Isolation and characterization of a new class of DNA aptamers specific binding to Singapore grouper iridovirus (SGIV) with antiviral activities

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ABSTRACT

The Singapore grouper iridovirus (SGIV), a member of the genus *Ranavirus*, is a major viral pathogen that has caused heavy economic losses to the grouper aquaculture industry in China and Southeast Asia. No efficient method of controlling SGIV outbreaks is currently available. Systematic evolution of ligands by exponential enrichment (SELEX) is now widely used for the *in vitro* selection of artificial ssDNA or RNA ligands, known as aptamers, which bind to targets through their stable three-dimensional structures. In our current study, we generated ssDNA aptamers against the SGIV, and evaluated their ability to block SGIV infection in cultured fish cells and cultured fish *in vivo*. The anti-SGIV DNA aptamers, LMB-761, LMB-764, LMB-748, LMB-439, LMB-755, and LMB-767, were selected from a pool of oligonucleotides randomly generated using a SELEX iterative method. The analysis of the secondary structure of the aptamers revealed that they all formed similar stem-loop structures. Electrophoretic mobility shift assays showed that the aptamers bound SGIV specifically, as evidenced by a lack cross-reactivity with the soft shell turtle iridovirus. The aptamers produced no cytotoxic effects in cultured grouper spleen cells (GS). Assessment of cytopathic effects (CPE) and viral titer assays showed that LMB-761, LMB-764, LMB-748, LMB-755, and LMB-767 significantly inhibited SGIV infection in GS cells. The *in vivo* experiments showed that LMB-761 and LMB-764 reduced SGIV-related mortality, and no negative effects were observed in orange-spotted grouper, *Epinephelus coioides*, indicating that these DNA aptamers may be suitable antiviral candidates for controlling SGIV infections in fish reared in marine aquaculture facilities.

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1. Introduction

Groupers, *Epinephelus* spp. are commercially important, popular, and economical seafood fish throughout China and Southeast Asia (Marino et al., 2001). In recent years, however, the rapidly developing aquaculture industry has been affected by outbreaks of infectious viral, bacterial, and parasitic diseases. Among these outbreaks, viral diseases, especially those caused by iridovirus and nodavirus, have caused high mortality and substantial economic losses (Chao et al., 2004; Lakra et al., 2010; Qin et al., 2003). Singapore grouper iridovirus (SGIV) is a member of the genus *Ranavirus* of the virus family *Iridoviridae*. The SGIV was first isolated from the brown-spotted grouper, *Epinephelus tauvina*, and has recently emerged as the etiological agent of serious outbreaks of systemic disease at grouper fish farms (Qin et al., 2001, 2003). Effective methods for controlling SGIV infections are urgently needed.

Vaccination against virus represents one potentially effective strategy. Most of the currently used viral vaccines consist of whole killed virus particles. However, the effectiveness of killed whole-virus vaccines is often limited by poor induction of cell-mediated immunity and the risk of incomplete inactivation (Davis and McCluskie, 1999). In addition, fish often need a higher dose than terrestrial animals to achieve a comparable level of protection, and the cost of producing inactivated vaccines often make

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them an economically nonviable strategy (Sommerset et al., 2005). Chemical strategies, such as treatments using formalin, binary ethylenimine, or β-propiolactone, has been used to inactivate viruses for vaccine production, and biophysical methods using UV irradiation, pressure, and heat have also been used (Burstyn and Hageman, 1996; Horowitz and Ben-Hur, 1995; Jiang et al., 2008; Lawrence, 2000). In a previous study, two intraperitoneally injected SGIV vaccines were developed using chemical methods, which produced limited protection against SGIV infection *in vivo* (Ouyang et al., 2012). Thus, currently available vaccines against SGIV are suboptimal.

Molecular probes that recognize a virus with a high level of specificity are essential for the development of diagnostics and treatments for viral pathogens. Since its first description in 1990, the systematic evolution of ligands by exponential enrichment (SELEX) technology has been widely applied to the development of unnatural nucleic acids or protein ligands, known as aptamers (Ellington and Szostak, 1990; Bunka and Stockley, 2006). Many of the aptamers reported in previous studies have been short single-stranded nucleic acid oligomers (ssDNA or RNA) that were characterized by complex structural features, such as stems, loops, hairpins, and pseudo knots (Zhou and Rossi, 2011). The unique three-dimensional shape of aptamers allows them to bind target molecules with high affinity and specificity.

Aptamers have the advantage of binding to many different types of targets, from single molecules to complex target mixtures, with higher affinity and greater specificity than conventional probes, such as antibodies. Aptamers are also associated with a lower incidence of toxicity, and are less immunogenic than antibodies. Because aptamers are synthetic, they are easily modified and manipulated (Fang et al., 2003; Guo et al., 2005). Aptamers have been applied to many areas, such as diagnostics, environmental analysis, food analysis (Tombelli et al., 2007), biosecurity (Fischer et al., 2007), pathogen detection (Torres-Chavolla and Alocilja, 2009), and cancer research (Phillips et al., 2008). Macugen (pegaptanib sodium injection), an anti-VEGF aptamer developed by Pfizer, was approved by the United States Food and Drug Administration in 2004 for the treatment of age-related macular degeneration (Ng et al., 2006).

Aptamers have been used in virus research for the analysis of virus replication, as diagnostic biosensors, and as antiviral agents (Torres-Chavolla and Alocilja, 2009; James, 2007). A previous study showed that aptamers inhibited viral infection at every stage of the viral replication cycle including viral entry, suggesting that aptamers might prevent infection *in vivo* (Binning et al., 2012). Jeon et al. (2004) showed that a number of DNA aptamers that targeted the hemagglutinin protein of influenza virus A inhibited viral infection effectively. In fish, RNA aptamers have been shown to provide protection against the viral hemorrhagic septicemia virus (VHSV) and the Hirame rhabdovirus (HIRRV) (Porntep et al., 2012; Hwang et al., 2012).

In our present study, we generated a panel of ssDNA aptamers against intact SGIV using a SELEX iterative method. The specificity of aptamer-SGIV binding was assessed *in vitro*. Aptamer-mediated cytotoxicity and the effects of the aptamers on SGIV infection were also evaluated in cultured fish cells and in orange-spotted grouper, *E. coioides*.

2. Materials and methods

2.1. Cells and viruses

Grouper spleen cells (GS) and fathead minnow cells (FHM) were grown and maintained in Leibovitz's L-15 medium supplemented with 10% Gibco fetal bovine serum (Life Technologies, Carlsbad, CA,

USA) at 28 °C, as described previously (Qin et al., 2006; Huang et al., 2011). The SGIV (strain A3/12/98) was propagated in GS, and the soft shell turtle iridovirus (STIV) was propagated in FHM. The virus titer was determined based on the 50% tissue culture infection dose (TCID$_{50}$) (Reed and Muench, 1938), and the viruses were purified by sucrose gradient ultracentrifugation as described previously (Song et al., 2004). The virus stocks were stored in TN buffer containing 0.01 M Tris–HCl (pH 7.2) and 0.85% NaCl.

2.2. Fish and rearing condition

Apparently healthy grouper, *E. coioides* weighing approximately 40 g were obtained from a fish farm on Hainan Island, China. The fish were maintained in 30% salinity seawater at 28 °C in tanks with a closed, circulating nonchlorinated-water system, and fed a daily diet of commercially available food. The fish were acclimatized to the laboratory conditions for 2 weeks before they were used in our experiments. Prior to the experiments, tissue samples from ten randomly selected fish were examined for the presence of SGIV using PCR and SGIV-sequence-specific primers, according to a previously described method (Ouyang et al., 2010). No specific band was detected in any of the tissue samples examined (data not shown).

2.3. SELEX library and primers

The SELEX library (Sigma–Aldrich, St. Louis, MO, USA) was 44.7 nmol of ssDNA that consisted of a central randomized 50-nt sequence (N50), in which the A, G, C, and T nucleotide bases were incorporated at each position at equivalent frequencies. The N50 sequence was flanked by two primer-hybridization sequences (5′-GACGCTTACTCAGGTGTGACTCG-N50-CGAAGGAC-GCAGATGAAGTCTC-3′). The N50 5′-primer (5′-GACGCTTACTC-AGGTGTGACTCG-3′) and the biotinylated N50 3′-primer (5′-biotin-GAGACTTCATCTGCGTCCTTCG-3′) were used in the PCR to generate the corresponding SELEX library.

2.4. SELEX protocol

The DNA aptamers were selected following the SELEX protocol as described by Pan et al. (1995) with some optimizations. The pooled ssDNA were denatured at 95 °C for 5 min. After cooling on ice for 10 min, 200 μl binding buffer containing 2.5 mM MgCl$_2$, 100 mM NaCl, and 20 mM Tris–HCl (pH 7.5) was added to the pool, and the mixture was passed through a prewetted 0.1 μm PVDF filter (EMD Millipore, Billerica, MA, USA). The filtrate was mixed with 100 μl of purified SGIV (10^8 TCID$_{50}$/ml), and incubated on ice for 40 min. The ssDNA-SGIV complexes were separated from the free ssDNAs by passing the mixture through the filter. The ssDNA-SGIV complexes were eluted from the filter by washing with TN buffer, and the eluate was transferred to a microcentrifuge tube. The ssDNA-SGIV mixture was heated at 95 °C for 5 min to dissociate the complexes, and was desalted afterward. The dissociated aptamers were amplified by PCR using the N50 primers. Thermal cycling was performed using 20 cycles of denaturation at 94 °C for 1 min, annealment at 55 °C for 30 s, and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 5 min. The PCR products were denatured by heating at 95 °C for 5 min, and cooled on ice for 10 min. The sense ssDNAs were separated from the biotin-conjugated antisense ssDNAs using Pierce streptavidin magnetic beads (Takara-Bio, Shiga, Japan) in a MiniMACS Separator (Miltenyi Biotec, Cologne, Germany). The sense ssDNAs were used for the next cycle of the SELEX procedure. To improve the affinity and specificity of the selected aptamers, the content of the ssDNA pool and SGIV was reduced serially.

2.5. Cloning and sequencing of DNA aptamers

After 10 rounds of selection, the enriched ssDNA pool was PCR-amplified with unlabeled N50 primers, and ligated into the pMD18-T vector (Takara-Bio). The isolated clones were sequenced, and the corresponding ssDNA aptamers were synthesized by Life Technologies. The secondary structure of the aptamers was predicted using the MFOLD computational program (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form).

2.6. Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) was used to demonstrate the specificity of aptamer-SGIV binding. The SGIV (10^8 $TCID_{50}$/ml) was combined with 30, 15, or 7.5 μg of each aptamer in binding buffer, and incubated on ice for 45 min. The binding mixture was passed through a prewetted 0.1 μm PVDF filter (EMD Millipore). The aptamer-SGIV complexes were eluted from the filter by washing with TN buffer, and the elute was transferred to a microcentrifuge tube. The aptamer-SGIV complexes were subjected to polyacrylamide gel electrophoresis on a 6% acrylamide nondenaturing gel, and the gel was stained with SYBR Green EMSA stain, according to the manufacturer's instructions (Life Technologies). The gel bands were visualized by UV epi-illumination at 312 nm. Aptamer binding to STIV (10^6 $TCID_{50}$/ml) was also evaluated using the EMSA to demonstrate binding specificity.

2.7. Cytotoxicity assays

The GS cells were cultured in 96-well plates, and incubated with the selected aptamers at concentrations ranging from 1 nM to 1 μM for 48 h at 28 °C. To assess cell viability, 20 μL of MTT solution
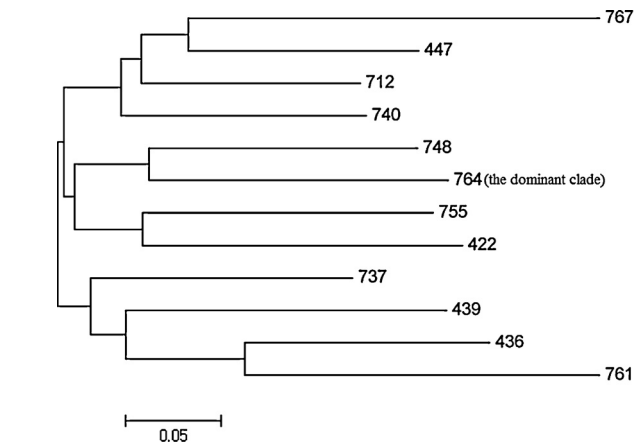

Fig. 1. Cluster analysis of the aptamer sequences. The most prevalent sequence, LMB-764, and the most unique sequence, LMB-748, were closely related.

(Takara-Bio) was added to each well, and the plates were incubated at 28 °C for 4 h. The color change was measured at 450 nm using an ELISA plate reader. The results of at least three independent cell viability assays were averaged for each aptamer.

2.8. Aptamers inhibit SGIV infection in vitro

To evaluate the effects of the aptamers on SGIV infection, 30 μg of each selected aptamer or 30 μg of the SELEX library was incubated with or without 10^5 $TCID_{50}$/ml SGIV in 100 μl of binding buffer for 45 min. The binding mixture was diluted with 400 μl of L-15 medium, and added to 24-well plates containing GS cell
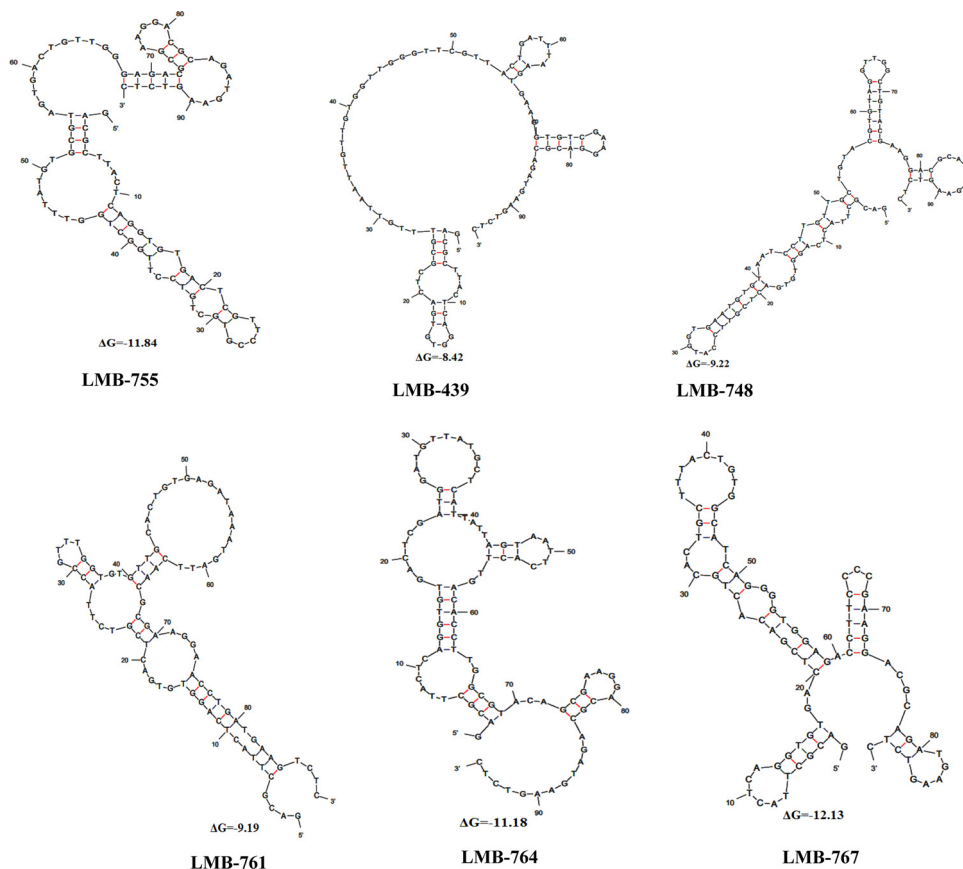

Fig. 2. The predicted secondary structures of six anti-SGIV ssDNA aptamers. The stability of the aptamer secondary structure was calculated as the free energy (ΔG).

monolayers. Similar experiments were performed using only 10^5 TCID$_{50}$/ml SGIV in the binding mixture. At 24 and 48 h postinfection, the GS cells were examined to identify CPE under light microscopy. Samples of the supernatant and cells were collected from each well, and transferred to a 96-well plate to determine virus titer using the TCID$_{50}$ assay. The data from three independent experiments were used to quantify the effects of each selected aptamer on virus infection.

2.9. Analysis of the effects of aptamers on SGIV infection in vivo

Grouper, *E. coioides* fry were starved for 24 h before receiving a 200-µl intraperitoneal injection containing 10^8 TCID$_{50}$/ml SGIV and 60 µg of a selected aptamer in L-15 medium. The fish in the control groups were injected with the same amount of the SGIV, aptamer, or L-15 medium alone. Thirty fish were used in each treatment group. Each group was transferred to a separate aquarium supplied with running seawater and ample aeration, and maintained as described in Section 2.2. Mortality was recorded daily, and dead fish were removed until day 10 post-injection, after which no additional mortalities occurred. The results from three independents for each group were averaged to quantify the effects of the aptamers on viral infection *in vivo*.

2.10. Histological analysis

Grouper, *E. coioides* fry were starved for 24 h before receiving an intraperitoneal injection containing 15, 30 or 60 µg of selected aptamer LMB-764, or the same volume of TN buffer (negative control). Normal fish were also served as controls. Thirty fish were used in each treatment group. Each group was transferred to a separate aquarium supplied with running seawater and ample aeration, and maintained as described in Section 2.2 for ten days. Tissue specimens were collected from the liver and spleen, and fixed in 10% neutral buffered formalin for 24 h. The fixed tissues were defatted by immersion in graded organics and alcohols, and embedded in paraffin. Thin sections were prepared, and stained with hematoxylin and eosin (H&E).

2.11. Statistical analysis

The experimental data are expressed as the mean ± standard deviation (SD). Intergroup differences were compared using a one-way analysis of variance. The results of comparisons with $P < 0.05$ were considered to represent statistically significant differences. The statistical analysis was performed using the SPSS, version 13.0, statistical software (IBM, Armonk, NY, USA).

3. Results

3.1. Characterization of ssDNA-aptamers

After 10 rounds of SELEX procedures, 12 ssDNA aptamers were isolated from a pool containing 10^{15} different ssDNAs (Table 1) based on highly specific SGIV binding. The LMB-764 aptamer comprised 21% of the aptamer pool, whereas each of the other aptamers was less prevalent in the pool. The cluster analysis showed that LMB-764 and LMB-748 were closely related, and clustered with LMB-755 and LMB-422 (Fig. 1). Some aptamers, such as LMB-761, LMB-767, and LMB-740, were shorter than the initial sequences, which might have resulted from nonspecific PCR amplification in the selection procedure. Six of the aptamers were randomly selected from the pool for further analysis.
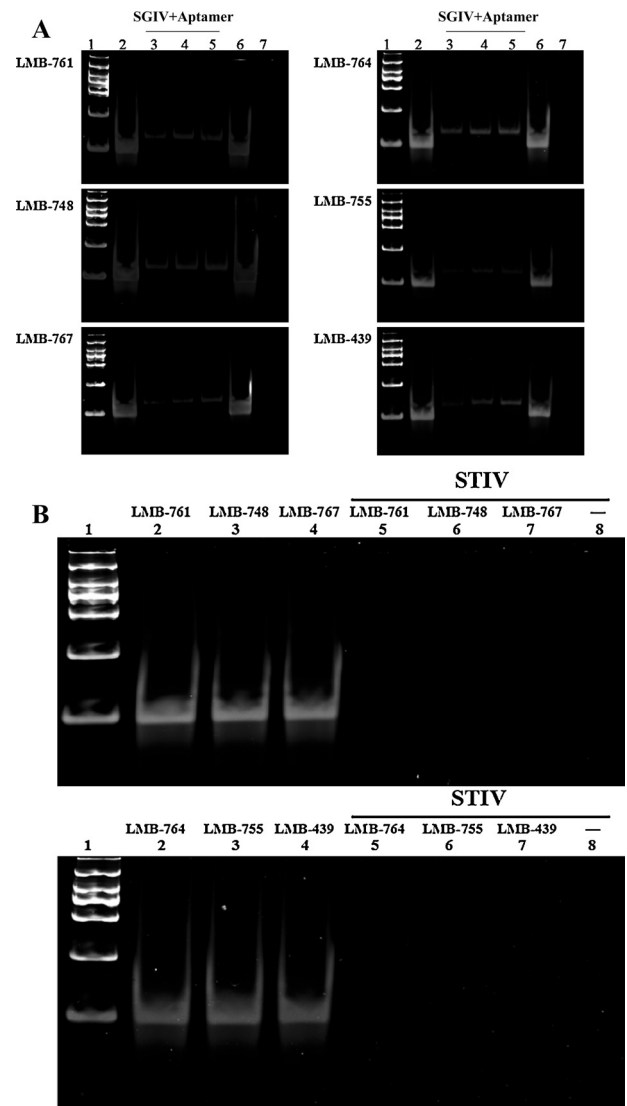

Fig. 3. Binding specificity analysis of the anti-SGIV ssDNA aptamers. (A) EMSA of selected aptamers incubated with SGIV (lane 1, molecular marker; lane 2, 7.5 µg aptamer only; lanes 3–5, SGIV incubated with 7.5, 15, or 30 µg of each aptamer, respectively; lane 6, 30 µg of aptamer only; and lane 7, SGIV only). (B) EMSA of selected aptamers incubated with STIV. Note that no bands were visible in lanes 5–8.

3.2. Secondary structure of the aptamers

According to free energy (ΔG) values that were calculated using the MFOLD program, the secondary structures of LMB-767, LMB-748, LMB-439, LMB-764, LMB-761, and LMB-755 were highly stable, among which LMB-767 was the most stable (Fig. 2). Compared with the other aptamers, LMB-439 had the most distinct secondary structure and the highest ΔG value.

3.3. Selected aptamers bound SGIV with a high level of specificity

The EMSA was used to demonstrate specific aptamer-SGIV binding. The free ssDNA aptamers were visible at the bottom of the gel (Fig. 3, lanes 2 and 6). When the aptamers were incubated with SGIV, only single bands were visible in the upper region of the gel (Fig. 3, lanes 3–5), indicating that the aptamers bound SGIV. The specificity of aptamer-SGIV binding was demonstrated in the analysis of the aptamers incubated with the STIV, in which the EMSA

Table 1
Identification of anti-SGIV ssDNA aptamers.

Aptamer	Central randomized sequences	Frequency (%)
LMB-764	ATGGATGTTATGCTCATTATTAGTAATTCACTTGACACCTTGGCGTACAG	21
LMB-761	TCTTACCGTTTGGTGTGTTGCACTGTGAGATAAATGATTCAACG	7
LMB-767	ACACTGCACTGCTTTACTGTGGCATCAGGGGTGGAGACCTTCCC	7
LMB-740	TGTTGAATTCTTGTTGTTGTGTATTTACATTTTGTTGGTACAGGTTA	7
LMB-447	TTATCCTTCTTGTTAATTATGTGACTTATCTAGCGTTGGCAGTCATTAGG	7
LMB-712	TTAGCTTGCTCTCTATGGTTGGTCTAGGATATGGGTGTGATGTGGTGCTA	7
LMB-436	GCATTGTTGTTTATGTTCTATTGCTCTATTCGTTCTACCGGACCCTCTGT	7
LMB-748	TTCCATGGTGAATGTGTAATCCTTGTTGCTGTACGTGTAGGTTGGCTGTA	7
LMB-755	TTCCGTGCTGTCCTTGGCTGGTTTATGTGCGTAGTGACTGTTGGGAGACG	7
LMB-422	TTGTAATCATGTTGTGCTCTTGGCCATGGAGTCTGTGTATGGGCTCTTCA	7
LMB-737	TTGTACTGTCTTGTCGTCTGGTATTGTTTCATCCTTCACTGTGTATGTTC	7
LMB-439	CGTTTGTTAATTGTTGTGGTTGGGTTCGTTACTGATTTAAGTGAAGGTGT	7

Frequency indicates the percentage of the tenth selected pool comprised by each aptamer.

produced no bands in the upper region of the gel, suggesting that the aptamers did not bind to STIV (Fig. 3B).

3.4. Selected aptamers exhibited no cytotoxic effects in cell culture

We evaluated cell viability using an MTT method to determine whether the selected aptamers were toxic to cells. The results showed that the viability of the GS cells was not significantly affected by any of the six selected aptamers at concentrations of 1 nM to 1 μM, suggesting that the aptamers were nontoxic to the fish cells (Fig. 4).

3.5. Inhibition effects of the aptamers on SGIV infection in cell culture

To explore whether the aptamers could inhibit SGIV infection, we incubated each of the six selected aptamers with SGIV, and

treated GS cells with the different aptamer-SGIV binding mixtures. As shown in Fig. 5A, the GS cells treated with the SELEX library or aptamers displayed normal growth, which is consistent with the results of the cell viability assays. In the presence of the aptamers, the replication of SGIV was partially inhibited. Significant CPE, including rounded and aggregated cells, were observed in the control groups treated with the SELEX library combined with SGIV or the SGIV alone (Fig. 5B). Compared to the control groups, all of the aptamers, except LMB-439, significantly reduced virus titers at 24 and 48 h postinfection (Fig. 5C). LMB-764 and LMB-761 exhibited the greatest inhibitory effects on viral replication, whereas LMB-439 exhibited the lowest level of protection among the tested aptamers.

3.6. Inhibitory effects of aptamers on SGIV infection in vivo

Based on the inhibitory effects of the aptamers in cell culture, LMB-764, LMB-761, and LMB-439 were chosen for use in the *in vivo*
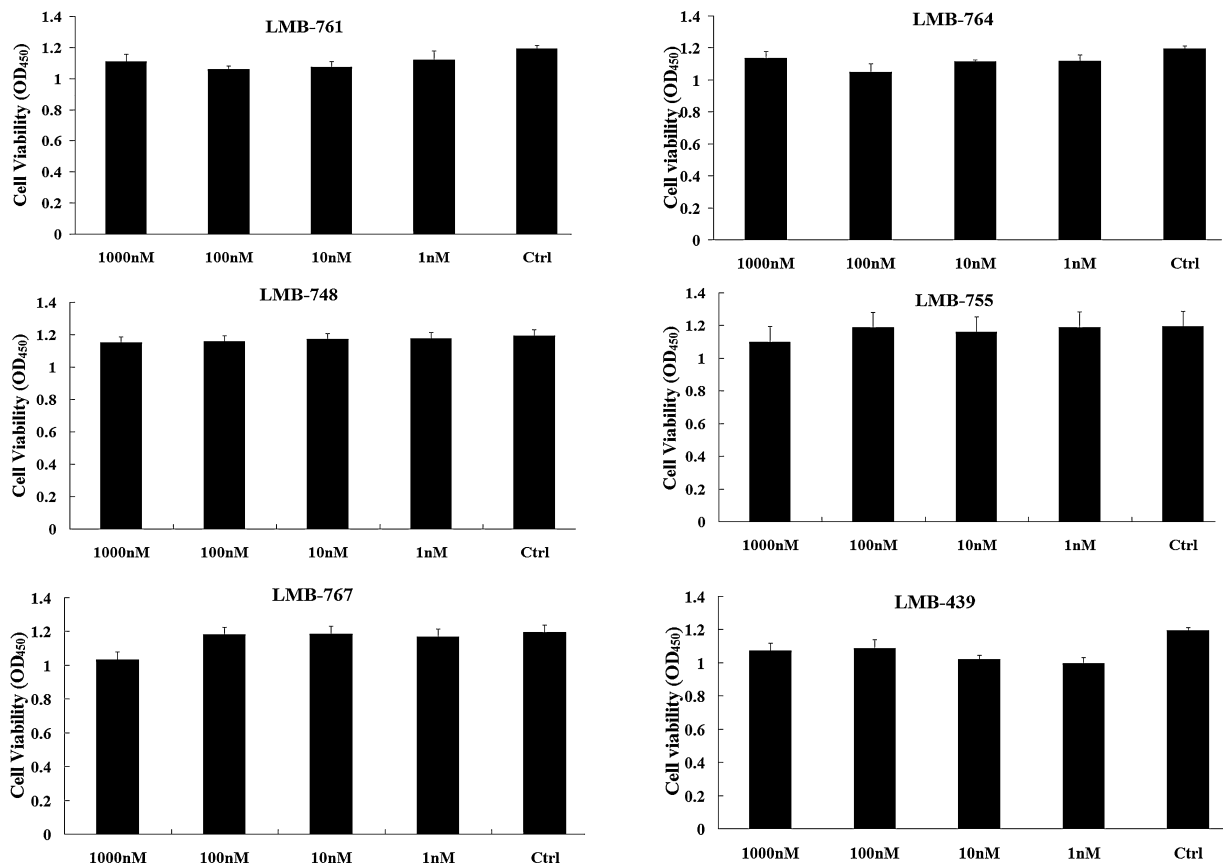

Fig. 4. Cytotoxicity of the DNA aptamers in GS cells. The results for each group are presented as the mean ± SD of three independent experiments.
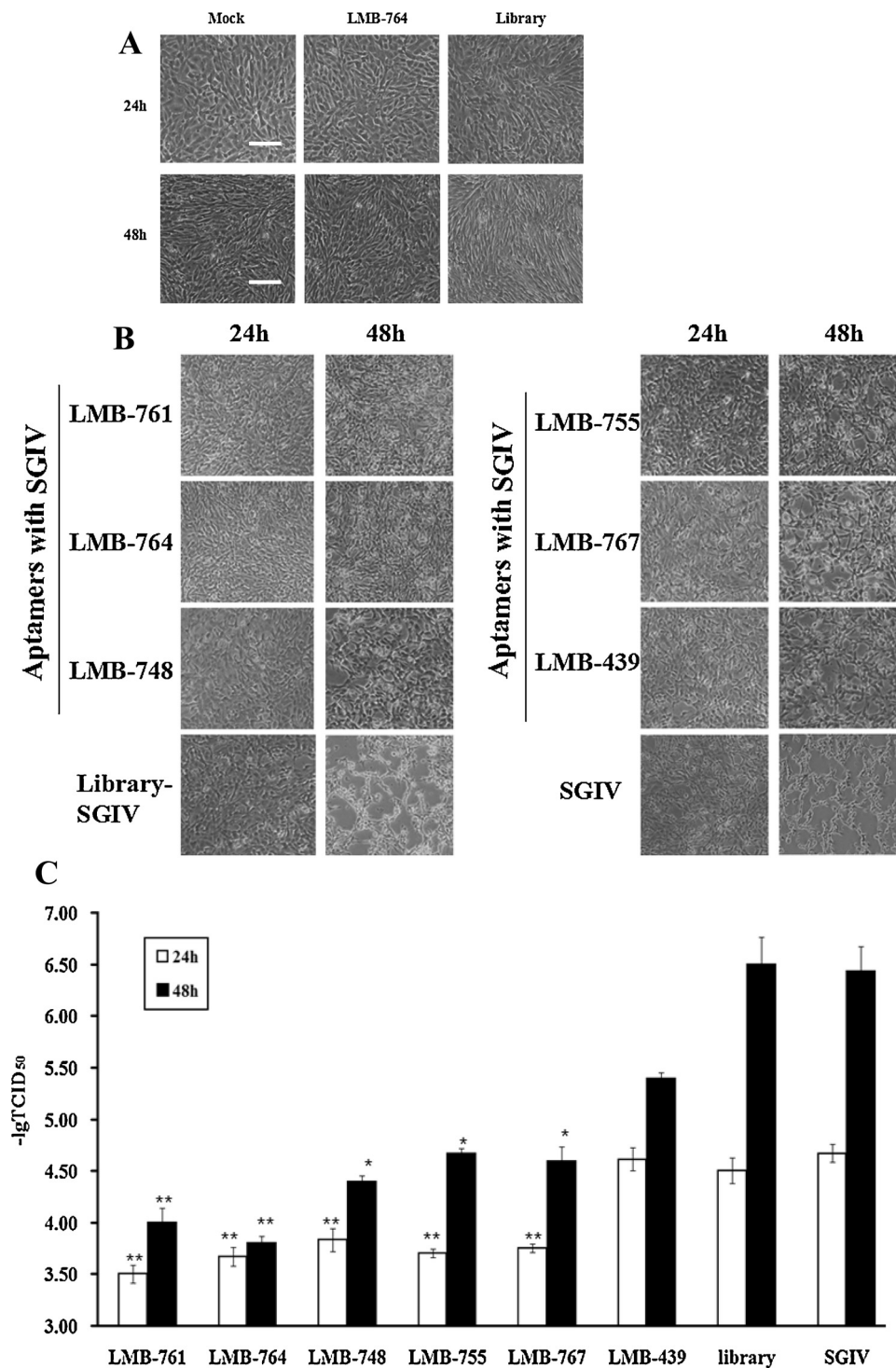
Fig. 5. Anti-SGIV ssDNA aptamers inhibit SGIV infection in cultured fish cells. (A) Morphology of GS cells following treatment with the SELEX library or selected aptamers. (B) Cell morphology and CPEs after treatment with SGIV previously incubated with or without selected aptamers. (C) Treatment with SGIV previously incubated with aptamers reduced virus titers in GS cells. The results for each group are presented as the mean ± SD of three independent experiments (**$P < 0.01$; *$P < 0.05$; bars = 100 μm).

experiments. Among the grouper treated with SGIV only, 10% mortality was observed on day 2 postinfection, and the cumulative mortality reached 90% on day 7 postinfection (Fig. 6). By contrast, in the groups treated with a binding mixture containing SGIV and LMB-761 or LMB-764, the cumulative mortalities were 50% and 60%, respectively, indicating that LMB-761 and LMB-764 provided protection against SGIV infection *in vivo*. Among the grouper treated with SGIV and LMB-439, the cumulative mortality reached 80%, indicating that LMB-439 provided a lower level of protection

against SGIV infection than that observed for LMB-761 and LMB-764.

3.7. Aptamers caused no histopathology in grouper, E. coioides

No fish died in all aptamer treatment and control groups after ten days post-injection. Compared to the negative controls and normal fish, the histological analysis revealed no pathological changes
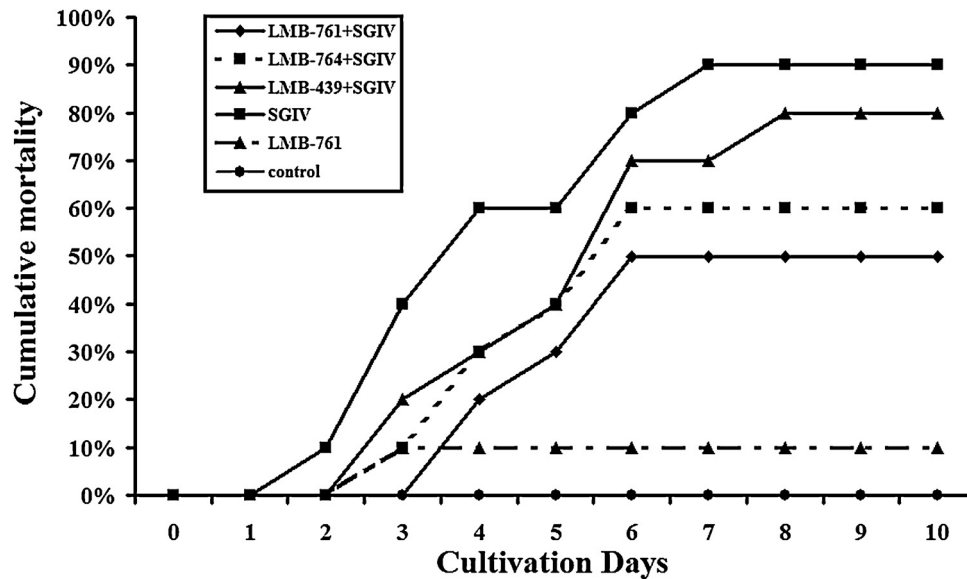
Fig. 6. Anti-SGIV ssDNA aptamers inhibit SGIV infection *in vivo*. The cumulative mortality of grouper, *E. coioides* intraperitoneally injected with an SGIV and aptamer mixtures, culture medium (control), or SGIV or aptamer alone was recorded daily for ten days.

in the all aptamer-injected fish liver and spleen, suggesting that the aptamers produced no cytotoxic effects *in vivo* (Fig. 7).

4. Discussion

The SGIV has caused high mortality to grouper aquaculture (Qin et al., 2003). Conventional therapeutic methods include chemical and antibiotic treatments. However, the development of drug resistance and adverse environmental effects of such treatments have raised concerns (Kümmerer, 2008). Vaccination has been shown to be an effective strategy for the prevention of viral diseases in

fish. However, no commercially prepared vaccine against SGIV is currently available. Hence, the development of an environmental friendly, commercially available anti-SGIV strategy would improve efforts to prevent outbreaks of SGIV infection.

In present study, ssDNA aptamers were generated using a SELEX iterative process and purified SGIV. Aptamers bind target molecules through various combinations of interactions between the structural features of each, including aromatic-ring stacking, electrostatic interactions, and hydrogen bonding (Hermann and Patel, 2000). The analysis of the secondary structures of the aptamers produced in our study revealed similarities in their
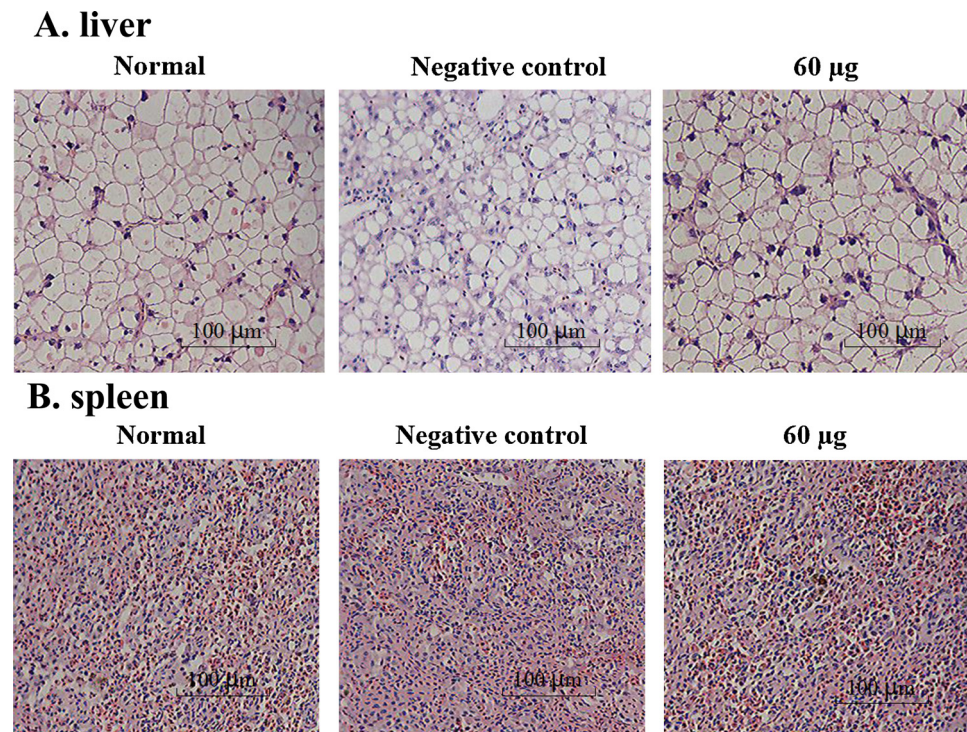

Fig. 7. Histological analysis of tissues. Paraffin-embedded thin sections of tissues collected from the (A) liver or (B) spleen of grouper, *E. coioides* intraperitoneally injected with 60 μg of aptamers and TN buffer, respectively, were stained with H&E to evaluate their cytotoxic effects *in vivo*.

stem-loop structures. These results indicate that these structural features may have formed the virus-binding sites that mediated the inhibition of SGIV infection observed for LMB-761 and LMB-764, which had similar ΔG values. By contrast, LMB-439 produced a lower level of protection against SGIV infection, and had the most distinct secondary structure, consisting primarily of a large loop.

The differences in the inhibitory effects of LMB-761, LMB-764, and LMB-439 also suggest that they may have different tertiary or quaternary structures, such as kinks, bulges, pseudo knots, and so on. A previous study revealed that aptamers bound their targets at 4 °C, but not at 28 °C, indicating that increasing temperature may change the secondary structure of aptamers, thereby limiting their application to disease control (Mallikaratchy et al., 2010). It is therefore important to generate aptamers with structures that remain stable under different experiment conditions. Previous studies have shown that aptamers bound their targets with high affinity and a high level of specificity, and were not immunogenic when introduced into organisms. The combination of such characteristics, suggests that aptamers might be useful for a variety of applications in basic pharmaceutical research, drug development, and medical therapy.

Aptamers have been developed against a number of pathogens, and are being evaluated as candidates for the development of antiviral therapeutics (Yan et al., 2005). Aptamers have generated promising results in animal studies and clinical trials, including studies of cancers and HIV infection (Zhou and Rossi, 2010). In this study, intact virus was used to select aptamers. In contrast to the purified protein-based SELEX method, intact virus-based SELEX can generate aptamers that bind unknown targets or protein complexes on the surface of the virus, which might have critical roles in viral infection. In addition, the natural structure and function of viral surface proteins are maintained using the intact-virus method of aptamer selection (Pan et al., 1995).

Porntep et al. (2012) reported that RNA-aptamers might bind to the antigenic receptors of VHSV, and prevent the virus from invading the host cell. Hwang et al. (2012) reported that HIRRV-RNA aptamers might bind to glycoproteins on the virus surface that mediate virus entry into the cell *via* receptor-mediated endocytosis. A DNA aptamer was also shown to block the receptor-binding region of the influenza hemagglutinin protein (Jeon et al., 2004). Aptamers obtained in this study bound SGIV with a high level of specificity, and reduced its replication in cultured fish cells. It is therefore possible that the aptamers developed in our study bound molecules on the surface of the SGIV. Future investigations of the binding sites of these anti-SGIV aptamers are warranted to identify the viral mechanisms that they disrupt upon binding.

The inhibitory effects of our aptamers on viral infection varied between the cell culture and *in vivo* experiments. Although LMB-761 and LMB-764 displayed significant inhibitory effects on viral infection in cultured GS cells, the level of protection they provided against SGIV infection *in vivo* was comparatively lower. These inconsistencies may have been caused by complex factors in the environment that were absent in cell culture. Such environmental factors might destabilize the structures of aptamers, degrade them, or inhibit aptamer-virus binding. To further explore the potential of our aptamers for inhibiting viral infection in *vivo*, our future studies will focus on delivering aptamers *via* the oral route. We will also optimize the aptamer dosage and enhance the stability of aptamers by introducing amino or fluoro modifications at the 2′ position of pyrimidines (Green et al., 1995; Ruckman et al., 1998; Zhou et al., 2009).

5. Conclusion

The DNA aptamers that bound SGIV with a high level of specificity were generated, and the selected aptamers could inhibit SGIV replication and infection in fish cell cultures and cultured fish *in vivo*. To our knowledge, this is the first report of the development of DNA aptamers targeting a fish virus. The aptamers produced in our study, especially LMB-761 and LMB-764, may represent candidate therapeutic agents for blocking SGIV infection in fish.

Acknowledgments

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