RESEARCH ARTICLE

New Therapeutic Schedule for Prostatic Cancer-3 Cells with ET-1 RNAi and Endostar

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Abstract

Background: Endothelin-1 and Endostar are both significant for the progression, proliferation, metastasis and invasion of cancer. In this paper, we studied the effect of ET-1 RNAi and Endostar in PC-3 prostatic cancer cells. Materials and Methods: The lentiviral vector was used in the establishment of ET-1 knockdown PC-3 cells. Progression and apoptosis were assessed by CKK-8 and flow cytometry, respectively. Transwell assay was used to estimate invasion and signaling pathways were studied by Western blotting. Results: ET-1 mRNA and protein in ET-1 knockdown PC-3 cells were reduced to 26.4% and 22.4% compared with control group, respectively. ET-1 RNAi and Endostar both were effective for the suppression of progression and invasion of PC-3 cells. From Western blotting results, the effects of ET-1 regulation and Endostar on PC-3 cells were at least related to some signaling pathways involving PI3K/Akt/Caspase-3, Erk1/2/Bcl-2/Caspase-3 and MMPs (MMP-2 and MMP-9). Furthermore, combined treatment of ET-1RNAi and Endostar was found to be more effective than single treatment. Conclusions: Both ET-1 RNAi and Endostar can inhibit the progression and invasion of PC-3 cells, but combined treatment might be a better therapeutic schedule.

Keywords: ET-1 RNAi - endostar - PC-3 cells - apoptosis - proliferation - invasion

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Introduction

Prostatic cancer, as a malignant tumor in older men, is the second most common cancer in men and the number of people killed is only after lung cancer in west Countries (Liu et al., 2013). At present, the effect of drug and surgery is undesirable. So more efficient and safer treatment methods are needed for this disease.

The endothelin axis is found to be an important role in tumorigenesis and progression in many types of human cancers (Rotondo et al., 2012; Shao et al., 2013; Tamkus et al., 2013; Wang et al., 2013a). ET-1, as an important role in endothelin system, has the potential to be examination indicator (Kalles et al., 2012) and therapy target (Ling et al., 2013; Rosano et al., 2013) for cancers. According to the results in many papers, the abundant expression of ET-1 could result in cancer cells proliferation, anti-apoptosis, angiogenesis, invasion and metastasis (Ha et al., 2011; Bagnato and Rosano, 2012; Hinsley et al., 2012; Kalles et al., 2012). Thereby the suppression of the ET-1 is an key role for the therapy of tumors. The suppression of ET-1 which was carried out by siRNA delivered by lentiviral vector was not reported by previous literature. and it was meaningful to inhibit the expression of ET-1 directily for therapy of cancer.

Endostar, as a kind of recombinant human endostatin, could induce regression of tumors (Folkman, 2006; Zhang et al., 2013). The anti-tumor effect of Endostar is carried out by suppressing angiogenesis by down regulating the activity or expression of VEGA (Ling et al., 2007), MMP-2 (Kim et al., 2000), integrin $\alpha5\beta1$ (Sudhakar et al., 2003) and so on. However, there were some papers had reported that Endostar had a direct effect on cancer cells by inducing apoptosis, blocking cell cycle progression, reduce the capacity of migration and adhesion (Nyberg et al., 2003; Zhang et al., 2009; Dong et al., 2013). But Endostar was only effective directly for some special cancer cells (O'Reilly et al., 1997; Dhanabal et al., 1999), not all. The direct effect of Endostar on prostatic cancer is needed to be studied.

According to the above, it is meaningful to study the direct inhibition of ET-1 and the direct effect of Endostar for therapy of cancer. The suppression of ET-1 and Endostar could be used to the therapy of tumors. According to prior reports, the therapy of ET-1 RNAi and Endostar either alone for cancer involved in multiple stages in the development of cancer (Wu et al., 2001; Te Velde et al., 2005; Xu et al., 2007; Yokoyama and Ramakrishnan, 2007; Szarvas et al., 2012; Wang et al., 2013b; Irani et al., 2014), so it would be interesting to

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study the combined therapy of ET-1 RNAi and Endostar.

In this paper, the lentiviral vector was used to deliver the siRNA into the PC-3 cells to inhibit the expression of ET-1. The direct effect of ET-1 RNAi and Endostar either alone on prostatic cancer were studied, and it was compared with combined effect. Our paper showed that either ET-1 RNAi or Endostar could inhibit the growth and progression of prostatic cancer cells (PC-3), but the combined effect was significant better than either alone.

Materials and Methods

Cell culture

The prostatic cancer cells (PC-3 cells) were purchased from ATCC, and maintained in DMEM (Life Tech) with 10% fetal bovine serum (FBS, Gibco), penicillin (1%), and streptomycin (1%) at 37°C in a water-saturated environment (5%CO_2).

The establishment of ET-1 knockdown PC-3 cells

The ET-1-siRNA sequence, TGCCAATGTGCTAGCCAAA, was designed by JRDUN Biotechnogy (Shanghai) co.Ltd. The establishment process of lentiviral vector was similar to LI (Zheng Li et al., 2014).

After the establishment of lentiviral containing ET-1 siRNA, they were transduced into PC-3 cells. Real-time RT-PCR and western-blot were used to evaluate the silence rate of siRNA. The process of real-time RT-PCR was as follows: Trizol (Life Tech) was used to extract total RNA. The cDNA synthesis kit (BIO-RAD) was used to prepare First-strand cDNA and a SYBRGreen PCR kit (Thermo) was used for the amplification. The primers of ET-1 and GAPDH were as follows:

ET-1, Primer Forward 5' GCCTGTCTGAAGCCATAG 3', Primer Reverse 5' GCTGAGAGGTCCATTGTC 3';

GAPDH, Primer F 5' CACCCACTCCTCCACCTTTG 3', Primer R 5'CCACCACCCTGTTGCTGTAG 3'.

The analysis of all samples were carried out according to the instructions. The operation of western-blot was similar to that in part Western blot.

Cell proliferation assay

CKK-8 assay (Bogoo, Shanghai) was used to determine the cell proliferation. PC-3 cells (2000 cells/well) were seeded in 96-well plates overnight. After treatment, the plates with 10μl of CKK-8 were incubated for another 4 hours, then measured with a microplate reader (BioTek, USA) at 450nm.

Apoptosis detection

PC-3 cells were seeded in 6-well plates in DEME with 10% FBS, and then in DEME without 10%FBS for another 12 hours after PC-3 cells reached 70% confluence. Then the PC-3 cells were treated for 24 hours before harvesting. Annexin V- Propidium Iodide (PI) staining kit was used to analyze the apoptosis rate of PC-3 cells. The operation of staining was completed according to the manual. The detection was carried out using a FACSCalibur cytometer (Becton Dickinson).

Invasion detection

The transwell assay with Matrigel (Millipore) was used to test the invasion of PC-3 with different treatments. The medium DEME with 10% FBS was added to the bottom 24-well plates. The PC-3 cells ($1x10^4$ cells per transwell) were incubated in DEME without FBS for 24 hours. Same concentration of medicine was added into the medium in the bottom 24-well plates or transwell. Then the number of cells which traversed the filter was detected by staining with crystal violet.

Western blot

The PC-3 cells ($5x10^5$ cells) were seeded in 6-cm Petri dishes with 10% FBS DEME and then in the DEME without FBS for 12 hours after PC-3 cells reached 80% confluence. Then the PC-3 cells were treated for 24 hours. The PC-3 cells were washed 2 times with ice PBS buffer before harvesting. Cells were lysed on ice with lysis buffer (Beyotime Ins. Biotechnology) contained 1% 1mg/ml protease inhibitor (PMSF, Beyotime Ins. Biotechnology). The western blot was carried out by reference to the method published in paper (Dhanabal et al., 1999).

Statiscal analyses

SPSS Version 16.0 was used to analyze the statistical difference. Mean±SEM was the expression form of results. $P<0.05$ was the criterion of statistical significance.

Results

Establishment of ET-1 knockdown PC-3 cells

To evaluate the down-regulation effect of ET-1 mRNA by siRNA, the lentiviral was transduced into PC-3 cells. The amount of ET-1 mRNA was assayed by real-time RT-PCR. The results were shown in Figure 2A. We found that the mRNA of ET-1 in RNAi group was reduced to 26.37% compared with control or blank vector group. It indicated that RNAi had an significant interference effect

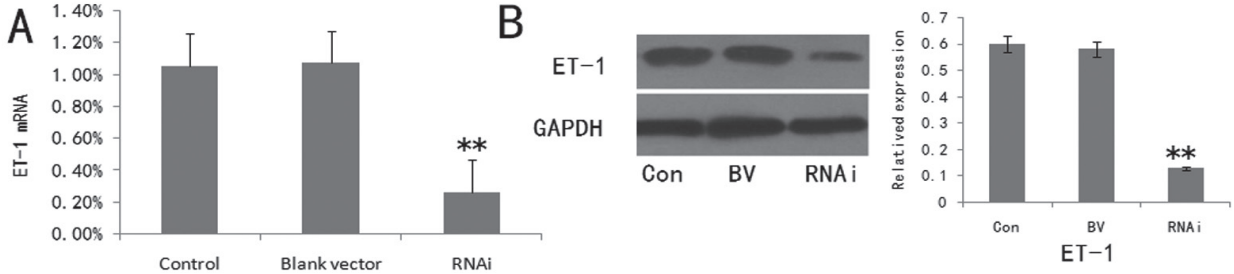

Figure 1. Establishment of ET-1 Knockdown PC-3 Cells. A) The results of real-time RT-PCR. **B)** The expression of ET-1 determined by western-blot.(Con-Control, BV-blank vector, RNAi-RNA interference. $^{**}P<0.01$)

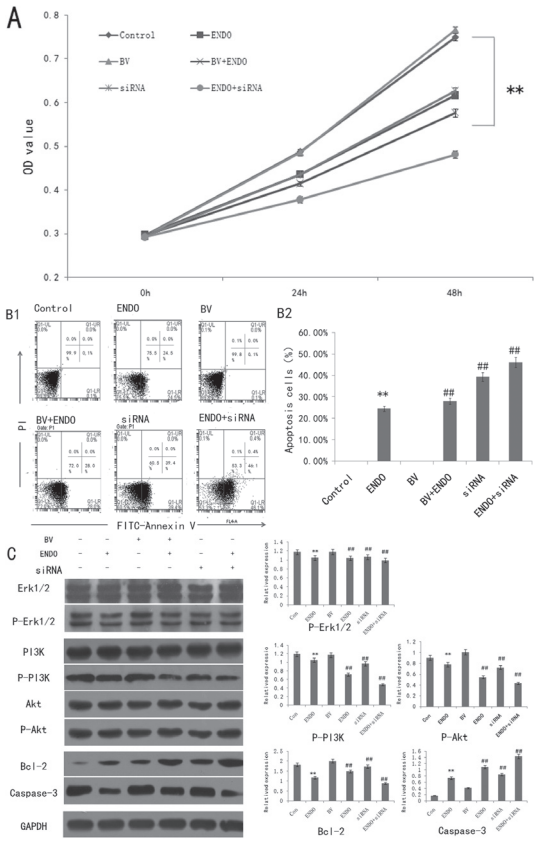

Figure 2. Influence of Proliferation and Apoptosis of PC-3 Cells and Modulation of Signaling Path in PC-3 Cells by the Treatment of ET-1 RNAi and Endostar. A) The inhibitions of proliferation of PC-3 cells were determined over time by CKK-8 assay. **B)** The promotion of apoptosis of PC-3 cells was determined by flow cytometry. C) The modulation of signaling path in PC-3 cells was determined by western blotting. (Con-Control, ENDO-Endostar, BV-blank vector, $^{**}P<0.01$, compared with Control. $^{\#\#}P<0.01$, compared with Blank vector)

to inhibit the transcription of ET-1 gene. The expression of ET-1 protein in RNAi group was also reduced to 22.4% compared with blank vector group (Figure 1B). It indicated that siRNA interfered the expression of ET-1 significantly.

Through the above analysis, we could know that the establishment of the ET-1 knockdown PC-3 cells was successful and they could be used for further analysis.

ET-1 RNAi and Endostar both inhibited progression of PC-3 cells

CKK-8 and Annexin V-Propidium Iodide (PI) staining kit were used to assess the proliferation and apoptosis of PC-3 cells. The results of CKK-8 assay (Figure 2A) showed that Endostar and ET-1 RNAi both suppressed the proliferation of PC-3 cells notably, and the suppression effect became stronger over time in 48 hours. In addition, the combined effect of Endostar and ET-1 siRNA was much stronger than the effect of single treatment ($P<0.01$). In the results of apoptosis assays (Figure 2B), we found that either Endostar and ET-1 RNAi induced apoptosis of PC-3 cells significantly, the apoptosis rate reached 24.5% and 39.4%, respectively. However, the combined treatment of Endostar and ET-1 siRNA was the most

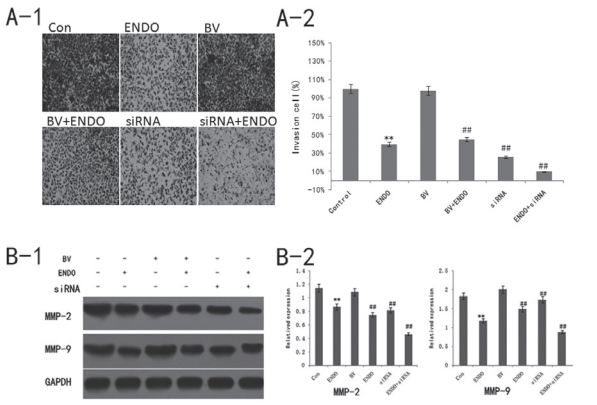

Figure 3. Suppression of Invasion of PC-3 Cells and Down-Regulation of Related Signaling Protein in PC-3 Cells with the Therapy of ET-1 siRNA and Endostar. A) The results of transwell assay for the invasion of PC-3 cells. **B)** The variation of the expression of MMP-2 and MMP-9 in PC-3 cells. (Con-Control, ENDO-Endostar, BV-blank vector, $^{**}P<0.01$, compared with Control. $^{\#\#}P<0.01$, compared with Blank vector)

effective to promote apoptosis, and the apoptosis rate reached 46.1%. By above analysis, we might safely draw the conclusion that Endostar and ET-1 siRNA could suppress proliferation and induced apoptosis of PC-3 cells, and these two treatment were better to combination for more efficiency.

To confirm the modulation of signaling pathways in PC-3 cells by the treatment of ET-1 RNAi and Endostar, the proteins of PC-3 were extracted for western-blot. As shown in Figure 3C, ET-1 RNAi and Endostar both suppressed the expression of Bcl-2 and Caspase-3 via suppressing phosphorylation of PI3K/Akt and Erk1/2 (Figure 2C). It indicated that PI3K/Akt/caspase-3 and Erk1/2/Bcl-2/caspase-3 was at least two important signaling ways affected notably by ET-1 RNAi and Endostar in PC-3 cells. In addition, we found that the combined effect of ET-1 RNAi and Endostar on affecting the two signaling ways was stronger than the effect of single. It explained why combined treatment had a better effect. It indicated that ET-1 RNAi and Endostar might have an cooperation therapeutic effect on suppressing proliferation and inducing apoptosis of PC-3 cells.

The invasion of PC-3 cells was suppressed by ET-1 RNAi and Endostar

The invasion of PC-3 cells was evaluated by transwell assay. The number of cells through the member were reduced significantly with the treatment of Endostar and ET-1 RNAi (Figure 4). It indicated that Endostar and ET-1 RNAi had the effect to reduce invasion of PC-3 cells. In addition, the combined effect of these two treatments was stronger than that of single. According to previous reports, MMP-2 and MPP-9 played the important role in the invasion of cancer cells (Fiorentini et al., 2014; Puzovic et al., 2014). In this study, we found that the expression of MMP-2 and MMP-9 were both reduced obviously in the treatment groups. What's more, the expression in the combined treatment group was lower compared with in the single treatment group. It indicated that the stronger therapeutic effect by the combined effect was related to

the lower expression of MMP-2 and MMP-9. Through the above analysis, combined treatment also showed its advantage in inhibiting invasion of PC-3 cells.

Discussion

Prostate cancer, as the second leading cause of tumor-related death in men, has very high metastatic rate (Wynder et al., 1971). Therefore, drugs which wanted to be successful in the treatment of cancer also should have the function to suppress the progression and metastasis of prostate cancer. ET-1, as an important part of ET axis, has been proved to be a therapeutic target for cancer (Maffei et al., 2014). The growth and metastasis of some kinds of cancers, such as ovarian, renal, colorectal. brain tumors, were promoted by the high expression or activation of ET-1 (Hsu and Pfahl, 1998; Ali et al., 2000; Zhou et al., 2008; Kalles et al., 2012). Some medicines had been proved to be effective for cancer treatment by suppressing the ET-1 (De Jesus-Gonzalez et al., 2012; Leon et al., 2014). In this paper, the ET-1 expression of PC-3 cells were interfered directly by siRNA which was delivered by lentiviral vectors. This gene therapy showed a good effect on the suppression of progression and metastasis of PC-3 cells. In addition, through the analysis of signaling pathways, we found that the reduction of ET-1 inhibited progression of PC-3 cells through modulating the signaling pathway of PI3K/Akt/caspase-3 and Erk1/2/Bcl-2/caspase-3 and invasion through modulating MMP-2 and MMP-9.

Endostar (Endostatin) could induce tumor regression by the function of anti-angigenesis (Wei et al., 2010). It was reported that the inhibition of cell proliferation and migration by Endostar was non-specific for endothelial cells (Wang et al., 2013c). Endostar could inhibit human non small cell lung cancer cell and breast cancer cell invasion (Lu et al., 2008; Ni et al., 2009), and promote ovarian cancer cells apoptosis (Meimei Liu et al., 2007.). In the present study, the data of Endostar also demonstrated that it could inhibit the progression and invasion of PC-3 cells. The western-blot analysis showed the signaling pathways affected by Endostar was similar to that by ET-1 RNAi.

According to the results, we found that combined treatment of ET-1 RNAi and Endostar had an synergistic therapeutic effect, it indicated that combined treatment might be a better choice for inhibiting progression and invasion of PC-3 cells. As we all known, multi-drug resistant (MDR) is a main cause of the failure for the medicine to cure the cancer. One mechanism for MDR appeared in tumor cells is the increase of exogenetic Bcl-2 (Stassi et al., 2003). RNAi is a method of gene therapy to reverse MDR by inhibiting Bcl-2 (Xue xue et al., 2010). In this study, we found that ET-1 RNAi could inhibit the expression of Bcl-2, it indicated that the combined treatment of ET-1 RNAi and Endostar not only enhanced the therapeutic effect directly, but also might inhibit the appearance of MDR. While the reversion effect of MDR should be further researched. Furthermore, according to prior papers, Endostar and ET-1 RNAi are likely to change the microenvironment in vivo to inhibit the progression of tumor (Johan Dixelius et al., 2000; Spinella et al., 2014), it indicated that the combined treatment of Endostar and ET-1 RNAi would be more effective in vivo. Therefore, although it still had a lot of research work to do, it had the potential to be a new therapeutic view for prostatic cancer.

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