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ORIGINAL ARTICLE

LncRNA HOX transcript antisense RNA mediates hyperglycemic-induced injury in the renal tubular epithelial cell via the miR-126-5pAkt axis

Qiong Jiang | Ting Yang | Yan Zou | Mingjie He | Qingchun Li | Xiaohui Chen | Aimin Zhong

Jiangxi Provincial Key Laboratory of Nephrology, Jiangxi Provincial People's Hospital, The First Affiliated Hospital of Nanchang Medical College, Nanchang, China

Correspondence
Qiong Jiang, No. 152, Patriotism Road, Donghu District, Nanchang 330006, China.
Email: 34214483@qq.com

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Abstract

Objective: To investigate the involvement of HOX transcript antisense RNA (HOTAIR) in the injury of renal tubular epithelial cells induced by high glucose.

Results: In high glucose-induced HK-2 cells, the expression of HOTAIR was upregulated, resulting in suppressed cell proliferation. Meanwhile, HOTAIR upregulates the expression of pro-apoptotic proteins Bax and cleaved caspase-3, while downregulating the expression of the anti-apoptotic protein Bcl-2. Luciferase reporter assays revealed that HOTAIR could target miR-126-5p. Additionally, it was found that the PI3K/Akt signaling pathway serves as a downstream target of miR-126-5p. Knockdown of HOTAIR relieved apoptosis, whereas further inhibition of miR-126-5p led to apoptosis in HK-2 cells.

Conclusions: HOTAIR plays a regulatory role in mediating high glucose-induced injuries in HK-2 cells, specifically affecting apoptosis and cell viability, via the miR-126-5p/PI3K/Akt signaling pathway.

KEYWORDS
diabetic nephropathy (DN), HOX transcript antisense RNA (HOTAIR), miR-126-5p

1 | INTRODUCTION

The prevalence of diabetes mellitus (DM) is increasing, particularly among the elderly population.[1] As a consequence, diabetic nephropathy (DN), a common complication of DM, is more frequently observed in aging patients with DM. A prospective observational cohort study reported that 63% of patients had an estimated glomerular filtration rate below 60 mL/min/1.73m^2.[2] Tubulointerstitial injury plays a critical role in the initiation and progression of DN.[3] High glucose, a risk factor for DN, can cause damage to renal tubular epithelial cells.[4] It is widely acknowledged that high glucose can accelerate the aging process of renal tubular epithelial cells, thereby leading to renal interstitial injury in DN.[5] However, for frail older patients with DN, merely controlling glycemic levels provides limited benefits.[6] Therefore, investigating the molecular mechanism underlying tubular cell injury induced by high glucose may facilitate the development of an effective therapeutic approach for DN.

Long noncoding RNAs (lncRNAs) have emerged as crucial players in the initiation and progression of DN and hold potential as biomarkers and therapeutic targets.[7,8] Unlike kidney biopsies,

the noninvasive nature of lncRNAs makes them particularly valuable in elderly patients to assess DN progression. HOX transcript antisense RNA (HOTAIR) has been identified as a trans-acting intergenic lncRNA. Previous studies have revealed HOTAIR's pivotal regulatory role in cancer, influencing cell growth, migration, invasion, and apoptosis.[8–11] More recently, studies have uncovered additional regulatory functions of HOTAIR in endometrial fibrosis[12] and osteoarthritis.[13] Abnormal expression of HOTAIR is associated with fat metabolism, vascular calcification, and other diabetes-related complications.[14,15] Bioinformatic analysis has indicated that miR-126-5p, a potential biomarker for DN,[16–18] may be targeted by HOTAIR and exhibits downregulated expression in DN-related mesangial cells,[19] suggesting the involvement of HOTAIR in DN. However, the role of HOTAIR in regulating miR-126-5p and its impact on renal tubular epithelial cell injury in DN remains to be elucidated.

In this study, our objective was to examine the involvement of HOTAIR in the injury of renal tubular epithelial cells induced by high glucose. To test this, we utilized HK-2 cells to establish a model of hyperglycemic-induced renal tubular epithelial cell injury. Subsequently, our study focused on elucidating the regulatory mechanism of HOTAIR and the involvement of miR-126-5p in the injury process. Additionally, we aimed to investigate whether miR-126-5p could mediate the PI3K/Akt signaling pathway and contribute to HK-2 injury.

2 | MATERIALS AND METHODS

2.1 | Materials

Human renal cortex proximal convoluted tubule epithelial cells HK-2 (Procell Life Science & Technology Co., Ltd.); DMEM-12 medium (KGM12500S; KeyGen bioTECH); Lipofectamine Reagent 3000 Transfection (L3000015; Invitrogen); Cell Counting Kit-8 (KGA317; KAIJI Biotech); Reporter plasmid pmirGLO, Plasmid Extraction Kit (DP103-02; TIANGEN Biotech); Annexin V-FITC/PI Apoptosis Kit (AP101-100-kit; MULTI SCIENCES); internal reference antibody: rabbit anti-β-actin (AF7018, Affinity); target primary antibodies: anti-p-PI3K (rabbit, AF3241, Affinity), anti-p-AKT (rabbit, AF0832, Affinity), anti-Bax (rabbit, AF0120, Affinity), anti-Bcl-2 (rabbit, AF6139, Affinity), and anti-Cleaved Caspase-3 (rabbit, AF7022, Affinity).

2.2 | Cell culture and cell transfection

HK-2 cells were divided into five groups as follows: the normal control (control group), the c (HG 60 mmol/L group), the model with small-interfering RNA (siRNA)-NC treatment group (HG 60 mmol/L + NC group), the model with HOTAIR siRNA treatment group (HG 60 mmol/L + siRNA group), and the model with HOTAIR siRNA and miR-126-5p inhibitor treatment group (HG 60 mmol/L + siRNA + inhibitor group). Hk-2 cells were cultured using the medium containing the treatments mentioned above.

In the progression of siRNA transfection, HK-2 cells were initially cultured with a serum-free medium. Two mixtures later, 125 μL Opti-MEM + 5 μL Lipofectamine 3000 and 125 μL Opti-MEM + 0.25 nmol siRNA, were added respectively in two tubes and incubated for 5 minutes. Then, they were mixed with the two reagents, we waited for another 15 minutes, and added them to the medium. Four to 6 hours later, 1 mL 20% serum-supplemented culture medium was added to the medium.

2.3 | Cell Counting Kit-8

Cell Counting Kit-8 (CCK-8; KGA317; KAIJI Biotech) was used to test cell viability. HK-2 cells (3×10^3 cells/well) were seeded in a 96-well plate. When HK-2 cells adhered to the dish, 100 μL/well high-glucose mediums of different concentrations (30, 45, 60, 75, and 90 mc) were added to the medium. Seventy-two hours later, 10 μL CCK-8 reagent was added to each well for another 1 hour of incubation. Finally, detecting the optical density values at 450 nm with a microplate reader.

2.4 | Flow cytometry

HK-2 cells (1×10^6) were resuspended by 300 μL Binding Buffer, prepared by diluting 5 × Binding Buffer with double distilled water. Subsequently, 5 μL Annexin V-FITC and 10 μL PI were added into wells and incubated for 10 minutes at room temperature, and protected from light exposure. Finally, 200 μL pre-cooled 1 × Binding Buffer was added and the mixed sample was processed for flow cytometry assay.

2.5 | Real-time quantitative polymerase chain reaction analysis

RNA isolation was performed using a DNA/RNA Extraction Kit (DNA/RNA Extraction Kit). A TaqMan RNA Reverse Transcription Kit (Thermo Fisher) was used for the RNA reverse transcription of cDNA. Quantitative polymerase chain reaction was performed on a 7500 Real-Time PCR System. β-actin served as the internal control. The relative expression of the target gene was calculated with the $2^{-\Delta\Delta Ct}$ method. The primers are listed in Table 1.

2.6 | Western blot

Scraping the HK-2 cells with a cell scraper into a marked tube by a pipette and then removing the deposit at 2000 rpm for 10 minutes. Total proteins were collected from the supernatant and the protein concentration was determined by a BCA Protein Assay Kit. The proteins were denatured, loaded, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 2 hours. Then,

TABLE 1 The sequences of primers.

	Forward primer	Reversed primer
β-Actin	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA
HOTAIR	ATAGGCAAATGTCAGAGGGTT	ATTCTTAAATTGGGCTGGGTC
Bax	GGATGCGTCCACCAAGAA	AAAGTAGAAAAGGGCGACAAC
Bcl-2	GAGGATTGTGGCCTTCTTTG	GCCGGTTCAGGTACTCAGTC
Caspase-3	AGCGAATCAATGGACTCTGG	GACTTCTACAACGATCCCCTCT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
miR-126-5p	GCGCGCATTATTACTTTTGG	AGTGCAGGGTCCGAGGTATT

Note: U6 snRNA and β-actin were set as controls for miRNA qPCR and qPCR, respectively.
HOTAIR, HOX transcript antisense RNA; miRNA, microRNA; qPCR, quantitative polymerase chain reaction; snRNA, small nuclear RNA.

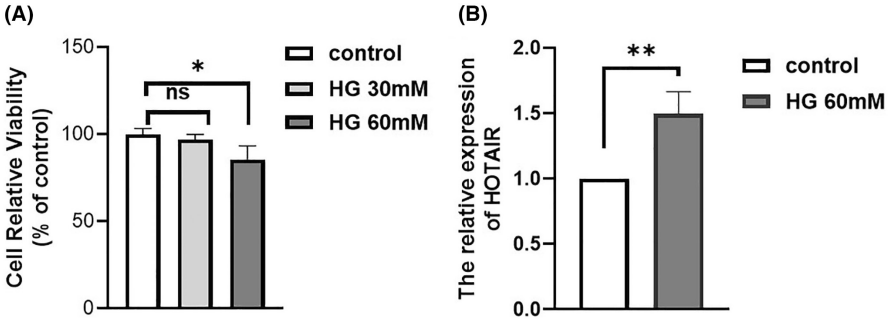

FIGURE 1 The expression level of HOTAIR increased in renal cell injury induced by high glucose. (A) CCK8 assay was used to determine the optimal concentration of the high glucose-induced injury model; (B) HOTAIR mRNA expression was upregulation under high glucose. $^{*}P<0.05$, $^{**}P<0.01$. CCK-8, Cell Counting Kit-8; Control, normal saline culture; HG 30 mmol/L, high glucose 30 mmol/L glucose culture; HG 60 mmol/L, high glucose 60 mmol/L glucose culture; HOTAIR, HOX transcript antisense RNA; mRNA, messenger RNA; ns, not significant.

transferring the separated proteins to a polyvinylidene difluoride (PVDF) membrane at 300 mA for 80 minutes, and then incubating with antibody at 4°C overnight, followed by hybridization with the secondary antibodies for 2 hours. The PVDF membrane was exposed to electrochemiluminescence reagents and protein bands were detected on the Gel Imaging System. Gray values of the bands were measured by Image-J software.

2.7 | Luciferase reporter assay

HOTAIR and mutant HOTAIR 3'-UTR reporter vectors were purchased from BoYuan. Luciferase reporter vectors were delivered into 1×10^{6} HK-2 cells at 70% confluence, along with the treatment of five groups. After 2 days, the cells were collected to analyze the luciferase signals using a dual-luciferase assay system (Promega).

2.8 | Statistical analysis

The data were presented as mean value ± SD . One-way analysis of variance and unpaired *t* test were adopted for multiple-group comparisons, with $P<0.05$ considered to be statistically significant. Data processing of the study was performed using Graph Prism version 9.0.

3 | RESULTS

3.1 | LncRNA HOTAIR exhibits upregulated in hyperglycemic-induced HK2 cells injury

To establish the high-glucose model, we set a concentration gradient of glucose and found that 60 mmol/L glucose could inhibit the proliferation of HK2 cells, depicted in Figure 1A. Under 60 mmol/L glucose, the level of lncRNA HOTAIR significantly increased, as presented in Figure 1B.

3.2 | Hyperglycemic-induced HK2 cells injury depends on HOTAIR

To identify whether hyperglycemic-induced injury depended on HOTAIR, we knocked down HOTAIR in HK2 cells. The efficiency of siRNA-mediated interference was presented in Figure 2A. CCK-8 assay showed that inhibition of lncRNA HOTAIR rescued the proliferation of HK2 cells, as illustrated in Figure 2B. Furthermore, the apoptosis rate of HK2 cells significantly increased with inhibiting LncRNA HOTAIR, displayed in Figure 2C,D. Simultaneously, Western blotting showed that silencing of HOTAIR led to a reduction in Bax and Cleaved Caspase-3 proteins while elevating the levels of Bcl-2 proteins, as revealed in Figure 3B.

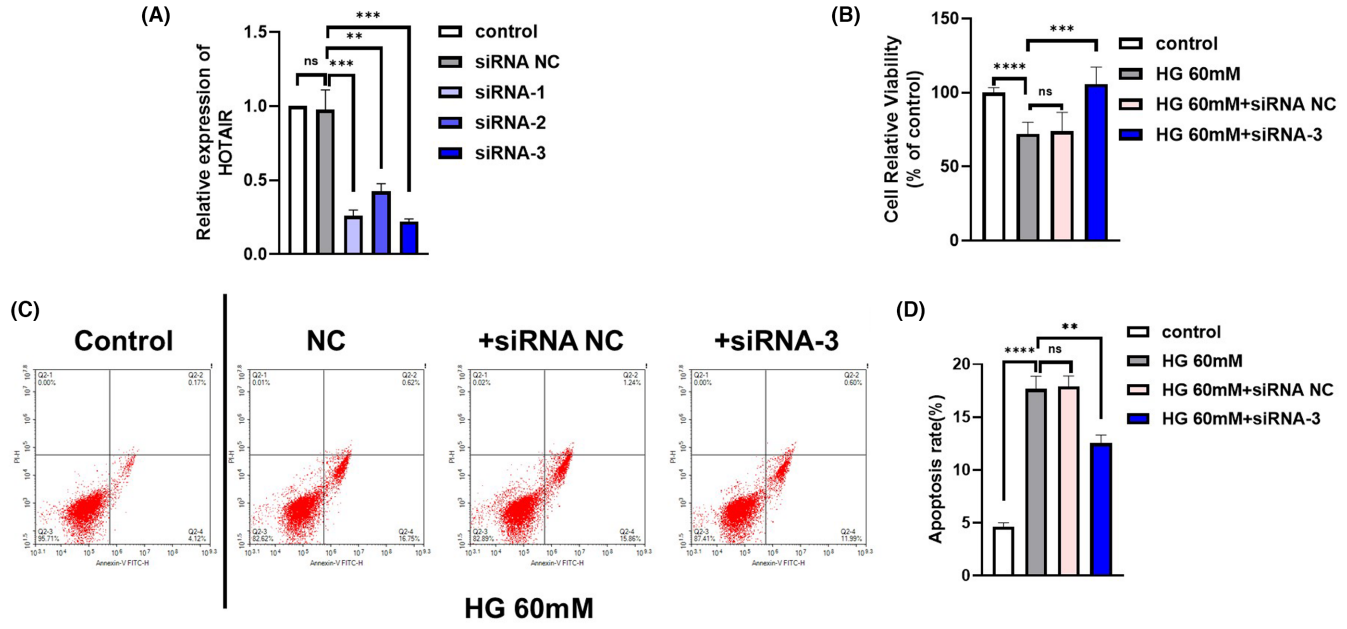

FIGURE 2 The injury of HK-2 cells induced by high glucose is lncRNA HOTAIR-independent. (A) The interference efficiency of lncRNA HOTAIR siRNAs. (B) The cell relative viability of HK-2 under control, HG 60 mmol/L, HG 60 mmol/L + siRNA NC, and HG 60 mmol/L + siRNA-3 group. (C, D) The apoptosis rate of HK-2 under control, HG 60 mmol/L, HG 60 mmol/L + siRNA NC, and HG 60 mmol/L + siRNA-3 group. (C) Is the statistic of (D). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Control, normal saline culture; HG 30 mmol/L, high glucose 30 mmol/L glucose culture; HG 60 mmol/L, high glucose 60 mmol/L glucose culture; HOTAIR, HOX transcript antisense RNA; lncRNA, long noncoding RNA; siRNA, small-interfering RNA.

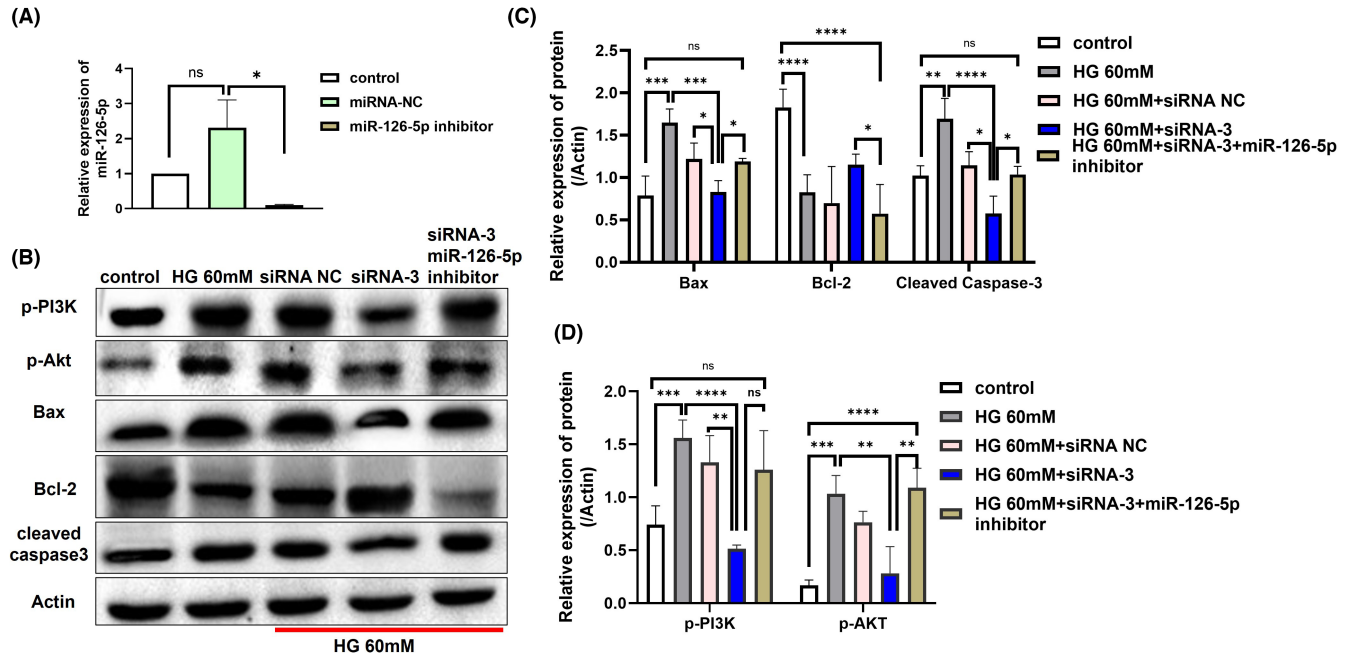

FIGURE 3 LncRNA HOTAIR regulates the injury of HK-2 cells through miR-126-5p/PI3K/AKT axis. (A) The interference efficiency of lncRNA HOTAIR siRNAs. (B) The result of p-PI3K, p-AKT, Bax, Bcl-2, and cleaved caspase-3 with the treatment of normal saline culture, HG 60 mmol/L, HG 60 mmol/L + siRNA NC, HG 60 mmol/L + siRNA-3, and HG 60 mmol/L + siRNA-3 + miR-126-5p inhibitor. (C, D) Is the statistic of (B). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Control, normal saline culture; HG 30 mmol/L, high glucose 30 mmol/L glucose culture; HG 60 mmol/L, high glucose 60 mmol/L glucose culture; HOTAIR, HOX transcript antisense RNA; lncRNA, long noncoding RNA; siRNA, small-interfering RNA

3.3 | LncRNA HOTAIR promotes apoptosis in HK2 cells by inhibiting miR-126-5p

Given that lncRNA HOTAIR could act as a "molecular sponge" to reduce the number of miRNAs, we used bioinformatic tools to identify its target miRNA. A dual luciferase reporter assay depicted that luciferase activity significantly decreased in the HOTAIR-WT + miR-126-5p mimic group compared to HOTAIR-WT + mimic NC group ($P = 0.016$). However, there was no significant difference between HOTAIR-mut + mimic NC and HOTAIR-mut + miR-126-5p mimic groups, illustrated in Figure 4A,B. These results proved that miR-126-5p was a target of HOTAIR. Furthermore, upon inhibition of HOTAIR activity, miR-126-5p was significantly upregulated, as displayed in Figure 4C. Notably, the simultaneous inhibition of both HOTAIR and miR-126-5p resulted in increased expression of Bax and cleaved caspase-3, along with decreased expression of Bcl-2, as shown in Figure 3B.

3.4 | LncRNA HOTAIR regulates the injury of HK-2 cells through miR-126-5p/PI3K/AKT axis

Previous studies[20] reported that PI3K/Akt was involved in high glucose-induced progenitor cell injury. Our result showed that high glucose could induce the activation of the PI3K/Akt signaling pathway, via phosphorylating PI3K and Akt. Then, the knockdown of HOTAIR led to a downregulation of the PI3K/Akt signaling pathway and two pro-apoptosis markers, Bax and cleaved caspases-3, as displayed in Figure 3B. Additionally, to investigate whether miR-126-5p could regulate the activation of the PI3K/Akt signaling pathway, we inhibited both miR-126-5p and HOTAIR. The efficiency of the miR-126-5p inhibitor is illustrated in Figure 3A. We found that compared with the knockdown of HOTAIR, inhibition of miR-126-5p could rescue the PI3K/Akt signaling pathway and the expression levels of Bax and cleaved caspases-3, suggesting that HOTAIR regulated PI3K/Akt pathway through miR-126-5p.

4 | DISCUSSION

With a growing elderly population, the incidence of senile DN is increasing yearly.[21] Although glycemic control is the major treatment for DN, merely controlling blood glucose gives little benefit to senile DN.[6,22] Consequently, elucidating the mechanism underlying glucose-induced renal injury is crucial to enhancing DN's therapeutic efficacy in elderly patients. we found that the levels of Bax and cleaved caspase-3 increased in the HG model, whereas the anti-apoptotic factor Bcl-2 was inhibited by high glucose. These findings replicate previous studies demonstrating that high glucose inhibits proliferation and induces apoptosis in HK2 cells.[23,24] At the same time, we first found that lncRNA HOTAIR was induced by high glucose in a dose-dependent manner, and could regulate the apoptosis of HK2 cells. Abnormal activation of apoptosis is implicated in glomerular injury in diabetic kidneys, contributing to the development of DN.[25,26] Podocyte apoptosis reduces podocyte numbers and subsequently results in proteinuria.[27,28] Renal tubular apoptosis would cause tubular atrophy and the loss of renal function.[29,30] Therefore, HOTAIR would be a potential target to inhibit apoptosis in renal

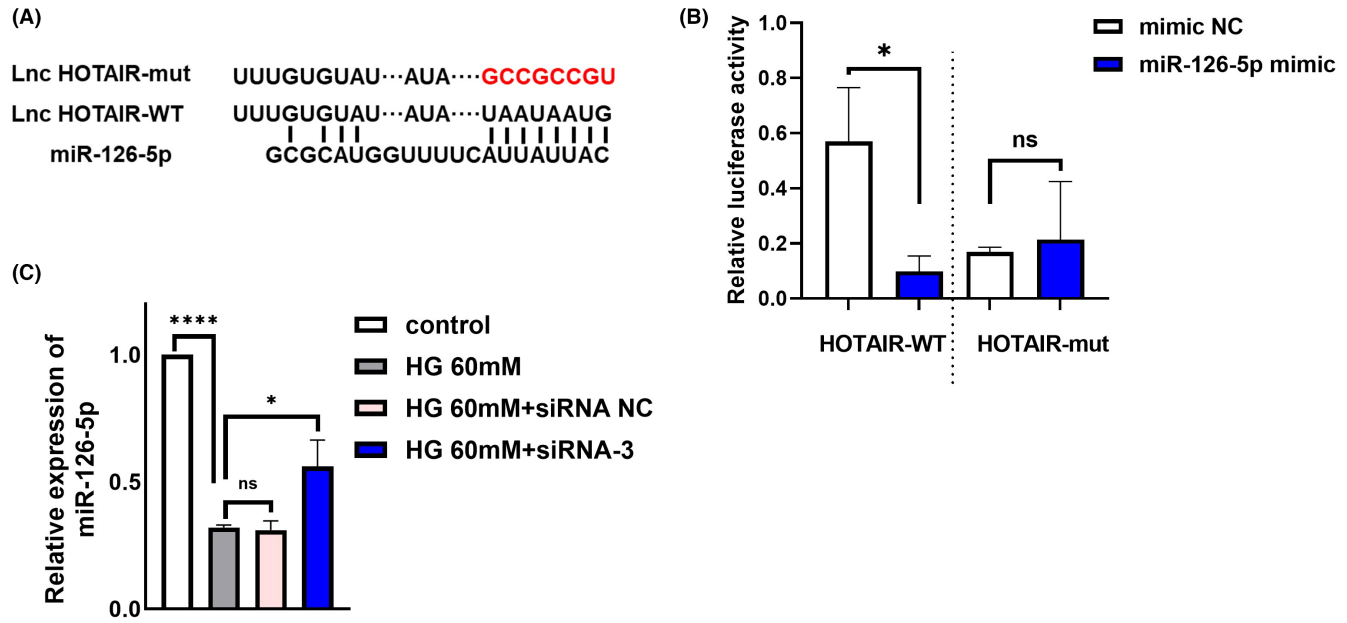

FIGURE 4 LncRNA HOTAIR regulates the level of miRNA-126-5p in the high glucose injury HK2 model. (A) The binding sites of lncRNA HOTAIR and miR-126-5p. (B) The results of dual luciferase reporter assay of lncRNA HOTAIR and miR-126-5p. (C) The relative expression of miR-126-5p under control, HG 60 mmol/L, HG 60 mmol/L + siRNA NC, and HG 60 mmol/L + siRNA-3 group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Control, normal saline culture; HG 30 mmol/L, high glucose 30 mmol/L glucose culture; HG 60 mmol/L, high glucose 60 mmol/L glucose culture; HOTAIR, HOX transcript antisense RNA; lncRNA, long noncoding RNA; miRNA, microRNA.

tubular cells, thereby mitigating the progression of renal damage in DN.

The association between lncRNA and elderly patients with DN remains poorly understood. As a classical lncRNA, HOTAIR is more commonly reported to play a regulatory role in developing various malignant tumors, but evidence of its effect on DN is scarce.[10,31,32] Our findings revealed that HOTAIR is upregulated in HK-2 cells under conditions of high-glucose injury, suggesting its involvement in DN development. In tumor cells, HOTAIR can interact with epigenetic modifiers, such as PRC2 and LSD1, which act as chromatin modifiers.[33,34] Additionally, it can also act as an RNA sponge to inhibit miRNAs and facilitate ubiquitin-mediated proteolysis.[35,36] Our study found that HOTAIR exerted detrimental effects on HK-2 cells by acting as a sponge for miRNA-126-5p. Interestingly, unlike in tumor cells, HOTAIR promoted apoptosis in HK-2 cells.[37,38] However, the upstream mechanism of HOTAIR is unclear and further research is required to clarify this.

A previous study, which included 80% of patients with DN, demonstrated a potential association between angiogenic miRNAs and cognitive decline in older individuals with DN.[39] However, the specific miRNAs that are specifically related to kidney injury in DN are yet to be determined. A previous investigation revealed decreased expression of miR-126-5p in the peripheral blood of patients with DN, suggesting an increased risk in these individuals.[19] Moreover, miR-126 negatively correlates with kidney function in patients with DN and exerts anti-apoptosis and anti-inflammation in DN in vitro through the PI3K/AKT pathway.[19,40] Our observations confirmed the downregulation of miR-126-5p in response to high glucose exposure, further supporting its role in DN. Notably, a previous study had identified miR-126-5p as a potential target site for HOTAIR.[16] Moreover, HOTAIR can decrease the levels of miR-126-5p. Therefore, miR-126-5p exhibits a protective effect in DN and may serve as a potential marker for assessing tubulointerstitial injury in elderly patients with DN.

PI3K/Akt signaling pathway is involved in regulating cell proliferation, growth, and viability.[41,42] It consists of the catalytic subunit p110 and the regulatory subunit p85, which work together to activate Akt by recruiting it to the plasma membrane through the binding of the PH domain.[43] Aberrant activation of the PI3K/Akt pathway has been observed, in which it regulates inflammation, apoptosis, and cell cycle.[25,44] Meanwhile, some evidence identifies that high glucose is a risk factor for promoting the activation of the PI3K/Akt signaling pathway.[45,46] Previous studies showed that miR-126 could target PI3K/Akt signaling pathway.[20,40] Consistently, our result also suggested that PI3K/Akt was downstream of miR-126-5p under high-glucose injury. However, both inhibition of HOTAIR and miR-126-5p, HK-2 cells still promote AKT activation and suppress Bcl-2 expression under high glucose stimulation, shown in Figure 3B–D. Activated PI3K/Akt induces ECM deposition in the glomerulus and is involved in the process of renal tubule injury.[47–49] Taken together, we concluded that HOTAIR causes high glucose-induced tubule cell injury through PI3K/Akt via targeting miR-126-5p.

This study reveals that HOTAIR exerts a pro-apoptotic influence by sponging miR-126-5p, thereby leading to heightened PI3K/Akt signaling activity. Whereas the precise mechanism governing the regulation of PI3K/Akt by HOTAIR necessitates further exploration, this discovery paves the way for novel therapeutic avenues in the management of DN and the identification of potential biomarkers for elderly patients with DN.

AUTHOR CONTRIBUTIONS

Qiong Jiang proposed the idea and experimented. Ting Yang and Yan Zou analyzed data and performed statistical analysis. Mingjie He wrote the manuscript. Qingchun Li and Xiaohui Chen are responsible for reagent management. Aimin Zhong reviewed and revised the manuscript. All authors have given consent to the publication of this study.

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CONFLICT OF INTEREST STATEMENT

Nothing to disclose.

DATA AVAILABILITY STATEMENT

The raw data and the images of the original Western blots presented in this study are openly available in Figshare at 10.6084/m9.figshare.21972503.

ORCID

Qiong Jiang https://orcid.org/0000-0003-1936-9302

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