Mechanism of EGCG promoting apoptosis of MCF-7 cell line in human breast cancer

CHAO-YOU HUANG[1], ZHENG HAN[1], XI LI[2], HUI-HUA XIE[1] and SHAN-SHAN ZHU[1]

[1]Department of Breast and Thyroid Surgery, Hexian Memorial Hospital of Panyu, Guangzhou, Guangdong 511400; [2]Department of Breast and Thyroid Surgery, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong 510630, P.R. China

Abstract. The aim of the present study was to investigate the effects of epigallocatechin-3-gallate (EGCG) on the apoptosis of the human MCF-7 cancer cell line and the underlying mechanism. MCF-7 cells were divided into the control group and EGCG groups. The proliferation of MCF-7 cells in the two groups was determined using MTT and apoptosis was examined using flow cytometry. Western blot analysis and qRT-PCR were used to analyze P53 and Bcl-2 expression levels. The silencing effect of specific siRNA was evaluated using RT-PCR and western blot analysis. P53 and Bcl-2 expression levels were determined using western blot analysis in the si-P53, EGCG and EGCG-combined si-P53 groups. EGCG inhibited the proliferation of MCF-7 cells in a concentration-dependent manner and IC_{50} was 37.681 mol/l. The apoptotic rates were 1.37 and 5.83% (t=8.9, p=0.0124) in the blank control and treatment groups after treatment with 30 μmol/l EGCG. The RT-qPCR and western blot results demonstrated that the effect of siRNA interference was evident. The expression of P53 in the EGCG-combined si-P53 group was higher than that of the si-P53 group, but lower than the EGCG group. The Bcl-2 expression level in the EGCG-combined si-P53 group was lower than that of the si-P53 group and higher than that of the EGCG group. In conclusion, EGCG suppressed the proliferation of human MCF-7 breast cancer cells and promoted apoptosis. In addition, the underlying mechanism may be related to the P53/Bcl-2 signaling pathway.

Introduction

Green tea is one of the main tea species in China. It contains tea polyphenols, catechins, chlorophyll, caffeine, amino acids, vitamins and other active ingredients (1). Epigallocatechin-3-gallate (EGCG) content is very high in green tea (1). EGCG has anti-aging, sterilization, anti-inflammatory and anticancer properties, and it also can inhibit the growth of a variety of malignant cells and induce apoptosis (2-4). It can also inhibit the angiogenesis, invasion and metastasis of malignant tumor cells, in various types of cancer (5).

Breast cancer is one of the common malignancies in women. It is crucial to identify new effective drugs with minimal side effects to treat breast cancer. Liang *et al* (6) identified that EGCG inhibited the proliferation of breast cancer cells by repressing cyclin-dependent protein activity and also inhibited the growth and metastasis of breast cancer cells by downregulating the expression of VEGF and MMP-9 (7-9). However, the mechanism by which EGCG promoted breast cancer apoptosis has rarely been studied.

The present study provides a theoretical reference for the development of breast cancer target by investigating the mechanism of apoptosis of EGCG on breast cancer.

Materials and methods

Materials. EGCG and thiazolyl blue were purchased from Sigma (St. Louis, MO, USA). RPMI-1640 basal medium and fetal bovine serum (FBS) was produced by HyClone (Logan, UT, USA). RPMI-1640 basal medium and EGCG were used to prepare a 10 mmol/l storage solution. It was sterilized by filtration (0.22 μm) and stored at -80°C in the dark. MTT was dissolved in phosphate-buffered saline (PBS) solution at 5 mg/ml, and was stored at -20°C in the dark after sterilization. Mouse anti-human P53, Bcl-2 and GAPDH were all purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and the RT-PCR kit was purchased from Takara (Otsu, Shiga, Japan). P53, Bcl-2 and GAPDH primers were produced by Invitrogen (Carlsbad, CA, USA) (Table I) and si-P53 was supplied by Gemma (Shanghai, China).

Cell line. MCF-7 cells were purchased from the Shanghai ATCC cell bank. The cells were cultured in RPMI-1640 medium containing 10% FBS (with 100 U/ml green-strepto-mycin) and incubated under 5% CO_2 at 37°C.

Methods
MTT detection of the sensitivity of MCF-7 cells to EGCG. The EGCG concentration gradient was 0, 2, 4, 8, 16, 32, 64

Correspondence to: Dr Zheng Han, Department of Breast and Thyroid Surgery, Hexian Memorial Hospital of Panyu, 2 Qinghe East Road, Panyu, Guangzhou, Guangdong 511400, P.R. China
E-mail: kh80sk@163.com

Table I. Primers of P53, Bcl-2 and GAPDH used in RT-qPCR.

Gene	Forward primer	Reverse primer
P53	5'-TTCCCTGGATTGGCCAGACT-3'	5'-ACCATCGCTATCTGAGCAGC-3'
Bcl-2	5'-CGACTTCGCCGAGATGCCAGCCAG-3'	5'-ACTTGTGGCCCAGATAGGCACCCAG-3'
GAPDH	5'-AGAAGGCTGGGGCTCATTTG-3'	5'-AGGGGCCATCCACAGTCTTC-3'

and 128 μmol/l. MCF-7 cells ($2x10^3$) were seeded in a 96-well plate with blank control wells. After cells adhered to the walls, EGCG of the concentration gradient of 0, 2, 4, 8, 16, 32, 64 and 128 μmol/l was added. After incubation for 72 h, the supernatant was discarded and 20 μl of MTT was added to each well. After incubation for 4 h, the liquid was discarded and 150 μl of dimethyl sulfoxide (DMSO) was added to each well of the EGCG. The optical density (OD) of each well at 490 nm was measured by a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The inhibition rate was calculated as: (control group OD - experimental group OD/control group OD) x 100%.

Effect of EGCG on MCF-7 cell apoptosis measured by flow cytometry (FCM). MCF-7 cells were seeded in a 6-well cell culture plate. After adherence, 30 μmol/l EGCG was added and the cells were stained after 48 h with Annexin V-FITC kit. The change in apoptotic rate was measured, and the experiment was repeated three times.

RT-qPCR and western blot analysis determine how EGCG treatment on MCF-7 affects the mRNA and protein levels of P53 and Bcl-2. The experiment was conducted on the control and EGCG groups. EGCG was added to fresh MCF-7 cells in the experimental group after cell adhesion (EGCG at a final concentration of 30 μmol/l), but the control group was not treated. After 72 h of incubation, total RNA was extracted using TRIzol reagent. The RT-qPCR assay was performed using the Takara kit, and the *GAPDH* gene was used as an internal control. The conditions for RT-qPCR were: Preheating at 95˚C for 2 min, denaturation at 95˚C for 30 sec, annealing at 58˚C for 30 sec and extension at 72˚C for 40 sec. The experiment was repeated three times. The results were expressed as $2^{-\Delta\Delta Cq}$. MCF-7 cells were treated with 30 μmol/l EGCG for 48 h. The cells were then rinsed three times with PBS and 100 μl of RIPA lysate was added. The cells were placed on ice for 30 min and centrifuged at 12,000 x g for 20 min at 4˚C. The supernatant was collected and stored at -80˚C. Protein concentration was determined using the BCA protein method. The protein extract was subjected to SDS-PAGE (10%) and transferred to a PVDF membrane. The membrane was blocked with 3% BSA solution for 2 h. Then, primary rabbit monoclonal P53 antibody (dilution, 1:500; cat. no. ab32049), rabbit polyclonal Bcl-2 antibody (dilution, 1:500; cat. no. ab59348) and rabbit polyclonal GAPDH antiboody (dilution, 1:500; cat. no. ab37168), purchased from Abcam (Cambridge, MA, USA), were added at 1:1,000 and incubated at 4˚C overnight. The membrane was washed with PBS three times (5 min each time). Then secondary goat anti-rabbit (HRP) IgG antibody (dilution,

1:2,000; cat. no. ab6721) was added followed by 2-h incubation and washing with PBS three times (5 min each time). Finally, it was placed on the ECL for exposure and development.

RT-qPCR and western blot analysis of si-P53 interference effect. The cells were divided into the si-P53, si-NC and NC groups (si-P53 was added with small fragments of interference P53, with si-NC as a positive control and NC as a negative control). Cells in the logarithmic growth phase were inoculated into the culture flask and transiently transfected according to the protocol of the Lipofectamine™ 2000 kit. After 48 h, the RNA was extracted and purified according to the kit protocol, and RNA purity and concentration was determined by a spectrophotometer (Hitachi, Tokyo, Japan). The P53 mRNA expression was evaluated using RT-qPCR, and the P53 protein expression level was measured using western blot analysis. The three groups were compared and the transfection efficiency was calculated.

Alterations in the expression of P53 and Bcl-2 proteins in MCF-7 cells transfected with si-P53 detected by western blot analysis. The cells were divided into the si-P53, EGCG and EGCG + si-P53, (si-P53 plus interference P53 small fragments, si-P53 for the positive control, NC for the negative control) groups. The P53 and Bcl-2 protein expression levels in the three groups were evaluated using western blot analysis using the same method explained before.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) and SPSS 20.0 (Chicago, IL, USA) statistical software. The measurement data were presented as mean ± standard deviation. Comparison between two groups was conducted using a t-test. The one-way analysis of variance (ANOVA) test was used for comparison among three groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

MTT detection of the sensitivity of MCF-7 cells to EGCG. The sensitivity of EGCG to MCF-7 was evaluated using MTT assay. As shown in Fig. 1, 10 μmol/l EGCG significantly inhibited the proliferation of MCF-7 cells and the inhibition was enhanced with the increase of drug concentration. The IC_{50} was 37.684 μmol/l. We selected 30 μmol/l as the follow-up dose (Table II).

Effect of EGCG on MCF-7 cell apoptosis measured using FCM. As shown in Fig. 2, the apoptotic rates of the EGCG

Table II. Different concentrations of EGCG and the inhibitory rate of MCF-7 cells using MTT.

Concentration	OD (mean ± SD)	Inhibition rate (%)
0	1.187±0.049	0
10	0.979±0.035	17.52
20	0.726±0.021	38.85
40	0.589±0.027	50.39
60	0.427±0.012	64
80	0.309±0.017	73.92
100	0.219±0.016	81.53
120	0.116±0.011	90.23

EGCG, epigallocatechin-3-gallate.

Figure 1. Different concentrations of EGCG of the inhibitory rate of MCF-7 cells using MTT. EGCG, epigallocatechin-3-gallate.

Table III. The effect of 30 μmol/l EGCG on the apoptotic rate of MCF-7 after 48 h was detected using a flow cytometer.

Group	Apoptosis rate (%) (mean ± SD)	t	P-value
NC	1.38±0.19		
EGCG	5.65±0.64	8.9	0.0124

EGCG, epigallocatechin-3-gallate; NC, negative control.

Table IV. The effect of 30 μmol/l EGCG on the Cq and F value of P53 and Bcl-2 in MCF-7 cells.

Gene	$2^{-\Delta\Delta Cq}$ (mean ± SD) NC	$2^{-\Delta\Delta Cq}$ (mean ± SD) EGCG	t	P-value
P53	4.289±0.831	11.352±0.992	9.451	<0.001
Bcl-2	12.013±1.082	4.465±1.228	7.985	<0.001

EGCG, epigallocatechin-3-gallate; NC, negative control.

and control groups were 1.37 and 5.83%, respectively, with a significant difference (t=8.9, p=0.0124). These results suggested that EGCG promoted apoptosis in MCF-7 cells (Table III).

Effects of EGCG treatment on the expression of P53 and Bcl-2. After treatment with 30 μmol/l EGCG, P53 mRNA and protein expression levels were significantly higher, while the Bcl-2 mRNA and protein expression levels decreased significantly (Fig. 3; Table IV).

si-P53 interference effect. The RNA interference technique was used to silence P53 expression. The interference effect was studied using RT-qPCR and western blot analysis. As shown
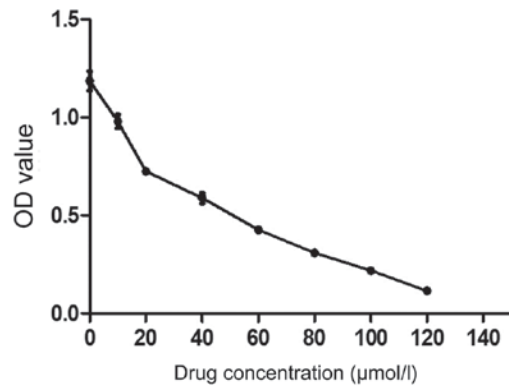
in Fig. 4, the interference effect was significant and could be further tested.

Changes in expression levels of P53 and Bcl-2 proteins in MCF-7 cells transfected with si-P53. P53 and Bcl-2 expression levels in si-P53-transfected MCF-7 cells treated with EGCG were detected using western blot analysis. As shown in Fig. 5, the expression of P53 in the EGCG + si-P53 group was higher than that in the si-P53 group but lower than that in the EGCG group. The expression of Bcl-2 in the EGCG + si-P53 group was lower than that in the si-P53 group but higher than that in the EGCG group. The results suggested that EGCG promoted the expression of P53 (Table V).

Discussion

P53 is an important tumor suppressor gene. It is involved in tumor occurrence and development through DNA repair, cell cycle regulation, angiogenesis inhibition and cell apoptosis (10). The *P53* gene has been found to be mutated in more than 50% of malignant tumors (11). Mutations and deletions of the *P53* gene have been reported to be closely related to breast cancer (12-15). Apoptosis is a spontaneous cell death process regulated by a series of genes. The proto-oncogene Bcl-2 is a member of the Bcl-2 family. The oncoproteins located in the nuclear membrane, part of the endoplasmic reticulum and mitochondrial outer membrane, can negatively regulate cell apoptosis and prolong cell life (16). Previous findings have shown that P53 can inhibit the expression of Bcl-2 protein (17,18).

EGCG is the most active substance in green tea, which has great developmental value, and its anticancer effect has been widely studied. Previous results have shown that EGCG can promote apoptosis in a variety of malignant tumor cells, including pancreatic and gastric cancer cells (18,19). In the present study, we showed that the apoptotic rate in MCF-7 cells treated with EGCG was significantly higher than that of the control group. In order to explore the mechanism of apoptosis, P53 and Bcl-2 expression levels were determined. The RT-qPCR and western blot results showed that EGCG induced the expression of P53 but inhibited the expression of Bcl-2. The EGCG + si-P53 group had a higher P53 expression level compared with the si-P53 group. The P53 expression level was
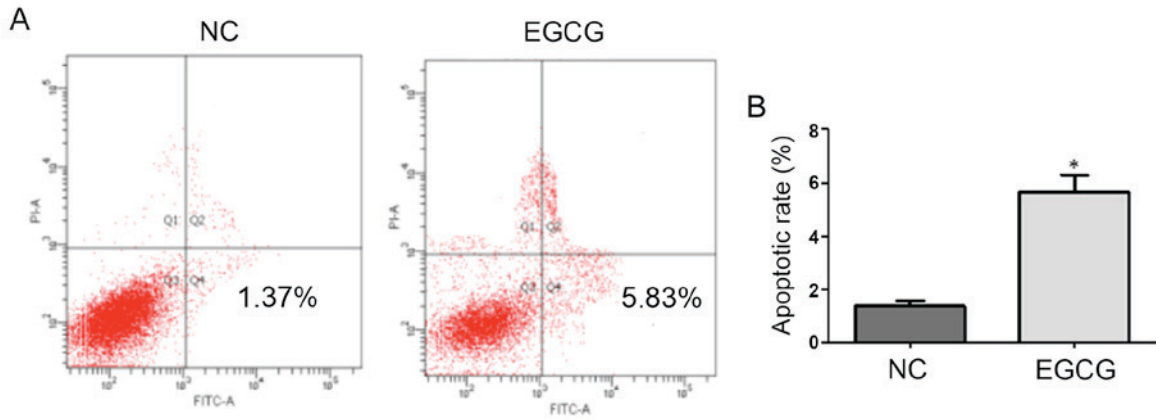
Figure 2. (A) The effect of 30 μmol/l EGCG on the apoptosis rate of MCF-7 after 48 h was detected by flow cytometer. (B) Statistics of apoptosis rate. *P<0.05, compared to the NC group. EGCG, epigallocatechin-3-gallate; NC, negative control.
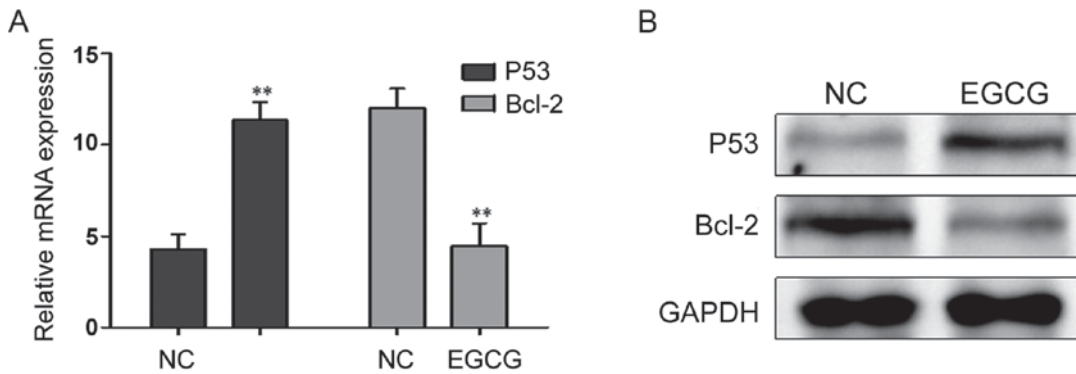

Figure 3. (A) The effect of 30 μmol/l EGCG on the mRNA expression of P53 and Bcl-2 in MCF-7 cells. **P<0.001 compared to the NC group. (B) The effect of 30 μmol/l EGCG on the protein expression of P53 and Bcl-2 in MCF-7 cells. EGCG, epigallocatechin-3-gallate; NC, negative control.
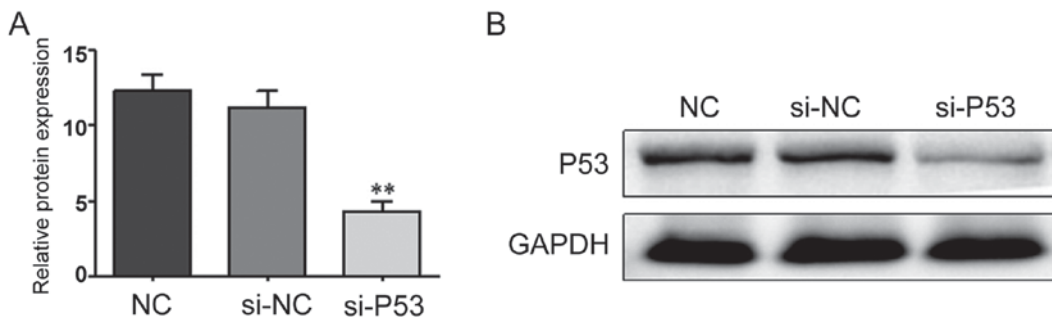

Figure 4. (A) The Cq value of P53 in the MCF-7 cells after transfection with si-P53 was measured using RT-qPCR. (B) The P53 protein expression in the MCF-7 cells after transfection with si-P53 was detected by western blot analysis. **P<0.001 compared to the NC group. NC, negative control.
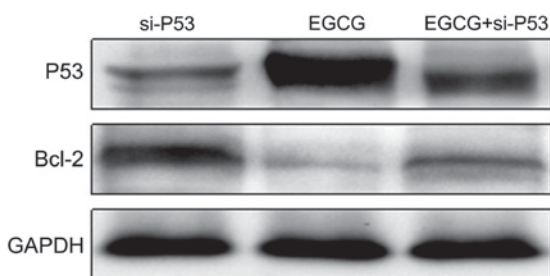

Figure 5. Protein expression of P53 and Bcl-2 was examined using western blot analysis on MCF-7 cells treated with EGCG and transfected with si-P53. EGCG, epigallocatechin-3-gallate.

Table V. The Cq value of P53 was detected by RT-qPCR after transfection with si-P53.

Group	$2^{-\Delta\Delta Cq}$ (mean ± SD)	F	P-value
NC	12.332±1.063		
si-NC	11.219±1.097		
si-P53	4.329±0.658	61.450	<0.001

NC, negative control.

lower than that of the EGCG group, while the Bcl-2 expression was lower than that in the si-P53 group but higher than that in the EGCG group. These results suggest that EGCG inhibited the expression of Bcl-2 and promoted the expression of P53.

In conclusion, the findings show that EGCG inhibited the proliferation of MCF-7 cells and promoted apoptosis in these cells. The underlying mechanism involved may be related to the P53/Bcl-2 signaling pathway, which can provide a theoretical basis for EGCG to be used in the new target drug development for breast cancer.

References

1. Yang CS and Wang ZY: Tea and cancer. J Natl Cancer Inst 85: 1038-1049, 1993.
2. Tan XH, Zhou DY and Zhang YL: Advances in research on anti-cancer mechanism of tea. Foreign Medical Oncology 25: 4, 1998.
3. Yang GY, Liao J, Kim K, Yurkow EJ and Yang CS: Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. Carcinogenesis 19: 611-616, 1998.
4. Ahmad N, Feyes DK, Nieminen AL, Agarwal R and Mukhtar H: Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. J Natl Cancer Inst 89: 1881-1886, 1997.
5. Zhang G, Wang Y, Zhang Y, Wan X, Li J, Liu K, Wang F, Liu K, Liu Q, Yang C, et al: Anti-cancer activities of tea epigallo-catechin-3-gallate in breast cancer patients under radiotherapy. Curr Mol Med 12: 163-176, 2012.
6. Liang YC, Lin-Shiau SY, Chen CF and Lin JK: Inhibition of cyclin-dependent kinases 2 and 4 activities as well as induction of Cdk inhibitors p21 and p27 during growth arrest of human breast carcinoma cells by (-)-epigallocatechin-3-gallate. J Cell Biochem 75: 1-12, 1999.
7. Sartippour MR, Shao ZM, Heber D, Beatty P, Zhang L, Liu C, Ellis L, Liu W, Go VL and Brooks MN: Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. J Nutr 132: 2307-2311, 2002.
8. Masuda M, Suzui M, Lim JT, Deguchi A, Soh JW and Weinstein IB: Epigallocatechin-3-gallate decreases VEGF production in head and neck and breast carcinoma cells by inhibiting EGFR-related pathways of signal transduction. J Exp Ther Oncol 2: 350-359, 2002.
9. Farabegoli F, Papi A and Orlandi M: (-)-Epigallocatechin-3-gallate down-regulates EGFR, MMP-2, MMP-9 and EMMPRIN and inhibits the invasion of MCF-7 tamoxifen-resistant cells. Biosci Rep 31: 99-108, 2011.
10. Levine AJ, Momand J and Finlay CA: The p53 tumour suppressor gene. Nature 351: 453-456, 1991.
11. Pakos EE, Kyzas PA and Ioannidis JP: Prognostic significance for TP53 tumor suppressor gene expression and mutations in human motations in human osteosarcoma: A meta-analysis. Clin Cancer Res 10: 6208-6214, 2004.
12. Barnes DM, Dublin EA, Fisher CJ, Levison DA and Millis RR: Immunohistochemical detection of p53 protein in mammary carcinoma: An important new independent indicator of prognosis? Hum Pathol 24: 469-476, 1993.
13. Dastjerdi MN, Rarani MZ, Valiani A and Mahmoudieh M: The effect of adenosine A1 receptor agonist and antagonist on p53 and caspase 3, 8, and 9 expression and apoptosis rate in MCF-7 breast cancer cell line. Res Pharm Sci 11: 303-310, 2016.
14. Synnott NC, Murray A, McGowan PM, Kiely M, Kiely PA, O'Donovan N, O'Connor DP, Gallagher WM, Crown J and Duffy MJ: Mutant p53: A novel target for the treatment of patients with triple-negative breast cancer? Int J Cancer 140: 234-246, 2017.
15. Darb-Esfahani S, Denkert C, Stenzinger A, Salat C, Sinn B, Schem C, Endris V, Klare P, Schmitt W, Blohmer JU, et al: Role of TP53 mutations in triple negative and HER2-positive breast cancer treated with neoadjuvant anthracycline/taxane-based chemotherapy. Oncotarget 7: 67686-67698, 2016.
16. Schinzel A, Kaufmann T and Borner C: Bcl-2 family members: Integrators of survival and death signals in physiology and pathology. Biochim Biophys Acta 1644: 95-105, 2004.
17. Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P and Moll UM: p53 has a direct apoptogenic role at the mitochondria. Mol Cell 11: 577-590, 2003.
18. Zhu BH, Zhan WH, Li ZR, Wang Z, He YL, Peng JS, Cai SR, Ma JP and Zhang CH: (-)-Epigallocatechin-3-gallate inhibits growth of gastric cancer by reducing VEGF production and angiogenesis. World J Gastroenterol 13: 1162-1169, 2007.
19. Qanungo S, Das M, Haldar S and Basu A: Epigallocatechin-3-gallate induces mitochondrial membrane depolarization and caspase-dependent apoptosis in pancreatic cancer cells. Carcinogenesis 26: 958-967, 2005.