Journal of Translational Medicine

RESEARCH

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Bioinformatics analysis and experimental validation of potential targets and pathways in chronic kidney disease associated with renal fibrosis

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Abstract

Background Chronic kidney disease (CKD) has emerged as a major health problem worldwide. Previous studies have shown that specific miRNA expression profiles of patients with CKD are significantly changed. In this study, we aim to elucidate the role of miRNAs as potential biomarkers in CKD progression by integrating bioinformatics analysis with experimental validation, thereby providing medical evidence for the prevention and treatment of CKD.

Method Bioinformatics analysis was used to identify potential targets and pathways in CKD-associated renal fibrosis through randomly obtaining miRNA microarray data related to CKD patients in the Gene Expression Omnibus (GEO) database according to the inclusion and exclusion criteria, conducting pathway enrichment analysis and constructing protein-protein interaction (PPI) networks and miRNA-mRNA network by Cytoscape 3.8.0. In vitro experiments were employed to verify the role and mechanism of miR-223-3p in human renal tubular epithelial cells (HK2) through Quantitative real-time PCR assays, Western blot, Immunofluorescence analysis and Double luciferase reporter gene experiment. Multi-group one-way analysis of variance (ANOVA) and the Dunnett-t test were uesd to analyze the results by SPSS24.0.

Results 10 up-regulated and 11 down-regulated miRNAs of CKD patients were screened out. Phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) was the first pathway of pathway enrichment analysis. MiR-223-3p (logFC=-2.047, $p=0.002$) was one of the four hub miRNAs. Furthermore, we observed a reduction in α-smooth muscle actin (α-SMA) ($p=0.001$) and Collagen type I alpha 1 (Col1-a1) ($p=0.023$) levels upon miR-223-3p overexpression, which aligned with our bioinformatics predictions. This downregulation was attributed to the inhibition of nuclear factor kappa-B (NF-κB) nuclear translocation and subsequent decrease in the secretion of inflammatory cytokines, such as interleukin-6 (IL-6) ($p=0.005$). Conversely, when CHUK was further overexpressed, the inhibitory effect of miR-223-3p on epithelial-mesenchymal transition (EMT) was attenuated, confirming the specific interaction between miR-223-3p and CHUK.

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Conclusion Our findings provide compelling evidence that miR-223-3p acts as a suppressor of EMT in CKD by specifically targeting the CHUK and modulating the PI3K/Akt pathway, which holds great promise as a novel therapeutic target for CKD treatment. Additionally, this study offers a potential avenue for the development of future interventions aimed at halting or reversing the progression of CKD.

Keywords Chronic kidney disease, Bioinformatics analysis, miR-223-3p, Epithelial-mesenchymal transition, PI3K/Akt pathway

Introduction

Chronic Kidney Disease (CKD), one of the major health concerns worldwide [1], is caused by various factors, including diabetes, hypertension, obstructive nephropathy, infectious nephropathy, autoimmune nephropathy and some nephrotoxic drugs [2]. The prevalence rate of CKD is as high as 10–14% and causes heavy medical economic burden for both individuals and the society [3].

Renal fibrosis (RF) is the common pathological alterations and pathways leading to the progression of CKD towards end-stage renal disease (ESRD) [4]. The pathogenic mechanisms underlying RF mainly include haemodynamic alterations change, inflammation, epithelial-mesenchymal transition (EMT), endothelial-mesenchymal transition, oxidative stress, macrophage-to-myofibroblast transition (MMT) and energy metabolism changes [5–9], suggesting that RF is an extremely complex process [5]. However, the mechanism of RF in CKD has not been fully elucidated, and no effective preventive or therapeutic measures have been established [10]. Previous studies have shown that specific microRNA (miRNA) expression profiles of patients with CKD are significantly changed [11], which not only aids in identifying potential biomarkers of CKD biology and uncovering the pathogenesis of CKD, but also provides new possibilities for early diagnosis, prevention and treatment of CKD.

MiRNA, with about 22 nucleotides, is the most abundant and representative small non-coding RNAs and plays an important role in gene regulation and many biological processes [12]. Certain miRNAs in plasma and/or urine of CKD patients have been potential diagnostic biomarkers [13]. For instance, urinary miR-185-5p acts as a biomarker for RF in IgA nephropathy [14], plasma miR-186-5p functions as a biomarker for focal segmental glomerulosclerosis (FSGS) associated with nephrotic proteinuria [15], and plasma level of miR-130b serves as a biomarker for RF in diabetic nephropathy [16]. Additionally, the role of specific miRNAs in the pathogenesis of CKD has also been demonstrated, such as miR-34a promotes RF in CKD mice and EMT in human renal tubular epithelial cells (HK2) by targeting Klotho mRNA [17]. MiR-214-3p accelerates CKD-associated RF by destroying the oxidative phosphorylation of renal tubule mitochondria [18]. MiR-429-3p can inhibit branchchain amino acid catabolism, thereby promoting iron death in CKD [19]. MiR-30-3p targets binding protein phosphatase 3 catalytic subunit alpha (ppp3ca) and nuclear factor 5 of activated T-cells (NFAT5) c3 to inhibit the activation of calcineurin signaling pathway, thereby inhibiting the occurrence of cardiac hypertrophy, which is a cardiovascular complication of CKD [20]. All of which indicate that microRNAs are useful in the onset and progression of CKD. In this regard, revealing all dysregulated miRNAs in the kidneys of CKD patients and deeply exploring the relationship between specific miRNAs and the complex mechanism of CKD-associated RF are urgent matters for us to solve.

Gene expression profiling and bioinformatics analysis have become increasingly important tools for yielding mechanistic insights of various diseases [21]. In addition to being used to screen differentially expressed genes (DEGs) and identifying the interactions of DEGs via the in-depth study of Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, bioinformatics analysis can also be used to select hub genes and explore the relationship between gene expression and disease phenotypes [22]. However, to understand the complex interactions between miRNAs and the genome comprehensively, bioinformatics analysis must be supplemented by validation experiments [23].

To elucidate the underlying mechanism of CKD-associated RF, this study employs a combination of bioinformatics analysis and validation experiments to identify and analyze the key microRNAs, mRNAs and pathways of patients with CKD. Moreover, in vitro experiments are conducted to validate previously uninvestigated targets and pathways. The fundings of this study will provide an experimental foundation and scientific evidence for utilizing miR-223-3p as a new potential target for the diagnosis and treatment of CKD, the experimental flow of this study is shown in Fig. 1.

Methods

Bioinformatics analysis

Obtaining microRNA microarray data of CKD

The Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) was utilized to obtain microRNA microarray data associated with CKD using the keyword "chronic kidney disease", "Homo sapiens", "microRNA" and "renal tissue" [24]. GSE80247 was

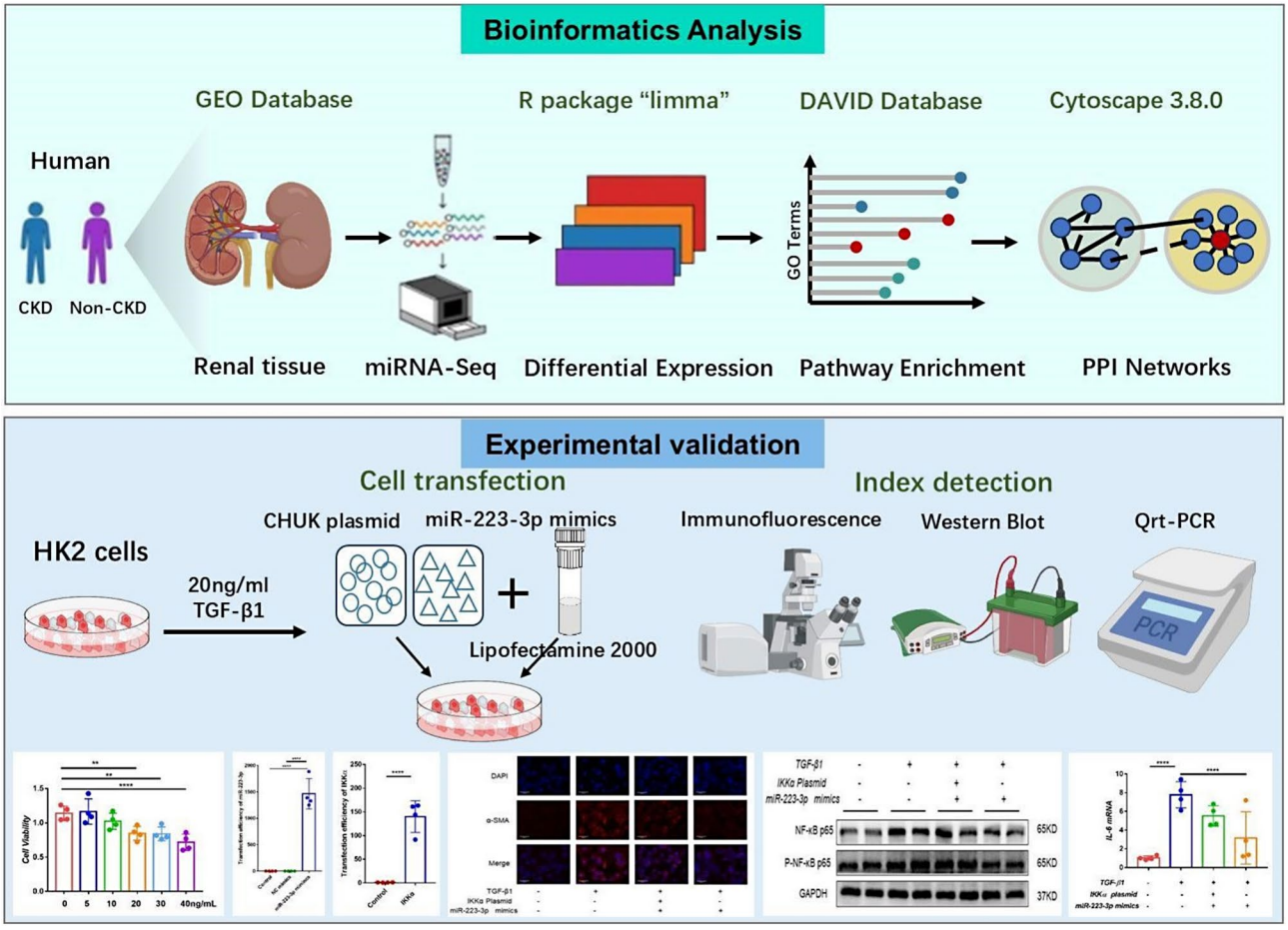

Fig. 1 The experimental flow

chosen for further analysis. GSE80247 is based on the GPL17537 platform (nCounter Human miRNA Expression Assay, V2), subjects in GSE80247 were patients developing CKD after radical nephrectomy (RN) for renal cancer (RCC) and patients who did not develop CKD. The inclusion criteria of the subjects were: (a) age > 18 and < 80 years; (b) clear-cell carcinoma (RCC) histology diagnosis; (c) resectable RCC and absence of metastatic within the initial 12 months from diagnosis; (d) no history of other malignancies; (e) normal renal function prior to radical nephrectomy (RN); (f) absence of primitive, secondary, hereditary or acquired glomerulopathies; (g) absence of kidney stone disease, myeloproliferative disorders and autoimmune diseases; (h) absence of chronic nephrotoxic drug therapy [25]. The exclusion criterion of this study was that datasets with incomplete data, and seven CKD patients and seven patients without CKD were included for further study.

Screening of differentially expressed miRNAs

R (version 4.1.2) was utilized to analyze the GSE80247 raw expression data, such as standardization, correction and gene name annotation. The R package "factoextra" was used for principal component analysis (PCA) [26]. The R package "limma", obtained from Bioconductor (https://www.bioconductor.org) [27], was used to an alyze differentially expressed microRNAs between CKD patients and patients without CKD. The up-regulated and down-regulated gene expression threshold were |log2 fold change| (log2FC) > 0.5 and $p < 0.05$.

Construction of the protein-protein interaction (PPI) network and screening of the core genes

Target genes of differentially expressed miRNAs were predicted based on the TargetScan (https://www.targetscan.org) [28], miRDB (https://mirdb.org) [29], and miRtarbase (https://mirtarbase.cuhk.edu.cn) [30] dat abases. Target genes, which were predicted by two of above three databases simultaneously, were then input into STRING (https://cn.string-db.org/) to generate PPI network with the limitation of organisms to homo sapiens [31]. Subsequently, the resulting PPI network was visualized using the Cytoscape software (version 3.8.0) [32]. The Cytoscape software can calculate multiple

attribute values, such as degree (DC), closeness centrality (CC) and betweenness centrality (BC), which reflect the significance of genes. Molecular Complex Detection (MCODE) plug-in in Cytoscape was used to analyze the cluster properties, and the analysis parameters were set as follows: degree cutoff = 2, node score cutoff = 0.2, K-core = 2, and max depth = 100 [33]. CytoHubba app was used to identify the top 30 nodes by several topological algorithms, such as Degree, Maximal Clique Centrality (MCC), Edge Percolated Component (EPC) and Clustering Coefficient [34].

Integration of the miRNA-mRNA regulatory network and screening of the hub miRNAs

Based on the target genes regulated by each miRNA and hub genes selected by PPI network, Venn analysis was used to choose hub genes regulated by each differentially expressed miRNA. Then the miRNA-mRNA regulatory network was visualized by the Cytoscape software (version 3.8.0). MiRNAs who regulated the largest number of hub genes were considered as hub miRNAs.

GO analysis and KEGG pathway enrichment

Target genes of downregulated miRNAs were subjected to GO analysis and KEGG pathway enrichment using Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.abcc.ncifcrf.gov/) with the restriction of "OFFICIAL_GENE_SYMBOL" and "homo sapiens". DAVID is a gene functional annotation tool that is helpful for understanding functional relationships between genes [35]. The criterion for determining the importance of the pathways was that under the condition of $p<0.05$, the greater the enrichment, the more important the pathway. The bioinformatics platform (http://www.bioinformatics.com.cn/) were used to generate statistical graphs for results of cellular component (CC), molecular function (MF), biological process (BP), and KEGG pathway.

In vitro experiment

Cell culture

Human renal tubular epithelial cells (HK2), purchased from Cell Bank of Chinese Academy of Sciences, were cultured in HK-2 cell specific culture medium (Pricella Life Technology Co., LTD) at 37℃ in a 5% CO_2 humidified atmosphere. HK-2 cell specific culture medium is consist of MEM medium, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The EMT of HK2 cell was induced by transforming growth factor-β1 (TGF-β1) (Sino Biological, Beijing, China). Lipofectamine 2000 (Invitrogen, USA) was used to transfect miR-223-3p mimics, NC mimics and CHUK plasmid, whose protein name is inhibitor of kappa B kinase (IKKα). The miR-223-3p mimics, NC mimics and CHUK plasmid were synthesized by Shanghai Shenggong Company, China.

6–10 generations of HK2 cells were seeded into 6-well plates (about 1×10^5 cells each well). After cells attached to the wall, they were divided into 4 groups: the control group, the model group (20ng/ml TGF-β1), the miR-223-3p overexpression group (20ng/ml TGF-β1 + 15pmol/l miR-223-3p mimics) and the miR-223-3p overexpression + CHUK overexpression group (20ng/ml TGF-β1 + 15pmol/l miR-223-3p mimics + 1.25 μg/ml CHUK plasmid). 24 h later, cells were collected for indicator detection. The concentration of TGF-β1, miR-223-3p mimics and CHUK plasmid were determined by Cell Counting Kit-8 (CCK8) or Quantitative real-time PCR (qRT-PCR) assay.

CCK8

The cell suspension was seeded into the 96-well plate (about 1×10^4 cells each well) and cultured in the incubator for 24 h. After the cells were attached to the wall, medium containing different concentrations of TGF-β1 (0ng/ml, 5ng/ml, 10ng/ml, 20ng/ml, 30ng/ml, 40ng/ml) was added to interfere with the cells. 24 h later, 10 μl CCK8 solution (KGA317, Nanjing, China) was added to each hole and incubated at 37℃ for 1 h, then the absorbance was detected at 450 nm using an enzyme-labeled instrument (Thermo Fisher, USA).

qRT-PCR

The mRNA levels of miR-223-3p, inhibitor of kappa B kinase (IKKα), alpha-smooth muscle actin (α-SMA), collagen type I alpha 1 (Col1-a1), tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and monocyte chemotactic protein-1(MCP-1) were detected by qRT-PCR. Total RNA was extracted from HK2 cells by RNA extraction kit (TIANGEN Biochemical Technology Co., LTD., Beijing, China, DP220706), and cDNA was generated by reverse transcription kit (Takara, Kusatsu, Japan, RR036A/RR037A). qRT-PCR was performed with TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Kusatsu, Japan, RR820A) on the real-time PCR system (Bio-Rad, USA). The sequence of qRT-PCR primers are shown in Table 1. The primers were synthesized by Shanghai Shenggong Company, China. The target mRNA level was normalized to GAPDH mRNA and the expression level of microRNA was normalized to U6.

Western blot (WB)

Total protein was extracted from HK2 cells with the total protein extraction kit (KGP250, Nanjing, China), and the concentration of protein was determined by the BCA protein assay kit (KGPBCA, Nanjing, China). The protein samples (20 μg per lane) were separated by the sodium

Table 1 Primers of qRT-PCR for human

Gene	Forward Primers (5′-3′)	Reverse Primers (5′-3′)
CHUK	CAGGGACAAAGGGCAGCAATG	CCAATGACACCAACCTCAGCATAG
α-SMA	CTTGGCTTGTCAGGGCTTGTCC	TGCGGGAGGCGTTGGAGAC
Col1-a1	TGACGAGACCAAGAACTGCC	GGGAGCAAAGTTTCCTCCGA
MCP-1	AGTCTCTGCCGCCCTTCTGTG	GGGTTTGCTTGTCCAGGTGGTC
TNF-α	AGCCGCATCGCCGTCTCC	TCGTCCTCCTCACAGGGCAATG
IL-6	CCAGAGCTGTGCAGATGAGT	GTGCCCATGCTACATTTGCC
miR-223-3p	GCGCGTGTCAGTTTGTCAAAT	ATCCAGTGCAGGGTCCGAGG
RT-Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGGGT	

dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene fluoride (PVDF) membrane, then the membrane was sealed with a rapid sealing solution (CEV in Innovative Biotechnology Co., LTD., Beijing, China, SW162-01) for 30 min, and incubated with the primary antibody at 4℃ overnight. IKKα, inhibitor kappa B alpha (IKB-α), p-IKB-α, phosphorylated nuclear factor kappa-B p65 (p-NF-κB p65), Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain- containing 3 (NLRP3) (1:1000, Abcam, Cambridge, UK), α-SMA (1:10000, Abcam, Cambridge, UK), NF-κBp65, Caspase-1 (1:1000, Wuhan Sanying Biotechnology Co., LTD., Wuhan, China), β-Actin (1:4000, Abcam, Cambridge, UK) and GAPDH (1:10000, Abcam, Cambridge, UK). After the membrane being washed in PBST for 3 × 5 min, secondary antibody was added (1:4000, Zhongshan Jinqiao Biotechnology Co., LTD., Beijing, China) and incubated at the room temperature for 1 h. The protein chemiluminescence was obtained by a chemiluminescence detector and signal capture by the bioanalytical imaging system.

Immunofluorescence analysis

HK2 cells were seeded in a confocal dish for 24 h. After the cells being washed in PBS for 3 × 5 min, 4% paraformaldehyde was used to fix cells for 20 min, 0.1% Triton X to permeate for 15 min and 3%BSA to seal antigen for 1 h at room temperature. Then the HK2 cells were incubated with the primary antibody at 4℃ overnight, α-SMA(1:500, Abcam, Cambridge, UK) and NF-κB p65 (1:100, Wuhan Sanying Biotechnology Co., LTD., Wuhan, China). After the cells being washed in PBST for 3 × 5 min, secondary antibody was added (1:500, Aibixin Biotechnology Co., LTD., Shanghai, China) at room temperature for 30 min. Finally, the cells were covered with the DAPI solution (Soleibao Technology Co., LTD., Beijing, China) for 15 min. A laser confocal scanning microscope was used to observe as soon as possible.

Double luciferase reporter gene system experiment

Wild type and mutant double luciferase reporter gene vectors were constructed by Shanghai Shenggong Company in China according to the binding sites of miR-223-3p and CHUK, which were predicted in TargetScan. Then we divided HK2 cells into 4 groups: the wide type vector + miR-223-3p mimics group, and the wide type vector + NC mimics group, the mutant type vector + miR-223-3p mimics group and the mutant type vector + NC mimics group. In the end, we prepared reaction solution using double luciferase reporter gene kit (MedChemexpress, USA), and detected luciferase activity by an enzyme-labeled instrument (Thermo Fisher, USA).

Statistical analysis

R (version 4.1.2) was utilized to analyze the GSE80247 raw expression data. |log2 fold change| (log2FC) > 0.5 and $p < 0.05$ were used to screen differentially expressed microRNAs. SPSS24.0 software (Chicago, IL, USA) was used to analyze the results of in vitro experiments. The Bartlett test was used to test the homogeneity of variance of the measurement data in vitro experiment. Multi-group one-way analysis of variance (ANOVA) was used to analyze the results with homogeneity of variance, and the Dunnett-t was used for the comparison between the control group and model group, the model and miR-223-3p overexpression group, and the model and miR-223-3p overexpression group + CHUK overexpression group. Kruskal-Wallis H test was uesd to analyze the results without homogeneity of variance. All data were presented as mean ± standard deviation (SD), $p < 0.05$ was considered statistically significant. Image J was used for quantitative analysis of protein band. GraphPad prism 9.3 software and the bioinformatics platform were used for mapping.

Results

Differentially expressed microRNAs and the heatmap

The result of principal component analysis was shown in Fig. 2A. Based on the analysis of GSE80247 by R, 21 differentially expressed miRNAs were discovered in CKD patients, among which there were 10 up-regulated miRNAs: miR-30a-5p, miR-30e-5p, miR-29a-3p, miR-16-5p, miR-429, miR-532-5p, miR-497-5p, miR-125a-3p, miR-501-3p and miR-548a, and 11 down-regulated miRNAs: miR-132-3p, miR-135b-5p, miR-28-5p, miR-361-3p,

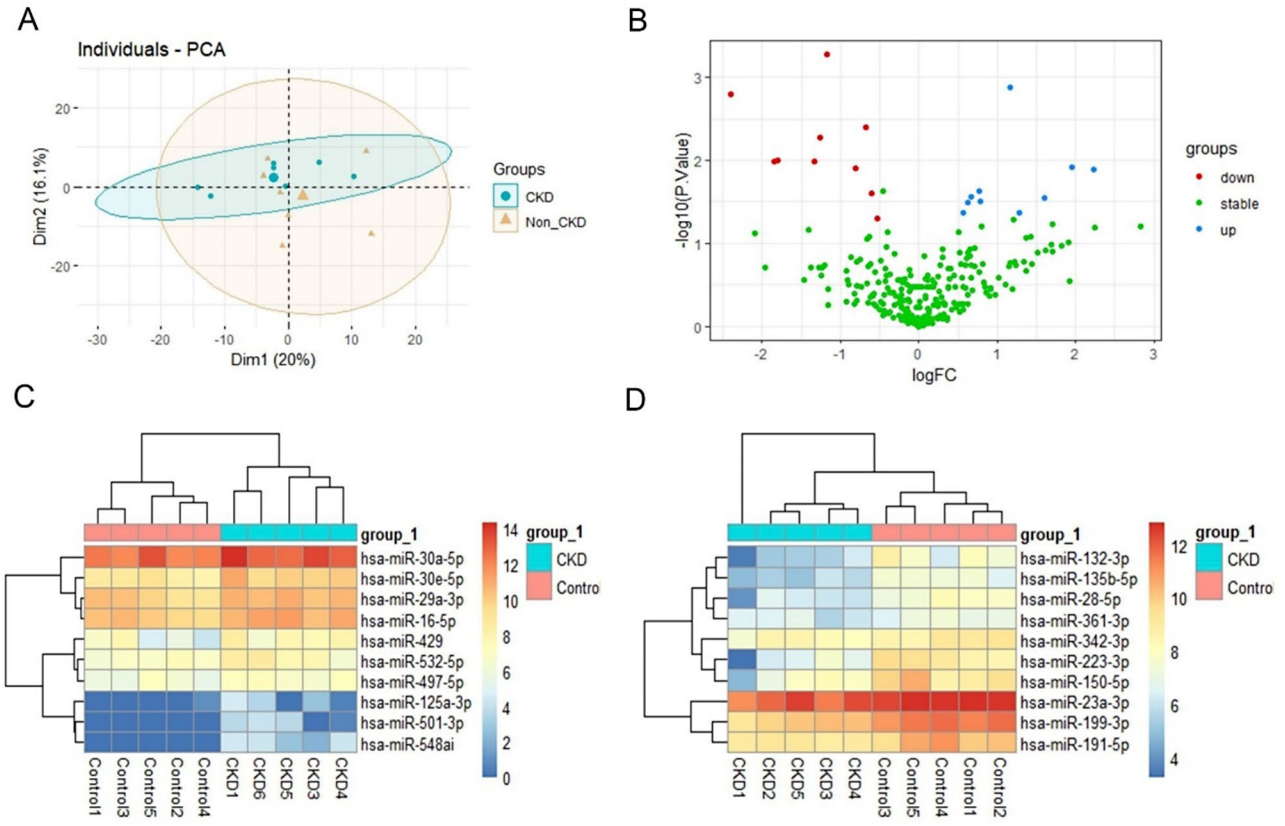

Fig. 2 Differentially expressed miRNAs in chronic kidney disease. (**A**) Principal component analysis. (**B**)Volcano map. (**C**) Heatmap of up-regulated miRNAs. (**D**) Heatmap of down-regulated miRNAs

miR-342-3p, miR-223-3p, miR-150-5p, miR-23a-3p, miR-199-3p (miR-199a-3p, miR-199b-3p) and miR-191-5p (Fig. 2B and D).

GO function and KEGG pathway enrichment analysis

To further investigate the main function and mechanism of down-regulated miRNAs in CKD, 332 common target genes of all down-regulated miRNAs except miR-135b-5p were imported into DAVID to conduct GO analysis and KEGG pathway enrichment, of which 7 target genes were not identified by DAVID (supplementary material 1). 143 items of BP, 28 items of CC, 48 items of MF and 79 KEGG pathways were identified using screening criterion of pvalue < 0.05 (supplementary material 2–5). MiR-135b-5p was not found in the TargetScan database.

In terms of KEGG pathway enrichment analysis, except pathways related to human diseases and cellular processes system, phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway (Enrichment: 8.31%), mitogen-activated protein kinase (MAPK) signaling pathway (Enrichment: 7.38%), and renin angiotensin system (Ras) signaling pathway (Enrichment: 4.92%) ranked the top 3 (Fig. 3A and C). Additionally, Wnt signaling pathway (Enrichment: 3.38%), NF-kappa B signaling pathway (Enrichment: 1.87%) and TGF-β signaling pathway (Enrichment: 1.85%) also played important roles. The disease-pathway (the top 20)-target network was construct by Cytoscape software (Fig. 3D).

The top 3 terms related to BP were positive regulation of transcription by RNA polymerase II (Enrichment: 18.46%), negative regulation of transcription by RNA polymerase II (Enrichment: 15.69%) and regulation of transcription by RNA polymerase II (Enrichment: 18.46%). The top 3 MF terms were protein binding (Enrichment: 82.77%), DNA-binding transcription factor activity (Enrichment: 9.85%) and RNA polymerase II cis-regulatory region sequence-specific DNA binding (Enrichment: 15.38%). The top 3 CC terms were nucleoplasm (Enrichment: 37.85%), nucleus (Enrichment: 48.31%) and chromatin (Enrichment: 15.69%) (Fig. 3B).

PPI network analysis

To futher explore the interactions of target genes and identify the core genes in CKD, we imported 332 common genes into STRING and generated a PPI network containing 272 nodes and 1187 edges (Fig. 4A). Then, we set Degree ≥ 10 as the screening condition and acquired a relatively central PPI network comprising 77 nodes and 641 edges (Fig. 4B). Last, we acquired a central PPI network comprising 34 nodes and 318 edges with the

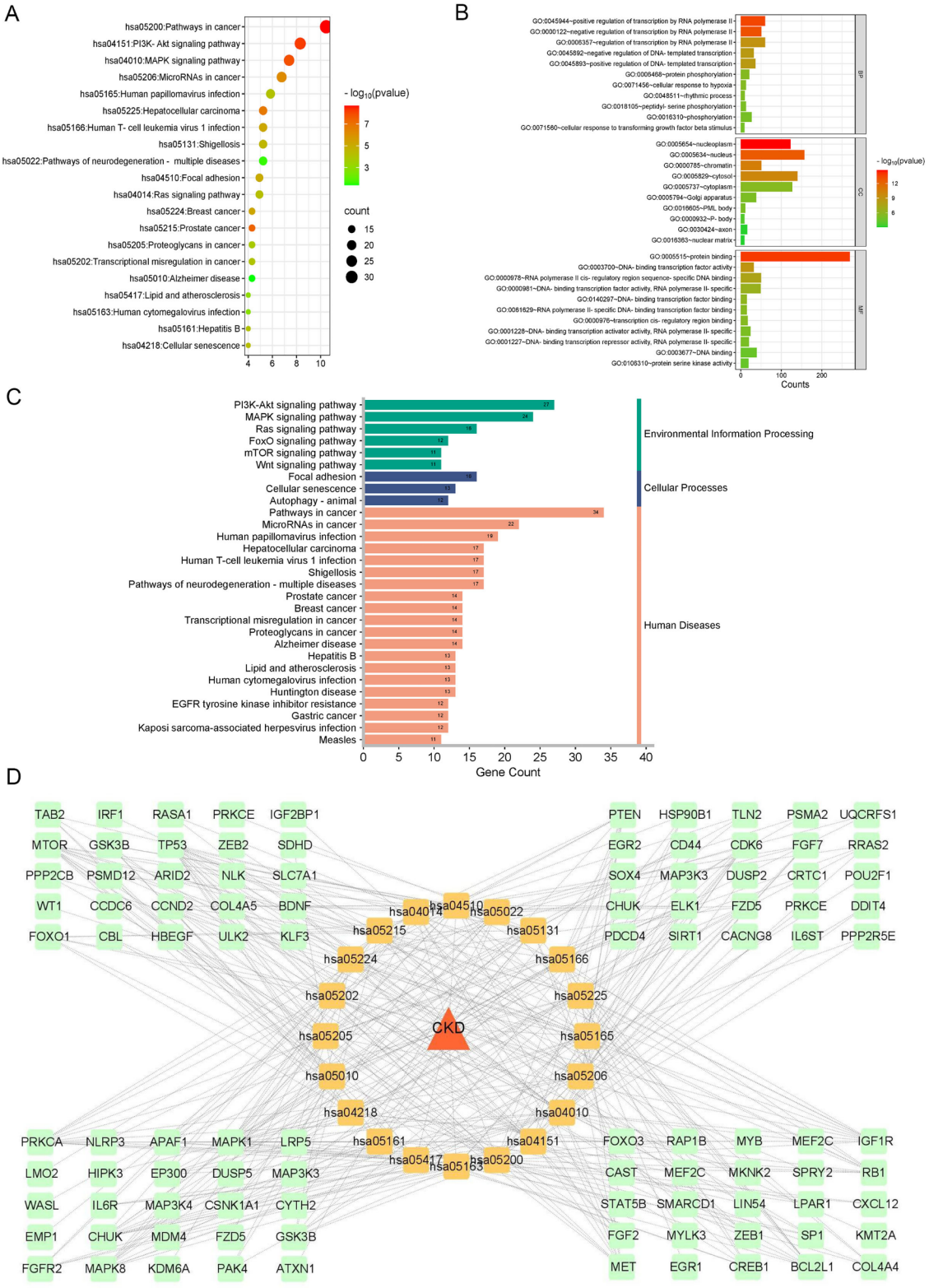

Fig. 3 Function and pathological mechanism of down-regulated microRNAs in chronic kidney disease. (**A**) Bubble plot of KEGG pathway enrichment. (**B**) Bar chart of GO function analysis. (**C**) Results of KEGG pathway enrichment (different colors represent different systems of action). (**D**) Disease- Pathway (the top 20) - Target network

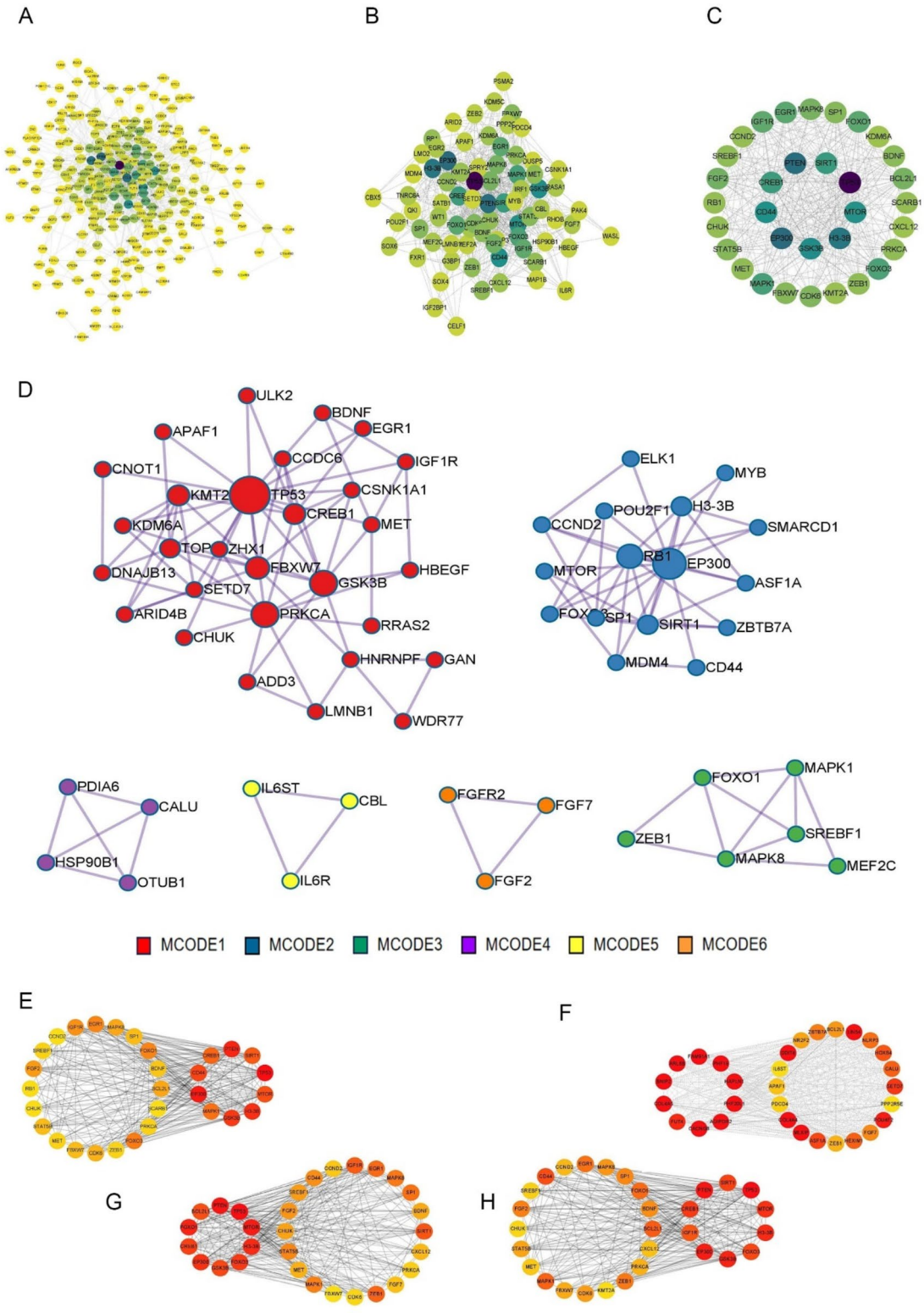

Fig. 4 PPI network analysis and screening of the core genes. (**A**) PPI network of all target genes of down-regulated miRNAs. (**B**) PPI network of target genes whose Degree value ≥ 10. (**C**) PPI network of target genes whose Degree value ≥ 20. (**D**) Cluster analysis by MCODE in Cytoscape Software. The top 30 core genes selected by Degree (**E**), Clustering Coefficient (**F**), Maximal Clique Centrality (MCC) (**G**), Edge Percolated Component (EPC) (**H**) algorithm in Cytohubba of Cytoscape Software

screening condition of Degree ≥ 20 (Fig. 4C and supplementary material 6). Cluster analysis was performed using the MCODE application, and all target genes were divided into 6 clusters (Fig. 4D). In addition, the Degree, MCC, EPC and Clustering Coefficient algorithm in CytoHubba app were used to discovered the top 30 core genes (Fig. 4E and H). These results revealed that PTEN, SIRT1, TP53, GSK3B, MTOR, CREB1, HB-3B, CHUK, et al. were the core genes in regulating the progression of CKD.

miRNA-mRNA network analysis

The regulatory relationship between down-regulated miRNAs and core genes were imported into Cytoscape software and obtained the miRNA-mRNA network (Fig. 5A). MiR-223-3p regulated the largest number of core genes: IGF-1R, SP1, FOXO1, SCARB1, CHUK, FBXW7, TP53, FOXO3, CDK6 and PTEN (Fig. 5B). MiR-199a-3p regulated 8 core genes: TP53, MET, FGF2, SP1, CD44, MAPK1, MAPK8 and MTOR. MiR-132-3p and miR-150-5p regulated 7 core genes respectively. So, we chose miR-223-3p, miR-199a-3p miR-132-3p and miR-150-5p as hub miRNAs (Fig. 5B). As shown in Fig. 5C, miR-223-3p may regulate PI3K/Akt pathway through targeting CHUK, PTEN, FOXO3, TP53, IGF1R.

Mir-223-3p inhibited EMT of HK2 cells

As shown in Fig. 6A and C, with the increase of TGF-β1 concentration, cell viability and the level of miR-223-3p decreased gradually, while the expression of IKKα mRNA increased little by little. Compared with the control group, 20ng/ml TGF-β1 lowered cell viability and the expression level of miR-223-3p with significant statistical differences (P<0.05). So, we induced EMT of HK2 cells by 20ng/ml TGF-β1.

24 h after HK2 cells were transfected with 15 pmol/ml miR-223-3p mimics, the level of miR-223-3p in HK2 cells elevated by 1259.76 times (Fig. 6D). IKKα mRNA level in HK2 cells increased by 152.89 times after being transfected with 1.25 μg/ml CHUK plasmid (Fig. 6E). The basis for selection of transfection concentrations were shown in Supplymentory material 7. After 20ng/ml TGF-β1 stimulation for 24 h, expression levels of α-SMA protein and mRNA in HK2 cells were significantly increased (P<0.01). Overexpressing miR-223-3p, the level of α-SMA protein reduced by 11.04% and α-SMA mRNA droped to 17.76% compared with the model group. However, when overexpressing miR-223-3p and CHUK at the same time, the inhibitory effect of miR-223-3p on α-SMA expression was weakened (Fig. 6F, H and J). In addition, overexpression of miR-223-3p inhibited Col1-a1 mRNA level significantly (P<0.05) (Fig. 6I).

Mir-223-3p alleviated the inflammatory response of HK2 cells

As shown in Fig. 7A and C, after the stimulation of TGF-β1, the mRNA expression levels of inflammatory factors MCP-1, TNF-α and IL-6 in HK2 cells increased 8.24, 11.17 and 6.64 times, respectively. However, overexpression of miR-223-3p lowered levels of MCP-1, TNF-α and IL-6 mRNA by 74.36%, 83.78% and 59.39%. When overexpressing miR-223-3p and IKKα at the same time, the mRNA levels of MCP-1, TNF-α and IL-6 decreased, but the differences were not statistically significant (P>0.05).

Mir-223-3p targeted IKKα mRNA and inhibited the NF-κB signaling pathway

Transfection of miR-223-3p mimics improved the expression level of miR-223-3p significantly (P<0.01), furtherly overexpressing CHUK, miR-223-3p level was decreased (Fig. 8A). In addition, overexpression of miR-223-3p reduced the expression level of IKKα protein by 34.02% (Fig. 8B and C), which suggested that miR-223-3p may target IKKα mRNA. TGF-β1 stimulation enhanced the expression levels of NF-κB p65, p-NF-κBp65, p-IKB-α, IKB-α, NLRP3 and Caspase-1 proteins in HK2 cells, while overexpression of miR-223-3p reduced levels of NF-κB p65, p-NF-κB p65, p-IKBα, IKBα, NLRP3 and Caspase-1 by 33.37%, 18.40%, 24.05%, 58.01%, 40.80% and 29.73%, respectively. On the contrary, overexpression of miR-223-3p and CHUK simultaneously reversed the downward trend of NF-κB p65, p-NF-κB p65, p-IKBα, IKBα, NLRP3 and Caspase-1 (Fig. 8D and L). Immunofluorescence results showed that the expression level and nuclear transfer of NF-κB p65 were declined after overexpression of miR-223-3p (Fig. 8M). In addition, the binding site of miR-223-3p and CHUK was predicted by Targetscan (Fig. 8N), and the results of double luciferase reporter gene experiment showed that the relative activity of luciferase decreased significantly (P<0.01) after co-transfection of miR-223-3p mimics and wild-type CHUK vector, while co-transfected miR-223-3p mimics and mutant CHUK vectors showed no significant effect on the relative activity of luciferase (P>0.05) (Fig. 8O), indicating that miR-223-3p could target IKKα mRNA to inhibit downstream NF-κB signaling pathway and suppress the EMT of HK2 cells.

Discussion

CKD is the most prevalent chronic disease in nephrology department all over the world. Patients with CKD often face high risks of many adverse outcomes, including cardiovascular events, requirements for dialysis or kidney transplantation and even death [36]. However, such a widespread and serious disease has no effective treatment in clinical practice [10]. Therefore, it is essential to identify potential biomarkers involved in biological pathways

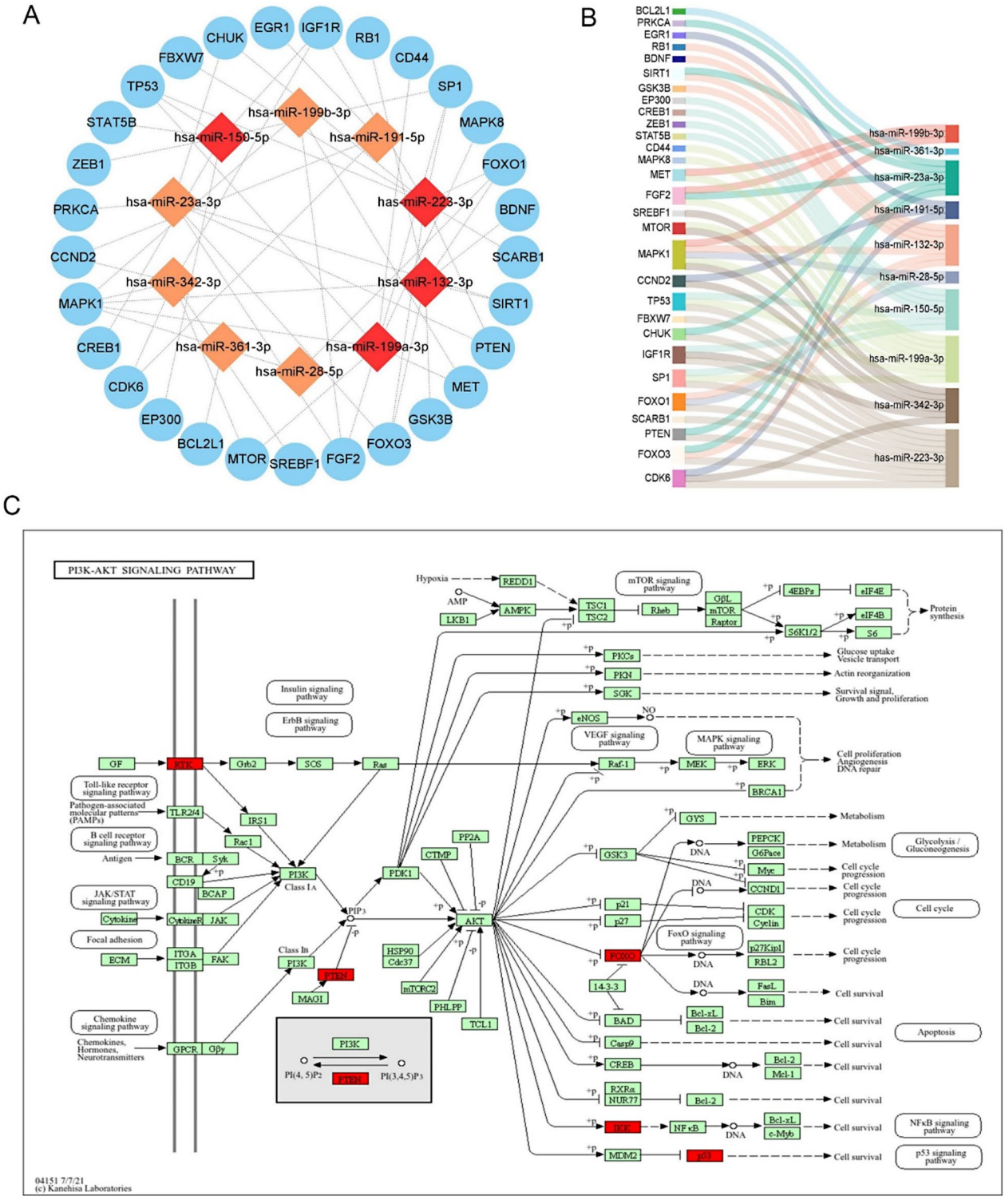

Fig. 5 miRNA-mRNA regulatory network analysis and screening of the hub miRNAs. (**A**) miRNA-mRNA regulatory network of down-regulated miRNAs and core genes selected by PPI network. (**B**) The contribution of each down-regulated miRNA. (**C**) PI3K/Akt signaling pathway (red represent target genes which are regulated by miR-223-3p)

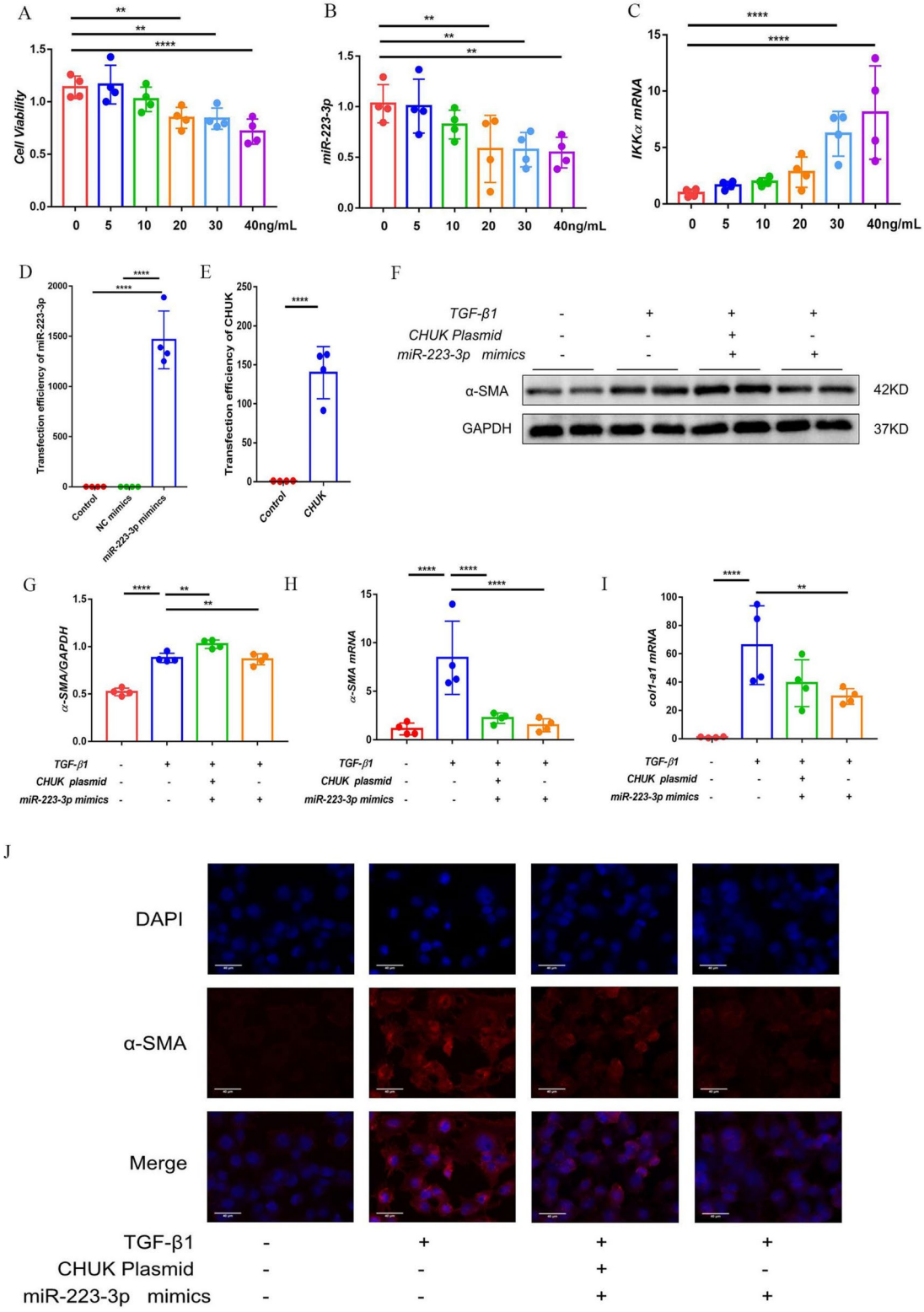

Fig. 6 miR-223-3p inhibited EMT of HK2 cells. (**A**) Cell activity. (**B**) The expression of miR-223-3p. (**C**) The expression of IKKα mRNA. (**D**) Transfection efficiency of miR-223-3p. (**E**) Transfection efficiency of CHUK. (**F**-**G**) Western blot images and quantitative band density analyses of α-SMA in HK2 cells. (**H**-**I**) mRNA expression of α-SMA and Col-1a in HK2 cells. (**J**) Immunofluorescence staining of α-SMA in the HK2 cells (at 400×magnification). $^{**}P < 0.05$ vs. Group (TGF-β1+, miR-223-3p-, CHUK plasmid-). **** $P < 0.01$ vs. Group (TGF-β1+, miR-223-3p-, CHUK plasmid-)

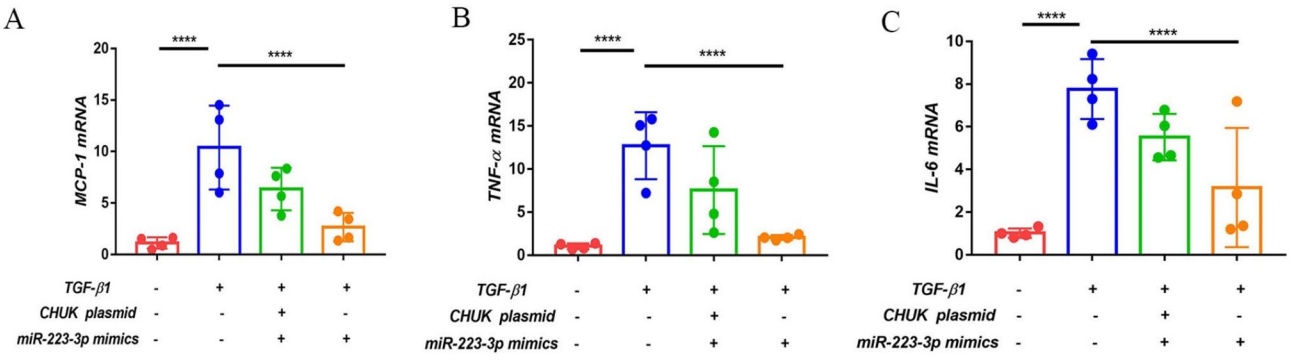

Fig. 7 miR-223-3p inhibited inflammatory response of HK2 cells. (**A**) mRNA expression of MCP-1 in HK2 cells. (**B**) mRNA expression of TNF-α in HK2 cells. (**C**) mRNA expression of IL-6 in HK2 cells. ** $P < 0.05$ vs. Group (TGF-β1+, miR-223-3p-, CHUK plasmid-). **** $P < 0.01$ vs. Group (TGF-β1+, miR-223-3p-, CHUK plasmid-)

and elucidate the mechanisms of CKD-associated RF at a deeper level.

In this study, we obtained differentially expressed miRNAs from CKD related microarray datasets of GEO database and found 10 up-regulated miRNAs and 11 down-regulated miRNAs using bioinformatics methods. Next, the TargetScan, miRDB and miRtarbase database were utilized to predict target genes of 11 down-regulated miRNAs, while DAVID was employed to conduct pathway enrichment analysis. It was discovered that the target genes of 10 of the down-regulated miRNAs were significantly enriched in the PI3K-Akt, MAPK, and Ras signaling pathways. MCODE clustering analysis and topological algorithms in CytoHubba are crucial methods for identifying core genes [37–39]. Therefore, MCODE and cytoHubba plug-in in Cytoscape software was utilized to select core genes, and PTEN, SIRT1, TP53, GSK3B, MTOR, CREB1, HB-3B, CHUK, et al. were considered as core genes in CKD. Then through miRNA-mRNA network, miR-223-3p, miR-132-3p, miR-150-5p and miR-199-3p were identified as the hub miRNAs. Finally, in vitro experiment was conducted to verify the role and function of miR-223-3p in CKD-associated RF.

MiR-223-3p, used to be called miR-223, is a highly conservative miRNA, and plays an important regulatory role in a variety of diseases, such as viral myocarditis [40], sepsis [41] and nonalcoholic fatty liver disease (NAFLD) [42]. In CKD, miR-223-3p has been verified to reduce ferroptosis in podocytes by targeting histone deacetylase 2 (HDAC2) and downregulating the phosphorylation of signal transducer and activator of transcription 3 (STAT3) [43]. Meanwhile, miR-223-3p overexpression has been shown to lighten calcium oxalate nephrocalcinosis-stimulated renal inflammation and oxidative injury [44]. Also, miR-223-3p upregulation suppresses renal tubular epithelial cell pyroptosis to alleviate lipopolysaccharide (LPS)-induced acute kidney injury [45]. In addition, previous studies have shown that the serum miR-223-3p level of mice with CKD is decreased [46], and the serum miR-223-3p level of patients with stage III and later CKD is also decreased significantly [47], which is consistent with the results of this study. Moreover, the serum miR-223-3p level is positively correlated with the survival rate and vascular complications of patients with CKD [47]. However, whether the abnormal expression of miR-223-3p is related to the pathogenesis of renal fibrosis in CKD is still under constant exploration. In addition, the results of miRNA-mRNA network showed that miR-223-3p was one of the four hub miRNAs and targeted three-tenths core genes of CKD, among which CHUK, IGF1R, FOXO3, PTEN and TP53 were related to the PI3K/Akt pathway. So, we choose miR-223-3p/CHUK as target for further study.

KEGG pathway enrichment analysis showed that target genes of 10 down-regulated miRNAs were enriched in PI3K-Akt, MAPK and Ras signaling pathways. Ras signaling pathways is a hormonal system that regulates blood pressure and fluid balance [48]. Renal Ras activation is associated with the development and progression of CKD through both blood pressure dependent and independent mechanisms [49, 50]. Ras is composed of a variety of components, including Angiotensinogen (AGT), renin, angiotensin-converting enzyme (ACE), angiotensin II (Ang II), type 1 and type 2 receptors (AT1 and AT2). Ang II is the main effector peptide of Ras, acting when binding to AT1 and AT2 receptors [51]. At present, one of the main clinical treatments for CKD is the use of anti-Ras drugs, such as ACE inhibitors (ACEI), AT1 receptor blockers (ARB), which have been proven to be beneficial to patients with CKD [51]. However, a considerable number of patients still develop to ESRD. Therefore, finding other targets may bring new hope for the treatment of CKD.

Several studies have reported that the PI3K-Akt signaling pathway is closely related to the pathological process of CKD. On one hand, activated PI3K/Akt can promote the transfer of NF-κB from cytoplasm to nucleus and secrete inflammatory factors, leading to the persistent

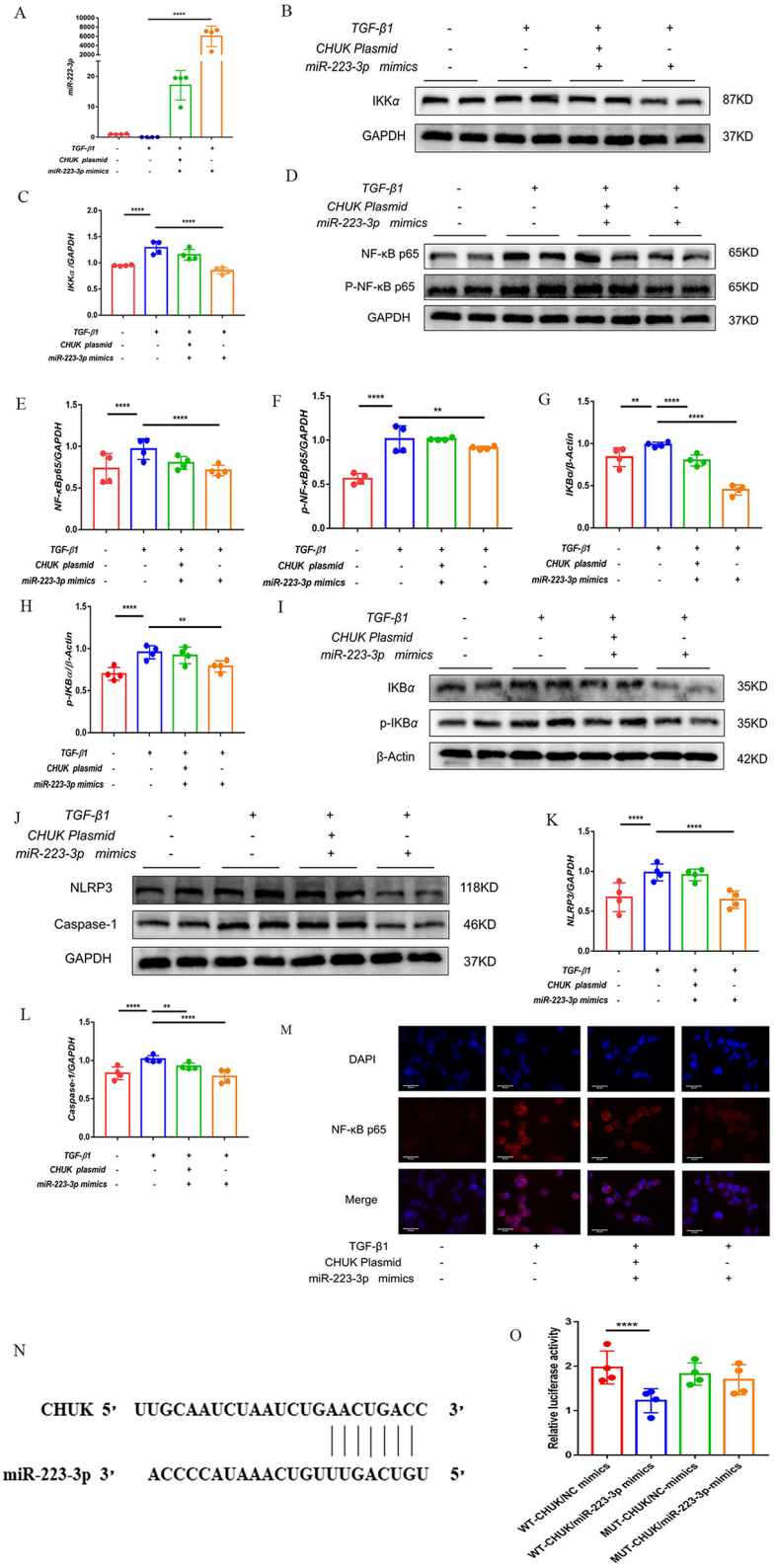

Fig. 8 miR-223-3p targeted IKKα mRNA and inhibited the NF-κB signaling pathway. (**A**) The level of miR-223-3p in HK2 cells. Western blot images and quantitative band density analyses of IKKα (**B**-**C**), NF-κBp65 and p-NF-κBp65 (D-F), P-IKBα and IKBα (**G**-**I**), NLRP3 and Caspase-1 (**J**-**L**) in different groups. (**M**) Immunofluorescence staining of NF-κB p65 in HK2 cells (at 400×magnification). (**N**) The binding site of miR-223-3p and CHUK predicted by TargetScan. (**O**) Relative luciferase activity. $^{**}P < 0.05$ vs. Group (TGF-β1+, miR-223-3p-, CHUK plasmid-). **** $P < 0.01$ vs. Group (TGF-β1+, miR-223-3p-, CHUK plasmid-)

chronic inflammatory state in kidney, which is one of the main factors for the occurrence and development of CKD [52, 53]. On the other hand, previous studies have confirmed that tubular EMT is the key pathogenesis of CKD [54]. Activated PI3K/Akt signaling pathway can accelerate the progression of EMT by increasing the expression of a-SMA and reducing the level of E-cadherin, leading to the sclerosis of renal tubules and the decline of renal function [55, 56]. Moreover, the PI3K/Akt signaling pathway is also closely related to renal oxidative stress [57], cellular aging [58], autophagy [58] and lipid metabolism [59].

The MAPK family includes extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. Previous studies have confirmed that the ERK pathway is involved in renal fibrosis, ERK is a profibrotic factor, and necessary for ECM deposition, myofibroblast accumulation and EMT progression [60]. The relationship between Ras, MAPK and PI3K/Akt signaling pathway in CKD is shown in Fig. 9. These 3 signaling pathways intersect with each other, and the enrichment of PI3K/Akt pathway in CKD was the largest. Therefore, the PI3K/Akt signaling pathway was selected as the focus of in vitro experiment validation.

TGF-β1 is a key factor in regulating renal fibrosis. Inhibiting the expression of TGF-β1 alleviates renal fibrosis and EMT significantly in UUO rats and renal tubular epithelial cells [61]. In vitro experiment of this study, the cell viability of HK2 cells was significantly decreased by 20ng/ml TGF-β1stimulation, meanwhile, the expression level of miR-223-3p decreased and the IKKα mRNA level increased obviously. Therefore, we used 20ng/ml TGF-β1 to induce EMT of HK2 cells, which was consistant with a study from Nanjing University of Chinese Medicine, Nanjing, China [62]. The level of α-SMA and Col1-a1 mRNA of HK2 cells were increased 24 h after stimulated by TGF-β1, indicating that the EMT model of HK2 cells was successfully created [63]. However, overexpression of miR-223-3p reduced levels of α-SMA and Col1-a1 in HK2 cells, which suggested miR-223-3p could restrain EMT. Conversely, overexpressed CHUK furtherly, α-SMA and Col1-a1 levels up-regulated, which implied that miR-223-3p might inhibit EMT by regulating CHUK. Therefore, we predicted the binding site of miR-223-3p and CHUK in TargetScan database, and verified whether miR-223-3p and CHUK were bound directly through double luciferase reporter gene experiment, and the results showed that miR-223-3p could target CHUK directly.

In order to investigate the mechanism of miR-223-3p on the EMT of HK2 cells, we detected the expression levels of IKKα protein, and IKBα, p-IKBα, NF-κB p65, p-NF-κB p65, NLRP3, Caspase-1, which are in the downstream of IKKα. Data of our research showed that overexpression of miR-223-3p lowered the expression levels of IKKα, IKBα, p-IKBα, NF-κB p65, p-NF-κB p65, NLRP3, Caspase-1 protein, and overexpressed IKKα furtherly, the level of above proteins up-regulated, which suggested again that miR-223-3p functions through CHUK. IKKα is a subunit of IKK, which phosphorylates IKBα, makes IKBα lose its inhibitory effect on NF-κB and activates NF-κB pathway [64]. The NF-κB signaling pathway is

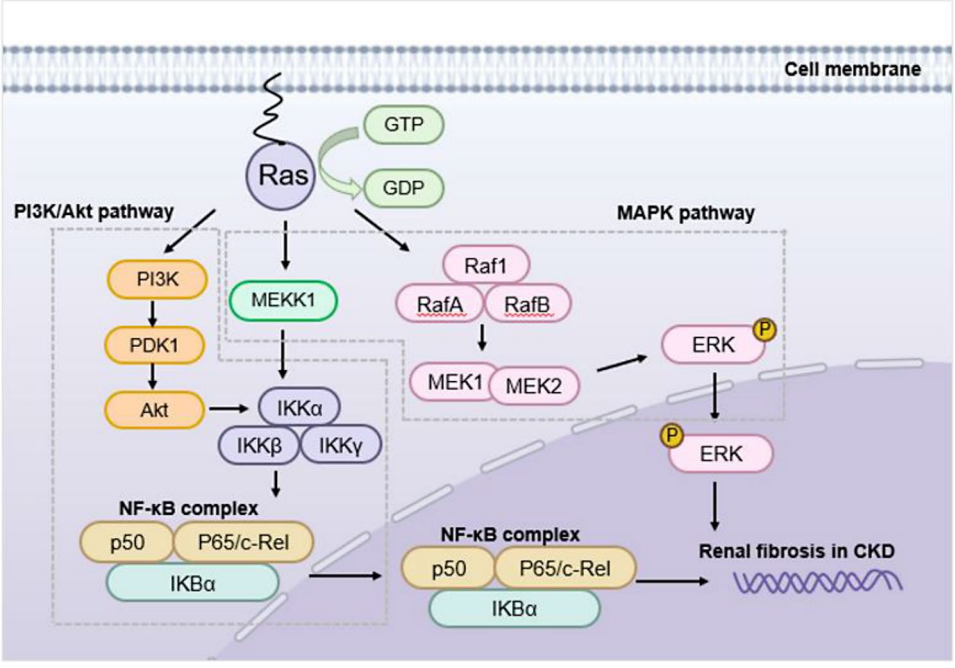

Fig. 9 The relationship between Ras, MAPK and PI3K/Akt signaling pathway in CKD

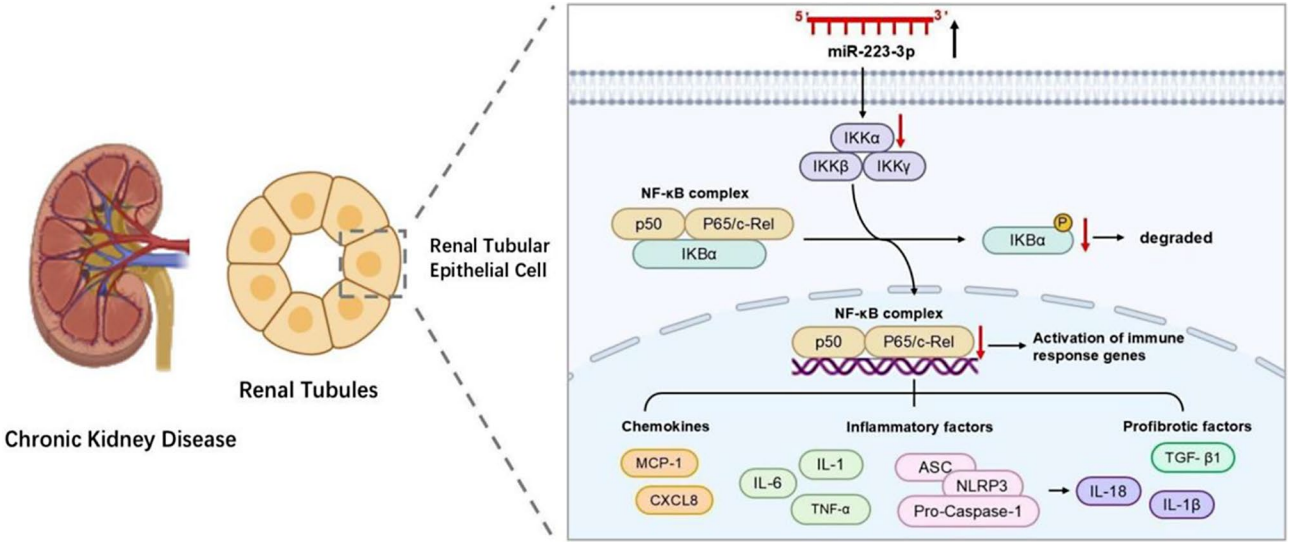

Fig. 10 The mechanism by which miR-223-3p alleviated EMT of HK2 cells

an important pathway in renal inflammatory response, inhibiting the activation of NF-κB obviously inhibits renal inflammatory response [65], as a result, levels of inflammatory factors like IL-6, IL-1, TNF-α [66], and chemokines like IL-8, MCP-1 [67] are decreased. While chemokines can recruit monocytes from bone marrow to the renal interstitium to amplify the inflammatory response [67], and produce a substantial amount of profibrotic factors like TGF-β1 to promote the progression of renal fibrosis [68]. In addition, the assembling of NLRP3-ASC-pro-Caspase-1 inflammasome is inhibited and the secretion of IL-18 and IL-1β are decreased [69]. In this study, the expression of IL-6, TNF-α, MCP-1 mRNA and NLRP3, Caspase-1 protein down-regulated, suggesting that miR-223-3p inhibited the EMT of HK2 cells through NF-κB signaling pathway. In brief, miR-223-3p inhibits EMT of HK2 cells by enhancing the anti-inflammatory ability (Fig. 10).

The process of RF is mainly divided into three stages: inflammatory response stage, fibrosis formation stage and scar formation stage [70]. In inflammatory response and fibrosis formation stage, although the function of the damaged renal cells has changed, they still retain some of their original functions. Therefore, appropriate therapeutic interventions are able to stop the transformation of damaged cells into myofibroblasts and reverse them into normal cells in both stages. Whereas, in scar formation stage, renal fibrosis is irreversible [71]. Inflamatory response [5], oxidative stress [9], energy metabolism changes [72], ferroptosis and lipid peroxidation [73] all play crucial roles in renal fibrosis of CKD, they interact with each other to disrupt kidney homeostasis. MiR-223-3p can enhance the anti-inflammatory ability of HK2 cells, which is the first stage of RF. For the purposes of delaying renal fibrosis and treating CKD, miR-223-3p plays a certain role. The effect of miR-223-3p on inflammation of CKD is similar with miR-451 and miR-146a, both of which can regulate NF-κB pathway and inhibit renal inflammation [74, 75].

However, except CHUK, miR-223-3p can also target HDAC2 [43], NLRP3 [44], and TNF receptor associated factor 6 (**TRAF6**) [40]. The ability of miRNAs to bind multiple targets can potentially lead to off-target effects, affecting genes other than the one intended [76]. In addition, the expression and function of miRNAs can be influenced by various factors, such as the cellular microenvironment, developmental stage, and disease state [77]. Therefore, the challenges to using miR-223-3p to prevent or treat CKD is ensuring the safe delivery and maintaining its stability. To address these challenges, researchers have developed several strategies to mitigate off-target effects and optimize the specificity of miRNAs. The first strategy is sequence optimization, where the miRNA sequence is modified to enhance its specificity and reduce off-target effects [78]. The second is the design of the delivery system, targeted delivery approaches, such as the use of tissue-specific promoters or ligand-receptor interactions, can help to direct the miRNA to the desired cell type or tissue, reducing the risk of off-target effects in non-target cells [79]. The third is local delivery of miRNAs, which can achieve the desired gene silencing due to higher bioavailability, such as direct injection of miRNA vectors to target tissues [80]. The last is the use of combinatorial miRNA approach, such as a multifunctional nanoparticle co-delivering miRNA, siRNA and mRNA cocktails, which is also helpful to improve the specificity and efficacy of miRNAs [79]. Nowadays, miRNAs have been used to generate vaccine strains, such as poliovirus, influenza virus, and tick-borne encephalitis [80], which brings hope to use miR-223-3p in the treatment of CKD.

In this study, we explored the role and mechanism of miR-223-3p in EMT of human renal tubular epithelial cells. However, renal tissue is made up of a variety of cells which interact and work together to maintain renal homeostasis. So, renal organoids may be a better way to explore the role and mechanism of miR-223-3p. Simultaneously, CKD can be caused by multiple causes, the role and mechanism of miR-223-3p in different types of CKD can be explored in the future, such as diabetic kidney disease. In addition, due to the limited GEO datasets related to miRNAs of patients with CKD, we only screened differentially expressed miRNAs of CKD patients in GSE80247 without using a validation set to validate the conclusion, transcriptomic sequencing of a larger sample size of CKD patients may address this issue.

Conclusion

In summary, we identified that miR-223-3p, miR-132-3p, miR-150-5p and miR-199-3p were four hub miRNAs in the occurrence and development of CKD, and target genes of 10 down-regulated miRNAs in the kidney of patients with CKD were enriched in PI3K-Akt, MAPK and Ras signaling pathways through bioinformatics method. In vitro experiment confirmed that miR-223-3p could inhibit EMT of HK2 cells by targeting CHUK and regulating downstream NF-κB protein, providing a new target for the treatment of CKD. Additionally, this study offers a potential avenue for the development of future interventions aimed at halting or reversing the progression of this debilitating disease.

Abbreviations

CKD	Chronic kidney disease
PPI	Protein-protein interaction
RF	Renal fibrosis
ESRD	End-stage renal disease
EMT	Epithelial-mesenchymal transition
MMT	Macrophage-to-myofibroblast transition
MiRNA	MicroRNA
HK2	Human renal tubular epithelial cell
DEGs	Differentially expressed genes
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
GEO	Gene Expression Omnibus
PCA	Principal component analysis
DC	Degree
BC	Betweenness centrality
MCODE	Molecular Complex Detection
MCC	Maximal Clique Centrality
EPC	Edge Percolated Component
DAVID	Database for Annotation, Visualization, and Integrated Discovery
CC	Cellular component
MF	Molecular function
BP	Biological process
FBS	Fetal bovine serum
TGF-β1	Transforming growth factor-β1
CCK8	Cell Counting Kit-8 assay
IKKα	Inhibitor of kappa B kinase
α-SMA	Alpha-smooth muscle actin
Col1-a1	Collagen type I alpha 1
TNF-α	Tumor necrosis factor
IL-6	Interleukin-6
MCP-1	Monocyte chemotactic protein-1
IKB-α	Inhibitor kappa B alpha
p-NF-κB p65	Phosphorylated nuclear factor kappa-B p65
NLRP3	Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain- containing 3

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-024-06058-x.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Author contributions

JJ-Y designed the research and revised the manuscript. HM-C drafted the first version of the manuscript and conducted bioinformatics analysis, YX-Z, P-W and H-L conducted the in vitro experiments. W-G and N-L were responsible for data analysis. All authors read and approved the final version of the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (No. 82060597) and Natural Science Foundation of Ningxia (2023A0854).

Data availability

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Declarations

Consent for publication

Not applicable.

Competing interests

None declared.

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Received: 11 September 2024 / Accepted: 24 December 2024
Published online: 02 April 2025

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