

Effects of hepatitis C virus gene NS2 on the expressions of Bcl-2 and Bax in HepG2 cells

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Abstract

Objective: To study the effects of hepatitis C virus gene nonstructural protein 2 on the expressions of Bcl-2 and Bax in liver hepatocellular cells.

Method: The study was conducted at the Department of Infectious Diseases, the First Affiliated Hospital of Medical School, Xi'an Jiaotong University, Xi'an, China, from March 2012 to April 2013. Negative controls pEGFP-C3-NS2, pEGFP-C3-C and pEGFP-C3 were transiently transfected into liver hepatocellular cells and expressions of Bcl-2 and Bax were detected by Western blot 24h post-transfection. SPSS 13 was used for statistical analysis.

Results: After transfected with NS2 gene, expression of Bcl-2 in liver hepatocellular cells was slightly higher than the non-transfected cells, and the expression of Bax was significantly higher than the non-transfected cells.

Conclusion: Hepatitis C virus non-structural protein 2 gene plays a role in adjusting the proto-oncogene Bcl-2 and tumour suppressor gene Bax.

Keywords: Bax, Bcl-2, Hepatitis C virus, Liver cancer cell, NS2. (JPMA 64: 1127; 2014)

Introduction

Hepatitis C virus (HCV), a pathogen of hepatitis C, can be spread through blood transfusion, syringes, sweat and saliva etc. Hepatitis C caused by infection is distributed globally.¹ HCV belongs to genus flavivirus whose viral genome is a single-stranded positive-sense ribonucleic acid (RNA) containing a 5' un-translated region (UTR), an open reading frame (ORF), a 3' UTR and comprising 9600 monomeric nucleotides. It can encode 3010-3030 amino acids to constitute protein precursor.^{2,3} This protein may be cleaved by host and viral proteases to produce at least 10 mature viral protein products, including Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B.^{4,5} Among this, HCV core protein (C protein) can trans-regulate intracellular signalling pathway molecules or transcription factors to regulate cell growth, proliferation and apoptosis, so it is considered to be related to the occurrence of cirrhosis and cancer induced by HCV infection. C protein can not only increase the expression of proto-oncogene Bcl-2, but also elevate the expression of tumour suppressor gene Bax.^{6,7} In recent years, researchers have isolated HCV non-structural protein 2 (NS2) from the serum of patients infected with HCV, and found that in patients with chronic hepatitis and HCV-induced liver cancer, the quantity of NS2 and positive rate of HCV gene sequence were significantly elevated,

indicating that NS2 may be associated with the occurrence and development of liver cancer.^{8,9}

Liver hepatocellular cell (HepG2) represents a pure cell line of human liver carcinoma, often used as hepatocellular carcinoma model due to the absence of viral infection and low level of NS2.¹⁰ This study aims at detecting the effects of HCV NS2 on oncogene expression, including intracellular Bcl-2 and Bax, by transfecting HepG2 cell with NS2 and C gene eukaryotic expression vectors, so as to explore the role of NS2 in the occurrence of cirrhosis and cancer induced by HCV infection.

Materials and Methods

The study was conducted at the Department of Infectious Diseases, the First Affiliated Hospital of Medical School, Xi'an Jiaotong University, Xi'an, China, from March 2012 to April 2013. Material used was: 1-C and pcDNA3.1-NS2 (SantaCruz, USA), Polymerase chain reaction (PCR) reagents (ComWin Biotech Co., Ltd., Beijing); endonuclease and Marker (Amresco USA); T4DNA polymerase (Ruixianghe Biotech Co., Ltd., Beijing); Lipofectamine LTX and plasmid extraction kit (Sigma, USA); nucleic acid extraction kit (Kangpu Huiwei Technology Co., Ltd., Beijing); cell lysis kit (Jianglai Biotech Co., Ltd., Shanghai); Nitrocellulose (NC) membrane, beta-actin, Bcl-2 monoclonal antibody, Bax monoclonal antibody and enzyme-labelled secondary antibody (Fermentas China).

For the construction of recombinant expression vectors

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pEGFP-C3- NS2 and pEGFP-C3-C, the gene sequence of NS2 was amplified using the following two primers by PCR with plasmid pcDNA3.1-NS2 gene as the template: upstream primer P1: 5'-AGCTTGTGCCAGGTTGGAGATAC-3' containing HindIII restriction sites; and downstream primer P2: 5'-CATAGGGTTCACCGAAGTTCACCTTCT-3' containing EcoRI restriction sites. The NS2 gene was amplified and target fragment was extracted using gel purification kit. HindIII and EcoRI double digestion was conducted on pEGFP-C3 plasmid (with green fluorescent protein [GFP] tag). NS2 gene fragment and pcDNA3.1-C plasmid were used for the extraction of restriction fragments. Next, in 30 μ l of reaction system the concentrations of extracted target genes NS2 and C with vectors 1:3 were made to react under the action of T4 deoxyribonucleic acid (DNA) ligase at 20 $^{\circ}$ C overnight. The ligation products were transformed into competent *E. coli* TG1. Positive clones were screened out from Luria agar plates containing kanamycin to extract plasmids by shaking bacteria. HindIII and EcoRI double digestion was used to identify recombinant plasmids, digestion products were subjected to 10g/L agarose gel electrophoresis, and bacterial liquid with preliminarily correct analysis was sent for sequencing. The plasmid bacterial liquids with correct sequencing result were saved in which the plasmids were named pEGFP-C3-NS2 and pEGFP-C3-C.¹¹

For the transfection HepG2 cells with pEGFP-C3- NS2, pEGFP-C3-C and pEGFP-C3, HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 50ml/L foetal bovine serum, incubated in 24-well plate one day before transfection, 3 \times 10⁵ cells in each well, and cultured in incubator at 37 $^{\circ}$ C and 5% carbon dioxide (CO₂). Transfection was conducted according to the instructions of Lipofectamine 2000 Reagent. The medium was replaced with normal medium after 12h, and the expression of GFP was observed by fluorescence microscope 24h after transfection. The expressions of NS2 and C were detected by Western blot.

For the detection of Bcl-2 and Bax expressions in cells, the proteins were extracted using Kgp cytoplasmic protein extraction kit, then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to NC membrane, and incubated with the indicating primary and secondary antibodies. X-ray films were developed for strip analysis.

For cell cycle detection, both the non-transfected cells in healthy growth and liver cancer cells transfected with pEGFP-C3-NS2, pEGFP-C3-C and pEGFP-C3 were digested with trypsin to adjust the cell density to 3 \times 10⁶/L, washed

with phosphate buffer 3 times, fixed in 60% ethanol overnight at 4 $^{\circ}$ C and digested with 0.5 ng/ml ribonuclease (RNase) at 37 $^{\circ}$ C for 30min. Then 10 μ g/ml of propidium iodide (PI) was added, and cells were incubated in dark at 4 $^{\circ}$ C for 60min. The cells were analysed by flow cytometry in BD FACSCanto II flow cytometer (BD Bioscience).

All numerical data from three or more independent experiments were expressed as mean \pm standard error of mean (SEM) and processed using SPSS 13.0. Two-tailed paired Student's t-test was used for analysing two groups. One-way analysis of variance (ANOVA) was used for analysing three or more groups. Statistical significance was set at $p < 0.05$.

Results

Using pcDNA3.1-NS2 plasmid as the template, the 618bp fragment amplified by PCR contained the expected NS2 fragment at 562bp (Figure-1A). HindIII and EcoRI double

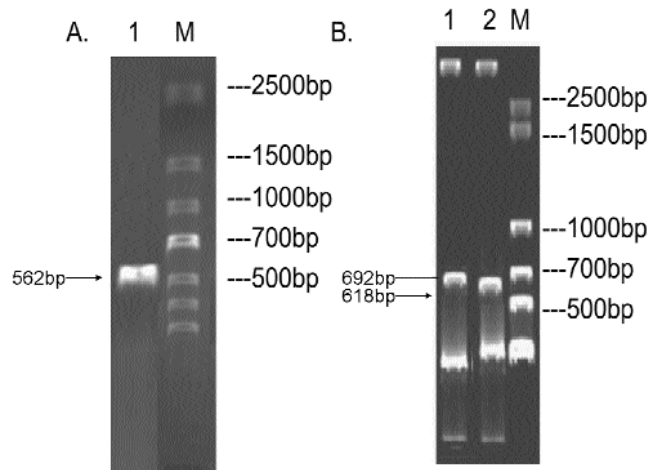


Figure-1: Construction of expression vectors. (A) Agarose gel electrophoresis of the PCR products of HCV1b-type NS2 gene. M: DNA marker (DL2000); 1: PCR amplification products of NS2 gene (562 bp). (B) Agarose gel electrophoresis of pEGFP-C3-NS2 (618 bp) and pEGFP-C3-C (692 bp) after double digestion. M: DNA marker (DL2000); 1: pEGFP-C3-C plasmid AFTER HindIII and EcoRI double digestion; 2: pEGFP-C3-NS2 plasmid after HindIII and EcoRI double digestion.

Table: Effects of NS2 and C protein on cell cycle and apoptosis (%).

Group	Early stage of DNA synthesis	DNA synthesis	Late stage of DNA synthesis	Apoptotic rate
Untransfected	72.56 \pm 3.57	27.18 \pm 2.49	22.51 \pm 2.15	5.92 \pm 0.68
pEGFP-C3	71.29 \pm 3.49	25.24 \pm 2.13	23.49 \pm 2.18	5.37 \pm 0.59
pEGFP-C3-C	67.45 \pm 3.19	26.34 \pm 2.19	30.26 \pm 2.46*	0.94 \pm 0.11*
pEGFP-C3-NS2	64.58 \pm 3.08	27.82 \pm 2.29	31.81 \pm 2.51*	0.98 \pm 0.12*

Comparison between untransfected cells and those transfected with empty vector, * $P < 0.05$. DNA: Deoxyribonucleic acid.

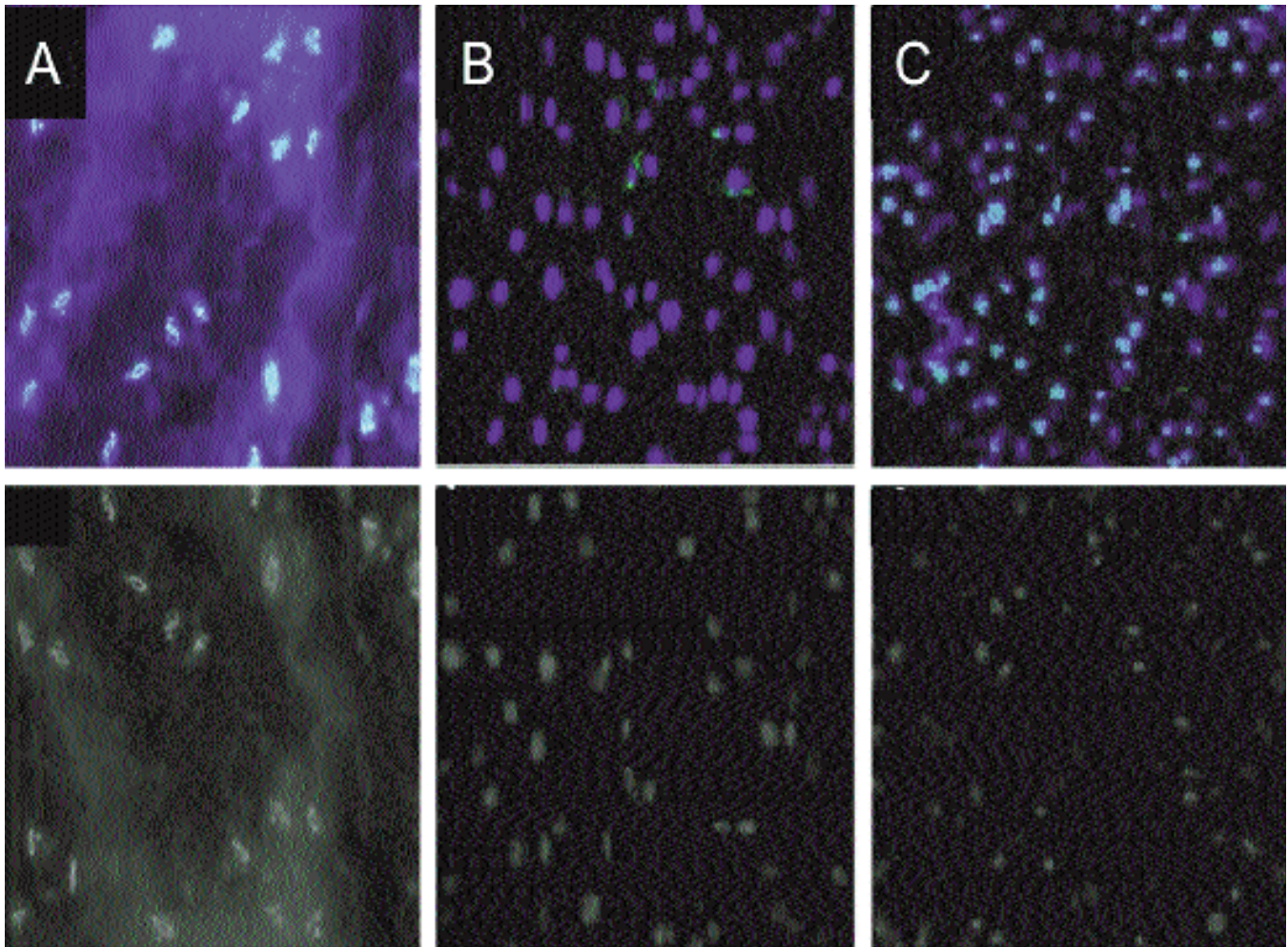


Figure-2: Expressions of NS2 and C protein under fluorescence microscope (x100). (A) Cells transfected pEGFP-C3 plasmid; (B) Cells transfected pEGFP-C3-C plasmid; (C) Cells transfected pEGFP-C3-NS2 plasmid. Upper lane: blue excitation light; lower lane: natural light.

digestion was conducted on recombinant plasmid pEGFP-C3-NS2 and pEGFP-C3-C, and restriction fragments were observed at 618bp and 692bp by agarose gel electrophoresis, which were consistent with those of the expected target fragments (Figure-1B). DNA sequencing results also showed that NS2 and C gene fragments were successfully inserted into the pEGFP-C3 plasmid.

Besides, 24h after HepG2 cells were transfected, GFP was expressed in HepG2 cells (Figure-2). The transfection efficiencies of pEGFP-C3-NS2 and the pEGFP-C3-C recombinant plasmids were about 65% and 60% respectively. Western blot analysis verified that both HCV NS2 and C proteins were expressed in HepG2 cells, with the protein molecular weights in accordance with the expected 19 kD and 26 kD (Figure-3A).

HepG2 cells transfected with pEGFP-C3-NS2, pEGFP-C3-C and pEGFP-C3 plasmids and non-transfected HepG2 cells were detected by Western blot. The expression of Bcl-2 in HepG2 cells transfected with NS2 gene was slightly higher than that of the non-transfected cells, and the expression of Bax was significantly higher than that of the non-transfected cells. In HepG2 cells transfected with C gene, both the expressions of Bax and Bcl-2 were higher than those of the non-transfected cells and the cells transfected with pEGFP-C3 plasmid (Figure-3B).

Flow cytometry was used to detect the non-transfected cells and the cells transfected with pEGFP-C3, pEGFP-C3-C and pEGFP-C3-NS2 plasmids. The results of cell cycle showed that NS2 and C proteins inhibited cell cycle (Table).

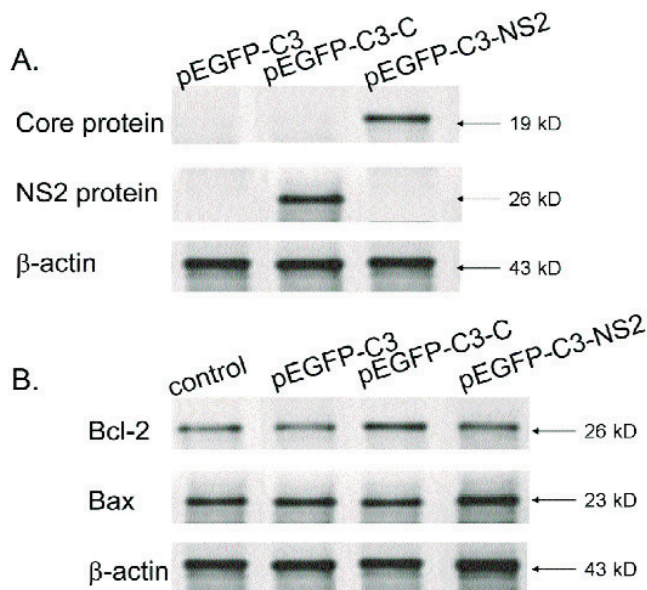


Figure-3: Western blot assay of normal and transfected HepG2 cells. (A) Expressions of NS2 and C protein in transfected HepG2 cells. Left lane: Cells transfected with pEGFP-C3 plasmid; middle lane: Cells transfected with pEGFP-C3-C plasmid; right lane: Cells transfected with pEGFP-C3-NS2 plasmid. (B) Up-regulated expressions of Bcl-2 and Bax by NS2.

Discussion

NS2 is encoded by gene located at sites 2530-3360nt of the HCV whole genome sequence, containing 217 amino acids (810-1026aa), with a molecular weight of approximately 23kDa. The C-end of NS2 is closely connected with the N-end of NS3 to form a complex protein with enzymatic activity. This C protein can cut NS2/NS3 connection site, and its mode of action is mainly autocatalysis. NS2 is a transmembrane protein in which the C-end is inserted into the endoplasmic reticulum (ER) lumen, and the N-end is located within the cytoplasm. Some researchers found that this protein is distributed around the cell nucleus with GFP as the marker.¹² The N-end of NS2 has 38 amino acids, which enables the NS2 gene to play an important cis-regulatory role in the expression of other genes of HCV, and suggests that the NS2 may have a similar function with the C protein.^{13,14} Western blot analysis found that NS2 could increase Bcl-2 slightly, while enhanced Bax more remarkably, suggesting that the common first 54 amino acids of NS2 and C proteins may be involved in the regulation. As NS2 can competitively inhibit the expression of C protein in liver cancer cells, it was found in the study that after pEGFP-C3-NS2 and pEGFP-C3-C plasmids were transfected into HepG2 cells at the same time, the expression of Bcl-2 and Bax had no significant difference with those in HepG2 cells transfected with the two plasmids respectively.

Another mechanism of liver cancer caused by HCV chronic infection is the induction of cell cycle arrest. Stapleford et al. found that cell proliferation and growth inhibition rate of cells with stable expression of NS2 was 50%-60%; the proportion of cell that stably expressed NS2 gene was increased significantly in the S phase of cell cycle, suggesting that NS2 protein can induce cell cycle to be arrested in the S phase.¹⁵

HCV C protein, as a trans-activate protein, can not only activate the activity of Bcl-2 promoter, up-regulate the expression of Bax, and react with 14-3-3 ϵ protein to release Bax to activate apoptosis, but also inhibit the proliferation of T cells and the generation of interferon- γ (IFN- γ), hinder the clearance effect of the body on HCV-infected liver cells so as to prompt HCV infection chronicity and the occurrence of liver cancer.¹⁶⁻¹⁸ Ma et al. reported that C protein can significantly induce Bax protein, considering that Bax signalling pathway plays an important part in inducing apoptosis.¹⁹

In recent years, the research of NS2 structure and function has experienced a rapid progress. Not only NS2's molecular structure has been parsed, but also many of its important functions have been found. It is observed that NS2 can interfere with the antiviral response of host cells, regulate gene transcription, inhibit apoptosis, arrest cell cycle, and can also play a key role in HCV infection process.²⁰ With more research on NS2 protein, there are bound to be more new discoveries about its structure and functions.

Conclusion

HCV NS2 gene plays a role in adjusting the proto-oncogene Bcl-2 and tumour suppressor gene Bax.

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