Tropical Journal of Pharmaceutical Research May 2022; 21 (5): 951-956
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v21i5.7

Original Research Article

Effect of glaucocalyxin B on the protein expressions of PTEN, Beclin1 and LC3 in a mouse model of transplanted cervical cancer, and its significance

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Sent for review: 31 December 2021 *Revised accepted: 27 April 2022*

Abstract

Purpose: *To determine the effect of glaucocalyxin B (GLB) on the protein expressions of PTEN, Beclin1 and LC3 in a mouse model of transplanted cervical cancer, and its significance.*
Methods: *A mouse model of transplanted cervical cancer was established in female BALB/C mice. The model mice were divided into control group, low-dose GLB group and high-dose GLB group. Mice in low-dose and high-dose groups were given intraperitoneal injection of low-dose GLB and high-dose GLB, respectively. The volume and weight of transplanted tumor were measured and compared between the two groups. Serum levels of CEA and CA125 were assayed by enzyme-linked immunosorbent assay (ELISA). The expressions of phosphatase and tensin homolog (PTEN), autophagy-related factor microtubule-associated protein-1 (Beclin-1), microtubule-associated protein 1 light chain 3 (LC3), apoptosis-related protein p53, and Bax were determined using SABC immunohistochemical operation.*
Results: *On days 5, 10 and 15, the volume and weight of transplanted tumor, and levels of CA125 and CEA in low- and high-dose GLB groups were significantly and dose-dependently lower than those in control group ($p < 0.05$). Results from immunohistochemistry showed that the protein expression levels of PTEN, Beclin-1, LC3, p53 and Bax were significantly and dose-dependently higher in low- and high-dose GLB groups than in the control group ($p < 0.05$).*
Conclusion: *Glaucocalyxin B significantly and dose-dependently induces apoptosis of cervical cancer cells and inhibits their growth by regulating the protein expressions of PTEN, Beclin1 and LC3. Thus, glaucocalyxin B is a potential adjunct therapy in the management of cervical cancer.*

Keywords: *Glaucocalyxin B, Mouse, Cervical cancer transplanted tumor, PTEN, Beclin1, LC3*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Cervical cancer is one of the most common gynecological malignant tumors in the world: it ranks third in incidence of malignant tumors in females, and it often occurs in people over 40 years old [1]. In recent years, several studies have shown that the main cause of cervical cancer is persistent infection with high-risk human papilloma virus (HPV) [2]. In China, HPV infection rate has increased to a certain extent, and the incidence of cervical cancer has

increased significantly among the younger population as a result of changes in sexual concepts, multiple sex partners, and early exposure to sexual activity [3]. Traditional Chinese Medicine (TCM)-related research on anti-cervical cancer therapy has become popular in recent years. Blue calyx fragrant tea herb is a genus of the fragrant tea herb in labiaceae which is widely distributed in nature, and it is used in TCM for *clearing heat* and detoxification, *activating* blood circulation and removing blood stasis, and as an anti-tumor agent [4]. Glaucocalyxin B (GLB) is a TCM compound extracted from Blue calyx. It has been reported that GLB inhibited the growths of tongue squamous cell carcinoma, breast cancer and other malignant tumors, although there are limited studies on its effect on cervical cancer [5]. In this study, the effects of GLB on protein expressions of PTEN, Beclin-1 and LC3 were determined in a female mouse model of BALB/C cervical cancer graft.

EXPERIMENTAL

Animals

Sixty (60) female BALB/C mice aged 4 - 6 weeks and weighing 18 – 22 g, were obtained from Beijing Stomach-Pain Lihua Experimental Animal Technology Co. Ltd (certificate Nos. = 114007000633088 & SCXK (Beijing 2012-0001)). The nude mice were maintained in an animal house at an average temperature of 23 ± 2 ºC, and humidity of 50 - 60 % in an environment with 12-h light/12-h dark photoperiod. The nude mice were allowed *ad libitum* access to laboratory diet and clean drinking water. This research was approved by the Animal Ethical Committee of Sinopharm Dongfeng General Hospital according to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [6] (the approval number was 20200932).

Glaucocalyxin B

Glaucocalyxin B (GLB) was supplied by Nanjing Puyi Biotechnology Co. Ltd. (specification: 20 mg; Batch no. 20180621).

Cell culture

Human cervical cancer HeLa cell line was provided by the Cancer Institute of the Third Affiliated Hospital of Harbin Medical University and thawed in a 37 ºC incubator and centrifuged at 12000 rpm for 3 min. The supernatant was discarded, and a suspension of the cytoplasmic single cells was cultured in an incubator at 37 ºC. Cells at logarithmic growth were washed with 0.9 % normal saline, followed by incubation with 1 mL of trypsin for 1 min. Following detachment, the cells were centrifuged at 13000 rpm for 2 min, and the supernatant was discarded. Then, 10 mL of PBS solution was added to the cells, and the cell density adjusted to 5×10^7 cells/mL.

Establishment of mouse cervical cancer transplanted tumor model and treatments

A total of 60 female BALB/C mice were selected for establishment of mouse model of transplanted cervical cancer. Following routine disinfection of the dorsal root of the right thigh of each mouse, 0.2 mL of single cell suspension was injected subcutaneously. The mice were randomly divided into control group, low-dose GLB group and high-dose GLB group. The control group was not treated. Mice in low-dose and high-dose groups were given GLB at doses of 0.15 and 0.20 μmol/g, respectively *via* the intraperitoneal route, once every 2 days.

Determination of treatment indicators

Growth inhibition of transplanted tumor

On days 1, 5 and 15 after drug administration, the long and short diameters of transplanted tumor were measured with vernier caliper, and the volume of transplanted tumor was calculated at each time point. On days 1, 5 and 15 post-GLB administrations, the mice were sacrificed *via* decapitation. The transplanted tumor was carefully removed and weighed in a balance.

Assay of serum levels of CEA and CA125

At 24 h after the last GLB administration, mice in all groups were fasted and deprived of drinking water for 6 h, and blood was taken from the retro-orbital plexus of each mouse. Following the blood samples were kept at room temperature for 20 min prior to centrifugation at 3000 rpm for 10 min. The resultant serum samples were carefully separated and stored at -70 ºC to avoid repeated freezing and thawing. Serum levels of CEA and CA125 were measured using enzyme-linked immunosorbent assay (ELISA).

Immunohistochemistry

The expressions of PTEN, Beclin1, LC3, autophagy-related p53 protein and apoptosis-related Bax protein were determined with SABC immunohistochemistry. The transplanted tumor in each group was divided into 4-μm sections which were oven-heated. The sections were dewaxed in xylene Ⅰ and xylene Ⅱ, and

dehydrated in gradient alcohol, washed in PBS buffer, treated with 3 % hydrogen peroxide, and incubated in the dark. Then, the sections were rinsed in PBS, microwaved for antigen repair, PBS-washed, and incubated with 5 % BSA at 37 ℃ for 40 min. Thereafter, the sections were incubated with primary antibodies at 37 ℃ for 40 min, washed with PBS, and incubated with SABC solution at 37 ℃ for 40 min. After PBS-washing, the sections were stained with DAB working solution, re-stained with hematoxylin, dehydrated in gradient alcohol, cleared in xylene I and xylene II, and sealed with neutral gum. Then, the sections were examined under an optical microscope. A total of 10 high-power fields were examined closely in each section, and the proportion of positive cells was calculated according to the intensity of staining.

Statistical analysis

The SPSS 20.0 software package was used for statistical analysis of the data in this study. The volume and weight of transplanted tumor, as well as the levels of CA125, CEA, PTEN, Beclin1, LC3, p53 and Bax of the transplanted tumor in mice were all in line with normal distribution. Measurement data are expressed as mean ± standard deviation (SD). One-way ANOVA was used for comparison amongst multiple groups, while SNK- Q test was used for pairwise comparison. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Volume of transplanted tumor

On days 5, 10 and 15, the volumes of transplanted tumor in low-dose GLB and high-dose GLB groups were significantly and dose-dependently lower than those in control group ($p < 0.05$). These results are shown in Table 1.

Transplanted tumor weight

On days 5, 10 and 15, the weights of transplanted tumor in low- and high-dose groups were significantly and dose-dependently lower than the corresponding values in the control group ($p < 0.05$). These data are shown in Table 2.

Levels of tumor markers

As shown in Table 3, the expression levels of CA125 and CEA in low- and high-dose GLB groups were significantly and dose-dependently lower than those in control group ($p < 0.05$).

Table 3: Levels of tumor markers in each group

Group	CA125 Xi (Chen U/mL)	CEA (ng/mL)
Control	12.65±0.98	75.64±10.89
Low dose	7.64±0.15[a]	10.69±0.77[a]
High dose	3.06±0.21[ab]	6.05±0.45[ab]
F	1344.152	760.7813
P-value	<0.001	<0.001

[a]$P < 0.05$, compared with control group; [b]$p < 0.05$, compared with the low-dose GLB group

Protein expression levels of PTEN, Beclin-1 and LC3

Results from immunohistochemistry showed that the protein levels of PTEN, Beclin1 and LC3 in low- and high-dose GLB groups were markedly and dose-dependently higher than the corresponding levels in the control group ($p < 0.05$). These results are presented in Table 4.

Table 1: Comparison of transplanted tumor volume between the 2 groups (cm^3)

Group	Day 1	Day 5	Day 10	Day 15
Control	0.25±0.12	0.62±0.15	1.25±0.26	1.74±0.31
Low-dose	0.26±0.08	0.41±0.08[a]	0.89±0.21[a]	1.26±0.25[a]
High-dose	0.24±0.11	0.30±0.07[ab]	0.61±0.08[ab]	0.97±0.16[ab]
F	0.180	46.924	52.294	49.262
P-value	0.833	<0.001	<0.001	<0.001

[a]$P < 0.05$, compared with control group; [b]$p < 0.05$, compared with the low-dose GLB group

Table 2: Comparison of transplanted tumor volume between the 2 groups (g)

Group	Day 1	Day 5	Day 10	Day 15
Control	18.52±0.76	21.85±0.65	24.05±0.34	26.15±0.38
Low-dose GLB	18.25±0.64	20.46±0.41[a]	21.79±0.25[a]	23.65±0.21[a]
High-dose GLB	18.61±0.52	19.06±0.61[ab]	19.42±0.36[ab]	20.81±0.18[ab]
F	1.670	121.293	1045.221	1938.943
P-value	0.196	<0.001	<0.001	<0.001

[a]$P < 0.05$, compared with control group; [b]$p < 0.05$, compared with the low-dose GLB group

Table 4: Protein expression levels of PTEN, Beclin-1 and LC3 in each group

Group	PTEN	Beclin-1	LC3
Control	21.52±1.25	20.56±1.68	23.64±0.85
Low dose	70.64±5.61[a]	76.39±1.85[a]	77.54±1.54[a]
High dose	95.34±12.88[ab]	90.64±12.85[ab]	93.68±12.74[ab]
F	425.894	480.333	487.982
P-value	<0.001	<0.001	<0.001

[a]$P < 0.05$, compared with control group; [b]$p < 0.05$, compared with the low-dose GLB group

Protein expression levels of p53 and Bax

The immunohistochemistry results demonstrated that protein expressions of p53 and Bax in low- and high-dose GLB groups were significantly and dose-dependently higher than those in control group ($p < 0.05$, Table 5).

Table 5: Protein expression levels of PTEN, Beclin1 and LC3 in each group

Group	P53	Bax
Control	19.85±2.16	12.74±1.79
Low-dose GLB	57.64±6.52[a]	58.94±6.38[a]
High-dose GLB	80.26±5.64[ab]	91.56±8.56[ab]
F	707.611	803.122
P-value	<0.001	<0.001

[a]$P < 0.05$, compared with control group; [b]$p < 0.05$, compared with the low-dose GLB group

DISCUSSION

Cervical cancer is a malignant tumor and a serious threat to the lives and health of women. Currently, surgery and radiotherapy are the main treatment strategies used for cervical cancer. Surgical treatment is the first choice of treatment for young women with surgical indications, while adjuvant radiotherapy is based on the presence of medium- and high-risk factors [7]. However, due to the numerous side effects and severe pain associated with radiotherapy, young women exhibit poor compliance with radiotherapy, thereby resulting in unsatisfactory prognosis [8]. Therefore, it is important to identify more efficient and low-toxicity antitumor drugs for treatment of cervical cancer.

Glaucocalyxin B (GLB) is extracted from cymbidium blue, and it exerts anti-inflammatory, antioxidant and anti-tumor effects [9]. A study has demonstrated that GLB inhibited the proliferation and apoptosis of tongue squamous cell carcinoma cells, possibly *via* inhibition of the activation of NF-κB signaling pathway [10]. In another study, it was reported that GLB effectively interfered with the epithelial-mesenchymal transformation process in breast cancer cells by inhibiting the proliferation, migration and invasion of triple-negative breast cancer cells via modulation of signal transduction in the p38MAPK/FOXO3 pathway [11]. In this study, the volume and weight of transplanted tumor in low- and high-dose groups were significantly and dose-dependently lower than those in control group on the 5th, 10th and 15th days post-drug administration. These results suggest that GLB inhibited the growth of cervical graft tumor. This finding is similar to the results of an earlier study [12]. It has been reported that CA125 and CEA are common clinical tumor markers which are abnormally expressed in various malignant tumors such as ovarian cancer, colon cancer and lung cancer. A study has demonstrated that changes in levels of CA125 and CEA are helpful in evaluating the status of cervical cancer [13]. In the present study, the levels of CA125 and CEA in low- and high-dose groups were significantly and dose-dependently lower than those in control group. This suggests that GLB suppressed cervical cancer. Excessive up-regulation of autophagy promotes apoptosis and affects tumor growth. Phosphatase and tensin homolog (PTEN), a phosphatase tumor suppressor gene, regulates autophagy and apoptosis by regulating the expressions of PI3K/AKT/mTOR signaling pathway-associated proteins [14]. A study has demonstrated that inhibition of PTEN expression induced epithelial-mesenchymal transformation in non-small cell lung cancer cells via regulation of Akt phosphorylation [15]. Beclin-1 regulates autophagy, apoptosis and cell proliferation. A study has shown that high expression of Beclin-1 inhibited the growth of transplanted breast cancer via Beclin-1-associated regulation of autophagy [16]. It is known that LC3 is a landmark factor in mammalian autophagosomes, and changes in its level reflect the autophagic capacity of cells. A study has reported that LC3 expression level in gastric cancer tissues was significantly lower than that in adjacent tissues, indicating that LC3 may promote lymph node metastasis of gastric cancer [17]. Cyclin acts as a tumor suppressor gene which repairs cells by acting on the cell cycle. The tumor suppressor gene p53 is a negative regulator of cell proliferation which induces reversion of cancer cells to normal state [18]. The most common pro-apoptotic cell in clinical application is Bax, and its overexpression induces apoptosis. In this study, immunohistochemical results showed that the protein expression levels of PTEN, Beclin-1,

LC3, p53 and Bax in low- and high-dose groups were significantly and dose-dependently higher than those in control group. These results suggest that GLB regulated the protein expressions of PTEN, Beclin-1 and LC3, thereby inducing apoptosis of cervical cancer cells.

CONCLUSION

The results obtained in this study demonstrate that GLB significantly and dose-dependently induce apoptosis of cervical cancer cells and inhibits their growth by regulating the protein expressions of PTEN, Beclin1 and LC3. Glaucocalyxin B exhibits potentials as adjunct therapy in the management of cervical cancer.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Feixiang Yang designed the study, supervised the data collection, and analyzed the data. Zheng Zhao and Zhengyang Han interpreted the data and prepared the manuscript for publication. Zheng Zhao, Zhengyang Han and Cong Huang supervised the data collection, analyzed the data and reviewed the draft of the manuscript. Zheng Zhao and Zhengyang Han contributed equally to this work and should be treated as co-first authors.

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