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ORIGINAL ARTICLE

Circulating miR-103 family as potential biomarkers for type 2 diabetes through targeting CAV-1 and SFRP4

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Abstract

Aims MicroRNA-103 (miR-103) family plays important roles in regulating glucose homeostasis in type 2 diabetes mellitus (DM2). However, the underlying mechanisms remain poorly characterized. The objective of this study was to test the hypothesis that circulating miR-103a and miR-103b, which regulate CAV-1 and SFRP4, respectively, are novel biomarkers for diagnosis of DM2.

Methods We determined the predictive potential of circulating miR-103a and miR-103b in pre-DM subjects (pre-DM), noncomplicated diabetic subjects, and normal glucose-tolerance individuals (control) using bioinformatic analysis, qRT-PCR, luciferase assays, and ELISA assays.

Results We found that both miR-103a and miR-103b had high complementarity and conservation, modulated reporter gene expression through seed sequences in the 3′UTRs of CAV-1 and SFRP4 mRNA, and negatively regulated their mRNA and protein levels, respectively. We also found that increased miR-103a and decreased miR-103a in plasma were significantly and negatively correlated with reduced CAV-1 levels and elevated SFRP4 levels in pre-DM and DM2, respectively, and were significantly associated with glucose metabolism, HbA1c levels, and other DM2 risk factors for progression from a normal individual to one with pre-DM. Furthermore, we demonstrated that the reciprocal changes in circulating miR-103a and miR-103b not only provided high sensitivity and specificity to differentiate the pre-DM population but also acted as biomarkers for predicting DM2 with high diagnostic value.

Conclusions These findings suggest that circulating miR-103a and miR-103b may serve as novel biomarkers for diagnosis of DM2, providing novel insight into the mechanisms underlying pre-DM.

Keywords Type 2 diabetes mellitus · Pre-diabetes · Circulating microRNA · Caveolin-1 · Secreted frizzled-related protein 4

Abbreviations

DM2	Type 2 diabetes mellitus
miR-103	MicroRNA-103
NCDM	Noncomplicated diabetic subjects
pre-DM	Pre-diabetes mellitus
CAV-1	Caveolin 1
SFRP4	Secreted frizzled-related protein 4
FPG	Fasting plasma glucose

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Mao Luo and Chunrong Xu have contributed equally to this work.

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2hPG	2-hour post-load glucose
OGTT	Oral glucose-tolerance test
BMI	Body mass index
WC	Waist circumference
TC	Total cholesterol
TG	Triglyceride
HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
HbA1c	Hemoglobin A1c

Introduction

Type 2 diabetes mellitus (DM2), a cluster of chronic and progressive metabolic disorders that are characterized by hyperglycemia and insulin resistance, accounts for approximately 90–95% of all diabetes cases and has reached epidemic levels worldwide [1–3]. Pre-diabetes mellitus (pre-DM) is the precursor stage of DM2 that is characterized by a metabolic condition and is a growing global problem [4, 5]. Pre-DM has been reported to be closely associated with obesity and increased risks for diabetes and cardiovascular disease [5, 6]. Further, studies have also noted that pre-DM is a reversible state, suggesting that preventing the onset of diabetes at the pre-DM stage is a crucial step [7]. Several methods have been gold standards for diagnosing diabetes for decades including fasting plasma glucose (FPG) levels, 2-hour post-load glucose (2hPG) levels, and HbA1c levels. However, because of the individual differences and test implementation, there is a certain proportion of high deviation, low reproducibility, and possible misdiagnosis. Therefore, screening and identification of novel biomarkers with high specificity and sensitiveness for diagnoses and prognoses of pre-DM and DM2 are a current focus and future challenge of clinical research.

MicroRNAs (miRNAs) have been reported to play important roles in multiple aspects of the regulation of a whole series of diseases including pre-DM and DM2 [8–10]. Since being discovered, miRNAs in pre-DM and DM2 have been shown to play crucial roles in regulating several metabolic processes including insulin release, glucose homeostasis, lipid metabolism, and carbohydrate tolerance [9, 11, 12]. Among all miRNAs, the miR-103 family consists of two isoforms (miR-103a and miR-103b) that are highly conserved from mouse to human and have been widely investigated in the pathogenesis of various diseases, including cancers [13], diabetes mellitus [14], and cardiovascular diseases [15]. Recent studies have reported that the miR-103 is highly enriched in the circulatory system from patients with DM2 that contributes to the maintenance of insulin sensitivity and glucose homeostasis, thereby acting as a potential biomarker for the diagnosis and treatment of DM2 and obesity [14, 16–20]. In our previous study, we found that platelet-derived miR-103b is a novel biomarker for the early diagnosis of type 2 diabetes [19]. Furthermore, other studies have reported that miR-103a influences glucose homeostasis and insulin sensitivity in DM2 and obesity [14, 21]. Overall, the miR-103 family has a close relationship to the occurrence and development of DM2 through regulating the expression of its target genes. To date, however, characteristics of circulating miR-103 family members are still poorly understood in the development of DM2. Moreover, the relative contribution of circulating miR-103 family members to screening pre-DM and newly diagnosing DM2 remains to be determined.

In the current study, we focused on thoroughly studying the characteristics, co-expression, and clinical significance of the circulating miR-103 family (miR-103a and miR-103b) and its targets in plasma from patients with pre-DM and DM2. We expected to determine the potential of circulating miR-103 family members as novel biomarkers for the early diagnosis of patients with DM2.

Materials and methods

Subjects and study design

All study subjects were Han Chinese individuals who received an oral glucose-tolerance test (OGTT) and other health checkups at the Department of Endocrinology, Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, China, from August 2018 to January 2019. Subjects were grouped as normal glucose-tolerance individuals (control, $n = 50$), pre-DM subjects (pre-DM, $n = 47$), and noncomplicated diabetic subjects (NCDM, $n = 48$). The diagnosis of DM2 was confirmed as subjects with fasting glucose ≥ 7 mmol/L (126 mg/dL) by OGTT and 2-hour oral glucose-tolerance test glucose levels of ≥ 11.1 mmol/L (200 mg/dL). The subjects had a clinical diagnosis of the disease based on the guidelines by the World Health Organization (WHO). The patient characteristics and detailed methods are given in supporting material in Table 1.

The process of this study is shown in Fig. 1. The predictive potential of circulating miR-103 family (miR-103a and miR-103b) in pre-DM subjects, NCDM subjects and control individuals were assessed using bioinformatic analysis, qRT-PCR, luciferase assays, ELISA assays, Pearson's correlation analysis, and receiver operating characteristic (ROC) curve analysis, respectively. The circulating miR-103a and miR-103b with the best diagnostic value were measured as potential biomarkers for pre-DM. Exclusion criteria are tumor, kidney or liver dysfunction, any other clinically systemic acute or chronic inflammatory diseases, autoimmune

Table 1 Characteristics of the research subjects recruited for the study

Characteristics	Control	pre-DM	NCDM2
Number, *n*	50	47	48
Gender			
Male (%)	26 (52.00)	24 (51.06)	26 (54.17)
Female (%)	24 (48.00)	23 (48.94)	22 (45.83)
Age, years	45.62 ± 8.58	49.23 ± 6.45	52.6 ± 9.13
BMI, kg/m^2	24.56 ± 3.40	25.47 ± 3.02	25.52 ± 2.89
WC, cm	79.26 ± 10.15	87.58 ± 10.36[a]	102.73 ± 11.26[a, b]
TC, mmol/L	4.89 ± 0.33	5.12 ± 0.38	5.28 ± 0.29
TG, mmol/L	1.32 ± 0.15	1.96 ± 0.23[a]	2.12 ± 0.29[a]
HDL-C, mmol/L	1.45 ± 0.32	1.44 ± 0.34	1.36 ± 0.35
LDL-C, mmol/L	2.16 ± 0.76	2.51 ± 0.83	2.62 ± 0.96[a]
Blood Pressure			
SBP, mmHg	120.35 ± 10.14	136.26 ± 11.37[a]	141.59 ± 12.26[a, b]
DBP, mmHg	79.36 ± 9.83	80.15 ± 10.22	81.69 ± 11.12
Blood GLU			
FPG, mmol/L	5.01 ± 0.09	6.53 ± 0.25[a]	8.76 ± 0.68[a, b]
2 h PG, mmol/L	5.65 ± 0.64	9.42 ± 1.03[a]	13.62 ± 2.06[a, b]
HbA1c (%)	5.15 ± 0.32	6.78 ± 0.73[a]	9.16 ± 1.05[a, b]

BMI body mass index, *WC* waist circumference, *TC* total cholesterol, *TG* triglyceride, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *FPG* fasting plasma glucose, *2hPG* 2-hour plasma glucose, *HbA1c* hemoglobin A1c

[a]$p < 0.05$ versus controls; [b]$p < 0.05$ versus pre-DM subjects

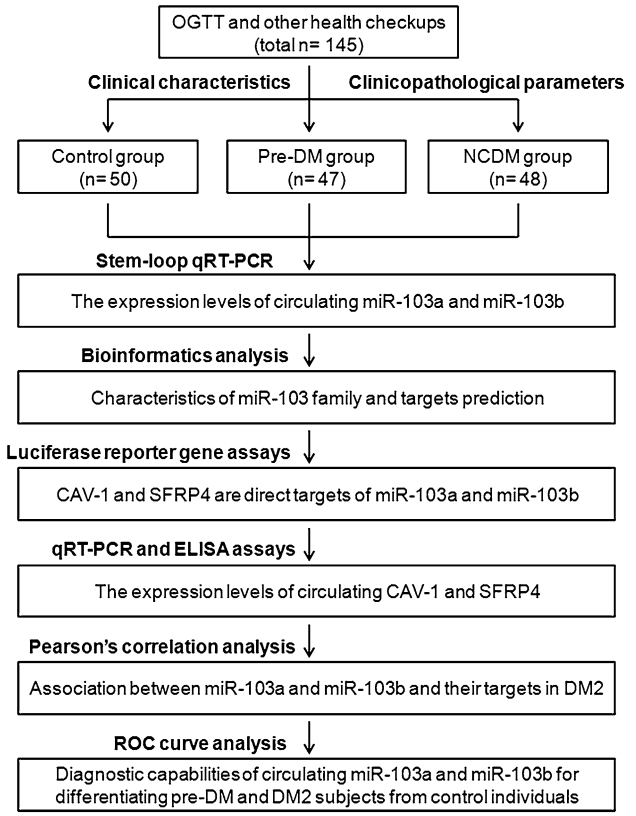

Fig. 1 Schematic representation. Flowchart shows the process of the present study

disease, untreated hypertension, and other endocrine disease except DM2.

Bioinformatic analysis

The basic composition characteristics of miR-103 family members and their precursors in different species were obtained from the miRBase online software (http://www.mirbase.org/) [22]. Then, the motif enrichment analysis of miR-103 family members and their precursors were performed using MEME 5.0.3 Suite software (http://meme-suite.org/) [23]. Sequences homologous to miR-103 family members were analyzed by Geneious software (Biomatters, Auckland, NZ) [24]. Moreover, the conservation and evolutionary relationships of precursor sequences of the miR-103 family among different species were also determined using Geneious (Biomatters). The Gene Ontology terms analysis of hsa-miR-103 was analyzed using QuickGO software (https://www.ebi.ac.uk/QuickGO/) [25]. Furthermore, we searched for putative target sites for the hsa-miR-103 family (miR-103a and miR-103b) using TargetScan 7.1 human software (http://www.targetscan.org/vert_71/) [26], and the results were further cross-compared by more efficient programs including miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/) [27], miRWalk (http://mirwalk.uni-hd.de/) [28], and miRDB (http://www.mirdb.org/) [29]. Then, the obtained intersection results were submitted to compare against known target genes whose protein functions contribute to glucose homeostasis. Finally, predicted binding sites of miR-103a in caveolin-1 (CAV-1) mRNA 3′-UTR and miR-103b in secreted frizzled-related protein 4 (SFRP4) mRNA 3′-UTR were selected. The RNA secondary structures of CAV-1 3′-UTR and SFRP4 3′-UTR were constructed using RNAfold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi).

3′-UTR luciferase reporter gene assay

Based on the predicted miRNA binding sites of target genes using bioinformatics exploration, the fragments of CAV-1 mRNA 3′-UTR and SFRP4 mRNA 3′-UTR that contained putative or mutated miR-103a and miR-103b binding sites were amplified by RT-PCR and cloned into psi-CHECK2TM vectors (Promega) downstream of the Renilla luciferase coding sequence. The wild-type plasmids (pMIR-Luc-miR-103a-CAV-1 mRNA 3′-UTR and

pMIR-Luc-miR-103b-SFRP4 mRNA 3′-UTR) and corresponding mutant recombinant plasmids were cotransfected with miR-103a or -103b mimics, miR-103a or -103b inhibitors, and the corresponding negative controls into HEK 293 cells using Lipofectamine 2000 (Invitrogen). After co-transfection for 48 h, cells were lysed, and luciferase activity was measured using the Dual-Luciferase Reporter (DLR™) Assay System (Promega, E1910) in an Orion II microplate luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany).

Plasma preparation, RNA isolation, and quantitative real-time PCR assay

Whole blood samples (3 ml) were collected and centrifuged at 3000*g* for 10 min at 4 °C. The upper yellow plasma samples were carefully transferred into fresh EP tubes, followed by a second centrifugation at 3000*g* for 10 min at 4 °C. Plasma samples were transferred to RNase/DNase-free tubes and kept at − 80 °C for long-term storage until further analysis.

Total RNA extraction was performed from the cryopreserved plasma specimens with TRIzol (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized from 1 μg of total RNA isolated above using an NCode™ miRNA First-Strand cDNA Synthesis Kit (Invitrogen) and M-MLV Reverse Transcription Kit (Promega Corp., Madison, WI, USA). The expression levels of circulating miR-103a and miR-103b were validated by stem-loop qRT-PCR with the designed miRNA-specific stem-loop RT primers, and then the mRNA levels of the corresponding target genes CAV-1 and SFRP4 were measured by qRT-PCR in an ABI PRISM 7900 (Applied Biosystems, Foster City, USA) using SYBR Premix Ex Taq II (Takara Bio, Japan). RNU68 (for miRNA) and 18S ribosomal RNA (for mRNA) were employed as internal controls for normalization. The expression levels of circulating miR-103a and miR103b as well as the levels of their targets CAV-1 and SFRP4 were quantified by the $2^{-\Delta\Delta CT}$ method as described previously. Primer sequences are provided in Supplementary Table S1.

Assay of CAV-1 and SFRP4 antigen levels

After the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA), CAV-1 and SFRP4 antigen levels were measured using ELISA assay kits (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's recommended instructions from the same plasma samples isolated above.

Statistical analyses

Statistical analyses of data were performed with SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA), and graphs were generated using GraphPad Prism version 6.0 software (GraphPad Software, Inc., CA, USA). Data are expressed as the mean ± SEM. One-way analysis of variance (ANOVA) for variance *p* values was employed to determine the difference in clinical characteristics between normal glucose-tolerance individuals (control group), pre-DM subjects, and NCDM subjects where appropriate. The relative levels of circulating miRNAs as well as their target mRNAs were calculated using the $2^{-\Delta\Delta CT}$ method. Comparisons between two groups were made by unpaired *t* test, and comparisons between more than two groups were made by one-way ANOVA followed by a Bonferroni posttest. Moreover, correlations between circulating miRNAs and their target expression profiles were calculated using Pearson's correlation. The motif enrichment analyses were measured as *E* values using bioinformatics methods. Furthermore, receiver operating characteristic (ROC) curves and the areas under the ROC curve (AUCs) were established to assess the diagnostic accuracy of circulating miRNAs among the control, pre-DM, and NCDM groups. Then, the sensitivity and specificity of the best statistical cut-off point were calculated. *p* values were considered significant when less than 0.05 for all tests.

Results

Clinical characteristics

The clinical characteristics and clinicopathological parameters were investigated in normal glucose-tolerance individuals (control, $n = 50$), pre-DM subjects ($n = 47$), and NCDM subjects ($n = 48$), as shown in Table 1. There were no statistically significant differences in sex distribution, age, body mass index (BMI), diastolic blood pressure (DBP), high-density lipoprotein cholesterol (HDL-C), and total cholesterol (TC) among the control, pre-DM, and NCDM subject groups ($p > 0.05$). Furthermore, we found that subjects (pre-DM) with significantly higher fasting blood glucose concentrations (FPG and 2hFPG) as detected by OGTT had a larger waist circumference (WC) and higher systolic blood pressure (SBP), triglycerides (TG), and glycosylated hemoglobin (HbA1c) than subjects (control) with normal glucose concentrations ($p < 0.05$); the highest levels were reached in NCDM subjects ($p < 0.05$). The results suggest that these above indexes can be risk factors for pre-DM development.

Bioinformatics analysis of the miR-103 family in different species

Mature miRNAs and their precursors are generally evolutionarily conserved among distantly related species [30]. To investigate the molecular characteristics of the mature miR-103 family and corresponding precursors among different species, e.g., *Homo sapiens* (hsa), *Mus musculus* (mmu), *Danio rerio* (dre), *Gallus gallus* (gga), *Sus scrofa* (ssc), etc., miR-103 family members and precursor sequences were downloaded from miRBase (http://www.mirbase.org/) (Supplementary Table S2). Subsequently, the homologous sequence and motif enrichment analysis of the mature miR-103 family and precursors was conducted using Geneious and MEME Suite software, respectively (Fig. 2A, B, and C, Supplementary Fig. S1, and Supplementary Fig. S2A). Hsa-miR-103 family members are widely present in approximately 23 species and mainly include two subtypes, hsa-miR-103a and hsa-miR-103b, which are located on human chromosome 20p13 and 5q34, respectively (Supplementary Table S2). Moreover, hsa-miR-103a and hsa-miR-103b are located in the introns of pantothenate kinase 2 (PANK2) and pantothenate kinase 3 (PANK3) from the PANK family, respectively (Supplementary Table S2).

As shown in Fig. 2A, B, and C, although hsa-miR-103a and hsa-miR-103b are a class of sense and antisense pairs, the sequence homologs of mature hsa-miR-103a and hsa-miR-103b, which contain two motif enrichments with respective *E* values of 1.4e−550 (a) and 4.6e−002 (b), are highly conserved between *Homo sapiens* and other species.

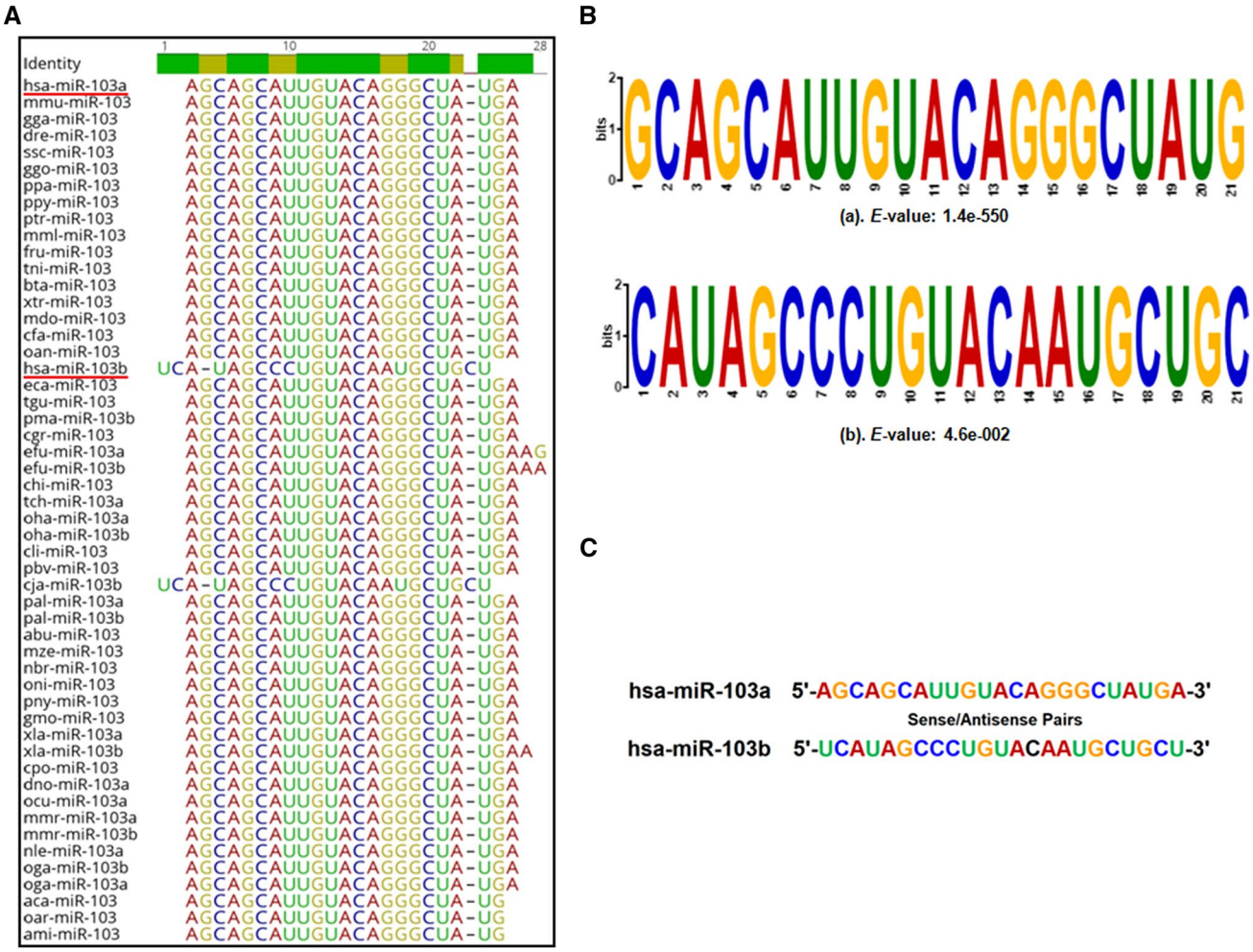

Fig. 2 Bioinformatics analysis of miR-103 family in different species. **A** Sequence homologous of miR-103 family members in different species (e.g., *Homo sapiens*, *Mus musculus*, *Danio rerio*, etc.) was analyzed by Geneious software. Furthermore, the motif enrichment analysis of mature miR-103 family members was identified by the MEME Suite software, as shown in **B** including (a) and (b). *E* value is the measure of enrichment in this case. **C** hsa-miR-103a and hsa-miR-103b (also named miR-103-as) are sense/antisense gene pairs. Four colors represent A, U, C and G that reveal the relationship significance of sequence variation among miR-103 family members (color figure online)

Furthermore, we also found that the precursor sequence similarities between the hsa-miR-103 family and other miR-103 family members, e.g., hsa-pre-miR-103a and mmu-pre-miR-103, hsa-pre-miR-103b and cja-pre-miR-103b, are similar to each other (Supplementary Fig. S1). The sequence homologs of hsa-pre-miR-103a and hsa-pre-miR-103b, which contain three motif enrichments (*E* values 8.1e−034 (a), 3.4e−170 (b), and 2.9e−1644 (c), respectively), are highly conserved among *Homo sapiens*, *Mus musculus*, and other species (Supplementary Fig. S2A). The phylogenetic trees demonstrated that both hsa-pre-miR-103a and hsa-pre-miR-103b have a high degree of sequence similarity with other species, suggesting that the miR-103 family confers critical biological functions in organisms (Supplementary Fig. S1). The gene ontology terms analysis of the hsa-miR-103 family was performed using QuickGO software, and it indicated that hsa-miR-103 regulates glucose homeostasis (Supplementary Fig. S2B).

Expression levels of circulating miR-103a and miR-103b in NCDM patients, pre-DM subjects, and control individuals

To determine the potentials of circulating miR-103a and miR-103b as novel biomarkers, the plasma levels of miR-103a and miR-103b were detected in NCDM, pre-DM, and control individuals. As shown in Fig. 3A and B, the levels of circulating miR-103a were progressively upregulated from subjects classified as pre-DM and NCDM subjects, with significant increases (approximately 2- and 3-fold) compared with control individuals; they were significantly greater in the NCDM subjects compared with other groups (Fig. 3A). By contrast, circulating miR-103b showed significant decreases (approximately 65.0% and 64.1%) in the pre-DM and NCDM groups compared to the control group. Interestingly, there were no significant differences in miR-103b levels between pre-DM and NCDM subjects (Fig. 3B).

CAV-1 and SFRP4 are direct targets of miR-103a and miR-103b

To investigate possible miRNA-target interactions, targets of miR-103a and miR-103b were predicted using various bioinformatics-based approaches as described above. Predicted binding sites of miR-103a in CAV-1 mRNA 3′-UTR as well as miR-103b in the SFRP4 mRNA 3′-UTR were selected for the current study (Figs. 4A and 5A). The RNA secondary structures of the 3′-UTRs of CAV-1 and SFRP4 were constructed using RNAfold software; the single miR-103a- or miR-103b-binding sites are marked in red in Figs. 4B and 5B.

Subsequently, to assess whether miR-103a and miR-103b directly regulate CAV-1 and SFRP4 expression, luciferase reporter plasmids carrying the 3′-UTRs of CAV-1 and SFRP4 mRNAs with the putative miR-103a- or miR-103b-binding sites or mutant binding sites were cotransfected with miR-103a or -103b mimics, miR-103a or -103b inhibitors, and the corresponding negative controls into HEK 293 cells. An empty vector was used as a blank control (Figs. 4A and 5A). We found that transfection with miR-103a or -103b mimics significantly suppressed luciferase activity compared with the respective blank control group and miR-mimic NC group (Figs. 4C and 5C). Conversely, the miR-103a or -103b inhibitors increased the relative luciferase activity of the CAV-1 and SFRP4 3′-UTRs compared with the blank control group and miR-inhibitor NC

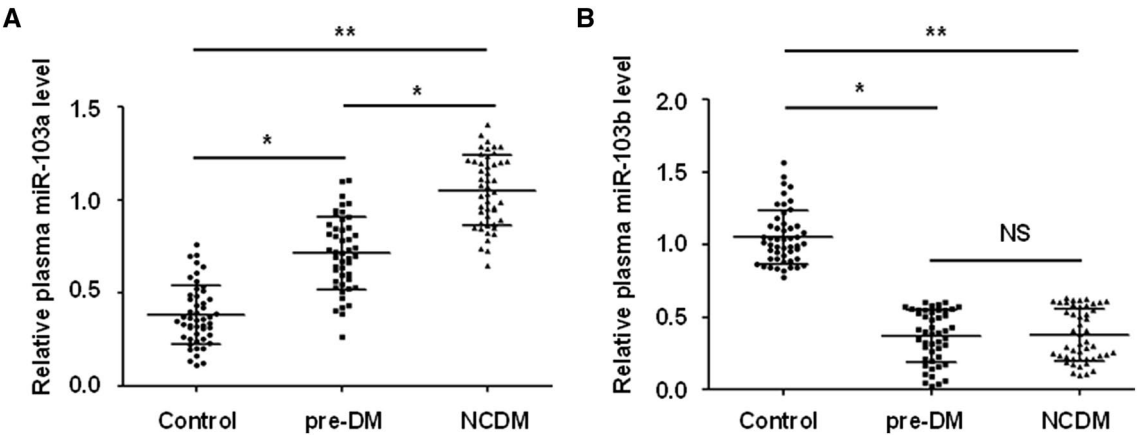

Fig. 3 Expression levels of circulating miR-103a and miR-103b. **A** and **B** confirm circulating miR-103a and miR-103b expression levels in normal glucose-tolerance individuals (control, $n=50$), pre-diabetic mellitus subjects (pre-DM, $n=47$), and noncomplicated diabetic subjects (NCDM, $n=48$), as determined by stem-loop qRT-PCR, respectively. The data were normalized to U6 RNA in each sample. Group data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by a Bonferroni posttest, $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$. NS, not significant

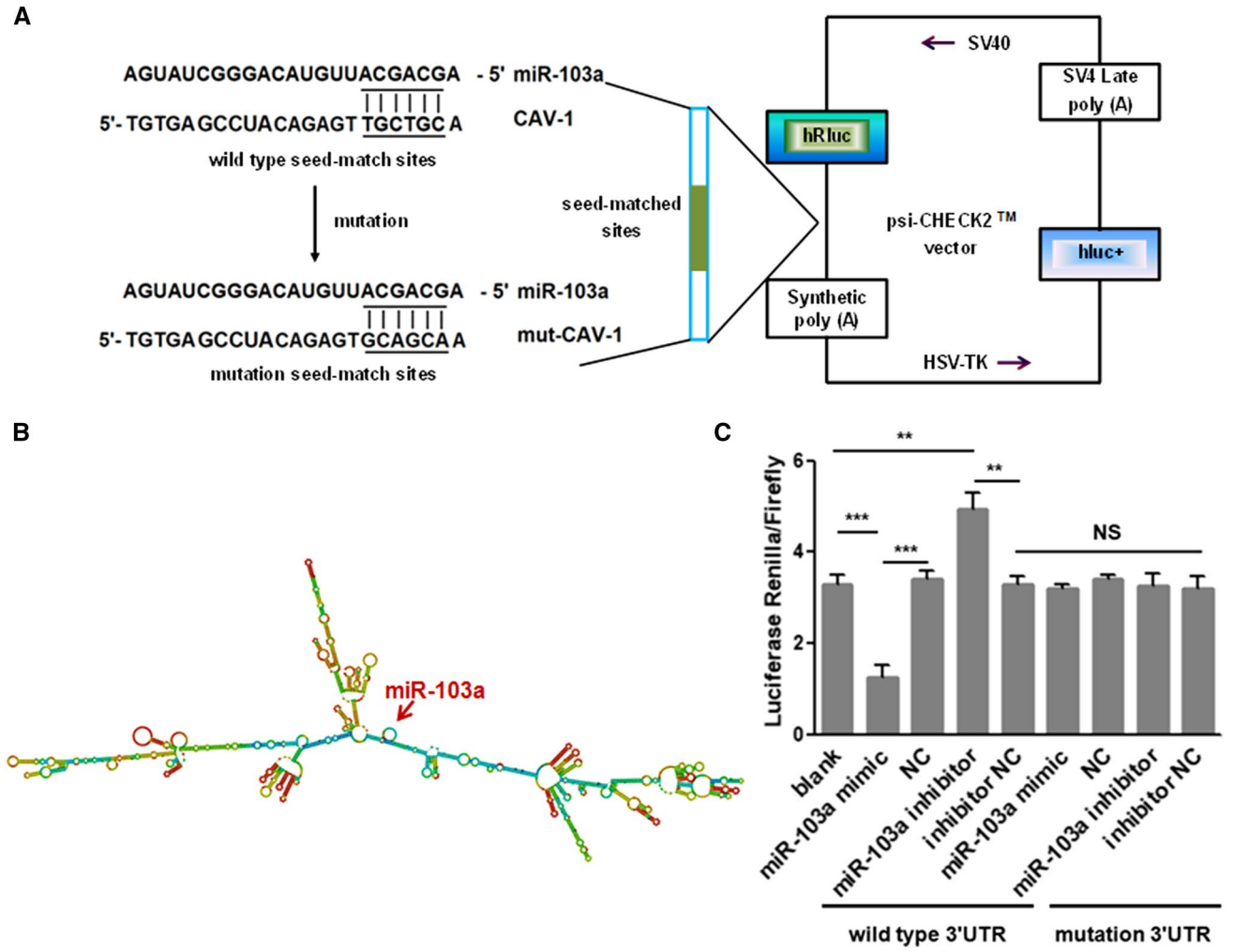

Fig. 4 CAV-1 is a direct target of miR-103a. **A** shows the structure of psi-CHECK2™ -CAV-1-3′-UTR- (wild type and mutation) luciferase reporter plasmid. Predicted (upper) and mutated (lower) binding sites of miR-103a in CAV-1 mRNA 3′-UTR were cloned into the 3′ end of the luciferase reporter plasmids. The seed sequences and corresponding mutations shown as underlined were designed. **B** shows the predicted RNA secondary structure of the CAV-1 3′-UTR that contains the binding area of miR-103a sequences using the RNAfold web server. **C** shows the relative luciferase activity analysis in HEK293 cells 48 h after co-transfection with the psi-CHECK2™ -CAV-1-3′-UTR- (wild type and mutation) luciferase reporter plasmid and the indicated miR-103a mimic (30 nmol/L), miR-103a inhibitor (100 nmol/L) and respective negative controls (NCs). All data are presented as the mean ± SEM of triplicate independent experiments. $^{**}p < 0.01$, and $^{***}p < 0.001$, two-way ANOVA followed by Bonferroni posttest

group. The effects of the miR-103a or -103b mimics and inhibitors were indeed substantially abrogated when mutated 3′-UTR psi-CHECK2 constructs were used (Figs. 4C and 5C). The above results indicate that miR-103a and miR-103b modulate reporter gene expression through respective seed regions and sequences in the 3′-UTRs of CAV-1 and SFRP4 mRNAs, respectively, and directly negatively regulate their expression.

Expression levels of circulating CAV-1 and SFRP4

To test whether there were reciprocal changes between miR-103a or miR-103b and CAV-1 or SFRP4 mRNA and protein levels, respectively, we identified changes in the CAV-1 and SFRP4 mRNA levels by qRT-PCR in the previous cohort of plasma. The CAV-1 mRNA expression levels were progressively reduced and significantly lower in the NCDM subjects compared with other groups (Fig. 6A). Conversely, the expression levels of SFRP4 mRNA were significantly elevated in pre-DM and NCDM subjects compared with control individuals (Fig. 6B).

Furthermore, the total amount of circulating CAV-1 and SFRP4 protein antigens were measured by ELISA in blood samples. As shown in Fig. 5C and Fig. 6D, the average amount of CAV-1 antigen was progressively reduced by approximately 32.5% and 41.0% in pre-DM and NCDM

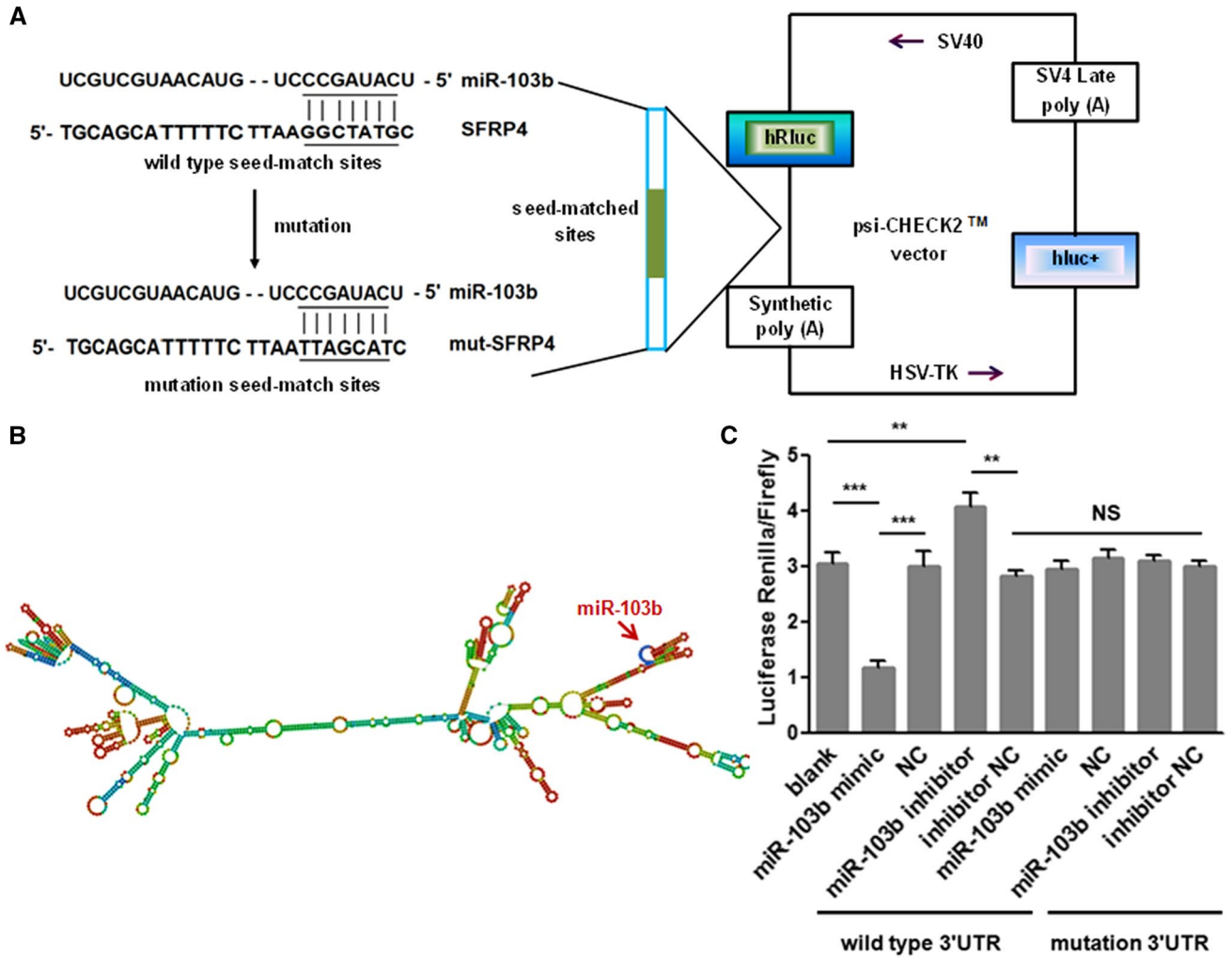

Fig. 5 SFRP4 is a direct target of miR-103b. **A** shows the schematic representation of luciferase reporter plasmid construction within the seed-binding region for miR-103b in the 3′-UTR of SFRP4 mRNA and its mutation was inserted into the 3′ end of the luciferase cDNA. **B** shows the predicted RNA secondary structure of the SFRP4 3′-UTR containing the binding sequences of miR-103b by using the RNAfold web server. **C** Luciferase activity was assessed in HEK293 cells transfected with the psi-CHECK2™ - SFRP4-3′-UTR- (wild type and mutation) luciferase reporter plasmid and the indicated miR-103b mimic (30 nmol/L), miR-103b inhibitor (100 nmol/L) and respective negative controls (NCs). Group data (three independent experiments) are expressed as mean ± SEM. $^{**}p < 0.01$, and $^{***}p < 0.001$

subjects compared with control individuals (Fig. 6C). Reversely, the average amount of SFRP4 antigen was significantly increased by approximately 3.02- and 3.35-fold in pre-DM and NCDM compared to the control group (Fig. 6D). Additionally, there were no significant differences in SFRP4 mRNA and protein levels between pre-DM and NCDM subjects (Fig. 6B and D).

Association between circulating miR-103a and miR-103b and changes of their targets in DM2

To further explore the role of circulating miR-103a and miR-103b in pre-DM and DM2, the association between their expression and CAV-1 and SFRP4 levels were evaluated by Pearson's correlation analysis among the control, pre-DM and NCDM groups. Interestingly, as shown in Fig. 7A-F, we found that circulating miR-103a expression levels were significantly and negatively correlated with changes in CAV-1 levels in NCDM subjects ($r = -0.9883$, $p < 0.0001$) (Fig. 7C), pre-DM subjects ($r = -0.7974$, $p < 0.0001$) (Fig. 7B), and control individuals ($r = -04848$, $p = 0.0004$) (Fig. 7A). Moreover, circulating miR-103b was significantly and negatively correlated with SFRP4 in both NCDM subjects ($r = -0.7001$, $p < 0.0001$) (Fig. 7F) or pre-DM subjects ($r = -0.9885$, $p < 0.0001$) (Fig. 7E), while circulating miR-103b showed no significant correlation with SFRP4 in control individuals ($r = 0.1390$, $p = 0.3357$) (Fig. 7D).

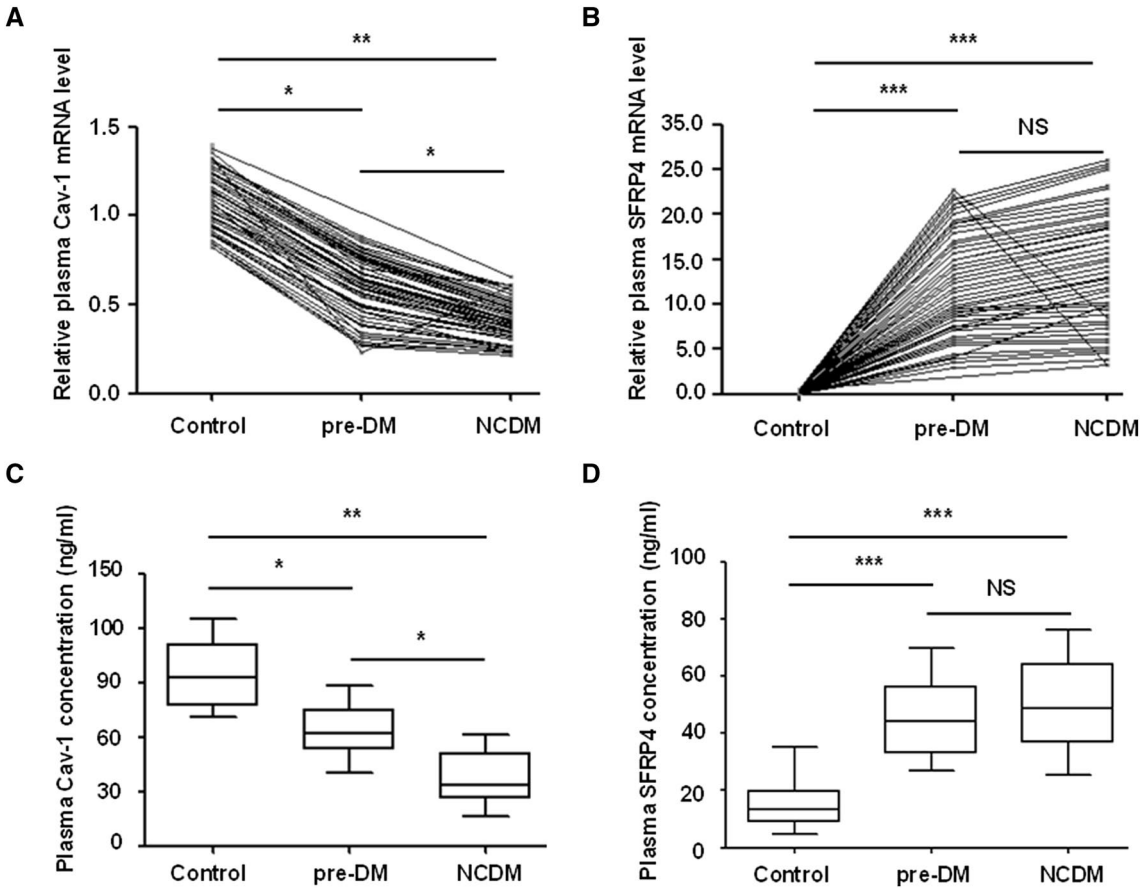

Fig. 6 Expression levels of circulating CAV-1 and SFRP4. **A** and **B** show upregulation and downregulation of CAV-1 mRNA (as a direct target of miR-103a) and SFRP4 mRNA (as a direct target of miR-103b) in normal glucose-tolerance control individuals ($n=50$), pre-DM subjects ($n=47$), and NCDM subjects ($n=48$), as measured by qRT-PCR, respectively. Total RNA extraction was performed using TRIzol reagent. All data were normalized to 18S rRNA in each sample. **C** and **D** show circulating CAV-1 and SFRP4 antigen levels, as determined by ELISA following a BCA assay, respectively. Group data are expressed as mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$, two-way ANOVA followed by Bonferroni posttest. NS, not significant

Diagnostic performance of circulating miR-103a and miR-103b for pre-DM and DM2

Subsequently, ROC curve analysis was used to evaluate the diagnostic capabilities of circulating miR-103a and miR-103b for differentiating pre-DM and DM2 subjects from control individuals. As shown in Fig. 8A–F, in the analysis of pre-DM subjects and control individuals, circulating miR-103a had the cut-off value of 0.5195 with 85.10% sensitivity and 80.00% specificity with an AUC of 0.901 [95% confidence interval (CI) 0.842–0.960; $p<0.001$] (Fig. 8A). Correspondingly, circulating miR-103b had the cut-off value of 0.7955 with 98.00% sensitivity and 97.90% specificity with an AUC of 0.999 (95% CI 0.997–1.0; $p<0.001$) (Fig. 8D).

In the analysis of NCDM patients and control individuals, the AUC was 0.998 for circulating miR-103a (95% CI 0.993–1.0; $p<0.001$) with a cut-off value of 0.7148 with 97.90% sensitivity and 98.00% specificity (Fig. 8B). Moreover, the AUC was 0.964 for circulating miR-103b (95% CI 0.920–1.0; $p<0.001$) with a cut-off value of 0.8203 with 98.00% sensitivity and 91.70% specificity (Fig. 8E). Furthermore, in the analysis of pre-DM and NCDM subjects, circulating miR-103a had a cut-off value of 0.8464 with 87.50% sensitivity and 74.50% specificity with an area under curve (AUC) of 0.890 [95% confidence interval (CI) 0.828–0.952; $p<0.001$] (Fig. 8C). The AUC was 0.474 for circulating miR-103b (95% CI 0.355–0.593; $p<0.001$) that had a cut-off value of 0.3216 with 61.70% sensitivity and 50.00% specificity (Fig. 8F).

Discussion

Pre-DM, an intermediate state between normal metabolism and DM2, is a risk factor for the development of DM2 and its complications [5, 7]. Many epidemiologic investigations

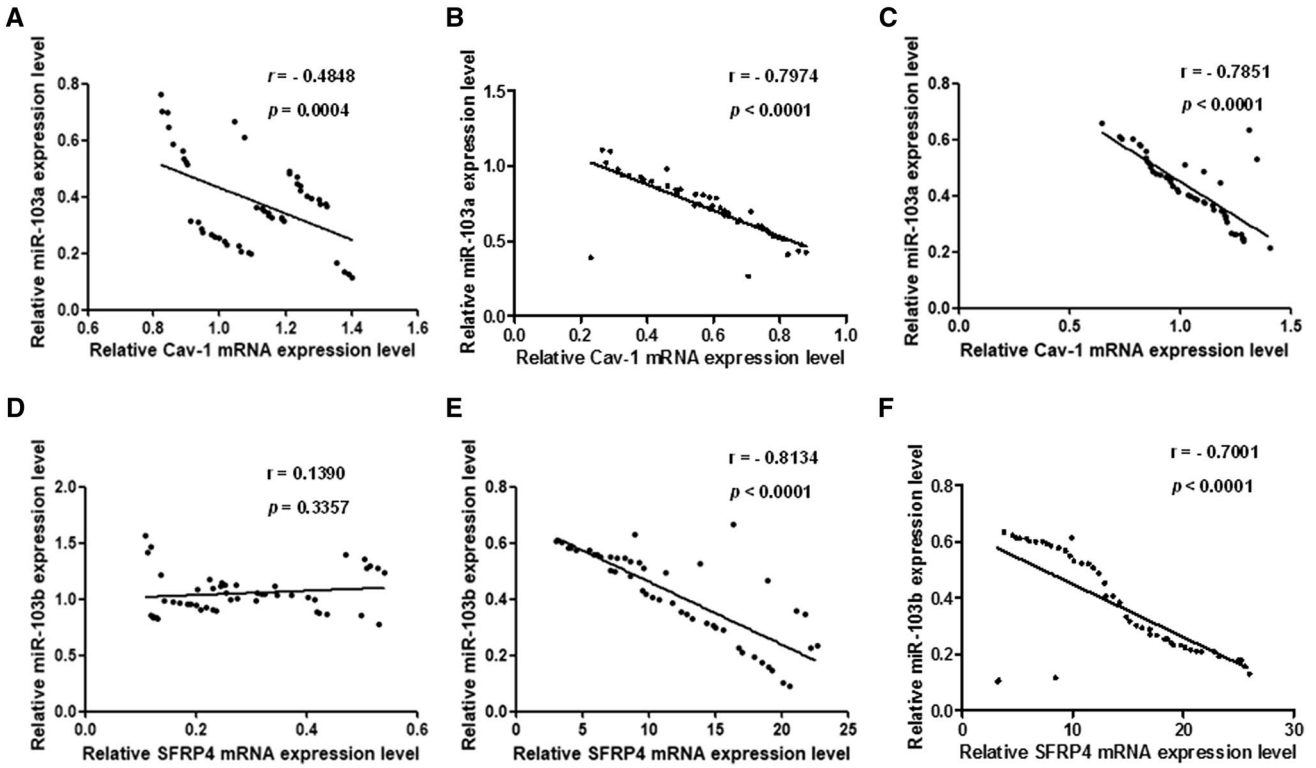

Fig. 7 Circulating miR-103a and miR-103b expression correlates with CAV-1 and SFRP4 I levels in DM2. **A**–**F** show correlation analyses between circulating miR-103a expression and CAV-1 levels (**A, B** and **C**), miR-103b expression and SFRP4 levels (**D, E** and **F**) in control individuals, pre-DM and NCDM subjects. **A** and **D** show correlation plots for control individuals group. **B** and **E** show correlation plots for pre-DM subjects group. **C** and **F** show correlation plots for NCDM subjects group

have found that more than two-thirds of pre-DM individuals develop DM2 [31–34]. Pre-DM is a complicated process of polygene-related action, and these kinds of genetic changes may indicate the onset of pre-DM, suggesting that genetic testing for pre-DM can lead to early detection of changes that may be ignored or undetected by histological examination. Previous studies have shown that circulating miRNAs present in plasma/serum are characteristically altered in several pathological disorders and have been identified as diagnostic markers for specific disease processes, including DM2 [9, 10, 35–38].

In this study, using individual plasma, we showed that hyperglycemia-induced upregulation of miR-103a and downregulation of miR-103b with detectable levels at baseline in our subjects. Their levels were significantly associated with glucose metabolism, HbA1c levels, and other DM2 risk factors among pre-DM subjects, DM2 patients, and control individuals. We also showed that circulating miR-103a and miR-103b were significantly and negatively correlated with CAV-1 and SFRP4 levels in pre-DM and DM2 subjects, as determined using Pearson's correlation analysis. Moreover, we further demonstrated that circulating miR-103a and miR-103b were quite rare for novel biomarkers in distinguishing DM2 patients, pre-DM subjects, and control individuals, as assessed by ROC analysis. The findings in our study suggested that circulating miR-103a and miR-103b, with reciprocal expression changes, may serve as valuable diagnostic biomarkers and mediate posttranscriptional regulation in the early stage of DM2.

The bioinformatic results in this and previous studies have consistently shown that the miR-103 family and their respective precursors are highly conserved between *Homo sapiens* and other species, and the family includes two subtypes: miR-103a and miR-103b. Additionally, we also demonstrated that hsa-miR-103a and hsa-miR-103b, located on human chromosome 20p13 and 5q34 in the introns of PANK2 and PANK3, respectively, may regulate glucose homeostasis, as forecasted using bioinformatics analysis. We further demonstrated that miR-103a and miR-103b had high complementarity and high conservation with respect to binding sites within the 3′-UTR seed sequences of CAV-1 and SFRP4 mRNAs, respectively, and directly and negatively regulated their mRNA and protein expression levels. Previous studies have shown that the miR-103 family plays crucial roles in the onset and development of various tumors as well as in glycolipid metabolism and the pathological progress of diabetes [13, 14, 19, 20]. Recent studies have found that the glycometabolic disorder of patients with DM2 is usually

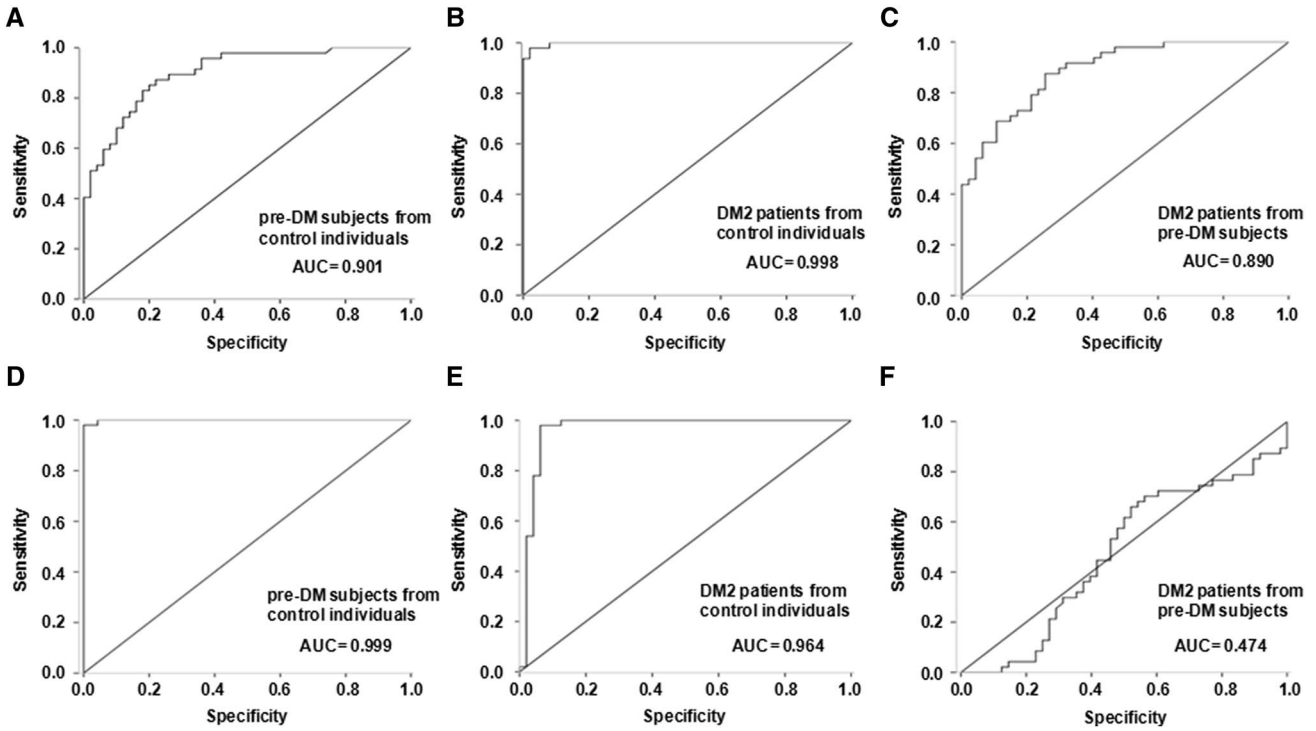

Fig. 8 Diagnostic performance of circulating miR-103a and miR-103b between the cases of DM2 subjects, pre-DM and control individuals. **A** and **D** show diagnostic abilities of circulating miR-103a (**A**) and miR-103b (**D**) for the cases of pre-DM subjects and normal glucose-tolerance (control) individuals using the ROC curve analysis. **B** and **E** show diagnostic abilities of circulating miR-103a (**B**) and miR-103b (**E**) for the cases of DM2 subjects and control individuals. **C** and **F** circulating miR-103a (**C**) and miR-103b (**F**) discriminate the cases of DM2 subjects and pre-DM individuals

accompanied by abnormal expression of miRNAs, where miR-103a and miR-103b are closely correlated with glucose metabolism [14, 19, 20, 39]. Although there have been several studies that have demonstrated that there is abundant miR-103a/b in circulation, the mechanisms by which circulating miR-103a/b regulate biological pathways in DM2 have largely remained unclear.

Caveolin-1, an essential structural, scaffolding, and regulatory protein of caveolae, is known for its role in regulating signal transduction by linking signaling molecules and thus modulating their downstream activity [40, 41]. Some studies have reported that Cav-1 participates in the development of obesity-related diabetes and contributes to signal transduction, lipid metabolism, and endocytosis [42–45]. Previous studies have also demonstrated that miR-103a regulates glucose homeostasis and insulin sensitivity by targeting CAV-1 expression in patients with DM2 [14]. Here, we demonstrate that Cav-1 is a direct target of miR-103a and increased circulating miR-103a in plasma is significantly and negatively correlated with decreased Cav-1 levels in pre-DM and DM2.

Secreted frizzled-related protein 4 (SFRP4), a modulator of Wnt signaling, is overexpressed in DM2 and closely correlated with insulin secretion [46, 47]. Some studies have revealed that increased SFRP4 levels in the blood could be a potential strong-risk biomarker for pre-DM [46]. Data obtained in the present studies showed that there was a significant increase in the expression of SFRP4 mRNA and protein in patients with pre-DM and DM2 and that this increase was significantly and negatively correlated with reduced expression of miR-103b.

Prior studies have demonstrated that in addition to blood glucose levels, metabolic factors such as SBP, TG, and HbA1c could be risk factors for the development of DM2 [48, 49]. Clinical data obtained in the present studies demonstrated that the above factors including FPG, 2hFPG, WC, SBP, TG, and HbA1c could be critical risk indicators for the progression from a normal individual to a pre-DM patient. Other studies have reported that increased TG leads to lipid metabolism disorders [50]. Cross-referencing the results obtained in our study, we pointed out that changes in TG levels could provide preventive monitoring for pre-DM patients, suggesting that control of lipid metabolism disorders should be considered as a strategy for controlling the prevalence of DM2. Collectively, these data indicate that these increased risk factors in pre-DM and DM2 could be one significant cause of the abnormal expression of miR-103a and miR-103b that significantly and negatively regulate the expression of their target genes.

A number of studies have revealed that abnormal expression of circulating miRNAs may occur even before diabetic patients have obvious clinical symptoms, indicating that circulating miRNAs can be employed as potential biomarkers for early diagnosis of DM2 [9, 10, 35–38, 51–54]. For example, Yan et al. explored that plasma miR-1249, miR-320b, and miR-572 were differentially expressed during the pathogenesis of DM2, which yielded AUCs of 0.784, 0.946, and 0.843 discriminating DM2 patients from control groups, respectively, while the AUCs were 0.887, 0.635, and 0.69 discriminating pre-DM patients from control groups, respectively [37]. Al-Muhtaresh and Al-Kafaji revealed peripheral blood miR-375 and miR-9 were expressed at higher levels in pre-DM subjects and progressively more enriched in DM2 patients, and they reported that miR-375 and miR-9 were earlier blood biomarkers for detecting pre-DM and DM2, which yielded the AUCs of 0.77 and 0.50 discriminating DM2 patients from controls, respectively, while the AUCs were 0.76 and 0.63 discriminating pre-DM subjects from controls, respectively [38]. Yang et al. explored low serum levels of miR-23a, let-7i, miR-486, miR-96, miR-186, miR-191, miR-192, and miR-146a in DM2, and they revealed that serum miR-23a was a potential biomarker for early detection of DM2, which yielded AUCs of 0.690 and 0.835 in discriminating pre-DM and DM2 subjects from control individuals, respectively [51]. Al-Kafaji et al. revealed that peripheral blood miR-15a is a potential biomarker for pre-DM and DM2 with AUCs of 0.852 and 0.864 in discriminating pre-DM and DM2 subjects from control groups [52]. Liu et al. found that serum miR-126 was significantly lower in pre-DM and DM2 subjects compared with healthy controls, and they revealed serum miR-126 was a novel biomarker for screening pre-DM and newly diagnosed DM2 [53]. Párrizas et al. revealed circulating miR-192 and miR-193b were markers of pre-DM and were modulated by an exercise intervention [54].

In the present study, we also demonstrated the feasibility of using circulating miR-103a and miR-103b to discriminate DM2, pre-DM, and control individuals. Our data indicated that circulating miR-103 family was better in terms of diagnosis performance than other miRNAs which have been identified as biomarkers of DM2. Our results identified significantly increased levels of miR-103a in plasma, which yielded AUCs of 0.901 and 0.998 in discriminating pre-DM and DM2 subjects from control individuals, respectively, while the AUC was 0.890 for discriminating DM2 patients from pre-DM subjects. We also detected significantly reduced levels of miR-103b in plasma, which yielded AUCs of 0.999 and 0.964 for discriminating pre-DM and DM2 subjects from control individuals, respectively, while the AUC was 0.474 for discriminating DM2 patients from pre-DM subjects. Collectively, these data indicate that reciprocal changes of circulating miR-103a and miR-103b not only provide high sensitivity and specificity to differentiate the pre-DM population but also act as biomarkers for predicting DM2 patients with high diagnostic value.

In conclusion, we report a novel pathway for circulating miR-103a and miR-103b that regulates CAV-1 and SFRP4 in subjects with early DM2, which suggests that circulating miR-103a and miR-103b could be novel biomarkers for screening pre-DM and newly diagnosing DM2. Although there are several limitations of our present studies, these findings provide direction for future studies. For example, the biomarker potential of circulating miR-103a and miR-103b for diagnosing DM2 was conducted in a relatively small sample size of subjects and controls, and the precise mechanisms by which interactions between the upstream and downstream factors of miR-103a and miR-103b contribute to the development of DM2 remain largely unclear. Future studies should center on further evaluation in a large clinical study. Moreover, additional experiments are necessary to further define the precise regulatory network of miR-103a and miR-103b in DM2. Overall, our present work demonstrates that miR-103a and miR-103b can be efficiently measured in circulating blood. It provides an impetus for assessing the role of miR-103a and miR-103b as novel biomarkers for new diagnoses of early DM2. These findings will ultimately provide novel insights into the mechanisms underlying pre-DM.

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Authors' contributions The original idea of this study was proposed by QW. ML and CX performed experiments and analyzed the data and wrote the first draft of this manuscript. YL and GW performed and interpreted the experiments. JW and QW edited subsequent drafts. All authors have read and approved the final version of the manuscript for submission.

Compliance with ethical standards

Conflict of interest All the authors including Mao Luo, Chunrong Xu, Yulin Luo, Gang Wang, Jianbo Wu, and Qin Wan declare that they have no conflict of interest.

Ethical approval All human subjects used in the study ''Circulating miR-103 Family as Potential Biomarkers for Type 2 Diabetes through targeting CAV-1 and SFRP4'' have been reviewed by the Research Ethics Committee of the Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan Province, P. R. China and have been performed in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All samples were collected with informed consent of all subjects. There is no security and privacy violation to the patient's health in our study.

Human and animal rights All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the 1975 Helsinki declaration, as revised in 2008 (5).

Informed consent Informed consent was obtained from all individual participants included in the study.

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