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Gene expression profiling in the skin of zebrafish infected with *Citrobacter freundii*

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ABSTRACT

Skin is considered the largest immunologically active organ, but its molecular mechanism remains unclear in fish. Here, Affymetrix Zebrafish GeneChip was used to assess gene expression in the skin of zebrafish (*Danio rerio*) infected with the bacterium *Citrobacter freundii*. The results showed that 229 genes were differentially expressed, of which 196 genes were upregulated and 33 genes were downregulated. Gene Ontology and KEGG pathway analyses indicated 88 genes significantly associated with skin immunity involved in complement activation and acute phase response, defense and immune response, response to stress and stimulus, antigen processing and presentation, cell adhesion and migration, platelet activation and coagulation factors, regulation of autophagy and apoptosis. When compared with transcriptional profiles of previously reported carp (*Cyprinus carpio*) skin, a similar innate immunity (e.g., interferon, lectin, heat shock proteins, complements), and several different acute phase proteins (transferrin, ceruloplasmin, vitellogenin and alpha-1-microglobulin, etc.) were detected in zebrafish skin. The validity of the microarray results was verified by quantitative real-time PCR analysis of nine representative genes. This is first report that skin play important roles in innate immune responses to bacterial infection, which contribute to understanding the defense mechanisms of the skin in fish.

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1. Introduction

Skin is an essential protective barrier for fish and functions as a first line of defense against invading pathogens. Several lines of evidence have recently shown that skin is a peripheral immune organ in teleosts [1–4]. Cytokines act in the local signaling network of considerable importance for the initiation, effectuation and modulation of skin immune responses [5,6]. Unlike that of higher vertebrates, fish skin is not keratinized and is more susceptible to infection [7]. It is the most common site of colonization and infection for pathogens, especially under intensive aquaculture conditions.

Although skin is an important component of the mucosal immune system, only a few studies have targeted the transcription profile in fish skin. One of the earliest investigations analyzed the gene expression in the skin of channel catfish (*Ictalurus puntatus*) [8]. Subsequently, several studies have shown regulation of immune-relevant genes expression in rainbow trout (*Oncorhynchus mykiss*) skin following ectoparasitic infections [9–11]. Recently, a transcript analysis was carried out in common carp (*Cyprinus carpio*) challenged by the parasite *Ichthyophthirius multifiliis* [12], eighty-two immune-related genes were found out of a total of 3500 expressed sequence tags (ESTs) derived from two skin cDNA libraries, and the expressions of several genes such as complement components (C7, FP, FD), interferon (IFN) and CC chemokine were described, all of which may play regulatory roles in skin immune system of fish. More recently, microarray analysis of Atlantic salmon (*Salmo salar*) skin after ectoparasitic infection, showed changes in the expression of genes belonging to immune response, oxidative stress, protein folding and cytoskeletal proteins [13]. Very recently, the transcriptional profiles of selected twenty-six candidate genes with putative functions in the skin immune defense were evaluated in Atlantic cod (*Gadus morhua*), and showed high expression of genes involved in antibacterial activity, antiviral response, cytokine, stress response and antiapoptotic activity [5]. Nevertheless, the little information is available for this organ with regard to immune response mechanisms and associated gene expression in response to bacterial infection.

Zebrafish has become a useful model organism for studies in genetics, development, cancer and immunology. Its well-developed innate and adaptive immune systems make zebrafish an ideal model for the study of infectious diseases [14]. Recently, infection of zebrafish has been successfully demonstrated with some bacterial pathogens [15–17]. Very recently, zebrafish as a model for the

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evaluation of pathogenicity of *Citrobacter freundii* has been performed by our group, which exhibited features with the developed diffuse bleeding on the skin [18]. In this study, microarray analysis was used to identify transcription profiles of the skin of *C. freundii*-infected zebrafish, with the aim of clarifying the mechanism of skin immune responses against bacterial pathogens in fish.

2. Materials and methods

2.1. C. freundii infection in zebrafish

Adult zebrafish were infected by immersion with *C. freundii* as previously described [18]. Zebrafish were acclimatized in aerated fresh water tanks for 15 d prior to the experiment at a water temperature of 25 °C, and were fed with commercial dry feed distributed manually twice a day. Control fish were treated with 0.65% PBS. At 7 h post-infection, forty infected fish and forty control fish were randomly selected, and the skin sections were aseptically excised from the dorsal regions along the two sides of the fish. The mixed skin samples were immediately frozen in liquid nitrogen, and then stored at −80 °C for total RNA extraction.

2.2. RNA preparation, labeling and hybridization

Total RNA was extracted from the skin using Trizol reagent (Invitrogen), and was further purified using an RNeasy Mini kit (Qiagen) according to the manufactures' instructions. The RNA quality was assessed by formaldehyde agarose gel electrophoresis and was quantitated spectrophotometrically. The RNA samples were hybridized separately with the same Affymetrix GeneChip probe arrays (containing 14,900 transcripts). Two replicates were performed in order to compare the gene expression profiles between the infected and the control samples. Briefly, two μg of total RNA was used to synthesize single- and double-stranded cDNA, and then purified dscDNA was converted into the biotin-labeled cRNA using MessageAmp™ II cRNA Amplification Kits (Ambion). Ten μg amounts of labeled cRNA were fragmented and hybridized to Affymetrix Zebrafish Genome Array. Hybridization was performed at 45 °C with rotation for 16 h on an Affymetrix GeneChip Hybridization Oven 640 (Affymetrix). The GeneChip arrays were washed and then stained (streptavidin–phycoerythrin) on an Affymetrix Fluidics Station 450 followed by scanning on a GeneChip Scanner 3000 (Affymetrix).

2.3. Microarray analysis

The hybridization data were analyzed using GeneChip Operating Software (GCOS 1.4). The scanned images were first assessed by visual inspection then analyzed to generate raw data files saved as CEL files using the default setting of GCOS 1.4. A global scaling factor of 500 was used to normalize the different arrays. The detection calls (present, absent, or marginal) for the probe sets were made by GCOS. The differentially expressed genes were identified using SAM (Significant Analysis of Microarray) software, and thus were selected on the basis of their fold changes ($\geq$2-fold or $\leq$0.5-fold) as compared with the control sample.

The categorization of biological process GO was analyzed using Gene Ontology project (http://www.geneontology.org). The pathway analysis was carried out using KEGG (Kyoto Encyclopedia of Genes and Genomes) database (http://www.capitalbio.com). Fisher's test and $\chi 2$ test were used to classify the GO category and pathway analysis, and the false discovery rate (FDR) was calculated to correct the *P*-value. *P*-value < 0.05 and FDR < 0.05 were used as a threshold to select significant GO categories and KEGG pathways.

2.4. Quantitative real-time PCR

Microarray data were validated by quantitative real-time PCR (qRT-PCR), and nine pairs of primers were designed using primer 5 program (Table 1). qRT-PCR was done as described before [19]. Briefly, qRT-PCR was performed by amplifying cDNA with the SYBR Green qRT-PCR kit (Finnzymes) in a Chromo 4 Real-Time System (MJ Research). Amplification conditions were 94 °C for 4 min, followed by 40 cycles of 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 20 s. The expression of the candidate genes was normalized using β-actin as a housekeeping gene. Each gene was run in triplicate. After completion of the PCR amplification, the relative fold change after stimulation was calculated based on the $2^{-\Delta\Delta CT}$ method.

3. Results

3.1. Gene expression profiling

Only gene expressions with more than 2-fold or less than 0.5-fold change are considered significant regulation in skin immune responses. In general, a total of 229 genes were obtained according to the microarray analysis, of which 196 genes were upregulated

Table 1
The primers of selected genes for quantitative real-time PCR.

GenBank number	Gene title	Primer sequence (5′–3′)	Size (bp)
BM036389	Complement component c3c	F: CCAGTTGCCGAGTGAGTTT R: CAGTCATGCCGATGTAGCG	189
BM957464	Transferrin	F: TGTTATCAGAGGTCTGGAGGCT R: GCACAGGTTTGGGTATTTGG	168
BC048037	Ceruloplasmin	F: AGGCAGGAGATAGACAAACC R: TGTGCGTGGAGGGAGTAAG	125
BQ284855	Mannose-binding lectin 1	F: CTATTTGAAGGACTGGGAGA R: CAGGGAGCCGTTATTGACC	241
BC045371	Activating transcription factor 3	F: CTGTGGGCATCTGTGAATC R: AGGCACCCGTGTTTAGTCC	112
BG303658	Vitellogenin 2	F: GCTGGCTTCTGGATACTCT R: GTTGGAATGAACTCGGTAA	106
CD014745	α-1-Microglobulin	F: TGCTGTTTCTGCCTCTGCT R: ATGGCTGCGTCTCCTTTAT	169
AW019105	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor	F: CCATTCTCACCTTCCCTAA R: GCCACTACACTGCTCCTTT	131
AW117084	Annexin A4	F: TTCCAGAGGGTCTTGGTGTC R: TGTCCTCAATGTCTCGTCCA	226
AF057040	β-Actin	F: CGAGCAGGAGATGGGAACC R: CAACGGAAACGCTCATTGC	102

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