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Original Research

Carnosol inhibits Hedgehog signaling pathway in both LNCaP and DU145 prostate cancer cell lines

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Abstract: To investigate the effect of carnosol on the Hedgehog (HH) signaling pathway in human hormone-dependent prostate cancer cell line LNCaP and hormone-independent prostate cancer cell line DU145. The expression levels of glioma-associated oncogene homolog 1 (Gli1) and Sonic hedgehog (Shh) in human prostate cancer tissues were detected by immunohistochemistry. After treated with carnosol (0.25-16 μmol/L), the cell survival of LNCaP and DU145 cells were detected by MTT assay. The expression levels of Gli1 and Shh mRNA and protein in the two cells were detected by qRT-PCR and western blot, respectively. The apoptosis was determined by the caspase-3 activity assay. Results showed that Shh and Gli1 were upregulated in cancer tissues. The inhibitory effect of carnosol on cell survival was enhanced with concentration, suggesting both LNCaP and DU145 cells were sensitive to carnosol. The inhibitory effects of carnosol on Gli1 and Shh mRNAs in the hormone-dependent LNCaP prostate cancer cell was stronger than that in the hormone-independent DU145 prostate cancer cells. Carnosol downregulated the expression of Gli1 in nucleus, and Shh in cells. Greater carnosol concentration resulted in lower levels of Gli1 and Shh. Carnosol increased caspase-3 activity in a dose-dependent manner, suggesting that carnosol promotes cell apoptosis. Thus, carnosol can inhibit the proliferation and induce the apoptosis of prostate cancer cells *in vitro*, and its mechanism might be associated with the inhibiting of HH signaling pathway. Although the inhibitory effect of carnosol on hormone-dependent LNCaP prostate cancer cells is stronger than hormone-independent DU145 prostate cancer cells, carnosol might be a potential drug for hormone-independent prostate cancer.

Key words: Carnosol; Prostate cancer; Hedgehog signaling pathway; Gli1; Shh.

Introduction

Prostate cancer is one of the most common malignant tumors in male, and it ranked the first in tumors that endangers the health of men (1, 2). The modulation of prostate cancer from hormone-dependent to non-dependent is a leading cause of recurrence and metastasis of prostate cancer (3, 4). Most patients with prostate cancer will experience the process from hormone-dependent to hormone-independent state (5, 6). There is still lack of effective treatment for the metastasis in those patients with hormone-independent prostate cancer (1, 3, 7).Considering the disadvantages of traditional chemotherapy drugs, such as side effects, poor compliance and poor efficacy, numerous researches have focused on the development of new drugs, especially the low toxicity and effective anticancer drugs for the patients with hormone-independent prostate cancer(1, 7). In recent, it has showed that carnosol serves as an anti-tumor agentin a variety of tumors, such as lung cancer, prostate cancer, colon cancer, ovarian cancer and breast cancer (8-11). Carnosol inhibited the tumor development by affecting the angiogenesis and the metabolism of carcinogenic substances *in vivo*(12-14), but has litter side effects on normal cells, which is of great interests (15-18).

Hedgehog (HH) signaling pathway is involved in the development of various cancers and plays an important role in the invasion and metastasis of cancers (19, 20). Sonic hedgehog (Shh) is a well-studied ligand of the vertebrate pathway. Most of the knowledge about HH signaling has been established by studying Shh signaling (21, 22). Shh is associated with CD138, which also plays important role in metastasis (23, 24).In addition, glioma-associated oncogene homolog 1 (Gli1) acts as a transcription factor of the HH signaling pathway to activate and transcribe most of the target genes that involved in tumor invasion and metastasis(25-28). In the modulation process of prostate cancer from hormone-dependent to non-dependent, the prostate cancer cells become more invasive and metastatic. To improve the diagnosis and treatment of metastatic prostate cancer, the mechanism of modulation of prostate cancer from hormone-dependent to hormone-independent is one of the major problems that need to be identified urgently in clinic.

Thus, we investigated the differences in changes of HH signaling in the treatment of carnosol using hormone-dependent prostate cancer cell line LNCaP and hormone-independent prostate cancer cell line DU145, and detected the cell apoptosis by examining the caspase-3 activity.

Materials and Methods

Clinical data and tissue specimens

A total of 42 patients with prostate cancer who were admitted to the Central Hospital of Dalian from June 2010 to December 2011 were enrolled in this study. The

patients underwent prostate biopsy, transurethral resection of the prostate (TURP) and radical prostatectomy. The age was 61-89 years old, with a median age of 73 years. Patient didn't conduct radiotherapy, chemotherapy and other related treatments before surgery. Pathological grade: Gleason score of 2-4 were 8 cases, 5-7 were 15 cases, 8-9 were 19 cases. TMN staging: T1+T2 were 18 cases, T3+T4 were 24 cases. The control group was prostatic hyperplasia and adjacent prostate normal tissues. Ethics approval for this project was granted from the medicine ethics committee of affiliated hospital of Dali Medical University and all patients signed written informed consent.

Immunohistochemistry

Fresh cancer and adjacent normal tissues, and prostatic hyperplasia tissues were immersed and fixed in 10% neutral formaldehyde for 24h.Sections of 4 μm thickness were cut from each sample using Hemo-De (Fisher, USA), followed by an alcohol series and washing in PBS. The slides were then incubated in 3% H_2O_2 for 15 min to block peroxidase, and antigens were retrieved by boiling the slides in a microwave oven in 50 mM citrate buffer. After blocking with goat serum, the slides were incubated with primary anti-Gli1 and Shh (1:200, Sigma, USA), and then counterstained with hematoxylin. Three random fields were captured for each tumor section at 100× magnification.

Cell culture

The LNCaP and DU145 cells were purchased from ATCC, USA. Cells were cultured in DMEM medium (Gibco, USA) containing 10% fetal bovine serum, 0.1% penicillin and streptomycin and maintained at 37°C, 5% CO_2. The cells in logarithmic growth phase were used in the following experiments. Carnosol (Cayman, USA) was diluted to indicated concentration in 10%DMSO. The medium and DMSO were used as controls.

Cell survival

Cells were seeded in 96 well-plate at 1×10^4 /well and cultured for 24h. Then, 0.25, 0.5, 1, 2, 4, 8, and 16 μmol/L carnosol (in medium with 2% fetal bovine serum) were used to treat cells. After 24h, 20 μL MTT (5 mg/mL) were added. After 4h, add 150 μL DMSO and shook for 15 min to solubilize the internalized MTT. The absorbance of the released dye was detected at 490 nm. Cell survival rate was calculated as ratio of sample emission and control group. The experiment was repeated 3 times and averaged.

qRT-PCR

Total RNA from cells were extracted by Trizol (Invitrogen, USA) and dissolved in 20μL DEPC treated water. The A260/A280 ratio was determined by ultraviolet spectrophotometer. The concentration of RNA was determined by GeneQuant II (Pharmacia, Sweden) and mRNA was reverse transcribed using a Revert Aid First-Strand cDNA Synthesis Kit (Thermo, USA) according to the manufacturer's instructions. Then, qRT-PCR was performed with SYBR Green Mix (Thermo, USA) ON A LightCycler 480II (Roche). The primers used were: Shh 5'-GGAGTGAAACTGCGGGTGA-3', and 5'-GCGGTCCAGGAAAGTGAGG-3'; Gli1 5'-TTCCTACCAGAGTCCCAAGT-3', and 5'-CCCTATGTGAAGCCCTATTT-3'; β-actin 5'-AGCGAGCATCCCCCAAAGTT-3', and 5'-GGGCACGAGGGCTCATCATT-3'. The reaction conditions were as follows: 94°C for 5 min, 94°C for 30s, 55°C for 30s, 72°C for 30s, 30 cycles and then at 72°C for 7min. The data were normalized to that of β-actin. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ methods.

Western blot

Total protein was extracted using RIPA protein lysis buffer (Beyotime, China) with freshly added 1% protease inhibitor cocktail and 1mM PMSF. Cell fractions were prepared using a Nuclear Protein Extraction Kit (Pierce, IL) according to the manufacture's protocol. In total, 50 μg proteins were separated by SDS-PAGE and transferred onto PVDF membranes. After rinse, blocked in 5% fat-free milk solution for 2h, the PVDF blots were incubated with primary antibodies against Gli1 and Shh (1:500, Sigma, USA) overnight, 4°C and then with HRP-conjugated secondary antibody for 1 h. The β-actin was set as control. Reactive bands wre visualized with ECL reagent (Pierce, IL) and analyzed.

Caspase-3 activity

The cells were collected and the caspase-3 activities were detected by using caspase-3 activity assay kit (Clontech, USA) according to the instructions. The fluorescence values of enzyme-catalyzed cleavage reaction of substance were detected using the HTS700 fluorescence photometer (PE, USA)at 360/465 nm.

Statistical analysis

SPSS 17.0 statistical software was used for the statistical analyses. The significance was evaluated by using repeated-measures one-way ANOVA or a two-tailed Student's *t*-test. Data was shown in Mean ± SD. $P<0.05$ was considered significantly difference.

Results

Levels of Shh and Gli1 were up-regualted in prostate cancer tissues

There were significant differences in expression levels of Shh and Gli1 between prostate cancer and adjacent normal tissues (Figure 1). The Shh positive cells showed brown granules in the cell membrane and cyto-

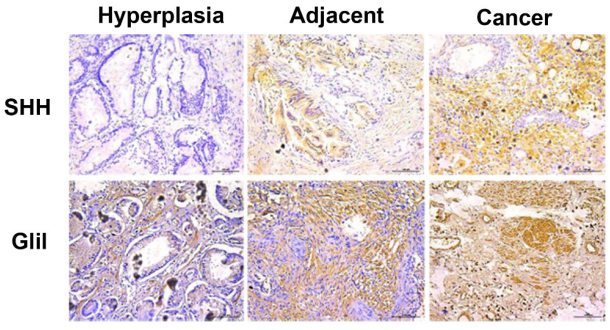

Figure 1. Levels of Shh and Gli1 in prostate cancer and adjacent normal tissues. IHC staining of Shh and Gli1 were performed in both prostate cancer and adjacent normal tissues. The tissues from patients with prostatic hyperplasia were used as control. (A) Shh; (B) Gli1.

plasm. The Gli1-positive cells showed brown granules in the cytoplasm and nucleus. The levels of Gli1 and Shh were lower in adjacent normal tissues than in cancer tissues, although big difference between Gli1 and Shh in the cancer tissues. In the hyperplasia tissues, the levels of Shh and Gli1 were lower than those in the cancer tissues. Thus, Shh and Gli1 were upregulated in cancer tissues.

Carnosol inhibits cell survival of LNCaP and DU145

MTT assay showed that cell growth was inhibited by carnosol for 24h (Figure 2). The inhibitory effect of carnosol on cell survival was enhanced with concentration in LNCaP and DU145 cells, suggesting both LNCaP and DU145 cells were sensitive to carnosol.

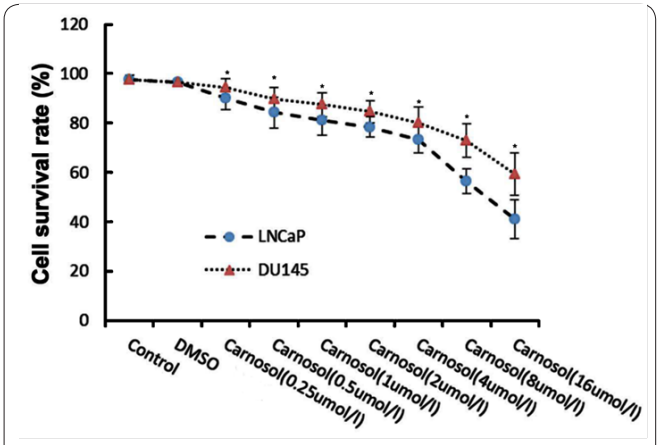

Figure 2. Effect of carnosol on cell survival of LNCaP and DU145 cells. After treated with different concentrations of carnosol for 24 h, the cell survivals were detected by MTT assay. **P*<0.05 vs. DU145 vs. LNCaP.

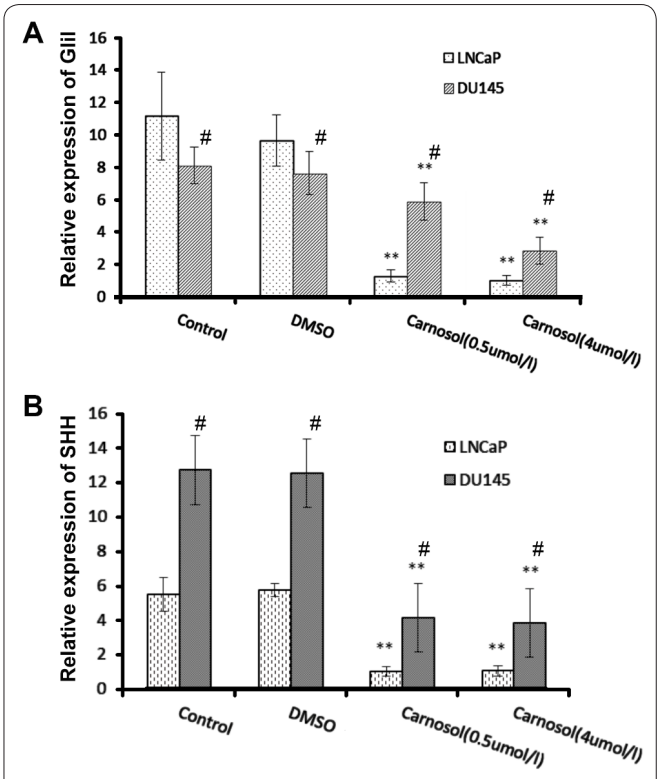

Figure 3. Effect of carnosol on mRNA expression levelsof Gli1 and Shh in LNCaP and DU145 prostate cancer cells**.** After treated with different concentrations of carnosol for 24 h, the mRNA levels of Gli1 (A) and Shh (B) in cells were detected, respectively. ***P*<0.01 vs. control; # *P*<0.05 vs. DU145 cells.

Carnosol inhibits mRNA expressionof Gli1 and Shh

Carnosol down-regulates mRNA levels of Gli1 and Shh in LNCaP and DU145 cells**,** respectively (Figure 3). The inhibitory effect of carnosol on transcription of Gli1 and Shh in the hormone-dependent LNCaP prostate cancer cell was stronger than that in the hormone-independent DU145 prostate cancer cells. Thus, carnosol inhibits the mRNA expression of Gli1 and Shh.

Carnosol inhibits the protein levels of Gli1 and Shh in LNCaP and DU145 prostate cancer cells

After treated with different concentrations of carnosol (0, 0.5 and 4μmol/L) for 24 h, the protein levels of Gli1 and Shh in nucleus and cells were detected by Western blot, respectively (Figure 4).Results showed that carnosol downregulated the expression of Gli1 in nucleus, and that of Shh in cellular. Greater the carnosol concentration, lower levels of Gli1 and Shh. Thus, carnosol inhibited the expression of Gli1 and Shh.

Carnosol promoted caspase-3 activity of prostate cancer cells

The activity of caspase-3 in LNCaP and DU145 cells was significantly increased by carnosol after 24h (Figure 5). The caspase-3 activity in cells treated with 4 μmol/L carnosol were significantly greater than that treated with 0.5 μmol/L. Thus, carnosol promoted caspase-3 activity with concentration. It was suggested that carnosol promotes cell apoptosis.

Discussion

Carnosol is a core component of Rosmarinus extract, which has obvious anticancer activity against a variety of cancers with no obvious side effects on normal cells (11, 13, 14). The application value of carnosol is one of the hot spots of clinical concern (10, 11). The present study was conducted to investigate whether there are differences in HH signaling pathway between hormone-dependent and hormone-independent prostate cancer cells in the treatment of carnosol. We found that carnosol inhibited the HH signaling pathway in both

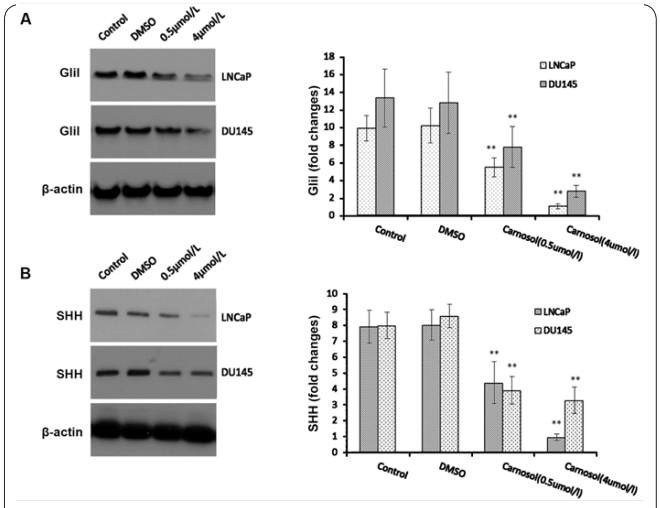

Figure 4. Effect of carnosol on protein expression levels of Gli1 and Shh in LNCaP and DU145 prostate cancer cells. After treated with different concentrations of carnosol (0, 0.5, 4μmol/L) for 24 h, the protein expression levels of Gli1 (A) and Shh (B) in LNCaP and DU145 cells were detected by Western blot. ***P*<0.01 vs. control.

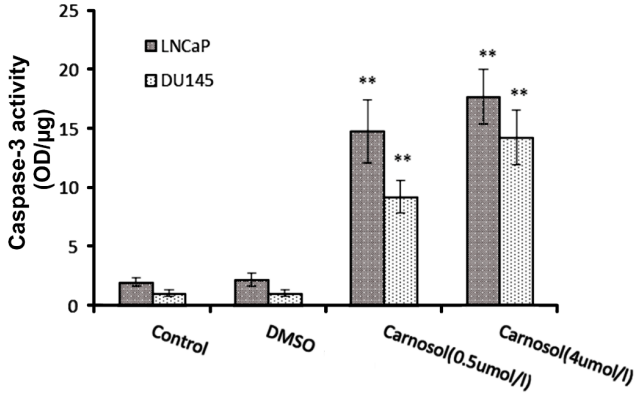

Figure 5. Effect of carnosol on caspase-3 activity in LNCaP and DU145 prostate cancer cells. After treated with different concentrations of carnosol (0, 0.5, 4μmol/L) for 24 h, the caspase-3 activity in the LNCaP and DU145 prostate cancer cells were detected. **P*<0.01 vs. control.

hormone-dependent and hormone-independent prostate cancer cells, and promoted the cell apoptosis, which can provide a novel evidence for treatment of prostate cancer by carnosol.

In the modulation process of prostate cancer from hormone-dependent to hormone-independent, HH signaling plays important roles (29-31). Gli1 acts as a transcription factor of the HH signaling pathway to activate and transcribe most of the target genes of the HH signaling pathway (32, 33). By detecting the expression of Gli1 and Shh in prostate cancer tissues, we found that Gli1 and Shh were highly expressed in the cancer tissues, and lowly expressed in the adjacent normal tissues, indicating the activation of HH signaling pathway in prostate cancer, and might associated with the modulation of prostate cancer from hormone-dependent to hormone-independent. To further investigate the different in HH signaling pathway between hormone-dependent and hormone-independent prostate cancer, and whether cells will modulate from hormone-dependent to hormone-independent in the treatment of carnosol, two cell lines including the hormone-dependent cell line LNCaP and the hormone-independent cell line DU145 were treated with carnosol.

Shh is the key HH signaling pathway that affects the biological characteristics of cancer cells. We examined the Shh expression in both hormone-dependent and hormone-independent prostate cancer cells in the treatment of carnosol. The carnosol concentration used in different researches were not the same. In the present study, we tested the effect of carnosol on cell survival with different concentrations firstly. By treating with 0, 0.25, 0.5, 1, 2, 4, 8, 16 μmol/L carnosol for 24 h, cell survival in LNCaP and DU145 were significantly inhibited. Greater the concentration, greater the inhibitory effects. It was suggested both the hormone-dependent cell line LNCaP and the hormone-independent cell line DU145 are sensitive to the carnosol. For further investigation on the expression of Shh and Gli1, 0.5 and 4 μmol/L were used to treat the cells.

The expression of Shh and Gli1 in both mRNA and protein levels were detected in this study. We found carnosol downregulated the expression of Gli1 mRNA and the Gli1 in the nucleus. Also, Shh in mRNA and protein levels were downregulated, suggesting the activity of HH signaling pathway were inhibited by carnosol. The inhibitory effect of carnosol on HH signaling pathway in hormone-dependent prostate cancer cell LNCaP was stronger than the hormone-independent prostate cancer cell DU145. This is consistent with the clinically observation that hormone-independent prostate cancer prone to metastasis, with higher degree of malignant. In the presence of Shh, Gli-1 is transcriptionally activate, and hedgehog/Gli-1 signaling pathways were involved in the inhibitory effect of resveratrol on human colorectal cancer HCT116 cells(34). Shh significantly inhibited cell apoptosis in human colorectal cancer HCT116 cells(34).It can be concluded that carnosol may play an anti-tumor effect by inhibiting the activity of HH signaling pathway, thereby inhibiting its proliferation and inducing its apoptosis, which might be one of the important mechanisms of carnosol against prostate cancer.

In conclusion, carnosol can inhibit the proliferation and induce apoptosis of prostate cancer cells *in vitro* and its mechanism might be associated with the inhibiting of HH signaling pathway. Whether Shh and Glil responsible for the cell apoptosis induced by carnosol needs to further studied. Moreover, the inhibitory effect of carnosol on hormone-dependent LNCaP prostate cancer cells is stronger than hormone-independent DU145 prostate cancer cells, but its specific mechanism needs to be further studied.

Compliance with Ethical Standards

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were carried out after obtaining approval from the Dalian Central Hospital Ethics Committee, and were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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