



# Hepatitis C Virus Nonstructural 5A Protein (HCV-NS5A) Inhibits Hepatocyte Apoptosis through the NF- $\kappa$ B/miR-503/bcl-2 Pathway

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The nonstructural protein 5A (NS5A) encoded by the human hepatitis C virus (HCV) RNA genome is a multifunctional phosphoprotein. To analyse the influence of NS5A on apoptosis, we established an Hep-NS5A cell line (HepG2 cells that stably express NS5A) and induced apoptosis using tumour necrosis factor (TNF)- $\alpha$ . We utilised the MTT assay to detect cell viability, real-time quantitative polymerase chain reaction and Western blot to analyse gene and protein expression, and a luciferase reporter gene experiment to investigate the targeted regulatory relationship. Chromatin immunoprecipitation was used to identify the combination of NF- $\kappa$ B and miR-503. We found that overexpression of NS5A inhibited TNF- $\alpha$ -induced hepatocellular apoptosis via regulating miR-503 expression. The cell viability of the TNF- $\alpha$  induced Hep-mock cells was significantly less than the viability of the TNF- $\alpha$  induced Hep-NS5A cells, which demonstrates that NS5A inhibited TNF- $\alpha$ -induced HepG2 cell apoptosis. Under TNF- $\alpha$  treatment, miR-503 expression was decreased and cell viability and B-cell lymphoma 2 (bcl-2) expression were increased in the Hep-NS5A cells. Moreover, the luciferase reporter gene experiment verified that bcl-2 was a direct target of miR-503, NS5A inhibited TNF- $\alpha$ -induced NF- $\kappa$ B activation and NF- $\kappa$ B regulated miR-503 transcription by combining with the miR-503 promoter. After the Hep-NS5A cells were transfected with miR-503 mimics, the data indicated that the mimics could reverse TNF- $\alpha$ -induced cell apoptosis and bcl-2 expression. Collectively, our findings suggest a possible molecular mechanism that may contribute to HCV treatment in which

NS5A inhibits NF- $\kappa$ B activation to decrease miR-503 expression and increase bcl-2 expression, which leads to a decrease in hepatocellular apoptosis.

**Keywords:** bcl-2, HCV-NS5A, hepatocyte apoptosis, miR-503, NF- $\kappa$ B

## INTRODUCTION

Chronic hepatitis C virus (HCV) infection results in progressive liver fibrosis leading to cirrhosis and liver cancer (Lim et al., 2014). According to 2015 statistical data from the World Health Organization (WHO) (2015), 130-150 million people are infected with HCV, and 500,000 people with an illness associated with HCV die every year. To date there is no effective HCV or hepatitis B vaccine that can be widely used (Weil et al., 2016). Current therapy relies on a combination of different types of drugs. Lawitz et al. (2014) reported that a fixed-dose combination of sofosbuvir and ledipasvir alone or with ribavirin has the potential to cure most patients with genotype-1 (GT-1) HCV. Charlton et al. (2015) reported that the combination of ledipasvir, sofosbuvir and ribavirin administered for 12 weeks produced high rates of SVR12 (sustained virological response at 12 weeks) in patients with advanced liver disease, including those with decompensated cirrhosis before and after liver transplantation.

HCV is a single-stranded RNA with a length of about 9.6

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KB, and it can be coded in at least four structural proteins (core protein, E1, E2 and p7) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and 55B). HCV nonstructural protein 5A (HCV-NS5A) is phosphorylated protein that is composed of about 447 amino acids; it has two hydrophilic alpha helix structures and three domains in the N-terminal. While the NS5A protein plays a critical role in the replication of HCV, its specific regulatory mechanism is unclear (Gao et al., 2010). The most recent study suggested that NS5A was a key factor in HCV treatment (Ikeda et al., 2016). Moreover, many NS5A inhibitors have been proven to effectively treat HCV (Gane et al., 2016), such as BMS-790052 (Gao et al., 2010), MK-8742 (Coburn et al., 2013; Yu et al., 2016), MK-8408 (Ling et al., 2016), ABT530 (Lin et al., 2015), GS-5816 (Mogalian et al., 2015), etc. Because drugs are expensive and have a resistance barrier, it is essential to identify the NS5A inhibition mechanism that can offer a new strategy for the future treatment of HCV. Furthermore, Miyasaka and Watanabe (2003) found that HCV-NS5A could inhibit TNF- $\alpha$ -induced Huh7 cell apoptosis. Majumder et al. (2002a) and Park et al. (2002) reported that NS5A impaired TNF- $\alpha$ -mediated apoptosis by interfering with NF- $\kappa$ B activation in 293 cells. Blocking the apoptosis of host liver cells is an important part of the pathological mechanism of HCV infection, and this can prevent an immune response caused by hepatocyte death. Thus, this present study aimed to investigate the mechanism of NS5A on TNF- $\alpha$ -induced hepatocyte apoptosis.

MicroRNA (miRNA), is a kind of non-coding short-chain RNA widely found in animals and plants (Bartel, 2004). Recently, studies have showed that miR-122 (Trebicka et al., 2013), miR-491 (Ishida et al., 2011), miR-146-5p (Bandiera et al., 2016) and miR-130a (Li et al., 2014) are involved in HCV-induced fibrosis and HCV replication. It has been reported that miR-503 is abnormally expressed in various type of cancers, including oral cancer, unicellular carcinoma, parathyroid carcinoma and nonidentical carcinoma (Yin et al., 2015). This suggests that miR-503 might play a complicated and tissue-specific role in cancer. Moreover, NF- $\kappa$ B-dependent miR-503 (Zhou et al., 2013) was found to be a pro-apoptotic gene (Xiao et al., 2016), and NS5A was reported to inhibit hepatocyte apoptosis and NF- $\kappa$ B activation (Majumder et al., 2002b), speculating that miR-503 may be involved in NS5A-inhibited hepatocyte apoptosis.

The present study aimed to study the mechanism of NS5A on TNF- $\alpha$ -induced hepatocyte apoptosis. It demonstrated that NS5A inhibited TNF- $\alpha$ -induced NF- $\kappa$ B activation in a dose-dependent manner. NF- $\kappa$ B controlled miR-503 transcription and downregulated bcl-2 expression, which led to cell apoptosis. Thus, these findings provide a potential mechanism for HCV infection.

## MATERIAL AND METHODS

### Plasmid cloning

HCV RNA was extracted from the serum of HCV patients using reverse transcription to obtain the NS5A sequence, which was cloned into a pcDNA3.1 carrier (Invitrogen, USA) using standard techniques, resulting in pcDNA-NS5A. Plas-

mids were transfected using Lipofectamine® 2000 reagent (Invitrogen) according to the manufacturer's instructions. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University. Written informed consent to obtain the samples was secured from all the patients.

### Cell culture and transfection experiment

The HepG2 cells were maintained at 37°C with 5% CO<sub>2</sub> in DMEM Medium containing 10% foetal bovine serum (FBS), 50 U of penicillin G and 50 ug/ml of streptomycin.

To establish stable cell lines (HepG2 cells that stably expressed NS5A), the cells were cultured in DMEM, 10% FBS in a 6-well culture plate. When the cultured cell density reached approximately 40%, the medium was aspirated and the cell lines were washed twice with phosphate buffered saline (PBS). The recombinant pcDNA-NS5A (1/2.5/5  $\mu$ g) was transfected, respectively, and as a control, an empty vector was also transfected. The stable transfectants were screened using neomycin (Invitrogen). The transfected cells were treated with neomycin for 5-10 days. The NS5A stable transfected cell lines were screened out successfully, and labelled Hep-NS5A. Hep-mock cell was used as the negative control.

Lipofectamine RNAi MAX (Invitrogen) was used to transfect the HepG2 cells with miR-503 mimic, and its negative control (miRNC), according to the manufacturer's instructions.

### Luciferase assay

To investigate whether miR-503 directly regulates bcl-2, luciferase reporter constructs were transfected into the Hep-NS5A and Hep-mock cells together with either miR-503 mimic or a scrambled oligonucleotide sequence (control). The cells were cultured for 48h and assayed with the Luciferase Reporter Assay System (Promega, US).

To investigate whether NF- $\kappa$ B binds to the miR-503 promoter and regulates its transcription, a pNF- $\kappa$ B-Luc plasmid, constructed by the luciferase reporter constructs spanning the putative NF- $\kappa$ B-binding sites of the miR-503 promoter, was obtained from Stratagene (USA). The transfections were performed according to the manufacturer's instructions. Briefly, the Hep-NS5A and Hep-mock cells ( $1 \times 10^5$  cell/well) were seeded in 96-well plates, and the Hep-mock cells were divided into two groups. The cells were transfected with plasmids for each well, and then incubated. After 24 h, the cell culture medium was removed and replaced with fresh medium containing dihydromyricetin for 12 h, followed by 10 ng/ml of TNF- $\alpha$  treatment for another 12 h. Luciferase activity was determined using a Luminoskan™ Ascent Microplate Luminometer (Thermo, USA).

### Measurement of cell viability via MTT assay

After undergoing various treatments, the Hep-NS5A and Hep-mock cells were seeded in 96-well plates and treated with TNF- $\alpha$ . At the end of the experiment, the media was removed and the cells were cultured with MTT solution (5 mg/ml) (Sigma, USA) for 4 h. The viable cells converted MTT to formazan, which generated a blue purple colour after

dissolving in 150  $\mu$ l of dimethyl sulfoxide (DMSO). The absorbance at 570 nm was measured using a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, Inc., USA).

### Western blot analysis

After undergoing various treatments, the cells were collected and lysed in protein lysis buffer. Proteins samples (30  $\mu$ g) were separated using sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE), and then the PAGE was transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Scientific, Rockford, USA). Primary mouse monoclonal antibodies against c-caspase, bcl-2, nuclear NF- $\kappa$ B p65 (np65), and their respective  $\beta$ -actin and lamin B levels (Abcam, USA), and secondary antibody peroxidase-conjugated rabbit anti-IgG (Sigma, USA) were used in the Western blot analysis. Band intensity was quantified using Quantity One software. The protein expression of c-caspase and bcl-2 was normalised to the  $\beta$ -actin levels, and the protein expression of np65 was normalised to the lamin B levels.

### Reverse real-time quantitative polymerase chain reaction (RT-qPCR)

After undergoing various treatments, total RNA samples were extracted from the cultured cells using Trizol (Invitrogen) according to the manufacturer's instructions. The TaqMan microRNA Reverse Transcription Kit and the TaqMan Universal Master Mix II with the TaqMan MicroRNA Assay of miRNAs (Applied Biosystems, USA) were used to test the miR-503 expression levels of the miRNAs in the tissues and cells. The bcl-2 levels were calculated relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (internal control) via the  $2^{-\Delta\Delta C_t}$  method using a real-time PCR system according to the manufacturer's instructions in SYBR® Green Master Mix (Applied Biosystems, USA).

### Chromatin immune co-precipitation

Lysates of the cells were generated under the addition of proteinase. The total protein of the lysates was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, USA) and analysed using an Eppendorf Master Photometer (Eppendorf, Germany). Then, 2% aliquots of the lysates were used as an input control and the remaining lysates were immunoprecipitated with either normal rabbit IgG or np65 antibody.

### Apoptosis assays

Cell apoptosis was analysed with an Annexin V-FITC Apoptosis Detection Kit (BD, Pharmingen, USA) according to the manufacturer's instructions. Briefly, after incubation, the cells were collected by centrifugation and resuspended in binding buffer containing HEPES, NaCl and CaCl<sub>2</sub> in pH 7.4. The supernatant cells were incubated with Annexin V-FITC and propidium iodide (PI) for 10 min at room temperature in the dark. The samples were analysed via flow cytometry using an FACScan Flow Cytometer (Becton Dickinson, USA). Cell Quest software was used to analyse the data (Becton Dickinson, USA).

### Statistical analysis

SPSS 17.0 software (SPSS Inc., USA) was used for the statistical analyses. All experiments were repeated three times, and all data were presented as mean  $\pm$  standard deviation. Statistically significant differences between the groups were defined as p-values less than 0.05.

## RESULTS

### NS5A inhibited TNF- $\alpha$ -induced HepG2 cell apoptosis

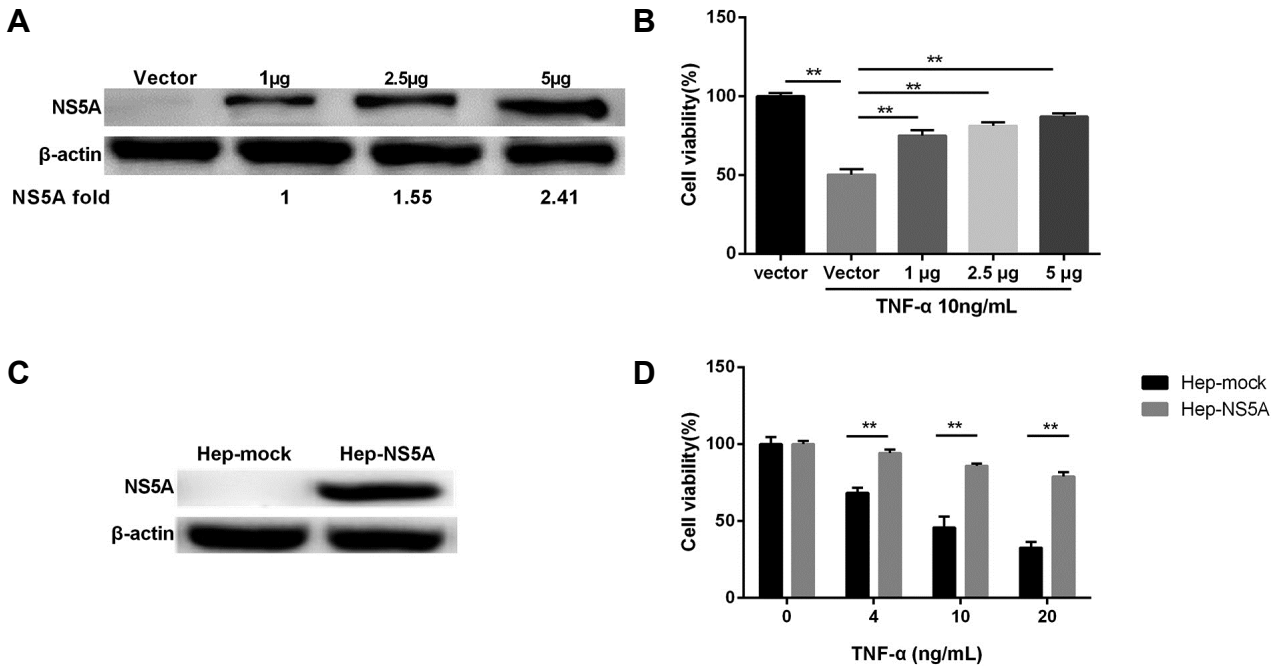
To confirm the effect of HCV-NS5A mRNA expression on TNF- $\alpha$ -induced HepG2 cell apoptosis, total RNA was extracted from the patients' serum using reverse transcription to obtain the NS5A sequence, which was cloned into pcDNA3.1 carrier, resulting in pcDNA-NS5A. The HepG2 cells were transfected with different concentrations of pcDNA-NS5A, and the NS5A protein expression was analysed via Western blot. As shown in Fig. 1A, as the concentrations of pcDNA-NS5A that were transfected into the cells increased, the NS5A protein expression increased. Then, TNF- $\alpha$  was used to induce cell apoptosis. As shown in Fig. 1B, TNF- $\alpha$  reduced cell viability; However, as the concentrations of pcDNA-NS5A transfected into the cells increased, cell viability gradually increased. For the next experiment, two HepG2 cell lines were constructed, an Hep-NS5A cell line with stable NS5A expression and a Hep-mock cell line as the negative control. The NS5A protein expression in the two established cell lines is shown in Fig. 1C. As seen in Fig. 1D, as the TNF- $\alpha$  level (0-20 ng/ml) increased, the Hep-mock cell viability decreased; however, the Hep-NS5A cell viability was greater than the Hep-mock cell viability, which further suggests that NS5A can inhibit HepG2 cell apoptosis induced by TNF- $\alpha$ .

### The effect of NS5A and TNF- $\alpha$ on cell viability and apoptotic-related protein expression

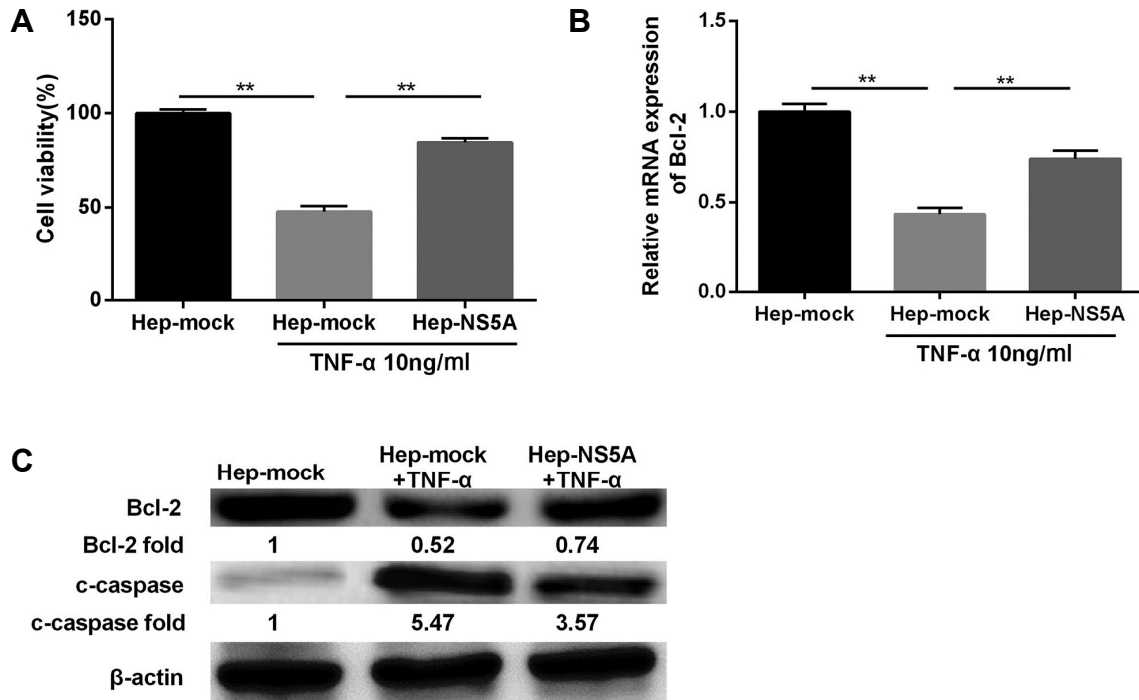
After being induced by TNF- $\alpha$ , Hep-NS5A cell viability significantly increased in comparison to the Hep-mock cell viability (Fig. 2A). As shown in Fig. 2B, TNF- $\alpha$  downregulated bcl-2 expression in the Hep-mock cells, but it increased in the Hep-NS5A cells. Moreover, the bcl-2 and c-caspase protein expressions were assayed by Western blot. Because bcl-2 is an anti-apoptotic gene and c-caspase is a pro-apoptotic gene, the bcl-2 protein expression trend was consistent with bcl-2 mRNA, but the trend for c-caspase protein expression was opposite to the trend for bcl-2 expression (Fig. 2C).

### NS5A inhibited TNF- $\alpha$ -induced NF- $\kappa$ B activation

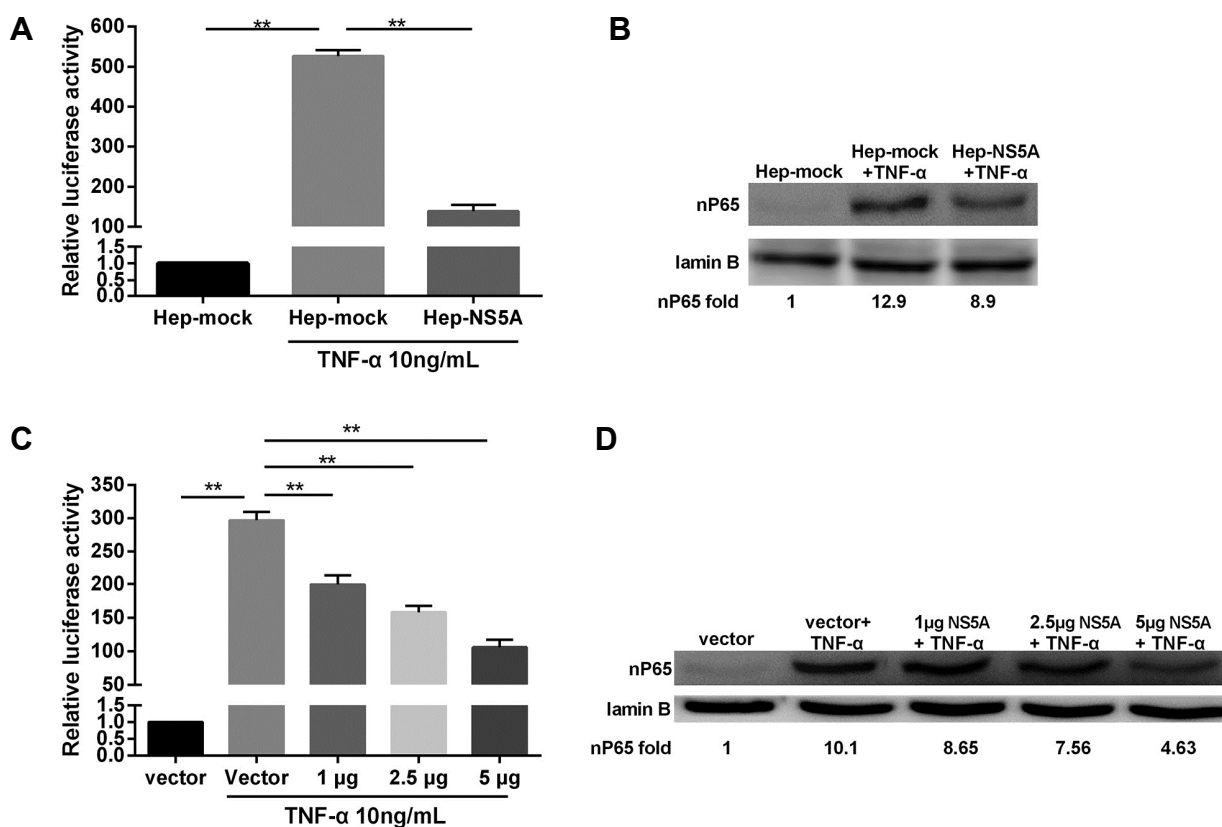
The Hep-NS5A and Hep-mock cells transfected with pNF- $\kappa$ B-luc plasmid were incubated with TNF- $\alpha$ , and a luciferase experiment was utilised to analyse whether NS5A stopped TNF- $\alpha$ -induced NF- $\kappa$ B activation. As shown in Fig. 3A, TNF- $\alpha$  enhanced the luciferase activity in the Hep-mock cells, but luciferase activity was obviously weakened in the Hep-NS5A cells. Nuclear factor kappaB p65 (np65) is one of the key regulators of transcription for a variety of genes (Vermeulen et al., 2003). The Western blot result showed that np65 protein expression increased in the TNF- $\alpha$ -induced Hep-mock cells and decreased in the TNF- $\alpha$ -induced Hep-NS5A cells (Fig. 3B).



**Fig. 1. NS5A inhibited TNF- $\alpha$ -induced HepG2 cell apoptosis.** Cells were transfected with different concentrations of pcDNA-NS5A, and then incubated in DMEM culture containing 10 ng/ml TNF- $\alpha$  for 6 h. (A) Protein expression level of NS5A. (B) Cell viability. HepG2 cell line with stable NS5A expression (Hep-NS5A) and Hep-mock cells as the negative control. (C) Protein expression level of NS5A in the Hep-NS5A and Hep-mock cells. (D) Cell viability of the Hep-NS5A and Hep-mock cells, which were incubated under different concentrations of TNF- $\alpha$ . The IC<sub>50</sub> of the Hep-NS5A and Hep-mock cells were 84.5 ng/ml and 8.821 ng/ml, respectively. \*P < 0.05, \*\*P < 0.01.



**Fig. 2. The effect of NS5A and TNF- $\alpha$  on the expression of apoptotic-related proteins.** The Hep-NS5A and Hep-mock cell lines were cultured with 10 ng/ml TNF- $\alpha$ . (A) Cell viability. (B) The bcl-2 mRNA expression. (C) Protein expression of bcl-2 and c-caspase. \*P < 0.05, \*\*P < 0.01



**Fig. 3. NS5A inhibited TNF- $\alpha$ -induced NF- $\kappa$ B activation.** (A) The luciferase activity in the Hep-NS5A cells transfected with pNF- $\kappa$ B-luc plasmid. TNF- $\alpha$ -induced NF- $\kappa$ B activation in the Hep-mock cell lines, but it decreased in the Hep-NS5A cells. (B) The np65 protein expression. (C) The luciferase activity in the TNF- $\alpha$ -induced HepG2 cells transfected with different concentrations of pcDNA-NS5A. (D) The np65 protein expression in the TNF- $\alpha$ -induced HepG2 cells transfected with different concentrations of pcDNA-NS5A. \*P < 0.05, \*\*P < 0.01

To further explore the influence of NS5A on TNF- $\alpha$ -induced NF- $\kappa$ B activation, HepG2 cells were co-transfected with pNF- $\kappa$ B-luc plasmid and different concentrations of pcDNA-NS5A. The results showed an increase in the pcDNA-NS5A concentration accompanied by a gradual decrease in luciferase activity (Fig. 3C), and a decrease in np65 expression (Fig. 3D). In general, TNF- $\alpha$  induced NF- $\kappa$ B activation, which may have been inhibited by the high expression of NS5A in the HepG2 cells.

#### The effect of NS5A and TNF- $\alpha$ on miR-503 expression by NF- $\kappa$ B binding to the miR-503 promoter and regulating its transcription

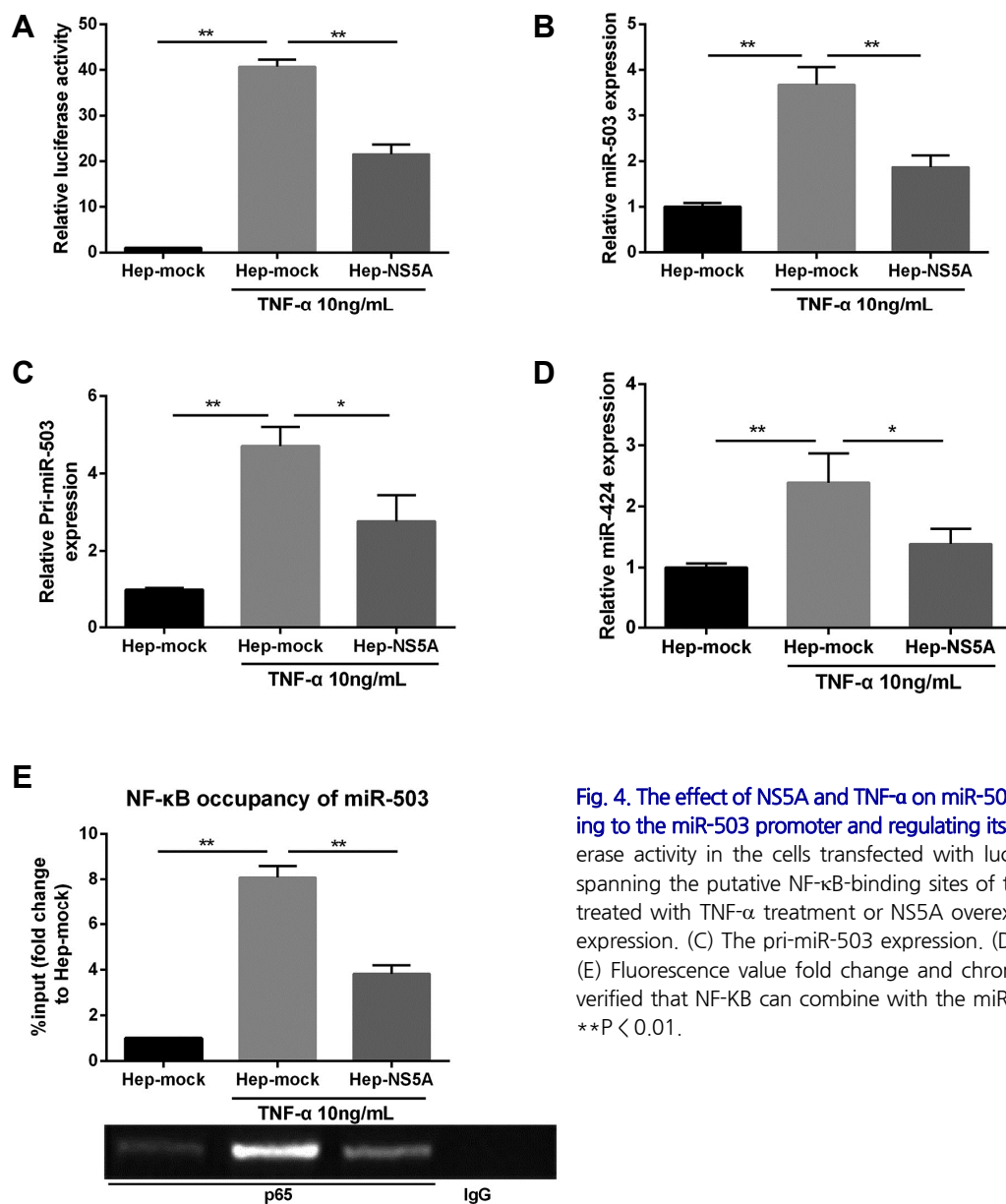
The cells were transfected with luciferase reporter constructs spanning the putative NF- $\kappa$ B-binding sites of the miR-503 promoter, and they then underwent TNF- $\alpha$  treatment or NS5A overexpression. Luciferase activity was measured and presented as a fold-change in comparison to the control. In Fig. 4A, TNF- $\alpha$  treatment of the HepG2 cells induced a significant upregulation in luciferase activity of the reporter construct containing the NF- $\kappa$ B p65 binding site within the miR-503 promoter sequence. Under the TNF- $\alpha$  treatment, NS5A overexpression in the HepG2 cells induced the oppo-

site result: an obvious downregulation in the luciferase activity of the reporter construct containing the NF- $\kappa$ B p65 binding site within the miR-503 promoter sequence.

TNF- $\alpha$  induced miR-503 expression while being controlled by NS5A. As seen in Fig. 4B, TNF- $\alpha$  upregulated miR-503 expression in the Hep-mock cells, but its expression obviously decreased in the Hep-NS5A cells. miR-424 and miR-503 are derived from a polycistronic precursor mir-424-503 (Chen et al., 2012). Thus, primary transcription of miR-503 (Pri-miR-503), and its cluster miR-424, was tested in the cells treated with TNF- $\alpha$ /NS5A. The result showed that TNF- $\alpha$  upregulated the expression of miR-503, Pri-miR-503 and miR-424 in the Hep-mock cells, but the expression of each of these factors was decreased in the Hep-NS5A cells (Figs. 4C and 4D).

In addition, we observed an increase in the binding of np65 to the miR-503 promoter in the TNF- $\alpha$ -induced Hep-mock cells, but the binding decreased in the Hep-NS5A cells. Chromatin immunoprecipitation was carried out to identify if NF- $\kappa$ B binds to the promoter regions of miR-503, and an IgG-precipitated sample was used as negative control (Fig. 4E). Combined with previously mentioned data presented in this paper, these data suggested that NF- $\kappa$ B can bind to the miR-503 promoter and regulate its transcription.





**Fig. 4. The effect of NS5A and TNF- $\alpha$  on miR-503 expression by NF- $\kappa$ B binding to the miR-503 promoter and regulating its transcription.** (A) The luciferase activity in the cells transfected with luciferase reporter constructs spanning the putative NF- $\kappa$ B-binding sites of the miR-503 promoter and treated with TNF- $\alpha$  treatment or NS5A overexpression. (B) The miR-503 expression. (C) The pri-miR-503 expression. (D) The miR-424 expression. (E) Fluorescence value fold change and chromatin immunoprecipitation verified that NF- $\kappa$ B can combine with the miR-503 promoter. \*P < 0.05, \*\*P < 0.01.

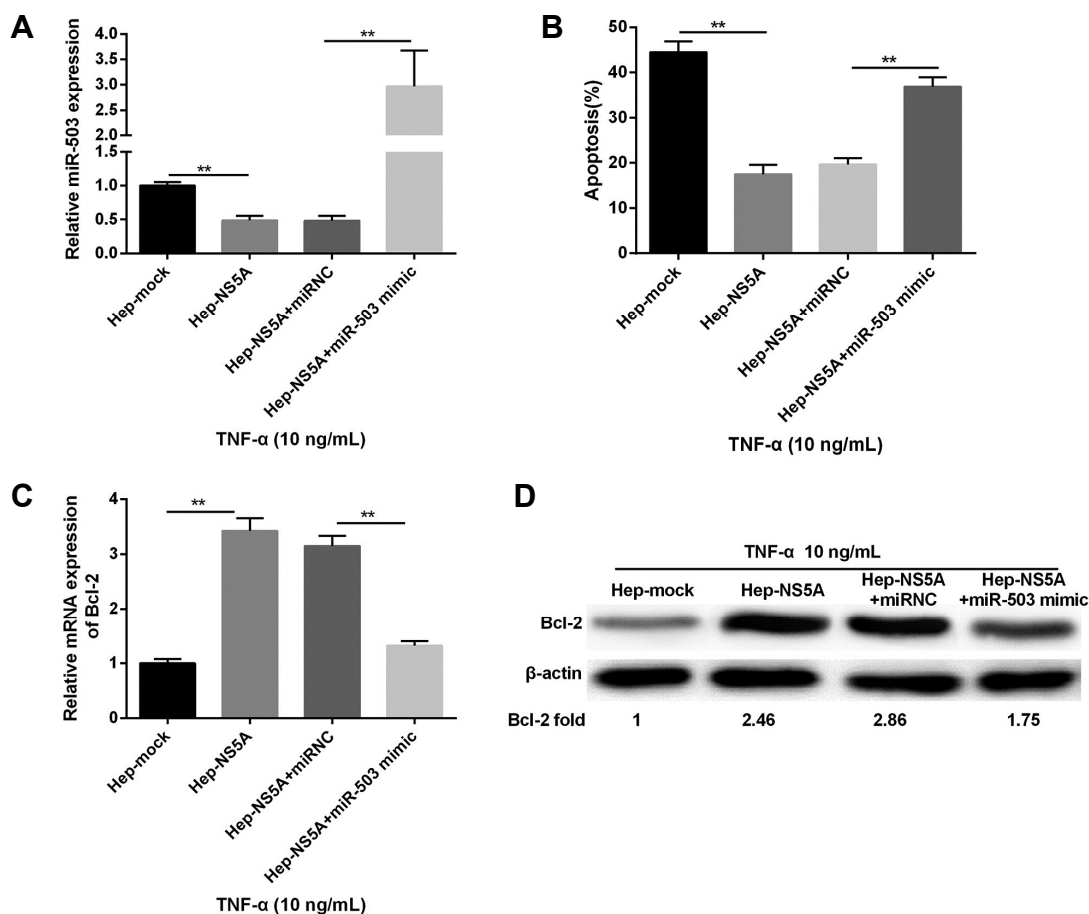
### Upregulated miR-503 reserved TNF- $\alpha$ -induced Hep-NS5A cell apoptosis

Previously mentioned research has shown that TNF- $\alpha$  can induce HepG2 cell apoptosis and NF- $\kappa$ B activation, increase miR-503 expression and decrease bcl-2 expression, and that NF- $\kappa$ B can regulate miR-503 transcription; moreover, all of this can be reversed by NS5A gene overexpression. To further explore the effect of miR-503 on TNF- $\alpha$ -induced Hep-NS5A cell apoptosis and bcl-2 expression, we conducted another experiment. As seen in Fig. 5A, NS5A inhibited TNF- $\alpha$ -induced miR-503 expression, and the miR-503 mimic improved miR-503 expression in the Hep-NS5A cells. Although Hep-NS5A cell apoptosis under the TNF- $\alpha$  treatment was significantly less than apoptosis in the Hep-mock cells, the apoptosis in the Hep-NS5A cells transfected with miR-503

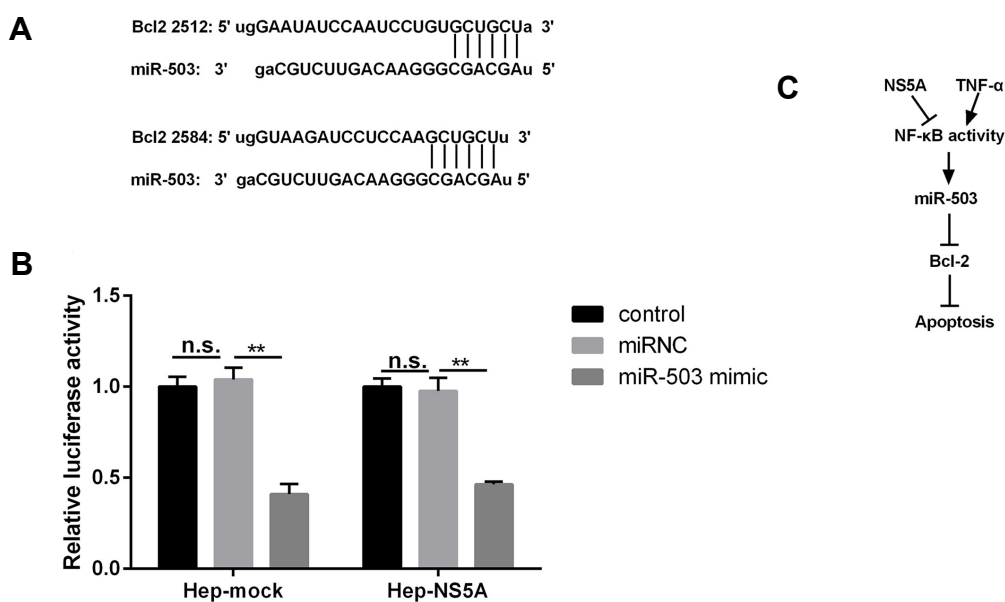
mimic was significantly greater than the apoptosis in the cells transfected with miRNC; this implies that upregulated miR-503 prevented TNF- $\alpha$ -induced Hep-NS5A cell apoptosis (Fig. 5B). Both bcl-2 mRNA and protein expression were detected (Figs. 5C and 5D). NS5A upregulated bcl-2 mRNA and protein expression, which was reversed by the miR-503 mimic in the TNF- $\alpha$ -induced Hep-NS5A cells.

### Bcl-2 was a direct target of miR-503

According to the miRNA target analysis tool, Starbase 2.0, two binding sites, bcl-2 to miR-503, were predicted (Fig. 6A). The luciferase reporter assay was used to identify if bcl-2 was a direct target of miR-503. In the first step, the Hep-NS5A and Hep-mock cells were transfected with luciferase reporter constructs containing the bcl-2 binding sites of the miR-503,



**Fig. 5. Upregulated miR-503 reserved TNF- $\alpha$ -induced Hep-NS5A cell apoptosis.** (A) The cell apoptosis rate. (B) The miR-503 expression. (C, D) The bcl-2 mRNA and protein expressions. \*P < 0.05, \*\*P < 0.01.



**Fig. 6. The luciferase reporter gene used to validate bcl-2 was a direct target of miR-503.** (A) Predicted binding site of miR-503 to bcl-2. (B) The luciferase activity in the Hep-NS5A and Hep-mock cells transfected with the miR-503 mimic and miRNA-NC. \*P < 0.05, \*\*P < 0.01. (C) Diagram of the signalling cascade identified in this study.

and then treated with the miR-503 mimic. As shown in Fig. 6B, the luciferase activity decreased in both the Hep-NS5A and Hep-mock cells after transfecting with the miR-503 mimic; this demonstrates that miR-503 directly targeted bcl-2 in the HepG2 cells.

Figure 6C presents a diagram of the signalling cascade identified in this study. NS5A inhibited TNF- $\alpha$ -induced HepG2 cell apoptosis through the inhibition of NF- $\kappa$ B activation, which reduced miR-503 transcription and upregulated bcl-2 expression.

## DISCUSSION

The NS5A protein plays a critical role in the replication of HCV. Given that NS5A can inhibit TNF- $\alpha$ -induced Huh7 cell apoptosis (Miyasaka and Watanabe, 2003) and interfere with NF- $\kappa$ B activation (Majumder et al., 2002a; Park et al., 2002), this present study found that cell viability and np65 expression were affected by TNF- $\alpha$  and NS5A. We found that as the levels of pcDNA-NS5A that were transfected into the cells increased, the TNF- $\alpha$ -induced cell viability gradually increased. As the TNF- $\alpha$  level increased in the Hep-NS5A cell line, its cell viability was greater than the cell viability of the Hep-mock cells, which clearly proves that NS5A inhibited TNF- $\alpha$ -induced HepG2 cell apoptosis. TNF- $\alpha$  has a wide range of actions in inflammation, infection and immunity. Nuclear transcription factor NF- $\kappa$ B is an important signalling molecule of TNF- $\alpha$  downstream, and its activation can promote hepatocyte apoptosis. Our results suggest that NS5A plays a role in inhibiting TNF- $\alpha$ -mediated apoptosis in HepG2 cells via disturbed NF- $\kappa$ B activation. We conducted an experiment on a luciferase reporter gene to further confirm whether or not NS5A could suppress NF- $\kappa$ B activation in hepatocyte cells; the luciferase activity and np65 protein expression were gradually reduced with increased concentrations of pcDNA-NS5A in the HepG2 cells.

Caspases and bcl-2 are both related to hepatocyte apoptosis. Caspases are a family of cysteine-aspartic proteases, and they play an essential role in pro-apoptosis; the bcl-2 protein can repress a number of apoptotic death programs (Mitupatum et al., 2016; Zeng et al., 2015). previously mentioned data have shown that upregulated anti-apoptotic protein Bcl-2 prevented cellular apoptosis, and the caspase family has always had high expression in apoptotic cells. Consistent with the findings reported in those studies, our results showed that bcl-2 expression was significantly lower in the TNF- $\alpha$  induced Hep-mock cells, while c-caspase expression was higher in the Hep-mock cells without TNF- $\alpha$  treatment. We also studied the effect of NS5A on bcl-2 and caspase expression. The results suggest that NS5A promotes bcl-2 mRNA and protein expression and inhibits c-caspase protein expression.

MiRNA can play a regulatory function by degrading RNA and inhibiting protein translation through complete or incomplete matching target genes. We noted that miR-503 expression increased with TNF- $\alpha$  treatment but it decreased when NS5A was overexpressed in the HepG2 cells, while opposite results were obtained for the bcl-2 expression. Qiu et al. (Qiu et al., 2013) reported that miR-503 targeted

3'UTR of bcl-2 in lung cancer cells. In this present study, we also demonstrated that bcl-2 was a direct target of miR-503 in the HepG2 cells. Xiao et al. (2016) found that miR-503 inhibited hepatocellular carcinoma cell growth, which is consistent with our results in which the Hep-mock cells had low cell viability with high miR-503 expression under the condition in which TNF- $\alpha$  was introduced for cell cultivation. Previous studies have demonstrated that TNF- $\alpha$  can activate NF- $\kappa$ B (Liu et al., 2015; Zhao et al., 2015), and NF- $\kappa$ B can promote miR-503 transcription (Caporali et al., 2015). Our results support those findings; we found that the expression of miR-503 and np65 were upregulated in the TNF- $\alpha$ -induced Hep-mock cells. We performed a luciferase reporter gene experiment and chromatin immunoprecipitation to confirm whether or not NF- $\kappa$ B could bind to the miR-503 promoter and regulate its transcription in hepatocyte cells. Moreover, we found that NS5A upregulated bcl-2 expression, which could have been blocked by the miR-503 mimic in the TNF- $\alpha$ -induced Hep-NS5A cells.

In conclusion, we observed decreased cell viability, upregulated miR-503 expression, reduced bcl-2 expression with TNF- $\alpha$  treatment in the Hep-mock cells, but all of these were reversed in the Hep-NS5A cells. Furthermore, we verified that NS5A inhibited TNF- $\alpha$ -induced NF- $\kappa$ B activation, NF- $\kappa$ B bound to the miR-503 promoter and regulated its transcription and bcl-2 was a direct target of miR-503. Our data suggest a possible molecular mechanism that contributes to HCV infection: NS5A inhibited NF- $\kappa$ B activation to decrease miR-503 expression and increase bcl-2 expression, which lead to a decrease in hepatocellular apoptosis.

## ACKNOWLEDGEMENTS

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