Fish and Shellfish Immunology 73 (2018) 288–296

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Identification of immune-related genes in gill cells of Japanese eels (*Anguilla japonica*) in adaptation to water salinity changes

Jie Gu[a,1], Shuya Dai[a,1], Haitao Liu[a], Quanquan Cao[b], Shaowu Yin[b], Keng Po Lai[c], William Ka Fai Tse[d,*], Chris Kong Chu Wong[e], Haifeng Shi[a,**]

[a] Institute of Life Science, Jiangsu University, Zhenjiang, Jiangsu, 212000, China
[b] College of Life Sciences, Key Laboratory of Biodiversity and Biotechnology of Jiangsu Province, Nanjing Normal University, Nanjing, Jiangsu, 210023, China
[c] Department of Chemistry, City University of Hong Kong, Kowloon, Hong Kong
[d] Faculty of Agriculture, Kyushu University, Fukuoka, Japan
[e] Department of Biology, Hong Kong Baptist University, Kowloon, Hong Kong

ARTICLE INFO

Keywords:
Immune-related genes
Adaptive immune response
Environmental salinity
RNA-seq

ABSTRACT

The changes in ambient salinity influence ion and water homeostasis, hormones secretion, and immune response in fish gills. The physiological functions of hormones and ion transporters in the regulation of gill-osmoregulation have been widely studied, however the modulation of immune response under salinity changes is not determined. Using transcriptome sequencing, we obtained a comprehensive profile of osmo-responsive genes in gill cells of Japanese eel (*Anguilla japonica*). Herein, we applied bioinformatics analysis to identify the immune-related genes that were significantly higher expressed in gill pavement cells (PVCs) and mitochondrial-rich cells (MRCs) in freshwater (FW) than seawater (SW) adapted fish. We validated the data using the real-time qPCR, which showed a high correlation between the RNA-seq and real-time qPCR data. In addition, the immunohistochemistry results confirmed the changes of the expression of selected immune-related genes, including C-reactive protein (CRP) in PVCs, toll-like receptor 2 (TLR2) in MRCs and interleukin-1 receptor type 2 (IL-1R2) in both PVCs and MRCs. Collectively our results demonstrated that those immune-related genes respond to salinity changes, and might trigger related special signaling pathways and network. This study provides new insights into the impacts of ambient salinity changes on adaptive immune response in fish gill cells.

1. Introduction

Environmental salinity is an important factor for aquaculture and could affect physiological responses of aquatic organisms. In fish, environmental salinity changes influences osmoregulation, hormonal control, energy metabolism and growth [1–3]. Japanese eel is cultured in aquaculture ponds, and is an important commercial fish in East Asia. In addition to dietary consumption, some proteins isolated from the fish are used for medical purposes. For example, a fluorescent protein called UnaG isolated from muscles of Japanese eel could be applied for screening toxins that can trigger liver disease [4]. Although the salinity is an important factor considered for the growth of Japanese eel [5], it is difficult to avoid salinity alterations in freshwater of open-air culture due to natural (e.g raining or infiltration of groundwater) or man-made (e.g feeding or drugs) causes.

It suggests that environmental salinity not only affects these physiological processes (i.e osmoregulation, ion transport and hormonal control), but also alters the immune system in fishes [6,7]. In gilthead seabream (*Sparus aurata* L.), a significant increase in plasma IgM level was observed after acclimation at high salinity condition for 14 days, while enhancement peroxidase content and complement activity in plasma were detected after a longer term of acclimation (100 days) [6]. In brown trout (*Salmo trutta*), phagocytic activities of the pronephric leucocytes and the lysozyme concentrations were significantly increased after transfer from freshwater to seawater [8]. In addition, acute exposure of tilapia to hyperosmotic conditions was found to have immunostimulatory effects on its cellular immune reactions (phagocytosis and respiratory burst activity) and humoral responses (lysozyme activity and complement activity) [9]. Moreover, fresh water to seawater transfer in rainbow trout showed sustained elevation in total white blood cell counts, increased plasma but decreased mucus lysozyme, and enhanced head kidney macrophage

* Corresponding author. Attached Promotive Center for International Education and Research of Agriculture, Faculty of Agriculture, Kyushu University, Fukuoka, Japan.
** Corresponding author. Xuefu Road 301, Zhenjiang, Jiangsu, China.
E-mail addresses: kftse@agr.kyushu-u.ac.jp (W.K.F. Tse), shihf@ujs.edu.cn (H. Shi).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.fsi.2017.12.026
Received 24 August 2017; Received in revised form 6 December 2017; Accepted 17 December 2017
Available online 18 December 2017
1050-4648/

respiratory burst activity [10]. Increased activity and proliferation of immune cells were found in pipefish (*Syngnathus typhle*) after acute salinity change, but the expression of the immune genes granulocyte colony-stimulating factor precursor (*GRCSF*) and interleukin 10 (*IL10*) were significantly decreased with increasing salinity on day one [11]. In kidney of striped catfish (*Pangasianodon hypophthalmus*, Sauvage), chronic hyperosmotic stress inhibited toll-like receptors (TLRs) expression, and the down-regulations of TLRs were aggravated when exposed to bacterial infection [52]. Taken together, acute and chronic salinity change could stimulate both cellular and humoral immune activities; but also suppress the expressions of some immune-related genes.

In this report, we would like to understand if the immune-related genes are responsive to salinity changes. Recently, our group had obtained the transcriptome profiles of gill cells from Japanese eel (*Anguilla japonica*) adapted in freshwater and seawater environments [12,13]. Besides identifying the new osmo-responsive genes and pathways in the study, we also identified several enhanced immune-related pathways such as cytokine-related pathways such as IL-6, IL-8 and IL-9 signaling pathways under different osmotic environment [12,13]. In this study, we combined transcriptomics approach, RT-qPCR and immunohistochemistry to declare the effect of environmental salinity changes on immune-related genes involved in adaptive immune responses in gill cells of Japanese eels. The results of this study provide an important resource for future investigations on the mechanisms of adaptive immune response of gill cells.

2. Materials and methods

2.1. Animals and isolation of gill cells

Japanese eels (*Anguilla japonica*) weighing 500–600 g were purchased and kept in a 40 L glass tanks supplied with charcoal-filtered aerated fresh water (FW) at 18–20 °C under a 12 L:12D photoperiod for 3 weeks before the experiments. The fish were then kept in FW (n = 4) or acclimated to seawater (SW) (34 ppt) (n = 4) for another 3 weeks. Ten liters of water was changed every 3 days. The experiment was repeated at least three times. The fishes were anesthetized by 0.1% MS222 in a plastic container and the gills were perfused with buffered saline (130 mM NaCl, 2.5 mM KCl, 5 mM $NaHCO_3$, 2.5 mM glucose, 2 mM EDTA, and 10 mM Hepes, pH 7.7) to remove blood cells from gills. Gill arches were excised and washed. The gill arches were cut into small fragments and subjected to two cycles of trypsin digestion (0.5% trypsin + 5.3 mM EDTA), each for 20 min at room temperature in a rotator (300 rpm). The cell suspension was then filtered, washed, and underwent a three-step Percoll gradient of 1.09, 1.06, and 1.03 g/ml in PBS and centrifuged at 2000 g at 15 °C for 20 min. Cells at the interface of 1.03 and 1.06 g/ml Percoll solution were regarded as pavement cells (PVCs), while at the interface of 1.06 and 1.09 g/ml were mitochondria rich cells (MRCs). The identity of MRCs was confirmed by mitochondria staining (Mitotracker, CMTMRos-H2, Molecular Probes) and Na^+/K^+-ATPase staining (mouse anti-Na^+/K^+-ATPase α-subunit antiserum) (1:50, Developmental Studies Hybridoma Bank, the University of Iowa).

2.2. Library construction and illumina RNA-seq

The overall workflow described as before [12,13]. Briefly, total RNA extracted from FW PVCs, FW MRCs, SW PVCs and SW MRCs (each n = 2) by mirVanaTM miRNA isolation kit (Applied Biosystems). RNA quality with a RNA Integrity Number (RIN) > 8 (assessed using the Agilent 2100 Bioanalyzer system) were used for RNA library construction. The cDNA libraries were constructed according to manufacturer's instruction and index codes were ligated as identification to individual samples as previous described. Briefly, 8 cDNA libraries were constructed (2 × 4 groups), each prepared from 300 ng total RNA. Then purified mRNA from the total RNA using poly-T oligo-attached magnetic beads (Illumina, San Diego, USA) to remove the ribosomal RNA. Then the mRNA was fragmented by divalent cations in Illumina proprietary fragmentation buffer at 94 °C for 1 min. First strand cDNAs were synthesized using random oligonucleotides and SuperScript II, and the second cDNAs were synthesized using DNA polymerase I and RNase H. Overhangs were blunted by using exonuclease/polymerase, followed by 3′ end adenylation. After adenylation, DNA fragments Illumina were ligated with PE adapter oligonucleotides. DNA fragments that ligated with adaptor molecules on both ends were selectively enriched by Illumina PCR Primer Cocktail in a 15 cycles PCR reaction. Libraries were purified using AMPure XP system and quantified using the KAPA Library Quantification Kits. Before start sequencing, the libraries were normalized and pooled together in a two single lane on an Illumina MiSeq platform and 150 bp paired-end reads were generated. Adapters and reads containing poly-N were first trimmed and the sequence-reads were dynamically trimmed according to BWA's-q algorithm [14]. Briefly, a running sum algorithm was executed in which a cumulative area-plot is plotted from 3′-end to the 5′-end of the sequence reads and where positions with a base-calling Phred quality lower than 30 cause an increase of the area and vice versa. Such plot was built for each read individually and each read was trimmed from the 3′-end to the position where the area was greatest. Read-pairs were then synchronized such that all read-pairs with sequence on both sides longer than 35 bp after quality trimming were retained. Any singleton read resulting from read trimming was removed [14]. All the downstream analyses were based on quality trimmed reads.

2.3. De novo transcriptome assembly and annotation

Obtained reads (Forward and reverse) from all the libraries/samples were pooled and subjected to transcriptome de novo assembly using Trinity (version r20140413p1) with “min_kmer_cov” set to 2; “SS_lib_type” set to RF, and all other parameters set to default [15]. Trinity uses fixed kmer to generate an assembly and it is efficient in recovering full-length transcripts as well as spliced isoforms. The open reading frames (ORF) were identified by Transdecoder [16] using the following criteria: (1) the longest ORF was identified within each transcript; (2) from the longest ORFs extracted, a subset of the longest ones was identified and randomized to provide a sequence composition corresponding to non-coding sequences before being used to parameterize a Markov model based on hexamers; and (3) all the longest ORFs were scored according to the Markov Model to identify the highest scoring reading-frame out of the six possible reading-frames. These ORFs were then translated to protein sequences and subjected to (1) BLASTp search against UniProtKB/Swiss-Prot with a cut-off e-value [17,18] of 1.0×10^{-6}, (2) protein domain search via HMMScan, (3) transmembrane helicase prediction by TMHMM, and (4) signal peptide prediction by SignalP.

2.4. Differential expression, gene ontology (GO) and pathway enrichment analysis

Differential gene expression and TMM-normalized FPKM gene expression were calculated by RSEM pipe-line with edgeR package [19]. Samples from identical salinity condition were considered to be biological replicates. Genes with B&H corrected p-value < .05 and log2 (fold change) > 1 were considered be statistically significant differentially expressed (FW PVCs Vs SW PVCs and FW MRCs Vs SW MRCs). The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used for functional annotation clustering analysis with the classification stringency as Benjamini-Hochberg corrected P-value (P < .05) [20]. The dysregulated transcripts with human homologs of the assembled contigs were identified by the IPA software to find functional canonical pathways and functions (www.qiagen.com/ingenuity) with the significance level set at Benjamini-Hochberg corrected P-value (P < .05) [20]. The dysregulated transcripts (FW PVCs