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Sequencing of the first ayu (*Plecoglossus altivelis*) macrophage transcriptome and microarray development for investigation the effect of LECT2 on macrophages

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ABSTRACT

Macrophages play an important role in first-line host defense of innate immune in fishes. However, it is difficult to investigate cellular mechanism of immune response in fish species with little genomic information available. Here we present the first use of RNA-Sequencing to study the macrophage transcriptome of ayu, *Plecoglossus altivelis*, which is an economically important fish in East Asia. *De novo* assembly generated 49,808 non-redundant consensus sequences, among which 23,490 transcripts found respective coding sequences. 15,707 transcripts are predicted to be involved in known metabolic or signaling pathways. The sequences were then used to develop a microarray for measurement the effect of recombinant LECT2 on ayu macrophages. LECT2 altered expression of a variety of genes mainly implicated in actin cytoskeleton, pattern recognition receptors and cytokines. Meanwhile, LECT2 enhanced phagocytosis, bacterial killing, and respiratory burst in ayu macrophages, which supported the thought derived from the microarray data that LECT2 activates macrophages. In conclusion, our results contribute to understanding the specific regulation mechanism of LECT2 in macrophage activation, and the combination of transcriptome analysis and microarray assay is a good method for screening a special tissue or cell response to a stimulus or pathogen in non-model fish species.

1. Introduction

Ayu, *Plecoglossus altivelis*, the only member in the genus *Plecoglossus* of the family Plecoglossidae, is an economically important fish species cultured in East Asia. Its delicate and delicious tastes make ayu a great treasure in fishery. However, the development of ayu culture has been challenged seriously by bacterial and viral infection which has resulted in both production and animal welfare problems [1]. Limited available genomic information of ayu has hampered the understanding of the complex molecular mechanisms that involved in disease resistance in this species.

Fish occupies a vital position during the evolution of the innate immune system and the appearance of the adaptive immune response [2]. Some important and highly conserved molecular components, somehow, are missing in certain strains of fish. For example, the major histocompatibility complex (MHC) II cannot be found in Atlantic cod and hagfish [3,4]. This indicates that there is a necessity to investigate the versatile and complicated immune system in different fishes. Macrophages are an important component of the innate immune system, and it might play a more important role in fish than in mammal [5]. Therefore, the studies on fish macrophages will contribute to the understanding of the evolution of the immune system from lower vertebrate to mammals and will facilitate the research of the molecular mechanisms associated with disease resistance in fish.

Leukocyte cell-derived chemotaxin 2 (LECT2) is firstly isolated from human T cell line as a neutrophil chemotactic factor [6]. Proteins homologous to LECT2 have been found in many fishes, such as carp (*Cyprinus carpio*) [7], rainbow trout (*Oncorhynchus mykiss*) [8], zebrafish (*Danio rerio*) [9], croceine croaker (*Larimichthys crocea*) [10], longtooth grouper (*Epinephelus bruneus*) [11], and red-spotted grouper (*Epinephelus akaraa*) [12], and it is dramatically up-regulated after microorganism infection [9]. Most recently, we expressed and purified recombinant ayu LECT2 and test its bioactivity based on its ability to induce chemotaxis of ayu

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head kidney-derived macrophages and alter some gene expression [13]. However, little was known about the functions of LECT2 on ayu macrophages and corresponding signaling pathway.

Recently, next-generation sequencing (NGS) technologies emerge as a powerful and cost-effective approach to perform transcriptome analysis in tissues or cells. Since the transcriptome of zebrafish was obtained in 2009 [14,15], several economically or scientifically important fish species have been analyzed at transcriptome level, such as Japanese seabass (*Lateolabrax japonicus*), European eel (*Anguilla anguilla*), orange-spotted grouper (*Epinephelus coioides*), hagfish (*Eptatretus burgeri*), gilthead seabream (*Sparus aurata*), Atlantic salmon (*Salmo salar*) [3,16–20]. Development of transcriptome sequencing in these species provides access to functional and evolutionary analyses previously restricted to genetic model organisms. DNA microarrays can measure the individual transcript level of tens or even hundreds of thousands of genes simultaneously, thus providing a high-throughput mean to analyze gene expression levels at the whole-genome scale [21,22]. Recently, an affordable approach towards expression analysis for organisms lacking genome sequence information was developed by combining the advantages of NGS and microarray technology [23].

In this study, we sequenced and characterized the first transcriptome of ayu macrophages, and developed a microarray for profiling gene expression patterns in macrophages affected by LECT2. We found that LECT2 activated ayu macrophages by altering mRNA expression of a variety of genes mainly implicated in actin cytoskeleton, pattern recognition receptors and cytokines by microarray-based assay, which was supported by the evidence that LECT2 enhanced phagocytosis, bacterial killing, and respiratory burst in ayu macrophages.

2. Materials and methods

2.1. Ayu head kidney-derived macrophage culture

The ayu fish were anaesthetized (0.03% (v/v) ethylene glycol monophenyl ether) and sacrificed to obtain head kidney. Macrophages were isolated as previously described [13]. After 12 h incubation at 24 °C, non-adherent cells were washed off and the remaining adherent cells were incubated with RPMI 1640 medium containing 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin throughout the experiment. Greater than 95% of adherent cells were macrophages according to morphological characteristics observed after Giemsa staining.

2.2. cDNA library construction and sequencing

Total RNAs were isolated with TRIzol (Invitrogen, Shanghai, China) from the macrophages according to the manufacturer's instructions. RNAs were treated with RNase-free DNase I for 30 min at 37 °C (New England BioLabs, Beverly, USA) to remove residual DNA. Oligo (dT) beads were used to isolate poly(A)-mRNA. After the mRNA was fragmented as templates, the cDNA first-strands were synthesized using random hexamer-primer and reverse transcriptase (Invitrogen). Second-strands were synthesized using RNase H (Invitrogen) and DNA polymerase I (New England BioLabs). Paired-end libraries with about average insert lengths of 200 base pairs were synthesized according to Illumina protocols. Transcriptome sequencing of ayu macrophages was performed at Beijing Genomics Institute (BGI)-Shenzhen, China using the Illumina HiSeq™ 2000 platform.

2.3. Assembly

Data quality control and sequence filtering of repetitive, low-complexity, and low-quality reads prior to assembly of sequenced reads for non-redundant consensus were performed using FASTX_-Tookit (http://hannonlab.cshl.edu/fastx_toolkit) and PRINSEQ software [24]. The default parameters were used when using Trinity (2011-07-13) for the first assembly, and parameters "-new_ace -minmatch 12 -minscore 20 -repeat_stringency 0.9" were set for the Phrap software. Markov Cluster Algorithm, MCL (http://micans.org/mcl, Stijn van Dongen), was performed as follows. First, similarity of 52,029 contigs was detected using Blastn. Sequences with more than 90% identity and more than 60% coverage were filtered out. Subsequently, these similar sequences were used by TRIBE_MCL algorithm. The longest sequences were selected as the representative transcripts. High-throughput RNA-Seq data was deposited in compliance with MINSEQE requirements and submitted to the NCBI SRA database (accession number is SRA047923.1).

2.4. BLAST against sequence databases and functional annotation

Sequence homology searches were performed using BLASTX programs against sequences in NCBI non-redundant (nr) protein database and in SwissProt database (E-value $<1e-5$). Genes were tentatively identified according to the best hits against known sequences. The non-redundant consensus sequences identified either in nr or SwissProt database were used to mapped to GO and were analyzed further using KEGG.

2.5. Production of recombinant ayu LECT2

Recombinant ayu LECT2 proteins containing no endotoxin were produced from *Escherichia coli* as previously described [13].

2.6. Microarray development and assay

Agilent 60-mer oligonucleotide probes of ayu macrophage genes were designed by eArray (Agilent, Palo Alto, USA) for each transcript. Probes that had potential cross-hybridization were removed. Total 49,077 probes were finally used and Agilent in situ synthesis probe microarrays were then made (Agilent). Macrophages in a 100-mm culture dish (2×10^6 cells/ml) were treated with 5 μg/ml LECT2 for 3.5 h, and RNA was extracted from ayu macrophages using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA quality was analyzed by agarose gel electrophoresis and was quantified via spectrophotometry using NanoDrop ND2000 (Thermo Scientific, Waltham, USA). RNA was subsequently amplified and labeled using Agilent LowInput Quick Amp Labeling Kit. Then fluorescence dye labeled cRNA was hybridized to Agilent array. Hybridization and scanning were performed on Agilent's Microarray Platform according to Agilent's Standard Protocols. The threshold of differentially expressed genes was set at fold change >2 and $P < 0.05$.

2.7. Real-time quantitative PCR (RT-qPCR)

The ayu macrophage samples were collected and preserved at −80 °C after saline or LECT2 treatment. The total RNA was extracted and purified from ayu macrophage samples. After deoxyribonuclease I treatment, the isolated RNAs from ayu macrophages were reverse transcribed with M-MLV Reverse Transcriptase (TaKaRa, Kyoto, Japan). Specific primer pairs of selected genes were designed (Table 1). The reaction mixture was incubated for 300 s at 95 °C, followed by 37 amplification cycles of 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C, in a StepOne-Real Time PCR platform (Applied Biosystems, Foster City, USA). The threshold cycle data (Ct) and baselines were determined using auto settings. Ct values of genes for ayu macrophage samples were normalized to β-actin using the ΔCt method as previously described [13].