

The effect of beta-elemene on alpha-tubulin polymerization in human hepatoma HepG2 cells

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Objective: To investigate the impact of beta-elemene injection on the growth and alpha-tubule of human hepatocarcinoma HepG2 cells.

Methods: Cell proliferation was assessed by MTT assay. Cell cycle distribution was detected by flow cytometry (FCM). The mRNA expression of alpha-tubulin was measured by RT-PCR. Western blot analysis was used to determine protein expression of alpha-tubulin and the polymerization of tubulin.

Results: Beta-elemene injection inhibited HepG2 cells proliferation in a dose- and time-dependent manner; FCM analysis indicated beta-elemene injection induced cell cycle arrested at S phase. RT-PCR and western-blot analysis showed that beta-elemene injection down-regulated alpha-tubulin at both mRNA and protein levels, presenting a dose-dependent manner. Moreover, beta-elemene injection reduced the polymerization of microtubules in a dose-dependent manner.

Conclusions: Beta-elemene injection can inhibit the proliferation of hepatoma HepG2 cells and induce cell apoptosis, the mechanism might be partly related to the down-regulation of alpha-tubulin and inhibition of microtubular polymerization.

Keywords: Alpha-tubulin; beta-elemene injection; hepatocellular carcinoma (HCC); microtubules; tubulin polymerization



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Hepatocellular carcinoma (HCC) is a severe life threatening disease as the fifth most common cancer worldwide (1,2). It belongs to chemotherapy nonsensitive cancer, clinical reports show that the benefits of both chemotherapy and target antitumor drugs for HCC are not satisfactory (3-6). Therefore searching for new effective agents against hepatoma is anxiously expected.

Beta-elemene is a non-cytotoxic antitumor drug, which is extracted from a traditional Chinese medical herb *Rhizoma zedoariae*. It has been clinically used in China for decades. Clinical data shows it can effectively inhibit the growth of various tumors without severe side effects, especially for HCC (7-10). Studies show that beta-elemene can kill tumor cells by inducing apoptosis of tumor cells, blocking the cell

cycle and inhibiting signal transduction (10-16). However, the relationship between beta-elemene and microtubule is rarely reported.

Microtubule, polymerized by alpha-tubulin and beta-tubulin, is an important component of the cytoskeleton. It plays an important role in cell growth, maintenance of cell shape, cell signal transduction and mitotic processes etc., and acts as an essential tumor target. The important anti-tumor drugs, such as paclitaxel, docetaxel, and vincristine, inhibit cancer growth by promoting the over-dimerization or over-destabilization of microtubule in the cells (17,18).

This study is designed to investigate the impact of beta-elemene injection on the growth and alpha-tubulin of human hepatocarcinoma HepG2 cells, and at the same

time discussed the inhibiting mechanism of beta-elemene injection on HCC.

Materials and methods

Agents and implements

Beta-elemene injection (Dalian Jingang Pharmaceutical Corporation, China, more than 99%, #0508031), MTT (Biosharp Company, USA), dimethyl sulfoxide (DMSO) (Amersco Company, USA), RT-PCR kit (TaKaRa Company, Japan), alpha-tubulin mouse anti-human monoclonal antibody (Biyuntian Biotechnology Research Institute), horseradish peroxidase-labeled Goat anti-Mouse IgG (Beijing Zhongshan Jinqiao biological Corporation), PVDF membrane.

Cell lines and culture conditions

Human hepatoma HepG-2 cells (Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured with IMDM medium, supplemented with 10% (v/v) fetal calf serum (FCS). The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂.

MTT assay for cell proliferation

5,000 cells/well were seeded in 96-well plates. After 24 h of incubation, the treated group were added to beta-elemene injection with different concentrations (0.1, 0.08, 0.06, 0.04, 0.02, 0.01 mg/mL perspective). After incubation for 24, 48, 72 h, 20 μL of MTT dye solution (5 g/mL, in phosphate buffer pH 7.4) was added to each well and incubated at 37 °C for 4 h. The medium was removed and formazan was dissolved in 150 μL of DMSO and the absorbance (A) at 570 nm of each group was measured by enzyme-labeled instrument. Results were expressed as the mean percentage of tumor growth inhibition rate (IR) and half inhibition concentration (IC₅₀). IR% = (A_{control} - A_{treated})/(A_{control} - A_{blank}) × 100%. The experiments were repeated three times.

Flow cytometry (FCM) analysis for tumor cell cycle distribution

A total of 2×10⁶ HepG-2 cells were seeded and added to beta-elemene injection with different concentrations (0.02, 0.04, 0.08 mg/mL) for 24 h. Then they were harvested, washed and fixed in 70% ice cold ethanol at 4 °C overnight. Afterwards, cells were washed twice and resuspended in

1 mL of propidium iodide (PI) stain solution (1 μg/mL PI, 1% TritonX-100 in PBS) containing 2 mg/mL RNase A at 37 °C in the dark for 30 min. The cells were analyzed and fluorescence detected immediately by FCM of their apoptosis and cell cycle (excitation wave 562 nm, emission wave 630 nm). The experiments were repeated three times.

RT-PCR detection of alpha-tubulin mRNA expression

Cells in the exponential growth phase (5×10⁵ cells/well) were seeded in 24-well plates, and cultured for 24 h. Beta-elemene injection with different concentrations (0.02, 0.04, 0.08 mg/mL) were added to the treated group. After cultured for 24 h, the cells were collected. Total RNA was isolated from cells using Trizol (Invitrogen Company, USA). RT-PCR was performed following the instruction of TaKaRa RNA PCR Kit (AMV) Ver3.0. Primer pairs used for alpha-tubulin (158 bp) were 5'-ATGGAGCCCTGAATGTTGAC-3', 5'-CTCAAAGCAAGCATTGGTGA-3'. As to GAPDH (226 bp), its Primer pairs were 5'-GAAGGTGAAGGTCGGAGT-3', 5'-GAAGATGGTGATGGGATTTC-3', PCR reaction conditions were as follows: initial denaturation temperature of 95 °C for 5 min, denaturation temperature of 95 °C for 30 s, annealing temperature of 60 °C 30 s, extension temperature of 72 °C for 30 s (cycling 30 times).

The amplified products were analyzed by agarose gel electrophoresis using 1% gel with ethidium bromide staining. The bands were analyzed with Jim-250. The experiments were repeated three times.

Western Blot analysis for alpha-tubulin protein expression

HepG-2 cells were treated with different concentrations of beta-elemene injection (0.02, 0.04, 0.08 mg/mL) for 24 h before they were collected and washed. Then the treated groups were added with 200 μL lysis buffer [1.5 M Tris-HCl (pH 6.8) 41.5 μL/L, 20% SDS 100 μL/L, glycerine 100 μL/L, dithiothreitol 0.0155 g/L, bromophenol blue 0.000125 g/L] on the ice for 10 s. After collected and boiled in water at 100 °C for 7 min, the content of the total protein was determined using the method of Bradford. Equal amounts of protein (50 μg/hole) were subjected to SDS-PAGE on 8-12% separation gel for electrophoresis. Afterwards, the proteins were electrophoretically transferred to PVDF membranes, blocked at room temperature for 1 h, and then treated with

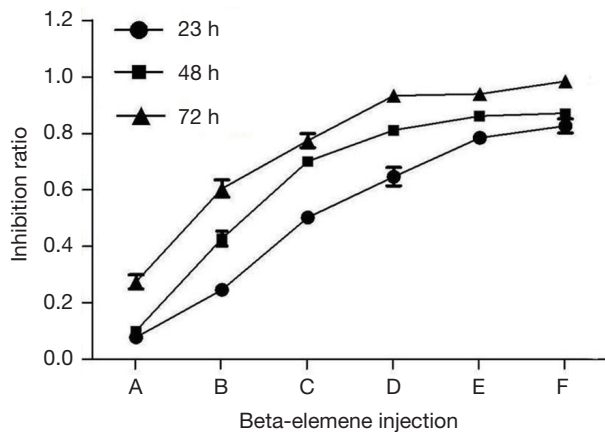


Figure 1 Time- and dose-dependent effect of beta-lemene injection on the inhibition rate (IR) of human hepatoma HepG-2. For the IR assay, HepG-2 cells were seeded in 96-well plates at 5,000 cells/well and added to beta-lemene injection with different concentrations. The cell proliferation after the indicated amounts of time was determined by MTT assay. The data are presented as the means \pm SD of three independent experiments.

alpha-tubulin mouse anti-Human monoclonal antibody overnight. After washed by PBS/T, the membranes were incubated in horseradish peroxidase-labeled goat antimouse IgG (1:5000) at room temperature for 1h, and then treated with the ECL reagent. Finally, X-ray films were exposed for 1-5 min, developed, dried, scanned and photographed. The experiments were repeated three times.

Detection of polymerized microtubules

The proportion of polymerized of microtubules was detected based on the protocol as previously described (19). HepG-2 cells were treated with different doses of beta-lemene injection (0.02, 0.04, 0.08 mg/mL) for 24 h before they were collected and washed. Then the treated groups were added with 200 μ L hypotonic lysis buffer (MgCl₂ 1 mmol/L, EGTA 2 mmol/L, 0.5% NP40, PMSF 2 mmol/L, 10 μ L of protease inhibitor, Tris-HCl (pH 6.8) 20 mmol/L) at 37 °C in dark for 10 min. After centrifuged at 14,000 r/min for 10 min. The supernatant and the precipitate were collected respectively. The supernatant containing soluble tubulin, while the precipitate was resuspended with 100 μ L hypotonic buffer, this portion of the supernatant containing the original polymerized tubulin. Both the polymerized tubulin and soluble tubulin

were detected at protein level, using Western blot analysis with the former (Considering tubulin protein content decreased because of drug effects, we cautiously increased the amount of protein samples in drug treatment groups).

Statistical analysis

Statistical analysis was performed using SPSS14.0 statistical software. All experimental data are presented as mean \pm SD. The data between multiple groups was compared by single-factor analysis of variance, and $P < 0.05$ was considered statistically significant.

Results

Beta-lemene inhibited HepG2 cells proliferation

MTT assay showed that after 24, 48, 72 h interference of beta-lemene injection with different concentrations, IR of beta-lemene injection on HepG2 cells were increased when interference time and drug concentration increased, illustrating time- and dose-dependent ways (Figure 1).

Flow cytometry analysis of tumor cell cycle distribution

After treated by beta-lemene injection with different concentrations (0.02, 0.04, 0.08 mg/mL) for 24 h, HepG-2 cells were analyzed by FCM. The rate of HepG-2 cells at S phase increased when interference dose of beta-lemene injection increased (Figure 2), significantly higher than that of the control group ($P < 0.05$), illustrating a dose-dependent way (Table 1).

Detection of alpha-tubulin mRNA expression by RT-PCR

After 24 h interference of beta-lemene injection with different concentrations, the relative mRNA level of alpha-tubulin was determined by RT-PCR. Corresponding to beta-lemene injection with 0.02, 0.04, 0.08 mg/mL respectively, the relative alpha-tubulin mRNA level (alpha-tubulin mRNA/GAPDH mRNA) were 0.746 ± 0.018 , 0.456 ± 0.035 , 0.274 ± 0.025 , significantly lower than that of the control group (0.923 ± 0.009 , $P < 0.05$), illustrating a dose-dependent way (Figure 3).

Detection of alpha-tubulin protein expression by Western Blot

After 24 h interference of beta-lemene injection with

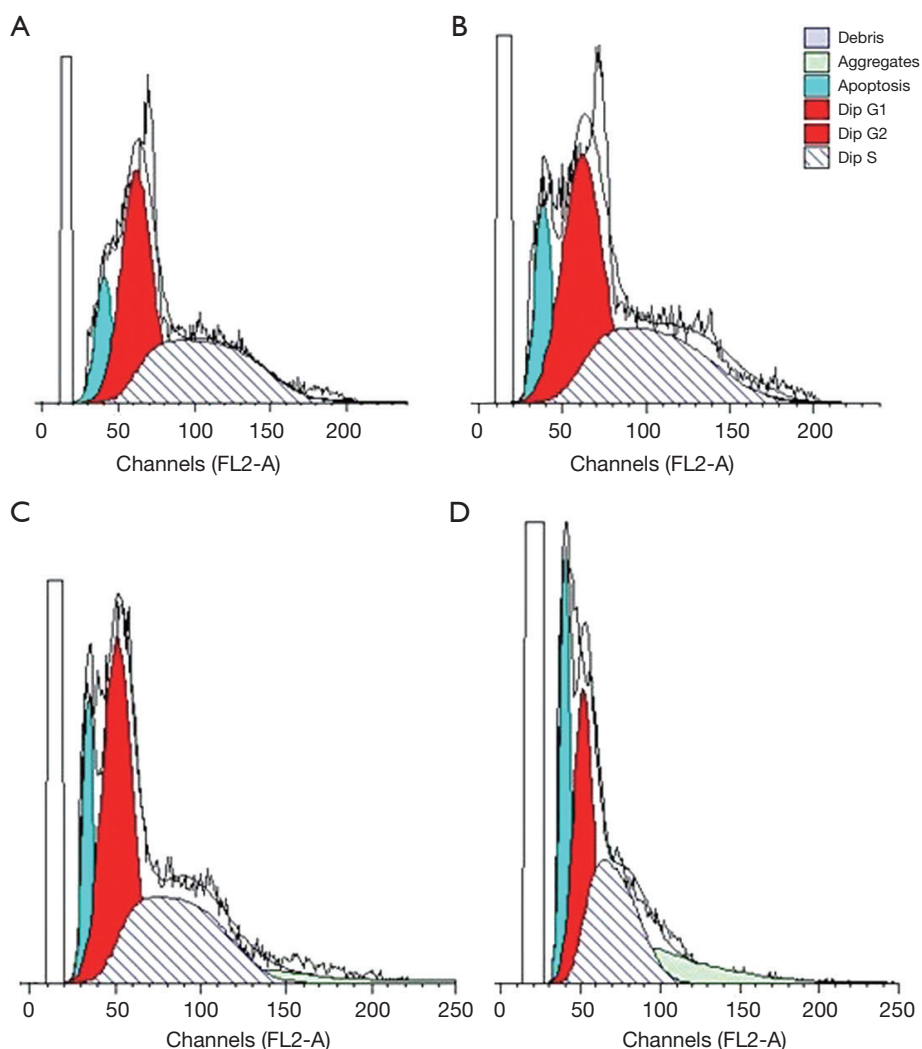


Figure 2 Cell cycle distribution of human hepatoma HepG-2 cells treated by beta-elemene injection. Cells were analyzed by FCM after added to beta-elemene injection with different concentrations for 24 h.

Table 1 Cell cycle distribution of human hepatoma HepG-2 treated by beta-elemene injection

	Control (%)	Beta-elemene 0.02 mg/mL (%)	Beta-elemene 0.04 mg/mL (%)	Beta-elemene 0.08 mg/mL (%)
G1	39.74	38.53	40.58	45.62
S	30.14	39.23*	44.18*	52.89*
G2/M	30.12	22.24	15.24	1.49

The data are obtained from three representative results. *P<0.05 when compared to negative control group.

different concentrations, the relative protein level of alpha-tubulin was detected by Western Blot. Corresponding to beta-elemene injection with 0.02, 0.04, 0.08 mg/mL respectively, the relative alpha-tubulin protein level (alpha-

tubulin protein/GAPDH protein) were 0.575 ± 0.006 , 0.286 ± 0.014 , 0.224 ± 0.015 , significantly lower than that of the control group (0.710 ± 0.010 , $P < 0.05$), illustrating a dose-dependent way (Figure 4).

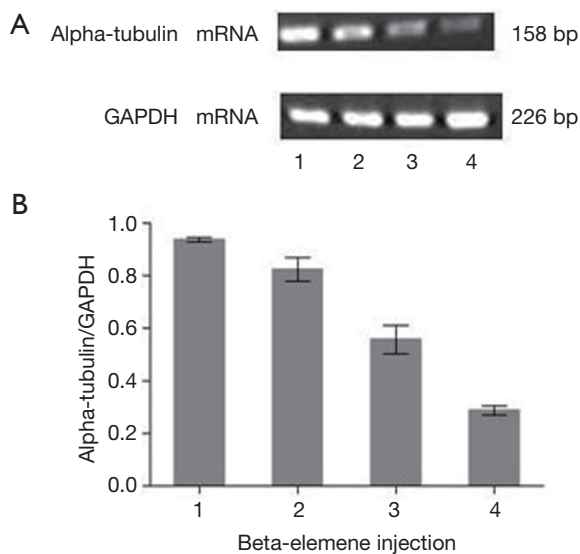


Figure 3 Detection of alpha-tubulin mRNA in human hepatoma HepG-2 cells. (A,B) Cells were treated by beta-lemene injection with different concentrations for 24 h. The expression levels of alpha-tubulin mRNA were detected by RT-PCR and semi-quantitative analysis. Data are shown as the means \pm SD of three independent experiments. * $P < 0.05$ when compared to negative control group.

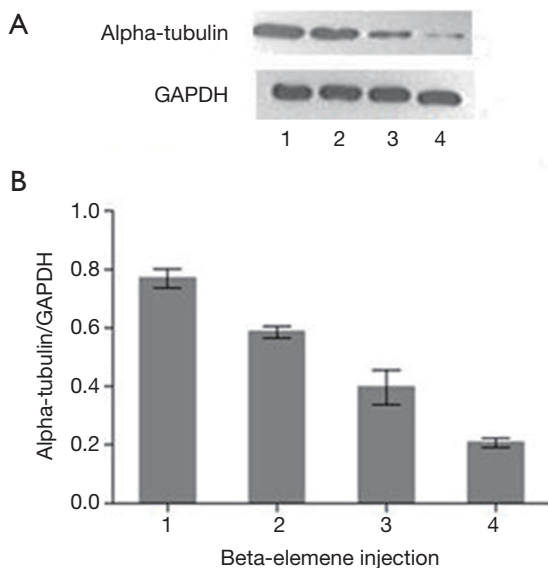


Figure 4 Detection of alpha-tubulin protein expression in human hepatoma HepG-2 cells. (A,B) Cells were treated by beta-lemene injection with different doses for 24 h. The expression levels of alpha-tubulin protein were detected by Western-blot and semi-quantitative analysis. Data are shown as the means \pm SD of three independent experiments. * $P < 0.05$ when compared to negative control group.

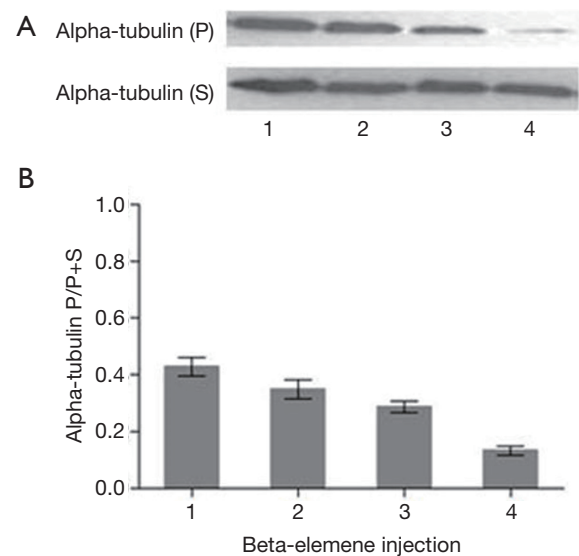


Figure 5 Analysis of polymerized alpha-tubulin protein expression in human hepatoma HepG-2 cells. (A,B) Cells were treated by beta-lemene injection with different doses for 24 h. The expression levels of polymerized alpha-tubulin protein (P) and soluble alpha-tubulin protein (S) were detected by Western-blot and semi-quantitative analysis. Data are shown as the means \pm SD of three independent experiments. * $P < 0.05$ when compared to negative control group.

Analysis of polymerized microtubules by Western blot

After 24 h interference of beta-lemene injection with different concentrations, both the relative polymerized tubulin and soluble tubulin were detected at protein level by Western Blot. With the concentration of beta-lemene injection increasing, the relative ratio of polymerized tubulin to total tubulin (polymerized tubulin + soluble tubulin) gradually decreased (In the detection of microtubules, considering tubulin protein content decreased because of drug effects, we cautiously increased the amount of protein samples in drug treatment groups. As a result, it looked like that the total soluble alpha-tubulin protein level did not change too much). The treated group was significantly lower than that of the control group ($P < 0.05$; Figure 5).

Discussion

Nowadays, drugs for HCC are far below satisfactory. Clinical reports show that the partial response (PR) of hepatoma to chemotherapy is less than 20% up to now (3,4). Also, as the most promising target antitumor drug for HCC,

sorafenib only shows approximate three months survival advantage over the untreated group (5,6). Therefore, drugs with new mechanism are anxiously expected in HCC therapy.

Beta-elemene exhibits anticancer effects in various human tumors such as HCC, ovarian cancer and cervical cancer without severe side effects (20-23). No bone marrow suppression has been observed; on the contrary, patient immunity was improved (24). Study show that beta-elemene and cisplatin may be a potential combinatorial therapy to circumvent cisplatin drug resistance in patients with HCC, ovarian cancer and other malignancies (10).

Current studies show that, the antitumor mechanisms of beta-elemene involve apoptosis induce, cell cycle arrest, signal transduction interference, tumor angiogenesis and metastasis inhibition (10-16). However, the impact of beta-elemene on tumor microtubule system is rarely reported.

In this study, we showed that beta-elemene injection can inhibit human hepatoma HepG2 cells growth, induce cell apoptosis, and induce cell cycle arrested at S phase. It can also down-regulated alpha-tubulin at both mRNA and protein levels, presenting a dose-dependent manner.

Microtubule is an essential component of the cytoskeleton. It is involved in multiple processes such as cell growth, maintenance of cell shape, cell signal transduction and mitotic processes. Interfering with microtubule polymerization and decomposition is an important mechanism for tumor drug therapy. Some tumor drugs, such as paclitaxel and vincristine, kill carcinoma cells by Interrupting tubulin synthesis and induce cell cycle arrested at G2/M phase (10,11). There are also compounds, such as cyclic ethyl ester series derivatives, natural products Artelastin and beta-Valley sterol ect., inhibit microtubule assembly and induce cell cycle arrested at S phase (25-28), which is consistent with our conclusion. The researchers have explained the S phase-arrested mechanism of the compounds on tumor as follows: drugs interfere the combination of mitochondria with microtubules, and then interrupt ATP/ADP conversion and energy supply, and further cause tumor cells arrested at S phase. However, the mechanism of beta-elemene injection inducing HCC arrested at S phase needs further research.

In summary, our study suggested beta-elemene injection can inhibit the proliferation of hepatoma HepG2 cells and induce cell apoptosis, the mechanism might be partly related to the down-regulation of alpha-tubulin and inhibition of microtubular polymerization. Beta-elemene is a promising drug on HCC therapy.

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