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Short communication

Molecular characterization and expression analysis of the interleukin 1b gene in Pacific cod (*Gadus macrocephalus*)

Biyang Hu[1], Bing Chen[1], Mingguang Mao, Mingkang Chen, Xia Liu, Qianjin Cui, Yang Liu, Chen Jiang*

Key Laboratory of Mariculture & Stock Enhancement in North China's Sea, Ministry of Agriculture, Dalian Ocean University, Dalian, 116023, China

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ABSTRACT

Interleukin 1b (IL-1b) is a member of the cytokine family that serves as major mediators for early pro-inflammatory responses. During artificial breeding of Pacific cod (*Gadus macrocephalus*), outbreaks of NNV in the early development stages could lead to high mortality. Recent research reported that IL-1b participated in the host-virus interaction against NNV infection, however, no IL-1b gene has been identified from Pacific cod so far. In this study, we described the identification and characterization of a IL-1b from Pacific cod transcriptome at both transcript and protein level. Sequence alignment and phylogenetic analysis were conducted to determine and confirm their evolutionary relationship. Expression analysis revealed that IL-1b was expressed in all tested tissues and the western blot analysis confirmed the same expression profiles using purified recombinant IL-1b protein. In addition, significant up-regulation was detected in IL-1b transcripts during early development stages at 30dph and 50dph. After challenged with poly I:C, IL-1b was significantly induced after 24 h. Taken together, these results strongly suggested that IL-1b might play an essential role in innate defense mechanism of Pacific cod.

1. Introduction

Interleukins are a subset of cytokines that were first found as signal transmissions between leukocytes, which play significant roles in initiating and regulating the inflammatory process (Dinarello, 1997). Interleukins are mainly produced by Th cells, lymphocytes, monocytes, macrophages, dendritic cells, natural killer cells, neutrophils, endothelial cells, smooth muscle cells, fibroblasts and many other cell types (Bird et al., 2002b; Ridker et al., 2000; Huising et al., 2004). They can be secreted rapidly in response to stimulus, such as an infectious agent (Park et al., 2005). When interleukins are synthesized, they move and bind to the target cell through the receptor of the cell surface, a cascade of signals within the target cell are then triggered to alter the cell's behavior (Ridker et al., 2000; Schmitz et al., 2005). 37 different types of interleukins are known for now, from interleukin 1 to 37 (Akdis et al., 2011), they are particularly involved in regulating immune responses, physiological functions and pathological process.

Interleukin-1b (IL-1b) is one of most important secretory pro-inflammatory cytokines, which was produced by many different types of cells including blood monocytes and tissue macrophages, neutrophils, B-lymphocytes, T-lymphocytes and cells of the central nervous system (Arend et al., 1989; Dinarello 1997; Huising et al., 2004; Oppenheim et al., 1986). It is a 17-kDa polypeptide derived from 31-kDa biologically inactive precursor prointerleukin 1b (proIL-1b) (Kominato et al., 1995), and the activation was processed by a cysteine protease caspase-1 (previously known as interleukin-1β-converting enzyme or ICE) (Siegmund et al., 2001). The bioactive IL-1b possesses a wide range of biological functions including cell survival, inflammatory, metabolic, physiologic and immunological properties (Okamoto et al., 2010; Wang et al., 2003). Mammalian IL-1b are characterized by a conserved family motif with the consensus pattern: [FC]-x-S-[ASLV]-x (2)-P-x (2)-[FYLIV]-[LI]-[SCA]-T-x (7)-[LIVM], present in the C-terminal region which spans β-sheets 9–11 of IL-1b. IL-1b was initially discovered within mice (Gray et al., 1986; Telford et al., 1986) and humans (Bensi et al., 1987; Nishida et al., 1987), over the last 30 years IL-1b has been characterized within a wide variety of species, including many teleost such as seabass (Scapigliati et al., 2001), gilthead seabream (Pelegrin et al., 2004), haddock (Corripio-Miyar et al., 2007), goldfish, rainbow turbot (Zou et al., 1999a, 1999b).

Pacific cod (*Gadus macrocephalus*) is one of the most important

* Corresponding author.
E-mail address: jiangchen@dlou.edu.cn (C. Jiang).
[1] These authors contributed equally.

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deep-sea marine fish in China, as well as an ecologically important species (Hunt Jr et al., 2002; Laurel et al., 2008; Mao et al., 2015a). A number of studies have been conducted on Pacific cod, such as reproduction biology, early development, and digestive enzyme activities (Wei et al., 2012; Gong et al., 2013; Jiang et al., 2012). Recently years, Pacific cod stock has been threatened due to environmental changes, overfishing and outbreak of infectious diseases. In order to recover the situation, artificial breeding of Pacific cod has been conducted and carried out for 9 years in our lab (Key Laboratory of Mariculture and Stock Enhancement in the North China's sea, Ministry of Agriculture, and Dalian, China). However, the breakout of nervous necrosis viruses (NNV) during early developmental stages led to the high mortality of up to 90% (Mao et al., 2015b). Unfortunately, little was known about the molecular mechanism of innate immunity responses expect for interferon regulatory factor 7 (Sun et al., 2016), rag 1 and Igmu (Mao et al., 2015a). IL-1b has been confirmed to be involved in the immune responses against NNV infection in orange-spotted grouper, suggesting its important role in the antiviral defense (Wu et al., 2012). Therefore, the interleukin gene IL-1b was identified and characterized in this study. In addition, the expression profiles of the IL-1b gene were determined in various healthy tissues, during different developmental stages and after the induction of an interferon inducer polycytidylic acid (poly I: C).

2. Materials and methods

2.1. Database mining, gene identification and sequence analysis

In order to identify IL-1b gene, the previously assembled transcriptome of Pacific cod (Mao et al., 2015a) were searched using all available sequences of IL-1b protein sequences, including human, cattle, mouse, chicken, lizard, zebrafish, fugu, common carp, channel catfish, Atlantic salmon, turbot, Japanese seabass and Atlantic cod retrieved from the NCBI and Ensembl database. TBLASTN was used to pool the initial set of IL-1b sequences for Pacific cod. The retrieved sequences were then used to predict their amino acid sequences by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Furthermore, the predicted amino acid sequences were confirmed by BLASTP against the NCBI non-redundant protein sequences database (NR). The simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/) (Letunic et al., 2012; Schultz et al., 1998) was used to identify conserved domains of IL-1b gene and confirmed by conserved domain prediction from BLASTP. Putative signal peptides were predicted using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The putative isoelectric point was predicted using compute pl/Mw tool (https://web.expasy.org/compute_pi/). Protein molecular weight was predicted by SMS software (http://www.bio-soft.net/sms/index.html).

2.2. Phylogenetic analysis

For further identification of Pacific cod IL-1b gene, phylogenetic analysis was conducted using all the amino acid sequences of IL-1b gene from Pacific cod and selected vertebrate species retrieved from Ensembl and GenBank, including those from human (*Homo sapiens*), cattle (*Bos taurus*), mouse (*Mus musculus*), chicken (*Gallus gallus*), lizard (*Anole lizard*), frog (*Xenopus tropicalis*), zebrafish (*Daino rerio*), tilapia (*Oreochromis niloticus*), medaka (*Oryzias latipes*), fugu (*Takifugu rubripes*), common carp (*Cyprinus carpio*), turbot (*Scophthalmus maximus*), Atlantic salmon (*Salmo salar*), Europen seabass (*Dicentrarchus labrax*), silver carp (*Hypophthalmichthys molitrix*), channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), green spotted pufferfish (*Tetraodon nigroviridis*) and Atlantic cod (*Gadus morhua*). Alignment of multiple IL-1b protein sequences were performed using Muscle v3.8 (multiple sequence comparison by log-expectation) (Edgar, 2004) with default parameters. The phylogenetic tree of IL-1b was then constructed in MEGA7 (Tamura et al., 2011) using Maximum likelihood method. Bootstrapping with 1000 replications was applied to evaluate the phylogenetic tree.

2.3. Sample collection and challenge experiment

Adult Pacific cods were caught from Dalian Lvshun sea area and kept in aerated seawater tanks at 6 °C for one week before sampling. Tissues of healthy fish were collected for the detection of the expression profiles in different tissues, including spleen, kidney, gill, heart, intestine, liver, muscle, and ovary. Pacific cod larvae were sampled from artificial breeding site located at the Key Laboratory of Mariculture Stock Enhancement in Dalian Ocean University. Five to ten larvae were collected in 1.5 ml tube at 1, 5, 10, 20, 30, 40, 50, 60 and 70 day-post hatching (dph) in three replicates. All the samples were flash-frozen in liquid nitrogen and stored at −80 °C for later RNA extraction.

Before the poly I:C challenge experiment, larvae at 12 dph were kept in 200 L tanks at 10 °C with a 12/12 h dark/illumination cycle. The challenge was conducted using Pacific cod larvae artificially bred by our laboratory as previously described (Sun et al., 2016). Briefly, one group of larvae was challenged with poly I:C (60 mg/L), the larvae in this challenge group were collected at 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h post treatment. Meanwhile, untreated age-matched larvae were also collected as controls. All samples were collected in three replicates, flash-frozen in liquid nitrogen, and then stored at −80 °C until RNA extraction.

2.4. RNA extraction and quantitative real-time PCR analysis

Total RNA were extracted from adult tissues and larvae using RNAprep PureTissue Kit (Tiangen Biotech, Beijing, China) following the manufacturer's protocol. RNA concentrations were determined by measuring their absorbance at a wave length of 260 nm, and its quality was monitored using the criterion of A260/A280 > 1.8. First strand cDNA was synthesized by PrimeScipt™ RT reagent Kit with gDNA Eraser (Perfect Real Time) according to manufacturer's instructions. The primers used in quantitative real-time PCR were listed in Table S1 and the β-actin was selected as the internal reference gene based on previous study (Hu et al., 2010). The cDNA products were subjected for quantitative real-time PCR reaction using a 7500 Real Time PCR System (Applied Biosystems, FosterCity, CA) and Top Green qPCR SuperMix (TransGen Blotech, Beijing, China). The quantitative real-time PCR procedure was as follows: 95 °C for 30s, followed by 40 cycles of denaturation at 95 °C for 5s, followed by annealing and primer extension at 60 °C for 30s, respectively.

Data from the quantitative real-time PCR were analyzed using the Relative Expression Software Tool (REST) version 2009 based on the cycle threshold (Ct) values (Pfaffl et al., 2002). For the analysis of gene expression in healthy fish tissues, Ct values of IL-1b gene in intestine was used as control group, β-actin was used as reference for normalization of the relative expression, and the relative expression level of other tissues were then calculated based on the control group using REST software. For the analysis of gene expression in different developmental stages, the relative fold changes in IL-1b gene was determined by comparison with 1 dph with normalization using β-actin as internal reference gene. To determine the relative fold change following challenge with Poly I:C, the expression of IL-1b gene at each time point were compared with the corresponding expression in untreated control groups. For all the statistical analyses, expression differences were considered significant when p-value < 0.05. Quantitative real-time PCR reaction analysis was repeated in triplicate runs to confirm expression patterns.

2.5. Prokaryotic expression and western blot analysis of IL-1b protein expression profiles

According to the IL-1b gene sequence, specific primers (IL-1b-F1, IL-

1b-R1) were designed to amplify the sequence encoding the full-length of IL-1b. *Nde*I and *Xho*I sites were added to both ends of the primers. The amplified fragment was digested by the restriction enzymes *Nde*I and *Xho*I, and then inserted into the expression vector pET-21a. The recombinant plasmid was transformed into *E. coli* BL21 cells for recombinant protein expression. The target protein was induced using IPTG. Then recombinant protein was purified with affinity chromatography method.

The purified recombinant protein was used to immunize rabbits and blood samples were collected, polyclonal antibody was then purified. Detection of anti-serum titer was carried out by enzyme-linked immunosorbent assays (ELISA). Total proteins from all collected healthy Pacific cod tissues were separated for Western blot detection, including spleen, kidney, gill, heart, intestine, liver, muscle, and ovary. These protein samples were analyzed in SDS-PAGE gel (12% separating gel, 5% stacking gel) and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated and immersed in antiIL-1b antibody at a dilution of 1:1000 at 4 °C overnight, followed by incubation at room temperature for 30 min. Then the membrane was incubated in HRP-conjugated goat anti-rabbit IgG (BBI) at a dilution of 1:8000 for 1 h. After washing five times in TBST, the membrane was detected with ECL substrate reagent.

3. Results and discussion

3.1. Gene identification and sequence analysis

The Pacific cod IL-1b gene was successfully identified from the transcriptome database (Mao et al., 2015a) following the strict screening step. The cDNA sequences of IL-1b gene was submitted to GenBank with accession numbers of MH136556. The cDNA sequence of IL-1b contained an open rending frame of 762 bp nucleotide, predicted to encode a 253 amino acid protein (Table S2). The predicted protein sequence of IL-1b contained only one functional domain, named IL-1 domain, which was highly conserved across zebrafish, tilapia, medaka, fugu, platyfish and Atlantic cod (Fig. 1a). In addition, the predicted protein sequence contained an IL-1 family signature motif [FCL]-x-S-[ASLV]-xx-[PRS]-xx-[FYLIV]-[LI]-[SCAT]-T-xxxxxxx-[LIVMK] as in all other teleost (Bird et al., 2002b). As shown in Fig. 1a, no signal peptide was found in all listed vertebrate IL-1b genes, indicating that these secretory molecules are secreted through a non-classical pathway not involving the golgi/endoplasmic reticulum route (Bird et al., 2002b; Rubartelli et al., 1990). In mammals, IL-1b remains inactive until it is proteolytically cleaved and transported out of the cell. Release of the mature IL-1b protein is facilitated by the cleavage of precursor protein after the aspartic acid^{116} in exon 5 by the IL-1b-converting enzyme (ICE) (Huising et al., 2004; Secombes et al., 2011). Similarly, fish IL-1b are also shown to be made as a precursor and cleaved subsequently (Hong et al., 2004; Pelegrin et al., 2004). However, the ICE cut site (aspartic acid) was absent in all known teleost IL-1b genes, leaving the mechanism of cleavage unknown. The predicted translation of IL-1b amino acids showed good homology with other IL-1b genes from the multiple alignment results, whose identity ranged from 27.47% to 48.19% compared to that in Pacific cod, with the highest amino acid identity to Atlantic cod IL-1b. The translated molecule of Pacific cod IL-1b was predicted to be 28.5 kDa, similarly, the full-length IL-1b in trout (Zou et al., 1999a) and carp (Fujiki et al., 2000) is predicted to be 28 kDa (Scapigliati et al., 2001) while the translated molecule of yellow-fin sea bream IL-1b was predicted to give a protein with the MW of 28.5 kDa (Jiang et al., 2008) and Nile tilapia is 27.5 kDa (Lee et al., 2006). According to our experiment, the molecular weight of IL-1b was not shocked to be 28.5 kDa, indicating that the obtained recombinant protein was consistent with our prediction.

3.2. Phylogentic analysis

The construction of phylogenetic tree on IL-1b gene allowed determination of the degree of evolutionary similarity among Pacific cod, other teleost, amphibians, and mammals. As shown in Fig. 1b, the maximum likelihood phylogenetic tree was built using all IL-1b amino acid sequences from various species. Pacific cod IL-1b was placed into a proper clade to form an IL-1b clade. The sequences of IL-1b in mammals and the teleost segregated into two separate clusters with high bootstrap confidence value within their own clade. The further subdivision of the teleost sequences generally followed the established phylogeny: Pacific cod IL-1b was more closely to Atlantic cod IL-1b than to the other teleost, indicating that Pacific cod and Atlantic cod shared close relations through evolution.

3.3. Tissue distribution of IL-1b

The basal expression analyses of IL-1b gene was conducted using both qRT-PCR and western blot in various tissues of healthy Pacific cod (Fig. 2ab), including intestine, muscle, gill, liver, heart, ovary, kidney, and spleen. In healthy fish, the expression of IL-1b mRNA was detected across all tested tissues but with different expression pattern (Fig. 2a). IL-1b was statistically significantly expressed in kidney and spleen compared to that in intestine, and lower expression were detected in other six tested tissues, while the highest expression was observed in the spleen with extremely high fold change. To characterize the tissue distribution of Pacific cod IL-1b protein, specific antibodies were prepared in rabbit immunized with the purified recombinant protein. Western blotting revealed that the rabbit anti-IL-1b serum reacted with the purified Pacific cod IL-1b protein in each tested tissue (Fig. 2b), indicating that the expression of IL-1b protein was consistent with those of its transcript. Similar expression profiles were also observed in many fish species, even though no basal expression of IL-1b observed in some mammals or fish species (Corripio-Miyar et al., 2007; Zou et al., 1999b). In carp, constitutive in vivo expression of IL-1b mRNA was found by qRT-PCR in head kidney, spleen, gill and brain, and confirmed by in situ hybridizations with specific DIG-labelled anti-sense probes in the head kidney (Engelsma et al., 2001). Basal expression of IL-1b was also detected in gilthead seabream (*Sparus aurata* L.) from most of the healthy tissues (Engelsma et al., 2001), including brain, peritoneal exudate, blood, head kidney and gill. The two tandemly duplicated copies of IL-1b genes in channel catfish exhibited constitutive expression from various normal tissues (Wang et al., 2006), however, different expression profiles were detected between the two genes. IL-1ba was expressed highly in the liver, head kidney, spleen, intestine, and muscle, but its expression was lower in the stomach, brain, ovary, skin, and trunk kidney. While IL-1bb was expressed uniformly in all tested tissues except that it was expressed at lower levels in the brain.

3.4. Transcription profiles of IL-1b gene during early development period

In order to reveal the roles that IL-1b took during Pacific cod early development period, the expression of IL-1b gene was examined in eight early development periods, including 1, 10, 20, 30, 40, 50, 60, and 70 dph using quantitative real-time PCR. As displayed in Fig. 2c, IL-1b gene was expressed throughout the early development states, and significant up-regulation was observed at 30 dph and 50 dph. The IL-1b gene was induced the most at 30 dph with 22.02-fold, and decreased its expression to 6.30-fold at 50 dph. A number of researches have shown that innate immune system play important roles in early developmental stage of vertebrates (Mulero et al., 2007). When zebrafish infected with *Edwardsiella tarda* during embryotic stage, increased expression was detected and reached its peak at 4 h post exposure (Pressley et al., 2005). IL-1b in orange-spotted grouper metamorphosis-stage larvae was reported to be significantly elevated after nervous necrosis virus infection, and was positively correlated with viral content (Wu et al.,

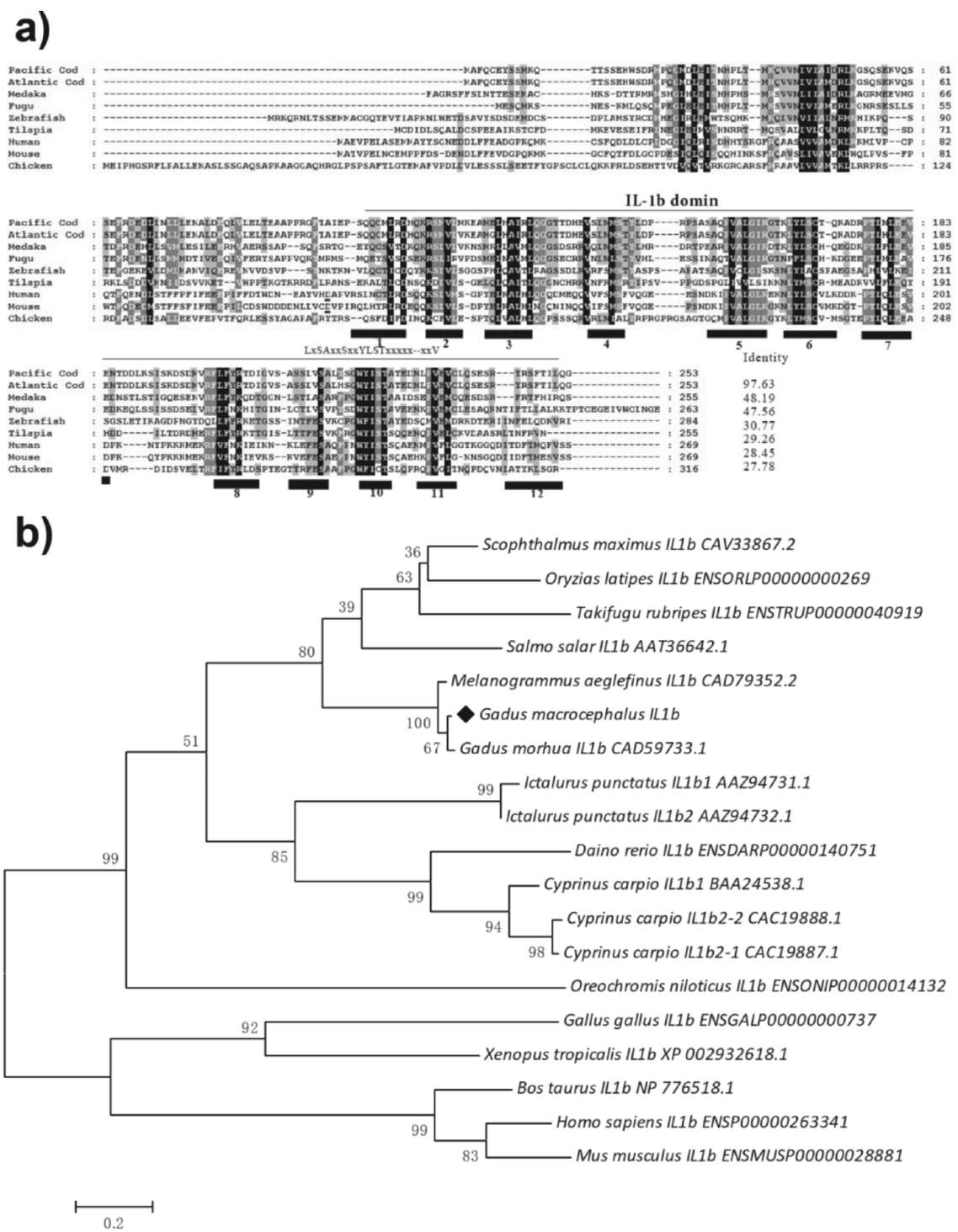

Fig. 1. Molecular characterization and phylogenetic analyses of IL-1b. (a) Multiple alignment of IL-1b amino acid sequences. The amino acid alignment was generated by Clustal Omega and further edited in GeneDoc. Shading was performed using the conserved mode (black, dark, grey, light grey shading set to 100%, 80%, 60% amino acid conservation, respectively). The ICE site (aspartic acid) in all the mammalian sequence were underlined. The 12 β-sheets predicted in IL-1b were marked with bars below the alignment and numbered from 1 to 12. The GenBank accession number of the IL-1b protein sequences used in the alignment were: Atlantic cod, CAD59733; Medaka, ENSORLP00000000269; Fugu, ENSTRUP00000040919; Zebrafish, ENSDARP000000140751; Tilapia, ENSONIP00000014132; Human, ENSP00000263341; Mouse, ENSMUSP00000028881. Chicken, ENSGALP00000000737. (b) Phylogenetic tree of IL-1b members constructed using amino acid multiple alignments. The phylogenetic tree was constructed using the Muscle and MEGA 7 software packages and was bootstrapped 1000 replications. The number on each node represented bootstrap probability. The accession number for each amino acid sequences were provided after the species name. Pacific cod IL-1b molecule identified in this study was marked with a black rhombus sign.

2012). Consistent with the observations above, IL-1b gene in Pacific cod was also induced in the early development stages, the highest expression appeared at 30dph, following with 50dph, suggesting drastic immune responses were emerged during these two time points. This observation conformed with our artificial breeding trial (Mao et al., 2015b), where large outbreaks of NNV were taken place around 30dph

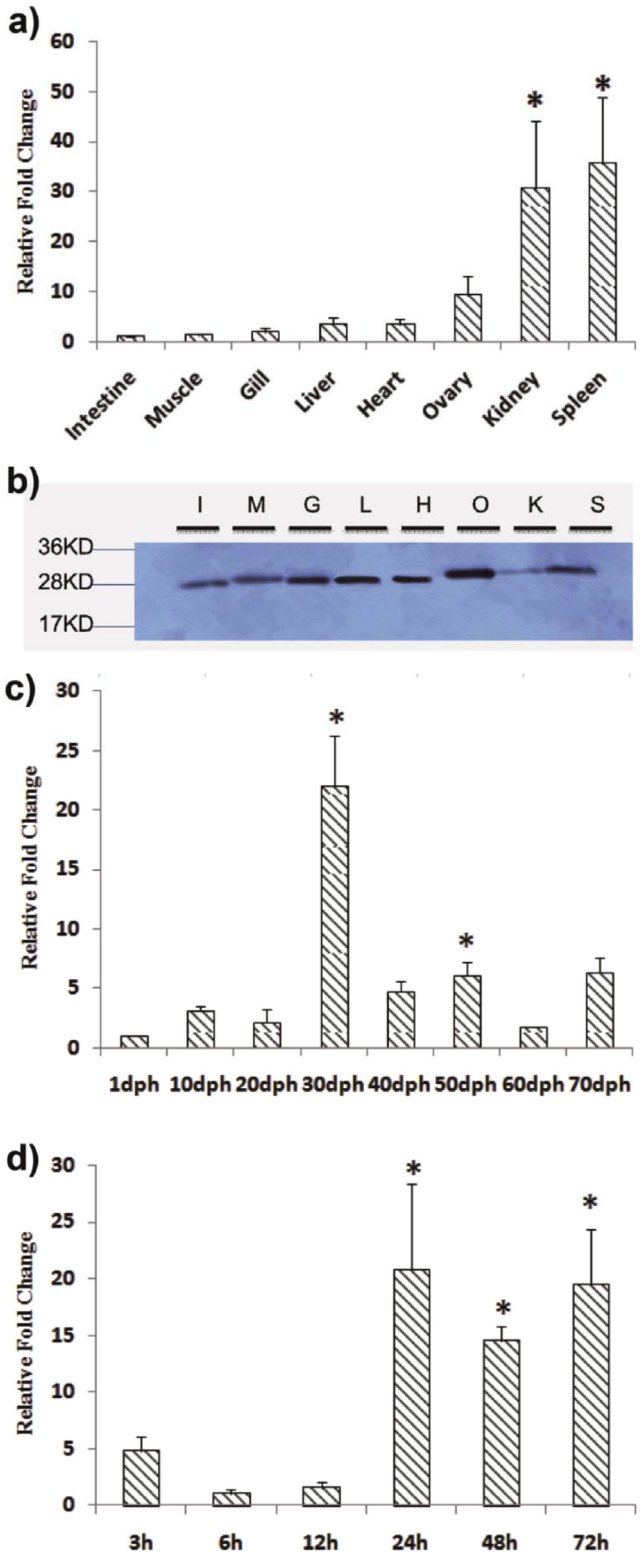

Fig. 2. Expression analysis of Pacific cod IL-1b gene. (a) Expression of IL-1b gene was normalized to that of the housekeeping gene, β-actin. Relative expression values were calculated using intestine expression value as reference. Asterisks indicated statistical significant difference ($p < 0.05$). (b) Western blot analysis of the expression profiles of Pacific cod IL-1b in normal tissues. The selected healthy tissues including I (intestine), M (muscle), G (gill), L (liver), H (heart), O (ovary), K (kidney), and S (spleen). (c) Expression analysis of IL-1b during early development period. Quantitative real-time PCR was used to detect expression profiles from 1dph to 70 dph. The expression level of IL-1b was presented as mean ± SE of fold change as normalized with the housekeeping gene, β-actin. Asterisks indicated statistical significant difference ($p < 0.05$). (d) Expression analysis of IL-1b following poly I:C challenge. Quantitative real-time PCR was used to detect expression profiles at the time points of 3 h, 6 h, 12 h, 24 h, 48 h and 72 h post challenge. The expression level of IL-1b was presented as mean ± SE of fold change after poly I:C challenge to the control as normalized with β-actin. Asterisks indicated statistical significant difference ($p < 0.05$).

and 50dph, indicating that the enhancement of IL-1b gene expression might give rise to survival of the viral attack.

3.5. Transcription profiles of IL-1b gene upon stimulation with poly I: C

In mammals, IL-1b is produced in response to many stimuli, which include bacterial, viral, numerous microbial products, cytokines and immune complexes (Secombes et al., 2011). To determine the antiviral roles of IL-1b genes in Pacific cod larvae, expression patterns of IL-1b gene was investigated followed by poly I:C stimulation. As shown in Fig. 2d, IL-1b was not significantly induced until 24 h after poly I:C challenge, the level of the IL-1b induction remained significant in the latter two time points with slight fluctuation. The highest expression was detected after 24 h with 20.73-fold, declined to 14.54-fold after 48 h and went up to 19.43-fold after 72 h. In *Xenopus*, IL-1b was induced in vivo following injection with LPS, its expression was clearly detectable by RT-PCR in kidney, liver and spleen, with the lowest expression level observed in brain (Zou et al., 2000a). In rainbow trout, IL-1b was expressed in head kidney leucocytes and isolated macrophages after stimulation with LPS for 4 h (Zou et al., 1999b, 2000b). IL-1b was also shown to be induced following injection with LPS in small spotted catshark (*Scyliorhinus canicula*) using RT-PCR in the spleen and testes, tissues known to contain a variety of leucocytes (Bird et al., 2002a). The expression of the IL-1b gene in haddock was discovered in the head kidney, spleen, liver and gill by using RT-PCR, however, the results showed that the IL-1b was not constitutively expressed in the non-stimulated fish. The haddock IL-1b was not expressed constitutively, the fish have to be stimulated to induce the expression of this gene. A significant induction was found in head kidney, spleen and gill in haddock using different treatments, while its expression was stimulant dependent (Corripio-Miyar et al., 2007). Overall, these results showed that the interleukin-1b gene is up-regulated in response to poly I:C in Pacific cod, but its role during the immune response should be further investigated.

4. Conclusion

Overall, the present study characterized an interleukin gene, IL-1b in Pacific cod. Our results suggested that IL-1b shared high homology with the corresponding genes from various fish. IL-1b was detectable in all examined tissues during transcription and translation. It was also highly induced in vital developmental periods and after poly I:C challenge. Our results collectively indicated that IL-1b was a crucial antiviral-related gene involved in innate immune response, these finding could provide new strategy for artificial breeding of Pacific cod.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://

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