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# Identification of urinary microRNA biomarkers for *in vivo* gentamicin-induced nephrotoxicity models

Byung-Suk Jeon <sup>1</sup>, Soo-ho Lee <sup>1</sup>, So-Ryeon Hwang <sup>1</sup>, Hee Yi <sup>1</sup>,  
Ji-Hyun Bang <sup>1</sup>, Nga Thi Thu Tham <sup>1</sup>, Hyun-Kyoung Lee <sup>2</sup>, Gye-Hyeong Woo <sup>3</sup>,  
Hwan-Goo Kang <sup>3,\*</sup>, Hyun-Ok Ku <sup>1,\*</sup>

<sup>1</sup>Toxicological Evaluation Laboratory, Animal and Plant Quarantine Agency, Gimcheon 39660, Korea

<sup>2</sup>Animal Pathodiagnostic Laboratory, Animal and Plant Quarantine Agency, Gimcheon 39660, Korea

<sup>3</sup>Department of Clinical Laboratory Science, Semyung University, Jecheon 27136, Korea

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\*Corresponding authors:

Hyun-Ok Ku

Toxicological Evaluation Laboratory, Animal and Plant Quarantine Agency, 177 Hyeoksing 8-ro, Gimcheon 39660, Korea.  
E-mail: kuho@korea.kr

Hwan-Goo Kang

Department of Clinical Laboratory Science, Semyung University, 65 Semyung-ro, Jecheon 27136, Korea.  
E-mail: toxfree@gmail.com

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ORCID iDs

Byung-Suk Jeon 

<https://orcid.org/0000-0002-7981-9507>

Soo-ho Lee 

<https://orcid.org/0000-0002-5041-1874>

So-Ryeon Hwang 

<https://orcid.org/0000-0002-6947-0241>

Hee Yi 

<https://orcid.org/0000-0003-0684-1882>

Ji-Hyun Bang 

<https://orcid.org/0000-0003-2212-7056>

Nga Thi Thu Tham 

<https://orcid.org/0000-0001-8396-3299>

## ABSTRACT

**Background:** Although previous *in vivo* studies explored urinary microRNA (miRNA), there is no agreement on nephrotoxicity-specific miRNA biomarkers.

**Objectives:** In this study, we assessed whether urinary miRNAs could be employed as biomarkers for nephrotoxicity.

**Methods:** For this, literature-based candidate miRNAs were identified by reviewing the previous studies. Female Sprague-Dawley rats received subcutaneous injections of a single dose or repeated doses (3 consecutive days) of gentamicin (GEN; 137 or 412 mg/kg). The expression of miRNAs was analyzed by real-time reverse transcription-polymerase chain reaction in 16 h pooled urine from GEN-treated rats.

**Results:** GEN-induced acute kidney injury was confirmed by the presence of tubular necrosis. We identified let-7g-5p, miR-21-3p, 26b-3p, 192-5p, and 378a-3p significantly upregulated in the urine of GEN-treated rats with the appearance of the necrosis in proximal tubules. Specifically, miR-26-3p, 192-5p, and 378a-3p with highly expressed levels in urine of rats with GEN-induced acute tubular injury were considered to have sensitivities comparable to clinical biomarkers, such as blood urea nitrogen, serum creatinine, and urinary kidney injury molecule protein.

**Conclusions:** These results indicated the potential involvement of urinary miRNAs in chemical-induced nephrotoxicity, suggesting that certain miRNAs could serve as biomarkers for acute nephrotoxicity.

**Keywords:** Acute kidney injury; biomarker; gentamicin; microRNAs; nephrotoxicity

## INTRODUCTION

The kidney is a primary target for chemical-induced toxicity. Especially the renal proximal tubules are frequently affected due to their role in the excretion of toxicants and toxicant-derived metabolites. However, it is still challenging to predict proximal tubular injury in both preclinical and clinical studies. Blood urea nitrogen (BUN) and serum creatinine (sCr) have been commonly used as traditional biomarkers for nephrotoxicity [1], but their low sensitivity and limited applicability for early pathogenesis represent a hindrance that should be overcome. Urinary proteins, such as kidney injury molecule-1 (KIM-1) has been considered

Hyun-Kyoung Lee   
<https://orcid.org/0000-0002-1984-8939>  
Gye-Hyeong Woo   
<https://orcid.org/0000-0003-2301-4184>  
Hwan-Goo Kang   
<https://orcid.org/0000-0002-6689-0080>  
Hyun-Ok Ku   
<https://orcid.org/0000-0002-7890-7919>

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#### Conflict of Interest

The authors declare no conflicts of interest.

#### Author Contributions

Conceptualization: Ku HO, Kang HG; Data curation: Jeon BS, Lee SH, Hwang SR; Formal analysis: Hwang SR, Bang JH; Funding acquisition: Ku HO; Investigation: Jeon BS, Lee SH, Hwang SR; Methodology: Lee SH, Hwang SR, Tham NTT; Project administration: Yi H, Jeon BS; Resources: Ku HO; Software: Hwang SR, Bang JH; Supervision: Ku HO, Kang HG; Validation: Hwang SR, Jeon BS; Visualization: Jeon BS, Lee HK, Woo GH; Writing - original draft: Jeon BS, Lee SH, Ku HO; Writing - review & editing: Yi H, Ku HO, Kang HG.

as a sensitive biomarker for nephrotoxicity [1,2]. Another class of biomolecules, microRNAs (miRNAs) has been recently explored as a novel biomarker type for nephrotoxicity [3-7]. In toxicity testing of chemicals, the combination with multiple biomarkers enable to improve the prediction of toxicity and facilitate the refinement of testing methods.

MiRNAs are endogenous noncoding RNA molecules ( $\leq 22$  nucleotides) that regulate the expression and function of the genome by targeting multiple mRNAs [8,9]. Different cell types display distinct miRNA expression profiles, and certain miRNAs are associated with the pathology of organ injury [10-12]. These miRNAs are relatively stable and readily detectable in biofluids (blood or urine) after injury [13,14]. This fact indicated that miRNA biomarkers could be employed as an efficient tool for the prediction of toxicity. Our previous studies suggested that certain miRNAs may have the potential of toxicological biomarkers for the identification of chemical hazards [15]. Pavkovic et al. [2] reported that miR-21 might have urinary biomarker potential for the detection of drug-induced acute kidney injury in humans by combining it with the urinary KIM-1 protein. Several studies have explored urinary miRNA biomarkers for nephrotoxicity in cisplatin or gentamicin (GEN)-treated rats [3-7]. It was reported that urinary miRNAs, such as miR-192 [3,5] or miR-378a, 1839, 140, 26b, and let-7g [3,6], were commonly upregulated in *in vivo* models of tubular injury induced by cisplatin. However, there was inconsistency in urinary miRNAs upregulated in GEN-induced nephrotoxicity models [4,7]. Overall, there was no agreement on urinary miRNA biomarkers between both *in vivo* models, despite showing similar tubular injury.

This study aimed to assess the potential of urinary miRNAs as biomarker for nephrotoxicity. To reduce animal use under animal welfare considerations, candidate urinary miRNAs were identified by reviewing previous studies with potential leads ( $> 1.5\text{-log}_2$  fold change and reproducibility). Here, GEN sulfate was employed as an inducing agent for *in vivo* nephrotoxicity models. GEN is an aminoglycoside antibiotic that has been in use for decades [16], and is generally administered parenterally because of its poor oral absorption [17]. GEN causes tubular damage through necrosis of tubular epithelial cells, predominantly in the proximal tubule [18]. It is often used to induce animal models of tubular injury [4,7,19]. In this experiment, rats were injected with  $< 50\%$  of the subcutaneous median lethal dose (from 817 to 893 mg/kg) of GEN [20]. The expression of miRNAs was determined by real-time reverse transcription-polymerase chain reaction (RT-PCR) in 16 h pooled urine from GEN-treated rats, and then compared between single and repeated-dose models, or between high and low-dose models to determine their potential as biomarkers for nephrotoxicity.

## MATERIALS AND METHODS

### Chemicals

GEN sulfate was purchased from Sigma-Aldrich Chemicals (USA). The test substances were freshly prepared in vehicle (sterile saline) on the day before treatments.

### Literature search for candidate miRNAs and chemical-induced acute kidney injury

A scientific literature search of the PubMed online database was performed using the keywords “microRNA” and “acute kidney injury.” The identified references were filtered to include only articles pertaining to chemical-induced acute tubular injury in rat models. We analyzed articles describing candidate miRNAs upregulated in acute kidney injury

and confirmed by the presence of necrosis in renal tubules. MiRNAs showing significant alteration with  $> 1.5$ -log<sub>2</sub> fold change and reproducibility in expression levels were selected for further analysis.

### Animal treatment

Eight-week-old female Sprague-Dawley rats (CrI:CD(SD); Orientbio Inc., Korea) were acclimatized to the facility environment for at least 5 days before treatment. They were maintained under controlled conditions, including temperature ( $22 \pm 3^\circ\text{C}$ ), relative humidity ( $55 \pm 10\%$ ), and alternating 12-h light and dark cycle. Water and an irradiated pellet diet (LabDiet, USA) were given *ad libitum* throughout the acclimatization.

Four rats per group were subcutaneously injected with a single dose or repeated doses (3 consecutive days) of GEN (412 mg/kg GEN base), or with repeated doses of a lower dose (137 mg/kg GEN) or vehicle (sterile saline), using a volume of 0.5 mL/100 g of body weight per injection. For urine collection, rats were transferred to metabolic cages 16 h prior to necropsy and fasted but given *ad libitum* access to drinking water. Urine was pooled for 16 h and collected at 24 h after final treatment in individual bottles on wet ice and centrifuged (16,000 g for 10 min at  $4^\circ\text{C}$ ) to remove debris. To obtain serum, whole blood was collected from the abdominal aorta of each rat under an inhalation anesthesia at 24 h after final treatment, allowed to clot for 1 h at room temperature and centrifuged at 1,900 g for 10 min at  $4^\circ\text{C}$ . All biofluid samples (urine and serum) were stored at  $-80^\circ\text{C}$  until further analysis. To confirm GEN-induced nephrotoxicity in rats, both kidneys were removed, sliced longitudinally, and fixed in 10% neutral buffered formalin for histopathological evaluation. Liver samples were also prepared for histopathology to obtain additional toxicological information. All animal experimental procedures were conducted in accordance with the Animal and Plant Quarantine Agency's Institutional Animal Care and Use Committee Guidelines (Study approved No. 2018-393 [2018.05.09]).

### Clinical biochemistry and histopathology

The rat serum and urine samples were tested for clinical biomarkers, including BUN, sCr, and urinary KIM-1 protein, as general biomarkers of nephrotoxic damage, along with urinary creatinine for normalizing KIM-1 protein. Clinical biochemistry parameters (BUN, sCr, and urinary creatinine) were measured using an Olympus AU400 Chemistry Analyzer (Diamond Diagnostic, USA) and biochemical reagents (Beckman Coulter Inc., USA). Urinary KIM-1 protein was assayed using a rat KIM-1 (TIM-1) ELISA kit (Abcam, UK) and a multi-spectrophotometer (FlexStation III; Molecular Devices, USA), and the values were normalized by urinary creatinine. Kidney tissues were further processed for microscopic examination by a qualified veterinary pathologist. Microscopic findings were graded as 0 (absent), 1 (minimal;  $< 5\%$  of the area with changes), 2 (mild; 5–25%), 3 (moderate; 25–50%), or 4 (severe;  $\geq 50\%$ ) for classifying renal lesions in toxicological pathology [4,7].

### RNA preparation and miRNA analysis

Total RNA was isolated from 200  $\mu\text{L}$  urine samples by using a modified small RNA purification protocol with the miRNeasy Serum/Plasma Kit (Qiagen, Germany). A synthetic miRNA (lyophilized *Caenorhabditis elegans* miR-39) was added to each sample during RNA isolation. Samples were eluted in 20  $\mu\text{L}$  of water to 10 sample equivalents per  $\mu\text{L}$ . The quantity and purity of the RNA preparations were determined by an ND-1000 spectrophotometer (NanoDrop Technologies, USA).

The isolated RNAs were reverse transcribed with a miScript II RT kit (Qiagen), and the complementary DNA product was used for miRNA analysis. The expression of miRNAs was analyzed by quantitative real-time RT-PCR with the CFX Connect Real-Time PCR System (Bio-Rad, USA) using an individual miScript Primer assay (Qiagen) and miScript SYBR Green PCR kit (Qiagen). The  $C_t$  value of each miRNA was normalized to that of the spike-in control genes. The values were expressed as treatment/vehicle ratios.

### Statistics

The data of the 4 animals per group are expressed as mean  $\pm$  SE. The significant differences between the treatments and vehicle control was determined by the Student's *t*-test for a single dose or analysis of variance with a post-hoc test for multiple doses;  $p < 0.05$  and  $p < 0.01$  were considered statistically significant. In the quantitative RT-PCR analysis, alteration by  $> 1.5\text{-log}_2$  fold change with  $p < 0.05$  or by  $> 3\text{-log}_2$  fold change without  $p < 0.05$  were considered.

## RESULTS

### Candidate urinary miRNAs in chemical-induced acute kidney injury

A scientific literature search performed with the keyword “microRNA” and “acute kidney injury” retrieved 257 articles. Only 6 studies on drug-induced acute kidney injury in rat models were thoroughly reviewed (Table 1). Acute kidney injury in rat models was confirmed by the presence of tubular necrosis. The urinary miRNAs upregulated with significant changes in response to drug-induced acute kidney injury are summarized in Table 1. There were no urinary miRNAs simultaneously upregulated in both cisplatin- and GEN-induced acute kidney injury. However, we identified several genes such as miR-192-5p [3,5], or let-7g-5p, miR-26b-3p, 140-3p, 378a-5p, and 1839-5p [3,6], that were reproducibly upregulated by  $> 1.5\text{-log}_2$  fold change in rat models (at least 2 studies) of tubular injury induced by cisplatin, a representative nephrotoxicant. In contrast, there was no consistency among urinary miRNAs with significant changes in GEN-induced acute kidney injury models (3 studies)

**Table 1.** Urinary miRNA candidates elevated in chemical-induced acute kidney injury

Toxicant	Traditional biomarker	Histology	MiRNA expression	Quantification	Reference
Cisplatin	BUN, sCr, urinary KIM-1/clusterin	Necrosis, proximal tubules/basophilic tubules/hyaline casts, tubules	miR-335, <b>miR-378a-5p</b> , miR-183-5p, miR-328a-3p, <b>miR-1839-5p</b> , let-7a-1-3p, miR-93-5p, <b>miR-192-5p</b> , miR-20b-5p, miR-17-5p, <b>miR-140-3p</b> , miR-340-5p, miR-191a-5p, <b>mmu-let-7g-5p</b> , miR-193-5p, miR-7a-1-3p, miR-130b-3p, <b>miR-26b-3p</b> , mmu-miR-7a-1-3p	qPCR	Kanki et al. [3]
Cisplatin	KIM-1	Degeneration/necrosis, proximal tubules/basophilic tubules	miR-16, miR-20a, <b>miR-21</b> , miR-34a, miR-141, miR-185, <b>miR-192</b> , miR-196c, miR-200b, miR-210	qPCR	Pavkovic et al. [5]
Cisplatin	BUN, sCr, KIM-1	Necrosis, renal tubule	<b>miR-378a</b> , <b>miR-1839</b> , <b>miR-140</b> , <b>miR-26b</b> , <b>let-7g</b> , miR-22	MiRNA sequencing	Wolenski et al. [6]
GEN	Urinary protein, $\beta$ 2-microglobulin, KIM-1	Degeneration/necrosis, proximal tubules/basophilic tubules	miR-134-5p, miR-342-3p, mmu-miR-494-3p, mmu-miR-207, mmu-miR-345-3p, mmu-miR-193b-3p	qPCR	Nassirpour et al. [4]
GEN	Urinary protein, $\beta$ 2-microglobulin, KIM-1	Degeneration/necrosis, proximal tubules/basophilic tubules	<b>miR-378a-3p</b> , miR-30e-3p, miR-125b-2-3p, miR-320-5p, miR-100-5p, <b>miR-21-3p</b> , miR-455-5p	MiRNA sequencing	Nassirpour et al. [4]
GEN	BUN, sCr, urinary KIM-1/clusterin	Degeneration/necrosis, proximal tubules / basophilic tubules/hyaline casts, tubules	mmu-miR-16-1-3p, miR-138-5p, <b>miR-140-3p</b> , miR-342-3p, miR-423-3p, miR-484, mmu-miR-1971, mmu-miR-218-1-3p, mmu-miR-28a-3p, mmu-miR-345-5p, mmu-miR-690, miR-489, miR-539-5p	qPCR	Zhou et al. [7]

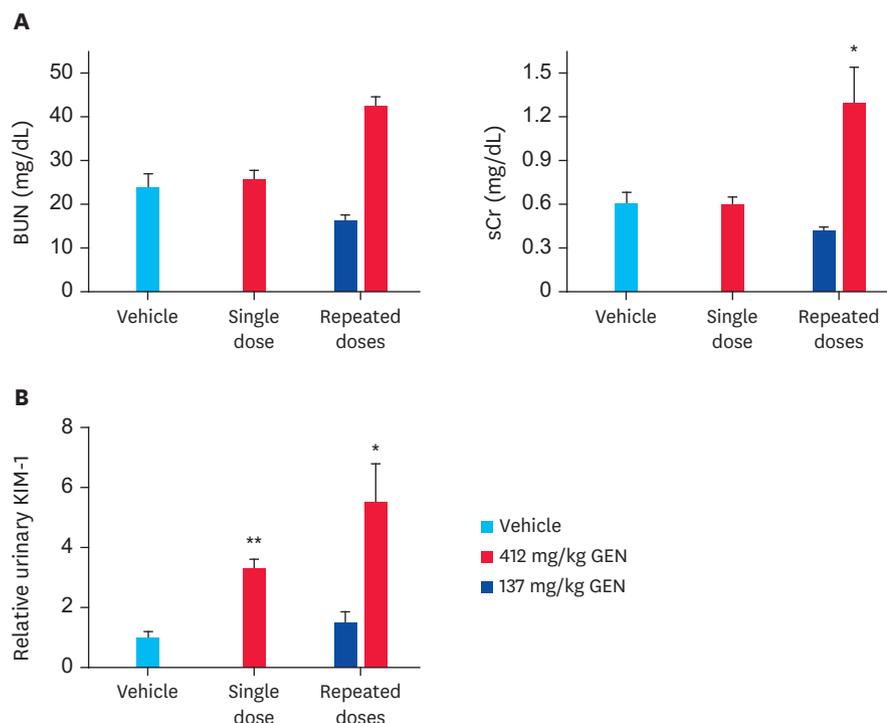
MiRNA candidates were selected in urine of rats with acute tubular injury by reviewing the previous studies and were cut-off by more than  $1.5\text{-log}_2$  fold change with statistical significance; some of the miRNAs (bold) were in agreements with the published articles.

MiRNA, microRNA; BUN, blood urea nitrogen; sCr, serum creatinine; KIM-1, kidney injury molecule-1; GEN, gentamicin; qPCR, quantitative polymerase chain reaction.

[4,7]. Nevertheless, we decided to investigate whether miRNA candidates, including miR-21-3p, 140-3p, and 378a-3p, simultaneously identified in both rat models of cisplatin- and GEN-induced acute kidney injury (at least one study) were reproducibly upregulated in GEN-induced nephrotoxicity models.

### GEN-induced acute tubular injury

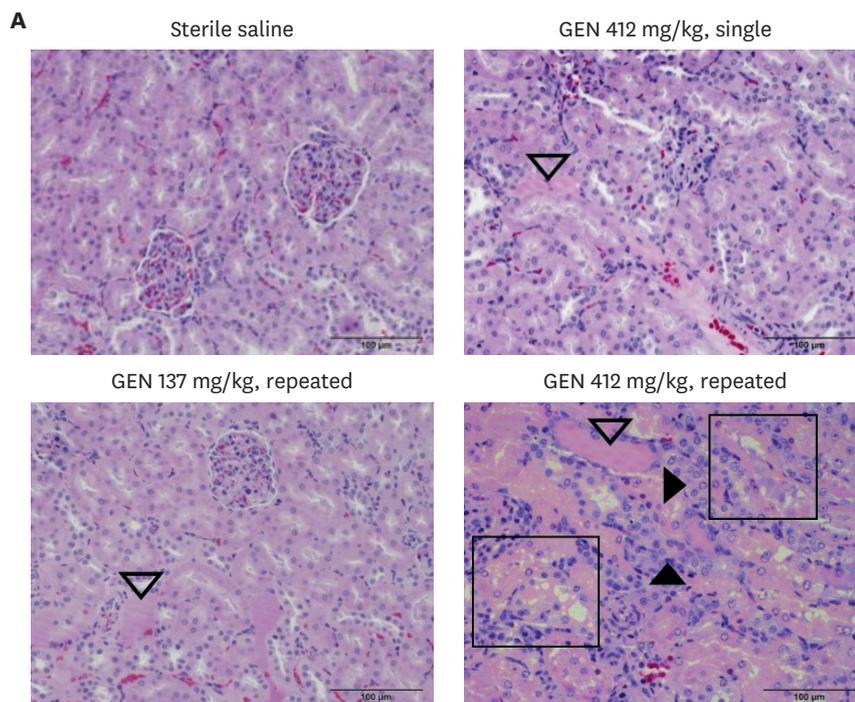
To establish a model for GEN-induced acute kidney injury, female Sprague-Dawley rats were sacrificed 24 h after the single and repeated (3 consecutive days) administration of GEN (137 or 412 mg/kg). In our rat models, there were differences in the serum and urine biomarkers for nephrotoxicity. The levels of the traditional biomarkers BUN and sCr were biologically or significantly increased after repeated administration of GEN (412 mg/kg) but not after the single and lower dose, compared with the vehicle group (**Fig. 1A**). However, higher levels of KIM-1 protein, a urinary biomarker for kidney injury, were observed after the single and repeated administration but not after the lower dose (**Fig. 1B**). GEN-induced nephrotoxicity was confirmed by histopathological examination of formalin-fixed kidney sections (**Fig. 2A**). The repeated GEN-dose treatment (412 mg/kg) mildly induced tubular degeneration and necrosis in the cortex and outer medulla area, along with the formation of hyaline (proteinaceous) casts that were observed in the same areas. In addition, basophilic tubules, representing regeneration, were observed in the cortex of the multiple-dose treatment group (**Fig. 2B**). Furthermore, hyaline casts were also detected after the single GEN-dose and the lower GEN-dose. In contrast, there were no detectable differences in the glomeruli and liver



**Fig. 1.** Levels of clinical biomarkers after single and repeated administration of GEN. CD rats were subcutaneously administrated with single dose or repeated doses (3 consecutive days) of GEN (137 or 412 mg/kg) or vehicle (sterile saline), and sacrificed 24 h after the final treatment. Urine samples were individually collected for 16 h prior to necropsy. (A) Clinical parameters in rat serum: BUN and sCr. (B) Relative expression of KIM-1 in rat urine, normalized to urinary creatinine content (ng/mg) and then expressed as treatment-to-vehicle ratios. Values indicate the mean  $\pm$  SE (n = 4).

BUN, blood urea nitrogen; sCr, serum creatinine; KIM-1, kidney injury molecule-1; GEN, gentamicin.

\* $p < 0.05$  and \*\* $p < 0.01$  compared with the vehicle control.



**B**

Dose	Degeneration/necrosis, proximal tubules in cortex & outer medulla	Basophilic tubules in cortex	Hyaline (proteinaceous) casts in cortex & outer medulla
Sterile saline (n = 4)	0	0	0
GEN 137 mg/kg, repeated (n = 4)	0	0	1.0 (3)
GEN 412 mg/kg, single (n = 4)	0	0	1.3 (3)
GEN 412 mg/kg, repeated (n = 4)	2.0 (4)	1.0 (4)	1.1 (4)

**Fig. 2.** Histopathological changes in kidney tissues after single and repeated administration of GEN. CD rats were subcutaneously administered with single dose or repeated doses (3 consecutive days) of GEN (137 or 412 mg/kg) or vehicle (sterile saline), and sacrificed 24 h after the final treatment. (A) Microscopic images (×200) of hematoxylin and eosin-stained formalin-fixed kidney sections (approximately 5 μm in thickness). Degeneration/necrosis (square areas) with basophilic tubules (arrowheads) were noted in the repeated treatment of GEN (412 mg/kg). Hyaline casts (arrowhead blank) in the tubules were observed in all GEN-treated groups. (B) Microscopic findings were graded on a scale of 0 (absent), 1 (minimal), 2 (mild), 3 (moderate), and 4 (severe). Data are shown as number of animals observed. GEN, gentamicin.

among the GEN- and vehicle-treatment groups. These data indicated that the serum and urine biomarkers were consistent with the kidney histopathology results.

### Urinary miRNAs upregulated in GEN-induced acute tubular injury

The levels of candidate miRNAs (let-7g-5p, miR-21-3p/5p, 26b-3p/5p, 140-3p/5p, 192-5p, 378a-3p/5p, and miR-1839-3p/5p) were determined by real-time RT-PCR in the rat urine samples of the single and repeated administration of GEN (412 mg/kg) and compared with those in the vehicle group (Table 2). The miR-26b-3p levels were significantly upregulated by more than a 1.5-log<sub>2</sub> fold change in the rat urine samples of GEN- treated groups, compared with those of the vehicle group. A statistically significant increase of the urinary let-7g-5p, miR-21-3p, and miR-192-5p level was only detected in the repeated GEN-dose group. The results for the urinary miR-378a family were not consistent; the miR-378a-3p level was

**Table 2.** Expression of candidate miRNA biomarkers in urine after single and repeated administration of GEN

Gene symbol	Sterile saline	GEN-single dose	GEN-repeated doses
let-7g-5p	1.0 ± 0.4	3.7 ± 2.2	4.1 ± 0.9*
miR-21-3p	1.0 ± 0.1	3.1 ± 1.0	3.2 ± 0.3**
miR-21-5p	1.0 ± 0.5	6.6 ± 4.1	5.7 ± 2.1
miR-26b-3p	1.0 ± 0.3	7.9 ± 1.7**	16.7 ± 2.8**
miR-26b-5p	1.0 ± 0.3	1.9 ± 1.0	1.0 ± 0.3
miR-140-3p	1.0 ± 0.4	4.7 ± 2.5	8.5 ± 5.9
miR-140-5p	1.0 ± 0.4	3.8 ± 2.0	2.8 ± 0.6*
miR-192-5p	1.0 ± 0.4	2.2 ± 0.5	16.0 ± 5.5*
miR-378a-3p	1.0 ± 0.3	7.0 ± 1.1**	17.1 ± 6.7
miR-378a-5p	1.0 ± 0.3	1.4 ± 0.4	2.1 ± 0.9
miR-1839-3p	1.0 ± 0.3	3.3 ± 1.7	1.0 ± 0.2
miR-1839-5p	1.0 ± 0.2	1.9 ± 0.4	5.9 ± 3.6

CD rats were subcutaneously administrated with a single dose or repeated doses (3 consecutive days) of GEN (412 mg/kg) or vehicle (sterile saline). Urine samples were individually collected for 16 h prior to necropsy at 24 h post-dose and used for quantitative real-time reverse transcription-polymerase chain reaction analysis. Treatment-to-vehicle ratios (fold changes) were calculated from the  $C_t$  value of each miRNA normalized to that of the synthetic spike-in control miR-39. Values indicate the mean ± SE (n = 4).

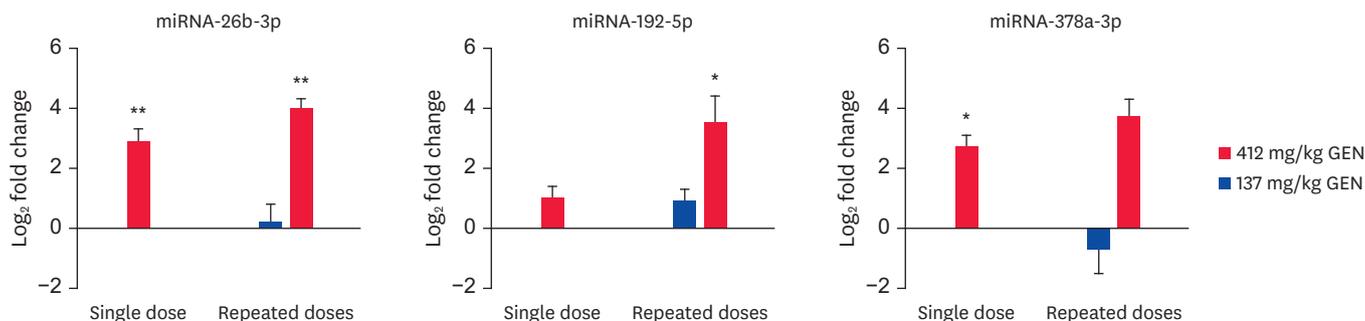
MiRNA, microRNA; GEN, gentamicin.

\* $p < 0.05$  and \*\* $p < 0.01$  compared with the vehicle control.

significantly increased but not the miR-378a-5p level. Other candidate miRNAs—miR-21-5p, 140-3p, and 1839-5p—were biologically increased by  $> 1.5\text{-log}_2$  fold change but showed no significant changes. Thus, our analysis indicated that let-7g-5p, miR-21-3p, 26b-3p, 192-5p, and 378a-3p could be used as reproducible biomarkers, consistent with clinical biomarkers and histological findings. Additionally, the levels of miR-26b-3p, 192-5p, and 378a-3p upregulated by  $> 3\text{-log}_2$  fold change, were compared with those in a lower GEN-dose (137 mg/kg) but there were no significant changes (Fig. 3).

## DISCUSSION

Recent studies have focused on the potential of miRNAs as a predictive biomarker in both nonclinical and clinical field. In toxicity testing for the identification of chemical hazards, the use of multiple biomarkers, including miRNAs, can help define pathology of organ injury. To date, it has been reported that there were no circulating miRNAs consistently upregulated



**Fig. 3.** Comparison of miR-26b-3p, miR-192-5p, and miR-378a-3p highly expressed in GEN-induced acute tubular injury. Female Sprague-Dawley were subcutaneously administrated with single dose or repeated doses (3 consecutive days) of GEN (137 or 412 mg/kg) or vehicle (sterile saline). Urine samples were individually collected for 16 h prior to necropsy at 24 h post-dose and used for quantitative real-time reverse transcription-polymerase chain reaction analysis. The treatment-to-vehicle ratios (fold changes) were calculated from the  $C_t$  value of each miRNA normalized to that of a synthetic spike-in control RNA, miR-39, and transformed to  $\log_2$ . Values indicate the mean ± SE (n = 4).

MiRNA, microRNA; GEN, gentamicin.

\* $p < 0.05$  and \*\* $p < 0.01$  compared with the vehicle control.

in *in vivo* models of kidney injury [6,19]. However, it is hypothesized that miRNAs that are affected by kidney injury may be directly released into the urine from tissues.

In this study, we assessed whether urinary miRNAs could be employed as biomarkers for nephrotoxicity. For this, we identified miRNA candidates that were significantly upregulated in response to chemical-induced acute kidney injury by reviewing previous studies [3-6]. The rat model of acute tubular injury was confirmed by the occurrence of tubular necrosis, but there was no agreement on specific miRNA biomarker candidates (**Table 1**). Here, candidate urinary miRNAs (let-7g-5p, miR-21-3p/5p, 26b-3p/5p, 140-3p/5p, 192-5p, 378a-3p/5p, and miR-1839-3p/5p), which were reproducibly upregulated in an appropriate rat model, were selected for further study.

Prior to miRNA analysis, we confirmed a GEN-induced acute tubular injury model in female Sprague-Dawley rats. Acute tubular injury was reproduced only in the repeated GEN-dose treatment (412 mg/kg), showing tubular degeneration and necrosis in proximal renal tubules (**Fig. 2A and B**). However, there were differences in the response of toxicological biomarkers to kidney injury. The levels of traditional biomarkers BUN and sCr level were elevated only in the repeated GEN-dose treatment (412 mg/kg) (**Fig. 1A**), consistent with the histopathological results, but the urinary KIM-1 protein level was significantly increased after the single and repeated administration of GEN (412 mg/kg) (**Fig. 1B**). The present study demonstrated that known nephrotoxicity biomarkers in serum and urine relatively correlate with the kidney histopathology results, consistent with previous studies [4,7]. Furthermore, it has been shown that the combination of multiple biomarkers could be useful in assessing nephrotoxicity potential of chemicals.

To evaluate candidate miRNA biomarkers, their levels were determined by real-time RT-PCR in 16 h pooled urine samples from rats treated with single or repeated doses of GEN (412 mg/kg). The present study demonstrated that let-7g-5p, miR-21-3p, 26b-3p, 192-5p, and 378a-3p were upregulated by  $> 1.5\text{-log}_2$  fold change with statistical significance in GEN-induced nephrotoxicity models, consistent with the presence of tubular necrosis (**Table 2**). Consistent with previous studies [4,5], miR-21-3p was significantly upregulated in the GEN-induced kidney injury model. It has been indicated that miR-21 could be strongly associated with renal pathogenesis in acute kidney injury [2,21,22]. We demonstrated that miR-21-3p upregulated in cisplatin- and GEN-induced tubular injury was also upregulated in our GEN-induced tubular injury model but not sensitive ( $< 3\text{-log}_2$  fold change). We demonstrated that let-7g-5p and miR-26b-3p, commonly upregulated in rat models of cisplatin-induced acute tubular injury [3,6], were also significantly increased in our GEN-induced tubular injury model. Our results showed that miR-26b-3p might be a useful urinary biomarker for nephrotoxicity of chemicals. The expression of miR-26b-3p in urine has also been identified as a significantly upregulated by  $> 3\text{-log}_2$  fold change, while that of let-7g-5p was increased by  $> 1.5\text{-log}_2$  fold change. Moreover, miR-26b-3p were also upregulated in rats after administering a single GEN dose, indicating that could be detectable earlier than the appearance of clinical effects such as histopathology. Hence, it could be considered sensitive urinary biomarkers for detecting renal tubular injury induced by chemicals. The present study demonstrated that miR-192-5p was upregulated in the urine of rat models of kidney injury, confirmed by the presence of tubular necrosis. It has been reported that miR-192 is abundantly expressed in the human and animal kidney [23,24]. In previous *in vivo* studies, miR-192-5p was upregulated in cisplatin-induced tubular injury but not in GEN [2,3]. However, our results indicated that urine miR-192 levels were in parallel with histopathology results in acute kidney injury. Interestingly,

miR-192-5p was expressed in the liver and proposed as a biomarker for acute liver injury in a previous study [15]. Thus, it is likely that miR-192-5p is a toxicological biomarker for organ injury but not specific to kidney injury. Our results with the urinary miR-378a family were not consistent with previous studies [3,4,6]. It has been reported that miR-378a-5p was upregulated in acute tubular injury induced by cisplatin [3,6]. However, the present study showed that only miR-378a-3p was significantly increased in GEN-induced acute tubular injury, same as another study [4]. These results indicated that miR-378a family could play a potential role in the pathogenesis of chemical-induced acute tubular injury. Zhang et al. [25] reported the miR-378 was differentially expressed in cisplatin-induced acute kidney injury models and could affect regulation of inflammation and cell apoptosis. Furthermore, the expression of these miRNAs was compared with lower GEN doses (**Fig. 3**). Specifically, high expression of miR-26b-3p, 192-5p, and 378a-3p was observed in the urine of rats with GEN-induced acute tubular injury, which was in parallel with the kidney histopathology results. These miRNAs were considered to have sensitivities comparable to clinical biomarkers, such as BUN, sCr, and urinary KIM-1 protein.

In conclusion, we identified miR-26b-3p, 192-5p, and 378a-3p with highly expressed levels in the urine of rats with GEN-induced acute tubular injury, consistent with clinical endpoints (elevated levels of BUN, sCr, and urinary KIM-1, along with the presence of tubular necrosis). Our results indicated that urinary miRNAs could potentially serve as biomarkers in chemical-induced nephrotoxicity, although further studies investigating different types of acute kidney injury are still needed.

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