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Weight Change Alters the Small RNA Profile of Urinary Extracellular Vesicles in Obesity

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Extracellular vesicle · RNA · Small untranslated · Obesity

Abstract
Introduction: Various kidney diseases reportedly show different urinary extracellular vesicle (EV) RNA profiles. Although obesity is one of the main causes of chronic kidney disease, the expression pattern of urinary EV RNA in obesity is uncertain. Our aim was to sequence the small RNA profiles of urinary EVs in obese patients before and after weight reduction and compare them to those of healthy volunteers (HVs). ***Methods:*** We recruited age-sex-matched obese patients and HVs. The small RNA profiles of urinary EVs were analyzed using RNA sequencing. To evaluate the effect of weight reduction, small RNA profiles of urinary EVs 6 months after bariatric surgery were also analyzed. ***Results:*** The proportion of urinary EVs transfer RNA and microRNA of obese patients differed from that of HVs. Obese patients showed differential expression of 1,343 small RNAs in urinary EVs compared to HVs (fold change ≥2 and p value <0.05). Among those, 61 small RNAs were upregulated in obese patients and downregulated after weight reduction, whereas 167 small RNAs were downregulated in obese patients and upregulated after weight reduction. RNA sequencing revealed the correlation between the specific urinary EV small RNAs and clinical parameters including body weight, low-density lipoprotein cholesterol, triglyceride, high-density lipoprotein cholesterol, serum glucose, estimated glomerular filtration rate, and albuminuria. ***Conclusion:*** Obese patients showed distinct urinary EV small RNA profiles compared to HVs. Weight reduction altered urinary EV small-RNA profiles in obese patients.

Introduction

Obesity is a rapidly increasing health problem worldwide [1] that, through increased insulin resistance, represents one of the major risk factors for developing type 2

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diabetes mellitus (T2DM). In addition, obesity itself, or concomitant T2DM, leads to the development and progression of chronic kidney disease (CKD) [2, 3]. Previous studies have reported that weight reduction in obese patients can reduce the risk of CKD by leading to weight loss and subsequent T2DM remission [4–6].

Small RNAs are a class of typically noncoding RNAs, which is divided into distinct subclasses with lengths of less than 200 nucleotides [7]. Many types of small RNAs have been discovered, including microRNAs (miRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), P-element-induced wimpy testis-interacting RNAs (piRNAs), Y RNAs, and small nucleolar RNAs (snoRNAs). Small RNAs are critical components of various cellular pathways [8]. Extracellular vesicles (EVs) are membrane-bound vesicles released from cells into the extracellular space. The role of EV pathways in the selective transfer of cell cargo is increasingly recognized as an essential process for intercellular communication [9]. EVs carry various types of proteins and nucleic acids, including small RNAs. Small RNAs in EVs have been evaluated as potential biomarkers of various diseases [10]. Each human biofluid is suggested to contain different compositions of small RNAs [11, 12]. Among human biofluids, urine contains EVs from nephron cells, and various kidney diseases show different urinary EV RNA profiles [13]. Containing a large number of EVs that carry diverse small RNAs, and with its ease of collection, urine is a key body fluid in clinical molecular biology [14, 15].

As one of the leading causes of CKD, obesity is expected to show a distinct urinary EV small RNA profile. However, only a few previous studies reported the urinary miRNAs in obesity and metabolic syndrome [16–18], and insufficient data regarding small RNAs in urinary EVs of obesity are available. In this current study, we aimed to investigate the small RNA profile of urinary EVs in obese patients and compare them to healthy volunteers (HVs). Furthermore, urinary EV small RNAs of obese patients after 6 months from bariatric surgery were analyzed to determine the effect of weight reduction in obese patients.

Materials and Methods

Participants

We prospectively recruited age-sex-matched HVs and patients with morbid obesity (n = 6, respectively) between January 2016 and August 2017. Both groups consisted of 3 females and 3 males. The participants ranged from 30 to 59 years of age. Patients with a body mass index (BMI) ≥35 kg/m^2 or ≥30 kg/m^2 with comorbidities related to obesity, including inadequately controlled obstructive sleep apnea and obesity-related arthropathy, were classified as having obesity. All 6 patients were diabetes. Three of them had hypertension, and 1 patient had dyslipidemia. Patients with decreased estimated glomerular filtration rate (eGFR) (Chronic Kidney Disease Epidemiology Collaboration equation <60 mL/min/1.73 m^2) or resistant hypertension were excluded. Patients with obesity subsequently underwent bariatric surgery. The patients returned for follow-up assessments and data collection 6 months after surgery.

Collection of Urine

Urine was collected over 24 h. Urinary cells and debris were removed by centrifugation at 2,000 *g* at room temperature for 20 min. The supernatants were transferred to clean tubes with a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and stored at −80°C until use.

Characterization of Urinary EVs and RNA Isolation

Small RNA isolation was modified according to the manufacturer's protocol (exoRNeasy Serum/Plasma Maxi Kits, QIAGEN 77064, Hilden, Germany). Commercial kits, which we have used in this study, were validated for urinary EV miRNAs and small RNAs analyses [19, 20]. EVs were isolated from 6 mL of urine. The quantity and quality of the RNA extractions were determined using the Agilent Bioanalyzer 2100 with an RNA Pico Chip and Small RNA Chip to examine the size distribution of EVs RNAs (Agilent Technologies, Santa Clara, CA, USA). CD63 levels were measured using the ExoELISA-ULTRA CD63 kit (System Biosciences, Palo Alto, CA, USA) according to the manufacturer's protocol. Transmission electron microscopy was taken by protocol according to Thery et al. [21] and Rikkert et al. [22]. A droplet of exosome solution was put on a Parafilm, and the Formva carbon-coated nickel grid (200 meshes, TED PELLA, Redding, CA, USA) was floated on the drop to absorb the sample at room temperature. After 10 min, the exosome was fixed with 2.5% glutaraldehyde and stained using 1% uranyl acetate. The sample was washed with d.w and dried under light. The grid was observed on an electron microscope operating at 75 kV (H-7000B, Hitachi, Japan) (online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000521730).

Small RNA Library Construction and Sequencing

A total of 10 ng of RNA isolated from each sample was used to construct sequencing libraries with the SMARTer smRNA-Seq Kit for Illumina (Takara Bio, Shiga, Japan), following the manufacturer's protocol. Sequencing libraries were constructed by polyadenylation, cDNA synthesis, and PCR amplification. The resulting library cDNA molecules included sequences required for clustering on an Illumina flow cell.

The libraries were gel-purified and validated by checking the size, purity, and concentration on the Agilent Bioanalyzer. The libraries were quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualified using the TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany). The libraries were pooled in equimolar amounts and sequenced on an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) instrument to generate 51 base reads. Image decomposition and quality value calculations were performed using the modules of the Illumina pipeline. All procedures for next-generation sequencing analysis were performed by Macrogen (Seoul, Korea). We have analyzed data with our previous study methods [23].

Table 1. Clinical characteristics of obese patients and HVs

	HVs ($n = 6$)	Obese patient at the baseline ($n = 6$)	Obese patient after weight reduction ($n = 6$)	p value
Age, years	35 (29, 38)	36.5 (30, 39)	36.5 (30, 39)	P1 = 0.589 P2 = 1.000 P3 = 0.589
Height, cm	164.5 (162, 174)	166.5 (162, 183)	166.5 (162, 183)	P1 = 0.589 P2 = 1.000 P3 = 0.589
Weight,*,#,§ kg	58.85 (53, 71.4)	119.5 (97, 166)	89 (75, 132)	P1 = 0.002 P2 = 0.027 P3 = 0.026
BMI,* #,§ kg/m^2	22.1 (20.2, 24.6)	42.2 (36, 48.7)	32 (28.6, 39.4)	P1 = 0.002 P2 = 0.028 P3 = 0.015
FPG,* mg/dL	93.5 (90, 99)	139 (117, 199)	104.5 (94, 117)	P1 = 0.002 P2 = 0.249 P3 = 0.065
AST,* U/L	15.5 (14, 18)	21.5 (17, 38)	18 (14, 22)	P1 = 0.041 P2 = 0.172 P3 = 0.485
ALT,* U/L	12 (10, 16)	23 (19, 61)	14.5 (12, 26)	P1 = 0.009 P2 = 0.074 P3 = 0.310
Albumin, g/dL	4.6 (4.3, 4.8)	4.6 (4.5, 4.6)	4.6 (4.1, 5.0)	P1 = 0.818 P2 = 0.916 P3 = 0.818
Uric acid,*,§ mg/dL	4 (3.7, 5)	5.95 (5.1, 7.8)	6.65 (5.3, 7.6)	P1 = 0.015 P2 = 0.600 P3 = 0.041
Total cholesterol, mg/dL	185.5 (148, 195)	211 (149, 230)	178.5 (140, 197)	P1 = 0.240 P2 = 0.249 P3 = 0.818
LDL cholesterol, mg/dL	109.5 (99, 117)	153 (96, 172)	118 (91, 132)	P1 = 0.240 P2 = 0.173 P3 = 0.589
HDL cholesterol,*,§ mg/dL	71.5 (63, 76)	43.5 (35, 48)	45.5 (42, 49)	P1 = 0.015 P2 = 0.400 P3 = 0.015
TG,*,§ mg/dL	83.5 (56, 91)	167.5 (120, 207)	131.5 (119, 173)	P1 = 0.009 P2 = 0.141 P3 = 0.015
Creatinine, mg/dL	0.72 (0.68, 0.75)	0.82 (0.65, 0.92)	0.795 (0.69, 0.99)	P1 = 0.589 P2 = 0.674 P3 = 0.485
eGFR, mL/min	108.725 (93.91, 116.64)	114.855 (71.67, 124.29)	106.005 (79.13, 127.39)	P1 = 0.937 P2 = 0.917 P3 = 0.937
24HU albumin,*,#,§ mg	2.55 (2, 3)	122.1 (26.4, 188)	37.8 (9.3, 91)	P1 = 0.002 P2 = 0.028 P3 = 0.004

P1 = p value between HVs and obese patients. P2 = p value between obese patient before and after weight reduction. P3 = p value between HVs and weight reduced obese patients. FPG, fasting plasma glucose; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDL, low-density lipoprotein; HDL, high-density lipoprotein. * $p < 0.05$ in HVs versus obese patients before weight reduction. # $p < 0.05$ in obese patients before weight reduction versus obese patients after weight reduction. § $p < 0.05$ in HVs versus obese patients after weight reduction.

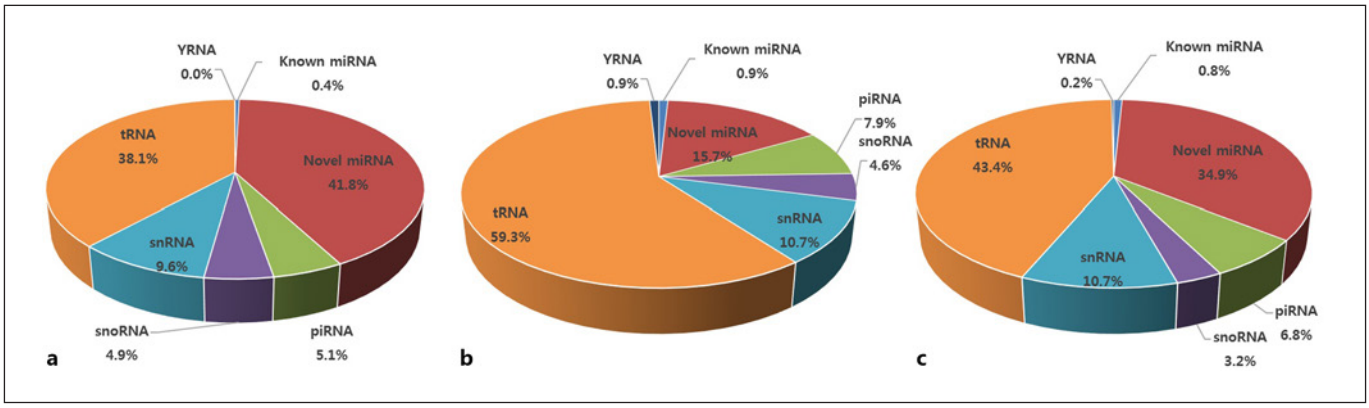

Fig. 1. Proportions of small RNAs in urinary EVs from each group. Analysis of urinary EV small RNA composition by NGS. **a** HV group. **b** Obese-patient group at baseline. **c** Obese-patient group after weight reduction. NGS, next-generation sequencing.

Identification of Known miRNA Reads and Novel miRNA Prediction

Sequence alignment and detection of known and novel miRNAs were performed using the miRDeep2 software algorithm [24]. The reference genome of *Homo sapiens*, released hg19, was retrieved from the University of California Santa Cruz genome browser. The reference genome was indexed, and ribosomal-RNA-filtered reads were mapped using Bowtie (1.1.2) [25]. Ribosomal-RNA-filtered reads and mature miRNAs were aligned to the precursor miRNAs of *H. sapiens* obtained from miRBase v21 [26] using the miRDeep2 quantifier module. Novel miRNAs were predicted from the mature, star, and loop sequences according to the RNAfold algorithm using miRDeep2. The RNAfold function uses the nearest-neighbor thermodynamic model to predict the minimum free-energy secondary structure of an RNA sequence.

Proportion of miRNAs and Other RNA Categories

Uniquely clustered reads were then sequentially aligned to the reference genome, miRBase v21, and noncoding RNA database RNAcentral release 10.0 [27] to identify known miRNAs and other types of RNA for classification.

Statistical Analysis of Differential miRNA Expression

Raw data (the reads for each miRNA) were relative log expressions normalized with DESeq2 [28]. For preprocessing, miRNAs with a zeroed count across more than 51% of all samples were excluded, leaving 507 mature miRNAs to be analyzed. We used a regularized log transformation to construct various plots. A statistical hypothesis test for comparison of two groups was conducted using the "nbinomWaldTest" in DESeq2. Differentially expressed miRNAs between the two groups were determined by the criteria fold change ≥2 and p value <0.05. All data analysis and visualization of differentially expressed genes were conducted using R 3.6.1 (http://www.r-project.org).

Statistical Analysis of Clinical Variables and Parameters

Statistical analyses of clinical variables and parameters were conducted using SPSS version 14.0 software (SPSS Inc., Chicago, IL, USA). Clinical variables are expressed as the median with interquartile range. The Mann-Whitney U test was used to compare clinical variables between HVs and obese patients, and the Wilcoxon signed-rank test was used to compare obese patients before and after weight reduction. The relationship between the urinary EV small RNA and clinical parameters were assessed using Spearman's correlation analysis. p values of <0.05 were considered statistically significant.

Identification of miRNA Target Genes and Their Molecular Pathways

We uploaded the miRNAs that were differentially regulated in the healthy, before surgery, and after surgery groups into popular prediction programs, such as DIANA-miRPath v.3 (http://snf-515788.vm.okeanos.grnet.gr) and miRSystem ver. 20160513 (http://mirsystem.cgm.ntu.edu.tw), for further analyses. Gene ontology analyses were performed using Database for Annotation, Visualization and Integrated Discovery v6.8 [29, 30].

Results

Clinical Characteristics of the Study Participants

Clinical characteristics of HVs and obese patients are shown in Table 1. Weight and the BMI of obese patients were higher than that of HVs (119.5 kg vs. 58.85 kg, p = 0.002; 42.2 kg/m^2 vs. 22.1 kg/m^2, p = 0.002, respectively). Among the laboratory data, there were significant differences in fasting plasma glucose, aspartate aminotransferase, alanine aminotransferase, uric acid, high-density lipoprotein cholesterol, triglyceride (TG), and 24 h urine albumin between the two groups. Among the obese patients, 3 patients underwent a single anastomosis gastric bypass, and 3 patients underwent Roux-en-Y gastric bypass surgery. Six months after surgery, weight and mean BMI were significantly decreased in obese patients (119.5 kg vs. 89 kg, p = 0.027; 42.2 kg/m^2 vs. 32 kg/m^2, p = 0.028, respectively).

Small RNA Composition of Urinary EVs

Next-generation sequencing revealed differential expression of small RNAs in urinary EVs. The total number of small RNAs was 5,205 (online suppl. Fig. 2). The proportions of tRNA, miRNA (known miRNA + novel miRNA), and snRNA were 46.9%, 31.5%, and 10.3%, respectively. Analysis of the small RNA composition of each group (HVs, obese patients at baseline, and obese patients after weight reduction) was conducted (Fig. 1). Compared to HVs, the proportions of tRNA and miRNA were different in obese patients (p value 0.0289 for tRNA and 0.0266 for miRNA; online suppl. Fig. 3). In obese patients, the small RNA composition was altered after weight reduction; however, the difference was not statistically significant. When analyzed by gender, differences were found in urinary EV small RNA profiles (online suppl. Fig. 4).

Comparison of Urinary EV Small RNA Expression between HVs and Obese Patients

The differential expression of small RNAs in each group is presented in Figure 2. The expression patterns of small RNAs in urinary EVs were compared between each group. Compared to HVs, obese patients showed the differential expression of 1,343 small RNAs in urinary EVs (fold change ≥2 and p value <0.05). After weight reduction, 372 small RNAs were changed in obese patients. Among those, compared to HVs, 61 small RNAs (3 piRNAs, 4 snRNAs, and 54 tRNAs) were upregulated in obese patients and downregulated after weight reduction, and 167 small RNAs (2 miRNAs, 12 piRNAs, 7 snoRNAs, 131 snRNAs, and 15 tRNAs) were downregulated in obese patients and upregulated after weight reduction.

Analysis of miRNAs and tRNAs in Urinary EVs

Following miRNA profiling, we identified 6 miRNAs (hsa-miR-6124, hsa-miR-4644, hsa-miR-7110-5p, hsa-miR-3141, hsa-miR-574-5p, and hsa-miR-1273a) that were downregulated in obese patients compared to HVs. Among these, 2 miRNAs (hsa-miR-6124 and hsa-miR-3141) were upregulated after weight reduction (online suppl. Fig. 5). To evaluate the clinical significance of miRNA pathways in obese patients, miRSystem analysis was performed. Table 2 shows enriched biological pathways in obese patients compared to HVs. Among them, some pathways regarding glucose metabolism like diabetes pathway, regulation of insulin secretion, integration of energy metabolism, metabolism of carbohydrates, insulin synthesis, and processing pathways were identified. Analyzing the interactions with the Kyoto Encyclopedia of Genes and Genomes, statistically significant miRNA pathways of obese patients compared with those after weight reduction are presented in Table 3. Fatty-acid biosynthesis and bile secretion were notable pathways regarding obesity and metabolism in the Kyoto Encyclopedia of Genes and Genomes pathways. Among tRNAs in urinary EVs, mitochondrially encoded tRNA tyrosine (MT-TY) is a weight reduction-responsive tRNA that showed upregulation before surgery (7.51-fold, p value <0.01) and downregulation after surgery (−5.11-fold, p value <0.01).

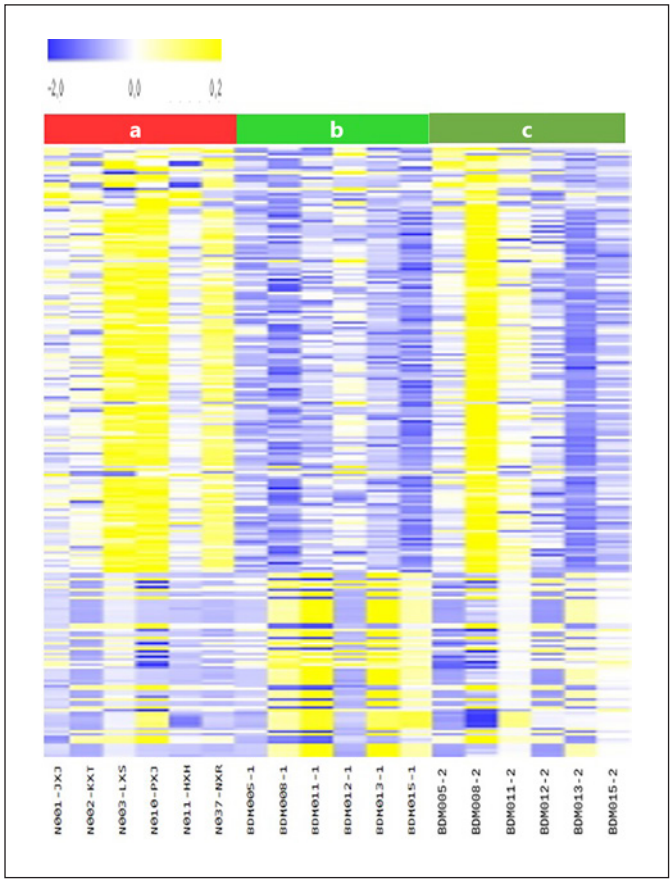

Fig. 2. Differential expression of small RNAs in urinary EVs from each group. Heat map showing z score of small RNAs in urinary EVs from each group (fold change ≥2 and p value <0.05). Obese patient group showed differential expression of 1,343 small RNAs from HVs. After weight reduction, 372 small RNAs were changed in the obese patient group. Upregulation and downregulation are represented in yellow and blue colors, respectively. **a** HVs group. **b** Obese-patient group at baseline. **c** Obese-patient group after weight reduction.

Relationship between Urinary EV Small RNAs and Clinical Parameters

Correlation between urinary EV small RNAs and clinical parameters are presented in Table 4. Significant

Table 2. Biological pathways in obese patients compared to HVs by miRSystem

Term	Total genes of the term	Union targets in the term	Score
Post NMDA receptor activation events	33	2	0.348
Activation of the NMDA receptor upon glutamate binding and postsynaptic events	37	2	0.332
Transmission across chemical synapses	190	3	0.237
L1CAM interactions	94	2	0.213
Diabetes pathways	229	3	0.208
Regulation of insulin secretion	98	2	0.208
Downstream signaling of activated FGFR	100	2	0.206
Signaling by EGFR	109	2	0.196
Signaling by FGFR	114	2	0.191
Transmembrane transport of small molecules	427	4	0.19
Axon guidance	266	3	0.186
Integration of energy metabolism	125	2	0.181
Metabolism of carbohydrates	126	2	0.18
Neuronal system	289	3	0.175
Host interactions of HIV factors	135	2	0.173
Insulin synthesis and processing	135	2	0.173
Neurotransmitter receptor binding and downstream transmission in the postsynaptic cell	136	2	0.172
Developmental biology	494	4	0.168
Calmodulin induced events	26	1	0.167
CAM pathway	26	1	0.167

NMDA, N-methyl-D-aspartate; L1CAM, L1 cell adhesion molecule; FGFR, fibroblast growth factor receptor; EGFR, epidermal growth factor receptor; HIV, human immunodeficiency virus; CAM, crassulacean acid metabolism.

Table 3. Statistically significant miRNA pathways of obese patients compared with after weight reduction by mirPath, interactions with KEGG

KEGG pathway	*p* value	Genes	miRNAs
Fatty-acid biosynthesis	5.20E−17	2	2
Drug metabolism – cytochrome P450	1.36E−05	8	5
Thyroid hormone synthesis	0.00016047	15	8
Tyrosine metabolism	0.00082303	6	3
Mucin type O-glycan biosynthesis	0.00301283	6	5
Primary bile acid biosynthesis	0.0339921	3	2
ECM-receptor interaction	0.0339921	10	4
Bile secretion	0.0339921	16	7
Estrogen signaling pathway	0.04922105	17	6

KEGG, Kyoto Encyclopedia of Genes and Genomes; ECM, extracellular matrix.

negative associations with body weight were found in hsa-miR-3141, snRNA U6 spliceosomal RNA (URS0000646FD6), snRNA U6 spliceosomal RNA (URS000065F260), and snRNA U6 spliceosomal RNA (URS00006AAB7B). Low-density lipoprotein cholesterol negatively correlated with snRNA U6 spliceosomal RNA (URS0000646FD6). Negative correlations between TG were found in hsa-miR-3141, piR-61289, snRNA U6 spliceosomal RNA (URS00006AC55A), snRNA U6 spliceosomal RNA (URS0000646FD6), and snRNA U6 spliceosomal RNA (URS000065F260). High-density lipoprotein cholesterol positively correlated with hsa-miR-6124, piR-53536, piR-61289, snRNA U6 spliceosomal RNA (URS0000646FD6), and snRNA U6 spliceosomal RNA (URS000065F260). The serum glucose level inversely correlated with hsa-miR-3141, hsa-miR-6124, piR-53536, piR-32313, piR-61289, and snoRNA U13-related. The eGFR negatively correlated with snRNA U6 spliceosomal RNA (URS00006AAB7B), and albuminuria inversely correlated with hsa-miR-6124.

Table 4. Correlation between small RNAs of urinary EVs and clinical parameters

Small RNAs	Weight	TC	LDL	HDL	TG	eGFR	Glucose	U24 albumin
hsa-miR-3141								
R	−0.649*	−0.347	−0.307	0.291	−0.537*	−0.108	−0.711*	−0.084
p value	0.004	0.158	0.216	0.242	0.022	0.669	0.001	0.741
hsa-miR-6124								
R	−0.362	0.081	0.059	0.496*	−0.436	−0.098	−0.546*	−0.524*
p value	0.140	0.751	0.817	0.036	0.071	0.699	0.019	0.026
piR-53536								
R	−0.344	−0.006	−0.077	0.544*	−0.437	−0.185	−0.633*	−0.405
p value	0.163	0.981	0.760	0.020	0.070	0.463	0.005	0.096
piR-32313								
R	−0.424	−0.280	−0.276	0.365	−0.370	−0.183	−0.588*	−0.136
p value	0.079	0.261	0.268	0.136	0.131	0.468	0.010	0.590
piR-61289								
R	−0.267	0.060	−0.032	0.610*	−0.647*	−0.176	−0.474*	−0.467
p value	0.284	0.813	0.900	0.007	0.004	0.484	0.047	0.051
snRNA U6 spliceosomal RNA (URS00006AC55A)								
R	−0.451	−0.198	−0.236	0.427	−0.476*	−0.284	−0.421	−0.281
p value	0.060	0.430	0.345	0.077	0.046	0.254	0.081	0.259
snRNA U6 spliceosomal RNA (URS0000646FD6)								
R	−0.583*	−0.394	−0.515*	0.637*	−0.542*	−0.414	−0.438	−0.314
p value	0.011	0.105	0.029	0.004	0.020	0.088	0.069	0.204
snRNA U6 spliceosomal RNA (URS000065F260)								
R	−0.480*	−0.240	−0.414	0.557*	−0.470*	−0.455	−0.434	−0.236
p value	0.044	0.338	0.088	0.016	0.049	0.058	0.072	0.347
snRNA U6 spliceosomal RNA (URS00006AAB7B)								
R	−0.523*	−0.327	−0.408	0.392	−0.464	−0.536*	−0.198	−0.250
p value	0.026	0.185	0.093	0.108	0.053	0.022	0.430	0.317

TC, total cholesterol; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol. * *p* value <0.05.

Discussion

This study identified the different urinary EV small RNA profiles between obese patients and HVs. Besides, weight reduction by bariatric surgery was found to alter the small RNA profile of obese patients. These results suggest that weight change can affect small RNAs in urinary EVs.

Although most current related studies are focused on miRNAs, other small RNAs and their composition in each body fluid needs to be identified because these are suggested to play a role in regulating specific diseases [31, 32]. Furthermore, considering that urine contains a relatively small portion of miRNAs compared to other body fluids [12], it is advantageous to analyze whole small RNAs of urine than just miRNAs to identify the molecular mechanisms.

In our study, we identified changes in small RNAs of urinary EVs according to the body weight change. Obesity is one of the leading risk factors of CKD, yet molecular biological studies lack associative evidence. Bariatric surgery is known to improve renal function and alter RNA expression [23, 33]. A recent study comparing gastric bypass surgery and calorie restriction in obese T2DM patients showed similar metabolic benefits for both weight loss strategies [34]. Therefore, the investigation of urinary EV small RNA profiling before and after bariatric surgery can provide information on the connection between obesity and kidney disorders. Our study revealed that the proportion of small RNAs in urinary EVs of obese patients was different from HVs. Although the proportion of small RNAs in EVs did not change significantly after weight reduction in obese patients, the level of specific small RNAs has changed. Considering that urinary exosomal miRNAs have been suggested as novel biomarkers of renal disorders [35, 36], the current study provides novel molecular insight into the effects of weight reduction on preventing CKD progression.

Interestingly, the proportion of tRNAs in EVs in obese patients differs from that of HVs. In view of tRNA func-

tion in stress sensing [37, 38], changes in specific tRNAs in obese patients after weight reduction can be hypothesized as an amelioration of stress response due to weight loss. Furthermore, our study suggested weight reduction-responsiveness expression of the MT-TY gene. While the association between mitochondrial tRNAs and obesity has been previously reported [39, 40], the role of the MT-TY gene has remains to be elucidated. Some studies have implicated MT-TY in neuromuscular disorders [41, 42]. However, the association between the MT-TY gene and metabolic disorders or kidney diseases has not been reported previously. The results of the present study warrant future studies investigating the role of the MT-TY gene in metabolic disorders and kidney disease for potential use as a clinical biomarker.

We identified specific miRNA changes after bariatric surgery-induced weight change. Compared to HVs, 2 miRNAs, hsa-miR-6124, and hsa-miR-3141 were downregulated initially in obese patients and upregulated after weight reduction. A recent study on serum circulating miRNAs and hyperlipidemia reported that hsa-mir-3141 was upregulated in hyperlipidemia [43]. On the other hand, our current study identified that has-mir-3141 negatively correlated with serum TG and glucose levels. However, there are very few reports of these miRNAs regarding kidney diseases or metabolic disorders, including obesity.

Interestingly, another of our previous reports regarding serum exosomal miRNAs of obesity identified different miRNAs [44]. One possible reason for these conflicting results may be due to the intrinsic differences in biofluids as our current study was conducted with urine. Furthermore, these conflicting results may suggest that urinary EVs contain distinct miRNAs from serum and warrants further investigation. There have been previous reports regarding other urinary miRNAs and kidney diseases. Decreased levels of miR-29 and miR-200 family members in the urinary exosome of CKD patients compared to healthy controls have been found; miR-29 positively and negatively correlated with the eGFR and tubulointerstitial fibrosis [45], respectively. Furthermore, miRNA-181a was suggested as the most suitable biomarker for CKD because it decreased by about 200-fold in urinary exosomes of CKD patients [46]. Recently, Zang et al. [36] reported the upregulation of miR-21-5p and downregulation of miR-30b-5p in T2DM patients with diabetic kidney disease. Alkandari et al. [16] tried to identify the molecular effect of bariatric surgery on the regulation of renal function by comparing urinary miRNAs in obese patients before and after bariatric surgery; among the urinary miRNAs, miR-192 and miR-200 were upregulated after surgery.

In our study, there were gender differences in the urinary EV small RNA profile. Luan et al. [47] recently published a study regarding gender differences of urinary exosomal circular RNA participating in the pathogenesis of IgA nephropathy. However, there are not many studies regarding gender difference in urinary EV small RNAs yet. Although a relatively small sample size was not enough to conclude the gender differences in our study, it is interesting that gender difference was shown. Future studies enrolling more samples focusing on the gender difference may be needed.

Canonical pathways regarding diabetes, insulin, energy metabolism, and biosynthesis were enriched in obese patients through analysis with popularly used bioinformatic tools, DIANA-miRPath, and miRSystem [48]. Additionally, pathways regarding N-methyl-D-aspartate receptors and the neuronal system were differentially expressed in HVs and obese patients and are reportedly related to leptin function and obesity [49, 50]. Changes in the bile secretion pathway, which were noted before and after bariatric surgery in the current study, were reported in a study that involved Roux-en-Y gastric bypass surgery in obese diabetic rats [51]. Changes in miRNA profiling after weight reduction and differences in those pathways would suggest the function of urinary EV miRNAs in energy metabolism. Further studies regarding the role of miRNA pathways in obesity metabolism are required.

Our study has some limitations. First, the study sample size was relatively small and not all patients underwent the same surgical procedure. Obese patients were all diabetics, and 3 of them were taking angiotensin II receptor antagonists for their hypertension, and 1 patient was taking a statin for dyslipidemia. It is possible that metabolic components other than pure obesity have affected the results. Nevertheless, we have sequenced a vast number of urinary EV small RNAs, which were not reported previously. Considering that there is no previous report on the total small RNAs of urinary EVs in obesity and after weight reduction, our report could be a good reference study for upcoming studies. Second, we could not identify tRNA fragments. Since tRNA fragments are increasing as potential kidney disorder biomarkers [52, 53], profiling tRNA fragments in urinary EVs may give more information on the effects of obesity and weight reduction on the progression of CKD. Future studies are needed to determine tRNA fragments in urinary EVs. Third, most miRNAs we profiled are presumed miRNAs according to their length. Fourth, we could not show information

about EV dynamics and the total number of EVs due to our limited resources. In characterizing EVs, commercial kits were used instead of centrifugation protocols since our limited laboratory resources. However, since there is no single optimal separation method on characterizing EVs, variety of additional techniques can be used in characterization [54]. We used commercial kits which were validated previously in characterizing urinary EV small RNAs analyses [19, 20]. Finally, we did not investigate the function of these small RNAs.

In conclusion, we have identified urinary EV small RNAs in obese patients and their changes after weight reduction. Weight gain and reduction could affect the small RNA profile of urinary EVs in humans. Future studies involving a more extensive study cohort with a longer follow-up period are needed to reveal the function of urinary EV small RNAs and their potential use as novel biomarkers to identify CKD in obese patients.

Statement of Ethics

The study was approved by the Soonchunhyang University Seoul Hospital Institutional Review Board (Number 2015-11-020) and conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Written informed consent was obtained from each participant.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Soon Hyo Kwon, Yun-Ui Bae, and Dughyun Choi designed the study. Sang Hyun Kim, Dong Cheol Han, Hyoungnae Kim, and Haekyung Lee provided resources. Jin Seok Jeon and Hyunjin Noh provided equipment. Yun-Ui Bae, Sewon Kim, and Jeyoung Woo extracted the urinary EV small RNA and provided data. Ho Chan Cho and Jong-Soo Choi visualized the data. Soon Hyo Kwon and Yun-Ui Bae and Dughyun Choi conducted statistical analysis. Hyoungnae Kim performed formal analysis. Dughyun Choi wrote the manuscript with input from all the authors. All the authors reviewed and approved the final version of the manuscript.

Data Availability Statement

Some or all datasets generated during the current study are available from the co-corresponding authors (Yun-Ui Bae and Soon Hyo Kwon) on reasonable request.

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