



## Full length article

# Identification, ontogeny and expression analysis of a novel laboratory of genetics and physiology 2 (LGP2) transcript in Asian seabass, *Lates calcarifer*



Anutosh Paria<sup>1</sup>, A. Deepika, K. Sreedharan, M. Makesh, Aparna Chaudhari, C.S. Purushothaman, K.V. Rajendran\*

ICAR-Central Institute of Fisheries Education (CIFE), Off-Yari Road, Versova, Mumbai, 400 061, India

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## ABSTRACT

*LGP2* (laboratory of genetics and physiology 2) is an important member of the retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs), which plays a significant role in antiviral innate immunity. In this study, we have cloned the full-length cDNA sequence of *LGP2* from Asian seabass, *Lates calcarifer* (*AsLGP2*). The complete *AsLGP2* cDNA sequence consisted of 2586 nucleotides encoding a putative protein of 681 amino acids with a molecular mass of 77.6 kDa. From the *AsLGP2* protein, four different conserved domains were predicted: a DExDc (DEAD/DEAH box helicase domain), a bacterial type III restriction enzyme domain (RES III), a HELICc (Helicase superfamily c-terminal domain and a RIG-I<sub>C</sub>-RD (RIG-I C-terminal regulatory domain). The transcript of *AsLGP2* could be detected in all the 11 tissues tested in healthy animals with high expression noticed in tissues facing external environment such as gill, hindgut and skin. The ontogenic expression profile of *AsLGP2* implies a possible maternal transfer of this gene as it has been detected in all early embryonic developmental stages along with unfertilized eggs. Viral analogue, poly I:C, injection resulted in rapid up-regulated expression in different tissues with the highest modulation of expression observed in kidney followed by liver and gill. A rapid response of *AsLGP2* expression was also observed in the different tissues of *Vibrio alginolyticus*-injected *L. calcarifer*, while significant change in expression was noticed following *Staphylococcus aureus* infection. Similarly, exposure to different pathogen-mimicking microbial analogues such as poly I:C, LPS and PGN resulted in enhanced expression of *AsLGP2* in SISK cell-line. Taking together, these observations suggest that *AsLGP2* can act as both antiviral and antibacterial cytosolic receptor and may play a significant role in embryonic and larval development in marine euryhaline teleosts like Asian seabass.

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## 1. Introduction

Retinoic acid-inducible gene 1- (RIG-I)-like receptors (RLRs) are a group of cytoplasmic pattern recognition receptors belonging to the family of DExD/H RNA helicases [1]. These helicases mainly sense intracellular viral nucleic acids and play a key role in building an effective protection strategy against invading infectious pathogens [2]. Three highly similar proteins constitute the RLRs family: RIG-I, melanoma differentiation-associated gene 5 (*MDA5*) and the

laboratory of genetics and physiology 2 gene (*LGP2*). All the three members of the RLR family contain a central DExD/H-box helicase domain and a C-terminal regulatory domain (CTD) [3–5]. The CTD domain exerts its function by regulating the RLR signalling and recognising specific foreign RNA substrates [6–8]. Two members of the RLR family, i.e., RIG-I and *MDA5*, contain two N-terminal caspase activation and recruitment domains (CARD), whereas *LGP2* does not have any N-terminal domain [6,9,10]. The N-terminal CARD domains are required for downstream signalling by interacting and recruiting the adaptor protein MAVS (Mitochondrial antiviral signalling protein). The MAVS is the molecule which starts a downstream signalling cascade to activate different transcription factors like NF- $\kappa$ B and IRFs, and finally result in the production of type I IFNs and pro-inflammatory cytokines [5,11]. The *LGP2* shares a close structural similarity with RIG-I and *MDA5*, and lacks a N-terminal

\* Corresponding author.

E-mail address: [rajendrankv@hotmail.com](mailto:rajendrankv@hotmail.com) (K.V. Rajendran).

<sup>1</sup> Present address: ICAR-Indian Institute of Agricultural Biotechnology, Ranchi, 834 010, India.

domain. Moreover, as *LGP2* possesses strong affinity towards dsRNA, it can be hypothesized that *LGP2* interferes in RLR signalling or may have separate function altogether from its other RLR family members [10,12–14]. Both *RIG-I* and *MDA5* have almost similar function in antiviral immunity but they differ in the type of molecules they detect; *RIG-I* detects relatively shorter double-stranded RNAs (dsRNAs) (<1 kb) along with free 5' triphosphate end structures and RNAs with complex secondary structures, whereas, *MDA5* detects long dsRNAs (>3 kb) and RNA molecules with "non-self" - 5' termini [15–20]. In contrast to the size-dependent detection of RNA molecules exhibited by *RIG-I* and *MDA5*, *LGP2* can detect both dsRNA and single-stranded RNA (ssRNA) in a size- and 5'-triphosphate-independent manner [3,21,22].

The specific role exhibited by *LGP2* in innate immunity is still unclear. Initially, the *LGP2* was identified as a negative regulator of RLR-signalling [9,23,24]. However, recent studies proved that *LGP2* can have a positive role in antiviral innate immunity [25,26] in higher vertebrates. Moreover, it has been found that *LGP2* can potentially enhance the *MDA5*-mediated antiviral signalling [14]. Thus, *LGP2* acts as an inhibitory as well as stimulatory molecule in antiviral innate immunity; but these mechanisms are not mutually exclusive [10,27]. Apart from having a role in RLR-signalling, *LGP2* also plays important functional roles in antiviral T-cell regulation [28] and adversely contributes in cancer cell resistance to ionizing radiation [29].

Cloning and functional characterization of *LGP2* have been reported in many species of finfish including olive flounder, *Paralichthys olivaceus* [30], grass carp, *Ctenopharyngodon idella* [31], rainbow trout, *Oncorhynchus mykiss* [32], channel catfish, *Ictalurus punctatus* [33], common carp, *Cyprinus carpio* [34], seaperch, *Lateolabrax japonicus* [35] and large yellow croaker, *Larimichthys crocea* [36]. Almost all these previous studies on teleosts showed a positive regulatory role played by *LGP2* in antiviral signalling against RNA and DNA viruses or viral analogue, poly I:C *in vivo* or *in vitro*. However, it has also been reported that the transcript level of *LGP2* can be modulated by bacterial infection as well [33].

Asian seabass, *Lates calcarifer* is a highly valuable candidate species for aquaculture in tropical marine, brackish and freshwater throughout the South-Eastern Asia and Australia because of its resistance to salinity changes, high stocking density, high growth and fecundity [37]. However, one of the major concerns in the culture of this species is its susceptibility to different viral, parasitic and bacterial pathogens including nodavirus, *Streptococcus iniae*, marine vibrios and *Cryptocaryon irritans* [38–41]. Only limited information is available on the immune repertoires of *L. calcarifer* and how the immune system performs against different pathogenic invaders, especially in immune-gene level. Recently, few groups of antimicrobial peptides, copper zinc superoxide dismutase and DC-SCRIPT have been characterized, and immune gene expression profiling against a parasite, *Cryptocaryon irritans*, been studied in *L. calcarifer* [42–45]. In a recent study, *NLRC3*, a pivotal cytosolic receptor with a possible antimicrobial activity has been cloned and characterized in *L. calcarifer* [46]. Here, we have cloned the full-length cDNA sequence of *LGP2*, an important intracellular PRR from Asian seabass, *L. calcarifer* (*AsLGP2*), and also evaluated the phylogenetic relationship and ontogenetic developmental profile. We have also investigated the expression profile of *AsLGP2* in different tissues of healthy and bacteria-infected or poly I:C-injected animals *in vivo*. Further, the response of *AsLGP2* towards different ligands such as poly I:C, lipopolysaccharide (LPS) and peptidoglycan (PGN) *in vitro* has been investigated. The findings of this study would give a better insight on the functional role played by *LGP2* in a euryhaline teleost model and also will provide a novel biological phenomenon of ontogenetic development of RLRs in bony fishes.

## 2. Materials and methods

### 2.1. Identification and cloning of full-length cDNA sequence of *AsLGP2*

Total RNA was extracted from the kidney tissue of healthy Asian seabass using RNeasy mini kit (Qiagen, USA) following the manufacturer's protocol. One microgramme of the extracted RNA was reverse-transcribed using First Strand cDNA Synthesis Kit (Thermo Scientific, USA). For amplifying an initial sequence of *AsLGP2*, primers (*LGP2*-F1 & *LGP2*-R1) were designed based on the conserved sequence of the available finfish sequences including *P. olivaceus* *LGP2* mRNA (HM070372.1), *Salmo salar* *LGP2* (NM\_001140177.1) and *O. mykiss* *LGP2* (FN396359.1). The PCR was performed following the PCR condition consisting of an initial denaturation step of 3 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 40 s and extension at 72 °C for 1 min, and final elongation step at 72 °C for 7 min. The amplified product was cloned into pTZ57R/T vector (Thermo Scientific, USA) and sequenced in both the directions using universal vector-specific primers. From the generated sequence, specific primers for 5' and 3'-RACE PCR were designed. RACE was performed using SMARTer™ RACE cDNA amplification kit (Clontech, USA) following the manufacturer's protocol. Specific fragments amplified in the RACE PCR were purified and cloned into pTZ57R/T vector (Thermo Scientific, USA). Positive recombinant clones were sequenced in both directions using M13 forward and reverse universal primers. All the primers used in the study are tabulated in Table 1.

### 2.2. Bioinformatics tools used for sequence annotation and phylogenetic tree construction

The complete cDNA sequence of *AsLGP2* was analysed with the BLAST (<http://www.ncbi.nlm.nih.gov/blast>) programme to get other homologous sequences and the homology percentage was predicted. The multiple alignment of *AsLGP2* along with other *LGP2* sequences of animals belonging to different taxa was done using the ClustalW2 programme (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The deduced amino acid sequence was analysed by Expert Protein Analysis System (ExPaSy) tools (<http://www.expasy.org/tools/>) and the protein domains were predicted by Simple

**Table 1**  
Primers used in the study.

Primers	Sequence (5'-3')
<b>Gene cloning</b>	
LGP2-F1	CGACCGGAGGTGGAAGAC
LGP2-R1	CCGGCGATGCAGTGAGA
LGP2-F2	CAGATCCTGGGTCTCACTG
LGP2-R2	TCATGTAAGTGATGCCGGTG
LGP2-F3	GCCACCGGCACCTACTACAT
LGP2-R3	GGGACATCCTCCACTCTCTCA
<b>RACE</b>	
LGP2-5'-R1	TGTCCTCGGTGATGCAGTGA
LGP2-5'-NR1	CCTGACTGGCACCAGCTTGT
LGP2-3'-F1	GTACAAGAAGATCGCCTGTGCC
LGP2-3'-NF1	TGGCAGGACAAGTGTGAAGAAGTG
UPM-long	CTAATACGACTCACTATAGGGCAAGCAG TGATCAACGGCAGAGTCTAAT ACGACTCACTATAGGGC AAGCAGTGGTATCAACGCAGAGT
<b>Real-time PCR</b>	
<i>AsLGP2</i> -F	TGTCACCACACCACAAGGA
<i>AsLGP2</i> -R	ATGCTCCAGCAGCTTTTCCA
EF-1 $\alpha$ -F	GTTGCCITTTGTCCTCATCTC
EF-1 $\alpha$ -R	CTTCCAGCAGTGTGGTTCCA
$\beta$ -actin-F	TACCACCGGTATCGTCATGGA
$\beta$ -actin-R	CCACGCTCTGTCAGGATCTTC

Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de/>). The molecular mass (MM) and theoretical isoelectric point (pI) of the protein were calculated based upon its deduced amino acids by the Compute pI/Mw software ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)), and N-linked glycosylation sites in the amino acid sequence were predicted using NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/NetNGlyc> 1.0 Server). A neighbour-joining phylogenetic tree was constructed using the MEGA 7 programme following the Poisson model with bootstrapped 1000 times.

### 2.3. Experimental animals and in vivo challenge experiments

Healthy Asian seabass juveniles (~15–20 g) were procured from nearby commercial farms and maintained at 5‰ seawater with a commercial diet for two weeks before conducting the experiment. For the tissue distribution study of *AsLGP2* in healthy animals, nine fishes divided into three separate pools were sacrificed and tissues including gill, heart, spleen, liver, midgut, hindgut, stomach, head kidney, trunk kidney, brain and skin were collected. To study whether *AsLGP2* responds to bacterial infection, fishes were injected with 100  $\mu$ L of  $1 \times 10^6$  CFU/mL *Vibrio alginolyticus* (ATCC<sup>®</sup> 17749) or 100  $\mu$ L of  $1 \times 10^7$  CFU/mL *Staphylococcus aureus* (ATCC<sup>®</sup> 11632) suspended in PBS. For poly I:C stimulation, fish were injected with 100  $\mu$ L of 1 mg/mL poly I:C suspended in PBS. After injection, six animals each were sacrificed at 3, 8, 24, 72 h and 5 days post-injection (dpi), and tissues such as gill, heart, spleen, kidney, liver and intestine were collected. Fish injected with sterile PBS were sampled similarly at each time-point and used as control in all the challenge studies. Tissues from two animals constituted a single pooled sample and three such pools were used to study the gene expression.

### 2.4. Developmental stages and sample collection

Different embryonic and larval developmental stages of Asian seabass were collected from the Asian seabass hatchery of the Central Institute of Brackishwater Aquaculture, Chennai. Six different embryonic developmental stages: unfertilized eggs (Unf), fertilized eggs at 0 h post-spawning (0 hps), blastula (5 hps), gastrula (7 hps), neurula (9 hps), early embryo (12 hps) and seven different larval developmental stages: hatchlings (0 day post-hatching) and six subsequent larval developmental stages (1, 2, 3, 5, 10 and 30 day post-hatching) were collected. The developmental stages were collected from three separate larval rearing units and the pooled sample from each rearing unit was treated as a single sample.

### 2.5. In vitro ligand stimulation

Asian seabass kidney cell line (SISK) was sourced and maintained in L-15 medium supplemented with 10% FBS (Gibco, USA) [47] at 28 °C. After 2–3 passages, the cells were transferred to 6-well plates ( $1 \times 10^5$  cells/well) for induction study with different ligands. After 24 h of culture in 6-well plates, the cells were exposed to different ligands such as poly I:C (50  $\mu$ g/mL, Sigma P1530), LPS (25  $\mu$ g/mL, Sigma L2630) and PGN (25  $\mu$ g/mL, Sigma 77140). The cells were harvested at 3, 8, 24, 48 and 72 h post-induction and the cells from two wells were pooled. Three such pools were used as sample replicates for a given time-point. Hanks' balanced salt solution (HBSS)-treated cells were harvested similarly at each time-point and used as the control.

### 2.6. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from different tissues, developmental stages and cultured cells, and subsequently cDNA was synthesized

as described in Section 2.1. The qPCR was performed in ABI 7500 RT-PCR system (Applied Biosystems, USA) using SYBR<sup>®</sup> Premix ExTaq<sup>™</sup>II (Tli RNaseH Plus) (Takara Bio Inc., Japan). The primers used for qPCR are listed in Table 1. PCR efficiency for all the primers used was determined by generating standard curves using a serial 10-fold dilution of spleen cDNA. The efficiency of the primers ranging between 95% and 105% was considered significant for use in expression analysis. The calculation of PCR-efficiency was done using the formula  $E (\%) = (10^{-1/\text{slope}} - 1) \times 100$  [48]. The reaction was done in duplicates in a total volume of 25  $\mu$ L reaction mixture containing 12.5  $\mu$ L of 2X SYBR Green master mix, 0.5  $\mu$ L of ROX, 1  $\mu$ L of each primer (10  $\mu$ M), 2  $\mu$ L of 100 ng cDNA and 8  $\mu$ L of nuclease-free water. The default thermal profile was used for PCR amplification and it consisted of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melt curve analysis of the amplification products was performed at the end of each PCR reaction to confirm that only the specific PCR product was amplified and detected. Elongation factor 1- $\alpha$  (*EF-1 $\alpha$* ) was used as the housekeeping gene for data normalization. For ontogeny experiment, a two-housekeeping gene normalization method using *EF-1 $\alpha$*  and  $\beta$ -actin was employed [49]. Fold-change in expression in the treated group compared to control group was determined using  $2^{-\Delta\Delta CT}$  method [50]. The data obtained from the three individual biological replicates were analysed using one-way analysis of variance (ANOVA) to find out the significance level between the groups.  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Molecular characterisation and sequence analysis of *AsLGP2*

The full-length cDNA sequence of *AsLGP2* was amplified and cloned from kidney tissue of healthy Asian seabass. The complete *AsLGP2* cDNA sequence consisted of 2586 nucleotides encoding a putative 681 amino acid-protein with a molecular mass of 77.6 kDa and isoelectric point of 6.38 (GenBank Accession No. KX268683.1). The open reading frame encoding this protein is of 2046 bp length with 88 bp 5' un-translated region (UTR) and 452 bp 3' UTR (Fig. 1A). From the deduced amino acid sequence of *AsLGP2*, four different conserved domains could be predicted by the SMART programme. At the extreme N-terminal end, it has a DExDc (DEAD/DEAH box helicase domain, 2 to 221 AA) which also overlapped with a bacterial Type III restriction enzyme domain (RES III, 3 to 176 AA). *AsLGP2* also harboured a HELICc (Helicase superfamily c-terminal domain, 382 to 477 AA) and RIG-I\_C-RD (RIG-I C-terminal regulatory domain, 553 to 674 AA) (Fig. 1B).

### 3.2. Sequence homology and phylogenetic tree construction

The *AsLGP2* amino acid sequence showed the highest identity with *L. japonicus* (84%), *Oplegnathus fasciatus* (82%), *L. crocea* (82%) and *P. olivaceus* (80%), whereas the lowest identity could be found with *Mus musculus* (45%). Among the predicted conserved domains, DExDc domain showed a relatively high amount of conserved sequences across the species, while sequence similarity was found to be less in RIG-I\_C-RD domain (Table 2). From the alignment of amino acids pertaining to the RIG-I\_C-RD domain, four highly conserved cysteine residues forming two separate RNA-binding loops were identified. The first loop consisting of four amino acids flanked with cysteine moieties was highly conserved, whereas the second loop was found to be varied across the species (Fig. 2).

To find out the phylogenetic relationship of *AsLGP2* with *LGP2* from other animals, a neighbour-joining phylogenetic tree was created. *AsLGP2* was found to be clustered distinctly with other



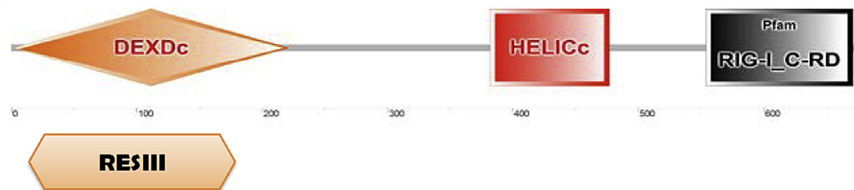
A

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1 cttgaccacactgtgaggtgttagtggacgagagagaagctgaaataccacacggttccctacagctgctggagtaaa 377
1 M A D F G L Y S Y Q E E V V E R A L R G E N T I I 1216
82 gtgtagcagattttggactgtattcataccagcagaggaagtggttgaagggtcttagggagagAACAAATAT 404
26 W L P T G G G K T R A A V Y V A K R H L E T T O K A K 1297
163 CTGGCTGCCGACGGAGGTGAAGACCCGTCGCTGTATGTAGCCAAAAGACACCTGGAGACCACACAGAAAGCTAA 431
53 V V V L V N K V H L V D O H Y T K E F K P H L G Y D Y 1378
244 GGTTGGTCTCTGGTAAACAAGGTTACCTGTAGACACACATTACACAAAGAGTTAAACCTCACTGGGTTATGACTA 458
80 K L V P V S G E S E E K D F F G K V V Q D S D L V I C 1459
325 CAAGCTGGTCCAGTCACTGGAGAGTGGAGAGAGGACTCTTTGAAAAGTGGTGCAGGACTCAGACCTGGTCACTG 485
107 T A Q I L Y N A L I N T E E T K H V E L S D I T L L I 1540
406 CACAGCAGACATCTTATAATAATGCCCTGATTACACAGGAGAAACCAACATGTTGAGCTCTCAGATATCACTACTGAT 512
134 I D E C H H T H K E S V Y N K I M R C Y V E K K L R R 1621
487 AATCGATGAGTTCACACACCAAGAGTCACTCAGCAGGATTAATGAGTCTGCTGAGAAAAGTTGAGACG 539
161 E H P L P Q I L I G L T A S P G T G G A K I L E K A V E 1702
568 GGAACATCCATGCCACAGTCTGGTCTCACTCAGTCAACAGGACAGGGGGCCGCAAGATCCTGGAAAAGGCTGTGA 566
188 H V L Q I C A N L D S A I V S T K N Y A P E L K K K V 1783
649 GCATGCTCCTGAGATCTGTAACTGGACTCAGCAGTATGTTCACTAAAACATATGCCCTGAGCTGAAGAAGAAGT 593
215 P R P I K T E D I V E K R H E D P F G D H L K W M M Q 1864
730 CCCCAGACCCATCAAGACATTTGACATTTGGAGAAAAGGACATGAGGATCCATTTGGGATCCTCAAGTGGATGATGCA 1620
242 L I H E Y M N L P P D V K L R E C G T Q E Y E A D V V 1945
811 GCTGATCCATGATGATGAACTACCACAGCTCAAACTGAGAGAGTGGTGCACACAGAGATGAGGACAGATGTGGT 647
269 I L E Q R G V I V N N R L L A Q C A L H L R Q Y N D A 2026
892 GATTTAGAGCAGCGGGGTAATAGTGAACACAGACTGCTGCACAGTGTGCCTCCACTCAGACAGTACAACGACGC 674
296 L L I N D T L R M M D A Y R S L E E F Y I T K S D T A 2107
973 CCTGCTCAATGACACCCCTGAGAATGATGATGCTTATGCTCCCTAGAAGAATTCATACACAAAAGTCAGACACAG 2188
323 I D G T D F F L V G L F Q E N Q V E L R K L A M D S H 2269
1054 CATCGATGGAACAGACTTCTCTGTTGGGACTTTTCCAAAGAAATCAGGTGGAGCTGAGGAAACTGCAATGGATTCTCA 2350
350 Y E N P K M G K L E S V L L N Q P F G H G S K E S R G I 2431
1135 CTATGAGAACCAGAAATGGTAACTTGAGAGTGTCTACTCAATCAATTTGGTCATGGATCAAAAGAAATCAAGAGGGAT 2512
L F S K T R K S T H C L K D W V V T T E P Y S K L D P
TCTCTTCAGTAAAACCTGTAAGACCCACCTGCTTAAAGACTGGGCTTACACACAGAACCTACAGCAAGCTGGATCC
G S I S L G L A T G I T Y M T Q N E Q A D T I R N F R
AGGACAGTCTCACTGGGCTGGCCACCGGCATCACTTACATGACACAGACAGCAGCAGACACAACTCCGCAATTCGC
Q G K L N L L I S T S V A E E G L D I P E C N L V V R
CCAGGGTAACTCAACCTTCTGATCCACAGTGGCTGAAGAAGCCCTGACATCCCTGAGTGAACCTGGTGTGGG
Y G L L T N E I A Q Q Q A S G R A R A R D S Q Y S V V
TTATGGACTCTTACAAATGAGATTGCCAGCAGCAGCCAGTGGACCTGCCAGAGCCGAGACAGCCAGTATTCAGTGGT
A V K G G R E E R R E L T N E Y L E E L T G K A I A Q
CGCTGTAAGAGTGAAGGAGAGCCGAGAACTCAACATGAATACCTGGAAGAGCTCACTGGAAAAGCCATCGCTCA
V Q D M S L R E F C R K I T E L Q T E A V V T S K V A
GGTCAAGATATGACCTCCGAGAGTTTTGCAAGAAATAACTGAACACAGACAGAGCAGTGTTCACAGTAAAGTTGC
E T R K I E K R S R Y A A S S I O L L C R N C F K P V
AGAGACCCGAAAATGAGAAGAGGAGTCTCACTGCTTCCAGTATCCAGCTCTGTGTCGAAAATTTGTTCAAGCCTGT
A S G S D I K L V D N A H Y V N V N P D F K R H Y R
GGCCTCTGGCAGTACATCAAACCTGTTGACAATGCGCACTATGTCATGTCATCTCACTCAAGACACTACAGAGT
G G P V I L E R S F E D W E P G C T I N C N N G N C N
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L O W G F E I K Y K K I A L E L P N L A I K N F A L E T
CTTCAATGGGATTCGAGATCAAGTACAAGAGATGCCCCTGCTGCCAATTTAGCCATAAAGAAATTTGCCCTGGAGAC
P D G R T T V K K W K D V L F T V E D F S F E O Y C Q
CCCTGATGGCAGCAACTGTGAAGAAGTGAAGGATGTCCTTTCCAGCTGAGGACTTTAGCTTTGAGCAATACTGCCA
T C P P L F D *
AGACACCTCCCTGACCTCTTCGATTCaaccatcaagagagctcttgcaaggttttttcccaactcacgtcagctt
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ctgtaactgaacttgaaccatgaaacttgaagtctcatgataaaaaaataaaaaaataaaaaaataaaaaa

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B



**Fig. 1.** The sequence and schematic representation of the domain structures of *AsLGP2*. (A) Complete nucleotide and deduced amino acid sequence of *AsLGP2* cDNA. The start codon (ATG), stop codon (TGA) boxed and N-linked glycosylation sites are marked with bold. 5' and 3' UTR sequences are in lower case. 3' UTR mRNA instability motif (ATTTA) is marked with bold-italics. The DEXDc domain is highlighted in dark-grey (2–221 AA), HELICc domain is highlighted in light-grey (382–477 AA) and RIG-I\_C-RD domain (554–671 AA) is underlined. The RESIII domain (3–176 AA) within the DEXDc domain is wavy underlined. (B) Schematic description of the domain topologies of *AsLGP2*.

marine finfishes such as large yellow croaker, striped beakfish, seaperch and Japanese flounder. Among the piscine homologues of *LGP2*, seaperch showed the maximum phylogenetic homology, whereas elephant shark appeared to be distantly related to *AsLGP2*.

Table 2

Structural homology (%) of *AsLGP2* complete amino acid and domains with other animals.

Animals	<i>LGP2</i> (AA)	DExDc	HELICc	RIG-I_C-RD
<i>Lates calcarifer</i>	100	100	100	100
<i>Lateolabrax japonicus</i>	84	90	77	81
<i>Oplegnathus fasciatus</i>	82	89	72	83
<i>Larimichthys crocea</i>	82	88	74	81
<i>Paralichthys olivaceus</i>	80	87	73	75
<i>Stegastes partitus</i>	79	84	75	74
<i>Xiphophorus maculatus</i>	78	82	77	77
<i>Oryzias latipes</i>	78	85	74	74
<i>Takifugu rubripes</i>	73	79	71	74
<i>Oreochromis niloticus</i>	72	80	77	76
<i>Oncorhynchus mykiss</i>	70	78	73	60
<i>Cyprinus carpio</i>	66	76	70	54
<i>Ctenopharyngodon idella</i>	65	74	67	54
<i>Danio rerio</i>	65	73	68	54
<i>Ictalurus punctatus</i>	64	75	67	50
<i>Xenopus tropicalis</i>	52	60	56	—
<i>Homo sapiens</i>	47	56	63	45
<i>Mus musculus</i>	45	53	62	44

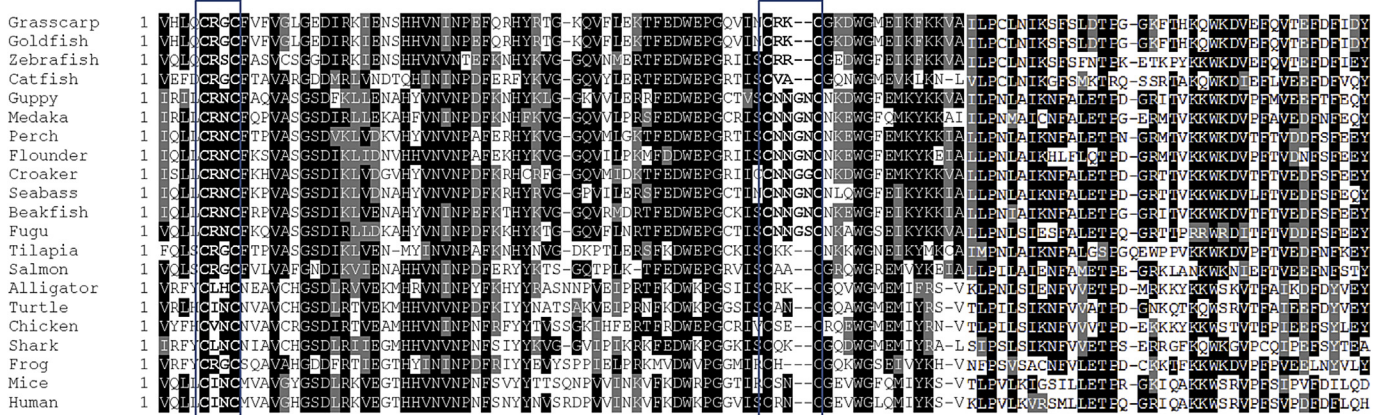
As, the *LGP2* gene from selected mammals, reptiles, amphibians and birds were formed a separate distant clad from piscine homologues of *LGP2*, thus treated as outgroup in the phylogenetic tree (Fig. 3).

### 3.3. Tissue distribution of *AsLGP2* in healthy animals

The expression profile of *AsLGP2* was determined in 11 different tissues of healthy *L. calcarifer* by qPCR using *EF-1 $\alpha$*  as the internal control. The mRNA transcript of *AsLGP2* could be detected in all the tissues tested with the highest expression observed in gill followed by hindgut. Moderate level of *AsLGP2* expression could be detected in skin, trunk kidney, liver, head kidney, midgut, brain and spleen, whereas low transcript abundance was observed in stomach (Fig. 4).

### 3.4. Ontogenetic profile of *AsLGP2*

To study the ontogeny of *AsLGP2*, the expression level was estimated in 13 different embryonic and larval developmental stages starting from unfertilized eggs to 30 days post-hatched (30 dph) larvae (Fig. 5) by qPCR using two-gene (*EF-1 $\alpha$*  &  *$\beta$ -actin*) data normalization method. Initial embryonic developmental stages including fertilized eggs, blastula and gastrula along with unfertilized eggs showed almost the same level of transcript. However,



**Fig. 2.** Multiple alignments of the deduced amino acid sequence pertaining to RIG-1\_C-RD domain of *LGP2* from different species. Dashes (-) indicate gaps, black shadow indicates identical residues and grey shadow indicates similar residues in the aligned amino acid sequences. Four conserved cysteine residues and intermittent sequence motifs were boxed within the domain. The deduced amino acid sequence obtained from *LGP2* sequence of different animals available in GenBank – Asian seabass, *Lates calcarifer* (Accession No. AOV82294.1), Grass carp; *Ctenopharyngodon idella* (Accession No. AFQ93565.1), Goldfish; *Carassius auratus* (Accession No. AEN04474.1), Zebrafish; *Danio rerio* (Accession No. AKA09351.1), Channel catfish; *Ictalurus punctatus* (Accession No. NP\_001265738.1), Guppy; *Poecilia reticulata* (Accession No. XP\_008415285.1), Medaka; *Oryzias latipes* (Accession No. XP\_004071339.1), Seaperch; *Lateolabrax japonicus* (Accession No. ALE66118.1), Japanese flounder; *Paralichthys olivaceus* (Accession No. ADI75503.1), Yellow croaker; *Larimichthys crocea* (Accession No. KKF29873.1), Striped beakfish; *Oplegnathus fasciatus* (Accession No. AHX37213.1), Fugu; *Takifugu rubripes* (Accession No. XP\_003964848.1), Tilapia; *Oreochromis niloticus* (Accession No. XP\_019213271.1), Atlantic salmon; *Salmo salar* (Accession No. XP\_014059582.1), Alligator; *Alligator sinensis* (Accession No. XP\_006032816.1), Green turtle; *Chelonia mydas* (Accession No. EMP40502.1), Chicken; *Gallus gallus* (Accession No. NP\_001305337.1), Elephant shark; *Callorhynchus milii* (Accession No. XP\_007907694.1), Frog; *Xenopus tropicalis* (Accession No. XP\_002939087.2), Mice; *Mus musculus* (Accession No. NP\_084426.2), Human; *Homo sapiens* (Accession No. NP\_077024.2).

subsequent stages such as neurula and early embryo showed a comparatively higher expression of *AsLGP2* followed by a low transcript level during hatching. *AsLGP2* mRNA expression level was not very stably expressed in larval stages as the expression level was found to be varied till 30 dph. In 3 dph and 5 dph stages, relatively low *AsLGP2* expression was detected and the level of expression was similar to that of the expression detected during hatching (Fig. 5).

**3.5. Temporal expression pattern of *AsLGP2* in different tissues following poly I:C stimulation in vivo**

Time-dependant expression pattern of *AsLGP2* in poly I:C-injected *L. calcarifer* was determined in six different tissues by qPCR using *EF-1α* as internal control and PBS-injected animals served as experimental control. Among the tissues, gill, kidney and liver showed significant up-regulation as early as 3 h post-injection (hpi). At this point, liver showed 55-fold up-regulation in *AsLGP2* expression (Fig. 6). Though the level showed a decline at 8 hpi, the expression showed a significant up-regulation (34-fold) and the elevated expression declined further and attained normal level at 5 days post-injection (dpi) ( $p < 0.05$ ). In gill and kidney, significant up-regulation could be observed from 24 hpi with 29-fold and 64-fold, respectively ( $p < 0.05$ ). However, in kidney, the expression increased further and attained the highest level (189-fold;  $p < 0.05$ ) at 72 hpi. Although the expression showed a decline at 5 dpi, the expression was found to remain at a highly elevated level (77-fold) (Fig. 6). In spleen, a late significant up-regulation (5-fold) at 72 hpi and 6-fold change ( $p < 0.05$ ) at 5 dpi was observed. *AsLGP2* mRNA expression in heart and intestine did not show any significant change at early time-points. However, these tissues showed significantly high expression (38-fold and 29-fold, respectively) at 5 dpi (Fig. 6).

**3.6. Response of *AsLGP2* to bacterial challenge in vivo**

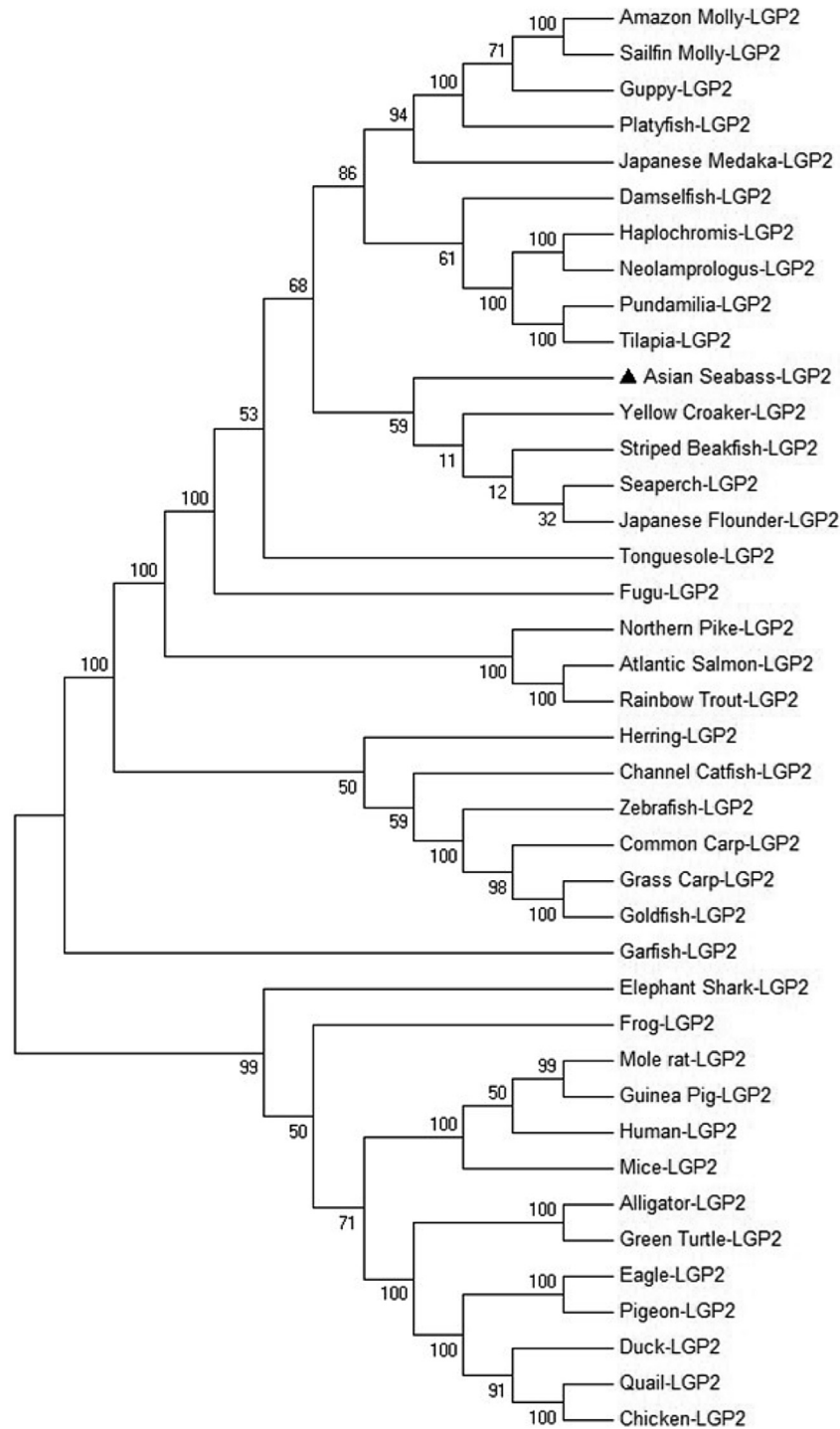
To investigate, whether *AsLGP2* expression can be modulated by bacterial infection, the spatial expression pattern of *AsLGP2* was determined following *V. alginolyticus* and *S. aureus* challenge in six

different tissues over a period of 5 days. *V. alginolyticus* challenge resulted in an early up-regulation of *AsLGP2* in most of the tissues tested. At 3 hpi, a significant increase in expression level was observed with 12-fold in gill and heart, 24-fold in kidney, 36-fold in liver and 40-fold in intestine ( $p < 0.05$ ) compared to PBS-injected control group (Fig. 7A). Following initial up-regulation, the expression level was found to be maintained either at similar level or increased further at 8 hpi with 17, 13, 2.5, and 34-fold ( $p < 0.05$ ) in gill, heart, spleen and liver, respectively (Fig. 7A). However, apart from the significant up-regulation of 6-fold at 24 hpi in gill and 9-fold at 5 dpi in heart, no significant change in the expression ( $p < 0.05$ ) of *AsLGP2* was observed at later time-points in any other tissues tested (Fig. 7A).

In *S. aureus*-challenged animals, spleen and kidney showed significant up-regulation only at 3 hpi with 4.2-fold and in intestine with 8-fold at 24 hpi ( $p < 0.05$ ) whereas, at other time-points, the expression level was found to be similar to the control in these tissues (Fig. 7B). Similarly, as observed in intestinal tissue, expression level was found to be significantly up-regulated with 3.2-fold in heart and 2.8-fold ( $p < 0.05$ ) in liver at 24 hpi. In contrast, the expression level showed significant down-regulation at 3, 8 and 72 hpi in heart and at 72 hpi in liver ( $p < 0.05$ ). In gill, although modulation in *AsLGP2* expression was observed at some time-points compared to PBS-injected control, the change in expression was found to be not significant ( $p < 0.05$ ) (Fig. 7B).

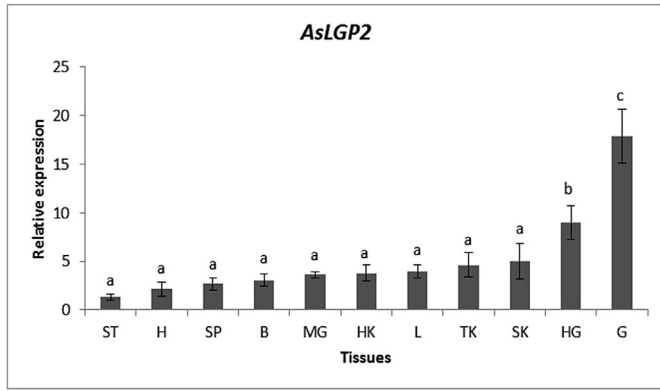
**3.7. *AsLGP2* expression in response to different ligand stimulation in vitro**

The expression pattern of *AsLGP2* in Asian seabass kidney cell line (SISK) exposed to different ligands such as poly I:C, LPS and PGN was determined over a period of 72 h compared to HBSS-treated control cells (Fig. 8). *AsLGP2* mRNA expression was found to be significantly increased (3.4-fold) at 24 hpi and reached peak level at 48 hpi with 8.8-fold increase ( $p < 0.05$ ) in poly I:C-treated cells. Subsequently, the elevated expression level attained almost normal level at 72 hpi. In LPS-induced cells, the expression level showed gradual increase at 24 hpi (2.9-fold) and reached the maximum level of 5.7-fold increase at 72 hpi ( $p < 0.05$ ). However,



**Fig. 3.** Phylogenetic tree of deduced amino acid sequences of *LGP2* (Asian seabass *LGP2* – *AsLGP2* highlighted). Numbers next to the branches indicate bootstrap values. Accession numbers of the *LGP2* sequences used for phylogenetic tree construction are as follows: AOV82294.1 (Asian seabass, *Lates calcarifer*), ALE66118.1 (Seaperch; *Lateolabrax japonicus*), AHX37213.1 (Striped beakfish; *Oplegnathus fasciatus*), KKF29873.1 (Croaker; *Larimichthys crocea*), ADI75503.1 (Japanese flounder; *Paralichthys olivaceus*), XP\_007558174.1 (Amazon molly; *Poecilia formosa*), XP\_008415285.1 (Guppy; *Poecilia reticulata*), XP\_014893336.1 (Sailfin molly; *Poecilia latipinna*), XP\_008283077.1 (Damselfish; *Stegastes partitus*), XP\_005796753.1 (Platyfish; *Xiphophorus maculatus*), XP\_004071339.1 (Japanese medaka; *Oryzias latipes*), XP\_005920418.1 (*Haplochromis burtoni*), XP\_006798306.1 (*Neolamprologus brichardi*), XP\_003964848.1 (Fugu; *Takifugu rubripes*), XP\_005726147.1 (*Pundamilia nyererei*), XP\_019213271.1 (Tilapia; *Oreochromis niloticus*), XP\_008327470.1 (Tongue sole; *Cynoglossus semilaevis*), XP\_014059582.1 (Atlantic salmon; *Salmo salar*), XP\_010873108.1 (Northern pike; *Esox lucius*), CAZ27718.1 (Rainbow trout; *Oncorhynchus mykiss*), AIX47137.1 (Common carp; *Cyprinus carpio*), AFQ93565.1 (Grass carp; *Ctenopharyngodon idella*), AEN04474.1 (Goldfish; *Carassius auratus*), AKA09351.1 (Zebrafish; *Danio rerio*), NP\_001265738.1 (Channel catfish; *Ictalurus punctatus*), XP\_012672250.1 (Herring; *Clupea harengus*), XP\_006638307.1 (Garfish; *Lepisosteus oculatus*), XP\_002939087.2 (Frog; *Xenopus tropicalis*), XP\_007907694.1 (Elephant shark; *Callorhynchus milii*), XP\_010583905.1 (Eagle; *Haliaeetus leucocephalus*), XP\_005513651.1 (Pigeon; *Columba livia*), XP\_006032816.1 (Alligator *sinensis*), XP\_015741127.1 (Quail; *Coturnix japonica*), NP\_001305337.1 (Chicken; *Gallus gallus*), AHA15372.1 (Duck; *Cairinamo schata*), EMP40502.1 (Green turtle; *Chelonia mydas*), EHB02819.1 (Mole rat; *Heterocephalus glaber*); NP\_084426.2 (Mice; *Mus musculus*), NP\_077024.2 (Human; *Homo sapiens*), XP\_013005131.1 (Guinea pig; *Cavia porcellus*).





**Fig. 4.** Normal expression profile of *AsLGP2* in different tissues of healthy Asian seabass. Data are shown as relative expression, Mean  $\pm$  SE in 11 different tissues; ST – Stomach, H – Heart, SP – Spleen, B – Brain, MG – Midgut, HK – Head kidney, L – Liver, TK – Trunk kidney, SK – Skin, HG – Hindgut, G – Gill.

PGN-induced cells exhibited no significant change in expression except for a transient 5.4-fold up-regulation at 3 hpi (Fig. 8).

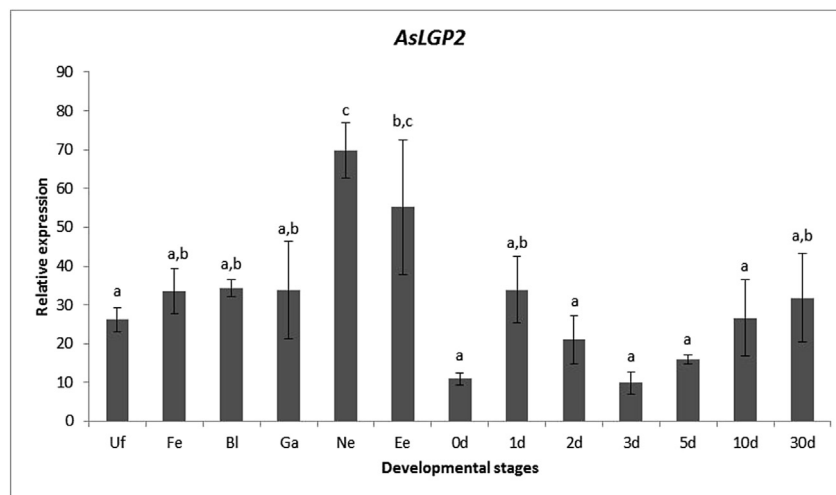
#### 4. Discussion

RLRs, the group of cytoplasmic viral sensors, have become a focused area of research as an important component of innate immunity in higher vertebrates as well as lower vertebrates like fish. Among the three members of the RLR family, the specific role of *LGP2* in the RLR signalling is not fully elucidated and it needs a great deal of attention to establish its common role as an immune molecule [51,52]. In the present study, we have cloned and sequenced an *LGP2* transcript from Asian seabass, *L. calcarifer*. The predicted structure of *AsLGP2* harbours four conserved domains: N-terminal DEXD/H-box helicase domain and a bacterial type III RES domain, a central HELIC domain and a C-terminal regulatory domain. Each of these domains has a specific role to play in the RLR-signalling cascade. DEXD/H helicase domain, which contains putative MG++ binding motif and ATP binding site, has catalytic activity to bind and unwind dsRNA in an ATP hydrolysis-dependent manner [9]. The bacterial type RES III domain has a specific role in the cleavage of unmethylated double-stranded foreign DNA and

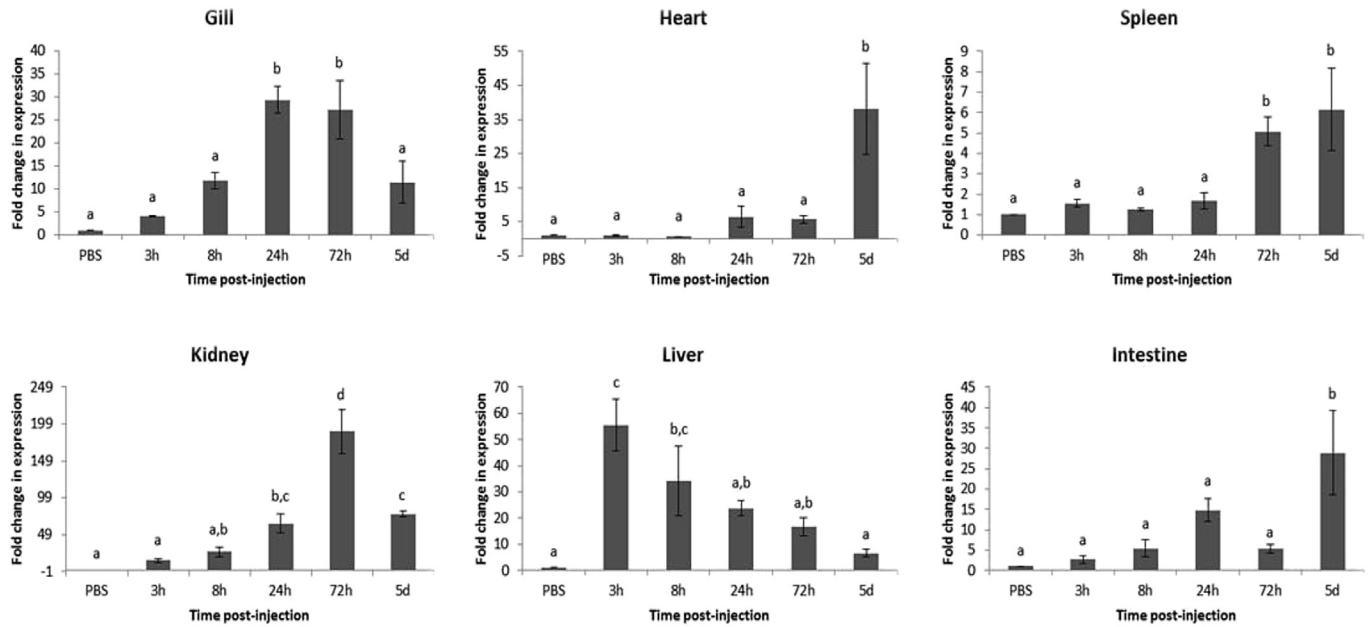
protection of self DNA from damage by methylation by virtue of its conserved sub-units [53,54]. The C-terminal RD domain of *LGP2* is responsible for the detection and binding of both ssRNA and dsRNA preferably with 5'-triphosphate containing RNA [7,55]. It has also been shown that RD of *LGP2* regulates the self-association and signalling of RIG, thus acting as a switch for the regulation of antiviral immunity [7]. With these conserved functions exhibited specifically by these putative domains, the structural organisation of this receptor is very less likely to vary across the animal kingdom. This may be the reason for having these conserved domains with similar structure across different animals. When compared with other animals, *AsLGP2*-specific domains show high similarity with the *LGP2* reported from marine finfishes. Phylogenetic tree also showed that *AsLGP2* forms a separate clad with other marine finfishes including seaperch, croaker, beakfish and flounder.

Four highly conserved cysteine moieties could be detected in the C-terminal RD domain of *AsLGP2*. These cysteine moieties along with other amino acids formed two looped structures in separate positions. The first looped structure is highly conserved across the species with four amino acid residues (CRNC), whereas the second loop connecting two cysteine moieties (CNGNC) is varied across the species in terms of its residue length. The second loop of the C-terminal of *LGP2* identified in *L. calcarifer* showed similarity in the loop structure reported from other fish species such as guppy, medaka, seaperch, Japanese flounder, croaker, beakfish and fugu, as they all have six amino acid residues. However, only four amino acid residues have been reported from cyprinids, catfish, tilapia, sharks, reptiles and mammals. Interestingly, the species with longer cysteine-bound looped structure mostly do not have *RIG-1* in their genome [36]. As mutation in cysteine residue results in the instability of CTD and this domain is believed to function as dsRNA sensor [22]; the longer amino acid loop connecting cysteine may have a different functional role to play in species deprived of *RIG-1*.

The *AsLGP2* mRNA was constitutively expressed in all the 11 tissues tested in healthy *L. calcarifer*. The transcript abundance was found to be higher in gill, hindgut and skin than in other tissues. Similar tissue distribution pattern of *LGP2* was observed in *C. carpio* with high expression in gill and intestine [34], and in gill, skin along with muscle in *L. crocea* [36]. However, in *L. japonicus*, the highest expression was detected in liver followed by heart, intestine and gill [35]. In contrast, *P. olivaceous* and *I. punctatus* *LGP2* are highly expressed in kidney, spleen and heart tissue [30,33]. Along with



**Fig. 5.** Expression of *AsLGP2* embryonic and larval developmental stages of Asian seabass. Data are shown from unfertilized eggs until 30 dph as relative expression, Mean  $\pm$  SE; Uf: Unfertilized eggs, Fe: Fertilized eggs (0 hps), Bl: Blastula (5 hps), Ga: Gastrula (7 hps), Ne: Neurula (9 hps), Ee: Early embryo (12 hps), Od: Hatchlings (0 dph), 1 d: 1 dph, 2 d: 2 dph, 3 d: 3 dph, 5 d: 5 dph, 10 d: 10 dph, 30 d: 30 dph. Abbreviation, hps: Hours post-spawning; dph: Days post-hatching.



**Fig. 6.** Expression profile of *AsLGP2* in various tissues of Asian seabass at different time-points following poly I:C stimulation *in vivo*. Different letters indicate significant differences ( $p \leq 0.05$ ) between different time-points within a treatment group compared to control.

kidney and spleen, the mRNA expression was also found to be high in intestine and skin tissue of grass carp [31]. Although spleen and heart showed high transcript abundance of *LGP2* in some of the finfishes, *AsLGP2* is very lowly expressed in these tissues. However, the expression in skin was found to be high, unlike the low expression pattern reported in *I. punctatus* and *C. carpio*. Further, the constitutive expression of *LGP2* observed in various tissues of different teleosts indicates that *LGP2* expression is not tissue-specific; rather, it is species-specific based on the environment it inhabits.

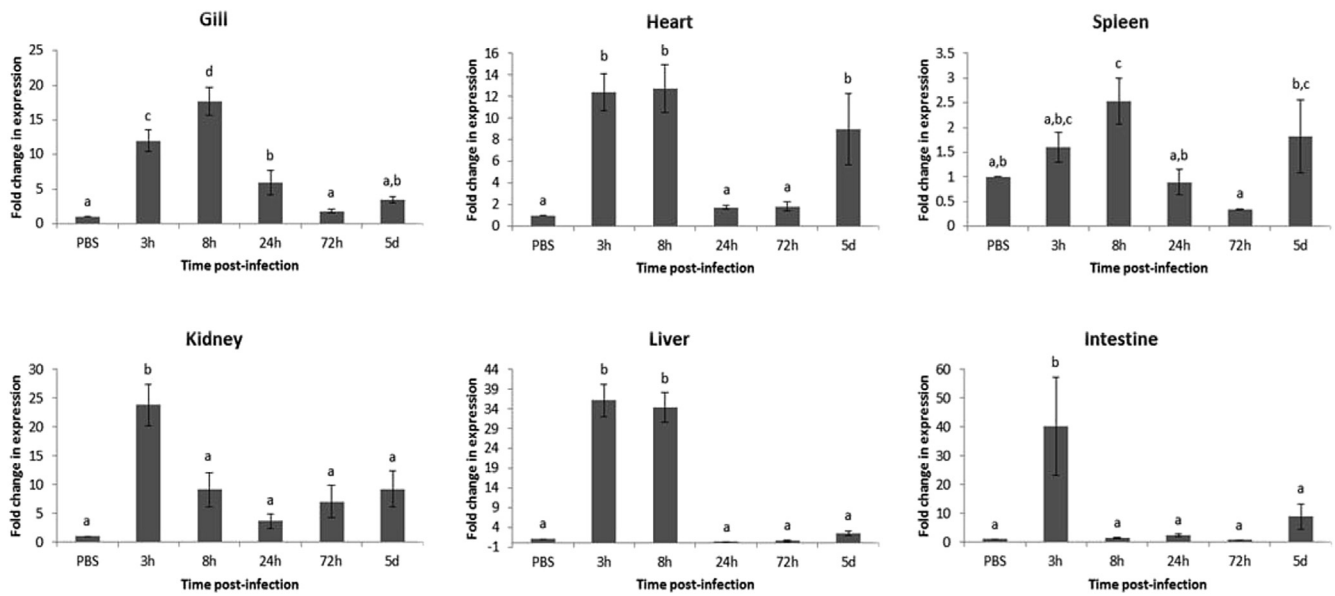
The role of innate immune genes is of great importance for larval survival, as the products of these genes such as protein and mRNA get transferred maternally to offspring through egg in teleosts [56,57]. Recently, the ontogenetic developmental profiles of different innate immune-related genes including cytokines, transferrin and caspase-1 have been studied in two marine fish models, i.e., European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) [58]. The transcript levels of these genes are differentially expressed in the embryonic and larval development process in these marine teleosts. The interleukin 1 beta and tumour necrosis factor alpha could not be detected in eggs while other genes such as cyclooxygenase-2, caspase-1, and transferrin were found to be maternally transferred in eggs. Similar expression profile of transferrin gene could be observed in *Megalobrama amblycephala* [59]. In an earlier study in Atlantic cod, it has been reported that the antibacterial innate immune genes like lysozyme and cathelicidin are maternally transferred while other antimicrobial molecules like hepcidin, pentraxins and *LGP2* could not be detected in unfertilized eggs. The transcript levels of these non-maternally transferred genes could be detected only in the gastrula stage [60]. Contrasting to this, the *AsLGP2* transcript had shown a ubiquitous expression in all the embryonic and larval developmental stages including unfertilized eggs, and the highest transcript level was detected in the neurula stage. Similarly, a maternal transfer of saccin gene was observed in loach, *Misgurnus anguillicaudatus*, but interestingly, the transcript level could not be detected in the later stages of embryonic development from blastula to neurula and reappears in hatchlings [61]. In zebrafish, the potent antiviral innate immune molecule Interferon- $\gamma$  (IFN- $\gamma$ ) has

shown a maternal transfer trend as detected in unfertilized eggs and expressed in all the succeeding developmental stages with a potential to respond towards bacterial infection [62]. The difference in the transcript abundance of *LGP2* in embryonic developmental stages may be due to the difference in incubation period before hatching as Atlantic cod eggs hatch in ~20 days, whereas the eggs of *L. calcarifer* hatch within 16–18 h post-fertilization. As *L. calcarifer* has a very short incubation period, before the maternal transcript gets exhausted, the advanced embryo (neurula and early embryo stages) might be capable of producing its own transcript of *LGP2*. The findings of the ubiquitous expression of *AsLGP2* in this study and the presence of active transcript of IFN- $\gamma$  in zebrafish embryo provides the indication of the active role played by RLR-mediated signalling in the embryonic and larval developmental stages of teleosts.

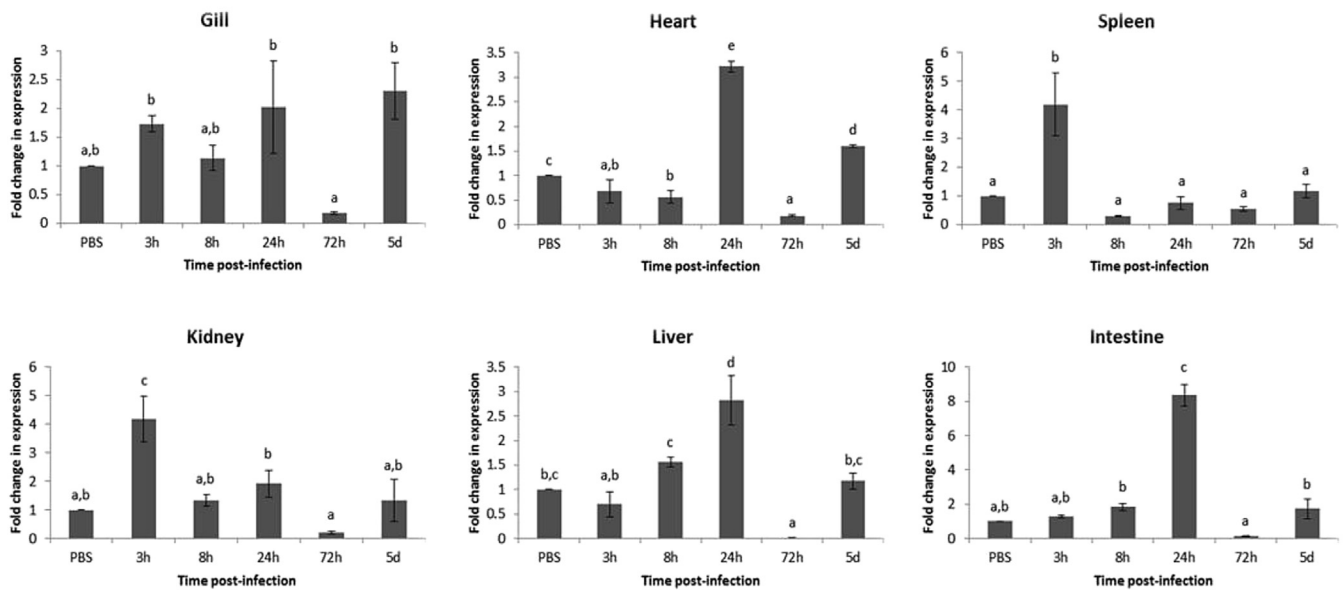
*LGP2* has the highest RNA binding potential among the genes belonging to RLR family, but lacks a signal communicator, CARD at the N-terminal end. So the signalling pathway followed by the *LGP2* to exhibit an antiviral function is not clear. From the available findings on the functional role played by *LGP2* in different animals, it can be speculated that this gene can act as a positive or negative regulator of innate immune system [3,21,63]. The initial reports described *LGP2* as a negative regulator of RLR-signalling. The overexpression of *LGP2* impeded the function of other RLRs in viral detection similar to a mutant exhibiting *RIG-I* without CARD. Even, some of the viruses interact with *LGP2* for a potential inhibition of *RIG-I*-mediated antiviral signalling. Moreover, mutant mice lacking *LGP2* gene showed a more effective immune response against vesicular stomatitis virus [7,64–66]. In contrast, recent studies in different animal models and *in-vitro* cell line study showed a possible positive role played by *LGP2*. Many reports showed that *LGP2* may have a major role in IFN- $\beta$  expression and viral recognition [26,67]. The piscine *LGP2* homologues are highly modulated following poly I:C or viral challenge in different teleosts such as grass carp, flounder, rainbow trout, channel catfish, seaperch and yellow croaker [30–33,35,36,68]. Similarly, in this study, *AsLGP2* expression was highly modulated by poly I:C in the different tissues of *L. calcarifer* or SISK cell line *in vitro*. Among the tissues, kidney, liver and gill, showed relatively high level of *AsLGP2* with kidney



A



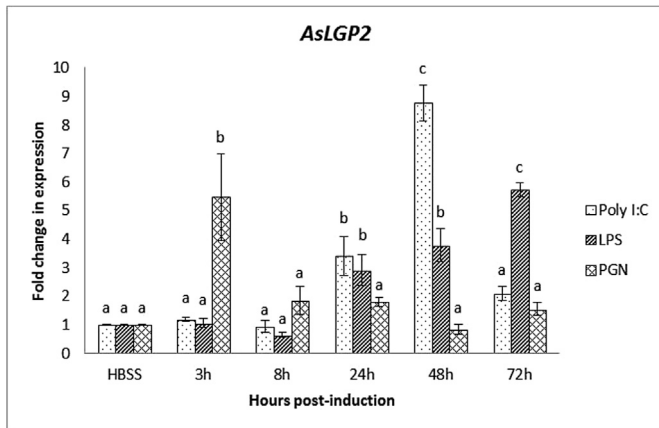
B



**Fig. 7.** Expression profile of *AsLGP2* in various tissues of Asian seabass at different time-points following bacterial challenge with (A) *V. alginolyticus* and (B) *S. aureus* *in vivo*. Different letters indicate significant differences ( $p \leq 0.05$ ) between different time-points within a treatment group compared to control.

showing the highest up-regulation of expression followed by liver. Grass carp challenged with GCRV also showed a similar pattern of expression with the highest modulation of expression observed in kidney and gill [31]. However, in seaperch and yellow croaker, high constitutive expression has been noticed in liver and kidney following NNV infection or poly I:C stimulation [35,36]. Significant up-regulation of *LGP2* in different tissues indicates a positive regulatory role played by the gene in finfish with kidney and liver as the most potent sites of viral RNA recognition. Similar to *in vivo* study, poly I:C induction also resulted in the up-regulated

expression of *AsLGP2* in SISK cell line. However, the magnitude of up-regulation is not comparable with the *in vivo* experiment as the highest fold-change observed was only 8.8 at 48 hpi. In a study in *L. japonicus*, the cells infected with NNV showed a rapid up-regulation with almost 50-fold increase, whereas poly I:C stimulation could only modulate the *LGP2* expression by maximum 2.5 folds. From these different response patterns of *in vitro* model, it can be assumed that the cells as a biological replica of live animals are not as responsive towards synthetic analogue as its live counterpart.



**Fig. 8.** Expression pattern of *AsLGP2* in SISK cell line following induction with poly I:C, LPS and PGN at different time intervals. Different letters indicate significant differences ( $p \leq 0.05$ ) between different time groups within a treatment group compared to control.

There are evidences that RLRs can also mount a cellular immune response against different bacteria and viruses with DNA as nucleic acid rather than the usual viral RNA sensing [34,69]. The mutant *LGP2*-deficient mice infected with the bacterium *Listeria monocytogens* showed enhanced bacterial colonization and reduced production of different cytokines [70]. The antibacterial potential of *LGP2* in fish was earlier elucidated in *I. punctatus* challenged with the intracellular bacterium, *Edwardsiella ictaluri*. A consistently significant up-regulation of *LGP2* expression has been recorded throughout the experiment (till 6 d) [33]. In the current study, injection of Gram-negative intracellular bacterium, *V. alginolyticus*, showed a rapid increase of *AsLGP2* transcript at 3 hpi in almost all the tissues tested except spleen. However, the rapid increase was found to be sustained only up to 8 hpi in most of the tissues except for gill (up to 24 hpi) and heart (up to 5 dpi). Gram-positive bacterium, *S. aureus*, could also transiently modulate *AsLGP2* at certain time-points in different tissues. Similarly, *AsLGP2* responded towards LPS stimulation; but PGN-treated cells showed a significant up-regulation only at 3 hpi *in vitro*. However, the magnitude of up-regulation of *AsLGP2* exhibited by poly I:C-injected animals was found to be relatively higher than those of bacteria-challenged animals. From the previous reports along with the observations made in the present study, it is quite evident that apart from its primary role of sensing viral RNA, *LGP2* can also respond to bacterial infection. However, this response, as reported by Pollpeter et al. (2011), might not be a DNA-mediated response [70]. Further, it has been suggested that *LGP2* can involve in the RNA polymerase III-mediated signalling pathway by recognising the transcription products to trigger antimicrobial response [70]. Nevertheless, further research on the specific antibacterial activity exhibited by RLRs is required to unravel the possible mechanism or molecules involved in this response.

In conclusion, we have cloned and characterized a full-length cDNA sequence of *LGP2* from Asian seabass, *L. calcarifer*. *AsLGP2* was constitutively expressed in the tissues of healthy animals and the expression is highly modulated in poly I:C-treated animals. The ubiquitous expression of *AsLGP2* transcript in embryonic developmental stages suggests a possible maternal transfer of this gene. *AsLGP2* is also found to be responsive towards different bacterial invaders, but this mechanism needs to be elucidated further. From the observations made in this study, it can be concluded that *AsLGP2* may act as a positive regulatory molecule with both antiviral and antibacterial functions, and this molecule could play a significant role in the larval survival against viral infection in Asian seabass.

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