Review Article

Urinary Biomarkers for Chronic Kidney Disease with a Focus on Gene Transcript

Lin-Li Lyu, Ye Feng, Bi-Cheng Liu

Department of Nephrology, School of Medicine, Zhongda Hospital, Southeast University, Nanjing, Jiangsu 210009, China

Abstract

Objective: In the upcoming era of precision medicine, searching for the early, noninvasive biomarkers has been the cornerstone and major challenge in the management of chronic kidney disease (CKD). Urine contains rich biological information which could be the ideal source for noninvasive biomarkers of CKD. This review will discuss the recent advance in urinary biomarker.

Data Sources: This review was based on data in articles published in the PubMed databases up to June 20, 2017, with the following keywords: "Chronic kidney disease", "Biomarker", and "Urine".

Study Selection: Original articles and important reviews on urinary biomarker were selected for this review.

Results: Urinary biomarker studies of CKD mainly focused on urine sediment, supernatant, and urinary extracellular vesicles. The gene transcript (microRNA [miRNA], messenger RNA [mRNA]) biomarkers have been recently shown with diagnostic potential for CKD reflecting kidney function and histological change. However, challenges regarding technique and data analysis need to be resolved before translation to clinic.

Conclusions: Different fractions of urine contain rich information for biomarker discovery, among which urine (extracellular vesicles) mRNA, miRNA, might represent promising biomarker for CKD.

Key words: Biomarkers; Chronic Kidney Disease; Extracellular Vesicle; Gene Transcript; Urine

Introduction

Chronic kidney disease (CKD) has become a major health burden worldwide. In China, a recent study has revealed that China's adult prevalence rate of CKD has reached 10.8%.[1] The increasing prevalence of diabetes may contribute to further enhanced prevalence of CKD due to the increased proportion of patients with diabetic kidney disease.[2] The main obstacle in the management of CKD patients is the absence of early clinical signs before the kidney enters an irreversible dysfunction stage. CKD is a progressive disease from inflammation to fibrosis.[3] Currently, the most common biomarkers for CKD are serum creatinine, urea nitrogen, and proteinuria. However, those biomarkers are influenced by many factors such as patient age, diet, and infection conditions, especially which could not accurately reflect the severity of renal fibrosis in early stage. Although renal biopsy could clearly demonstrate the pathologic change of kidney, it could not be repeatedly performed for severe patients and routine follow-up of patients by biopsy is not possible due to its invasive nature. Moreover, kidney biopsy is not suited for screening test in large populations for its association with discomfort and a risk of major complications. Consequently, in the upcoming era of precision medicine, searching for the early, noninvasive biomarkers will be the urgent issue for fighting with CKD.[4]

Urine is generated by the kidneys; it is apparently a source for noninvasive biomarker discovery. Through differential centrifugation, urine can be separated into different fractions, including urine sediment, supernatant, and a recently identified structure, called extracellular vesicles (EVs). These different fractions contain a variety of biological

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Address for correspondence: Prof. Bi-Cheng Liu, Institute of Nephrology, School of Medicine, Zhongda Hospital, Southeast University, Nanjing, Jiangsu 210009, China
E-Mail: liubc64@163.com

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information, such as cellular component, proteins, and nucleic acids. In this brief review, we will discuss the recent advances in urinary biomarker study with special focus on gene transcript. The advantages and disadvantages of biomarkers from different urine fractions were listed in Table 1.

Urine Contains Rich Biological Information: An Ideal Source for Noninvasive Biomarker

Urine sediment

Urine sediment analysis has been a long-standing practice for diagnosis of kidney disease. However, a recent study showed that there is a large amount of information to be explored. First of all, there is a variety of shedding cells, especially those facing up the urinary tract, including podocyte, tubular epithelial cell, immune cells, and stem/progenitor cells in the urine. Urinary cells can possibly be used to noninvasively explore the cellular change of damaged kidney.

Kopetschke *et al*. showed that, in systemic lupus erythematosus (SLE) patients with active renal disease activity, the number of urinary $CD4^+$ and $CD8^+$ T-cells is high. Moreover, $CD8^+$ and $CD4^+$ T-cells yielded the highest diagnostic value for diagnosing proliferative lupus nephritis (LN) among SLE patients.[5] Urinary excretion of viable podocytes was quantified in rat models with transient and continuous glomerular injury. It was shown that the number of glomerular Wilms' tumor-1-positive podocytes presents a characteristic time course in each disease model.[5]

Besides counting cell numbers from urine sediment, RNA could be extracted from urine sediment cells and analyzed for the messenger RNA (mRNA) levels. The first study trying to measure urine sediment mRNA for diagnosis of kidney disease was reported in 2001. It was shown that the levels of perforin and granzyme B mRNA were significantly higher in the patients with an episode of acute rejection than that in the other groups, ROC curve showed that the two mRNAs could efficiently identify acute rejection.[6] Studies from Szeto's group showed that urine cytokine mRNA was correlated with tubulointerstitial fibrosis (TIF) and glomerulosclerosis score.[7] The same group conducted a prospective study and demonstrated that urine hepatocyte growth factor mRNA was an independent predictor of CKD progression.[8] More recently, we found that genes of fibrosis-related markers such as a-SMA, fibronectin, MMP9, and FSP1 mRNA were significantly increased in patients with diabetic nephropathy (DN).[9] Moreover, we demonstrated that podocyte-related mRNAs were significantly increased in DN patients which correlated with the decline of renal function.[10] Besides mRNA, microRNA (miRNA) could also be detected from urine sediment. Wang *et al*. showed that in LN, urinary miR-146a and miR-155 could be used as potential markers for diagnosis, disease activity, and therapeutic response.[11]

Since a complex molecular network was involved in CKD progress, it is reasonable to screen those molecules in high-throughput approach. We have fabricated polymerase chain reaction (PCR) array by including molecules involved in CKD progression for biomarker study. Using this PCR array, we tested samples from DN patients and found a few genes that were differently expressed in DN patients compared with controls.[12] In a further validation study, vimentin mRNA showed impressive high level of expression in CKD patients, and the level is correlated with renal function and renal fibrosis score.[13] Besides, by feature selection and classification of microarray data, we identified a four-mRNA signature, including TGFβ1, MMP9, TIMP2, and vimentin, as important features of TIF.[14]

Urine supernatant

Inflammatory cytokines in urine supernatant are important source of new biomarkers for CKD. A recent study revealed that, in LN, urine monocyte chemoattractant protein-1 (MCP-1) levels were highest at the time of relapse compared to prerelapse levels and decreased in response to treatment. Urine MCP-1 was related with renal function and lupus disease activity.[15] Protein microarray is the high-throughput analysis approach for urine cytokine. We previously constructed protein microarray for detection of

Table 1: Advantages and disadvantages of biomarkers from different urine fractions

Urine fractions	Types of biomarker	Advantages and disadvantages		References
Urine sediment	Cell number	Complexity to obtain samples	+	[5-14]
	mRNA	Complexity to extract RNA	+	
	miRNA	NA stability	+	
		Information reflecting active renal cell status	+	
		Complexity to detect cell specific-RNA	+	
Urine supernatant	Cytokines, chemokines	Complexity to obtain samples	+	[15-18]
	Urine peptide	RNA stability	+	
		Information reflecting active renal cells status	++	
		Complexity to detect cell specific-RNA	+++	
Urinary extracellular vesicle (ECV)	mRNA	Complexity to obtain samples	+++	[23-28, 43]
	miRNA	Complexity to extract RNA	++	
	lncRNAs	RNA stability	+++	
		Information reflecting active renal cells status	++	
		Complexity to detect cell specific-RNA	++	

+, ++, +++ represent less, more and most respectively.

inflammatory cytokines and found differential expression of cytokines in CKD patients. However, due to the low sensitivity, the performance of protein microarray in biomarker discovery is not as good as PCR array for mRNA biomarker discovery.[16] Another high-throughput approach for analysis of urine protein is proteomic study by two-dimensional differential gel electrophoresis coupled with tandem mass spectrometry analysis. Urine peptide CKD273 is a representative proteomic biomarker. It has been reported to be well suited for early detection of CKD and for prognosis of progression.[17] A large multi-center study is currently ongoing for its validation which enrolled over 3000 patients. We are expecting the result coming out to support its translation to clinic.[18]

Urinary extracellular vesicle

Cell-free DNA was first identified and studied as circulating biomarkers in biofluid. Recently, cell-free RNA was found packaged into extracellular vesicles which are shed from cellular surfaces into the biofluid. In the recent years, many interests have been focused on urinary EVs as a source for biomarker discovery in humans. Urine EVs are small particles originating from cells of different nephron segments or of the urinary tract. It is released with cytoplasmic proteins, lipids, and nucleic acids which makes urinary EVs a unique source of information for diagnostic purposes.[19-21] According to the difference in size and formation, EVs could be divided into exosome, microvesicle, and apoptic bodies. Among those structures, exosome has been the focus of many studies.[22] A landmark study for urine exosome is reported in 2010. It showed that urinary exosome contains mRNA transcripts encoding specific genes from various regions of kidney. This provided the possibility for exploring the mRNA biomarkers from urine exosome. We have recently isolated exosome released from podocyte in the urine, the structure was positive for the markers of both exosome and podocyte that is CD9, AQP2, and nephrin. Further study showed that CD2AP mRNA from exosome was correlated with both kidney function and fibrosis score.[23]

Besides mRNA, exosome contains high levels of miRNA. Our study has demonstrated that exosome miRNA was stable despite repeated frozen-thaw cycles and long-term storage.[24] The stability and enrichment of miRNA in exosome make it a promising candidate biomarker for kidney disease. By reverse transcription-PCR (RT-PCR), we have measured several miRNAs in CKD patients and found that miR-29c from urinary exosome was significantly reduced in CKD patients and inversely correlated with renal fibrosis scores.[25] Ben-Dov *et al*. profiled miRNA in autosomal dominant polycystic kidney disease (ADPKD) and non-ADPKD-derived kidney epithelial cells and urine specimens from patients. They identified the role of mir-1 (4) and mir-133b (2) members in the pathogenesis of ADPKD, and their potential use as biomarkers for ADPKD.[26] In streptozotocin-induced DN model, using pilot small RNA sequencing combined with qPCR confirmation, Mohan *et al*. found that urine exosome miR-451-5p may hold prognostic value as an early and sensitive noninvasive indicator of DN.[27] Ramezani *et al*. recently conducted a pilot study using Affymetrix GeneChip® miRNA arrays and qRT-PCR and found that patients with minimal change disease and focal segmental glomerulosclerosis had distinct circulating and urinary miRNA expression profiles.[28] However, the diagnostic and prognostic potential of miRNAs in different types of kidney disease warrants further studies.

The NIH Common Fund Extracellular RNA Communication Program has funded 10 UH2/UH3 grants to identify extracellular RNA biomarkers in multiple conditions including kidney disease. The project is currently in its discovery phage which has identified potential biomarkers that might advance to next phage.[29]

Future Directions of Urinary Gene Transcript as Biomarker Candidate

Previously, renal biomarkers are limited to urinary protein analysis and changes in the glomerular filtration rate. Biomarkers at the gene transcript levels are underestimated, partly because this requires the invasive procedure of kidney biopsy. However, analysis of cell and EVs in urine offers a new opportunity to understand renal disease. Challenges and future directions of gene transcript biomarker study are discussed below.

Approaches for urinary gene transcript biomarker discovery

The detection and identification of gene transcript can be performed using RNA sequence, microarray technologies, or reverse transcription-quantitative real-time PCR (qRT-PCR). Currently, expression of microarrays could detect more than 1000 mature human miRNA sequences listed in the miR database (miRBase), and the next-generation sequencing (NGS) allows the screening of deregulated transcript levels of miRNAs. Subsequently, the deregulated miRNAs deduced from the array or NGS data can be validated by qRT-PCR in single assays.[30] However, advantages and disadvantages were associated with those different approaches for detecting gene transcript. Nassirpour *et al*. reported that there is a minimal agreement between NGS and qRT-PCR for low-yield urinary miRNA analysis. miRNA-seq may not detect low abundant miRNAs in urine samples which could be identified by qRT-PCR. Whereas the qRT-PCR analysis was not able to detect the miRNA isoforms, which could be detected by NGS.[31] Microarrays are available for screening RNAs; however, as microarrays use only known sequences as targets, the ability to detect novel sequences is not possible.

Besides, data normalization is an important issue in gene transcript biomarker study, especially for data from different platforms. The External RNA Controls Consortium (ERCC)

has developed universal RNA standards to aid validation of research findings from diverse platforms. Recently, Devonshire *et al*. showed that ERCC RNA standards provide an efficient means for evaluating different aspects of platform performance and can provide information on the technical variation associated with quantification of biomarkers.[32] Moreover, due to RNA degradation in urine, RNA normalization was specially warranted. Galichon *et al*. found that GAPDH and UPK1A are preferable to 18S or HPRT RNA to suppress the effect of RNA degradation.[33] Besides, in case of miRNAs, the selection of an endogenous reference gene to normalize the relative levels of the miRNA detected by qRT-PCR is a further confounding factor.[30]

Hence, the major challenges in gene transcript biomarker study are the sensitivity, specificity, and particularly the analytical variables derived from different analysis approaches. Extensive studies are needed to establish consensus normalization methods for biomarker analysis.

Cellular-specific RNAs in urine may represent more specific biomarker

Although previous studies have found promising urinary RNA as biomarkers of kidney disease, the cellular origin of urinary RNAs remains obscure and could potentially affect the significance of the results.

For intracellular RNA biomarker studies, most of the previous studies targeted on the RNA from the whole sediment of cellular component which may obscure the exact role of the gene transcript as biomarkers. EVs of urine are originated from kidney intrinsic cells and cells facing urinary tract, it might be used as a "surrogate tissue" for kidney biopsy and analysis.[34] With the discovery that EVs contain genetic material in the form of RNA (EV-RNA), vesicles have received increasing interest for their potential use as sources of disease biomarkers.[35] However, it is still difficult to purify EVs from specific kidney intrinsic cells in urine. Currently, most of the studies choose RNA from total EVs in urine for biomarker detection. Thus, it would be interesting to make analysis of those RNAs from separated cellular/EVs fractions, especially RNA from kidney intrinsic cells other than total cellular/EVs in urine. As approaches for analysis and isolation of individual EVs are rapidly improving, the opportunities to investigate the EV-RNA cargo of single EVs may be realized in the near future.[35]

Urinary noncoding RNA may serve as novel biomarker for chronic kidney disease

Among all ncRNAs, miRNAs are the most exploited and widely described ncRNAs both regarding its role in the pathogenesis of CKD and its levels in urine of kidney disease patients.[36] miRNAs is surprisingly resistant to RNA-degrading enzymes in biofluid due to two mechanisms. One is the encapsulation of miRNAs into extracellular vesicles with protection from external RNases by the surrounding membrane, and the other form of protection is that miRNAs conjugation with a variety of proteins, nucleophosmin 1, high-density lipoprotein, and Argonaute-2.[37] EVs have been demonstrated to be the major fraction of miRNA in biofluid.[38] EVs may protect RNA during urine passage which is more stable than RNA extracted from whole urine. Moreover, these EVs miRNAs are physiologically functional and have been shown to be transferred to target cells.[39] Consequently, it is reasonable to speculate that EVs miRNA may represent promising novel biomarker for kidney disease. We and others have described few pilot studies that showed miRNA from EVs may be effective in reflecting the severity of kidney injury and renal function,[25,27,40] more studies are needed to demonstrate the possibility for translating to clinic.

Recent studies have drawn attention to the other new class of noncoding RNA, the long noncoding RNA and circRNAs. This led us to ask whether lncRNA and circRNAs could also be detected in urine, and whether they could serve as putative biomarker molecules for kidney disease. Zhou *et al*. used RNA sequencing to identify lncRNAs related to renal inflammation and fibrosis in obstructive nephropathy and found *Arid2-IR* as a novel lncRNA that functions to promote NF-κB-dependent renal inflammation.[41] The authors suggested that blockade of *Arid2-IR* may represent a novel and specific therapy for renal inflammatory disease. It would be interesting to detect its expression level in urine to explore its potential to act as a novel biomarker for renal inflammation. Iyer *et al*. curated 7256 RNA-Seq libraries from tumors, normal tissues, and cell lines from 25 independent studies, they nominated 7942 lineage- or cancer-associated lncRNA genes. The lncRNA landscape may shed light into normal biology and be valuable for future biomarker development.[42] Lorenzen *et al*. conducted a global lncRNA expression analysis on RNA from urine of patients with acute T-cell-mediated renal allograft rejection and control transplant patients. The data revealed that lncRNAs are strongly altered in urine of patients with acute rejection, and urinary RP11-354P17.15-001 may serve as a novel biomarker of acute kidney rejection.[43] As for cirRNA, Memczak *et al*. observed that hundreds of circRNAs are much higher expressed than corresponding linear mRNAs. The findings suggest that circRNAs could be used as biomarker molecules in standard clinical blood samples.[44] However, there is no study regarding the circRNAs expression profiles in urine of CKD patients. It would be interesting to conduct further studies on urine lncRNA and circRNAs profiling and to find the novel markers for kidney disease.

Unique transcriptional biomarker may yield novel insights into the molecular mechanism of kidney disease

Although previous studies have demonstrated a number of differential expression genes as potential biomarkers, the exact roles of these genes in the development of disease and the mechanisms of their release from affected cells into urine are yet to be understood. However, the transcriptional analysis of different experimental models or clinic samples may yield the molecular candidates that are important for the development of kidney disease. Xu *et al*. identified

distinctive gene expression patterns in human urine as potential biomarkers of either extracellular fluid volume or intrinsic kidney injury. It has been suggested that molecular analysis should clarify our current definitions of acute changes in kidney excretory function.[45] By analyzing kidney transcriptome of a DN mouse model, Rubin *et al*. found unique biomarkers and canonical pathways which may hold the key to understand the mechanisms of DN pathobiology. The data suggest that mitochondrial dysfunction and oxidative stress are principal events in DN.[46]

EVs' biogenesis and release are signal and stimuli dependent, factors such as environmental stress and calcium concentration affect microvesicle release.[47] Besides, RNAs are not passively loaded into EVs, but that certain populations of RNAs become enriched in EVs compared to parental cells, mainly because of the existence of an active sorting mechanism that occurs at the RNA level.[48] Thus, to clarify the selective loading mechanism of EVs, RNAs in biomarker study may be helpful for understanding the contribution of EV-derived miRNAs and mRNAs to CKD progression.

Conclusions

In summary, different fractions of urine contain rich information for biomarker discovery, among which urine (EVs) mRNA, miRNA, might represent promising "fluid biopsy" for kidney disease diagnosis. For urine gene transcript biomarker, consensus analysis and normalization methods need to be established. Moreover, the cellular origination and role of packaged RNAs of EVs in the pathologic process of kidney disease need to be studied before the specific RNA markers move to advanced validation stage for clinic translation.

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Conflicts of interest

There are no conflicts of interest.

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