

Yiqi Yangyin Tongluo prescription targets lncRNA VIM-AS1 to regulate FOXK2/mTOR to promote autophagy and inhibit renal tubular epithelial cell apoptosis

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Abstract

Diabetic nephropathy (DN) is one dominating reason for death in diabetic patients, and its incidence is high. It has been reported that Yiqi Yangyin Tongluo prescription (YYTP) can relieve inflammation, and it owns better clinical effects in the treatment of DN. However, the molecular mechanisms of YYTP in the treatment of DN still keep unclear, and deeply investigations are needed. In this study, it firstly was manifested that YYTP can repress the lncRNA VIM-antisense 1 (VIM-AS1) expression in high glucose (HG)-evoked HK-2 cells. Overexpression of VIM-AS1 roll-backed the inhibitive impacts of YYTP on cell apoptosis in HG-triggered HK-2 cells. Additionally, it was uncovered that the attenuated autophagy of LC3B in HG-triggered HK-2 cells was counteracted after 20% YYTP treatment, but this phenomenon was further attenuated after VIM-AS1 amplification. Besides, VIM-AS1 can pull down FOXK2 protein, and overexpression of VIM-AS1 counteracted the suppressive effects of YYTP on forkhead box K2 (FOXK2)/mammalian target of rapamycin (mTOR) in HG-mediated HK-2 cells. In conclusion, it was firstly disclosed that YYTP targeted lncRNA VIM-AS1 to regulate FOXK2/mTOR to promote autophagy and inhibit cell apoptosis in DN progression. This discovery hinted that YYTP may be one valid drug for DN therapy.

Keywords: Autophagy, Diabetic nephropathy, VIM-AS1, Yiqi Yangyin Tongluo prescription

1. Introduction

Diabetic nephropathy (DN) is the dominating complication for diabetic patients, and its incidence is enhance year by year [1]. It is prevalently manifested as positive-urine protein, hypertension and damaged renal function [2]. The pathogenesis of DN is closely associated with the imbalance of oxidation, inflammation, autophagy and other factors [3,4]. At present, the treatments of DN chiefly contain controlling levels of blood glucose, blood pressure and blood lipid, but that cannot

prevent it eventually forming into end-stage renal disease [5,6]. Therefore, it is critical to search effective therapeutic targets and drugs for DN treatment.

According to Chinese traditional medicine, DN is a syndrome of mixed deficiency and excess. In the initial stage, it is mainly featured by Yin deficiency, dryness and heat; over time, it can result into both Qi and Yin deficiency; in the later stage, it can lead to both Yin and Yang damage. Therefore, adjusting Yin and Yang, invigorating Qi and nourishing Yin, tonifying spleen and kidney are the major treatment methods for DN. Yiqi Yangyin Tongluo prescription

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(YYTP) owns good clinical effects in some diseases. This prescription owns replenishing Qi, nourishing Yin, dredging collaterals, and relieving pain effects, and ameliorates the symptoms of various patients. YYTP has been discovered to participate into rheumatoid arthritis, and results demonstrated that YYTP can modulate the ASIC1a/NLRP3 pathway to alleviate chondrocytes pyroptosis [7]. Importantly, YYTP can effectively weaken inflammatory response and relieve renal injury, thereby ameliorating DN progression [8,9]. However, the molecular mechanisms of YYTP in DN progression still need deeply investigations.

In conclusion, the objective of this work is to explore the regulatory impacts and molecular targets of YYTP in DN treatment. It was firstly disclosed that YYTP can repress the lncRNA VIM-AS1 expression in HG-evoked HK-2 cells. And, YYTP targeted lncRNA VIM-AS1 to regulate FOXK2/mTOR to promote autophagy and inhibit cell apoptosis in DN progression. This work may supply novel opinions on YYTP and associated molecular mechanisms in DN therapy.

2. Materials and methods

2.1. YYTP serum

The Wistar rats (male, 180–200 g, $n = 12$) were gained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), and kept with free food and water at 12/12 h light/dark cycle for one week. Two groups: saline group and YYTP group. In YYTP group, these rats were treated with YYTP (0.6 g/mL) by oral administration for twice/day for 7 days, according to our previous experiments. In saline group, rats were treated with equal volumes of saline.

The blood was collected to obtain serum. Fasting for 12 h, 1 h after the last gavage, rats were anesthetized. Under sterile condition, blood was gathered from the abdominal aorta, next the whole blood was allowed to stand at 4 °C for 4 h. After centrifugation (3000 rpm, 15 min), the serum was separated. The serum was inactivated at 56 °C for 30 min, filtered through a microporous membrane, and stored in a refrigerator with −20 °C. The serums collected from the saline group and the YYTP group were prepared into a culture medium with 20% serum concentration for next experiments.

2.2. Cell culture

Human renal tubular epithelial cells (HK-2) were acquired from Cell Bank of Chinese Academy of

Sciences (Shanghai, China), and incubated in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Invitrogen) in the moist incubator at 37 °C with 5% CO₂. The cells were induced with high glucose (30 mM) to induce DN cell model. 20% YYTP serum was utilized to dispose HK-2 cells.

2.3. Cell transfection

The adenovirus-mediated VIM-AS1 over-expression (ad-VIM-AS1) with its negative control (ad-NC) were acquired from Hanbio (Shanghai, China). Then, transfection into HK-2 cells was done through using Lipofectamine 2000 (Invitrogen, USA).

2.4. RT-qPCR

The TRIzol reagent (Invitrogen, USA) was employed to extract total RNA from HK-2 cells. The PrimeScript® RT reagent Kit (Takara, Dalian, China) was utilized for reverse transcription (from RNA to cDNA). The SYBR Green PCR kit (TaKaRa, Dalian, China) was adopted for doing quantitative real-time PCR. The lncRNA VIM-AS1 expression was confirmed through the $2^{-\Delta\Delta C_t}$ method.

The primer sequences:

VIM-AS1:

forward, 5'-GCACTGGTCCTCTTCATCATCTC-3',
reverse, 5'-TGTGGTATTTGCTGCTGTTCCC-3';

GAPDH (internal reference):

forward, 5'-CTGGGCTACACTGAGCACC-3',
reverse, 5'-AAGTGGTCGTTGAGGGCAATG-3'.

2.5. Flow cytometry

After rising, HK-2 cells were resuspended. Next, in the dark, HK-2 cells were mixed with Annexin V-FITC (Beyotime, Shanghai, China) and propidium iodide (PI). At last, cell apoptosis was inspected through the flow cytometer (BD Biosciences, San Jose, CA, USA).

2.6. Western blot

HK-2 cells were lysed by using RIPA lysis buffer to obtain proteins. Next, proteins were segregate by SDS-PAGE, and then migrated to PVDF membranes. After sealing, membranes was placed with primary antibodies including Bax (ab182734, 1/1000, Abcam, Shanghai, China), Cleaved-caspase 3 (ab2302, 1/500, Abcam), Caspase 3 (ab32351, 1/5000, Abcam), Bcl-2 (ab182858, 1/2000, Abcam), LC3B

(ab192890, 1/2000, Abcam), P62 (ab91526, 1 µg/mL, Abcam), p-mTOR (ab109268, 1/1000, Abcam), mTOR (ab134903, 1/10000, Abcam), FOXK2 (ab309489, 1/1000, Abcam) and GAPDH (ab8226, 1 µg/mL) at 4 °C for overnight. Then, membranes were placed with secondary antibody (ab6721, 1/2000, Abcam). At last, the chemiluminescent detection kit (Thermo Fisher Scientific) was adopted for examining the protein blots.

2.7. Immunofluorescence (IF) assay

HK-2 cells (1×10^5) were put on coverslips, and fixed with using 4% paraformaldehyde. After sealing with 5% BSA, HK-2 cells were permeabilized with Triton X-100, and mixed with primary antibody against LC3B (ab192890, 1 µg/mL, Abcam, Shanghai, China). Next, FITC-labeled secondary antibody (ab7149, 1/1000, Abcam) was further mixed. The fluorescent images were gained under the Olympus BX53 microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

2.8. RNA pull down assay

Biotin-labelled RNAs was *in vitro* transcribed with HiScribe™ T7 Quick High Yield RNA Synthesis Kit (NEB, USA) with Biotin-16-UTP (Roche, Basel, Switzerland). Next, RNAs (5 µg) were mixed with cell lysate, followed by culturing with Dynabeads M-280 Streptavidin (Invitrogen) at 4 °C. Post washing, the pull-down complexes were eluted, and detected by Western blot.

2.9. Statistical analysis

Data were exhibited as the mean $\pm$ standard deviation (SD). SPSS 20.0 (SPSS, Chicago, USA) was utilized for data analysis. Differences were subjected to the one-way analysis of variance (ANOVA). $p < 0.05$ was set as statistically significant.

3. Results

3.1. Yiqi Yangyin Tongluo prescription repressed the lncRNA VIM-AS1 expression

Firstly, it was revealed that the lncRNA VIM-AS1 expression was uplifted after HG induction, but this change was alleviated after 20% YYTP treatment (Fig. 1), indicating that YYTP can repress the lncRNA VIM-AS1 expression in HG-evoked HK-2 cells.

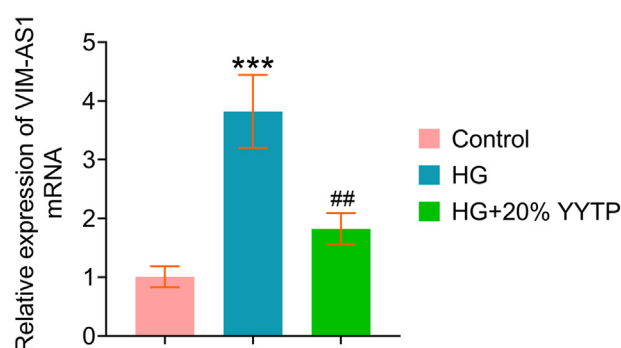


Fig. 1. Yiqi Yangyin Tongluo prescription repressed the lncRNA VIM-AS1 expression. The expression of lncRNA VIM-AS1 was verified in the Control, HG and HG + 20% YYTP groups through RT-qPCR. *** $p < 0.001$ vs the Control group; ## $p < 0.01$ vs the HG group.

3.2. Overexpression of VIM-AS1 reversed the inhibitive effects of Yiqi Yangyin Tongluo prescription on cell apoptosis in HG-triggered HK-2 cells

The elevated VIM-AS1 expression in HG-triggered HK-2 cells was attenuated after 20% YYTP treatment, but this impact was offset after VIM-AS1 overexpression (Fig. 2A). In addition, the augmented cell apoptosis in HG-evoked HK-2 cells was weakened after 20% YYTP treatment, but this phenomenon was reversed after VIM-AS1 up-regulation (Fig. 2B–C). Next, through Western blot, it was discovered that the increased Bax and Cleaved caspase-3/Caspase-3 protein expressions as well as the decreased Bcl-2 protein expression in HG-triggered HK-2 cells were reversed after 20% YYTP treatment, but these changes were further neutralized after VIM-AS1 amplification (Fig. 2D). Taken together, overexpression of VIM-AS1 reversed the inhibitive effects of YYTP on cell apoptosis in HG-triggered HK-2 cells.

3.3. Overexpression of VIM-AS1 rescued the promotive effects of Yiqi Yangyin Tongluo prescription on autophagy in HG-evoked HK-2 cells

The receded LC3-II/LC3-I protein expression and aggrandized P62 protein expression in HG-evoked HK-2 cells were offset after 20% YYTP treatment, but these impacts were further rescued after VIM-AS1 amplification (Fig. 3A). Moreover, the lessened fluorescence intensity of LC3B in HG-triggered HK-2 cells was counteracted after 20% YYTP treatment, but this phenomenon was further attenuated after VIM-AS1 amplification (Fig. 3B). The strengthened

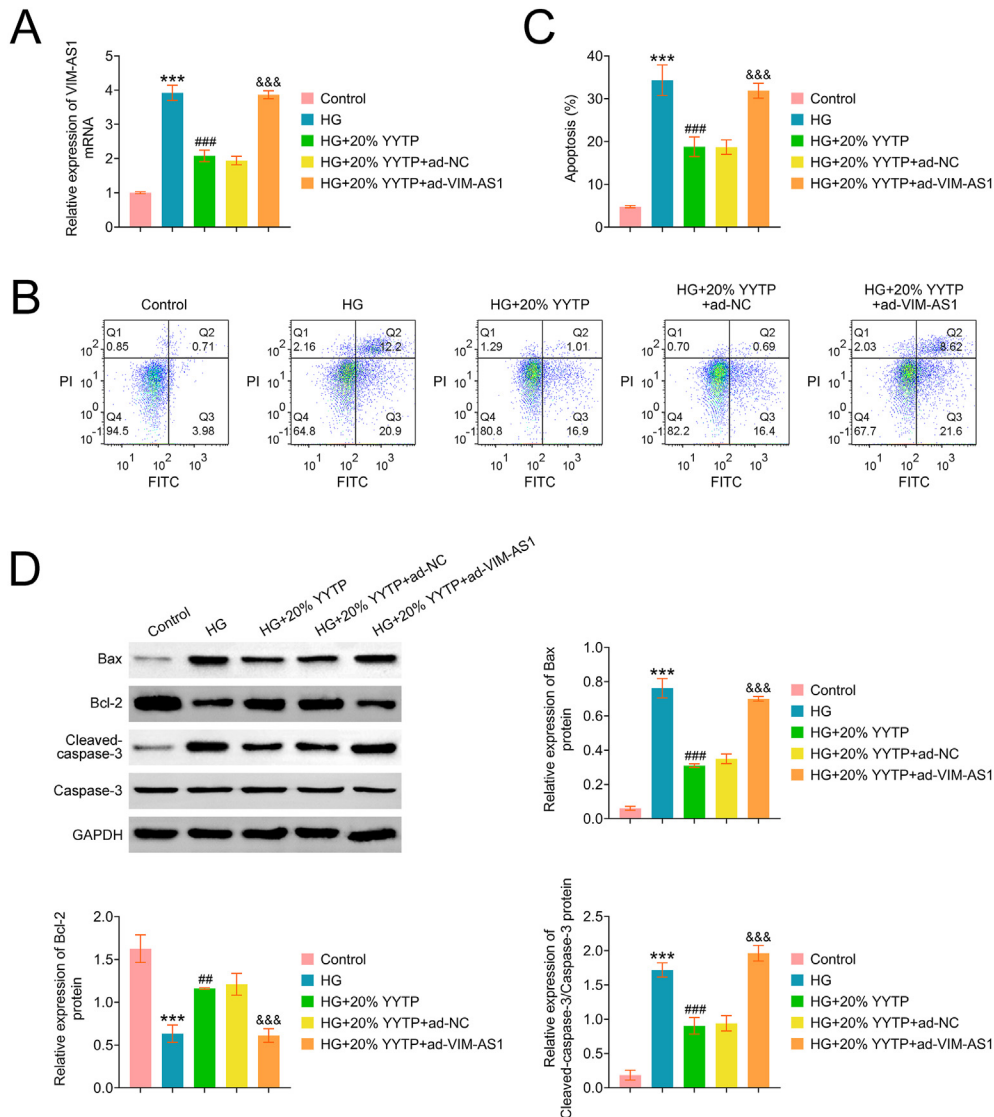


Fig. 2. Overexpression of VIM-AS1 reversed the inhibitive effects of Yiqi Yangyin Tongluo prescription on cell apoptosis in HG-triggered HK-2 cells. Groups were separated into the Control, HG, HG + 20% YYTP, HG + 20% YYTP + ad-NC and HG + 20% YYTP + ad-VIM-AS1 group. (A) The expression of lncRNA VIM-AS1 was confirmed. (B–C) The cell apoptosis was examined through flow cytometry. (D) The protein expressions of Bax, Bcl-2, Cleaved caspase-3 and Caspase-3 were determined through Western blot. *** $p < 0.001$ vs the Control group; ### $p < 0.01$, ### $p < 0.001$ vs the HG group; &&& $p < 0.001$ vs the HG + 20% YYTP + ad-NC group.

cell apoptosis mediated by HG induction was relieved after 20% YYTP treatment, but this impact was reversed after 3-MA treatment (Fig. 3C). Additionally, the increased Bax and Cleaved-caspase-3/caspase-3 as well as the decreased Bcl-2 protein expression stimulated by HG treatment were offset after 20% YYTP treatment, and cell apoptosis was also heightened after 3-MA induction (Fig. 3D). In general, overexpression of VIM-AS1 rescued the promotive effects of YYTP on autophagy in HG-evoked HK-2 cells.

3.4. Overexpression of VIM-AS1 counteracted the suppressive effects of Yiqi Yangyin Tongluo prescription on FOXK2/mTOR in HG-excited HK-2 cells

As shown in Fig. 4A, through RNA pull down assay, VIM-AS1 can pull down FOXK2 protein. Moreover, the enhance protein expressions of p-mTOR/mTOR and FOXK2 were attenuated after 20% YYTP treatment, but these changes were further reversed after VIM-AS1 amplification

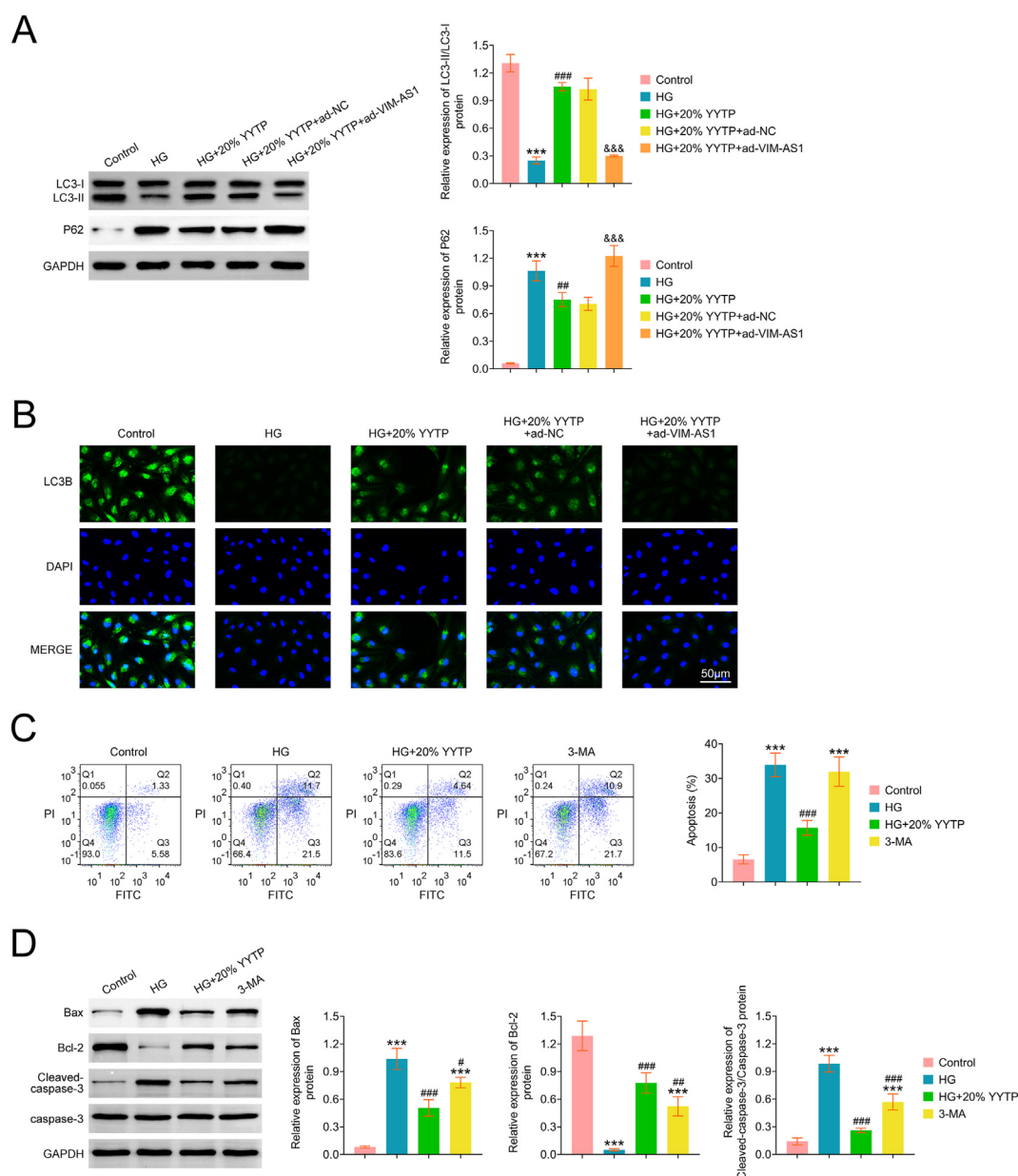


Fig. 3. Overexpression of VIM-AS1 rescued the promotive effects of Yiqi Yangyin Tongluo prescription on autophagy in HG-stimulated HK-2 cells. Groups were separated into the Control, HG, HG + 20% YYTP, HG + 20% YYTP + ad-NC and HG + 20% YYTP + ad-VIM-AS1 group. (A) The protein expressions of LC3-I, LC3-II and P62 were tested through Western blot. (B) The fluorescence intensity of LC3B was assessed through IF assay. (C) The cell apoptosis was examined through flow cytometry in the Control, HG, HG + 20% YYTP and 3-MA groups. (D) The protein expressions of Bax, Bcl-2, Cleaved-caspase-3 and caspase-3 were measured through Western blot in the Control, HG, HG + 20% YYTP and 3-MA groups. *** $p < 0.001$ vs the Control group; ## $p < 0.01$, ### $p < 0.001$ vs the HG group; &&& $p < 0.001$ vs the HG + 20% YYTP + ad-NC group.

(Fig. 4B–C), manifesting that overexpression of VIM-AS1 counteracted the suppressive effects of YYTP on FOXK2/mTOR in HG-excited HK-2 cells.

4. Discussion

Many Chinese prescriptions have been ascertained to take part into the progression of DN. For example, TangShenWeiNing Formula affects the SIRT1/HIF-1 α pathway to protect podocytes,

thereby resisting DN progression [10]. Moreover, JinChan YiShen TongLuo Formula modulates HIF-1 α /PINK1/Parkin pathway to improve mitochondrial dysfunction in DN [11]. Additionally, Yishen Huashi Granules can improve glomerular filtration barrier to relieve the progression of DN [12]. Moreover, Buyang Huanwu Decoction restrains renal fibrosis and inflammation to ameliorate STZ-evoked DN [13]. YYTP can relieve inflammation,

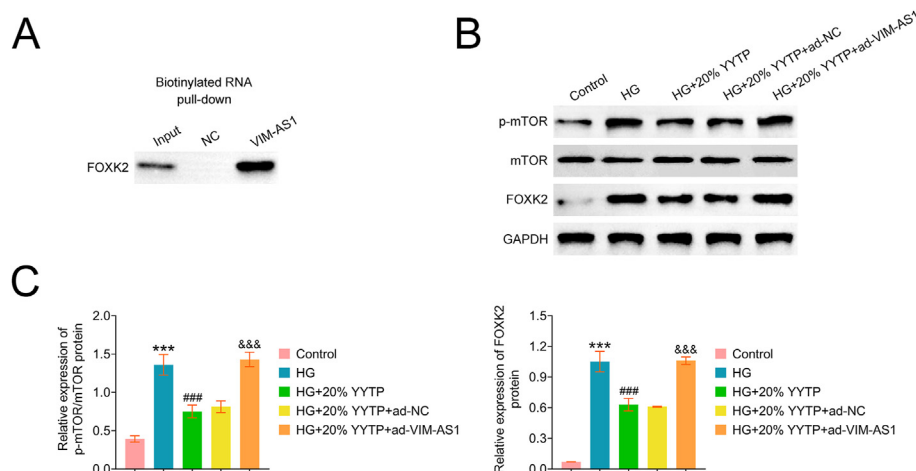


Fig. 4. Overexpression of VIM-AS1 counteracted the suppressive effects of Yiqi Yangyin Tongluo prescription on FOXK2/mTOR in HG-mediated HK-2 cells. Groups were separated into the Control, HG, HG + 20% YYTP, HG + 20% YYTP + ad-NC and HG + 20% YYTP + ad-VIM-AS1 group. (A) The relationship between VIM-AS1 and YYTP was confirmed through RNA pull down assay. (B–C) The protein expressions of p-mTOR, mTOR and FOXK2 were inspected through Western blot. *** $p < 0.001$ vs the Control group; ### $p < 0.001$ vs the HG group; &&& $p < 0.001$ vs the HG + 20% YYTP + ad-NC group.

and it owns better clinical effects in the treatment of DN [8,9]. However, the molecular mechanisms of YYTP in the treatment of DN still keep unclear, and deeply investigations are needed.

LncRNA VIM Antisense RNA 1 (VIM-AS1) has been confirmed to be one promoter in cancers and participate into other diseases. For instance, lncRNA VIM-AS1 modulates HMGCS1 stabilization in prostate cancer to aggravate cell proliferation and enhance enzalutamide resistance [14]. In addition, lncRNA VIM-AS1 triggers the Wnt/ β -catenin pathway to facilitate cell proliferation and epithelial-mesenchymal transition (EMT) process in gastric cancer [15]. LncRNA VIM-AS1 can modulates EMT process to exhibit pivotal functions in preeclampsia [16]. Importantly, lncRNA VIM-AS1 suppresses the development of diabetic retinopathy through targeting miR-29 [17]. However, the relationship between YYTP and lncRNA VIM-AS1 in DN progression keep dimness. In this study, it was manifested that YYTP can repress the lncRNA VIM-AS1 expression in HG-evoked HK-2 cells. Overexpression of VIM-AS1 reversed the inhibitive impacts of YYTP on cell apoptosis in HG-excited HK-2 cells.

Autophagy has been verified to be one key process in DN progression [18,19]. For example, inhibition of Risa aggrandizes autophagy to alleviate podocyte injury in DN [20]. Furthermore, astragalus polysaccharide influences lncRNA Gm41268/PRLR axis to aggravate autophagy, thereby ameliorating DN progression [21]. Isoorientin can stimulate autophagy to against DN progression [22]. In addition, Yiqi Jiedu Huayu Decoction strengthens autophagy to relieve renal damage in DN progression [23]. In this work, it

was uncovered that the attenuated autophagy of LC3B in HG-triggered HK-2 cells was counteracted after 20% YYTP treatment, but this phenomenon was further attenuated after VIM-AS1 amplification. Next, it was uncovered that the strengthened cell apoptosis mediated by HG induction was relieved after 20% YYTP treatment, and cell apoptosis was also heightened after 3-MA treatment. Interestingly, FOXK2 serves as a downstream regulator of mammalian target of rapamycin (mTOR) pathway [24]. But, the regulatory impacts of YYTP and lncRNA VIM-AS1 in DN progression remain dimness. In this work, it was revealed that VIM-AS1 can pull down FOXK2 protein, and overexpression of VIM-AS1 counteracted the suppressive effects of YYTP on FOXK2/mTOR in HG-provoked HK-2 cells.

In conclusion, it was firstly disclosed that YYTP targeted lncRNA VIM-AS1 to regulate FOXK2/mTOR to promote autophagy and inhibit cell apoptosis in DN progression. This work also owns some limitations, and more experiments for investigating YYTP in DN progression will be further proceeded in the future.

Ethics approval

Ethical approval was obtained from the Ethics Committee of The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine (Approval no. 2024-N(A)-0212).

Data availability

The authors declare that all data supporting the findings of this study are available within the paper

and any raw data can be obtained from the corresponding author upon request.

Authors contribution

Rucui Yu designed the study and carried them out, Rucui Yu, Ruiying Wu, Tingting Chen, Jingwei Xu supervised the data collection, Rucui Yu, Ruiying Wu, Tingting Chen, Jingwei Xu analyzed the data, Rucui Yu, Ruiying Wu, Tingting Chen, Jingwei Xu interpreted the data, Rucui Yu, Ruiying Wu, Tingting Chen, Jingwei Xu prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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Conflicts of interest

The authors state that there are no conflicts of interest to disclose.

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