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Identification of n-3 PUFA-modulated miRNA Expression Pattern in Diet Induced Obesity Rats*

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ABSTRACT Objective: To determine the effect of n-3 PUFA in the miRNA level in diet induced obese (DIO) rats. **Methods:** A total of 10 DIO rats were randomly divided into two groups: n-3 PUFA-containing group and PBS-containing group (control group). Body weight, body length and food intake were recorded once a week. Small RNA was extracted from PBMCs of the two groups for miRNA array assay. Four of the identified differentially expressed miRNAs were selected and verified using real-time PCR. Then we predicted the target genes of the miRNAs with miRWalk. The predicted target genes were analyzed by using DAVID database and Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathway using the molecular annotation. **Results:** The lee's index of two groups in the 10th week had statistical difference. 29 up-regulated miRNAs and 31 down-regulated miRNAs were identified in the PBMCs. The expression of rno-miR-200 and rno-miR-211 in the peripheral blood mononuclear cells (PBMCs) were up-regulated in rats of n-3 group, while rno-miR-29b and rno-miR-92b down-regulated. Target genes of the four miRNAs were significantly involved in neurotrophin, adipocytokine, chemokine, and insulin signaling pathways. **Conclusion:** n-3 PUFA can regulate the miRNA level on adipose tissue metabolism in DIO rats.

Key words: N-3 polyunsaturated fatty acids; Diet Induced Obesity; MiRNA; Rats

Chinese Library Classification: Q95-3; **R589.2** **Document code: A**

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Introduction

Obesity, led by high-fat diets, has currently become a global epidemic in both developed and developing countries. It is the major risk factor associated with the genesis or development of various metabolic disorders including hypertension, hyperlipidemia, nonalcoholic fatty liver diseases, insulin resistance and cardiovascular disease[1]. It is also associated with type 2 diabetes characterized by impaired glucose and lipid metabolism [2]. Chronic con sumption of a high fat and high cholesterol diet may induce hyperlipidemia, hepatic lipid accumulation, lipid peroxidation and hepatotoxicity[3]. Food components with adipogenesis-suppressive ability may be helpful in the prevention of obesity[4].

The n-3 polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are known as anti-obesity factors. Fish oil containing high concentrations of DHA and EPA is considered a good source of these n-3 PUFA. Weight loss and decreased fat deposition are observed in mice fed a diet containing a high concentration of DHA and EPA [5].The SREBP-1 mRNA is also decreased by DHA in porcine adipocytes [6,7]. Dietary n-3 PUFA supplementation combined with very low calorie intake enhances weight loss in obese women [8]. The n-3 PUFA mainly exert their fat-lowering effect through extensive regulation of lipid metabolism by inhibiting lipogenesis, promoting lipolysis and fatty acid oxidation, and suppressing preadipocyte differentiation[9].

MiRNAs are single stranded RNAs of 18-24 nt in length, generated from an endogenous transcript containing a local hairpin structure by the action of an RNase-III type enzyme Dicer. MiRNAs suppress translation by a non-perfect pairing of 6-8 nucleotides with target mRNAs and subsequent formation of RISC complex resulting in degradation of target mRNAs[10]. It is currently estimated that up to 30% of human genes may contain miRNAs' binding sites, which suggested a potential role of miRNAs as central regulators in the control of gene expression. MiRNAs play important regulative roles in almost all major biomolecular processes, including metabolism[11], cell proliferation[12] and apoptosis, development and morphogenesis [13], stem cell maintenance, and tissue differentiation[14].

Therefore, the aim of the present study was to reveal changes in the miRNA level of n-3 PUFA on adipose tissue metabolism to help investigate the possible underlying mechanisms. The objective was realized through measuring miRNA expression profile of blood cells in the experimental rats.

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1 Materials and methods

1.1 Animals and diets

30 male Wistar rats (150-200 g) were procured from Experimental animal center of Shandong university. The animals were housed in standard polypropylene cages and maintained under controlled room temperature (22± 2℃) and humidity (55%± 5%) with 12 h light and 12 h dark cycle. All the rats were provided with commercially available rodent chow diet and tap water ad libitum. After 1 week of acclimatization with free access to rodent chow diet and water, animals were used in the study. All animal procedures were performed in accordance with National Institutes of Health (NIH) Guidelines for animal care and use of laboratory animals (National Research Council, 1985). A high fat diet (HFD) was composed of 20% normal feed, 5% egg yolk powder, 10% milk powder, 65% defatted soybean meal,Vitamin A (50000 U/100g) and Vitamin D (5000 U/100 g). The rats were fed HFD for 4 weeks and 10 DIO (Diet Induced Obesity) rats were selected. They were divided into two groups, each consisting of 5 rats: control group (Standard feed and fill the stomach 10 g PBS every rat per week) and n-3 group (Standard feed and fill the stomach 10g fish oil every rat per week). The rats were fed the corresponding diet and water ad libitum for 10 weeks. Body weight and body length were measured once a week. Food intake was recorded weekly. The diets were stored at 4℃ cold chamber.

1.2 Preparation of PBMC samples

After 10 weeks, the two group rats were killed with an overdose of CO_2. Blood samples were collected in sterile vacutainer tubes with liquid K3 EDTA. PBMCs from the rats were isolated from 3mL EDTA-anticoagulated blood samples by density gradient separation using Separation of Lymphatic Fluid of Rats (Tian Jin Hao Yang biological manufacture CO.,LTD, China).

1.3 miRNA extraction and reverse transcription

Small RNA, including miRNAs, was extracted from PBMCs using RNAiso for Small RNA (TAKARA, Dalian, Japan) following the manufacturer's instructions. The RNA concentration was quantified using Eppendorf 22331spectrophotometry (Eppendorf Technologies Inc.). An aliquot of 0.25μg of Small RNA was converted to cDNA using the RevertAidTM First Stand cDNA Synthesis Kit (Fermentas,Canada). Other chemicals and reagents available were from local commercial sources.

1.4 miRNA array assay

6 qualified RNA samples were further detected by miRNA array at KangChen Bio-tech Inc. (Shanghai, China) to analyzed global miRNA expression using miRCURY LNA Array 6th gen (Exiqon, Denmark) that covered all rat miRNAs in miRBase release v.16.0. Raw data was generated to further analysis by using significance analysis of microarrays (SAM) [15] and target prediction software [16].

1.5 qRT-PCR analysis

Stem-loop reverse transcription (RT) primers set were provided by the manufacturer (RiboBio, Guangzhou, China) and the RT reaction was performed according to manufacturer's protocol. Real-time PCR was performed using FastStart Universal SYBR Green Master (ROX) purchased from Roche, according to the manufacturer's directions on Rotor-Gene 3000 (Corbett Research Inc). The expression of miRNAs was normalized using the U6 small RNA as the endogenous control.

2 Results

2.1 Physical changes of subjects

The Lee's index is a rapid means of determining obesity. It consists of dividing the cube root of the body weight by the naso-anal length. Lee's index=$10*(weight_{kg}/lengthcm)^{0.33}$ As shown in Fig. 1, obesity level of control group was obviously higher than that of n-3 group rats, but the food consumptions of two groups were equal. It means that n-3 PUFA has inhibitory effect on fat metabolism, consistent with the literature.

2.2 Changes of PBMCs miRNA profile

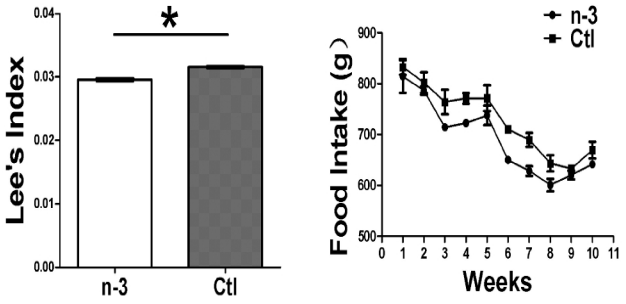

Fig. 1 Lee's index[$10*(weightkg/lengthcm)^{0.33}$] and, food intake weekly of the two groups rats

Note: Data are means± SEM obtained from 5 rats for each group and representative of 5 times of independent experiments. *Significant difference between the two groups. The letters over the bars represent significant differences (P<0.05).

Microarray data was analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (locally-weighted regression) to remove system-related variations. A miRNA to be listed as detectable must meet at least two conditions: signal intensity higher than 3× (background standard deviation) and spot CV<0.5, where CV is calculated as (standard deviation)/(signal intensity). In addition, where signals were detected for <4 of the repeats, they were considered unreliable and excluded from sets of detected miRNAs. In the dual-sample experiments, the ratio of the two sets of detected signals (log2 transformed, balanced) and t-test P-values were calculated. Differentially detected signals were those with P-values less than 0.01.

To identify specific miRNAs that might function in Obesity of n-3 PUFA, the array data was further analyzed using SAM software. Based on the fold-changes observed, 29 up-regulated miRNAs and 31 down-regulated miRNAs were identified in the PBMCs. The SAM analysis plots were shown in Figure 2, and a hierarchical clustering image is shown in Fig. 3.

2.3 Validation of the differentially expressed miRNAs by qRT−PCR

To confirm the microarray data, we selected 4 of the 60 differentially expressed miRNAs for qRT-PCR analysis. The selected miRNAs are rno-miR-29b, rno-miR-92b, rno-miR-200b, and rno-miR-211 which represented a range of apparent abundances but included those that corresponded to the highest signals produced by the microarray probes. As shown in Fig. 4, the results of qRT-PCR were consistent with that from the microarray analysis.

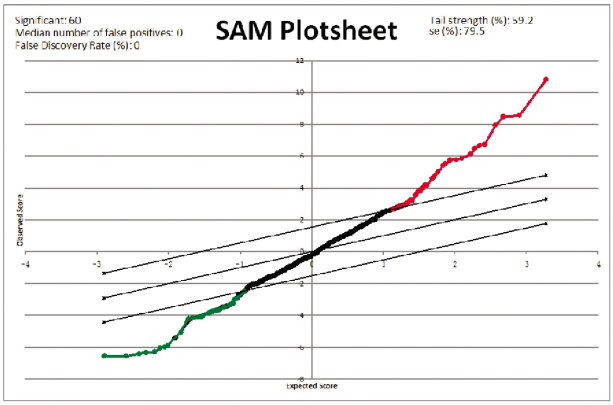

Fig. 2 SAM SAM plotsheet outputs under the four sets of criteria: Δ = 0.25, fold change = 2

Note:Conditions are indicated at the upper right corner of each plotsheet. The red, green, and blackdots represent upregulated, downregulated, and insignificantlychanged miRNAs, respectively. The upper and lower 45° degree lines indicate the Δ threshold boundaries. The number of significant miRNAs, median number of false positives, and false discovery rate(FDR) are indicated at the upper left corner of the plotsheet.

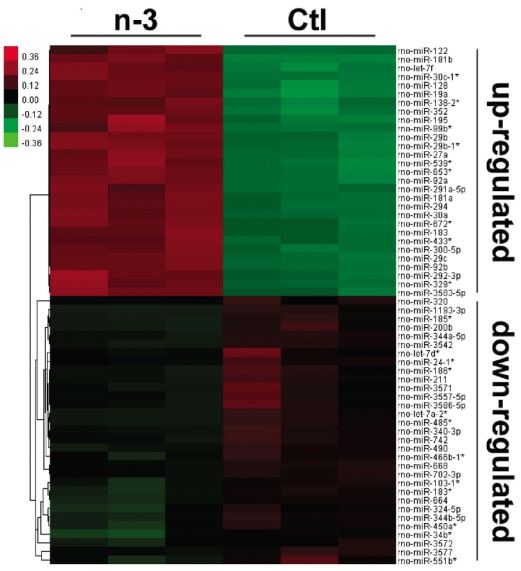

Fig. 3 Heat map of altered miRNA expression

Note:A heat map was generated using the expression ratios of 60 miRNAs that differed significantly in PBMCs of n-3 group compared to control, according to significance analysis of microarrays (SAM). Red, overexpressed miRNAs; green, underexpressed miRNAs compared to counterparts. Relatedness in miRNA expression across samples is shown by a hierarchical tree on the Y axis through standard linkage.

2.4 Target prediction

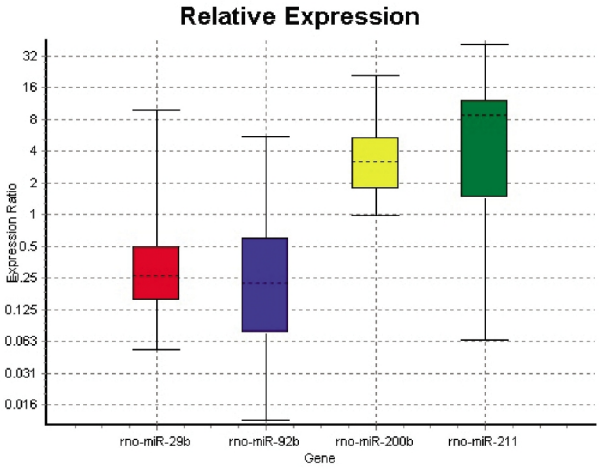

Fig. 4 Validation of microarray data using Real-time RT-PCR

Note:The levels of rno-miR-200b, rno-miR-211 are significantly increased (Expression Ratio>1), while the levels of rno-miR-29b,rno-miR-92b are significantly decreased (Expression Ratio<1) in the PBMCs of n-3 group compared to control. Real-time RT-PCR analysis of these miRNAs using small RNA isolated from the PBMCs showed similar results with miRNA expression.

Predicted miRNA-targets were generated by using miRWalk database. The miRNA-mRNA interactions were built into a bipartite network (the miRNAome) (Fig.5).

The predicted targets were analyzed by using DAVID

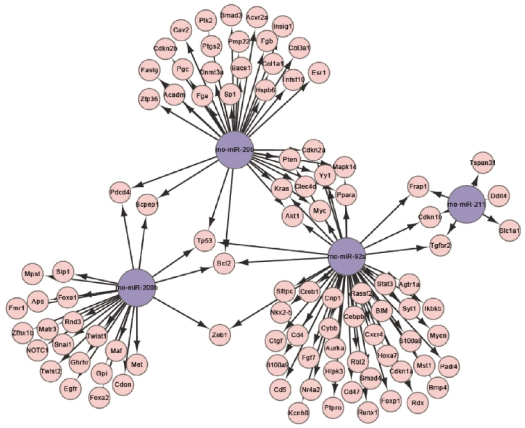

Fig. 5 Targets prediction of miRNAs

Note: miRNA-mRNA interactions were built into a bipartite network (the miRNAome). Blue circles indicate the verified miRNAs and red circles indicate the target mRNAs.

database and Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathway using the molecular annotation. As shown in Table 1, these target genes were significantly involved in neurotrophin, adipocytokine, chemokine, and insulin signaling pathways. It demonstrated that miRNAs with significant change are involved in regulation of target genes related to the development of obesity.

Table 1 Target genes prediction

Category	Term	Fold Enrichment	P Value	Genes
KEGG_PATHWAY	rno04722:Neurotrophin signaling pathway	6.089325	8.21E-04	AKT1, KRAS, MAPK14, BCL2, TP53, FASLG, IKBKB
KEGG_PATHWAY	rno04920:Adipocytokine signaling pathway	6.543752	0.0021603	AKT1, PPARA, IKBKB, STAT3
KEGG_PATHWAY	rno04062:Chemokine signaling pathway	3.204908	0.006558	AKT1, KRAS, CXCR4, IKBKB, STAT3
KEGG_PATHWAY	rno04910:Insulin signaling pathway	2.491087	0.0331409	AKT1, KRAS, IKBKB

3 Discussion

Obesity is a worldwide problem. It is tightly associated with dys lipidemia, type 2 diabetes and cardio vascular diseases, all posing huge threats to human health. The n-3 PUFA, are known as anti-obesity factors. Since obesity is characterized by an increased size and/or number of adipocytes, elucidating the molecular events governing adipogenesis is of utmost importance. Recent findings indicate that miRNAs-small non-protein-coding RNAs that function as post-transcriptional gene regulators are involved in the regulatory network of adipogenesis. This study provided a miRNA expression profile in PBMCs of n-3 PUFA treated and PBS treated rats by miRNA microarray. It has been reported miR-143 expression levels were up-regulated in mesenteric fat of mice fed high-fat diet [17]. miR-335 was also up-regulated in liver from diabetic model mice [18]. miR-27b overexpression impairs adipocyte differentiation of hMADS cells [19]. miR-27a is a negative regulator of adipocyte differentiation via suppressing PPARγ expression [20]. In our study, miRNA expression profiling and qRT-PCR analysis revealed that rno-miR-200b and rno-miR-211 expression were significantly up-regulated while rno-miR-29b and rno-miR-92b were down-regulated in PBMCs of n-3 PUFA treated rats. And the target genes were significantly involved in neurotrophin, adipocytokine, chemokine, and insulin signaling pathways. It demonstrated that miRNAs with significant change are involved in regulation of the development of obesity. Despite the cellular functions of many miRNAs remaining unknown, we assume that further miRNA studies will contribute to gene therapy of obesity.

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n-3 多不饱和脂肪酸调节饮食诱导肥胖大鼠 miRNA 表达变化 *

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摘要 目的: 研究 n-3 多不饱和脂肪酸(polyunsaturated fatty acids, PUFA)饮食对饮食诱导肥胖大鼠的 miRNA 表达影响。**方法:** 将 10 只饮食诱导肥胖(diet induced obese, DIO)大鼠随机分成两组:n-3PUFA 添加组和安慰剂添加组(对照组);每周记录两组老鼠的体重、体长和进食量。对外周血 miRNA 的表达并进行分析和预测。**结果:** 两组老鼠 Lee 指数有统计学差异($P<0.05$);与对照组相比,在 n-3 组的外周血单核细胞中,29 个 miRNA 上调,31 个下调;其中 rno-miR-200 和 rno-miR-211 的表达量上调,rno-miR-29b 和 rno-miR-92b 的表达量下调, 其靶基因预测结果与神经营养因子, 脂肪细胞因子, 趋化因子和胰岛素信号通路有关。**结论:** n-3PUFA 能够调节 DIO 大鼠的 miRNA 水平,其中有些与脂肪代谢相关。

关键词: n-3 多不饱和脂肪酸;肥胖;miRNA;大鼠

中图分类号: Q95-3;R589.2 **文献标识码:** A **文章编号:** 1673-6273(2014)35-6830-05

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