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Review Article

Circulating microRNA as a diagnostic marker in populations with type 2 diabetes mellitus and diabetic complications

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Abstract

Diabetes mellitus (DM) is a global health care issue resulting from hyperglycemia-mediated life-threatening complications. Although the use of glucose-lowering agents is routinely practiced, high dependence on medication leads to poor quality of life for DM patients. While it is still not feasible to precisely determine the critical timing when DM is truly established, perhaps the best way to reduce DM-associated mortality is to prevent it. To this end, an exploration of prognostic molecules sensitive enough to detect early physiological alteration at the initiating stage would be required. Recently discovered small noncoding molecules, microRNAs (miRs), in body fluid seem promising to be utilized as a biomarker to monitor DM initiation and progression, as it is believed that expression of circulating miRs reflects disease pathology. Current DM-related miRs were often referred to miRs differentially expressed in insulin target organs (liver, muscle, and adipose tissues) or circulating blood (peripheral blood) in diabetic patients compared to their control counterparts, although these miRs could merely be resultant nucleotides from DM-induced organ impairment instead of the indicators of onset/progression of DM. In the current review, studies showing circulating miRs associated with type 2 DM and its complications are summarized, and future scope of using miRs as biomarkers for disease prognosis/diagnosis is also emphasized.

Keywords: biomarkers; complications; diabetes mellitus; diagnosis; microRNAs

1. Introduction

Diabetes mellitus (DM), a global public health issue, was estimated to affect ~450 million people, and the economic cost is projected at $490 billion/year by 2030.[1] The disease results from insufficient production of the pancreatic hormone insulin (type 1 DM, T1DM) or from ineffective insulin action (type 2 DM, T2DM).[2] As for T1DM, in addition to conventional insulin injection, whole pancreas/islet transplantation has been considered the most promising clinical procedure to restore normoglycemia. The shortage of cadaveric islets for transplantation, however, has been the main obstacle for this treatment.[3] T2DM is more common, comprising 85–90% of total DM cases, and has been considered a progressive metabolic disorder characterized by reduced insulin sensitivity and eventual pancreatic β-cell dysfunction.[4] Aberrant physiological conditions often lead to adverse effects on multiple organs as well as life-threatening complications, accounting for major DM-related mortality including cardiovascular disease, kidney

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failure (nephropathy), blindness (retinopathy), and nerve damage (neuropathy). While prescription/use of a number of insulin sensitizers (e.g., rosiglitazone) to promote insulin sensitivity of the insulin target organs is often practiced, tight glycemic control by routine exercise and diet is also a major way of lessening the risk of DM-associated mortality.[5,6]

Although clinical procedure for treatment of DM patients has been fairly well established, to completely cure DM, there remains a critical need to better understand the underlying mechanisms of the onset and progression of the disease in order to develop advanced therapeutic strategies. While various pathophysiological stages of DM development have been defined, it is still not feasible to precisely determine the critical timing when DM is truly established.[7] This may be due to the lack of prognostic molecules sensitive enough to monitor the dynamic changes of pre-DM status, namely impaired fasting glucose (IFG)/impaired glucose tolerance (IGT), to insulin resistance and finally to frank DM condition. In this regard, perhaps the best way to "cure" DM is to prevent it; specific clinical biomarkers with the potential to revolutionize diagnosis and treatment of DM are therefore required. Recent studies taking advantage of a metabolomic analysis uncovered possible metabolic molecules to differentiate IFG from overt T2DM.[8] However, the analysis is time- and labor-consuming and may not be objective enough, leaving this new method to be further evaluated prior to its translation to the bedside.

2. Biogenesis of (circulating) microRNA and its association with DM

In clinics, an ideal biomarker should be easily accessible by using a minimally invasive sampling procedure, making routine blood, urine, or saliva examination an excellent source of choice. Blood carries a wide array of biomolecules including nutrients, hormones, and molecules secreted by cells with specific biological functions.[9] Among these molecules, circulating nucleic acids such as microRNAs (miRs) have recently been recognized to be present in the circulation system, providing useful diagnostic indicators.[10] These miRs were first detected in blood serum/plasma in 2008[11] and subsequently shown to also be present in urine, saliva, tears, and breast milk.[12,13] These comprise a group of small, single-stranded, 22–25-nucleotide-long, noncoding RNA molecules that normally bind to the 3′untranslated region of their target mRNA, leading to translational inhibition and/or mRNA degradation.[14] It has been reported that distinct miR expression profiles regulate various physiological and pathological conditions.[15] The miRs are initially transcribed as long primary miRs sequentially processed to a hairpin-like fragment, termed precursor miR, in the nucleus by an RNase III enzyme Drosha and the double-stranded RNA-binding protein DGCR8. Precursor miRs are then exported to the cytoplasm by Exportin-5 protein, followed by the cleavage of miR duplexes by another RNase III enzyme Dicer. The miR duplexes are then unwound and the guide strand, which has complementarity to target mRNAs, is loaded into RNA-induced silencing complex (RISC) containing Argonaute 2, Dicer, and TRBP proteins for gene silencing. If the complementarity of a miR to target an RNA is perfectly matched, RISC cleaves the target mRNA. Most miRs exhibited mismatches with their target mRNAs, resulting in RISC-induced translational repression of target genes by hybridization of miRs to their 3′untranslated region, leading to repression of the initiation and elongation steps of translation, thereby decreasing protein expression. In addition to their role in post-transcriptional repression, miRs are also implicated in transcriptional gene silencing by targeting the promoter regions.[16–18] Over 1000 miRs have been discovered in the human genome, and it has been estimated that they could regulate 74–92% of all protein-encoding mRNAs in a tissue- and/or cell-specific manner.[19] Since miRs can regulate a wide range of biological events, including cell growth and proliferation, differentiation, organogenesis, metabolism, stress response, and tissue remodeling, detection of miR expression can be implicated in physiological/pathological changes and it can even be useful for the prognosis or diagnosis of the onset/progression of diseases.[20–23]

Due to various pathogeneses of different DM types, the differential regulatory roles of miRs leading to disease outcomes, including β-cell deficiency and insulin resistance, have recently been defined. For T1DM, the role of miRs in controlling β-cell genesis, β-cell death (e.g., miR-21), insulin production (e.g., miR-30d, miR-204, and miR-124a), and α/β-cell mass balance (miR-375) or its susceptibility to immune-mediated β-cell destruction has been described.[24–29] When the possibility that a different set of miRs could control compensatory β-cell mass under pre-DM or gestational DM (GDM) condition was recently examined, the results showed that expression of miRs in omental adipose tissues (miR-222), placentas (miR-518d), or isolated rodent islets (miR-338-3p) are differentially expressed in GDM compared to controls,[30–33] suggesting that these miRs are important for regulating β-cell mass in response to physiological demand. In regard to T2DM, a number of review articles have provided a broad overview of the possible functions of different miRs in regulating insulin sensitivity and glucose utilization activity in insulin target tissues, including adipose, muscle, liver, and pancreatic β cells, from T2DM as well as obese individuals.[34–37] In general, miRs related to insulin resistance were often identified based on the differential expression of candidate miRs in insulin target cells of DM and non-DM individuals. It is interesting that most of insulin resistance-related miRs are also key regulators of cellular homeostasis. In brief, insulin sensitivity-related miRs in adipocytes (miR-21, miR-29, miR-93, miR-103, miR-143, and miR-320),[38–42] muscle (miR-1, miR-106b, miR-133a, and miR-223),[42–44] and liver (Let-7, miR-130a-3p, miR-143, miR-181a, and miR-802)[45–49] were essential in maintaining physiological homeostasis and energy balance. Taken together, miRs could be differentially expressed in various types of DM, suggesting their potential to be DM indicators.

3. MicroRNAs serve as circulating DM biomarkers

The miRs present in the circulation system represent a remarkably stable form, making them ideal materials to

monitor disease progression. Indeed, recent reports suggested that expression patterns of circulating miRs are reflective of underlying pathological/physiologic processes.[10,50,51] Based on the fact that RNases are abundant in circulating blood, the requirement of distinct mechanism(s) to protect miRs from degradation was proposed. El-Hefnawy and colleagues[52] first showed that plasma RNA is protected from degradation by its inclusion in protein or lipid vesicles. Depending on their size and mode of release from cells, these particles are classified as exosomes (50–100 nm), microvesicles (0.1–1 mm), or apoptotic bodies (0.5–2 mm).[53] Circulating miRs can also be released in complexes with RNA-binding proteins such as Argonaute 2 or lipoprotein complexes with high-density lipoprotein.[54,55] Although the precise cellular release mechanisms of miRs remain largely unknown, a recent study revealed that these circulating miRs may be delivered to recipient cells, where they can regulate translation of target genes, suggesting that circulating miRs can serve as extracellular communicators.[56,57]

The miRs present in the serum or plasma seem promising for utilization as biomarkers to identify disease initiation and progression. In agreement with findings of other diseases such as cancer and cardiovascular disease, it was recently recognized that there are distinct profiles of circulating miRs in patients with DM and T2DM complications compared to non-DM patients.[58] Studies have shown that specific miR profiles are correlated to DM pathology, while all candidate miRs are involved in regulating insulin production machinery, insulin sensitivity, glucose homeostasis, or lipid metabolism implicated in T2DM pathology.[59–61] Regarding the involvement of miRs to predict patients at risk for DM complications, various miRs were described as playing a crucial role to regulate homeostasis of tissues where DM complications occur.[20] Interestingly, a recent investigation revealed an ethnicity-specific DM-related miR profile. Among 14 selected miRs (miR-15a, miR-20, miR-21, miR-24, miR-29b, miR-126, miR-144, miR-150, miR-197, miR-223, miR-191, miR-320a, miR-486-5p, and miR-28-3p) in plasma from 19 Iraqis and 14 Swedes diagnosed with T2DM and 119 control patients, the expression of miR-24 and miR-29b was significantly different between the T2DM patients and the control group after adjustment for age, sex, waist circumference, a family history of T2DM, and a sedentary lifestyle. A higher expression of miR-144 was significantly associated with T2DM in Swedes but not in Iraqis, implying that miR-144 is an ethnicity-specific DM-associated miR.[62] Moreover, by using an unusual DM cat as the model, expression of circulating miR-122, miR-193b, and miR-483 in serum was found to be significantly greater in newly diagnosed DM cats than that in healthy lean cats and cats in diabetic remission, suggesting an important evolutionary role of circulating miRs in regulating DM pathophysiology.[63]

Zampetaki and colleagues[64] first reported that miR-20b, miR-21, miR-24, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, and miR-486 were downregulated, while miR-28-3p was upregulated in plasma of T2DM patients in comparison with non-DM individuals. Further follow-up study showed that normoglycemic patients who developed DM over a 10-year follow-up period could be identified using the five most significant differentially expressed miRs (miR-15a, miR-126, miR-320, miR-223, and miR-28-3p), indicating that these miRs could be potential biomarkers for the diagnosis or even prediction of DM. More recent studies also detected significantly increased levels of circulating miR-146a[65] and miR-126[66] in newly diagnosed T2DM patients compared with healthy controls. In addition to plasma miRs, serum miRs have also been used in different studies to explore DM biomarkers. It was shown that seven candidate miRs (miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a, and miR-375) were significantly increased in serum from newly diagnosed T2DM patients compared with T2DM-susceptible individuals with normal glucose tolerance in a Chinese cohort, implying their potential as biomarkers for T2DM.[67] Interestingly, a similar pattern of these miRs was observed in pre-DM (IGT/IFG) and T2DM-susceptible individuals with normal glucose tolerance, suggesting that during the pathogenesis of T2DM, DM-related miRs are not significantly changed in serum. A report of further study aiming to explore potential diagnostic circulating miRs in genetic-susceptible DM, HNF1A-MODY (m*aturity onset diabetes of the young* due to a mutation in HNF1A), recently implied that HNF1A-MODY pathophysiology is associated with the overexpression of miR-103 and miR-224.[68] The miR expression in T2DM using whole-blood samples was also recently highlighted. Karolina et al[69] analyzed eight miRs (miR-144, miR-146a, miR-150, miR-182, miR-192, miR-29a, miR-30d, and miR-320a) in IGT and T2DM, and found that all eight tested miRs were differentially expressed. Among them, increased circulating miR-144 was found to correlate with its decreased potential target gene insulin receptor substrate 1, a key molecule in insulin signaling.

Taken together, differential miR expression profiles can be good indicators for DM prediction or disease progression; incoherent DM-related miRs from different studies, however, this would need to be clarified further. Inconsistency in results from different investigations could possibly be due to ethnic variance of samples or different inclusion/exclusion criteria in different trials, thereby leading to difficult interpretation and comparison between various investigations. Furthermore, studies using whole-blood samples that might contain circulating cells that affect miR signature could also be a concern when comparing results using plasma or serum.

4. Biological significance of DM-associated circulating miRs

While different sets of miRs related to DM pathophysiology were identified in various studies, it is essential to further define the specific roles of these miRs in controlling insulin activity and maintaining normal physiology of insulin target organs under DM condition. In addition, miR-mediated regulations in different biological materials by various experimental methods should be clarified.

4.1. Profiles of miR in peripheral blood cells among DM patients

The distinct miR profile in peripheral blood mononuclear cells (PBMCs) from DM patients may correlate with disease pathology. Recent studies showed that miR-126, miR-146a, and miR-155 were downregulated in PBMCs in response to hyperglycemic/diabetic conditions, suggesting that miR-mediated impaired proangiogenic effect and deregulated cellular metabolic control could be underlying molecular mechanisms for T2DM pathogenesis.[70,71] A more recent report showing differential miR expression in PBMCs from T1DM, T2DM, and GDM revealed that PBMCs could be used as reporter cells to characterize different DM manifestations. The study indicated that nine tested miRs, including miR-126, miR-1307, miR-142-3p, miR-142-5p, miR-144, miR-199a-5p, miR-27a, miR-29b, and miR-342-3p, were shared among T1DM, T2DM, and GDM as the miR profiles in T1DM (miR-1274a, miR-1274b, and let-7f), T2DM (miR-222, miR-30e, and miR-140-3p), and GDM (miR-181a and miR-1268) were distinctly expressed, representing molecular signatures for each type of DM.[71] On a molecular basis, the studies further identified potential links between individual DM miRs and disease manifestation. For example, miR-126 expression in DM inhibited endothelial progenitor cell (EPC) proliferation and migration, and might induce apoptosis, thereby leading to DM-mediated cardiovascular diseases.[72] As for the modulation of DM-mediated intracellular signals, miR-144 was shown to target insulin receptor substrate 1, a pathway highly involved in glucose homeostasis and insulin-mediated cell growth, suggesting its significance in contributing to pathophysiology in both T1DM and T2DM patients.[69] Furthermore, the miR-29 family was also shown to be essential to maintain cellular physiology, including insulin-stimulated glucose uptake of β cells, signal transmission of neuronal cells, and cellular fibrosis in kidney—all of which are potential features resulting in DM complications.[73–75]

4.2. Correlation of miR expression with T2DM-related vascular dysfunction

Among the vast array of long-term DM-associated complications, almost 80% of DM-associated mortality is due to cardiovascular diseases, resulting mainly from endothelial dysfunction. Despite all efforts and advancement made to address this global burden, the challenge to clinicians remains large.[76] Recent studies suggested that several miRs play important roles in regulating endothelial function and angiogenesis under condition of DM cardiovascular complications. For example, it was shown that exposure to hyperglycemic conditions decreased the expression of *c-kit*, the receptor for stem cell factor, and deregulated migration of human umbilical vein endothelial cells through induction of miR-221.[77] Additionally, it was also found that DM condition decreased the number and impaired the function of EPCs, the cells important for endothelial regeneration in response to pathological challenges.[78,79] Another study demonstrated that miR-126 expression was downregulated in EPCs from DM patients compared to the control group, whereas deregulated miR-126 level impaired EPC-mediated vascular function by controlling its target Spred-1 and downstream Ras/ERK/VEGF and PI3K/Akt/eNOS signaling pathways.[72] More recently, it was further reported that elevated miR-21 could be detected in primary cultured EPCs from DM patients compared to those from control individuals, and that increased miR-21 could protect EPCs from apoptosis via the regulation of downstream target DAXX.[80] Taken together, the detection of deregulated miR profile in circulating peripheral blood cells or vascular cells may potentially be associated with T2DM pathological conditions.

4.3. Extracellular indicators of DM complication

Regarding involvement of miRs in predicting DM patients at risk for complications, the number of miRs has been found to play a key physiological role in tissues where DM complications occur. However, whether these miRs are involved in DM-associated tissue damage requires further clarification.[20] Taking diabetic nephropathy (DN) as an example, recent efforts have been directed toward elucidating potential DN-associated miRs. DN is the leading cause of kidney failure, affecting the glomerulus and resulting in progressive kidney scarring and fibrosis.[81,82] A recent study defined the role of miRs in renal disease and showed the importance of specific miRs in normal renal development and function, suggesting that specific renal miRs might be ideal candidates for monitoring DN initiation and progression.[83] Several potential miRs have been proposed to serve as DN miRs. For example, miR-192 was found to be specifically expressed in the kidney, and its expression is upregulated in streptozotocin-induced DM and db/db mice.[84] In this study, it was also reported that miR-192 suppressed the translation of SIP1/E-box repressor ZEB2, a transcriptional repressor that binds to the E-box in the collagen1a2 (col1a2) gene, leading to increased col1a2 expression *in vitro* and elevated collagen deposition *in vivo*, suggesting a role of miR-192 in the development of the matrix accumulation observed in DN.[85] In addition to DN, miR-192 has also been correlated with disease severity and progression in patients with immunoglobulin A nephropathy and hypertensive nephrosclerosis, indicating that miR-192 may also play a role in renal diseases.[20] Other DN-associated miRs such as miR-21 and miR-29 were also described. Expression of miR-21 was downregulated in response to early DN. Ectopic miR-21 expression *in vivo* resulted in decreased albuminuria in db/db mice, suggesting its role in maintaining renal functions in response to high-glucose condition as well as in preventing renal fibrosis and regulating renal cell death.[20,85] Recent reports also linked miR-29 to renal fibrosis based on the detection of decreased expression of miR-29 in fibrotic kidney of obstructive nephropathy. Knockdown of miR-29c significantly reduced albuminuria and kidney mesangial matrix accumulation, and prevented high-glucose-induced cell apoptosis in db/db mice, whereas forced expression of miR-29c strongly induced podocyte apoptosis.[86]

Most previous studies aiming to identify DN miRs were performed using either *in vitro* cultured renal cells or diabetic

Table 1
DM-associated circulating microRNAs.

Sample type	DM miRs	Impact (significance, complications)	Refs
T2DM; plasma	↑ miR-20b, miR-21, miR-24, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, and miR-486 ↓ miR-28-3p	Elucidation of potential underlying molecular cues for impaired peripheral angiogenic signaling in patients with pre-DM condition/T2DM	66
T2DM; plasma	↑ miR-146a, miR-126	Identification of possible predictors for newly onset T2DM	67,68
T2DM; serum	↑ miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a, and miR-375		69
HNF1a-MODY; serum	↑ miR-103 and miR-224	Definition of pathological molecular players and potential predictor for HNF1a-MODY	70
IGF/T2DM; whole blood	↑ miR-144, miR-150, miR-192, miR-29a, and miR-320 ↓ miR-146, miR-182, and miR-30d	Link of circulating microRNAs with intracellular signaling pathway in pre-DM/T2DM patients	71
T2DM; PBMCs	↓ miR-126, miR-146a, and miR-155	Significant correlation of microRNA profiles with clinical parameters (HbA1C, BMI, and glucose level)	72
T1DM/T2DM/GDM; PBMCs	miR-1274a, miR-1274b, and let-7f (T1DM specific) miR-222, miR-30e, and miR-140-3p (T2DM specific) miR-181a and miR-1268 (GDM specific)	Identification of differential expression of microRNAs in different DM manifestations	73
T2DM; EPCs	↓ miR-126 ↑ miR-21	Explore the role of microRNA in regulating EPC-mediated endothelial dysfunction in DM patients	80,81
T2DM; urine	miR-29a (correlated with albuminuria)) miR-29b (associated with carotid intima-media thickness)	Definition of biomarkers for monitoring progression of diabetic nephropathy	90
T2DM; urine	↑ miRNA-377, miRNA-192, miRNA-216/217, and miRNA-144 ↓ miRNA-21 and miRNA-375		93

BMI = body mass index; DM = diabetes mellitus; EPC = endothelial progenitor cell; GDM = gestational diabetes mellitus; HNF = hepatocyte nuclear factor; IGF = insulin-like growth factor; miR = microRNA; MODY = maturity onset diabetes of the young; PBMC = peripheral blood mononucleated cell; T1DM = type 1 diabetes mellitus; T2DM = type 2 diabetes mellitus.

animal models, whereas the examination of prognostic/diagnostic potential for circulating DN-susceptible miRs from humans was lacking. To this end, in addition to serum or plasma, urine might also be an alternative sampling material to determine DN-mediated renal dysfunction, although only limited studies have been reported. While most urinary nucleotides were wrapped in exosomes, an advanced procedure to efficiently isolate urinary exosomes was recently suggested.[87] By taking advantage of this experimental procedure, it was recently described that 27 urinary miRs were differentially expressed at different stages of untreated DN in T1DM patients, as these miRs were mapped to overlapping pathways of growth factor signaling and renal fibrosis known to be targeted in DN.[88] In addition, it was found that urinary miR-29a correlated with albuminuria as urinary miR-29b correlated with carotid intima-media thickness in T2DM patients, revealing their potential to serve as biomarkers for DN and atherosclerosis in T2DM.[89] The regulatory roles of miR-29a and miR-29b were also demonstrated in proximal tubular *cell* HK2 cells *in vitro* and in mice with established obstructive nephropathy *in vivo*, respectively. The results showed that miR-29a could suppress collagen IV expression and miR-29b could block progressive renal fibrosis, confirming the antifibrotic properties of miR-29a and miR-29b in DN.[90,91] Further analysis of DN-associated regulations of candidate urinary miRs, including miR-377, miR-192, miR-216/217, and miR-144 (upregulated in DN patients), as well as miR-21 and miR-375 (downregulated in DN patients),[92] would be required to better define DN miRs.

In conclusion, the potential DM miRs are summarized in Table 1.[66–73,80,81,90,93] Current DM-associated miRs are often referred to as miRs differentially expressed in insulin target organs or circulating blood in DM patients compared to their control counterparts, although these miRs could merely be resultant nucleotides from DM-induced organ impairment instead of the indicators of onset/progression of DM. In order to define DM- or DM-complication-associated miRs more precisely, more stringent recruitment criteria need to be considered. For example, differentiating key miRs distinctly expressed in patients with a DM risk factor (e.g., obesity) and those with overt T2DM can be very difficult. A recent study proposed a solution for this issue via comparison of miR profiles between obese, T2DM, and obese T2DM patients.[93] Lastly, further examination using larger population-based cohort to accurately correlate clinical biochemical variables will also be required.

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