

Validation and Evaluation of SPP1 as a Candidate Biomarker for Disease Monitoring in Focal Segmental Glomerulosclerosis

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Background: Secreted phosphoprotein 1 (SPP1), a glycoprotein encoded by the SPP1 gene, can be detected in body fluids and tumor tissues of various diseases, representing a promising candidate biomarker. However, its application in focal segmental glomerulosclerosis (FSGS) remains at the exploratory stage.

Patients and Methods: In a small-scale cohort, Bulk-RNA sequencing was employed to screen for core differentially expressed genes in urinary cells of FSGS patients, with SPP1 identified as a key candidate. RT-qPCR and ELISA were subsequently used to detect SPP1 expression in clinical urine samples. An adriamycin (ADR)-induced FSGS mouse model was established, and renal histopathological changes were evaluated using hematoxylin-eosin (HE), periodic acid-Schiff (PAS), and Masson staining. Immunohistochemistry and immunofluorescence were performed to examine the expression of SPP1, fibronectin, and F4/80 in renal tissues, with assessment of the effects of prednisone intervention.

Results: Urinary SPP1 expression was significantly elevated in FSGS patients, particularly in those with CKD stage III. In the ADR mouse model, as glomerulosclerosis progressed, albuminuria levels increased, accompanied by enhanced expression of SPP1 and F4/80-positive macrophages. Prednisone treatment attenuated these parameters.

Conclusion: SPP1 is involved in the progression of FSGS and is closely associated with renal immune inflammation and fibrosis. Prednisone exerts renoprotective effects by downregulating SPP1 expression and inhibiting macrophage infiltration, suggesting that SPP1 represents a promising molecular marker for disease monitoring and targeted intervention in FSGS.

Keywords: focal segmental glomerulosclerosis, Bulk-RNA sequencing, SPP1, prednisone

Introduction

Focal segmental glomerulosclerosis (FSGS) is a chronic glomerular disease characterized by massive proteinuria. Pathologically, FSGS demonstrates sclerotic lesions affecting segments of individual glomeruli in a focal distribution pattern.¹ FSGS can result from various etiologies, including primary FSGS, hereditary FSGS, and secondary FSGS caused by factors such as obesity and viral infections.² FSGS represents one of the leading causes of end-stage renal disease (ESRD), with its incidence showing a rising trend annually.³ Moreover, the recurrence rate following renal transplantation in FSGS patients ranges from 20% to 50%, significantly compromising long-term allograft survival.⁴ Therefore, early and accurate diagnosis, assessment of disease progression, and prediction of prognosis are essential for the clinical management of FSGS.

Currently, renal biopsy remains the gold standard for FSGS diagnosis.⁵ However, as an invasive procedure, it carries risks of complications including hemorrhage, infection, and arteriovenous fistula formation. Additionally, sampling errors may occur due to the focal distribution of FSGS lesions, potentially leading to missed diagnoses on single biopsies. Contraindications such as coagulation disorders and solitary kidney, along with patient compliance issues, further limit its applicability.^{6,7} More importantly, renal biopsy cannot be performed frequently for dynamic monitoring of disease progression or assessment of therapeutic response and long-term prognosis. The 2021 Kidney Disease: Improving Global Outcomes (KDIGO) clinical practice guidelines explicitly stated that FSGS diagnosis should not rely solely on renal biopsy, emphasizing the urgent need to develop safer, reproducible noninvasive diagnostic methods to assist in etiological diagnosis, guide treatment decisions, and predict treatment response and long-term outcomes.^{8,9}

In this context, urine, as the direct filtrate of the kidney, offers an ideal source for identifying noninvasive biomarkers, as its compositional changes can reflect the pathophysiological status of glomeruli and renal tubules.¹⁰ Compared with traditional blood biomarkers, urinary markers offer advantages including convenient sampling, the ability for continuous monitoring, and direct reflection of local renal pathological changes. In recent years, with the advancement of transcriptomic technologies, urinary sediment cell RNA detection has opened new avenues for noninvasive diagnosis of kidney diseases. Studies have demonstrated that urinary sediment cells contain kidney-derived cellular components including tubular epithelial cells and immune cells, and analysis of cellular gene expression profiles can reveal disease pathophysiology at the molecular level.^{11,12} Latt et al¹³ employed single-cell RNA sequencing to analyze urinary cells from FSGS patients and identified immune-inflammatory gene expression signatures highly consistent with renal biopsy tissues. Furthermore, Lyu et al¹⁴ highlighted in their review that urinary gene transcript biomarkers, including urinary sediment cell mRNA, demonstrate considerable diagnostic potential in chronic kidney disease. Therefore, screening and validating FSGS-specific biomarkers based on urinary transcriptomics holds significant importance for achieving early diagnosis, monitoring disease activity, and assessing prognosis in FSGS.

Secreted phosphoprotein 1 (SPP1), also known as osteopontin (OPN), is a multifunctional glycoprotein extensively involved in numerous physiological and pathological processes, including inflammation, bone remodeling, cell survival, fibrosis, tumorigenesis, and metastasis.^{15,16} In the kidney, SPP1 is primarily secreted by tubular epithelial cells and participates in regulating biological processes such as cell adhesion, migration, survival, and immune-inflammatory responses.¹⁷ In recent years, substantial evidence has indicated that SPP1 plays a critical role in the pathogenesis and progression of kidney diseases, particularly in renal fibrosis, glomerulosclerosis, and chronic kidney disease progression.

From a pathophysiological perspective, SPP1 may be mechanistically linked to FSGS pathogenesis through multiple pathways. First, SPP1 serves as a key regulator of renal fibrosis. Ding et al¹⁸ demonstrated that Spp1 promotes fibroblast activation through the transforming growth factor-beta 1 (TGF- β_1)/Smad signaling pathway, contributing to renal fibrosis. Liu et al¹⁹ further emphasized in their review that SPP1 regulates extracellular matrix homeostasis and plays a central role in the transition from acute kidney injury to chronic kidney disease. Second, SPP1 is closely associated with podocyte injury. Fu et al²⁰ identified significant upregulation of SPP1 expression in glomerular endothelial cells in a podocyte depletion-induced glomerulosclerosis mouse model, with *in vitro* experiments demonstrating that SPP1 promotes angiogenic responses in endothelial cells, suggesting its potential involvement in glomerulosclerosis progression. Endlich et al²¹ found that SPP1 is an important regulatory molecule enabling podocytes to respond to mechanical stress, enhancing podocyte adaptation to mechanical stress through cytoskeletal remodeling. Furthermore, SPP1 acts as a critical mediator of macrophage infiltration and inflammatory responses. Vegting et al²² identified SPP1-positive lipid-associated macrophages (SPP1+ LAMs) as playing a key role in renal inflammation and fibrosis in antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis. Fan et al²³ demonstrated that SPP1+ macrophages promote chronic kidney disease progression through intercellular communication. Collectively, these studies suggest that SPP1 participates in multiple pathophysiological mechanisms of FSGS, including promoting extracellular matrix deposition, mediating podocyte injury, and regulating macrophage infiltration and inflammatory responses.

As a biomarker, SPP1 has demonstrated promising diagnostic and prognostic value in various kidney diseases.^{24,25} In longitudinal biopsy and follow-up studies of patients with lupus nephritis, transcript subset analysis revealed that SPP1 abundance is closely associated with disease activity.²⁶ Our previous study also indicated²⁷ that urinary SPP1 may serve as a potential noninvasive diagnostic marker for FSGS. However, research on urinary SPP1 in FSGS remains at

a preliminary stage, and its value as a marker for disease progression and treatment response assessment requires further validation. Based on this background, the present study aimed to systematically evaluate the clinical utility of urinary SPP1 as an FSGS biomarker through expanded-sample urinary cell transcriptome sequencing, combined with clinical sample validation and animal experiments. We hypothesized that urinary SPP1 could serve as a noninvasive biomarker for assessing FSGS disease progression and treatment response, with its expression levels correlating with glomerulosclerosis severity, renal function impairment, and disease progression. Through this study, we hope to provide a novel molecular marker for FSGS clinical management and offer new insights into understanding FSGS pathophysiology.

Materials and Methods

Patient Selection Criteria

Patients with biopsy-confirmed kidney diseases and healthy controls were recruited from the Second Affiliated Hospital of Guangxi Medical University between 2022 and 2023. For FSGS patient selection, exclusion criteria included: (1) malignancy, severe hepatic impairment, or other rheumatic diseases; (2) secondary FSGS caused by diabetes, Henoch-Schonlein purpura, multiple myeloma, malignancy, or systemic lupus erythematosus; and (3) severe cardiac, pulmonary, or cerebral diseases, or malignant wasting conditions. Inclusion criteria were: (1) biopsy-confirmed FSGS diagnosis by professional pathologists; (2) absence of gross hematuria; and (3) no prior renal biopsy or treatment before urine sample collection. Similar selection criteria were applied for patients with membranous nephropathy (MN), minimal change disease (MCD), and diabetic nephropathy (DN). This exploratory study was conducted with a relatively small sample size. The Bulk-RNA sequencing cohort comprised 7 FSGS patients and 13 healthy controls. The ELISA validation cohort included 43 patients with glomerular diseases and 14 healthy controls. These two cohorts were independent.

Bulk-RNA Sequencing of FSGS Patient Urinary Cells

Based on preliminary work, urinary cells were collected from 7 FSGS patients and 13 healthy controls at the Second Affiliated Hospital of Guangxi Medical University for Bulk-RNA sequencing. Urine samples intended for Bulk-RNA sequencing were centrifuged at 490 g for 10 minutes at 4°C, and the supernatant was retained. Cells were resuspended in 1× Dulbecco's phosphate-buffered saline (DPBS) and centrifuged at 485 g for 5 minutes. This process was repeated twice. Total RNA was extracted from urinary cells using the AccuraCode HTP OneStep RNAseq Kit (1071065, Singleron, China). Cells were lysed in AccuraCode lysis buffer for 6 minutes, followed by one-step reverse transcription and PCR amplification [42°C for 1 hour, 95°C for 3 minutes, then 4 cycles (98°C for 20 seconds, 65°C for 45 seconds, 72°C for 3 minutes), followed by 10 cycles (98°C for 20 seconds, 67°C for 20 seconds, 72°C for 3 minutes), and finally 72°C for 5 minutes, with termination at 4°C]. Amplified complementary DNA (cDNA) was purified using Ampure XP magnetic beads, followed by fragmentation, adapter ligation, and PCR enrichment [98°C for 30 seconds, then 2 cycles (98°C for 10 seconds, 65°C for 75 seconds), 65°C for 5 minutes, with termination at 4°C]. Final libraries were size-selected and quality-controlled using Qubit and AATI, with qualified libraries used for sequencing.

Clinical information was collected according to the 2012 KDIGO clinical practice guideline for CKD evaluation and management.²⁸ FSGS patients were stratified into CKD stages based on glomerular filtration rate (GFR): (G1: GFR ≥ 90 mL/min/1.73m², G2: GFR 60–89 mL/min/1.73m², G3: GFR 30–59 mL/min/1.73m²). G1 and G2 were combined as CKD stages I–II, and G3 as CKD stage III. Detailed patient clinical characteristics are presented in Table 1.

Transcriptomic Data Analysis of FSGS Patient Urinary Cells

The “limma” package²⁹ was used for data cleaning, preprocessing, and differential expression analysis of urinary cell RNA sequencing data. For Gene Set Enrichment Analysis (GSEA), urinary cell expression profiles from the FSGS group and Normal group were divided into two groups. The c2.cp.kegg.v7.4.symbols.gmt reference gene set was downloaded from the Molecular Signature Database (<http://www.gsea-msigdb.org/gsea/downloads.jsp>), and visualization was performed using Sangerbox (<http://sangerbox.com/home.html>). Screening criteria were |normalized enrichment score (NES)| > 1 and P < 0.05. CKD staging data were processed using the same method.

Table 1 Clinical Characteristics of the Bulk-RNA Sequencing Cohort of Urinary SPPI in FSGS Patients

Group	Healthy group (n = 13)	FSGS group (n = 7)		p-value
		CKD I-II (n=4)	CKD III (n=3)	
Age, mean±SD	40.15±19.47	27.75±7.37	46.67±9.29	0.606
Gender (male/female)	6/7	2/2	2/1	-
Scr (μmol/L)	70.46±20.50	88.67±33.71	145.33±4.04	0.0057**
GFR (mL/min per 1.73 m ²)	119.31±48.40	85.37±35.78	43.90±5.90	0.0415*
Hb (g/L)	139.08±13.64	128.67±13.87	117.67±19.40	0.0452*
Uric acid (μmol/L)	311.62±101.73	384.33±212.70	311.62±101.73	0.0801

Note: *P < 0.05, **P < 0.01.

RT-qPCR Detection of SPPI in FSGS Patient Urine

Urine samples were collected from 7 FSGS patients and 10 healthy individuals at the Second Affiliated Hospital of Guangxi Medical University for preliminary RT-qPCR experiments. Fresh urinary cell samples were processed for total RNA extraction using RNAiso Plus reagent (9108, Takara, China), with phenol-chloroform purification. RNA concentration and purity were measured using a micro-spectrophotometer. Only samples with 260/280 ratios between 1.8–2.0 and 260/230 ratios greater than 1.8 were used for further analysis.³⁰ For all samples, 1 μg of total RNA was reverse transcribed using PrimeScript RT Master Mix (RR036A, Takara, China) under identical conditions. Quantitative polymerase chain reaction was performed using 2× RealStar Fast SYBR qPCR Mix (A301, GenStar, China). The qPCR cycling conditions were: 95°C for 2 minutes, followed by 40 cycles (95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds), with termination at 4°C. Gene expression was normalized to β-actin and quantified using the 2^{-ΔΔCT} method. Primer sequences used were: β-actin (forward: TCACCATGGATGATGATATCGC, reverse: CCACATAGGAATCCTTCTGACC) as the reference gene, and SPPI (forward: CAGTGATTGCTTTTGCC, reverse: AGATGGGTCAGGGTTTAG) as the target gene.

ELISA Detection of Urinary SPPI in Patients with Glomerular Diseases

Urine samples were collected from 43 patients with glomerular diseases (18 FSGS, 7 DN, 9 MN, 9 MCD) and 14 healthy controls (Normal) at the Second Affiliated Hospital of Guangxi Medical University for ELISA clinical validation. Upon obtaining urine samples, they were centrifuged at 1000 g for 20 minutes at 4°C, and the urinary supernatant was collected. Human urinary SPPI detection was performed according to the Human Osteopontin ELISA Kit (EK1135, Multi Sciences, China) protocol. Mouse urinary SPPI detection was performed according to the Mouse Osteopontin ELISA Kit (SEA899Mu, Cloud-Clone, China) protocol. Detailed patient clinical characteristics are presented in Table 2.

FSGS Mouse Model

Animal experimental protocols were approved by the Animal Ethics Committee of the Second Affiliated Hospital of Guangxi Medical University. Eight-week-old male C57BL/6J mice weighing 18–20 g (specific pathogen-free [SPF] grade, Certificate No. SCXK [Yue] 2022–0063) were purchased from Charles River Laboratories Animal Technology Co,

Table 2 Clinical Characteristics of the ELISA Test Cohort for SPPI in the Urine of Patients with Glomerular Diseases

Group	Healthy Group (n = 14)	FSGS (n=18)	DN (n=7)	MN (n=9)	MCD (n=9)
Age, mean±SD	46.71±19.22	39.72±17.89	56.29±4.92	55.44±12.54	37.89±17.02
Gender (male/female)	7/7	10/8	5/2	6/3	8/1
Scr (μmol/L)	71.43±21.04	425.11±385.87**	620.43±328.31**	139.33±86.74*	143.67±114.20
GFR (mL/min per 1.73 m ²)	96.69±18.30	41.78±43.82***	9.89±5.73***	59.94±31.05*	77.42±38.90
Hb (g/L)	136.14±14.86	116.39±29.04*	97.57±23.80**	108.00±29.04*	130.33±31.65
Uric acid (μmol/L)	347.43±97.44	417.78±122.58	443.29±143.88	434.11±99.98	357.11±125.90

Note: *P < 0.05, **P < 0.01, ***P < 0.001.

Ltd, Guangdong, China. All mice had free access to water and standard chow and were randomly divided into control group (n = 6), ADR group (n = 6), and ADR+prednisone group (n = 6). For modeling, ADR mice received two tail vein injections of 10.0 mg/kg adriamycin (D1515, Sigma-Aldrich, Germany) at a concentration of 0.5 mg/mL with a 12-hour interval; control mice received saline instead of ADR.³¹ For treatment, mice in each group received gavage treatment starting at week 4 after tail vein injection. ADR+prednisone group mice received daily gavage of 5 mg/kg³² prednisone (HY-B0214, MCE, USA), while ADR and control group mice received equivalent volumes of saline. At the end of week 13, mice were anesthetized by intraperitoneal injection of 0.2 mL/10 g of 1.25% tribromoethanol solution (M2910, Aibei Bio, China). After confirming adequate anesthesia depth by loss of righting reflex and absence of toe withdrawal reflex, euthanasia was performed by cervical dislocation. Mouse kidneys were collected after confirming death by cessation of respiration and heartbeat. All procedures followed the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020 Edition) and institutional animal care regulations.

HE, PAS, and Masson Staining

Paraffin sections were dewaxed and rehydrated. Briefly, for HE staining, sections were stained with hematoxylin for 5 minutes followed by 0.5% eosin for 10 seconds. For PAS staining, sections were treated with 1–3% periodic acid for 10 minutes, PAS solution for 10 minutes, and hematoxylin for 5 seconds. Masson staining was performed according to the Masson Trichrome Kit (DTA02, DeepMind, China) protocol. Sections were finally mounted with neutral resin. Images were acquired using a digital pathology slide scanner (KF-PRO-020, KFBIO Technology, China).

Mouse Urinary Albumin and Creatinine Detection

Twenty-four-hour urine was collected from mice using metabolic cages, centrifuged at 1000 g for 15 minutes, and the supernatant was retained. Subsequent experimental procedures followed the protocols of the albumin detection kit (G1208W, Geruisi Biotechnology, China) and creatinine detection kit (G1204W, Geruisi Biotechnology, China).

Immunohistochemistry

Paraffin sections were dewaxed, rehydrated, and subjected to antigen retrieval using EDTA antigen retrieval buffer with high-pressure heat treatment. After incubation with 3% hydrogen peroxide (H₂O₂), sections were blocked with 3% bovine serum albumin (BSA). Primary antibodies were then applied: recombinant anti-fibronectin antibody (1:2000, ab268020, Abcam, UK), recombinant anti-osteopontin antibody (1:2000, ab283656, Abcam, UK), and recombinant anti-F4/80 antibody (1:2000, YM8779, ImmunoWay, USA), incubated overnight at 4°C in the dark. The following day, horseradish peroxidase (HRP)-conjugated secondary antibody was applied, followed by 3,3'-diaminobenzidine (DAB) chromogenic reaction in the dark, hematoxylin counterstaining, and final mounting with neutral balsam. Images were acquired using a digital pathology slide scanner (EasyScan, China), and positive area quantification analysis was performed using ImageJ.

Immunofluorescence

Paraffin section antigen retrieval procedures were identical to immunohistochemistry. Subsequent steps followed the dual-label triple-color multiplex fluorescence staining kit (AFIHC033, AiFang Biological, China) protocol. Primary antibodies were applied as a mixture of anti-osteopontin rabbit polyclonal antibody (1:400, AFRP0012, AiFang Biological, China) and anti-F4/80 rabbit monoclonal antibody (1:1000, YM8779, ImmunoWay, USA). Images were acquired using a digital pathology slide scanner (KF-FL-020, KFBIO Technology, China).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 10.0. For comparisons between two groups, independent samples *t*-test or Mann–Whitney *U*-test was used. For comparisons among three groups, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was employed. Biological replicates were performed more than 3 times, with technical replicates of 3. Clinical data are presented as mean ± standard deviation (SD). Statistical significance was defined as: ns for no significant difference, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Results

Transcriptomic Data Analysis of FSGS Urinary Cells and Validation of SPP1 Gene

Analysis of FSGS urinary cell transcriptome sequencing data, with $|\log_2\text{fold change (FC)}| \geq 1$ and false discovery rate (FDR) < 0.05 as differentially expressed gene (DEG) screening criteria, identified upregulated SPP1 expression among DEGs (Figure 1A). Heatmap visualization displayed the top 14 DEGs (Figure 1B). GSEA analysis results (Figure 1C) demonstrated that compared with the control group, the FSGS group was mainly enriched in sensory perception transduction, glycosphingolipid biosynthesis-ganglio series, hematopoietic cell lineage, lysine degradation, and calcium signaling pathways. Furthermore, comparison of key gene expression in urine revealed significantly elevated SPP1 expression in the FSGS group, consistent with previous findings²⁷ (Figure 1D). Differential analysis of FSGS urine stratified by CKD stage identified upregulated SPP1 expression (Figure 1E). SPP1 in urine could distinguish between CKD stages I–II and CKD stage III, with $P < 0.05$, showing statistical significance (Figure 1F). Receiver operating characteristic (ROC) analysis results showed an area under the curve (AUC) value of 0.833 for SPP1 (Figure 1G). Therefore, we hypothesized that increased urinary SPP1 correlates proportionally with FSGS kidney injury.

SPP1 mRNA and Protein Expression in FSGS Urine and Diagnostic Value Assessment

RT-qPCR measurement of SPP1 mRNA levels in random urine samples from FSGS patients showed significantly elevated urinary SPP1 mRNA levels in the FSGS group compared with the Normal group, with $P < 0.01$, showing statistical significance (Figure 2A). ROC analysis of urinary cell SPP1 mRNA (Figure 2B) demonstrated an AUC of 0.957, $P < 0.05$, suggesting that urinary cell SPP1 has diagnostic value for FSGS assessment. Urinary supernatant from healthy controls and patients with glomerular diseases was collected for ELISA detection. Results showed significantly elevated urinary SPP1 protein levels in the FSGS group compared with the Normal group, with $P < 0.05$, showing statistical significance. However, no statistically significant differences were observed between the DN, MCD, and MN groups compared with the Normal group (Figure 2C). Using FSGS patients as positive samples and the Normal group as negative samples, ROC curves were plotted (Figure 2D). ROC analysis results indicated that the optimal cutoff value for urinary SPP1 was 0.7169 $\mu\text{g/mL}$, with AUC = 0.726, $P < 0.05$, sensitivity of 61.1%, and specificity of 85.7%. ROC analysis suggests that when urinary SPP1 concentration exceeds 0.7169 $\mu\text{g/mL}$, it may assist in the diagnosis or screening of FSGS.

Progression of Focal Segmental Sclerotic Lesions in ADR Mice

To investigate SPP1 expression changes in renal tissue, we first established an adriamycin-induced FSGS model (ADR mice). Through HE, PAS, and Masson staining, we observed that control mice had clear and intact overall renal tissue architecture. At week 4 after drug injection, ADR mice began to show glomerular lesions (Figure 3A). HE staining revealed adhesion of Bowman's capsule, retraction and collapse of capillary loops, with segmental aggravation. PAS staining showed glomerular capsular adhesion. Masson staining demonstrated segmental green clumps of collagen fiber deposition within glomeruli. The urinary albumin/urinary creatinine ratio increased in ADR mice compared with controls, indicating decreased renal function (Figure 3B). At week 13 after drug injection, the focal sclerotic lesion area expanded in ADR mice, with interstitial fibrosis observed (Figure 4A).

Prednisone Treatment Downregulates SPP1 Expression and Attenuates Glomerulosclerosis in ADR Mice

We administered prednisone, the first-line treatment for FSGS patients, to ADR mice by gavage to assess whether SPP1 was downregulated after treatment. HE, PAS, and Masson staining results showed that compared with the ADR group, ADR+prednisone group mice exhibited alleviation of capillary loop retraction and collapse and capsular adhesion, with reduced lesion areas of unstructured magenta matrix clumps and green collagen fiber deposition (Figure 4A). The urinary albumin/urinary creatinine ratio in ADR mice decreased after prednisone treatment, indicating recovery of renal function (Figure 4B). Subsequent ELISA detection of SPP1 in mouse urine revealed that prednisone treatment achieved the goal of reducing elevated SPP1 in ADR mice (Figure 4C). Immunohistochemistry showed that compared with the control

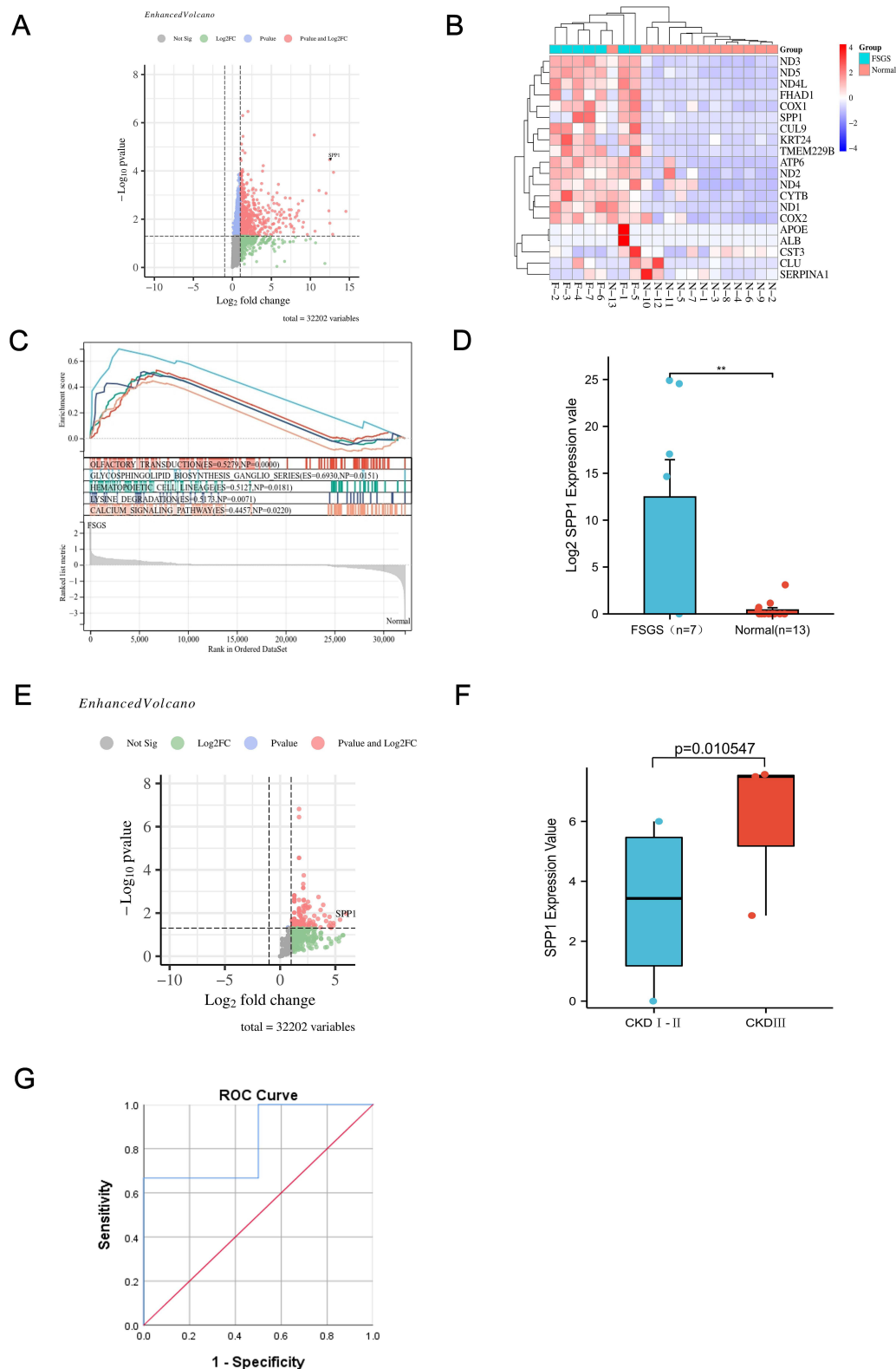


Figure 1 Transcriptomic data analysis of urinary cells and expression and diagnostic efficacy of key genes in FSGS urine (FSGS group n=7, Normal group n=13, CKD I-II group n=4, CKD III group n=3, **P < 0.01). **(A)** Volcano plot of differentially expressed genes (DEGs) between FSGS patients and controls. **(B)** Heatmap of top 14 DEGs expression. **(C)** Gene set enrichment analysis showing 5 enriched pathways in the FSGS group. **(D)** SPP1 expression levels in urine between FSGS and normal controls. **(E)** Volcano plot of DEGs between CKD stages I-II and CKD stage III. **(F)** Urinary SPP1 expression levels between CKD stages I-II and CKD stage III patients. **(G)** Diagnostic efficacy of urinary SPP1.

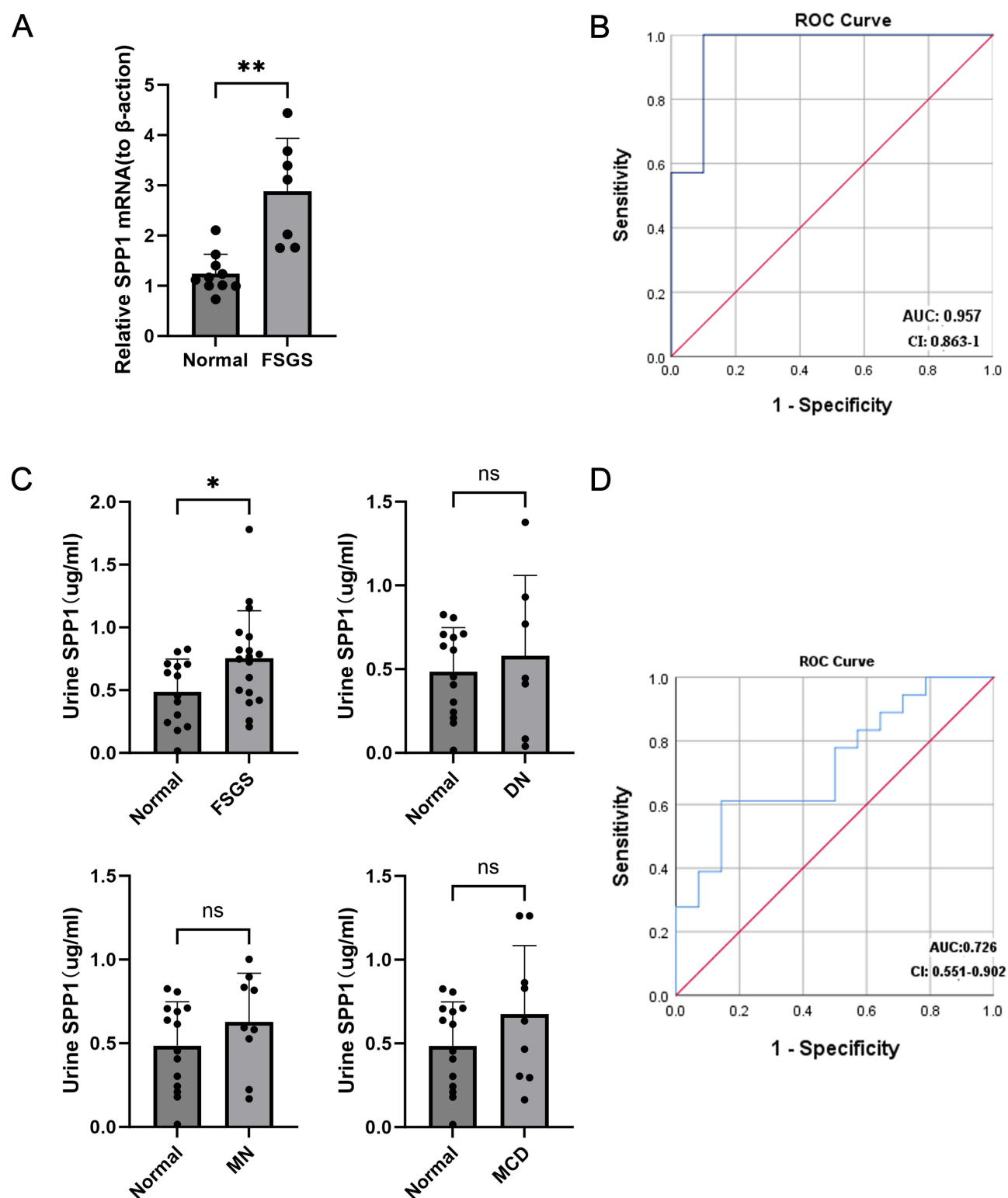


Figure 2 SPP1 expression in FSGS urine (ns indicates no significant difference, * $P < 0.05$, ** $P < 0.01$). **(A)** Urinary SPP1 mRNA expression levels between FSGS and control groups (FSGS group $n=7$, Normal group $n=10$). **(B)** Diagnostic efficacy evaluation of urinary SPP1 mRNA in FSGS. **(C)** Urinary SPP1 expression levels in different types of glomerular diseases and controls (FSGS group $n=18$, DN group $n=7$, MN group $n=9$, MCD group $n=9$, Normal group $n=14$). **(D)** Diagnostic efficacy evaluation of urinary SPP1 in FSGS.

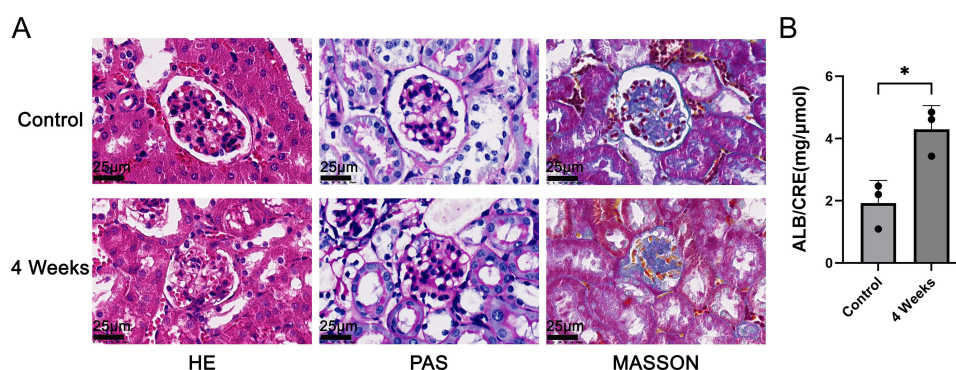


Figure 3 Glomerulosclerosis in renal cortex sections of ADR mice during the experimental course (Control group n=3, 4 Weeks group n=3, *P < 0.05). **(A)** Glomerular lesion assessment by hematoxylin-eosin (HE), periodic acid-Schiff (PAS), and Masson staining of renal tissue sections from ADR mice at week 4 after Adriamycin tail vein injection. **(B)** Urinary albumin/urinary creatinine ratio in ADR mice at weeks 4.

group, the fibrotic factor fibronectin³³ demonstrated diffuse strong positivity in ADR mouse renal tissue (Figure 4D), while SPP1 was highly expressed in tubular regions of glomerular lesions (Figure 4F). In the ADR+prednisone group, the focal sclerotic area in glomeruli was reduced, fibrosis was decreased (Figure 4E), and SPP1 expression was also downregulated (Figure 4G). In summary, SPP1 expression is inversely proportional to treatment effects in ADR mice. Prednisone effectively alleviates glomerulosclerosis and fibrotic lesions in ADR mice, suggesting that SPP1 has potential for assessing FSGS treatment response.

Correlation Between SPP1 and Immune Infiltration in ADR Mice Before and After Prednisone Treatment

Immunohistochemistry results for the macrophage marker F4/80³⁴ in ADR mouse tissues showed that F4/80 aggregated in renal sclerotic lesion areas (Figure 5A), and prednisone could reduce renal F4/80 expression (Figure 5B). Immunofluorescence co-expression results (Figure 5C) demonstrated that SPP1 signals were mainly distributed in tubular regions of renal lesions and showed some regional correlation with F4/80-positive cells in certain interstitial lesion areas, though overall cellular-level overlap was limited. Compared with the control group, ADR mice showed enhanced SPP1 and F4/80 signals in close proximity; compared with the ADR+prednisone group, ADR mice showed downregulated SPP1 and F4/80 signal intensity. These findings suggest the possibility of interaction between SPP1 enriched in glomerular and tubular lesions and macrophage immune infiltration, indicating that SPP1 may have potential in promoting immune infiltration in ADR mouse glomerulosclerosis.

Discussion

In our previous study,²⁷ we identified elevated urinary SPP1 expression in FSGS patients through bulk RNA sequencing combined with single-cell sequencing data. The present study further explored SPP1 expression characteristics and its association with disease progression in FSGS through expanded-sample Bulk-RNA sequencing (7 FSGS patients and 13 healthy controls), ELISA validation, and ADR mouse models.

The core pathogenic mechanism of FSGS primarily involves podocyte injury and detachment, which is often accompanied by functional abnormalities in renal tubular damage.³⁵ Regarding the intrinsic connection between podocyte injury and tubular lesions in FSGS, SPP1 appears to serve as an important bridge. As a multifunctional glycoprotein, SPP1 participates in inflammatory and tissue remodeling processes in various tissues including brain, kidney, liver, and bone,^{17,36,37} and exerts strong chemotactic effects on immune cells.^{38,39} When renal tubular injury induces upregulation of SPP1 inflammatory signals,⁴⁰ SPP1 may chemoattract macrophages toward glomeruli, exacerbating podocyte injury and causing SPP1 release from damaged cells into urine.^{23,41} In glomerular lesions with epithelial cell proliferation, SPP1 promotes FSGS progression by recruiting macrophages to invade damaged glomeruli, and detection of SPP1 in glomeruli and urine may help assess FSGS prognosis.⁴² Thus, urinary SPP1 theoretically has the potential to reflect FSGS kidney injury.

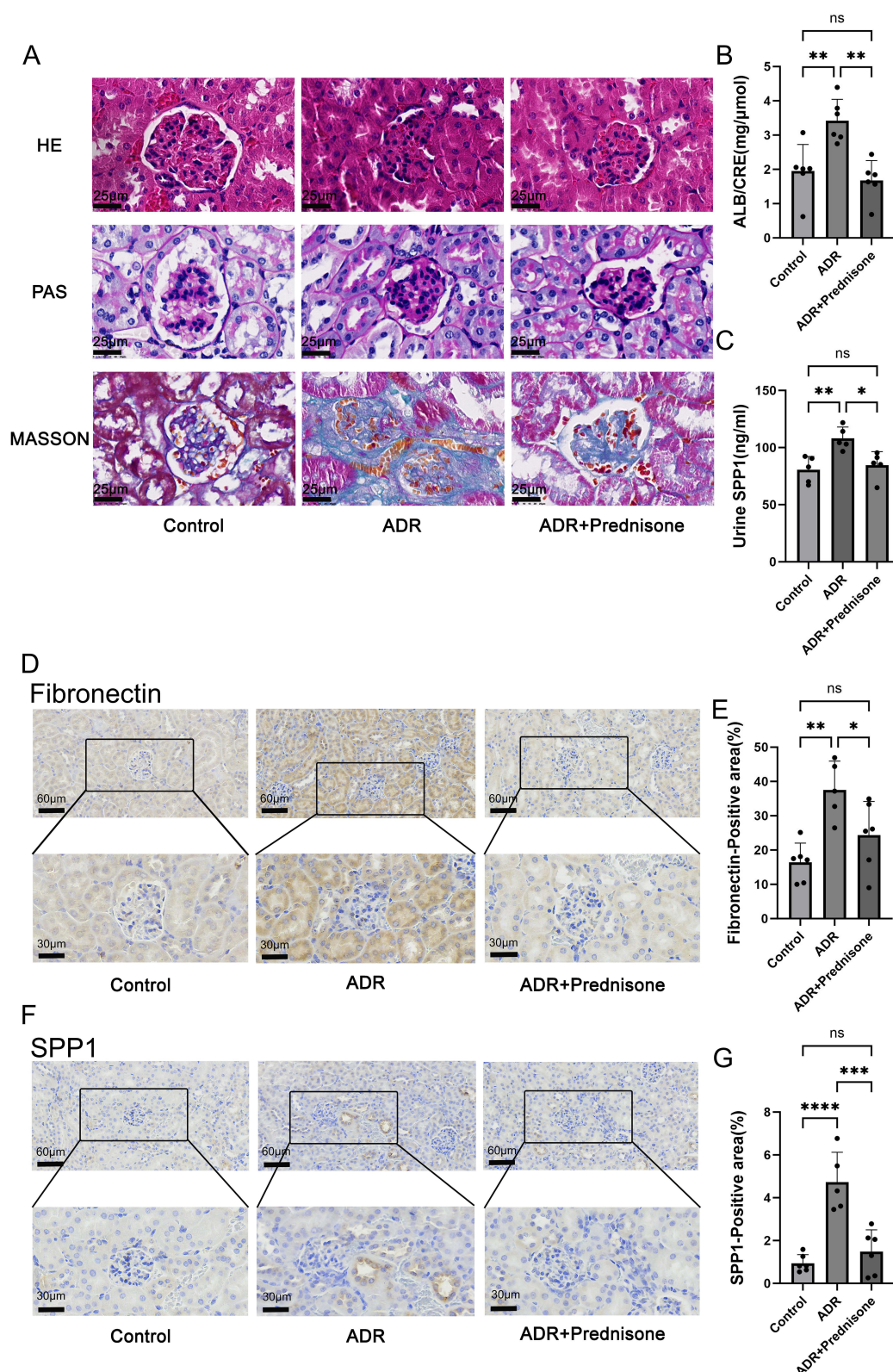


Figure 4 SPP1 expression in ADR mice before and after prednisone treatment (Control group n≥5, ADR group n≥5, ADR+Prednisone group n≥5, ns indicates no significant difference, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). **(A)** Glomerular lesion assessment by HE, PAS, and Masson staining of renal tissue sections from ADR mice before and after prednisone treatment. **(B)** Urinary albumin/urinary creatinine ratio in ADR mice before and after prednisone treatment. **(C)** Urinary SPP1 expression in ADR mice before and after prednisone treatment. **(D-G)** Immunohistochemistry showing expression of renal fibrosis marker fibronectin and SPP1 in mouse kidneys.

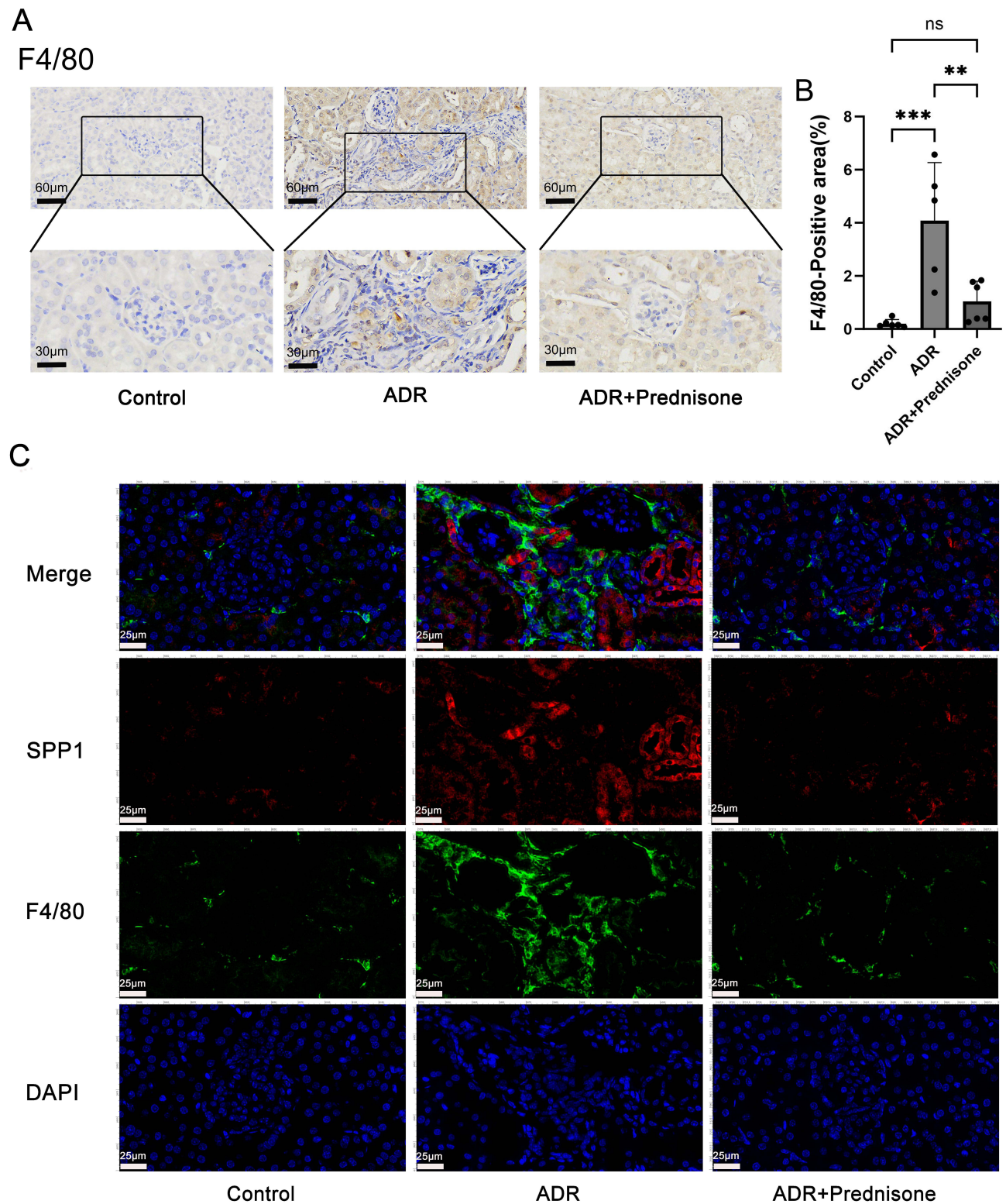


Figure 5 Correlation between SPP1 and immune infiltration in ADR mice before and after prednisone treatment (Control group n=6, ADR group n=5, ADR+Prednisone group n=6, ns indicates no significant difference, ** $P < 0.01$, *** $P < 0.001$). (A and B) Immunohistochemistry showing expression of macrophage marker F4/80. (C) Immunofluorescence double-label staining detecting co-expression and spatial distribution characteristics of SPP1 and F4/80 in renal tissue.

In this study, Bulk-RNA sequencing results showed differential expression of urinary SPP1 in FSGS patients, consistent with previous findings.²⁷ Additionally, we found that urinary SPP1 was significantly elevated in FSGS patients, particularly in CKD stage III, with urinary SPP1 showing an increasing trend with disease progression and correlation with renal function indicators. This finding suggests that urinary SPP1 may have potential for reflecting FSGS disease progression. ELISA results showed that urinary SPP1 was significantly elevated in FSGS patients compared with healthy controls, with statistical significance, but no statistically significant differences were observed in MN, DN, or MCD diseases compared with healthy controls. Although this result suggests that urinary SPP1 in this study cohort reflects FSGS-related kidney injury, the current data are insufficient to determine whether SPP1 is an FSGS-specific marker or a general indicator reflecting broader kidney injury. In previous studies, SPP1, as an immune regulatory factor, has been involved in regulating immune responses, renal inflammation, and fibrosis processes in kidney diseases including FSGS, MN, and DN,^{42–44} as a renal fibrosis mediator, it promotes fibrosis progression in fibrotic kidney diseases such as renal ischemia-reperfusion injury, hydronephrosis, and DN.^{18,45–47} Thus, SPP1 shows elevated expression trends in various kidney diseases. Therefore, SPP1 may be more suitable as a disease progression monitoring indicator for FSGS, used to track changes in disease status in the same patient, while its ability for initial differential diagnosis between FSGS and other kidney diseases requires further investigation. Given that the clinical discriminatory ability of single biomarkers is usually limited, future studies should further evaluate the value of SPP1 combined with other urinary markers and renal function indicators to improve the accuracy of disease monitoring and enhance the specificity of clinical assessment.^{48,49} Multi-marker combination strategies may better reflect the complex pathophysiological processes of FSGS.

Renal biopsy is not the preferred method for primary or secondary endpoint assessment in FSGS patient treatment.^{50,51} Therefore, we selected renal tissue from ADR mice after prednisone treatment for FSGS treatment prognosis assessment. Considering the lack of electron microscopy examination of renal tissue in the mouse modeling process, we performed multi-faceted validation using HE, PAS, and Masson staining. Results showed that glomerular lesions began to appear in ADR mice at week 4 after adriamycin tail vein injection, and prednisone treatment was initiated at this time point. After prednisone treatment, ADR+prednisone group mice showed significant alleviation of glomerulosclerosis, capsular adhesion, and glycoprotein and collagen fiber proliferation progression, accompanied by decreased SPP1 expression in renal tissue. This finding suggests that SPP1 expression changes may be associated with glomerulosclerosis and fibrosis, and prednisone treatment may exert renoprotective effects by reducing SPP1 expression, suggesting that SPP1 may become an effective molecule for FSGS disease progression and treatment prognosis assessment in the future.

Furthermore, numerous studies have indicated that SPP1 participates in shaping the immune microenvironment by regulating immune cell infiltration in various tumors and inflammatory diseases. For example, SPP1 promotes macrophage profibrotic phenotype through the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway, accelerating pulmonary fibrosis progression.⁵² SPP1+ tumor-associated macrophages limit CD8+ T cell infiltration and function,⁵³ reducing programmed cell death protein 1 (PD-1) inhibitor efficacy. In experimental validation of ADR mouse renal tissue, SPP1 and the macrophage marker F4/80 showed spatial co-expression in sclerotic glomeruli and renal interstitial regions, suggesting that SPP1 may participate in FSGS immune microenvironment remodeling by recruiting and activating macrophages. However, inherent differences exist between phenomena observed in animal models and human diseases. Therefore, the regulatory effects of prednisone on SPP1 and their clinical significance in humans require further validation through clinical studies.

This study has several limitations. First, regarding sample size, the Bulk-RNA sequencing cohort included 7 FSGS patients and 13 healthy controls, which is reasonable for preliminary exploratory research, but the robustness and generalizability of the findings require confirmation in larger independent validation cohorts. Second, regarding disease specificity, this study did not include other types of glomerular diseases (such as MCD, DN, MN) as controls, and the collected sample size was small; therefore, it remains unclear whether elevated SPP1 is specific to FSGS or a common feature of various glomerular diseases. Third, regarding mechanistic research, this study was primarily based on clinical observation and animal models, without in-depth exploration of the specific molecular mechanisms of SPP1 in FSGS pathogenesis, such as how SPP1 is regulated and released, the interaction mechanisms between SPP1 and macrophages, and the respective roles of SPP1 in podocyte injury and tubular lesions, which require further elucidation. Finally, this study mainly adopted a cross-sectional design with single measurement of urinary SPP1 levels, without assessing its

dynamic changes in relation to disease progression; the prognostic value of the biomarker requires validation through longitudinal cohort studies. Future research should expand sample sizes, conduct multi-center prospective studies to validate these findings, perform longitudinal follow-up studies to assess the association between urinary SPP1 dynamic changes and disease progression and treatment response, deeply explore the role of SPP1 in FSGS pathophysiology, and investigate the possibility of SPP1 combined application with other biomarkers.

Conclusion

Through integration of urinary transcriptome sequencing, clinical sample validation, and animal experiments, this study identified elevated urinary SPP1 levels in FSGS patients, with levels correlating with disease progression. In the FSGS mouse model, SPP1 expression was closely associated with glomerulosclerosis, macrophage infiltration, and fibrosis, and prednisone treatment reduced SPP1 expression and improved renal pathological changes. These results suggest that urinary SPP1 may be a promising candidate indicator for reflecting FSGS disease progression, and its potential value in disease monitoring and treatment response assessment warrants further research and validation.

Abbreviations

SPP1, secreted phosphoprotein 1; FSGS, focal segmental glomerulosclerosis; CKD, chronic kidney disease; GFR, glomerular filtration rate; RT-qPCR, reverse transcription quantitative real-time polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; GSEA, gene set enrichment analysis; NES, normalized enrichment score; DEG, differentially expressed gene; FDR, false discovery rate; ROC, receiver operating characteristic; AUC, area under the curve; HE, hematoxylin and eosin; PAS, periodic acid-Schiff; ADR, adriamycin; DPBS, Dulbecco's phosphate-buffered saline; cDNA, complementary DNA; BSA, bovine serum albumin; DAPI, 4',6-diamidino-2-phenylindole; HRP, horseradish peroxidase; DAB, 3,3'-diaminobenzidine; SPF, specific pathogen-free; AVMA, American Veterinary Medical Association; ANCA, antineutrophil cytoplasmic antibody; JAK2, Janus kinase 2; STAT3, signal transducer and activator of transcription 3; PD-1, programmed cell death protein 1; TGF- β_1 , transforming growth factor-beta 1; MN, membranous nephropathy; MCD, minimal change disease; DN, diabetic nephropathy; ESRD, end-stage renal disease; KDIGO, Kidney Disease: Improving Global Outcomes; Scr, serum creatinine; Hb, hemoglobin; SD, standard deviation; ANOVA, analysis of variance.

Data Sharing Statement

The data supporting the findings of this study are included in the manuscript and its supporting information. The urinary cell Bulk-RNA sequencing expression matrix supporting this study is available in [Supplementary Material Data 1](#).

Ethics Approval

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Guangxi Medical University and conducted in accordance with the ethical principles of the Declaration of Helsinki (2022-KY-0623). Human experimental protocol approval number: 2022-KY-0336; Animal research protocol approval number: 2023-IACUC(002); Clinical trial number: not applicable.

Informed Consent and Publication

Study participants underwent clinical sample collection after signing informed consent forms. This study is not applicable for consent to publish identifiable images or other personal or clinical details of participants.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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