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ORIGINAL ARTICLE

Cytokeratin 8 is increased in hepatitis C virus cells and its ectopic expression induces apoptosis of SMMC7721 cells

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Abstract

AIM: To investigate cytokeratin 8 (*CK8*) overexpression during hepatitis C virus (HCV) infection and its pathogenesis, and the effect of ectopic *CK8* expression on hepatoma cell lines.

METHODS: We successfully established an *in vitro* HCV cell culture system (HCVcc) to investigate the different expression profiles of *CK8* in Huh-7-HCV and Huh-7.5-HCV cells. The expression of *CK8* at the mRNA level was determined by real-time polymerase chain reaction (RT-PCR). The expression of *CK8* at the protein level was evaluated by Western blotting. We then constructed a eukaryotic expression combination vector containing the coding sequence of human full length *CK8* gene. *CK8* cDNA was amplified by reverse transcription-PCR and inserted into pEGFP-C1 and the positive clone pEGFP-*CK8* was obtained. After confirming the sequence, the recombinant plasmid was transfected into SMMC7721 cells with lipofectamine2000 and *CK8* expression was detected using inverted fluorescence microscopy, RT-PCR and Western blotting. Besides, we identified biological function of *CK8* on SMMC7721 cells, including cell proliferation, cell cycle and apoptosis detection.

RESULTS: RT-PCR showed that the expression level of *CK8* in Huh-7-HCV and Huh-7.5-HCV cells was 2.88 and 2.95 times higher than in control cells. Western blot showed that *CK8* expression in Huh-7-HCV and Huh-7.5-HCV cells was 2.53 and 3.26 times higher than that in control cells, respectively. We found that *CK8* at mRNA and protein levels were both significantly increased in HCVcc. *CK8* was up-regulated in SMMC7721 cells. *CK8* expression at the mRNA level was significantly upregulated in SMMC7721/pEGFP-*CK8* cells. *CK8* expression in SMMC7721/ pEGFP-*CK8* cells was 2.69 times higher than in SMMC7721 cells, and was 2.64 times higher than in SMMC7721/pEGFP-C1 cells. *CK8* expression at the protein level in SMMC7721/pEGFP-*CK8* cells was 2.46 times higher than in SMMC7721 cells, and was 2.29 times higher than in SMMC7721/pEGFP-C1 cells. Further analysis demonstrated that forced expression of *CK8* slowed cell growth and induced apoptosis of SMMC7721 cells.

CONCLUSION: *CK8* up-regulation might have a functional role in HCV infection and pathogenesis, and could be a promising target for the treatment of HCV infection.

Key words: Hepatitis C virus cell culture system; Cytoker-

atin 8; Up-regulation; Eukaryotic expression; Apoptosis

Core tip: In this study, we observed that cytokeratin 8 (*CK8*) levels are elevated in hepatitis C virus (HCV) cell culture system and its ectopic expression decreased the proliferation and induced apoptosis of SMMC7721 cells. *CK8* up-regulation might have a functional role in HCV infection and pathogenesis, and could be a promising target for the treatment of HCV infection.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a significant global healthcare burden[1]. Current estimation suggests that a minimum of 3% of the world's population is chronically infected, with a prevalence of up to 170 million people[2,3]. However, the mechanism of HCV infection is not fully understood. Recently, the development of HCV replicon technology has accelerated the understanding of the mechanism underlying HCV infection[4,5]. It has been reported that there were more than 100 abnormal expression proteins in HCV infected cells and hepatitis C patients[6-10]. Studies determining the changes in protein expression associated with HCV infection will help elucidate host/virus interactions, and provide further insight to HCV pathogenesis.

Cytokeratin 8 (*CK8*) is the major component of the intermediate filament cytoskeleton, belonging to the type-Ⅱ keratin, and is primarily expressed in the epithelia of liver, intestine, and exocrine pancreas[11,12]. *CK8* plays a crucial role in maintaining the structural integrity and the mechanical properties of cells[13]. Recent studies have suggested that *CK8* is involved in several liver diseases. *CK8* knock-out mice develop liver hemorrhage and are more susceptible to liver injury[14,15]. Some variants of *CK8* are associated with disease severity and progression in patients with chronic liver diseases[16,17]. Thus, we hypothesized that *CK8* contributed to cellular pathological processes and the infection and pathogenesis of HCV, leading to liver injury and chronic liver diseases.

In this study, we established an *in vitro* HCV cell culture system (HCVcc) and investigated whether HCV affects *CK8* levels. Simultaneously, we established eukaryotic expression recombination vector containing the full length coding sequence of *CK8*, then transfected into hepatoma cells *in vitro* and investigated the biological and functional role of *CK8* in hepatoma cells.

MATERIALS AND METHODS

Construction and identification of HCVcc

Huh-7 and Huh-7.5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 0.1 mmol/L nonessential amino acids and 1 × penicillin-streptomycin-glutamine. Plasmid pFL-J6/JFH, containing a chimeric full length HCV genome, was kindly provided by Professor Charles M Rice from Rockefeller University. Plasmid pFL-J6/JFH, containing a single Xba I restriction site and T7 RNA polymerase start site, is the chimera of HCV J6 strain (5'-NCR-NS2) and JFH strain (NS3-3'-NCR). Subsequently, plasmid pFL-J6/JFH encoding the full length HCV chimeric genome was transcribed to HCV RNA *in vitro*. HCVcc was established by electroporation of HCV RNA into Huh-7 and Huh-7.5 cells.

Huh7 and Huh-7.5 were used as negative controls of HCVcc. Huh-7-HCV and Huh-7.5-HCV cells were maintained under the same condition as Huh-7 and Huh-7.5 cells. Cells were cultured in an incubator at 37 ℃ supplemented with 5% CO_2. During the cell culture, the supernatant of cell culture was collected at 24, 48, 72 and 96 h after electroporation in order to determine the HCV copies. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine HCV copy number. At approximately 72 h after transfection, cells were washed three times with 1 × phosphate-buffered saline (PBS) and then harvested. In addition, indirect immunofluorescence was used to observe the expression of HCV core protein. Mouse monoclonal HCV core protein antibody (Novus Biologicals, United States) was used as the primary antibody, and goat anti-mouse conjugated with Fluorescein Isothiocyanate (FITC) was used as the secondary antibody. The harvested cells were fixed with 3% glutaraldehyde at 4 ℃ for 24 h, then washed twice by 0.1 mol/L arsenic acid dimethyl sodium buffer (pH 7.4) at 4 ℃, fixed by 1% osmium tetroxide for 1 h, gradient acetone dehydration, embedded by Epon812, sliced by ultra-thin LKB-V slicer. H-7650 transmission electron microscope (HITACHI, Japan) was also used to observe the morphology of the viral particles and intracellular ultrastructure changes.

Total RNA isolation, cDNA synthesis and RT-PCR

Total RNA was extracted from cells by TRIzol reagent (Invitrogen, United States) according to the manufacturer's protocol. A two-step reverse transcription PCR was performed. The first-strand cDNA was synthesized from 1 μg of total RNA with AMV Reverse Transcriptase[b] (TAKARA, Japan). To investigate the expression of *CK8* at the mRNA level, the expression of *CK8* and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) genes was quantified by RT-PCR, and GAPDH was used as an internal control. A total of 20 ng cDNA was used as template in the reaction. All RT-PCR assays were performed

Table 1 Primers used for real-time polymerase chain reaction and high fidelity

Name	Forward primer (5′-3′)	Reverse primer (5′-3′)
CK8 (172 bp)	AGCTGGAGTCTCGCCTGGAA	TGTGCCTTGACCTCAGCAATG
GAPDH (138 bp)	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
CK8 (1465 bp)	ATGTCGACATGTCCATCAGGGTGAC	TAGGATCCCTTGGGCAGGACGTC

CK8: Cytokeratin 8; PCR: Polymerase chain reaction; GAPDH: Glyceraldehydes-3-phosphate dehydrogenase.

in triplicate using SYBR green incorporation method with Bio-Rad IQ5 Multicolor RT-PCR Detection System (Bio-Rad, United States) based on the manufacture's protocol. Table 1 shows the sequences of the primer sets for *CK8* and GAPDH. Briefly, following a denaturation at 95 ℃ for 5 s, RT-PCR was carried out with 50 cycles at a melting temperature of 95 ℃ for 30 s, an annealing temperature of 65 ℃ for 30 s, and an extension temperature of 72 ℃ for 10 s. Data analysis was performed using the Sequence Detector System software. The relative quantification was calculated by the $2^{-\Delta\Delta Ct}$ method with GAPDH as the housekeeping gene and the control cells as the baseline, and the results were expressed as fold-change.

Protein extraction, SDS-PAGE and Western blotting

Total proteins were prepared by RIPA cell lysate. Proteins of interest were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% polyacrylamide gel, and 1 mg/mL protein was loaded onto a SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes and then detected by Western blotting under the recommended conditions. Mouse anti-human *CK8* IgG (Abcam, United States) was used as the primary antibody, goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) was used as the secondary antibody, and GAPDH was used as the control. The antigen-antibody complex was detected by an enhanced chemiluminescence (ECL) kit following the manufacturer's protocol. The experiments were repeated in triplicate. The chemiluminescent signal of each band was analyzed by gel image analysis system (Syngene, United States).

Construction of pEGFP-CK8 recombination vector

The *Bam*H I and *Sal* I restriction sites were introduced into the *CK8* coding sequence (CDS) by high fidelity PCR (Thermo, United States). Sequences for the primers are listed in Table 1, with the amplified product being 1465 bp. The *CK8* CDS was purified by gel extraction. *CK8* CDS and pEGFP-C1 vector (TAKARA, Japan) were digested respectively by the restriction enzyme *Sal* I and *Bam*H I (TAKARA, Japan). The digestion products were examined on 1% agarose gel by electrophoresis. The ligation reaction (Ligation Kit, TAKARA, Japan) was carried out between both of the DNA fragments, followed by transformation into competent *Escherichia coli* DH5α cells at 37 ℃ overnight (12-16 h). Colony selection was performed by PCR, and the amplicons were examined on 1% agarose gel by electrophoresis. Plasmid extraction (E.Z.N.A.® Endo-Free Plasmid Midi Kit, Omega, United States) was carried out for positive colonies, and then sequenced and matched by Blast method.

Transfection of pEGFP-CK8 vector into SMMC 7721 cells

SMMC7721 cells were seeded in 6-well plates in 4 mL of growth medium for 24 h prior to transfection. In each well, 0.8×10^5-4.0×10^5 adherent cells were seeded. Four microgram (4.0 μg) of DNA (pEGFP-*CK8* vector or pEGFP-C1 vector) was diluted in 250 μL of serum-free DMEM. Lipofectamine2000 (Millipore, United States) was added (10 μL) to the diluted DNA and mixed immediately by pipetting. The mixture was incubated for 25 min at room temperature. The lipofectamine2000/DNA mixture (500 μL) was added dropwise to the four wells containing the pEGFP-*CK8* plasmid, and another two wells to control cells containing the pEGFP-C1 plasmid. The plate was then gently rocked to achieve even distribution of the complexes and incubated at 37 ℃ in a 5% CO_2 incubator.

Detection assay

The expression and distribution of *CK8* was observed under an inverted fluorescence microscope (Nikon eclipse Ti, Japan) 24 h after transfection. Forty-eight hours after transfection, cellular RNA and total cellular proteins were determined by RT-PCR and Western blotting, respectively. Total RNA was extracted from SMMC7721, SMMC7721/ pEGFP-C1, and SMMC7721/pEGFP-*CK8* cells by TRIzol reagent. Total proteins were prepared by RIPA cell lysate. Real time PCR assays (SYBR® *Premix Ex Taq*™ Ⅱ, TAKARA, Japan) were performed in triplicate with Bio-Rad iQ5 Multicolor RT-PCR Detection System according to the manufacture's protocol. Rabbit anti-human IgG (Santa, United States) was used as the primary antibody, goat anti-rabbit IgG conjugated with HRP was used as the secondary antibody and β-actin (Abcam, United States) was used as control. Cells were collected after 24, 48 and 72 h transfection to perform a proliferation assay by MTT reaction (MTT cell proliferation Assay kit, Trevigen, United States). Cells were also collected 48 h after transfection to detect apoptosis (Annexin V-FITC Apoptosis Detection Kit, Abcam, United States) using Flow Cytometry (guava easyCyte HT, Millipore, United States).

Statistical analysis

All experiments were performed in triplicate. Representative graphical data are presented as mean ± SD. Statistical analyses were performed using the SPSS 10.0 software

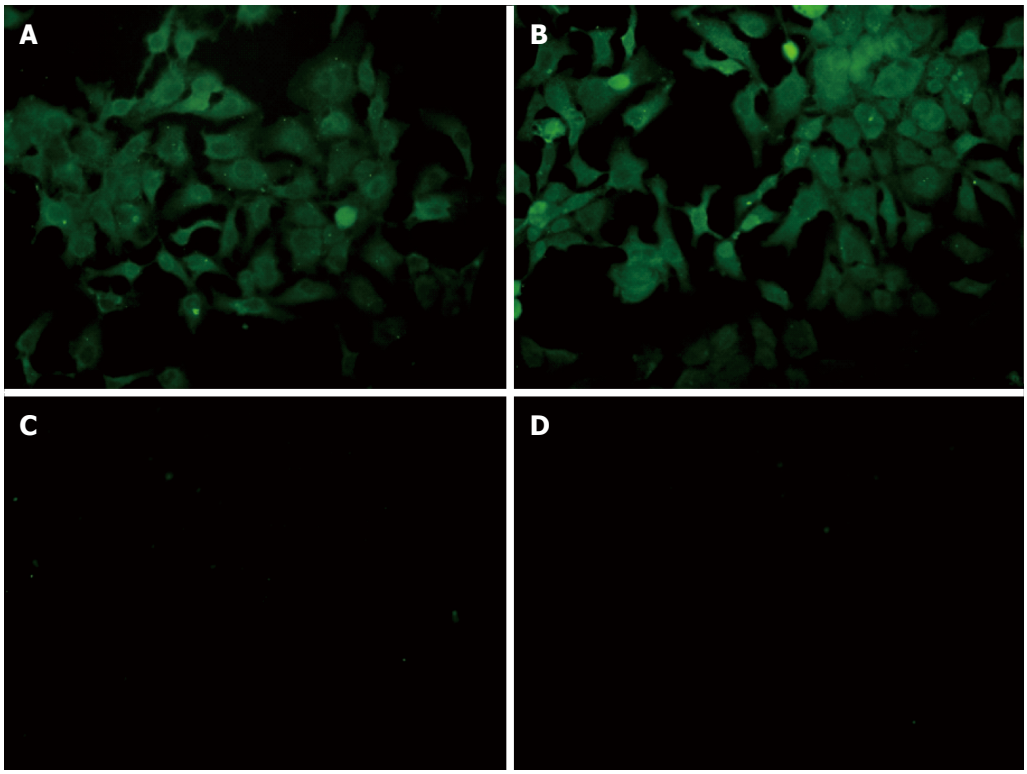

Figure 1 Indirect immunofluorescence detection of hepatitis C virus core proteins (400 ×). A: Huh-7-HCV cells appeared bright green fluorescent, they are HCV core proteins which were labeled with GFP; B: Huh-7.5-HCV cells appeared bright green fluorescent, they are HCV core proteins which were labeled with GFP; C and D: In Huh-7 and Huh-7.5 control cells, there were no green fluorescent, HCV core proteins were not expressed in them.

Table 2 Detection of hepatitis C virus at RNA level in transfected cellular supernatant

	HCV RNA in supernatant of Huh-7-HCV cells	HCV RNA in supernatant of Huh-7.5-HCV cells
24 h	5.73×10^5	9.48×10^5
48 h	1.38×10^6	6.40×10^6
72 h	3.00×10^4	9.29×10^4
96 h	6.62×10^3	1.43×10^4

HCV: Hepatitis C virus.

package (SPSS Inc.). We used Student's *t* test. *P* values below 0.05 were considered to be significant.

RESULTS

Detection of HCV RNA copies, HCV core protein, and HCV particles

We determined HCV RNA copy number by performing qRT-PCR of viral supernatants obtained from HCV-transfected cells. High-level viral copies in the supernatant of transfected cells were observed at different time-points and reached its peak value at 48 h after transfection (Table 2). Indirect immuno-fluorescence also showed high expression of HCV core protein in the HCV-transfected cells. Huh-7-HCV and Huh-7.5 HCV cells were also labeled with GFP, further indicating that HCV core protein has been expressed in these cells compared to control cells (Figure 1). Transmission electron microscopy (TEM) revealed a large number of enveloped or unenveloped virus-like particles (VLPs) in HCVcc. Some characteristic structures of *Flaviviridae* virus infection were observed, including an increased number of endoplasmic reticulum, mitochondrial swelling, cristae disappearance, and cytoplasmic vacuolar structures. Also, a large number of HCV nucleocapsid-like particles of inclusion body were presented in HCVcc cells (Figure 2). Viral-like particles were not seen in the control cells. Moreover, hyperplasia, vacuolar membrane structure, and formation of inclusion bodies were not observed in the control cells.

Increased CK8 levels in HCVcc cells by RT- PCR

Extracted total cellular RNA was examined by electrophoresis on a 0.8% non-denaturing agarose gel. A 172 bp fragment of *CK8* was successfully amplified by PCR without unspecific amplification. The melting and amplification curves of *CK8* expression indicated that the primers were properly designed. *CK8* expression in Huh-7-HCV cells was 2.88 times higher than that in Huh-7 cells, and *CK8* expression in Huh-7.5-HCV cells was 2.95 times higher than that in Huh-7.5 cells (Figure 3). Therefore, *CK8* was significantly highly expressed in HCVcc cells.

Increased CK8 levels determined by Western blotting of HCVcc cells

By Western blotting, we showed that the ratio of *CK8*/GAPDH was 0.079 ± 0.004 and 0.031 ± 0.003 in Huh-7-HCV cells and Huh-7 cells, respectively, which was 2.53

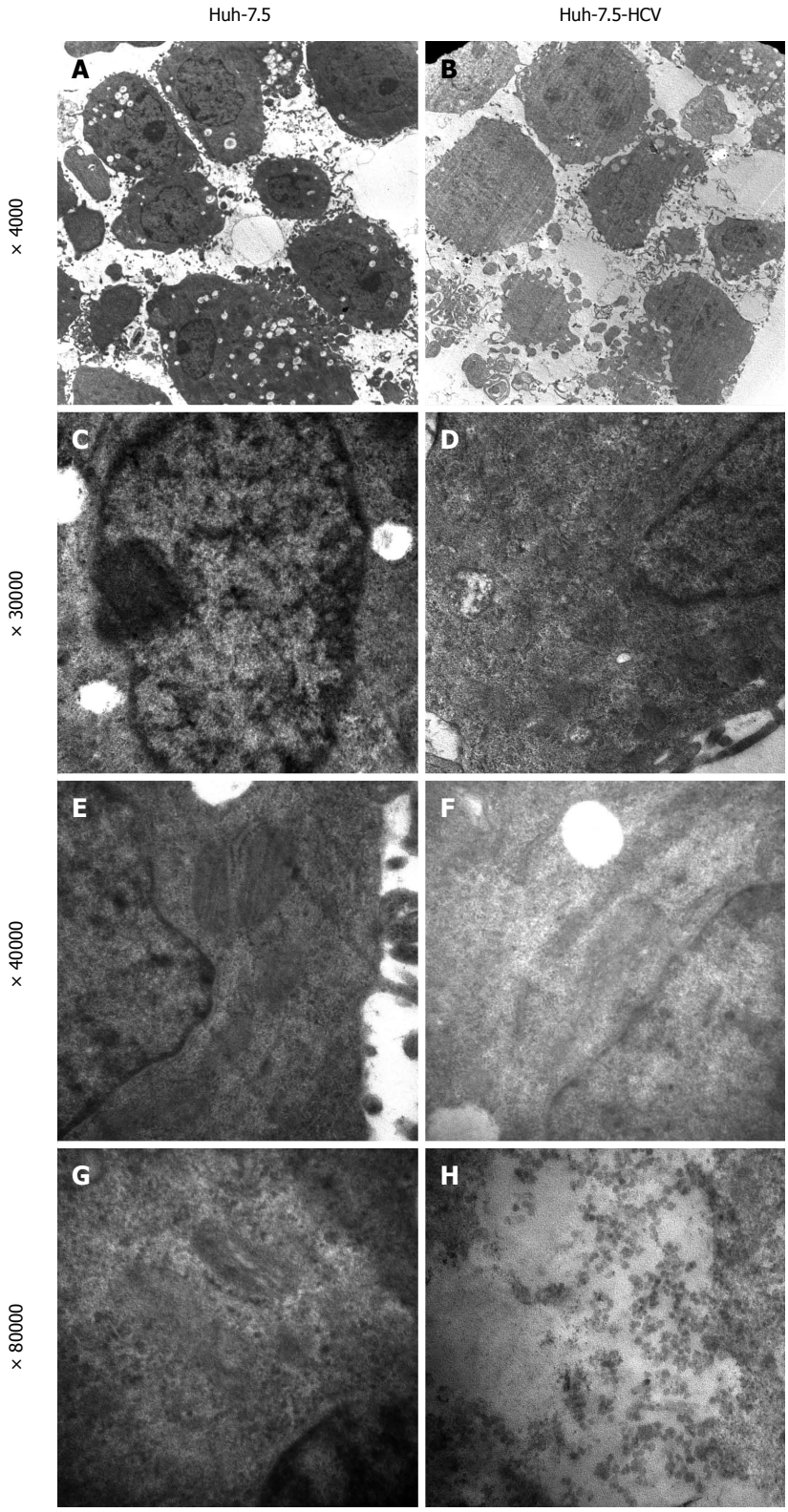

Figure 2 Transmission electron microscopy of hepatitis C virus-transfected Huh7.5 cells (× 4000, × 30000, × 40000, × 80000). A, C, E, G: Control human hepatoma cells, no virus-like particles, mitochondrial and endoplasmic reticulum are normal; B, D, F, H: HCV-transfected human hepatoma cells, human hepatoma cells have large deformed nuclei, and cultured cells prone to exist large vacuoles; D shows mitochondrial swelling and cristae disappearance; F shows the rough endoplasmic reticulum increased; H shows spherical structures of electron density, diameter is between 30-50 nm.

times higher. Furthermore, the ratio of *CK8*/GAPDH was 0.105 ± 0.004 in Huh-7.5-HCV cells, which was significantly higher than in Huh-7.5 cells (0.032 ± 0.002) and expression was 3.26 times higher (Figure 4). Therefore, we confirmed that HCVcc cells do have increased *CK8* expression.

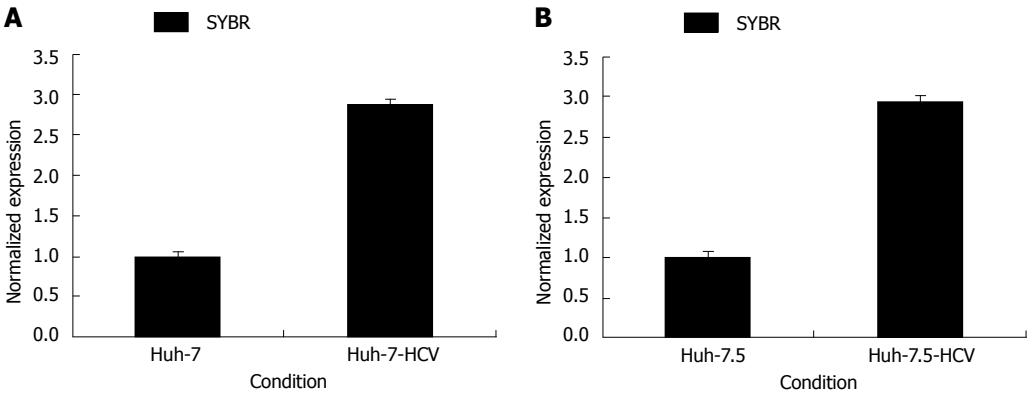

Figure 3 Relative cytokeratin 8 mRNA expression in Huh-7 and Huh-7- hepatitis C virus cells (A), or Huh-7.5 and Huh-7.5- hepatitis C virus cells (B).

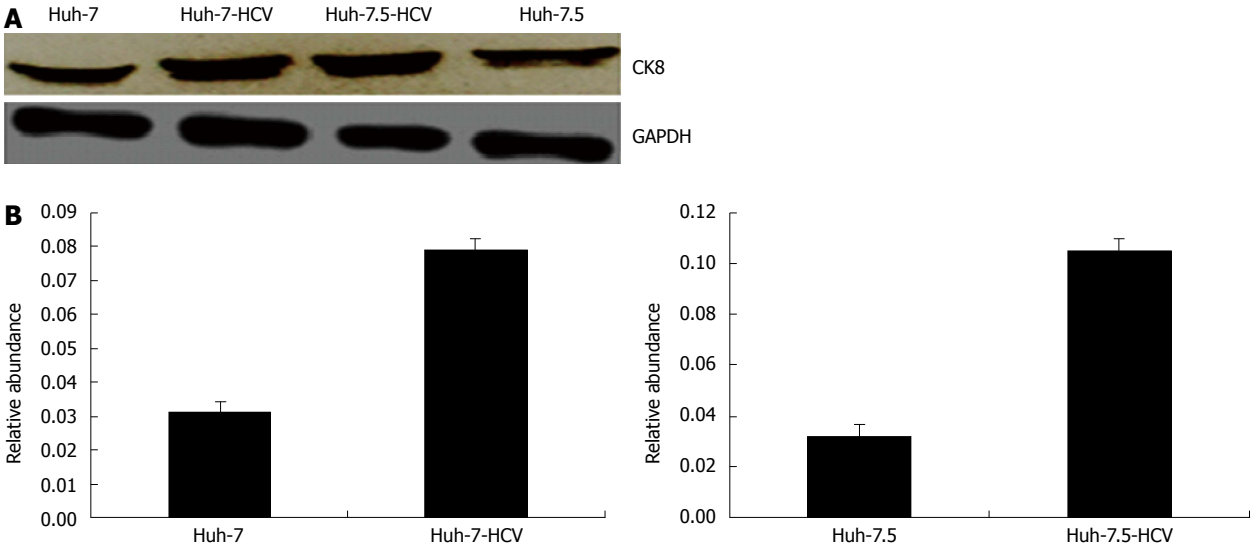

Figure 4 Cytokeratin 8 expression determined at the protein level by Western blotting. A: CK8 expression in Huh-7, Huh-7-HCV cells, Huh-7.5 and Huh-7.5-HCV cells; B: Relative CK8 expression normalized to GAPDH. HCV: Hepatitis C virus; CK8: Cytokeratin 8; GAPDH: Glyceraldehydes-3-phosphate dehydrogenase.

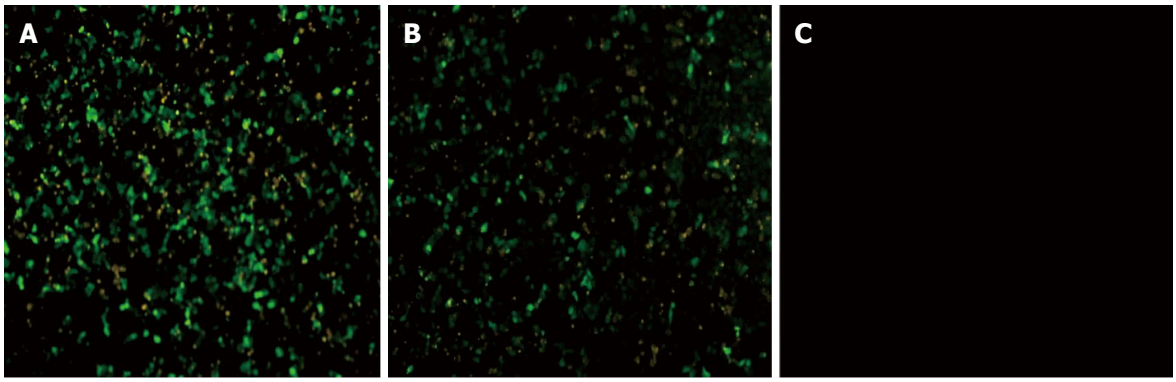

Figure 5 Inverted fluorescence microscopic observation 24 h after cytokeratin 8 transfection (× 200). A: SMMC7721 cells transfected by pEGFP-CK8 recombination vector; B: SMMC7721 cells transfected by pEGFP-C1 vector; C: SMMC7721 cells without transfection. CK8: Cytokeratin 8.

Inverted fluorescence microscopic observation

We next ectopically expressed *CK8* in SMMC7721 cells. We confirmed the overexpression of *CK8* in cells under inverted fluorescence microscope 24 h after transfection. Since the *CK8* expression vector contains an EGFP marker, we observed that SMMC7721/pEGFP-C1 and SMMC7721/pEGFP-*CK8* cells appeared bright green compared to control SMMC7721 cells (Figure 5). This data indicated that ectopic expression of *CK8* was achieved in SMMC7721 cells.

CK8 mRNA expression by qRT- PCR

The $2^{-\Delta\Delta Ct}$ value of *CK8* mRNA levels in SMMC7721, SMMC7721/pEGFP-C1, and SMMC7721/pEGFP-*CK8*

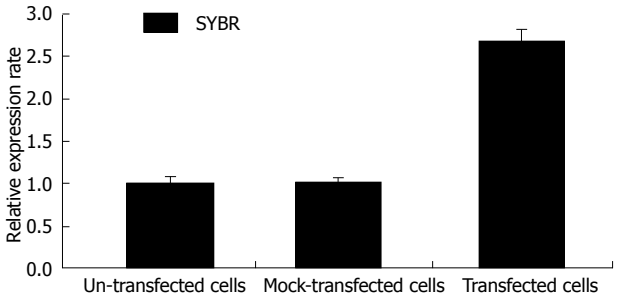

Figure 6 Cytokeratin 8 relative expression at the mRNA level.

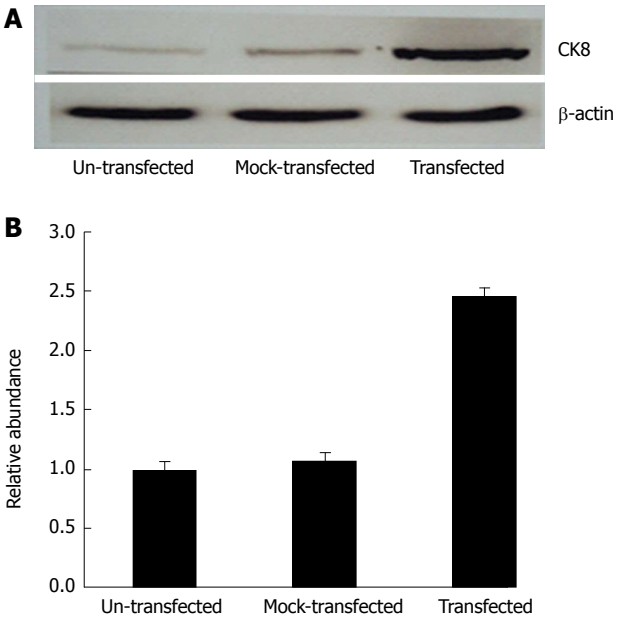

Figure 7 Cytokeratin 8 expression at the protein level 48h after transfection. CK8: Cytokeratin 8.

cells are shown in Figure 6. Beta-actin was used as the housekeeping gene, while SMMC7721 cells were used for baseline detection. The results were expressed as fold-change. *CK8* expression at the mRNA level was significantly upregulated in SMMC7721/pEGFP-*CK8* cells. *CK8* expression in SMMC7721/pEGFP-*CK8* cells was 2.69 times higher than in SMMC7721 cells, and was 2.64 times higher than in SMMC7721/pEGFP-C1 cells.

Ectopic expression of CK8 determined by Western blot analysis

Using Western blotting, we compared the chemiluminescent signals of *CK8* and β-actin in SMMC7721, SMMC7721/pEGFP-C1, and SMMC7721/pEGFP-*CK8* cells. The ratio between *CK8* and β-actin were reflective changes in *CK8* expression. *CK8* expression in SMMC7721/pEGFP-*CK8* cells was 2.46 times higher than in SMMC7721 cells, and 2.29 times higher than in SMMC7721/pEGFP-C1 cells. This demonstrated that ectopic expression of *CK8* was observed at the protein level in SMMC7721/pEGFP-*CK8* cells (Figure 7). Therefore, we confirmed that *CK8* expression was increased

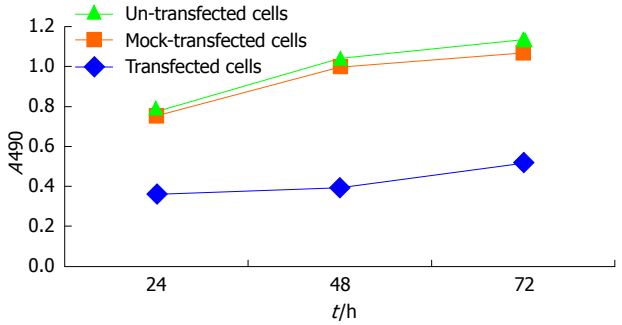

Figure 8 Growth chart after transfection in three groups of cells.

in SMMC7721 cells after transfection with pEGFP-*CK8* vector.

Effects of ectopic CK8 overexpression on cell proliferation

Using MTT detection, we determined the effects of ectopic *CK8* expression on SMMC7721 cells 72 h after transfection. *CK8* overexpression decreased the growth and proliferation of SMMC7721 cells compared to control cells and mock-transfected cells (Figure 8). This data indicated that ectopic *CK8* expression decreased cell growth and proliferation of SMMC7721 cells.

Effects of ectopic CK8 expression on the apoptosis of SMMC7721 cells

We determined the effects of ectopic *CK8* expression on the apoptosis of SMMC7721 cells 48 h after transfection. Using flow cytometry, ectopic *CK8* expression increased the apoptotic rate of SMMC7721 cells, compared to untransfected and mock-transfected cells (Figure 9).

DISCUSSION

In this study, we established a full-length HCV genomic replication in Huh-7 and Huh-7.5 cells. Lohmann *et al*[18] reported that subgenomic HCV RNA replicons are capable of autonomously replicating in Huh7 cells. These dicistronic replicons include the neomycin-resistant gene, making them selectable by G418, and most or all of the viral nonstructural genes[19,20]. This system provides a novel and powerful tool for the study of HCV replication mechanisms and for study of the interaction between host and viral factors involved in viral progression[21,22]. In our study, we transfected Huh-7 and Huh-7.5 cells to express HCV RNA and generated the HCVcc cell line. We used qRT-PCR, immunofluorescence, and TEM to detect HCV RNA, HCV core protein, and HCV particles, respectively. The results confirmed that HCV expression in Huh-7 and Huh-7.5 cells led to the production of HCV particles.

CK8 is a cytoskeletal intermediate filament protein that abundantly expresses in hepatocytes to maintain cell integrity, and prevent mechanical and non-mechanical cell injury[23,24]. Previous studies showed that *CK8* was upregulated in HBV-infected liver tissues fromp21-HBx mice[25],

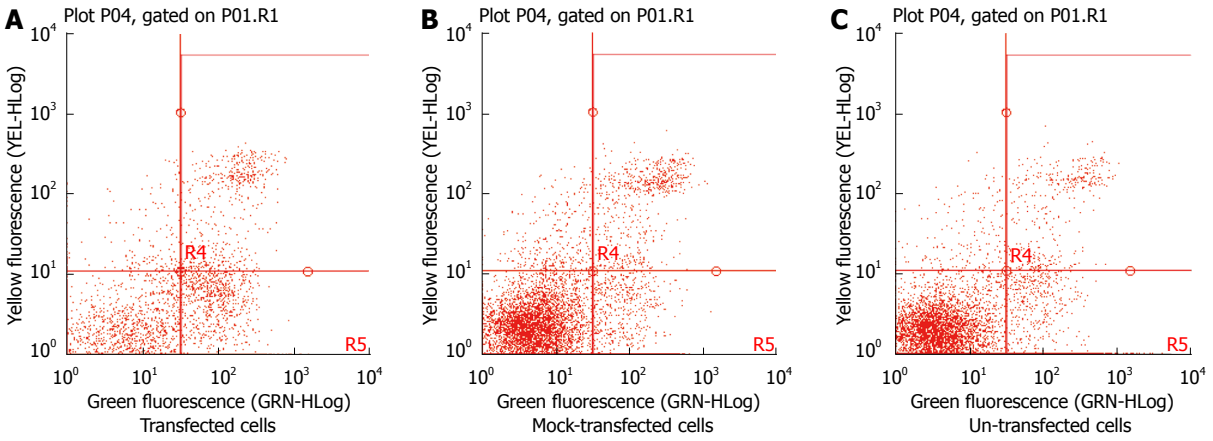

Figure 9 Cell flow cytometry. A: SMMC7721/ pEGFP-cytokeratin 8 cells; B: SMMC7721/ pEGFP-C1 cells; C: SMMC7721 cells without transfection. Cells were collected and washed twice with PBS, suspended in 200 μL binding buffer and 10 μL annexin V-FITC for 20 min in the dark, and thereafter, 300 μL binding buffer and 5 μL propidium iodide (PI) were added to each sample. The apoptotic cells were determined using a flow cytometer by staining with Annexin V-FITC. Representative dot plots show annexin V-FITC staining in cells. Results are representative of 3 independent experiments. FITC: Fluorescein isothiocyanate; PBS: Phosphate-buffered saline.

and that its upregulation contributed to the development and progression of HCC-induced HBV. Tai DI found that *CK8* was focally positive in a patient with a malignant liver patient infected with HCV[26]. Toivola *et al*[17] found that in chronic HCV infection, *CK8* phosphorylation is a progression marker during HCV progression and regression. Furthermore, Strnad *et al*[27] found that a number of *CK8* gene variants are increased in patients with chronic HCV infection. However, it is unclear about the relation between *CK8* expression and HCVcc cells. We observed a concomitant increase in *CK8* levels, which was confirmed by RT-PCR and Western blot analysis. *CK8* mRNA expression in Huh-7-HCV and Huh-7.5-HCV cells was 2.88 and 2.95 times higher than in Huh-7 and Huh-7.5 cells, respectively. At the protein level, *CK8* expression was 2.53 and 3.26 times higher in Huh-7-HCV and Huh-7.5-HCV cells, respectively, than Huh-7 and Huh-7.5 cells. This suggests that HCV up-regulates *CK8* expression in HCVcc cells, and that *CK8* expression is significantly associated with HCV.

CK8 plays a role in maintaining cellular structural integrity, signal transduction, and cellular differentiation[28-32]. Snider NT demonstrated that acetylation of *CK8* was up-regulated in diabetic human livers[33]. We showed that HCV up-regulates *CK8* expression in HCVcc cells. However, the biological function of ectopic *CK8* in tumor cells is not fully elucidated. To further investigate the biological function of aberrant *CK8* expression, we cloned the full length CDS of *CK8* to establish the eukaryotic expression recombination vector pEGFP-*CK8*. To study the biological function of increased *CK8* on cells independently, we chose another cell line called SMMC7721 cells in our laboratory. SMMC7721 cells were transfected by pEGFP-*CK8* recombination vector, and under an inverted fluorescence microscope we observed the expression and distribution of GFP-tagged *CK8*. In addition, by RT-PCR and Western blot analysis, we found that *CK8* mRNA levels in SMMC7721/pEGFP-*CK8* cells was 2.69 and 2.64 times higher than in SMMC7721 cells and SMMC7721/pEGFP-C1 cells, respectively. At the protein level, *CK8* expression in SMMC7721/pEGFP-*CK8* cells was 2.46 and 2.29 times higher than in SMMC7721 and SMMC7721/pEGFP-C1 cells, respectively. These observations showed that *CK8* gene was transcribed and expressed in SMMC7721 cells.

CK8 abnormal expression and mutations can lead to acute or sub-acute liver injury and promote tumor cells apoptosis[34,35]. The persistent expression of *CK8* can induce tumor cell apoptosis through a number of transcription factors that regulate a large number of oncogenes[36]. In SMMC7721 transfected by pEGFP-*CK8*, we further observed the biological effects of increased *CK8* on cells. We detected proliferation and apoptosis by MTT reaction and flow cytometry, respectively. We found that ectopic *CK8* expression decreased cell growth and proliferation, and increased apoptosis of SMMC7721 cells. Therefore, we concluded that the abnormal expression of *CK8* regulates cellular pathological injury. However, it is unclear what the mechanisms are by which *CK8* affects cell cycle and apoptosis. In conclusion, these results suggest *CK8* up-regulation might have a functional role during HCV infection and pathogenesis, and it could be a promising target for the treatment of HCV infection.

In summary, we successfully established and identified HCVcc and observed that *CK8* is upregulated in HCVcc. Overexpression of *CK8* in SMMC7721 cells inhibited cell proliferation and induced apoptosis. *CK8* could be a potential target for the treatment of HCV infection. Future studies will (1) identify the interactions of *CK8* with other proteins to mediate its effects; (2) assess how *CK8* expression regulates a number of known oncogenes in HCV; and (3) determine how *CK8* promotes apoptosis.

COMMENTS

Background

Currently, several proteins have been identified to be overexpressed during hepatitis C virus (HCV) infection and pathogenesis. Studies have suggested

that cytokeratin 8 (*CK8*) is closely related to a number of liver diseases. *CK8* knock-out mice develop liver hemorrhage and are more susceptible to liver injury. However, it remains unknown whether HCV affects *CK8* levels in their established *in vitro* HCV cell culture system (HCVcc) and the biological and functional role of *CK8* in hepatoma cells.

Research frontiers

It has been reported that there are more than 100 abnormal proteins expressed in HCV-infected cells and hepatitis C patients. Studies determining the changes in protein expression associated with HCV infection will help to elucidate host/virus interactions, and provide further insight to HCV pathogenesis. *CK8* plays a crucial role in maintaining the structural integrity and the mechanical properties of cells. Recent studies have suggested that *CK8* is involved in several liver diseases. Much interest is shown to understand *CK8* overexpression during HCV infection and to investigate the role of ectopic *CK8* expression in hepatoma cell lines.

Innovations and breakthroughs

In this study, the authors transfected Huh-7 and Huh-7.5 cells to express HCV RNA and generated the HCVcc cell line. Previous studies showed that *CK8* is upregulated in HBV-infected liver tissues from p21-HBx mice and in a patient with a malignant liver infected with HCV. However, it is unclear what the relation between *CK8* expression and HCVcc cells is. The authors observed a concomitant increase in *CK8* levels by real-time Polymerase chain reaction and Western blot analysis. The results show that HCV up-regulates *CK8* expression in HCVcc cells. However, the biological function of ectopic *CK8* in tumor cells is not fully elucidated. The authors found that ectopic *CK8* expression decreased cell growth and proliferation, and increased apoptosis of SMMC7721 cells. Therefore, the authors concluded that the abnormal expression of *CK8* regulates cellular pathological injury.

Applications

The results of this study suggest that *CK8* up-regulation might have a functional role during HCV infection and pathogenesis, and it could be a promising target for the treatment of HCV infection.

Peer review

This is a very well written manuscript. In this paper, the authors show the overexpression of *CK8* in an *in vitro* HCV cell culture system. Large-scale proteome analyses of the *in vitro* HCV infection model have also been performed. Thus new hopes characterize the HCV field and new advances are reasonably expected. Here, *CK8* is found up-regulated in Huh7 and Huh7.5 cells infected with chimeric full length HCV genome. The methodology is acceptable. The conclusions are interesting.

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