

Synthesis of ribozyme against vascular endothelial growth factor₁₆₅ and its biological activity *in vitro*

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Abstract

AIM: To investigate the designation, synthesis and biological activity of against vascular endothelial growth factor₁₆₅ (VEGF₁₆₅) ribozyme.

METHODS: The ribozyme against VEGF₁₆₅ was designed with computer. The transcriptional vector was constructed which included the anti-VEGF₁₆₅ ribozyme and 5', 3' self-splicing ribozymes. The hammerhead ribozyme and substrate VEGF₁₆₅ mRNA were synthesized through transcription *in vitro*. The cleavage activity of the ribozyme on target RNA was observed in a cell-free system.

RESULTS: The anti-VEGF₁₆₅ ribozyme was released properly from the transcription of pGEMRz212 cleaved by 5' and 3' self-splicing ribozymes which retained its catalytic activity, and the cleavage efficiency of ribozyme reached 90.7%.

CONCLUSION: The anti-VEGF₁₆₅ ribozyme designed with computer can cleave VEGF₁₆₅ mRNA effectively.

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INTRODUCTION

Ribozyme (Rz) is one kind of RNA with site-specific ligation and cleavage activities. Being sequence-specific binding and cleaving specific RNA of ribozyme, the target gene expression can be destructed by an artificially designed and synthesized ribozyme^[1-5]. A great attention has been attracted into the field of gene therapy for cancers with the hammerhead ribozyme, by the virtue of its simple structure, small molecule, easy designation, site-specific mRNA cleavage activity, and catalytic potential^[6-13]. Many studies have showed that the growth, metastasis and prognosis of solid tumors critically related to the angiogenesis. Tumor angiogenesis is a complex process. Among all of the known factors of tumor angiogenesis, vascular endothelial growth factor (VEGF) is a vascular endothelial cell-specific mitogen and the most important and direct one that can stimulate

tumor angiogenesis. VEGF₁₆₅ is the most effective angiogenic factor in the VEGF family^[14-22]. Conversely, inhibition of VEGF expression and tumor angiogenesis to inhibit tumor growth and metastasis become a new hot spot of tumor treatment^[23-34]. In this study, the ribozyme against VEGF₁₆₅ site 212 was designed with computer, the transcriptional vector including the anti-VEGF₁₆₅ ribozyme and self-splicing ribozymes were synthesized and constructed, and the cleavage effect of the ribozyme on target mRNA in cell-free system was observed.

MATERIALS AND METHODS

Vectors

The vector pGEM-3zf(+)VEGF (carrying full length amino acids cDNA of VEGF₁₆₅) was kindly provided by Dr. Abraham (Columbia University, USA). Vector pGEMRzHBV and bacterium JM 109 were gift from Dr. Li (Department of Infectious Disease, Tangdu Hospital, Fourth Military Medical University, China).

Ribozyme design

The cleavage sites of ribozyme anti-VEGF₁₆₅ were designed by computer analysis of the secondary structure of VEGF₁₆₅ mRNA with a computer program (Chen Nong-an, Shanghai Institute of Biochemistry, Academia Sinica). According to Symon's hammerhead ribozyme structural model, the sequences of the cleavage active core of ribozyme and the flanking sequences around the cleavage sites were designed.

Ribozyme synthesis

The hammerhead ribozyme was synthesized by 35 amplification cycles of PCR (TakaRa Biotechnology Co. Ltd. Dalian, China) with the following primer: 5' TGAAGATGCTGATGAGTCCGT GAGGACGAAACTCGAT 3' and purified by electrophoresis on a 100 g/L denaturing polyacrylamide gel.

Plasmid construction

In the down stream of T7 promoter, plasmid pGEMRzHBV included 5' cis-self-splicing ribozyme, RzHBV and 3' cis-self-splicing ribozyme in order. The pGEMRzHBV was digested with *Xba* I and *Aat* II, then the linear vector was purified by 10 g/L agarose gel electrophoresis by using plasmid purification kit (Gibco, USA) according to the manufacturer's instruction. The two complementary strands of designed ribozyme gene cDNA ends were prepared by adding linkers to create sticky ends (*Xba* I and *Aat* II). The double-stranded DNA was then subcloned into the multicloning site (at *Xba* I and *Aat* II) of pGEM by using T4 DNA ligase (Promega, USA) to create the self-splicing transcriptional plasmid pGEMRz212 (Figure 1). After being transformed competent JM109 cells with pGEMRz212 and blue-white screening, the plasmids were extracted from the positive colonies. The sequences of the VEGF₁₆₅ ribozyme and self-splicing ribozymes were confirmed by restriction enzyme and DNA sequencing.

Ribozyme transcription and cleavage activity *in vitro*

The pGEM-3zf(+)VEGF was cut by *Eco*R I, and pGEMRz212 by *Xba* I and *Aat* II. The ribozyme and substrate RNAs were

prepared from the cDNA templates with T7 RNA polymerase (Gibco-BRL, USA) with [α - 32 P]UTP (Yahui Co., Beijing) by *in vitro* transcription. Both the ribozyme and substrate mRNA were synthesized by using T7 *in vitro* transcriptional kit (Gibco-BRL, USA). Equal amounts of ribozyme and substrate were mixed in 10 μ L of reaction buffer (10 mmol/L MgCl₂ and pH 7.6, 75 mmol/L Tris-HCl) at 95 °C for one minute and cooled in ice immediately. The mixture was then reacted at 37 °C for 2 h. The reaction was quenched with EDTA. The cleavage products were detected by autoradiography after 60 g/L denaturing polyacrylamide gel electrophoresis.

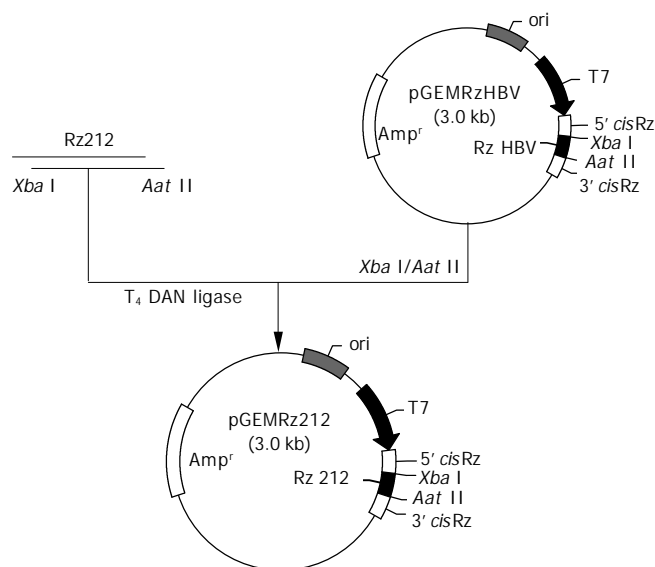


Figure 1 Diagram of construction of the vector pGEMRz212.

RESULTS

Ribozyme designation with computer

The topography of the substrate RNA could be simulated by analyzing the cleavage site and the region surrounding the cleavage site by using a RAN secondary structure folding program. In this way, it might be possible to determine whether or not the target site is buried within an obvious thermodynamically stable region of secondary structure. Among the VEGF₁₆₅ mRNA, there were four hammerhead ribozyme cleavage sites. The site 212 was selected as the optimal cleavage site due to its in single chain region of substrate RNA secondary structure and its both binding arms forming a loop structure to expose for ribozyme cleavage interaction as well as site 212 creating the ribozyme essential core (Figure 2). We called the ribozyme RZ212.

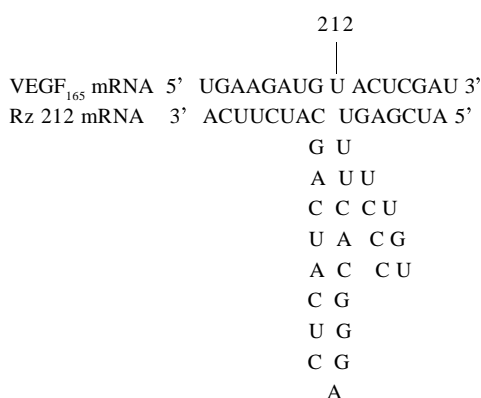


Figure 2 Sequencing of the RZ212 and the target mRNA of VEGF₁₆₅.

Synthesis and transcription RZ212

The synthesized ribozyme was confirmed by restriction enzyme and sequencing analysis. The ribozyme molecule was transcribed *in vitro*. Autoradiography showed three fragments after electrophoresis. The fragments were 5' *cis* ribozyme (64 nt), 3' *cis* ribozyme (54 nt) and RZ212 (47 nt), respectively (Figure 3). The results indicated synthesized ribozyme presenting self-cleavage and releasing the desired ribozyme.

Cleavage activity of RZ212

The cleavage reaction was carried out *in vitro* in a cell-free system. RZ212 cleaved the substrate VEGF₁₆₅ mRNA into 2 fragments (259 nt and 380 nt) (Figure 4) consistent with anticipation. After being cleaved by ribozyme, the density was analyzed by using laser density scanner and the substrate residue was just about 9.3%, indicating that the substrate was nearly cleaved completely by ribozyme.

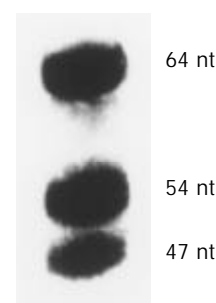


Figure 3 Transcription of pGEMRz212.

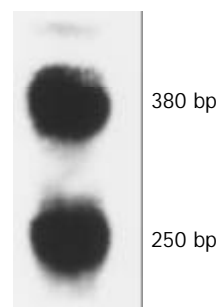


Figure 4 Cleavage activity of VEGF₁₆₅ mRNA with RZ212.

DISCUSSION

The cleavage site and both binding arms must be considered attentively while designing ribozyme. First of all, the cleavage site should be in the important functional region of the target gene assuring the corresponding protein function lost after being cleaved. In addition, the flanking sequences around the cleavage site should be as conserved as possible so that the ribozyme cleavage spectrum become broader. The ribozyme arms sequence context can also influence cleavage rate significantly. In a simple term, the longer the binding arms, the lower the turnover in cleavage of short substrate^[35]. Results from various studies have indicated that ribozyme activity is closely related to the arms length and this depends somewhat on the sequence context^[36,37]. The ribozyme design program we used was approved and improved continuously by experiments. It can resolve the cleavage site design and the sequence surrounding the cleavage site as well as the predicting of ribozyme secondary structure. In this study, we designed successfully the ribozyme target VEGF₁₆₅ mRNA site 212 using the program, synthesized RZ212 and constructed self-cleavage plasmid pGEMRz212. *In vitro* transcription and cleavage

experiment showed that the designed ribozyme was released and cut the target mRNA into two fragments.

Practically, ribozyme gene was constructed in the transcriptional and eukaryotic vector. The ribozyme molecule was transcribed in cells. But there are some long additional sequence in the both arms of ribozyme. The long additional sequence has a strong secondary structure and influenced ribozyme catalytic core, resulted in forming the incorrect secondary structure, even blocking ribozyme binding site. The long additional sequence also influenced ribozyme dissociation from the cleaved target mRNA and reduced cleavage rate^[38,39]. In order to cleave the long additional sequence, we designed and constructed the trimming plasmid pGEMRz212, which included 5' *cis*-self-splicing ribozyme, Rz212 and 3' *cis*-self-splicing ribozyme in order. The long additional sequence was cleaved while pGEMRz212 transcription *in vitro* and VEGF₁₆₅ mRNA specific ribozyme released without long additional sequence. The result showed 5' and 3' *cis* ribozyme neither cleaved the substrate and nor influenced Rz212 cleavage efficacy.

Compared with antisense RNA, ribozyme can not only block target mRNA but also cleave the target mRNA in a sequence-specific manner. Ribozyme has received much attention for their potential use due to their inherent simplicity, relatively small size, repetition use and the ability to be incorporated into a variety of flanking sequence motifs without changing site-specific cleavage capacities^[40-42]. In this experiment, the cleavage efficacy of ribozyme we designed and synthesized reached up to 90.7%. It can suppress effectively the expression of substrate. This research may facilitate the development of ribozyme anti-angiogenesis gene therapy for the treatment in the tumors. Further studies are required for therapeutic application of anti-angiogenesis in human cancer.

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