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*GASTRIC CANCER*

# Construction of retroviral vector of $p^{125FAK}$ specific ribozyme genes and its effects on BGC-823 cells

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## Abstract

**AIM:** To construct the retroviral vector of $p^{125FAK}$ specific ribozyme genes and to explore the feasibility of ribozyme in BGC-823 gene therapy *in vitro*.

**METHODS:** A hammerhead ribozyme DNA targeting $p^{125FAK}$ mRNA from nt 1010 to nt 1032 was synthesized and recombinated into the retroviral vector pLXSN forming pLRZXSN recon. Using the lipofectin-mediated DNA transfection technique, pLRZXSN was introduced into BGC-823 cells. The effects of ribozyme on the growth of BGC-823 cells and apoptosis were studied by cell colony assay, flow cytometry (FCM), reverse transcriptase-polymerase chain reaction (RT-PCR), detection of DNA fragmentation and electron microscopy.

**RESULTS:** The number of BGC-823 cell colonies was inhibited by 56% after the cells were treated for 48 h. The cell proliferation was inhibited effectively by $p^{125FAK}$ ribozyme and the inhibitory effect depended on the concentration and the time of incubation. The expression of $p^{125FAK}$ mRNA and protein $P^{125}$ decreased sharply in BGC-823 cells treated with $p^{125FAK}$ ribozyme. The characteristics of apoptosis, namely sub-G1 peak, DNA fragmentation and morphological changes, were revealed in BGC-823 cells treated with $p^{125FAK}$ ribozyme.

**CONCLUSION:** $p^{125FAK}$ ribozyme decreases $p^{125FAK}$ gene expression and induces apoptosis of human gastric cancer cells *in vitro*.



**Key words:** Ribozyme; $p^{125FAK}$ gene; Stomach neoplasm; Apoptosis

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## INTRODUCTION

$p^{125FAK}$ is a non-receptor cytoplasm protein tyrosine kinase (PTK) with a molecular weight of 125 ku , which has unique structural and functional characteristics . Focal adhesion kinase (FAK) regulates multiple cellular processes including growth, differentiation, adhesion, motility and apoptosis[1-4]. The high expression of $p^{125FAK}$ is possibly a part of cell incidents contributing to the invasion and metastasis of tumors[5-7]. Ribozyme (RZ) is defined as a kind of small RNA molecule that has catalytic activity and can inhibit the translation process of mRNA after RZ combines with the complementary sequences of mRNA and incises it[8-11]. In this study, a hammerhead ribozyme DNA targeting $p^{125FAK}$ mRNA from nt 1010 to nt 1032 was synthesized according to $p^{125FAK}$ cDNA sequences, and recombinated into the retroviral vector pLXSN forming pLRZXSN recon. Using the lipofectin-mediated DNA transfection technique, pLRZXSN was introduced into BGC-823 cells to study the expression of RZ and its effect on BGC-823 cells.

## MATERIALS AND METHODS

### *Materials*

BGC-823 cells were purchased from Shanghai Cytobiology Institute of Chinese Academy of Medical Sciences. *E. coli* DH-5 were donated by Hematopathy Laboratory of Fujian Medical University. Restriction enzymes (HindⅢ, pstⅠ, BamHⅠ, XhoⅠ)and $T_4$DNA ligase, X-gal, ITPG were all from Promega. pBluescript@SK plasmid was from Stratagene. RT-PCR kit was from Promega, Lipofectin™ was from Gibco/BRL, $p^{125FAK}$ (H-1) was from American Santa Cruz. S-P kit and DAB kit were from Maxim Biotechnology and Zymed Lab Inc, respectively.

### *Design and synthesis of RZ template*

GUU triplets of nt 1010 to nt 1032 in $p^{125FAK}$ cDNA base sequences were used as cleavage sites. Two small nucleotide sequences complementary to the cleavage

**Table 1 Primer sequence and fragment length**

| Primer | Sequence | Fragment length (bp) | Annealin Temp (℃) |
|---|---|---|---|
| $p^{125FAK}$ | A: 5′TTCTTCTATCAACAGGTGAAG3′<br>B: 5′CTGCGAGGTTCCATTCACCAG3′ | 632 | 55 |
| β-actin | A: 5′GGCATGGGTCAGAAGGATTCC3′<br>B: 5′ATGTCACGCACGATTTCCCGC3′ | 500 | 55 |

Note: A and B represent the sense and anti-sense primers, respectively.

sites of target RNA were put at the two ends of the conservative core sequence-(the hammerhead structure) to form a typical active incisive secondary structure of RZ as previously described[12]. The two limbs and complementary ribonucleotides of target RNA were all 10 nt. RZ gene consisted of 2 completely complementary trains called trains A and B, consisting of 60 bases respectively. Two cleavage sites and 3 protective bases were synthesized by Shanghai Sangon Technology Corporation. The sequences of RZ gene were 5'CGGACTCATCAGCAAGCTGGAT AAGCTTCGT-3' for train A and 3'GCCTGAGTAGTCG TTCGACCTATTCGAAGCA-5' for train B.

### *Constructtion of sequencing vector and DNA sequence analysis*

Plasmid extraction, restrictive enzymolysis reaction, ligase coupled reaction, preparation of competent germ, *E coli* transfection, agarose gel electrophoresis and DNA fragment retrieval were routinely carried out. DNA sequencing was undertaken by Shanghai Sangon Technology Corporation.

### *Construction of pLRZSN recon*

Sequencing of a fragment of 81 bp was carried out with Bam HI and Xho I, retrieved by polyacrylamide gel electrophoresis(PGE), recombinated into pLXSN cleavage sites of Bam H I and Xho I and then labeled as pLRZSN. The pLRZSN from QIAGEN was exactly quantified and sub-packaged for later use.

### *Transfection of BGC-823 cells*

BGC-823 cells in log growth phase were divided into BGC 823 cell blank group, BGC-823 cells + Lipofectin™ group, BGC-823 cells + pLXSN group and BGC-823 cells + pLRZSN group. Liquor A was obtained by adding 4 μg DNA (pLXSN or pLRZSN) to 100 μL serum-free RPMI1640, while liguor B was obtained by adding 10μL Lipofectin™ to 90 μL serum-free RPMI1 640. Liguors A and B were mixed after 30 min and stood for 15 min. At the same time, $1\times10^6$ BGC-823 cells in log growth phase were washed. After resuspension in 0.8mL serum-free RPMI1640, the mixture of liquors A and B was added into the suspension and incubated at 37 ℃ in an atmosphere containing 5% $CO_2$ for 6 h . Four mL RPMI1640 containing 10% serum was added into the suspension and incubated for 48-72 h and then the BGC823 cells were collected.

### *Colony forming experiment*

After incubated for 48 h, the 4 groups of cells were digested in monoplast suspensions and inoculated into 24-well plates (200/well). At the same time, 3 parallel wells were being designed. After being incubation for 7 d, colonies formed when the number of cells was over 50. The number of colonies was calculated under microscope and the colony forming efficiency was determined.

### *Detection of $p^{125FAK}$ protein*

After being incubated for 48 h, the 4 groups of cells were digested in monoplast suspensions and inoculated onto slides. When the slides were open-air dried and fixed in cold acetone for 10 min, immunochemistry dyeing was carried out by S-P method. Positive cell cytoplasm was buffy, nuclei and negative cells were not stained. $p^{125FAK}$ positive cell labeling index (PI = $p^{125FAK}$ positive cell number under one field of vision/1000×100%) was calculated. After being incubated for 48 h, the 4 groups of cells were digested in monoplast suspensions and prepared into specimens. $p^{125FAK}$ protein was detected by flow cytometry (Bio-Rad, Bryte-HS).

### *Evaluation of $p^{125FAK}$ mRNA*

Total RNA was extracted from the cells after incubation for 48 h. cDNA synthesis and PCR amplification were performed as previously described[13]. $p^{125FAK}$ gene nucleotide sequence to be amplified the primers used are listed in Table 1.

### *Detection of apoptosis of BGC-823 cells*

After being incubated for 48 h, the 4 groups of cells were digested in monoplast suspensions, poached with PBS, centrifuged and incubated for 30 min in Kenesis50 kit (BioRad) and the DNA contents were analyzed by FCM. The ultra-structure of BGC-823 cells was observed under transmission electron microscope. The 4 groups of cells incubated for 48 h were prepared and photographed. Extraction and electrophoresis of DNA apoptotic fragments were carried out following the directions of apoptotic DNA ladder kit (Roche Corporation).

### *Statistical analysis*

The data were expressed as mean ± SD. The variance analysis and significant difference test were crried out by SPSS 10.0.

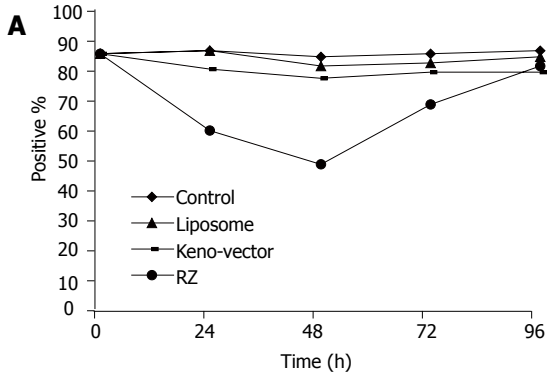
## RESULTS

### *Effect of RZ on colony forming efficiency (CFE) of BGC-823 cells*

The results of colony forming experiment are summarized in Table 2. RZ could inhibit colony formation of BGC-823 cells ($P < 0.05$).

### *Effect of RZ on expression of BGC-823 $p^{125FAK}$ protein*

After the cells were incubated for 48 h, the expression rate of $p^{125FAK}$ protein was 88.4% in control group, 79.55% in liposome group, 77.08% in kenovector group, and 46.09% in ribozyme group. A significant difference was found among the 4 groups. The positive number of BGC823 cells expressed in the $p^{125FAK}$ protein decreased sharply and the intensity of expression also weakened. PI was obviously smaller in RZ group than in other three groups



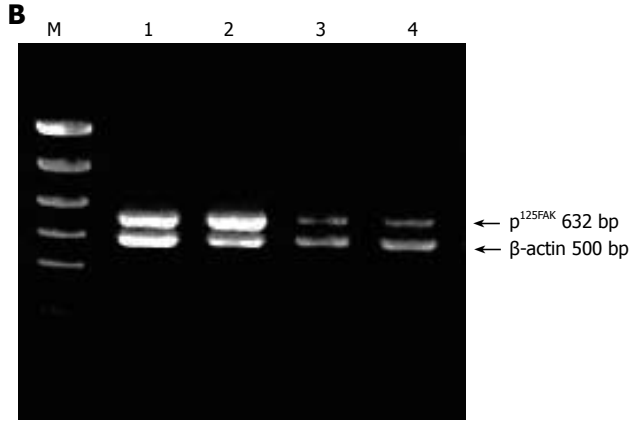




**Figure 1** Expression of $p^{125FAK}$ protein **(A)** and mRNA **(B)** in BGC 823 cells M: Marker; lane 1: control; lane 2: liposome; lane 3: keno-vector; lane 4: RZ

**Table 2 Effect of RZ on colony formation of BGC-823 cells**

| Groups | CFE (%) | CIE (%) |
|---|---|---|
| Control group | 51.24±6.8[a] | |
| Liposome group | 46.43±8.6[a] | 9.38 |
| Kenovector group | 45.32±5.2[a] | 11.55 |
| Ribozyme group | 16.14±3.5[a] | 68.50 |

[a]$P<0.05$ *vs* other groups.

(Figure 1A).

### *Effect of RZ on $p^{125FAK}$ mRNA expression in BGC 823 cells*

PCR product electrophoresis showed 632 bp and 500 bp bands. The fluorescence intensity value of $p^{125FAK}$ and β-actin was 1.23 in control group, 0.98 in liposome group, 0.92 in keno-vector group, and 0.38 in RZ group, indicating that the level of $p^{125FAK}$ mRNA in BGC-823 cells decreased sharply than that in other three groups (Figure 1B).

### *Apoptosis of BGC-823 cells induced by RZ*

Apoptotic peak (sub-diploid peak) appeared in RZ group but not in other three groups (Figure 2).

Karyopynosis, chromatin margination, complete caryotheca, condensed kytoplasm with deep staining were found in RZ group. Larger karyoplasmic ratio, puffed chromotin, clear plasmosome, and abundant cytoplasm were seen in other three groups. These cells were poorly-differentiated BGC-823 cells which did not undergo apoptosis (Figure 3).

Typical DNA fragments were seen in RZ group but not in other three groups (Figure 4).

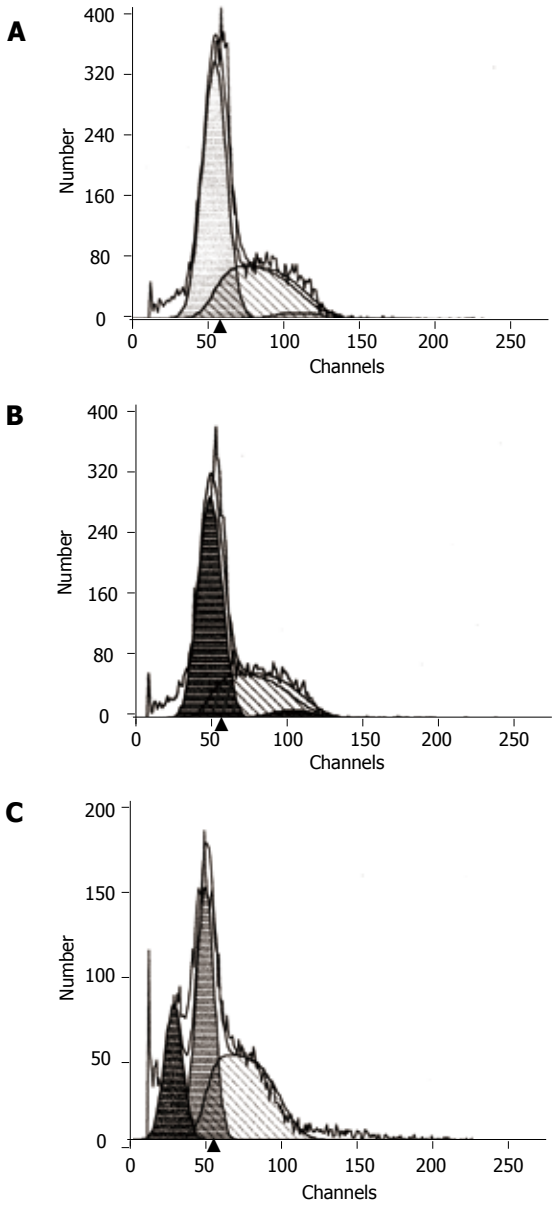



**Figure 2** Cell cycle analysis of cells cultivated for 48 h in liposome group **(A)**, keno-vector group **(B)** and RZ group **(C)**.

## DISCUSSION

Recent studies showed that when malignant cells adhere to extra-cellular matrix (ECM), $p^{125FAK}$ tyrosine self phosphorylation takes place and its activity increases[14-16]. The precise mechanism of $p^{125FAK}$ underlying apoptosis is not clear. But when malignant cells spread or migrate , $p^{125FAK}$ regulates the formation of adhesion plaque or takes part in signal cascade conduction and inhibits apoptosis by informing karyoplasts to that cells are ECM-anchored[17,18]. Anti-sense oligonucleotides of $p^{125FAK}$ have been used to

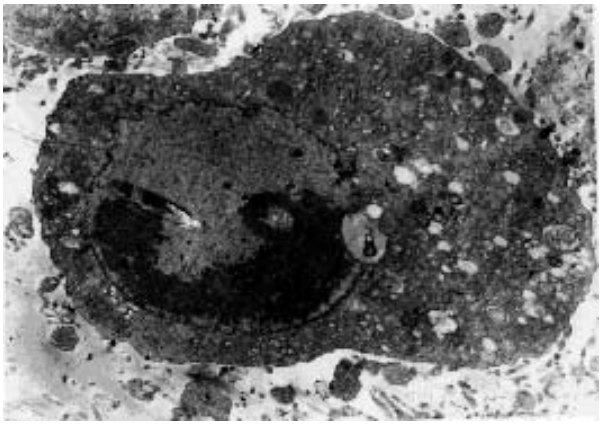


**Figure 3** Apoptotic changes of BGC-823 cells in RZ group.

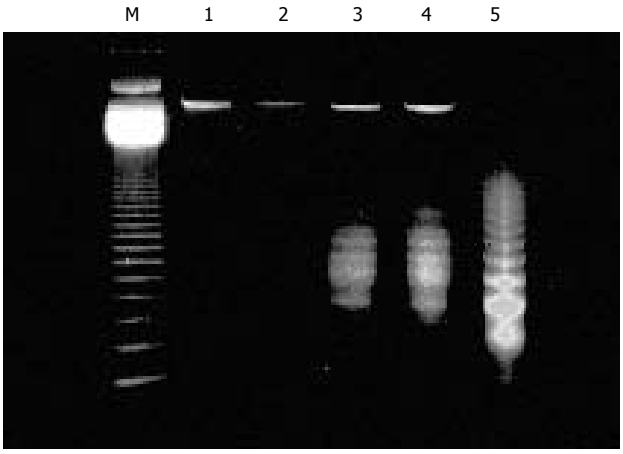



**Figure 4** DNA fragments of BGC-823 cells in different groups M: 123 bp DNA ladder; lane1: liposome group; lane 2: keno-vector group; lane 3: RZ group for 24 h; lane 4: RZ group for 48 h; lane 5: RZ group for 72 h.

inhibit the expression of $p^{125FAK}$ gene in order to induce apoptosis of malignant cells[19,20], but the result is not satisfactory because the number of target RNAs is so larger that complete blocking is impossible and anti-sense oligonucleotides are so easy to be degraded. RZ may solve the above problems. Ribozyme is a small RNA molecule that has catalytic activity. On the one hand, it can combine with complementary sequences of mRNA to block the translation of mRNA. moreover, it can incise mRNA and promote the degradation of mRNA[10,21]. Symons[8] reported that RZ can be used in treatment of viral disease and tumor. RZ genes such as c-erbB-2, P53, Ras, TGF-β, can be used in oncotherapy[22-25]. Qian *et al* [26] reported that FGFR3 RZ gene could inhibit the growth of myeloma cells and promote their apoptosis. But studies on $p^{125FAK}$ RZ gene are relatively fewer. In the present study, a hammerhead ribozyme DNA targeting $p^{125FAK}$ mRNA from nt 1010 to nt 1032 was synthesized according to $p^{125FAK}$ cDNA sequences, and recombinated into the retroviral vector pLXSN forming pLRZXSN recon. Using the lipofectin-mediated DNA transfection technique, pLRZXSN was introduced into BGC-823 cells to inhibit the growth of BGC-823 cells to observe the typical changes of apoptosis under electron microscope. The results demonstrate that $p^{125FAK}$ RZ gene is sequence specific and can be used in treatment of gastric carcinoma.

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