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Short communication

Grass carp *Ctenopharyngodon idella* Fibulin-4 as a potential interacting partner for grass carp reovirus outer capsid proteins

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ABSTRACT

Mammalian EGF containing fibulin-like extracellular matrix protein 2 (*Fibulin-4*/*EFEMP2*), an extracellular matrix(ECM) protein and a member of the fibulin family, is involved in elastic fiber formation, connective tissue development and some human diseases. In a yeast-two hybrid screening of host proteins interacting with outer capsid protein of grass carp reovirus (GCRV), a grass carp homologue of Fibulin-4 (designated as GcFibulin-4) is suggested to hold the potential to bind VP7, VP56 and VP55, the outer capsid protein encoded by type I, II, III GCRV, respectively. *GcFibulin-4* gene of grass carp was cloned and sequenced from the cDNA library constructed for the yeast two-hybrid screening. Full-length cDNA of *GcFibulin-4* contains an open reading frame (ORF) of 1323 bp encoding a putative protein of 440 amino acids. Phylogenetic analysis of GcFibulin-4 indicated that it shared a high homology with zebra fish Fibulin-4 protein. Transcriptional distribution analysis of *GcFibulin-4* in various tissues of healthy grass carp showed that *GcFibulin-4* was highly expressed in muscle, moderately expressed in the intestine and brain, and slightly expressed in other examined tissues; the expression pattern is consistent with tissue tropism of GCRV resulting in hemorrhage symptom in the corresponding tissues. Our results suggested that Fibulin-4 might enable free GCRV particles, the pathogen for grass carp hemorrhagic disease, to target fish tissues more efficiently by interacting with viral outer capsid proteins.

The grass carp *Ctenopharyngon idellus* is an important freshwater fish cultured in eastern Asia fisheries. The frequent outbreak of grass carp hemorrhagic disease caused by grass carp reovirus (GCRV) has resulted in substantial economic losses. GCRV (genus *Aquareovirus*, family *reoviridae*) is a dsRNA virus with 11 genomic fragments that encode 12 structure or non-structure proteins [1–3]. GCRV can be classified into three major genotypes based on the VP6 sequence, which were represented by strains GCRV-873 (GCRV-JX01), GCRV-HZ08 (GCRV-JX02), and HGDRV [4,5]. The genotypes have less than 20% similarity and the characteristics such as cytopathic effect, virulence, pathogenesis, and antigenicity of each genotype are very different [4]. Given the differences between the genotypes, studies to understand the interactions between the GCRV outer capsid proteins and host proteins that contribute to determining the tissue tropism of infection and disease prevention are critical. Little is known on the cellular interaction partner(s) of outer capsid proteins of GCRV.

The Fibulin family of proteins consists of eight isoforms that share overlapping calcium-binding consensus sites and a diverse array of protein ligands [6,7]. This creates the potential for broad array of overlapping interactions and functions between Fibulin proteins. For example, they interact with several basement-membrane proteins including tropoelastin, fibrillin, fibronectin, and proteoglycans [8,9]. In humans, tissue fibrosis, a life-threatening autoimmune disease is related to Fibulin family proteins [10]. Fibulin-4 in particular is associated with elastic fiber formation and connective tissue development, and is an essential component of the extracellular matrix (ECM) [11,12]. To date, the role of Fibulin-4 in fish immune or autoimmune disease remains unknown due to the lack of investigation; however, its mammalian homologue has been linked to many diseases, such as an autosomal recessive cutis laxa syndrome, cancer, inherited eye disorders, and sepsis [8,13]. It's reasonable to speculate that fish Fibulin-4 might

List of abbreviations: GCRV, Grass Carp Reovirus; dsRNA, Double Stranded RNA; Y2H, Yeast 2 Hybrid; ECM, Extra Cellular Matrix.

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hold the potential to be involved in innate host response to pathogen challenge.

In this study, we have cloned and expressed outer capsid proteins of all three GCRV genotypes (VP55 [HGDRV segment 7], VP56 [GCRV-JX02 segment 7], and VP7 [GCRV-JX01 segment 11]) in *Saccharomyces cerevisiae* (AH109) in an effort to identify interacting partner using yeast two-hybrid (Y2H) screening assay. The GCRV-JX01 and GCRV-JX02 isolates used have been previously published [14]. The HGDRV isolate was obtained from the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences. The viruses were amplified and purified as previously described [15]. The bait plasmid for the Y2H screen was constructed from the *VP55* gene amplified by RT-PCR (PrimeScript™ II 1st stand cDNA Synthesis Kit; TaKaRa, Japan) from grass carp infected with HGDRV (pGBKT7-VP55). Additional bait plasmids were constructed in a similar manner for *VP56* (pGBKT7-VP56) and *VP7* (pGBKT7-VP7). The primers are provided in Table 1. The construction of the cDNA library from grass carp kidney tissue for GAL4-based Y2H assay has been previously published [16]. Potential interacting partners were identified in AH109 yeast transformed with pGBKT7-VP55, using the Yeastmaker™ Yeast Transformation System 2 (Clontech, USA). Yeast transformants were grown on medium with X-alpha-gal, but without histidine, leucine, adenine, and tryptophan (SD/-Trp-Leu-His-Ade/X-α-gal, Clontech, USA) to screen positive clones. Interaction between pGBKT7-NS26 and pGADT7-CiLITAF, shown as reported previously [16], served as a positive control in the screening, while the empty bait/prey plasmids served as a negative control. All of the plasmids tested in the Y2H assay had been transformed into AH109 first to detect self-activation.

The results of the Y2H assay are presented in Fig. 1.(in the web version) Five positive clones were identified and sequenced for screening the interacting partners of VP55. Of these, two proteins were identified. One shared more than 90% similarity with the zebra fish small ubiquitin-related modifier E2-conjugating enzyme Ubc9 protein. The second protein was a homologue of the zebra fish Fibulin-4 precursor. Notably, the screenings assays using *VP56* or *VP7* as bait gene also identified the Fibulin-4 homologue.

Little is known on the sequence or tissue distribution of *Fibulin-4* in aquatic organisms. To better understand the role of GcFibulin-4 in GCRV infection, we further cloned and characterized the *GcFibulin-4* nucleotide and amino acid sequences; and analyzed the tissue mRNA distribution of *GcFibulin-4* by quantitative RT-PCR. Total RNA was isolated (TRIzol; Invitrogen USA) from 100 mg grass carp tissue sample. The partial sequence of GcFibulin-4 was obtained from a positive clone in the Y2H assay. Based on the partial grass carp sequence and conserved nucleotide sequences from *Danio rerio* (NM_001008587), the primers (Table 1) were designed to amplify a fragment of the 3′ region of *GcFibulin-4*. A 1548 bp fragment of the *GcFibulin-4* gene was cloned into the pMD™19-T Vector (Takara, Japan) using the TA cloning approach and sequenced. Using the SMARTER™ RACE cDNA Amplification Kit (Clontech, USA), the 5′ rapid amplification of *GcFibulin-4* cDNA end (RACE) was carried out as previously described [17], and another 1098 bp fragment was obtained and sequenced. The fragments were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA). The nucleotide and putative amino acid sequences were compared to known sequences using the National Center for

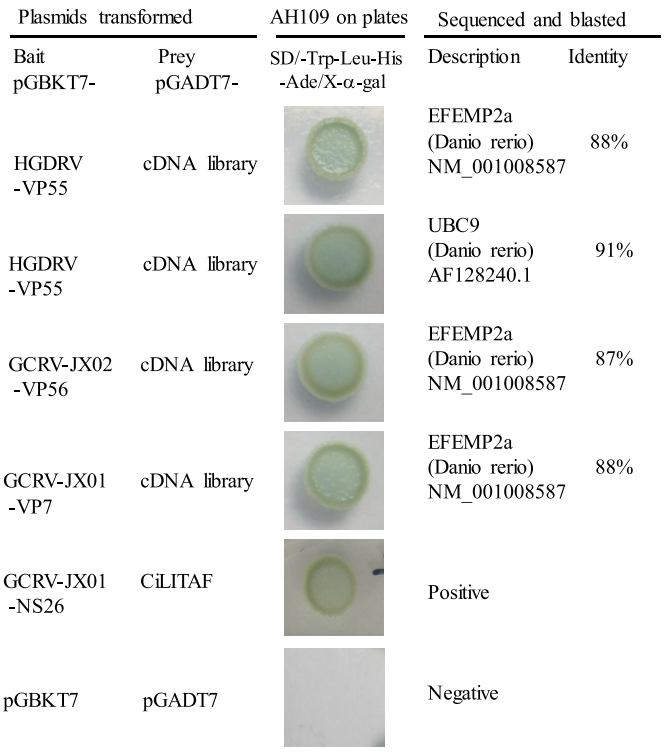

Fig. 1. Interaction between GcFibulin-4 and the GCRV outer capsid proteins in yeast. The results of the yeast 2 hybrid screening assay are shown. The bait and prey plasmids transformed into the AH109 yeast cells are shown in the left hand columns. Representative images of the yeast transformants grown on SD/-Trp-Leu-His-Ade/X-α-gal plates are shown in the center columns. The blue colonies indicated a positive interaction between the bait and prey. The right hand columns show the related genes and accession numbers. The percent sequence identity refers to the similarity ratio from blasting in the Genbank database. Accession number: VP55 [JN967635.1], VP56 [KU161132], VP7 [JQ042807](For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 1
Primers used in this study for cloning and quantitative RT-PCR.

Gene	Primer (5′-3′)	Application
F-VP55	CCG*GAATTC*ATGGACGATCAAGCGCTCGCA	pGBKT7 cloning
R-VP55	CGC*GGATCC*GGCAAGTGACAGGCCGCCACC	pGBKT7 cloning
F-VP56	CATG*CCATGG*AGATGGCCACTCGTGACAG	pGBKT7 cloning
R-VP56	CGC*GGATCC*GGTACTTACAGCAAACTACCG	pGBKT7 cloning
F-VP7	GGAATTC*CATATG*ATGCCACTTCACATGATTCCG	pGBKT7 cloning
R-VP7	CCG*GAATTC*ATCGGATGGCTCCACATGCAAG	pGBKT7 cloning
GcFibulin-4	GACAGACACAGGAGAATTTGCCGTG	5′RACE
F-GcFibulin-4	CACGGCAAATTCTCCTGTGTCTGTC	Cloning
R-GcFibulin-4	TTATCACCTCTAAATCAGTTCTC	Cloning
qF-18S rRNA	ATTTCCGACACGGAGAGG	qRT-PCR
qR-18S rRNA	CATGGGTTTAGGATACGCTC	qRT-PCR
qF-GcFibulin-4	TCATTCAACTTGTGCCCTCC	qRT-PCR
qR-GcFibulin-4	GCCCACTTTGCGATACCC	qRT-PCR

Note: Restriction enzyme recognition sites are marked with italicized text.