

Hepatocyte transformation and tumor development induced by hepatitis C virus NS3 c-terminal deleted protein

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Abstract

AIM: To study the effect of hepatitis C virus nonstructural protein 3 c-terminal deleted protein (HCV NS3-5') on hepatocyte transformation and tumor development.

METHODS: QSG7701 cells were transfected with plasmid pRcHCNS3-5' (expressing HCV NS3 c-terminal deleted protein) by lipofectamine and selected in G418. The expression of HCV NS3 gene and protein was determined by PCR and immunohistochemistry respectively. Biological behavior of transfected cells was observed through cell proliferation assay, anchorage-independent growth and tumor development in nude mice. The expression of HCV NS3 and *c-myc* proteins in the induced tumor was evaluated by immunohistochemistry.

RESULTS: HCV NS3 was strongly expressed in QSG7701 cells transfected with plasmid pRcHCNS3-5' and the positive signal was located in cytoplasm. Cell proliferation assay showed that the population doubling time in pRcHCNS3-5' transfected cells was much shorter than that in pRcCMV and non-transfected cells (24 h, 26 h, 28 h respectively). The cloning ratio of cells transfected with pRcHCNS3-5', pRcCMV and non-transfected cells was 33 %, 1.46 %, 1.11 %, respectively, the former one was higher than that in the rest two groups ($P < 0.01$). Tumor development was seen in nude mice inoculated with pRcHCNS3-5' transfected cells after 15 days. HE staining showed its feature of hepatocarcinoma, and immunohistochemistry confirmed the expressions of HCV NS3 and *c-myc* proteins in tumor tissue. The positive control group inoculated with HepG2 also showed tumor development, while no tumor developed in the nude mice injected with pRcCMV and non-transfected cells after 40 days.

CONCLUSION: 1. HCV NS3 c-terminal deleted protein has transforming and oncogenic potential. 2. Human liver cell line QSG7701 may be used as a good model to study HCV NS3 pathogenesis.

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INTRODUCTION

HCV infection is a major worldwide health problem. Persistent infection with HCV is a critical risk for the development of hepatocellular carcinoma (HCC)^[1-3]. It has been reported that the Core protein, NS3, NS4B, and NS5A have oncogenic potential^[2, 4-6]. HCV NS3 protein (nucleotide 3 420 to 5 312 with 631 amino acid residues) is a multi-functional viral protein. In addition to serine proteinase activity, which is located in the one-third of the NS3 protein at the N-terminal end, helicase and nucleotide triphosphatase activities are identified in the c-terminal half of the NS3 protein^[7]. HCV NS3 that plays a key role in the life cycle of virus and interacts with host cellular protein has been one of hot spots in recent research. We found that the NIH3T3 cell has stronger telomerase activity after transfected with HCV NS3 plasmid, indicating that HCV NS3 may be an important part in the hepatocarcinogenesis^[8]. Most studies on NS proteins have been carried out by expression of single or multiple NS proteins in cultured none hepatocyte, despite the fact that HCV is a hepacivirus. In order to reflect the relation between HCV NS3 and host cell transformation more clearly, here we tried to transfect human liver cell line QSG7701 with eukaryotic cell plasmid pRcHCNS3-5' (expressing NS3 c-terminal deleted protein), and then inoculated nude mice with transfected cells to investigate its biological behaviors and carcinogenesis.

MATERIALS AND METHODS

Materials

Hepatocyte cell line QSG7701 was got from Shanghai Institute of Cell Biology, Chinese Academe of Sciences (CAS) and HepG2 cell line was got from Cell Center of Central South University. Nude mice (BALB/cA-nude) were got from Shanghai Laboratorial Animal Center, CAS. The plasmid pRcHCNS3-5' (expressing HCV NS3 c-terminal deleted protein) was the kind gift from professor Takegami^[9]. Non-expressive plasmid pRcCMV was purchased from Sigma Com USA. LipofectaminTM reagent, G418 and Dulbecco's modified Eagle medium (DMEM) were products of GIBCO BRL (Germany). *Xba*I, buffer and PCR kit, marker were bought from Sino-American Biotechnology INC (Shanghai, P.R.China). Anti-HCV NS3 protein MAb, anti-c-myc protein MAb and S-P detection kit were purchased from Boshide Com (Wuhan, P. R.China) and Maxim Biotech INC (Fuzhou, P.R.China), respectively. New-born calf serum was got from Sijiqing Bioengineering Ltd. (Hangzhou, P.R.China). PCR primers for amplifying HCVNS3-5' gene were synthesized at Shanghai Sangon Com (Shanghai, P.R.China).

Methods

Experimental groups Group 1: QSG7701 parental cells; Group 2: QSG7701 transfected with blank plasmid pRcCMV; Group 3: QSG7701 transfected with plasmid pRcHCNS3-5'; Group 4: HepG2 cell line used as positive control.

Cell culture Cell lines were maintained in DMEM with 10 % heat-inactivated new-born calf serum, 100 unit/ml penicillin and 100 unit/ml streptomycin at 37 °C in a humidified atmosphere of 5 % CO₂.

Preparation, purification and identification of plasmids pRcHCNS3-5' and pRcCMV Plasmids pRcCMV and pRcHCNS3-5' were transferred into competent *E coli* JM109 respectively. JM109 was cultured to amplify the two plasmids. Little plasmids were prepared from JM109 to identify the specificity of the plasmids. The plasmid pRcHCNS3-5' was digested with *Xba*I, resolved with agarose gel electrophoresis, and stained with ethidium bromide (EB). As shown in Figure 1, the electrophoresis analysis revealed the major 886bp fragment of the plasmid pRcHCNS3-5' as being expected. Then the plasmids were massively extracted and purified for transfecting QSG7701 cells.

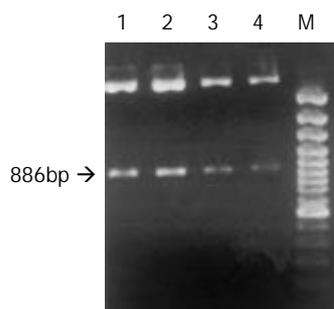


Figure 1 pRcHCNS3-5' digested with *Xba*I. Lane M:DNA marker (Generuler™ 100bpDNA). Lane: 1, 2, 3, 4 plasmid pRcHCNS3-5' with 886bp fragment. (×100).

Transfection of QSG7701 cells with plasmids pRcHCNS3-5' and pRcCMV QSG7701 cells were transfected with the plasmids pRcHCNS3-5' and pRcCMV, respectively according to the instruction of Lipofectamine reagent. Cells were seeded into selection medium containing 400 µg/ml G418 until G418-resistant clones were obtained, and then cells were maintained in G418 with concentration of 200 µg/ml (Figure 2). QSG7701 parental cells were used for the parallel control. To detect cDNA in stable transfectants, total genomic DNA was extracted according to standard methods and subjected to PCR and agarose gel electrophoresis analysis. The primers for amplifying HCVNS3-5' gene were based on published sequences^[9]. PCR conditions were 35 cycles of three steps (94 °C 30 sec, 57 °C 30 sec, 72 °C 40 sec) and then 72 °C 5 min in a 50 µl reaction mixture containing 5 µl 10×buffer, 5 µl 2 mM dNTPs, 0.5 µl of each primer (25 pmol/µl), 1 µl (100 ng)DNA, 0.5 ul 5U/µl Taq DNA polymerase, and 37.5 µl distilled water. PCR products were subjected to electrophoresis on a 0.8 % agarose gel, visualized by EB staining. As shown in Figure 3, 257bp fragment was specifically amplified from DNA of the QSG7701 cells transfected with plasmid pRcHCNS3-5', but no fragment was amplified from DNA of the pRcCMV transfected cells and parental cells.

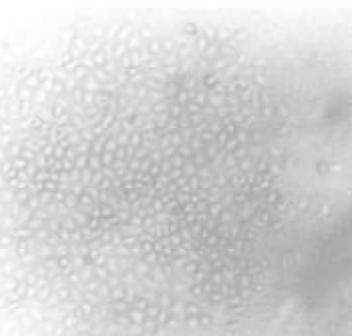


Figure 2 Colony formation of pRcHCNS3-5' transfected QSG7701 cells in G418 (×100).

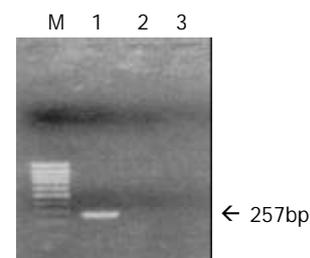


Figure 3 PCR analysis of DNA from QSG7701 transfected with pRcHCNS3-5' and pRcCMV; Lane M: 100bp marker; Lane 1: pRcHCNS3-5' transfected cells; Lane 2: pRcCMV transfected cells; Lane 3: QSG parental cells.

Immunohistochemistry S-P method was used to detect the expression of HCV NS3 protein in parental QSG7701 cells and QSG7701 cells transfected with plasmid pRcHCNS3-5', pRcCMV. PBS substituting Mabs was used as blank control. HCC expressing HCV NS3 protein was used as positive control. **Cell proliferation assay** QSG parental cells and cells transfected with pRcHCNS3-5', pRcCMV (6×10^4 cells per well) were seeded in 24-well plates and counted daily with a haemocytometer respectively. Experiment was lasted for 7 days and was repeated three times.

Anchorage-independent growth Cells were suspended at a density of 2×10^3 /ml containing 0.3 % granulated agar. The mixture was added over a layer of 0.7 % agar in medium. After 4 weeks, the morphology of transfected cells was analysed microscopically. Colonies including more than 50 cells were scored. Experiment was repeated three times.

Tumor development in athymic nude mice 12 nude mice (BALB/cA-nude, females, 4-6 weeks, 18-22 g) were divided into 4 groups of 3 nude mice each and inoculated subcutaneously in the right flank with pRcHCNS3-5', pRcCMV and parental cells (1×10^7 cells in 0.2 ml of phosphate-buffered saline) and monitored for tumor development. HepG2 cells were used as the positive control. Tumor size and animal weight were measured weekly. The nude mice were sacrificed and the tumors were removed 40 days after inoculating. Tumor tissue was fixed by 10 % formalin, embedded with paraffin wax, sliced successively 5 µm per section and stained by HE. Immunohistochemistry S-P method was used to detect the expressions of HCV NS3 protein and *c-myc* protein in tumor tissue.

Statistical analysis

F test was used. *P* value of less than 0.05 was considered as statistically significant.

RESULTS

HCV NS3 protein expression in QSG7701 cells transfected with plasmid pRcHCNS3-5'

Immunohistochemical staining showed that QSG7701 cells transfected with pRcHCNS3-5' strongly expressed HCV NS3 protein. The positive signal was in cytoplasm (Figure 4). The positive signal was also found in the positive control group, but not in the blank and negative control groups.

Cell proliferation assay

The population doubling time in cells transfected with pRcHCNS3-5' (12 h) was much shorter than that in those transfected with pRcCMV and parental QSG7701 cells (26 h, 28 h respectively). (Figure 5).

Anchorage-independent growth

The cloning efficiencies in pRcHCNS3-5', pRcCMV

transfected cells and parental cells were 33 %, 1.46 %, 1.11 %, respectively ($P < 0.01$, the former vs the rest two).

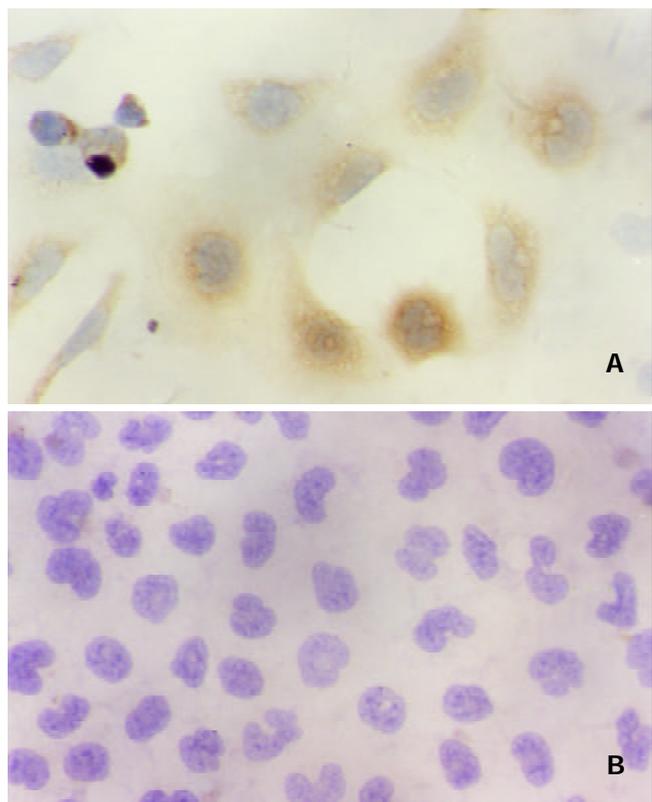


Figure 4 Immunohistochemistry analysis (S-P) of HCV NS3 protein expression in pRcHCNS3-5' transfected cells (up) and the negative control without positive signal (down) ($\times 400$).

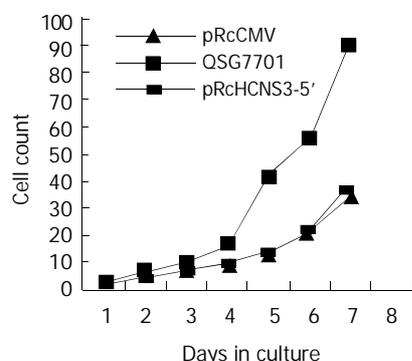


Figure 5 Proliferation rate of cells transfected with pRcHCNS3-5', pRcCMV and parental QSG7701 cells.

Tumor development in nude mice

In the nude mice inoculated with pRcHCNS3-5' transfected cells, the first evidence of well-defined and distinct subcutaneous tumors appeared on day 15. The mean tumor size and weight on day 40 were 3.08 cm³ and 3.13 g, respectively. The HepG2 cells, as positive control, also induced tumor in the nude mice. But no tumor developed in the mice inoculated with pRcCMV and non-transfected QSG7701 cells until 40 days after inoculating (Figure 6). Macroscopically, the tumors induced by pRcHCNS3-5' showed irregular yellow-brown node and appeared to be encapsulated. Central necrosis could be seen on the cut surface. Microscopically, the tumor cells exhibited similarity to hepatocyte except for heterogenesis and they arranged as trabecular with intervening sinusoids. Cancer cell nests with abundance granular necrosis were separated by fibrous tissue (Figure 7a, 7b). They showed the

histologically feature of hepatocellular carcinoma. Immunohistochemical staining showed that tumor tissue expressed HCV NS3 protein and *c-myc* protein. The positive signal was located in cytoplasm (Figure 7c, 7d).

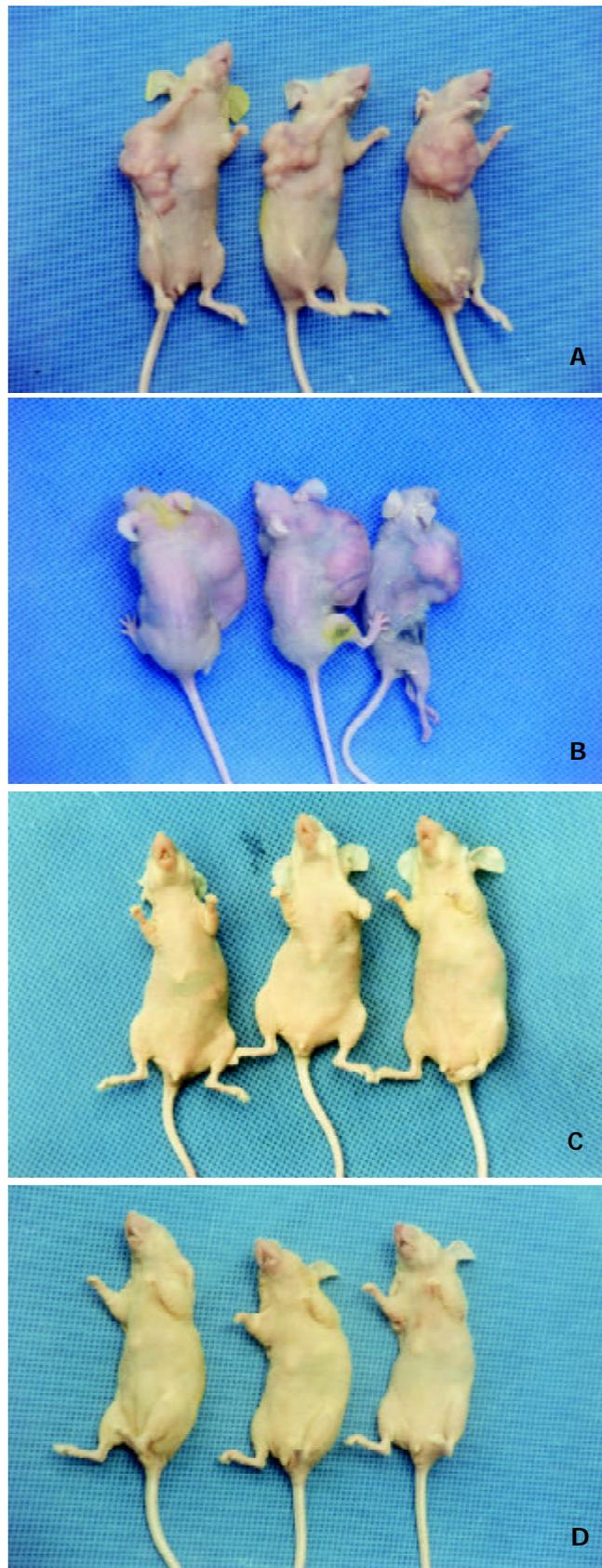


Figure 6 Tumor development in nude mice, 6a, 6b, 6c and 6d show inoculated with pRcHCNS3-5' transfected QSG7701 cells, HepG2 cells, QSG7701 cells transfected with pRcCMV and parental QSG7701 cells, respectively.

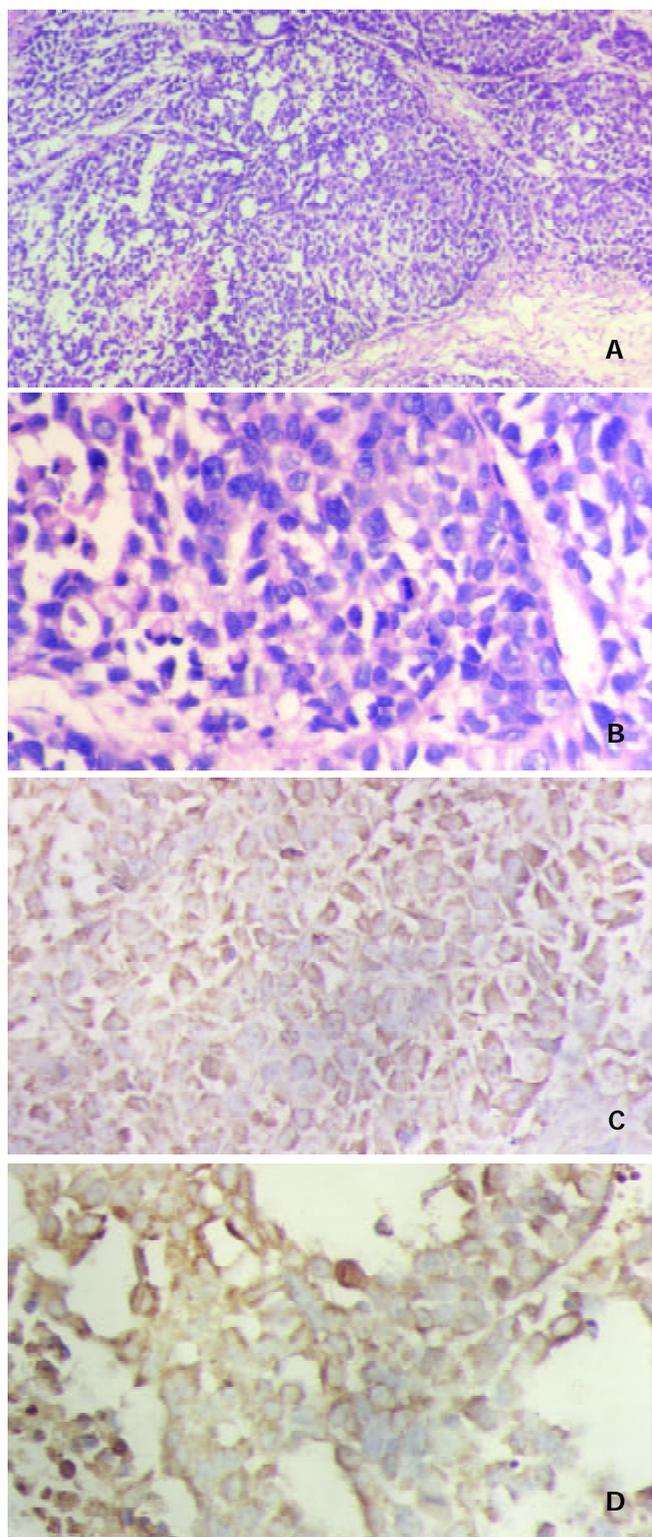


Figure 7 pRcHCNS3-5' induced hepatocellular carcinoma (7a HE \times 100, 7b HE \times 400), HCV NS3 (7c) and *c-myc* (7d) protein expressing in the tumor tissue by immunohistochemistry (S-P), the positive signals are both in cytoplasm (\times 400).

DISCUSSION

It has been reported that persistent infection with HCV is associated with HCC, and HCV replication and protein expression can be observed in HCC tissue^[1, 10-11], although the pathogenesis of HCV infection-associated HCC is still unknown. HCV, as a RNA virus without any RT activity, is replicated in the cytoplasm and does not integrate with host genome like HBV. Furthermore, unlike EBV or HPV, the HCV genome itself, does not contain any known oncogene. Most

studies indicate that the interaction between the HCV protein and liver cell gene/protein has been involved in hepatocarcinogenesis. Zemel *et al* reported significant mutations of amino acids that occur at the catalytic domain of the NS3 serine protease gene isolated from HCC tissue will affect the activity and substrate specificity of serine protease^[12]. Sakamuro^[9] and Zemel^[2] *et al* founded that HCV NS3 could transform NIH3T3 cell and non-tumorigenic rat fibroblast (RF) cell respectively. HCV NS3 can suppress actinomycin D induced apoptosis^[13], inhibit cAMP-dependent protein kinase^[14] and repress P53 function^[15-17]. But the cell lines used by most researchers were not based on HCV natural host cell-hepatocyte. Here non-tumorigenic human liver cell line QSG7701 was used in our study. QSG7701 cells that were immortal normal hepatocytes came from liver tissue 6 cm far from HCC and their karyotype number was 57^[18]. QSG7701 cells with normal hepatocyte phenotype were stably transfected with an expression vector containing cDNA for NS3 proteinase activity. The transfected cells grew rapidly, showed anchorage -independent growth in soft agar and induced significant tumor formation in nude mice. Our study firstly showed that the protease-coding gene of HCV induced malignant transformation of nontumorigenic human liver cells. Moreover injecting pRcHCNS3-5' transfected cells into nude mice led to significant tumor formation. The tumor obtained from the nude mice showed the histologically feature of hepatocellular carcinoma. The results suggest the involvement of proteinase activity of HCV NS3 N terminal peptide in cellular transforming and oncogenic potential.

Current studies show that HCV NS3 may be directly involved in hepatocarcinogenesis by disturbing the regulation of cell proliferation. But the mechanism of carcinogenesis is still perplexing. Carcinogenesis, including HCC, is the result of the abnormal proliferation of cancer cells. It is reported that various oncogenic products are related to functional abnormalities of intracellular signal transduction pathway, which have been proved to be one of the proliferative mechanism of cancer cells. Great progress has been made that HCV proteins regulate signal transduction in the recent studies. Ras/Raf/MAPK pathway is regarded to have close relation to HCV associated HCC.

Constitutive activation of the Ras/Raf/MAPK pathway is important for the transformation of mammalian cells^[19,20]. Indeed, studies have demonstrated that HCC development and progression is associated with the activation of Ras/Raf/MAPK pathway in humans and rodents^[21,22]. Our lab also found that MAPK activity is higher in HCC than that in the adjacent noncancerous lesions, suggesting a progression of HCC through Ras/Raf/MAPK activation^[23]. Recently, the development of anticancer drugs that target signaling proteins of MAPK pathway has been a major goal in the cancer treatment^[24,25]. There are several reports concerning the relationship between HCV protein and Ras/Raf/MAPK signal transduction pathway. For example, some evidences suggest that HCV C protein directly or indirectly activates Ras/Raf/MAPK signal transduction pathway elements (including Raf-1, ERK, MEK, Elk, SRE, AP-1) in different cells such as HepG2, CCL13, COS7, MCF-7, BALB/3T3 and NIH3T3^[26-31]. Borowski *et al* reported an arginine-rich domain located in the NS3 region (residues1487 to 1500 of HCV polyprotein) which strongly resembles the autoinhibitory sequence of the PKA R subunit. It binds to the C subunit of PKA and inhibits the translocation of C subunit into nucleus. Consequently, PKA functions are arrested^[32]. Marshall identified that specific sites between Ras and Raf-1 are essential to plasma membrane localization and Raf activation. The formation of Ras/Raf-1 complex is negatively regulated by PKA through phosphorylation of Raf-1 N terminal serine 43, which is believed to cause an N-terminal cap structure to cover the Ras docking site which ultimately leads to inhibit Raf-1 activation^[33,34]. HCV NS3 may activate Raf-1 by interfering the function of PKA. We

previously demonstrated that HCV NS3 N-terminal peptide (residues 1020-1295) positively regulates ERK1/2 phosphorylation. Because the N terminal peptide of HCV NS3 used in our study does not include the arginine-rich domain. We preclude that either HCV NS3 interacts with PKA through other domain or HCV NS3 involves in Ras/Raf/MAPK signal transduction pathway through other targets. Nuclear transcriptional factors *c-myc* as a target factor regulated by downstream of the MAPK cascade may increased when MAPK is activated. Overexpression of *c-myc* in tumor tissue of nude mice was detected which was consistent with other's report^[35]. It is supposed that HCV NS3 may be involved in hepatocarcinogenesis through Ras/Raf/MAPK/*c-myc* signal transduction.

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