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ORIGINAL ARTICLE

Peripheral Blood miR-139 May Serve as a Biomarker for Metabolic Disorders by Targeting FoxO1 and FoxP1

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SUMMARY

Background: **The current study mainly evaluated whether peripheral blood miR-139 could be used as a biomarker to screen patients with metabolic disorders.**
Methods: **The peripheral blood was collected with patients with hyperglycemia and high triglycerides (TG) combined with high total cholesterol (TC) as well as healthy control. Real time PCR was carried out to determine the relative peripheral blood miR-139 level in patients with metabolic disorders and healthy individuals. Receiver operating characteristic curve (ROC) analysis and Spearman's correlation coefficient were carried out to evaluate the possible application of miR-139 as a potential biomarker for metabolic disorders. Dual luciferase reporter assay was performed to identify the possible target genes of miR-139.**
Results: **First, miR-139 was highly upregulated (19.9 ± 12.67) in the peripheral blood of hyperglycemia patients. Meanwhile, compared with healthy controls (1 ± 0.66), the level of miR-139 was much higher (50.28 ± 26.26) in the peripheral blood of high TG combined with TC patients. ROC analysis showed that the peripheral blood levels of miR-139 may be used to differentiate subjects with hyperglycemia or high TG and TC from healthy controls. Furthermore, peripheral blood miR-139 level positively correlated with serum glucose level ($r = 0.592$, $p < 0.001$) as well as total serum TG/TC levels ($r = 0.423$, $p < 0.001$). Dual luciferase reporter assay indicated that miR-139 significantly suppressed the relative luciferase activity of pmirGLO-FoxO1-3'UTR or pmirGLO-FoxP1-3'UTR.**
Conclusions: **In summary, enhanced circulating miR-139 level may be a potential biomarker for patients with metabolic disorders via suppressing the expression of FoxO1 and FoxP1.**
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KEY WORDS

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INTRODUCTION

Metabolic syndrome is characterized by a constellation of physiological, biochemical, clinical, and metabolic factors that results in the increased risk of atherosclerosis, type 2 diabetes, and all-cause mortality [1]. The pathogenesis of metabolic syndrome is still not fully elucidated. It is indicated that the interaction between obesity, insulin resistance, and inflammation exerts an important role in its development [2]. Among obese

people, the accumulation of free fatty acids in the liver, adipocytes, skeletal muscles, and the pancreas results in impaired insulin signaling and subsequent insulin resistance, which then increases glucose production in the liver [3]. Furthermore, hyperinsulinemia induces the transcription of genes related to lipogenesis in the liver, thereby increasing triglyceride (TG) production [4]. To maintain human physiology and health, it is especially important to properly control metabolic homeostasis. Recent studies have shown that there are complicated regulatory networks that are involved in the monitoring and response to changes in environmental conditions and physiological states [5-7]. Among the regulatory networks, microRNAs (miRNAs) have recently been shown to interact with downstream target genes in guiding metabolic homeostasis [8,9]. For instance, miR-27a has been shown to improve the progression of non-alcoholic fatty liver disease (NAFLD) by targeting fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD1) [8]. MiR-33a and b have been well established as key regulators in cholesterol and lipid metabolism via suppressing sterol regulatory element binding protein-1c (SREBP-1c) [9]. More importantly, emerging evidence has indicated that miRNAs can be secreted from cells and stably exist in the circulating system since they are sensitive to degradation by RNases [10,11]. Hence, circulating miRNAs are desirable candidates as both endocrine signaling molecules and disease markers.

In the present study, we mainly focused on miR-139, which was shown to be dysregulated in different cancers, including bladder cancer, osteosarcoma, and breast cancer [12-14]. However, whether circulating miR-139 was involved in the progression of metabolic disorders has been poorly understood. Here, for the first time, we showed that peripheral blood miR-139 level was increased in patients with metabolic syndrome. Receiver operating characteristic curve (ROC) analysis indicated that miR-139 could screen patients with high glucose, TG, and TC from healthy controls. Further study revealed that FoxO1 and FoxP1 were the target genes of miR-139. These findings indicate that circulating miR-139 could be promising non-invasive biomarkers to evaluate the progression of metabolic disorders.

MATERIALS AND METHODS

Patient samples

Peripheral blood from patients with hyperglycemia (fasting blood-glucose higher than 6.1 mM, n = 27), high TG (fasting blood TG higher than 1.7 mM) combined with high total cholesterol (TC, fasting blood TC higher than 6.0 mM) (n = 14) or healthy controls (n = 16) was collected at their annual physical examination at Beijing Hospital. The application for patient-derived materials was approved by the Research Ethics Committee of Beijing Hospital, and written consent was obtained from all of the patients. Physical examinations, biochemical measurements, and body mass index (BMI) calculations were listed in Table 1.

Sample acquisition and handling

A 5 mL aliquot of blood was collected from each participant directly into an anticoagulation tube containing ethylenediaminetetraacetic acid (EDTA).

RNA isolation

Total RNA was isolated with RNAVzol LS (Vigorous, Beijing, China) according to the specific instructions to isolate small RNAs. Quality, quantity, and integrity of RNA were monitored using a NanoDrop spectrophotometer (ND-1000, Nanodrop Technologies).

qPCR validation

RNA was reverse transcribed into cDNA using the Prime-Script one-step qRT-PCR kit (C28025-032, Invitrogen). Detailed qRT-PCR procedure was described as follows: 95°C for 10 minutes followed by 50 cycles of 95°C for 10 seconds, 55°C for 10 seconds, 72°C for 5 seconds; 99°C for 1 second; 59°C for 15 seconds; 95°C for 1 second; then cooling to 40°C. U6 was used as an internal control. The relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method and experiments were repeated in triplicate. The primers used in the current study were listed as follows:

miR-139-RT:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGACACTGG;
U6-RT:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGACAAATATG;
miR-139-F:
GCGCTCTACAGTGCACGTGTCT;
U6-F:
GCGCGTCGTGAAGCGTTC;
Universal reverse primer:
GTGCAGGGTCCGAGGT.

Cell culture

293 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM; Gibco) containing 10% heat-inactivated fetal calf serum (FCS; Gibco), streptomycin (100 mg/mL; Gibco, Grand Island, NY, USA), and penicillin (100 units/mL; Gibco) to a density of 2 x 10^3cells/cm^2 in flasks at 37°C with 5% CO_2.

MiRNA target prediction and dual-luciferase reporter assay

TargetScan (https://www.targetscan.org) was applied to determine the potential target gene of miR-139. The 3' untranslated region (3'UTR) of forkhead box O1 (FoxO1) and forkhead box P1 (FoxP1) containing the binding site of miR-139 was cloned into the pmirGLO plasmid. After 293 cells were seeded for 24 hours, miR-139 or scramble were cotransfected with blank pmirGLO or pmirGLO-FoxO1-3'UTR or pmirGLO-FoxP1-3'UTR using vigofect (Vigorous, Beijing, China) according to the instructions. The luciferase activity was analysed

Table 1. Biochemical index for patients and healthy controls.

Variable	Healthy Controls	Hyperglycemia	High TG and TC
Total subjects (n)	16	27	14
Gender (male/female)	7/9	12/15	7/7
Age (years)	54.7 ± 6.22	59.5 ± 4.06	57.3 ± 3.52
BMI (kg/㎡)	24.47 ± 2.48	26.80 ± 3.27 *	26.03 ± 4.06
GLU (mM)	4.90 ± 0.90	7.98 ± 1.22 ***	5.80 ± 0.88 **
HBA1C (%)	5.53 ± 0.25	6.26 ± 0.63 *	6.01 ± 0.39
AST (U/L)	17.35 ± 4.83	22.05 ± 10.58	18.90 ± 4.57 *
ALT (U/L)	12.58 ± 5.50	19.37 ± 11.58 **	14.27 ± 6.32 **
HDL-cholesterol (mM)	4.30 ± 0.60	1.42 ± 0.28 ***	1.63 ± 0.34 **
LDL-cholesterol (mM)	2.30 ± 0.33	3.22 ± 0.76 *	3.59 ± 0.22
Total cholesterol (mM)	4.30 ± 0.50	5.20 ± 0.76 ***	7.35 ± 1.27 ***
Triglycerides (mM)	0.85 ± 0.24	1.63 ± 1.03 ***	1.96 ± 2.38 ***

*** p < 0.05, ** p < 0.01, *** p < 0.001 vs. healthy controls.**

with the Dual-Luciferase Reporter Assay System (E1910; Promega).

Statistics

The data are represented as the mean ± standard error (SD). The two-tailed unpaired Student's *t*-tests were used for comparisons of two groups. ROC curves were used to assess miR-139 as a biomarker, and the area under the curve (AUC) was reported (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA). Spearman's correlation coefficient was used to test the correlation between the expression of miRNA-139 and clinical index (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA). $p < 0.05$ was considered significant.

RESULTS

Upregulation of miR-139 in the peripheral blood of patients with hyperglycemia and TG/TC

First, real time PCR was carried out to evaluate the peripheral blood level of miR-139 in patients with hyperglycemia or high TG/TC as well as controls. As shown in Figure 1A, the level of miR-139 was 1 ± 0.66 in the peripheral blood of healthy controls, but miR-139 was increased to 19.9 ± 12.67 in the peripheral blood of hyperglycemia patients. Furthermore, compared with miR-139 in healthy controls (1 ± 0.66), the level of miR-139 was much higher in the peripheral blood of high TG combined with TC patients (50.28 ± 26.26) (Figure 1B). These data indicated the abnormal upregulation of miR-139 in patients with metabolic disorders.

miR-139 could screen patients with metabolic disorders from healthy controls

We then evaluated whether miR-139 could be a potential biomarker for patients with metabolic disorders. ROC analysis showed that the peripheral blood levels of miR-139 may be used to differentiate subjects with hyperglycemia from healthy controls, with an ROC curve area of 0.942 (95% confidence interval: 0.858 - 1.000; $p < 0.001$) (Figure 2A). Meanwhile, the peripheral blood levels of miR-139 could be applied to differentiate subjects with high TG and TC from healthy controls, with a ROC curve area of 0.912 (95% confidence interval: 0.819 - 1.000; $p < 0.001$) (Figure 2B).

Peripheral blood miR-139 positively correlated with serum GLU and TG/TC levels

Furthermore, the correlation between peripheral blood miR-139 and serum GLU as well as TG/TC levels were analyzed. Our data showed that peripheral blood miR-139 level positively correlated with serum glucose level ($r = 0.592$, $p < 0.001$) (Figure 3A). Meanwhile, significant correlation between total serum TG/TC levels and peripheral blood miR-139 level was identified ($r = 0.423$, $p < 0.001$) (Figure 3B).

FoxO1 and FoxP1 were target genes of miR-139

Hence, we examined the possible target genes of miR-139. Based on TargetScan, two different conserved binding sites were identified in the 3'UTR of FoxO1 and FoxP1, which are well recognized as key regulators in glucose and lipid metabolism [15,16]. Then, the 3'UTR containing the binding sites of miR-139 was cloned into pmirGLO plasmid (Figure 4A and 4B). Dual luciferase reporter assay indicated that miR-139 significantly suppressed the relative luciferase activity of

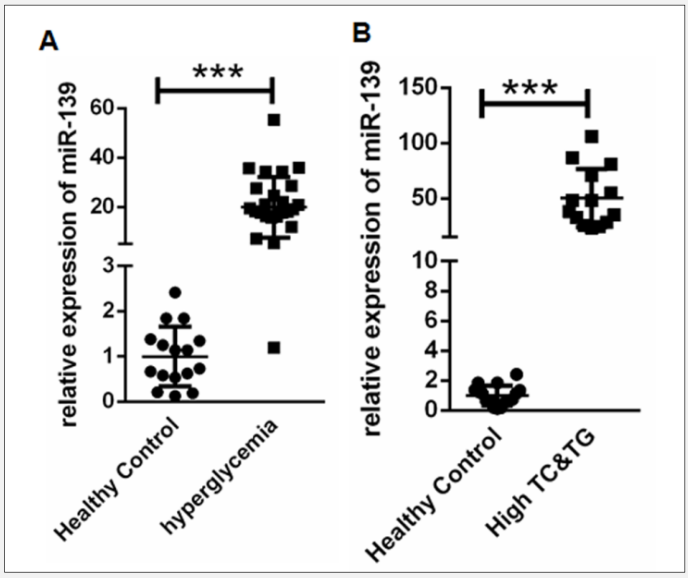

Figure 1. Peripheral blood miR-139 was increased in patients with metabolic disorders.

(A) miR-139 was highly upregulated in the peripheral blood of hyperglycemia patients. (B) Compared with miR-139 in healthy controls (1 ± 0.66), the level of miR-139 was much higher in the peripheral blood of high TG combined with TC patients. *** p < 0.001 vs. healthy controls.

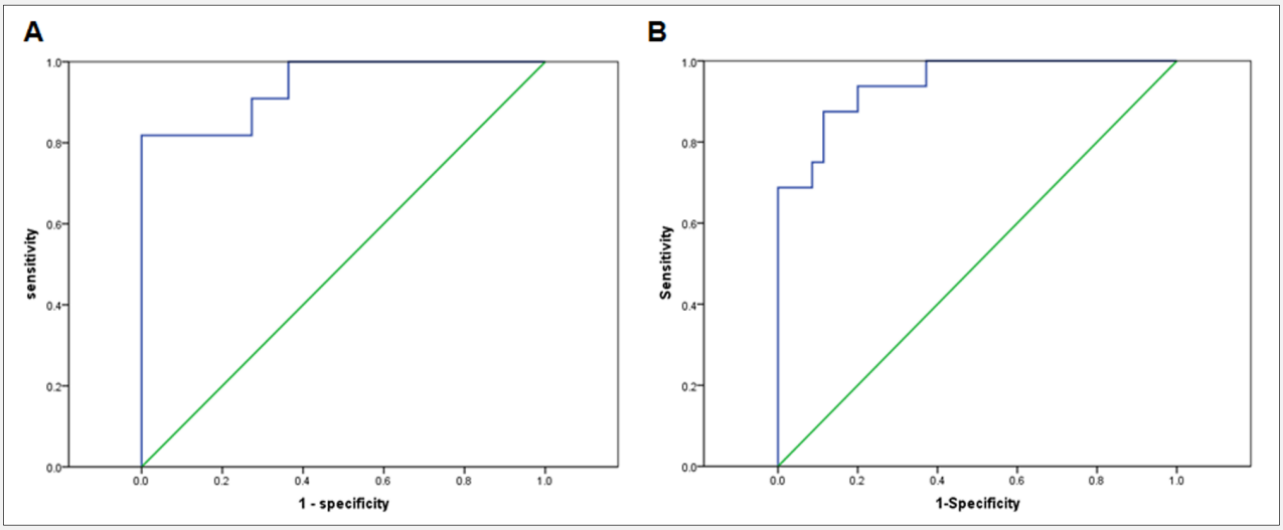

Figure 2. miR-139 could screen patients with metabolic disorders from healthy controls.

(A) ROC analysis showed that the peripheral blood levels of miR-139 may be used to differentiate subjects with hyperglycemia from healthy controls. (B) The peripheral blood levels of miR-139 could be applied to differentiate subjects with high TG and TC from healthy controls. *** p < 0.001 vs. healthy controls.

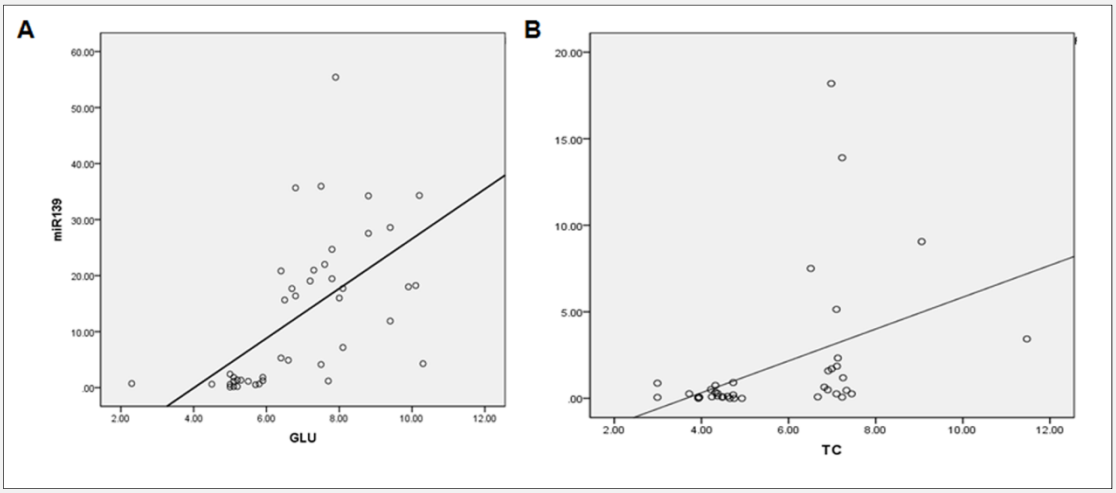

Figure 3. Peripheral blood miR-139 positively correlated with serum GLU and TG/TC levels.

(A) Peripheral blood miR-139 level positively correlated with serum glucose level (r = 0.592, p < 0.001). (B) Significant correlation between total serum TG/TC levels and peripheral blood miR-139 level was identified. *** p < 0.001 vs. healthy controls.

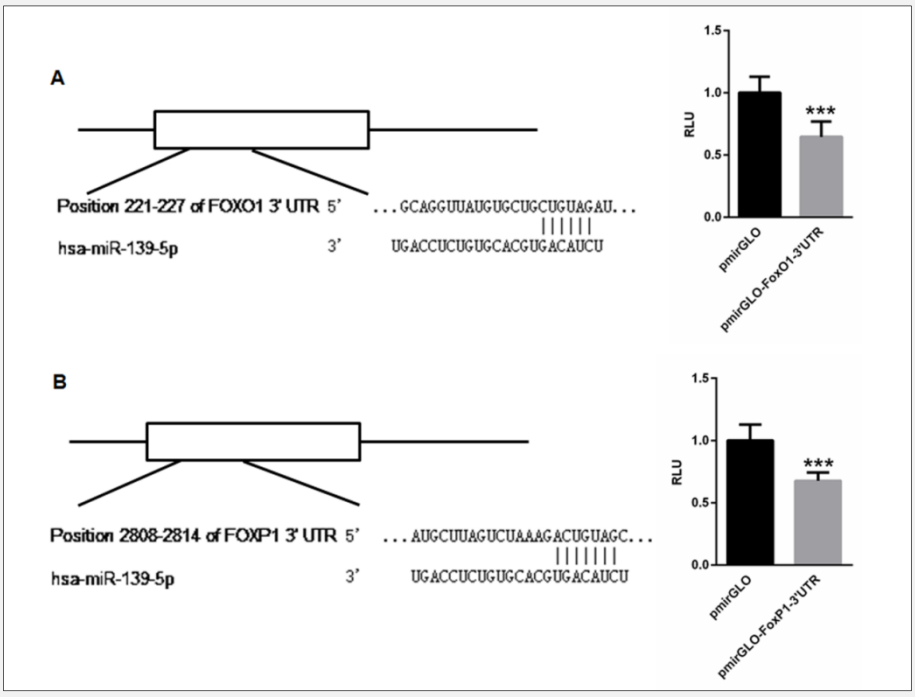

Figure 4. FoxO1 and FoxP1 were target genes of miR-139.

Dual luciferase reporter assay indicated that miR-139 significantly suppressed the relative luciferase activity of pmirGLO-FoxO1-3'UTR (A) or pmirGLO-FoxP1-3'UTR (B). *** p < 0.001 vs. blank pmirGLO plasmid.

pmirGLO-FoxO1-3'UTR or pmirGLO-FoxP1-3'UTR (Figure 4A and 4B). These data indicated that both FoxO1 and FoxP1 were target genes of miR-139.

DISCUSSION

At present, overweight and obesity are prevalent in developed and developing countries, which then leads to an increased risk of insulin resistance and type 2 diabetes mellitus [17,18]. Multiple studies have shown the abnormal expression of miRNAs in metabolically important organs among obese individuals [19,20]. These miRNAs act as mediators of disease via regulating various signaling pathways, including insulin signaling, inflammation response, adipogenesis, and lipid metabolism [19,20]. Hence, miRNA-based therapeutics may be a potential and innovative treatment modality.

Furthermore, the presence of miRNAs in human biofluids, including peripheral blood, serum, urine, and tears, has led to the pursuit of miRNA-based biomarkers for different diseases [21-23]. Peripheral blood miRNAs are characterized by high stability at room temperature [24]. In the current study, we evaluated whether miR-139 could be used as a biomarker for metabolic diseases. Compared with healthy controls, miR-139 demonstrated a statistically high upregulation in patients with hyperglycemia and high TG combined with TC. ROC analysis also showed that miR-139 could screen patients with metabolic disorders from healthy controls. Meanwhile, enhanced peripheral blood miR-139 positively correlated with serum glucose and TG/TC levels. These data indicated that peripheral blood miR-139 appears to be a biomarker candidate for metabolic disorders.

It is well known that miRNAs exert their role mainly through repressing various target genes [25,26]. Hence, we evaluated the possible target genes of miR-139. Interestingly, two important metabolic regulators, FoxO1 and FoxP1, were shown to have conserved binding sites for miR-139. FoxO1, a downstream mediator of insulin signaling, has been implicated in glucose and lipid homeostasis [27]. Studies have shown that the activity of FoxO1 is mediated by protein kinase B (Akt) and other kinases [28,29]. Meanwhile, FoxO1 is also shown to regulate hepatic lipid metabolism via integrating multiple pathways and suppressing genes related to lipid metabolism, such as sterol regulatory element binding protein 1c (SREBP-1c) and glucokinase [29,30]. FoxP1, a transcriptional repressor, is also shown to be a key mediator in the regulation of systemic glucose homeostasis [15]. In diabetic mice, FoxP1 is found to be physically interacted with FoxO1 thereby interfering expression of genes related to gluconeogenesis [15]. Here, we showed that both FoxO1 and FoxP1 were target genes of miR-139. Then, we proposed that enhanced circulating miR-139 level may lead to synergistic effects of glucose and lipid metabolism disorders in multiple organs via suppressing the expression of FoxO1 and FoxP1.

CONCLUSION

Compared with other therapies, miRNA-based therapies in the circulating system demonstrate some distinct advantages since they target multiple mRNAs in different organs, thereby exerting synergistic effects that are positive for therapy [31]. Thus, it is interesting to evaluate the antagomiR-based silencing of miR-139 in clinical application due to the significant upregulation of miR-139 in peripheral blood. However, there are still many challenges since unmodified miRNAs are rapidly degraded and off-target effects may exist. Hence, improved strategies for precise and efficient tissue-delivery of miRNAs are required.

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Declaration of Interest:
We declare no conflicts of interest.

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