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Identification of differentially-expressed genes in early developmental ovary of Yellow River carp (*Cyprinus carpio* var) using Suppression Subtractive Hybridization

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ABSTRACT

Ovary development appears to be under polygenic control, and is influenced by multiple genetic factors that may vary from organism to organism. To gain a better insight into the molecular mechanisms of carp ovary development, Suppression Subtractive Hybridization (SSH) DNA libraries in two species of Yellow River carp were analyzed. Primordial gonads and stage II ovaries were used as testers, and adult ovaries as drivers. One hundred and fifty differentially-expressed candidate genes were examined by Southern blot microarray hybridization. We identified 41 differentially-expressed genes in the PG (Primordial gonad) library and 37 in the stage II ovary library. Gene Ontology Biological Pathway analysis showed the genes were involved in signal transduction, proteolysis process, cell differentiation, TGF-β signal and other biological responses. Twenty-two candidate genes were selected and further characterized using qRT-PCR. *Pvalb, epd*, and *MYH* were found specifically expressed in PG, while *bmp2b*, *desmin* and *fp1* were specifically expressed in stage II ovary. Our results indicate that these genes could be used as biomarkers of the early development of carp ovary. This finding will provide a basis for further understanding of the complex gonad developmental molecular mechanisms in Yellow River carp.

1. Introduction

Carp is one of the most important cyprinid species; it accounts for 10% of the global freshwater aquaculture production [1]. The Yellow River carp (*Cyprinus carpio* var.) is a member of this group, with a long history of breeding and high-yield production rates in China [2]. After gonad differentiation, female carps grow significantly faster than males. This growth difference might involve gonad developmental mechanisms. A comprehensive understanding of carp ovary development might allow growth differences to be exploited and facilitate carp cultivation.

The ovary is a dynamic organ that undergoes histological changes during the reproductive cycle [3]. Multiple studies on differentiation and early carp ovary development have been performed [4]. During ovary development, cells of the germ line exhibit significant differences in their characteristics and functions. The primordial gonad contains a considerable number of primordial germ cells (PGCs), which will later become oogonia and undertake ovary differentiation. In the adult ovary, proliferation and growth of oocytes results in the formation of the follicle.

Ovarian function is tightly regulated by a large number of genes [5]. The pattern of ovarian gene expression can reflect the status of gonadal development. Phenotype can be transformed through targeted gene disruption. We previously identified a critical dose-sensitive sex-reversal adrenal hypoplasia congenital region on the X chromosome of carp. This region, named gene 1 (*dax1* or *Nr0b1*; GenBank accession no. KF703999), is involved in a wide range of developmental processes and in the sex differentiation process of vertebrates. The genes that are essential for primordial follicle formation (*Figla*, *Wnt4* and *TrkB*) and that are required for follicle recruitment and growth (*Foxo3*, *Gdf9* and *Bmp15*) have been identified [6]. After comparing gene expression of carp ovary and testis, several genes (*ZP3C*, *Psmb2*, *Tektin-1*, *GAPDS*, *FGFIBP*, and *IGFBP-5*) playing key roles during carp growth (both in the gonad and other tissues) were identified [2]. However, the validation of genes during different developmental stages of ovary has been limited [7]. The identification of the set of genes involved in ovary development is important for assessing gonadal development in fish, as is the identification of genes that are up-regulated during the early stages of ovary development.

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Suppression Subtractive Hybridization (SSH) is a simple and powerful technique for comparing transcripts that are differentially-expressed in two RNA samples. SSH has been used successfully in scientific fields such as developmental biology, physiology, and toxicology [8]. To date, multiple differentially-expressed genes have been investigated using SSH [9]. Differential gene expression profiling of carp ovary and testis was conducted by Chen [2], however, the developmental mechanisms of early carp ovary development are still not well defined.

Thus, the purpose of this study was to identify differentially-expressed mRNAs in carp un-differentiated ovaries, in stage II ovaries, and in adult ovaries, using SSH technology. The expression of genes that seemed to be involved in the early development of the ovary was further confirmed using Southern blot hybridization and quantitative real-time PCR (qRT-PCR). The results of this study will help to identify new gene transcripts that are regulated during carp ovarian growth and development, which can be used in the future for investigating the molecular mechanisms of developmental processes. The findings may also help to fuel studies on carp breeding practices and reproductive regulation of female carp.

2. Materials and methods

2.1. Fish and histology of gonads

Yellow River carps were obtained from Henan Academy of Fishery Science (Henan, P. R. China). During the initial acclimation period, fish were maintained at the Genetic Laboratory (Xinxiang, Henan) in through-flow water tanks at 25 ± 2 °C under a natural photoperiod. The fish were fed with fish manure twice a day. No fish died during the experiment.

For histology analysis, the reproductive gland was dissected under the microscope, gonads from 50 fish were mixed after the presence of a primordial was confirmed by histological analysis. Juvenile ovaries were collected from 30 to 80dph (days post hybridization) fish, and the presence of a primordial stage II ovary was confirmed by histological analysis. Adult ovarian samples were collected from 10 sexually-mature fish (3 years old). The presence of an ovary in stage V was confirmed by histological analysis. Fish were handled according to Chinese ethics law (ethics approval no. SCXK (YU) 2005-0001). A piece of ovary was fixed with paraffin and the remaining tissue was snap-frozen in liquid nitrogen for RNA isolation and SSH.

2.2. RNA extraction

Total RNA was extracted using the RNA extraction kit and RNAiso reagent (TaKaRa, Shiga, Japan). Approximately 20 mg of tissue was homogenized using a mortar containing liquid nitrogen. An aliquot of each extract was used for spectrophotometry to determine the quality and concentration of RNA. Three biological replicates were collected from three developmental stages. RNA samples with an OD260/280 ratio of 1.9–2.2, an OD260/230 ratio ≥2, and a 28S:18S ratio of approximately 2:1 were considered satisfactory and used for further analysis. For SSH, cDNA was synthesized from 1 μg of total pooled mRNA, using the SMARTer™ PCR cDNA Synthesis Kit (Clontech Laboratories, Mountain View, CA, USA). For gene expression profile analysis, cDNA was synthesized using PrimeScript Reverse Transcriptase with 1 μg of total RNA, according to the manufacturer's instructions.

2.3. SSH cDNA library construction

SSH was performed using a PCR-Select cDNA subtraction kit (Clontech Laboratories), following the instructions of the manufacturer. Two subtractions were performed in parallel to construct tester-1 and tester-2 subtracted cDNA libraries. Subtracted cDNA in the tester-1 library was made using pooled PG mRNA as the tester and adult ovary cDNA as the driver. cDNA in the tester-2 library was made from pooled stage II ovary mRNA as the tester and cDNA from adult ovary mRNA as the driver. Two rounds of hybridization and PCR amplifications were performed and the subtraction efficiency was evaluated. Subtracted cDNA samples were cloned into pMD-19 vector (TaKaRa) and transformed into competent *Escherichia coli*. Recombinant white clones were selected randomly in ampicillin/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/isopropylβ-D-1-thiogalactopyranoside agar plates and amplified by PCR using nested PCR primers (1-5′TCGAGCGGCCGCCCGGGCAGGT3′ and 2R-5′ AGCGTGGTCGCGGCCGAGGT3′) to construct the corresponding SSH cDNA library.

2.4. Southern blot microarray hybridization

The plasmids obtained from recombinant clones from the forward and reverse SSH libraries were isolated and subjected to Southern blot hybridization. Plasmid DNA was spotted onto two Hybond-N1 nylon membranes (Solarbio, Beijing, China) at the same position after denaturation. The second round of PCR products from the forward and reverse subtracted cDNA libraries were separately labeled using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Mannheim, Germany). After hybridization with the two different probes, the cloned inserts were compared by contrasting the hybridization signals on the two membranes. Analysis of gray density variance and single-sample t-tests were performed to identify genes that were differentially expressed, which would be later isolated and sequenced.

2.5. Sequence analysis and gene ontology (GO) annotation

Positive clones showing significant differences in hybridization signals were sequenced at the Shanghai Bioengineering Institute (Shanghai, China). After removing adaptor sequences and repeated clones, the DNA sequences were compared against the GenBank database using the BLASTN and BLASTX programs at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). For functional annotation of the genes identified, GO Annotation was performed according to the PANTHER classification system (http://www.pantherdb.org).

2.6. Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR was performed using a detection system (LightCycler 96® Roche) based on SYBR Green fluorescent label. The reaction mixture consisted of the tissue cDNA as template, 2× Ultra SYBR Mixture (TaKaRa), and 0.2 μl of each primer (Table 1) in a final reaction volume of 10 μl. Each assay was performed in triplicate and included a negative control. The cycling parameters were as follows: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, plus 1 min at 60 °C. For each sample, a dissociation step was performed to verify a single specific melting temperature for each primer set. The amplification temperature was gradually increased from 65 °C to 95 °C at a rate of 0.2 °C/s. The $2^{-\Delta\Delta Ct}$ method was used to analyze gene expression levels. Each sample was tested in triplicate to normalize the system and pipetting error, using a standard curve method with the *40S* gene (40S ribosomal protein S11) as reference [10].