

## EGCG sensitizes human nasopharyngeal carcinoma cells to TRAIL-mediated apoptosis by activation NF- $\kappa$ B

P. LI, S. LI\*, D. YIN, J. LI, L. WANG, C. HUANG, X. YANG

*Department of Otolaryngology, Head and Neck Surgery, the Second Xiangya Hospital, Central South University, Changsha 410011, Hunan, PR China*

\*Correspondence: [lissdoctor@sina.com](mailto:lissdoctor@sina.com)

Received July 5, 2016/ Accepted September 12, 2016

Aim of presented study was to investigate whether EGCG could sensitize TRAIL resistant NPC cells to TRAIL-mediated apoptosis. Three human NPC cell-lines CNE-1, CNE-2, C666-1 and a non-transformed nasopharyngeal epithelium cell-line NP-69 were treated with EGCG or/and TRAIL. The apoptosis and TRAIL receptors were determined by flow cytometric analysis. The protein expression was determined by western blotting. Mitochondrial transmembrane potential was determined by DiOC<sub>6</sub> (3). C666-1 cell-line was the only one that resistant to TRAIL and selected to be treated with EGCG. EGCG could sensitize C666-1 to TRAIL. Combinatorial treatments led to decrease expression of Bcl-XL, Bcl-2, FADD and FLIP and enhance activation of capase-3, -8, -9. The DiOC<sub>6</sub> (3) negative cell rate was increased and p65 of NF- $\kappa$ B, XIAP and survivin expression was reduced by the combination treatment.

In summary, EGCG sensitizes NPC cells to TRAIL-mediated apoptosis via modulation of extrinsic and intrinsic apoptotic pathways and inhibition of NF- $\kappa$ B activation.

*Key words: nasopharyngeal carcinoma, TRAIL, EGCG, apoptosis, NF- $\kappa$ B*

Tumor necrosis factor (TNF)- $\alpha$ -related apoptosis inducing ligand (TRAIL) is a type II transmembrane homotrimer cytokine of TNF family. It is capable of binding death receptors and inducing selective, P53-independent apoptosis of tumor cells, while producing negligible effects on normal cells [1]. Previous researches have revealed that some cancer cells including nasopharyngeal carcinoma (NPC) cells are resistant to the apoptotic effects of TRAIL [2, 3]. However, chemotherapeutic drugs combine with TRAIL could sensitize these cells to induced apoptosis [4]. Which indicated that the resistance of cancer cells to TRAIL can be overcome by combinatorial treatment. Thus, to find efficient treatment methods to sensitize cancer cells to undergo TRAIL-mediated apoptosis are of paramount important.

Incidence of nasopharyngeal carcinoma (NPC) varies largely in different area of the world. The cancer is endemic in certain regions, which accounted for 1.34% of all new cancer cases and 1.03% of all cancer-related deaths in 2010 in China [5]. Radiotherapy alone was the standard treatment for almost all stages of NPC until 1990 and got a good track record. However, the outcome with radiotherapy alone is disappointing,

since 5-year survival rates are 34–52% [6]. Study has found that the addition of chemotherapy to radiotherapy significantly improved the survival rate [7]. And developing novel chemotherapy options for NPC has become an important medical need. Death receptors have been detected in biopsy specimens of neoplasm but not in the surrounding tissues of head and neck squamous cell carcinoma [8]. This finding supports the notion that the presence of death receptors may result in a greater susceptibility to TRAIL-mediated apoptosis in these cancers. Studies have shown robust TRAIL induced apoptosis in the NPC cell-line TW02 [9], while others have found it is difficult to induce apoptosis in some NPC cell-lines by TRAIL [10]. The reason for this discrepancy remains unknown. However, our prior studies showed that overexpression of Bcl-2 and PI3K/Akt activation may contribute to TRAIL resistance in NPC cell lines [3].

Tea polyphenols are being investigated as possible neoadjuvant and adjuvant therapy for cancer due to their ability to inhibit multiple signaling pathways. A major component of tea polyphenols are the polyphenol (-) epigallocatechin-3-gallate (EGCG) [11]. EGCG impairs cancer cell growth by a variety of

mechanisms, including up-regulation of adhesion molecules, suppression of gelatinases activity and induce apoptosis of cancer cells [12].

This study aims to investigate whether EGCG could sensitize TRAIL resistant NPC cells to TRAIL-mediated apoptosis. The possible molecular mechanisms of these effects were also being established. Here, we present the evidence that EGCG could reverse the resistance of NPC cells to TRAIL. We also revealed that both intrinsic and extrinsic pathways are involved in the EGCG-mediated apoptosis of TRAIL-resistant NPC cells, and also made it clear that the EGCG-mediated NF- $\kappa$ B suppression coursed the synergism.

## Materials and methods

**Cell lines and reagents.** The study used three human NPC cell-lines CNE-1, CNE-2, and C666-1. CNE-1 is a well-differentiated nasopharyngeal squamous cell carcinoma cell-line. CNE-2 is a poorly differentiated cell-line. C666-1 is the NPC cell-line expressed EBV constantly. NP-69 is a non-transformed nasopharyngeal epithelium cell-line derived from human nasopharynx. We used RPMI-1640 (Invitrogen, Carlsbad, USA) media with 10% fetal bovine serum (Hyclone, Logan, USA) for cell culture and antibiotics (100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin). Cultures were maintained at 37 °C and 5% CO<sub>2</sub> in a fully humidified atmosphere. TRAIL was obtained from Pharmacia Corporation, Pfizer, Inc.; G. D. Searle and Co. EGCG was obtained from Biomol Corporation, China. EGCG was dissolved in DMSO and stored at -20°C until used.

**Cell viability assay.** We used 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to determine the effect of EGCG (20  $\mu$ M pretreatment for 24 hours) and TRAIL (0 to 100 ng/ml for 24 hours or 100 ng/ml for 4 to 24 hours) on the viability of cells. Cells were seeded into 96-well plates (3 $\times$ 10<sup>3</sup> cells/well) with 100  $\mu$ L of medium. And treated with each compound alone or in combination after 24 hours of culture, and incubated for another 24 hours. We used MTT assay to determine the cell viability by adding 10  $\mu$ L of 5 mg/mL MTT solution (Sigma) into each well. EGCG (200  $\mu$ L of DMSO solution) was added into each well after 3 hours of incubation. The optical density (OD) of each well was measured at 570 nm with a microplate reader (Viability =  $OD_{\text{treat}}/OD_{\text{control}}$ ).

**Flow cytometric analysis.** Annexin V-FLUOS staining kit was used to determine the effect of EGCG and TRAIL on the apoptotic rate of cells. Cells were treated with each compound alone or in combination and the cells were harvested 24 hours later. The cells were dissolved in 500  $\mu$ L binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>) at a density of 1 $\times$ 10<sup>6</sup>/mL. Samples were incubated in the dark for 5 min with 5  $\mu$ L PI and 1  $\mu$ L Annexin V-FITC at room temperature. Then the cells were measured by a FACSCalibur cytometer (Becton Dickinson, USA). After correction to the spectral overlap, PI and Annexin V-FITC

fluorescence were respectively detected in the FL-1 (green) and FL-2 (red) channels. We used CellQuest software (Becton Dickinson, Country) for Data were analyzing.

**Flow cytometric analysis of TRAIL receptors.** Cells (1 $\times$ 10<sup>6</sup> cells) were isolated and pelleted (1000r/m for 10 min) from the culture media and dissolved in 500  $\mu$ L of the collected medium. Then the cells were incubated with 5  $\mu$ L of anti-DR4 or anti-DR5 polyclonal goat antibody (1:100, Sigma-Aldrich Co., St. Louis, MO) for 1 hour. Then FITC-conjugated rabbit anti-goat polyclonal antibody (1:200, Sigma-Aldrich Co., St. Louis, MO) was added into the cell suspension after eluted by PBS, and incubated for 1 hour on ice. After rinsed by PBS, the samples were detected by a FACSsort flow cytometer (Becton Dickinson, USA). We used CellQuest software (Becton Dickinson, Country) for Data were analyzing.

**Western blotting.** Cells were lysed with lysis buffer (150 mM NaCl, 100 mM NaF, 20 mM Na<sub>2</sub>PO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 1% aprotinin and 2 mM Na<sub>3</sub>VO<sub>4</sub>). Proteins were separated by electrophoresis on a dodecyl sulfate-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. The membranes were pre-treated with specific primary antibodies. Then, membranes were incubated with the secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit, all the antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA.). Then the protein was detected with enhanced chemiluminescence (ECL).

**NF- $\kappa$ B activation analysis.** For nuclear extracts, C666-1 cells were seeded in 10cm plates and incubated with combination of EGCG and TRAIL. After the incubation process, cells were transfer to PBS and pelleted (1200 r/m for 5 min). The pellets were lysed by 100  $\mu$ L lysis buffer A (PMSF 2 mM containing cocktails protease inhibitors, HEPES 10 mM pH 7.9, EDTA 1mM pH 8.0, KCl 60 mM, N-P40 0.2%, all from Sigma-Aldrich, Italy) and then incubated on ice for 5 min. The presence of active NF- $\kappa$ B was detected by specific antibodies for p65 subunits by Western blotting in nuclear extracts.

**Assessment of mitochondrial transmembrane potential.** The cationic lipophilic fluorochrome 3,3'-dihexyloxacarbocyanide iodide (DiOC<sub>6</sub> (3)) (460 ng/mL, Molecular Probes, Eugene, OR, USA) was used to assess the mitochondrial transmembrane potential ( $\Delta\psi$ m). Cells were treated with TRAIL and/or EGCG for 30 min at 37.8 °C in complete media with DiOC<sub>6</sub> and then analyzed by FACSsort flow cytometer.

**Statistical analysis.** All tests were performed in triplicates and the authors used SPSS 19.0 for statistical analyses. Descriptive statistics was expressed as means $\pm$ SD and Student's t-tests were used to analyze significant differences between paired datasets. An alpha value of p<0.05 was considered statistically significant.

## Results

**EGCG sensitizes NPC cells to TRAIL.** First, we assessed the growth inhibition effects of TRAIL against different NPC

cell-lines by MTT assay. TRAIL induced cell death in a dose – and time-dependent manner. C666-1 cells were highly resistant to TRAIL (Figure 1A). Annexin V/PI staining and flow cytometry were used for further analyzed the apoptosis rate to confirm whether the difference in cell death was due to different apoptotic responses. The apoptotic effect of TRAIL was also time – and dose-dependent. The percentage of apoptotic cells was lowest in C666-1 cells (Figure 1B). We selected C666-1 as resistant cell for the following experiments. The results of MTT showed that C666-1 cells were sensitized to TRAIL by 20uM EGCG pretreatment for 24 hours. In order to confirm whether the enhancements of cell death were due to increased apoptosis, we use flow cytometric analysis. Combination of 20uM EGCG to 100ng/mL TRAIL also increased apoptosis rate that was observed by FCM (Figure 2).

**EGCG sensitized NPC cells to TRAIL by modulation of extrinsic and intrinsic apoptotic pathway.** To investigate the possible molecular mechanism underlying the effects of EGCG-mediated apoptosis of TRAIL-resistant, several molecules relate to the initiation and execution apoptotic procedure were studied. Combination of TRAIL to the ligand of death receptors, DR4 and DR5, promotes apoptosis [13]. Figure 3 reveals expression of DR4 was upregulated synergisti-

cally when the cells were simultaneously treated with EGCG and TRAIL, but not DR-5. Studies revealed that caspase-8 and Fas-associated death domain (FADD) recruited the DR-TRAIL complex to form a signaling complex (death-inducing signal complex, DISC) which induce cell apoptosis [14]. Then the apoptotic signal is transmitted through both the extrinsic and intrinsic pathways. Precious studies revealed that FLICE-inhibitory protein (FLIP) is an endogenous regulator of death receptor signaling pathway, which interacts with FADD and promotes cell survival [15]. Expression of FADD and FLIP were significant inhibited with TRAIL treatment, and more obvious in the cells with combinative treatment of EGCG and TRAIL (Figure 4A). Activation of caspases-3 and -9 was downstream of activation of caspase-8. And a synergistic activation of initiator caspase-9 and effector caspases-3 were observed after combinative treatment (Figure 4B). Proapoptotic Bax and Bak protein expression level were demonstrated to be upregulated by EGCG and TRAIL treatment specialty and this upregulation was augmented with combinative treatment (Figure 5A). Protein expressions of prosurvival Bcl-2 and Bcl-XL were observed to be inhibited by EGCG and TRAIL and synergistically inhibited by in combination. Furthermore, the DiOC6 (3) negative cell rate was higher in cells treated with

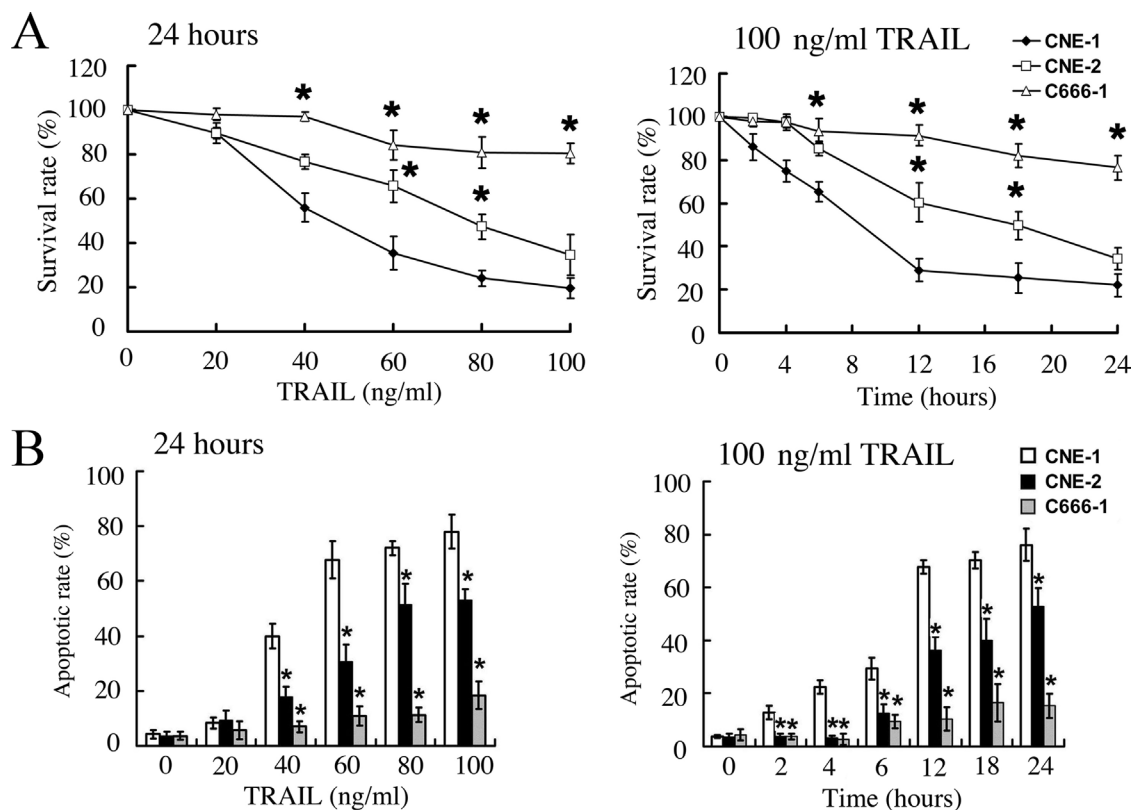


Figure 1. The sensitivity to TRAIL varied among NPC cells. A) CNE-1, CNE-2 and C666-1 cells were treated with escalating doses of TRAIL for 24 hours and 100 ng/mL TRAIL with different time period and cell viability was determined by MTT assay. B) Cells were treated with escalating doses of TRAIL for 24 hours and 100 ng/mL TRAIL with different time period and apoptotic rate was determined by flow cytometric analysis. Apoptosis rate included early apoptosis (Annexin-V positive) and late apoptosis (PI positive).

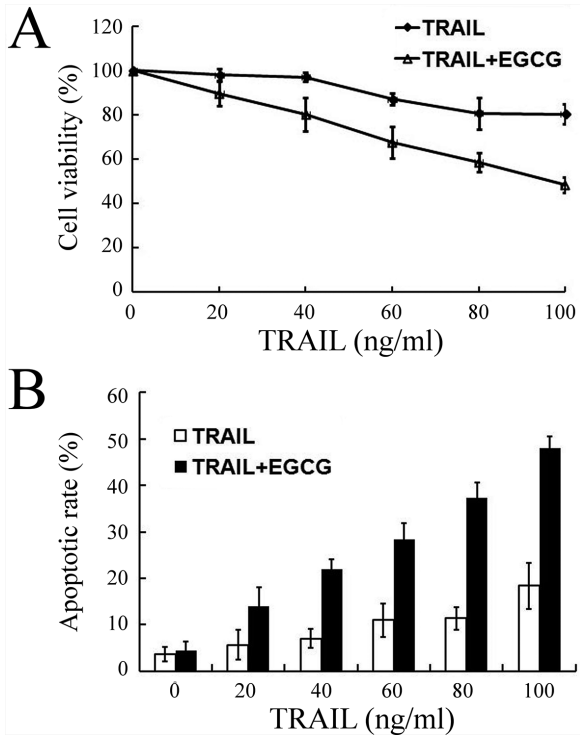


Figure 2. EGCG sensitizes NPC cells to TRAIL. A) C666-1 cells were treated with escalating doses of TRAIL for 24 hours following pretreatment for EGCG and cell viability was determined by MTT assay. B) Cells were treated with escalating doses of TRAIL for 24 hours following pretreatment for EGCG and apoptotic rate was determined by flow cytometric analysis. Apoptosis rate included early apoptosis (Annexin-V positive) and late apoptosis (PI positive).

combinative treatment than cells treated with TRAIL single treatment (Figure 5B). These results suggested that TRAIL-induced extrinsic apoptotic pathway and intrinsic apoptotic pathway were enhanced by EGCG.

**EGCG inhibits NF-κB nuclear translocation in C666-1 cells.** The effect of EGCG and TRAIL on NF-κB expression in C666-1 cells was evaluated. P65 is a subunit of NF-κB and the expression of p65 was detected by Western blotting in nuclear extracts. EGCG clearly reduced p65 expression in nuclear extracts, which suggested a possible inhibition of NF-κB nuclear translocation. However, TRAIL alone (100 ng/ml) had no effect on NF-κB activation. The combinative treatment reduced p65 expression in nuclear extracts synergistically (Figure 6A).

**Effects of EGCG on the expression of anti-apoptotic proteins.** Changes of expression of members of the Bcl-2 family and inhibitor of apoptosis (IAP) family result in chemotherapy resistance of NPC cells [16, 17]. NF-κB inhibits apoptosis via its ability to bind to anti-apoptotic genes, and change the expression of anti-apoptotic proteins, like Bcl-2, Bcl-XL, and IAPs family [18]. Hence, the expression of survivin and XIAP were evaluated by Western blotting in EGCG and/or TRAIL treated

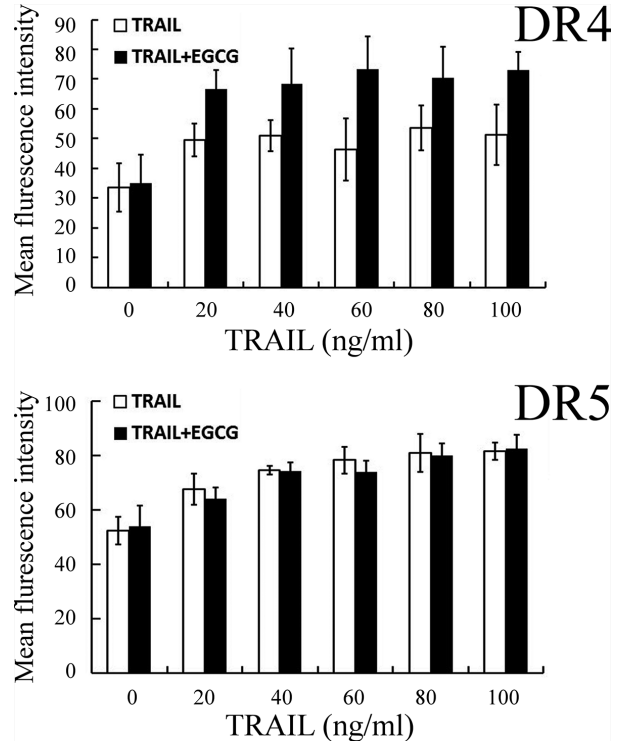


Figure 3. C666-1 cells were treated with escalating doses of TRAIL for 24 hours following pretreatment for EGCG and expressions of DR4 and DR5 were measured by flow cytometry analysis.

C666-1 cells. Figure 6B reveals that TRAIL treated alone had no effect on protein expression, while EGCG treated alone inhibited XIAP expression after 24 hours of incubation but not survivin. EGCG in combination with TRAIL inhibited expression levels of XIAP and survivin synergistically.

**Effects of EGCG on TRAIL-induced apoptosis in normal cells.** One important advantage is that normal cells naturally

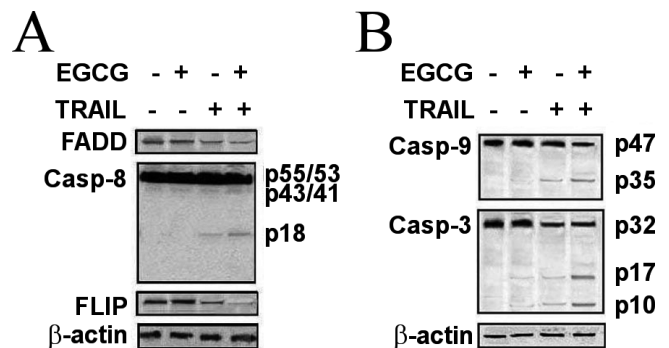


Figure 4. A) Cleavage of caspases-8 and expression of FADD and FLIP was assessed by Western blot during TRAIL and EGCG treatment. B) Cleavage of caspases-3 and -9 was assessed by Western blot during TRAIL and EGCG treatment. Caspase-3: p32-proform, p17, p10-cleavage fragments; caspase-8: p55/53-proform, p43/41, p18-cleavage fragments; caspase-9: p47-proform, p35-cleavage fragments.

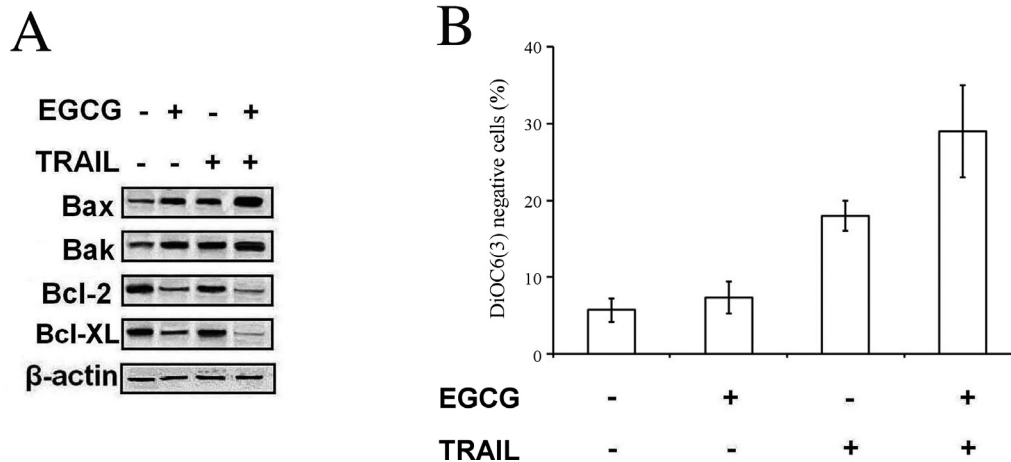


Figure 5. A) Expression of Bax, Bak, Bcl-2 and Bcl-XL was assessed by Western blot during TRAIL and EGCG treatment. B) Mitochondrial depolarization was measured by DiOC6(3) fluorescence during T TRAIL and EGCG treatment.

resistance to TRAIL. In order to investigate whether EGCG could sensitize normal cells to TRAIL, we used NP69, human nontransformed nasopharyngeal epithelium cell line, to detected the toxicity of the combinative therapy. No alter of TRAIL-induced apoptosis rate was observed under combinative treatment in benign nasopharyngeal epithelium cell.

## Discussion

In this study, we report EGCG sensitized NPC cells to TRAIL-induced apoptosis. EBV positive NPC cell line C666-1 is more resistance to TRAIL-induced apoptosis compared to the other two EBV negative NPC cell lines. This result suggests that EBV plays a restrictive role in TRAIL-induced apoptosis. EBV could up-regulate the expression of several anti-apoptotic genes through induction activation of NF- $\kappa$ B, which provide survival signal to cells [19]. This may be the one of the molecular mechanism that EBV positive NPC cells escape from apoptosis and more resistant to TRAIL.

TRAIL is a cytotoxic protein that induces apoptosis by binding to its death domain-containing receptors, DR4

and DR5 [14]. Caspase-8 and fas-associated death domain (FADD) recruited the DR-TRAIL complex to form a signaling complex which induce cell apoptosis. This apoptotic signal is further transmitted through both the intrinsic and extrinsic pathways. The intrinsic signal pathway is activated in a mitochondrial-dependent manner. Once cleaved by caspase-8, the pro-apoptotic protein BH3-interacting domain death agonist (Bid) translocates to the mitochondria and activates the Bcl-2 family members Bax and Bak, which then causes mitochondrial outer membrane permeabilisation and depolarize mitochondria. Apoptogenic factors such as cytochrome c are released into the cytosol to activate caspase-3 to induce apoptosis [20]. On the contrary, the extrinsic pathway is activated in a mitochondrial-independent mechanism. Caspase-8 is a well-characterized initiator of death receptor is triggered by the external stimulus. Once activated, it induces direct activation of downstream effectors like caspase-3 to induce apoptosis [21]. Previous researches have revealed

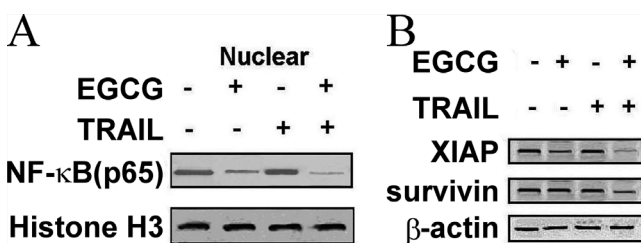


Figure 6. A) Expression of subunit p65 of NF- $\kappa$ B was assessed by Western blot in nuclear extracts during TRAIL and EGCG treatment. B) Expression of XIAP and survivin was assessed by Western blot during TRAIL and EGCG treatment.

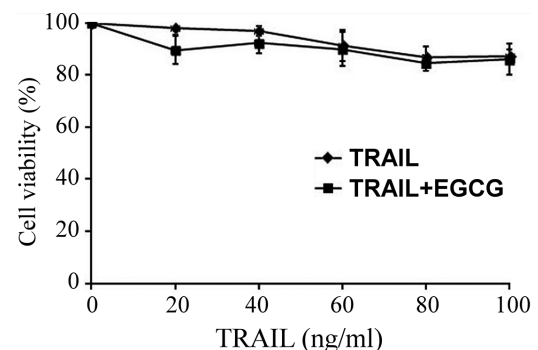


Figure 7. NP69 cells were treated with escalating doses of TRAIL for 24 hours following pretreatment for EGCG and cell viability was determined by MTT assay.



that some cancer cells are resistant to the apoptotic effects of TRAIL named as TRAIL resistance. However, chemotherapeutic drugs combine with TRAIL could sensitize these cells to induced apoptosis, which indicated that the resistance of cancer cells to TRAIL can be overcome by combinatorial treatment. Drug resistance is one of the major difficult for cancer treatment, ideal treatment should be highly selectively to cancer cells without normal cells damage [22]. So new treatment strategies like nontoxic agents are warranted to achieve TRAIL sensitivity before application in clinical therapy. EGCG is a natural phytochemical which inhibit activation of NF- $\kappa$ B, previous studies suggests it is a potential cancer chemopreventive and therapeutic agent. We here investigate whether EGCG could sensitize TRAIL resistant NPC cells to TRAIL-mediated apoptosis.

Figures 2 shows that treatment of C666-1 cells with 40mM of EGCG enhanced the sensitivity to TRAIL. The results reveal that treatment of cells with TRAIL does not result in change of expression of antiapoptotic molecules such as Bcl-XL, and Bcl-2, however, EGCG decrease their expression. Interesting, the combinative treatment led to a synergistic decrease in the expression of Bcl-XL and Bcl-2 (Figures 5A), which suggested that both the compounds act synergistically in down-regulating their expression. Although TRAIL itself did not induce significant apoptosis in C666-1 cells, it significantly modulated the expression levels of DR4 (Figure 3) and Bak, Bax (Figure 5), activation of caspases-3, 8 and 9, and FLIP (Figure 4), but not DR5, XIAP and survivin (Figure 3, 6). The results demonstrate that TRAIL could induce modulation in expression of apoptosis-related protein. However, the prosurvival protein may be result in the resistance of TRAIL-induced apoptosis in C666-1 cells. Since EGCG treatment inhibited these prosurvival factors, the threshold for TRAIL resistance is overcome, which may be the reason of EGCG treatment synergistically increases the rate of TRAIL-induced apoptosis. The DiOC6 (3) negative cell rate was increased synergistically by the combination treatment suggesting that EGCG enhance TRAIL-induced apoptosis by modulation of intrinsic apoptotic pathway. Many cancers cells epigenetically down-regulate the expression of DR as a strategy for survival. After treated by EGCG, the expression of DR4 was increase. Previous studies reveal EGCG effects through DNA demethylation, which may associate to the up-regulated expression of DR4 [23]. The increased DR expression in C666-1 cells thereby making them sensitive to TRAIL-mediated apoptosis. Our results reveal that EGCG sensitizes C666-1 cells to TRAIL-mediated apoptosis through modulation of intrinsic and extrinsic apoptotic pathways.

The inhibitor of apoptosis (IAP) family contributes to TRAIL resistance. Since NF- $\kappa$ B inhibits apoptosis through down-regulating the expression of a variety of anti-apoptotic proteins such as IAPs family [24]. The expression of p65 NF- $\kappa$ B, XIAP and survivin were evaluated by Western blotting through nuclear extracts in combinative treated EGCG and TRAIL C666-1 cells. EGCG alone inhibited XIAP expression

and NF- $\kappa$ B activation. Synergistically effects were observed in EGCG and TRAIL (0.5 ng/ml) combinative cells.

In conclusion, the study revealed EGCG could sensitize TRAIL resistant NPC cells to TRAIL-mediated apoptosis. The intrinsic and extrinsic apoptotic signal pathways were modulated and inhibition of NF- $\kappa$ B activation is a potential mechanism. These results may provide a new strategy for eliminating TRAIL resistance in NPC cells.

Acknowledgments: The National Nature Science Fund of China 81402502 supported this research.

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