

# miR-34a and miR-125b are upregulated in peripheral blood mononuclear cells from patients with type 2 diabetes mellitus

YANXIN SHEN<sup>1\*</sup>, HUILING XU<sup>2,3\*</sup>, XIAOYUAN PAN<sup>2,4\*</sup>, WEIJIANG WU<sup>2</sup>, HUI WANG<sup>2</sup>,  
LINLIN YAN<sup>2</sup>, MIAOMIAO ZHANG<sup>2</sup>, XIA LIU<sup>2</sup>, SHENG XIA<sup>2</sup> and QIXIANG SHAO<sup>2</sup>

<sup>1</sup>Department of Laboratory Medicine, The Second People's Hospital of Taicang, Taicang, Jiangsu 215400; <sup>2</sup>Department of Immunology, Jiangsu Key Laboratory of Medical Science and Laboratory Medicine, School of Medicine, Jiangsu University, Zhenjiang, Jiangsu 212013; <sup>3</sup>Department of Laboratory Medicine, Shanghai Gongli Hospital, The Second Military Medical University, Shanghai 200135; <sup>4</sup>Department of Laboratory Medicine, The People's Hospital of Taicang, Taicang, Jiangsu 215400, P.R. China

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**Abstract.** Type 2 diabetes mellitus (T2DM) is a leading cause of blindness, non-traumatic amputation and end-stage renal disease, as well as a major cardiovascular risk factor. To determine whether miR-125b and miR-34a serve an important role in the development of T2DM, the current study investigated the expression profile of two microRNAs (miR-34a and miR-125b) and their relative genes in peripheral blood mononuclear cells from 73 patients with T2DM and 52 healthy donors by reverse transcription-quantitative polymerase chain reaction. In addition, the association between miR-34a, miR-125b and their relevant genes expression profile were analyzed with respect to the pathogenesis of T2DM. The present study demonstrated that the expression levels of miR-125b and miR-34a were elevated in peripheral blood mononuclear cell samples from patients with T2DM. Furthermore, miR-34a and miR-125b were positively correlated with low-density lipoprotein/high-density lipoprotein (HDL) and Foxp3 and negatively related to triglyceride/HDL. However, no correlation among miR-34a, miR-125b and the value of homeostasis model assessment of insulin resistance, homeostasis model assessment of  $\beta$ -cell function and the genes of B lymphocyte-induced maturation protein-1, interferon regulatory factor-4, P53 and

retinoid-related orphan receptor  $\gamma$ t were observed. These results indicate that the alteration of miR-34a and miR-125b exists in patients with T2DM, which may be involved in the pathogenesis of T2DM, and could be a potential novel biomarker of T2DM.

## Introduction

Type 2 diabetes mellitus (T2DM), formerly termed non-insulin-dependent diabetes mellitus or adult-onset diabetes, is a multifactor disease that involves complex interactions between genes (1-3), abnormalities of the immune system (4-6), environmental factors (7-10) and health-impacting behavior (11,12), and represents a serious public health problem in numerous developed countries (13). Current investigations have revealed a definite global increase in the incidence and prevalence of diabetes. In 2013, 382 million people worldwide were estimated to be diabetic by the International Diabetes Federation, which is expected to rise to 592 million cases in the year 2035 (14). As a result of the increasing rate of diabetes and its widespread societal and economic consequences, prevention of diabetes among people at high risk is an important public health issue in clinic practice. However, the extent to which multiple defects in the regulation of lipids, insulin secretion and action, and the immune system contribute to the pathogenesis of T2DM has yet to be elucidated.

MicroRNAs (miRNAs) are a class of small, single-stranded non-coding RNAs (~22 nucleotides) that are transcribed from the DNA of a gene, and modulate the expression of a network of mRNAs through binding to the 3'-untranslated region (3'-UTR), 5'-UTR or to the open reading frame of target mRNAs (15). Notably, each miRNA is able to target multiple mRNAs. Growing evidence indicates that miRNAs are involved in T2DM (16). However, the specific role of miRNAs in T2DM has yet to be elucidated. Previous studies have demonstrated that miR-34a directly targets p53 and serves a crucial role in p53-mediated biological processes, such as cell cycle arrest, apoptosis and senescence (17), functioning downstream of the p53 pathway and participating in the initiation and progression of certain types of cancer (18). Previous

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*Correspondence to:* Dr Xia Liu or Professor Qixiang Shao, Department of Immunology, Jiangsu Key Laboratory of Medical Science and Laboratory Medicine, School of Medicine, Jiangsu University, 301 Xuefu Road, Zhenjiang, Jiangsu 212013, P.R. China  
E-mail: 1984lxia@163.com  
E-mail: shao\_qx@ujs.edu.cn

\*Contributed equally

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research has reported that p53 is involved in T cell development (19), which is of note as T cells mediate the immune response and inhibit autoimmune disease (20,21). miR-125b is predicted to be able to bind to B lymphocyte-induced maturation protein-1 (Blimp-1) and interferon regulatory factor-4 (IRF-4) transcription factors, which are essential for plasma cell differentiation (22). In CD4<sup>+</sup> T cells, Blimp-1 attenuates T helper (Th)1 differentiation, and represses the formation of follicular helper T cells (22). Furthermore, Villeneuve *et al* (23) found that miR-125b is upregulated in vascular smooth muscle cells cultured from *db/db* mice with diabetes and increases expression of inflammatory genes, monocyte chemoattractant protein-1 and interleukin (IL)-6 via targeting *Suv39h1* in *db/db* vascular smooth muscle cells. Thus, we speculate that miR-34a and miR-125b may contribute to the development of diabetes. In the present study, the expression levels of miR-34a, miR-125b and their relevant genes in peripheral blood mononuclear cells (PBMCs) were detected in samples obtained from T2DM patients. The results suggest that miRNAs may be used as biomarkers for the diagnosis or prognosis of T2DM.

## Materials and methods

**Patients and healthy blood donors.** In the present study, 73 patients with T2DM (age, 56.81±11.85 years), including 35 women (47.9%) and 38 men (52.1%), were recruited between the March and December 2012 from the Department of Laboratory Medicine at The Second People's Hospital of Taicang. Patients were diagnosed according to the criteria of the American Diabetes Association (24). Fifty-two healthy control subjects were recruited from age- and gender-matched healthy blood donors. Clinical parameters are summarized in Table I. All subjects provided written informed consent prior to enrollment in the study, and approval for this study was obtained from the Medical Ethics Committee of the Jiangsu University (Jiangsu, China).

**Lipid measurement.** Low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels were measured in the serum of patients with T2DM and healthy donors using the LDL-C reagent and HDL-C reagent, respectively (Weifang 3V Bioengineering Group Co., Ltd., Weifang, China). All lipids were measured using the Beckman Coulter AU5800 chemistry analyzer (Beckman Coulter, Inc., Brea, CA, USA).

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** PBMCs from 73 patients with T2DM and 52 healthy donors were freshly isolated by density-gradient centrifugation at 600 x g at room temperature for 20 min over Ficoll-Hypaque (Tianjin Haoyang Biological Manufacture Co., Ltd., Tianjin, China) media (1.077±0.001 g/ml). Total RNA was extracted from PBMCs using a TriPure Isolation Reagent kit (Roche Diagnostics, Shanghai, China) and quantified using a spectrophotometer at 260 and 280 nm. The integrity of total RNA was verified by analyzing ~1 µg RNA on 1% (w/v) formaldehyde denaturing agarose gel. The cDNA templates for each specific gene mRNA and miRNA were generated by reverse transcription (RT) of 1 µg and 250 ng of total RNA, respectively using a RevertAid™ First Strand cDNA Synthesis kits (Thermo Fisher

Table I. Clinical feature profile in T2DM and control subjects.

Parameter	T2DM (n=73)	Control (n=52)
Disease duration (years)	4.54±5.41	-
FBG (mM)	8.62±2.91	5.42±0.45
HbA1C (%)	8.50±2.09	5.82±1.07
TG (mmol/l)	3.29±1.53	2.66±0.59
TC (mmol/l)	3.67±2.28	3.23±1.12
HDL (mmol/l)	1.15±0.45	1.30±0.36
LDL (mmol/l)	2.77±1.01	2.53±0.84
TG/HDL	3.36±1.89	2.05±0.76
LDL/ HDL	0.13±0.06	1.95±0.68
HOMA-IR	3.05±2.28	-
HOMA-β	30.82±40.78	-

FBG, fast blood glucose; HbA1C, hemoglobin A1C; TC, serum total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HOMA-IR, insulin resistance index; HOMA-β, insulin secretion index.

Scientific, Inc., Waltham, MA, USA). Briefly, the RT reaction mixture contained 4.0 µl 5X RT buffer, 2 µl 10 mM of each dNTP, 1 µl 2 µM oligo (dT)18 or stem-loop RT primer, 1 µl 40 U/µl RNase inhibitor, 1 µl 200 U/µl ReverTra Ace reverse transcriptase and 1 µg or 250 ng total RNA, and diethylpyrocarbonate-treated water was added to provide a total volume of 20 µl. An Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) was used to perform the RT reaction under the following conditions: 42°C for 60 min, 70°C for 15 min, and finally, held at 4°C. RT-qPCR was performed using a Bio-Rad Real-Time thermal cycler CFX 96 [Bio-Rad Laboratories (Shanghai) Co., Ltd., Shanghai, China] with miScript SYBR Green PCR kit (Qiagen China Co., Ltd., Shanghai, China). In accordance with the manufacturer's instructions, the PCR reaction mixture contained 1 µl cDNA, 5 µl 2X SYBR Green Mix, 0.6 µl 10 µM primer mix and 3.4 µl nuclease-free water. The reaction protocol was as follows: 94°C for 10 min, 40 amplification cycles of degeneration at 95°C for 10 sec, annealing for 30 sec, and extension at 72°C for 30 sec. All reactions were run in triplicate. The gene names, primer sequences and the annealing temperature are listed in Table II. To account for possible differences in the amount of starting RNA, RT-qPCR data were represented by the quantification cycle (Cq) value. The relative expression level (i.e., fold change) for each gene or miRNA was calculated using the 2<sup>-ΔΔCq</sup> method (25). Relative mRNA expression levels of retinoid-related orphan receptor γt (RORγt), Blimp-1, IRF-4, P53 and Foxp3 was normalized against the housekeeping gene β-actin. The relative expression of miR-34a and miR-125b were normalized to U6 snRNA.

**Statistical analysis.** Statistical analysis was performed with Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Correlations between variables were determined by Pearson's correlation coefficient. Target genes expression levels of T2DM patients were compared with healthy donors using the Mann-Whitney U test. P<0.05 was considered to indicate a statistically significant difference.

Table II. Sequences of primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')	Cycles	Annealing (°C)
$\beta$ -actin (genebank #NM-001101)	Sense: CACGAACTACCTTCAACTCC Antisense: CATACTCCTGCTTGCTGATC	40	57
blimp-1 (genebank #NM-182907.2)	Sense: CCGGGACTCCTACGCTTACTT Antisense: CGTTGTACGAGGGGATGAAAG	40	60
ROR $\gamma$ t (genebank #NM-001001523.1)	Sense: CAAGAGAGGTTCTGGGCAAG Antisense: CTGCTCTTGTTGTGGAGAAGG	40	57
IRF-4 (genebank #NM-001195286.1)	Sense: CATCGACAAGCCGGACCC Antisense: CTCCGCTCAACCAGTTCCT	40	59
P53 (genebank #NM-001126818.1)	Sense: GGCCCACTTCACCGTACTAA Antisense: GTGGTTTCAAGGCCAGATGT	40	56
Foxp3 (genebank #NM-001114377.1)	Sense: CAAGAGAGGTTCTGGGCAAG Antisense: CTGCTCTTGTTGTGGAGAAGG	40	64
U6 (genebank #NM-001207056)	Sense: CTCGCTTCGGCAGCACA Antisense: AACGCTTCACGAATTTGCGT	40	60
miR-34a (genebank #MIMAT0000260)	RT: GTCGTATCCAGTGCAGGGTCCO GAGGTATTTCGCACTGGATACG ACAACAAC Sense: CGGTATCATTGTCAGTGTCT Antisense: GTGCAGGGTCCGAGGT	40	60
miR-125b (genebank #MIMAT0000680)	RT GTCGTATCCAGTGCAGGGTCC CGAGGTATTTCGCACTGGATA CGACTCACA Sense: GCCGTAAAGTGCTGACAGT Antisense: GTGCAGGGTCCGAGGTAT	40	60

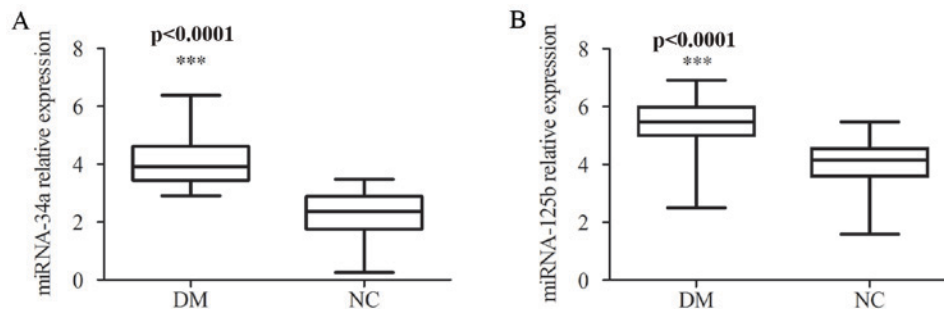


Figure 1. Relative expression levels of (A) miR-34a and (B) miR-125b in the PBMCs of patients with T2DM. PBMCs were isolated from subjects, and expression levels of miR-34a and miR-125b was assessed by RT-qPCR. Expression was normalized to the U6 reference gene. \*\*\* $P < 0.0001$ . PBMCs, peripheral blood mononuclear cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; T2DM, type 2 diabetes mellitus; DM, diabetes mellitus; NC, negative control.

## Results

*Higher expression of miR-34a and miR-125b in patients with T2DM.* Initially, RT-qPCR was employed to examine miR-34a and miR-125b expression levels in PBMCs obtained from 73 patients with T2DM and 52 healthy donors. The expression levels of miR-34a and miR-125b were significantly higher in the PBMCs of patients with T2DM compared with those of the PBMCs of healthy donors ( $P < 0.0001$ ; Fig. 1).

*Expression levels of IRF-4 and P53 were upregulated, whilst those of Blimp-1, ROR $\gamma$ t and Foxp3 were downregulated in*

*the PBMCs of patients with T2DM.* To further investigate the target or relevant genes of miR-34a and miR-125b in the pathological process underlying T2DM, the present study detected the expression levels of Blimp-1, IRF-4, P53, ROR $\gamma$ t and Foxp3, and found that the expression levels of IRF-4 and P53 in the PBMCs from patients with T2DM were higher compared with the PBMCs of healthy donors ( $P < 0.05$ ; Fig. 2), while Blimp-1, ROR $\gamma$ t and Foxp3 were lower compared with those of healthy donors ( $P < 0.05$ ).

*Association between the expression of miR-34a, miR-125b and the levels of Blimp-1, IRF-4, P53, ROR $\gamma$ t and Foxp3.*

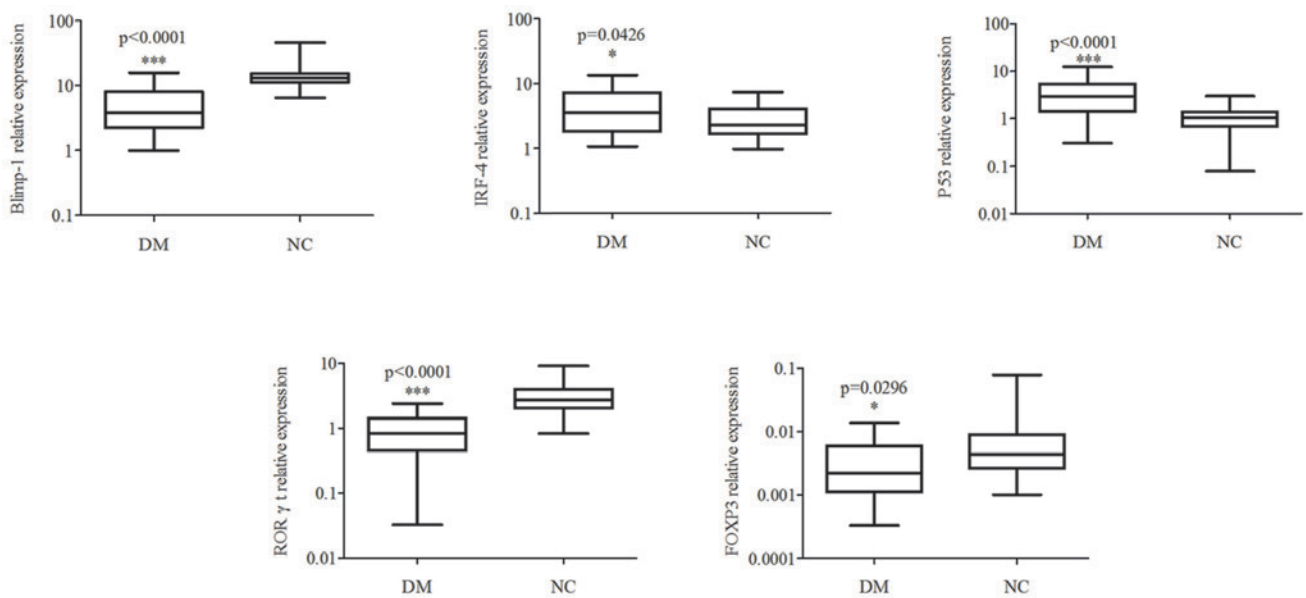


Figure 2. Relative expression levels of Blimp-1, IRF-4, P53, ROR $\gamma$ t and Foxp3 in PBMCs of T2DM patients. PBMCs were isolated from patients and the expression levels of Blimp-1, IRF-4, P53, ROR $\gamma$ t and Foxp3 were assessed by RT-qPCR. Expression was normalized to the housekeeping gene  $\beta$ -actin. \* $P < 0.05$  and \*\*\* $P < 0.001$ . IRF-4, interferon regulatory protein-4; ROR $\gamma$ t, retinoid-related orphan receptor  $\gamma$ t, PBMCs, peripheral blood mononuclear cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; T2DM, type 2 diabetes mellitus; DM, diabetes mellitus; NC, negative control.

Subsequently an analysis was performed to determine whether there was any correlation between miR-34a (Fig. 3A) and miR-125b (Fig. 3B) expression levels and Blimp-1, IRF-4, P53, ROR $\gamma$ t and Foxp3 genes in the PBMCs of patients with T2DM. The present study found that there was a significant positive correlation between the expression levels of miR-34a, miR-125b and the level of Foxp3 (Fig. 3;  $P < 0.05$ ). However, there was no significant correlation between miR-34a and miR-125b levels and Blimp-1, IRF-4, P53 or ROR $\gamma$ t (Fig. 3;  $P > 0.05$ ).

**Relationship between miR-34a and miR-125b levels and clinical features.** In addition, the correlation between miR-34a and miR-125b expression levels and clinical features was investigated. Positive correlations were identified between the expression levels of miR-34a, miR-125b and plasma (LDL/HDL) ratio, while the levels of miR-34a and miR-125b are negatively correlated with TG/HDL ratio (Fig. 4A;  $P < 0.05$ ). However, no correlation was observed between miR-34a, miR-125b and homeostasis model assessment of insulin resistance and homeostasis model assessment of  $\beta$ -cell function (Fig. 4B,  $P > 0.05$ ). The current results suggest that miR-34a and miR-125b may be involved in the pathogenesis of T2DM.

## Discussion

Recently, considerable progress has been made with regard to T2DM, which is a major health problem associated with excess morbidity and mortality. Growing evidence indicates that miRNAs are involved in the pathogenesis of T2DM, resulting in increased glucagon secretion and reduced incretin response (26-28). miRNAs have been implicated in the epigenetic regulation of key metabolic, inflammatory, and antiangiogenic pathways in T2DM. Zhang *et al* (29)

demonstrated that miR-34a could regulate mesangial proliferation and glomerular hypertrophy by directly inhibiting GAS1 in early diabetic nephropathy (DN). A further study has ascertained that miR-34a was able to inhibit osteosarcoma growth through the downregulation of *Eag1* expression (17). Recently, miR-34a has been shown to be one of the key mediators and downstream factors of p53 (30). It had been demonstrated that miR-34a is involved in pancreatic development, and can inhibit insulin secretion (31-33). Lovis *et al* reported that the expression level of miR-34a was higher in the islets of diabetic *db/db* mice, and was increased in the  $\beta$ -cell line, MIN6B1 and in pancreatic islets following prolonged exposure to saturated fatty acids (34). Kong *et al* found that miR-34a was significantly upregulated in the serum of patients with T2DM (35). An increase in miR-34a is associated with the activation of p53, and results in sensitization to apoptosis and impaired nutrient-induced secretion (34,36). The latter effect is associated with inhibition of the expression of vesicle-associated membrane protein 2, an important factor in  $\beta$ -cell exocytosis (34).

T2DM results from progressive  $\beta$ -cell dysfunction in the presence of chronic insulin resistance, leading to a progressive decline in plasma glucose control. Previous studies showed that the activation of T lymphocytes and cytokine-induced inflammatory responses serve a considerable role in the development of diabetes. The proinflammatory cytokines involved in  $\beta$ -cell damage are those secreted by CD4<sup>+</sup>T-cells (Th1 and Th17) and those by macrophages, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-1 $\beta$ , IFN- $\gamma$  and IL-17 (37-39). It also had been demonstrated that the Th1/Th2/Th17/T regulatory (Treg) paradigm skewed to Th1 and Th17 in patients with T2DM (5). Treg cells expressing Foxp3 have an anti-inflammatory role and maintain tolerance to self-components by contact-dependent suppression, or by releasing anti-inflammatory cytokines such as IL-10 and transforming growth factor- $\beta$ 1 (40), while



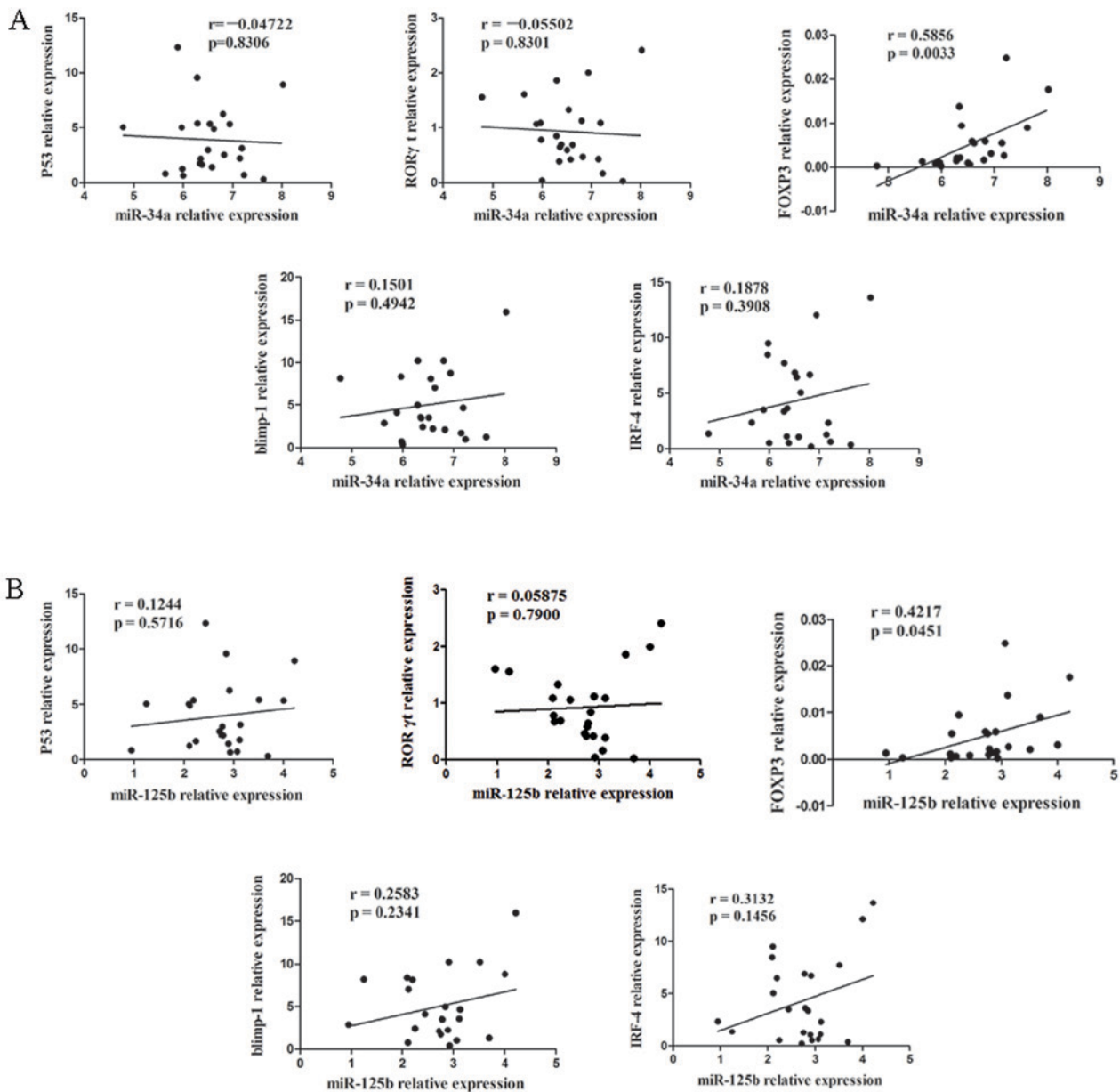


Figure 3. Association between the expression levels of (A) miR-34a or (B) miR-125b and the levels of Blimp-1, IRF-4, p53, ROR $\gamma$ t and Foxp3 in patients with T2DM. There was a positive correlation between the upregulation of miR-34a and miR-125b levels and genes of Foxp3 ( $P < 0.05$ ); There was no observed correlation between miR-34a and miR-125b with Blimp-1, IRF-4, P53 and ROR $\gamma$ t ( $P > 0.05$ ). IRF-4, interferon regulatory protein-4; ROR $\gamma$ t, retinoid-related orphan receptor  $\gamma$ t; PBMCs, peripheral blood mononuclear cells; T2DM, type 2 diabetes mellitus.

Th17 cells expressing ROR $\gamma$ t serve important roles in the development of autoimmunity, allergic reactions and in T2DM by producing IL-17, TNF- $\alpha$  and IL-6 (39,41). An imbalance of Th17/Treg is present in T2DM patients (4,5). Thus, it may contribute to the pathogenesis of inflammation and T2DM (42). miR-125b binds to the 3'-untranslated region of p53, Blimp-1 and IRF-4 messenger RNAs, and inhibits cell apoptosis by attenuating the expression of p53, or blocking B cell differentiation in germinal centers through decreasing the expression levels of Blimp-1 and IRF-4 (22,43). Blimp-1 is able to attenuate type 1 diabetes mellitus (T1DM) via suppressing the function of Th1 and Th17 (44). It has been demonstrated that the expression of Blimp-1 is required for the production of IL-10 by Treg cells that localize in mucosal sites, and for their tissue homeostasis. Furthermore, IRF-4 was essential for

Blimp-1 expression and for the differentiation of these Treg cells (45). Thus, we speculated that miR-125b may be involved in the pathological process underlying T2DM, and Blimp-1, IRF-4 and Foxp3 may also be downregulated in the disease.

In the present study, the expression levels of miR-125b and miR-34a were upregulated in PBMCs obtained from patients with T2DM. It had been demonstrated that miR-34a is the product of downstream of p53, and that p53 activates the expression of miR-34a, which subsequently attenuates E2F3 expression and induces cell apoptosis (46). In T2DM, miR-34a may contribute to  $\beta$  cell apoptosis and promote insulin resistance (33). Furthermore, miR-34a may be secreted in exosomes and migrate into PBMCs. miR-125b inhibits p53 (43), and prevents cells from undergoing apoptosis, and may serve an important role in vascular endothelial cell proliferation.

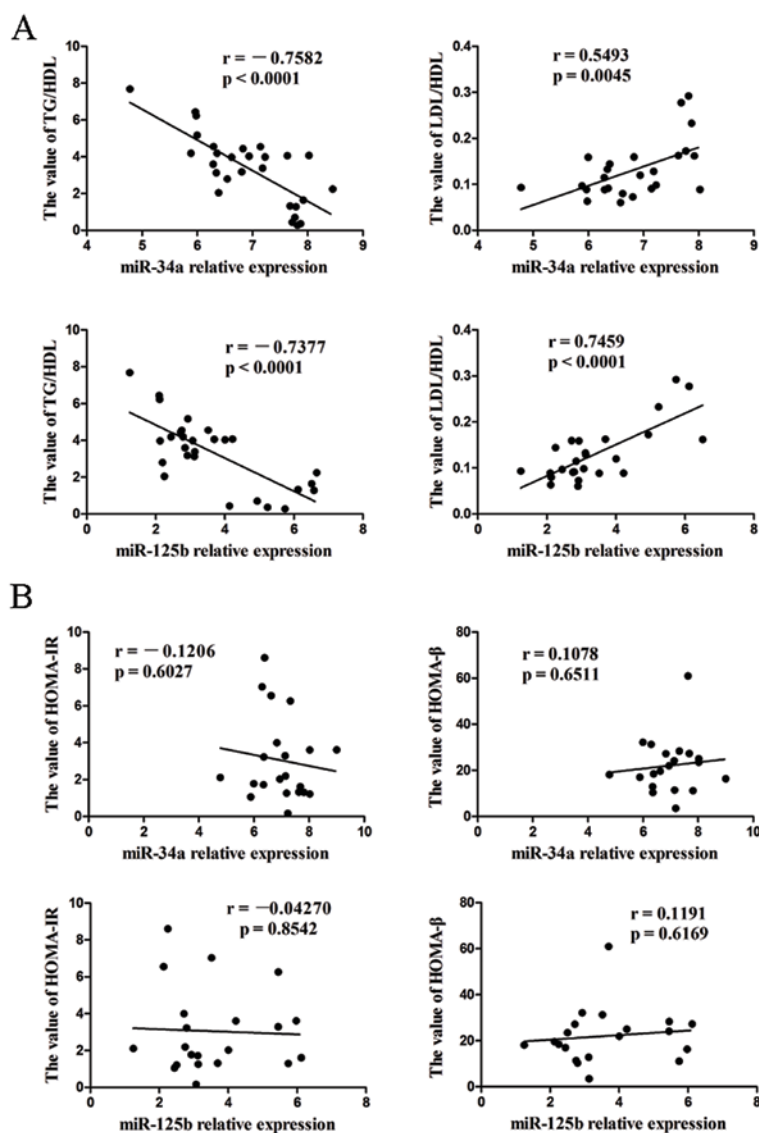


Figure 4. Association between the expression levels of miR-34a, miR-125b and the clinical feature profiles of patients with T2DM. (A) The levels of miR-34a and miR-125b are correlated to those of TG/HDL and LDL/HDL; (B) The levels of miR-34a and miR-125b are correlated to HOMA-IR and HOMA- $\beta$ . HOMA-IR, homeostasis model assessment of insulin resistance; HOMA- $\beta$ , homeostasis model assessment of  $\beta$ -cell function.

miR-125b is also able to transfer from vascular endothelial cells to T cells via exosomes. The migrated miR-125b inhibits the expression of Blimp-1 and IRF-4, which drives the conversion of Tregs and Th2 cells into Th17 cells (45). However, the expression levels of IRF-4 were observed to increase in the present study, which may be a result of the fact that IRF-4 is the critical transcript in IL-21-mediated induction, amplification, and stabilization of Th17 (47). In addition, it was observed that the expression of Foxp3 was not consistent with previous studies. It is probable that the expression of Foxp3 occurs in effector T cells as well as in Tregs. It has been reported that activated T cells can upregulate the expression of Foxp3 (48,49). There are several activated T cells, such as Th1, involved in PBMCs, which may indicate why the expression levels of Foxp3 are upregulated in PBMCs. However, it had been shown that the p53 negatively regulates autoimmune diseases via attenuating the STAT3-Th17 axis (21). In the present study, p53 was upregulated, and may be associated with  $\beta$  cell apoptosis.

In conclusion, the current investigation reveals that miR-34a and miR-125b are involved in T2DM. In addition, there was a positive correlation between the expression levels of miR-34a and miR-125b with LDL/HDL, and a negative correlation with TG/HDL. It has also been previously demonstrated that miR-34a was increased in a high-carbohydrate diet and is associated in the nonalcoholic fatty liver disease (50,51). The current findings may provide new insights into how miRNA is associated with the cholesterol metabolism of T2DM.

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