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Theanine from tea and its semi-synthetic derivative TBrC suppress human cervical cancer growth and migration by inhibiting EGFR/Met-Akt/NF-κB signaling

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ABSTRACT

Cervical cancer is the third most prevalent cancer among women worldwide. Theanine from tea and its derivatives show some anticancer activities. However, the role of theanine and its derivatives against human cervical cancer and the molecular mechanisms of action remain unclear. Thus, in this study, we aim to investigate the anticancer activities and underlying mechanisms of theanine and a theanine derivative, ethyl 6-bromocoumarin-3- carboxylyl L-theanine (TBrC), against human cervical cancer. *In vitro* and *in vivo* assays for cancer cell growth and migration have confirmed the inhibition of the cell growth and migration by TBrC and theanine in highly-metastatic human cervical cancer. TBrC displays much stronger activity than theanine on inhibition of the cell growth and migration as well as induction of apoptosis and regulation of related protein expressions in the human cervical cancer cells. TBrC and theanine greatly reduced endogenous and exogenous factors-stimulated cell migration and completely repressed HGF- and EGF+HGF-activated EGFR/Met-Akt/NF-κB signaling by reducing the phosphorylation and expressions of EGFR, Met, Akt, and NF-κB in cervical cancer cells. The enhancer of zeste homolog 2 (EZH2) knockdown decreased the cancer cell migration and NF-κB expression. The NF-κB knockdown reduced the cancer cell migration. TBrC and theanine reduced the EZH2 expression by more than 80%. In addition, TBrC and theanine significantly suppressed human cervical tumor growth in tumor-bearing nude mice without toxicity to the mice. Our results suggest that TBrC and theanine may have the potentials of the therapeutic and/or adjuvant therapeutic application in the treatment of human cervical cancer.

1. Introduction

Cervical cancer is the third most prevalent cancer among women worldwide. According to GLOBOCAN statistics on cervical cancer (Ferlay et al., 2010), there were approximately 529,000 new cases and 275,000 deaths in 2008, of which more than 85% occurred in developing countries. Epidemiologic and molecular studies support that persistent infections with high-risk types of human papillomavirus (HPV) are essential but not exclusive prerequisites for cervical carcinogenesis (Lazo, 1999). For cervical cancer, there are few effective therapeutic options for patients who progressed after first-line chemotherapy. Metastasis is the main cause of morbidity and mortality in the thousands upon thousands of patients with cervical cancer. The endless proliferation and migration of cancer cells are the important steps of cervical cancer metastasis. Clearly, an agent which could efficiently inhibit proliferation and migration would suppress cancer progression and metastasis and thus could reduce mortality.

Theanine as a characteristic amino acid found in tea has been widely used for many years. It is a safe food additive without

Abbreviations: TBrC, ethyl 6-bromocoumarin-3-carboxylyl L-theanine; BrC, ethyl 6-bromocoumarin-3-carboxylic acid; NF-κB, nuclear factor κB; HGF, hepatocyte growth factor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EZH2, enhancer of zeste homolog 2; LLC, Lewis lung carcinoma, Ly, Ly294002, Bay, Bay 11-7082; PARP, poly(ADP-ribose) polymerase; MTT, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl- tetrazolium bromide; Z, Z-VAD-FMK; PI, propidium iodide; Cyto C, cytochrome c

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limitation to its dose. Some experimental results showed the anticancer activities of theanine (Sugiyama and Sadzuka, 1998; Sugiyama et al., 1999; Zhang et al., 2001, 2002; Liu et al., 2009). Our previous study indicated that theanine and the sera from rats treated with theanine significantly repressed the *in vitro* and *ex vivo* invasion of rat hepatoma cells (Zhang et al., 2001) and the *in vivo* growth of rat hepatoma (Zhang et al., 2002). We also reported that theanine repressed the growth and migration of lung cancer cells (Liu et al., 2009). To develop new effective compounds to inhibit cancer, we previously synthesized some theanine derivatives such as ethyl 6-fluorocoumarin-3-carboxylyl L-theanine (TFC), ethyl 6-nitrocoumarin-3-carboxylyl L-theanine (TNC), and ethyl 6-bromocoumarin-3-carboxylyl L-theanine (TBrC) by using theanine from green tea, and confirmed that the theanine derivatives significantly suppressed the growth of the highly-metastatic mouse Lewis lung carcinoma (LLC) cells without toxicity to normal cells (Zhang et al., 2014; Ji et al., 2014;). In order to further explore the anticancer activity of theanine and TBrC and their molecular mechanisms of action against highly metastatic cervical cancer, here we have studied the effects of theanine from tea and TBrC on cell growth, migration, and tumor growth as well as the relevant signaling pathways in human cervical cancer cell lines HeLa and CaSki with papillomavirus (HPV) types 16 and 18 sequences.

2. Materials and methods

2.1. Chemicals and antibodies

The primary antibodies against human p-Akt (Ser473), Akt, p-NF-κB p-65 (Ser536), NF-κB (p-65), p-EGFR (Tyr1068), EGFR, p53, p-Met (Tyr1234/1235), Met, β-tubulin, GAPDH, EGF and HGF and the hourseradish peroxidase-conjugated second antibodies were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). The primary antibodies against human cyclin D1, caspase-3, PARP-1, Bcl-2, Bax, cytochrome c, EZH2 (ENX-1), and β-actin and the hourseradish peroxidase-conjugated second antibodies were purchased from Santa Cruz Technology Inc. (Shanghai, China). Z-VAD-FMK (Z) and Annexin V-FITC/PI Apoptosis Detection Kit were purchased from Beyotime, Haimen, China. Fibronectin and Boyden chambers were purchased from BD Inc. (BD Biosciences, San Jose, CA, USA) and Corning Inc. (Corning, NY, USA), respectively. 3-[4,5-Dimethylthiazol- 2-yl]-2,5-diphenyltetrazolium bromide (MTT), RPMI 1640 medium, penicillin, streptomycin, fetal bovine serum (FBS), trypsin/EDTA, propidium iodide, Ly294002 (Ly), Bay 11-7082 (Bay), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TBrC was synthesized by our laboratory as reported previously (Ji et al., 2014).

2.2. Cell culture and MTT cell viability assay

Human cervical cancer cell lines HeLa and CaSki (highly metastatic cells with human papillomavirus types 16 and 18 sequences) were purchased from the American Type Culture Collection. The cancer cells were maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), glutamine (2000 μM), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were incubated at 37 °C in a humidified incubator with 95% air/5% CO_2 atmosphere. The MTT cell viability assay was performed according to our previous method with slight modification (Wang et al., 2009; Luan et al., 2010; Jiang et al., 2010; Zhang et al., 2012). Cells were seeded in 96-well plates (Becton Dickinson, NJ, U.S.A.) at 2×10^3 per well and cultured overnight. The control group was treated with vehicle [0.1% of DMSO (v/v]. The cells were respectively cultured in RPMI 1640 medium supplemented with 10% FBS containing different concentrations of TBrC, theanine, Ly294002 (Ly), or Bay 11-7082 (Bay). Ly and Bay are the inhibitors of PI3K/Akt, and NF-κB, respectively. After 48 and 72 h of treatment, the cells were treated with 20 μL of MTT (5 mg/ml) for 3 h at 37 °C. After the cells were lysed with DMSO, the absorbance was detected under a wavelength of 570 nm with a reference wavelength of 630 nm using a Synergy H4 Hybrid Multi-Mode Microplate Reader (Bio-Tek, Instruments, Inc., Winooski, VT, USA).

2.3. In vitro migration assay

HeLa and CaSki cell migration was examined by detecting cell migration through fibronectin-coated polycarbonate filters, using modified transwell chambers according to our previous methods with slight modification (Liu et al., 2009; Wang et al., 2009; Zhang et al., 2012). In brief, the cancer cells (5×10^4) were added onto the upper chamber in 210 μL of serum-free RPMI 1640 medium containing different concentrations of TBrC, theanine, Ly, or Bay; the lower compartment was filled with 0.67 ml of RPMI 1640 medium supplemented with 10% of FBS, HGF (80 ng/ml), EGF (80 ng/ml), or EGF+HGF (80 ng/ml) (as the chemoattractant). The control group was treated with vehicle (0.1% of DMSO). The cells were incubated at 37 °C for 6 h. Then, the migrated cells were fixed and stained with propidium iodide. The migrated cells were counted and recorded for images under a fluorescent microscope (Nikon, TE2000-U, Japan).

2.4. Detection of cell apoptosis

The cell apoptosis was detected following the instructions from the manufacturer of Annexin V-FITC/PI Apoptosis Detection Kit (Zhang et al., 2014). In brief, cells were incubated for 48 h with various concentrations of TBrC, theanine, Ly294002, Bay 11-7082 and/or Z, Z-VAD-FMK. The apoptotic cell fraction was measured using the Annexin V- FITC/PI Apoptosis Detection Kit. The apoptotic ratio was detected by flow cytometry (Becton Dickinson FACS Vantage SE, San Jose, CA, USA).

2.5. Western blotting

Western immunoblotting experiments were performed as described previously with some modifications (Wang et al., 2009; Jiang et al., 2010; Zhang et al., 2012). In brief, HeLa cells were treated with different concentrations of TBrC or theanine (16–250 μM), Ly294002 (Ly, 16 μM), or Bay (3.2 μM). The control group was treated with DMSO vehicle [0.1% (v/v)]. The treated cells were harvested either at 30 min for detection of phosphorylation ratios of pEGFR/EGFR, pMet/Met, pAkt/Akt, and pNF-κB (p65)/NF-κB (p65) or at 48 h for detection of protein expressions of Bax, Bcl-2, PARP-1, procaspase/caspase-3, p53, cyclin D1, EZH2, NF-κB (p65), and cytosolic cytochrome c. For examining the effects of HGF and EGF+HGF on the protein expressions, HeLa cells were pretreated with theanine and TBrC for 30 min. Then, the cells were treated with HGF (80 ng/ml) and EGF (80 ng/ml) plus HGF (80 ng/ml) for 1 h, respectively. The cells were harvested and washed with PBS. The whole cell lysates and cytosolic fractions were prepared according to the instructions of the manufacturer (Beyotime, Haimen city, China). The protein concentration was measured by the Bradford method. Equal amounts of the cell lysates were resolved by electrophoresis in SDS-PAGE and probed with the primary antibodies to the detected proteins mentioned above, respectively. All samples were compared against β-actin, GAPDH, or β-tubulin as a reference protein. The dilutions of the WB antibodies were 1:1000 (the primary antibodies) and 1:2000 (the secondary antibodies), respectively according to the instructions of