Cancer Chemother Pharmacol (2008) 62:379–385
DOI 10.1007/s00280-007-0614-5

ORIGINAL ARTICLE

The effect of p53 gene expression on the inhibition of cell proliferation by paclitaxel

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Received: 28 February 2007 / Accepted: 18 September 2007 / Published online: 25 October 2007
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Abstract
Background/Aims We evaluated the relationship between p53 status and paclitaxel (PTX)-induced inhibition of the growth of human stomach cancer cells.
Materials and methods We made use of two human stomach cancer cell lines, MKN45 and MKN28. Growth inhibition in response to PTX was evaluated by MTT method. We used flow cytometry to monitor the cell cycle and western blot analysis to evaluate the expression of signaling molecules.
Results PTX inhibited the proliferation of both stomach cancer cell lines in a dose-dependent manner. However, PTX cytotoxicity was stronger in MKN28 cells. Flow cytometric analysis showed that 1 μM PTX enhanced the percentage of MKN 45 cells in the sub-G1 phase of the cell cycle, whereas it increased the percentage of MKN 28 cells arrested at G2/M phase. 1 μM PTX was found to increase cyclin B1 production in MKN28 cells, but not in MKN 45 cells. In contrast, PTX-treatment led to an increase in the cleaved form of caspase-3 in MKN45, but not MKN28 cells. An inhibitor of p53, pifithrin-α, antagonized the expression of the cleaved form of caspase-3 in MKN45 cells.
Conclusion Both p53 status and cyclin-B1 expression might be useful for predicting the therapeutic response of stomach cancer to PTX.

Keywords Paclitaxel · p53 Expression · Apoptosis · Caspase-3 · Cyclin-B1

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Introduction

Stomach cancer is one of the most common deadly types of cancer worldwide [1]. Chemotherapy is one of the most important strategies being investigated to inhibit metastasis and recurrence of this cancer. Indeed, randomized clinical trials have demonstrated the efficacy of chemotherapy to prolong survival, even for patients with advanced stomach cancer [2, 3]. Paclitaxel (PTX), isolated from the bark of the yew tree, has been used as an anti-mitotic drug against various human cancers since 1971 [4]. In three clinical trials of single-agent PTX for advanced stomach cancer [5–7], the overall response rates were reported to be 20–24%, which is comparable to the response rate of approximately 20% for agents such as 5FU, mitomycin C or doxorubicin [3]. PTX has been shown to inhibit growth of cells by inhibiting microtubule depolymerization [8, 9]. This leads to cell cycle arrest at the G1 and G2/M phases and subsequent activation of apoptotic cell death of cells exposed to PTX [10, 11].

The presence of a wild-type p53 gene increases the antiproliferative effects of commonly used chemotherapeutic agents [12, 13]. Mutant or deficient type of p53 gene expressed in cancer cells could not encourage chemotherapeutic agents-induced p53 gene dependent cell death pathways [14]. In fact, cancer patients with wild-type p53 are often considered better candidates for chemotherapy [15].

In contrast, clinical studies have shown that PTX-mediated cytotoxicity is independent of the presence of wild-type p53 [16]. The clinical experience is supported by studies in nine ovarian cancer cell lines expressing either wild-type or mutated p53 [17], which demonstrated that PTX cytotoxocity is equally effective or even stronger in cells harboring p53 mutations [16, 18]. However, the dependence of PTX cytotoxicity on p53 gene expression is yet poorly understood. In the present study, we evaluated the association of p53 expression with the ability of PTX to inhibit tumor cell growth of stomach cancer.

Materials and methods

Cell lines, culture medium

We used two human stomach cancer cell lines, MKN45 (expressing wild-type p53) and MKN28 (expressing mutated p53). Cells were maintained in RPMI-1640 (Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Sigma, St Louis, MO, USA) and antibiotic/antimyotic solution, in 5% CO_2 and 95% air at 37°C. For various tests, cells were harvested from the plates following trypsin–EDTA treatment and washing with Dulbecco's PBS (Japan Immunoresearch Laboratories Co. Ltd., Gunma, Japan), and replated and cultured for 24 h.

MTT assay

Cellular proliferation of stomach cancer cell lines was evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT) method, as described previously [19]. MTT (5 mg/ml) was dissolved in PBS and solution was stored at 2–8°C for frequent use after filtration though a 0.2 μm filter. Briefly, cells (1×10^4) were cultured in 96-well plates for 24 h. Then, the medium was removed, and the cells were treated with several concentrations (0.001–1 μM) of PTX (Taxol, Bristol-Meyers, Tokyo, Japan) for 6, 24, 48 and 72 h. After washing of the cells with PBS, they were incubated in MTT solution for 3 h. At the end of the incubation period the MTT solution was removed and the cell-associated sediment was dissolved by adding dimethylsulfoxide (DMSO: Wako, Osaka, Japan). The absorbance of the resulting solution was measured using a Microplate reader (BioRad, Tokyo, Japan) at a wavelength of 540 nm. The absorbance of the solution from control cells was designated as 100%. The experiments were performed in triplicate, and the mean ± SD of the data were calculated. Statistical analysis was performed using the Student's *t* test. A *P*-value of <0.05 was considered statistically significant.

Staining of apoptotic bodies with Hoechst 33342

Cells (1×10^4) were cultured on chamber slides for 24 h. After the medium was removed, the cells were treated with certain concentrations (0.001 or 1 μM) of PTX for 24 h. The culture medium was removed and the cells were fixed by treatment with acetic acid/methanol (1:3) solution for 10 min. The fixation solution was removed and the cells were air-dried for another 10 min. Cell was then stained with Hoechst 33342 stain solution (Dojindo Laboratories, Kumamoto, Japan) at room temperature for 30 min. After staining, the solution was removed, and the cells were washed three times with distilled water and then a drop of mounting solution (0.1 M citric acid:0.2 M disodium phosphate:glycerol, 1:1:2) was added before covering the cells with a cover slide. Then we analyzed the cells by fluorescence microscopy. Apoptotic cells showed chromatin condensation, nucleus fragmentation and apoptotic body formation. The apoptotic index was calculated following evaluation of 1,000 cells in each sample.

Flow cytometry

Cells (2×10^6) were cultured in 60-mm tissue culture dishes for 24 h. Cells were then treated with various concentrations (0.001 or 1 μM) of PTX for 24 h. Cells were collected and washed twice with PBS. After centrifugation (1,500*g*, 5 min) the cells were re-suspended in cold 70% ethanol at −20°C for 2 h. Ethanol-fixed cells were washed twice with cold PBS, re-suspended in PBS containing 0.25 mg/ml RNase A (Sigma) at 37°C for 30 min, and stained with 50 μg/ml of Propidium Iodide (Wako) for 30 min in the dark. Cell populations undergoing apoptosis, and those in the G0/G1, S and G2/M phases were analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry System, USA). The data were analyzed using Cell Quest software. In each sample 10,000 fluorescent cells were counted.

Western blot analysis

Cells (1×10^6) were cultured in six-well plates for 24 h. Cells were then treated with various concentrations (0.001 or 1 μM) of PTX for 24 or 48 h and cells were lysed in RIPA buffer [150 mM NaCl, 1.0% NP_4O, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris (pH 8.0)] containing phosphatase inhibitors (1 mM sodium orthovanadate, 30 mM NaF, 1 mM phenyl methylsulfonyl fluoride, and 30 mM NaPPi) and proteinase inhibitor. Twenty micrograms of protein from each sample was electrophoresed through a polyacrylamide-SDS gel and electroblotted onto nitrocellulose membranes in transfer buffer (50 mM Tris, 100 mM glycine, 0.01% SDS, and 20% methanol) for 3 h at

60 V. The proteins on the membranes were exposed to a monoclonal antibody against caspase-3 (Bioscience, San Diego, USA), cyclinB1 (Santa Cruz Biotechnology, CA, USA), cyclinD1 (Santa Cruz Biotechnology), and the interaction was detected with the enhanced chemiluminescence (ECL) system after the addition of anti-mouse or anti-rabbit IgG (Amersham Bioscience), as described previously [20].

Results

PTX inhibits the growth of stomach cancer cell lines

We first examined the effect of PTX on the growth of two stomach cancer cell lines, MKN45 and MKN28, expressing wild-type and mutant p53, respectively. Cells were exposed to several concentrations (0.001–1 μM) of PTX for 6–72 h. As shown in Fig. 1, we observed a dose- and time-dependent decrease in cell viability of both cell lines. The IC50 values for the cytotoxicity of PTX on MKN45 and MKN28 cell lines at 48 h was 0.118 ± 0.056 and 0.018 ± 0.011 μM, respectively ($P < 0.05$), suggesting that cells expressing mutant p53 were more sensitive to PTX-induced cytotoxicity. We next evaluated more carefully the differential response of the two cell lines to two different doses of PTX, 1 nM and 1 μM.

The mechanism of PTX-induced cell growth inhibition

Apoptotic cells were evaluated on the basis of morphological changes. After treatment with 1 nM or 1 μM PTX for 24 h, we monitored apoptotic characteristics including chromatin condensation, nucleus fragmentation and apoptotic body formation (Fig. 2). The apoptotic index of cells treated with 1 nM or 1 μM PTX was calculated to be 21.6 and 51.5%, respectively, in MKN 45 cells and 23.5 and 56.1%, respectively, in MKN 28 cells. Thus, we did not detect significant differences in the apoptotic index of the two cell lines at these concentrations of PTX despite the dose-dependent nature of the effect.

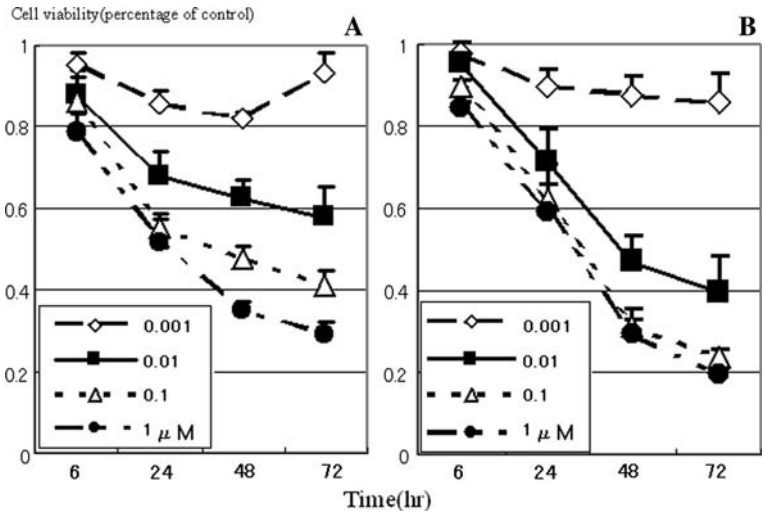


Fig. 1 The effect of PTX on the growth of stomach cancer cells. The effect of PTX on stomach cancer cell growth was examined using the MTT assay as described in "Materials and methods". Cells, MKN45 (**a**) and MKN28 (**b**), were exposed to 0.001–1 μM of PTX for 6–72 h

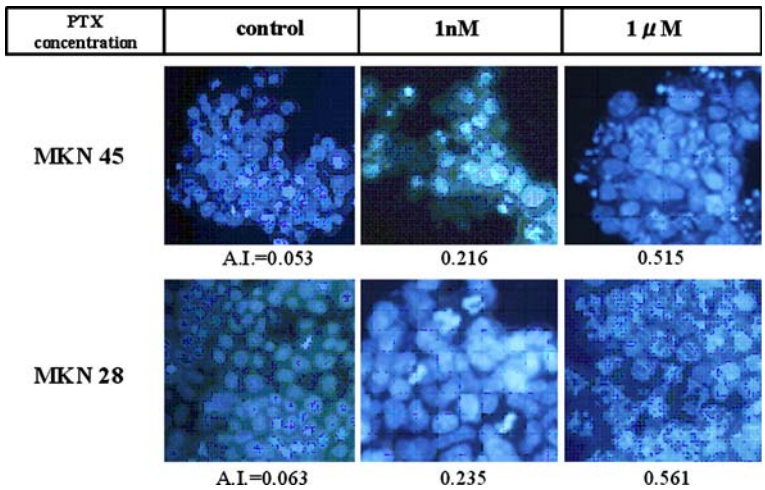
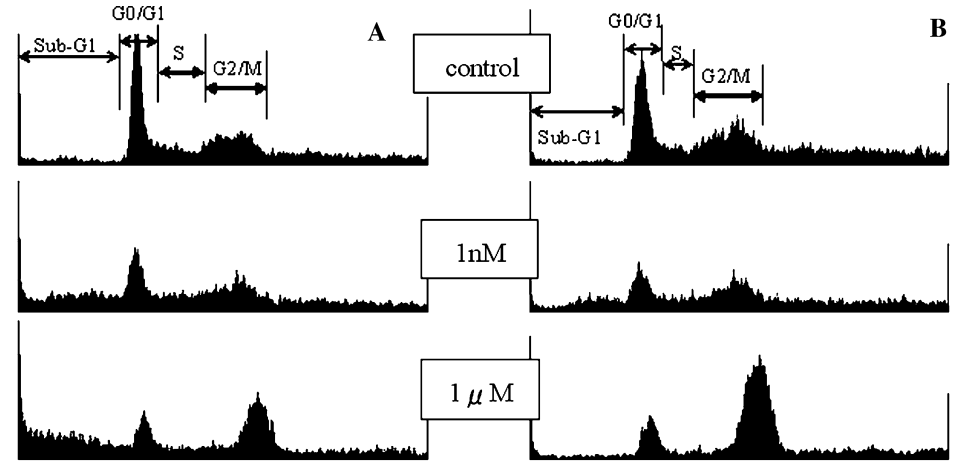


Fig. 2 Morphologic analysis of the induction of apoptosis in response to PTX treatment. Apoptotic cells were evaluated on the basis of morphological changes using Hoechst 33342 staining. After treatment with 1 nM or 1 μM PTX for 24 h, we monitored apoptotic characteristics including chromatin condensation, nucleus fragmentation and apoptotic body formation were detected of MKN45 (*upper panels*) or MKN28 cells (*lower panels*)

Using conventional DNA flow cytometry, we next compared the percentage of cells from each cell line in each phase of cell cycle (sub-G1, G0/G1, S, G2/M) in the presence of PTX (Fig. 3). We observed that treatment of cells with 1 nM PTX increased the percentage of cells in the sub-G1 phase in both cell lines (from under 5–15.63% and 19.55% for MKN45 and MKN28 cells, respectively). We further observed that treatment of the cells with 1 μM PTX increased the percentage of MKN45 cells in the sub-G1 phase to 25.78% and increased the percentage of MKN28 cells in the G2/M phase to 50.09%, suggesting that this concentration of PTX induced G2/M arrest.

We next evaluated the expression of cyclin-D1, cyclin-B1 and caspase-3 by western blot analysis of lysates of MKN45 and MKN28 cells treated with PTX. We did not observe any effect of exposure of either cell line to either 1 nM or 1 μM PTX on the expression of cyclin D1 (Fig. 4a). However we did detect an increase in the expression of cyclin-B1 in MKN28 cells treated with 1 μM PTX, whereas a similar increase was not detected MKN45 cells at this concentration (Fig. 4b). We did not detect an effect of 1 nm PTX on the expression of cyclin B1 in either cell line. Finally, we also detected an increase in the cleaved form of caspase-3 in MKN45, but not MKN28 cells, which was dependent upon the dose of PTX treatment (Fig. 4c).



MKN45	sub-G1	G0/G1	S	G2/M
control	4.91 ± 1.32	32.62 ± 4.07	13.99 ± 3.22	19.8 ± 0.63
PTX 1nM	15.63 ± 6.88	22.01 ± 4.08	10.48 ± 0.59	17.67 ± 0.77
PTX 1 μ M	25.78 ± 7.41 *	11.78 ± 0.22	7.08 ± 0.6	29.13 ± 2.71*

MKN28	sub-G1	G0/G1	S	G2/M
control	4.68 ± 1.99	33.46 ± 11.7	13.58 ± 3.66	18.95 ± 3.47
PTX 1nM	19.55 ± 5.76	15.98 ± 5.38	11.68 ± 3.81	21.63 ± 4.46
PTX 1 μ M	12.65 ± 6.06*	9.89 ± 1.2	5.25 ± 1.29	50.09 ± 4.35*

Fig. 3 PTX induced cell cycle arrest. Analysis of cell cycle phase (sub-G1, G0/G1, S, G2/M) by conventional DNA flow cytometry of MKN 45 (**a**) and MKN 28 (**b**) cells treated with PTX. The *lower panels* show the percentage of each cell line in each phase of the cell cycle

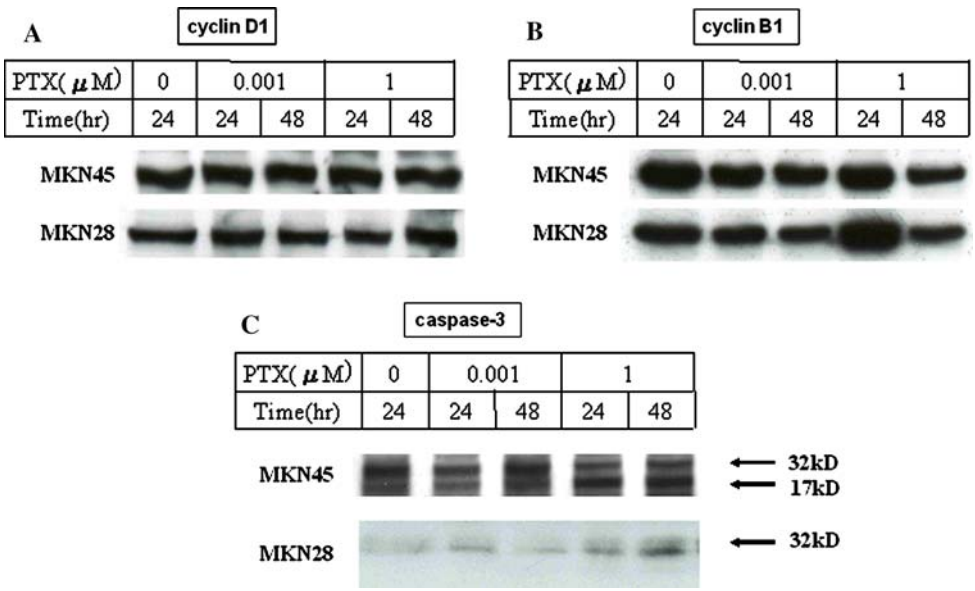


Fig. 4 The effect of PTX on cell signaling pathways. We evaluated the expression of cyclin-D1 (**a**), cyclin-B1 (**b**) and caspase-3 (**c**) in PTX-treated MKN45 and MKN 28 cells by western blot analysis as described in "Materials and methods"

Association of p53 expression with PTX-induced cellular responses

To evaluate the association of p53 expression with PTX-induced growth inhibition, we tested the effect of p53-inhibitor, pifithrin-α (Biomol) [21, 22], in the wild-type p53 expressing cell line, MKN 45 (Fig. 5). We observed that exposure of the cells to pifithrin-α inhibited the effect of 1 μM PTX on cleavage of caspase-3. In addition, we observed that pifithrin-α also antagonized PTX-induced cell cytotoxicity in a dose-dependent manner. In addition, the accumulation of cyclin B1 in MKN 28 cells in response to treatment with 1 μM PTX was not detected in cells treated with pifithrin-α (data not shown).

Discussion

The p53 gene is known to suppress tumorigenesis through a number of regulatory pathways, such as regulation of cell-cycle checkpoints, apoptosis, DNA repair, maintenance of genomic integrity and control of angiogenesis [23, 24]. However, because of its very short half-life, it is difficult to detect the expression of the wild type p53 protein, whereas the mutant protein is more readily detected due to its longer half-life and it can therefore monitored clinically [25]. Mutation of p53 abolishes its antitumor activity and is associated with over 60% of human carcinogenesis [26]. In contrast, cells expressing wild-type p53 have been reported to show high sensitivity to anti-tumor drugs [12, 13]. In the current study, we made use of two stomach cancer cell lines, MKN45 and MKN28, that were previously shown to express wild-type and mutated p53, respectively, based on their differential sensitivity to 5FU-CDDP therapies [15]. I want to emphasize that two cell lines I used this experiment were proved the character to be wild-type or mutated p53 in the previous our paper. The results suggested that p53-mediated apoptotic pathways play a critical role in the efficacy of 5FU-CDDP therapies, as has been suggested in other reports [27]. We thus made use of these two stomach cancer cell lines to evaluate the importance of p53 expression on PTX-induced cell growth inhibition.

PTX is well known to induce cell cycle arrest at G1 and G2/M phases by inhibiting microtubule depolymerization [8, 9], which activates the apoptotic death pathway [10, 11]. In addition, PTX was shown to act via both p53-dependent and p53-independent apoptotic pathways [28]. We also observed here that PTX could inhibit the growth of stomach cancer cells regardless of p53 status in a dose-dependent manner (Fig. 1). However, we found that PTX toxicity was stronger in cells expressing mutant p53.

PTX has been shown to have differential affects on different signaling pathways in a dose-dependent fashion. Cell death induced by low doses of PTX results from aberrant mitosis mediated by a Raf-1-independent signaling pathway, whereas cell cycle arrest at G2/M induced in response to a higher dose of PTX is mediated by a Raf-1-dependent signaling pathway [29]. Raf-1 is reported to be an intermediate of cellular signaling pathway [30], and can activate DNA fragmentation by PTX [31]. In the present study, low dose (1 nM) PTX increased the percentage of cells in the sub-G1 phase of the cell cycle regardless of p53 status, whereas higher dose (1 μM) PTX led to arrest of cells at the G2/M phase in cells expressing mutant p53 (Fig. 3). Functional p53 has been reported to enable cells to bypass agents-induced arrest of the cell cycle at G2/M [18]. It is thus possible that p53 might play a role in Raf-1-dependent inhibition of cell growth in response to PTX.

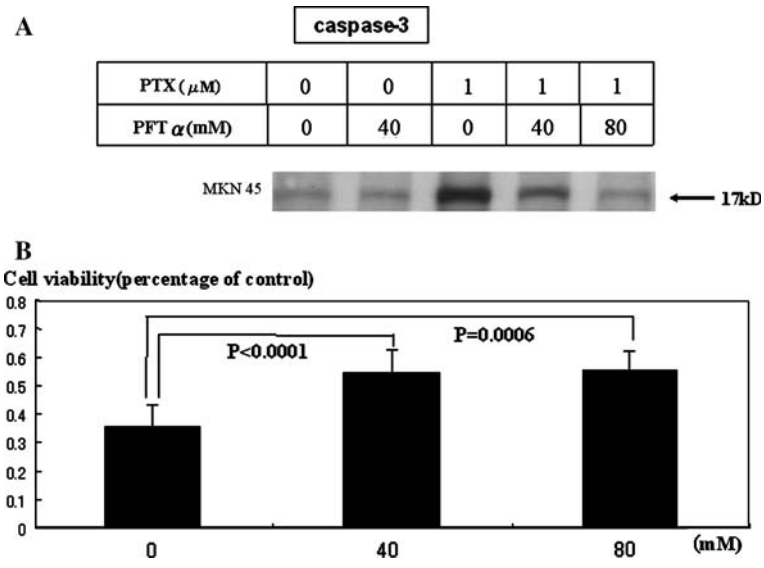


Fig. 5 The effect of a p53 inhibitor on PTX induced signaling. Western blot analysis of caspase-3 cleavage in MKN45 cells treated with PTX in the absence and presence of the p53 inhibitor, pifithrin-α (**a**). MTT assay performed on PTX-treated MKN 45 cells in the presence of increasing concentrations of pifithrin-α (**b**)

Indeed, a recent study has provided support for the association of p53 with Raf-1 dependent responses of cells to agents-induced cell death [32].

Cyclins are a group of proteins involved in cell cycle regulation. Numerous cyclins have been identified: A, B [1], C, D [1, 2], E F, G and H. Cyclin D1 is synthesized in the pre-DNA-synthetic gap (early G1 phase) just before the induction of S phase. Cyclin D1 is a key general regulator of cell cycle progression, and is often over-expressed in cancer cells [33, 34]. We did not observe accumulation of cyclin D1 following treatment of cells with low dose PTX (Fig. 4a), which leads to PTX-mediated sub G1 phase arrest [29]. On the other hand, cyclin B1 is synthesized in the late G2/M phase and is degraded during M phase [35]. Thus, increased expression of cyclin B1 is associated with cell-cycle blockage at the G2/M [36, 37], which appears to be critical to the effect of PTX treatment in gastric cancer cells expressing mutant p53 (Fig. 4b).

The p53 gene product was reported to be negative regulator of cyclin B1 transcription due to negative feedback regulation of cyclin B1 [38]. Moreover, a recent study has shown that p53 decreased intracellular levels of cyclin B1 and the absence of p53 lost its ability [39]. Taken together, these findings suggest that cyclin B1 may accumulate more readily in response to PTX in p53 mutant MKN 28 cells, thereby predisposing the cells to increased cell-cycle arrest-mediated toxicity. The present study also demonstrated that cells expressing mutant p53 did not express active caspase-3. Exposure of these cells to a p53 inhibitor demonstrated that the activity of p53 is important for caspase-3 dependent apoptotic pathway. However, PTX mediates apoptosis of stomach cancer cells regardless of p53 status (Fig. 2), suggesting that a caspase-3 independent pathway is involved in PTX-induced apoptosis. A recent study has suggested that PTX induces apoptosis by both caspase-dependent and caspase-independent pathways [40, 41]. And the still unknown pathway might be related with cell cycle arrest. Additional study of the relationship between regulation of the cell cycle and apoptosis is clearly needed.

Conclusion

As a summary of the present study, we have shown that both p53 status and cyclin-B1 expression might be useful for predicting the therapeutic response of stomach cancer to PTX.

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