Discovery of an integrative network of microRNAs and transcriptomics changes for acute kidney injury

Chan Gyu Lee[1], Jin Geol Kim[1], Hyun Joo Kim[1], Hyuk-Kwon Kwon[2], Il Je Cho[3], Dal Woong Choi[4], Woo Hyung Lee[1], Won Dong Kim[1], Se Jin Hwang[5], Sangdun Choi[2] and Sang Geon Kim[1]

[1]Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul, Korea; [2]Department of Molecular Science and Technology, Ajou University, Suwon, Korea; [3]College of Oriental Medicine, Daegu Haany University, Daegu, Korea; [4]Department of Public Health Science, Graduate School, College of Health Sciences, Korea University, Seoul, Korea and [5]College of Medicine, Hanyang University, Seoul, Korea

The contribution of miRNA to the pathogenesis of acute kidney injury (AKI) is not well understood. Here we evaluated an integrative network of miRNAs and mRNA data to discover a possible master regulator of AKI. Microarray analyses of the kidneys of mice treated with cisplatin were used to extract putative miRNAs that cause renal injury. Of them, miR-122 was mostly downregulated by cisplatin, whereas miR-34a was upregulated. A network integrating dysregulated miRNAs and altered mRNA expression along with target prediction enabled us to identify Foxo3 as a core protein to activate p53. The miR-122 inhibited Foxo3 translation as assessed using an miR mimic, an inhibitor, and a Foxo3 3′-UTR reporter. In a mouse model, Foxo3 levels paralleled the degree of tubular injury. The role of decreased miR-122 in inducing Foxo3 during AKI was strengthened by the ability of the miR-122 mimic or inhibitor to replicate results. Increase in miR-34a also promoted the acetylation of Foxo3 by repressing Sirt1. Consistently, cisplatin facilitated the binding of Foxo3 and p53 for activation, which depended not only on decreased miR-122 but also on increased miR-34a. Other nephrotoxicants had similar effects. Among targets of p53, Phlda3 was robustly induced by cisplatin, causing tubular injury. Consistently, treatment with miR mimics and/or inhibitors, or with Foxo3 and Phlda3 siRNAs, modulated apoptosis. Thus, our results uncovered an miR integrative network regulating toxicant-induced AKI and identified Foxo3 as a bridge molecule to the p53 pathway.

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Correspondence: *Sang Geon Kim, Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Gwanakno 599, Seoul 151-742, Korea. E-mail: sgk@snu.ac.kr*

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Acute kidney injury (AKI), which is most commonly caused by nephrotoxicants, markedly increases morbidity and mortality in hospitalized patients.[1,2] A variety of chemicals such as drugs and environmental materials can induce AKI.[3] Drugs have an ongoing etiological role in the occurrence of nephropathy for their direct damaging action on tubular cells. It is recognized as the most common cause of kidney-related problems because nontoxic concentration of the drug during treatment could reach toxic levels in the kidney. In particular, renal tubular cells are vulnerable to the toxic effects of drugs because of their role in concentrating and reabsorbing the glomerular filtrate. Despite the ongoing studies, prevalence of drug-induced AKI remains significantly high. As chemical-induced nephropathy accompanies the disruption of certain signal transduction pathways, understanding of the signaling and working molecules would be of value in finding a way to prevent toxicity and/or treat kidney injury.

The microRNA (miRNA) negatively regulates gene expression by base-pairing with the 3′-untranslated region (UTR) of target messenger RNA.[4] Abnormal levels of miRNA could be one of the mechanisms explaining dysregulated protein expression during kidney disease progression.[5] Although profiles of miRNA expression have been examined in renal diseases, such as polycystic disease, diabetic nephropathy, renal cancer, and fibrosis,[6–9] few studies have specifically described the identification of miRNAs and their roles in AKI. Moreover, little information was available on the network integrating miRNA dysregulation and altered mRNA expression that occur in nephropathy. Considering the fact that the correlation between transcriptome and proteome expression is not always linear because of posttranscriptional regulation of mRNA translation and protein modifications,[10] an integrative network of both miRNA and mRNA databases may provide key information in identifying the factors involved in drug-induced toxicity, shedding light on new strategies for the management of AKI.

Drugs such as cisplatin, aminoglycosides, and amphotericin B induce tubular injury. In particular, cisplatin is a widely prescribed agent; its main dose-limiting side effect is

AKI itself, and it is a direct inducer of proximal and distal tubular epithelial cell damage. Nephrotoxicity of cisplatin is a composite result of many presentations implicated in drug-induced nephrotoxicity, such as drug transport, activation of multiple pathways, and initiation of inflammatory response,[11] which makes cisplatin a suitable model for AKI.

This study investigated an integrative regulatory network of altered miRNA and transcript levels governing AKI induced by cisplatin treatment. In particular, we were interested in identifying the specific miRNAs that directly control a bridge molecule leading to cell death during AKI. Of the miRNAs differentially expressed in the miRNA microarrays, we found the most significant reciprocal changes in the levels of miR-122 and -34a during renal injury. Moreover, we identified their coordinated translational and posttranslational roles in activating Foxo3 as a molecule bridging to p53-dependent apoptosis. To assess the degree of nephropathy, a cascade of cell death pathway, and the underlying basis, both animal and cell models were utilized along with a bioinformatic approach using the outcomes of miRNA and mRNA microarrays. In addition, our findings uncovered the functional role of Phlda3, an effector molecule substantially induced downstream of p53, in tubular cell death.

RESULTS

miRNAs dysregulated in AKI caused by cisplatin

To identify the miRNAs dysregulated in AKI induced by cisplatin, renal miRNA microarray analyses were carried out in mice at day 3 after a single intraperitoneal (i.p.) injection of cisplatin (15 mg/kg). Seven miRNAs showed significant changes in expression on cisplatin treatment when a cutoff of $P<0.05$ was used; the expression of five was decreased, whereas that of two was increased (Figure 1a). Of them, miR-122 levels showed the greatest downregulation compared with vehicle-treated control, whereas expression of miR-34a was distinctly increased. Quantitative reverse transcription-PCR assays confirmed the changes (Figure 1b). In the time-course study, miR-122 levels decreased on day 1 after cisplatin treatment and remained low until day 3. At day 5, they were slightly increased from the trough (Figure 1c, left). However, miR-34a levels increased significantly at day 3 and were further increased at day 5 after treatment (Figure 1c, right). In a subsequent study, we narrowed our focus on dysregulation of miR-122 and -34a because the changes were the greatest.

a

Log2 (Cisplatin/vehicle) ratio of differentially expressed miRNAs that reached statistical significance by T-test ($P < 0.05$)

Downregulated miRNAs			Upregulated miRNAs		
miRNA	Log2 ratio	*P*-value	miRNA	Log2 ratio	*P*-value
miR-122	–3.39	0.0326	miR-34a	1.11	0.0213
miR-10b*	–1.16	0.0428	let-7g	0.27	0.0420
miR-30e	–0.44	0.0363			
miR-193	–0.26	0.0363			
miR-26a	–0.31	0.0293			

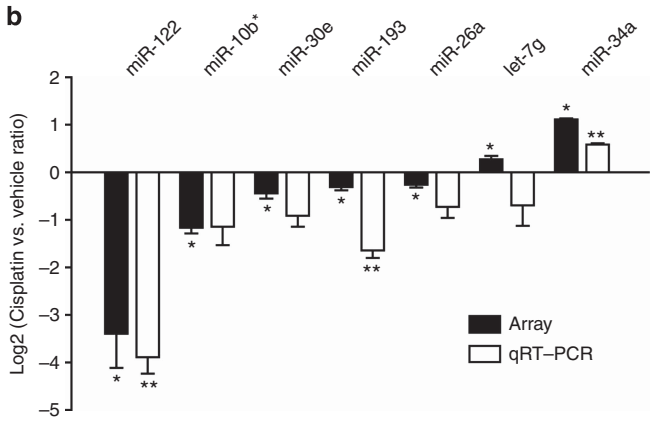

Figure 1 | The expression profiles of microRNAs (miRNAs) in the kidney after cisplatin treatment. (**a**) A list of differentially expressed miRNAs in the microarray analysis, including log2 ratio and *P* values. Microarrays were performed with miRNA samples extracted from the kidney at day 3 after a single administration of vehicle or cisplatin (15 mg/kg) to C57BL/6 mice. (**b**) Quantitative reverse transcription PCR (qRT-PCR) validation of differentially expressed miRNAs. Results are shown as mean of log2(cisplatin/vehicle) and are presented side-by-side with the respective array results. (**c**) The effects of cisplatin treatment on the levels of miR-122 and -34a. For **b** and **c**, values represent the mean ± s.e.m. (significantly different as compared with vehicle treatment according to array or qRT-PCR analysis, respectively, $*P<0.05$, $**P<0.01$; $N=3$ per group in miRNA array and $N=4$ per group in qRT-PCR).

An integrated regulatory network of dysregulated miRNAs and mRNAs

To facilitate the prediction of targets possibly interacting with miR-122 and -34a, we performed cDNA microarray analyses using the kidneys of mice treated with a single dose of cisplatin (15 mg/kg, at day 3), and the microarray profiles were compared (Figure 2a). Among those represented on the microarray, 36 genes were differentially expressed by cisplatin treatment when $P<0.01$ and a twofold change cutoff were used. Bioinformatic analyses using TargetScan algorithms allowed us to select the targets putatively regulated by miR-122 and -34a. In this approach, we focused on five candidate genes that have potential to be regulated by decreased miR-122 in association with cell death (Supplementary Table S1 online). Twelve genes were chosen as possible targets affected by increased miR-34a (Supplementary Table S2 online). Integrated analyses of miRNA and mRNA expression data