Identification of circulating miR-101, miR-375 and miR-802 as biomarkers for type 2 diabetes

Running title: Circulating miRNA in diabetes

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Abstract

Purpose. The unique circulating microRNAs (miRNAs) observed in patients with type 2 diabetes (T2D) are candidates as new biomarkers and therapeutic targets. In order to identify circulating miRNAs relevant to the disease process in case of type 2 diabetes, we performed the Illumina sequencing of miRNAs derived from the serum, liver and epididymal white adipose tissue (WAT) of diet-induced obese male C57BL/6J mice.

Basic procedures. We selected four miRNAs, miR-101, miR-335, miR-375, and miR-802, which are increased in the sera and tissues of obese mice, and measured the serum levels of miRNAs in T2D and subjects with normal glucose tolerance (NGT).

Main findings. The serum concentrations of miRNAs, $\log_{10}$miR-101, $\log_{10}$miR-375, and $\log_{10}$miR-802, were significantly increased in the T2D patients compared with NGT subjects (1.41±2.01 *v.s.* -0.57±1.05 ($P=1.36\times10^{-5}$), 0.20±0.58 *v.s.* 0.038±1.00 ($P=3.06\times10^{-6}$), and 2.45±1.27 *v.s.* 0.97±0.98 (P=0.014), respectively). The $\log_{10}$miR-335 values did not demonstrate any significant differences between the T2D and NGT groups (-1.08±1.35 *v.s.* -0.38±1.21 (P=0.25)). According to the stepwise regression analysis, the HbA1c was an independent predictor of miR-101. Regarding the serum miR-802 levels, eGFR, HbA1c and HDL-C values were identified as significant determinants.

Principal conclusions. The present findings demonstrated that the circulating miR-101, miR-375 and miR-802 levels are significantly increased in T2D patients versus NGT subjects and they may become the new biomarkers for type 2 diabetes.

Key words: miRNA, liver, adipose tissues, serum, diabetes

Abbreviations: BAT, brown adipose tissue; BMI, body mass index; Cr, serum creatinine; DPB, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HDL-C, HDL cholesterol; HFHS chow, high fat-high sucrose chow; hiPSCs, human induced pluripotent stem cells; IRI, immunoreactive insulin; LDL-C, LDL cholesterol; miRNA, microRNA; STD chow, standard chow; NGT, normal glucose tolerance; SBP, systolic blood pressure; T2D, type 2 diabetes, T-Cho, total cholesterol; TG, triglyceride; WAT, white adipose tissue

Introduction

MicroRNAs (miRNAs) have been identified as a new class of regulatory RNAs that are critically involved in the control of the expression of various genes. Mature miRNAs are short with 18-25 nucleotides and single-stranded RNAs derived from longer primary transcripts, pri-miRNAs, via sequential processing in the nucleus and cytoplasm. Based on the degree of complementarity between miRNAs and the 3'-untranslated region (UTR) sequences on target genes, miRNAs regulate the expression of the target genes via either mRNA degradation/cleavage or the inhibition of translation[1]. Recently, miRNAs have been reported to be stable in the serum and plasma[2], and miRNAs are actively secreted via microvesicles, exosomes, apoptotic bodies and lipoproteins. miRNAs are possibly transferred from donor cells to recipient cells where they alter the gene expression of recipient cells, suggesting their potential roles in intercellular communication[3, 4]. Identifying unique circulating microRNAs in patients with diabetes may be beneficial for discovering new biomarkers and therapeutic targets[5]. In such attempts, the profiling of circulating miRNAs has been reported in subjects with newly diagnosed type 1 diabetes[6], type 2 diabetes[7-10], vascular complications[11-13], obesity[14, 15] and metabolic syndrome[16]; however, the tissue sources and biological significance of these miRNAs remain entirely unknown.

In order to facilitate the identification of circulating miRNAs relevant to the disease process associated with type 2 diabetes, we performed expression profiling of miRNAs derived from the serum, liver and epididymal white adipose tissue (WAT) of male C57BL/6J mice fed with standard (STD) or high fat-high sucrose (HFHS) chow using Illumina sequencing. We next selected four miRNAs, miR-101, miR-335, miR-375 and miR-802, which are increased in the serum in association with their upregulation in the liver or WAT. Finally, we measured the serum levels of miRNAs and identified the circulating miR-101, miR-375 and miR-802 levels to be new biomarkers in patients with

type 2 diabetes.

Methods

Animals

Male C57BL/6J (Charles River Laboratories Japan, Yokohama, Japan) mice were housed in cages and maintained on a 12-hour light-dark cycle. The mice at 4 weeks of age were fed with STD (NMF; Oriental Yeast) or HFHS chow (D12331; Research Diet, New Brunswick, NJ), subsequently sacrificed at 20 weeks of age and subjected to the following studies. All animal experiments were approved by the Animal Care and Use Committee of the Department of Animal Resources, Advanced Science Research Center, Okayama University.

Expression profiling of miRNAs using Illumina sequencing

Total RNAs were isolated from the serum and various tissues using QIAamp Circulating Nucleic Acid Kit and miRNeasy Mini kit (Qiagen, Hilden, Germany), respectively. We pooled the mouse sera and extracted total RNA containing miRNA. The quality of total RNAs derived from various tissues was confirmed by measuring the ratio of 28S/18S using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNAs were then subjected to Illumina TruSeq Small RNA Sample Preparation protocol (Illumina, San Diego, CA), including 3'- and 5'- adapter ligation, reverse transcription, PCR amplification, and pooled gel purification to generate a library product. Sequencing was performed using Genome Analyzer IIx (Illumina) and the obtained data were mapped to mouse genome sequence and annotated (bowtie-0.12.7). In each group, the read numbers of known miRNAs were counted and compared. The whole raw and processed data are freely accessible in the Gene Expression Omnibus (GEO) under the accession number GSE61959.

Quantitative PCR of miRNA in the mouse sera

We sacrificed male C57BL/6 mice under the chow diet and those with diet-induced obesity at 20 weeks of age, and extracted total RNA including miRNA from various tissues, using miRNeasy Mini Kit. For the quantitative real time PCR analysis, cDNAs synthesized from 10 ng of total RNA were amplified in the presence of TaqMan Small RNA Assays using StepOnePlus Real Time PCR System (Applied Biosystems, Carlsbad, CA). The relative abundance of miRNA was standardized according to that of snoRNA202 (AF357327) and snoRNA234 (AF357329) using the geometric mean of these internal controls (Applied Biosystems) (**Supplemental Table 1**).

Cross-sectional clinical study

Japanese subjects with normal glucose tolerance (NGT) (n=49, 46.0 ± 9.67 years) and patients with type 2 diabetes (T2D) (n=155, 62.3 ± 13.2 years) were enrolled in this study. The patients were treated with metformin (n=57), insulin (n=56), α-glucosidase inhibitors (n=54), sulfonylureas (n=39), pioglitazone (n=33), glinides (n=30) and DPP-4 inhibitors (n=10). Patients with an estimated glomerular filtration rate (eGFR) < 15 ml/min/1.73 m^2 or under dialysis were excluded from the current study. All recruited NGT subjects and T2D patients agreed to undergo measurements of the serum levels of miRNA after providing their informed consent. The study was conducted in accordance with the ethical principle of the Declaration of Helsinki and approved by the ethics committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (#736).

Quantitative RT-PCR of miRNA in the human sera

Total RNA containing miRNA was extracted from the human sera using QIAamp

Circulating Nucleic Acid kit (Qiagen) and cleaned with RNeasy MinElute Cleanup kit (Qiagen). *C. elegans* spiked-in control miRNA with 50 fmol of cel-miR-39 (Applied Biosystems) in a 2.5-μl total volume of water was added to 1 ml of human serum. We then performed the purification procedures following the manufacturer's protocol, after which the miRNA was eluted with 40 μl of RNase free water. Reverse transcription reactions were performed using the Taqman miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied BioSystems) (**Supplemental Table 1**). The RT products were subjected to real-time PCR in duplicate using Taqman Universal PCR Master Mix (2X), No AmpErase UNG and TaqMan Small RNA Assay (Applied BioSystems), and quantitative real time PCR analysis was performed on the StepOnePlus Real Time PCR System (Applied Biosystems) as follows: 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds, 60°C for 1 minute, and soaked at 4°C.

Statistical analysis

The serum immunoreactive insulin (IRI) and miRNA levels did not show a normal distribution, while the log transformed data followed normal distribution as demonstrated by Shapiro-Wilk test. Therefore, we used the log transformed data of IRI and miRNAs for the parametric statistical analyses. The data are expressed as the mean ± standard deviation and analyzed using the unpaired Student's *t* test. Pearson correlation coefficients were used to evaluate whether the serum levels of the miRNAs correlated with various clinical parameters. To determine variables independently associated with serum levels of miRNAs in the T2D patients, a multiple regression analysis was performed by including age, HbA1c, postprandial glucose (PG), and body mass index (BMI) as independent variables for miR-101. For miR-802, age, eGFR, triglycerides (TG), high density lipoprotein cholesterol (HDL-C), HbA1c, and PG were employed as

independent variables. A *P* value of < 0.05 was regarded as being statistically significant. The data were analyzed with IBM SPSS Statistics (IBM, Armonk, NY), and the effect size (Cohen's *d*) and statistical power (1-β) were calculated by G*Power program (http://www.gpower.hhu.de/).

Results

Identification of miR-101, miR-335, miR-375 and miR-802 in the sera of the C57BL/6J fed with HFHS chow

Total RNAs were isolated from the serum and various tissues using QIAamp Circulating Nucleic Acid Kit and miRNeasy Mini kit. Sequencing was performed using Genome Analyzer IIx (Illumina), and the obtained data were mapped to the mouse genome sequence and annotated. The number of mapped reads reached more than 20,000,000 in the serum, liver and epididymal WAT (**Supplemental Table 2**). The total reads per million mapped reads was quite uniform in all experimental groups (**Supplemental Table 3**). Among the mapped RNAs in the serum samples, tRNAs were abundant, while miRNAs and small nucleolar RNAs (snoRNAs) were less abundant compared with that observed in the liver and WAT. We next searched the miRNAs in which the read numbers were upregulated in the serum and in either the liver or WAT in the male C57BL/6J mice fed with HFHS in comparison with the mice fed with STD. Consequently, the miR-101, miR335, miR-375 and miR-802 levels were identified, and the expression of these miRNAs was investigated in various tissues of the male C57BL/6J mice (**Supplemental Table 4**). The expression of miR-101 in the epididymal WAT samples, miR-335 in all WAT samples and brown adipose tissue (BAT) samples, miR-375 in BAT samples, and miR-802 in liver and BAT samples was significantly increased in the male C57BL/6J mice fed with HFHS (**Figure 1**). Interestingly, all miRNAs were abundantly expressed in the pancreatic tissues; however, no upregulation of these miRNAs was

observed in the pancreatic tissues in C57BL/6J mice fed with HFHS.

Serum levels of miRNAs in the NGT subjects and T2D patients

The serum concentrations of miRNA, log_{10}miR-101, were significantly increased in the T2D group versus NGT group (1.41±2.01 *v.s.* -0.57±1.05, P=1.36×10^{-5}) (**Table 1**). The Log_{10}miR-101 values positively correlated with age (R=0.186, P=0.025), BMI (R=0.197, P=0.019), HbA1c (R=0.331, P=4.61×10^{-5}), and PG (R=0.270, P=9.99×10^{-4}) (**Figure 2**). The multiple regression analysis employing age, BMI, HbA1c and PG as independent variables revealed that HbA1c was the only significant determinant for the serum miR-101 levels (**Supplemental Table 5**). The stepwise regression analysis also showed only HbA1c to be an independent variable (**Supplemental Table 5**).

The serum concentrations of miR-335 did not demonstrate any significant differences between the T2D and NGT groups (-1.08±1.35 *v.s.* -0.38±1.21, P=0.25) (**Table 1**). The log_{10}miR-335 values negatively correlated with both the PG (R=-0.191, P=0.034) and HbA1c (R=-0.267, P=2.88×10^{-3}) levels (**Figure 3a and 3b**). The upregulation of serum miR-335 observed in the DIO mice was not demonstrated in T2D.

The log_{10}miR-375 values were significantly increased in the T2D group versus the NGT group (0.20±0.58 *v.s.* 0.038±1.00, P=3.06×10^{-6}) (**Table 1**). The log_{10}miR-375 values demonstrated a negative correlation with age (R=-0.126, P=0.072), which did not reach a statistically significant level, and a positive correlation with the TG levels (R=0.172, P=0.014) (**Figure 3c and 3d**).

The log_{10}miR-802 values were significantly increased in the T2D group versus the NGT group (2.45±1.27 *v.s.* 0.97±0.98, P=0.014) (**Table 1**). The log_{10}miR-802 values positively correlated with age (R=0.129, P=1.82×10^{-3}), TG (R=0.276, P=8.24×10^{-5}), HbA1c (R=0.293, P=2.49X10^{-5}) and PG (R=0.248, P=3.78×10^{-4}), and negatively correlated with eGFR (R=-0.259, P=4.08X10^{-4}) and HDL-C (R=-0.271, P=1.20X10^{-4})

(**Figure 4**). The stepwise regression analysis revealed that eGFR, HbA1c and HDL-C were significant determinants of the serum miR-802 levels (**Supplemental Table 6**).

Discussion

In the current study, we found that the circulating miR-101, miR-375, and miR-802 levels to be increased in T2D patients and may be new biomarkers for type 2 diabetes. Among these miRNAs, miR-375 is well-described as a pancreatic islet-specific miRNA, that suppresses glucose-induced insulin secretion by inhibiting the expression of myotrophin[17]. miR-375 is highly expressed during human pancreatic islet development[18] and is essential for normal glucose homeostasis, β cell turnover, and adaptive β cell expansion in response to increasing insulin demand under a state of insulin resistance[19]. In addition, miR-375 promotes the pancreatic differentiation of human induced pluripotent stem cells (hiPSCs)[20]. Recently, Sun K *et al.* reported that the miR-375 promoter is hypomethylated in patients with type 2 diabetes and that the plasma levels of miR-375 are upregulated in these patients compared with controls exhibiting normal glucose tolerance[21]. The expression profile of miR-375 in the various tissues of the male C57BL/6J mice noted in the current study revealed an abundant expression of miR-375 in the pancreatic tissues; however, this parameter was not upregulated in the mice fed with the HFHS chow. In our experiments, miR-375 was significantly unexpectedly increased in the BAT samples in the mice fed with HFHS chow (**Figure 1c**). Hence, it remains unknown whether the source of increased circulating miR-375 in T2D is mainly from pancreas or other tissues.

In contrast to that observed for miR-375, there are scarce data regarding miR-101 and miR-802 in terms of the pathogenesis of type 2 diabetes. miR-101 targets EZH2 at the posttranscriptional level in the cell-lines of intraductal papillary mucinous neoplasm of the pancreas. The expression of miR-101 has been reported to be significantly lower,

while that of EZH2 mRNA is higher in malignant cell lines[22]. Although miR-101 is involved in the process of carcinogenesis in the pancreas, there are no reports of the involvement of miR-101 in onset of insulin resistance and development of diabetes[23]. With respect to the expression profile of miR-101 in various tissues in the male C57BL/6J mice, an abundant expression of miR-101 was demonstrated in the pancreatic tissues (**Figure 1a**). Again, it remains unknown whether the source of the increased circulating miR-101 level in the setting of T2D is thepancreas or other tissues; however, we speculate that miR-101 may be derived from WAT, since the expression of miR-101 was increased in the epididymal WAT of the male C57BL/6J mice fed with HFHS chow.

Recently, Kornfeld J-W et al. reported that the miR-802 levels are increased in the liver in high fat diet-fed mice, *db/db* mice and overweight subjects in a cohort of human individuals[24]. We also demonstrated the upregulation of miR-802 in the liver and BAT in male C57BL/6J mice fed with HFHS chow. In a previous report, the transgenic expression of miR-802 impaired glucose tolerance, a reduction in the miR-802 expression improves the insulin action, and the hepatic overexpression of miR-802-targeted *Hnf1b* improves the insulin sensitivity in *db/db* mice[24]. As the circulating miR-802 level well-correlates with the eGFR, HbA1c and HDL-C values, we further demonstrated that the circulating miR-802 level is a new biomarker for type 2 diabetes with metabolic syndrome.

Although we failed to confirm miR-335 as a circulating marker for type 2 diabetes, the expression of miR-335 was prominently upregulated in the adipose tissues of the male C57BL/6J mice fed with HFHS chow (**Figure 1b**). In previous studies, the upregulation of miR-335 was observed in the liver and adipose tissues of ob/ob, db/db and KKAy mice[25], and the possible role of miR-335 in the pathogenesis of adipose tissue inflammation has been postulated[26]. Furthermore, miR-335 has been shown to

be upregulated in the pancreatic islets of Goto-Kakizaki (GK) rats, an animal model of non-obese spontaneous type 2 diabetes[27]. In the present study, we compared lean mice to HFHS-chow-induced obese mice in animal experiments and compared subjects with normal glucose tolerance with patients with type 2 diabetes; BMI values in the human cohort were similar, and neither group was obese on average. The ability to extrapolate the results obtained in mice to humans is substantially limited, and further investigation of the circulating miR-335 levels in overweight and obese subjects is required to determine whether the circulating miR-335 level is a biomarker of obesity and/or metabolic syndrome.

The strength of the current investigation is that elevation of the serum miR101, miR375, and miR-802 levels is a common phenotype in both rodents and human patients with T2D. The establishment of gene-manipulated models in mice would facilitate functional analyses of these miRNAs and promote translational research for diagnosis and therapy for T2D. The weakness of this study lies in the analysis of statistical power and the effect size of the statistical tests. Although the serum levels of miR-101, miR-375 and miR-802 were significantly higher in the patients with T2D, the effect size (Cohen's *d*) and statistical power (1-β) were lower for the serum levels of miR-375 (**Table 1**). Similarly, the statistical power of simple correlations with miR-375 was also lower for age (1-β=0.436) and TG (1-β=0.695) (**Figure 3**). Another problem is the effect of multiple comparisons when comparing the expression of many genes between two groups[28, 29]. In order to avoid family-wise type I error, the Bonferroni adjustment may be used and a p value of 0.0125 may be significant, as four miRNAs were measured in the current investigation (0.05/4=0.0125). As shown in Table 2, the serum levels of miR-101 and miR-375 remained significantly higher in the patients with T2D, whereas significant differences were not observed for miR-802.

In conclusion, hundreds of miRNA are released into the circulation and may be used for the early detection of disease, including evaluations of insulin resistance and predictions of long-term complications in patients with T2D. In present study, large-scale sequencing of miRNAs in the serum, liver and WAT in the male C57BL/6J mice under HFHS chow identified miR-101, miR335, miR-375, and miR-802 as such candidates. Finally, we demonstrated that the circulating miR-101, miR-375, and miR-802 levels are significantly increased in patients with T2D compared with NGT and may be used as new biomarkers for T2D. Nevertheless, clinical questions as to whether the circulating miR-101, miR-375, and miR-802 levels can be used to detect glucose intolerance and predict the development of insulin resistance and vascular complications remain for investigation in future studies.

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Authors' contributions: C.H., J.W., and A.N. participated in the design of the study and J.E., S.T., M.K., A.K., K.M., D.O., S.S., and H.M. participated in the recruitment of the patients. A.N. and J.W. analyzed the results of Illumina sequencing, and C.H. and K.M. carried out quantitative RT-PCR of all samples. C.H., J.W., A.N., and H.M. and conceived of the study design, participated in coordination, performed the statistical analyses and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1 Quantitative PCR of miRNAs, miR-101, miR-335, miR-375, and miR-802, in various tissues of the male C57BL/6J mice under standard (STD; n=3) and high fat-high sucrose (HFHS; n=3) chow. WAT, white adipose tissue; BAT, brown adipose tissue. **P < 0.01, *P < 0.05; STD v.s. HFHS.

Figure 2 Serum $\log_{10}$miR-101 levels in the Japanese subjects with normal glucose tolerance (NGT) (n=49) and the patients with type 2 diabetes (T2D) (n=155). Simple correlations between the $\log_{10}$miR-101 values and various parameters: age (**a**), body mass index (BMI) (**b**), HbA1c (**c**), and postprandial glucose (PG) (**d**).

Figure 3 Serum $\log_{10}$miR-335 and $\log_{10}$miR-375 levels in the Japanese subjects with normal glucose tolerance (NGT) (n=49) and the patients with type 2 diabetes (T2D) (n=155). Simple correlations between the $\log_{10}$miR-335 values and postprandial glucose (PG) (**a**) and HbA1c (**b**) and between the $\log_{10}$miR-375 values and age (**c**) and triglycerides (TG) (**d**).

Figure 4 Serum $\log_{10}$miR-802 levels in the Japanese subjects with normal glucose tolerance (NGT) (n=49) and the patients with type 2 diabetes (T2D) (n=155). Simple correlations between the $\log_{10}$miR-101 values and various parameters: age (**a**),

445 estimated glomerular filtration rate (eGFR) (**b**), triglycerides (TG) (**c**), HDL-cholesterol
446 (**d**), HbA1c (**e**) and postprandial glucose (PG) (**f**).

Table 1 Clinical characteristics of the patients with type 2 diabetes (T2D) and subjects with normal glucose tolerance (NGT).

	T2D	NGT	Total	P value	Effect size (Cohen's *d*)	Statistical power (1-β)
Number (male/female)	155 (96/59)	49 (25/24)	204 (121/83)			
Age (years)	62.3±13.2	46.0±9.67	58.4±14.2	0.039*	1.40	1.00
BMI (kg/m^2)	25.9±4.97	23.6±4.05	25.3±4.85	0.33	0.50	0.87
SBP (mmHg)	130±16.4	123±16.4	128.5±16.6	0.94	0.43	0.74
DBP (mmHg)	75.2±11.4	76.7±10.9	75.6±11.2	0.97	0.13	0.13
PG (mmol/L)	8.67±3.05	5.18±0.55	7.80±3.10	9.54×10^{-13}**	1.60	1.00
HbA1c (%)	7.31±1.08	6.03±0.39	7.00±1.10	8.45×10^{-7}**	1.58	1.00
Log_{10}IRI (mU/L)	1.27±0.50	0.86±0.30	1.15±0.48	9.99×10^{-5}**	0.99	0.99
Cr (μmol/L)	76.0±41.9	63.0±12.5	72.4±37.6	0.023*	0.42	0.72
eGFR (mL/s)	1.22±0.37	1.43±0.26	1.25±0.36	0.091	0.66	0.98
T-Cho (mmol/L)	4.63±1.50	5.45±0.70	4.80±1.43	0.011*	0.70	0.99
TG (mmol/L)	1.54±0.79	0.98±0.56	1.41±0.78	0.027*	0.82	0.99
HDL-C (mmol/L)	1.57±0.47	2.05±0.57	1.69±0.53	0.24	0.92	0.99
LDL-C (mmol/L)	2.78±0.73	3.23±0.72	2.87±0.75	0.98	0.62	0.96
Log10miR-101	1.41±2.01	-0.57±1.05	0.96±2.02	1.36×10^{-5}**	1.23	1.00
Log10miR-335	-1.08±1.35	-0.38±1.21	-0.86±1.34	0.25	0.55	0.91
Log10miR-375	0.20±0.58	0.038±1.00	0.16±0.70	3.06×10^{-6}**	0.20	0.23
Log10miR-802	2.45±1.27	0.97±0.98	2.11±1.35	0.014*	1.30	1.00

BMI, body mass index; SBP, systolic blood pressure; DPB, diastolic blood pressure; Cr, serum creatinine; eGFR, estimated glomerular filtration ratio; T-Cho, total cholesterol; TG, triglycerides; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; *, $p < 0.05$; **, $p < 0.01$.