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Effects of TRPC6 on invasibility of low-differentiated prostate cancer cells

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ABSTRACT

Objective: To study the expression of TRPC6 among prostate cancer cells, establish high expression cell lines of TRPC6, and to provide potential cell mode for prostate cancer oncogenesis and development. **Methods:** Occurrence and development of prostate cancer cells, PC3, PC-3 m DU145, 22 rv1, LNCaP and normal prostate epithelial cells in the PrEC TRPC6 expression level were detected by QPCR method. Calcium phosphate transfection method was used to package retrovirus pLEGFP-N1-TRPC6 and pLEGFP-N1-vector and infect the prostate cancer cells, a stable high expression of TRPC6 prostate cancer cells. Stable cell lines of TRPC6, matrix metalloproteinase (MMP) 2, MMP9 expression was detected by QPCR and Western blot. Change of cell invasion ability was detected by Transwell. **Results:** The expression level of prostate cancer cells TRPC6 were higher than control group PrEC cells. Among TPRC6 the expression of cell line PC 3 transfer potential wre the lowest, and high transfer cell line PC-3M express was the highest. Real-time fluorescent quantitative PCR and western blot results showed that after filter, the seventh generation of cell TRPC6 protein and mRNA expression levels were higher than the control group obviously. Transwell experimental results showed that the overexpression of TRPC6 could promote the invasion ability of PC3 prostate cancer cells. **Conclusions:** TRPC6 expressed in prostate cancer cells is in disorder, and its action may be associated with the invasion and metastasis of prostate cancer cells; successful establishment of stable high expression of TRPC6 prostate cancer cells primarily confirm the invasion-trigger ability of TRPC6 on prostate cancer, and lay down the foundation for exploring the TRPC6's role in the occurrence and development of prostate cancer mechanism

1. Introduction

Prostate cancer (Pca) is one of most common malignant tumor for males, with the second highest mortality rate to lung cancer in the western countries. Pca is a kind of double stage cancer, with slow progress rate to detect in the early stage. The curative effect of combining treatment using surgery, radiation therapy and hormone is distinct[1–3]. But after a period of time after early treatment, Pca is mostly turn into a progress quickly, invasive ability strong AIPC independent on male hormones. proliferation There are

studies showing some molecular targets play a role in the treatment of castration Pca (CRPC)[4]. Denosumab et al found that RANKL antibody can hobble CRPC bone metastases[5]; M Bjorkman *et al* found the high expression of PHF8 in Pca is associated with the transfer of the tumor[6]. van der Horst et al proved that the peptide hormones alpha v integrin antagonist GLPG0187 can inhibit the bone metastases of Pca *in vivo* and *in vitro*[7]. In the Pca metastasis, most of them are bone metastases[8]. TRPC1 and TRPC6 are important moleculars of TRP family regulate calcium ion channel. Fabian et al suggested that TRPC1 using calcium regulating cell migration, concluded that TRPC1 and matrix metalloproteinases (MMPs) should have close relationship in the process of tumor metastasis[9]. In the research, TRPC6 shows increased expression in high transfer Pca cell lines and decreased expression in low transfer cell lines. The effects of TRPC6 on Pca expression disorders, development and transfer capability have not been studied. We will explore the induction role of TRPC6 in increased Pca cells,

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and the correlation between TRPC6 and MMP2, MMP9 and TRPC6 regarding the influence on the Pca cells transfer for providing a new basis for the clinical treatment of Pca.

2. Materials and methods

2.1. Cells and plasmid

PrEC prostate epithelial cells were from institute of Chinese academy of medical sciences tumor cell bank tumor hospital, PC-3 M, PC3, DU145, 22 rv1, LNCaP were from ATCC cell bank, 293FT, pLEGFP-N1-TRPC6 and pLEGFP-N1-vector) retroviral packaging cells systems presented in Guangzhou Cardiac Tumor Research Institute.

2.2. Reagent

Fetal bovine serum, RPMI-1640 medium, Transwell culture plate for GIBCO products; Matrigel glue from BD company; G418 were from Sigma; TRPC6, MMP2 and MMP9 primary antibody, and secondary antibody of sheep were purchased from Abcam company; Real-time fluorescent quantitative PCR kit for self restoring genes company; Other experiments conventional reagents and consumables were from Guangzhou Langri biological technology co., LTD.

2.3. Establishment of stable cell line PC3

Virus packaging was performed by calcium phosphate transfection method, retrovirus plasmids PIK were packed respectively with ppLEGFP-N1-TRPC6, pLEGFP-N1-TRPC6-vector and the PIKs plasmid to the packaging cells of 293FT. Five hours after transfection the medium was refreshed, poison was collected 24 h after transfection. It was filtered using 0.45 μ m filter and saved at -80°C . It was inoculated with infected cells PC3 cells, and when the fusion rate reached 70%, virus liquid was added for infection with polybrene at final concentration of 8 μ g/mL. After 48 h, cells underwent passage. After adherence, they were cultured in culture medium (Sigma) containing 0.5 μ g/mL G418. GFP intensity of the seventh generation of cells was observed under fluorescence microscope.

2.4. Transwell invasion experiment

Matrigel glue was dissolved overnight at 4°C , and was added to serum-free medium RPMI1640 1:9 to dilute Matrigel. One hundred μ L diluted Matrigel was placed evenly into the bottom of the Transwell cell, and then was placed in incubator until fully cohesion; cells at logarithmic

growth phase were digested and collected, washed by 1 \times PBS, resuspended in serum-free medium RPMI1640, and then incubated in Transwell cell at 1.0×10^4 . Every pore was 200 μ L, and 500 μ L RPMI1640 culture medium containing 10% FBS was added as chemotactic fluid invasion; after 24 h Transwell cell was taken out, and residual Matrigel was wiped out by cotton swab. After washed by 1 \times PBS, the cell was fixed in methanol for 15 min, stained by hematoxylin after cleaning for 3 min. Transmembrane cells were counted under microscopy for 10 fields, and the average was calculated. The difference was analyzed by using SPSS 17.0 statistical software between the two groups of cells, $P < 0.05$ was regarded as statistically significance.

2.5. TRPC6, MMP2, MMP9 mRNA expression after infection PC3 using QPCR

Trizol was used to produce the cells lysis in logarithmic growth phase. Total RNA was extracted with chloroform, the concentration was determined by ultraviolet spectrophotometer, then they were saved at -20°C . With beta Actin as the reference, expression of MMP9 and MMP2 in different cell lines was detected. Primer sequences were as Table 1.

2.6. Detection of TRPC6, MMP2, MMP9 expression using Western blot

Cells in log phase were lysized, protein concentration was determined by BCA protein concentration kit. Total 20 μ g protein was determined by 9% SDS-PAGE gel, 70 eV for 4 h, followed by transmembrane, blocking and antibody incubation. It underwent horseradish peroxidase-ECL method and X-ray film exposure, to determine TRPC6, MMP2, MMP9 protein expression levels.

2.7. Statistical analysis

All statistical data were analyzed using SPSS18.0 for Windows (IBM, standard version 18.0). Groups data were expressed by mean \pm SD. One-way analysis of variance was used to analyze difference among several samples. $P < 0.05$ (bilateral inspection) was regarded as statistical significance.

3. Results

3.1. Expression of TRPC6 protein and mRNA in Pca cell lines using QPCR and Western blot

QPCR and western blot showed that protein and mRNA

Table 1

Primer sequences.

Gene	Forward primer	Reverse primer
MMP2	5'CTGAGACCCGATGTCCACT 3'	5'GCCACGAGGAACAGGCTGTA 3'
MMP9	5'ATCCGGCACCTCTATGGTCTC 3'	5'GCACAGTACTGGCCGTAGAAG 3'
TRPC6	5'TCTGTGCCACAGATGTGAAG 3'	5'ACCGTGTGAGCGGTAGTAG 3'
β -Actin	5'TGGCACCCAGCACAATGAA-3'	5'CTAAGTCATAGTCCGCCTAGAAGCA-3'

expression of TRPC6 in 6 Pca cell lines were significantly higher than those of normal epithelial cells (Figure 1).

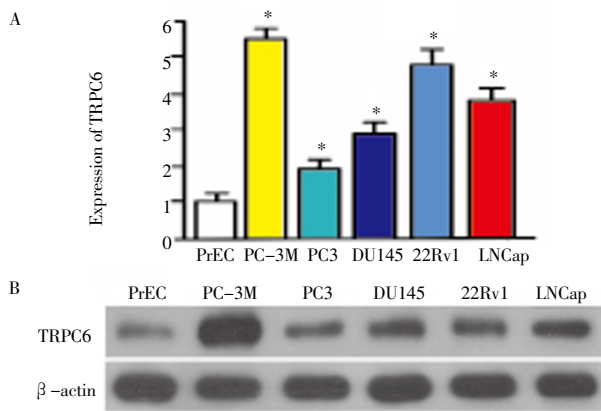


Figure 1. TRPC6 expression changes in QPCR(A) and Western blot(B) experiment.

3.2. Establishment of cell lines with stable expression of TRPC6

Virus with overexpressed TRPC6 gene was used to infect PC3 cells. After screened by G418 and continuous passage, the GFP expression was observed under fluorescence microscope. It was as Figure 2.

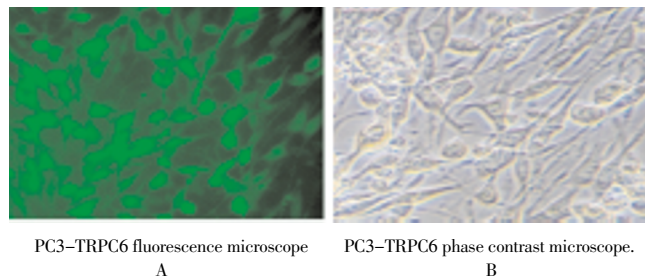


Figure 2. GFP expression in TRPC6 stable cells under fluorescence microscope (A) and phase contrast microscope (B).

3.3. Effect of TRPC6 on invasibility using transwell test

Transwell results showed that after transduction of TRPC6, number of PC3/TRPC6 cells through the basement membrane was significantly higher than that of control group (Figure 3); so overexpression of TRPC6 could promote cell's ability to go through the basement membrane, and cells invasion ability was increased with TRPC6 expression enhancement.

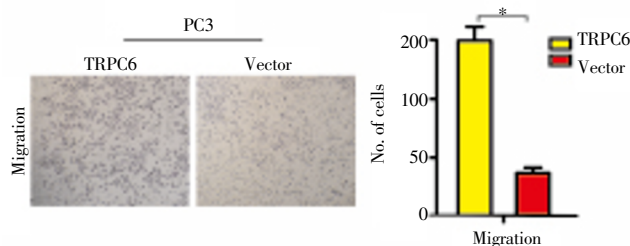


Figure 3. TRPC6 promotes PC3 cell invasibility changes.

3.4. Effect of Overexpression of TRPC6 on cell invasive ability

QPCR (Figure 4a) and Western Blot (Figure 4b) showed that after overexpression of TRPC6, TRPC6 in PC3 cells was positively correlated with MMP2 and MMP9. It indicated overexpression of TRPC6 could significantly enhance the capacity of invasion and metastasis of tumor cells.

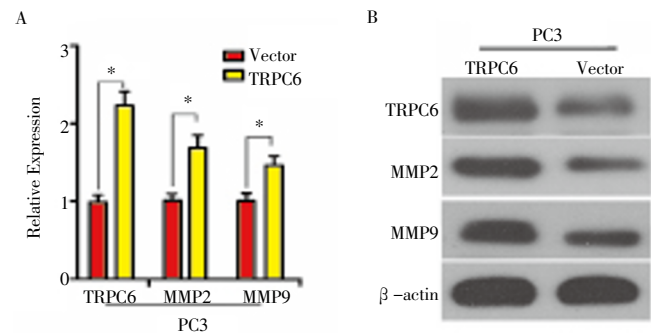


Figure 4. QPCR(A), Western Blot(B) determination of TRPC6, MMP2, MMP9.

4. Discussion

At present the major reason of tumor treatment failure is the tumor metastasis including adhesion, invasion and migration process. MMPs play a critical role in the process of malignant tumors, such as incoming transfer. In the process of tumor metastasis, the degradation and destruction of adhesion to extracellular matrix and basement membrane is the most critical.

Extracellular matrix (ECM) are the outer adhesion parts of cells, and the MMPs can degrade the ECM for most of the ingredients, adjust the ECM physiological dynamic balance and the integrity of the basement membrane, promote cancer cells to the surrounding normal tissues, and lead to the spread of the tumor metastasis. Kleiner^[17] noted that MMPs play an important role in three key biological infiltration process: the degradation of ECM structure, structure of ECM adhesion, and cell migration behavior.

TRP is located in the membrane of the voltage dependent nonselective cation channels. It can adjust the intracellular Ca^{2+} flow, promote cell proliferation, inhibit cell apoptosis^[18,19]. Distribution of the TRP channels members are widely distributed on the skin, cardiovascular system, respiratory system, gastrointestinal system, urogenital system and immune system, and plays an important role in cell proliferation and differentiation of apoptosis and migration process^[20-22]. TRPC6 is one of the members of TRP family^[23]. Overexpression of TRPC3 and TRPC6 was reported in prostatic smooth muscle, and in gliomas and ovarian cancer cells^[24,25]. Yue et al found TRPC6 in Pca with high expression can promote cell proliferation and boost the expression level; and Pca histologic stage, Gleason score and transfer scope positively correlated^[26-28]. However, the effect of TRPC6 in Pca cells on the tumor invasion and metastasis ability has not been reported before.

In this experimental study, highly transferring PC-3 in 5 strains of Pca expressed TRPC6 highly, and lowly transferring PC-3 had low expression. It is assumed that TRPC6 is related with the development, invasion and metastasis of Pca. Fluorescence microscope showed after overexpression of TRPC6, invasive were stronger than the blank control group obviously. QPCR and western blot experiments showed that TRPC6 and expression of MMP were correlated.

To sum up, this study preliminarily confirmed that abnormal expression of TRPC6 in the Pca cells can affect cell invasion and metastasis through influencing the matrix metalloproteinases. Using a retrovirus mediated trial, a steady high expression of TRPC6 Pca cell line PC3–TRPC6 and control cell line PC3–vector were successfully established, laying down the foundation for signaling pathway research on TRPC6's effect on the development and progress of Pca. This research will provide a basis for the prevention of Pca and targeted therapy.

Conflict of interest statement

We declare that we have no conflict of interest.

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