

Dihydromyricetin inhibits aberrant cGAS-STING signaling activation and renal fibrosis in diabetic nephropathy by promoting ubiquitin-mediated STING degradation

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ABSTRACT: This study aims to investigate whether dihydromyricetin (DHM) can alleviate diabetic nephropathy (DN) by promoting the ubiquitination and degradation of STING. Network pharmacology analysis indicates that the cGAS-STING pathway is a potential target. In a streptozotocin-induced DN mouse model, DHM improved renal function (as evidenced by reduced urine albumin-to-creatinine ratio, creatinine, and urea nitrogen), and alleviated fibrosis. In high glucose-treated HK-2 cells, DHM decreased the expression of cGAS, and specifically accelerated the degradation of STING protein through the ubiquitin-proteasome pathway. Mechanistically, DHM enhanced the K48-linked polyubiquitination of STING, leading to its degradation by the proteasome and subsequent inhibition of the downstream TBK1-IRF3 signaling pathway and of the production of inflammatory factors. In conclusion, DHM inhibits the cGAS-STING pathway by promoting the K48-ubiquitination and degradation of STING, thereby alleviating renal damage in DN.

KEYWORDS: dihydromyricetin, diabetic nephropathy, cGAS-STING, TANK-binding kinase, interferon receptor factor

INTRODUCTION

Diabetic nephropathy (DN) represents one of the most prevalent and severe microvascular complications arising from diabetes mellitus and has emerged as a leading contributor to end-stage renal disease globally, consequently placing a steadily escalating strain on public healthcare systems [1]. The characteristic pathological features of DN include excessive deposition of extracellular matrix components, glomerulosclerosis, and tubulointerstitial fibrosis. Current therapeutic strategies for DN in clinical practice are predominantly centered on rigorous glycemic control, effective blood pressure management, and administration of renin-angiotensin-aldosterone system inhibitors [2]. While these approaches can retard disease progression to a degree, a considerable proportion of patients ultimately develop an irreversible and progressive decline in renal function [3], underscoring the intricate nature of DN pathogenesis. This pathological process encompasses multiple interconnected mechanisms, such as hyperglycemia-induced oxidative stress, sustained inflammatory responses, renal interstitial fibrosis, and glomerulosclerosis. Among these, the establishment of a chronic inflammatory microenvironment within the kidney, coupled with progressive fibrotic changes, is considered a principal driver of the relentless deterioration in renal function [4]. Hence, there is an urgent need to move beyond conventional treatment paradigms, delving deeper into the fundamental molecular mechanisms governing DN progression to identify novel therapeutic targets and potential drug candidates.

Within this complex pathological landscape, the cyclic GMP-AMP synthase (cGAS, also designated MB21D1)-stimulator of interferon genes (STING) signaling axis, an innate immune pathway that has attracted considerable research interest recently, has been identified as playing a pivotal role in DN initiation and progression [5,6]. Physiologically, the cGAS-STING pathway functions to detect aberrant cytoplasmic double-stranded DNA, including microbial DNA or self-DNA released following cellular damage, subsequently triggering the expression of type I interferons and other inflammatory mediators as part of host defense [7]. However, under diabetic conditions, hyperglycemia can induce mitochondrial dysfunction, resulting in the escape of mitochondrial DNA (mtDNA) into the cytosol. This released mtDNA is recognized by cGAS, which catalyzes the formation of the second messenger cGAMP, leading to STING activation [8]. Upon activation, STING undergoes conformational rearrangement and translocates to the Golgi apparatus, where it recruits downstream effectors such as TANK-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3). This cascade ultimately drives the production and release of inflammatory molecules, including type I interferons (e.g., IFN- β), TNF- α , and IL-6, thereby amplifying the local renal inflammatory response [9]. Evidence from both preclinical models, such as db/db mice, and clinical specimens from DN patients demonstrates significant upregulation of cGAS and STING expression in renal tissues, with their activation levels positively correlated with biomarkers of renal injury and fibrosis [10]. Collectively, these observations position aberrant cGAS-STING pathway

activation as a critical nexus linking metabolic dysregulation to the progression of renal inflammation and fibrosis.

To prevent excessive immune activation and subsequent tissue injury, cGAS-STING signaling is tightly controlled through multiple layers of regulation. The ubiquitin-proteasome system (UPS) constitutes a vital post-translational regulatory mechanism, mediating the ubiquitination and subsequent proteolytic degradation of target proteins via an E1-E2-E3 enzymatic cascade, thereby fine-tuning STING protein stability and functional activity [11]. The STING protein, encoded by the TMEM173 gene, serves as a key mediator of host immune responses against pathogens [12]. A growing body of research has elucidated the involvement of various E3 ubiquitin ligases in the negative regulation of STING. For example, TRIM21 facilitates K48-linked ubiquitination of STING, targeting it for proteasomal degradation and effectively dampening pathway activity [13]; meanwhile, RNF5 mediates K27-linked ubiquitination, promoting autophagy-dependent clearance of STING [14]. Nevertheless, under the persistent metabolic stress characteristic of DN, STING ubiquitination levels are markedly diminished, leading to impaired degradation, enhanced protein stability, and sustained pathway hyperactivation, which exacerbates renal pathological damage [15]. Consequently, therapeutic strategies aimed at actively promoting STING ubiquitination and subsequent degradation may offer a more fundamental and specific approach compared to merely inhibiting its kinase function, yet exploration of this concept in the context of DN remains nascent.

Dihydromyricetin (DHM), a natural dihydroflavonol compound derived from plants like *Ampelopsis grossedentata*, has garnered widespread attention for its significant antioxidant, anti-inflammatory, and anti-fibrotic activities [16]. Prior investigations have demonstrated that DHM can ameliorate insulin resistance and glucose metabolic disturbances in diabetic animal models, and it exhibits renoprotective effects in various forms of kidney injury, potentially through mechanisms involving inhibition of the TGF- β /Smad signaling axis and attenuation of oxidative stress [17, 18]. Of particular relevance, recent reports indicate that DHM can modulate the cGAS-STING signaling pathway in models of viral pneumonia and hepatic fibrosis, suggesting its potential as a regulator of this pathway [19]. However, whether DHM exerts its beneficial effects in DN through modulation of this pathway, and specifically whether it can directly impact STING protein stability, remain to be elucidated. Given its chemical structure, which facilitates interactions with protein targets, DHM theoretically possesses the capacity to influence STING ubiquitination dynamics.

Based on the foregoing background and analysis, the central hypothesis guiding this investigation is that DHM may attenuate renal injury in DN by promoting K48-linked polyubiquitination of the STING

protein, thereby enhancing its degradation through the ubiquitin-proteasome pathway. This would result in negative regulation of aberrant cGAS-STING signaling activation, ultimately suppressing downstream inflammatory cytokine production and mitigating renal fibrogenesis. This study was designed to systematically test this hypothesis through a combination of *in vivo* and *in vitro* approaches. The findings are expected not only to provide direct evidence elucidating a novel mechanism underlying the renoprotective effects of DHM but also to pave the way for developing innovative therapeutic strategies for DN focused on modulating STING protein stability.

MATERIALS AND METHODS

Network pharmacology analysis

RNA-seq expression data (GSE142025)

To identify genes differentially expressed in DN, transcriptomic data were obtained from the Gene Expression Omnibus (GEO) repository under accession code GSE142025 (<https://www.ncbi.nlm.nih.gov/geo/>). This dataset consists of renal tissue expression profiles generated via the Illumina platform from streptozotocin (STZ)-induced DN mice and healthy controls. Specimens were clearly divided into DN and normal control groups. These sequencing data were subsequently used to identify transcripts significantly altered during DN pathogenesis, thereby establishing a foundation for downstream network analyses.

Drug target prediction (PharmMapper)

The molecular structure of DHM was retrieved as a two-dimensional structure from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>, accessed on 18 March 2025). To identify candidate protein targets, the PharmMapper web server (<https://lilab-ecust.cn/pharmmapper/>, accessed on 18 March 2025) was used; this platform applies reverse pharmacophore matching algorithms to predict interactions between small molecules and potential binding proteins. The analytical workflow involved uploading the DHM structure file (in .mol2 or .sdf format), selecting the human protein target subset for screening, and subsequently filtering results to retain only human-derived targets. Corresponding gene symbols were then retrieved from UniProt entries, yielding a final list of putative DHM-interacting proteins.

DN-related genes (GeneCards)

Genes implicated in DN were retrieved from the GeneCards database (<https://www.genecards.org/>, accessed 21 March 2025). This platform aggregates functional annotations from multiple sources, permits disease-based queries to identify relevant genes, and employs “relevance scores” to quantify the strength of association between individual genes and specific

pathological conditions. For the present analysis, “Diabetic Nephropathy” was entered as the search term. Differential expression analysis between DN and normal samples was conducted using the limma package in the R environment, applied to the transcriptomic data. Genes exhibiting a relevance score exceeding 10 were selected as high-confidence candidates, forming a preliminary DN-associated gene set. Criteria for defining differentially expressed transcripts were $|\log_2 \text{fold change}| > 1$ and adjusted p -value (adj. p) < 0.05 , with up-regulated and down-regulated genes segregated accordingly. Functional annotation was performed using Gene Ontology enrichment analysis, and pathway enrichment was assessed via the Kyoto Encyclopedia of Genes and Genomes database, with a significance threshold of $p < 0.01$. All graphical representations were generated using R software.

Experimental animals

A total of thirty male C57BL/6 mice, aged 6–8 weeks and weighing 20–22 g, were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China). All animals were specific pathogen-free and were maintained in a dedicated SPF facility at the Ningbo No.2 Hospital, Wenzhou Medical University Animal Center under controlled environmental conditions: a 12-h light/dark cycle, ambient temperature of $22 \pm 1^\circ\text{C}$, and relative humidity of 40–50%. Food and water were provided ad libitum throughout the study. All experimental procedures were performed in compliance with the National Act on the Use of Laboratory Animals (China) and received approval from the Animal Ethics Committee of Ningbo No.2 Hospital, Wenzhou Medical University.

Experimental methods

Following a one-week acclimatization period, mice were randomly allocated to three experimental groups ($n = 10$ per group): normal control (Normal), diabetic nephropathy model (DN), and dihydromyricetin-treated (DN + DHM). To induce diabetes, animals in the DN and DN + DHM groups received intraperitoneal injections of STZ (Sigma-Aldrich, USA) at a dose of 50 mg/kg body weight for five consecutive days after a 12-h fasting period. STZ was freshly dissolved in 0.1 mol/l citrate buffer (pH 4.5). The Normal group received equivalent volumes of citrate buffer alone. Seventy-two hours following the final injection, tail vein blood samples were collected, and random blood glucose concentrations were determined using a glucometer (OneTouch, USA). Successful induction of diabetes was defined as blood glucose levels ≥ 16.7 mmol/l [20]. After diabetes confirmation, animals were maintained for an additional 8 weeks, during which blood glucose was monitored weekly. At the end of the eighth week, 24-h urine specimens were collected to measure the urinary albumin-to-creatinine ratio (UACR) and related parameters. Upon

confirmation of successful DN model establishment, the DN + DHM group received daily oral gavage of DHM (purity $\geq 98\%$; Sigma-Aldrich) at 100 mg/kg body weight, whereas the Normal and DN groups received equivalent volumes of normal saline. This intervention continued for 8 weeks [21].

Renal function assay

At the study endpoint after the eight-week intervention, 24-h urine samples were collected from mice housed in metabolic cages (Beijing HFK Bioscience Co., Ltd., China), and total urine volume was recorded. Urinary albumin concentration was quantified in 100 μl aliquots using the Coomassie Brilliant Blue method (Beyotime, China, cat. no. P0018), enabling calculation of total 24-h albumin excretion. Blood samples were obtained via orbital sinus puncture, and serum was isolated by centrifugation at 3,000 rpm for 10 min at 4°C . Serum creatinine (Scr) and blood urea nitrogen (BUN) concentrations were measured using an automated biochemical analyzer. Urinary creatinine levels were determined via the sarcosine oxidase method, and the UACR was subsequently calculated (expressed in mg/g).

Renal tissue sample collection and processing

Following anesthesia induced by intraperitoneal injection of 10% chloral hydrate (3 ml/kg), mice underwent cardiac puncture for blood collection, after which both kidneys were promptly excised. One kidney was immediately placed into cryovials, flash-frozen in liquid nitrogen, and subsequently stored at -80°C for later RNA extraction and Western blot analysis. The contralateral kidney was immersed in 4% paraformaldehyde fixative (Solarbio, China) for 24 h at room temperature. After undergoing graded dehydration, clearing with xylene, and paraffin embedding, serial sections of 4 μm thickness were prepared. These sections were subsequently utilized for Hematoxylin and Eosin (H&E) staining, Masson's trichrome staining, and immunohistochemistry.

H&E staining

Paraffin-embedded kidney sections were first deparaffinized and rehydrated through sequential immersion in xylene and graded ethanol solutions, after which H&E staining was performed. In brief, sections were incubated with hematoxylin for 5 min, differentiated in acidified ethanol, and rinsed with tap water to achieve blueing. Following this, sections were counterstained with eosin for 2 min, dehydrated through an ascending ethanol series, cleared in xylene, and finally mounted using neutral balsam. Morphological assessment was conducted under a light microscope. Renal injury was quantified employing a semi-quantitative scoring system with the following criteria: 0, no damage; 1, affected area $< 25\%$; 2, $25\% \leq$ affected area $< 50\%$; 3, $50\% \leq$ affected area $< 75\%$; 4, affected area $\geq 75\%$.

75%. Parameters evaluated included glomerular atrophy, tubular dilation, proteinaceous cast formation, and inflammatory cell infiltration. For each animal, five non-overlapping fields at $\times 200$ magnification were randomly selected and scored, and the mean value was calculated to derive the final injury score.

Masson's trichrome staining

Following deparaffinization and rehydration, tissue sections were stained with Weigert's iron hematoxylin for 5 min and then rinsed with tap water. Subsequently, sections were incubated with Ponceau-acid fuchsin for 10 min, differentiated in 1% phosphomolybdic acid solution for 5 min, and counterstained with aniline blue for an additional 5 min. A brief treatment with 1% acetic acid for 1 min was applied, followed by dehydration through graded ethanol, clearing in xylene, and mounting with neutral balsam. To quantitatively assess interstitial collagen deposition, ImageJ software was employed. For each specimen, five randomly selected non-overlapping fields at $\times 200$ magnification were analyzed. The collagen volume fraction (CVF) was defined as the percentage of blue-stained collagen fiber area relative to the total field area, and the mean value from the five fields was subjected to subsequent statistical evaluation.

Immunohistochemistry staining

Paraffin-embedded sections were deparaffinized, rehydrated, and incubated with 3% H_2O_2 for 10 min at room temperature to quench endogenous peroxidase activity. Heat-induced antigen retrieval was performed using high-temperature and high-pressure treatment in citrate buffer (pH 6.0) for 15 min, after which sections were allowed to cool naturally. Non-specific binding sites were blocked with 5% bovine serum albumin for 30 min. Primary antibody incubation was performed overnight at 4°C using antibodies directed against α -smooth muscle actin (α -SMA; ab5694, Abcam, UK, 1:200), collagen type I (Col I; ab1138492, Abcam, 1:200), cGAS (ab252416, Abcam, 1:200), and STING (3337, Cell Signaling Technology, USA, 1:100). Following three washes with PBS (5 min each), sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (ab6721, 1:2000, Abcam) for 1 h at ambient temperature. Immunoreactivity was visualized using a DAB substrate kit (ZSGB-Bio, China), followed by counterstaining with hematoxylin. After dehydration, clearing, and mounting with neutral balsam, all sections were examined under light microscopy. Quantitative analysis of immunostaining was performed using ImageJ software. For each marker, five non-overlapping fields at $\times 200$ magnification were randomly selected, and the integrated optical density of positive staining was measured. Mean values were calculated for statistical comparisons.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total cellular RNA was isolated from mouse kidney tissues and cultured cells subjected to various treatments using Trizol reagent (Life Technology, USA) in accordance with the manufacturer's protocol. Complementary DNA (cDNA) was synthesized through reverse transcription of mRNA templates following the supplier's guidelines. qRT-PCR amplifications were conducted employing the SYBR GreenER qPCR SuperMix kit (Thermo Fisher Scientific, USA) on an ABI PRISM 7700 sequence detection system (Thermo Fisher Scientific). Each 25 μ l reaction mixture contained 0.2 μ g cDNA, 10 μ M gene-specific primers, and SuperMix components. Primer sequences for target genes are detailed in Table S1 and were synthesized by Invitrogen (USA) and Takara (China). Thermal cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. GAPDH served as the internal reference gene for normalization. Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method with ABI Prism 7700 SDS Software (Thermo Fisher Scientific).

Western blot analysis

Protein lysates were prepared from mouse kidney tissues and differentially treated cells using RIPA lysis buffer (Beyotime) according to the manufacturer's instructions. Protein concentrations in clarified supernatants were determined using the BCA protein assay kit (Beyotime). Equivalent amounts of protein were resolved by SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes. Following blocking of non-specific binding sites with 5% non-fat milk, membranes were incubated overnight at 4°C with primary antibodies recognizing the following targets: cGAS (ab252416, 1:1000, Abcam), STING (ab288157, 1:1000, Abcam), phospho-STING (Ser366; 19781, 1:1000, Cell Signaling Technology), phospho-TBK1 (D52C2, 1:1000, Cell Signaling Technology), α -SMA (ab5694, 1:1000, Abcam), phospho-IRF3 (4D4G, 1:1000, Cell Signaling Technology), Col I (ab138492, 1:1000, Abcam), fibronectin (ab45688, 1:500, Thermo Fisher Scientific), and GAPDH (ab213508, 1:1000, Abcam). After washing with PBS, membranes were incubated with HRP-conjugated secondary antibody (ab6721, 1:2000, Abcam) for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence substrate (ECL Western Blotting Substrate, Thermo Scientific Pierce, USA).

Cell culture and treatment

Human kidney-2 (HK-2) renal tubular epithelial cells, obtained from the Cell Bank of the Chinese Academy of Sciences, were maintained in DMEM/F12 medium

(Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Solarbio, China). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

At 70–80% confluence, cells were subjected to various experimental conditions as follows: normal glucose control (5.5 mmol/l D-glucose); high glucose (HG; 30 mmol/l D-glucose); HG with low-dose DHM (50 µmol/l); HG with medium-dose DHM (100 µmol/l); HG with high-dose DHM (200 µmol/l); HG with DHM (100 µmol/l) plus the proteasome inhibitor MG132 (10 µmol/l); and HG with DHM (100 µmol/l) plus the autophagy inhibitor chloroquine (CQ, 20 µmol/l). After 24 h of treatment, cells were harvested for subsequent analyses [22–24].

STING protein half-life assay

Following 24 h of the specified treatments, HK-2 cells were exposed to 100 µg/ml cycloheximide (CHX), a protein synthesis inhibitor, to block new protein production. Total protein extracts were prepared at 0, 2, 4, and 6 h after CHX addition. STING protein abundance at each time point was evaluated by immunoblotting and expressed as a percentage relative to the level at 0 h (defined as 100%). Degradation kinetics were analyzed using GraphPad Prism version 10.12, and the STING protein half-life was calculated from these data.

Co-immunoprecipitation (Co-IP) assay

Following collection, cells from each experimental group were lysed in IP buffer supplemented with protease and phosphatase inhibitors. Homogenates were maintained on ice during processing, then clarified by centrifugation at 12,000 rpm for 15 min at 4°C. The resulting supernatants were collected, and aliquots containing 500 µg of total protein were incubated overnight with 2 µg of STING-specific antibody at 4°C under gentle rotation. Subsequently, 30 µl of Protein A/G agarose beads (Santa Cruz, USA) were added, and the mixtures were incubated for an additional 4 h at 4°C. Bead-bound immune complexes were pelleted by centrifugation at 3,000 rpm for 5 min at 4°C, followed by five washes with IP buffer (5 min per wash). Captured proteins were eluted by resuspending the beads in 5× SDS loading buffer and boiling at 100°C for 5 min. Eluates were then subjected to immunoblot analysis. STING ubiquitination status was assessed using antibodies recognizing total ubiquitin (43124, 1:1000, Cell Signaling Technology) and K48-linked ubiquitin chains (8081, 1:1000, Cell Signaling Technology) [25].

Statistical analysis

Data are presented as the mean ± SD and were analyzed using GraphPad Prism V.10.12 software (GraphPad Software, San Diego, CA, USA). Differences between two groups were assessed using an unpaired Student's *t*-test (two-tailed). Comparisons of means

among multiple groups were performed using one-way or two-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test. All data met the assumptions of the statistical tests. A two-tailed *p*-value < 0.05 was considered statistically significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

RESULTS

Prediction of DHM targets in network pharmacology

A network pharmacology strategy was utilized to systematically investigate the potential mechanisms through which DHM exerts therapeutic effects in DN. Initial target prediction for DHM was conducted using the PharmMapper database. Concurrently, a comprehensive set of 1864 DN-associated genes was retrieved from the GeneCards database. Comparative analysis between these datasets identified an overlapping set of 82 putative targets common to both DHM and DN (Fig. 1A).

Transcriptomic profiling of renal tissues, using the criteria of $|\log_2 \text{fold change}| > 1$ and *adj. p* < 0.05, was employed to discern differentially expressed genes between DN model mice and normal controls. Visualization using a volcano plot illustrated the distribution of these differentially expressed transcripts, with upregulated genes denoted in red and downregulated genes in blue (Fig. 1B). Heatmap analysis corroborated distinct gene expression profiles between the experimental groups and confirmed satisfactory intra-group reproducibility, thereby validating the reliability of the dataset (Fig. 1C). To refine the focus onto central targets, the 82 intersecting genes were further cross-referenced with the transcriptome-derived differentially expressed gene set. This integrative approach culminated in the identification of six pivotal targets: NEDD4, MB21D1 (encoding cGAS), TMEM173 (encoding STING), COL1A1, ACTA2 (encoding α-SMA), and TGFβ1. Expression analysis demonstrated significant upregulation of both MB21D1 and TMEM173 in the DN group relative to controls, alongside elevated levels of the fibrotic markers COL1A1 and ACTA2 (Fig. 1D). These observations indicate concomitant upregulation of cGAS and STING within the renal milieu under DN conditions.

Subsequent GO enrichment analysis was performed to elucidate the biological processes associated with these core targets, revealing significant enrichment in pathways governing immune responses, inflammatory reactions, regulation of cytokine production, and oxidative stress responses (Fig. 1E). Complementarily, KEGG pathway enrichment analysis demonstrated substantial representation of these genes within inflammation-related cascades, prominently featuring the following signaling pathways: TNF, NOD-like receptor, JAK-STAT, and Toll-like receptor (Fig. 1F). Although the cGAS-STING pathway was not explicitly

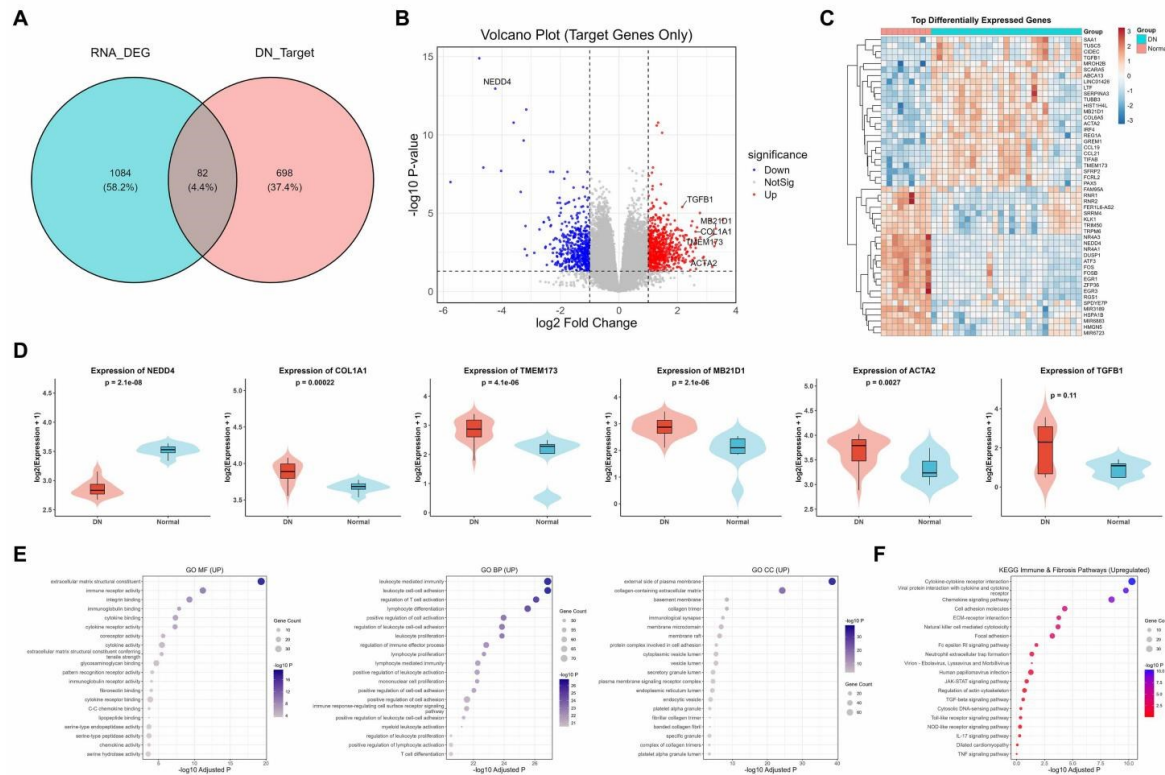


Fig. 1 The cGAS-STING signaling pathway is likely aberrantly activated in the pathogenesis of DN. (A) Venn diagram of relevant targets, (B) Volcano plot of significantly differentially expressed genes, (C) Heatmap of significantly differentially expressed genes, (D) Expression analysis of key target genes, (E) GO functional enrichment analysis, (F) KEGG pathway enrichment analysis. Data are presented as mean $\pm$ SD. $p < 0.05$ was considered statistically significant.

enriched in the KEGG analysis, the concurrent identification of its principal constituents—MB21D1 and TMEM173—as key differentially expressed genes is noteworthy. Furthermore, their expression patterns aligned consistently with the activation status of several enriched innate immune pathways. Collectively, these findings support the proposition that aberrant activation of the cGAS-STING signaling axis constitutes a feature of DN pathogenesis and likely participates in disease progression through interactions with other immune and inflammatory pathways.

DHM improves renal function and renal fibrosis in DN mice

Relative to the normal control group, mice in the DN model group displayed pronounced renal impairment, as evidenced by marked increases in 24-h urinary albumin excretion, UACR, Scr, and BUN. Administration of DHM significantly ameliorated these functional deficits; specifically, 24-h albuminuria and BUN were substantially reduced compared to the untreated DN group, while the UACR and Scr concentrations recovered to values approaching those observed in healthy controls (Table 1). Histological examination using H&E staining revealed preserved renal architecture in

control specimens, with regular glomerular structures, well-organized tubules, and absence of inflammatory infiltrates. Conversely, renal tissues from DN mice exhibited substantial glomerular atrophy, thickening of the glomerular basement membrane, pronounced tubular dilation accompanied by abundant intraluminal proteinaceous casts, and extensive interstitial inflammatory cell accumulation, resulting in a significantly elevated pathological injury score. DHM treatment markedly mitigated these histopathological alterations, as evidenced by attenuated glomerular shrinkage, diminished tubular dilation and cast formation, reduced inflammatory infiltration, and a concomitant decrease in the injury score relative to the DN group (Fig. 2A,B). Assessment of interstitial fibrosis using Masson's trichrome staining demonstrated minimal collagen deposition in the normal kidney interstitium, with a CVF of $3.5 \pm 0.4\%$. In contrast, DN kidneys showed extensive accumulation of collagen fibers, elevating the CVF to $28.6 \pm 2.5\%$. DHM intervention substantially reduced fibrotic deposition, lowering the CVF to $12.3 \pm 1.6\%$ (Fig. 2C,D). Immunohistochemical analysis further corroborated these observations. Compared with normal controls, renal tissues from DN mice displayed significantly elevated integrated optical

Table 1 Renal function parameters in Normal and DN mice (Mean $\pm$ SD; $n = 10$).

Group	Urine albumin (mg/24 h)	UACR (mg/g)	Scr (μ mol/l)	BUN (mmol/l)
Normal	12.5 $\pm$ 2.3	28.4 $\pm$ 5.1	18.6 $\pm$ 3.2	8.5 $\pm$ 1.1
DN	158.3 $\pm$ 25.6 ^{##}	385.7 $\pm$ 45.2 ^{##}	65.8 $\pm$ 8.9 ^{##}	32.4 $\pm$ 4.7 ^{##}
DN + DHM	65.1 $\pm$ 12.4 ⁺⁺	75.2 $\pm$ 15.3 ⁺⁺	25.3 $\pm$ 4.1 ⁺⁺	15.8 $\pm$ 2.6 ⁺⁺

^{##} $p < 0.01$ vs. Normal group; ⁺⁺ $p < 0.01$ vs. DN group.

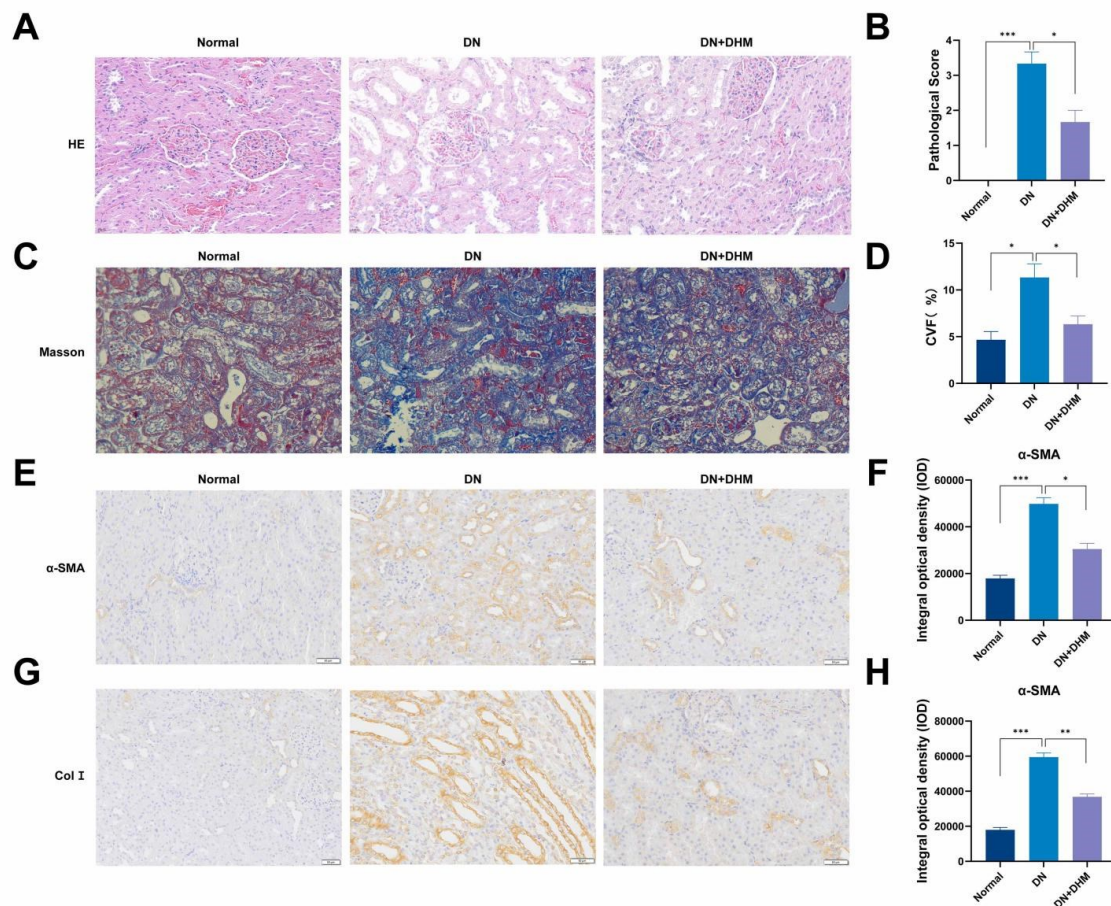


Fig. 2 DHM improves renal function and renal fibrosis in DN mice. (A, B) Representative images of H&E staining and quantitative analysis of pathological injury scores in mouse kidney tissues; (C, D) Representative images of Masson's trichrome staining and quantitative analysis of CVF in mouse kidney tissues; (E–H) Representative images of immunohistochemical staining and quantitative analysis of integrated optical density values for the fibrosis markers α -SMA and Col I in mouse kidney tissues. (A) to (H), ($n = 10$). Data are presented as mean $\pm$ SD. Comparisons were performed using one-way ANOVA. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

density values for the fibrosis markers α -SMA and Col I. DHM treatment resulted in a significant downregulation of both markers (Fig. 2E–H). Collectively, these findings demonstrate that DHM effectively suppresses the progression of renal fibrosis in a murine model of DN.

cGAS and STING are synergistically upregulated in the DN model and positively correlated with renal fibrosis

To elucidate the involvement of the cGAS-STING signaling axis in DN pathogenesis, transcriptomic profil-

ing combined with RT-qPCR validation was performed. These analyses demonstrated marked upregulation of both cGAS and STING mRNA transcripts in renal tissues obtained from DN model mice relative to controls. Consistent with these transcriptional changes, immunoblotting confirmed significantly elevated protein levels of cGAS, STING, and the activated phosphorylated form of STING (p-STING at Ser366) in the DN group (Fig. 3A–C), indicating coordinated activation of this signaling cascade under diabetic conditions. Immunohistochemical staining was employed to localize the expression of these molecules within the

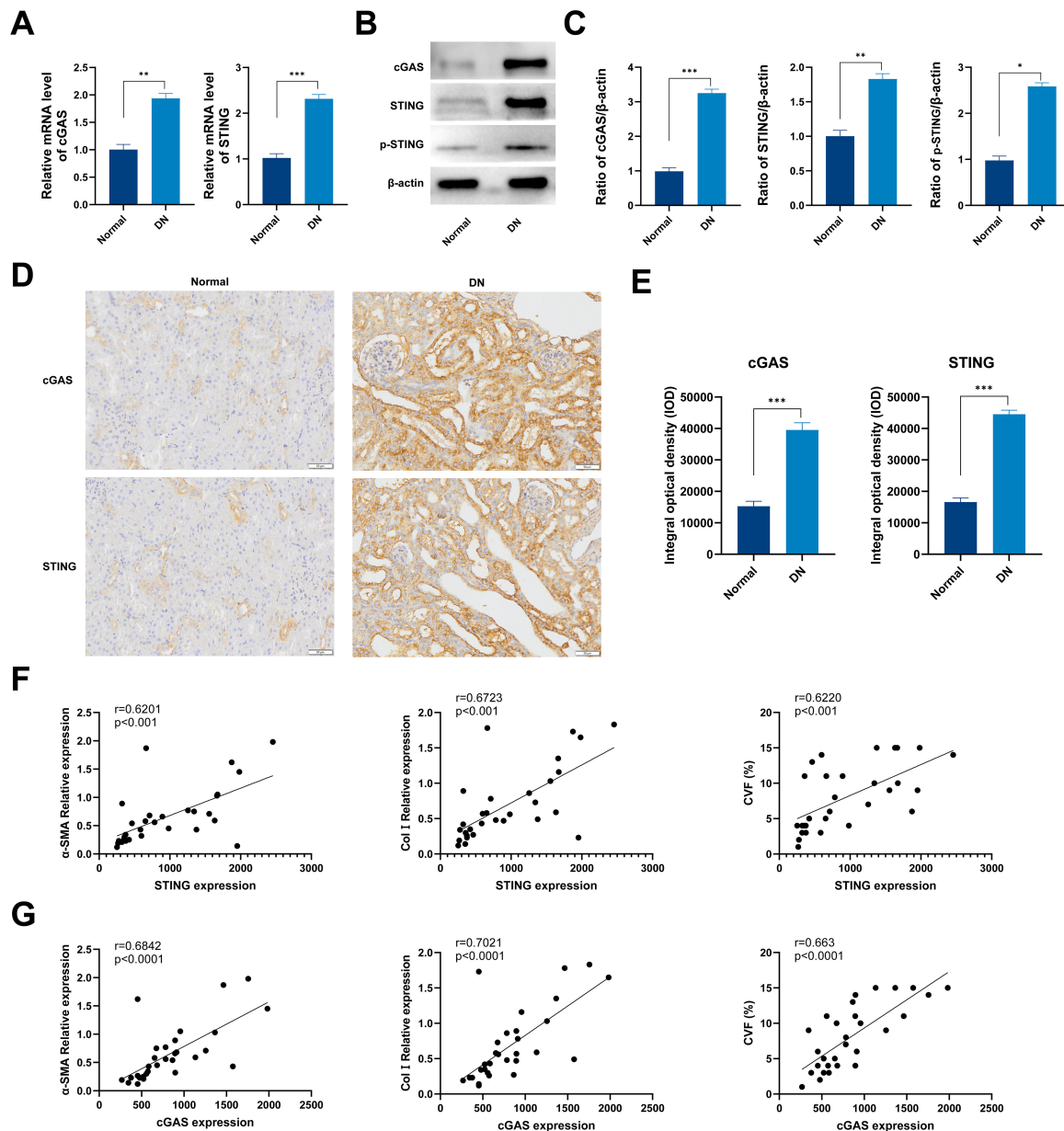


Fig. 3 Upregulated expression of cGAS and STING is significantly positively correlated with the degree of renal fibrosis in DN mice. (A) RT-qPCR analysis of cGAS and STING mRNA expression levels in mouse kidney tissues; (B, C) Western blot analysis of cGAS, STING, and phosphorylated STING (p-STING, Ser366) protein expression levels in mouse kidney tissues; (D, E) Immunohistochemical detection of cGAS and STING expression localization and quantitative analysis of integrated optical density values in mouse kidney tissues; (F, G) Correlation analysis between STING and cGAS protein expression levels and the protein expression levels of α -SMA and Col I, as well as CVF, in DN mouse kidney tissues. (A) to (G), ($n = 10$). Error bars represent at least three independent experiments. Data are presented as mean $\pm$ SD. Comparisons were performed using Student's t -test and one-way ANOVA. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

kidney. Positive signals for both cGAS and STING were predominantly detected in glomerular mesangial cells and interstitial cells. Quantitative analysis revealed substantially higher integrated optical density values for both proteins in DN specimens compared to normal

controls (Fig. 3D,E). Subsequent correlation analyses demonstrated a strong positive association between the expression levels of cGAS and STING and the extent of renal fibrosis. Specifically, cGAS expression exhibited correlation coefficients of 0.6842, 0.7021,

and 0.663 with the fibrotic markers α -SMA, Col I, and CVE, respectively. Similarly, STING expression correlated with these parameters, with coefficients of 0.6201, 0.6723, and 0.622, respectively (all $p < 0.001$; Fig. 3F,G). These observations collectively indicate that the activation status of the cGAS-STING pathway is closely linked to the severity of fibrotic pathology in DN.

DHM improves renal function, inhibits renal fibrosis and STING signaling activation

To explore the underlying mechanisms, the effects of DHM on renal function and fibrotic progression were assessed in the DN mouse model. Treatment with DHM resulted in marked functional improvement and reduced fibrotic pathology, as indicated by a lower CVP and diminished protein expression of fibrotic markers, including α -SMA, fibronectin, and Col I (Fig. 4A,B). Similarly, DHM specifically reduced the levels of c-GAS and STING proteins. This was accompanied by suppression of downstream signaling events: phosphorylation of TBK1 and IRF3, which were elevated approximately 3.8-fold and 3.5-fold, respectively, in untreated DN mice, decreased to 1.7-fold and 1.6-fold relative to control levels after DHM treatment (Fig. 4C,D). Additionally, DHM treatment significantly attenuated the DN-induced upregulation of mRNA transcripts for the pro-inflammatory cytokines IL-6, TNF- α , and IL-1 β (Fig. 4E-G). Collectively, these findings suggest that DHM mitigates renal fibrosis primarily by curtailing STING protein accumulation and subsequently inhibiting the hyperactivation of the TBK1-IRF3 signaling axis, thereby dampening the associated inflammatory response.

DHM drives STING protein degradation by promoting K48-linked polyubiquitination of STING

To investigate how DHM reduces STING protein levels, cultured human renal tubular epithelial cells (HK-2) exposed to HG (30 mmol/l D-glucose) were used for mechanistic studies. qRT-PCR analysis revealed that treatment with varying concentrations of DHM (50–200 μ M) did not alter STING mRNA expression (Fig. 5A), while Fig. 4D indicated that DHM significantly reduced the level of STING protein. This discrepancy between mRNA and protein levels strongly suggests that DHM does not act at the transcriptional level; therefore, our subsequent research focused on post-transcriptional regulatory mechanisms. To assess effects on protein stability, translational arrest was induced with CHX (100 μ g/ml), and the decay of existing STING protein was monitored. Under HG conditions, STING exhibited a half-life of approximately 1.87 h. Pre-incubation with 100 μ M DHM substantially reduced this value to 0.89 h ($p < 0.01$). Following 4 h of CHX exposure, residual STING protein in the HG + CHX group was 32.6%, whereas only 20.3%

remained in cells co-treated with DHM ($p < 0.01$; Fig. 5B), confirming accelerated protein turnover.

To determine whether the UPS or autophagy mediates this effect, selective pharmacological inhibitors were utilized. Co-administration of the proteasome inhibitor MG132 (10 μ M) completely abrogated DHM-induced STING loss, whereas treatment with the autophagy inhibitor chloroquine (CQ, 20 μ M) failed to prevent degradation (Fig. 5C). These observations identify the ubiquitin-proteasome pathway as the primary mechanism underlying DHM-mediated STING clearance, independent of autophagic or transcriptional control.

The impact of DHM on STING ubiquitination status was subsequently examined through co-immunoprecipitation experiments. Compared to control conditions, DHM treatment resulted in decreased total STING ubiquitination, an effect reversed by MG132 co-treatment (Fig. 5D). Examination of ubiquitin chain topology revealed that K48-linked polyubiquitination, a canonical signal for proteasomal targeting, was markedly reduced following DHM administration, and this reduction was also normalized upon MG132 addition (Fig. 5E). Collectively, these data demonstrate that DHM facilitates STING degradation through the ubiquitin-proteasome pathway by enhancing its K48-ubiquitination.

DISCUSSION

The present investigation, employing an integrative approach combining network pharmacology, *in vivo* animal studies, and *in vitro* molecular analyses, uncovers a previously unrecognized mechanism underlying the therapeutic efficacy of DHM in DN. The data demonstrate that DHM facilitates K48-linked polyubiquitination of the STING protein, thereby accelerating its proteasomal degradation. This process effectively suppresses aberrant activation of the cGAS-STING signaling cascade, culminating in attenuation of renal inflammatory responses and fibrotic progression. A schematic illustration of the proposed mechanism by which DHM inhibits diabetic nephropathy is shown in Fig. 6. These findings not only expand the understanding of DHM's renoprotective properties but also propose an innovative therapeutic paradigm for DN centered on modulating STING protein turnover.

Initial network pharmacology screening revealed significant enrichment of overlapping DHM and DN targets within immune-related pathways, including TNF, NOD-like receptor, and JAK-STAT signaling cascades. Of particular interest, pronounced upregulation of MB21D1 (encoding cGAS) and TMEM173 (encoding STING) was observed in DN renal tissue, corroborating recent evidence implicating cGAS-STING axis activation as a fundamental driver of DN pathophysiology [26, 27]. Hyperglycemia-induced mitochondrial dysfunction and subsequent mtDNA release are known to trigger sustained activation of this pathway, promot-

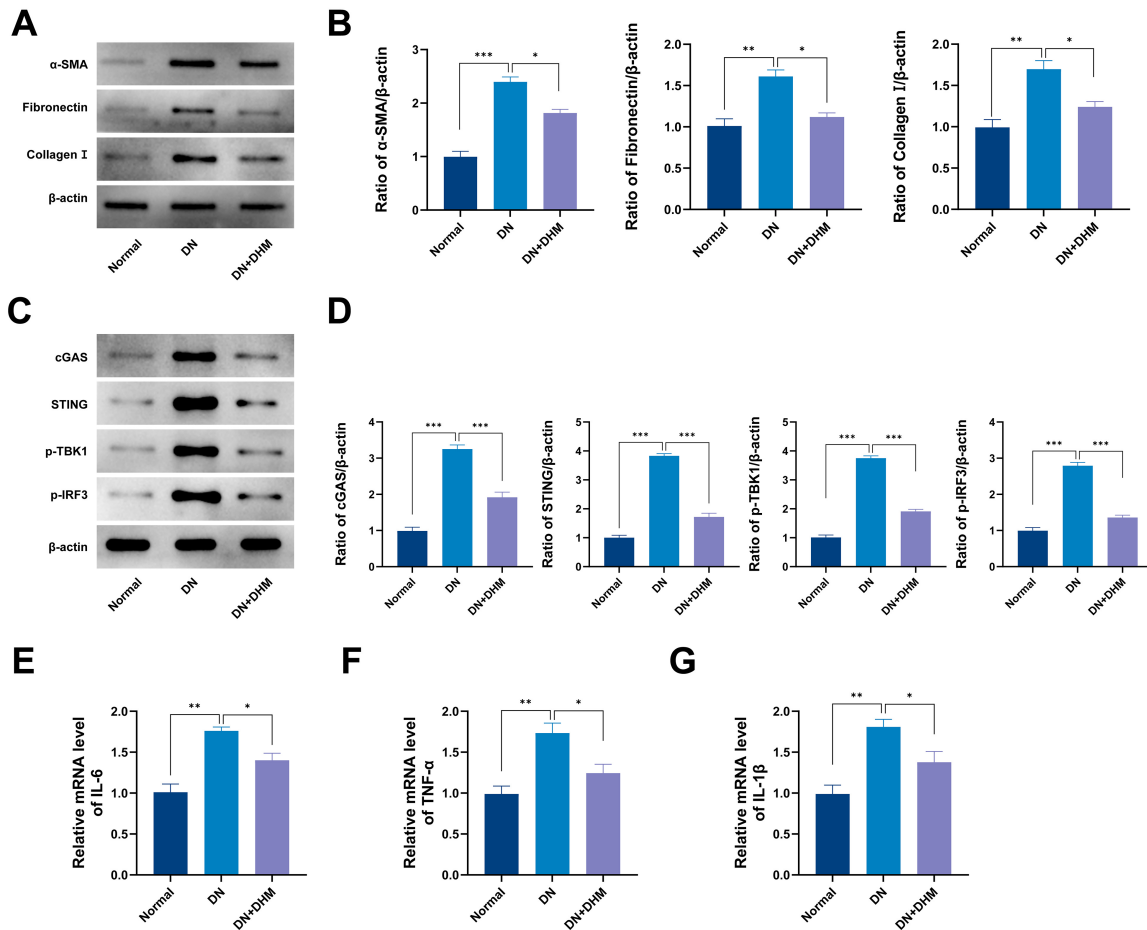


Fig. 4 Dihydromyricetin improves renal function, inhibits renal fibrosis and STING signaling activation. (A, B) Western blot analysis of the protein expression levels of fibrosis markers α -SMA, Fibronectin, and Collagen I in mouse kidney tissues; (C, D) Western blot analysis of the protein expression levels of cGAS, STING, p-TBK1, and p-IRF3; (E–G) RT-qPCR analysis of the mRNA expression levels of inflammatory factors IL-6, TNF- α , and IL-1 β in mouse kidney tissues. (A) to (G), ($n = 5$). Error bars represent at least three independent experiments. Data are presented as mean $\pm$ SD. Comparisons were performed using one-way ANOVA. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

ing type I interferon production and pro-inflammatory cytokine secretion (e.g., IL-6, TNF- α), thereby accelerating glomerulosclerosis and tubulointerstitial fibrosis [28]. The current work extends these observations by demonstrating significant elevation of cGAS, STING, and its activated phosphorylated form (p-STING Ser366) in kidneys of DN mice, with strong positive correlations established between these molecules and fibrotic indicators including α -SMA, Col I, and CVF. Meanwhile, DHM exhibits significant inhibitory effects on the pathways. It reduces the levels of cGAS and STING proteins, and simultaneously inhibits the downstream phosphorylation process of TBK1 and IRF3. This mechanism distinguishes DHM from conventional STING antagonists such as H-151, which function primarily through kinase inhibition and may potentially compromise innate immune surveillance

[29]. The degradation-focused strategy employed by DHM more closely resembles physiological regulatory processes, potentially offering enhanced safety and target specificity for clinical applications.

The UPS serves as a critical determinant of STING protein stability through coordinated actions of E3 ubiquitin ligases. Previous reports have documented that TRIM21 facilitates K48-ubiquitination leading to proteasomal degradation, whereas RNF5 mediates K27-linked ubiquitination promoting autophagic clearance [30]. Consistent with evidence that HG impairs STING ubiquitination [31], the present study observed diminished total STING ubiquitination under DN conditions, suggesting that compromised UPS function contributes to STING accumulation and sustained pathway activation. DHM treatment effectively reversed this pathological signature: in HK-

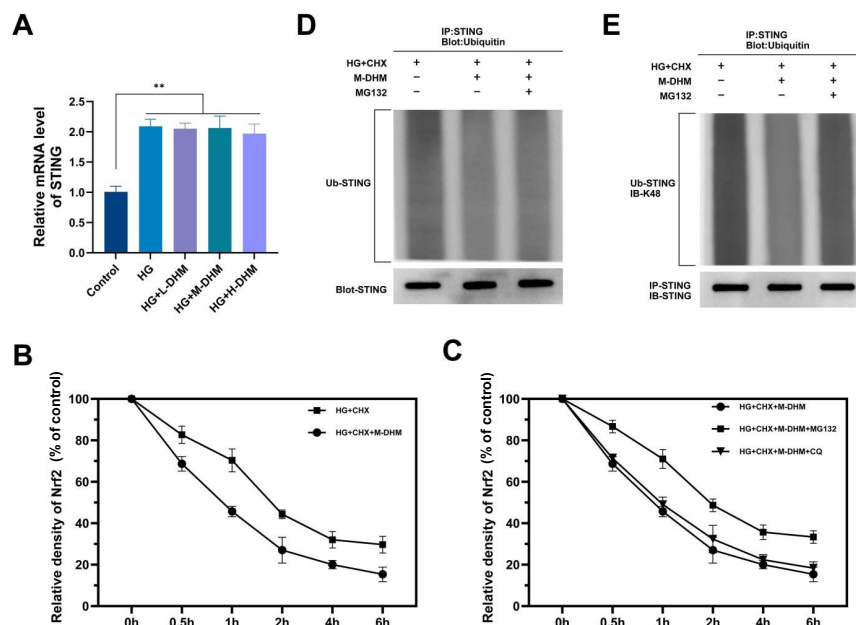


Fig. 5 Dihydromyricetin drives STING protein degradation by promoting K48-linked polyubiquitination of STING. In the HG HK-2 cell model (A) RT-qPCR analysis of TMEM173 mRNA expression levels in the cells treated with different concentrations of DHM (50–200 μ M) ($n = 5$); (B) Western blot analysis of STING protein degradation ($n = 5$); (C) Detection of STING protein degradation following intervention with the proteasome inhibitor MG132 (10 μ M) and the autophagy inhibitor chloroquine (20 μ M), respectively; (D) IP assay for the ubiquitination level of STING; (E) IP and Western blot analysis for the K48-linked polyubiquitination level of STING. Error bars represent at least three independent experiments. Data are presented as mean $\pm$ SD. Comparisons were performed using one-way ANOVA. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

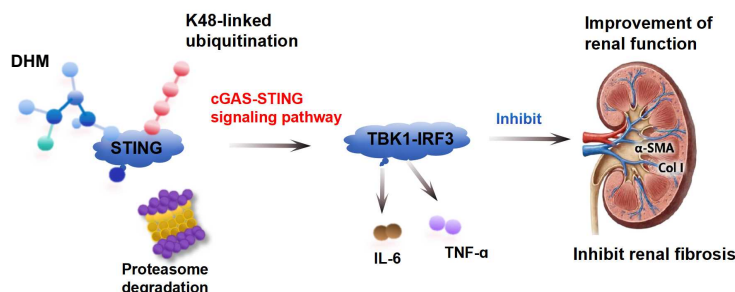


Fig. 6 Schematic diagram illustrating the mechanism of DHM in preventing diabetic nephropathy.

2 cells exposed to HG, DHM selectively accelerated STING turnover (half-life reduced from 1.87 to 0.89 h, $p < 0.01$) without altering TMEM173 transcription. Pharmacological inhibition studies confirmed proteasome dependence, as MG132 completely abolished DHM-induced degradation while chloroquine showed no effect. Subsequent co-immunoprecipitation analyses revealed that DHM specifically enhanced K48-ubiquitination, the canonical signal directing substrates to the proteasome [32]. These findings provide the first molecular-level evidence that DHM modulates STING stability through enhanced K48-ubiquitination, expanding current understanding of how natural compounds interface with the UPS. Whether DHM achieves this effect through activation of specific E3 ligases (e.g.,

TRIM21) or inhibition of deubiquitinating enzymes (e.g., USP18) represents an important question for future investigation. Systematic screening for DHM-interacting proteins involved in ubiquitination dynamics will help delineate the complete regulatory network.

Comprehensive evaluation of DHM therapeutic potential across functional, histological, and molecular dimensions yielded consistent findings. Renal function parameters including 24-h albuminuria, UACR, Scr, and BUN were substantially improved following DHM administration, with UACR and Scr approaching normal values. Histopathological assessment demonstrated marked amelioration of glomerular atrophy, tubular dilation, and protein cast formation, accom-

panied by reduction in CVF from $28.6 \pm 2.5\%$ to $12.3 \pm 1.6\%$ ($p < 0.01$). Molecular analyses confirmed downregulation of fibrotic markers (α -SMA, Col I) and inflammatory mediators (IL-6, TNF- α). These observations align with previously reported renoprotective effects of DHM in diverse injury models, including ischemia-reperfusion and cisplatin-induced nephrotoxicity [33, 34], suggesting that modulation of the cGAS-STING pathway may represent a common mechanism underlying its broad protective actions. At the same time, pyrroloquinoline quinone (PQQ) has been shown to reduce urinary albumin excretion and improve the tissue structure of the glomerular filtration barrier in diabetic mice, further supporting the protective effects of natural compounds against DN [35]. Similarly, other herbal extracts such as *Hedyotis diffusa* have also been shown to protect renal tubular epithelial cells from HG-induced injury, with mechanisms involving inhibition of the PI3K/AKT signaling pathway [36]. Regarding the potency of DHM relative to standard treatments, the present study did not include a positive control (e.g., an ACE inhibitor or SGLT2 inhibitor). However, comparison with published data from similar DN mouse models suggests that DHM at 100 mg/kg/day produces renoprotective effects—including reductions in UACR, Scr, BUN, and tubulointerstitial fibrosis—that are comparable to those reported for lisinopril (40 mg/kg/day) [37] and dapagliflozin (10 mg/kg/day) [38]. Notably, DHM's unique mechanism of promoting STING K48-linked ubiquitination and proteasomal degradation differs from the hemodynamic or glucosuric actions of conventional drugs, implying potential additive or synergistic benefits. Direct head-to-head comparisons between DHM and first-line agents in future studies will be necessary to fully establish its relative efficacy and clinical positioning. Nonetheless, the present findings support DHM as a promising complementary candidate for DN therapy, particularly in patients with residual inflammation or fibrosis despite standard treatment. Regarding the safety profile of DHM, both *in vitro* and *in vivo* assessments were considered. In the present study, no obvious signs of toxicity, such as significant body weight loss, abnormal behavior, or mortality, were observed in DHM-treated mice during the 8-week intervention period. Additionally, the purity of DHM used in this study was $\geq 98\%$, and the administered dose (100 mg/kg/day) was selected based on previous reports demonstrating its safety and efficacy in murine models [21]. *In vitro*, HK-2 cells treated with DHM at concentrations up to 200 μ M for 24 h exhibited no overt morphological changes or reduced viability compared to controls, consistent with prior studies indicating that DHM has a favorable safety window [39]. These observations suggest that the anti-fibrotic and anti-inflammatory effects of DHM are achieved at doses that do not elicit detectable adverse effects, supporting its potential as a safe therapeutic candidate

for DN. Nevertheless, future systematic toxicological evaluations, including assessments of chronic toxicity, organ-specific safety, and pharmacokinetic profiles, are warranted to fully establish the translational potential of DHM. Network pharmacology additionally identified NEDD4 and TGF β 1 as potential targets of interest. NEDD4, an E3 ubiquitin ligase known to suppress TGF- β /Smad signaling through Smad7 ubiquitination [40], was significantly downregulated in DN kidneys. Whether DHM exerts dual anti-fibrotic effects through coordinated regulation of NEDD4 and STING ubiquitination warrants further exploration. Future studies employing combinatorial approaches such as NEDD4 knockdown alongside DHM treatment may elucidate potential synergistic mechanisms.

Several limitations of the current study merit consideration. First, confirmation of DHM efficacy in additional DN models, particularly spontaneous diabetic models such as db/db mice, would strengthen the generalizability of findings. Second, while HK-2 tubular epithelial cells provided valuable mechanistic insights, examination of DHM effects on glomerular mesangial cells and podocytes—critical contributors to DN pathology—is warranted. Third, direct physical interaction between DHM and STING protein remains to be established; techniques including molecular docking and surface plasmon resonance could provide complementary evidence. Building upon these findings, several promising research directions emerge: investigation of potential synergistic effects with established DN therapeutics (e.g., SGLT2 inhibitors, ACE inhibitors); development of genetically modified models with STING knockout or K48 ubiquitination site mutations to definitively establish pathway essentiality; and structure-activity relationship studies to develop DHM analogs with optimized ubiquitination-modulating properties.

In conclusion, this study provides the first demonstration that DHM attenuates renal inflammation and fibrosis in DN by promoting K48-linked polyubiquitination and subsequent proteasomal degradation of STING, thereby suppressing aberrant cGAS-STING pathway activation. These findings establish a previously unrecognized mechanism contributing to the renoprotective properties of DHM and introduce a novel therapeutic strategy targeting STING stability through ubiquitination modulation. The study carries significant theoretical implications and offers promise for clinical translation in DN management.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at <https://dx.doi.org/10.2306/scienceasia1513-1874.2026.081>.

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Appendix A. Supplementary data

Table S1 Primer sequences for RT-qPCR.

Gene	Primer Sequence (5' → 3')
<i>cGAS</i>	Forward: 5'- CTGCGCATTCAAAAC TGGCT-3' Reverse: 5'- GTCCCACTGACTGTCTTGAGG-3'
<i>Sting</i>	Forward: 5'-TTGGCTGAGTGTGTGGAGTC-3' Reverse: 5'-TCATCTGCAGGTTCCGCTG-3'
<i>Il-6</i>	Forward: 5'-ACATAGACGGATCACAGTGC-3' Reverse: 5'-AGAAGGCAACTGGACCGAAG-3'
<i>Tnf-α</i>	Forward: 5'-GACAAGCCTGTAGCCCATGT-3' Reverse: 5'-GGAGGTTGACCTTGGTCTGG-3'
<i>Il-1β</i>	Forward: 5'-AGGCAACCTGCCTAACATGC-3' Reverse: 5'-ATCCCTCCGAGACACTGGAA-3'
<i>Gapdh</i>	Forward: 5'-GGGTGTGAACCACGAGAAAT-3' Reverse: 5'-ACTGTGGTCATGAGCCCTTC-3'