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Estramustine phosphate induces prostate cancer cell line PC3 apoptosis by down-regulating miR-31 levels

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Abstract. – OBJECTIVE: Prostate cancer seriously threats to patient's life and health. Estramustine phosphate (EP) is one of the most important drugs in the clinical treatment of prostate cancer. This study aims to explore the molecular mechanism of estramustine phosphate in regulating PC3 cell growth and survive through mediating miR-31.

MATERIALS AND METHODS: Estramustine phosphate was used to treat prostate cancer cell line PC3. Flow cytometry was applied to detect PC3 cell growth and apoptosis. RT-PCR was performed to test miR-31 level. Prostate cancer tissue and paracarcinoma tissue were collected to test miR-31 level. PC3 cells were transfected with miR-31 or control microRNA by lipofectamine, and followed treated by estramustine phosphate.

RESULTS: PC3 cell appeared growth restrain and apoptosis after treated by estramustine phosphate. MiR-31 level decreased after estramustine phosphate treatment. Prostate cancer tissue presented higher miR-31 level than paracarcinoma tissue. MiR-31 over-expression inhibited estramustine phosphate induced PC3 cell apoptosis.

CONCLUSIONS: Estramustine phosphate induces prostate cancer cell line PC3 apoptosis through reducing miR-31.

Key Words:
Prostate cancer, Estramustine phosphate, miR-31, PC3, Cell apoptosis, Cell viability.

Introduction

Prostate cancer is a type of cancer occurred in male. Its pathogenic site locates at the epithelial part of the prostate[1]. It can be classified as adenosquamous carcinoma, ductal adenocarcinoma, squamous cell carcinoma, acinous adenocarcinoma, and urothelial carcinoma according to the pathological types[2,3]. Prostate cancer has a high incidence at about 1/10000 in China, which accounts for the fifth in male[4]. The age of prostate cancer onset is from 56 years and reached top at 80. Prostate cancer seriously threats to male's health[5,6].

In clinic, the treatment of prostate cancer includes chemotherapy, radiotherapy, and surgery. Comprehensive treatment shows better effect[7,8]. Chemotherapy is a most commonly used method in the therapy of prostate cancer. Usual chemotherapy drugs mainly include estramustine phosphate (EP), paclitaxel, vincristine, cisplatin, mitoxantrone, etoposide, and gemcitabine[9,10]. Among them, EP is more widely used in clinical. However, the molecular mechanism of EP in treating prostate cancer remains needed to be further clarified[11,12]. Current research suggested that EP was metabolized to oestrone nitrogen mustard and estradiol in the body, while the latter two have strong affinity to prostate epithelial cells. Moreover, they also have strong toxic effect on cells proliferation. Therefore, EP shows the strongest toxicity to prostate cancer cells and becomes the first choice for prostate cancer patients[13-15]. However, the mechanism of EP in anti-prostate cancer at the molecular level still needs further investigation[16,17]. Unfortunately, EP also induces strong side effects to prostate cancer patients, such as vomiting, nausea, abnormal liver function, thrombosis symptoms, and so on. It needs to clarify the molecular mechanism of EP and carry out combined therapy of EP and molecular targets[18-20]. MicroRNAs are a type of small RNAs with the function of regulating cell activity, and tissue and organ development process[21,22]. However, at present, how microRNAs are regulated still needs to be further discussed[23,24]. Our preliminary microRNA screening showed that miR-31 level in tumor tissue changes after EP treatment, indicating that miR-31 may participate

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in prostate cancer occurrence and development. This study attempts to investigate the effect of EP on prostate cancer cell line PC3 *in vitro* and the related mechanism. Our results may provide scientific basis for EP clinical application.

Materials and Methods

Reagents

Prostate cancer cell line, PC3, was purchased from ATCC Cell Bank (Manassas, VA, USA). High-glucose Dulbecco minimum essential medium (DMEM) and fetal calf serum (FCS) were from Beijing Hualan Biotechnology Co., Ltd. (Beijing, China). Penicillin-streptomycin, phosphate buffered solution (PBS), dimethyl sulphoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), Hanks buffer, trypsin, and poly-L-lysine were obtained from Beijing Dingguo Biotechnology Co., Ltd (Beijing, China). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was purchased from Sangon Biotech., Co., Ltd., (Shanghai, China). Caspase-3 activity detection kit and FITC-Annexin V were obtained from Tiangen Biotech., Co., Ltd., (Beijing, China). MiR-31 siRNA and control siRNA were synthesized by Sun Biotech Co., Ltd. (Beijing, China). Their sequences were as follows: 5-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGT-3 and 5-ACACTCCAGCTGGGGTCCAGTTTTCCCAGGA-3, 5-CTCGCTTCGGCAGCACA-3 and 5-AACGCTTCACGAATTTGCGT-3. MiR-31 plasmid was constructed and preserved by our laboratory.

PC3 Cell Culture

Prostate cancer cell line PC3 was maintained at 5% CO_2 and 37°C.

PC3 Cell Transfection

MiR-31 and control microRNA were transfected to PC3 cells using Lipo2000 according to previous report[9]. Specially, PC3 cells were seeded in the plate at 60% density. MiR-31 and control microRNA were resuspended in Lipo2000 at room temperature for 6 min. Next, the mixture was added to the cells and cultured at 5% CO_2 and 37°C for 12 h[9]. After changing the medium, the cells were further cultured for 24 h.

MTT Assay

PC3 cell viability was tested by MTT according to the reports[10]. Specially, PC3 cells were seeded to the plate at 1×10^3 per well and cultured at 5% CO_2 and 37°C for 48 h. Next, MTT solution at 0.5 mg/ml was added to each well and cultured for 4 h. After treated by DMSO to stop reaction, the plate was incubated at room temperature for 6 min. At last, the plate was read on micro-plate reader at 492 nm to draw the growth curve[11].

Flow Cytometry

Flow cytometry was applied to test PC3 cell apoptosis according to the reports. Specially, PC3 cells transfected with miR-31 or control microRNA were collected. Then, the cells were added with 0.5 μl Annexin-V-FITC and 25 μl buffer to the total amount at 125 μl and incubated at room temperature avoiding light for 20 min. At last, flow cytometry was performed to detect PC3 cell apoptosis. The wavelength of exciting light and reception light was 484 nm and 560 nm, respectively[14].

Caspase-3 Activity Detection

Caspase-3 activity kit was adopted to determine PC3 cell apoptosis level. PC3 cells transfected with miR-31 or control microRNA were collected and resuspended in DMEM. Next, the cells were added with lysis and cracked on ice for 20 min. Then, the cells were treated by 1 mM Ac-DEVD-pNA substrate and incubated at 37°C for 10 min. At last the cells were read on micro-plate reader at 492 nm to calculate caspase-3 activity[17].

Statistical Analysis

All the statistical analysis was performed on SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The data were presented as mean ± standard deviation. Student's *t*-test was applied for comparison. $p < 0.05$ was considered as statistical significance.

Results

EP inhibited PC3 Cell Growth

As shown in Figure 1, MTT assay revealed that compared with untreated PC3 cells, 1 μg/ml EP treatment suppressed PC3 cell growth ($p = 0.017$).

EP Induced PC3 Cell Apoptosis

Figure 2 showed that EP treatment (2 μg/ml) significantly elevated phosphatidylserine eversion amount on PC3 cells compared with control ($p = 0.0074$).

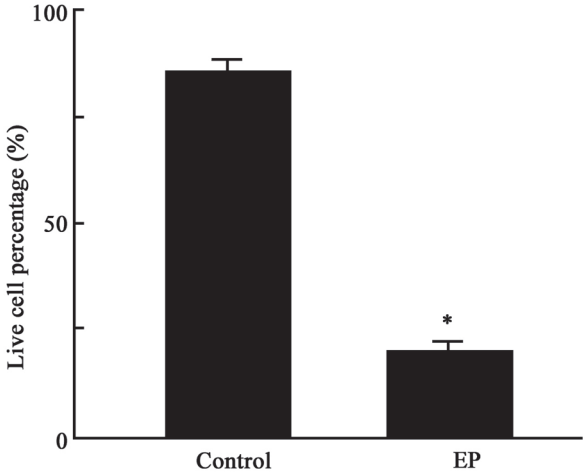

Figure 1. EP inhibited PC3 cell growth. *$p < 0.05$, compared with control.

EP Activated Caspase-3

Figure 3 results showed that the relative caspase 3 activity in EP group was significantly higher compared to the Control group. EP induced PC3 cell apoptosis (Figure 2, $p < 0.05$).

EP Down-Regulated miR-31 Level

PCR results showed that compared with control, miR-31 level obviously declined in PC3 cells treated by 2 μg/ml EP (Figure 4, $p = 0.013$).

MiR-31 Knockdown Enhanced EP induced PC3 Cell Apoptosis

Caspase-3 activity detection revealed that PC3 cells treated by 0.1 μg/ml EP after miR-31 inhibition presented significantly higher caspase-3 activity (Figure 5, $p = 0.021$).

MiR-31 Plasmid Transfection Suppressed EP Induced Cell Apoptosis

PC3 cells were transfected with miR-31 plasmid to upregulate miR-31 level and followed by 2 μg/ml EP treatment. Caspase-3 activity detection showed that its activity was markedly restrained after transfection compared with control (Figure 6, $p = 0.025$).

Discussion

Prostate cancer is characterized by high incidence, poor curative effect, and unclear pathogenesis[1]. This study discussed the impact and mechanism of EP in treating PC3 cells. The results showed that EP obviously inhibited PC3 cell growth and induced apoptosis, which was in agreement with the previously report[25,26]. In this paper, our results further revealed that EP played an anti-cancer role by inducing apoptosis.

This study also has shortcomings and insufficiency as follows: (1) how EP affect miR-31 level was not proved; (2) if EP can induce PC3 cell apoptosis through down-regulating miR-31 expression in animal model was not tested[27]; (3) clinical prostate cancer tissue examination to explore the relationship between EP treatment and miR-31 level was not permormed[28]; (4) the key downstream target

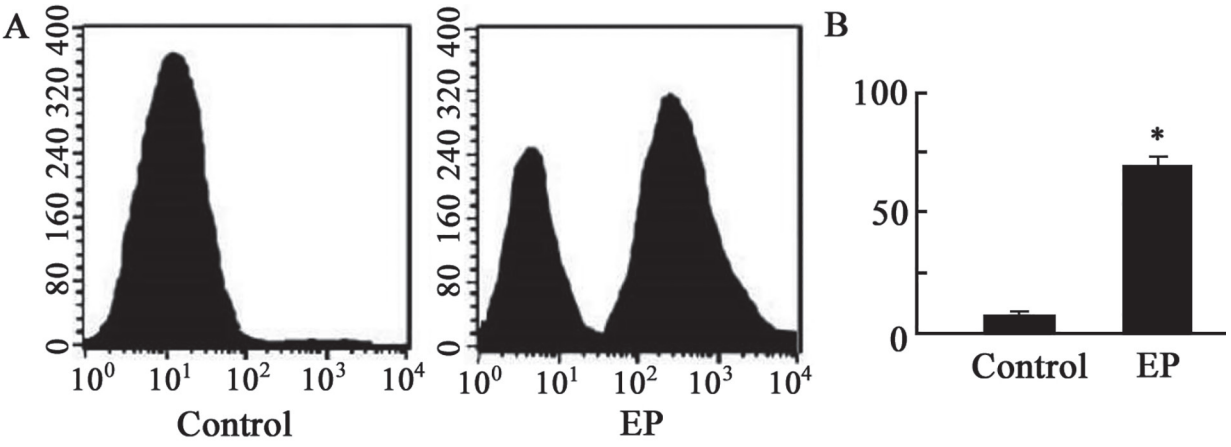

Figure 2. EP induced PC3 cell apoptosis. *$p < 0.05$, compared with control. Caspase-3 activity detection demonstrated that EP treatment (2 μg/ml) markedly enhanced PC3 cells caspase-3 activity compared with control ($p = 0.0067$).

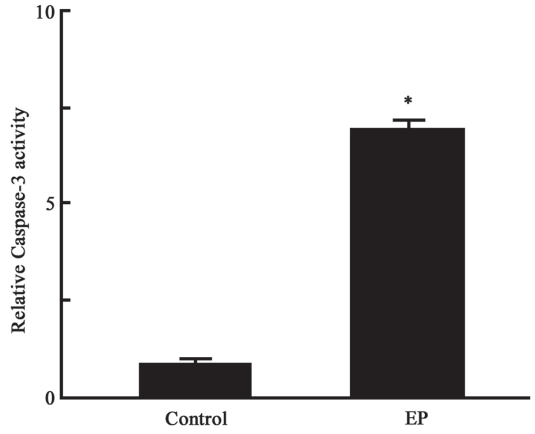

Figure 3. EP activated caspase-3 in PC3 cells. *$p < 0.05$, compared with control.

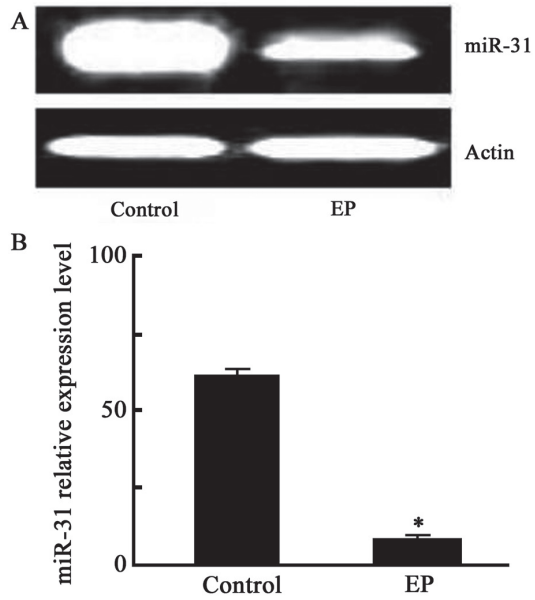

Figure 4. EP downregulated miR-31 level. *$p < 0.05$, compared with control.

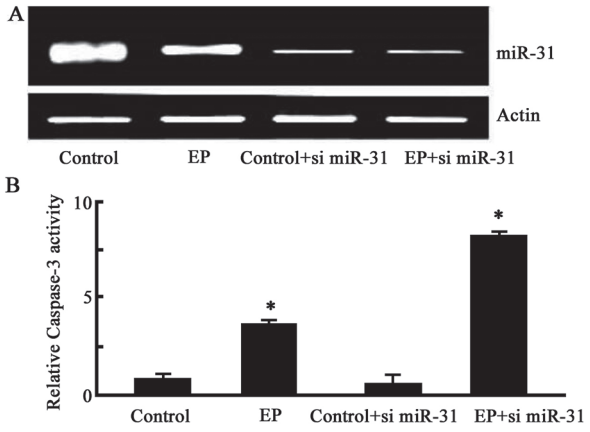

Figure 5. MiR-31 knockdown enhanced EP induced PC3 cell apoptosis. *$p < 0.05$, compared with control.

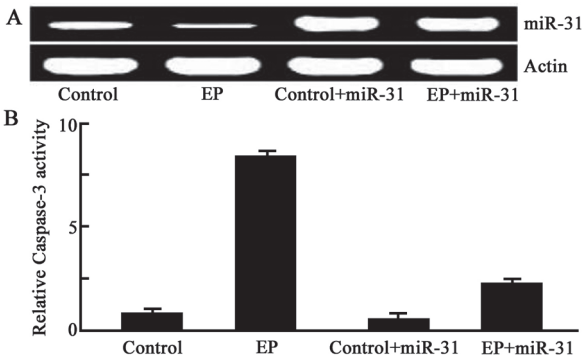

Figure 6. MiR-31 plasmid transfection suppressed EP induced cell apoptosis. *$p < 0.05$, compared with control.

protein affected by miR-31 was not verified. To sum up, our results presented that EP induced prostate cancer cell line PC3 apoptosis through reducing miR-31.

Conclusions

There are three main findings in this study: (1) EP can suppress PC3 cell growth, induce apoptosis, and down-regulate miR-31 level; (2) MiR-31 level was higher in prostate cancer tissue compared with para-carcinoma tissue; (3) MiR-31 over-expression restrained EP induced PC3 cells apoptosis. These results indicated that EP may induce PC3 cells apoptosis by declining miR-31.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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