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Characterization of the LECT2 gene and its associations with resistance to the big belly disease in Asian seabass

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ABSTRACT

Leukocyte cell-derived chemotaxin-2 (LECT2) is an important protein of the innate immune system for the defense against bacterial infection. We cloned and characterized the LECT2 gene from Asian seabass (*Lates calcarifer*). Its complete cDNA consisted of an open reading frame of 459 bp encoding a protein of 152 amino acids. The genomic DNA sequence of this gene consists of four exons and three introns. Quantitative real-time PCR revealed that the LECT2 gene was expressed predominantly in liver while its expression was moderate in spleen and heart, and weak in other tissues. The LECT2 transcript was up-regulated in the kidney, spleen and liver in response to a challenge with a pathogenic bacterium *Vibrio harveyi*. In addition, we identified three single nucleotide polymorphisms (SNPs) in the LECT2 gene, and found significant associations between these polymorphisms and resistance to the big belly disease. These results suggest that the LECT2 gene play an important role in resistance to bacterial pathogens in fish. The SNP markers in the gene associated with the resistance to bacterial pathogens may facilitate selecting Asian seabass resistant to bacterial diseases.

1. Introduction

Leukocyte cell-derived chemotaxin-2 (LECT2) is a multifunctional protein involved in cell growth, differentiation and autoimmunity [1]. LECT2 is a 16 kDa secreted protein with three intramolecular disulphide bonds. It is expressed mainly in the liver and secreted into the bloodstream [2]. LECT2 genes have been identified in a few fish species, such as common carp [3], rainbow trout [4], croceine croaker [5] and zebrafish [6]. However, it is not known whether polymorphisms in this gene are associated with resistance to diseases.

Single nucleotide polymorphisms (SNPs) in immune response genes may be associated with susceptibility to diseases [7,8]. SNPs in immune response genes may also influence susceptibility to infection or result in variation of individual response to pathogenic agents [9]. SNPs in immune response genes have been identified as markers for susceptibility to infectious disease in humans [10] and livestock [11]. SNPs in immune response genes have been identified and characterized in a few fish species, such as grass carp [12], common carp [13], Asian seabass [14], freshwater pearl mussel [15] and sea bream [16]. However, such studies on polymorphisms of LECT genes in fish species have not been conducted yet.

The Asian seabass (*Lates calcarifer*) is an important marine foodfish species and have been cultured for food in Southeast Asia since 1980s [17]. We have worked on traditional breeding and marker-assisted breeding of Asian seabass for growth and disease resistance since 1998 [18,19]. To accelerate genetic improvement, we have developed microsatellites [18,20], SNPs in genes [21–23], BAC and cDNA libraries [20,24], linkage and physical maps [19,25,26], a genetic tracing system [27], and transcriptomes [28,29]. We have analyzed the lysozyme genes and their antimicrobial function in Asian seabass [30]. Diseases are the major bottleneck for sustainable and profitable aquaculture. It is difficult to select fishes for disease resistance using conventional breeding approaches. In aquaculture of Asian seabass, several major diseases caused by viruses [31], bacterial pathogens [32] and parasites [33] led to huge economic loss. The big belly disease, is caused by a facultative intracellular Gram-negative bacterium [34]. It was also called skinny potbelly disease. However, it is not known which bacterium species caused this disease. It is present in hatcheries in Indonesia, Singapore and Malaysia. This disease causes serious clumping of internal organs,

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abdominal distension and muscular atrophy in juveniles (<5 g). The mortality rate of this disease exceeds 80% [34]. However, no effective approach in conventional breeding is available for selecting Asian seabass resistant to this disease.

The purpose of this study is to understand the functions of the LECT2 genes in innate immunity and to facilitate selective breeding for genetic improvement for disease resistance. We isolated cDNA and genomic DNA of the LECT2 gene in Asian seabass, and analyzed its expression profiles in normal individuals as well as in individuals challenged with a pathogen *Vibrio harveyi* (*V. harveyi*). In addition, we identified SNPs in the LECT2 gene, and found their significant associations with resistance against the big belly disease. This study could shed new insights on the defense function of the LECT2 gene in Asian seabass and supply DNA markers for selection of fishes resistant to the big belly disease.

2. Materials and methods

2.1. Fishes and ethics statement

Asian seabass individuals were cultured in the Marine Aquaculture Center (MAC), Singapore with a standard operation protocol (SOP) set by MAC. Forty five healthy individuals at the age of 90 days post-hatch (dph) with an average body weight of 40.1 ± 2.7 g were transported to a large tank containing 500 L seawater. The tank was located in the animal house of our institute for three weeks before the commencement of the experiment. These fishes were kept in the large tank, and were fed twice daily with pelleted feed (Biomar, Nersac, France). All handling of fishes was conducted in accordance with the guidelines on the care and use of animals for scientific purposes set up by the Institutional Animal Care and Use Committee (IACUC) of the Temasek Life Sciences Laboratory, Singapore. The IACUC has specially approved this study within the project "Breeding of Asian seabass" (approval number is TLL (F)-12-004). These fishes were used in studying expressions of the LECT2 gene in different tissues.

2.2. Isolation of LECT2 cDNA and genomic DNA sequences

The full-length cDNA for Asian seabass LECT2 was obtained from cDNA libraries constructed in a previous study [26]. The open reading frame (ORF) of the LECT2 gene was identified using the program Editseq in the DNASTAR package (DNASTAR, CA, USA). The amino acid sequence and isoelectric point (PI) of its protein were also analyzed using Editseq. The cDNA sequence of the gene was submitted to NCBI (GenBank accession no, EU136177). To obtain the genomic sequence of the LECT2 gene, primers were designed to amplify genomic DNA. The forward primers were located in the 5′ UTR, and the reverse primers were in the 3′ UTR (see primer sequences in Table 1). PCR was performed in a volume of 25 μl consisting of 10 ng of genomic DNA, 1 × PCR buffer with 1.5 mM $MgCl_2$, 0.2 μM of each primer, 50 μM of each dNTP and 1 unit of Taq DNA Polymerase (Fermentas, PA, USA). The PCR program consisted of the following steps: 94 °C for 2 min followed by 37 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s, then a final step of 72 °C for 5 min. The PCR products were cleaned using NucleoSpin Extract II Columns (Macherey-Nagel, Düren, Germany) and cloned into the pGEM-T vector. The cloned fragment was sequenced using M13F and M13R primers and Bigdye chemicals on an ABI 3730xl DNA sequencer (Applied Biosystems, CA, USA). The full-length genomic DNA sequence of the LECT2 gene was obtained by assembling sequences using software Sequencher 4.9 (Genecodes, MI, USA) and submitted to NCBI (GenBank accession no, KF717364). Exons and introns were identified by aligning the ORF with the genomic DNA sequence using Sequencher 4.9 (Genecodes, MI, USA).

Table 1
Primers used for amplifying cDNA and genomic DNA, analyzing expression, and genotyping SNPs for the LECT2 gene in Asian seabass.

Primer name	Primer sequence (5′–3′)	Annealing temperature (°C)	Application
LECT2-A2	GTGAGGCCGGACCGAACTTCTG	68	3′ RACE
LECT2-B2	CCGTAGCGACCTTCTCCCCAGGTG	71	5′ RACE
gLECT2-F1	TGAGACGTGTCATCGTTCTG	55	Genomic
gLECT2-R1	GTCCCCAGTTAGTTTCCAGGTCAT		DNA
rLECT2-F2	GTGAGGCCGGACCGAACTTCTG	60	Real-time
rLECT2-R2	GATCAGATCCCGAGCAGGTCAATC		PCR
rEF1α-F	AGGGGCAGCAGCTTTGGTGAAGAC	60	Real-time
rEF1α-R	GGAGTCGACATGGCCAATGACCAC		PCR
Lect2 snp F	TTGGGTGAGAAACTGAATCC	55	PCR for
Lect2 snp R	GACCGACCCTCTCCTCTC		SNP

2.3. Phylogenetic analysis of LECT2 genes

To evaluate the evolutionary relationships among LECT2 genes in Asian seabass and other species, we retrieved amino acid sequences of the LECT2 gene of the other species from GenBank. Phylogenetic analysis was carried out using the MEGA package [35] with the neighbor-joining method and bootstrap tests (10,000 times). Genetic distances among amino acid sequences were evaluated with the Poisson correction by MEGA.

2.4. Tissue distribution and expression profile of the LECT2 gene

Total RNA was isolated from five fishes at the age of three months using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Nine tissues were collected, including heart, liver, spleen, eye, gill, kidney, muscle, intestine and brain. The expression of the LECT2 gene of the Asian seabass in the different tissues was detected by Quantitative real-time PCR (qRT-PCR) with the elongation factor 1-alpha (EF1α) gene as an internal control [14]. The LECT2 cDNA were amplified with the primers in Table 1. PCR amplification was conducted in a total volume of 20 μl containing 1 × Maxima™ SYBR Green qPCR Master Mix (Fermentas, PA, USA), 0.3 μM of each primer and 1 μl (ca. 10 ng) template cDNA. The cycling conditions consisted of an initial, single cycle of 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at 55 °C and 20 s at 72 °C. After completion of the qRT-PCR, a melting-curve analysis was performed to confirm the specificity of the amplification. PCRs were performed in triplicates. The expression level of the LECT2 gene was analyzed using ΔΔCT method [36].

2.5. The expressions of the LECT2 gene in Asian seabass challenged with V. harveyi

Forty individuals (at the age of 110 days) maintained in our fish facility were divided into two groups. Twenty fishes were transferred into the test tank where each fish was injected intraperitoneally with 0.1 ml of *V. harveyi* ($\sim e^8$ copy/ml) in phosphate-buffered saline as described by Xia et al. [29]. In the control tank, another 20 fishes received an intraperitoneal injection of 0.1 ml of phosphate-buffered saline. Four fishes from each tank were sacrificed at 1, 3, 6, 12 and 24 h post-injection (hpi). The spleen, kidney and liver were taken and preserved in Trizol reagent (Invitrogen, CA, USA) at −80 °C until use. RNA extraction, cDNA synthesis, qRT-PCR and the data analysis were conducted as described above. The normalized values of expressions of the LECT2 gene for the fishes challenged with the bacteria *V. harveyi* were compared with the control level at the respective time and tissues.