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Identification of 21 novel immune-type receptors in miiuy croaker and expression pattern of three typical inhibitory members

Fanqiang Meng, Rixin Wang, Tianjun Xu *

Laboratory of Fish Biogenetics & Immune Evolution, College of Marine Science, Zhejiang Ocean University, Zhoushan 316022, China

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ABSTRACT

Novel immune-type receptor (NITR) genes belong to the immunoglobulin superfamily and are encoded by clusters of multigene families. NITRs encode type I transmembrane proteins and are only found in teleosts. In the current study, total 21 NITR genes are identified from miiuy croaker (*Miichthys miiuy*) and named as MmNITR1 to MmNITR21. Miiuy croaker NITR genes that encoded one or two extracellular immunoglobulin (Ig) domains, a transmembrane (TM) region, an immunoreceptor tyrosine-based inhibitor motif (ITIM) in the cytoplasmic (Cyt) region. The majority of MmNITRs possess cytoplasmic ITIM that can be classified as inhibitory receptors. However, a smaller number of NITRs (MmNITR8, MmNITR15 and MmNITR16) can be classified as activating receptors by the lack of cytoplasmic ITIMs and presence of a positively charged residue within their transmembrane domain. As typical inhibitory receptors, MmNITR1, MmNITR2 and MmNITR3 have different characteristics of the structure. In MmNITR1 gene, variable (V) and intermediate (I) domains are encoded by two separate exons. In contrast to MmNITR1, MmNITR3 gene encode V and I domains in a single exon. And MmNITR2 gene is characterized by the presence of only one Ig-like (V-type) extracellular domain and lack of J or J-like motifs. Also MmNITR2 gene displays an additional exon which is 48 bp long between the V domain and the TM region. Two and four potential N-link giycosylation sites (N-X-S/T) are present in the extracellular Ig domains. Real-time RT-PCR results showed that upon induction with *Vibrio anguillarum*, NITR gene expressions were induced by bacteria in kidney, liver and spleen. Meanwhile, NITRs are also primarily detected in different tissues. Phylogenetic analyses of NITR V domains indicate that MmNITR1 and MmNITR2 are more similar than MmNITR3.

1. Introduction

Ever-increasing attention is being directed to the role of innate processes within the immune system, the diversity and variety of immune receptors which interact with other surface molecules effect innate function, and the mechanisms whereby innate immune receptors transduct of intracellular signals (Hoffmann et al., 1999). As an ancient form of host defense, the innate immune system relies upon a wide range of non-rearranging encoded receptors including a large number of immunoglobulin superfamily (IgSF) receptors (Cannon et al., 2010; Janeway and Medzhitov, 2002). IgSF is an extensively diversified multigene family whose members share three conserved regions: an immunoglobulin (Ig) like domain, a transmembrane (TM) region and a cytoplasmic tail (Cyt). Depending on the Cyt, they can be subdivided into activating and inhibitory receptors. Generally speaking, short-tailed receptors generate activating signals, whereas inhibitory receptors possess long cytoplasmic tails which containing one or two immunoreceptor tyrosine-based inhibitory motifs (ITIM). In the last decade, with a similar molecular structure to the killer Ig-like receptors (KIRs), two gene families that encoding non-rearranging receptors, have been discovered in teleost fish, the novel immunoglobulin-like transcripts (NILTs) and the novel immune-type receptors (NITRs). As both KIRs and NITRs appear to stimulate the same signalling pathways, NITRs have been proposed to be functional homologs of mammalian KIRs (Yoder, 2009).

NITRs are a large and diverse polygenic family of putative inhibitory and stimulatory receptors (Hawke et al., 2001; Piyaviriyakul et al., 2007; Strong et al., 1999; Yoder et al., 2001, 2002). They belong to a subset of the IgSF and do not undergo rearrangement or somatic mutation (Litman et al., 2001; Yoder and Litman, 2000). In common with other immune genes, both natural killer receptors (NKRs) and NITRs appear to follow a "birth-and-death" model of evolution (Nei and Rooney, 2005).

* Corresponding author.
E-mail address: tianjunxu@163.com (T. Xu).

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The prototypic NITR cDNA was cloned more than many years ago from the Southern pufferfish (*Spheroides nephelus*) (Rast et al., 1995) and then named as "Sn193". This NITR cDNA encodes an N-terminal extracellular variable (V) domain adjacent to a joining (J) domain, a C-terminal extracellular intermediate (I) domain which previously termed V-like C2 or V/C2 domain, a TM region and a Cyt possessing an ITIM and a second ITIM-like (itim) sequence. Subsequent studies have identified additional NITR genes from several fish species. Twenty-six genes were first characterized in the Southern pufferfish (Strong et al., 1999), then with the isolation and description of four NITRs in the rainbow trout (*Oncorhynchus mykiss*) (Yoder et al., 2002), eighteen genes in the channel catfish (*Ictalurus punctatus*) (Evenhuis et al., 2007; Hawke et al., 2001), one transcript in the Japanese flounder (*Paralichthys olivaceus*) (Piyaviriyakul et al., 2007) and the identification of 24 families in medaka (*Oryzias latipes*) (Desai et al., 2008). Transcripts encoding NITR proteins have also been found in the Atlantic salmon, Atlantic halibut, stickleback, Atlantic cod and lake whitefish (Yoder, 2009). Interestingly, no such homologs have yet been identified in mammals (Litman et al., 2001). Several forms of NITRs were identified according to: (1) number of extracellular Ig domains (V-type and I-type) and exons encoding the ectodomains, (2) proteins that only possess a V-domain but lack an I domain can be classified as NITRs if their genes are present within an NITR gene cluster, (3) presence and location of J or J-like sequences and (4) presence of ITIM or itim motifs in the Cyt region. All NITRs possess one extracellular Ig domain of the V type, and most of them possess a second extracellular Ig domain of the I type. Recent crystal structures of NITR V domains demonstrate that they are highly similar to the V domains of antigen receptors (Yoder, 2009). I domain helps differentiate NITRs from other V domain receptors. NITRs that encoded a Cyt region which typically possesses activating or inhibitory signaling motifs. A smaller number of NITRs are considered to be activating receptors, cause they possess a positively charged residue within the TM region and possesses a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) (Wei et al., 2007; Yoder et al., 2007). Most NITRs possess cytoplasmic ITIMs and are classified as inhibitory forms. Moreover, like NKRs, the crystal structure of NITR Ig domains not only demonstrate their potential interaction with a polymorphic set of ligands, but also provide evidence that binding specificity for the allogeneic determinant resides in the V domain CDR1-analogous loop (Cannon et al., 2008). NITR transcripts are detected in diverse hematopoietic lineages supporting the assumption that NITRs function in immunity (Hawke et al., 2001; Evenhuis et al., 2007).

Between mammalian leukocyte receptor complex (LRC) and the NITR gene clusters, comparative genomic analysis showed only a weakly conserved synteny. This can be included that the presence of NITRs may be restricted to bony fish (Yoder, 2009). So far, the collected data demonstrate that NITRs are representative of a rapidly evolving and extraordinarily divergent gene family.

Miiuy croaker, *Miichthys miiuy* is not only an high-valued marine aquaculture fish but also has more in-depth research in immune and genetic background, some important immune genes and molecular markers have been reported (Cheng et al., 2010; Xu et al., 2010, 2011b). Nowadays, as lower vertebrates, miiuy croaker and other important cultured fish are afflicted by various diseases. It is very important to research miiuy croaker's own innate immune mechanism, because innate immunity plays a significant role in protecting the body from antigens infection (Xu et al., 2011a). In order to further elucidate the molecular immune mechanisms in miiuy croaker, series of immune response and evolution mechanism studies of the immune-related genes been carried out and reported (Cheng et al., 2011; Meng et al., 2012; Sun et al., 2011, 2012). In this paper, in order to understand the immune significance of NITR gene in this species, we report the identification of total 21 NITR genes which including inhibitory and activating forms in miiuy croaker. Based on NITR nomenclature, we named these NITRs as MmNITR1 to MmNITR21, miiuy croaker NITRs generated by repeated tandem duplication event, so they represent a highly diversified gene complex. Furthermore, three typical inhibitory NITRs (MmNITR1, MmNITR2 and MmNITR3) have been comprehensive analyzed and examined its expression pattern in various tissues in response to infection with pathogenic bacteria.

2. Materials and methods

2.1. Samples

Ten tissues (liver, spleen, kidney, intestines, heart, muscle, gill, brain, eye, and fin) of uninfected miiuy croaker and three immune tissues (liver, spleen and kidney) of challenged miiuy croaker injected with *Vibrio anguillarum* were sampled as described by Zhu et al. (2013). Total RNA was extracted from diverse tissues of individuals using RNAiso Reagent (Takara) according to the manufacturer's instructions and the cDNA template was transcribed by reverse transcriptase M-MLV (Takara).

2.2. Database mining and Primer design

To identify all the NITR genes from miiuy croaker, all annotation NITRs from GenBank were used to construct a query set, local BLASTp and tBLASTn programs were conducted to search for all NITR genes from miiuy croaker whole-genome scaffold sequences (unpublished). In addition, three partial NITR cDNA sequences (Xu et al., 2010) were used to construct a query set, local BLASTN program were used to search for full-length cDNA sequences and gene sequences (MmNITR1, MmNITR2 and MmNITR3) from transcriptome assembled sequences (Che et al., 2014) and whole-genome scaffold sequences. Three pairs of primers (HM-NITR1-RT-F/R, HM-NITR2-RT-F/R and HM-NITR3-RT-F/R) were designed to detect the expression level of NITR genes, primers β-actin-F/R were used as internal control for NITRs expression analysis (Supplementary Table 1).

2.3. Tissue specific expressions of NITR gene

Ten uninfected tissues were used to determine the tissue expression pattern of three MmNITRs. And, three immune tissues after *V. anguillarum* infection were used to determine the mRNA expression profile. Real-time quantitative PCR was conducted on a 7300 real time PCR system (Applied Biosystems, USA) using a RealMaster Mix kit (TIANGEN). The PCR was carried out in a total volume of 20 μl, including 9 μl SYBR Green Real-time PCR master mixtures, 1 μl cDNA sample, 1 μl sense primer, 1 μl anti-sense primer and 8 μl ddH_2O. Cycling conditions were as follows: 10 s at 95 °C, followed by 40 cycles consisting of 5 s at 95 °C and 34 s at 60 °C. Dissociation curve analysis was performed after each assay to determine target specificity. The data were analyzed using SPSS V13.0 software and 7300 System SDS Software v1.3.0 (Applied Biosystems, USA). The base line was set automatically by the software and the expression levels were determined by the 2-ΔΔCT method (Livak and Schmittgen, 2001).

2.4. Phylogenetic analyses

Predicted leader and TM regions of protein sequences were identified with SMART software (http://smart.embl-heidelberg.de/;) (Letunic et al., 2004). Homology models were viewed and decorated using the SWISS-Pdb Viewer (http://swissmodel.expasy.org/spdbv/). The NITR V and I domains were aligned by Clustal W in MEGALIGN software. Neighbor-joining tree were