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Research paper

Comparison of liver microRNA transcriptomes of Tibetan and Yorkshire pigs by deep sequencing

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ABSTRACT

MicroRNAs (miRNAs) play an important role in the modulation of various metabolic processes in the liver, yet little is known about the liver microRNAome (miRNAome) of the Tibetan pig. Here we used the Yorkshire pig as a control to analyze the Tibetan pig-specific liver miRNAome, and for preliminary investigation of differentially expressed miRNAs participating in metabolism. A comprehensive analysis of Tibetan and Yorkshire pig liver miRNAomes by small RNA sequencing identified 362 unique miRNAs. Among these, 304 were co-expressed in both libraries, and 10 and 48 miRNAs were specifically expressed. Differential expression analysis of miRNAs, miRNA target prediction and KEGG analysis revealed that differentially expressed miRNAs were associated mainly with the metabolism of glucose, lipid and protein. Six differentially expressed miRNAs (miR-34a, miR-326, miR-1, miR-335, miR-185 and miR-378) participating in the metabolism of glucose and lipid were identified. Additionally, qPCR results revealed that a lower expression of miR-34a in Tibetan pig liver may promote gluconeogenesis by increasing the expression of Sirtuin type 1 (Sirt1); a lower expression of miR-1 in Tibetan pig liver may promote the synthesis and accumulation of lipid by increasing the expression of Liver X receptor α (LXRα); and a lower expression of miR-185 in Tibetan pig liver may promote the uptake of cholesterol from blood and secretion of bile by increasing the expression of the scavenger receptor class B type I (SR-BI). Our results provide new information and understanding of porcine miRNA profiles, which may help explain the regulatory mechanisms of miRNAs in the metabolic functions of Tibetan pig liver, and provide new biomarkers to assist in the development of Tibetan pig breeding characteristics.

1. Introduction

MiRNAs are an expanding group of non-coding RNAs (ncRNAs) of ~22 nucleotides (nt), and are derived from ~70 nt long stem–loop precursors (pre-miRNAs) located in all mammalian autosomes and X chromosome (Carthew and Sontheimer, 2009; Winter et al., 2009). They are known to function in a sequence-specific manner to decrease translation of targeted gene products (Lai, 2005; Chan and Slack, 2006) at the post-transcriptional level by targeting the 3′ untranslated region of mRNAs (Nelson et al., 2003). The 2–8 nt at the 5′ end of an miRNA are considered as the functional 'seed' region for the recognition of target mRNAs. A single miRNA may potentially target hundreds of mRNAs (Selbach et al., 2008; Bartel, 2009; Friedman et al., 2009). Various miRNAs have been shown to play important regulatory roles in metabolism. Typically, up-regulation of the expression of miR-210 can promote glycolysis (Chen et al., 2010); however, an increase in the expression of miR-326 suppresses glycolysis (Dang, 2009; Kefas et al., 2010). In addition, miR-122 (Esau et al., 2006) promotes fatty acid oxidation, while miR-33 (Dávalos et al., 2011) and miR-370 (Iliopoulos et al., 2010) suppress fatty acid oxidation. MiR-378 promote fatty acid synthesis (Gerin et al., 2010), while the miR-103/7 cluster suppresses fatty acid synthesis (Chartoumpekis et al., 2012). While miR-143 (Takanabe et al., 2008), miR-103 (Ortega et al., 2010) and the miR-17-92 cluster (Wang et al., 2008) promote adipocyte differentiation, this is suppressed by miR-27a (Lin et al., 2009), miR-27b (Karbiener et al., 2009) and miR-15a (Andersen et al., 2010).

Liver is the largest digestive gland and most important metabolic organ. So it has often been chosen to study the regulating role of

Abbreviations: miRNA, microRNA; TPL, Tibetan pig liver; YKL, Yorkshire pig liver; qPCR, quantitative real-time PCR; Stem–loop qPCR, stem–loop quantitative real-time PCR; Sirt1, Sirtuin type 1; PKM2, M2 isoform of pyruvate kinase; LXRα, Liver X receptor α; SR-BI, scavenger receptor class B type I.

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miRNAs on metabolism. For example, obese diabetic model mice have relative higher expression of miR-335 in the liver than normal mice (Nakanishi et al., 2009). Nowadays, the miRNAome of various porcine tissues has been revealed by deep sequencing (Li et al., 2012a; Jideng et al., 2013; Siengdee et al., 2015). But little is known about liver miRNAome in pig.

Tibetan pig is a very valuable resource of genetic species, being the unique native pig breed of the Qingzang plateau of China. Compared to western breed, Tibet pig has strong disease resistance, thin sebum, lean meat and good adaptability to crude feed (Zhang et al., 1986; Megens et al., 2008; Li et al., 2012b). However, the study of liver miRNAome in Tibetan pig is still lacking. A comprehensive analysis of liver miRNAome in relation with the metabolic characteristics of different pig breeds is lacking.

Here, we analyzed the different miRNA expression patterns of Tibetan and Yorkshire pig livers (TPL and YKL resp.), screened out 6 differentially expressed miRNAs participating in metabolism. Three of the 6 miRNAs (miR-34a, miR-1 and miR-185) may have important effects on the metabolism characteristics of Tibetan pig. Our study extends the repertoire and understanding of porcine miRNAs, and the 3 differentially expressed miRNAs may be used as new biomarkers to select the hybrid progeny with corresponding metabolism traits in pig breeding process.

2. Materials and methods

2.1. Animal and sample collection

Six purebred pigs (3 Tibetan and 3 Yorkshire pigs, 90-day-old) were provided by the Sichuan Academy of Animal Science, Chengdu, China. The pigs used for this study are female with no blood relationship in three generations. They were feed with the same food and living in a standardized plant. The pigs were killed by electrocution and their livers were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until use. Animal experiments were performed according to Chinese animal welfare laws and regulations, and approved by the Institutional Animal Care and Use Committee in College of Life Science, Sichuan University, Sichuan, China under permit No. DKY-B20150123.

2.2. Small RNA library construction and sequencing

Total RNA was isolated from the liver, using the Trizol reagent (Invitrogen, USA), following the manufacturer's instructions. Total RNAs were tested for quality and purity using a NanoDrop ND-1000 spectrophotometer (Nano Drop, DE, USA) at 260/280 nm (ratio > 2.0). The integrity of total RNA was also monitored via analysis by the Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with RIN number > 6.0. Qualified RNA was prepared for sequencing samples as follows: equal quantities (4 μg) of total RNA isolated from three pigs were gently mixed by transfer liquid gun within each breed in an EP tube and prepared for Illumina sequencing. Both small RNA libraries were generated according to Illumina's sample preparation instructions and sequenced in the Illumina HiSeq 2000 system (Illumina, USA) following the vendor's instructions.

2.3. Sequencing data analysis

Raw sequence reads were generated by the Illumina Genome Analyzer at BIOMARKER-Beijing, China. To determine conserved miRNAs, the filtered sequences were initially used to search the miRBase 20.0 with BLASTN, allowing a maximum of two mismatches with gaps regarded as mismatches. These criteria were applied according to reported miRNA protocols. RNAFold (Sharbati et al., 2010) was used to predict the hairpin RNA structures of potentially novel miRNAs which did not map to miRBase 20.0.

2.4. MiRNA validation via stem–loop qPCR

Following assay for RNA integrity and concentration, reverse-transcription PCR was performed using Transcript One-Step gDNA Removal and cDNA synthesis SuperMix (Transgen, Beijing). Reverse transcribed the total RNAs (1–2 μg) with a combination of top 10 miRNA primers and U6 snRNA primers were run with the following program: 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and hold at 4 °C. The expression changes of 10 selected miRNAs were validated by SYBR Green Real-time PCR analysis by the iQ5 TM Real-Time PCR Detection System (Bio-Rad, CA, USA) and the stem–loop qPCR method. The mature miRNA and primer sequences are available in Supplemental Table S1. The DDCt method was used to determine the expression level differences between samples (Livak and Schmittgen, 2001) with significant level set to 0.05. Pig U6 snRNA was used as an internal control and all reactions were run in triplicate with the following program: a. Stage 1 (hot start): 95 °C for 2 min. b. Stage 2 (amplification): 95 °C for 5 s then 56 °C for 10 s (40 cycles).

2.5. Target genes of the differentially expressed miRNA validation by qPCR

Following assay for RNA integrity and concentration, reverse-transcription PCR was performed using Transcript One-Step gDNA Removal and cDNA synthesis SuperMix (Transgen, Beijing). Reverse transcribed the total RNAs (1–2 μg) were run with the following program: 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and hold at 4 °C. The expression changes of reported target genes were validated by SYBR Green Real-time PCR analysis on the IQ5 TM Real-Time PCR Detection System (Bio-Rad, CA, USA). All primer sequences are listed in Supplemental Table S2. Pig PPIA (peptidylprolyl isomerase A) was used as an internal control and all reactions were run in triplicate with the following program: a. Stage 1 (hot start): 95 °C for 5 min. b. Stage 2 (amplification): 95 °C for 10 s then 56 °C for 30 s (40 cycles). The relative expression of gene was calculated by the $2^{-\Delta\Delta CT}$ method. *T*-test was used for data analysis. Results were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Summary of deep sequencing data

The sequencing of two small RNA libraries from TPL and YKL yielded 10.13 M and 16.26 M high quality reads, respectively, of which 8.31 M and 13.03 M TPL and YKL sequences were regarded as clean reads after filtering out the counts that did not meet the accepted criteria. The clean tags were then annotated into different categories. The flow results of sequence data for the two libraries are listed in Supplemental Table S3, A.

Length distributions of the clean tags were then summed up. The size distribution of sequence lengths was similar in both libraries by following the law of normal distribution (Supplemental Table S3, B). The most abundant size class among the small RNA sequences was the 22 nt RNAs, accounting for 3.26 M (TPL, 39.18% of clean reads) and 5.13 M (YKL, 39.35% of clean reads), followed by the 21 and 23 nt RNAs, the number of 21–23 nt sequences being significantly greater than those shorter or longer. This distribution is consistent with the known specificity for Dicer processing and the features of mature miRNAs (Lau et al., 2001; Zhang et al., 2009).

Table 1
Conserved and novel miRNAs detected in TPL and YKL.

Group (number of miRNA/pre-miRNA)	TPL	YKL	Total
Conserved miRNAs	330/300	360/332	370/343
Novel miRNAs	60/55	77/73	80/76
Total miRNAs	390/355	437/405	450/419

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