Analysis of urinary microRNAs in chronic kidney disease

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Abstract
Kidney biopsy is the gold-standard diagnostic test for intrinsic renal disease, but requires hospital admission and carries a 3% risk of major complications. Current non-invasive prognostic indicators such as urine protein quantification have limited predictive value. Better diagnostic and prognostic tests for chronic kidney disease patients are a major focus for industry and academia, with efforts to date directed largely at urinary proteomic approaches. microRNAs constitute a recently identified class of endogenous short non-coding single-stranded RNA oligonucleotides that regulate gene expression post-transcriptionally. Quantification of urinary microRNAs offers an alternative approach to the identification of chronic kidney disease biomarkers.

Chronic kidney disease (CKD)
CKD is the 12th most prevalent cause of death, and 17th highest cause of disability worldwide [1]. Global prevalence of CKD is estimated at between 10 and 16%, with the incidence of end-stage renal disease increasing dramatically over the last decade [2–5]. CKD therefore represents a significant challenge to 21st Century global health policy [6], with serious implications for health and economic output [7].

A complex and progressive condition, CKD originates from both non-communicable and infectious diseases and is defined by the NKF (National Kidney Foundation) as either a decline in GFR (glomerular filtration rate) to <60 ml/min per 1.73 m² for at least 3 months, or the presence of kidney damage. Diabetes mellitus is a leading cause of CKD, and hypertension is a major contributor to the progression, as many kidney diseases result in increased blood pressure, which in turn promotes the progression of kidney disease. Other common CKD risk factors are the metabolic syndrome, cardiovascular disease and obesity, and the NKF has identified additional factors, including age >60 years, autoimmune disease, systemic infection, urinary tract infection and family history of kidney disease [8]. Infectious diseases such as malaria, schistosomiasis, HIV and hepatitis B may also lead to CKD [5,9,10].

CKD is typically divided into five stages on the basis of the amount of residual excretory function. The disease does not typically cause the patient to feel unwell until the kidney damage is advanced. Proteinuria, excessive urinary serum protein content, is a classic indicator of kidney damage, but may, for example, be absent from patients in whom tissue damage is restricted to the tubules and interstitium of the kidney rather than the glomeruli.

The NKF recommends that CKD screening measures should include the determination of GFR, assessment for the presence of proteinuria and screening for microalbuminuria (considered positive at >30 mg/24 h). Although these measures have undoubted utility in identifying patients with diminished kidney function, it is hard to predict individual outcome on the basis of currently available tests, and new measures for CKD testing are required.

Current CKD biomarkers
Fassett et al. [11] have described comprehensively the range of biomarkers that can be used to diagnose CKD and identify the effects of its progression on kidney function and structure, endothelial dysfunction, cardiovascular peptides, inflammation, fibrosis, metabolic factors and oxidative stress. Similarly, other reviews have focused on detection and evaluation of CKD [12], or on biomarkers in serum or urine that can determine the potential risk of kidney damage for acute, as well as chronic, kidney disease [13].

However, although it is clear from the above text that some biomarkers show promise, the necessity for effective biomarkers for early CKD identification remains. Current problems with CKD screening are related to the need for both more efficient screening strategies and the development of better screening tests for pre-clinical disease detection, facilitating earlier stage treatment and leading to a better outcome. The present mini-review focuses on the potential role of urinary miRNAs (microRNAs) as CKD biomarkers.

Urinary miRNAs as biomarkers for CKD

miRNAs
miRNAs are a recently identified class of endogenous non-coding single-stranded RNA oligonucleotides, of approximately 20–22 nt in length. miRNAs function as
important negative regulators of gene expression primarily by translational repression and mRNA degradation [14].

Currently more than 1000 human miRNAs have been identified. Many of these exhibit tissue-specific expression patterns, dysregulation of which has been associated with various diseases, including cancers [15,16], heart disease [17], autoimmune disease [18] and kidney disease [19,20].

**Potential of miRNAs as urinary biomarkers**
The present gold-standard diagnostic test for intrinsic renal disease is the kidney biopsy. This is an invasive procedure carried out to remove a small sample of renal tissue and carrying a 3% risk of major complications. Under homoeostasis and during disease, urine carries soluble waste from the body following renal filtration. The identification of routinely excreted urinary components that can be used as disease biomarkers is therefore potentially attractive.

**miRNA stability in biological fluids**
miRNAs appear to be very stable in tissues and biological fluids, even under adverse conditions such as extreme pH, long-term room temperature storage, multiple freeze–thaw cycles and RNase activity [21,22]. Primary RNA sequence structure may influence miRNA stability. Bail et al. [23] found that manipulation of the terminal seven 3’-nucleotides of miR-382 conferred increased RNA stability. In addition, Sethi and Lukiw [24] have proposed a correlation between the percentage of AU or UA dinucleotides and miRNA lability.

Protection of miRNAs from degradation may also occur as a result of packaging in microparticles such as exosomes, microvesicles and apoptotic bodies [25,26], or by the association of free miRNAs with RNA-binding proteins [27,28], or with lipoprotein complexes [29] (Figure 1).

**Microparticles, exosomes and miRNAs**
Microvesicles are formed by outward budding of the plasma membrane, while exosomes are manufactured within the endocytic tract in MVEs (multivesicular endosomes), and their secretion occurs following fusion of MVE compartments with the plasma membrane [30].

Exosomes are vesicles of approximately 30–100 nm diameter that are secreted by most, if not all, nucleated cells [31]. Exosomal protein content principally comprises membrane and cytosolic proteins, and is not generally representative of cellular compartments such as the nucleus, mitochondrion and endoplasmic reticulum [32]. Changes within the parent cell driven by hypoxia [33] or other forms of stress [34,35] are reflected in altered exosomal protein composition.

Both mRNAs and miRNAs have recently been identified in mast cell exosomes [25]. These exosomal RNAs were impervious to exogenous RNase activity, apparently protected by their intraluminal location, and the exosomal RNA repertoire did not correlate closely with that of the parent cell [25]. This study also showed that the mRNA present in mouse mast cell exosomes could be translated in corresponding human cell lines, encoding proteins that were often exosome-specific [25]. Similarly to proteins, exosomal RNA content is related to parent cell status, and changes in exosomal RNA content may result from environmental stress [36].

Physiological functions of exosomal RNAs, in particular miRNAs, are currently under investigation. In viral infection, exosomal delivery of viral miRNAs can exert transcriptional control in recipient cells [37,38]. Exosomal dissemination of functional miRNAs in health and disease has yet to be fully established, but cellular uptake of RNA via exosomal delivery has been posited as a general mechanism [23,39–41]. Indeed, recent work has suggested that plasma-borne miRNAs from patients with stable coronary artery disease, as well as acute coronary syndrome, are associated with microparticles, and only a small amount of this miRNA component is microparticle-free [42].

Differential ultracentrifugation has been used to purify exosomes from various biological fluids. Specimens including blood plasma [43], saliva [44], breast milk [45] and cancer-associated effusions [46] contain exosome-like vesicles that exhibit typical vesicle morphology and contain common exosome-associated proteins, including MVE markers. Ex vivo exosomes also contain RNAs [40], such preparations being of heterogeneous cellular origin. For example, plasma exosomes contain markers of platelets and lymphocytes [43], and exosomes from tumour effusions principally carry tumour markers [46]. This is a significant consideration with respect to the utility of exosomes as disease biomarkers, since selective isolation of exosome subpopulations is challenging.

**Urinary exosomes as a source of disease biomarkers**
The free availability and ease of collection of large volumes of urine has facilitated screening of urinary exosomes for biomarker discovery [47]. These exosomes originate from various sites associated with the urinary tract, including the bladder [47], prostate gland [48,49], and renal collecting ducts and proximal tubules [47]. However, exosomes from distant anatomical sites, such as hepatic exosomes, can also be found in urine [50].

Several primarily protein/proteomics studies have revealed the promise of urinary exosomes as a potential source of valuable disease biomarkers for bladder cancer [51], prostate cancer [48,49] and a broad range of renal diseases [52–55]. Acute renal injury is marked by rapid elevations in exosomal proteins such as fetuin-A [52] and ATF3 (activating transcription factor 3) [53], preceding changes in serum creatinine and creating opportunities for early intervention to minimize potential long-term tissue damage. Exosomes may also be diagnostically useful in distinguishing forms of renal pathology such as early IgA nephropathy and thin basement membrane nephropathy, with proteins such as aminopeptidase-N, vasorin precursor, α1-antitrypsin, and caeruloplasmin as putative distinguishing markers [54]. In addition, markers of polycystic kidney disease are also present on urinary exosomes [55].
miRNA biogenesis begins in the nucleus with RNA polymerase II-mediated transcription from the respective genomic loci of primary (pri)-miRNAs, which are then processed by the RNase III enzyme Drosha to one or more pre-miRNA hairpin precursors. Following export to the cytoplasm by exportin-5, another RNase III, Dicer, processes pre-miRNAs to miRNA duplexes of approximately 22 nt in length, from which the mature bioactive miRNAs are derived. The mature miRNA, ready to bind complementary miRNA target sequence, is incorporated into the RNA-induced silencing complex (RISC), which comprises multiple proteins including Dicer and Ago2. Perfect miRNA–mRNA binding with 100% sequence identity leads to miRNA cleavage (A); sequence identity <100% and imperfect miRNA–mRNA binding result in translational repression (B). miRNAs may be protected from degradation, secreted and released from the cell by association with lipoprotein complexes (C), DNA/RNA-binding proteins (D) or Ago protein complexes (E); or packaging into microparticles such as apoptotic bodies (F) or exosomes (G). UTR, untranslated region.

Exosomal miRNAs as disease biomarkers
There is considerable current interest in relating exosomal miRNA repertoire to disease, with data reported for cardiac [56] and renal [57] disease, but most efforts to date have focused on malignancy-associated exosomes. Among the earliest examples of the latter was a study of affinity-isolated EpCAM (epithelial cell adhesion marker)-positive exosomes in the circulation of lung cancer patients [58]. The authors identified miRNAs present in these tumour-derived exosomes that were not detected in the background EpCAM-negative exosome population in patient-matched specimens [58]. Additional studies on ovarian [59] and prostate cancer [60] have also examined changes in miRNAs with disease status. In general terms, differences in miRNA profiles could distinguish between cancer patients and unaffected individuals. However, exosomal miRNA profiles may not be so clear-cut in defining tumour stage [58], except perhaps for the most extreme scenarios such as metastatic disease [60].

Extra-exosomal miRNAs
A number of recent studies have emphasized that miRNAs are not always predominantly associated with microparticles. Arroyo et al. [27] used differential centrifugation and size-exclusion chromatography to show that the majority of circulating plasma and serum miRNAs are not vesicle-encapsulated, but reside within a ribonucleoprotein complex, with up to 90% of circulating miRNAs in a non-membrane-bound form. A significant proportion of circulating miRNAs were also detected by immunoprecipitation of Ago (Argonaute) 2 ribonucleoprotein complexes, suggesting that these complexes are responsible for plasma miRNA stability [27]. Another recent report has also shown that extracellular
miRNAs from plasma and conditioned medium from the culture of five human cell types are predominantly free of exosomes/microvesicles and are instead associated with Ago proteins, predominantly Ago2 [61]. Analysis of conditioned tissue culture medium from a different set of human cell types revealed the release of a significant number of RNA-binding proteins [62]. Of these, nucleophosmin 1 was highlighted as an miRNA-binding protein with a potential role in exportation, packaging and protection of extracellular miRNAs [62]. Results from other studies have suggested that extra-exosomal miRNAs may evade degradation by association, transport and delivery to target cells by high-density lipoprotein [29] or by binding to DNA/RNA-binding protein translin [28].

Concluding remarks

The seriousness of the problem of CKD is underlined by the fact that 2% of the National Health Service budget in the U.K. is accounted for by renal dialysis. The use of urinary biomarkers may provide a non-invasive, safe and cost-effective approach to obtaining important diagnostic and prognostic information, and is clearly attractive to clinical scientists and industry alike. The discovery of miRNAs in urine presents a potential new source of biomarkers, and their stability in other biological fluids bodes well for their potential use. Given the current technological limitations for isolating target exosome/microvesicle subpopulations from urine, the potential for stabilized microparticle-free miRNAs as accessible alternatives may point the way for future developments.

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References
