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Significant inhibition of two different genotypes of grass carp reovirus *in vitro* using multiple shRNAs expression vectors

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ABSTRACT

The hemorrhagic disease of grass carp (*Ctenopharyngodon idellus*), caused by grass carp reovirus (GCRV), is the most severe disease of the fish that leads to huge economic losses. GCRV, belonging to the genus *Aquareovirus* of the family *Reoviridae*, has been classified into three genotypes based on their phylogenetic relationship. It is essential to develop an effective method to inhibit the replication of different genotypes of GCRV simultaneously. In this report, two multiple-shRNAs expression vectors, named pMultishVP2/2 and pMultishVP6/7, were generated and investigated. pMultishVP2/2 targeted the VP2 gene of GCRV-JX0901 (genotype I) and the VP2 gene of HGDRV (Hubei grass carp disease reovirus; genotype III). pMultishVP6/7 targeted the VP7 gene of GCRV-JX0901 and the VP6 gene of HGDRV. These two multiple-shRNAs expression vectors can simultaneously, significantly inhibit the replication of GCRV-JX0901 and HGDRV *in vitro*. Compared to the positive control, CPE induced by GCRV-JX0901 or HGDRV in cell transfected with shRNA transcribing vector was significantly delayed. The quantitative PCR analysis of the GCRV genomic RNA revealed that the pMultishVP2/2 could simultaneously inhibit the GCRV-JX0901 and HGDRV VP2 coding genes by 89.02% and 89.84%, respectively. The pMultishVP6/7 could simultaneously inhibit the GCRV-JX0901 VP7 coding gene and HGDRV VP6 coding gene by 80.63% and 86.78%, respectively. Furthermore, compared to the positive control, the indirect immunofluorescence assay and western blot demonstrated that the protein expression of the two genotypes of GCRV decreased significantly. The results in this study indicated that this multiple-shRNAs expression system could be used as a cross-reactive antiviral agent for treating the hemorrhagic disease of grass carp caused by multiple genotypes of GCRV.

1. Introduction

The hemorrhagic disease of grass carp (*Ctenopharyngodon idellus*), which was caused by grass carp reovirus (GCRV), is the most severe disease of the fish in China and has caused huge economic losses (Chen and Jiang, 1983; Yang et al., 2013). GCRV has a non-enveloped double capsid shell with an icosahedral structure that consists of seven structural proteins (VP1–VP7) and a genome composed of 11 segments of double stranded RNA (Ke et al., 1990, 1992; Fang et al., 2000). Currently, there are more than 20 strains of GCRV isolated from grass carp. Based on the phylogenetic relationship of these isolates, there are three genotypes represented by isolates GCRV-873 and GCRV-JX0901 (genotype I), GCRV-HZ08 (genotype II) and HGDRV (Hubei grass carp disease reovirus, formerly grass carp reovirus strain 104, GCRV-104; genotype III) (Zeng et al., 2011). Specifically, HGDRV has been identified as a new species in the genus *Aquareovirus* that is distantly related to any known species within this genus (Fan et al., 2013). The levels of HGDRV nucleotide sequence identity that could be detected between homologous proteins of different reoviruses are low (19.2%) by the standards of other genetic groups. At the amino acid level, seven segments (Seg-1 to Seg-6, and Seg-8) matched with species in the genera *Aquareovirus* (15–46% identities), while for four segments (Seg-7, Seg-9, Seg-10 and Seg-11) no similarities in these genera were found (Wang et al., 2012; Fan et al., 2013). Recent investigations regarding the epidemiology of the disease revealed that multiple genotypes of GCRV existed simultaneously in many endemic areas (Wang et al., 2013). To date, there are no effective treatments available for GCRV disease. Effective therapies against multiple genotypes of GCRV infection need to be developed.

RNA interference (RNAi) is a process in which the gene expression can be silenced in a sequence-specific manner mediated by short hairpin RNA (shRNA), making it a powerful tool in

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Table 1
Oligonucleotides used to produce shRNA expression vectors.

Targets	sequences of shRNA (5′-3′)
GCRV-JX0901VP2 sense strand:	pGATCCG***GGACCAGCGCGTCATTTAT***TTCAAGAGA***ATAAATGACGCGCTGGTCC***TTTTTTGGAAA
GCRV-JX0901VP2 antisense strand:	pAGCTTTTCCAAAAAAGGACCAGCGCGTCATTTATTCTCTTGAAATAAATGACGCGCTGGTCCCG
GCRV-JX0901VP7 sense strand:	pGATCCG***CTGAGACCACCAACTTTGA***TTCAAGAGA***TCAAAGTTGGTGGTCTCAG***TTTTTTGGAAA
GCRV-JX0901VP7 antisense strand:	pAGCTTTTCCAAAAAACTGAGACCACCAACTTTGATCTCTTGAATCAAAGTTGGTGGTCTCAGCG
HGDRV VP2 sense strand:	pGATCCG***GCGCAATCCTCAAATTTCT***TTCAAGAGA***AGAAATTTGAGGATTGCGC***TTTTTTGGAACCAATTGCATGGCCCA
HGDRV VP2 antisense strand:	pAGCTTGGGCCATGCAATTGGTTCCAAAAAAGCGCAATCCTCAAATTTCTTCTCTTGAAAGAAATTTGAGGATTGCGCCG
HGDRV VP6 sense strand:	pGATCCG***GTGGCAGATATCACCTGAT***TTCAAGAGA***ATCAGGTGATATCTGCCAC***TTTTTTGGAACCAATTGCATGGCCCA
HGDRV VP6 antisense strand:	pAGCTTGGGCCATGCAATTGGTTCCAAAAAAGTGGCAGATATCACCTGATTCTCTTGAAATCAGGTGATATCTGCCACCG

Letters in bold and italics indicate oligonucleotides contained 19-mer hairpin sequences specific to the mRNA target; letters in boxes indicate transcription termination signal; letters in underline indicate loop sequence; letters in double-underline indicate restriction sites.

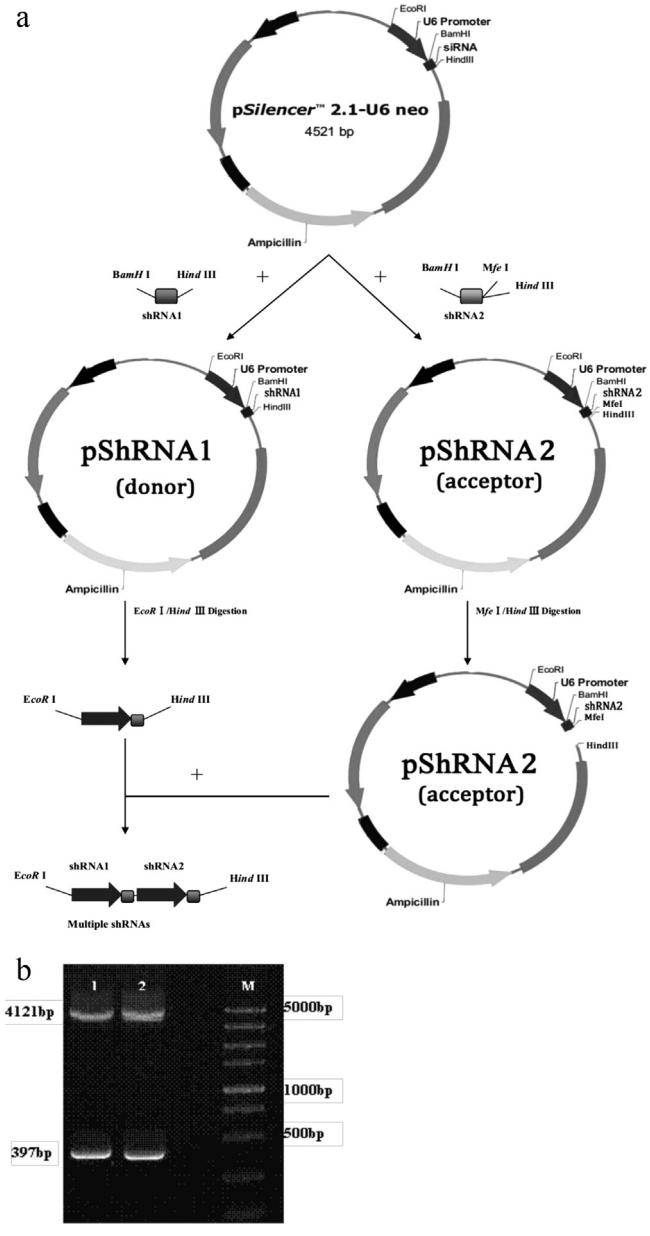

Fig. 1. (a) Schematic representation of the generation of the multiple-shRNAs expression vector. (b) Identification of Multiple-shRNAs vector (pMultishVP2/2 and pMultishVP6/7) with two shRNA-expressing cassettes. Digestion with *EcoR* I/*Hind* III have confirmed the presence of the 397 bp two shRNA-expressing cassettes. Line1: pMultishVP2/2; Line2: pMultishVP6/7; Line M: 5000 bp DNA ladder.

therapeutic applications (Paddison et al., 2002). Inhibition of virus replication by RNA interference has been reported for a number of viruses, including poliovirus, human immunodeficiency virus type 1 (HIV-1), hepatitis B, C and E virus (Gitlin et al., 2005; Hamasaki et al., 2003; Jacque et al., 2002; Lee and Rossi, 2004; Saulnier et al., 2006; Wilson and Richardson, 2005; Huang et al., 2010). We also have reported a plasmid-transcribed shRNA against grass carp reovirus (GCRV) (Ma et al., 2011). However, the single-shRNA expression vector has limitations in protecting from infection with GCRV because of the different genotypes that exist. Previous reports showed that after long-term culturing, some viruses (especially RNA virus), like human immunodeficiency virus (HIV), hepatitis C virus (HCV) and poliovirus, can evolve to escape recognition of RNAi machinery by accumulating point mutations that change the sequence of target site directly or the local secondary structure nearby to increase the difficulty for siRNA to bind (Das et al., 2004; Westerhout et al., 2005; Wilson and Richardson, 2005; Gitlin et al., 2005). This may lead to the speculation that different genotypes of virus with a conserved target site may have different susceptibility to the same siRNA because of RNA secondary structure, possibly influenced by sequences near the target. An alternative approach for preventing this emergence of escape mutants has been the simultaneous use of multiple shRNAs (Gitlin et al., 2005; ter Brake et al., 2006; Snyder et al., 2008). The hepatitis B virus (HBV) could be effectively inhibited by using dual shRNAs driven by the RNA polymerase III (Pol III) promoter, U6 (Wu et al., 2005; Snyder et al., 2008). A plasmid encoding three shRNAs driven by a single U6 promoter against HBV was more effective than a plasmid encoding single shRNA in cells (Chen and Mahato, 2008).

For the development of a durable gene therapy that blocks the replication of several GCRV genotypes simultaneously, we proposed to combine multiple shRNAs against both GCRV-JX0901 (genotype I) and HGDRV (genotype III). Different shRNAs targeting GCRV-JX0901 and HGDRV were screened and a single plasmid expressing multiple-shRNAs against GCRV-JX0901 and HGDRV was constructed. The multiple-shRNAs expression systems are capable of protecting against infection by silencing the sequences of multiple genotypes of GCRV. The results reported here highlight the

Table 2
Primers used for real-time quantitative RT-PCR.

Primers	Primer sequence (5′–3′)	Position in gene sequence
GCRV-JX0901 VP2-F	ATCAAGGATCCCATTCCGCCTTCA	1213
GCRV-JX0901 VP2-R	TTAGAGGATCGTGCCATTGAGGGT	1369
GCRV-JX0901 VP7-F	ACCACCAACTTTGATCACGCTGAG	124
GCRV-JX0901 VP7-R	AGCGTGGGAGTCTTGAATGGTCTT	240
HGDRV VP2-F	CTGATGAATGGTAAGCAAGCGA	490
HGDRV VP2-R	GGATGACCTGACTAACCGCAT	595
HGDRV VP6-F	GGCTACCCTCTTTGTCTCGC	240
HGDRV VP6-R	CCTCCGCAGGTACCACTGC	374
β-Actin-F	TTGCCGCACTGGTTGTTG	1635
β-Actin-R	TTTCCCTGTTGGCTTTGG	2046