Expression and clinical significance of miR-1 and miR-133 in pre-diabetes

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Abstract. Pre-diabetes represents an intermediate state of altered glucose metabolism between normal glucose levels and type 2 diabetes mellitus (T2D), and is considered a significant risk factor for the development of T2D and related complications. Early detection of pre-diabetes may allow for the use of timely and effective treatment strategies to prevent its progression. Circulating microRNAs (miRNAs/miRs) that reflect changes in diabetes-related tissues, including the pancreas, liver, skeletal and heart muscle, and adipose tissue are promising biomarkers of disease progression. In our previous study, it was demonstrated that the cardiac and skeletal muscle specific miR-1 and miR-133 are upregulated in the blood of patients with T2D and cardiovascular disease. Since both miRNAs have been shown to be implicated in insulin resistance, an important feature of pre-diabetes, we hypothesised that their expression may be increased prior to clinical diagnosis of T2D, and may thus serve as biomarkers for pre-diabetes. The expression levels of circulating miRNAs were evaluated by reverse transcription-quantitative PCR in whole blood samples from 55 subjects, including 28 pre-diabetes individuals with impaired fasting glucose (FG) and impaired glucose tolerance, and 27 healthy controls. The individuals with pre-diabetes exhibited significantly higher expression levels of miR-1 and miR-133 compared with the controls ($P<0.05$). Target prediction search revealed that both miR-1 and miR-133 target several pathways involved in the pathophysiology of diabetes. Pearson's correlation analysis revealed that the two miRNAs were positively correlated with blood glucose parameters, including FG, 2-h oral glucose tolerance test and glycated haemoglobin A1c levels, as well as with insulin resistance ($P<0.05$). Multivariate logistic regression analysis revealed that the two miRNAs were significantly and directly associated with pre-diabetes, and this association remained significant after adjustment for several confounding variables ($P<0.05$). Moreover, linear regression analysis showed that the Homeostatic Model Assessment-Insulin Resistance was the only significant predictor to be significantly associated with both miRNAs ($P<0.05$). In discriminating pre-diabetes individuals from healthy controls, the area under the curves (AUCs) of the receiver operating characteristic curves (ROCs) were 0.813 and 0.810 for miR-1 and miR-133 respectively ($P<0.05$). Despite the relatively small number of participants, the present study showed for the first time that circulating levels of miR-1 and miR-133 were increased in individuals with pre-diabetes, and they were associated with important features of pre-diabetes. Thus, they may serve as clinical biomarkers for the early-stages of T2D.

Introduction

Pre-diabetes represents a condition of intermediate hyperglycaemia in which blood glucose levels are higher than normal, but below the diagnostic levels of type 2 diabetes mellitus (T2D). It can be diagnosed using an impaired fasting glucose (IFG) test, impaired glucose tolerance (IGT) test and elevated glycated haemoglobin (HbA1c) levels (1). These glucometabolic abnormalities often precede T2D (2,3), and most individuals with pre-diabetes eventually develop T2D (2,3). In addition to the risk of progression to T2D, pre-diabetes is associated with cardiovascular disease (CVD) (4,5) and other vascular complications (6). The pathophysiology of pre-diabetes is similar to T2D in that the pathological basis is insulin resistance and impaired β-cell function (7). Insulin resistance occurs several years prior to the development of T2D, and is evident in individuals with both IFG and IGT results (7,8). This is followed by a compensatory increased rate of insulin secretion by the β-cells as an adaptive response. Over time, the β-cells fail to

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fully compensate for insulin resistance, leading to changes in their function accompanied by a reduction in insulin secretion, thus fasting and post-load glucose levels are not adequately maintained (7,8). When the function β-cells deteriorates, hyperglycaemia occurs, and pre-diabetes progresses to overt T2D (9). Lifestyle modifications and therapeutic interventions may reverse pre-diabetes and prevent it from proceeding to T2D, and other related devastating complications (10,11). Early and accurate identification of the pre-diabetic stage is the gold standard for preventing T2D and its consequences.

MicroRNAs (miRNAs/miRs) are an abundant class of small non-coding RNAs that act as post-transcriptional regulators of gene expression (12). They serve important roles in a wide range of cellular functions, such as proliferation, differentiation and other metabolic processes (13). A single miRNA can target multiple genes, and hence a large number of human genes are predicted to be regulated by miRNAs (14). miRNAs are increasingly being recognized as fine-tuning regulators of glucose metabolism, β-cell function and insulin signalling, and their dysfunction has been implicated in the development of T2D (15,16). miRNAs are also present in human bio-fluids, including in the blood in a highly stable form that is protected from endogenous RNase activity (17). These circulating miRNAs act as intercellular mediators, and can be used to identify the states of several cellular pathophysiological processes, and thus represent a further novel subclass of biomarkers (17,18). An altered circulating miRNA profile that reflects changes in diabetes-related tissues, such as the pancreas, liver, skeletal and heart muscle, and adipose tissue, has shown promise as biomarkers for diagnosis of T2D (19). Additionally, the alterations in circulating miRNAs may appear at early stages of diabetes progression, supporting their use as early candidate biomarkers for detection of a pre-diabetic state. For example, miR-15a and miR-375, which are associated with β-cell injury, have been suggested as potential biomarkers for diagnosis of pre-diabetes, as well as for evaluating the risk of developing T2D (20-22). In addition, the endothelial-specific miR-126, which is implicated in CVD, has been reported as a biomarker for screening for pre-diabetes and newly diagnosed T2D (23).

In our previous study, it was shown that miR-1 and miR-133 were upregulated in the peripheral blood of patients with T2D, and this upregulation was associated with the risk of coronary artery disease (24). These two miRNAs belong to the myomiR family, and are specifically expressed in skeletal muscle and cardiomyocytes, and are highly enriched in the latter (25). Notably, skeletal muscle is the major site of postprandial insulin-mediated glucose uptake, and insulin resistance in this tissue is considered as the primary defect prior to the development of T2D (26). Therefore, dysregulation in the expression of skeletal muscle-related mRNAs is central to the pathogenies of diabetes and other metabolic disorders (27). Studies in human and animal models have shown that changes in the circulating levels of miR-1 and miR-133 are associated with insulin resistance or T2D progression. Higher levels of miR-1 and miR-133 have been described in patients with T2D and in a murine model of insulin resistance (28). Progressive increases in the plasma levels of miR-133 have been reported in Zucker diabetic rats during the natural progression of T2D from pre-diabetes and initial hyperinsulinemia to β-cell failure and late-stage diabetes (29).

There is an urgent need for new options to treat T2D during the early stages due to the ineffective control of its development in patients. Given the role of circulating miRNAs as promising biomarkers, it was hypothesized that the increased expression of peripheral blood miR-1 and miR-133 may appear prior to the occurrence of T2D, and thus, they may serve as biomarkers for pre-diabetes. Therefore, the expression levels of miR-1 and miR-133 in whole blood from pre-diabetes individuals with IFG and IGT, as well as in healthy controls was quantified. Additionally, the relationship between these two miRNAs and clinical characteristics as well as their diagnostic values were evaluated.

Materials and methods

Study subjects. The present study included 55 subjects, 28 individuals with pre-diabetes with IFG and IGT (18 males and 11 females; mean age 50±5.6 years; age range 43-59 years; median age 49.5) and 27 healthy controls (13 males and 14 females; mean age 54±4.4 years; age range; 47-61 years, median age 50). The sample size was calculated as the minimum number of subjects in each group with sufficient statistical power (two-sided significance at 0.05 with a power of 80%) to detect a 20% difference between the mean miR-1 and miR-133 expression levels of the groups. Subjects were recruited from King Abdullah University Medical City in the Kingdom of Bahrain. They were randomly selected irrespective of age and sex. All diagnoses were confirmed by the FG test after 12 h of fasting, 2-h oral glucose tolerance test (OGTT) and glycated haemoglobin A1c (HbA1c) levels, according to the World Health Organization (30) and American Diabetes Association (31) criteria. Therefore, pre-diabetes was defined as IFG with FG levels between 5.6-6.9 mmol/l, IGT with 2-h OGTT glucose levels between 7.8-11.0 mmol/l and HbA1c between 5.7-6.4%. Healthy control subjects had FG levels between 4.8-5.2 mmol/l, 2-h OGTT glucose levels between 7.8-11.0 mmol/l and HbA1c <5.7%. The healthy controls were randomly selected during their routine check-up visits to the medical centre. Insulin resistance in the Homeostasis Model Assessment-Insulin Resistance (HOMA-IR) index was calculated using the following formula: [Fasting plasma glucose (mmol/l) x fasting serum insulin (mU/l)]/22.5, as described by Matthews *et al* (32). Accordingly, a low HOMA-IR was indicative of high insulin sensitivity, whereas a high HOMA-IR was indicative of low insulin sensitivity (insulin resistance). Other clinical parameters including age, sex, body mass index (BMI), mean blood pressure, low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), triglyceride and total cholesterol levels were collected from the medical records of participants. As the focus of the present study was the expression of circulating miR-1 and miR-133 in pre-diabetes, and as our previous study showed changes in the circulating levels of these two miRNAs in T2D patients with and without CVD (24), patients diagnosed with T2D and those with diabetes complications including CVD (evident by electrocardiogram, echocardiogram and other tests) were excluded from the present study.

Ethics statement. The present study was performed in accordance with the relevant ethical guidelines and regulations

of clinical research of Arabian Gulf University (agu.edu. bh/en/Research/Colleges/CMMS/Pages/Research-&-Ethics. aspx), and approved by the Medical Research and Ethics Committee at the College of Medicine and Medical Sciences, Arabian Gulf University, Kingdom of Bahrain. All participants were informed regarding the study procedures and provided written consent.

Blood collection and miRNA isolation. Whole blood samples from participants were collected in EDTA tubes (BD Biosciences). For pre-diabetes individuals, blood samples were collected at the time of diagnosis, prior to initiation of the treatment. To ensure RNA stabilization, RNA later (1.3 ml; Thermo Fisher Scientific, Inc.) was added to aliquots of 0.5 ml EDTA-blood, and blood samples were stored at -80°C until required for analysis. miRNAs were extracted from 200 μl whole blood and eluted in 30 μl RNase-free water using the miRNeasy Mini kit (Qiagen, Inc.) according to manufacturer's protocol and as previously described (21,22,24). The concentration and purity of RNA samples were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.).

cDNA synthesis and miRNA quantification. The miRNAs isolated from whole blood were reverse transcribed to cDNAs using an Applied Biosystems TaqMan MicroRNA RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction mix contained 20 ng RNA (5 μl), specific stem-loop RT primers (3 μl), 100 mM dNTPs (0.15 μl), 10X RT buffer (1.5 μl), 20 U/μl RNase inhibitor (0.19 μl), 50 U/ml MultiScribe™ Reverse Transcriptase (1 μl) and nuclease-free water (4.16 μl) to a final volume of 15 μl. The reaction mix was incubated in a GeneAmp® PCR system 9700 thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) at 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and 4°C. All cDNA samples were stored at -20°C until required. For miRNA quantification, reverse transcription-quantitative (RT-q)PCR was performed in duplicate using TaqMan microRNAs assays, with miR-1 and miR-133 as the targets and RNU6B as the reference (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction mix consisted of 1.33 μl cDNA, 10 μl TaqMan 2X Universal PCR MasterMix II, 1 μl gene-specific primers and 7.67 μl nuclease-free water to a final volume of 20 μl. PCR amplification was carried out in an ABI 7900HT Fast thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were: 95°C for 10 min; followed by 35 cycles of 95°C for 15 sec and 60°C for 60 sec. The sequences of the primers for miR-1, miR-133 and RNU6B are shown in Table I (24). The data were analysed using the Sequence Detection relative quantification software version 1.4, (Applied Biosystems; Thermo Fisher Scientific, Inc.). The fold change of relative expression was calculated as $2^{-\Delta\Delta Cq}$ (21,22,24).

miRNA target prediction. The miRDB online database for miRNA target prediction (mirdb.org/index.html) was used to search for target genes for human miR-1 (hsa-miR-1) and human miR-133 (hsa-miR-133). The miRNA sequences and nomenclatures were downloaded from miRBase for target prediction (33), and mRNA sequences and annotation files

Table I. Sequences of primers used for PCR amplification.

Target	Sequence, 5'-3'
miR-1	
Forward	CTCGACAAACGTCTAAATGCT
Reverse	TCAACTGGTGTCGTGGAGTC
miR-133	
Forward	CAGGTTTGGTCCCCTTCAA
Reverse	TCAACTGGTGTCGTGGAGTC
RNU6B	
Forward	GCTTCGGCAGCACATATACTAAAAT
Reverse	CGCTTCACGAATTTGCGTGTCAT

miR, microRNA.

were downloaded from NCBI. All transcript 3'UTR sequences and their isoforms from five species (human, mouse, rat, dog and chicken) were analysed from the GenBank files using BioPerl (bioperl.org).

Statistical analysis. All data were analysed using the SPSS version 27 (IBM Corp.). Differences in the expression of miRNAs and other clinical parameters between the prediabetes individuals and healthy controls were evaluated using an independent samples t-test for continuous variables and χ^2 test for categorical variables. Pearson's correlation coefficient analysis was used to assess the relationship between miRNAs and clinical variables. Multivariate logistic regression analysis was performed to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association of miR-1 and miR-133 with the presence of prediabetes. This analysis was performed before and after adjustment for possible confounders. Linear regression analysis with defined clinical variables was performed to identify the predictors of miRNAs. Receiver operating characteristic (ROC) analysis was performed to evaluate the diagnostic performances of miRNAs and the areas under the curve (AUCs) were calculated. The sensitivity and specificity for each miRNA were obtained from the optimal cut-off values of the ROC curves. The data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical characteristics of the study subjects. Table II illustrates the clinicopathological characteristics of the study subjects which included 28 pre-diabetes individuals and 27 healthy controls. A significant difference was observed in the mean age between the pre-diabetes and the control subjects (P=0.017), but not in the sex distribution (P>0.05). There was no significant difference in BMI between the pre-diabetes and the control subjects (P>0.05). However, the prediabetes individuals were slightly overweight (BMI, 25.4±4.5 kg/m²) compared with the healthy controls (BMI, 24.1 ± 4.4 kg/m²). Also, no significant difference was observed between the mean blood pressure in the study subjects (P>0.05). Blood glucose

Table II. Clinicopathological characteristics of the study subjects.

Characteristics	Healthy group[c]	Pre-diabetes group[c]	P-value
Number of subjects	27	28	
Age, years	54±4.4	50±5.6	0.017[a]
Sex			0.333
Male	13	18	
Female	14	11	
BMI, kg/m^2	24.1±4.4	25.4±4.5	0.209
Mean blood pressure, mmHg	87±4.2	88±5.4	0.19
FG, mmol/l	4.3±4.8	6.4±5.8	<0.001[b]
2-h OGTT, mmol/l	6.2±1.04	8.9±2.0	<0.001[b]
HbA1c, %	5.1±1.0	6.7±0.5	<0.001[b]
Insulin, mU/l	6.5±0.9	9.5±1.3	<0.001[b]
HOMO-IR	1.25±0.2	2.7±0.4	<0.001[b]
LDL-C, mmol/l	2.2±0.8	2.1±0.4	0.434
HDL-C, mmol/l	1.3±0.3	1.3±0.4	0.813
Triglyceride, mmol/l	1.5±0.6	1.5±0.6	0.654
Total cholesterol, mmol/l	4.3±0.6	4.1±1.3	0.402

[a]$P<0.05$, [b]$P<0.001$. [c]Data are presented as the n, or the mean ± standard deviation. BMI, body mass index; FG, fasting glucose; 2-h OGTT, 2 hour oral glucose tolerance test; HbA1c, glycated haemoglobin A1c; HOMO-IR, homeostasis model assessment-insulin resistance; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

parameters including FG, 2-h OGTT and HbA1c differed significantly between the study subjects, and were significantly higher in the pre-diabetes individuals than in the controls (all P<0.001). In addition, insulin sensitivity based on the HOMO-IR index were significantly higher in the pre-diabetes individuals compared with the healthy controls (P<0.001). For the lipid profile analysis, no significant differences were found in triglyceride, total cholesterol, LDL-C and HDL-C levels between the pre-diabetes and control subjects (P>0.05).

Expression of circulating miR-1 and miR-133. TaqMan-based RT-qPCR was used to determine the expression of circulating miR-1 and miR-133 relative to the expression of RNU6B in whole blood from individuals with pre-diabetes and healthy controls. Significant differences in the expression of both miR-1 and miR-133 were observed between the pre-diabetes and control subjects (Fig. 1). miR-1 expression levels were 2.8-fold higher in the pre-diabetes individuals compared with the controls (P<0.05). The mean fold increase in the relative expression of miR-1 was 9.09±1.17 in the pre-diabetes individuals compared with 3.13±0.86 in the control group. In addition, miR-133 expression was 2.2-fold higher in the pre-diabetes individuals compared with the controls (P<0.05). The mean fold increase in the relative expression of miR-133 was 12.13±0.95 in the pre-diabetes individuals compared with 5.64±1.5 in the control group.

miRNA target prediction. miRDB (mirdb.org/index.html) was used to identify targets for human miR-1 (hsa-miR-1) and human miR-133 (hsa-miR-133). In total >900 predicted targets

for hsa-miR-1 and >600 predicted targets were identified in the miRDB database. several of these targets were found to be related to T2D. These included IGF-1, SLC7A2, SLC45A4, SLC8A2, HSPD1 and CDK14 for hsa-miR-1; and IGF-1, SLC7A8, SLC46A1, SLC2A12 and CD47 for hsa-miR-133.

Correlation analysis. The relationship between circulating miR-1 and miR-133 and clinical parameters in the pre-diabetes subjects was evaluated using Pearson's correlation coefficient analysis (Table III). miR-1 was found to be positively correlated with FG (r=0.512; P<0.001), 2-h OGTT (r=0.411; P=0.002) and HbA1c (r=0.466; P<0.001). miR-1 was also positively correlated with insulin levels (r=0.426; P=0.001) and the HOMO-IR index (r=0.477; P=0.001). A similar trend of correlation was found for miR-133. It was positively correlated with FG (r=0.342; P=0.011), 2-h OGTT (r=0.39; P=0.032), HbA1c (r=0.299; P=0.009), insulin level (r=0.325; P=0.016) and the HOMO-IR index (r=0.342; P=0.017). No significant correlations were found for miR-1 or miR-133 with any of the other clinical parameters (P>0.05).

Multivariate logistic regression analysis. Multivariate logistic regression was used to explore the association between miR-1 or miR-133a and pre-diabetes outcomes. As shown in Table IV (Model 1) a direct association was found between miR-1 and pre-diabetes (OR, 1.200; 95% CI, 1.061-1.357; P=0.004). Similarly, a direct association was observed between miR-133 and pre-diabetes (OR, 1.160; 95% CI, 1.053-1.276; P=0.003). In order to establish whether the association between miR-1 or miR-133a and pre-diabetes could be influenced by possible

Table III. Correlation analysis.

Variables	miR-1		miR-133	
	r	P-value	r	P-value
Age, years	0.195	0.154	0.102	0.087
BMI, kg/m^2	0.008	0.954	0.041	0.766
Mean blood pressure, mmHg	0.097	0.483	0.056	0.689
FG, mmol/l	0.512	<0.001[c]	0.342	0.011[a]
2-h OGTT, mmol/l	0.411	0.002[b]	0.383	0.032[a]
HbA1c, %	0.466	<0.001[c]	0.299	0.009[b]
Insulin, mU/l	0.426	0.001[b]	0.325	0.016[a]
HOMO-IR	0.477	0.001[b]	0.342	0.017[a]
LDL-C, mmol/l	0.197	0.166	0.235	0.097
HDL-C, mmol/l	0.261	0.067	0.029	0.839
Triglyceride, mmol/l	0.004	0.975	0.218	0.117
Total cholesterol, mmol/l	0.1	0.944	0.16	0.243

[a]P<0.05, [b]P<0.01, [c]P<0.001. BMI, body mass index; FG, fasting glucose; 2-h OGTT, 2 hour oral glucose tolerance test; HbA1c, glycated haemoglobin A1c; HOMO-IR, homeostasis model assessment-insulin resistance; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

Figure 2. ROC analysis was used to evaluate the diagnostic values of circulating miR-1 and miR-133 as biomarkers for pre-diabetes. (A) AUC for miR-1 was 0.813. The sensitivity was 96.4% and the specificity was 81.5% for discriminating pre-diabetes individuals from healthy controls. (B) AUC of miR-133 was 0.810. The sensitivity was 89.3% and the specificity was 92.6% for discriminating pre-diabetes individuals from healthy controls. ROC, receiver operating characteristic; AUC, area under the curve; miR, microRNA.
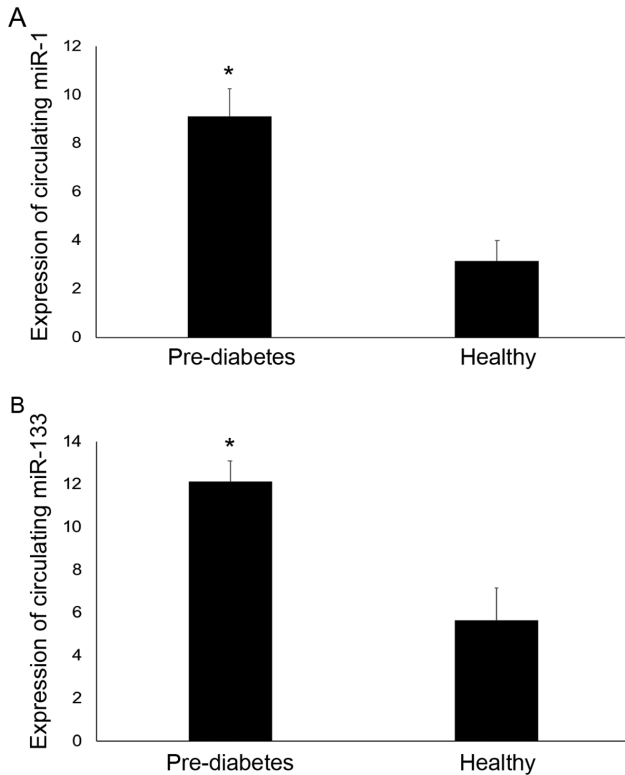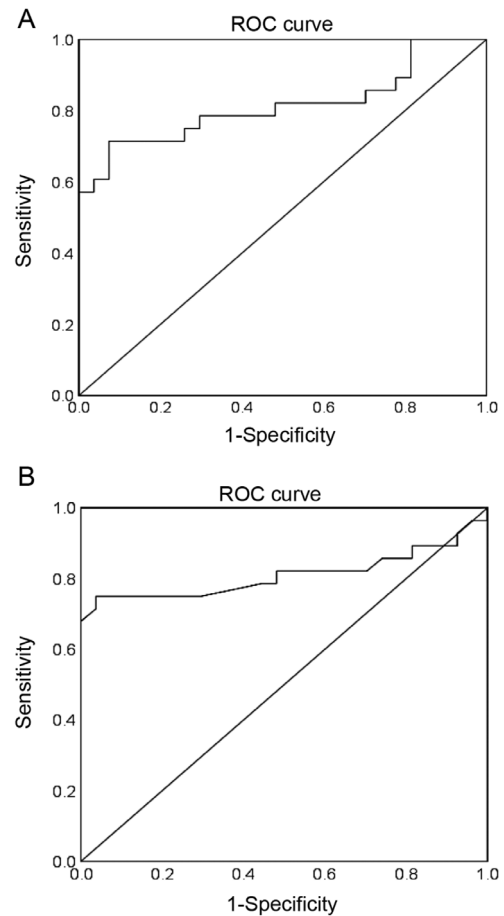

Figure 1. Expression of circulating miR-1 and miR-133 in whole blood from individuals with pre-diabetes (n=28) and healthy controls (n=27). Expression of circulating (A) miR-1 and (B) miR-133. miR, microRNA. *P<0.05 compared with healthy controls.

independent variables into Model 1. Adjustment for age and sex (Model 2), for age, sex, BMI and blood pressure (Model 3), and for age, sex, BMI, blood pressure, total cholesterol, triglyceride and LDL-C levels (Model 4) had no effect on the association between miR-1 and prediabetes (all P<0.05; Table IV). Similarly, the observed association between miR-133 and pre-diabetes remained statistically significant after the same adjustments in all 4 models (all P<0.05; Table IV)

Linear regression analysis. Linear regression analysis was performed to identify predictors of miR-1 and miR-133. In this analysis, miR-1 and miR-133 were used as dependent variables and all clinical parameters (Table V) were entered as independent variables. As shown in Table V, HOMO-IR (β=0.647, 95% CI, 0.503-5.268; P<0.001) was the only significant predictor that remained positively associated with miR-1. Similarly, the HOMO-IR index was found to be the only significant and positive predictor associated with miR-133 (β=0.406, 95% CI, 0.26-5.466; P=0.005).

ROC analysis. ROC analysis was performed to evaluate the diagnostic performance of circulating miR-1 and miR-133

confounders, associations were also adjusted for different clinical parameters, which were subsequently entered as

Table IV. Multivariate regression analysis.

Model	miR-1			miR-133		
	OR	95% CI	P-value	OR	95% CI	P-value
Model 1	1.2	1.061-1.357	0.004	1.16	1.053-1.276	0.003
Model 2	1.179	1.041-1.334	0.009	1.16	1.049-1.283	0.004
Model 3	1.235	1.063-1.4636	0.006	1.164	1.047-1.293	0.005
Model 4	1.251	1.070-1.462	0.005	1.175	1.048-1.319	0.006

Model 1, unadjusted; Model 2, adjusted for age and sex; Model 3, adjusted for age, sex, BMI and blood pressure; Model 4, adjusted for age, sex, BMI, blood pressure, total cholesterol, triglyceride and low-density lipoprotein-cholesterol levels. miR/miRNA, microRNA; OR, odds ratio; CI, confidence interval; BMI, body mass index.

Table V. Linear regression analysis.

miRNA	Variable	β	95% CI	P-value
miR-1	HOMO-IR	0.647	0.503-5.268	<0.001[b]
miR-133	HOMO-IR	0.406	0.26-5.466	0.005[a]

[a]P<0.05, [b]P<0.01. miR/miRNA, microRNA; CI, confidence interval; HOMO-IR, homeostasis model assessment-insulin resistance.

for pre-diabetes. The AUCs and 95% CIs were obtained. Cut-off points with the values of sensitivity and specificity considered to be maximal for miR-1 and miR-133 were also obtained. For miR-1, an AUC of 0.813 (95% CI, 0.693-0.934; P<0.001) was obtained for discriminating pre-diabetes from controls (Fig. 2A). At a cut-off value of 15.579, the sensitivity and specificity of miR-1 were 96.4 and 81.5% respectively. For miR-133, an AUC of 0.810 (95% CI, 0.680-0.940; P<0.001) was obtained for discriminating pre-diabetes individuals from controls (Fig. 2B). The sensitivity and specificity of miR-133 were 89.3 and 92.6% at a cut off value of 20.674.

Discussion

Circulating miRNAs have been widely investigated as possible biomarkers for T2D and disease progression. Profiling studies have shown that altered expression of circulating miRNAs can be detected during the early stages of diabetes, supporting their use as candidate biomarkers for pre-diabetes (20-23).

The present study investigated changes in the expression of circulating miR-1 and miR-133 before the clinical diagnosis of T2D, in individuals with IFG and IGT, and in healthy controls, and evaluated their clinical utility as biomarkers for pre-diabetes. Using RT-qPCR, the expression levels of circulating miR-1 and miR-133 in the blood were found to be significantly higher in the pre-diabetes individuals compared with the healthy controls. While no previous studies have investigated the circulating levels of miR-1 and miR-133 in pre-diabetes individuals, de Gonzalo-Calvo *et al* (28) found significant increases in the serum levels of miR-1 and miR-133 in T2D patients, and in a murine model of insulin resistance. Delic *et al* (29) reported progressive increases in the plasma

levels of miR-133 in Zucker diabetic rats from the early stages of pre-diabetes to progression towards T2D.

miR-1 and miR-133 are clustered on the same chromosomal loci and transcribed together as a single transcript, in a tissue-specific manner (25). These two miRNAs are members of the myomiR family. They are considered muscle-specific miRNAs due to their high expression in both cardiac and skeletal muscle, and serve a critical regulatory role in muscle development and remodelling (25). Thus, dysregulation of their expression is frequently observed in cardiac and muscle diseases (34-36). The target prediction performed in the present study supported this hypothesis. Both miR-1 and miR-133 were predicted to target several pathways involved in the pathophysiology of diabetes. A previous study by Granjon *et al* (27) showed that miR-1 and miR-133 are regulated by insulin in human skeletal muscle via sterol regulatory element-binding protein 1c and myocyte enhancer factor 2C, and both miRNAs were associated with impaired insulin response in the skeletal muscle of T2D patients. In the same study, it was also shown that insulin treatment in insulin-deficient mice resulted in the downregulation of both miRNAs (27). Moreover, miR-1 and miR-133 have been also found to be dysregulated in the skeletal muscle of insulin-resistant mice through altered insulin-like growth factor 1 (IGF-1)-mediated signalling (37). IGF-1 is one of the validated targets of miR-1 and miR-133, and both miRNAs negatively regulate its expression (38,39). Whereas miR-1 has been suggested as an early-stage marker for the development of insulin resistance through regulating the IGF-1 pathway in skeletal muscle (37), miR-133 has been shown to repress the expression of IGF-1R and signalling pathway during skeletal myogenesis, and is thus proposed as a potential therapeutic target for management of muscle diseases (38). IGF-1 is a polypeptide hormone that serves a key role in growth, development and metabolism (40,41). It shares amino acid sequence homology with insulin and exerts an insulin-like effect through the stimulation of glucose uptake by peripheral tissues (40,41). Notably, low IGF-1 levels are associated with insulin resistance, and with the subsequent development of IFG/IGT and T2D (42-44).

IFG and IGT are two states of abnormal glucose metabolism in the early stages of development of T2D (3,4). Individuals with both IFG and IGT exhibit β-cell dysfunction and insulin resistance, the core defects seen in T2D patients (7,8). Insulin

resistance serves a major role in the pathophysiology and development of pre-diabetes and is thought to precede the manifestation of T2D by several years (7-10). Particularly, insulin resistance in skeletal muscle is considered as the primary defect that is evident years before the development of T2D (26). As with all miRNAs (18), myomiRs can be released into the blood circulation (45), acting as cell-cell communicators to reflect several physiological and pathological processes.

In the present study, increased expression of miR-1 and miR-133 was observed in the blood of pre-diabetes individuals with IFG and IGT compared to the healthy controls, further supporting their potential involvement in insulin resistance and the pathogenesis of pre-diabetes. In our previous study, it was demonstrated that miR-1 and miR-133 levels were upregulated in the blood of T2D patients with and without CVD (24). These previous results and the data reported in the present study suggest that the higher expression levels of circulating miR-1 and miR-133 possibly reflect pathophysiological complications in skeletal muscle and cardiac tissue. These observations may also suggest that upregulated miR-1 and miR-133 levels in the blood circulation occurs during the pre-diabetic stage as a result of altered glucose metabolism, and may persist through to the development of T2D. It is worth noting that several post-transcriptional modifications can alter miRNA actions during circulation. Indeed, it has been recently shown that position-specific oxidation of miR-1 could serve as an epitranscriptional mechanism to coordinate pathophysiological redox-mediated gene expression (46). This observation should be further investigated in patients with prediabetes.

In the present study, the results of Pearson's correlation coefficient analysis indicated that circulating miR-1 and miR-133 levels, which were higher in the pre-diabetes individuals, were positively correlated with FG, 2-h OGTT and HbA1c levels. They were also positively correlated with insulin levels and the HOMO-IR index. The positive relationship between increased miR-1 and miR-133 with glycaemic parameters and insulin resistance suggests their contribution to the metabolic abnormalities associated with pre-diabetes.

Excess body fat is a potent factor in the development of glucose intolerance and T2D (47,48). The pre-diabetes individuals in the present study were slightly overweight with an average BMI of 25.4 kg/m^2. However, there were no significant correlations between miR-1 and miR-133 levels with BMI. However, changes in the levels of these two miRNAs have been reported in the skeletal muscle (37) and serum (28) of a mouse model of diet-induced obesity. It is worth mentioning that the period of high-fat diet employed to induce obesity in these animals as well as the degree of obesity can affect the expression of miR-1 and miR-133, and may account for the discrepancies between studies.

The logistical regression analysis performed in the present revealed a direct association between upregulated miR-1 and miR-133 levels with the presence of pre-diabetes. Interestingly, the association between miR-1 and miR-133 with prediabetes remained statistically significant after adjustment for age, sex, BMI, blood pressure, total cholesterol, triglyceride and LDL-C. These results indicate that higher miR-1 and miR-133 levels were independent of potential confounders in pre-diabetes. Additionally, the results of the linear regression analysis identified HOMO-IR as a significant and positive predictor of miR-1 and miR-133 levels amongst other clinical variables, suggesting a role of these two miRNAs in insulin resistance in pre-diabetes.

Pre-diabetes is a high-risk state for the development of T2D (2,3) and related vascular complications (4-6). Intervention strategies such as lifestyle modifications and medications can reduce the risk of progression of pre-diabetes to T2D (10,11). Early identification of pre-diabetes is therefore the gold standard for preventing disease progression. In the absence of reliable and accurate tests that can identify pre-diabetes, circulating miRNAs may serve as suitable biomarkers. Based on previous studies from our group and others, altered levels of circulating miRNAs that reflect changes in diabetes-related tissues, such as the pancreatic β cell-related miRNAs (miR-15a and miR-375) (20-22) and endothelium-enriched miRNAs (miR-126) (23) have been proposed as biomarkers for detection of pre-diabetes. In the present study, ROC curve analysis showed a promising ability of the cardiac and skeletal muscle specific miR-1 and miR-133 levels in whole blood samples to distinguish between pre-diabetes individuals and healthy controls, with high sensitivities and specificities, suggesting the potential clinical use of these two miRNAs as biomarkers for pre-diabetes.

Despite the small sample size of the participants, which limits the statistical power of the study, these results are promising as miR-1 and miR-133 could be developed as diagnostic biomarkers for the early identification of individuals with pre-diabetes. Therefore, these data should be further verified in larger clinical samples. Additional experiments using two blood samples from each participant are required to confirm the expression levels of these two miRNAs. Additionally, it would be valuable to evaluate the levels IGF-1 and other target genes of miR-1 and miR-133 in pre-diabetes and T2D patients, and correlate their expression levels to the levels of these two miRNAs.

In conclusion, the present study showed that circulating miR-1 and miR-133 levels in whole blood samples were significantly elevated in individuals with pre-diabetes compared with the controls. They were positively associated with important characteristics of pre-diabetes, including glycaemic abnormalities and insulin resistance, suggesting their involvement in disease pathogenesis. The present study also revealed that these two miRNAs could potentially act as clinical diagnostic biomarkers for pre-diabetes.

Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

GAK collected and analysed the data, and wrote and edited the manuscript. HAAM collected and analysed the data, and wrote the manuscript. AHS analysed the data and wrote the manuscript. GAK and HAAM confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Medical Research and Ethics Committee of the College of Medicine and Medical Sciences, Arabian Gulf University, Kingdom of Bahrain. All participants in the current study provided informed consent for the use of their blood samples and data.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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