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Original Article

A novel gain-of-function mutation in Transient Receptor Potential C6 that causes podocytes injury

Min Yu[1,#], Jian Hu[2,#], Tao Ju[1], Ren Wang[3], Meiqiu Wang[4], Chunlin Gao[3,*], Zhengkun Xia[3,*]

[1] Department of Neonatology, Taizhou People's Hospital, Nanjing Medical University, Taizhou, Jiangsu 225300, China
[2] Department of Pediatrics, The Affiliated Huai'an No.1 People's Hospital of Nanjing Medical University, Huai'an, Jiangsu 223300, China
[3] Department of Pediatrics, Jinling Hospital, Nanjing, Jiangsu 210002, China
[4] Department of Pediatrics, Affiliated Jinling Hospital, Medical School of Nanjing University, Nanjing, Jiangsu 210002, China

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Abstract

Podocyte injury plays a vital role in focal segmental glomerulosclerosis (FSGS), and apoptosis is one of its mechanisms. The transient receptor potential channel 6 (TRPC6) is highly expressed in podocytes and mutations mediate podocyte injury. We found TRPC6 gene mutation (N110S) was a new mutation and pathogenic in the preliminary clinical work. The purpose of this study was to investigate the potential mechanism of mutation in TRPC6 (TRPC6-N110S) in the knock-in gene mouse model and in immortalized mouse podocytes (MPC5). Transmission electron microscopy was used to evaluate renal injury morphology. We measured 24-hour urinary albumin-to-creatinine ratios and major biochemical parameters such as serum albumin, urea nitrogen, and total cholesterol. The results of CCK-8 assay and apoptosis experiments showed that the TRPC6-N110S overexpression group had slower proliferative activity and increased apoptosis than the control group. FluO-3 assay revealed increased calcium influx in the TRPC6-N110S overexpression group. Podocin level was decreased in TRPC6-N110S group, while TRPC6 and desmin levels were increased in TRPC6-N110S group. The 24 h uACR at 6 weeks was significantly higher in the pure-zygotes group than in the WT and heterozygotes groups, and this difference was found at 8 and 10 weeks.TRPC6 levels showed no significant difference between homozygote and WT mice. Compared to homozygote group, expression of podocin and nephrin were increased in WT, but levels of desmin was decreased in WT. Our results suggest that this new mutation causes podocyte injury probably by enhancing calcium influx and podocyte apoptosis, accompanied by increased proteinuria and decreased expression of nephrin and podocin.

Keywords: Calcium influx, FSGS, Injury, Mutation, Podocytes, TRPC6.

1. Introduction

Focal segmental glomerulosclerosis (FSGS) is a common pathological type of adult and child nephropathy, accounting for 20% of the cases of nephrotic syndrome in children. It is both a common lesion in progressive renal disease and a disease characterized by marked proteinuria and podocyte injury [1]. The incidence of FSGS is increasing over the years [2]. Primary FSGS that do not respond to glucocorticoid therapy often inevitably develop into end-stage renal disease (ESRD) [3]. Current treatments are limited, which cannot prevent the progression of glomerulosclerosis to ESRD. Therefore, it is necessary to find new therapeutic methods and targets.

FSGS is considered to be a kind of podocytosis. Podocytes are highly differentiated epithelial cells, which can be divided into cell body, primary processes and foot processes. The podocytes attach to glomerular basement membrane (GBM) in a regular finger-like pattern, forming slit diaphragms (SDs), and dynamically control the structure of podocytes by sending signals to actin cytoskeleton [4]. Podocyte dysfunction and cytoskeleton disorder lead to the fusion of these podocytes [5]. Podocytes cannot be repaired by cell division, which makes the damaged podocytes become exhausted due to detachment, apoptosis or necrosis, and podocyte effacement, which becomes an important reason for glomerulosclerosis [6]. Therefore, the damage to the filtration barrier, especially to podocytes, is a characteristic of many progressive kidney diseases. The pathogenesis of FSGS is still not fully defined, but the pathology is focal and segmental, showing sclerosis of the affected segments (increased thylakoid matrix, capillary occlusion, balloon adhesions, etc.), loss of podocyte pedicles, tubular atrophy, and interstitial fibrosis. formation, and interstitial fibrosis is a pathological indication of poor prognosis. It has been found that more than 20 podocyte gene mutations are closely related to the pathogenesis of FSGS or nephrotic syndrome, such as NPHS1, NPHS2, WT-1, LAMB2, CD2AP, TRPC6, ACTN4 and INF2.

* Corresponding author.
E-mail address: shuangmu34@163. com (C. Gao); njxzk@126.com (Z. Xia).
# These authors contributed equally
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FSGS caused by these single gene mutations provide an excellent model and possible intervention targets for human understanding of diseases.

Since the CRISPR/Cas9 system was used for gene editing in 2013, the continuously optimized CRISPR/Cas9 system has shown its unique advantages of stability, efficiency, specificity, and operability, and has been widely used in the preparation of human, animal, plant, bacterial, and viral models and gene editing research, and has revolutionized the construction of animal models such as gene knockout and knock-in. The specificity of CRISPR/Cas9 system editing depends on sgRNA. When the Cas9 nuclease and sgRNA enter the nucleus, the sgRNA recognizes the target. When the Cas9 nuclease and sgRNA enter the nucleus, the sgRNA recognizes the target gene and directs the Cas9 nuclease to create a DNA doublet at the When the Cas9 nuclease and sgRNA enter the nucleus, the sgRNA recognizes the target gene and directs the Cas9 nuclease to cause a DNA double-strand break at the target editing site.In the preliminary clinical work, a patient with steroid-resistant nephropathy (SRNS) and rapid progression to chronic kidney disease (CKD) stage 3-5 was detected by whole exon sequencing. The results showed that TRPC6 gene mutation (N110S) was a new mutation and pathogenic [7].

Human transient receptor potential cation 6 (TRPC6) gene is located on the long arm of chromosome 11 (11q22. 1) and encodes transient receptor potential cation channel (subfamily C, member 6), which allows calcium-dominated cation influx. TRPC channel has six transmembrane domains, which are S1-S6. Non-selective cation channels are formed between N-terminal S5 and S6 segments, allowing calcium and other cations to pass through the cell membrane. There are three or four anchored protein-like repeats at the N-terminal of TRPC channel, which can regulate the release of calcium ions in calcium pool by binding with anchored protein. TRPC channel is widely expressed in different types of human and mouse cells and tissues, which is related to cell growth, differentiation, apoptosis and migration [8]. In podocytes, TRPC6 is one component of the glomerular SD. As early as 2005, it was found that TRPC6 mutation was associated with FSGS [9].

Here we make TRPC6-N109S point mutation mice via Clustered Regularly Interspaced Short Palindromic Repeats-associated protein 9 (CRISPR/Cas9) system and performed a transcriptome analysis in mice according to human N110S point mutation. A lentivirus-encoded N110S mutation targeting TRPC6 mRNA was transferred in podocyte in vitro. The aim of this research was to address the impact of the TRPC6 N110S mutation on gene expression *in vivo and in vitro*.

2. Materials and Methods

2.1. Animals

The wild-type (WT) and Trpc6-N109S KI/KI (KI, knock-in) mice generated from *TRPC6-N109S KI/wt* mice using CRISPR/Cas9 system, under the background of the C57BL/6 strain, were obtained from GemPharmatech Company. The animals used in this study were SFP-grade healthy male 9-10 weeks old mice, which weigh 25-30g and are housed in pathogen-free conditions with free access to food and water under 12 h light-and-dark cycles. All experiments were undertaken with approval from the Ethics Committee of Nanjing Medical University.

For *in vivo* studies, a mixture of male and female homozygote and WT mice between 4 and 10 weeks of age were used. The mice were divided into the 4-week group (N =6), 6-week group (N =6), 8-week group (N =6) and 10-week group (N =6). We also established the WT group (N =6). The mice were sacrificed by intraperitoneal administration of barbiturates (200 mg/kg) at the corresponding age, and blood, urine and kidney samples were collected.

2.2. Cell Culture

Conditionally immortalized mouse podocytes (MPC5) purchased from Shanghai Yaji Biotechnology Co., LTD (Shanghai, China) were cultured as described previously [10]. Cells were cultured under growth-permissive conditions at 33°C with 5% CO_2 in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco), 10 U/mL mouse recombinant interferon-gamma (IFN-γ; PeproTech USA), and 100 U/mL penicillin plus 100 mg/mL streptomycin (Sigma). To induce differentiation, podocytes were maintained in nonpermissive conditions at 37°C in the absence of IFN-γ for at least 2 weeks and then were used for experiments. After serum starvation for 24 hours, differentiated podocytes were divided into two groups: negative control group (NC), and Trpc6-N110S overexpression group (Trpc6-N110S). After 48 hours, expression levels of podocin and desmin were measured using Western blot analysis.

2.3. Measurement of Biochemical Parameters and Urinary Albumin and Creatinine Concentrations

Serum albumin, serum creatinine (Scr), and total cholesterol (TC) were determined using a biochemical analyzer (Hitachi Ltd., Tokyo, Japan) at the Department of Clinical Laboratory, Affiliated Hospital of Nanjing Medical University. To determine urine albumin and creatinine (Ucr) concentrations, 24 h urine samples were collected. Urine protein levels were determined using urine protein test kit (C035-2, Nanjing Jiancheng Bioengineering Institute), and creatinine levels were determined using creatinine assay kit (C011-2-1, Nanjing Jiancheng Bioengineering Institute). 24 h urinary albumin-to-creatinine (uACR) was then calculated.

2.4. Light and Transmission Electron Microscopy

Body mass was weighed before sacrifice. Kidneys were harvested and fixed in 10% paraformaldehyde, dehydrated, and embedded in paraffin. Thin sections of tissues were created for periodic acid-Schiff base (PAS) staining. Pathological changes were examined using a light microscope. Renal specimens were fixed in 2. 5% glutaraldehyde and then dehydrated. After staining using uranyl acetate-lead citrate, the morphological characteristics of renal cortex sections (50 nm), including the thickness of GBM, and the condition of podocytes were measured using transmission electron microscopy. Renal specimens were photographed using a JEM-1200 transmission electron microscope (JeolLtd., Tokyo, Japan).

2.5. Western Blot

The homogenized renal cortex tissues and podocytes were washed twice with PBS before the addition of the lysis buffer (RIPA). The lysates were scraped and then centrifuged for 30 min at 12000 rpm. The protein concentration was measured by bicinchoninic acid assay. The protein

samples were separated by SDS-PAGE. Antibodies used were: TRPC6 (515837; Santa Cruz Biotechnology, USA), podocin (1: 1000, Abcam), Desmin (1: 100, Abcam) and β-actin (1: 1000, Abcam). Primary and secondary antibodies were incubated with the membranes using the standard technique. Immunodetection was accomplished using Immobilon Western Chemiluminescent HRP Substrate (P90719; Millipore, USA). Full-length western blots of each antibody with Protein Ladder (26616; Thermo Scientific, USA) were done to confirm antibody specificity. Western blot bands were quantified using ImageJ software and expressed as fold compared to internal loading control.

2.6. CCK-8

Cell proliferation was determined using a Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China). In brief, 2×10^4 cells/well were seeded in a 96-well plate for 24 h. Ten microliters of the CCK-8 solution were added into each well and incubated for 2 h. The absorbencies at each time point were measured at 450 nm by a microplate reader.

2.7. Measurement of Apoptosis

Apoptosis in vitro was quantified using flow cytometry (FCM) after cell staining with an Annexin V-PE/7-AAD Apoptosis Detection Kit (A213-01, Vazyme, China). Briefly, cells were washed twice with cold PBS and resuspended in binding buffer at 1×10^6 cells/mL. Then, 5 μL of Annexin V and 7-AAD was added to 100 μL cell suspensions for 15 min in the dark. Following the incubation, the mixture was diluted with 400 μL binding buffer and analyzed by a flow cytometer (Gallios, Beckman Coulter, USA).

2.8. Fluorescence measurement of intracellular free calcium

Podocytes were grown on 6-well plates and loaded in Hanks' balanced salt solution (HBSS) containing 5 μM Fluo-3/AM (CA1180; Solarbio, China) and 0. 03% Pluronic F-127 (CA1180; Solarbio, China) at 37 °C for 30 min. The fluorescence intensity of Fluo-3 in podocytes was recorded using flow cytometry (Gallios, Beckman Coulter, USA).

2.9. Statistical Analysis

We use GraphPad Prism (version 8. 0; GraphPad Software, San Diego, CA) for statistical analysis. One-way analysis of variance or the Student's *t*-test was used for comparisons between different groups. Data were presented as mean ± SEM, and P <0. 05 was considered statistically significant.

3. Results

3.1. The function of TRPC6 N110S mutation in vitro

CCK-8 assay showed that the proliferation activity of TRPC6-N110S overexpression group was slower than that of the control group (Fig. 1A). We use Annexin V-PE/7-AAD apoptosis Kit to detect apoptosis, suggesting increased apoptosis in the TRPC6-N110S overexpression group (Fig. 1B). Using FluO-3, AM calcium ion concentration detection, increased calcium ion inflow was found in TRPC6-N110S overexpressed group (Fig. 1C).

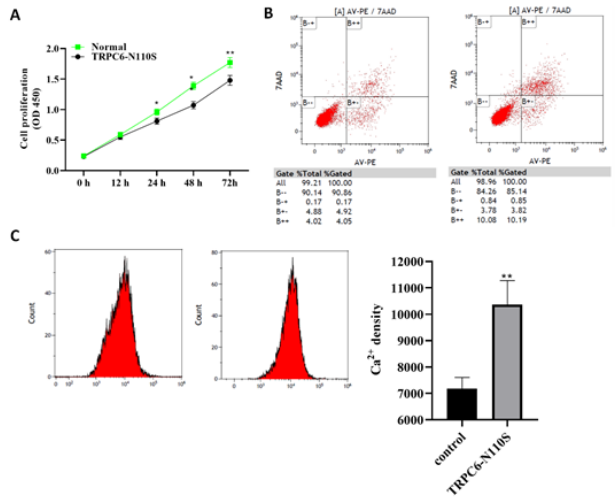

Fig. 1. The function of TRPC6 N110S mutation in vitro. (A) CCK-8 assay showed that the proliferation activity of TRPC6-N110S overexpression group was slower than that of the control group. (B) Flow cytometry showed increased apoptosis in the TRPC6-N110S overexpression group. (C) Increased calcium ion inflow was found in TRPC6-N110S overexpressed group. *P < 0. 05, **P < 0. 01.

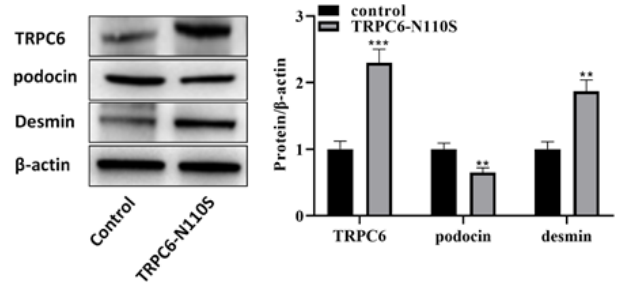

Fig. 2. TRPC6 and associated proteins in vitro. Western blot analyses of TRPC6, podocin, and desmin in TRPC6-N110S and control group. **P < 0. 01, ***P < 0. 01.

3.2. TRPC6 N110S mutation causes changes in podocyte markers and cytoskeletal protein in vitro

Podocin level was decreased in TRPC6-N110S group, while TRPC6 and desmin levels were increased in TRPC6-N110S group (Fig. 2).

3.3. Biochemical Parameters in vivo

Levels of serum albumin at 8 and 10 weeks were significantly lower in the homozygote group than in the WT and heterozygote groups; however, there were no significant differences at 4 and 6 weeks among the three groups. No significant differences were found in body weights and levels of total cholesterol among them. The homozygote group at 6 weeks had significantly greater 24 h uACR than did the WT and heterozygote groups, and this difference was found at 8 and 10 weeks. (Fig. 3A-D).

3.4. Light and transmission electron microscopy

The kidneys of the mice were analyzed, and PAS staining of the kidneys under light microscopy showed no obvious abnormalities (Fig. 4). We used transmission electron microscopy to determine changes of podocytes. We found that foot process fusion in mutant homozygous groups.

3.5. Western Blot Analysis of TRPC6 and associated

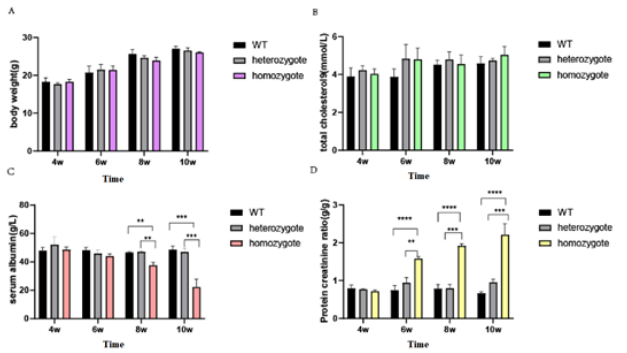

Fig. 3. Biochemical Parameters in vivo. (A) Body weights analysis of mutant and wt mice. (B) Total cholesterol analysis of mutant and wt mice. (C) Levels of serum albumin at 8 and 10 weeks were significantly lower in the homozygote group than in other groups. (D) 24 h uACR of 6-, 8- and 10-week mice was higher in the homozygote group. **P < 0. 01, **P < 0. 001.

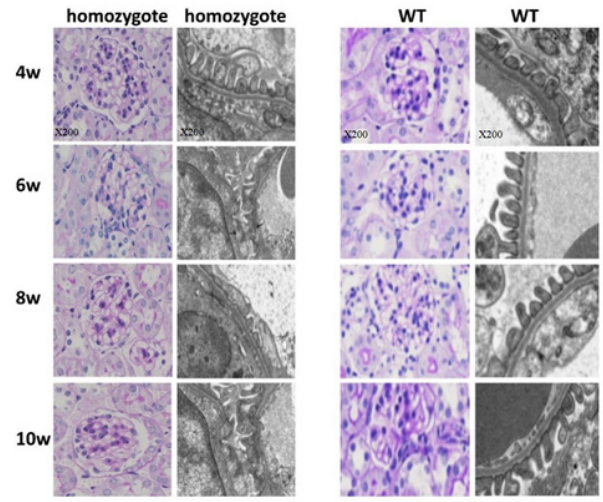

Fig. 4. Glomerular morphology and foot processes in homozygote and wt mice. PAS staining showed no difference in glomerular morphology among groups. Original magnification, X200. Transmission electron microscopy showed foot process effacement at 6, 8 and 10 weeks.

proteins in vivo

TRPC6 levels showed no significant difference between homozygote and WT mice. Compared to homozygote group, expression of podocin and nephrin were increased in WT, but levels of desmin was decreased in WT (Fig. 5).

4. Discussion

In the present study, we established a model of TRPC6 mutation and studied the effects in vivo and in vitro. Reduced cell proliferation, increased calcium ion inflow, and higher apoptosis rate were detected together with decreased expression of podocin. We found higher 24 h uACR and abnormal structures of renal tissues in TRPC6 mutation mice, such as foot process fusion. TRPC6-mediated increased calcium influx directly affects cytoskeletal integrity of podocytes and in turn, causes proteinuria.

The mechanisms underlying podocyte injury in FSGS remain incompletely understood. Researchers have proposed several mechanisms that lead to FSGS, including genetic mutations, glomerular hypertrophy, direct podocyte injury such as podocyte loss, and apoptosis [3]. TRPC6 plays an essential role in podocyte calcium influx pathway and interacts with podocin and nephrin in the slit diaphragm [11]. Consistently, our study established a model of TRPC6 mutation and found reduced cell proliferation, increased calcium ion inflow, and higher apoptosis rate together with decreased expression of podocin in this model. The renal filtration barrier is composed of fenestrated endothelial cells, GBM, and podocytes. TRPC6 interacts with podocin and nephrin in the podocyte [12]. Here, we used transmission electron microscopy to determine changes in podocytes. We found that foot process fusion in mutant homozygous groups. Podocin and nephrin are transmembrane proteins that are two components of the slit diaphragm and maintain the integrity of filtration barrier [13]. Lu and colleagues show that high glucose reduces podocin and nephrin protein levels via a TRPC6-dependent mechanism [14]. Nephrin plays an important role in the regulation of podocytes, in contact with podocin, which suppresses podocyte death [15]. Therefore, reduction of nephrin and podocin is closely related to degradation of the foot process [16].

To our knowledge, TRPC6 is also an upstream regulator of podocyte cytoskeleton. Desmin is one of the cytoskeleton intermediate filaments, which is not expressed or at a low level in the podocytes under normal circumstances. When podocyte damage occurs, desmin expression can increase for the possible reason of cytoskeleton rearrangement. Desmin can therefore be one of the proteins as a marker of podocyte injury. Increasing calcium influx through mutated TRPC6 might disrupt podocyte cytoskeletons, thereby causing proteinuria. Impaired cytoskeletal reorganization leads to podocyte damage, and apoptosis [17]. In the case of N110S mutation, this process would be enhanced. It is possible that TRPC6N110S-mediated calcium influx regulates the cytoskeleton through some pathways.

There are several limitations in our study. First, how TRPC6N110S leads to the decrease in podocin and nephrin will serve as an interesting direction for future study. Also, therapeutic strategies based on the inhibition of TRPC6 channels or targeting the signal pathways deserve our consideration.

5. Conclusions

In summary, we have CRISPR/Cas9 methods to knock in the Trpc6 mutant in mice and podocytes, resulting decrease in podocin and nephrin, and appears to be gain-of-function. Animals homozygous had more proteinuria and podocyte injury as assessed by multiple measures. Our results suggest that this new mutation causes podocytes

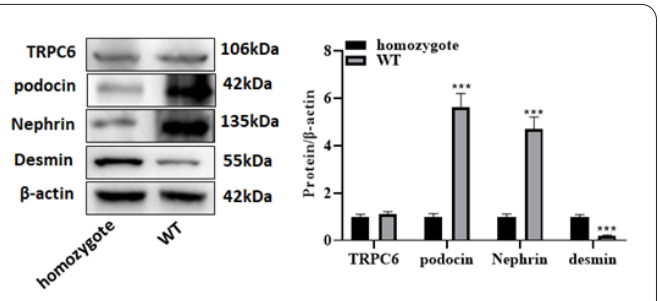

Fig. 5. TRPC6 and associated proteins in vivo. Western blot analyses of TRPC6, podocin, nephrin and desmin in homozygote and WT mice. *P < 0.05, **P < 0.01.

injury probably by enhancing calcium influx and podocyte apoptosis, accompanied by increased proteinuria and decreased expression of nephrin and podocin.

Conflict of Interests
The authors declare that there are no conflicts of interest.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
We have received approval from the Ethics Committee of Nanjing Medical University.

Informed Consent
Not applicable.

Availability of data and material
Data generated during the study can be obtained from the corresponding author under reasonable request.

Authors' contributions
XZ and GC contributed to the study conception and design. Experimental operation, data collection and analysis were performed by YM, HJ, JT, WR and WM. The first draft of the manuscript was written by YM and all authors commented on previous versions of the manuscript.

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