[Article ID] 1000 - 4718(2006)07 - 1324 - 06

Androgen responsive element decoy DNA inhibits the promoter of prostate specific antigen and induces apoptosis of LNCaP cells*

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[**ABSTRACT**] **AIM**: To observe the effect of exogenous androgen responsive element decoy on the promoter of prostate specific antigen (PSA) and the growth of LNCaP cells for searching the possibility of gene therapy for prostate cancer. **METHODS**: Firstly, pGL3 - PSA luciferase expression vector containing 640bp - promoter fragment of PSA gene was constructed. Then, a 23 - mer phosphorothioated ARE decoy based on the deduced ARE sequence at the promoter region of PSA gene was synthesized. pGL3 - PSA and ARE decoy DNA were cotransfected into PC3 - M cell by lipofectamine™ 2000. Through detecting the activity of luciferase, the effect of ARE decoy on the promoter of PSA was studied. Then the ARE decoy DNA was transfected into LNCaP cells. The effect of decoy DNA on the proliferation of LNCaP cells was examined by using MTT assay. The effects of apoptosis were detected by phase contrast microscopy, DNA agrose gel electrophoresis and flow cytometry. Meanwhile, the nuclear extract was prepared from LNCaP cells and DNA - protein interactions were examined by electrophoretic mobility shift assay (EMSA). **RESULTS**: The reporter assay showed that the activity of luciferase was significantly reduced in the ARE decoy - transfected cells, bnt not in the cells transfected with the control decoy. EMSA demonstrated specific binding of the ARE decoy to androgen receptor. The growth of LNCaP was remarkably inhibited and apoptotic morphological changes as well as DNA fragmentation were observed in the ARE decoy - transfected cells. The rate of apoptosis was 22.4% detected by FCM. **CONCLUSION**: The ARE decoy is capable of inhibiting the promoter of PSA gene and inducing the apoptosis in prostate cancer cells. It may become a potential therapeutic tool for prostate cancers.

[**KEY WORDS**] Androgen responsive element; Transcription factors; Genes, reporter; LNCaP cells; Apoptosis
[**CLC number**] R363　　[**Document code**] A

A series of studies have indicated that androgen receptor (AR) plays a key role in the development of prostate cancer by mediating androgen activity on target cells[1]. Activated by androgen binding, AR moves into the nucleus, binds to the specific DNA sequences or androgen responsive element (ARE) of AR target genes and regulates the transcriptional activity of these genes[2,3]. Prostate - specific antigen (PSA) is expressed specifically in epithelial cells of the prostate, and is regulated by androgens[4]. The PSA promoter contains one of the ARE sequences AGAACAGCAAGTGTC at position - 170bp from its transcription start site. Activated AR binds this responsive element[5] and enhances the transcription of PSA. It has been proposed that decreasing androgen and AR levels as well as blockade the function of AR could be an effective therapeutic strategy for prostate cancer[6]. In this study, we synthesized an ARE decoy, a phosphorothiolated double stranded oligonucleotide which contained the same sequence as the ARE sequence (near - 170 bp region) at the promoter of PSA. The ARE decoy was cotransfected into PC3 - M cells with constructed luciferase expression vector, pGL3 - PSA, containing 640 bp promoter fragment of PSA gene. Through detecting the activity of luciferase, the effect of ARE decoy on the promoter of PSA was studied. Then, the ARE decoy was transfected into LNCaP cells to explore the effects of blocking AR DNA binding domain on growth of LNCaP cells.

[**Received date**] 2004 - 09 - 29　　[**Accepted**] 2005 - 01 - 28
* [**Foundation item**] Supported by the National Natural Science Foundation of China (No. 30470820); Shandong Province Health Department Youth Science Foundation (2005JW2007)
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MATERIALS AND METHODS

1 Materials

All enzymes used were purchased from TaKaRa. RPMI - 1640 medium and fetal bovine serum were purchased from Gibico. Dual - Luciferase Reporter Assay System was obtained from Promega. Plasmid pGL3 - Basic, pGL3 - Control and pRL - TK were kindly provided by Professor Gong yao - qin. Plasmid PSA - pEGFP, the human AR expression plasmid (hAR/pSG5) and DIG Gel Shift Kit (Roche) were gifts from Dr. Young of Mayo Clinic/Foundation. The human prostate carcinoma cell line PC3 - M was provided from QiLu Hospital of Shandong University. Another human prostate carcinoma cell line LNCaP was purchased from Institute of Biochemistry and Cell Biology, SIBS, CAS. Phosphorothiolated ARE decoy DNA, control decoy DNA and E2F decoy DNA were synthesized by Boya company.

2 Cell culture

Androgen - independent prostate cancer cell line PC3 - M cells were maintained in RPMI - 1640 medium supplemented with 10% new born calf serum, 1×10^5 U/L penicillin, and 0.1 g/L streptomycin at 37 ℃ in a 5% CO_2 incubator. Androgen - responsive prostate cancer cell line LNCaP cells were maintained in RPMI - 1640 medium supplemented with 10% fetal bovine serum. Other culture conditions were the same as PC3 - M.

3 Decoy oligodeoxynucleotides synthesis and annealing

The ARE decoy was designed based on the DNA sequence (- 173 to - 153) of the ARE in the promoter region of the human PSA gene. A mutant ARE decoy which had two - base mutations in the consensus ARE sequence[7] was also synthesized and used in the control studies. The 14 - mer E2F decoy was synthesized according to the sequence in the *c - myc* promoter.

ARE decoy: 5' - TGC AGA ACA GCA AGT GCT AGC - 3';3' - ACG TCT TGT CGT TCA CGA TCG - 5'; control decoy: 5' - GTC TGA TAA AGG GTG TTC TTT TT - 3';3' - CAT ACT ATT TCC CAC AAG AAA AA - 5';E2F decoy: 5' - GAT CCG CGG GAA AT - 3';3' - CTA GGC GCC CTT TA - 5'.

To obtain double stranded DNA decoys, a pair of complementary oligo deoxynucleotides above were mixed in TEN buffer (10 mmol/L Tris - HCl, 0.1 mmol/L EDTA, 0.1 mol/L NaCl, pH 8.0) at equal molar concentrations at 95 ℃ for 10 min, then cooled down to room temperature slowly.

4 Nuclear extracts and electrophoretic mobility shift assay (EMSA)

The nuclear extracts were prepared from LNCaP cells as described[8]. The protein concentration of nuclear extracts was measured 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 ARE decoy to the LNCaP nuclear proteins. Firstly, ARE 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 - 8 μg) in a buffer containing 20 mmol/L HEPES, pH 7.9, 100 mmol/L KCl, 5 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 12% glycerol, 4 mmol/L DTT and 1 μg poly[d(A - T)]/poly [d(I - C)] for 30 min at 25 ℃. Then, the above DNA - protein complexes were separated by electrophoresis using a 6% native - PAGE at 25 V for 1 - 1.5 h. The bands in gels were transferred to the positively charged nylon membrane by electroblotting in 0.25 × TBE transfer buffer at 400 mA 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 ARE decoy DNA (125 fold and 250 fold molar excess) was added during DNA - protein incubation period. In nonspecific competition experiments, the unlabeled control decoy DNA and E2F decoy DNA (125 fold molar excess) were used. For antibody supershift studies, the anti - AR 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 ℃. Before incubation with the ARE decoy, poly [d(I - C)] was added.

5 The effect of ARE decoy DNA on the promoter of PSA

5.1 Plasmid construction To generate a 640 bp fragment of the PSA promoter, PSA - pEGFP was used as a template for the polymerase chain reaction (PCR). The PCR product containing the 640 bp PSA promoter fragment with *Sac* Ⅰ site at 5' end and *Kpn* Ⅰ site at 3' end, was digested with the two corresponding enzymes, purified from agarose gel and inserted into the upstream

of luciferase gene of the pGL3 – basic vector pre – cut with *Sac* Ⅰ and *Kpn* Ⅰ enzymes. The recombinant plasmid was transformed into competent *E. coli* DH – 5α. 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 – PSA.

5.2 Transfection experiments The day before transfection, PC3 – M cells were seeded at a density of 2×10^5/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 PC3 – M cells by lipofectamine™ 2000 according to the manufacturer's instructions. 0.4 – 1.6 μg plasmid/decoy DNA and 1 – 4 μL lipofectamine were used per well. The cells were incubated with 0.8 mL serum – free medium during the transfection. Plasmid pRL – TK containing renilla luciferase was used as an internal control. The ratio of pRL – TK and experimental vector was 1∶25.

5.3 Cells harvest for luciferase reporter assays Dual – luciferase Reporter Assay System was used for this procedure. The transfected cells were washed once by PBS and harvested following the addition of 1 × PLB (passive lysis buffer) by scrapping vigorously with a rubber policeman. The luminometer was programmed to perform a 2 – second premeasurement delay, followed by a 10 – second measurement period for each reporter assay. 100 μL Luciferase Assay Reagent (LAR) and 20 μL cell lysate were mixed in the luminometer tube and placed in the luminometer to initiate reading as M1. Then another 100 μL Stop& Glo reagent was added into the above tube. The renilla 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 three times.

6 The effect of ARE decoy DNA on the growth of LNCaP cells

6.1 Transfection experiments ARE decoy DNA was transfected into LNCaP cells by lipofectamine™ 2000 as above described. In this experiments, three groups were designed, of which one with 2 mg/L ARE decoy transfection was experimental group, one with 2 mg/L control decoy transfection was control group and another without any decoy transfection was blank group.

6.2 Measurement of LNCaP proliferation by MTT colorimetric assay LNCaP cells were seeded at a density of 1×10^4 cells/well into 96 – well plates. MTT (5 g/L) was added to the wells (10 μL/well) respectively at 24 h, 48 h and 72 h after transfection as before. After another 4 hours incubation at 37 ℃, the media in the wells were replaced with dimethylsulfoxide (DMSO) (150 μL/well). The plates were agitated at room temperature for 10 min. Absorbance (*A* value) of every well at 570 nm wavelengths was read on an ELISA reader.

6.3 Cell morphology and apoptosis assay by flow cytometry (FCM) At 48 h after transfection, the morphology of LNCaP cells was observed by phase contrast microscope and photographed. Meanwhile, about 1×10^6 cells were collected, fixed with 70% ethanol and sent to Jinan Military General Hospital for apoptosis assay by flow cytometer.

6.4 Apoptosis DNA fragmentation assay At 48 h after transfection, cell pellets (1×10^7 cells) were resuspended in 500 μL of lysis buffer (1% SDS, 1 mmol/L EDTA, 200 mmol/L NaCl, 200 mg/L protease K and 10 mmol/L Tris – HCl, pH 7.8) at 37 ℃ for 5 h and centrifuged at 12 000 × *g* for 10 min. The chromosomal DNA was then extracted twice with phenol/chloroform (1∶1), precipitated with ethanol, and resuspended in TE buffer (10 mmol/L Tris – HCl, pH 8.0, and 1 mmol/L EDTA). DNA was analyzed after separation by gel electrophoresis (1.5% agarose gel). DNA bands were visualized under ultraviolet illumination and photographed.

7 Statistical analysis

All numerical data were expressed as $\bar{x} \pm s$. Results were considered significant if $P < 0.05$ was obtained by appropriate analysis of variance (ANOVA) procedures and Student's *t* – test.

RESULTS

1 The specificity of ARE decoy DNA with protein

To examine whether the ARE decoy was able to bind to AR, electrophretic mobility shift assay was performed. The nuclear extracts from LNCaP cells were used for *in vitro* binding assay with double strands ARE decoy probes and the complex of DNA – protein was observed. In the competition study with a 125 – fold molar excess of the unlabeled ARE 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 ARE decoy to the LNCaP nuclear protein was specific. Furthermore, the band of the DNA – protein complex was supershifted by adding anti – AR antibody, indicating that the protein binding to the ARE decoy was AR (Fig 1).

	1	2	3	4	5	6	7	8
Labeled ARE decoy	+	+	+	+	+	+	+	+
Unlabeled ARE decoy	-	-	+	+	-	-	-	-
Control decoy	-	-	-	-	+	-	-	-
E2F decoy	-	-	-	-	-	+	-	-
Nuclear protein	-	+	+	+	+	+	+	+
Anti-AR antibody	-	-	-	-	-	-	+	+

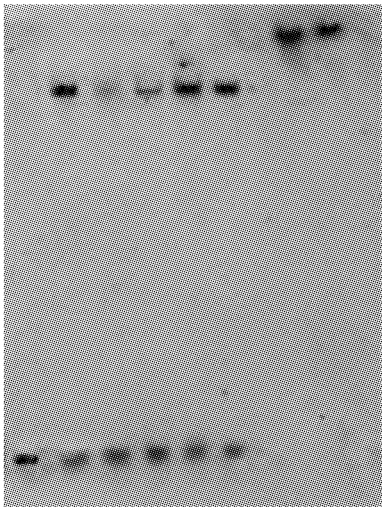

Fig 1 EMSA of ARE decoy DNA and prostatic nuclear extracts. 1: labeled ARE decoy without protein; 2: labeled ARE decoy with nuclear protein; 3, 4: lane 2 plus unlabeled ARE decoy (250 and 125 folds molar excess, respectively); 5: lane 2 plus unlabeled control decoy; 6: lane 2 plus unlabeled E2F decoy; 7, 8: lane 2 plus anti – AR antibody.

2 pGL3 – PSA construction and identification

A 640 bp promoter fragment of PSA gene was generated by using PCR. It was inserted into the upstream of Luc$^+$ gene of pGL3 – basic . The recombinant could be cut into 640 bp and 4.8 kb fragments by *Sac* I and *Kpn* I enzymes. The results of digestion and DNA sequencing were consistent with our anticipation.

3 Inhibitory effects of the ARE decoy DNA on the promoter of PSA

To ensure the suitable ratio of pGL3 – PSA and hAR expression plasmids, hAR vector at a fixed concentration of 1.5 mg/L was co – transfected with different doses of pGL3 – PSA and pRL – TK into PC3 – M. Then the pGL3 – PSA at 0.19 mg/L and pRL – TK 7.6 μg/L were co – transfected with different amounts of hAR into PC3 – M. In the end, 0.19 mg/L hAR, 0.19 mg/L pGL3 – PSA and 7.6 μg/L pRL – TK were used for co – transfection with different doses of ARE decoy DNA. It turned out that ARE decoy DNA could inhibit the activity of luciferase significantly at the dose of 1.98 mg/L, at which the inhibition rate reached 95% (Fig 2).

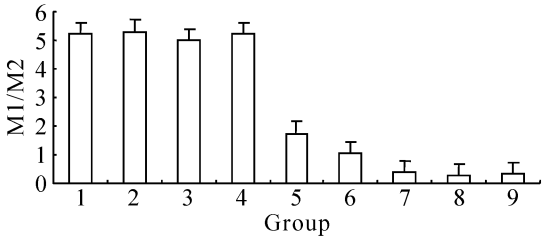

Fig 2 Dual – luciferase assay for detection the promoter activity. Measurement of luciferase activities in PC3 – M with the following transfections. Lane 1: treated without any decoy DNA; Lanes 2 – 4: treated with 0.66 mg/L, 1.32 mg/L, 1.98 mg/L control decoy DNA, respectively; Lanes 5 – 9: treated with 0.66 mg/L, 0.99 mg/L, 1.32 mg/L, 1.98 mg/L, 2.64 mg/L ARE decoy DNA, respectively. The results are presented as $\bar{x} \pm s$.

4 Proliferation measurement by MTT colorimetric assay

To study the effect of ARE decoy on the proliferation of LNCaP cells, MTT colorimetric assay was used. As shown in Fig 3, the proliferation of LNCaP cells was obviously inhibited by ARE decoy DNA, comparing with control group and blank group.

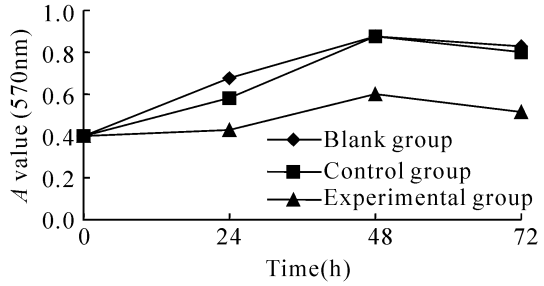

Fig 3 MTT colorimetric assay for proliferation detection. ARE decoy DNA inhibited the growth of LNCaP cell obviously.

5 Apoptotic effects

At 48 h after transfection, the morphology of LNCaP was observed by phase contrast microscope. Apoptotic morphological changes, such as cell shrinking, chromatin condensation and formation of apoptotic body were seen in the ARE decoy – transfected cells, but not in the control decoy – transfected cells. DNA fragmentation assay was done by agrose gel electrophoresis. DNA from the ARE decoy – transfected LNCaP cells

showed an internucleosomal DNA ladder characteristic of apoptosis, whereas DNA from the control and the blank group did not (Fig 4).

Furthermore, the rate of apoptosis was measured by FCM. At the result, the rate of apoptosis was 22.4% in the ARE decoy – transfected LNCaP cells, which was remarkably higher than the other two groups: 9.8%, 4.7%, respectively (Fig 5).

DISCUSSION

Androgen and androgen receptor play the important role in the development and progression of prostate cancer. Androgen exerts its function through androgen receptor. In the absence of androgens, AR stays in the cytoplasm and binds to heat – shock protein in an inactive form. When activated by binding to androgen, it changes its conformation to form dimmer, moves into nucleus and binds to a specific DNA sequence – androgen responsive element in the promoter of target genes (such as PSA gene) to initiate gene transcription. The androgen – signaling pathway is critical to prostate cancer and androgen ablation which induces apoptosis is a mainstay of therapy for this disease.

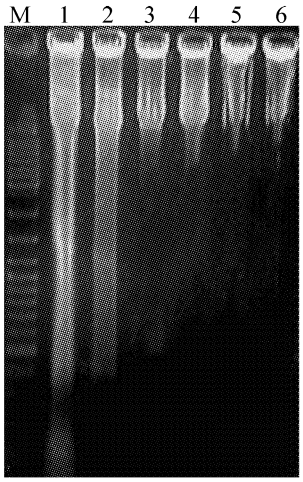

Fig 4 Apoptosis DNA fragmentation assay. M: 100 bp DNA ladder marker; 1, 2: 48 h after ARE decoy DNA transfection; 3, 4: 48 h after control decoy DNA transfection; 5, 6: 48 h after not any decoy DNA transfection.

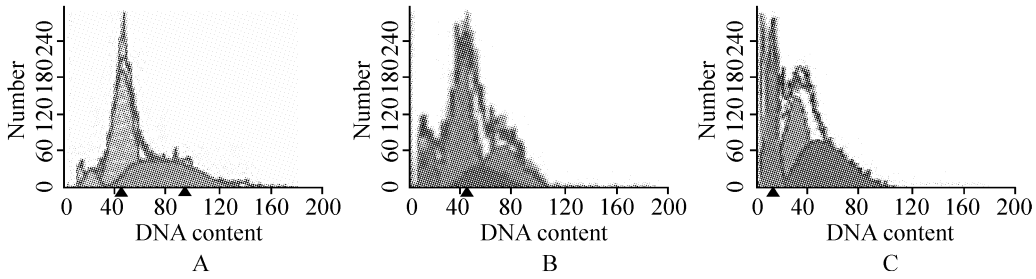

Fig 5 Apoptosis of LNCaP cells 48h after transfection. A: without any decoy DNA transfection, apoptotic rate was 4.7%; B: control decoy DNA transfection, apoptotic rate was 9.8%; C: ARE decoy DNA transfection, apoptotic rate was 22.4%.

Recently, transfection of cis – element double – stranded oligodeoxy – nucleotides (ODNs), referred to as "decoy" ODNs, has been reported to be a powerful tool in a new class of anti – gene strategies[9-11]. 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 experiment, we constructed luciferase gene eukaryotic expression vector by linking the PSA promoter to luciferase gene (a reporter gene). The effect of ARE decoy on the PSA promoter was studied after co – transfection with the PSA promoter vector into PC3 – M cells. The results showed that ARE decoy could suppress the transcriptional activity of AR by blocking its binding sites to the endogenous ARE sequence resulting in the inhibition of PSA promoter driving luciferase expression. Meanwhile, we transfected ARE decoy DNA into LNCaP cells, by which induction of apoptosis was demonstrated. In conclusion, ARE decoy DNA can inhibit the promoter of PSA and induce the apoptosis of prostate cancer cell line LNCaP through binding the AR competitively and inhibiting the function of AR. Decoy DNA strategy may provide a new method for therapy of prostate cancer.

Different cis – element decoy DNA can inhibit the correspondent transcription factor after transfected into cells so it can be used for studying the mechanisms of cis – element and transcription factor in transcriptional regulation as well as the interaction of cis – element and transcription factor. On the other hand, transcription of

decoy DNA can induce the suppression of cell growth and the cell apoptosis by inhibiting the expression of related genes such as survivin and Bcl - 2. Therefore, transcription factor "decoy" strategy can be used as a powerful tool for the study of transcriptional regulation mechanisms and for gene therapy of various diseases.

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雄激素应答元件陷阱 DNA 抑制 PSA 基因启动子的作用并诱导 LNCaP 细胞凋亡

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[**摘 要**] **目的**：研究雄激素应答元件陷阱 DNA（ARE decoy）对前列腺特异抗原（PSA）基因启动子的抑制作用及其对前列腺癌细胞 LNCaP 细胞生长活性的影响，为前列腺癌的基因治疗寻求新的策略。**方法**：联合运用报告基因和陷阱 DNA 策略，构建含 PSA 基因 5'侧启动子区 640 bp DNA 的荧光素酶表达载体 pGL3 - PSA，ARE 陷阱 DNA 共转染前列腺癌细胞株 PC3 - M。应用双荧光素酶测定系统，检测荧光素酶的表达活性。然后，应用 2 mg/L ARE decoy 转染 LNCaP 细胞，通过相差显微镜观察细胞超微结构变化，MTT 比色法检测细胞生长活性，DNA ladder 和流式细胞技术（FCM）检测细胞凋亡以研究 ARE decoy DNA 对前列腺癌细胞 LNCaP 细胞生长活性的影响。同时提取 LNCaP 细胞核蛋白，应用电泳迁移率变动分析（EMSA）检测 ARE decoy DNA 与雄激素受体的特异结合。**结果**：ARE decoy DNA 显著抑制报告基因荧光素酶的表达，抑制率可达 95%。EMSA 显示 ARE decoy DNA 能特异与核蛋白中雄激素受体结合。LNCaP 细胞转染 ARE decoy DNA 后，镜下观察部分细胞出现凋亡形态学的改变，细胞体外生长受到抑制，染色体 DNA 凝胶电泳可见明显梯形条带。转染 48h 的凋亡率为 22.4%。**结论**：实验表明 ARE decoy DNA 能竞争结合雄激素受体（AR），阻断 AR 的作用而诱导 LNCaP 细胞凋亡，有可能为前列腺肿瘤的治疗提供新的策略。

[**关键词**] 雄激素应答元件；转录因子；基因，报告；LNCaP 细胞；细胞凋亡

[**中图分类号**] R363 [**文献标识码**] A