RESEARCH ARTICLE

AKT1 Inhibitory DNAzymes Inhibit Cell Proliferation and Migration of Thyroid Cancer Cells

Le Yang[1], Jin-Ting He[2], Hong Guan[1], Ya-Dong Sun[1]*

Abstract

AKT1 is a member of the serine/threoine AGC protein kinase family involved in thyroid cancer metabolism, growth, proliferation and survival. It is overexpressed in thyroid tumors. In this study, we designed two AKT1 specific DNAzymes (DRz1 and DRz2) that target AKT1 mRNA. The results showed that DRz1 could decrease the expression of AKT1 by 58%. Furthermore, DRz1 significantly inhibited cell proliferation, induced apoptosis and inhibited invasion in SW597 cells. In addition, down-regulation of survivin expression was associated with decreased caspase-3, VEGF and MMP2 in SW597 cells after 24 h. In our study, the efficacy of DRz1 in decreasing AKT1 protein levels were better than DRz2. AKT1-DRz1 might have anti-tumorigenic activity and may provide the basis for a novel therapeutic intervention in thyroid cancer treatment.

Keywords: DNAzymes - proliferation - apoptosis - AKT1 - thyroid cancer

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Introduction

The serine/threonine kinase AKT (Protein kinase B, PKB) is a key effector of the phosphoinositide 3-kinase (PI3k)/AKT signal transduction pathway which regulates multiple cellular processes, such as cell proliferation, growth, survival, transformation and differentiation (Gijsen et al., 2010). AKT has three isoforms (Tang et al., 2011), AKT1, AKT2 and AKT3 (or PKB-α, PKB-β and PKB-γ). Activation of AKT is initiated by extracellular stimuli in a PI3k-dependent manner. AKT pathway can be propagated to a diverse array of downstream targets (Cheng, 2010; Ponce et al, 2010) that exert many cellular effects including the inhibition of apoptosis and promotion of cell survival. Further more, it has been shown that activation of AKT contributes to the cancer initiation and maintenance, confers resistance to chemotherapy and radiation and is a poor prognostic factor for many cancers (Mansley, 2010). Increasing evidences demonstrate that aberrant activation of AKT is one of the most common molecular events in human malignancies. Thus, AKT1 inhibition may exhibit pleiotropic effects on cancer cells and represent a very attractive cancer therapeutic strategy to target human cancers with high AKT kinase.

Thyroid cancer is the most common endocrine neoplasm, this represents nearly a 3-fold increase since the early 1970s, the reasons for the increased incidence are unclear. Prognosis is closely associated with stage at diagnosis, 5-year survival being 99% for patients with localized disease compared with 58% for patients with distant metastatic cancer (Zhao et al., 2012). The rate of neck lymph node metastasis was as high as 50%-70%. The vast majority of differentiated thyroid carcinoma can be suppressed by surgery, TSH secretion and iodine 131 treatment had good therapeutic effect. However, there are more than a third loss differentiation which displayed that the capacity of iodine 131 treatment were decreased in metastases, the invasion and transfer speed of thyroid cancer cell were enhanced (Kucukalic-Selimovic et al., 2012). It did not do conventional combination therapy in the loss of differentiation and low loss of differentiated thyroid cancer. Therefore, the further solution may rely on biological therapy.

Deoxyribozymes (DNAzymes or DRzs) are synthetic, single-stranded catalytic DNAs which can be engineered to bind by Watson–Crick base pairing to complement sequences in a target messenger RNA (mRNA) and cleave it at predetermined phosphodiester linkages (Li, 1997). The 10-23 model has been proposed as a general model for DNAzymes (Tian, 2005). A 10-23 DNAzyme has a catalytic domain of 15 deoxyribonucleotides, flanked by two arms, each with a 7-9 deoxyribonucleotide substrate-recognition domain. In vitro analysis has shown that this type of DNAzyme can cleave substrate RNA effectively at purine and pyrimidine junctions (Hollenstein, 2008). They have been widely shown, both in vitro and in vivo, to inhibit the expression of their target genes and dependent genes (Wang, 2008). They can also block the development of diverse pathologies in animal models and have potential utility as therapeutic tools.

In the present study, we designed two 10-23 DNAzymes against human AKT1 mRNA, investigated

*[1]Department of Endocrinology, The People's Hospital of Jilin Province, [2]Department of Neurology, China-Japan Friendship Hospital, Jilin University, Changchun, China *For correspondence: yangle800511@sina.com*

their function and examined their effectiveness as a tool for cancer gene therapy using thyroid cancer cell.

Materials and Methods

DNAzyme design

Briefly, a 10-23 model was designed by incorporating 8 bp arms complementary to the target gene at each side of the catalytic motif (Verma et al., 2010). and an inactive DNAzyme was designed based on the flip sequence of DRz1. All DNAzymes were synthesized from Sangon (Shanghai, IN, CHINA). DNAzymes targeting AKT1 were as follows (arrow indicated the cleavage point):
DNAzymes:
Template 1 5'-GGUACCCCA↓UGGACGAC -3'
DNAzyme 1 (DRz2) :
3'-CCATGGGGAGCAACATCGATCGGACCTGCTG-5'
Template2 5'-AUGAAUGAGUUUGAGU-3'
DNAzyme 2 (DRz1):
3'-TACTTACTAGCAACATCGATCGGAAACTCAT-5'
Inactive DNAzyme:
3'-GTACTCAAAGGCTAGCTACAACGATC ATTCAT-5'

Cell lines and cell culture

SW597 cell is human thyroid cancer cell line, which highly express AKT. All cells lines were cultured in 1640 medium supplemented with 10% fetal bovine serum (FBS, GIBCO), 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mmol/L L-glutamine. All cultures were incubated in a humidified atmosphere of 5% CO_2 at 37 °C (Verma et al., 2010). Cell counting was carried out using a Cedex (Innovatis AG, Bielefeld, Germany).

Transfection

Transfection of DNAzymes in vector system was performed with Lipofectamine 2000 reagent as described by the manufacturer. Cells were transfected with DNAzyme oligonucleotide using of Lipofectamine 2000 and serum-free medium (Opti-MEM), respectively. The concentration was selected accordance with previous reports (Verma et al., 2010). After 6 hour, the medium was replaced by serum-containing medium and incubated for an additional 24 hour or 48 hour. Transfection efficiency was determined before transfection of GFP-expressing plasmids and approximately 40% to 60% were estimated by fluorescence microscopy.

Real-time PCR analysis

PCR primers for human AKT1 and β-actin (housekeeping control) were designed using the Primer Express software from Perkin-Elmer Biosystems based on the published sequences: human AKT1(forward), 5'-ATGAGCGACGTGGCTATTGTGAAG-3'and (reverse), 5'-GAGGCCGTC AGCCACAGTCTGGATG-3'; ß-actin(forward),5'-ATCATGTTTGAGACCTTCAAC A-3' and (reverse), 5'-CATCTCTTGCTCGAAGTCCA-3'. Total RNA was isolated from Jurkat cells with the TRIzol reagent (Invitrogen, Rockville, MD) according to the manufacturer's instructions. To avoid DNA contamination, total RNA was treated with RNase-free DNase I (Takara, Kyoto, Japan) for 60min at 37 °C and extracted with the TRIzol reagent again (Mo et al., 2012). The purity of total RNA was determined by the 260/280 absorbance ratio and the RNA integrity by the intensity of the 28S and 18S rRNA bands after formaldehyde agarose gel electrophoresis. Two micrograms of total RNA was subjected to reverse transcription using RevertAidTM First-Strand cDNA Synthesis Kit (Fermentas, Lithuania) with random hexamer primer. One microliter of the cDNA solution was used for real-time PCR. The genes were amplified in a 25 μL reaction using SYBR Universal PCR Master Mix (Tiangen, CA) on Bio-Rad Opticon Monitor (Applied Biosystems). The conditions comprised an initial denaturation step at 95 °C for 5 minute, followed by 40 cycles each of 95 °C for 10 seconds, 54°C for 15 seconds and 68 °C for 10 seconds, before being subjected to melting curve analysis. Amplified products were also analyzed for specificity by agarose gel electrophoresisand were further verified by automated cycle sequencing. To ensure consistency in threshold cycle (Ct) values, duplicate reactions were performedand the mean Ct values were used for calculating the relative expression levels. The Ct values were analyzed as described previously and the normalized Ct values of each gene were subjected to Student's t-test with two-tailed distribution to determine the significance at the 95% confidence level. Reactions were run in triplicate and results were averaged. As an internal control of each sample, ß-actin was used for standardization.

Western blotting analysis

After transfection, cells were washed twice with cold PBS then 1×10^6 cells were treated with RIPA buffer (50 mmoL/L Tris (pH 8.0), 150 mmoL/L NaCl, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate) containing protease inhibitors (1% Cocktail and 1 mmoL/L PMSF). Total proteins were separated by 15%SDS-PAGE and transferred to PVDF membranes. The membrane was blocked for with Tris-buffered saline with 0.1% Tween 20 (pH 7.6, TBST) at room temperature 1 hour and immunoblotted with primary antibody solution (1:1000) at 4 oC overnight. After washing twice with TBST, the membrane was incubated with HRP-l abeled secondary antibody (Santa SC-6721) for 1 hour at room temperature and washed three times with TBST. Final detection was performed with enhanced chemiluminescence (ECL) western blotting reagents (Amersham Biosciences, Piscataway, NJ) and membranes were exposed to Lumi-Film Chemiluminescent Detection Film (Roche). Loading differences were normalized using a monoclonal AKT2 antibody. The antibodies used in the study include AKT1 (Santa SC-7345), VEGF (Santa SC-80437), MMP2 (Santa SC-80201) and ß-actin (Santa SC-130301).

Cell viability assay

The MTT method (Taskin et al., 2012) was used to assess the cytotoxic effects of DRzs. Briefly, cells growing logarithmically were grown to a density of 5×10^4 cells/ well and treated with DRzs in a 96-well plate for 24 or 48 hour. At the end of the treatment, media containing the DRzs were carefully removed and 200 μL medium with 20 μL MTT (5 mg/mL in PBS, St. Louis, MO, USA) was added to each well. After 4 hour incubation at 37 °C, the medium

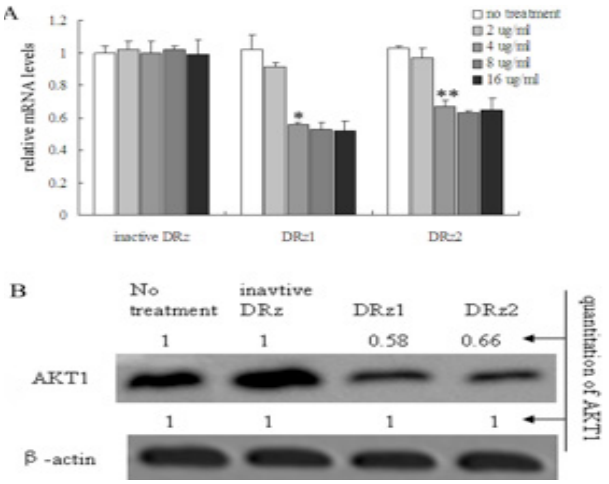

Figure 1. Effect of DRzs on the Expression of AKT1 in SW597 Cells. (A) mRNA expression of AKT1 was significantly inhibited by different concentrations DRzs. The cells were transfected with the indicated concentrations DRz1, DRz2 or inactive DRz for 24 h. Data shown are means + SD of normalized relative survivin mRNA levels (n ≥ 3). * denotes a significant difference to control ($P < 0.05$). (B) AKT1 protein expression was quantified via densitometry and compared with the "no treatment" control. The maximal grey value was set to 1 and all other results were expressed relative to this value (black) (n ≥ 3)

was removed and 100 μL DMSO was added to each well. The optical absorbance (A) of each well was then read at 490 nm. The percentage of viable cells was calculated as follows: (A of experimental group/A of control group) ×100%.

Flow cytometry by AnnexinV/PI staining

For quantitative assessment of apoptosis, Annexin V-FITC and PI double-staining followed by flow cytometry was used. Cells (1×10^5) were harvested and stained with Annexin V-FITC and a PI double staining kit (Kaiji Bio Co., Nanjing, China) according to the manufacturer's instructions. Cells were then immediately analyzed by flow cytometry. Signals from apoptotic cells were localized in the lower right quadrant of the resulting dot-plot graph.

Cell invasion and motility assays

Cell invasion assays SW597 cells were treated with DRzs (1 μg) for 24 h, and then harvested and their in vitro invasiveness determined using a Transwell chamber (Corning, NY, USA). Matrigel (Sigma, St Louis, MO, USA) was diluted with serum-free medium to a final concentration of 2 mg/mL, and used (20μl) to coat 8 μm pore polycarbonate membrane filters. Treated cells were then seeded into the upper chamber (2×10^4 cells/well in 100 μl of serum-free medium), and 1 ml medium containing 20% FBS (v/v) as a chemoattractant was added to the lower chamber. After incubation (24 h, 37 oC, 5% CO_2), the Matrigel coating on the upper surface of the filter was wiped with a cotton swab. Cells that invaded the lower surface of the filter were fixed with 95% paraformaldehyde and stained with Giemsa. Cell numbers were counted in three random fields (×100) per filter. The cell motility assay was conducted in a similar fashion in a Transwell chamber without a Matrigel coating and with 3×10^4 cells/well.

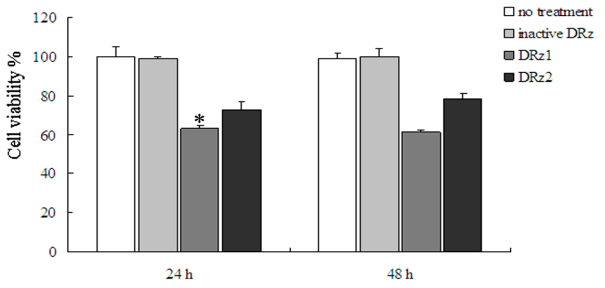

Figure 2. Growth Inhibitory Effects of DRzs on SW597 Cells were Analyzed by MTT Assay. Results are means ± SD of three separate experiments performed in triplicate wells. Cells were grown at a density of 5×10^4 cells/a 96-well plate for 24, 48 hours. At the end of treatment, added to each well with 20 μL MTT. After 4 h incubation and 100 μL DMSO was added to each well, the optical absorbance (A) of was read on 96 well at 490. The inhibitory rate of DRz1 was higher than inactive DRz in 24 hours treated, *$p< 0.05$ compared with a inactive DRz treated cells

Statistics

SPSS software was employed for statistical analyses and values are presented as means ± SD. ANOVA was used to compare the mean values. P values of less than 0.05 were taken to indicate statistically significant differences.

Results

DRzs reduce AKT1 mRNA expression

To analysis the DRzs and siRNA effects on transcriptional level of AKT1, Real-time PCR was used to detect their effect on the mRNA level. As shown in Figure 1, DRz1 and siRNA inhibited the AKT1 mRNA expression in Jurkat cells and DRz1 as well as A6730, an AKT1/2 kinase inhibitor (Sigma-Aldrich) had the more ability to down regulation AKT1 expression in transcriptional level.

DNAzymes and siRNA inhibit AKT1 protein expression in SW597 cells

In order to determine the DRzs effect on the target gene expression after transfection, we assayed for their effects on the AKT1 expression at the mRNA using Real-time PCR in various concentrations (Figure 1A) to determine the optimal incubation concentration. As shown in Figure 1A, DRz1 could inhibit the AKT1 mRNA expression, both DRz1 and DRz2 were able to inhibit AKT1 mRNA expression. These effects were concentration dependent. To evaluate the capacity of the DRzs to inhibit AKT1 protein expression in SW597 cells was treated with the DRzs for 24 h and AKT1 protein levels were evaluated on Western blot. As shown in Figure 1B, DRz1 could inhibit the AKT1 protein expression in SW597 cell and DRz1 can more significantly down regulation of the AKT1 expression than DRz2, while the control transfection and no treatment had no effect on the AKT1 expression. These experiments were repeated three times and representative results were shown.

Effect of DNAzymes on cell proliferation

To evaluate cytotoxicity of DRzs on the cell proliferation, SW597 cells were treated with DRzs for 24 and 48 hour, cell viability was detected by the MTT

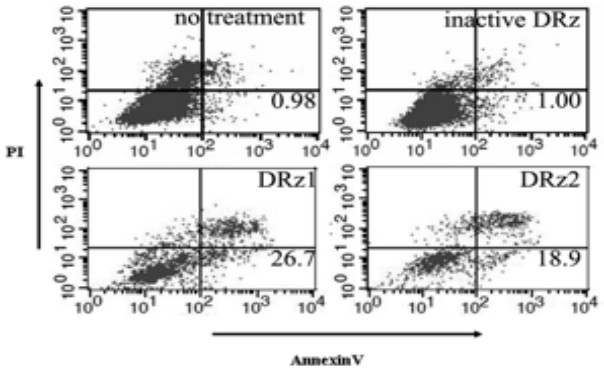

Figure 3. Representative Flow Cytometric Assessment of Apoptosis via Annexin V and Propidium Iodide (PI) Staining. SW597 cells were treated with 4 μg/ml DRz1 and DRz2, and kept in culture for 24 h. Every 24 h, ~105 cells were analyzed with flow cytometry following Annexin-V-FITC/PI staining to evaluate changes in the apoptotic population

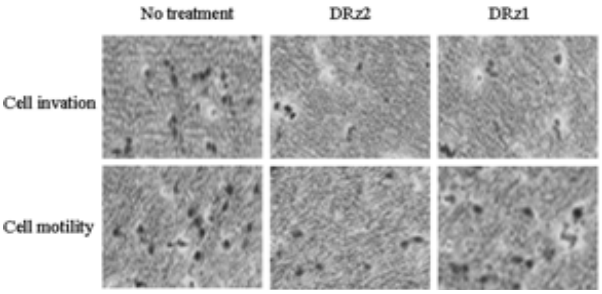

Figure 4. The Effects of DRzs on the Invasion and Motility of SW597 Cells. SW597 cells were treated with 4 μg DRz1 or DRz2 for 24 h or received no treatment (controls). Cells invading or moving into the lower chamber of Transwell Chambers were counted. A representative image showing inhibition of the invasion and motility of SW597 cells after treatments (magnification×200)

method. As shown in Figure 2, the inhibition rate of DRz1 on SW597 cells was 27.11% and 28.82% after 24 hour and 48 hour compared with the control cells, respectively. The inhibitory rate of DRz1 was higher than DRz2 treatment at 24 hour.

DNAzymes induced the apoptosis in SW597 cells

To assess apoptosis in SW597 cells treated with DRzs for 24 hour, we measured the cells using Annexin V-FITC and PI double-staining cytometry techniques. Signals from each group of cells were localized in the lower right quadrant of the dot-plot graph and results were showed in Figure 3. The proportion of apoptotic cells in DRz1 and DRz2 treated cells were 22.14%, 28.56% respectively.

Inhibition on invasion and motility of SW597 cells by DRz

DRz reduced the invasion and motility of SW597 cells (Figure 4) with only 61.3% of the invasive capability and 75% of motility capability retained after 4 μg DRz1 treatment for 24 h. Our results are supportive of the idea that DRz1 inhibits the invasion and motility migration of SW597 cells.

DRz inhibits caspase-3, VEGF and MMP-2 expression in SW597 cells

We investigated any DRzs-induced changes in

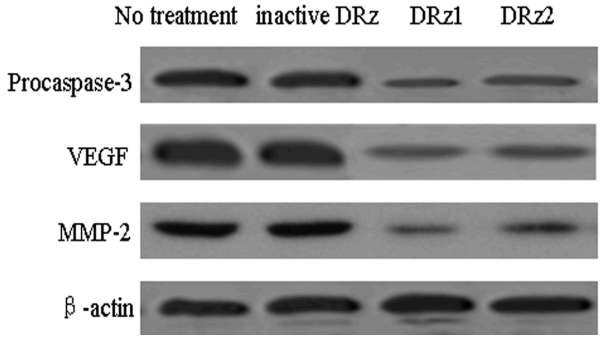

Figure 5. Inhibitory Effects of DRzs on the Expression of Caspase-3, VEGF and MMP-2 in SW597 Cells. SMMC-7721 cells were transfected with the DRzs, DRz1 and DRz2, caspase-3, VEGF and MMP-2 expression were determined by western blotting after 24 h. Results shown are representative of three repeated experiments. NIH imaging indicated that the protein signal densities were lower in DRz1 treated than in control cells (no treatment)

caspase-3, vascular endothelial growth factor (VEGF), matrix metalloproteinase-2 (MMP-2) expression in SW597 cells by western blotting analyses (Figure 5). To assess caspase-3, VEGF and MMP-2 protein expression in SW597 cells treated with DRzs for 24 h, we analyzed the cells using western blotting analysis (Figure 5), the protein level of caspase-3, VEGF and MMP-2 was decreased significantly in DRz1-treated cells, suggesting that down-regulation of VEGF and MMP-9 might be involved in the inhibition of invasion, motility of SW597 cells after DRz1 treatment. DRz1 showed the strongest caspase induction (Figure 5). These results suggested that DRz1 and 2 induce apoptosis in a caspase-dependent manner.

Discussion

The AKT kinase family is composed of three members, AKT1, Akt2 and Akt3. All are structurally homologous and share similar mechanisms of activation, but exhibit distinct features (Hou et al., 2007; Liu R et al., 2012). AKT is overexpressed in a number of cancers, including colon, thyroid, pancreatic, ovarian and some steroid hormone-insensitive breast cancers (Pitt, 2008).

To date, cancer persists as one of the most devastating diseases worldwide. Problems such as metastasis and tumour resistance to chemotherapy and radiotherapy have seriously limited the therapeutic effects of existing clinical treatments. Therefore, the successful treatment of cancer will have a significant impact on improving patient survival. To address these problems, cancer gene therapy has been developing over the past two decades. DNAzymes compared with small-molecule drogs, they are characteristic of minimal toxicity, high efficacy, specific cleave of target gene. Small DNAzymes were potential to be used as a therapeutic tool.

In this study, we used anti AKT1 DNAzyme to inhibit AKT1 expression, we found that DRz1 significantly downregulated the expression of AKT1 in SW597 cells in mRNA and protein level. Flow cytometry assays confirmed qualitatively and quantitatively that DRz1 could suppress the growth of cells. Meanwhile, anti AKT1 DRzs may exert inhibitory effects on the invasion and motility of

SW597 cells, anti-AKT1 DRzs could inhibit the invasion and motility of SW597 cells, the molecular effects of DRz treatments on SW597 cells were further analyzed by detecting alterations in the expression of several molecules relating to cancer invasion and metastasis, such as VEGF and MMP-2. These results showed that DRz1 and siRNA could induce cell apoptosis and invasion though inhibiting AKT1 expression. Thus, anti AKT1 DNAzyme would be potentially used in the therapy of thyroid cancer. We found that DRz1 can more significantly down regulation of the AKT1 expression than DRz1.

In summary, our reports demonstrated that anti-AKT1 DNAzyme could be used as a specific gene-targeting therapy to suppress progression of thyroid cancer. A handful of studies have shown the potential of DNAzymes against cancer both in cell culture suggesting that DNAzymes would become powerful therapeutics of cancer in the future.

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The author(s) declare that they have no competing interests.

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