



Original Article



Naringenin is a Potential Immunomodulator for Inhibiting Liver Fibrosis by Inhibiting the cGAS-STING Pathway

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Abstract

Background and Aims: Naringenin is an anti-inflammatory flavonoid that has been studied in chronic liver disease. The mechanism specific to its antifibrosis activity needs further investigation. This study was focused on the cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS) pathway in hepatic stellate cells and clarified the antifibrosis mechanism of naringenin. **Methods:** The relationship between the cGAS-stimulator of interferon genes (STING) pathway and liver fibrosis was analyzed using the Gene Expression Omnibus database. Histopathology, immunohistochemistry, fluorescence staining, Western blotting and polymerase chain reaction were performed to assess gene and protein expression levels associated with the cGAS pathway in clinical liver tissue samples and mouse livers. Molecular docking was performed to evaluate the relationship between naringenin and cGAS, and western blotting was performed to study the expression of inflammatory factors downstream of cGAS *in vitro*. **Results:** Clinical database analyses showed that the cGAS-STING pathway is involved in the occurrence of chronic liver disease. Naringenin ameliorated liver injury and liver fibrosis, decreased collagen deposition and cGAS expression, and inhibited inflammation in carbon tetrachloride (CCl₄)-treated mice. Molecular docking found that cGAS may be a direct target of naringenin. Consistent with the *in vivo* results, we verified the inhibitory effect of naringenin on activated hepatic stellate cells (HSCs). By using the cGAS-specific agonist double-stranded (ds)DNA, we showed that naringenin attenuated the activation of cGAS and its inflammatory factors affected by dsDNA. We verified that naringenin inhibited the cGAS-STING pathway, thereby reducing the secretion of inflammatory factors by HSCs to ameliorate liver fibro-

sis. **Conclusions:** Interrupting the cGAS-STING pathway helped reverse the fibrosis process. Naringenin has potential as an antihepatic fibrosis drug.

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Introduction

Liver fibrosis is a chronic disease generated by liver injuries caused by several factors, such as excessive alcohol consumption, virus infection (including hepatitis B and hepatitis C), nonalcoholic steatohepatitis (NASH), nonalcoholic fatty liver disease, and autoimmune hepatitis.¹⁻³ Multiple types of liver damage and disease have led to the high prevalence and mortality of liver fibrosis in China and worldwide.⁴ Without appropriate treatment, liver fibrosis gradually results in hepatocellular carcinoma.² As the driving center of liver fibrosis, hepatic stellate cells (HSCs) are implicated in hepatic inflammation⁵ and considered a main factor that promotes liver fibrosis.⁶ HSCs receive signals from leukocytes within the hepatic environment, amplify the signals, and produce molecules that then target and modulate leukocytes. Specifically, HSCs promote leukocyte chemotaxis and adherence, and may also regulate the activation of leukocytes within the hepatic environment by secreting immunoregulatory cytokines.⁷ Inhibition of HSC-associated inflammatory signals effectively ameliorates liver fibrosis.^{8,9} However, the specific regulatory mechanism of inflammation in HSCs needs further study.

As an inflammation-mediated pathway, the cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS) stimulator of interferon genes (STING) signaling pathway has been widely studied in recent years for its role in chronic liver disease, including liver fibrosis, which is an important process associated with chronic liver disease. cGAS is an inflammatory pathway activation sensor. When the cGAS-STING signaling pathway is activated, the expression of type I interferon and other inflammatory cytokines are induced, such as interferon regulatory factor 3 (IRF3), and triggers the innate immune response.¹⁰ STING

Keywords: Liver fibrosis; cGAS; Naringenin; Inflammation; Hepatic stellate cells.

Abbreviations: α-SMA, alpha smooth muscle actin; cGAS, cyclic guanosine monophosphate-adenosine monophosphate synthase; CCl₄, carbon tetrachloride; ECM, extracellular matrix; HSCs, hepatic stellate cells; STING, stimulator of interferon genes; WB, western blot.

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is widely expressed in various cell types, and recent studies have found that it can regulate different pathways of programmed cell death. Therefore, a deeper understanding of the cGAS-STING signaling pathway may provide a new method of treating chronic inflammatory diseases.¹¹ Recently, many studies have shown that the cGAS-STING signaling pathway is associated with the occurrence of liver fibrosis.¹² Thus, targeting the cGAS-STING pathway may be a potential therapeutic strategy for liver fibrosis.

Naringenin is a flavonoid compound, that has been studied in various liver damage models, induced by carbon tetrachloride (CCl₄), alcohol, N-methyl-N-nitro-nitroguanidine, lipopolysaccharide (LPS), and heavy metals *in vivo* and *in vitro*. In those studies, naringenin had a good hepatoprotective activity because of its antioxidant activity and its ability to inhibit inflammatory and fibrotic signaling pathways.¹³ Although naringenin has been reported to reduce inflammation and liver fibrosis, the mechanism underlying this action still needs to be fully revealed. Whether the cGAS-STING pathway is involved in inhibiting inflammation and the antifibrotic effect of naringenin has not been reported. Therefore, the aim of the present study was to explore the mechanism underlying the effects of naringenin in liver fibrosis treatment. We showed that naringenin affected the secretion of inflammatory factors and the phenotypic changes of HSCs by interfering with the cGAS-STING signaling pathway to alleviate liver fibrosis.

Methods

Gene Expression Omnibus (GEO) database analysis

After downloading the gene expression matrix of GSE99807 and GSE33650 (<https://www.ncbi.nlm.nih.gov/geo/>), the information and RNA expression data of four patients with liver cancer tissues and pericarcinoma tissues were extracted from GSE99807. The gene expression data of patients with normal liver tissues and HCV infected liver fibrosis tissues were extracted from GSE33650. The selection condition for differentially expressed genes between liver cancer tissues and adjacent normal tissues in GSE99807 was $p < 0.05$. The logFC values of genes of the samples (GSE99807) were used to draw a volcano map. The differentially expressed genes (GSE99807) were used for KEGG signal enrichment analysis and gene ontology biological processes (GOBP) enrichment analysis (<https://david.ncifcrf.gov> was used to obtain analysis results). TMEM173 and IRF3 gene expression analysis was performed based in the RNA expression data of GSE33650. The data was visualized by using the cloud platform <http://sangerbox.com/Tool>.

Collection of samples from patients with liver cancer

The liver cancer tissues and adjacent normal tissues of human liver cancer patients were collected in Jiangsu Provincial Hospital of Traditional Chinese Medicine from July 2018 to December 2021. This study was approved by the hospital ethics committee (ethics approval number: 2018-050). According to the inclusion and exclusion criteria, the study collected tumor tissues and adjacent normal tissues from three patients with liver cancer. The inclusion was surgical treatment with pathological diagnosis of liver cancer in tissue specimens. Before this visit, the patient had not received any radiotherapy, chemotherapy, or biological treatment. Patients with autoimmune diseases complicated by primary tumors in other organs other than liver cancer, with tumor metastases in organs other than the liver before and within 6

months after the operation, poor physical condition, and expected postoperative survival of <6 months were excluded.

Chemicals and antibodies

Naringenin (must-21032406) was purchased from CDMUST (Chengdu, China). Other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-alpha smooth muscle actin (α -SMA) (A7248), anti-cGAS (A8335), anti-IRF3 (A2172), anti-P-TBK1 (AP0847), anti-TBK1 (A3458), anti-IL8(A12452), and anti-STING (A20175) were purchased from ABclonal (Woburn, MA, USA). Anticollagen I (14695-1-AP), anti- β -actin (66009-1-Ig), anti-rabbit IgG (SA00001-2), and anti-mouse IgG (SA00001-1) were purchased from Proteintech Group (Rosemont, IL, USA). Anti-IL1 β (ab234437), anti-IL6 (ab229381), and anti-TNF α (ab215188) were purchased from Abcam (Cambridge, UK).

Animal experiments

Male C57BL/6J mice weighing 18–20 g were obtained from the Nanjing Qinglongshan Animal Co. Ltd. (Beijing, China). All animals were cared for humanely following National Institutes of Health (Bethesda, MD, USA) guidelines. When conducting experiments on animals, we followed the 3R principle and respected the highest ethical and animal welfare standards. Before the procedures, all animals were kept in an air-conditioned room at 24°C, with a 12-hour dark/light cycle for 1 week. All animals were free to get food and water during the study. The study was approved by the College Animal Care Committee of our university (approved No. SYLL-2021-002).

Eighteen mice were randomly divided into three groups of six each. Mice in group 1 were intraperitoneally injected with olive oil as the negative control, mice in group 2 were intraperitoneally injected with CCl₄ (1:9 v/v with olive oil) at a dose of 5 ml/kg for 8 weeks (3 times/week) to induce liver fibrosis, and mice in group 3 were given intragastric naringenin 100 mg/kg¹⁴ for 8 weeks (3 times/week) with CCl₄ modeling.

Serum index assays

Mouse blood was collected from the eye socket vein, for assay of serum liver biochemical indexes, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), Laminin (LN), hyaluronic acid (HA), procollagen type-III (PC-III) and collagen type-IV (IV-C) were analyzed using a Hitachi 7020 Chemistry Analyzer (Tokyo, Japan). The inflammatory factors were detected by ELISA kits, interleukin (IL)-1 β (YFXEM00028), IL-6 (YFXEM00045), IL18 (YFXEM00573), and tumor necrosis factor alpha (TNF α , YFXEM00031) purchased from Yi Fei Xue (Nanjing, China).

Histological examination

The liver tissue was fixed with 10% neutral buffer formalin and embedded in paraffin. Liver sections with a thickness of 5 μ m were prepared and stained with hematoxylin and eosin, and Masson pine trichromatic staining was performed by standard methods. For Sirius red collagen staining, sections were dewaxed, and stained with Sirius red for 1 h at room temperature. After washing, the slides were dehydrated in 100% ethanol and xylene and then mounted in

Permout. The photos were taken in a random area blindly.

Immunofluorescence staining

Immunofluorescence staining of LX2 cells or slides of liver mouse and human tissues was performed according as previously reported.¹⁵ Diamidino-phenyl-indole (DAPI) was used to stain the nucleus. In some immunofluorescence staining of liver tissues, cGAS-STING pathway-related molecule (cGAS, 1:100 diluted in 5% bovine serum albumin [BSA]) and activated HSC marker α -SMA (1:100 diluted in 5% BSA) were doubly stained. Pictures were taken in five random areas with a fluorescence microscope (Zeiss, Oberkochen, Germany).

Molecular docking

The binding of naringenin to cGAS protein (PDB 6047) was modeled with GLIDE software (Schrödinger, LLC, New York, NY, USA). The guide protein preparation method used with the Maestro workstation was to remove all water molecules from the original structure during protein preparation, adding electric charges and hydrogen atoms, and modify the geometry of all heterogroups separately. After that, the Prime tool was used to predict and fill in missing ring atoms and hydrogen bonds. Finally, IMPREF optimized the placement of hydrogen bonds and keeps all the atoms in place. The default constraints of OPLS_2005 force field and 0.3a of Root Mean Squared Error (RMSD) were used for energy minimization. The receptor mesh generation panel was used to generate grids under the silicon chip target screening, defining the receptor structure to exclude any other primitive compounds that may be present, and the method of settling the location and size of the active site by the receptor mesh was previously reported by Dai *et al*.¹⁶

Cell culture and assay of cellular viability

Human LX2 and L02 cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Invitrogen, Grand Island, NY, USA) supplemented with 1% fetal bovine serum and grown in a 5% CO₂ humidified atmosphere at 37°C. Dimethyl sulfoxide (DMSO) was selected as the carrier for dissolving naringenin. LX2 and L02 cells were treated with 0, 2, 5, 10, 20, or 40 μ M naringenin for 24 h. Cell viability indicative of cellular metabolic activity was measured using MTT assays as described by Wang *et al*.¹⁷ The spectrophotometric absorbance at 490 nm was determined using a SPECTRAmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Western blot (WB) analysis

Protein extracts were prepared from the liver and LX2 cells using RIPA buffer. Protein detection, WBs, and quantification were performed by standard methods. All primary antibodies were diluted 1:2,000 with 5% BSA, and secondary antibodies were diluted 1:10,000 with 5% skimmed milk powder. β -actin was used as a constant control for total protein equivalent loading.

Real-time PCR (PCR)

Trizol reagent (Sigma-Aldrich) was used to isolate total RNA

from LX-2 cells. RNA (2 μ g) was reverse transcribed by a reverse transcription-polymerase chain reaction kit to obtain cDNA. NanoDrop assays was used to quantify RNA. Primers were purchased from Tsingke Biotech (Nanjing, China). The mRNA of interest was normalized to GAPDH. GAPDH was used as endogenous control. The primers for the target genes were listed in Table 1. The relative expression changes were determined by the 2^{- $\Delta\Delta$ CT} method.

Statistical analysis

Values were reported as means and standard deviation. Between-group differences were compared by unpaired student *t*-tests. Multiple-group differences were compared by one-way analysis of variance with Bonferroni correction (Graph Pad Prism 9.0, San Diego, CA, USA). *P*-value <0.05 were considered statistically significant.

Results

TMEM173 (STING) and IRF3 are excessively expressed in liver fibrosis tissues from liver cancer patients

RNA microarray analysis data from liver cancer and adjacent normal tissues from eight patients showed that the gene expression levels of *TMEM173*, *COL1A1*, and *IRF3* were higher in cancer tissues than pericarcinoma tissues (Fig. 1A, B). The abnormally expressed genes in liver fibrosis tissues from liver cancer patients were enriched in multiple signaling pathways, including the primary immunodeficiency, and NF-kappa B signaling pathways, which have been reported to promote the development of chronic liver disease. Obviously, the signaling pathways reported by some studies were closely related to the functional regulation of STING in chronic liver disease and liver cancer,¹⁸ which may contribute to promoting the development of liver fibrosis (Fig. 1C). The top 12 enriched biological processes derived from the differentially expressed genes between liver cancer tissues and adjacent normal tissues in patients showed that the inflammatory response was involved in the malignant progression of liver fibrosis (Fig. 1D). The analysis based on GSE33650 showed that *TMEM173* (STING) and *IRF3* were highly expressed in liver fibrosis tissues from liver cancer patients (Fig. 1E, F). The liver fibrosis samples also had high expression of genes related to the cGAS-STING pathway (Supplementary Fig. 1A). The results showed that STING-related inflammatory pathways had an important role in the development of liver fibrosis, and that the inflammatory response signal was evoked along with STING.

STING-related proteins were significantly increased in liver fibrosis tissues from liver cancer patients

Among the samples collected from liver cancer patients, HE, Masson, and Sirius red staining confirmed the occurrence of liver fibrosis (Fig. 2A). The Masson and Sirius red staining results showed that collagen deposition and fibrotic lesions were more extensive in the area of the tumor sample, which represented the liver fibrosis area in the tumor tissue. Compared with adjacent normal tissues, immunofluorescence staining showed that the expression levels of α -SMA and cGAS (Fig. 2B) were significantly increased in liver fibrosis tissues from liver cancer patients. The results showed that the increase in liver fibrosis was accompanied by an increase in cGAS expression. The WB results showed that

Table 1. Primer sequences for real-time PCR

Genes		Sequences
GAPDH (Human)	Forward	5'-CCAACCGCGAGAAGATGA-3'
	Reverse	5'-CCAGAGGCGTACAGGGATAG-3'
GAPDH (Mouse)	Forward	5'-TGTGAACGGATTTGGCCGA-3'
	Reverse	5'-CAATCTCCACTTTGCCACTGC-3'
α -SMA (Human)	Forward	5'-ACTGCCTTGGTGTGTGACAA-3'
	Reverse	5'-TCCCAGTTGGTGATGATGCC-3'
α -SMA (Mouse)	Forward	5'-GTACCACCATGTACCCAGGC-3'
	Reverse	5'-GCTGGAAGGTAGACAGCGAA-3'
COL1 α 1 (Human)	Forward	5'-GTGAAGCTGGTCCCCAAGG-3'
	Reverse	5'-CAGCACTAGCAGGGCCAG-3'
COL1 α 1 (Mouse)	Forward	5'-TTCTCCTGGCAAAGACGGA-3'
	Reverse	5'-CCATCGGTCATGCTCTCTCC-3'
IL10 (Human)	Forward	5'-GTTGTTAAAGGAGTCCTTGCTG-3'
	Reverse	5'-TTCACAGGGAAGAAATCGATGA-3'
IL6 (Human)	Forward	5'-CACTGGTCTTTTGAGTTTGAG-3'
	Reverse	5'-GGACTTTTGTACTCATCTGCAC-3'
IL-1 β (Human)	Forward	5'-GCCAGTGAAATGATGGCTTATT-3'
	Reverse	5'-AGGAGCACTTCATCTGTTTAGG-3'
IL-8 (Human)	Forward	5'-AACTGAGAGTGATTGAGAGTGG-3'
	Reverse	5'-ATGAATTCTCAGCCCTCTTCAA-3'
NF- κ B (Human)	Forward	5'-TATTTGAAACACTGGAAGCACG-3'
	Reverse	5'-CCGGAAGAAAAGCTGTAAACAT-3'
cGAS (Human)	Forward	5'-AGATGGAGTCTCGCTCTGTACC-3'
	Reverse	5'-GAGGCAGGAGAATCGCTTGAACC-3'
STING (Human)	Forward	5'-GGGCTGAAGTAGAGTGGCACAATC-3'
	Reverse	5'-TGGGAGGCTAAGGCAGGAGAATC-3'

the protein levels of cGAS and STING were higher in the tumor tissues of patients than the adjacent normal tissues (Fig. 2C). The above results indicate that the occurrence and development of liver fibrosis were associated with the cGAS-STING pathway.

Naringenin reduces liver fibrosis in mice

To evaluate whether naringenin alleviated liver fibrosis, we used hematoxylin and eosin, Masson, and Sirius red staining to detect the level of liver damage and fibrosis in the liver tissue of naringenin-treated mice with CCl₄-induced liver fibrosis. Serum biochemical indicators (ALT, AST, ALP, ALT, LN, HA, PC-III, and IV-C) in mice were also assayed to determine changes in liver function. The immunohistochemistry results showed that naringenin significantly reduced liver tissue damage and liver fibrosis caused by CCl₄ (Fig. 3A). Serological testing showed that naringenin reduced the levels of ALT, AST, ALP, and ALT (Fig. 3B) as well as the levels of LN, HA, PC-III, and IV-C (Fig. 3C) in CCl₄-induced liver fibrosis. In addition, the ELISA results showed that naringenin significantly reduced the expression of inflammatory factors, such as IL1 β , IL6, IL18 and TNF α in CCl₄-induced liver fibrosis (Fig. 3D). The results indicated

that naringenin alleviated the symptoms of liver damage and liver fibrosis caused by CCl₄ and reduced the inflammatory response induced by chemical damage.

Naringenin targets cGAS in HSCs

HSC activation is closely associated with inflammation. cGAS is an important link in the process of inflammatory activation. Therefore, we performed fluorescence staining of the HSC activation indicators α -SMA and cGAS in mouse liver tissue sections. The results showed that α -SMA and cGAS were coexpressed in liver tissue and naringenin reduced the expression of α -SMA and cGAS in CCl₄-induced liver fibrosis (Fig. 4A).

Subsequently, we docked naringenin and the cGAS complex using the molecular modeling packages in Maestro workstation to explore the potential binding mode of naringenin and cGAS protein. cGAS crystal structure was obtained from the RCSB Protein Data Bank (PDB code: 6O47). The docking simulation results confirmed that naringenin bound to the hydrophobic pocket and partially overlapped the binding sites of cGAS, which disrupted the dimerization of cGAS (Fig. 4B). The result showed that naringenin fit into the hydrophobic pocket of cGAS. The docking score of the

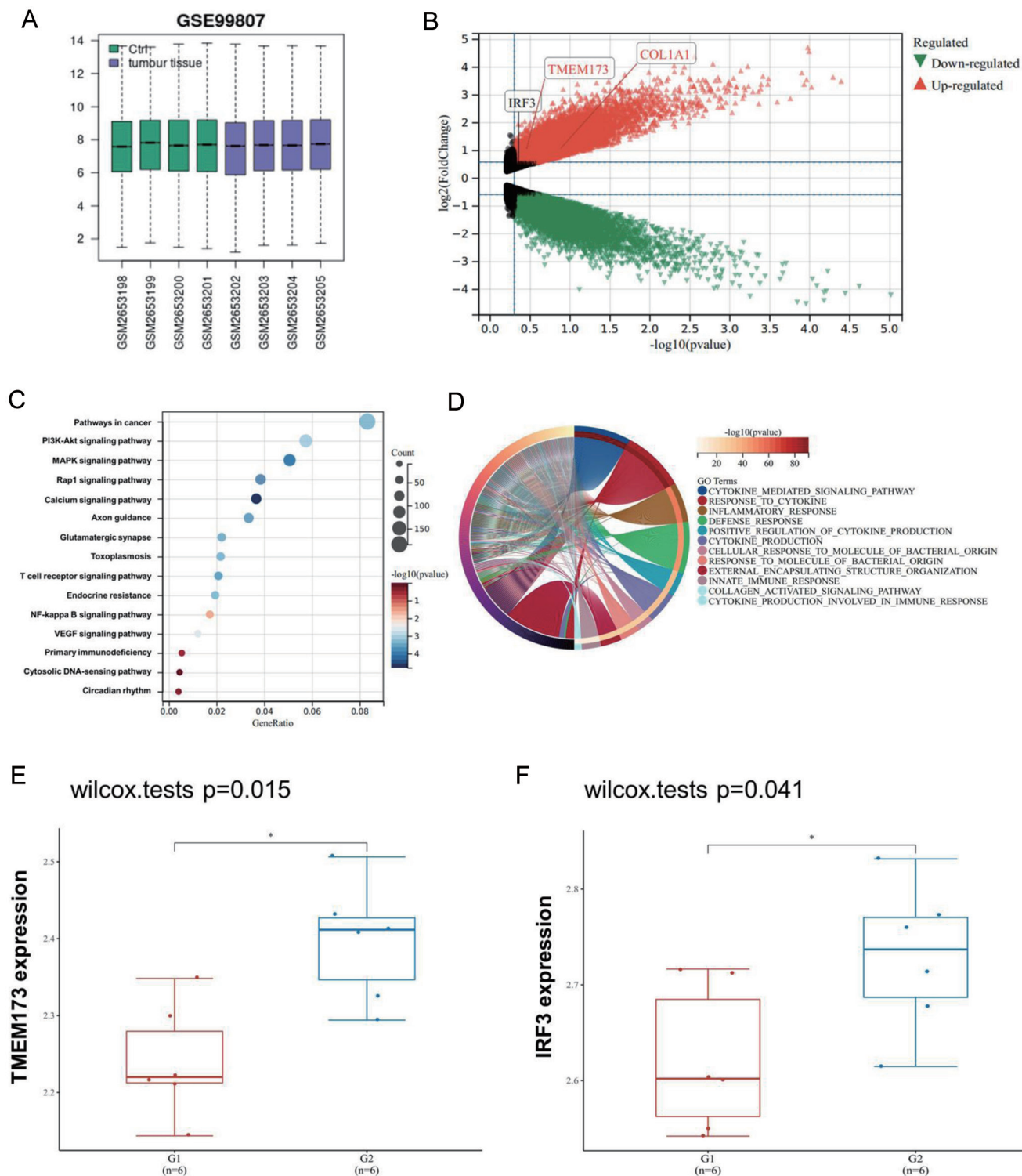


Fig. 1. TMEM173 (STING) and IRF3 are excessively expressed in liver fibrosis tissues from liver cancer patients. (A) The GSE number and sample quality for RNA microarray analysis of liver cancer tissue (tumor tissue, $n=4$) and normal adjacent tissue (ctrl, $n=4$) from GSE99807. (B) A volcano map derived from the differentially expressed genes (screening condition, $p<0.05$) between liver cancer tissue (tumor tissue, $n=4$) and normal adjacent tissue (ctrl, $n=4$) from GSE99807. (C) Using the differentially expressed genes between liver cancer tissue (tumor tissue, $n=4$) and normal adjacent tissue (ctrl, $n=4$) from GSE99807 to perform KEGG signal enrichment. (D) Using the differentially expressed genes in liver cancer tissue (tumor tissue, $n=4$) and normal adjacent tissue (ctrl, $n=4$) from GSE99807 to perform GOBP enrichment. (E and F) The expression distribution of TMEM173 and IRF3 genes in tissues (G1: normal, $n=6$; G2: liver fibrosis, $n=6$) from GSE33650, the upper left corner shows the significance p -value test method. COL1A1, collagen type I alpha 1 chain; STING, stimulator of interferon genes; IRF3, interferon regulatory factor 3.

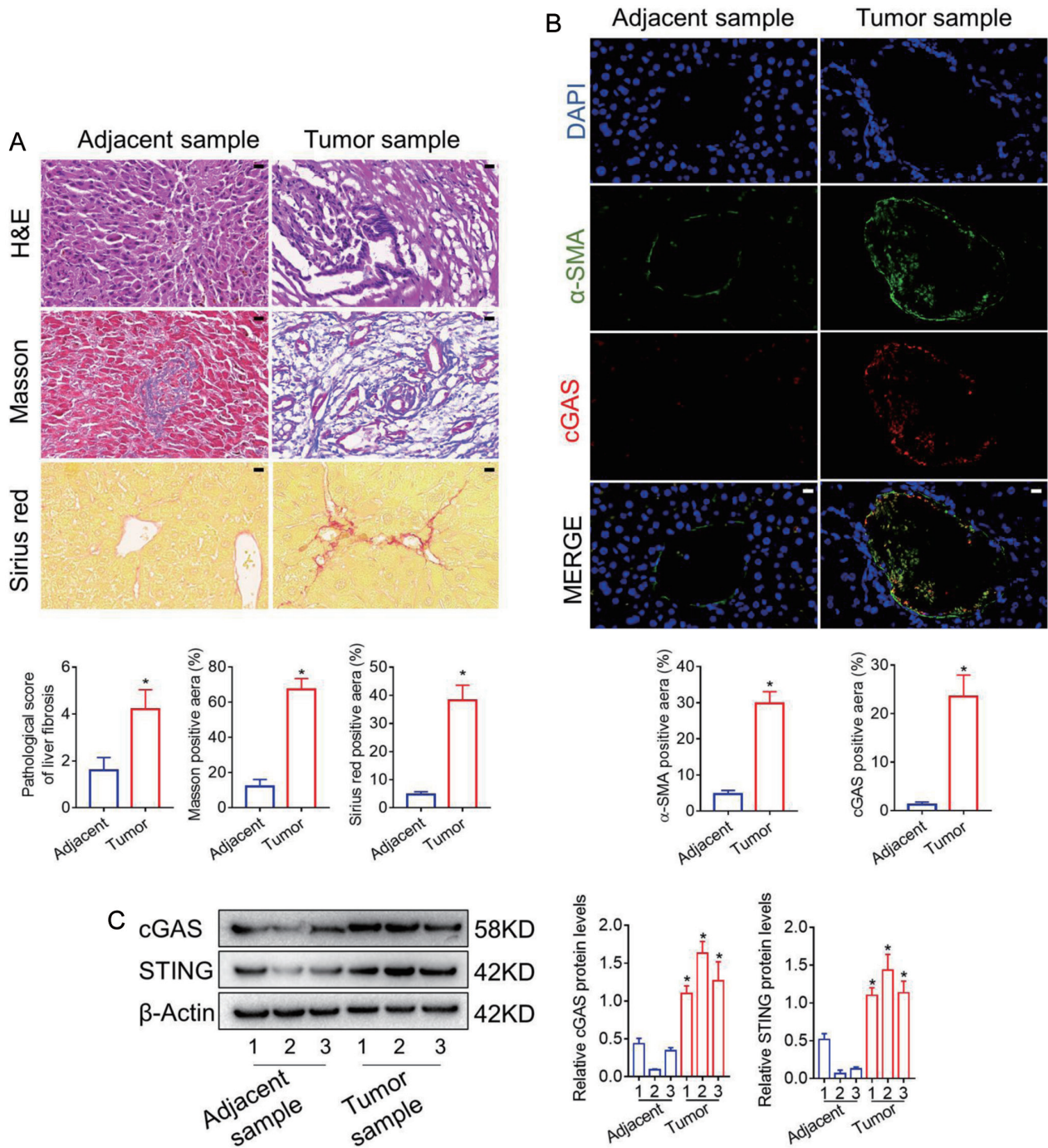


Fig. 2. STING-related proteins were significantly increased in liver fibrosis tissues from liver cancer patients. (A) Samples from liver cancer patients, HE, Masson, and Sirius red staining confirmed the occurrence of liver fibrosis in the samples. Scale bar 20 μ m. (B) Fluorescence staining of the HSC activation indicators α -SMA and cGAS in liver tissue sections. Scale bar 50 μ m. (C) WBs and quantitative analysis show high protein expression of cGAS and STING in liver tumor tissue. Adjacent sample versus tumor sample ($n=3$) * $p<0.05$. α -SMA, alpha smooth muscle actin; cGAS, cyclic guanosine monophosphate-adenosine monophosphate synthase; STING, stimulator of interferon genes.

two compounds was -6.179 .

Naringenin inhibits the activation of HSCs in vitro

We attempted to verify the antihepatic fibrosis effective-

ness and mechanism of naringenin in liver fibrosis *in vitro*. MTT assays showed that naringenin significantly inhibited fibrosis in LX2 cells at 20 μ M but had no effect in L02 cells (Fig. 5A). Therefore, in follow-up experiments, naringenin concentrations of 0, 10, 20, and 40 μ M were used to verify the dose-dependent influence. The real-time PCR results

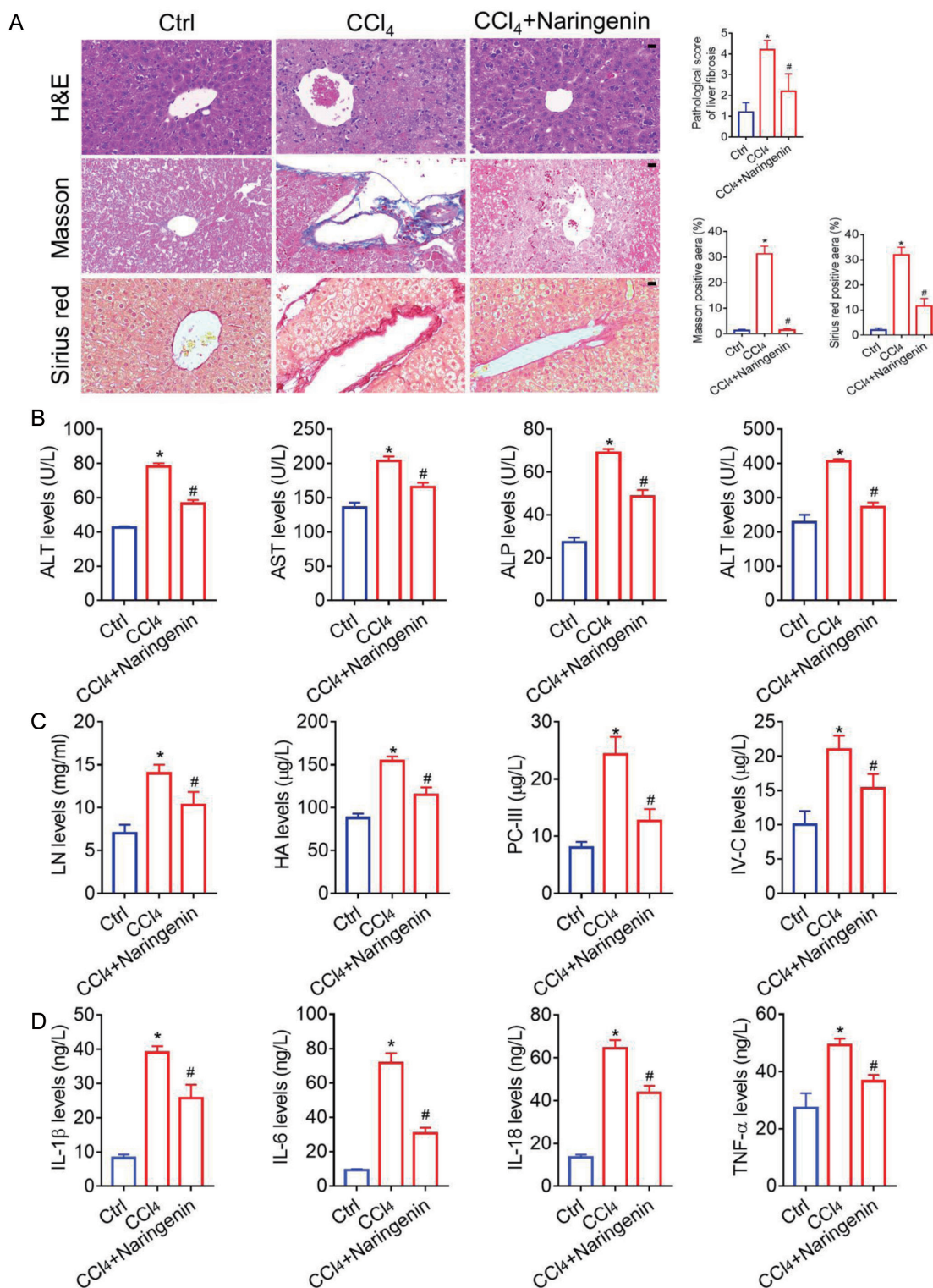


Fig. 3. Naringenin reduces liver fibrosis in mice. Mice with CCl₄-induced fibrosis were treated with naringenin (100 mg/kg) for 8 weeks (3 times/week). (A) HE, Masson, and Sirius red staining show levels of liver damage and fibrosis. Naringenin significantly reduced liver tissue damage and liver fibrosis that caused by CCl₄. Scale bar 20 μm. (B) Serological testing showed that naringenin reduced the levels of ALT, AST, ALP, and ALT in liver fibrosis mice caused by CCl₄. (C) Serological testing showed that naringenin reduced the levels of LN, HA, PC-III, and IV-C in CCl₄-induced liver fibrosis mice. (D) ELISA showed that naringenin significantly reduced the expression of inflammatory factors such as IL1β, IL6, IL18 and TNFα in CCl₄-induced liver fibrosis mice. Statistics for this figure: n=3/group, **p*<0.05, versus control; #*p*<0.05, versus CCl₄. CCl₄, carbon tetrachloride; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; LN, Laminin; HA, hyaluronic acid; PC-III, procollagen type-III; IV-C, collagen type-IV.

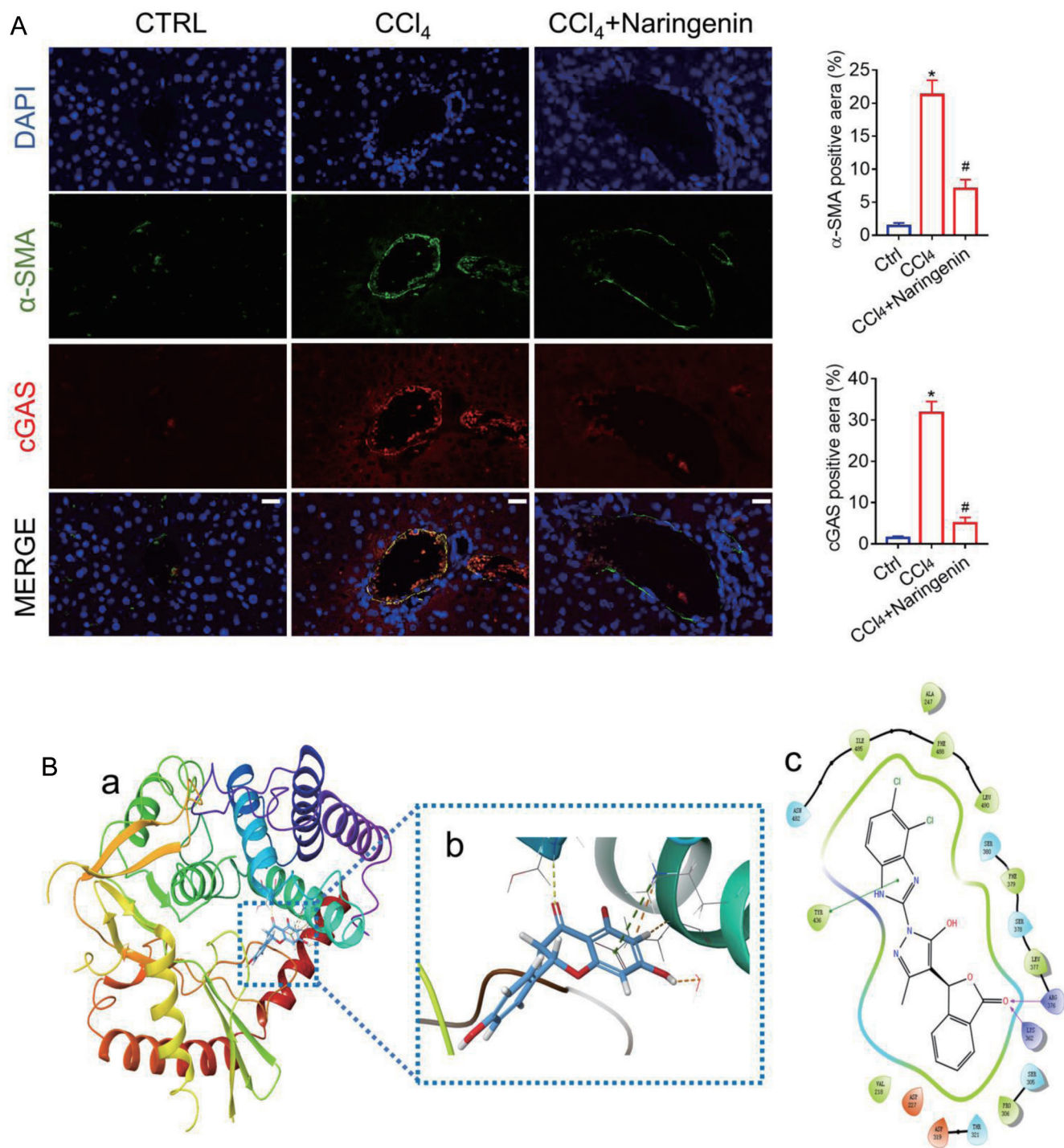


Fig. 4. Naringenin targets cGAS in HSCs. Mice with CCl₄-induced fibrosis were treated with naringenin (100 mg/kg) for 8 weeks (3 times/week). (A) Fluorescence staining of HSC activation indicators α-SMA and cGAS in liver tissue sections. α-SMA and cGAS were coexpressed in liver tissues, and naringenin reduced the expression levels of α-SMA and cGAS in CCl₄-induced liver fibrosis. Scale bar 20 μm. (B) Molecular docking of naringenin and cGAS using molecular modeling packages in the Maestro workstation. (A) cGAS, (B) naringenin, (C) Two-dimensional diagram after molecular docking. α-SMA, alpha smooth muscle actin; cGAS, cyclic guanosine monophosphate-adenosine monophosphate synthase.

showed that naringenin inhibited the expression of the LX2 activation markers α-SMA and α1-procollagen in a dose-dependent manner (Fig. 5B). The WB results also showed that naringenin dose-dependently reduced the α-SMA and col-

lagen1 protein in LX2 cells (Fig. 5C). Immunofluorescence staining further confirmed the above results (Fig. 5D). The results indicated that naringenin significantly inhibited HSC activation *in vitro*.

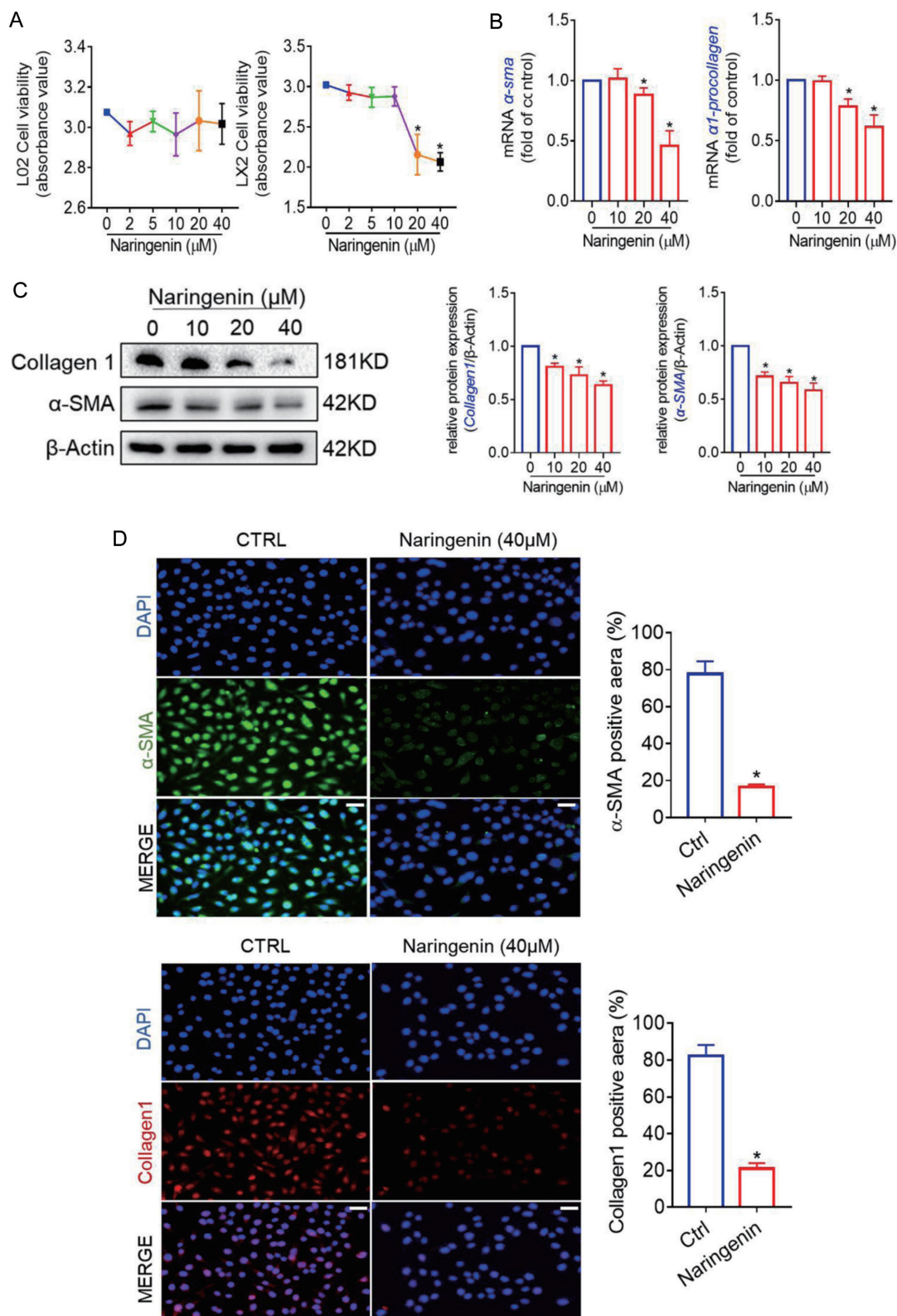


Fig. 5. Naringenin inhibits the activation of HSCs *in vitro*. HSC-LX2 were treated with naringenin for 24 h. (A) MTT assays showed that naringenin significant inhibited LX2 at 20 μM and had no effect on L02 cells. (B) Real-time PCR shows that naringenin inhibited expression of the LX2 activation indicators $\alpha\text{-SMA}$ and $\alpha1\text{-procollagen}$ in a dose-dependent manner. (C) WBs showed that naringenin dose-dependently reduced protein expression of $\alpha\text{-SMA}$ and collagen1 in LX2 cells, quantitative analysis, $n=3$. $*p<0.05$, versus naringenin (0 μM). (D) Immunofluorescence. Scale bar 20 μm . $*p<0.05$, versus naringenin (0 μM). $\alpha\text{-SMA}$, alpha smooth muscle actin.

Naringenin reduces the secretion of inflammatory factors in LX2 cells by inhibiting the cGAS pathway

To explore the mechanisms underlying the ability of naringenin to reduce liver fibrosis, we assayed the mRNA expression of cGAS and related inflammatory factors in LX2 cells by real-time PCR. The results showed that naringenin significantly reduced the expression of cGAS and STING mRNA in LX2 cells (Fig. 6A), and also inhibited the mRNA levels of inflammatory factors, such as IL1 β , IL6, NF-kappa B, and IL8 in LX2 cells (Fig. 6B). WB assays confirmed that naringenin dose-dependently reduced the expression cGAS, STING, and related inflammatory factors in LX2 cells (Fig. 6C), and also dose-dependently reduced the expression IRF3 protein (Supplemental Fig. 1B).

To clarify the mechanism underlying the effect of naringenin on the cGAS-STING pathway, we added exogenous dsDNA (1 μ g/ml) to LX2 cells to specifically stimulate the cGAS signal, and treated LX2 cells with naringenin (40 μ M) for 24 h. WB assays showed that dsDNA promoted the secretion of IL1 β , IL6, TNF- α , and IL8 by activating cGAS and STING and that naringenin suppressed those effects (Fig. 6D). Naringenin also offset the activation of IRF3 induced by dsDNA (Supplementary Fig. 1C). The results indicate that naringenin inhibited the cGAS-STING pathway in HSCs, thereby inhibiting the secretion of inflammatory factors and promoting the alleviation of liver fibrosis.

Discussion

Persuasive evidence has indicated that anti-inflammatory drugs block the progressive development of liver fibrosis.¹⁹ In this study, an analysis of the GEO database showed that the inflammation-related cGAS-STING pathway may be closely associated with the development of chronic liver disease and fibrosis. In clinical liver tissue samples, we found evidence that cGAS-STING is positively related to the expression of liver fibrosis indicators. In the CCl₄-induced mouse liver fibrosis model, we confirmed that naringenin primarily reduced symptoms of fibrosis by inhibiting inflammation. In particular, molecular docking experiments showed that the molecular structure of naringenin allowed binding with cGAS. The combined application of the cGAS-specific agonist dsDNA and naringenin confirmed the specific binding relationship between naringenin and cGAS at the cellular level. Binding inhibited the activation of the cGAS-STING pathway in activated HSCs, thereby reducing the secretion of inflammatory factors and ultimately improving liver fibrosis indicators.

Liver fibrosis is considered an inflammation-related disease,²⁰ and fibrotic livers present with a large accumulation of inflammatory factors. Although the emerging concept of immune metabolism mainly focuses on immune cells, HSCs represent a significant contribution to the convergence pathways of metastatic inflammation and tissue injury, especially in alcoholic and nonalcoholic steatohepatitis.⁸ HSCs produce and respond to inflammatory mediators, including cytokines and chemokines that amplify the liver's response to injury. The interaction between HSCs and inflammatory cells can drive inflammation and injury through paracrine signals. For example, HSCs interact with macrophages through the MER-TK receptor to cause fibrosis in experimental NASH.²¹

cGAS is a cytoplasmic pattern recognition receptor that acts as a DNA sensor to activate the NF-kappa B signaling pathway to produce inflammatory factors.²² STING can bind to activate NF-kappa B, which is the main regulator of inflammation and considered to play a central role in liver injury.²³ Many studies have confirmed that NF-kappa B is

closely related to the progression of liver fibrosis. For example, Liu *et al.*²⁴ reported that Liuweiwuling tablets improved BDL-induced liver fibrosis, mainly by inhibiting the NF-kappa B signaling pathway. Additional evidence from Liu *et al.*²⁵ showed that direct inhibition of NF-kappa B signaling pathway activation induced by drugs in activated HSCs inhibited secretion of downstream inflammatory factors, which had an antifibrosis effect in the liver. Many studies have focused on the ability of NF-kappa B to regulate the production of inflammatory factors in HSCs and have shown that inhibiting the NF-kappa B signaling pathway using drugs improves liver fibrosis, but studies on antifibrosis drugs related to cGAS are relatively rare. As an inflammation-mediated molecule that has received more attention in recent years, cGAS may have a key role in the secretion of inflammatory factors in HSCs. Persistent liver damage can cause liver cells to rupture, and their intracellular contents act as damage-related molecular patterns (DAMPs), thus causing additional leukocyte infiltration and amplifying the original damage. Necrosis-derived DNA can be recognized as a DAMP that activates liver nonparenchymal cells, such as HSCs.²⁶ As a DNA sensor, cGAS must play an important role in the progression of liver damage.

Naringenin is a flavonoid with good anti-inflammatory activity. In this study, we verified for the first time that naringenin and cGAS can directly combine. Naringenin can be used as a specific antagonist of cGAS to antagonize the effect of dsDNA, which attenuates the secretion of inflammatory factors in HSCs and inhibits the activation of HSCs. The results of this study provide important insights on the anti-inflammatory effects of naringenin and its potential pharmacological mechanism for the treatment of chronic liver disease.

In summary, the study shows that naringenin inhibited HSC activation and inflammation by disrupting the cGAS-STING signaling pathway in HSCs, thus preventing the further development of CCl₄-induced liver fibrosis in mice. Interruption of the cGAS-STING pathway was helpful in reversing the fibrosis. The results indicate that naringenin may help to prevent or reverse the progression of liver injury and may be used to develop a new therapeutic approach for chronic liver diseases.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Designed the study and supervised the data collection (LC), analyzed and interpreted the data (LC, SX, SW), provided material and technique support (YZ, FW, ZL, YL, DK, ZZ, JS, XX), and reviewed the draft of the manuscript and made

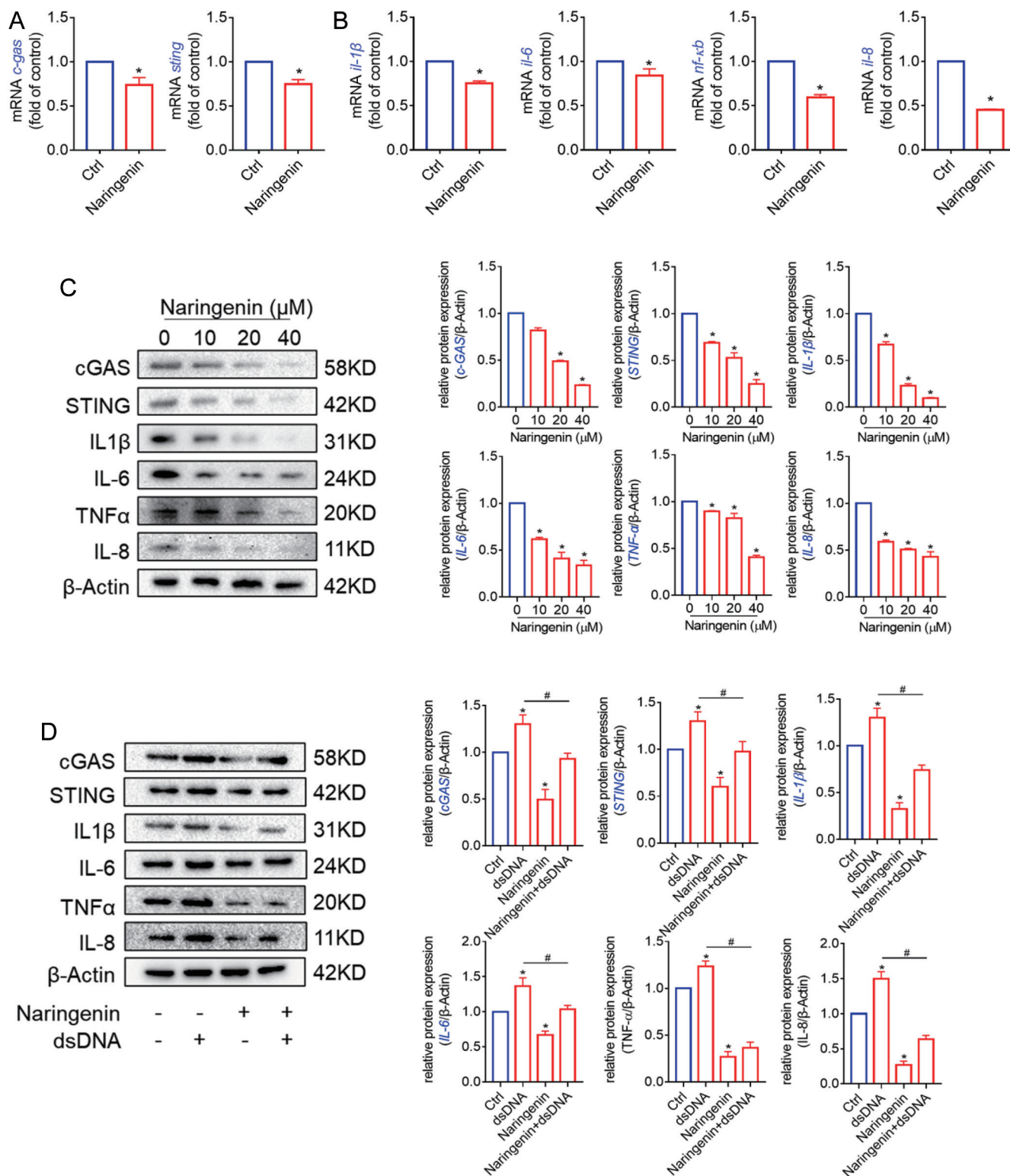


Fig. 6. Naringenin reduces the secretion of inflammatory factors in LX2 cells by inhibiting cGAS pathway. HSC-LX2 were treated with naringenin for 24 h. (A) Naringenin significantly reduced the mRNA levels of cGAS and STING in LX2 (B) Naringenin inhibited the mRNA expression of inflammatory factors, such as IL-1 β , IL-6, NF-kappa B, and IL-8 in LX2 cells. (C) WB assay show that naringenin dose-dependently reduced the protein expression of cGAS, STING, and related inflammatory factors in LX2 cells, quantitative analysis, $n=3$. * $p<0.05$, versus naringenin (0 μ M). (D) WB assay show that dsDNA promoted the secretion of IL-1 β , IL-6, TNF- α , and IL-8 by activating cGAS and STING, while naringenin suppressed those effects, quantitative analysis, $n=3$. * $p<0.05$, versus control; # $p<0.05$, versus dsDNA treatment. α -SMA, alpha smooth muscle actin; cGAS, cyclic guanosine monophosphate-adenosine monophosphate synthase; STING, stimulator of interferon genes.

critical revisions (FZ, SZ). All authors have read and approved the manuscript.

Ethical statement

Animal experimental procedures were approved by the Institutional and Local Committee on the Care and Use of Animals of Nanjing University of Chinese Medicine (approval No. SYLL-2021-002). All animals received humane care according to the National Institutes of Health (USA) guidelines. The liver cancer tissues and adjacent normal tissues of human liver cancer patients were collected in Jiangsu Provincial Hospital of Traditional Chinese Medicine from July 2018 to December 2021. This study was approved by the hospital ethics committee (ethics approval number: 2018-050), the patient and family members informed consent, and signed informed consent.

Data sharing statement

The raw data used to support the findings of this study are available from the corresponding author upon request.

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