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Original Research Article

MiR-192–5p targets cell cycle regulation in diabetic kidney disease via cyclin-dependent kinase inhibitor 3

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ABSTRACT

Diabetic kidney disease (DKD), a.k.a diabetic nephropathy, is a leading cause of end-stage renal disease. However, in a fair percentage of patients with type-2 diabetes, renal involvement also occurs due to non-diabetic reasons (non-diabetic kidney disease, NDKD). In this study, we identified miRNA-mRNA regulatory networks specific to human DKD pathogenesis. miRNA profiling of the renal biopsy from cases (DKD, n = 5), disease controls (T2DM with NDKD, n = 6), and non-diabetic, non-CKD controls (patients undergoing nephrectomy for renal cancer, n = 3) revealed 68 DKD-specific miRNA regulation. Sixteen target mRNAs of these DKD-miRNAs were found to have a negative association with the estimated glomerular filtration rate (eGFR) in patients with DKD. The renal gene expression and eGFR data of DKD patients (n = 10–18) in the NephroSeq database were used. Based on these findings, 11 miRNA-mRNA regulatory networks were constructed for human DKD pathogenesis. Of these, in-vitro validation of miR-192-5p- CDKN3 (Cell cycle-dependent kinase inhibitor 3) network was done as miR-192–5p exhibited a maximum number of target genes in the identified DKD regulatory networks, and CDKN3 appeared as a novel target of miR-192–5p in our study. We demonstrated that miR-192–5p overexpression or knockdown of CDKN3 attenuated high glucose-induced apoptosis, fibrotic gene expression, cell hypertrophy, and cell cycle dysregulation and improved viability of proximal tubular cells. Moreover, miR-192–5p overexpression significantly inhibited CDKN3 mRNA and protein expression in proximal tubular cells. Overall, 11 miRNA-mRNA regulatory networks were predicted for human DKD pathogenesis; among these, the association of miR-192-5p- CDKN3 network DKD pathogenesis was confirmed in proximal tubular cell culture.

1. Introduction

Diabetes mellitus (DM) is one of the most common causes of kidney disease, which is a major contributor to the Global Disease burden. Diabetic kidney disease (DKD) occurs in 30–40 % of diabetic patients [1]. However, 45–80 % of diabetics with kidney involvement who have undergone kidney biopsy are diagnosed to have non-diabetic kidney disease (NDKD) [2–5]. Progression of DKD is a major cause of end-stage renal disease (ESRD), a debilitating condition with poor outcomes. ESRD patients are left with the only choice of renal replacement therapy. Early diagnosis and therapeutic intervention could prevent or, at least, slow down the progression of DKD to ESRD. Identifying miRNA and their regulatory genes associated with the pathogenesis of DKD would facilitate early disease management.

miRNA (miR) plays an essential role in the development of kidney disease through its capacity to regulate cell transcriptome [6,7]. miRNAs are small noncoding RNAs consisting of 20–22 nucleotides each which inhibit the translation of the targeted mRNA, thereby regulating many biological and cellular processes [8]. Several miRNAs, including miR-21, miR-184, miR-377, miR-146a, and let-7 family miRNAs are associated with the kidney disease pathogenesis by regulating processes such as inflammatory responses and fibrogenesis [9–14]. Being upstream gene regulators, miRNA holds promise as early therapeutic targets for kidney disease management [15]. Interestingly, a single miRNA has the capability to modulate the expression of numerous target genes, consequently impacting a particular pathology by regulating entire disease-specific pathways and signaling cascades rather than individual genes. This distinctive function underscores the tremendous significance of these small molecules.

Hence, a functionally relevant miRNA-mRNA network is required to translate any physiological and pathological manifestation. Aberrant regulation of certain miRNAs and their target mRNAs has been reported in the pathogenesis of various human diseases, including kidney disease [16–18]. There are reports on miRNA dysregulation in DKD kidneys;

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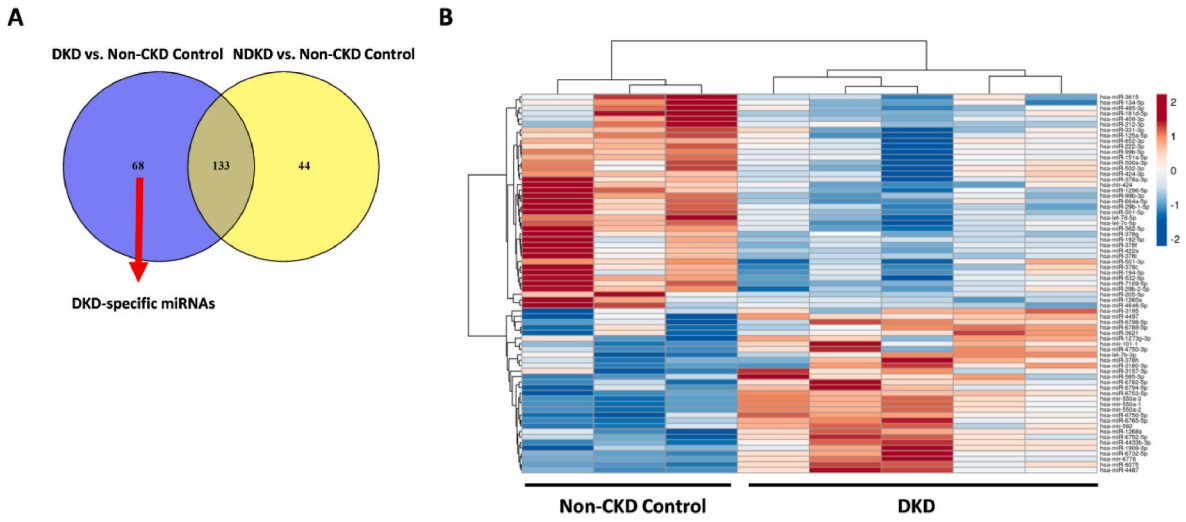


Fig. 1. Diabetic kidney disease-specific miRNAs in human kidneys
(A) Venn diagram showing the number of miRNAs regulated in renal biopsies in T2DM patients with diabetic kidney disease (DKD, n = 5) or disease control (NDKD, n = 6), relative to non-CKD controls (n = 3). The intersection shows miRNAs specific to DKD biopsies. (B) Heat maps showing hierarchical clustering of 68 miRNAs differentially expressed only in DKD biopsies. Red represents the upregulated miRNA, and blue represents the downregulated miRNA.

Table 1
Target Hub gene of DKD-associated miRNAs.

Upregulated miRNAs targets		Downregulated miRNAs targets	
Gene	Degree	Gene	Degree
TP53	241	TTK	667
CTNNB1	205	DLGAP5	586
HSP90AA1	178	CCNB2	567
JUN	147	BUB1B	547
PTEN	146	NCAPG	507
UBC	137	CDK1	489
NOTCH1	128	KIF20A	439
CCND1	121	ASPM	424
RHOA	114	CENPE	385
MTOR	112	MAD2L1	381
HIF1A	107	CENPF	379
CASP3	106	KIF2C	378
VEGFA	106	KIF15	372
CCNB1	102	NUF2	363
EZH2	101	DTL	343
MDM2	100	KIF23	342
ATM	98	MCM10	332
HSP90AB1	92	PLK4	319
FOS	87	CENPA	317
H2AFX	87	CEP55	311
CREB1	86	CDC6	301
SMAD2	81	KIF4A	297
GSK3B	80	FBXO5	294
APP	80	TRIP13	281
CHEK1	79	RACGAP1	275
SUMO1	77	SMC4	272
EPRS	77	NCAPG2	270
EEF2	76	PRC1	269
AGO2	76	CDKN3	266
RAD51	74	CDC20	264

Top 30 hub genes identified in the PPI network of DKD-associated miRNAs based on the degree of interaction.

however, in these reports differential miRNA expressions were evaluated relative to either healthy kidney tissues (non-CKD) [19–22] or disease controls (kidney disease other than DKD, NDKD) [23–26].

In sum, more studies are needed to understand various miRNA-mRNA regulatory networks for human DKD pathogenesis, which could help early disease management. In this study, we identified a few miRNA-mRNA regulatory networks for human DKD pathogenesis by comparing renal biopsy samples from type 2 DM (T2DM) patients with biopsy-proven DKD, disease controls (T2DM with kidney disease other than DKD) and non-CKD controls (non-diabetic patients undergoing nephrectomy for renal cancer). Although all networks need to be harnessed, one of such networks in DKD pathogenesis has been explored.

2. Materials & methods

2.1. Study population

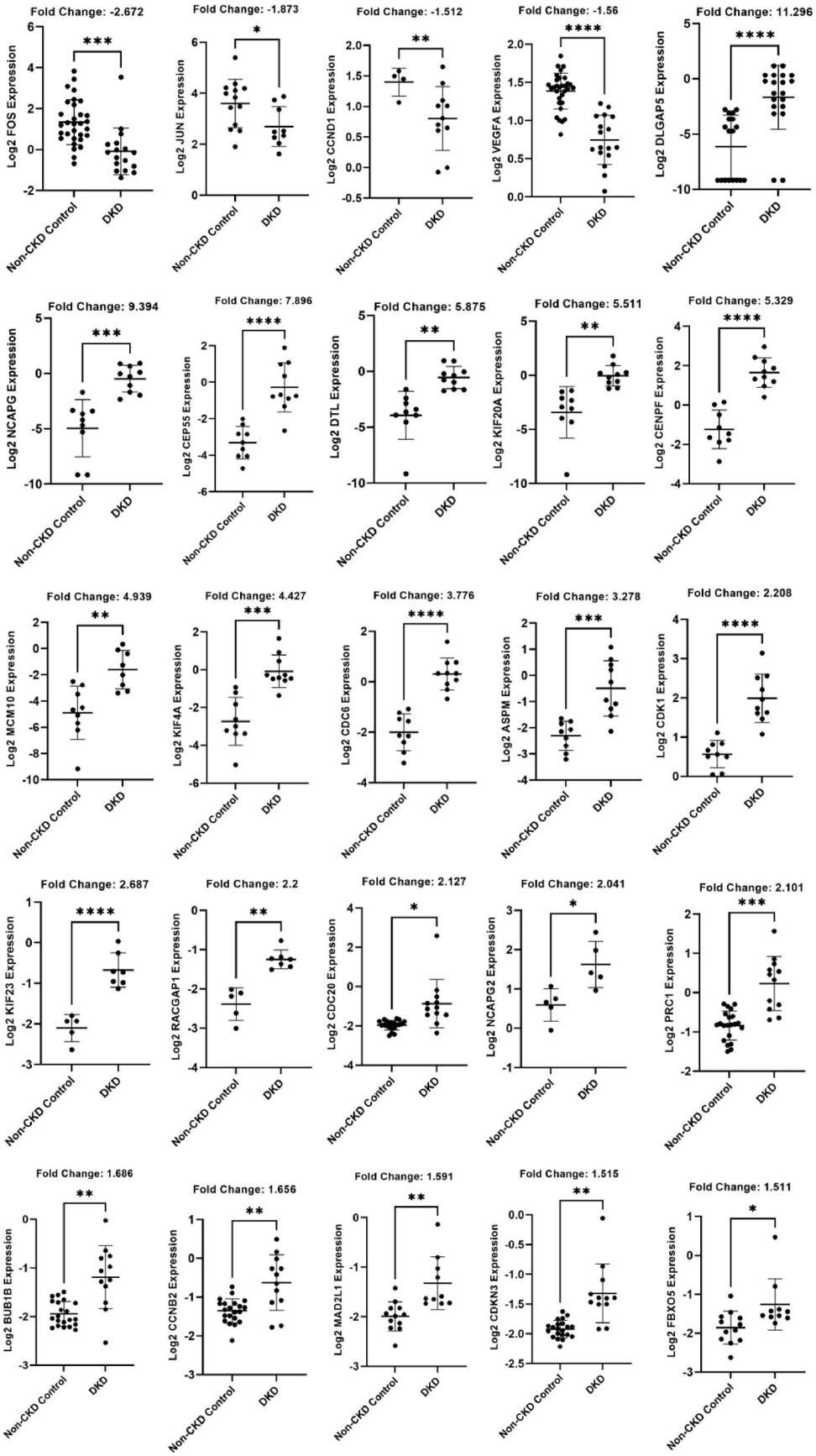
This study included two study populations, i.e., renal biopsies and data retrieval from the Nephroseq database.

2.2. Renal biopsies

The study was approved by the institutional human ethics committees of Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS, IEC Code 2018-139-EMP-106 and 2021-309-EMP-EXP-44), Lucknow, and Christian Medical College (CMC, IRB Min. No. I2243 (OTHER), dated September 25, 2019), Vellore, India. After written informed consent, renal biopsies were collected from kidney disease patients with T2DM. The biopsies were processed according to standard techniques for light microscopy, immune fluorescence, and electron microscopy and interpreted by renal pathologists. Based on histopathological findings, renal biopsies were classified as Diabetic kidney disease (DKD) or disease controls NDKD (MN, FSGS). In addition, non-CKD control biopsy samples, defined as renal cortical tissue from the normal region of the radical nephrectomy specimens from renal cancer patients, were collected from the Department of Urology, SGPGIMS. All Renal tissue samples were obtained for molecular analyses, and demographic information was also collected.

2.3. Data from nephroseq database

Gene expression and eGFR data were obtained from the Nephroseq v5 database (www.nephroseq.org). The Nephroseq database is a platform for integrated data analysis of genotype/phenotype data from humans and analysis tools specifically for kidney-related studies. Studies that met the following inclusion criteria were selected: 1) kidney tissue samples, 2) comparison of DKD with healthy controls with transcriptomic data. Normalized expression values were obtained directly from the database.



(caption on next page)

Fig. 2. Regulation of diabetic kidney disease-associated hub genes in human renal biopsies.
Scatter dot plot with line at mean with SD showing log2 gene expression of predicted hub genes in the renal biopsies collected from diabetic kidney disease (DKD) patients and non-CKD control biopsies in the NephroSeq database. Each dot in the scatter plot represents individual gene expression data. p*< 0.05, **< 0.01 were considered significant by the *t*-test.

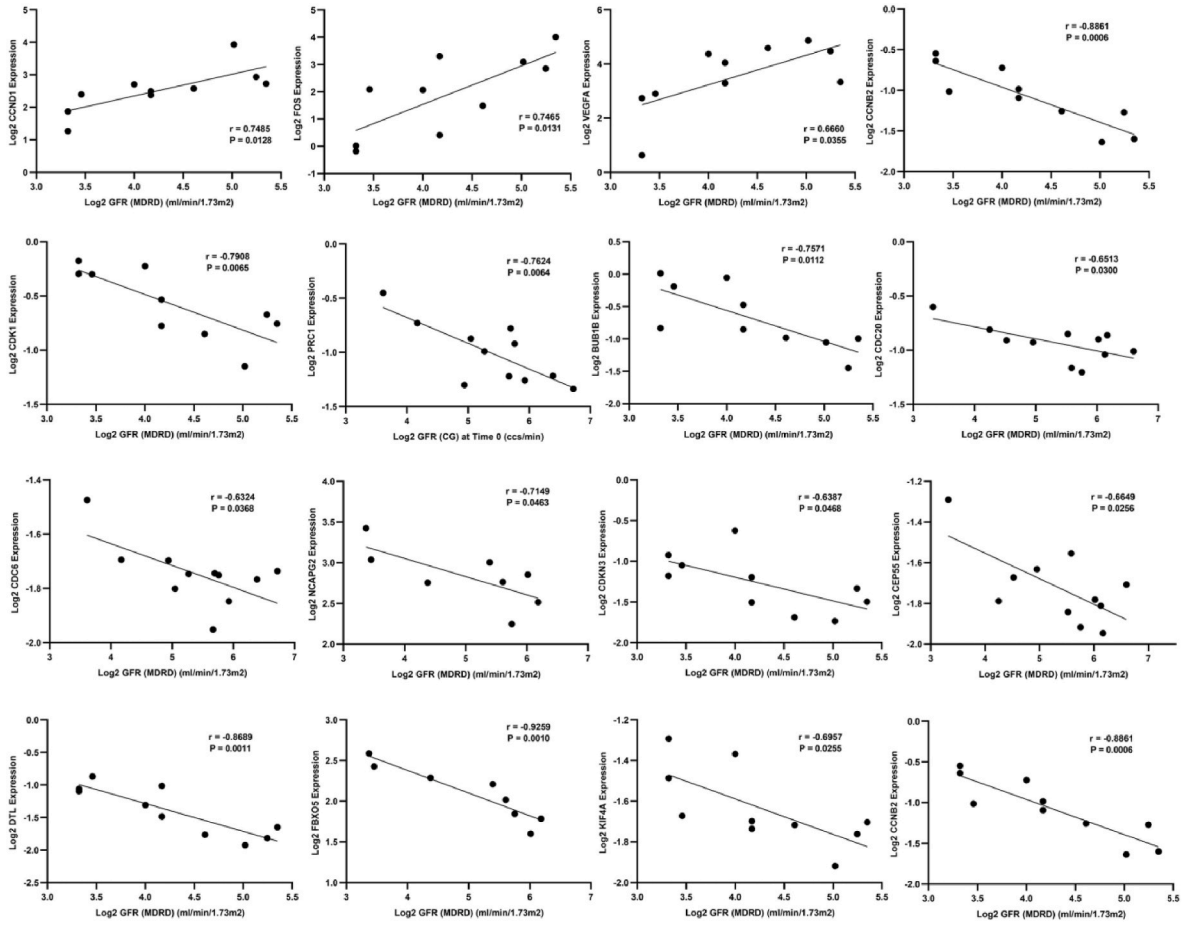


Fig. 3. Association of target hub genes with renal function impairment.
Correlation of hub gene expression in the kidney tissue with estimated GFR (eGFR) in patients with diabetic kidney disease in the Nephroseq database. Pearson correlation coefficient 'r' is given with its respective p-value; $p < 0.05$ was considered significant.

2.4. miRNA microarray and array data analysis

Total RNA (including miRNAs) was extracted from renal biopsy tissue using miRNeasy Micro Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. Microarray was performed according to Affymetrix GeneChip miRNA 4.0 array kit to find the differentially expressed miRNAs. For each sample, 120 ng of total RNA was labeled using the FlashTag™ Biotin RNA Labeling Kit. After this, the labeled RNA was fractionated and hybridized into the miRNA microarray. The hybridization was performed at 50 rpm for 16 h at 48 °C on an Affymetrix® 450 Fluidics Station. After washing the array, the chips were stained using the GeneChip Fluidics Station 450 (Affymetrix) and scanned using an Affymetrix GCS 3000 scanner (Affymetrix). The raw signal values were evaluated using the Transcriptome Analysis Console (TAC) software. A comparative analysis between the DKD vs. non-CKD control and disease control (NDKD) vs. non-CKD control samples was performed to find statistically significant differentially expressed miRNAs defined as p-value <0.05 and fold change < - 2 and > +2. A heatmap was constructed using the ClustVis web tool to display the miRNA expression profiles of the samples.

2.5. Target gene prediction and hub gene identification

miRNet (version 2.0) (https://www.mirnet.ca/) was used for target gene prediction. For hub gene identification, a protein-protein interaction (PPI) network of target genes was performed using the string (version 12.0) (https://string-db.org/) database and visualized in Cytoscape software (version 3.9.1). The Hub genes were identified by analyzing the degree in the networks.

2.6. Gene validation and correlation with renal function

The expression and regulation of the predicted hub genes and their association with estimated glomerular filtration rate (eGFR) were validated using DKD patient data available at the Nephroseq database (NephroSeqv5 database). Healthy living donor samples were used as control in the database. We used the 'Nephroseq V5' tool to determine the correlation between selected hub genes and renal function (eGFR) in DKD.

2.7. Gene ontology and pathway enrichment analysis

Enrichr (https://maayanlab.cloud/Enrichr/), an online tool, was

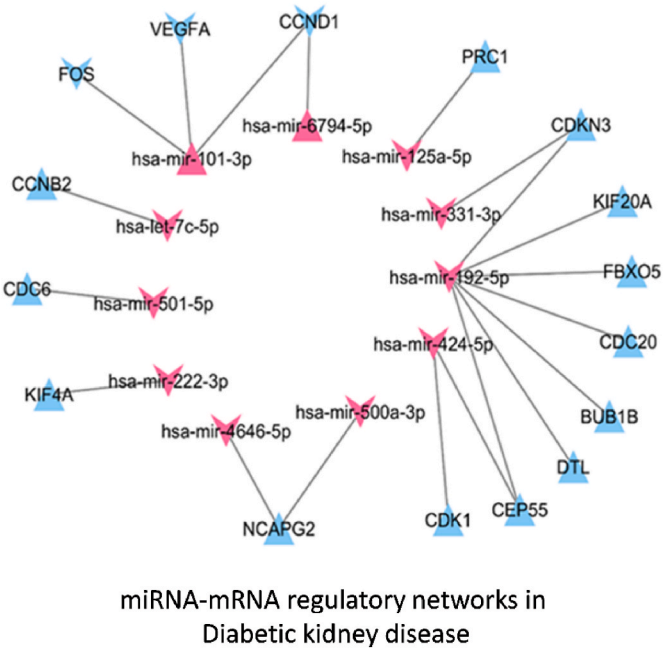


Fig. 4. miRNA-mRNA regulatory networks specific to diabetic kidney disease. Eleven DN-specific miRNA-mRNA regulatory networks show sixteen selected hub genes out of 9951 target genes. Pink represents miRNAs, blue represents the targeted mRNAs, and the line represents their interaction. The triangle shape and v shape represent upregulation and downregulation, respectively.

used to perform Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for the targeted genes of differentially expressed miRNAs (p-values <0.05 were considered statistically significant).

2.8. miRNA– mRNA network construction

Cytoscape software (version 3.9.1) was used to construct and visualize miRNA–mRNA regulatory networks for the identified DKD-specific miRNAs and their target hub genes.

2.9. RNA isolation

Total RNA was extracted from the kidney biopsy samples and HK-2 cells using PureLink™ RNA Mini Kit (Invitrogen), according to the manufacturer's instructions.

2.10. Quantitative real-time PCR

For expression analysis of miRNA, miRNA-specific stem-loop primers were designed by sRNAPrimerDB [27] and synthesized by IDT technologies. 10 ng of total RNA from kidney tissue/HK-2 cells were taken for cDNA preparation using Tqman cDNA synthesis kit (Applied Biosystems), according to the manufacturer's instructions. Next, qPCR was performed in a BioRad CFXmastro96Real-Time PCR system by SYBER Premix Ex Taq (Takara). The fold change of relative expression levels of miRNAs (relative to U6 as endogenous control) was analyzed by the $2^{-\Delta\Delta Ct}$ method.

To analyze the mRNA expression of predicted target genes, total RNA (2 μg) from kidney tissue/HK-2 cells was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit as per manufacturer's instructions using random primers (Applied BioSystems, USA). qPCR analysis for relative quantification of target genes was performed using SYBER Premix Ex Taq (Takara) in a BioRad CFXmastro96Real-Time PCR system. 18 S RNA was used as endogenous control. The relative gene expression was also analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.11. Cell culture and glucose treatment

HK-2 cells were purchased from the American Type Cell Collection (ATCC, Manassas, VA, USA, #CRL-2190) and cultured in Dulbecco's

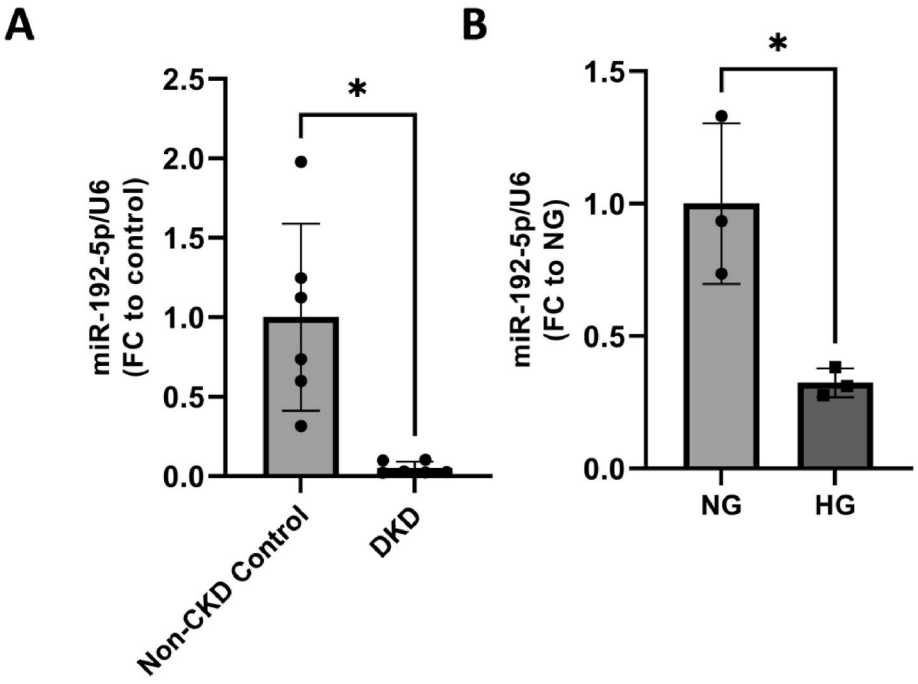


Fig. 5. miR-192–5p expression in human kidney.
Bar graph with scatter shows miR-192–5p expression in (A) renal biopsies in patients with DKD compared to non-CKD controls, and (B) proximal tubular cells (HK-2 cells) treated with high glucose (HG) compared to cells exposed in normal glucose (NG) media. Each dot in the scatter plot represents data from an individual patient's sample. The expression was analyzed by qPCR. *$p < 0.05$ by unpaired *t*-test.

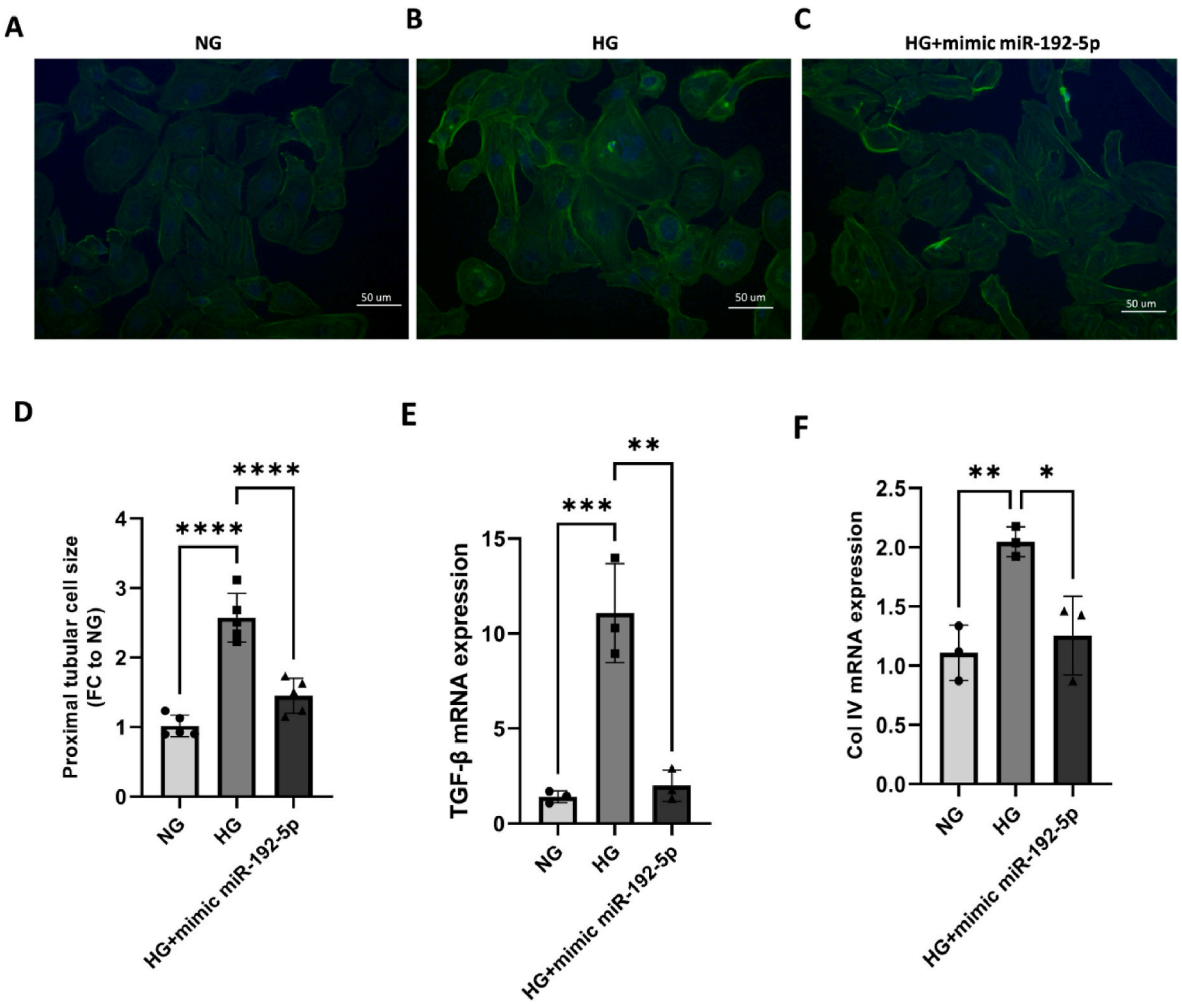


Fig. 6. MiR-192–5p overexpression attenuated high-glucose induced cell hypertrophy and fibrotic gene expression in HK-2 cells
Micrographs showing proximal tubular cell hypertrophy by F-actin staining (green) after treatment with (A) normal glucose (NG) or (B) high glucose (HG) media or (C) Cells transfected with miR-192–5p mimic cultured in HG media (HG + mimic miR-192–5p). (D) The bar with scatter plot shows the quantification of the cell size of HK-2 cells using ImageJ. Bar and scatter plots show (E) TGF –β, (F) Col IV mRNA expression (by qPCR) in the HK-2 cells cultured in normal glucose (NG), high glucose (HG) media, or miR-192–5p mimic transfected cells cultured in HG media (HG + mimic miR-192–5p). Each cell culture experiment was repeated three times. Each dot in the scatter plot represents data from an individual experiment.

modified Eagle medium (DMEM) supplemented with fetal bovine serum (FBS), streptomycin, and penicillin under a 5 % CO2 atmosphere at 37 °C. The culture medium was replaced every 48–72 h until the desired cell density was achieved. Upon reaching 70–80 % confluence within 3–5 days, the HK-2 cells were detached using trypsinization and passaged at a 1:3 ratio, or plated onto 6- or 24-well plates at the required density for the immediate experiment. Specifically, approximately 2*105 cells or 5*104 cells per well were seeded in 6- or 24-well plates, respectively. For the high glucose treatment, HK-2 cells were exposed to 30 mmol/L of glucose for 48 h, while cells designated for control treatment were cultured in 5.5 mmol/L of glucose for the same duration, referred to as normal glucose treatment.

2.12. Transfection

Cells were transfected in 24-well plates at 50 % confluence with miR-192–5p mimics or NC mimics (Thermo Scientific, USA) and CDKN3 siRNA or scrambled control siRNA (Santa Cruz Biotechnology, USA) using Lipofectamine RNAiMAX (Invitrogen, CA, United States), according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested for further analysis.

2.13. Immunofluorescence

In vitro, proximal tubular cell hypertrophy was evaluated by assessing cell size by measuring F-actin staining in the cytoplasm of cells using phalloidin-FITC Reagent (ab235137, Abcam, USA) following the manufacturer's instructions. Images were taken with an Olympus fluorescence microscope (Olympus, IX73, Japan).

2.14. Western blot

Western blotting was performed following the protocol outlined in our previous study [28]. In brief, proteins were separated using 10 % SDS-PAGE and then transferred onto polyvinylidene difluoride membranes through immunoblotting. After blocking with 5 % milk, the membranes were treated with primary antibodies specific to CDKN3 (A2061 Abclonal, USA) at a dilution of 1/1000, followed by secondary antibodies conjugated with horseradish peroxidase at a dilution of 1:2000 (ab6721, Abcam, USA). The detection of antigen-antibody complexes was visualized using a chemiluminescence-based detection system (ChemiDoc MP System, Bio-Rad, USA).

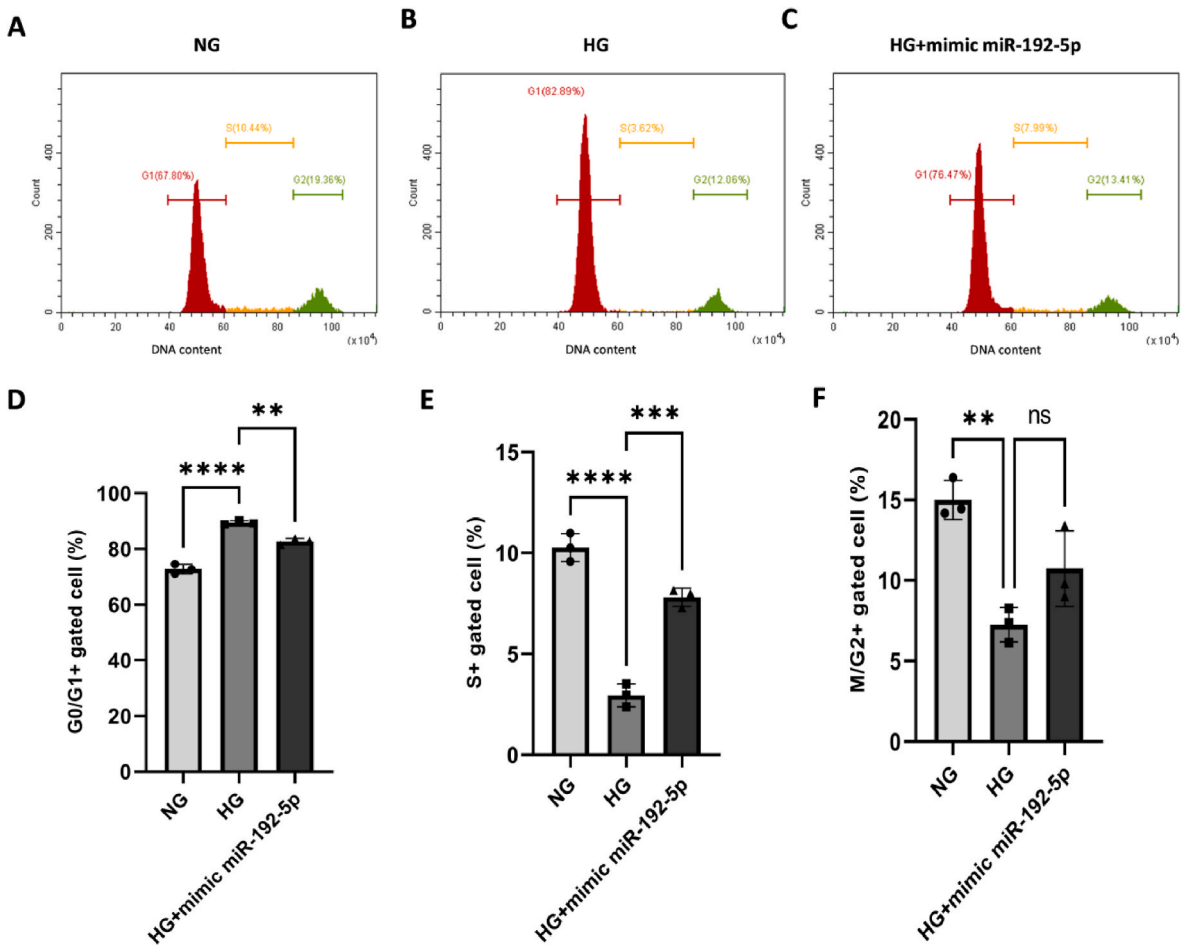


Fig. 7. Overexpression of miR-192–5p prevented high-glucose induced cell cycle dysregulation in HK-2 cells
DNA Histograms showing proximal tubular cells are detected in the G0/G1, S, or M/G2 phase of the cell cycle after treatment (A) normal glucose (NG) or (B) high glucose (HG) media or (C) Cells transfected with miR-192–5p mimic cultured in HG media (HG + mimic miR-192–5p) generated through Flow cytometric analysis. The bar and scatter plot shows the qualification of cells detected in the G0/G1 phase (D), S phase (E), or M/G2 phase (F) of the cell cycle. Each cell culture experiment was repeated three times. Each dot in the scatter plot represents data from an individual experiment.*$p < 0.05$ by one-way ANOVA followed by multiple comparisons by Tukey's multiple comparisons test.

2.15. Cell cycle analysis

Cellular DNA was labeled with propidium iodide to identify cells in various cell cycle stages using the Cell Cycle Assay Kit (E-CK-A351, Elabscience, USA) according to the manufacturer's instructions. Cell cycle Analysis was analyzed using a CytoFLEX Flow Cytometer (Beckman Coulter, USA), with data processed using Cytoexpert software (Version 2.5) from Beckman Coulter.

2.16. Apoptosis assay

Cells undergoing early apoptosis were identified using Annexin V and 7-amino actinomycin D (7-AAD) staining by Annexin V-FITC/7-AAD Apoptosis Kit (E-CK-A212, Elabscience, USA) as per the manufactures' instructions. Analysis of the cell cycle was carried out using a CytoFLEX Flow Cytometer (Beckman Coulter, USA), with data processed using Cytoexpert software (Version 2.5) from Beckman Coulter.

2.17. Cell counting Kit-8 (CCK-8) assay

The proliferation rate was measured using the Enhanced Cell Counting Kit 8 (E-CK-A362, Elabscience, USA) following the manufacturer's instructions for the assay. Briefly, HK-2 cells ($2*10^3$ cells) were seeded into 96 well plates according to the experimental design. After 48 h incubation, 10 μL of CCK-8 Buffer was added to each well and incubated for 2 h at 37 °C. The optical density of each well was assessed using a Microplate Reader (Synergy H1 microplate reader, BioTek, USA) at a wavelength of 450 nm.

2.18. Statistical analysis

The results were shown as mean ± standard deviation (SD) with scatter. A two-tailed Student's t-test was used to find the differences between the two groups. ANOVA followed by multiple comparisons was used to compare multiple groups. All p values < 0.05 were considered significant. All statistical analyses were conducted using GraphPad Prism9 Software (San Diego, CA).

3. Results

3.1. DKD-specific miRNAs in human kidneys

Microarray provided signal intensities for around 4603 miRNAs in the renal biopsies. A total of 201 miRNAs were differentially regulated in renal biopsies from DKD cases, relative to non-CKD control with log2 fold change $>\pm 2$ and $p < 0.05$. A venn diagram was generated to determine the relationship between the two sets of differentially expressed miRNAs. One hundred thirty-three miRNAs present in the

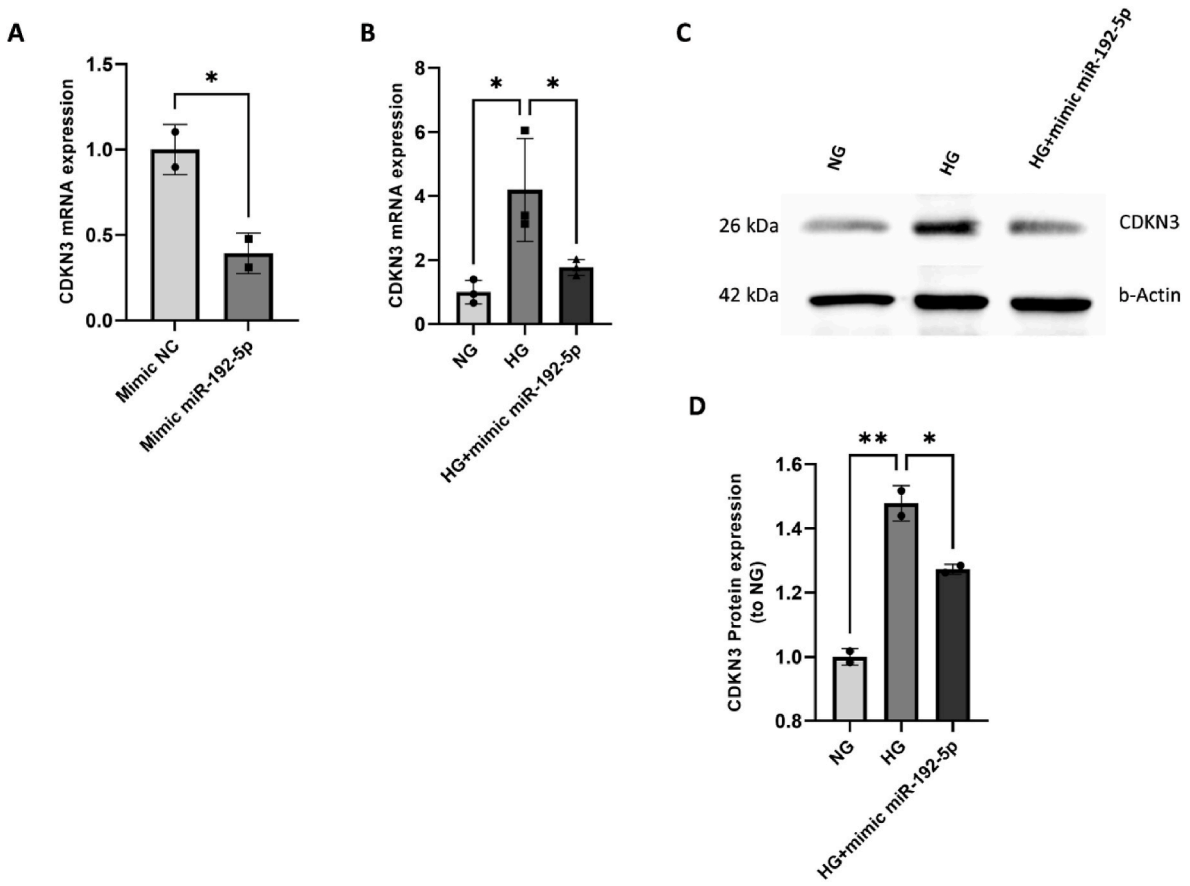


Fig. 8. miR-192–5p directly targets CDKN3, resulting in a decrease in its expression levels
Bar and scatter plots show CDKN3 mRNA expression (by qPCR) in the proximal tubular cells, (A) transfected with miR-192–5p mimic or control mimic (NC), or (B) cultured in normal glucose (NG), high glucose (HG) media or miR-192–5p mimic transfected cells cultured in HG media (HG + mimic miR-192–5p). (C) Representative immunoblot showing CDKN3 protein expression in the HK-2 cells of the three groups NG, HG, or HG + mimic miR-192–5p. Bar and scatter plots show (D) densitometric summary of the immunoblots (n = 2 p = 0.0022).*p < 0.05 by *t*-test. Each dot in the scatter plot represents average data from an individual experiment.*p < 0.05 one-way ANOVA followed by multiple comparison test by Tukey's multiple comparisons test.

intersection area of the two circles were commonly regulated in both DKD and disease controls relative to non-CKD controls. While 68 DKD-associated miRNAs fell outside the intersection/overlapping area were considered DKD-specific (Fig. 1A–B). Principal component (PCA) plot of miRNA profiles has been shown in Figure S1A & B. Heat maps were generated to show the hierarchical clustering of differentially expressed miRNAs in renal biopsies from cases and disease control, relative to non-CKD control (Figure S1 B & D). The Clinical and demographic parameters of the patients enrolled in the study are presented in Supplementary Table 1.

3.2. DKD specific miRNA-mRNA regulatory networks in humans

A total of 9951 predicted target genes were identified for the 68 DKD-specific miRNAs, miR-192–5p had the maximum number of predicted target genes (994 target genes), followed by hsa-let-7c-5p which had 516 target genes (Supplementary Table 2, Supplementary Figure 2). To construct DKD-specific miRNA-mRNA regulatory networks, target hub genes were selected from the total 9951 predicted target genes at two levels. At the first levels, the top 30 target genes for differentially regulated miRNAs with maximum interaction within the 9951 predicted target gene, identified through PPI network analysis, were selected (Supplementary Figure 3& Table 1). The selected hub genes were further filtered based on the relation of their expression levels in the Nephroseq database with the respective targeting miRNA levels, i.e., the genes showing inverse gene expression compared to their respective miRNA (Fig. 2). Further, the selected hub genes with validated expression were associated with kidney function impairment with the eGFR values in the NephroSeq database from the DKD patients. Sixteen (of 25) DKD-associated hub genes showed significant association with eGFR (Fig. 3). Accordingly, eleven miRNA-mRNA networks were constructed for human DKD pathogenesis (Fig. 4). Pathway analysis of the genes used in miRNA-mRNA networks generation indicated their role in AGE-RAGE signaling, relaxin signaling pathways, and cell cycle processes, including cellular senescence, p53 signaling pathw ay (Supplementary Figure 4 & 5).

3.3. Role miR-192–5p regulatory network in DKD pathogenesis

We validated miR-192–5p regulatory network through *in vitro* experiments as miR-192–5p had the maximum number of predicted mRNA target genes in our regulatory network (Fig. 4). Also, a robust 8.89-fold reduction in the expression of miR-192–5p was found in DKD biopsies relative to non-CKD controls in microarray data (Fig. 1B). The down-regulation of miR-192–5p fold expression of in renal biopsy samples of DKD patients was confirmed by qPCR (Fig. 5 A). Moreover, miR-192–5p was significantly lower in human proximal tubule cells, HK-2 cells exposed to high glucose (30 mM) for 48 h compared to cells cultured in normal glucose (5.5 mM) conditions (Fig. 5 B). We exposed HK-2 cells with 30 mM glucose to mimic diabetic conditions for in-vitro studies as described by others [29].

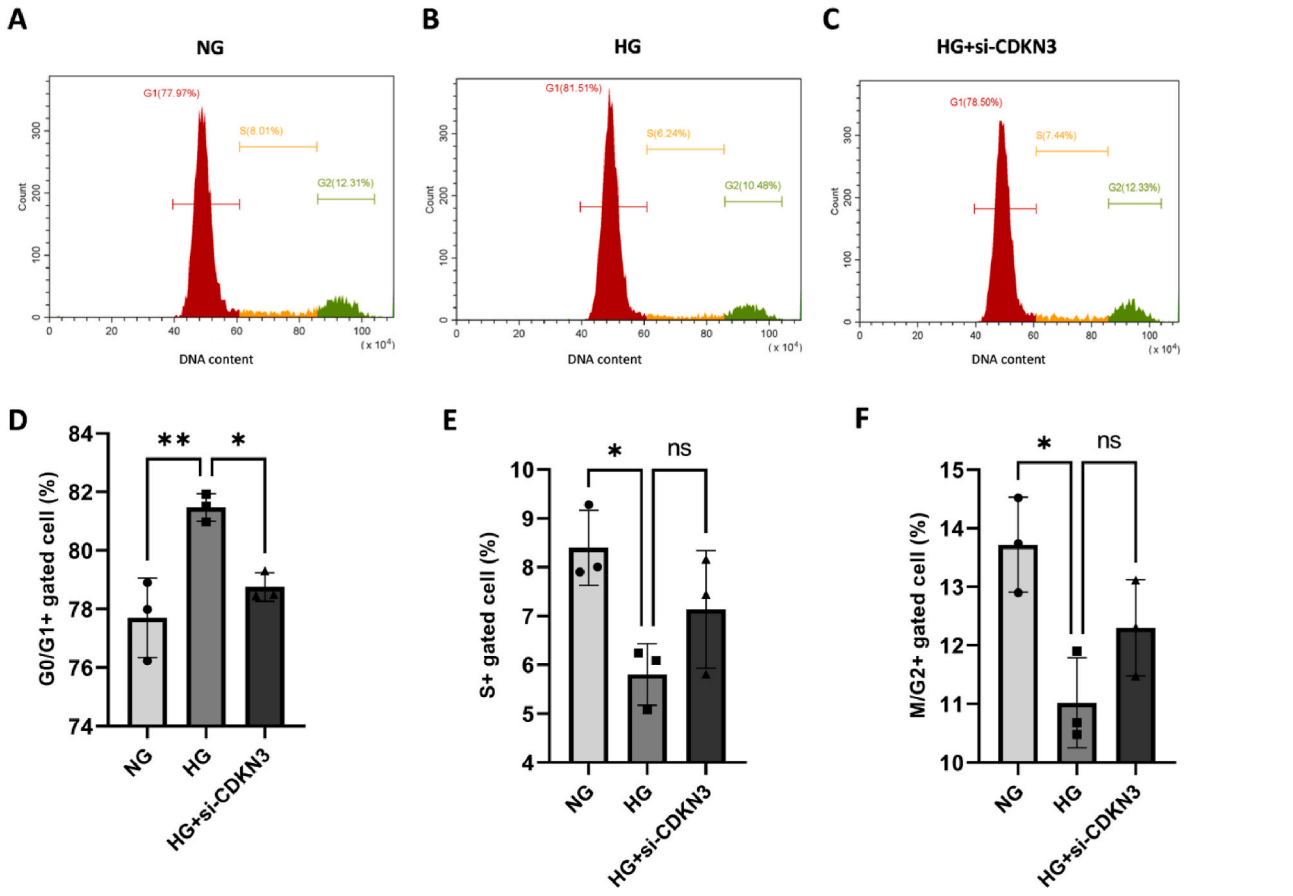


Fig. 9. CDKN3 knockdown prevented high-glucose induced cell cycle dysregulation in HK-2 cells
DNA Histograms showing proximal tubular cells are detected in the G0/G1, S, or M/G2 phase of the cell cycle after treatment (A) normal glucose (NG) or (B) high glucose (HG) media or (C) Cells transfected with si-CDKN3 cultured in HG media (HG + si-CDKN3) generated through Flow cytometric analysis. The bar and scatter plot shows the qualification of cells detected in the G0/G1 phase (D), S phase (E), or M/G2 phase (F) of the cell cycle. Each cell culture experiment was repeated three times. Each dot in the scatter plot represents data from an individual experiment.*$p < 0.05$ by one-way ANOVA followed by multiple comparison test by Tukey's multiple comparisons test.

3.4. miR-192–5p overexpression attenuated high-glucose induced cell hypertrophy and fibrotic gene expression in HK-2 cells

Hypertrophy of proximal tubular cells emerges as an early hallmark of diabetic renal involvement [30]. Treatment with high glucose led to an increase in the size of HK-2 cells compared to cells exposed to normal glucose conditions (Fig. 6A–B). However, transfection of HK-2 cells with miR-192–5p mimics attenuated high-glucose-induced cell hypertrophy (Fig. 6C–D). Similarly, overexpression of miR-192–5p attenuated the high glucose-induced upregulation of transforming growth factor beta (TGF-β) and collagen IV (Col IV) in HK-2 cells (Fig. 6E–F).

3.5. miR-192–5p overexpression or CDKN3 knockdown prevented high-glucose induced cell cycle dysregulation in HK-2 cells

Pathway analysis performed in this study indicated a role of 192–5p and its target genes in several DKD-associated pathways, for the miRNA-mRNA networks, indicated their role in various DKD-associated pathways, including cell cycle dysregulation, which is also commonly observed in DKD [31,32]. Moreover, prolonged exposure to high glucose has been shown to arrest proximal tubular cells in the G1 phase of the cell cycle, subsequently leading to cell hypertrophy [33]. Therefore, cell cycle analysis was carried out in HK-2 cells exposed to high glucose conditions. High glucose treatment induced cell cycle arrest in the G1 phase in HK-2 cells (Fig. 7A–B & D-E). Meanwhile, miR-192–5p transfected HK-2 cells attenuated high glucose-induced cell-cycle arrest in the G1 phase (Fig. 7C–D). Additionally, the number of cells treated with high glucose was reduced in the S and M/G2 phases.

The above findings suggest miR-192–5p as a major regulator of cell cycle progression, prompting us to delve deeper into the targets of miR-192–5p within the miRNA-mRNA regulatory networks identified for DKD pathogenesis (Fig. 4). CDKN3 (cyclin-dependent kinase inhibitor 3), a crucial participant in cell cycle regulation and proliferation, assumes pivotal roles through its interaction with cyclin proteins. This interaction triggers the dephosphorylation of CDK1 and CDK2 proteins, thereby halting the progression of the cell cycle [34–36]. We found that overexpression of miR-192–5p in HK-2 cells cultured in normal glucose condition led to a significant reduction in fold expression of CDKN3 (Fig. 8A). In comparison, the high glucose exposure resulted in significant upregulation in the expression of CDKN3, both the mRNA and protein levels (Fig. 8B–D). However, the overexpression of miR-192–5p was able to attenuate the high glucose-induced upregulation of CDKN3 in HK-2 cells (Fig. 8B–D). Thus, the observed effects of miR-192–5p may be mediated through CDKN3 regulation in HK-2 cells.

To confirm the role of CDKN3 in cell-cycle regulation in HK-2 cells, its expression was silenced using small interfering RNA (siRNA). Knockdown of CDKN3 resulted in a significant reduction in the proportion of cells in the G0/G1 phase compared to HK-2 cells without CDKN3 knockdown (Fig. 9). A significant decrease in CDKN3 levels in HK-2 cells treated with CDKN3 siRNA validated the successful knockdown (Supplementary Figure 6).

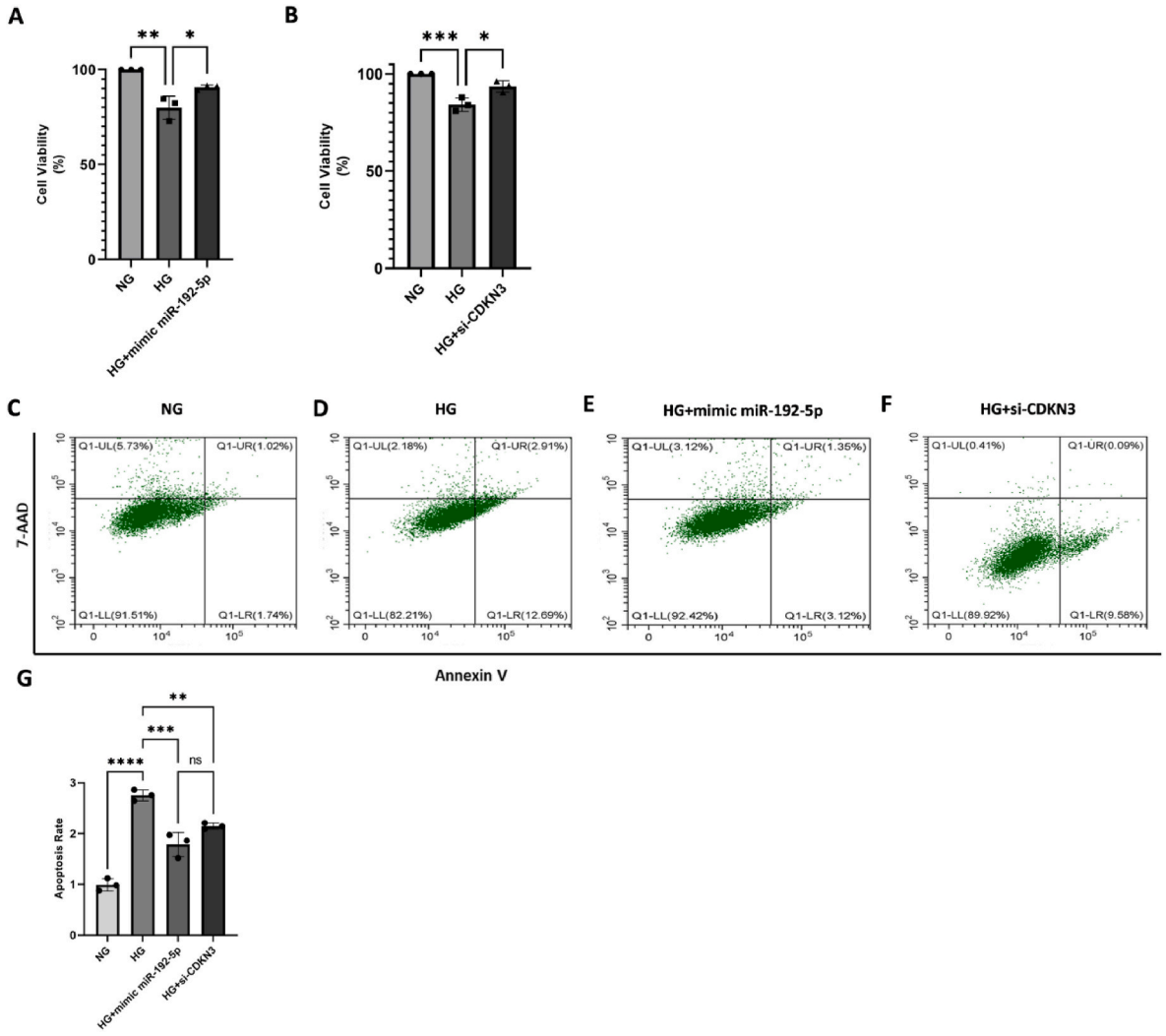


Fig. 10. miR-192–5p overexpression or CDKN3 knockdown improved cell viability, and attenuated apoptosis in high-glucose exposed HK-2 cells
Bar and scatter plots show the effect of (A) miR-192-5poverexpression or (B) si-CDKN3 treatment on cell viability. Figures (C–F) show representative images of scatter plots generated during flow cytometric analysis for apoptosis rate using 7-AAD/Annexin V assay. Cells were cultured in (C) normal glucose media (NG); (D) high glucose media (HG); (E) cells transfected with miR-192–5p mimic in HG media (HG + mimic miR-192–5p) or (F) si-CDKN3 treated cells in HG media (HG + si-CDKN3). (G) number of Annexin-positive cells. Each dot in the scatter plot represents average data from an individual experiment.).*$p < 0.05$ by one-way ANOVA followed by multiple comparisons by Tukey's multiple comparisons test.

3.6. miR-192–5p overexpression or CDKN3 knockdown improved cell viability, and attenuated apoptosis in high-glucose exposed HK-2 cells

Since miR-192–5p overexpression or CDKN3 knockdown was found to affect HK-2 cell cycle progression, which could have regulated HK-2 proliferation and apoptosis status. We next tested the effects of miR-192–5p overexpression or CDKN3 knockdown on high glucose-induced HK-2 proliferation and apoptosis status. The high glucose-induced reduction in HK2 cell viability was attenuated by miR-192–5p mimic transfection or si-CDKN3 treatment (Fig. 10A–B). The flow cytometry analysis to determine the apoptosis regulation indicated that HK-2 cells cultured in high glucose had a heightened apoptotic rate compared to those in normal glucose conditions. As anticipated, transfecting either with miR-192–5p mimic or CDKN3 siRNA mitigated this effect (Fig. 10C–G). These findings collectively suggested that high glucose downregulation of miR-192–5p could have triggered apoptosis in proximal tubule cells via CDKN3 induction.

4. Discussion

Kidney disease is prevalent in patients with diabetes miletus. Around 30–40 % of patients with type 2 Diabetes Miletus (T2DM) develop diabetic kidney disease (DKD), while 45–80 % of patients are diagnosed with non-diabetic kidney disease (NDKD) [1]. miRNA-mRNA regulatory networks in DKD pathogenesis could provide novel DKD-specific diagnostic and therapeutic targets [16–18]. In this study, we identified eleven miRNA–mRNA regulatory networks in human DKD pathogenesis. Certain miRNA-mRNA regulatory networks with potential role in kidney disease pathogenesis have been reported in a few studies [20,21,37]. However, in the majority of such studies, the networks are generated using bioinformatically predicted mRNA target genes [38,39]. Among the reports based on wet laboratory results, DKD kidney tissues were compared with healthy kidneys [40,41], lacking appropriate disease controls, which would be a non-DKD kidney disease in T2DM patients. In our study, the networks were generated using differentially expressed miRNA in renal biopsies of DKD patients relative to biopsies from patients with NDKD or without CKD. The predicted target hub genes used in the networks were thoroughly validated using DKD patients' data, available on the NephroSeq database. Accordingly, 11 DKD-specific networks included; miR-192-5p- CDKN3/BUB1B/CDC20/DTL/FBXO5/KIF20A/CEP55, miR-331-3p-CDKN3, miR-424-5p-CDK1/CEP55, miR-222-3p-KIF4A, miR-501-5p-CDC6, miR-125a-5p-PRC1,

miR-4646-5p-NCAPG2, miR-500a-3p-NCAPG2, miR-let-7c-5p-CCNB2, miR-101-3 P-CCND1/FOS/VEGFA and miR-6794-5p- CCND1 were identified.

The regulatory networks of miR-192–5p and its downregulation in the DKD kidneys were suggestive of their role in DKD pathogenic mechanisms, including dysregulation of the cell cycle, p53 signaling, and cellular senescence signaling pathways [42–47]. Downregulation of renal miR-192–5p in DKD was reported in humans and animals with DKD, though a biphasic regulation with disease progression was observed [48,49]. Nevertheless, in the case of human DKD, biopsies from disease controls (i.e., NDKD cases) were not considered, which is important to confirm the DKD-specific regulation of renal miR-192–5p [48]. Our study is the first to report DKD-specific regulation of renal miR-192–5p by including NDKD biopsy samples in addition to non-CKD control biopsies. Besides, we confirmed the DKD association of miR-192–5p network through our *in-vitro* experiments.

Our findings suggested a protective role of miR-192–5p in DKD, as overexpressing miR-192–5p attenuated high glucose-induced apoptosis, fibrotic gene expression, cell hypertrophy, and cell cycle dysregulation, and improved viability of proximal tubular cells. Proximal tubular hypertrophy stands out among the initial pathogenic events of DKD [50–52]. During the interphase of cellular growth, the G1 phase is distinguished from the S and G2 phases. Cells in the G1 phase of the cell cycle have several pathways available to them: they may (1) proceed to cell division, (2) undergo apoptosis, or (3) arrest at the G1/S transition, leading to an increase in cell size characterized by a rise in the protein: DNA ratio (hypertrophy) [30]. Cyclins, in conjunction with cyclin-dependent kinases, play a crucial role in positively regulating cell cycle progression. Notably, a high-glucose environment can impact the expression of p27Kip1, a cyclin-dependent kinase inhibitor responsible for halting cells in the G1 phase by inhibiting complexes formed by cyclin D-CDK4, cyclin D-CDK6, and cyclin E-CDK2 [33]. In this study, we introduce a novel mechanism of cell cycle disturbance in DKD progression/pathogenesis by miR-192–5p, which, by targeting CDKN3, regulates the transition of renal cell from the G1 to the S phase. CDKN3 is a critical player in cell cycle regulation and proliferation exerts pivotal functions through its interaction with cyclin proteins, leading to the dephosphorylation of CDK1 and CDK2 proteins and ultimately halting cell cycle progression. Moreover, the overexpression of miR-192–5p suppresses CDKN3, a vital contributor to cell cycle advancement [34–36]. Furthermore, our findings reveal that overexpression of miR-192–5p targets silenced CDKN3, thereby rescuing high-glucose-induced G1-phase arrest. This is the first report showing that CDKN3 is targeted by miR-192–5p in renal proximal tubule cells. Several studies reported that CDKN3 plays a role in cell proliferation and cell cycle in different cancer cells, including Esophageal Squamous Cell Carcinoma [53], prostate cancer [54].

Fibrosis represents a pivotal mechanism in diabetic kidney disease (DKD). The upregulation of pro-fibrotic gene expression was effectively mitigated by miR-192–5p overexpression. Consequently, we suggest that miR-192–5p plays a crucial role in the pro-fibrotic processes observed in HK-2 cells. Additionally, renal fibrosis is associated with heightened apoptosis of tubular cells [55]. Zhou et al. suggested that inhibiting apoptosis in renal tubular epithelial cells could be a promising strategy for anti-fibrosis treatment [56]. Our findings showed the potential of miR-192–5p to suppress high glucose-induced apoptosis in renal cells effectively.

Overall, this is the first report on DKD-specific miRNA-mRNA regulatory networks in renal biopsies of T2DM patients. Eleven miRNA-mRNA regulatory networks unique to DKD kidneys were identified in our study. Other miRNAs such as, miR-101 was upregulated in the diabetic kidney. A similar trend was found in the previous studies, which showed that inflammatory and immune processes are the most prominent pathways targeted by this miRNA [57]. The expression of miR-424–5p is lower in the diabetic kidney in our present study. Previous studies have shown that significantly lower expression of miR-424–5p was observed in the renal tissues of DN rats. Upregulation of miR-424–5p is involved in the inhibition of apoptosis in DN by targeting Caspase-3 and Bax [58]. Similarly, Li et al. also showed the downregulation of miR-424–5p in human renal mesangial cells. Restoring miR-424–5p alleviated high glucose-induced proliferation, inflammatory responses, and extracellular matrix accumulation in human renal mesangial cells via targeting WNT2B [59]. Additionally, our analysis suggested miR-205–5p could serve a reno-protective role in diabetic kidney disease. A study by Yun et al. verified that overexpressing miR-205–5p in high glucose-treated renal mesangial cells protects cell injury and cell dysfunction by directly targeting high-mobility group AThook 2 (HMGA2) [60]. While a few of the DKD-specific miRs, such as miR-331–3p, let-7c-5p, and miR-501–5p were studied in biofluids from DN patients, including serum, urine, or blood extracellular vesicles [61–63]. However, the miRNAs in these DKD networks have not been investigated in human renal biopsies to date. These networks may have a role in pathogenesis specific to DKD. Due to the limited number of biopsy samples, networks specific to NDKD subtypes in T2DM patients could not be identified. Nevertheless, the other DKD-specific networks identified in our study may be useful in disease management by providing specific therapeutic and prognostic targets for diabetic kidney disease. Further studies for direct targets and functional validation of the identified networks are warranted.

In conclusion, our results described herein reveal that miR-192-5p-CDKN3 is an important regulator of DKD progression. Our findings suggest hyperglycemia-induced downregulation of miR-192–5p in renal cells in DKD, which by relieving its suppressive effects on CDKN3 expression leads to cell cycle deregulation and subsequently promotes apoptosis and fibrosis.

CRediT authorship contribution statement

Biswajit Sahoo: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization, Validation. **Deendayal Das Mishra:** Methodology. **Swasti Tiwari:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Ethics approval and consent to participate

The study was approved by the institutional human ethics committee, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India (IEC Number 2018-139-EMP-106 and 2021-309-EMP-EXP-44), and Christian Medical College, Vellore, India (IEC number (CMC, IRB Min. No. I2243 (OTHER), dated September 25, 2019). Written informed consent was obtained from each participant.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study will be made available by the authors upon reasonable request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2024.11.003.

Abbreviations

T2DM	Diabetes mellitus
DN	Diabetic nephropathy
DKD	Diabetic kidney disease
NDKD	Non diabetic kidney disease
miR	micro RNA
mRNA	messenger RNA
GO	Gene Ontology
PCA	Principal component analysis
DEmiR	Differentially expressed miRNA

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