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Dietary Regulation of miR-33b and miR-29a in Relationship to Metabolic Biomarkers of Glucose and Lipids in Obese Diabetic Women: A Randomized Clinical Controlled Study

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Abstract

Background: MicroRNAs have recently been introduced as epigenetic regulators of glucose and lipid metabolic pathways, which are impaired in obesity and diabetes.
Objectives: We evaluated the effects of calorie-restricted diet therapy on the circulating levels of miR-33b and miR-29a in relationship to glucose and lipid metabolic parameters in obese patients with type 2 diabetes mellitus (T2DM).
Methods: This randomized clinical controlled trial was performed on 30 eligible obese women with T2DM, randomly divided into two groups (control group, n = 15; diet therapy group, n = 15) for 10 weeks. Ten healthy women with normal weight were enrolled at the baseline of the study as controls. Demographic information, dietary intake, and anthropometric and biochemical indices were obtained before and after the study. Circulating miR-33b and miR-29a were assessed for all subjects using quantitative RT-PCR, and the fold change of each circulating miRNA was compared between groups.
Results: The circulating levels of miR-29a and miR-33b in the diabetic women were higher (0.40-fold) and lower (1.43-fold), respectively, than normal levels. Diet therapy significantly increased the circulating level of miR-33b (P = 0.023, 0.97-fold upregulation) to normal levels. This increase was independently correlated with caloric restriction (95%CI: -0.004 to -0.0001, P = 0.022) and 2hPPBS (95%CI: -0.009 to -0.001, P = 0.035). No remarkable change was observed in circulating levels of miR-29a.
Conclusions: Our findings introduced a novel therapeutic effect of diet therapy on circulating miRNAs in obese patients with T2DM. MiR-33b is an important therapeutic target in the treatment and prevention of T2DM and its complications.

Keywords: Diet Therapy, microRNAs, Diabetes, Obesity

1. Background

Global estimates have reported an increasing incidence of type 2 diabetes mellitus (T2DM) over the past 15 years. T2DM and its debilitating micro- and macrovascular complications have profound effects on public health (1). It is a complex disorder resulting from interactions between genes and environmental factors, including obesity and dietary intake (2). Obesity (defined as a body mass index [BMI] of $\geq$ 30 kg/m^2) contributes to approximately half of the worldwide incidence of T2DM (3). Compared to the global incidence of T2DM (~13% of adults) in 2014, the rate in Iran was 26.3% in 2008 (4). The American diabetes association (ADA) has reported that nutritional factors play a critical role in the prevention and management of obesity and its related metabolic complications (5). Nutritional factors and a positive caloric balance can affect a variety of obesity- and diabetes-related epigenetic factors (6, 7). Interactions between nutrition, obesity, and gene expression may cause the higher incidence of T2DM among families and its more progressive pathology in the Middle East, including Iran, compared to other countries (8).

MicroRNA (miRNA), a key post-translational regulator of gene expression, is a class of non-coding small RNA (~22

nucleotides) (9) that inhibits mRNA translation by targeting the 3′ un-translational region (3'UTR) (10). MiRNAs stably exist in bodily fluids such as blood; they are disease-specific and their circulating levels reflect their expression patterns (11). Under healthy conditions, miRNAs regulate the expression of cognate target genes, but under unhealthy conditions, such as obesity, any disturbance in the expression of individual miRNAs can cause the dysregulated expression of target genes and disease-specific complications (12). The dysregulated circulating levels of miR-33b and miR-29a have recently been introduced as metabolic-disease-specific biomarkers in obese patients. MiR-33a and b are located within the introns of the sterol regulatory element-binding protein (SREBP), respectively SREBP-2 and SREBP-1c. This family is the key regulator of glucose homeostasis via targeting numerous related genes (13-15). Several studies have identified the regulatory roles of the miR-29 family (a, b, and c) in glucose metabolism and insulin signaling (15-17). The over-expression of miR-29a reduces insulin-dependent glucose uptake, reduces cellular and hepatic glucose production (16, 18), and suppresses the expression of Syntaxin 1A (Stx1a), a key regulatory gene of insulin synaptic exocytose from the β-cells (16).

The most recent evidence suggests the potential of miR-33b and miR-29a as targets for the early diagnosis and treatment of glucose homeostasis disruptions and the complications of T2DM (19). MiR-33b is encoded in humans and other large mammals (13, 20), but only limited studies have assessed it in human glucose homeostasis. Moreover, there are scarce studies exploring the impact of diet therapy on circulating miR-33b and miR-29a in obesity and T2DM. We hypothesized that if diet can affect circulating levels of microRNA, this interaction could explain the different pathogenesis of T2DM in various populations, including in Iran, and may make possible the development of new treatments to reduce the inheritability of T2DM.

2. Objectives

This study characterized the expression of miR-33b and miR-29a in obese female patients with T2DM compared to values from healthy, non-obese women. We also examined the effects of a calorie-restricted diet on circulating miR-33b and miR-29a levels in relationship to lipid and glucose metabolism biomarkers in obese women with T2DM.

3. Methods

3.1. Study Population

The sample size was calculated by considering the primary outcomes of miR-33b (21) on the basis of Pocock's sample size formula with a power of 80% and a 95% confidence interval (10 patients per study group), taking into account a probable 30% loss of subjects. The study was carried out in compliance with CONSORT guidelines, from February 2014 to January 2015. The approved format of the study was used after receiving an ethics code (92162) from the ethics committee of Tabriz University of Medical Sciences, as well as from the Iranian registry of clinical trials (IRCT) (code no IRCT2014011416223N1).

At baseline, all patients were provided with a detailed explanation of the study, and informed written consent was signed by each participant. The subjects were selected from 55 obese female volunteers with T2DM, based on the study's inclusion criteria, from the clinic of Emdadi hospital of Zanjan University of Medical Sciences, Zanjan, Iran. The T2DM patients were all undergoing drug therapy with Glucophage/glyburide. The participants consisted of 32 obese (BMI $\geq$ 30 kg/m^2) women aged 25 - 60 years with a diagnosis of T2DM based on world health organization criteria (22) for a minimum of two years, with a control group of 10 healthy age-matched women with normal BMI (18 - 25 kg/m^2). The diabetic subjects were sequentially numbered from the beginning of the study and randomized with a block size of 4 (1:1) by a statistician using RAS software (16 patients each in the treatment and control groups).

The exclusion criteria were pregnancy, lactation, diabetic ketoacidosis, acute presentation, heavy ketonuria, history of major comorbidity (cardiovascular disease, liver failure, renal disease, or malabsorption disorders), BMI of $\geq$ 45 kg/m^2, any change in drug use throughout the study, incomplete questionnaires, unwillingness to give blood samples, the use of any special dietary plan (including vegetarian or calorie-restricted diets) within the previous year, > 3 kg of weight change, the use of any type of dietary supplement within the previous 3 months, history of hormone therapy, insulin requirement, or a diagnosis of T1DM.

3.2. Dietary Intake

At the baseline, the validated 168-item semi-quantitative food-frequency questionnaire (FFQ) (23) was completed by a trained dietitian to determine the habitual diet of each subject. Household kitchen scales were delivered to the participants. Two-weekday and one-weekend dietary records were filled out by each participant. The portion sizes of the foods and beverages consumed were converted into grams using household measures (24), then coded and analyzed for energy content and other nutrients using the Iranian version of Nutritionist IV software.

3.3. Diet Planning

For each subject in the treatment group, the personalized caloric intake was calculated by subtracting 700 Kcal from the obtained total energy expenditure, using the standard equation for overweight and obese women over 19 years of age, after considering the patient's current weight and physical activity coefficient (25). Physical activity levels and coefficients were determined using the completed related questionnaires for each participant. During the study, the diabetic patients continued their habitual physical activity without any changes. Personalized calorie-restricted diets lasted 10 weeks and contained 17%, 53%, and 30% of energy as protein, carbohydrates, and fat, respectively, which was designed based on the concepts of low glycemic load and low glycemic index. Such diet therapies are healthy therapeutic interventions that promote the health of patients with no side effects. During the present study, the diabetic control group continued their habitual diet. Medications were held constant during the study, and the number of medications used in the two diabetic groups was adjusted by blocked randomization at the baseline of the study. Three days of dietary records were obtained at 5 and 10 weeks to estimate the subjects' adherence to the diet. All dietary measurements, planning, and follow-up were performed by a trained dietitian.

3.4. Demographic, Clinical, and Anthropometric Measurements

Demographic, clinical, and anthropometric questionnaires were completed for all participants by a trained dietitian at baseline. Clinical and anthropometric questionnaires were collected again for the diabetic patients 5 and 10 weeks after the study began. The demographic information included detailed medications, menopausal status, stress quality and severity, quantity of sleep, education level, job, physical activity, history of abortions or pregnancies, marital status, and a family history of obesity, diabetes, hypertension, or CVD. Waist circumference (WC) at the point between the lowest rib and the superior border of the right iliac crest, and hip circumference (HC) at the widest point around the greater trochanters, were measured to the nearest 0.1 cm with the measuring tape directly on the individual's skin (Seca, UK). The waist-to-hip ratio (WHR) was calculated as WC/HC. Body weight (to the nearest 0.1 kg) and visceral fat (%) were measured using a calibrated and validated Omeron body-composition scale (Seoul, Korea). Height was measured using a calibrated portable stadiometer (Seca, UK) to the nearest 0.1 cm, with the subject stretching to maximum height and the head positioned in the Frankfort plane. BMI was calculated as weight (kg)/height (m^2). The same trained researcher took all measurements, with the participant in the fasting state and wearing soft indoor clothing without shoes.

3.5. Biochemical Measurements

At the baseline point in the study (for the diabetic patients and healthy participants) and after 10 weeks (for the diabetic patients), 10-cc fasting blood samples were taken in simple and 7.5% K3-EDTA tubes (Becton Dickinson Vacutainer System, UK). The tubes were inverted five times, then respectively centrifuged for 10 min at 13,000 x g (Eppendorf 5702, US) and for a 40 minutes three-spin protocol (1500 rpm for 30 min, 3000 rpm for 5 min, and 4500 rpm for 5 minutes) (Eppendorf Vacufuge Plus, USA) to prevent any contamination by cellular nucleic acids. The serum and plasma samples were then stored at -80°C for further processing. Plasma samples were stored in RNase-free microtubes while awaiting the microRNA assays.

Fasting blood sugar (FBS) and two-hour postprandial blood sugar (2hPPBS) were measured with the GOD-PAP method, serum triglycerides (TG) were measured with GPO-PAP, and serum total cholesterol (TC) was measured by enzymatic endpoint. All measurements were performed using a Randox Laboratories kit (UK) (Hitachi 902, Roche Diagnostics, USA) under the same conditions. The homeostasis model assessment (HOMA) was estimated for assessing insulin resistance (HOMA-IR) and β-cell activity (HOMA-β) in response to serum glucose concentrations before and after the study (26). Fasting and 2-hour postprandial insulin levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (Monobind Pharmaceuticals, CA, USA; sensitivity 0.182 μIU/ ml; intra CV: 5.5%; inter CV: 9.1%). All analyses were done in duplicate under the same conditions in the central biotechnology laboratory at Shahid Beheshti University of Medical Sciences, Tehran, Iran, by a trained laboratory specialist.

3.6. Circulating RNA Extraction and cDNA Synthesis

For molecular assessments, plasma samples were collected from the diabetic participants before and 10 weeks after the study began. For the normal participants, samples were collected only at baseline. RNA extraction was performed from 500 mL of each patient's plasma samples stored in RNase-free microcentrifuge tubes according to the QIAzol RNA extraction protocol (Qiagen, USA). After confirming the integrity and quality of the RNA with a spectrophotometer (Eppendorf, Hamburg, Germany), DNase I treatment was performed following the manufacturer's protocol (Sigma-Aldrich, USA). RNA was stored at -80°C before use. Afterward, the extracted RNAs were reverse-transcribed to cDNA using stem-loop reverse transcriptase (RT) primers (for predicted miR-33b, miR-29a, and SNORD U6) and Expand^{TM} Reverse Transcriptase (Roche Diagnostics GmbH Mannheim, Germany), according to the manufacturer's instructions. The cDNA was stored at -20°C before use.

3.7. Quantitative Real-Time RT-PCR

Triplicate real-time reverse-transcription polymerase chain reactions (PCRs) were carried out for miR-33b and miR-29a (and some unreported miRNAs) in 12.5 μL PCR mixture volumes consisting of Maxima® Probe qPCR Master Mix (Fermentas, Lithuania), oligonucleotide primers and probe, and 2 μL of cDNA. Amplification was carried out in the LightCycler® rotor (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Reaction setups were done manually. Cycle threshold (Ct) values were calculated using LightCycler® software, version 3.5,2001 (Roche Molecular Biochemicals, Mannheim, Germany). The U6 gene was used as the reference gene and underwent the aforementioned procedure. The non-template control and RT-minus were used for the entirety of the experiments. MIQE guidelines were used in all of the manipulation steps. The fold changes of miR-33b and miR-29a expressions (in the diabetic participants compared with the normal controls before and after the intervention) were calculated using the relative expression software tool (REST®) as suggested by Pfaffl (27). RNA extractions and real-time RT-PCR were carried out in the molecular cell biology laboratory at the stem cell technology research department of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

3.8. Statistical Analysis

Statistical analyses were performed using SPSS software, version 15.0 (SPSS, Chicago, IL, USA). After testing the normality and homogeneity of the variances using the one-sample Kolmogorov-Smirnov descriptive analysis and the Chi-square test, the quantitative variables were matched with the Chi-square test. Variables were given and analyzed in a base of log10-transformation where necessary. Descriptive statistics were computed using mean $\pm$ standard deviation (SD) for continuous measurements. The microRNA data (based on the advised principals for reporting the most accurate molecular measurements (28) were expressed using mean $\pm$ standard error (SE). Alterations in total energy and macronutrients were examined with the repeated-measure analysis of variance (ANOVA). The independent t-test or the Mann-Whitney U test and the paired-T test or Wilcoxon's test were used for group comparisons. To adjust for the effects of multiple variables on the intervention, a univariate analysis of covariance (ANCOVA) was used. Multiple linear regression tests were carried out to assess the independent relationship of the miRNA fold changes with other biochemical, anthropometric, and nutritional factors in the study. For all of the analyses, a two-sided P value of < 0.05 was deemed statistically significant. All real-time PCR results were analyzed using REST® 2009 software, and only the significant fold changes were transferred to the SPSS file for further analysis. Graphical procedures were performed using Microsoft Excel 2007.

4. Results

4.1. Disposition, Demographic, and Clinical Characteristics of Diabetic Patients and Healthy Participants

During the study, one patient each from the control and treatment groups were lost to follow-up, while 30 diabetic women completed the study. Ten healthy women also participated at the baseline of the study in order to compare the expression levels of miRNA in obese patients with the corresponding normal amounts (Figure 1). Primarily, participants were frequency-matched for quantitative variables, including menopausal status, stress, quality and quantity of sleep, education level, job, physical activity, history of abortion or pregnancy, marital status, and a family history of obesity, diabetes, hypertension, or cardiovascular disease (exact $P > 0.05$). There were no differences in the number of healthy or diabetic participants with these characteristics, or between the two diabetic groups. The demographic and clinical characteristics of the healthy participants and diabetic patients are shown in Tables 1 and 2.

4.2. Significant Reduction of Caloric and Carbohydrate Intake in the Treatment Group

After testing the normal distribution, homogeneity, and sphericity, the results of the repeated-measure ANOVA test showed significant reductions of total energy intake (from 1882.27 $\pm$ 149.94 to 1595.1 $\pm$ 110.58 Kcal/day; P = 0.001), carbohydrate intake (from 231.48 $\pm$ 27.34 to 179.40 $\pm$ 33.08 g/day; P = 0.001), and fat intake (from 82.76 $\pm$ 10.87 to 72.88 $\pm$ 10.13 g/day; P = 0.019) during diet therapy in the treatment group. The results did not show any significant change in protein intake (from 52.87 $\pm$ 6.14 to 55.80 $\pm$ 9.30 g/day; P = 0.215). There was no significant change in energy intake in the diabetic control group (from 1787.64 $\pm$ 318.59 to 1787.1 $\pm$ 322.05 Kcal/day; P = 0.045).

There was significant caloric reduction at the 5th and 10th weeks compared to before the study (-168.17 $\pm$ 57.17, P = 0.013, and -286.752 $\pm$ 47.18, P < 0.001, respectively). There were also significant reductions in dietary carbohydrates at the 5th and 10th weeks of the diet therapy compared to before the study (-33.26 $\pm$ 9.29, P = 0.004, and -52.08 $\pm$ 11.08, P < 0.001, respectively). We observed significant reductions in dietary fat intake at the 10th week of diet therapy compared to before the study and from the 5th to the 10th weeks (-9.87 $\pm$ 3.24, P = 0.011, and -5.38 $\pm$ 2.38, P = 0.046, respectively).

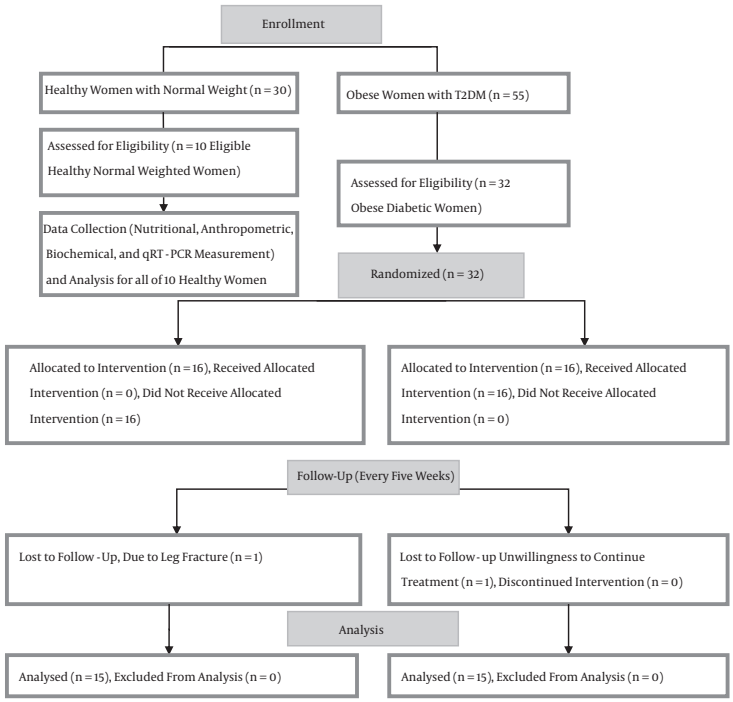


Figure 1. The Present Study's CONSORT Workflow

Table 1. Demographic and Clinical Characteristics of Participants at Baseline[a]

	Healthy Participants (n = 10)	Diabetic Patients (n = 30)	P Value[b]
Age, y	44.50 ± 5.79	49.60 ± 8.18	0.082
Weight kg	59.25 ± 6.21	81.95 ± 11.78	0.001[c]
BMI, kg/m^2	22.35 ± 2.67	33.38 ± 4.16	0.001[c]
Total body fat, %	28.16 ± 5.40	45.84 ± 3.83	0.001[c]
Visceral fat, level	6.00 ± 0.81	10.60 ± 2.04	0.001[c]
SBP, mmHg	11.20 ± 1.03	13.74 ± 1.88	0.001[c]
DBP, mmHg	7.50 ± 0.52	9.19 ± 2.04	0.015[d]
FBS, mg/dL	73.80 ± 5.20	193.72 ± 70.44	0.001[c]
Logarithm of triglyceride, mg/dL	1.85 ± 0.13	2.22 ± 0.25	0.001[c]
Total cholesterol, mg/dL	170.80 ± 23.99	193.32 ± 33.22	0.061
Logarithm of fasting insulin, μU/mL	-0.88 ± 0.13	1.02 ± 0.23	0.001[c]

Abbreviations: BMI, body mass index; CI, confidence interval; DBP, diastolic blood pressure; FBS, fasting blood sugar; HC, hip circumference; MD, mean differences; SBP, systolic blood pressure; WC, waist circumference.
[a] Values are expressed as mean ± SD.
[b] P value from independent t-test.
[c] Difference is significant (P ≤ 0.01).
[d] Difference is significant (P ≤ 0.05).

4.3. Significantly Low Levels of Circulating MiR-33b and High Levels of miR-29a in Diabetic Patients

To investigate the circulating levels of miR-33b and miR-29a in the obese T2DM subjects, the initial circulating miRNA levels at baseline were identified with RT-PCR assays and compared to those of healthy participants. Unlike miR-33b levels, which were significantly lower in the obese

Table 2. Demographic and Clinical Characteristics of Diabetic Patients[a]

	Control (n = 15)		Treatment (n = 15)		P Value[b]	
	Baseline	After the Study	Baseline	After the Study	Baseline	After the Study
Age, y	49.63 ± 9.57		49.28 ± 7.75		0.920	
Diabetes medications, n/d	3.15 ± 1.45		3.71 ± 1.63		0.764	
Duration of diabetes, y	6.45 ± 4.27		8.00 ± 4.64		0.401	
Weight, kg	82.80 ± 13.45	82.60 ± 14.18	81.28 ± 10.76	79.94 ± 11.65[c]	0.757	0.626
BMI, kg/m^2	34.57± 5.62	34.45 ± 5.97	32.45 ± 2.34	31.86 ± 2.40[c]	0.212	0.180
Visceral fat, level	11.09 ± 2.66	11.54 ± 2.84	10.33 ± 1.43	9.83 ± 1.46[d]	0.296	0.727
WC, cm	109.97 ± 14.09	111.50 ± 15.05	108.36 ± 8.61	104.17 ± 7.99[c]	0.727	0.154
HC, cm	114.91 ± 12.27	115.41 ± 12.05	113.08 ± 9.35	109.96± 8.99[d]	0.676	0.230
WHR	0.95 ± 0.06	0.96 ± 0.07	0.96 ± 0.05	0.94 ± 0.02	0.909	0.459
FBS, mg/dL	182.64 ± 69.40	172.82 ± 64.68	202.43 ± 72.60	148.50 ± 72.05[c]	0.497	0.417
2hPPBS, mg/dL	254.91 ± 119.39	257.36 ± 100.00	267.14 ± 88.92	192.92 ± 77.90[c]	0.771	0.098
Cholesterol, mg/dL	178.55 ± 36.21	190.36 ± 48.92	207.20 ± 24.68	203.00 ± 25.23	0.046	0.427
L-trigyceride mg/dL	2.24 ± 0.23	2.28 ± 0.18	2.22 ± 0.27	2.19 ± 0.24	0.946	0.379
L-fasting insulin, μU/mL	1.09 ± 0.28	0.97 ± 0.25	0.97 ± 0.17	0.94 ± 0.23	0.222	0.651
L-HOMA-IR	0.72 ± 0.34	0.57 ± 0.25	0.64 ± 0.28	0.46 ± 0.40[d]	0.384	0.225
HOMA-B, %	-30.91 ± 19.62	-35.81 ± 18.59	- 44.13 ± 6.21	- 38.60 ± 7.75[d]	0.026[d]	0.638

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HC, hip circumference; FBS, fasting blood sugar; HOMA-IR or β, homeostatic model assessment insulin resistance or β-cell function; L, logarithm; SBP, systolic blood pressure; WC, waist circumference; WHR, waist-to-hip ratio; 2hPPSB, 2-hour postprandial blood sugar.

[a] Values are expressed as mean ± SD.

[b] P value from independent t-test for non-logarithmic and exact p value from the Mann-Whitney U test for logarithmic values.

[c] P < 0.01.

[d] P < 0.05.for paired comparisons before versus after study (paired t-test for non-logarithmic and exact P value Wilcoxon's test for logarithmic values). (These data have been reported in our unpublished recent manuscript (29)).

T2DM patients, circulating levels of miR-29a were higher in these patients than in the healthy control group (Figure 2).

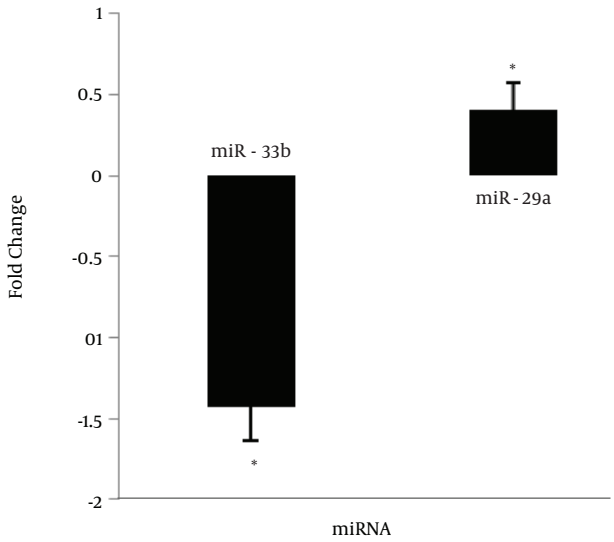
4.4. Circulating Levels of miR-33b Increased Significantly in Diabetic Patients During Diet Therapy

In order to evaluate the effects of a calorie-restricted diet on the expressions of miR-33b and miR-29a in the diabetic patients, circulating levels of these microRNAs were detected at baseline and at the 10th week of the study with RT-PCR assays. Interestingly, compared with those of the healthy normal-weight women, circulating levels of miR-33b increased significantly in the treatment group, up to normal levels (Figure 3). However, no significant change was observed in the circulating levels of miR-29a after the treatment (data not shown).

4.5. Independent Significant Negative Relationship of miR-33b Fold Change With Caloric Restriction and 2hPPBS in the Treatment Group

As reported in our previous manuscript (29), the results of the ANCOVA test showed that diet therapy independently improved most of the anthropometric measures (P < 0.05), FBS (P = 0.024, MD -37.47, 95% CI: -69.37 to -5.57 mg/dL), 2hPPBS (P = 0.007, -72,86; -123.34 to -22.3 mg/dL), and serum total cholesterol (P = 0.005; -28,10; -46.48 to -9.72 mg/dL) in the treatment group. In order to separate the independent effects of macronutrients on miRNA fold changes and the relationship of miRNA with glucose and lipid metabolism biomarkers, we assessed the correlations between the fold changes of miR-33b and miR-29a and the significant changes of anthropometric, biochemical, and dietary factors during the study in both diabetic groups, and reported the significant relationships. As shown in Table 3, unlike the results for miR-29a, we found important independent negative correlations of miR-33b fold changes with caloric intake and levels of 2hPPBS in the treatment group after adjusting all other measured indices in this study.

Figure 2. Fold Change of miR-33b and miR-29a in T2DM Patients (n = 30) Compared With Normal Women (n = 10) at Baseline, From the Results of Quantitative RT-PCR



Values are expressed as mean ± SE (standard error). *Fold change is significant ($P \leq 0.05$).

5. Discussion

The rising prevalence of T2DM and its complications, in tandem with obesity and nutritional factors, indicate the regulatory network among the epigenetic factors, including miRNAs, obesity, and nutrition (15, 30). Dysregulation of miRNA, as the post-transcriptional modification of target genes involved in glucose and lipid metabolic pathways, leads to the pathology of diabetes and its complications (15).

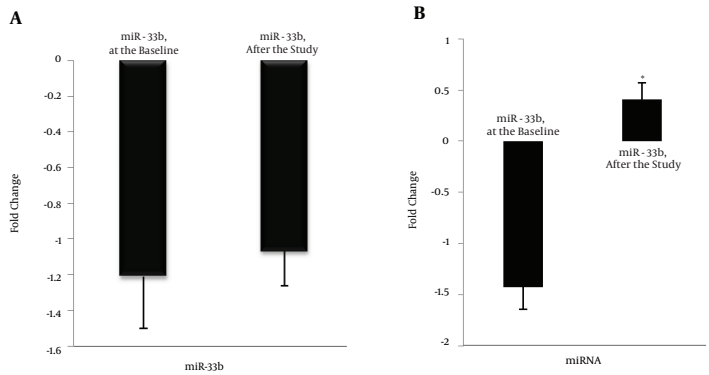
In this study, we demonstrated significantly decreased energy intake, total dietary fat intake, and carbohydrate intake in obese women with T2DM under the diet therapy. Based on the evidence from this nutritional analysis, the mean caloric restriction in the treatment group was approximately 230 Kcal/day during the study. Therefore, the adherence of the patients in this study to the treatment diet was approximately 35%. It has been observed that diabetic patients' average adherence to therapeutic procedures in developed countries is < 50% (31).

To investigate the effects of diet therapy on post-transcriptional epigenetic modulations of glucose and lipid metabolic pathways, we examined the impact of a calorie-restricted diet on circulating levels of miR-33b and miR-29a in obese women with T2DM. Initially, the quantitative RT-PCR assays indicated that the circulating levels of miR-33b and miR-29a were lower (-143%) and higher (40%), respectively, in obese T2DM patients compared to healthy normal-weight women (Figure 2). We found that a diet that controlled calories from total fat and carbohydrate intake can mediate a significant upregulation of miR-33b (~ 97%), toward normal circulating levels, in obese T2DM women (Figure 3). Interestingly, a significant negative correlation was identified between the fold change of miR-33b and the amount of restricted calories in the treatment group (Table 3). This novel observation introduces diet therapy as an important epigenetic modulator in the regulation of miR-33b expression to normal levels.

MiR-33b is encoded in humans and large mammals, but not in rodents, with a two-nucleotide difference from miR-33a. While the same target activities have already been suggested for miR-33a and miR-33b, they have different patterns of evolutionary conservation (13, 20). Studies exploring the effects of human miR-33b on glucose and lipid metabolism pathways are scarce. Considering the significant miR-33b downregulation (-143%) in the obese T2DM patients and a recent report on the suppressed expression of SREBP-1c in the hypertrophic adipose tissues of ob/ob mice (32), we can propose that insulin resistance in obesity may cause downregulation of SREBP-1c and a consequential downregulation of miR-33b. Induction of miR-33b expression in patients on diet therapies has been demonstrated along with improved glucose and lipid metabolism. As shown in Table 3, we demonstrated a significant correlation between increased circulating miR-33b and decreased 2hPPBS in T2DM patients under the diet therapy. This is in agreement with a previous study that reported the inverse effect of miR-33b on glycemic control in hepatocytes through the inhibition of miR-33b's targets, including pyruvate carboxykinase (PCK1) and glucose-6-phosphatase (G6PC), two key regulatory enzymes of hepatic gluconeogenesis (19). MiR-33b also regulates cholesterol homeostasis by targeting key transcriptional regulators of lipid metabolism, including steroid receptor co-activator 1 (SRC1), steroid receptor co-activator 3 (SRC3), nuclear transcription factor Y subunit gamma (NFYC), and nuclear receptor-interacting protein 140 (RIP140) (19). Also, we did not see any significant correlation between miR-33b fold changes and lipid metabolism biomarkers in obese women with T2DM.

To further evaluate the effects of a calorie-restricted diet on the expression of the involved miRNAs in metabolic pathways, we also detected circulating levels of miR-29a before and after the diet therapy in the diabetic patients. An increased circulating level of miR-29a (40%) was observed in the obese T2DM patients compared to healthy normal-weight women (Figure 2). This finding was in line with new evidence (18, 33) but in contrast to an animal study (34). However, our study did not show any marked effect of calorie-controlled diet therapy on miR-29a expres-

Figure 3. Fold Change of miR-33b in T2DM Patients During the Study Compared With Healthy Women, From the Results of Quantitative RT-PCR



Values are expressed as mean ± SE (standard error). (A) Diabetic control group (n = 15) vs. healthy women (n = 10) (exact P value from Wilcoxon analysis = 0.613). (B) Diabetic treatment group (n = 15) vs. healthy women (n = 10) (exact P value from Wilcoxon analysis = 0.011).

Table 3. Relationship Between miR-33b Fold Change and Changes in Calorie Intake During the Study in the Treatment Group

	Fold Change of miR-33b		
	β[a]	95% CI[a]	P Value[a]
Calorie intake	-0.619	-0.004 to -0.001	0.022[b]
2hPPBS	-0.603	-0.009 to -0.001	0.035[b]

Abbreviation: 2hPPBS, 2-hour post-prandial blood sugar.
[a] Results of multiple linear regression analysis, adjusted for anthropometric, biochemical, and dietary indices in the study.
[b] Correlation is significant at the 0.05 level (2-tailed).

sion. Previous reports have shown the roles of the miR-29 family in the reduction of glucose production, decreased glucose tolerance, and increased insulin synaptic exocytose in human INS-1E β cells (17, 35) and in animal models (18). These findings suggest, to some extent, that there is a compensatory role of miR-29a in offsetting high circulating glucose and insulin resistance in obese diabetic humans. Considering the pathophysiology of T2DM (36), since miR-29a affects the upstream genes of insulin secretion from the β-cells (17, 18), we propose that more time is required to assess the probable results of diet therapies on the expression of miR-29a in obese T2DM subjects.

Our findings introduce moderate restricted caloric intake from fat and carbohydrate as an epigenetic modulator of miR-33b expression in the treatment of obesity and diabetic complications. We suggest that a balanced expression of miR-33b and miR-29a is necessary to achieve the ideal regulation of genes involved in glucose and lipid metabolism and in complications of diabetes in obese T2DM patients.

With regard to conducting comprehensive research and identifying probable independent relationships, one advantage of the present study is that it is one of the few molecular human studies that have considered the different nutritional, anthropometric, biochemical, and molecular aspects of glucose and lipid metabolism in one framework, using participants in a similar geographic region with matched demographic characteristics. Dietary interventional studies on human miRNA levels are in their infancy, and further studies are necessary in order to obtain detailed knowledge about the therapeutic effects of diet protocols on miR-29a levels in obese diabetic patients. Because of the need to keep the patients' medications constant throughout the study, we could not continue the intervention for more than 10 weeks under stable conditions.

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Footnotes

Author's Contribution: Somayeh Mohammadi conceived and designed the study and experiments, per-

formed the experiments and data acquisition, and prepared the manuscript. Mehrangiz Ebrahimi-Mameghani and Seyed Rafie Arefhosseini participated in the design of the study and its coordination. Parviz Fallah, Sepideh Zununi, Masoud Soleimani, and Mehdi Banitalebi Dehkordi made substantial contributions to qRT-PCR. Mohammad Asghari Jafarabadi participated in the analysis and interpretation of data and in the design of the study. Hossein Ghanbarian designed and coordinated the qRT-PCR and substantially helped to revise the manuscript's draft. All authors read and approved the final manuscript.

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