Investigation of umbilical cord serum miRNAs associated with childhood obesity: A pilot study from a birth cohort study

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INTRODUCTION
Childhood obesity is increasing globally and has become a serious problem worldwide1,2. It is reported that the incidence of obesity from 5 to 14 years was related to the body weight at the age of 5 years3. Furthermore, early adiposity rebound (AR) has become the focus of attention as a factor related to adult obesity4. Body mass index (BMI) rises from birth to around 1 year of age and then declines. After that, it generally rises again at the age of 6–7 years. This second rise is called AR.

MicroRNAs (miRNAs) are now attracting substantial attention as useful biomarkers of various diseases5–7. The fetal environment is thought to influence the likelihood of developing diseases later in life. To predict childhood obesity and subsequent adulthood obesity, it is important to explore useful markers reflecting the fetal environment. We hypothesized that umbilical cord serum miRNAs could be biomarkers acting as early predictors of childhood obesity. Here, we examined the association between umbilical cord serum miRNAs and the risk of obesity.

MATERIALS AND METHODS
Participants
This study was performed as an adjunct study of the Japan Environment and Children’s Study (JECS). The JECS protocol has been described previously8. The JECS is a birth cohort study comprising 15 study regions including Chiba. In this study, the participants followed by the Chiba Regional Center were included. We extracted groups at high and low risk of obesity. Early AR was considered to reflect a high risk of obesity. Those who had a higher BMI at 3 years than at 1.5 years were identified as having early AR9. Children with early AR and whose BMI at 5 years was over the 95th percentile were considered as the high-risk group. Children without early AR and whose BMI at the age of 5 years was within the 25th–75th percentile were classified into the low-risk group. The BMI percentile was determined according to the BMI percentile chart for Japanese children10. Of the 2,716 children whose BMI...
data were available at ages 1.5, 3, and 5, 66 (2.5%) were in the high-risk group for obesity and 1,279 (47%) were in the low-risk group. Five children were randomly selected from each group for comprehensive miRNA analysis. Moreover, the study included 28 children for whom umbilical cord serum could be prepared in the high-risk obesity group and 28 children who were randomly selected from the low-risk obesity group. Quantitative miRNA PCR was performed on these 33 children in each group.

### Umbilical cord serum miRNA extraction

Umbilical cord blood was obtained immediately after birth. Cord blood was centrifuged, and cord serum was obtained by J ECS and then stored at −20°C. miRNA was isolated from some of these samples using the mi RNeasy Mini Kit (Qiagen, Hilden, Germany).

### Comprehensive umbilical cord serum miRNA profiling

The serum levels of miRNAs were analyzed using the 3D-Gene® Human miRNA Oligo Chip (Toray Industries, Inc., Tokyo, Japan), which was designed to detect 2,632 miRNA sequences registered in miRBase release 20. The expression levels of miRNAs were globally normalized using the background-subtracted signal intensity of the entire miRNAs in each microarray11,12. Serum levels of miRNA were compared between the high- and low-risk groups. The top 10 miRNAs with the most significant findings (in terms of the P-value) were used for further analysis.

### Analysis of individual miRNAs

The serum levels of selected miRNAs were examined by quantitative real-time PCR. The TaqMan® microRNA assay (Thermo Fisher Scientific, San Jose, CA, USA), TaqMan® Reverse Transcription kit (Thermo Fisher Scientific), and TaqMan® Universal PCR Master Mix II (Applied Biosystems, Foster City, CA, USA) were used in accordance with the manufacturers’ instructions. Real-time PCR was performed in 96-well plates using ABI StepOne Plus Thermal Cycler (Applied Biosystems). Each PCR reaction was performed in triplicate. Fold changes in miRNA levels were calculated using the 2^ΔΔCt method and Spike-In cel-miR-39 was used as a normalization control13.

### Statistical analysis

All the reported data are expressed as mean ± standard deviation. To compare the data between groups, we performed a t-test or Wilcoxon’s rank-sum test according to their distribution, using GraphPad Prism 7. The Benjamini–Hochberg method was used to control for multiple testing.

#### RESULTS

### Participants’ characteristics

The clinical characteristics of the children enrolled in this study are shown in Table 1. At 5 years, height was 106.6 ± 4.7 and

| Table 1 | Clinical characteristics in children at low and high risk of obesity |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| miRNA           | Fold change     | P-value         |
| hsa-miR-516b-3p | 1.34085         | 0.0036          |
| hsa-miR-6721-5p | 1.26432         | 0.0255          |
| hsa-miR-4672    | 1.41865         | 0.0260          |
| hsa-miR-130a-3p | 3.05748         | 0.0284          |
| hsa-miR-3065-3p | 0.63102         | 0.0292          |
| hsa-miR-1260b   | 1.8545          | 0.0310          |
| hsa-miR-4709-3p | 0.87565         | 0.0349          |
| hsa-miR-194-3p  | 0.86472         | 0.0371          |
| hsa-miR-3907    | 1.73265         | 0.0399          |
| hsa-miR-612     | 1.26418         | 0.0426          |
| hsa-miR-671-5p  | 0.77169         | 0.0442          |
| hsa-miR-8055    | 0.74269         | 0.0457          |
| hsa-miR-4,286   | 1.44223         | 0.0480          |

Fold change: log2 fold change (children at high vs low risk of obesity).

Data are expressed as mean ± standard deviation. P-values are from Welch’s two-sample t-test. P-values <0.05 are shown in bold.
107.5 ± 4.4 cm, while BMI was 15.4 ± 0.5 and 19.1 ± 1.5 kg/m² in the low- and high-risk groups, respectively. Birth weight was 3,042 ± 268 and 3,073 ± 420 g, respectively.

**Selection of candidate umbilical cord serum miRNAs from the profiling**

To select candidate miRNAs for subsequent examination, the serum levels of the 2,632 miRNAs were evaluated and compared between five subjects from the high-risk group and five from the low-risk group. Of these miRNAs, 13 showed a crude (unadjusted for multiple tests) P-value <0.05 (Table 2).

**miRNA expression levels measured by qRT-PCR**

We selected the top 10 most significant miRNAs for qRT-PCR assay. However, miR-6721-5p, miR-3065-3p, miR-3907, and miR-612 could not be detected using this protocol. Therefore, we evaluated the expression levels of miR-516b-3p, miR-4672,
miR-130a-3p, miR-1260b, miR-4709-3p, and miR194-3p. The serum levels of miR-516-3p and miR-130a-3p were higher in the high-risk group than in the low-risk group. In contrast, the serum levels of miR-1260b, miR-4709-3p, and miR194-3p were lower in the high-risk group than in the low-risk group (Table 3, Figure 1). For miR-1260b, the results of comprehensive analysis and quantitative PCR showed the opposite patterns.

DISCUSSION
The present study identified five umbilical cord serum miRNAs that were differentially expressed between groups at high and low risk of obesity. Recently, many studies have revealed that nutrient and environmental exposure during the fetal period impacts on postnatal growth and diseases such as metabolic syndrome later in life through epigenetic mechanisms including altered microRNA expression. It is reported that a specific placental miRNA profile was related to prenatal and postnatal growth parameters. In addition, Marcondes et al. demonstrated that altered miR-181a in umbilical cord blood cells could be adopted as a biomarker for childhood obesity. Moreover, in the past few years, it has been reported that circulating miRNAs may also play a variety of biological roles, such as in energy homeostasis and metabolic processes. Three of the candidate miRNAs in this study have been reported to be associated with metabolism. Interestingly, the hepatic exosome-derived miR-130a-3p regulates energy metabolism in adipose tissues. Moreover, miR-1260b directly targets the 3'UTR of growth differentiation factor 11 and miR-194 was also reported to suppress the synthesis of glucagon-like peptide-1 in L cells. Taken together, our data suggest that umbilical cord serum miRNAs may be associated with the biological process of childhood obesity and could be new biomarkers for the early identification of future obesity. Our study this time has a limited number of samples. Therefore, future studies with larger sample sizes are required to verify our findings.

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DISCLOSURE
This study was supported by a grant from the Yamada Bee Company Inc. The sponsor had no control over the interpretation, writing, or publication of this work. Approval of the research protocol: The JECS protocol was approved by the Ministry of the Environment’s Institutional Review Board on Epidemiological Studies (Registration number: 2021-012) and by the ethics committees of all participating institutions. Approval date of registry of this adjunct study by Biomedical Research Ethics Committee of the Graduate School of Medicine, Chiba University was August 30, 2019 and registry no. of the study trial: 995(958). Informed consent: Written informed consent was obtained from all participating women in accordance with the Declaration of Helsinki. In conducting this adjunct study, we confirmed the consent of the participants by opt-out. Animal studies: Not applicable.

REFERENCES
1. de Onis M, Blössner M, Borghi E. Global prevalence and trends of overweight and obesity among preschool children. Am J Clin Nutr 2010; 92: 1257–1264.
2. Güngör NK. Overweight and obesity in children and adolescents. J Clin Res Pediatr Endocrinol 2014; 3: 129–143.
3. Cunningham SA, Kramer MR, Venkat Narayan KM. Incidence of childhood obesity in the United States. N Engl J Med 2014; 370: 403–411.
4. Rolland-Cachera MF, Deheeger M, Maillot M, et al. Early adiposity rebound: causes and consequences for obesity in children and adults. Int J Obes (Lond) 2006; 30(Suppl. 4): S11–S17.
5. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. PNAS 2008; 105: 10513–10518.
6. Vasu S, Kumano K, Darden CM, et al. MicroRNA signatures as future biomarkers for diagnosis of diabetes states. Cell 2019; 8: 1533.
7. Xie JX, Fan X, Drummond CA, et al. MicroRNA profiling in kidney disease: Plasma versus plasma-derived exosomes. Gene 2017; 627: 1–8.
8. Kawamoto T, Nitta H, Murata K, et al. Rationale and study design of the Japan Environment and Children’s study (JECS). BMC Public Health 2014; 14: 25.
9. Arisaka O, Sairenchi T, Ichikawa G, et al. Increase of body mass index (BMI) from 1.5 to 3 years of age augments the degree of insulin resistance corresponding to BMI at 12 years of age. J Pediatr Endocrinol Metab 2017; 30: 455–457.
10. Kato N, Takimoto H, Sudo N. The cubic functions for spline smoothed L, S and M values for BMI reference data of Japanese children. Clin Pediatr Endocrinol 2011; 20: 47–49.
11. Giovannetti E, van der Velde A, Funel N, et al. High-throughput microRNA (miRNAs) arrays unravel the prognostic role of MiR-211 in pancreatic cancer. PLoS One 2012; 7: e49145.
12. Sudo K, Kato K, Matsuoka J, et al. Identification of serum microRNAs predicting the response of esophageal squamous-cell carcinoma to nivolumab. Jpn J Clin Oncol 2020; 50: 114–121.
13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-delta delta C(T)} method. Methods 2001; 25: 402–408.
14. Desai M, Jellyman JK, Ross MG. Epigenomics, gestational programming and risk of metabolic syndrome. Int J Obes (Lond) 2015; 39: 633–641.

15. Carreras-Badosa G, Bonmatí A, Ortega F-J, et al. Dysregulation of placental miRNA in maternal obesity is associated with pre- and postnatal growth. JCEM 2017; 102: 2584–2594.

16. de Castro Marcondes JP, Andrade PFB, Sávio ALV, et al. BCL2 and miR-181a transcriptional alterations in umbilical cord blood cells can be putative biomarkers for obesity. Mutat Res-Gen Tox En 2018; 836: 90–96.

17. Tsukita S, Yamada T, Takahashi K, et al. MicroRNAs 106b and 222 improve hyperglycemia in a mouse model of insulin-deficient diabetes via pancreatic β-cell proliferation. EBioMedicine 2017; 15: 163–172.

18. Zhu J, Wang C, Zhang X, et al. Correlation analysis of microribonucleic acid-155 and microribonucleic acid- with type 2 diabetes mellitus, and the prediction and verification of target genes. J Diabetes Invest 2021; 12: 165–175.

19. Wu J, Dong T, Chen T, et al. Hepatic exosome-derived miR-130a-3p attenuates glucose intolerance via suppressing PHLPP2 gene in adipocyte. Metabolism 2020; 103: 154006.

20. Seong M, Kang H. Hypoxia-induced miR-1260b regulates vascular smooth muscle cell proliferation by targeting GDF11. BMP Rep 2020; 53: 206–211.

21. Wang J, Zhao D, Ding C-Z, et al. MicroRNA-194: a novel regulator of glucagon-like peptide-1 synthesis in intestinal L cells. Cell Death Dis 2021; 12: 113.