Molecular Characterization of Severin from *Clonorchis sinensis* Excretory/Secretory Products and Its Potential Anti-apoptotic Role in Hepatocarcinoma PLC Cells

Xueqing Chen1,2,9, Shan Li1,2,9, Lei He1,2, Xiaoyun Wang1,2, Pei Liang1,2, Wenjun Chen1,2, Mengyu Ren1,2, Jinsi Lin1,2, Chi Liang1,2, Jin Xu1,2, Zhongdao Wu1,2, Xuerong Li1,2, Yan Huang1,2,*, Xinbing Yu1,2,*

1 Department of Parasitology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, People’s Republic of China, 2 Key Laboratory of Tropical Diseases Control at Sun Yat-sen University, Ministry of Education, Guangzhou, People’s Republic of China

Abstract

**Background:** Clonorchiasis, caused by the infection of *Clonorchis sinensis* (*C. sinensis*), is a kind of neglected tropical disease, but it is highly related to cholangiocarcinoma and hepatocellular carcinoma (HCC). It has been well known that the excretory/secretory products of *C. sinensis* (CsESPs) play key roles in clonorchiasis associated carcinoma. From genome and transcriptome of *C. sinensis*, we identified one component of CsESPs, severin (C severed), which has three putative gelsolin domains. Its homologues are supposed to play a vital role in apoptosis resistance of tumour cell.

**Methodology/Principal Findings:** There was significant similarity in tertiary structures between human gelsolin and C severed by bioinformatics analysis. We identified that C severed expressed at life stage of adult worm, metacercaria and egg by the method of quantitative real-time PCR and western blotting. C severed distributed in vitellarium and intraterine eggs of adult worm and tegument of metacercaria by immunofluorescence assay. We obtained recombinant C severed (rC severed) and confirmed that rC severed could bind with calcium in circular dichroism spectrum analysis. It was demonstrated that rC severed was of the capability of actin binding by gel overlay assay and immunocytochemistry. Both Annexin V/PI assay and mitochondrial membrane potential assay of human hepatocarcinoma cell line PLC showed apoptosis resistance after incubation with different concentrations of rC severed. Morphological analysis, apoptosis-associated changes of mitochondrial membrane potential and Annexin V/PI apoptosis assay showed that co-incubation of PLC cells with rC severed in vitro led to an inhibition of apoptosis induced by serum-starved for 24 h.

**Conclusions/Significance:** Collectively, the molecular properties of C severed, a molecule of CsESPs, were characterized in our study. rC severed could cause obvious apoptotic inhibition in human HCC cell line. C severed might exacerbate the process of HCC patients combined with *C. sinensis* infection.

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* E-mail: huang66@mail.sysu.edu.cn (YH); yuxb@mail.sysu.edu.cn (XY)
* These authors contributed equally to this work.

Introduction

*Clonorchis sinensis* (*C. sinensis*) has been proven to be the causative agent of clonorchiasis, which is endemic in China, Korea and Vietnam [1,2,3]. As an important food-borne parasite, *C. sinensis* has afflicted more than 35 million people in world and approximately 15 million in China, creating a socio-economic burden in endemic regions [4]. Most clonorchiasis cases are due to the consumption of raw freshwater fish containing infective *C. sinensis* metacercariae, which excyst in the duodenum until they grow into juvenile *C. sinensis* and then migrate into the bile ducts of their host [5,6]. Both experimental and epidemiological evidence have implied that long-term infections with liver flukes lead to chronic pathological changes, including hepatomegaly, hepatic fibrosis, cholangitis, cholecystitis, adenomatous hyperplasia, and cholangiocarcinoma (CCA) [7,8,9]. Furthermore, *C. sinensis* was recently classified along as a Group I biological carcinogen by the World Health Organization [10,11]. In endemic area of China, 16.44% of HCC patients were infected with *C. sinensis*, while 2.40% were infected in non-tumor patients. The OR value and 95% CI in HCC group were 8.00 and 4.34–14.92 [12,13,14], so that we should pay high attention to the relationship between primary hepatocellular carcinoma and the infection of *C. sinensis*. It has been well known that the excretory/secretory products of *C. sinensis* (CsESPs) can cause histopathological changes such as bile duct dilatation, inflammation and fibrosis, and adenomatous...
Author Summary

Clonorchis sinensis (C. sinensis) has afflicted more than 35 million people in world and approximately 15 million in China, creating a socio-economic burden in epidemic regions. The infection of C. sinensis is highly related to cholangiocarcinoma and hepatocellular carcinoma (HCC). It has been documented that excretory/secretory products of C. sinensis (CsESPs) involved in the pathogenesis of HCC. Csseverin, expressed at life stage of egg, metacercaria and adult worm, was a component of CsESPs. In the current study, we characterized the properties of Csseverin such as sequence signature, active and calcium binding activity. In addition, we demonstrated that Csseverin could cause apoptotic inhibition in spontaneously apoptotic human HCC cell line PLC cells by using morphological analysis, detection of the apoptosis-associated change of mitochondrial membrane potential (MMP) as well as Annexin V/PI apoptosis assay. Our study provided an exploratory sight view of mechanism involved in progress of carcinoma associated with the infection of C. sinensis and Csseverin might exacerbate the process of C. sinensis infected HCC patients.

proliferation of the biliary epithelium [15]. In the present studies, from the published genome [16] and transcriptome [17,18] of C. sinensis, we identified one component of CsESPs, Csseverin, which has three putative gelsolin domains.

The gelsolin superfamily is conserved in mammalian as well as in non-mammalian organisms and takes the leading role in controlling actin organization or actin filament remodeling. The family has some specific and apparently non-overlapping particular roles in several cellular processes, including cell motility, control of apoptosis and regulation of phagocytosis [19]. Initial evidence of anti-apoptotic effect of gelsolin was provided by the observation that a point mutation in mouse gelsolin confers on this protein tumor-suppressor activity against H-ras oncogene transformed NIH-3t3 cells [20,21]. Direct evidence of the inhibitory role of gelsolin was provided by Ohtsu et al., who generated Jurkat transfectants expressing up to threefold gelsolin than wild-type cells. These transfectants exhibited a phenotype more resistant to apoptosis induced by several stimuli [22]. Moreover, it has been reported that human cytoplasmic gelsolin can prevent apoptotic mitochondrial changes such as mitochondrial membrane potential loss by binding to mitochondrial voltage-dependent anion channel (VDAC) [23].

Large-scale gene sequencing efforts have revealed gelsolin homologues in the majority of parasitic phyla [24,25,26,27,28]. In the current study, we presented for the first time the molecular characteristics of Csseverin. We described the detection of recombinant Csseverin (rCsseverin) binding to cytoskeletal actin filament of human hepatocarcinoma PLC cells and investigated its potential anti-apoptotic role on PLC cells as an ingredient of CsESPs in vitro. The present study is a cornerstone for researches on biological characterization of Csseverin in the future. In addition, our work will provide an exploratory sight view of mechanism involved in progress of carcinoma associated with the infection of C. sinensis.

Materials and Methods

Ethics statement

C. sinensis flukes were isolated from naturally infected cats (Guangdong Province, China) for sample preparation. Animals in experiments were all purchased from animal center of Sun Yat-sen University and raised carefully in accordance with National Institutes of Health on animal care and the ethical guidelines. All experimental procedures were approved by the animal care and use committee of Sun Yat-sen University (Permit Numbers: SCXK/Guangdong 2009-0011).

Cell culture

PLC and human normal hepatocyte L-02 cells were a gift from Dr. Wang Shutong and Dr. Xie wenxuan (the first affiliated hospital of Sun Yat-Sen University) and routinely cultured in high glucose DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and penicillin–streptomycin (100 units/ml) in 5% CO2 at 37°C. Serum-starved PLC were prepared by incubating the cells in high glucose DMEM medium at 37°C and 5% CO2 with fetal bovine serum deprivation for at least 24 h.

Sequence analysis of Csseverin

The gene (GenBank accession No. GAA30384.2) predicted encoding homologue of severin was screened from C. sinensis genome by blastx and Open Reading Frame (ORF) Finder program at NCBI (http://www.ncbi.nlm.nih.gov). The alignment of its deduced amino acid sequences with homologues from other species were analyzed and shown with Vector NTI. Proteomics bioinformatics tools such as Motif-Scan, InterPro-Scan and Swiss-Model were used to analyze the protein characteristics including physicochemical parameters, conserved domains and spatial structure. The phylogenetic tree was constructed online (http://www.ebi.ac.uk/Tools/clustalw/index.html).

Preparation of anti-Csseverin IgG

The ORF of severin was amplified using the following primers: sense: 5’- ATAGGATCCGATGGCGGAGTACT -3’ (underlined, BamHI) and antisense: 5’- CGGAAGCTTTCATTTGGAGAACCG-3’ (underlined, Hind III). The PCR was carried out for 32 cycles at 94°C for 45 s, 51°C for 45 s, and 72°C for 45 s, and extension for 10 min at 72°C after the last cycle in a DNA-Thermal Cycler (Biometra, Germany). PCR products were purified and digested with BamHI and Hind III, and then subcloned into prokaryotic expression vector 6×His tag pET28a(+) (Novagen, Germany). After digestion with BamHI and Hind III, the recombinant plasmid was confirmed by DNA sequencing and then transformed into E. coli, BL21 (Promega, USA). The expression of rCsseverin was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h at 37°C. After induction, the bacteria were harvested by centrifuging at 4°C for 15 min at 8,000×g and suspended in lysis buffer (0.5 M NaCl, 20 mM Tris–HCl, 5 mM imidazole, pH 8.0), sonicated on ice, and centrifuged at 10,000×g for 15 min at 4°C. The fusion protein was batch-purified using His Bind Purification kit (Novagen, USA) and the eluted fractions containing rCsseverin were pooled and dialyzed with phosphate-buffered saline (10 mM phosphate buffer, 27 mM KCl, 157 mM NaCl, pH 7.4). Protein samples were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coo massie brilliant blue G-250, the concentration was measured by a Bicinchoninic acid assay (BCA, Novagen, USA) according to manufacturer’s instructions. Then, 100/50 μg of rCsseverin were mixed with an equal volume of incomplete Freund’s adjuvant and injected subcutaneously to six-week-old male Sprague-Dawley (SD) rats (purchased for experiments under the Guide for the Care and Use of Laboratory Animals). Boost injections were given at 2 and 5 weeks after first injection. Anti-serum was collected at 1 week after the second booster, then aliquoted and stored in...
Western blotting

GSEPs and sera from GSEPs immunized rat were obtained by referring to previous study [29]. 10 µg of rCseverin or GSEPs were subjected to 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. Successively, the membranes were blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS) overnight at 4°C, washed five times with PBS-0.05% Tween 20 (PBS-T, pH 7.4), and incubated with His-tag monodonal antibody, sera from naive rats, rCseverin immunized rats, C. sinensis-infected rats or GSEPs immunized rats (1:100 dilutions) followed by HRP-conjugated goat anti-mouse/rat IgG (Promega; dilution of 1:2000) and 0.4% lidene fluoride (PVDF) membranes. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rat IgG in 1:2000 dilutions (Proteintech, USA) at 37°C for 1 h. Detection was then carried out by enhanced chemiluminescence (ECL) method.

Immunohistochemical localization of Cseverin in C. sinensis adults and metacercariae

Fresh adult worms and metacercariae of C. sinensis were fixed with 4% formaldehyde, embedded with paraffin wax, and sliced into 4-µm-thick sections. After dewaxing and dehydration, slides were blocked with goat serum overnight at 4°C, and incubated with anti-rCseverin sera (1:100 in 0.1% PBS-T) at room temperature for 2 h. Sera from naive rats were used as a negative control. The slides were washed twice and incubated with goat anti-rat IgG labeled with red fluorescent Cyanine dye 3 (Cy3, Proteintech; 1:100 in 0.1% PBS-T). Fluorescence microscopy was used in visualization of antibody staining.

Circular Dichroism (CD) measurements

As the protein contains a potential Ca²⁺-binding domain, Ca²⁺-binding will change its conformation of secondary structure which can be detected by CD [34,35,36]. CD measurements were carried out on a J-810 Circular Dichroism Spectrometer (Jasco, Japan) with the Jasco Spectra Manager software at room temperature. Three samples were assayed: purified rCseverin in PBS, purified rCseverin in PBS containing 1 µM CaCl₂, and purified rCseverin in PBS containing 1 µM EDTA to remove combined Ca²⁺ during expression of rCseverin in bacteria and purification in solutions. Secondary structure was analyzed using Jasco Spectra Manager Secondary Structure Analysis program. Far-UV CD spectrum was acquired using a 0.2 mm path length cell at 0.2 nm intervals over the wavelength range from 190 to 250 nm. Three scanning values were averaged for each sample and were corrected by subtracting buffer contribution from parallel spectra in the absence of Cseverin. The concentration of Cseverin was kept at 1 µM in 10 mM sodium phosphate buffer pH 7.4 and then the CD data were converted to molar units.

Actin binding activity of rCseverin

Gel overlay assay and immunocytochemistry were employed to investigate the actin binding activity of rCseverin. F-actin (from rabbit muscle, 99% similar to human F-actin, Sigma-Aldrich) and its fragments digested with 0.25% trypsin (Sigma-Aldrich, USA) at 37°C for 1 h, were separated on 12% SDS-PAGE and electro-phoretically transferred onto PVDF membranes. Membranes were blocked with PBS-T (25 mM Tris-HCl, pH 7.2, 50 mM NaCl, 0.5% Tween-20) containing 5% BSA overnight at 4°C and washed (3 times, for 15 min each) in PBS-T. Then, membranes were incubated with 0.1 mg/ml rCseverin in PBS-T for 1 h at room temperature. After washing extensively, membranes were incubated with anti-Gseverin rat serum (1:100 dilutions) in PBS-T for 1 h at room temperature. The membranes were incubated with 1:2000 HRP-conjugated secondary antibodies against rat IgG in PBS-T for 1 h at room temperature after washing. Following extensive washing in PBS-T, the membranes were incubated with 0.1% diaminobenzidine substrate solution to develop color after washing again [37].

In immunocytochemistry assay, the PLC cells were seeded into sterile Petri dish (Nest, diameter of 15 mm) which is special for the detection of laser scan confocal microscopy, at a density of 2 × 10⁴.
cells per well and then cultured for 24 h. The PLC cells were washed four times with PBS and then fixed with 2 ml of 4% paraformaldehyde solution in PBS at room temperature for 30 min, then treated with 50 mM NH₄Cl for 10 min, to reduce aldehyde groups. The cells were permeabilized for 4 min at 4°C with 0.5% Triton X-100 in PBS. At the next step, cells were incubated in PBS buffer containing 3% of BSA for 1 h, followed by coating with rCsseverin overnight at 4°C. To visualize cytoskeleton, cells were incubated overnight at 4°C with mouse anti human F-Actin monoclonal antibody (AbD Serotec, UK) diluted 1:100, then subsequently incubated overnight at 4°C with rat anti-rCsseverin serum (1:100) for 12 h at 4°C. The incubation with secondary antibodies was carried out at RT for 2 h, using fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Proteintech, USA) diluted 1:200 and Cyanine dye 3 (Cy3)-conjugated goat anti-rat IgG (Proteintech, USA) diluted 1:400 at the same time. All antibodies were diluted with 1% BSA in PBS buffer and all steps described above were preceded by intensive washes in PBS. After finally washing with water, cover dishes were mounted on slides with Hoechst 33258 (Sigma, USA). By contrast, to visualize whether rCsseverin could bind with cytoskeletal actin filaments in vitro, PLC cells were serum-starved overnight after incubating 24 h in standard conditions, and coated with rCsseverin in DMEM with 2% FBS for 48 h before fixed with 4% paraformaldehyde solution. The following steps were similar with that mentioned above previously. Images were finally obtained with the LSM 710 laser scanning confocal microscope (Zeiss).

**Apoptosis assays**

After being induced spontaneous apoptosis by serum-starved for 24 h and treated with rCsseverin at different concentrations of 10, 20, 40, 80 µg/ml and PBS for 48 h, 1–5×10⁵ PLC cells were collected by centrifugation, and then incubated with Annexin V/propidium iodide (PI), provided by the Apoptosis Detection Kit (Lankebio, China). The cells were washed twice in PBS and resuspended in 500 µl of 1×Binding Buffer before being incubated with 5 µl of Annexin V and 10 µl of PI. The cells were then analyzed by using flow cytometry after incubation for 5–10 min in dark. Early apoptotic cells were stained with Annexin V alone whereas necrotic and late apoptotic cells were stained with both Annexin V and PI.

PLC cells (5×10⁴ cells per well) were seeded into a 6-well culture plate and cultured as described above. After treatment with Apoptosis Inducers (Beyotime, China), the cells were washed twice with PBS, permeabilized with 0.3% Triton in PBS, and stained with Hoechst 33258 for 3 min in dark. Morphologic changes in apoptotic nuclei were observed and photographed under the inverted fluorescence microscope (Leica DM1000B, Germany) with emission wavelength at 460 nm and excitation wavelength at 350 nm.

**Assessment of mitochondrial membrane potential (MMP) by flow cytometry and immunofluorescence**

MMP assay kit (Beyotime, China) with JC-1 probe was used to measure MMP in PLC cells. Briefly, cells were seeded in six-well plates overnight and serum-starved for 24 h, then treated with various concentration of rCsseverin for 48 h. The cells were then washed with ice-cold PBS and incubated in a 5% CO₂ humidified incubator at 37°C for 20 min after adding 1 ml of JC-1 working solution. The supernatant was then discarded and the cells were washed twice with JC-1 staining buffer. Next, 2 ml medium was added to each well and MMP was monitored using an inverted fluorescence microscope (Leica DM1000B, Germany) and laser scanning confocal microscope (Zeiss LSM 710, Germany). The red JC-1 fluorescence was observed at 525 nm excitation (Ex)/590 nm emission (Em) and the green cytoplasmic JC-1 fluorescence was observed at 405 nm Ex/530 nm Em.

Quantitative changes of MMP at the early stage of cell apoptosis were measured by flow cytometry with JC-1 probe. After being incubated with 10, 20, 40 and 80 µg/ml of rCsseverin for 48 h, 1–5×10⁵ cells were harvested and resuspended with ice-cold PBS (1,500 rpm×5 min). Then, the cell suspensions were incubated with 0.5 ml JC-1 working solution in 0.5 ml DMEM for 20 min at 37°C. The staining solution was removed by centrifugation. The cells were washed with JC-1 (1×) washing buffer twice, then resuspended in 500 µl JC-1 (1×) staining buffer and detected by flow cytometer (Bechman Coulter Gallios, USA).

**Statistical analysis**

All of the experiments were repeated at least three times. Experimental values were obtained from three independent experiments with a similar pattern and expressed as means ± standard deviation (SD). Statistical analyses were performed using SPSS software package 17.0. Data were analyzed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) for comparison between control and treatment groups. Significance was set at p value<0.05.

**Results**

**Sequence analysis of Csseverin**

The ORF of Csseverin contained 1077 base pairs (bp) encoding a protein of 358 amino acids (predicted MW 40.88 kDa, pi 5.24). Blastx analysis showed that the deduced amino acid sequence was homologous to gelsolin of Schistosoma mansoni, Schistosoma japonicum, Saburitis domuncula, Echinococcus granulosus, Strongyloides stercoralis and Hydra magnipapillata with 54%, 65%, 50%, 65%, 48%, 47% identities respectively. The amino acid sequence had no N-terminal signal peptide or transmembrane domain. According to MotifScan and InterproScan prediction, there were three gelsolin domains (aa51–133, aa171–247, aa278–354) indicating that Csseverin might have similar role with gelsolin superfamily. Furthermore, we inferred that the location of putative actin binding surface of Csseverin was from 50 to 150 amino acids by Gene Ontology analysis (http://www.geneontology.org/). The nuclear magnetic resonance (NMR) derived structure of human (Homo sapiens) gelsolin (PRF: 225304) was used as the template to build a molecular model of Csseverin. The two proteins shared 36% identity among their gelsolin core domains and there was significant similarity between their tertiary structures (Figure S1).

**Phylogenetic relationships**

Csseverin grouped very closely with Schistosoma japonicum (Figure S2), a parasite that increases the risk of HCC incident when associated with positive hepatitis B surface antigen [30]. The Csseverin was also closely relative to severin/gelsolin from Echinococcus granulosus, followed by Dictostelium discoideum, but far from those of H. sapiens and M. musculus.

**Prokaryotic expression and purification of rCsseverin**

The soluble rCsseverin was expressed with 6×His-tag in E. coli BL21 after induced by 1 mM IPTG at 37°C for 5 h. The purified recombinant protein showed a single band around 45 kDa (including His-tag sequence) in 12% SDS-PAGE, consistent with the predicted molecular mass (Figure S3, lane 7). The final protein concentration was 0.8 mg/L. The anti-rCsseverin serum was collected from immunized rat.
level of Cseverin was consistent with the transcriptional level. Egg has the highest expression level of Cseverin protein, followed by adult worm and metacercaria (Figure 2B).

Immunolocalization of Cseverin in the adult worm and metacercaria of C. sinensis

The analysis of immunofluorescence localization by using rat anti-Cseverin serum showed that in C. sinensis adult intensive fluorescences were observed in vitellaria while scattered fluorescences were detected in tegument. In metacercaria, specific fluorescences were only deposited in tegument. In addition, intensive fluorescences were presented in intraterine eggs of adult worm (Figure 3D, F and J). By comparison, no specific fluorescence was detected either in adult worm or in metacercaria when treated with serum from naïve rat (Figure 3B, H).

Analysis of circular dichroism (CD) spectrum

According to the profile of CD spectrum, the secondary structure of rCseverin changed from the presence of Ca$^{2+}$ shifted to the absence of Ca$^{2+}$ (presence of EDTA) (Figure 4). With Ca$^{2+}$, the secondary structure of rCseverin contained 23.6% α-helix, 56.6% β-sheet, and 19.8% random loop. While with equivalent EDTA, it changed to 21.5% α-helix, 41.2% β-sheet, and 37.3% random loop. The conformation of the purified rCseverin was between the two conditions with 24.6% α-helix, 49.9% β-sheet, 25.5% random loop. Ca$^{2+}$-binding altered the conformation of EF-hand domain from α-helix to β-sheet. The purified rCseverin partially combined Ca$^{2+}$ during the processes of expression and purification. We showed that rCseverin was easily to precipitate when calcium was added into the solution, and can be resolved by adding EDTA.

Interaction of rCseverin with actin

The binding of rCseverin to F-actin and its fragments were examined using gel overlay assay as described above. After incubation with rCseverin, F-actin and its fragments were blotted by anti-rCseverin serum (Figure 5A, pane b, lane 1–2 and pane c, lane 1). While incubation with BSA or without rCseverin (Figure 5A, pane b, lane 2–3), F-actin couldn’t be probed by anti-rCseverin serum. Whether PLC cells were incubated with rCseverin before or after fixation and permeabilization, both the green fluorescence (FITC–conjugated affinipure goat anti-mouse IgG reacted with anti-F-actin monoclonal antibody) and the red fluorescence (Cy3–conjugated affinipure goat anti-rat IgG reacted with anti-rCseverin serum) were observed. The locations of green fluorescence were mostly coincident with those of the red fluorescence (Figure 5B, pane a and b). There was no red fluorescence or green fluorescence in negative control group (Figure 5B, pane c and d). Thus, we suspected that rCseverin might enter into PLC cells and bind to actin.

Apoptosis assay

To identify the effect of rCseverin on PLC cells, we tested the total percentage of Annexin V+/PI− and Annexin V+/PI+ cells by flow cytometry. As shown in Figure 6A, incubation of PLC cells with different dosages of rCseverin (10, 20, 40, and 80 μg/ml) for 48 h after induced spontaneous apoptosis by serum-starved for 24 h decreased the percentage of Annexin V+/PI− and Annexin V+/PI+ cells in a dose-dependent manner (30.63, 26.98, 14.36, and 9.68%, respectively), as compared to the PBS-treated controls, which showed 40.74% Annexin V+/PI− and Annexin V+/PI+ cells. The results showed that rCseverin exhibited potent anti-apoptotic activity on PLC cells in concentration-dependent
manner. We also tested the effect of rCs2everin on human normal hepatocyte L-02 cells. No significant decrease of Annexin V+/PI− and Annexin V+/PI+ cells was observed (Figure 6B).

We also compared the morphology of PLC cells in the presence of 80 μg/ml rCs2everin to that of PBS-treated cells under the inverted phase-contrast microscopy. Hoechst staining of PBS-treated cells after induced spontaneous apoptosis by serum-starved for 24 h revealed marked morphological changes, such as cell shrinkage, vesicular degeneration, threadlike morphology, nuclear condensation, and nuclear fragmentation, which are typical features of apoptotic cell death. While morphological changes of the PLC cells in presence of 80 μg/ml rCs2everin after treatment with serum-starved for 24 h were not significant (Figure 6C).

Recovery of the mitochondrial membrane potential (MMP) in rCs2everin treated PLC cells

To further investigate the molecule events triggered by rCs2everin inhibition, we measured MMP in the PLC cells by using flow cytometry and JC-1 staining in situ. The decline of MMP is considered as a symbolic event of early cellular apoptosis. Changes in MMP can be assessed by monitoring JC-1, which accumulates in mitochondria forming red fluorescent aggregates at high membrane potential and exits mainly in cytosol forming a green fluorescent monomer, presenting a collapse of the membrane [39]. In our study, rCs2everin-treated cells showed reduction of green fluorescence and production of an obvious red fluorescence. The treatment of rCs2everin recovered the MMP in a concentration-dependent manner (Figure 7, A and B), as indicated by an increase of red (JC-1 aggregates)/green (JC-1 monomers) ratio. At 48 h, the percentage of 80 μg/ml rCs2everin and PBS treated PLC cells which emitted green fluorescence was 15.42 and 9.63%, respectively, indicating the recovery of mitochondrial membrane depolarization. The PLC cells that treated with apoptosis introducers exhibited mitochondrial green fluorescence with little red fluorescence, suggesting the cells in depolarization state. The red fluorescence in PLC cells increased, as monitored by in situ JC-1 staining, after the treatment of 10, 20, 40, 80 μg/ml rCs2everin as compared with the PBS group (Figure 7C).

Discussion

In the present study, we identified that Cs2everin, which expressed at life stage of egg, metacercaria and adult worm was a component of Cs-ESPs. We also demonstrated its ability of binding with calciumion and actin filaments. Furthermore, co-incubation of PLC cells with rCs2everin in situ led to an inhibition of apoptosis induced by serum-starved for 24 h, by using morphological analysis of PLC, detection of the apoptosis-associated change of mitochondrial membrane potential as well as Annexin V/PI apoptosis assay. We inferred that rCs2everin may play an intracellular protective role via preventing apoptotic mitochondrial changes (the loss of mitochondrial membrane potential), just like endogenous human gelsolin did [40].
Gelsolin family is found in a diverse range of organisms including bacteria, invertebrates, plants, primates, rodents and vertebrates. The superfamily in mammals consists of seven different proteins: gelsolin, adseverin, villin, capG, advillin, supervillin and flightless I. All of them contain three or six homologous repeats of a domain named gelsolin-like (G) domain [41]. Bioinformatics analysis showed that Csseverin comprised three gelsolin homology domains, calciumion and actin binding motifs. The amino acid sequence of Csseverin shared 36% identity with that of human gelsolin, but there was significant similarity between their tertiary structures. Our phylogenetic analysis suggested that a majority of gelsolin proteins do not form clades.

Figure 5. Interaction of rCsseverin with actin. (A) The bindings of rCsseverin to F-actin and its fragments were examined using gel overlay assay as described in materials and methods. In pane a, actin and its fragments were separated on 12.5% SDS-PAGE. Protein molecular weight markers (M), F-actin at 37 °C for 1 h (lane 1), F-actin digested with 0.25% trypsin at 37 °C for 1 h (lane 2), purified rCsseverin (lane 3), 0.25% trypsin at 37 °C for 1 h (lane 4). In pane b, rCsseverin binding to F-actin and its fragments were examined using gel overlay assay. The membrane was incubated with rCsseverin then rat anti-rCsseverin serum. Protein molecular weight markers (M), F-actin at 37 °C for 1 h (lane 1), F-actin digested with 0.25% trypsin at 37 °C for 1 h (lane 2), purified rCsseverin (lane 3). In pane c, the membrane was incubated with rCsseverin then rat anti-rCsseverin serum (lane 1), the membrane was incubated with BSA then rat anti-rCsseverin serum (lane 2), the membrane was incubated with rCsseverin then pre-immune rat serum (lane 3). (B) The bindings of rCsseverin to cytoskeletal actin filaments of PLC cells by immunocytochemistry. Pane a, cells were coated with rCsseverin before fixed with 4% paraformaldehyde and successively with rat anti-rCsseverin serum and mouse anti-F-actin monoclonal antibody, and then a mixture of FITC (green fluorescence) and Cy3 (red fluorescence)-labeled goat anti mouse/rat IgG. Pane b, cells were coated with rCsseverin after permeabilized with 0.3% Triton X-100 and successively with rat anti-rCsseverin serum and mouse anti-F-actin monoclonal antibody, and then a mixture of FITC (green fluorescence) and Cy3 (red fluorescence)-labeled goat anti mouse/rat IgG. Pane c, cells incubated with monoclonal anti-F-actin antibody without coated with rCsseverin as negative control. Pane d, cells were coated with rCsseverin before fixed with 4% paraformaldehyde, then incubated only with anti-rCsseverin serum after permeabilized with 0.3% Triton X-100 as another negative control. The images were taken under a LSM 710 Zeiss confocal microscope, with an oil immersion objective (63×, 1.40 numerical aperture).

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Figure 6. Apoptosis inhibition of PLC cells with rCsseverin in a concentration-dependent manner. (A) Flow cytometry analysis of PLC cells treated with PBS, apoptosis inducer (positive control) and 10, 20, 40, and 80 μg/ml rCsseverin for 48 h after induction of spontaneous apoptosis. Representative dot plots of cell apoptosis were showed after AnnexinV/PI dual staining. Apoptotic rate was represented as a percentage of total cell populations. The proportion of dead cells (Annexin V−/PI+), live cells (Annexin V−/PI−), early apoptotic cells (Annexin V+/PI−) and late apoptotic/necrotic cells (Annexin V+/PI+) was respectively measured for comparison. (B) Flow cytometry analysis of L-02 cells with the same treatment. (C) Morphologic changes in apoptotic PLC cells. Following treatment with PBS (negative control) or 80 μg/ml rCsseverin for 48 h, apoptotic nuclei were condensed and brightly stained with Hoechst 33258 then nuclear morphology was photographed and visualized with a Leica DMI4000B (Magnification×400). Each experiment was performed in triplicate.

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based on taxonomic groupings but rather according to protein functions. The individual gelsolin domains from human gelsolin form distinct clades with homologues from other species, supporting the notion that these proteins have evolved to perform distinct functions in different organisms.

Increased Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels is the major determinant of cell injury following excitotoxicity [42,43]. The activity of these channels is modulated by dynamic changes in the actin cytoskeleton [44,45], which may occur, in part, through the actions of gelsolin [46]. We obtained soluble and stable rCsseverin. CD measurements actually showed that rCsseverin could bind to calcium. It has been documented that gelsolin family is of actin-regulatory function [47]. Cytoskeletal actin filaments are dynamic structures that form membranous networks interacting with cell surface receptors and intracellular effectors [48,49]. Gel overlay and immunocytochemistry assay indicated the binding activity of rCsseverin.

Gelsolin expression in certain tumors correlates with poor prognosis and therapy-resistance. In vitro, human gelsolin has anti-apoptotic and pro-migratory functions and is critical for invasion of some types of tumor cells [50,51,52,53]. We found that gelsolin was highly expressed at tumor borders infiltrating into adjacent liver tissues [54]. In Jurkat lymphoblastoid T-cell line, gelsolin has been shown to inhibit apoptosis, and the overexpression of gelsolin inhibits the loss of mitochondrial membrane potential and cytochrome c release from mitochondria [55]. Additionally, in several models of neuronal cell death, endogenous gelsolin has been demonstrated that has an anti-apoptotic property which
correlates to its dynamic actions on the cytoskeleton mediated by inhibition of mitochondrial permeability transition [56].

Here we also showed that rCsC severin could cause obvious apoptotic inhibition in the human HCC cell line. Flow cytometry was used to evaluate rCsC severin-inhibited apoptosis after dual staining of cells with Annexin V and PI. Due to that Annexin V binding is based on the transposition of phosphatidylserine from the inner to the outer face of the cell membrane during the early stages of apoptosis [57]. This method has been widely used to discriminate between normal cells (AnnexinV−/PI−), early apoptotic cells AnnexinV+/PI−, late apoptotic cells (AnnexinV+/PI+), and necrotic cells (AnnexinV−/PI+). Compared with PBS-treated group (negative control), there were less typical apoptotic changes in rCsC severin-treated PLC cells after induced spontaneous apoptosis by serum-starved for 24 h in morphology analysis. We also measured the changes in mitochondrial membrane potential (MMP) using a JC-1 probe that gives a red fluorescence when MMP is high and green fluorescence when MMP is low that occurs in early apoptosis cells. We found that interact directly with rCsC severin led to the recovery of mitochondrial membrane potential in PLC cells.

Moreover, rCsC severin could be probed by sera from rats infected with C. sinensis besides anti-GESPs serum that confirmed CsC severin was a molecular of GESPs. Although it is still unclear about the mechanism of uptake or internalization of GESPs by host cells, internalized GESPs could play roles in the interaction between the host and parasite. These data demonstrated that CsC severin, as an anti-apoptotic molecule to carcinoma cell, might be a pathogenic factor in GESPs, contributing to the development of a protumorigenic environment that was conductive to HCC.

Tissue-specific distribution of CsC severin in muscular locations such as teguments of adult worm and metacercaria, as well as its actin binding activity, we inferred that CsC severin might involve in regulating the contraction of smooth muscle and movement of worm body [38,39,60]. What was more, relative high transcript/regulating the contraction of smooth muscle and movement of CsCs such as teguments of adult worm and metacercaria, as well as its CsCs factor in anti-apoptotic molecule to carcinoma cell, might be a pathogenic factor in GESPs, contributing to the development of a protumorigenic environment that was conductive to HCC.

Overall, we presented the molecular characteristics of CsC severin, a molecule of GESPs. Recombinant CsC severin (rCsC severin) could bind to Ca2+ and cytoskeletal actin filaments and cause obvious apoptotic inhibition in human HCC cell line. By promoting apoptosis inhibition, CsC severin might exacerbate the process of HCC patients combined with C. sinensis infection. More experiments should be further conducted. The current study may provide a novel insight in understanding the pathogenesis of carcinoma associated with the infection of C. sinensis, which was an inducing factor that cannot be ignored in the process of the development of primary hepatic carcinoma. Since gelsolin has actin-regulatory functions, modulation of the actin network might be responsible for the inhibition of apoptosis, the actin cytoskeleton may be a target to protect from apoptosis [61]. The anti-apoptotic mechanism of CsC severin are worthy of studying in the future.

Supporting Information

Figure S1 Sequence analysis of severin of Clonorchis sinensis (CsC severin). (DOC)

Figure S2 Neighbor joining phylogenetic tree for the gelsolin core domains from a range of phyla. (DOC)

Figure S3 Prokaryotic expression and purification of rCsC severin by 12% SDS-PAGE. (DOC)

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Author Contributions

Conceived and designed the experiments: YH XY. Performed the experiments: XC SL. Analyzed the data: XC YH CL ZW JX MR. Contributed reagents/materials/analysis tools: XW PL WC LH SL XL JL MB. Wrote the paper: XC YH.

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