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Reversal MDR in Breast Carcinoma Cells by Transfection of Ribozyme Designed According the Secondary Structure of mdr1 mRNA

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Abstract

Multidrug resistance (MDR) is a major obstacle in cancer chemotherapy. The present study aims to investigate whether the ribozyme could reverse MDR in breast carcinoma cells. In this study, two GUC sites (GUC106 and GUC135) on the surface of mdr1 mRNA were selected according to the secondary structure of the 5'-region of mdr1 mRNA. The ribozyme gene RZ_{106} and RZ_{135} complementary to two sides bases of the target GUC were synthesized and cloned into the plasmid pEGFP -C1 which has EGFP (Enhanced Green Fluorescence Protein) as report gene and Kan/Neo as selection gene. After transfection with the recombinant plasmid and selected by G418, the stable cell clones were produced and used for detection. The alteration of mdr1 mRNA and P-gp in the treated cells was detected by RT-PCR, flow cytometry and Rh123 retention. The reversal efficiency of the drug resistance for adriamycin was determined by MTT assay. The results showed that after transfection with RZ_{106} and RZ_{135}, the amount of the mdr1 mRNA and P-gp decreased significantly and the efflux function of P-gp was inhibited accordingly. Nine-fold and 16-fold reduction of resistance for adriamycin was observed in the two groups of treated cells. These results suggested that both ribozymes can reverse the MDR phenotype by inhibiting the expression of mdr1 mRNA and P-gp, and the RZ_{135} showed the better cleavage efficiency. The ribozyme strategy designed according the secondary structure of the target RNA could be a useful therapy for reversal of MDR.

Key Words: multidrug resistance, reversal, ribozyme

Introduction

Multidrug resistence (MDR) is a common problem in cancer chemotherapy and has become a major obstacle for clinical treatment (12). This phenomenon means tumors could be refractory to a variety of antineoplastic drugs with different structures or different target sites. One of the main mechanisms of MDR involves multidrug resistence gene 1 (mdr 1), which encode a 170-kD membrane protein, P-glycoprotein (P-gp). P-gp is an ATP-binding transmembrane transporter which serves as a drug efflux pump. The overexpression of P-gp causes an increase in drug excretion from cells and results in the resistance to chemotherapeutic drugs (19). In human cancers, the overexpression of P-gp is often seen in colon carcinoma (21), liver carcinoma (9), kidney carcinoma (23) which derived from the tissues that normally express P-gp. Moreover, the overexpression is also seen in breast carcinoma (28), and leukemia (18), which derived

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from tissues that not express P-gp. Some strategies like chemical compounds or monoclonal antibody have been used to overcome the MDR phenotype. For example, some chemical drugs such as verapamil, cyclosporine A have been used in clinical trials in combination with chemotherapeutic drugs and have been proved useful (17). However, the innate toxicities of these compounds are severe and must be carefully considered (7). Less toxic strategy for example antisense gene therapy including antisense oligonucleotide (ASODN) and ribozyme need to be developed.

ASODN is short sequence of DNA which could combine with complementary single-strand mRNA and inhibit the expression of target gene. ASODN had showed the function of down-regulating the expression of P-gp in several studies (14), while hammerhead ribozyme was thought more powerful in inhibition the synthesis of the protein (25). Ribozymes are RNA molecules with catalytic to recognize the GUC site on target mRNA and to cleave it specially. Many studies have proven its usefulness as specific gene silencing tools. The inhibition of HIV gene expression by ribozyme through down-regulation of gag, env gene has been proved successful (20). After introduction to P-gp positive tumor cells, anti-mdr 1 ribozyme can decrease resistance to chemotherapeutic agents in leukemic cells, colon carcinoma cells, hepatocellular carcinoma cells (3). But, it is still difficult to predict the biological activity of individual ribozymes, because of the complexity of the target mRNA (27). The target mRNA in tumor cells is the 3-dimensinal structure. It is has been demonstrated that the GUC site of the target mRNA is more accessible when it is near the surface than in the inner structure. In the present study, by using a computer RNA-folding program, two kinds of anti-mdrl ribozymes were synthesized. Both of them were complementary to the GUC site proximate to the surface of mdr1 mRNA. These two ribozymes were cloned into a plasmid respectively, which had EGFP as report gene. After transfection of the ribozyme plasmids into breast MDR cells, the alteration of MDR phenotype in the treated cells was investigated.

Materials and Methods

Cell Lines and Cell Culture

The parental breast carcinoma cell line MCF-7, which was sensitive to adriamycin, and the MDR subline MCF-7/ADR (more than 100-fold resistant to adriamycin) were obtained from American National Cancer Institute. They were maintained in RPMI-1640 culture medium (Gibco, Los Angeles, CA, USA) containing 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2. The MCF-7/ADR cells express P-gp and its MDR phenotype is stable in adriamycin-free medium for at least 4 months.

Synthesis of Ribozymes Gene

Hammerhead ribozymes can recognize and cleave RNA sequences containing GUX (X=A, U, C, the best catalytic efficiency appears when X is C). Before being cleaved, the target site must be accessible to combination. It is thought that site accessibility is mainly determined by secondary structure of mRNA. By using a computer RNA-folding analysis program (m-fold 3.0) (6) provided by Dr. M. Zucker (www. bioinfo.rpi.edu/~zukerm/rna/) and referencing several related studies (11, 16), the mdr1 mRNA was analyzed and the secondary structure of 5'-region of mdr 1 mRNA was obtained (Fig 1). According to the secondary structure, the GUC sites at 106 and 135 were chosen. Besides that two GUC sites were proximate to the surface of mdr 1 mRNA, they were in the loop region and easy to be cleaved.

The sequences of deoxyoligonucleotides for the construction of anti-mdr 1 ribozymes were showed in Table I. Two deoxyoligonucleotides were synthesized contained RNA polymerase transcription termination signal, with the EcoR I and Sal I site at the 5' and 3' end. The two DNA strands were heated at 80°C for 2 min and slowly cooled to room temperature.

Construction of Ribozyme Vector

The plasmid pEGFP-C1 with EGFP as report gene and Kan/Neo as selection gene was used to construct ribozyme-expression vector.

The two ribozymes were cloned into the EcoR I and Sal I restriction sites of pEGFP respectively. After introducing into E.coli DH5α, the positive recombination plasmids were identified by standard methods (10). Then the E.coli containing recombinant plasmids were amplified and the plasmids were extracted and named pEGFP-RZ_{106}, pEGFP-RZ_{135} respectively.

Transfection of Breast Carcinoma MDR Cell

The MDR cells were seeded in 6-well plates at the density of 1×10^6 cell per well and incubated at 37°C in an atmosphere with 5% CO_2 for 12 h. For each well, 5 μl (1 mg/ml) of lipofectamine (Invitrogen, Carlsbad, CA, USA) and 5 μl (1 mg/ml) of ribozyme plasmid were diluted into 250 μl of 1640 culture medium without serum respectively and incubated for 5 min at room temperature. The diluted plasmid and lipofectamine were mixed together and incubated for 20 mins. Before adding the complexes directly into MDR cells, the growth medium of MDR cells

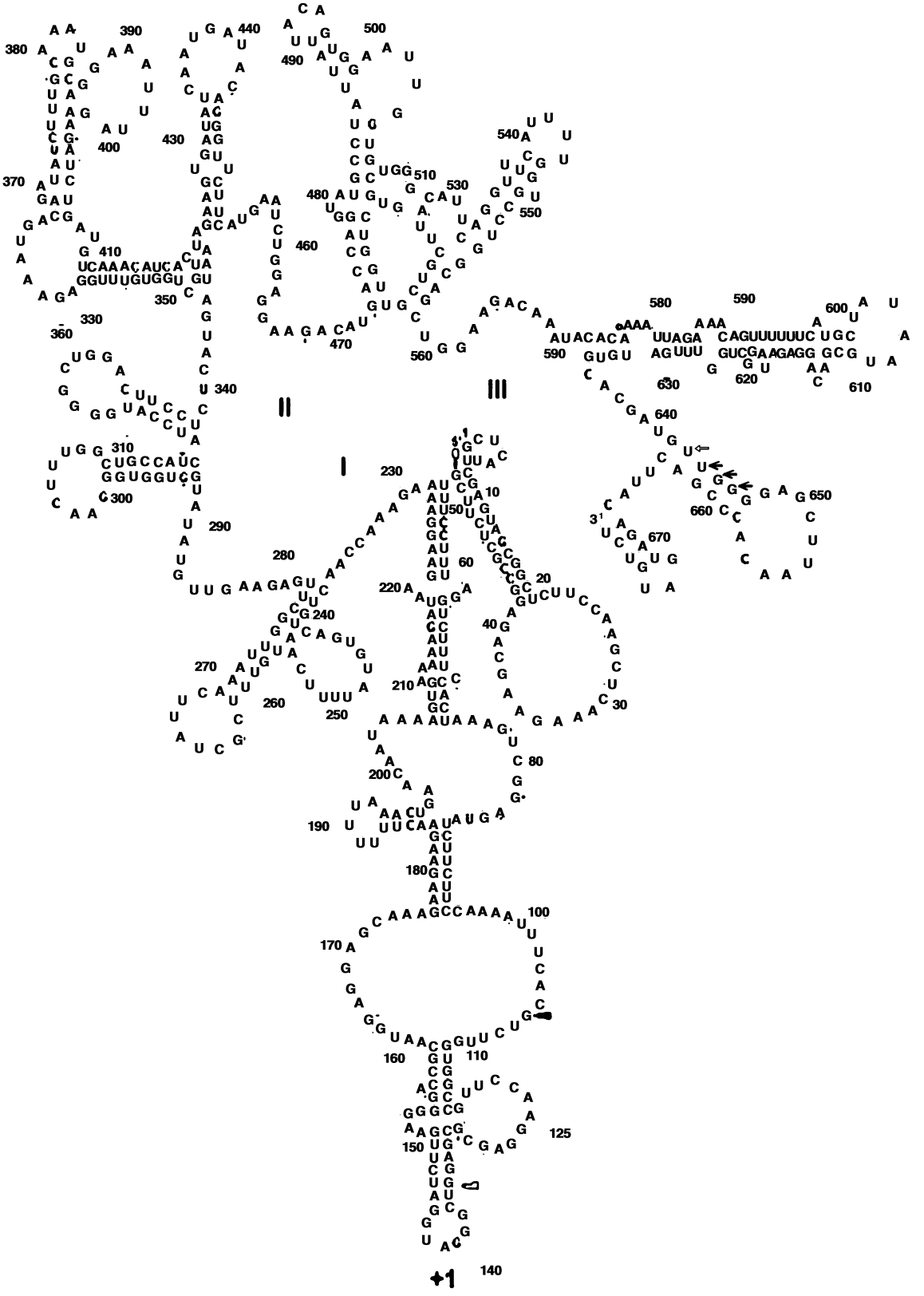

Fig. 1. The secondary structure of 5'-region of mdr 1 mRNA. The 106 and 135 GUC were selected as target site.

was removed and replaced with 0.5 ml of medium without serum. Six h later, the medium was replaced with normal 1640 culture medium. Twenty four h after transfection, the expression of EGFP was

Table I. The sequences of deoxyoligonucleotides for synthesize of anti-mdr1 ribozymes

106(+)5'-AATTCCGGTGGTTCTGATGAGTCCGTGAGGACGAACACTTTAACGTTTTTGGG-3'
106(-)5'-GGCCACCAAGACTACTCAGGCACTCCTGCTTGTGAAATTGCAAAAACCCAGCT-3'
135(+)5'-AATTCTCCATCCCCTGATGAGTCCGTGAGGACGAACTCGCGCTCGTTTTTGGG-3'
135(-)5'-GAGGTAGGGGACTACTCAGGCACTCCTGCTTGAGCGCGAGCAAAAACCCAGCT-3'

RNA polymerase transcription termination signal is underlined.

observed by fluorescence microscopy (Olympus, Japan) and the transfection rate was detected by flow cytometry (Becton Dickinsin, San Jose, CA, USA). After selected by G418 (500 μg/ ml) for 3 weeks, stable clones were grown to confluency and tested for their drug resistance character.

Detection of Mdr 1 mRNA by Reverse Transcription Polymerase Chain Reaction

Total RNA from cells was extracted as described (5) and converted to single-strand cDNA using a random 9 mers (Takara, Japan). The reaction was carried out at 30°C for 1 min, 45°C for 30 min, 99°C for 5 min and 5°C for 5 min. PCR was performed using thermal cycler (M J Research, Waltham, MA, USA), thirty cycles were carried out at 94°C for 2 min, 55°C for 45 sec, 72°C for 90 sec. The sequence of the sense and anti-sense primers for mdr 1 mRNA is: 5'-ACTGAGCCTGGAGGTGAAGA-3' and 5'-CCACCAGAGAGCTGAGTTCC-3'. To confirm that the same amount of RNA was used for the assay, β-actin was used as control set. Ten μl of each reaction mixture was analyzed by electrophoresis on a 2% agarose gel. Each band of the result was quantitated by gel figure analysis system (Gel Doc 2000, Hercules, CA, USA). The ratio of mdr1 /β-actin by scanning densitometry was named mdr1 index.

Detection the Amount of P-gp by Flow Cytometry

The amount of P-gp was analyzed quantitatively by fluorescence-activated cell sorting. The cells were washed once with PBS (pH 7.4) and combined with the anti-mdr1monoclonal antibody MRK16 for 30 min on ice. Then the cells were washed with PBS and incubated with the second antibody conjugated to fluorescein isothiocyanate (Sigma, St. Louis, MO, USA). The cells were again washed with PBS and re-suspended in PBS containing 3% FBS. The fluorescent intensity (FI) of the cells was detected by using flow cytometry and was analyzed quantitatively.

Detection the P-gp Function by Rh123-Retention Assay

One million cells were seeded in 6-well plates and cultured for 12 h. The cells were incubated with 200 ng/ml Rh123 at 37°C for 1 h. After washed, the cells were cultured in Rh123-free culture medium at 37°C for 30 min and harvested for measurement of Rh123 efflux. The sample was subjected to flow cytometry (Becton Dickinson, San Jose, CA, USA) for measurement of mean fluorescence intensity. All analyses were performed in triplicate in 3 separate experiments and the results were expressed as the mean fluorescence intensity, which reflected cellular content of the dye retained.

Drug Resistance Test in Vitro

The 3-(4.5-dimethylthiazed-2-yl)-2,5-diphenylterazolium bromide (MTT) assay was used to detect drug resistance *in vitro* (15). Briefly, 4×10^3 cells were seeded in 96-well culture plates and incubated for 24 h. Adriamycin was delivered at various concentrations and incubated for another 48 h in a humidified 5% CO_2 air atmosphere. At the end of incubation, 10 μl MTT solution (4 mg/ml) and 10 μl of 0.1 M sodium succinate were added to each well and the cells was incubated for 4 h at 37°C in the dark. After the MTT solution was aspirated from the wells, the formazan crystals produced were solved by addition of 150 μl DMSO (Sigma). After shaking for 5 min, the absorbance of each well was immediately measured by Bio-Rad microplate reader (Hercules, CA, USA) at 540 nm. The relative drug resistance was determined by comparing the IC_{50} which defined as the concentration of cytotoxic drug causing 50% inhibition of cell growth. The value of relative drug resistance was the IC_{50} of the treated cells relative to MCF-7.

Statistical Analysis

Paired Student's *t*-test was used to evaluate the difference between two groups. *P* value less than 0.05 was considered to be statistically significant.

Results

Identification of Recombinant Plasmid

The plasmids were digested with restrictive enzyme EcoR I, Sac I and were electrophoresed on a 2% agarose gel. The 53 bp of DNA fragment, which

Table II. The expression of P-gp in transfectant cells measured by flow cytometry

cell	FI	Relative level of P-gp expression
MCF-7	0.16 ±0.01	1
MCF-7/ADR	4.63 ± 0.02	28.9
MCF-7/ADR treated with RZ_{106}	0.62 ±0.02	3.8
MCF-7/ADR treated with RZ_{135}	0.43 ± 0.02	2.7

Values of FI are expressed as the means ±SD of 3 independent experiments. Relative levels are relative to the P-gp expression of MCF-7 cells. Compared with the high FI of MCF-7/ADR cells, the cells treated by RZ_{106} and RZ_{135} were both shown a decreased FI ($P < 0.05$).

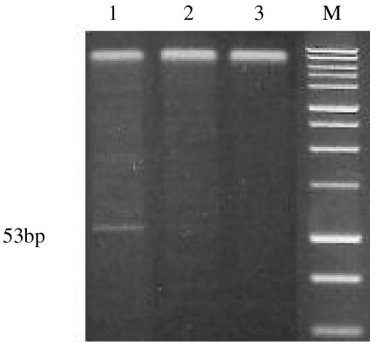

Fig. 2. The identification of recombinant plasmid. M was marker, sample 1 was the positive recombinant plasmid. Both sample 2 and sample 3 were empty plasmids.

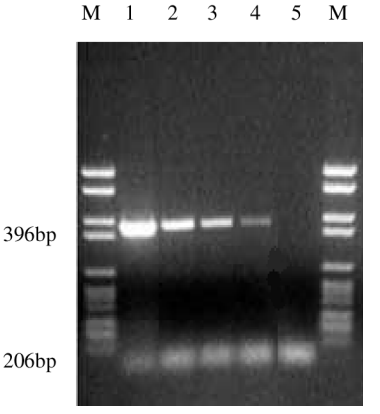

Fig. 3. The alteration of mdr1 mRNA detected by RT-PCR. M was marker, sample 1 was the MDR cell MCF-7/ADR, sample 2 was the MDR cell treated with RZ_{106}, sample 3 was the MDR cell treated with RZ_{135}, sample 4 was the sensitive cell MCF-7, sample 5 was the empty control without mdr1 primers.

was the ribozyme gene, was seen in positive recombinant plasmid (Fig. 2).

Expression of EGFP in the Transfected Cells

Twenty four h after transfection, EGFP could be seen in the cytoplasm of the treated cells by fluorescent microscopy. The transfection rate in cells treated by pEGFP-RZ_{106} and pEGFP-RZ_{135} was 15.8% and 16.3% respectively. The expression of EGFP can also be observed in the stable clones after selected by G418.

Alteration of Mdr1 mRNA in the Transfectant Cells

The amplification product of mdr1 and β-actin by RT-PCR was 396bp and 206bp respectively. The mdr1 index of the MDR cells was 2.20 ± 0.06. In the cells transfection with RZ_{106} and RZ_{135}, the decreased expression of mdr1 mRNA was observed (Fig. 3) and the mdr1 index was 1.40 ±0.04 and 0.75 ± 0.05 respectively. Significant difference was showed either between the MDR cells and the treated cells or between the cells treated with RZ_{106} and the cells treated with RZ_{135} (t was 31.9, 19.2, 17.3, respectively, $P < 0.05$).

Alteration of P-gp Amount in the Transfectant Cells

P-gp expression was analyzed by using a FITC-conjugated antibody and the FI detected by flow cytometry was shown in Table II. In the sensitive cell line MCF-7, the FI was very low, representing the low expression of P-gp. Compared with the high FI of the MDR cells, the cells treated by RZ_{106} and RZ_{135} were both observed a decreased FI ($P < 0.05$).

Alteration of P-gp Function in Transfectant Cells

The function of P-gp was measured by Rh123-retention test. In this study, the fluorescent intensity of Rh123 in MCF-7 was set as 100%. After 30 min of incubation in Rh123-free culture medium, a rapid reduction of intracellular Rh123 was observed in MDR cell MCF-7/ADR, about decreased by 78%. But in the cells transfection with RZ_{106} and RZ_{135}, it only decreased by 44% and 30% respectively (Fig. 4). This suggested that the transfectant cells don't have an active efflux as MCF-7/ADR.

Alteration of Drug Resistance

The MDR cells MCF-7/ADR demonstrated an

Table III. IC_{50} and the relative drug resistance of the transfectant cells

cell	IC_{50}	Relative drug resistance
MCF-7	0.11 ± 0.01	1
MCF-7/ADR	12.30 ±0.03	112
MCF-7/ADR treated with RZ_{106}	1.36 ±0.02	12.4
MCF-7/ADR treated with RZ_{135}	0.78 ±0.01	7.1

The value of relative drug resistance was the IC_{50} of the treated cells relative to MCF-7. In the cells treated with RZ_{106} and RZ_{135}, 9-fold and 16-fold reduction of resistance for adriamycin was showed respectively.

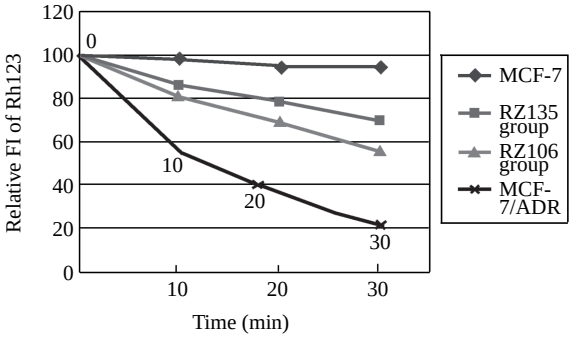

Fig. 4. The relative fluorescent intensity (FI) of Rh123 was used to assess the function of P-gp. 30 min after incubation in Rh123-free culture medium, the FI in MCF-7/ADR decreased by 78%, while the FI in the cells treated with RZ_{106} and RZ_{135} decreased by 44% and 30%, respectively.

IC50 for adriamycin of 12.3 μM. After transfection with RZ_{106} and RZ_{135}, the IC_{50} was lowered to 1.36 μmol/l and 0.78 μM (Table III), equivalent to 9-fold and 16-fold reduction of resistance for adriamycin respectively. This suggested the MDR cells could be chemosensetized after transduction with RZ_{106} and RZ_{135}.

Discussion

At present, several strategies have been developed for the restoration of the chemotherapeutic sensitivity in MDR cells. Compared with chemical drugs such as verapamil-cyclosporin-A, the antisense technology seems less toxic and more exciting. In the last 10 years, ribozymes have emerged and been thought a more powerful strategy in suppressing the expression of certain gene. Ribozymes are catalytic RNAs that can cleave specific RNA sequences. In contrast to other known ribonucleases, ribozyme catalyze highly sequence-specific reactions determined by RNA-RNA interaction between the ribozyme and its substrate molecules (1). At present, more than seven kinds of ribozymes have been discovered and the hammerhead ribozyme is the smallest and the most attractive one (2). The helices I and III of hammerhead ribozyme can recognize and bind the substrate by Wastson-Crick base pairing and they both could be modified according to different target RNA without losing catalytic ability (24). It is possible that any RNA molecule can be cleaved by a ribozyme, provided that it contains a cleavage site GUC. Besides that hammered ribozymes have been successfully used in controlling expression of several genes such as HIV-I, bcl-abl, c-erbB-2, some reports have shown that the anti-mdr1 hammerhead ribozyme is an useful approach for reversing MDR. Moreover, Kiehntopf's study demonstrated that the ribozyme was more effective than the ASODN in restoring the chemosensitivity of tumor cells (13).

During the study of using ribozyme strategy to reverse MDR, one factor must be considered. That is the selection of target sites in mdr1 mRNA, which is 3-dimensional structure in cells. The efficiency of the ribozymes, which also have a secondary structure with hammerhead domain, is mainly decided by the accessibility of the target GUC sites. The cleavage occurs on the condition that the target sites are accessible and then could be bind *via* the sequences of the bases on both sides. It is important and necessary to get the structure information of the substrate RNA to solve the accessibility problem. The computer RNA-folding algorithms have been used to predict or determine RNA secondary structures. Among them, m-fold 3.0 from Dr. Zucker is the most widely used on the internet. By using it and referencing the related study of Elena V (16), the secondary structure of 5'-region of mdr1 mRNA was obtained and used to select the GUC sites on the surface. Among the candidate GUC sites, those sites in the double-stranded regions or in the inner structure were excluded from the potential target sites. The GUC sites both on the surface and in the loop regions were reserved.

In this study, MCF-7/ADR cells which was more than 100-fold resistant to adriamycin compared with the parent cells MCF-7 were used. After transfection with RZ_{106} and RZ_{135} respectively and selected by G418, the decreased expression of mdr1 mRNA and P-gp in treated cells was observed. The mdr1 index decreased from 2.20 to 1.40 and 0.75 and the relative

level of P-gp expression decreased from 28.9 to 3.8 and 2.7 respectively. t demonstrated that the expression of mdr1 and the synthesis of P-gp were inhibited.

The function of P-gp was analyzed by Rh123 retention. The fluorescent dye Rh123 is a substrate pumped by P-gp specially (8). If the function of P-gp in MDR cells was inhibitory, higher fluorescent intensity in the cells should be detected. In our study, the fluorescent intensity in the transfectant cells increased significantly compared to the untreated MDR cells. Therefore, the function of P-gp may produce an inhibition on the treated cells. The results of MTT assay showed that the resistance for adriamycin of the two treated cells was reduced by 9-fold and 16-fold, respectively. The drug resistance between the cells transfection with RZ_{106} and the cells transfection with RZ_{135} was significantly different, which suggested that the ribozyme targeting to the 135 GUC site should be effective in cleaving mdr 1 mRNA.

In this study, EGFP was used as a marker protein .EGFP is an artificial variant of GFP, which comes from *Aeguorea victoria* and it is 35-fold brighter than GFP (22). EDFP can emit green fluorescence by appropriate UV illumination with no substrates or cofactors. This protein is quite stable even in the condition of 65°C, pH 11 and treated with formaldehyde (4). Because of its expression in heterologous species, this novel reporter has attracted much interest for its potential as an *in vivo* marker of gene expression (26). In our study, the cells were observed the expression of EGFP 24 hr after transfection with pEGFP-RZ_{106} or pEGFP-RZ_{135}. And the expression was rather stable in the cell colonies selected by G418. We considered the ribozyme plasmid with EGFP as report gene was rather convenient to determine if the plasmid does express in the transfectant cells, especially when the reversal experiment is undertaken *in vivo*.

In summary, this study has demonstrated that both the RZ_{106} and RZ_{135} have the ability to cleave mdr 1 mRNA and the RZ_{135} has the better cleavage efficiency. The ribozyme vector with EGFP as report gene was convenient to be observed and might be useful in the *in vivo* study. We conclude that the ribozyme strategy may be a useful therapy in reversal MDR phenotype of the tumor cells and RZ_{135} is a good selection for the cleavage of mdr1 mRNA.

Acknowledgments

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References

1. Bevilacqua, P.C. Mechanism of catalytic RNA. *Biopolymers* 73: 69-70, 2004.
2. Bratty, J., Chartrand, P., Ferbeyre, G. and Cedergren, R. The hammerhead RNA domain, a model ribozyme. *Biochim. Biophys. Acta*. 1216: 345-359, 1993.
3. Cech, T.R. Ribozymes, the first 20 years. *Biochem. Soc. Trans*. 30: 1162-1166,2002.
4. Chalfie, M. Green fluorescent protein. *Photochem. Photobiol*. 62: 651-656,1995.
5. Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem*. 162: 156-159, 1987.
6. David, H. M., Sabina, J. and Zuker, M. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J. Mol. Biol*. 288: 911-940,1999.
7. Fojo, T. and Bates, S. Strategies for reversing drug resistance. *Oncogene* 22: 7512-7523, 2003.
8. Fruehauf, S., Breems, D.A. and Knaan-shanzer, S. Frepuency analysis of multidrug resistency-1 gene transfer into human primitive hematopoietic progenitor cells using the cobblestone area froming cell assay and detection of vector-mediated p- glycoprotein expression by rhodamine123. *Hum. Gene. Ther*. 7: 1219-1231, 1996.
9. Grude, P., Conti, F., Mennecier, D., Louvel, A., Houssin, D., Weill, B. and Calmus, Y. MDR1 gene expression in hepatocellular carcinoma and the peritumoral liver of patients with and without cirrhosis. *Cancer Lett*. 186: 107-113, 2002.
10. He, T.C., Zhou, S., Costa, L.T., Yu, J., Kinzler, K.W. and Vogelstein, B. A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. U.S.A*. 95: 2509-2514, 1998.
11. Ho, S.P., Britton, D.H., Stone, B.A., Behrens, D.L., Leffet, L.M., Hobbs, F.W. Miller, J.A. and Trainor, G.L. Potent antisense oligonucleotides to the human multidrug resistance-1 mRNA are rationally selected by mapping RNA-accessible sites with oligonucleotide libraries. *Nucleic Acids Res*. 24: 1901-1907, 1996.
12. Kaur, P. Multidrug resistance: Can different keys open the same lock? *Drug Resist. Updat*. 5: 61-64, 2002.
13. Kiehntopf, M., Esquivel, E.L., Brach, M.A. and Herrmann, F. Clinical applications of ribozymes. *Lancet* 345: 1027-1031, 1995.
14. Kobayashi, H., Takemura, Y. and Miyachi, H. Novel approaches to reversing anti-cancer drug resistance using gene-specific therapeutics. *Hum. Cell*. 14: 172-184, 2001.
15. Kobayashi, H., Takemura, Y. and Ohnuma, T. Relationship between tumor cell density and drug concentration and the cytotoxic effects of doxorubicin or vincristine: mechanism of inoculum effects. *Cancer Chemother. Pharmacol*. 31: 6-10, 1992.
16. Kostenko, E.V., Beabealashvilly, R.S., Vlassov, V.V. and Zenkova, M.A. Secondary structure of the 5'-region of PGY1/MDR1 mRNA. *FEBS Lett*. 475: 181-186, 2000.
17. Krishna, R. and Mayer, L.D. Modulation of P-glycoprotein (PGP) mediated multidrug resistance (MDR) using chemosensitizers: recent advances in the design of selective MDR modulators. *Curr. Med. Chem. Anti-Canc. Agents* 1: 163-174, 2001.
18. Mahon, F.X., Belloc, F., Lagarde, V., Chollet, C., Moreau-Gaudry, F., Reiffers, J., Goldman, J.M. and Melo, J.V. MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. *Blood* 101: 2368-2373, 2003.
19. Marzolini, C., Paus, E., Buclin, T. and Kim, R.B. Polymorphisms in human MDR1 (P-glycoprotein): recent advances and clinical relevance. *Clin. Pharmacol. Ther*. 75: 13-33, 2004.
20. Mcknight, K.L. and Heinz, B.A. RNA as a target for developing antivirals. *Antivir. Chem. Chemother*. 14: 61-73, 2003.
21. Pfrunder, A., Gutmann, H., Beglinger, C. and Drewe, J. Gene expression of CYP3A4, ABC-transporters (MDR1 and MRP1-MRP5) and hPXR in three different human colon carcinoma cell

lines. *J. Pharm. Pharmacol*. 55: 59-66, 2003.
22. Reichel, C., Mathur, J., Eckes, P., Langenkemper, K., Koncz, C., Schell, J., Reiss, B. and Maas, C. Enhanced green fluorescence by the expression of an Aequorea victoria green fluorescent protein mutant in mono- and dicotyledonous plant cells. *Proc. Natl. Acad. Sci. U.S.A.* 93: 5888-5893. 1996.
23. Reinecke, P., Schmitz, M., Schneider, E.M., Gabbert, H.E. and Gerharz, C.D. Multidrug resistance phenotype and paclitaxel (Taxol) sensitivity in human renal carcinoma cell lines of different histologic types. *Cancer Invest.* 18: 614-625, 2000.
24. Scott, W.G. and Klug, A. Ribozymes: structure and mechanism in RNA catalysis. *Trends Biochem. Sci*. 21: 220-224, 1996.
25. Sioud, M. Ribozyme- and siRNA-mediated mRNA degradation: a general introduction. *Methods Mol. Biol*. 252: 1-8, 2004.
26. Stewart, C.N. The utility of green fluorescent protein in transgenic plants. *Plant Cell Rep*. 20: 376-382, 2001.
27. Turner, P.C. Ribozymes: Their design and use in cancer. *Adv. Exp. Med. Biol*. 465: 303-318, 2000.
28. Zampieri, L., Bianchi, P., Ruff, P. and Arbuthnot, P. Differential modulation by estradiol of P-glycoprotein drug resistance protein expression in cultured MCF7 and T47D breast cancer cells. *Anticancer Res.* 22: 2253-2259, 2002.