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Original Article

Expression of LncRNA KCNQ1Ot1 in diabetic nephropathy and its correlation with MEK/ERK signaling pathway

Xuangeng Huang[1*], Junhua Tan[2*], Yingrong Li[1], Hongye Su[1], Meiying Huang[2], Feifan Huang[2], Peng Huang[2]

[1]Department of Endocrinology and Metabolism, The Second Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China; [2]Department of Nephrology, The Affiliated Hospital of Youjiang Medical University for Nationalities, Baise, Guangxi, China. [*]Equal contributors.

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Abstract: Objective: To explore the expression of LncRNA KCNQ1OT1 in diabetic nephropathy (DN), and its correlation with MEK/ERK signaling pathway. Methods: 148 patients with type 2 diabetes in our hospital were selected as research subjects, including 83 patients with simple type 2 diabetes (T2D group) and 65 patients with type 2 diabetes with DN (DN group). Another 50 non-diabetic patients were enrolled as the control group. The expressions of LncRNA KCNQ1OT1 and MEK/ERK signaling pathway related molecules in peripheral blood mononuclear cells (PBMCs) of the three groups of subjects were detected and their correlations were analyzed. In addition, 30 Wistar rats were divided into a control group, diabetes group and DN model group, and the expression of LncRNA KCNQ1OT1 and MEK/ERK signal pathway-related molecules in kidney tissue of the three groups was detected and compared. Results: The relative expression of LncRNA KCNQ1OT1, MEK-5 and ERK2 in the control group was lower than that of the T2D group and DN group ($P<0.05$), and the relative expression of LncRNA KCNQ1OT1 in T2D group was lower than that of DN group ($P<0.05$). The expression of LncRNA KCNQ1OT1 was positively-correlated with MEK-5 and ERK2 ($P<0.05$). The relative expression of LncRNA KCNQ1OT1, MEK-5, and ERK2 in renal tissues of the DN group was higher than those in the control group and diabetes group ($P<0.05$). Conclusion: The expression of LncRNA KCNQ1OT1 in PBMCs of DN patients is abnormally increased, and may be a biomarker for the diagnosis and treatment of the disease. In addition, an abnormal increase of LncRNA KCNQ1OT1 is associated with the activation of the MEK/ERK signaling pathway.

Keywords: LncRNA KCNQ1OT1, diabetic nephropathy, MEK/ERK signaling pathway

Introduction

Diabetic nephropathy (DN) is a common complication of diabetes and the main cause of end-stage renal disease (ESRD). The treatment for DN is usually more difficult than for other kidney diseases. Early prevention and timely treatment are of great significance to delay the progression of DN [1]. The pathologic features of DN include glomerular mesangial dilatation and hypertrophy, extracellular matrix (ECM) protein deposition, and podocyte apoptosis [2]. Patients with DN subsequently develop glomerulosclerosis and tubular interstitial fibrosis, which eventually lead to renal failure.

Recent research has shown that the abnormal expression of long non-coding RNA (LncRNA) exerts a crucial role in a variety of diseases including DN. However, the function and mechanism of LncRNA in inflammatory process of DN remain unclear [3]. LncRNA is a functional RNA molecule whose transcript length exceeds 200 nucleotide units and does not encode proteins. Its structure is similar to that of mRNA. LncRNA regulates gene expression at the transcriptional, post-transcriptional, and epigenetic levels, including chromatin imprinting, binding to epigenetic modified complexes or transcription factors to regulate transcription levels, and binding with miRNA, mRNA or proteins to exert regulation at the post-transcriptional level. LncRNA can also regulate cell growth by regulating cell apoptosis and cell cycle [4]. LncRNAs usually have secondary

structures and stable expression in body fluids including blood and urine, enabling LncRNAs to be novel specific molecular diagnostic markers or targets for drug therapy [5, 6].

Recent studies have revealed that LncRNA expression is closely associated with various endocrine and metabolic diseases, but the study of DN is still in its infancy [7, 8]. There are reports [9] that LncRNA KCNQ1 overlap transcript 1 (LncRNA KCNQ1OT1) is greatly elevated in serum of DN patients, but no specific regulatory mechanism has been reported. MEK/ERK signaling pathway regulates the process of transduction of extracellular signals into cells, and it may also be a target for medical treatment of diabetes and its complications [10]. This study analyzed the expression of LncRNA KCNQ1OT1 in DN patients and its correlation with expressions of MEK/ERK signaling pathway related molecules, aiming to provide a basis for clarifying the regulatory mechanism of LncRNA KCNQ1OT1 in DN.

Materials and methods

Clinical materials

This is a retrospective study. 148 patients with type II diabetes, who admitted during March 2018 and March 2020, were enrolled in the study. There were 83 patients were simple type II diabetes (T2D group) and 65 patients were type II diabetes complicated with DN (DN group), and the other 50 non-diabetic volunteers who had a healthy physical examination in our hospital were selected as control group. The study was carried out under approval of the ethics committee. All three groups of subjects voluntarily signed the informed consents.

Inclusion and exclusion criteria

Inclusion criteria: (1) Patients with type II diabetes met the diagnostic criteria of the World Health Organization in 1999 [11]; (2) The DN patients met the diagnostic criteria of *Clinical Guidelines for Diabetes and Chronic Kidney Disease* recommended by 2007 American Kidney Association [12].

Exclusion criteria: (1) Patients with abnormal liver function; (2) Patients with type I diabetes, or other types of diabetes; (3) Patients who experienced with other types of renal disease; (4) Patients with infectious diseases or thyroid diseases; or (5) Patients during pregnancy or lactation.

Laboratory animals and reagents

Thirty healthy 8-week-old male Wistar rats, weighing 180~220 g, were purchased from Experimental Animal Center of Guangxi Medical University. Streptozotocin was purchased from Sigma, USA; Lymphocyte separation solution was purchased from Sigma Company, USA; Trizol reagent was purchased from Invitrogen USA.

Preparation of animal models

After 1 week of acclimatization, the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate, and their right kidneys were removed. Two weeks after right nephrectomy, the rats were randomly divided into control group, T2D group and DN model group, with 10 rats in each group. The DN model group was intraperitoneally injected with 50 mg/kg STZ (STZ dissolved in 10 mmol/L citrate buffer, pH 4.3), while the control group was intraperitoneally injected with the same volume of citrate solution without STZ. The rats were put back into the cage after fully recovered from anesthesia. The model was successfully established if the blood glucose of rat's posterior tail tip was higher than 16.7 mmol/L 72 h later. After modeling, the rats were fed for 8 weeks before the experiment. In addition, the diabetic rats (did not undergo right nephrectomy) were continuously given a high-glycemic and high-fat diet for 8 weeks, injected with a small dose of STZ (20 mg/kg), and then continued to be fed with high glucose and fat diet for 4 weeks. The rats with fasting blood glucose of more than 11.1 mmol/L were deemed as a successful model establishment.

Methods

Extraction of peripheral blood mononuclear cells: The fasting venous blood of each group was collected in the morning, and the peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation. 5 ml lymphocyte separation liquid Ficoll was added into a 15 ml centrifuge tube, and 5 ml of anticoagulant venous blood and 5 ml sterile PBS were fully mixed at a ratio of 1:1. The

Table 1. Primer sequences

Primer	Sequence
LncRNA KCNQ1OT1	Forward primer: 5'-CCCAGAAATCCACACCTCGG-3'
	Reverse primer: 5'-TCCTCAGTGAGCAGATGGAGA-3'
MEK-1 mRNA	Forward primer: 5'-CTGCTGGCGTCTAAGTGT-3'
	Reverse primer: 5'-TACCACCAAATFTCAAAGAA-3'
MEK-2 mRNA	Forward primer: 5'-CAACTCGCCGTACATCGTG-3'
	Reverse primer: 5'-TCCTCGGGAATCCTCTTGG-3'
MEK-5 mRNA	Forward primer: 5'-TCGCCCGTCCAGTTAGGT-3'
	Reverse primer: 5'-GTGGGTTGTTCTCCTTGGTT-3'
ERK1 mRNA	Forward primer: 5'-GCAGTTCTGGAATGGAAGGT-3'
	Reverse primer: 5'-GGGTTTGAATGAGATGAGGG-3'
ERK2 mRNA	Forward primer: 5'-TACGACCCGAGTGACGAG-3'
	Reverse primer: 5'-AGGGCTGCCAGGAGGAAT-3'
GAPDH	Forward primer: 5'-TGACTTCAACAGCGACACCCA-3'
	Reverse primer: 5'-CACCCTGTTGCTGTAGCCAAA-3'

pipette was slowly superimposed on the stratified liquid surface along the wall of tube, and the final volume ratio of peripheral blood, PBS and lymphocyte separation solution was 1:1:1. The liquid was centrifuged horizontally for 400 g ×30 min. After centrifugation, the tube was divided into three layers: the upper layer was plasma and PBS, the lower layer was erythrocyte and granulocyte, and the middle layer was lymphocyte isolate; and a narrow band of white cloud layer at the interface of upper and middle layers was the mononuclear cells. After feeding for 12 weeks, the kidney of rats was removed and preserved in liquid nitrogen. Total RNA from PBMCs and rat kidney tissues were extracted using TRIzol reagent, and the concentration and purity were determined. Later the RNA was subjected to reverse-transcription into cDNA. Using cDNA as template, PCR was amplified in accordance with the instructions of the real-time fluorescence quantitative PCR detection kit. The reaction conditions were as follows: pre-denaturation at 94°C for 2 min, denaturation at 94°C for 20 s, and annealing and extension at 60°C for 30 s, with a total of 35 rounds. With GAPDH as the internal reference gene, the mRNA expression level of the target gene was calculated by $2^{-\Delta\Delta Ct}$ method. Each sample was repeated for 3 times. The design and synthesis of primers were completed by Shanghai Sangong Engineering Co., Ltd with sequences shown in **Table 1**.

qPCR: After 12 weeks of rearing, the kidneys of the rats were stored in a -80° refrigerator. 3 g of frozen kidney tissues were placed in RIPA lysis buffer to extract total protein, and the protein concentration was measured with a BCA kit. The obtained protein was boiled for 10 min, denatured, and loaded, and subjected to SDS-PAGE electrophoresis for 2 h, followed by wet transfer for 45 min. The protein was added with 5% skimmed milk powder and sealed for 1 h. After washing the membrane with TBST, the protein was added with primary antibody solution and incubated at 4°C overnight; Similarly, after washing the membrane with TBST, the protein was added with secondary antibody solution and incubated at room temperature for 2 h. The protein was exposed in the gel imaging system. Taking β-actin as the internal reference, the gray value of the strips was detected by Image J software.

Western blot: The slices were placed in a constant temperature oven at 65°C for 1 h, dewaxed and hydrated. A pipette was used to absorb a small amount of PBS solution that containing 0.3% Triton, and drop onto the slices until PBS was completely immersed on the surface. The slices were placed at room temperature to break the membrane for about 30 min. Subsequently, the PBS containing 0.3% Triton was discarded, and the slices were rinsed 3 times with PBS for 10 min each time. 5% bovine serum albumin (BSA) solution with PBS was prepared, absorbed by pipette and dropped onto the slices until the solution was completely immersed on the surface, and placed at room temperature for 1 h. The slices were incubated overnight at 4°C with a prepared primary antibody. The slices were removed and rinsed with PBS for three times by 10 min each time. The prepared secondary antibody was used to incubate the slices at room temperature in the dark for 1 h. After the incubation, the slices were rinsed with PBS in the dark for 3 times by each time for 10 min. A tablet containing DAPI was slowly dropped onto the slices under dark, incubated at room temperature for 5 min, carefully sealed with cover glass, and then observed and photographed by fluorescence microscope. When immunofluorescence was used to detect LncRNA KCNQ1OT1, the dewaxed and hydrated

Table 2. Comparison of clinical data of three groups of subjects

Clinical data	Control group (n=50)	T2D group (n=83)	DN group (n=65)	Statistics	*P*
Gender					
Male	21	38	28	0.209	0.901
Female	29	45	37		
Age (y, $\bar{x}$±s)	58.39±7.29	59.17±8.33	58.26±7.05	0.302	0.739
Triglyceride (mmol/L, $\bar{x}$±s)	1.52±0.50*	1.98±0.61*	3.09±0.89	81.861	<0.001
FBG (mmol/L, $\bar{x}$±s)	5.68±0.79*	8.30±1.56*	9.17±1.64	88.327	<0.001
Course of diabetes (y, $\bar{x}$±s)	-	14.27±5.20	15.13±6.12	0.933	0.357
BMI (kg/m^2, $\bar{x}$±s)	24.89±4.17	25.28±4.12	26.03±5.10	0.994	0.372
Proteinuria (mg/24 h, $\bar{x}$±s)	2.19±0.79*	7.80±2.67*	1560.28±129.32	9606.800	<0.001
Serum creatinine (mmol/L, $\bar{x}$±s)	58.92±12.01*	61.29±12.18*	148.39±37.48	302.159	<0.001
Glycosylated hemoglobin (%, $\bar{x}$±s)	3.08±0.79*	8.89±1.73*	9.93±1.84	298.268	<0.001

Note: In the table, the three groups of counted data were compared by rank sum test, the three groups of measured data were compared by analysis of variance, and the two groups of measured data were compared by t test. Compared to the DN group, using post-hoc analysis, *P<0.05.

slices were incubated with RNA probes, denatured on a hot plate at 80°C for 10 min, and then hybridized in a wet box at 37°C overnight. The next day, the slices were washed with PBS for 3 times by 10 min each time, and DAPI of appropriate concentration was added to stain the nuclei for sealing and observation under the microscope.

H&E staining: After the establishment of the rat model, the kidneys were removed, fixed, and embedded in conventional paraffin and sliced at 4 μm. The slices were routinely dewaxed with xylene, washed with gradient alcohol, stained with hematoxylin for 5 min, and rinsed with tap water. Differentiation of ethyl hydrochloride for 30 s, soaking in tap water for 15 min, placing in eosin solution for 2 min, routine dehydration, transparency and sealing.

Immunofluorescence staining: Frozen sections of rat kidney tissues in each group were placed at room temperature for 15 minutes, fixed with -20°C pre-cooled iced propanol for 15 minutes, washed with phosphate washing solution 3 times, 3 minutes each time. we used 0.5% volume fraction of Triton X-100 and incubate at room temperature for 19 minutes, wash 3 times with phosphate buffer, 5 minutes each time. We added the primary antibody, incubated overnight at 4°C, and washed 3 times with phosphate buffer, 3 minutes each time. We added diluted fluorescein isothiocyanate-labeled goat anti-rabbit IgG as a secondary antibody, and kept it in the dark for 30 minutes at room temperature, and rinsed with phosphate buffer 3 times, 3 minutes each time. We washed with distilled water for 3 times, and observed each group of pictures under a fluorescence microscope (Olympus, Japan) after mounting.

Statistical analysis

SPSS 25.0 was applied for data processing and analysis in this research. The comparison of measured data between the two groups was conducted by t-test, and among three groups it was conducted by the analysis of variance. The comparison of the enumerated data between the two groups was conducted by χ^2, and the comparison of enumerated data among three groups was by the rank-sum test. The correlation analysis was conducted by Pearson correlation analysis. *P<0.05* was considered a significant difference.

Results

Clinical data

The differences in gender, age, and BMI of the three groups of subjects were not significant (*P>0.05*). There was no difference in the course of diabetes between T2D group and DN group (*P>0.05*). The differences in triglyceride, fasting blood glucose (FBG), proteinuria, serum creatinine and glycosylated hemoglobin among the three groups were significant (*P<0.05*), as shown in **Table 2**.

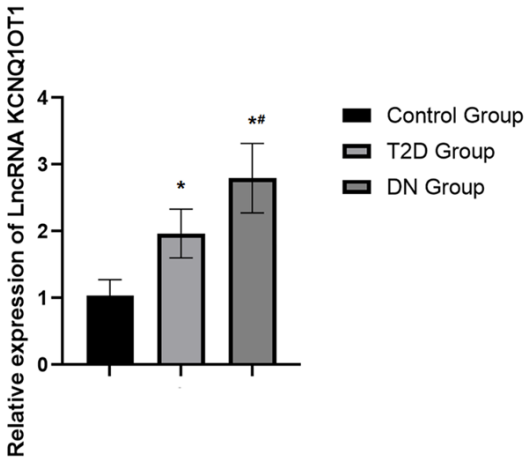


Figure 1. Relative expression of LncRNA KCNQ1OT1 in the three groups. Note: Compared to the control group, *$^{*}P<0.05$; compared to the T2D group, $^{\#}P<0.05$.

Comparison of lncRNA KCNQ1OT1 expression

The relative expression of LncRNA KCNQ1OT1 among the three groups had significant difference (*P<0.05*), of which the control group had significantly lower LncRNA KCNQ1OT1 expression than the T2D and DN group (*P<0.05*), and T2D group had apparently lower LncRNA KCNQ1OT1 expression than the DN group (*P<0.05*), as shown in **Figure 1**.

Expression of MEK/ERK signaling pathway related molecules among three groups

There was no significance among the three groups in the relative mRNA expression of MEK-1, MEK-2 and ERK1 (*P>0.05*). There were significant differences in relative expression of MEK-5 and ERK2 (*P<0.05*). Specifically, the relative mRNA expression of MEK-5 and ERK2 in the control group were lower than those in T2D group and DN group (*P<0.05*), and those in the T2D group were lower than in the DN group (*P<0.05*), as shown in **Figure 2**.

Correlation analysis

The analysis of correlation between LncRNA KCNQ1OT1 and MEK/ERK signaling pathway in DN patients showed that LncRNA KCNQ1OT1 was not significantly correlated with MEK-1, MEK-2, and ERK1 expression (P>0.05), but was positively correlated with MEK-5 and ERK2 (*P<0.05*), as shown in **Figure 3** and **Table 3**.

Comparison of the expression of LncRNA KCNQ1OT1 and MEK/ERK signaling pathway-related molecules in renal tissues of the two groups

The H&E staining of renal tissues of the three groups of rats is shown in **Figure 4**. Normal glomeruli and renal tubules were seen in the control group and T2D group. Moderate proliferation of glomerular mesangial cells can be seen in the model group. The epithelial cells of the renal tubules showed vacuole-like changes, the mesangial matrix was widened, and the basement membrane of the glomerular capillaries was thickened. The relative expression of LncRNA KCNQ1OT1, MEK-5, and ERK2 in renal tissues of the DN model group were higher than those of the control group and T2D group (*P<0.05*) (**Figure 4A** and **4B**), and the protein expression of MEK-5 and ERK2 in DN model group was higher than that of the control group and T2D group (*P<0.05*), as shown in **Figure 4C**. Immunofluorescence results showed that the expression of LncRNA KCNQ1OT1, MEK-5, and ERK2 in the kidney tissue of DN model group were higher than those of thecontrol group and T2D group, as shown in **Figure 5**.

Discussion

Diabetes-related nephropathy has become the leading cause of chronic nephrosis in China, and its incidence has exceeded primary glomerulonephritis-related chronic kidney disease [13]. The molecular network and mechanism of the occurrence and development of DN is quite complicated, and the in-depth study on its pathogenesis is conductive for searching reliable biomarkers and new therapeutic targets [14]. LncRNA KCNQ1OT1 is an imprinted gene that only expresses the parent allele, and the abnormal expression of imprinted genes is usually associated with a variety of diseases. A series of studies in recent years have gradually revealed that the KCNQ1OT1 gene exerts key role in pathological processes such as malignant tumors, inflammatory diseases, and vascular diseases. However, studies on the role of LncRNA KCNQ1OT1 in the pathogenesis of DN are quite few [15-17].

The outcomes in this study revealed that the relative expression of LncRNA KCNQ1OT1 in the T2D group and DN group was significantly

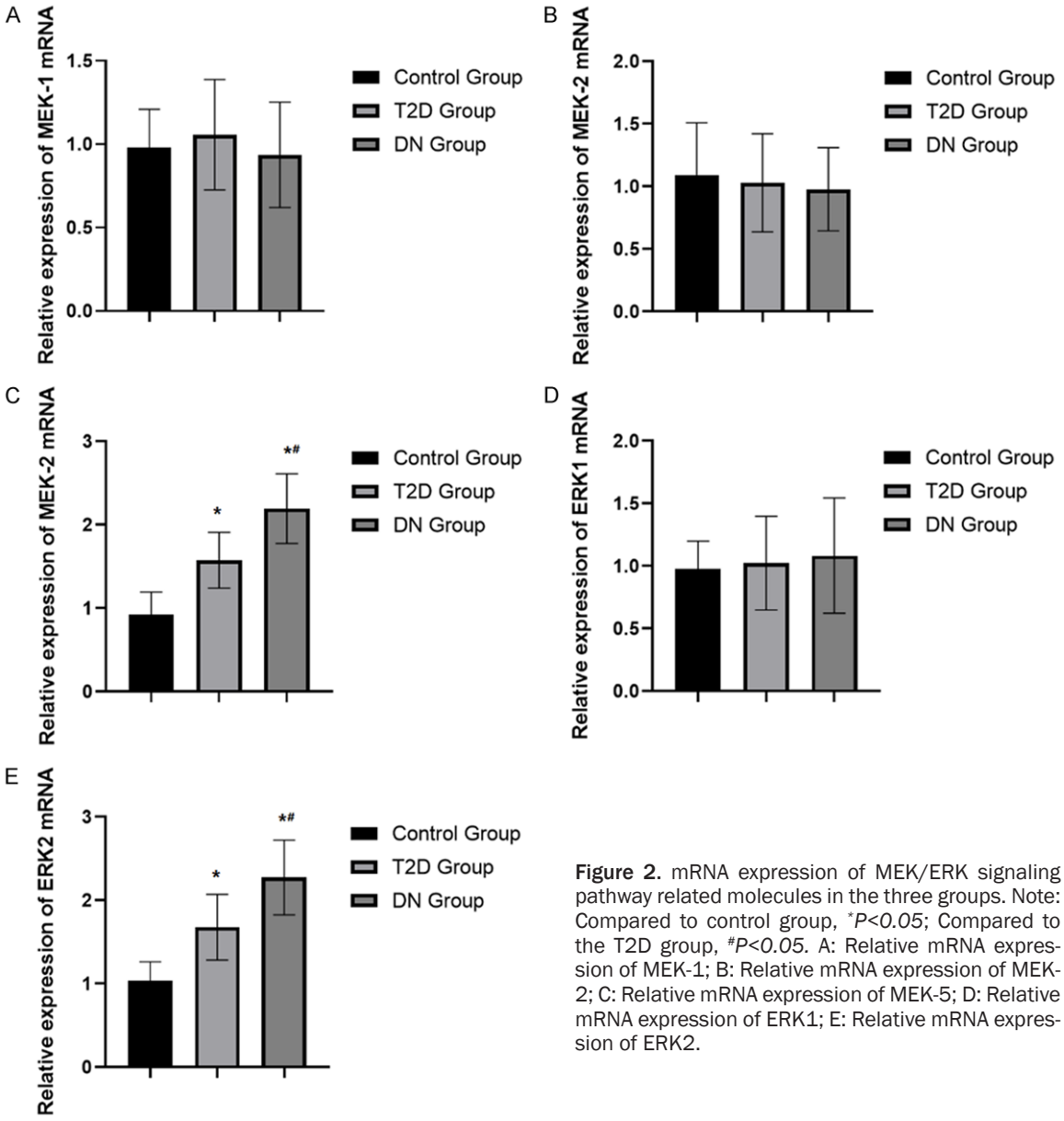


Figure 2. mRNA expression of MEK/ERK signaling pathway related molecules in the three groups. Note: Compared to control group, $^{*}P<0.05$; Compared to the T2D group, $^{\#}P<0.05$. A: Relative mRNA expression of MEK-1; B: Relative mRNA expression of MEK-2; C: Relative mRNA expression of MEK-5; D: Relative mRNA expression of ERK1; E: Relative mRNA expression of ERK2.

lower than that in healthy controls, and T2D group had apparently lower LncRNA KCNQ1OT1 expression than the DN group, which was similar to the results of other studies. This suggests that LncRNA KCNQ1OT1 may be connected to the occurrence of DN [18, 19].

MEK/ERK signaling pathway is a significant participant in diabetes and its complications, and the intensity of its protein expression has a certain correlation with the severity of DN [20, 21]. This study investigated and analyzed the expression of LncRNA KCNQ1OT1 in DN patients, as well as its correlation with the expression of MEK/ERK signaling pathway-related molecules. The expression of key molecules MEK-1, MEK-2, MEK-5, ERK1, and ERK2 in theMEK/ERK pathway was analyzed. The results illustrated that there were no statistically significant differences in the relative expression of MEK-1, MEK-2, and ERK1 among the three groups, while there were significant differences in relative expression of MEK-5 and ERK2 among the three groups. This indicates that MEK/ERK signaling pathway plays as key regulatory role in the occurrence of DN, which is mainly related to MEK-5 and ERK2. The correlation analysis showed that LncRNA

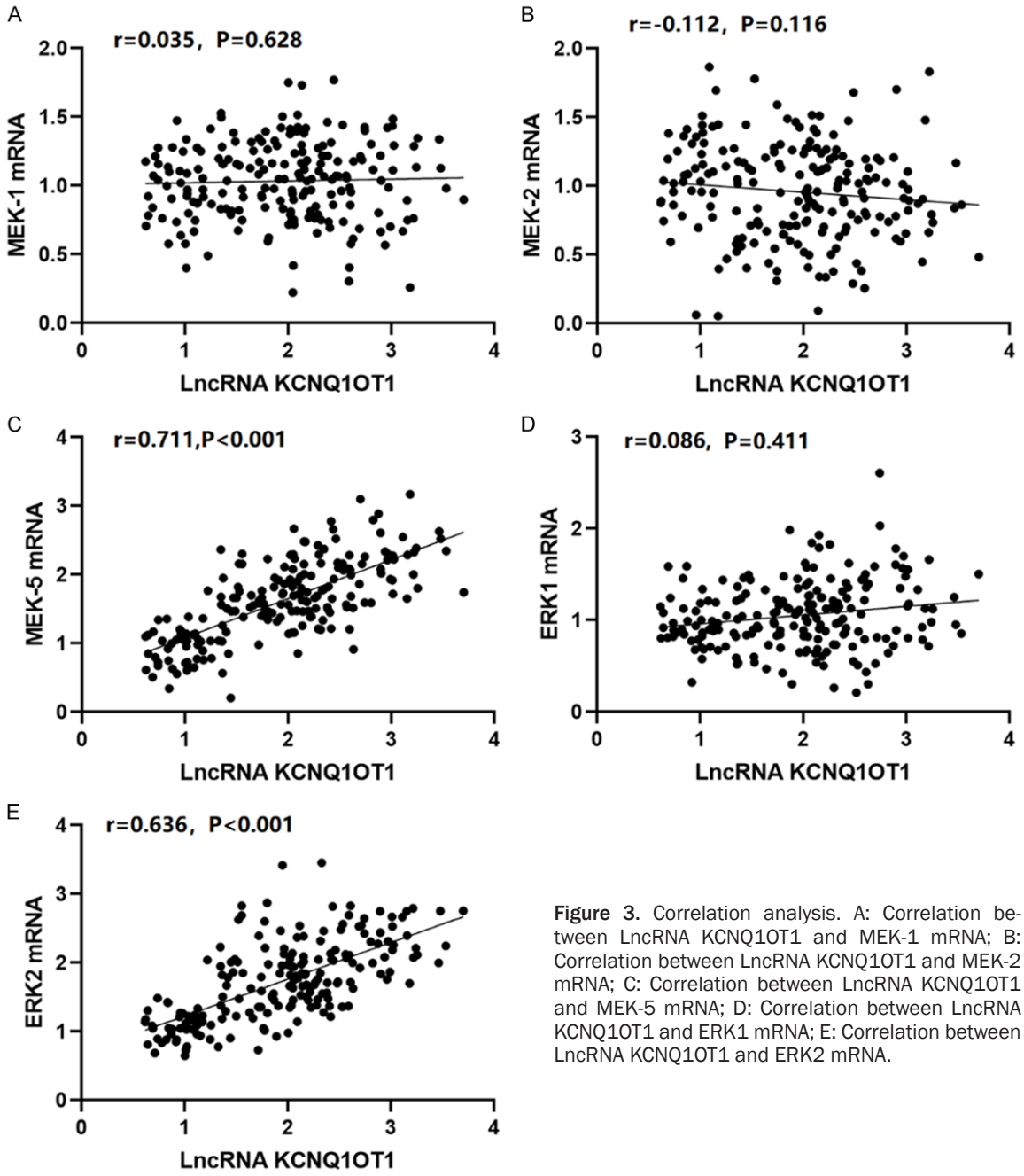
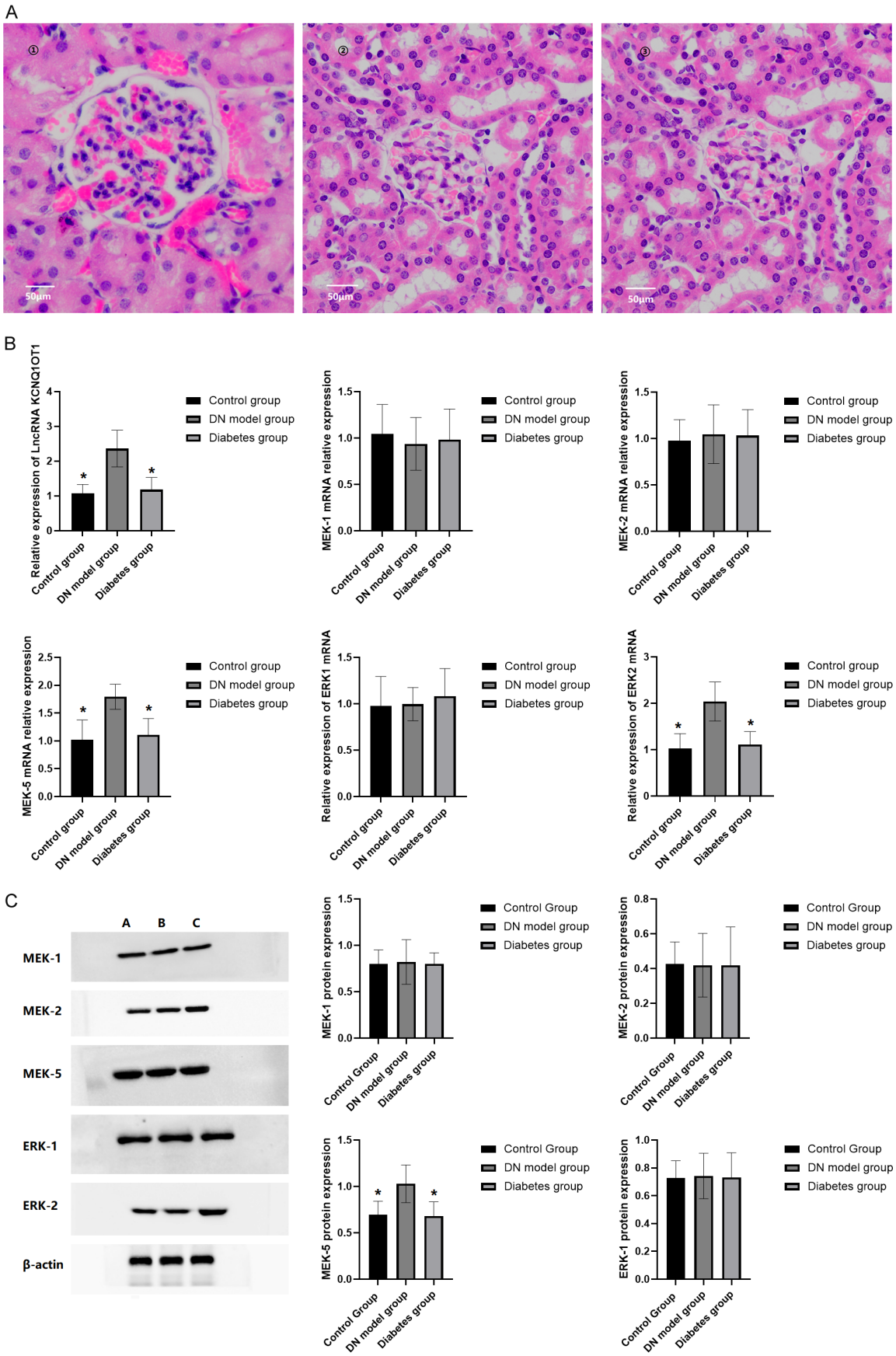


Figure 3. Correlation analysis. A: Correlation between LncRNA KCNQ1OT1 and MEK-1 mRNA; B: Correlation between LncRNA KCNQ1OT1 and MEK-2 mRNA; C: Correlation between LncRNA KCNQ1OT1 and MEK-5 mRNA; D: Correlation between LncRNA KCNQ1OT1 and ERK1 mRNA; E: Correlation between LncRNA KCNQ1OT1 and ERK2 mRNA.

Table 3. Correlation analysis

	Statistics	MEK-1 mRNA	MEK-2 mRNA	MEK-5 mRNA	ERK1 mRNA	ERK2 mRNA
LncRNA KCNQ1OT1	r	0.035	-0.112	0.711	0.086	0.626
	P	0.628	0.116	<0.001	0.411	<0.001

KCNQ1OT1 had a positive correlation with the expression of MEK-5 and ERK2, indicating that the abnormal expression of LncRNA KCNQ1OT1 in DN patients may strongly correlate to the activation of the MEK/ERK signaling pathway [22, 23]. In addition, we also received analogous results from animal models; that is, the relative expression of LncRNA


A
①
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Relative expression of LncRNA KCNQ1OT1
MEK-1 mRNA relative expression
MEK-2 mRNA relative expression
MEK-5 mRNA relative expression
Relative expression of ERK1 mRNA
Relative expression of ERK2 mRNA
Control group
DN model group
Diabetes group
C
A
B
C
MEK-1
MEK-2
MEK-5
ERK-1
ERK-2
β-actin
MEK-1 protein expression
MEK-2 protein expression
MEK-5 protein expression
ERK-1 protein expression
Control Group
DN model group
Diabetes group

Figure 4. Expression of LncRNA KCNQ1OT1 and MEK/ERK signaling pathway in kidney tissue. A. H&E staining of renal tissue of the rats in three groups (①: Normal glomeruli and renal tubules were seen in the control group; ②: There were no obvious abnormalities in the glomeruli and tubules in the diabetic group; ③: Moderate proliferation of glomerular mesangial cells can be seen in the model group. The epithelial cells of the renal tubules showed vacuole-like changes, the mesangial matrix was widened, and the basement membrane of the glomerular capillaries was thickened). B. Comparison of expressions of LncRNA KCNQ1OT1 and MEK/ERK signaling pathway related molecules in renal tissues of rats in the three groups (Note: Compared to the control group, $^{*}P<0.05$). C. Comparison of protein expression of MEK/ERK signaling pathway-related molecues in renal tissue of rats in the three groups (①: Control group; ②: T2D group; ③: DN model group. Compared to the control group, $^{*}P<0.05$).

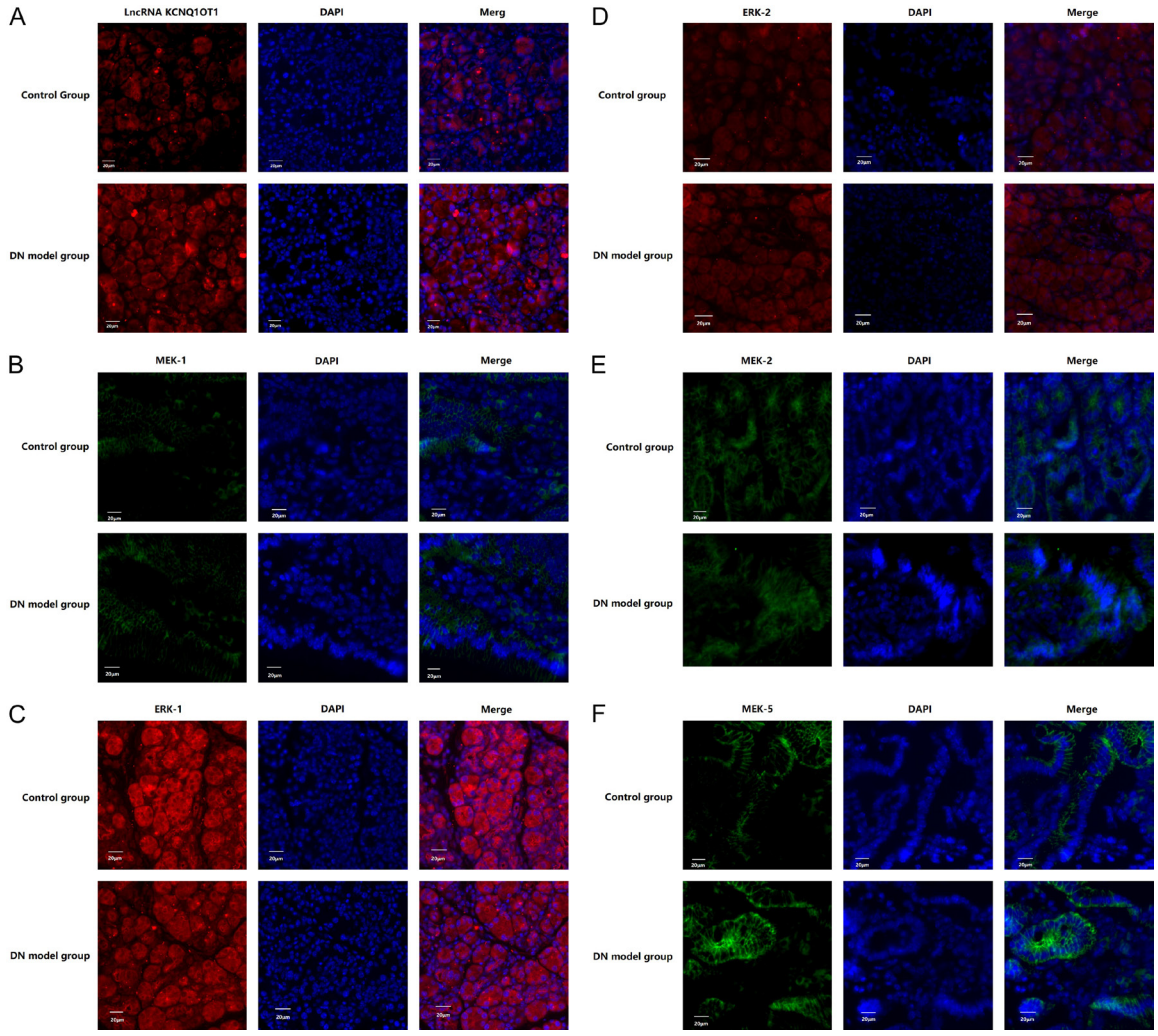

Figure 5. Immunofluorescence detection of LncRNA KCNQ1OT1 and MEK/ERK pathway-related molecules in rat kidney tissues. A: LncRNA KCNQ1OT1. B: MEK-1. C: ERK-1. D: ERK-2. E: MEK-2. F: MEK-5.

KCNQ1OT1, MEK-5 and ERK2 in renal tissue of DN model group was dramatically higher than that of the control group. The mechanism may be that the MEK/ERK pathway is activated by various pathogenic factors during the occurrence and progression of diabetic nephropathy. LncRNA KCNQ1OT1 may affect the progression of diabetic nephropathy through the regulation of MEK/ERK pathway.

However, the sample size included in this study was limited, and the specific mechanism of action of LncRNA KCNQ1OT1 was explored in depth. Therefore, it is necessary to conduct further research to enrich the pathogenesis of DN, which helps to provide new targets for DN target gene therapy, and a basis for the search for biomarkers for diagnosis and prognosis of DN as well as new therapeutic strategies.

In summary, the expression of LncRNA KCNQ1OT1 in peripheral blood of DN patients is abnormally increased, which is expected to be a biomarker for the diagnosis and treatment of DN. The abnormal increase of LncRNA KCNQ1OT1 is associated with activation of the MEK/ERK signaling pathway.

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Disclosure of conflict of interest

None.

Address correspondence to: Peng Huang, Department of Nephrology, The Affiliated Hospital of Youjiang Medical University for Nationalities, No. 18 Zhongshan 2nd Road, Baise, Guangxi, China. Tel: +86-0776-2855284; E-mail: 780777@qq.com

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