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Research paper

Dysregulated miR-103 and miR-143 expression in peripheral blood mononuclear cells from induced prediabetes and type 2 diabetes rats

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ABSTRACT

The progression from normal glucose tolerance (NGT) to type 2 diabetes (T2D) occurs through an intermediate state of glucose intolerance known as pre-diabetes. This transition is usually a gradual phenomenon that occurs over 5–10 years. Among the routinely practiced T2D screening criteria, like, FPG, IFG, IGT or HbA1c, still the issue of a preferable one is debated. The newly emerged microRNAs are created much hope to act as a class of suitable diabetes gene signature detectable at an early stage of the disease development. Although T2D related miRNA fluctuations are reported from the main insulin target organs, sampling of these organs for the sake of screening due to its invasive nature is not practicable. Peripheral blood mononuclear cells (PBMCs) are in constant touch with all body organs hence may exhibit the trace of miRNA changes which take place in insulin target organs.
In this study we have evaluated miR-103 and miR-143 expression in three groups of rats namely; normal control, high fat diet (HFD) which is corresponding to prediabetes state, and high fat diet/streptozotocin (STZ) induced T2D.
Quantitative real time PCR was used for profiling the selected miRNA expression at various time intervals of the three defined groups of rats. In prediabetes and overt diabetes stages, miR-103 showed significantly elevated expression in PBMC specimens compared to the normal healthy control group. Overexpression pattern of mir-143 was statistically significant in T2D compared to non-diabetic controls. However in HFD (prediabetic) rats also we observed an increasing pattern of miR-143 compared to the normal controls but it was not statistically significant.

1. Introduction

Diabetes is the most prevalent chronic metabolic disorder caused by defect in insulin production, secretion and insulin function. The number of people with type 2 diabetes mellitus (T2D) shows almost two fold increase globally during the past three decades. According to reports, approximately 366 million people were diagnosed with diabetes in 2011 and this is predicted to rise to 552 million by 2030 (Whiting et al., 2011). The global health expenditure on diabetes is expected to increase from 376 billion dollars in 2010 to 490 billions in 2030 (Abdul-Ghani and DeFronzo, 2009).

Insulin resistance, an inefficient respond of target tissues to concentration of circulating insulin, is an early event in the course of T2D development. Reduction in pancreatic insulin secretion, creates a state of relative insulin deficiency, resulting in hyperglycemia (Abdul-Ghani and DeFronzo, 2009; Muoio and Newgard, 2008). Elevated blood glucose level over time leads to serious multi organ damages such as kidney disease, retinopathy and blindness, and nervous and blood vessel damages (Schlienger, 2013). Furthermore diabetes becomes a global economic challenge because of its long term medical cares without a well defined cure. Asymptomatic early diagnosis of the disease is currently considered as the hallmark of T2D morbidity and mortality control. However, without a suitable set of biomarkers with desired level of sensitivity this goal would not be achievable. First-degree relatives (FDRs) of individuals with diabetes are at higher risk of developing glucose intolerance and diabetes, contributing to annual rise of affected cases. Having a reliable and sensitive biomarker at disposal could help in the timely diagnosis of at risk FDRs which certainly help in reduction of annual affected rate.

The causes of T2D consist of a very complex group of genetic and epigenetic system interaction and environmental effects (Romao and Roth,

Abbreviations: NGT, normal glucose tolerance; T2D, type 2 diabetes; FPG, fast plasma glucose; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; HbA1c, hemoglobin A1c; PBMCs, peripheral blood mononuclear cells; HFD, high fat diet; STZ, streptozotocin; FDRs, first-degree relatives; 3′UTR, 3′ untranslated region; NFD, normal fat diet; cDNA, DNA complementary to RNA; LNA, locked nucleic acid; qRT-PCR, quantitative reverse transcriptase PCR; OGTT, oral glucose tolerance test; Cav1, caveolin-1; ob/ob mice, leptin-deficient mice.

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2008). In recent years several studies have been conducted to identify the key components that play crucial role in the onset of diabetes and its progression. Gene expression and protein studies have been used to uncover the processes and impaired signaling pathways in the development of the disease, but the molecular mechanisms underlying the pathogenesis of diabetes still remain far from clear (Sanghera and Blackett, 2012; Billings and Florez, 2010; Gerich, 1998). The discovery of microRNAs as the post transcriptional regulators of many mammalian genes sheds new light on the development of insulin resistance and T2D (Pandey et al., 2009; Shantikumar et al., 2012; Bartel, 2004). MicroRNAs are a class of small endogenous noncoding RNAs approximately 19–22 nucleotides in length that have been conserved among vast array of species (Bartel, 2004; Kloosterman and Plasterk, 2006). They mediate posttranscriptional regulation of protein-coding genes by binding to the 3′ untranslated region (3′UTR) and sometimes to the 5′UTR or coding regions of target mRNAs (Xie et al., 2005). Their mode of action involves either by transcript degradation or translation inhibition depending on complete or partial match between microRNA's seed, a short segment of few critical nucleotides which is directing specific miR–mRNA binding, and its target site (Behm-Ansmant et al., 2006; Engels and Hutvagner, 2006). Since their discovery, microRNAs have become the focus of research and identified as key regulators of cellular physiological process and different biological functions such as differentiation, proliferation and apoptosis of cells, and hormone secretion (Kloosterman and Plasterk, 2006; Ambros, 2004; Aravin and Tuschl, 2005). mRNA must be translated into protein to exert a biological effect whereas, microRNAs can themselves act as an active element. In addition a single microRNA targets a cluster of functionally related mRNAs hence implies great impact on control of complex developmental or metabolic pathways (Bartel, 2009). Therefore in multifactorial disorders, single gene expression profiling reflects an individual segment of a broad network, whether single microRNA, due to its multiple action on a cohort of related mRNAs, and provides more specific and conclusive signature (van Iterson et al., 2013; Selbach et al., 2008). Deregulation of microRNA expression has been implicated in many diseases including multifactorial complex diseases like cancers, neurodegeneration and cardiovascular diseases (Ha, 2011; Lu et al., 2008). Recent reports indicate that microRNAs also can play an important role in the establishment of diabetes through the impairment of insulin sensitivity, insulin production, insulin secretion and development of obesity (Poy et al., 2004; Tang et al., 2009; He et al., 2007; Ling et al., 2009; Rottiers et al., 2011; Zhao et al., 2009).

Available reports are well supporting this idea that microRNA expression profile in diabetes-related tissues, like the pancreas, liver, skeletal muscles and adipose tissues, could serve as a sensitive marker for early assessment of disease onset (Herrera et al., 2010; Esguerra et al., 2011). However, the main obstacle in utilizing this sort of early diagnostic/screening markers is the harsh and invasive nature of sampling which is accomplish through biopsy procedure. In recent years free microRNAs in circulatory systems or PBMCs gained much importance for pre-symptomatic non-invasive screening purposes [(Hoekstra et al., 2010; Keller et al., 2009; Zeng et al., 2013; Frampton et al., 2013) 46–49]. Blood has been proposed as a 'sentinel tissue' being in continuous contact with various organs, molecular profiling of circulating blood transcriptome might reflect physiological and pathological events occurring in different tissues of the body (Mohr and Liew, 2007). It is believed that secretory microRNAs are acting as a versatile communication tool delivered to another cells and can be functional in recipient cells as well as incipient cells (Iguchi et al., 2010; Simons and Raposo, 2009; Valadi et al., 2007).

To evaluate the microRNA expression modification in PBMCs of diabetes cases as a potential non-invasive early diagnosis biomarker, we performed a careful in silico functional analysis. On the basis of a certain criterion like involvement in multiple stages leading to insulin secretion impairment, insulin targeting and resistance, adipocyte development and obesity (John et al., 2004; Lewis et al., 2005; Dweep et al., 2011), two microRNAs, miR-143 and miR-103, were short listed for this study. Changing profile of these two microRNAs was evaluated in PBMCs among normal (NFD), pre-diabetic (HFD) and high fat diet/streptozotocin induced type 2 diabetes rat models. Using animal model in this study provided us with an opportunity to control all variables including, lipid profile, glycemic control, medication used, and so on, which might influence the outcome of the research.

2. Material and method

2.1. Animal care and sample collection

Male Wistar rats, aged 6 weeks (n = 16; body weight 150 ± 5 g) were used for this study. The animals were handled according to the guidelines of the Isfahan University of Medical Sciences. All animals were housed in standard cages (three rats in each one) and maintained under controlled room temperature and humidity with 12/12-hour light–dark cycle and provided with commercially available rat normal diet (ND). T2D was induced according to protocols of Reed et al., Srinivasan et al. and Yuan Wang et al. with slight modifications (Reed et al., 2000; Srinivasan et al., 2005; Wang et al., 2003). After one week of adaptation, sixteen rats were randomly divided in two equal groups. One group was fed with normal fat diet (NFD) comprised of 66% carbohydrates, 22% protein and 12% fats for 10 weeks. Another group was fed with high fat diet (HFD), consisting of 15% carbohydrates, 25% protein and 60% fat (Wang et al., 2003). After 10 weeks, rats were weighted and, fasting plasma glucose and serum insulin, cholesterol and TG levels were recorded using their blood samples. This was done to confirm the development of prediabetic state in HFD rats. Subsequently all eight rats on HFD that were now converted into prediabetic were received intraperitoneal injection of low dose (35 mg/kg) of streptozotocin (STZ) prepared in citrate buffer (pH 4.4) after an overnight fast. Animals in normal control group (n = 8) were injected with vehicle buffer only. Both groups of animals were continued on their own specific diet (NFD or HFD) for another two weeks. FBG and NFBG of rats were daily measured. Using prolonged high fat diet to induce insulin resistance and a subsequent low dose of STZ to induce hyperglycemia is exactly comparable to the natural course of T2D development in human beings.

2.2. PBMC isolation

2.5 mL of peripheral blood was collected in EDTA tubes from NFD and HFD rats, as well as T2D induced rats (STZ injection of HFD rats). Ficol gradient density (Ficoll–Hypaque Sigma, St. Louis, MO) was used for the isolation of PBMCs according to the standard protocol. Finally we subjected isolated PBMCs from all three groups of rats, i.e. NFD, HFD and T2D to RNA isolation.

2.3. Total RNA extraction

Total RNA was extracted using the TRIZOL reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions with some modifications. Briefly 1 ml TRIZOL reagent, and 1μl UniSp6 spike-in were added to cell samples. After 5 min room temperature incubation, 200 ml chloroform was added and incubated at 4 °C for 3 min, vortexed for 5 s and then centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant containing RNA was transferred into a new tube, 500 ml isopropanol was added and incubated at −80 °C for 1 h, the mixture was centrifuged at 12,000rpm for 15 min at 4 °C again, supernatant was discarded and the RNA pellet was washed twice in 75% ethanol, air-dried and then dissolved in 25 μl RNase-free water and stored at −80 °C. The purity of isolated RNA was determined by OD260/280 using a Nano drop (ND-1000 Thermo Scientific, Worcester, MA), and the quality was examined by 1% agarose gel electrophoresis.

2.4. cDNA synthesis

All microRNAs were converted into poly adenylated molecules and reverse transcribed into cDNA in a single reaction step using Exiqon's miRCURY LNA™ cDNA Synthesis Kit (Cat# 203300). A poly-A tail was added to the mature microRNA template, cDNA synthesized using a poly-T primer with a 3′ degenerate anchor and a 5′ universal tag. The cDNA template was then amplified using microRNA-specific and LNA™-enhanced forward and reverse primers. Equal quantities of purified RNA (200 ng) were used in a 20 μL cDNA reaction (normalization by amount). Expression of microRNA was assessed by one-step SYBR Green I relative real-time PCR using the Exiqon SYBR Green Master Mix (Cat# 203450). PCR reaction mixture (10 μl) which included 4 μl reverse transcript products (1:10 dilution), was set to amplify RT product using specific LNA™ qPCR primer sets for miR-103a-2 and miR-143. PCR was performed using step one plus Applied Biosystems: a 10 min preincubation at 95 °C, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. All samples were run in duplicate. Data were analyzed using the comparative ΔΔct method. To normalize experimental qRT-PCR data the unisp6 RNA spike-in was used.

2.5. Serum metabolites measurement

Serum samples were prepared from the blood obtained from the tail of all three groups of rats, i.e. NFD, HFD and T2D. Blood glucose level was measured using a portable glucometer (EmpEror, manufactured by ISOTECH CO., LTD), and serum insulin level (Mercodia Rat Insulin ELISA kit Cat#10-1250-01), triglyceride, total cholesterol and LDL (Crystal Chem Rat LDL-Cholesterol Assay Kit cat# 80096, USA) were measured.

2.5.1. Oral glucose tolerance test (OGTT)

To assess oral glucose tolerance, animals were fasted overnight (12 h) then orally administered (by gavage) with solution of 20% glucose (2 g/kg). Blood glucose concentration was subsequently measured at 0 (just prior to oral gavage), 30, 60, 90 and 120 min after administration of glucose.

2.5.2. Statistical analysis

All data are presented as mean ± SEM. Differences between groups were analyzed using Student's paired t test or one-way ANOVA with post hoc multiple comparisons. Statistical analysis was performed using SPSS version 20 and statistical significance was defined as $P \leq 0.05$.

3. Results

3.1. Induction of T2D in male Wistar rats

Rats were kept on high fat diet (HFD) for ten weeks. Compared to the control rats which were fed normal fat diet (NFD) for the same period of time, HFD rats weighed approximately 20 g more, with higher serum levels of triglycerides and fasting blood glucose (5.83 mmol/l).

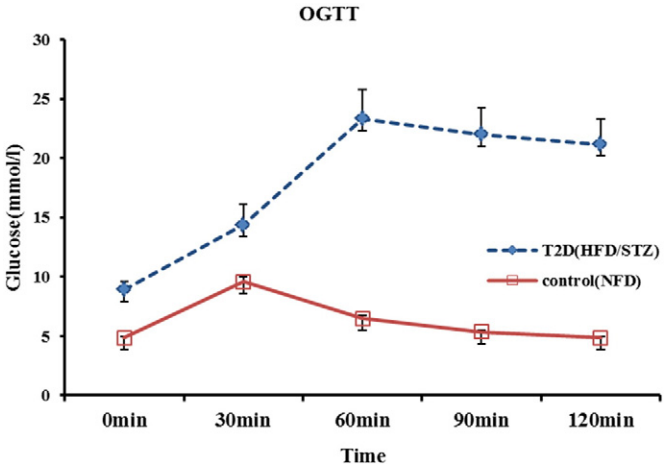

Fig. 1. Oral glucose tolerance test (OGTT) in NFD and HFD/STZ groups. Data presented as mean ± SEM with n = 8 for each group. $P \leq 0.05$ considered as statistically significant.

However, the serum cholesterol and LDL remained at comparable level with no significant differences between the two groups (Table 1). Fig. 1 shows the serum glucose levels after OGTT. Rats on NFD showed a peak increase in their serum glucose concentration at 30 min (9.5 mmol/L) after glucose intake but dropped quickly to normal level within 2 h (5 mmol/L). Conversely, serum glucose concentration of HFD rats increased rapidly and remained at high level even at 2 h after glucose intake (21 mmol/L). High fat fed animals treated with STZ showed impair glucose tolerance following a glucose challenge. Elevated blood glucose was observed obviously in HFD/STZ group. FBG of HFD/STZ animals were significantly higher compared to the NFD and HFD groups; the level was between 9.5 and 11 mmol/l and the mean of NFBG was ≥19 mmol/l.

3.2. miR-103 and miR-143 expression in peripheral blood mononuclear cells

All assays of the samples from the 8-NFD rats, 8-rats in pre-diabetes (HFD) and overt diabetes stages were successfully performed in the PBMC specimens. Both microRNAs were detectable in PBMC samples from three groups; as shown in Fig. 2A, *miR-143* expression in the PBMC specimens was significantly elevated in T2D rats compared to NFD rats (P value = 0.005), while expression level between HFD rats and healthy rats was not statistically significant (P value = 0.37). The expression pattern of *miR-143* in HFD rats was lower than T2D but the difference was not significant (P value = 0.37). *miR-103* expression was significantly elevated in PBMC samples of pre-diabetes (HFD) subjects and T2D rats compared with the normal group; P value = 0.024 and P value = 0.01, respectively (Fig. 2B). No significant difference was found between *miR-103* levels in PBMC samples of the pre-diabetes (HFD) and T2D groups (P value = 0.25) (Fig. 2B).

Table 1
Serum metabolites of rats fed on high fat diet and normal fat diet.

	Before diet intervention (just after dividing rats into two groups)		After diet intervention (ten weeks later)	
	Control (NFD)	HFD	Control (NFD)	HFD (prediabetic)
Weight (g)	145.75 ± 1.99	144.75 ± 3.06	191.6 ± 6.51	*223.5 ± 3.2
TC (mmol/l)	1.5 ± 0.13	1.51 ± 0.08	1.53 ± 0.13	1.55 ± 0.09
TG (mmol/l)	1.23 ± 0.16	0.88 ± 0.08	1.18 ± 0.10	*2.14 ± 0.29
LDL (mmol/l)	0.9 ± 0.34	0.9 ± 0.36	0.91 ± 0.33	0.88 ± 3.7
FBG (mmol/l)	4.91 ± 0.28	5.05 ± 0.17	5.34 ± 0.43	*5.83 ± 0.10
Insulin (μg/l)	1.4 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	0.5 ± 0.03

NFD, normal diet healthy controls; HFD, high fat diet; TC, total cholesterol; LDL, low-density lipoprotein cholesterol; FBG, fasting blood glucose; Values are mean ± SEM with n = 8 for each group, n = 6 for insulin. Significant differences are tested at $P \leq 0.05$ significance, denoted by*.

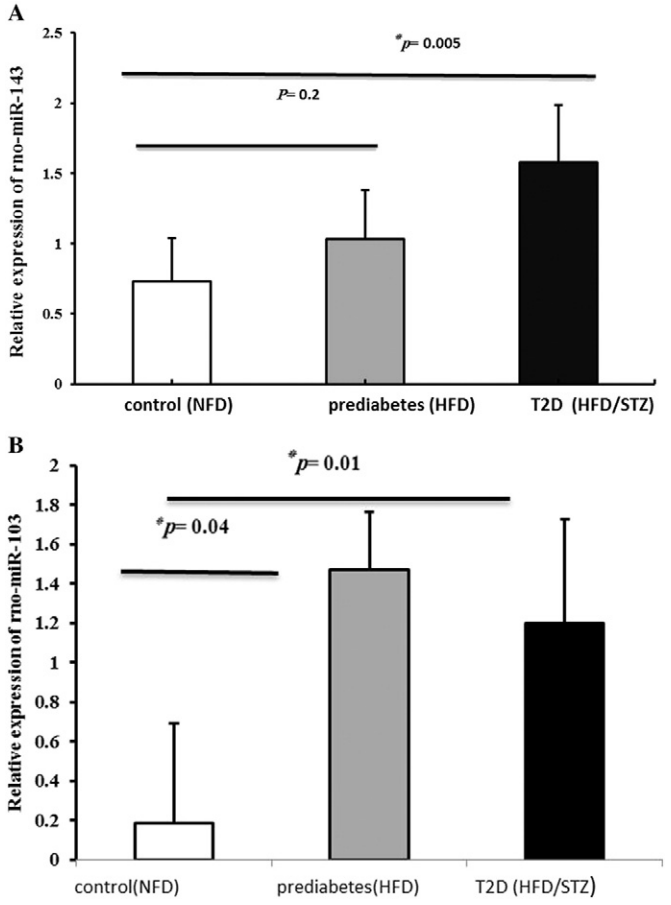

Fig. 2. Quantitative real-time RT-PCR analysis of the two microRNAs in the PBMCs from three groups (NFD, n = 8; pre-diabetes, n = 8; T2D, n = 8) shown as the mean ± SEM in log2 scale. Expression levels of *miR-103* and *miR-143* were significantly higher in T2D rats. *miR-103* also showed significantly elevated expression in pre-diabetes (HFD). Expression was measured in duplicate, and P values were generated by one-way ANOVA *P < 0.05.

4. Discussion

Identification of the primary molecular defects contributing to the pathophysiology of T2D is an important scientific and clinical goal (Jin et al., 2011; Herder et al., 2011; Lyssenko et al., 2008). There are a number of tests now in use for T2D but still the selection of any one as a gold standard is highly debated. Tests like fasting plasma glucose (FPG), oral glucose tolerance test (OGTT), and HbA1c for diabetes detection and prediction are suffering from serious limitations like costs, inconveniency, poor reproducibility, required long time to perform, and considerable intra-individual variation. Similarly, T2D remains undiagnosed for many years because hyperglycemia is usually not severe enough to provoke noticeable symptoms, unlike T1D which often presents with keto-acidosis requiring admission to hospital for correction and initiation of treatment. But hyperglycemia can cause significant pathological and functional changes, which can cause organ damage before the diagnosis of T2D is made. So all diagnostic/screening procedures that function on the basis of high glycemia remain unsuitable for early diagnosis.

Previous studies have demonstrated association between the aberrant microRNA expression and T2D development. The proposed involvement of these small molecules in T2D opens up new windows to explore and highlight some candidate biomarkers in T2D. These studies have mainly focused on microRNA expression in T2D related tissues such as the liver, adipose tissue, pancreas and skeletal muscles (Herrera et al., 2009, 2010); however, it is difficult to sample these tissues for the purpose of genetic diagnosis. There are increasing evidence that microRNAs are not only found in tissues but also in the blood of human and other animals both as freely circulating and within mononuclear cells (Chen et al., 2008; Hunter et al., 2008). Existence of some sort of intra-organs communications through exchange of microRNAs as well as continuous shedding of nucleic acids, including microRNAs from various organs into the circulatory system, specifically from those with impaired physiological status, represents a suitable non-invasive source equivalent to the main organ with primary deregulated expression profile (Iguchi et al., 2010; Simons and Raposo, 2009; Valadi et al., 2007). The findings indicate that exosomal secretory microRNAs as a silence master of gene expression can make multi translation inhibitory leading to the creation of specific biological events (Kosaka et al., 2010). Peripheral blood mononuclear cells (PBMCs) circulating throughout the body maintain close contact with various body organs and may be considered as an appropriate source for disease related microRNA expression signature. This has led to the development of novel methods to probe an individual's health status by identifying and monitoring blood circulating microRNAs (Hoekstra et al., 2010; Keller et al., 2009; Frampton et al., 2013). Zampetaki et al. provided the first evidence of plasma microRNA deregulation in patients with T2D (Zampetaki et al., 2010). Circulating diabetes-related microRNA differential expression patterns have been demonstrated in serum and whole blood of T2D patients as well as T2D animal models. The results were in concordance to those from the pancreas and the insulin-target tissues (Karolina et al., 2011; Kong et al., 2011). Relatively less information is available on microRNA expression patterns of blood cells derived from T2D patient (Collares et al., 2013; Corral-Fernandez et al., 2013).

For the present study, based on publications and in silico evaluations and available information from appropriate data bases like TARGETSCAN (www.targetscan.org), miRWalk (www.umm.uni-heidelberg.de/apps/zmf/mirwalk/micrornapredictedtarget.html), miranda (www.microrna.org) and miRDB (http://mirdb.org/miRDB), miR-103 and miR143 as two important key regulators of multiple genes in insulin signaling pathway and pancreatic function in insulin production and secretion were selected. The expression patterns of these miRNAs in PBMCs of control (NFD), pre-diabetes (HFD) and T2D were evaluated in a rat model of induced T2DM. Sabine et al. showed increased level of hepatic *miR-143* expression and its effectiveness in hepatic insulin action and systemic glucose homeostasis in two different obesity mouse models. They described a central role for *miR-143* as an integrator of metabolic signaling. This upregulation has been documented later in the liver, heart and pancreas of db/db mice. Induced overexpression of *miR-143* impairs glucose metabolism via affecting *AKT* activation and hence triggering insulin resistance (Jordan et al., 2011). *miR-143* was reported to be an important regulator of adipocyte differentiation (Esau et al., 2004; Xie et al., 2009). The role of *miR-103* is implicated in multiple mRNA regulations which are involve in cellular acetyl-CoA and lipid metabolism (Wilfred et al., 2007). Silencing of *miR-103* in ob/ob mice led to increased insulin sensitivity in adipocytes. *Caveolin-1* (*Cav1*) gene as one of the experimentally approved targets of *miR-103* is a key component of caveolae, an important mediator of insulin signaling (Trajkovski et al., 2011). It is also reported that *miR-103* was significantly up-regulated in the liver of ob/ob mice compared with normal and STZ-treated mice (Li et al., 2009).

In this study the data obtained on *miR-103* changes is indicative of its high capability to differentiate between normal and prediabetic cases. Hence according to our study outcome, evaluation of this miR in PBMCs can act as a suitable non-invasive tool for very early detection of T2DM in at risk individuals. Moreover as in other cases that inhibition of an overexpressed miRNA could significantly convert disease phenotype to the normal one (Sharifi et al., 2014), and also there are chances to revert or attenuate T2D phenotype by inhibition of these two miRNAs.

Conflict of interest

None.

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