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Tannic acid-induced apoptosis in FaDu hypopharyngeal squamous cell carcinoma

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Tannic acid (TA) is a water-soluble polyphenol compound found in various herbal plants. We investigated the chemopreventive effects of TA on FaDu hypopharyngeal squamous carcinoma cells. In an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, TA showed dose-dependent cytotoxicity with a half maximal inhibitory concentration (IC50) of 50 μM. Cell cycle analysis and immunofluorescence imaging demonstrated that under low-dose (25 μM) treatment, FaDu cells were arrested in G2/M phase, and as the dose of TA was increased, apoptosis was induced with the increase of cell population at sub-G1 phase. The expressions of various cyclins, including cyclin D1 and cyclin-dependent kinases (CDK-1 and CDK-2), were down-regulated at low doses of TA, whereas apoptotic effectors such as cleaved caspase 3, cleaved caspase 7, and poly (ADP-ribose) polymerase (PARP) were expressed in a dose-dependent manner in Western blotting. In addition, TA-induced apoptosis of FaDu cells might be mediated by the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase pathway, with the upregulation of p-AKT/p-PKB (phosphorylated protein kinase B) and p-ERK. Overall, our data support the hypothesis that TA is a potential candidate agent for the treatment of hypopharyngeal cancer.

Keywords: Apoptosis, Hypopharyngeal cancer, FaDu cells, Tannic acid

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a group of cancer mostly arising in head and the region comprising epithelial layer of oral cavity, nasal cavity, larynx and pharynx [1,2]. It is not clear what causes the mutations in squamous cells leading to HNSCC but alcohol use and tobacco smoking have been identified as risk factors. Statistical research in the UK showed that more than 75% of mouth and oropharyngeal cancers are attributed to the consumption of alcohol and tobacco [3,4]. In addition these factors have synergistic effects that people who use both alcohol and tobacco are at higher risk of oral cancer because alcohol has dehydrating effect on cell membrane inducing specific carcinogens in tobacco to be absorbed into oral cells [5]. Other risk factors which contribute to the incidence of HNSCC in community include human papilloma virus infection, chronic immunodeficiency and excessive sunlight exposure [3]. Despite the extensive efforts for early diagnosis and development in therapeutics, over half of patients with HNSCC were diagnosed at advanced stage and high proportion of them developed cancer [6]. Conventional treatment was not successful in improving five-year survival rate of HNSCC patients which remains in the ranges of 40% to 50%. Surgery or radiotherapy was applied for the treatment of

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the early stage while multimodal treatment such as combination of radiotherapy with chemotherapy and/or molecular-targeted therapies using monoclonal antibodies were applied for the advanced HNSCCs [7]. Especially hypopharyngeal cancer which shows the worst prognosis and the lowest 5-year survival rate (25% to 40%) [8,9] is in urgent need for the efficient therapeutics.

Tannic acid (TA), also called gallotannin, is a water-soluble polyphenols with multi-gallic acid moieties in chemical structure which is found in various plants. Especially grape and green tea contain high level of TA, and in the production of commercial wine and tea TA plays a critical role for the generation of color and flavor. TA is also used as a flavoring agent in food industry or as additives in ink manufacturing [10]. Previous reports showed that TA has various therapeutic effects of anti-inflammation [11], antioxidation [12,13], and anti-tumor [14,15].

For anti-tumor effects, TA suppressed cell proliferation on cancer cell lines such as prostate cancer [16], breast cancer [17,18], and malignant glioma [19]. Interestingly, TA effects on tumor cell lines were cell-type dependent. In breast cancer cells, TA induced G1-arrest and apoptosis through regulating both canonical and non-canonical signal transducer and activator of transcription (STAT) pathway [18] whereas TA inhibited cell cycle arrest at G2/M phase and stimulated apoptosis through p53 induction in T98G glioma cell lines [20]. In case of gingival cancer cells, TA exhibited apoptosis by cell cycle arrest at G1 phase by inhibiting Jak2/STAT3 pathway [21]. Thus, our intent in this study was to evaluate whether TA has any specific cellular effects on hypopharyngeal squamous cancer cells and if so what would be the underlying mechanism to elucidate. Here, we report that TA suppresses the proliferation of FaDu hypopharyngeal squamous cells by arresting cell cycle at G2/M phase and induces apoptosis possibly through the signaling pathway of extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and AKT.

Materials and Methods

1. Materials and reagents

Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), TA was purchased from Sigma Aldrich (St Louis, MO, USA). Ten percent fetal bovine serum (FBS), 0.05% trypsin-EDTA (ethylene diamine tetraacetic acid) and antibiotic-antimycotic solution were obtained from WELGENE (Gyeongsan, Korea). Primary antibodies of cyclins, cyclin-dependent kinases (CDKs), pro-caspase 3 and caspase 9 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and cleaved caspase 3, cleaved caspase 7 and phosphorylated-ERK (p-ERK) were from Cell Signaling Technology Inc. (Danver, CA, USA). Anti-tubulin, anti-pericentrin and cleaved-PARP (poly [ADP-ribose] polymerase) antibodies were purchased from Abcam (Cambridge, MA, USA). Secondary antibodies were purchased from Novus Biologicals (Centennial, CO, USA).

2. Cell culture

FaDu hypopharyngeal squamous cell carcinoma was obtained from Korean Cell Line Bank (Seoul, Korea) and cultured in minimun essential media (MEM) media containing 10% FBS and 1% amoxicillin and streptomycin. The cells were seeded at the density of 2×10^5 cells/mL and incubated in 37℃, 5% CO_2 for 24 hours.

3. MTT assay

Two $\times 10^4$ cells/well were seeded in 96-well plate and incubated for 24 hours. After treating with various concentrations of TA for 24 hours, the media were replaced by 200 μL of MTT solution (0.5 mg/mL). The cells were further incubated for 4 hours at 37℃ until formazan crystals were formed. The old media were aspirated and 200 μL of DMSO was added to dissolve formazan. The absorbance was measured at 540 nm by using Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

4. Cell cycle analysis

FaDu cells were seeded in 100 mm culture dish for 24 hours and then followed by TA treatment at a concentration range from 0 to 100 μM. After 24 hours, cells were trypsinized, washed and fixed in 70% ethanol. The fixed cells were washed by phosphate buffered saline (PBS) to remove ethanol trace, treated with ribonuclease (200 μg/mL) and stained with propidium iodide (50 μg/mL). Flow cytometer was used to identify DNA content of 10,000 cells per sample and the data was analyzed by Cellquest software (BD Biosciences, San Jose, CA, USA).

5. Western blotting

After TA treatment on FaDu cells, cells were collected to carry out protein extraction. The total proteins were separated in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with skim milk 5% in PBS with Tween 20 for 1 hour, incubated overnight at 4℃ with primary antibody of a target protein. After washing (×3) with tris buffered saline (TBS) 0.1% Tween-20 for 15 minutes, the membrane was incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 hours at room temperature, and then followed by additional washing (×3). Proteins were exposed with chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA, USA) and detected by imaging system (Kodak image station; Bruker BioSpin, Billerica, MA, USA).

6. Immunofluorescence imaging

FaDu cells (1 × 10^5/well) were seeded in coverslip containing 24-well plate and incubated for 24 hours prior to TA treatment. After treating with TA, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 minutes at room temperature. The chamber slides were incubated with antibodies of tubulin or pericentrin overnight at 4℃. Prior to the treatment with secondary antibodies, cells were completely washed with TBS plus 0.1% Tween to eliminate residual unconjugated antibodies. For nuclei staining DAPI (4',6-diamino-2-phenylindole) was added and incubated for 20 minutes at room temperature. After washing with TBS, cells on coverslips were finally observed by a fluorescence microscopy (Zeiss International, Oberkochen, Germany).

Results

1. The cytotoxicity of tannic acid on FaDu cell

The cytotoxicity of TA on FaDu cell growth was examined by an MTT assay. TA significantly inhibited cell growth dose-dependently in the range of 0 to 200 μM with 50 μM of IC50. Treating cells with 200 μM of TA for 24 hours caused 80% of cell death (Fig. 1).

2. Tannic acid induced cell cycle arrest and apoptosis

Flow cytometry was used to examine the cell cycle distribution under the TA treatment. The analysis on cell cycle revealed that under the low concentration of TA (25 μM) cells were significantly inhibited in cell cycle progression with 30% of cells arrested at G2/M phase. Meanwhile, cell population at sub-G1 phase was dose-dependently increased indicating that high dose of TA induced apoptosis on FaDu cells (Fig. 2). To further confirm the changes in cell division and cellular nucleus, the effect of TA on cell cycle progression was examined by immunofluorescence imaging. Under the treatment of 25 μM TA for 24 hours, the number of cells in division process was increased with doubled centrosomes and bipolar mitotic spindles while at higher doses cells were in apoptosis with nuclei condensed

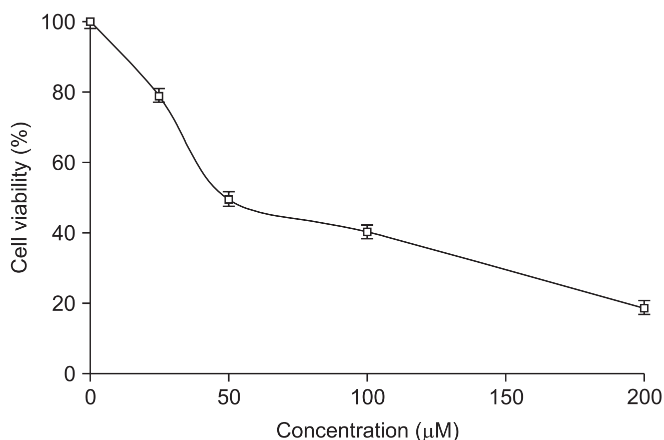

Fig. 1. Tannic acid (TA) inhibited the growth of FaDu cells. Cells were treated with various concentrations of TA and incubated for 24 hours. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out to determine the cell viability. Vertical bars indicate means and standard errors (n = 3).

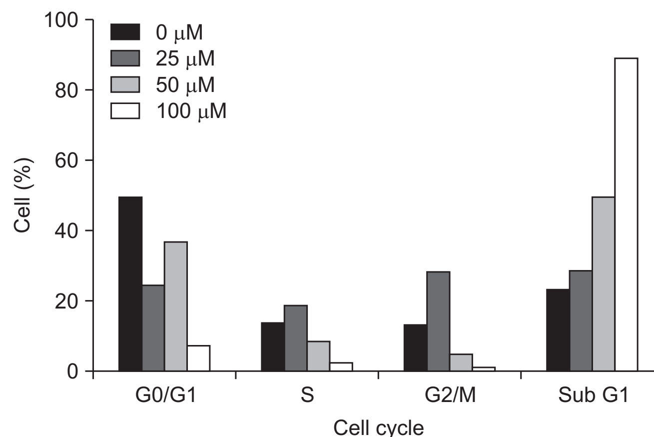

Fig. 2. Tannic acid (TA) regulated cell cycle distribution. FaDu cells were treated with TA (0, 25, 50, and 100 μM) and incubated for 24 hours. TA treated cells were stained with propidium iodide and analyzed by flow cytometry.

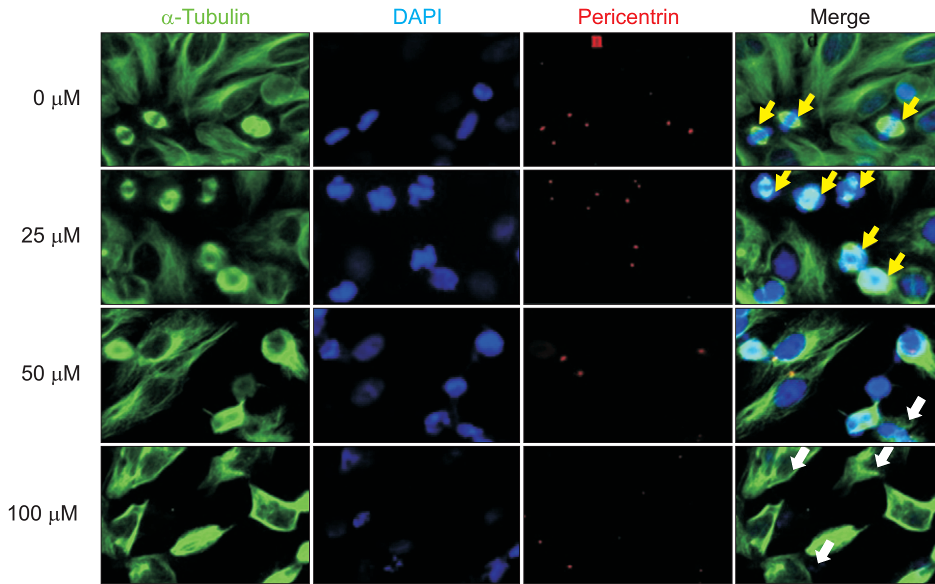

Fig. 3. Immunofluorescence staining images of FaDu cells. After tannic acid treatment for 24 hours, cells were fixed with paraformaldehyde and stained for the following detection: α-tubulin for microtubules, DAPI for nuclei and pericentrin antibody for centrosomes. The yellow arrows indicate the cell at G2/M phase and the white arrows the cells in apoptosis (×400).

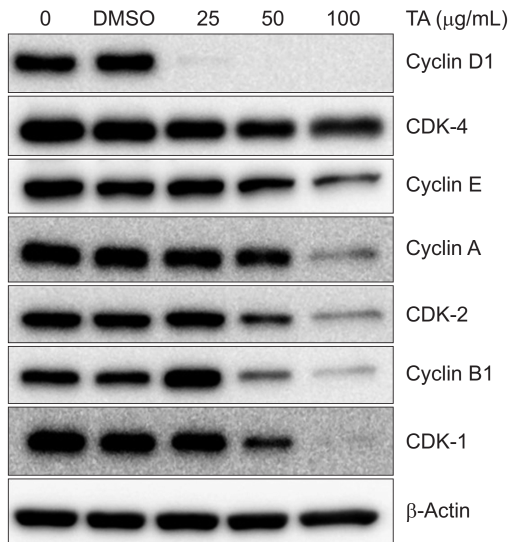

Fig. 4. Tannic acid (TA) suppressed the expression of cell cycle regulatory proteins. The cells were treated with 25, 50, 100 μM of TA for 24 hours and Western blotting was carried out as described in Materials and Methods. DMSO, dimethyl sulfoxide; CDK, cyclin-dependent kinase.

and fragmented (Fig. 3).

3. Tannic acid suppressed cell cycle regulatory proteins

The effects of TA were further investigated by Western blotting to elucidate the expression of regulatory proteins in cell cycle. As shown in Fig. 4, TA inhibited the expression of cyclins and CDKs. Noticeably cyclin D1 was dramatically suppressed at low dose (25 μM) condition while the expression of cyclin D1 dependent kinase CDK-4 was slightly at 25 μM and gradually decreased at higher doses condition. Other cyclins (cyclin A, B1, and E) and CDKs (CDK-1 and CDK-2) were also down-

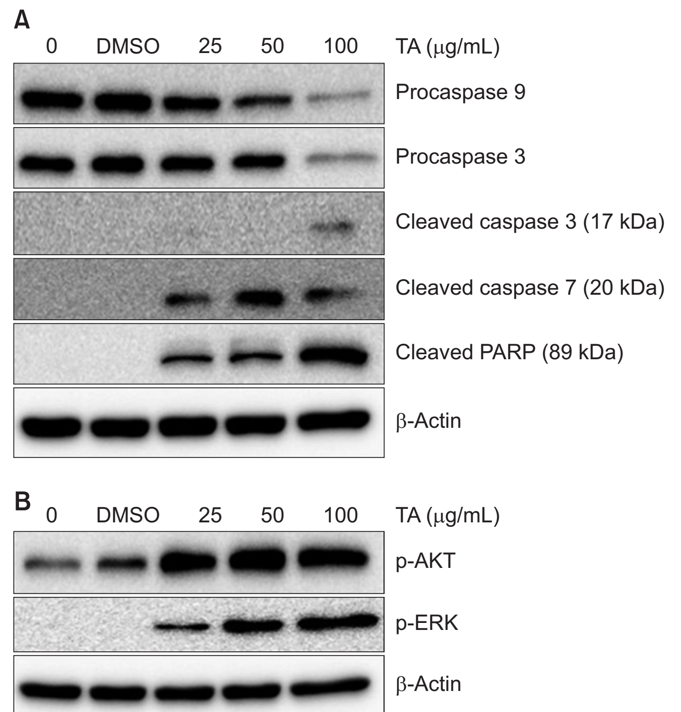

Fig 5. Tannic acid (TA) activated caspase-dependent apoptosis (A) and phosphorylated both AKT and ERK/MAPK (B). FaDu cells were treated with various concentrations of TA for 24 hours and various proteins were detected by Western blotting.
DMSO, dimethyl sulfoxide; PARP, poly (ADP-ribose)polymerase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase.

regulated dose-dependently.

4. Tannic acid-induced apoptosis was through caspase-dependent pathway

TA-induced apoptosis was further investigated to elucidate the underlying molecular mechanism. As shown in Fig. 5A, the apoptosis was mediated by caspase-dependent pathway. Under TA treating condition, the expression of procaspase 9 and procaspase 3 was dose-dependently decreased in Western blotting while the active forms of apoptotic effectors, cleaved caspase-3 and caspase-7 as well as cleaved PARP were increased at higher doses of TA. Under the same condition both AKT and ERK were highly phosphorylated indicating that TA-induced apoptosis was mediated through the signaling pathway of AKT and ERK/MAPK (Fig. 5B).

Discussion

In this study we investigated the cellular effect of TA on FaDu hypopharyngeal squamous cell carcinoma to elucidate TA-induced apoptosis and its underlying mechanism. TA treatment for 24 hours reduced the viability of FaDu cells by arresting cell cycle at G2/M phase at low dose (25 μM) and inducing apoptosis at higher dose (50 to 100 μM). Immunofluorescence imaging and cell cycle analysis revealed that at low dose condition cells were at G2/M phase in cell division but at higher doses undergone into apoptosis with nuclei condensed and fragmented. The expression of various cell CDK and cyclins were reduced under TA treatment. Especially the suppression of cyclin D1 and overexpression of cyclin B1 at 25 μM of TA were dramatic. Considering that cyclin D1 overexpression has been associated with cancer onset and tumor progression [22], the strong suppression of cyclin D1 might be the main contributor against cell division, consequently arresting cell cycle at G2/M phase. Other cyclins (cyclin A and B1) and CDKs (CDK-1 and CDK-2) also contributed to cell cycle arrest at G2/M phase since cyclin A/CDK-2 complex which drives cells from S phase to G2/M phase, and mitotic cyclin/CDK-1 complex (A/CDK-1 and cyclin B1/CDK-1) which promotes G2/M phase entry, were not affected at the condition of 25 μM of TA (Fig. 4). The decreased expression of cyclin E and CDK-2 might be associated with the poor activity of cyclin E/CDK-2 complex which is responsible for G2/M phase transition. Contrarily, at higher dose (50 to 100 μM) condition programmed cell death was overtaken with rapidly increasing cell population at sub-G1 phase (Fig. 2). Sequential activation of caspase 9, caspase 7 and caspase 3 were confirmed by Western blotting, and the activated forms of these proteolytic caspases catalyzed the cleavage of PARP generating cleaved PARP (89 kDa), which has the catalytic function on DNA fragmentation in nuclear and protein degradation in cytosol thus leading to programmed cell death.

Interestingly TA-induced apoptosis was through the up-regulation of ERK and AKT pathways with dose-dependent increase of p-ERK and p-AKT. It is unclear how TA-induced apoptosis was mediated through the signaling pathway of AKT and ERK/MAPK. ERK/MAPK was known to be associated with various functions of cell proliferation, differentiation, migration, senescence and apoptosis [23,24]. AKT activation in response to extracellular stimuli generally promoted cell survival, metabolism and proliferation [25,26]. However, there have been other reports demonstrating that AKT activation and its relocalization into nucleus induce apoptosis when cells were treated with antitumor drugs, methotrexate and docetaxel [27]. Reactive oxygen species also promoted apoptosis through the activation of AKT in prostate cancer cells [28].

TA effects on hypopharyngeal cancer cells have been rarely reported. The most recent report by Schmidt et al. [29] shows TA effects on FaDu cells by using the extract of *Osmunda regalis* root, which contains tannin and other phytochemicals. The root extract inhibited the cell proliferation, motility and invasion which were accompanied by various gene modulations involved in adhesion and metastasis. However it was unclear whether these inhibition effects were attributed by tannin itself. From this context, our results provide the convincing evidence of anti-tumor effect by TA action on hypopharyngeal cancer cells. Whether or not TA has other effects on cell signaling pathway including cell motility remains to be answered in future study. Overall our data support that TA may be a potential candidate as an alternative for the treatment of hypopharyngeal cancer.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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