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Original Article

Study on inhibitory effect of paclitaxel on MEK and ERK protein overexpression and activation in different breast cancer cell lines

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Abstract: Objective: To investigate the mechanism of the proliferation of different types of breast carcinoma cells and the protein expression of MEK and ERK through studying the inhibitory effect of paclitaxel on the protein expression and activation of MEK and ERK in four kinds of human breast cancer cell lines. Methods: Culture normal mammary epithelial cells and breast carcinoma cells in a routine way. Use flow cytometry to detect the proliferation ability of normal mammary glandular celland four kinds of breast carcinoma cells. Use quantitative PCR and Western blot to test the protein expression, phosphorylation of MEK and ERK and the effect of paclitaxel on the protein expression and activation of MEK and ERK. Results: Compared with normal mammary epithelial cells, the apoptosisin the different types of breast cancer cell linesdecreased. And the levels of MEK and ERK protein expression and phosphorylation were significantly increased. After being treated with paclitaxel, degrees of apoptosis and inhibitions of the overexpression of ERK and MEK protein in different types of breast cancer cell lines became different. And it had no such effect on normal mammary epithelial cells. Conclusion: Paclitaxel can inhibit the protein overexpression and activation level of MEK, ERK in different breast cancer cell lines, and there is no significant cytotoxicity to normal mammary epithelial cells.

Keywords: Paclitaxel, breast carcinoma cells, MEK, ERK

Introduction

Breast cancer is one of the most common malignant tumors in women. According to the latest statistics, the incidence of breast cancer accounts for 7%-10% of all kinds of malignant tumorsin the body [1]. Such malignant tumor usually occurs in the mammary gland epithelial tissue [2]. And it is one of the most common malignant tumors which can seriously affect women's physical and mental health, even endanger women's lives. At present, the etiology of breast cancer has been not clearyet, and the treatment has been mainly based on surgery and supplemented by radiotherapy and chemotherapy [3]. Breast cancer is very easy to relapse. But clinically, there are no specific drugs that can inhibit the proliferation of breast carcinoma cells. Thus, it is especially crucial to find a specific drug for the treatment of breast cancer [4, 5].

Paclitaxel is a kind of diterpenoid alkaloids. In 1971, it was isolated and extracted from the bark of taxus brevifolia by Wani and others, and later it was named "paclitaxel". Paclitaxel is a kind of effective phytogenic anticarcinogen which can act oncell microtubules and inhibit the depolymerization of tubulin, thus making thecancer cells unable to split then dead [6]. Some studies found that paclitaxel could act on macrophages, next inhibit the production and the release of tumor necrosis factor α, then promote the release of interleukin I and interferon, and finally the effect of anti cancer cells emerged.

Mitogen-activated protein kinase (MAPK) is a kind of serine threonine kinase and it can be activated by different extracellular stimulation such as cytokine, neurotransmitter, hormone, etc. It is one of the most important signal transduction pathways in living organism and partici-

pates in the regulation of cell cycle. Particularly, it plays a key role in cell proliferation, differentiation and apoptosis [8]. MAPK can promote the proliferation of vascular endothelial cells and induce the formation of new blood vessels, which provides the essential oxygen and nutrition for the growth of tumor cells, accelerates tumor growth and promotes the proliferation of cancer cells. Extracellular regulated kinase (ERK) and its upstream kinase MEK are two important signaling molecules in this pathway [9]. MEK can activate the downstream signal ERK, further activate the downstream substrates, thereby inducing the expression of certain genes and leading to cell proliferation [10-12].

This experiment took the normal mammary epithelial cell line MCF10A as a control, investigated the expression and activation of extracellular regulated kinase (ERK), which was involved in MAPK signal transduction pathway, and its upstream kinase (MRK) in human breast cancer cell line MCF 7, HCC 1937, HCC 38 and BT-549, as well as detected the inhibitory effect of paclitaxel on the proliferation of breast cancer cell lines and the protein expression and activation of MEK and ERK, so as to explore the therapeutic effect and related mechanisms of paclitaxel on the occurrence and development of human breast cancer.

Materials and methods

Materials

Human normal mammary epithelial cell line MCF 10A [13], human breast cancer cell line MCF 7 [14] and HCC 1937 [15], human breast ductal carcinoma cell line HCC 38 [16] and BT549 [17] were purchased from iCell Bioscience Inc. Fetal bovine serum and RPMI1640 cul-ture medium were purchased from GBICO Company. Paclitaxel was purchased from Tai Chi Group Sichuan Tai Chi Pharmaceutical Co., Ltd. BCA Protein Concentration Assay Kit (enhanced) was purchased from Shanghai biyuntian Biological Technology Co. Ltd., PE Annexin V Apoptosis Detection Kit I (BD559763) was purchased from BD Company. RIPA lysates was purchased from Shanghai Boguang Biology Co. Ltd., rabbit antibody MEK, rabbit antibody ERK, rabbit antibody phosphorylation MEK, rabbit antibody phosphorylation ERK antibodies and GAPDH were all purchased from Aibokang Company.

Methods

Cell culture: The purchased cell lines were resuspended in a constant-temperature water bath. After resuscitated, the cell lines were cultured inwell-mixed DMEM medium (i.e., complete medium) containing 10% FBS, penicillin and streptomycin double anti (100 U/mL penicillin and 100 U/mL streptomycin). Then, the cell lines were put in 1000 rpm centrifuge for 5 min, then the supernatant was abandoned, nextthe complete medium was added, mixed well and put in a sterile 25 cm^2 flask whose concentration was 1*10^6/ml, finally they were incubated in a 37° incubator containing 5% CO_2. When it comes to experiments, the cell lines in the logarithmic growth phase were taken to act the cell plating.

Detection of apoptosis: The apoptotic ratio of each cell line before and after paclitaxel treatment was tested by Annexin V and 7AAD kit (BD559763) through flow detection. The cultured cells were washed twice with ice PBS, centrifuged at 1000 rpm for 5 minutes, and resuspended in 1× Binding Buffer (10 ^ 6/ml), took 100 μl to 1.5 ml of EP tube, added 5 [mu] l of Annexin V and 5 [mu] l of 7AAD, after vortexing, incubated them for 15 min at room temperaturein the dark, added 400 μl of Binding Buffer, and performed flow pattern detection.

DNA breakage detection: Extracted the total DNA from each cell line and selectively extracted the small molecular weight DNA for agarose gel electrophoresis, observed and recorded the number of DNA fragments.

Expression of MEK and ERK and the detection of phosphorylation level: After all the cell lines were stimulated by paclitaxel and scrubbed twice in ice-cold PBS, RIPA lysates containing 1% PMSF was added to lyse the cells. Then cell scraping was used to collect the cells in 1.5 ml EP tube accompanied with 5-minute standing on the ice and 20-minute centrifugation at the speed of 12000 rpm/min. Protein concentration in supernatant was determined by BCA method. 20 μg of each sample was performed SDS-PAGE electrophoresis in effect of 5 times buffer. After electrophoresis, the protein on gel

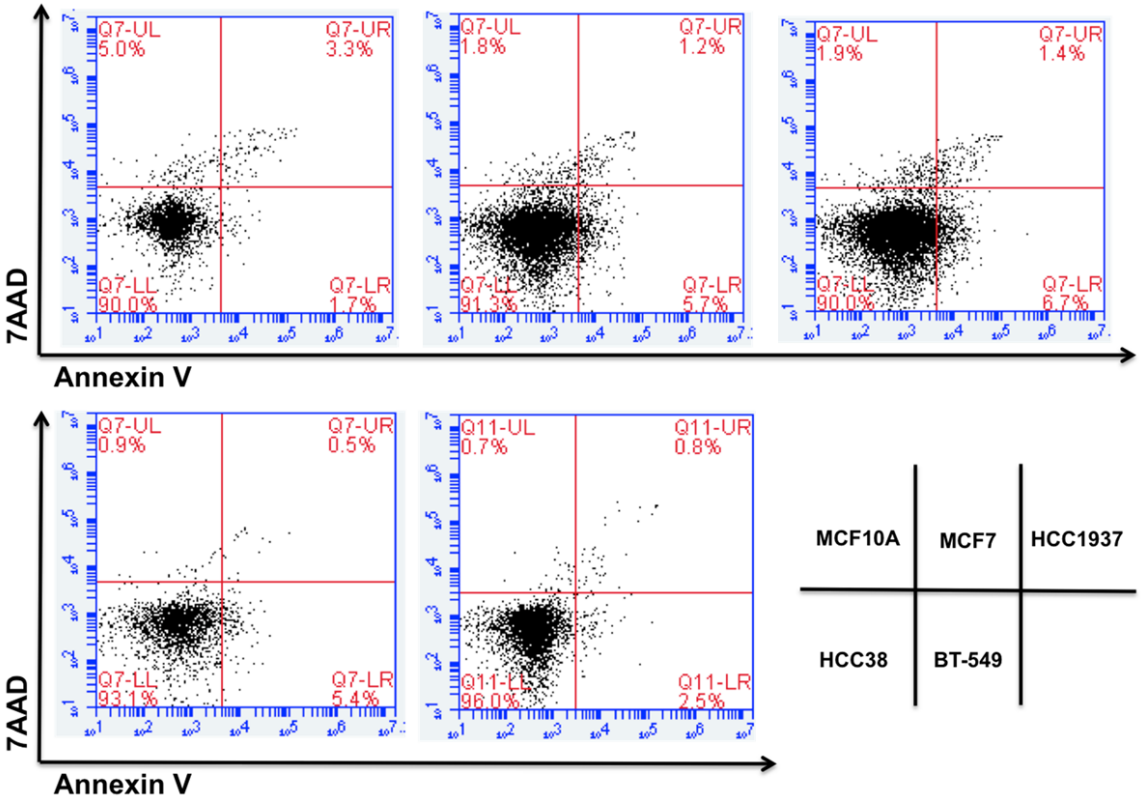

Figure 1. Normal mammary epithelial cells and proliferation and apoptosis of different types of breast carcinoma cells.

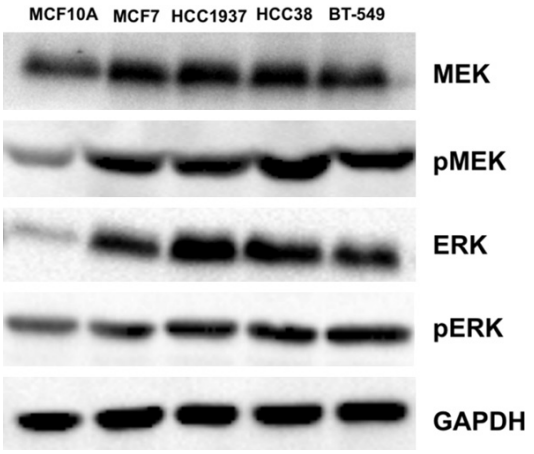

Figure 2. Normal mammary epithelial cells and the over-expression and phosphorylation level of MEK, ERK protein from different types of breast carcinoma cells.

was electrically transferred to polyvinylidene fluoride (PVDF membrane) and closed with 5% skim milk powder, primary antibodies (rabbit anti MEK, rabbit anti phosphorylated MEK, rabbit anti ERK and rabbit anti phosphorylated ERK to a working concentration of 1: 1000) were dropped to incubate overnight at 4°C. Apply secondary antibodies (labeled sheep anti-rabbit IgG in concentration of 1: 3000) after it was washed with TBST and incubate for 90 min at room temperature. Washing the membrane, afterwards, the ECL chemiluminescence method was used for detection with the results recorded.

Results

Compared with normal breast epithelial cells, breast cancer cell lines had faster proliferation and less apoptosis

Stream analysis displayed that apoptosis rate of normal breast epithelial cells was 3.3%, however, it dropped in different degrees when it came to other four kinds of breast cancer cell lines. Among them, the apoptosis rate of MCF was 1.2%, HCC 19379 was 1.4%, HCC 38 was 0.5% and BT-549 was 0.8% (see **Figure 1**).

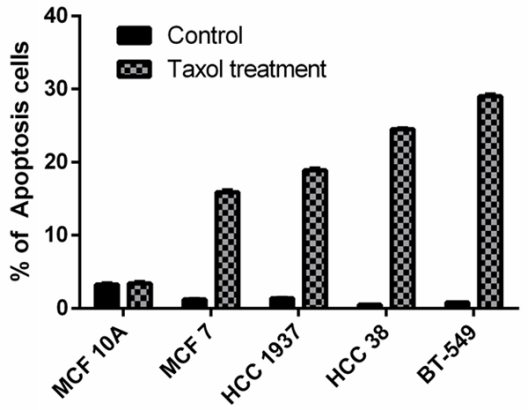

Figure 3. Paclitaxel promotes apoptosis of different types breast carcinoma cells.

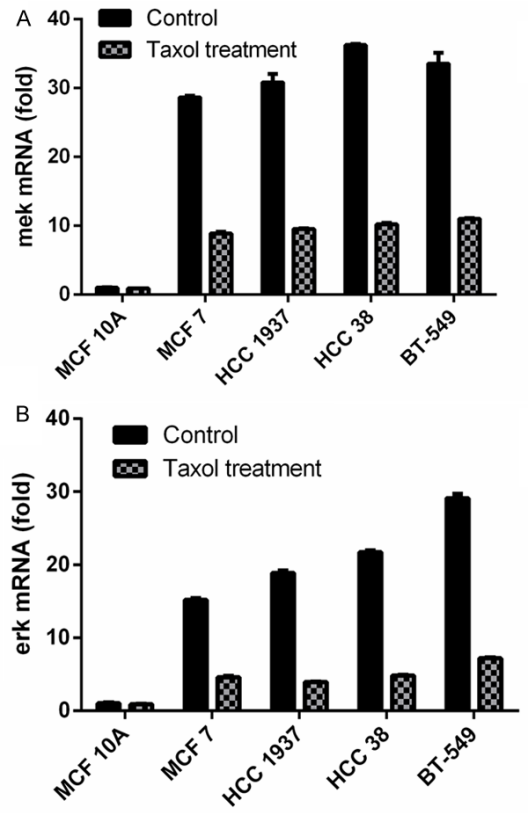

Figure 4. Inhibitory effects of paclitaxel on MEK and ERK expression in the different types breast carcinoma cells. A: The expression level of MEK mRNA in different breast carcinoma cells treated by paclitaxel; B: The expression level of ERK mRNA in different breast carcinoma cells treated by paclitaxel.

Expression and activation of MEK and ERK in breast carcinoma cells

Analyzed by Western blot, it indicated that four different types of breast carcinoma cells have significant increased level of MEK and ERK protein expression and phosphorylation compared with mammary epithelial cells (see **Figure 2**).

Impact on apoptosis of breast cancer cell by paclitaxel

After done with paclitaxel, the proportion of apoptotic cells in MCF7 and HCC 1937 breast carcinoma cells rapidly grew nearly 20 times, while BT-549's apoptotic cells were 30 times more than before. The differences were statistically significant. There was no obvious growth happening to the apoptotic cells of normal mammary epithelial cells (see **Figure 3**). Meanwhile, from the DNA breakage ratio detection, paclitaxel could cause DNA breakage of four types of breast carcinoma cells increased nearly 10 times more and the apoptosis of cells ($P<0.05$). The differences were also statistically significant. Due to the DNA breakage ratio was 2-3% before, there was no statistically significant exited in this differences (**Table 1**) after the performance of paclitaxel to normal mammary epithelial cells ($P>0.05$).

Paclitaxel can reverse the protein expression of different types of breast carcinoma cells' MEK and ERK

Real-time PCR indicated that after paclitaxel performed, the MEK and ERK protein expression level of four different types of breast carcinoma cellswas obviously lower than that of the untreated group. As for normal mammary epithelial cells, there was no significant decline before and after the work of paclitaxel ($P>0.05$). The differences were not statistically significant (see **Figure 4**). It means that paclitaxel could distinctively inhibit protein expression of MEK and ERK in different types of breast carcinoma cells without suppressing MEK and ERK protein expression in normal mammary epithelial cells. It implied that paclitaxel had the safe and effective characteristics.

Discussion

MAPK is a multigene family, which can be activated by a variety of extracellular signals. Activated by mitogen activating protein kinase (MAPK), it can cause cell proliferation, differentiation and death [18, 19]. MAPK signal transduction pathway plays a key role in regulating cell proliferation and apoptosis, which has a close relationship with multiple tumor or proliferative diseases [20].

Table 1. Increase in DNA fragmentation in different cell types

Cell lines	% DNA fragmentation		
	Ctrl	DMSO	Taxol
MCF 10A	2.1 ± 0.08	3.4 ± 0.16	2.8 ± 0.40
MCF 7	1.2 ± 0.04	1.5 ± 0.8	18 ± 2.7*
HCC 1937	1.1 ± 0.06	1.9 ± 0.9	24 ± 1.2*
HCC 28	1.5 ± 0.12	2.1 ± 0.6	32 ± 1.1*
BT-549	0.8 ± 0.01	1.4 ± 0.04	25 ± 0.8*

Note: *, P<0.05, vs. MCF 10A. Data represent mean of three independent experiments. Percent of DNA fragmentation refers to the ratio of DNA in the supernatant to the total DNA recovered in the supernatant and pellet.

Extracellular signal regulated protein kinase (extracellular signal regulated protein kinase 1/2, ERK)pathway is one of the most widely and thoroughly studied MAPK signal transduction pathways thus far. It participates in signal transduction of vairous of growth factors, cell factors, mitogens and activated hormone receptor. In addition, it plays an crucial role in regulating in several regards, i.e., cell split, survival, migration and tumor invasion. What's more, it participates in the survival and proliferation oftumor cells [21, 22]. Topreliminary explore the therapeutic prospect of paclitaxel in the treatment of different types of breast cancer, different types of breast cancer cell lines in vitro production model were used in this study to observe the effect of paclitaxel on the proliferation and the activation expression of MEK, ERK of different breast cancer cells. The results showed that compared with normal breast epithelial cells, breast carcinoma cells had the characteristics of faster proliferation, less apoptosis, higher expression, activation levels of MEK/ERK protein, but paclitaxel could inhibit these effects. We found that the proportion of different breast carcinoma cells apoptosis after paclitaxel treatment significantly increased ten times with significant statistical difference. However, there was no significant change in the apoptotic rate of normal breast epithelial cells,indicating that paclitaxel could inhibit the proliferation of breast carcinoma cells, promote cell apoptosis, without affecting the normal breast epithelial cells. In other words, paclitaxel has a significant antitumor effect and no cell toxicity.

At present, it has been reported that the expression of ERK protein was correlated with chemotherapeutic drugs. In the practical application, we can conjecture growth inhibitory state of tumor cells by testing protein levels of MRK, ERK [23]. By using isotope labeling and immuno agglutination inspection, blagosklonny and other authors confirmed that paclitaxel could induce bcl-2 protein phosphorylation and activate Raf-1 kinase to induce apoptosis of human breast cancer cell lines MCF-7.

In conclusion, paclitaxel can inhibit the overexpression and phosphorylation level of MEK, ERK protein of different types of breast cancer cell lines, promotebreast carcinoma cells apoptosis. However, it doesn't have obvious cytotoxicity to normal mammary epithelial cells.

Disclosure of conflict of interest

None.

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