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Identification and profile of microRNAs in Xiang pig testes in four different ages detected by Solexa sequencing

Zhi-Yu Luo [a, 1], Xin-Lan Dai [b, 1], Xue-Qin Ran [a, *], Yong-Xiu Cen [a], Xi Niu [b], Shi-Hui Huang [a], Jia-Fu Wang [b, c, **]

[a] College of Animal Science, Guizhou University, Guiyang, China
[b] Institute of Agro-Bioengineering, Guizhou University, Guiyang, China
[c] Tongren University, Tongren, China

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ABSTRACT

To further understand the role of microRNA (miRNA) during testicular development, we constructed four small RNA libraries from the testes of the Chinese indigenous Xiang pig at four different ages, which were sequenced using high-throughput Solexa deep sequencing methods. It yielded over 23 million high-quality reads and 1,342,579 unique sequences. At two and three months of age, the proportion which represented miRNAs was the most abundant class of small RNAs, but it was gradually replaced by the category that represented piRNAs in adult testes. We identified 543 known and homologous conserved porcine miRNAs and 49 potential novel miRNAs. There were 306 known miRNAs which were co-expressed in four libraries. Six miRNAs and three potential novel miRNAs were validated in testes and sperms of Xiang pig by RT-qPCR method. Many clusters of mature miRNA variants were observed, in which let-7 family was the most abundant one. After comparison among libraries, 204 miRNAs were identified as being differentially expressed and likely involved in the development and spermatogenesis of pig testes. This work presented a general genome-wide expression profile of the testes-expressed small RNAs in different ages of pig testes. Our results suggested that miRNAs performed a role in the regulation of mRNAs in puberty pig testes while piRNAs likely functioned mainly in sexually mature pig testes.

1. Introduction

The testes are the most important reproductive organs in male mammals as they are the site of testosterone production and spermatogenesis [1]. Spermatogenesis is the primary biological process of the development and differentiation of germ cells in the seminiferous tubules of the mammalian testes [2,3]. This process is hugely complicated which involves a series of cellular differentiation and cell biological activities [4,5]. Evidence shows that miRNAs have emerged as a major mechanism of translational regulation during spermatogenesis and other biological activities in human and animals [6–9]. Many testicular miRNAs have been cloned by various groups [10–16]. Several studies have found that numerous miRNAs are exclusively or preferentially expressed in the testes or male germ cells of human and mouse [8,17]. Further studies indicated that some testicular miRNAs are involved in the regulation of gene expression during spermatogenesis process [13,18].

Pigs are an important species in the animal production industry and are also model animals for biological and medical studies due to their similarity to humans in terms of anatomy, physiology, metabolism, genome and diet [19]. Worldwide, domestic pigs can be divided into two main clades: Asian- and European-types. It is generally considered that current European and Asian pig breeds are domesticated from different ancestors that might have different genome wide diversity and extent of linkage disequilibrium (LD) [19]. Independent geographical origins have resulted in many phenotypically different pig breeds [19,20]. Particularly, the reproductive and meat production and quality traits have shown marked differences between Asian and European pig breeds.

* Corresponding author.
** Corresponding author. Institute of Agro-Bioengineering, Guizhou University, Guiyang, China.
E-mail addresses: xqran@gzu.edu.cn (X.-Q. Ran), jfwang@gzu.edu.cn (J.-F. Wang).
[1] These two authors contributed equally to this work as joint first authors.

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Recently, many studies using deep-sequencing approaches have reported that miRNAs are differentially expressed between breed groups [21,22]. Breed-specific miRNAs could be potentially associated to certain phenotypes [23]. miRNAs expressed during embryonic, postnatal and adult life in testes, ovary and spermatic cells of the pig are discussed in recent papers providing a valuable resource for investigators interested in the regulation of spermatogenesis in pigs and other animals [11,15,22,24–26].

The Xiang pig is a rare kind of Chinese miniature breed known for its small size, early sexual maturity and lower litter size. Most boars initiate the puberty after two months old and grows to sexually adulthood in only six months. In China, they are raised mainly for food and as an experimental animal model for human medical research [27]. However, very limited knowledge exists regarding the overall and specific expression status of miRNAs during postnatal development of the Xiang pig testes. To obtain more insights on the roles of miRNAs in Xiang pig testes development and spermatogenesis, we utilized a next generation sequencing approach to investigate the miRNA expression profiles of juvenile and adult Xiang pig testes. By comparing the miRNA expression profiles among four ages, we identified differentially expressed and novel miRNAs. Our data indicate that miRNAs are likely to play an important role in testes development and spermatogenesis in Xiang pig breeds.

2. Materials and methods

2.1. Animals and histological assay

Twelve purebred male Xiang pigs provided by the Guizhou Dachang pig breeding company (Guizhou province, China) were used in this study. The testes samples were collected with surgery at two, three, six and twelve months of ages. A part of them were fixed in 10% neutral phosphate buffered formalin and the others were immediately frozen in liquid nitrogen, and stored at −80 °C for RNA preparation. Testis tissues were cut at 4–5 μm in thickness and stained with hematoxylin and eosin (H&E) solution [28]. The semen samples of ten Xiang boars in seven and 24 month of ages were cellected artificial by the gloved-hand technique with percentage of motile sperm between 86% and 92%. All animal procedures were approved by the Institutional Animal Care and Use Committee of Guizhou University, and were conducted in accordance with the National Research Council Guide for Care and Use of Laboratory Animals.

2.2. RNA preparation

Total RNA was extracted individually from the testes samples with Trizol (TIANGEN, China) according to the manufacturer's instructions. The quantity and integrity of the total RNAs were assessed using the Agilent 2100 Bio-analyzer (Agilent Technologies, USA). Total RNAs were stored at −80 °C until subsequent analysis. The same sample was used to both the sequencing and RT-qPCR analysis.

2.3. Small RNA library construction and high throughput sequencing

Four small RNA libraries were constructed using the pooled total RNAs from three Xiang pig testes at four ages. Total RNAs were prepared for small RNA Sequencing-by-Synthesis according to the procedure and standards of the Illumina Sample Preparation Protocol. The small RNA fragments between 15 and 35 nt were excised and isolated from denaturing 12.5% polyacrylamide gel electrophoresis. Subsequently, the small RNA molecules were ligated to 5′ and 3′ RNA adaptors at both ends of the small RNAs in two separated reactions using T_4 RNA ligase and then converted to cDNA by reverse transcription followed by PCR amplifications. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced using Illumina 1 G Genome Analyzer (Illumina, San Diego, CA, USA) according to the manufacturer's protocols at the Beijing Genomies Institute (BGI), Shenzhen, China.

2.4. Small RNA analysis

The raw sequence reads were generated by the Illumina Genome Analyzer. After masking the adaptor sequences and removing contamination, the clean reads were mapped to the ssc genome (Ensembl, 2014, *Sus scrofa*) using SOAP v2.20 software (http://soap.genomics.org.cn/). The sequences with perfect matches were retained for further analysis. The sequence reads that matched to mRNA in NCBI Genbank, non-coding RNAs (rRNA, tRNA, snoRNA, snRNA and others) in Rfam (http://rfam.xfam.org/) and repetitive sequence elements in RepBase (http://www.girinst.org/repbase/) databases were removed before further analysis. The sequencing results were compared to all available miRNA sequences in miRBase v21.0 (http://www.mirbase.org/) using local Blast method. Parameters were set to 100% identity and up to 4 mismatches allowed at the end of the sequences to assume variability at 3′ and 5′ ends [29].

Novel miRNA candidates were identified by folding the flanking genome sequence from each mapping locus of unique small RNAs using MIREAP (https://sourceforge.net/projects/mireap/) and RNA folding (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form) described previously [30]. The sequences with more than 5 reads were used for further analyses as novel miRNA candidates.

To identify the differentially expressed miRNAs in the four libraries of Xiang pig testes, the expression of miRNA in the libraries was normalized to obtain expression level of the transcript per million (normalized expression = 1,000,000 × (Actual miRNA reads counts/Total counts of clean reads)) as described previously [31]. The IDEG6 program was used to identify the differentially expressed miRNAs based on the normalized expression (http://telethon.bio.unipd.it/bioinfo/IDEG6/). The selection methods for differential expression were Audic and Claverie, Fisher's exact test and chi-squared 2×2, with the selection threshold of 0.01 [31]. Log2 fold change between groups were calculated using normalized data and scatter plots were presented.

The hierarchical clustering of miRNA expression was performed using PermutMatrix software with Pearson distance [32]. The relative expression frequency for each miRNA was calculated as the number of sequences for each miRNA in a library divided by the total of number of sequence reads from library.

To understand the function of the miRNA found in Xiang pig testes, potential target sequences for the abundant expression miRNAs were predicted by integrating three databases (microinspector, Miranda, mirtarget2, nbmirtar, pictar, pita, rnahybrid, targetscan). Furthermore, Gene ontology (GO) annotation and KEGG pathway analysis were performed to identify the functional modules regulated by the miRNAs using Kobus online [15].

2.5. Real-time quantitative RT-PCR

Differentially expressed miRNAs in testes were validated using relative real-time quantitative RT-PCR according to the manufacturer's protocol. Real-time quantitative PCR was performed using the CFX96 real-time PCR system (Bio-Rad, USA) with SYBR Premix ExTaq™ kit (TaKaRa Biotechnology Co. Ltd. Japan, DRR081A). The primers of selected miRNAs and internal control gene (U6 snRNA)

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