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Cloning and preliminary functional studies of the *JAM-A* gene in grass carp (*Ctenopharyngodon idellus*)

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ABSTRACT

Grass carp (*Ctenopharyngodon idellus*) is a very important aquaculture species in China and other South-East Asian countries; however, disease outbreaks in this species are frequent, resulting in huge economic losses. Grass carp hemorrhage caused by grass carp reovirus (GCRV) is one of the most serious diseases. Junction adhesion molecule A (JAM-A) is the mammalian receptor for reovirus, and has been well studied. However, the *JAM-A* gene in grass carp has not been studied so far. In this study, we cloned and elucidated the structure of the *JAM-A* gene in grass carp (*GcJAM-A*) and then studied its functions during grass carp hemorrhage. *GcJAM-A* is composed of 10 exons and 9 introns, and its full-length cDNA is 1833 bp long, with an 888 bp open reading frame (ORF) that encodes a 295 amino acid protein. The GcJAM-A protein is predicted to contain a typical transmembrane domain. Maternal expression pattern of *GcJAM-A* is observed during early embryogenesis, while zygote expression occurs at 8 h after hatching. *GcJAM-A* is expressed strongly in the gill, liver, intestine and kidney, while it is expressed poorly in the blood, brain, spleen and head kidney. Moreover, lower expression is observed in the gill, liver, intestine, brain, spleen and kidney of 30-month-old individuals, compared with 6-month-old. In a *GcJAM-A*-knockdown cell line (CIK) infected with GCRV, the expression of genes involved in the interferon and apoptosis pathways was significantly inhibited. These results suggest that GcJAM-A could be a receptor for GCRV. We have therefore managed to characterize the *GcJAM-A* gene and provide evidence for its role as a receptor for GCRV.

1. Introduction

Grass carp (*Ctenopharyngodon idellus*) is a very important aquaculture species that accounts for 20% of the freshwater aquaculture production in China. However, disease outbreaks in this species are frequent, resulting in huge economic losses, which severely restrict the development of grass carp farming. Grass carp hemorrhage caused by grass carp reovirus (GCRV) is one of the most serious diseases. Hence, there is a need to study the pathogenesis of GCRV infection and look for preventative measures to promote sustainable culture of grass carp.

GCRV is a non-enveloped icosahedral virus that belongs to the family Reoviridae, genus *Aquareovirus*, and it has a dsRNA genome [1]. Reoviruses infect most mammalian species [1], and the receptor for reovirus in humans and other mammals is junction adhesion molecule A (JAM-A) [2,3], a member of the immunoglobulin superfamily. JAM-A regulates the formation of intercellular tight junctions [4] and is involved in platelet activation, leukocyte transmigration and angiogenesis [5,6]. Reovirus recognizes structural features that are present in JAM-A, but not in JAM-B or JAM-C [7]. The crystal structure of the extracellular region of JAM-A reveals the presence of two concatenated Ig-type domains (D1 and D2) with a pronounced bend at the domain interface. Two JAM-A molecules form a dimer that is stabilized by extensive ionic and hydrophobic contacts between the D1 domains. Binding and infection experiments using chimeric and domain-deletion mutant receptor molecules indicate that the amino-terminal D1 domain of JAM-A is required for reovirus attachment, infection and replication in mouse [8–10].

Mammalian reovirus and GCRV belong to different genera [11], however, they share some structural similarities. Although the functions of mammalian JAM-A have been studied in viral infection, no functional studies have been carried out on fish JAM-A.

Abbreviations: *JAM-A*, junction adhesion molecule A; GcJAM-A, grass carp junction adhesion molecule A; GCRV, grass carp reovirus; TRAIL, TNFγ-related apoptosis-inducing ligand; RACE, rapid amplification of cDNA ends.

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This study is therefore the first to clone the *JAM-A* gene in grass carp and study its functions during grass carp hemorrhage. Our results suggest that GcJAM-A, like mammalian JAM-A, could also be a receptor for GCRV.

2. Materials and methods

2.1. Experimental animals

Grass carp (average weight, 9.6 g) were adapted to the conditions in a 55.5 × 45.0 × 34.8 cm^3 aquarium with a water temperature of 28.5 ± 0.5 °C, pH 7.0, and dissolved oxygen concentration of 5.5 ± 0.3 mg O_2/L dechlorinated and aerated water. The grass carp were fed twice daily, at 9:00 AM and 5:00 PM, during the experimental period. After an acclimatization period of 3 days, the fish were challenged with GCRV.

2.2. Cloning and sequencing of grass carp JAM-A (GcJAM-A)

The total RNA of grass carp liver was purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and treated with RNase-free DNase (Promega, Madison, WI, USA). First-strand cDNA was synthesized from RNA using moloney murine leukemia (M-MLV) reverse transcriptase (Toyobo, Osaka, Japan). Sequence alignment of *JAM-A* nucleotide sequences from a variety of species was performed with the ClustalX 1.83 multiple-alignment software. The primers were designed based on the conserved nucleotides of the sequences of seven species reported before: *Homo sapiens* (NP_058642.1) *Pan troglodytes* (XP_001172741.1), *Canis familiaris* (XP_536132.2), *Bos taurus* (NP_776520.1), *Mus musculus* (NP_766235.1), *Rattus norvegicus* (NP_446248.1), *Danio rerio* (NP_001004667.1) (Table 1). A 750 bp fragment of the *JAM-A* gene was amplified from grass carp liver cDNA using the primer pair 25/34 (Table 1). The resulting fragments were separated on 1.0% agarose gel and purified using the Axygen DNA gel extraction kit (Axygen, Union City, CA, USA). The purified fragments were cloned into the pMD-18T vector (Takara, Dalian, China) by the TA cloning strategy and sequenced (BGI, Shenzhen, China). The 750 bp fragment was confirmed to be a homologous sequence of the *JAM-A* gene from *D. rerio* by BlastX. The 5′ and 3′ ends of the *JAM-A* cDNA were obtained using the rapid amplification of cDNA ends (RACE) approach (Zhuan Dao, Wuhan, China).

2.3. GcJAM-A genomic DNA sequence amplification and organization analysis

Grass carp genomic DNA was extracted from the tail fin using phenol-chloroform. The primers 79/75, 74/71 and 70/68 (Table 1) were designed to amplify the *GcJAM-A* genomic DNA sequence according to the full-length cDNA of *GcJAM-A*. PCR amplification was carried out using LA *Taq*™ Polymerase (Takara, China) under the following cycling parameters: 94 °C for 5 min; followed by 36 cycles of 30 s at 94 °C, 40 s at 62 °C, and 7 min at 72 °C; and a final extension step for 10 min at 72 °C. The resulting fragments were purified and sequenced, and the genomic DNA sequence obtained was analyzed to determine the exons and introns based on the cDNA sequence of *GcJAM-A*.

2.4. Analysis of nucleotide and amino acid sequences

The nucleotide and predicted amino acid sequences of *GcJAM-A* were analyzed using DNA figures software (http://www.bio-soft.net/sms/index.html). The similarity of *JAM-A* from carp with *JAM-A* from other organisms was analyzed using the BLASTP search program of NCBI (http://www.ncbi.nlm.nih.gov/blast). The domain structures were predicted using the SMART program (http://smart.embl-heidelberg.de/). The alignment was compared with that of multiple amino acid sequences of *JAM-A* reported, using ClustalX 1.83 (http://www.ebi.ac.uk/clustalW/) and the GeneDoc software. The phylogenetic tree was constructed using the MEGA 3.1 software (http://megasoftware.net).

Table 1
Sequences of the primers used in this study.

Primer	Sequence	Amplicon length (bp)	Use
25	5′-ACACCCAGAGTAGAATGG AAGTT-3′		
34	5′-ACACCACAAAAGATGATTTCT GTCTGAA-3′	750	GcJAM-A cloning
79	5′-TTTCGTCTTTGTGTGCCTCTCTATCTC-3′		
75	5′-AAATGTGGGGAACTTGCTGGGAT-3′	1627	GcJAM-A cloning
74	5′-ATCCCAGCAAGTTCCCCACATTT-3′		
71	5′-CCCTTTTTAGTGGCAAACCAGAGAG-3′	2834	GcJAM-A cloning
70	5′-ATTGCTCCTCTTTGCTCTCTGGTTT-3′		
68	5′-CATTCATAGTACCTTCTTTTCCCCAGA-3′	1170	GcJAM-A cloning
93S	5′-TGTGGCTTTGCTGGCAGTAG3′		
93A	5′-TGGCTTGCTTTCTGCTATTTTT-3′	91	qRT-PCR for JAM-A
120S	5′-GGATGATGAAATTGCCGCACTGG-3′		
120A	5′-ACCGACCATGACGCCCTGATGT-3′	136	qRT-PCR for *β-actin*
107S	5′-GGGGTACCATGTTGACTTTCGT CTTTGTGTGCC-3′		
107A	5′-CCCAAGCTTCCAAAGCAGGCTACA CCACAAAAG-3′	898	Vector constructing
108S	5′-CGGGGTACCTCTGTGGATAACCG TATTACCGCC-3′		
108A	5′-CGGGATCCATGTTGACTTTCGT CTTTGTGTGCC-3′	1555	Vector constructing
112	5′-GATCTTAAAATAAAGCAATAGCA TCACAAATTTC ACAAATAAAGCATTTTTTTCACTGCA-3′		
113	5′-AGCTTGCAGTGAAAAAAATGCTT TATTTGTGAAATT		
RF230	5′-ACTACACTGAACACCTGCGGAA-3′		
RR231	5′-GCATCTTTAGTGCGGGCG-3′	106	qRT-PCR for RIG-1
132S	5′-CCAGCATCAAACTTCACTACCG-3′		
132A	5′-TTACTGAGCGTGTGTCTCCAAA-3′	97	qRT-PCR for JAK1
138S	5′-ATCTGCGATGGGCTTTTGTC-3′		
138A	5′-GGCTTGGTCTCGTTTGGTTTC-3′	87	qRT-PCR for caspase 8
140S	5′-CAACCGAAAAGGCACTGAGAA-3′		
140A	5′-CGAGAGGACACAGCAGACAAAA-3′	170	qRT-PCR for caspase 9
142S	5′-AGTGAGGAAGATGCGGCTATTT-3′		
142A	5′TGTTGAGGGCACAGCGAAG-3′	116	qRT-PCR for IRF9
145S	5′-GACACATACAGTAGGATATTCACTCGC-3′		
145A	5′-TTGCCTGGGAAGTAGTTTTCTTG-3′	122	qRT-PCR for IFNγ
147S	5′-TGCCTGGATTGAGAAAAGAAACT-3′		
147A	5′-TTCCATCACTGCCGAACATTAT-3′	167	qRT-PCR for PKR
148S	5′-GCAGGGGACAAAAAGAGATTATAGA-3′		
148A	5′-AGCCAACTTAGGAATAGTAGCAAAAC-3′	134	Mxa
158S	5′-TGAGCAAAGACCCCAACGAG-3′		
158A	5′-TCGACTGCAGAATTCGAAGCT-3′	139	EGFP

2.5. RT-qPCR analysis of GcJAM-A mRNA expression profiles

For tissue distribution analysis of *GcJAM-A*, total RNA was extracted from the gill, liver, spleen, kidney, head kidney, brain, intestine and blood of 6-month-old and 30-month-old healthy