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Curcumin downregulates homeobox gene *NKX3.1* in prostate cancer cell LNCaP[1]

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Key words

curcumin; prostate cancer cell; NKX3.1; androgen receptor; androgen responsive element

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Abstract

Aim: To elucidate the effect and the mechanisms of curcumin on the expression of the human homeobox gene *NKX3.1* in the prostate cancer cell LNCaP. **Methods:** The expression change of *NKX3.1* in cells incubated with varying concentrations of curcumin was observed by Western blotting and RT-PCR. A dual luciferase reporter assay was used to test the effect of curcumin on the activity of the *NKX3.1* 1040 bp promoter. Curcumin-treated cells disposed to a designated amount of androgen analog R1881 and the androgen receptor (AR) antagonist flutamide, then the expression of *NKX3.1* or the activity of the *NKX3.1* promoter were investigated by Western blotting or reporter gene assay, respectively. Finally, Western blotting and electrophoretic mobility shift assay were performed to demonstrate the effect of curcumin on the expression of AR and its binding activity to the androgen response element (ARE). **Results:** Curcumin downregulated the expression of *NKX3.1* and the activity of the *NKX3.1* 1040 bp promoter in LNCaP cells. R1881 increased the expression of *NKX3.1*, and the AR antagonist flutamide decreased the expression of *NKX3.1* in LNCaP cells, while curcumin could inhibit androgen-AR mediated induction of *NKX3.1* expression. Curcumin decreased the expression of AR and the binding activity to ARE directly. **Conclusion:** Curcumin could downregulate *NKX3.1* expression in LNCaP cells. It could also inhibit the androgen-AR mediated induction of *NKX3.1* expression by downregulating AR expression and blocking its DNA binding activity.

Introduction

Prostate cancer is the most common cancer in males, and the second leading cause of male cancer deaths in developed countries[1]. Current prostate cancer therapies such as surgery, chemotherapy, and radiation therapy are of limited efficacy and may not avoid the significant side-effects. Androgen reduction therapy is commonly used to control hormone-sensitive tumor cells; however, hormone refractory clones often emerge after hormonal therapy[2]. Advanced hormone refractory prostate cancer is almost incurable[3]. Therefore, novel effective therapies, including biotherapy, are urgently needed to be developed.

In a search for alternative and preventive therapies for prostate cancer, attention has been focused on plant polyphenolic compounds, which are rich in nutritional supplements such as soybeans, garlic, and green tea, and have been used to augment anticancer therapies[4,5]. Curcumin, the active component of turmeric, is a dietary constituent that has received a great deal of attention recently as a chemoprotective agent[6,7]. Several recent observations have shown that curcumin has antioxidant and anti-inflammatory activities, as well as anticarcinogenic activity in colon cancer, breast cancer[8,9], and leukemia[10]. The molecular mechanisms of the actions of curcumin are beginning to be elucidated, including the effects of suppressing tumor initiation and promotion in animal models. Curcumin is a potent inhibitor of cycloxygenase-2, lipoxygenase, ornithine decar-

boxylase[11–13], c-Jun/AP-1, nuclear factor-κB[14,15], c-Jun N-terminal kinase, and protein kinase C[16,17]. Curcumin inhibited epidermal growth factor receptor activity in various tumors, including prostate carcinoma[18]. Curcumin can cause a marked decrease in cell proliferation and microvessel density, and an increase in apoptosis in prostate tumors[19]. By virtue of its multiple effects, curcumin has potential clinical application in the prevention of prostate cancer.

NKX3.1 has been identified as the androgen-regulated NK-class homeobox gene that is largely specific to prostate for expression, and is thought to play an important role in normal prostate organogenesis and carcinogenesis[20–23]. Human *NKX3.1* has been mapped to human chromosome 8p21, a locus that experiences a frequent loss of heterozygosity in human prostate cancer, raising the possibility that *NKX3.1* may be a tumor suppresser gene[22–24]. It has been reported that the loss of a single allele may predispose to prostate carcinogenesis.

As a gene regulated by androgen, the maintaining expression of *NKX3.1* in the prostatic epithelium is dependent on androgen signaling[20,22–23]. However, it is not very clear whether this regulation occurs directly through the interaction of the androgen receptor (AR) and the *NKX3.1* promoter.

In this study, an experimental investigation was undertaken to characterize the effect of curcumin on *NKX3.1* expression in the prostate cancer cell LNCaP, and to investigate the mechanisms in which curcumin downregulates the homeobox gene *NKX3.1* in the prostate cancer cell.

Materials and methods

Cell culture and treatments The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (Manassas, Virginia, USA). This cell line was established from a lymph node metastasis of a prostate cancer patient and expressed mutant, but functional on AR. The LNCaP cells were seeded in 35 mm culture dishes in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 5% CO_2 at 37 °C until reaching approximately 50%–70% confluence. The cells were maintained in serum-free RPMI 1640 medium for a further 24 h before the experiments. The cells were then treated with designated curcumin, R1881, or flutamide in RPMI 1640 medium containing 5% FBS. Curcumin (No C1386, Sigma, St Louis, MO, USA) and flutamide (No F9397-1G, Sigma, St Louis, MO, USA) were dissolved in dimethyl sulfoxide, which is also a control vehicle.

RT-PCR analysis Total RNA was extracted from the LNCaP cells with Trizol reagent (MBI Fermentas, Burlington, ON, Canada) following the manufacturer's instructions, and the expression of *NKX3.1* mRNA was determined by RT-PCR using M-MuL V reverse transcriptase in the presence of random hexamer primers. The PCR primers for *NKX3.1* mRNA were as follows: 5'-GTACCTGTCGGCCCCTGAACG-3' (sense), and 3'-GGACCAGAGGCACATA TTGTCG-5' (antisense). A 500 bp β-actin mRNA was amplified and used to normalize the quantity of the *NKX3.1* mRNA in RT-PCR. The primers for the AR were as follows: 5'-TCTCAAGAG-TTTGGATGGCTCC-3' (sense) and 5'-TCACTGGGTGTGG-AAATAGATG -3' (antisense)[25]. The PCR profiles consisted of initial denaturation at 94 °C for 3 min, followed by 28 cycles of denaturation at 94 °C for 40 s, primer-annealing at 61 °C for 40 s, and primer extension at 72 °C for 40 s. The final primer extension was performed at 72 °C for 10 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide and photographed under UV light.

SDS-PAGE and Western blot analysis The LNCaP cells were harvested at designated times, and nuclear extraction was prepared using the protocol described by a nuclear extraction kit (Active Motif, Carlsbad, CA, USA). Protein concentrations of cell nuclear extracts were quantified by the bicinchoninic acid method. For Western blot analysis, 40 μg of protein was separated on 10% SDS-PAGE and then transferred to the nitrocellulose membrane. After being blocked and washed, the membrane was incubated with human specific anti-*NKX3.1* antibody or anti-AR antibody (gifts from Dr Charles YOUNG, Department of Urology Research, Mayo Clinic, Rochester, MN, USA) at 4 °C for 12 h, followed by incubation with horseradish peroxidase-labeled second antibody for 1 h at room temperature. Immunoreactive bands were then visualized by enhanced chemiluminescence (Santa Cruz, San Diego, CA, USA). β-tubulin or β-actin (Sigma, St. Louis, MO, USA) was used to normalize the quantity of the protein on the blot.

Electrophoretic mobility shift assay (EMSA) Nuclear extracts were prepared from the LNCaP cells using a nuclear extraction kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Equal amounts of sense and antisense oligonucleotides were mixed and annealed in a buffer (10 mmol/L Tris-HCl, pH 8.0, 200 mmol/L NaCl, and 1 mmol/L EDTA) by heating to 95 °C for 5 min and slowly cooling to room temperature. The corresponding oligonucleotides were labeled with digoxigenin (DIG). The following oligonucleotides were used for the EMSA experiments: the ARE decoy based on the deduced ARE sequence at the promoter region of the human prostate-specific antigen (PSA) gene[26], and the E2F decoy which contained the 8 bp

cis-element (underlined) that was identified in the c-myc promoter[27].

ARE: sense 5'-TGCAGAACAGCAAGTGCTAGC-3', antisense 5'-GCTAGCACTTGCTGTTCTGCA-3';
NF-κB: sense 5'-GCCTGGGAAAGTCCCCTCAACT-3', antisense 5'-AGTTGAGGGGACTTTCCCAGGC-3';
E2F: sense 5'-TGATTTCCCGCGGAT-3', antisense 5'-ATCCGCGGGAAATCA-3'.

Binding reactions were carried out at room temperature for 30 min in a mixture containing 4% glycerol, 1 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 50 mmol/L NaCl, 10 mmol/L Tris·HCl, 2 μg poly (dI-dC), 10 μg nuclear extracts, and a DIG-labeled oligonucleotide probe. Then the reaction mixtures were subjected to electrophoresis in 5% non-denaturing polyacrylamide gels in 0.25×Tris/borate/EDTA buffer. Based on the instructions of the DIG Gel Shift Kit (Roche Co, Basel, Switzerland), electroblotting and chemiluminescent detection were performed. The specificity of AR-ARE binding was confirmed by adding 125 fold excess of unlabeled ARE\E2F\NF-κB probe to the assay.

Transient transfection assay The LNCaP cells were plated in a 24 well plate until they reached a confluency of 90%. The cells were transiently transfected with a 1040 bp *NKX3.1* promoter-luciferase reporter plasmids pGL3-*NKX3.1*[28] using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA), while pRL-TK (Promega, Madison, WI, USA) plasmids were used as a internal control. Twenty-four hours after the transfection, the cells were treated with different concentrations of curcumin for an additional 12 h. Whole cell extracts were prepared and a luciferase assay was performed according to the manufacturer's instructions (Promega, USA) for the *NKX3.1* promoter/luciferase transfection. Each transfection was done 3 times and standard deviations were calculated.

Statistical analysis All the measurement data were analyzed and expressed as the mean±SEM. Results were considered significant if $P<0.05$ was obtained by an appropriate ANOVA procedure and Student's *t*-test.

Results

Curcumin inhibited the expression of the *NKX3.1* gene To determine whether *NKX3.1* expression levels changed with curcumin treatment in the androgen-sensitive prostate cancer cell LNCaP, expression of *NKX3.1* was determined in serum-starved LNCaP cells exposed to varying concentrations of curcumin for 24 h by RT-PCR and Western blotting. As shown in Figure 1A, *NKX3.1* mRNA expression was significantly downregulated by 40 μmol/L of curcumin treatment (by ~5 fold), and less effect was observed in the cells treated with 10 and 20 μmol/L curcumin.

To further confirm the inhibitory effect of curcumin on *NKX3.1* protein expression, Western blotting was carried out. Since *NKX3.1* is a nuclear protein and functions in the nucleus, we prepared the nuclear extracts for Western blotting after the LNCaP cells were exposed to curcumin for 24 h. The results in Figure 1B show that the expression of the *NKX3.1* protein dramatically decreased by curcumin in a dose-dependent manner, similar to its mRNA as shown in Figure 1A.

To test whether the inhibitory effect of curcumin on *NKX3.1* expression occurs at the transcription level, we transfected a vector containing a 1040 bp *NKX3.1* promoter fragment in the upstream of a luciferase reporter gene into the LNCaP cells. As seen in Figure 1C, for the LNCaP cells exposed to varying amounts of curcumin for 24 h, the *NKX3.1* promoter gave a gradual inhibition of the luciferase activity (representing *NKX3.1* promoter activity) in a dose-dependent manner.

Taken together, curcumin could inhibit the expression of *NKX3.1* at the promoter, mRNA, and protein levels.

Curcumin inhibited androgen-mediated induction of *NKX3.1* expression Because *NKX3.1* is known as an androgen upregulated gene, we examined the nonmetabolizable androgen R1881 and the AR antagonist flutamide to see if they can influence expression of the *NKX3.1* protein in LNCaP cells exposed to curcumin. In Figure 2, we show that the expression of *NKX3.1* could be enhanced by R1881 (lane 2), which was partially inhibited by the AR antagonist flutamide (lane 3) without curcumin treatment of LNCaP cells. The results demonstrated that *NKX3.1* protein expression was upregulated by androgen and AR activity without curcumin treatment. When the LNCaP cells were exposed to curcumin (30 μmol/L), the *NKX3.1* protein had a notable decrease (lane 4), and R1881 no longer stimulated *NKX3.1* protein expression significantly (lane 5), which suggests that curcumin may have disrupted the AR function so that R1881 lost its stimulation on *NKX3.1* expression. Furthermore, curcumin and flutamide together could further decrease the *NKX3.1* protein (lane 6). Our data suggests that the expression of *NKX3.1* was partially dependent on the AR signaling pathway, and curcumin could repress androgen and AR-mediated induction of *NKX3.1* expression.

Curcumin inhibited expression of the AR gene To further elucidate the role of AR in curcumin-induced *NKX3.1* depression, we evaluated the effect of curcumin on AR expression in LNCaP cells directly. Both RT-PCR and Western blot analysis gave very similar results (Figure 3), indicating that AR expression significantly decreased in mRNA

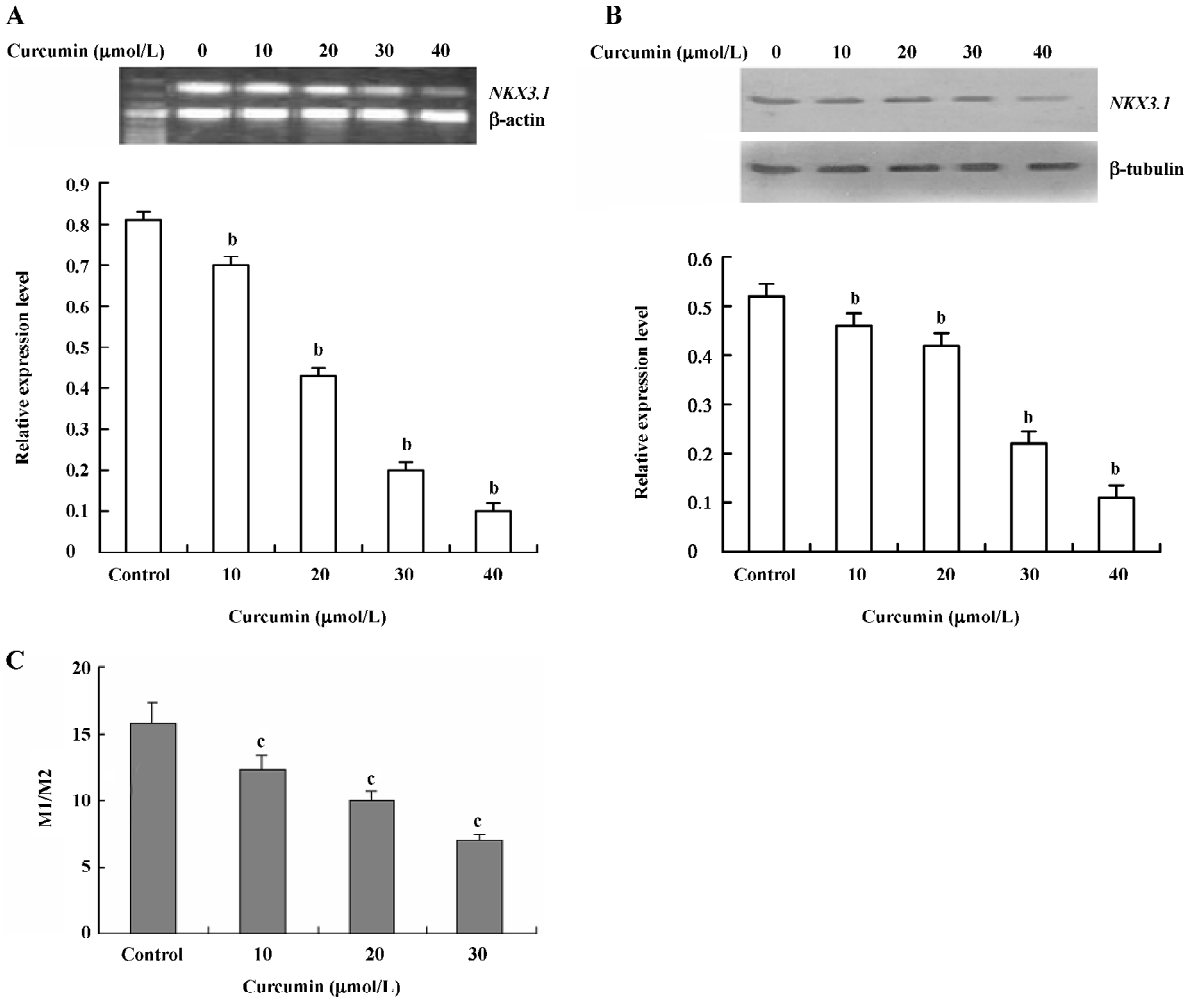


Figure 1. Curcumin inhibits the expression of the *NKX3.1* gene. Serum-starved LNCaP cells were incubated in the presence and absence of the indicated doses of curcumin for 24 h. (A) RT-PCR analysis for the *NKX3.1* gene. The β-actin gene was used as an internal control. (B) Western blot analysis for the *NKX3.1* protein (i), β-tubulin reblotted by anti-β-tubulin antibody in same membranes was used as an internal control for protein loading and transfer efficiency (ii). The densitometric measurements of *NKX3.1* mRNA and *NKX3.1* protein levels were normalized to the internal control, respectively, and the ratio was regarded as a relative number which is expressed as mean±SEM. n=3. $^bP<0.05$ *vs* control. (C) Curcumin inhibits the promoter activity of the *NKX3.1* gene. LNCaP cells were transiently cotransfected with pGL3-*NKX3.1* promoter and pRL-TK (internal control) with Lipofectamine 2000. Then, the serum-starved LNCaP cells were incubated in designated concentrations of curcumin for 24 h. All cells were harvested for the dual-luciferase activity assay after 48 h of transfection. Relative luciferase activity is represented by the ratio of firefly luciferase activity (M1) in pGL3 to Renilla luciferase activity (M2) in pRL-TK. The results are presented as mean±SEM (n=3). $^cP<0.01$ *vs* control.

(Figure 3A) and protein (Figure 3B) levels by curcumin in a dose-dependent manner. Remarkably, the effect of curcumin on AR expression was almost identical to that on *NKX3.1* expression, as shown in Figure 1.

Curcumin decreased ARE binding activity The above results led us to further investigate whether the function of the AR could be affected by curcumin. We used the gel band-shift technique as an in vitro functional assay to determine the AR DNA binding activity. The results in Figure 4 show that ARE binding activity was dramatically inhibited after 24 h of treatment with 30 and 40 μmol/L of curcumin when compared to the control. The bands were confirmed to be a result of specific binding for ARE, because the DNA-protein complex was competed out by a 125 fold molar excess of unlabeled ARE and could not be blocked by a 125 fold excess of unlabeled E2F and NF-κB oligonucleotides.

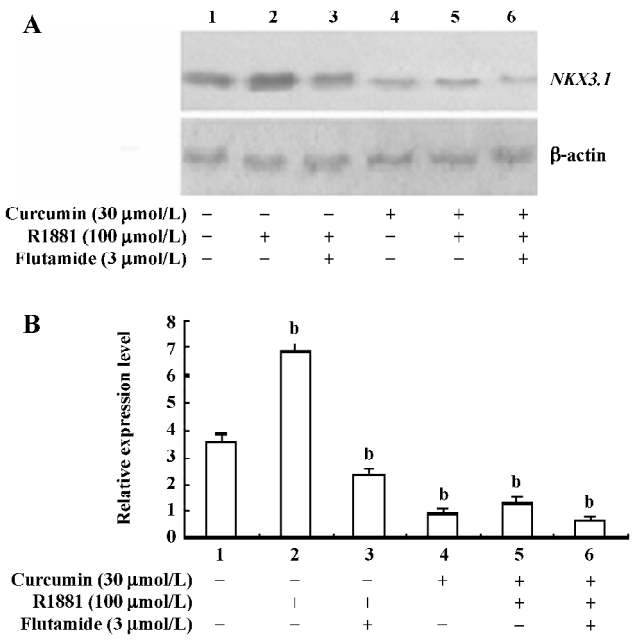


Figure 2. Curcumin inhibits androgen-induced *NKX3.1* expression. The expression of the *NKX3.1* was determined in serum-starved LNCaP cells incubated in 30 μmol/L curcumin for 12 h followed by R1881 (1×10^{-7} mol/L) and flutamide (3 μmol/L) treatment for an additional 12 h. For the Western blot analysis, 40 μg of nuclear extract was separated on 10% SDS-PAGE, and human specific anti-*NKX3.1* antibody was used to detect the *NKX3.1* protein. β-actin was used to normalize the experiment. The relative expression levels are presented as the ratio of the band densities of *NKX3.1* to β-actin. 1, control without any treatment; 2, with 100 nmol/L R1881 treatment; 3, with 100 nmol/L R1881 and 3 μmol/L flutamide treatment; 4, with 30 μmol/L curcumin treatment; 5, with 100 nmol/L R1881 and 30 μmol/L curcumin treatment; and 6, with 30 μmol/L curcumin, 100 nmol/L R1881, and 3 μmol/L flutamide treatment. The results are expressed as mean±SEM (n=3). $^bP<0.05$ *vs* column 1.

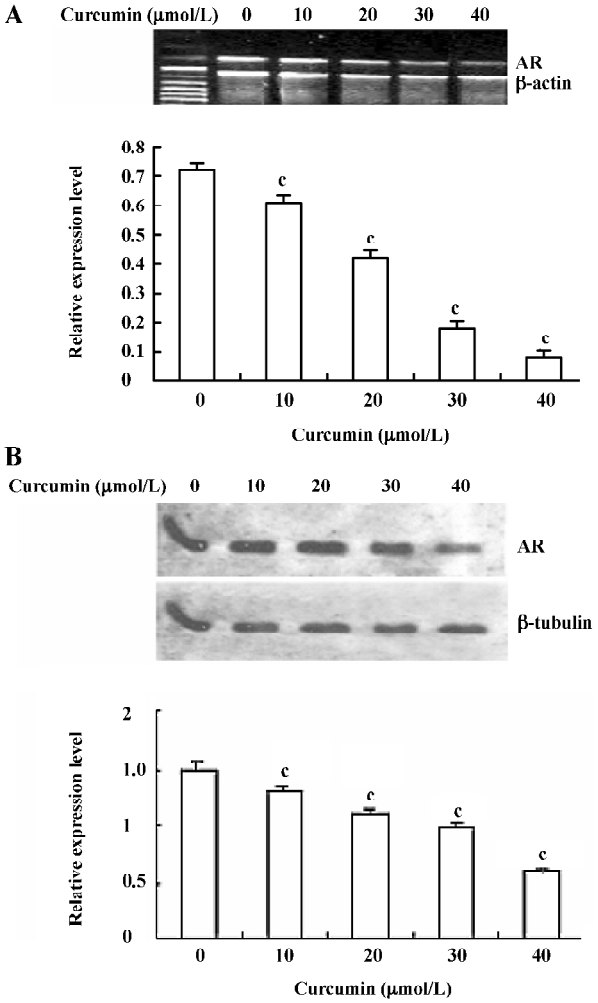
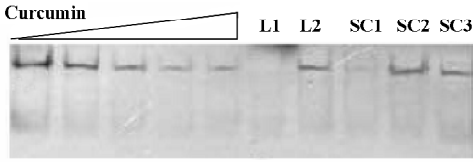


Figure 3. Curcumin inhibits the expression of the AR gene. Serum-starved LNCaP cells were incubated in the presence and absence of the indicated doses of curcumin for 24 h. (A) RT-PCR analysis was carried out for AR mRNA. β-actin was used as an internal control. (B) Western blot analysis was carried out for AR protein (i). Same membranes were reblotted with an anti-β-tubulin antibody to control protein loading and transfer efficiency (ii). Each column of the histogram represents a densitometric value of AR/internal control (β-actin or β-tubulin) ratio from 3 independent experiments. Mean±SEM. $^cP<0.01$ *vs* control.

Discussion

Curcumin, used as a food additive and a herbal medicine in Asia, is associated with a plethora of beneficial effects on human health, predominant among which are the anti-inflammatory and cancer chemoprophylaxis activities. Early works have shown that curcumin could suppress the activation of novel eukaryotic transcriptional factors, such as AP-1, NF-κB and AR in prostate cancer[29,30], and significantly inhibit the growth of prostate cancer cells *in vivo*[19] and *in vitro*[18]. The AR is a member of the nuclear receptor superfamily of transcription factors[31]. It is activated by its androgen ligand or by a ligand-independent manner[32–34]. Subsequently, the activated receptor homodimerizes and interacts with specific androgen response elements in the regulatory regions of androgen target genes, resulting in the stimulation of gene expression. *NKX3.1* is an androgen-regulated homeobox gene in the prostate[22]. In the present study, we first demonstrated the inhibitory effects of curcumin on *NKX3.1* expression in LNCaP cells and investigated the mechanisms.

In this study, we used 10–40 μmol/L of curcumin to treat the prostate cancer cell LNCaP that expresses both *NKX3.1* and the AR. With RT-PCR, Western blotting, and luciferase reporter analysis, we found that curcumin downregulated



	1	2	3	4	5	6	7	8	9	10
Labeled ARE	+	+	+	+	+	+	+	+	+	+
Nucleic extracts	+	+	+	+	+	–	+	+	+	+
Unlabeled ARE	–	–	–	–	–	–	–	+	–	–
Unlabeled E2F	–	–	–	–	–	–	–	–	+	–
Unlabeled NF-κB	–	–	–	–	–	–	–	–	–	+
Curcumin (μmol/L)	0	10	20	30	40	0	0	0	0	0

Figure 4. Effect of curcumin on the ARE DNA binding activity assay by EMSA. DIG-labeled ARE oligonucleotides were incubated without nucleic extracts (lane 6/L1) or with nucleic extracts from curcumin-untreated (lanes 1,7/L2) and curcumin-treated LNCaP cells with 10, 20, 30, and 40 μmol/L curcumin, respectively (lanes 2–5). SC, Specific competition experiments were carried out with a 125 fold excess of unlabeled ARE (lane 8/SC1), E2F (lane 9/SC2), and NF-κB (lane 10/SC3) oligonucleotides.

the expression of the *NKX3.1* gene at both mRNA and protein levels in a dose-dependent manner. To further investigate whether or not this inhibitory effect is androgen-AR mediated, the LNCaP cells were treated with curcumin only or curcumin and synthetic androgen analog R1881. The results in Figure 2 show that curcumin could decrease the *NKX3.1* protein and inhibit the stimulation of *NKX3.1* expression by androgen, suggesting that curcumin could repress the androgen-mediated induction of *NKX3.1* expression. Furthermore, the effects of curcumin on AR expression and its DNA binding activity were detected by Western blotting and EMSA to demonstrate the mechanisms in which curcumin inhibits androgen-mediated induction of *NKX3.1* expression. The results show that curcumin decreased AR expression significantly at both mRNA and protein levels, and blocked AR-ARE binding activity in EMSA. Our data suggests that curcumin can repress the androgen-mediated induction of *NKX3.1* expression by inhibiting AR gene expression and blocking its DNA binding activity.

In the EMSA experiment, we used the known ARE sequence in the upstream of the PSA gene to show that curcumin could block AR DNA binding activity and make AR lose its function, disrupting the androgen-AR signaling pathway. *NKX3.1* is proved to be a prostate-specific and androgen-regulated gene, and it was reported that there are several potential AR binding sites in the regulatory region of the *NKX3.1* gene[35,36], but so far, there is no identification of functional ARE in *NKX3.1* to be reported. Further investigation will be required to detect functional ARE in the regulatory regions of the *NKX3.1* gene and to determine if the ARE is the actual function target of curcumin.

The inhibition of *NKX3.1* gene expression by curcumin seems to be inconsistent with the classical idea that the antitumor agent increases the expression of the tumor suppressor gene. *NKX3.1* is supposed to be a tumor suppressor gene; however, its function is mainly related to the development and differentiation of normal prostate. Evidence of *NKX3.1* being involved in the prostate tumor and progression mainly come from animal models[37–41]. The reports on the roles of *NKX3.1* in human prostate cancer are pendent, and some reports are inconsistent. Bowen *et al* showed that loss of *NKX3.1* expression in human prostate cancers correlated with tumor progression[27], while Aslant *et al* reported that the decline of *NKX3.1* expression was not correlated with prostate cancer progression and was not associated with advanced stage. Thus, *NKX3.1* expression is not a clinically-valuable prognostic factor[42]. However, overexpression of *NKX3.1* in prostate cancer also has been reported[43]. So the roles of *NKX3.1* on prostate cancer in clinics needs to be further studied.

In summary, our results demonstrate that curcumin, as an anticancer agent, can downregulate the expression of *NKX3.1* which is a prostate-specific and androgen-regulated homeobox gene, and can intervene the AR signaling pathway as well as repress the androgen-mediated induction of *NKX3.1* expression by inhibiting AR gene expression and blocking its DNA binding activity.

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Correction

1) Acta Pharmacol Sin 2006 Jul; 27(7): 888–94
Molecular nature of sulfhydryl modification by hydrogen peroxide on type 1 ryanodine receptor.
Hong-mei HAN, Ri-sheng WEI, Anthony F LAI, Chang-cheng YIN
The name of the third author appears in the authorship list incorrectly as Anthony F LAI and it should be corrected to F Anthony LAI.

2) Acta Pharmacol Sin 2006 Dec; 27 (12): 1630–6
Antisense oligonucleotides targeting midkine induced apoptosis and increased chemosensitivity in hepatocellular carcinoma cells.
The symbol legends for Figure 9A and Figure 9B had been incorrectly deleted. The correct symbol legends should be added as ▲ 5-Fu; ○ 5-Fu+ASODN 0.1 μmol/L; ■ 5-Fu+ASODN 0.2 μmol/L; × 5-Fu+ASODN 0.4 μmol/L for Figure 9A and ▲ ADM; ○ ADM+ASODN 0.1 μmol/L; ■ ADM+ASODN 0.2 μmol/L; × ADM+ASODN 0.4 μmol/L for Figure 9B.
The symbol legend for Figure 9C should be ▲ DDP; ○ DDP+ASODN 0.1 μmol/L; ■ DDP+ASODN 0.2 μmol/L; × DDP+ASODN 0.4 μmol/L, rather than ▲ 5-Fu; ○ 5-Fu+ASODN 0.1 μmol/L; ■ 5-Fu+ASODN 0.2 μmol/L; × 5-Fu+ASODN 0.4 μmol/L. The correct Figure 9 as list:

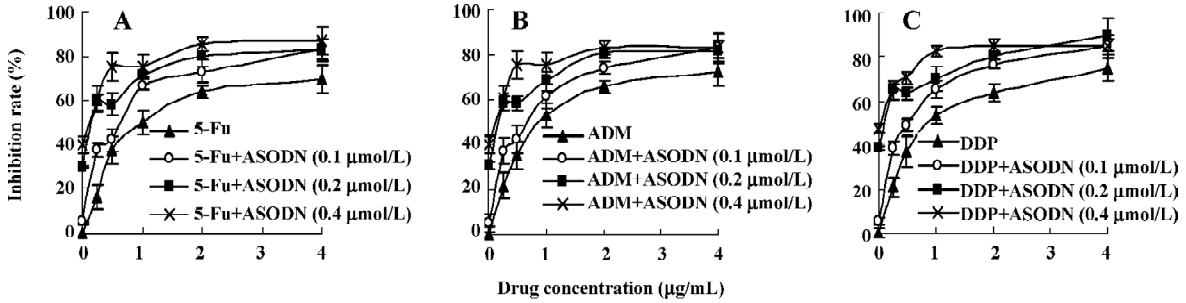

Figure 9. Cell proliferation of HepG2 cells after combination treatment with ASODN5 and chemotherapeutic drugs. (A: 5-Fu; B: ADM; C: DDP). Experiments were carried out after combination treatment for 24 h.