Selection and characterization of novel DNA aptamers specifically recognized by Singapore grouper iridovirus-infected fish cells

Pengfei Li,1,2 Shina Wei,1 Lingli Zhou,1,2 Min Yang,1 Yepin Yu,1,2 Jingguang Wei,1 Guohua Jiang3 and Qiwei Qin1,2

1Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, PR China
2University of Chinese Academy of Sciences, 19 Yuquan Road, Beijing 100049, PR China
3Analytical and Testing Center, Beijing Normal University, Xinjiekouwai Street, Beijing 100875, PR China

Singapore grouper iridovirus (SGIV) is a major viral pathogen of grouper aquaculture, and has caused heavy economic losses in China and South-east Asia. In this study, we generated four ssDNA aptamers against SGIV-infected grouper spleen (GS) cells using SELEX (systematic evolution of ligands by exponential enrichment) technology. Four aptamers exhibited high affinity to SGIV-infected GS cells, in particular the Q2 aptamer. Q2 had a binding affinity of 12.09 nM, the highest of the four aptamers. These aptamers also recognized SGIV-infected tissues with high levels of specificity. Protease treatment and flow cytometry analysis of SGIV-infected cells revealed that the target molecules of the Q3, Q4 and Q5 aptamers were trypsin-sensitive proteins, whilst the target molecules of Q2 might be membrane lipids or surface proteins that were not trypsin-sensitive. The generated aptamers appeared to inhibit SGIV infection in vitro. Aptamer Q2 conferred the highest levels of protection against SGIV and was able to inhibit SGIV infection in a dose-dependent manner. In addition, Q2 was efficiently internalized by SGIV-infected GS cells and localized at the viral assembly sites. Our results demonstrated that the four novel aptamers we generated were specific for SGIV-infected cells and could potentially be applied as rapid molecular diagnostic test reagents or therapeutic drugs targeting SGIV.

INTRODUCTION

*Epinephelus* is a genus of groupers, which are a commercially important and popular fish in China and South-east Asian countries (Marino et al., 2001). The grouper aquaculture industry has grown rapidly in recent years, but has been seriously threatened by viruses, bacteria and parasites. *Singapore grouper iridovirus* (SGIV) is a member of the genus *Ranavirus* within the family *Iridoviridae*. It was first isolated from the brown-spotted grouper *Epinephelus tauvina*. SGIV is considered a major pathogen of groupers and has caused heavy economic losses (Qin et al., 2001, 2003). Molecular probes for the specific diagnosis of SGIV infection and effective therapies against the virus are urgently needed.

Aptamers are synthetic nucleic acids, such as ssDNA or RNA, or protein ligands, which are selected and generated using SELEX (systematic evolution of ligands by exponential enrichment) technology. This technique was first reported in 1990 and has since been widely used for many applications (Ellington & Szostak, 1990; Syed & Per-vaiz, 2010). Aptamers usually have a distinct three-dimensional structure and are characterized by complex structural features, such as stem–loops, hairpins and pseudoknots (Zhou & Rossi, 2011, 2012). Aptamers are known to be highly specific, most with low immunogenicity, and some are non-toxic; therefore, they have been used as probes in diagnostics, pathogen detection and cancer research (Balogh et al., 2010; Bunka et al., 2010; Chou et al., 2005; Liang et al., 2011; Xiao & Farokhzad, 2012). In fish, with purified virus particles used as targets, RNA aptamers with therapeutic potential were generated against viral hemorrhagic septicemia virus and Hirame rhabdovirus (Hwang et al., 2012; Punnarak et al., 2012). In a previous study, we selected a panel of DNA aptamers against purified SGIV particles, and demonstrated their inhibitory effects on viral infection in vitro and in vivo (Li et al., 2014).

During virus replication, modifications occur in cell membranes during infection (Abós et al., 2015; Gerold & Pietschmann, 2014; Karst et al., 2015; Seeger & Mason,
2015; Verdaguer et al., 2014). Based on these modifications, or other markers of virus-infected cells, highly specific molecular probes could be designed. However, the number of known SGIV target molecules in cell membranes is low, thereby limiting the development of biomarker probes via traditional antibody-based techniques. Aptamers have been used to recognize targets on and within cell membranes via Cell-SELEX technology (Shangguan et al., 2008a, b; Simaeys et al., 2014; Yang et al., 2014). Shangguan et al. (2008a) applied Cell-SELEX technology to leukaemia research and found that protein tyrosine kinase 7 was highly expressed in a series of leukaemia cell lines. Simaeys et al. (2014) observed that the aptamer TOV6 was able to specifically recognize ovarian cancer cells and found that its target in the membranes of these cells was stress-induced phosphoprotein 1, which could then be used as a potential biomarker for ovarian cancer. Cell-specific aptamers could also be applied in targeted delivery by decorating aptamers on delivery vehicles for target cells, thereby decreasing potential toxicities of drugs and increasing the therapeutic efficacy (Zhou & Rossi, 2011). For example, Chu et al. (2006) reported the decoration of aptamers with small interfering RNA targeting prostate-specific membrane antigen.

In this study, a panel of DNA aptamers that specifically recognized SGIV-infected cells was generated. Internalization and the binding specificity of the aptamers were investigated in SGIV-infected cells in vitro and in SGIV-infected tissues in vivo. Aptamer-mediated cytotoxicity and the effects of aptamers on SGIV infection were evaluated in cultured fish cells and in the grouper Epinephelus coioides. Certain aptamers displayed high potential for use as molecular probes in diagnostics and as delivery vectors for drugs that control SGIV infection.

RESULTS

Cell-SELEX and characterization of ssDNA aptamers

Flow cytometry was used to monitor the progress of SELEX. As selection cycles were in progress, an obvious increase in fluorescence for SGIV-infected grouper spleen (GS) cells was observed (Fig. 1a), with no distinct changes in fluorescent intensity seen for uninfected cells (Fig. 1b). After 16 rounds of SELEX, the fluorescent intensity for SGIV-infected GS cells was maximal, indicating that this pool of aptamers displayed the highest specificity for SGIV-infected GS cells. Four ssDNA aptamers were isolated from this pool (Table 1). Aptamers Q2, Q3, Q4 and Q5 comprised 35, 31, 18 and 16 % of the aptamer pool, respectively. The Mfold program was applied to calculate secondary structures and free energy ($\Delta G$) values of Q2, Q3, Q4 and Q5. These aptamers contained stem–loop structures, with Q4 exhibiting the lowest $\Delta G$ value of $-25.14$ kJ mol$^{-1}$ (Fig. 1c).

Selected aptamers bound to SGIV-infected GS cells and tissues

Flow cytometry and fluorescence imaging results showed that the four aptamers were able to bind to SGIV-infected GS cells (Fig. 2a, b). The binding affinities of our aptamers were calculated using flow cytometry, with the four aptamers demonstrating a high affinity for SGIV-infected GS cells. The calculated binding affinities ($K_d$) for Q2, Q3, Q4 and Q5 were 12.09, 23.5, 25.6 and 24.35 nM, respectively (Fig. 2c).

We treated SGIV-infected GS cells with FITC-conjugated aptamers at 2, 4, 8 and 12 h post-infection (p.i.). According to the levels of fluorescence intensity recorded, binding of Q2, Q3, Q4 and Q5 occurred $\sim$8 h p.i. (Fig. 2d). These results were consistent with those for our reverse transcription (RT)-PCR assays targeting the late structural gene of SGIV MCP, which encodes the major structural protein of the virus (Fig. 2e). The four generated aptamers were able to detect and distinguish between SGIV-infected and uninfected cells from 8 h p.i. onwards. Fluorescence images indicated that the aptamers specifically bound to SGIV-infected cells of the spleen and liver (Fig. 2f, g) with a high level of specificity, but not to uninfected tissues, consistent with our flow cytometry and RT-PCR results (Fig. 2a, b).

Aptamers failed to exert cytotoxic effects in vitro and in vivo

We incubated Q2, Q3, Q4 and Q5 with cells, and then evaluated cell viability. We did not observe any significant changes between the control and treatment groups, even when the concentration of aptamers approached 1000 nM, suggesting that they were non-toxic to fish cells (Fig. 3a). At 10 days after the injection of aptamers, fish appeared healthy with no deaths recorded amongst the control or aptamer treatment groups. Compared with controls, no pathological changes were seen in the liver and spleen tissues of aptamer-injected fish, suggesting that the aptamers investigated in this study exert no cytotoxic effects in vivo (Fig. 3b).

Inhibitory effects of aptamers on SGIV infection in vitro

The GS cells exposed to the SELEX library or aptamers maintained normal growth characteristics and kinetics (Fig. 4a). Extensive cytopathic effects (CPEs) were observed in the GS cells treated with mixture of SGIV and the initial library or GS cells infected with SGIV only. When mixtures of aptamers (500 nM) and SGIV (m.o.i. 0.5) were simultaneously added to cells, CPE was markedly reduced, indicating that the aptamers partially inhibited virus replication (Fig. 4b). Virus titres of untreated, SGIV-infected cells or SGIV-infected cells treated with the unselected library reached $10^{6.53}$ and $10^{6.54}$ TCID$_{50}$ ml$^{-1}$, respectively, whereas those treated with Q2, Q3, Q4 and
Q5 were $10^{5.33}$, $10^{5.35}$, $10^{5.99}$ and $10^{6.07}$ TCID$_{50}$ ml$^{-1}$, respectively (Fig. 4c). Amongst the four aptamers, Q2 displayed the best inhibitory effects and reduced the titre $\geq$10-fold. Furthermore, using Q2, we observed that inhibition of SGIV infection took place in a dose-dependent manner (Fig. 4d).

Table 1. Identification of ssDNA aptamers

<table>
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<tr>
<th>Aptamer</th>
<th>Central randomized sequences</th>
<th>Frequency (%)</th>
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<tr>
<td>Q2</td>
<td>TATGTCCATGGCCGATATTGGGAAGGTTGGTTTGGACTATGTGGAAGTT</td>
<td>35</td>
</tr>
<tr>
<td>Q3</td>
<td>TGGCTCTTTATGTTTTTGGGAGGGTTGGCTCGTATGT</td>
<td>31</td>
</tr>
<tr>
<td>Q4</td>
<td>TTTCGTGTTATGCTCCTCTTTATTGTCAGCTGAGTTTCTGCAGTG</td>
<td>18</td>
</tr>
<tr>
<td>Q5</td>
<td>TATTCGGGTTATTGCTCCTCTTTATTGTCACCTGGATGATGTATGATG</td>
<td>16</td>
</tr>
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Frequency indicates the percentage of the pool comprised by each aptamer after 16 rounds of selection.
Novel DNA aptamers against SGIV-infected cells

(a) Flow cytometry analysis of Q2 and Q3.
(b) Fluorescence microscopy images of SGIV-infected GS cells compared to normal GS cells.
(c) Binding affinity curves for Q2 and Q3.
(d) Flow cytometry analysis of Q4 and Q5.
(e) Relative mRNA expression for Q2, Q3, Q4, and Q5.
(f) Fluorescence microscopy images of SGIV-infected spleen cells compared to normal spleen cells.
(g) Fluorescence microscopy images of SGIV-infected liver cells compared to normal liver cells.
SGIV is the most serious pathogen of grouper aquaculture, resulting in high mortality rates (Qin et al., 2001; 2003). Currently, no commercial vaccine against SGIV is available; therefore, effective strategies to combat SGIV infection are required. Highly sensitive and specific aptamers have been applied in many different ways (Bunka & Stockley, 2006). Aptamers have been developed as promising candidates for antiviral therapeutics, given the impressive results seen in animal-based studies and clinical trials (Bunka & Stockley, 2006; Bunka et al., 2010; Zhou & Rossi, 2011, 2012). In the current study, four ssDNA aptamers targeting SGIV-infected GS cells were generated successfully. To the best of our knowledge, this is the first report of DNA aptamers being developed against a viral pathogen of commercially important fish.

Aptamers have complex three-dimensional structures, such as hairpins and stem–loops, which are maintained by hydrogen bonding, electrostatic interactions and hydrophobic forces. These complex structures form the basis through which aptamers are able to bind tightly to their targets (Ellington & Szostak, 1990). By competing for targets, aptamers could play a role in affecting and interfering with various interactions, such as virus–cell attachment. Hwang et al. (2012) speculated that aptamers bind to glycoproteins, which are the only proteins exposed at the surface of rhabdoviruses, and then block entry of the virus into the host cell. Aptamers are highly specific molecular probes: the fluorophore added to the aptamers may affect the three-dimensional structures and the specificity/affinity of aptamer binding to targets. In order to eliminate the possible effects of fluorophore labelling on the three-dimensional structures of aptamers, we used fluorophore-labelled primers to amplify each round of the selected pool by PCR; then fluorophore-labelled sense ssDNAs were used in the next selection until specific aptamers were selected. The aptamers selected in this study had stem–loops in their putative secondary structures. Q4 had the lowest ΔG value of −25.14 kJ mol⁻¹, indicating that its secondary structure was the most stable of the four aptamers. As aptamers are able to inhibit viral infection through certain mechanisms, we speculated that the stem–loops of the selected aptamers formed target-binding sites, which allowed aptamers to bind to targets and interfere with SGIV infection.

The key to developing therapeutic reagents for in vivo application lies in reducing the side-effects of non-specific drug targeting. Aptamers can act as highly specific probes and are promising candidates to guide therapeutic reagents to target cells (Chu et al., 2006; Xiao et al., 2008; Zhang et al., 2012). Our findings confirmed that aptamers Q2, Q3, Q4 and Q5 had a high affinity for SGIV-infected GS cells and SGIV-infected grouper tissues. To the best of our knowledge, these are the first ssDNA aptamers to target SGIV-infected cells, and could inhibit SGIV infection given that they recognize SGIV-infected cells in vitro and...
Fig. 3. Cytotoxicity analysis of aptamers Q2, Q3, Q4 and Q5. (a) After being incubated with each aptamer at various concentrations (1–1000 nM), cell viability was analysed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method; uninfected cells that were not treated with aptamers served as control. The results proved that the selected aptamers exhibited no cytotoxic effects in cell cultures. Results for each group are presented as the mean±SD of three independent experiments. (b) Treatment with the aptamers did not result in any cytotoxic effects in vivo. Bar, 20 μm.
Fig. 4. Aptamers Q2, Q3, Q4 and Q5 inhibited SGIV infection in vitro. (a) GS cells treated with aptamers or the initial library. (b) Light microscopy images of cells, showing obvious CPE. (c) The virus titres of the control groups of SGIV treated with library or SGIV only reached $10^{6.53}$ and $10^{6.46}$ TCID$_{50}$ ml$^{-1}$, respectively. Treatment of SGIV with each of the aptamers resulted in a significant reduction of virus titres at 48 h p.i., $10^{5.35}$, $10^{5.99}$ and $10^{6.07}$ TCID$_{50}$ ml$^{-1}$ for Q2, Q3, Q4 and Q5, respectively. Results for each group are presented as the mean $\pm$ SD from three independent experiments. **$P<0.01$; *$P<0.05$. Bar, 100 μm. (d) Inhibitory effects of Q2 on SGIV infection at various concentrations. Q2 was able to inhibit SGIV infection in a dose-dependent manner. Bar, 100 μm.
in vivo. The four ssDNA aptamers we generated for this study could be developed commercially for use as detection probes for the early and rapid diagnosis of SGIV infection.

The molecular characteristics of cells infected by virus, especially at the proteomic level, are critical in understanding viral pathogenesis and designing targeted therapies (Gerold & Pietschmann, 2014; Karst et al., 2015; Seeger & Mason, 2015; Verdaguer et al., 2014). Our aptamers recognized SGIV-infected cells as early as 8 h p.i., indicating that the targets of Q2, Q3, Q4 and Q5 were apparent at the surface of SGIV-infected cells from 8 h p.i. at least. mAbs have been made for isolating target proteins. Aptamers also have been used to recognize targets on and within cell membranes via Cell-SELEX technology (Shangguan et al., 2008a, b; Simaeys et al., 2014; Yang et al., 2014). Yang et al. (2014) applied aptamers in acute myelogenous leukaemia detection and determined the target protein of aptamer K19 was sialic acid-binding Ig-like lectin 5 (Siglec-5), which could then be used as a potential biomarker for acute myelogenous leukaemia. Shangguan et al. (2008a) applied Cell-SELEX technology to leukaemia research and found that protein tyrosine kinase 7 was highly expressed in a series of leukaemia cell lines. Further analysis following protease treatment of cells revealed that the target molecules of Q2 were likely membrane lipids or surface proteins that were not sensitive to trypsin. The target molecules of Q3, Q4 and Q5 might be directly or indirectly related to surface proteins anchored on the infected cell membrane, which were trypsin-sensitive. As aptamers could block virus infection after interaction with virions, it was possible that targets recognized by aptamers may be virus-encoded proteins, which appeared on the membranes of infected cells and subsequently became part of the SGIV envelope during its release. More work should be done to identify the targets of aptamers in further studies. Our aptamers specifically recognized different types of SGIV-infected cells (GS and FHM cells); therefore, we speculated that SGIV could invade different cells through similar pathways or that it causes similar modifications in the membranes of infected cells (data not shown), which was consistent with Wang et al. (2014) who reported that SGIV invaded cells by macropinocytosis and endocytosis mediated by clathrin. Future investigations into Q3, Q4 and Q5 are warranted to identify their target proteins on the surface of SGIV-infected cells.

We also analysed the cellular internalization of Q2. We first revealed that the internalization of Q2 was active, because the internalization could be blocked by incubation at 4 °C (Fig. 6a). It was further verified by the flow cytometry results in Fig. 6(b) that fluorescence values of Q2 incubated with SGIV-infected cells at 28 °C were higher than those at 4 °C, which means Q2 not only bound to

![Fig. 5. Characterization of target molecules recognized by aptamers. (a) Changes in fluorescence intensities of aptamers that bound to SGIV-infected GS cells treated with trypsin. Black, FITC-initial library that bound to SGIV-infected cells; red, FITC-aptamers bound to SGIV-infected cells; green, FITC-aptamers bound to trypsin-treated SGIV-infected cells. (b) After being incubated with FITC-aptamers, the SGIV-infected cells were trypsinized and then imaged with a fluorescence microscope. Left, bright-field images; right, fluorescence images. Bar, 100 μm.](http://jgv.microbiologyresearch.org)
the targets on the SGIV-infected cell membrane, but also internalized into the cells; thus, we could be sure that internalization of aptamer was active. Our results show that Q2 was efficiently and specifically internalized by SGIV-infected GS cells for 2 h at 28 °C (Fig. 6c). Internalized Q2 was located around viral assembly sites (Fig. 6a). The four aptamers did not exhibit cytotoxicity in vitro and in vivo, consistent with previous reports (Li et al., 2014; Liang et al., 2012). Therefore, it would appear that Q2 could be safely applied for the treatment of SGIV infection by including this aptamer on delivery vehicles, thereby reducing side-effects and increasing therapeutic efficacy (Chu et al., 2006; Xiao et al., 2008; Zhang et al., 2012).

Fig. 6. Cell-specific internalization of aptamer Q2. (a) Fluorescence images of TAMRA-Q2 following incubation with SGIV-infected cells for 2 h at 28 °C. (b) Fluorescence intensities of TAMRA-Q2 following incubation with SGIV-infected cells for 2 h at 28 °C (green). Library (200 nM) incubated with infected cells at 28 °C for 2 h (black), Q2 (200 nM) incubated with normal GS cells at 28 °C for 2 h (blue), Q2 incubated with SGIV-infected cells for 2 h at 4 °C (red) served as controls. (c) Internalization kinetics of Q2. Cells were incubated with FITC-aptamers (200 nM) at 28 °C for 10, 20, 30, 60, 90 and 120 min; uninfected GS cells incubated with FITC-aptamers (200 nM) at 4 °C for 10, 20, 30, 60, 90 and 120 min served as controls. The fluorescence values of Q2 at each time point proved that aptamer Q2 could be internalized by SGIV-infected cells at a constant rate for 2 h. Bar, 20 μm.
In conclusion, the DNA aptamers that we generated were able to bind to SGIV-infected cells with a high level of specificity. The selected aptamers were able to recognize SGIV-infected cells in vitro and in vivo, and could inhibit SGIV infection in fish cell cultures. We also showed that aptamer Q2 was directly internalized by SGIV-infected cells without external assistance. To the best of our knowledge, the results from our current study are the first to report the development of DNA aptamers that target fish cells infected with SGIV. The aptamers produced in our study represent candidates for understanding SGIV pathogenesis, drug development and therapies against SGIV infection.

**METHODS**

**Viruses and cell lines.** SGIV strain A3/12/98 was propagated in GS cells (Qin et al., 2001, 2003). GS and FHM cells were grown and maintained in Leibovitz’s L-15 medium supplemented with 10 % FBS (Life Technologies) at 28 °C, as described previously (Huang et al., 2011a; Qin et al., 2006). TCID₅₀ was used as an indication of virus titre (Reed & Muench, 1938).

**Initial library and primers for SELEX.** The SELEX library was constructed by Sigma-Aldrich as described previously (Li et al., 2014). We used 44.7 mol ssDNA and two primer-hybridization sequences (5′-GACGCTTACTCAGGTGTGACTCG-3′ and 5′-CGAAGGACCGCAGATGAAGTCTC-3′), with a central randomized 50 nt sequence (N₅₀). The 5′ primer was labelled with FITC or TAMRA (5′-FITC-GACGCTTACTCAGGTGTGACTCG-3′ or 5′-TAMRA-GACGCTTACTCAGGTGTGACTCG-3′), whilst the 3′ primer was biotinylated (5′-biotin-GAGACTTCTCAGGCTCCFTCG-3′).

**SELEX protocol.** The ssDNA aptamers were selected based on the SELEX protocol described by Shangguan et al. (2006) with some modifications. GS cells were grown to 100 % confluence in 60 mm cell culture dishes (Corning) and infected with SGIV at m.o.i. 5 at 28 °C for 24 h. For the first cycle of selection, ssDNA (10 nmol) was denatured at 94 °C for 10 min and cooled on ice for 10 min. The ssDNA was then dissolved in 1 ml binding buffer (5 g glucose l⁻¹, 10 % PBS (Life Technologies), 1.0 g BSA l⁻¹ (Solarbio), 0.1 mg yeast tRNA ml⁻¹ (Invitrogen) and 5 mM MgCl₂) and cooled on ice for 10 min. We then incubated ssDNA with SGIV-infected GS cells for 60 min at 4 °C and 60 r.p.m. Cultures were then washed (5 g glucose l⁻¹, 9 g NaCl l⁻¹, 10 mM Tris/HCl and 5 mM MgCl₂), and cells were collected and heated to 95 °C for 5 min to dissociate the bound ssDNA. Dissociated aptamers were amplified by PCR. The thermal cycling profile used in these PCRs involved 25 amplification cycles (94 °C for 1 min, 60 °C for 30 s, 72 °C for 45 s), followed by a final extension step at 72 °C for 5 min. The resulting PCR products were heated to 95 °C for 5 min and then cooled immediately on ice for 10 min. Pierce streptavidin magnetic beads (100 μl; TakaraBio) were added to separate the biotin-conjugated antisense ssDNA from the sense ssDNA in a MiniMACS Separator (Miltenyi Biotec). Sense ssDNAs were collected and used for further selection. To enhance the specificity and affinity of candidate aptamers to be selected, we decreased the incubation time, and serially diluted the quantity of infected cells and ssDNA. From the fourth cycle and the subsequent selection cycles, we incorporated counter-selection into the process. Counter-selection was performed by incubating candidate DNA aptamers with uninfected GS cells for 20 min at 4 °C and 60 r.p.m.; the supernatant was then collected for the next round of selection.

**Cloning and sequencing of DNA aptamers.** The ssDNA pool was collected and subjected to PCR with unlabelled primers after 16 rounds of selection. Amplicons were ligated into the pMD18-T vector (TaKaRa Bio) and transformed into Escherichia coli DH5α, as described previously (Shangguan et al., 2006). We picked 100 clones for sequencing, with DNA sequences that were present in more than two clones serving as aptamer candidates; FITC-aptamers and TAMRA-aptamers were then synthesized by Life Technologies. The secondary structure of the aptamers was predicted using Mfold (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form), as described previously (Li et al., 2014).

**Flow cytometry.** We used flow cytometry to evaluate the specificity of aptamer candidates (Li et al., 2012). The FITC-labelled ssDNA pool was denatured at 94 °C for 10 min and cooled on ice for 10 min. After washing three times with PBS (2 mM KH₂PO₄, 10 mM Na₂HPO₄, 137 mM NaCl and 1 ‰ NaN₃), 5 × 10⁵ SGIV-infected cells were incubated with the FITC-labelled ssDNA pool, in 200 μl binding buffer on ice for 40 min. Cells were then washed twice with PBS and resuspended in 400 μl PBS. FITC fluorescence was assessed with a FACScan (BD) by counting 20 000 events. The initial FITC-labelled ssDNA library and uninfected GS cells serving as controls.

**Fluorescent imaging of cells.** We used fluorescence microscopy to evaluate the specificity of aptamer candidates (Shangguan et al., 2008a). Following denaturation at 94 °C for 10 min and cooling on ice for 10 min, FITC-labelled aptamers (300 nM) were incubated with SGIV-infected cells seeded on coverslips within six-well plates at 4 °C for 1 h in the dark. Cells were fixed with 4 % paraformaldehyde and then washed with PBS three times. Fluorescence was determined with a Leica DMRXA fluorescence microscope, with the initial FITC-labelled ssDNA library and uninfected GS cells serving as controls.

**Experimental infection of groupers and fluorescent imaging of tissue sections.** Groupers (E. coioides) were infected with SGIV as described previously (Li et al., 2014). There were three groups and each treatment group contained 30 fish. Prior to the commencement of the experiment, fish were made to fast for 24 h. Fish in the first group were intraperitoneally injected with 100 μl SGIV (10⁶ TCID₅₀ ml⁻¹). Fish received an intraperitoneal injection of PBS (100 μl) and normal fish without any treatment served as control groups. Each group was transferred to a separate aquarium supplied with running seawater and ample aeration for 10 days. Fish in each group were anaesthetized in ethyl 3-aminobenzoate methanesulfonate solution (40 mg l⁻¹). Liver and spleen tissue specimens were collected and fixed in 10 % neutral-buffered formalin for 24 h. Fixed tissues were then frozen and stored. TAMRA-labelled aptamers (300 nM) were incubated with frozen tissue sections at 4 °C for 1 h in the dark and then washed with PBS. The TAMRA-labelled initial library (300 nM) incubated with frozen tissue sections served as control. Fluorescence in tissue sections was visualized with a Leica DMRXA fluorescence microscope. Our animal experiment was conducted in accordance with the guidelines issued by the Ethics Committee of the University of Chinese Academy of Sciences.

**Aptamer binding affinity analysis.** The binding affinity of aptamers was assessed using a protocol described by Tang et al. (2009), with some modifications. FITC-labelled aptamers at various concentrations (0–2000 nM) were incubated with SGIV-infected GS cells in the dark for 40 min on ice. FITC-labelled aptamers were also incubated with uninfected GS cells to serve as controls. Cells were washed with PBS and the mean fluorescence value for each aptamer candidate was determined with a FACSscan by counting 20 000 events. After subtracting the mean fluorescence values for the control groups according to the following equation:

$$ Y = B_{\text{max}} X / (K_D + X) $$
SigmaPlot was used to calculate $K_d$ for interactions between aptamers and SGIV-infected GS cells (Liang et al., 2013). Results for each aptamer are presented as the mean ± SD of three independent experiments.

**Cytotoxicity and histological analysis.** Cytotoxicity analysis of the selected aptamers was based on assays described by Liang et al. (2012). GS cells in 96-well plates were incubated with each aptamer at various concentrations (1–1000 nM) at 28 °C for 48 h. Uninfected GS cells that were not treated with aptamers served as the control group. To assess cell viability, 20 μl MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (TaKaRa Bio) was added to each well and allowed to incubate for 4 h at 28 °C. The $A_{570}$ in each well was measured using a microplate reader (Thermo). Results from at least three independent assays were used for each aptamer.

Histological analysis of the four aptamers was performed as described previously (Li et al., 2014). Liver and spleen tissue specimens taken after the animal experiment, described above, were fixed with 10% neutral-buffered formalin, embedded in paraffin and sectioned. Sections were stained with haematoxylin and eosin for histological analysis.

**In vitro inhibition of SGIV infection by aptamers.** Assessment of the inhibitory effects of each aptamer upon SGIV infection was performed as described previously (Liang et al., 2012; Li et al., 2014). GS cells were cultured in 24-well plates. Each aptamer (500 nM) and SGIV (m.o.i. 0.5) were simultaneously added to GS cells. The SELEX library (500 nM) and SGIV (m.o.i. 0.5) simultaneously added to cells served as the control (library group). In addition, SGIV (m.o.i. 0.5) alone added to cells was served as another control. At 48 h p.i., light microscopy was used to examine GS cells and identify any CPE. Culture supernatant and cells from each well were collected to determine the virus titre according to the method of Reed & Muench (1938). Data from three independent experiments were used to quantify the effects of each selected aptamer on virus infection.

**Quantitative PCR assays.** We used RT-PCR assays to determine the presence of SGIV in samples, with 18S mRNA serving as an internal control (Huang et al., 2011b). Uninfected normal GS cells and SGIV-infected cells in 24-well plates were collected at 2, 4, 8 and 12 h p.i. An SV Total Isolation kit (Promega) was used to extract total RNA according to the manufacturer’s instructions. RNA was transcribed into cDNA using a ReverTra Ace kit (Toyobo). We used oligonucleotide primers (5’-GGCAACGGGAGCACTA-3’ and 5’-GCACGCTTCTCTCACCTTCA-3’) specific for the gene (ORF072) encoding the SGIV MCP protein.

**Trypsin treatment of SGIV-infected GS cells.** We digested 5 × 10⁶ SGIV-infected GS cells with 1 ml 0.25% trypsin (Thermo Scientific HyClone) at 28 °C for 10 min. We used L15 medium supplemented with 10% FBS to inhibit the activity of trypsin. After washing with PBS, trypsin-treated cells were incubated with FITC-labelled aptamers. Fluorescence was assessed with a FACSscan cytometer by counting 20 000 events as described previously (Xiao et al., 2008).

**Determination and visualization of aptamer internalization.** The internalization of aptamers was performed as described previously, with some modifications (Xiao et al., 2008; Zhang et al., 2012). GS cells were grown to 100% confluence on coverslips placed in the wells of six-well plates. Cells were infected with SGIV (m.o.i. 10) for 8 h at 28 °C. After washing with PBS, cells were incubated with TAMRA-aptamers (200 nM) in L15 medium at 28 °C for 2 h. Cells were then stained with Hoechst 33342 (Sigma-Aldrich) at a final concentration of 1 μg ml⁻¹. After being quickly washed with NaOH (200 mM), the cell surface receptors were removed, and cells were then washed with PBS and visualized with a Leica DMRXA fluorescence microscope. Infected cells incubated with TAMRA-aptamers (200 nM) at 4 °C for 2 h, normal GS cells incubated with TAMRA-aptamers (200 nM) at 28 °C for 2 h and infected cells incubated with TAMRA-library (200 nM) at 28 °C for 2 h served as controls.

The internalization of aptamers was also proved by flow cytometry. GS cells were cultured to 100% confluence in six-well plates and then infected with SGIV (m.o.i. 10) for 8 h at 28 °C. After washing with PBS, cells were incubated with FITC-aptamers (200 nM) in L15 medium at 28 °C for 2 h. Infected cells incubated with FITC-aptamers (200 nM) at 4°C for 2, 20, 30, 60, 90 and 120 min. Cells were washed with PBS at 4°C and placed on ice; the internalization of aptamers into live cells was stopped at various time points. Fluorescence was assessed with a FACScan cytometer by counting 20 000 events. Uninfected GS cells incubated with FITC-aptamers (200 nM) at 4°C for 10, 20, 30, 60, 90 and 120 min served as controls. Following subtraction of mean fluorescence values for control groups, we examined the kinetics of aptamer internalization. All experiments were repeated at least three times.

**Statistical analysis.** Statistical analysis of data was performed with SPSS version 13.0 (IBM). Inter-group differences were compared using one-way ANOVA. $P<0.05$ was considered statistically significant.

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Novel DNA aptamers against SGIV-infected cells


