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Differential expression of microRNAs in sexually immature and mature canine testes

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ABSTRACT

Mammalian testis exhibits spatiotemporal gene expression patterns that are essential for successful and continuous spermatogenesis. Although microRNAs (miRNAs) modify gene expression through translational repression and degradation of target messenger RNAs, the precise molecular mechanisms of these regulatory processes are unclear. We used canine miScript miRNA polymerase chain reaction (PCR) Array technology to elucidate the repertoire of canine testis miRNAs and compared their expression patterns between sexually immature (prepubertal) and mature (adult) dog testes. Eighty-four well-characterized canine miRNAs were customized in this study. The data were analyzed by RT^2 Profiler PCR Array Data Analysis (version 3.5). Results identified upregulation of 32 and considerable downregulation of 12 miRNAs in adult dog testis. In conclusion, the two developmental stages had significantly different miRNAs expression patterns. The finding provides fundamental information of miRNAs which may help to elucidate their role in spermatogenesis and male infertility in this species. To the best of our knowledge, this study is the first to offer comparative profile of the miRNA transcriptome in prepubertal and adult canine testes using miRNA PCR array approach.

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1. Introduction

Highly tissue-specific expression and distinct temporal expression patterns during embryogenesis suggest that microRNAs (miRNAs) play a key role in the differentiation, development, maintenance, and functions of various tissues. Mammalian spermatogenesis is a series of complex processes, including morphologic and functional changes of different spermatogenic cells [1,2], which are likely precisely regulated by miRNA-mediated pathways [3,4]. However, biological functions of many miRNAs involved in spermatogenesis and testicular function are largely unknown. Furthermore, no study has been performed on the miRNA transcriptome of developing canine testes. Numerous miRNAs are preferentially expressed in the testis and male germ cells of humans and mice [5–9]. Conditional knockout of the Dicer gene disrupts the proliferation and differentiation of mouse spermatogenic germ cells [10–16]. Expressions of miRNAs are stage specific; some are upregulated, several are downregulated, and a number are novel to the developmental stages [17–19]. In mouse testis, pachytene, round, and elongated spermatocytes showed the highest levels of miRNA expressions. By regulating mRNA degradation and disrupting mRNA translation [20,21], these miRNAs can control meiosis and thus spermatogenesis [7]. Using the miRNA Array technology to extend the repertoire of dog testis miRNAs, the purpose of this study was to compare the expression patterns between sexually immature and mature canine testes.

2. Materials and methods

2.1. Study population

Testes from healthy Labrador-mix dogs undergoing elective castrations under general anesthesia were used. The dogs

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were classified into two age groups: young (2.2 ± 0.13 months; $n = 4$) and adult (11 ± 1.0 months; $n = 4$) were included. This study was carried out in strict accordance with the ethics and use of the animal species models for research. The protocol was approved by the Institutional Animal Care and Use Committee of the Washington State University (Protocol Number: 04070–001). All the surgeries were performed under general anesthesia, and all efforts were made to minimize suffering. Owner's consent was obtained to use testicular tissue in this study.

2.2. RNA preparation

Approximately 100 mg of testicular tissue in 1-mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was homogenized in a 5-mL Falcon round-bottom tube (Becton Dickinson, San Jose, CA, USA) using a handheld tissue homogenizer (Fisher Scientific, Pittsburg, PA, USA). Homogenized samples were transferred into a 1.5-mL microcentrifuge tube (Fisher Scientific) and incubated for 5 minutes at room temperature to allow dissociation of the nucleoprotein complexes. After addition of chloroform (VWR Corp., Aurora, CO, USA) and centrifugation ($\times 12000g$; 20 minutes at 4 °C), the aqueous phase containing RNA was isolated. The RNA precipitated by isopropyl alcohol (Decon Laboratories Inc., King of Prussia, PA, USA) and washed with 75% ethanol (Decon Laboratories Inc.). The RNA pellet was dissolved in RNAse-free water (Invitrogen, Green Island, NY, USA) at 60 °C. A NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., West Palm Beach, FL, USA) was used to measure RNA concentration and determine its quality. Sample absorbance was measured at 260 and 280 nm and the ratio was approximately 2.0 for all samples.

2.3. Reverse transcription

RNA samples were diluted to a concentration of 400 ng/μL. Micro Script II RT kit (Qiagen, Frederick, MD, USA) was used for reverse transcription. Micro Script HiSpec buffer (5X) was used to prepare complementaryDNA for mature miRNA profiling, which was performed with miRNome miScript miRNA polymerase chain reaction (PCR) arrays. Template RNA, 10X miScript Nucleics Mix, 5X miScript HiSpec Buffer, and RNase-free water were combined into 20 μL of reverse transcription reaction mix, and 1 μg of template RNA was added. The reverse transcription reaction mix was incubated at 37 °C for 60 minutes, at 95 °C for 5 minutes to inactivate miScript Reverse Transcriptase Mix, and then placed on ice. For whole canine miRNome profiling, the mix was diluted with 90 μL of RNase-free water and stored at −20 °C.

2.4. Canine mature miRNA expression profiling using real-time PCR

Mature miRNA profiling was performed using miScript miRNA PCR Arrays in combination with the miScript SYBR Green PCR Kit (Qiagen), which contains miScript Universal Reverse Primer and QuantiTect SYBR PCR Master Mix [22]. Reaction mix for canine miRNome miScript miRNA PCR Arrays was prepared with 2X QuantiTect SYBR Green PCR Master Mix, 10X miScript Universal Reverse Primer, RNAse-free water, and template DNA. Twenty-five microliter of the reaction mix was added to each well of a 96-well plate, miScript miRNA PCR Array Dog miFinder (MIFD-001Z; Qiagen). To activate HotStar Taq DNA Polymerase, the plate was heated at 95 °C for 15 minutes. Forty cycles of three-step cycling that included denaturation at 94 °C for 15 seconds, annealing at 55 °C for 30 seconds, and the extension at 70 °C for 30 seconds were carried out. Fluorescence data were collected during the extension step. Using a StepOne Plus instrument (Applied Biosystems, Inc., Carlsbad, CA, USA), the 40 cycles were carried out. Specificity and identity were verified by dissociation curve analysis. Baseline and threshold were set automatically for all PCR runs. Threshold cycle (CT) values were exported as an Excel file for further analyses.

2.5. Dog miRNA PCR Array and analysis

The Canine miScript miRNA PCR Array plate (Qiagen) includes primers for 84 mature miRNAs and controls (Supplementary Table 1) [22]. The controls are cel-miR-39-3p (H01 and H02), SNORD61 (H03), SNORD68 (H04), SNORD72 (H05), SNORD95 (H06), SNORD96A (H07), RNU6-2 (H08), miRTC (H09 and H10), and PPC (H11 and H12). SNORDs and RNU6-2 serve as internal normalizers [22]. Two reverse transcription controls and two positive controls ensure the efficiency of the array, the reagents, and the instrument.

2.6. Data handling and analyses

Raw CT data in Excel Version 1997 to 2003 (.xls file format) were uploaded to the Web-based software, http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php. Data quality control section was reviewed to assess the PCR reproducibility and reverse transcription efficiency and detect genomic DNA contamination in amplified samples of both the groups. SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, and RNU6-2 were selected as housekeeping genes and normalized by arithmetic mean. Data were reviewed for distribution of threshold cycle values and the raw data average in each group. Average delta CT, $2^{\wedge -\text{delta CT}}$, fold change, P value, and fold regulation were calculated by the software, and the fold change and P value results were included in all subsequent graphical analyses.

3. Results

Eighty-four well-characterized canine miRNAs were customized in the experiment (Supplementary Table 1). The fold regulation of 84 customized canine miRNA between prepubertal and adult canine testis is shown in valcano plot (Supplementary Fig. 1). We found that 32 miRNA species are upregulated (Fig. 1) and 12 miRNAs are downregulated (Fig. 2) in adult compared with the prepubertal testis. The miRNA identifications, fold changes (upregualtion values >1; downregulation values from 1 to 0), and P values were given in Table 1. The fold regulation for upregulated miRNAs in adult testis ranged from 2 ($P < 0.001$) to 1175 ($P < 0.000000$), and for downregulated miRNAs in adult testis, it ranged from −2 ($P < 0.0003$) to

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