CXCL16/ERK1/2 pathway regulates human podocytes growth, migration, apoptosis and epithelial mesenchymal transition

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Abstract. Primary nephrotic syndrome (PNS) is the commonest glomerular disease affecting children. Previous studies have confirmed that CXC motif chemokine ligand 16 (CXCL16) is involved in the pathogenesis of PNS. However, the exact mechanisms underlying the pathogenesis of PNS remain to be elucidated. Thus, the present study aimed to elucidate the role of CXCL16 in PNS. It was found that the expression of CXCL16 and extracellular signal-regulated kinases 1 and 2 (ERK1/2) were significantly increased in clinical PNS renal tissues using reverse transcription-quantitative PCR, western blot analysis and immunohistochemistry. lentivirus overexpression or short hairpin RNA vector was used to induce the overexpression or knockdown of CXCL16 in podocytes, respectively. Overexpression of CXCL16 in podocytes could decrease the cell proliferation and increase the migration and apoptosis, whereas CXCL16 knockdown increased cell proliferation and decreased cell migration and apoptosis. Results of the present study further demonstrated that ERK2 protein expression was regulated by CXCL16. The knockdown of ERK2 expression reversed the effects of CXCL16 on the proliferation, apoptosis, migration and epithelial mesenchymal transition (EMT) of podocytes. Collectively, the findings of the present study highlighted that the CXCL16/ERK1/2 pathway regulates the growth, migration, apoptosis and EMT of human podocytes.

Introduction

Primary nephrotic syndrome (PNS) is a crucial factor leading to chronic kidney disease in children. The most common pathological types are minimal change disease and focal segmental glomerular stiffness (1,2). Although the majority of affected children benefit from hormone therapy and exhibit a good prognosis, some children do not tolerate hormone therapy. If they are not treated with other drugs in time, they may eventually develop glomerulosclerosis and end-stage renal disease. To date, the mechanisms through which PNS develops into glomerulosclerosis remain to be elucidated. Therefore, enhancing the basic research of PNS, searching for PNS-related pathogenic molecules and further researching associated drug targets may provide early intervention treatment for PNS with important clinical significance.

The main pathological mechanisms associated with PNS are podocyte and glomerular injury (3,4). The most typical feature of podocyte injury is podocyte shedding or apoptosis from the outside surface of the glomerular basement membrane (4,5). The rearrangement of the cytoskeleton leads to phenotypic transformation, which leads to podocytes losing their epithelial features and acquiring the ability of movement and migration (6,7). The occurrence and development of glomerular sclerosis can cause podocyte damage. Proteinuria is a key clinical feature of PNS and podocyte damage is also a cause of proteinuria. Therefore, the present study approached the pathogenesis of PNS from the perspective of podocyte injury.

CXCL motif chemokine ligand 16 (CXCL16), as a member of the CXC chemokine family, is closely associated with kidney diseases. Studies have demonstrated that CXCL16 expression is upregulated in several kidney injury models from
animals (8,9), as well as blood and urine samples of patients with kidney disease (10,11). Blocking the mRNA and protein expression of CXCL16 has been shown effectively improve the occurrence and development of kidney disease (12,13).

In addition, when cells are overwhelmed by oxidative stress, genes involved in cell death signaling are activated to induce apoptosis or necrosis to remove irreversibly damaged cells (14). Therefore, an increase in the level of apoptosis may induce apoptosis or necrosis to remove irreversibly damaged occurrence and development of kidney disease (12,13).

Biotech Co., Ltd.) at 33˚C with 5% CO₂, RPMI-1640 medium (MilliporeSigma) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Cat. No. DCS05; Vazyme Biotech Co., Ltd.), using the following thermocycling conditions: 35 cycles at 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 15 sec; extension at 72˚C for 1 min; and maintained at 4˚C. The restriction enzymes were used to perform a double digest of the plasmid and the PCR gel purification products. The products were separated by 1% agarose gel electrophoresis, purified and incubated with T4 ligase overnight at 4˚C. The products were transformed into Escherichia coli, and a single colony was randomly selected. The plasmid was extracted using a plasmid extraction kit (cat. no. DC201; Vazyme Biotech Co., Ltd.). The plasmids were subsequently sent to GenScript Biotech for sequencing.

**Lentivirus packaging and transduction.** 293T cells (Conservation Genetics CAS Kunming Cell Bank) were cultured in a 10 cm cell culture dish at 37˚C with 5% CO₂, overnight. At 80% confluence, 293T cells were co-transfected with lentiviral plasmids (5.94 µg lentivirus-CXCL16, 5.94 µg lentivirus-CXCL16 shRNA1, 2, 5.94 µg lentivirus-ERK2 shRNA, 4.86 µg helper plasmid psPAX2 and 9.0 µg pMD2.G using 40 µg Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) to produce the recombinant lentivirus. The 4.86 µg empty vectors pCDH and pLKO-1 were also transfected as control. At 10 h post-transfection, the cell culture medium was replaced with DMEM medium containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Following transfection for 48 h, the medium was collected. The lentiviral titer was approximately 1x10⁸ to 1x10⁹ transduction units (TU)/ml.

**Cell proliferation assay.** Different groups of HPCs (5x10⁵ cells/well) were cultured in 96-well plates. After the HPCs were allowed to adhere to the plate (12 h after cell plating), cell viability was detected at 0, 1, 3 and 5 days. Briefly, the procedure used for the Cell Counting Kit-8 (CCK-8) reagent kit (cat. no. C0038, Beyotime, Institute of Biotechnology) involved the addition of 10% reconstituted CCK-8 reagent in 100 µl of culture medium and returning to an incubator at 37˚C for 1 h. The optical density (OD) value determined using a microplate reader (BioTek Instruments, Inc.) at 450 nm.

**Transwell migration assay.** Briefly, 200 µl HPC (2x10⁵ cells) suspension was seeded into the culture insert, while 500 µl RPMI-1640 medium containing 10% FBS were added to the lower chamber. The Transwell chamber was incubated at 37˚C for 12 h. The inner surface of the basement membrane was cleaned. The membrane was then dried, inverted. Images were captured from six random of fields (magnification, 2x200) using an light microscope (BX54; Olympus Corporation).
Table I. Baseline demographic and clinical characteristics.

| Characteristic | PNS          | Normal         |
|----------------|--------------|----------------|
| Sex, male/female| 9/6          | 7/8            |
| Age, mean ± SD, years | 8.53±2.47   | 7.07±3.61      |
| Albumin, mean ± SD, g/l | 25.19±6.84   | 43.27±1.95     |
| Cholesterol, mean ± SD, mmol/l | 10.33±3.81   | 3.99±0.44      |
| 24-hour urine, protein, mean ± SD, mg/kg | 118.44±79.8 | -              |

Flow cytometry. Cell apoptosis was measured using an Apoptosis Detection kit [cat. no. 40302ES20; Yeasen Biotechnology (Shanghai) Co., Ltd.]. The different groups of HPCs were detached with 0.25% trypsin (cat. no. T1320, Beijing Solarbio Science & Technology Co., Ltd.), centrifuged at 200 x g for 5 min at room temperature and washed three times with cold 4˚C PBS. Subsequently, 200 µl binding buffer were used for re-suspension, followed by the addition of 5 µl PI reagent and 5 µl Annexin-V-FITC. The mixture was reacted in an incubator at 25˚C for 15 min in the dark and 400 µl binding buffer were then added. Finally, the cells were detected using a FACSflow cytometry (BD Biosciences) within 2 h at wavelengths of 488 and 615 nm. Stained cells were analyzed using the FACSflow cytometry system (BD Biosciences). The apoptotic rate was calculated as the percentage of early + late apoptotic cells.

Reverse transcription-quantitative PCR (RT-qPCR). mRNA was extracted from cells (1x10^7/ml) and tissues (20 mg) using TRIzol® reagent (Thermo Fisher Scientific, Inc.) and was reverse transcribed into cDNA using the PrimeScript Reverse Transcription kit (Vazyme Biotech Co. Ltd), the temperature protocol used for reverse transcription was as follows: 50˚C for 20 min and 85˚C for 2 min. qPCR was performed using the ABI-7300 System (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a total volume of 20 µl according to the manufacturer’s protocols. The primers sequences used were as follows: CXCL16 forward, 5'-GAATGATTGGTGCAGCACAGGTAT-3' and reverse, 5'-GGGTTGTGAAGTGCTCAA-3'; ERK2, forward, 5'-GATCTGGACTTTGGCTGG-3' and reverse, 5'-GTCGGTGACTGGTCAAGA-3'; β-actin, forward, 5'-GGCATCCTACCCCTGAAGTA-3' and reverse, 5'-CCCTAGGCTGCTGGATGAGA-3'. The following thermocycling conditions were used for qPCR: 95˚C for 5 min; 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. Relative gene expression was determined using the 2^{-ΔΔCq} method (25) using β-actin as an internal control.

Western blot analysis. The HPCs in the different groups were harvested and extracted using RIPA lysis buffer (Thermo Fisher Scientific, Inc) supplemented with protease and phosphatase inhibitors(Thermo Fisher Scientific, Inc). Total protein was quantified using the BCA Protein Assay kit (Beyotime Institute of Biotechnology). Total protein (equal, 30 µg/lane) was separated on 10% SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were then incubated with 5% non-fat milk (Beijing Solarbio Science & Technology Co., Ltd) at room temperature for 1 h. Finally, the membranes were incubated with the following primary antibodies at 4˚C overnight: CXCL16 (1:1,000; cat. no. ab119350, Abcam) and ERK1/2 (cat. no. ab17942, Abcam) at 4˚C overnight. The following day, the sections were washed three times and then incubated with goat anti-rabbit IgG (1:10,000; cat. no. ab205718, Abcam) or anti-mouse IgG (1:10,000; cat. no. ab6789, Abcam) at room temperature for 2 h. The membranes were detected using ECL reagent (Thermo Fisher Scientific, Inc) and images of each protein band were obtained using an enhanced chemiluminescence detection system (4600; Tanon Science and Technology Co., Ltd.).

Immunohistochemistry (IHC). The clinical tissues were provided by Shandong Provincial Hospital for IHC and were then fixed in 10% formaldehyde for 24 h, immersed in 75% ethanol for 2 h followed by tissue dehydration: 80% ethanol 20 min, 90% ethanol 20 min, 95% ethanol 20 min, 100% ethanol I 10 min, 100% ethanol II 10 min, xylene I 10 min, xylene II 10 min, xylene III 10 min, paraffin I 40 min, paraffin wax I 1 h, Paraffin III 1 h and embedded in paraffin. The tissue was sectioned at 5 µm and attached to slides. After blocking endogenous peroxides and non-specific proteins, the tissues sections were incubated with primary antibodies, CXCL16 (cat. no. ab119350, Abcam) and ERK1/2 (cat. no. ab17942, Abcam) at 37˚C for 30 min. The tissues sections were then stained with diaminobenzidine for 3 min and the nuclei were counterstained with hematoxylin for 2 min at room temperature. The CXCL16 and ERK1/2 antibodies were used at a dilution of 1:200. IHC images of each tissues were randomly obtained using a light microscope (BX53M; Olympus Corporation), then were analyzed using the Image-Pro Plus 6.0 image analysis system (Media Cybernetics, Inc.).

Statistical analysis. Values were presented as the mean ± standard deviation. Differences between two groups were analyzed using a Student’s t-test. One-way analysis of variance and Tukey or LSD post hoc test were used for comparisons between multiple groups. The correlation between ERK1/2 and CXCL16 was measured by Spearman’s correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

CXCL16 is upregulated in PNS. The present study first evaluated CXCL16 expression levels in renal tissues from patients from PNS and healthy individuals. As illustrated in Fig. 1A, CXCL16 expression in podocyte of the renal tissues of the 15 patients with PNS was significantly upregulated compared with that in the paired normal tissues (Pc=0.001). Accordingly, five tissues from each group were randomly selected for...
CXCL16 regulates the growth, migration and apoptosis of human podocytes. Previous studies have indicated that CXCL16 promotes mouse podocyte migration and regulates the progression of PNS; however, there are still areas of uncertainty. To clarify this, the present study examined the effects of CXCL16 after overexpression or knockdown on human podocytes. HPCs were transfected with lentivirus to overexpress or knockdown CXCL16. Western blot analysis confirmed the overexpression or knockdown of CXCL16 (P<0.01, Fig. 2A and B). The overexpression of CXCL16 suppressed the growth of human podocytes and the knockdown of CXCL16 promoted the proliferation of podocytes (P<0.01, P<0.001; Fig. 2C and D). Consistent with this finding, CXCL16 overexpression promoted HPC apoptosis and the knockdown of CXCL16 suppressed the apoptosis of HPCs (P<0.05; Fig. 2E and F). In addition, Transwell assays were performed on HPCs stably transfected with CXCL16 or CXCL16 shRNA. The results indicated that CXCL16 overexpression promoted HPC migration (P<0.001; Fig. 2G), whereas CXCL16 knockdown significantly suppressed the migration of
Figure 2. CXCL16 regulates the growth of podocytes. (A and B) The expression of CXCL16 proteins after transduction with lentivirus-CXCL16 or lentivirus-CXCL16 shRNA1 and 2 detected by western blotting. (C and D) CCK8 assay was used to detect the growth of CXCL16 overexpressed and knockdown HPC. (E and F) Apoptosis was detected by flow cytometry. The early-apoptotic cells (Q1), late-apoptotic (Q2), cell debris (Q3) and live cells (Q4) are indicated. The numbers of apoptotic cells within quadrants Q2 and Q3 were counted for statistical analysis. (G and H) Cell migration in immortalized human podocytes was analyzed using Transwell assay (magnification, x200). *P<0.05, **P<0.01, ***P<0.001. CXCL16, CXC motif chemokine ligand 16; sh, short-hairpin RNA.
Overall, these findings demonstrated that CXCL16 regulated the progress of PNS by inhibiting the growth and promoting the apoptosis of podocytes, which caused podocytes to lose the characteristics of normal cells and obtain a higher migration and movement ability.

**ERK1/2 expression is upregulated in PNS.** In order to reveal the potential downstream targets of CXCL16, a literature search was performed. The results demonstrated that ERK1/2 may be the potential downstream target of CXCL16 and may be regulated by CXCL16 (26,27). To verify the association between CXCL16 and ERK1/2, the expression of ERK1/2 in PNS was verified. The ERK2 mRNA and ERK1/2 protein levels were evaluated in the same renal tissues from patients with PNS using qPCR (P<0.001, Fig. 3A), western blot analysis (P<0.01, Fig. 3B) and IHC (P<0.001, Fig. 3C). Notably,
compared with normal tissues, the expression of ERK1/2 in
PNS tissues was also upregulated and there was a positive
association between ERK2 and CXCL16 expression (Fig. 3D).
The expression of CXCL16 and ERK1/2 was then verified in
HPCs in which was stably overexpressed or knocked down. As
shown in Fig. 3E and F, ERK1/2 expression increased with the
overexpression of CXCL16 expression and decreased with the
knockdown of CXCL16 (P<0.01). The aforementioned results
suggested that ERK1/2 may be a potential downstream target
of CXCL16.

Figure 4. Knockdown of ERK1/2 expression in podocytes overexpressing CXCL16 could prevent the inhibition of podocyte proliferation, the increase of
podocyte apoptosis rate and migration ability. (A) Western blotting detected ERK1/2 expression in CXCL16 overexpressed HPC with ERK1/2 knockdown
(referred to as: CXCL16-ERK1/2 shRNA). (B) CCK8 assay was used to detect the growth of CXCL16-ERK1/2 shRNA. (C) Apoptosis was detected by flow
cytometry. (D) Cell migration in CXCL16-ERK1/2 shRNA was analyzed using Transwell assay (magnification, x200). **P<0.01, ***P<0.001. ERK1/2, extracel-
lar signal-regulated kinases 1 and 2; CXCL16, CXC motif chemokine ligand 16; HPC, immortalized human podocytes; sh, short-hairpin RNA.
ERK1/2 is a downstream protein of CXCL16 and is involved in regulating podocyte growth, migration, apoptosis and EMT. To define the role of ERK1/2 upregulation in PNS, the recombinant lentivirus plasmid of ERK1/2 shRNA was first constructed and the expression of ERK2 was knocked down in HPCs overexpressing CXCL16 (P<0.001, Fig. 4A). The effects of this knockdown were then evaluated on the ability of cell proliferation and apoptosis. As indicated in Fig. 4B and C, the knockdown of ERK1/2 expression reversed the inhibitory effects of CXCL16 on HPC proliferation and reduced the apoptosis of HPCs which was induced by CXCL16 (P<0.5, P<0.01 and P<0.001). Subsequently, the effects of ERK2 knockdown on podocyte migration were evaluated and the results were consistent with the expectations. The knockdown of ERK2 inhibited the increased migratory ability of HPCs induced by CXCL16 (P<0.01 and P<0.001; Fig. 4D). EMT of HPCs is the main pathway leading to HPC dysfunction, proteinuria and glomerulosclerosis (28,29). Therefore, the present study detected the expression of N-cadherin, P-cadherin, vimentin and zonula occludens-1 (ZO-1) proteins related to EMT. Of note, it was found that the knockdown of ERK1/2 expression in CXCL16-overexpressing HPCs reversed the CXCL16-induced expression of protein molecules associated with EMT (P<0.5 and P<0.01; Fig. 5A and B).

Discussion

PNS is a kidney disease. The clinical features of PNS are the following: Large amounts of proteinuria, hypoproteinemia, hyperlipidemia and varying degrees of edema (4). The pathogenesis of PNS has not yet been fully elucidated. At present, podocyte and glomerular injury are considered the main pathological characteristics of PNS (2,3). Podocytes are terminally differentiated cells lining the outside surface of the glomerular basement membrane (30). Podocyte injury can lead to podocyte fusion, which causes podocyte apoptosis (31). It also causes cytoskeleton rearrangement (32), which causes podocytes to acquire the ability of movement and migration, resulting in glomerular sclerosis and proteinuria (5). In the present study, a high CXCL16 expression was found in human PNS samples and podocytes; CXCL16 was also found to regulate the proliferation, apoptosis and migration of podocytes, which is closely associated with podocyte injury and PNS pathogenesis.

ERK is a member of the mitogen-activated protein kinase (MAPK) family, which is widely involved in regulating cell growth, cell cycle, apoptosis and differentiation processes. ERK1/2 expression in PNS renal tissue has rarely been reported. The activation of ERK1/2 has been shown to be responsible for the beneficial effects of adrenocorticotropic hormone on the glomerular filtration barrier in patients with nephrotic syndrome (33). The complement membranous attack complex can induce the phosphorylation of JNK and ERK1/2 and AS-IV is able to block the complement-induced phosphorylation of JNK and ERK1/2 (34). The present study detected the ERK2 expression in PNS and normal renal tissue samples. The results revealed that ERK2 expression in renal tissue samples from children with PNS was upregulated at both mRNA and protein levels and it was thus hypothesized that ERK2 may be involved in the pathogenesis of PNS. CXCL16 has been reported that to be involved in acute kidney injury by regulating ERK1/2 to induce the apoptosis and inhibit the proliferation of renal tubular epithelial cells (35). In the present study, it was found that the knockdown of ERK2 expression in podocytes overexpressing CXCL16 significantly prevented the inhibition of podocyte proliferation, podocyte apoptosis and the increase in podocyte migratory ability induced by CXCL16; this suggested that CXCL16 inhibits podocyte proliferation and induces podocyte apoptosis and migration via ERK2. This study also has some shortcomings. Due to the poor phosphorylation of ERK1/2, only the expression of total ERK1/2 protein was detected and the changes in ERK1/2 phosphorylation should be detected. The next step is to supplement the changes in ERK1/2 phosphorylation and explore related signaling pathways.
Recent studies have demonstrated that the EMT of podocytes is involved in various disease processes, such as podocyte dysfunction, proteinuria and glomerulosclerosis (36,37). Under certain conditions, podocyte injury leads to phenotypic transition, which causes podocytes to lose their epithelial characteristics, such as the downregulation of P-cadherin and ZO-1, so as to obtain the characteristics of interstitial cells, such as an increase in N-cadherin and vimentin expression (38); podocytes acquire the ability of movement and migration, which renders podocytes unable to maintain the glomerular filtration barrier, eventually leading to the occurrence of kidney diseases (39,40). For example, Shi et al. (41) report that ginsenoside Rg1 alleviates diabetic nephropathy (DN) caused by podocyte injury by inhibiting the EMT pathway of podocytes. Xing et al. (42) report that phosphatase and tensin homolog inhibited the EMT process of podocytes and was an underlying target for the therapy of DN. In addition, some studies have confirmed that CXCL16 promotes the occurrence of breast cancer by regulating ERK1/2 protein and inducing EMT (26,43). In the present study, CXCL16 was found to induce podocyte EMT by regulating ERK1/2.

In conclusion, the findings of the present study suggested that the CXCL16/ERK1/2 pathway regulated human podocytes growth, migration, apoptosis and EMT. In addition, the CXCL16/ERK1/2 axis was found to be involved in the pathophysiological mechanisms of PNS. These findings may provide an underlying target for further and more in-depth research of PNS.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YC designed the present study. YC, ZW, QL and MT performed the experiments. YC and SS analyzed the data. YC and ZW drafted the manuscript and analyzed data. YC, ZW, QL, MT, YZ, LY and JW collected and interpreted data, and revised the final manuscript. YC and SS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethical approval and consent to participate

This study was approved by the clinical research ethics committee of Shandong Provincial Hospital (approval number SWYX:NO.2020-152). All samples were obtained with the written informed consent from the patients (or signed by the child's guardian) with PNS.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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