科技部補助

大專學生研究計畫研究成果報告

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＊ 計 畫 ＊
＊ ： 軟骨素生成酵素(CHSY1)在肝細胞癌的表現與功能 ＊
＊ 名 稱 ＊
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Abstract

Hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer and the third leading cause of cancer-related deaths worldwide. Because of limited effectiveness of current treatments, investigation of precise molecular mechanisms and identify new therapeutic target underlying HCC progression are very important to treat this aggressive malignancy. Chondroitin sulfate (CS) proteoglycans, one of major glcosaminoglycans existing on animal cells and extracellular matrix, plays important roles in cell growth, migration, and tissue morphogenesis. It has been reported that CS proteoglycans are increased in human HCC, particularly in poor differentiated HCC tissues. However, which enzyme regulates aberrant CS chains formation and the biological functions of CS chains in HCC progression remain unclear. This research is aimed to explore the expression and functions of chondroitin sulfate synthase 1 (CHSY1) in HCC tissues and cell lines. Searching from public database, we found that CHSY1 is significantly up-regulated in cirrhosis liver, which is a crucial risk factor for HCC development. Immunohistochemistry analysis on CHSY1 expression in the HCC tissue arrays showed that increased expression of CHSY1 was associated with poor differentiated HCC ($n=98$, $p<0.01$). Survival assessment revealed that HCC patients with increased CHSY1 expression were significantly associated with shortened survival ($n=98$, $p<0.01$). Cell proliferation and metastasis are typical features of a malignant tumor. The Western blot and transwell assay demonstrated that inhibition of CHSY1 could reverse epithelial-mesenchymal transition and decrease migratory capability, but enhance cell growth rate of HCC cells. These findings indicated that CHSY1 could be a crucial enhancer in HCC progression. Overexpressed CHSY1 increases the synthesis of CS chains during HCC progression, thereby could promote the invasive/migratory capability of HCC. Therefore, targeting CHSY1 may serve as a potential candidate in HCC therapy.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer and the third leading cause of cancer-related deaths worldwide (1). US and European clinical practice guidelines proposed five therapeutic recommendations: resection; transplantation; radiofrequency ablation; chemoembolization; and sorafenib treatment. Resection, Transplantation, and thermal ablation are applicable for the 30% patients, and relapse rates are up to 50% (2). Systemic chemotherapy for HCC has been associated with low response rates and no survival benefit (2). Because of late stage diagnosis and limited therapeutic options, the prognosis of HCC patients after medical treatments remains disappointing (3). Therefore, investigation of precise molecular mechanisms and identification new therapeutic target underlying HCC progression is extremely important for treatment of this aggressive malignancy.

Glycosaminoglycans (GAG) are linear polysaccharide chains, which disaccharide repeating unites consist of alternating amino sugar and hexuronic acid or galactose. The different repeating disaccharide units and sulfation status construct various types of GAGs. The GAG chains covalently link to a core protein, such as aggrecan family, and are known as proteoglycans. Proteoglycans and glycosaminoglycans are generally present on animal cells and extracelluar matrix (ECM). They play important roles in cell growth, differentiation, morphogenesis, cell migration, and bacterial and viral infections (4). Chondroitin sulfate (CS) chains, one of major GAGs, are composed by turns of N-acetyl-D-galactosamine (GalNAc) and glucuronic acid (GlcA). In human, CS chains are initiated by the transfer of N-acetylgalactosamine to the linkage tetrasaccharide by CSGALNACT1 or CSGALNACT2, and the polymerization step is catalyzed by a group of bifunctional enzymes (chondroitin sulfate synthase：CHSY1, CHSY2, and CHSY3) that have both β1–3 glucuronosyltransferase and β1–4 N-acetylgalactosaminyltransferase activities.

CS chains can undergo a complex sulfation process, which mainly at the C2 position of uronic acid residues and at the C4 and/or C6 positions of GalNAc residues, forming diverse disaccharide units (4). CS chains recently have been found interacting with a wide spectrum of proteins, including growth factors, cytokines, chemokines and adhesion molecules.

In 2012, Jia et al, reported that expression of chondroitin sulfate proteoglycans are increased in rat hepatocellular carcinoma tissues (5). A recent study revealed that targeting on a GAG core protein, glypican-3, via a conformation-specific antibody have therapeutic effects in hepatocellular carcinoma (6). In addition, it has been reported that up-regulated CS in poorly differentiated human HCC and the sulfation pattern is altered in different differentiation status (7). However, which enzyme regulates CS chain formation and the biological functions of CS chains in HCC progression remain unclear.

As attempts to answer these questions, the present study is firstly used the ONCOMINE database and computerized image analysis system to systemically examine the CHSY1 expression in the in liver cirrhosis and human HCC tissues. Secondly, the aberrant expression of CHSY1 if regulates cell-surface CS change and epithelial-mesenchymal transition (EMT)-associared proteins expression of HCC cells. HA22T cells were further analyzed by using flower cytometric analysis and Western blots.

Finally, in order to detect the effects of aberrant CHSY1 expression on HCC cells, the performance of cellular behavior such as proliferation, and migration were further assessed.

Materials and Method

Immunohistochemistry of HCC tissue array

Paraffin-embedded normal human liver and HCC tissue microarrays were purchased from SUPER BIO CHIPS (Seoul, Korea) and Biomax (Rockville, MD). Arrays were incubated with anti-CHSY1 monoclonal antibody (GeneTex Inc, Irvine, CA) in 5% BSA/PBS and 0.1% Triton X-100 (Sigma) for 16 h at 4℃. After rinsing twice with PBS, Super Sensitive™ Link-Label IHC Detection System (BioGenex, San Ramon, CA) was used and the specific immunostaining was visualized with 3,3-diaminobenzidine liquid substrate system (Sigma). All sections were counterstained with hematoxylin (Sigma). The correlation between CHSY1 expression and pathological characteristics was evaluated according to the information provided by supplier.

Plasmid construction and stable clone selection.

CHSY1 pLKO-shRNA plasmids (National RNAi Core Facility, Taiwan) were transfected into HA22T cells to establish stable knockdowned clones, and were selected by puromycin for 10 days. CHSY1 expression was analyzed by Western blot.

Flow cytometry analysis of cell-surface CS change

To study CHSY1-mediated cell-surface chondroitin sulfate changes, chondroitin sulfate specific antibody (CS-56) was used for flow cytometry on CHSY1 knockdowned cells. Acquire suspended HA22T cells in FACS buffer and adjust to give cell concentration of 1×10^6 cells/ml. After incubating with CS-56 (1:100) on ice for 30 minutes, pellet cells by certification at 2000 rpm for 5 minutes. Repeat twice of washing resulting pellet with FACS buffer, centrifugation and discarding supernatant. Resuspend cells, stain with FITC-Goat anti-mouse IgM (1:100) and incubate on ice for 30 minutes in the dark. Then, wash, centrifugate and resuspend stained cells in FACS buffer and acquire data on a flow cytometer. The analysis of cell-surface CS

change was performed using a FACSCalibur (Becton-Dickinson Immunocytometry Systems) equipped with a 488 nm laser and Cell Quest software (10000 cells analyzed for each sample) was used for fluorocytometric data analysis and graphic display.

Western blotting analysis

HA22T cells were subjected to Western blot analysis for evaluating mesenchymal-epithelial transition. Total proteins were extracted in lysis buffer, followed by centrifugation at 15000 rpm for 15minutes at 4℃ to remove insoluble materials. Preceding with 10% SDS-PAGE, separated proteins were electroblotted onto a polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were then blocked with 2% bovine serum albumin and probed with Epithelial-Mesenchymal Transition Antibody Sampler Kit (1∶2000；Cell Signaling Technology) and antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1∶20000；GeneTex, Irvine, CA, USA) as internal control. After incubation with primary antibodies, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10000 ； Bethyl Laboratories, Montgomery, TX, USA). The chemiluminescence images were visualized with ECL solution (Millipore, Temecula, CA, USA) and captured by Multi-function Gel Image System.

Cell proliferation assay

HA22T cells were seeded at a density of 1×10^5 cells per well into 24-well plates. After incubation at 37℃ for 24, 48 and 72 hours separately, cells of each well were harvested by trypsin/EDTA and cell number was determined by microscopic counting, using a Burker-Turk counting chamber (Erma, Tokyo, Japan).

Cell migration assay

Transwell migration assays were performed using transwell chambers (8-uM pore size; Costar). Cells were starved in serum-free media for 3 hours, detached by trypsin/EDTA, and seeded on upper transwell membranes at a density of 3×10^5 cells/ml in serum-free media. The lower chambers were filled with 10% FBS media as attractant. After incubation for 24 hours, membranes were fixed in 100% methanol and stained with 0.1% crystal violet. The cells in the upper side of the insert membrane were rubbed with a cotton swab. The migrated cells on the underside were pictured under a 200x magnification field followed by quantitation using Image J. Software.

Statistical analysis

Two-sided Fisher's exact test or log-rank test were used to analyze acquired patients' data. The statistical significance was considered if $P < 0.05$.

Result

CHSY1 is up-regulated in liver cirrhosis

To determine which chondroitin sulfate synthases regulate abnormal expression of CS chains in HCC, we searched the ONCOMINE database and found that expression of CHSY1 in cirrhosis liver is significantly higher than that in normal liver (Fig. 1; t test, *p<0.05). Liver cirrhosis is the pathogenic hallmark of advanced liver injury and general risk factor of HCC development. Therefore, dysregulated expression of CHSY1 could be responsible for abnormal formation of CS in human HCC.

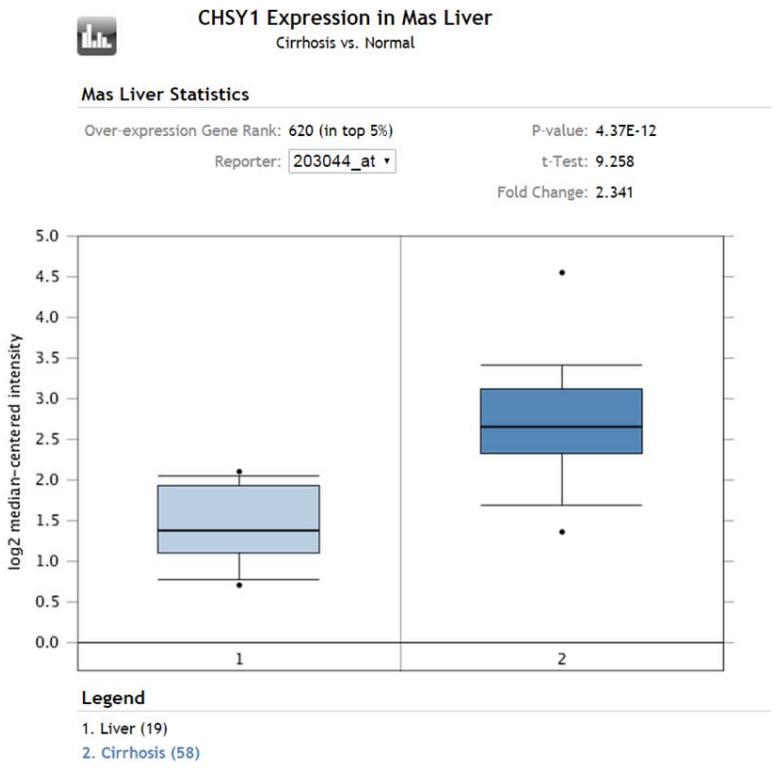

Figure 1. CHSY1 is up-regulated in liver cirrhosis. Expression of CHSY1 in cirrhosis liver is significantly higher than that in normal liver. This data was from ONCOMINE (https://www.oncomine.org/resource/login.html) database.

Expression of CHSY1 was up-regulated in high histological grade HCC tissues and associated with poor survival rate

To investigate the expression of CHSY1 in human HCC tissues and assess the correlation between CHSY1 expression and pathological characteristics, immunohistochemical staining was applied on HCC tissue array. We divided HCC patients into two groups based on the degree of CHSY1 expression. The results showed that CHSY1 expressed at cytoplasm of parenchymal cells, and significantly up-regulated in moderately and poorly differentiated HCC tissues (Fig. 2A and Table 1). In addition, up-regulation of CHSY1 was associated with poor overall survival

(Fig. 2B and Table 1, Kaplan-Meier analysis, n=98, **p<0.01). These results suggested that CHSY1 could be a worse prognostic marker of HCC.

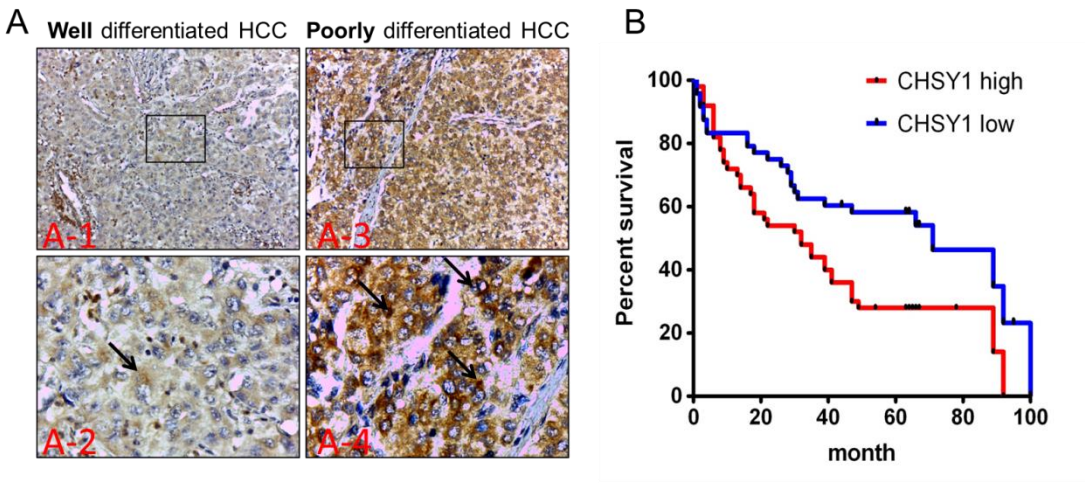

Figure 2. Expression of CHSY1 in human HCC tissues and comparison of survival rate of HCC patients. Immunohistochemistry showing CHSY1 in well (Fig. 2A left) and poorly (Fig. 2A right) differentiated HCC tissues. In well differentiated HCC tissue, the CHSY1 was located in cytoplasm (Fig. 2A-2, arrow). In poorly differentiated HCC tissue, the CHSY1 expression was drastically increased (Fig. 2A-4, arrows). Cumulative overall survival curves are shown for HCC patients based on high or low CHSY1 expression (Fig. 2B).

		CHSY1 expression			
Factor		**Low (n = 48)**	**High (n = 50)**	***P* value**	**Method**
Sex	Male	38	38	0.810	Two-sided Fisher's exact test
	Female	10	12		
Age	< 55 years	24	22	0.686	
	≧ 55 years	24	28		
Histology grade	well	15	3	0.002*	
	moderately and poorly	33	47		
Tumor stage	T1 + T2	24	26	1.000	
	T3 + T4	24	24		
Metastasis	No	36	41	0.465	
	Yes	12	9		
Overall survival				0.009	Log rank

* *P*< 0.05 was regarded as statistically significant.

Table 1. Comparison of patient characteristics between low and high CHSY1 expression.

CHSY1 regulated cell-surface CS change and epithelial-mesenchymal transition (EMT) in HCC cells.

After establishing stable knockdowned clones by transfecting CHSY1 shRNA plasmids into HCC cell lines, we performed flow cytometry to study cell-surface CS change. The histogram showed that fewer amount of cell-surface CS of CHSY1 knockdowned cells than that of CHSY1 control cells (Fig. 3A).

In order to evaluate whether CHSY1 participates in HCC progression, CHSY1 knockdowned cells were applied to analyze EMT by Western blot. Western blots confirmed that the expression of CHSY1 was suppressed. We examined expression of several epithelial and mesenchymal protein markers, and observed that knockdown of CHSY1 enhanced a tight junction molecule, Claudin-1, but suppressed several mesenchymal proteins, including β–catenin, Snail and Vimentin expression (Fig. 3B).

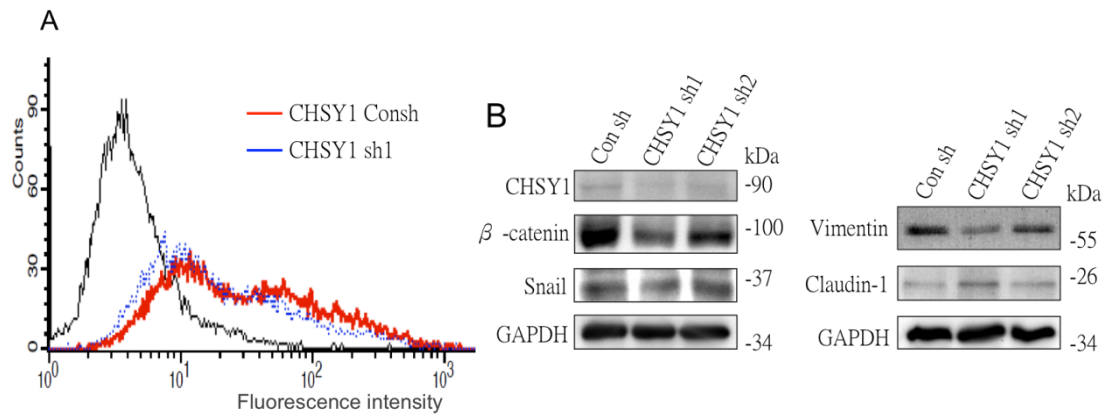

Figure 3. Silencing of CHSY1 altered the CS expression and reversed epithelial-mesenchymal transition (EMT) in HA22T cells. Flow cytometric analysis (Fig. 3A) showing that cell-surface CS change on CHSY1-transfected cells. Western blots showing CHSY1 and EMT-associated proteins expression in control shRNA (Con sh) and two CHSY1 specific shRNA (CHSY1 sh1 and sh2) of transfected HA22T cells. Epithelial marker: Claudin-1; Mesenchymal marker:

β–catenin, Snail, and Vimentin.

Knockdown of CHSY1 enhanced HCC cells proliferation

To further confirm the role of CHSY1 on HCC progression, CHSY1 knockdowned cells were applied to cell proliferation study. We counted cell number after incubation for various periods of time, in the presence of 10% FBS or in serum-free condition. Except for groups in serum-free condition, the number of CHSY1 control cells and CHSY1 knockdowned cells increased in a time-dependent manner. After incubation for 48hr in the presence of 10% FBS media, however, we found that both the number of CHSY1 knockdowned cells were greater than CHSY1 control cells, indicating the rate of cell growth increased after silencing CHSY1 (Fig. 4).

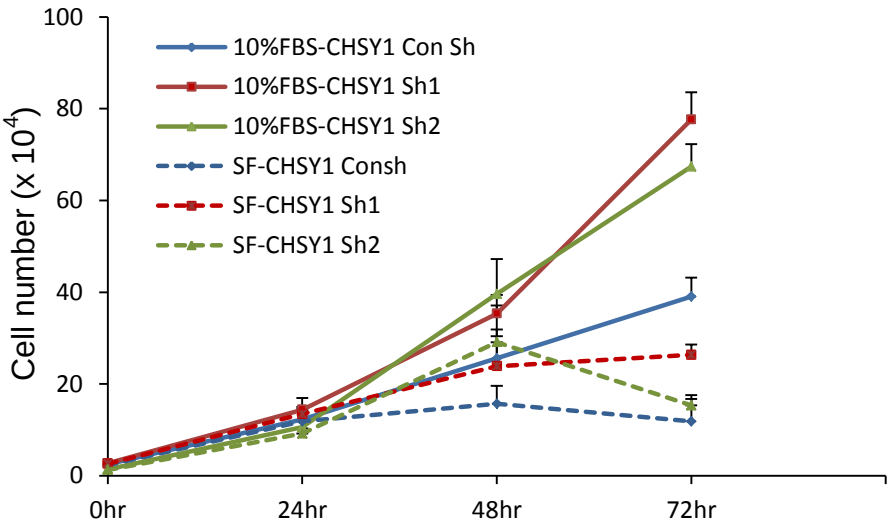

Figure 4. Silencing of CHSY1 elevates cell growth of HA22T cells. Proliferation curves of HA22T cells incubated for various periods of time in 10% FBS or serum-free (SF) media.

Knockdown of CHSY1 suppressed HCC cells migration

To further explore the function of CHSY1, based on its regulation on EMT from previous finding, we performed transwell assay to evaluate the effect of CHSY1 on the migratory capacity of HCC cells. The results showed that the number of migratory HA22T cells of CHSY1 knockdown groups were less than control group, with significant difference between control and Sh2 groups (Fig. 5).

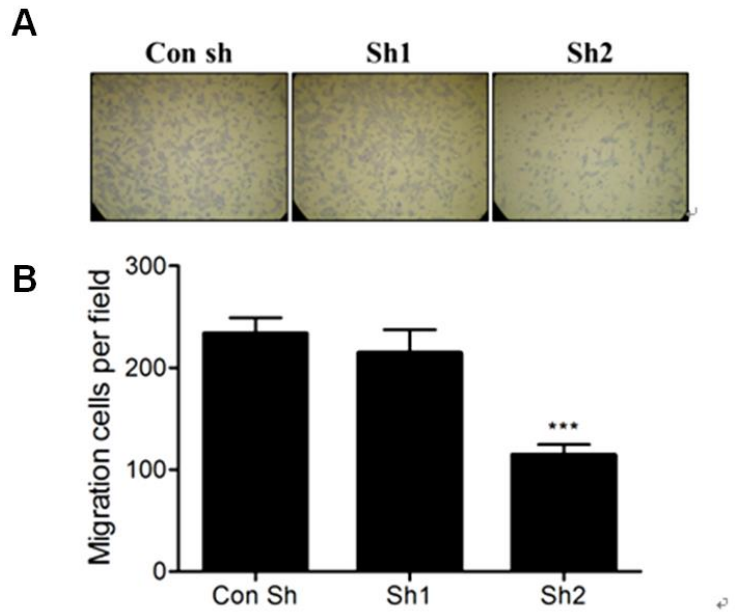

Figure 5. The migration of HA22T cells were attenuated by knockdown of CHSY1. Fig.5A：representative images of CHSY1 control and two CHSY1 knockdowned HA22T cells after transwell migration assay. Fig. 5B: Quantitative results are illustrated for top panel. *$P < 0.001$, vs control.

Discussion

GAGs are linear polymers attached to a core protein and distributed as side chains of proteoglycans (PGs) in the extracellular matrix or at the cell surface of animal tissues. Although the polysaccharide backbones of GAGs are simple, repetitive linear chains, extensive modification involving sulfation and uronate epimerization contributes to tremendous structural variation, which is the basis for the

wide variety of domain structures with biological activities (4). Major GAGs include chondroitin sulfate/dematan sulfate (CS/DS) and heparan sulfate. The roles of heparan sulfate in developmental processes and signaling pathways have been well-investigated, but CS chains have been considered to participate only in structural stabilization and attracted less attention. Until recently, accumulating evidence implies several crucial biological functions of CS chains in cytokinesis, cell proliferation, differentiation, migration, tissue morphogenesis, organogenesis, infection, and wound repair (8-10). The roles of CS chains in physiological and pathological condition have been widely discussed.

Although the biological role of CS in the progression has not been determined, an association between the accumulation of CS and cancer progression has been demonstrated. Abnormally high concentration of CS has been found in tumor stroma and tumor fibrotic tissues (11), accompanied by specific structural modification (12, 13). Increased expression of CS in rat hepatocellular carcinoma tissues has been reported (5). In the fibrous lesions of liver cirrhosis, a pathogenic hallmark of liver cancer, a remarkable elevation of CS content as well as altered sulfation pattern and different constituent units of CS chains have been observed (14). Abnormal elevated amount and sulfation pattern variation of CS chains were also reported in poorly differentiated human HCC (7). Placental-like CS chains, which are associated with the ability of trophoblasts to invade the uterine tissue and promote rapid cell proliferation during normal placental implantation process (15), can be anchored by a parasite-derived protein during placental malaria pathogenesis (16, 17). Recently, these specific placental-like CS chains are found highly expressed on liver hepatocellular carcinoma (18). Besides, hepatocyte growth factor and heparin-binding EGF-like growth factor, which are CS binding proteins, are involved in liver fibrosis, cirrhosis and carcinogenesis (8). These findings suggest that abnormal expression and

structural variation of CS chains in liver are influential in the progression of hepatocellular malignancy. Therefore, recognition of malignancy-associated glycosaminoglycan modification and targeting at the metabolism of abnormal CS chains may be a potential anti-tumor strategy.

Ranging from core protein-linkage region to repeating disaccharide region, many functional enzymes participate sequentially in the biosynthesis of CS chains. Recent studies demonstrate that aberrant activity of enzymes and synthesized specific sequences of CS chains are associated with the regulation of tumor development and progression. Knockdown of GalNAc 4-sulfate 6-O-sulfotransferase in Lewis lung carcinoma cell, an experimental model for pulmonary metastasis, suppressed its pulmonary colonization (19). A gene for chondroitin 4-O-sulfotransferase-1, *CHST11* was reported overexpressed in aggressive human breast cancer cell lines and in malignant tissues of breast cancer patients (20). These findings indicate that aberrant activity of CS biosynthetic enzymes correlates with different type of tumors and their malignant behavior. Regarding liver tumor, we found significantly higher expression of CHSY1 in liver cirrhosis and poorly differentiated human HCC tissues, and observed fewer cell-surface CS of CHSY1 knockdowned cells. The increased amount of CHSY1 may correlate with previous finding about increased CS expression in rat hepatocellular carcinoma tissues (5) and influence subsequent tumor progression and patients' shortened survival.

Epithelial–mesenchymal transitions (EMT) are mainly marked by the loss of cell–cell interactions and of epithelial apico-basal polarity with acquisition of mesenchymal markers and enhanced cell motility (21). These characteristics are of relevance for various developmental processes, including gastrulation, neural crest formation and carcinoma progression. Until recently, EMT has been extensively studied and validated in the progression of various carcinomas. The characteristics of

EMT are observed at hepatocytes derived from cirrhotic livers, implicating for the progression to HCC (22). Driven by the stimuli of numerous extracellular proteins and subsequent activation of intracellular signaling pathways as well as transcription program switching, the activity of EMT is accompanied by the dynamic change of various biomarkers (23). In our study, we observed that silencing of CHSY1 of HCC cells reversed EMT by upregulation of epithelial marker and downregulation of mesenchymal markers. These findings indicate that abnormally overexpressed CHSY1 in liver cirrhosis and HCC tissues increases the amount of CS chains on the surface of hepatocytes and at surrounding extracellular matrix. Aberrant elevation of CS chains thereby induces the activity of EMT by interacting with various proteins at local microenvironment. Consequently, activation of EMT leads to a shift to mesenchymal phenotype and promotes the invasive/migratory capability of HCC.

Conclusion

Our study made it clear that abnormal increased expression of CHSY1 plays a vital role in regulating EMT during HCC progression and is absolutely associated with patient's shortened survival. Taking into account a wide variety of proteins interacting with CS chains in liver, developing medicines targeting CS biosynthetic enzymes is a promising therapeutic strategy in HCC treatment.

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