Original Article
Alpinia oxyphylla Miq. extract changes microRNA expression profiles in db-/db- mouse livers

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Abstract: MicroRNAs (miRNAs) involvement in the pathology of obesity and type 2 diabetes has received limited research attention to date. Emerging evidence suggests that *Alpinia oxyphylla* may exhibit antioxidant and anti-diabetic activities. To determine the microRNA expression profiles in the livers of db-/db- mice treated with *Alpinia oxyphylla* extract (AOE). Male diabetic mice were administered 500 mg/kg per day AOE via the intragastric route once a day for 8 weeks. Euthanized mice were dissected and miRNA was extracted from the liver, sequenced using standard methodology, and confirmed by qRT-PCR. Cluster analysis was used to predict target genes. Treatment with AOE led to significantly lower concentrations of blood glucose, serum triglyceride, and serum cholesterol levels, compared with the AOE untreated group. Sequencing showed that, of the identified miRNAs, 19 were statistically significantly differentially regulated in the livers of DB/DB mice group, and 7 were differentially regulated in the livers of db-/db-AOE500 mice group, compared to db-/db-H_2O mice group. mmu-miR-423-5p and mmu-miR-598-3p were identified in both groups. Functional bioinformatic analysis showed that the target genes of these miRNAs included those related to the lysosome cellular pathway, the MAPK and Ras signaling pathways, and glycosphingo-lipid biosynthesis. *Alpinia oxyphylla* Miq. extractmay serve as a potential natural anti-diabetic and anti-obesity agent because it changes the expression of specific miRNAs involved in biologically significant signaling pathways.

Keywords: MicroRNA, db-/db- mice, *Alpinia oxyphylla* Miq., liver

Introduction

Obesity, defined as an excessive proportion of body fat, has become a growing medical concern because it increases the risk for several diseases, particularly type 2 diabetes mellitus (T2DM) [1]. Increased calorie intake and decreased physical activity both contribute to the development of obesity and insulin resistance [2], which arelargely the consequence of adipose tissue inflammation and adipokine dysregulation [3]. Insulin resistance leads to an increase in free fatty acid levels in the circulation through the promotion of adipose tissue lipolysis. This results in an increase in the delivery of free fatty acids to the liver, as well as to the synthesis of excess triglycerides and the accumulation of excess liver fat [2]. Consequently, non-alcoholic fatty liver disease (NAFLD) is highly prevalent in patients with T2DM [2].

MicroRNAs (miRNAs) are small, non-coding, regulatory RNAs (comprising 20-22 nucleotides) involved in numerous biological processes as well as in the pathology of various diseases. Their expression profiles have been examined in a range of previous studies. For example, circulating miRNAs have been studied in diabetes mellitus patients [4, 5] and miRNA expression profiles have been investigated in a range of different animal tissues [6, 7]. Several dysregulated miRNAs are reportedly involved in obesity [8-10]. A previous meta-analysis identified 40 miRNAs that displayed significantly aberrant expression profiles in T2DM patients [11]. Administration of miR-346 has been found to downregulate SMAD3/4 expression and ameliorate renal function in diabetic mice [12], and a separate mouse study has shown that suppressing miR-144 expression may help decrease oxidative stress in the hearts of diabetic individuals

[13]. Although significant numbers of miRNAs have been identified in such studies, only a few are likely to be important therapeutic targets.

Alpinia oxyphylla Miq. is regarded as a sought-after drug in the herbal medicine arena and is widely distributed in south China. Its fruits are used in traditional Chinese medicine to treat intestinal disorders and dementia. The plant is rich in sesquiterpenes, diterpenes, flavonoids, and diarylheptanoids. Sesquiterpenes have been shown to inhibit nitric oxide (NO) production in lipopolysaccharide- and interferon-gamma-induced mouse peritoneal macrophages [14, 15]. A growing body of evidence suggests that extracts from *A. oxyphylla* fruits possess significant anti-adipocyte differentiation activity [16] and enhance the proliferation of human adipose tissue-derived stromal cells [17]. We previously found that *A. oxyphylla* extract (AOE) exhibits antioxidant and anti-diabetic activities [18, 19]. However, the molecular mechanisms underlying the anti-diabetic effects are not yet understood.

Against this background and given the established importance of miRNAs in the development of T2DM and obesity, we hypothesized that miRNAs may play a role in the observed beneficial effects of AOE on these conditions. The db-/db- mouse model is a well-established rodent model of T2DM that exhibits early insulin resistance followed by extreme hyperglycemia associated with obesity, occurring 4-8 weeks after birth [20]. The present study investigated the hepatic miRNA expression profiles of normal DB/DB mice and compared these-with the profiles of AOE-treated or untreated db-/db- mice, using miRNA sequencing.

Materials and methods

Preparation of the plant extract

The ripe fruit of A. oxyphylla were purchased from a market specializing in herbs (Haikou, Herb Market, China) in Jan of 2015. The plant was authenticated by Dr. Qiang Liu of the Department of Pharmacognosy, Hainan Medical College, Haikou, China. A. oxyphylla was extracted with 640 ml of water for 16 hours at 90°C, two times. The water extract was then lyophilized and stored at room temperature until use. The dry yield was 8% (w/w). With Good reproducibility, the polysaccharide content (about 8%) provided an effective method for quality control. The dry powder was dissolved directly by water to proper concentration.

Animals

In this study, we strictly obeyed the animal protocols approved by the Ethics Committee of Hainan Medical College for Animal Care and Use. For the care and use of animals utilized in this research, we monitored the animals twice per week, and none of animals showed severe ill, died or moribund during the whole experiments.

A total of 24 3 to 4 week-old male mice, including 8 DB/DB mice and 16 db-/db- (The mice carry a mutation in the leptin receptor gene) mice on a C57BL/Ks background, were obtained from the Model Animal Research Center of Nanjing University, China. All mice were allowed to acclimatize for 1 week before the 8 weeks experimental period. The animal room was maintained under a constant 12 h light/dark cycle at a temperature of 23 ± 3°C and a relative humidity of 70 ± 10% throughout the experimental period. All mice were housed in group cages with two animals per cage. They were given free access to standard feeding pellets and water. The mice were divided into 3 groups with 8 animals in each group. DB/DB mice group and db-/db-H_2O group were administered placebo (saline) only, db-/db-AOE group was administered with 500 mg/kg of AOE via the intragastric route once a day for 8 weeks (approximately, 0.2 ml in volume).

At the end of the 8-week period, individual mice were placed in metabolic cages to obtain 24-h urine collections. Then, the mice were euthanized under chloral hydrate anesthesia, and blood and liver samples were collected for analysis. Blood samples were collected from the hepatic portal vein into a tube for EDTA anticoagulation and centrifuged (3000 rpm for 15 minutes at 4°C) for separating the plasma. The plasma was then frozen at -70°C for biochemical analysis. The liver were excised, weighed and homogenized in a 3:1 v/w of 0.25 M sucrose, 10 mM HEPES, 1 mM EDTA (pH 7.5) buffer. Samples were homogenized for 30 sec at 6.45 m/s in an Omni Bead Ruptor (OMNI International IM, GA, USA). The protein concentration in each sample was determined using
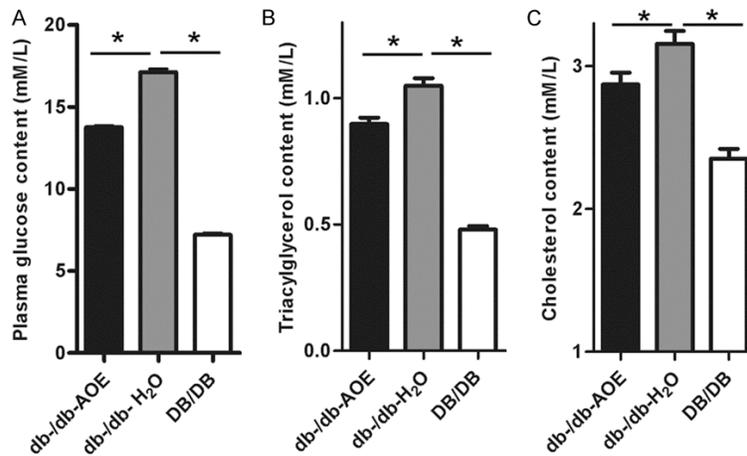
Figure 1. Effects of AOE treatment on blood glucose levels (A), triglyceride concentrations (B), and cholesterol concentrations (C) of db-/db- mice. Data represent the mean ± SD (n = 8). *P < 0.05.

Bradford protein assay kit (TIANGEN Biotech, Beijing).

Measurement of concentration of glucose, albumin and creatinine. These parameters were measured using commercial kits (Jian Cheng Biotechnology Company, Nanjing, China), according to the manufacturer's instructions.

RNA isolation

Total miRNA was extracted from mice liver using the mirVana MiRNA Isolation kit (Applied Biosystems, USA) according to the manufacturer's instructions.

Sequencing and reads processing

For small-RNA sequencing, complementary small-RNA libraries were prepared by ligating different adaptors to the total RNA followed by reverse transcription and polymerase chain reaction (PCR) amplification. Sequencing was performed using the Illumina HiSeq 2000 sequencer (Illumina, USA) with 50-bp single-end reads according to the manufacturer's standard protocol. The removal of poor quality sequences and trimming of adaptor sequences from the raw sequence data was carried out using cutadapt [21], trimmed sequences shorter than 18 nt was discarded. The clean sequencing data were mapped to the mouse genome (release GRCm37.p1, from NCBI genome database) and Rfam database v11 (http://www.sanger.ac.uk/Software/Rfm/). Reads aligned in the genome, excluding those

matching tRNAs, rRNAs, snRNA, and snoRNAs, were used for further analysis. All known mature miRNAs and their precursors were retrieved from miRBase (version 21; http://www.mirbase.org).

miRNA identification and qualification

The remaining reads were used to predict novel miRNAs and do quantitative analysis through the miRDeep2 [22]. The frequency of miRNAs from different libraries was normalized by total clean reads of miRNAs in each sample. If the normalized read count of a given miRNA is zero, the expression value was modified to 1 for further analysis. The pairwise t-test was applied to filter differentially expressed miRNAs and mRNAs for the two groups. For each miRNA, reads number was normalized. False discovery rate (FDR)-adjusted P values (P, 0.05) and an absolute fold change of 1 were set as the cutoff values.

Hierarchical clustering

Hierarchical clustering was applied to both axes using the weighted pair-group method with centroid average as implemented in the program Cluster (M. Eisen; http://www.microarrays.org/software). The distance matrixes used were Pearson correlation for clustering the arrays and the inner product of vectors normalized to magnitude 1 for the genes (this is a slight variant of Pearson correlation; see Cluster manual available at http://www.microarrays.org/software/for computational details). The results were analyzed with Tree View (M. Eisen; http://www.microarrays.org/software) [23].

Validation of differentially expressed MiRNAs

Quantitative real time (qRT)-PCR was performed to confirm the differential expression of miRNAs identified by sequencing. Briefly, cDNA synthesis and qRT-PCR were performed using NCode™ VILO™ miRNA cDNA synthesis Kit and NCode™ EXPRESS SYBR® GreenER™ miRNA qRT-PCR Kits (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Each Reverse transcription reactions

Table 1. Statistical comparison of miRNA expression level in liver tissues of DB/DB mice compared with that of db-/db-H_2O mice (P < 0.05 denotes significant differences in expression)

miRNA	DB/DB vs db-/db- (FC log2)	p-value
mmu-let-7k	2.04	0.0325
mmu-let-7e-5p	1.85	0.0003
mmu-miR-320-3p	1.51	0.0002
mmu-miR-127-3p	1.42	0.0059
mmu-miR-1948-3p	1.39	0.0499
mmu-miR-485-5p	1.34	0.0066
mmu-miR-125a-5p	1.31	0.0363
mmu-miR-541-5p	1.23	0.0011
mmu-miR-423-5p	1.22	0.0012
mmu-miR-598-3p	1.12	0.0003
mmu-miR-382-3p	1.02	0.0023
mmu-miR-365-3p	-1.06	0.0490
mmu-miR-193a-5p	-1.17	0.0356
mmu-miR-802-5p	-1.29	0.0025
mmu-miR-802-3p	-1.38	0.0118
mmu-miR-383-5p	-1.45	0.0243
mmu-miR-676-3p	-1.76	0.0004
mmu-miR-676-5p	-2.12	0.0000
mmu-miR-34a-5p	-2.17	0.0002

Table 2. Statistical comparison of miRNA expression level in liver tissues of db-/db-H_2O mice compared with that of db-/db-AOE500 mice (P < 0.05 denotes statistically significant differences in expression)

miRNA	db-/db-H_2O vs db-/db-AOE500 (FC Log2)	p-value
mmu-miR-223-3p	1.33	0.0092
mmu-miR-184-3p	1.27	0.0305
mmu-let-7d-3p	-1.11	0.0113
mmu-miR-423-5p	-1.16	0.0010
mmu-miR-122-3p	-1.16	0.0061
mmu-miR-598-3p	-1.31	0.0154
mmu-miR-378d	-4.24	0.0001

should be comprised of 4.3 μL H_2O, 1 μL 10X Reverse-Transcription Buffer, 0.2 μL RNase-Inhibitor (20 U/μL), 1 μL 10 mM dNTPs with dTTP, 0.5 μL Multiscribe Reverse Transcriptase, 1 μL RT primer, 2 μL RNA. RT reactions using the following conditions: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, hold at 4°C. RT products are stored undiluted at -20°C prior to running the real-time PCR.

Real-time PCR reactions are performed in duplicate, in scaled-down (5 μL) reaction volumes using 2.5 μL TaqMan 2X Universal PCR Master Mix, 0.25 μL miRNA-specific primer/probe mix, and 2.25 μL diluted RT product per reaction. mmu-miR-423-5p Reverse primer: CTCAACTG-GTGTCGTGGAGTCGGCAATTCAGTTGAGAAAGT-CTC, mmu-miR-423-5p Forward primer: ACA-CTCCAGCTGGGTGAGGGGCAGAGAGCGA, mmu-miR-598-3p Reverse primer: CTCAACTGGTG-TCGTGGAGTCGGCAATTCAGTTGAGTAACGATG, mmu-miR-598-3p Forward primer: ACACTCCA-GCTGGGTACGTCATCGTCGTCA, U6 Forward primer: CTCGCTTCGGCAGCACA, U6 Reverse primer: AACGCTTCACGAATTTGCGT. Real-time PCR is carried out on an Applied BioSystems 7300 Real-Time PCR System (Applied Biosystems, Inc.) using the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by a hold at 4°C. Raw data can then be analyzed with SDS Relative Quantification Software version 2.2.3 (Applied BioSystems, Inc.), generally using the automatic cycle threshold (Ct) setting for assigning baseline and threshold for Ct determination. The Ct values for miRNAs were normalized against U6 RNA as an internal control and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Predication of the potential target miRNAs

There is no one algorithm that outperforms the others in terms of sensitivity and specificity. The potential miRNAs target genes were identified using each of miRanda, Sanger miRDB, RNAhybrid, and Targetscan, from the most commonly used prediction website (http://www.umm.uni-heidelberg.de/apps/zmf/mir-walk/predictedMiRNAs gene.html) [24]. Gene function was assigned based on Database for Annotation, Visualization and Integrated Discovery (DAVID).

Statistical analyses

The concentration of glucose, albumin and creatinine are presented as the mean ± standard deviation (SD). Data were analyzed by the Statistical Product and Service Solutions (SPSS) program (Version 16) (SAS Institute Inc., Cary, NC). Comparisons of multiple groups were done with ANOVA with corrections for multiple comparisons. Differences of P < 0.05 were considered statistically significant.
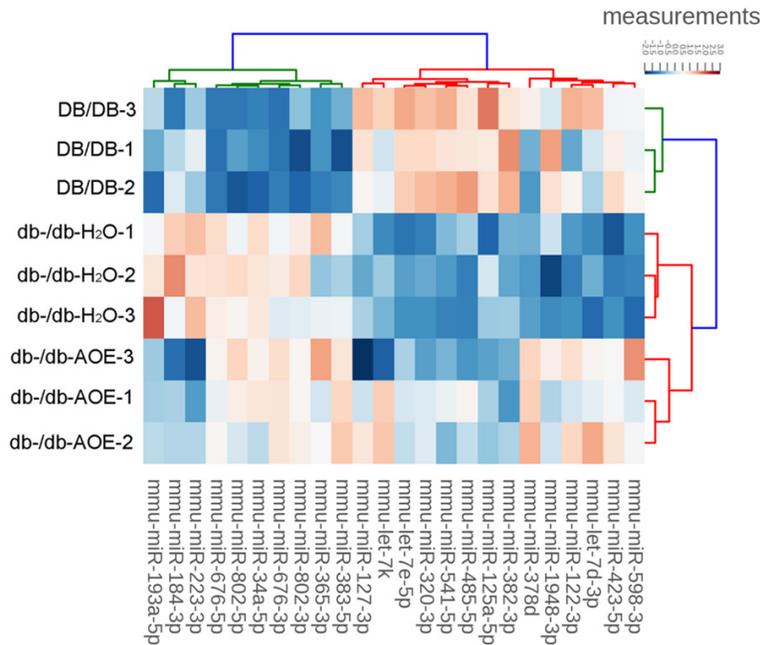
Figure 2. Hierarchical clustering analysis of miRNA expression in liver tissues from DB/DB and db-/db-H$_2$O mice treated or untreated with AOE. Liver tissue was clustered according to the expression profiles of 24 differentially expressed miRNAs among db-/db- mice, DB/DB mice and db-/db-H$_2$O mice treated and untreated with AOE. The analyzed samples are reported in columns and the miRNAs are presented in rows. The miRNA clustering tree is shown on the left, and the sample clustering tree appears at the top. The color scale shown at the top indicates the relative expression levels of miRNAs, with red representing a (relatively) high expression level and blue a (relatively) low expression level.
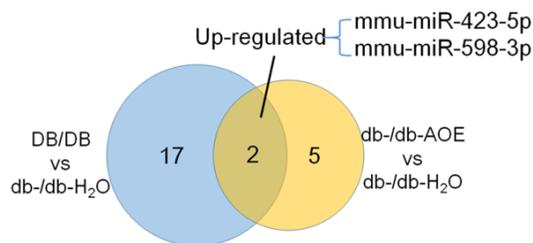

Figure 3. Venn diagrams showing the overlap between DB/DB group vs db-/db-H$_2$O group and db-/db-AOE group vs db-/db-H$_2$O group.

Results

Body weight gain after AOE administration for 8 weeks in DB/DB and db-/db-H$_2$O mice did not differ between the groups (data not shown). Plasma glucose decreased significantly (by 28%, P < 0.001) in db-/db-AOE500 group mice compared with db-/db-H$_2$O mice group (**Figure 1A**). In addition, the plasma concentrations of triglyceride and of cholesterol in db-/db-

AOE500 group mice decreased by 15.0% and 10%, respectively (**Figure 1B** and **1C**).

To detected miRNA expression patterns in mice treated with AOE, the deep Sequencing was performed. miRNA expression profiles among the treatment groups were compared by plotting log2-ratio figures and scatter plots. We identified 19 miRNAs that were differentially expressed in the livers of DB/DB mice compared with db-/db-H$_2$O mice (**Table 1**). In them, 8 miRNAs (mmu-miR-365-3p, mmu-miR-193a-5p, mmu-miR-802-5p, mmu-miR-802-3p, mmu-miR-383-5p, mmu-miR-676-3p, mmu-miR-676-5p, and mmu-miR-34a-5p) were down-regulated, while 11 miRNAs (mmu-let-7k; mmu-let-7e-5p, mmu-miR-320-3p, mmu-miR-127-3p, mmu-miR-1948-3p, mmu-miR-485-5p, mmu-miR-125a-5p, mmu-miR-541-5p, mmu-miR-423-5p, mmu-miR-598-3p, and mmu-miR-382-3p) were upregulated. 7 miRNAs that were differentially expressed in the livers of AOE500 treated db-/db- mice compared with untreated db-/db- mice (**Table 2**). In them, 5 miRNAs (mmu-let-7d-3p, mmu-miR-423-5p, mmu-miR-122-3p, mmu-miR-598-3p and mmu-miR-378d) were down-regulated, while 2 miRNAs (mmu-miR-223-3p and mmu-miR-184-3p) were upregulated. Moreover, two miRNAs (mmu-miR-423-5p and mmu-miR-598-3p) were identified in both DB/DB mice group vs db-/db-H$_2$O mice group and db-/db-AOE500 mice group vs db-/db-H$_2$O mice group.

The expression profiles of all identified miRNAs were used to perform hierarchical cluster analysis. The results showed that 9 miRNAs were either down- or up-regulated in both DB/DB mice group and db-/db-AOE500 mice group compared with the db-/db- mouse group. The results further revealed that the expression levels of 15 miRNAs showed similar regulation between the DB/DB mice group and db-/db-
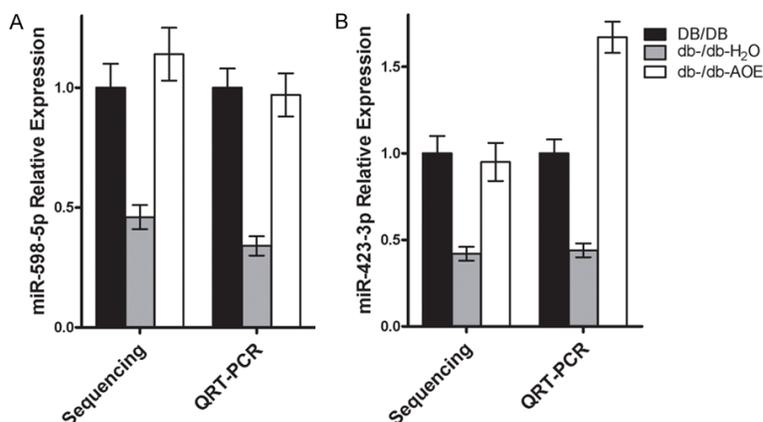
Figure 4. Quantitative real-time polymerase chain reaction (qRT-PCR) validation of the identified miRNAs. The expression of (A) miR-598-5p, (B) miR-423-3p in DB/DB groups (white column), db-/db-H$_2$O (gray column) and db-/db-AOE (black column) detected by QRT-PCR consist with sequencing. Data represent the mean ± SE, The experiment repeated three times.

myeloid leukemia, malaria, glycosphingolipid biosynthesis (globo series), and gastric acid secretion (**Table 3**).

Discussion

The present study aimed to understand the effect of dietary AOE supplementation on miRNA expression levels in the livers of diabetic (db-/db-) mice and to consequently determine the role of the implicated miRNAs in the observed therapeutic actions of AOE. To this end, we used miRNA deep-sequencing technology, qRT-PCR, and functional analyses to investigate miRNA expression and function in the db-/db- mouse liver.

Obesity is the most common cause of the insulin-resistant state. The increased fat stores that are a feature of obesity lead to aberrant lipid metabolism, inflammatory alterations in white adipose tissue and ectopic sites of fat deposition, all of which result in insulin resistance and irregularities in insulin signaling pathways [25]. In the present study, we observed that AOE treatment reduced plasma triglyceride and cholesterol levels as well as blood glucose levels. Additionally, hepatic triglyceride and cholesterol levels were decreased in db-/db-AOE500 mice group. These results suggest that AOE functions by altering lipid metabolism.

Liver abnormalities are associated with the deregulation of various normal metabolic processes. Aberrant miRNA expression patterns underlie the pathogenesis of liver disease. Therefore, we assessed the status of miRNA expression in db-/db- mice livers. Our results revealed that 19 miRNAs showed significant differential regulation in db-/db-AOE500 mice group compared to db-/db-H$_2$O mice group. In the former group, 8 miRNAs were found to bedown-regulated, while 11 found to be up-regulated compared with db-/db-H$_2$O mice group.

The status of hepatic miRNAs in db-/db- mice with a C57BL/6J background has been reported in a recent study [26, 27]. Several of the dif-

AOE500 mice groups, while compared with db-/db-H$_2$O mice group (**Figure 2**). In a permutation test, the overlap between the altered miRNAs expression levels between DB/DB mice group and AOE500-treated db-/db- mice was found to be significant.

After initial identification of AOE-regulated miRNAs by Expdiff and hierarchical clustering analysis, the 2 miRNAs identified in both DB/DB mice group and db-/db-AOE500 mice group compared with the db-/db-H$_2$O mouse group (**Figure 3**) were analyzed in order to predict their corresponding target genes, which were further subjected to functional enrichment analysis. Subsequent qRT-PCR validation of mmu-miR-423-5p and mmu-miR-598-3p confirmed the sequencing data (**Figure 4**).

The miRWalk database was used to predict the miRNA target genes. In total, 612 putative target genes were selected according to stringent standards (minimum seed length = 10, P < 0.01). To gain insights into the biological implications of the differential expression of miRNAs, DAVID tools were used to deter mine KEGG pathway enrichment analysis for the differentially expressed genes. The results suggested that the following pathways might be altered with changes in the expression of mmu-miR-423-5p and mmu-miR-598-3p alteration: regulation of actin cytoskeleton, lysosomes, MAPK signaling pathway, Ras signaling pathway, proteoglycans in cancer, bladder cancer, acute

Table 3. Biological pathways enriched by the differentially expressed miRNAs in the liver tissues of AOE-treated db-/db- mice

KEGG class	KEGG description	Odds ratio	P value
Cellular Processes	Regulation of actin cytoskeleton	2.05	0.0142
	Lysosome	2.25	0.0267
Environmental Information Processing	MAPK signaling pathway	2.12	0.0059
	Ras signaling pathway	1.76	0.0474
Human Diseases	Proteoglycans in cancer	2.17	0.0092
	Bladder cancer	3.34	0.0404
	Acute myeloid leukemia	2.74	0.0454
	Malaria	3.39	0.0219
Metabolism	Glycosphingolipid biosynthesis	10.37	0.0014
Organismal Systems	Gastric acid secretion	2.52	0.0413

ferentially expressed miRNAs that were identified in that study match with those identified in the present study, including mmu-miR-676, mmu-miR-34a-5p, and mmu-let-7e-5p. It has also been demonstrated that miR-34a levels were increased in steatosis-induced hepatocytes and in fatty liver tissue in mice, and miR-34a has been suggested to decrease lipid accumulation and the degree of steatosis [28]. It is thought that miR-34a promotes lipid accumulation in HepG2 cells [27]. Therefore, it is plausible that miR-34a may be therapeutic against NAFLD by regulating target PPARalpha and SIRT1 genes [29, 30]. The differentiation markers WT1, Pax2, and Wnt4 have been shown to be down-regulated when the let-7e miRNA is silenced, indicating that miR-let-7e regulates adipose-derived stem cell differentiation [30]. In the present study, miR-34a levels in db-/db-H$_2$O mice were higher while let-7e levels were lower than those in DB/DB mice.

Nevertheless, most of the differentially expressed miRNAs identified in the present study, namely: miR-320; miR-382; miR-485; miR-541; and miR-802, have also been reported to be differentially expressed in other models of diabetes or obesity. High-glucose exposure decreased the expression of miR-320, while transfection of miR-320 mimics partly restored the high-glucose response in human umbilical vein endothelial cells in a study reported by Feng *et al.* in 2012 [31]. In a separate study, miR-320 was found to regulate insulin resistance in adipocytes through insulin-PI3-K signaling pathways [32], and miR-382 has been implicated in the regulation of cholesterol homeostasis and inflammatory reactions [33]. Another of the

miRNAs identified in our study, miR-485, has been shown to modulate hypertriglyceridemia through post-transcriptional down-regulation of APOA5 [34]. Han *et al.* reported 3 novel genetic variations of miR-541 in type 1 diabetes, while the diabetes-associated gene neurogenin 3 (NGN-3) was found to be a target for miR-541 [35]. The expression of miR-802 was found to be up-regulated in T2DM patients and obese mice [36]. Further, Kornfeld reported that miR-802 is involved in glucose metabolism by targeting the *Hnf1b* gene and might control hepatic insulin sensitivity [37].

In addition to the identification of these differentially expressed miRNAs and to our above discussion of their possible biological significance, our results represent, to our knowledge the first demonstration of elevated levels of mmu-let-7k, mmu-miR-127-3p, mmu-miR-1948-3p, mmu-miR-125a-5p, mmu-miR-423-5p, mmu-miR-598-3p, and the first demonstration of inhibited levels of mmu-miR-365-3p, mmu-miR-193a-5p, mmu-miR-383-5p in the livers of db-/db-H$_2$O mice. Our study may therefore add to the current knowledge of the effects of miRNA expression on liver metabolism in diabetes.

Many of the identified miRNAs, namely, mmu-let-7d-3p, mmu-miR-423-5p, mmu-miR-122-3p, mmu-miR-598-3p, and mmu-miR-378d, were down-regulated in db-/db-AOE500 mice group. Other researchers have shown that the expression of let-7a and let-7d was increased in the skeletal muscle of T2DM patients, and it is known that these miRNA are direct translational repressors of the *IL-13* gene, which regulates

glucose uptake and metabolism [38]. The hepatocyte-specific miRNA miR-122 has been found to show higher expression levels in patients with NAFLD than in those without, and this has been related to liver steatosis and obesity [39, 40]. The miRNA miR-378 is also significant because it is highly induced during adipogenesis [41] and may be a novel target for controlling adipose tissue inflammation [42]. Mice over-expressing miR-378 exhibit insulin resistance and miR-378-knockout mice display hypoglycemia and hypertriglyceridemia with enhanced insulin sensitivity [43].

By contrast, in the present study, mmu-miR-223-3p and mmu-miR-184-3p were significantly up-regulated in the livers of db-/db-AOE500 mice. The miR-184, which is abundant in pancreatic beta cells, regulates insulin secretion by repressing the Slc25a22 gene [44, 45]. On the other hand, miR-223 is a crucial regulator of macrophage polarization and protects against diet-induced adipose tissue inflammatory responses and systemic insulin resistance [46]. The over-expression of miR-223 significantly attenuates lipid accumulation, which can be reversed by anti-miR-223 inhibitor transfection [47]. It has also been shown that miR-223 downregulates cytochrome $b5$ expression in the human liver, modulating the activities of P450 enzymes involved in fatty acid desaturation [48].

Interestingly, miR-598 and miR-423, whose expression in the present study was inhibited in db-/db-H$_2$O mice, were up-regulated after AOE treatment. Ortega *et al.* reported a marked decrease in levels of miR-423 in patients with morbid obesity and T2DM [49, 50]. Further, this miRNA has been implicated in angiogenesis and fibrosis in the vitreous body in patients with proliferative diabetic retinopathy [51]. In order to gain insight into the function of miR-598 and miR-423, KEGG pathway analyses were performed to predict their target genes. Genome annotation in KEGG annotation showed that genes involved in glycosphingolipid biosynthesis, lysosome cellular processes, the MAPK signaling pathway, and the Ras signaling pathway were significantly enriched. Inhibiting glycosphingolipid synthesis has been reported to improve insulin sensitivity in the Zucker diabetic fatty rat and to ameliorate hepatic steatosis in obese mice [52, 53]. Further, extracellular lysosome-associated membrane protein-1 re-

portedly mediates autoimmune disease progression in type 1 diabetes [54]. Luo *et al.* reported that adiponectin stimulates the proliferation of human osteoblasts and their differentiation via the MAPK signaling pathway [55]. Additionally, Kikkawa *et al.* demonstrated the important role of protein kinase C-MAPK activation in the development and progression of diabetic nephropathy, and suggested that PKC inhibitors and/or MAPK inhibitors might be useful therapeutics for treating diabetic nephropathy [56]. Lin *et al.* showed that Ras modulation of superoxide activates extracellular signal-regulated kinase-dependent fibronectin expression in diabetes-induced renal injuries [57]. Collectively, the results indicate that AOE may regulate miR-598 and miR-423 in T2DM by modulating the MAPK and Ras signaling pathways.

In conclusion, we identified 19 miRNAs in DB/DB mice and 7 miRNAs in AOE-treated db-/db- mice that were differentially expressed compared to db-/db-H$_2$O mice group. Most of the miRNAs identified in this study have previously been implicated in obesity or diabetes. In the case of 2 of the identified miRNAs (miR-598 and miR-423), their expression was inhibited in db-/db-H$_2$O mice but restored by AOE treatment. These miRNAs may be involved in several signaling pathways, including the MAPK and Ras signaling pathways. However, the target genes of these miRNAs need further experimental validation and the detailed functions of these genes in the process of AOE therapy need further investigation.

Disclosure of conflict of interest

None.

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