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Overexpression of miR-29b in Plasma of Javanese Type 2 Diabetes Mellitus Patients in Semarang, Indonesia

ABSTRACT

Objective: In diabetes, the miR-29b, also known as redoximiRs, has been shown to regulate oxidative pathways. The aim of this study was to observe the circulation of miR-29b in plasma of Javanese type 2 diabetes mellitus patients at the Primary Health Care Center in Semarang, Indonesia, and its correlation with fasting blood glucose, cholesterol, and triglyceride.

Methods: The expression of miR-29b in type 2 diabetes mellitus groups and control groups was measured by reverse transcriptase polymerase chain reaction (RT-PCR). The $2^{-\Delta\Delta Cq}$ method was used to determine the relative amount of miR-29b. The miR-29b expression was compared with an independent *t*-test. Furthermore, the correlation between miR-29b expression was analyzed by the Pearson correlation test using SPSS 17.0. Fold change was positive at 14.12 (i.e., upregulation).

Results: There was a significant negative correlation between age and miR-29b expression ($P = .000$, $r = -0.627$). Moreover, fasting blood glucose and miR-29b also had a negative correlation ($P = .000$, $r = -0.504$).

Conclusion: miR-29b overexpression occurred in plasma of Javanese type 2 diabetes mellitus patients at the Primary Health Care Center in Semarang, Indonesia, i.e., 14.12-fold overexpression. The level of miR-29b expression was negatively correlated with age and fasting blood glucose.

Keywords: Diabetes mellitus, Indonesia, micro-RNAs, miR-29b, plasma

Introduction

In Indonesia, noncommunicable and communicable diseases (infectious disease) have the same high prevalence. More precisely, cases of noncommunicable diseases such as stroke, hypertension, diabetes mellitus (DM), tumor, and heart disease have continually increased. A total of 13% population in Indonesia has DM. Approximately 80%-90% of DM cases are type 2 diabetes mellitus (T2DM).[1] There were 10.7 million T2DM patients in Indonesia, according to The International Diabetes Federation (IDF) in 2019.[2] The Indonesian Ministry of Health has reported an increased number of T2DM cases from 1.1% in 2007 to 2.1% in 2013.[3] Javanese is the major population in Central Java province. DM incidence is the second highest after hypertension.[4] Javanese is the biggest ethnic group in Indonesia and has a cultural preference for consuming sweet foods that trigger a high risk of DM.

Diabetes mellitus is a disease characterized by a variety of symptoms such as hyperglycemia. Type 2 diabetes mellitus is a metabolic and endocrine disorder caused by insulin resistance and pancreatic β-cell dysfunction. Insulin resistance occurs due to inadequate insulin receptors in cells. Thus, cells are unable to use the glucose, and blood glucose levels remain high. Insulin resistance mainly occurs in insulin-sensitive tissues, such as muscle, adipose, liver, gastrointestinal tract, central nervous system, and pancreatic cells.[5] Insulin resistance pathophysiology involves several factors, including mitochondrial dysfunction, inflammation, obesity, and oxidative stress.[6]

The hyperglycemia condition in T2DM causes protein glycation, glucose autoxidation, lipid peroxidase, and glucose metabolic imbalance. The polyol pathway is involved in glucose metabolic imbalance. This mechanism causes the formation of free radicals that leads to the imbalance ratio of free radicals and antioxidants in the body. Moreover, it induces oxidative stress. Oxidative stress causes the damage of DNA and proteins in various tissues and is responsible for the development of T2DM and its complications.[7] micro-RNA has the capability to increase reactive oxygen species or decrease the antioxidant expression in T2DM.

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micro-RNA is a noncoding RNA consisting of 18-25 nucleotides that bind to the 3′-untranslated region of the target gene. Small micro-RNA molecules are actively or passively released into the circulation. It can be used as a biomarker for the onset and progression of some diseases such as DM. In diabetes, micro-RNA has a role in oxidative stress mechanism, inflammation, or insulin signaling pathway that responds to the development of insulin resistance. A previous study suggests the associations of miR-29 with insulin resistance in Goto Kakizaki mice. The expression of miR-29a and miR-29b significantly increases in 3T3-L1 cells after incubating with high glucose and various doses of insulin.[8] miR-29a and miR-29b are associated with caveolin 2, insulin-induced gene 1, and phosphatidylinositol 3-kinase regulatory subunit-α in the insulin signaling pathway.[9,10]

miR-29b, also known as redoximiRs, is a dominant micro-RNA in regulating genes and oxidative pathways associated with the incidence of DM.[11-13] In this study, we investigate the circulation of miR-29b in plasma of Javanese T2DM patients at the Primary Health Care Center in Semarang, Indonesia, and its correlation with fasting blood glucose (FBG), cholesterol, and triglyceride.

Materials and Methods

Research Design

The case–control study was performed by a consecutive sampling of T2DM patients in the Chronic Disease Management Program (PROLANS) program at the Primary Health Care Center in Semarang, Indonesia. The inclusion criteria were Javanese T2DM patients for the case group and non-T2DM with age 30-70 years for the control group. The exclusion criteria were having a history of cardiovascular disorders such as stroke, heart failure, and acute myocardial infarction. A total of 30 patients for the case group and 17 patients for the control group were used in this study. All respondents had signed the informed consent as a sign of willingness to participate in this study. This work was approved by the Ethics Commission of the Faculty of Medicine, Universitas Muhammadiyah Semarang, with Number 040/EC/FK/2019.

Sample Analysis

Blood samples were obtained from the vein to examine the fasting blood sugar level and lipid profile (triglycerides and cholesterol). The plasma was used for micro-RNA examination. The RNA extraction was conducted using miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany) (cat. no. 217204). RNA was transcribed into cDNA using the miRCURY LNA RT Kit (cat. no. 339340, Qiagen). Quantitative real-time PCR was performed using ExiLent SYBR Green master mix reagent (Qiagen, cat. no. 339345).

After RNA was extracted, a total of 2 µL RNA was transcribed into cDNA by mixing with 4.5 µL nuclease-free water, 2 µL RT Rx buffer, 1 µL RT enzyme mix, and 0.5 µL UniSp6 RNA spike. The solution was mixed with free water, 2 µL RT Rx buffer, 1 µL RT enzyme mix, and 0.5 µL UniSp6 RNA spike. For PCR amplification, the solution was conducted in a ProFlex thermal cycler at 42°C for 60 minutes, 95°C for 5 minutes, and immediately cool to 4°C. miR-29b expression was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR), by mixing 3 µL cDNA, 1 µL free water, 5 µL ExiLent SYBR Green master mix, 1 µL PCR primary mix, and 0.05 µL Rox reference dye. The RT-PCR Program was performed on 7500 fast machines, with initial denaturation at 95°C for 2 minutes, followed by amplification for 40 cycles at 95°C for 10 seconds, 60°C for 60 seconds (melting curve 60°C-95°C). Hsa-mir-29b-3p and U6 snRNA were used as primers for RT-PCR.

micro-RNA amplification values were analyzed using the 7500 Fast RT-PCR system v2.3 software to obtain the value of Ct, the melt curve, and the melt peak curve. An increase or decrease in comparison in the target expression was analyzed using the LIVAK method is the $2^{-\Delta\Delta Cq}$ method used to calculate the relative quantification of microRNA expression in plasma of T2DM patients compared to control with U6 snRNA as a reference gene.

Statistical Analysis

miR-29b expression was presented as mean ± SD and compared to the T2DM group and control group using an independent *t*-test. The correlations between miR-29b expression with fasting blood sugar, cholesterol, and triglycerides were measured using a Pearson correlation test with a significance of $P < .05$ and CI = 95%. Multivariate analysis was conducted using a multiple regression test to determine the most important factor on miR-29b expression. All data were analyzed using the Statistical Package for Social Sciences, version 17.0 software (SPSS Inc.; Chicago, IL, USA).

Results

A total of 30 T2DM patients and 17 patients for control was used in this study. The highest levels of FBG, cholesterol, and triglycerides were 199.70 ± 14.88 mg/dL, 200.27 ± 7.69 mg/dL, and 201.97 ± 22.42 mg/dL in the T2DM group. We found that the highest body mass index (BMI) was 24.55 ± 0.92 in the control group. There were significant differences between the T2DM and control groups with $P < .005$, that is, for the age parameters, FBG, and triglycerides. There was no significant difference between sex, BMI, and cholesterol (Table 1).

The miR-29b relative expression is presented in Table 2. We found that the fold change (2^{-We}) value was positive with a value of 14.12

Table 1. Sample Characteristics and Biochemical Data

Variable	T2DM Group (n = 30) Mean ± SD	Control (n = 17) Mean ± SD	*P*
Age (years)	57.60 ± 1.39	45.35 ± 1.59	.000**
Gender			
Female	24	12	.493*
Male	6	5	
Body mass index	24.33 ± 0.57	24.55 ± 0.92	.929**
Fasting blood glucose (mg/dL)	199.70 ± 14.88	96.41 ± 2.55	.000**
Cholesterol (mg/dL)	200.27 ± 7.69	182.53 ± 11.06	.084**
Triglyceride (mg/dL)	201.97 ± 22.42	141.71 ± 18.87	.043**

T2DM, type 2 diabetes mellitus; SD, standard deviation.
*Chi-square.
**Mann–Whitney *U*-test.

MAIN POINTS

- miR-29b overexpression occurred in the plasma of Javanese type 2 diabetes mellitus patients.
- miR-29b expressions were negatively correlated with age.
- miR-29b expressions were negatively correlated with fasting blood glucose.

Table 2. Expression of miR-29b in T2DM Patients and Control Group

	T2DM (n = 30)	Control (n = 17)			Fold Change	
Variable	Mean ± SD	Mean ± SD	***P***	**ΔΔCt miR-29b**	2^{-ean}	**Regulation**
ΔCT	−1.38 ± 1.94	2.44 ± 2.28	.000	−3.82	14.12	Upregulation

T2DM, type 2 diabetes mellitus; SD, standard deviation.

according to the LIVAK method. It revealed that the expression of plasma miR-29b in T2DM patients was increased compared to the control group. miR-29b levels between the T2DM and control groups were significantly different with $P = .000$ (Figure 1).

Furthermore, there was a significant negative correlation between age and miR-29b variables ($P = .000$, $r = -0.627$) and between FBG and miR-29b variables ($P = .000$, $r = -0.504$). There were no significant correlations among BMI, cholesterol, and triglycerides (Table 3).

Based on the multivariate analysis results (Table 4), it was found that the variables with $P < .005$ were age and FBG, with $P = .000$ and $P = .021$, respectively. These results showed that age was the most important risk factor of the miR-29b levels.

Discussion

In the present study, we investigated the relationship between the miR-29b and DM incidence, especially in Javanese ethnicity. Based on the Household Life Aspect Survey data, the highest incidence of diabetes in Indonesia was Javanese and Madura ethnicity, so this study focuses on the incidence of diabetes in this ethnic group.[14]

micro-RNA has been proven to affect the oxidative stress in T2DM through the increase of reactive oxygen species or a decrease in antioxidant expression. Micro-RNA impact oxidative stress in diabetes pathogenesis through several mechanisms, including Nrf2 is (nuclear factor erythroid 2-related factor 2) –Keap1 (Kelch ECH associating protein 1) pathway, Sirtuin-1 (SIRT1), PGC is peroxisome proliferator-activated receptor-gamma coactivator-1α, superoxide dismutase, catalase, and NADPH is nicotinamide adenine dinucleotide phosphate hydrogen oxidase 4 (NOX4).[15-18] Nuclear facto r-erythroid-2-related factor 2 (Nrf-2) regulates the gene expression of antioxidant Nuclear factor-erythroid-2 (NF-E2)-related factor 2 (Nrf-2) regulates the expression of antioxidant and cytoprotective genes against oxidative stresses.[19] Nrf-2 interacts with Kelch-like golgi apparatus membrane protein-like protein ECHIDNA (ECH)-associated protein (KEAP1) that triggers the proteasomal degradation and increases along with the reduced cellular antioxidant responses. miR-29b is a KEAP1 expression regulator. Reduced expression of miR-29b occurs in diabetes and increases NRF-2 expression and oxidative stress.[20,21] These results are contradictory to our findings.

We demonstrated an increased expression of miR-29b in plasma of Javanese T2DM patients. These results were in line with a study conducted by Hung[22] using T2DM mice liver that observed miR-107, miR-148b, miR-222, miR-802, miR-34a, and miR-29a/b/c. Our study showed that miR-29b expression in T2DM was upregulated to 1.5-fold from normal conditions. In contrast, another study conducted by Zampetaki et al[23] showed a decrease of miR-29b, miR-126, and miR-223 expression in T2DM plasma.

miR-29b was overexpressed in this study. This is similar to a research by He et al that examined miR-29a/b/c in the muscle, fat, and liver of diabetic mice. An elevated level of miR-29b caused insulin resistance and decreased insulin-induced glucose uptake in adipocyte 3T3-L1 cells. Of the 3 miR-29 families, only miR-29a and miR-29b were modulated by hyperinsulinemia and hyperglycemia in DM.[8] Another study demonstrated that miR-29a and miR-29c had a role in T2DM patients to regulate glucose of skeletal cells. An increase in expression only occurs in miR-29a and miR-29c, while miR-29b has no change in expression. This is contradictory to our results. An increase in miR-29a and miR-29c expression was the major cause of glucose reduction by 20% not in miR-29b.[24]

Furthermore, miR-29b expression decreases in diabetes and increases the expression of proteasome activator and its activity in

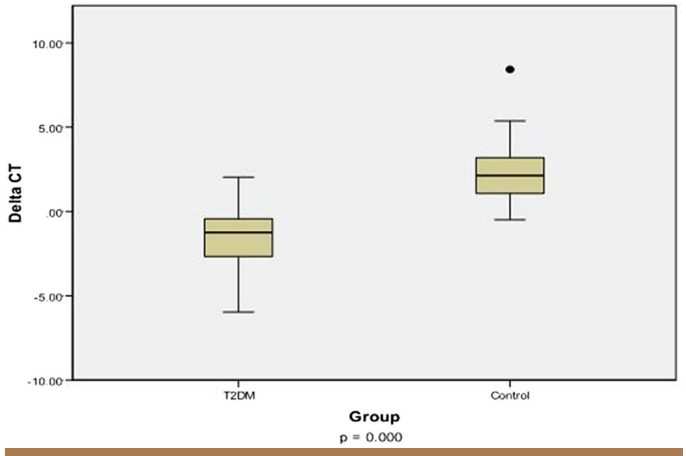

Figure 1. ΔCT comparison of miR-29 expression in plasma of T2DM group (n = 30) and control group (n = 17) with $P = .00$. T2DM, type 2 diabetes mellitus.

Table 3. Correlation Between Parameters and miR-29b Expression

Variable	miR-29b *P*-value	*r*
Age	.000	−0.627
Body mass index	.749	0.048
Fasting blood glucose	.000	−0.504
Cholesterol	.056	−0.280
Triglyceride	.108	−0.238

Table 4. Multivariate Analysis to Determine Risk Factors of miR-29b Expression

Variable	*B*	SE	*P*
Age	−0.143	0.037	.000
Body mass index	−0.041	0.094	.662
Fasting blood glucose	−0.010	0.004	.021
Cholesterol	−0.007	0.007	.331
Triglyceride	0.000	0.003	.744

SE, standard error.

endothelial cells. Proteasome has an important role in the degradation of nuclear factor kappa B (NFκB α) inhibitor. The elevated activity of proteasome will increase the activity of transcript factors NFκB and NOX. They trigger oxidative stress.[25]

Another study using Cmah-null mice showed that miR-29b-3p was significantly upregulated in the pancreas and liver through PI3K/AKT in the insulin signaling pathway.[26] Overexpression of miR-29b inhibits the insulin-induced phosphorylation of AKT in the hepatocyte. It reveals that micro-RNA acts as a negative regulator of insulin signaling.[27] 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside is also involved in insulin signaling that regulates hepatic glucose and lipid metabolism. This pathway involves miR-29b downregulated against the p85 target genes that inhibit AKT insulin-induced phosphorylation in wild-type C57BL/6 mouse hepatocytes.[28] This is in line with our study that the overexpression of miR-29b may inhibit the insulin signaling pathway in T2DM patients.

The role of miR-29b in insulin resistance involves a facilitated glucose transporter member 4 (GLUT4) along with hexokinase-2 (HK2). In skeletal muscle and white adipose tissue, glucose uptake is facilitated by GLUT4 through Akt activation.[29,30] If glucose gets into the cells, it will be phosphorylated by HK2 into glucose-6-phosphate and affect further metabolism. A decreased expression of GLUT4 in the muscle cells was commonly found in diabetic mice and humans.[31,32] Moreover, there was a decreased expression of Slc2a4/GLUT4 and Hk2/HK2 and increased expression of mir-29b-3p.[33] An increased expression of miR-29b-3p and miR-29c-3p was found in streptozotocin (STZ), a compound that has a toxicity toward pancreatic β cells-diabetic rats. GLUT4 and HK2 were negatively correlated with miR-29b-3p.

The miR-29b roles in DM are also associated with other proteins, including Secreted Protein Acidic Rich in Cysteine (SPARC). Secreted Protein Acidic Rich in Cysteine is a protein produced by adipocytes that regulate adipogenesis, adipocyte differentiation, and hyperplasia.[34] Obesity and insulin resistance have an impact on SPARC expression in T2DM.[35] miR-29s also regulates SPARC by binding with 3′-UTR. Overexpression of SPARC increases insulin secretion in beta cells and GLUT4 production in the 3T3-L1 adipocyte.[36] Song et al[37] suggest that the overexpression of miR-29 (consisted of miR-29 a,b,c) downregulates SPARC that leads to GLUT4 downregulation in the 3T3-L1 adipocyte. It induces insulin resistance due to the decrease in glucose uptake. This could be one of the theories that are in line with our findings. SPARC is involved in miR-29b overexpression in T2DM.

This study also analyzed the correlation of miR-29 levels with patient characteristics and clinical chemical parameters. The results showed that there was a negative correlation between age and miR-29b (the most important factor) and between fasting blood glucose and miR-29b. The miR-29b level is correlated with fasting blood glucose. The higher the expression of miR-29b, the fasting blood glucose level will decrease. This is in line with Liang's study that demonstrates that overexpression of miR-29a-c in rat liver decreases fasting blood glucose through negative regulation of hepatic gluconeogenesis. The decrease is mediated by the downregulation of PGC-1a and G6Pase proteins that are targets of miR-29a-c. The decrease in PGC-1a and G6Pase proteins causes a reduction in liver glucose production.[38] However, Zampetaki et al[23] showed that many micro-RNAs were inversely correlated with FBG, except miR-29b and miR-320.

miR-29b levels are considered to decrease with increase in age. Circulating miRNA is simultaneously detected in the blood as a biomarker, and its level is influenced by several factors, including age. Moreover, some miRNAs such as miR-24 and miR-130b-5p are negatively correlated with age.[39] However, levels of plasma miR-29b related to age have never been reported, although increasing levels of miR-29b with age in mice and human brains have been discovered.[40] Therefore, this study can be used as a reference that miR-29b levels are correlated with the age of T2DM patients.

This study did not simultaneously measure miR-29b with the target protein such as NRF2-KEAP1, Sirtuin 1 (SIRT1), Toll-like Receptor 7 (TLR7), GLUT, PI3K/AKT, SPARC, PGC-1a, and G6Pase so that further research may consider specific protein targets.

Conclusions

miR-29b overexpression occurred in plasma of Javanese T2DM patients at the Primary Health Care Center in Semarang, Indonesia, that is, 14.12-fold overexpression. The level of miR-29b expression was negatively correlated with age and FBG.

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Ethics Committee Approval: This research has received ethical approval from the Ethics Commission of the Faculty of Medicine, Universitas Muhammadiyah Semarang, with Number 040/EC/FK/2019.

Informed Consent: Written informed consent was obtained from the patients who participated in this study.

Peer-review: Externally peer-reviewed.

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Declaration of Interests: The authors declare that they have no competing interest.

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