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Characterization and expression analysis of BCA2 gene in large yellow croaker, *Larimichthys crocea*

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ABSTRACT

BCA2, as an E3 ubiquitin ligase, is an important anti-virus immune factor in mammals. Up to date, there are not any related reports on BCA2 protein in fishes yet. In the present investigation, BCA2 in large yellow croaker *Larimichthys crocea* (named as LcBCA2) was identified and characterized. The full-length cDNA of LcBCA2 was 1571 bp, including an ORF of 888 bp encoding a polypeptide of 295 amino acids. The putative LcBCA2 protein contained a RING-H2 motif at C terminal. The LcBCA2 transcripts were broadly distributed in all detected tissues, with high expression in muscle, moderate in blood, skin, heart, liver and spleen, weak in other tissue as indicated by qPCR analysis. Significant increases were observed in skin, gill and spleen after infection of *Cryptocaryon irritans*, and in spleen and head-kidney after inactivated *Vibrio. parahaemolyticus*, LPS and Poly I:C stimulations. Tissue localization by *in-situ* hybridization showed that LcBCA2 mainly expressed in the spleen of the fish in the test group. Our findings showed that LcBCA2 inclined to sharply increase in immune organs, especially in head-kidney after bacterial and viral stimulations, while in locations (skin and gill) of parasites infections, suggesting that BCA2 may play an important role in fish defense against bacteria, virus and parasites infections, but the immune mechanisms is are different.

1. Introduction

Innate immunity is the first line of host defense against pathogen invasion and has been regarded as the base to activate adaptive immunity [1,2]. Fish, as lower vertebrate, the adaptive immunity is influenced by many factors, such as water temperature, photoperiod, pH and dissolved oxygen, etc. [3]. Therefore, the innate immunity is of paramount importance for fish to defense against pathogen infection. There are many innate immunity factors, among which, E3 ubiquitin ligase was paid more and more attention. There are growing evidences showed that ubiquitination mediated by E3 ubiquitin ligase can not only regulate the development of immune system, but also initiate effective immune responses [4–6]. Furthermore, prokaryote may utilize the host ubiquitin system to invade, although prokaryote itself lacks classical ubiquitin system [7].

Breast cancer associated gene 2 (BCA2), also referring to as ring finger protein 115 (RNF115) or Rabing7, belongs to E3 ubiquitin ligase family with ring finger domain. In mammals, BCA2 has been reported to be involved in proliferation of breast cancer cells, and obviously inhibited Epidermal Growth Factor (EGF) degradation [8–10]. More recently, Miyakawa and his collaborators showed that BCA2 is a potential antiviral factor, which interacts with the cytoplasmic tail of tetherin to facilitate the internalization and lysosomal degradation of HIV-1 particles, and only promotes tetherin-dependent restriction of HIV-1 release from infected cells [11]. Subsequently, Nityanandam and Serra-Moreno revealed that BCA2 also possesses tetherin-independent antiviral activity. The N-terminus of BCA2 physically interacts with the Matrix region of HIV-1 and other retroviral Gag proteins and promotes their ubiquitination, redistribution to endo-lysosomal compartments and ultimately lysosomal degradation [12]. However, there are not any reports on BCA2 in fishes at the present and whether BCA2 in fishes involves in immunity function or not remains unknown.

Large yellow croaker *Larimichthys crocea* is a commercially important fish species, mainly distributed in the eastern and southern area of China [13,14]. Unfortunately, with the rapid

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development of the *L. crocea* culture industry, farmed croakers are extremely vulnerable to various marine pathogens, including bacteria (*vibrio* etc.), viruses (*iridovirus* etc.) and parasites (*Cryptocaryon irritans* etc.), resulting in enormous losses [15–18]. Therefore, it is urgent to investigate the molecular mechanism of immune responses of *L. crocea* to pathogens, and to develop effective methods to control diseases.

In this investigation, what and how BCA2 in the *L. crocea* responds to various pathogenic stimuli were investigated, including *C. irritans*, inactivated vibrio, and two pathogen-associated molecular patterns (PAMPs): LPS and Poly I:C. The differences of BCA2 responses to the parasite and the other immune stimulations were compared. Finally, the precise position and change of the gene expressions in spleen — the main immune tissue was further detected via *in-situ* hybridization (ISH) to further understand whether BCA2 is related to immune responses in fish or not.

2. Materials and methods

2.1. Sample collection and immune challenge

Healthy *L. crocea* (average mass: 130 g) were obtained from a mariculture farm in Ningde, Fujian, China. The fish were acclimatized 10 days at salinity 25–26 and temperature 23–26 °C, and fed with a commercial feed. Tissue samples of brain, heart, gill, liver, spleen, kidney, head-kidney, stomach, intestine, skin, muscle and blood cells were collected from five fish, and preserved in liquid nitrogen for RNA extraction.

The *L. crocea* were infected with *C. irritans* according to previously described procedures [19]. *C. irritans* was originally isolated from *L. crocea*. The fish were divided into two groups, a control group and a test group. The test group was infected with 25,000 *C. irritans* theronts per fish for 6 h. Subsequently the fish were transferred into a new cement pool containing fresh seawater. To avoid the auto-reinfection, the fish were transferred into a new pool at the day 3 post primary infection. At day 7 post primary infection, the fish were transferred into a new pond again and infected with the same dose and method as primary infection. At 6 h, 12 h, 1 d, 2 d, 3 d, 5 d, 7 d, 10 d and 14 d post primary infection, the skin, gill, spleen and head-kidney tissues were collected. The control group was uninfected with *C. irritans*, and the procedure of treatment was the same as that of the test group and the counterpart tissues were collected. Five individuals were sampled from each group at each time point.

Besides *C. irritans* infection, *L. crocea* were challenged with 250 μl formalin-inactivated Gram-negative bacteria suspensions of *Vibrio. parahaemolyticus* (10^8 cfu ml^{-1}), 250 μl Poly I:C (27,472,901, GE, 1 mg ml^{-1}) and 250 μl LPS (L2,880, Sigma, 1 mg ml^{-1}) by intraperitoneal injection, respectively [20]. Fish injected with 250 μl PBS was used as control. Liver, spleen and head-kidney of each group were collected at 3, 6, 12, 24, 48 and 72 h after injection. Five fish were sampled from each group at each time point.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted with Trizol reagent (Invitrogen, USA) following the instructions of manufacturer. The RNA was then incubated with RNase-free DNaseI (Promega, USA) at 37 °C for 30 min to remove any contaminating DNA. First strand cDNA was synthesized from an aliquot (1 μg) of the total RNA with PrimeScript Reverse Transcriptase (TaKaRa, Japan).

2.3. Identification of BCA2 cDNA sequence

The full length cDNA sequence of BCA2 was obtained from previous transcriptomic sequences of various tissues from large yellow croaker in our laboratory (unpublished data). Two specific primers BCA2F/R (Table 1) for RT-RCR were designed based on the sequence of BCA2. PCR was performed at annealing temperatures 58 °C. The RT-RCR product was cloned into pMD18-T vector (TaKaRa, Japan) and sequenced (Invitrogen, USA).

2.4. Amino acid sequence analysis, multiple sequence alignment and phylogenetic tree analysis

Sequence homology analysis was performed using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The deduced amino acid sequences were analyzed with EXPASY (http://www.expasy.org/) and the protein domain features were predicted by SMART (http://smart.embl-heidelberg.de/). Multiple alignments of amino acid sequences were performed using ClustalX 1.83 (http://www.ebi.ac.uk/clustalw/). A phylogenic tree was reconstructed using the MEGA 5.0 program (http://www.megasoftware.net/mega5/mega.html).

2.5. Real-time PCR analysis of LcBCA2 mRNA expression

Tissue-specific expression profile of LcBCA2 in various tissues including brain, heart, gill, liver, spleen, kidney, head-kidney, stomach, intestine, skin, muscle and blood cell, as well as temporal expression in the skin, gill, spleen, and head-kidney challenged with *C. irritans*, and temporal expression in the spleen, head-kidney and liver stimulated with *V. parahaemolyticus*, Poly I:C, LPS and PBS were investigated by Quantitative Real-time PCR (qPCR) on ABI 7500 Real-time Detection System (Applied BioSystems, USA). Gene-specific primers BCA2QF/R (Table 1) were used to amplify a 109-bp fragment by reverse transcript PCR. Then the PCR product was sequenced to verify the specificity of RT-PCR. The housekeeping gene β-actin was used as an internal control for cDNA normalization. PCR reactions without template were used as blank control. Each reaction was performed in triplicate. Real-time PCR was performed by 95 °C for 1 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. After the PCR program, data was analyzed with ABI 7500 SDS software. To maintain consistency, the baseline was set automatically by the software. The relative mRNA expression of BCA2 compared to the reference gene was calculated with the $2^{-\Delta\Delta CT}$ method [21]. SPSS software (version 16.0) was used for the significance test between the test group and the control group. Data was expressed as mean ± SE. $P < 0.05$ was considered statistically significantly different.

2.6. In-situ hybridization

In-situ hybridization was performed as described by De Boer et al. with some modifications [22]. The specific BCA2HF/R (Table 1) primers were designed to amplify DIG-labeled sense and antisense

Table 1
Primers used for BCA2 cDNA identification, expression and hybridization in situ analysis.

Primer	Sequence (5′–3′)	Purpose
BCA2F	ACTTGATTTTTGGAGCACTGAC	cDNA identification
BCA2R	CAGTGATGCGTGACTCAAATGC	
BCA2QF	AAGCAACTCTACCTCCTTCACTCAC	qRT-PCR
BCA2QR	CAGTGATGCGTGACTCAAATGC	
β-actin-F	TTATGAAGGCTATGCCCTGCC	qRT-PCR
β-actin-R	TGAAGGAGTAGCCACGCTCTGT	
BCA2HF	TCCTCGTTCTCAGAGTTATGGC	ISH
BCA2HR	ATGACCTCTCTTGCTCCGACTG	