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ORIGINAL ARTICLE

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Differential sensitivity of paclitaxel-induced apoptosis in human esophageal squamous cell carcinoma cell lines

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Abstract *Purpose*: Paclitaxel is a highly effective chemotherapy agent against adenocarcinomas and squamous cell carcinomas of the esophagus. However, its precise effects in human esophageal cancer cells are not well understood. This study was designed to examine the relationship between cell-cycle phases of paclitaxel-activated checkpoints and to elucidate the molecular pathway of the effect of paclitaxel in human esophageal squamous cell carcinoma (ESCC) cell lines. *Methods*: The three human ESCC cell lines—TE-2, TE-13 and TE-14—were examined for their response to paclitaxel. ESCC cells were treated with various concentrations of paclitaxel for 1–3 days using MTT assay. The cell-cycle progression and apoptosis were examined by flow cytometry. DNA fragmentation assay was carried out to confirm the fragmented cells as hallmark for apoptotic cells. In additional, the expression of apoptosis-related proteins in ESCC-treated cells was then examined by Western blot analysis. *Results*: TE-14 cells demonstrated the highest sensitivity among all cells. G2/M cell-cycle arrest occurs prior to paclitaxel-induced apoptosis in ESCC cells. The fragmentation of chromatin was observed in drug treated TE-13 and TE-14 cells by flow cytometry and DNA ladder formation. In contrast, the measurement for TE-2 cells was more suggestive of phenotype a resistant in response to paclitaxel treatment. Western blot analysis results showed that the mitochondrial pathway might be involved in paclitaxel-induced apoptosis in ESCC cell lines. *Conclusion*: Differential sensitivity was observed in human ESCC cell lines in response to paclitaxel treatment. G2/M arrest occurs with a prior to paclitaxel-induced apoptosis and might be mediated by the mitochondrial (intrinsic) apoptosis pathway in human ESCC cells.

Keywords Paclitaxel · Esophageal squamous cell carcinoma cell lines · Flow cytometry · DNA fragmentation assay · Apoptosis-related proteins

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Introduction

One of the most lethal gastrointestinal tract malignancies is esophageal carcinoma. Despite recent advances in therapy and management, the overall 5-year survival rate remains at less than 50% [1]. Randomized studies have suggested that neo-adjuvant chemo-radiation followed by surgery may achieve survival rates in esophageal cancer that are superior to the results of surgery alone [2, 3].

Paclitaxel is an anti-mitotic drug has been used for the treatment of various human cancers. Paclitaxel was originally isolated from the bark of the yew tree, and its anti-tumor effects have been known since 1971 [4]. Paclitaxel inhibits tumor cell division by its action on microtubule assembly [5]. This drug does not damage DNA; instead, it stabilizes the polymerization of tubulin, resulting in cell-cycle arrest during mitosis followed by apoptotic cell death [6, 7]. Recently, several studies have shown that paclitaxel is effective against various malignant tumor cells, such as melanoma [8], breast [9], ovarian [10] and prostate cancers [11, 12], as well as brain tumors [13]. Although paclitaxel is an active agent against adenocarcinomas and squamous cell carcinomas of the esophagus [14], the mechanism of paclitaxel-induced apoptosis in human ESCC cell lines remains unclear.

Triggering apoptotic cell death by chemotherapy agent, such as paclitaxel, leads to the activation of a family of cysteine proteases that are called caspases [15]. Pro-apoptotic factors, such as Bax, are released followed by cytochrome c (Cyt c), Apaf-1 and pro-caspase-9 for activation of caspase-9, which is connected with the activation of caspase-3, to execute apoptotic cell death, DNA fragmentation and chromatin condensation [16, 17]. Caspase-3 and caspase-9 have been shown to cleave the 112 kDa nuclear protein Poly (ADP-ribose) polymerase (PARP) into an 85 kDa apoptotic fragment [18, 19]. In contrast, anti-apoptotic factors, such as the X-linked inhibitor of the apoptosis protein (Xiap), inhibit caspase-3 and caspase-9 activation [20, 21]. In addition, two groups reported a role for Hsp60 in helping caspase-3 maturation, suggesting that the chaperone function of Hsp60 is involved in the apoptosis pathway [22, 23]. Based on these considerations, we performed a viability, flow cytometry, DNA fragmentation assay and Western blot analysis on paclitaxel-treated cells. Three human ESCC cell lines were used and analyzed to elucidate the correlation between paclitaxel-induced mitotic arrest and paclitaxel-induced apoptosis. Further, we investigated the possible involvement of the apoptosis pathway in the mediation of paclitaxel-induced apoptosis.

Materials and methods

Cell lines

Three human ESCC cell lines (TE-2, TE-13 and TE-14) were used. All cancer cell lines were derived from human ESCC with varying degrees of differentiation [24]. The TE cell lines were cultured in RPMI-1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U ml^{-1} penicillin and 100μg ml^{-1} streptomycin).

Drug

Paclitaxel was purchased from Wako Pure Chemicals (Osaka, Japan) and received as sterile lyophilized powder. A stock solution of 5 mg/ml was made in dimethylsulfoxide (DMSO) and stored at 4°C, the further dilutions were made in RPMI-1640 medium (Sigma) to obtain the desired concentrations when the cells reached approximately 80% confluency. In flow cytometry, DNA fragmentation assay and Western blotting, we used IC_{50} doses for TE-13 and TE-14 cells. For TE-2, since it did not reach the IC_{50}, a paclitaxel peak plasma concentration of 5 μM was used [25].

Drug sensitivity assay

Cell proliferation analysis was performed on ESCC cells in the presence of increasing concentrations of paclitaxel (Wako Pure Chemical Industries, Japan) by the tetrazolium assay using MTT. Briefly, ESCC cells (2×10^4 /well) were plated in 96-well plates, 100μl for each well. At 24 h after the initial cell seeding, various paclitaxel concentrations (0, 0.5, 2.5, 5, 10, 25 μM) were incubated for another 24 h [26] and wash with fresh medium. WST-8 assay (Dojindo Laboratories, Tokyo, Japan), 10 μl of the cell counting solution was added to each well for control, 24, 48, and 72 h of the plates and incubated in a humidified 5% CO_2 atmosphere at 37°C for 3 h. The formazan was dissolved in 100 μl/well 1 N HCl, and the absorbance of the solution was read at 450 nm using a microtiter plate reader (Becton Dickinson, Franklin Lakes, NJ, USA). All experiments were performed in triplicate. After pulsed exposure, the 50% inhibitory concentration (IC_{50}) was calculated as percentage of control cultures which were not exposed to paclitaxel by interpolate logarithmic concentration curve. Results were derived from at least three independent sets of triplicate experiments.

Flow cytometry analysis

Cell sample preparation and propidium iodide (PI) staining was performed according to the methods described by Nicoletti et al. [27]. At approximately 80% confluency, the cells were treated with the IC_{50} for 24 h and wash with fresh medium. Cells were harvested as control, 24, 48, and 72 h for cell-cycle distribution which was determined using FACScan Coulter EPICS XL Flow Cytometer (Coulter Corp., USA) with an argon laser set to excite at wave length 488 nm [28].

DNA fragmentation assay

Soluble DNA was extracted from both floating and attached cells after 24 h paclitaxel treatment with each IC_{50}. Cultured cells following no treatment and treatment were harvested at the time indicated, washed with cold phosphate buffered saline (PBS) and pelleted by centrifugation at 300 *g* for 10 min. Cell pellets were re-suspended in lysis buffer (5 mM Tris at pH 7.5, 0.5% Triton X-100, and 20 mM EDTA) and kept on ice for 20 min. RNAse A was added to final concentration of 15μg/ml and incubated for 20 min at 37°C, followed by an addition of NaCl to 1 M, with further incubation at 4°C for 2 h. The samples were centrifuged at 13,000 *g* for 30 min at 4°C and the DNA was extracted with phenol–chloroform and precipitated with 2 volume of ethanol at −20°C for 24 h. The precipitated DNA was centrifuged at 13,000 *g* for 30 min at 4°C and allowed to air dry. The DNA was then re-suspended in TE buffer (10 mM Tris at pH 7.5 and 10 mM EDTA) and quantified by absorbance at 260 nm (Beckman DU 640, USA). Five microgram of DNA was applied to 2% agarose gel and electrophoresed at 50 V for 45 min and

the gels were stained with ethidium bromide. The DNA bands were visualized using UV transluminator and photographed with polaroid film.

Cell extraction and Western blot analysis

Lysates from exponentially growing cell lines were prepared in a buffer (20 mM Tris–HCl, ph 7.6, 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40, 1% aprotinin, 1 mM phenylmethylsulphonyl fluoride, 1 mM sodium vanadate). The protein concentration was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein (40μg) from each cell line was loaded in a sodium dodecyl sulphate (SDS) sample buffer (100 mM Tris–HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS) containing 1 mM dithiothreitol, boiled for 5 min and subjected to a 5–20% Tris–Tricine ReadyGel (Bio-Rad, Tokyo, Japan). Proteins were electrotransferred to a hybond-enhanced chemiluminescence nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Antibodies to Xiap and PARP (PharMingen Labs); Bax, Caspase-9, and Caspase-3 (Santa Cruz Labs, CA, USA); Hsp60 (StressGen, BC, Canada) to detect protein level were used in Western blotting. The bands were detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Proteins were re-blotted using antiβ-actin (Sigma) and served as the control. Horizontal scanning densitometry was performed on Western blots by using acquisition into Adobe Photoshop (Apple, Inc., Cupertino, CA, USA) and analysis by the NIH Image Program.

Statistical analysis

Statistical analysis was performed using the Stat View software program (Version 5, SAS Institute, NC, USA). Results are expressed as mean ± SD. The statistical significance of differences between the experimental groups was analyzed using t test; differences were considered significant when $P < 0.05$.

Results

Drug sensitivity assay

The differential sensitivity of the TE-2, TE-13, and TE-14 cell lines to paclitaxel was observed in this study. We examined the differences in the sensitivity of these three human ESCC cells to microtubule agents that are commonly used in the treatment of human cancer. Treatment with an active microtubule interfering agent, paclitaxel (0.5–25 μM) produced a dose- and time-dependent reduction in cell growth in three ESCC cell lines (Fig. 1a–c). TE-14 cells were more highly sensitive to paclitaxel than TE-13 cells (IC_{50}: 2.5 μM; IC_{50}: 5.76 μM), whereas TE-2 cells showed more resistance (Fig. 2 and Table 1). In fact, TE-2 cells, unlike the other cells, did not reach IC_{50} at doses inducing growth arrest, even after 72 h exposure.

Flow cytometry analysis

To investigate whether paclitaxel induces apoptosis in esophageal cancer cells, flow cytometric cell-cycle analysis of exponentially grown cells treated with IC_{50} of paclitaxel for 24 h was performed. As shown in Fig. 3, treatment with paclitaxel-induced G2/M blocks began as early as 24 h and reached the highest levels (53.4–86.5%) in all cell lines. At 48 h after treatment, the population of G2/M began to decrease, followed by an increase in the number of sub-G1 (hypodiploid) cells, which refer to the cells that underwent apoptosis. These results suggest that the cells were undergoing apoptosis after prolonged mitotic blockage. By 48 h after treatment, the number of sub-G1 (hypodiploid) cells was markedly increased and reached the peak level at 72 h (18–57%). TE-14 cells demonstrated the highest sensitivity among all cell lines, with a high sub-G1 population at 72 h after treatment (57.2%). In contrast, TE-2 cells demonstrated the lowest sensitivity, with a low sub-G1 population. G2/M arrest still occurred at a high rate even at 72 h after treatment. The kinetics of the cell-cycle distribution of cells treated with paclitaxel is shown in Fig. 3.

DNA fragmentation assay

DNA fragmentation assay was performed to confirm the results of MTT assays and flow cytometric analysis. Treatment with paclitaxel resulted in the degradation of chromosomal DNA into small internucleosomal fragments, as evidence by the formation of 180–200 bp DNA ladders on 2% agarose gels. Highly sensitive TE-14 cells exhibited a clear and marked DNA ladder pattern at 48 h after paclitaxel exposure and increased over time as compared with TE-13 and TE-2 cells (Fig. 4).

Western blot analysis

Figure 5 shows different levels of the apoptosis-related protein. The role of the mitochondrial pathway in mediating cell death in human ESCC cells exposed to paclitaxel was investigated by analyzing the expression of pro-apoptotic factor: Bax, caspase-9, caspase-3, PARP and anti-apoptotic factor: Xiap. The levels of Bax, caspase-9 as an initiator caspase of the mitochondrial pathway induced processing of the 32 kDa procaspase-3 zymogen to an active enzyme form as an executor caspase were increased by 24 h after treatment. The activation of caspase-3 was accompanied by cleavage caspase-3 substrate protein poly (ADP-ribose)

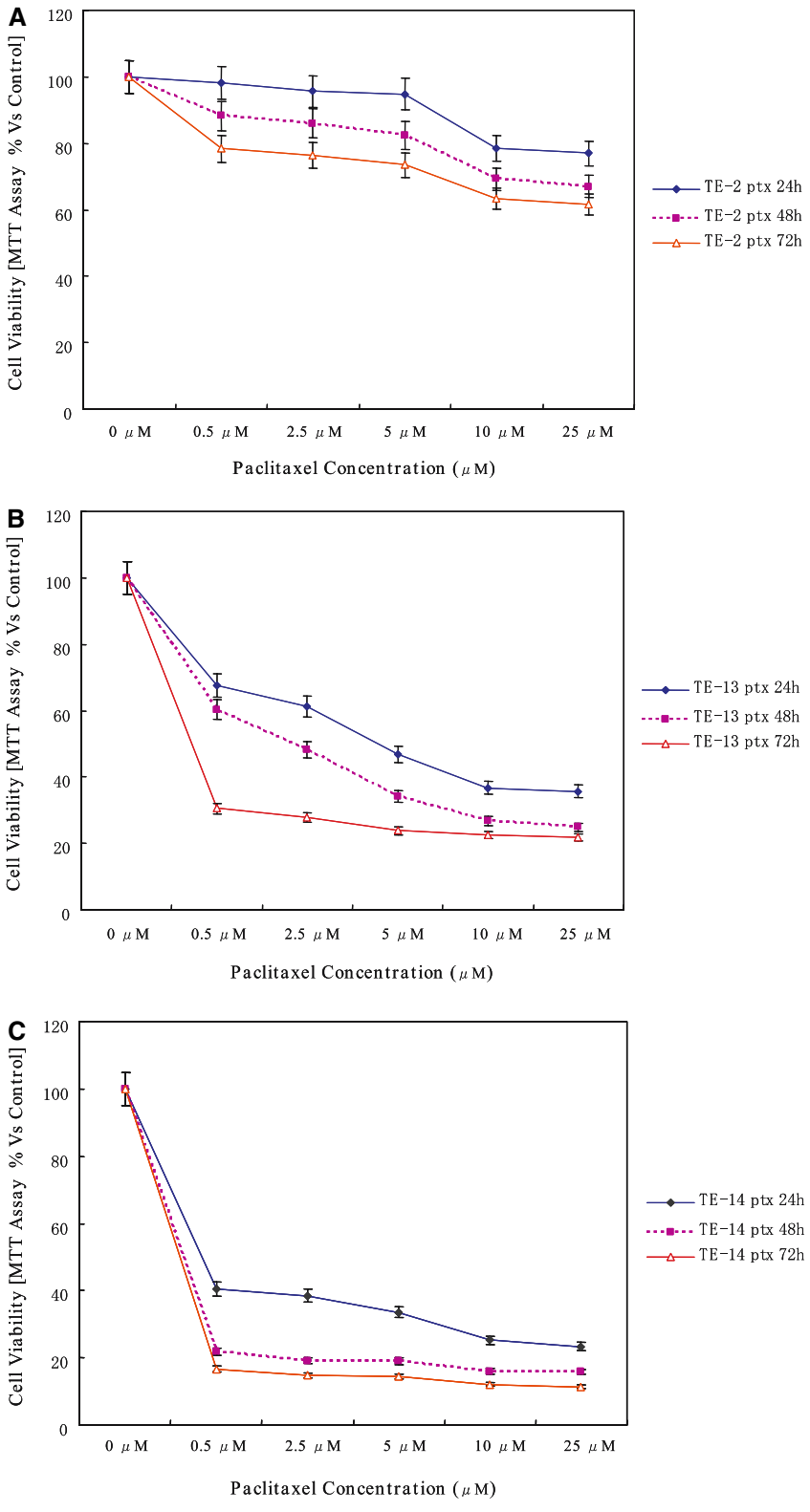

Fig. 1 Differential sensitivity among three of human ESCC cell lines after treatment with paclitaxel. **a** TE-2 cells, **b** TE-13 cells, and **c** TE-14 cells, the dose- and time-related activity of paclitaxel-treated cells is shown

polymerase 85 kDa, respectively. The anti-apoptosis regulator protein, Xiap, was then analyzed. The results showed that Xiap expression was markedly decreased in all cells after paclitaxel treatment. In addition, we analyzed the Hsp60 protein as a chaperone that helps caspase-3 maturation. Hsp60 expression was increased in TE-13 and TE-14 cell, but not in TE-2 cells. All of these results indicate that the mitochondrial pathway might be involved in response to paclitaxel-induced apoptosis in human ESCC cells.

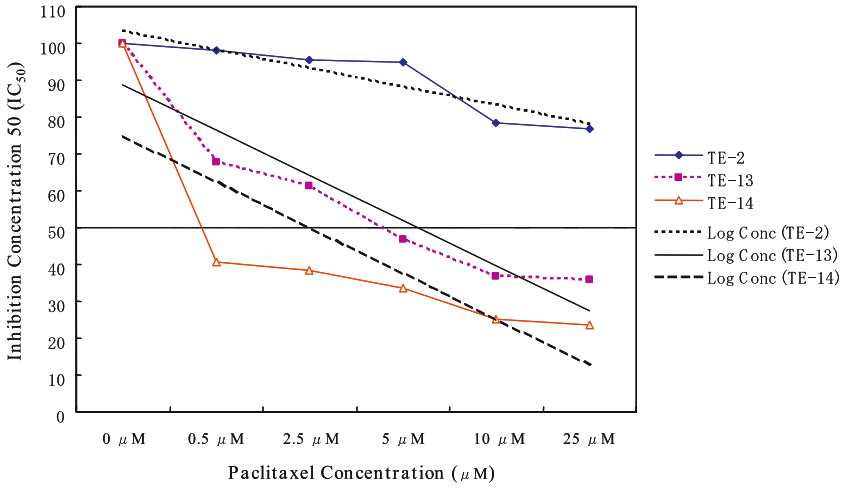

Fig. 2 Analysis of paclitaxel sensitivity was observed after 24 h exposure and the IC_{50} was evaluated by interpolate logarithmic curve. The value of IC_{50} determined for each cell lines is shown in Table 1

Table 1 Sensivity of human an ESCC cells. Its expressed drug's concentration to achieve 50% growth inhibition (IC_{50}) after 24th treatment. Values are means of three experiments

	TE-2 cell line	TE-13 cell line	TE-14 cell line
Paclitaxel (IC_{50} μ M ± SD)	Not reached IC_{50}^{a}	5.76 ± 3.4	2.5 ± 3.7

SD standard deviation; [a]we used the peak plasma level of paclitaxel 5 μM

Discussion

Using flow cytometry, we found that paclitaxel treatment arrested cells mainly at the G2/M phase (Fig. 3). The same effects have been observed in human breast, lung and gastric cancer cells [29, 30]. The accumulation of apoptosis cells—the sub-G1 DNA content has been shown to correlate with apoptotic cell death [31]—after paclitaxel treatment was confirmed with the DNA fragmentation assay. The results of both of these experimental studies were consistent (Fig. 4).

In this study, we showed different sensitivity effects of paclitaxel on human ESCC cell lines in dose- and time-dependent manners. TE-14 cells showed the most sensitivity among three ESCC cells. One possible explanation for this result, Shibata et al. [32] reported, is that the checkpoint of FHA and ring finger (*Chfr*) gene was silenced in TE-14 cells . The implication of this finding is that the cells that lose the *Chfr* gene by hypermethylation of the promoter region have a great impact on the microtubule agent, paclitaxel, by inducing cells to undergo apoptosis as result of the failure of the checkpoint function. In contrast, TE-2 cells, which had positive *Chfr* expression, showed a tendency to resist paclitaxel treatment [32]. However, because the details of this mechanism are not fully understood, further study will be needed to elucidate the role of *Chfr*-paclitaxel-induced cell apoptosis in vitro and to take advantage of its beneficial role in cancer therapies.

Apoptosis has been shown to be a significant feature of cell death following paclitaxel treatment in a variety of tumor types. Two key pathways for apoptosis induction are well known: the mitochondrial (intrinsic) and the death receptor (extrinsic) pathway. In the mitochondrial pathway, a cell death signal through the Bcl-2 family (Bcl-2, Bcl-xl and Bax) has been implicated to induce the release of Cyt c [33, 34], which then binds to the Apaf-1 and results in the eventual recruitment of pro-caspase-9. Activation of this complex can trigger the caspase pathway by activating downstream caspase-3 [35, 36]. Caspase-3 acts as the executor in this pathway. In addition, in this study, the protein expression that may be involved in mediating cell death in cells exposed to paclitaxel was also explored. We investigated the role of the mitochondrial pathway by analyzing the expression of Bax, caspase-9, and caspase-3 proteins. Paclitaxel altered the expression of Bax, which was induced caspase-9, and then activates caspase-3 followed by PARP cleavage in TE-13 and TE-14 cells. A high number of apoptosis cells in these two ESCC cells were accompanied by an increased level of active cleaved fragment of downstream caspase effector and executor that cleaves specific substrate such as PARP, suggesting that the mitochondrial (intrinsic) signaling pathway may play a role in mediating cell death by paclitaxel. In contrast, apoptosis may also occur by the down-regulation of the Xiap protein because an increased number of apoptotic cells were accompanied by a marked decrease in the expression of Xiap. Recent studies have demonstrated that Xiap may play a role in controlling both receptor-mediated cell survival and death signaling by inhibiting the principle effectors of apoptosis, downstream caspases [37, 38].

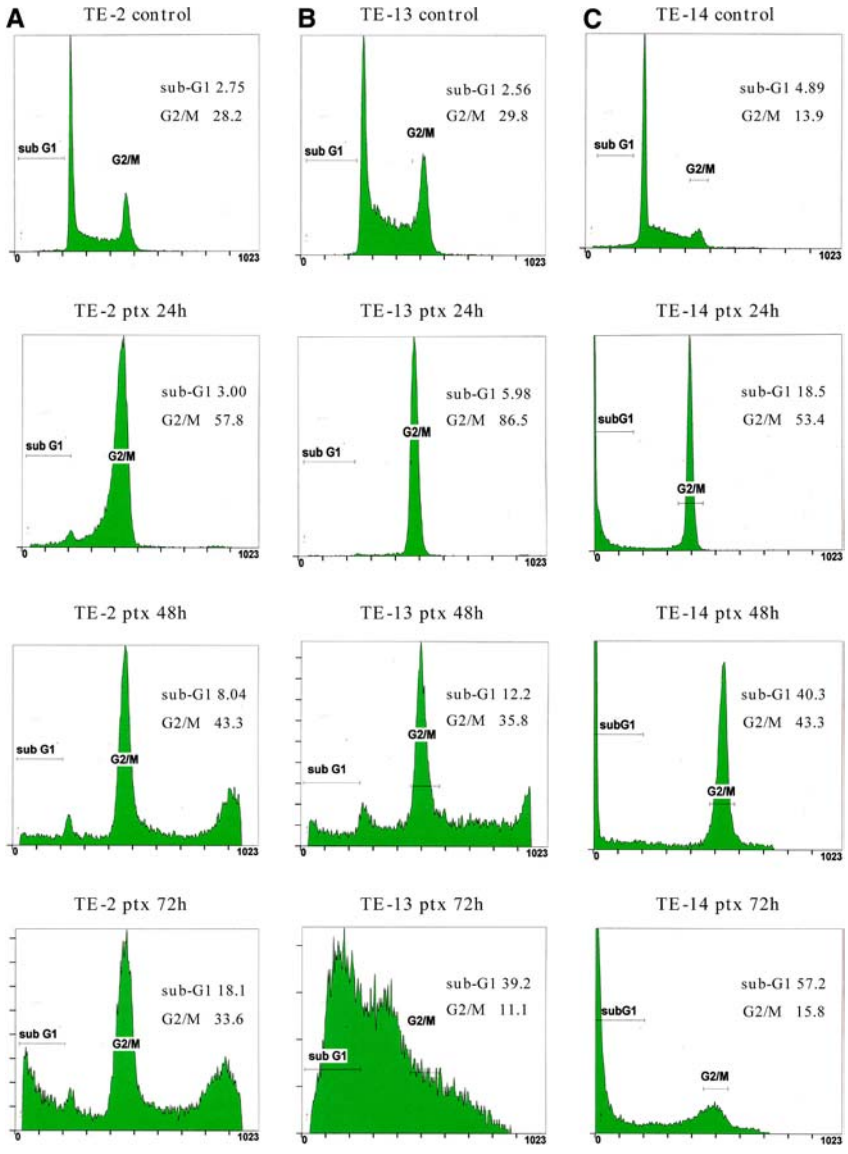

Fig. 3 Effect of paclitaxel on the cell-cycle progression. **a** TE-2 cells, **b** TE-13 cells, and **c** TE-14 cells as control and after a 24 h treatment then harvested at different time point (24–72 h), analyzed by flow cytometry analysis. The percentage of cells in each cell cycle phase is indicated within each plot. The data presented is representative of at least three independent experiments with similar results

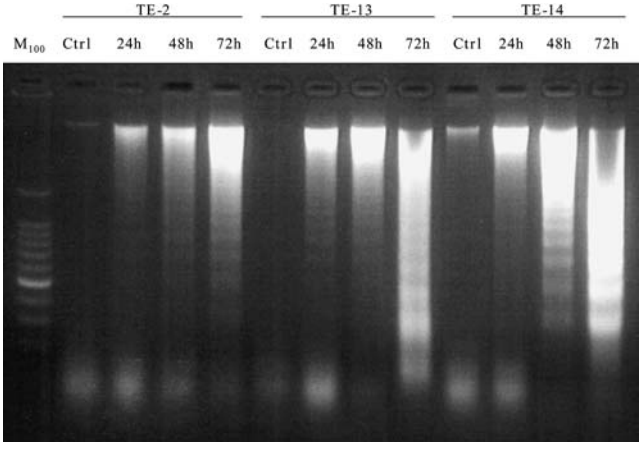

Fig. 4 DNA fragmentation assay of three human ESCC cell lines; TE-2 cells, TE-13 cells and TE-14 cells were treated with or without paclitaxel (by IC_{50}) for various periods. After incubation, total DNA was extracted and electrophoresed in 2% agarose gel containing ethidium bromide. *M* size marker of 100 bp DNA ladder

In addition, we confirmed the expression of Hsp60, which is related to apoptotic activity in human ESCC [39]. Previous reports by two groups have suggested a role for Hsp60 in helping the induction of apoptosis by acting as a chaperone to pro-caspase-3 and aiding in its maturation into active caspase-3 [22, 23]. In this study, Hsp60 expression increased concomitant with caspase-3 expression in TE-13 and TE-14 cells. In contrast, TE-2 treated cells, which showed a resistant tendency, did not express Hsp60. Although Hsp60 can be expressed in untreated TE-2 cells, Hsp60 was not activated following paclitaxel treatment. This result suggests that in the TE-2 cell line, which was more suggestive of a resistant phenotype in response to paclitaxel, the Hsp60 function to help caspase-3 maturation may not play a major role, which raises the possibility that Hsp60 activity might act differently from other cell types.

In conclusion, there we have demonstrated a differential sensitivity among human ESCC cell lines in

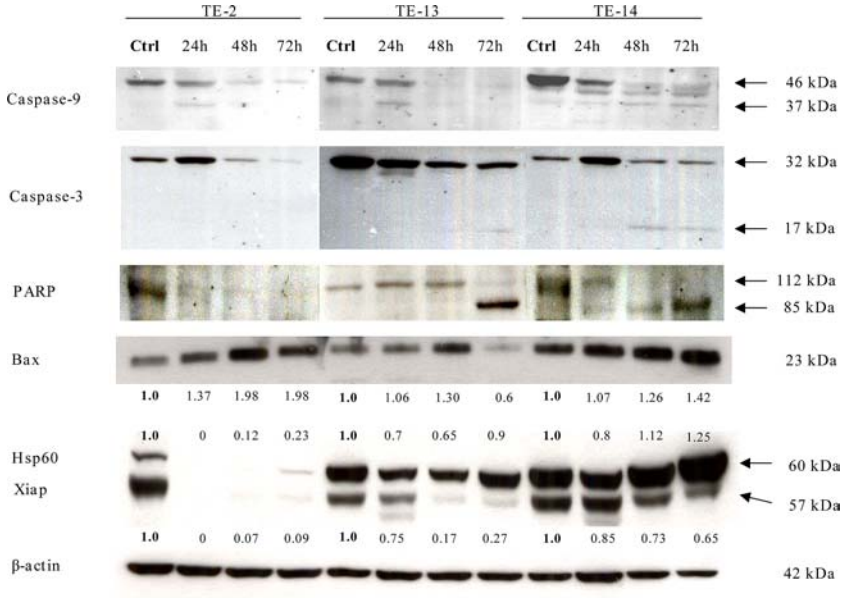

Fig. 5 Western blotting analysis of caspase-9, caspase-3, PARP, Bax, Hsp60, and Xiap expression in human ESCC cells treated for 24 h with paclitaxel and harvested for the indicated time. For Bax, Hsp60 and Xiap proteins, the values underneath and or above the bands represent the densitometric estimation of the density of the bands. The levels of β-actin served as the loading control

response to paclitaxel. This agent leads to mitotic cell-cycle arrest. Cells arrested at this phase might eventually undergo apoptosis. In other words, the apoptotic cell is considered as a secondary event following G2/M arrest. Furthermore, paclitaxel-induced apoptosis in ESCC cell lines might due in part to the mitochondrial (intrinsic) signaling pathway.

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