

## Epigallocatechin gallate inhibits the growth of salivary adenoid cystic carcinoma cells via the EGFR/Erk signal transduction pathway and the mitochondria apoptosis pathway

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ACC is one of the most malignant tumors in salivary gland, and of poor prognosis. A critical role in ACC development and progression is played by EGFR family members including EGFR. EGCG, a low molecular weight polyphenol contained in green tea, has broad anticancer properties, but whether EGCG regulates activity of ACC is unknown. In the present study, the effects of EGCG were investigated *in vitro* with particular attention to the pathway of EGFR/Erk and mitochondria apoptosis in SACC-83 cell lines. The results of MTS assay and flow cytometry demonstrated that EGCG (20-80  $\mu$ M) could inhibit proliferation and promote apoptosis of SACC-83 cells. Furthermore, by Western blotting with antibodies specific for EGFR, Erk 1/2 (p-Erk 1/2), Mek (p-Mek), Bcl-2, and Bax, it was demonstrated that EGCG could reduce the expression of EGFR, inhibit phosphorylation of Erk 1/2 and Mek, downregulate Bcl-2, and upregulate Bax. In addition, it was also shown that EGCG could inhibit mRNA expression of P90 RSK by RT-PCR. In conclusion, the results suggest that EGCG might be a potential therapeutic or adjuvant strategy for the treatment of patients with ACC, by inhibiting proliferation and inducing the apoptosis of the tumor cells.

*Key words:* EGCG, ACC, EGFR, MAPK, Bcl-2

ACC is one of the most malignant tumors in salivary gland and the predominant histologic type among malignancies of the minor salivary glands and the submandibular gland [1]. Although it grows slowly, it is strongly invasive. It often grows along nerves and blood vessels and tends to recur and metastasize [2, 3]. Studies have reported that the long-term disease control is poor in spite of a good 5-year survival rate [4]. H. Kokemueller et al. reported that survival rates of ACC at 5, 10 and 15 years were 71%, 54% and 37% with a mean overall survival of 11.2 years [5]. Nowadays ACC is mainly treated with wide tumor-free surgical margin excision and radiation, or chemoradiotherapy [6, 7]. However these treatments are not ideal with poor prognosis. Because of the strong aggression of its character and the particular anatomy of oral and maxillofacial region, surgery may not be the best approach. Choice of enlargement resection and removing all the adjacent peripheral nerves result in sacrificing the facial nerves or other critical structures or organs. And if the nerves nearby are preserved, tumor tends to recur after surgery. ACC has a high

recurrence rate, with 5-year recurrence rates of 35% to 50% [8]. For preventing recurrence and metastasis, post-operative modalities including radiotherapy and chemotherapy should be implemented. Though the curative effect of conventional chemotherapy should be affirmed, its side effects and the drug resistance of tumor cells are still hard problems in clinic [9]. Researching a non-toxic method which can inhibit tumor cell growth would be a better strategy.

Tea polyphenols has a good anti-tumor activity in animal models and humans [10]. EGCG is the most abundant and active phenolic constituent of tea polyphenols and has strong antioxidant properties, possessing chemotherapeutic and chemopreventive roles [11, 12]. Previous studies showed that EGCG can inhibit the growth and metastasis of oral cancer, breast cancer, hepatic cancer, colorectal cancer and other cancer cells [13-16] via a variety of ways. Many anticancer mechanisms of EGCG have been mentioned such as antioxidant activity, cell cycle stationary, induction of apoptosis, inhibition of DNA methylation, and effect on small RNA,

protease, telomerase, dihydrofolate reductase and regulation of cell signaling pathways [17-19].

EGCG could inhibit receptors in cell membrane such as EGFR [20, 21], HGFR [22], VEGFR [23], and action sites in cells such as MMPs [24, 25], P90 RSK [21], Bcl-2 family [26], and Erk, P38 MAPK, JNK, Akt which belong to MAPK family [21]. These action sites and others that have not been found or confirmed form a complicated signal transduction network, by which EGCG affects biological behaviors of tumor cells including proliferation, apoptosis, invasion and metastasis.

In the present study, the effects of EGCG were investigated in SACC-83 cell lines. MTS assay and flow cytometry were performed to evaluate the effect of EGCG on proliferation and apoptosis. The protein expression levels of the pathway of EGFR/Erk and mitochondria apoptosis-related Bcl-2, Bax were evaluated using Western blotting analysis and the mRNA levels of P90 RSK was measured by RT-PCR.

## Materials and methods

**Cell culture.** The human salivary gland adenoid cystic carcinoma cell line, SACC-83, was a gift from the College of Stomatology, Beijing University, which was cultured in PRMI 1640 media (PAA Laboratories GmbH, AUS), supplemented with 10% fetal bovine serum (GIBCO, USA), 1% antibiotic solution containing 10,000 units/mL penicillin and 10 000 µg/mL streptomycin (GIBCO), 1% sodium pyruvate (GIBCO), and 1% MEM non-essential amino acids (GIBCO). All cells were grown in a humid atmosphere of 5% CO<sub>2</sub> at 37 °C.

**Cell proliferation analysis.** SACC-83 cells were seeded in 96-well plates (5000 cells/well) and cultured overnight, then treated with 0, 5, 10, 20, 40, 80 µM EGCG (Sigma-Aldrich, USA). Cell proliferation was evaluated after 24 h, 48 h, and 72 h respectively using the MTS reagent (Promega, USA).

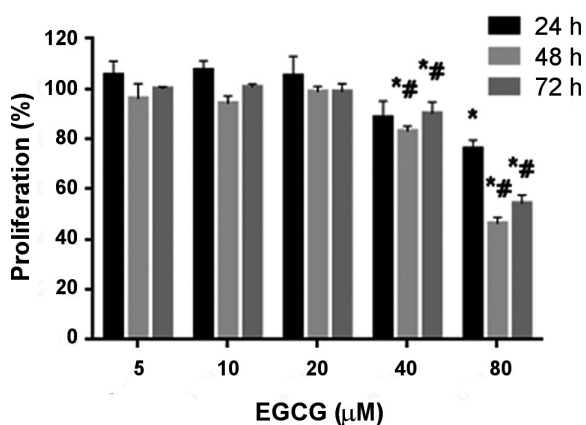


Figure 1. Effect of EGCG on the proliferation of SACC-83 cells.

Notes: Cells were treated with EGCG (0-80 µM) for 24 h, 48 h or 72 h, and proliferation was assessed by MTS assay. Data were normalized with control (EGCG 0) and given by percentage (means ± SD, n=3). Significant difference from the control was determined by ANOVA at P < 0.05 (denoted as \*) and from the lower concentration group at P < 0.05 (denoted as #).

Absorbance was detected at 490 nm. Results were normalized with control (EGCG 0) by the formula: cell proliferation rate =  $OD_{test} / OD_{ctrl} \times 100\%$ .

**Cell apoptosis analysis.** The effect of EGCG on cell apoptosis was determined by flow cytometry after staining the cells with Annexin V-FITC and PI (Annexin V/PI apoptosis kit, BD Biosciences, USA). SACC-83 was seeded in 6-well plates ( $2 \times 10^5$  cells/well) overnight, and then treated with desired concentrations of EGCG. Both the floating and adherent cells were collected using 0.25% trypsin (Gibco) after 24 h, 48 h, and 72 h respectively. ( $1 \sim 5$ )  $\times 10^5$  cells were collected and stained with Annexin-V-FITC solution and PI. Then the cells were evaluated on flow cytometer (Becton Dickinson, Mountain View, CA).

**Western blotting.** Cell cultures were prepared in the same way as described previously in apoptosis analysis. After treated with a certain concentration of EGCG for 48 h, cells were lysed to extract proteins, and the total proteins were quantified by the BCA method. The sample proteins were separated by electrophoresis on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated with various primary antibodies overnight at 4 °C and with HRP-linked secondary antibodies for 1 h at 25 °C. Immunoreactive bands were visualized by chemiluminescent HRP substrate (Millipore Corporation, USA). Optical densities of the band were quantitated by Quantity One software.

**RT-PCR.** Cell cultures were prepared in the same way as described previously. Total RNA was isolated by Trizol (Life Technologies, UK). Complementary DNA was synthesized by reverse transcription of total RNA using iScript cDNA Synthesis Kit (TaKaRa Bio, Japan). Quantitative RT-PCR was performed using ABI 7900ht system (Applied Biosystems, USA) with iTaq Universal SYBR Green Supermix (Bio-Rad, USA). The GAPDH was used to normalize the expression levels. Relative mRNA expression was calculated using the  $\Delta\Delta Ct$  method. Genes with a fold change of  $\leq -1.25$  or  $\geq 1.25$  and P < 0.05 were considered statistically significant. The primers used were as follows:

Gene	Forward	Reverse
GAPDH	AGAAGGCTGGGGCT-CATTTC	AGGGGCCATCCACA-GTCTTC
P90 RSK	GAGACTGACTGCT-GCTCTTGTG	GGTTCCAAAAGTGGT-GACTGAT

**Statistical analysis.** All experiments were performed three times. Results were expressed as mean ± standard deviation (SD). Statistical significance was determined by a one-way analysis of variance (ANOVA). The significance level was set at P < 0.05.

## Results

**EGCG treatment inhibits proliferation of SACC-83 cells *in vitro*.** An MTS was performed to evaluate the effect of EGCG on the proliferation of SACC-83 cells (Figure 1.)

EGCG treatment at lower concentrations (0 to 20  $\mu\text{M}$ ) had no significant effect on cell proliferation. But at doses of 40 to 80  $\mu\text{M}$ , EGCG affected cell proliferation in a dose-dependent manner. After being treated with 40  $\mu\text{M}$  EGCG for 48 h and 72 h, cell viability decreased to  $(83.2 \pm 1.8) \%$  and  $(90.2 \pm 4.3) \%$ , respectively (Figure 1). Exposed to 80  $\mu\text{M}$  EGCG for 24 h, 48 h, and 72 h, cell proliferation reduced to  $(76.2 \pm 3.4) \%$ ,  $(46.6 \pm 2.5) \%$ , and  $(54.4 \pm 3.2) \%$ , respectively (Figure 1). After 48 h and 72 h, cell proliferations of 80  $\mu\text{M}$  are lower than that of 40  $\mu\text{M}$ , and 40  $\mu\text{M}$ 's are lower than 20  $\mu\text{M}$ 's.

**Induction of cell apoptosis.** EGCG-induced apoptosis was measured using flow cytometry. Annexin V-FITC/PI assay was used to distinguish apoptotic cells from viable ones. Cells were differentiated to viable (Annexin V-negative and PI-negative), early apoptotic (Annexin V-positive, PI-negative), and late apoptotic (Annexin V-positive and PI-positive) parts. The range of apoptosis was quantified as percentage of Annexin V-positive cells.

Incubation with EGCG at 0 to 10  $\mu\text{M}$  scarcely alter the percentage of apoptotic cells, while apoptotic rate was increased after being treated with EGCG at 20 to 80  $\mu\text{M}$  compared with control ( $P < 0.05$ ) (Figure 2). We did not detect the difference among 20 to 80  $\mu\text{M}$  ( $P > 0.05$ ). The results demonstrate higher apoptosis rates in EGCG treatment groups compared with the control group that EGCG induces cell apoptosis significantly at the concentration of 20 to 80  $\mu\text{M}$ .

**Effect of EGCG on protein expression of EGFR/Erk downstream pathways and Bcl-2, Bax in SACC-83 cells.** To elucidate the molecular mechanisms of EGCG, we examined the protein expression of EGFR and ERK downstream pathways in SACC-83 cells by Western blotting. As shown in Figure 3, the level of total EGFR (Cell Signaling, USA) was decreased in a dose-dependent manner after being treated with EGCG (5-80  $\mu\text{M}$ ) for 48 h in SACC-83 cells. Additionally, Phospho-Erk 1/2 (Thr202/Tyr204, Cell Signaling) and Phospho-Mek (Ser217/221, Cell Signaling) proteins were downregulated by EGCG (20-80  $\mu\text{M}$ ) after 48 h without altering the total levels of Erk 1/2 (Cell Signaling) and Mek (Cell Signaling) proteins. Bcl-2 (Epitomics, USA) was inhibited by EGCG. On the contrary, the pro-apoptotic protein Bax (Epitomics) was activated ( $P < 0.05$ ) by EGCG.

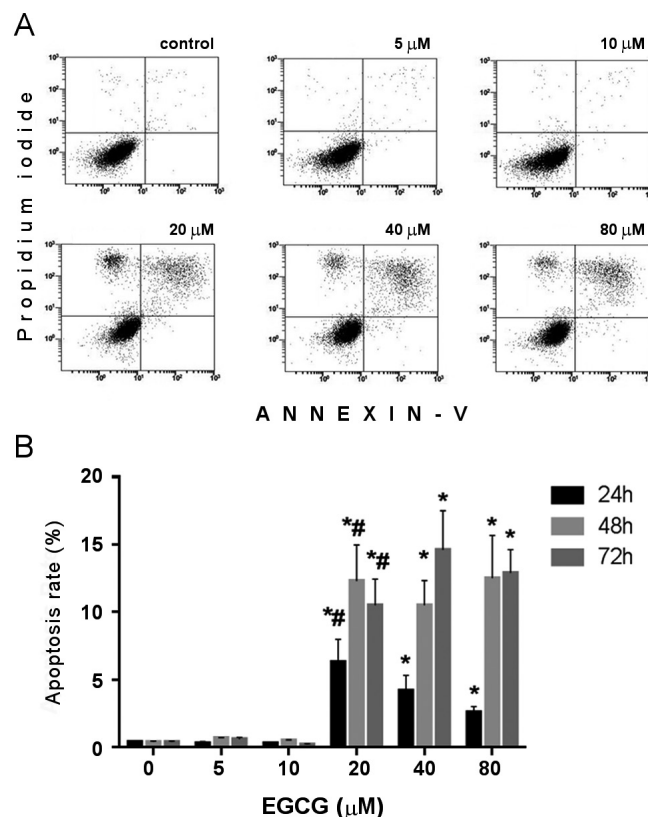
**Effect of EGCG on P90 RSK gene expression in SACC-83 cells.** To further explore the mechanism of EGCG in SACC-83 cells, we next researched whether P90 RSK expression would be downregulated by EGCG in gene level. The fold change data are presented in Figure 4. As shown in Figure 4, EGCG did not have effect on mRNA levels of P90 RSK gene at small dose (5-10  $\mu\text{M}$ ,  $P > 0.05$ ). While concentration of EGCG increased to 20-80  $\mu\text{M}$ , mRNA levels of P90 RSK gene was distinctively reduced ( $P < 0.05$ ).

## Discussion

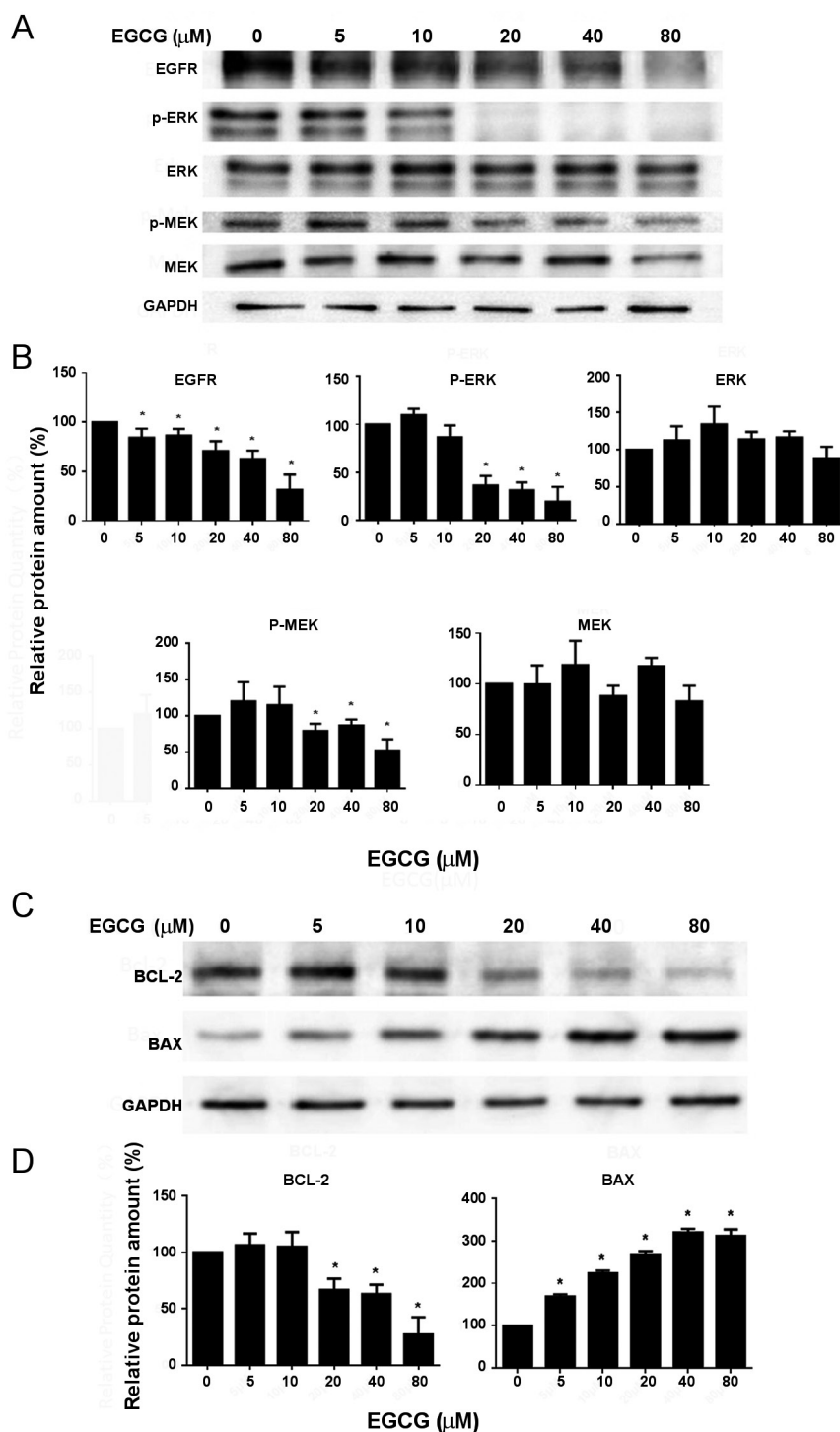
ACC is one of the most malignant tumors of salivary gland, representing about 1% of all head and neck malignancies

[28] and about 10% of salivary gland neoplasms [29]. But the prognosis is still poor even the treatment of wide tumor-free surgical margin excision and radiation, or chemoradiotherapy are performed [7] because of tumor character of a locally infiltrative growth along perineural and blood vessel invasion and high rates of local recurrence and distant metastasis[30]. After the operation and radiotherapy, the quality of the life is also influenced seriously.

With the increasing understanding of the molecular biology and the mechanism of head and neck cancer, more and more therapeutic targets have been developed [31]. To search for molecular targets for therapeutic intervention, the importance is to find the biochemical and molecular differences between normal cells and cancer cells. These changes may be associated with the tumor suppressor genes, growth factors and their receptors, tumor angiogenesis factors, protein kinases and so on. Targeted therapies against any of these changes may represent therapeutic targets [32]. However, the characteristics of multiple genes, multiple factors and multiple stages of carcinoma



**Figure 2.** Effect of EGCG on the apoptosis induction of SACC-83 cells. Notes: (A) Dot plot representation of cell viability after being treated with EGCG (0-80  $\mu\text{M}$ ) for 48 h by flow cytometry. (B) Apoptosis rate of SACC-83 cells after treated with EGCG (0-80  $\mu\text{M}$ ). Cells were cultured with EGCG (0-80  $\mu\text{M}$ ) for 24, 48 and 72h and marked with Annexin V-FITC/PI. Values are shown as means  $\pm$  SD of 3 replicates. Significant difference compared with control (EGCG 0) can be found at \* ( $P < 0.05$ ), and with lower concentration group at # ( $P < 0.05$ ).



**Figure 3.** Effect of EGCG on the regulation of EGFR, Erk, Mek, Bcl-2 and Bax of SACC-83 cells.

**Notes:** (A) Representative Western blotting analysis of EGFR and Erk 1/2 (p-Erk 1/2), Mek (p-Mek) in SACC-83 cells. (B) Quantification of Western blotting band of EGFR and Erk 1/2 (p-Erk 1/2), Mek (p-Mek). (C) Representative Western blotting analysis of Bcl-2 and Bax in SACC-83 cells. (D) Quantification of Western blotting band of Bcl-2 and Bax. SACC-83 cells were treated for 48 h with EGCG (0-80 μM) and equal amounts of total protein were immunoblotted with anti-EGFR, anti-Erk 1/2, anti-p-Erk 1/2, anti-Mek, anti-p-Mek, anti-Bcl-2 and anti-Bax antibodies. GAPDH was used as the internal control. Dates were obtained from 3 independent experiments and normalized with GAPDH. Symbol \* illustrated there was difference between the drug group and control group ( $P < 0.05$ ), respectively. As the increase of EGCG (0-80 μM), total amount of EGFR protein decreased ( $P < 0.05$ ). And at the treatment of EGCG (20-80 μM), the phosphorylation of Erk 1/2 and Mek were inhibited compared with control ( $P < 0.05$ ). Received the treatment of EGCG (20-80 μM), the Bcl-2 was inhibited compared with control ( $P < 0.05$ ). And as the increase of EGCG (0-80 μM), Bax was upregulated ( $P < 0.05$ ).

determine the multiple therapeutic targets other than single target. The pathogenesis of ACC is unclear, until now relatively few genetic alterations critical to the ACC development have been recognized. The MYB-NFIB translocation has recently been identified in ACC and may play an important role in the development of ACC [33, 34], and the MYB-NFIB translocation may evolve as another potential therapeutic targets in the future [35]. Report showed that targeted therapies directed against the EGFR and PTEN proteins or their constitutive pathways may be valuable in the treatment of ACC [36].

EGCG, the most major constituent of tea, has broad anticancer properties, including those for cancers of the skin, lung, oral cavity, stomach, small intestine and so on [37]. Studies have shown that the mechanisms of its anticancer properties are very complex, such as enhancement of apoptosis, suppression of cell proliferation, inhibition of angiogenesis and so on [38-40]. Additionally, it is likely that multiple molecular mechanisms, rather than a single target, are involved [41]. However, there is no relevant evidence for its efficacy in the treatment of ACC. Thus, we hypothesized that EGCG may has a good anti-tumor activity in ACC.

In the present study, it was shown that EGCG had an effective anti-tumor activity on SACC-83 cell lines. According to the MTS and flow cytometry, EGCG was able to inhibit proliferation and promote apoptosis of ACC cells, as was confirmed in multiple cell lines, such as renal cell carcinoma (RCC) cells and human villous trophoblasts (HVT) [42, 43]. Uncontrolled growth is a necessary step for the development of all cancers. EGFR plays critical roles in ligand-activated signaling pathways that regulate cell proliferation and death [44], and involves in the development of many malignant tumors, such as ACC, colon cancer and non-small cell lung cancer [45-47]. It was shown that EGFR was overexpressed in the ACC and owned with biologic aggressiveness and poor prognosis of malignant character [48]. Activating mutations and overexpression of receptor tyrosine kinases just like EGFR represent an important source of therapeutic resistance in salivary gland adenoid cystic carcinoma [45]. So it was deduced that EGFR played an essential role in anti-tumor of the ACC treated by EGCG. Previous studies revealed that MAPK signaling is a key pathway of cellular proliferation and apoptosis regulation, and a major downstream signaling route of the MAPK signaling is via the Ras-Raf-Erk kinase pathway and at last regulates the cell proliferation, survival, apoptosis and transformation [49]. In the present study, it was demonstrated that the expression of EGFR was reduced, phosphorylation of Erk and Mek was inhibited. Furthermore, it was also found that mRNA level of P90 RSK was suppressed and RSK was a downstream mediator of the MAPK pathway that regulates proliferation and apoptosis in a variety of cancer cell lines [50]. RSK phosphorylates multiple signaling effectors to play an essential role in a number of cellular functions, including regulation of gene expression by phosphorylation of transcriptional regulators, including c-Fos and CREB; regulation of cell cycle by phosphorylating and inhibiting Myt1 [51]; and regulation

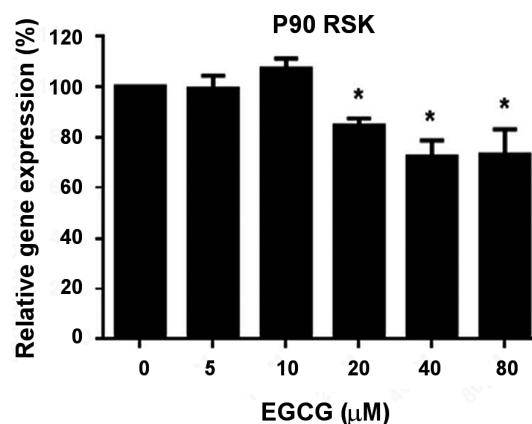


Figure 4. Relative P90 RSK gene expression level of SACC-83 after cells were treated with EGCG (0-80 μM) for 48 h.

Notes: P90 RSK gene expression level was detected by Quantitative RT-PCR. GAPDH was used to normalize the expression levels. Relative mRNA expression was calculated using the  $\Delta\Delta Ct$  method. Genes with a fold change of  $\leq -1.25$  or  $\geq 1.25$  and  $P < 0.05$  were considered statistically significant. Significant difference compared with control (EGCG 0) can be found at  $^*(P < 0.05)$ , EGCG (20-80 μM) distinctively reduced mRNA levels of P90 RSK gene.

of cell survival by phosphorylating Bad, Bim, and DAPK to protect cells from apoptosis [52]. So it may concluded EGCG inhibits proliferation and promotes apoptosis of SACC-83 cells *in vitro* via an EGFR dependent signaling transduction mechanism and regulates SACC tumor cell activity through the MAPK mediated pathway.

Accumulating evidence indicates that the anti-tumor effect of EGCG is also depending on promoting cell apoptosis. There are two major signaling pathways induce apoptotic cell death, the mitochondrial (the intrinsic) pathway and the death receptor (the extrinsic) pathway [52]. Bcl-2 family is divided into two subgroups according to their functions: the anti-apoptotic Bcl-2 family members and the pro-apoptotic members [53]. Bcl-2 and Bax are the most representative anti-apoptotic and pro-apoptotic protein in the Bcl-2 family while Bax is the main regulator of Bcl-2 activity [53]. Studies have shown that EGCG has the effect to downregulate the expression of Bcl-2 protein and to upregulate Bax. In OSCC, compared with oral epithelium, there is a decreased Bcl-2 expression by EGCG [27]. In B lymphoma cells, EGCG upregulated the protein expression of Fas and Bax while downregulating Bcl-2 to induce B lymphoma cells apoptosis [54]. Indirectly inducing up-regulation of pro-apoptotic signals or directly inhibiting the activity of anti-apoptotic proteins are two strategies that may target the evasion of cancer cells for treatment [55]. According the results of our research (Figure 3), we conclude that EGCG initiates the mitochondrial pathway to induce apoptosis of SACC-83 cells. Compared to cause necrosis of tumor cells, inducing the apoptosis of tumor cells can promote tumor ablation or even elimination, at the same time reduce the inflammatory reaction and other side effects of surrounding normal tissues

[56]. Therefore, the drugs which induce apoptosis of tumor cells are better than chemotherapeutic drugs which cause cell necrosis. According to the strategy of inducing apoptosis for cancer therapy, EGCG which can upregulate pro-apoptotic protein and inhibit the expression of anti-apoptotic proteins would be a potential therapeutic drug for the patients with SACC tumors.

In conclusion, the present study proposes that EGCG would exert anticancer effects on SACC-83 cells and the evidences demonstrate that EGCG is able to inhibit growth and induce apoptosis of SACC-83 cells by reducing expression of EGFR, which can active EGFR/Erk/P90 RSK pathway, and initiating mitochondria apoptosis pathway. It also suggests EGCG may be a potential therapeutic or adjuvant strategy for the treatment of patients with SACC tumors. However, it is unclear how EGCG acts on these pathways, and whether these signaling pathways relate to other beneficial effects of EGCG.

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