科技部補助專題研究計畫報告

研究硫酸軟骨素生成酵素在肝細胞癌的惡化與抗藥性之功能(第3年)

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本研究具有政策應用參考價值：■否 □是，建議提供機關
（勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
本研究具影響公共利益之重大發現：□否 □是

中 華 民 國 109 年 10 月 28 日

中 文 摘 要 ： 肝細胞癌（HCC）在男性中佔90.73%，在女性中佔85.42%，是全世界導致癌症死亡的第三大原因。手術切除，經動脈化學栓塞，射頻消融和肝移植為治愈HCC提供了機會。但是，這些方法僅適用於30%的患者，復發率高達50%。癌細胞的浸潤和轉移是肝癌高複發率的主要因素。越來越多的證據表明，基質細胞，免疫細胞，細胞外蛋白和糖基化的變化建立了促進HCC進程的腫瘤微環境（TME）。癌細胞和TME之間的複雜相互作用很難直接解釋腫瘤細胞的基因組信息。因此，探索HCC中癌細胞與TME相互作用的潛在機制可能為臨床診斷和治療提供重要意義。

糖胺聚醣（GAGs）是細胞外基質和細胞表面的未分支多醣鏈，參與TME中的各種生物學功能。與核心蛋白共價連接的GAG被稱為蛋白聚醣（PGs），某些類型的GAG可以以自由鏈的形式存在，例如透明質酸。重要的是，最近的研究表明，異常的GAG經常積存在肝癌的腫瘤微環境中，這不僅可能與疾病進展有關，而且某些特定的GAG修飾也可以用作疾病診斷和藥理靶標的生物標記。硫酸軟骨素（CS）是GAG的主要類型之一。過去，CS鏈的功能僅在結構穩定化時才考慮。最近，由於各種生長因子，已發現蛋白酶，細胞因子，趨化因子和粘附分子與CS鏈相互作用，因此對CS鏈在疾病進展中的重要性進行了重新評估。

我們最近的研究表明，這些CHSY和CS修飾酶具有組織特異性表達譜，並在調節不同類型癌細胞的惡性行為中發揮獨特作用。我們最近發現，CHSY1在肝細胞癌（HCC）和神經膠質瘤組織中表達上調，其上調與陰性患者預後相關。 C5差向異構酶DSE在人神經膠質瘤中也經常上調，其表達增加與預後較差有關。相反，DSE在HCC組織中通常被下調。

中 文 關 鍵 詞 ： 肝細胞癌, 基質細胞, 腫瘤微環境, 糖胺聚醣, 硫酸軟骨素, 糖基化

英 文 摘 要 ： Hepatocellular carcinoma (HCC) accounts for 90.73% in men and 85.42% in women of liver cancer, and it’s being the third-leading cause of cancer death worldwide. Surgical resection, transarterial chemoembolization, radiofrequency ablation, and liver transplantation provide an opportunity for a cure for HCC. However, these approaches are only applicable for 30% patients, and relapse rates are up to 50%. Invasiveness and metastasis of cancer cells are main contributors for the high relapse rate of HCC. Accumulating evidence pointed that the changes of stromal cells, immune cells, extracellular protein, and glycosylation established tumor microenvironment (TME) that facilitates HCC progression. The complex interactions within cancer cells and TME are difficult to directly interpret form genomic information of tumor cells. Thus, the exploration underlying mechanisms of cancer cells and TME interactions in HCC could provide important significance for clinical diagnosis and treatment.

Glycosaminoglycans (GAGs) are unbranched polysaccharide

chains in extracellular matrix and cell surface, which participate in various biological functions in TME. GAGs covalently link to core protein are known as proteoglycans (PGs), and certain types of GAGs can exist as free chains, such as hyaluronan. Importantly, recent studies indicated that aberrant GAGs are often accumulated in the tumor microenvironment of HCC, which may not only be associated with disease progression, certain specific modification of GAGs could also be used as biomarkers for disease diagnosis and pharmacological targets. Chondroitin sulfate (CS) is one of major types of GAG. In the past, functions of CS chains were considered only in structure stabilization. Recently, due to various growth factors, proteases, cytokines, chemokines, and adhesion molecules have been found interacting with CS chains, the importance of CS chains in disease progression has been reevaluated.
The biosynthesis of CS chains begins with the formation of a link between N-acetylgalactosamine (GalNAc) and a common tetrasaccharide structure at a serine residue on the core protein. The next polymerization (elongation) step is catalyzed by a group of bifunctional enzymes (CHSY1, CHPF, CHPF2, and CHSY3), that have β1-3 glucuronosyltransferase and β1-4 N-acetylgalactosaminyltransferase activities. A single CS chain can consist of up to 50 repeating GlcA-GalNAc subunits, which are modified with sulfate groups at various positions. Depending on the spectrotemporal expression of the polymerization and modification enzymes, a single CS chain usually consists of a series of variably sulfated units.
Our recent studies revealed that these CHSYs and CS-modifying enzymes have tissue-specific expression profiles and play distinct roles in regulating the malignant behavior of different types of cancer cells. We have recently identified that CHSY1 is up-regulated in hepatocellular carcinoma (HCC) and glioma tissues, and its up-regulation is associated with negative patient outcomes. The C5 epimerase DSE is also frequently up-regulated in human gliomas, with its increased expression being associated with a worse prognosis. By contrast, DSE is often down-regulated in HCC tissues.

英文關鍵詞：hepatocellular carcinoma; chondroitin sulfate; CHSY1; CHPF; tumor microenvironment

一、前言

The long-term goal of our lab is to development a new treatment for human hepatocellular carcinoma (HCC) by exploring functions of glycosaminoglycan (GAG) in HCC progress, drug resistance, and immunoregulation. The objectives of this project will investigate functions of chondroitin sulfate synthase in HCC cells. Our preliminary data indicated that expression of chondroitin sulfate synthase 1 (CHSY1) may associate with worse HCC prognosis, and it may involve in Sorafenib resistance. Therefore, chondroitin sulfate synthase-mediated drug resistance and underlying mechanism will be studied in this project.

According to the least survey in Taiwan, liver cancer is the second-leading cause of cancer death in male and forth-leading cause of cancer death in female (*1*). In addition, it's being the third-leading cause of cancer death worldwide (*2*). Hepatocellular carcinoma (HCC) accounts for 90.73% in men and 85.42% in women of liver cancer. The high mortality of this disease mainly attributes to late diagnosis and limited treatment for advanced HCC.

Effective treatment for HCC, including surgical resection, transarterial chemoembolization, radiofrequency ablation, and liver transplantation may provide an opportunity for a cure. However, these approaches are only applicable for 30% patients, and relapse rates are up to 50% (*3*). Systemic chemotherapy for HCC has been associated with low response rates and no survival benefit (*4*). Currently, a multi-kinase inhibitor, Sorafenib (Nexavar; Whippany, NJ), is the first and the only approved treatment for advanced HCC. Although Sorafenib was shown to improve overall survival (prolongs 2.3 months compared with placebo groups), the outcome of the treatment is still disappointing (*5-7*).

Currently, various small-molecule inhibitors or monoclonal antibodies for target therapy of HCC are under clinical trials as an alternative to sorafenib or after sorafenib failure (*8*). Like sorafenib, many on trial drugs are multi-protein kinase inhibitors, such as Sunitinib, Brivanib, and Regorafenib (*9-12*). However, the results of most recent trials are failures or only provide very mild survival benefit in phase III clinical trials. One reason of these failures may due to most ongoing trials were conducted without any molecular selection criteria. These failures also reflect our lack of understanding of molecular mechanisms in HCC progression (*13, 14*). In addition, interactions between cancer cells and tumor microenvironment (TME) may induce both soluble-factor-mediated drug resistance and cell adhesion-mediated drug resistance in various types of cancer (*15*). However, these drug resistance mechanisms are largely unexplored in HCC. To development new therapeutic strategies for HCC, it is an urgent need to elucidate interaction between TME and HCC cells.

1.2 Importance of chondroitin sulfate in cancer progression

Changes in expression and composition of extracellular matrix (ECM) molecules in TME are hallmarks of cancer (*16*). Glycosaminoglycans (GAGs) are unbranched polysaccharide chains in ECM which have been found participating in various biological functions in TME. GAGs covalently link to

core protein are known as proteoglycans (PG); GAGs also exist as free chains. GAG and PG are present in the ECM as well as on the cell surface and inside the cell. The complex interactions within cancer cells, stromal cells, and immune cells to extracellular proteoglycans, free GAG chains, and glycoprotein are difficult to directly interpret form genomic information of tumor cells.

The different repeating disaccharide units construct the structure of hyaluronan, dermatan sulfate, keratan sulfate, heparin, heparan sulfate, and different sulfation status of chondroitin sulfate (CS). Accumulated knowledge about GAGs and PG demonstrated that they can regulate cell growth, differentiation, morphogenesis, cell migration, and bacterial/viral infections (*17*). Recent studies also indicated that altered structure of GAG is associated with cancer progression and can be taken as biomarkers for disease diagnosis as well as pharmacological targets (*18-21*).

CS chain is one of major types of GAG. In the past, functions of CS chains were considered only in structure stabilization. **Recently, due to various growth factors, proteases, cytokines, chemokines, and adhesion molecules have been found interacting with CS chains, the importance of CS chains in disease progression has been reevaluated (*22-24*).** Accumulating evidence indicated that CS chains participate in cancer progression. Increasing of CS chains in melanoma cells could enhance MMP2 activation, and promote angiogenesis, proliferation, as well as cell invasion (*25, 26*). Highly sulfated CS chains on cell surface could promote metastatic process of lung cancer cells (*27*) and osteosarcoma cells (*28*). Chondroitin sulfate-E was found strongly expressed in ovarian carcinoma and promoted VEGF binding (*29*). In addition, CS chains were reported as P-selectin ligand on breast cancer cells (*30*).

1.3 Biosynthesis of chondroitin sulfate and their possible roles in tumor microenvironment

In human, the biosynthesis of CS chains initiate from N-acetylgalactosamine linking to a tetrasaccharide structure by CSGALNACT1 or CSGALNACT2 transferases. Next, the polymerization step is catalyzed by a group of bifunctional enzymes (CHSY1, CHSY2/CHPF, and CHSY3) that have both β1–3 glucuronosyltransferase and β1–4 N-acetylgalactosaminyltransferase activities. These GlcA-GalNAc repeats are esterified by sulfate at various positions by a sulfurtransferase family (4-O-Sulfotransferase, 6-O-Sulfotransferase, and 2-O-Sulfotransferase).

Abbreviations of typical disaccharide units are **CS-O, CS-A, CS-C, CS-D**, and **CS-E**-units consist of GlcA-GalNAc(non-sulfate), GlcA-GalNAc(4-O-sulfate), GlcA-GalNAc(6-O-sulfate), GlcA(2-O-sulfate)-GalNAc(6-O-sulfate), GlcA-GalNAc(4,6-O-disulfate), respectively. In addition, C5 epimerase exist for epimerization of glucuronic acid to iduronic acid which consider as dermatan sulfate (also called **CS-B)** (*31*).

CS is one of major components in extracellular matrix, and CS is important in both maintaining structural integrity and regulating cellular activities of the tissue. **In structurally,** CS is a major component of cartilage; loss of CS in cartilage is a major cause of osteoarthritis. CS also can be a physically barrier to prevent pathogen dispersal and nerve regeneration. Using Chondroitinase ABC to

digest CS chains deposit in the lesioned dorsal columns promotes functional recovery of spinal injuries (*32*). In addition, degradation of glioma ECM by oncolytic viral expressing bacterial Chondroitinase ABC enhanced virus spread and anti-tumor efficacy of these virus (*33*). **In cellular regulatory,** due to negative charges of CS chains, they are able to interact with various proteins in the extracellular matrix with different affinity. They can form complexes with growth factors and mediate bioactive of certain proteinase, such as MMP2, MMP7. Consequently, CS chains and CS-proteoglycans (CSPG) have been proposed involved in cancer cell proliferation, adhesion, migration, and invasion (*34-36*).

In HCC, it has been reported that expression of CS chains are increased in a rat hepatocarcinogenesis model (*37*). A previous study also indicated that CS chains overexpressed in HCC, and altered sulfation status were associated with poorly histological grade (*38*). Importantly, a recent study reported that a distinct modification of "oncofetal CS chains" is highly expressed on many types of cancer, including HCC, which can be used as a marker for cancer diagnosis or target therapy (*21*). **Although the expression of CS chains in HCC has been investigated in some groups, the biological functions of CS chains in HCC progression remain obscure.**

二、研究目的

Hypothesis and specific aims

According to literature review and our preliminary results, **we hypothesize that CHSY1 could not only promote malignant phenotypes of HCC cells, but also enhance drug resistance of hepatocellular carcinoma by enhancing growth factors binding on CS chains in tumor microenvironment.** Thus, we suppose that inhibit CHSY1 expression or degrade CS chains in tumor could sensitize effects of antitumor drugs, which could develop into a novel therapy for patients with HCC.

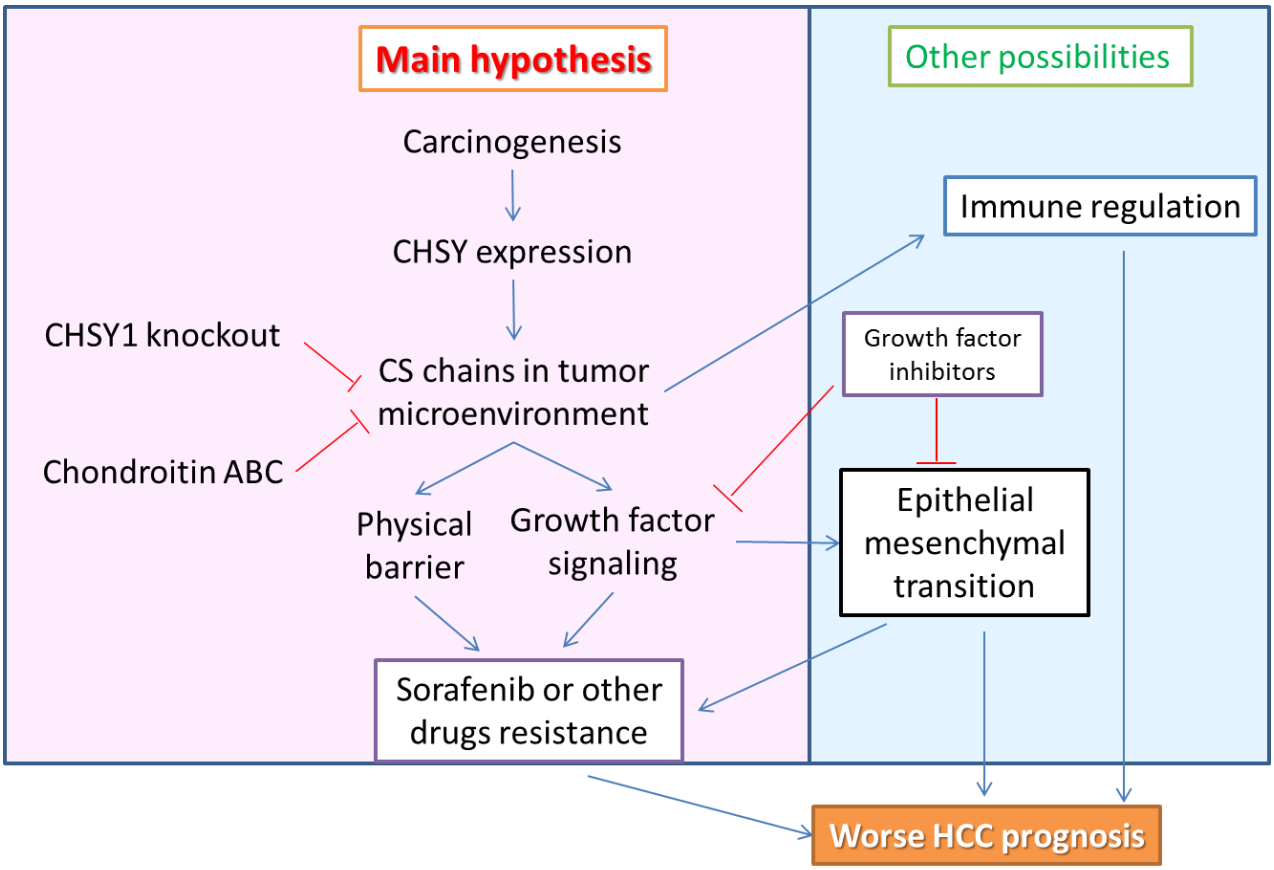
To testify this hypothesis, we propose three specific aims in this project as following.

Aim 1 will elucidate CHSY1-regulated malignant phenotypes of HCC cells *in vitro* and *in vivo*.

Aim 2 will investigate CHSY1-mediated drug resistance and its underling mechanisms in HCC cells.

Aim 3 will examine whether silencing CHSY1 or suppressing chondroitin sulfate in tumor could sensitize Aim 2-tested drugs *in vivo*.

3.7 Flow chart of experimental design



三、研究發法

Reagents and antibodies

Recombinant TGF-β protein was purchased form PeproTech. Full length Chpf cDNA clone were purchased from OriGene. Mouse monoclonal antibody against CHPF (sc-376183) was purchased from Santa Cruz. Antibodies against p-Smad2/3, Smad2/3, p-AKT, AKT, p-p38, p38, p-ERK1/2, ERK1/2, and ZO-1 were purchased from Cell Signaling Technology, Inc. Goat polyclonal anti-decorin (R&D Systems, catalog #AF143) was used to examine expression of Decorin. Antibodies against actin were purchased from GeneTex, Inc. TGF- inhibitor, LY364947 (Cayman Chemical) was dissolved in DMSO (1.0 mg/ml) and applied to inhibit TGF-β receptor. Chondroitinase ABC, -D-xylopyranoside, and CCK8 reagent was purchased from Sigma-Aldrich. Heparinase II was purchased from R&D Systems.

Human tissue samples

Post-surgery frozen and paraffin-embedded HCC tissues for western blots and immunohistochemistry were obtained from the Chung Shan Medical University Hospital (Taichung, Taiwan). This study was approved by the Ethical Committees of Chung Shan Medical University Hospital, and all patients gave

informed consent to have their tissues before collection (CSMUH No:CS218075). Commercial human paired HCC tissue microarray with survival data (HLiv-HCC180Su) was purchased form Shanghai Outdo Biotech and Pantomics, Inc.

Immunohistochemistry

The array and the HCC patients' tissues were incubated with anti-CHPF antibody (1:200) in 5% bovine serum albumin/PBS and 0.1% Triton X-100 (Sigma) for 16 h at 4 ℃. UltraVision Quanto Detection System (Thermo Fisher Scientific Inc.) was used. The specific immunostaining was visualized with 3,3-diaminobenzidine and counterstained with hematoxylin(Sigma). Using microscopy, the distribution and positive intensity of CHPF were graded by two scorers blinded to the clinical parameters. The staining results were scored based on the percentage of positive tumor cells in the tumor tissue (0, <5%; 1, 5-20%; 2, 20-50%; 3, >50%), as well as staining intensity (0 and 1, for low expression; 2 and 3, for high expression).

Cell culture

Liver cancer cell lines, HA22T, PLC5, HepG2, HA59T, Heb3B, and Hepa1-6, were purchased from Bioresource Collection and Research Center in the year 2014 (Hsinchu, Taiwan). HCC36 cells were ungrudgingly furnished by Prof. Lei Wan (China Medical University). Cells above were cultured in DMEM containing 10% FBS in 5% CO_2 at 37 ℃.

Transfection and RNA interference

For overexpression experiments, Hepa1-6 cells and HA22T cells will be transfected with CHPF plasmids using Lipofectamine-3000 (Invitrogen), and empty pCMV6 plasmid was used as mock transfectant. Cells were selected with 400 μg/ml of G418 for 14 days. For knockdown of CHPF, ON-TARGETplus SMARTpool siRNA against CHPF and control siRNA were purchased from Dharmacon. RNAiMAX (Thermo Fisher Scientific Inc.) were used for transfection.

Cell invasion and migration assay

Transwell inserts for 24-well plate (Corning) with uncoated porous filters (pore size 8 mm) were used to evaluate cell migration, and Matrigel (BD Biosciences) coated porous filters were used to examine cell invasion. 2×10^4 Hepa1-6, HA22T cells, and 5×10^4 HA59T cells in 0.1 ml serum-free DMEM were seeded into inserts, and 0.6 ml DMEM containing 10% FBS was added in lower part of the well. Hepa1-6 cells, HA22T cells and HA59T cells were incubated for 24 h. Cells moving to the other side of the filters were stained by crystal violet and counted. Values were shown as the average number of cells per microscopic field over three fields of each filter. Independent experiments were repeated for three times.

Cell proliferation and colony formation

Cells (2×10^3) were seeded into 96-well plates with culture medium. Living cells were analyzed by CCK8 at 0, 24, 48, and 72 h. For anchorage dependent colony formation assay, 500 cells were seeded in 6-well plates and incubated for 20 days. Colonies were fixed in methanol and stained with 0.1% crystal violet (Sigma) for counting.

Immunofluorescence

Cells on cover slips and frozen sections of Hepa1-6 tumor tissue were stained with antibody against CS (CS56; GeneTex, Inc.), Ki67 (Abcam), and CHPF. Cell Navigator^{TM} F-actin Labeling Kit (1:1000, AAT Bioquest Inc.) was used to visualize F-actin. DAPI was used for nuclear staining. Images were captured by ZEISS Axio Imager A2 microscope.

Metastasis and tumor growth mice models

Male SCID mice, 5 weeks of age, were purchased from National Laboratory Animal Center (Tainan, Taiwan). For tumor growth analysis, 3×10^6 of Hepa1-6 mock transfectants and equal number of CHPF transfectants were subcutaneously and respectively inoculated into left and right flank (n = 5, both group). Tumor volumes were monitored for 15 days. Excised tumors were weighed and analyzed. For tumor metastasis model, Hepa1-6 stable transfectants were injected into tail veins of mice (1×10^6 cells/mouse, n = 3 for each group). Mice were sacrificed 5 weeks after inoculation. The lungs were excised and surface nodules were counted. Excised tissues were paraffin- embedded for H&E stain and IHC staining.

All animal experiments in this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Chung Shan Medical University Experimental Animal Center.

Statistical analysis

All data analysis was performed using GraphPad Prism 6. Student t-test was used for statistical analyses. Paired t-test was used for the analyses of paired Hepa1-6 tumors. Two-sided Fisher exact test was used for comparisons between CHPF expression and clinicopathologic features. Kaplan-Meier analysis and the log-rank test were used to estimate overall survival. $P < 0.05$ was considered statistically significant.

結果與討論

Part 1. CHSY1 enhances hedgehog signaling

Several growth factors have been proposed interacting with chondroitin sulfate, and among these growth factors, HGF (hepatocyte growth factor) and SHH (sonic hedgehog) induced pathways were crucial for EMT in HCC cells (Chen et al, 2011; Thelin et al, 2012; Whalen et al, 2013; Yamada & Sugahara, 2008). Thus, we investigated whether CHSY1 could affect these signaling pathways. In addition, a strong EMT inducer, TGF-β, was also been analyzed (Giannelli et al, 2016; van Zijl et al,

2009). Recombinant TGF-β, HGF, and SHH protein were treated to mock and CHSY1-overexressed HCC cells. Our results indicated that CHSY1 enhanced SHH-induced Ptch1 and Gli-2 expression (Figure 1). In contrast, CHSY1 expression did not significantly alter TGF-β and HGF induced signaling (Figure 2). These results suggest that CHSY1 selectively enhances the SHH signaling pathway.

To investigate the role of the SHH signaling pathway in CHSY1-enhanced malignant phenotypes, we treated HCC cells with vismodegib, a U.S. Food and Drug Administration approval drug for blocking SHH pathway (Sekulic et al, 2012). We found that 3 μM of vismodegib did not significantly suppress cell growth in CHSY1 overexpressed cells (data not shown). In contrast, results of trans-well assays showed that CHSY1- enhanced cell migration and invasion were significantly inhibited by the blockade of SHH pathway (Figure 3A and 3B). We next analyzed whether CHSY1 expression enhanced SHH binding to HCC cells. Confocal microscopy revealed that CHSY1 enhanced SHH binding on cell surface when cells were treated with His-tag SHH for 30 minutes (Figure 4). In addition, most of cell surface SHH were co-localized with chondroitin sulfate, revealed by CS56 staining. These results suggest that CHSY1 could activate SHH pathway by enhancing SHH binding on HCC cells. CS is important in both maintaining structural integrity and regulating cellular activities of tumor. In structurally, CS can be a physically barrier to limit oncolytic virus spread in glioma (Dmitrieva et al, 2011).

Discussion of part 1

In cellular regulatory, due to negative charges of CS chains, they are able to interact with various proteins in the extracellular matrix with different affinities, and form complexes with growth factors or shape activity of proteinase, such as MMP2, MMP7 (Mizumoto et al, 2015; Theocharis et al, 2014; Theocharis et al, 2010). In this study, we found that CHSY1 regulated CS expression on surface of HCC cells, and CHSY1-mediated CS formation enhanced SHH binding to cell surface as well as activated downstream pathway. Several studies proposed that CS and heparin sulfate are necessary for proper hedgehog signaling in development, and the structure SHH-CS complex has been determined by crystal packing analysis (Cortes et al, 2009; Whalen et al, 2013; Yan & Lin, 2009). Our data revealed that inhibiting hedgehog pathway with vismodegib significantly suppressed CHSY1-induced migration and invasion in HCC cells. Although we cannot exclude other growth factors or proteinase may also involve in our experimental conditions, it is possible that CHSY1-mediated tumor malignancy is mainly through activating SHH pathway.

Part 2. Knockdown of CHSY1 promotes drug efficacy in HCC cells.

It is well known that CHSY1 modulating malignant cell behaviors and the molecular mechanism might be relevant to SHH signaling pathway. Although, how CHSY1 modulate cell proliferation and invasion by regulating HH pathway is unknown, CHSY1 still offers a potential therapeutic target for HCC. We used western blot to examining drug sensitivity in PLC5. The data indicated that Doxorubicin (Dox)

and Gant61 treatments increased caspase 3 cleavage in CHSY1-knockdown cells. (Figure 5). This result suggested that knockdown of CHSY1 could enhance Dox and Gant61 induced cell apoptosis. In addition, we used CCK8 to analyze cell viability, and found that CHSY1-knockdown cells were more sensitive to Dox treatment (Figure 6) Initial results indicated that CHSY1 could enhance Dox and GANT61 sensitivity in PLC5 cells. The extensive studies in other HCC cell lines is necessary in our future works. In addition, the change of key SHH signaling pathway molecule Gli1 and Gli2 should be further examine. Therefore, it is worth to get an insight into the connection between CHSY1 and the downstream signaling molecules of SHH pathway.

Discussion of Part 2

Recent studies have shown that aberrant hedgehog signaling has been implicated in several human malignancies including HCC (Huang et al, 2006; Sicklick et al, 2006). Hedgehog signaling activity correlates with poorly histologic differentiation, aggressive invasion, and chemoresistance in HCC (Cheng et al, 2009b; Marijon et al, 2011; Omenetti et al, 2011). Our data showed that CHSY1 can increase SHH binding on cell and promote Ptch1 and Gli2 expression. Targeting hedgehog signaling is considered to be an attractive strategy for treating many human cancers, including HCC (Jiang & Hui, 2008; Wang et al, 2013). Therefore, a complete understanding of the mechanisms by which the structure and function of hedgehog signaling are regulated is crucial to improve the effect of target therapies in human cancers. This study provides novel insights into the role of aberrant CS in modulating hedgehog pathway, but not HGF- or TGFβ-mediated signaling, suggesting the selectivity of CHSY1-mediated CS structure toward certain growth Figure 2 factors. Because hedgehog signaling activity has been supposed to regulate chemoresistance and autophagy in HCC cells, it will be important to further investigate whether changes in cellular CS expression can affect HCC cell sensitivity toward other therapeutic drugs or chemotherapy drugs (Chen et al, 2011; Wang et al, 2013). Additionally, we found that using SHH inhibitor suppressed around 50% of CHSY1-induced migration and invasion, and did not have significant effects on cell growth. These results suggested that including hedgehog signaling, other unknown mechanisms may also involve in CHSY1-mediated malignant phenotypes. Thus, targeting CHSY1 or degrading CS in tumor microenvironment could have effects similar to those drugs targeting multiple kinases in cancer cells.

Part 3. CHPF associates with survival of hepatocellular carcinoma patients and regulates malignant phenotypes of cancer cells

Our recent studies revealed that these CHSYs and CS-modifying enzymes have tissue-specific expression profiles and play distinct roles in regulating the malignant behavior of different types of cancer cells. We have recently identified that CHSY1 is up-regulated in hepatocellular carcinoma (HCC) and glioma tissues, and its up-regulation is associated with negative patient outcomes (*39, 40*). The C5 epimerase DSE is also frequently up-regulated in human gliomas, with its increased expression being associated with a worse prognosis (*41*). By contrast, DSE is often down-regulated in HCC tissues

(*42*).

In the part 3, we focus on the CHPF that build the elemental structure of CS. We evaluate the correlation between clinicopathological features and expression of CHPF in HCC patients, and explored their possible molecular mechanisms in cancer progression.

Part3 結果暫不公開

Figure legends

Figure 1. Effects of CHSY1 on SHH-induced signaling. Hepa1-6 stable transfectants were treated with SHH (0.4 μg/ml) or PBS (solvent) for 48 hours. The mRNA levels of Ptch1, Gli1, and Gli2 were analyzed by real-time RT-PCR. Expression fold change (2^ΔΔCt) to Mock-PBS groups was shown. Experiment was done in triplicate and mean ± SD was shown.

Figure 2. Time course of TGF-β-induced signaling (A)and HGF-induced signaling (B) in mock and CHSY1-overexpressed cells. Hepa1-6 cells were starved for 3 hours and then treated with TGF-β (10 ng/mL) or HGF (25 ng/mL) for 5, 30, and 60 minutes. Cell lysates were analyzed by Western blotting with various antibodies, as indicated.

Figure 3. (A) Effects of SHH pathway inhibitor, vismodegib, on CHSY1-enhanced cell migration and (B) invasion. Cells were treated with vismodegib (3 μM) or DMSO (solvent control) during the migration and invasion assays. Data were represented as means ± SD from three independent experiments. *, $P < 0.05$; **, $P< 0.01$; ***, $P< 0.001$.

Figure 4. Confocal microscopy analysis of recombinant His-SHH (red) and chondroitin sulfate (CS56, green). Recombinant SHH (0.4 μg/ml) was added to cells for 30 minutes. Increased SHH binding (indicated by white arrows) on CHSY1 overexpressed HCC cells was observed. Scale bar, 25 μm.

Figure 5. Knockdown of CHSY1 increased drug sensitivity Examine cell cleaved-caspase 3 (c-Caspase 3) between CHSY1-siRNA and CHSY1 Control-siRNA transfected PLC5 cells by western blots. Doxorubicin (1.25μg/ml) and Gant61 (2.5μg/ml) was added for 24 hours. Actin was used as internal control.

Figure 6. Knockdown of CHSY1 promotes sensitivity of Doxorubicin in PLC5 cells. CCK8 cell viability assay was used to measure toxicity of Doxorubicin (Dox) for 24 hour incubation.

Figure 7. Western blots of CHPF in paired HCC patients.

Figure 8. Immunohistochemistry of CHPF in HCC tissue sections.

Figure 9. A. Representative images of CHPF staining intensity in HCC tissue. B. A Kaplan-Meier survival analysis of HCC patients according CHPF staining.

Figure 10. A. Immunohistochemistry of CHPF in commercial HCC tissue array. B. Survival analysis of tissue array patients according CHPF intensity.

Tables

Table 1. Correlation of CHPF expression with clinicopathological features of HCC cases.

Factor		CHPF expression		*P* value (Two-sided Fisher's exact test)
		Low	High	
Tissue types	Non-tumor	15	34	0.0275*
	Tumor	40	38	
Sex	Male	31	29	1.00
	Female	9	9	
Age	< 55 years	10	7	0.5869
	≥ 55 years	30	31	
Tumor stage	T1 + T2	20	29	0.0202*
	T3 + T4	20	9	
Metastasis	No	1	0	1.00
	Yes	39	38	

*$P < 0.05$ was considered as statistically significant.

Table 2. Correlation of CHPF expression with clinicopathological features of HCC tissue array.

Factor		CHPF expression		*P* value (Two-sided Fisher's exact test)
		Low	High	
Tissue types	Non-tumor	22	66	0.0045*
	Tumor	37	52	
Sex	Male	29	45	1.00
	Female	6	9	
Age	< 55 years	20	24	0.2820
	≥ 55 years	15	30	
Tumor stage	T1 + T2	17	34	0.2433
	T3 + T4	15	16	
Metastasis	No	35	54	1.00
	Yes	0	0	

*$P < 0.05$ was considered as statistically significant.

Figures

Figure 1

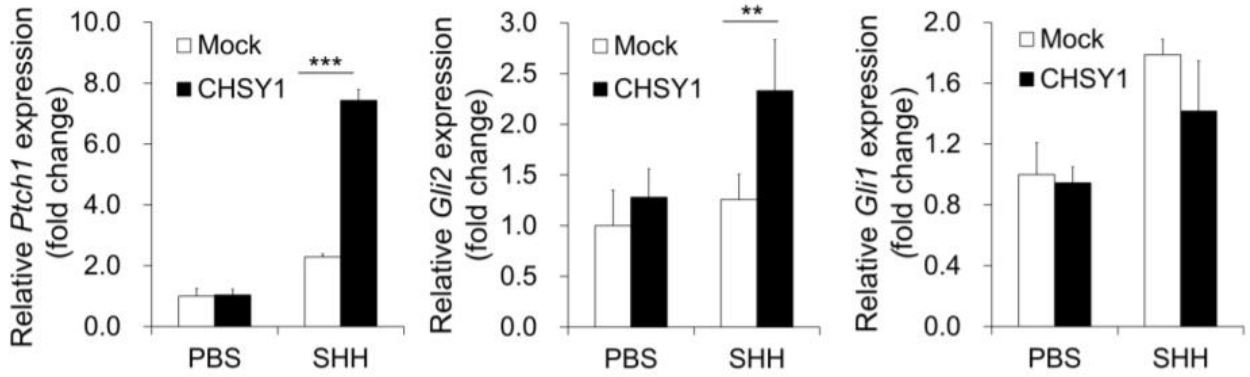
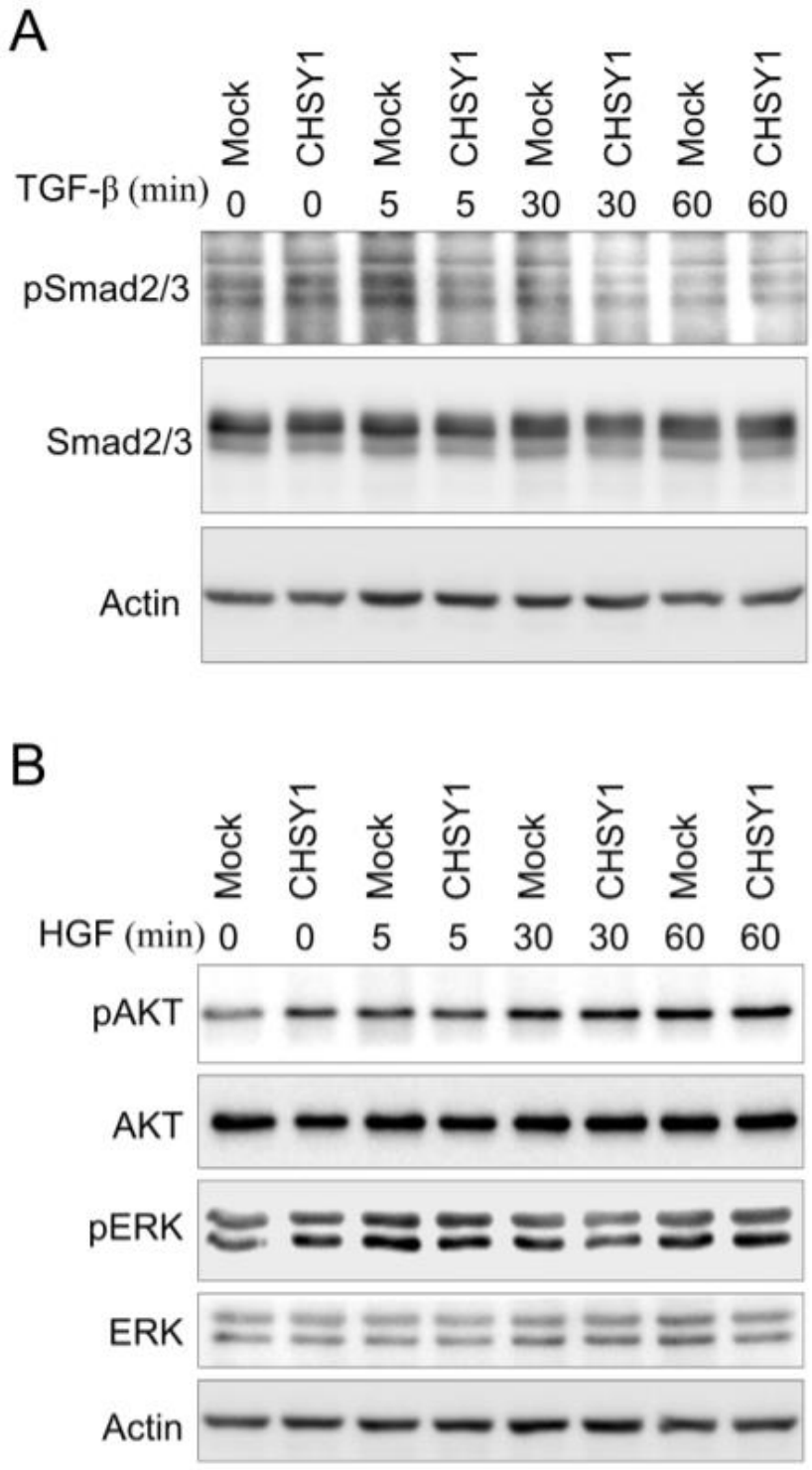


Figure 2


A
Mock
CHSY1
Mock
CHSY1
Mock
CHSY1
Mock
CHSY1
TGF-β (min)
0
0
5
5
30
30
60
60
pSmad2/3
Smad2/3
Actin
B
Mock
CHSY1
Mock
CHSY1
Mock
CHSY1
Mock
CHSY1
HGF (min)
0
0
5
5
30
30
60
60
pAKT
AKT
pERK
ERK
Actin

.

Figure 3

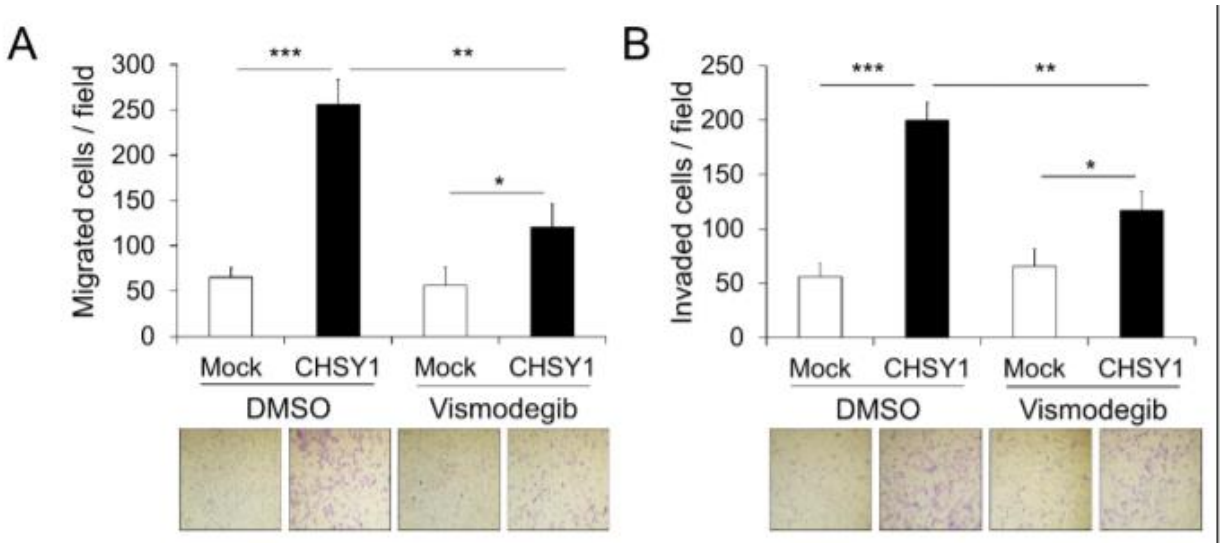


Figure 4

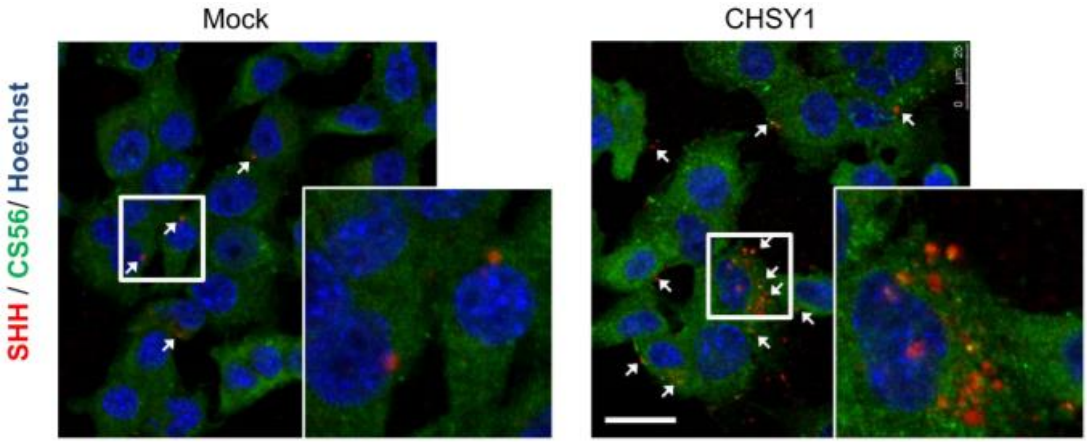


Figure 5

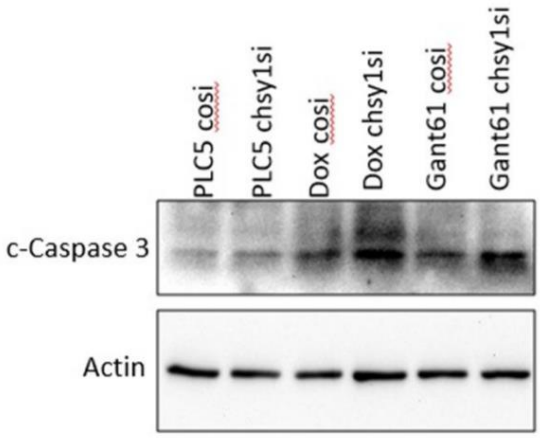


Figure 6

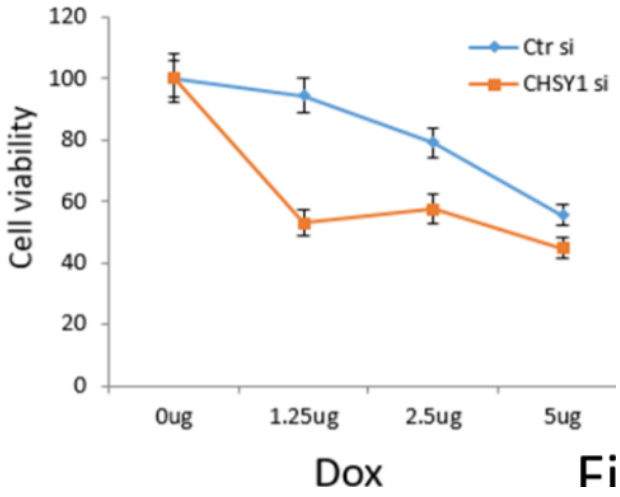


Figure 7

Figure 8

Figure 9

Figure 10

Reference:

1. H. P. A. M. O. H. A. W. TAIWAN, CANCER REGISTRY ANNUAL REPORT, 2013 TAIWAN. *HEALTH PROMOTION ADMINISTRATION MINISTRY OF HEALTH AND WELFARE TAIWAN*, (2016).
2. L. Laursen, A preventable cancer. *Nature* **516**, S2 (Dec 4, 2014).
3. A. Forner, J. M. Llovet, J. Bruix, Hepatocellular carcinoma. *Lancet* **379**, 1245 (Mar 31, 2012).
4. A. Villanueva, V. Hernandez-Gea, J. M. Llovet, Medical therapies for hepatocellular carcinoma: a critical view of the evidence. *Nat Rev Gastroenterol Hepatol* **10**, 34 (Jan, 2013).
5. M. A. Worns, P. R. Galle, HCC therapies-lessons learned. *Nat Rev Gastro Hepat* **11**, 447 (Jul, 2014).
6. A. L. Cheng *et al.*, Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled

trial. *The Lancet. Oncology* **10**, 25 (Jan, 2009).

7. J. M. Llovet *et al.*, Sorafenib in advanced hepatocellular carcinoma. *The New England journal of medicine* **359**, 378 (Jul 24, 2008).
8. M. P. Brizzi, D. Pignataro, M. Tampellini, G. V. Scagliotti, M. Di Maio, Systemic treatment of hepatocellular carcinoma: why so many failures in the development of new drugs? *Expert review of anticancer therapy*, (Aug 22, 2016).
9. A. L. Cheng *et al.*, Sunitinib versus sorafenib in advanced hepatocellular cancer: results of a randomized phase III trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **31**, 4067 (Nov 10, 2013).
10. P. J. Johnson *et al.*, Brivanib versus sorafenib as first-line therapy in patients with unresectable, advanced hepatocellular carcinoma: results from the randomized phase III BRISK-FL study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **31**, 3517 (Oct 1, 2013).
11. A. X. Zhu *et al.*, Ramucirumab versus placebo as second-line treatment in patients with advanced hepatocellular carcinoma following first-line therapy with sorafenib (REACH): a randomised, double-blind, multicentre, phase 3 trial. *The Lancet. Oncology* **16**, 859 (Jul, 2015).
12. J. Bruix *et al.*, Regorafenib as second-line therapy for intermediate or advanced hepatocellular carcinoma: multicentre, open-label, phase II safety study. *European journal of cancer* **49**, 3412 (Nov, 2013).
13. A. X. Zhu, Systemic therapy of advanced hepatocellular carcinoma: how hopeful should we be? *The oncologist* **11**, 790 (Jul-Aug, 2006).
14. J. M. Llovet, V. Hernandez-Gea, Hepatocellular carcinoma: reasons for phase III failure and novel perspectives on trial design. *Clin Cancer Res* **20**, 2072 (Apr 15, 2014).
15. M. B. Meads, R. A. Gatenby, W. S. Dalton, Environment-mediated drug resistance: a major contributor to minimal residual disease. *Nature reviews. Cancer* **9**, 665 (Sep, 2009).
16. D. Hanahan, R. A. Weinberg, Hallmarks of cancer: the next generation. *Cell* **144**, 646 (Mar 4, 2011).
17. J. D. Esko, K. Kimata, U. Lindahl, in *Essentials of Glycobiology,* A. Varki *et al.*, Eds. (Cold Spring Harbor Laboratory Press

The Consortium of Glycobiology Editors, La Jolla, California, Cold Spring Harbor (NY), 2009).

18. N. Afratis *et al.*, Glycosaminoglycans: key players in cancer cell biology and treatment. *Febs J* **279**, 1177 (Apr, 2012).
19. R. Q. Zhang *et al.*, Biomarker-based prognostic stratification of young adult glioblastoma. *Oncotarget* **7**, 5030 (Jan 26, 2016).
20. A. D. Theocharis *et al.*, Insights into the key roles of proteoglycans in breast cancer biology and translational medicine. *Biochimica et biophysica acta* **1855**, 276 (Apr, 2015).
21. A. Salanti *et al.*, Targeting Human Cancer by a Glycosaminoglycan Binding Malaria Protein. *Cancer cell* **28**, 500 (Oct 12, 2015).

22. K. Sugahara *et al.*, Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate. *Curr Opin Struct Biol* **13**, 612 (Oct, 2003).
23. K. Akita *et al.*, Expression of multiple chondroitin/dermatan sulfotransferases in the neurogenic regions of the embryonic and adult central nervous system implies that complex chondroitin sulfates have a role in neural stem cell maintenance. *Stem cells* **26**, 798 (Mar, 2008).
24. S. Yamada, K. Sugahara, Potential therapeutic application of chondroitin sulfate/dermatan sulfate. *Curr Drug Discov Technol* **5**, 289 (Dec, 2008).
25. E. M. Denholm, Y. Q. Lin, P. J. Silver, Anti-tumor activities of chondroitinase AC and chondroitinase B: inhibition of angiogenesis, proliferation and invasion. *European journal of pharmacology* **416**, 213 (Mar 30, 2001).
26. J. Iida *et al.*, Cell surface chondroitin sulfate glycosaminoglycan in melanoma: role in the activation of pro-MMP-2 (pro-gelatinase A). *The Biochemical journal* **403**, 553 (May 1, 2007).
27. F. Li *et al.*, Involvement of highly sulfated chondroitin sulfate in the metastasis of the Lewis lung carcinoma cells. *The Journal of biological chemistry* **283**, 34294 (Dec 5, 2008).
28. Basappa *et al.*, Involvement of chondroitin sulfate E in the liver tumor focal formation of murine osteosarcoma cells. *Glycobiology* **19**, 735 (Jul, 2009).
29. G. B. ten Dam *et al.*, Antibody GD3G7 selected against embryonic glycosaminoglycans defines chondroitin sulfate-E domains highly up-regulated in ovarian cancer and involved in vascular endothelial growth factor binding. *The American journal of pathology* **171**, 1324 (Oct, 2007).
30. C. A. Cooney *et al.*, Chondroitin sulfates play a major role in breast cancer metastasis: a role for CSPG4 and CHST11 gene expression in forming surface P-selectin ligands in aggressive breast cancer cells. *Breast cancer research : BCR* **13**, R58 (Jun 09, 2011).
31. K. Sugahara, T. Mikami, Chondroitin/dermatan sulfate in the central nervous system. *Current opinion in structural biology* **17**, 536 (Oct, 2007).
32. E. J. Bradbury *et al.*, Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* **416**, 636 (Apr 11, 2002).
33. H. Wilke *et al.*, Ramucirumab plus paclitaxel versus placebo plus paclitaxel in patients with previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (RAINBOW): a double-blind, randomised phase 3 trial. *The Lancet. Oncology* **15**, 1224 (Oct, 2014).
34. S. Mizumoto, S. Yamada, K. Sugahara, Molecular interactions between chondroitin-dermatan sulfate and growth factors/receptors/matrix proteins. *Current opinion in structural biology* **34**, 35 (Oct, 2015).
35. A. D. Theocharis, S. S. Skandalis, G. N. Tzanakakis, N. K. Karamanos, Proteoglycans in health and disease: novel roles for proteoglycans in malignancy and their pharmacological targeting. *Febs J* **277**, 3904 (Oct, 2010).
36. A. D. Theocharis *et al.*, Cell-matrix interactions: focus on proteoglycan-proteinase interplay

and pharmacological targeting in cancer. *Febs J* **281**, 5023 (Nov, 2014).

37. X. L. Jia *et al.*, Increased expression of chondroitin sulphate proteoglycans in rat hepatocellular carcinoma tissues. *World J Gastroenterol* **18**, 3962 (Aug 14, 2012).
38. H. Lv *et al.*, Elevate level of glycosaminoglycans and altered sulfation pattern of chondroitin sulfate are associated with differentiation status and histological type of human primary hepatic carcinoma. *Oncology* **72**, 347 (2007).
39. C. H. Liu, C. T. Lan, J. F. Chou, T. J. Tseng, W. C. Liao, CHSY1 promotes aggressive phenotypes of hepatocellular carcinoma cells via activation of the hedgehog signaling pathway. *Cancer letters* **403**, 280 (Sep 10, 2017).
40. W. C. Liao *et al.*, Chondroitin sulfate synthase 1 enhances proliferation of glioblastoma by modulating PDGFRA stability. *Oncogenesis* **9**, 9 (Feb 4, 2020).
41. W. C. Liao *et al.*, DSE promotes aggressive glioma cell phenotypes by enhancing HB-EGF/ErbB signaling. *PloS one* **13**, e0198364 (2018).
42. W. C. Liao *et al.*, DSE regulates the malignant characters of hepatocellular carcinoma cells by modulating CCL5/CCR1 axis. *American journal of cancer research* **9**, 347 (2019).

106年度專題研究計畫成果彙整表

計畫主持人：劉炯輝	計畫編號：106-2320-B-040-009-MY3
計畫名稱：研究硫酸軟骨素生成酵素在肝細胞癌的惡化與抗藥性之功能	

成果項目			量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文	0	篇	
		研討會論文	3		第34屆生物醫學聯合學術年會 108/03/23~台灣地區(原國內)-國防醫學院 108/03/24 Nerve Decompression Improves Spinal Synaptic Plasticity of Toll-like Receptor Subtype 5 for Pain Relief 第33屆生物醫學聯合學術年會台灣地區(原國內)-國防醫學院107/03/24~107/03/25 Function of chondroitin sulfate synthase in hepatocellular carcinoma 第三十四屆生物醫學聯合學術年會台灣地區(原國內)-國防醫學院108/03/23~108/03/24 Artocarpin Suppresses TGF-β1-Induced Epithelial-to-Mesenchymal Transition, Migration and Invasion of A549 Lung Cancer Cells
		專書	0	本	
		專書論文	0	章	
		技術報告	0	篇	
		其他	0	篇	
國外	學術性論文	期刊論文	6	篇	Chiung-Hui Liu, Hung-Ming Chang, Yin-Shuo Yang, Yu-Ta Lin, Ying-Jui Ho, To-Jung Tseng, Chyn-Tair Lan , Wen-Chieh Liao (2020, Mar). Melatonin promotes nerve regeneration following end-to-side neurorrhaphy by accelerating cytoskeletal remodeling via the melatonin receptor-dependent pathway. Neurosciences, Available online 2 November 2019. MOST 106-2320-B-040-009-MY3. 本人為第一作者.

					2. Wen-Chieh Liao, Chih-Kai Liao, To-Jung Tseng, Ying Jui Ho, Ying-Ru Chen, - Kuan-Hung Lin, Te Jen Lai, Chyn-Tair Lan, and Kuo-Chen Wei, Chiung-Hui - Liu* (2020, Feb). Chondroitin sulfate synthase 1 enhances proliferation of glioblastoma by modulating PDGFRA stability. Oncogenesis, 9(9) page 1-11. MOST 106-2320-B-040-009-MY3. 本人為通訊作者. 3. Chiung-Hui Liu, Chyn-Tair Lan, Li-You Chen, Wen-Chieh Liao, Miau-Hwa Ko, To-Jung Tseng (2019, Mar). Phosphorylation of extracellular signal-regulated kinase 1/2 in subepidermal nerve fibers mediates hyperalgesia following diabetic peripheral neuropathy. Neurotoxicology, Volume 71, Pages 60-74. 本人為第一作 者. 4. Wen-Chieh Liao, Hung-Rong Yen, Chih-Kai Liao, To-Jung Tseng, Chyn-Tair Lan, Chiung-Hui Liu* (2019, Jan). DSE regulates the malignant characters of hepatocellular carcinoma cells by modulating CCL5/CCR1 axis. American Journal of Cancer Research, 2019; 9(2): 347–362. MOST 106-2320-B-040-009- MY3. 本人為通訊作者. 5. Wen-Chieh Liao, Chih-Kai Liao, You-Huan Tsai, To-Jung Tseng, Li-Ching Chuang, Chyn-Tair Lan, Hung-Ming Chang, Chiung-Hui Liu* (2018, Jun). DSE promotes aggressive glioma cell phenotypes by enhancing HB-EGF/ErbB signaling. Plos One, 3(6): e0198364. MOST 106-2320-B-040-009-MY3. 本人為 通訊作者. 6. Chiung-Hui Liu, Chyn-Tair Lan, Jui-Feng Chou, To-Jung Tseng*, Wen-Chieh

					Liao* (2017, Sep). CHSY1 promotes aggressive phenotypes of hepatocellular carcinoma cells via activation of the hedgehog signaling pathway. Cancer Letters, Volume 403, Pages 280-288. MOST 105-2320-B-040-033-MY2. 本人為第一作
		研討會論文	2		第十一屆歐洲神經醫學聯會其他地區(原國外)-德國柏林 107/07/07~107/07/11 Therapeutic potential and signaling mechanisms of melatonin-mediated cytoskeleton remodeling in nerve sprouting end-to side neurorrhaphy 第十一屆歐洲神經醫學聯會其他地區(原國外)-德國柏林107/07/07~107/07/11 DSE promotes aggressive phenotypes of glioma cells by enhancing HB-EGF / ErbB signaling
		專書	0	本	
		專書論文	0	章	
		技術報告	0	篇	
		其他	0	篇	
參與計畫人力	本國籍	大專生	1	人次	李承翰
		碩士生	0		
		博士生	0		
		博士級研究人員	0		
		專任人員	3		莊立勤, 洪岳慈, 朱殷弘
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士級研究人員	0		
		專任人員	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）					