RESEARCH COMMUNICATION

Antitumor Activity of NF-κB Decoy Oligodeoxynucleotides in a Prostate Cancer Cell Line

Yongqi Fang[1&], Hongli Sun[2&], Jing Zhai[1], Yuanying Zhang[1], Shuying Yi[1], Gangping Hao[1], Tao Wang[1*]

Abstract

Background: Nuclear factor-kappaB (NF-κB), a transcription factor, is abundantly expressed in prostate cancer and regulates many tumor-related genes. Given the important roles of these genes in tumor control, the present study was conducted to test the hypothesis that there was different expression of NF-κB in androgen-dependent or androgen-independent prostate cancer cells. In addition NF-κB decoy oligodeoxynucleotides (ODNs) were transfected into two prostate cancer cells to determine affects on growth and apoptosis. Methods: First, NF-κB decoy ODNs were designed according to the NF-κB elements in the promoter region of c-myc gene. Then, NF-κB and control decoy ODNs were transfected with lipofectamine. Their influence on prostate cancer cell line proliferative activity was detected by MTT assay. Cell apoptosis was determined by flow cytometric(FCM) analysis and AO/EB study. Thirdly, nuclear extracts were prepared from PC-3M cells and DNA-protein interactions were examined by electrophoretic mobility shift assay (EMSA). Lastly, 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-κB decoy ODNs into PC-3M cells with lipofectamineTM2000. Expression levels of related endogenous genes were assessed by western blotting. Results: We found overexpression of NF-κB in the androgen-independent prostate cancer cell line PC-3M compared to the androgen-independent LNCaP. Treatment with NF-κB decoy ODNs resulted in strong suppression of proliferation, especially in the PC-3M case. Induction of apoptosis of PC-3M was observed in FCM and AO/EB studies. Activity of luciferase was significantly reduced in the NF-κB decoy-transfected cells, but not in cells transfected with a control decoy. Furthermore, we found that expression of some endogenous genes was reduced, while other genes transcripts were induced. EMSA demonstrated specific binding of the NF-κB decoy to NF-κB protein. Conclusions: These findings indicate that NF-κB activation plays an important role in evolution of androgen-independent prostate cancer via manipulating expression of target genes. Inhibitors of NF-κB may thus offer promise as a therapeutic approach for the treatment of androgen-independent prostate cancer. NF-κB decoy ODNs may allow development of therapeutic and investigative tools for human malignancies.

Keywords: NF-κB - decoy oligodeoxynucleotides - prostate cancer - apoptosis

Asian Pacific J Cancer Prev, **12**, 2721-2726

Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous in western countries (Tsing et al., 2000). In the United States, it is the second leading cause of cancer death in men following lung cancer (Jemal et al., 2003). Prostate cancer initiation and progression are processes involving multiple molecular alteration (Morton and Isaacs, 1993). Genomic alterations, combined with changes in the tissue microenvironment, lead inevitably to altered levels of expression of many individual genes in tumor cells. Identification of these genes represents a critical step toward a through understanding of prostate carcinogenesis and an improved management of prostate cancer patients. Of particular biological and clinical interest are those genes that are consistently overexpressed or lower-expressed in the vast majority of prostate cancers. Such genes and their products, besides providing possibly valuable insight into the etiology of prostate cancer, may have important utility as diagnostic and treated markers in the disease.

With the development of microarray technology, a number of genes have been identified that are consistently up-regulated or down-regulated in prostate cancer with reference to nomal prostate and benign prostate hyperplasia. These genes have the potential to complement PSA as new and perhaps better diagnostic marker for prostate cancer. One of these genes was a nuclear factor-kappaB (NF-κB)(Karin and Lin, 2002; Kumar et al., 2004). As transcription factor, NF-κB plays a pivotal

*[1]School of Basic Medicine, [2]School Hospital, Taishan Medical University, China [&]Equal contributions *For correspondence: wangtaofch@yahoo.com.cn*

role in the regulation of cell-cycle and cell proliferation (Walmsley et al., 2005; Maxwell et al., 2007; Rius et al., 2008; Oliver et al., 2009) . During androgen-independent tumorigenesis in the prostate, NF-κB expression is elevated at both mRNA and protein level, as evidenced by real-time PCR and western blot, sugguesting novel therapeutic strategies based on this gene and/or pathway (Doi et al., 1996; Domingo-Domenech et al., 2005; 2006; Shimizu et al., 2006; Benitez et al., 2009; Bollrath and Greten, 2009; Lee et al., 2009).

In this study, we synthesized an NF-κB Decoy Oligodeoxynucleotides(ODNs), a phosphorothiolated double-stranded oligonucleotides which contained the same sequence as the specific DNA sequences in the promoter of target genes. The NF-κB Decoy ODNs was transfected into two kinds of prostate cancer cell line to explore the effects of blocking NF-κB DNA binding domain on growth of prostate cancer cell line and expression of related genes.

Materials and Methods

All the enzymes were purchased from Takara. RPMI1640 was the product of Hyclone. Antibody and Western-blotting Luminol Reagent were obtained from Beijing Zhongshang.

Cell culture

Androgen-independent prostate cancer cell lines PC-3M, Androgen-dependent prostate cancer cell lines LNCaP were provided by our laboratory. Four kinds of prostate cancer cell lines were maintained in RPMI-1640 medium supplemented with 5% cattle bovine serum, 100 units/ml penicillin, and 0.1mg/ml streptomycin at 37°C in a 5% CO_2 incubator.

Decoy oligodeoxynucleotides synthesis and annealing

The NF-κB decoy was designed according to the NF-κB elements in the promoter region of the human c-myc gene. By contrast, Control-1 decoy ODNs, two mutated version of the decoy that differed by two base pair did not bind the activated NF-κB protein. The Control-2 decoy ODNs had sequeneces of unrelated NF-κB element sequences and was used in the control studies. TEN buffer (10mmol/L Tris-HCl, 0.1mmol/L EDTA, 0.1mol/L NaCl, PH8.0) diluted oligodeoxynucleotides respectively. Complement oligodeoxynucleotides were mixed as equal mol, 95°C, 10min, then cooled to room temperature.

NF-κB decoy ODNs:

5'-CCTTGAAGGGATTTCCCTCC- 3'

3'-GGAACTTCCCTAAAGGGAGG-5'

Control-1 decoy ODNs:

5'-CCTTGAAGAGATTTCTCTCC- 3'

3'-GGAACTTCTCTAAAGAGAGG-5'

Control-2 decoy ODNs:

5'-GTCTGATAAAGGGTGTTCTTTTT- 3';

3'-CATACTATTTCCCAC AAGAAA AA-5'

Suppression prostate cancer cell proliferation by NF-κB decoy ODNs

Measurement of prostate cancer cell lines in vitro proliferation by MTT colorimetric assay. Four kinds of prostate cancer cell lines were seeded at a density of 1×10^4 cells/well into 96-well plates. MTT (5 g/L) was added to the wells (10μL/well) respectively at 24h, 48h and 72h after transfection as before. After another 4 hours incubation at 37°C, the media in the wells were replaced with dimethylsulfoxide(DMSO)(150μL/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.

Apoptosis assay of prostate cancer cell

Two different assays were used to determine the cell apoptosis. Analysis of DNA fragmentation was carried out as previously described(Barnett et al, 2000). After transfection of 48h, Four kinds of prostate cancer cell lines were harvested and DNA were extracted. The degradation of DNA was investigated by DNA gel electrophoresis.

The apoptotic level of cells incubated with decoy ODNs was determined by FACS analysis after PI6 staining. Approximately 10 cells (70% confluent monolayer) were transfected with decoy ODNs in culture medium without antibiotics at 37°C in a CO_2 incubator. After transfection of 48h, Four kinds of prostate cancer cell lines were harvested and then fixed with ice-cold 70% ethanol for cytornetric analysis. DNA content of the subdiploid peak, which indicated apoptotic cells,were determined by FACS analysis.

The effect of NF-κB decoy ODNs on the promoter of the endogenous gene

Human genomic DNA was used as a template for the polymerase chain reaction (PCR) to get a fragment of the c-myc promoter. The PCR product was digested, purified, and then inserted into the upstream of luciferase gene of the pGL3-Enhancer vector pre-cut with Kpn and Sma enzymes. The recombinant plasmid was transformed into competent *E.coli* . The plasmid was first screened and verified by DNA sequencing. When prostate cancer cells were seeded at a density of $2x10^5$/per well and reached 90%-95% confluence, the double stranded decoy ODNs and plasmids as indicated were co-transfected into prostate cancer cells by lipofectamineTM2000 according to the manufacturer's instructions. Plasmid pSV-Gal was used as an internal control. After the addition of 1xPLB (passive lysis buffer), The transfected cells were washed and harvested. 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 efficiency of transfection, the ratio M1/M2 was used for representing the luciferase relative activity. The experiments were repeated at least three times.

Reverse Transcriptase-PCR(RT-PCR) Analysis

Expression of NF-κB gene was determined by RT-PCR.Total RNA was extracted using TRIzol

Reagent(GIBCOBRL). For oligonucleotide treated cells, RNA expression was performed. Two mcrogram of total RNA were reverse transcribed using 0.2ug of random hexamer primer and 200 units of murine leukemia virus reverse transcriptase (MBI) under recommended conditions.one-fourth of this cDNA was used as the template in a PCR. The amplification conditions were denaturation at 94°C for 60sec, annealing at 51°C for 50sec, extension at 72°C for 60sec for 28cycles, followed by 10min apostextension at 72°C. Each PCR product was run on an 1.5% gel electrophoresis at 100V for 30 min and the gel was scanned.

Western blot Analysis

Cell lysates were prepared and Western blots were performed by using total protein, as described previously (Rius et al., 2008. A 85% confluent 1640 culture flask of prostate cancer cells were harvested in a 1:10 solution of trypsin-EDTA and HBSS, centrifuged and resuspended in 350 ul of 1×lysis buffer (5×, premoga), incubated for 20 min, the supernatant recentrifuged and transferred to a fresh tube. An aliquot of 35 ul of the cell lysate was mixed with 30 ul of SDS gel loading buffer and resolved by 10% SDS-PAGE. The gel was transferred to a PDVF membrane and Western blot analysis was performed according to the procedure provided with primary antibody, briefly, the membrane was incubated in a room temperature for 1h in a 5% non-flat milk powder solution in 0.05% PBS-T (Sigma) to block non-specific reactivity. The blot was then washed three times for 5 min each time with PBS-T alone. It was then incubated for 1h at a room temperature in PBS-T containing a 1:100 dilution of mouse-reactive monoclonal antibody(Santa Cruz Biotechnology, Inc). After another series of 3-5min washes, the blot was incubated for 45 min with a 1:500 dilution of horseradish peroxdase-conjugated goat anti-mouse secondary antibody (Dako). The blot was again washed three times in PBS-T then once in PBS alone for 5 min. Protein detection was performed using ECL chemiluminsescent reagents(Amersham), followed by rapid audtoradiography at room temperature.

Electrophoretic mobility shift assay (EMSA)

The nuclear extracts were prepared from PC-3M cells as described [4]. 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 manufacture's instruction to confirm specific binding of the NF-κB decoy ODNs to the PC-3M nuclear proteins. Firstly, NF-κB 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 (5-8μg) 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 1.2 h. 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 an 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-κB decoy ODNs (100 fold and 200 fold molar excess) was added during DNA-protein incubation period. In nonspecific competition experiments, the unlabeled control decoy ODNs (100 fold molar excess) were used.

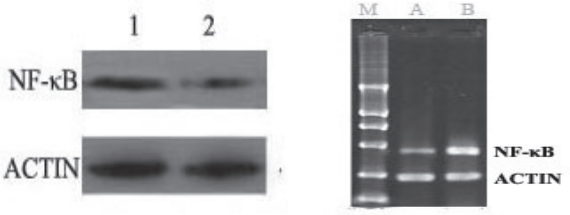

Figure 1. Expression of NF-κB by RT-PCR and Western Blot Analysis

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. $P< 0.05$ and $P\leq 0.01$ were considered significant.

Results

Expression of NF-κB in PC-3M cells and LNCaP cells

To characterize involvement of NF-κB in PC-3M cells and LNCaP cells, we checked expression of NF-κB by RT-PCR and Western blot analysis.The results analysis (Figure 1A, 1B) showed a marked increase of NF-κB in PC-3M cells compared to LNCaP cells. These results suggested that NF-κB play an important role in evolution of Androgen-independent prostate cancer.

Specificity of NF-κB decoy ODNs with protein

To examine whether the NF-κB decoy ODNs was able to bind to NF-κB, electrophretic mobility shift assay was performed. The nuclear extracts from PC-3M cells were used for in vitro binding assay with double strands NF-κB decoy probes and the complex of DNA-protein was observed. In the competition study with a 1, 2, 3-fold molar excess of the unlabeled NF-κB decoy ODNs, the formation of this complex was inhibited, though not with the same molar excess of the unlabeled control-1 decoy and NF-κB decoy ODNs, indicating that the binding to the PC-3M nuclear protein was specific.

NF-κB decoy ODNs inhibits proliferation of prostate cancer cell

When PC-3M cell lines was treated with NF-κB decoy ODNs, control-1 decoy ODNs and Control-2 decoy ODNs and analyzed for viability, the results demonstrated that PC-3M cells were quite sensitive to NF-κB decoy ODNs and inhibited while being relatively resistant to Control-1 and Control-2 decoy ODNs.

NF-κB decoy ODNs induces apoptosis of prostate cancer cells via inhibition of NF-κB

Apoptosis was measured after cells were exposed

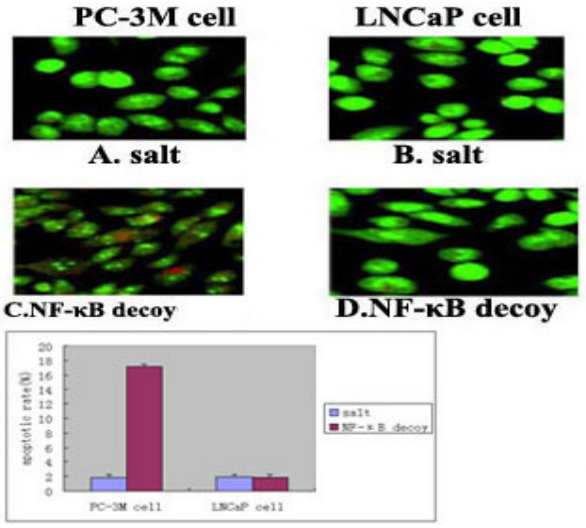

Figure 2. NF-κB Decoy ODNs Induce Apoptosis of PC-3M Cells by AO-EB Stain Analysis

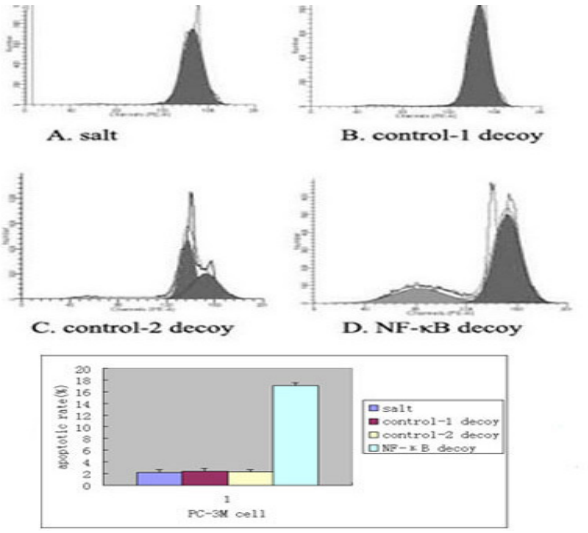

Figure 3. NF-κB Decoy ODNs Induce Apoptosis of LNCaP Cells by FCM analysis

to NF-κB decoy ODNs, control-1 decoy ODNs and Control-2 decoy ODNs for 48h using the AO/EB assay and the FACS analysis. Figure 2 shows the percentage of apoptotic cells after treatment with NF-κB decoy ODNs was increased in PC-3M cells (53.7%, respectively) compared to that treated with salt (3.3%, respectively) and compared to LNCaP cells (3.9%, 3.5%, respectively). As shown in Figure 3 using FACS analysis, the percentage of apoptotic cells after treatment with NF-κB decoy ODNs was increased in PC-3M cells (25.1%, respectively) compared to that treated with salt, control-1 decoy, and control-2 decoy (0.47%, 0.51%, 0.86%, respectively) and compared to LNCaP cell (0.97%, 1.01%, 1.01%, 5.53%, respectively).

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. The different doses of NF-κB decoy ODNs were co-transfection with 0.2mg/L pGL3-enhancer and 8.0μg/L pSV-β-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 2.0mg/L, at which the inhibition rate reached 63.5%, 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.

Decrease of CyclinD1, C-myc, A1/Bf1-1 and c-IAP2 is associated with enhanced apoptosis induced by NF-κB decoy ODNs

To characterize involvement of NF-κB decoy ODNs in the apoptotic process, we looked for an NF-κB-induced modulation of proliferation-related molecules such

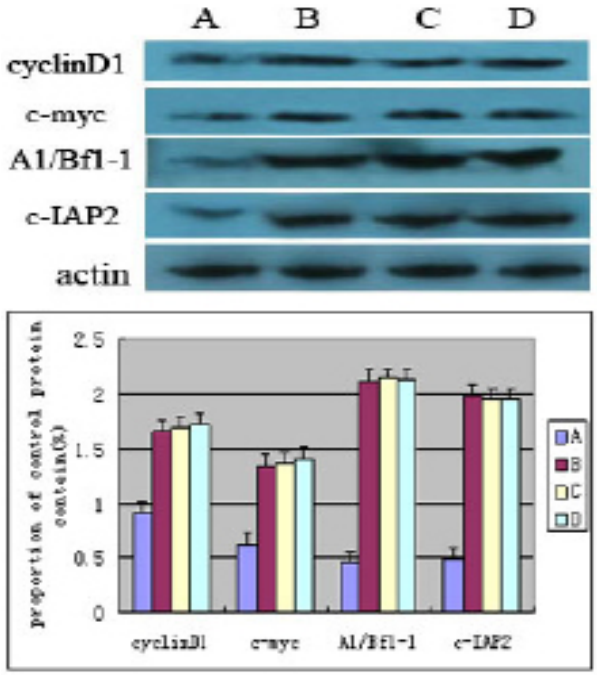

Figure 4. Decrease of Cyclin D1, C-myc, A1-Bf1-1 and c-IAP2 with Enhanced Apoptosis in PC-3M cells

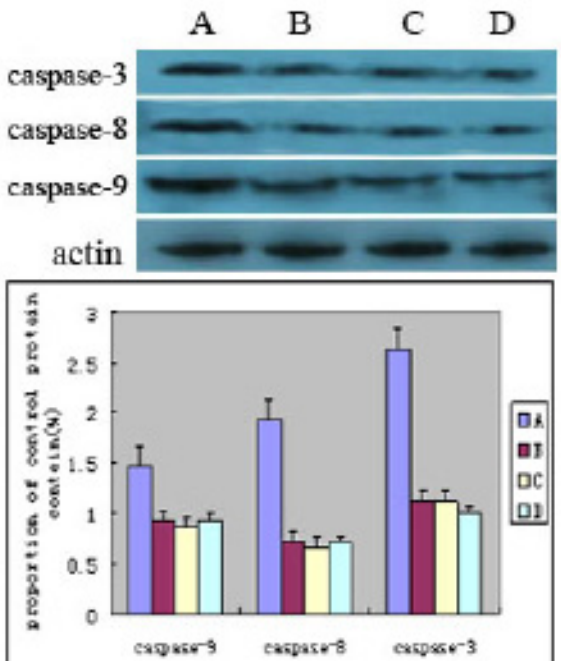

Figure 5. Increase of Caspase Forms is Associated with Enhanced Apoptosis in PC-3M cells

as CyclinD1, C-myc, A1/Bf1-1 and c-IAP2. Western blot analysis (Figure 4) showed a marked decrease of CyclinD1, C-myc, A1/Bf1-1 and c-IAP2 in apoptotic cells with NF-κB decoy ODNs compared to treatment with salt, control-1 decoy ODNs and Control-2 decoy ODNs. These results suggested that with enhanced apoptosis, the treatment of PC-3M cells with the NF-κB decoy ODNs caused an decreased expression of the cyclinD1, C-myc, A1/Bf1-1 and c-IAP2 in the PC-3M cells.

Increase of caspase-3, caspase-8, and caspase-9 is associated with enhanced apoptosis induced by NF-κB decoy ODNs

To futher characterize involvement of NF-κB decoy ODNs in the apoptotic process, we more studied an NF-κB-induced modulation of apoptosis-related molecules such as caspase-3, caspase-8, and caspase-9.Western blot analysis (Figure 5) showed a marked increase of caspase-3, caspase-8, and caspase-9 in apoptotic cells with NF-κB decoy ODNs, compared to treatment with salt, control-1 decoy ODNs and Control-2 decoy ODNs. These results suggested that with enhanced apoptosis, the treatment of PC-3M cells with the NF-κB decoy ODNs caused an increased expression of the caspase-3, caspase-8, and caspase-9 in the PC-3M cells.

Discussion

NF-κB, a family of structurally related DNA-binding proteins, has been implicated in the regulation of cell growth and oncogenesis by inducing proliferative and anti-apoptotic gene products (Beg et al., 1996; Perkins et al., 2000) . As transcription factor, NF-κB was discovered in 1986 (Sen et al., 1986). A series of studies have indicated that NF-κB expression and activity are involved in proliferation and pathogenesis of tumors, which activated, transfered into the nucleus, and then combined with the specific DNA sequences of certain related gene promoter or enhancer to regulate and control a variety of gene expression Baldwin et al., 1996).The seqence elements, which NF-κB binds to, is present in the promoters of a number of genes that are known and presumed to be important for limiting regulator of DNA synthesis and inflammatory factor, cell-cycle, proto-oncogenes, tumor suppressor genes, including IL-8, CXCR1, c-myc, cyclinD1, caspase-3 and so on (Huang et al., 2008).

As an attractive approach to manipulating transcription factors and regulating the expression of the desired target genes, transfection of double-stranded ODNs corresponding to the cis sequence will result in the attenuation of authentic cis-trans interaction, leading to the removal of transcription factors from the endogenous cis-element and the suppression of the expression of regulated genes (Tomita et al., 2007; Edwards et al., 2009). Transfection of decoy ODNs has been reported to be a powerful therapeutic strategy against hyperplasia, acute and chronic inflammation and tumour, there several attractive advantages of decoy method over the other gene therapeutic approaches (Yamasaki et al., 2003; Shimizu et al., 2006).

In our works, firstly, to characterize involvement of NF-B in PC-3M cells and LNCaP cells, we checked expression of NF-κB by RT-PCR analysis and Western blot analysis.We found a marked increase of NF-κB in PC-3M cells compared to LNCaP cells. These results suggested that NF-κB played an important role in evolution of Androgen-independent prostate cancer. Second, we synthesized double-strands oligodeoxynucleotides (ODNs), which had consensus sequence with endogenous NF-κB cis-element and high-affinity to the NF-κB transcription factor. NF-κB decoy ONDs was transfected into LNCaP cell and PC-3M cell. Firstly, the results showed that NF-κB decoy ODNs remarkably supressed proliferation and induced apoptosis in the androgen-independent prostate cancer cell lines PC-3M, compared to the androgen-independent prostate cancer cell lines LNCaP. To confirm the specific effect of NF-κB decoy ODNs targeting to the NF-κB to inhibit activity of the promoter, NF-κB decoy ODNs was co-transfected with pGL3-enhancer, pSV-β-Gal.The results showed that NF-κB decoy could effectively suppress the transcriptional activity of NF-κB by specifically blocking its binding sites to the NF-κB sequence resulting in the inhibition of endogenous gene promoter driving luciferase expression. the results of EMSA study indicated that the NF-κB decoy ODNs specifically competes with the NF-κB consensus DNA sequence for binding to the NF-κB transcription factor, also suggesting that the NF-κB transcription factor has a strong binding affinity towards the decoy ODNs. These results obtained from western bloting study indicated that, in the case of treatment with NF-κB decoy ODNs leading to decreased proliferation and increased apoptosis of PC-3M cells, the mechanism of proliferation inhibition and apoptosis induction following inhibition of NF-κB signaling is probably multifactored. One possible mechanism involves the fact that inactivation of NF-κB by NF-B decoy ODNs leads to block of cyclinD1, c-myc, A1/Bf1-1 and c-IAP2 expression regulated by NF-κB to inhibit proliferation and induce apoptosis. Another possible mechanism is related to the fact that NF-κB decoy ODNs leads to increased expression of caspase-3, caspase-8 and caspase-9, apoptotic genes.

In the conclusion, our studies suggested that NF-κB played an important role in development of prostate cancer, specially in androgen-independent prostate cancer. These results suggested that, in the case of androgen-independent prostate cancer evolution, the mechanism of evolution following NF-κB signaling is probably multifactored. One possible mechanism involves the fact that activation of CyclinD1, C-myc, A1/Bf1-1 and c-IAP2 genes by NF-κB leads to androgen-independent prostate cancer evolution. Another possible mechanism is related to the fact that NF-κB leads to inactivation of caspase-3, caspase-8s and caspase-9 genes, apoptotic genes. We also reported here that local NF-κB inhibition was associated with proliferation inhibition and apoptosis induction of androgen-independent prostate cancer cell lines PC-3M with attenuated pro-oncogene expression and increased apoptotic genes expression. inhibitors of NF-κB may offer promise as a therapeutic approach for the treatment of androgen-independent prostate cancer. 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 oligodeoxynucleotides.

Acknowledgements

We thank Dr. Jian-Ye Zhang, Director, for being very supportive and giving all the encouragement to carry out this work. The authors declare that they have no competing interest. Yong-qi Fang and Hong-li Sun performed most of the experiments and prepared the manuscript. Yuan-Ying Zhang and Shu-Ying Yi participated in performing experiments and helped to draft the manuscript. TW conceived the study, participated in its design and coordination, corrected the manuscript and supervised the project. All authors read and approved the final manuscript. This work was supported by the Shandong Technology Development Plan of Medicine and Health Grant 2011HZ107, China.

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