Original Article

New Mutation of Coenzyme Q_{10} Monooxygenase 6 Causing Podocyte Injury in a Focal Segmental Glomerulosclerosis Patient

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Abstract

Background: Focal segmental glomerulosclerosis (FSGS) is a kidney disease that is commonly associated with proteinuria and the progressive loss of renal function, which is characterized by podocyte injury and the depletion and collapse of glomerular capillary segments. The pathogenesis of FSGS has not been completely elucidated; however, recent advances in molecular genetics have provided increasing evidence that podocyte structural and functional disruption is central to FSGS pathogenesis. Here, we identified a patient with FSGS and aimed to characterize the pathogenic gene and verify its mechanism.

Methods: Using next-generation sequencing and Sanger sequencing, we screened the causative gene that was linked to FSGS in this study. The patient's total blood RNA was extracted to validate the messenger RNA (mRNA) expression of coenzyme Q_{10} monooxygenase 6 (COQ6) and validated it by immunohistochemistry. COQ6 knockdown in podocytes was performed *in vitro* with small interfering RNA, and then, F-actin was determined using immunofluorescence staining. Cell apoptosis was evaluated by flow cytometry, the expression of active caspase-3 was determined by Western blot, and mitochondrial function was detected by MitoSOX.

Results: Using whole-exome sequencing and Sanger sequencing, we screened a new causative gene, *COQ6*, NM_182480: exon1: c.G41A: p.W14X. The mRNA expression of COQ6 in the proband showed decreased. Moreover, the expression of COQ6, which was validated by immunohistochemistry, also had the same change in the proband. Finally, we focused on the *COQ6* gene to clarify the mechanism of podocyte injury. Flow cytometry showed significantly increased in apoptotic podocytes, and Western blotting showed increases in active caspase-3 in si-COQ6 podocytes. Meanwhile, reactive oxygen species (ROS) levels were increased and F-actin immunofluorescence was irregularly distributed in the si-COQ6 group.

Conclusions: This study reported a possible mechanism for FSGS and suggested that a new mutation in *COQ6*, which could cause respiratory chain defect, increase the generation of ROS, destroy the podocyte cytoskeleton, and induce apoptosis. It provides basic theoretical basis for the screening of FSGS in the future.

Key words: Apoptosis; Coenzyme Q_{10} Monooxygenase 6 Mutation; Focal Segmental Glomerulosclerosis; Podocyte

Introduction

Nephrotic syndrome (NS) is a chronic kidney disease that is defined by significant proteinuria, with resulting hypoalbuminemia and edema. NS is classified into steroid-sensitive, steroid-dependent, and steroid-resistant forms according to the response to intensive steroid therapy. Most steroid-resistant NS (SRNS) patients have a histological presentation of focal segmental glomerulosclerosis (FSGS).[1] FSGS is a clinicopathological entity that is characterized by focally sclerous glomeruli and the effacement of podocyte foot processes. The pathogenesis of FSGS has not been completely demonstrated. However, recent advances in molecular genetics have provided growing evidence that

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disruptions in podocyte structure and function are crucial to FSGS pathogenesis.

Podocytes are highly differentiated cells with complex actin cytoskeletal architectures that play vital roles in maintaining the integrity of the glomerular filtration barrier (GFB). Therefore, podocyte loss certainly disturbs glomerular function. Apoptosis is one of the main causes of podocyte loss and consequent proteinuria. Mounting evidence has shown that podocyte apoptosis is one of the most significant mechanisms in the pathogenesis of many kidney diseases, including FSGS,[2] diabetic nephropathy,[3,4] and chronic kidney diseases.[5] To date, 39 dominant and recessive genes have been discovered in patients with SRNS.[6] All of the known monogenic mutations that cause NS exist in podocyte-specific genes, suggesting that podocytes are significant for normal GFB function.[7]

The enzyme coenzyme Q_{10} monooxygenase 6 (COQ6) is an evolutionarily conserved flavin-dependent monooxygenase that is required for the biosynthesis of coenzyme Q_{10} (CoQ_{10}, also referred to as ubiquinone) and is thought to catalyze one or more ring hydroxylation steps. Heeringa *et al*.[8] confirmed that *COQ6* mutations cause SRNS. Recently, we used next-generation sequencing (NGS) to identify mutations in an FSGS patient and found a heterozygous stop-gain mutation in the *COQ6* gene. Moreover, we verified the function of the pathogenic gene *in vivo* and *in vitro*.

Methods

Ethical approval

The study was conducted in accordance with the *Declaration of Helsinki* and was approved by the Ethics Committee of Chinese People's Liberation Army General Hospital (No. S2014-012-01). Informed written consent was obtained from all patients before their enrollment in this study.

Subjects

The proband was a 16-year-old girl, and she was admitted to our department because of macroalbuminuria. Her physical examination showed chronic renal injury and hypertension. Laboratory examinations revealed that her serum creatinine level had increased (158.6 μmol/L, 1 μmol/L = 884 mg/L) and that her 24-h urine protein level was high (6.8 g/d). No history of proteinuria or hematuria was reported for the proband or her father; however, her mother, whose renal biopsy showed FSGS, had a history of proteinuria. Therefore, we collected blood samples from both the subject and her parents, and the venous blood, used for the experiments, was obtained following a morning fast. The control was her father, who was in good health and for whom renal dysfunction, hypouricemia, albuminuria, and hematuria were excluded; his blood sample was obtained as described above.

Next-generation sequencing

Whole-exome sequencing (WES) was performed by the Beijing Annoroad Company (Beijing, China) using an Ion Torrent sequencing system. High-quality genomic DNA was obtained with the super-multiple polymerase chain reaction (PCR) amplification technology, and 294,000 primer pairs from 12 super-multiple PCR primer pools covering approximately 97% of the consensus coding sequences were used to amplify the genomic DNA. Next, DNA-coding exons were sequenced.

Immunohistochemistry

Immunohistochemical staining for the detection of COQ6, CUBN, and APOL1 protein expression in renal tissue was performed on formaldehyde-fixed and paraffin-embedded tissues using the avidin-biotin-immunoperoxidase method. Kidney sections were blocked and labeled with CUBN (Abcam, MA, USA), APOL1 (Abcam, MA, USA), and COQ6 (Proteintech Group Inc., Wuhan, China). After incubation with horseradish peroxidase-conjugated secondary antibodies (ZSGB-BIO, Beijing, China), the sections were treated with an avidin-biotin-peroxidase conjugate (ZSGB-BIO, Beijing, China). The reaction was visualized using a 3, 3′-diaminobenzidine chromogen (Dako, Denmark) following tissue counterstaining with hematoxylin.

Cell culture

The conditionally immortalized mouse podocyte cell (MPC) line was cultured as described previously.[9] Cells were cultured at 33°C in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD, USA) and recombinant interferon-gamma (IFN-γ) (Sigma, MO, USA) in a humidified incubator with 5% CO_2 in air. To induce differentiation, podocytes were reseeded and cultured at 37°C coated with 12 mg/ml type I collagen (Sigma, MO, USA) and in RPMI-1640 medium supplemented with 10% FBS, without IFN-γ for 10–12 days. Cultures were provided with fresh medium three times every week.

Transfection of small interfering RNA

For the targeted downregulation of protein expression in podocytes, we used small interfering RNA (siRNA). Small interfering negative control (si-NC) and si-COQ6 (targeting COQ6) were designed and synthesized by GenePharma Co. (Shanghai, China). The siRNA sequences were as follows: *si-NC 5′-UUC UCC GAA CGU GUC ACG UTT-3′ and si-COQ6 5′-GGA AGG ACU UAG GCU CCA UTT-3′.* When the podocytes were 80% confluent, the siRNAs were transfected into podocytes using the jetPRIME® reagent (Polyplus-transfection® SA, France) according to the manufacturer's protocol. We conducted subsequent experiments after transfections for 24 h.

Real-time polymerase chain reaction

Total RNA was extracted using Trizol (Invitrogen, CA, USA). A ProtoScript® II First Strand cDNA Synthesis Kit (NEB, USA) and a Programmable Thermal Controller (MJ Research, USA) were used to generate cDNA. Gene expression analysis was conducted by quantitative real-time PCR. The following synthesized primers (BGI, Shenzhen, China) were used for real-time PCR: MusCOQ6, forward 5′-GGTCCAAAGAAAGCCCTGGA-3′, reverse 5′-TGGTCCCATGCACCAAAACT-3′; Mouse GAPDH,

forward 5′-AATTGAGCCCGCAGCCTCCC-3′, reverse 5′-CCAGGCGCCCAATACGACCA-3′; Human COQ6, forward 5′-CCCGAGGAGGCAAGGATATG-3′, reverse 5′-GAGCTGACCCTGTTGCTGTA-3′; and Human GAPDH, forward 5′-AATTGAGCCCGCAGCCTCCC-3′, reverse 5′-CCAGGCGCCCAATACGACCA-3′. Each reaction was amplified in triplicate and the fold change in expression of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method, using GAPDH as the internal control.

Western blot analysis

The cells were lysed by RIPA buffer (Beyotime® Biotechnology, Shanghai, China). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used for separating proteins. Proteins were transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). The primary antibodies (1:1000 diluted in Tris-buffered saline with 0.1% tween, TBST) were used to detect COQ6, nephrin, cleaved caspase-3 (Abcam, MA, USA), F-actin (Abcam, MA, USA), and β-actin (Abcam, MA, USA). β-actin was used as an internal control.

Immunofluorescence

After treatments with siRNAs for 24 h, podocytes were fixed with 4% v/v paraformaldehyde and 0.2% v/v Triton X-100 in sequence. The primary rabbit polyclonal antibodies specific for nephrin and COQ6 were diluted 1:100 v/v. Then, the secondary antibodies were added and conjugated to Cy3 (Jackson ImmunoResearch Laboratories, ME, USA). Confocal laser scanning microscopy (Olympus America Inc., Center Valley, PA, USA) was performed. FITC-phalloidin, applied for conjugation to F-actin, was incubated with the podocytes according to the manufacturer's protocol (YEASEN, Shanghai, China).

Immunofluorescence staining for the detection of nephrin expression in renal tissue was performed. Kidney sections were blocked and labeled with nephrin. The subsequent steps were basically the same as those used for nephrin immunofluorescence in cells.

Cell apoptosis assay

Cell apoptosis was assessed by nuclear staining or Annexin V staining. The cell grouping was the same as described above. At 24 h of incubation, Hoechst 33258 was added to the cellular suspensions according to the manufacturer's instructions (Beyotime® Biotechnology, Shanghai, China). Confocal laser scanning microscopy was performed immediately. For Annexin V-FITC/PI staining, cells were harvested 24 h after transfection, and Annexin V-FITC and PI (Miltenyi Biotec., Bergisch Gladbach, Germany) were added to the cellular suspension according to the manufacturer's instructions. Then, the samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences, CA, USA).

Mito-reactive oxygen species production assay

The MitoSOX™ Red mitochondrial superoxide indicator (Thermo Fisher Scientific, Shanghai, China) was added to the cellular suspension to detect mitochondrial reactive oxygen species (ROS) generation according to the manufacturer's protocol. Briefly, after 24 h of incubation, the cells were covered with 1.0 ml of a 5-μmol/L MitoSOX™ reagent working solution, and then, the cells were incubated for 10 min at 37°C. Confocal laser scanning microscopy was immediately performed.

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism 5 software (Vision 5.0, San Diego, CA, USA). A value of $P < 0.05$ was considered as statistically significant. All data are presented as the mean ± standard deviation (SD). All experiments were performed at least three times.

Results

Next-generation sequencing and Sanger sequencing of mutated genes related to focal segmental glomerulosclerosis

Data from WES led to the identification of 11 genes that were mutated in association with FSGS in the proband [Table 1]. All of the potential disease-causing variants that were identified above were confirmed by Sanger sequencing [Table 2]. The proband shared six identical gene mutations with both parents and one gene mutation with her father, even though her father was in good

Table 1: Results of Whole-exome sequencing in proband*

Gene	GenBank number	Exonic function	AAChange
NPHS1	NM_004646	Nonsynonymous SNV	Exon3:c.G349A:p.E117K
APOL1	NM_001136541	Nonsynonymous SNV	Exon5:c.G394A:p.E132K
COQ2	NM_015697	Nonsynonymous SNV	Exon1:c.G196T:p.V66L
COL4A3	NM_000091	Nonsynonymous SNV	Exon7:c.T422C:p.L141P
COL4A3	NM_000091	Nonsynonymous SNV	exon9:c.A485G:p.E162G
COQ6	NM_182480	Stop gain	Exon1:c.G41A:p.W14X
CRB2	NM_173689	Nonsynonymous SNV	Exon10:c.A2905G:p.T969A
CUBN	NM_001081	Nonsynonymous SNV	Exon11:c.C1165A:p.P389T
CUBN	NM_001081	Nonsynonymous SNV	Exon8:c.T758C:p.F253S
CFH	NM_000186	Nonsynonymous SNV	Exon9:c.C1204T:p.H402Y
TTC21B	NM_024753	Nonsynonymous SNV	Exon8:c.A826G:p.T276A

*The results showed 11 potential disease-causing variants on FSGS. SNV: Single nucleotide variation; FSGS: Focal segmental glomerulosclerosis.

Table 2: Results of Sanger sequencing*

Gene	The proband	The proband's father	The proband's mother
NPHS1	T/T	T/T	C/T
APOL1	A/A	G/G	G/G
COQ2	A/A	A/A	A/A
COL4A3	C/C	C/C	C/C
COL4A3	G/G	G/G	G/G
COQ6	A/G	G/G	A/G
CRB2	G/G	G/G	G/G
CUBN	T/T	T/G	T/T
CUBN	G/G	A/G	G/G
CFH	T/T	T/T	T/T
TTC21B	C/C	C/C	C/C

*The proband shared six identical mutated genes with her parents and one mutated gene with her father. Therefore, we screened out three mutated genes: COQ6, CUBN, and APOL1. COQ6: Coenzyme Q10 monooxygenase 6. CUBN: Cubilin. APOL1: Apolipoprotein L1.

health. Therefore, three mutated genes (*COQ6*, *CUBN*, and *APOL1*) were screened.

Coenzyme Q_{10} monooxygenase 6 expression is downregulated in both the kidney tissues and blood samples of the proband

To verify the expression of the mutated genes, we conducted immunohistochemistry of COQ6, CUBN, and APOL1 in control renal tissues and in the renal tissues of the proband and her mother. The results demonstrated the cellular localizations of the three genes' protein. The immunohistochemical results showed that the COQ6 and CUBN expression levels of both the proband and her mother were significantly lower than those of the control group [Figure 1a]. However, the APOL1 expression of the subject was much higher than that of the control group [Figure 1a], which contradicted the findings in the literature.[10] Therefore, the pathogenicity of APOL1 was excluded. Moreover, CUBN is mainly expressed in renal tubules,[11] while FSGS is a clinicopathological entity that is characterized by the effacement of podocyte foot processes. Therefore, the pathogenicity of CUBN was also excluded, and COQ6 was confirmed. We then examined the levels of COQ6 in whole blood using reverse transcription PCR (RT-PCR). Our results showed that the messenger RNA (mRNA) expression levels of COQ6 were significantly decreased compared to those of the control group (her father) [Figure 1b].

Coenzyme Q_{10} monooxygenase 6 mutation causes podocyte injury

To verify the function of the pathogenic gene *in vitro*, we suppressed the *COQ6* gene expression in the MPC line with siRNA. The inhibitory efficiency was measured by RT-PCR and compared with the normal and negative controls. This experiment showed that *COQ6* gene expression was approximately 78% inhibited [Figure 2a]. This strategy established a successful cell model of *COQ6* gene mutation.

Western blot analysis of the si-COQ6 and si-NC MPCs was performed to assess whether the COQ6 protein

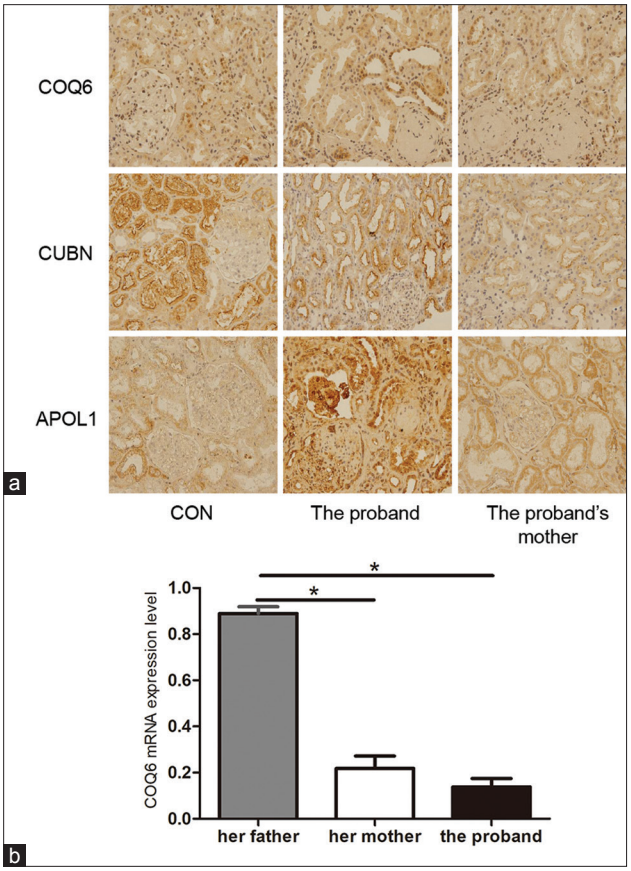

Figure 1: Immunohistochemistry of COQ6, CUBN, and APOL1 in renal tissues. Original magnification ×400. (a) Expression of COQ6, CUBN, and APOL1 in kidney tissues of CON, the proband, and the proband's mother. The staining for COQ6 and CUBN in the tissues of the proband and the proband's mother is negative. The expression of COQ6 is mainly localized to the glomeruli, while CUBN is mainly localized to the renal tubules. APOL1 is overexpressed in the proband compared to the mother and healthy control. (b) The RT-PCR results showed that compared with the control (the proband's father), the COQ6 mRNA expression levels of the proband and the proband's mother were significantly decreased, respectively (*$P < 0.01$). In CON, or the healthy control, the renal biopsy showed a normal glomerular structure. In the proband, the renal biopsy showed FSGS. In the proband's mother, the renal biopsy showed FSGS. COQ6: Coenzyme Q_{10} monooxygenase 6; RT-PCR: Reverse transcription polymerase chain reaction; FSGS: Focal segmental glomerulosclerosis; mRNA: Messenger RNA; si-NC: Small interfering negative control; CON: Healthy control.

was differentially expressed. After incubation with the anti-COQ6 antibody, a distinct band at approximately 51,000 was detected in each lane. Consistent with the SDS-PAGE analysis, Western blot analysis also showed decreased COQ6 expression in the si-COQ6 group. These alterations in COQ6 protein abundance between the normal and si-COQ6 podocytes were consistent with the immunohistochemistry and PCR results. Together, these results suggested that the same effects were achieved by siRNA-COQ6 in MPCs as those achieved by the mutation in the FSGS subject. To better understand the molecular mechanisms underlying the deleterious effects of COQ6, Western blot analysis of nephrin and F-actin was conducted, and the expression levels of both

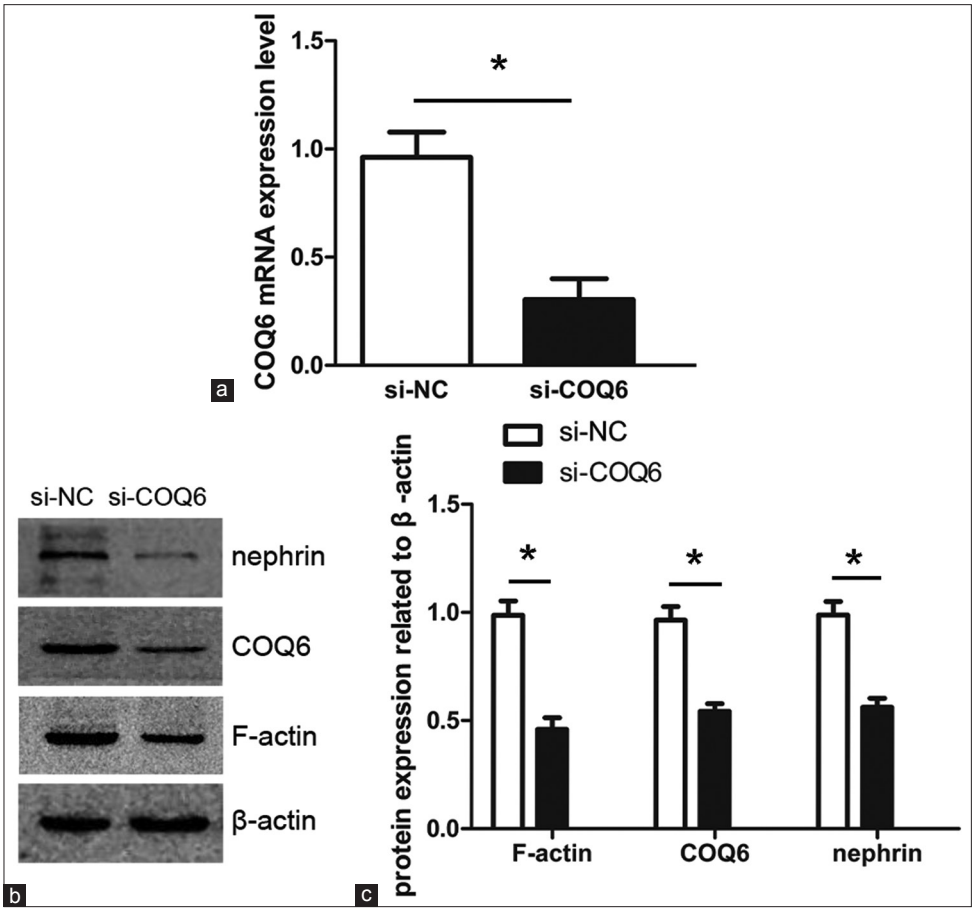

Figure 2: *COQ6* mRNA expression and cell injury protein expression levels in MPCs incubated with siRNA-COQ6. (a) RT-PCR revealed the downregulation of the *COQ6* gene expression in the podocytes incubated with siRNA compared with the negative control (*$P < 0.05$). (b) The expression levels of COQ6, nephrin, and F-actin were determined by Western blot in si-NC and si-COQ6 cells. The protein levels of COQ6, nephrin, and F-actin were all significantly lower in the si-COQ6 cells than in the control cells. (c) Relative quantification of the COQ6, nephrin, and F-actin levels in (b), which were normalized to the levels of β-actin (*$P < 0.05$). COQ6: Coenzyme Q_{10} monooxygenase 6; mRNA: Messenger RNA; MPCs: Mouse podocyte cells; siRNA: Small interfering RNA; RT-PCR: Reverse transcription polymerase chain reaction; si-NC: Small interfering negative control.

proteins were significantly lower in the si-RNA-COQ6 group than in the control group [Figure 2b and 2c].

Immunofluorescence of COQ6 was applied to further evaluate the effect of COQ6 downregulation after siRNA-COQ6 incubation with MPCs. As predicted, COQ6 immunofluorescence was negatively affected by the siRNA-COQ6 transfection [Figure 3a]. We also performed immunofluorescence for nephrin and F-actin to further confirm the damaging effects of COQ6, and the results showed that the nephrin and F-actin immunofluorescence levels were lower in the MPCs incubated with siRNA-COQ6 than in the control MPCs [Figure 3b and 3c]. In addition, F-actin immunofluorescence in the si-COQ6 group was irregularly distributed [Figure 3c].

Coenzyme Q_{10} monooxygenase 6 mutation causes renal damage

We performed immunofluorescence staining of nephrin in healthy control renal tissues (living donor, normal paracancerous tissues from tumor surgery), in renal tissues from the proband, and in renal tissues without the COQ6 mutation (renal biopsy revealed FSGS). The results showed that the nephrin expression levels were lower in the proband and in the non-COQ6 mutation group than in the control group [Figure 4].

Coenzyme Q_{10} monooxygenase 6 knockdown in cultured podocytes induces mitochondrial dysfunction and apoptosis

COQ6 is required for the biosynthesis of CoQ_{10} (also referred to as ubiquinone). COQ10 operates as a redox carrier in the mitochondrial respiratory chain, shuttling electrons from respiratory chain complexes RCCI and RCCII to complex RCCIII.[12] Therefore, we measured ROS production with MitoSOX™ [Figure 5a]. Consistent with the results from previous studies, ROS levels were increased when the MPCs were incubated with si-COQ6 compared to the normal group.

Heeringa *et al*.[8] found that COQ6 knockdown in murine podocytes via siRNA resulted in an increase in apoptosis. To further verify whether the COQ6 mutation promoted apoptosis, we conducted a series of experiments examining apoptosis and determined the apoptotic rates of MPCs using

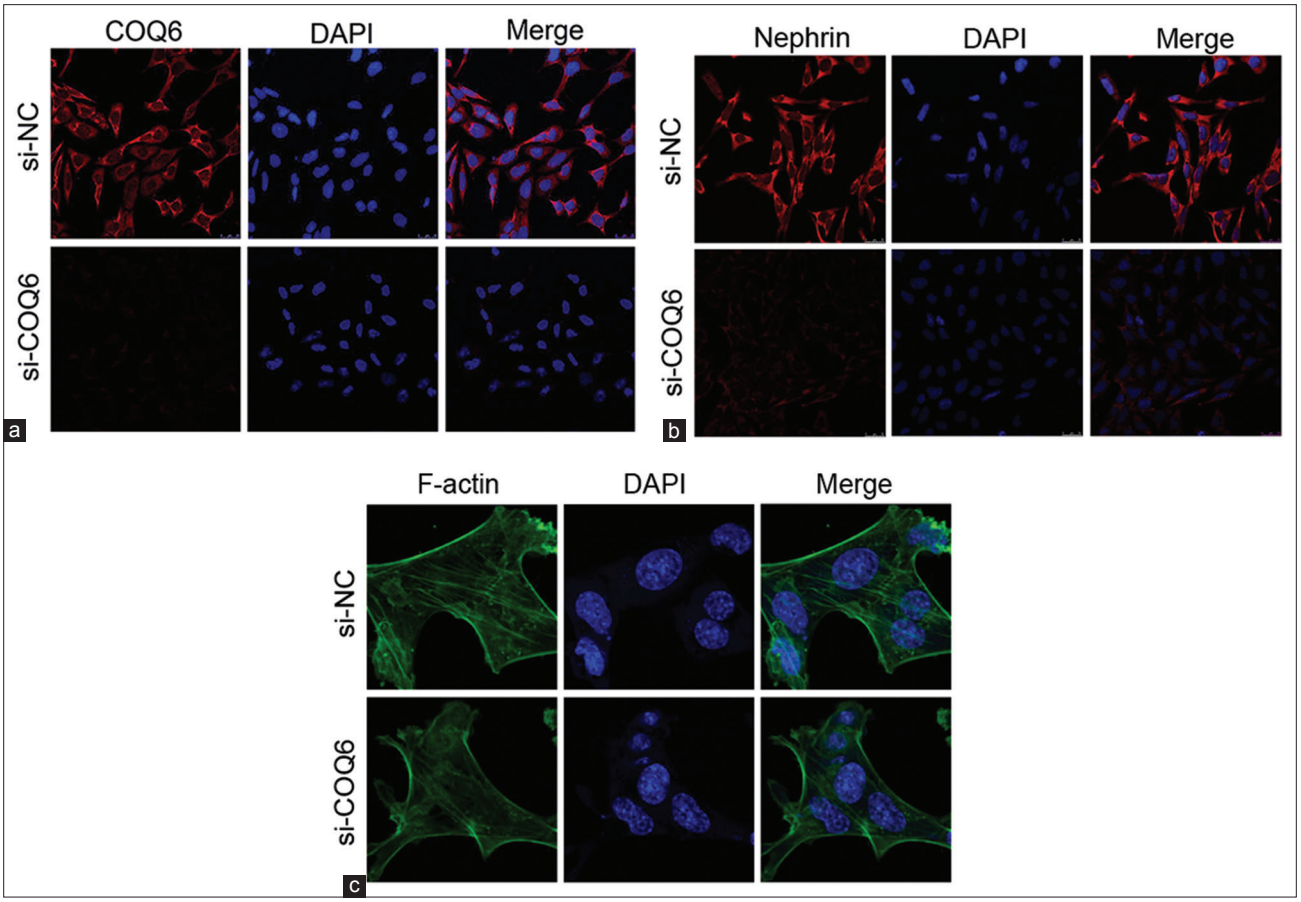

Figure 3: Fluorescence of COQ6, nephrin, and F-actin. Immunofluorescence staining of si-NC and si-COQ6 cells showed that the expression of nephrin and COQ6 in si-COQ6 cells was remarkably decreased compared to that in si-NC cells. COQ6 (a) and nephrin (b) expression was detected with a CY3-labeled antibody (original magnification, ×600). F-actin (c) was detected with FITC-phalloidin (original magnification, ×1800). Nuclei were counterstained with DAPI. The structure of F-actin in si-COQ6 cells was irregular. The results represent three independent experiments. si-NC: Small interfering negative control; COQ6: Coenzyme Q_{10} monooxygenase 6.

flow cytometry. As shown in Figure 5b, the number of Annexin V^+/PI^+ and V^+/PI^- cells was significantly increased in the si-COQ6 group. Moreover, we examined caspase-3, the final downstream executioner caspase of different apoptotic signaling pathways,[13] and evaluated cleaved caspase-3 protein expression in the si-NC and si-COQ6 groups. An increase in cleaved caspase-3 was observed in the si-COQ6 group compared to the control group [Figure 6a and 6b].

Morphological changes to nuclear chromatin during the cell apoptotic process can be classified into three different phases: I, rippled or creased nuclei are observed, with or without chromatin condensation; IIa, nuclear chromatin is highly condensed and marginalized; and IIb, the cell nucleus breaks into fragments, and apoptotic bodies appeared. Therefore, we performed DAPI staining in the si-COQ6 and si-NC groups with Hoechst 33258, a popular cell-permeable nuclear counterstain that emits blue fluorescence when bound to dsDNA, to detect DNA fragmentation [Figure 7a and 7b]. Interestingly, we observed different nuclear chromatin morphological stages of apoptosis in the si-COQ6 group, which served as additional confirmation that si-COQ6 induced apoptosis. The result was consistent with that of the flow cytometric analysis. These results suggested that the *COQ6* mutation carries out damaging effects on podocytes by inducing cell apoptosis, increasing cellular oxidative stress, and destroying the cytoskeleton. Thus, the effects that the *COQ6* gene mutation has on podocyte injury and hence on FSGS have been verified by *in vivo* and *in vitro* investigations.

Moreover, we observed the podocyte foot processes of both the proband and the healthy control with transmission electron microscopy [Figure 8]. The subject showed obvious damage to the podocytes [Figure 8a], but the control had normal podocyte structures [Figure 8b].

Discussion

In this study, we identified a new mutation in *COQ6* (NM_182480: exon1: c.G41A: p.W14X) using NGS, and we determined that the new mutation was a heterozygous nonsense mutation of UGG (Trp) to UGA (X) at codon 41 in *COQ6*, as the cause of SRNS. NGS technology has recently been applied to genetic research in various fields, including hematology,[14] intellectual disability,[15] and oncology.[16] To examine the pathogenic mechanism of this gene, we

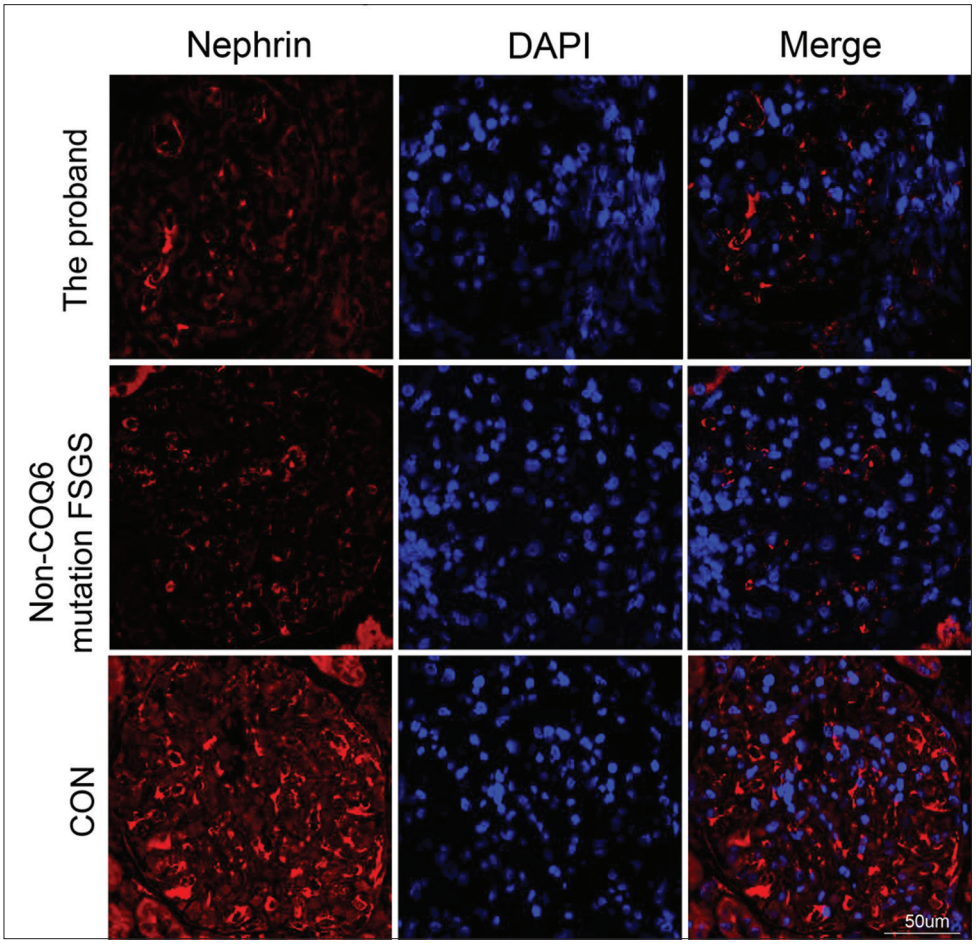

Figure 4: Expression of nephrin in kidney tissues by immunofluorescence. The results showed that the expression levels of nephrin were lower in the proband and the non-COQ6 mutation group than in the control group. The Cy3-stained nephrin in podocytes is indicated in red; the DAPI-stained nuclei are indicated in blue. COQ6: Coenzyme Q_{10} monooxygenase 6.

conducted a series of *in vivo* and *in vitro* experiments, which showed that the *COQ6* mutation played a vital role in FSGS.

Glomeruli are functional filtration units comprising a capillary network of endothelial cells and mesangial cells, which are separated from podocytes by a basement membrane.[17,18] Podocyte injury and loss contribute to proteinuria and glomerulosclerosis.[7,19-23] Numerous podocyte gene products, such as nephrin (NPHS1),[24] podocin (NPHS2),[25] laminin beta-2 (LAMB2),[26,27] α-actinin-4 (ACTN4),[28] Wilms' tumor suppressor gene 1 (WT1),[29] and inverted forming 2 (INF2),[30] are required to construct the podocyte body and foot processes. All of the known monogenic mutations that cause NS reside in podocyte-specific genes, suggesting that podocytes are essential for normal GFB function.[7] The complex structural podocyte composition is also achieved by sophisticated metabolic and energy requirements, such as autophagy.[31] Enzymes and kinases involved in the mitochondrial respiratory transport chain (COQ6,[8] COQ2,[32] and aarF domain containing kinase 4 [ADCK4][33]) are also implicated in podocyte integrity.

Mutations in *COQ6*, which is required for the biosynthesis of CoQ10, were first reported in human patients with steroid-resistant FSGS in 2011.[8] CoQ10, also known as ubiquinone, is a lipid-soluble component of virtually all cell membranes, in which CoQ10 is thought to play a crucial antioxidant role and to transport electrons from complexes I and II to complex III in the respiratory chain of the mitochondrial inner membrane.[34] CoQ10 also serves as a lipid-soluble antioxidant and protector against ROS-mediated cell damage.[35] The biochemical pathway of CoQ10 biosynthesis is complex and has not been completely elucidated. To date, approximately 15 genes have been identified as required for the biosynthesis of CoQ10, and mutations in 8 of these genes (*PDSS1*, *PDSS2*, *COQ2*, *COQ4*, *COQ6*, *ADCK3*, *ADCK4*, and *COQ9*) have been reported to cause primary CoQ10 deficiency in humans.[36] Combined phenotypes of SRNS/FSGS have been associated with mutations in 4 of these genes (*COQ6*, *COQ2*, *ADCK4*, and *PDSS2*).[8,32,33,37]

Mitochondrial dysfunction leads to a decrease in ATP production, alterations in cellular function and structure, and the loss of renal function.[38] That ROS stimulates the intrinsic mitochondrial apoptotic pathway, which further triggers caspase-dependent or caspase-independent signaling events, which is universally acknowledged.[39] Previous studies with similarities to the present study have

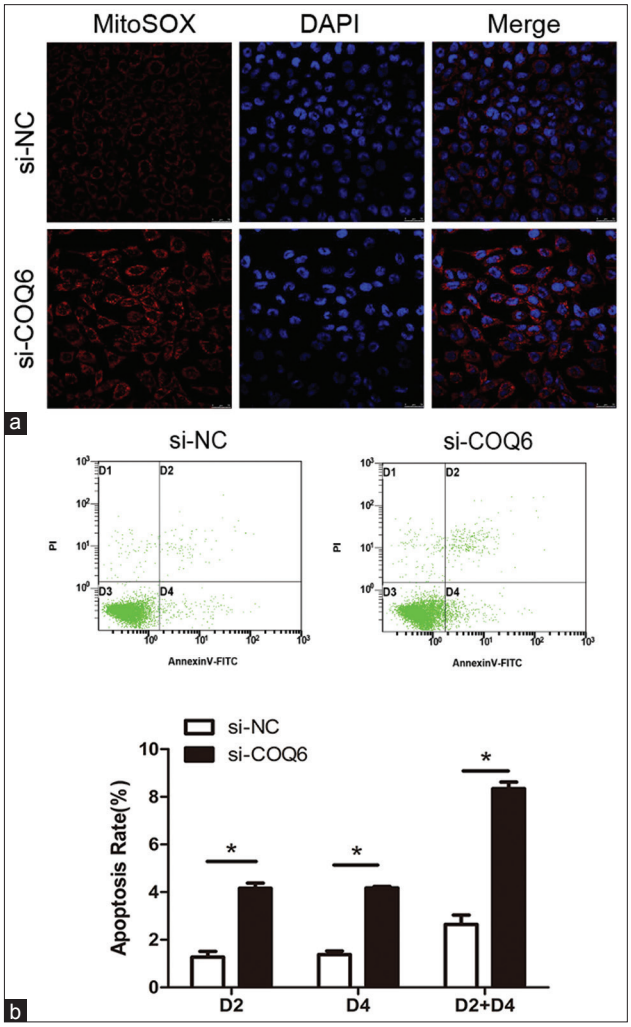

Figure 5: COQ6 knockdown in cultured podocytes augments ROS generation and induces apoptosis. (a) The results show that ROS generation, which was detected with MitoSOX™ (original magnification, ×600), in the si-COQ6 group was significantly increased. The MitoSOX-stained mitochondria of the podocytes are indicated in red, which represent the generated ROS. Hoechst 33258-stained nuclei are indicated in blue. (b) Flow cytometry for the apoptosis of podocytes (*$P < 0.05$). Podocytes were incubated as described above. The results represent three independent experiments. ROS: Reactive oxygen species; COQ6: Coenzyme Q_{10} monooxygenase 6; si-COQ6: Small interfering COQ6.

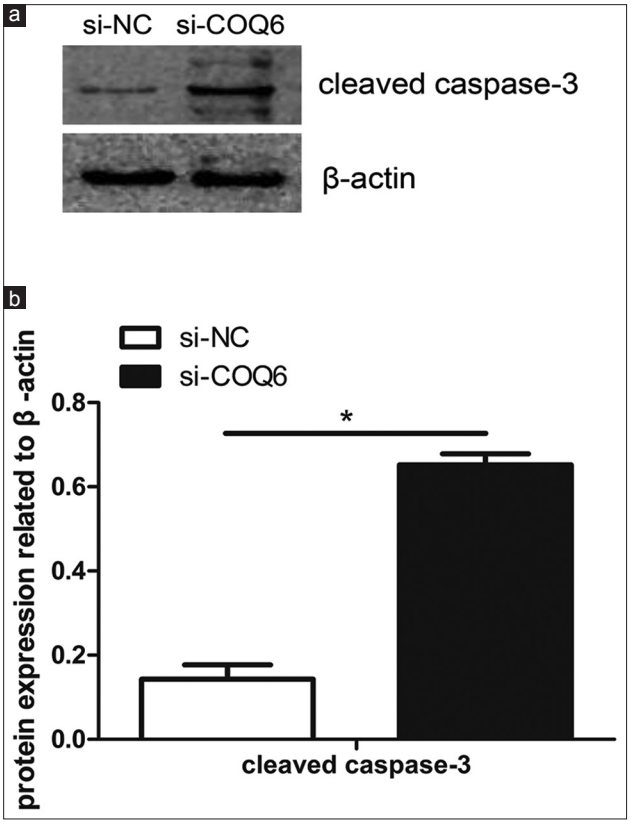

Figure 6: COQ6 knockdown in cultured podocytes induces apoptosis. (a) Western blot for cleaved caspase-3 in the si-NC and si-COQ6 groups. We found that the transfection of siRNA-COQ6 significantly decreased cleaved caspase-3 compared to the negative control. (b) Relative quantification of cleaved caspase-3 (a), normalized to β-actin (*$P < 0.01$). COQ6: Coenzyme Q_{10} monooxygenase 6; si-NC: Small interfering negative control; siRNA: Small interfering RAN.

been performed[8,40] and have reported *COQ6* mutations in patients with steroid-resistant FSGS and sensorineural hearing loss. However, the proband in our study did not show hearing loss, a difference that may be partly attributed to the heterozygous mutation. To further verify the function of the mutation, we performed *in vitro* experiments. We established an *in vitro* cell model with MPCs transfected with siRNA. Cell protein pellets were collected and examined, and the results of the experiments were consistent with those obtained *in vivo*. Our data indicated that the *COQ6* mutation led to cytoskeletal disorder and that the downregulation of F-actin caused FSGS.[41] In our study, we did not detect any gene mutations for F-actin. Thus, we consider the F-actin effect to be a secondary change, accelerating the disease progression. Therefore, the dysfunction of the *COQ6* mutation is responsible for podocyte injury and FSGS.

Fortunately, the specific nature of CoQ10 deficiency allows patients to respond well to oral supplementation with CoQ10, making FSGS the only treatable mitochondrial disorder. High-dose oral CoQ10 supplementation can stop or ease the progression of renal manifestations in patients with *COQ6*,[8] *ADCK4*,[33] and *COQ2*[42] mutations. However, when severe kidney damage is established, the contribution of CoQ10 supplementation to recovery is minimal.[42] The proband in our study continues to be followed up in our department. The proband began CoQ10 supplementation with ubidecarenone tablets (purchased from Eisai China Inc.) according to specific instructions after the genetic diagnosis was obtained. At the beginning of the CoQ10 treatment, the 24-h urinary protein quantity was 6.8–7.4 g/d. After 6 months of treatment and at the time this article was written, the parameter had decreased to 4.0–4.3 g/d. However, the number of patients suffering this kind of disease was still reported low. Therefore, clinical doctors should pay sufficient attention to this condition. When a mutation in *COQ6* is identified, CoQ10 treatment should be administered as soon as possible. The identification and

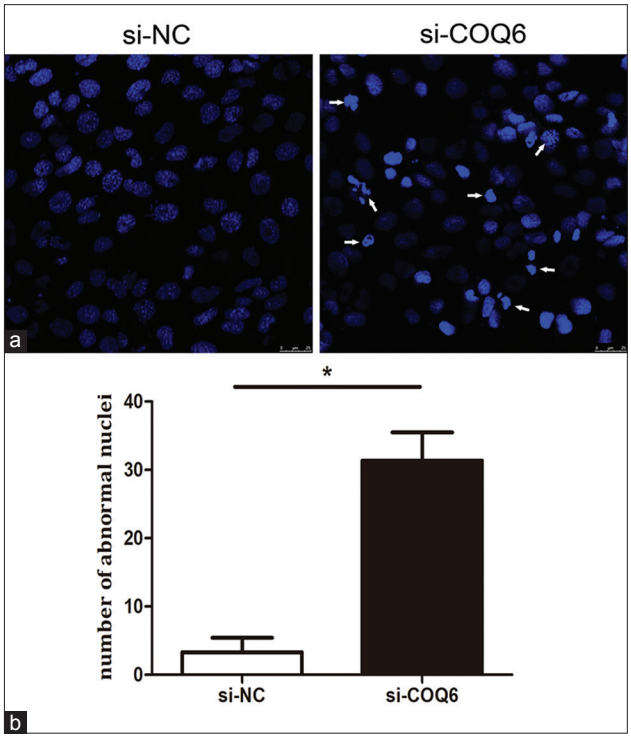

Figure 7: Nuclear staining of podocytes. (a) Nuclear staining of podocytes with Hoechst 33258 in the si-NC and si-COQ6 groups (original magnification, ×600). The results represent three independent experiments. (b) Relative quantification of abnormal nuclear morphology (*$P < 0.01$). si-NC: Small interfering negative control; si-COQ6: Small interfering coenzyme Q_{10} monooxygenase 6.

reporting of further patients with CoQ10 deficiency will lead to a better understanding of the clinical manifestation and pathogenesis of the disease.

Human genetic studies over the past few decades have confirmed that FSGS is primarily a podocytopathy and that more than 20 mutated genes are implicated in the pathogenesis of NS/FSGS.[43] NGS is rapidly transforming the genetic testing of FSGS.[44] WES will likely be available as a clinical diagnostic at a much lower cost, which will enable the convenient analysis of multiple FSGS-related podocyte genes and the exploration of genetic factors in the pathogenesis of FSGS.

In conclusion, we have identified a new mutation in *COQ6* in an FSGS patient. This mutation can destroy the podocyte cytoskeleton and increase ROS generation, eventually leading to cell apoptosis activation and death. Based on these results, we have confirmed that the new *COQ6* mutation causes FSGS and provides a possible pathogenic mechanism for FSGS.

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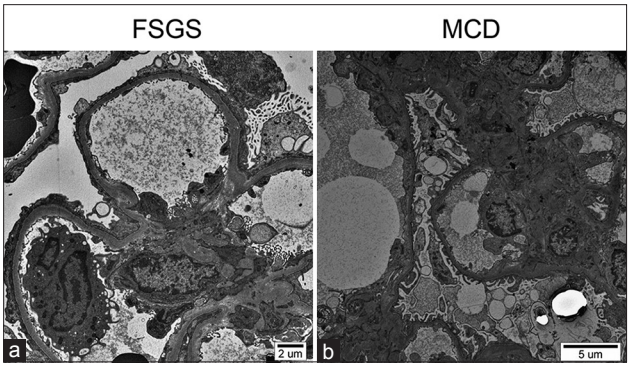

Figure 8: Transmission electron microscopy for podocytes shows podocyte foot processes (a). The proband shows the effacement of podocyte foot processes, resulting in a continuous electron-dense deposition (b). CON (MCD) shows normal podocyte foot processes. FSGS: Focal segmental glomerulosclerosis. MCD: Minimal change nephrosis.

Conflicts of interest

There are no conflicts of interest.

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COQ6突变导致FSGS患者足细胞损伤的新突变

摘要

背景: 局灶节段性肾小球硬化(focal segmental glomerularsclerosis，FSGS)是一种常见的肾病，常与蛋白尿和渐进性肾功能衰竭有关，其特征为足细胞损伤，肾小球毛细血管袢的缺失和塌陷。然而，FSGS的发病机制尚未完全阐明，近年来分子遗传学的进展提供了越来越多的证据表明足细胞的结构和功能紊乱成为FSGS发病机制的关键。在本研究中，通过围绕一例FSGS患者旨在找出致病基因并验证其致病机制。

方法: 采用新一代测序技术(next-generation sequencing，NGS)和Sanger测序筛出与FSGS相关的致病基因。后提取先证者全血检测COQ6-mRNA表达水平，并通过免疫组织化学验证。体外实验中，利用siRNA敲除足细胞中的COQ6基因，并利用免疫荧光技术检测F-actin表达。通过流式细胞检测细胞凋亡率及检测凋亡蛋白caspase3的表达来验证细胞凋亡情况，并利用MitoSOX检测线粒体功能。

结果: 通过全外显子测序和Sanger测序筛选出COQ6一种新致病突变，NM_182480: exon1: c.G41A:p.W14X。先证者全血COQ6-mRNA检测结果及免疫组化结果均显示COQ6表达明显下降，最终明确COQ6为足细胞损伤的致病基因。通过siRNA抑制足细胞COQ6表达后，流式细胞仪检测到凋亡细胞数明显增多，同时活性Capase-3的表达明显增加，并且线粒体产生的ROS水平明显升高，免疫荧光示F-actin排列紊乱。

结论: 本研究为FSGS发病机制提供了一种新的思路，并且发现了COQ6的一种新突变位点，该突变会致线粒体呼吸链受损，从而ROS生成过多，破坏足细胞骨架，诱导足细胞凋亡。本研究为今后的FSGS的筛查提供了基本的理论依据。