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The characterization and initial immune functional analysis of SCARA5 in turbot (*Scophthalmus maximus* L.)

Chao Li[a,1], Mengyu Tian[a,1], Lu Zhang[a], Qiang Fu[a], Lin Song[b], Fei Chen[c], Ning Yang[a,*]

[a] Marine Science and Engineering College, Qingdao Agricultural University, Qingdao, 266109, People's Republic of China
[b] College of Marine Science and Biological Engineering, Qingdao University of Science & Technology, Qingdao, 266011, People's Republic of China
[c] Weifang Animal Health Supervision Institute, Weifang, 261031, People's Republic of China

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ABSTRACT

Scavenger receptors (SRs) are a group of membrane-bound receptors that could bind to a variety of ligands including endogenous proteins and pathogens. SRs have been recognized to play vital roles in innate immune response against pathogen infection in both vertebrates and invertebrates. In this regard, one *Sm*SCARA5 gene was captured in turbot (*Scophthalmus maximus*). The full-length *Sm*SCARA5 transcript contains an open reading frame (ORF) of 1494 bp. *Sm*SCARA55 showed both the highest identity and similarity to half-smooth tongue sole (*Cynoglossus semilaevis*), and a high degree of conservation of genomic structure to the teleost species. In addition, the phylogenetic tree analysis showed *Sm*SCARA5 had the closest relationship to half-smooth tongue sole, the syntenic analysis revealed a relatively conserved synteny pattern of *Sm*SCARA5 to other species. Moreover, *Sm*SCARA5 was ubiquitously expressed in all the examined tissues, with the highest expression level in brain and the lowest expression level in blood. And it was significantly down-regulated in intestine following Gram-negative bacteria *Vibrio anguillarum*, and Gram-positive bacteria *Streptococcus iniae* challenge. Finally, the recombinant *Sm*SCARA5 showed the highest affinity to lipopolysaccharide (LPS), followed by peptidoglycan (PGN) and lipoteichoic acid (LTA), as well as the strong inhibition effect on the growth of *V. anguillarum*. Taken together, our results suggested *Sm*SCARA5 plays vital roles in innate immune response in teleost, further studies should be carried out to better understand its regulatory mechanism for innate inflammation response in teleost.

1. Introduction

Scavenger receptors (SRs) are a group of membrane-bound receptors that could bind to a variety of ligands including endogenous proteins and pathogens. The first description of SRs was in macrophages in the 1970s by Brown and Goldstein in the study of low-density lipoprotein (LDL) [1]. According to their structure features, SRs are classified into 10 eukaryote families, defined as Classes A-J [2], and participate in a wide range of biological functions including endocytosis, phagocytosis and adhesion. SRs are predominantly expressed in myeloid cells, including mammalian monocytes and macrophages, as well as invertebrate hemocytes. As a group of ligand-binding proteins, SRs could recognize a wide range of microbial ligands, which suggested their vital roles in host immune responses to microbial pathogens [3].

SRs have been recognized to play vital roles in innate immune response against pathogen infection in both vertebrates and invertebrates. The first identification of SRs in teleost was reported in pufferfish (*Tetraodon nigroviridis*), which was acting as pattern recognition receptors, involved in negative regulation of NF-κB activation [4]. Currently, SRs are available in more and more aquatic species, including rainbow trout (*Oncorhynchus mykiss*) [5], large yellow croaker (*Larimichthys crocea*) [6], common carp (*Cyprinus carpio*) [7], zebrafish (*Danio rerio*) [8], red drum (*Sciaenops ocellatus*) [9], Zhikong scallop (*Chlamys farreri*) [10], swimming crab (*Portunus trituberculatus*) [11]. As pattern recognition receptors, SRs have been recognized to play vital roles in innate immunity in aquatic species. For example, in mud crab (*Scylla paramamosian*), SR could promote bacteria clearance by enhancing phagocytosis and attenuates virus proliferation [12]. In pufferfish and zebrafish, SRs could inhibit the HMGB1mediated inflammation [13]. However, the knowledge of the immune roles of SRs are still limited in a small number of species in aquaculture.

Turbot (*Scophthalmus maximus* L.), one of the most extensively maricultured species in China, suffers from the bacterial disease including *Vibrio anguillarum* and *Streptococcus iniae*, which have resulted

* Corresponding author.
E-mail address: yangning04@163.com (N. Yang).
[1] All these authors contributed equally to this work.

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in significant economic losses to turbot industry. Many efforts have been made to understand the activities of immune genes during the infection [14–18], in order to facilitate the development of disease control and prevention measures. In addition, as living in pathogen-rich aquatic environment, the mucosal immune system plays crucial roles in the establishment and maintenance of mucosal homeostasis between the host and the outside environment [19]. There are urgent need to better understand mucosal immune system in aquaculture research to improve immunity of fish via immersion or feeding [20]. In addition, SRs are divided into 8 classes, termed A to H [21]. As a member of class A scavenger receptors, SCARA5 was reported to be presented in populations of epithelial cells [22]. In addition, SCARA5 is capable of recognizing pathogen associated molecular patterns (PAMPs), and triggering the innate immune responses for pathogen clearance in their entry points [4]. In this regard for the first time, we identified and characterized SCARA5 gene in turbot, and investigated its expression patterns in mucosal barriers following different bacterial infection, as well as microbial ligand-binding and bacteriostatic activities.

2. Materials and methods

2.1. Sequence identification and analysis

In order to capture SCARA5 gene sequences in turbot, the SCARA5 protein sequences from other species were collected as query sequences to BLAST against our transcriptome database [23]. The retrieved candidate sequences were then translated using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted ORF sequences were further verified against NCBI non-redundant protein sequence database by BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The conserved domains and signal peptides were further identified by the simple modular architecture research tool (SMART; http://smart.embl-heidelberg.de/). The theoretical pI, molecular mass and N-glycosylation sites were captured in ExPASy server [24]. The intron and exon structures were predicted in Splign program [25].

2.2. Sequence alignment and phylogenetic analysis

A neighbor-joining phylogenetic tree was generated using the Molecular Evolutionary Genetics Analysis (MEGA 6) software package [26]. Clustal Omega program was utilized to perform the multiple protein sequence alignment [27]. The phylogenetic tree was constructed based on the amino acids sequences of SCARA5 from various species retrieved from GenBank, including human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), green sea turtle (*Chelonia mydas*), western clawed frog (Xenopus tropicalis), channel catfish (*Ictalurus punctatus*), Atlantic salmon (*Samlo salar*), large yellow croaker (*L. crocea*), half smooth togue sole (*Cynoglossus semilaevis*), fugu (*T. rubripes*), spotted gar (*Lepisosteus oculatus*) and turbot (*S. maximus*). Based on full-length amino acid sequence alignments, phylogenetic analyses were performed using the neighbor-joining method with 1000 bootstrapping replications, Poisson correction model, and complete or partial deletion to remove gaps.

2.3. Syntenic analysis

In order to further verify the characterization of turbot SCARA5 gene, syntenic analysis was performed with human, mouse, chicken, catfish and zebrafish. Briefly, using FGENESH program, the protein sequences of neighbor genes of the turbot SCARA5 was predicted from the turbot scaffold. The identified protein sequences were annotated against NCBI non-redundant (nr) database by BLASTP. Ensembl database and Genomicus were used to determine the conserved syntenic pattern of SCARA5 gene in other species [28].

2.4. Bacteria challenge and sample collection

In order to characterize the roles of SCARA5 gene in the host immune defense against bacterial infection, the Gram-negative bacteria *V. anguillarum* and Gram-positive bacteria *S. iniae* were selected to conduct the bath challenge. Turbot fingerlings (average body weight: 15.6 g and average body length = 5.5 cm) were obtained from the turbot hatchery (Haiyang, Shandong, China), and acclimated in the laboratory in a flow-through system for at least two weeks prior to challenge. After a pre-challenge, the bacteria was re-isolated from single symptomatic fish and biochemically confirmed before cultured. During challenge, symptomatic fish were confirmed to be infected with *V. anguillarum* and *S. iniae*, respectively. During the experiments, the fish were immersed for 2 h and then were transferred in fresh water. The fish were euthanized with tricaine methanesulfonate (MS-222) at 200 mg/L (buffered with sodium bicarbonate) for sample collection. At each time point following challenge, skin, gill and intestine samples were collected from 15 fish (5 fish per pool) from the appropriate aquaria.

Briefly, the *V. anguillarum* was inoculated in LB broth and incubated in a shaker (180 rpm) at 28 °C overnight. During challenge, the fish were immersed at a final concentration of 5×10^7 CFU/ml for 2 h, while the control fish were immersed in sterilized media alone. Aquaria were randomly assigned for 2 h, 6 h, 12 h and 24 h post-treatment and 0 h control with thirty fish in each aquarium for sample collection.

The *S. iniae* isolate was inoculated in LB medium in a shaker incubator at 28 °C overnight. The fish were equally divided into five aquariums, four treated groups (2 h, 4 h, 8 h and 12 h) and one control group. For the challenge, the fish were immersed for 2 h at a final concentration of 5×10^6 CFU/mL, while control fish were immersed in sterilized media alone. All samples from both experiments were flash-frozen in liquid nitrogen and then stored in a −80 °C ultra-low freezer until preparation of RNA.

2.5. Total RNA extraction and cDNA synthesis

Prior to RNA extraction, tissue samples were homogenized under liquid nitrogen using mortar and pestle under liquid nitrogen. Total RNA was extracted using Trizol® Reagent (Invitrogen, USA) according to the supplied protocol. The quality and quantity of RNA of each sample were measured on a Nanodrop 2000 (Thermo Electron North America LLC, USA). All extracted samples had an A260/280 ratio greater than 1.8, and were diluted to 250 ng/μl.

2.6. Real-time PCR analysis

Gene specific primers were designed using online software Primer3 based on the turbot SCARA5 sequence. And 18S rRNA gene was used as a reference gene. First strand cDNA was synthesized by PrimeScript RT reagent Kit (TaKaRa, China) according to manufacturer's protocol (500 ng RNA per 10 μl reaction). Quantitative real-time PCR (qPCR) was performed on a LightCycler 96 system (Roche Applied Science, USA) using the SYBR ExScript qRT-PCR Kit (Takara, China) following the manufacturer's instructions. The reaction systems for all real-time PCR were as follows: 1.0 μL of each primer (5 μM), 5.0 μL SYBR Green supermix, 2.0 μL RNase/DNase-free water, and 1.0 μl 200 ng/μL cDNA. The thermal cycling profile was performed as follows. The PCR reaction mixture was denatured at 95 °C for 30 s and then subjected to 40 cycles of 95 °C for 5 s, 58 °C for 5 s and followed by dissociation curve analysis, 5 s at 65 °C, then up to 95 °C at a rate of 0.1 °C/s increment, to verify the specificity of the amplicons. Results were analyzed using Relative Expression Software Tool (REST) to capture the significance at the level of $P < 0.05$ [29]. In order to determine the gene expression patterns in turbot healthy tissues, the tissue with the lowest Ct values were used as control. The mRNA expression levels of all samples were normalized to the levels of 18S ribosomal RNA gene in the same samples. A no-template control was run on all plates.