

ORIGINAL ARTICLE

## Regulatory roles of KDR antisense oligonucleotide on the proliferation of human prostate cancer cell line PC-3

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### Summary

**Purpose:** To explore the regulatory roles of antisense oligonucleotide (ASODN) of vascular endothelial growth factor receptor 2/ VEGFR2 (kinase insert domain-containing receptor, KDR) on the proliferation of PC-3 human prostate cancer cell line.

**Methods:** Different concentrations of synthetic sense and antisense KDR oligonucleotides were transfected into PC-3 human prostate cancer cell line. The inhibitory effects of oligonucleotides on tumor cell proliferation, KDR mRNA expression, cell cycle distribution and cell apoptosis were analyzed.

**Results:** The inhibitory effects of ASODN on tumor cell proliferation reached the peak 48 hrs after transfection,

and its intensity was positively related to the ASODN concentration. The expression of KDR mRNA was reduced in different degrees after transfection with ASODN, with significant differences in different concentration groups. After transfection of ASODN, apoptosis took place in different degrees but the cell cycle distributions were not significantly different in different concentration groups.

**Conclusion:** KDR gene plays a certain role in promotion of human prostate cancer PC-3 cells' proliferation. It is expected to become a molecular target for the treatment of androgen-independent prostate cancer.

**Key words:** antisense oligonucleotide, KDR, PC-3 cell, prostate cancer, vascular endothelial growth factor receptor

### Introduction

Prostate cancer is a common malignancy in European and American male populations. It ranks second in mortality among malignant tumors, and is only second to lung cancer [1-3]. Due to the aging of the population, diversified living environment, and changes in diet and other factors, the incidence of prostate cancer shows an obvious increasing trend in elderly men in China [4]. When prostate cancer metastasizes to bone, lung and other organs or after 18-24 months of continuous endocrine therapy, the cancer cell type may be transformed from androgen-dependent to androgen-independent, and the effectiveness of the endocrine therapy decreases remarkably, leading to therapeutic difficulties. Up until now, there is no effective treatment method for endocrine-inde-

pendent prostate cancer [5,6].

Gene therapy is a new method for the treatment of malignant tumors, which is achieved by inhibiting or promoting the expression of some genes [7,8]. Many studies have reported that VEGF is highly expressed in a variety of tumor tissues, and it is closely related to the occurrence and development of tumors [9-11]. VEGF has 4 receptors, including fms-like tyrosine kinase-1 (Flt-1), KDR, fms-like tyrosine kinase-4 (Flt-4) and neuropilin-1 [12]. KDR is mainly distributed in vascular endothelial cells and mediates the proliferation of vascular endothelial cells, while increasing the vascular permeability [13,14]. In addition, KDR mediates the tumor cell proliferation and angiogenesis at the tumor initiation and development stage. It is the main functional receptor of VEGF [15,16]. However, the expression of KDR gene in

prostate cancer tissue is rarely reported, and its role in the occurrence and development of prostate cancer is still not clear.

In this study, different concentrations of KDR ASODN were transfected into PC-3 human prostate cancer cell line. The inhibitory effects of ASODN on cell proliferation, KDR mRNA expression, cell cycle distribution and cell apoptosis were investigated.

## Methods

### Cell line

Prostate cancer cell line PC-3 was kindly endowed from the Department of Urology, Affiliated Hospital of Xi'an Jiaotong University, Shaanxi, P.R. China. This study was conducted in accordance with the declaration of Helsinki and was conducted after approval from the Ethics Committee of Affiliated Hospital of Xi'an Jiaotong University. Written informed consent was obtained from all participants.

### Design and synthesis of oligonucleotides

Sense and antisense oligonucleotides targeting the translation initiating sites of the KDR mRNA [11] were designed by Primer3 and BLAST software. The following specific oligonucleotides were used: (antisense) 5'-GGAAACGCAGCGACCACACA-3' and (sense) 5'-TGTGTGGTTCGCTGCGTTTCC-3'. Both sense and antisense oligonucleotides were fully modified by phosphorothioate and synthesized by Invitrogen® Life Technologies Corp. (California, USA).

### Cell culture

PC-3 prostate cancer cells were cultured and subcultured in RPMI1640 complete medium supplemented with 10% fetal bovine serum (FBS) (Gibco Inc., Montana, USA), 10 g/L L-Glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin and incubated in 5% CO<sub>2</sub> atmosphere at 37 °C.

### Inhibition of cell proliferation

PC-3 cells in the exponential growth phase were collected and inoculated in 96-well flat-bottom microtiter plates at a density of  $5 \times 10^5$  cells/well and then cultured in an incubator (5% CO<sub>2</sub>, 37 °C). As the cells adhered completely, the medium was replaced with serum-free medium allowing growth arrest at the G1 phase. Six hrs later, the medium was changed back to the complete culture medium with 10% FBS. Cells were divided into 4 groups: Group A: PC-3 cells in normal culture. Group B and C: PC-3 cells transfected with ASODN and SODN, respectively. Group B and C were divided into subgroups with 0.01, 0.05, 0.1, 0.2 and 0.4 μmol/L concentrations. Group D: PC-3 cells treated with lipofectamine 2000 only. Transfection was per-

formed according to the manufacturer's instructions of lipofectamine 2000. The experiments in all groups were carried out in triplicate. After incubation in 5% CO<sub>2</sub> atmosphere at 37 °C for 4 hrs, the medium was replaced with normal RPMI1640 medium and cultured continually. At 12, 24, 48 and 72 hrs after changing the medium, 20 μl MTT (0.5 mg/ml) were added into each well and the incubation continued at 37 °C for 4 hrs. Then the medium was removed and 150 μl DMSO (Sigma Corp., New York, USA) was added into each well. After oscillation for 10 min, the optical density (OD) was measured at 450 nm using a Microplate Reader (Bio-Rad Laboratories Inc., California, USA). The inhibitory rate of cell proliferation was determined through the following formula:

$$\text{Cell inhibitory rate (\%)} = (\text{OD}_{\text{blank}} - \text{OD}_{\text{test}}) / \text{OD}_{\text{blank}} \times 100\%$$

### RNA extraction and real-time PCR

Cells transfected with different concentrations of ASODN were collected for RNA extraction at 48 hrs after transfection. PC-3 cells in normal culture were used as blank control. All experiments were performed in triplicate. RNA extraction was performed using the Trizol method according to the manufacturer's instructions. The following primer pair specific to KDR gene was used: (forward) 5'-TGTTGAAAGAAGGAGCAACA-3', and (reverse) 5'-GCAGAATTCACAATCACCAT-3'. The PCR product (synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China) size was 152 bp. PCR was performed in a total volume of 30 μl (containing 2 μl cDNA, 50 pmol forward and reverse primers respectively, 0.4 mmol/L dNTP, 3 μl 25mM MgCl<sub>2</sub>, 5 μl 10×Buffer and 2.5 IU Taq polymerase) by using GeneAmp® PCR System 9700 (Applied Biosystems Inc., California, USA). All samples were run in cycles as follows: denaturing at 94 °C for 10 sec, annealing at 50 °C for 30 sec, and extending at 72 °C for 1 min. After 30 cycles, the reaction was continually extended at 72 °C for 5 min and then cooled to 4 °C. Fluorescence intensity was collected at the annealing period of each cycle. Cycle threshold (Ct) value was determined according to the kinetic curves of each sample. The relative copy number of each cDNA sample was calculated by the following formula:  $X = y^{\text{Ctn} - \text{Ctx}} \times N$ , where  $y = \text{root}$ , Ctn=basic design of the numerical,  $N = \text{Ct}$  value of the template copy number of Ctn specimens.

### Measurement of cell cycles and apoptosis by flow cytometry

Cells used for the measurement of cell cycles and apoptosis were similar to those used for RNA extraction. Cells were collected and fixed in 70% ethanol overnight at -20 °C. After washing with PBS, cells were stained with 50 μg/ml propidium iodide for 20 min. Washed by PBS, cells were detected on a FACSaria cell sorter (Media Cybernetics, Inc., Maryland, USA). Random ELITE (Eppendorf Co, Germany) software was used to analyse the apoptotic rate and MULTICYCLE

(Eppendorf Co, Germany) software was used to analyze cell cycles.

### Statistics

Data are shown as mean±standard deviation (SD) and analyzed by the SPSS software program v12.0 (Chicago, IL, USA). Differences among groups were compared using one-factor analysis of variance. Pairwise comparison between groups was carried out using SNK test method. Significant difference was determined with a p value less than 0.05.

## Results

### *KDR ASODN inhibits effectively the proliferation of PC-3 cells*

In order to examine the effects of KDR ASODN on the proliferation of PC-3 cells, transfections with different concentrations of KDR ASODN into PC-3 cells were performed and then the inhibitory rate was detected using MTT assay. We found that KDR ASODN could significantly inhibit the proliferation of PC-3 cells even in low concentrations; this activity was enhanced with the increase of the concentration. At 48 hrs after transfection, the inhibitory rate reached the peak. However, KDR SODN and L2000 showed no effect on the proliferation of PC-3 cells, which was significantly different from KDR ASODN ( $p < 0.05$ ); (Tables 1,2).

### *KDR mRNA was reduced by ASODN in a concentration-dependent manner*

At 48 hrs after transfection, the expressions of KDR mRNA in different concentration groups were measured by real-time PCR. The expression of KDR mRNA was reduced by ASODN in a concentration-dependent manner. No significant dif-

ferences were noticed between the concentration groups ( $p < 0.005$ ; Table 3).

### *ASODN promoted apoptosis of PC-3 cells but had no effect on cell cycle*

In order to investigate the mechanism of the inhibitory roles of ASODN on the proliferation of PC-3 cells we analyzed their cell cycles and apoptosis after transfection with ASODN. The cell cycles were not affected by ASODN despite the increase of ASODN concentration (Table 4). However, apoptosis of PC-3 cells was promoted by ASODN and was positively correlated with the concentration of ASODN (Table 5).

## Discussion

The occurrence and development of prostate cancer are closely related to the androgen levels. After endocrine therapy, androgen-dependent prostate cancer is transformed into androgen-independent prostate cancer, a new condition that causes a lot of therapeutic difficulties. At present, there is no effective treatment method for androgen-independent prostate cancer [17,18]. Gene therapy is a new effective treatment modality for malignant tumors. In this study, the antisense nucleic acid technology was adopted to block the expression of KDR in PC-3 cells in patients with androgen-independent prostate cancer. The result was growth inhibition and apoptosis of PC-3 cells. KDR ASODN could inhibit the proliferation of PC-3 cells in different degrees. The inhibitory effect appeared 12 hrs after transfection, and reached the peak at 48 hrs. The inhibition rate of ASODN on cell proliferation was related to its concentration. Fluorescence quantitative PCR showed that with the action of KDR ASODN the intracellular KDR

**Table 1.** Inhibitory effect of ASODN on PC-3 cells at different time points (mean± SD)

Group	Number	Control	0.01 $\mu\text{mol/L}$	0.05 $\mu\text{mol/L}$	0.1 $\mu\text{mol/L}$	0.2 $\mu\text{mol/L}$	0.4 $\mu\text{mol/L}$
12 hrs	3	10.9±3.8	13.9±4.2	21.7±2.7	31.5±3.4	31.1±2.8	35.7±4.3
24 hrs	3	13.9±2.4	18.5±1.2	23.5±2.6	28.2±0.8	33.6±3.7	42.3±2.6
48 hrs	3	12.3±4.7*	29.6±1.7*	50.5±3.2*	59.6±0.6*	63.9±3.2*	71.1±2.3*
72 hrs	3	15.2±3.1*	20.5±4.6*	46.8±2.6*	60.2±2.6*	65.8±1.6*	73.3±3.2*

\* $p < 0.05$  vs 12 hrs and 24 hrs group; SD: standard deviation

**Table 2.** Inhibitory effect of ASODN, SODN and L2000 on the PC-3 cells at 48 hrs (mean± SD)

Group	Number	Control	0.01 $\mu\text{mol/L}$	0.05 $\mu\text{mol/L}$	0.1 $\mu\text{mol/L}$	0.2 $\mu\text{mol/L}$	0.4 $\mu\text{mol/L}$
ASODN	3	12.3±4.7*	29.6±1.7*	50.5±3.2*	59.6±0.6*	63.9±3.2*	71.1±2.3*
SODN	3	11.4±3.5	12.7±3.4	12.6±2.6	14.0±2.0	17.5±1.9	14.3±3.2
L2000	3	13.7±2.5	8.0±2.6	14.1±1.9	13.8±3.7	16.3±3.4	14.1±2.1

\* $p < 0.05$  vs SODN and L2000 groups

**Table 3.** Expression of KDR mRNA in different concentration groups (mean±SD)

Group	Control	0.01 $\mu\text{mol/L}$	0.05 $\mu\text{mol/L}$	0.1 $\mu\text{mol/L}$	0.2 $\mu\text{mol/L}$	0.4 $\mu\text{mol/L}$
Ct value	128.3±31.2	122.8±35.6*	115.6±41.3*	102.4±38.7*	93.5±45.8*	85.6±42.5*

\*p &lt; 0.05 vs the control

**Table 4.** Cell cycle distribution in different concentration groups

Group	Cell cycle (mean±SD)		
	G1	S	G2
Control	65.9±5.2	5.0±3.1	29.2±4.8
0.01 $\mu\text{mol/L}$	58.4±6.3	13.6±4.7	28.0±5.5
0.05 $\mu\text{mol/L}$	55.8±3.8	10.7±3.2	33.6±4.3
0.1 $\mu\text{mol/L}$	67.0±6.2	2.5±1.2	30.3±6.2
0.2 $\mu\text{mol/L}$	62.3±5.4	7.1±2.3	30.6±4.3
0.4 $\mu\text{mol/L}$	63.8±5.6	2.5±1.8	33.6±3.2

p &gt; 0.05 vs the control in all categories, SD: standard deviation

**Table 5.** The apoptotic rate in different concentration groups

Group	Apoptosis (mean±SD)
Control	3.5±1.3
0.01 $\mu\text{mol/L}$	10.2±3.4
0.05 $\mu\text{mol/L}$	9.2±2.5
0.1 $\mu\text{mol/L}$	20.9±3.9
0.2 $\mu\text{mol/L}$	43.3±4.8
0.4 $\mu\text{mol/L}$	48.6±7.3

p &lt; 0.05 vs the control in all categories, SD: standard deviation

mRNA level was reduced, and the reduced degree was related to ASODN concentration. This indicates that KDR ASODN can be incepted by PC-3 cells and inhibit the expression of KDR mRNA and the proliferation of PC-3 cells. Flow cytometry results showed that cell apoptosis differed in the experimental groups. The apoptosis rate was positively correlated with the ASODN concentration, but the cell cycle distribution did not show an obvious change. In addition, after transfection with KDR ASODN, the proliferation of PC-3 cells was inhibited, and the expression of KDR mRNA was down-regulated, with obvious cell apoptosis in different degrees. This finding correlates with the results of previous researches [9-11].

The results of this study suggest that, blocking the expression of KDR can promote the apoptosis of PC-3 cells. This method is expected to be an effective therapeutic modality for the treatment of androgen-independent prostate cancer. In the present study, only blocking KDR expression with antisense nucleic acid technology was adopted to promote the apoptosis of PC-3 cells, without using other methods such as interfering the dimerization of receptor using VEGF heterodimer [19,20], competitively inhibiting the combination of VEGF with natural receptor using soluble receptor, and blocking KDR signal transduction using small-molecular protein kinase inhibitor [21]. In addition, the aforementioned methods have not been used to block the expression of KDR in

another androgen-independent prostate cancer cell (DU145), and observe the cell apoptosis. This needs to be clarified in our next work, accompanied with animal experiments.

Our previous study [22] has investigated the expression of KDR in one androgen-dependent prostate cancer cell line (LNCap) and two androgen-independent prostate cancer cell lines (DU-145, PC-3). Results showed that there was KDR mRNA expression in three cell lines, and the expression in PC-3 cells was the strongest, followed by DU-145 cells. The expression in LNCap cells was the weakest. In clinical practice, there is no effective method for the treatment of androgen-independent prostate cancer. In this study, PC-3 cells with the strongest KDR expression were selected as research objects and the results showed that KDR ASODN could indeed be incepted by PC-3 cells and inhibit the expression of KDR mRNA and cell proliferation. In addition, KDR ASODN can cause different degrees of apoptosis of PC-3 cells at each stage. Should the therapeutic effect be confirmed in animals or humans, this method can undoubtedly fill the gaps of treatment of endocrine-independent prostate cancer.

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