Research Paper

Effect of Esomeprazole on Biological Characteristics of Hepatoma Cells by Regulating Protein Kinase B/ Forkhead Box O3 Pathway

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To explore the effect of esomeprazole on the biological characteristics of hepatoma cells by regulating protein kinase B/forkhead box O3 pathway. Hepatoma cell lines were cultured and subcultured and divided into model group, low dose group and high dose group. The changes of proliferation, colony formation and apoptosis of hepatoma cells in each group and the expression levels of proliferation, apoptosis related proteins and protein kinase B/forkhead box O3 pathway related proteins were detected. Esomeprazole inhibited tumor cells in a time and dose-dependent manner and the difference was statistically significant ($p<0.05$). With the increase of drug dose, the number of liver cancer cell colony formation decreased gradually and the difference was statistically significant ($p<0.05$). Esomeprazole induced apoptosis of tumor cells in a time and dose-dependent manner and the difference was statistically significant ($p<0.05$). Compared with the model group, the expression levels of cyclin-dependent kinase 1, cyclin-dependent kinase 2, cyclin A2 and cyclin E2 decreased with the increase of esomeprazole dose ($p<0.05$). Compared with the model group, the level of apoptotic protein procaspase-9 in high and low dose groups decreased significantly, while the level of cleaved caspase-9 increased in a dose-dependent manner ($p<0.05$). The level of protein kinase B in high dose group was lower than that in model group and low dose group, while the level of forkhead box O3 protein increased; the difference was statistically significant ($p<0.05$). Esomeprazole can affect the biological characteristics of tumor cells such as proliferation and apoptosis by activating protein kinase B/forkhead box O3 pathway, which provides a new method for the treatment of liver cancer.

Key words: Esomeprazole, protein kinase B/forkhead box O3 pathway, hepatoma cells, tumor, hepatitis

The liver cancer is a malignant tumor occurred in the liver and it could be divided into the primary and secondary cancer[1]. The primary liver cancer refers to the malignant tumor occurred in the hepatocyte or intrahepatic bile duct epithelium. The secondary liver cancer is caused by the diffusion or transfer of malignant tumors in other organs, which is more common than the primary cancer[2]. The exact cause and pathogenesis of the primary liver cancer are not clear and it may be associated to the viral hepatitis, alcohol drinking, moldy food and genetics[3]. It may be cured in the early stage, but it is complex for treatment of the liver cancer in the middle and later stage. The most applied method in clinical treatment is the surgery, including hepatectomy and liver transplantation.

However, it has some restrictions and the synthetic therapy shall be adopted[4]. With the development of cancer research, it is found that the cancer is caused by the imbalance of the cell signal pathway, leading to the imbalance of cell proliferation control. In view of the research on anti-tumor drugs, it has been transferred from the traditional cytotoxic drugs to targeted drugs and it is featured as accurate treatment, low toxicity and remarkable curative effect, focusing on the biological characteristics of tumor cells[5]. In recent years, the effect analysis of therapeutic drugs for other diseases in the field of tumor has become a research hotspot and there are more researches on the Proton Pump Inhibitor

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(PPI) [6]. The PPI is an important drug in inhibiting the gastric acid secretion and it could inhibit the tumor cell proton pump to play an anti-tumor role. It is found from the research that PPI has the special impact on the biological characteristics of the tumor cells[7]. This research adopts the esomeprazole to regulate the Protein Kinase B/Forkhead Box O3 (AKT/FOXO3a) pathway and it aims at analyzing its influence on the biological characteristics of hepatoma cells.

MATERIALS AND METHODS

General data:

Hepatoma cell line Huh7 was purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences and was passaged and preserved in our laboratory.

Key reagents:

Minimal Essential Medium (MEM) culture medium containing 10 % of fetal calf serum; primary liquid of 30 % esomeprazole; phosphate buffer solutions; 4 % paraformaldehyde solution and 0.6 % cell permeabilization solution.

Test methods:

Cell culture techniques: Place the hepatoma cell line Huh7 in the MEM culture medium containing 10 % fetal calf serum and culture in 36° and 4 % carbon dioxide cell incubator. Cell recovery was done by taking the culture medium out of the cell incubator and stand at room temperature for 30 min. Then, place 8 ml culture medium in the sterile centrifuge tube, take the frozen cells out at -70° and quickly place in a 36° water bath for shaking, so that the cell suspension could be centrifuged in the containing medium containing the centrifuge tube. After removing the clear liquid, add 5 ml of culture medium into the centrifuge tube again and place it in the cell incubator for culture. Cell passage was when the cells grow to 80 % of the culture medium; passage and culture the cells. After washing with the phosphate buffered solution and observe under the microscope add 2 ml 0.25 % trypsin in the culture medium. When the cells become round and bright, pour out trypsin and add the culture medium. Inoculate the cell suspension into 2-4 culture flasks with 6 ml culture medium. Place it in the cell incubator for further culture. Cell cryopreservation was done by transferring the cell suspension to the centrifuge tube. After removing the clear liquid, transfer the precipitated cells to the cryopreservation tube for labeling, and store at -70°.

Cell Counting Kit-8 (CCK-8) test: After taking the cells in the logarithmic phase for the routine digestion count, dilute it to the corresponding cell concentration with the full culture medium. Add the cell suspension in 96-well plates, enable its number of cells as 2×10^4, 8×10^3 and 7×10^3 after 24 h, 48 h and 72 h, and place it in the incubator for culture; after 24 h culture, add MEM complete medium in the model group and add the culture medium with the low dose and high dose concentration of esomeprazole to the corresponding medication administration groups for further culture. The treatment at 48 h and 72 h is same to that at 24 h; take it out after culturing for 24 h, 48 h and 72 h, and remove the culture medium and then, add CCK-8 solution for culture and determination.

Cell colony formation: Carry out the plate colony forming test, add the culture medium and place in the incubator. Then, terminate the culture after observing the colony formation. After pouring out the culture solution, wash and dry it, fix with formaldehyde and count with the software.

Cell apoptosis: Detect it with the flow cytometry. After routine digestion of cells and grouping according to different drug doses, place it in the incubator for culture of 24 h, 48 h and 72 h. Then, centrifuge the culture medium. After removing the clear liquid and adding the primary liquid for shaking, detect with the flow cytometry.

Western blot test: After cell dissociation, conduct the grouping based on the drug dose for culture. Add the protein lysate for centrifugation and take the Western blot to detect the protein expression level at each group.

Statistical methods:

In this research, Statistical Package for the Social Sciences (SPSS) 20.0 software package is adopted for the analysis of statistical data and the measurement data comply with the normal distribution, expressed by ($\bar{x}\pm s$). The independent sample t-test is used for comparison between groups and the paired sample t-test is used for comparison before and after treatment. If $p<0.05$, the difference is statistically significant.

RESULTS AND DISCUSSION

CCK-8 is a detection method in analyzing the cell proliferation and toxicity simply and accurately and it gradually replaces the traditional detection method due to its high sensitivity. After esomeprazole acts on hepatoma cells for 24 h, 48 h and 72 h, the inhibition rate of proliferation of hepatoma cells would improve

with the increase of action time and drug dose. The difference is statistically significant ($p<0.05$), as shown in Table 1.

The colony forming test could reflect that the single cell has the strong proliferative capacity and the cell colony forming capacity is an index to detect the efficacy of the anti-cancer drugs. With the increase of the drug dosages, the number of the colony forming of the hepatoma cells would be decreased accordingly and the difference is statistically significant ($p<0.05$), as shown in Table 2.

Annexin V-Fluorescein Isothiocyanate (FITC)/ Propidium Iodide (PI) is taken to detect the impact of esomeprazole on apoptosis of hepatoma cells. After esomeprazole acts on hepatoma cells for 24 h, 48 h and 72 h, the apoptosis rate of hepatoma cells would be improved with the increase of action time and drug dose. The difference is statistically significant ($p<0.05$), as shown in Table 3.

Expression of esomeprazole on hepatoma cell proliferation and cell cycle related genes could be found in the Western-blot test; compared with the model group, the protein expression level of Cyclin-Dependent Kinase 1 (CDK1), Cyclin-Dependent Kinase 2 (CDK2), cyclin A2 and cyclin E2 decreases significantly as the increase of esomeprazole dosage. The difference is statistically significant ($p<0.05$), as shown in Table 4.

Impact of esomeprazole on expression of apoptotic protein caspase-9 of hepatoma cells is found in the Western-blot test that compared with the model group; esomeprazole could decrease the expression of the apoptotic protein procaspase-9 level and increase the protein level expression of cleaved caspase-9, showing the dose dependence. The difference is statistically significant ($p<0.05$), as shown in Table 5.

Comparison of protein expression level of the hepatoma cell AKT/FOXO3a in each group as AKT protein level in the high dose group is significantly lower than that in the model group and low dose group and the FOXO3a protein level is higher than the model group and low dose group significantly. The difference is statistically significant ($p<0.05$). Phosphorylated (p)-AKT and p-FOXO3a protein levels in the high dose group and low dose group has the decreasing trend, as shown in Table 6.

TABLE 1: COMPARISON OF PROLIFERATION INHIBITION RATES OF THREE GROUPS AT DIFFERENT TIME ($\bar{x}\pm s$)

Group	n	Inhibition ratio (%)		
		24 h	48 h	72 h
Model group	3	1.25±0.34	4.37±1.23	10.15±4.51
Low dose group	3	6.52±1.04	17.24±5.63	40.11±7.32
High dose group	3	48.35±8.41	79.32±9.41	82.23±10.79
F		83.34	118.72	62.00
p		<0.001	<0.001	0.000

TABLE 2: COMPARISON OF NUMBER OF COLONY FORMING OF HEPATOMA CELLS IN THREE GROUPS ($\bar{x}\pm s$)

Group	n	Number of cell colony forming (×105)
Model group	3	57.23±10.35
Low dose group	3	35.21±9.57
High dose group	3	5.12±2.18
F		30.27
p		0.001

TABLE 3: COMPARISON OF APOPTOSIS RATE IN THREE GROUPS AT DIFFERENT TIME ($\bar{x}\pm s$)

Group	n	Apoptosis rate (%)		
		24 h	48 h	72 h
Model group	3	10.21±8.54	19.32±9.75	28.37±10.21
Low dose group	3	25.65±9.54	32.02±11.48	49.87±12.34
High dose group	3	38.34±11.23	49.36±12.07	74.23±13.75
F		6.16	5.49	10.63
p		0.035	0.044	0.011

TABLE 4: HEPATOMA CELL PROLIFERATION AND EXPRESSION LEVEL OF CELL CYCLE RELATED GENES (x̄±s)

Group	n	CDK1	CDK2	Cyclin A2	Cyclin E2
Model group	3	2.98±1.97	2.97±1.68	2.87±1.74	2.95±1.67
Low dose group	3	0.41±0.36	0.45±0.89	0.48±0.75	0.39±0.68
High dose group	3	0.12±0.14	0.31±0.25	0.15±0.03	0.13±0.02
F		5.53	5.49	5.52	6.72
p		0.043	0.044	0.044	0.029

TABLE 5: EXPRESSION LEVEL OF HEPATOMA CELL PROCASPASE-9 AND CLEAVED CASPASE-9 PROTEIN (x̄±s)

Group	n	Procaspase-9	Cleaved caspase-9
Model group	3	2.54±1.39	0.25±0.36
Low dose group	3	0.54±0.36	1.05±0.75
High dose group	3	0.35±0.27	2.95±1.47
F		6.28	6.07
p		0.034	0.036

TABLE 6: COMPARISON OF PROTEIN EXPRESSION LEVEL OF HEPATOMA CELL AKT/FOXO3A IN EACH GROUP (x̄±s)

Group	n	AKT	p-AKT	FOXO3a	p- FOXO3a
Model group	3	1.89±0.95	0.49±0.87	0.40±0.11	0.25±0.36
Low dose group	3	1.34±0.31[a]	0.20±0.54	1.32±0.25[a]	0.21±0.25
High dose group	3	0.19±0.03[a]	0.02±0.01	1.84±0.87[a]	0.20±0.14
F		6.78	0.48	5.76	0.03
p		0.029	0.639	0.04	0.971

Note: Compared with the model group, [a]$p<0.05$

The liver cancer, also known as malignant tumor, is one of the most common malignant tumors in China. The malignant tumor data issued by the National Cancer Center showed that the incidence rate of liver cancer ranks 4th in malignant tumors and its mortality rate ranks 2nd in malignant tumors. Men are prone to that with a male:female ratio of 3.5:1[8]. In the clinical treatment, the liver cancer is not sensitive to the chemotherapy and radiotherapy. Early diagnosis and comprehensive treatment based on surgical resection is the key to improve the efficacy of the long-term treatment. PPI is an important drug to inhibit gastric acid secretion after Histamine Type 2 (H_2) receptor blocking drugs. With wide application in the gastric acid related diseases, it is currently the strongest class of drugs to inhibit gastric acid secretion[9]. Mainly adopted to treat the disease at the initial period and long term treatment, esomeprazole could be taken for the long term maintenance treatment of esophagitis and symptomatic treatment of gastroesophageal reflux disease. It could prevent the recurrence of the esophageal reflux disease efficiently, but there are few studies on the role of PPI on the hepatoma cell[10]. In most normal cells, a small number of cells could grow, divide and proliferate, and the tumor cell has the significant biological characteristics, including self-growth, insensitive to growth inhibition and periodic proliferation, so that the tumor cell proliferation may threaten the life[11]. In the research, the CCK-8 test is carried out to detect the influence of esomeprazole on the hepatoma cell proliferation. The results show that esomeprazole could inhibit the proliferation of tumor cells more with the increase of time and drug dose. The colony forming could reflect the proliferative capacity of the single cell and it could detect the anticancer drugs effectively. The plate colony forming test is one of the effective methods to detect the proliferation of cultured cells[12]. The results showed that esomeprazole could inhibit the formation of the tumor cell colony in a dose-dependent manner. The increase in the number of tumor cells is related to cell proliferation and apoptosis. Apoptosis refers to the independent and orderly death of cells controlled by genes, to maintain the stability of the internal environment[13]. The researches show that the tumor cells have obvious characteristics of avoiding apoptosis. This study takes Annexin V-FITC/PI technology for detection. The results indicate that esomeprazole could significantly induce the apoptosis

of hepatoma cells. Apoptosis should also be activated by related protein. It is found in this test that esomeprazole affects the expression of the apoptotic protein caspase-9 after acting on the hepatoma cells. The apoptotic protein is activated. Compare with the model group, the protein expression level of CDK1, CDK2, cyclin A2 and cyclin E2 decreases significantly as the increase of dosage of esomeprazole, indicating that it could further inhibit the proliferation and apoptosis induction of the hepatoma cell.

The research further analyzes the pathways through which esomeprazole affects the biological characteristics of tumor cells. The Phosphoinositide 3-Kinases (PI3K)/AKT is mainly used as an important pathway connecting intracellular and extracellular signals and AKT is the main key point of this pathway, which is activated by phosphorylation, so as to affect the biological characteristics of cells[14]. FOXO3a is an important transcription factor in the downstream signal pathway, and it obviously changes the biological characteristics and has the impact on that of tumors after being activated by the protein kinase and factor inhibitor. It could be found in the researches that AKT/FOXO3a pathway participates in the cell proliferation, apoptosis and other processes[15]. The research results show that the AKT protein level in the high dose group is significantly lower than the model group and dosage group, indicating that the high dose drugs significantly activate AKT/FOXO3a signaling pathway.

To sum up, esomeprazole inhibits the tumor cell proliferation and induces apoptosis by activating the AKT/FOXO3a signal pathway, to provide the important foundation and to study the liver cancer treatment. However, due to the limited sample size and short observation time, it is necessary to accumulate samples to further explore the effect of esomeprazole on the biological characteristics of hepatoma cells by regulating AKT/FOXO3a pathway.

Conflict of interests:

The authors declared no conflicts of interest.

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