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Class B CpG-ODN2006 is highly associated with IgM and antimicrobial peptide gene expression through TLR9 pathway in yellowtail *Seriola lalandi*

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ABSTRACT

The purpose of this study was to characterize the TLR9 gene from yellowtail (*Seriola lalandi*) and evaluate its functional activity using the class B Cytosine-phosphate-guanine-oligodeoxynucleotide2006 (CpG-ODN2006) in an *in vivo* experiment after one-week immunostimulation. The gene expressions of TLR9, Immunoglobulin M (IgM), antimicrobial peptides and cytokines were evaluated by real time PCR, and humoral immune parameters were analyzed in serum. The TLR9 nucleotide sequence from yellowtail was obtained using the whole-genome shotgun sequencing method and bioinformatics tools. The yellowtail full-length cDNA sequence of *SlTLR9* was 3789 bp in length, including a 66-bp 5′-untranslated region (UTR), a 3′-UTR of 528 bp, and an open reading frame (ORF) of 3192 bp translatable to 1064 amino acid showing a high degree of similarity with the counterparts of other fish species and sharing common structural architecture of the TLR family, including LRR domains, one C-terminal LRR region, and a TIR domain. Gene expression studies revealed the constitutive expression of TLR9 mRNA in all analyzed tissues; the highest levels were observed in intestine, liver and spleen where they play an important role in the fish immune system. The expression levels of TLR9 after B class CpG-ODN2006 (the main TLR9-agonist) was significantly up-regulated in all analyzed tissues, with the high expression observed in spleen followed by intestine and skin. The CpG-B has been shown as a potent B cell mitogen, and interestingly, IgM mRNA transcript was up-regulated in spleen and intestine, which was highly correlated with TLR9 after CpG-ODN2006 stimulation. The antimicrobial peptides, piscidin and NK-lysine, were up-regulated in spleen and gill after CpG-ODN2006 injection with a high correlation ($r \geq 0.82$) with TLR9 gene expression. Cytokine genes were up-regulated in spleen, intestine and skin after CpG-ODN was compared with the control group. No significant correlation was observed between TLR9 and IL-1β, TNF-α and Mx gene expressions. The results showed that CpG-ODN2006 intraperitoneal injection enhanced lysozyme, peroxidase and superoxide dismutase activities in serum and demonstrated that CpG-ODN2006 can induce a specific immune response via TLR9 in which IgM and antimicrobial peptides must have an important role in the defense mechanisms against infections in yellowtail.

1. Introduction

The innate immune system is evolutionally conserved protecting the host from invading microbial pathogens. Bacterial DNA is known to activate the innate immune system. Synthetic oligodeoxynucleotides (ODNs) containing dinucleotides with unmethylated cytosine-phosphate-guanine (known as CpG motifs) are a type of pathogen-associated molecular patterns (PAMPs) commonly present in the genomes of microbial pathogens [1]. The recognition of such CpG motifs results in the activation of various cells of the innate immune system. The cells responsible for detecting unmethylated CpG motifs include dendritic cells (DC), monocytes, macrophages and neutrophils [2]. Differences in CpG-DNA sequence, secondary structures and their stimulatory impact on human peripheral blood mononuclear cells led to the characterization of different CpG-DNAs: CpG-A, CpG-B and CpG-C [3]. Particularly, Class-B ODNs, completely designed with phosphorothioate backbone, have much stronger stimulatory effect on B lymphocytes and Immunoglobulin production [4,5]. Based on B-cell activation and proliferation, the human CpG motif was identified, and an oligonucleotide was developed (CpG ODN 2006) that induced the mitogen-activated

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protein (MAP) kinase pathway and nuclear factor kappa beta (NF-kβ) translocation in purified B cells that turned out to be a potent adjuvant to support humoral immune responses in primates [6]. Furthermore, expression of antimicrobial peptides like human β-defensin-2 gene can be induced by CpG-DNA in human B cells [7].

Toll-like receptors (TLRs) are well-known innate immune-recognition receptors for microbial pathogens [8,9]. Among them, TLR9 recognizes the unmethylated CpG motifs present at high frequency in bacteria and viruses [10]. The TLR9 receptor is localized in the endoplasmatic reticulum, late endosomal and lysosomal compartments of the intracellular milieu. Thus, internalization of pathogen-derived DNA is required for TLR9 triggering, an outcome that results from either intracellular infection or uptake of bacterial/viral particles by immune cells [11]. Stimulation via TLR9 results in the rapid activation of the innate immune system that in turn supports the induction of an adaptive immune response. This series of effects provides a mechanism by which CpG ODN are being explored in various possible applications as immunomodulators or vaccine adjuvants [12]. Overall, Antigen Presenting Cells (APCs) triggered by CpG-ODNs up-regulate the expression of co-receptor molecules and secrete a variety of cytokines, including IL-12, IL-6, IL-1 and TNF-α [13–15].

In teleost fish, moreover, CpG ODN treatments stimulate leucocyte immune activities [16–18]. Furthermore, increased disease resistance has also been demonstrated as a consequence of their adjuvant properties [19]. Recently, our research group evaluated the immunostimulatory properties of B-class CpG ODN 1668 in an *in vivo* experiment in the Pacific red snapper *Lutjanus peru* after infection with *Vibrio parahaemolyticus* [18]. Interestingly, these results indicated that CpG ODN 1668 activates innate immune response and upregulates the TLR9 and IgM-mediated immune response. Although a large number of studies on immunostimulatory effects of CpG ODNs had been conducted in mice and TLR9 is well characterized, information regarding the fish immune system is limited and that regarding humoral innate response is scarce in yellowtail. Moreover, despite several *Seriola* species are being farmed, little has been done to understand their immune system and few immune related genes have been characterized, specially in *S. lalandi* [20–22]. This paper describes the isolation of full-length cDNA, phylogenetic relationship and tissue-specific mRNA expression distributions of the yellowtail TLR9. In addition, the response to B class CpG-ODN2006 was evaluated in an *in vivo* experiment and gene expression of TLR9, IgM, antimicrobial peptides and cytokines were analyzed in gill, skin, intestine, liver and spleen after one week of stimulation compared with control group. Finally, we analyzed the humoral immune parameters changes in serum in response to stimulation by CpG-ODN2006.

2. Materials and methods

2.1. Experimental fish

Healthy yellowtail *Seriola lalandi* specimens (150 ± 50 g mean body weight) were randomly placed in six running seawater tanks (three fish per tank) (flow rate 1500 l h^{-1}). Quarantine was induced for a month before the start of the study without showing any disease. Fish received commercial feed (Skretting, Vancouver, Canada; crude protein: 55%, crude fat: 15%, crude fiber: 1.5%, phosphorus: 1%), two times daily (2% live body weight/day) during quarantine (one month) and experimental (one week) periods. Water quality was monitored weekly and water temperature was maintained at 26 °C (with a 12 h dark/12 h light photoperiod), dissolved oxygen at 4.3–6.9 mg l^{-1} and pH at 7.7–8.1. Total ammonia and nitrite concentration remained below 0.02 mg l^{-1}.

2.2. CpG-ODN2006

Synthetic unmethylated CpG ODN2006 (T*C*G*T*C*G*T*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T) was purchased from Eurogentec (USA). CpG ODN2006 was resuspended in sterile phosphate buffer at 1 mM.

2.3. CpG-ODN2006 in vivo stimulation

Two groups were used for *in vivo* stimulation: (1) CpG-ODN2006 and (2) Control or PBS. One group was injected intraperitoneally with 0.1 ml of CpG-ODN2006 (10 μg ml^{-1}) and the other group was injected with 0.1 ml phosphate buffered saline (PBS) per fish as control group. Three fish from each tank (n_{total} = 9 fish/treatment) were randomly sampled at week one after injection. Fish were anesthetized in diluted clove oil solution (50 mg l^{-1}). Blood samples were taken from the caudal vein with a 27-gauge needle and 1-ml syringe and allowed to clot at 4 °C for four h. Serum was obtained by centrifugation (2000 g, 4 °C, 10 min) and then stored at −20 °C until used for antioxidant and humoral immune parameter determination. Gill, skin, intestine, liver and spleen fragments were also sampled and immediately stored at −80 °C in TRIzol Reagent (Invitrogen) for RNA extraction.

2.4. SlTLR9 full-length cDNA

The nucleotide sequence of *Sl*TLR9 was identified from the transcriptomic data obtained by next-generation sequencing (NGS); mRNA was isolated from the leukocytes of *S. lalandi*; cDNA synthesis and library preparation was performed using Truseq stranded mRNA sample preparation kit (Illumina); NGS analysis was performed using MiSeq reagent kit v.2 (MiSeq) with MiSeq (Illumina), and the reads were assembled into contigs with the Trinity program [23]. The sequences homologous to TLR9 were identified from the contigs using tBLASTn program [24].

2.5. DNA sequence and structure analysis

Homology analysis was performed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). Amino acid sequences were analyzed with the Expert Program Analysis System (http://www.expasy.org). Protein domains were predicted by the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) and phosphorylation sites were predicted using PROSITE (http://prosite.expasy.org/). Multiple sequence alignment was carried out using CLUSTAL Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and a phylogenetic tree was constructed using neighbor-joining method in MEGA 6 program (http://www.megasoftware.net).

2.6. Analysis of gene expression

The expression of TLR9 gene mRNA was examined in gill, skin, intestine, liver and spleen by Real-Time PCR (qPCR) analysis and the 2-< DELTA > < DELTA > CT (2^{-DDCT}) method according to Livak and Schmittgen [25]. Similarly, the expression of TLR9, Immunoglobulin M (IgM), piscidin, NK-lysin, Interleukin-1 β (IL-1β), Tumor nuclear factor (TNF-α), and interferon-inducible Mx protein (Mx) genes were analyzed after CpG-ODN2006 stimulation (Table 1). RNA extraction from tissues was carried out using TRIzol reagent (Invitrogen, Cat. 15596026). Genomic DNA contamination from RNA extractions was removed by DNase I (Invitrogen, Cat. 18068015) treatment. Then, one mg of total RNA was used to synthesize complementary DNA (cDNA) using the oligo-dT18 primer and SuperScript III reverse transcriptase (Invitrogen,