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• ESOPHAGEAL CANCER •

Resveratrol induces apoptosis in human esophageal carcinoma cells

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Abstract

AIM: To investigate the apoptosis in esophageal cancer cells induced by resveratrol, and the relation between this apoptosis and expression of Bcl-2 and Bax.

METHODS: In *in vitro* experiments, MTT assay was used to determine the cell growth inhibitory rate. Transmission electron microscope and TUNEL staining method were used to quantitatively and qualitively detect the apoptosis status of esophageal cancer cell line EC-9706 before and after the resveratrol treatment. Immunohistochemical staining was used to detect the expression of apoptosis-regulated gene Bcl-2 and Bax.

RESULTS: Resveratrol inhibited the growth of esophageal cancer cell line EC-9706 in a dose-and time-dependent manner. Resveratrol induced EC-9706 cells to undergo apoptosis with typically apoptotic characteristics, including morphological changes of chromatin condensation, chromatin crescent formation, nucleus fragmentation and apoptotic body formation. TUNEL assay showed that after the treatment of EC-9706 cells with resveratrol (10 mmol· L^{-1}) for 24 to 96 hours, the AIs were apparently increased with treated time ($P<0.05$). Immunohistochemical staining showed that after the treatment of EC-9706 cells with resveratrol (10 mmol· L^{-1}) for 24 to 96 hours, the PRs of Bcl-2 proteins were apparently reduced with treated time ($P<0.05$) and the PRs of Bax proteins were apparently increased with treated time ($P<0.05$).

CONCLUSION: Resveratrol is able to induce the apoptosis in esophageal cancer. This apoptosis may be mediated by down-regulating the apoptosis-regulated gene Bcl-2 and up-regulating the expression of apoptosis-regulated gene bax.

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INTRODUCTION

The Bcl-2 family plays a crucial role in the control of apoptosis. The family includes a number of proteins which have homologous amino acid sequence, including anti-apoptotic members such as Bcl-2 and Bcl-x_L, as well as pro-apoptotic members including Bax and Bad. In *in vitro* experiments, overexpression of Bcl-2 has been shown to inhibit apoptosis, but overexpression of Bax has been shown to promote apoptosis.

Resveratrol, a phytoalexin found in grapes, fruits, and root extracts of the weed *Polygonum cuspidatum*, is an important constituent of Chinese folk medicine. Indirect evidence suggests that the presence of resveratrol in white and rose wine may be helpful to reduce risks of coronary heart disease which would be achieved by moderate wine consumption. This effect has been attributed to the inhibition of platelet aggregation and coagulation, in addition to the antioxidant and anti-inflammatory activity of resveratrol. Moreover, a recent report shows that resveratrol is a potent cancer chemopreventive agent in three major stages of carcinogenesis. The anti-tumor activity of resveratrol might be related to induce the apoptosis of tumor cells but the precise mechanism of antitumor activity is not well understood.

MATERIALS AND METHODS

Materials

Resveratrol and MTT were obtained from Sigma Chemical Co. Ltd. *In situ* cell detection kit, anti-Bcl-2 monoclonal antibody and anti-Bax monoclonal antibody were purchased from Beijing Zhongshan biotechnology Co. Ltd. Stock solution of resveratrol was made in dimethylsulfoxide (DMSO) at a concentration of 100 mmol· L^{-1}. Working dilutions were directly made in the cell culture medium. Human esophageal carcinoma cell line EC-9706 was obtained from Professor Ming-Rong Wang in Chinese Scientific Academy.

Methods

Cell culture EC-9706 cells were incubated in RPMI 1640 supplemented with 100 ml· L^{-1} fetal bovine serum, 100 kU· L^{-1} penicillin, 100 mg· L^{-1} streptomycin and 2 mmol· L^{-1} L-glutamine under 50 ml· L^{-1} CO_2 in a humidified incubator at 37 ℃. EC-9706 cells were incubated for different time periods in the presence of resveratrol at 0.1, 1, 10 and 100 mmol· L^{-1}.

MTT assay 1×10^5 cells/well in a 96-well plate after incubation for 24 hours were treated with different concentrations of resveratrol (0.1, 1, 10, 100 mmol· L^{-1})for 24, 48, 72, 96 hours respectively. 10 μL of 5 g· L^{-1} of MTT was added to the medium in three wells at every dose and incubated for 4 hours at 37 ℃. Culture media were discarded followed by adding 0.2 ml DMSO and vibrating for 10 minutes. The absorbance (OD) was measured at 570 nm using a microplate reader. The cell growth inhibitory rate was calculated as follows: [(OD of control group -OD of experimental group)/(OD of control group-OD of blank group)]×100 %.

Transmission electron microscopy Cells treated with 10 mmol· L^{-1} resveratrol were harvested after incubation for 24 hours. Subsequently the cells were fixed in 4 % glutaral and immersed with Epon 821, imbedded for 72 hours at 60 ℃, the cells were prepared into ultrathin section (60 nm) and stained with uranyl acetate and lead citrate. Cell morphology was observed by transmission electron microscopy.

TUNEL assay Apoptosis of EC-9706 cells was evaluated by using an *in situ* cell detection kit. The cells were treated in the presence or absence of 10 mmol· L^{-1} resveratrol for 24 to 96 hours and fixed in ice-colded 80 % ethanol for up to 24 hours, treated with proteinase K and then 0.3 % H_2O_2, labeled with fluorescein dUTP in a humid box for 1 hour at 37 ℃. The cells

were then combined with POD-Horseradish peroxidase, colorized with DAB and counterstained with methyl green. Controls were received the same management except the labeling of omission of fluorescein dUTP. Cells were visualized with light microscope. The apoptotic index (AI) was calculated as follows: AI=(Number of apoptotic cells/Total number)×100 %.

Immunohistochemical staining Immunohistochemical staining was done by an avidinbiotin technique. EC-9706 cells treated in the presence or absence of 10 mmol· L^{-1} resveratrol for 24 to 96 hours were grew six-well glass slides and were fixed by acetone. After washing in PBS, the cells were incubated in 0.3 % H_2O_2 solution at room temperature for 5 minutes. The cells were then incubated with anti-Bcl-2 or anti-Bax monoclonal antibody at a 1:300 dilution at 4 ℃ overnight. After washing in PBS, the second antibody, biotinylated antirat Ig G, was added and the cells were incubated at room temperature for 1 hour. After washing in PBS, ABC compound was added and then incubated at room temperature for 10 minutes. DAB was used as the chromagen. After ten minutes, the brown color signifying the presence of antigen bound to antibodies was detected by light microscopy and photographed at ×200. Controls were managed the same as the experimental group except the incubation of the primary antibody. The positive rate (PR) was calculated as follows: PR=(Number of positive cells/Total number)×100 %.

Statistical analysis

Datas were analyzed by the paired two-tailed Student *t* test, and significance was considered when $P<0.05$.

RESULTS

MTT assay

EC-9706 cells were exposed to increasing concentrations (0.1 mmol· L^{-1} to 100 mmol· L^{-1}) of resveratrol for 24 to 96 hours, respectively. EC-9706 cells showed the mortality in a dose- and time-dependent manner. The data were summarised in Table 1.

Table 1 The inhibitory effect of resveratrol on EC-9706 cells

Time(h)	Inhibitory rate(%)				
	RPMI-1640	Resveratrol (mmol·L^{-1})			
		0.1	1	10	100
24	0.0	9.2[a]	18.4[b]	21.8[b]	34.4[b]
48	0.0	17.1[b]	21.3[b]	36.7[b]	45.5[b]
72	0.0	23.9[b]	37.5[b]	48.6[b]	59.9[b]
96	0.0	36.6[b]	45.7[b]	56.6[b]	88.8[b]

[a]$P<0.01$, [b]$P<0.001$ *vs* the control group.

Morphological changes

After treatment of EC-9706 cells with resveratrol (10 mmol· L^{-1}) for 24 hours, some cells appeared apoptotic characteristics including chromatin condensation, appearance of chromatin crescent, nucleus fragmentation and of formation apoptotic body which could be seen by transmission electron microscope.

TUNEL assay

Apoptotic cell death was determined by TUNEL assay according to the manufacture' s instructions. Positive staining located in the nucleus (Figure 1). After treatment with resveratrol (10 mmol· L^{-1})for 24 to 96 hours, AIs were apparently increased with treated time ($P<0.05$) (Table 2).

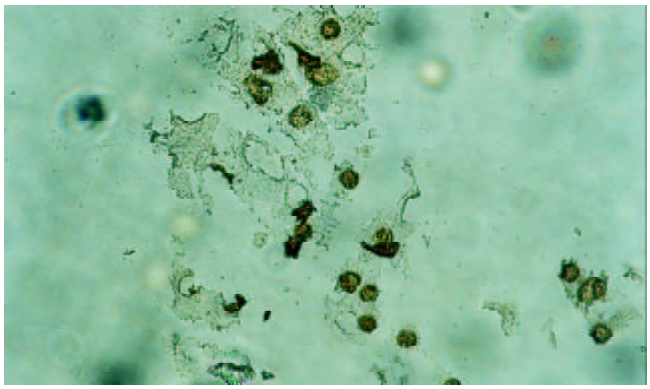

Figure 1 TUNEL assay of apoptotic cells induced by resveratrol with (×200).

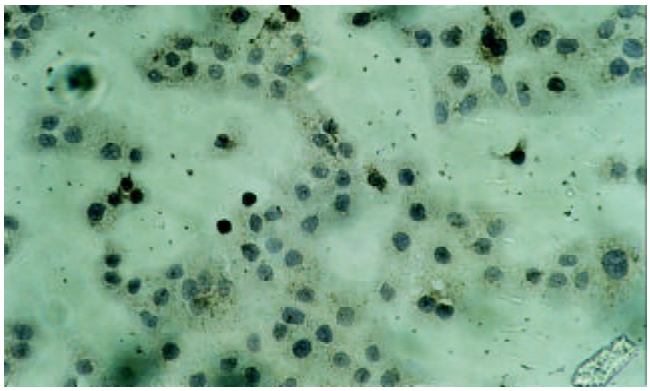

Figure 2 Immunohistochemical staining of the expression of Bcl-2 (×200).

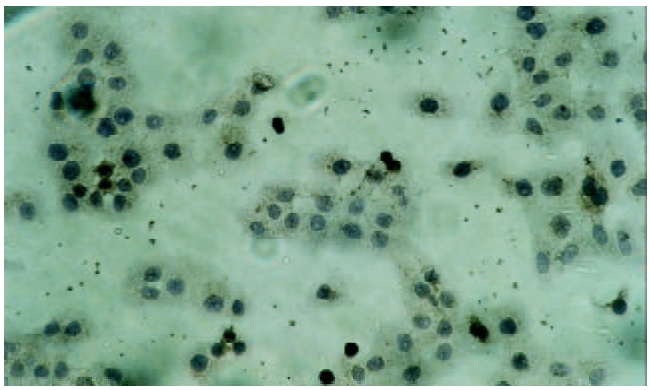

Figure 3 Immunohistochemical staining of the expression of Bax (×200).

Table 2 Apoptotic Index (AI) of treated EC-9706 cells by resveratrol

Time(h)	AI(%)
0	1.46±2.51
24	4.96±2.67[a]
48	16.64±1.87[a]
72	27.94±3.32[b]
96	37.46±3.96[b]

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

Expression of Bcl-2 proteins

Positive staining located in the cytoplasm (Figure 2). After treatment with resveratrol (10 mmol· L^{-1})for 24 to 96 hours, PRs of Bcl-2 proteins were apparently reduced with treated

time (P<0.05) (Table 3). This suggested that resveratrol could down-regulate the expression of Bcl-2.

Expression of bax proteins

Positive staining located in the cytoplasm (Figure 3). After treatment with resveratrol (10 mmol· L^{-1})for 24 to 96 hours, PRs of Bax proteins were apparently increased with treated time (P<0.05) (Table 4). This suggested that resveratrol could up-regulate the expression of Bax.

Table 3 Positive rate of Bcl-2 on treated EC-9706 cells by resveratrol

Time (h)	PR(%)
0	35.64±3.95
24	20.50±2.79[a]
48	10.76±2.46[a]
72	6.82±1.78[b]
96	3.88±1.24[b]

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

Table 4 Positive rate of Bax on treated EC-9706 cells by resveratrol

Time(h)	PR(%)
0	10.66±2.26
24	19.68±2.67[a]
48	30.77±3.76[a]
72	41.56±6.14[a]
96	59.96±5.34[a]

[a]P<0.01 *vs* the control group.

DISCUSSION

Currently, only few chemotherapeutic drugs take effect in the treatment of human primary esophageal carcinoma and it clearly need to look for new anti-esophageal carcinoma drugs. Resveratrol, a polyphenol found in various fruits and vegetables and is rich in grapes. The root extracts of the weed *Polygonum cuspidatum*, an important constituent of Chinese folk medicine, is also an ample source of resveratrol[1,2]. Several studys in last several years have shown that resveratrol have cardioprective and chemopreventive effects[3-5]. This constituent may account for the reduced risk of coronary heart disease in humans which may be achieved by moderate wine consumption[6]. Resveratrol is able to inhibit the growth of a wide variety of tumor cells, including leukemic, prostate, breast and hepatic cells[7-11]. The anti-tumor activity of resveratrol might be related to the induction of tumor apoptosis of tumor cells[12-22].

The Bcl-2 family plays a crucial role in the control of apoptosis. The family includes a number of proteins which have homologous amino acid sequences, including anti-apoptotic members such as Bcl-2 and Bcl-x_L, as well as pro-apoptotic members including Bax and Bad[23-26]. Overpression of Bax has the effect of promoting the cell death[27-31]. Conversely, Overpression of antiapoptotic proteins such as Bcl-2 will repress the function of Bax[32-36]. Thus, the ratio of Bcl-2 / Bax appears to be a critical determinant of a cell' s threshold for undergoing apoptosis[37].

In this study resveratrol could reduce Bcl-2 expression and improve Bcl-2 expression. The ratio of Bcl-2 /Bax was decreased when EC-9706 cells were treated with resveratrol. The decreased ratio could triggered the apoptosis of EC-9706 cells.

Our results demonstrated resveratrol is able to induce the apoptosis in esophageal cancer. This apoptosis may be mediated by down-regulating the expression of apoptosis-regulated gene Bcl-2 and up-regulating the expression of apoptosis-regulated gene Bax. Resveratrol may be potentially used as a chemotherapeutic drug in the anti-esophageal carcinoma chemptherapy.

REFERENCES

1 **Yoon S,** Kim Y, Ghim S, Song B, Bae Y. Inhibition of protein kinase CKII activity by resveratrol, a natural compound in red wine and grapes. *Life Sci* 2002; **71**: 2145

2 **Gao X,** Xu YX, Divine G, Janakiraman N, Chapman RA, Gautam SC. Disparate *in vitro* and *in vivo* antileukemic effects of resveratrol, a natural polyphenolic compound found in grapes. *J Nutr* 2002; **132**: 2076-2081

3 **Bhat KP,** Pezzuto JM. Cancer chemopreventive activity of resveratrol. *Ann N Y Acad Sci* 2002; **957:** 210-229

4 **Kuwajerwala N,** Cifuentes E, Gautam S, Menon M, Barrack ER, Reddy GP. Resveratrol induces prostate cancer cell entry into s phase and inhibits DNA synthesis. *Cancer Res* 2002; **62**: 2488-2492

5 **Joe AK,** Liu H, Suzui M, Vural ME, Xiao D, Weinstein IB. Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. *Clin Cancer Res* 2002; **8**: 893-903

6 **Wang Z,** Zou J, Huang Y, Cao K, Xu Y, Wu JM. Effect of resveratrol on platelet aggregation *in vivo* and *in vitro*. *Chin Med J (Engl)* 2002; **115**: 378-380

7 **Ferry-Dumazet H,** Garnier O, Mamani-Matsuda M, Vercauteren J, Belloc F, Billiard C, Dupouy M, Thiolat D, Kolb JP, Marit G, Reiffers J, Mossalayi MD. Resveratrol inhibits the growth and induces the apoptosis of both normal and leukemic hematopoietic cells. *Carcinogenesis* 2002; **23**: 1327-1333

8 **Kampa M,** Hatzoglou A, Notas G, Damianaki A, Bakogeorgou E, Gemetzi C, Kouroumalis E, Martin PM, Castanas E. Wine antioxidant polyphenols inhibit the proferation of human prostate cancer cell lines. *Nutr Cancer* 2000; **37:** 223-233

9 **Bove K,** Lincoln DW, Tsan MF. Effect of resveratrol on growth of 4T1 breast cancer cells in vitro and in vivo. *Biochem Biophys Res Commun* 2002; **291**: 1001-1005

10 **Tian XM,** Zhang ZX. The anticancer activity of resveratrol on implanted tumor of HepG2 in nude mice. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 161-164

11 **Sun ZJ,** Pan CE, Liu HS, Wang GJ. Anti-hepatoma activity of resveratrol *in vitro*. *World J Gastroenterol* 2002; **8:** 79-81

12 **Pervaiz S**. Resveratrol-from the bottle to the bedside? *Leuk Lymphoma* 2001; **40**: 491-498

13 **Dorrie J,** Gerauer H, Wachter Y, Zunino SJ**.** Resveratrol induces extensive apoptosis by depolarizing mitochondrial membranes and activating caspase-9 in acute lymphoblastic leukemia cells. *Cancer Res* 2001; **61**: 4731-4739

14 **She QB,** Bode AM, Ma WY, Chen NY, Dong Z**.** Resveratrol-induced activation of p53 and apoptosis is mediated by extracelluar-signal-regulated protein kinases and p38 kinase. *Cancer Res* 2001; **61:** 1604-1610

15 **Tsan MF,** White JE, Maheshwari JG, Bremner TA, Sacco J. Resveratrol induces Fas signaling-independent apoptosis in THP-1 human monocytic leukemia cells. *Br J Haematol* 2000; **109:** 405-412

16 **Szende B,** Tyihak E, Kiraly-Veghely Z. Dose-dependent effect of resveratrol on proliferation and apoptosis in endothelial and tumor cell cultures. *Exp Mol Med* 2000; **32**: 88-92

17 **Bernhard D,** Tinhofer I, Tonko M, Hubl H, Ausserlechner MJ, Greil R, Kofler R, Csordas A**.** Resveratrol causes arrest in the S-phase prior to Fas- independent apoptosis in CEM-C7H2 acute leukemia cells. *Cell Death Differ* 2000; **7**: 834-842

18 **Mouria M,** Gukovskaya AS, Jung Y, Buechler P, Hines OJ, Reber HA, Pandol SJ**.** Food-derived polyphenols inhibit pancreatic cancer growth through mitochondrial cytochrome C release and apoptosis. *Int J Cancer* 2002; **98**: 761-769

19 **Shih A,** Davis FB, Lin HY, Davis PJ. Resveratrol induces apoptosis in thyroid cancer cell lines via a MAPK- and p53-dependent mechanism. *J Clin Endocrinol Metab* 2002; **87:** 1223-1232

20 **Mahyar-Roemer M,** Katsen A, Mestres P, Roemer K**.** Resveratrol induces colon tumor cell apoptosis independently of p53 and precede by epithelial differentiation, mitochondrial proliferation

and membrane potential collapse. *Int J Cancer* 2001; **94**: 615-622

21 **Lin HY,** Shih A, Davis FB, Tang HY, Martino LJ, Bennett JA, Davis PJ. Resveratrol induced serine phosphorylation of p53 causes apoptosis in a mutant p53 prostate cancer cell line. *J Urol* 2002; **168**: 748-755

22 **She QB,** Huang C, Zhang Y, Dong Z. Involvement of c-jun NH (2)-terminal kinases in resveratrol-induced activation of p53 and apoptosis. *Mol Carcinog* 2002; **33**: 244-250

23 **Konopleva M,** Konoplev S, Hu W, Zaritskey AY, Afanasiev BV, Andreeff M. Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. *Leukemia* 2002; **16**: 1713-1724

24 **Van Der** Woude CJ, Jansen PL, Tiebosch AT, Beuving A, Homan M, Kleibeuker JH, Moshage H**.** Expression of apoptosis-related proteins in Barrett's metaplasia-dysplasia-carcinoma sequence: A switch to a more resistant phenotype. *Hum Pathol* 2002; **33**: 686-692

25 **Panaretakis T,** Pokrovskaja K, Shoshan MC, Grander D**.** Activation of Bak, Bax and BH3-only proteins in the apoptotic response to doxorubicin. *J Biol Chem* 2002; [epub ahead of print]

26 **Bellosillo B,** Villamor N, Lopez-Guillermo A, Marce S, Bosch F, Campo E, Montserrat E, Colomer D**.** Spontaneous and drug-induced apoptosis is mediated by conformational changes of Bax and Bak in B-cell chronic lymphocytic leukemia. *Blood* 2002; **100**: 1810-1816

27 **Matter-Reissmann UB,** Forte P, Schneider MK, Filgueira L, Groscurth P, Seebach JD**.** Xenogeneic human NK cytotoxicity against porcine endothelial cells is perforin / granzyme B dependent and not inhibited by Bcl-2 overexpression. *Xenotransplantation* 2002; **9**: 325-337

28 **Lanzi C,** Cassinelli G, Cuccuru G, Supino R, Zuco V, Ferlini C, Scambia G, Zunino F. Cell cycle checkpoint efficiency and cellular response to paclitaxel in prostate cancer cells. *Prostate* 2001; **48:** 254-264

29 **Mertens HJ,** Heineman MJ, Evers JL**.** The expression of apoptosis-related proteins bcl-2 and ki67 in endometrium of ovulatory menstrual cycles. *Gynecol Obstet Invest* 2002; **53**: 224-230

30 **Mehta U,** Kang BP, Bansal G, Bansal MP**.** Studies of apoptosis and bcl-2 in experimental atherosclerosis in rabbit and influence of selenium supplementation. *Gen Physiol Biophys* 2002; **21**: 15-29

31 **Chang W,** Yang K, Chuang H, Jan J, Shaio M**.** Glutamine protects activated human T cells from apoptosis by up-regulating glutathione and bcl-2 levels. *Clin Immunol* 2002; **104**: 151

32 **Chen GG,** Lai PB, Hu X, Lam IK, Chak EC, Chun YS, Lau WY**.** Negative correlation between the ratio of Bax to Bcl-2 and the size of tumor treated by culture supernatants from Kupffer cells. *Clin Exp Metastasis* 2002; **19**: 457-464

33 **Usuda J,** Chiu SM, Azizuddin K, Xue LY, Lam M, Nieminen AL, Oleinick NL. Promotion of photodynamic therapy-induced apoptosis by the mitochondrial protein Smac / DIABLO: dependence on Bax**.** *Photochem Photobiol* 2002; **76:** 217-223

34 **Sun F,** Akazawa S, Sugahara K, Kamihira S, Kawasaki E, Eguchi K, Koji T. Apoptosis in normal rat embryo tissues during early organogenesis: the possible involvement of Bax and Bcl-2. *Arch Histol Cytol* 2002; **65**: 145-157

35 **Jang M,** Shin M, Shin H, Kim K, Park H, Kim E, Kim C. Alcohol induces apoptosis in TM3 mouse Leydig cells via bax-dependent caspase-3 activation. *Eur J Pharmacol* 2002; **449:** 39

36 **Tilli CM,** Stavast-Koey AJ, Ramaekers FC, Neumann HA**.** Bax expression and growth behavior of basal cell carcinomas. *J Cutan Pathol* 2002; **29:** 79-87

37 **Pettersson F,** Dalgleish AG, Bissonnette RP, Colston KW. Retinoids cause apoptosis in pancreatic cancer cells via activation of RAR-gamma and altered expression of Bcl-2 / Bax. *Br J Cancer* 2002; **87**: 555-561

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