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Original Article

Roles of ARA55 and ARA55-estrogen receptor β signaling pathway on the prostate cancer

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Abstract: Objective: To investigate the effect of ARA55 over-expression on the prostate cancer proliferation, aggression and apoptosis; to investigate whether ARA55 can regulate the expression of TGF-β or Smad7 in prostate cancer cells, and its relationship with estrogen receptor. Methods: The expression of ARA55, estrogen receptor α (ERα), ERβ, TGF-β and Smad7 was detected in human prostate cancer tissues, benign prostate hyperplasia tissues and normal prostate tissues using immunochemistry. *In vitro*, the human prostate cancer cell lines, LNCaP and DU-145, were cultured, and transfected with pEGFP-ARA55, then the bio-behaviors of the ARA55 over-expression cells were evaluated, including the proliferation, aggression and apoptosis. Lastly, the expression of TGF-β and Smad7 in prostate cancer cells with both ARA55 over-expression and ERβ knockout was detected by western blot. Results: The immunochemical results indicated that the expression of ARA55 was significantly lower in prostate cancer tissues than that in the benign prostate hyperplasia and normal prostate tissues. The cell experiment revealed that, both in the LNCaP cells and DU-145 cells, the proliferation and aggression of cells with ARA55 over-expression was much lower than the control groups, respectively; while the apoptosis rate of cells with ARA55 over-expression was higher than the control groups, suggesting that ARA55 could inhibit the prostate cancer proliferation and aggression. The expression of TGF-β was decreased when the ARA55 over-expression in DU-145 cells; whereas the down-regulation of TGF-β was eliminated, when the ERβ was knocked out in the cells with ARA55 over-expression. Conclusion: In prostate cancer, the expression of ARA55 was decreased, while the expression of ERα/β, TGF-β and Smad7 was increased. Furthermore, ARA55 could inhibit the TGF-β level via the ERβ signaling pathway, to inhibit the cancer cell proliferation and aggression. Therefore, ARA55 could be considered as one of the treatment targets.

Keywords: Androgen receptor associated protein 55, prostate cancer, estrogen receptor, transforming growth factor β

Introduction

Prostate cancer is one of the most common cancers, which also is the second cancer leading to death in European and American men [1]. However, the specific cause and the mechanism of tumor progression of prostate cancer are still unclear. Previous studies have indicated that prostate cancer is kind of multiple endocrine neoplasias, and its prostate carcinogenesis is related to the androgen/androgen receptor (ER) signaling pathway [2]. The animal experiments showed that high androgen level could promote the prostate tumor growth, but removal of androgens would cause tumor regression in a short period of time, whereas the independent prostate cancer would be developed after 12-18 months of castration [3-7]. Therefore, we speculate that, in addition to androgen receptor signaling pathway, there are other signaling pathways involved in the prostate cancer development and progression.

Androgen receptor associated protein 55 (ARA55) is a factor involved in the development and progression of prostate cancer. ARA55 is protein with 55 kD, containing 444 amino acids, in which the C-terminal contains LIM domain, a cysteine-rich zinc finger. ARA55 can regulate the androgen receptor activity. Previous study on the mice found that a protein called HIC-5 with high homology to ARA55 could activate Ras, induce the prostate carcinogenesis [8, 9]. Besides, ARA55 could play a role in prostate

cancer in other signaling pathways, such as transforming growth factor-β (TGF-β) signaling pathways. Studies have found that TGF-β could interact with ARA55 to regulate the androgen receptor activity together. TGF-β is a member of TGF superfamily, which can regulate the transcription and translation of a variety of target genes, and be involved amounts of cell behaviors, including cell proliferation, differentiation, apoptosis and mesenchymal epithelial interaction [10, 11]. Moreover, TGF-β contributes to cancer carcinogenesis, progression and metastasis, especially in the prostate cancer [12, 13]. Actually the expression of TGF-β was higher in the prostate cancer than that in cancerous peripheral tissues. In addition, estrogen receptor signaling pathway is also involved in the procession and development of prostate cancer. The recent studies have indicated that estradiol injection could induce prostate cancer in mice, and the prostate tumor was regressed when the estrogen receptor was inhibited [5]. In addition, other study reported that ARA70 could up-regulate the estrogen receptor signaling to promote breast cancer cell proliferation. However, it is still unknown whether there is a relationship between ARA55 and estrogen receptor signaling pathway, especially in the prostate cancer. It needs to be focus on the ARA55 signaling pathway in the prostate cancer in the further study.

In this study, we will evaluate the expression of ARA55, ERα & β, TGF-β and Smad7, and their correlations in the prostate cancer. Furthermore, we also will investigate the effect of ARA55 over-expression on the prostate cancer bio-behavior, including proliferation, aggression, apoptosis, and so on. Lastly, we will investigate whether ARA55 can regulate the expression of TGF-β or Smad7 in prostate cancer cells, and its relationship with estrogen receptor. Our study will provide a new clue for explaining the mechanism of tumor progression and development of prostate cancer.

Materials and methods

General information

30 cases of prostate cancer samples were collected in our hospital from January, 2010 to December, 2011; meanwhile, 15 cases of normal prostate tissues and 15 cases of benign prostatic hyperplasia tissues were included as control. The average age of patients with prostate cancer was 69.2±6.8 years; the Gleason score of 7 cases was 8-10 points (23%), the Gleason score of 11 cases was 7 points (37%), and that of 12 cases was 6 points (40%). According to TNM 1997 staging criteria, the clinical staging of prostate cancer was as followings: 5 cases were T1 staging, 21 cases were T2 staging, 4 cases were T3-4 staging.

Immunohistochemistry

The samples were fixed with 10% formalin, embedded with paraffin, and cut into 5 μm serial sections. Immunohistochemistry was performed with peroxidase labeled streptomycin avidin staining method, the first antibodies were rabbit-anti-human ERα, goat-anti-human ERβ, mouse-anti-human Smad7 and mouse-anti-human TGF-β (Santa Cruz, 1:200), respectively. PBS was used as blank control. The sections were observed under microscope and the integrated optical density (IOD) value was analyzed based on immunohistochemical results.

Cell culture and plasmid transfection

Human prostate cancer cell line LNCaP and DU-145 were purchased from Research Institute of Zoology, Shanghai. The cells were cultured in RPMI1640 media with 10% FBS at 37°C, 5% CO_2 incubator. When the cells reached 80% confluence, the cells were transfected with pEGFP-ARA55 plasmid, pEGFP plasmid and negative control plasmid by Lipofectamine™ 2000 for 48 h. Then the cell proliferation, apoptosis and invasion were detected.

Flow cytometry

The LNCaP and DU-145 cells were collected with 0.25% trypsin and washed with PBS, twice. Then 500 μl binding buffer, 5 μl Annexin V-EGFP and 5 μl Propidium Iodide were added into the cells in succession; then incubated at 4°C, for 30 min, in the dark. After washing with PBS again, the cell apoptosis was detected by flow cytometry.

Cell proliferation, invasion and scratch assay

1) The cell proliferation was determined by CCK-8 method. 2) Firstly, the Matrigel was coated on the microporous membrane of Boyden transwell at 37°C for 30 min, and then the tran-

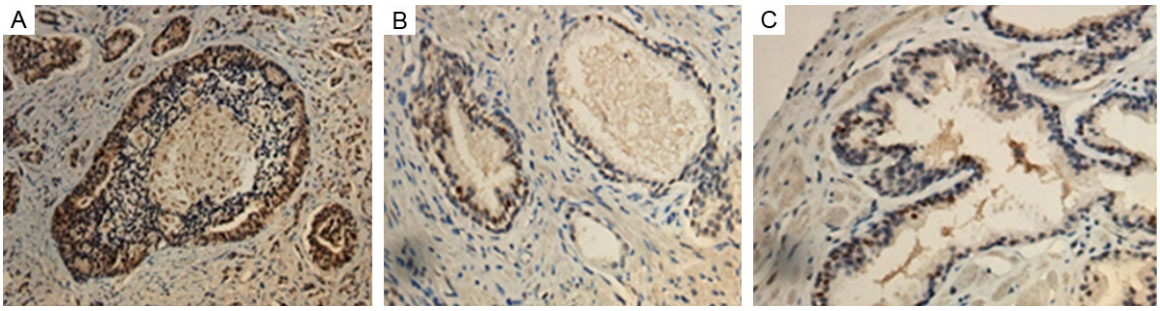

Figure 1. The immunohistochemical staining of ARA55 in prostate cancer (A), benign prostatic hyperplasia (B) and normal prostate tissues (C). The ARA55 was located in cell nucleus.

Table 1. The IOD of Smad7, ERα/β, ARA55 and TGF-β in prostate tissues

Group	ERα	ERβ	TGFβ	smad7	ARA55
Normal	72.81±3.03	33.90±2.58	21.63±1.77	481.84±30.26	6408.50±318.01
Hyperplasia	93.24±3.00#	59.13±6.04#	4371.93±130.93*	4646.88±301.04*	3683.548±251.52*
Cancer	5081.99±217.67*	6132.24±208.55*	7131.83±492.74*	9771.21±171.62*	1337.49±229.85*

*P<0.01, #P<0.05.

swell was placed on a 24-well plate. Then 0.2 ml LNCaP and DU-145 cells were added into upper chamber, and 0.5 ml medium were added into lower chamber. There were 3 wells in each group. Cells were cultured with 5% CO_2, at 37°C for 32 h, then the membrane was removed and fixed with 75% paraformaldehyde for 30 min, stained with coomassie blue for 30 min, and the number of cell penetrating membrane was counted under microscope. 3) 1.0×10^5 cells were seeded on 24-well plate and then performed cell scratch assay, then cell movement was analyzed under microscope (×100).

Transfection of DU-145 cells with ERβ-siRNA

Firstly, the ERβ-siRNA were constructed verified, the sequence was as followings: 5'-CGCGTCCCCAGAAGCATTCAAGGACATATTCAAGAGATATGTCCTTGAATGCTTCTTTTTTGGAAAT-3', 5'-CGATTTCCAAAAAAGAAGCATTCAAGGACATATCTCTTGAATATGCCTTGAATGCTTCTGGGGA-3'. Then the human prostate cancer cell line DU-145 were cultured and transfected with ARA55 plasmid and ERβ-siRNA in succession for 48 h.

Western blot

The proteins were extracted from DU-145 cells treating with ARA55 plasmid or ERβ-siRNA. Then the protein was run polyacrylamide gel electrophoresis (PAGE), and transferred to PVDF membrane. After blocking, the PVDF membrane was washed with TBST for 5 min 3 times; then incubated with anti-human ERβ, Smad7 and TGF-β antibody overnight and secondary antibody for 1 h, respectively. Lastly, the PVDF membrane was developed with ECL chemiluminescence.

Statistical analysis

All data were analyzed with SPSS 16.0 statistical software, the χ^2 test and t test were used to analyze data between groups. A P<005 was considered as significant difference.

Results

The expression of ARA55, ERα/β, TGF-β and Smad7 proteins

The immunohistochemical results showed that the expression of ARA55 was positive in prostate cancer tissues, and it was also expressed in benign prostatic hyperplasia and normal prostate tissues. The ARA55 was located in cell nucleus (**Figure 1**). However, the statistical results of IOD value showed that the expression of ARA55 was lower in prostate cancer tissues than that in the other two groups (**Table 1**); while the expression of ERα/β, TGF-β and Smad7 was higher in in prostate cancer tissues than that in the other two groups (**Table 1**), suggesting there was a negative correlation

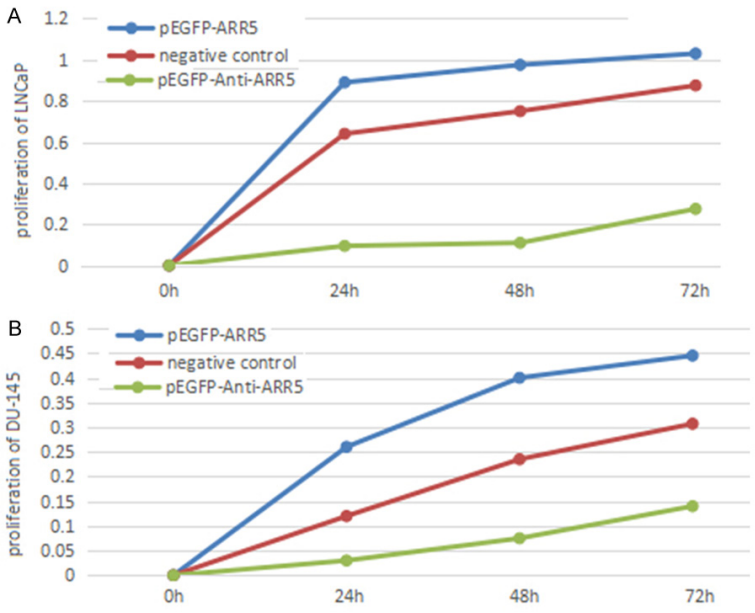

Figure 2. Detection of cell proliferation with CCK-8 method. A. LNCaP cells; B. DU-145 cells.

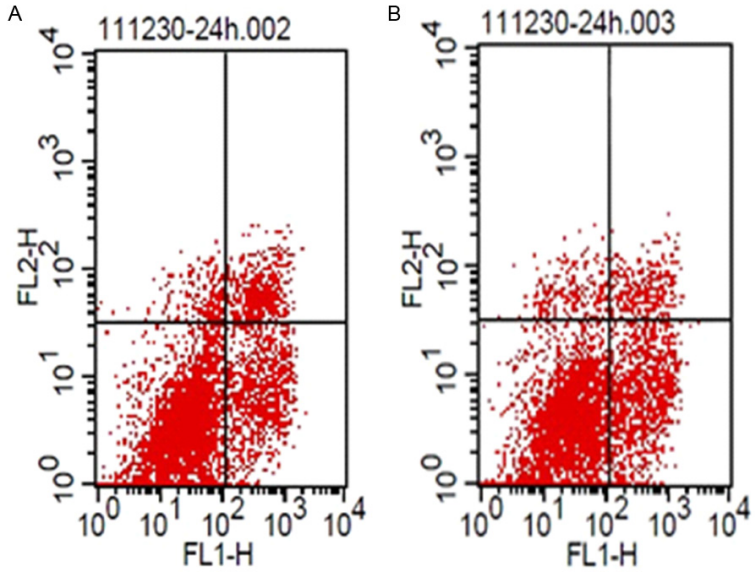

Figure 3. Detection of cell apoptosis transfected with ARA55 plasmid by flow cytometry. A. LNCaP cells; B. DU-145 cells.

between the expression of ARA55 and the expression of ERα/β, TGF-β and Smad7.

The cell proliferation after over-expression of ARA55

The LNCaP and DU-145 cells were transfected with pEGFP-ARA55 plasmid, pEGFP-anti-ARA55 plasmid and negative control, respectively. At 0, 24, 48 and 72 h, the cell proliferation was detected by CCK-8. The results showed that over-expression of ARA55 could inhibit significantly the proliferation of both LNCaP and DU-145 cells, especially at 72 h, suggesting ARA55 had different proliferating ability at different time points (**Figure 2**).

The cell apoptosis after over-expression of ARA55

The living cells cannot be combined with annexin V or PI, the early apoptotic cells show positive annexin V and negative PI, the late apoptotic cells show positive annexin V and PI. After transfection of ARA55 plasmid for 24 h, the apoptotic rate of LNCaP cells was 29.83±2.25%, which was significantly higher than control group (10.21±0.35%) and pEGFP group (7.43±0.67%, $P<0.05$). Similarly, the apoptotic rate of DU-145 cells was 17.36±1.63%, which was significantly higher than control group (5.47±2.18%) and pEGFP group (11.18±3.47%, $P<0.05$). The flow cytometry results showed that over-expression of ARA55 could induce LNCaP and DU-145 cell apoptosis (**Figure 3**).

The cell aggression after over-expression of ARA55

The invasion assay results showed the number of LNCaP cells transfected ARA55 (47.5) passed through the Transwell membrane was less than that of LNCaP cells transfected with control plasmid (53.4); while the number of DU-145 cells transfected ARA55 (73.5) passed through the Transwell membrane was less than that of DU-145 cells transfected with control plasmid (124.2), suggesting ARA55 could inhibit prostate cancer cell aggression (**Figure 4A**, **4B**). The cell scratch assay results showed that the LNCaP cells transfected with

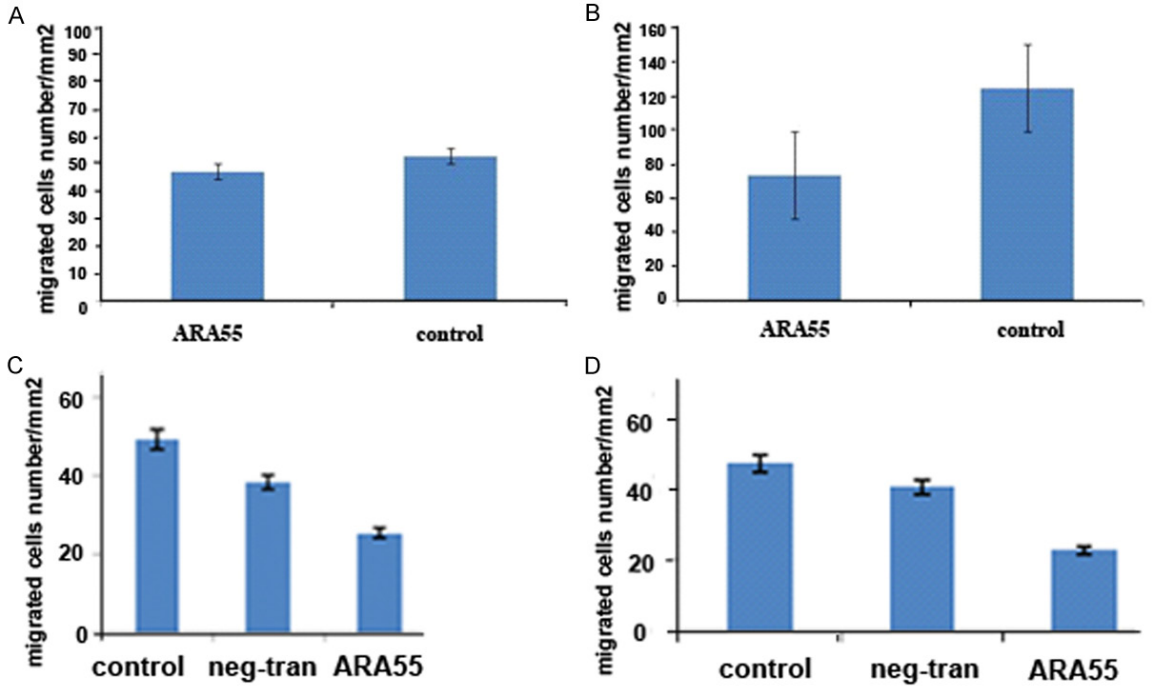

Figure 4. The prostate cancer cell aggression determined by cell invasion and scratch assay. A. The cell invasion assay with LNCaP cells transfected ARA55; B. The cell invasion assay with DU-145 cells transfected ARA55; C. The cell scratch assay with LNCaP cells; D. The cell scratch assay with DU-145 cells.

control plasmid migrated to the blank area after 18 h, while there was no significant change in LNCaP cells transfected with ARA55 plasmid ($P<0.05$). The above situation was consistent with DU-145 cells (**Figure 4C**, **4D**).

The ARA55-TGF-β signaling pathway

When the DU-145 cells transfected ARA55 plasmid, the expression of ERβ and TGF-β was significantly decreased; however, the expression of Smad7 had no significant change (**Figure 5A**). Therefore, we speculated that ARA55 could regulate the TGF-β signaling pathway. Next, we detected the expression of related factors in TGF-β signaling pathway after the DU-145 cells transfected with ARA55 and ERβ-siRNA together. The resulted revealed that the expression of TGF-β was significant lower in cells transfected with ARA55 and ERβ-siRNA than that in cells transfected with ARA55 alone; however, the expression of Smad7 still had no significant changes (**Figure 5B**). The results suggested that in the prostate cancer cells, the ARA55 could up-regulate the TGF-β expression via estrogen receptor pathway.

Discussion

In this study, we found that ARA55 was lowly expressed in prostate cancer, which was mainly located in the nucleus; moreover, with the development of prostate cancer, the rise of Gleason score, the lower expression of ARA55, suggesting ARA55 was involved in the incidence and progression of prostate cancer. Interesting, our correlation analysis found that there was a negative correlation between ARA55 and ERβ, suggesting that synergy of ARA55 and ERβ contributed to the development and progression of prostate cancer. It has been proved that androgen played an important role in prostate cancer, and removal of androgen would promote prostatic atrophy; while injection of androgen and chemical inducer could induce prostate cancer in rat [14]. It has been confirmed that blocking androgen promoted prostate cancer shrink early, however, with the development of cancer, the cancer could growth without androgen [3].

Recent studies found that the prostate cancer was not only depended on androgen, as the prostate cancer would turn into androgen-independent cancer without androgen. Therefore,

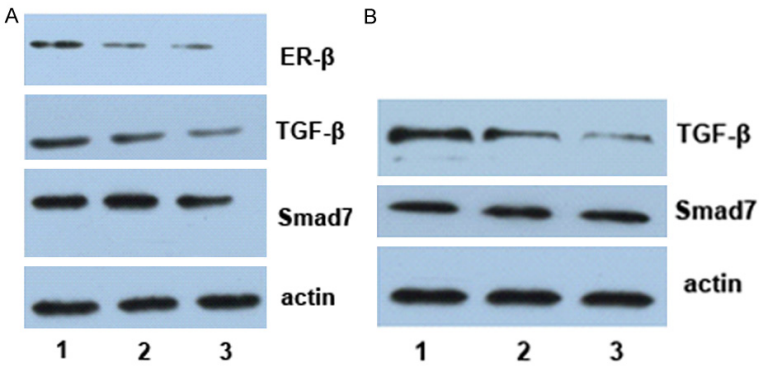

Figure 5. ARA55 up-regulated the TGF-β expression via estrogen receptor pathway. A. TGF-β signaling pathway after the over-expression of ARA55 in DU-145 cells, 1: control group; 2: pEGFP group; 3: pEGFP-ARA55 group. B. TGF-β signaling pathway after the over-expression of ARA55 and knock-out ERβ in DU-145 cells, 1: control group; 2: pEGFP-ARA55 group; 3: pEGFP-ARA55 + ERβ-siRNA group.

we speculate there are other signaling pathways involved in prostate cancer. Mestayer *et al* [15] found that the expression of ARA55 was lower in prostate cancer tissues than that in normal prostate tissues and benign prostatic hyperplasia tissues, which was consistent with our study. It also has been proved that ARA55 could up-regulate the TGF-β expression in cancer cells [8]; other study found that estrogen could increase the TGF-β expression in breast cancer cells, to promote breast cancer proliferation, invasion and metastasis [16]. The estrogen is extensively involved in breast cancer, ovarian cancer, and prostate cancer, and so on. The estrogen interacted with estrogen receptor to initial downstream signaling pathway, and eventually playing a physiological or pathological role. The ERβ was detected in LNCaP and DU-145 cells, while the ERα and ERβ were detected in prostate cancer cell line PC-3 cells. In addition, studies have found that ERβ was increased when the androgen-independent prostate cancer occurred [17], suggesting ERβ was associated with the progress of prostate cancer.

LNCaP cell is androgen-independent prostate cancer cell, and DU-145 is androgen-dependent prostate cancer cell. In our study, the two kinds of cells had the same biological behavior, suggesting ARA55 might be involved in the transformation process of androgen-independent of prostate cancer cells, which was consistent with other studies [18-21]. TGF-β was increased in prostate cancer tissues, which could promote the prostate cancer cell proliferation and invasion [23, 24]. The low expression of ARA55 could activate the downstream signaling pathway and promote tumor aggression; therefore, the over-expression of ARA55 could inhibit the TGF-β expression via estrogen receptor pathway, thereby inhibiting the prostate cancer cell proliferation and aggression.

Conclusion

In prostate cancer, the expression of ARA55 was decreased, while the expression of ERα/β, TGF-β and Smad7 was increased, which indicated that theses factors must be involved in the prostate cancer carcinogenesis and progression, and were able to be considered as the biomarkers to provide the prognosis of patients with prostate cancer. Furthermore, ARA55 could inhibit the TGF-β level via the ERβ signaling pathway, to inhibit the cancer cell proliferation and aggression. Therefore, ARA55 could be considered as one of the treatment targets. The prostate cancer could be controlled via increasing the ARA55 level in the cells.

Disclosure of conflict of interest

None.

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