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A RING finger protein 114 (RNF114) homolog from Chinese sturgeon (*Acipenser sinensis*) possesses immune-regulation properties via modulating RIG-I signaling pathway-mediated interferon expression

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ABSTRACT

Ubiquitin ligases play important roles in immune regulation. The human RNF114 (RING finger protein 114), an ubiquitin ligase, was recently reported to be involved in immune response to double-stranded RNA in disease pathogenesis. Here, we identified a RNF114 homolog in Chinese sturgeon (*Acipenser sinensis*) and investigated its potential role in immune response. The full-length cDNA of Chinese sturgeon RNF114 (csRNF114) contains an open reading frame (ORF) of 681 nucleotides coding a protein of 227 amino acids. csRNF114 shares the highest identity of 76% at amino acid level to other RNF114 homologs, clustering with bony fish RNF114s based on phylogenetic analysis. The main structural features of csRNF114, including a C_3HC_4 (Cys3-His-Cys4) RING domain, a C_2HC (Cys2-His-Cys)-type zinc finger motif, a C_2H_2 (Cys2-His2)-type zinc finger motif, and a UIM (ubiquitin-interacting motif), take csRNF114 as an ubiquitin ligase. csRNF114 mRNA was widely expressed in various tissues and significantly up-regulated in poly(I:C)-treated Chinese sturgeon. Over-expression of csRNF114 in HEK293T cells significantly promoted both basal and poly(I:C)-induced activation of interferon regulatory transcription factor 3 (IRF3) and nuclear factor-κB (NF-κB) downstream retinoic acid inducible gene I (RIG-I) signaling pathway and expression of target genes type I interferon (IFN), which was nearly abolished by knockdown of RIG-I with specific human siRNA and by mutation of the C_3HC_4 RING domain (C28A/C31A) in csRNF114 as well. Furthermore, csRNF114 associated with ubiquitinated proteins in HEK293T cells, for which the C3HC4 RING domain was essential. These data suggested that an ubiquitin ligase RNF114 homolog with a potential role in antiviral response possibly through modulating RIG-I signaling pathway was cloned from Chinese sturgeon, which might contribute to our understanding of the immune biology of Chinese sturgeon.

1. Introduction

The immune system in fish, like in mammals, consists of innate and adaptive immunity, of which the innate immune response is well known to constitute the first line of defense against pathogen infection and play a vital role along with the adaptive immune response in preventing diseases in fish [1,2]. To initiate an innate immune response against invading pathogens, multicellular organisms generally utilize a variety of germline-encoded pattern recognition receptors (PRRs) [3] which can be functionally divided into soluble endocytic PRRs, bridging PRRs and signaling PRRs [4,5]. The signaling PRRs involved in cell activation in response to diverse microbial pathogen-associated molecular patterns, such as proteins, glycans, lipopolysaccharide and nucleic acids [4], include toll-like receptors, nucleotide-oligomerization domain-like receptors, and the retinoic acid inducible gene I-like receptors [6]. Among them, RIG-I plays crucial roles in the induction of type I IFN signaling and dsRNA-induced antiviral responses in both fish and mammals [7,8]. Evidence from both fish and mammalian immunology shows that it is a combinational system, of which innate immune response generally precedes and activates the adaptive immune response, thus determining the nature of the adaptive immune response and co-operating with adaptive immune response in the maintenance of homeostasis [9]. Immune responses successfully achieve largely dependant on many kinds of modification for the regulation of immune signaling pathways, among which ubiquitination plays an important role in the regulation of immune responses by modifying the activity or promoting

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the degradation of many signaling molecules involved in immune signaling pathways [10].

Ubiquitination of proteins requires three classes of proteins, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). These three kinds of enzymes function sequentially during ubiquitination. E1 binds to ubiquitin and transfers it to the active site of E2 which then interacts with E3, followed by promoting the attachment of ubiquitin to lysine residues on target proteins and formation of a polyubiquitin chain as a signal for degradation [11]. Among these three kinds of enzymes, the ubiquitin ligases are crucial for ubiquitination by selecting substrates and interacting with E2 proteins. The presence of an E2-interacting domain, of which the RING (really interesting new gene), HECT (homologous with E6-associated protein C-terminus) and U-box domains are the best characterized [12], is usually adopted for recognizing the ubiquitin ligases. Given its versatility, ubiquitination has been reported to be involved in regulation of immune response in many aspects. For example, ubiquitination has been demonstrated in regulating the internalization and degradation of the T cell receptor (TCR) [13], the activation of NF-κB [14] and the down-modulation of ζ-chain-associated protein kinase of 70 kDa [15]. As being ubiquitin ligases, members of the well known zinc finger protein gene family play important roles in the regulation of immune response via ubiquitination [10]. This family can be divided into many subfamilies including C_2H_2, C_8, C_6, C_3HC_4, C_2HC, C_2HC_5, C_4, C_4HC_3, C_3H and CCCH subfamily based on their unique and highly conserved consensus amino acid sequences [16]. Recently, a novel subfamily of zinc finger protein with similar domain architecture was identified in human, including RNF125/TRAC-1, RNF166, RNF138, and RNF114/ZNF313 [17]. Among these four RNF proteins, RNF125 and RNF138 have been shown to display strong ubiquitin ligase activity [17,18]. RNF125 was reported to contribute to the ubiquitination and proteasomal degradation of the RIG-I and melanoma differentiation-associated gene 5 (MDA5) innate antiviral receptors [19], thus regulating the cascade through a negative feedback mechanism since RIG-I and MDA5 signaling-induced type I IFN up-regulates RNF125 expression. Unlike RNF125, RNF114 was reported to be involved in the positive regulation of RIG-I and MDA5 signaling [20]. However, there is little research about ubiquitin ligase in regulating fish immune response.

Chinese sturgeon (*Acipenser sinensis*), called the living fossil of aquatic biology for its maintaining some evolutionary characters between chondrichthyes and osteichthyes, is one of the most primitive Actinopterygii species and belongs to Acipenseriformes. Chinese sturgeon diverged from the Acipenseriformes around 184 million years ago [21] and currently is distributed mainly in the East China Sea and the Yangtze River in China. It was listed as the first-class protected animal in China due to its academic value and its exiguity due to over-fishing, destruction of their spawning grounds and other anthropogenic interferences [22]. To elucidate the regulation of immune response and understand the immunology and conservation of Chinese sturgeon, we isolated the RNF114 cDNA from Chinese sturgeon and analyzed the potential function of csRNF114 in immune response.

2. Materials and methods

2.1. Chinese sturgeon and immune challenge

Juvenile Chinese sturgeon (*A. sinensis*), weighing 315 ± 30 g, was purchased from a local Chinese sturgeon breeding base, and maintained in clean tanks. Chinese sturgeon was stimulated by intraperitoneal injection of 200 μl poly(I:C) (GE, USA) in PBS at concentration of 1.0 mg/ml. Tissues including kidney, spleen, liver, heart, intestine and brain were then dissected from pre-anaesthetized Chinese sturgeon of each group at 0, 0.5, 1, 2, 4 and 7 days after immune stimulation and stored in liquid nitrogen immediately for RNA extraction and quantitative real-time RT-PCR analysis. Chinese sturgeon injected with 200 μl sterilized PBS was used as control.

2.2. cDNA library construction and EST sequencing

Total RNA was extracted from various tissues with RNeasy Plus Mini Kit (Qiagen, Germany) following the manufacturer's protocol. The obtained total RNA was used for cDNA library construction and RT-PCR after being analyzed by detecting the 28S and 18S bands and assaying the ratio of OD_{280}/OD_{260}. To construct a cDNA library with full length insertion, the SMART cDNA library construction kit (Clontech, USA) was used following the manufacturer's instructions. All cDNAs larger than 500 bp were ligated into pDNR-LIB vector, and then transformed into DH10B competent cells (Invitrogen, USA). Different cDNA clones were picked out randomly and inoculated directly into 96-well plates containing 1 ml YT broth and cultured in 37 °C overnight. DNAs were extracted using Vitagene 96-easy plasmid Miniprep Kit (Vitagene, Italy). The 5′-terminal sequencing of each cDNA was conducted in an automated ABI Prism 3730 sequencer using T7 primer.

2.3. Bioinformatics analysis

The csRNF114 amino acid sequence was deduced using BioXM 2.6 software. The multiple sequence alignment of csRNF114 was performed using ClustalW program (http://www.ebi.ac.uk/clustalw/). The percentage of identity was calculated between csRNF114 and the known RNF114 sequences using GeneDoc (http://www.psc.edu/biomed/). The protein domain was predicted with the simple modular architecture research tool (SMART) version 4.0 program (http://www.smart.Emblheidelberg.de/). The phylogenetic tree was constructed with MEGA program version 6 based on amino acid sequences alignment. The phylogenetic tree was tested for reliability using 1000 bootstrap replications.

2.4. Real-time quantitative RT-PCR

Total RNA extracted from various tissues of the experimental Chinese sturgeon was treated with RNase-free DNase I (TaKaRa). Equal amount of total RNA was used for the real-time quantitative RT-PCR analysis with primers csRNF114-qF (5′-ATTCGTCTGCCCCG-TATGCCTTG-3′) and csRNF114-qR (5′-AGGTGAAGGGAAGCGGTTTGGC-3′) using the THERMOcript OneStep qRT-PCR kit (Aidlab Biothechnologies Co. Ltd, China). The RT-PCR was performed on LightCycler 480 (Roche, Germany) and the program was 50 °C for 30 min, 94 °C for 5 min, 30 cycles of 94 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s. Concentration of cDNA in each sample was calculated from the standard curve. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The β-actin gene was used as internal standard. All reactions were carried out in triplicate.

2.5. Plasmid construction

The coding sequence of csRNF114 was obtained by amplifying the gene cDNA from a pDNR-LIB-csRNF114 clone using two primers (Forward primer: 5′-GGGATCCATGGCGATGTTTCGGAGCTTGGAC-3′ and Reverse primer: 5′-GCTCGAGCTAGTTGTCCAGCATGGAGCGCTG-3′) and subcloned into pCMV-N-Flag vector (Beyotime, China) between *Bam*HI and *Xho*I sites to obtain Flag-csRNF114. The csRNF114C28A/C31A construct (Flag-csRNF114Mut) was obtained using the Quick-Change Site-Directed Mutagenesis kit (Stratagene, USA) with two set of primers (For C28A: 5′-ATATCAGAA-