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Tea polyphenols induced apoptosis of breast cancer cells by suppressing the expression of Survivin

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To study the mechanism of tea polyphenols (TP)-induced apoptosis of breast cancer cells. Proliferation of MCF-7 and SK-BR-3 cells was evaluated by MTT assays. Cellular ultrastructure was examined by electron microscopy. Apoptosis was detected by TUNEL. PCNA、Cyclin D1、Cyclin E and Survivin expression was measured by Western blot. Cell proliferation was significantly inhibited by TP. Spindle and round cells were loosely distributed with increased particles after TP treatment. Increased cell size, frequent nuclear atypia and a collapse of apoptosis were observed. The nucleus was pushed towards one side, while the cytoplasm was rich in free ribosome. The membrane of mitochondria was thickening, and the cell apoptotic body was observed. TP treated cells experienced significantly enhanced apoptosis compared with 5-Fu treated or control groups. The expression of survivin was downregulated by TP. To conclude, TP can inhibit cell growth and induce apoptosis through downregulating the expression of survivin in breast cancer.

Phytochemicals has been introduced to support the immune system or fight against diseases[1]. Green tea and its constituents are important components of diet-based strategies to prevent various malignancies[1]. The anti-carcinogenic and anti-mutagenic activities of green tea may provide protection and reduce cancer prevalence[1]. The pharmacological features of green tea are derived from polyphenols including epigallocatechin, epigallocatechin-3-gallate, epicatechin, and epicatechin-3-gallate[1]. Green tea and its components effectively mitigate cellular damages from oxidative stress[1]. A large number of studies have shown that tea polyphenols (TP) can enhance human immunity so that protect from a variety of diseases such as cardiovascular events and cancer, owing to its anti-oxidant, anti-radiation, antibacterial, antiviral, anti-diabetic, and anti-aging functions[2–4]. Accumulating evidence indicates that TP can scavenge free radicals, induce detoxification enzymes, and regulate immune function[5,6]. TP can induce apoptosis of cancer cells through caspases cascade and p53[5,7]. Further investigation on TP-induced apoptosis of tumor cells may provide a theoretical basis for the development of novel antitumor drugs.

Breast cancer is the most common malignancy in women with increased incidence worldwide. Although anti-tumor activity of TP in breast cancer has been indicated, its molecular mechanism is yet to be clarified[8]. Poor prognosis of breast cancer is partially attributed to multiple-drug resistance and anti-apoptosis of cancer cells[9]. Survivin, an inhibitor of apoptosis is highly expressed in most cancers and closely related to multiple-drug resistance, increased tumor recurrence, and reduced survival of patients, making it an attractive target for cancer treatment[10]. Survivin is highly expressed in breast cancer tumor compared with the normal breast tissue[11]. Survivin participates in recurrence and progression of breast cancer, and is an important prognostic factor for clinical outcome of breast cancer[12]. In this study, we investigated whether TP can induce apoptosis and its downstream signaling pathway in human breast cancer cells, in order to clarify the mechanism by which TP can exert inhibitory effect on breast cancer.

Results

Effect of TP on cell proliferation. Proliferation of SK-BR-3 and MCF-7 cells treated with TP was significantly inhibited in a dose-dependent manner, compared to control cells ($p < 0.0001$, Figure 1).

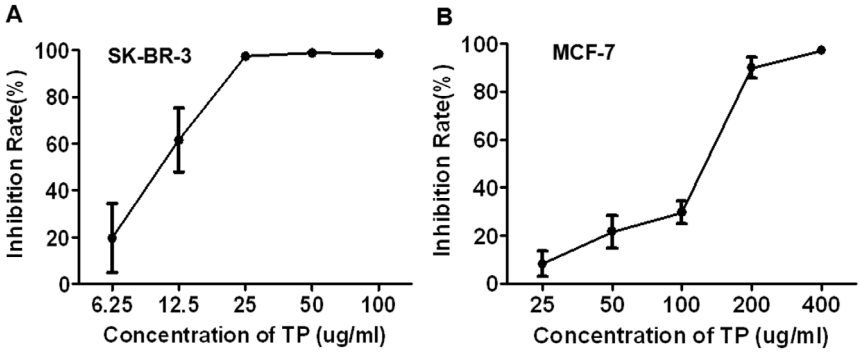

Figure 1 | **Effects of TP on cell proliferation.** Inhibition rate was dramatically increased as a dose-dependent manner in response to TP treatment in (A) SK-BR-3 and (B) MCF-7 cells.

Effect of TP on cell morphology. Under an optical microscope MCF-7 cells presented swollen, polygonal or round shapes, with strong refraction and clear boundaries. MCF-7 cells treated by 5-Fu (at concentration of 125 ug/ml) for 48 hours became round and spindle, loosely distributed, with a few cells dissolved. MCF-7 cells treated by TP at concentration of 50 ug/ml for 48 hours became spindle and round, loosely distributed, with a majority of cells containing particles (Figure 2A).

Under electron microscope MCF-7 cells which were treated by 5-Fu at concentration of 125 ug/ml for 48 hours showed enlarged cell size, partial loss of villi and moderate degeneration. MCF-7 cells treated by TP at a concentration of 50 ug/ml for 48 hours presented changes in structure of the nucleus and organelles. We observed apoptotic chromatin condensation and clumping, concentrated cytoplasm, loose endoplasmic reticulum, as well as fusion of the membrane and formation of bubbles (Figure 2B).

Apoptosis detected by Situ 3'-end labeling (TUNEL). Very few apoptotic cells were observed in control and treated by 5-Fu (at a concentration of 125 ug/ml) treated cells which were stained as a light blue polygon. MCF-7 cells treated by TP (concentration of 50 ug/ml) for 48 hours showed increased apoptosis, which was represented by a large amount of round or spindle-shaped cells, with a majority of cells stained as brown ($p < 0.05$, Figure 3).

Survivin gene expression. Western blot showed that TP efficiently inhibits survivin expression compared with control groups in breast cancer cells SK-BR-3 and MCF-7 ($p < 0.05$, Figure 4). However, the expression of PCNA, Cyclin D1, and Cyclin E was not significantly downregulated by TP (Figure 5).

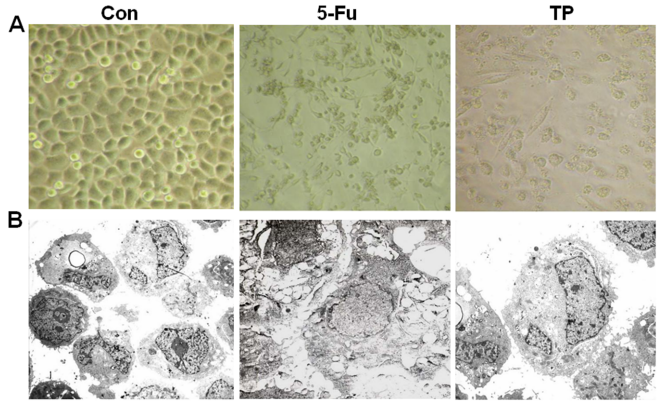

Figure 2 | **Effect of TP on cell morphology.** (A) Morphological changes of MCF-7 cells after 5-Fu or TP treatment. (B) Ultramicrostructure changes of MCF-7 cells induced by 5-Fu or TP.

Effects of TP on cell growth of MCF-7 xenograft tumors in nude mice. On days 10 and 17 after inoculation, the tumor volumes with treatment of TP in the mice began to shrink. On days 24, 31 and 38 after treatment, the tumor volumes were significantly reduced ($p < 0.05$).

Survivin protein expression in xenograft tumors in mice treated with TP for 38 days was lower than that in control tumors (0.35 ± 0.01 vs. 0.39 ± 0.01, respectively; $p < 0.05$, Figure 6).

Discussion

In current study, we have demonstrated new molecular mechanism of TP induced apoptosis in breast cancer cells. TP is able to induce cell apoptosis by regulating a variety of signal transduction pathways in breast cancer[13]. Our data may help to provide theoretical basis for development of novel anticancer drugs.

TP fights against tumor by inhibiting cell proliferation, urokinase activity, neovascularization, cancer metabolism, nitrification, as well as inducing apoptosis of tumor cells[14,15]. Targeting molecules involved in anti-apoptosis may provide new ideas in the aspect of screening and development of antitumor drugs. TP regulates a variety of cellular signaling pathways to induce apoptosis of tumor cells. TP inhibits the activity of protein kinase C and CDKs by producing H_2O_2, upregulates the expression of IkBα, which is the inhibitor of transcription factor activator NF-kβ, and initiates caspases cascade

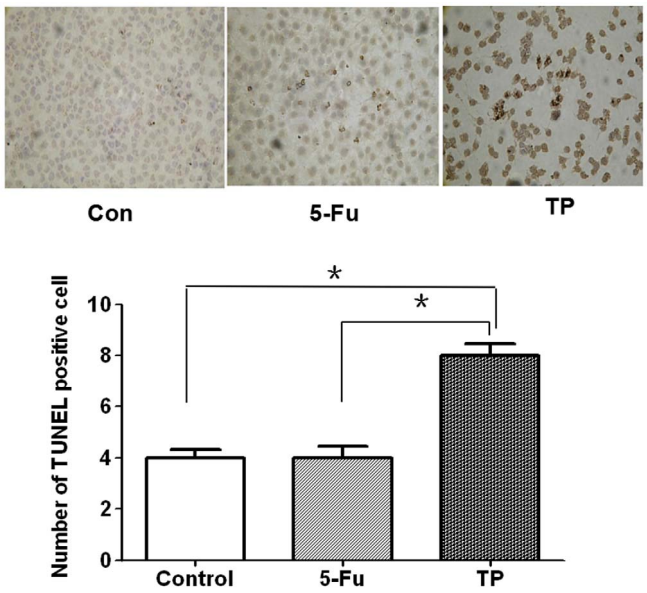

Figure 3 | **TUNEL staining of MCF-7 cells treated by 5-Fu or TP (*$P <$ 0.05).**

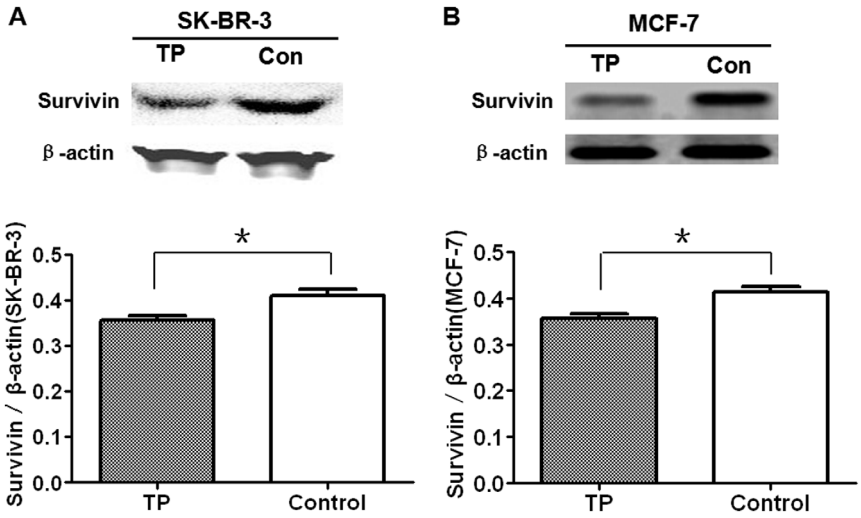

Figure 4 | Expression of Survivin in SK-BR-3 and MCF-7 cells after TP treatment (*$P < 0.05$). C: control; T: TP.

in mitochondria[16]. Moreover, TP exerts its cancer chemoprevention by blocking the mitogenic and differentiating signals through modulating EGFR—MAPK signaling, as well as c-myc, c-jun and c-fos expression[17].

We observed decreased cell proliferation in response to TP in breast cancer cells. Our data show that both 5-Fu and TP can inhibit proliferation of MCF-7 and SK-BR-3 cells. 5-Fu is a widely used antitumor drug. However, its toxicity may limit its efficacy in clinics. To the contrary, TP has minimum side effect.

To elucidate the mechanism of TP against breast cancer, we observed cellular ultrastructure, detected apoptotic cells, and measured the expression of anti-apoptotic gene survivin. Comprehensive ultrastructure analysis demonstrated that 5-Fu treated cells experienced necrosis, whereas TP treated cells experienced apoptosis.

Programmed cell necrosis occurs under either normal/physiological or pathological conditions[18]. We observed TP-induced cell death was significantly increased. Survivin, a new member of anti-apoptotic protein (IAP) family, is the strongest inhibitor of apoptosis that have ever been found. Survivin is overexpressed in breast cancer and participates in the development and progression of breast cancer by affecting cell proliferation and apoptosis[19]. We detected down-regulation of survivin in MCF-7 cells treated by TP. The data indicates that TP can efficiently induce apoptosis through inhibiting the expression of survivin. Antioxidant potential of green tea derives from its immunopotentiating properties. Its mode of action against malignant behaviors of cancer cells demonstrates its potential as a chemopreventive agent against breast cancer.

In summary, we have confirmed the anti-tumor activity of TP in breast cancer cells and demonstrated one of the mechanisms by which TP is able to induce apoptosis of breast cancer cells.

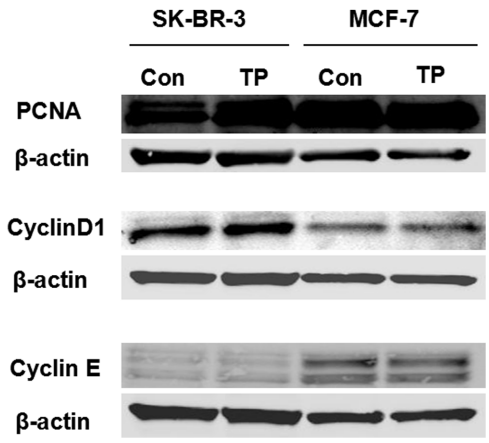

Figure 5 | Expression of PCNA, Cyclin D1, and Cyclin E in SK-BR-3 and MCF-7 cells after TP treatment.

Methods

Cell lines, reagents, drugs and antibody. Human breast cancer cell line MCF-7 and SK-BR-3 was a gift from Dr. Song at Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, The University of Rhode Island, USA. Tea Polyphenols was purchased from Sigma (USA). The 5-fluorourocil was purchased from Shanghai Xudong Pharmaceutical. Thiazolyl blue tetrazolium bromide was purchased from Fluka. Rabbit anti- human β-actin polyclonal antibody was purchased from Boster (Wuhan, China). Rabbit anti-human Cyclin D1, Cyclin E, PCNA and Survivin polyclonal antibody was purchased from Santa Cruz (USA).

Cell culture. MCF-7 and SK-BR-3cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin, and incubated at 37°C in a humidified atmosphere of 5% CO_2.

MTT assay. Breast cancer MCF-7 and SK-BR-3 cells in logarithmic growth phase were digested with trypsin, harvested, adjusted to a density of 2×10^4 cells/mL and transferred to 96-well plates at a volume of 100 μL per well. After 24 h when cells formed a monolayer, drugs at different concentrations were added to the medium. After incubated for 24 h, MTT solution (5 mg/mL) was added (20 μL/well). Cells were incubated at 37°C for another 4 h. Then the culture supernatant was removed and DMSO was added (100 μL/well). Cells were incubated in a shaker at 37°C for 10 min until crystals were completely dissolved. The absorbance at 490 nm was determined using a microplate reader. Each experiment was performed in quintuplicate.

Electron microscopy. Breast cancer MCF-7 cells treated with TP were recovered from cultures and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde, and then observed in a Zeiss transmission electron microscope.

TUNEL. The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method was used for detection of apoptotic cells, with an *in situ* cell death detection assay (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. MCF-7 cells were incubated with drugs for 48 hours. The number of positive cells in five randomly selected areas was counted under the microscope (original magnification, ×200).

Western blot. Breast cancer cells MCF-7 and SK-BR-3 were digested with trypsin, harvested, total protein concentration in each sample was determined using the Bradford assay. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed at a constant voltage of 100 V for 1 h. After electrophoresis, proteins were electrically transferred onto a nitrocellulose membrane at a constant voltage of 45 V for 1 h. After washing for three times (5 minutes each) in TBST buffer with shaking at room temperature, the membrane was incubated overnight with the blocking solution (1% bovine serum albumin) at room temperature. The membrane was then incubated with primary antibody (1 : 500 dilution) for 2 h at room temperature, followed by washing with TBST buffer three times (10 minutes each).

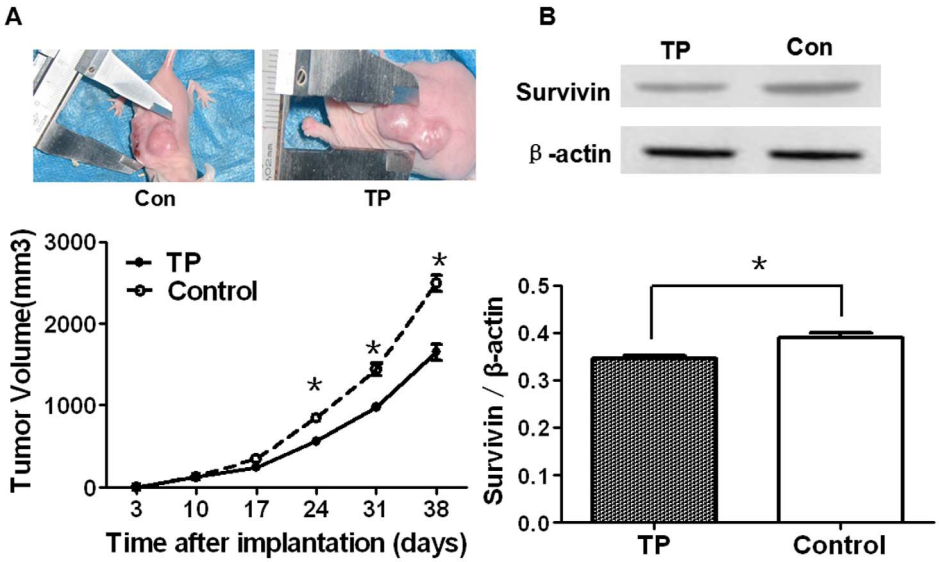

Figure 6 | Effects of TP on the growth of subcutaneous xenograft tumors derived from MCF-7 cells in nude mice (*$P < 0.05$). (A) Subcutaneous xenograft tumor-bearing nude mice treated with TP for 38 days and tumor volumes at various time points after tumor implantation. (B) Expression of survivin in MCF-7 cells treated with TP for 38 days.

Subsequently, the membrane was incubated with alkaline phosphatase-labeled goat anti-rabbit secondary antibody (1 : 500) at room temperature for 1 h, followed by washing with TBST for three times (10 minutes each) and TBS twice (10 minutes each). BCIP/NBT substrate solution was used for color development to detect the expression of survivin protein. Images were photographed using the Bio-Rad gel imaging system (Bio-Rad, USA).

Nude mouse tumor xenograft model. Athymic BALB/c female nude mice (Chinese National Academy of Sciences) were housed in laminar flow cabinets under specific pathogen-free conditions and used at 6–8 weeks of age. All animal protocols were approved by the Institutional Animal Care and Use Committee the Third Affiliated Hospital of Harbin Medical University. MCF-7 breast cancer cells with the highest Survivin expression were adjusted to a density of 2.5×10^7 cells/mL using serum-free RPMI 1640 medium. Trypan blue staining indicated the ratio of viable cells was >95%. Tumor cells (5×10^6 in 0.2 mL of HBSS) were suspended in serum free-RPMI/Matrigel mixture (1 : 1 volume) and injected into the back of the nude mice by using a 22-gauge needle. Strict aseptic technique was followed during the entire process. The mean volume of the implanted tumors at the time of inoculation was about 5 mm^3, without differences among various groups. A total of 5 mice per group were used. Three days after the injection and every fifth day thereafter, tumor volume was recorded. The length and width of the tumors that developed were measured. Three days after inoculation, TP (0.5%, 100 ul) were given every day. Mice were sacrificed at 38 days. Tumor volume was calculated as (length/2) × ($width^2$). Tumors were harvested; half of each tumor was frozen in liquid nitrogen and stored at −80°C; half was fixed in 4% paraformaldehyde and stored at 70% ethanol. All procedures were carried out according to the guidelines approved by the ethics committee at the Third Affiliated Hospital of Harbin Medical University.

Statistical analysis. The ANOVA test was used to examine the differences in numerical variables between the multiple groups. The statistical analyses were conducted using the SPSS12.0 software. A p value of <0.05 was considered statistically significant.

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Author contributions

X.S.C., Y.L., Q.S.L., L.C. and X.Q.D. were responsible for experimental design. X.S.C., Y.L. and Q.S.L. developed methodology. X.S.C., Y.L., Q.S.L., Y.W., H.S., J.W. and G.Q.C. carried out the experiments. X.S.C., Y.L., Q.S.L., Y.W., H.S., J.W. and G.Q.C. interpreted the results, performed data analysis and prepared the figures and tables. X.S.C., Y.L., Q.S.L., Y.W., H.S., J.W., G.Q.C., L.C. and X.Q.D. wrote, reviewed, and revised the manuscript. L.C. and X.Q.D. provided administrative, technical, or material support. L.C. and X.Q.D. supervised the study.

Additional information

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