Profiling of MicroRNA Expression in Obese and Diabetic-Induced Mice for Biomarker Discovery

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ABSTRACT MicroRNAs (miRNAs) are short (-22 nucleotides) regulatory RNAs involved in many fundamental biological processes. They are involved in post-transcriptional regulation of gene expression. Dysregulated expression of microRNAs has been associated with a variety of diseases, including obesity and diabetes. Obesity is a potential risk factor contributing to the development of type 2 diabetes. Meanwhile, diabetes is one of the most prevalent chronic diseases, affecting 6.4% of the world's adult population. The aim of this study is to identify microRNAs that are differentially expressed in obese, diabetic and control C57BL/6 mice by using small RNA sequencing. Total RNAs were extracted from the serum of the target groups of animals. Next, the small RNAs were sequenced using the TruSeq small RNA Library Prep Kit in a MiSeq Illumina sequencer. A total of 52 up-regulated and 54 down-regulated miRNAs were identified based on the comparison of the log2 fold change of obese and diabetic (with normal mice as control; FC ≥ 2). The obese groups showed 22 up-regulated and 25 down-regulated microRNAs. Meanwhile, in the diabetic group, 32 microRNAs were up-regulated and 29 were down-regulated. This finding will help better understand the mechanism of metabolic disorders and may influence future approaches for the diagnosis and treatment of obesity and diabetes.

KEYWORDS: MicroRNA, Gene expression, Biomarker Obesity, Diabetes, C57BL/6N

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INTRODUCTION

Obesity is a significant risk factor leading to the development of type 2 diabetes. According to Kopelman (2000), the progression of type 2 diabetes and cardiovascular disease is often associated with excessive weight that increases their risks and complications. Nonetheless, growing evidence also suggests that deviant genetic expression, particularly in microRNA genes, may play a major influence and causal role in their pathogenesis (Krützfeldt & Stoffel, 2006).

MicroRNA-stranded non-coding regulatory RNAs that are 18-25 nucleotides in length. They are involved in many fundamental biological processes including the post-transcriptional regulation of gene expression. Dysregulated expression of microRNAs has been associated with a variety of diseases, including obesity and diabetes (Griffiths, 2006). Generally, there are many miRNAs that are located inside the cell and these are termed as intracellular miRNA. Meanwhile, some miRNAs are situated outside the cell and are commonly known as circulating or extracellular miRNA. The presence of miRNAs in human or animal biofluids (plasma, serum, urine, tears, saliva) has been observed to trigger the involvement of miRNA-based biomarkers (miRNA signatures) for multiple diseases that include obesity and cardiometabolic disease (Chen *et al*., 2008).

One of the rapid techniques to identify miRNAs is thorough next generation sequencing of small RNAs. This deep sequencing procedure yields an incredibly rich amount of data, which can determine not only the expression level of microRNAs, but also the level of other small RNA species such as Piwi-interacting RNA (piRNA) and small nucleolar RNAs (snoRNAs) as well as discovering novel small RNAs. Deep sequencing utilises a massive parallel sequencing, generating millions of small RNA sequence reads from a given sample (Chu & Corey, 2012).

Recent studies have indicated that miRNAs are detectable and quantifiable in blood serum (Gallo *et al*., 2012). This allows for its potential use as circulating biomarkers of diseases, in particular obesity and diabetes. As such, the aim of this study was to identify microRNAs that were differentially expressed in obese, diabetic and control C57BL/6 mice. It is expected that systemic miRNA expression could be utilized to detect and classify disease, determine prognosis, and predict response to existing therapies.

METHODOLOGY

Animals

Eight weeks old C57BL/6N mice were purchased from Invivos Pte Ltd-(Singapore) and randomly divided into three groups of 10 animals each. The first group was fed a high-fat diet (Bio Serv, Product# F3282) containing 60 kcal% fat to induce obesity while the other two groups were given normal diets. The second group in the age of 20 weeks was injected with alloxan monohydrate in the concentration of 150 mg/kg to induce type 2 diabetes (Xie *et al*., 2011). The last group was used as a negative control. Once the all three groups of mice reached the age of 24 weeks, they were sacrificed and blood serum, liver and pancreas were collected.

Collection Of Blood And Serum Separation

Approximately, 1 ml of blood of each mouse was collected from the posterior vena cava after cervical dislocation and placed in a BD vacutainer ® blood collection tube. The blood was allowed to clot by leaving it undisturbed at room temperature for 30 min. The sample was then centrifuged at 1,500 x g for 10 minutes in 4°C to obtain the serum.

Total RNA Extraction

Total RNAs were extracted from the serum of the target groups of animals using the miRNeasy Serum/Plasma Spike-in Control with RNase-Free DNase (Qiagen). The protocol was modified from Moret *et al*. (2013). The integrity of the RNAs was analyzed on a 1.2% agarose gel and only high quality RNA with an A_{260}/A_{280} spectrophotometry ratio at 1.8 to 2.0 was used for further analysis.

Small RNA Library Construction

For each of the animal groups, the cDNAs was pooled to a final concentration of 5µg and subsequently the libraries were sequenced using the TruSeq small RNA Library Prep Kit in a MiSeq Illumina sequencer. The sequencing service was provided by Macrogen, Korea.

Bioinformatics Analysis

The obtained reads were processed according to Hirst & Marra (2010) to produce a library consisting of 18 nt to 25 nt of small RNA reads. MiRNAs were then mined through homology search against mature miRNA in the miRNA Registry (miRBase, Release 21.0). Genome mapping result, known/novel miRNA predicted by miRDeep2 and blast result based on Rfam database are used to classify the type of RNA as stated by Friedlander *et al.,* (2008).

RESULT AND DISCUSSION

High quality total RNAs were isolated from the mice serum of 30 individuals, representing the three groups. The RNAs was then converted to cDNAs and all samples for each group of animals were pooled to be in the highest concentration. The cDNAs had undergone a quality control test to prove their quality (Figure 1). The template sizes for all three groups were in the range of 145 bp to

160 bp which was in agreement with the expected size reported by Lopez *et al.*, (2015). The pooling of cDNA has the advantage that it could detect miRNAs which have been expressed in the majority of subjects within the group.

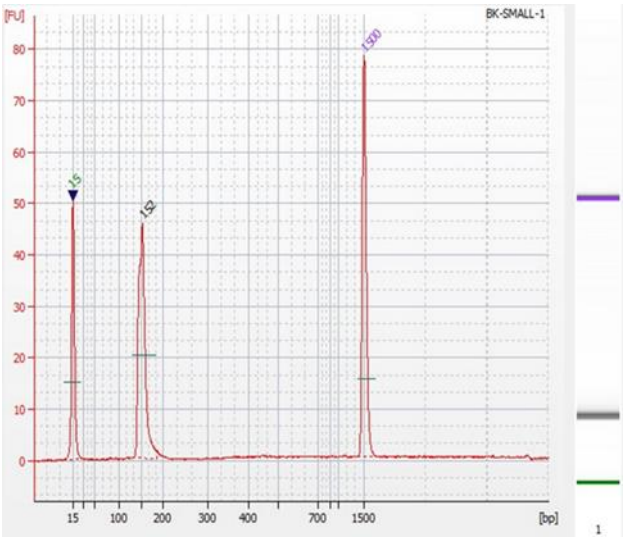

Figure 1. Electropherograms of PCR size enriched fragments, the template size distribution by running on an Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip.

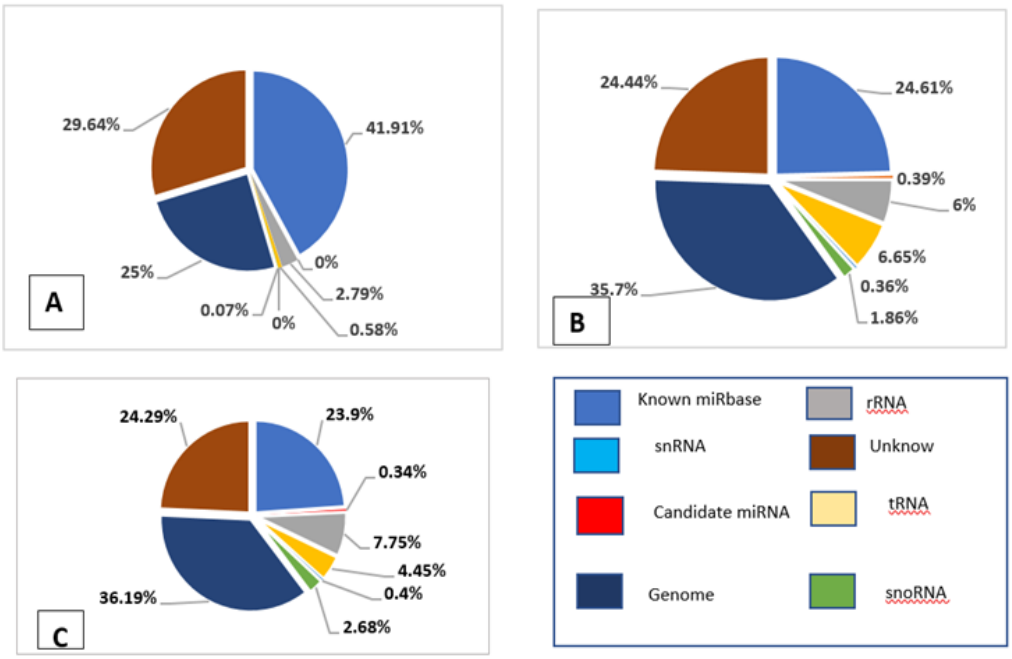

Figure 2. Small RNA categories in clustered reads count to the Genome mapping that predicted by miRDeep2 and blast based on Rfam database; (A) presented control group sample; (B) Obese and (C) Diabetic.

For each group, unique clustered reads were sequentially aligned to the reference genome and miRbase v21. This resulted in a list of known and novel miRNAs which was predicted by miRDeep2 and blast based on the Rfam 9.1 database. We have also classified the various types of RNA to sieve out the known miRNA from other types such as, tRNA, snRNA, snoRNA etc., as shown in Figure 2. The categories of the small RNA clustered read in the three different groups of animals, showed variances in the percentage of reads. For instance, known miRNAs were found in 41.9%, 24.61% and 23.9% of the control, obese and diabetes groups, respectively. Meanwhile, Obese

and diabetic groups only contain candidate miRNAs in 0.39% and 0.34%, singly. The candidate novel miRNA could be biomarkers of the two diseases i.e. diabetes and obesity.

By using a bioinformatics analyses pipeline, a total of 1,915 mature miRNAs were found. The Normalize-Quantiles method by Bolstad *et al*., (2003) indicated that only 127 were significant when a comparison was made between the obese, diabetic and control groups. A total of 52 up-regulated and 54 down-regulated miRNAs were identified based on the comparison of the log2 fold change of obese and diabetic with controlled normal mice; (FC ≥ 2). The obese groups showed 22 up-regulated and 25 down-regulated microRNAs. Meanwhile, in the diabetic group, 32 microRNAs were up-regulated and 29 were down-regulated.

Interestingly, the distribution of expression level between obese and diabetic group were almost comparable (Figure 3). These results seem to indicate the development of a diabetogenic phenotype in C57BL/6 mice fed high fat diet. Kopelman (2000), reported that the exposure of the developing obesity is thought to trigger changes to the body's metabolism. These changes cause fat tissue (adipose tissue) to release fat molecules into the blood, which can affect insulin responsive cells and lead to reduced insulin sensitivity and thus, lead to the formation of diabetes mellitus (T2D). In addition, circulating and biofluid-based miRNAs measures can provide insight into infuse tissue miRNA changes, circulating macrovesicle and lipoprotein miRNA enrichment and can be used as biomarkers or 'miRNA signatures' of disease. This is the first report where differential miRNA expression in obese and diabetic animal mice is observed in comparison with healthy individuals and among themselves.

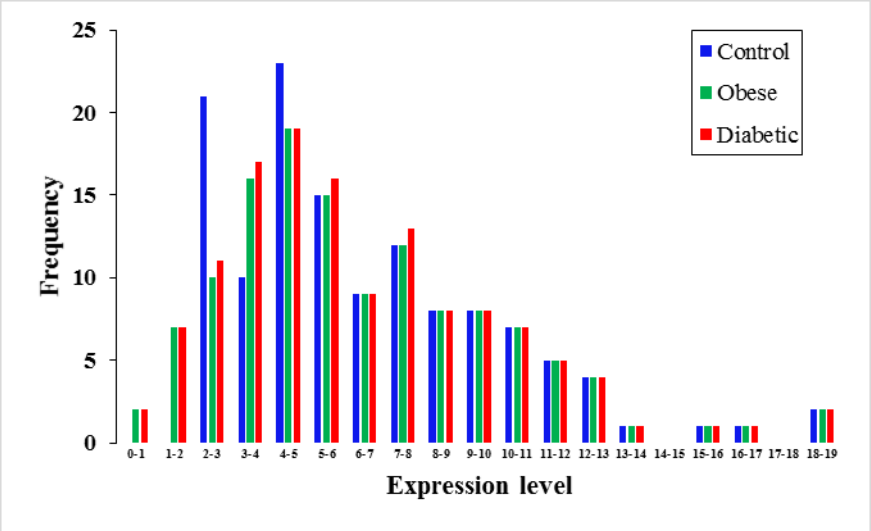

Figure 3. Comparison of the distribution of normalized log2(RPM+1) for each group of animals.

CONCLUSION

This study identified 127 significant miRNAs from the serum of C57BL/6N mice that induce obesity and diabetes mellitus T2D. The presence of miRNAs in the blood serum of mice has resulted in the pursuit of miRNA-based biomarkers (miRNA signatures) for multiple diseases including obesity and T2D. This may lead to the discovery of new biomarkers to monitor the disease progression and the response to the experimental therapies.

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