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Influence of *Ginkgo biloba* extract on the proliferation, apoptosis of ACC-2 cell and Survivin gene expression in adenoid cystic carcinoma of lacrimal gland

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ABSTRACT

Objective: To explore the influence of extract of *Ginkgo biloba* (EGB) on the proliferation, apoptosis of ACC-2 cell and Survivin gene expression in adenoid cystic carcinoma (ACC) of lacrimal gland. **Methods:** ACC-2 cell in human with ACC of lacrimal gland was in vitro cultured. MTT method was used for cell proliferation detection. Annexin V/PI double-staining flow cytometer was used to detect cell apoptosis and cell cycle. Survivin gene expression was analyzed by RT-PCR and Western blotting. **Results:** EGB had inhibitory effect on the proliferation of ACC-2 cell with significant dose-effect relationship, and there was statistical difference when compared with the control group ($P<0.01$). The inhibitory concentration 50 % (IC_{50}) is 88 mg/L. The flow cytometer test indicated that EGB can gradually increase ACC-2 cell in G_0-G_1 stage and decrease it in G_2-M and S stage. With the increase of dose, the apoptosis rate of ACC-2 cell was obviously increased ($P<0.05$ or $P<0.01$). EGB had certain inhibitory effect on Survivin gene expression of ACC-2 cell, and Survivin gene expression was decreased with the increasing of the EGB concentration ($P<0.01$). **Conclusions:** EGB can effectively inhibit Survivin gene expression of ACC-2 cell in human with ACC of lacrimal gland, induce the apoptosis of ACC-2 cell and inhibit tumor cell proliferation.

1. Introduction

Adenoid cystic carcinoma (ACC) of lacrimal gland is the most common and most malignant tumor in malignant epithelial tumour of lacrimal gland. The incidence rate of ACC of lacrimal gland takes up 29% of epithelial tumour of lacrimal gland, ranking as the second following pleomorphic adenoma[1,2]. ACC of lacrimal gland is insensitive to conventional radiotherapy and chemotherapy, therefore surgical treatment is mainly used. However, ACC of lacrimal gland has high invasion growing along nerve[3]. It can't be eradicated by operation, and the recurrence rate after operation is high with poor long-term effect. Therefore, exploring new treatment method has become the new direction of ACC research.

Extract of *Ginkgo biloba* (EGB) has inhibitory effect on tumor cell proliferation, induces apoptosis of various tumor cells[4,5], and strengthens the antitumor effect of chemotherapeutic drugs. However, there is no report about the antitumor effect of EGB on ACC nor about its mechanism. Survivin, a new apoptosis inhibitor found in recent years, has the apoptosis inhibition effect through inhibiting the activity of Caspas-3 and Caspas-7 in the downstream of Caspas cascade reaction, which is also a hot spot in tumor related gene research[6,7]. This study explored the possible antitumor mechanism of EGB by observing the influence of EGB on proliferation and apoptosis of ACC-2 cell and Survivin gene expression in ACC of lacrimal gland, which provided experimental and theoretical basis for further clinical application.

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2. Materials and methods

2.1. Materials

2.1.1. Drugs and reagents

Extract of *Ginkgo biloba* (EGB761, trade name: Ginaton) was produced by Dr Willmar Schwabe Pharmaceutical Factory (Germany, batch number: 1140808). RT-PCR kit was purchased from Germany Qiagen Company. Annexin V-FITC apoptosis detection kit was purchased from Beijing Baosai Biotechnology Co., Ltd. Survivin antibody was from America Santa Cruz Company; RPMI-1640 culture solution and fetal calf serum were purchased from American Gibco Company. MTT, dimethyl sulfoxide (DMSO) and agarose were from America Amresco Company; Trizol total RNA extraction reagent was from Invitrogen Company; Taq DNA polymerase, Oligod and RNAsin were purchased from Promage Company; Primers were synthesized by Shanghai Sangon Biotechnology Co., Ltd.

2.1.2. Cell strains

ACC-2 cells- human ACC of lacrimal gland were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Under the condition of 37 ℃, 5% CO_2 and saturated humidity, the cells were cultured in RPMI-1640 culture solution with 10% fetal calf serum and 2% Glutamine.

2.1.3. Instrument and equipment

CO_2 incubator (MCO175, SANYO); inverted microscope (IX-70, Olympus); horizontal flow clean bench (ZHJH-2112); flow cytometry (FACS Calibur, Becton Dickinson); PCR amplifier (Roche); ELX800 enzyme-labeling instrument.

2.2. Methods

2.2.1. Cell proliferation detected by MTT method

2×10^7/mL cell suspension was made with ACC-2 cells in logarithmic growth phase. Then the cell suspension was inoculated into a 96-well culture plate with 100 μL in each well for 24-hour culture. 100 μL EGB was added to make the final concentration was 0, 10, 20, 40, 80 and 160 mg/L, respectively. At the same time, control well and zero well was set, and there were 6 parallel holes in each group. After another 48-hour culture, 20 μL MTT solution (5 g/L) was added into each well. The supernatant was absorbed after 4-hour culture. Then, 200 μL DMSO was added into each well. The culture plate was fully oscillated. After the reaction stopped, absorbance value (A value) at 490 nm was detected by enzyme-labeling instrument. The cell proliferation inhibition rate was calculated by the following formula.

Inhibition rate= (A value of the control group-A value of the drug group)/A value of the control group×100%

2.2.2. Cell apoptosis rate detected by Annexin V/PI double staining flow cytometry

1×10^6/mL cell suspension was made with ACC-2 cells in logarithmic growth phase. Then the cell suspension was inoculated into a 12-well culture plate with 2 mL in each well. 100 μL EGB was added to make the final concentration was 0, 10, 40 and 160 mg/L, respectively. After cultured for 48 hours, the cell concentration was regulated to 1×10^6/mL. At 4 ℃, 1 mL cell suspension was centrifuged at the speed of 1 000 r/min for 10 minutes, and then the supernatant was abandoned. 1 mL cold PBS was added to resuspend cells and repeat centrifugation and supernatant abandon. 200 μL binding buffer was added for resuspension, then 10 μL Annexin V-FITC and 5 μL PI were added and well mixed. After dark incubation at room temperature for 15 minutes, 300 μL binding buffer was added. Cell apoptosis rate was detected immediatedly by flow cytometry, and the cell cycle was analyzed.

2.2.3. Survivin mRNA expression detected by RT-PCR

ACC-2 cells in logarithmic phase were cultured for 24 hours. EGB was added to make the final concentration was 0, 10, 40 and 160 mg/L, respectively. After cultured for another 48 hours, total RNA was extracted by Trizol reagent. RNA was detected by electrophoresis. cDNA was synthesized by reverse transcription. Survivin was amplified by PCR with cDNA as the template and β-action as the internal reference gene. The upstream primer of Survivin: 5′-GGCACCA- GAGGCAGTAACCA-3′, downstream primer: 5′-GGACCTTCGGTGACTGATGATCTAA-3′; The upstream primer of β-action: 5′-AACGGATTTGGTCGTATTGGG-3′, downstream primer: 5′-AGTCTT- TCTGGGTGGCAGTGAT-3′. PCR was run for 35 cycles with 5 minutes per 95 ℃ predenaturation, 30 s/ 94 ℃ denaturation, 30 s/58 ℃ annealing and 30 s/72 ℃ elongation; 12 μL PCR product was conducted agarose gel electrophoresis with 2% agarose. Gel imaging analysis system was used to observe and record the results.

2.2.4. Survivin gene expression detected by Western blotting

1×10^7 ACC-2 cells after 48 hours reaction with 0, 10, 40 and 160 mg/L EGB, respectively were collected. 200 μL protein lysis solution was added. After 1 hour lysis at 4 ℃, 20 minutes centrifugation at 4 ℃ with the speed of 12 000 r/min was conducted, and then the supernatant was taken. Protein concentration was detected by Coomassie Brilliant Blue protein detection method. After 10 minutes total protein denaturation of the 80 μg sample, SDS-PAGE gel electrophoresis was conducted. The isolated protein was transferred to PVDF membrane by electrotransfer. It was sealed for 1 hour with 5% BSA at 37 ℃. Then anti-Survivin monoclonal antibody was added and incubated for 16 hours at 4 ℃, and then the membrane was washed three times. Then the goat anti mouse secondary antibody was added and incubated for 1 hour at 37 ℃, and then the membrane was washed three times. Chemiluminescence technique was used to detect and analyze the results. Relative quantitative analysis was conducted for the gray scale of the protein strips using β-action strip as the reference. Relative content of Survivin=gray value of Survivin/gray value of β-action, Inhibition ratio=1- Relative content of Survivin in drug group/ Relative content of Survivin in control group.

2.2.5. Statistical analysis

Statistical treatment was conducted by SPSS15.0 software. Measurement data was expressed as mean±SD. *t* test or single factor analysis of variance was used. *Chi*-square test was used for the rate comparison. A P value of <0.05 was considered as statistical significance.

3. Results

3.1. Inhibitory action of EGB on ACC-2 cell proliferation

EGB in different concentration has inhibitory effect on the in vitro proliferation of ACC-2 cell, and with the increase of EGB concentration, the inhibitory action was also increased, showing significant dose-effect relationship. There was statistical difference between the drug group in different doses and the control group (t=11.67, 16.31, 22.01, 24.18, 26.68, P<0.01). The IC_{50} was 88 mg/L by Logit method (Table 1).

Table 1
Inhibitory action of EGB on ACC-2 cell proliferation (mean±SD, n=6).

Groups		A value	Inhibitory rate (%)
Control group		0.835±0.037	-
EGB group	10 mg/L	0.643±0.016[b]	22.99
	20 mg/L	0.561±0.018[b]	32.81
	40 mg/L	0.457±0.020[b]	45.27
	80 mg/L	0.415±0.021[b]	50.30
	160 mg/L	0.382±0.019[b]	54.25

[b]: P<0.01 *vs*. control group.

3.2. Influence of EGB on ACC-2 cell cycle and apoptosis

EGB can gradually increase ACC-2 cell in G_0-G_1 stage and decrease that in G_2-M and S stage. With the increase of dose, the apoptosis rate of ACC-2 cell was obviously increased (χ^2=2.97, 5.62, 7.96, P<0.05 or P<0.01, Table 2).

Table 2
Influence of EGB on ACC-2 cell cycle and apoptosis.

Groups		G_0-G_1 stage (%)	G_2-M stage (%)	S stage (%)	Apoptosis rate (%)
Control group		46.37	8.11	45.52	8.68
EGB group	10 mg/L	52.97	7.15	39.88	17.96
	40 mg/L	56.68	5.93	37.39	21.72[a]
	160 mg/L	61.38	4.59	34.03	24.53[b]

[a]: P<0.05, [b]: P<0.01 *vs*. control group.

3.3. Influence of EGB on Survivin gene expression by Western blotting analysis

Survivin gene expression significantly reduced as the EGB concentration increases, showing obvious dose-effect relationship (Figure 1).

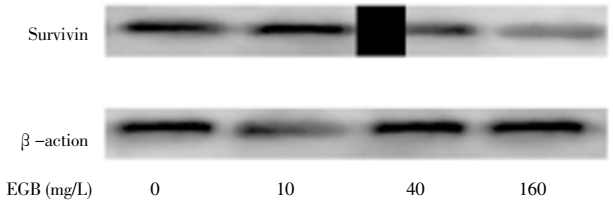

Figure 1. Influence of EGB on Survivin gene expression analyzed by Western blotting.

3.4. Influence of EGB on the relative content of Survivin gene in ACC-2 cell and inhibitory rate

EGB in different concentration has inhibitory effect on the Survivin gene of ACC-2 cell, and with the increase of EGB concentration, the inhibitory action was also increased, showing significant dose-effect relationship. There was statistical difference between the drug group in different doses and the control group (t=8.77, 10.04, 15.59, P<0.01, Table 3).

Table 3
Influence of EGB on the relative content of Survivin gene in ACC-2 cell and inhibitory rate (mean±SD, n=6).

Groups		Relative content	Inhibitory rate (%)
Control group		0.85±0.06	
EGB group	10 mg/L	0.52±0.07[b]	38.82
	40 mg/L	0.44±0.08[b]	48.24
	160 mg/L	0.31±0.06[b]	63.53

[b]: P<0.01 *vs*. control group.

4. Discussion

ACC of lacrimal gland is a highly invasive tumor with poor prognosis, the pathogenesis and treatment of which is an important topic demanding prompt solution in clinic. After years of research, no effective therapeutic tool has been found. Having the advantages like multi-channel, multi-target and low toxicity, the traditional Chinese medicine has become a hot spot in tumor research. EGB is the active principle separated and purified from *Ginkgo biloba*, mainly containing flavonoid and lactone components. EGB is mainly used for cardiovascular and cerebrovascular disease and peripheral circulation disorder[8,9]. In recent years, some researches proved that EGB has certain antineoplastic activity, having lethal effect on gastric cancer[10], liver cancer[11], intestinal cancer[12] and breast cancer[13], but the precise mechanism of antitumor effect was unclear. EGB in different concentration has inhibitory effect on the *in vitro* proliferation of ACC-2 cell with significant dose-effect relationship (P<0.01). The IC_{50} was 88 mg/L by Logit method.

Some research indicated that the development of tumor is not only related with the abnormal proliferation and differentiation of cell, but also related with apoptosis[14,15]. Cell apoptosis is a physiological cell suicide process in order to maintain organism balance, which is regulated by gene. In this study, the Annexin V/PI double staining flow cytometry detection indicated that EGB promoted the apoptosis of the ACC-2 cell and changed the cell cycle distribution of ACC-2 cell. After 48h effect of EGB, the percentage of cells in G_0-G_1 stage was significantly increased while the percentage of cells in S stage was significantly reduced, and this retarding effect has certain dose dependency. This indicated that the proliferation inhibitory effect of EGB on ACC-2 cell maybe related with EGB changed the cell cycle distribution, which is, stagnating most cells in cell differentiation stage (G_1 stage) and blocking the transform to S stage which reduced the DNA synthesis and mitosis at the same time promoted

the differentiation.

Survivin is one of the cell regulatory factors having the strongest anti-apoptosis effect. Located in Chromosome17q25 and composed by 3 introns and 4 exons, Survivin can encode 142 amino acids[16-19] with the length of 14.7 kb. Survivin is a member of Inhibitor of apoptosis proteins (IAPs). Survivin can inhibit the activation of Caspase-3 and block cell apoptosis consequently, the overexpression of which maybe the main factor for the tumor development and poor prognosis[20,21]. Researchers found that[22,23] Survivin selectively express in malignant tumor, and with increase of the malignant degree, Survivin expression increases. But Survivin doesn't or seldom express in normal tissue. In this study, using Survivin as the research target, EGB in different concentration was acted on ACC-2 cell with ACC of lacrimal gland. Survivin gene expression was analyzed by Western blotting. The results indicated that Survivin gene expression obviously decreased as the EGB concentration increased, showing significant dose-effect relationship. This study presumed that the mechanism and molecular basis of EGB inducing ACC-2 apoptosis maybe as follows: First, EGB inhibits Survivin gene expression in cell apoptosis pathway, then Caspase activity is inhibited accordingly, at last, signal transduction pathway of cell apoptosis is activated.

Conflict of interest statement

We declare that we have no conflict of interest.

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