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REVIEW ARTICLE

An insight of dysregulation of microRNAs in the pathogenesis of diabetic kidney disease

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Abstract

Background: Worldwide, diabetic kidney disease (DKD) remains the leading cause of chronic kidney disease (CKD) and its successor, the end stage renal disease, both of which constitute major morbidity and mortality concerns. *Content:* The residual risk of disease progression remains despite the advert of newer therapeutic modalities and current biomarkers. Meanwhile, microRNAs (miRNAs) are small non-coding RNAs, which regulate gene expression post-translationally by binding to specific mRNAs. Circulating miRNAs are increasingly recognised as novel biomarker or therapeutic targets, owing to their unique characteristics, such as their resilience to degradation by endogenous RNases, multiple downstream targets, involvement in biological processes, some degree of tissue specificity, relatively easy access and quantification. Unlike proteins, there are far less miRNAs and mature miRNAs are highly stable, structurally less complex without post-translational modification with high degree of conservation across species. Aberrant expression of miRNAs has been established in both *in vitro* and *in vivo* models of DKD. An up-to-date compilation of previous studies involving selected circulating miRNAs in blood and urine samples of DKD patients is discussed herein. *Summary:* This review highlights the unmet clinical challenges and dysregulation of miRNAs in the pathogenesis of DKD.

Keywords: Diabetic kidney disease, clinical challenges, pathogenesis, microRNAs

INTRODUCTION

Diabetic kidney disease (DKD) continues to be the leading cause of chronic kidney disease (CKD) worldwide. Through its progressive nature to end stage renal disease (ESRD), CKD is becoming a major morbidity and mortality concern. To this day, despite the advent of newer therapies, the residual risk of disease progression remains. Hence, there is a growing need to further unravel the underlying mechanisms of DKD in search for novel biomarkers and therapeutic targets. This review aims to discuss the unmet clinical challenges and the identification of microRNAs as potential modulators in epigenetic mechanisms and regulators of gene expression in DKD, whilst giving a brief current overview of the disease.

DIABETIC KIDNEY DISEASE (DKD)

The disease burdens

Diabetes mellitus (DM) is rapidly becoming a global epidemic, currently affecting 463 million people (9.3% of adults) in 2019.[1] As many as 700.2 million (10.9%) people are estimated to be diagnosed by the year 2045 worldwide.[1] In Malaysia, the prevalence of DM continued to steadily increase from 15.2% (2703 out of 17783 respondents) in 2011[2] to 17.5% (3489 out of 19935) in 2015[3] and 18.3% (1915 out of 10462) in 2019.[4] Thus, currently Malaysia has surpassed the global projected prevalence for 2045 of 10.9% global population.

The prevalence of CKD remained the highest among individuals with DM among the U.S adult population surveyed in 2015-2018, accounting for 39% of patients (304,379 out of 783,925) with

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ESRD and costing Medicare USD130 billion in 2018.[5] Due to the increasing prevalence of DM[5], that of ESRD has been projected to substantially increase to about 30% more between 2015 and 2030.[6]

Likewise, the prevalence of CKD among the Malaysian adult population continued to rise from 9.07% (80 out of 876 respondents) in 2011[7] to 15.48% (138 out of 890 respondents) in 2018[8] in parallel with the increasing prevalence of its underlying risk factors.[4] It is of no surprise that DKD remained the leading cause for new dialysis for the past 2 decades, accounting for 69.2% (5834 out of 8431) in 2018.[9] Ultimately, the prevalence of ESRD is projected to more than double, from about 44,000 in 2018[9] to almost 110,000 in 2040.[10]

CKD and its consequent ESRD impose a major economic burden, both personally, as 30% of dialysis was self-funded[9] and to the government, with increasing public sector's total health expenditure from 2.95% [MYR 572 million (USD 135 million);USD 1 = MYR 4.22] to 4.2% [MYR 1.12 billion (USD 265 million)] in 2010 and 2016, respectively.[11] At this rate, the total cost of ESRD is estimated to keep rising, from MYR 2 billion (USD 476 million) in 2020 to MYR 4 billion (USD 947 million) in 2040.[12] Furthermore, not only CKD imposes a higher mortality rate, mainly via promotion of cardiovascular disease (CVD)[13], it is a major cause of morbidity both to patients and caregivers.[12]

Physiology: The Kidney

In brief, the bean-shaped kidneys, each surrounded by fibrous renal capsule, are made up of two regional parts, the outer cortex and the inner medulla.[14] The glomerulus, together with its encompassing Bowman's capsule, is known as renal corpuscle. The nephron, which is made up of renal corpuscle and tubule, forms the urine-producing functional unit of the kidney.[14] Comprising a network of capillaries at the beginning of a nephron, the glomerulus filters 20% of circulating blood daily, across its filtration barrier.[14] The three main resident cell types therein are glomerular endothelial cells (GECs), glomerular mesangial cells (GMCs) and podocytes. The glomerular basement membrane (GBM) sits in between GEC and podocytes and together they form the glomerular filtration barrier (GFB) which serves as charge and size barriers to macromolecules. The glomerulus is connected to the proximal tubule which eventually leads to the collecting tubule.[14]

Glomerular filtration rate (GFR), or the rate at which blood is filtered at the glomerulus, represents the best measure of kidney function.[15] At the glomerulus, larger proteins, such as albumins, being too large to cross the GFB, remain within the circulation. However, in an event of glomerular or tubular damage, abnormal amount of proteins may appear in urine. Thus, proteinuria is suggestive of kidney damage and the amount of albumin, which is normally about 25% of total protein in urine, progressively increases as protein excretion increases.[15]

Pathogenesis

In susceptible cells, in which glucose uptake is mediated via insulin-independent GLUT-1 transporter, hyperglycaemic milieu leads to intracellular hyperglycaemia which initiates a series of metabolic and haemodynamic changes.[16] DKD is thought to begin in the glomeruli, whereby hyperglycaemia-induced activation of intracellular signaling pathways eventually culminates in renal fibrosis.[16,17] The pathological paradigm has since expanded to recognise the roles of tubular injury and several new key players.[17]

Hyperglycaemia activates Protein Kinase C (PKC) signalling pathway which in turn leads to increased expression of growth factors such as Transforming growth factor-beta (TGF-β) and Vascular endothelial growth factor (VEGF); altered activities of enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and endothelial nitric oxide synthase (eNOS); activation of transcription factors such as Nuclear factor-kappa B (NF-κβ) and activation of signalling proteins such as mitogen-activated protein kinase (MAPK).[18] Hyperglycaemia also results in production of reactive oxygen species (ROS) and production of advanced glycation end products (AGEs), both of which also activate PKC pathway.[16,18] Tissue-specific alteration in the expression of pro-fibrotic and pro-inflammatory growth factors and cytokines then activates transcription factors, leading to altered gene expression involved in various cellular processes including inflammation, fibrosis, cell cycle and cell death.[16]

Several growth factors and cytokines have been shown to be involved in DKD. By increasing the expression of extracellular matrix (ECM) proteins, TGF-β, which activates MAPK[19] is said to play a key part in mesangial fibrosis and hypertrophy. Activation of key effector transcription factors such as Janus kinase-STAT

(JAK–STAT) and NF-κβ leads increased output of pro-inflammatory cytokines, inducing an influx of macrophages, which via their production of ROS, further stimulates the resident cells.[20]

Hyperglycaemia also initiates haemodynamic changes such as impaired tubulo-glomerular autoregulation leading eventually to increased intraglomerular pressure.[20,21] This in turn increases mechanical stress and hyperfiltration leading to capillary rarefaction and proteinuria with subsequent tubular cell injury, respectively.[20] Hyperglycaemia also causes upregulation of vasoactive factors, namely RAS and endothelin-1, and downregulation of vasodilation endothelial nitric oxide (NO), thus compounding haemodynamic imbalance.[20]

Once initiated, these processes are further promoted by underlying conditions, such as an underlying genetic susceptibility and CVD risk factors including dyslipidaemia with its metabolic burden of excess fatty acids and hypertension.[16,21] Furthermore, it is believed that the underlying fundamental diabetic milieu of insulin resistance also plays a role in diabetic complication with evidence of tissue selective insulin resistance observed in glomerular cells such as GMCs and podocytes.[16]

Once established, these complex interlinked pathways become self-perpetuating leading to progressive disease.[16] For example, activation of vasoactive RAS and endothelin, in combination with insulin resistance-induced reduction in vasodilating NO, leads to endothelial dysfunction, which increases vascular susceptibility to oxidative stress.[21,22] The resultant haemodynamic imbalance, glomerular hypertension and subsequent capillary rarefaction eventually lead to reduced blood flow, and ultimately hypoxia, thus, predisposing renal glomeruli to acute kidney injuries, which can further exacerbate DKD.[20-21] Furthermore, hypoxia arising from oxidative stress and increased oxygen demand by tubular cells compounded by impaired oxygen supply due to interstitial fibrosis further promotes DKD progression.[20]

Increasing evidence suggests that mitochondrial dysfunction may play a key role in the diabetic complications, driven by its overproduction of ROS.[16,21] In addition, non-mitochondrial source such as cytosolic NADPH oxidase, and inflammatory cells such as macrophages also produce ROS and thus contribute to oxidative stress.[16,21] Cellular senescence has recently become increasingly recognised as one of the new players in DKD.[23] Senescence, which can results from DNA damage secondary to oxidative stress via ROS production, mediates auto- and paracrine effects via a series of proteins termed senescence-associated secretory phenotype, in a cell- and context-specific manner.[23] Dysregulation of autophagy such as that of podocytes leading to loss of podocytes, has also been implicated in DKD pathogenesis.[20,21]

Not only DKD is a disease of multiple and complex underlying mechanisms, observations that only a handful of diabetic patients progress to ESRD and that not all patients develop DKD despite poor glycaemic control as well as familial clustering of DKD and ESRD, suggest a role of genetic susceptibility in the development of these conditions.[17,22,24] Indeed higher prevalence rates of DKD and albuminuria are reported in certain ethnicities, for example in African Americans and Asians, respectively, compared to Caucasians.[17,22,25] Findings that many of the genetic studies could not be replicated among different ethnic groups, further suggested an ethnic-specific association between genetic polymorphism and DKD.[24] On the other hand, DKD develops in some patients despite good glycaemic control, suggesting a role of metabolic memory in DKD.[16,20-22]

Recently, epigenetic modification during the early stages of DKD has been suggested to play a part in DKD.[22] Epigenetic modification is also thought to be the key mechanism underlying the self-perpetuation of diabetic complication pathways[16] as well as the phenomenon of metabolic memory, whereby, despite subsequent glycaemic control, epigenetic changes mediate lasting long-term expression of diabetes-related genes and phenotypes induced by prior hyperglycaemia.[16,20-22] Furthermore, it is hoped that elucidation of the epigenome will help fill the gap left by the absence of a conclusive genomic candidate[17] in explaining part of the ethnic variation and familial clustering of DKD, albuminuria and ESRD.[18,22,25] Figure 1 depicts the pathogenesis of DKD as discussed.

Clinical presentation

Clinically, DKD is a syndrome characterised by decreased kidney function and increased renal albumin excretion, measured as GFR and albuminuria, respectively. Stage 1 is characterised by glomerular hyperfiltration, detected as increased GFR. GFR remains elevated or returns to normal with progressive glomerular injury, manifest clinically as microalbuminuria

during Stage 2.[17,26] In Stage 3, glomerular injury has progressed to macroalbuminuria, often with hypertension; whilst increasing albuminuria, hypertension and decreasing GFR characterise Stage 4.[17,26] Stage 5 is when GFR falls to <10mL/min/1.73 m^2, with global sclerosis, which eventually requires renal replacement therapy.[17,26]

Laboratory Evaluation

Albuminuria

Urinary albumin excretion is traditionally classified as normo-, micro- and macroalbuminuria. "Moderately increased albuminuria" and "severely increased albuminuria" are newly-introduced terms for microalbuminuria and macroalbuminuria, respectively, as recommended by the 2012 Kidney Disease Improving Global Outcomes (KDIGO) guidelines for CKD.[27] The former refers to low levels of albumin in the urine, which is undetectable by conventional urine dipstick.

Glomerular Filtration Rate (GFR)

Creatinine is the most commonly measured endogenous marker of GFR, either as its serum concentration or renal clearance. Although creatinine clearance is more sensitive than the former, it requires a timed urine collection, which is subject to collection error and inconvenient.[28] The three most used creatinine-based estimated GFR (eGFR) equations in adults are the Cockcroft–Gault, the Modification of Diet in Renal Disease (MDRD) Study and the Chronic Kidney Disease-Epidemiology (CKD-EPI) equations. Although it is mathematically simple, as it estimates urinary creatinine clearance, the

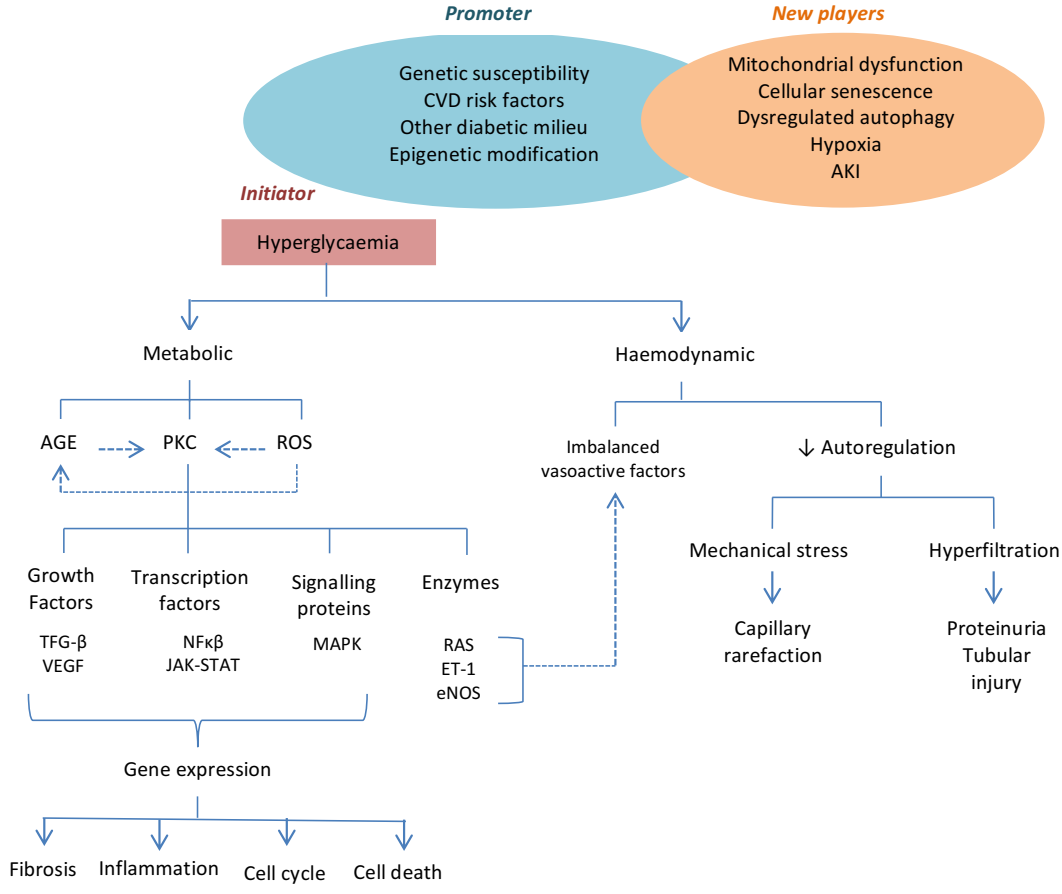

FIG. 1: Pathogenesis of diabetic kidney disease. CVD: cardiovascular disease; AKI: acute kidney injury; AGE: advanced glycation end product; PKC: Protein Kinase C; ROS: reactive oxygen species; TGF-β: Transforming growth factor-beta; VEGF: vascular endothelial growth factor; NFκβ: Nuclear factor kappa-B; JAK-STAT: Janus kinase-STAT; MAPK: mitogen-activated protein kinase; RAS: renin-angiotensin system; ET-1: Endothelin-1; eNOS: endothelial nitric oxide synthase.

Cockcroft–Gault equation overestimates GFR because of tubular secretion of creatinine.[28] Also, inclusion of 'weight' in the equation may limit its clinical utility.[15] Meanwhile, as it is developed in a CKD cohort, the MDRD Study tends to underestimate GFR greater than 60 mL/min/1.73 m^2 but is more accurate at lower ranges.[15] The CKD-EPI equation involved some healthy potential kidney donors and therefore performs better than the other equations.[15]

Histological staging

A recently proposed histological classification was based on glomerular changes, with separate classification of interstitial and vascular lesions.[29] Glomerular lesions are believed to best reflect the natural course of progressive DKD[29] and that tubulointerstitial changes are not specific for DKD.[30] In short, DKD is thought to progress from GBM thickening (Class I) to mesangial expansion (Class II), Kimmelstiel–Wilson lesions (Class III) and global glomerulosclerosis (Class IV).[29]

Treatment

The pharmacological modalities of DKD anchor on 4 major aspects, namely CVD risk reduction, blood pressure control, renal function preservation via RAS inhibition and glycaemic control. As DKD substantially increases one's CVD risk, aggressive attempts at CVD risk reduction include lipid-lowering and blood pressure control. Although dyslipidaemia is thought to promote DKD pathogenesis, there is inconclusive evidence of renoprotective effect of lipid-lowering agents.[25] Regardless, its use in CKD is purposefully for its evident cardioprotective effect.[25] The cornerstone of blood pressure-lowering treatment is via RAS inhibition, either with angiotensin-converting-enzyme inhibitor (ACE-I), or angiotensin-receptor-blocker (ARB), has been established to be renoprotective.[16,25] However, dual RAS inhibition does not improve outcome but incurs increased rates of adverse effect and therefore not recommended.[25]

Although intensive glycaemic control can lower the risk of developing DKD and slows its progression, particularly in type 1 DM (T1DM), this occurs at an increased risk of hypoglycaemic events.[25] The benefit of tighter glycaemic control seems to diminish at advanced stages of DKD. Neither it completely eliminates the risk of DKD occurring, nor lowers the risk of progression to ESKD.[25] Thus, while the optimum glycaemic control is said to be of glycated haemoglobin A1c (A1c) less than 7.0%, target should be individualised to keep hypoglycaemic risk at bay.[31] Recently, glycaemic control has been revolutionised by the advert of a new glucose-lowering agent, sodium-glucose cotransporter-2 inhibitors (SGLT2-I) which exhibits renoprotective effects including reduction in renal endpoints.[25] Meanwhile, non-pharmacological interventions, such as weight control, increased physical activity, salt intake reduction and tobacco cessation are regarded as part of a holistic approach in managing DKD patients.[25]

The clinical challenges

Currently, diagnosis and monitoring of DKD is achieved by means of clinical parameters namely albuminuria and GFR. However, accumulating evidence suggests that clinical presentation, clinical course and progression rate of DKD vary substantially among DKD patients, particularly in type 2 DM (T2DM).[32] The classical natural history of DKD heralded by microalbuminuria and hyperfiltration which slowly progress to macroalbuminuria and reduced GFR, respectively, is nowadays changing.

The classical progression of albuminuria is not always seen[17,25,33,34] and the rates of regression of albuminuria are greater than previously thought.[25,34] Although the latter could partly be due to increasing use of RAS blockers, this phenomenon is also observed in the lack of it.[34] Furthermore, the progression rates vary amongst DKD patients, whereby in general, one-third of patients in each albuminuria category will progress to the next stage.[17] Progressive decline in GFR has been thought to mirror changes in albuminuria. However, population studies have shown that reduction of GFR occurred despite normoalbuminuria[32,35] and a substantial number of patients with DKD are now presenting with non-proteinuric low GFR.[17] Thus, albuminuria represents a dynamic and fluctuating rather than a linearly progressive phenomenon and is therefore not a sensitive marker for DKD progression.[17,35] Furthermore, microalbuminuria lacks specificity for DKD, as it also occurs in other non-DM CKD such as glomerulonephritis. In addition, microalbuminuria is a known CVD risk factor in person with and without diabetes and is also inherently confounded by conditions such as fever, infection, physical exercise and obesity.[34]

Meanwhile, GFR may be reduced by 50% before serum creatinine exceeds the reference

range.[15,28] Thus, its reciprocal exponential relationship with GFR renders serum creatinine a relatively insensitive marker of mild renal impairment, as seen in the early stage of DKD.[15] Being a metabolic product of muscle, serum creatinine level reflects muscle mass and recent protein intake. Therefore, not only creatinine is a non-ideal marker of GFR, it does not reflect the histological lesions seen in DKD.[28] Also, serum creatinine is inherently affected by its physiological renal and extra-renal handling, whereby creatinine is freely filtered at the glomerulus as well as being secreted by the tubules.[15] The latter increases at low GFR and is inhibited by certain drugs; whilst its extra-renal clearance by intestinal bacteria also increases at low GFR.[15]

Although creatinine-based eGFR equations correct for some of these non-GFR variability of serum creatinine, making them more superior at estimating renal function than serum creatinine *per se*, these equations are still susceptible to many of the inherent limitations of serum creatinine.[15] In addition, these equations are unable to accurately estimate hyperfiltrating stage of DKD, as values above 60ml/min/1.73m^2 are not numerically reported.[28] Histological findings by means of kidney biopsy, although is considered a gold standard in establishing a diagnosis and prognosis of DKD, is invasive and expensive with a 3 % risk of major complications and therefore rarely performed in routine clinical practice; most often being indicated by atypical clinical presentation.[25,33] In addition, the changes in T2DM DKD are more heterogeneous than those in T1DM[32], particularly in those with normoalbuminuria.[34] On this ground, the authors recommended a separate histological classification for T2DM DKD, arguing whether the current classification[29] is representative of the heterogeneity in T2DM and whether its heterogeneity is reciprocally predictable from the classification.[32] It was argued that the heterogeneity in clinical course and histological changes seen in T2DM may represent different time-points in the disease, which in turn is reflective of the uncertain disease onset in T2DM, or contributions of different factors such as ageing, hypertension and higher atherosclerotic burden in T2DM or due to heterogeneity of T2DM patients in general.[32,34]

Furthermore, the histological changes in DKD often precede clinical abnormalities and were highly variable amongst this group as a whole.[36] Interestingly, finding of a correlation between albuminuria and tubulointerstitial injury but not with the severity of DKD, supported the notion that tubular injury occurs early in DKD[17]; whereby during the early stage of clinically silent albuminuria, the excess albumin was successfully reabsorbed by the initially healthy tubular epithelial cells.[36] The eventual loss of tubular cell reabsorption capacity caused by inflammatory and fibrotic injuries imposed by albumin leading to clinically apparent albuminuria[37] could thus explain the severe glomerular histological changes that precede albuminuria onset.[36] In this light, it is thus suggested that albumin is not just a marker of glomerular damage but a pathological stimulus itself.[37]

In all, these evidences suggest that the two current routine clinical parameters, namely urine albuminuria and GFR, are not sensitive markers of DKD as their changes are not reflective of and non-parallel to the pathological changes seen in kidney biopsy. This highlights the need of an early recognition of DKD and more specific marker, as severe renal lesions become progressively less susceptible to treatment.[32] Furthermore, the early stage of DKD itself is not without risk as it is associated with increased CVD risk.[38]

Meanwhile, although current therapeutic approaches to DKD continues to target the four main domains, as discussed earlier, epidemiological evidences showed that tight glycaemic control is preventive of DKD progression before its onset but not otherwise; albeit at a greater risk of acute hypoglycaemia.[35] While RAS inhibition remains one of the cornerstone therapeutic regimes in DKD, there has been new advances in the development of therapeutic approaches such as the introduction of SGLT2-I. Nevertheless, the residual risk of disease progression still exists, reflecting the multiple pathophysiological mechanisms involved in DKD.[39]

The role of genetic susceptibility has long been recognised in DKD. Yet there is paucity in finding a conclusive genomic candidate.[17] Epigenetic modification has been implicated in the early stages[22] and in self-perpetuation of DKD pathways[16] as well as the phenomenon of metabolic memory in DKD progression.[22] Furthermore, epigenetic modification may be partly responsible for the ethnic variation and familial clustering of DKD and its associated conditions.[22,25] One of the epigenetic candidates of interest is microRNA (miRNA). miRNA is a family of small non-coding RNAs of 19-22

nucleotides which regulates gene expression post-translationally by binding to specific mRNA.[40]

microRNAs

Biogenesis and functions

Biogenesis of miRNA begins in the nucleus as primary miRNA (pri-miRNA), which is then processed by a nuclear RNase, Drosha, into precursor-miRNA (pre-miRNA).[40] A second cytoplasmic RNase, Dicer, cleaves pre-miRNA into mature duplexed strands, one of which then complexes with Argonaute proteins, forming RNA-induced silencing complexes (RISCs).[40,41] Subsequently, miRNAs are released either as vesicle-free RNA protein complexes such as RISCs or conjugated with lipoprotein; or vesicle-embedded such as exosomes or apoptotic bodies.[40-42] Readers are referred to previous reviews[40-42] for further details.

The mature miRNA helps identify the sequences within the 3' UTR of the target mRNA, called the seed sequence, with often a perfect Watson-Crick complementarity to the 5' end of the miRNA.[43] By either perfect or partial complementarity to the seed sequences of those mRNAs, miRNA then causes mRNA destabilisation or translational repression, respectively, or a combination of the two.[43]

Potential as a biomarker or therapeutic target

While the number of non-modified human proteins has been estimated to be about 20,000, up to 100 different proteins are potentially produced from a single gene.[44,45] In comparison, there are currently 38589 miRNAs in the human genome [http://www.mirbase.org/]. Unlike proteins, miRNAs have less complex structure without post-processing modification[46] and its highly regulated biogenesis produces stable mature miRNAs.[40] Furthermore, miRNAs display a high degree of conservation across species.[40]

Findings of circulating miRNAs in extracellular fluid (ECF) suggested that miRNAs may represent a type of cell-to-cell communication.[41] Indeed, miRNAs are released from cells following injury, inflammation or apoptosis before being taken up by target cells and thereby, exert their biological functions.[40] Of particular importance is that their association with either vesicles or protein-complexes renders them to be stable and protected from degradation by endogenous RNases.[41]

miRNAs have been implicated in several biological processes, such as cell proliferation and differentiation[40] and their levels and compositions correlate with pathophysiological conditions.[46] Indeed, specific signatures of dysregulated miRNAs in human cancer have been established since the breakthrough findings in the early 2000s.[40]

As each miRNA is potentially capable of targeting and regulating several mRNAs by partial complementarity binding to the seed sequences of these mRNAs[47], it follows that each miRNA can potentially regulate multiple aspects of one or more pathways.[48] Restoring the expression of a miRNA may therefore reinstitute those of the downstream targets.[47] Thus far, experimental restoration of miRNA expression by means of knock-ins and knock-offs has been encouraging.[47]

Detection and quantification of miRNAs in ECF is relatively easier compared to proteins with the availability of commercial isolation kits, such as column purification method, and high-throughput quantification methods, such as RT-PCR.[41,46] The use of synthetic complementary oligonucleotide offers sufficient analytical specificity whilst DNA amplification methods increase sensitivity.[46] In addition, following a recommended sample processing of within 2 hours of collection[41,49], miRNAs in ECF remain stable following long-term storage at -80°C.[49] Therefore, owing to their resilience, some degree of tissue specificity, stability and easy access, circulating miRNAs are increasingly recognised as potential new biomarkers.[47]

miRNAs in DKD

Previous studies have established aberrant expression of miRNAs and their involvement in cellular mechanism of DKD.[19] Recently, expression of tissue-specific miRNA has been shown in the glomeruli of several types of renal diseases including DKD.[50] Interestingly, although some overlaps were seen, differential expression of certain miRNAs, such as downregulation of miR-30a-5p, was only significant in DKD.[50] This further suggests a potential role of miRNAs in DKD.

Aberrant expression of glomeruli-enriched miRNAs has been previously observed in experimental models of kidney injury[51] and DKD.[52] Among miRNAs involved in kidney injury were those in the miR-200 family, -217, -216 and -377[51]; whilst downregulation of miR-25, -26a, -30b, -141-3p, -196a and -let-7; and

Table 1. Expression studies of selected miRNAs in blood samples of DKD patients

Author	Lv *et al.* (2015) (56)	Motawi *et al.* (2018) (57)	Bai *et al.* (2016) (58)	Mohammad *et al.* (2017) (59)	Liu *et al.* (2017) (60)	Al-Kafaji *et al.* (2016) (61)	Chien *et al.* (2016) (62)	Ma *et al.* (2016) (63)	Akhbari *et al.* (2018) (64)
Sample	Serum	Serum	Plasma	Serum	Serum	Whole Bood	Serum	Serum	Serum
Control	Non-DM	Non-DM	Non-DM	Non-DM		Non-DM		Non-DM	Non-DM
Gp1	NA T2DM	NA	DKD	NA		Non-DKD	Non-DKD	NA	Non-DKD
Gp2	MIC	MIC		MIC		MIC	MIC	MIC	MIC
Gp3	MAC			MAC		MAC	MAC	MAC	MAC
Extract kit	miRcute	QIAamp (Qiagen)	Trizol	Trizol	Trizol	Norgen	miRVana	miRcute	miRCURY
cDNA kit	miRcute	MicroRNA RT kit			MMLV	TaqMan	TaqMan	miRcute	Universal kit Exiqon
miR	-130b	-130b	-130b	-155	-25	-126	-21, -29a, -29b, -29c, -192	-192	-93
HKG	cel-miR-39	RNU6B	RNU6B	RNU48	Ath-miR-156a	RNU6B	RNU6B	cel-miR-39	miR-16
Method	RT PCR miRcute SYBR	RT PCR (Applied Biosystem)	miScript PCR system	RT PCR ABI Prism 7900	RT PCR AB7300	RT PCR Applied Biosystem	RT PCR ABI 7500	RT PCR miRcute SYBR	RT PCR LNA primer ExiLENT SYBR
Normalisation	cel-miR-39	RNU6B	RNU6B	Mean of non-DM	ath-miR-156a or RNU6B	RNU6B	RNU6B	cel-miR-39	Mean of Non-DM

Notes:
DM, diabetes mellitus; DKD, diabetic kidney disease; T2DM, type 2 diabetes mellitus; NA, normoalbuminuria; MIC, microalbuminuria; MAC, macroalbuminuria; RT PCR, real time PCR; HKG, housekeeping gene; SYBR, Sybr Green.

Table 2: MiRNA profiling studies in urine of DKD patients

Author	Argyropolous *et al*. 2013 (65)	Barutta *et al*. 2013 (66)	Delić *et al*. 2016 (67)	Cardenas-Gonzalez *et al*. 2017 (68)	Prabu *et al*. 2018 (69)
DM	T1	T1	T2	T1 & T2	T2
Group	1. NA v MAC 2. IMA v PMA	1. NA 2. PMA 3. C	1. NA 2. DKD (3NA,5 MIC) 3. C	1. Discovery 2. Confirmation 3. Replicate	1. Non-DM 2. NA 3. MIC 4. MAC
Type	Free	Exosome	Exosome	Free	EV
Panel/ Method	Exiqon miRNA qPCR panels 1 and 2 (Version 1)	Human TaqMan miRNA Array A	Agilent´s miRNA microarrays.	Global urinary miRNome	Human Urine Exosome Focus microRNA PCR Panel (Exiqon)
No. of miRNAs	748	377	2006 (miRBase 19.0)	2402 (miRBase 21.0)	87
Result	1. PMA v baseline (NA) urine Upregulated 214-3p, 92b-5p, 765, 429, 373-5p, 1913, 638 Downregulated 323b-5p, 221-3p, 524-5p, 188-3p 2. PMA v IMA: Upregulated 17-5p, 323b-5p, 433, 222-3p, 628-5p Downregulated 92a-3p, 589-5p, 373-5p, 520h 3. NA v MAC: Upregulated 335-5p, 141-3p, 619, 552, 1912, 1224-3p, 424-5p, 29b-1-5p Downregulated 221-3p	22 miRNAs: dysregulated 1. PMA v NA: Upregulated: 130a, 145 Downregulated: 155, 424 2. NA v C: Upregulated: 145, 130	16 miRNAs: dysregulated in DKD v C & DM -320c, 6068, -1234-5p, 6133, 4270, 4739, 371b-5p, 638, 572, 1227-5p, 6126, 1915-5p, 4778-5p, 2861, 30e-5p, 30d5p. -29 & -200 families ↓ in DM ↓↓ in DKD	5 miRNAs: Upregulated: miR-4536, -6747 Downregulated: miR-2861, -1915-3p, -4532	↑miR30a-5p in MAC ↑ miR27b-3p, let7i-3p, miR-24-3p ↓ miR 15b-5p in MIC

Notes:
DM, diabetes mellitus; T1, type 1; T2, type 2; NA, normoalbuminuria; MAC, macroalbuminuria; IMA, intermittent microalbuminuria; PMA, persistent microalbuminuria; DKD, diabetic kidney disease; C, healthy Control; MIC, microalbuminuria; EV, extracellular vesicles.

Table 3: Differential expression studies of specific DKD-related miRNAs in urine

Author	Peng *et al.* (2013) (70)	Lv *et al.* (2013) (71)	Jia *et al.* (2016) (72)	An *et al.* (2018) (73)	Sayilar *et al.* (2016) (74)
DM	T2	CKD biopsy (3 DKD) vs Healthy	T2	(Not specified) Biopsy-proven DKD	T1 & T2 & HTN
Group	NA vs MAC		1. NA 2. MIC 3. MAC 4. Healthy		1. CKD Stg 2 2. CKD Stg 5 3. Healthy
Type	Free	exosome	EV	Free	Free
miRNA	miR29a-c	miR-29a-c miR-200a-c	miR-192, -194, -215	miR-196a	-192, 195, 451, 21, -124
Results	miR-29a and miR-29c > miR-29b in urine T2DM miR29a in MAC > NA	miR-29 & miR-200 family in urinary exosomes ↓↓ in CKD	miR-192 ↓ in MAC	Urinary free miR-196a correlated with many clinical & pathological parameters.	miR-195 ↓Stg 3 &5 CKD miR-192 ↑Stg 3 & 5 CKD

Notes:
DM, diabetes mellitus; T2, type 2; CKD, chronic kidney disease; T2, type 2; HTN, hypertension; NA, normoalbuminuria; MAC, macroalbuminuria; MIC, microalbuminuria; Stg, stage; EV, extracellular vesicles.

Table 4: Patterns of dysregulation of circulating miRNAs in DKD

	Upregulation	**Downregulation**
Serum	miR-155[59] miR-21[62] miR-29a[62] miR-192[62]	miR-130b[56-58] miR-25[60] miR-126[61] miR-192[63]
Urine	miR-335-5p[65] miR-141-3p[65] miR-619[65] miR-552[65] miR-1912[65] miR-1224-3p[65] miR-424-5p[65] miR-29b-1-5p[65] miR-130a[66] miR-145[66] miR-4536[68] miR-6747[68] miR-30a-5p[69] miR-27b-3p[69] let-7i-3p[69] miR-24-3p[69] miR-29a[70] miR-192[74**]	miR-221-3p[65] miR-155[66] miR-424[66] miR-29 family[67,71*] miR-200 family[67,71*] miR -2861[68] miR -1915-3p[68] miR -4532[68] miR-15b-5p[69] miR-192[72] miR-195[74]

Notes:
CKD, chronic kidney disease; DKD, diabetic kidney diseaseT1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; HTN, hypertension; * In CKD patients (including 3 DKD patients); ** In CKD patients (including T1DM, T2DM and HTN patients).

upregulation of miR-744, -146a and -34a were reported in DKD.[52]

miRNA profiling studies in DKD patients have been done in various populations, as previously reviewed.[53-55] An up-to-date compilation of previous studies involving selected circulating miRNAs in blood samples of T2DM with DKD are summarised in Table 1[56-64]; and those involving urinary miRNAs in DKD patients in Table 2[65-69] and Table 3.[70-74] The patterns of dysregulation of circulating miRNAs in these studies are summarised in Table 4.

As shown in Table 1, a downregulation of serum miR-130b levels in T2DM patients compared with control, with further decrease in patients with micro- and macroalbuminuria has been shown.[56] The authors concluded that serum miR-130b may be a prospective biomarker for detecting early stage of DKD in T2DM.[56] Similar significant downregulation of serum miR-130b in T2DM patients compared to healthy controls, more so in micro- than in normoalbuminuric group was later observed.[57] Interestingly, these findings concurred with a previous finding of a downregulated miR-130b in plasma of biopsy-proven DKD patients compared with normal controls, which correlated with impaired renal function.[58]

A significant difference in miR-155 and serum neutrophil-gelatinase associated lipocalin (sNGAL) levels between T2DM patients with DKD and healthy non-DM groups was also shown.[59] Meanwhile, a downregulation of miR-25 was shown in the sera of diabetic patients with and without DKD, compared to healthy controls.[60] Also, an association between downregulated miR-126 with the development of DKD in T2DM patients led to a suggestion that miR-126 may be a prospective circulating biomarker of risk estimation in DKD.[61]

Significant upregulation of miR-21, miR-29a and miR-192 in the macroalbuminuric group compared with microalbuminuria groups led to a conclusion that these miRNAs could reflect DKD pathogenesis and therefore potentially be biomarkers for DKD progression.[62] Meanwhile, miR-192 was shown to be significantly lowest in macro- compared to micro- and normoalbuminuric groups[63]; whilst miR-93 was concluded as a good diagnostic marker for DM but not able to distinguish between the diabetics with and without DKD.[64]

Profiling studies involving a large number of free or exosomal urinary miRNAs, ranging from 87[69] to 2402[68] miRNAs, showed several dysregulated urinary miRNAs in DKD patients, as shown in studies summarised in Table 2[65-69]. As shown in Table 2, a profiling study of urinary free miRNA in Type 1 diabetic patients with and without overt DKD (macroalbuminuria vs normoalbuminuria) and patients with incipient DKD (intermittent vs persistent microalbuminuria) revealed differential expression of 27 miRNAs between these groups, with each group having unique expression pattern.[65] Meanwhile, upregulation of miR-130a and miR-145 and downregulation of miR-155 and miR-424 in urinary exosomes was shown in T1DM patients with and without incipient DKD (persistent microalbuminuric vs normoalbuminuric).[66]

16 dysregulated miRNAs in urinary exosomes of DKD patients compared to non-albuminuric T2DM patients[67]; whilst 5 dysregulated miRNAs in DKD patients compared to non-diabetic healthy controls, were discovered in a profiling study of free urinary miRNAs of T1 and T2 diabetic patients with biopsy-proven DKD with evidence to suggest their roles in fibrotic pathophysiology in DKD, particularly miR-2861, miR-1915-3p and miR-4532.[68] Meanwhile, a subset of miRNA which could differentiate micro- from normoalbuminuric and macro- from normoalbuminuric T2DM patients was also found in a profiling study of a number of DKD-related urinary extracellular vesicular (EV) miRNAs.[69] Interestingly, a genome-wide profiling of free, EV and EV-depleted urinary fraction of miRNAs in T1DM patients found that miRNA spectra of urine and EVs were mostly unique.[75] The authors concluded that comparing between non-DKD (normoalbuminuric) and overt DKD (macroalbuminuric) patients, certain miRNAs showed differential expression in urine whilst others in urinary EV fraction.[75]

Subsequently, studies involving urinary miRNAs investigated specific miRNAs which have been previously studied in DKD (Table 3).[70-74] As shown in Table 3, a study examining urinary miR-29 family showed a significantly higher expression of cell-free urinary miR29a in macro- than normoalbuminuric inT2DM patients and that it correlated significantly with urinary albumin excretion rate.[70] Another study showed significant downregulation of urinary exosomal miR-29 and miR-200 families in biopsy-proven CKD patients compared to healthy controls.[71] The majority of patients, however, had IgA nephropathy with only 3 DKD patients.[71] The authors concluded

that miR29c levels could reflect renal function and the degree of renal fibrosis.[71]

In a study involving T2DM patients, it was shown that, compared to healthy controls and normoalbuminuric, extravesicular (EV) urinary miRNA-192, -194, and -215 were upregulated in microalbuminuric, but downregulated in macroalbuminuric patients.[72] They also showed that urinary EV miR-192 correlated with albuminuria and could distinguish between normo- and microalbuminuric patients.[72] In a study examining the expression of 5 cell-free miRNAs in plasma and urine of stage 3 and stage 5 CKD patients with underlying DKD showed that miR-192 showed significant overexpression in plasma and urine of Stage 3 CKD patients compared to healthy controls.[74] In a prospective study of biopsy-proven DKD patients, urinary free miR-196a showed several correlations with various clinical and pathological parameters.[73] The authors concluded that urinary free miR-196a could be utilized as a progression marker of renal fibrosis in DKD.[73]

In general, as evidenced in Tables 1-3, profiling studies of circulating miRNAs in blood and urine, varied substantially in the study design, methodology and miRNAs being measured. As a result, certain miRNAs may be found to be upregulated in one study and downregulated in another, for example miR-192, as shown in Table 4. Interestingly, similar pattern of dysregulation of miR-192 was observed both in serum and urine (Table 4). The differences in the study design include type of diabetes, definition of study and control groups, type of sample, methods of extraction and quantification of miRNA and normalization method in RT-PCR data analyses, as previously highlighted.[54,69,76] Nevertheless, the difference observed could be attributable to the heterogeneity of the study population, possibly reflecting ethnic-specific differences in miRNA expression, as previously suggested.[69,77]

CONCLUSION

There remains a need for better diagnostic and prognostic biomarkers for DKD with improved sensitivity at earlier disease stages and increased specificity of disease progression. Likewise, there is still a growing need for better alternative treatments that may arise from a better understanding of the underlying mechanisms of DKD. Although much discovery has been made in the roles of miRNAs in DKD, there are certain inherent issues pertaining to miRNA studies that still require further improvement prior to their clinical utilization in DKD, either as novel biomarkers or therapeutic targets. Nevertheless, research in miRNAs will continue to contribute towards improving our understanding of the disease for the betterment of the outcome of DKD.

Abbreviations

DKD, diabetic kidney disease; CKD, chronic kidney disease; ESRD, end stage renal disease; RNAs, ribonucleic acids; miRNAs, microRNAs; DM, diabetes mellitus; CVD, cardiovascular disease; GBM, glomerular basement membrane; GFB, glomerular filtration barrier; GFR, glomerular filtration rate; ROS, reactive oxygen species; PKC, protein kinase C; AGEs: advanced glycation end-products; ECM, extracellular matrix; RAS, renin-angiotensin system; KDIGO, Kidney Disease Improving Global Outcomes; eGFR, estimated GFR; T1DM, type 1 DM; T2DM, type 2 DM; mRNA, messenger RNA; RNase, ribonuclease; ECF, extracellular fluid; NA, normoalbuminuria; MIC, microalbuminuria, MAC, macroalbuminuria

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