INHIBITORY EFFECT OF CELECOXIB ON PROLIFERATION, APOPTOSIS AND CELL CYCLE ARREST OF LARYNGEAL CARCINOMA CELLS THROUGH COX-2 RELATED PATHWAY

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ABSTRACT

Objective: *To investigate the effect and mechanism of celecoxib on inhibiting proliferation, inducing apoptosis and cell cycle arrest of laryngeal carcinoma tumour cells through cyclooxygenase-2 (COX-2) related pathway.*

Methods: *Hep-2 cells were treated with different concentrations of celecoxib medium. Namely, 10 µm, 30 µm, 50 µm, 70 µm and 100 µm celecoxib groups and a blank control group were cultured for 12 h, 24 h and 48 h. The effects of celecoxib on cell activity, apoptosis, cell cycle and related proteins (COX-2, Bcl-2, p16^{INK4a}, p27^{KIP}, p 21$^{waf/cip1}$) were observed.*

Results: *Compared with the control group at 12 h, 24 h and 48 h, the cell inhibition rate of celecoxib group (10 µm, 30 µm, 50 µm, 70 µm, 100 µm) was significantly higher than that of the control group (P<0.05). Compared with the control group, the apoptosis rate of celecoxib group (10 µm, 30 µm, 50 µm, 70 µm, 100 µm) increased significantly (P<0.05) in a dose-dependent manner. Compared with the control group, the proportion of G0/G1 in celecoxib group (10 µm, 30 µm, 50 µm, 70 µm, 100 µm) increased significantly, while the proportion of S decreased significantly in a dose-dependent manner. Compared with the control group, the expression of COX-2, Bcl-2 protein in celecoxib group (30 µm, 50 µm, 70 µm, 100 µm) decreased significantly, while the expression of p27KIP protein in p16INK4A group was significantly higher than that in the control group (P<0.05). There was no significant difference in p21waf/cip1 protein expression between the celecoxib group and the control group (P>0.05).*

Conclusion: *The inhibitory effect of celecoxib on the proliferation, apoptosis and cell cycle arrest of laryngeal carcinoma cells may be achieved through a COX-2 related pathway.*

Keywords: *Celecoxib, COX-2, laryngeal carcinoma, proliferation activity, apoptosis, cell cycle.*

DOI: 10.19193/0393-6384_2020_6_572

Received *November 30, 2019;* ***Accepted*** *January 20, 2020*

Introduction

Laryngeal carcinoma is a head and neck malignant tumour, which is divided into primary and secondary types. There is no exact cause of the disease, and it is considered that smoking, drinking, industrial development, increasing air pollution, and long-term exposure to toxic substances can accelerate the development of laryngeal carcinoma[1]. The early symptoms of the disease are not significant. Most patients had seen a doctor because of obvious symptoms, but the condition had already progressed to the middle and late stages, where the lymph node metastasis and distant metastasis of some patients had already occurred[2]. Hoarseness, cough, dysphagia and the like are the main manifestations of laryngeal carcinoma, and surgical treatment is mainly adopted for early patients, where the effect is more obvious. If patients who are in the middle and advanced stages are treated with an operation, the clinical curative effect is not ideal, and patients lose the sound production function. As a result, radiotherapy and chemotherapy[3] are supported. Cyclooxygenase (COX) is a two-functional enzyme, which is an important speed-limiting enzyme for the participation of arachidonic acid (AA) in the synthesis of prostaglandins. COX-1 and COX-2 are the main forms, in which COX-2 is difficult to detect in normal cells, The increase in the level of COX-2 expression is increased[4] under multiple signal pathway mediat-

ed by stimulation of the production of the product. The clinical study shows that COX-2 is abnormally expressed in the head and neck malignant tumour, and the expression level of COX-2 is closely related to the recurrence of the patient[5]. The non-opioid, anti-inflammatory agent has anti-inflammatory, analgesic and anti-tumour effects.

Celecoxib is a drug capable of effectively controlling pain, which can selectively inhibit the activity of COX-2, and the adverse reaction is light[6]; it is reported that it has a better effect in the treatment of cancer such as gastric cancer[7]. Therefore, the study on the expression of COX-2 by celecoxib in this study is to explore its mechanism of inhibiting the proliferation of laryngeal cancer cells, inducing apoptosis and cell cycle arrest.

Materials and methods

Experimental cell

Laryngeal Carcinoma cell line Hep-2 was purchased from Shanghai Zishi Biotechnology Co., Ltd.

Reagents and instrument reagents

Reagent

Tetramethyl azo blue (Shanghai Huzhen Industrial Co., Ltd); culture solution (Shanghai Yubo Biotechnology Co., Ltd.); trypsin (Nanjing Dawsff Biotechnology Co., Ltd.); Cell cryopreservation pipe (Beijing Jihuibo Biotechnology Co., Ltd.); celecoxib (Sichuan Vikic Biotechnology Co., Ltd.); Rabbit anti-human monoclonal COX-2 antibody (Beijing Bolsey Technology Co., Ltd.); rabbit anti-human polyclonal Caspase antibody and rabbit anti-human monoclonal AIF antibody (Xiamen Research Biotechnology Co., Ltd.); rabbit anti-human monoclonal BAX antibody (Xiamen Huijia Biotechnology Co., Ltd.); mitochondrial dye (Tianjin Taize Xingye Biotechnology Co., Ltd.). The cell cycle detection kit (Beijing Donggebo Biotechnology Co., Ltd.); the mitochondrial extraction kit (Anhui Jingke Biotechnology Co., Ltd.); the first anti-second antibody dilution solution (Hangzhou Xiaoyou Biotechnology Co., Ltd.); PBS powder (Tianjin Taize Xingye Biotechnology Co., Ltd.); PVDF membrane (Qiyi Biotechnology (Shanghai) Co., Ltd.); and the cell lysate (Shanghai Yuanmu Biotechnology Co., Ltd.).

Instruments

Inverted incineration microscope (Guangzhou Mingmei Optoelectronic Technology Co., Ltd.); low-speed, ordinary centrifuge (Shanghai Jolai Experimental instrument Co., Ltd.); Super-clean working platform (Su Jie Medical device (Suzhou) Co., Ltd.); Microliquid remover (Shanghai High Pigeon Industry and Trade Co., Ltd.); enzyme labelling instrument (Kehua Biology); flow Cytometer (Beckman, USA); Fluorescent gel imaging system (Nanjing Shiyan instrument and equipment Co., Ltd.)

Methods

• Laryngeal squamous cell carcinoma cell line Hep-2 was cultured and digested with trypsin when the cells were adherent to the appropriate value, and then continued to be cultured. The logarithmic cells were digested, then centrifuged and discarded, mixed with DMSO, refrigerated at 4°C for 1 h, and then stored in liquid nitrogen.

The liquid nitrogen was taken out for rewarming, moved to the centrifuge tube, centrifuged and discarded in the culture medium, then mixed with the culture medium and cultured. 24 h later, the observation was carried out.

• The logarithmic growth cells were digested and cultured on 96 well plate. After overnight culture, the cells were cultured in 10 µm celecoxib group, 30 µm celecoxib group, 50 µm celecoxib group, 70 µm celecoxib group, 100 µm celecoxib group and blank control group for 12 h, 24 h and 48 h.

• MTT method: MTT was added 4 h before celecoxib disappeared, then cultured for 4 h, dimethyl sulfoxide (DMSO), was added into the culture medium to make full concussion, and the optical density (OD) value of parallel pores was measured and calculated by enzyme labelling instrument.

The formula for calculating the inhibition rate was (the average OD value of the control group-the average OD value of the drug group) / (the average OD value of the control group-the OD value of the blank control group).

• The apoptosis rate was detected by flow cytometry: the cells in each group were digested, and the cells were suspended with PBS and centrifuged. The cells were collected and resuspended with buffer. Celecoxib group was incubated with Annexin-FITC for light avoidance, then PI staining solution was added for 10 min, and the cell suspension was taken for detection.

• Flow Cytometry was used to detect the cell cycle: the cells in each group were washed with PBS solution, centrifuged and discarded, the cell concentration was adjusted to the appropriate value, and then the cells were centrifuged and discarded by centrifugation. PI/Triton X ≥100 staining solution was

added to the cell cycle, and the cells were resuspended and stained for 30 min. Cell cycle analysis was carried out by BD software.

• Western Blot: the cells were pre-treated, the total protein was extracted, and the concentration was measured. After electrophoresis, the cells were closed after being transferred to the membrane. Then rinsed with PBST, incubated at 4°C for the night and incubated with the second antibody for 1 h. The colour reaction was carried out by adding colour reagent, and then detected by machine after the reagent was sucked dry.

Statistical software

The econometric data were expressed by (x̄±s), and the variance analysis was used in the comparison between the two groups ($P<0.05$), and there was a significant difference between the two groups ($P<0.05$).

Results

The effect of different doses of celecoxib on cell activity

Compared with the control group at 12 h, 24 h and 48 h, the cell inhibition rate of the celecoxib dose group (10 mm, 30 mm, 50 mm, 70 mm and 100 mm) was significantly increased ($P<0.05$) in a time-dose dependent manner. See table 1.

Group	Inhibition rate (%)		
	12 h	24 h	48 h
Control group	2.22±0.42	5.67±0.63	7.93±0.59
10 µm celecoxib dose group	7.78±1.48[a]	25.43±2.65[a]	30.12±3.00[a]
30 µm celecoxib dose group	29.13±1.60[a]	40.78±2.46[a]	49.56±3.56[a]
50 µm celecoxib dose group	44.25±3.49[a]	59.63±2.77[a]	65.97±1.69[a]
70 µm celecoxib dose group	53.50±5.28[a]	65.92±4.00[a]	80.92±3.48[a]
100 µm celecoxib dose group	67.19±3.71[a]	78.48±4.41[a]	87.00±4.72[a]

Table 1: Effect of celecoxib on cell activity (x̄±s).
Note: compared with the control group, [a]P<0.05.

Effects of different doses of celecoxib on apoptosis

Compare with the control group, celecoxib dose group (10 µm, 30 µm, 50 µm, 70 µm, 100 µm) was significantly higher than that of the control group ($P<0.05$), and in a dose-dependent manner. See table 2.

Group	Apoptosis rate
Control group	1.31±0.45
10 µm celecoxib dose group	8.91±1.71[a]
30 µm celecoxib dose group	14.61±2.25[a]
50 µm celecoxib dose group	20.41±2.52[a]
70 µm celecoxib dose group	29.01±2.85[a]
100 µm celecoxib dose group	32.81±3.07[a]

Table 2: Effects of different doses of celecoxib on apoptosis (x̄±s).
Note: compared with the control group, [a]P<0.05.

Effect of different doses of celecoxib on the cell cycle

The proportion of G0/G1 in the Celecoxib dose (10 µm, 30 µm, 50 µm, 70 µm, 100 µm) was significantly increased as compared to the control group, and G2/M, S accounted for a significant decrease ($P<0.05$) and dose-dependent. See Table 3.

Group	G0/G1 (%)	S (%)	G2/M (%)
Control group	33.01±5.31	42.71±5.31	28.81±4.21
10 µm celecoxib dose group	37.81±5.31[a]	39.12±3.31[a]	23.31±4.01[a]
30 µm celecoxib dose group	46.01±4.81[a]	38.51±4.01[a]	11.61±2.41[a]
50 µm celecoxib dose group	61.01±6.31[a]	34.51±5.01[a]	4.81±0.81[a]
70 µm celecoxib dose group	71.31±4.91[a]	26.91±3.61[a]	2.11±0.51[a]
100 µm celecoxib dose group	75.48±3.95[a]	23.21±3.56[a]	1.56±0.32[a]

Table 3: Effect of different doses of celecoxib on the cell cycle (x̄±s).
Note: compared with the control group, [a]P<0.05.

Effects of different doses of celecoxib on expression of cell-related proteins

Compared with the control group, the expression of COX-2, BCL-2 protein in celecoxib (30 µm, 50 µm, 70 µm, 100 µm) group cells was significantly decreased, and the expression of p16INK4A, p27KIP protein was considerably increased ($P<0.05$), which was dose-dependent. There was no significant difference in p21waf/cip1 protein expression compared with the control group ($P>0.05$). See figure 1 and 2.

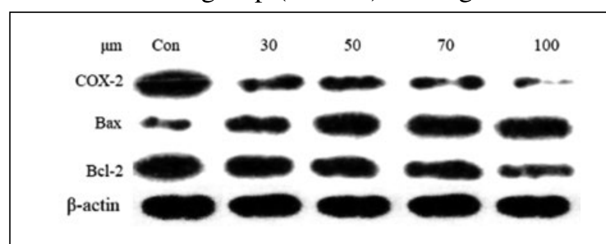

Figure 1: Effects of celecoxib at different doses on the expression of COX-2, Bax and Bcl-2.

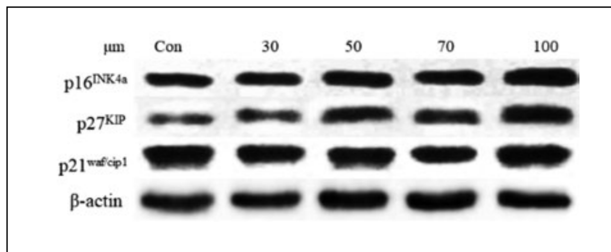

Figure 2: Effects of different doses of celecoxib on expression of cell-related proteins $p16^{INK4a}$, $p27^{KIP}$, $p21^{waf/cip1}$.

Discussion

The incidence of laryngeal cancer in China is slightly lower than that in foreign countries, but it cannot be ignored. It has been reported that laryngeal cancer is more common in men and more in the north than in the south. With the development of medical knowledge and the deepening of the study of laryngeal carcinoma, the possible pathogenesis of laryngeal carcinoma has been gradually discovered, and the 5-year survival rate of patients has been improved. However, there are still many patients with recurrence and metastasis after an operation, so early diagnosis and effective and reasonable treatment is the key to prolonging the survival time of patients with laryngeal carcinoma[8].

At present, celecoxib has become the focus of antitumour research. As a selective inhibitor of COX-2, celecoxib has been used in tumour prevention and treatment[9]. COX-2 is highly expressed in a variety of cancers. In addition, it is also highly expressed in oesophageal cancer, breast cancer and other cancers[10-11]. It has been reported that if patients with chronic pain use non-steroidal anti-inflammatory drugs for a long time, the incidence of colon cancer is significantly lower than that of patients without use[12]. At present, it is recognized that celecoxib can act at the cell and tissue level, and finally play an anti-tumour role[13]. Bax and Bcl-2 are the key genes in the apoptosis-signalling pathway, which have 'gated' function in the process of apoptosis[14]. Bax belongs to an apoptosis-promoting gene, whereas Bcl-2 belongs to apoptosis-inhibiting gene. In the study of liver cancer, it is found that celecoxib can induce apoptosis, and its mechanism may be to increase the ratio of Bax/Bcl-2 by downregulating the expression of COX-2[15]. During the cell cycle, the G/S and G2/M phases are more critical. $P16^{INK4a}$ belongs to the cell cycle tumour inhibitor gene, which plays an important role in slowing cell division and prolonging division time. P21waf/cip1 is a negative regulator, which can participate in the regulation of the cell cycle and play an important role. P27KIP can inhibit cell proliferation, and P27KIP can inhibit the activation of cyclinD and other genes.

In this experiment, cancer cells were treated with different doses of celecoxib. The results showed that the cell inhibition rate of celecoxib group (10 µm, 30 µm, 50 µm, 70 µm, 100 µm) was significantly higher than that of the control group at 12 h, 24 h and 48 h ($P<0.05$). The results showed that celecoxib had a significant inhibitory effect on cell activity at a certain time and dose. Compared with the control group, the apoptosis rate of the celecoxib group (10 µm, 30 µm, 50 µm, 70 µm, 100 µm) increased significantly ($P<0.05$) in a dose-dependent manner. The results showed that celecoxib could induce apoptosis in a certain dose. Compared with the control group, the proportion of G0/G1 in celecoxib group (10 µm, 30 µm, 50 µm, 70 µm, 100 µm) increased significantly. In contrast, the proportion of G2/M and S decreased significantly (P<0 05), and the proportion of S also decreased markedly in a dose-dependent manner compared with the control group (10 µm, 30 µm, 50 µm, 70 µm, 100 µm). It is suggested that celecoxib can block the cell cycle in G0/G1 phase at a certain dose. Compared with the control group, the expression of COX-2, Bcl-2 protein in the celecoxib group (30 µm, 50 µm, 70 µm, 100 µm) decreased significantly, whereas the expression of p27KIP protein in the p16INK4A group was considerably higher than that in the control group ($P<0.05$). There was no significant difference in p21waf/cip1 protein expression between the celecoxib group and the control group ($P>0.05$). It is suggested that celecoxib can inhibit the proliferation activity, induce apoptosis and cell cycle arrest of laryngeal carcinoma cells through COX-2 related pathway, and the mechanism of the celecoxib-inducing cell cycle may be achieved by up-regulating the expression of $p16^{INK4a}$ and p^{27KIP}.

In conclusion, the inhibitory effect of celecoxib on the proliferation activity, apoptosis and cell cycle arrest of laryngeal carcinoma tumour cells may be realized through the COX-2 related pathway. Celecoxib has certain potential for the treatment of laryngeal carcinoma and is expected to become one of the main chemotherapeutic drugs for the treatment of laryngeal carcinoma.

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