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• BASIC RESEARCH •

Maxizyme-mediated specific inhibition on mutant-type p53 *in vitro*

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Abstract

AIM: To evaluate the specific inhibition of maxizyme directing against mutant-type p53 gene (mtp53) at codon 249 in exon 7 (AGG→AGT) *in vitro.*

METHODS: Two different monomers of anti-mtp53 maxizyme (maxizyme right MzR, maxizyme left MzL) and control mutant maxizyme ($G^5 \rightarrow A^5$) were designed by computer and cloned into vector pBSKU6 (pBSKU6MzR, pBSKU6MzL). After being sequenced, the restrictive endonuclease site in pBSKU6MzR was changed by PCR and then U6MzR was inserted into pBSKU6MzL, the recombinant vector was named pU6Mz and pU6asMz (mutant maxizyme). Mtp53 and wild-type p53 (wtp53) gene fragments were cloned into pGEM-T vector under the T7 promoter control. The ^{32}p-labeled mtp53 transcript was the target mRNA. Cold maxizyme transcripts were incubated with ^{32}p-labeled target RNA *in vitro* and radioautographed after denaturing polyacrylamide gel electrophoresis.

RESULTS: In cell-free systems, pU6Mz showed a specific cleavage activity against target mRNA at 37 ℃ and 25 mM $MgCL_2$. The cleavage efficiency of pU6Mz was 42 %, while pU6asMz had no inhibitory effect. Wtp53 was not cleaved by pU6Mz either.

CONCLUSION: pU6Mz had a specific catalytic activity against mtp53 in cell-free system. These lay a good fundation for studying the effects of anti-mtp53 maxizyme in HCC cell lines. The results suggest that maxizyme may be a promising alternative approach for treating hepatocellular carcinoma containing mtp53.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major causes of mortality worldwide[1,2]. Several risk factors are associated with the development of HCC including chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), exposure to genotoxic environmental agents such as aflatoxins. The high incidence of HCC has been observed in areas such as sub-saharan Africa, Thailand and the Southern region of China (Qidong) where concomitant infection occurs with HBV and high intake of aflatoxins[3-5]. Mutational inactivation of tumor suppressor gene p53 is very common in hepatocellular carcinoma. Indeed, p53 gene mutations, deletions or loss is a very important step during carcinogenesis and might participate in all stages of HCC development[6-9]. AGG to AGT transversion in codon 249 (the third base of codon 249) of exon 7 of p53 gene occurs in over 50 % of HCC from endemic regions, where both chronic infection with HBV and exposure to carcinogens such as aflatoxin B1(AFB1) prevail[10-13]. The p53 tumor suppressor gene product plays a crucial physiological role as a "cellular gatekeeper" by exerting a variety of effects following DNA damage[14,15]. Study of p53 as a tumor suppressor gene has attracted a large number of top scientists worldwide. But mutant p53 has drawn much less attention. Mutant p53 may not be an inactivated tumor suppressor gene, it appears to be one of the most prominent members of a new family of oncogenes[16,17]. Inactivation of p53 contributes not only to tumor progression but also to resistance of cancer cells to chemotherapy. Mutant p53 protein may possess transforming ability and can cooperate with other oncogenes in the transformation of normal cells. Mutant p53 protein has a prolonged halflife of 2 to 12 hours, resulting in higher intracellular concentrations than the wild-type protein[18,19]. Loss of ability to suppress transformation and gain of transforming potential and tumorigenicity are the properties of mutant p53 gene product.

Ribozyme is a kind of catalytic RNA which can catalyze the cleavage of sequence-specific RNA[20]. Among different types of ribozymes discovered, hammerhead ribozyme has been studied extensively for the treatment of disorders ranging from cancer to infectious disease[21-29]. However, because the limited number of cleavable sequences on target RNA, in some cases conventional ribozyme does not have precise cleavage specificity. This shortcoming may greatly limit the utility of hammerhead ribozyme[30,31]. A minizyme (minimised ribozyme) is a hammerhead ribozyme with short oligonucleotide linker instead of stem II. It has lower cleavage activity compared with that of wild-type parental hammerhead ribozyme[32,33]. Two minizymes could form an active dimeric structure. The dimmers can be homodimeric (with two identical binding sequences) or heterodimer (with two different binding sequences). In order to distinguish monomeric forms of conventional minizymes that have extremely low activity from novel heterodimer with high-level activity, the latter was designated as "maxizyme"[34-36]. Maxizyme stands for minimized, active, heterodimeric, and intelligent ribozyme. Some study showed that maxizyme could cleave chimeric genes, such as BCR-ABL mRNA, which cauases chonric myelogenous leukemia (CML)[37]. In this study, we designed maxizyme targeting mtp53 mRNA by computer and examined its cleavage activity *in vitro*.

MATERIALS AND METHODS

Materials

In vitro transcription kit and pGEM-T vector were purchased from Promega Company. Restriction endonucleases, T4 DNA ligase, RNase inhibition and DNA marker were purchased from TaKaRa Company. [α-^{32}p] dUTP was purchased from Beijing Yahui Company. *E.coli* DH5α was maintained in our laboratory. pBSKU6 vector was a generous gift from Dr.You-Xin Jin. pCMV-mtp53 (or wtp53) plasmid was kindly provided by Pro.Bert Vogelstein (Howard Hughs Medical Institute). Materials used were of analytical purity.

Maxizyme design

Maxizyme targeting mutant-type p53 (249 codon) was designed. Only after binding mutant-type p53 in codon 249 (the third base of 249 codon, AGG→AGT) can the maxizyme cleave mtp53 in 201bp site. The oligonucleotide sequences included Xba I and BamH I linker sites and were as follows: *MzL*: 5' CTA GAG AGG ATG GCT GAT GAG CGA AAG GTC TGG 3' ; 5' GAT CCC AGA CCT TTC GCT CAT CAG CCA TCC TCT3' . *MzR*: 5' CTA GAA GTT TCC ACT GAT GAG CGA AAC TCC GGG 3' ; 5' GAT CCC CGG AGT TTC GCT CAT CAG TGG AAA CTT 3' . A mutant maxizyme was designed with a sequence almost identical to that of the maxizyme except for G^5 to A^5 mutation within the catalytic core. This mutant maxizyme was expected to have no cleavage activity. They were chemically synthesized in Beckman Oligo 1000-DNA synthesizer. The structure of maxizyme against mtp53 is shown in Figure 1.

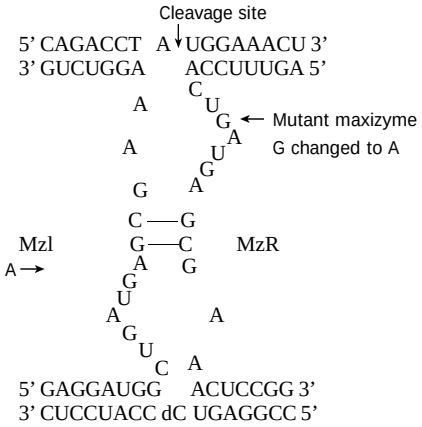

Figure 1 Structure of maxizyme against mtp53.

Methods

Construction of cell-free transcription plasmid for target RNA Mtp53 and wtp53 gene containing a cDNA fragment with 986 bases was amplified by PCR from pCMV-p53mt249 and pCMV-wtp53 vector. The PCR products were analyzed and purified by 2 % agarose gel and cloned into the transcription vector pGEM-T downstream of the T7 bacteriophage RNA promoter. The reconstructed plasmids were named as pmtp53 and pwtp53. The primers used were: upstream primer 5' GAT TCT CTT CCT CTG TGC 3' and downstream primer 5' CTT TCC ACG ACG GTG ACA 3' .

Construction of cell-free transcription plasmid for maxizyme Maxizyme targeting mtp53 was designed according to the computer software from professor Chen Nong-An (Shanghai Institute of Biochemistry of Chinese Academy of Sciences). The homologous possibility with the gene of humanbeing was excluded by consulting with RNA sequences of human cell from NCBI Genebank. The *in vitro* transcription vector pBSKU6 was digested with *Xba* I and *BamH* I restriction enzymes. The linearlized vector pBSKU6 was purified by 1 % agarose gel electrophoresis. After annealed, oligonucleotides of MzL and MzR were cloned into pBSKU6. The reconstructed plasmids were named as pBSKU6MzL and pBSKU6MzR. After being sequenced, the restrictive endonuclease site in pBSKU6MzR was changed by PCR and then U6MzR was inserted into pBSKU6MzL, the recombinant was named as pU6Mz. pBSKU6 vector contained the T7 RNA polymerase promoter for driving transcription of Mz *in vitro*. The steps of pU6Mz construction was shown in Figure 2.

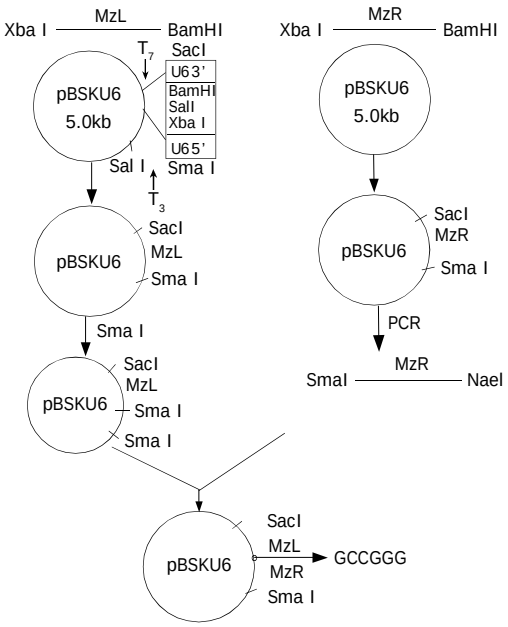

Figure 2 Steps of pU6Mz construction.

***In vitro* transcription of target RNA and maxizyme** Templates pmtp53 and pwtp53 were linearlized with Sal, while pU6Mz was linearlized with Sma I. The linearlized templates were transcribed in the presence of [α-^{32}p]dUTP (10 μl) using T7 RNA polymerase according to the manufacturer's protocol. Transcription was performed for three hours at 37 ℃ in a 50 μl final volume. The samples were purified by cutting off the autoradiograph bands after running on 8 % polyacrylamide (8 mol/l urea) gel and soaking in NES (0.5 M NH_4Ac, 0.1M EDTA, 0.1 % SDS pH 5.4) at 42 ℃ overnight. The products were precipitated by ethanol, washed once by 70 % ethanol, dissolved in DEPC-treated water and kept at -20 ℃.

***In vitro* cleavage reaction** The products of maxizyme and target RNA were quantified by measuring their radioactive cpm in 1 ul solution. The cleavage reaction was performed for 90 minutes at 37 ℃ in 25 mmol/l Tris-Cl (pH7.5) and 25 mmol/l $MgCl_2$ with cold maxizyme to [α-^{32}p] dUTP-labeled substrate ratio of 4:1 and stopped by adding 1 ul RNA loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 20 mmol/l EDTA and saturated urea) and heated for 3 minutes at 95 ℃. The cleaved products were separated by 8 % polyacrylamide gel electrophoresis(PAGE) with 8M urea. The cleavage efficiency was calculated from cpm values of the bands of substrates (S) and products (P): cleavage efficiency=[P/(S+P)]×100 %.

RESULTS

Construction of cloning vector pU6Mz

Mz gene was successfully cloned into the vector pBSKU6. The gene complex was treated with restriction endonuclease *Sma* I and *Sac* I and analyzed by 2 % agarose gel (Figure 3). The DNA sequence analysis showed that chimeric U6 maxizyme was correct.

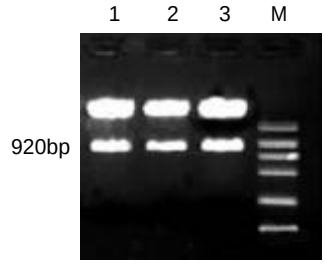

Figure 3 Restrictive enzyme analysis of recombinant plasmid pU6Mz by Sma I and Sac I. M was DNA Marker (DL2000). 1, 2, 3 were selected clones.

Identification of transcripts of pU6Mz and target RNA

The lengths of RNA transcribed from Sal I-linearlized templates of pmtp53 and pwtp53 shoud be 1002nt (Figure 4A). The transcription of pU6Mz from Sma I-linearlized templates including U6 and Mz bases shoud be 910nt (Figure 4B). The results showed that our design was correct.

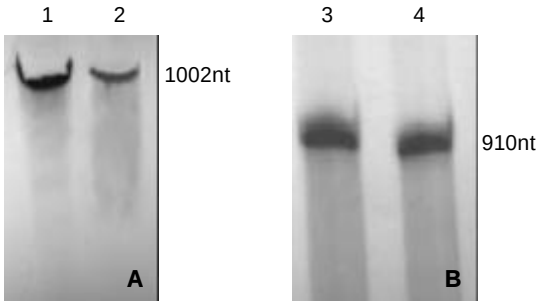

Figure 4 *In vitro* transcripts. (A) *In vitro* transcripts of wtp53 and mtp53 (1002nt). (B) *In vitro* transcripts of maxizyme and control maxizyme (910nt). lane 1: mtp53; lane 2: wtp53; lane 3: maxizyme; lane 4: control maxizyme.

In vitro cleavage reaction of Mz

Target RNA was ^{32}p-labeled. The cleavage results showed that the designed maxizyme had correct structure, it cleaved mtp53 mRNA efficiently, giving cleavage products with the expected size of 874nt and 128nt under conditions at 37 ℃ and high Mg concentration (25 mM), the cleavage efficiency was 42 %. More encouraging was that no cleavage was found with mutant maxizyme under the same conditions, and wtp53 was not cleaved by maxizyme (Figure 5).

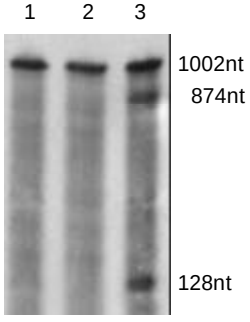

Figure 5 *In vitro* cleavage reaction. lane 1: cleavage of wtp53 by cold maxizyme; lane 2: cleavage of mtp53 by inactive cold maxizyme; lane 3: cleavage of mtp53 by cold maxizyme.

DISCUSSION

Ribozyme is a class of small catalytic RNA molecules that recognize specific substrate RNA by their complementary nucleotide sequence, and cleave the substrate RNA as an endoribonuclease at enzymatic rate. It has been demonstrated that potential utility in attenuating eukaryotic gene expression was studied in preclinical gene therapy models. Among different types of ribozymes discovered, the hammerhead ribozyme has received a great deal of attention in recent years because of its application in treatment of malignant and infectious diseases[38,39]. Many different hammerhead ribozymes targeting oncogene and HIV, HBV have been developed. The hammerhead ribozyme could cleave almost any RNA molecules containing the 3 base target recognition sequence NUX (N=any base, X is any nucleotide except G). The basic structure of a hammerhead ribozyme is composed of a catalytic core of 24 conserved bases containing helix II and two self-associating helices, I and III which are antisense to the substrate and position of ribozyme to catalyze cleavage. However, because the limited number of cleavable sequences on target RNA, in some cases conventional hammerhead ribozyme does not have precise cleavage specificity. Some scientists found that the stem II (loop II) region of hammerhead ribozyme did not appear to be directly involved in catalysis. For development of chemically systhesized ribozyme as potential therapeutic agent, it would certainly be advantageous to remove surplus nucleotides. This consideration led to the production of minizymes, which are conventional hammerhead ribozyme with a deleted stem II region. But the activities of minizyme are two or three orders of magnitude lower than those of the parental hammerhead ribozyme, suggesting that minizymes might not be suitable as gene inactivating reagents. But Kinetic and NMR analysis indicated that the minizyme was essentially inactive as a monomer but exhibited strong catalytic activity as a dimer. This dimeric structure is called maxizyme[40-42]. The maxizyme is also a metalloenzyme, its activity depends on the presence or absence of the correct formation of Mg ion-binding pocket. The maxizyme could bind to two different substrate-binding sites, one substrate-binding site functions as the "eye" that identifies the specific mRNA, wheareas the other serves as the "scissors" and cleaves the target mRNA. Some studies showed that only after binding to the target gene can the maxizyme form a cavity that captures the Mg ions.

As a novel class of ribozyme, maxizyme that targets different chimeric genes has been studied[43,45]. The chimeric gene is generated as a result of reciprocal chromosomal translocation. A well known chimeric gene is BCR-ABL gene which causes chronic myelogenous leukemia(CML). In the design of ribozyme that might cleave chimeric mRNA, it is necessary to avoid the cleavage of normal mRNA. There have been many attempts to specifically cleave chimeric BCR-ABL gene using ribozymes, but it is very difficult to cleave only chimeric gene without affecting the normal genes, such as BCR or ABL gene. Kuwabara *et al*[44] designed a maxizyme directly against BCR-ABL mRNA. The results showed that the maxizyme had extremely high specificity and high level activity not only *in vitro* but also in cultured cells. p53 mutation in 249 codon was observed in 50 % of hepatocellular carcinoma. Some studies have suggested that p53 has a gain of transforming function after mutation in addition to loss of tumor suppressor activity. Several groups have attempted to develop gene therapy methods to treat HCC via introduction of wild-type p53 cDNA into cancer cells. Unfortunately, these approaches did not result in regulated expression of p53 gene and did not reduce the expression of mutant p53 that was overexpressed in HCC cells. These shortcomings may greatly limit the utility of this gene replacement approach.

We designed a maxizyme directing against mutant-type p53 (249 codon). The cleavage results showed that the maxizyme cleaved mtp53 target mRNA efficiently. More encouraging

was that no cleavage was found in wild-type p53 under the same conditions. The activity of maxizyme must have originated from the formation of active heterodimers. So control of inactive maxizyme was also made by the mutation of a functionally indispensable ($G^5 \rightarrow A^5$) in the catalytic core. The *in vitro* cleavage reaction showed that the inactive maxizyme had no effects on target mRNA. This suggests that the cleavage effects of active maxizyme are clearly originated from the chemical cleavage. For the application of maxizyme to gene therapy, they must be expressed *in vivo* under the control of a strong promoter. Development of an efficient system for the expression of a small piece of RNA in the cell, such as antisense RNA and ribozyme, is a major challenge in nonviral gene therapy. Recently, U6 RNA has been explored to drive the expression of antisense RNA and oligonucleotide. It is much efficient than the CMV promoter which is used often[46,47]. In our study, the U6 expression system was explored for the construction of maxizyme plasmid. These lay a good fundation for the study in HCC cell lines.

In conclusion, the results of the present study showed that chimeric U6 maxizyme could cleave mtp53 mRNA *in vitro* with high efficiency. Anti-mtp53 maxizyme may be a promising tool for the treatment of hepatocellular carcinoma with an oncogenic mutation in codon 249 of p53 gene.

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