Fish and Shellfish Immunology 74 (2018) 559–566

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Molecular characterization of caspase members and expression response to Nervous Necrosis Virus outbreak in Pacific cod

Ming-Guang Mao[a,b], Jie-Lan Jiang[b], Zhi-Qiang Jiang[b], Rui-Ting Liu[b], Qi-Ya Zhang[a,b], Jian-Fang Gui[a,b,*]

[a] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China
[b] Key Laboratory of Mariculture and Stock Enhancement in North China's Sea, Ministry of Agriculture, Dalian Ocean University, Dalian 262306, China

ARTICLE INFO

Keywords:
Caspase
Pacific cod
NNV
Apoptosis
Development

ABSTRACT

Multiple functions of caspases include normal cell turnover, proper development and function of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell injury. During artificial propagation of Pacific cod, *Gadus macrocephalus*, high mortality occurred during early development stages. Here, we performed various analyses on the cDNA and protein sequences of six different *G. macrocephalus* caspases namely *GmCasp3*, *6*, *7*, *8*, *9* and *10*, and tried to investigate the contributions of caspase family to the development and Nervous Necrosis Virus (NNV) resistance. Sequence analysis of *GmCaspase* proteins showed that each caspase shared conserved domains like "HG", "QACXG (X for R, G or Q)" and "GSWF" except *GmCasp10*. Sequence alignment and phylogenetic tree showed that *GmCasp8 and GmCasp10* were quite different from those of other fishes. 3-D models indicated that structure of *GmCasp3* is very conservative, but *GmCasp6*, *7*, *8*, *9* and *10* are less conservative. Tissue distribution analysis showed that six *Gmcaspases* mRNA transcripts were detected in tissues of intestine, gill, thymus, head-kidney and spleen with different abundance, but *Gmcasp7* were not detected in the brain. *GmCasp3* transcript was kept at very low level in the early development stages, while the expression levels of *GmCasp6*, *7*, *8*, *10* were different at various development stages. *GmCasp8* level seemed to be much higher than other caspases in the heads of 65dph and 75dph juveniles. To understand the role of caspases during NNV outbreak, modulation in expression of each *Gmcaspases* were investigated. The results showed that *GmCasp3* transcript level increased significantly when NNV broke out, while *GmCasp7*, *8*, *9 and 10* in cod heads decreased obviously at 69dph and 77dph. The results suggest that caspases in Pacific cod should be diverse in their structure and function, and their unique features and response to NNV outbreak add more evidences for the specificity of immune system in Pacific cod.

1. Introduction

Pacific cod (*Gadus macrocephalus*) is a transoceanic species, occurring at depths from shoreline to 500 m. As one of the 25 major marine capture species, Pacific cod was harvested a quantity of 464 367 tons in 2013 and 474 498 tons in 2014 separately [1]. However, its capture amount in Yellow Sea of northern China has decreased year by year, although artificial propagation has been carried out in China and Japan since ten years ago [2,3]. During breeding, over 90% mortality happened frequently from 20-day post hatching (dph) to 90dph because of nervous necrosis virus (NNV) outbreak [4]. NNV discovered in *G. macrocephalus* belongs to the Barfin flounder NNV (BFNVV) genotype, one of betanodavirus family. Previous report indicated that variable region of betanodavirus RNA2 could determine host specificity and the products were considered as key factors to evoke fish caspase activity and induce cell apoptosis [5]. However, the apoptosis information is still unknown in Pacific cod.

Apoptosis is considered as vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death [6]. To date, research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. Caspases have been identified and broadly categorized into initiators (caspase-2,-8,-9,-10), executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) [7]. In teleost, caspase-3 has been widely investigated in European sea bass (*Dicentrarchus labrax*), large yellow croaker (*Pseudosciaena crocea*), rock bream (*Oplegnathus fasciatus*), zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*), and Medaka (*Oryzias latipes*) [8–12], and it possesses immune-related functions in these fishes.

* Corresponding author. State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China.
E-mail address: jfgui@ihb.ac.cn (J.-F. Gui).

https://doi.org/10.1016/j.fsi.2018.01.004
Received 7 October 2017; Received in revised form 1 January 2018; Accepted 5 January 2018
Available online 06 January 2018
1050-4648/

Table 1
Primers used in the experiment.

Primer name	Primer sequence (5′-3′)	Purpose
Casp3-F	TCCCGTGACCGTCCTTACA	Cloning of *GmCasp3*
Casp3-R	GTTCATCTCCAGAGCACCTTG	
Casp6-F	CGTCTCAGTTTGTGCTGGTAA	Cloning of *GmCasp6*
Casp6-R	TTTAGGTAAACGAAAGTGGGAC	
Casp7-F	ATCTCGTTGAGACGTCGTTA	Cloning of *GmCasp7*
Casp7-R	AGTCCCAGTCTCCGTCAGTCA	
Casp8-F	GTGCCCAGGCTCCTCGTA	Cloning of *GmCasp8*
Casp8-R	AGTTGAGCAGTAGCACCGTTA	
Casp9-F	CCTGCAACCGTGCGAATA	Cloning of *GmCasp9*
Casp9-R	GTTGACAACAGCCATAAAGATG	
Casp10-F	GCGGAACTTAGGCTACTGATG	Cloning of *GmCasp10*
Casp10-R	CAGGTCCAAGGTCGGTTTA	
qCasp8-F	GGCAAAGTGATGGAGAAA	qPCR
qCasp8-R	CCACGAGCTTGATTATGAG	
qCasp9-F	CATTGCTCCGATCTATCC	qPCR
qCasp9-R	TTGGGTTCAAACTTCACG	
qCasp10-F	TCGTTTACTGCGTGAGTT	qPCR
qCasp10-R	TCCAAGGCTAAGAAGACC	
qCasp6-F	GCGAAGGTGAACGAGGTG	qPCR
qCasp6-R	CGAAGCTACGCAGGGTGT	
qCasp7-F	CTTTGAGGATAAGACAGGGAT	qPCR
qCasp7-R	GCTCTGGTCGTTGTAGACG	
qCasp3-F	GCAGAGGAGCGAGGAGACA	qPCR
qCasp3-R	AGGTTCCGATGCTTGGGT	
qActin-F	CCCAAAGCCAACAGGGAGA	qPCR
qActin-R	GAGGCGTACAGGGACAGCA	

However, documented studies on other caspases in teleost are relatively scarce.

Although high-throughput sequencing and bioinformatics methods have gained more and more information about fish genomes, the annotated gene functions remain largely unknown due to species differences. In this study, Pacific cod caspases including Caspase-3 (*GmCasp3*), Caspase-6 (*GmCasp6*), Caspase-7 (*GmCasp7*), Caspase-8 (*GmCasp8*), Caspase-9 (*GmCasp9*) and Caspase-10 (*GmCasp10*) were cloned to shed light on their structural conservation and uniqueness. Their expression profiles in various tissues and in the early developmental stages were examined. However, the question we are most concerned about is how caspases respond to the virus outbreak.

2. Materials and methods

2.1. Fish and sampling

During breeding season, adult Pacific cods weighing 1.5–4 kg were caught from Yellow Sea near Lvshun, Dalian. Various tissues including gill, spleen, intestine, thymus, kidney and brain were collected for gene cloning and gene expression analysis. The fertilization and breeding work was conducted in the breeding system of Key Laboratory of Mariculture & Stock Enhancement in North China's Sea, Ministry of Agriculture. Whole fish of the apparent healthy cods, without typical NNV symptom, were collected at 5dph (day-post hatching), 15dph, 25dph, 35dph and 40dph, and the heads of cods at 65dph and 75dph were also sampled. When Nervous Necrosis Virus (NNV) broke out at 24dph, 37dph, 46dph, 69dph and 77dph, fish with the typical NNV symptom [4] were collected for gene expression analysis, and the apparent healthy fish were set as control.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from samples collected in section 2.1 using Tripure (Roche). First strand cDNA was synthesized from 1 μg of total RNA by Primescript™ 1st cDNA Synthesis Kit (Takara).

2.3. Cloning and sequencing of caspase-3, 6, 7, 8, 9, 10

Partial sequences of caspase-3, 6, 7, 8, 9 and 10 were acquired from the Pacific cod transcriptome of our previous work [13]. Gene-specific primers were designed based on the known sequences (Table 1). PCR products were cloned into the pMD18-T vector (TaKaRa). Three positive clones for each product were sequenced at Sangon (Shanghai, China).

2.4. Sequence analysis

Sequences homology were obtained using BLAST program at National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) and the deduced amino acid sequences of *GmCaspase*-3, 6, 7, 8, 9, 10 were analyzed using software DNAsisst version 2.2. Multiple alignments analysis of each protein were performed using the ClustalW2 Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). The molecular mass (MM) and theoretical Isoelectric point (pI) of the proteins were calculated based upon their deduced amino acids by the ProtParam tool (http://www.expasy.ch/tools/protparam.html). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0.

2.5. 3-D homology modeling

The three dimensional protein structures were predicted by Protein

Table 2
Information of *GmCaspases* and amino acid identity among species from online BLAST.

Caspases	Full length (bp)	Predicted protein (aa)	Theoretical pI	Mw	Query coverage	Identity	Species	GenBank No.
GmCasp3 (KY652105)	1555	287	5.94	32 027.68	100%	61%	*Paralichthys olivaceus*	XP_019946087.1
					95%	57%	*Oncorhynchus mykiss*	CEO43908.1
					98%	57%	*Chaetura pelagica*	NP_001290581.1
GmCasp6 (KY652110)	1073	321	6.01	35 654.37	89%	76%	*Salmo salar*	XP_014065132.1
					89%	72%	*Paralichthys olivaceus*	XP_019956800.1
					86%	67%	*Ovis aries*	XP_006930979.1
GmCasp7 (KY652106)	1235	317	5.21	35 672.19	95%	90%	*Lates calcarifer*	XP_018554240.1
					94%	91%	*Paralichthys olivaceus*	XP_019965790.1
					95%	74%	*Gallus gallus*	XP_421764.3
GmCasp8 (KY652107)	1948	399	5.91	44 501.08	99%	52%	*Labrus bergylta*	XP_020505530.1
					99%	48%	*Maylandia zebra*	XP_014263672.1
					69%	39%	*Bos mutus*	XP_005904304.1
GmCasp9 (KY652108)	2710	421	5.30	47 081.65	96%	72%	*Paralichthys olivaceus*	XP_019944605.1
					96%	70%	*Takifugu rubripes*	XP_003978524.1
					95%	49%	*Gallus gallus*	XP_424580.5
GmCasp10 (KY652109)	1742	348	5.47	38 529.54	100%	97%	*Gadus morhua*	ADG85728.1
					54%	55%	*Sebastes schlegelii*	AKM76972.1
					51%	37%	*Ailuropoda melanoleuca*	XP_011218837.1