

Original Article

MiR-21 as a potential biomarker for renal dysfunction induced by cadmium exposure

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Abstract: Cadmium (Cd) is a serious environmental contaminant. It accumulates in the kidneys, potentially affecting human health at low concentrations. However, the relationship between miRNAs and renal dysfunction associated with Cd exposure remains undefined. Stem-loop real-time PCR was used to investigate expression of kidney tissue miRNAs in rats exposed to Cd. It was also used to validate expression of circulating miRNAs in the plasma of 31 humans with renal dysfunction, associated with low-level exposure of Cd, and 24 healthy controls. Results showed that expression of miR-21 and miR-29b were both upregulated in the Cd-exposed group (both $P < 0.01$). Only miR-21 was significantly differentially expressed in the plasma of renal dysfunction patients ($P < 0.001$). In conclusion, miR-21 was elevated in renal dysfunction patients associated with Cd-exposure, indicating that miR-21 might serve as a biomarker of renal dysfunction development and progression associated with Cd exposure.

Keywords: MiR-21, cadmium, kidney, general population, biomarker

Introduction

Heavy metal pollution has been known to induce many health problems. They are easily incorporated into biological molecules and exert their toxic effects by displacing essential metals of a lower binding power in biologically active molecules or by acting as noncompetitive inhibitors of enzymes. This situation has become more and more serious in developing countries and some developed countries [1]. Most heavy metals cannot be metabolized by the body. Excessive accumulation in the body disturbs the normal function of cells. Kidneys are the keys to eliminating heavy metals from the body. Certain heavy metals have an additive effect of inducing nephrotoxicity. For example, the effects of cadmium (Cd), in causing renal damage, have been shown in the Chinese general population [2]. It is considered highly toxic and progressively bio-accumulated in the organism, with a biological half-life in humans estimated to be decades [3]. Long-term exposure to Cd leads to kidney accumulation, which

may induce proximal tubule damage. It causes renal tubular dysfunction, as assessed by increased urinary excretion of low molecular weight proteins, such as N-Acetyl-beta-(D)-Glucosaminidase (NAG) [4].

MicroRNA (miRNA) is a small non-coding RNA molecule (containing about 22 nucleotides) found in plants, animals, and some viruses. They function in RNA silencing and post-transcriptional regulation of gene expression. It has been reported that miRNAs are involved in regulation of gene expression and many crucial biological processes, including development, differentiation, apoptosis, and proliferation [5, 6].

Many studies have focused on comparing miRNA expression between pathologic samples and normal tissues. For example, expression changes of miRNAs have been reported in renal tumors [7] and in the kidneys of a small number of patients with lupus nephritis [8], immunoglobulin A nephropathy [9], and acute rejection after renal transplantation [10]. However, only

few studies have evaluated changes in miRNA expression in response to environmental stimuli [11], such as Cd exposure. It would be very interesting to determine whether miRNA is involved in the pathogenetic processes of kidney dysfunction induced by Cd exposure. In the present study, it was hypothesized that expression of miRNAs in Cd-exposed people may be abnormal. The purpose of this study was to identify differentially expressed miRNAs in the kidney tissues of Cd-exposed rats and humans. The Cd-exposed rat model was established by daily subcutaneous injections of CdCl₂ solution at the ratio of 2.0 mg CdCl₂ per kg body weight for two weeks. This study screened differentially expressed miRNAs using miRNA arrays, followed by verification using real-time-PCR. Moreover, expression of these miRNAs was detected in the plasma from humans with kidney dysfunction associated with Cd exposure. These miRNAs may serve as biomarkers for kidney dysfunction caused by Cd exposure.

Materials and methods

Animals and treatment

Male Sprague-Dawley (SD) rats, weighing 180-200 g, were obtained from Experimental Animal Center of Military Medical Sciences (Beijing, China). The animals were housed in the Experimental Animal Center of Shanxi Medical University. They were maintained in an environmentally controlled room under a 12-hour light/dark cycle. Food and water were provided ad libitum. Twenty-four SD rats were randomly divided into the control group (n=12) and Cd-exposed group (n=12), with two rats per cage. The Cd-exposed group received daily subcutaneous injections of CdCl₂ solution at a ratio of 2.0 mg CdCl₂ per kg body weight for two weeks. Rats injected with normal saline were used as the control group. Body weights of each rat were recorded every day. Animal welfare and experimental procedures were performed strictly in accordance with the Guidelines for the Care and Use of Laboratory Animals. Experimental protocol and procedures were approved by the Institutional Animal Ethics Committee of Shanxi Medical University. All animals were taken care of in accordance with guidelines provided by the committee (Certificate number: 2012009).

Subject recruitment

A total of 55 subjects were recruited from April 2016 to July 2016. Of these, 31 subjects lived in a Cd-contaminated environment with hyper-UNAG and higher urinary cadmium, while 24 healthy subjects lived in a no-exposed area with lower urinary cadmium. The concentration of Cd in the soil of the contaminated area was 0.208 mg Cd/Kg. The concentration of Cd in the soil of the healthy area was 0.077 mg Cd/Kg.

Based on registry information available from the local authorities, characteristics of the patients (age, sex distribution, and lifestyle) were available for the two areas (Cd-polluted area and control area). All patients were previously informed concerning the objectives of this study, providing informed consent. Ethical permission was given by the Ethical Committee of Shanxi Medical University (No. 2012009).

Collection and analysis of rat and patient samples

Two weeks after Cd administration, the rats were placed in metabolic cages (Suzhou Fengshi Laboratory Animal Equipment Co, LTD. Suzhou, China) to collect urine for 24 hours. The rats were sacrificed, then kidney tissues were rapidly removed, weighted, and quickly placed in liquid nitrogen for 2 minutes. They were then stored at -80°C in a freezer.

For patients, venous blood was obtained via antecubital venipuncture in the sitting position. Whole blood (5 mL) was placed in EDTA-containing tubes and centrifuged (1200 g for 10 minutes). The supernatant was collected. Plasma was then obtained and 1 mL was rapidly subjected to miRNA extraction.

Urine samples from rats were collected from metal-free polyethylene bottles. These bottles were washed with diluted nitric acid followed by deionized water and stored at -20°C until analysis. Each urine sample was divided into two aliquots. The first was acidified with concentrated nitric acid and used for detection of concentrations of Cd. The second was used to measure NAG, albumin (ALB), kidney injury molecule-1 (KIM-1), and creatinine (Cr). UCd concentrations were measured by Inductively Coupled Plasma Mass Spectrometry (ICPMS). UN-

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AG was assessed, as described by Tucker et al. [12]. UALB were analyzed by ELISA. Creatinine was determined by the Jaffe reaction method. All urinary parameters were standardized to concentrations of Cr in urine [4].

RNA extraction and miRNA array

Total RNA was extracted from rat kidney tissues. Briefly, 50 µg of total RNA was purified using SanPrep Column microRNA Mini-Preps Kit (Sangon Biotech, Shanghai, CHINA). RNA from Cd-exposed rat kidney tissues and control tissues were subjected, in triplicate, to Affymetrix microRNA Gene Chip array analysis. The miRNA labeling, hybridization assay, and data analysis were performed using LuxScan-3.0 image analysis software (Capital Biology Corporation National Engineering Center for Beijing Biochip Technology), as described previously [13]. For comparison analysis, a two-class unpaired method was applied using SAM 2.1 (significance analysis of microarray 2.1) software to identify differentially expressed miRNAs. The screening condition was FDR-controlled within 5% with no less than a 2.0-fold change or no more than a 0.5-fold change in expression.

For human subjects, total RNA was extracted from plasma using the TRIzol LS RNA isolation kit (Invitrogen, USA), according to manufacturer protocol. Total RNA quality was tested using the NanoDrop 1000 system (ThermoFisher Scientific, Wilmington, DE, USA) and the RNA was immediately stored at -80°C.

Real-time PCR

Stem-loop RT-PCR was performed by using the HaiGene miRNA SYBR Green PCR kit (cat. No. APO1108) to detect expression of miRNAs. To generate a miRNA cDNA library, the first strand of cDNA was synthesized using 2 µg of RNA in 20 µL of reaction buffer. The mix was incubated at 37°C for 60 minutes, followed by 95°C for 5 minutes. Subsequently, real-time quantification was performed using iCycle Real Time PCR system (BioRad Laboratories, USA), according to manufacturer recommendations. The 20 µL PCR reaction system consisted of 2 µL RT product, 0.5 µL miRNA forward primer, 0.5 µL miRNA reverse primer, 4 µL 5 × Golden HS SYBR Green qPCR Mix, and RNase-free water (HaiGene). The mixtures were incubated at

95°C for 15 minutes, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. All reactions were run in triplicate. Data were analyzed using the $2^{-\Delta\Delta CT}$ method and fold changes of miRNA expression were normalized to U6.

Gene set enrichment analysis of miRNA targets in different pathways

MicroRNA (miR-21, miR-29b) functional enrichment analysis was performed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (<http://www.genome.jp/kegg/pathway.html>), using Molecule Annotation System 3.0 (MAS 3.0) (<http://bioinfo.capitalbio.com/mas3/>). Significantly changed KEGG pathways were identified based on whether they had at least 3 DEGs with a hypergeometric test of $P < 0.01$.

Statistical analysis

Data are presented as mean ± SD. Significance was determined using Student's t-tests for comparisons between groups.

Population research data were double-entered into Epi info version 3.5.1 (CDC, Atlanta, GA, USA), which reduces error in creating electronic data sets before statistical analysis. Differences in the distribution of characteristics between the exposed group and control group were evaluated by Chi-squared test for categorical variables (sex, smoking status) and Student's t-test for continuous variables (age, BMI, blood pressure, and miRNA expression levels). For abnormally distributed continuous variables (UNAG, UALB, and UCd), two-sided Mann-Whitney U-test was used to evaluate differences between the exposed group and healthy controls. Correlation between UCd and variables (UNAG and miRNA-21) was evaluated with Spearman's rank correlation test. All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA, version 19.0 for Windows). All *P*-values reported were two-sided and are considered statistically significant at $P < 0.05$.

Results

Significant body and kidney weight reduction in Cd-exposed rats

As shown in **Table 1**, after exposure to Cd for one week, body weights of Cd-exposed rats

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Table 1. Body weight and kidney weight measurements of Cd-exposed rats and saline treated rats (n=6)

Time	Body weight (g)		P	Kidney/body ratio ($\times 1000$)		P
	Control	Cd-exposed		Control	Cd-exposed	
0 week	193.29 \pm 14.58	199.25 \pm 13.90	0.432	--	--	--
1 week	220.43 \pm 18.02	209.75 \pm 21.77	0.017	8.43 \pm 0.47	9.50 \pm 0.73	0.002
2 weeks	253.43 \pm 20.72	211.63 \pm 14.94	0.001	7.31 \pm 0.47	8.69 \pm 0.46	<0.001

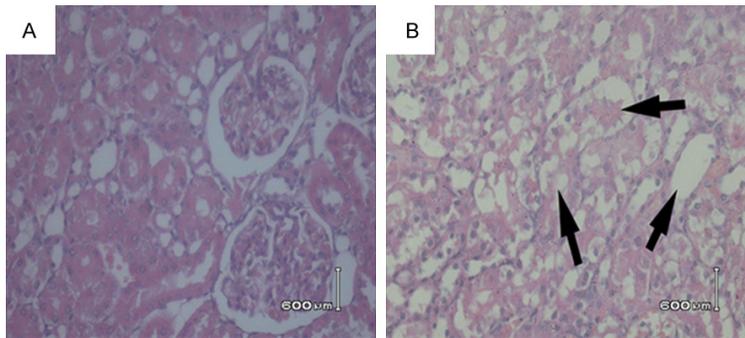


Figure 1. Histological observations of kidneys between normal rats and Cd-exposed rats. A. Control group, normal glomeruli, and tubules. B. 2.0 mg/kg Cd treated rat kidney for two weeks. Tubular swollen, necrosis and dilated, epithelial cells exfoliated (arrows). No changes were noted in the animals exposed to 2.0 mg/kg Cd for one week (not shown).

Table 2. Concentrations of NAG, KIM-1, and ALB in Cd-exposed group and control group (n=6)

Biomarkers	Control group	Exposed group	P
UNAG (pg/ μ mol Cr)	8.97 \pm 5.46	27.20 \pm 11.17	0.005
UALB (ng/ μ mol Cr)	17.93 \pm 9.00	49.89 \pm 33.75	0.019
UKIM-1 (pg/ μ mol Cr)	13.24 \pm 6.89	21.97 \pm 6.05	0.071

were obviously reduced, compared to control rats. Especially after two weeks, body weights of Cd-exposed rats dropped further. Similarly, weights of kidneys of Cd-exposed rats were also significant decreased, compared to control rats. In addition, the weight ratio of kidney/body in the Cd-exposed group was significantly higher than the control group at both 1 week and 2 weeks of treatment. Moreover, histological observations showed the tubular swollen, necrosis and dilated, and epithelial cells exfoliated (**Figure 1**) after Cd treatment for two weeks. No changes were noted in the animals exposed to 2.0 mg/kg Cd for one week (not shown).

Urinary NAG, KIM-1, and ALB analysis

To evaluate kidney function in Cd-exposed rats, concentrations of NAG, KIM-1, and ALB were

detected. As shown in **Table 2**, concentrations of NAG and ALB were significantly increased in Cd-exposed rats, compared to the control group. There were no significant differences in concentrations of UKIM-1 between the Cd-exposed group and control group.

Differentially expressed miRNAs in the kidneys of Cd-exposed rats

As shown in **Figure 2** and **Table 3**, miRNA array showed that four miRNAs were differentially expressed in the 2.0 mg/Kg-bw Cd-exposed rats, compared to controls. Of these, three miRNAs (rno-miR-29b, rno-miR-21, rno-miR-29c) were upregulated and one miRNA (rno-miR-206) was downregulated. This study further confirmed whether these

above mentioned differentially expressed miRNAs were differentially expressed in the kidneys of Cd-exposed rats. As shown in **Figure 3**, relative expression of rno-miR-29b, rno-miR-21, and rno-miR-29c in the kidneys of Cd-exposed rats was significantly higher than in controls. However, there were no significant differences in miR-206 expression between Cd-exposed rats and control rats.

Confirmation of differentially expressed miRNAs in human subjects

This study further confirmed expression of differentially expressed miRNAs in human plasma with kidney dysfunction correlated with Cd-exposure. **Table 4** shows all subject characteristics. Concentrations of UNAG and UCd were statistically higher in the Cd-exposed group than in controls. Expression levels of the miR-

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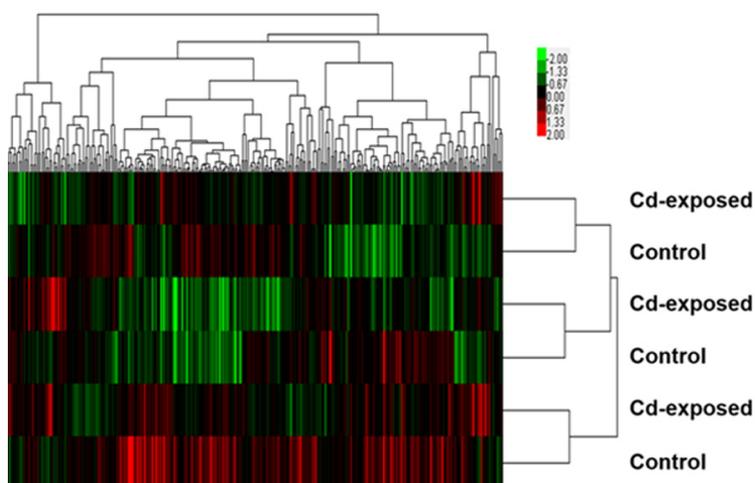


Figure 2. This heatmap shows miRNAs array results between Cd-exposed rats and controls.

Table 3. Differentially expressed miRNAs in the kidneys of Cd-exposed rats

Accession number	Name	Fold change*	Expression condition
MIMAT0000801	Rno-miR-29b	3.42	Upregulation
MIMAT0000790	Rno-miR-21	2.41	Upregulation
MIMAT0000803	Rno-miR-29c	2.23	Upregulation
MIMAT0000879	Rno-miR-206	0.18	Downregulation

*: Fold changes no less than a 2.0 or no more than a 0.5 in expression are considered significant.

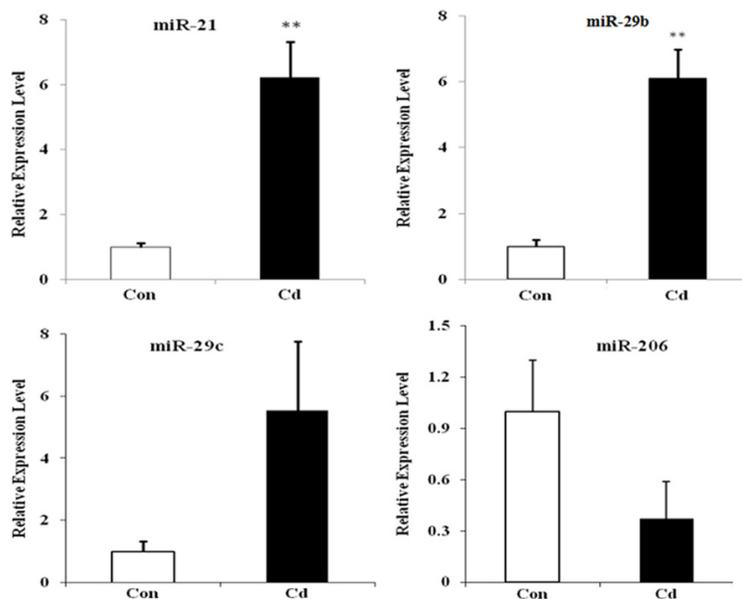


Figure 3. Relative expression of rno-miR-29b, rno-miR-21, rno-miR-29c, and miR-206 in the kidneys of Cd-exposed rats compared to control rats.

NAs were then measured in kidney dysfunction patients and healthy controls. As shown in

Figure 4, levels of miR-21 were significantly higher in the kidney dysfunction population, compared with healthy controls ($P < 0.001$). In contrast, that of miR-29b showed no significant differences ($P = 0.137$). Regarding correlation between markers of kidney function, UCd and miRNA, UCd showed significant positive correlation with UNAG and miR-21 ($r_s = 0.402$, $P = 0.002$; $r_s = 0.345$, $P = 0.010$, respectively). Positive correlation was revealed between UNAG and miR-21 ($r_s = 0.364$, $P = 0.006$). UCd was not associated with UALB and miR-29b.

Discussion

Cd is a serious environmental contaminant. It has been established that cadmium can accumulate in many organs, including the liver, kidneys, pancreas, and testis, adversely affecting the function of these organs. Of these, the kidneys have been recognized as a major target of cadmium-induced toxicity, especially proximal tubular cells. Cd accumulates in the kidneys, potentially affecting human health at relatively low concentrations [14, 15]. The toxic effects of cadmium on proximal tubular cells cause decreased reabsorption of low molecular weight (LMW) proteins. This results in increased urinary excretion of these proteins, so-called 'tubular proteinuria' [1, 16, 17]. The present study found increased excretion of LMW protein-UNAG in urine both in populations with Cd-exposure and in rats exposed to Cd. NAG is a lysosomal enzyme that is excreted in urine during tubular damage.

Thus, it is a specific indicator of renal tubular membrane dysfunction [18]. The result means

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Table 4. Clinical characteristics of Cd-exposed with kidney dysfunction and healthy controls

	Controls (n=31)	Cd-exposed (n=24)	P
Age (years)	60.81±7.74	62.29±12.11	0.583
Gender (male/female)	12/19	12/12	0.402
Smoking status (yes/no)	6/25	12/12	0.016
BMI (Kg/m ²)	25.06±3.14	25.26±4.27	0.844
Systolic blood pressure (mmHg)	139.58±21.00	134.08±18.23	0.313
Diastolic blood pressure (mmHg)	83.29±11.97	77.67±10.07	0.070
UNAG (U/gCr) ^a	14.82 (12.65, 19.03)	22.18 (17.93, 33.04)	0.003
UALB (mg/gCr) ^a	9.91 (5.57, 17.38)	11.04 (7.13, 13.46)	0.757
UCd (µg/gCr) ^a	0.47 (0.12, 0.62)	1.25 (0.69, 3.40)	0.003
MiR-21 expression level	10.73±1.86	14.63±2.21	<0.001
MiR-29b expression level	7.46±4.18	9.30±4.72	0.137

P<0.05 is considered significant. ^a: The values presented are median (5-95th percentiles). Abbreviations used were: BMI = body mass index; UNAG = urinary N-acetyl-β-glucosaminidase; UALB = urinary albumin; UCd = urinary cadmium; Cr = creatinine.

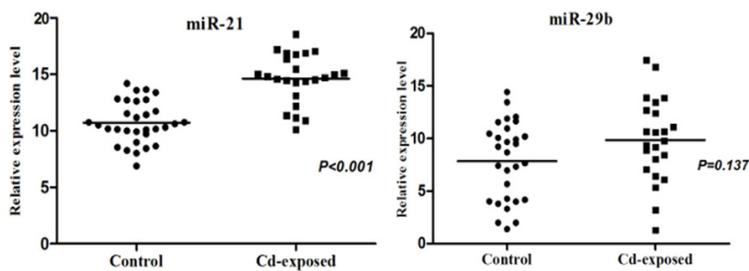


Figure 4. Relative expression levels of peripheral blood miRNAs in Cd-exposed population with renal dysfunction and healthy controls. Horizontal lines indicate means. P-values were calculated using two-sided Student t-tests.

Table 5. Pathways enriched in predicted targets of miR-21 and miR-29b (Top 10)

TERM	P-Value
MAPK signaling pathway	4.24E-28
Cytokine-cytokine receptor interaction	8.29E-26
TGF-beta signaling pathway	3.41E-24
Prostate cancer	1.59E-20
Long-term depression	1.50E-16
Focal adhesion	3.10E-16
Melanoma	3.79E-16
Colorectal cancer	5.33E-16
Small cell lung cancer	8.65E-16
Pancreatic cancer	7.79E-15

that renal tubular function could be affected by cadmium exposure both in the general population and experimental animals. UALB had been also detected. No association was found between cadmium and excretion of albumin. Albumin has a molecular size of about 65 kDa,

above the threshold size of the pores in the glomerular basement membrane. Elevated levels of UALB indicate damage of the integrity of the glomerular filtration barrier [19]. Results suggest no evidence of elevated UALB due to cadmium exposure in this study population. Results indicate that levels of Cd-exposure were not enough to damage glomerulus in this population. Significant increases in rats exposed to Cd were found, however. The 2.0 mg/Kg-bw Cd-exposure could induce the dysfunction of the glomerulus.

Recent studies have demonstrated that miRNAs can be detected in the blood and that they may serve as potential biomarkers for many diseases. MicroRNA arrays are a newly developed high-throughput screening technology that can be used to detect different expression levels of miRNAs in patients and healthy subjects [20]. In this study, a miRNA profile of the kidney tissue of SD rats was obtained. After comparing kidney tissue miRNAs levels of the rats, it was observed that some miRNAs were differentially expressed between Cd-exposed and control rats. Two miRNAs (rno-miR-21 and rno-miR-29b) that were differentially expressed in kidney tissue samples were identified. Results were confirmed by quantitative reverse-transcription PCR. Furthermore, this study examined expression levels of these two circulat-

ing miRNAs in the Cd-exposed population, comparing the kidney dysfunction group with healthy controls. The abundance of miR-21 was greatly increased in rats and confirmed in kidney dysfunction subjects. Present results are consistent with other studies of chronic kidney disease patients [21]. In another study of ischemia-reperfusion injury, similar elevation of renal miR-21 was observed [22]. This result suggests the pathological roles of miR-21 in renal diseases. miR-21 are considered useful because their levels are relatively stable in serum. It plays important roles in the progression of renal fibrosis [23]. The potential of miRNAs as biomarkers for kidney disease has been investigated recently. In unilateral ureteral obstruction (UUO) kidneys, miR-21 demonstrated the greatest increase. Enhanced expression of miR-21 was located mainly in distal tubular epithelial cells. Remarkably high levels of circulating miR-21 were found in patients with severe interstitial fibrosis and tubular atrophy [24, 25]. To the best of our knowledge, the current study is the first to examine the relationship of miRNAs, NAGuria, and Cd exposure in humans and rats. This study suggests that miR-21 may be a biomarker of renal dysfunction induced by Cd exposure. UNAG showed a significant correlation with miR-21 ($r_s=0.364$). Present results suggest that miR-21 might be involved in the pathogenetic mechanisms of hyperNAGuria. It has been reported that overexpression of miR-21 can significantly repress the production of superoxide dismutase 2 in cells, resulting in increased intracellular ROS levels [26]. Expression of miR-21 is part of a response aimed at limiting injuries from ROS [27]. Cd exposure induces apoptotic cell death via ROS. The present study observed an upregulation of miR-21 expression after Cd-exposure. This may reflect a nonspecific response to ROS production in blood due to increased Cd-induced oxidative stress. The ability of toxic heavy metals, like cadmium, to elicit "oxidative stress" and to alter metal homeostasis may lead to augmentation of defense mechanisms involving heme-oxygenase-1 (HO-1) to protect kidneys against injurious cadmium-induced oxidative stress and development of chronic kidney disease (CKD). The first sign of renal damage is usually proteinuria with increased excretion of biomarker proteins like NAG [28]. However, future studies are necessary to elucidate the connection between miR-21 expression

profiles and mechanisms of kidney tubular dysfunction induced by cadmium.

This study further carried out gene set enrichment analysis to identify potential targets of these miRNAs. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis results are shown in **Table 5**. Mitogen-activated protein kinase (MAPK) signaling pathways and transforming growth factor- β (TGF- β) signaling pathways were enriched with predicted targets of rno-miR-21 and rno-miR-29b that were differently expressed in kidney tissues of different Cd exposure. It has been established that MAPK pathways are involved in the stress response to environmental stimuli. Cd directly activates p38 MAPK in a variety of cell types [29]. Cd induces oxidative stress which contributes to Cd-induced toxicity in the tubular cells, activating MAPK pathways. The function of MAPKs is to respond to extracellular stimuli and regulate a variety of cellular activities, such as differentiation, proliferation, cell survival, and apoptosis [30]. miR-21 can regulate and control MAPK signaling pathways. Regulation is independent of different tissue or cell types [31]. miR-21 was demonstrated to have an aberrant role in cell proliferation and the promotion of miR-21 increased proliferation. In support of present results, hsa-miR-21 has been associated with several key processes involved in the progression of renal dysfunction induced by cadmium exposure. Present results suggest that stimuli of procedures that induce renal stress result in miRNA-mediated activation of MAP kinase activity, leading to renal dysfunction.

Recently, *in vitro* and *in vivo* models of diabetic nephropathy (DN) have shown that TGF- β 1 also positively or negatively regulates expression of several miRNAs. These, in turn, amplify TGF- β 1 signaling to further promote renal fibrosis [32, 33]. Numerous studies have demonstrated that TGF- β regulates specific miRNAs, influencing renal fibrosis in kidney disease. miR-21 is the most significant miRNA involved in fibrotic disorders. Its levels are upregulated in human kidney disease [34]. During renal fibrosis, TGF- β induces many fibrogenic genes, such as extracellular matrix (ECM) proteins, via Smads, Smad3, or MAPKs [35]. Although the function of miR-21, especially its role in renal dysfunction, has not been extensively studied, present

results suggest that miR-21 may serve as a potential indicator of renal dysfunction caused by Cd-exposure. However, more in-depth work is necessary.

Compared with other studies on the role of miRNAs as biomarkers of various diseases, this study contained a relatively small sample size, including only 31 renal dysfunction subjects with Cd exposure and 24 healthy controls. Therefore, current results are more suitable for a pilot study. It is expected that these results will be validated by studies on larger populations. Future research on this potential biomarker is warranted.

Conclusion

In conclusion, present findings reveal that cadmium exposure may increase the risk of renal dysfunction. Additionally, miR-21 was found to associate with both. This study suggests that miR-21 might serve as a biomarker of renal damage induced by cadmium exposure. However, additional prospective studies with larger sample sizes are necessary to validate present results.

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Disclosure of conflict of interest

None.

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