

Deciphering the role of paclitaxel in the SKGT4 human esophageal adenocarcinoma cell line

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Abstract. Paclitaxel (taxol) has been used for the treatment of various human tumors and is an exceedingly efficient chemotherapy agent against esophageal cancer. However, the precise molecular mechanisms of paclitaxel effects on human esophageal adenocarcinoma cells are not well understood. MTT assay and cell cycle analysis were performed to examine the mechanism of antiproliferative and cell viability effects of paclitaxel in human esophageal adenocarcinoma cancer cells. Western blotting was also used to examine the cell cycle- and apoptosis-related proteins. Paclitaxel inhibited the proliferation of SKGT4 cells in a dose- and time-dependent manner with G₂/M arrest. In addition, paclitaxel induced apoptosis through the activation of caspase-3 followed by PARP degradation. In conclusion, our results suggest that paclitaxel leads to mitotic cell cycle arrest following G₂/M arrest and induces apoptosis via a caspase-3 pathway in SKGT4 cells.

Introduction

Upper gastrointestinal tumors involving the esophagus are a serious public health problem worldwide. In particular, the west has seen a dramatic increase in the incidence of esophageal adenocarcinoma in the past three decades (1). In the United States alone, it was estimated that approximately 15,000 people died of esophageal adenocarcinoma in 2010 (2,3) because it often presents at an advanced stage upon which the 5-year survival rate has been reduced to less than 10% (4-6). Although surgery is the best curative treatment for esophageal adenocarcinoma, chemotherapy is most commonly recommended for patients (7,8). Since paclitaxel is known as an active antitumor agent,

it is often used for the treatment of various tumors including melanoma, breast, prostate, and esophageal cancer (9-14). The combination of 5-fluorouracil and paclitaxel is currently used against esophageal adenocarcinoma. Clinically, paclitaxel as a single agent has been reported to be an active agent for the treatment of esophageal cancer (15). In fact, apoptotic effects by paclitaxel in human esophageal squamous cell carcinomas cell lines *in vitro* have been studied with differential sensitivity to paclitaxel (16,17). However, no information is available regarding the effects of paclitaxel on esophageal adenocarcinoma cells *in vitro*.

Chemotherapeutic agents usually achieve their anticancer effect through G₁/S or G₂/M cell cycle arrest. Paclitaxel has also been shown to induce G₂/M block in various human cancer cells *in vitro* (16,17). Cell cycle regulators, specifically cyclins, bind to cyclin-dependent kinases (CDKs) and regulate cell cycle progression whereas cyclin-dependent kinase inhibitors (CDKIs) negatively regulate CDKs activities (18-20). Apoptosis (programmed cell death) often occurs in response to chemotherapeutic agents (21). In particular, chemotherapeutic agents have been reported to cause activation of caspases. The activated caspase-3 or -9 play an important role for cell death via activation of poly-(ADP-ribose) polymerase (PARP), a cleaved form of cellular substrates (22,23). Based on these considerations, our present study set out to investigate the cell cycle related antitumor mechanisms of paclitaxel in human esophageal adenocarcinoma cells.

Materials and methods

Reagents. Paclitaxel was obtained from Sigma-Chemical Co. (St. Louis, MO, USA) dissolved in DMSO at 5 mg/ml as a stock solution. This dissolved mixture was stored at -20°C until use.

Cell culture. Human esophageal adenocarcinoma cell line, SKGT4, was used in this study and was obtained from Dr Izzo (University of Texas M.D. Anderson Cancer Center). SKGT4 cells were grown in DMEM-F12 medium (Gibco, Grand Islands, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, PAA Laboratories Inc., USA), 100 mg/ml streptomycin and 100 IU/ml penicillin (Gibco, Grand Island,

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NY, USA) as a monolayer in 100 mm dishes (BD, USA) under standard conditions (37°C, 5% CO₂, humidified atmosphere). Each confluent monolayer was washed with phosphate-buffered saline (PBS; Gibco) and detached with 0.05% trypsin/0.02% EDTA solution (Gibco) to transfer or passage the cell lines.

Cell growth inhibition assay by MTT. Cell viability of paclitaxel on SKGT4 cells was determined by MTT assay (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide). To the test wells, 100 μ l of serial diluted paclitaxel was added and 100 μ l of cell suspension was then plated in 96-well microtitre plates (SPL, Korea) at 1×10^4 cells per well. After exposure to the drug for 1, 2, and 3 days, 50 μ l of MTT solution (2 mg/ml in PBS) was added to each well and the plates were incubated for an additional 3 h at 37°C. MTT solution in medium was aspirated off. To achieve solubilization of the formazan crystals in viable cells, 200 μ l of dimethylsulphoxide (Sigma) was added to each well. The plates were shaken for 30 min at room temperature and the absorbance was read immediately at a wavelength of 540 nm with a scanning multiwall spectrophotometer (Spectra MAX 340, Molecular Devices Co., Sunnyvale, CA).

Cell cycle analysis. Cell sample preparation and propidium iodide (PI, Sigma) staining was performed according to the manufacturer's protocol. Briefly, 1×10^6 cells were incubated with or without paclitaxel. Cells were subsequently washed with PBS and incubated with PI (10 μ g/ml) containing RNase A at 37°C for 30 min. The percentage of cells in the different phases of the cell cycle was measured with a FACstar flow cytometer (Becton-Dickinson), and analyzed using Becton-Dickinson software (Lysis II, Cellfit).

Western blotting. Paclitaxel treated cells and non-treated cells were harvested and suspended in lysis buffer (Intron Biotechnology, Inc.). After centrifugation for 5 min at 4°C at 13,000 g, the supernatant was collected. The protein concentration was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Whole lysate (20 μ g) was resolved on a 10% SDS-PAGE gel, transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) by electroblotting and then probed with ~0.1 μ g/ml of rabbit anti-human phospho-cdc2, mouse anti-human cyclin A, rabbit anti-human cyclin B1, rabbit anti-human CIP1/WAF1-p21, rabbit anti-human cleaved caspase-3, rabbit anti-human cleaved caspase-9, and rabbit anti-human cleaved PARP antibodies (Cell Signaling Technology, Beverly, MA, USA). The membrane was then washed with TBS-T (10X TBS and 0.1% Tween-20) and incubated for 1 h with HRP-linked anti-rabbit and anti-mouse antibodies (Cell Signaling Technology). The blot was developed by using the enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL, USA).

Statistical analysis. Data are presented as the means \pm SD. Data were analyzed by Student's t-test. P-values <0.05 were considered statistically significant.

Results

Cytotoxic effect of paclitaxel on SKGT4 cells. Cell proliferation assay (MTT) was performed to investigate the effect of paclitaxel

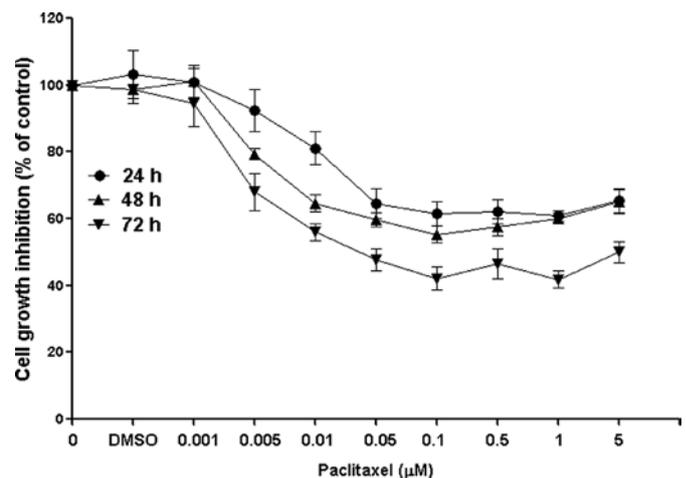


Figure 1. Effect of paclitaxel on growth inhibition of human esophageal cancer cells. Logarithmically growing human esophageal cancer cell line SKGT4 was treated with various concentrations of paclitaxel for 24, 48 and 72 h. Cell growth inhibition was assessed by MTT assay. Results are expressed as percentage of control plates containing no paclitaxel. Each point represents the mean (\pm SD) of at least three independent experiments with triplicate dishes.

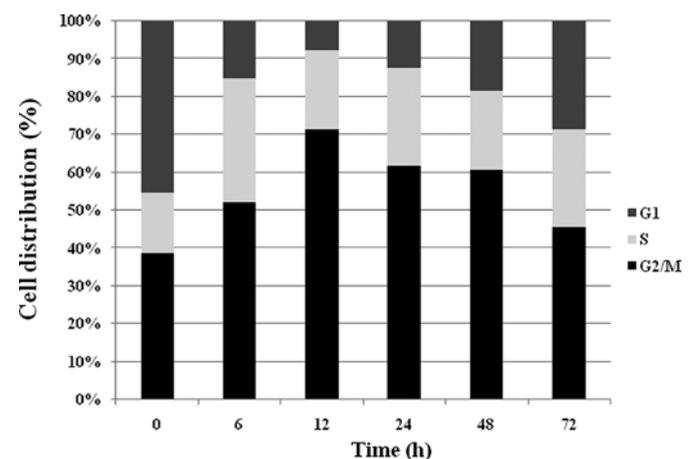


Figure 2. Cell cycle analysis of SKGT4 cells cultured with paclitaxel. Cells were incubated with 50 nM of paclitaxel for different time-points (0, 6, 12, 24, 48 and 72 h). Control represents SKGT4 cells which are not exposed to paclitaxel (0 h). Cell cycle distribution was calculated as the percentage of cells in the G₁, S and G₂/M phase.

on the SKGT4 cell line. Paclitaxel induced cell growth inhibition in a dose- and time-dependent manner. The IC₅₀ of paclitaxel was ~50 nM (Fig. 1). Based on these data we assumed that paclitaxel has potent antitumor effects, specifically with regards to inhibiting the proliferation of SKGT4 cells *in vitro*.

Cell cycle regulation by paclitaxel. To elucidate whether paclitaxel causes growth inhibition in the SKGT4 cells, cell cycle distribution was determined by the fluorescence-activated cell sorting (FACS) analysis. The cell cycle fraction was measured at 0, 6, 12, 24, 48, and 72 h after treatment of 50 nM paclitaxel. As shown in Fig. 2, at 12 h after treatment, the population of G₂/M was significantly increased from 34.38 to 54.66% (P=0.05). The G₂/M population reached the highest level at 12 h, and decreased thereafter. At the same time, the percentage of cells in the S phase was slightly increased from 14.02 to 15.96%. These

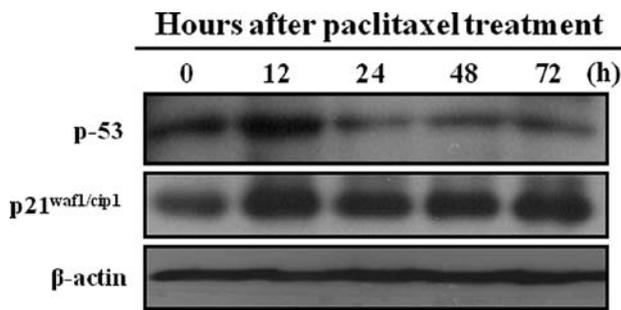


Figure 3. Effect of paclitaxel on p21^{waf1/cip1} in SKGT4 cells. Cells were harvested at the indicated times after incubation with 50 nM of paclitaxel. Cells were then lysed, and the supernatants were subjected to Western blot analysis. Aliquots of 20 μg of protein of extracts were analyzed by 12% SDS-PAGE, transferred to a PVDF membrane and immunoblotted with the indicated antibodies. β-actin was used as an internal control.

results indicated that paclitaxel induced G₂/M phase arrest in SKGT4 cells by 12 h after treatment.

Cell cycle regulatory protein expression by paclitaxel. Since paclitaxel induced G₂/M arrest in the SKGT4 cell line, we next investigated whether there were alterations of cell cycle regulatory proteins such as CDKIs, p21 and p53 after treatment with paclitaxel (50 nM) in SKGT4 cells. The p53 protein levels were drastically increased at 12 h and gradually decreased from 24 h (Fig. 3). The p21 protein expression was also increased by 12 h after paclitaxel treatment (Fig. 3). Since G₂ to M phase progression is regulated by a number of the Cdk/cyclin family including cdc2/cyclin B1 complex, we next examined the protein expression levels of phospho-p34^{cdc2} (Tyr15), p34^{cdc2}, cyclin B1, phospho-Wee1 (Ser642), and cyclin A. As shown in Fig. 4, phospho-Wee1 (Ser642) protein expression was increased at 12 h and then gradually decreased by 72 h after paclitaxel treatment. Although, the expression of p34^{cdc2} which is involved in cell cycle arrest in the G₂ phase was unchanged after paclitaxel treatment as compared to control, while its phosphorylated form, p34^{cdc2} (Tyr15) was significantly increased at 12 h and decreased thereafter. Cyclin A protein expression was also increased at 12 h while no change was observed in the level of cyclin B1 at all time-points after paclitaxel treatment. In addition, the expression of phospho-histone H3, which induces cell cycle arrest in the M phase, was significantly increased by 24 h, then gradually decreased (Fig. 4). Therefore, these results suggest that paclitaxel induces the G₂/M cell cycle arrest in SKGT4 cells.

Induction of apoptosis by paclitaxel in SKGT4 cells. As shown in Fig. 5A, paclitaxel (50 nM) treated SKGT4 cells showed an increased sub-G₁ population in a time-dependent manner (control; 1.33%, paclitaxel at 72 h; 20.65%). We further investigated apoptosis-related proteins (cleaved-caspase-9, -3 and -PARP) using Western blotting. As seen in Fig. 5B, the expression of the cleaved form of initiator caspase-9 and -3 gradually increased in a dose-dependent manner at 48 h after paclitaxel treatment. In addition, a cleaved form of poly-(ADP-ribose) polymerase (PARP) protein, a major substrate for active caspase-3 and a hallmark of apoptosis, was observed to gradually increase in a dose-dependent manner (Fig. 5B). This result

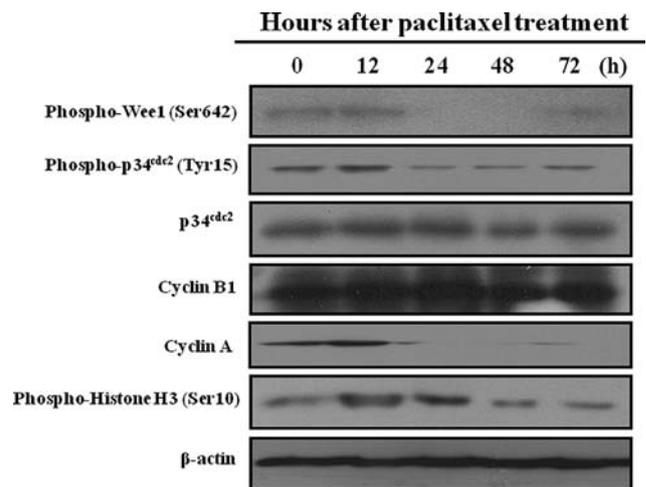


Figure 4. Paclitaxel affected cell cycle-related proteins in SKGT4 cells. Western blot analysis of phospho-Wee1 (Ser642), phospho-p34^{cdc2} (Tyr15), p34^{cdc2}, Cyclin B1, Cyclin A, phospho-Histone H3 (Ser10) in human SKGT4 cells treated with 50 nM paclitaxel at different time-points (0, 12, 24, 48, and 72 h). β-actin was used as an internal control.

indicated that paclitaxel induced apoptotic cell death in SKGT4 cells.

Discussion

The purpose of the present study was to investigate the cytotoxic effect of paclitaxel in human esophageal adenocarcinoma cells to elucidate the biochemical mechanism of cell death. The main result of this set of experiments is the observation that paclitaxel leads to G₂/M cell cycle arrest and induces apoptosis via a caspase-3 pathway in SKGT4 cells.

We used MTT and FACs analysis to ensure paclitaxel induced cell cycle arrest and antiproliferative effect on the human esophageal adenocarcinoma cell line. Paclitaxel induced a time- and dose-dependent inhibition of cellular proliferation of SKGT4 cells. Previously published reports demonstrated similar effects of paclitaxel observed in human esophageal squamous cell carcinoma cells (16). Although, there were different sensitivity effects of paclitaxel on esophageal squamous cell carcinoma cells versus esophageal adenocarcinoma cells, the tendency of paclitaxel to exhibit antiproliferative effects against esophageal cancer appeared to be consistent. In this study, cell cycle analysis revealed that paclitaxel induced the highest G₂/M growth arrest in SKGT4 cells after exposure to 50 nM of the paclitaxel at 12 h. These observations are in agreement with earlier studies demonstrating paclitaxel induced G₂/M phase cell cycle arrest in human breast, lung, gastric, and esophageal squamous carcinoma cells (16,17,24,25). Our results suggest that paclitaxel may induce cells to undergo apoptosis as result of the failure of the cell cycle checkpoint function by accumulation of cells in the G₂/M phase in human esophageal adenocarcinoma cells. We further investigated the cell cycle regulatory molecule after the treatment of paclitaxel to elucidate the precise molecular mechanism of cell cycle regulation. Because p53 is concerned with the regulation of the cell cycle, it is possible that p53 protein could alter cell sensitivity to paclitaxel treatment. The results showed paclitaxel increased p53 protein expression followed by activation of p21^{WAF-1/CIP1} expression at 12 h. Our findings suggest that

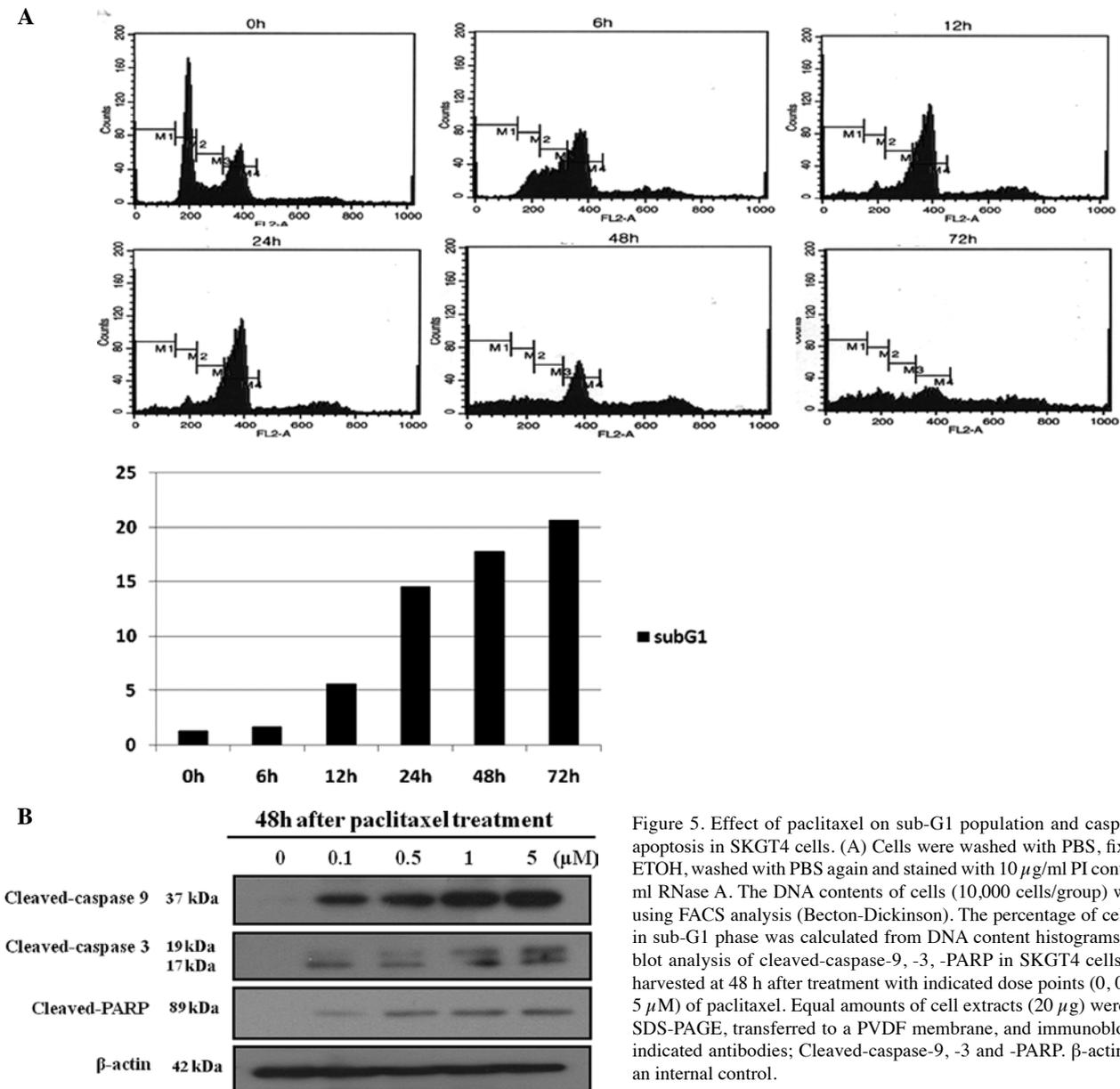


Figure 5. Effect of paclitaxel on sub-G1 population and caspase-mediated apoptosis in SKGT4 cells. (A) Cells were washed with PBS, fixed with 75% ETOH, washed with PBS again and stained with 10 μ g/ml PI containing 10 μ g/ml RNase A. The DNA contents of cells (10,000 cells/group) were analyzed using FACS analysis (Becton-Dickinson). The percentage of cell populations in sub-G1 phase was calculated from DNA content histograms. (B) Western blot analysis of cleaved-caspase-9, -3, -PARP in SKGT4 cells, which were harvested at 48 h after treatment with indicated dose points (0, 0.1, 0.5, 1 and 5 μ M) of paclitaxel. Equal amounts of cell extracts (20 μ g) were subjected to SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with the indicated antibodies; Cleaved-caspase-9, -3 and -PARP. β -actin was used as an internal control.

paclitaxel may induce DNA damage and this causes the increase of p53 and p21 protein levels.

In light of the fact that paclitaxel could be related to the G₂/M checkpoints, we performed Western blot analysis to determine the expression patterns of cell cycle related proteins including phospho-Wee1, phospho-p34^{cdc2}, p34^{cdc2}, cyclin B1, and cyclin A. It is well known that cdc2/cyclin B1 complex starts to form at the beginning of S phase and accumulates with cyclin B protein level. It is usually held in an inactive form until G₂ phase because of phosphorylation on Thr14 and Tyr15 of cdc2 by the Wee1 and myt1 protein kinases (26). This helps explain the increased amounts of Tyr15-phosphorylated p34^{cdc2} found to be coupled with G₂-arrested cells after DNA damage in several systems (27-29). Our results showed that paclitaxel induced augmentation of Tyr15-phosphorylated p34^{cdc2} expression following and increase in the expression of phospho-Wee1 for 12 h. However, the expression of cyclin B1 was not affected. Histone H3 phosphorylation at Ser10 plays an important role in mitotic chromosome condensation (30,31). In the present study,

the levels of phospho-histone H3 was increased from 12 to 24 h which suggests that paclitaxel may induce cell cycle arrest in the M phase. Taken together, our results suggest that paclitaxel affects cell cycle arrest through the regulation of multiple regulators in the progression of the G₂/M phase.

Paclitaxel treatment in SKGT4 cells induced the sub-G₁ population at various incubation time-points. Especially, the sub-G₁ population was markedly detected after 24 h treatment, suggesting paclitaxel may induce apoptotic cell death. Since sub-G₁ populations were increased in SKGT4 by paclitaxel, we next measured the levels of apoptosis-related proteins to investigate the molecular mechanism involved in apoptosis by paclitaxel in SKGT4 cells. Apoptosis is a significant feature of cell death that occurs in response to paclitaxel treatment in many cancers. The intrinsic (mitochondrial) and the extrinsic (death receptor) apoptosis induction pathways are well known (16). A cell death signal in the mitochondrial pathway has been implicated to induce the pro-caspase-9 (16,32,33). It has been reported that caspase-9 activates procaspase-3 as part of the

apoptotic pathway (34). In this study, it was found that the induction of apoptosis was accompanied by up-regulation of cleaved-caspase-9, and -3. Furthermore, the cleaved form of PARP protein, a major substrate for active caspase-3 enzymatic protein, was activated in a dose-dependent manner by paclitaxel. Taken together, these results provide strong evidence that the increased cleaved forms of caspase-3, -9, and -PARP indicated that paclitaxel lead to mitotic cell cycle arrest which might ultimately undergo apoptosis.

In conclusion, paclitaxel markedly inhibits proliferation of esophageal adenocarcinoma cells via G₂/M phase cell cycle arrest and induces apoptosis via a caspase-3 pathway in SKGT4 cells. Although our findings are consistent with previous studies, further examination of other esophageal adenocarcinoma cell lines is needed.

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