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THE ASSOCIATION OF PLASMA LEVELS OF miR-34a AND miR-149 WITH OBESITY AND INSULIN RESISTANCE IN OBESE CHILDREN AND ADOLESCENTS

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Abstract

Context. MicroRNAs (miRNAs) are short noncoding RNAs involved in posttranscriptional regulation of gene expression that influence various cellular functions including glucose and lipid metabolism and adipocyte differentiation.

Objective. The aim of this study was to evaluate the levels of miR-34a and miR-149 and their relationship with metabolic parameters in obese children and adolescents.

Design. Seventy children and adolescents were enrolled in the study. Plasma levels of microRNAs were evaluated by real-time PCR using SYBR green and analyzed by ΔCt method. Plasma concentrations of visfatin and insulin were measured by ELISA method. Glucose and lipid profile were determined colorimetrically. HOMA-IR was calculated and used as an index of insulin resistance (IR).

Results. miR-34a was significantly lower in subjects with insulin resistance compared to obese children with normal insulin sensitivity. There was an inverse relationship between miR-34a levels and both insulin and HOMA-IR. On the other hand, miR-149 was significantly correlated with visfatin. There was no significant difference in miR-34a and miR-149 between obese and normal weight subjects.

Conclusions. miR-34a is associated with insulin and HOMA-IR and thus seems to be involved in IR. miR-149 is inversely associated with visfatin levels which could be indicative of anti-inflammatory effect of this miRNA.

Key words: obesity, miR-34a, miR-149, insulin resistance.

INTRODUCTION

The World Health Organization (WHO) has reported obesity as the global pandemic of the 21st century (1). Childhood obesity participates in metabolic abnormalities which lead to disorders such as insulin resistance, type 2 diabetes, metabolic syndrome (MetS) and cardiovascular disease (2). Obesity is accompanied by increased adipose tissue mass. Adipose tissue is not only a storage tissue for lipids but also it functions as an endocrine organ and releases various adipokines such as visfatin that influence other tissues and cause changes in gene expression patterns and metabolic homeostasis throughout the body (3). Visfatin, also known as pre-B-cell colony-enhancing factor (PBEF) or nicotinamide phosphoribosyl transferase (NAMPT), has been shown to be implicated in obesity and its long-term consequences including insulin resistance. Altered visfatin levels have been reported in obesity, diabetes mellitus, cardiovascular disorders, kidney diseases and bone disorders and different types of cancers (4, 5).

MicroRNAs (miRNAs) are endogenous ~23-nt RNA molecules that regulate gene expression by pairing to the 3'- untranslated region (UTR) of target mRNAs of protein-coding genes to direct their post transcriptional suppression (6). MicroRNAs play key roles in controlling metabolic homeostasis and regulation of insulin signaling and therefore are involved in inflammation, dyslipidemia and insulin resistance. Hundreds of miRNAs are actively or passively released into the circulation and can be used to assess health condition and disease development (7). The expression of miRNAs is influenced by obesity and may be effective in promoting obesity-associated metabolic abnormalities such as insulin resistance and type 2 diabetes.

MicroRNA-34a (miR-34a) is a part of the p53 tumor suppressor network (8). The mature miR-34a is one of the major miRNAs involved in insulin production, pancreatic development and glucose homeostasis (9). A number of studies have shown that elevated miR-34a decreases NAD^+ levels by directly targeting

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nicotinamide phosphoribosyltransferase (NAMPT) (4, 5). NAMPT, which is secreted from adipose tissue as an adipokine called visfatin, has been shown to be elevated in obesity (10). MiR-34a levels are highly elevated in the fatty livers of both diet-induced obese mice and the leptin-deficient ob/ob mice (11, 12). Circulating levels of miR-34a correlate with hepatic disease severity in patients with chronic hepatitis C infection (CHC) or non-alcoholic fatty-liver disease (NAFLD) and diabetes mellitus (13, 14).

The aim of this study was to investigate the association of plasma levels of miR-34a and miR-149 with obesity and its associated parameters in children and adolescents.

MATERIALS AND METHODS

Subjects

Seventy children between the ages of 8-16 years old (35 obese and 35 normal subjects) were enrolled in this cross-sectional study. After a routine medical history acquisition and health examination, the subsequent factors were assessed: waist circumference (WC), hip circumference (HC), waist to hip ratio (WHR), weight, height, systolic (SBP) and diastolic (DBP) blood pressure. Body mass index (BMI) was calculated using the formula weight [kg]/ (height)^2 [m^2] and BMI z-score and percentiles were determined according to sex and age of each subject. WC-SD (standard deviation), SBP-SD and DBP-SD values were calculated by the Anthropometric Calculator for normal children 0-20 years of age developed by the Canadian Pediatric Endocrine Group based on NHLBI 2004 guidelines (15) and US Centers for Disease Control and Prevention (CDC) 2000 growth charts, according to the age and gender of each subject (16). BMI percentiles were also determined according to the sex-specific 2000 growth charts of CDC (17). Those with BMI $\geq 95^{th}$ percentile were considered to be obese and those with BMI between 5^{th} and 85^{th} percentile were enrolled as the control group. None of the subjects had current illness and any history of disease. Those who were taking medications were excluded from the study.

The homeostatic model assessment of insulin resistance HOMA-IR was used to evaluate IR and was determined with the formula (fasting insulin [μIU/mL] × fasting glucose [mg/dL])/405 (18). Those with HOMA-IR-values greater than 3.16 were classified as having IR (19).

MetS was diagnosed based on the presence of abdominal obesity (WC above 90^{th} percentile for age and sex) (20), and two or more other clinical features including elevated glucose, TG, SBP or DBP, or low HDL-C, according to the IDF consensus definition of MetS in children and adolescents (21).

Written informed consent was obtained from subjects and/or their parents. This study was approved by the Ethics Committee of Iran University of Medical Sciences.

Sample collection and biochemical measurements

Venous blood samples were drawn after an overnight fast. Plasma samples were prepared in RNase-free tubes at 4°C and kept frozen at -80°C for microRNA extraction. Serum samples were used for other biochemical measurements.

Fasting plasma glucose (FPG), triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) were measured by enzymatic methods using calorimetric kits (Parsazmoon, Iran). Insulin was measured using Enzyme-Linked Immunosorbent Assay (ELISA) kit (Monobind, USA). In order to measure visfatin, plasma samples were separated immediately at 4°C. Visfatin was measured by an ELISA kit (AdipoGen, Switzerland).

MicroRNA Isolation and cDNA synthesis

The RNA fraction isolated from plasma contains small RNA species less than 100 nucleotides. The total RNA was extracted from 200 μL of plasma sample using the miRNeasy serum/plasma Kit (QIAGEN GmbH, Hilden, Germany) that uses phenol/guanidine-based lysis of samples and silica membrane–based purification of total RNA. QIAzol Lysis Reagent was used to inhibit RNase and also to eliminate most of the cellular DNA and proteins from the lysate by organic extraction. Similar amounts of miRNeasy Serum/Plasma Spike-In Control (1.6 x10^8 copies/μL) were added to each sample prior to extraction to monitor the extraction efficiency.

Poly(A) Polymerase was used to add a poly (A) tail and elongate miRNAs. Reverse transcriptase from RevertAid first strand cDNA synthesis kit (Thermo Scientific, USA) was used for the synthesis of cDNA with a primer containing complementary sequence for the poly (A) tail and an adapter sequence (Table 1).

Real-time PCR

Real-time qPCR was performed for the measurement of miRNA levels using SYBR green (TaKaRa, Shiga, Japan) using both miRNA-specific

Table 1. Sequences of primers

Primers	Sequence
34a	5'- GTGGCAGTGTCTTAGCTGGTT-3'
149	5'- CTGGCTCCGTGTCTTCACTC-3'
16	5'- GGGTAGCAGCACGTAAATATTGG-3'
Universal	5'- GCGAGCACAGAATTAATACGACTC-3'
Adaptor	5'- GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTAG -3'

Table 2. The baseline experimental characteristics and demographic variables

Characteristics	Control group	Obese group	P value
Female/male	20 /15	22/13	n.s.
Age (years)	11.42 ± 2.18	10.95 ± 2.73	n.s.
BMI (kg/m^2)	18.41 ± 2.64	28.12 ± 5.7	< 0.001
BMI z-score	0.683 ± 0.91	2.09 ± 0.45	< 0.001
WC (cm)	61.65 ± 6.57	84.80 ± 13.45	< 0.001
WC-SD	-0.44 ± 0.8	1.55 ± 0.4	< 0.001
HC (cm)	78.37 ± 8.1	97.62 ± 13.62	< 0.001
WHR	0.78 ± 0.47	0.87 ± 0.89	< 0.001
SBP (mmHg)	95.43 ± 11.96	101.14 ± 14.50	< 0.001
SPB-SD	-0.69 ± 1.1	-0.33 ± 1.3	n.s.
DBP (mmHg)	68.57 ± 8.7	72.57 ± 12.21	< 0.001
DBP-SD	0.73 ± 0.7	0.71 ± 1.3	n.s.
FPG (mg/dL)	90.0 ± 4.92	92.05 ± 6.54	< 0.001
TG (mg/dL)	73.94 ± 31.93	126.6 ± 69.0	0.001
TC (mg/dL)	148.65 ± 19.06	171.54 ± 24.97	0.001
LDL-C (mg/dL)	75.80 ± 13.14	92.48 ± 16.81	0.024
HDL-C (mg/dL)	54.60 ± 7.50	47.65 ± 9.5	0.036
Insulin (μIU/dL)	7.74 ± 10.13	19.77 ± 9.64	< 0.001
HOMA-IR	1.75 ± 2.40	4.50 ± 2.22	< 0.001
Visfatin (ng/mL)	1.49 ± 0.87	2.08 ± 1.033	< 0.001

Values are expressed as mean ± SD. BMI: body mass index; WC, waist circumference; WC-SD, waist circumference standard deviation; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; TG, triglycerides; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance.

and universal primers. PCR program contained an initial denaturation at 95˚C for 15 s, followed by 40 cycles of 95˚C for 3 s and 60˚C for 30 s with the Rotor-Gene 6000™ (Corbett Research). The sequences of primers are shown in Table 1.

Normalization of the miRNA levels was carried out by assessing miR-16 expression. A primer for Cel-miR-39-1 (miScript Primer Assay, Qiagen, USA) was used for measuring the previously added Spike-In control to ensure reproducibility. A standard curve containing 10^3 - 10^6 copies of Cel-miR-39 cDNA was generated independent of a serum/plasma sample and RNA purification procedure, allowing estimation of the recovery of miRNeasy Serum/Plasma Spike-In Control.

A melt curve was also generated after each reaction. Relative expressions of miRNA levels were determined by ΔCt using the equation Ct normalizer - Ct specific miRNA.

Statistical analysis

Analysis of the results was performed by Mann-Whitney test for non-parametric variables and independent samples t-test for parametric variables and the chi-square (for gender distribution) using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). Normal distribution of data was evaluated by Kolmogorov–Smirnov test. Association between different parameters was analyzed by Pearson's and Spearman's correlation analysis for parametric and non-parametric variables, respectively. P values below 0.05 were considered to be significant.

RESULTS

The baseline experimental features and demographic variables of the study groups are presented in Table 2. There were no statistically significant differences in age and gender distribution of the case and control groups. Both HC and WC and their ratio as well as WC-SD were significantly higher in

obese subjects compared to normal subjects. SBP and DBP were also significantly higher in obese subjects. However, no significant difference was observed for SBP-SD and DBP-SD between obese and normal subjects.

Significant differences were observed in lipid profile between cases and controls. All of the indices of glycemic control including FPG, insulin and HOMA-IR were higher in obese subjects. Visfatin levels were also significantly elevated in the obese group compared with the control group.

Among obese subjects 31% were diagnosed as having MetS and 74% were found to be insulin resistant.

Semi-quantitative real-time PCR was used for the evaluation of plasma miR-34a and miR-149 expressions in both groups. Analysis by Δct method showed that there were no significant differences in the levels of miR-34a and miR-149 in control and obese groups.

There was also no statistically significant differences in the levels of these microRNAs in obese subjects with or without MetS. Plasma levels of miR-34a were found to be significantly reduced (P=0.001) in subjects with IR compared to the control group (Fig. 1). However, there was no statistically significant difference in the level of miR-149 in these two groups.

The correlation analysis showed that the plasma miR-149 level was negatively correlated with the serum visfatin level (r = -0.302, p = 0.001).

On the other hand, serum miR-34a level was negatively correlated with insulin (r=-0.285, p=0.022) and HOMA-IR (r=-0.281, p=0.023) (Figures 2A and 2B). No association was found between miR-149 and the other variables.

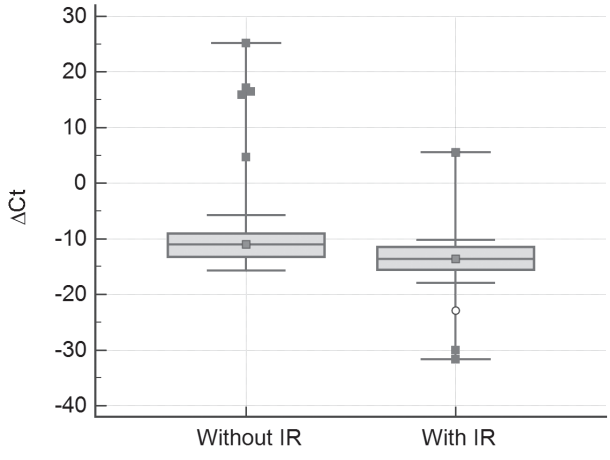

Figure 1. Real-time quantitative RT-PCR analysis of plasma miR-34a in groups with and without insulin resistance. miR-34a levels were calculated after normalizing with miR-16. P<0.001 compared to subjects without insulin resistance.

DISCUSSION

Childhood obesity causes a wide range of severe problems and augments the risk of premature disease. In obesity, elevation of inflammatory factors such as visfatin may lead to IR, impaired glucose tolerance and consequently type 2 diabetes (22).

Some miRNAs are related to adipocytokines and insulin function. Recently, researchers have focused on the link between miRNAs and disorders such as obesity and diabetes. In the present study, we showed that the plasma miR-34a had significant

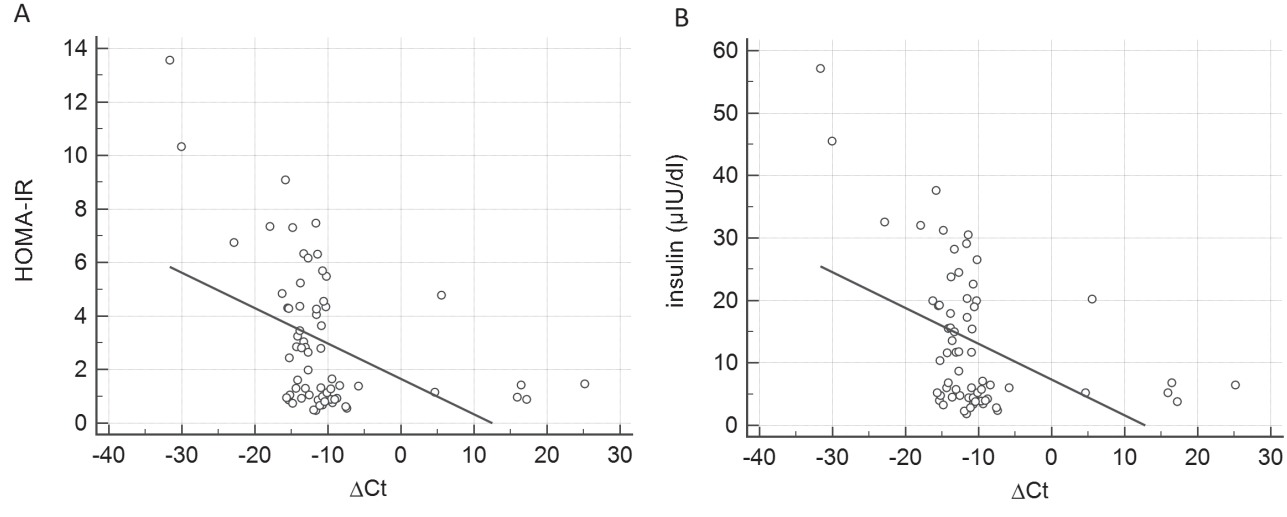

Figure 2. (A) The negative correlation between miR-34a levels and insulin (r=-0.450, p=0.001). (B) The negative correlation between miR-34a levels and HOMA-IR (r=-0.447, p=0.002).

negative correlations with insulin and HOMA-IR. No association was found between miR-34a, miR-149 and the other related variables. Also, there was no significant difference in the levels of miR-34a and miR-149 in obese and control groups, but miR-34a expression was significantly lower in those with IR compared to the obese subjects with normal insulin sensitivity.

MicroRNA-34a has various roles in insulin secretion, p53-dependent apoptosis, regulation of sirtuins 1 expression, hepatic lipid homeostasis, cancer and cardiovascular disease (23). Previous studies have shown that miR-34a is overexpressed in non-alcoholic steatohepatitis (NASH) in mice (13). Some studies have also reported significantly increased miR-34a in patients with non-alcoholic fatty liver disease (NAFLD) (24) which may be related to the tissue damage induced by lipid disorders, because there is a link between liver cell apoptosis, miR-34a and NAFLD severity (25).

The most prominent finding of the current study is the association of miR-34a with insulin resistance in obese children. MicroRNA-34a has been previously found to be associated with diabetes. The expression of miR-34a is augmented in the islets of non-obese diabetic mice during development of pre-diabetic IR (26). On the other hand, increased miR-34a expression leads to sensitization of β cells to apoptosis and destruction in nutrient-induced insulin secretion (27). MicroRNA-34a decreases the expression of VAMP2, a sensitive factor attachment protein receptor (SNARE) that is essential for insulin exocytosis (28). Therefore, decreased miR-34a levels may lead to increased insulin secretion which is consistent with our findings.

Although visfatin is a direct target of miR-34a and reduction of visfatin activity by miR-34a has been reported (29), we did not find a significant correlation between these two parameters which may be the result of interaction of several other factors and shows that *in vitro* relationships between miRNAs and their targets are not necessarily reflected in the blood circulation. On the other hand, miR-149 showed a significant negative correlation with visfatin. MicroRNA-149 inhibits poly (ADP-ribose) polymerase-2 (PARP-2) and subsequently increases cellular NAD^+ levels and SIRT-1 activity (30) suggesting a relationship between miR-149 and visfatin. It has also been reported that miR-149 inhibits inflammatory cytokines, tumor necrosis factor-α (TNF-α) and IL-6 (31) which increase the production of visfatin (32). Thus miR-149 may indirectly reduce visfatin, exerting an anti-inflammatory function.

Other miRNAs, such as miR-494 are also involved in reducing insulin secretion which may be attributable to resistin, and contribute to pathogenesis of diabetes (33).

In conclusion, according to the results of the present study, the plasma miR-34a is correlated with insulin and HOMA-IR, suggesting a role for miR-34a in the pathogenesis of diabetes and its potential as an early biomarker for identification of insulin resistance. Further studies are needed to clarify the link of miR-149, miR-34a and obesity-associated metabolic abnormalities.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgment

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