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Inhibitor RNA blocks the protein translation mediated by hepatitis C virus internal ribosome entry site *in vivo*

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Abstract

AIM: To investigate the inhibitory effect of hepatitis C virus internal ribosome entry site (HCV IRES) specific inhibitor RNA (IRNA) on gene expression mediated by HCV IRES *in vivo*.

METHODS: By using G418 screening system, hepatoma cells constitutively expressing IRNA or mutant IRNA (mIRNA) were established and characterized, and HCV replicons containing the 5' untranslated region (5'UTR) were constructed by using the same method. Cotransfection of pCMVNCRluc containing HCV 5'UTR-luc fusion genes and eukaryotic vector of IRNA into human hepatic carcinoma cells (HepG2) was performed and the eukaryotic expression plasmid of IRNA was transfected transiently into HCV replicons. pCMVNCRluc or pCDNA-luc was cotransfected with pSV40-β Gal into IRNA expressing hepatoma cells by using lipofectamine 2000 *in vitro*. Then the reporting gene expression level was examined at 48 h after transfection by using a luminometer and the expressing level of HCV C antigen was analysed with a confocal microscope.

RESULTS: Transient expression of IRES specific IRNA could significantly inhibit the expression of reporter gene and viral antigen mediated by HCV IRES by 50% to 90% *in vivo*, but mIRNA lost its inhibitory activity completely. The luciferase gene expression mediated by HCV IRES was blocked in the HHCC constitutively expressing IRNA. At 48 h after transfection, the expression level of reportor gene descreased by 20%, but cap-dependent luciferase gene expression was not affected. IRNA could inhibit the HCV replicon expression 24 h after transfection and the highest inhibitory activity was 80% by 72 h, and the inhibitory activity was not increased until 7d after transfection.

CONCLUSION: IRNA can inhibit HCV IRES mediated gene expression *in vivo*.

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INTRODUCTION

Hepatitis C virus (HCV) is the primary causative agent of parenterally transmitted non-A, non-B hepatitis and affects a significant part of the world population. HCV infection frequence leads to chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma. The genome of HCV is a single-stranded, plus-polarity RNA. The 5' untranslated region (UTR) of HCV RNA is approximately 340 nt long, and contains multiple AUG codons. The 5' UTR is highly conserved among different strains of HCV. Nucleotides 40 to 370 of the 5' UTR of HCV have been shown to contain an internal ribosome entry site (IRES)[1-3]. The presence and stability of IRES play an important role in virus life cycle, so the region has become the target of antivial gene therapy[4-16]. Coward and Dasgupta found that gene expression mediated by polio virus (PV) IRES was inhibited by one 60 nt long RNA which is called inhibitor RNA (IRNA). Because HCV and PV IRES elements bound to similar polypeptides[16-20], it was reasoned that IRNA might also interfere with HCV IRES-mediated translation. Using transient transfection of hepatoma cells and a hepatoma cell line consititutively expressing IRNA, we demonstrated specific inhibition of HCV IRES-mediated translation by IRNA *in vivo*.

MATERIALS AND METHODS

Materials

Vectors pcRz-IRNA and pcRz-mIRNA were constructed by our laboratory, which introduced the sequences of 5' and 3' *cis*-self cleavage ribozyme into both sides of IRNA or mIRNA sequence[21]. pCMVNCRluc contain full sequence of HCV 5' UTR and 66 nt core gene, and was fused with lucferase gene (generous gift of professor Alt). pcHCVcluc was constructed by our laboratory containing full sequence of HCV 5' UTR and partial sequence of core region, and could express in cells stably.

Methods

Cell culture Human hepatocarcinoma cell (HHCC) HepG2 was grown in RPMI1640 medium supplemented with 100 mL/L newborn calf serum.

Plasmid construction By using subcloning methods, IRNA and mIRNA sequence were cloned into the pcDNA3 vector, yielding pcRz-IRNA and pcRz-mIRNA which introduced the ribozyme sequence over both sides of IRNA and mIRNA to generate the correct side of IRNA and mIRNA[16]. In brief, by using PCR methods the sequences of target RNA were generated from pGRz-IRNA or pGRz-mIRNA which was constructed by our laboratory. Then the PCR product was cloned into the *BamH*I-*Apa*I sites of the pcDNA3 vector.

Establishment of stable hepatoma cell line expressing IRNA or cloning HCV replicon Plasmids pcRz-IRNA, pcRz-mIRNA, pcDNA3 and pcHCVcluc were transfected into HHCC respectively by using Lipofectamine 2000 reagent (GIBCO) and screened for neomycin resistance with 300 μg/mL of geneticin (G418) (Invitrogen) per milliliter for 4 weeks. The antibiotic-resistant cell clones were harvested and further screened by dilution titer.

Detection of IRNA in cell lines IRNA or mIRNA expression in the cells was measured by isolating total RNA from these cells and IRNA or mIRNA were detected by reverse transcriptase (RT)-mediated PCR (RT-PCR) by using IRNA or mIRNA specific oligonucleotide primers. One to 2 μg of total RNA isolated from the IRNA or mIRNA expressing cells, and 2 μg total RNA from HHCC control cells were reversely transcribed by murine leukemia virus RT using random hexamer primers in 20 μL reaction mixture according to the TaKaRa RNA PCR kit protocol. Twenty pmol of each primer (corresponding to 5' nt 1 to 20 and 3' nt 1 to 20 of the IRNA or mIRNA sequence) was used to amplify the 60-nt fragement in 100 μl PCR reaction. The cycling parameters were as follows: denaturation at, 95 ℃ for 1 min, annealing at, 65 ℃ for 1 min, extrension at, 72 ℃ for 1 min, a total of 50 cycles, then total extrension at, 72 ℃ for 10 min. Twenty microliters of each reaction product were loaded onto 20 g/L gel and visualized by ethidium brominde staining.

Detection of HCV core protein expression in HHCC HCV core protein was detected by using indirect immune fluorescence method. HCV replicon cells were plated on a cover glass and fixed with pure ethanol for 10 min. Monoclonal antibody of HCV core protein was properly diluted (1:100) and covered on the glass with HCV replicon cells for 1 h at 37 ℃, and then the glass was washed 3 times with PBS (10 min each). Then FITC labeled second antibody was covered on the glass at 37 ℃ for 1 h and the glass was washed 3 times again with PBS. At last the cells were examined by using fluorescence microscopy or laser confocal microscopy.

DNA transfection For each transfection assay, 1×10^6 HHCC cells in 30-mm-diameter plates were transfected with 15 μL of lipofectin (GIBCO) and 2 to 5 μg of plasmid DNA. At 16 h post transfection, cell lysates were prepared according to the luciferase assay kit protocol (Promega) and assayed for both β-galactosidase (β-Gal) and luciferase expression.

RESULTS

Inhibitor effecty of IRNA transient expression on HCV IRES-mediated translation

To test the possibility that IRNA interfered with HCV IRES-mediated translation, human hepatocellular carcinoma cells (HepG2) were transiently cotransfected with three plasmids: a reporter gene expressing luciferase programmed by the HCV IRES element (pCMVNCRluc), pSV-β-galactosidase to measure transfection efficiency, and the plasmid expressing IRNA (pcRz-IRNA). All transfections were done in triplicate and contained equal amounts of the luciferase reporter and β-Gal plasmid. Increasing concentrations of plasmid pcRz-IRNA were used in various reactions, and the total amount of DNA in each reaction was kept constant by addition of an appropriate amount of a nonspecific DNA(pcDNA3). Following transfection, luciferase activity was measured in cell extracts at 48 h. At the lowest concentration of the IRNA plasmid, inhibition of luciferase activity from plasmid pCMVNCRluc was approximately 50% compared to the control. However, at the highest concentration, 92% of luciferase activity was inhibited. Translation of luciferase from a control plasmid (pCDNA-luc) without HCV IRES was not significantly inhibited by IRNA, (P>0.05,Table 1).

In order to determine the inhibitor effect of IRNA on HCV IRES-mediated translation further, the HHCCs were transfected with pCMVNCRluc or cotransfected with pcRz-IRNA and pCMVNCRluc. Following transfection, the HCV core protein programmed by HCV IRES was detected by using laser confocal microscopy. HCV core protein could express efficiently in the HHCC cells as shown in Figure 1. But IRNA plasmid cotransfection could inhibit HCV core protein expression. The pels density of HCV core protein was different between the two groups (58.05±42.24 *vs* 15.56±8.54). The inhibitory rate was plotted by 1- pels density of IRNA transfection group/pels density of control ×100%.

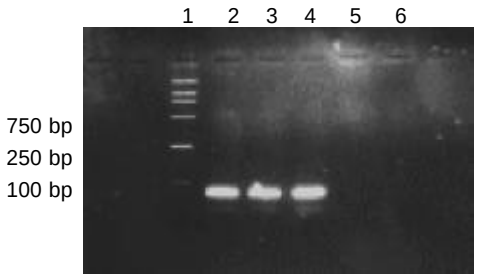

Figure 1 Stable expression of IRNA and mIRNA in HHCC cells. 1: DL2000 DNA marker; 2, 3: IRNA RT-PCR; 4: mIRNA RT–PCR; 5: IRNA PCR.

Construction of hepatoma cell line expressing IRNA or mIRNA

To determine the long-term effect of RNA expression in HepG2, the cell line constitutionally expressing IRNA was generated by using a pcDNA-based vector as described in Material and Methods. In order to obtain both the correct and stable sites of the expressed IRNA, ribozyme sequences were introduced into both sides of IRNA and mIRNA. IRNA or mIRNA was examined by RT-PCR using appropriate primers. IRNA and mIRNA were expressed stably in the stable cell lines as shown in Figure 2.

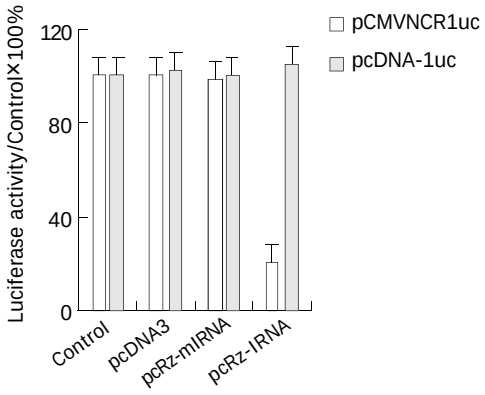

Figure 2 Inhibitory effect of IRNA on luciferase expression mediated by different mechanism.

Table 1 Inhibitory effect of IRNA on HCV IRES mediated gene translation

Sample pcRz-IRNA μg	Activity of luciferase programmed by cap-dependent mechanism (IU / U)	Activity of luciferase programmed by HCV IRES (IU / U)	Inhibitory rate (%)	
			pcDNA-luc	pCMVNCRluc
0	55.5±3.11	52.49±2.31		
2	60.1±2.31	27.0±0.740	0	50
4	58.3±1.89	14.7±0.380	0	72
6	52.4±2.12	4.03±0.120	4	92

HCV IRES-mediated gene expression in IRNA expressing HepG2 cells

IRNA or mIRNA expressing HepG2 cells and empty vector pcDNA3 expressing cells or control cells were cotransfected with pCMVNCRluc and transfection efficiency control plasmid pSV-β-Gal. At 48 h post-transfection cell extracts were used to measure the activities of both luciferase and β-galactosidase. The result was plotted as percent of control after normalized for β-Gal activity and protein concentration. The pcRz-IRNA cells showed approximately 80% inhibition of luciferase activity compared to the control. Both pcRz-mIRNA cells and pcDNA3 cells showed less than 5% inhibition activity. No significant inhibition of cap-dependent translation from the pCDNA-luc construct was observed in cell lines expressing IRNA, (P=0.05, Figure 3).

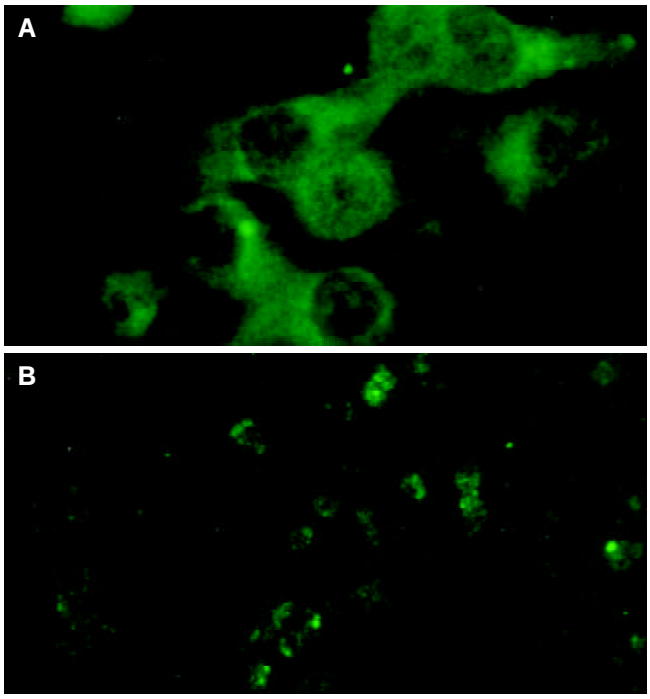

Figure 3 Inhibitory effect of IRNA on HCV Core expression mediated by HCV IRES(A: 40×10 *vs* B: 25×10). A: HCV Core protein of pCMVNCRluc transfection group, B: HCV Core protein of pcRz-IRNA and pCMVNCRluc cotransfection group.

Construction of HCV replicon containing HCV IRES

The results demonstrated that HCV core programmed by HCV IRES was positive in about 90% of HHCC cells (Figure 4).

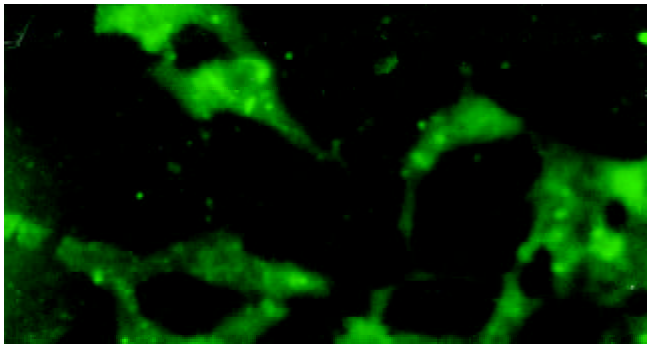

Figure 4 Detection of HCV core protein in HHCC lines stably expressing pcHCV Cluc (25×10).

Interference of IRNA with HCV replicon translation

To confirm the result obtained in IRNA expressing cells, HCV replicon containing HCV IRES was transfected with IRNA expressing plasmid, and luciferase activity was determined at different time following transfection. The result was that at 24 h HCV IRES-mediated luciferase gene translation decreases by 15% compared to the control HHCC cells, and along with time extending, the inhibitory effect of IRNA on HCV IRES-mediated luciferase gene translation increased and reached 80% at 72 h. On d 7, the inhibitory effect was still 80%. But mIRNA and nonspecific short RNA did not show any inhibitory effect on HCV IRES-mediated gene translation (Figure 5).

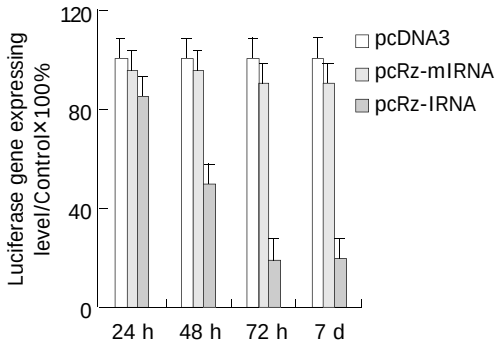

Figure 5 Interference of IRNA with HCV replicon translation.

DISCUSSION

IRES-dependent protein translation mechanism was first discovered in picornavirues, including PV, rhinovirus and hepatitis A virus, as well as certain flavivirues, such as hepatitis C virus[23-28]. Although there is very little sequence homology between these different IRES elements, structural similarity does appear to exist. In fact, in order to keep the activity of IRES, it was more important to maintain the secondary structure than to maintain the integrality of certain genome sequences[1-3,7,8]. IRES is the key structure for some viral RNA replication, so it has become the target for antiviral infection. We have constructed the self-cleavage plasmid of IRNA, and affirmed that IRNA can inhibit IRES-dependent protein translation *in vitro*[22]. In order to further confirm the effect of long-term expressing IRNA on cellular protein and viral protein translation, we established a HHCC line stably expressing IRNA, and confirmed that long-term expressing IRNA could significantly inhibit IRES mediated protein translation compared to the control cells and mIRNA expressing cells. Das *et al* prepared the human hepatoma (Huh-7) cell lines expressing IRNA by using the similar methods. They found that HCV IRES-mediated cap-independent translation was markedly inhibited in cells constitutively expressing IRNA compared to control hepatoma cells[29].

Alt *et al* designed the vector pCMVNCRluc fusing the luciferase gene to HCV core gene 66 nt site, and the gene expression was mediated by HCV 5' UTR, so we could dertermine the inhibitor effect of new strategies on HCV 5' UTR by examining the activity of luciferase. In this study, three plasmids pCMVNCRluc, pcRz-IRNA expressing IRNA and transfection efficiency control plasmid pSV-βGal were cotransfected into HHCC cells and luciferase activity (light units) was expressed as percentage of the control after normalized for β gal activity. When the effect of transfection efficiency and transient expression efficiency where excluded, the results of this study showed that IRNA could specifically inhibit HCV IRES mediated gene expression *in vivo*. The results of our study suggested that HCV 5' UTR-mediated translation was specifically inhibited by IRNA transient expression in hepatoma cells (50% to 92%), whereas cap-dependent translation of luciferase from the control plasmid

lack of HCV IRES element was not significantly affected by IRNA. To confirm the result obtained by using transient transfection, the vector containing HCV IRES was transfected into human hepatoma cells expressing IRNA constitutively and the results demonstrated that stably expressing IRNA could inhibit HCV IRES-mediated translation. By using a bicistronic construct containing CAT and luciferase genes flanked by the HCV 5' UTR Das *et al* found that IRNA could significantly inhibit HCV IRES-mediated gene expression *in vitro*. Further, they studied the IRNA effect *in vivo* and obtained the similar result to our study[29,30].

In order to determine the IRNA inhibitor effect *in vivo* further, we used the HCV replicon containing the full length of HCV 5' UTR to investigate the IRNA activity; the results demonstrated that IRNA could inhibit HCV 5' UTR mediated gene expression *in vivo*, but IRNA could not completely block HCV 5' UTR mediated gene expression.

To rule out the nonspecific effect of nonspecific short RNA regiment on HCV IRES –mediated gene expression, plasmids pCDNA3 and pCMVNCRluc were cotransfected into human hepatoma cells and the results showed that nonspecific RNA regiment didn' t have the inhibitor effect on HCV IRES-mediated translation.

In summary, IRNA can significantly inhibit HCV IRES-mediated translation.

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