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

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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\(^3,4\). 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\(^5-9\). 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\(^10,11\). The p53 tumor suppressor gene product plays a crucial physiological role as a “cellular gatekeeper” by exerting a variety of effects following DNA damage\(^12,13\). 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\(^21-25\). However, because of 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\(^26,27\). 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\(^28,29\). 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”\(^30-32\). Maxizyme stands for minimized, active, heterodimeric, and intelligent ribozyme. Some study showed that maxizyme could cleave chimeric sequences, such as BCR-ABL mRNA, which causes chronic myelogenous leukemia (CML)\(^33\). In this study, we designed maxizyme targeting mtp53 mRNA by computer and examined its cleavage activity in vitro.

METHODS: Two different monomers of anti-mtp53 maxizyme (maxizyme right MzR, maxizyme left MzL) and control mutant maxizyme (G\(^\rightarrow\)A) 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 (pU6asMz). Mtp53 and wild-type p53 (wtp53) gene fragments were cloned into pGEM-T vector under the T7 promoter control. Mtp53 and wtp53 were transcribed in vitro using T7 RNA polymerase. The 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 °C 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 foundation 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.

## Basic Research

### 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→A) 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 (pU6asMz). Mtp53 and wild-type p53 (wtp53) gene fragments were cloned into pGEM-T vector under the T7 promoter control.

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**CONCLUSION:** pU6Mz had a specific catalytic activity against mtp53 in cell-free system. These lay a good foundation 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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[1] AGT, J.I., et al., 1989. Nature, 342(6245), 847-849.

[2] World J Gastroenterol 2003;9(7):1571-1575

[3] World Journal of Gastroenterology
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-32p]dUTP was purchased from Beijing Yahuai 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 Hughes 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’ AAG ACA GTT TCC ACT GAT 3’; MzR: 5’ CTA GAA GTT TCC ACT GAT 3’. A mutant maxizyme was designed with a sequence almost identical to that of the maxizyme except for G to A 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.

Methods

Construction of cell-free transcription plasmid for target RNA

Templates pmt53 and pwtp53 were linearized with SalI, while pU6Mz was linearized with SmalI. The linearized templates were transcribed in the presence of [α-32p]dUTP (10 µl) using T7 RNA polymerase according to the manufacturer’s protocol. Transcription was performed for three hours at 37 °C in a 50 µl final volume. The samples were purified by cutting off the autoradiograph bands after running on 8 % polyacrylamide gel electrophoresis (PAGE) with 8M urea. 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 °C in 25 mmol/l Tris-Cl (pH7.5) and 25 mmol/l MgCl2, with cold maxizyme to [α-32p]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 °C. 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 SmalI and SacI and analyzed by 2 % agarose gel (Figure 3). The DNA sequence analysis showed that chimeric U6 maxizyme was correct.
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. 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 synthesized 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 parent 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. 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, whereas 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. 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. 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→A) 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[4,6]. 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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REFERENCES

1. Tang ZY. Hepatocellular carcinoma-cause, treatment and metastasis. World J Gastroenterol 2001; 7: 445-454
2. Tang ZY. Advances in clinical research of hepatocellular carcinoma in China. Huaren Xiaohuazhizhi 1998; 6: 1013-1016
3. Lin NF. Tang J, Ismad HS. Study on environmental etiology of high incidence areas of liver cancer in China. World J Gastroenterol 2000; 6: 572-576
4. Zhai SH, Liu JB, Liu YM, Zhang LL, Du ZH. Expression of HBSAg, HCV-Ag and AFP in liver cirrhosis and hepatocarcinoma. Shijie Huaren Xiaohuazhizhi 2000; 8: 524-527
5. Li Y, Tang ZY, Ye SL, Lin YK, Chen J, Gao DM, Bao WH. Establishment of cell clones with different metastatic potential from the metastatic hepatocellular carcinoma cell line MHCC97L. World J Gastroenterol 2001; 7: 630-636
6. Qin LL, Su J, Li Y, Yang C, Ban KC, Yuan RQ. Expression of IGF-II, p53, p21 and HBsAg in precancerous events of hepatocarcinogenesis induced by ABF1 and/or HBV in tree shrews. World J Gastroenterol 2000; 6: 138-139
7. Blandino G, Levine AJ, Oren M. Mutant p53 gain of function: differential effects of different p53 mutants on resistance of cultured cells to chemotherapy. Oncogene 1999; 18: 477-485
8. Sohn S, Jaitovitch-Groisman I, Benlilamme N, Galipou J, Batist G, Alouai-jamal MA. Retroviral expression of the hepatitis B virus x gene promotes liver cell susceptibility to carcinogen-induced site-specific mutagenesis. M utation Res 2000; 460: 17-28
9. Ghebranious N, Selm S. Hepatitis B injury, male gender, aflatoxin, and p53 expression each contribute to hepatocarcinogenesis in transgenic mice. Hepatology 1998; 27: 383-391
10. Bressac B, Kew M, Wands J, Ozurtak M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. Nature 1991; 350: 429-431
11. Prost S, Ford JM, Taylor C, Doig J, Harrison DJ. Hepatitis B x protein inhibits p53-dependent DNA repair in primary mouse hepatocytes. J Bio Chem 1998; 273: 33327-33332
12. Liu H, Wang Y, Zhou Q, Gu SY, Li X. The point mutation of p53 gene exon 7 in hepatocellular carcinoma from Anhui Province, a non HCC prevalent area in China. World J Gastroenterol 2002; 8: 480-482
13. Niu ZS, Li BK, Wang M. Expression of p53 and C-myc genes and its clinical relevance in the hepatocellular carcinomatous and pericarcinomatous tissues. World J Gastroenterol 2002; 8: 822-826
14. Levine AJ. p53, the cellular gatekeeper for growth and division. Cell 1997; 88: 323-331
15. Qin LX, Tang ZY, Ma ZC, Wu QZ, Zhou XD, Ye QH, Ji Y, Huang LW, Jia HL, Sun HC, Wang L. p53 immunohistochemical scoring: an independent prognostic marker for patients after hepatocellular carcinoma resection. World J Gastroenterol 2002; 8: 459-463
16. Doppert W, Gohler T, Koga H, Kom E. Mutant p53: ‘gain of function’ through perturbation of nuclear structure and function? J Cell Biochem Suppl 2000; 35(Suppl): 115-122
17. Lee YL, Lee S, Das GC, Park US, Park SM, Lee YL. Activation of the insulin-like growth factor II translocation by aflatoxin B1 induced mutant p53 249 is caused by activation of transcription complexes; implications for a gain-of-function during the formation of hepatocellular carcinoma. Oncogene 2000; 19: 3717-3726
18. Koga H, Doppert W. Identification of genomic DNA sequences bound by mutant p53 protein(Gly245→Ser) in vivo. Oncogene 2000; 19: 4178-4183
19. Zheng SX, Liu LJ, Shao YS, Zheng QP, Ruan YB, Wu ZB. Relationship between ras p53 gene RNA and protein expression and HCC metastasis. Huaren XIAOAHUAZHI 1998; 6: 104-105
20. Phylactou LA. Ribozyme and peptide-nucleic acid-based gene therapy. Adv Drug Deliv Rev 2000; 44: 97-106
21. Song YH, Lin JS, Liu NZ, Kong XJ, Xie N, Wang NX, Jin YX, Liang KH. Anti-HBV hairpin ribozyme-mediated cleavage of target RNA in vitro. World J Gastroenterol 2002; 8: 91-94
22. Tanaka M, Kijima H, Itoh J, Matsuda T, Tanaka T. Impaired expression of a human septin family gene Bradeion inhibits the activities of dimeric hammerhead minizymes. J Bio Chem 2000; 275: 13332-13337
23. Tokunaga T, Abe Y, Tsuchide T, Hatanaka H, Oshika Y, Tomisawa M, Yoshimura M, Ohtsuki Y, Kijima H, Yanazaki H, Ueyama Y, Nakamura M. Ribozyme-mediated cleavage of a catalytic core. Association between ras p53 gene RNA and protein expression and hepatocellular carcinoma. Shijie Huaren Xiaohuazhizhi 2000; 8: 524-527
24. Qu Y, Liu S, Liu B. Attenuation of telomerase activity by hammerhead ribozyme targeting the 5′-end of hTERT mRNA. Zhonghua Yixue Yichuangxue Zazhi 2002; 19: 389-392
25. Blount LF, Grover NL, Molker V, Belderman L, Uhlenbeck OC. Steric interference modification of the hammerhead ribozyme. Nucleic Acids Res 2002; 30: 2274-2281
26. Huesker M, Mor J, Schmidt M, Fulda C, Blum HE, Hafkemeyer P. Reversal of drug resistance of hepatocellular carcinoma cell lines by adenoviral delivery of anti-MDR1 ribozymes. Hepatology 2002; 36: 874-884
27. Sullenger BA, Glibbo E. Emerging clinical applications of RNA. Nature 2002; 418: 252-258
28. Xu R, Lin J, Zhou X, Xie Q, Jin Y, Yu H, Liao D. Activity identification of anti-caspase-3 mRNA hammerhead ribozyme in both cell-free condition and BRL-3A cells. Chinese Medical Journal(Engl) 2001; 114: 606-611
29. Yu YC, Mao Q, Gu CH, Li OF, Wang YM. Activity of HDV ribozymes to trans-cleave HCV RNA. World J Gastroenterol 2002; 8: 694-698
30. Zhang YA, Nemunaitis J, Tong AW. Generation of a ribozyme-adenoviral vector against k-ras mutant human lung cancer cells. Mol Biotechnol 2000; 15: 39-49
31. Hammad C, Lilley DM. Folding and activity of the hammerhead ribozyme. ChemBiochem 2002; 3: 690-700
32. Sioud M, Opstad A, Hendry P, Lockett TJ, Jennings PA, McCall MJ. A minimised hammerhead ribozyme with activity against interferlin-2 in human cells. Biochem Biophys Res Comm 1997; 231: 397-402
33. Amontov S, Nishioka S, Taira K. Dependence on Mg2+ and RNA of expression of a human septin family gene Bradeion inhibits the activities of dimeric hammerhead minizymes. FEBS Lett 1996; 386: 99-102
34. Iyo M, Kawasaki H, Taira K. Allosterically controllable ribozyme. J Cell Biochem Suppl 2000; 35(Suppl): 115-122
35. Tani K. Target therapy for CML-applying ribozyme. Nippou Rinsho 2001; 59: 2439-2444
Kuwabara T, Hamada M, Warashina M, Taira K. Allosterically controlled single-chained maxizymes with extremely high and specific activity. Biomacromolecules 2001; 2: 788-799

Kuwabara T, Tanabe T, Warashina M, Xiong KX, Tani K, Taira K, Asano S. Allosterically controllable maxizyme-mediated suppression of progression of leukemia in mice. Biomacromolecules 2001; 2: 1220-1228

Morino F, Tokunaga T, Tsuchida T, Handa A, Nagata J, Tomii Y, Kijima H, Yamazaki H, Watanabe N, Matsuzaki S, Ueyama Y, Nakamura M. Hammerhead ribozyme specifically inhibits vascular endothelial growth factor gene expression in a human hepatocellular carcinoma cell line. Int J Oncol 2000; 17: 495-499

Ozaki I, Zern MA, Liu S, Wei DL, Pomerantz RJ, Duan L. Ribozyme-mediated specific gene replacement of the alphal-antitrypsin gene in human hepatoma cells. J Hepatol 1999; 31: 53-60

Kuwabara T, Warashina M, Taira K. Cleavage of an inaccessible site by the maxizyme with two independent binding arms: an alternative approach to the recruitment of RNA helicases. J Biochem (Tokyo) 2002; 132: 149-155

Kuwabara T, Warashina M, Taira K. Allosterically controllable maxizymes cleave mRNA with high efficiency and specificity. Trends Biotechnol 2000; 18: 462-468

Nakayama A, Warashina M, Kuwabara T, Taira K. Effects of cetyltrimethylammonium bromide on reactions catalyzed by maxizymes, a novel class of metalloenzymes. J Inorg Biochem 2000; 78: 69-77

Hamada M, Kuwabara T, Warashina M, Nakayama A, Taira K. Specificity of novel allosterically trans- and cis-activated connected maxizymes that are designed to suppress BCR-ABL expression. FEBS Lett 1999; 461: 77-85

Kuwabara T, Warashina M, Tanabe T, Tani K, Asano S, Taira K. A novel allosterically trans-activated ribozyme, the maxizyme, with exceptional specificity in vitro and in vivo. Mol Cell 1998; 2: 617-627

Suwanai H, Matsushita H, Kobayashi H, Ikeda Y, Kizaki M. A novel therapeutic technology of specific RNA inhibition for acute promyelocytic leukemia: improved design of maxizymes against PML/RALpha mRNA. Int J Oncol 2002; 20: 127-130

Kuwabara T, Warashina M, Orita M, Koseki S, Ohkawa J, Taira K. Formation of a catalytically active dimer by tRNA(Val)-driven short ribozymes. Nat Biotechnol 1998; 16: 961-965

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 2003; 3: 169-177

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