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Transcriptome profiling of developing spleen tissue and discovery of immune-related genes in grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

Grass carp *Ctenopharyngodon idella* is an important freshwater aquaculture species. However, studies regarding transcriptomic profiling of developing spleen tissue in the grass carp are lacking. Here, the transcriptome sequencing from the spleen tissue of one-year-old (cis1) and three-year-old (cis3) grass carp was performed using Illumina paired-end sequencing technology. The *de novo* assemblies yielded 48,970 unigenes with average lengths of 1264.51 bp from the two libraries. The assembled unigenes were evaluated and functionally annotated by comparing with sequences in major public databases including Nr, COG, Swiss-Prot, KEGG, Pfam and GO. Comparative analysis of expression levels revealed that a total of 38,254 unigenes were expressed in both the cis1 and cis3 libraries, while 4356 unigenes were expressed only in the cis1 library, and 3312 unigenes were expressed only in the cis3 library. Meanwhile, 1782 unigenes (including 903 down-regulated and 879 up-regulated unigenes) were differentially expressed between the two developmental stages of the grass carp spleen. Based on GO and KEGG enrichment analysis, these differentially expressed genes widely participated in the regulation of immunity and response in the grass carp. Moreover, the main components of six immune-related pathways were identified, including complement and coagulation cascades, Toll-like receptor signaling, B-cell receptor signaling, T-cell receptor signaling, antigen processing and presentation, and chemokine signaling. Finally, two identified transcripts including TLR 8 and complement component C8 were validated for reliability by RT-PCR. Collectively, the results obtained in this study will provide a basis for the study of molecular mechanisms in grass carp spleen development.

1. Introduction

The spleen in teleosts is a primary hematopoietic and peripheral lymphoid organ [1], and is closely located to the stomach in the dorsal mesentery. This organ consists of splenic sinusoids, surrounded by lymphoid cells that contain red blood cells. Its functional unit is the ellipsoid corpuscle, comprised of a capillary surrounded by melano-macrophages [2]. Therefore, an important function of the spleen is the storage and production of erythrocytes (erythropoiesis) and the destruction of aged and affected blood cells [3]. Meanwhile, the spleen of teleosts contains antibody-producing cells and is the major source of immunoglobulins [4]. For this reason, spleen also has a role in antigen presentation and initiation of the adaptive immune response [5,6], its main tasks include the development of B cells, antigen processing, and MHC class II expression. In addition, fish spleen acts as a second lymphoid organ to filter blood-borne pathogens and is the main site where defense against microbes takes place [7,8]. Therefore, the spleen is an eminently suitable organ for assessing immune responses and identification of immune-relevant genes in fish. In recent years, with the outbreak of infectious diseases, the research around the spleen function of fish has been increasingly valued. Previously, information on the fish spleen is largely restricted to a

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few species of teleosts (e.g., zebrafish [9], sea bass [10], catfish [11], Australian lungfish [12]) and has focused on morphological features. However, the molecular basis of fish spleen development is relatively unknown.

The term transcriptome refers to the entire set of RNA transcribed from a genome. At present, the rapid development of high-throughput sequencing technologies has provided a powerful platform for the rapid and nearly complete characterization of transcriptomic events in various species. For species with the lack of whole-genome sequence information, high-throughput sequencing of the transcriptome offers a large amount of bioinformatics data from the complex whole transcriptome. Thus, transcriptomic profiling analysis is considered a perfect method for genome study and functional gene identification. In recent years, high-throughput sequencing of the spleen transcriptome has been performed in several aquaculture fish species, including zebrafish (*Danio rerio*) [13], orange-spotted grouper (*Epinephelus coioides*) [14], large yellow croaker (*Pseudosciaena crocea*) [15], Atlantic cod (*Gadus morhua*) [16], common carp (*Cyprinus carpio*) [17], tiger grouper (*Epinephelus fuscoguttatus*) [18], Asian seabass (*Lates calcarifer*) [19,20], channel catfish (*Ictalurus punctatus*) [21], *Schizothorax prenanti* [22], and Japanese flounder (*Paralichthys olivaceus*) [23]. These studies provided a new insight into the molecular biology of the spleen in fish. However, most of these largely focused on the role of the spleen in host–pathogen interactions. The knowledge about the molecular biology of normal development processes in the spleen of fish, especially large-scale studies at the transcriptome levels, are relatively scarce.

The grass carp *Ctenopharyngodon idella* is one of the most important freshwater aquaculture species in China. Over the years, infectious diseases induced by viruses and bacteria can cause tremendous economic losses and are emerging as one of the most prominent problems in grass carp aquaculture. Therefore, research regarding the immune system and its function have received considerable attention in the grass carp. In the past decade, research of the grass carp immune system mainly focused on identification of immune-relevant genes [24,25], and development of genomic markers [26,27]. In recent years, a few studies regarding the immune system of grass carp at the transcriptome level largely involved the head kidney [28] as well as the spleen under the conditions of host–pathogen interaction [29–32]. Up to now, the molecular basis of the grass carp immune system is still vague. To our knowledge, no studies have reported transcriptomic profiling during the normal development process in the spleen of grass carp. Considering the importance of the spleen in grass carp, we selected it as the target sample to conduct transcriptome sequencing in both one-year and three-year-old grass carp. We analyzed the characterization of transcriptome profiling in developing spleen tissue and identified the main components of pathways related to the immune system. The study aims were to provide a new insight into the molecular understanding of spleen development as well as a valuable resource for future research on the immune system in the grass carp and other closely related species.

2. Materials and methods

2.1. Experimental animals

The investigation was approved by the Ethics Committee of the Freshwater Fisheries Research Institute of Henan Province and did not involve endangered or protected species. All animal experiments were performed using cultivated grass carp *C. idella*. The spleen tissues were collected from one-year (cis1) and three-year-old (cis3) grass carp, respectively, and six individuals were dissected at each developmental stage. The collected samples were stored at −80 °C for preservation before use.

2.2. RNA extraction and cDNA library construction

Total RNA from the grass carp spleen tissues was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Equal amounts of high-quality RNA samples from different individuals were mixed, and the mixed RNA sample at each developmental stage was used for cDNA library construction. Two cDNA libraries for the spleen tissue were constructed using a Truseq™ RNA sample prep Kit (Illumina); one from one-year-old grass carp and the other from three-year-old grass carp. Briefly, poly(A) mRNA was isolated from 5 μg of initial total RNA using oligo (dT) magnetic beads (Invitrogen), and then the mRNA was randomly interrupted into ~200 bp short fragments with fragmentation buffer. The cleaved mRNA fragments were used as templates, first-strand cDNA was synthesized using random hexamer primers and reverse transcriptase (Invitrogen), and then second-strand cDNA was synthesized. The double-stranded cDNA was end repaired, a-tailed and indexed adapters were ligated. Finally, the cDNA library was enriched with 15 cycles of PCR.

2.3. Sequencing and de novo assembly

The fragments were separated using 2% certified low-range Ultra Agarose (Bio-Rad) by electrophoresis, and the concentration was determined using TBS380 Picogreen (Invitrogen). The clusters were generated using a Truseq PE Cluster Kit v3-cBot-HS (Illumina) by bridge-PCR amplification. The paired-end cDNA library was sequenced, at Majorbio (Shanghai, China) on an Illumina HiSeq4000 sequencing platform that generated paired-end reads of 151 nucleotides. Raw reads generated by Illumina Hiseq 4000 were cleaned using SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) software by removing reads with adaptors, reads with more than 10% Q < 20 bases (those with a base quality less than 20) and low-quality sequences (reads with ambiguous bases 'N'). *De novo* assembly of all cleaned reads was carried out with short reads assembling program-Trinity (version: numbertrinityrnaseq-r2014-04-13) at default parameters [33].

2.4. Functional annotation

Functional annotations were performed by homology searches against major public databases. All assembled transcripts were compared with sequences in the Nr (NCBI non-redundant protein sequences) [34], Swiss-Prot (a manually annotated and reviewed protein sequence database) [35], COG (Clusters of Orthologous Groups of proteins) [36], Pfam (protein family) [37], KEGG (Kyoto Encyclopedia of Genes and Genomes) [38] and GO (Gene Ontology; http://www.geneontology.org/). Nr, COG, Swiss-Prot, and KEGG used BLASTx analysis with a cut-off E-value of 10^{-5}, Pfam used HMMER3 (http://hmmer.org/) and GO used Blast2GO [39]. The putative functions of the given transcripts were defined by first subject hits, and the BLAST results of the best hit were extracted for transcript description. Based on the Nr annotations, the Blast2GO program was used to obtain GO annotations of unique assembled transcripts to describe biological processes, molecular functions, and cellular components.

2.5. Differential gene expression analysis

All clean reads from each of the two libraries (cis1 and cis3) were mapped to reference sequences (unigenes from the assembled transcriptome data) using Bowtie2 software [40], respectively.