# Apoptosis Induction by E2F Decoy DNA of the Prostate Cancer Cell Line

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## ABSTRACT

*To observe effect of E2F decoy DNA on proliferation and apoptosis of androgen-independent prostate cancer cell line PC-3M the binding specificity of the E2F decoy DNA to the PC-3M nuclear protein was detected by electrophoretic mobility shift assay (EMSA). E2F decoy DNA, ARE decoy DNA and Control decoy DNA were respectively transfected into PC-3M cells with lipofectamine.Their influence on cell proliferative activity was detected by MTT assay.The cell apoptotic rate was determined by flow cytometric(FCM) analysis and chromosome DNA ladder was detected by DNA gel electrophoresis.The change of mRNA expression of C-myc and CyclinD1 were detected by RT-PCR.The change of mRNA expression of C-myc and CyclinD1 were detected by Western-blot. EMSA demonstrated specific binding of the E2F decoy to E2F transcription factor.The PC-3M cell growth was inhibited after transfection. The apoptotic rate was 26.35% and DNA ladder could be observed after transfection.The expression of C-myc and CyclinD1 were inhibited. All these results indicated that E2F decoy DNA induced apoptosis of androgen-independent prostate cancer cell lines PC-3M and inhibited cell proliferation via inhibiting expression of C-myc and CyclinD1.*

**Key words:** Decoy DNA, transcription factors, PC-3M cells, apoptosis

## INTRODUCTION

A series of studies have indicated that E2F expression and activity are involved in proliferation and pathogenesis of tumors.The transcription factor E2F plays a pivotal role in the regulation of cell-cycle and cell proliferation. The seqence elements,which E2F 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 cell-cycle,proto-oncogenes,tumor suppressor genes,including DNA polymer α,PCNA,c-myc,b-myb,cdc2,cyclinD1,and Rb and so on(Xin et al,2001; Yamasaki  et al, 1996; Kurt et al, 2001). In this study, we synthesized an E2F decoy DNA, a phosphorothiolated double-stranded oligonucleotide which contained the same sequence as the specific DNA sequences (5'-TTTNNCGC-3'N=C or G) in the promoter of target genes. The E2F decoy DNA was transfected into PC3-M cells to explore the effects of blocking E2F DNA binding domain on growth of PC-3M cells and expression of related genes.

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## MATERIALS AND METHODS

### Materials
All the enzymes were purchased from Takara. RPMI1640 was the product of Hyclone.C-myc antibody CyclinD1 antibody and Western-blotting Luminol Reagent were obtained from Beijing Zhongshang. The human prostate carcinoma cell line PC-3M was provided by our laboratory.

### Decoy oligodeoxynucleotides synthesis and annealing
The E2F decoy was designed according to the E2F elements in the promoter region of the human C-myc gene.the ARE decoy was designed based on the DNA sequence of the ARE in the promoter region of the human PSA gene. The control decoy had three mutations in the consensus E2F 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 oligo deoxynucleotides respectively. Complement oligo deoxynucleotides were mixed as equal mol, 95 $^0$C, 10min, then cooled down to room temperature slowly.

**E2F decoy: 5'-TGATTTCCCGCGGAT-3'**
**3'-ACTAAAGGGCGCCTA-5'**

**ARE decoy:**
**5'-TGCAGAACAGCAAGTGCTAGC-3'**
**3' -ACGTCTTGTCGTTCACGATCG-5'**

**Control decoy:**
**5'-TGATTACGAGCGGAT-3'**
**3'-ACTAATGCTCGCCTA-5'**

### Electrophoretic mobility shift assay (EMSA)
The nuclear extracts were prepared from PC-3M cells as decribes (Rita et al, 2002). 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 E2F decoy to the PC-3M nuclear proteins. Firstly, E2F decoy DNA 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$^0$C. Then, the DNA-protein complexes were separated by electrophoresis using a 6% native-PAGE at 25V for 90mins. 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 control experiments, the unlabeled E2F decoy DNA (100 fold and 200 fold molar excess) was added during DNA-protein incubation period. In nonspecific control experiments, the unlabeled control decoy DNA (100 fold molar excess) were used.

### Cell cultures
Human prostate carcinoma cell line PC-3M were cultured in RPMI-1640 medium supplemented with 10% new born calf serum, 1×105 U/V penicillin, and 0.1 g/L streptomycin at 37 $^0$C in a 5% $CO_2$ incubator. Cells between the 37th and 49th generation were used in these experiments. For transfection, cells were grown in phenol red-free and serum free RPMI-1640.

### Transfection experiments
The day before transfection, PC3-M cells were seeded at a density of $2×10^5$ cell/cm$^2$. twenty-four hour later, when cells reached 70-80% confluency, the decoy ODNs was transfected into PC-3M cells by Lipofectamin$^{TM}$2000 according to the manufacturer's instructions. PC-3M cells were divided into four groups. E2F decoy was transfected into one group, ARE decoy was transfected into second group, Control decoy was transfected into third group, the fourth group was used as a internal control. 0.8-1.6μg /100μl decoy DNA and 2-4μl/100μl lipofectamin were diluted into 100μl medium without serum. Then the above two were combined and layed at room temperature for 20 mins. The complexes were added onto the cells cultured with serum-free medium. 6 hour later, the medium with serum was added.After 24-72h, PC-3M cells were detected.

### Cell viability assay
Measurement of PC-3M cells in vitro proliferation by MTT colorimetric assay. PC-3M cells were seeded at a density of 1×104 cells/well into 96-well plates. MTT (5 g/L) was added to the wells (10μL/well) at 24, 48 and 72h respectively after transfection as before. After another 4h incubation at 37 $^0$C, the medium 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 was read on an ELISA reader.

## Apoptosis assay

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, PC-3M 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 DNA was determined by FACS analysis after PI staining. Approximately $10^6$ cells (70% confluent monolayer) were transfected with decoy DNA in culture medium without antibiotics at 37°C in a $CO_2$ incubator. After transfection of 48h, PC-3M cells were harvested and then fixed with ice-cold 70% ethanol for cytornetric analysis.DNA content of the subdiploid peak,which indicated apoptotic cells,was determined by FACS analysis.

## Reverse transcriptase-PCR (RT-PCR) Analysis

Expression of C-myc gene and CyclinD1 gene was determined by RT-PCR.Total RNA was extracted using TRIzol Reagent(GIBCOBRL). For oligonucleotide treated cells, RNA expression was performed 6 or 24 h after termination of oligonucleotide treatments.Two mcrogram of total RNA were reverse transcribed using 0.2μg 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 using the following upstream and downstream primers:C-myc,
5'-CCCAGCGAGGACATCTGGAAGAA-3'
(upstream primer) and 5'-GAGAAG CCGCTCCACATGCAGTC-3'(downstream primer) (expected PCR product,268bp); CyclinD1,
5'-CTGTCGCTGGAGCCCGTGAAAAAG
-3' (upstream primer) and 5'-GAAGTTGTTGGGGCTCCTCAGGT T-3'(downstream primer) (405bp); β-actin,
5'-GTGGGGCGCCCAGGCACCAC-3'(upstream primer) and 5'-CTCCT TA ATGTCACGCACGATTT-3' (downstream primer) (500bp). All primers used flank at least one intron.The amplification conditions were denaturation at 94 $^0$C for 60 sec, annealing at 51 $^0$C for 50 sec,extension at 72 $^0$C for 60 sec for 28 cycles, followed by 10 min apostextension at 72 $^0$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 (Sen-hong et al, 1998).A 85% confluent 1640 culture flask of PC-3M cells was harvested in a 1:10 solution of trypsin-EDTA and HBSS, centrifuged and resuspended in 350μl 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 μl 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 at room temperature for 1h in a 5% non-flat milk powder solution in 0.05% PBS-T (Segma) 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 room temperature in PBS-T containing a 1:100 dilution of mouse-reactive c-Myc 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 5min. Protein detection was performed using ECL chemiluminsescent reagents(Amersham), followed by rapid audtoradiography at room temperature.

## Statistical analysis

All numerical data were expressed as x±s. Results were considered significant, if $P<0.05$ was obtained by appropriate analysis of variance (ANOVA) procedures and Student's-test.

# RESULTS

## Specificity of E2F decoy DNA with protein

To examine whether the E2F decoy DNA was able to bind to E2F, electrophretic mobility shift assay was performed. The nuclear extracts from PC-3M cells were used for in vitro binding assay with double strands E2F decoy probes and the complex of DNA-protein was observed. In the competition

study with a 100-fold molar excess of the unlabeled E2F decoy, the formation of this complex was inhibited, though not with the same molar excess of the unlabeled control decoy and E2F decoy, indicating that the binding of the E2F decoy DNA to the PC-3M nuclear protein was specific (Fig. 1).

## E2F decoy DNA inhibits proliferation of PC-3M cells

When PC-3M cell lines was treated with E2F decoy DNA,ARE decoy DNA and Control decoy DNA and analyzed for viability, the results (Fig. 2) demonstrated that PC-3M cells were quite sensitive to E2F decoy DNA and inhibited while the PC-3M cells were relatively resistant to ARE decoy DNA and Control decoy DNA. E2F decoy DNA had a direct inhibitory effect on the proliferation of PC-3M cells in vitro.

## E2F decoy DNA induces apoptosis of PC-3M cells via inhibition of E2F

Apoptosis was measured after cells were exposed to E2F decoy DNA, ARE decoy DNA and Control decoy DNA for 48h using the DNA fragmentation assay and the FACS analysis. Fig. 3 shows the electrophoretic result of extracted DNA from PC-3M cells. it clearly indicated DNA degradation after a 48h incubation with E2F decoy DNA. No such degradation was observed when PC-3M cells were untreated or incubated with ARE decoy DNA and Control decoy DNA.As shown in Fig. 4 using FACS analysis,the percentage of apoptotic cells after treatment with E2F decoy DNA was increased in PC-3M cells (26.35%)compared to that untreated or treated with ARE decoy DNA and Control decoy DNA(2.85,2.91 and 0.40%,respectively).
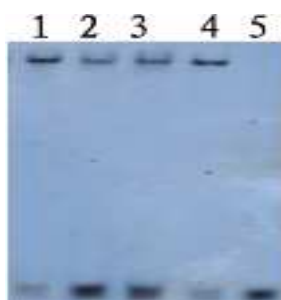


**Figure 1** - EMSA of E2F decoy DNA and E2F transcription factor.
1. 4 plus unlabeled Control decoy DNA
2. 4 plus unlabelde E2F decoy DNA
3. 4 plus unlabelde E2F decoy DNA
4. labeled E2F decoy DNA with nuclear extracts
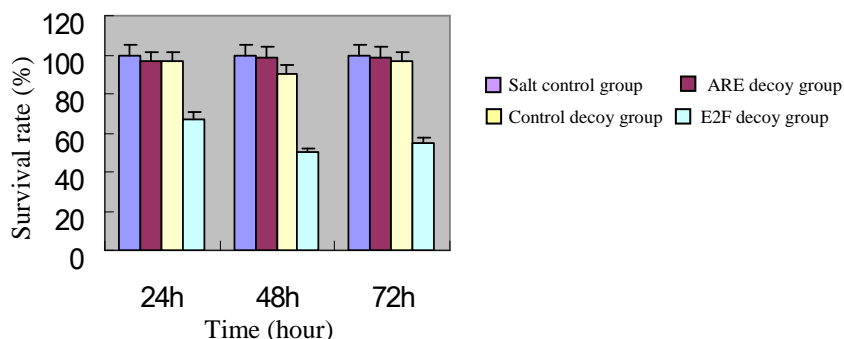5. labeled E2F decoy DNA



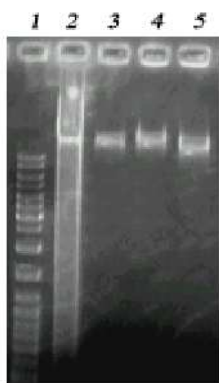**Figure 2** - Cell livability by MTT detection

**Figure 3** - DNA gel electrophoresis. 1 - Marker 2 - treated with E2F decoy DNA 3 -treated with ARE decoy DNA 4 - treated with control decoy DNA 5 - treated without any decoy DNA.

**Decrease of C-myc and CyclinD$_1$ levels Is associated with enhanced apoptosis induced by E2F decoy DNA**

To characterize the involvement of E2F decoy DNA in the apoptotic process, we studied an E2F-induced modulation of apoptosis-related molecules such as C-myc, CyclinD$_1$.RT-PCR analysis (Figs. 5 and 6) showed a marked decrease of C-myc and CyclinD$_1$ mRNA levels in apoptotic cells with E2F decoy DNA with respect to untreated cells and treatment with ARE decoy DNA and Control decoy DNA. The expression of these proteins was also probed by Western blotting with specific antibodies.In fact, an decrease in C-myc and CyclinD$_1$ protein expression from PC-3M cells with E2F decoy DNA in Western blot we observed (Figs. 7 and 8). Comparingly no significant changes in C-myc and CyclinD$_1$ expression were observed in these untreated cells and cells treated with ARE decoy DNA and Control decoy DNA.
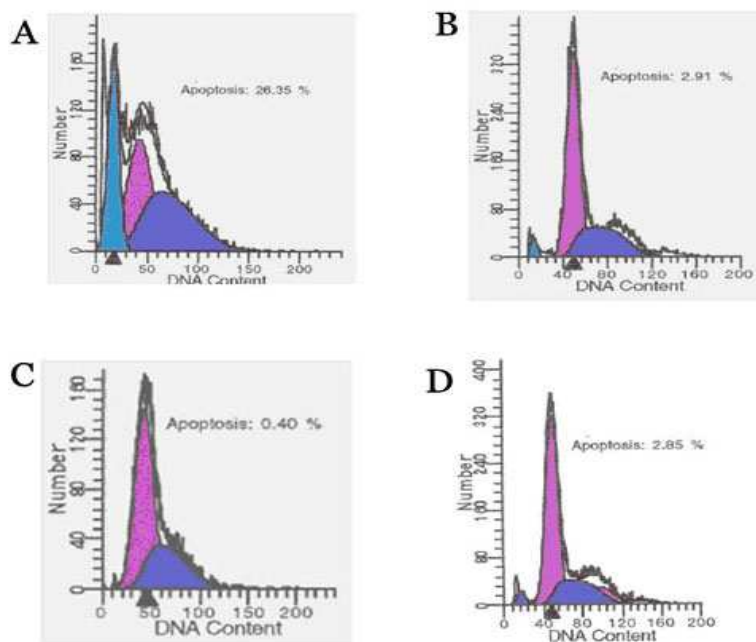


**Figure 4** - Apoptotic rate in PC-3M cells treated with. A. E2F decoy DNA. B. ARE decoy DNA. C. control decoy DNA. D. treated without any decoy DNA.
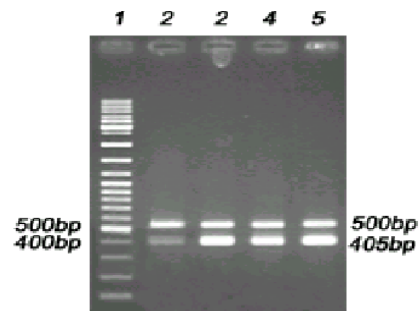
**Figure 5** - CyclinD1 expressiong inhibition of E2F decoy DNA in PC-3M cells were analyzed by RT-PCR after 24h of transfection. 1 - Marker 2 - treated with E2F decoy DNA 3 - treated with ARE decoy DNA 4 - treated with control decoy DNA 5 -treated without any decoy DNA.
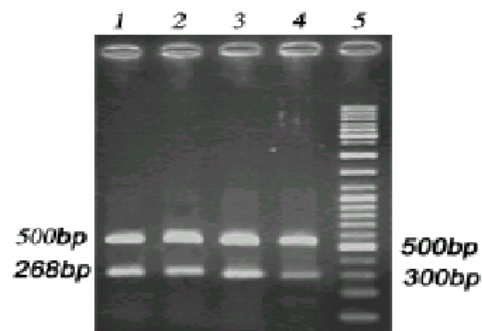


**Figure 6** - C-myc expressiong inhibition of E2F decoy DNA in PC-3M cells were analyzed by RT-PCR after 6h of transfection. 1 - treated without any decoy DNA. 2 - treated with Control decoy DNA. 3 -treated with ARE decoy DNA. 4 - treated with E2F decoy DNA. 5 - Marker.
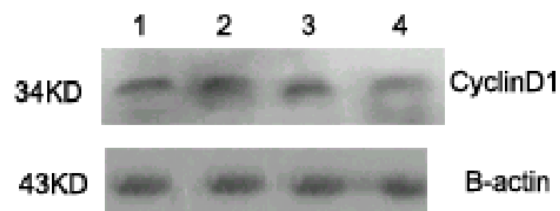


**Figure 7** - CyclinD1 expression inhibition of E2F decoy DNA in PC-3M cells were analyzed by Western blotting after 24h of transfection. 1- treated without any decoy DNA. 2-.treated with control decoy DNA. 3-treated with ARE decoy DNA. 4-treated with E2F decoy DNA.
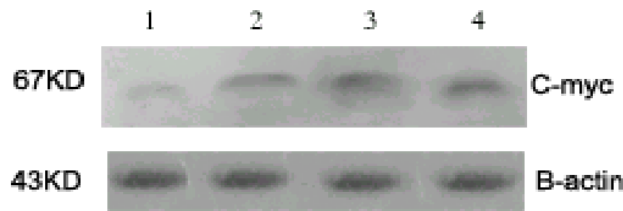
**Figure 8** - C-myc expression inhibition of E2F deocy DNA in PC-3M cells were analyzed by Western blotting after 24h of transfection. 1-treated with E2F decoy DNA. 2-ARE decoy DNA. 3-control decoy DNA. 4-treated without any deocy DNA.

## DISCUSSION

E2F transcription factor plays an important role in progression into S-phase and that this probably coincides with capacity to stimulate transcription. Lines of evidence implicate the E2F transcription factor as an important component of cell proliferation control. First, E2F binding sites are found in the promoters of genes responsive to proliferation signals and the level of E2F binding activity increases at a time when many of these genes are activated. Second, the tumour suppressor protein Rb, as well as the related p107 protein,complexes with E2F, resulting in an inhibition of E2F transcriptional activity.Third, oncogenic products of the DNA tumour viruses can dissociate these E2F complexes.

Transfection of decoy ODNs has been reported to be a powerful therapeutic strategy against hyperplasia□acute and chronic inflammation and tumour (Morishita et al, 1998; Tomita et al, 2003; Yamasaki et al, 2003). 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. In this experiments, we synthesized double-strands oligodeoxynucleotides (ODNs), which had consensus sequence with endogenous E2F cis-element and high-affinity to the E2F transcription factor. E2F decoy DNA was transfected into androgen-independent prostate cancer cells line PC-3M by which induction of apoptosis was demonstrated and competed with the endogenous E2F cis-element for binding to the E2F transcription factor. In conclusion, E2F decoy DNA coud inhibit proliferation of PC-3M cell and induced the apoptosis of PC-3M cells through binding the E2F competitively and inhibiting the function of E2F on expression of C-myc and CyclinD$_1$. Decoy DNA strategy could provide a new method for therapy of prostate cancer.

## ACKNOWLEDGMENTS

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