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Molecular cloning, characterization and expression profiling of galectin-9 gene from *Labeo rohita* (Hamilton, 1822)

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ABSTRACT

Galectin-9 is a b-galactoside-binding tandem repeat galectin that regulates many cellular functions, ranging from cell adhesion to pathogen recognition. In spite of extensive study of mammalian galectin importance in immune system, little is known about that of fish. To study the normal expression and immune response of *Labeo rohita* to pathogens, a tandem-repeat galectin-9 from *Labeo rohita* was identified and named *LrGal-9*. Its full-length cDNA was 1534 bp encoded 291 amino acids (35.12 KDa), shared the highest 81% identity with the galectin-9 of *Danio rerio*. *LrGal-9 identified* in this study lacked signal peptide and a transmembrane domain like galectin-9 members reported in other fishes. Quantitative PCR showed that *LrGal-9* was lowly expressed in gill, muscle, heart, highly expressed in tested immune tissues (intestine, kidney, liver, spleen) in normal body. After *Aeromonas hydrophila* challenge, *LrGal-9* was remarkably increased in all tested immune tissues in a time-dependent manner. These results suggest that *LrGal-9* plays a role in innate immunity in *Labeo rohita*.

1. Introduction

Galectins constitute an evolutionary conserved family of beta-galactoside binding lectins, ubiquitous in eukaryotic taxa, including the parazoa (sponges) and both protostome and deuterostome lineages of metazoans, and fungi. Based on structural features, galectins have been classified in three types: “proto”, “chimera”, and “tandem-repeat” (TR). Galectins were initially thought to only bind endogenous (“self”) glycans and mediate developmental processes, including cell differentiation and tissue organization, and more recently, regulation of immune homeostasis. Galectins also bind glycans on the surface of potentially pathogenic microbes and parasitic worms, and mediates recognition and effector functions in innate immunity [1]. In the past years it has been shown that galectins participate in regulation of both innate and adaptive immunity [2]. The proposed roles of galectins in immune functions have been further supported by their ability to directly recognize microbial pathogens [2]. Thus, the potential role of galectins as pattern recognition receptors (PRRs) has become an area of increased attention.

Galectins could be a good candidate for disease control in aquaculture which suffers losses due to bacterial and viral diseases [3]. Previous studies have shown that galectins characterized from aquatic animals have shown potent anti-bacterial, anti-fungal antiviral and anti protozoan activity [4] [5] [6] [7]. With the recognized role of galectins for innate immunity and since no study has characterized the immune roles of galectins in Indian major carps, the present study was undertaken to identify and characterize galectin gene from an Indian major carp *Labeo rohita*.

2. Materials and methods

2.1. Experimental animals

Clinically healthy *Labeo rohita* (rohu) of average size of 8–10 cm without any apparent disease lesions were procured from fish farm, located in Maharashtra, India for carrying out the experiment. The stock was acclimatized under aerated conditions for a period of 15 days and was fed *ad-libitum* with commercial fish diet twice daily.

2.2. Experimental design

Expression profiles of galectin-9 gene in different tissues of healthy *Labeo rohita*, along with the modulation of expression of the gene in animals experimentally challenged with *A. hydrophila* were investigated. To study the tissue level expression of the gene eight different tissues viz., brain, gill, heart, liver, spleen, kidney, intestine,

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muscle and Intestine collected from nine fishes were used. Tissues were pooled from three animals and a total of three pools were analyzed for the gene expression. For *in vivo* pathogen challenge study, 110 fishes were given intra peritoneal injection randomly distributed in one challenged and one control group, in triplicates, following a completely randomized design (CRD). Challenged group were injected with 0.2 mL *A. hydrophila* suspended in PBS (1×10^8 CFU/mL LD_{50} dose) and the control group was injected with same volume of PBS alone. After challenge, the fishes were divided into 2 groups of 18 animals each and maintained. Nine animals from each control and challenged groups were sacrificed at each time–point post challenge and samples were collected at six time points (0, 6, 12, 24, 48, and 72 h). Tissues collected from three animals from each replicate were pooled and three such pools are analyzed at each time point.

2.3. Total RNA isolation and first strand cDNA conversion

Total RNA from the control and infected fish were extracted using trizol® (Invitrogen, USA), according to the manufacturer's protocol with slight modifications [8]. First strand cDNA conversion was carried out using 2.0 µg of RNA by RevertAid first strand cDNA kit according to manufactures recommendation (Thermo Scientific, Lithuania). The prepared cDNA was stored at −20 °C for further analysis.

2.4. Cloning of LrGalectin9 cDNA

Degenerate primers were designed for amplifying *LrGal-9* cDNA based on the multiple alignments of *Sinocyclocheilus rhinocerous* (XM_016573382.1), *Danio rerio* (NM_200072.1), and *Cyprinus carpio* (XM_019116442.1) galectin-9 sequences. The degenerate PCR amplification for partial fragment of *LrGal-9* was performed by using the following PCR condition: an initial denaturation step of 3 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and 1 min of extension at 72 °C, and final elongation step at 72 °C for 5 min. Amplified PCR products were cloned into pTZ57R/T (Thermo Scientific, Lithuania) and sequenced. Rapid amplification of cDNA ends (RACE) was performed to obtain 5′UTR (untranslated region) and 3′UTR of *LrGal-9* cDNA by using SMARTer™ RACE kit (Clontech, USA). The full-length cDNA of *LrGal-9* was cloned into pTZ57R/T and sequenced at M/s Bioserve Biotechnologies Pvt. Ltd. India.

2.5. Bioinformatics analysis

The nucleotide sequence and deduced amino acid sequence of the *LrGal-9* cDNA were analyzed using the BLAST algorithm (https://www.ncbi.nlm.nih.gov/BLAST/). Open reading frame (ORF) was predicted using ORF finder tool in NCBI (https://www.ncbi.nlm.nih.gov/gorf/gorf.html). The putative protein features were obtained with ExPaSy tools (http://www.expasy.org/tools/), and simple modular architecture research tool (SMART; http://smart.embl-heidelberg.de/) was used to analyze the protein domain topology. A Multalin alignment was made with similar protein sequences after BLASTp analysis with different organisms in the evolution-scale, and then the Neighbor-joining tree was performed with MEGA software version 7.0 [9]. The phosporylation sites, glycosylation sites were retrieved with NetPhos 3.0, NetNGlyc servers, respectively.

2.6. Relative quantification of gene expression using qRT-PCR

Quantitative RT-PCR was performed on Roche LC96 Lightcycler™ (Roche, Switzerland) using a 96 well plate layout. The following volumes were used per reaction (total volume 10 µl): 5 µl of 2X SybrGreen reaction mix (Takara Bio, Japan), 0.2 µl each of 10 pmol/µl forward and reverse primers, 1 µl of 100 ng cDNA and nuclease free water to make up the volume. The thermo cycling conditions were: 1 cycle of 5 min at 95 °C to activate the polymerase, 40 cycles of 10 s at 95 °C and 1 min at 59 °C for amplification. The primers for the internal control gene were designed from *L. rohita* β-actin sequence. Expression of genes of interest was normalized to the mean of β actin as the house keeping gene. Comparative C_T method was used to estimate the relative expression of mRNA [10].

2.7. Statistical analysis

All data were subjected to one way analysis of variance (ANOVA) using SPSS V.22.0 software. The results were expressed as mean ± SEM (standard error of mean). $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Bio-informatics analysis

Full length cDNA of *LrGal-9* was found to be 1534 bp, including a 109 bp of 5′ untranslated region (UTR), a 876 bp of open reading frame (ORF), and a 552 bp of 3′ UTR. The ORF encoded 291 amino acids (35.12 KDa) (Fig. 1). Nine potential tyrosine phosphorylation sites and three N-linked glycosylation sites were identified in *LrGal-9*. The molecular mass and theoretical isoelectric point (pI) of the *LrGal-9* found to possess a pI of 9.07 with a predicted molecular weight of 32.54 kDa. Neither signal peptide nor was transmembrane region found in *LrGal-9*. Prosite motifs analysis by SMART program showed that *LrGal-9*

Fig. 1. Nucleotide and deduced amino acid sequence of *LrGal-9* cDNA. The start codon (ATG) is marked in red and the stop codon (TAA) with asterisk. In the deduced amino acid sequence CARD 1 is marked in light blue color and underlined with the same color, CARD 2 is marked in green color and underlined with the same color. The sugar binding sites in both CARD domains are highlighted in yellow color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)