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The effect and significance on Akt and PTEN expression in melanoma B16 cells with chamaejasme extract*

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Abstract *Objective:* The aim of this study was to investigate the effect on Akt and PTEN expression and the mechanism of proliferation and apoptosis of melanoma B16 cells treated with chamaejasme extract. *Methods:* The expressions of Akt and PTEN of B16 cells treated with different concentrations of chamaejasme extract were detected with immunohistochemical method, cell apoptosis index (AI) was calculated with in situ labeling method. *Results:* Akt expressions of B16 cells treated with different concentrations of chamaejasme extract were significantly lower than the control group, while the PTEN expressions significantly up-regulated, and the effects appeared to be dose-related ($P < 0.05$). The Akt/AI of B16 cells treated with different concentrations of chamaejasme extract was significantly lower than the control group, all the parameters had extremely difference ($F = 24.58$, $P < 0.05$). *Conclusion:* Chamaejasme extract can inhibit proliferation and induce apoptosis of malignant melanoma B16 cells by down-regulating the expression of Akt and up-regulating the expression of PTEN.

Key words malignant melanoma; chamaejasme extract; Akt chamaejasme; PTEN; immunocytochemistry

The incidence of malignant melanoma has increased year by year with an annual growth rate of about 3%. which represents the third-leading causes of the skin malignant tumor [1]. Numerous studies have confirmed that the poor prognosis of malignant melanoma was primarily attributed to its high incidence of distant metastasis and strong capacity of invasion. Therefore, the development of more effective and revolutionary new drugs for inhibiting metastasis is currently the most pressing problem for treatment of malignant melanoma [2]. Recent studies have shown that cancer gene Akt (serine/threonine protein kinase B) and tumor suppressor gene PTEN (phosphatase and tensin homology deleted on chromosome ten) through PI3K/Akt pathway could regulate cell proliferation and apoptosis, and the formation model has the very vital significance on its diagnosis and treatment of malignant melanoma [3]. This research adopted the immunocytochemistry method to detect Akt and PTEN protein expression and apoptosis index (AI) of malignant melanoma B16 cells treated with different concentrations of chamaejasme extract. We investigated the activities of chamaejasme extract against mice highly metastatic B16 cell line and its mechanism with the proliferation and apoptosis.

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Materials and methods

Cell culture

Malignant melanoma B16 cells in mice were obtained from the cell center of Beijing union medical college. The cells were cultured with EMDM culture medium containing 10% fetal bovine serum and streptomycin and penicillin (100 μg/mL) at 37 ℃ in a humidified incubator with 5% CO_2 atmosphere, the logarithmic phase of cells used in the experiment.

Preparation of chamaejasme extract

The chamaejasme extract was chosen a euphorbia fischeriana steud from Linyi as the preparation raw material. The chamaejasme was mixed with 88% ethanol by 1:10 after crushing, then constant stirred in 50 ℃ for 2 h, filtered with filter paper after cold precipitation and made for 2 mg/mL (Chinese medicine) preparations.

Table 1 The expression of Akt and PTEN in malignant melanoma B16 cells in different concentration of chamaejasme extract

Groups	*n*	Akt		PTEN	
		–	+	–	+
Blank control group	15	2	13	11	4
Low dose of chamaejasme	15	6	9	7	8
Medium dose of chamaejasme	15	8	7	4	11
High dose of chamaejasme	15	9	6	2	13

Reagents and cell processing

Antibodies of p-Akt, PTEN and Tunel kit were bought from Dako company (Denmark). The melanoma B16 cells were collected in the logarithmic phase, when cell count had been completed the cells were inoculated in 96-well culture plate per hole 3×10^3. After 24 h, respectively, the cells were added to a final concentration of 0.8, 1.2, 1.6 mg/mL of chamaejasme extract samples, meanwhile setted up blank control group, each drug concentration of 15 holes. They were continued at 37 ℃ in a humidified incubator with 5% CO_2 atmosphere and tested after 24 h.

Expression of Akt and PTEN protein

Immunocytochemistry method Envision system adopted two footwork dyeing. We used the known positive biopsy as the positive contrast, phosphate buffered saline (PBS) instead of primary antibody as the negative control. The detailed steps were following: (1) the section dewaxing hydration and later repaired antigen by high pressure hot, (2) rinsing by distilled water, (3) putted it in the PBS 10 min, (4) plus the primary antibody 37% hatching 1–2 h, (5) plus Envision TM hatching 20 min, (6) rinsing by PBS, (7) DAB coloration, (8) initial survey by light microscope, (9) rinsing by distilled water, (10) redyeing by hematoxylin, (11) dehydrate making it transparent, (12) microscopic examination of the sealing piece.

Detect AI

The experimental steps were done according to product manuals of Tunel apoptosis detection kit. We used the Well label Solution as the negative control instead of Tunel reagents, used the untreated B16 cells as the positive control.

Observation

In Envision method Akt and PTEN in tan granule cells were positive cells, positive cells < 10% was (–), > 10% was (+). Akt slices at the same time record Akt index, the formula was the number of positive cells/total number of cells × 100% in each section. AI: brown granules in cell nucleus were apoptotic cells as the positive, random reading 1000 cells took positive cell number/total cell number × 100% as AI.

Table 2 Akt index/AI ($\bar{\chi} \pm s$) of the malignant melanoma B16 after different concentration of chamaejasme extract processing

Groups	*n*	Akt/AI
Blank control group	15	4.41 ± 0.30
Low dose of chamaejasme	15	2.34 ± 0.23
Medium dose of chamaejasme	15	2.07 ± 0.27
High dose of chamaejasme	15	1.09 ± 0.14

Statistical processing

Using SPSS 13.0 statistical software for data processing, measurement data were in, $\bar{\chi} \pm s$ used the test to compare the sample rate, used correlation analysis to record the expression results of Akt and PTEN, used the variance analysis of group design to compare sample mean.

Results

Immunocytochemistry method marking

Akt positive signals mainly locate in cytoplasm, and a small express in the cell nucleus. PTEN positive signal locate in the cytoplasm. Upon detection, the positive rate of Akt were lower than the blank control group ($P < 0.05$) when the groups had a high, medium and low dose of the chamaejasme extract. And the positive rate of PTEN were higher than blank control group ($P < 0.05$; Table 1).

Correlation analysis of the concentration and the expression

According to the correlation analysis, the chamaejasme extract concentration and the expression of Akt and PTEN in processed malignant melanoma B16 cells had correlation, correlation coefficient r was –0.7163 and 0.8024 respectively ($P < 0.05$); the Akt and PTEN expression in B16 cells after the extract processing was negatively related (correlation coefficient $r = 0.9548$).

Akt index/AI

In order to more accurately reflect the relationship of the Akt expression and apoptosis, this experiment was examined the ratio of the Akt index (Akt positive cell percentage) and the AI to measure different concentration of the chamaejasme extract having what kind of influence on the malignant melanoma B16 apoptosis and Akt expression. As shown in Table 2, along with the extract concentration increased, Akt/AI had a gradually reduce and the mean differences between groups was statistically significant ($F = 24.58$, $P < 0.05$).

Discussion

The occurrence and development of tumor is a multifactor, multi-step and multi-gene interactive process. The different mechanisms, such as the activation of on-

cogenes, the inactivation and mutation of tumor suppressor gene, antiapoptosis and so on are involved in [4]. The process of signal transmission of apoptosis involves many steps of phosphorylation and dephosphorylation. Akt/protein kinase B is a serine/threonine protein kinase, is phosphatidylinositol 3 hydroxy kinase downstream key effector molecules, after phosphorylation and activation it can promote tumor growth and invasion by inactivating many apoptosis effector molecules, regulating cell cycle, activating telomerase activity and promoting angiogenesis and migration [5]. PTEN was discovered in 1997, the first suppressor gene with phosphatase activity, it located in chromosome heterozygosity missing parts of 10 q 23.3, unlike many tumor suppressor genes mainly involved in apoptosis, PTEN also has growth inhibition function [6]. PTEN can make PIP3 (phosphatidyl inositol 3, 4, 5 triphosphate) to the dephosphorylation and weaken the activated signals from PI3K, inhibit Akt function indirectly, and it also can be directly to dephosphorylation inactivatin on adhesion kinase and inhibition of cell migration and cell skeleton, inhibiting signal transmission mediated by growth factor receptor to suppress tumor occurrence and development [7].

Through analysis that the chamaejasme extract preparations containing a number of chemical compositions of rock spurge lactone (jolkinolide) A, B, flavonoids, stellera spurge A, B (fischeriana A, B), spurge alcohol (euphol), saponins, cardiac glycoside, sterol, phenolic compounds and tannins, etc. The research adopt the immunochemical method to detect the influence of the chamaejasme extract to the malignant melanoma B16 cells in the expression of Akt and PTEN protein and the ratio of Akt index/AI, We find that the influence is the most obvious when the concentration of chamaejasme extract is 1.6 mg/mL. With the increase of drug concentration, the expression of PTEN up-regulated and the expression of Akt protein down-regulated. Akt index/AI ratio along with the increase of the chamaejasme extract reduce gradually and significantly, and present the dose-response relationship and the difference is statistically significant ($P < 0.05$). In conclusion, the experimental results show that the chamaejasme extract have a significant inhibition of proliferation and induce cell apoptosis to the melanoma B16 cell lines. These results demonstrate that the up-regulation of PTEN protein ratios and down-regulation of p-Akt protein may be one of molecular mechanisms of action of chamaejasme extract suppressing the B16 cells growth.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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