EFFECT OF POMEGRANATE EXTRACT GALLIC ACID ON THE PROLIFERATION OF PROSTATE CANCER CELLS BY PROMOTING THE EXPRESSION OF IGFBP7

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Abstract. Objective: Pomegranate extract gallic acid inhibits the proliferation of prostate cancer PC-3 cells by promoting IGFBP7 expression. Methods: PC-3 cells were divided into six groups: control group (NC), blank control group (BL), IGFBP7 overexpression group (IGFBP7), Pomegranate extract gallic acid group (5.0 μmol/L, 10.0 μmol/L, 20.0 μmol/L). Cell proliferation was detected by MTT assay; apoptosis of each group was analyzed by flow cytometry; the expression levels of IGFBP7, AKT and mTOR were assessed by Western blot. Results: The proliferative plural of PC-3 cells in IGFBP7 group and pomegranate gallic acid group were significantly lower than those in NC and BL groups ($P < 0.05$). The pomegranate gallic acid group showed dose- dependence. The results of Western blot analysis showed that IGFBP7 group and The levels of IGFBP7 in the pomegranate extract gallic acid group were significantly higher than those in the NC and BL groups ($P < 0.05$), while the levels of AKT and mTOR plural were significantly lower than those in the NC and BL groups ($P < 0.05$). Pomegranate extract gallic acid showed dose-effect further elaborated within each group. Conclusion: Pomegranate extract gallic acid can inhibit the expression level of AKT/mTOR protein by enhancing the expression level of IGFBP7, thereby inhibiting the AKT/mTOR signaling pathway, the result is an inhibitory effect on the proliferation of prostate cancer PC-3 cells.
Keywords: *pomegranate extract gallic acid, IGFBP7 protein, AKT/mTOR protein, PC-3 cell, prostate cancer*

Introduction

Pomegranates (*Punica granatum* L.) are widely distributed and are cultivated in large quantities in China, India and Asia, Africa, Europe along the Mediterranean Sea, and California, USA. Among them, the key production areas of Chinese pomegranates are mainly fruit production, including Lintong, Ganxian, and Sanyuan in Shanxi Province, Zaozhuang in Shandong Province, Suzhou, Nanjing, Xuzhou, Pi xian in Jiangsu Province, Mengzi, Qiaojia, Jianshui, Chenggong in Yunnan Province, Yecheng Pomegranate in Xinjiang, Huili in Sichuan, Huaiyuan in Anhui Province, Xiaoxian, Suixi, Chaoxian (now Chaohu area of Hefei City), etc., as a common fruit, has many biological uses in areas like edible, medicine and health care, especially against cancer, cardiovascular and cerebrovascular diseases, liver disease and inflammatory infection certain treatment and prevention effects. The effective components of pomegranate are mainly the polyphenols in its fruit, and the pomegranate extract gallic acid is one of the main phenolic substances. It had many biological activities such as antibacterial, anti-inflammatory, anti-oxidation, liver function protection and anti-tumor. Function (Feng,

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2014; Lee, 2017; Pang, 2017; Rasool, 2010). Recent studies have shown that polyphenols have a positive significance in inhibiting the proliferation and migration of tumor cells (Panth, 2017; Momtaz, 2017). At present, prostate cancer is the primary factor that endangers male health in the world. The incidence of males is the second in the world, and the incidence of developed countries is the first. It ranks among the highest rates of male genitourinary malignant tumors (Wang, 2019). In this study, the mechanism of action of pomegranate extract gallic acid on prostate cancer tumor cells was explored. Different concentrations of pomegranate extract gallic acid were used to treat prostate cancer PC-3 cells. This provides a theoretical basis for the development and application of pomegranate extract gallic acid.

Materials and methods

Cell line

The prostate cancer PC-3 cell line was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences.

Experimental drugs and related reagents

Pomegranate extract gallic acid (GA) was purchased from Nanjing Surang Pharmaceutical Technology Development Co., Ltd., with a mass fraction of 99%(China); fetal bovine serum, DMEM high glucose medium, and trypsin were purchased from Sigma (USA); rabbit anti-human IGFBP7 was purchased from Abcam (UK); Annexin V/PE Apoptosis Detection Kit, MTT Cell Proliferation and Cytotoxicity Assay Kit from Shanghai Biyuntian Biotechnology Co., Ltd (China).

Experimental methods

PC-3 cells were divided into six groups: control group (NC), blank control group (BL), IGFBP7 overexpression group (IGFBP7), 5.0 μmol/L pomegranate extract gallic acid group (5.0 μmol/L GA), 10.0 μmol/L pomegranate extract gallic acid group (10.0 μmol/L GA), and 20.0 μmol/L pomegranate extract gallic acid group (20.0 μmol/L GA). The NC group was cultured in DMEM high-glucose medium; the BL group was transfected with blank plasmid in PC-3 cells and cultured in DMEM high glucose medium (Johnson, 2018; Zephania, 2019); IGFBP7 overexpression group was transfected with IGFBP7 plasmid on PC-3 cells. The cells were cultured in DMEM high glucose medium. The pomegranate extract gallic acid group was not transfected with PC-3 cells. The concentration of pomegranate extract gallic acid in the medium was 5.0 μmol/L, 10.0 μmol/L and 20.0 μmol/L, respectively. The culture medium of each group was incubated for 24 h in an incubator with 5.0% CO_2 and a temperature of 37 °C.

IGFBP7 transfected cells (Hu, 2017)

PC-3 cells were inoculated into 24-well plates, and the cells were observed to be in good condition after 48 h, that is, adherent growth, when the cell fusion rate reached 60% to 80%, prepared for transfection. Lipofectamine TM 2000 transfection kit (Invitrogen, USA), IGFBP7 and internal reference primers were designed and synthesized by Shanghai Shenggong Biotech Co., Ltd. to transfect PC-3 cells and stably express IGFBP7 gene.

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MTT assay to detect cell proliferation (Maleki, 2020)

Six groups of cells were taken, trypsinized, and inoculated into a 96-well plate to adjust the cell density to 1.5×10^4/well, and continue to incubate. The cultures were stopped at 12, 24 and 48 h, and 20 μL of MTT was added. The culture was continued for 4 h. 150 μL of dimethyl sulfoxide was added and shaken for 10 min. The OD value of each well was measured by a microplate reader at 570 nm.

Apoptosis detection apoptosis rate (Guo, 2016)

PC-3 cells in logarithmic growth phase were inoculated into 96-well plates for 24 h. The NC group, the BL group and the IGFBP7 transfection group were cultured in DMEM medium, and the treatment group was added with 5.0 μmol/L, 10.0 μmol/L and 20.0 μmol/L pomegranate gallic acid for 24 h, and the Annexin V/PE apoptosis detection kit was used. The cells were prepared in strict accordance with the instructions and the apoptosis rate of the above six groups of prostate cancer PC-3 cells was analyzed by BD FACSCanto II flow cytometry.

Western blot detection of total protein extracted from each group of cells (Wei, 2016)

The protein concentration was determined by the BCA method. After treatment with different concentrations of pomegranate gallic acid for 24 h, PC-3 cells were collected and washed twice with cold PBS. The resulting cells were then lysed on ice for 10 min. After centrifugation at 12000 × g for 10 min at 4 °C, the supernatant was transferred to a fresh tube and stored at -70 °C. The protein concentration was determined by using a BCA test kit, and 50 μg of each well was loaded, and the sample was separated by SDS-PAGE at a concentration of 12%, transferred, blocked, and added with a primary antibody at 4 °C overnight. Wash the membrane, add the secondary antibody, incubate for 1 h at room temperature, and wash the membrane with TBST. The band gradation value was determined by adding the illuminant using ImageJ software.

Statistical processing and analysis (Guo, 2016)

The results were processed and statistically analyzed using the SPSS19.0 statistical software package. The mean ± standard deviation ($\overline{X} \pm s$) was used. The pairwise comparison was performed by LSD-t test; the count data was expressed by the rate value. The comparison was performed using the χ 2 test. $P < 0.05$ indicates that the difference was statistically significant.

Results

PC-3 cell inhibition results

The results of six groups of cells after different treatments inhibited PC-3 cells. The IGFBP7 overexpression group and the three GA groups (5.0 μmol/L group, 10.0 μmol/L group and 20.0 μmol/L group) were significantly higher than the BL and NC group; and compared with the BL and NC groups, there are statistical differences between the three GA groups ($P < 0.05$), IGFBP7 overexpression group and the three GA groups showed obvious time-effect relationship and dose-effect relationship (see *Table 1*).

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Table 1. *Inhibition of PC-3 cells in six groups after different treatments ($\bar{x} \pm s$)*

Groups	Number of samples	12 h	24 h	48 h
		OD value	OD value	OD value
NC group	5	0.45 ± 0.02	0.56 ± 0.03	0.87 ± 0.03
BL group	5	0.46 ± 0.02	0.57 ± 0.03	0.85 ± 0.04
IGFBP7 transfection group	5	0.21 ± 0.03a,b	0.25 ± 0.08a,b	0.33 ± 0.08a,b
5.0 μmol/L group	5	0.54 ± 0.03a,b	0.59 ± 0.08a,b	0.69 ± 0.08a,b
10.0 μmol/L group	5	0.39 ± 0.02a,b,aa	0.45 ± 0.08a,b,aa	0.48 ± 0.08a,b,aa
20.0 μmol/L group	5	0.26 ± 0.04a,b,aaa	0.30 ± 0.05a,b,aaa	0.46 ± 0.05a,b,aaa

a: $P < 0.05$, the difference from the NC group was statistically significant
b: $P < 0.05$, the difference from the BL groups was statistically significant
aa: $P < 0.05$, the difference was statistically significant compared with the 5.0 μmol/L GA group
aaa: $P < 0.05$, the difference was statistically significant compared with the 10.0 μmol/L GA group

Apoptosis detection

Compared with the NC group (4.84 ± 0.45) and the BL group (5.16 ± 0.38), the apoptosis rates of PC-3 cells in the IGFBP7 overexpression group and the three GA groups (40.52 ± 1.76, 15.98 ± 1.89, 30.36 ± 1.7 and 39.04 ± 1.43) increased significantly. Compared with NC group and BL group, the difference between IGFBP7 overexpression group and the three GA treatment group was statistically significant ($P < 0.05$), and there was a significant dose-effect relationship between with the three GA groups. The result is shown in *Figure 1*.

Comparison of six groups of IGFBP7 protein levels

Western blot method was used to detect the effect of different treatments on the expression of IGFBP7 protein in PC-3 cells. Compared with NC and BL groups, the results showed that the expression levels of IGFBP7 protein by IGFBP7 overexpression group and the three GA groups (5.0 μmol/L group, 10.0 μmol/L group and 20.0 μmol/L group) was significantly increased; and compared with BL and NC groups, the three GA groups are statistical differences between groups ($P < 0.05$). The result is shown in *Figure 2*.

Comparison of six groups of AKT protein levels

Western blot method was used to detect the effect of different treatments on the expression of AKT protein in PC-3 cells. Compared with NC and BL groups, the results showed that the expression levels of AKT protein by IGFBP7 overexpression group and the three GA groups (5.0 μmol/L group, 10.0 μmol/L group and 20.0 μmol/L group) were significantly reduced. And compared with the BL and NC groups, the three GA groups are statistical differences between groups ($P < 0.05$). The result is shown in *Figure 3*.

Comparison of six groups of mTOR protein levels

Western blot method was used to detect the effect of different treatments on the expression of mTOR protein in PC-3 cells. Compared with NC and BL groups, the results showed that the expression levels of mTOR protein by IGFBP7 overexpression group and the three GA groups (5.0 μmol/L group, 10.0 μmol/L group and 20.0 μmol/L group) were significantly reduced. And compared with the BL and NC groups, the three GA groups are statistical differences between groups ($P < 0.05$). The result is shown in *Figure 4*.

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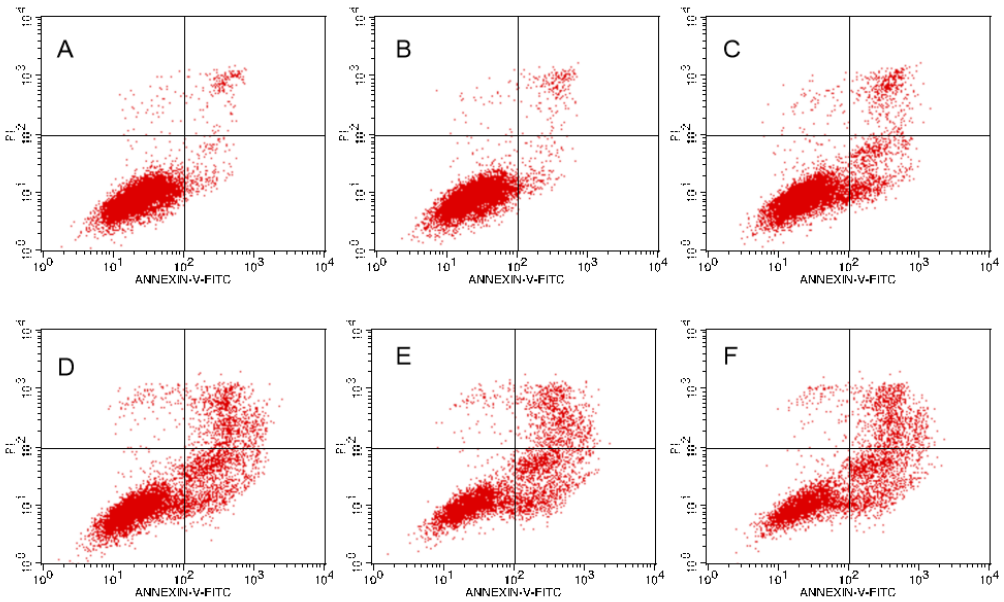
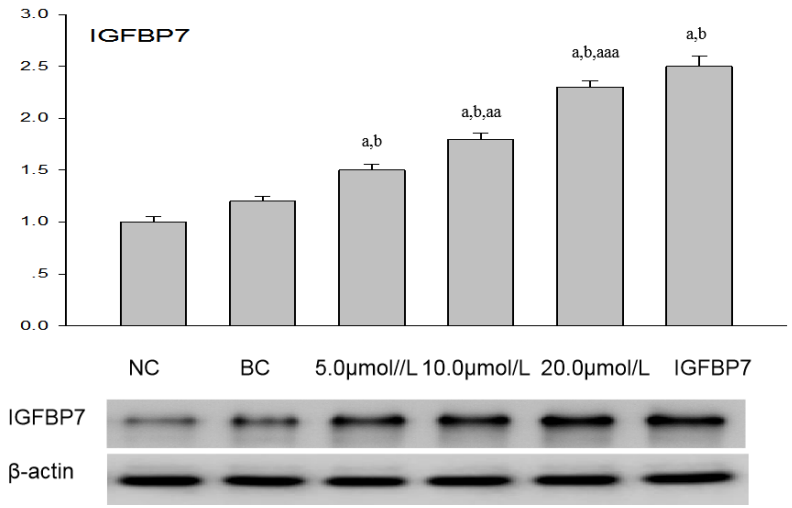


G

groups	NC group	BL group	IGFBP7 group	5.0μmol/L group	10.0μmol/L group	20.0μmol/L group
UL(%)	0.61±0.03	0.48±0.03	1.86±0.12	0.92±0.03	1.6±0.05	1.99±0.05
UR+LR(%)	4.84±0.45	5.16±0.38	40.52±1.76^{a,b}	15.98±1.89^{a}	30.36±1.76^{ab,aa}	39.04±1.43^{ab,aa}
LL(%)	94.55±1.89	94.36±2.14	57.61±2.01	83.1±2.33	66.03±2.98	59.07±2.17

a: P<0.05, the difference from the NC group was statistically significant
b: P<0.05, the difference from the BL groups was statistically significant
aa: P<0.05, the difference was statistically significant compared with the 5.0μmol/L GA group
aaa: P<0.05, the difference was statistically significant compared with the 10.0 μmol/L GA group

Figure 1. *Comparison of apoptosis in six groups. (A is NC groups; B is BL groups; C is IGFBP7 transfection group; D is 5.0 μmol/L GA group; E is 10.0 μmol/L GA group; F is 20.0 μmol/L GA group)*



a: P < 0.05, the difference from the NC group was statistically significant
b: P<0.05, the difference from the BL groups was statistically significant
aa: P<0.05, the difference was statistically significant compared with the 5.0μmol/L GA group
aaa: P<0.05, the difference was statistically significant compared with the 10.0 μmol/L GA group

Figure 2. *Comparison of six levels of IGFBP7 protein levels*

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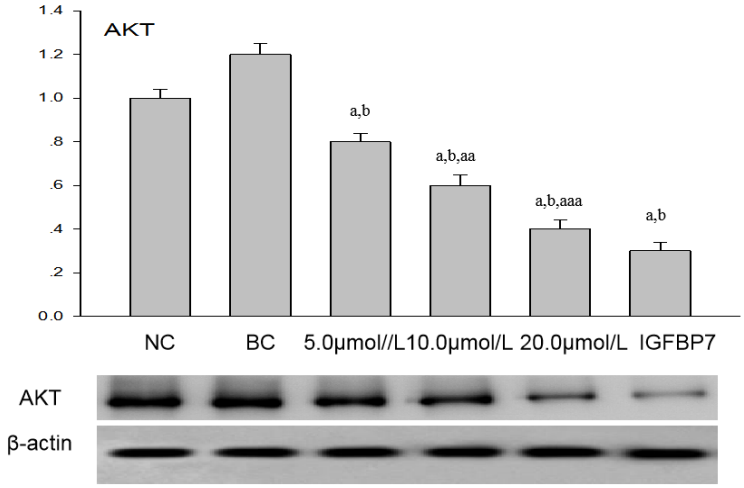
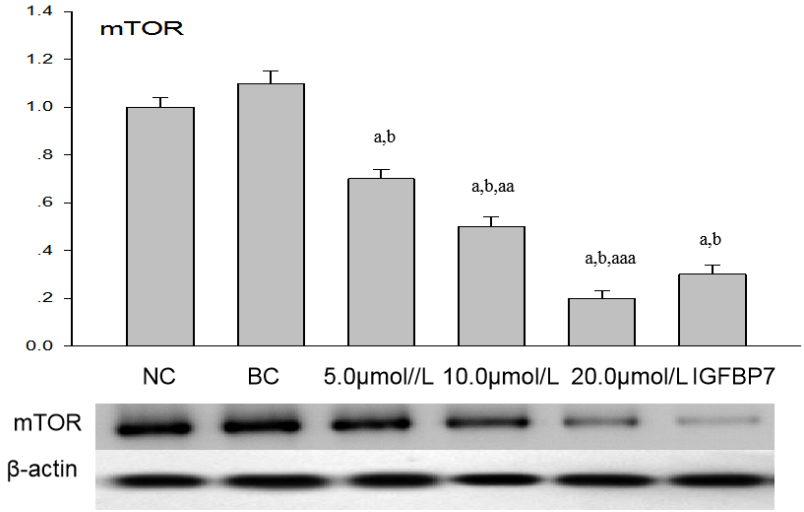


a: P < 0.05, the difference from the NC group was statistically significant
b: P<0.05, the difference from the BL groups was statistically significant
aa: P<0.05, the difference was statistically significant compared with the 5.0μmol/L GA group
aaa: P<0.05, the difference was statistically significant compared with the 10.0 μmol/L GA group

Figure 3. *Comparison of six levels of AKT protein levels*



a: P < 0.05, the difference from the NC group was statistically significant
b: P<0.05, the difference from the BL groups was statistically significant
aa: P<0.05, the difference was statistically significant compared with the 5.0μmol/L GA group
aaa: P<0.05, the difference was statistically significant compared with the 10.0 μmol/L GA group

Figure 4. *Comparison of six levels of mTOR protein levels*

Discussion

IGFBP7, as a member of the IGFBP superfamily, plays an extremely important role in metabolic processes such as cell differentiation, proliferation and growth. A large number of studies have confirmed that the expression of IGFBP7 is related to the occurrence of various cancers (Wei, 2016; Chen, 2017; Kim, 2018; Akiel, 2017; Benassi, 2015), which is Many tumor suppressor factors. The data shows that IGF can bind to PI3K to produce protein kinase phosphorylation, activate the Akt/mTOR signaling pathway, and thereby promote cell proliferation, while IGFBP7 is an IGF binding protein 7 factor, which inactivates PI3K protein, inhibits Akt/mTOR signaling

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pathway, and produces cell inhibition role (Zhao, 2017; Xu, 2013). The results of this study suggest that the level of expression of IGFBP7 protein in prostate cancer PC-3 cells after treatment with different concentrations of pomegranate extract gallic acid group (5.0 μmol/L group, 10.0 μmol/L group and 20.0 μmol/L group) and IGFBP7 overexpression group was significantly higher than the untreated group, but the corresponding AKT protein and mTOR protein expression levels were significantly lower than the untreated group. This result confirmed that the mechanism of pomegranate extract gallic acid inhibiting the proliferation of prostate cancer PC-3 cells may firstly enhance the expression of IGFBP7 protein, and then weaken the expression levels of AKT protein and mTOR protein, causing negative feedback of AKT/mTOR signaling pathway. It is related to regulation and eventually produces the inhibitory effect of tumor cells. However, whether the regulation of AKT/mTOR signaling pathway by gallic acid from pomegranate extract also blocks PI3K protein expression still needs further verification.

In the process of cell proliferation, differentiation, apoptosis and invasion, the AKT/mTOR signaling pathway plays a key role, and is an important signaling pathway in the process of tumor development (Wang, 2019; Zhao, 2019; Gasparri, 2018; Li, 2019). Data show that in the signal pathway of P13K/Akt/mTOR, the upstream pathway of mTOR, P13K is phosphorylated by AKT protein to activate mTOR gene expression and promote tumor cell apoptosis (Gao, 2016; Johnson, 2018). The results suggest that the apoptosis rate of PC-3 cells treated with IGFBP7 protein expression group and pomegranate extract gallic acid group (40.52 ± 1.76, 15.98 ± 1.89, 30.36 ± 1.76, 39.04 ± 1.43) was significantly higher than the untreated NC and BL groups (4.48 ± 0.45, 5.16 ± 0.38), this result indicates that PC-3 cell apoptosis is related to AKT/mTOR signaling pathway. In addition, the inhibitory effect on prostate cancer PC-3 cells has a significant dose-effect and time-effect relationship with the concentration and action time of pomegranate extract gallic acid. There were also some defects in this paper. Considering that pomegranate gallic acid also has certain toxicity to cells, the concentration is relatively low, and whether 20 μmol/L was its maximum effective concentration remains to be further discussed.

Conclusion

The study found that naturally-derived polyphenolic compounds play an important role in inhibiting the proliferation and migration of tumor cells, but there are certain differences in the reports of their mechanism of action (Wang, 2017; Estrela, 2017; Amani, 2017). This experiment used different concentrations of pomegranate extract gallic acid to treat prostate cancer PC-3 cells. It was found that pomegranate extract gallic acid can inhibit the expression level of AKT/mTOR protein by stimulating the expression of IGFBP7 and inhibit the AKT/mTOR signaling pathway Therefore, it has the effect of inhibiting the proliferation ability of PC-3 cells, and there is a significant dose-effect and time-effect relationship between this action trend and its concentration and action time. Studies have shown that compared with the NC and BL groups, the proliferation of PC-3 cells in the IGFBP7 transfection group and the three groups of pomegranate extract gallic acid was significantly reduced. The proliferative capacity of cells, as for the molecular mechanism of this result needs to be further explored.

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