

Research on effect of minor bupleurum decoction of proliferation and apoptosis of esophageal cancer cell strain eca-109 cell

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Abstract: The research protocol is MTT (Methyl Thiazolyl Tetrazolium) method, Hoechst33342 staining method and flow cytometry detection to observe the effect of minor bupleurum decoction on proliferation inhibition and apoptosis-inducing of esophageal cancer cell strain Eca-109 cell and its purpose is to discuss the effect. The result of MTT method shows that minor bupleurum decoction can obviously inhibit proliferation of esophageal cancer cell strain Eca-109 cell. Apoptosis number of esophageal cancer cell increased with the increase of concentration of tetrandrine by the Hoechst 35528 staining experiment of cancer cell in three different concentrations. Flow cytometry detection result showed that cells in cell cycle G0/G1 of esophageal cancer cell strain Eca-109 cell increased obviously and cell in S period decreased significantly. This research proved that minor bupleurum decoction had anti-tumor effect and can influence proliferation and apoptosis of esophageal cancer cell strain Eca-109 cell.

Keywords: Minor bupleurum decoction; Esophageal cancer; Proliferation and apoptosis.

INTRODUCTION

Esophageal cancer is a kind of common malignant cancer, which accounts for more than 90% of tumors of esophagus. It is second in all malignancy cancer death review investigation and just secondary to stomach cancer (Zeb *et al.*, 2008). Distribution of people who have esophageal cancer is related to age, gender, profession, race, territory, surroundings, diet habit, genetic predisposition, etc. The existing investigation shows that esophageal cancer may be caused by various diseases. The pathogeny is as follows: 1. chemical pathogeny; 2. biological pathogeny; 3. deficiency of some microelement; 4. deficiency of vitamin; 5. smoking, drinking, hot food, hot drink, oral unclean, etc; 6. esophageal cancer genetic predisposition. Treatment of esophageal cancer is operation, radiotherapy and chemotherapy. However, they all have boundedness and shortcomings. The combination of traditional Chinese medicine and modern medicine can draw on each other's strength to improve treatment effect.

Minor bupleurum decoction comes from Treatise on Febrile and Miscellaneous Diseases of *Zhongjin Z* from Eastern Han, which is the famous representative prescription of traditional Chinese medicine and *Shaoyang X*. It is composed of bupleurum, scutellaria baicalensis, licorice, rhizoma pinellinae praeparata, ginseng, ginger and Chinese date (Xudong *et al.*, 2005). This prescription has a wide clinical application and can be applied in curing alternating episodes of chills and fever and noncausal periodic fever. It can also cure gastrointestinal stagnation of Qi, diet accumulation and

hypochondriac pain and epigastric oppression caused by chills and fever. Someone use it for hepatitis vertigo and diabetes abdominal distention in modern times. In recent ten years, the effect of this prescription on cancer treatment is more and more obvious, which bring hope for patients (Ying, 2012). This research aimed to study the effect of minor bupleurum decoction on esophageal cancer cell strain Eca-109 cell and clinical practice.

MATERIALS AND METHODS

Materials

Cells

Esophageal cancer cell strain eca-109 cell.

Primary reagents

Minor bupleurum decoction: 12g bupleurum 9g scutellaria baicalensis, 9g ginger. 6g ginseng, 6g honey-fried licorice root, 4 Chinese dates. Add 800ml water and mix the liquid. It was heated to condensed to 400ml. Remove the sediment. And heat it to 1gdrug/ml.

Instruments

Incubator, ELISA (Enzyme-Linked Immuno Sorbent Assay) instrument, high speed centrifuge, ordinary centrifuge, DK-8D electric heating thermostatic water tank (Shanghai Jinhong Laboratory Equipment Co., Ltd.), UNO- II Gene Amp PCR (Polymerase Chain Reaction) System (BIOMETRA Co.), DYY- III TB horizontal electrophoresis (American BIORAD), DYY-8B steady voltage and current electrophoresis apparatus (Nanjing Kege Biological Science and Technology Co., Ltd), flow cytometry (American Guave), water-jacket heating thermostat (Shanghai Daping Instrument Co., Ltd),

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oscillating mixer (German Heidolph), constant temperature oven (German Memmert), etc.

Methods

Cell culture

Microtubes of esophageal cancer Eca-109 strains was picked out from liquid nitrogen and placed into 37°C water bath for rapid thaw. Then cells were regularly cultured in RPMI 1640 nutrient solution containing 10% inactivated newborn bovine serum, 100U/ml streptomycin and 100U/ml penicillin. Then it was placed into 3°C and 5% CO₂ incubator. Observe the situation of cell wall and change nutrient solution every 3 days. Subculture when cells covered culture bottles to 80%-90%. The culture solution should be absorbed in subculture and washed for one time by PBS (Phosphate Buffer Solution). Then mixed digestive juice of 0.25% pancreatin and 0.02% EDTA (Ethylene Diamine Tetraacetic Acid) was added to cover the bottom of the bottle. The digestion process was conducted for 1 to 2 min. The cells were observed under microscope. When the cells turned to be round, then culture solution was added to terminate the digestion and straw was used to blow and beat cell lightly for complete separation of adherence. Cell suspension was moved into centrifuge tube for 5min centrifuge in 1000rpm. Supernatant was abandoned and culture solution was added for suspension cell again and subculture. At last, minor bupleurum decoction was respectively prepared into concentration of 0.5mg/ml, 1.0mg/ml, 1.5mg/ml and 2.0mg/ml for experiment. All experiment selected cells are in logarithmic phase.

Cell proliferation survival rate detection by MTT method

First, esophageal cancer cell strain Eca-109 cell was digested by pancreatin and centrifuged. Then it was put into medium with 10% serum to adjust cell density to 5×10⁴/L. Then it was inoculated in 96-hole board. There were 5×10³ cells in every hole. The board was then placed in condition of 37°C, 5% CO₂ and saturation humidity for 24 h. Medicine of different concentration was added after adherence of cells. Then the 96-hole board was taken out. The medium was absorbed and abandoned. Then 20µl of 5mg/ml MTT (Methyl Thiazolyl Tetrazolium) solution was added into every hole for 4 h of cultivation. Then 150µl of DMSO (Dimethylsulfoxide) was added for 10 min of shocking. OD value of every hole was detected by microplate reader under 490 nm after full dissolution of crystal. At last, the proliferation survival rate of cells in groups was calculated according to survival rate (%) = (experimental group OD value - blank control group OD value) / (control group OD value - blank control group OD value) × 100%, inhibition rate (%) = 100% - survival rate.

Cellular morphology observation by fluorescence microscope

Esophageal cancer cell strain Eca-109 processed by

different concentration of minor bupleurum decoction was placed in cover glass and fixed with 95% ethyl alcohol for 10 min. Then stationary liquid was absorbed was abandoned. 0.5 ml Hoechst33342 staining fluid was added for staining away from light for 5 min. Then staining liquid was absorbed and abandoned. A drop of anti-fluorescence quenching mounting solution was dropped on cover glass. A cover glass with cells was covered on it so that cells can contact mounting solution and bubble should be avoided as possible. Then we observed cellular morphology under fluorescence microscope.

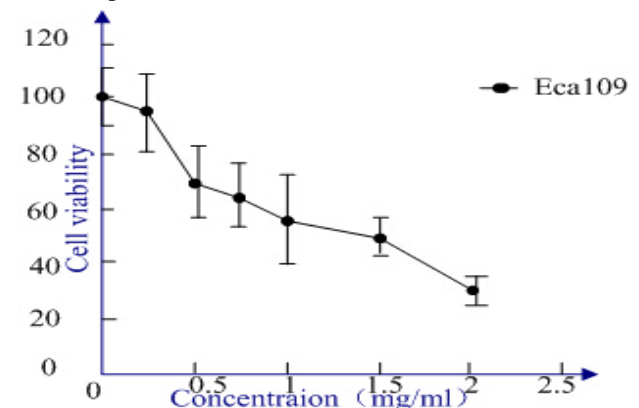


Fig. 1: 24 h processing of minor bupleurum decoction of different concentration on esophageal cancer cell strain Eca-109

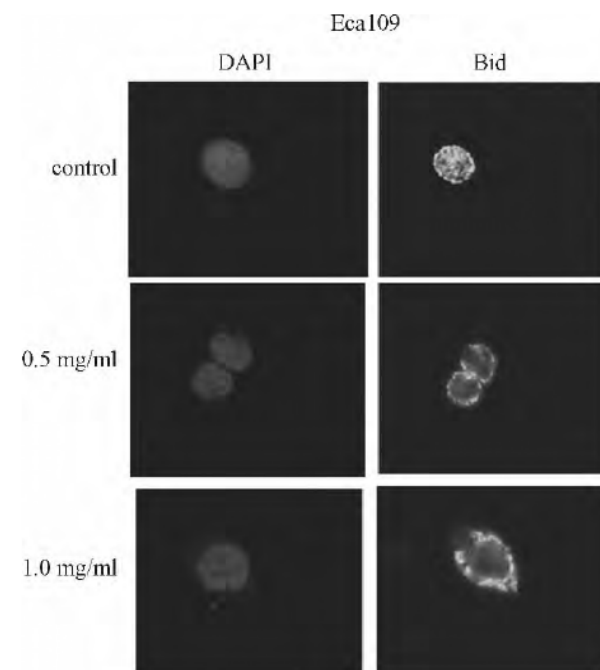


Fig. 2: Fluorescence figure of esophageal cancer cell strain Eca-109 after staining by Hoechst33342

Cell cycle, proliferation index and apoptosis rate detection by flow cytometry

Esophageal cancer cell strain Eca-109 processed by

different concentration of minor bupleurum decoction was placed in 6-hole board and washed for twice. The liquid was absorbed and abandoned. Then it was fixed by 70% ethyl alcohol for 10 min. Then it was centrifuged for 5 min in 1000 rpm to remove ethyl alcohol. 3×10^5 cells were collected for subsequent experiment. 500 μ l of Bindingbuffer was added for cell suspension and 5 μ l of Annexin V-FITC was also added and mixed. 5 μ l of PI dye liquid was added for 30 min processing away from light. At last, flow cytometry was used for detection of cell apoptosis rate, proliferation index and periodic distribution. Then we made a data analysis.

STATISTICAL METHOD

Experimental data was expressed as mean \pm standard deviation. SPSS16.0 statistical software was adopted to make t test analysis on experiment result. $P \leq 0.05$ is judged that difference has statistical meaning.

RESULTS

Expression of cell proliferation survival rate detection result

MTT (Methyl Thiazolyl Tetrazolium) method was applied to detect absorbance of experimental groups. Absorbance value was converted into cell activity. Inductivity of minor bupleurum decoction of different concentration on Eca-109 cell apoptosis can be calculated according to the following formula: survival rate (%) = (experimental group OD value - blank control group OD value) + (control group OD value - blank control group OD value) \times 100%, inhibition rate (%) = 100% - survival rate. The calculation result shows that survival rate of cell proliferation decrease with the increase of minor bupleurum decoction. fig. 1 shows that compared to control group, groups of different concentration of minor bupleurum decoction all had statistical difference. In addition, cell proliferation survival rate decrease obviously 24 h after 0.5mg/ml, 1.0mg/ml, 1.5mg/ml and 2.0mg/ml minor bupleurum decoction processing on cell strain Eca-109.

Morphology observation of minor bupleurum decoction on inducing eca-109 apoptosis

Esophageal cancer cells of different gradient were cultivated for 48 h. Then we conducted cell slide and stained with Hoechst 33342. Hoechst 33342 is a kind of blue fluorescent dye that can penetrate cytomembrane and stain DNA. When cells were in apoptosis, Hoechst 33342 dye absorbed would increase because membrane permeability changed and the chromatin was highly centralized. As a result, it was observed as a granular strong blue fluorescence under fluorescence microscope. Esophageal cancer cell strain Eca-109 cell was placed under fluorescence microscope for observation after

staining. As shown in fig. 2, control group shows uniform faint blue fluorescent while medicine administration team shows stronger granular blue fluorescence. It further illustrated that minor bupleurum decoction can effectively induce apoptosis of Eca-109 *in vitro* condition.

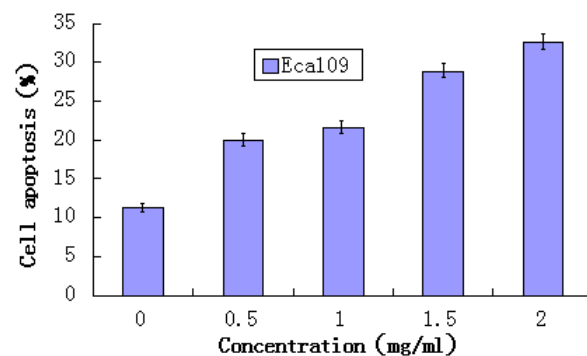


Fig. 3: Apoptosis rate after 24h processing of minor bupleurum decoction of different concentration

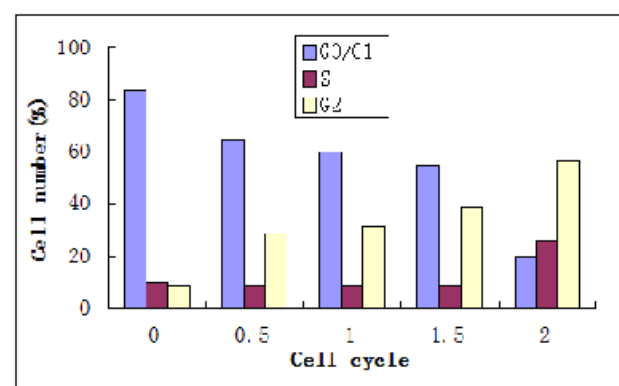


Fig. 4: The effect of minor bupleurum decoction on Eca-109 cell cycle distribution

Effect of minor bupleurum decoction on apoptosis rate of esophageal cancer cell strain eca-109

Through morphology observation, we found that minor bupleurum decoction can effectively induce apoptosis of esophageal cancer cell strain Eca-109. We conducted quantitative research on apoptosis in order to prove that minor bupleurum decoction have the ability of inducing apoptosis of esophageal cancer cell strain Eca-109. fig. 3 shows apoptosis rate of Eca-109 24h after processing of minor bupleurum decoction in different concentration with adoption of Annexin V/PI double staining method that detected by flow cytometry. The result shows that minor bupleurum decoction had obvious apoptosis inducing function after 24h processing of different concentration decoction on Eca-109. Compared to control group, it illustrates that minor bupleurum decoction can induce apoptosis of Eca-109 cell strains. The number of apoptosis cells increased with the increase of

concentration of minor bupleurum decoction and showed obvious dose dependence.

Periodic change of eca-109 strain after function of minor bupleurum decoction

Flow cytometry was applied to detect the periodic change of Eca-109 strain. Cell cycle distribution situation after 24 h processing of minor bupleurum decoction of different concentration is shown in fig.4. Compared to control group, ratio of cell number accounting for total amount in G0/G1 period increased gradually and ratio of cell number accounting for total amount in s period gradually decreased with the increasing of minor bupleurum decoction concentration. Ratio of cells number in G0/G1 period from 2.0mg/ml minor bupleurum decoction group and total cell amount was obviously different compared to control group. It illustrates that minor bupleurum decoction in certain concentration can block cell cycle of Eca-109 in G0/G1 period.

DISCUSSION

Cancer is closely related to out of control of cell proliferation, differentiation obstacle, apoptosis blocking and transduction means of cell signal. Cell apoptosis is regulation of body. It is the critical step of maintaining refresh and proliferation of normal cell and also a normal physiological process of eliminating harmful cell and preventing excessive proliferation of tumor cell (Yanli *et al.*, 2006; Jin and Bao'en, 2006; Weidong and Ruifeng, 2004). It shows the importance of cell apoptosis in occurrence and development of cancer. Meanwhile, chemotherapy, radiotherapy and biotherapy are also damage and kill cancer cell by inducing apoptosis. Some scholars found that minor bupleurum decoction in small dose group had obvious inhibit function on sarcoma rat S180 cell. In addition, minor bupleurum decoction of different doses all can induce apoptosis of sarcoma rat S180 cell and had obvious difference compared to control group. Minor bupleurum decoction of small dose and medium dose can decrease cell number in s period and S180 cell proliferation index and increase cell number in S180 period. Scholars abroad found that minor bupleurum decoction can induce the change of hepatoma carcinoma cell morphology and block cancer cell in stationary phase of cell cycle. Hu Yalin (Yalin, 2008) found that minor bupleurum decoction can make cancer cell form develop into normal cell, strengthen SDH (Succinate Dehydrogenase) activity, lower LDH (Lactate Dehydrogenase) activity and block cell cycle in G0/G1 period. The relationship of minor bupleurum decoction and esophageal cancer cell strain is seldom reported in many research results. *Epidemiology of Esophageal Cancer* observed the anti-tumor function of minor bupleurum decoction on esophageal cancer cell strain (Eslick, 2009). This paper found that cell cycle block played important function in the process of minor

bupleurum decoction inducing esophageal cancer cell apoptosis.

MTT (Methyl Thiazolyl Tetrazolium) method is used for detecting the survival and growth situation of cells. Detection of OD value can indirectly reflect number of alive cells. Amount of MTT (Methyl Thiazolyl Tetrazolium) crystal and cell amount show direct ratio within certain cell amount scope. The result shows that minor bupleurum decoction had obvious inhibition function on survival of esophageal cancer cell strain Eca-109 cell. Inhibition rate increase with the increase of medicine solubility. Minor bupleurum decoction show dose dependence on proliferation of Eca-109 cell. In the process of occurrence and development of cancer, cell proliferation and apoptosis was closely related. Cell apoptosis block was an important mechanism of cancer incidence. Therefore, inducing of cancer cell apoptosis has become the main means and target in cancer treatment (Yang and Xiaohang, 2010). The result shows that minor bupleurum decoction can effectively induce apoptosis of esophageal cancer cell strain Eca-109 cell and the function is dependent on the concentration of minor bupleurum decoction. Apoptosis amount of esophageal cancer cell strain Eca-109 cell would be more with the increase of concentration of minor bupleurum decoction.

So far, regular chemotherapy has weak effect on esophageal cancer. Exploration of a medicine that can specific targeting kill esophageal cancer cells has been a research hotspot. Through *in vitro* experiment, this paper initially showed that minor bupleurum decoction had obvious inhibition on proliferation of esophageal cancer cells and it may be a kind of hopeful anti-cancer Chinese traditional medicine (Xiangfan, 2003). Development of molecular biological technique is bound to advocate the research on anticancer inhibition function of minor bupleurum decoction and similar medicine. Research for these mechanism is hopeful to develop new anticancer medicine.

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REFERENCES

- Eslick GD (2009). Epidemiology of esophageal cancer. *Gastroenterol. Clin. of North Am.*, **38**(1): 17-25.
- Jin Z and Bao'en P (2006). Research progress of anticancer immunological regulating effect and action mechanism of traditional Chinese medicine. *Chin. J. of Immunology*, **22**(4): 385-388.
- Weidong C, Ruifeng C and Jianxiong Z (2004). Research progress of traditional Chinese medicine inducing apoptosis of liver cancer cell. *Gansu J. of Trad. Chin.*

- Medi.*, **17**(4): 44-47.
- Xudong H, Xiaojiang W, Hong Q and Rufeng J (2005). Effect of six common anticancer traditional Chinese medicines on liver cancer cell strain BEL-7402. *J. of Jiangxi. Medil. College*, **17**(4): 47-48.
- Xiangfan X (2003). Research on anti-cancer oral liquid *in vitro* on relative genetic function of stomach MGC 803 cell strain. *Chin. J. of Composite Clinical Medi.*, **5**(1): 14-15.
- Ying Z (2012). Research progress of anti-cancer pharmacological effect of traditional Chinese medicine. *Modern J. of integrated Chin. Medi. and Western Medi.*, **33**: 3752-3753.
- Yanli W, Shuixing H and Jinyan L (2006). Research progress of anti-cancer mechanism of saikoside. *J. of integrated Chin. Medi. and Western Medi.*, **1**(4): 98-100.
- Yalin H (2008). Research on minor bupleurum decoction inducing differentiation of SMMC-7721 cell and its action mechanism. *Chengdu: Basic Medi. College of Sichuan Univer. of Chin. Medi. Master's Thesis.*
- Yang X and Xiaohang Z (2010). LAP family molecular and cancer target therapy. *Bio. Sci.*, **2**: 161-168.
- Zeb A, Rasool A and Nasreen S (2008). Cancer incidence in the districts of Dir (North West Frontier Province), Pakistan: A preliminary study. *J. of the Chin. Medi. Association*, **71**(2): 62-65.