

RESEARCH COMMUNICATION

Antitumor Activity of Decoy Oligodeoxynucleotides Targeted to NF- κ B *in vitro* and *in vivo*

Tao Wang¹, Qing-Hua Li², Gang-Ping Hao³, Jing Zhai^{1*}

Abstract

Background: Nuclear factor-kappaB (NF- κ B), a transcription factor, is abundantly expressed in many tumors and regulates many tumor-relative genes such as c-myc and caspase-8, which NF- κ B-mediated genes activation serves as an anti-tumor pathway by regulating expression of these tumor-relative genes. Given the important roles of these genes in tumor control, the present study was to test the hypothesis that NF- κ B decoy ODNs transfected into tumor cells *in vitro* and *in vivo* might affect growth and apoptosis. **Methods:** First, NF- κ B decoy oligodeoxynucleotides were designed according to the NF- κ B elements in the promoter region of c-myc gene. Then, suppression effects of decoy ODNs on proliferation of five carcinoma cell lines were assessed by MTT assay. Apoptosis of carcinoma cells was determined by chromosome DNA ladder and flow cytometric analysis (FCM). Thirdly, suppression effects of NF- κ B decoy ODNs on proliferation of carcinoma *in vivo* were investigated in nude mice. To confirm mechanisms of action, a pGL3-C-MYC luciferase expression vector containing a fragment of the c-myc promoter was constructed and co-transfected with NF- κ B decoy ODNs into SKOV-3 cells by lipofectamineTM2000. Expression levels of endogenous c-myc and caspase-8 genes were assessed by northern blotting. Lastly, nuclear extracts were prepared from SKOV-3 cells and DNA-protein interactions were examined by electrophoretic mobility shift assay (EMSA). **Results:** Treatment of cancer cell lines with NF- κ B decoy ODNs resulted in strong suppression of proliferation, especial of the SKOV-3 ovarian cancer cell line *in vitro* and *in vivo*. Induction of apoptosis of SKOV-3 was observed in DNA gel electrophoresis and FCM. Activity of luciferase was significantly reduced in the NF- κ B decoy-transfected cells, but not in cells transfected with a control decoy. Furthermore, we found that transcripts of endogenous c-myc gene were reduced, while caspase-8 transcripts were induced. EMSA demonstrated specific binding of the NF- κ B decoy to NF- κ B protein. **Conclusions:** These findings indicated that NF- κ B activation plays an important role in proliferation in many cancers, especially ovarian carcinomas. Inhibitors of NF- κ B may thus offer promise as a therapeutic approach for the treatment of tumors via manipulating expression of desired target genes. NF- κ B decoy ODNs may allow development of therapeutic and investigative tools for human malignancies.

Key Words: NF- κ B - decoy oligodeoxynucleotides - ovarian cancer - antitumor effects

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Introduction

Human malignant tumors are very frequently diagnosed diseases and the second leading cause of diseases death that affect millions of people worldwide. The risk of malignant tumor increases steeply with age in older men as fewer die of cardiovascular disease. The majority of patients selected for treating clinically localized disease with radical tumorectomy, chemotherapy and radiation continue to harbor pathologically more advanced disease. Hence surgical, radiation therapy and chemotherapy as monotherapy or in combination do not appear to be adequate to control locally clinical or progressive stage human malignant tumor.

The concept that human malignant tumor is a genetic disease is based on observations that normal gene expression is frequently altered. Aberrant gene expression may develop due to various reasons, including inherited

or acquired gene gain, loss or mutation as cells become malignant. This altered gene expression may affect normal cellular functions, leading to the phenotypic and characteristic changes associated with malignancy. It is well documented that the altered gene expression in human malignant tumor is characterized by overexpression of proto-oncogenes and inactivation of tumor suppressor genes. Therefore, it is conceivable that resuming normal gene expression at one or more of these points in the pathway to fatal disease may reverse, arrest or delay this progression. The regulation of genes expression include transcriptional regulation, post-transcriptional regulation (mRNA splicing and editing), translational regulation and so on.

Decoy strategy was recently developed into a powerful tool for studies of gene regulation *in vivo* and *in vitro* (Tomita et al., 2000; Mann et al., 2005), with more efficiency and selectivity than the antisense

¹Institution of Biochemistry, Department of Basic Medicine, Taishan Medical University, ²Affiliated Hospital of Tai Shan Medical College, Taishan, Guangdong, China *For Correspondence: neuroman@163.com

oligonucleotide. Via competitively binding to specific transcription factor, decoy ODNs could suppress its binding to the promoter of its target gene, thereby reduce expression of its target genes (Feeley et al., 2000; Crinelli et al., 2002). In this study, we synthesized an NF- κ B decoy ODNs, a phosphorothiolated double stranded Oligodeoxynucleotides. Its proliferation inhibition and apoptosis induction of tumor cells was evaluated in vivo and in vitro. The specificity of its function and mechanism of NF- κ B decoy ODNs were studied.

Materials and Methods

Cell culture

Ovarian carcinoma cell lines OVCAR-3 and SKOV-3, hepatocarcinoma cell lines SMMC-7721, Lung cancer cell lines Lewis, colorectal carcinoma cell lines SW480 were purchased from Institute of Shanghai Biochemistry and Cell Biology.

Five kinds of carcinoma cell lines were maintained in RPMI-1640 or DMEM medium supplemented with 5% cattle bovine serum, 100 units/ml penicillin, and 0.1mg/ml streptomycin at 37°C in a 5% CO₂ incubator.

Decoy oligodeoxynucleotides synthesis and annealing

NF- κ B decoy oligodeoxynucleotides was designed according to the NF- κ B elements in the promoter region of c-myc gene. The NF- κ B decoy oligodeoxynucleotides bound specifically to activated NF- κ B and blocked binding of NF- κ B to a NF- κ B binding element. By contrast, Control-1 decoy ODNs, two mutated version of the decoy that differed by two base pair did not bind the activated NF- κ B protein. The Control-2 decoy ODNs had sequences of unrelated NF- κ B element sequences and was used in the control studies TEN buffer (10mmol/L Tris-HCl, 0.1mmol/L EDTA, 0.1mol/L NaCl, PH 8.0) diluted oligodeoxynucleotides respectively. Complement oligodeoxynucleotides were mixed as equal mol, 95°C, 10min, then cooled down to room temperature slowly.

NF- κ B decoy ODNs:

5'-CCTTGAAGGGATTTCCCTCC- 3'
3'-GGAAGTCCCTAAAGGGAGG-5'

Control-1 decoy ODNs:

5'-CCTTGAAGAGATTTCTCTCC- 3'
3'-GGAAGTCTCTAAAGAGAGG-5'

Control-2 decoy ODNs:

5'-GTCTGATAAAGGGTGTCTTTTT- 3';
3'-CATACTATTTCCAC AAGAAA AA-5'

Transfection experiments

The day before transfection, Five kinds of carcinoma cells were seeded at a density of 2x10⁵ cell/cm². 24 hour later, when cells reached 70%-80% confluency, the decoy ODNs was transfected into carcinoma cells by LipofectaminTM2000 according to the manufacturer's instructions. cells were divided into four groups. NF- κ B decoy ODNs was transfected into one group, Control-1 decoy ODNs was transfected into second group, Control-2 decoy ODNs was transfected into third group, the fourth group was used as a internal control. 0.8-1.6ug /100 μ l decoy ODNs and 2-4 μ l/100 μ l lipofectamin were diluted

into 100 μ l medium without serum respectively. Then the above two were combined and layed in room temperature for 20 minute. The complexes were added onto the cells cultured with serum-free medium. 6 hour later, the medium with serum was added. After transfection, carcinoma cells were detected.

Anti-tumor spectrum analysis of NF- κ B decoy ODNs

The effect of NF- κ B decoy ODNs on five kinds of carcinoma cells proliferation were Measured by MTT colorimetric assay. Ovarian carcinoma cell line (OVCAR-3 and SKOV-3), lung carcinoma cell line (Lewis), colorectal carcinoma cell line (sw480), hepatoma carcinoma cell line (SMMC-7721) were seeded at a density of 1x10⁴ cells/well into 96-well plates. 4ug /100 μ l NF- κ B decoy ODNs and 4 μ l/100 μ l lipofectamin were diluted into 100 μ l medium without serum respectively. Then the above two were combined and layed in room temperature for 20 minute. The complexes were added onto the cells cultured with serum-free medium 6 hour later, the medium with serum was added. MTT (5mg/mL) was added to the wells (10 μ L/well) respectively at 72h after transfection as before. After another 4 hours incubation at 37°C, the media in the wells were replaced with dimethylsulfoxide(DMSO)(150 μ L/well). The plates were agitated at room temperature for 10min. Absorbance (A value) of every well at 570nm wave lengths was read on an ELISA reader.

SKOV-3 cells Cell viability assay

Proliferation of SKOV-3 cells in vitro was firstly measured by MTT colorimetric assay. SKOV-3 cells were seeded at a density of 1x10⁴ cells/well into 96-well plates. NF- κ B decoy ODNs was added to each well and the final concentration should be 2, 4, 6, 8 μ g/mL. The incubation duration for each concentration group should be 4h. Each group should include 6 wells. 6 wells were taken as normal cell group and another 6 wells were taken as blank group (only cell culture fluid included). The plate was incubated at 37°C in a humidified incubator, 5%CO₂ for 24 hours. Then MTT (5 mg/mL) was added into each well (10 μ L/well) and the plate was incubated at 37 °C for 4 hours. the medium in the wells were replaced with dimethylsulfoxide (DMSO) (150 μ L/well).The plates were agitated at room temperature for 10min. Absorbance (A value) of every well at 570nm was read on an ELISA reader.

Second, SKOV-3 cells were cultured in 6-well plates at a density of 2x10⁵ cells per well, washed and incubated with 10 μ M of the NF- κ B decoy ODNs or control ODNs. After four hours of preincubation, cells were either incubated with DMEM medium supplemented with 5% cattle bovine serum for 24h. Thereafter, cells were washed and The change of cell morphology was observed by inverted phase contrast microscope.

Apoptosis assay

Two different assays were used to determine the cell apoptosis. Analysis of DNA fragmentation was carried out according to manufacturer's instructions. After transfection of 48h, SKOV-3 cells were harvested and DNA was extracted. The degradation of DNA was

investigated by DNA gel electrophoresis.

The apoptotic level of cells incubated with decoy ODNs was determined by FCM analysis after PI staining. Approximately 106 cells (70% confluent monolayer) were transfected with decoy ODNs in culture medium without antibiotics at 37°C in a CO₂ incubator. After transfection of 48h, SKOV-3 cell cells were harvested and then fixed with ice-cold 70% ethanol for cytometric analysis. DNA content of the subdiploid peak, which indicated apoptotic cells, was determined by flow cytometric analysis (FCM). NF- κ B decoy ODNs suppressing tumor growth in nude mice

T-cell immune deficiency nude mice (18~22 g, 6 weeks, female) were purchased from the Experimental Animal Center of Chinese Medicine and Biological Products Test Institute. Nude mice were housed in laminar airflow racks under pathogen-free conditions.

SKOV-3 cells were grown as a monolayer in RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum, antibiotics, and antimycotics at 37°C in a humidified 95% air, 5% CO₂ atmosphere. Tumor cells growing exponentially were harvested by brief incubation with 0.25% trypsin-EDTA solution (GIBCO). Xenografts of SKOV-3 were initiated by injection of 1x10⁷ cells into the left flanks of seven female nude mice.

SKOV-3 tumors resulting after 8 weeks of growth were aseptically dissected and mechanically minced; 3-mm³ pieces of each tumor tissue were transplanted by trocar needle into female nude mice under methoxyflurane anesthesia. Ten days after transplantation, tumor-bearing mice were divided into four groups of four animals each. The three control groups were respectively injected with control-1 decoy ODNs, control-2 decoy ODNs and lipofectamine and the experimental group was treated with 1mg/kg of NF- κ B decoy ODNs once a day. The treatment was continued for 30 days. The tumors were measured once a week with microcalipers, and the tumor volume was calculated as length x width x height x 0.5236. At the end of the experiment, mice were anesthetized with methoxyflurane and sacrificed by decapitation. Body weights were recorded and the tumors were carefully dissected, cleaned, and weighed, and samples of each tumor were taken for weighing.

The effect of NF- κ B decoy ODNs on the promoter of the endogenous gene^{oo}

To generate a -1279/+36 bp fragment of the c-myc promoter, human genomic DNA was used as a template for the polymerase chain reaction (PCR). The PCR product containing the 1315 bp c-myc promoter fragment with single Kpn₁ site at 5' end, was digested with the corresponding enzymes, purified from agarose gel and inserted into the upstream of luciferase gene of the pGL3-Enhancer vector pre-cut with Kpn₁ and Sma₁ enzymes. The recombinant plasmid was transformed into competent *E. coli* DH-5_α. The plasmid was first screened and isolated by using double digestion with the above restriction enzymes and gel electrophoresis. It was further verified by DNA sequencing. The recombinant construct was named after pGL3-C-MYC.

The day before transfection, SKOV-3 cells were

seeded at a density of 2x10⁵/per well in a 12-well plate. 18-24 hours later, when cells reached 90%-95% confluence, the double stranded decoy ODNs and plasmids as indicated were co-transfected into SKOV-3 cells by lipofectamineTM2000 according to the manufacturer's instructions. 0.8-3.2μg plasmid/decoy ODNs and 2-8μL lipofectamine were used per well. The cells were incubated with 0.8 mL serum-free medium during the transfection. Plasmid pSV₂-Gal was used as an internal control. The ratio of pSV₂-Gal and experimental vector was 1x25.

Dual-luciferase Reporter Assay System was used for this procedure. The transfected cells were washed once by PBS and harvested following the addition of 1xPLB (passive lysis buffer) by scrapping vigorously with a rubber policeman. The luminometer was programmed to perform a 2-sec ODNs premeasurement delay, followed by a 10-sec ODNs measurement period for each reporter assay. 100μL Luciferase Assay Reagent (LAR) and 20μL cell lysate were mixed in the luminometer tube and placed in the luminometer to initiate reading as M1. Then another 100μL Stop&Glo reagent was added into the above tube. The luciferase activity was measured as M2. To eliminate the influence of the efficiency of transfection, the ratio M1/M2 was used for representing the luciferase relative activity of each sample. The above experiments were repeated at least threetimes.

Efficacy of NF- κ B decoy oligodeoxynucleotides on c-myc and caspase-8 expression in SKOV-3 cells in vitro analyzed by Northern blot Analysis

The SKOV-3 cells were seeded at a density of 4x10⁴ cells/ml and treated with the NF- κ B decoy oligodeoxynucleotides. As controls, some SKOV-3 cells were treated with control decoy oligodeoxynucleotides, lipofect, in parallel experiments. The cells were harvested and treated for 24 h with NF- κ B decoy. Total RNA was harvested using an RNeasy kit (Qiagen) according to the manufacturer's instructions. For each sample, 15 μg of total RNA was loaded on 1.2% agarose/formaldehyde gels and transferred on to Nytran Super Charge membranes (Schleicher and Schuell, Dassel, Germany). Membranes were hybridized overnight at 65 °C and then washed three times for 30 min each at 65 °C in buffer with the gene-specific primers which was 32P-labelled using the Rad Prime DNA Synthesis random-primed DNA labeling kit (Life Technologies). Detection was performed according to standard procedures as specified by the manufacturer (Roche).

Electrophoretic mobility shift assay (EMSA)

The nuclear extracts were prepared from SKOV-3 cell according to the manufacturer's instructions. The protein concentration of nuclear extracts was measured by using Bradford assay. EMSA was performed by using DIG gel shift kit (Roche) according to the manufacturer's instruction to confirm specific binding of the NF- κ B decoy ODNs to the SKOV-3 cell nuclear proteins. Firstly, NF- κ B decoy ODNs were labeled with DIG-11-ddUTP at 3' end by terminal transferase as the probes. 30-60 fmol labeled probes were incubated with the above nuclear

extracts (5-8 μ g) in a buffer for 30min at 25°C. Then, the DNA-protein complexes were separated by electrophoresis using a 6% native-PAGE at 25V for 1.2h. The bands in gels were transferred to the positively charged nylon membrane by electroblotting in 0.25_TBE transfer buffer at 400mA for 40min. Afterwards, the membrane was baked to fix the bands and incubated with blocking reagent anti-DIG-AP, Fab fragment and CSPD in turn. Finally, the membrane was sealed into hybridization bag and exposed to x-ray film in darkroom. In competition experiments, the unlabeled NF- κ B decoy ODNs (100 fold molar excess) was added during DNA-protein incubation period. In nonspecific competition experiments, the unlabeled control-1 decoy ODNs and Control-2 decoy ODNs (100 fold molar excess) were used. For antibody supershift studies, the anti-NF- κ B rabbit monoclonal antibody was used. The nuclear extract was preincubated with the antibody in the absence of poly[d(I-C)] for 30 min at 37°C. Before incubation with the NF- κ B decoy ODNs, poly [d(I-C)] was added.

Statistical analysis

Data of anti-tumor spectrum, proliferation, apoptosis, and luciferase assays were analyzed using the SPSS 13.0 software package. All variables were expressed as mean (M) and standard deviation (SD). Multiple group comparison experiments were validated by ANOVA. Each experiment was repeated at least three times. $P < 0.05$ was considered significant.

Results

Anti-tumor spectrum analysis of NF- κ B decoy ODNs

One of the characteristics of tumor cells is malignant proliferation. NF- κ B decoy ODNs, through specific binding to transcription factor NF- κ B, changed expression of downstream gene to inhibit the proliferation of malignant tumor cells. the determination of NF- κ B decoy ODNs on the inhibition of different tumor cell proliferation (Figure 1). The results showed that, there are different degrees of inhibition amongs Ovarian Epithelial carcinoma cell line (SKOV-3 and OVCAR-3), lung carcinoma cell line (Lewis), colorectal carcinoma cell line (sw480), hepatoma carcinoma cell line (SMMC-7721). Compared with orther kinds of carcinoma cells, the Ovarian carcinoma cell line SKOV-3 Cell showed a significantly inhibited proliferation, inhibition rates reacheding 80%. Lung carcinoma, colorectal carcinoma, hepatoma carcinoma has a moderate inhibitory rate of 50% or so. This also indicates that NF- κ B decoy in vitro can inhibit the growth of multi-carcinoma cell lines. Ovarian carcinoma cell line SKOV-3 was selected as the further experimental object.

NF- κ B decoy ODNs inhibits proliferation of SKOV-3 cells in vitro

Shape of SKOV-3 Cells gradually became from spindle or polygonal into round, some cells shrinking, some cells falling off from the adherent state. SKOV-3 cells were quite sensitive to NF- κ B decoy and inhibited by NF- κ B decoy ODNs, while relatively resistant to

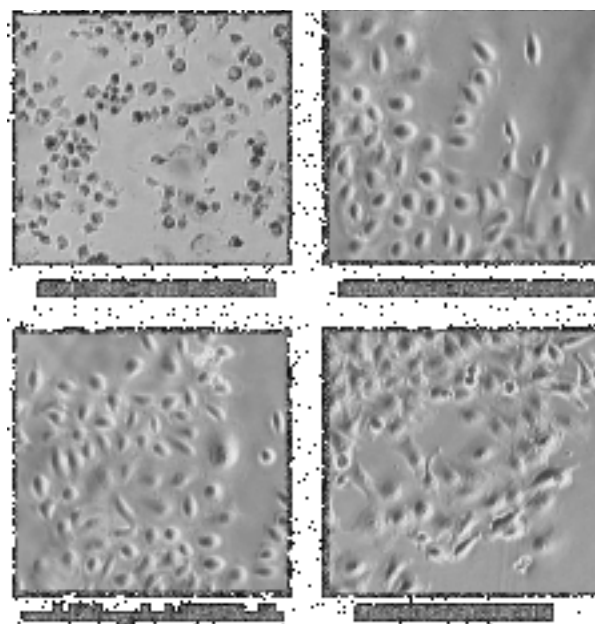


Figure 1. Inhibitory Effects of NF- κ B decoy ODNs on Cancer Cells *in vitro*. Five kinds of carcinoma cells were seeded in 96 well plates. After 24 h, the cells were treated with NF- κ B decoy ODNs, control ODNs or lipofect control using lipofectamine. The cell viability was determined using MTT assay

control decoy ODNs and lipofect.

When SKOV-3 cell lines was treated with NF- κ B decoy ODNs, control decoy ODNs and analyzed for viability by MTT assay, the results demonstrated that with treatment of control,the SKOV-3 cells lines normally grew. Treated with NF- κ B decoy ODNs, the proliferation of SKOV-3 Cells was significantly inhibited, which growth rate decreased. The results indicated that NF- κ B decoy ODNs had a direct inhibitory effect on the proliferation of SKOV-3 cells in vitro, a dose-dependent inhibition of proliferation with maximum inhibition in SKOV-3 Cell lines at a dose of 8 μ g/mL.

NF- κ B decoy ODNs induces apoptosis of SKOV-3 cells in vitro

Apoptosis of SKOV-3 cells was measured after cells were exposed to NF- κ B decoy ODNs, control decoy ODNs and lipofect for 48h using the DNA fragmentation assay and the FCM analysis. Figure 2(A) showed the electrophoretic result of extracted DNA from SKOV-3 cells. it clearly indicated DNA degradation after a 48h incubation with NF- κ B decoy ODNs. No such degradation was observed when SKOV-3 cells were untreated or incubated with control decoy ODNs.As shown in Figure 2(B) using FCM analysis, the percentage of apoptotic cells after treatment with NF- κ B decoy ODNs was increased in SKOV-3 cells (39.1% \pm 10.6%) compared to that treated with lipofect(10.4 \pm 13.7%).

Inhibiting effect of NF- κ B decoy ODNs on Growth of SKOV-3 tumor in nude mice

After 15 days of treatment, the volume of SKOV-3 tumors in the group receiving NF- κ B decoy ODNs was significantly ($P < 0.05$) decreased to 0.18 \pm 0.06 cm³ as compared with the control groups and lipofect groups

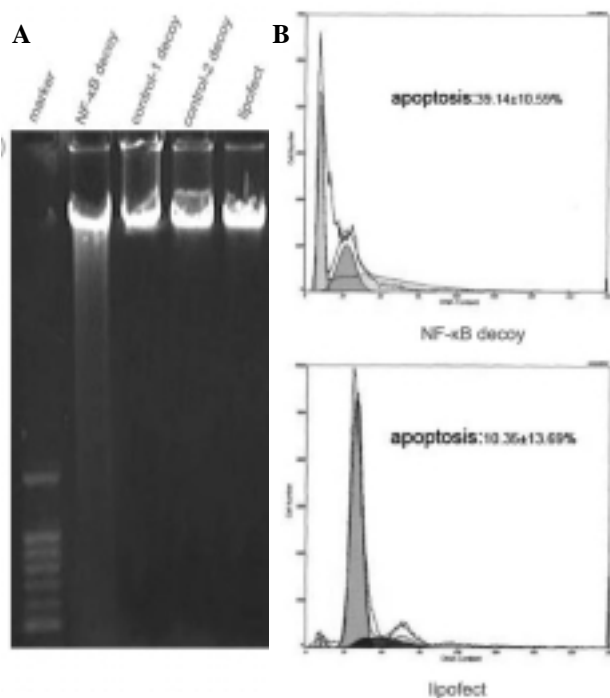


Figure 2. Apoptosis of SKOV-3 Cells Induced by NF-κB decoy ODNs. DNA degradation after a 48h incubation with NF-κB decoy ODNs was observed, no such degradation in SKOV-3 cells untreated or incubated with control decoy ODNs (A). Meanwhile, the percentage of apoptotic cells after treatment with NF-κB decoy ODNs was increased in SKOV-3 cells (39.1%±10.6%) compared to that with treatment of lipofect(10.35±13.69%)(B).

(0.53 ± 0.18 cm³) corresponding to a 66.1% reduction (Figure 3), After 20 days, the final tumor volume was significantly (P < 0.01) diminished in animals treated with NF-κB decoy ODNs to 0.28 ± 0.07 mm³ compared with controls, which measured 1.43 ± 0.15 mm³, corresponding to a 80.4% decrease. At the end of the experiment, there also was a 66.8% (P < 0.01) reduction in tumor weight in the group injected with NF-κB decoy ODNs (112.5 ± 37.1 mg) as compared with the control group (338.0 ± 24.3 mg). There were no significant differences in body weights between the treated (32.7 ± 1.57 g) with control decoy ODNs and treated with lipofect (32.8 ± 2.35 g) animals. Inhibiting effects of the NF-κB decoy on the promoter of endogenous gene regulated by NF-κB

In order to prove sequence specificity and rates of NF-κB decoy ODNs inhibiting, different doses of NF-κB

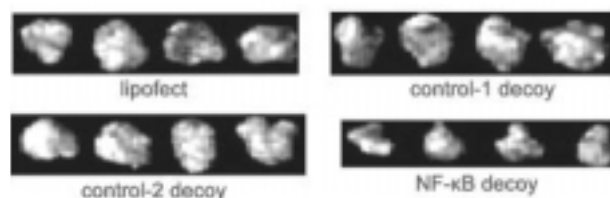


Figure 3. Inhibitory Effect of NF-κB Decoy ODNs on SKOV-3 Tumor-bearing Nude Mice. Human SKOV-3 tumor-bearing nude mice were established as described in materials and methods. The mice were intratumorally injected daily with NF-κB decoy ODNs, control ODNs or lipofect for 30 days, and the tumor volumes and weights were measured at the indicated day

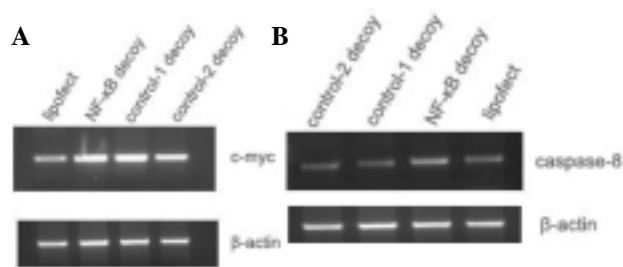


Figure 4. Efficacy of NF-κB Decoy Oligodeoxynucleotides on c-myc and caspase-8 Expression in vitro. SKOV-3 cells were treated with the NF-κB decoy, lipofect and control ODNs. Total RNA was isolated from cells after 24h following the treatment. The level of c-myc and caspase-8 mRNA was determined by Northern blot Analysis. The NF-κB decoy ODNs treatment-inhibited c-myc transcription (A) and induced accumulation of caspase-8 (B)

decoy ODNs were co-transfection with 0.19mg/L pGL3-enhancer and 7.6_g/L pSV-_-Gal. It turned out that NF-κB decoy ODNs could significantly inhibit the activity of luciferase, a dose-dependent inhibition of proliferation with maximum inhibition at a dose of at the dose of 2.4mg/L, at which the inhibition rate reached 80%, while control decoy ODNs and lipofect not significantly inhibiting. The result also showed that with obvious sequence specificity, NF-κB decoy ODNs could inhibit the activity of the promoter.

The NF-κB decoy oligodeoxynucleotides treatment caused an inhibition in expression of the c-myc gene or an increase in expression of the caspase-8 in SKOV-3 cells

We first determined (Figure 4.A) the NF-κB decoy ODNs treatment transcription of endogenous c-myc gene. The SKOV-3 cells were treated with the NF-κB decoy ODNs. As controls, some SKOV-3 cells were treated with the control ODNs and lipofect. The cells were harvested and total RNA was isolated. Northern blot assay was then performed to determine the level of endogenous c-myc mRNA in each RNA sample. When treated with the control ODNs and lipofect, no significant decrease in the level of endogenous c-myc mRNA was observed in the SKOV-3 cells. When treated with the NF-κB decoy ODNs, however, significant decreases in the level of the c-myc mRNA were detected in the SKOV-3 cells.

We also determined (Figure 4.B) that the NF-κB decoy ODNs treatment effectively increases the transcription of endogenous caspase-8 genes in the SKOV-3 cells compared to control decoy ODNs and lipofect.

These results suggested that the treatment of SKOV-3 human ovarian carcinoma cells with the NF-κB decoy ODNs caused an decreased expression of the endogenous c-myc genes in the SKOV-3 cells and an increased expression of the endogenous caspase-8 genes in the SKOV-3 cells.

The specific binding of NF-κB decoy ODNs with protein

To examine whether the NF-κB decoy ODNs was able to bind to NF-κB protein, electrophoretic mobility shift assay was performed. The nuclear extracts from SKOV-3 cells were used for in vitro binding assay with double

control-1 decoy	-	-	+	-	-	-
control-2 decoy	-	-	-	-	+	-
anti-NF- κ B antibody	-	-	-	-	-	+
unlabeled NF- κ B decoy	-	-	-	+	-	-
labeled NF- κ B decoy	+	+	+	+	+	-
protein	-	+	+	+	+	+

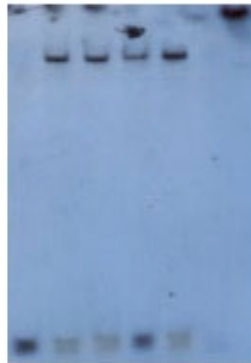


Figure 5. Determination of Specific Binding of NF- κ B Decoy Oligodeoxynucleotides to NF- κ B *in vitro*. The radioactively labeled NF- κ B decoy ODNs was incubated with SKOV-3 nuclear extracts at room temperature for one hour and then analyzed by electrophoretic mobility shift (EMSA)

strands NF- κ B decoy probes and the complex of DNA-protein was observed. In the competition study with a 100-fold molar excess of the unlabeled NF- κ B decoy ODNs, the formation of this complex was inhibited, though not with the same molar excess of the unlabeled control-1 decoy and control-2 decoy ODNs, indicating that the binding of the NF- κ B decoy ODNs to the SKOV-3 nuclear protein was specific (Figure 5). Furthermore, the band of the DNA-protein complex was supershifted by adding anti-NF- κ B antibody, indicating that the protein binding to the NF- κ B decoy ODNs was NF- κ B.

Discussion

NF- κ B is a nuclear transcription factor, first discovered by Sen and Baltimore in 1986 (Sen et al., 1986). It is widely found in the cytoplasm of a variety of cells, with a combination of inhibitory I κ B proteins in non-active state. As a variety of stimulation from outside, NF- κ B is activated, transferred into the nucleus, and then combined with the specific DNA sequences of certain related gene promoter or enhancer (κ B sites) to regulate and control a variety of gene expression (Shah et al., 2001). Subsequent research has shown that dysregulation of NF- κ B transcription machinery and constitutive expression of chemotactic cytokines are factors thought to be common early events in malignant tumor progression (Baldwin et al., 1996). NF- κ B (nuclear factor- κ B), a family of structurally related DNA-binding proteins, has been implicated in the regulation of cell growth and oncogenesis by inducing proliferative and antiapoptotic gene products (Beg et al., 1996; Feeley et al., 2000; Perkins et al., 2000).

Decoy oligonucleotide provide an attractive approach to manipulating transcription factors and regulating the

expression of the desired target genes (Gambari et al., 2004; Cutroneo et al., 2006; Isomura et al., 2006; Tomita et al., 2007; Edwards et al., 2009). Theoretically, when a decoy oligonucleotide containing the consensus sequence of a specific transcription factor is introduced into the cells, the presence of high levels of the decoy oligonucleotide will compete with the endogenous gene targets for binding to the transcription factor, which will lead to the removal of the transcription factor from the endogenous gene targets and cause alteration in transcription of the target genes.

There are several attractive advantages of decoy method over the other gene therapeutic approaches (Yamasaki et al., 2003; Shimizu et al., 2006). First, decoy ODNs, as a small DNA molecule, can be easily delivered to specific tissues and transfected into cells, and directly abrogate the activated transcript factors. Second, a growing number of transcript factors with their promoter sequences have been found, which made the potential drug targets plentiful and readily identifiable. Third, the synthesis, storage and transportation of decoy ODNs are much simpler than other approaches. Finally, decoy ODNs have been documented to be more effective than antisense ODNs in blocking constitutively activated transcript factors (Morishita et al., 2004).

In this work, we first added NF- κ B decoy ODNs into five kinds of cancer cells to determine the effect of NF- κ B decoy ODNs on proliferation of many kinds of carcinoma cells. The results showed that NF- κ B decoy ODNs effectively suppressed proliferation of five kinds of carcinoma cells, especially proliferation of the ovarian carcinoma cell lines SKOV-3 *in vitro*. To confirm the above inhibitory effect and mechanism of NF- κ B decoy ODNs on carcinoma cells in culture, we used the ovarian carcinoma cell lines SKOV-3 to assay the *in vivo* and *in vitro* efficacy of NF- κ B decoy ODNs. Compared to lipofect and control decoy ODNs, NF- κ B decoy ODNs significantly inhibited growth of SKOV-3 cells *in vitro*, *in vivo* as well as remarkably induced apoptosis in SKOV-3 cells. In this experiment, we have also studied the effect of NF- κ B decoy ODNs targeting the NF- κ B transcription factor on the promoter of c-myc gene and the possibility of using NF- κ B decoy ODNs to influence expression of the endogenous gene. we constructed luciferase gene eukaryotic expression vector by linking the c-myc promoter to luciferase gene (a reporter gene). The effect of NF- κ B decoy ODNs on the c-myc promoter was studied after co-transfection with the c-myc promoter vector into SKOV-3 cells. The results showed that NF- κ B decoy could suppress the transcriptional activity of NF- κ B by blocking its binding sites to the endogenous NF- κ B sequence resulting in the inhibition of c-myc promoter driving luciferase expression. Meanwhile, the results obtained from northern blotting study indicated that the NF- κ B decoy ODNs effectively inhibited expression of c-myc gene. The results obtained from our NF- κ B decoy ODNs treatment studies also showed that the treatment of the SKOV-3 cells with the NF- κ B decoy ODNs results in an increase in transcriptions of the caspase-8 gene. Furthermore, the results obtained from our *in vitro* protein-DNA binding study (electrophoretic mobility shift assay, EMSA) indicated that the NF- κ B decoy ODNs specifically

competes with the NF- κ B consensus DNA sequence for binding to the NF- κ B transcription factor, also suggesting that the NF- κ B transcription factor has a strong binding affinity towards the decoy ODNs. These results suggested that, in the case of treatment with NF- κ B decoy ODNs leading to decreased proliferation and increased apoptosis of SKOV-3 cells, the mechanism of proliferation inhibition and apoptosis induction following inhibition of NF- κ B signaling is probably multifactored. One possible mechanism involves the fact that inactivation of NF- κ B by NF- κ B decoy ODNs leads to block of c-myc expression regulated by NF- κ B to inhibit proliferation and induce apoptosis. Another possible mechanism is related to the fact that NF- κ B decoy ODNs leads to increased expression of caspase-8 genes, a apoptotic gene.

In conclusion, we report here that local NF- κ B inhibition was associated with proliferation inhibition and apoptosis induction of carcinoma cells with attenuated c-myc expression and increased caspase-8 expression. Our studies suggested that inhibitors of NF- κ B may offer promise as a therapeutic approach for the treatment of tumor. In addition, the knowledge obtained from this study may also lead to innovative strategies for the treatment of many other disease conditions via manipulating expression of the desired target genes through the decoy oligonucleotides.

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