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Urinary microRNAs in environmental health: biomarkers of emergent kidney injury and disease

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Abstract

Purpose of review: There is a critical need for sensitive biomarkers of renal disease and progression. Micro(mi)RNAs are attractive as next-generation biomarkers in kidney disease, particularly as urine miRNAs can inform kidney function and cellular integrity. This review summarizes recent epidemiologic and toxicologic advances using urinary miRNAs and exosomal miRNAs as novel biomarkers of chemical exposure and of kidney damage and disease.

Recent findings: Urine miRNA biomarkers offer improved stability over protein in stored samples, relative ease of collection and quantitation, and conserved sequence homology across species. Particularly in the case of emergent environmental health threats such as chronic kidney disease of unknown origin, urinary miRNAs hold promise as biomarkers of disease and/or exposure.

Summary:

We present evidence to address scientific knowledge gaps, comment on the relevance of urine-derived miRNAs in environmental health research, and discuss limitations and recommendations for future directions needed to advance miRNA biomarker strategies.

Keywords

microRNA; exosome; environmental toxicant; chemicals; acute kidney injury; chronic kidney disease; glomerular filtration rate

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Introduction

Acute and chronic kidney disease contribute to a high public health burden, and patients requiring subsequent renal replacement therapy experience a mortality rate of 50–80% [1], even in high resource medical settings. While once thought to be discrete syndromes, there is increasing evidence that acute kidney injury (AKI) is a substantial risk factor for the development of chronic kidney disease (CKD) [2]. Furthermore, emerging health threats, such as the epidemic of chronic kidney disease of unknown origin in agricultural workers lacking classic risk factors of diabetes mellitus or hypertension, has sparked global interest in kidney health surveillance and assessment of environmental risk factors associated with farming practices and agrochemicals, heat stress, and water quality [3].

In order to advance surveillance of kidney disease in patient populations, there is a critical need for sensitive and specific biomarkers of disease and progression [4]. A key diagnostic challenge in AKI or early stage CKD is that conventional kidney injury biomarkers, including serum creatinine (sCr) and blood urea nitrogen, do not elevate outside the normal range until there is a relatively high degree of underlying injury to the kidney tissue. In addition, traditional measures such as the glomerular filtration rate (GFR) for assessing renal insufficiency is complicated by the need for specialized facilities. The estimated GFR is a measure that is more accessible, however this measure is indirect as compared to the “true” GFR [5]. Serum creatinine is particularly insensitive owing to a long lag time prior to sCr elevation. Further, increases in sCr can be also caused by decreased glomerular filtration arising from non-renal causes including intravascular volume depletion, increased abdominal pressure, or reduced cardiac function [6]. The challenges inherent in standard AKI and early stage CKD monitoring have sparked advances in kidney biomarker research [6]. Urine is an optimal non-invasive and accessible biofluid for biomarker measurements and urine-based biomarkers are derived directly from the kidney and urogenital tract [7].

Encouragingly, regulatory qualification efforts have successfully introduced urine-based protein biomarkers following kidney injury for use in nonclinical rat safety assessments [8]. Urinary concentrations of several protein biomarker levels inform underlying renal histopathology, and preliminarily hold some added diagnostic potential for clinical surveillance of renal injury in a hospital setting. Two of these markers, urinary kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) are widely assessed markers of kidney damage [9], yet limited clinical data and defined boundaries of normal levels exist for other urine proteins. Moreover, protein-based biomarkers have limited sensitivity, may not be evident until later stages of disease progression, are difficult/expensive to measure, and exhibit poor stability in stored samples [10]. As such, discovery efforts to identify non-protein urinary biomarkers of kidney disease that may be more sensitive to detect early and subclinical injury are critical. Omics approaches to urinary proteins, transcripts, and metabolites for detection of kidney disease have expanded extensively in the last decade (reviewed elsewhere [11–13]), yet urinary microRNAs (miRNAs) remain largely unexplored in environmental health discovery efforts towards exposure-associated renal diseases.

Urinary miRNAs offer a potential alternative to urinary protein biomarkers for detection. MiRNAs are small (approximately 22 nucleotides), non-coding RNAs that play an essential role in post-transcriptional regulation of gene expression. Particularly in the case of emergent environmental health threats, urinary miRNAs hold promise as biomarkers of disease and/or exposure owing to their known mechanistic roles as endocrine signaling molecules in stress response, their relative stability in biofluids during tissue injury, and their ease of measurement in resource-poor regions. Yet knowledge gaps remain around utility of these biomarkers that the field must address. This review aims to summarize recent epidemiologic and toxicologic studies using urinary miRNAs as novel biomarkers of kidney disease and nephrotoxic exposures, and comment on their relevance in the nascent field of nephrotoxicant environmental health research.

Characteristics of miRNAs

MiRNAs are small, endogenous RNA molecules approximately 21–25 nucleotides in length. MiRNAs, like proteins, are detected in a variety of biofluids including urine, blood, cerebrospinal fluid, saliva, and tears among others. MiRNAs have a functional role within the cell to regulate post-transcriptional gene expression via transcript inhibition or destabilization [14]. In this role, miRNAs regulate a wide variety of transcriptional pathways, both homeostatic and during pathogenic response to perturbation. However, there are key characteristics of miRNAs that offer advantages over protein biomarkers. In whole blood, plasma and serum, miRNAs are stable up to 24 h, although specific miRNAs are more or less resilient depending on whether they are complexed with proteins or encapsulated in membrane-bound vesicles [15, 16]. Importantly for translatability across species (for example, rat to human), miRNA sequences are conserved across mammalian species, meaning that the same detection assay can often be utilized. miRNAs are easily measured using sensitive transcriptomic detection assays such as RT-PCR, and can be quantified using standard curves and synthetic miRNA standards [17]. Further, miRNAs exhibit a wide dynamic concentration range when measured in an extracellular biofluid [18, 19].

Extracellular miRNAs released into urine are packaged in a variety of ways that improve their stability and protect from degradation by endogenous nucleases. Cells can secrete miRNAs packaged as protein-miRNA complexes or secrete miRNAs through exocytosis of extracellular vesicles (EVs) [20–22]. Exosomes are a subclass of small endosome-derived EVs that are 30–150 nm in diameter [23, 24] and play essential roles in cell communication, performing paracrine-and endocrine-like messaging functions [25, 26]. Urinary EVs, exosomes, and miRNAs released from renal epithelial cells can serve as a “liquid biopsy” to determine cellular status in distinct regions of the renal nephron. In this way, these biomarkers can either be released actively during intracellular signaling processes or passively as a cellular leakage marker when the cell membrane is compromised (as occurs during cellular membrane leakage and cell death) [25]. EVs, including exosomes, regulate intercellular homeostasis and facilitate intercellular communication along the nephron and represent a separate compartment of the urine miRNA complement. Moreover, exosomal-contained miRNAs originating from kidney-specific cell types may be directly responsible for physiologic changes in blood pressure in animal models [27, 28]. While miRNAs and

exosomal miRNAs have been the focus of intense study in the blood compartment, they are comparatively unexplored in urine, particularly in exposure-associated molecular-and systems-based networks in renal and urinary disease pathophysiology.

Urinary microRNA in environmental health

A growing body of literature has examined tissue-specific or blood-based miRNA expression in relation to a number of environmental contaminants including cigarette smoke, air pollution, endocrine disruptors, metals, benzene, trichloroethylene, and organic chemical mixtures [29–33]. In contrast, the urinary compartment has received limited attention. A recent review by Kotsyfakis *et al.* included a single study of exposure-associated urine miRNA biomarkers [31]. To our knowledge, no study to-date has specifically examined urinary miRNAs contained within EVs with respect to environmental toxicant exposure and more work is needed in this area to identify functional biomarkers.

Two studies have investigated whole urine miRNAs associated with chemical exposures without performing enrichment for EVs or exosomes. A cross-sectional study of 27 parent/child, farmworker/non-farmworker pairs in Washington State assessed 384 urine miRNAs using an array-based PCR assay and found that six miRNAs were associated with farmworker status [34]. Five of those miRNAs showed a suggestive dose-response relationship with organophosphate pesticide metabolites in farmworkers including miR-223, −518d-3p, −597, −517b, and −133b [34]. Several identified miRNAs had target genes associated with neurotransmitter receptor binding and activity, including the acetylcholinesterase and cholinesterase pathways -known pathways impacted by organophosphate pesticides. In another cross-sectional study of 83 children in Mexico co-exposed to arsenic, cadmium, chromium, fluoride and lead, examined pair-wise associations with three urine miRNAs [35] selected *a priori* as previously identified biomarkers of kidney injury in adults: miR-200c, miR-423, and miR-21 [36]. MiR-200c and miR-423 as well as KIM-1 were associated with the highest tertile of chromium exposure [35]. Although the two studies varied by environmental exposures of interest and scope of miRNAs assessed, taken together they highlight the utility of urinary miRNAs as promising non-invasive biomarkers of exposure and biological response.

Additionally, we identified two studies of clinically-relevant exposures and urine miRNA biomarkers. In a study comparing extracellular miRNA from 10 healthy children to 10 children hospitalized for acetaminophen overdose and also to those receiving therapeutic acetaminophen doses (n=8), elevated levels of urine miR-375, −940, −9–3p and −302a were observed among those in the overdose group compared to the other groups [37]. Intriguingly, serum levels of miR-375 were also associated with acetaminophen adduct levels along with serum miR-122 (a known biomarker of hepatotoxicity [38], suggesting urine miRNA may be useful biomarkers of acetaminophen exposure or hepatotoxicity. Potential nephrotoxic effects were not reported. A separate proof-of-concept study using cultured proximal tubule cells as well as mouse and human urine samples demonstrated the translational utility of urinary miRNA as biomarkers of radiation nephropathy [39]. Specifically, among 600 miRNAs analyzed with a hybridization-based nCounter platform, 12 irradiation-responsive miRNAs were identified in mouse urine, of which miR-1224–3p and miR-21 were pursued

for validation in urine from leukemia patients undergoing total body irradiation [39]. The findings suggest that miR-1224 may be a biomarker involved in the acute molecular response to tissue injury and inflammatory response, whereas miR-21 may serve as a non-specific late molecular responder to radiation exposure [39]. Taken together, these studies highlight the promise of miRNAs as biomarkers in the clinical context for a variety of organ injuries. While additional research and validation is needed, these studies highlight insights to be leveraged from urinary miRNAs in clinical translation and toxicant-dependent contexts.

Urinary miRNA as clinical biomarkers

MiRNAs have been investigated as a complementary approach to detect AKI more generally, which is associated with high inpatient mortality, particularly in pediatric populations [40, 41]. AKI results from an abrupt decrease in renal function as indicated by increased sCr level and is an early risk factor for CKD and recurrent episodes of AKI may additionally play a role in development of CKD and irreversible kidney disease [42]. Individuals with underlying injury to the tubule cells may be more susceptible to toxic, inflammatory, or hemodynamic challenges versus their healthy counterparts [43].

MiRNAs hold particular utility as early stage responsive molecules in kidney injury and disease [44–46]. In one of the first published studies of miRNA biomarkers for AKI, urine samples pooled from 6 healthy controls compared to 6 patients with AKI, 378 miRNA of 1809 profiled were stably detected in all 12 samples. Notably, 4 were significantly different between the AKI and control group including miR-21, miR-200c, and miR-423, and miR-4640 [36]. AKI is a frequent complication of major cardiac surgery and these patients provide an opportunity to investigate AKI-linked miRNA biomarkers. Abundance of miR-21 in the blood was reduced in 42 patients that experienced postoperative AKI and these patients furthermore had a significantly increased mortality over the 2.9-year follow-up period [47]. Thus, miR-21 has been proposed as a biomarker to stratify high-risk patients following major cardiac surgery. While there remains more work to be done to investigate miRNA profiles in urine during AKI in the clinic, candidate biomarkers that merit further development have been evaluated in kidney biopsy tissue. An example includes miR-182–5p, which was found to be elevated in expression in kidney in patients with post-transplant AKI [48].

Similarly, the field is beginning to put effort behind identifying miRNA biomarkers associated with ongoing CKD. Recent investigations on urinary miRNAs changing with kidney injury in humans include the derivation of an extracellular miRNA signature in CKD in humans based on association with a reduced glomerular filtration rate [49, 46]. Notably in a recent study in which RNA-seq was used to examine several classes of noncoding RNAs contained in urinary exosomes of 15 patients of various stages of CKD compared to 10 healthy controls' exosomes, miR-181a was identifed as a robust and stable biomarker for CKD [46]. Additionally, urinary concentrations of miR-1825 and miR-1281 have been found to be elevated in the urine and plasma of patients with eGFR<30 [49]. In a mouse model of diabetic nephropathy, miR-415–5p was found to be elevated in urinary exosomes three weeks prior to significant albuminuria and three weeks before glomerulosclerosis and

tubulointerstitial nephritis were observed histologically [50]. Confirmation of these miRNA biomarkers in the clinic will require follow-up studies.

Urine miRNA biomarker development in experimental animals (acute kidney injury)

Critical to urine miRNA biomarker discovery is the ability to utilize experimental model systems in which urinary biomarker concentrations can be benchmarked to underlying histopathology to build confidence in disease-biomarker associations. There is interest in utilizing a miRNA biomarker strategy to detect kidney injury in nonclinical settings, such as safety pharmacology studies in pharmaceutical development, and a concerted effort has been made in this area. Importantly, because of conservation in miRNA sequences across mammalian species, there are translational implications of animal studies for biomarker applications in the clinic.

Notably, biomarker discovery efforts in rats – led by the Health and Environmental Sciences Institute (HESI) Emerging Systems Toxicology for the Assessment of Risk (eSTAR) Committee's Biomarkers of Nephrotoxicity Working Group – has revealed a number of potential biomarker candidates. These cross-laboratory efforts represent a partnership between academia, the pharmaceutical industry, and government and has the goal of identifying miRNA biomarkers that leak out into the urine during damage to specific regions of the renal nephron (glomerulus, proximal tubule, loop of Henle, or collecting duct).

This effort has resulted in a number of studies and publications, which are the subject of a forthcoming meta-analysis. Here, we highlight several of the most promising candidate biomarkers. A rat study of doxorubicin exposure, a chemotherapeutic drug which targets the glomerulus, identified increased urinary miR-34c-3p as a potential biomarker that increased prior to observing albuminuria [19]. Because samples were collected temporally following exposure, it was possible to observe urinary elevations in miR-34c-3p prior to observations of glomerular injury via renal histopathology, indicating that the miRNA was a particularly sensitive biomarker for detecting early stage kidney injury. Furthermore, expression of miR-34c-3p was found to be preferentially expressed in the glomerulus versus the tubular cells, which suggests that this miRNA species may be a site-specific marker for preferential detection of glomerular injury.

Similarly, a rat study evaluating cisplatin, a chemotherapeutic drug and proximal tubular toxicant, identified several urinary miRNAs were increased earlier than blood urea nitrogen and sCr [51] and miRNAs were comparable to KIM-1 with respect to timing of release into the urine. In a related study in rats exposed to three toxic agents, urinary miR-210–3p was elevated with exposure to collecting duct toxicant N-phenylanthranylic acid (NPAA) [52] and also in response to proximal tubular toxicant cisplatin [51]. Furthermore, miR-335 urinary levels were altered after exposure to either puromycin or cisplatin [53]. Nassirpour *et al.* showed that several urinary miRNAs were elevated in urine samples in two rat models of glomerular injury: puromycin exposure and a Heymann nephritis model [54].

In a rat study that was conducted independently of the HESI eSTAR efforts, longitudinal miRNA profiling of urinary exosomes showed that miRNA concentration tracks with progression of AKI [55]. Specifically, the exosomal miRNAs miR-16, miR-24, and

miR-200c were increased in urine acutely following kidney injury, and target mRNA expression was reduced preferentially in the kidney medulla [55]. Additional miRNAs were identified in urine during AKI recovery stages; miR-9a, −141, 200c, and −429 and their target mRNAs within the TGF-β signaling pathway were altered, suggesting a potential regulatory mechanism in AKI progression [55].

Throughout the course of the cross-partner eSTAR committee projects, it became evident that nephrotoxic agents most frequently targeted more than one cell type or nephron segment, which complicated observation of a “pure” lesion and assessment of nephron regional site-specificity. In the doxorubicin study, for example, primary glomerular lesions were observed along with secondary tubular lesions [19]. Further, there are clearly miRNAs that are expressed across sites in the kidney, which may have utility broadly as kidney biomarkers but less utility for assessing site of the pathological lesion. Depending on the application, this may not pose a deterrent to their use as functional kidney biomarkers. For a full list of HESI eSTAR committee publications on miRNA biomarkers, visit https://hesiglobal.org/emerging-systems-toxicology-for-assessment-of-risk-committee/.

Challenges and Future Directions

More investment is required to identify specific miRNAs altered in urine as kidney injury markers across species, or more generally as acute or chronic kidney damage markers in animals or humans. Although many studies have reported changes in urinary concentrations of miRNAs following kidney damage, they are mostly separate exploratory studies, represent acute or chronic kidney injury induced with reference toxicants or associated with certain diseases, may or may not consider EV-derived miRNAs, and use varied miRNA isolation and detection platforms and analytical methods [44]. Comparative evaluations of animal and human studies on urinary miRNAs changes in conjunction with AKI [56], or diabetic nephropathy [57] and renal fibrosis [58] have recently been reported. However, the number of currently known human mature miRNAs is 2693, nearly three times that of the 769 known rat miRNAs (http://www.mirbase.org) [59], which cautions for an appreciation for the limitations of animal studies and against an over-reliance on translational animal studies for biomarker discovery.

Increasing the number of rigorous meta-analyses across studies reporting urinary miRNA changes in animal kidney injury models and/or in human patients with kidney injury associated with drug/chemical exposure or certain disease states, differentiating between acute or chronic settings, are still needed. These evaluations may be followed by specifically designed measurements and exploration of several platform and analytical approaches to measure miRNA expression including reverse transcription quantitative PCR, microarray hybridization, or small RNA sequencing. While each method has respective limitations, RNA-seq offers advantages for biomarker discovery by inherently allowing for discovery of new sequences and sequence variants [60, 61]. Such investigations may require collaborations across stakeholders to yield miRNA measurements in a reasonable time frame for use in focused kidney injury settings. For the purposes of environmental surveillance and epidemiological studies, miRNA biomarker stability and measurement techniques in low-resource settings are of particular importance.

Understanding of tissue-enriched and tissue-specific miRNA expression patterns are particularly important when considering miRNA abundance in blood. Because many tissues are accessible to the circulating blood compartment, teasing out the tissue-of-origin for a specific miRNA is often challenging and best done in a panel with other miRNAs and protein biomarkers as a unified weight of evidence approach. Some established exosomal markers including Alix and tumor susceptibility gene-101 (Tsg101) [62, 63] can reliably distinguish EV heterogeneity and the originating cell source type, however advances in surface protein markers for tissue of origin identification are greatly needed [64]. New methods, including exosome surface protein profiling for differentiation, identification and quantification of a large number of exosomes [23], are showing promise towards realizing the “liquid biopsy” ideal. Although circulating miRNAs are attractive biomarker candidates of kidney disease in the clinic, it must be kept in mind that comorbidities, medication used, and even some demographic features such as age and sex may also affect miRNA expression profiles [65] and may complicate interpretation. Particularly in clinical studies conducted to date, it is unclear whether these miRNA candidates are tissue and disease specific or represent more general pathologies such as inflammation [66]. An example is miR-21–5p, which is highly expressed across multiple tissues beyond the kidney in the rat, according to rat miRNA body atlas data collected in the RATEmiRs web portal (https://connect.niehs.nih.gov/ratemirs/) [67, 68].

More research likewise needs to be done to evaluate measurement characteristics and best practices for the clinic. A key concern for biomarkers is the clearance half-life and temporal release of the biomarker into the urine from the damaged kidney. There have been few time course studies to date on urinary miRNA biomarkers and this should be an area of intense focus. In humans, there is evidence of seasonal variation in pesticide-associated urinary miRNAs that suggests transitory response [34]. In animal models, toxicant-induced urinary miRNA response was observed 3–5 days following treatment and preceded blood urea nitrogen and sCr changes [51]; urinary exosomal miRNA tracked with AKI progression and recovery following ischemia/reperfusion injury observed 1–14 days after treatment [55]. Relatedly, stability of urinary miRNA has been investigated and researchers have observed differences in stability of different miRNA species [69, 70]. This observation indicates that biomarker qualification efforts should be individualized to the specific miRNA biomarker rather than relying on stability measures for the entire miRNA fraction. An understanding of the relative miRNA abundance in urine cell sediment, EVs and extracellular protein-bound fractions may have relevance to interpretation of biomarker levels [20, 65], as well as other factors such as daily variation in biomarker levels and timing of urine void [65].

The urinary compartment provides an advantage for biomarker measurements relative to underlying kidney injury, which is that the urine is a directly accessible compartment to the kidney and urinary tract. Thus, interpretation of miRNA levels in the urine becomes more straightforward and relevant specifically to kidney injury (as compared to blood which perfuses multiple organs), thereby expanding the number of miRNA candidates that could be considered as diagnostic for kidney injury. There are examples where environmental toxicant studies assessed chemical exposure in urine, and reported plasma-based miRNA changes [33]. Depending on the chemical of interest, urine and blood miRNA measurements may be measured in concert to assess local effects in the kidney as well as systemic effects to other

organs; such an investigation would require measuring a broader miRNA panel that is diagnostic across a variety of organ systems. More work is needed to draw conclusions regarding specificity of miRNA biomarker candidates. This will require moving miRNA biomarkers from discovery investigations to intentional validation campaigns. As reported above, the HESI eSTAR committee is systematically approaching miRNAs altered upon induction of acute kidney injury.

Given the relatively nascent field of urine miRNA for environmental health surveillance and clinical use, researchers should be aware of and acknowledge study limitations and corresponding challenges when interpreting findings. As in all molecular epidemiologic investigations, scientists should avoid overgeneralizations about novel systemic communication pathways or mechanisms between cells and tissues. Toxicologic models in nonclinical species will be helpful in elucidating mechanisms associated with miRNA release and with benchmarking of urinary miRNA profiles to underlying histopathology changes. Translational studies between nonclinical species and humans will be an important validation step and should be attempted on a case-by-case basis depending on the intended biomarker context of use.

Conclusions

Taken together, miRNA biomarkers have potential to provide important health surveillance data for epidemiologic studies and for clinical decision making. A likely approach for deployment may include a panel of miRNA biomarkers in combination with traditional measures of kidney injury, including sCr and, where feasible, GFR. Given resources and dedicated research, validated biomarkers have the potential to impact public health strategies to monitor for emerging human health threats at the nexus of exposure-response and disease in environmental health, such as heat stress and CKD of unknown origin.

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Abbreviations:

AKI	acute kidney injury
CKD	chronic kidney disease
eGFR	estimated glomerular filtration rate
EV	extracellular vesicle
miRNA	microRNA
sCr	serum creatinine

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*Annotated references of importance of importance:

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• In a rat model of AKI, this study identified different exosomal miRNA expression in accordance with injury (miR-16, −24, and −200c) and recovery stages (miR-9a, −141, 200c, and −429). Upregulated target mRNAs were identified in the TGF-β signaling pathway, suggesting a potential regulatory mechanism in AKI progression.

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