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BRIEF REPORT

Identification of (-)-epigallocatechin-3-gallate as a potential agent for blocking infection by grass carp reovirus

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Abstract Grass carp reovirus (GCRV), the representative strain of the species *Aquareovirus C*, serves as a model for studying the pathogenesis of aquareoviruses. Previously, epigallocatechin gallate (EGCG) was shown to inhibit orthoreovirus infection. The aim of this study was to test its potential in blocking infection by GCRV. We show that adhesion to the CIK (*Ctenopharyngodon idellus* kidney) cell surface by GCRV particles is inhibited in a dose-dependent manner by EGCG, as well as by a crude extract of green tea. We also evaluated the safety of EGCG and green tea extract using CIK cells, and the results suggest that EGCG is a promising compound that may be developed as a plant-derived small molecular therapeutic agent against grass carp hemorrhagic disease caused by GCRV infection. As the ligand for the 37/67-kDa laminin receptor (LamR), EGCG's blocking effect on GCRV attachment was associated with the binding potential of GCRV particles to LamR, which was inferred from a VOPBA assay.

Keywords EGCG · Grass carp · GCRV · Reovirus

Introduction

Aquareoviruses have been isolated from a number of fish and shellfish species, and genetic analysis has revealed great variability among isolates. Hemorrhage is a typical pathogenic effect resulting from aquareovirus infection, but the majority of aquareoviruses isolated from apparently healthy individuals have low pathogenicity [22]. Grass carp reovirus (GCRV), the type strain of the species *Aquareovirus C*, is currently one of the most virulent pathogens for grass carp (*Ctenopharyngon idellus*) [41], the most economically important fish species cultivated in China, with an annual production of more than 3.6 million metric tons. The frequent outbreaks of hemorrhagic disease due to GCRV infection have resulted in significant economic losses in the grass carp cultivation industry [1, 7]. Because grass carp plays a very important role in the Chinese aquaculture industry, the vaccination approach has been heavily investigated as a means of GCRV control [20, 26, 32]. Vaccination of grass carp with inactivated virions or attenuated live virus is effective in preventing viral hemorrhagic disease in grass carp, but it has many limitations, such as regional variability, high costs, and the fact that it is difficult to administer in an aquatic environment [15, 16, 20, 32]. So far, more than 20 strains of GCRV have been reported, and ten of them have known complete genome sequences [26]. Additionally, Wang et al. [37] reported the co-existence or co-infection of two genetically distant genotypes. Based on current evidence, vaccination strategies against one genotype of GCRV would be not adequate in disease prevention. Thus, there is a clear need to develop effective anti-GCRV drugs, which should be environmentally friendly.

GCRV is a double-stranded RNA virus belonging to the genus *Aquareovirus*, family *Reoviridae* [9, 25]. *Aquareovirus* and *Orthoreovirus* are the two dominant genera in the family *Reoviridae*. Most recently, EGCG has been reported to inhibit various virus infections including orthoreovirus infection [5]. It is our interest to test the inhibitory effect of EGCG on GCRV, which is the

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prototype aquareovirus. Polyphenolic compounds known as catechins are major active ingredients of green tea. EGCG is the major catechin found in green tea, which accounts for approximately 50 % of the total catechins. Catechin has demonstrable benefits, including antitumor, anti-oxidative, and antiviral effects [17]. Green tea is produced from the leaves of the evergreen plant *Camellia sinensis* [28]. Various antiviral activities have been reported for EGCG. It has been reported to be mediated mainly by preventing the envelope glycoproteins of viruses, including HSV and HCV, from binding to their surface receptors [17]. Studies have shown that GTE (green tea) and EGCG are efficient HBV inhibitors, but EGCG alone is not as efficient as GTE [38]. EGCG acts as a strong inhibitor of HIV replication in cultured peripheral blood cells [8]. Distinct antiviral effects of EGCG have been reported for influenza virus. The antiviral effect of catechins on influenza virus is mediated not only by interfering with hemagglutination; the compounds also exert inhibitory effect on neuraminidase and affect viral RNA synthesis at high concentration [28]. We investigated the potential of EGCG to block infection by GCRV, which might function by inhibiting viral attachment.

The CIK cell line, derived from the kidney of grass carp (*Ctenopharyngon idellus*) [42] was cultured in a 25-cm^2 cell culture flask in medium 199 (GibcoBRL) supplemented with 10 % fetal bovine serum (GibcoBRL) and 1 % penicillin-streptomycin. The cells were incubated at 27 °C without additional CO_2. The GCRV-JX01 strain, which is adapted to grow in CIK cells, was used in all experiments. The purified GCRV particles were prepared as reported previously [15], and the titer was determined by a plaque assay [35]. EGCG compounds were obtained from Sigma-Aldrich Shanghai Trading (China). GTE (green tea) compounds and the crude green tea extract were purchased from Tian Yuan Company (China). Other chemicals were purchased from Sang-Gon Chemical Reagents Company (China). The structures of EGCG and GTE compounds were confirmed by HPLC (high-performance liquid chromatography). HPLC analysis was performed as described [3] with an Agilent 1100 system consisting of a double pump, an auto-injector, a column temperature tank, and a UV radiation detector with the excitation and emission wavelengths set at 280 nm. Cellular viability studies were performed 1 h after EGCG or GTE treatment by cytofluorimetric assay as described previously [34]. Cytofluorimetric assay was performed using a Muse Count & Viability Kit (Millipore). The effect of EGCG and GTE compounds on GCRV was tested as follows: CIK cells were grown in six-well plates and incubated until the cell number reached 1×10^6 cells. Three extra wells were prepared for counting cells in the same way. Cell counts were determined by using a Muse Cell Analyzer (Millipore) according to the manufacturer's protocol. The cells were then incubated with the small molecule EGCG, GTE or vehicle at various doses at 4 °C for 1 h. After the incubation, the cells were washed twice with PBS and then exposed to GCRV at a multiplicity of infection MOI of 1 for 60 min. Thereafter, the supernatant was replaced with fresh medium, and the infected cells were incubated for a further 24 h. The cell monolayers were viewed under a light microscope, and the level of infectious virus in the growth medium was assayed by a standard $TCID_{50}$ assay [15]. Virus binding inhibition assay was performed as

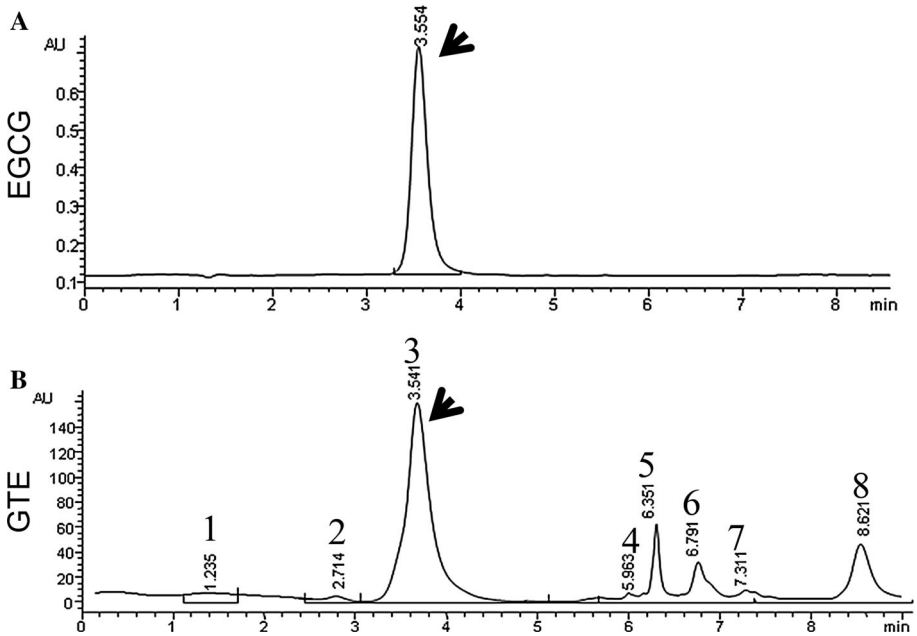

Fig. 1 Reverse-phase HPLC analysis of EGCG (A) and GTE (B). The detection wavelength was 280 nm. Arrows indicate the highest peak

follows: A total of 10^4 CIK cells were grown in 24-well microplates. The cells were incubated at 4 °C for 1 h at various doses of EGCG, GTE or vehicle. After the incubation, cells were washed twice with PBS, and viral adsorption was measured using purified GCRV-JX01 virus at an MOI of 10 at 4 °C for 1 h. After viral adsorption, extracellular viruses were removed by washing three times with PBS. The cells were then fixed with 4 % paraformaldehyde in PBS for 30 min at room temperature. The cells were blocked for 30 min in PBS containing 5 % goat serum. The cells were then analyzed by IFA assay with an anti-VP5 polyclonal antibody at 1:100 dilution [15]. The experiment was performed with three biological replicates.

The structures of EGCG and GTE were confirmed by HPLC. Fig. 1A shows that the retention time of standard EGCG was 3.554 min. At least eight components were present in the GTE sample according to HPLC. The highest peak was the third one, which had the same retention time as the standard sample of EGCG (Fig. 1B). These data indicated that the main component of GTE was EGCG. As shown in Fig. 2A and Fig. 3A, EGCG and GTE had low toxicity to CIK cells. The concentration of EGCG and GTE that were used in this study did not alter cell viability.

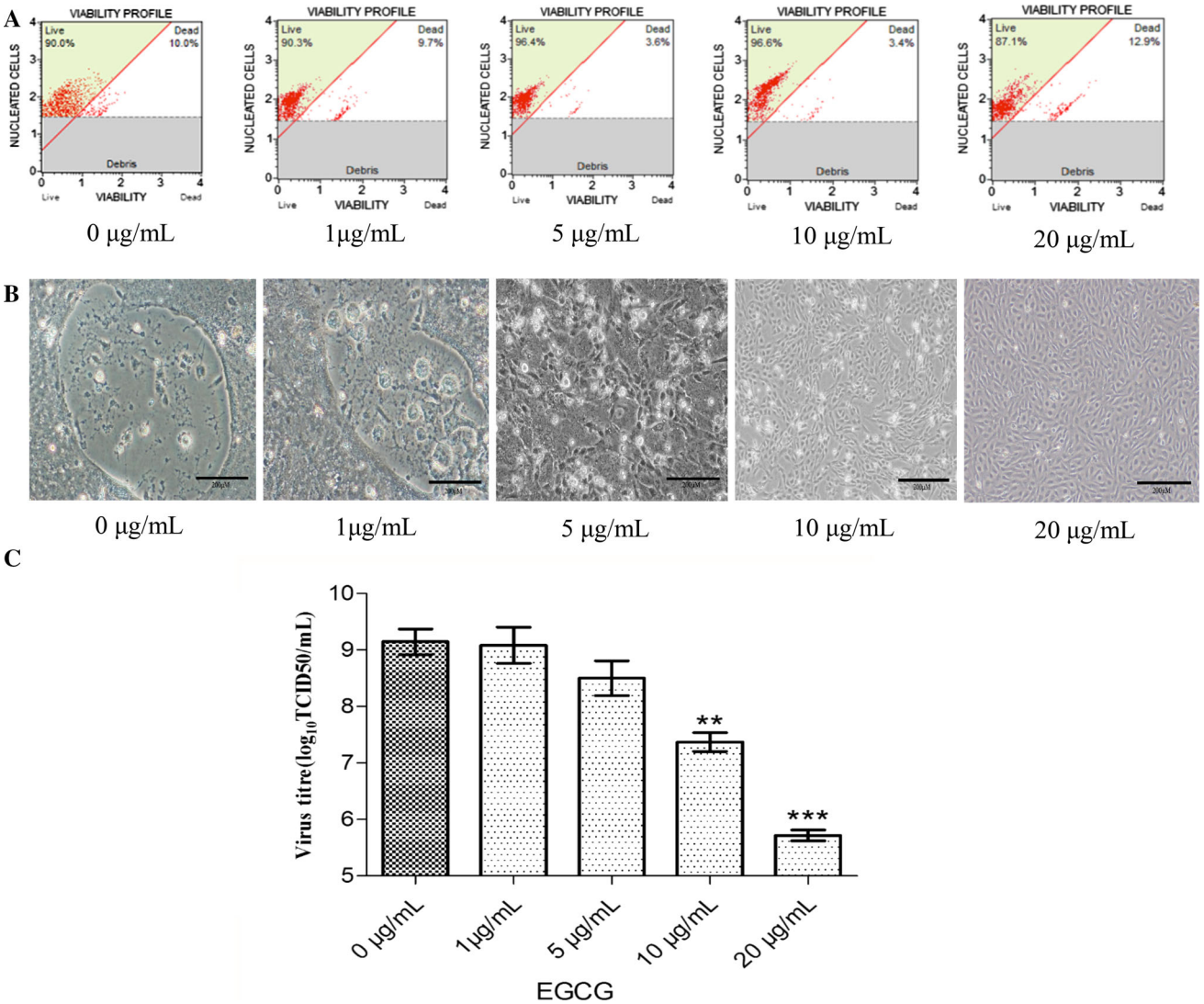

Fig. 2 Effect of pretreating CIK cells with EGCG on the infection with GCRV. (A) cell viability assessed using a Muse™ Count and Viability Kit (Millipore, Poland). (B) Blocking of GCRV infection by EGCG. Cells were blocked with EGCG at the indicated concentrations before being infected with GCRV for 1 h at 4 °C. CPE due to GCRV infection assessed using a visible light phase microscope. Bars, 200 μm. (C) $TCID_{50}$ assay of virus yield in supernatants. Each experimental point is the sum of triplicate experiments with two titer determinations. Error bars represent standard errors of the mean. Asterisks represent a significant difference from the control (one-sample *t*-test)

EGCG or GTE was incubated with CIK cells at various concentrations before GCRV infection. Infected cells were observed daily for CPE, and progeny viruses in the supernatant were titrated simultaneously. Fig. 2B and Fig. 3B show the CPE in CIK cells pretreated with various doses of the selective ligands at 24 h postinfection, and their suppression of virus replication in infected supernatants was quantitatively analyzed using a real-time PCR assay. Untreated CIK cells demonstrated a typical CPE profile, including extensive cell fusion and necrosis, while treatment with either EGCG or GTE resulted in a dose-dependent protection of the cells from viral infection (Fig. 2B and Fig. 3B). Consistently, titration of supernatants from the infected cultures revealed that viral replication was suppressed in a dose-dependent manner in CIK cells treated with either EGCG or GTE (Fig. 2C and Fig. 3C). Notably, treatment of CIK cells with 10 μg of EGCG per ml resulted in 80 % inhibition of viral replication when compared to cells without drug treatment, while complete inhibition of GCRV infection could be achieved by EGCG at a dose of 20 μg/ml. We also tested the effect of a crude extract of green tea (GTE, containing 50 % EGCG) in inhibiting viral infection in tissue culture. The results showed that EGCG from green tea conferred a protective effect to CIK cells against GCRV infection similar to that of the standard EGCG (Fig. 3C). To investigate whether suppression of viral replication resulted from the inhibition of viral adhesion to CIK cells, a binding

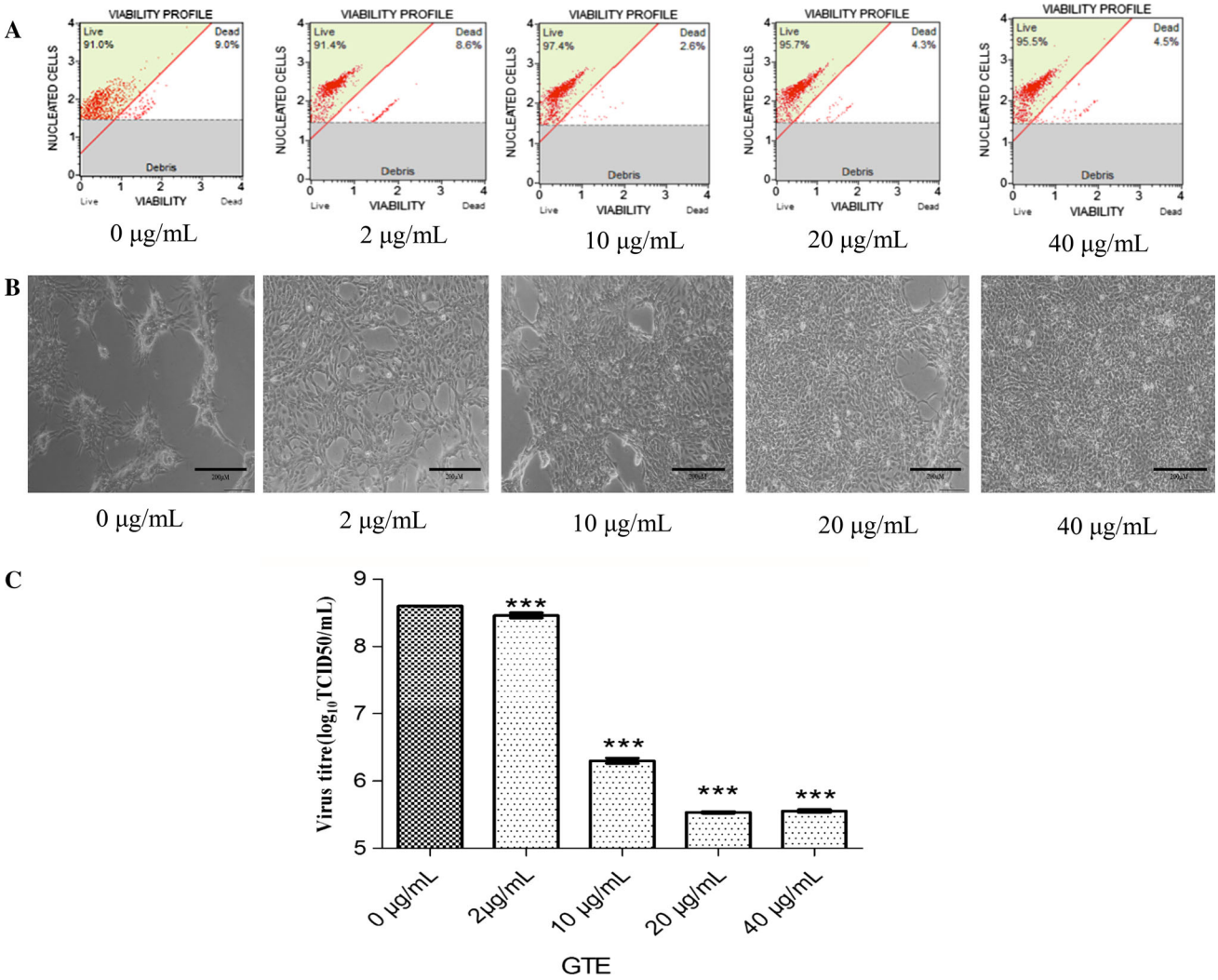

Fig. 3 Effect of pretreating CIK cells with GTE on infection with GCRV. (A) cell viability assessed using a Muse™ Count and Viability Kit (Millipore, Poland). (B) Blocking of GCRV infection by GTE. Cells were blocked with EGCG at the indicated concentrations before being infected with GCRV for 1 h at 4 °C. CPE due to GCRV infection assessed using a visible light phase microscope. Bars 200 μm. (C) $TCID_{50}$ assay of virus yield in supernatants. Each experimental point is the sum of triplicate experiments with two titer determinations. Error bars represent standard errors of the mean. Asterisks represent a significant difference from the control (one-sample *t*-test)

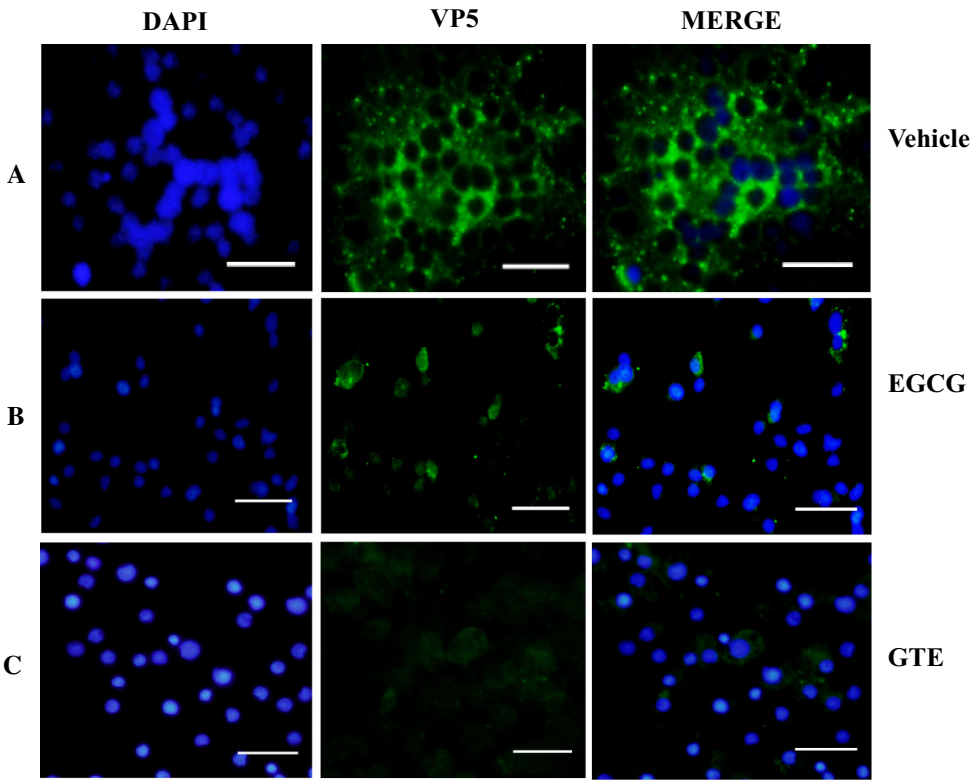

Fig. 4 Blocking of GCRV infection by EGCG or GTE. CIK cells were preincubated either with EGCG or GTE prior to infection with GCRV-JX01 at a MOI of 10. The binding of GCRV to CIK cells was visualized by subsequent and successive incubation with a monoclonal antibody directed against the GCRV-VP5 and an FITC-conjugated anti-mouse monoclonal antibody. Nuclei were stained with DAPI. The samples were viewed under a fluorescence microscope. Magnification, ×200. Bars, 100 μm

assay was performed on CIK cells treated with either EGCG or GTE. In contrast to control cells treated with vehicle (Fig. 4A), viral adhesion to CIK cells treated with 20 μg of EGCG or 40 μg of GTE per ml was completely abolished (Fig. 4B and C). Thus, EGCG is an ideal candidate for the development of a naturally obtained small-molecule drug against GCRV infection.

EGCG has anti-cancer activity that has been shown to be mediated through the laminin receptor [29]. It was worth noting that LamR is a receptor for prion protein (PrP) [14], dengue virus [31], adeno-associated virus [2], Sindbis virus [36], Venezuelan equine encephalitis virus [21], shrimp yellow head virus, and infectious myonecrosis virus [4]. Thus, we speculate that EGCG might serve a multifunctional antiviral agent against various viruses, including GCRV, by blocking viral attachment. If LamR is involved in the blocking of GCRV infection by EGCG as suggested, GCRV should have the ability to bind to LamR. To test this hypothesis, we conducted a virus overlay protein binding assay (VOPBA). The membrane protein fraction extracted from CIK cells was subjected to 10 % SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then incubated with 10^7 purified GCRV particles in GTNE buffer (50 mM Tris-Cl, 20 mM glycine, 100 mM NaCl, 1 mM EDTA, pH 7.5) at room temperature overnight and then washed three times with PBS-T buffer. Subsequently, the membrane was incubated with an anti-VP5 polyclonal antibody[15] at a dilution of 1:100 in PBS-T buffer containing 5 % skim milk. The virus-binding band was visualized by incubation with a secondary goat anti-mouse IgG. The signal was developed as described above for Western blot analysis. The presence of LamR in the membrane extract was confirmed by Western blot with a monoclonal antibody against LamR (EPR8469, Santa Cruz Biotechnology) (Fig. 5, lane 1). After incubation with free GCRV particles, the nitrocellulose membrane was probed with an anti-VP5 polyclonal antibody, and a single protein band at the expected position for LamR was detected, confirming GCRV binding (Fig. 5, lane 2). Thus, the VOPBA results indicated that LamR was the only membrane-associated protein with detectable binding affinity for GCRV on the nitrocellulose membrane. To exclude the possibility of false positive signals, the Western blot of the sample incubated without GCRV was probed with an anti-VP5 polyclonal antibody, and no signal was detected. Collectively, the VOPBA results supported the potential binding of GCRV to LamR.

EGCG has been extensively studied for various biological activities. Previous reports have demonstrated that EGCG prevents influenza virus and HIV from binding to their cell-surface receptors [6, 11, 12]. LamR is a nonintegrin laminin receptor that is overexpressed on the surface of various tumor cells [23]. EGCG has also been shown to target to the cell-surface receptor 67LR [10, 33]. EGCG

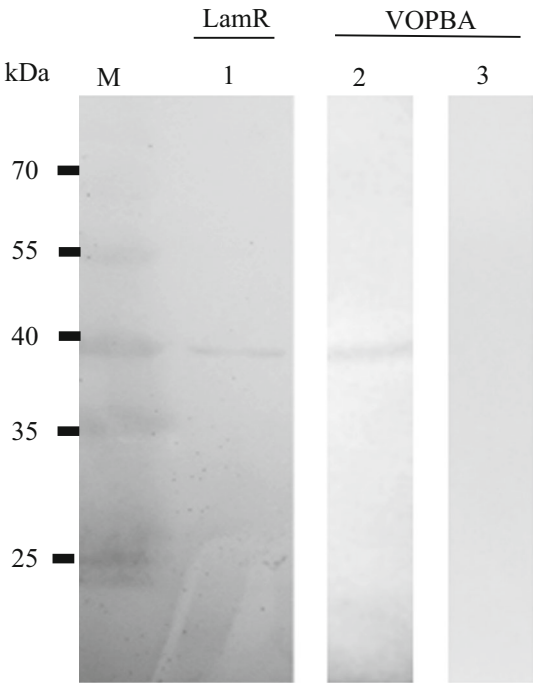

Fig. 5 VOPBA analysis of purified GCRV particles on CIK cell membrane proteins. CIK cell membrane proteins were extracted and separated on a denaturing gel. The membrane proteins were transferred to nitrocellulose membranes and incubated with GCRV particles. Virus-binding protein bands were detected using a polyclonal antibody against the outer capsid VP5 protein (lane 2). Control reactions were identical, except that no virus was included in the hybridization mix (lane 3). To confirm that GCRV bound to LamR on the membrane, the same membrane without incubation of virus was reacted with a monoclonal antibody specific for LamR (lane 1). M, protein molecular standards as noted on the left of the figure

from green tea extract is a natural antioxidant that confers strong resistance against oxidation and free radicals [19]. Surface plasmon resonance experiments have demonstrated the binding of EGCG to LamR with a K_d value in the nanomolar range [29]. Since then, the involvement of LamR in metastasis formation has been intensively investigated. EGCG is now regarded as a multifunctional molecule with antioxidative [40], anti-cancer [39] and immunity-enhancing [27] properties. The potential of EGCG as a drug for blocking infection of grass carp reovirus was demonstrated by a dose-dependent suppression of GCRV infection by EGCG in the present study (Fig. 2, Fig. 3).

In summary, we demonstrated here that the small molecule EGCG can inhibit the infection of CIK cells by GCRV in a dose-dependent manner, and the inhibition of viral replication resulted from the blockage of viral adhesion to CIK cells. A key finding of the current work was that a low-molecular-weight plant compound, EGCG, could effectively block GCRV infection *in vitro*. The results thus suggest a novel prophylactic and therapeutic small-molecule approach for treating GCRV infection. Although our data indicated that GCRV might bind LamR, the question whether LamR serves as the receptor or a co-receptor factor for GCRV requires further biochemical analysis with both viral and host factors. However, the present study did suggest that the blocking effect of EGCG is associated with LamR.

Even though green tea has been considered a health-promoting beverage for a thousand years [24], basic research findings on its main active component, EGCG, have proven to be extremely promising in numerous fields in recent years. Furthermore, EGCG as a natural agent has low toxicity even at very high doses [13] and low impact on the environment [18]. Thawonsuwan et al. [30] have reported the efficacy of EGCG (a commercial product containing more than 94 % EGCG) *in vivo* as a feed additive for fish both in terms of its antioxidant function and its immunostimulatory potential. As EGCG provides many benefits for fish, EGCG could be used as a feed additive or antiviral agent in grass carp culture. Therefore, further *in vivo* investigation is necessary to confirm that EGCG is useful as an herbal biomedicine against GCRV infection.

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Compliance with ethical standards

Conflict of interest Author Hao Wang declares that he has no conflict of interest. Author Weisha Liu declares that she has no conflict of interest. Author Fei Yu declares that he has no conflict of interest. Author Liqun Lu declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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