Original Paper

Mucin 4 Gene Silencing Reduces Oxidative Stress and Calcium Oxalate Crystal Formation in Renal Tubular Epithelial Cells Through the Extracellular Signal-Regulated Kinase Signaling Pathway in Nephrolithiasis Rat Model

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Key Words

Mucin 4 • Extracellular signal-regulated kinase signaling pathway • Oxidative stress • Calcium oxalate crystal formation • Renal tubular epithelial cell • Nephrolithiasis

Abstract

Background/Aim: Nephrolithiasis plagues a great number of patients all over the world. Increasing evidence shows that the extracellular signal-regulated kinase (ERK) signaling pathway and renal tubular epithelial cell (RTEC) dysfunction and attrition are central to the pathogenesis of kidney diseases. Mucin 4 (MUC4) is reported as an activator of ERK signaling pathway in renal tubular epithelial cells. In this study, using rat models of calcium oxalate (CaOx) nephrolithiasis, the present study aims to define the roles of MUC4 and ERK signaling pathway as contributors to oxidative stress and CaOx crystal formation in RTEC. **Methods:** Data sets of nephrolithiasis were searched using GEO database and a heat flow map was drawn. Then MUC4 function was predicted. Wistar rats were prepared for the purpose of model establishment of ethylene glycol and ammonium chloride induced CaOx nephrolithiasis. In order to assess the detailed regulatory mechanism of MUC4 silencing on the ERK signaling pathway and RTEC, we used recombinant plasmid to downregulate MUC4 expression in Wistar rat-based models. Samples from rat urine, serum and kidney tissues were reviewed to identify oxalic acid and calcium

contents, BUN, Cr, Ca^{2+} and P^{3+} levels, calcium crystal formation in renal tubules and MUC4 positive expression rate. Finally, RT-qPCR, Western blot analysis, and ELISA were employed to access oxidative stress state and CaOx crystal formation in RTEC. **Results:** Initially, MUC4 was found to have an influence on the process of nephrolithiasis. MUC4 was upregulated in the CaOx nephrolithiasis model rats. We proved that the silencing of MUC4 triggered the inactivation of ERK signaling pathway. Following the silencing of MUC4 or the inhibition of ERK signaling pathway, the oxalic acid and calcium contents in rat urine, BUN, Cr, Ca^{2+} and P^{3+} levels in rat serum, p-ERK1/2, MCP-1 and OPN expressions in RTEC and H_2O_2 and MDA levels in the cultured supernatant were downregulated, but the GSH-Px, CAT and SOD levels in the cultured supernatant were increased. Moreover, MUC4 silencing or ERK signaling pathway inactivation may decrease the formation of CaOx crystals. **Conclusion:** Taken together, silencing of MUC4 can inactivate the ERK signaling pathway and further restrain oxidative stress and CaOx crystal formation in RTEC. Thus, MUC4 represents a potential investigation focus target in nephrolithiasis.

Introduction

Urolithiasis is a common disease of the urinary system, the prevalence rate is about 1-20%, incidence is increasing in the whole world, and its recurrence rate was more than 50% after treatment, which brings great challenge to medicine and social economy [1, 2]. Calcium bearing stones are the most common stones, accounting for about 75% of all urinary calculi, which might be pure calcium oxalate (CaOx) (50 %) or calcium phosphate (5 %) or calcium oxalate and calcium phosphate (45 %) [3, 4]. Kidney stones are solid stones or crystalline aggregates formed by minerals in the urine [5]. Most idiopathic CaOx stones are formed in related to sub-epithelial formation of calcium phosphate (CaP), which need supersaturation leading to crystallization, and regulated by a variety of crystalline regulators to adapt to the status of the urinary system [6]. Previous studies have shown that renal tubular epithelial cells forming in oxidative stress are important reasons for the formation of calcium oxalate crystals [7]. In other studies, we found that inhibition of ERK signaling pathway could induce tubular apoptosis in autosomal dominant polycystic kidney disease and related to renal dysfunction and attrition [8, 9]. Evidence showed silencing of Mucin 4 (MUC4) could decrease the activation of Extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway.

MUC4 is large heavily glycosylated proteins which consists of a mucin-type subunit (MUC4alpha) and a transmembrane growth factor-like subunit (MUC4beta), and overexpressed in many epithelial malignancies, indicating a possible role in the pathogenesis of these cancers [10]. MUC4 expression is correlated with proliferation, blocking apoptosis, metastasis and gemcitabine resistance, and high expression of MUC4 in cancer related to poor prognosis [12]. The high expression of ERK signaling pathway causes damage to renal tubules, while downregulation of ERK signaling pathway is beneficial to the treatment of nephrolithiasis [13]. Incubated cells with ERK pathway inhibitor U0126 could alleviate mucin 3 (MUC3) expression, and MUC3 could suppressed activation of ERK signaling pathway, speculated that MUC4 can also inhibit the activation of ERK signaling pathway [14]. There is not much studies on MUC4 mediated ERK pathway in nephrolithiasis rat model. Therefore, our study employed rat models of CaOx nephrolithiasis to test the hypothesis that MUC4 silencing induced ERK signaling pathway may suppress oxidative stress and CaOx crystal formation in RTEC.

Materials and Methods

Data retrieval

GEO database (<https://www.ncbi.nlm.nih.gov/gds/>) was used to retrieve the relative sequence chip of kidney stone, with the kidney stone as the key word and finally we found GSE73680 data set. Limma and DESeq2 of R language were adopted to analyze the difference of GSE73680 between the disease group and the normal group, with the $|\log FC| > 2$ and $p < 0.05$ as the screen criteria. Ten genes of maximum difference multiplier screened with heatmap of R language were treated with a heat flow map.

Function prediction for MUC4 gene

MGI (<http://www.informatics.jax.org/>) is the international database resource for the laboratory mouse, providing integrated genetic, genomic, and biological data to facilitate the study of human health and disease. We used the database and searched with MUC4 as the key word to confirm the position in which MUC4 belonged to. Then we employed KEGG (<http://www.kegg.jp/>), which is a database resource for understanding high-level functions and utilities of the biological system, to further obtain an understanding of the function of MUC4.

Study subjects and model establishment

A total of 60 male Wistar rats at age of 8-9 weeks housed in specific pathogen-free (SPF) facilities were divided into two groups after 1 weeks of adaptive period. In the normal control group ($n = 10$), rats were given normal saline by gavage (2 ml/d). In the model group ($n = 50$), rats were given the stone forming agent. Ethylene glycol and ammonium chloride were used as a stone forming agent to establish CaOx nephrolithiasis models. The experimental period was 30 days. During the experiment, the rats in each group were free to feed drinking water during the experiment. Feed intake, water intake, behavioral activities and body hair changes were observed and recorded daily in rats. Standards of model establishment [15]: the hind limbs of rats were unable to crawl, anorexia was reduced, and the body hairs were progressive dull and lackluster. The experiments in this study were conducted according to the Biomedical Research Ethics Committee of Xuzhou Central Hospital, Medical College of Xuzhou University.

Hematoxylin-eosin (HE) staining

In 30 days after model establishment, two rats from the normal control and model groups were taken respectively and were sacrificed by cervical dislocation. Rat's kidney tissues were collected, and fixed in 10% neutral formalin solution. After 24-h fixation, alcohol dehydration, the sections were paraffin-embedded. After 12 h the sections were cut with a thickness of 5 μ m. The sections were spread at 45°C, and taken out. Then, these sections were baked at 60°C, and dewaxed with xylene. The sections were washed with distilled water twice (each time for 1 min) after soaked in 100%, 95%, 80%, 70% gradient alcohol respectively for 2 min. After dehydration, the sections were stained in HE (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 3 min, and washed with tap water for 5 min. Then dehydration of 70% ethanol and 80% ethanol for 5 min, xylene I and xylene II transparency for 5 min were followed, and then all sections were sealed with neutral balsam. The morphologic variations of renal structure in rats were observed under the microscope (XP-330, Shanghai Bingyu Optical Instrument Co., Ltd., Shanghai, China).

Plasmid construction, screening and grouping

According to the MUC4 (NM_080457.3) sequence on Genebank website and following the design principles of RNA sequence, unrelated sequences and interference fragments were designed for MUC4, which was synthesized by Shanghai Genechem Company. All of the fragments ensure specificity after Blast gene homology analysis. The amplified target fragment and the expression plasmids (pcDNA3.1(-); Invitrogen Inc., Germany) that were linearized by BamH I and Hind III enzyme (Invitrogen Inc., Carlsbad, CA, USA) were interacted overnight at 16°C under the action of T4 DNA ligase. The linked products were converted into DH5 α receptive cells (CB101; Tiangen biotech Co., Ltd., Beijing, China) and positive clone were picked out for sequence verification. The normal rat renal tubular epithelial cell (RTEC) line NRK52E was purchased from the China Center for Type Culture Collection. Before the three plasmids were

transfected into cells, logarithmic phase cells were seeded in 6-well plates. When the cell density reached 80 ~ 90%, it was transferred to serum-free medium Opti-DMEM (Gibco Company, Grand Island, NY, USA). Cells were transfected in accordance with the lipofectamin 2000 (Invitrogen Inc., Carlsbad, CA, USA) instruction. The Lipo solution (11668-027; Shanghai KanWin Biotechnology Co., Ltd., Shanghai, China) (244 μ L serum medium + 6 μ L lipo, a total volume of 250 μ L) was incubated for 5 min. The mixture of Lipo and plasmid solution (248 μ L serum medium + 2 μ g plasmids, a total volume of 250 μ L) were placed for 20 min at room temperature, then dropped into the well, followed by a gentle shaking of plates to mix the solution. After incubation of 5 ~ 6 h at 37°C and with 5% CO₂, complete medium was used instead. After further cultivation for 24 ~ 48 h, total protein and total RNA were extracted. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analysis were used to detect the relative mRNA and protein expression of MUC4, and the group with best interference effect was selected for subsequent experiments (Table 1).

In 30 days after modeling, the rats were divided into 6 experimental groups: the normal group (normal rats), the blank group (model rats), the normal control group (injection of unrelated plasmid in model rat), the shMUC4 group (injection of shMUC4 plasmid in rat model group: rats were anesthetized with 2% pentobarbital sodium and fixed on their backs on the operating table; and then 0.1 mL plasmid (2 μ L) was intravenously injected in a swift manner into rats), the UO126 group (injection of ERK1/2 signaling pathway inhibitor UO126 in model rats: daily intraperitoneal injection of 10 mg/kg of signaling pathway inhibitor UO126 (30 mg/kg) (BCP201706205458, Shanghai biochempartner Co., Ltd., Shanghai, China)), the shMUC4 + UO126 group (After 5 days of plasmids injection, 30mg/kg of ERK signaling pathway inhibitor UO126 (10 μ mol/L) was intraperitoneally injected for consecutive three days; the other groups were injected equal amount of normal saline.)

Contents of urine oxalic acid and urine calcium in each group

In the seventh, fourteenth, twenty-first and twenty-eighth days of grouping, the rats were placed in the cage for the collection of urine within 24 h (8 rats were collected randomly in each group), and the oxalic acid and calcium were detected in the urine. The concentration of oxalic acid in urine was calculated according to the calculation formula: $C_{\text{oxalic acid}} = (\text{Blank tube} - \text{sample tube}) / (\text{Black tube} - \text{standard tube}) \times 0.05$ (μ mol/mL). The EDTA complexometric method was used for the determination. The calcium concentration in urine was calculated according to the calculation formula: $C_{\text{calcium}} = V_{\text{sample}} \times 0.001/1000/0.5 \times 10^6$ (μ mol/mL). After the urine collection for the first time, the rats were killed, and blood from the heart and kidneys were collected. Some of them were used for functional cultivation of glomerular epithelial cells, while others were fixed in 10% neutral formalin solution. After 24 h, the kidney tissues were dehydrated with gradient alcohol and embedded in paraffin for later usage.

Serum biochemical analysis of rats in each group

The blood samples were collected from the abdominal aorta, and the contents of BUN, Cr, Ca²⁺ and P³⁺ in serum were measured by biochemical analyzer. After the blood was drained, the left kidney was stored in the liquid nitrogen, partially fixed in 4% polycondensation, followed by paraffin embedding and slicing.

Masson's Trichrome Staining

Paraffin embedded left kidney tissues of two rats were sliced at a thickness of 5 μ m followed by conventional dewaxing with xylene I and xylene II for 10 min respectively and gradient alcohol dehydration (100%, 95%, 80% and 70%) respectively for 2 min. The slices were washed with phosphate-buffered saline (PBS) twice for 5 min each time. After stained with 2% silver nitrate impregnation for 20 ~ 60 min under the sunlight, the slices were washed with distilled water for 5 min, and 5% aqueous solution of sodium thiosulfate for 2 min, and then washed with tap water for 5 min. The slices were stained with 0.1% nuclear fast red for 2 min, and washed with distilled water for 10 s. Then conventional gradient alcohol dehydration and xylene transparency were performed, and all sections were mounted with neutral balsam. Calcium crystallization of rat's renal

Table 1. Sequences of siRNA

SiRNA	Sequence(DNA)
shRNA MUC4-1	GCAGCTATGCTTCCCATTAGG
shRNA MUC4-2	GCTACTGTGTTTGCTACTTTG
shRNA MUC4-3	GCAACTGACATTCTAGCAAGC

tubule was observed under the optical microscope (XP-330, Shanghai Bingyu Optical Instrument Co., Ltd., Shanghai, China).

Immunohistochemistry (IHC)

The sections were conventionally dewaxed by xylene I and xylene II for 10 min respectively and hydrated with gradient alcohol (100%, 95%, 80% and 70%) respectively for 2 min, and washed with phosphate-buffered saline (PBS) twice and 5 min each time. The sections were immersed in 3% H₂O₂ for 10 min, and washed with PBS for two times, 5 min per wash. High-pressure antigen retrieval was conducted for 90 s. After the slices had cooled down to room temperature in a cold-water bath, the slices were rinsed with PBS. The 5% bovine serum albumin (BSA) was added for incubation at 37°C for 30 min. The sections were then added with the 50 µL of rabbit anti-rat primary antibodies (MUC4; ab60720; Abcam, Cambridge, MA, USA) (1: 100) at 4°C overnight. Washed with PBS for 2 min and with addition of 50 µL of biotin-labeled secondary rabbit IgG (RXE0155, Shanghai rongchuangsw Co., Ltd. Beijing, China) (1: 100), the sections were incubated at 37°C for 30 min, added with SAB solution, stained with DAB, re-stained with hematoxylin for 5 min, and washed with tap water for 10 min. Then gradient alcohol dehydration and xylene transparency were performed. All sections were mounted with neutral balsam and observed under the optical microscope. The primary antibodies were replaced with PBS in the normal group. The expression was positive when there were more than 10% of the positive cells, and the coloring was located in the cytoplasm or cell membrane and vascular endothelium were brown. Five fields were randomly selected to observe the positive expression rate of MUC4. The experiment was repeated 3 times.

Fractional cultivation of rat renal tubular epithelial cells

The rats were anesthetized and fixed on the operating table with addition of dabbed iodine on the rat's abdomen. The abdomen was opened following the "laparotomy". The kidney was perfused in situ from the abdominal aorta with normal saline, until the renal tissue became pale white, following dissociation and removal of the renal pedicle, perirenal fat and renal capsule. The renal cortex was cut to 1 mm³, soaked into Hank balanced salt solution containing 1 mg/ml type I collagenase, and digested in 37 °C for 30 min. After percussing, mixing and filtering, the digested tissue block with DMEM-F12 medium, the cell suspension was obtained. Following centrifugation at 37°C for 3 h, non-adherent cells were selected, centrifuged for 5 min with 1200 r/min, and cell precipitation was resuspended with DMEM-F12 medium. After centrifugation for another 5 min, the precipitation was suspended with 45% Percoll solution and centrifuged at 4°C for 30 min with 1200 r/min. The second layer of cell aggregation at the bottom of the proximal tube was the renal tubular epithelial cells (containing a small amount of renal tubule). The cell density of DMEM-F12 medium (containing 10% fetal bovine serum) was adjusted to 5 × 10⁵/mL for subculture.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The cells to be cultured were washed with PBS three times, and each well was added with 1 mL Trizol placed still for 10 min. Cells were treated with blowing until they became completely non-adherent, and then transferred to a 1.5 mL non-enzyme EP tube. After standing for 10 min, 200 µL chloroform was added to the EP tube and mixed with cells. Following another 10 min of standing, centrifugation at 12000 r/min was carried out for 5 min at 4°C. Then 400 µL supernatant was absorbed to another EP tube and mixed with the same volume of isopropanol, followed by 10 min of standing. After centrifugation for 10 min at 12000 r/min at 4°C, the supernatant was discarded. Following the addition of 1 mL of 75% ethanol (ethyl alcohol reagent grade: DEPC, 3: 1), the tube was put upside down to suspend RNA precipitation. The supernatant was removed after centrifugation of the suspension at 7500 r/min at 4°C for 5 min. In the case of extracting RNA from primary cells, this step was repeated twice. Next, after the solution was centrifuged at 7500 r/min at 4°C for 1 min, the supernatant was completely sucked out. After standing for 5 min, RNA was dried moderately and was dissolved with a proper amount of Diethyl-pyrocabonate (DEPC) water for the detection of RNA concentration. The density of all samples with the purity of A260/A280 = 1.8 ~ 2.0 was adjusted to 50 ng/µL. The RNA was converted to cDNA (50 ng/µL) through reverse transcription with PrimeScript™ RT reagent kit (Takara, RR047A, Bei Jing Think-Far Technology Co., Ltd, Beijing, China), and the reverse transcription system was 10 µL. According to the instructions, the reaction conditions were as follows: 37°C for 45 min

(reverse transcriptase), 85°C for 5 s (reverse transcriptase inactivation reaction), and reserved at -80°C. Designed primers by Primer Premier 5.0 software were synthesized by Beijing Tsingke Biological Technology Co., Ltd (Beijing, China) and primer sequences are displayed in Table 2. According to the two-step method, RT-qPCR was conducted using ABI 7900HT Real-Time PCR system (Shanghai Pudi Biotechnology Co., Ltd., Shanghai, China). GAPDH was used as the internal reference. The reaction conditions were as follows: pre-denaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s, and annealing at 58°C for 30 s and extension at 72 °C for 15 s. The $2^{-\Delta\Delta Ct}$ method was applied to calculate the relative mRNA expression of MUC4, ERK1/2, MCP-1, and OPN. All repeated experiments were set for every gene of each sample. The experiment was repeated 3 times.

Western blot analysis

The cells at logarithmic growth period were centrifuged at 3000 rpm at 4°C for 20 min, then the supernatant was discarded. PCV (compact cell volume) centrifugation was estimated, each 20 µL of cells counted at PCV were cracked in ice bath for 30 min, 100 µL lysate and 1 µL enzyme inhibitors (1111111, Beijing Jiamay Biotechnology Co., Ltd., Beijing, China) following centrifugation at 12000 r/min for 10 min at low temperature, the quantitative detection of protein was conducted on upernatant protein. The protein (50 µg) was dissolved into 2 × SDS sample buffer, then the sample was boiled at 100°C for 5 min, transferred to the polyvinylidene fluoride (PVDF) membrane through 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The membranes were blocked with 5% skim milk for 1 h at room temperature, washed with PBS for 3 times, and incubated with rabbit anti-rat at 1: 1000, 1: 1000, 1: 2000, 1: 1000 and 1: 1000 (MUC4, ERK1/2 (p-ERK1/2), MCP-1, OPN, and p-ERK1/2) (ab60720, ab17942, ab25124, ab8448, ab8245; Abcam Inc., Cambridge, MA, USA). Washed with Tris-buffered saline containing Tween-20 (TBST) three times, the membranes were incubated with HRP-labeled secondary antibody of goat anti-rabbit (1: 5000) for 1 h. After TBST washing three times, 5 min per time, the membranes were developed with electrochemiluminescence (ECL), exposed with X-ray and photographed. The absorbance was analyzed by FluorChem Q imaging system (ProteinSimple, CA, USA). The relative expression of the protein was obtained by the average absorbance of the sample was divided by the average absorbance of the relative internal reference. Then statistical chart was drawn about the relative content of each sample protein. The experiment was repeated 3 times.

Enzyme-linked immunosorbent assay (ELISA)

After the cells were seeded in 12-well plates after 48 h incubation, cell supernatant was collected to detect the content of H₂O₂, malondialdehyde (MAD), superoxide dismutase (SOD), catalase (CAT) and glutathione catalase (GSH-Px) in the cell culture supernatant following strictly the kit instructions. The kit number of H₂O₂, SOD, CAT, GSH-Px, and MAD was 69-22748, 69-30017, 69-210385, 69-21219 (Wuhan Meitake Biotech, Co., Ltd, Wuhan, China) and HY22002E (Shanghai Yubo Biotechnology Co., Ltd., Shanghai, China) respectively.

Statistical analysis

All data were analyzed using the statistical analysis SPSS 21.0 software (IBM Corp. Armonk, NY, USA). The measurement data were expressed as the mean ± standard deviation (SD). Comparisons between two groups were analyzed using *t*-test and comparisons among groups were analyzed with one-way analysis of variance (ANOVA). *P* < 0.05 was indicative of significant statistical difference.

Table 2. Primer sequences for RT-qPCR. Notes: RT-qPCR, reverse transcription quantitative polymerase chain reaction

Gene	Sequence
MUC4	upstream primer: 5'-GCTTGGACATTTGGTGATCC -3' downstream primer: 5'-GCCCCGTTGAAGGTGTATTTG -3'
ERK1/2	upstream primer: 5'-CTGGAATGGAAGGGCTATGA -3' downstream primer: 5'-CAGGATGAGTAGGGCAGAGC -3'
MCP-1	upstream primer: 5'-CATCAACCCTAAGGACTTCAGC -3' downstream primer: 5'-TCTACAGAAGTGCTTGAGGTGC -3'
OPN	upstream primer: 5'-GATGAACAGTATCCTATGCC -3' downstream primer: 5'-CCCTCTGCTTATAGTCTTGGGA -3'
GAPDH	upstream primer: 5'-TGATGGCAACAATGTTC -3' downstream primer: 5'-AGACAGCGTATCTTC -3'

Results

MUC4 has an influence on the process of nephrolithiasis

Initially, we conducted difference analysis of GSE73680 and found 30 different genes. Then 10 genes with maximum difference multiplier were treated with a heat flow map (Fig. 1A). Among all the different genes, MUC4 was expressed at a much higher level in the nephrolithiasis group and exhibited the highest difference multiplier. In addition, GO items that MUC4 belonged to was searched in MGI database. As shown in Fig. 1B, the items mainly included transporter, establishment of localization and so on. Furthermore, meta

1. MUC4 affects the process of nephrolithiasis. A, abscissa represented sample number and ordinate represented difference genes; the diagram in the upper right represented the expressions of different genes, and the expression lowered gradually, during which the rectangle corresponded the expression of one sample; each line represented the expressions of all difference gene samples; the dendrogram in the left represented giant thunder analysis of different genes of different samples; the color bar in the upper represented the type of sample, where blue referred to nephrolithiasis sample and red referred to normal samples; B, the results of GO items MUC4 belonging to searched by MGI database, where the blue block referred to the main GO items MUC4 belonging to, in which the three bars were Molecular Function, Biological Process and Cellular Component (three different GO items); C, MUC4 function noted through KEGG database, in which Gene name referred to the frequently used name, Definition KO referred to the item number that MUC4 belonged to, Organism referred to the belonging species that the target gene, and Brite referred to the notes of MUC4 function.

pathway of MUC4 was correlated with membrane transport pathway using KEGG database (Fig. 1C). Recent studies showed that the transportation of materials was closely relevant with the development of nephrolithiasis [16, 17]. For example, TRPV5 overexpression transported by calcium can induce calcium oxalate crystallization in rats' kidney [18]. The above literature and findings demonstrated that MUC4, as transporter related protein, showed a strong correlation with the development of nephrolithiasis. There is also data showed that the ERK signaling pathway correlated with nephrolithiasis induced by calcium oxalate [19, 20]. In epithelial cancer, MUC4 can form a complex, and then activated the ERK signaling pathway, triggering cell proliferation and inhibiting cell apoptosis [21]. In H12 knockdown cell line of pancreatic cancer, MUC4 can mediate cell migration through the ERK signaling pathway [22]. Also, there are many studies demonstrating that MUC4 can affect cell metabolism through the ERK signaling pathway [10, 23, 24]. The above results showed that MUC4 can have an influence on the disease progression via the ERK signaling pathway. However, in nephrolithiasis, little data showed the role of MUC4 and its relationship with the ERK signaling pathway in this disease has seldom been reported.

Glyoxylic acid stimulation affects organizational structure and morphology of kidney tissues

A total of 60 Wistar rats were selected for analysis. From the beginning after the successful model establishment, no obvious changes in the life characteristics of normal rats were observed. Model rats showed inability to move their hind limbs, anorexia and dull fur without burnish. No rats died during the model establishment. Images of kidney tissues after HE staining are shown in Fig. 2. No expansion was observed in kidney tissues of normal rats. Glyoxylic acid stimulated kidney tissues showed changes in the organizational structure and morphology with different degrees: the proximal renal tubules dilated, the renal tubular epithelial cells were swollen, denatured and necrotic, chronic inflammatory cell infiltrated in the renal interstitium, the basement membrane exposed gradually.

ShMUC4-1 presents relatively high interference effect against MUC4 expression

RT-qPCR and Western blot analysis were employed to determine the expression of MUC4 in DH5α cells transfected with shMUC4-1, shMUC4-2, shMUC4-3. Fig. 3 showed that the mRNA and protein expression of MUC4 in the shMUC4-3 groups were relatively high, while those in the shMUC4-1 were relatively low. The results indicate that shMUC4-1 presents relatively high interference effect against MUC4 expression, thus shMUC4-1 was selected for the following experiments.

MUC4 deficiency or ERK signaling pathway inactivation reduces oxalic acid and calcium contents in rat urine

The results of oxalic acid and calcium contents in rat urine at the 7th, 14th, 21st, 28th day are shown in Table 2. Table 2 and Fig. 3 showed that compared with the normal group, the same time, the other groups revealed higher oxalic acid and calcium contents ($p < 0.05$). Oxalic acid and calcium contents were not significantly

Fig. 2. Histological changes of kidney tissues of normal and model rats following HE staining.

Fig. 3. mRNA and protein expression of MUC4 in DH5α cells were the highest in the shMUC4-3 group and the lowest in the shMUC4-1 group; A, the histogram of mRNA expression of MUC4 in three groups; B, histogram of protein expression MUC4 in 3 groups; C, protein bands observed after Western blot analysis; *, $p < 0.05$, compared with the shMUC4-1 group; MUC4, mucin 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; sh, short hairpin.

Table 3. Detection result of oxalic acid content in rat urine among 6 groups. Note: *, $p < 0.05$, compared with the normal group; #, $p < 0.05$, compared with the blank and NC groups; NC, negative control; MUC4, mucin 4; sh, short hairpin

Group	n	7 d	14 d	21 d	28 d
Normal	8	0.020 ± 0.001	0.050 ± 0.003	0.060 ± 0.005	0.070 ± 0.006
Blank	8	$0.085 \pm 0.007^*$	$0.100 \pm 0.006^*$	$0.110 \pm 0.005^*$	$0.163 \pm 0.007^*$
NC	8	$0.090 \pm 0.008^*$	$0.124 \pm 0.007^*$	$0.120 \pm 0.008^*$	$0.171 \pm 0.009^*$
shMUC4	8	$0.060 \pm 0.001^{\#}$	$0.100 \pm 0.001^{\#}$	$0.122 \pm 0.006^{\#}$	$0.131 \pm 0.007^{\#}$
UO126	8	$0.060 \pm 0.002^{\#}$	$0.090 \pm 0.001^{\#}$	$0.114 \pm 0.004^{\#}$	$0.123 \pm 0.007^{\#}$
shMUC4 + UO126	8	$0.040 \pm 0.001^{\#}$	$0.090 \pm 0.004^{\#}$	$0.080 \pm 0.003^{\#}$	$0.090 \pm 0.003^{\#}$

Table 4. Detection result of calcium content in rat urine among 6 groups. Note: *, $p < 0.05$, compared with the normal group; #, $p < 0.05$, compared with the blank and NC groups; NC, negative control; MUC4, mucin 4; sh, short hairpin

Group	n	7 d	14 d	21 d	28 d
Normal	8	0.93 ± 0.05	1.23 ± 0.07	1.32 ± 0.05	1.35 ± 0.09
Blank	8	$2.58 \pm 0.12^*$	$2.87 \pm 0.14^*$	$3.24 \pm 0.18^*$	$3.35 \pm 0.15^*$
NC	8	$2.65 \pm 0.13^*$	$2.76 \pm 0.12^*$	$3.19 \pm 0.16^*$	$3.23 \pm 0.17^*$
shMUC4	8	$2.03 \pm 0.07^{\#}$	$2.21 \pm 0.11^{\#}$	$2.78 \pm 0.15^{\#}$	$2.93 \pm 0.14^{\#}$
UO126	8	$2.02 \pm 0.06^{\#}$	$2.37 \pm 0.10^{\#}$	$2.65 \pm 0.11^{\#}$	$2.84 \pm 0.11^{\#}$
shMUC4 + UO126	8	$1.49 \pm 0.07^{\#}$	$1.71 \pm 0.09^{\#}$	$2.21 \pm 0.12^{\#}$	$2.36 \pm 0.10^{\#}$

Fig. 4. MUC4 silencing in rat DH5α cells significantly reduced oxalic acid and calcium contents in rat urine; A, line chart showing oxalic acid content in rat urine; B, line chart showing calcium content in rat urine; *, $p < 0.05$, compared with the normal group; #, $p < 0.05$, compared with the blank and NC groups; MUC4, mucin 4; sh, short hairpin.

different between the blank group and the NC group. In contrast to the blank and NC groups, the shMUC4, UO126 and shMUC4 + UO126 groups showed a decrease in oxalic acid and calcium contents ($p < 0.05$). These findings show that the silencing of MUC4 and the inactivation of the ERK signaling pathway inactivation decreased oxalic acid and calcium contents in rat urine.

MUC4 silencing or ERK signaling pathway inactivation reduces BUN, Cr, Ca^{2+} and P^{3+} levels in rat serum

The results of BUN, Cr, Ca^{2+} and P^{3+} levels in rat serum are shown in Table 5. Compared with the normal group, BUN, Cr, Ca^{2+} and P^{3+} levels in rat serum were elevated in the other five groups ($p < 0.05$). We found no significant difference in BUN, Cr, Ca^{2+} and P^{3+} levels in rat serum between the blank and NC group ($p > 0.05$). BUN, Cr, Ca^{2+} and P^{3+} levels in rat serum were downregulated in the shMUC4, UO126 and shMUC4 + UO126 groups when compared with those in the blank and NC groups ($p < 0.05$). The findings indicate that MUC4 silencing or ERK signaling pathway inactivation can lead to the decline of BUN, Cr, Ca^{2+} and P^{3+} levels in rat serum.

MUC4 silencing or ERK signaling pathway inactivation reduces formation of calcium crystals in proximal tubules of kidney tissues

We employed von Kossa's staining to observe the formation of calcium crystals in proximal tubules of kidney tissues and the images after the staining are shown in Fig. 5. As we can see, the area of calcium salt deposition is black with red background. The normal group presented no formation of calcium crystals in proximal tubules of kidney tissues. Following the stimulation of glyoxylic acid, the model groups showed the formation of calcium crystals in proximal tubules. The blank and NC groups revealed significantly increased calcium salt deposition with exposed basement membrane. The shMUC4, UO126 and shMUC4 + UO126 groups had a relatively small number of calcium crystals. Taken together, MUC4 silencing

Table 5. BUN, Cr, Ca^{2+} and P^{3+} levels in rat serum among six groups. Note: *, $p < 0.05$, compared with the normal group; #, $p < 0.05$, compared with the blank and NC groups; NC, negative control; MUC4, mucin 4; sh, short hairpin

Group	n	BUN (mmol/L)	Cr (μ mol/L)	Ca^{2+} (mmol/L)	P^{3+} (mmol/L)
Normal	8	5.12 $\pm$ 0.43	39.23 $\pm$ 3.22	1.71 $\pm$ 0.15	2.15 $\pm$ 0.17
Blank	8	15.12 $\pm$ 0.91*	86.83 $\pm$ 6.21*	4.28 $\pm$ 0.32*	4.30 $\pm$ 0.32*
NC	8	16.73 $\pm$ 1.01*	84.74 $\pm$ 5.78*	4.32 $\pm$ 0.32*	4.24 $\pm$ 0.28*
shMUC4	8	9.88 $\pm$ 0.83*#	68.37 $\pm$ 5.37*#	3.54 $\pm$ 0.25*#	3.49 $\pm$ 0.23*#
UO126	8	9.95 $\pm$ 0.70*#	70.23 $\pm$ 5.32*#	3.47 $\pm$ 0.26*#	3.47 $\pm$ 0.20*#
shMUC4 + UO126	8	7.55 $\pm$ 0.60*#	53.87 $\pm$ 4.22*#	2.54 $\pm$ 0.22*#	2.81 $\pm$ 0.17*#

Fig. 5. MUC4 silencing or ERK signaling pathway inactivation reduces formation of calcium crystals observed after Kossa's staining among six groups; NC, negative control; MUC4, mucin 4; sh, short hairpin.

or ERK signaling pathway inactivation restrained formation of calcium crystals in proximal tubules of kidney tissues.

MUC4 positive expression is lower in the shMUC4, UO126 and shMUC4 + UO126 groups

IHC assay was conducted to test the positive expression of MUC4 and results are shown in Fig. 6. MUC4 was expressed in the cytoplasm and positive granules were brownish yellow. The positive expression rate of MUC4 in the kidney tissues was higher in the other five groups than that in the normal group ($p < 0.05$). There was no difference shown in the positive expression rate of MUC4 between the blank and NC group ($p > 0.05$). In contrast to the blank and NC groups, the shMUC4, UO126 and shMUC4 + UO126 groups showed lower positive expression rate of MUC4 ($p < 0.05$). The results above suggest that silenced MUC4 or suppressed ERK signaling pathway lead to lower positive expression of MUC4.

MUC4 silencing downregulates the levels of ERK1/2, MCP-1 and OPN in RTECs

The functional mechanism of MUC4 silencing on oxidative stress and CaOx crystal formation in RTECs was subsequently analyzed using RT-qPCR and Western blot analysis (Fig. 7). In comparison with the normal group, the other five groups showed upregulated mRNA and protein levels of MUC4, ERK1/2, MCP-1 and OPN, as well as the extent of ERK1/2 phosphorylation in RTECs ($p < 0.05$). No significant differences were found in the mRNA and protein levels of MUC4, ERK1/2, MCP-1 and OPN, as well as the extent of ERK1/2 phosphorylation in RTECs were found between the blank and NC group ($p > 0.05$). In contrast to the blank and NC groups, the shMUC4, UO126 and shMUC4 + UO126 groups showed lower mRNA and protein levels of MUC4, ERK1/2, MCP-1 and OPN, as well as the extent of ERK1/2 phosphorylation in RTECs ($p < 0.05$). The results demonstrated that MUC4 silencing may inactivate the ERK signaling pathway, and further inhibit oxidative stress and CaOx crystal formation in RTEC.

MUC4 silencing inhibits oxidative stress in RTEC

ELISA was employed in order to determine the levels of oxidative stress parameters in RTEC (Table 6). H_2O_2 and MDA levels increased, while SOD, CAT and GSH-Px levels decreased in the other five groups than those in the normal group ($p < 0.05$). No obvious differences in H_2O_2 , SOD, CAT, GSH-Px and MDA levels were observed between the blank and NC groups ($p > 0.05$). Compared with the blank and NC groups, the shMUC4, UO126 and shMUC4 + UO126 groups showed lower H_2O_2 and MDA levels and higher SOD, CAT and GSH-Px levels ($p < 0.05$). These findings indicated that MUC4 silencing may inhibit oxidative stress in RTEC.

Fig. 6. The positive expression rate of MUC4 in the kidney tissues was lower in the shMUC4, UO126 and shMUC4 + UO126 groups; A, images of kidney tissues after IHC ($\times 400$); B, positive expression rate of MUC4 in the kidney tissues among six groups; *, $p < 0.05$, compared with the normal group; #, $p < 0.05$, compared with the blank and NC groups; NC, negative control; MUC4, mucin 4; sh, short hairpin; IHC, immunohistochemistry.

Fig. 7. MUC4 silencing downregulates the levels of ERK1/2, MCP-1 and OPN in RTECs; A, the relative expression of ERK1/2, MCP-1 and OPN in RTECs; B, the protein levels of ERK1/2, p-ERK1/2, MCP-1 and OPN in RTECs; C, the protein bands of related proteins detected by Western blot analysis. *, $p < 0.05$, compared with the normal group; #, $p < 0.05$, compared with the blank and NC groups; ERK1/2, extracellular-regulated kinases; MCP-1, monocyte chemoattractant protein-1; OPN, osteopontin; NC, negative control; MUC4, mucin 4; sh, short hairpin.

Table 6. H_2O_2 , SOD, CAT, GSH-Px and MAD levels in the cultured supernatant. NC, negative control; MUC4, mucin 4; sh, short hairpin; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; MAD, malondialdehyde

Group	H_2O_2 (mmol/L)	SOD (U/mg)	CAT (U/mg)	GSH-Px (U/mg)	MAD (nmol/mg)
Normal	10.64 ± 0.83	136.84 ± 5.54	107.64 ± 3.64	55.13 ± 4.73	3.47 ± 0.24
Blank	$21.55 \pm 1.30^*$	$73.58 \pm 4.76^*$	$40.47 \pm 1.42^*$	$23.25 \pm 1.42^*$	$9.37 \pm 0.83^*$
NC	$23.14 \pm 1.32^*$	$71.34 \pm 4.61^*$	$42.84 \pm 1.41^*$	$23.86 \pm 1.36^*$	$9.75 \pm 0.83^*$
shMUC4	$18.21 \pm 1.14^{\#}$	$91.31 \pm 5.14^{\#}$	$67.14 \pm 2.67^{\#}$	$32.73 \pm 2.61^{\#}$	$7.63 \pm 0.61^{\#}$
U0126	$17.85 \pm 1.27^{\#}$	$89.38 \pm 4.81^{\#}$	$65.72 \pm 4.81^{\#}$	$32.14 \pm 2.54^{\#}$	$7.02 \pm 0.62^{\#}$
shMUC4 + U0126	$14.43 \pm 0.86^{\#}$	$110.15 \pm 5.14^{\#}$	$84.36 \pm 6.91^{\#}$	$42.63 \pm 3.87^{\#}$	$5.31 \pm 0.37^{\#}$

Discussion

Nephrolithiasis is regarded as a prevalent disease with a high incidence [25]. Most of all, pediatric nephrolithiasis presents increasing morbidity for the past several decades, meanwhile, a continuous alarming rise in health care costs and burden to the children population suffering from this disease [26]. Increasing evidence shows that the ERK signaling pathway and RTEC dysfunction and attrition are central to the pathogenesis of kidney disease [27]. MUC4 is reported as an activator of ERK signaling pathway in epithelial cells [28]. In the present study, it was found that silencing of MUC4 can inactivate the ERK signaling pathway and further restrain oxidative stress and CaOx crystal formation in RTEC, indicating that MUC4 may represent a potential investigative focus target in nephrolithiasis. Our study found high expressions of MUC4 in kidney tissues and RTEC of nephrolithiasis model rats. Mucins are heavily glycosylated proteins (glycoconjugates) with high molecular weight, extracted from epithelial tissues in most animals and functioned as dependable molecular biomarkers for adverse diagnosis and prognosis of human cancers [29, 30]. Some mucins play roles in controlling mineralization, involving bone formation in vertebrates, calcification in echinoderms and nacre formation in mollusks. MUC4 and MUC1, two transmembrane mucins, have been involved in wide-ranging studies in relation to their pathological implication in malignancies and other disease processes due to their over- or mis-expression [31, 32]. The epithelium separates multicellular animals from the external environment by means of its identity of a transversely connected layer of cells with apical-basal polarity [33]. Mucins were secreted early in the evolution of metazoan as part of transmembrane structures functioning in the protection, repairing and survival

of epithelia in vertebrates [34]. Mucins limits the activation of the inflammatory reaction at the interface with the environment [35]. Deregulation of mucin production has therefore provided an important link between inflammation and disease. Combined with our results, the exploitation of MUC4 function therefore seems to be a strikingly common theme for the damage of RTEC during nephrolithiasis process.

In view of RTEC damage in nephrolithiasis, we determined the impacts of silencing MUC4 on oxidative stress and CaOx crystal formation in RTEC of nephrolithiasis rats, and the observations substantiated that silencing MUC4 mobilized factors to take part in relieving RTEC damage. OPN, mediated by AngII, is one of the macromolecules in CaOx crystals and facilitates its immobilization and promotion of crystal attachment [36]. Xi et al. reported that downregulated OPN can markedly decrease CaOx crystal attachment to normal rat kidney cells, and hypercalciuria may induce the attachment of crystals to kidney epithelial cells. OPN expression [37]. However, OPN expression in RTEC is mediated by the production of reactive oxygen species (ROS), RAS activation and the expression of transforming growth factor-beta 1 (TGF- β 1) [38]. ROS has also been reported to mediate CaOx crystal-induced expression in MCP-1 [39]. In a study by Masanori et al., adiposity in Otsuka Long-Evans Tokushima fatty rats elevated expressions of MCP-1 and OPN, mediated by ROS production, resulting in the increasing of CaOx crystal deposition in the kidney *via* accelerating the processes of crystal retention and the inflammatory [40]. As discussed, CaOx crystal deposition in the kidneys has a bearing on the development of oxidative stress, epithelial injury and inflammation [41]. We found that silencing MUC4 can regulate oxidative stress-related factors in RETC, reducing H₂O₂ and MDA levels and increasing SOD, CAT and GSH-Px levels. SOD, CAT and GSH-Px are essential parts of antioxidant defense systems, functioning in preventing tissue injury with the help of free oxygen radical molecules [42]. A negative correlation was proved between GSH and each one of oxidative stress biomarkers, MDA & H₂O₂ [43].

Additionally, our study demonstrated that silencing of MUC4 can inactivate the ERK signaling pathway, which may help to understand the mechanisms of MUC4 in oxidative stress and CaOx crystal formation in RTEC. ERK1/2 can regulate cellular behavior through both anti-adhesion functions on cell-cell adhesion and cell-cell interactions and serving as an intramembranous ligand for the receptor tyrosine kinase ErbB2 [28]. The canonical Erk/MAP kinase pathway has been demonstrated to be involved most frequently in the regulation of MUC4 expression [44]. The ERK signaling pathway may be critically influenced by both cell-matrix and cell-cell adhesion [45]. ERK1/2 is one of the most important members of the mitogen-activated protein kinase (MAPK) family and its activity presents detrimental effects due to its activation enhancing oxidative stress and inflammation [46]. LIU et al. have carried out an experiment in proximal tubule epithelial cells (HK-2 cells) and found that *Shen-Kang*, a traditional Chinese herbal medicine, protects rats against renal injury *via* inhibiting oxidative stress through the MAPK signaling pathways [47]. Similarly, a study regarding chronic kidney diseases reported that matrix metalloproteinase-9 secretion was stimulated in RTEC through the activation of the ERK signaling pathway [48].

Our previous study has proved that oxidative stress and renal tubular cell injury, especially mitochondrial damage, induce the early stage of CaOx crystal formation in mice [7]. Collectively, the key findings were supported by results suggesting that MUC4 silencing may inhibit oxidative stress in RTEC by inactivating the ERK signaling pathway and the reduction of oxidative stress effectively suppressed the formation of CaOx crystals. The mechanism that MUC4 mediates ERK signaling pathway is shown in Fig. 8. MUC4 represents a potential new mechanism for nephrolithiasis progression and candidates for therapeutic use for human nephrolithiasis. Our findings can help provide the medical and surgical support needed for nephrolithiasis patients and offer key opportunities to learn more about nephrolithiasis, thereby fueling the much-needed research in this field. However, various factors were associated with the formation and recurrence of CaOx crystal in the kidney, and more in-depth studies are required in order to elucidate the specific mechanisms by which MUC4 eases nephrolithiasis [49, 50].

Fig. 8. The mechanism map about MUC4 regulating ERK signaling pathway.

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Disclosure Statement

The authors declare no conflicts of interest regarding the publication of this article.

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