

Induction of Huh-7 cell apoptosis by HCV core proteins via CK1 α -p53-Bid signaling pathway

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Abstract. Hepatitis C virus (HCV)-infected liver cells sensitize host cells to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced cell apoptosis; however, the precise mechanisms are unknown. In the present study, flow cytometry demonstrated that the Annexin V-positive Huh-7 cell number was higher in groups transfected with core proteins when compared with the pcDNA3.1 group. The mRNA and protein expression levels of B-cell lymphoma 2 (Bcl-2) were negatively associated, while Bcl-2-associated X protein (Bax) were positively correlated, with cell apoptotic rate, which, were verified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting. There were no significant differences in the expressions of casein kinase 1 (CK1)- ϵ , CK1 γ or CK1 δ ; however, the mRNA and protein levels of CK1 α were markedly higher in groups transfected with the T (those derived from the HCV-J6 strain), NT (those derived from non-tumor tissues) and C191 (those derived from tumor tissues) HCV core proteins than in mock group. When compared with the Mock and Negative Control (control known-down) groups, the mRNA and protein levels of CK1 α were lower in the CK1 α known-down group, and there were no marked Huh-7 cell morphological changes among the 3 groups. There was more sensitivity to cell apoptosis in CK1 α -silenced, however, not in non-CK1 α -silenced, Huh-7 cells. BH3 interacting-domain death agonist (Bid) protein levels in CK1 α -silenced Huh-7 cells were higher when compared with non-CK1 α -silenced Huh-7 cells, and the level of p53 that translocated to the nucleus increased. Chromatin immunoprecipitation-PCR demonstrated that p53 bound to human Bid gene promoter. The level of the Bid promoter in CK1 α -silenced Huh-7 cells was significantly higher than in the non-CK1 α -silenced Huh-7 cells. Electron microscopy indicated that p53 knockdown decreased HCV core protein and TRAIL-induced cell apoptosis. Bid/caspase-8 protein

levels in CK1 α -silenced Huh-7 cells that were transfected with p53 siRNA were lower than in the control group. The present study demonstrated that HCV core proteins sensitize host cells to TRAIL-induced cell apoptosis by activating the CK1 α -p53-Bid dependent pathway.

Introduction

Hepatitis C virus (HCV) is the major cause to HCC and liver failure worldwide, moreover, China is considered as a high endemic area of HCV infection (1-4). HCV which belongs to the *Flaviviridae* family has only a positive single-stranded RNA genome encoding a precursor polyprotein of about 3000 amino acid residues (5). HCV core protein plays an important role in the regulation of cell growth and host expression of genes that were crucial for infectivity, for instance, cell apoptosis (6,7). After the infection of host cells by HCV, they initiate the defense ability which named as cell apoptosis. However, HCV core protein has evolved to inhibit the ability of host-mediated cell apoptosis (8). It has been revealed that, apart from forming virus, HCV core proteins can modulate gene transcription, cell proliferation, cell apoptosis, and progression to HCC. Generally, HCV core protein appears to exert multiple effects on cell apoptosis which rely on the apoptotic stimuli as well as the cell type and microenvironment (9). Though there are numerous reports describing the functions of HCV core proteins in cellular apoptosis, the mechanisms and impacts of these proteins in Huh-7 cell apoptosis have not so far been studied or reported.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), also named Apo2L, belongs to the TNF superfamily and induces cell apoptosis typically in a variety of transformed cells but not healthy cells (10). Moreover, it is reported that TRAIL also induces cell apoptosis in virus-infected cells, for instance, cells that are infected by HCV (11). Therefore, TRAIL may serve an immune surveillance factor by selectively killing virus-infected cells.

It is well known that the tumor promoter protein p53 plays a key role in cell apoptosis, while the functional inactivation of p53 is often a crucial stage during tumorigenesis (12). Through sirt1, when p53 protein is deacetylated, its DNA binding activity is impaired, consequently leading to a reduction in p53-mediated cell apoptosis in response to DNA damage (13,14). BH3 interacting-domain death agonist (Bid), whose promoter has p53-binding sites and whose expression is regulated by p53,

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takes part in numerous apoptotic processes (15,16). Be cleaved by other proteases or caspase-8, activated Bid translocates to the mitochondrial outer membrane and leads to the activation of Bcl-2-associated X protein (Bax)/Bak (17).

In the present study, we focus on HCV core proteins of 3 different strains to explore the possible mechanisms and hunt for a novel therapeutic target for HCV infection.

Materials and methods

Plasmids. The plasmids of pcDNA3.1, pcDNA3.1-C191, pcDNA3.1-NT and pcDNA3.1-T were conserved by our lab in Laboratory of Infectious Disease, Affiliated Hospital of Xuzhou Medical University.

Cell culture and DNA transfection. The human hepatoma cell line Huh-7 was purchased from ATCC. Huh-7 cells were cultured in Dulbecco Minimum Essential Medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (both Gibco; Thermo Fisher Scientific, Inc.) in an incubator at the temperature of 37°C and 5% CO₂.

Transfection was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as recommended by the manufacturer. The p53-specific siRNA was purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA (sc-29435). The siRNA was transfected into cells that were cultured in 6-well plates by Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at the concentration of 100 pmol/well, or transfected into cells in 24-well plates with HiPerFect transfection reagent (Qiagen, Inc., Valencia, CA, USA) at the concentration of 37.5 ng/well.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was purified from cultured Huh-7 cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's instructions. Reverse transcription was performed by random primer and Moloney murine leukemia virus reverse transcriptase (Promega Corp., Madison, WI, USA), and cDNA was used as a template for RT-PCR. RT-qPCR was carried out using SYBR master mix (Toyobo Life Science, Osaka, Japan) and ABI7300. PCR conditions were as followed: 95°C for 60 sec followed by 40 cycles of 95°C for 15 sec, 60°C for 15 sec, and 72°C for 45 sec. The mRNA levels of casein kinase (CK)1 α , CK1 β , CK1 γ , CK1 δ and GAPDH (internal control) in Huh-7 cells were measured using the following primers: CK1 α (sense, 5'-AGTGGCAGTGAA GCTAGAATCT-3' and antisense, 5'-CGCCCAATACCCATT AGGAAGTT-3') CK1 ϵ (sense, 5'-TCCAAAGCCGAACCTCGTT GT-3' and antisense, 5'-GATGCCCAGATAAACGTCTCC-3'), CK1 γ (sense, 5'-ATGGACCATCCTAGTAGGGAAAA-3' and antisense, 5'-CACATCCTATCTTCTTGCCAACC-3'), CK1 δ (sense, 5'-CAGGAGAAGAGGTTGCCATCA-3' and antisense, 5'-CAAGCAGCAGGACGGTTTTG-3').

Electron microscope. The Huh-7 cells from each group were collected into different centrifuge tubes, washed with phosphate-buffered saline (PBS; pH 7.4), carefully transferred into 0.25% glutaraldehyde using a pipette. Then, they were incubated at 4°C overnight. Subsequently, samples were fixed

with 3% glutaraldehyde and 1% osmiumtetroxide. The fixed samples were dehydrated with a gradient series of acetone and embedded in Epon-812 agar (Shell Chemicals, Deer Park, TX, USA). Finally, the embedded samples, which were constructed into ultrathin sections by automatic semi-thin rotary microtome (Leica, Wetzlar, Germany) and stained with uranyl acetate/lead citrate, were observed under a Hitachi H-600IV transmission electron microscope (Hitachi, Tokyo, Japan). Images were captured accordingly.

FACS analysis. Apoptosis assays were performed as manufacturer's instructions (BD Biosciences, Franklin Lakes, NJ, USA). For each sample, 0.3x10⁶ cells were seeded per 35-mm well and analyzed at 18 h (for apoptosis) or 72 h (for survival) after treatment, respectively. Annexin V-FITC/PI staining for apoptosis assays were conducted according to the manufacturer's protocols.

Induction of apoptosis by TRAIL. Huh-7 cells (60-70% confluent) were seeded into 6-well plates. Three days post transfection, the Huh-7 cells were treated with recombinant human TRAIL (Perprotech, London, UK) for 2 h. Subsequently, cells were harvested, washed with PBS and stained with Annexin V as recommended by the manufacturer. The proportion of apoptotic cells was determined with flow cytometry.

Western blot analysis. At 24 h after the cells were harvested, the protein expression levels were measured by WB analysis. Equal amounts of protein samples were resolved by 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), and transferred onto a nitrocellulose membrane (Amersham; GE Healthcare, Chicago, IL, USA). The membranes were probed using a polyclonal antibody against CK1 α (1:1,000 ab108296; Abcam, Cambridge, UK), CK1 ϵ (1:1,000; sc-373912; Santa Cruz Biotechnology, Inc.), CK1 γ (1:1,000; ab64829), CK1 δ (1:1,000; ab85320; both Abcam), Bid (1:1,000; 8762), caspase-8 (1:1,000; 4927; both Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight. Thereafter, membrane was washed with TBST for 3 times, and incubated with secondary antibody at room temperature for 1 h. After wash for 3 times, blots were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc.). GAPDH (1:8,000; G5262; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used as an internal control for the comparison of protein load.

Statistical analysis. The results were analyzed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and are expressed as the mean \pm standard deviation of at least 3 independent experiments. Comparisons between groups were performed using Student's t-test and one-way analysis of variance with Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

HCV core proteins induced Huh-7 cell apoptosis. The ratios of Annexin V/PI-positive human Huh-7 cells in different groups were quantified by flow cytometry. Compared with Huh-7 cells that were transfected with pcDNA3.1, total number of Annexin V-positive Huh-7 cells was significantly increased after transfection with three different core proteins (Fig. 1A).

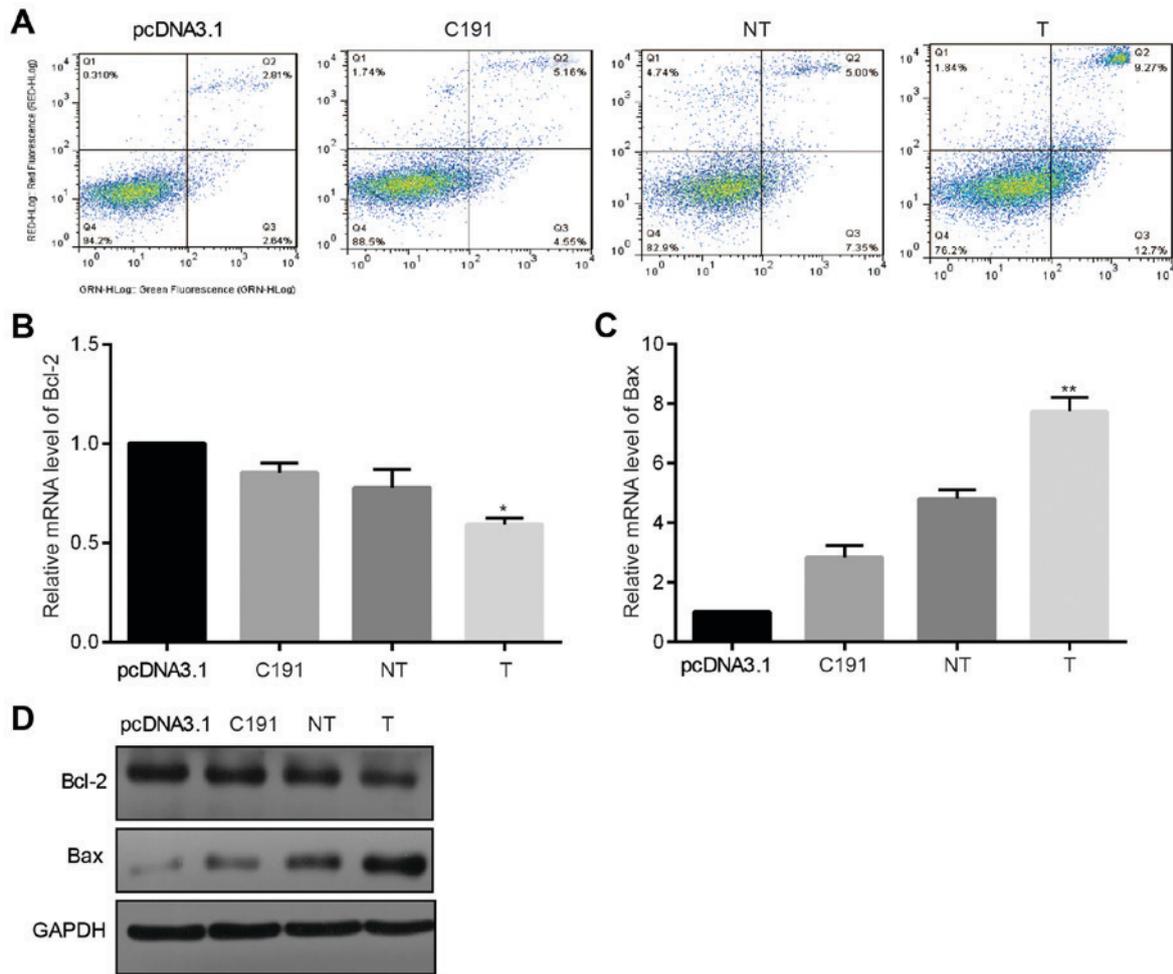


Figure 1. Apoptotic and anti-proliferation effects of HCV core proteins in Huh-7 cells. Huh-7 cells were transiently transfected with three core proteins, and Huh-7 cells that were transfected with pcDNA3.1 were used as the negative control. (A) The ratio of Annexin V-positive Huh-7 cells was measured by flow cytometry (n=3). HCV core proteins were transiently transfected into Huh-7 cells. Following 48 h, mRNA levels of (B) Bcl-2 and (C) Bax (n=5) were measured. (D) Correspondingly, following 48 h, the protein levels of BAX and Bcl-2 were evaluated (n=5). *P<0.05 and **P<0.01 vs. pcDNA3.1. HCV, hepatitis C virus; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; T, core proteins derived from tumor tissues; NT, core proteins derived from non-tumor tissues; C191, core proteins from the HCV-J6 strain.

Thereafter, we measured mRNA and protein levels of BAX and B-cell lymphoma 2 (Bcl-2) in Huh-7 cells that were transfected with three different HCV core plasmids 48 h later. As shown in Fig. 1B-D, Bcl-2 was significantly downregulated while BAX was significantly upregulated at both mRNA level and protein level in Huh-7 cells in groups transfected with core proteins than in group transfected with pcDNA3.1.

These results exhibited that all the different HCV core proteins induced Huh-7 cell apoptosis.

Different strains of HCV core proteins elevated expression of CK1 α in Huh-7 cells specifically. To explore the function of different strains of HCV core proteins (T, NT, C191), we firstly examined the changes of CK1 isoforms (CK1 α , CK1 ϵ , CK1 γ and CK1 δ) in Huh-7 cells after transfection with HCV core proteins. There were no statistical significance for changes of CK1 ϵ , CK1 γ and CK1 δ mRNA levels, while mRNA level of CK1 α in Huh-7 cells that transfected with HCV core protein was statistically increased compared with mock group (Fig. 2A). Protein expression of CK1 isoforms was in accordance with mRNA level changes (Fig. 2B).

In conclusion, CK1 α played a pivotal role in responding to the transfection of HCV core proteins into Huh-7 cells. It was unknown whether there were down-stream molecules that could be regulated or affected by CK1 α .

Successful construction of stable CK1 α knock-down Huh-7 cells. To investigate the function of CK1 α in Huh-7 cells which were transfected with different strains of HCV core proteins, virus-mediated shRNA CK1 α was adopted to infect Huh-7 cells. Compared to the MOCK group and control group, mRNA level of CK1 α was statistically decreased in the CK1 α knock-down group (Fig. 3A). Protein level of CK1 α was in accord with changes of mRNA (Fig. 3B). There were not obvious morphological changes of Huh-7 cells among MOCK group, NC group and CK1 α knockdown group (Fig. 3C).

Taken together, we obtained the stable CK1 α knock-down Huh-7 cells. We aimed to further identify the influence of CK1 α knock-down on Huh-7 cell behaviors and the related molecules.

Knockdown of CK1 α increased HCV core protein-induced cell apoptosis. We transfected 3 different HCV core proteins

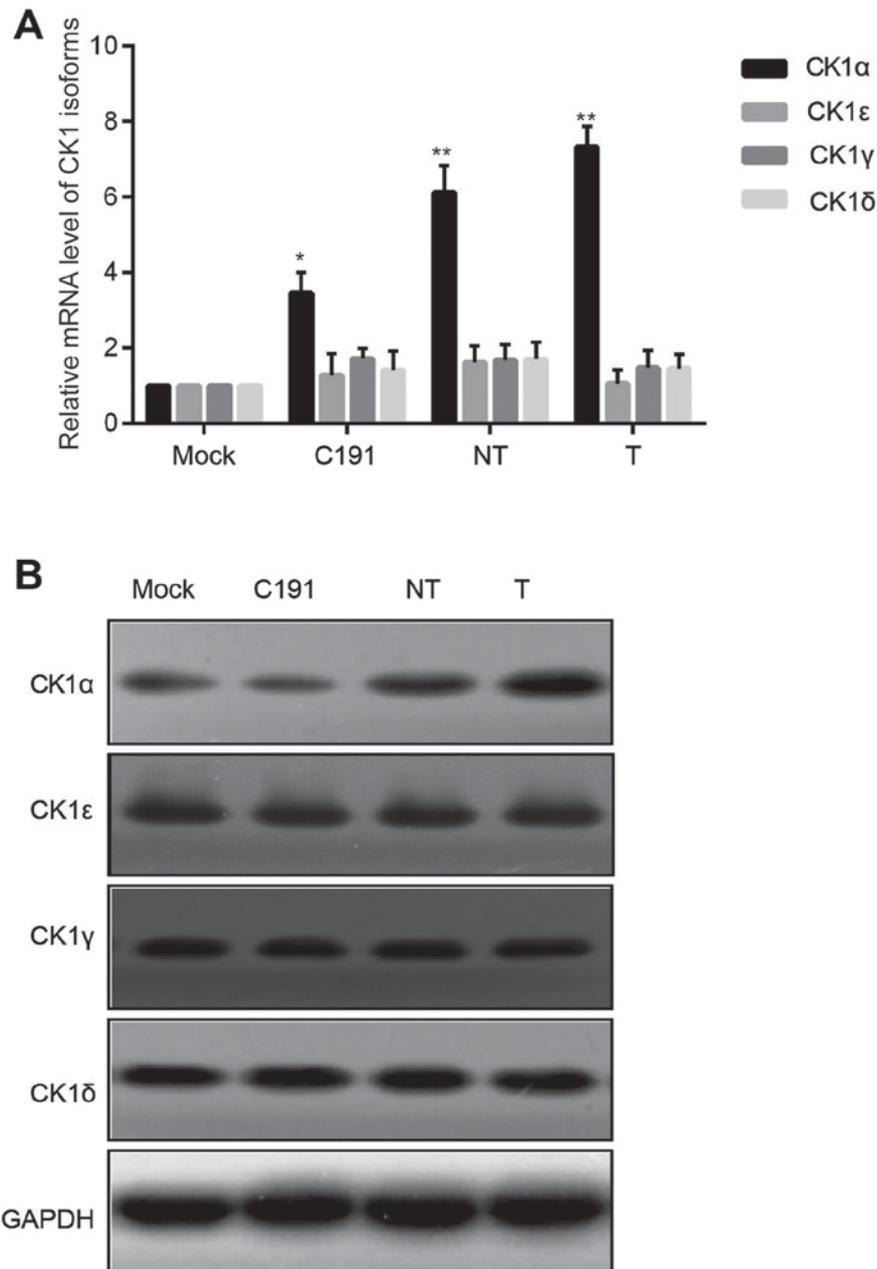


Figure 2. Transfection of HCV core proteins markedly elevated the expression levels of CK1 α in Huh-7 cells. The (A) mRNA and (B) protein level of CK1 isoforms following transfection of HCV core protein of different strains. * $P < 0.05$ and ** $P < 0.01$ vs. mock. HCV, hepatitis C virus; CK1, casein kinase 1; T, core proteins derived from tumor tissues; NT, core proteins derived from non-tumor tissues; C191, core proteins from the HCV-J6 strain.

into non-CK1 α -silenced and CK1 α -silenced Huh-7 cells. Compared with non-CK1 α -silenced Huh-7 cells, CK1 α -silenced Huh-7 cells were more sensitive to cell apoptosis. Cell apoptotic ratio in the non-CK1 α -silenced Huh-7 cells was 14.8% (C191), 14.74% (NT) and 17.13% (T), while cell apoptosis in the CK1 α -silenced group was 18.2% (C191), 24.81% (NT) and 31.11% (T), respectively (Fig. 4A).

The protein levels of Bid and caspase8 in CK1 α -silenced Huh-7 cells were notably higher than in non-CK1 α -silenced Huh-7 cells (Fig. 4B).

We could draw the conclusion that, knockdown of CK1 α upregulated HCV core protein-induced cell apoptosis. Whereas, the corresponding molecular mechanism was still unknown.

CK1 α knockdown upregulated HCV core protein levels and TRAIL-induced cell apoptosis. Since Trail could induce virus-infected cell apoptosis, we further tested whether HCV core protein-transfected CK1 α -silenced Huh-7 cells were sensitive to TRAIL. Flow cytometry revealed that, in non-CK1 α -silenced Huh-7 cells transfected with 3 different HCV core protein, the cell apoptosis ratio was 24.35% (C191), 26.6% (NT) and 31.65% (T), which was significantly lower than that in CK1 α -silenced group was 24.9% (C191), 46.6% (NT) and 50.77% (T) (Fig. 5A).

To investigate the corresponding mechanism, western blot analysis was adopted and exhibited that protein level of Bid in CK1 α -silenced Huh-7 cells was obviously higher than in non-CK1 α -silenced Huh-7 cells which was tightly associated

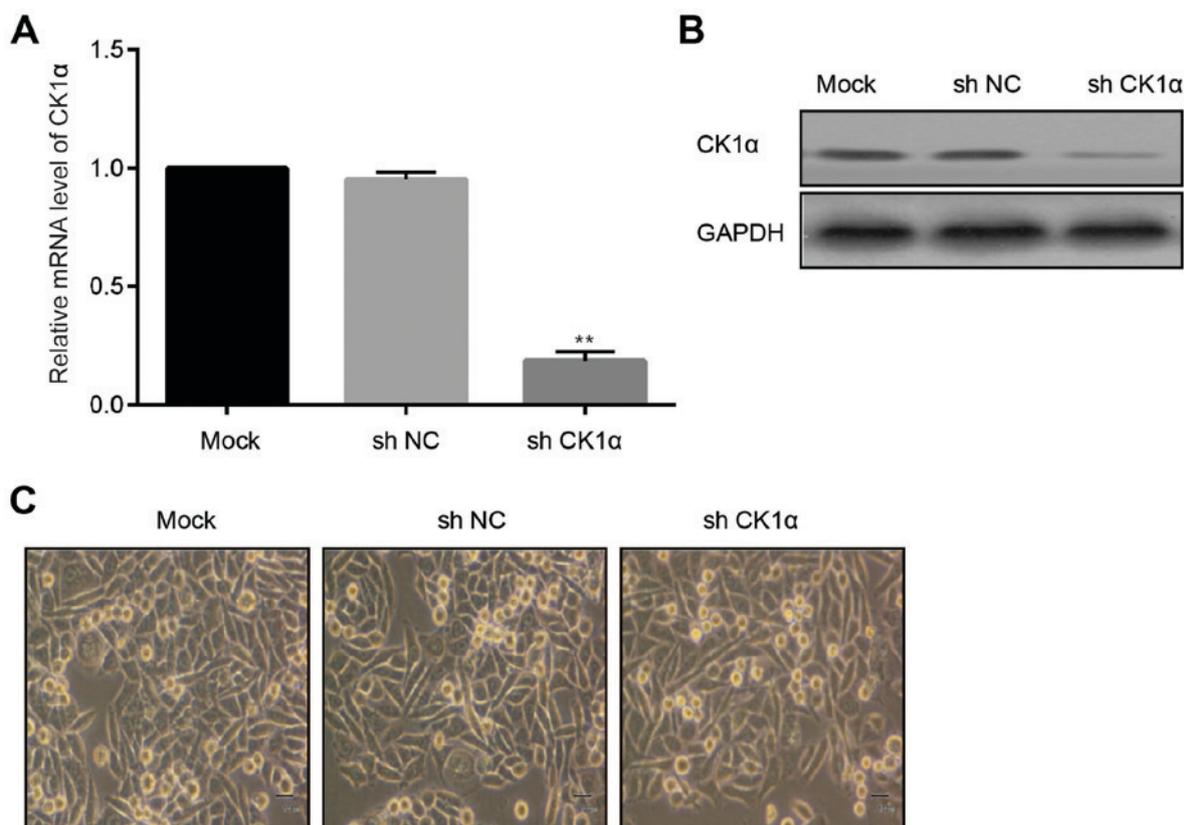


Figure 3. Successful construction of stable CK1 α knockdown Huh-7 cells. (A) mRNA and (B) protein levels of CK1 α among mock, NC and CK1 α knockdown Huh-7 cells. (C) Cellular morphology of Huh-7 cells in different groups (magnification, x100). **P<0.01 vs. mock. HCV, hepatitis C virus; CK1, casein kinase 1; NC, negative control.

with HCV core proteins of different strains. p53 that translocated to nuclear was also increased (Fig. 5B), indicating that translocation of p53 to nuclear initiated the transcription of Bid.

ChIP-PCR amplification demonstrated that p53 bound to the promoter regions of human Bid gene in Huh-7 cells (Fig. 5C).

We further measured the amount of Bid promoter precipitated by p53 in CK1 α -silenced Huh-7 cells with RT-qPCR, and found that it was significantly higher than in non-CK1 α -silenced Huh-7 cells which was also correlated with HCV core proteins of different strains (Fig. 5D).

Knockdown of p53 decreased HCV core protein and TRAIL-induced cell apoptosis. To verify the function of p53 in TRAIL-induced cell apoptosis in the CK1 α -silenced Huh-7 cells that were transfected with HCV core proteins, we further transfected p53 siRNA or control siRNA into CK1 α -silenced Huh-7 cells which were transfected with T core protein. Results were measured by electron microscope, indicating that knockdown of p53 decreased HCV core protein and TRAIL-induced cell apoptosis (Fig. 6A). The protein levels of Bid and caspase-8 in CK1 α -silenced Huh-7 cells that transfected with p53 siRNA were significantly lower than in control group (Fig. 6B).

In sum, we demonstrates that HCV core proteins sensitize host cells to TRAIL-induced cell apoptosis by activating CK1 α -P53-Bid dependent pathway.

Discussion

As the major cause of HCC and liver failure, HCV seriously affected the health of people worldwide (1-4). Therefore, it is urgent to explore effective treatments for HCV infection. In the past several years, the role of HCV core proteins in cell apoptosis has been debated, whereas, the mechanisms and impacts of HCV core proteins in Huh-7 cell apoptosis have not been reported yet (18). The research data showed that HCV core proteins function as both promoter and suppressor during the process of apoptosis. In the present study, we aimed to provide an improved understanding of the effects of HCV core proteins on Huh-7 cell apoptosis, and we studied 3 different HCV strains to explore the corresponding possible mechanisms for the first time.

As acknowledged, CK1 was an ubiquitously serine/threonine protein kinase, furthermore, human CK1 was reported to take part in the controlling of cell differentiation as well as cell proliferation (19,20). In current study, we found that all of the 3 HCV core proteins of different strains significantly upregulated the ratio of apoptotic Huh-7 cells, and they affected CK1 α in Huh-7 cells specifically, which were in consistent with previous studies.

As p53 acting as a direct transcriptional activator of the apoptosis related genes (12). In current study, we also found that knockdown of CK1 α increased different HCV core protein-induced cell apoptosis and enhanced p53 translocating to nucleus, which were in line with a former reported research

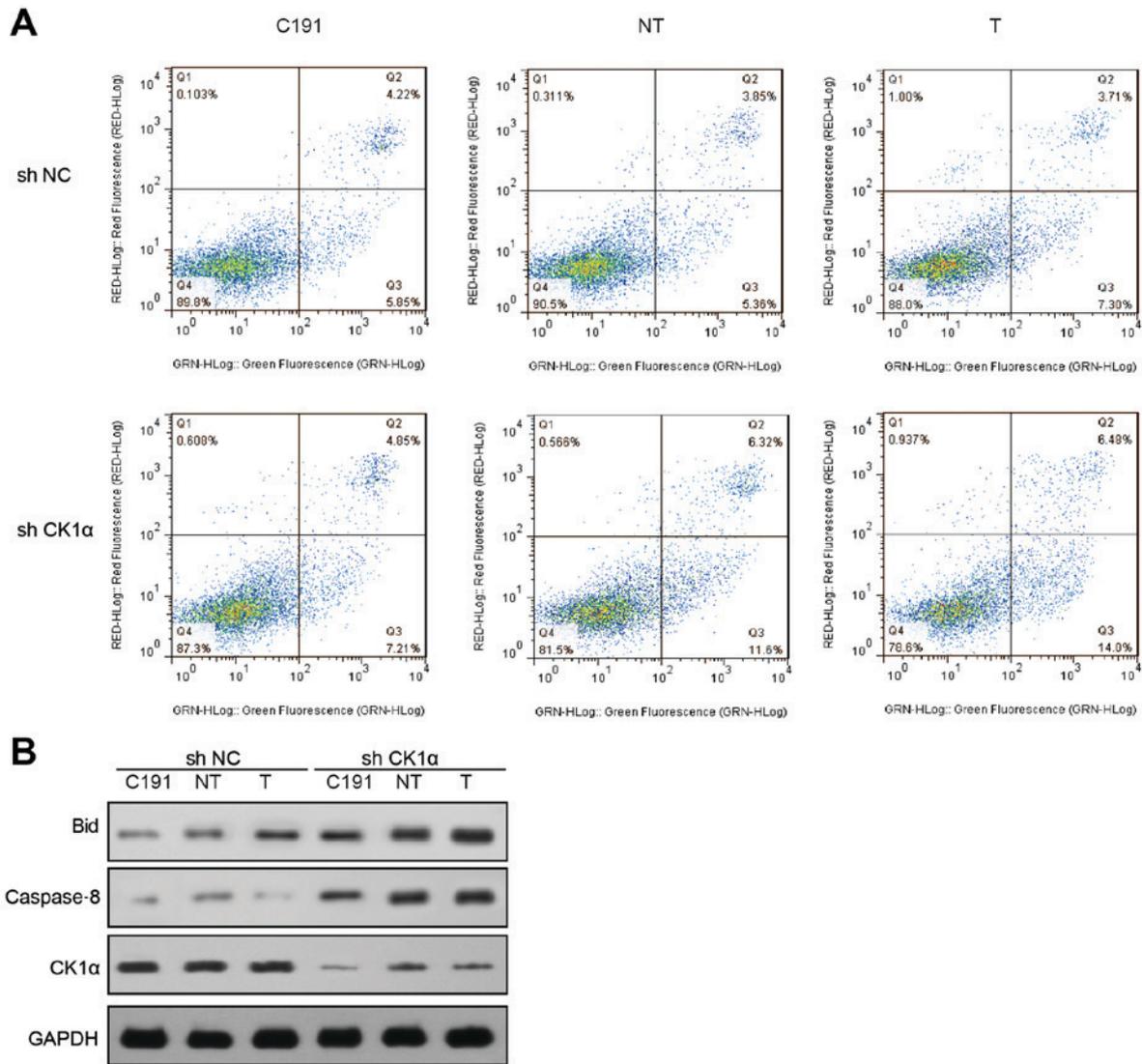


Figure 4. Knockdown of CK1 α increased HCV core protein-induced cell apoptosis. (A) Cell apoptosis was measured by flow cytometry combined with Annexin V-fluorescein isothiocyanate/propidium iodide staining in control and CK1 α knockdown cells. (B) Western blot analysis for the expression status of Bid and caspase-8 in control and CK1 α knockdown cells that transfected with different HCV core proteins. HCV, hepatitis C virus; CK1, casein kinase 1; Bid, BH3 interacting-domain death agonist; NC, negative control; T, core proteins derived from tumor tissues; NT, core proteins derived from non-tumor tissues; C191, core proteins from the HCV-J6 strain.

which demonstrated that CK1 α knockdown activated p53 in cultured cells thus inducing obvious cell death (21).

Whereas, under physiological circumstances, CK1 α exhibits a more complex function during the process of tumor development (22). A recent mouse model with tissue-specific knockout of CK1 α in the gut revealed that CK1 α depletion triggered p53 activation, induced cellular senescence and inhibited cell invasion significantly (23). CK1 α depletion, nevertheless, likewise led to stabilization of oncoprotein β -catenin, augmented cell proliferation and activation of DNA damage signals. The *in vivo* results consequently agree with the role of CK1 α in inhibiting p53 function via interacting with MDMX, but p53 activation after CK1 α depletion may affect additional mechanisms, for instance, oncogenic stress and DNA damage signaling pathways.

Because there were p53-binding sites in the promoter region of Bid, consequently, the expression of Bid was modulated by p53, and Bid participated in apoptotic processes (15,16). Be

cleaved by caspase-8, activated Bid could lead to the activation of Bax/Bak (17). We carried out experiments to test their expression changes, and results revealed that during the progression of transfecting HCV core proteins, the expression levels of both Bid and caspase-8 were obviously upregulated. By CHIP assay, we found that the amount of Bid promoter which was associated with HCV core proteins of different strains was much higher in CK1 α -silenced Huh-7 cells than in non-CK1 α -silenced Huh-7 cells. Different HCV core proteins of different strains showed different abilities to induce cell apoptosis.

As acknowledged, TRAIL induced cell apoptosis in various transformed cells and HCV infected cells (10,11). Therefore, TRAIL might selectively kill virus-infected cells. If HCV core protein enhances TRAIL-induced cell apoptosis, HCV-infected cells should preferentially be eliminated. To address the aforementioned question, we studied the impact of TRAIL on CK1 α -silenced Huh-7 cells that were transfected

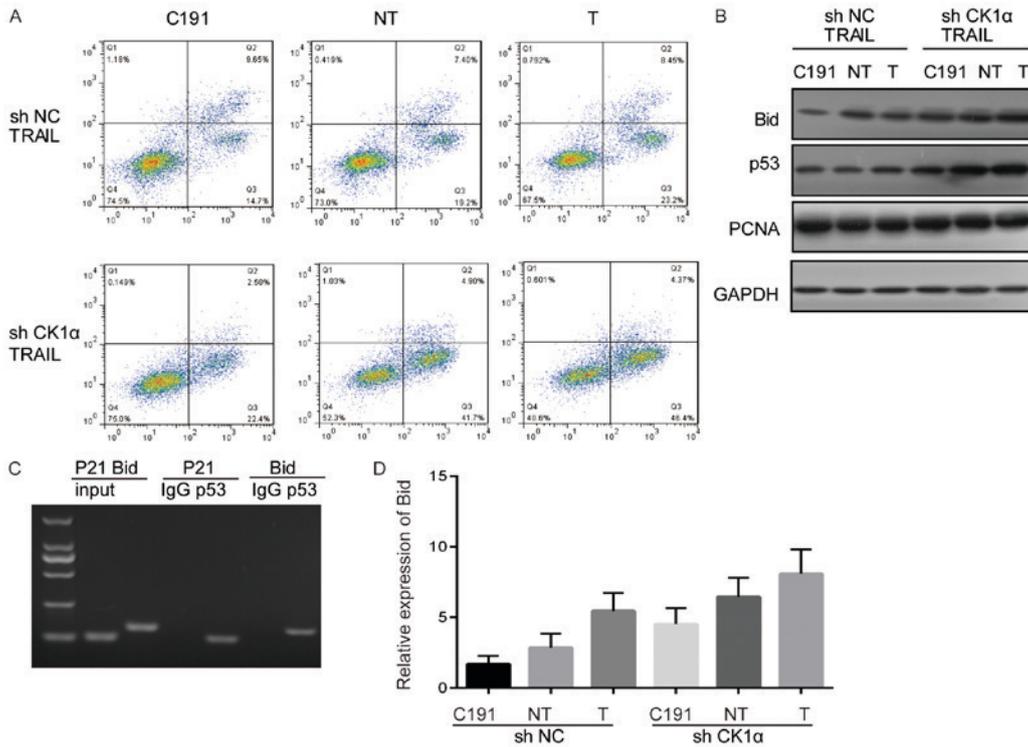


Figure 5. Knockdown of CK1α increased different HCV core proteins and TRAIL-induced cell apoptosis. (A) Cell apoptosis was tested by flow cytometry in control cells and CK1α knockdown cells that were induced by TRAIL (A). (B) Western blot analysis for the expression of Bid, p53 and PCNA in control and CK1α knockdown cells. (C) CHIP-PCR amplification demonstrated binding of p53 to the promoter regions of human Bid in Huh-7 cells. PCR was performed in input, p21-positive controls and IgG negative controls. (D) The quantitative PCR method was carried out for detecting the CHIP. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; HCV, hepatitis C virus; CK1, casein kinase 1; CHIP-PCR, chromatin immunoprecipitation-polymerase chain reaction; Bid, BH3 interacting-domain death agonist; NC, negative control; T, core proteins derived from tumor tissues; NT, core proteins derived from non-tumor tissues; C191, core proteins from the HCV-J6 strain; PCNA, proliferating cell nuclear antigen.

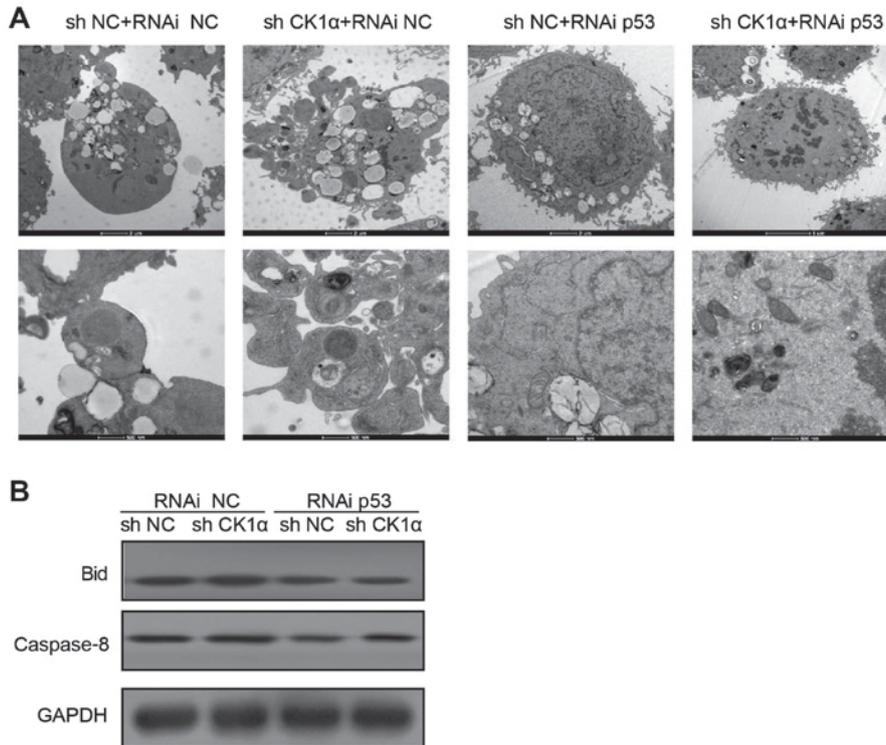


Figure 6. Knockdown of p53 increased HCV core protein and TRAIL-induced cell apoptosis. (A) Cell apoptosis in Huh-7 cells that were transfected with control siRNA or p53 siRNA was measured by electron microscopy. Scale bars, top row=2 μm; bottom row=500 μm. (B) Western blot analysis for the expression level of Bid in control and p53 knockdown cells that were transfected with different HCV core proteins. HCV, hepatitis C virus; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; CK1, casein kinase 1; Bid, BH3 interacting-domain death agonist; NC, negative control; siRNA, small interfering RNA; RNAi, RNA interference.

with 3 different HCV core proteins. The results showed that CK1 α played a crucial role in the aforementioned progression.

Therefore, it can be concluded from our findings that 3 different HCV core proteins induced Huh-7 cell apoptosis via the CK1 α -p53-Bid signaling pathway, with CK1 α acting as a key factor.

Whereas, there was only one cell line used in the present study, we will use more than one cell line as a model for the *in vitro* studies to confirm results in our future work.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SS and CL carried out the experiments and analyzed the data. MD analyzed the data, and XY designed the experiments, obtained funding and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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