科技部補助

大專學生研究計畫研究成果報告

計畫名稱：	Investigation of CHPF-modified decorin in hepatocellular carcinoma

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執行計畫學生： 許維成
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指 導 教 授 ： 劉烱輝

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1. Introduction

Liver cancers rank sixth in incidence cases of cancers and third in common causes of cancer-related death worldwide. Hepatocellular carcinoma (HCC) composes approximately 75 to 85% of cases of liver cancers [1]. Chronic inflammation inducted by liver fibrosis or cirrhosis are related to 90% of HCC development [2]. Viral hepatitis, metabolic disorders, and alcoholic or non-alcoholic fatty liver disease which are usually accompanying with abnormal changes in the extracellular matrix (ECM) also facilitate hepatocarcinogenesis [3, 4]. Therefore, a full understanding of the molecular mechanisms underlying HCC progression is crucial for developing new treatments of this fatal disease.

Glycosaminoglycans (GAGs) are unbranched polysaccharides consisting of repeated disaccharide units. Some of GAGs bind to the certain core protein to form proteoglycans (PGs), while some of which occur as free chains. GAGs or PGs surround to cell surfaces can interact with the cytokine, chemokine in tumor microenvironment (TME), and in turn affects tumor progression or supporting tumor-suppression immune response [5]. Chondroitin sulfate (CS) is one of the major types of GAGs, and CS can bind to a core protein to form chondroitin sulfate proteoglycan (CSPG), which has complex function in interacting with diverse proteins in the ECM and TME [6, 7]. Decorin (DCN), a kind of CSPG in the ECM with chondroitin sulfate or dermatan sulfate attached, is down-regulated in tumor tissue compared to the non-tumorous adjacent tissue in primary HCC of human patients. In C57BL/6 mice, DCN gene delivery diminishes liver carcinogenesis [8]. In colon cancer and breast cancer, DCN act as a tumor suppressor via inhibiting epithelial-mesenchymal transition (EMT) [9, 10], which is of great importance in early steps of metastasis due to losing of cell-cell contacts, and thus cells acquire motility to spread in carcinoma [11]. DCN is also found to be interacting with transforming growth factor-β (TGF-β) and ErbB receptor tyrosine kinases (RTKs), and they both are involved in cancer progression [12].

The biosynthesis of CS chains on proteoglycans requires a group of bifunctional enzymes (CHSY1, CHPF, CHPF2, CHSY3) [13]. The enzymes involved in the polymerization process might determine the length of CS chains and sulfated position on disaccharide units [14].

According to our preliminary data, low expression level of CHPF is correlated with a better prognosis in HCC patients. We also found that CHPF suppressed the aggressive phenotypes both in vitro and in vivo. Based on our hypothesis that CHPF-modified decorin might affect downstream signals and in turn altar the behaviors of HCC, we managed to purify DCN in different sizes from different cells. Furthermore, we also purchased paired tumor/peri-tumor tissue of 60 HCC patients from CSMU hospital to analyze the correlation between decorin expression, molecular weight and pathophysiological characteristics (The study of HCC patients 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)).

2. Materials and Methods

Reagents and antibodies

Decorin Human cDNA with N-His-tag and Decorin Human cDNA with C-His-tag were purchased from SinoBiological (catalog #HG10189-NH and # HG10189-CH). Transfection was performed using Toolstrong (in HEK293) or Lipofectamine 3000 (in HA59T). Goat polyclonal anti-decorin (R&D Systems, catalog

#AF143) was used to examine expression of Decorin; Mouse monoclonal anti-His-tag (Proteintech, catalog #66005-1-Ig) was used to detect His-tag-binding decorin.

Cell culture

Liver cancer cell lines, HA22T, HA59T, Hep3B, HCC36, PLC5, HepG2, Hep1-6 were cultured in DMEM containing 4.0 mM L-glutamine and 10% FBS in 5% CO_2 at 37℃. In experiments that require conditioned medium, cells were cultured in serum-free medium in 5% CO_2 at 37℃. Conditioned medium was collected after 24h of incubation.

Western blotting

Total protein lysates (10-30 μg) were separated by 8% SDS-PAGE and transferred to PVDF membrane. Total protein was measured by stain-free technology (Bio-Rad, Hercu- les, CA, USA).30 ug total protein will be used for each sample. Antibodies against decorin and His-tag were used.

Human tissue samples

Post-surgery frozen paired tumor/peri-tumor tissue of HCC patients 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: CS2-21116).

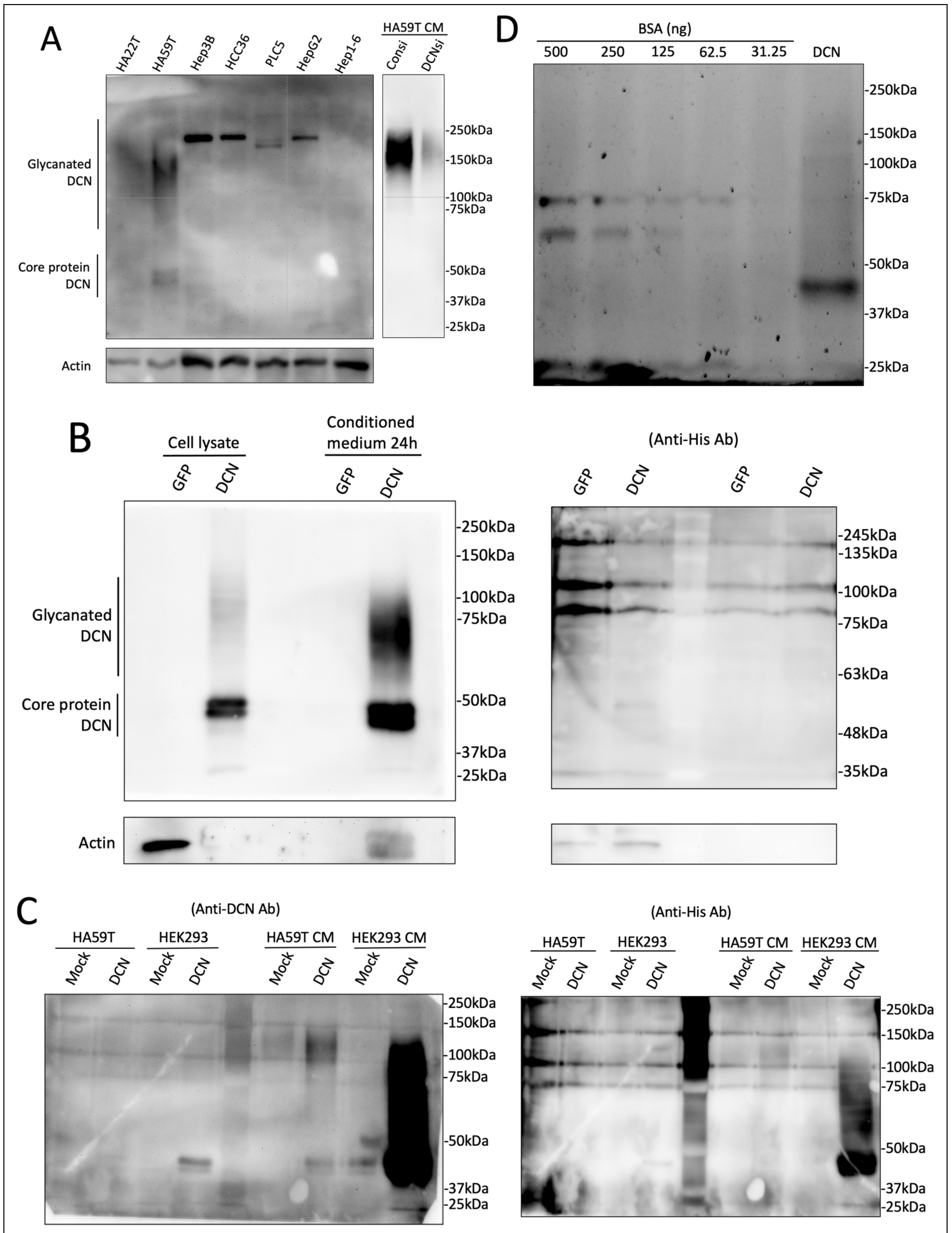
Decorin purification

Decorin in concentrated conditioned medium were purified via nickel affinity chromatography. Nuvia™ IMAC Resin and Chromatography Column were purchased from Bio-Rad (catalog # 7800800 and #7311550).

3. Results

In order to find a proper producer of decorin, we use western blot to analyze decorin expression in 7 HCC cell lines. High level of decorin was detected in both cell lysate and condition medium of HA59T, and the molecular weight of glycanated decorin was about 150kDa (Fig. 1A). To purified decorin protein from culture cells or medium, the his-fusion deccorin expressing plasmid was used. We transiently transfected DCN cDNA with His-tag on the N-terminal into HEK293 cells. Transfection efficiency was confirmed by western blot (Fig. 1B left), however, His-tag was not detected by anti-His immunoblotting (Fig. 1B right). On the other hand, we transfected DCN cDNA with His-tag on the C-terminal into HA59T and HEK293 cell. Expression pattern of His-tag resembled that of decorin in conditioned media suggesting that His-tags might be attached to secretory decorin (Fig. C). We then performed nickel affinity chromatography to purify the conditioned medium of decorin-overexpressed HEK293 cell (Fig. 1D). However, the purified decorin from HEK293 was mainly core protein, and the purification of decorin form HA59T cells have not success yet.

Figure 1. Decorin purification.



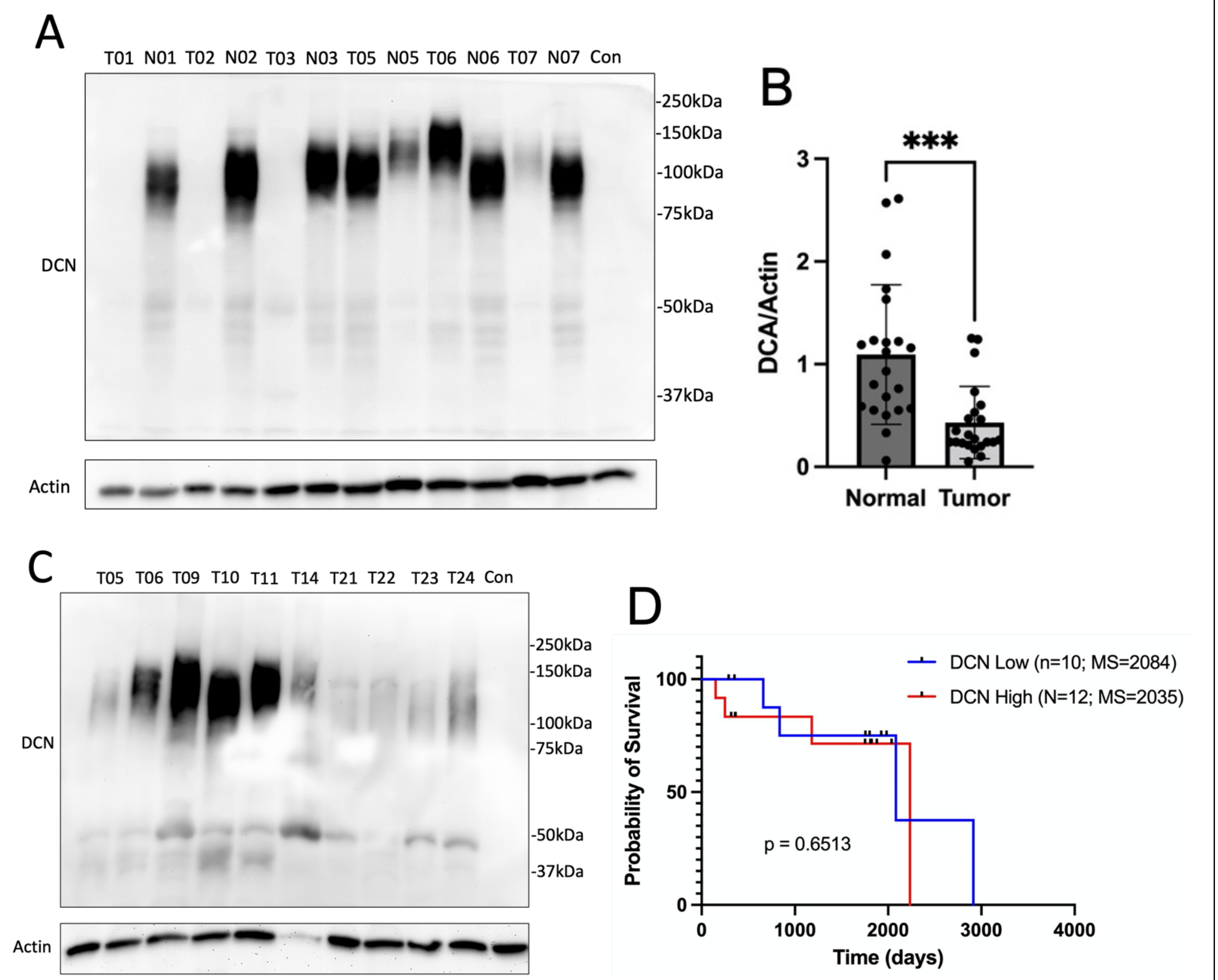
(A) Expression level of decorin in cell lysates of 7 HCC cell lines and the conditioned medium of siRNA-interfered HA59T were collected after the treatment of serum-free medium for 24 hours.

(B) Decorin expression of HEK293 cell transfected with N-his decorin cDNA were detected by anti-DCN (left) and anti-His (right) antibodies.

(C) Decorin expression of HA59T and HEK293 cell transfected with C-his decorin cDNA were detected by anti-DCN (left) and anti-His (right) antibodies.
(D) Purified conditioned medium of DCN-overexpressed HEK293. BSA was used for quantification. Total protein was measured.

For the purpose of understanding the correlation between decorin expression and clinicopathological features, we acquired paired tumor tissue/peri-tumor normal liver tissue of patients from CSMU hospital. We conducted western blot to detect decorin expression level (Fig. 2A). Compared to peri-tumor normal tissues, decorin level was consistently down-regulated in tumor tissues (Fig. 2B). We then discovered that decorin level was also varied among tumor tissues of HCC cases (Fig. 2C). In decorin high expression cases and low expression cases, survival rate showed no statistical significance (Fig. 2D).

Figure 2. DCN expression and in HCC patients



(A) Expression level of decorin in paired tumor (T) and peri-tumor normal (N) tissues of 22 HCC patients. 20μg of HA22T protein lysate was used as a loading control. Representative image was shown.
(B) DCN expression was quantified and analyzed with paired T test. $P^{***} = 0.0002$.
(C) Expression level of decorin in tumor (T) tissues of 22 HCC patients. 20μg of HA22T protein lysate was used as a loading control. Representative image was shown.
(D) Kaplan–Meier analysis of overall survival of HCC patients. The analyses were conducted according to

the western blotting (Fig. 2C) of DCN low expression (T21, T22, T23 etc.) and high expression (T06, T09, T10, T11etc.); $p = 0.6513$ (NS). MS, median survival.

Table 1. DCN expression and clinicopathological features in HCC cases

Factor	Feature	DCN expression		P value (Two-sided Fisher's exact test)
		Low	High	
Sex	Male	6	8	>0.9999
	Female	4	4	
Age	< 60 years	7	3	0.0836
	≥ 60 years	3	9	
Grade	Grade 2	2	8	0.0427*
	Grade 3	8	4	
Tumor stage	T1 + T2	2	2	>0.9999
	T3 + T3	2	2	
Lymphovascular invasion	No	5	6	>0.9999
	Yes	5	6	
Liver capsular invasion	No	6	9	0.6517
	Yes	4	3	
HBV	No	6	9	0.6517
	Yes	4	3	
HCV	No	6	3	0.1920
	Yes	4	9	

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

4. Discussion

The functions of decorin were described in several studies. Decorin is often expressed by fibroblasts and myofibroblasts which are crucial contributors of the extracellular matrix. In different types of tissues and organs, decorin was considered to be necessary for collagen formation, stability and organization [15, 16]. On

the other hand, decorin also interact with various receptors, growth factors and ECM components and in turn affects biologic and physiologic processes, including cell proliferation and differentiation. Changes in localization and expression level can also be observed in liver cancers [17].

In our study, decorin in different sizes were detected in HA59T (larger) and HEK293 (smaller) cells. We purified decorin secreted by HEK293 cell (Fig. 1D) for further in vitro experiments. Owing to the difficulty we've been facing in HA59T stable transfection, we'll perform co-transfection of CHPF and C-His decorin into HEK293 according to our previous finding of DCN modifying CS chain on decorin [18]. As long as we get the purified decorin in both sizes, we'll culture HCC cells in media with additional decorin in different sizes. Phenotypic analysis, including CCK-8 proliferation assay, colony formation and cell migraton/invasion assay, will be conducted, so that we get to examine the effect of the diversity of decorin glycosylation on HCC cells. Also, the experiments and analysis of HCC tumor tissues are not complete. Only 22 out of 60 pairs of tissues have been homogenized and analyzed with western blot. In spite of the fact that decorin level in tumor tissue was significantly lower than that in peri-tumor normal liver tissues, correlation between decorin expression and most of clinicopathological features did not reach statistical significance (Table 1). More comprehensive experiments should be done before we jump to conclusion. The rest of 38 HCC tissues will be included in further analysis.

In addition to decorin level, we noticed that the molecular weight of decorin in tumor tissue varied from case to case. It was reported that changes in proteoglycan glycosylation affected TGF-β and osteoblast differentiation in mouse [19]. Decglycanation of decorin led to failing collagen assembly in adipose tissue and skeletal muscle [20]. These studies revealed that the GAG on decorin was crucial for the functions of decorin. Therefore, we will devide HCC cases according to decorin molecular weight detected by western blot. The correlation between glycosylation of decorin and clinicopathological features will be our focal point. In conclusion, our study can broaden the understanding of decorin glycosylation and therapeutic target of HCC.

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