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Taiwanin E inhibits proliferation and enhances apoptosis in arecoline and 4-NQO-induced oral cancer cells

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Abstract

Oral cancers can be life threatening if not diagnosed and treated early. Areca nut chewing is very popular in Taiwan and in other parts of Asia and chronic exposure to arecoline carcinogens in causes genetic changes in the epithelial cells of the oral mucosa. The use of herbs as alternative cancer therapies has attracted a great deal of attention owing to their lower toxicity. Therefore, the purpose of this study was to investigate the anti-cancer effect of Taiwanin E on arecoline and 4-NQO-induced oral cancer cell lines.The OSCC model in C57BL/6J Narl mouse is generated by 0.5 mg/mL arecoline plus 0.2mg/mL 4-NQO carcinogen in drinking water for 8 and 28 weeks to mimic the etiology of oral cancer patient in Asia. Mice were sacrificed and cells were cultured as T28 cancer cells. Taiwanin E used in this study was extracted from Taiwania cryptomerioides Hayata woud.Taiwanin E significantly inhibited the cell viability of T28 cells in a dose dependent manner, but no cytotoxicity was observed in N28 normal cells. Taiwanin E activated p21 and p27 proteins and reduced cell cycle regulatory proteins like Cyclin D1 and Cyclin E and thus resulted in G0/G1cell cycle arrest in T28 cells. Annexin V-FITC staining and terminal transferase-mediated dUTP nick end-labeling (TUNEL) staining showed Taiwanin E strongly enhanced apoptosis in a dose-and time- dependent manner. Taiwanin E also decreased anti-apoptotic protein Bcl-xL and increased pro-apoptotic protein Bax, and down-regulated p-PI3K, p-Akt survival protein levels in T28 oral cancer cells.

Materials and Methods

Cell culture

The OSCC model in C57BL/6J Narl mice is generated by 0.5mg/mL arecoline plus 0.2mg/mL 4NQO carcinogen in drinking water for 8 and 28 weeks to mimic the etiology of oral cancer patient in Asia. Mice were sacrificed and cell were cultured as T8 and T28 cancer cells .T8 and T28 Were grown in Dulbecco's modified Eagle's medium (DMEM).supplemented with 10%FBS.Cells were seeded in cell culture flasks and maintained in a humidified incubator at 37 °C with 5% CO_2 .

MTT assay

[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltertrazolium-bromide]
Adherent cells in 24 well plates. Make a solution of 5 mg/ml MTT dissolved in PBS and filter sterile. Add 500 ul of MTT solution from step one to each well containing cells. Incubate the plate in a CO_2 incubator at 37 °C for 2 hours. Remove media with pipette. Add 500 ul of isopropanol to each well and pipette up and down to dissolve crystals. Transfer to plate reader and measure absorbance at 570nm.

DAPI staining and TUNEL assay

Taiwainin E were treated cells cultured in 24-well plates were fixed by 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 (in 0.1% sodium citrate for 2 min. After cellular nuclei were stained by 4', 6-diamidino-2-phenylindole (DAPI),(blue), Cells with TUNEL-positive nuclei (green) were identified by fluorescence microscopy. Three independent experiments were then averaged and statistically analyzed.

Annexin V and PI staining

Taiwainin E were treated cells cultured in 6-well plates were treated Taiwanin E for 24 h. Apoptotic cells were monitored by FACS Canto flow cytometry using the Annexin V-FITC Apoptosis Detection Kit .

Western blotting

The proteins of cell lysates were analyzed by 8%~12% SDS-PAGE, and proteins were transferred to nitrocellulose(or PVDF) by electrophoresis. Residual protein sites were blocked in Tween/Tris-buffer saline (TBS) containing 5% skin milk. The filters were incubated with primary antibodies in TBS plus 2.5% skin milk at recommended concentrations for 2h or at 4 °C overnight and incubated with secondary antibodies for 1h at room temperature. Antibody reaction was visualized with enhanced chemiluminescence (ECL) reagent for Western blotting.

Statistical analyses

Result were expressed as mean+SEM,and data were analyzed one-way ANOVA followed by Student's t test for significant difference. P-value<0.05 was considered statistically significant.

Results

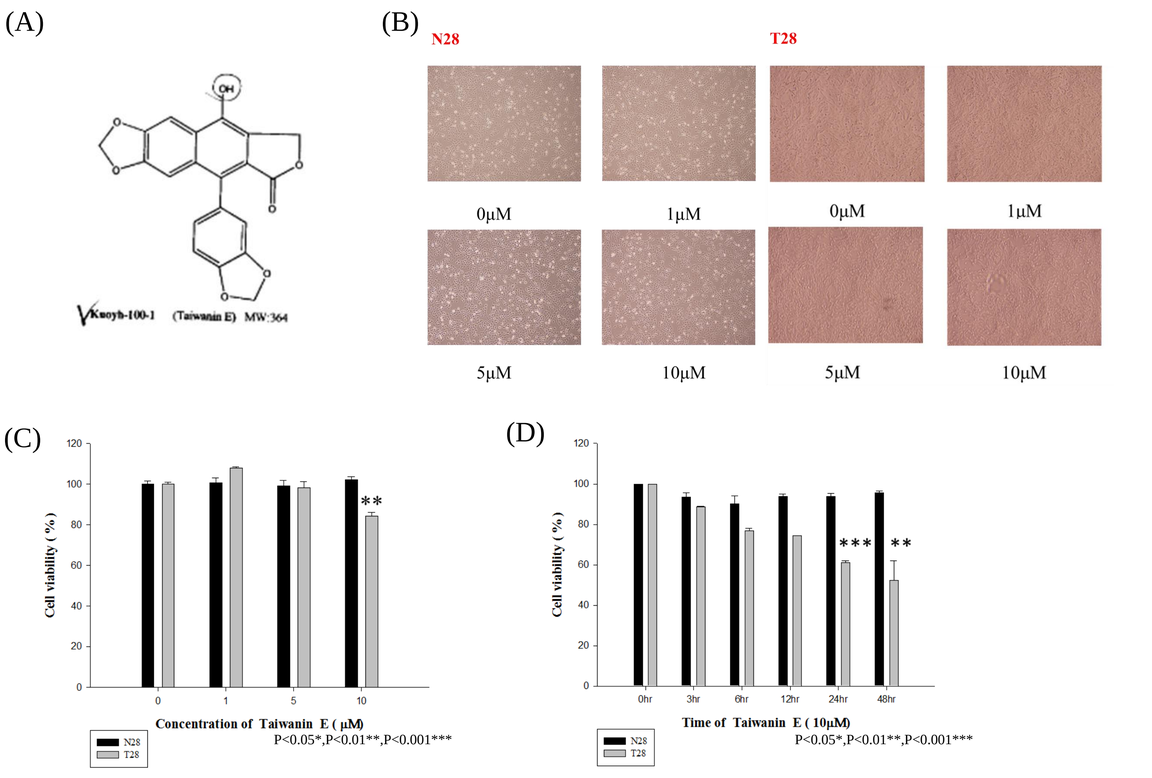

Fig.1 Taiwanin E inhibits oral cancer cell proliferation.(A)The molecular structure of Taiwinin E.(B)Representative photos to show the normal oral cells N28 and oral cancer cell T28 morphology after 24hr treated with Taiwanin E at 0,1,5,10μM individually. The cell after treated with Taiwanin E demonstrated the cell number decrease at dose dependent manner ,but were not effected in normal oral cells (N28).(C)Normal oral cells N28 and oral cancer cells T28 were treated with different concentrations of Taiwanin E at 0, 1, 5, 10μM individually for 24 h.****p < 0.01 represent significant differences when compared with the N28 control group.**(D)Normal oral cells N28 and oral cancer cells T28 were treated with different time of 10μM Taiwanin E at 0,3,6,12,24 and48 hours. ****p < 0.01 and ***p < 0.001 represent significant differences when compared with the N28 control group.**

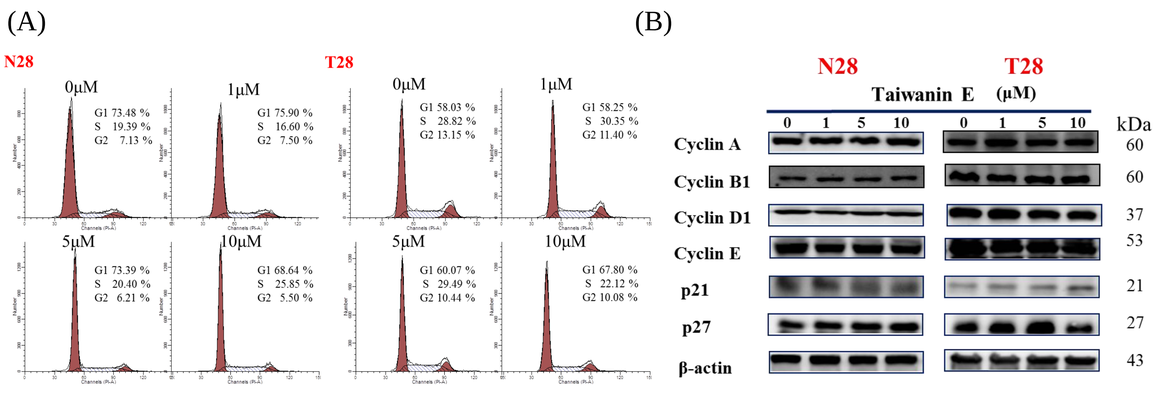

Fig.2 Modulation of proteins involved in the cell cycle process following Taiwanin E treatment. Normal oral cells N28 and oral cancer cells T28 were treated with Taiwanin E at 0,1,5,10μM for 24 h. (A)The results were showing Taiwanin E effect on inducing G0/G1phase cell cycle arrest in T28 oral cancer cells.(B)The Western blot analysis showing decreased cyclin D , cyclin E and increased p21 and p27 protein expression in oral cancer cells T28.(C)T28 cells were cultured and incubated with different time of 10μM Taiwanin E at0,3,6,12,24 and48 hours.The results were showing Taiwanin E effect on inducing G0/G1phase cell cycle arrest in T28 oral cancer cells.(D)Normal oral cells N28 and oral cancer cells T28 were treated with different time of 10μM Taiwanin E at 0,3,6,12,24 and48.The Western blot analysis showing decreased cyclin D,cyclin E and increased p21 and p27 protein expression in oral cancer cells T28.

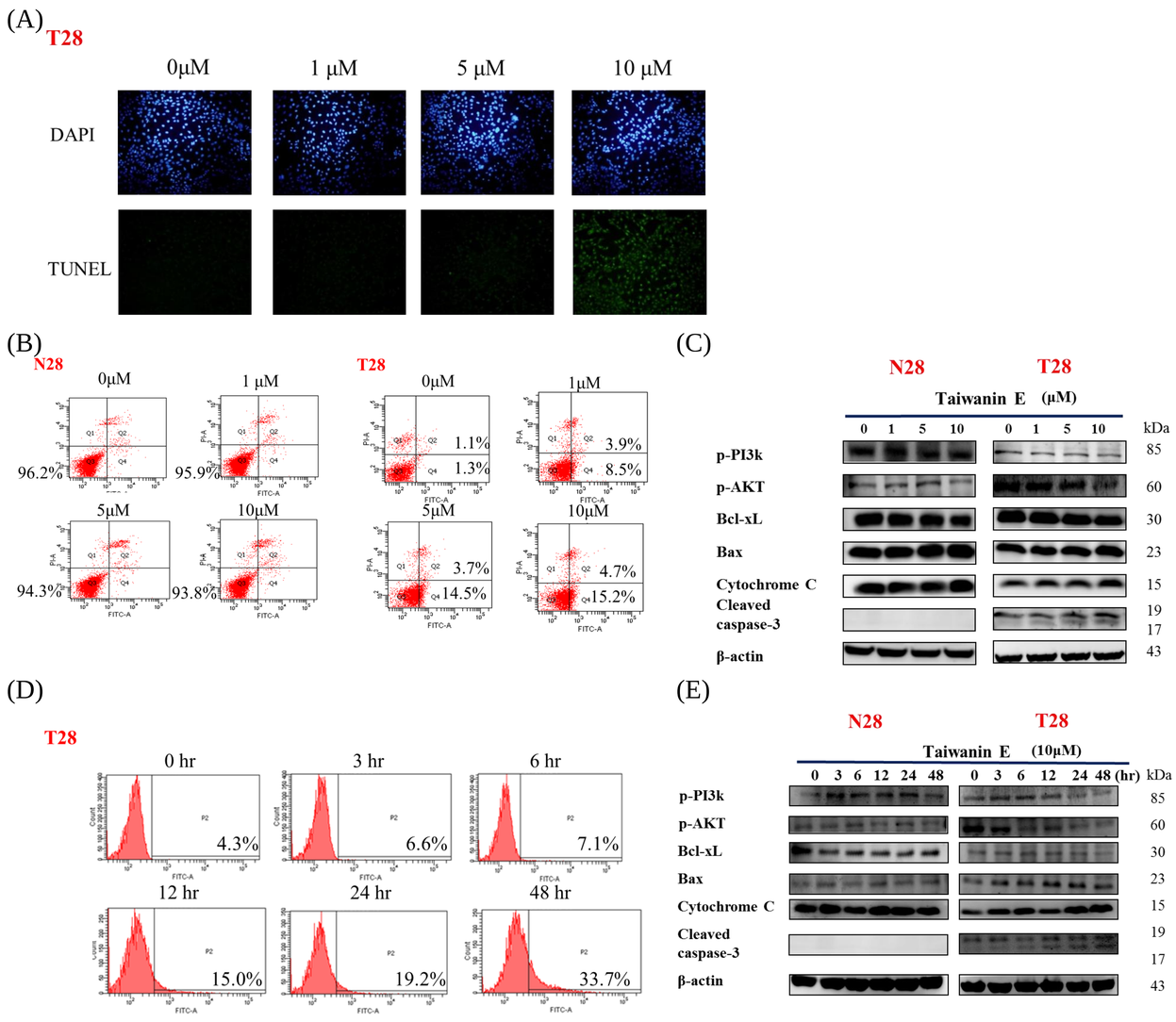

Fig.3 Taiwanin E induces cell apoptosis in T28 oral cancer cells. T28 oral cancer cells were treated with Taiwanin E at 0, 1, 5, 10μM for 24 h.(A)Taiwanin E did induce apoptosis in a dose-dependent manner on T28 oral cancer cells.(B)The annexin V-FITC and PI analysis results showingTaiwanin E did induce apoptosis in a dose-dependent manner on T28 oral cancer cell .(C)The Western blot analysis showing enhanced Bax , down-regulated Bcl-xL and induced Cytochrome C released on T28 oral cancer cells in dose dependent manner, but no significant effect on primary epithelial cells N28.
Normal oral cells N28 and oral cancer cells T28 were treated with different time of 10μM Taiwanin E at 0,3,6,12,24 and48 hours.(D)Taiwanin E did induce apoptosis in a time-dependent manner on T28 oral cancer cell as well.(E)The Western blot analysis showing enhanced Bax , down-regulated Bcl-xL and induced Cytochrome C released on T28 oral cancer cells in time-dependent manner, but no significant effect on primary epithelial cells N28

Summary

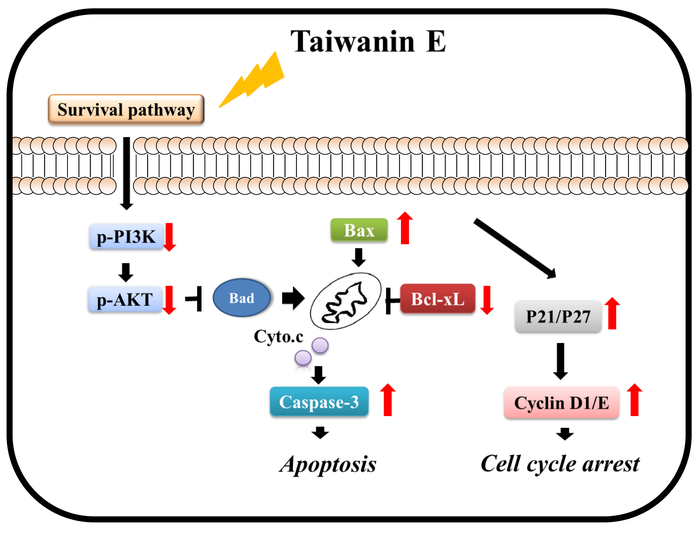

Fig.4 The proposed signal pathways of Taiwanin E – induced G0/G1 arrest and enhanced apoptosis in T28 oral cancer cells.