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 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...
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 3β (GSK3β) 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% CO₂ 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, 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 (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, discontinuation 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-μm polarized filters prior to incubation with the cells. SF268 cells were seeded in 6-well plates at a density of 4x10⁵ cells/well for 24 h prior to inoculation. After washing three time with 1X PBS, 200 μl 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 (6x10⁵ 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® 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 μl) 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’-TTGGTGAAAGTCATCGGATCC-3’; outer reverse (OR2), 5’-TGAGTTGCCAATGCGGGTTG-3’; inner forward (IF3), 5’-TTGGCCGACCTCATG GGTGATC-3’; and inner reverse (IR4), 5’-GGACACGTT CATCATACTATCCA-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.
**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 µg) 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.) and 5.00 (Fig. 3) in seven medium samples. HCV RNA load and affirm RNA replication. The mean RNA content of transfusion blood or use of blood products.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>No.</th>
<th>Age</th>
<th>Gender</th>
<th>Viral RNA (IU/ml)</th>
<th>Viral genotype</th>
<th>Core</th>
<th>Risk factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>JX17ZCF</td>
<td>1</td>
<td>43</td>
<td>Female</td>
<td>1.23x10⁶</td>
<td>2a</td>
<td>+</td>
<td>Otherα</td>
</tr>
<tr>
<td>JX17LXD</td>
<td>2</td>
<td>38</td>
<td>Male</td>
<td>1.77x10⁶</td>
<td>3a</td>
<td>+</td>
<td>IDUβ</td>
</tr>
<tr>
<td>JX17HLY</td>
<td>3</td>
<td>57</td>
<td>Male</td>
<td>2.23x10⁶</td>
<td>1b</td>
<td>+</td>
<td>Bloodδ</td>
</tr>
<tr>
<td>JX17LSB</td>
<td>4</td>
<td>29</td>
<td>Male</td>
<td>3.20x10⁶</td>
<td>1b</td>
<td>+</td>
<td>Bloodδ</td>
</tr>
<tr>
<td>JX17LMS</td>
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<td>62</td>
<td>Male</td>
<td>7.39x10⁶</td>
<td>2a</td>
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<tr>
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<td>43</td>
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<tr>
<td>JX16WSJ</td>
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<td>39</td>
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<td>3.47x10⁶</td>
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- Other, tattooing, piercing, acupuncture, hemodialysis, etc.;  
- IDU, patients with a history of intravenous drug use;  
- Blood, patients with a history of transfusion blood or use of blood products.

For descriptive purposes; quantitative variables are presented either as the mean ± 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 (± standard deviation) of 44±11.4 years. HCV RNA levels (IU/ml) varied between 1.23x10⁶ and 1.29x10⁷. 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 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)

<p>| Table I. Demographics and risk factors of patients associated with hepatitis C virus subtypes. |
|---------------------------------|-----------------------------|-----------------|-----------------|</p>
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protein levels were assessed at day 4 post-infection. As shown in Fig. 4, AKT (Ser473) phosphorylation was increased in cells expressing HCV core proteins. Phosphorylation of GSK3β, 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 of 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/GSK3β 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 Claudin-1) (23). In the current study, HCV-inoculated SF268 cells were positive for HCV RNA on day 4 post-infection;
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-α (TNF-α) 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)-1β, IL-6 and TNF-α] 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-1α, IL-1β, TNF-α, 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 to 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, YYY, YPL and YYZ 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.

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References

Hepatitis C virus hypervariable region 1
Akt plays a critical role in
Hepatitis C virus enters human
EASL recommendations on treatment of


