

Effect of hepatitis C virus core protein on modulation of cellular proliferation and apoptosis in hilar cholangiocarcinoma

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BACKGROUND: Hepatitis C virus (HCV) is believed to be an important human pathogen causing carcinoma. But the effect of HCV infection on the alteration of cellular proliferation and apoptosis and the relationship between the effect and the development of hilar cholangiocarcinoma are largely unknown. The aim of this study was to assess the effect of HCV core protein on proliferation and apoptosis of hilar cholangiocarcinoma.

METHODS: HCV core protein (HCV C protein) was detected by peroxidase-antiperoxidase assay in surgical specimens from 48 patients with hilar cholangiocarcinoma. The apoptosis index (AI) and PCNA index (PI) in hilar cholangiocarcinoma were detected by *in situ* end labeling assay and streptavidin-biotin assay respectively.

RESULTS: The expression of HCV C protein was observed in 32 (67.7%) of the 48 specimens of hilar cholangiocarcinoma. The mean \pm standard deviation for AI and PI was $3.52\% \pm 0.64\%$ and $46.24\% \pm 11.46\%$ respectively. The AI of hilar cholangiocarcinoma specimens with HCV C protein expression was significantly lower than that of HCV C protein negative specimens ($P < 0.01$), whereas the PI of HCV C protein positive specimens was significantly higher than that of HCV C protein negative specimens ($P < 0.01$).

CONCLUSION: HCV C protein may promote the cellular proliferation of hilar cholangiocarcinoma and inhibit its cellular apoptosis.

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KEY WORDS: hilar cholangiocarcinoma;
hepatitis C virus; core protein;
apoptosis; proliferation

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Introduction

Hilar cholangiocarcinoma is ranked the second in cancers of the hepatobiliary system. Its incidence and mortality are increasing yearly and about 3000 new patients are found in the USA each year. The expressions of HCV RNA and HCV antigens in cholangiocarcinoma^[1,2] have provided some insights into the pathogenesis of cholangiocarcinoma. Hepatitis C virus (HCV) is recognized as a serious infectious source detrimental to the health of humans, even leading to cancer.^[3] Moreover, its core protein could act as a transcriptional regulator of viral and cellular promoters to potentially disrupt normal cellular functions. It has been confirmed that HCV infection and alteration of homeostasis of cellular proliferation and apoptosis concurrently occurred in hilar cholangiocarcinoma tissue.^[4,5] Nevertheless, there is a paucity of study about the effect of HCV C protein on cellular proliferation and apoptosis and the subsequent effect on the development of hilar cholangiocarcinoma. The aim of this study is to explore the effect of HCV C protein on cellular proliferation and apoptosis in hilar cholangiocarcinoma by detecting HCV C protein expression, apoptotic index (AI) and proliferating cell nuclear antigen (PCNA) index (PI) of hilar cholangiocarcinoma.

Methods

Subjects and specimens

Surgical specimens from 48 patients with hilar cholangiocarcinoma were studied. The patients were treated at the Memorial Hospital of Sun Yat-Sen University of Medical Sciences, Guangzhou and the Tongji Hospital, Wuhan, China in the period from September 1996 to April 2004. Of the 48 patients, 32 were male and 16 female; the youngest was 38 years old and the oldest 76 years old. Tumors of Bismuth I were noted in 14 patients, Bismuth II in 14, Bismuth III in 16, and Bismuth IV in 4. Tumor diameters in 31 patients were ≥ 2 cm, and in another 17 patients < 2 cm. Histological examination showed tubular adenocarcinoma in 26 patients, papillary

adenocarcinoma in 12, undifferentiated carcinoma in 8, and mucinous carcinoma in 2, Twenty-one patients had well-differentiated carcinoma, 10 moderately differentiated carcinoma, and 17 poorly differentiated carcinoma. And lymph node metastasis was found in 30 patients (62.5%). The surgical specimens were fixed by 10% formalin, paraffin-embedded and cut into continuous sections of 4 μm thickness.

Agents

Monoclonal mouse anti-HCV C protein (Santa Cruz Biotech Co., USA) was diluted to 1:50. Cell apoptosis detection kit (Beijing Zhongshan Biological Technology Co., China), monoclonal mouse anti-human PCNA (Zhongshan, China), PAP kit (Zhongshan, China), and LSAB kit (Dako Co., USA) were used.

Methods

The expression of HCV C protein was detected by immunohistochemical peroxidase antiperoxidase assay (PAP), cellular apoptosis by ISEL assay (*in situ* end labeling), and the expression of PCNA by streptavidin-biotin (SLAB) assay. The sections were stained with 3, 3'-diaminobenzidine muriate (DAB) and with haematoxylin as counterstain, and the slides were sealed by neutral gum. Hepatoma tissue was used as positive control, and phosphate buffer solution (PBS) was used to take the place of first antibody as negative control.

Assessment

In cells positive with HCV C protein, small pale brown particles were observed in the cytoplasm or the nucleus. It was assessed positive with HCV C protein if the cytoplasm or the cytoplasmic membrane of 5% or above of the cells was stained pale brown.

The nucleus of apoptotic cell was stained pale brown. The apoptotic cells of 3000 cells were counted in 5 non-necrosis regions of each specimen, and the frequency of apoptotic cell in the 3000 cells was taken as apoptosis index (AI).

PCNA was assessed positive if the nucleus of 5% or above of the cells were stained pale brown. The frequency of PCNA positive cells in the total adenocytes of 10 representative high power fields of each specimen was taken as PCNA index (PI).

Statistical analysis

Student's *t* test was used.

Results

Characteristics and positive frequency of HCV C protein

Positive signal was observed in the nucleus and cytoplasm of cells of hilar cholangiocarcinoma with granulo-well-

distribution. The positive cells scattered in the cancer nests (Fig. 1). The positive rate of HCV C protein in the 48 patients with hilar cholangiocarcinoma was 67.7% (32/48).

Apoptotic cells and AI

Scatter positive cells and cluster positive cells were both observed in the tissues of hilar cholangiocarcinoma. The frequency of apoptotic cells in well-differentiated carcinoma was significantly higher than those in poorly differentiated carcinoma and metastatic carcinoma. The apoptotic cells were distributed in a lamellar or massive pattern in well-differentiated carcinoma (Fig. 2). The apoptosis rate of the 48 specimens of hilar cholangiocarcinoma was 83.33% (40/48). The AIs ranged from 0.28% to 8.6%, and their mean \pm SD of AIs was $3.52\% \pm 0.64\%$.

Expression of PCNA and PI

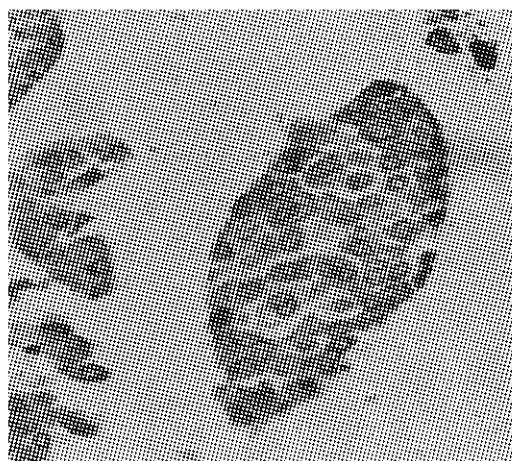


Fig. 1. The positive expression of HCV C protein in hilar cholangiocarcinoma tissue (PAP, original magnification $\times 400$).

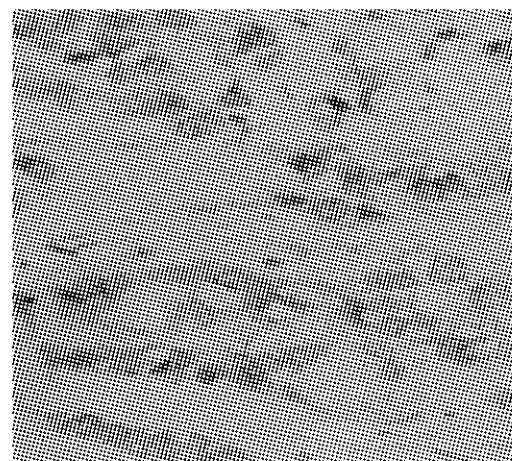


Fig. 2. The positive expression of PCNA in hilar cholangiocarcinoma tissue (SLAB, original magnification $\times 200$).

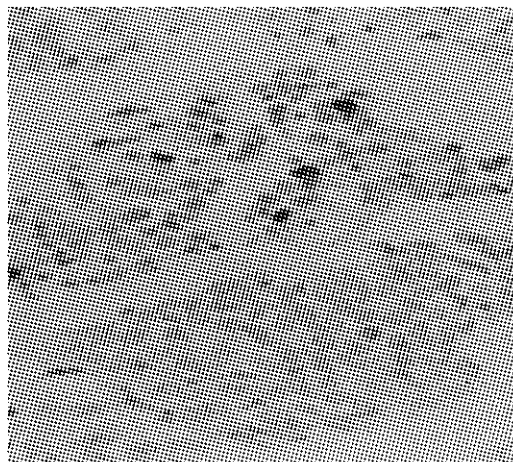


Fig. 3. Apoptosis cells in hilar cholangiocarcinoma tissues (ISEL, original magnification $\times 100$).

Table. The relationship between the HCV C protein expression and cellular proliferation and apoptosis (mean \pm SD)

Group	n	AI	PI
Positive with HCV C protein	32	3.16 \pm 0.12 *	54.28 \pm 8.34 *
Negative with HCV C protein	16	5.94 \pm 0.76	33.46 \pm 7.66

* : $P < 0.01$, between positive and negative groups.

The positive rate of PCNA in the 48 patients with hilar cholangiocarcinoma was 91.67% (44/48). The mean (SD) of PI was 46.24% \pm 11.41% (Fig. 3).

The relationship between the HCV C protein expression and the cellular proliferation and apoptosis

The AI of the group positive with HCV C protein was significantly lower than that of the group negative with HCV C protein ($P < 0.01$). And the PI of the group positive with HCV C protein was significantly higher than that of the group negative with HCV C protein ($P < 0.01$) (Table).

Discussion

The canceration of bile vessel epithelial cells is the genesis of cholangiocarcinoma. It is well known that many diseases are associated with the development of hilar cholangiocarcinoma, such as cholelithiasis, distoma hepaticum infection, and chronic enteritis.^[6] However, it is not uncommon for patients with hilar cholangiocarcinoma without such kinds of prior diseases, suggesting that there are other carcinogenic agents associated with the development of hilar cholangiocarcinoma.^[7] The main mechanisms of canceration of hilar bile vessel epithelial cells^[8-11] include: ① alteration of the homeostasis of cellular proliferation and apoptosis, disturbance of cell cycle modulation; ② activation of proto-oncogenes and

inactivation or mutation of anti-oncogenes; and ③ abnormality of intracellular signal transduction. It has been confirmed that HCV infection is associated with the development of hilar cholangiocarcinoma, in which there is alteration of the homeostasis of cellular proliferation and apoptosis.^[12,13] The effect of HCV infection on the alteration of cellular proliferation and apoptosis and the relationship between the effect and the development of hilar cholangiocarcinoma, however, are largely unknown. Thus, whether or not HCV C protein influences the percentage of proliferated cells and apoptotic cells remains to be an important issue in elucidating the possible carcinogenic mechanism of the HCV C protein.

HCV is believed to be an important human pathogen causing carcinoma. HCV C protein is one of the structural proteins of HCV, which are encoded by the HCV genome. It is a peptide with 191 amino acids. The gene encoding the HCV C protein is a relatively conservative region of the HCV genome,^[14,15] but HCV C protein has potential carcinogenic effect. In the study of Moriya et al,^[16] mice transgenic for the HCV core gene (1b genotype) developed histological features of chronic hepatitis C. After the age of 16 months, these transgenic mice developed HCC. Their features closely resembled those of the early stage of HCC in patients with chronic hepatitis C. In our early study, HCV C protein expression was higher in hilar cholangiocarcinoma than in the tissue around the tumor, thus we concluded that HCV infection was associated with the development of hilar cholangiocarcinoma. Studies mentioned above suggest that HCV C protein could potentially regulate cellular growth and differentiation, which is the direct evidence of the carcinogenic effect of HCV.

Not only is the abnormality of cellular proliferation and differentiation associated with the development of human carcinoma, but also may the abnormality of apoptosis be involved in the pathogenesis of carcinoma. Apoptosis occurs in untreated carcinoma tissue. Spontaneous apoptosis is one of the protecting mechanisms of anti-carcinoma, also the main way to eliminate virus out of the human body. In our study, the AI of the group positive with HCV C protein was significantly lower than that of the group negative with HCV C protein, whereas the PI of the group positive with HCV C protein was significantly higher than that of the group negative with HCV C protein. It is suggested that HCV may promote cellular proliferation and inhibit apoptosis in hilar cholangiocarcinoma. HCV C protein is a multi-functional protein, and the target enzyme of signal transduction. It can disturb the signal transduction system, resulting in proliferation of tumor cells, alteration of apoptosis homeostasis, and subsequent development of carcinoma.^[17-20] Giambartolomei et al^[21] considered that the expression of C protein in hepatocytes that infected with HCV interfered the normal signal transduction process, which is the main mechanism to develop carcinoma of these in-

ected hepatocytes. Spontaneous apoptosis is one of the protecting mechanisms of anti-carcinoma, also the main way to eliminate virus out of the human body. HCV infection may promote cellular proliferation and inhibit apoptosis in hilar cholangiocarcinoma, thereby the survival duration of infected cells is prolonged and the immune defence of body is attenuated, accordingly carcinoma occurs at last.

Recent studies have demonstrated that HCV core protein expresses with nuclear translocation in hepatocellular carcinoma (HCC), which is supposed to be associated with the development of HCC.^[22-24] Our study revealed that HCV C protein highly expressed in hilar cholangiocarcinoma tissue; moreover, positive signal was observed in the nucleus, namely the nuclear translocation expression of core protein also occurred in hilar cholangiocarcinoma tissue, which confirmed the carcinogenic effect of HCV C protein.^[25]

This study proposes a novel concept for the carcinogenic effect of HCV C protein; however, more and more studies are concentrated on the elucidation of the underlying molecular mechanism.

Competing interest

The author or authors do not choose to response to the statements listed in Instructions for Authors.

References

- 1 Watashi K, Shimotohno K. The roles of hepatitis C virus proteins in modulation of cellular functions; a novel action mechanism of the HCV core protein on gene regulation by nuclear hormone receptors. *Cancer Sci* 2003;94:937-943.
- 2 Zhai SH, Liu JB, Liu YM, Zhang LL, Du ZP. Expression of HBsAg, HCV-Ag and AFP in liver cirrhosis and hepatocarcinoma. *Shijie Huaren Xiaohua Zazhi* 2000;8:524-527.
- 3 Wang WL, Wang CJ, Wang BF. Significance of HCV gene and its antigen expression in human primary intrahepatic cholangiocarcinoma. *Shijie Huaren Xiaohua Zazhi* 2001;9:542-545.
- 4 Chen Rf, Zou SQ, Zhan XP. Expression of hepatitis C virus core gene in hilar cholangiocarcinoma and implication. *Chin J Exp Surg* 2000;17:223-224.
- 5 Liu XF, Zou SQ, Qiu FZ. Construction of HCV-core gene vector and its expression in cholangiocarcinoma. *World J Gastroenterol* 2002;8:135-138.
- 6 Tsuchida A, Kasuya K, Endo M, Saito H, Inoue K, Nagae I. High risk of bile duct carcinogenesis after primary resection of a congenital biliary dilatation. *Oncol Rep* 2003;10:1183-1187.
- 7 Zou SQ, Liu XF, Guo RX, Li CL, Zhou XS, Zhu XG. The retrospective analysis of HBV and HCV infection in cholangiocarcinoma. *Zhonghua Wai Ke Za Zhi* 2003;41:417-419.
- 8 Tullo A, D'Erchia AM, Honda K, Kelly MD, Habib NA, Saccone C. New p53 mutations in hilar cholangiocarcinoma. *Eur J Clin Invest* 2000;30:798-803.
- 9 Yamamoto K, Katayose Y, Suzuki M, Unno M, Sasaki T. Adenovirus expressing p27KIP1 induces apoptosis against cholangiocarcinoma cells by triggering Fas ligand on the cell surface. *Hepatology* 2003;50:1847-1853.
- 10 Holzinger F, Z'graggen K, Buchler MW. Mechanisms of biliary carcinogenesis; a pathogenetic multi-stage cascade towards cholangiocarcinoma. *Ann Oncol* 1999;10:122-126.
- 11 Lu H, Ye MQ, Thung SN, Dash S, Gerber MA. Detection of hepatitis C virus RNA sequences in cholangiocarcinomas in Chinese and American patients. *Chin Med J (Engl)* 2000;113:1138-1141.
- 12 Chen RF, Li ZH, Chen JS, Zou SQ. HCV C protein activates the signal transduction pathway mediated by NF- κ B in bile duct cancer cells. *Chin J Exp Surg* 2003;20:227-228.
- 13 Li ZH, Cen RF, Xie DR, Xin XG, Liu TH. The effect of hepatitis C virus core protein on apoptosis and cell cycle of hilar cholangiocarcinoma cells. *Sun Yat-Sen University (medical sciences)* 2004;25:119-121.
- 14 Suzuki R, Matsura Y, Suzuki T. Molecular basis of subcellular localization of HCV core protein. *Liver* 1996;16:221-224.
- 15 Cai Z, Liang TJ, Luo G. Effects of mutations of the initiation nucleotides on hepatitis C virus RNA replication in the cell. *J Virol* 2004;78:3633-3643.
- 16 Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K. The hepatitis C virus core protein induce hepatocellular carcinoma in transgenic mice. *Nat Med* 1998;4:1065-1067.
- 17 Tai DI, Tsai SL, Chen YM, Chuang YL, Peng CY, Sheen IS. Activation of nuclear factor κ B in hepatitis C infection; implications for pathogenesis and hepatocarcinogenesis. *Hepatology* 2000;1:656-664.
- 18 Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C, Strauss M. NF- κ B function in growth control; regulation of cyclin D1 expression and G0/G1-to-S-phase transition. *Mol Cell Biol* 1999;19:2690-2698.
- 19 Yang SH, Lee CG, Lee CW, Choi EJ, Yoon SK, Ahn KS, et al. Hepatitis C virus core inhibits the Fas-mediated p38 mitogen activated kinase signaling pathway in hepatocytes. *Mol Cells* 2002;30:452-462.
- 20 Chung YM, Park KJ, Choi SY, Hwang SB, Lee SY. Hepatitis C virus core protein potentiates TNF- α -induced NF- κ B activation through TRAF2-IKK β -dependent pathway. *Biochem Biophys Res Commun* 2001;284:15-19.
- 21 Giambartolomei S, Covone F, Levrero M, Balsano C. Sustained activation of the Raf/MEK/Erk pathway in response to EGF in stable cell lines expressing the hepatitis C virus (HCV) core protein. *Oncogene* 2001;20:2606-2610.
- 22 Chen SY, Kao CF, Chen CM, Shih CM, Hsu MJ, Chao CH. Mechanisms for inhibition of hepatitis B virus gene expression and replication by hepatitis C virus core protein. *J Biol Chem* 2003;3:278:591-607.
- 23 Falcon V, Acosta-Rivero N, Chinea G, de la Rosa MC, Mendez I, Duenas-Carrera S. Nuclear localization of nucleocapsid-like particles and HCV core protein in hepatocytes of a chronically HCV-infected patient. *Biochem Biophys Res Commun* 2003;10:310:54-58.
- 24 Yoshida H, Kato N, Shiratori Y, Otsuka M, Maeda S, Kato J. Hepatitis C virus core protein activates nuclear factor κ B-dependent signaling through tumor necrosis factor receptor-associated factor. *J Biol Chem* 2001;276:16399-16405.
- 25 Kato N, Yoshida H, Kioko Ono-Nita S, Kato J, Goto T, Otsuka M. Activation of intracellular signaling by hepatitis B and C viruses; C-viral core is the most potent signal inducer. *Hepatology* 2000;32:405-412.

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