

Inhibition of hepatitis C virus-transfected cholangiocarcinoma by antisense oligodeoxynucleotide in nude mice

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BACKGROUND: The inhibitory effect of antisense oligodeoxynucleotide (asODN) on the replication and expression of hepatitis C virus (HCV) *in vitro* is not well elucidated. This study was to assess the effect of asODN on HCV in cholangiocarcinoma.

METHODS: The QBC939 cells transfected by a recombinant HCV containing HCV core gene cloned in vector of PBK-CMV (PBK-HCV_C) were treated by 14-mers phosphorothioate ODN complementary to the HCV core genomic region. The variation of HCVmRNA level was detected by RT-PCR. Moreover, the inhibitory effect of asODN was observed in nude mice.

RESULTS: HCVmRNA was detected in transfected-QBC939 cells. The 14-mers complementary phosphorothioate ODN showed effective inhibition on HCVmRNA and unexpression HCVmRNA at 6 μmol/L. The tumorigenicity of the transfected-QBC939 cells incubated with asODN in nude mice was greatly inhibited.

CONCLUSION: The results suggest a potential therapy of asODN for HCV infected cholangiocarcinoma.

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KEY WORDS: cholangiocarcinoma; hepatitis C virus; core gene; HCVmRNA; antisense oligodeoxynucleotide; nude mice

Introduction

Hepatitis C virus (HCV) is recognized as a kind of serious infectious disease detrimental to the health of the human being.^[1] It could ultimately lead to cancerization. Recent studies, however, have sug-

gested that HCV may be related to the pathogenesis of cholangiocarcinoma.^[2-4] Successful use of antisense oligodeoxynucleotide (asODN) to inhibit the replication and expression of HCV *in vitro* has been reported recently.^[5-11] These effects are probably due to an unspecific binding of ODNs to intracellular and extracellular proteins, resulting in the alteration of cellular processes.

To study the inhibitory effect of asODN on hepatitis C virus in cholangiocarcinoma and find out a new therapy for HCV infected cholangiocarcinoma, we transfected QBC939 cells by a recombinant HCV containing HCV core gene cloned in vector of PBK-CMV (PBK-HCV_C), a recombinant HCV containing HCV core gene. They were treated by 14-mers phosphorothioate ODN complementary to the HCV core genomic region. The variation of HCVmRNA level was detected by RT-PCR. Moreover, the inhibitory effect of asODN was also observed in nude mice.

Methods

Materials

PBK-HCV_C transfected cholangiocarcinoma cells

PBK-HCV encompassing the core and envelope genomic regions of HCV, containing from 330nt to 2020nt of HCV II strain, was provided as a gift by Professor Zhi Chen, Institute of Infectious Disease, Zhejiang University, with restriction sites of *Pst* I and *Eco*R I. The prokaryotic expressing vector of PBK-CMV containing multiple cloning site (MCS), the neomycin- and kanamycin-resistance gene, SV40 poly (A), was purchased from Stratagene Co., USA. In our study, recombinant plasmid of the HCV-core gene was constructed by molecular cloning. It was identified by restriction enzymes, and then transfected into QBC939 cells with lipofection. After it was selected with G418 made by the presence of neomycin and kanamycin resistance gene, resistant colonies were obtained. The QBC939 cells (a cholangiocarcinoma cell line) were a generous gift from Professor Shu-Guang Wang, Third Military Medical University, China. The QBC939 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640, supplemented

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with 100 ml/L FBS and incubated at 37 °C in an atmosphere of 50 ml/L CO₂.

Nude mice

Twenty-five male nude mice were purchased from the Experimental Animal Center of Hubei province, Wuhan, China.

Reagents

Lipofection was provided by Boehringer Mannheim Co., Germany, and Trizol reagent was purchased from Gibco BRL Co., USA.

Antisense oligodeoxynucleotide

14-mers phosphorothioate ODN complementary to the HCV core genomic region was synthesized by Shanghai GeneCore Bio-Technologies Co., Shanghai, China. Complementary sites varied from 341nt to 354nt of HCV II strain; 5'-TTTGAGGTTTAGGA-3'.

PCR Primers

Two primers were designed according to the sequence of HCV core genomic regions and synthesized by Shanghai GeneCore Bio-Technologies Co., Shanghai, China; primer 1: 5'-TGAGCACGAATTTCTAAC-3', primer 2: 5'-GCGCCGACAAGCGGAATGT-3'; and β -actin mRNA primer 1: 5'-CAGAGCAAGAGAGGCATCCT-3', primer 2: 5'-GGATAGCACAGCCTGGATAG-3'. The products of HCV and β -actin RT-PCR were 403bp and 250bp.

Methods

RT-PCR for HCVmRNA

Total RNA was prepared from the QBC 939 cell line with the Trizol reagent (Gibco Brl, USA) according to the manufacturer's instructions. RT-PCR was performed using the RT-PCR kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol. cDNA synthesis was carried out with 2 μ g of total RNA. PCR reactions of 20 μ l were processed in a MJ. A peltier thermal cycler-100 (PTC-100) Thermocycler was used under the following conditions: 60 °C for 30 minutes, 94 °C for 1 minute, 55 °C for 30 seconds, 72 °C for 1 minute for a total of 35 cycles, and 72 °C at 10 minutes. The RT-PCR products were analyzed on 2% agarose gel. Amplified human β -actin served as a control for sample loading and integrity.

RT-PCR for HCVmRNA after adding asODN

After adding different concentration of ODNs (0, 1.0, 2.0, 4.0, 5.0, 6.0 μ mol/L), RT-PCR amplification was done formerly.

Test of proliferation effect on tumor

The 25 male nude mice (Balb/c) of 6-8 weeks

were divided into 3 groups; 10 mice for injection of QBC939 cells transfected of PBK-HCV_C vector, 5 mice for inoculation of QBC939 cells, and 10 mice for injection of PBK-HCV_C+asODN. Each mouse was injected subcutaneously at two sites (neck and back), each of which was inoculated 5 \times 10⁷ cells/0.1 ml. Six weeks after inoculation, the mean tumor size and number were measured. The differences between the 3 groups were analyzed by the *t*-test.

Results

Expression of HCVmRNA core protein in PBK-HCV_C transfected QBC939 cells

The cell sections transfected with PBK-HCV_C and control plasmid (PBK-CMV) were amplified by RT-PCR. The QBC939 cells of PBK-HCV_C transfected were seen approximately 400bp (HCVmRNA) and 250bp (β -actin mRNA). The cells transfected with control plasmid (PBK-CMV) were only seen approximately 250bp (β -actin mRNA) (Fig. 1).

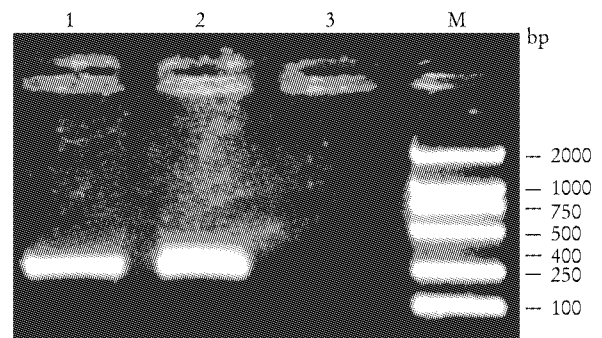


Fig. 1. Electrophoretic analysis of RT-PCR product. M; marker DL2000; Lane 1; PBK-CMV transfected QBC939 cells; Lane 2; PBK-HCV_C transfected QBC939 cells; Lane 3; negative control.

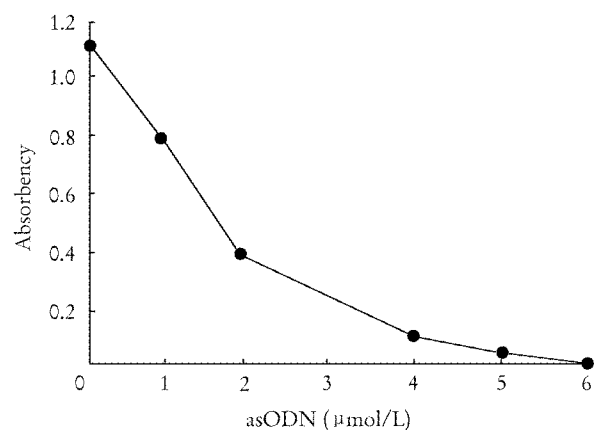


Fig. 2. Different concentration of asODN on HCVmRNA expression.

Table. asODN restraining effect on cholangiocarcinoma cells in nude mice

Group	Cases	Occurrence/seeding	Occurrence ratio (%)	Appearance time (d)	Tumor size (cm)
Control	5	6/10	60	12.0 ± 1.31 **	0.61 ± 0.14 *
PBK-HCV _C	10	13/20	65 *	10.1 ± 1.14 **	0.82 ± 0.22 *, **
asODN+PBK-HCV _C	10	5/20	25 *	15.3 ± 1.20 **	0.38 ± 0.16 **

* : $P < 0.05$; ** : $P < 0.01$.

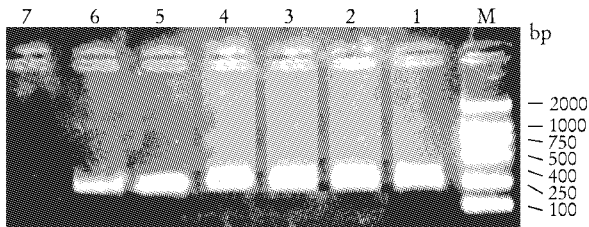


Fig. 3. RT-PCR products of HCVmRNA after action of asODN. M; marker DL2000; Lane 1; control; Lane 2-6; after action of asODN; Lane 3; negative control.

Expression of HCVmRNA after use of ODN

After adding ODNs (0-6 $\mu\text{mol/L}$), the ratio of HCVmRNA to β -actin mRNA was gradually decreasing. At 6 $\mu\text{mol/L}$ ODNs, the ratio was zero (Figs. 2 and 3).

ODN restraining effect of cholangiocarcinoma cells on nude mice

Tumors were found in the groups of PBK-HCV_C transfection, control and PBK-HCV_C+asODN ($P < 0.05$) at rates of 60% (6/10), 65% (13/20) and 25% (5/20). The time of tumor appearance was 10.1 days in the PBK-HCV_C transfection group, 12.0 days in the control group and 15.3 days in the PBK-HCV_C+asODN group. The time in the PBK-HCV_C transfection group was earlier than that in the control group and PBK-HCV_C+asODN group ($P < 0.01$, $P < 0.01$). The mean tumor size in the PBK-HCV_C transfection group was larger than that in the control group ($P < 0.05$) and PBK-HCV_C+asODN group ($P < 0.01$, Table). It was suggested that the tumorigenicity in nude mice was greatly depressed after action of ODN.

Discussion

Hepatitis C virus infection, an important cause of morbidity and mortality worldwide, produces a spectrum of liver disease ranging from an asymptomatic carrier state to end-stage liver disease. The HCV core protein could act as a transcriptional regulator of various viral and cellular promoters to potentially disrupt normal cellular functions.^[12-17] The core protein may cooperate with ras oncogene and transform primary rat embryo fibroblasts

to a tumorigenic phenotype.^[18-21] It may also cause anti-apoptosis by reversion of the tumor suppressor gene-p53 and activation of NF- κ B and implicate a mechanism by which HCV may evade the host's immune surveillance, leading to viral persistence and possibly to carcinogenesis.^[22-29] Thus, the HCV core protein plays a major role in the malignant transformation of cells.

Cholangiocarcinoma is the second cancer of the hepatobiliary system with an increasing incidence and mortality. Approximately 3000 new patients are diagnosed in the USA each year, but the pathogenesis of cholangiocarcinoma is not clear enough. Previously HCV was considered hepatotropic virus, and viral replication and cellular injury are largely confined to the liver. Recent studies, however, have suggested that HCV may replicate in tissues other than hepatocytes. Researchers reported the presence of HCV RNA and HCV-antigens in the epithelial cells of lymph nodes, pancreas, ovary, kidney, heart and bile duct.^[30-34] The infection of HCV could lead to damage and loss of the bile duct, which are defined as epithelial, lymphocytic infiltration^[35-38] and steatosis. The higher rate of HCV infection in the bile duct provides an evidence that epithelial cells of the bile duct could be an important reservoir of HCV and contribute to the pathogenesis of cholangiocarcinoma. Recent laboratory and epidemic studies found that the infection of HCV is also related to the development of cholangiocarcinoma.^[39-45]

In our study we observed the inhibitory effect of asODN on HCV in cholangiocarcinoma. The QBC939 cells transfected by PBK-HCV_C, a recombinant HCV containing the HCV core gene, were treated by 14-mers phosphorothioate ODN complementary to the HCV core genomic region. The variation of HCVmRNA level was detected by RT-PCR. Moreover, the inhibitory effect of asODN was observed in nude mice. HCVmRNA was detected in the transfected-QBC939 cells. The 14-mers complementary asODN showed effective inhibition on HCVmRNA and unexpression of HCVmRNA at 6 $\mu\text{mol/L}$. The tumorigenicity of transfected-QBC939 cells incubated with asODN in nude mice was greatly inhibited. These data have shown that ODNs directed against the HCV RNA specifically and significantly inhibit viral translation in vitro. Completely phosphorothioate-modified ODNs are currently undergoing phase I, II and III clinical trials for the translation inhibition of various RNAs such as cytomegalo virus

(CMV) in HIV patients. Our results suggest the feasibility of asODN in the treatment of HCV-infected cholangiocarcinoma.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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