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Loss of neuraminidase 1 inhibits the activation of hepatic stellate cells through TGF-β/Smad3 signaling

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ABSTRACT

Objective(s): Liver fibrosis is an abnormal wound-healing response. Neuraminidase 1 (NEU1) is a sialidase that has been reported to be involved in the development of cancers and metabolic diseases. However, the role of NEU1 in liver fibrosis remains unreported. This study explored the potential role of NEU1 in liver fibrosis.

Materials and Methods: Liver fibrosis was induced in C57BL/6J mice by using carbon tetrachloride (CCl_4) and thioacetamide (TAA). The CCl_4 group was established by intraperitoneal injection of CCl_4 (1.0 µl/g body weight, 1:4 dilution in olive oil) twice weekly for six weeks. In the TAA group, mice were provided drinking TAA water at 300 mg/l for 12 weeks. The expression of NEU1, Collagen-1, α-SMA and TIMP1 was detected by western blotting. The expression of NEU1 was measured by immunohistochemistry. Bioinformatics analysis was performed to explore the correlation between NEU1 and liver fibrosis in the GSE84044 dataset. Western blot analyses were performed to investigate the molecular mechanisms of NEU1 in hepatic stellate cells (HSCs).

Results: NEU1 expression was up-regulated in liver fibrosis tissues compared with normal liver tissues. The level of NEU1 was positively correlated with liver fibrosis in Chronic Hepatitis B (CHB) patients according to bioinformatics analysis. NEU1 levels were increased after stimulation with TGFβ *in vitro*. Knocking down NEU1 decreased the activation of HSCs by suppressing TGF-β/Smad3 signaling.

Conclusion: This study showed that NEU1 plays a crucial role in activating HSCs via TGF-β/Smad3 signaling. Therefore, it may be a potential therapeutic target for liver fibrosis.

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Introduction

Liver fibrosis is characterized by the activation of hepatic stellate cells (HSCs) and the deposition of the extracellular matrix (ECM) (1). HSC activation is considered an essential process in liver fibrosis, leading to excessive ECM accumulation (2). Liver fibrosis may progress to cirrhosis and even hepatocellular carcinoma, which may lead to a relatively poor prognosis (3). However, there is no effective drug therapy for liver fibrosis worldwide (4), and identifying novel targets to inhibit its progression is important.

Neuraminidases (NEUs) are also called sialidases, which cleave α-glucoside-linked sialic acid residues from glycolipids and glycoproteins (5). Four types of NEUs have been identified, namely, NEU1, NEU2, NEU3, and NEU4 (6). NEU1 is not only localized to lysosomes but can also be transported to the cell surface, where it regulates cell-surface receptors (6). A bioinformatics analysis was performed to investigate the relationship between NEU1 and liver fibrosis, and the results revealed that high NEU1 expression was positively associated with poor outcomes in patients with liver fibrosis (7). In addition, NEU1 is involved in the regulation of metabolic diseases, including obesity, insulin resistance, and nonalcoholic fatty liver disease (8-10). However, the role and function of NEU1 in liver fibrosis remain unclear.

In our study, we detected NEU1 expression in patients with liver fibrosis and in fibrotic mice. Bioinformatics analysis was performed to analyze the relationship between NEU1 and the progression of liver fibrosis. In addition, the molecular mechanism of NEU1 was investigated. We aimed to explore the role of NEU1 in liver fibrosis.

Materials and Methods

Human liver tissue collection

Normal human liver tissues were collected from patients with intrahepatic bile duct stones, and liver fibrosis tissues

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were collected from patients with liver fibrosis. All liver tissues were obtained from the Pathology Department of the First Affiliated Hospital of Nanchang University. All subjects provided written informed consent. This study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Nanchang University (2024) CDYFYYLK(11-044).

Liver fibrosis models

C57BL/6J mice were obtained from GemPharmatech Co., Ltd. These mice had free access to water and food at the Animal Center of the First Affiliated Hospital of Nanchang University. Liver fibrosis models were established using carbon tetrachloride (CCl_4) and thioacetamide (TAA) (11, 12). In the CCl_4 group, C57BL/6J mice were given intraperitoneal injections of CCl_4 (1.0 μl/g body weight, 1:4 dilution in olive oil) twice weekly for six weeks. In the control group, C57BL/6J mice were given intraperitoneal injections of olive oil (1.0 μl/g body weight) twice weekly for six weeks. In the TAA group, C57BL/6J mice were allowed to drink water containing TAA at 300 mg/l for 12 weeks. In the control group, C57BL/6J mice drank water for 12 weeks. All the mice were sacrificed, and liver tissues were collected after the model was established. The Animal Ethics Committee of the First Affiliated Hospital of Nanchang University approved all procedures.

The analysis of the GEO database

The microarray data GSE84044 were downloaded from the GEO database. The GSE84044 microarray data contained 124 chronic hepatitis patients who underwent liver biopsy. The sex, age, and liver stages of these participants were publicly available. The liver fibrosis stage was assessed using the Scheuer scoring system (13). The first group was divided into the non-cirrhosis (liver fibrosis stage≤3) and cirrhosis (liver fibrosis stage>3) groups. In addition, the second group was divided into the liver fibrosis stage<2 group and the liver fibrosis stage≥2 group. Univariate and multivariate logistic regression analyses were performed to identify independent risk factors for a liver fibrosis score ≥2.

Cell experiments

Human hepatic stellate LX2 cells were obtained from Procell Life Science & Technology Co., Ltd. LX2 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics. In addition, LX2 cells were exposed to NEU1 siRNA for 24 hr with Lipofectamine™ 3000 transfection reagent and then treated with 10 ng/ml TGF-β1 for 24 hr. The NEU1 siRNA sequences used were as follows: si-NEU1: sense GCCCGAAACCAGAACAACUTT and antisense AGUUGUUCUGGUUUCGGGCTT.

Immunohistochemistry

NEU1 expression was assessed by immunohistochemistry (IHC). Liver tissue sections were pretreated with 3% hydrogen peroxide for 8 min to block endogenous peroxidase activity. The sections were heated in 10 mM sodium citrate for 15 min to retrieve antigen. Then, the sections were incubated with anti-NEU1 (1:200) primary antibodies overnight at 4 °C. Diaminobenzidine (DAB) was used for positive staining of liver sections. Three random pictures were selected for each liver section.

Pathological staining

Liver sections were fixed in 4% formaldehyde. For HE staining, the slides were incubated in an eosin alcohol solution for 5 min. For Sirius red staining, the sections were stained with saturated aqueous picric acid for 1 hr. Then, the liver sections were dehydrated. A microscope was used to capture the images; three random pictures were selected for each liver section, and the positive area was determined via ImageJ software.

Western blot analysis

Total protein was extracted from cells and liver tissues. Total protein was subjected to SDS-PAGE, followed by transfer to a PVDF membrane. The membranes were subsequently blocked with 5% skim milk and incubated with primary antibodies, including NEU1 (Santa Cruz Biotechnology, sc-166824), Collagen-1 (Abcam, ab270993), α-SMA (Abcam, ab5694), TIMP-1 (Bioss, bsm-10895 m), GAPDH (cst, cst5174), Smad3 (cst, cst9523), and P-smad3 (cst, cst9520T), overnight at 4 °C. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hr the next day. The protein bands were visualized via a chemiluminescence system. ImageJ software was used for quantitative analysis.

Statistical analysis

All the data are presented as the means ± standard deviations (SDs). Student's t-test was used to assess the differences between the two groups. ANOVA was used to analyze the differences among multiple groups. The GEO dataset was analyzed using R. A value of $P<0.05$ was considered significant.

Results

Expression of NEU1 is up-regulated in liver fibrosis

Two well-constructed liver fibrosis models were used to explore the relationship between NEU1 and liver fibrosis. In the CCl_4-induced liver fibrosis model, HE and Sirius Red staining revealed significant fibrotic progression (Figure 1A). NEU1 expression was subsequently significantly increased in the CCl_4 group (Figure 1B). Furthermore, Western blot analysis confirmed that NEU1 protein levels were significantly increased in the CCl_4 group (Figure 1C). In the TAA-induced liver fibrosis model, fibrotic progression was determined by HE and Sirius Red staining of liver samples (Figure 1D). Moreover, the fibrotic liver tissues presented greater NEU1 expression than the normal liver tissues did (Figure 1E, F). NEU1 expression was subsequently compared between human liver fibrosis tissues and normal liver samples. As shown in Figure 1G, significant fibrotic progression was visualized by HE and Sirius Red staining in liver fibrosis tissues. Furthermore, IHC analysis confirmed that NEU1 expression was increased in human fibrotic liver tissues compared with normal liver samples. Taken together, these results confirmed that NEU1 expression was elevated in liver fibrosis.

Associations between NEU1 expression and liver fibrosis in CHB patients

In the GSE84044 dataset, as shown in Figure 2A, the expression of NEU1 was greater in the cirrhosis group than in the noncirrhosis group ($P<0.01$). Moreover, we found that NEU1 expression gradually increased with increasing liver fibrosis stage and was positively correlated with liver fibrosis ($r=0.24$, $P<0.01$) (Figure 2B). In addition, as shown

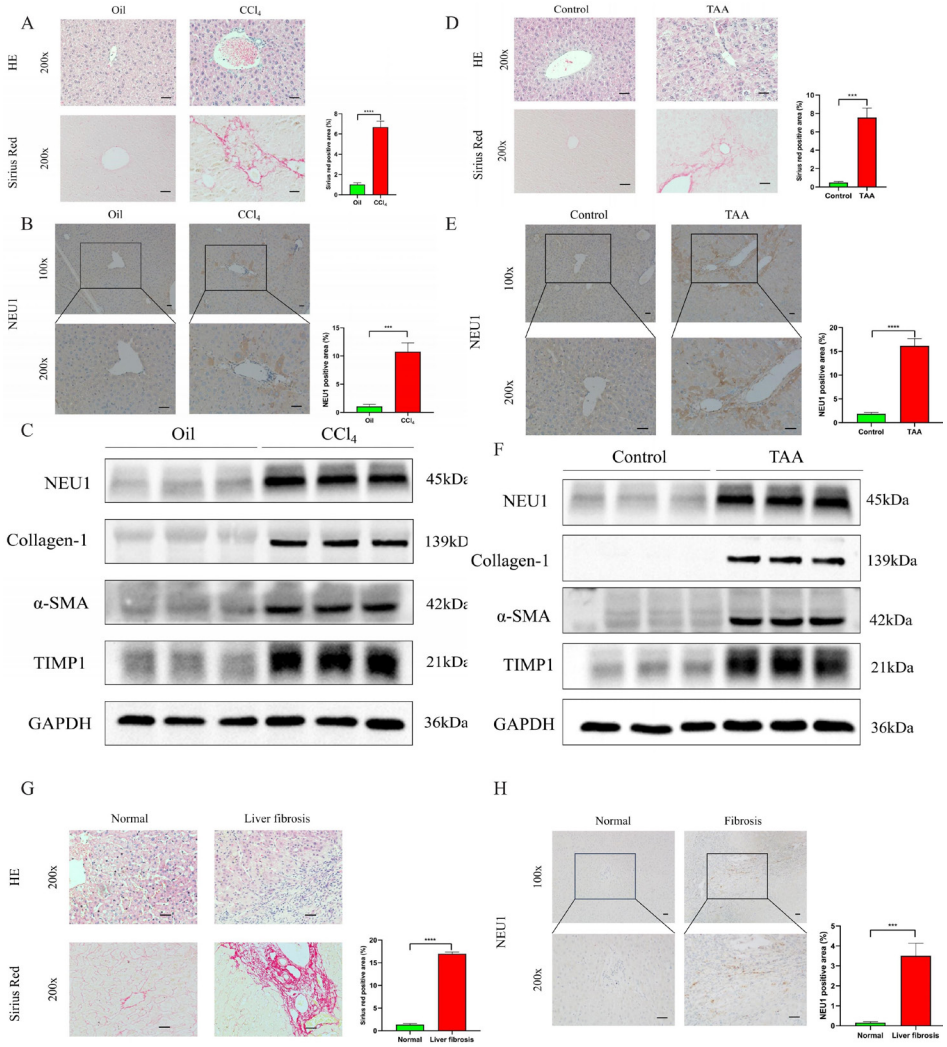

Figure 1. NEU1 expression is up-regulated in mice with liver fibrosis
(A) Representative images of HE and Sirius Red staining in CCl_4-induced fibrotic mice and the quantification of Sirius Red staining. Scale bars, 10 μm. (B) Representative images of immunohistochemical (IHC) staining of NEU1 in CCl_4-induced fibrotic mice and quantification analysis. Scale bars, 10 μm. (C) Western blot analysis of NEU1, Collagen-1, α-SMA, and TIMP1 in the Oil and CCl_4 groups. (D) Representative images of HE and Sirius Red staining in TAA-induced fibrotic mice and the quantification of Sirius Red staining. Scale bars, 10 μm. (E) Representative images of IHC staining of NEU1 in TAA-induced fibrotic mice and quantification analysis. Scale bars, 10 μm. (F) Western blot analysis of NEU1, Collagen-1, α-SMA, and TIMP1 in the control and TAA groups. (G) Representative images of HE and Sirius Red staining in human liver tissues and the quantification of Sirius Red staining. Scale bars, 10 μm. (H) Representative images of IHC of NEU1 in human liver tissues and quantification analysis. Scale bars, 10 μm. NEU1: Neuraminidase 1; TAA: Thioacetamide; HE: Hematoxylin-eosin; IHC: Immunohistochemical

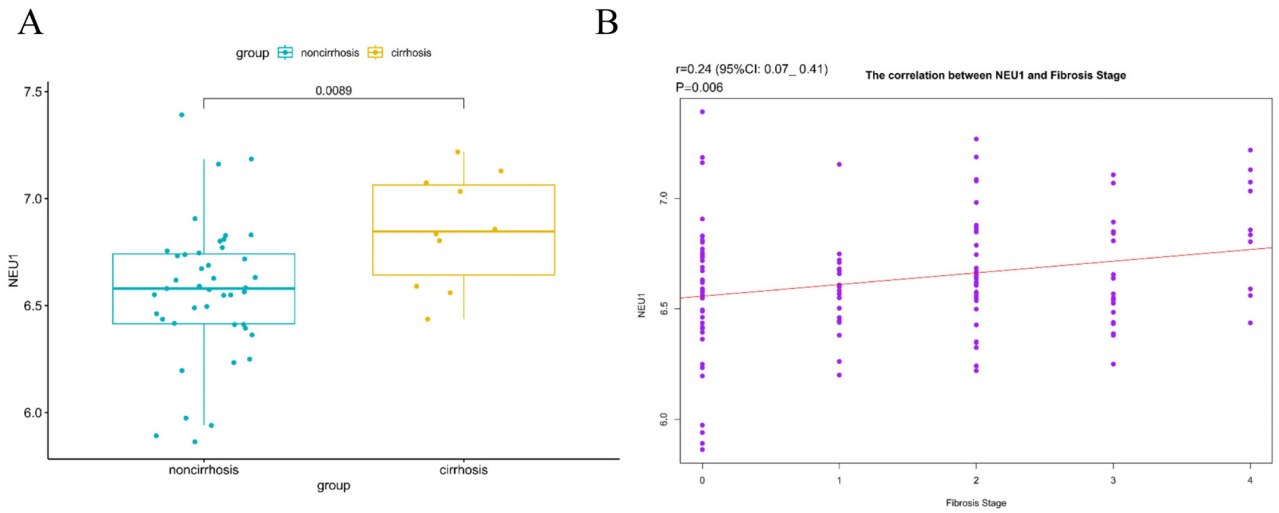

Figure 2. Relationship between NEU1 expression and liver fibrosis in CHB patients
(A) Comparison of the expression of NEU1 between the non-cirrhosis and cirrhosis groups. (B) Correlation between the NEU1 level and fibrosis stage. NEU1: Neuraminidase 1; CHB: Chronic hepatitis B

Table 1. Univariate and multivariate logistic regression analyses of variables associated with liver fibrosis stage≧ 2 in GSE84044 dataset

Variables	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*- value	OR (95% CI)	*P*- value
Age (year)	1.051 (1.017-1.085)	0.003	1.046 (1.012-1.082)	0.007
Male sex	0.595 (0.272-1.304)	0.195		
NEU1	5.616 (1.411-22.345)	0.014	4.487 (1.068-18.845)	0.040

Notes: In univariate analysis, *P* value <0.05 were subjected to multivariate analysis and was indicated in bold; In multivariate analysis, *P* value <0.05 was considered significant and was indicated in bold.

in Table 1, high NEU1 expression was a risk factor for liver fibrosis stage ≥2 (OR: 4.487, 95% CI: 1.068–18.845; $P<0.05$). These results indicated that the NEU1 level was positively correlated with liver fibrosis in CHB patients.

NEU1 knockdown suppresses the activation of HSCs through the TGF-β/Smad3 pathway

As shown in Figure 3A, we used TGFβ to stimulate LX2 cells, and TGFβ strongly promoted an increase in fibrosis markers such as Collagen-1, α-SMA and TIMP-1, and NEU1 was also up-regulated. To explore the functional role of NEU1 in liver fibrosis, NEU1 was knocked down in LX2 cells via siRNA. As shown in Figure 3B, TGFβ was used to stimulate LX2 cells when NEU1 was knocked down, and we found that knocking down NEU1 decreased the protein expression of Collagen-1, α-SMA and TIMP-1. Consistently, the results revealed a decrease in P-smad3/smad3 in NEU1-knockdown LX2 cells. In summary, the results showed that NEU1 knockdown in LX2 cells inhibited the TGF-β/Smad3 pathway and thereby reduced profibrotic gene expression.

Discussion

In the present study, NEU1 was confirmed for the first time as a positive regulator of liver fibrosis. NEU1 expression is up-regulated in both patients with liver fibrosis specimens and tissues from CCl_4-induced and TAA-induced liver fibrosis mice. In addition, we demonstrated that the NEU1 level was positively correlated with liver fibrosis in CHB patients. Mechanistically, inactivation of NEU1 blocked the TGF-β/Smad3 pathway by suppressing the phosphorylation of Smad3. These findings revealed that NEU1 may be a novel therapeutic target for liver fibrosis.

Liver fibrosis, a hallmark of chronic liver disease, is a major clinical concern worldwide (14). Liver fibrosis is characterized by the excessive accumulation of ECM within the liver (15). The activation of HSCs was confirmed to be critical for the occurrence of liver fibrosis (16). Transforming growth factor-β (TGF-β) is a well-known profibrotic cytokine that plays a vital role in HSC activation and ECM production (17). When damage occurs, TGF-β secretion increases, and Smad2 and Smad3 are activated via TGF-β

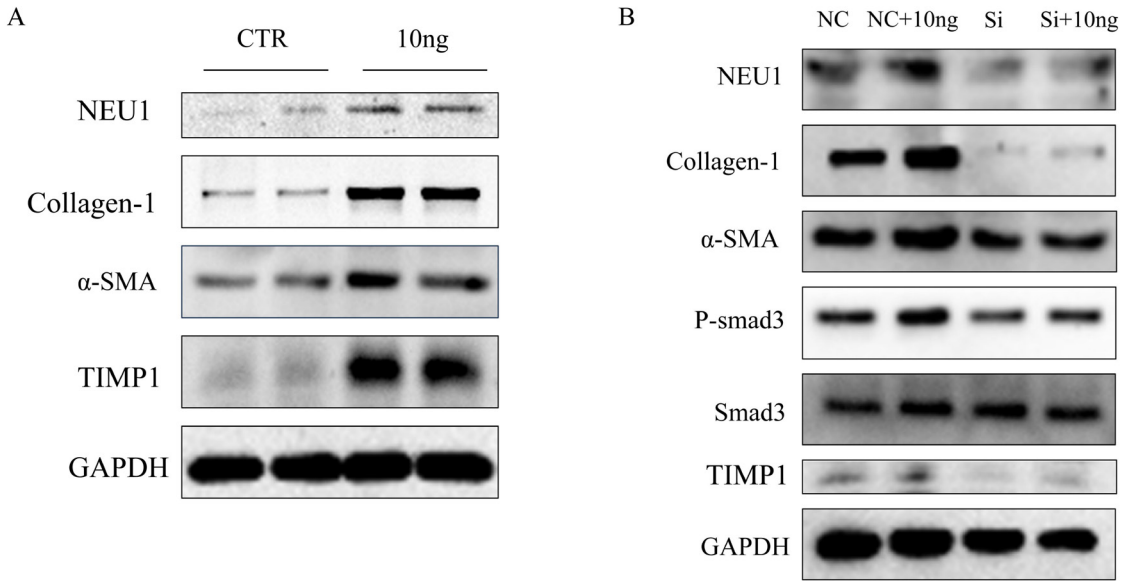

Figure 3. NEU1 knockdown suppresses HSC activation via the TGF-β/Smad3 pathway in mice
(A) Western blot analysis of NEU1, Collagen-1, α-SMA and TIMP1 in control and LX2 cells treated with TGF-β (10 ng/ml) for 24 hr. (B) Western blot analysis of NEU1, Collagen-1, α-SMA P-smad3, Smad3, and TIMP1 in NC and LX2-si-NEU1 cells treated with or without TGF-β (10 ng/ml) for 24 hr.
NEU1: Neuraminidase 1; HSC: Hepatic stellate cell

receptor 1/2 (TGFBR1/2) in the fibrotic microenvironment (18). However, because the TGF-β/Smad signaling pathway is universally expressed in the body, no drugs targeting TGF-β signaling are currently available (19). Therefore, new therapeutic targets to interfere with the TGF-β/Smad signaling pathway in HSCs are urgently needed.

NEU1 is involved in the development of the inflammatory response, atherosclerosis, and heart failure (20). NEU1 deficiency is related to sialic acid-rich macromolecular storage, which may lead to lysosomal disorders (21). Chen *et al.* confirmed that NEU1 was a critical driver of cardiac hypertrophy and that NEU1 inhibition prevented cardiomyocyte hypertrophy (22). Another study revealed that NEU1 inhibition protected against diabetes-related cardiac fibrosis (23). Recent studies have shown that NEU1 expression is up-regulated in a renal fibrosis model and that NEU1 suppression effectively protects mice from renal fibrosis (24). These studies revealed that NEU1 is closely related to fibrosis-related diseases. In our study, we confirmed that NEU1 was significantly elevated in fibrotic human and mouse liver tissues and that NEU1 knockdown suppressed HSC activation *in vitro*.

This study has several limitations. First, the in vitro overexpression of NEU1 was not examined. Second, this study did not investigate NEU1 knockout in fibrotic mice.

Conclusion

In summary, this study confirmed that NEU1 is a positive regulator of liver fibrosis and that NEU1 deficiency inhibits HSC activation via the TGF-β/Smad3 pathway.

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Availability of Data and Materials

The datasets in the current study are available from the corresponding author upon reasonable request.

Ethical Approval

The study protocol was approved by the Ethics Committee of First Affiliated Hospital of Nanchang University (2024) CDYFYYLK(11-044). The study was performed in accordance with the Declaration of Helsinki.

Authors' Contributions

Y Zh, Y S and Y N wrote the original draft, X Zh developed the methodology, and BM L and Ch H reviewed the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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