS to investigate novel N-glycan changes involved in lymphatic metastasis in the murine HCC cell lines Hca-F and Hca-P with high and low metastatic potential in the lymph nodes (31). To identify sialylated N-glycans associated with the metastatic potential of human HCC, we used an MS method and analyzed the composition profiling of N-glycans. In addition, the abundance ratios of oligosaccharides can be obtained from a comparison of relative ion intensities corresponding to individual glycans. Comparison of the total N-glycans from MHCC97H and MHCC97L cell lines showed a dramatic difference in N-glycan composition profiles between these two groups (Fig. 1, Table I). A majority of N-glycans detected in MHCC97H and MHCC97L cells had high-mannose structures (peaks 4, 5, 7, 10, and 13). Peaks 9 and 27 were exclusively detected in the high-metastasis MHCC97H cell line. Major peaks (peaks 12, 14, 16, 18, 19, 22, 28, 31, and 33) corresponding to sialylated oligosaccharides originating from MHCC97H cells showed a significant increase (≥2-fold).

Moreover, peaks 26 and 29 corresponding to sialylated oligosaccharides originating from MHCC97L cells also showed a significant increase (≥2-fold). Therefore, monitoring of the sialylated N-glycan profile would be important in the diagnosis of tumor metastasis and chemosensitivity.

A aberrant sialylation is also correlated with the invasive potential of various types of cancer (32, 33). Sialyltransferases are essential in the biosynthetic pathway of sialylated glycan. In this study, using real-time PCR analysis, we revealed that the expression profiles of 20 ST genes were remodeled between MHCC97H and MHCC97L. ST family expression was highly regulated, with 7 (out of 20) glycogenes (at least 2-fold; Fig. 2) significantly differentially expressed among the two cell lines. Relative to MHCC97L cells, MHCC97H cells showed up-regulated expression of ST6GAL1 (4.35-fold), ST3GAL5 (3.84-fold), ST6GAL6 (2.89-fold), and ST6GALNAC6 (2.71-fold) mRNA (Fig. 2E), suggesting that high-metastastic-potential cells were active in α-2,3- and α-2,6-linked sialylation (core sialylation). Furthermore, MHCC97L cells showed higher expression levels of ST8SIA2 (5.86-fold), ST6GALNAC2 (2.45-fold), and ST6GALNAC6 (2.16-fold). The ST family genes with altered expression patterns in the two cell lines might be more important as indicators and functional regulators of tumor metastasis and chemosensitivity.

Whether the ST gene family and its related proteins affect tumor invasion, proliferation, and chemosensitivity remains a question. ST6GAL1 is expressed ubiquitously and catalyzes the α-2,6-sialylation of aterminal Gal residues of Galβ1–4GlcNAc disaccharide, and high ST6GAL1 levels are associated with increased metastasis and poor patient prognosis (34–36). Our previous report indicated that the remodeled levels of ST6GAL1 mediated the invasive properties of murine
Modification of Sialylation Mediates Invasion and Chemosensitivity

A

B

C

D

E

Molecular & Cellular Proteomics 13.2

533
Modification of Sialylation Mediates Invasion and Chemosensitivity

HCC cell lines to lymph nodes (31). ST8SIA2 encodes N-acetylgalactosaminidase-2,8-sialyltransferase II (ST8Sia II), which catalyzes the polycondensation of α-2,8-linked sialic acid required for the synthesis of polysialic acid and is involved in the modulation of the adhesive properties of neural cell adhesion molecule. Interfering with polysialyltransferase ST8Sia2 mRNA inhibits neurite growth during early hippocampal development (37). Our previous work also demonstrated that differential expression of ST6GAL1 and ST8SIA2 was associated with the metastatic potential of human HCC cell lines (38). The present study targeted ST6GAL1 and ST8SIA2, which were differentially expressed in MHCC97H and MHCC97L cells. The altered level of ST6GAL1 or ST8SIA2 led to changed invasive properties, proliferative ability, and chemoresistance in the two cell lines, both in vitro and in vivo (Figs. 3–6 and supplemental Fig. S1). ST6GAL1 or ST8SIA2 products were also altered remarkably in HCC cell lines labeled with FITC-SNA or FITC-Siglec-7 lectin, whereas the FUT8 product detected by FITC-LCA lectin didn’t change (supplemental Fig. S2). We also elucidated the direct effect of sialylation in the invasion, proliferation-related phenotypes, and sensitivity to 5-FU of MHCC97H in vitro by removing the sialic acid with sialidase treatment. Sialidase treatment resulted in the occurrence of defective sialylation in MHCC97H cells (supplemental Fig. S4). These results clearly demonstrate that sialylation confers increasing invasion and decreasing chemosensitivity in HCC cell lines.

The present study also investigated the molecular mechanism by which the ST6GAL1- or ST8SIA2-mediated PI3K/Akt signaling pathway regulates cell invasiveness and chemoresistance. ST6Gal I adds an α-2,6-linked sialic acid to the N-glycans of CD133 membrane proteins that may stabilize CD133 (39). CD133-expressing liver cancer cells following radiation exposure show increased activation of the MAPK/PI3K signaling pathway (40). Our study discovered a novel mechanism by which the invasion and chemosensitivity of HCC cells can be altered through activation of the PI3K/Akt pathway by sialylation modification. The high-metastatic-potential cell line MHCC97H exhibited higher PI3K/Akt activity than the low-metastatic-potential line, which was in accordance with the invasive phenotype. Altered expression of ST6GAL1 or ST8SIA2 markedly modulated the activity of the PI3K/Akt pathway in HCC cell lines. In addition, inhibition of the PI3K/Akt pathway with the Akt-specific inhibitor wortmannin or Akt gene silencing by shRNA pretreatment reversed the invasive properties and chemoresistance of MHCC97H cells (Fig. 8). These results indicated that ST6GAL1- or ST8SIA2-modulated HCC cell invasion and chemosensitivity were, at least in part, PI3K/Akt-dependent.

### Table IV

**Expressional profiles of ST gene family in HCC and transitional tissues**

| Gene             | Relative mRNA expression (#10^6) | HCC tissues | Transitional tissues | p value |
|------------------|----------------------------------|-------------|----------------------|---------|
| ST3GAL1          | 47.791 ± 16.123                  | 49.343 ± 16.052 |                      | 0.494   |
| ST3GAL2          | 19.332 ± 9.874                   | 21.416 ± 9.109 |                      | 0.121   |
| ST3GAL3          | 14.125 ± 8.246                   | 12.612 ± 6.507 |                      | 0.149   |
| ST3GAL4          | 13.612 ± 7.282                   | 12.240 ± 6.517 |                      | 0.159   |
| ST3GAL5          | 38.134 ± 18.665                  | 29.542 ± 13.389 |                      | 0.001*  |
| ST3GAL6          | 23.019 ± 16.306                  | 15.928 ± 10.316 |                      | 0.001*  |
| ST6GAL1          | 32.735 ± 18.932                  | 18.662 ± 9.891 |                      | 0.001*  |
| ST6GAL2          | 7.032 ± 5.114                    | 8.016 ± 6.227  |                      | 0.221   |
| ST6GALNAC1       | 8.703 ± 6.581                    | 7.902 ± 4.949  |                      | 0.329   |
| ST6GALNAC2       | 6.639 ± 5.221                    | 8.908 ± 5.418  |                      | 0.003*  |
| ST6GALNAC3       | 0.004 ± 0.003                    | 0.005 ± 0.004  |                      | 0.146   |
| ST6GALNAC4       | 18.981 ± 8.128                   | 21.339 ± 9.193 |                      | 0.055   |
| ST6GALNAC5       | 0.013 ± 0.008                    | 0.011 ± 0.007  |                      | 0.061   |
| ST6GALNAC6       | 17.865 ± 8.985                   | 14.604 ± 8.281 |                      | 0.008*  |
| ST8SIA1          | 2.389 ± 1.391                    | 2.105 ± 1.127  |                      | 0.113   |
| ST8SIA2          | 4.356 ± 2.102                    | 6.032 ± 4.774  |                      | 0.002*  |
| ST8SIA3          | 0.008 ± 0.006                    | 0.010 ± 0.009  |                      | 0.064   |
| ST8SIA4          | 18.941 ± 11.432                  | 22.068 ± 15.517|                      | 0.105   |
| ST8SIA5          | 0.009 ± 0.007                    | 0.011 ± 0.008  |                      | 0.06    |
| ST8SIA6          | 0.006 ± 0.005                    | 0.007 ± 0.004  |                      | 0.118   |

* p < 0.05 versus transitional tissues.
Modification of Sialylation Mediates Invasion and Chemosensitivity

Sialyltransferases with altered mRNA expression in carcinoma tissues are regarded as prognostic factors and potential targets for therapeutic approaches (41, 42). In this study, 20 sialyltransferase subtype genes were used to probe the differential expression of the ST family in HCC and transitional tissues (Table IV). ST3GAL5, ST3GAL6, ST6GAL1, and ST6GALNAC6 were expressed at a high level in HCC tissues, whereas ST6GALNAC2 and ST8SIA2 expression was at a high level in transitional tissues. Once again, the findings from the clinical samples confirmed that the altered levels of these genes are probably associated with tumorigenesis and the progression of HCC. Based on the above results, it might be possible to utilize ST6GAL1 or ST8SIA2 as a useful biomarker for the clinical diagnosis of HCC metastasis and as potential targets in future therapeutic approaches.

In conclusion, by analyzing the sialylated N-glycans and expression patterns of ST genes in HCC cell lines and in HCC patients, we found that ST6GAL1 and ST8SIA2 regulation affects the unusual properties of invasion and chemosensitivity in HCC cells by modulating the PI3K/Akt signaling pathway. Although our results demonstrate that the modification of ST6GAL1 and ST8SIA2 primarily mediates invasion and chemosensitivity phenotype, there could be other potential effects of ST family alteration. Therefore, the molecular bases of tumor invasion and chemosensitivity-associated phenotype remained to be further investigated.

* This work was supported by grants from the National Key Basic Research and Development Program (973 Program) of China (Grant No. 2012CB822100) and from the National Natural Science Foundation of China (Grant Nos. 81071415 and 81271910).

** To whom correspondence should be addressed: Li Jia, College of Laboratory Medicine, Dalian Medical University, 9 Lushun Road Xiduan, Dalian 116044, Liaoning Province, China. Tel.:lege of Laboratory Medicine, Dalian Medical University, 9 Lushun

References

1. Dall’Olio, F., and Chiricolo, M. (2001) Sialyltransferases in cancer. Glycoconjug. J. 18, 841–850
2. Harvey, B. E., Toth, C. A., Wagner, H. E., Steele, G. D., Jr., and Thomas, P. (1992) Sialyltransferase activity and hepatic tumor growth in a nude mouse model of colorectal cancer metastases. Cancer Res. 52, 1775–1779
3. Majuri, M. L., Niemela, R., Tilsala, S., Renkonen, O., and Renkonen, R. (1999) Expression and function of alpha 2,3-sialyl- and alpha 1,3,1,4-fucosyltransferases in colon adenocarcinoma cell lines: role in synthesis of E-selectin counter-receptors. Int. J. Cancer. 63, 551–559
4. Ogosewaran, G., and Saik, P. L. (1981) Metastatic potential is positively correlated with cell surface sialylation of cultured murine tumor cell lines. Science 212, 1514–1516
5. Dennis, J., Waller, C., Timpl, R., and Schirmacher, V. (1982) Surface sialic acid reduces attachment of metastatic tumour cells to collagen type IV and fibronectin. Nature 300, 274–276
6. Kim, Y. J., Kim, K. S., Kim, H. H., Ko, J. H., Choe, I. S., Taulji, S., and Lee, Y. C. (1996) Molecular cloning and expression of human Gal beta 1,3GalNAc alpha 2,6-sialyltransferase (ST3Gal II). Biochem. Biophys. Res. Commun. 228, 324–327
7. Harduin-Lepers, A., Mollicone, R., Delannoy, P., and Oriol, R. (2005) The animal sialyltransferases and sialyltransferase-related genes: a phylogenetic approach. Glycobiology 15, 805–817
8. Krzewinski-Recchi, M. A., Julien, S., Juliant, S., Teintier-Lelièvre, M., Samyn-Petit, B., Montiel, M. D., Mir, A. M., Cerutti, M., Harduin-Lepers, A., and Delannoy, P. (2003) Identification and functional expression of a second human beta-galactosidase alpha2,6-sialyltransferase, ST6Gal II. Eur. J. Biochem. 270, 950–961
9. Takashima, S., Tsuji, S., and Tsujimoto, M. (2002) Characterization of the second type of human beta-galactosidase alpha 2,6-sialyltransferase (ST6Gal II), which sialylates Galbeta 1,4GlcNAc structures on oligosaccharides preferentially. Genomic analysis of human sialyltransferase genes. J. Biol. Chem. 277, 45719–45728
10. Pérez-Garay, M., Arteta, B., Pagès, L., de Llorens, R., de Bólos, C., Vidal-Vanaclocha, F., and Peracaula, R. (2010) alpha2,3-sialyltransferase ST3Gal III modulates pancreatic cancer cell motility and adhesion in vitro and enhances its metastatic potential in vivo. PLoS One 5, e15254
11. Jun, L., Yuanshu, W., Yanying, X., Zhougfa, X., Jian, Y., Fengling, W., Xia, L., Q., Kokuo, N., Wei, T., Weixia, Z., and Shuxiang, C. (2012) Altered mRNA expressions of sialyltransferases in human gastric cancer tissues. Med. Oncol. 29, 84–90
12. Seales, E. C., Jurado, G. A., Brunson, B. A., Wakefield, J. K., Frost, A. R., and Bellis, S. L. (2005) Hypersialylation of beta1 integrins, observed in colon adenocarcinoma, may contribute to cancer progression by upregulating cell motility. Cancer Res. 65, 4645–4652
13. Le Marer, N., and Strehmel, D. (1995) High level alpha 2,6-sialylation of N-acetyllactosamine sequences in ras-transformed rat fibroblasts correlates with high invasive potential. Glycobiology 5, 219–226
14. Lise, M., Belluco, C., Perera, S. P., Patel, R., Thomas, P., and Ganguly, A. (2000) Clinical correlations of alpha2,6-sialyltransferase expression in colorectal cancer patients. Hybridoma 19, 281–286
15. Julien, S., Adriaenssens, E., Ottenberg, K., Furlan, A., Courtrand, G., Verchère, F., and Poues, C. (1999) Identification and functional expression of a ST6GalNAc I expression in MDA-MB-231 breast cancer cells greatly modifies their O-glycosylation pattern and enhances their tumourigenicity. Glycobiology 16, 54–64
16. Schneider, F., Kemmner, W., Haensch, W., Franke, G., Gretschel, S., Karsten, U., and Schlap, P. M. (2001) Overexpression of sialyltransferase-type c-myc:alphaGalbeta1, 3GalNAc-R alpha6-Sialyl-transferase is related to poor patient survival in human colorectal carcinomas. Cancer Res. 61, 4605–4611
17. Bos, P. D., Zhang, X. H., Nadal, C., Shu, W., Gomis, R. R., Nguyen, D. X., Minn, A. J., van de Vijver, M. J., Gerald, W. L., Foekens, J. A., and Massagué, J. (2000) Genes that mediate breast cancer metastasis to the brain. Nature 459, 1005–1009
18. Steenackers, A., Vanbeselaere, J., Cazet, A., Bobowski, M., Rombouts, Y., Colomb, F., Le Boursis, X., Guéradel, Y., and Delannoy, P. (2012) Accumulation of unusual gangliosides GQ3 and GP3 in breast cancer tissues correlated with expression of the G3(s) synthesizing enzymes. Molecules 17, 9559–9572
19. Engelman, J. A., Luo, J., and Cantley, L. C. (2006) The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat. Rev. Genet. 7, 606–619
20. Bellacosa, A., Kumar, C. C., Di Cristofano, A., and Testa, J. R. (2005) Activation of AKT kinases in cancer: implications for therapeutic targetting. Adv. Cancer Res. 94, 29–86
21. Grabinski, N., Ewald, F., Hofmann, B. T., Stauffer, K., Schumacher, U., Nashan, B., and Jücker, M. (2012) Combined targeting of AKT and mTOR synergistically inhibits proliferation of hepatocellular carcinoma cells. Mol. Cancer 11, 85
22. Gedaly, R., Angulo, P., Chen, C., Creasy, K. T., Spear, B. T., Hunder, J., Daily, M. F., Shah, M., and Evers, B. M. (2012) The role of PI3K/mTOR inhibition in combination with sorafenib in hepatocellular carcinoma treatment. Anticancer Res. 32, 2531–2536
23. Fang, Y., Xue, J. L., Shen, Q., Chen, J., and Tian, L. (2012) MicroRNA-7 inhibits tumor growth and metastasis by targeting the phosphoinositide 3-kinase/Akt pathway in hepatocellular carcinoma. Hepatology 55, 1852–1862
24. Lee, E. K., Kim, H. J., Lee, K. L., Lee, H. J., Lee, J. S., Kim, D. G., Hong, S. W., Yoon, Y., and Kim, J. S. (2011) Inhibition of the proliferation and invasion of hepatocellular carcinoma cells by lipocarnin 2 through blockade of JNK and PI3K/Akt signaling. Int. J. Oncol. 38, 325–333
25. Li, Q. L., Gu, F. M., Wang, Z., Jiang, J. H., Yao, L. Q., Tan, C. J., Huang, X. Y., Ke, A. W., Dai, Z., Fan, J., and Zhou, J. (2012) Activation of PI3K/AKT and MAPK pathway through a PDGF(R)β-dependent feedback loop is involved in rapamycin resistance in hepatocellular carcinoma.
Modification of Sialylation Mediates Invasion and Chemosensitivity

PLoS One 7, e33379

26. Chen, K. F., Chen, H. L., Tai, W. T., Feng, W. C., Hsu, C. H., Chen, P. J., and Cheng, A. L. (2011) Activation of phosphatidylinositol 3-kinase/Akt signaling pathway mediates acquired resistance to sorafenib inhepatocellular carcinoma cells. J. Pharmacol. Exp. Ther. 337, 155–161

27. Domon, B., and Aebersold, R. (2006) Mass spectrometry and protein analysis. Science 312, 212–217

28. Ishihara, T., Fukuda, I., Morita, A., Takinami, Y., Okamoto, H., Nishimura, S., and Numata, Y. (2011) Development of quantitative plasma N-glycoproteomics using label-free 2-D LC-MALDI MS and its applicability for biomarker discovery in hepatocellular carcinoma. J. Proteomics 74, 2159–2168

29. Zhang, H., Liu, A. Y., Loriaux, P., Wollscheid, B., Zhou, Y., Watts, J. D., and Aebersold, R. (2007) Mass spectrometric detection of tissue proteins in plasma. Mol. Cell. Proteomics 6, 64–71

30. Zhang, H., Loriaux, P., Eng, J., Campbell, D., Keller, A., Mose, P., Bonnaud, R., Zhang, N., Zhou, Y., Wollscheid, B., Cooke, K., Yi, E. C., Lee, H., Peskind, E. R., Zhang, J., Smith, R. D., and Aebersold, R. (2006) UniPep, a database for human N-linked glycosites: a resource for biomarker discovery, Genome Biol. 7, R73

31. Zhang, Z., Sun, J., Hao, L., Liu, C., Ma, H., and Jia, L. (2013) Modification of glycosylation mediates the invasive properties of murine hepatocarcinoma cell lines to lymph nodes. PLoS One 8, e65218

32. Sawada, M., Moriya, S., Saito, S., Shineha, R., Satomi, S., Yamori, T., Tsuruo, T., Kannagi, R., and Miyagi, T. (2002) Reduced sialidase expression in highly metastatic variants of mouse colon adenocarcinoma 26 and retardation of their metastatic ability by sialidase overexpression. Int. J. Cancer 97, 180–185

33. Lin, S., Kemmer, W., Grigull, S., and Schlag, P. M. (2002) Cell surface alpha 2,6 sialylation affects adhesion of breast carcinoma cells. Exp. Cell Res. 276, 101–110

34. Recchi, M. A., Hebbbar, M., Horenczy, L., Harduin-Leipers, A., Peyrat, J. P., and Delannoy, P. (1998) Multiplex reverse transcription polymerase chain reaction assessment of sialyltransferase expression in human breast cancer. Cancer Res. 58, 4066–4070

35. Harvey, B. E., Toth, C. A., Wagner, H. E., Steele, G. D., Jr., and Thomas, P. (1992) Sialyltransferase activity and hepatic tumor growth in a nude mouse model of colorectal cancer metastases. Cancer Res. 52, 1775–1779

36. Vierbuchen, M. J., Fruedt, W., Brackrock, S., Krause, K. T., and Zienkiewicz, T. J. (1995) Quantitative lectin-histochemical and immunohistochemical studies on the occurrence of alpha(2,3)- and alpha(2,6)-linked sialic acid residues in colorectal carcinomas. Relation to clinicopathologic features. Cancer 76, 727–735

37. Brocco, M. A., and Frasch, A. C. (2006) Interfering polysialyltransferase ST8SiaI/STX mRNA inhibits neurite growth during early hippocampal development. FEBS Lett. 580, 4723–4726

38. Guo, R., Cheng, L., Zhao, Y., Zhang, J., Liu, C., Zhou, H., and Jia, L. (2013) Glycogenes mediate the invasive properties and chemosensitivity of human hepatocarcinoma cells. Int. J. Biochem. Cell Biol. 45, 347–358

39. Zhou, F., Cui, C., Ge, Y., Chen, H., Li, Q., Yang, Z., Wu, G., Sun, S., Chen, K., Gu, J., Jiang, J., and Wei, Y. (2010) Alpha2,3-sialylation regulates the stability of stem cell marker CD133. J. Biochem. 148, 273–280

40. Li, Z. (2013) CD133: a stem cell biomarker and beyond. Exp. Hematol. Oncol. 2, 17

41. Wang, P. H., Lee, W. L., Juang, C. M., Yang, Y. H., Lo, W. H., Lai, C. R., Hsieh, S. L., and Yuan, C. C. (2005) Altered mRNA expressions of sialyltransferases in ovarian cancers. Gynecol. Oncol. 99, 631–639

42. Lopez-Morales, D., Velazquez-Marquez, N., Valenzuela, O., Santos-Lopez, G., Reyes-Cordova, J., and Vallejo-Ruiz, V. (2009) Enhanced sialyltransferases transcription in cervical intraepithelial neoplasia. Invest. Clin. 50, 45–53