# Serum-derived hepatitis C virus can infect human glioblastoma cell line SF268 and activate the PI3K-Akt pathway

GUOSHENG YUAN<sup>1\*</sup>, LIANG RONG<sup>2\*</sup>, JUNWEI LIU<sup>1</sup>, ZHENZHEN ZHANG<sup>2</sup>, CHENGGUANG HU<sup>1</sup>, MINGXIAO CHEN<sup>2</sup>, LING MA<sup>2</sup>, YONG-YUAN ZHANG<sup>3</sup>, YI-PING LI<sup>2</sup> and YUANPING ZHOU<sup>1</sup>

<sup>1</sup>Department of Infectious Diseases and Hepatology Unit, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515; <sup>2</sup>Institute of Human Virology and Key Laboratory of Tropical Disease Control of Ministry of Education, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, Guangdong 510080, P.R. China; <sup>3</sup>HBVtech, Frederick, MD 20874, USA

Received October 16, 2018; Accepted February 8, 2019

DOI: 10.3892/mmr.2019.10063

Abstract. Extra-hepatic manifestations are frequently observed in hepatitis C virus (HCV)-infected patients; however the underlying mechanisms remain largely unknown. In the present study, the human glioblastoma SF268 cell line (the precise origin of the cell type is not clear) was infected with HCV using HCV-positive serum, and viral replication was assessed by immunofluorescence, reverse transcription-polymerase chain reaction (PCR), quantitative PCR and western blotting following infection. HCV core protein and HCV RNA were detected in HCV-positive serum-infected SF268 cells at day 4 post-infection, while no infection was observed in cells exposed to HCV-negative serum. The mean HCV RNA levels at day 4 post-infection were up to 5.00 IU/ml log10; however, HCV RNA and immunostaining for core protein were negative when cultured to day 6 or longer. The data suggest that human glioblastoma SF268 cells were transiently infected with HCV. AKT serine/threonine kinase phosphorylation was also detected in HCV-infected SF268 cells at day 4 post-infection. To the best of our knowledge, this is the first demonstration that a human glioblastoma cell line can be infected with serum-derived HCV. The results provide evidence that HCV infection can occur in cells of the central nervous system. Neurological disorder-associated phosphoinositide 3-kinase-AKT signaling pathway was activated in parallel with HCV infection, suggesting that SF268

*Correspondence to:* Professor Yuanping Zhou, Department of Infectious Diseases and Hepatology Unit, Nanfang Hospital, Southern Medical University, 1,838 North Guangzhou Ave, Guangzhou, Guangdong 510515, P.R. China E-mail: yuanpingzhou@163.com

\*Contributed equally

may serve as an *in vitro* model for investigating HCV-nervous system cell interactions.

### Introduction

As a public health problem worldwide, ~71 million people are chronically infected with hepatitis C virus (HCV), accounting for ~1% of the global population (1-3). HCV is a small positive-sense single-stranded RNA virus and belongs to the genus *Hepacivirus* of the family Flaviviridae (4,5), which includes certain well-known neurotropic viruses, including yellow fever, dengue, zika and tickborne encephalitis viruses.

Human hepatocytes are the main target cells for HCV infection, which can cause life-threatening liver diseases, including chronic hepatitis, fibrosis and hepatocellular carcinoma (6-9). In addition, extra-hepatic manifestations, including abnormalities in the central nervous system (CNS), are also observed in HCV-infected patients. Previous studies have reported that 50-60% of individuals chronically-infected with HCV had increased incidence of nervous system disorders, including chronic fatigue syndrome, depression and cognitive dysfunction, which may persist (10-13), even after spontaneous or treatment induced peripheral virus clearance. Furthermore, two studies (9,14) have identified that HCV can target microglia cells and astrocytes in the CNS, as HCV RNA and proteins were detected in these two types of CNS cells using laser capture microscopy, but not detected in oligodendrocytes or neurons. In addition, alteration of striatal dopaminergic neurotransmission has been reported in HCV-infected subjects (15). Taken together, emerging evidence suggests that the CNS appears vulnerable to HCV infection.

HCV entry involves interactions between the viral surface-resident glycoproteins (E1 and E2) and several host factors (16-19): Scavenger receptor class B type I (SR-B1), CD81, claudin-1 and occluding. Many brain-derived cell lines are known to express the above receptors (20,21). Previous studies have reported the cell culture-derived HCV replication in primary astrocytes, human SVG astrocyte cell line and SKNMC peripheral neuroblastoma cell line (22,23). However, there is no report on HCV infection in glioblastoma cells. The origin of glioblastoma is not clear, as neural stem cells, glial

*Key words:* hepatitis C virus, glioblastoma, SF268, replication, phosphoinositide 3-kinase-AKT serine/threonine kinase signaling pathway

progenitors (including oligodendrocyte progenitor cells) and astrocytes may all be associated with glioma development (24). In the current study, it was demonstrated that serum-derived HCV could transiently replicate in the human glioblastoma cell line SF268 and activated the AKT serine/threonine kinase/glycogen synthase kinase  $3\beta$  (GSK $3\beta$ ) pathway.

## Materials and methods

*Cell culture*. Human glioblastoma cell line SF268 (25-27) was used for current study (a gift from Professor Bao Zhang, Nanfang Hospital, Southern Medical University, Nanfang, China; the cell line was authenticated by STR profiling). SF268 cells originate from human neuromuscular cells and developed as glioblastoma cell line/anaplastic astrocytoma (non-epithelial) cell line (28-30). SF268 were cultured at 37°C in a humidified incubator with 5% CO<sub>2</sub> and maintained in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 100 U/ml penicillin G and 100 U/ml streptomycin (Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum.

Serum samples. A total of 128 chronic HCV patients (aged 24-59; 63.3% were male) and 14 healthy donors (age 26-51; 57.1% were male) were enrolled from January 2016 to December 2017 in Nanfang Hospital (Southern Medical University, Guangzhou, China). Blood specimens were collected and serum samples were stored at -80°C prior to further analysis. Laboratory results were recorded from the patient database; demographics and commercial blood donation history were obtained using a standardized questionnaire. Potential HCV infection routes were identified for each patient, including blood transfusion, intravenous drug use and procedures using shared medical equipment, such as dental treatment, hemodialysis, non-sterile tattooing or piercing, unsafe acupuncture, etc. (Table I).

Diagnosis of chronic HCV infection was based on detection of HCV RNA in serum or plasma by using the Cobas AmpliPrep/Cobas TaqMan HCV test, version 2.0 (lower limit of detection, 15 IU/ml; Roche Diagnostics GmbH, Mannheim, Germany), which was used according to the manufacturer's protocols (31) and guidelines (3,32,33). Samples were determined to be HCV positive via a HCV assay, which was conducted as described (34). Anti-HCV antibodies were detected using the Architect HCV assay (Abbott Japan Co., Ltd., Tokyo, Japan). Exclusion criteria were as follows: Coinfection with HBV or HIV, renal transplantation, discontinued treatment, incomplete data, or loss in follow-up during 12 weeks post-treatment.

The use of patient serum samples were both approved by the ethical Committee of Nanfang Hospital, Southern Medical University (no. NFEC-2014-079) and the Medical Ethics Committee at the Zhongshan School of Medicine, Sun Yat-sen University (no. 2014-072). The experiments were performed in accordance with the approved guidelines and informed consent was obtained from all subjects.

*HCV infection*. Serum was filtered through 0.22- $\mu$ m polarized filters prior to incubation with the cells. SF268 cells were seeded in 6-well plates at a density of 4x10<sup>5</sup> cells/well for 24 h

prior to inoculation. After washing three time with 1X PBS,  $200 \,\mu$ l HCV-positive serum and 1 ml serum-free culture media were added to each well. The control cells were mock-infected with the HCV-negative serum from a healthy donor. At 12 h post-infection, the medium was replaced with 2-3 ml fresh growth medium. The cells were maintained and harvested at the indicated time points (day 2, 4, 6, 8 and 10 post-infection). The supernatants were collected every other day and stored at -80°C prior to analysis. Cells were passaged every 2 days.

Immunofluorescence analysis. Infected cells ( $6x10^3$  cells/well) in 96-well plate were fixed with 3.7% paraformaldehyde for ~10 min at 37°C, permeabilized with 0.2% Triton X-100 in PBS for 10-15 min, and blocked with 1% bovine serum albumin for 40-45 min at room temperature, followed by overnight incubation with anti-HCV core monoclonal antibody C7-50 (1:400; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; sc-57800) at 4°C. The cells were washed three times with PBS, incubated with Alexa Fluor<sup>®</sup> 488 goat anti-mouse IgG (1:250; H+L; Thermo Fisher Scientific, Inc; A28175) and DAPI (10 µg/ml) at room temperature for 2 h. The percentage of HCV infected cells in the wells was estimated using a laser scanning confocal microscope (LSM 710; Leica Microsystems GmbH, Wetzlar, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative detection of HCV RNA. Serum HCV RNA was extracted from plasma (140  $\mu$ l) using using the QIAamp Viral RNA mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from 20 µl RNA with Superscript III-First-Strand Synthesis System (Thermo Fisher Scientific, Inc.) with the following conditions: 25°C for 10 min, 50°C for 50 min, then a final cycle at 85°C for 5 min. Primers that contained partial E1 region (reference strain H77 nucleotide positions 729-1,322) for nested-PCR were described previously (23): Outer forward (OF1), 5'-TTGGGTAAGGTCATCGATACC C-3'; outer reverse (OR2), 5'-TGATGTGCCAACTGCCGT TGGT-3'; inner forward (IF3), 5'-TTCGCCGACCTCATG GGGTACAT-3'; and inner reverse (IR4), 5'-GGACCAGTT CATCATCATATCCCA-3'. LA Taq (RR002A; Takara Bio, Inc., Otsu, Japan) was used for nested PCR. First round PCR (primers OF1 and OR2) was performed with the following conditions: 94°C for 2 min, 35 cycles of 94°C for 30 sec, 58°C for 1 min and 72°C for 40 sec, then a final cycle at 72°C for 7 min. Second round PCR (primers IF3 and IR4) was executed with the same conditions with the annealing temperature was changed to 56°C for 35 sec. The products were separated on a 1.0% agarose gel dyed with 2.5 µl Gel-red (1:10,000; cat. no. 41000; Biotium, Inc., Fremont, CA, USA), and the positive samples (PCR strip located at 594 bp) were subjected to sequencing (Shanghai Invitrogen Biotechnology Co., Ltd., Shanghai, China). To avoid potential contamination, all experimental procedures were all performed in parallel with the appropriate positive and negative controls. Sequencing was performed with IF3 and IR4 primers in both directions using ABI Prism Big Dye 3.0 terminators on an ABI Prism 3500 genetic analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). HCV RNA in the cell culture supernatant was detected quantitatively as aforementioned.

Patient ID	No.	Age	Gender	Viral RNA (IU/ml)	Viral genotype	Core	Risk factor
JX17ZCF	1	43	Female	1.23x10 <sup>6</sup>	2a	+	Other <sup>a</sup>
JX17LXG	2	38	Male	$1.77 \times 10^{6}$	3a	+	$IDU^{b}$
JX17HLY	3	57	Male	2.23x10 <sup>6</sup>	1b	+	Blood <sup>c</sup>
JX17LSB	4	29	Male	$3.20 \times 10^{6}$	6a	+	IDU
JX17LMS	5	62	Male	7.39x10 <sup>6</sup>	3a	+	Blood
JX16GSQ	6	43	Male	$1.29 \times 10^{7}$	1b	+	Blood
JX16WSJ	7	39	Male	$3.47 \times 10^{6}$	6a	+	IDU

Table I. Demographics and risk factors of patients associated with hepatitis C virus subtypes.

<sup>a</sup>Other, tattooing, piercing, acupuncture, hemodialysis, etc.; <sup>b</sup>IDU, patients with a history of intravenous drug use; <sup>c</sup>Blood, patients with a history of transfusion blood or use of blood products.

Western blotting and antibodies. Western blotting analysis was performed as previously described with minor modifications (35). At the indicated time points after infection, cells were washed with PBS and incubated with lysis buffer (A8261, Promega Corporation, Madison, WI, USA) for 45 min on ice and boiled for 10 min. The protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (cat. no. 2634, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and equal amounts of protein samples  $(40 \,\mu g)$  were run through 10% Bis-Tris SDS-polyacrylamide precasted gels for 1 h 30 min at 150 V. Subsequently, separated proteins were transferred to the polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.) by wet electroblotting (XCell SureLock Mini-Cell; Invitrogen; Thermo Fisher Scientific, Inc.) at constant current for 1 h. Membranes were then washed with PBS plus 1% Tween-20, and blocked with PBS plus 1% Tween-20 and 3% bovine serum albumin (cat. no. 11021029, Gibco; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The membranes were incubated overnight at 4°C with anti-HCV core C7-50 (1:200; sc-57800, Santa Cruz Biotechnology, Inc.) or AKT (1:1,000; cat. no. 4691, Cell Signaling Technology, Inc., Danvers, MA, USA), phosphorylated (p)AKT (1:1,000; cat. no. 4060; Cell Signaling Technology, Inc.), GSK3β (1:1,000; cat. no. 9315; Cell Signaling Technology, Inc.) and pGSK3β (1:1,000; cat. no. 5558; Cell Signaling Technology, Inc.), or anti β-tubulin (1:1,000; cat. no. 86298; Cell Signaling Technology, Inc.) with agitation. Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 680 (1:40,000; a32729; Thermo Fisher Scientific, Inc.) or Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 680 (1:40,000; a32734; Thermo Fisher Scientific, Inc.) were added to the membrane and incubated for 1 h at room temperature prior to treatment with an enhanced chemiluminescence kit (GE Healthcare, Chicago, IL, USA). Finally, protein bands were visualized using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA), while the quantification of immunodetected protein bands was performed using ImageJ software (Version: k 1.45; National Institutes of Health, Bethesda, MD, USA).

*Statistical analysis.* Statistical analysis was performed with the SPSS software, version 22.0 (IBM Corp., Armonk, NY, USA). At least three independent experiments were performed.

For descriptive purposes; quantitative variables are presented as either the mean  $\pm$  standard deviation or medians and ranges, as appropriate. Categorical variables are presented as number and percentages. Statistical analysis was performed with two-tailed unpaired Student's t-test. P<0.05 was considered to indicated a statistically significant difference.

#### Results

*Demographic and clinical characteristics.* Of the seven samples tested, six (85.7%) were from male patients (Table I). Their ages ranged from 29 to 62 years with a mean ( $\pm$  standard deviation) of 44 $\pm$ 11.4 years. HCV RNA levels (IU/ml) varied between 1.23x10<sup>6</sup> and 1.29x10<sup>7</sup>. HCV genotypes included 1b, 2a, 3a and 6a. The transmission routes are listed in Table I.

HCV replication in human glioblastoma cell line SF268. HCV core protein was detected in cells infected with HCV-positive serum on the day 4 post-infection (~1% of the cells exhibited efficient HCV infection; Fig. 1). Total RNA was extracted from SF268 cells exposed to with HCV-positive or -negative serum, and reverse transcribed to cDNA. HCV-negative strand-specific PCR was performed to detect the virus replicative intermediate in the SF268. Each of seven HCV-positive serum infected SF268 cells was HCV RNA positive on day 4, while it remained negative in the HCV-negative serum-infected SF268 (Fig. 2). qPCR was performed in cell culture supernatant-derived RNA samples in order to quantify viral RNA load and affirm RNA replication. The mean RNA levels (IU/ml, log10) on day 4 post-infection were between 2.68 and 5.00 (Fig. 3) in seven medium samples. HCV RNA and core protein were undetectable when the culture was extended to day 6, 8 and 10 (data not shown). These data indicate that human SF268 glioblastoma cell line can be infected with serum-derived HCV, but the infection was transient.

Activation of phosphoinositide 3-kinase (PI3K)-AKT signaling. Previous studies reported that HCV utilizes the PI3K/AKT pathway to facilitate viral entry and replication in human hepatocytes (10,36). However, little is known about the effect of HCV infection on the AKT signaling pathway in neuronal cell lines. To explore the effect of HCV infection on AKT pathway in neuronal cell lines, AKT and pAKT (Ser473)



Figure 1. Expression of HCV core protein in SF268 cells exposed to HCV-infected serum (patient JX17LSB). On day 4 post-infection, cells were stained with anti-core antibody C7-50 for indirect immunofluorescence (green, HCV core; blue, nuclei stained by DAPI). Images are representative of three independent experiments. Scale bar,  $10 \mu$ m. HCV, hepatitis C virus.



Figure 2. RT-PCR detection of HCV infection in SF268 cells. RT-PCR was performed to detect viral RNA extracted from HCV infected SF268 cells. C-E1 region (594 bp) of HCV was amplified. Data are representative of three independent experiments. HCV, hepatitis C virus; PC, positive control used serum sample of confirmed chronic HCV-infected patient; NC1 and 2, negative control 1 and 2 used serum samples of healthy controls; HCV, hepatitis C virus.

protein levels were assessed at day 4 post-infection. As shown in Fig. 4, AKT (Ser473) phosphorylation was increased cells expressing HCV core proteins. Phosphorylation of GSK3 $\beta$ , a downstream target of AKT, was also activated by HCV infection. These data demonstrate that HCV infection of glioblastoma cells activated the AKT pathway and downstream signaling.

### Discussion

To the best our knowledge, this is the first study demonstrating that the human glioblastoma cell line SF268 can be infected by HCV, and to detect activation of the AKT/GSK3 $\beta$  pathway following HCV infection.

Glioblastoma (World Health Organization grade IV glioma) is the most lethal primary brain tumor, with increasing prevalence. It was previously reported that four human glioblastoma multiform tumor cell lines, U373MG, T98G, DBTRG and U87MG, could not be infected with cell culture-derived HCV, even though these cell lines express certain key factors involved in HCV entry (scavenger receptor class B type 1 and



Figure 3. qPCR for HCV RNA. On day 4 post-infection, cell supernatant was collected and subjected to qPCR for the detection of HCV RNA. The mean HCV RNA levels (IU/ml, log10) on day 4 post-infection were 4.11 for JX17ZCF (patient 1), 3.26 for JX17LXG (patient 2), 2.68 for JX17HLY (patient 3), 4.01 for JX17LSB (patient 4), 3.65 for JX17LMS (patient 5), 5.00 for JX16GSQ (patient 6) and 3.82 for JX16WSJ (patient 7). bbHCV, blood-borne HCV; qPCR, quantitative polymerase chain reaction; HCV, hepatitis C virus.

Claudin-1) (23). In the current study, HCV-inoculated SF268 cells were positive for HCV RNA on day 4 post-infection;



Figure 4. Activation of phosphoinositide 3-kinase-AKT signaling pathway following HCV infection. AKT and pAKT (Ser473), and GSK3 $\beta$  and pGSK3 $\beta$  (Ser9) protein levels were analyzed and compared between HCV-positive and negative groups on day 4 post-infection. \*P<0.05 vs. normal. HCV, hepatitis C virus; NC1 and 2, negative control 1 and 2 used serum samples of healthy controls; p, phosphorylated; GSK3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; AKT, AKT serine/threonine kinase; Core, HCV core protein.

however, the HCV RNA titers were lower than the input viral RNA levels by ~3 logs, suggesting two possible scenarios: i) Residual HCV inoculum was not completely removed; ii) cells were infected, but with low replication. To distinguish the two possibilities, HCV core protein expression was also assessed in the HCV-infected SF268 on day 4, 6, 8 and 10 post-infection. Supporting with the PCR amplification results, HCV core protein was detectable on day 4 post-infection, suggesting that there was established HCV replication; however, only ~1% of the cells exhibited efficient HCV infection and both HCV RNA and core protein expression became undetectable beyond day 4, indicating that HCV infection in SF268 was transient and the replication was low. Overexpression of microRNA-122 (miR-122) in SF268 cells may serve to improve transfection efficiency in future studies, as miR-122 has been reported to stimulate HCV replication (37). In contrast with the study by Bürgel et al (23), the data of the current study support that SF268 glioblastoma cells can be infected by serum-derived HCV. This discrepancy could be due to the difference between serum-derived HCV and cell culture-derived HCV interactions with cells, the concentration of viral particles used in our study was much lower and there have been no previous studies using HCV with the SF268 cell line. However, the current data indicate that human SF268 glioblastoma cell line supports HCV replication, and this response may fluctuate with respect to different patient samples, as the presence of various known and unknown inflammatory factors such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in the patient serum may promote viral entry. Other studies have reported HCV replication in primary astrocytes inoculated with serum-derived HCV (38). Additionally, other studies have indicated that HCV RNA sequences isolated from brain and cerebrospinal fluid samples were different to the serum-derived viruses, suggesting that HCV could replicate in the brain and adapt in a tissue-specific manner (39-41). HCV infection has been associated with the incidence of Parkinson's disease in a number of epidemiological studies (42-44). Together, these findings suggest that HCV may spread across the brain-blood barrier and infect neurological cells, which may have important implications for viral neurological pathogenesis.

It has been reported that HCV infection activates AKT at the early stage of infection to enhance its entry, and this activation is mediated by interaction between the HCV E2 envelope protein and its co-receptors, Claudin-1 and CD81 (36). In line with this observation, the PI3K-AKT signaling pathway was activated in HCV-infected SF268 cells in the current study. Other viruses, such as paramyxoviruses has also been reported to regulate the pathway to promote their replication (45,46). Influenza A virus also activates this pathway to enhance viral replication at a post-entry step, and its NS1 protein can also activate PI3K to suppress apoptosis (47). Human cytomegalovirus (48), coxsackievirus B3 (49), and varicella-zoster virus (50) also require PI3K/AKT activation for efficient infection. It has been reported that the HCV NS5A protein binds to PI3K and activates the signaling pathway. In addition, the HCV core protein can induce the AKT Ser473 phosphorylation, but with no apparent effect on Thr308 phosphorylation, and also impair the insulin signaling pathway (51). The data of the current study revealed that AKT phosphorylation in SF268 cells occurred at day 4 post-infection in parallel with detection core protein, indicating an association between HCV infection and activation of AKT.

The activation of the PI3K/AKT pathway may also lead to CNS immune responses and subsequently cause neuronal injury. A previous study investigated the effects of HCV core protein on microglia, neurons and astrocytes (38), indicating that HCV core protein triggered immune activation of glial cells and was neurovirulent by inducing the expression of pro-inflammatory cytokines [including interleukin (IL)-1 $\beta$ , IL-6 and TNF- $\alpha$ ] and chemokines (including C-X-C motif chemokine 10 and interleukin-8). The current study suggests that the PI3K/AKT pathway was activated by HCV infection in SF268 cells, a human glioblastoma cell lines derived from glial cells. This serum-derived HCV infection of SF268 cells may provide a novel culture system for the study of HCV-induced neuro-immune activation and potential neuronal injury.

To the best of our knowledge, no previous studies have investigated the role of HCV in glioblastoma tumorigenesis; however, the findings of the present study suggest that HCV may have a role in the carcinogenesis of glioblastoma by inducing a chronic inflammatory state, which creates a pro-carcinogenic environment. Long-term infection with HCV creates an oncogenic environment through a combination of viral protein expression, persistent inflammation, oxidative stress and chronically deregulated signaling events, which accumulate to cause genetic instability. According to the study by Bokemeyer et al (52), HCV infection may induce neuroinflammation and carcinogenesis as choline, creatine and myo-inositol, which are usually indicate glial activation and macrophage infiltration in chronic inflammation, were all significantly higher in HCV-infected patients than in controls. Additionally, compared with HCV-negative controls, HCV-positive patients were demonstrated to exhibit significantly higher levels of pro-inflammatory cytokines, including IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , IL-12 and IL-18 (53). Furthermore, post-mortem studies of HCV quasispecies and replicative intermediates also indicated that microglia may be an infection locus, leading to neuroinflammatory activity (54,55). Finally, HCV core protein has been reported to trigger activation of the extracellular signal-related kinase/signal transducer and activator of transcription 3 system via Toll-like receptor 2 in the CNS (56), which may have a role in neurodegeneration. In summary, HCV may have a role in glioblastoma carcinogenesis by inducing a chronic inflammatory state, and this may be an important research direction.

There are certain limitations in the present study. Firstly, the sample size was relatively small as this was a single center study, though HCV infection of SF268 cells was clearly demonstrated. Additionally, identification of cellular factors that restricted long-term HCV infection in SF268 cells was not performed and will be a focus of further studies. Furthermore, additional studies are required investigate HCV infection of neurons, as glioblastoma cell lines are not neuronal cells, although the SF268 cell line appears to be a useful tool for studying HCV infection in the CNS. The current study suggests novel methods for establishing a HCV infection system to investigate HCV neuropathogenesis.

In summary, to the best of our knowledge, the current study is the first to demonstrate transient HCV infection in a human glioblastoma cell line. The results provide evidence that HCV is able to enter and spread in a non-liver cancer cell line *in vitro* and may explain extra-hepatic symptoms in patients with chronic HCV infection. Additionally, the PI3K-AKT signaling pathway was activated in HCV-infected SF268 cells, suggesting that SF268 cells may be used as a model for investigating HCV-nerve cells interactions.

#### Acknowledgements

## Not applicable.

## Funding

This study was supported by the grants from the National Natural Sciences Foundation of China (grant nos. 81470856, 81772923 and 31700150). The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

GY, ZZ, YYZ, YPL and YZ conceived and designed the research. GY, JL and CH collected the samples. GY, LR, ZZ and JL performed the experiments. GY, LR, ZZ, LM and MC analyzed experimental results. GY and ZZ wrote the manuscript. YZ, YYZ and YPL supervised the project and edited the manuscript.

#### Ethics approval and consent to participate

Not applicable.

#### **Patient consent for publication**

Not applicable.

## **Competing interests**

The authors declare that they have no competing interests.

#### **Authors' information**

ORCID: Guosheng Yuan, orcid.org/0000-0002-2118-4553; Yuanping Zhou. orcid.org/0000-0002-2493-396X.

#### References

- 1. European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu: EASL recommendations on treatment of hepatitis C 2016. J Hepatol 66: 153-194, 2017.
- 2. Mohd Hanafiah K, Groeger J, Flaxman AD and Wiersma ST: Global epidemiology of hepatitis C virus infection: New estimates of age-specific antibody to HCV seroprevalence. Hepatology 57: 1333-1342, 2013.
- AASLD/IDSA HCV Guidance Panel: Hepatitis C guidance: AASLD-IDSA recommendations for testing, managing, and treating adults infected with hepatitis C virus. Hepatology 62: 932-954, 2015.
- 4. Dorner M, Horwitz JA, Donovan BM, Labitt RN, Budell WC, Friling T, Vogt A, Catanese MT, Satoh T, Kawai T, et al: Completion of the entire hepatitis C virus life cycle in genetically humanized mice. Nature 501: 237-241, 2013
- Lorenz IC, Marcotrigiano J, Dentzer TG and Rice CM: Structure of the catalytic domain of the hepatitis C virus NS2-3 protease. Nature 442: 831-835, 2006.
- Ahmad J, Eng FJ and Branch AD: HCV and HCC: Clinical update and a review of HCC-associated viral mutations in the core gene. Semin Liver Dis 31: 347-355, 2011.
- Vandenbulcke H, Moreno C, Colle I, Knebel JF, Francque S, Sersté T, George C, de Galocsy C, Laleman W, Delwaide J, *et al*: Alcohol intake increases the risk of HCC in hepatitis C virus-related compensated cirrhosis: A prospective study. J Hepatol 65: 543-551, 2016.
- 8. Wang XF and Korangy F: Intrahepatic landscape of regulatory T-cell subsets in chronically HCV-infected patients with cirrhosis and HCC. Hepatology 60: 1461-1462, 2014.
- Wilkinson J, Radkowski M and Laskus T: Hepatitis C virus neuroinvasion: Identification of infected cells. J Virol 83: 1312-1319, 2009.
- Mannová P and Beretta L: Activation of the N-Ras-PI3K-Akt-mTOR pathway by hepatitis C virus: Control of cell survival and viral replication. J Virol 79: 8742-8749, 2005.

- 11. Mariotto S, Ferrari S and Monaco S: HCV-related central and peripheral nervous system demyelinating disorders. Inflamm Allergy Drug Targets 13: 299-304, 2014.
- Pham TN, MacParland SA, Mulrooney PM, Cooksley H, Naoumov NV and Michalak TI: Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C. J Virol 78: 5867-5874, 2004.
- Radkowski M, Gallegos-Orozco JF, Jablonska J, Colby TV, Walewska-Zielecka B, Kubicka J, Wilkinson J, Adair D, Rakela J and Laskus T: Persistence of hepatitis C virus in patients successfully treated for chronic hepatitis C. Hepatology 41: 106-114, 2005.
- 14. Letendre S, Paulino AD, Rockenstein E, Adame A, Crews L, Cherner M, Heaton R, Ellis R, Everall IP, Grant I, *et al*: Pathogenesis of hepatitis C virus coinfection in the brains of patients infected with HIV. J Infect Dis 196: 361-370, 2007.
- Weissenborn K, Ennen JC, Bokemeyer M, Ahl B, Wurster U, Tillmann H, Trebst C, Hecker H and Berding G: Monoaminergic neurotransmission is altered in hepatitis C virus infected patients with chronic fatigue and cognitive impairment. Gut 55: 1624-1630, 2006.
- 16. Bankwitz D, Steinmann E, Bitzegeio J, Ciesek S, Friesland M, Herrmann E, Zeisel MB, Baumert TF, Keck ZY, Foung SK, et al: Hepatitis C virus hypervariable region 1 modulates receptor interactions, conceals the CD81 binding site, and protects conserved neutralizing epitopes. J Virol 84: 5751-5763, 2010.
- Grigorov B, Reungoat E, Gentil Dit Maurin A, Varbanov M, Blaising J, Michelet M, Manuel R, Parent R, Bartosch B, Zoulim F, *et al*: Hepatitis C virus infection propagates through interactions between Syndecan-1 and CD81 and impacts the hepatocyte glycocalyx. Cell Microbiol 19, 2017.
   Lavie M, Sarrazin S, Montserret R, Descamps V, Baumert TF,
- Lavie M, Sarrazin S, Montserret R, Descamps V, Baumert TF, Duverlie G, Séron K, Penin F and Dubuisson J: Identification of conserved residues in hepatitis C virus envelope glycoprotein E2 that modulate virus dependence on CD81 and SRB1 entry factors. J Virol 88: 10584-10597, 2014.
- Tong Y, Zhu Y, Xia X, Liu Y, Feng Y, Hua X, Chen Z, Ding H, Gao L, Wang Y, et al: Tupaia CD81, SR-BI, claudin-1, and occludin support hepatitis C virus infection. J Virol 85: 2793-2802, 2011.
- Duffy HS, John GR, Lee SC, Brosnan CF and Spray DC: Reciprocal regulation of the junctional proteins claudin-1 and connexin43 by interleukin-1beta in primary human fetal astrocytes. J Neurosci 20: RC114, 2000.
- 21. Husemann J and Silverstein SC: Expression of scavenger receptor class B, type I, by astrocytes and vascular smooth muscle cells in normal adult mouse and human brain and in Alzheimer's disease brain. Am J Pathol 158: 825-832, 2001.
- 22. Rajalakshmy AR, Malathi J and Madhavan HN: Serum-derived hepatitis C virus 1a infection of human astrocyte cell line SVG. J Viral Hepat 23: 211-216, 2016.
- 23. Bürgel B, Friesland M, Koch A, Manns MP, Wedemeyer H, Weissenborn K, Schulz-Schaeffer WJ, Pietschmann T, Steinmann E and Ciesek S: Hepatitis C virus enters human peripheral neuroblastoma cells-evidence for extra-hepatic cells sustaining hepatitis C virus penetration. J Viral Hepat 18: 562-570, 2011.
- Zong H, Verhaak RG and Canoll P: The cellular origin for malignant glioma and prospects for clinical advancements. Expert Rev Mol Diagn 12: 383-394, 2012.
- 25. Al Hassan M, Fakhoury I, El Masri Z, Ghazale N, Dennaoui R, El Atat O, Kanaan A and El-Sibai M: Metformin treatment inhibits motility and invasion of glioblastoma cancer cells. Anal Cell Pathol (Amst) 2018: 5917470, 2018.
- 26. Shih SR, Weng KF, Stollar V and Li ML: Viral protein synthesis is required for Enterovirus 71 to induce apoptosis in human glioblastoma cells. J Neurovirol 14: 53-61, 2008.
- 27. Stein S, Zhao R, Haeno H, Vivanco I and Michor F: Mathematical modeling identifies optimum lapatinib dosing schedules for the treatment of glioblastoma patients. PLoS Comput Biol 14: e1005924, 2018.
- 28. Chan JF, Yip CC, Tsang JO, Tee KM, Cai JP, Chik KK, Zhu Z, Chan CC, Choi GK, Sridhar S, *et al*: Differential cell line susceptibility to the emerging Zika virus: Implications for disease pathogenesis, non-vector-borne human transmission and animal reservoirs. Emerg Microbes Infect 5: e93, 2016.
- Perazzoli G, Prados J, Ortiz R, Caba O, Cabeza L, Berdasco M, Gónzalez B and Melguizo C: Temozolomide resistance in glioblastoma cell lines: Implication of MGMT, MMR, P-Glycoprotein and CD133 expression. Plos One 10: e140131. 2015.

- 30. Westphal M, Harsh GR IV, Rosenblum ML and Hammonds RJ Jr: Epidermal growth factor receptors in the human glioblastoma cell line SF268 differ from those in epidermoid carcinoma cell line A431. Biochem Biophys Res Commun 132: 284-289, 1985.
- 31. Pas S, Molenkamp R, Schinkel J, Rebers S, Copra C, Seven-Deniz S, Thamke D, de Knegt RJ, Haagmans BL and Schutten M: Performance evaluation of the new Roche cobas AmpliPrep/cobas TaqMan HCV test, version 2.0, for detection and quantification of hepatitis C virus RNA. J Clin Microbiol 51: 238-242, 2013.
- 32. European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu; European Association for the Study of the Liver: EASL recommendations on treatment of hepatitis C 2018. J Hepatol 69: 461-511, 2018.
- 33. Omata M, Kanda T, Wei L, Yu ML, Chuang WL, Ibrahim A, Lesmana CR, Sollano J, Kumar M, Jindal A, *et al*: APASL consensus statements and recommendations for hepatitis C prevention, epidemiology, and laboratory testing. Hepatol Int 10: 681-701, 2016.
- 34. Huang H, Yuan G, Du Y, Cai X, Liu J, Hu C, Liang B, Hu G, Tang X and Zhou Y: Effects of preventive therapy for latent tuberculosis infection and factors associated with treatment abandonment: A cross-sectional study. J Thorac Dis 10: 4377-4386, 2018.
- 35. Zhang Z, Liu L, Jiang X, Zhai S and Xing D: The essential Role of Drp1 and its regulation by S-nitrosylation of parkin in dopaminergic neurodegeneration: Implications for parkinson's disease. Antioxid Redox Signal 25: 609-622, 2016.
- Liu Z, Tian Y, Machida K, Lai MM, Luo G, Foung SK and Ou JH: Transient activation of the PI3K-AKT pathway by hepatitis C virus to enhance viral entry. J Biol Chem 287: 41922-41930, 2012.
- 37. Li YP, Gottwein JM, Scheel TK, Jensen TB and Bukh J: MicroRNA-122 antagonism against hepatitis C virus genotypes 1-6 and reduced efficacy by host RNA insertion or mutations in the HCV 5' UTR. Proc Natl Acad Sci USA 108: 4991-4996, 2011.
- 38. Vivithanaporn P, Maingat F, Lin LT, Na H, Richardson CD, Agrawal B, Cohen EA, Jhamandas JH and Power C: Hepatitis C virus core protein induces neuroimmune activation and potentiates human immunodeficiency Virus-1 neurotoxicity. PLoS One 5: e12856, 2010.
- Fishman SL, Murray JM, Eng FJ, Walewski JL, Morgello S and Branch AD: Molecular and bioinformatic evidence of hepatitis C virus evolution in brain. J Infect Dis 197: 597-607, 2008.
- 40. Laskus T, Radkowski M, Bednarska A, Wilkinson J, Adair D, Nowicki M, Nikolopoulou GB, Vargas H and Rakela J: Detection and analysis of hepatitis C virus sequences in cerebrospinal fluid. J Virol 76: 10064-10068, 2002.
- 41. Radkowski M, Wilkinson J, Nowicki M, Adair D, Vargas H, Ingui C, Rakela J and Laskus T: Search for hepatitis C virus negative-strand RNA sequences and analysis of viral sequences in the central nervous system: Evidence of replication. J Virol 76: 600-608, 2002.
- 42. Boyd JT, Wangensteen KJ, Krawitt EL, Hamill RW, Kao CH and Tsai HH: Hepatitis C virus infection as a risk factor for Parkinson disease: A nationwide cohort study. Neurology 87: 342, 2016.
- 43. Tsai HH, Liou HH, Muo CH, Lee CZ, Yen RF and Kao CH: Hepatitis C virus infection as a risk factor for Parkinson disease: A nationwide cohort study. Neurology 86: 840-846, 2016.
- 44. Chen HH, Liu PF, Tsai HH, Yen RF and Liou HH: Re: Wangensteen et al. of a letter on 'Hepatitis C virus infection: A risk factor for Parkinson's disease'. J Viral Hepat 23: 560, 2016.
- 45. Sun M, Fuentes SM, Timani K, Sun D, Murphy C, Lin Y, August A, Teng MN and He B: Akt plays a critical role in replication of nonsegmented negative-stranded RNA viruses. J Virol 82: 105-114, 2008.
- 46. Zhirnov OP: Biochemical variations in cytolytic activity of ortho- and paramyxoviruses in human lung tumor cell culture. Biochemistry (Mosc) 82: 1048-1054, 2017.
- Preusse M, Schughart K and Pessler F: Host genetic background strongly affects pulmonary microRNA expression before and during influenza A virus infection. Front Immunol 8: 246, 2017.
- Peppenelli MA, Arend KC, Cojohari O, Moorman NJ and Chan GC: Human cytomegalovirus stimulates the synthesis of select Akt-dependent antiapoptotic proteins during viral entry to promote survival of infected monocytes. J Virol 90: 3138-3147, 2016.
- 49. Chen Z, Yang L, Liu Y, Tang A, Li X, Zhang J and Yang Z: LY294002 and Rapamycin promote coxsackievirus-induced cytopathic effect and apoptosis via inhibition of PI3K/AKT/mTOR signaling pathway. Mol Cell Biochem 385: 169-177, 2014.

- 50. Liu X and Cohen JI: Varicella-zoster virus ORF12 protein activates the phosphatidylinositol 3-kinase/Akt pathway to regulate cell cycle progression. J Virol 87: 1842-1848, 2013.
- 51. Banerjee S, Saito K, Ait-Goughoulte M, Meyer K, Ray RB and Ray R: Hepatitis C virus core protein upregulates serine phosphorylation of insulin receptor substrate-1 and impairs the downstream akt/protein kinase B signaling pathway for insulin resistance. J Virol 82: 2606-2612, 2008.
- 52. Bokemeyer M, Ding XQ, Goldbecker A, Raab P, Heeren M, Arvanitis D, Tillmann HL, Lanfermann H and Weissenborn K: Evidence for neuroinflammation and neuroprotection in HCV infection-associated encephalopathy. Gut 60: 370-377, 2011.
- 53. Wilkinson J, Radkowski M, Eschbacher JM and Laskus T: Activation of brain macrophages/microglia cells in hepatitis C infection. Gut 59: 1394-1400, 2010.
- 54. Grover VP, Pavese N, Koh SB, Wylezinska M, Saxby BK, Gerhard A, Forton DM, Brooks DJ, Thomas HC and Taylor-Robinson SD: Cerebral microglial activation in patients with hepatitis C: In vivo evidence of neuroinflammation. J Viral Hepat 19: e89-e96, 2012.
- 55. Forton DM, Hamilton G, Allsop JM, Grover VP, Wesnes K, O'Sullivan C, Thomas HC and Taylor-Robinson SD: Cerebral immune activation in chronic hepatitis C infection: A magnetic resonance spectroscopy study. J Hepatol 49: 316-322, 2008.
- resonance spectroscopy study. J Hepatol 49: 316-322, 2008.
  56. Paulino AD, Ubhi K, Rockenstein E, Adame A, Crews L, Letendre S, Ellis R, Everall IP, Grant I and Masliah E: Neurotoxic effects of the HCV core protein are mediated by sustained activation of ERK via TLR2 signaling. J Neurovirol 17: 327-340, 2011.