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URINE miRNAs: POTENTIAL BIOMARKERS FOR MONITORING PROGRESION OF EARLY STAGES OF DIABETIC NEPHROPATHY

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Abstract

With a steep increase in the incidence of type 1 and 2 diabetes globally, diabetic nephropathy (DN) has now become the leading cause of renal failure in the world. There are no suitable biomarkers for the diagnosis of early stages of DN. In recent years, tremendous efforts are being made worldwide to delineate the role of micro RNAs in the pathogenesis of DN. Circulating miRNAs in serum, plasma, urine and other body fluids, which reflect a response to various pathophysiological stresses, are being investigated in the context of diabetic nephropathy. Delineation of the changes in miRNA levels in patients with DN may lead to a better understanding of the progression of the disease. We present here an exhaustive survey of the miRNA literature, highlighting various studies performed over the last decade. The aim is to assess if changes in various miRNAs could correlate with the progression of diabetic nephropathy. Based on the survey, we found that miRNA-377, miRNA-192, miRNA-216/217 and miRNA-144 are increased in body fluids of patients with DN, while miRNA-21 and miRNA-375 are decreased. Overall, there are a very few miRNAs that are kidney specific, and although significant differences were observed in the urinary excretion of certain miRNAs, they were not correlative to their levels in the blood or plasma. Thus, it is completely plausible that urine-specific miRNAs could serve as novel biomarkers for the diagnosis of early stages of diabetic nephropathy.

Keywords

miRNA; diabetic nephropathy; biomarkers

INTRODUCTION

Early stages of diabetic nephropathy (DN) are characterized by hyperfiltration, nephron enlargement and mesangial cell hypertrophy, which later on progress to glomerulosclerosis [1]. Initial stages of DN are associated with mild proteinuria that is traditionally described as

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microalbuminuria (albumin excretion: 30 – 300 mg/day), but with progression to overt nephropathy there is an associated increased risk of vascular disease and patient mortality [2]. Major pathophysiological mechanisms associated with DN complicate the outcome of DN, including activation of the renin-angiotensin-aldosterone system (RAAS), the protein kinase C (PKC) pathway, and upregulation of pro-inflammatory cytokines and various growth factors [3]. Angiotensin II and transforming growth factor-β (TGF-β) are significant molecular mediators that influence DN pathogenesis, and have been suggested as potential targets for the development of therapeutics [4]. The generation of advanced glycation end products (AGEs) and increased oxidative stress in high glucose ambience are additional pathobiological processes that further exacerbate hyperglycemia-induced renal injury.

Currently, DN is the most common cause of end-stage renal disease (ESRD) worldwide, and approximately 40% of patients require renal replacement therapy. Early identification of patients who are prone to develop renal complications would be an important step for their better management during the clinical course of this disease process [5]. Microalbuminuria has been the standard method for diagnosis of early stages of DN, however, this method has some drawbacks. Microalbuminuria can develop when advanced changes have already set in, as assessed by renal biopsy examination [6]. Also, the immunoassay that measures microalbuminuria can only detect the immunoreactive form of albumin, and its non-immunoreactive forms are undetectable by this method [7]. The quantitation of immunoreactive albumin by RIA (radioimmunoassay) fails to detect approximately 80% of the total albumin; less than 28% of the non-immunoreactive albumin in urine could be accounted for by the total combination of all other proteins measured by ELISA [8]. During the past decade, proteomics have become a powerful tool for the discovery of biomarkers for various disease processes. However, these procedures are less than ideal to monitor the progression of DN because they are very time-consuming and restricted to a select group of proteins, excluding those that are highly hydrophobic. Thus, novel improved biomarkers are necessary to monitor the progression of early stages of diabetic nephropathy [9]. MicroRNAs comprise 21 to 23 nucleotides, and bind to the 3′-untranslated regions (UTRs) of their target mRNAs in a stable manner [10]. MiRNAs modulate a wide range of biological functions, including oncogenesis, apoptosis, cardiac development and insulin secretion [11][12][13][14][15]. MiRNAs are present in wide variety of body fluids, and their levels in these fluids usually reflect a tissue specific injury or expression, which makes them ideal candidates for potential biomarkers [16]. For example, serum miRNA-21 is a novel biomarker for the diagnosis of esophageal squamous cell carcinoma [17]. In addition miRNAs are potential biomarkers for smoking-related interstitial fibrosis [18]. This suggests a strong relationship between the expression of tissue miRNAs and their levels in body fluids. MiRNAs in are found at high concentrations in body fluids [20]. Further, miRNAs are extremely stable and notably less susceptible to RNase. This protection from RNase degradation has been shown to be a result of packaging within cytoplasmic microvesicles, exosomes and apoptotic bodies, and also due to specific interactions with protective proteins [21].

URINE-SPECIFIC miRNAs

We hypothesized that the unique stability of miRNAs in various body fluids would reveal urine-specific miRNAs that would have some promise to serve as biomarkers. As urine is an easily available source for molecular markers such as RNA; novel, highly sensitive, and specific urine-based diagnostic tools are particularly attractive. An analysis of miRNAs in urine from bladder cancer patients showed higher ratios of miR-126:miR-152 and miR-182:miR-152 [22]. Urinary miRNAs can be derived from glomerular ultrafiltrate or excreted by the renal tubules. Oftentimes, their levels may be reflective of intrinsic tissue injury in kidney or urinary tract. Melkonyan *et al.* detected 22 different urinary miRNAs, but

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none were kidney-specific, suggesting these miRNAs were transported in the plasma and were filtered across the glomerular capillary barrier; their isolation in an intact form from the urine indicated their potential to serve as biomarkers [23][24]. In recent years, many studies have shown that miRNAs play an important role in regulating glucose and lipid metabolism in diabetes, and their levels in blood or urine should be of some value in stratifying a given stage of the disease process and predicting its clinical course [25]. Argyropoulos *et al.* found micro-albuminuria is associated with decreased levels of miR-323b-5p and increased urine concentration of miR-429 in patients with long standing type 1 diabetes [26]. Interestingly, miR-323b-5p regulates Claudin-16, a key component of the tight junction in the thick ascending limb, [27], while miR-429 correlates with the level of proteinuria and renal function in immunologically-mediated renal diseases such as IgA nephropathy [28]. Finally, the five most significant miRNAs were found to be of immense value in classifying cases of DN with a high degree of significance [29].

MiRNAs AS POTENTIAL DISEASE BIOMARKERS

There are 2214 and 848 known miRNAs in humans and mice, respectively (Data from miRBase Release 19.0: 2013.02.at http://microRNA.sanger.ac.uk). Recent clinical studies have shown that human serum contains large amounts of stable miRNAs derived from various cells and tissues, and their altered expression profile in body fluids of type 2 diabetic patients is considered of high clinical value [30].

MiRNA-377 in diabetic nephropathy and pulmonary neoplasms

MiR-377 seems to be epigenetically regulated [31]. It is normally expressed in human lung tissues, and is upregulated in lung tumors, and thus could serve as a potential biomarker for the management of patients with pulmonary neoplasms [32]. Interestingly, in mouse and in vitro models for DN, elevated miR-377 levels were shown to mediate decreased expression of p21-activated kinase (PAK1) and superoxide dismutase (SOD), enhancing production of the fibronectin protein. PAK1 plays an important role in tumor formation [33] and can lead to fibronectin production via Smad activation in DN. Superoxide dismutase genes are related to oxidative stress, and may be associated with vascular damage in patients with diabetic mellitus [34]. Therefore, miR-377 may play a critical role in the pathobiology of mesangial cells since they are known to undergo oxidative stress under high glucose ambience.

MiRNA-192 in renal disease, hepatotoxicity and colon tumors

The value of miR-192, a liver-enriched miRNA, as a potential blood-based biomarker has been elucidated recently in a mouse model of acetaminophen (APAP)-induced hepatotoxicity [35]. The expression level of miR-192 is significantly decreased in colon tumors compared with normal tissues, suggesting that it could be used as a diagnostic tool to assess hepatotoxicity and neoplasms of the gastro-intestinal tract [36]. Urinary levels of miR-192 are downregulated in patients with IgA nephropathy [37]. Also, correlation of miRNA-192 levels with derangements in renal functional parameters and incremental increase in blood pressure has been recently reported [38]. More importantly, increased glomerular expression of miR-192 was found to be associated with heightened activity of renal TGF-β in mouse models of DN. Furthermore, miR-192 could target ZEB1/2 in PTCs and enforced expression of E-cadherin while TGF-β led to a reduction in miR-192. ZEB1 (zinc finger E-box binding homeobox 1) and ZEB2 are E-Box–binding proteins and an important early stage of EMT [39]. E-cadherin expression was increased and remained after 96 hours of incubation with TGF-β in PTC (proximal tubular epithelial cells) clones overexpressing miR-192 [40]. Interestingly, specific inhibition of renal miR-192 decreases renal fibrosis and dampens the proteinuric response [41]. Further support of miRNA-192 in the pathobiology of the kidney is derived from studies in patients having early stages of

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diabetic nephropathy who had a higher expression of miR-192 compared to the late stages of clinical course of this disease process [42].

MiRNA-216 and miRNA-217 in DN and pancreatic ductal adenocarcinoma

MiR-216 and miR-217 are characteristically expressed in the pancreatic tissue. A uniquely controlled expression of miR-217 in the pancreatic tissues may be an additional mechanism for acinar cells to balance their β-catenin expression levels and secretory functions. miR-216 and miR-217 play important roles in early detection of pancreatic ductal adenocarcinoma, a condition known for its difficulty to diagnose [43]. The relevance of miR-216 and miR-217 in chronic kidney diseases has also been described (reviewed in [44]). Interestingly, miR-216 and miR-217 also play a role in the activation of Akt kinase, a key mediator of diabetic nephropathy. Specifically, TGF-β was shown to activate Akt kinase by inducing miR-216 and miR217, in turn downregulating phosphatase and tensin homologue (PTEN). Overexpression of PTEN has been observed in early stages of DN, adding significance to the role of miR-216 and miR-217. Intriguingly, these miRNAs are also upregulated by miRNA-192, a finding that is also relevant in the pathogenesis of diabetic nephropathy [45].

miRNA-144 in type 2 diabetes and pancreatic cancer

miRNA-144 has been shown to play a role in regulation of cell growth [46] and apoptosis [47]. MiR-144 is a regulator of genes that modulate embryonic-hemoglobin, and is downregulated in pancreatic cancer [48]. RT-qPCR analyses showed that miR-144 is overexpressed in colorectal cancerous tissues, thus yielding the possibility to serve as a clinical diagnostic marker [49]. Increased circulating levels of miR-144 also correlate with the downregulation of its predicted target: insulin receptor substrate 1 (IRS1), at both mRNA and protein levels; thus suggesting that miR-144 may also be a potential biomarker for type 2 diabetes (T2D) [50].

MiRNA-21 in DN and solid tumors

MiRNA-21 is commonly upregulated in solid tumors of the lung, breast, stomach, prostate, colon, brain, head and neck, esophagus, pancreas, and kidney [51]. Its overexpression inhibits proliferation of mesangial cells, and decreases urinary excretion of albumin in diabetic db/db mice. This suggests that miR-21 plays a protective role in glomerular hypertrophy and the onset of early DN. What is more interesting is that like miR-216a and miR-217, PTEN is also a potential target of miR-21 [52]. In addition, miR-21 prevented mesangial hypertrophy by targeting the PTEN/PI3K/Akt pathway. The main biological function of PTEN is to block PI3K signaling, a crucial pathway involved in metabolic responses to insulin [53]. Binding of insulin to the insulin receptor activates the PI3K pathway, while defects in PI3K signaling have been demonstrated in type 2 diabetes mellitus [54]. Altered PTEN expression is associated with diabetic nephropathy (DN), and miR-21 expression is downregulated in early stages of DN. Thus, it is conceivable that overexpression of miR-21 could prevent renal mesangial cell hypertrophy via downregulating PTEN in diabetic nephropathy [55].

MiRNA-375 in type 2 diabetes and colorectal cancer

While miRNA-375 expression is decreased in colorectal cancerous tissues, there is no significant correlation between the expression of miRNA-375 with tumor size, histological grade, or the tumor stage [56]. Like miRNA-144, miRNA-375 also inhibits tumor growth and metastasis of esophageal squamous cell carcinoma by repressing insulin-like growth factor 1 receptor (IGF-R1) [57]. Another study reported that pancreatic miR-375 expression was increased in type 2 diabetic (T2D) patients, and upregulated in pancreatic islet amyloid formation and A-cell deficit [24]. Of great interest here is that miR-375 seems to be an

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important regulator of insulin secretion. It is a pancreatic islet cell specific miRNA in mice, and it upregulates insulin secretion by targeting myotropin [58]. Furthermore, a decrease of miR-375 levels has been seen in high glucose ambience. Such a regulation of miR-375 expression by glucose is also observed in rat islet cells[59]. In addition, miR-375 regulates PDK1 protein levels, by interacting directly with the 3′ UTR, resulting in modulation of glucose stimulatory action on insulin gene expression and DNA synthesis. This is significant as PDK1 can regulate cell growth and organ development [60]. Finally, miR-375 expression is decreased in diabetic GK rat islets, thus emphasizing the significance of miR-375 in the pathophysiology of diabetes in rodents and humans [61] (Table 1).

This suggests that kidneys are not involved in the physiological urinary clearances of circulating miRNAs. The only exception to this is miR-638.. In general, it seems that in patients with severe chronic renal failure, the blood circulating levels of total and specific miRNAs are reduced in comparison to patients with mild renal impairment or normal renal functions. Intriguingly also the rates of *ex vivo* microRNA degradation of blood/plasma miR-210, miR-16 and miR-21 were higher in patients with end-stage renal disease (ESRD) compared to those individuals with normal renal functions [62].

HYPOTHESIS

The above reviewed literature suggests that miRNAs play an important role in the pathogenesis of diabetic nephropathy and in the initiation of renal glomerular mesangial cell dysfunctions. Conceivably, miRNAs can modulate the pathogenesis of DN by affecting various different pathways. We hypothesize that urinary excretion of miR-377, miR-192, miR-216/217 and miR-144 increase in patients with diabetic nephropathy as they may exacerbate the perturbations in body homeostasis in a hyperglycemic milieu. On the other hand, the urinary excretion of miR-21 and miR-375 may decrease as they bind to their various tissue targets and henceforth serve to preserve body homoestasis in patients with diabetic nephropathy. In line with this contention is the study by Melkonyan *et al.*, where 22 different urinary miRNAs were identified, but none of them specific for kidney tissues [19]. It is likely that miRNAs in urine versus in blood has no significant impact on a given disease characteristics, except miRNA-638 [56], which showed a significant increase in the urine of patients with Stage 4 CKD compared to normal and Stage 3 CKD patients. Neal *et al.* recently found a striking reduction in the overall levels of circulating miRNAs in patients with severe chronic kidney disease (CKD) having marked impairment in renal functions [62].

CONCLUSIONS

Several miRNAs have now been identified, which may be upregulated or downregulated in the progression of DN, and their detection in very early stages may be of value in predicting the disease course. In addition, increasing the threshold of detection of miRNAs by various amplification methods and at the same time delineating miRNA tissue-restricted expression profiles will be very helpful in advancing this field by comprehensively determining their relevance in the pathogenesis of diabetic nephropathy.

Urine proteomic profiling studies have identified normoalbuminuric subjects with type 2 diabetes who subsequently develop diabetic nephropathy. As a further step, in this review we discuss a group of miRNAs that could serve as biomarkers in DN, including: miR-377, miR-21, miR-192, miR-216a, miR-217, miR-375 and miR-144. The blood levels of some of these miRNAs are increased in DN, suggesting they may be of some value in monitoring the progress of diabetic nephropathy. Whereas some of the other miRNAs in blood circulation are consumptively decreased as they bind to their targets in various tissues so as to maintain

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body homeostasis in early stages of DN. Of course, there are also some changes in the urinary excretion of certain miRNAs in patients with diabetic nephropathy; for instance, there is an upregulation of miR-638 in the urine specimens. Further studies are needed to characterize miRNAs that are highly specific to DN in order to understand their role in the pathogenesis of diabetic nephropathy.

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Table 1

Disease-associated miRNAs

miRNA	Location	Change	Disease Associations	Reference
miRNA377	Lung	Upregulation	Pulmonary neoplasm	Melkamu *et al.* [26]
	Kidney		Diabetic nephropathy	Zhao *et al.* [25]
miRNA192	Blood	Upregulation	Hepatotoxicity	Wang *et al.* [30]
	Colon		Colonic tumors	Karayvaz *et al.* [31]
	Urine		IgA nephropathy	Wang *et al.* [32]
	kidney		Diabetic nephropathy	Krupa *et al.* [37]
miRNA216/217	Pancreas	Upregulation	Pancreatic ductal adenocarcinoma	Szafranska *et al.* [38]
	Kidney		Diabetic nephropathy	Kato *et al.* [40]
miRNA144	Colorectal tissue	Upregulation	Colorectal cancer	Kalimutho *et al.* [42]
	Blood		Type 2 diabetes	Karolina *et al.* [43]
miRNA21	Lung, breast, stomach, prostate, colon, etc.	Upregulation	Lung, breast, stomach, prostate, colon tumor	Chow *et al.* [44]
	Kidney	Downregulation	Diabetic nephropathy	Mahimainathan *et al.* [48]
miRNA375	Colorectal tissue	Downregulation	Colorectal cancer	Dai *et al.* [49]
	Esophageal tissue		Esophageal squamous carcinoma	Kong *et al.* [50]
	islet cells		Type 2 diabetes	El Ouaamari *et al.* [53]
miRNA638	kidney	Upregulation	Chronic kidney disease	Neal *et al.* [54]