Effect of antisense oligodeoxynucleotide targeted against NF- κ B/P65 on cell proliferation and tumorigenesis of gastric cancer

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Running title: NF- κ B antisense oligodeoxynucleotide

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ABSTRACT

Gastric cancer is still a major health problem worldwide due to its frequency, poor prognosis and limited treatment options. To study the inhibitory effect of nuclear transcription factor kappa B(NF- κ B) antisense oligodeoxynucleotide(ASODN) on the growth and tumorigenesis of human gastric cancer, we synthesis and transfect ASODN of NF- κ B/P65 to gastric cancer cell line. The effect of ASODN of NF- κ B/P65 on the proliferation of gastric cancer cells was measured by MTT method. The subcutaneous xenograft model of human gastric cancer was established in nude mice and the tumor growth curve was observed. The cell proliferation was significantly inhibited in P65 ASODN transfected group *in vitro* ($P<0.05$). *In vivo*, tumor formation test showed that the tumor volume in nude mice in ASODN group was obviously smaller than in other groups ($P<0.05$), the apoptosis index (AI) was significantly higher ($P<0.001$). Simultaneously, MVD in ASODN group was markedly lower than in other groups ($P<0.01$). Our findings suggest that P65 ASODN can inhibit gastric cancer cells proliferation and tumorigenesis by inducing cell apoptosis and inhibiting tumor angiogenesis. NF- κ B can be used as a new biological therapeutic target of gastric cancer.

INTRODUCTION

NF- κ B is activated by a variety of cancer-promoting agents^[1]. Recent studies showed that as a multifunctional factor, NF- κ B involved in a variety of physiological and pathological processes such as immunity, inflammation, gene expression, cancer cell migration invasion, cell apoptosis and proliferation^[2-6]. The mechanism of NF- κ B activation in tumor cells is not well elucidated, but it is apparently complex and varies in different tumor types^[7]. NF- κ B constitutive activation was identified in some cancer cells such as breast cancer, liver cancer, prostate cancer, pancreatic cancer, and gastric cancer^[8]. Also, we have previously shown that there exists NF- κ B constitutive activation in gastric cancer cell^[9,10]. Antisense reagents and technology have developed as extraordinarily useful tools for analysis of gene function. The capacity of antisense to reduce expression of RNA has led to the concept of using antisense molecules as drugs to treat cancer. On behalf of all your co-authors, you are prepared to accept this condition should your paper be published. You may also use this text box for any additional comments about your submission. (ASOs) inhibit translation or induce degradation of complementary target RNA, and both Phase I and Phase II trials such as antisense anti-IGF-I^[11], antisense oligonucleotide GTI-2040^[12], antisense oligonucleotide ISIS 5132^[13] are either completed or in progress. ASOs have proved their efficacy as targeted therapy in preclinical studies, have been well tolerated and able to modulate target protein expression in clinical studies^[14,15]. Here we examined the impact that antisense oligonucleotide NF- κ B on cell proliferation and tumorigenesis of gastric cancer in nude mice.

MATERIALS AND METHODS

Cell line and reagents

SGC-7901 gastric cancer cell line was obtained from Committee on Type Culture Collection of Chinese Academy of Sciences(Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco BRL, Life Technologies Inc., USA) including 10% inactivated fetal bovine serum, 2mM L-glutamine, 100U/ml penicillin, and 100 U/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO₂.

Synthesis of antisense oligonucleotide

P65 antisense oligonucleotide and misantisense oligonucleotides (MSODN)sequence was from the literature^[16]. Antisense sequence: 5' GGG GAA CAG TTC GTC CAT GGC 3', target sequence: 5' GCC ATG GAC GAA CTG TTC CCC 3', missense sequence: 5' GTA CGC GGT GAA GCT GCG ATC 3'. According to Genbank, ASODN and MSODN do not match with any known gene. They were synthesized by Takara Biotechnology Co., Ltd (Dalian, China) with phosphorathioate oligonucleotide technology followed by HPLC purification.

Transfection of human gastric cancer cell

Cells would be 90% confluent at the time of transfection, the liposome was respectively mixed with antisense and missense oligonucleotides in serum-free medium in accordance with DOTAP Liposomal Transfection Reagent(Roche, Swiss) instructions to form liposome-oligonucleotide complexes, which were then added into culture plate. The final concentration of oligonucleotide was 160nmolL⁻¹. Seventy-two hours after transfection, cells were harvested for Western Blot, cell immunofluorescence.

MTT examination

The cell proliferating activity was measured by MTT method. During logarithmic

growth phase, cells were digested by 0.05% trypsin containing 0.02% EDTA. Cells were seeded in 96-well plates at a density of 4,000 cells/well and there were triplicate wells in each experimental and control group. After cells transfected by P65 ASODN, MSODN, liposome and incubate for 24h, 48h, 72h and 96 hours, 20 μ l MTT (Sigma, USA) at a final concentration of 5 mg/ml was placed in each well, and incubated for 4 hours at 37°C. The reaction was stopped by adding 100 μ l DMSO(Sigma, USA), and the absorbance was measured at 490 nm in a microplate reader (μ Quant, Bio-Tek, Instruments, Inc. UAS). After treatment, cells were treated with MTT (5 mg/ml) and incubated for 4 h at 37 °C.

Preparation of nuclear extracts

After transfection, cells were washed with cold phosphate buffered saline (PBS), harvested, and pelleted, then resuspended in buffer A (10mM Hepes, pH 7.9, 10mM KCL, 0.6% Nonidet P-40, 1mM EDTA, 1mM PMSF and 1mM DTT) and incubated on ice for 15 minutes. After centrifugation at 14000 rpm for 15 minutes, the supernatant cytoplasmic protein was collected. The remnants were resuspended in buffer B (20mM Hepes, pH 7.9, 400mM KCL, 20% glycerol, 1mM EDTA, 1mM PMSF and 1mM DTT) and incubated on ice for 30 minutes, and stirred discontinuously every 10 minutes, then centrifugated at 14000 rpm for 15 minutes, the supernatant nuclear extract was collected. All proteins were stored at -80°C. Protein concentration was determined by BCA kit (Pierce Biotechnology Inc, USA).

SDS-PAGE and Western blot analysis

The nuclear extracts were boiled for 10 min, resolved by SDS-PAGE on an 10% polyacrylamide gel, and blotted onto nitrocellulose membrane. The membranes were incubated in blocking solution (5% non-fat dry milk in 20 mM Tris-HCl 150 mM NaCl and 0.1% Tween-20). After washing with TBS, membranes were incubated by primary antibodies solution (p65 monoclonal antibodies, Santa Cruz, USA, 1:500) for 3 hours at room temperature. Membranes were washed and incubated by the goat anti-mouse secondary antibody conjugated to alkaline phosphatase (1:1000, Santa Cruz, USA) for 1 hour. Then the membranes were then incubated with

HRP-conjugated secondary antibodies and visualized by ECL Plus system (Amersham Life Sciences, Piscataway, NJ). The difference of NF- κ B expression was judged according to the brightness of lanes.

Nude mouse gastric cancer models

BALB/c nude mice (4weeks old) were purchased from Shanghai Laboratory Animal Center(Shanghai, China). Mice were kept in a pathogen-free environment. The different cells transfected by P65 ASODN, liposome and P65 MSODN were trypsinized, washed twice with DMEM and resuspended in 0.1 ml PBS for transplantation in nude mice. 2×10^6 cells were injected into nude mice subcutaneously. The date on which the first grossly visible tumor appeared for subcutaneous injection was recorded, and the tumor size was measured every 3 days. Two-dimensional measurements were taken with an electronic caliper after injection, and tumor volume was calculated. Tumor volume was calculated by the following formula: volume (mm³) = $a^2 \times b / 2$, where a is the width in mm and b is the length in mm. Mice were sacrificed and dissected 4 weeks after cell inoculation.

Apoptosis detection by TUNEL assay

The reagent kit for apoptosis detection, TdT-FragEL DNA fragmentation detection kit was bought from ONCOGENE. Test procedures consisting of the following sections were provided in the brochure of the kit. Tumor tissues were fixed in 4% formaldehyde, dehydrated with gradient ethanol, and embedded in paraffin. Paraffin-embedded tissue samples were cut at 4 μ m and examined on a coated slide glass. The specimens were deparaffinized and hydrated gradually, and rinsed with 1 $\times$ TBS, then incubated with proteinase K (20g/mL in 10 mmol/L Tris-HCl) for 20 min. After immersed in 30 mL/L H₂O₂ at room temperature for 5 min and in TdT labeling reaction mixture at 37 $^{\circ}$ C for 1.5 h, specimens were covered with 1 $\times$ conjugate for 30 min, visualized by DAB and counterstained by hematoxylin. TBS took the place of primary antibodies as a negative control. After being mounted, sections were observed under microscope. The results of staining were analyzed and evaluated with American Image-Pro Plus software. The percentage of positive cells

with TUNEL staining in five 400×sights served as apoptosis index (AI).

HE staining and MVD detection

MVD was detected by HE staining and counted according to Weidner method. Areas of highest neovascularization were found by scanning the tumor sections at low power (×100 total magnification). After the area of highest neovascularization was identified, individual microvessel counts were made on a ×200 field. Any highlighted endothelial cell or endothelial cell cluster clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements was considered a single, countable microvessel.

Statistical analysis

The Statistical Package for the Social Science (SPSS) for Microsoft Windows 11.0 was used to complete the analysis of the collected data. All data were expressed as means ± SEM. The means of the different groups were compared using one-way analysis of variance (ANOVA) test. Significant differences were accepted when $P < 0.05$.

RESULTS

ASODN inhibits P65 expression

To determine the effect of ASODN on expression of P65 in gastric cancer cells, we transfected SGC-7901 gastric cancer cells with P65 ASODN. Simultaneously, P65 MSODN as a control. We observed the protein expression of p65 by Western blot analysis. The results showed that the expression of P65 was lower in the ASODN groups than the control groups ($P<0.001$, Fig 1). There is no difference among the control groups.

P65 ASODN inhibits SGC-7901 gastric cancer cell growth

MTT assay was performed at different time period (at 24, 48, 72, 96hrs) after transfection. Decreased cell survival rate was observed in the ASODN group(P65 ASODN working concentration is $0.4\mu\text{mol/l}$). The inhibition rates ranged from 30.06% to 45.12%, and the results are shown in Figure In the other groups, cells'viability is well. There was statistically significant difference in P65 ASODN group when compared to control group($P<0.05$, Fig2). The difference was not statistically significant among the other groups. There is no time-dependent difference in four groups.

P65 ASODN suppresses gastric tumor growth in vivo

Because it showed that P65 ASODN has a significant inhibitory effect on the growth of gastric cancer cells in vitro, we next studied the effect of ASODN on tumor growth in vivo. For this, we injected gastric cancer cell transfected by P65 ASODN into nude mice. We periodically monitored the growth of tumor for 24 days. In the P65 ASODN group, the tumor volume is smaller than that in P65 MSODN-treated mice as reflected in Fig. 3 and Tab1. ($P<0.05$ at day 9, 12, 15, 18, 24, 21 and 24, respectively). To evaluate a potential toxicity of liposome vector, we found the tumor volume were similar in liposome and the control group without transfection of liposome vector ($P>0.05$), indicating that liposome does not have notable toxicity to mice.

P65 ASODN reduce the microvessel density(MVD) and induce apoptosis in vivo

At the endpoint, mice were sacrificed and examined for angiogenesis in tumor by MVD analysis and cell apoptosis by TUNEL. MVD in the P65 ASODN group was reduced significantly (Fig. 4A, left panels). The ASODN group had a mean MVD of 3.8 ± 0.8 , while MVD in control, liposome, MSODN group was 7.4 ± 1.5 , 6.8 ± 0.8 , 6.4 ± 1.1 microvessels respectively. There was statistically significant difference between ASODN group and the other groups ($P < 0.01$). The difference among MSODN, liposome and control group had no statistically significance ($P > 0.05$). P65 ASODN suppresses the gastric tumor growth, it maybe related to reducing angiogenesis. For induction of apoptosis in the xenograft model, TUNEL-positive cells were distinguished by brown-stained nuclei (Figure 4B, right panels). The number of apoptotic cells in P65 ASODN groups had increased. The mean AI in ASODN group was $21.67 \pm 2.52\%$, AI in control, liposome, MSODN group was $15.33 \pm 0.58\%$, $7.33 \pm 1.53\%$, $9.0 \pm 1.73\%$ respectively. The apoptosis index of the P65 ASODN group were significantly higher than that in the control groups ($P < 0.001$). It showed P65 ASODN maybe induce gastric cancer cells apoptosis in vivo. These results strongly indicate that the inhibition of P65 ASODN induced cell apoptosis and eventually led to tumor growth suppression, which is consistent with our in vitro data.

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DISCUSSION

In 1986, Baltimore first discovered transcription factor nuclear (NF- κ B) in mature B cell nucleus^[17]. It was one of the nuclear proteins, which bind with DNA enhancer of immunoglobulin κ light chain. In mammalian cells, it was a kind of dimeric protein complex, composed of homodimers and heterodimers derived from five distinct subunits, including NF- κ B1 (p50), NF- κ B2 (p52), RelA (p65), Rel B, and c-Rel. P65 contains transcription activating area, and participates in original regulation of gene transcription. Simultaneously, p65 facilitates the combination of p50 with DNA. All family members share a highly conserved Rel homology domain (RHD, ~300aa) responsible for DNA binding, dimerization domain, and interaction with I κ Bs, the intracellular inhibitor of NF- κ B^[18,19]. In most quiescent normal cells, the majority of NF- κ B complexes are kept predominantly as an inactive form cytoplasmic and in an inactive form by binding to a family of inhibitory proteins, the I κ Bs. Many divergent extracellular stimuli including proinflammatory cytokines such as TNF- α , interleukin-1, lipopolysaccharide (LPS), double-stranded RNA, and physical and chemical stresses can induce rapid phosphorylation and degradation of I κ B, allowing nuclear translocation of NF- κ B^[20]. This process is called activation of NF- κ B. The signaling pathways involved in NF- κ B constitutive activation remain largely unknown. Mechanism of constitutive activation of NF- κ B may be involved in activation of tyrosine kinase, NIK, and IKK^[21]. NF- κ B is involved in cancer development, modulating NF- κ B activation pathways has important implications in cancer prevention and therapy. Due to the complexity of NF- κ B roles in different cancers, careful evaluation of NF- κ B in each cancer type is crucial in this regard. More cancer cell-specific NF- κ B inhibiting means are desired for improving anticancer efficacy and reducing systemic toxicity. In this study, it was suggested that P65 ASODN could inhibit gastric cancer cell proliferation, it maybe related to the decreased expression of P65. Many cell cyclin gene expression is NF- κ B-dependent^[22]. The best explored link between NF- κ B activation and cell cycle

progression involves cyclin D1, a cyclin which is expressed relatively early in the cell cycle and which is crucial to commitment to DNA synthesis. Experiments in many cell types now indicate that NF- κ B acts through increasing the abundance of cyclin D1 and thus the activity of the cyclin D1 kinase holoenzyme complex. The role of NF- κ B factors in controlling the cell cycle and cyclin D1 has been well shown in investigations that used the I κ B “super repressor” (I κ B-SR), to inhibit NF- κ B activity. I κ B-SR effectively blocks the serum-induced increase in nuclear NF- κ B DNA binding activity in mouse embryo fibroblasts and delays S-phase entry when the cells are growing synchronously^[23]. The regulator of cell-cycle progression cyclin D1 is upregulated in a variety of cancer cells by mechanisms involving mainly transcriptional activation. Analysis of the human cyclin D1 promoter has revealed the presence of two κ B sites located at -840 and -33, designated κ B1 and κ B2, respectively, and mutational analysis demonstrated that p50/p65 binding to the -33 κ B2 site was important for cyclin D1 transcriptional regulation during proliferation induced by serum or growth factors^[24].

And also in vivo, we found P65 ASODN inhibited tumor growth which lead to apoptosis increasing and angiogenesis reducing. In vitro and in vivo, both liposome only and MSODN can also inhibited gastric cancer cells proliferation and inhibited tumorigenesis, which related to slight toxicity of liposome, there is no statistic difference. It was well known that tumor angiogenesis is dependent on many proinflammatory cytokines and growth factors such as IL-8, TNF- α and VEGF. NF- κ B plays a pivotal role in secreting the angiogenesis factors^[25,26]. Constitutive NF- κ B activation in cancer cells also triggers autocrine of angiogenic chemokines, and NF- κ B inhibition substantially suppresses tumor growth and angiogenesis^[27]. Previously, there reported NF- κ B-mediated IL-8 and angiogenin expression was involved in this process^[28]. In this experiment, we noticed that P65 ASODN as inhibitor of NF- κ B leads to an decrease in tumor angiogenesis in nude mice.

The evidence that cancer development is a complex and multistep process, characterized by alterations of genes involved in the regulation of proliferation, apoptosis and angiogenesis, has led to development of new therapeutic strategies

based on the use of agents able to selectively inhibit key molecules of these pathways. Although these agents have shown considerable promise for antitumoral therapy, treatment with ASODN used as single agent does not seem particularly promising because of the multigenic alterations of tumors.

Transcription factors have potential as targets for an individualized therapeutic approach to cancer^[29]. Hopefully, in the next few years several NF- κ B inhibitors that can increase the therapeutic efficiency of chemotherapy and radiotherapy will be successfully employed in treatment of gastric cancer patients.

Conflict of Interest

No potential conflicts of interest were disclosed.

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FIGURE LEGENDS

Fig 1. Changes in the expression of P65 in gastric cancer cells treated after transfecting ODN.

A. Western blot analysis of P65 expression. Scans of original blots with GAPDH used as loading controls. A representative blot is shown from three independent experiments with identical observations. **B.** The expression of P65 was significant lower in the P65 ASODN transfected group when compared to control, liposome and MSODN group($P<0.001$).

Fig2. Inhibitory effects of P65 ASODN on SGC-7901 gastric cancer cell growth examined by MTT assay.

A . The cell proliferating activity on 24, 48, 72 and 96h in the experimental group was significant inhibited when compared to control, MSODN, liposome group ($P<0.01$). The data are represented as mean $\pm$ standard deviation. **B.** Green fluorescent in gastric cancer cells after transfection with P65 ASODN. Photograph show that P65 ASODN was tansfected into cancer cell.

Fig3. Comparson of tumor size among different groups and tumor growth curve in nude mice.

A. All mice were monitored for survival for 24 days. At 24th day, mice were killed and tumors were removed. Representative photographs show tumor size in different

groups. **B.** Tumor volume curve. Tumor volume (mm^3) was also measured by external calipers twice per week and calculated as described under Materials and methods. Each point represents the mean for $n=15$ animals. The results represent the mean $\pm$ standard deviation. The experiment was repeated independently at least three times with similar results. Significant inhibition in MSODN group versus in other groups at the termination of the experiment($P<0.05$).

Fig4. Comparison of MVD and AI in nude mice.

A. Blood vessel morphology in tumor xenografts($\times 200$). Blood vessels were stained by HE staining in light microscopy at $200\times$ magnification. Arrows show blood vessel. Tumors characterized by large and thick, singly distributed vessels, often with a visible lumen were assigned as large vessels, while tumors characterized by small and thin vessels forming networks without a visible lumen were assigned as small vessels. The mean MVD were 7.4 ± 1.5 , 6.8 ± 0.8 , 6.4 ± 1.1 , 3.8 ± 0.8 in control, liposome, MSODN and ASODN group respectively. **B.** Detection of apoptotic cells in tumor tissue by TUNEL assay($\times 200$). Arrows show TUNEL-positive cells. The mean apoptosis index were $15.33\pm 0.58\%$, $7.33\pm 1.53\%$, $9.0\pm 1.73\%$, $21.67\pm 2.52\%$ respectively. **C.** Histograms show AI in different groups. There was statistically significant difference between ASODN group and the other groups($P<0.001$). **D.** Histograms show MVD in different groups. There was statistically significant difference between ASODN group and the other groups($P<0.01$).

A

B

Fig1

Fig2.

A

B

Fig3.

Fig4.

TABLE

Tab1. The tumor volume in different groups and in different time

Groups	Tumor volume (mm^3 , $\bar{X} \pm SD$)						P value
	9d	12d	15d	18d	21d	24d	
control(A)	69±52	150±112	369±291	776±353	930±448	1048±486	
liposome(B)	75±60	207±124	382±221	709±320	841±337	1067±350	
MSODN (C) **	52±32	102±56	252±170	625±163	776±175	911±229	>0.05
ASODN (D)*	27±24	61±54	171±118	265±213	334±247	382±270	<0.05

* The tumor volume in group D is smaller than the other groups($P < 0.05$).

** There is no difference among tumor volume in group C and A, B($P > 0.05$).