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Characterization of E3 ubiquitin ligase neuregulin receptor degradation protein-1 (Nrdp1) in the large yellow croaker (*Larimichthys crocea*) and its immune responses to *Cryptocaryon irritans*

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ABSTRACT

Neuregulin receptor degradation protein-1 (Nrdp1) was recently identified in humans as an important immune factor responding to the challenge of virus, LPS or cytokine. Its role in fish immune defense and whether it is involved in anti-parasite immunity have not been proven yet. In this report, the full-length cDNA sequence and genomic structure of Nrdp1 in the large yellow croaker *Larimichthys crocea* (LcNrdp1) were identified and characterized. The full-length cDNA of LcNrdp1 was 1248 bp, including a 5′ untranslated region (UTR) of 32 bp, a 3′ UTR of 259 bp and an open reading frame (ORF) of 937 bp, encoding a polypeptide of 318 amino acid residues. The full-length genomic DNA sequence of LcNrdp1 was composed of 2635 nucleotides, including four exons and three introns. The putative LcNrdp1 protein had no signal peptide sequence and contained a characteristic Nrdp1 consensus motif C_3HC_3D ring finger and a Coiled-coil domain. Phylogenetic analysis showed that Nrdp1 in fish was closer with that in other vertebrates (79%–90% amino acid identity) than in invertebrates and bacteria (27%–65%). In fishes, Nrdp1 in large yellow croaker was closer with that in *Takifugu rubripes*. The expression profile showed that LcNrdp1 was constitutively expressed in all tested tissues, especially highly expressed in brain, muscle and kidney. Post-infection (PI) with *Cryptocaryon irritans*, an increased expression of LcNrdp1 was induced in infection sites (skin and gill), whereas in immune organs, the expression of LcNrdp1 was up-regulated in spleen (except the 1st d and 10th d PI) but suppressed in head kidney. These results suggested that LcNrdp1 might play an important immune role in the finfish *L. crocea* in the defense against the parasite *C. irritans*.

1. Introduction

Neuregulin receptor degradation protein-1 (Nrdp1), an E3 ubiquitin ligase, also referring to as ring finger protein 41(RNF41) or fetal liver ring finger (FLRF), belongs to the family of single ring finger-containing proteins (Wauman et al., 2011). It has been demonstrated to be associated with cell growth or development (Diamonti et al., 2002; Qiu and Goldberg, 2002), apoptosis (Qiu et al., 2004; Zhong et al., 2005) and oxidative stress (Zhang et al., 2011; Yu and Zhou, 2008). More recently, Nrdp1 was found to be a key immunity factor. In innate immune response, Nrdp1 negatively regulated MyD88-dependent activation of NF-kB and AP-1, but it potentiated TRIF-dependent activation of TBK1 and IRF3 in the TLR response. Eventually, Nrdp1 inhibited TLR-induced production of pro-inflammatory cytokines. However, it promoted the production of type I interferon in macrophages. Nrdp1 transgenic (Nrdp1-TG) mice showed resistance to LPS-induced liver injury and vesicular stomatitis virus (Wang et al., 2009). Moreover, Nrdp1 interacted with transcriptional factor C/EBPβ and enhanced C/EBPβ-triggered transcriptional activation of the Arg1 reporter gene in M2 Macrophage. In addition, other distinctive M2 markers, such as Fizz1, Ym1, and MR, were also greatly up-regulated in Nrdp1-TG peritoneal macrophages after IL-4 stimulation (Ye et al., 2012). In adaptive immune response, Nrdpl regulates the function of CD8 + T cells, and thus may be involved in the pathogenesis of autoimmune diseases mediated by T cells, and the mechanisms are different to the other E3 ubiquitin ligases (Yang, 2012). These studies showed that Nrdp1 is active in responses to virus, LPS and cytokine stimulus. Yet whether Nrdp1 can respond to parasites infection is not reported to date.

The culture industry of large yellow croaker, *Larimichthys crocea*, one of the most commercially important marine finfish in China (Liu and

Abbreviations: Nrdp1, neuregulin receptor degradation protein-1; LcNrdp1, *Larimichthys crocea* Nrdp1; Nrdp1-TG, Nrdp1 transgenic; RNF41, ring finger protein 41; FLRF, fetal liver ring finger; EAE, experimental allergic encephalomyelitis; UTR, untranslated region; ORF, open reading frame; cDNA, DNA complementary to RNA; DNase, deoxyribonuclease.

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Han, 2011), has suffered significant economic losses due to the "marine white spot disease", one of the most damaging diseases in mariculture, caused by an obligate parasitic ciliate protozoan, *Cryptocaryon irritans* (Colorni, 1987; Yambot et al., 2003; Wright and Colorni, 2002a). This parasite has become a frequent and most devastating pest in marine fish farms (Colorni and Burgess, 1997; Wright and Colorni, 2002b). It incurred the outbreak of epidemic disease for marine cultured fish, especially for fish seedling, leading to the infection rate and mortality of the fish up to 100% (Wang et al., 2010; Li et al., 2011a, 2011b, 2014a, 2014b; Zuo et al., 2012; Niu et al., 2013). Due to its huge damage, the Ministry of Agriculture of the People's Republic of China classified this disease, namely, the white spot disease, as a Class II animal epidemic disease in 2008 (Ministry of Agriculture of the People's Republic of China, 2008). Therefore, it is crucial to develop effective methods to control *C. irritans*, and improving fish's own defense system is thought to the best choice because of its safety, low cost and negligible environmental impact.

Nrdp1 has been identified in humans, mice and other vertebrates, and its immune functions have been deeply investigated. Although a variety of fish Nrdp1 genes in fish genomes can be retrieved from the GenBank database, only two fish Nrdp1 genes were reported. A study showed that Nrdp1 from zebrafish was involved in differentiation of the melanocyte lineage (Maddirevula et al., 2011). Nrdp1 from grass carp was reported but its function was not investigated yet (Wei et al., 2013). Therefore, it is worthwhile to identify whether Nrdp1 participates in fish immune responses to parasite infection or not.

In this study, the full-length cDNA and the genomic structure of Nrdp1 in large yellow croaker (LcNrdp1) were investigated and its expression in healthy fish tissues has been analyzed as well. To better understand its potential role in fish immune responses, especially in response to parasite infection, the expression profiles of the gene were investigated post-infection with *C. irritans* as compared with the control.

2. Materials and methods

2.1. C. irritans tomont collection and theront incubation

Ten large yellow croakers seriously infected with *C. irritans* were bred in a small aerated cement pond, filled with a little water in it. Plates were placed on the bottom of the cement pond. The plates were lightly picked out when they were full of tomonts two days later. Subsequently, the tomonts were transferred into several beakers and incubated in a dark room for 2.5 days, with temperature of 25–26 °C. The density of theronts incubated from the tomonts was detected under an optical microscope.

2.2. Infection of large yellow croaker with C. irritans and sample collection

Healthy large yellow croakers (130 g ± 15 g) were obtained from the Fishery Extension Station of Ningde, Fujian Province, China. The fish were kept in a cement pond full of oxygen, at salinity of 25–26 psu and temperature of 23–26 °C, fed with commercial feed. The water was exchanged twice a day. The fish were acclimatized for 10 days prior to sampling.

The large yellow croakers were infected with *C. irritans* as described by Li et al. with minor modification (Li et al., 2011a). Ninety healthy large yellow croakers were exposed to *C. irritans* at a dose of 25,000 theronts per fish in 1000 L seawater for 6 h, and subsequently transferred into a new cement pond containing fresh seawater. The water was exchanged twice a day. The fish were observed daily for the presence of trophonts. On days 3–4 post-infection, trophonts emerged out from the host and formed tomonts which released theronts three days later. To avoid the auto-reinfection, the fish were transferred into a new pond at day 3 post-infection. At day 7 post primary infection, the fish were transferred into a new pond again and infected with the same dose and method as primary infection. Four challenged large yellow croakers were selected randomly and sacrificed at 6 h, 12 h, 1 d, 2 d, 3 d, 5 d, 7 d, 10 d and 14 d after primary infection. Then the skin and gill where the trophonts infected, as well as immune organs, spleen and head kidney, were collected for mutational expression analysis. The samples of the negative control group that were uninfected with *C. irritans* were also collected at the same time points. All samples were stored in liquid nitrogen until the RNA isolation started.

2.3. RNA isolation and cDNA synthesis

The total RNA was extracted from each tissue using Trizol reagent (Invitrogen) following the manufacturer's protocol. Total RNA was incubated with RNase-free Dnase (Promega, China) to remove genomic DNA. Subsequently, the first strand cDNA was synthesized using 1 μg total RNA and oligodT primer by PrimeScript Reverse Transcriptase (TaKaRa) according to the manufacturer's instructions.

2.4. Cloning of LcNrdp1 cDNA from the large yellow croaker

Partial cDNA sequence of Nrdp1 was obtained from the mixed tissue transcriptome database (unpublished data). To obtain the full length cDNA of Nrdp1, gene-specific primers (Table 1) for 3′ rapid amplification of cDNA ends (RACE-PCR) were designed based on the partial cDNA sequence. The 3′-end RACE PCR was performed with spleen cDNA template from the infected large yellow croaker using the gene-specific primer Nrdp13F1 and the adapter primer AOLP for the first round followed by nested PCR using primers Nrdp13F2 and AP. The first round PCR was carried out at 94 °C 2 min, 5 cycles at 94 30 s and 72 °C 2 min, 5 cycles at 94 30 s, 70 °C 30 s and 72 °C 2 min, 25 cycles at 94 °C 30 s, 66 °C 30 s and 72 °C 1 min, followed by an terminal extension for 10 min at 72 °C. Then the nested PCR was performed with 94 °C 2 min, 30 cycles at 94 °C 30 s, 68 °C 30 s and 72 °C 1 min, followed by a terminal extension for 10 min at 72 °C. The PCR product was cloned into pMD18-T vector (TaKaRa) and sequenced (Invitrogen).

To confirm the full cDNA sequence of LcNrdp1, two primers Nrdp1F/R (Table 1) for RT-RCR were designed based on the known cDNA sequence. PCR was performed at 94 °C 2 min, 30 cycles at 94 °C 30 s, 57 °C 45 s and 72 °C 1 min, followed by a 10 min extension at 72 °C. The RT-RCR products were sequenced as above.

2.5. Analysis of the genomic sequence of LcNrdp1 gene

To obtain the genomic structure and splicing patterns of LcNrdp1, the cDNA sequence of LcNrdp1 has been aligned to the assembled genomic sequence of the large yellow croaker in our lab (unpublished data) by blast program (Kent, 2002). By the standard of the longest intron less than 750,000 bp, we analyzed and visualized the genomic structure and potential splicing site of the cDNA sequence by identifying exons and introns from the genome sequence. For multiple sequence alignment, we

Table 1
Primers used for LcNrdp1 cloning and expression analysis.

Primers	Sequence (5′–3′)	Purpose
Nrdp13F1	CAGAGTCACACGCTGGGGAGGCAT	3′ RACE method
Nrdp13F2	GCGCACGGAGTGGAGGAGATCTTA	
AOLP	GGCCACGCGTCGACTAGTACTTTTTTTTTTT	General primers for RACE
AP	GGCCACGCGTCGACTAGTAC	
Nrdp1F	TCGTGGCTCCCAGTGATGTAC	cDNA sequence
Nrdp1R	ACTTGTCTCTGGATCAGCTACG	identification
Nrdp1QF	CAGGCGGTGGTGGTGATG	mRNA expression
Nrdp1QR	AACCTACTCATCACAGCAAACCC	
β-Actin-F	TTATGAAGGCTATGCCCTGCC	mRNA expression
β-Actin-R	TGAAGGAGTAGCCACGCTCTGT	

compared the coverage ratio of the cDNA, and ultimately selected the result with the highest homology identity.

2.6. Amino acid sequence analysis and construction of a phylogenetic tree

Sequence similarity analysis was performed using BLAST program at the website of National Center of Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The protein domain features were predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). The signal peptide was predicted using the signal 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignment was performed using the CLUSTALX program (http://www.ebi.ac.uk/clustaw/). A phylogenic tree was constructed using the MEGA5.0 program.

2.7. Tissue expression analysis of LcNrdp1

Tissue-specific expression profiles of LcNrdp1 in various tissues including the brain, heart, gill, liver, spleen, kidney, head-kidney, stomach, intestine, skin, muscle, and blood cell were analyzed by Real-time PCR, and mutational expression in the skin, gill, spleen, and head-kidney that were challenged with *C. irritans* was analyzed by Real-time PCR as well. Nrdp1 gene-specific primers Nrdp1QF/R (Table 1) were used to amplify 188 bp fragments by reverse transcript PCR. Then the PCR product was sequenced to verify the specificity of RT-PCR. β-Actin was amplified as an internal control to determine the concentration of each template with the primers β-actin-F/R (Table 1). PCR reactions without the addition of the template were used as blank control. Each reaction was performed in triplicate. Real-time PCR was performed on ABI 7500 Real-time Detection System (Applied BioSystems, USA) using SYBR Green I Real-time PCR Master Mix (TOYOBO, Japan). PCR cycling conditions were 95 °C for 1 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. After the PCR program, data was analyzed with ABI 7500 SDS software. To maintain consistency, the baseline was set automatically by the software. The relative mRNA expression of Nrdp1 compared to the reference gene was calculated with the $2^{-\Delta\Delta CT}$ method. Two-tailed Student's *t*-test was used for the significance test between the experimental group and the control group. Data was expressed as mean ± SE. The P-value of <0.05 was considered significantly different in statistic.

3. Results

3.1. Characterization of LcNrdp1 cDNA

A 986 bp cDNA fragment was obtained from our transcriptome database, showing 99% identity with that of *Oreochromis niloticus* and *T. rubripes* based on blastx analysis. Based on the above cDNA fragment, a fragment of 286 bp was amplified through 3′ RACE PCR. Subsequently, a 1014 bp fragment was obtained by RT-RCR with primers Nrdp1F and Nrdp1R, which was consistent with the sequence in our database. Finally, a 1248 bp complete cDNA of the large yellow croaker Nrdp1 (LcNrdp1) was assembled with a polyA tail (accession number: KJ748455). BLASTX

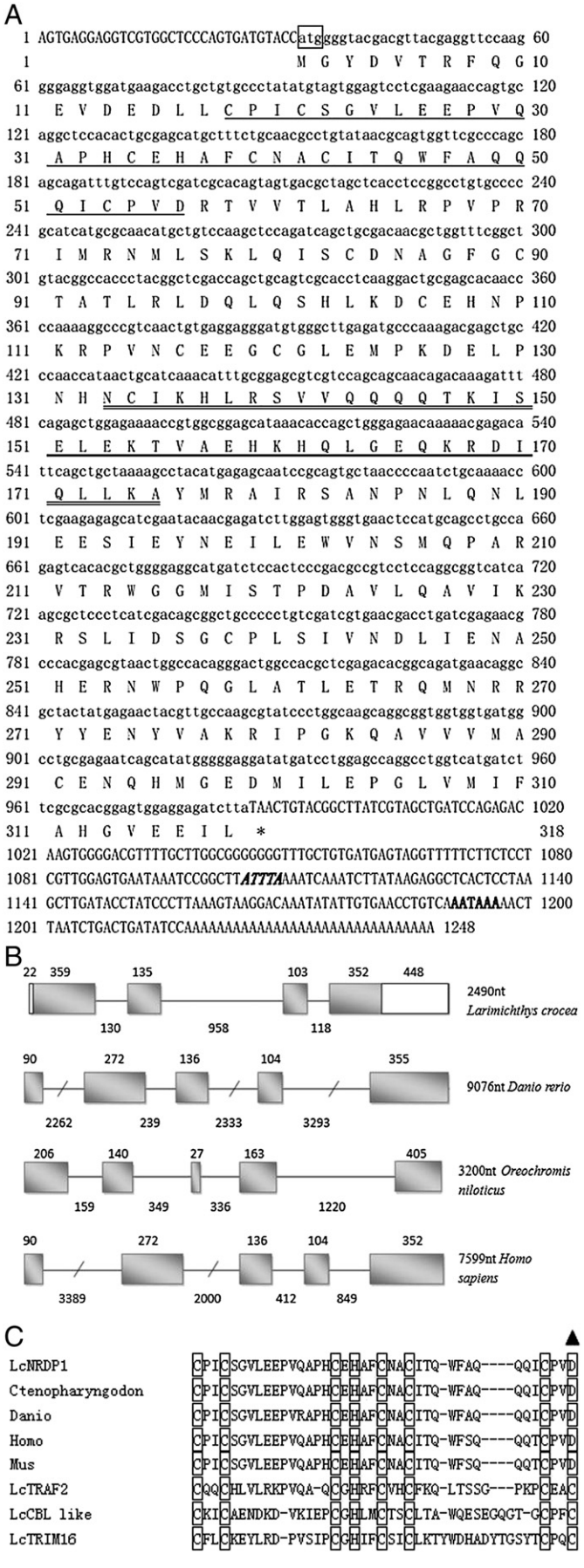

Fig. 1. Structural features of LcNrdp1 genes. (A) Nucleotide sequence and deduced amino acid sequence of LcNrdp1 cDNA. The start codon (ATG) was boxed and the stop codon (TGA) was represented with *. The polyadenylation signal motif (AATAAA) was in bold. The motif associated with mRNA instability (ATTTA) was in bold italic. The ring finger motifs were underlined (18–56 aa). The Coiled-coil domain was double underlined (133–175 aa). (B) Genomic structure of LcNrdp1. Exons were represented by boxes, shaded areas represent the coding region of the gene. Introns were indicated with horizontal lines and lengths in base pairs were shown on the bottom of each line and exon lengths are on the top. (C) The ring finger domains of Nrdp1 from *Larimichthys crocea*, *Ctenopharyngodon idella*, *Danio rerio*, *Homo sapiens* and *Mus musculus* were aligned with TRAF2, TRIM16 and Cbl like from *Larimichthys crocea*. The eight residues forming the ring finger domains were boxed. Point mutation was indicated by arrow heads above the alignment. TRAF2, TRIM16 and Cbl like gene from *L. crocea* were cloned by our team, and the accession numbers were not opened.

analysis suggested that LcNrdp1 is homologous with its counterparts in other fishes.

The full-length cDNA sequence of LcNrdp1 contained an open reading frame of 957 bp that encoded 318 amino acid residues, a 5′ untranslated region (UTR) of 32 bp, and a 3′ UTR of 259 bp including a consensus polyadenylation signal (AATAAA) at the 1191 bp downstream of the stop codon (TAG), a putative ATTTA instability sequence and a polyA tail (Fig. 1A).

3.2. Identification of LcNrdp1 genomic organization

A 2635 bp genomic DNA was obtained through mapping LcNrdp1 cDNA to the genome of the large yellow croaker. Comparison of the cDNA and genomic sequences, the Nrdp1 genome sequence consisted of four exons (381 bp, 135 bp, 103 bp, 807 bp) and three introns (130 bp, 958 bp, 118 bp) from the initial codon (Fig. 1B). The 5′ and 3′ terminals of the introns showed the typical splicing motifs (GT/intron/AG). The full length genomic sequence of LcNrdp1 was deposited to GenBank with accession number of KJ748456.

3.3. Amino acid sequence comparison and evolutionary analysis

The calculated molecular mass of deduced LcNrdp1 polypeptide was 36.156 kDa and the isoelectric point was 5.76. Prediction of protein domains by the signal 4.1 program revealed that LcNrdp1 had no signal peptide, so it was a non-secreted protein. The alignment of LcNrdp1 with closely related sequences and SMART prediction revealed a characteristic Nrdp1 consensus motif C_3HC_3D containing a ring finger domain (17–56 aa) in N-terminal and a predicted Coiled-coil domain (133–175 aa) in C-terminal (Fig. 1A). The ring finger domain of LcNrdp1 closely resembled those of TRAF2, TRIM16 and Cbl like, but the eighth conserved residue in the Ring of Nrdp1 was Asp (D56) instead of the usual Cys (Fig. 1C).

Phylogenetic analysis revealed that the large yellow croaker was clustered with other fish species and had the closest phylogenetic relationship with *T. rubripes* based on the amino acid sequences of Nrdp1 (Fig. 2). Nrdp1 was widely distributed in most living organisms, ranging from bacteria to parasites, insects, coelenterates, echinoderms, shellfishes, fishes, birds, amphibians and mammalians, based on their phylogenetic relationship as revealed by the phylogenetic tree which was consistent with their taxonomic classification. The observed relationships within this cluster reflected the relative taxonomic positions of the species.

3.4. Tissue expression profile of LcNrdp1

QPCR analysis was performed to investigate the mRNA expression pattern of LcNrdp1 in healthy fish tissues. As shown in Fig. 3, LcNrdp1 mRNA was expressed in all tested tissues, with the highest level in the brain, followed by blood, muscle and kidney. The lowest expression level of LcNrdp1 was detected in the stomach.

3.5. Expression modulation of LcNrdp1 under the C. irritans infection

3.5.1. Clinical signs of the large yellow croaker exposure to C. irritans

On the 2nd day post-collection of the tomonts, the bottom of plates was full (Fig. 4A–B). During the subsequent infection, white spotted trophonts were observed on the fins of the large yellow croakers (Fig. 4C), which can be obviously observed under a microscope (Fig. 4D). Moreover, white spots spread on the whole body of the fish after infection (Fig. 4E). The clinical symptoms demonstrated that the large yellow croakers were successfully infected with *C. irritans*.

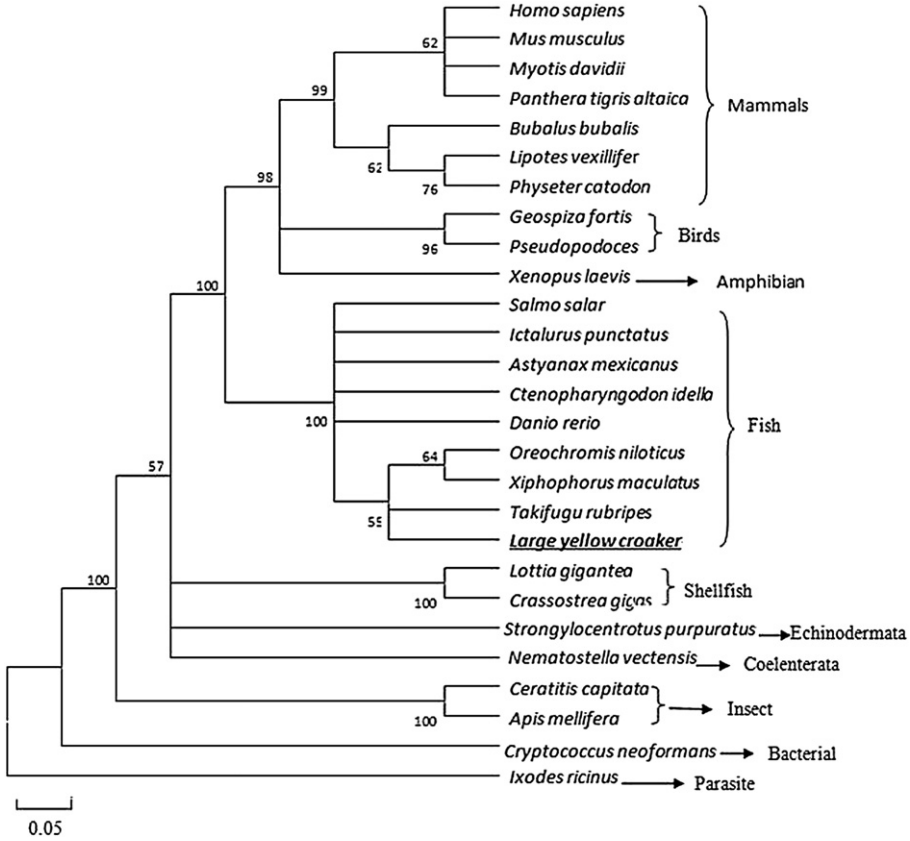

Fig. 2. Phylogenetic tree of LcNrdp1 sequences. Complete amino acid sequences were aligned by using Clustal X and the tree was constructed with neighbor-joining method in MEGA5.0 and a bootstrap analysis was performed using 1000 replicates to test the relative support for particular clades. GenBank accession numbers of these genes are listed in Table 2. The scale bar is 0.05.

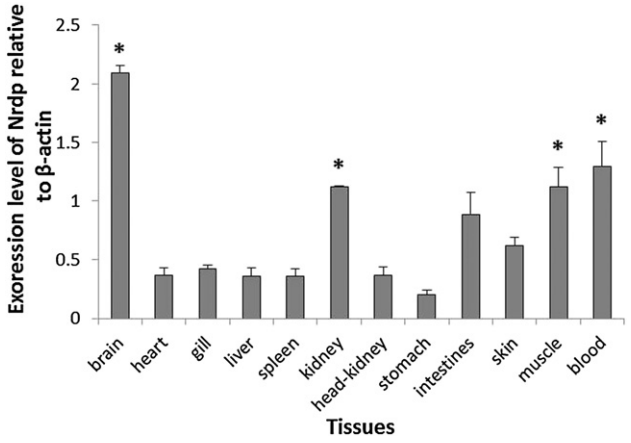

Fig. 3. Expression profiles of Nrdp1 in various tissues of the healthy large yellow croaker by Real-time PCR. The mRNA expression level was normalized against β-actin. Each experiment was performed in triplicate. Data (mean ± SE, n = 4) are indicated with significant difference (*, $P < 0.05$).

3.5.2. Expression profiles of LcNrdp1 mRNA after C. irritans challenge

To understand the impact of *C. irritans* infection on LcNrdp1 expression, Real-time PCR was applied to detect the changes of LcNrdp1 expression levels of the infected skin and gill, and the immunity organs spleen and head kidney of the large yellow croaker after *C. irritans* challenge. Transcriptional regulation of LcNrdp1 after infection in skin was shown in Fig. 5A. Overall, LcNrdp1 gene expression levels showed an increased trend from 6 h to 14 d post-infection (PI), and significantly up-regulated at 12 h (3.4 fold), 2 d (4.3 fold), 3 d (7.7 fold) and 7 d (8.5 fold) ($P < 0.05$) PI as compared to the control. In the gill (Fig. 5B), LcNrdp1 transcripts were up-regulated from 6 h to 7 d post the primary infection, with the expression levels rapidly reaching higher levels at 6 h (3.5 fold) and 12 h (2.7 fold) as compared with their controls, respectively. Subsequently, the expression levels were down-regulated at days 10 and 14 when *C. irritans* re-infected. In the spleen (Fig. 5C), LcNrdp1 transcripts showed a significant increase at 6 h and 12 h after primary infection with a peak value of 5.2 times at 6 h as much as the control group, and temporarily down-regulated at 1 d followed by up-regulation until the 7th day when the first infection ended. Interestingly, the LcNrdp1 transcript was down-regulated at the second infection, the 10th day, and then, up-regulated again at the 14th day. In the head kidney (Fig. 5D), LcNrdp1 transcripts were down-regulated at most time points post-infection, and reached the significantly lower points at 6 h (49%), 12 h (45%), 5 d (22%), 10 d (36%), and 14 d (32%) as compared to the control.

4. Discussion

In the present study, LcNrdp1 was cloned and characterized. The full-length cDNA of LcNrdp1 was 1248 bp, including an ORF of 957 bp

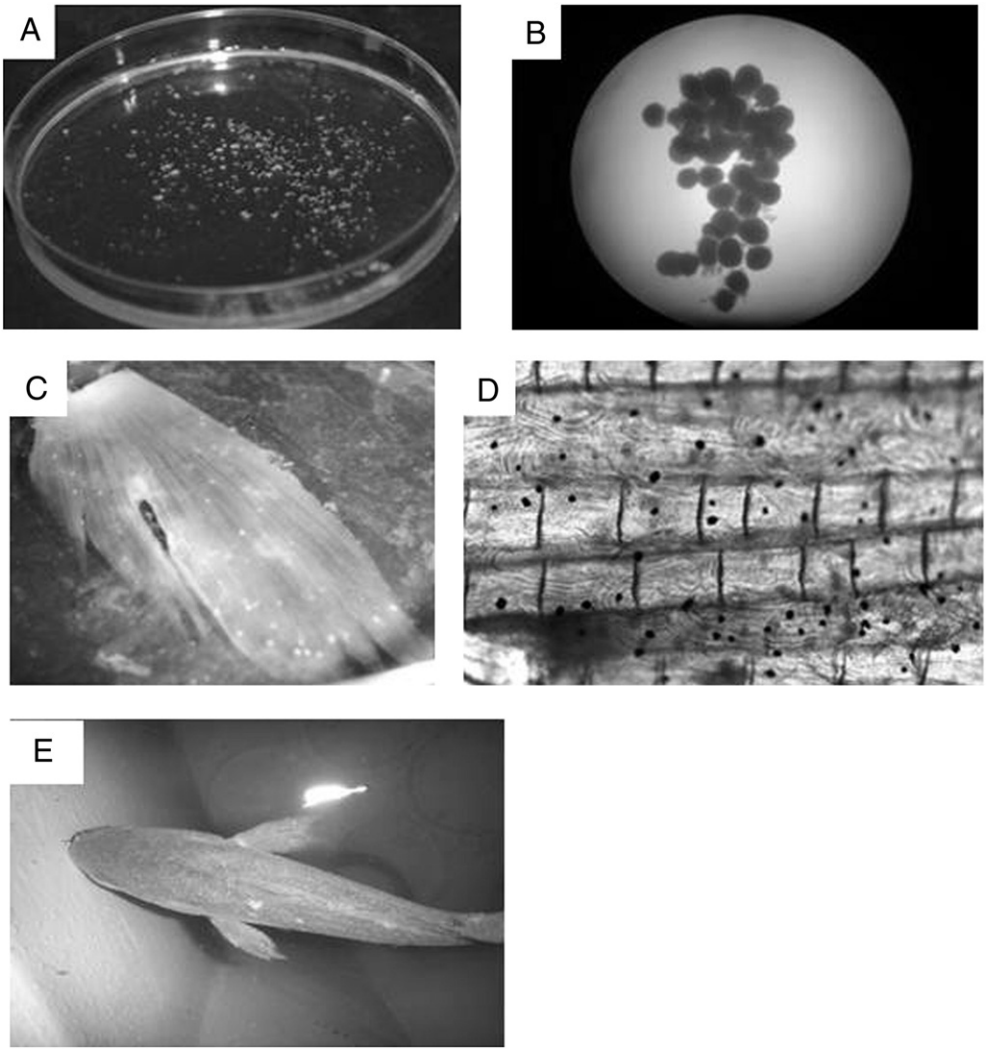

Fig. 4. Clinical signs of the large yellow croaker exposure to *C. irritans*. (A) White tomont collection. (B) Tomont under microscope (×10). (C) White spotted trophonts on the fin of large yellow croaker. (D) Trophonts on the fin under microscope (×10). (E) Heavy infection with trophonts on the entire body of the large yellow croaker.

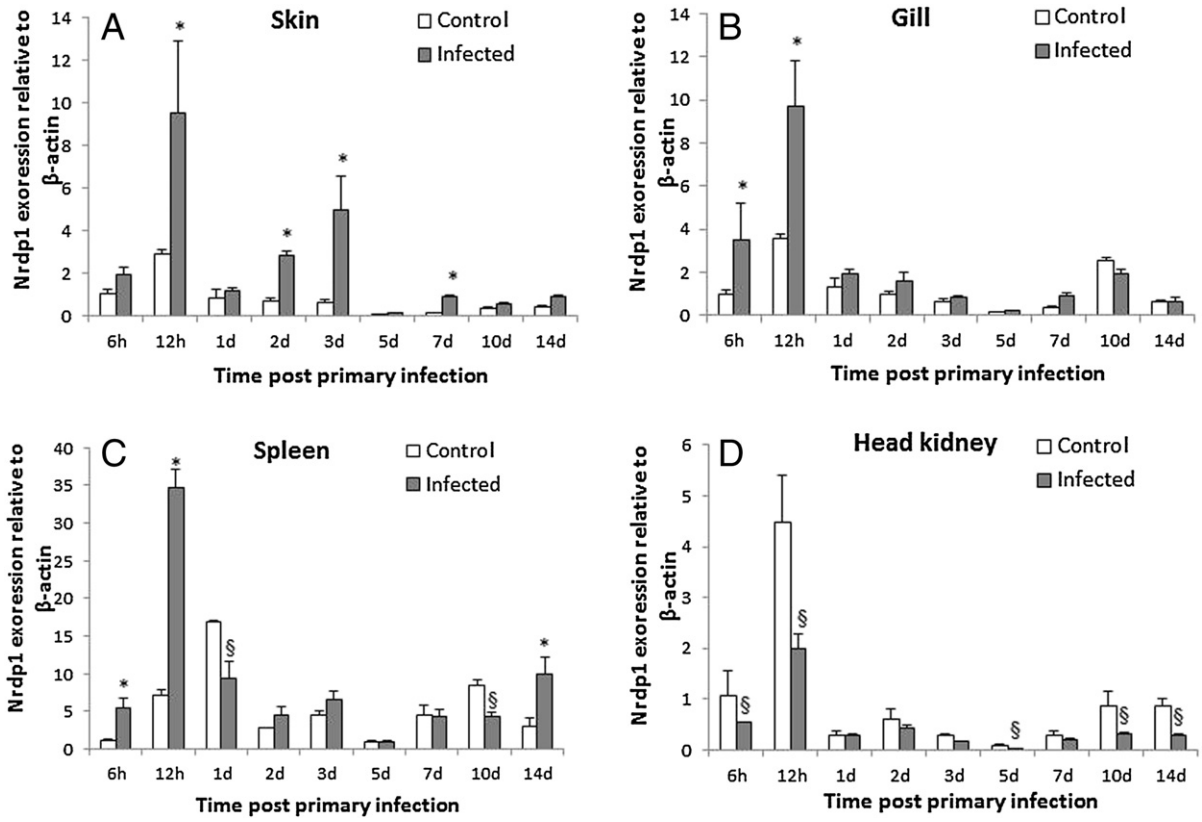

Fig. 5. Expression modulation of Nrdp1 gene in skin (A), gill (B), spleen (C) and head kidney (D) infected by *C. irritans* with Real-time PCR at various time points post-challenged. The mRNA expression level was normalized against β-actin. Data shown as the mean ± SE (N = 4). Each experiment was performed in triplicate. Significant differences of expression between control and *C. irritans* infection groups at each time point are indicated with * (significant increase, $P < 0.05$) or § (significant decrease, $P < 0.05$).

encoding a polypeptide of 318 amino acids. In the deduced amino acid sequence, conserved ring finger domain C_3HC_3D was identified at the position of the 17th to 56th amino acids (Fig. 1A, C). Ring finger domain was defined by eight conserved Cys and His residues that coordinate two zinc ions, with a His in the fourth coordination site and either a Cys (C_3HC_4 Ring) or a His ($C_3H_2C_3$ Ring) in the fifth position (Wiertz et al., 1996). The eighth conserved residue in the Ring of LcNrdp1 was Asp (D56) instead of the usual Cys compared to TRAF2, TRIM16 and CBL like (Fig. 1C). Therefore, we speculated that an Asp in this position may be functionally equivalent to a Cys. It was demonstrated that the Asp in the ring finger is critical for degradation of ErbB3, D56V mutant failed to stimulate ErbB3 degradation in the 293 T cells (Qiu and Goldberg, 2002). More interestingly, the genomic organization of LcNrdp1 had just four exons and three introns (Fig. 1B), different from Nrdp1 of other fish and humans which had five exons and four introns (Abdullah et al., 2001). Phylogenetic analysis showed that the LcNrdp1 sequence was grouped together with Nrdp1 in the other teleosts, while Nrdp1 from the amphibians, reptiles, birds, mammals was clustered into the same subgroup. Other lower animals formed their own clusters, respectively. Obviously, fish Nrdp1 had closer relationship with amphibian, reptile, bird, and mammal Nrdp1 with amino acid identity being 79%–90%, however, only 27%–65% for lower organisms (Table 2). These observations suggested that the functions of Nrdp1 may be diverse in various species, and LcNrdp1 may play a similar role in mammals as the amino acid identity is high up to 90%.

LcNrdp1 transcripts were broadly expressed in all detected tissues of the large yellow croaker. The highest expression was found in the brain, followed by blood, muscle and kidney and the lowest expression was found in the stomach (Fig. 3). The tissue expression profile of LcNrdp1 was partly similar to that of adult humans which was predominant in the brain and skeletal muscle (Diamonti et al., 2002). Moreover, zebrafish Nrdp1 transcripts were prevalent in the neural crest cells, nervous system and skeletal muscle throughout the embryogenesis (Maddirevula et al., 2011). Grass carp Nrdp1 gene expression was quite abundant in the brain and heart, and was also detected in the muscle and blood at a lower level (Wei et al., 2013). These various

Table 2
Amino acid sequence identities of LcNrdp1 with various organisms.

Species	GenBank accession number	Amino acid identity %
Homo sapiens	NP_005776	90
Mus musculus	NP_080535	90
Myotis davidii	XP_006777926	90
Panthera tigris altaica	XP_007081555	90
Bubalus bubalis	XP_006075097	90
Lipotes vexillifer	XP_007467793	84
Physeter catodon	XP_007129419	90
Geospiza fortis	XP_005431385	79
Pseudopodoces humilis	XP_005533904	91
Xenopus laevis	NP_001089261	90
Salmo salar	NP_001134731	96
Ictalurus punctatus	AHH40475	99
Astyanax mexicanus	XP_007235792	97
Ctenopharyngodon idella	AGL46978	97
Danio rerio	NP_998681	97
Oreochromis niloticus	XP_003438948	99
Xiphophorus maculatus	XP_005798968	99
Takifugu rubripes	XP_003963514	99
Lottia gigantea	ESO87978	65
Crassostrea gigas	EKC25754	60
Strongylocentrotus purpuratus	XP_780194	64
Nematostella vectensis	XP_001641586	60
Ceratitis capitata	XP_004518720	60
Apis mellifera	XP_395055	56
Cryptococcus neoformans	AGV14597	36
Ixodes ricinus	JAB81601	27

expression profiles might due to different species, immunological status, developmental stage, or genetic background. From previous reports, Nrdp1 gene existed in a wide range of species and was expressed in almost all tissues, although expression profiles were still different in the same tissue from different species. These discoveries indicated that Nrdp1 probably had important functions in biology processes.

Nrdp1 was involved in various biological reactions including innate immunity and inflammation by inducing the pro-inflammatory cytokines via interacting with MyD88 and TBK1 of the TLR signal pathway. In previous studies, Nrdp1 had been shown to respond to virus, LPS and cytokine stimulations. Changing expressions of LcNrdp1 transcripts were detected in the skin, gill, spleen and head kidney of the large yellow croaker post *C. irritans* infection in the present study (Fig. 5A–D). In the skin and gill (Fig. 5A–B), LcNrdp1 transcripts were up-regulated post *C. irritans* infection, with the exception of days 10 and 14 under the secondary infection when a slight down-regulation was observed in the gill. The up-regulation of LcNrdp1 in the skin and gill illuminated that the skin and gill are the first defense line of anti-pathogen, similar to other immune related gene responses against *C. irritans*, such as piscidin-like, CD8 β, IL-1β, IL-8, C-type lectin and transferrin (Wang et al., 2010; Li et al., 2011a; Zuo et al., 2012; Mohd-Shaharuddin et al., 2013). In the spleen (Fig. 5C), LcNrdp1 expression levels remained elevated except a temporary decline at 1 d and 10 d post-infection. In the head kidney (Fig. 5D), LcNrdp1 expression was significantly down-regulated. Thus, it was speculated that the increased expression level of LcNrdp1 in the skin and gill also probably was due to the migration of expressing cells from the spleen or head kidney, where gene expression level declined, this appearance was similar to TLR response in *Epinephelus tarda* (Takano et al., 2007). In addition, recent studies have also shown that TLR was involved in immune responses against *C. irritans* infections (Li et al., 2011a, 2012). And it also demonstrated that Nrdp1 regulated the TLR signal pathway after virus or LPS injection. However, whether Nrdp1 would regulate the TLR signal pathway in response to *C. irritans*, and the migration of LcNrdp1 expressing cells occurred post parasite infection need further studies for validation.

Using the SMART program, we have predicted that a ring finger and Coiled-coil domains existed in LcNrdp1. The similar structure was also identified in other fish Nrdp1 (Fig. 1A). Ring fingers characterized as zinc-binding domains were thought to mediate a variety of protein–protein interactions and were found in a subclass of E3 ubiquitin ligases (Lorick et al., 1999). Coiled-coil regions also mediated protein–protein interactions and often mediated the homo-dimerization of proteins. Taken together its structure and function, we speculated that LcNrdp1 may interact with specific proteins of the large yellow croaker or *C. irritans* which made LcNrdp1 play an essential role in anti-*C. irritans* process. Recent studies have also found that unmethylated CpG motifs also existed in protozoan parasite genomes, which was mitogenic for B lymphocytes like the bacterial CpG motif that stimulated expression of IL-12, IL-18, TNFa, type I IFN and NO by macrophage and dendritic cells in the host innate immune responses (Shoda et al., 2001; Brown and Corral, 2002). Two reports have showed that fish with intraperitoneal injection of CpG oligodeoxynucleotide CpG-ODN 1668 got an increased resistance to protozoan parasites *Neoparamoeba pemaquidensis* (Page) and *Philasterides dicentrarchi* (Ciliophora: Scuticociliatia) infection (Bridle et al., 2003; Lee and Kim, 2009). In a previous study, Nrdp1 expressions in RAW264.7 mouse macrophages and primary peritoneal macrophages were increased five to ten fold by CpG ODN stimulation (Wang et al., 2009). However, whether Nrdp1-dependent signaling pathways would mediate CpG ODN has yet to be determined. Further studies are required to identify what molecules within the large yellow croaker or *C. irritans* are recognized by LcNrdp1 and how the protein triggers the downstream immunological functions.

In conclusion, we have identified a full-length large yellow croaker Nrdp1 cDNA and its genomic sequence, and found it to be widely expressed in all tested tissues. The expression profile of LcNrdp1 in the skin, gill, spleen and head kidney changed significantly in response to *C. irritans* challenge, indicating that LcNrdp1 might play an important role in finfish defense against parasite infection. The mechanism of its anti-parasite needs to be further studied.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2014.11.024.

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