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Evaluation of differentially expressed immune-related genes in intestine of *Pelodiscus sinensis* after intragastric challenge with lipopolysaccharide based on transcriptome analysis

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ABSTRACT

Pelodiscus sinensis is the most common turtle species that has been raised in East and Southeast Asia. However, there are still limited studies about the immune defense mechanisms in its small intestine until now. In the present research, histological analysis and transcriptome analysis was performed on the small intestine of *P. sinensis* after intragastric challenge with LPS to explore its mechanisms of immune responses to pathogens. The result showed the number of intraepithelial lymphocytes (IELs) and goblet cells (GCs) in its intestine increased significantly at 48 h post-challenge with LPS by intragastrical route, indicating clearly the intestinal immune response was induced. Compared with the control, a total of 748 differentially expressed genes (DEGs) were identified, including 361 up-regulated genes and 387 down-regulated genes. Based on the Gene Ontology (GO) annotation and the Kyoto Encyclopedia of Genes and Genomes (KEGG), 48 immune-related DEGs were identified, which were classified into 82 GO terms and 14 pathways. Finally, 18 DEGs, which were randomly selected, were confirmed by quantitative real-time PCR (qRT-PCR). Our results provide valuable information for further analysis of the immune defense mechanisms against pathogens in the small intestine of *P. sinensis*.

1. Introduction

The Chinese soft-shelled turtle, *Pelodiscus sinensis*, is a high-value commercial freshwater species and widely cultured in East and Southeast Asia, with annual production over 341,000 tons in mainland China in 2014 [1]. However, owing to intensive cultivation and environmental deterioration in recent decades, it frequently suffered from serious infectious diseases caused by bacteria, viruses, parasites and other pathogens, which resulted in great economic loss every year [2]. Consequently, understanding its immune defense mechanisms may contribute to the development of novel management strategies for disease control and the long-term sustainability of the industry. In animal immune system, the small intestine is a very important immune organ because of its gut-associated lymphoid tissue (GALT), a major compartment of the immune system. Just like in mammals, the intestine immune system of *P. sinensis* involves innate, cell-mediated and humoral compartments and can protect the organism against parasitic and pathogenic infections. Though some immune-related cytokine genes in *P. sinensis*, such as IFN-γ, IL-1β, TNFα, IL-6, IL-12β and some immunoglobulin like IgM, IgY, IgD have been studied [3–6], there are still limited studies about the mechanisms of immune responses to pathogens of its small intestine. Chen et al. studied the morphological characteristics of the cells involved in intestinal mucosal immunity in *P. sinensis*, and identified several immune related cells in its epithelium and lamina propria, such as lamina propria lymphocytes (LPLs), intraepithelial lymphocytes (IELs), plasma cells, macrophages and some leucocytes [7]. However, in consideration of lower evolutionary grade in vertebrate and the absence of microfold cells (M cells) and lymph nodules, we suppose that the immune response of the small intestine in *P. sinensis* may be different from it in mammalian and birds.

Transcriptome profiling analysis is a rapid and effective approach for genome survey, massive functional gene and molecular maker identification due to its comprehensive view of gene expression profiling. It has been widely used in the research of aquatic animal recently, such as blue tilapia (*Oreochromis aureus*) [8], Pacific oyster (*Crassostrea gigas*) [9], rainbow trout (*Oncorhynchus mykiss*) [10], *Litopenaeus vannamei* [11], zebrafish (*Danio*

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rerio) [12], Atlantic salmon (*Salmo salar*) [13] and Atlantic cod (*Gadus morhua)* [14]. In the present study, we performed the transcriptome analysis on the small intestine of *P. sinensis* after challenged with lipopolysaccharide (LPS), which is the cell wall component of gram-negative bacteria and a powerful stimulator that activates innate immune responses of intestine through multiple signaling pathways [15]. The purpose of our research is to reveal the profiles of differentially expressed immune-related genes in the intestine of *P. sinensis* after challenge with LPS. The result can help us understand the immune defense mechanisms of small intestine better in *P. sinensis*, and contribute to the development of novel management strategies for its disease control and prevention.

2. Materials and methods

2.1. Experimental animal and treatments

Healthy Chinese soft-shelled turtles with an average body weight of 36.59 ± 5.24 g, were obtained from a breeding farm in Hangzhou, Zhejiang Province, China. All were acclimated in 40 L plastic tank with flowing aerated freshwater in a constant temperature laboratory room at 30 °C for one week before the experiment, and were fed with commercial forage (Kesheng Feed Stock Co., LTD, Zhejiang) twice a day. All experiments were according to "the rules of the Animal Care and Use Committee of Zhejiang University (Hangzhou, China)".

Ten turtles were randomly selected and divided into two groups: five were intragastrically given LPS (Sigma, 0.8 mg/100 g) which were dissolved in total 200 μL saline solution (0.75%) prior to use, and other five were intragastrically given the same volume saline solution (0.75%) as control group. Each turtle was anesthetized using diethyl ether and killed by decapitation at 48 h post challenge, and the middle part of small intestine was collected and separated into two parts: one was kept in the 4% paraformaldehyde for histological examination and the other was stored in −80 °C for RNA isolation.

2.2. Histological examination

Five intestine samples from challenge and another five from control group were fixed in 4% paraformaldehyde for 48 h respectively, and then rinsed several times in 70% ethanol, dehydrated with a graded series of ethanol, cleaned with xylol and embedded in paraffin wax. Subsequently, 4 μm sections were prepared for histological staining in triple. Hematoxylin-eosin (HE) and periodic acid-schiff (PAS)staining were used for observing intraepithelial lymphocytes (IELs) and goblet cells (GCs) respectively according to the method mentioned by Q. Sun et al. [16]. The number of IELs and goblet cells from 20 different intestinal villus of each section and 3 sections for each individual were counted.

2.3. RNA isolation

Three samples from challenge and control group were selected randomly for RNA isolation respectively. Total RNA was extracted from the frozen intestine sample using the RNeasy mini kit (QIAGEN, USA),and then treated with DNase I (QIAGEN) to remove genomic DNA contamination. The concentration of the purified RNA was determined by a Qubit2.0 fluorometer (Invitrogen, USA). RNA integrity was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies Inc., USA).

2.4. RNA-seq library preparation and illumina sequencing

RNA-Seq Libraries construction and Illumina sequencing were performed by Guhe Information Technology Company (Hangzhou, China). 1 μg of total RNA from each sample was collected for RNA-Seq library preparation and sequencing. cDNA library was prepared using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA) according to the manufacturer's instructions after mRNA purified and fragmented. The samples were clustered and sequenced using the Illumina HiSeq 2500 system with a 100-cycle paired-end run in duplicate.

2.5. RNA-seq data analysis

RNA-Seq reads were assessed for its quality with FastQC (version 0.10.1; http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Raw sequence data were transformed by base calling into sequence data, were stored in fastq format, and were cleaned by removing adapter sequences, empty reads, and low quality sequences (The phred quality score was less than Q30). Cleaned reads were then mapped to the reference genome of *P. sinensis* (GenBank: AGCU00000000.1) using HISAT [17]. The transcript abundances were estimated as fragments per kilobase of exon per million fragments mapped (FPKM) by Cufflinks 2.1.1 [18]. Cuffdiff was then used to determine differential gene expression of control and LPS challenged samples with an FDR<0.05 and a log2 ratio $\geq$ 1, which was divided into up-regulated and down-regulated lists of transcripts [19].

2.6. Functional enrichment analysis

Blast2GO (https://www.blast2go.com/) were performed to retrieve biological process, molecular function, and cellular component terms, to obtain GO annotations for all genes related to immunity with a Fisher's Exact Test (filtered with FDR $\leq$0.05). After getting GO annotations, the Web Gene Ontology Annotation Plot (WEGO) (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) was used to categorize all genes based on function. The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to assign and predict putative functions and pathways associated with the differential expressed genes (DEGs).

2.7. Verification of DEGs using quantitative real-time PCR (qRT-PCR)

To validate RNA-seq data and expression profiles, 7 immune-related DEGs and 11 other DEGs were randomly selected for verification using qRT-PCR. β-actin was used as the internal standard. The primer sequences and related information are shown in Supplemental File 1. The RNA samples used for qRT-PCR amplifications were the same with those used to construct RNA-Seq library mentioned above. The qPCR was performed using the LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland) with SYBR Green I Master. The reaction mixture (10 μL) comprised 2.5 μL cDNA, 0.5 μL (10 nM) forward primer and 0.5 μL (10 nM) reverse primer, 1.5 μL PCR grade water and 5 μL Master Mix. All samples were run in triplicate. The relative expression level of each gene was calculated according to $2^{-\Delta\Delta Ct}$ method and normalized to the endogenous control gene β-actin. Following normalization, the ratios were expressed as fold changes compared with the samples from the control group.

2.8. Statistical analysis

Experimental data were analyzed by a one-way analysis of