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• VIRAL HEPATITIS •

Activity of HDV ribozymes to trans-cleave HCV RNA

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Abstact

AIM: **To explore whether HDV ribozymes have the ability to trans-cleave HCV RNA.**

METHODS: **Three HDV genomic ribozymes were designed and named RzC1, RzC2 and RzC3. The substrate RNA contained HCV RNA 5'-noncoding region and 5'-fragment of C region (5'-NCR-C). All the ribozymes and HCV RNA 5'-NCR-C were obtained by transcription in vitro from their DNA templates, and HCV RNA 5'-NCR-C was radiolabelled at its 5'-end. Under certain pH, temperature, appropriate concentration of Mg^{2+} and deionized formamide, these ribozymes were respectively or simultaneously mixed with HCV RNA 5'-NCR-C and reacted for a certain time. The trans-cleavage reaction was stopped at different time points, and the products were separated with polyacrylamide gel electrophoresis (PAGE), displayed by autoradiography. Percentage of trans-cleaved products was measured to indicate the activity of HDV ribozymes.**

RESULTS: **RzC1 and RzC2 could trans-cleave 26 % and 21.8 % of HCV RNA 5'-NCR-C under our reaction conditions with 2.5 mol·L^{-1} deionized formamide respectively. The percentage of HCV RNA 5'-NCR-C trans-cleaved by RzC1, RzC2 or combined usage of the three ribozymes increased with time, up to 24.9 %, 20.3 % and 37.3 % respectively at 90 min point. Almost no product from RzC3 was observed.**

CONCLUSION: **HDV ribozymes are able to trans-cleave specifically HCV RNA at certain sites under appropriate conditions, and combination of several ribozymes aiming at different target sites can trans-cleave the substrate more efficiently than using only one of them.**

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INTRODUCTION

Ribozymes are sorts of small RNAs which have catalytic activity, and some of them can bind specifically through Watson-Crick base pairs with and trans-cleave substrate RNA in appropriate target sites[1-4]. Hammerhead ribozymes and hairpin ribozymes have been tried widely to cleave many target RNAs, such as pathogenic microbes RNA[5-10], oncogene mRNA[11-15] and other kinds of pathogenity-associated functional mRNA[16-18]. A great deal of experiments have demonstrated that these ribozymes, if well-designed, could be applied as antiviral or antitumor gene therapeutic drugs. Yet there are still few researches showing whether HDV ribozymes, owning a kind of pseudoknot-like secondary structure, have the ability to kill pathogenic virus or not. HDV ribozymes include genomic ribozymes (g.Rz) and antigenomic ribozymes (ag.Rz) , of them the latter is duplicate intermediates of HDV[19,20]. This study was to evaluate the ability of g.Rz to trans-cleave HCV RNA at molecular levels.

MATERIALS AND METHODS

Reagents

All the cDNA of ribozymes were synthesized in a DNA synthesizer and purified with 160 g·L^{-1} denatured (7 mol·L^{-1} urea) polyacrylamide gel electrophoresis in Shanghai Sangon Bioengineering Company. Plasmid pHCV-neo was kindly provided by Dr. Wang working in our institute. Polymerase chain reaction (PCR) reagents and T7 transcription kit (Ribomax™) were purchased from Sino-American Bioengineering Company. Agarose Gel DNA Purification Kit, calf intestinal alkaline phosphatase (CIP) and T4 polynucleotide kinase were bought from Boehringer Mannheim Co. γ-^{32}P-ATP was the product of Beijing Yahui Co,and KODAK X-ray film was chosen to do autoradiography.

Preparation of template for transcription of substrate in vitro

pHCV-neo contains full length of HCV RNA 5'-NCR (341nt), translation-initiating codon AUG and 5'-fragment of C region (90nt). Sequence of HCV RNA 5'-NCR-C has been proved to be identical with that of HCV strains isolated from Chinese people reported by Bi et al. pHCV-neo has T7 phage promoter sequence ahead of HCV cDNA. Transcription template of HCV RNA 5'-NCR-C was prepared by the PCR method. The upper primer was T7 phage promoter sequence: 5'-TAA TAC GAC TCA TAG-3', and the reversed primer identified with the sequence from 413th to 383th nucleotide (nt) of HCV genome: 5'-GCG*GGATCC*CCGGAACTTGACGTCCTG-3' (the italic letter was cleavage site of BamH I designed for future cloning). Route PCR process was adopted. PCR products were purified by Agarose Gel DNA Purification Kit, and the unpaired A at 3' end was cut off with Klenow enzyme. All these procedures were made according to the kit guidebook. After that, PCR products were regained by phenol (pH8.0)/chloroform/ isoamyl alcohol (volume ratio 25:24:1) extraction, precipitated with ethanol, and redissolved with RNAase-free water.

Design of ribozymes and preparation of their transcription templates

Based on the structure of our formerly reportedg.Rz55, we designed here three kinds of HCV RNA-targetting g.Rzs named RzC1, RzC2 and RzC3 respectively. The target sequences and their location in HCV RNA 5'-NCR-C are shown in Table 1. Routine PCR process was adopted to prepare the transcription templates of the ribozymes. PCR templates were formerly synthesized cDNA of these ribozymes. The upper primer contained T7 phage promoter sequence (Italic letters): 5’-TAATACGACTCACTATAG TCTAGAGTCCCAGCCTCCTCGCTGGC-3’, and the reversed primer was 5’-CTCGGATCCGTCCCATTCGCCATTCCG AAGAATGTTGCCC-3’. Purification of PCR products and treatment of their 3’-end were the same as that mentioned above.

Table 1 Target sequences and their location in HCV RNA 5’-NCR-C

Target sequence	Location in HCV RNA 5’NCR-C	Ribozyme
5’…CGU ↓ GCAGCCU…3’	107-113 nt	RzC1
5’…GUU ↓ GGGUCGC…3’	268-274 nt	RzC2
5’…CAU ↓ GAGCACG…3’	345-351 nt	RzC3

Transcription in vitro

Each transcription reaction was done under 37 ℃ for 4 h, with a total volume of 20 μl, containing template DNA 1.5 μg, rNTP (preservation concentration 25 mmol·L^{-1}) 6 μl, 5× transcription buffer 4 μl, T7 RNA polymerase 2 μl (preservation concentration 1 μ·μl^{-1}). Digestion of DNA templates, purification and quantification of transcription products were executed according to the kit guidebook.

Radiolabel of substrate RNA at 5’-end

Phosphate at the 5’-end of substrate RNA was deleted with CIP, then the substrate RNA was radiolabelled at the 5’-end with T4 polynucleotide kinase and γ-^{32}P-ATP. After that, substrate RNA was purified by routine phenol (pH4.5)/ chloroform/ isoamyl alcohol (volume ratio 25:24:1) extraction, precipitated with ethanol, and redissolved with RNAase-free water and then quantitified, and stored at -20 ℃. All operations were made according to the guidelines.

Trans-cleavage reaction and measurement of effects-time relationship

Trans-cleavage reaction was done under conditions with or without deionized formamide having a final concentration of 2.5 mol·L^{-1}. Each reaction system contained radiolabelled substrate RNA 50 nmol·L^{-1}, ribozymes 5 μmol·L^{-1}, Tris.cl (pH7.5) 50 mmol·L^{-1}, with a total volume of 10 μL. The standard reaction protocol was as follows: heat the tube containing the reaction mixtures to 95 ℃ for 3 min → place on ice for 10 min → dip into 37 ℃ water for another 10 min → add prewarmed $MgCl_2$ to the final concentration of 20 mmol.L^{-1} → keep the reaction temperature at 37 ℃ for 2 h → separate reaction products by electrophoresis on a 80 g·L^{-1} polyacrylamide gel containing 7 mol·L^{-1} urea → display the products by autoradiography at -70 ℃ (the X-ray film was exposed for 0.5 μs beforehand) for about 24 h → measure A value of the images with Gel Documentation-Analyzing Systems (Gel Doc^{TM} 2000, Bio-Rad) and then calculate the percentage of substrate cleaved by the ribozymes.

Under the optimized cleavage conditions, following reactions were made in four tubes containing 15 μl mixtures each, of which three reactions were designed for each ribozyme to trans-cleave the target RNA,separately, and the fourth reaction for all the three ribozymes to trans-cleave the substrate RNA simultaneously and cooperatively. The reaction procedures were the same as that mentioned above, except that after $MgCl_2$ was added to the mixtures, the reactions were terminated at 10, 30 and 90 min time points. Five μL solution was removed from each tube at different time points for investigating the effects-time relationship.

Percentage of cleavage (%) =A value of cleaved substrate RNA / (A value of cleaved substrate RNA+A value of uncleaved substrate RNA)×100%

RESULTS

Effects of the ribozymes to trans-cleave substrate RNA under different conditions

Transcription templates of RzC1, RzC2, RzC3 and HCV RNA 5’-NCR-C were proven to be obtained successfully by 20 g·L^{-1} agarose gel electrophoresis. The ribozymes and substrate RNA were successfully synthesized by transcription *in vitro* known from 80 g·L^{-1} denatured (7 mol·L^{-1} urea) polyacrylamide gel electrophoresis.

According to the design, HCV RNA 5’-NCR-C should be trans-cleaved into 106nt (5’ product) and 324nt (3’ product) fragments by RzC1, 267nt and 163nt fragments by RzC2, and 344nt and 86nt fragments by RzC3. As HCV RNA 5’-NCR-C was radiolabelled at its 5’-end, only the 5’-products were displayed in X-ray films after autoradiography. The results showed that RzC1 and RzC2 are able to trans-cleave HCV RNA 5’-NCR-C under the selected reaction conditions, and the length of their cleavage products was set in accordance with the design. Percentage of substrate RNA cleaved by RzC1 and RzC2 under the conditions without deionized formamide at 2 h time point was 4.2 % and 3.5 %, respectively. With the addition of deionized formamide to the trans-cleavage reaction systems to a final concentration of 2.5 mol·L^{-1}, percentage of cleaved substrate RNA reached up to 26 % and 21.8 %, respectively at the same time point (Figure 1). It was surprising that under both conditions with or without deionized formamide, RzC3 almost had no trans-cleavage activity to HCV RNA 5’-NCR-C. Based on the whole experiment, we think that the reaction conditions with deionized formamide was better than that without it, thus being adopted in the future investigations.

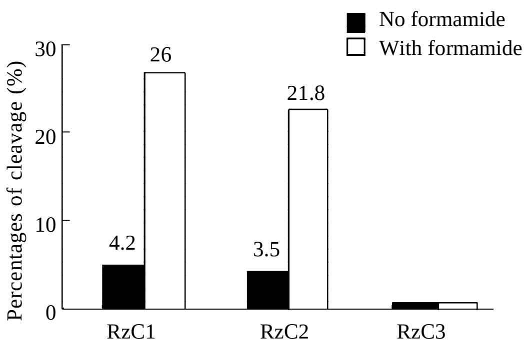


Figure 1 Effects of RzC1, RzC2 and RzC3 to trans-cleave HCV RNA 5’-NCR-C under different conditions (results came from image analysis).

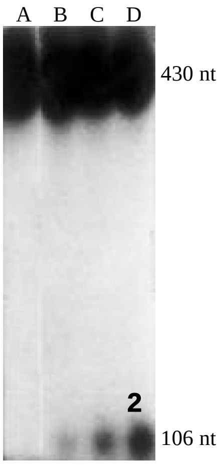
Roles of the three ribozymes to trans-cleave substrate RNA at different time points

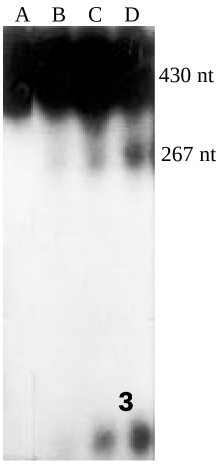
Under the optimized reaction conditions, fragments of HCV RNA 5’-NCR-C trans-cleaved by RzC1 and RzC2 were produced at the fixed time points (Figures 2 and 3). When the three ribozymes were added to one tube to trans-cleave HCV RNA 5’-NCR-C simultaneously, images of 106nt and 267nt fragments corresponding to the cleavage products of RzC1 and RzC2 respectively were observed in the same X-ray film (Figure 4), yet no marked image of 344nt fragment meaning the substrate RNA cleaved by RzC3 turned up at the same time.

Effects-time relationship of the ribozymes to trans-cleave substrate RNA

Percentage of cleaved substrate RNA by RzC1 and RzC2 was

6.1% and 4.6 % at 10 min time point, 14.0 % and 11.7 % at 30 min time point, and 24.9 % and 20.3 % at 90 min time point respectively. These results showed that the percentage of RzC1 and RzC2 to trans-cleave HCV RNA 5' -NCR-C increased with time. At the same time, nearly no effects of RzC3 to trans-cleave HCV RNA 5' -NCR-C was observed. The percentage of these ribozymes to trans-cleave HCV RNA 5' -NCR-C in one tube turned out to be 8.4 % at 10 min, 19.5 % at 30 min and 37.3 % at 90 min time point respectively (Figure 5). These results demonstrated that the combination of ribozymes aiming at different target sites could be applied to cleave substrate RNA more efficiently than using only one of them.





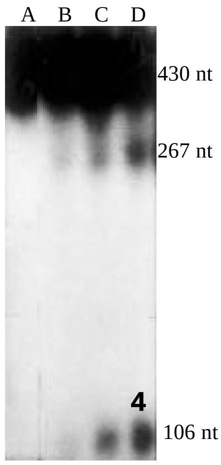


Figure 2 Results of trans-cleavage by RzC1. A: HCV RNA 5'-NCR-C in the system without ribozymes; B,C,D: Results of HCV RNA 5'-NCR-C trans-cleaved by RzC1 10, 30 and 90 min time points£¬ respectively.

Figure 3 Results of trans-cleavage by RzC2. A: HCV RNA 5'-NCR-C in the system without ribozymes; B,C,D: Results of HCV RNA 5'-NCR-C trans-cleaved by RzC2 at 10, 30 and 90 min time points,respectively.

Figure 4 Results of trans-cleavage by combination of RzC1, RzC2 and RzC3. A: HCV RNA 5'-NCR-C in the system without ribozymes; B,C,D: Results of HCV RNA 5'-NCR-C trans-cleaved by the ribozymes at 10, 30 and 90 min time points,respectively.

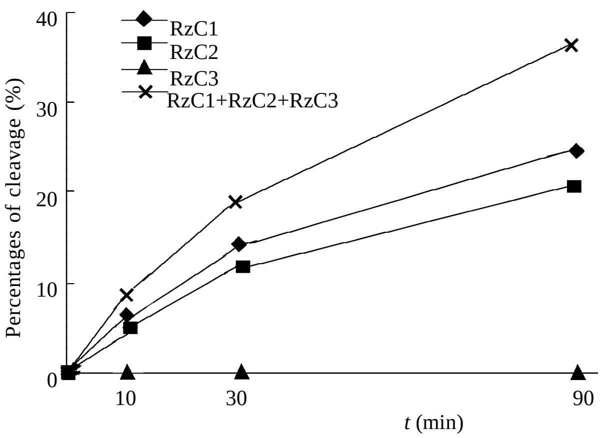


Figure 5 Effects-time relationship of RzC1, RzC2 and RzC3 to trans-cleave HCV RNA 5'-NCR-C.

DISCUSSION

HDV ribozymes were first reported for their self-cleavage activity, also named cis-cleavage, which meant splicing reaction occurring within the catalytic RNA molecule itself, i. e. intramolecular cleavage[19,21]. Several years later, it was found that a self-cleavage HDV ribozyme could also be divided into two parts, one part ("ribozyme" component) still maintained the catalytic activity and the other part (homologous "substrate" component) could be cleaved by the former intermolecularly when they were taken together again. This kind of intermolecular cleavage was called trans-cleavage, and the "ribozyme" components having trans-cleavage activity were usually described as trans-HDV ribozymes[22]. Our previous studies have shown that trans-HDV genomic ribozyme g.Rz55 was able to trans-cleave its homologous substrate S87 with a percentage of about 69 % under conditions Tris-Cl 50 mmol· L^{-1} (pH7.5) and $MgCl_2$ 20 mmol.L^{-1}[23]. In recent years, some researches have found that the substrate-binding region of trans-HDV ribozymes might be changeable to some extent[24]. These findings are the theoretical and experimental bases for reconstruction and application of HDV ribozymes to trans-cleave HCV RNA 5' -NCR-C.

Many studies have shown that HDV ribozymes owned a kind of special pseudoknot-like secondary structure which was different from that of hammerhead ribozymes and hairpin ribozymes[25-29], so did their requirements to the target sequences of substrate RNA[30-36]. Roy *et al*. reported that trans-ag.Rz was able to cleave 814nt HDAg mRNA efficiently at several target sites, and concluded that all the sequences having the characteristic of $5' \cdots R_{-4}R_{-3}R_{-2}\ Y_{-1} \downarrow G_1\ N_2\ N_3\ (A/C/U)_4\ N_5\ N_6\ N_7 \cdots 3'$ (R=A or G,Y=U or C) in HDAg mRNA might be the most possible sites to be cleaved by trans-ag.Rz[37]. The results are of significant value to design trans-g.Rz, because the secondary structure of g.Rz and ag.Rz is similar.

Five principles have been abided by during our selection of target sites of trans- g.Rz in HCV RNA 5' -NCR-C. Firstly, the target sequence was in accordance with the basic requirements of g.Rz, i.e., the first base G at 3' -end of the cleavage site was designed to form G· U wobble pair with the g.Rz. Secondly, the best first base at 5' -end of the cleavage site in substrate RNA was U. It should be noted that at any time G must not be selected when determine the first base at 5' -end of the cleavage site, and also it is necessary to avoid that more than two consecutive C located at 5' -end of the cleavage site. Thirdly, the more G-C or C-G base pair in the 7bp target sequences, the better for the ribozymes to bind the substrate. Fourthly, regions forming long intramolecular Watson-Crick base pairs in the reported HCV RNA 5' -NCR-C secondary structure were avoided as possible as it could. Finally, target sequences were located in the important functional regions of HCV RNA 5' -NCR-C such as internal ribosome entry site (IRES) and translation-initiating codon. These were the most important basic principles to design trans-HDV ribozymes and determine the target sites to be cleaved.

According to the theories and rules mentioned above, we tried to design trans-genomic ribozymes RzC1, RzC2 and RzC3 to cleave their heterologous substrate RNA, i.e. HCV RNA. The results showed that under the optimized cleaving reaction conditions, RzC1 and RzC2 exhibited the ability to trans-cleave HCV RNA 5' -NCR-C, and the percentage of cleaved substrate were increased with time, up to 24.9 % and 20.3 % respectively at 90 min time point. Combination of all these ribozymes resulted in 37.3 % substrate RNA to be cleaved at the same reaction conditions and time. From these results, we got to know that rationally designed trans-HDV ribozymes had the activity to cleave HCV RNA 5' -NCR-C which was the heterologous substrate to such ribozymes, and the cleavage effects could be improved by applying different trans-HDV ribozymes to cleave HCV RNA at multiple target sites simultaneously.

In comparison of the self-cleavage and trans-cleavage activity of HDV ribozymes at natural target sites in homologous substrate RNA and other target sites in HDV mRNA, the activity of RzC1 and RzC2 to trans-cleave HCV RNA 5' -NCR-C was a little lower, and RzC3 had almost no cleavage activity. By comparing the sequences and structure of RzC1, RzC2 and RzC3 to cis-HDV ribozymes and trans-HDV ribozymes reported by others, and the differences among the target RNAs, we suppose that the possible reasons may lie in several respects. Firstly, the target sites in HCV RNA 5' -NCR-C and the structure of trans-HDV ribozymes should be further optimized by more methods. For example, the calculation and application of the free energies required for forming pseudoknot and binding of ribozyme to substrate[38], or other strategies to map the accessible sites in substrate for ribozyme to bind[39,40]. Secondly, it is likely that the activity of trans-HDV ribozymes might be weakened if the A-U or U-A base pair just adjoined the G· U wobble pair when A-U or U-A and C-G or G-C base pairs co-existed in the binding regions of ribozymes and substrate RNA. Thirdly, the special secondary structure of HCV RNA 5' -NCR-C might impede the cleavage activity of trans-HDV ribozymes[31,41]. Fourthly, other sequences at both ends of trans-HDV ribozymes might diminish their cleavage activity[42]. Finally, different reaction conditions such as the kind of buffer might significantly influence the cleavage effects sometimes[43]. Certain denaturing agents such as deionized formamide in an appropriate concentration may reduce the formation of complex secondary structure of substrate RNA, thus improve the trans-cleavage activity to some extent[41]. A suitable pH of solution was very important for the trans-cleavage to occur too, and pH7.0-7.5 was commonly used during researches *in vitro*. Divalent cations such as Mg^{2+}, Ca^{2+} and Mn^{2+} could bind and interact with the ribozymes' phosphate-pentose skeleton full of negative charges[44,45], thus facilitating the ribozymes to fold into and maintain active structure, and/or take part in trans-cleavage reaction directly[46-49].

HCV is an important pathogenic factor of chronic hepatitis, and related to the formation of cirrhosis and occurrence of hepatocarcinoma or cholangiocarcinoma[50]. Finding new ways to control HCV infection is difficult but necessary. IRES and translation-initiating codon of HCV RNA are usually chosen as the trans-cleave target sequences not only because they are the important functional regions, but also they are very conservative in each HCV variant[51]. As a result, ribozymes aiming at the two regions will have a universal cleavage effects on all of the HCV variants. Effects of HDV ribozymes to trans-cleave HCV RNA 5' -NCR-C at extracellular molecular levels are not completely in accordance with that exhibited intracellularly, because intracellular conditions and factors that influence the ribozyme activity are far more complicated than the extracellular ones. On the other hand, HDV ribozymes are the only kind of viral ribozymes which exist in mammalian cells naturally, especially in human hepatocytes. Based on these opinions and facts, it is expected that the intracellular location and cleavage activity of HDV ribozymes might be more efficient than that of hammerhead ribozymes and hairpin ribozymes, thus it is necessary and valuable to assess the roles of HDV ribozymes in trans-cleaving HCV RNA intracellularly.

REFERENCES

1 **Curtis EA**, Bartel DP. The hammerhead cleavage reaction in monovalent cations. *RNA* 2001; **7**: 546-552
2 **Lilley DM**. Structure, folding and catalysis of the small nucleolytic ribozymes. *Curr Opin Struct Bio* 1999;**9**:330-338
3 **Tanner NK**. Ribozymes: The characteristic and properties of catalytic RNAs. *FEMS Microbio Rev* 1999;**33**:257-275
4 **Welch PJ**,Barber JR,Wong-Staal F.Expression of ribozymes in gene transfer systems to modulate target RNA levels. *Curr Opin Biotechnol* 1998;**9**:486-496
5 **Jia ZS**, Zhou YX, Lian JQ, Feng ZH, Li YG, Zhang WB. Computerized design of hepatitis C virus RNA-directed hammerhead ribozymes. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 300-302
6 **Li JG**, Zhou YX, Lian JQ, Jia ZS, Feng ZH. Inhibitory effect of ribozyme on HBeAg in human HCC cells. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 28-30
7 **Weinberg M**,Passman M,Kew M,Arbuthnot P.Hammerhead ribozyme-mediated inhibition of hepatitis B virus X gene expression in cultured cells.*J Hepatol* 2000;**33**:142-151
8 **de-Feyter R**, Li P. Technology evaluation: HIV ribozyme gene therapy, Gene Shears Pty Ltd. *Curr Opin Mol Ther* 2000; **2**: 332-335
9 **Cagnon L**, Rossi JJ. Downregulation of the CCR5 beta-chemokine receptor and inhibition of HIV-1 infection by stable VA1-ribozyme chimeric transcripts. *Antisense Nucleic Acid Drug Dev* 2000; **10**: 251-261
10 **Han S**, Wu Z, Yang H, Wang R, Yie Y, Xie L, Tien P. Ribozyme-mediated resistance to rice dwarf virus and the transgene silencing in the progeny of transgenic rice plants. *Transg Res* 2000; **9**: 195-203
11 **Zhang CS**, Wang WL, Peng WD, Hu PZ, Chai YB, Ma FC. Promotion of apoptosis of SMMC-7721 cells by Bcl-2 ribozyme. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 417-419
12 **Lui VW**, He Y, Huang L. Specific down-regulation of HER-2/neu mediated by a chimeric U6 hammerhead ribozyme results in growth inhibition of human ovarian carcinoma. *Mol Ther* 2001; **3**: 169-177
13 **Ludwig A**,Saretzki G,Holm PS,Tiemann F,Lorenz M, Emrich T, Harley CB, von-Zglinicki T. Ribozyme cleavage of telomerase mRNA sensitizes breast epithelial cells to inhibitors of topoisomerase. *Cancer Res* 2001;**61**:3053-3061
14 **Kijima H**, Scanlon KJ. Ribozyme as an approach for growth suppression of human pancreatic cancer. *Mol Biotechnol* 2000; **14**: 59-72
15 **Tokunaga T**, Tsuchida T, Kijima H, Okamoto K, Oshika Y, Sawa N, Ohnishi Y, Yamazaki H, Miura S, Ueyama Y, Nakamura M.Ribozyme-mediated inactivation of mutant K-ras oncogene in a colon cancer cell line. *Br J Cancer* 2000; **83**: 833-839
16 **Hu WY**, Fukuda N, Nakayama M, Kishioka H, Kanmatsuse K. Inhibition of vascular smooth muscle cell proliferation by DNA-RNA chimeric hammerhead ribozyme targeting to rat platelet-derived growth factor A-chain mRNA. *J Hypertens* 2001; **19**: 203-212
17 **Leavitt MC**,Yu G,Zhou C,Barber JR. Inhibition of interleukin-1beta (IL-1beta) production in human cells by ribozymes against IL-1beta and IL-1beta converting enzyme (ICE).*Antisense Nucleic Acid Drug Dev* 2000;**10**:409-414
18 **LaVail MM**, Yasumura D, Matthes MT, Drenser KA, Flannery JG, Lewin AS, Hauswirth WW. Ribozyme rescue of photoreceptor cells in P23H transgenic rats: long-term survival and late-stage therapy. *Proc Natl Acad Sci USA* 2000; **97**: 11488-11493
19 **Yu YC**, Gu CH, Mao Q, Li QF, Wang YM. *In vitro* self-cleavage activity of hepatitis delta virus ribozymes with different length and its significance. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 39-41
20 **Diegelman AM**, Kool ET. Mimicry of the hepatitis delta virus replication cycle mediated by synthetic circular oligodeoxynucleotides. *Chem Biol* 1999; **6**: 569-576
21 **Wadkins TS**, Shih I, Perrotta AT, Been MD.A pH-sensitive RNA tertiary interaction affects self-cleavage activity of the HDV ribozymes in the absence of added divalent metalion. *J Mol Biol* 2001; **305**: 1045-1055
22 **Shih IH**, Been MD. Ribozyme cleavage of a 2,5-phosphodiester linkage: mechanism and a restricted divalent metal-ion requirement. *RNA* 1999; **5**: 1140-1148
23 **Yu YC**, Gu CH, Mao Q, Li QF, Wang YM. Trans-cleavage

activity of hepatitis delta virus ribozymes. *J Med Coll PLA* 2000; **15**: 237-239
24 **Nishikawa F**,Roy M,Fauzi H,Nishikawa S.Detailed analysis of stem I and its 5' and 3' neighbor regions in the transacting HDV ribozyme.*Nucleic Acids Res* 1999; **27**: 403-410
25 **Wilson TJ**, Zhao ZY, Maxwell K, Kontogiannis L, Lilley DM. Importance of specific nucleotides in the folding of the natural form of the hairpin ribozyme. *Biochemistry* 2001; **40**: 2291-2302
26 **Pinard R**, Lambert D, Heckman JE, Esteban JA, Gundlach CW, Hampel KJ, Glick GD, Walter NG, Major F, Burke JM. The hairpin ribozyme substrate binding-domain: a highly constrained D-shaped conformation. *J Mol Biol* 2001; **307**: 51-65
27 **Zhao ZY**, Wilson TJ, Maxwell K, Lilley DM. The folding of the hairpin ribozyme: dependence on the loops and the junction. *RNA* 2000; **6**: 1833-1846
28 **Michiels PJ**,Schouten CH,Hilbers CW,Heus HA.Structure of the ribozyme substrate hairpin of Neurospora VS RNA: a close look at the cleavage site. *RNA* 2000; **6**: 1821-1832
29 **Fedor MJ**. Structure and function of the hairpin ribozyme. *J Mol Biol* 2000; **297**: 269-291
30 **Nakano S**,Chadalavada DM,Bevilacqua PC.General acid-base catalysis in the mechanism of a hepatitis delta virus ribozyme. *Science* 2000; **287**:1493-1497
31 **Ferre-D'Amare AR**, Doudna JA. Crystallization and structure determination of a hepatitis delta virus ribozyme: use of the RNA-binding protein U1A as a crystallization module. *J Mol Biol* 2000; **295**: 541-556
32 **Matysiak M**, Wrzesinski J, Ciesiolka J. Sequential folding of the genomic ribozyme of the hepatitis delta virus: structural analysis of RNA transcription intermediates. *J Mol Biol* 1999; **291**: 283-294
33 **Wadkins TS**,Perrotta AT, Ferre-D'Amare AR, Doudna JA, Been MD. A nested double pseudoknot is required for self-cleavage activity of both the genomic and antigenomic hepatitis delta virus ribozymes. *RNA* 1999; **5**: 720-727
34 **Mercure S**, Lafontaine D, Ananvoranich S, Perreault J. Kinetic analysis of delta ribozyme cleavage. *Biochemistry* 1998; **37**: 16975-16982
35 **Walter NG**, Chan PA, Hampel KJ, Millar DP, Burke JM. A base change in the catalytic core of the hairpin ribozyme perturbs function but not domain docking. *Biochemistry* 2001; **40**: 2580-2587
36 **Perez-Ruiz M**, Barroso-DelJesus A, Berzal-Herranz A. Specificity of the hairpin ribozyme. Sequence requirements surrounding the cleavage site. *J Biol Chem* 1999; **274**: 29376-29380
37 **Roy G**,Ananvoranich S,Perreault J.Delta ribozyme has the ability to cleave in trans an mRNA. *Nucleic Acids Res* 1999; **27**: 942-948
38 **Isambert H**, Siggia ED. Modeling RNA folding paths with pseudoknots: application to hepatitis delta virus ribozyme. *Proc Natl Acad Sci USA* 2000; **97**: 6515-620
39 **Wrzesinski J**, Legiewicz M, Ciesiolka J. Mapping of accessible sites for oligonucleotide hybridization on hepatitis delta virus ribozymes. *Nucleic Acids Res* 2000; **28**: 1785-1793
40 **Nyholm T**, Andang M, Bandholtz A, Maijgren C, Persson B, Hotchkiss G, Fehniger TE, Larsson S, Ahrlund-Richter L. Interaction between hammerhead ribozyme and RNA substrates measured by a surface plasmon resonance biosensor. *J Biochem Biophys Methods* 2000; **44**: 41-57
41 **Ferre-D'Amare AR**, Doudna JA. RNA folds: insights from recent crystal structures. *Annu Rev Biophys Biomol Struct* 1999; **28**: 57-73
42 **Chadalavada DM**, Knudsen SM,Nakano S,Bevilacqua PC. A role for upstream RNA structure in facilitating the catalytic fold of the genomic hepatitis delta virus ribozyme. *J Mol Biol* 2000; **301**: 349-367
43 **Perrotta AT**, Shih I, Been-MD. Imidazole rescue of a cytosine mutation in a self-cleaving ribozyme. *Science* 1999; **286**: 123-126
44 **Maderia M**, Hunsicker LM, DeRose VJ. Metal-phosphate interactions in the hammerhead ribozyme observed by 31P NMR and phosphorothioate substitutions. *Biochemistry* 2000; **39**: 12113-12120
45 **Butcher SE**, Allain FH, Feigon J. Determination of metal ion binding sites within the hairpin ribozyme domains by NMR. *Biochemistry* 2000; **39**: 2174-2182
46 **Wittberger D**,Berens C,Hammann C,Westhof E,Schroeder R. Evaluation of uranyl photocleavage as a probe to monitor ion binding and flexibility in RNAs. *J Mol Biol* 2000; **300**: 339-352
47 **Nishikawa F**,Nishikawa S. Requirement for canonical base pairing in the short pseudoknot structure of genomic hepatitis delta virus ribozyme.*Nucleic Acids Res* 2000;**28**:925-931
48 **Hunsicker LM**,DeRose VJ.Activities and relative affinities of divalent metals in unmodified and phosphorothioate-substituted hammerhead ribozymes. *J Inorg Biochem* 2000; **80**: 271-281
49 **Hammann C**, Cooper A, Lilley DM. Thermodynamics of ion-induced RNA folding in the hammerhead ribozyme: an isothermal titration calorimetric study. *Biochemistry* 2001; **40**: 1423-1429
50 **Liu XF**, Zou SQ, Qiu FZ. Construction of HCV-core gene vector and its expression in cholangiocarcinoma. *World J Gastroenterol* 2002;**8**:135-138
51 **Kruger M**, Beger C, Li QX, Welch PJ, Tritz R, Leavitt M, Barber JR, Wong-Staal F. Identification of eIF2B gamma and eIF2gamma as cofactors of hepatitis C virus internal ribosome entry site-mediated translation using a functional genomics approach. *Proc Natl Acad Sci USA* 2000; **97**: 8566-8571

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