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Short communication

Identification and characteristic analysis of TLR28: A novel member of the TLR1 family in teleost

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ABSTRACT

Toll-like receptors (TLRs) play a critical role in the innate immune response of fish to recognize microorganisms. Fish TLRs have significant variety and distinct features. This study focuses on a novel TLR member that belongs to the TLR1 family and was first discovered in miiuy croaker (designated as TLR28, mmiTLR28). In phylogenetic analysis, the mmiTLR28 clustered in the TLR1 family. Further characteristic analysis showed a high homology with TLR2 despite some differences between them. The predicted tertiary structure of mmiTLR28 possesses a hydrophobic pocket in the ectodomain region. Expression analysis showed the high expression level in the liver of miiuy croaker. Further functional experiments on the liver after *Vibrio anguillarum*, *Staphylococcus aureus*, lipopolysaccharides (LPS), and poly (I:C) stimulation showed significant upregulation; these results indicate the potential role of mmiTLR28 in immune response. For LPS stimulation in miiuy croaker leukocytes, mmiTLR28 also displayed significant upregulation. The discovery of mmiTLR28 will enrich the information on TLR family; the functional experiments have shown the role of mmiTLR28 in immunity. The results of this study lay the foundation for future research on fish immune systems.

1. Introduction

Vertebrates have evolved systems of immune defenses to eliminate infective pathogens in the body. A vertebrate's immune system protects it from the constant threat of invasion of microorganisms in the environment; this immune system can be classified into the innate immune system and the adaptive immune system (Akira et al., 2006). Microorganisms that invade a vertebrate host are initially recognized by the innate immune system through germline-encoded pattern-recognition receptors (PRRs) (Werling and Jungi, 2003). These PRRs recognize microbial components, known as pathogen-associated molecular patterns (PAMPs), such as lipoproteins, lipopeptides, LPS, flagellin, dsRNA, ssRNA, and CpG DNA motifs (Akira and Takeda, 2004; Janeway and Medzhitov, 2002). As important members of PRRs, Toll-like receptors (TLRs) play an indispensable role in PAMP recognition.

In 1997, the first mammalian homologue of Toll (now known as TLR4) was cloned in humans; 13 TLRs (TLR1–13) have been identified in mammals to date, although TLR11–13 have been identified only in mice (Medzhitov et al., 1997; Hopkins and Sriskandan, 2005). However, fish possess various TLRs; TLR18–20 and TLR23–27 are found only in fish, and several non-mammalian TLRs have been detected (Boudinot et al., 2014). According to the classification approach by Roach et al. (2005) TLRs are classified into six major gene families through phylogenetic analysis, namely, TLR1, TLR3, TLR4, TLR5, TLR7, and TLR11 (2005). TLR1 major family members, including TLR1, TLR2, TLR6, and TLR10, play pivotal roles in recognizing PAMPs from bacteria; several fish-specific TLRs, such as TLR14 and 18, also belong to this family (Zhang et al., 2013). However, TLR6 and TLR10 are absent in fish possibly because of gene loss or pseudogenization.

In the present study, a novel TLR member (called TLR28) was identified in the TLR1 family to enrich the knowledge on fish TLRs. The phylogenetic analysis, syntenic relationship, and tertiary structure of TLR28 were studied to better understand the characteristic of TLR28. Further functional experiments were performed to detect the role of TLR28 in immunity. These characteristic analyses and the functional experiments of TLR28 provide additional information about the TLR family for functional studies in the future.

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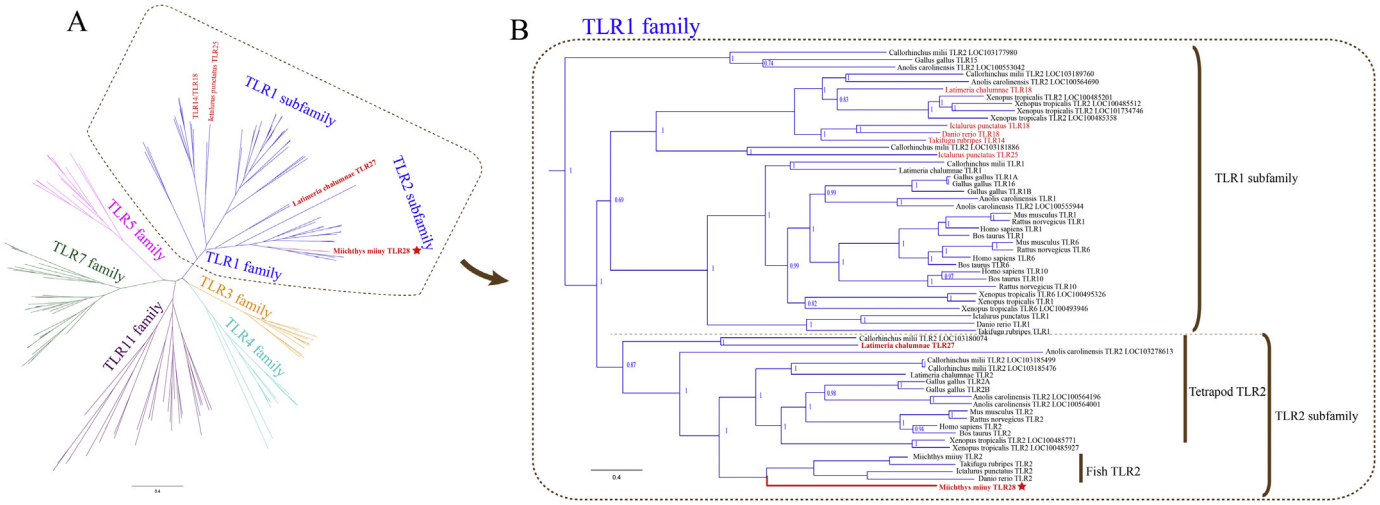

Fig. 1. Phylogenetic relationships of the vertebrate TLRs and identification of mmiTLR28. (A) The unrooted tree was constructed with MrBayes based on the known vertebrate TLRs from Chondrichthyes to Mammalia. Each family of TLRs is shown in different colors; mmiTLR28 is labeled in red. (B) Amplified clades of TLR1 family. Several fish-specific TLRs are marked in red, and the mmiTLR28 is marked with a red five-pointed star. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Samples

After one week of feeding, the selected non-deformed healthy fish (mean weight of 800 g) with abundant physical strength and no technical damage were used for pathogen infection experiment. Four group were injected with 1 ml *Vibrio anguillarum* (1.5×10^8 CFU/ml), 1 ml *Staphylococcus aureus* (1.5×10^8 CFU/ml), 1 ml lipopolysaccharides (LPS, 1 mg/ml, *Escherichia coli* 055:B5, SIGMA), and 1 ml poly (I:C) (2.5 mg/ml, InvivoGen) respectively. Additional details are described in previous studies (Zhu et al., 2013; Xu et al., 2012). In brief, the infected fish were dissected at specific time points after injection. The infected tissues of three individuals were sampled in each group and then stored at −80 °C for future use. The fish injected with physiological saline served as the internal control group. A total of 10 healthy tissues (liver, spleen, head kidney, intestine, blood, skin, eye, gill, brain, and heart) of three non-injected fish were also sampled.

2.2. Isolation of leukocytes

For the extraction of miiuy croaker leukocytes, miiuy croaker livers from three individuals were removed aseptically and placed in ice-cold L-15 cell culture medium containing high-concentration antibiotics (400 IU/ml penicillin and 400 μg/ml streptomycin). The livers were filtered aseptically using a 100 μm pore size cell strainer in L-15 medium with streptomycin (100 IU/ml), penicillin (100 μg/ml), 2% fetal bovine serum (FBS), and heparin (20 U/ml). The suspension was placed on a 51% Percoll separating medium and centrifuged at 400 × g for 40 min at 4 °C. Then, the cells at the interface were collected and washed twice in L-15 medium. Leukocytes were seeded in 6-well plates at a density of 4×10^7 cells per well in L-15 containing 0.1% FBS, and then cultured at 26 °C, 4% CO_2. The next day, the cells were cultured with fresh complete L-15 medium (with 20% FBS), and 6 h later, the cells were stimulated with LPS (10 μg/ml, SIGMA). Cells with no stimulation were collected as the control for the experiment; each treatment and control had three replicates.

2.3. Identification of mmiTLR28 and sequence analysis

To identify the TLR genes from miiuy croaker, annotations of TLRs from GenBank were used to construct a query set. A cDNA database of the assembled miiuy croaker transcriptome (Che et al., 2014) and a database of miiuy croaker whole-genome sequences

Table 1
The similarity and identity of miiuy croaker TLR28 with TLRs in TLR1 family of other species.

Species	Complete amino acid sequences of miiuy croaker TLR28		TIR domain of miiuy croaker TLR28	
	Similarity (%)	Identity (%)	Similarity (%)	Identity (%)
1. Miiuy croaker TLR2	61.0	39.2	73.1	53.2
2. Japanese pufferfish TLR2	59.8	38.2	76.3	54.5
3. Zebrafish TLR2	63.4	44.3	81.5	65.1
4. Zebrafish TLR18	49.5	29.6	67.8	48.6
5. Catfish TLR18	47.0	27.9	67.3	45.6
6. Coelacanth TLR27	53.3	31.1	74.0	51.4
7. Mouse TLR1	51.6	28.9	70.5	48.6
8. Mouse TLR2	63.1	40.8	84.2	67.1
9. Human TLR1	52.5	29.2	69.9	47.9
10. Human TLR2	62.2	40.9	83.6	67.1
11. Rat TLR1	50.8	28.2	69.9	47.9
12. Rat TLR2	63.4	40.1	84.2	67.1