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Research paper

Identification and differential expression of microRNAs in testis and ovary of Amur sturgeon (*Acipenser schrenckii*)

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ABSTRACT

Background: MicroRNAs (miRNAs) cooperate with sex-related genes in post-transcriptional regulation and play extremely important roles in the establishment of sexually dimorphic traits in animals. However, the gonad miRNAs and expression patterns of miRNAs in sturgeon have not been investigated.

Methods: In the present study, we used high-throughput small RNA sequencing (RNA-Seq) to discover gonad miRNAs from the ovaries and testes of Amur sturgeons (*Acipenser schrenckii*). Further, microarray and real-time PCR assays were performed to identify the expression patterns of gonad miRNAs.

Results: As a result, a total of 679 conserved and 51 novel miRNAs were successfully discovered in the gonads of *A. schrenckii*. Moreover, we found wide sequence variations (isomiRs) in gonad miRNAs, including 5′ and 3′ isomiRs. Our microarray analysis further characterized the 730 miRNAs expression profiles, which indicated that 117 differentially expressed miRNAs were detected with sex-biased patterns: 71 testis-biased and 46 ovary-biased miRNAs. Based on bioinformatics prediction, we found that there were functional differences between the testis-biased and ovary-biased miRNA targets involved in reproductive-related GO and KEGG pathways. Further, the association of the differentially expressed miRNAs and sex-related target mRNAs was uncovered. Finally, the expression patterns of 11 sex-biased miRNAs and 7 sex-related targets were validated in testes and ovaries using real-time PCR. Putative, negatively expressed miRNA-mRNA relationships were confirmed, such as Dmrt1 and asc-miR-2779, AR and asc-miR-203b-3p, foxl2 and asc-miR-30d.

Conclusion: This study provides information regarding the gonad miRNAs in sturgeon. The differential expression miRNAs in the gonads will help us to further understand the role of miRNA-mediated post-transcriptional regulation in the ovary and testis of sturgeon.

1. Introduction

Sex determination is a process through which the gonad is committed to differentiate into a testis or an ovary. Both the testis-determining and ovary-determining pathways have active gene networks that must be maintained throughout life by suppression of the opposing pathways at the transcriptional level. The key genes involved in testis and ovary development are widely conserved in most vertebrates (Eggers et al., 2014; Matson and Zarkower, 2012). For example, the critical genes of testis development mainly include Sox9 (SRY-box containing gene 9) and Dmrt1 (doublesex and Mab-3 related transcription factor 1), whereas ovarian development lacks a single genetic switch; however, some genes, such as folx2 (forkhead box L2) and Cyp19a (aromatase), are essential for correct ovary development. In particular, the appropriate regulation of sex-related gene expression also results from a controlled balance in post-transcriptional mechanisms mediated by non-coding RNAs. MicroRNAs (miRNAs) are endogenous, small non-coding RNAs (19–25 nucleotides in length) that function as post-transcriptional repressors of gene expression by binding to complementary sites in the 3′-untranslated region (3′UTR) of target mRNAs. Evidence of the importance of gonad-specific miRNAs involved in the process of gonad development is increasing (Imbar et al., 2014). For example, in mice, miR-124 is involved in regulating the fate of developing ovarian cells by preventing the expression of Sox9 (Real et al., 2013). Sox9 also regulates the expression of miR-202-5p/3p, a conserved miRNA that functions in the gonad during the early

Abbreviations: Dmrt1, doublesex and Mab-3 related transcription factor 1; Sox9, SRY-box containing gene 9; VASA, VASA-like protein; Foxl2, forkhead box L2; AR, androgen receptor; ER, estrogen receptor; Vtg, vitellogenin; Cyp19a, cytochrome P450, family 19, subfamily A; Cyp11b, cytochrome P450, family11 subfamily B; SRA, Sequence Read Archive; dah, days after hatch

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development of the testis (Wainwright et al., 2013). Furthermore, the sexual regulator Dmrt1 has been identified as a direct target of miR-19a/b during the sex reversal process (Liu et al., 2015). These studies show that gonad development in animals is regulated by numerous of molecules and signaling networks at transcriptional and post-transcriptional levels.

Sturgeons are polyploid chondrostean fish that originated during the Devonian period; as such, they constitute important model species for studying development and evolution (Saito et al., 2014). Although sturgeons are a sexually dimorphic fish species, it is difficult to distinguish females from males using morphological characteristics at the larval, juvenile or even adult stages. Therefore, it is important to establish the sexing techniques at an early gonad developmental stage in sturgeon aquaculture. However, although many studies have suggested that the sex of sturgeon may be genetically determined (Amberg et al., 2013), no sex chromosomes have yet been found, and the regulatory mechanism in the process of sexual differentiation in sturgeon is poorly understood (Keyvanshokooh and Gharaei, 2010). Using high-throughput sequencing technologies, transcriptomes from several sturgeon species, such as Amur sturgeon (*Acipenser schrenckii*) (Zhang et al., 2016a), Chinese sturgeon (*A. sinensis*) (Yue et al., 2015) and Adriatic sturgeon (*A. naccarii*) (Vidotto et al., 2013), have been recently reported, which provide the genetic resource for further study on gonad development in sturgeon. Meanwhile, a recent report showed that over 70% (481/663) miRNAs can be detected in the gonads of both sexes of sturgeon, and microarray methods have shown that there is high miRNAs sequence conservation among the different fish species (Zhang et al., 2016b). Therefore, it is necessary to identify miRNAs from the gonads (testis and ovary) of sturgeon using a combination of methods, which will further help us to understand the role of miRNA-mediated post-transcriptional regulation in the gonads of sturgeon.

This study was aimed at identifying and characterizing miRNAs in the gonads from *A. schrenckii*, which is a critically endangered and economically important aquaculture species (Wei et al., 2011). The 3-year-old *A. schrenckii* individuals are at the gonad II stage of gametogenesis (onset of meiotic activity of the spermatogonia of spermatogenesis in the testes and at the dominant period of the primary oocyte of oogenesis in the ovaries). The small RNA libraries were constructed from the testes and ovaries and the Illumina platform was employed for miRNA high-throughput sequencing. Then, the expression profiles of all the gonad miRNAs were analyzed using microarray analysis, and expression patterns for selected miRNAs were validated by stem-loop real time PCR. To understand functional differences, putative targets for the testis-biased and ovary-biased miRNAs were predicted and functional enrichment analyses were performed (GO and KEGG pathway) using bioinformatics methods. Finally, the interactions between differentially expressed miRNAs and putative sex-related targets were revealed, and their negative expression relationships were confirmed.

2. Material and methods

2.1. Ethics approval and consent to participate

All experimental animal procedures followed the principles of Guide for Care and Use of Laboratory Animals and were approved by the Animal Experimental Ethical Committee of Guangdong Institute of Applied Biological Resources (NO: 2014008).

2.2. Experimental animals and sample preparation

A total of twelve healthy Amur sturgeons, including six 3-year-old individuals (three males and three females) and six 3.4-year-old individuals (three males and three females), were collected from the Engineering and Technology Center of Sturgeon Breeding and Cultivation of the Chinese Academy of Fishery Science (Beijing, China). All experimental Amur sturgeon individuals were anaesthetized with 10^{-4} (v/v) eugenol in the water for 1–3 min, following the AVMA guidelines (2013) for use (Leary et al., 2013). The development stage of ovaries and testes from 3-year-old Amur sturgeon individuals was evaluated using histological analysis according to established methods (Hochleithner and Gessner, 1999; Grandi and Chicca, 2008; Rzepkowska et al., 2014). The ovary tissue was filled with the advantaged primary oocyte with bigger diameter (100–500 μm) and abundant nucleolus, whereas the testis tissue was alveolate seminiferous lobules filled with spermatogonia cells, differentiated primary spermatocyte and secondary spermatocyte (Additional file 1: Fig. S1).

Total RNA was extracted from each gonad sample using RNAiso reagent (TaKaRa, Tokyo, Japan). RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using a Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The RNA quality criteria for the RNA samples were RIN ≥ 8.0 (RNA Integrity Number) and OD 260/280 > 1.8. Qualified RNAs were used for Illumina sequencing, miRNA microarray analysis and expression validation using real time PCR.

2.3. Library preparation, clustering and small RNA sequencing

The small RNAs libraries were constructed from ovaries and testes of 3-year-old *A. schrenckii* individuals, respectively. According to the protocol of the NEBNext® Multiplex Small RNA (sRNA) Library Preparation Kit (Illumina, USA), 3 μg total RNA per sample was used as input material for the sRNA library. Briefly, 3′ SR Adaptor and 5′ SR Adaptor were specifically ligated to 3′ end and 5′ end of sRNA in two separated subsequent steps, respectively. Then first strand cDNA was synthesized using M-Mulv Reverse Transcriptase (RNase H^-). PCR amplification was performed using Long Amp Taq 2× Master Mix, SR primer for illumina and index primer. PCR products were purified on a 8% polyacrylamide gel and the products of 140 bp to 160 bp (with adaptors on both sides) were recovered and library quality was assessed on the Agilent Bioanalyzer 2100 system using DNA high sensitivity chips. The quantified criteria of library quality were concentration (nM) > 2, main peaks in 140–160 bp and no adapter pollution.

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq SR Cluster Kit v3-cBot-HS (Illumina, USA). After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2500 platform and 50 bp single-end reads were generated. sRNA sequence data were deposited in the NCBI Sequence Read Archive (SRA) database with accession number SRR3180645, SRR3180649, SRR3180651 and SRR3180713.

2.4. Filtering sRNA reads and miRNAs identification

Raw data (raw reads) of the FASTQ format were first processed through custom Perl and python scripts. Briefly, the irregular sequences from raw data were removed, including reads with 5′ adapter contaminants and 3′ adapter or insert tags, reads containing poly-N, poly A, T, G or C, smaller than 18 nt and longer than 35 nt and low quality reads. Then high-quality clean data (clean reads) were obtained. All the clean reads were aligned onto the gonad transcriptome reference sequences from *A. schrenckii* (Jin et al., 2015) using the program Bowtie (Langmead et al., 2009) without mismatches. The sequence assembly of transcriptome from *A. schrenckii* was previously deposited in SRA database with accession number SRR1131121. Finally, mapped sRNA reads with the length of 18–35 nt were further analyzed for identification of conserved and novel miRNAs.

For annotating conserved miRNAs, mapped sRNA reads were aligned to the whole metazoan mature miRNA sequence in miRBase 21 (http://www.mirbase.org/index.shtml). Then, the modified miRDeep2 (Friedlander et al., 2012) and sRNA-tools-cli program (http://srna-tools.cmp.uea.ac.uk/) were used to analyze conserved miRNA and