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# Cytokeratin 8 was over-expressed in cells harboring in vitro-transcribed full length hepatitis C virus 1b RNA, but down-expressed in HCV patients' serum

Meng Xun<sup>1</sup>, Haifeng Wang<sup>2</sup>, Burong Li<sup>3</sup>, Hongyan He<sup>4</sup>, Qian He<sup>3</sup>, Yonglie Chu<sup>1</sup>

<sup>1</sup>Department of Immunology and Microbiology, Medical School of Xi'an Jiaotong University, Xi'an, Shannxi, China

<sup>2</sup>Department of Clinical Laboratories, Xi'an Central Hospital, Xi'an, Shannxi, China

<sup>3</sup>Department of Clinical Laboratories, Second Affiliated Hospital of Medical School of Xi'an Jiaotong University, Xi'an, Shannxi, China

<sup>4</sup>Department of Medical Technology, Xi'an Medical College, Xi'an, Shannxi, China

## Email address:

xunmeng@mail.xjtu.edu.cn (Meng Xun)

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**Abstract:** Objectives: Previous proteome analysis conducted by our group has demonstrated that cytokeratin 8 was overexpressed in HuH-7 cells harboring *in vitro* transcribed HCV 1b full length RNA (HuH-7-HCV). Present study was aim to verify the results of proteomics, and obtain the clinical data of CK8 expression levels in HCV patients. Methods: The expression level of CK 8 in HuH-7-HCV cells was examined by Real time PCR and Western blotting. The concentration of CK8 in HCV patients' serum was measured by enzyme-linked immunosorbent assay (ELISA). Results: The results showed expression level of CK8 transcript in HuH-7-HCV cells was 2.3 fold higher than that in HuH-7 mock cells ( $P<0.01$ ). The protein expression of CK8 in HuH-7-HCV cells was approximately 3 fold higher than that in HuH-7 mock cells ( $P<0.01$ ). However, results of ELISA demonstrated the serum CK8 concentration was significantly reduced in chronic HCV patients compared to normal healthy controls ( $P<0.01$ ). And there was a negative linear correlation between serum CK8 concentration and HCV RNA titer ( $r=-0.380$ ,  $P<0.01$ ). Conclusion: Our present study supports the hypothesis that in response to HCV infection, expression of CK8 was increased, which may contribute to the essential cytoprotection provided by CK8 in the liver. Altered CK8 expression pattern could be an important event in the pathogenesis of HCV infection. CK8 have potential use as surrogate markers of liver injury.

**Keywords:** Hepatitis C Virus, Cytokeratin 8, HuH-7-HCV, Serum, Expression Levels

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## 1. Introduction

Hepatitis C is a major worldwide health problem with a worldwide prevalence of about 3% (around 170 million people). In total, 70%–90% of people who become infected failed to remove hepatitis C virus and remained chronically infected with the risk of developing liver cirrhosis and hepatocellular carcinoma[1]. Thus far there is no prophylactic vaccine to prevent HCV infection, and the current treatment,  $\alpha$ -interferon in combination with ribavirin, is not satisfactory because of significant side-effects and resistance[2]. And the mechanism of HCV pathogenesis is not well understood. In order to elucidate the components of HCV replication and the cellular responses to HCV replication, and identify candidates for HCV

infection-associated proteins, in our previous study, we identified changes in the proteome of the cells in the presence of HCV replication. The proteomic techniques have been applied to globally analyze the differential protein expression profiles of a human hepatocarcinoma cell line HuH-7 harboring *in vitro* transcribed HCV 1b full length RNA (HuH-7-HCV) including the X tail. The results showed cytokeratin 8(CK8) was upregulated after HCV-RNA transfection[3].

In this study, to verify the results of 2DE and MS, the expression level of CK 8 in HuH-7-HCV cells was examined by Real time PCR and Western blotting. Moreover, to obtain the clinical data of CK8 expression levels in HCV patients, the expression levels of CK8 in HCV patients' serum was detected and analyzed to understand the relationship between CK8 and HCV pathogenesis.

## 2. Materials and Methods

### 2.1. Cells

HuH-7 cells were grown in the presence of 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium with high levels of glucose (0.45%) supplemented with 10% fetal calf serum and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin).

### 2.2. RNA Preparation and Transfection

A recombinant expression plasmid, pSP72-HCV, containing the full-length HCV 1b genome, including the 98 nucleotides at the 3' end was constructed as described[4][4]. The plasmid DNA was extracted by phenol/chloroform extraction and ethanol precipitation. The HCV genomic RNA was transcribed by using MEGAscript TM T7 kit (Ambion, USA) in accordance with the manufacturer's protocol. HuH-7 cells were transfected with the RNA by electroporation and designated as HuH-7-HCV[3]. Mock-transfected (electroporation without HCV genomic RNA) cells were used as a control and designated as HuH-7 mock. The culture media of both HuH-7-HCV and HuH-7 mock cells were removed completely after 24 h. The cells were washed twice with PBS prior to refeeding with fresh culture media. The HuH-7-HCV and HuH-7 mock cells were collected at 96 h post-transfection. 3 samples from different plates were collected.

### 2.3. Real-Time RT-PCR Analysis for Cytokeratin 8 Expression Levels in HuH-7-HCV and HuH-7 Mock Cells

Total mRNA of HuH-7-HCV and HuH-7 mock cells was extracted with 1 mL TRIzol reagent (Invitrogen, USA) and precipitated with 500  $\mu$ L isopropanol. RNA pellets were washed once with 75% ethanol and dissolved in RNase-free water. To remove the residual amount of template DNA, RNA preparations were extracted once with acid phenol, precipitated with ethanol, and resuspended in RNase-free water. The integrity of total RNA was examined by electrophoresis on a 0.8% denaturing agarose gel. The cDNA was synthesized by random hexamer primed reverse transcription using the RevertAid first strand cDNA synthesis kit (Fermentas, Canada). To verify the quality of synthesized cDNA, CK8 and GAPDH gene were firstly amplified by PCR using primers (Table 1). PCR was performed in a 25  $\mu$ L reaction system containing the following components: 12.5  $\mu$ L 2 $\times$ Taq Master Mix (Tiagen, China), 1  $\mu$ L cDNA template, 1  $\mu$ L forward primer (10 mM), 1  $\mu$ L reverse primer (10 mM) and 9.5  $\mu$ L ddH<sub>2</sub>O. PCR was carried out with 35 cycles consisting of 4 min at 94 °C, 45 sec at 60 °C, and 45 sec at 72 °C. The reaction mixtures were heated at 72 °C for 10 minutes after the last cycle. The product were examined on 1.5% agarose gel by electrophoresis.

Table 1. Primers and PCR condition.

Gene name	Primers (5' to 3')	Annealing (°C)	Product (bp)
CK8	Forward: CTTCTAGGATCTCCGCCTGGTTC	64	91
	Reverse: GACACCTTGTAAGACTTCTGGGTCA		
GAPDH	Forward: GCACCGTCAAGGCTGAGAAC	64	138
	Reverse: TGGTGAAGACGCCAGTGGA		

Table 2. Median levels of CK8 concentration in different groups.

Group	Number	CK8 concentration ( $\mu$ g/L) Median (1 <sup>st</sup> quartile-3 <sup>rd</sup> quartile)	$\chi^2$	P	$\chi^2$	P
HCV RNA titer ( $\times 10^5$ - $10^7$ )	38	10.50 (7.86-15.69)	38.402 <sup>a</sup>	0.000		
HCV RNA titer ( $\times 10^3$ - $10^4$ )	32	12.02 (11.42-16.40)	6.820 <sup>b</sup>	0.009	44.636	0.000
control	36	42.86 (33.72-83.64)	21.005 <sup>c</sup>	0.000		

P values obtained by performing Kruskal-Wallis test for association between median CK8 levels of different groups. a: HCV RNA titer ( $\times 10^5$ - $10^7$ ) group vs control; b: HCV RNA titer ( $\times 10^5$ - $10^7$ ) group vs HCV RNA titer ( $\times 10^3$ - $10^4$ ) group; c: HCV RNA titer ( $\times 10^3$ - $10^4$ ) group vs control.

Real-time PCR was performed using the ABI 7500 real-time PCR system and SYBR premix ex Taq (Perfect real time; TaKaRa, Japan), primarily following the manufacturers' protocol. In brief, the reaction mixture (25  $\mu$ L total volume) contained 500 ng cDNA, primers at 0.2  $\mu$ mol/L final concentration and 12.5  $\mu$ L SYBR premix ex Taq. Thermal cycling conditions were 95 °C for 10 sec and 40 cycles at 95 °C for 5 sec and 60 °C for 34 sec.

Dissociation stage were 95 °C for 15 sec, 60 °C for 34 sec and 95 °C for 15 sec. Reactions were performed in duplicate, and 3 independent preparations of cDNA were studied. Human GAPDH gene was amplified as an endogenous control in the same reaction with target genes. The relative expression of each transcript was calculated by the  $2^{-\Delta\Delta C_t}$  method[5]. CK8 expression levels were estimated after being normalized to the endogenous control gene.

#### 2.4. Western Blotting Analysis for Cytokeratin 8 Expression Levels in HuH-7-HCV and 2.5 HuH-7 Mock Cells

Keratin 8 protein were detected by Western blotting. HuH-7-HCV and HuH-7 mock Cells were lysed in RIPA buffer with protease inhibitors. Both HuH-7-HCV and HuH-7 mock cells were separated by 12% SDS-PAGE, electrotransferred to nitrocellulose membranes (Millipore, USA), and blocked with 5% non-fat milk in TBST. The membranes were immunoblotted with rabbit anti-keratin 8 (1:1000) polyclonal antibody (Abcam, United Kingdom). Secondary goat antirabbit antibodies conjugated to horseradish peroxidase (BIOS, China) were applied at a dilution of 1:1000.  $\beta$ -actin was used as an internal control. The rabbit anti- $\beta$ -actin polyclonal antibody (BIOS, China) was diluted at 1:500. The signals were detected by an ECL detection reagent (Pierce, USA).

#### 2.5. Serum Samples

Serum samples of 70 patients with chronic HCV infection and 36 normal physical examinees (control group) were collected. HCV-RNA titers in HCV patients' serum were analyzed by a HCV fluorescence PCR diagnostic kit (DaAn Gene, China).

#### 2.6. ELISA Analysis for Cytokeratin 8 Levels in HCV Patients' Serum

Concentrations of CK8 in chronic HCV patients and normal physical examinees were determined by human CK8 ELISA kit (R&D Systems, USA) according to the manufacturer's protocol. All samples were assayed in duplicate.

#### 2.7. Statistical Analysis

Normal distribution test of all measurements was carried out by method of Kolmogorov-Smirnov. The differences in CK8 mRNA and protein expression levels between

HuH-7-HCV and HuH-7 mock cells were analyzed by Student's *t*-test or Mann-Whitney *U*-test. The differences of CK8 levels in serum among HCV patients and normal physical examinees were evaluated by Kruskal-Wallis test. When a significant difference was obtained in Kruskal-Wallis test, Mann-Whitney *U*-test was used to identify specific group differences. Spearman's rank correlation coefficients were obtained to determine the relationship between the HCV RNA titers and CK8 expression levels. The software used for statistical calculations was SPSS 17.0 (SPSS Inc., Chicago, IL, USA).  $P < 0.05$  was considered statistically significant.

### 3. Results

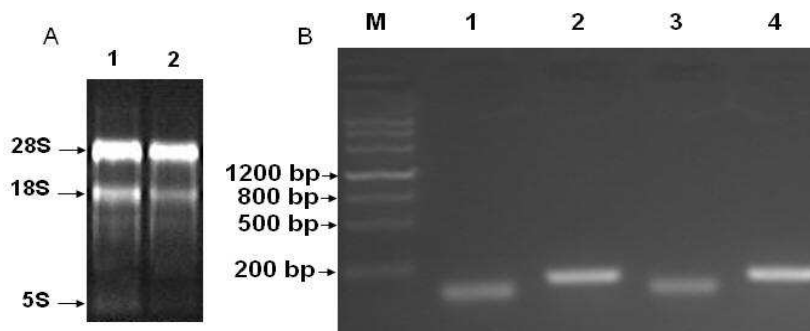
#### 3.1. CK8 Transcript was Overexpressed in HuH-7-HCV Cells

The extracted total RNA was examined by electrophoresis on a 0.8% denaturing agarose gel (Figure 1A). A 91 bp fragment of CK8 and a 138 bp fragment of GAPDH was successfully amplified by PCR (Figure 1B).

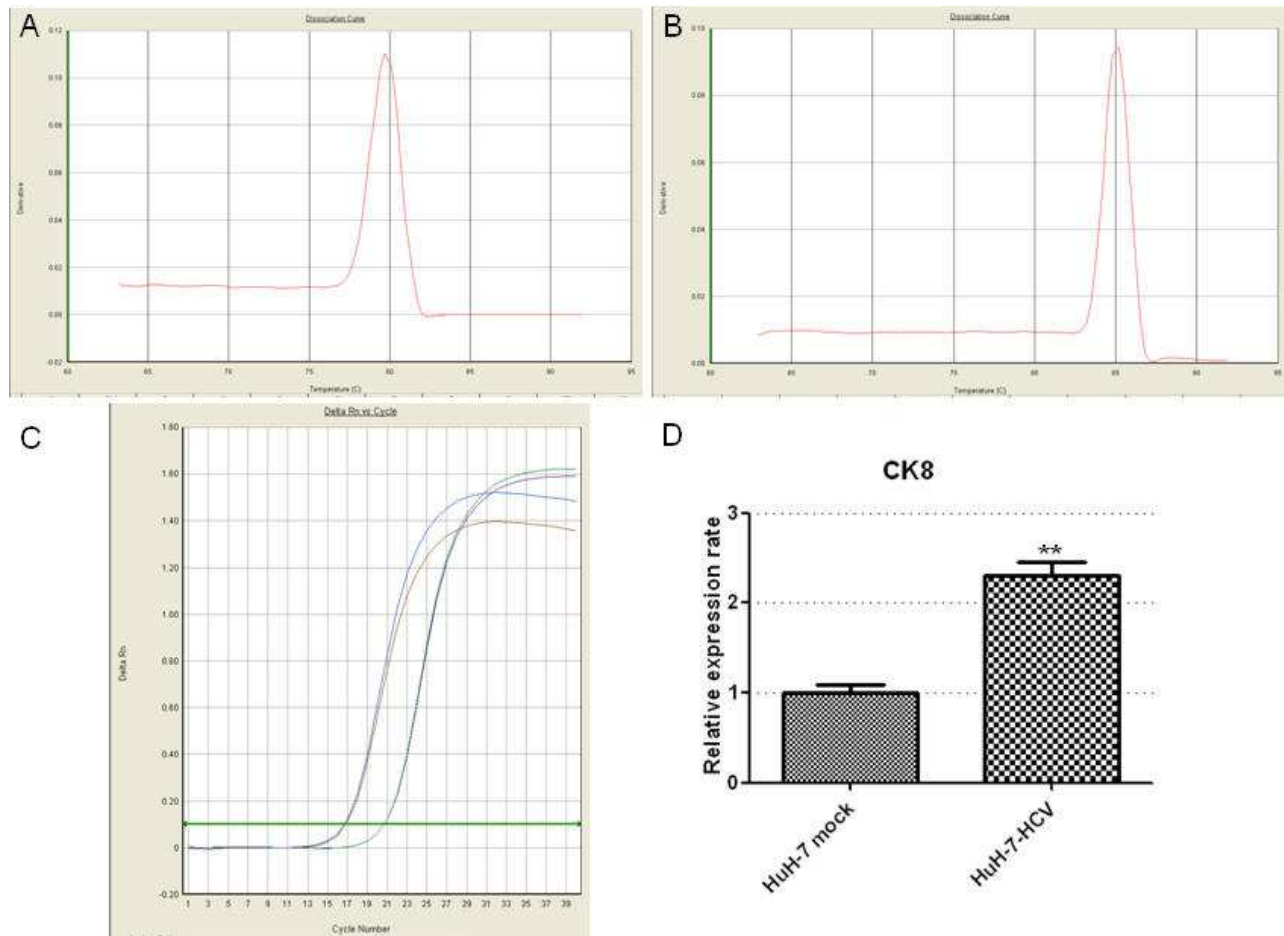
The mRNA expression of CK8 were determined by real-time PCR. Successful amplification of both targets (CK8 and GAPDH) was monitored by melt curve analysis showing two distinct peaks (Figure 2A-2B). Relative quantification of CK8 transcript was calculated by the  $2^{-\Delta\Delta Ct}$  method where the HuH-7 mock control levels were normalized to 1. The expression level of CK8 transcript in HuH-7-HCV cells was 2.3 fold higher than that in HuH-7 mock cells ( $P < 0.01$ ) (Figure 2D). CK8 transcript was overexpressed in HuH-7-HCV cells.

#### 3.2. CK8 protein was overexpressed in HuH-7-HCV cells

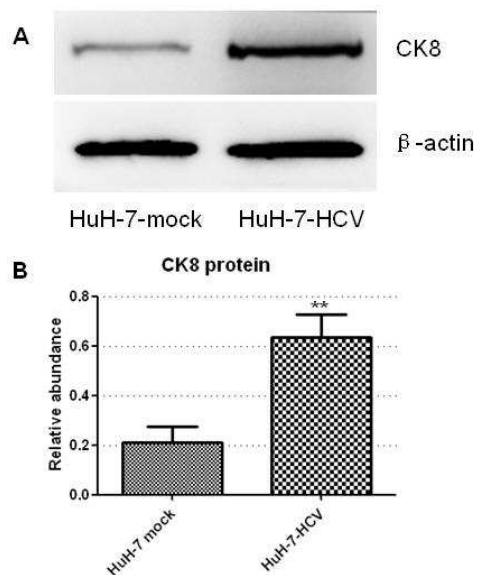
To examine the CK8 expression in HuH-7-HCV cells, Western blot analysis was performed (Figure 3). The protein expression of CK8 in HuH-7-HCV cells was approximately 3 fold higher than that in HuH-7 mock cells ( $P < 0.01$ ). CK8 protein was overexpressed in HuH-7-HCV cells.



**Figure 1.** Quality verification of extracted total RNA and synthesized cDNA. A: extracted total RNA was separated a 0.8% denaturing agarose gel. 1: HuH-7-HCV, 2: HuH-7 mock; B: CK8 and GAPDH were amplified by PCR. M: DNA Marker, 1: CK8 amplified in HuH-7-HCV cells, 2: GAPDH amplified in HuH-7-HCV cells, 3: CK8 amplified in HuH-7 mock cells, 4: GAPDH amplified in HuH-7 mock cells.



**Figure 2.** Verification of CK8 gene expression in HuH-7 mock and HuH-7-HCV cells by real-time RT-PCR. A: Melt curve of CK8 gene; B: Melt curve of GAPDH gene; C: Amplification curve of CK8 in HuH-7 mock and HuH-7-HCV cells; D: CK8 mRNA relative expression level in HuH-7 mock and HuH-7-HCV cells. Results are expressed as fold increase and GAPDH was served as an internal control. \*\* $P < 0.01$  versus the HuH-7 mock cells. CK8 mRNA expression was increased in HuH-7-HCV cells.



**Figure 3.** Western blot analysis of the CK8 protein expression in HuH-7-HCV and HuH-7 mock cells. A: The protein expression of CK8 was determined by western blot.  $\beta$ -actin was used as an internal control. B: Quantification analysis of the protein levels as shown in part B using the quantity one software. \*\* $P < 0.01$  versus the HuH-7 mock cells. Results

show that the CK8 expression was increased in HuH-7-HCV cells.

### 3.3. CK8 was Down-Expressed in Serum of Chronic HCV Patients

Total chronic HCV infection patients was categorized into two groups based on serum HCV RNA titer. The two HCV RNA titer groups were comprised HCV RNA  $\times 10^3$ - $10^4$  and  $\times 10^5$ - $10^7$ . The levels of serum CK8 were comparable between HCV RNA titer ( $\times 10^5$ - $10^7$ ) group, HCV RNA titer ( $\times 10^3$ - $10^4$ ) group and normal healthy controls. The median concentration of serum CK8 in HCV RNA titer ( $\times 10^5$ - $10^7$ ) group was 10.50 ng/L, in HCV RNA titer ( $\times 10^3$ - $10^4$ ) group was 12.02 ng/L and in control was 42.86 ng/L (Talbe 2). The serum CK8 concentration was found to be significantly reduced in chronic HCV patients compared to normal healthy controls ( $P < 0.01$ ). And the CK8 concentration between HCV RNA titer ( $\times 10^5$ - $10^7$ ) group and HCV RNA titer ( $\times 10^3$ - $10^4$ ) group were seen to be significantly different ( $P < 0.01$ ). Moreover, the result of Spearman's rank correlation test showed significant association between serum CK8 concentration and HCV RNA titer. There was a negative linear correlation between serum CK8 concentration and HCV RNA titer ( $r = -0.380$ ,  $P < 0.01$ ).



## 4. Discussion

In order to establish protein expression profiles and to characterize HCV infection-related proteins, HuH-7 cells harboring *in vitro*-transcribed full-length hepatitis C virus 1b RNA (HuH-7-HCV) were analyzed by 2DE and MS. The results showed cytokeratin 8 were upregulated after HCV-RNA transfection. In the present study, the differential expression of CK8 in HuH-7-HCV cells were validated by real-time PCR and Western blotting. The changes in the expression level of CK8 were consistent with the 2DE results.

Cytokeratins (CKs) are the largest subgroup among the most diverse family of intermediate filament proteins which have a conserved central coiled-coil  $\alpha$ -helical rod domain that is flanked by N-terminal 'head' and C-terminal 'tail' domains[6]. CKs are grouped into 2 sub-classes on the basis of their molecular weight and isoelectric points: relatively basic type-II (CK1-8, CK71-80) and relatively acidic type-I (CK9-28), which are both needed to form obligate heteropolymers[7, 8].

CK8 together with CK18 are the first cytokeratins expressed during embryogenesis and are expressed in simple epithelial cells found in the liver, the gastrointestinal tract, exocrine pancreas and mammary gland, from which many carcinomas arise[9]. Adult hepatocytes are unique in that they produce only CK8/18 and the molar ratio of CK8 and CK18 is 1:1[10, 11]. CK8/18 are highly abundant proteins in post-natal liver. Under physiological conditions, the total amount of cellular CK8 is kept at a stable level. The function of CK8/18 in the liver is protection from mechanical and non-mechanical forms of stress that can lead to cell death. Stress affect not only cytokeratins expression levels, but also expression profiles and posttranslational modification[12].

In response to liver injury, expression of CK8/18 increases up to 3-fold, which may contribute to the essential cytoprotection provided by CK8 and CK18 in the liver[13]. In addition, liver CK8/18 protein increases 2-4 fold in patients with primary biliary cirrhosis[14]. The study of Anna Kakehashi imply that CK8/18 overexpression, those two cytokeratins complex formation associated with histone type 2 H2aa3 up-regulation and intermediate filament reorganization may drive neoplastic transformation of GST-P positive foci during rat hepatocarcinogenesis leading to the formation of hepatocellular carcinomas[15]. And CK8/18 is a novel reliable marker of preneoplastic lesions arising during mouse hepatocarcinogenesis which might be used for prediction of tumor development[16]. K8-null mice, which manifest 94% embryoletality with extensive liver hemorrhage and susceptibility to liver injury, spontaneously develop anti-mitochondrial antibodies (AMA) and have altered hepatocyte mitochondrial size and function[17]. In humans, CK8/18 variants predispose to development of

end-stage liver disease and acute liver failure (ALF)[18]. CK8/18 variants also associate with development of liver fibrosis in patients with chronic hepatitis C[19].

CK8/18-deficient animals exhibit a marked susceptibility to tumor necrosis factor (TNF)-induced cell death and Fas-induced apoptosis[20]. It has been documented that CK8 and CK18 are in resistance to tumor necrosis factor (TNF) family receptors- and Fas-induced apoptosis[21]. CK8 was shown to modulate TNF-induced apoptosis by its ability to bind to the cytoplasmic domain of tumor necrosis factor receptor 2 (TNFR2)[22]. It was reported that CK8 was involved in the trafficking of Fas from the Golgi to the apical surface of polarized epithelia[23]. During apoptotic cell death, the protective resistance provided by CK8/CK18 heterodimers is mechanistically linked to a microtubule dependent modulation of Fas targeting to the surface membrane[21]. Gilbert et al. have reported that CK8/CK18 modulate Fas density at surface membranes, and the loss of CK8/CK18 leads to an increased density of Fas on the membrane[23]. There is increasing evidence suggesting that liver cell damage in chronic HCV infection is mediated by the induction of apoptosis[24, 25]. Engulfment of apoptotic bodies by hepatic stellate cells stimulates the fibrogenic activity of these cells and may be one mechanism by which hepatocyte apoptosis promotes fibrosis[26]. Thus our findings indicated that the upregulation of CK8 agreed with this proposed mechanism. CK8 was presumed to play a cytoprotective role against HCV infection and modulate the cellular response to proapoptotic signals to resist hepatocyte apoptosis and fibrosis. CK8 may be a marker for predicting liver disease severity, treatment response and fibrosis progression in patients with chronic HCV infection.

Cytokeratins has been usually used in serology diagnostics of some kind epithelial cell-associated carcinoma, including those involving breast, colorectum, lung, and bladder. The most commonly used markers are tissue polypeptide antigen (TPA; a mixture of CK8, CK18, and CK19), which are released from carcinoma cells into blood. The main clinical uses of TPA are to monitor treatment responses and tumor recurrence and to provide potential prognostic information for some cancers[12]. Furthermore, CK8 was also elevated in nonmalignant diseases. However, serum CK8 levels in HCV patients are not reported before. In our present study, ELISA was used to detected the CK8 levels in serum of chronic HCV patients. The results demonstrated serum CK8 was down-expressed in HCV patients, which is not in conformity with CK8 levels in cancers. And there was a negative linear correlation between serum CK8 concentration and HCV RNA titers. The reason of CK8 down-expression observed in serum of chronic HCV patients is unclear. Additional studies using different methods are needed to test larger numbers of patients and appropriate clinical controls, to analyze samples of different course of disease.

## 5. Conclusion

In summary, the progression of HCV infection involves various cellular and molecular mechanisms. The obtained data support our hypothesis that altered CK8 expression pattern could be an important event in the pathogenesis of HCV infection. CK8 have potential use as surrogate markers of liver injury. Additional studies will help us to better define the therapeutic and prevention options of CK8 in hepatitis C.

## Disclosure of Interest

The authors declare that they have no conflicts of interest concerning this article.

Meng Xun and Haifeng Wang contributed equally to this work and are joint first authors. This study was supported by the Fundamental Research Funds for the Central Universities (xjj2012054).

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