Resistance to paclitaxel therapy is related with Bcl-2 expression through an estrogen receptor mediated pathway in breast cancer

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Abstract. Taxanes are approved for the treatment of breast cancer that has spread to the lymph nodes, following surgery and doxorubicin containing chemotherapy. Taxanes have improved the survival of breast cancer patients, especially in estrogen receptor (ER) negative population in clinical settings. This time we examined the relationship between chemosensitivity to Taxanes and expresson of ERα in breast cancer cell lines. *In vitro* effects of paclitaxel in 4 ER-positive and 3 ER-negative breast cancer cell lines were investigated by MTT assay. We also investigated members of Bcl-2 family by Western blotting and RT-PCR to clarify their role in paclitaxel resistance both in ER-positive and in ER-negative cells. ER-negative cell lines were more sensitive to paclitaxel than ER-positive cells. ER-negative KPL-4 and ZR-75-30 cells, which were sensitive to paclitaxel, became resistant when they were treated with demethylation agent, 5-aza-2'-deoxycytidine. Analysis of proapoptotic (Bax) and antiapoptotic (Bcl-2) molecules suggested that Bcl-2 is likely to have a role in the resistance of ER-positive cells. Bcl-2 expression was increased in a time-dependent manner after treatment of ER-positive cell lines with estrogen (E2). On the other hand, Bcl-2 was not detected in ER-negative cell lines. However, no significant difference was detected for Bax mRNA levels before and after E2 treatment in ER-positive and negative cell lines. Activation of ER gene expression in ER-negative KPL-4 cells by 5-aza-2'-deoxycytidine resulted in up-regulation of Bcl-2 mRNA. To support our data, we examined paclitaxel sensitivity in ER-negative MDA-MB-231 and ER stable transfectant cells S30 and JM6. This experiment also showed ER-negative cells were sensitive to paclitaxel but ER-positive cells were resistant to it. These results suggest that ER influenced chemosensitivity to paclitaxel through regulation of Bcl-2 family and regulation of the pathway may be crucial to increase the efficacy of taxanes in ER-positive breast cancer.

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Introduction

Adjuvant chemotherapy with anthracycline-based regimens has been proven to decrease the risk of relapse and cancer-related mortality in breast cancer patients. The taxanes, paclitaxel and docetaxel, have been successfully incorporated into several adjuvant chemotherapy regimens in recent studies. The available studies reveal that the addition of taxanes after surgery and doxorubicin containing chemotherapy clearly shows a benefit in the adjuvant treatment of breast cancer especially in the lymph node-positive cases. Taxanes act by shifting the dynamic equilibrium between tubulin and microtubules in the direction of microtubule assembly. The cells become blocked during the G2 and M cell cycle phases and cannot form a normal mitotic spindle and divide. Essentially, these microtubules are excessively stable and therefore dysfunctional (1).

Paclitaxel, first marketed drug of taxanes affecting the integrity of microtubules was also shown to induce Bcl-2 phosphorylation in various cancer cell lines (2). Bcl-2 is a key member of a family of proteins that control apoptosis by preventing cancer cell death and that have been involved in resistance to chemotherapy and radiotherapy. Estradiol (E2) treatment significantly increased Bcl-2 protein expression and tamoxifen blocked Bcl-2 expression (3-5). The ability of E2 to act as a survival factor for breast cancer is not well

understood, but a substantial part of the effects is estimated to occur through the prevention of programmed cell death (apoptosis).

Estrogen receptor (ER) and its ligand, estradiol, play a significant role in the development, progression, treatment, and outcome of breast cancer. Estrogen-induced proliferation of mammary and uterine epithelial cells is primarily mediated by ER. The ER CpG islands are unmethylated in normal breast tissue and most ER-positive tumor cell lines, whereas it is methylated in about 50% of unselected primary breast cancers and most ER-negative breast cancer cell lines (6,7). The methylation of these CpG cluster sites is associated with either reduced or absent ER expression. Hypermethylation of CpG islands in the 5'-region of the ERα gene has been found in different tumor types, including breast cancer and its expression has been demonstrated to reactivate after treatment with 5-aza-2'-deoxycytidine (8). One of the promising predictive factors for paclitaxel treatment was reported to be the expression of ER. In this study, we investigated the relationship between chemosensitivity to taxanes and expression of ERα in breast cancer cell lines.

Materials and methods

Cell lines, reagents and treatments. KPL-1 and KPL-4 cells were kindly provided by Dr Junichi Kurebayashi (Kawasaki Medical School, Okayama, Japan) (9). MDA-MB-231 cell line was obtained from ATCC. S30 and JM6 cells were kindly provided by Dr V. Craig Jordan (Fox Chase Cancer Center, USA). ZR-75-1, ZR-75-30, MCF7 and T47D cells were obtained from Dainippon Sumitomo Pharma, Tokyo, Japan. ZR-75-1, ZR-75-30 and T47D cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), HEPES, streptomycin and penicillin. KPL-1 and KPL-4 cell lines were cultured in DMEM supplemented with 10% FBS, streptomycin and penicillin. MCF7 cell line was cultured in MEM containing 10% FBS, non-essential amino acid (NEAA), 1 mM sodium pyruvate, streptomycin and penicillin. MDA-MB-231 cell line was cultured in Levovitz supplemented with 10% FBS, streptomycin and penicillin. S30, JM6 were cultured in MEM (phenol red-free) supplemented with 10% Charcoal/Dextran treated FBS (Hyclone), L-glutamine, NEAA, antibiotic-antimycotic solution, insulin and G418. 5-aza-deoxycytidine, MTT and 17ß-estradiol were obtained from Sigma.

Immunostaining. Estrogen receptor expressed in human breast cancer cell lines was detected using monoclonal mouse anti-human estrogen receptor, clone 1D5 (Dako). Cells were seeded on 2-well chamber slides (Nalge Nunc) and cultured for 48 h. Cells were fixed in 4% paraformaldehyde at 4˚C for 20 min, washed in PBS. Endogenous peroxidase was blocked by incubating slides with 3% H_2O_2 in methanol for 10 min, and slides were washed in PBS. After blocking of non-specific staining for 10 min (Protein Block Serum-Free, Dako), slides were incubated 1 h at room temperature with anti-ERα antibody and washed in PBS. Slides were then incubated with secondary antibody (Envision+System-HRP Labeled Polymer Anti-mouse, Dako) and washed in PBS. Immunostaining was developed using DAB Substrate-Chromogen (Liquid DAB+Substrate Chromogen System, Dako) and counter-staining was performed with Mayer's haematoxylin. Slides were dehydrated in a series of alcohol and xylene.

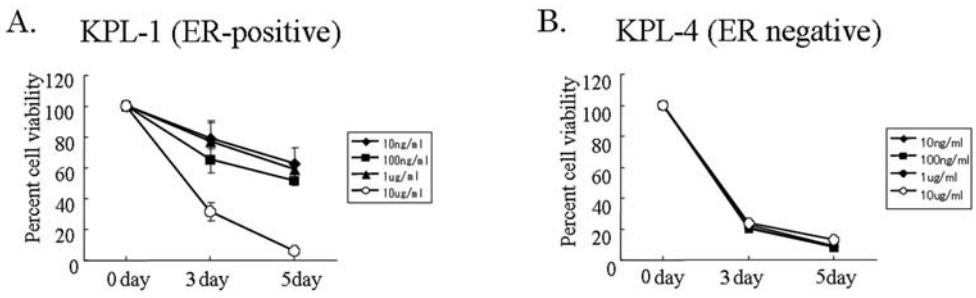
MTT assay for cell viability. Human breast cancer cell lines seeded in 96-well plates (5,000 cells/well) in 0.1 ml medium. Cells were cultured for 48 h before the addition of the drug. Paclitaxel, tamoxifen and 17ß-estradiol (E2) was diluted into 10% FBS-containing medium and added to each well in a volume of 0.1 ml. Cells were incubated at 37˚C for 24, 72 and 120 h. MTT (20 μl) (5 mg/ml in PBS) was added to each well and incubated for ~3-4 h at 37˚C. The medium was removed, the blue formazan dye was dissolved in 100 μl 0.04 N HCl isopropanol and absorption was calculated at dual wavelength (540/620 nm) using microplate reader.

Apoptosis assay. For quantitation of apoptosis induced by paclitaxel, we used CPP32/Caspase-3 Fluorometric Protease Assay Kit (MBL). Caspase-3 activity assay was performed according to the protocol recommended by the manufacturer. Briefly, cells were seeded onto 6-well plates at $1x10^5$ cells/well and precultured at 37˚C for 48 h. Then cells were treated with paclitaxel for 36 h, washed once with PBS, and collected by careful scraping. Treated cells were suspended in cell lysis buffer at 4˚C for 40 min and protein concentrations were determined using the Bio-Rad protein determination method (Bio-Rad Laboratories, Hercules, CA). Protein (25 μg) in 50 μl of cell lysis buffer was added with 50 μl of reaction buffer and 5 μl of the 1 mM DEVD-AFC substrate and incubated at 37˚C for 1 h. Samples were read in a fluorometer (Spectrofluorophotometer RF-1500; Shimadzu, Kyoto, Japan) with a 390 nm excitation filter and a 510-nm emission filter.

5-aza-2'-deoxycytidine treatment. Cells were seeded at $5x10^5$ cell/100-mm culture dish. After 48 h of incubation, cells were treated with 5-aza-2'-deoxycytidine at concentrations ranging from 50 ng/ml to 5 μg/ml for 96 h. Cells treated with 5-aza-2'-deoxycytidine were exposed to RT-PCR, MTT assay and immunostaining.

RT-PCR. RNA was extracted from the cells treated with E2 by using the RNAZol. RT-PCR was performed using Perkin-Elmer GeneAmp RT PCR Kit. PCR products were analyzed by agarose (1%) gel electrophoresis and visualized by ethidium bromide staining, and the sizes were estimated by comparison to DNA molecular weight marker. Oligonucleotide sequences of PCR primers for Bcl-2, Bax were previously reported (4). Primers for Bid was designed in our laboratory: Bcl-2, sense 5'-GGT GCC ACC TGT GGT CCA CCT G-3'; antisense 5'-CTT CAC TTG TGG CCC AGA TAG G-3'. Bax, sense 5'-ATG GAC GGG TCC GGG GAGCAG C-3'; antisense 5'-CCC CAG TTG AAG TTG CCG TCA G-3'. Bid, sense 5'-GCA GGC CTA CCC TAG AGA CA-3'; antisense 5'-GTC CAT CCC ATTT TCT GGC TA-3'.

Western blotting. Total cell protein was extracted from ~20,000 cells with E2 for up to 0, 12, 24 and 48 h. Total protein (40 μg), as determined by Bio-Rad assay, was subjected to 12% SDS-PAGE and transferred to membrane (Amersham



C. IC50 Values

ER positive		ER negative	
ZR-75-1	>1000 ng/ml	ZR-75-30	413 ng/ml
KPL-1	>1000 ng/ml	MDA-MB-231	37 ng/ml
T47D	>1000 ng/ml	KPL-4	0.01 ng/ml
MCF7	518 ng/ml		

Figure 1. Cytotoxic effects of paclitaxel in (A) ER-positive and (B) ER-negative cell lines by using MTT assay. (C) All of the ER-positive cells were resistant to paclitaxel, whereas ER-negative cells were sensitive to the drug.

Life Science Hybond-P). The membrane was blocked with 5% skim milk in 0.2% Tween-20 in TBS (TBST) for 1 h at room temperature and then incubated with primary antibody (Bcl-2, Bax, Santa Cruz Biotechnology Inc., Bid, BD) in TBST at 4˚C overnight. The membrane was washed three times with TBST and incubated with a peroxidase-linked secondary antibody at room temparature for 1 h by shaker incubator and then washed in TBST three times. ECL Western Blotting Detection System (GE Healthcare) was used to detect secondary probes.

PowerBlot Western array analysis. Protein was extracted according to the Becton-Dickinson protocol, and 400 μg of protein were loaded in one big well across the width of the SDS-polyacrylamide gel. The gel was run overnight, and was transferred to an Immobilon-P membrane (Millipore). After transfer, the membrane was blocked for 1 h with 5% milk and clamped with a Western blotting manifold that isolates 45 channels across the membrane. In each channel, a complex antibody mixture was added and allowed to hybridize for 1 h. The blot was removed from the manifold, washed and hybridized for 30 min with secondary antibody. The membrane was developed with chemiluminescence (SuperSignal West Pico, Pierce). Data analysis includes raw and normalized digital data from each blot with changes >1.25-fold indicated. The expression data are reported as a confidence level based on fold change, reproducibilty and signal intensity.

Results

Paclitaxel-induced cytotoxicity. First we compared the cytotoxic effects of paclitaxel in 4 ER-positive and 3 ER-negative cell lines by using MTT assay. All of the ER-positive cells were resistant to paclitaxel, whereas ER-negative cells were sensitive to the drug. Each of the ER-positive cells showed IC_{50} level of >500 ng/ml, while it was 413 ng/ml, 37 ng/ml and 0.01 ng/ml for the each of ER-negative ZR-75-30, MDA-MB-231 and KPL-4 cells, respectively (Fig. 1). This result suggests that ER expression may influence chemosensitivity to paclitaxel.

Paclitaxel-induced apoptosis. To quantify the induction of apoptosis, KPL-1 (ER-positive) and KPL-4 (ER-negative) cells were treated with paclitaxel (with a range from 1 ng/ml to 1000 ng/ml) for 36 h and caspase-3 assay was performed. A dose-dependent increase in the activity of caspase-3 in KPL-4 cells was detected, while no such effect was shown in KPL-1 cells (Fig. 2). No caspase-3 activity was detected in the dose of 1,000 ng/ml in ER-negative KPL-4 cells possibly due to extensive cell death. These data suggest that at least a part of paclitaxel cytotoxicity occurs through apoptotic pathway.

Induction of ER expression and its effect to the paclitaxel chemosensitivity by 5-aza-2'-deoxycytidine in breast cancer cell lines. Methylation of ER promoter is one of the major mechanisms for the loss of ER expression in breast cancer cells. To restore ER expression, we treated two ER-negative cell lines, KPL-4 and ZR-75-30, with 5-aza-2'-deoxycytidine for 96 h and MTT assay for cell proliferation was performed. Immunostaining of KPL-4 cells demonstrated the restoration of ER expression after treatment with 5-aza-2'-deoxycytidine (Fig. 3a). The paclitaxel-sensitive KPL-4 cells became resistant in a dose-dependent manner when treated with 5-aza-2'-deoxycytidine, suggesting that reactivation of ER expression results in the gain of resistance in the cells (Fig. 3b). Similar data were obtained in ZR-75-30 cells (data not shown).

Protein expression of proapoptotic and antiapoptotic molecules after paclitaxel treatment in ER-positive and negative cells. To investigate the differences in the protein expression of proapoptotic and antiapoptotic genes, we performed Western blotting in the ER-positive (ZR-75-1, T47D, MCF7 and KPL-1) and -negative cells (ZR-75-30,

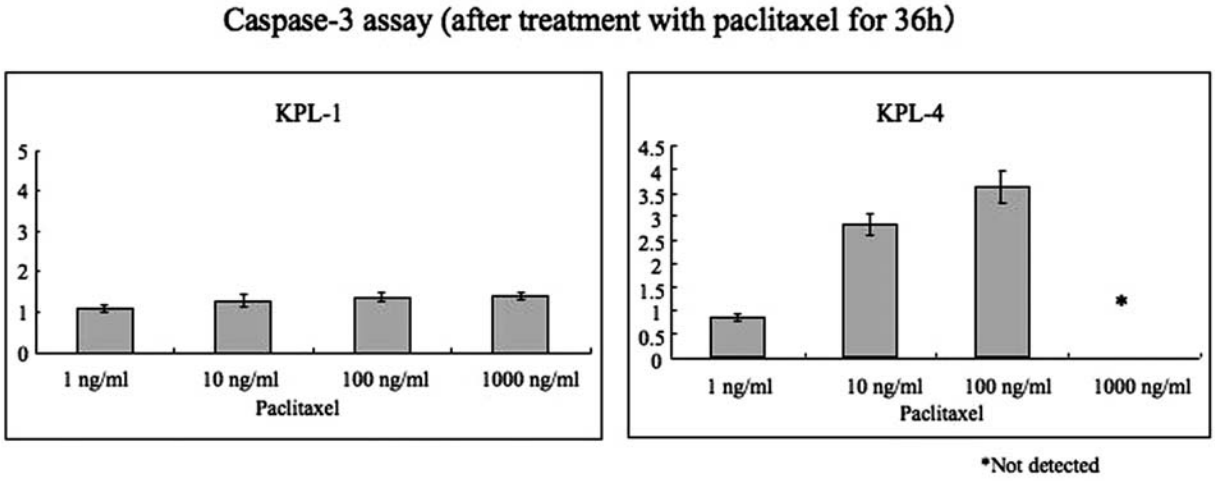


Figure 2. Caspase-3 assay after treatment with paclitaxel. A dose-dependent increase in the activity of caspase-3 in KPL-4 cells was detected, while no such effect was shown in KPL-1 cells.

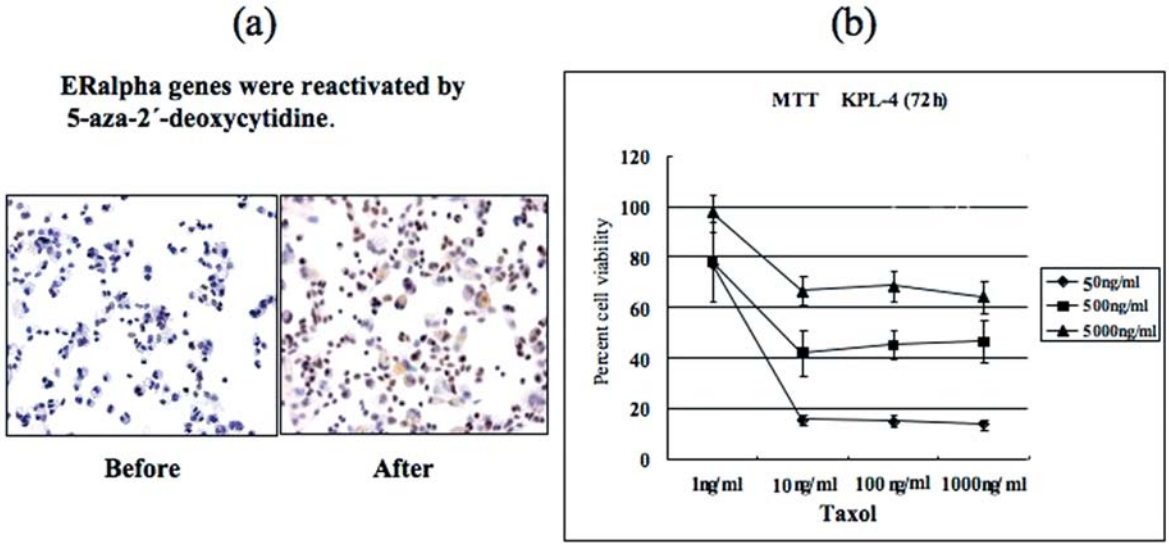


Figure 3. Induction of ER expression and its effect to the paclitaxel chemosensitivity by 5-aza-2'-deoxycytidine in breast cancer cell lines (a) and cell proliferation by MTT assay (b). Immunostaining of KPL-4 cells demonstrated the restoration of ER expression after treatment with 5-aza-2'-deoxycytidine (a). The paclitaxel-sensitive KPL-4 cells became resistant in a dose-dependent manner when treated with 5-aza-2'-deoxycytidine (b).

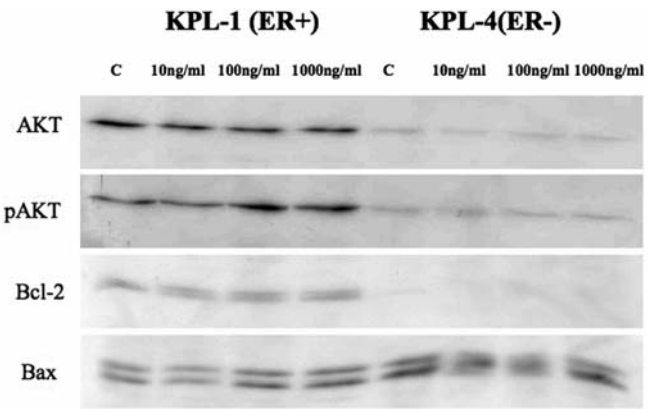


Figure 4. Protein expression of proapoptotic and antiapoptotic genes in ER-positive and -negative cells. Although proapoptotic molecule Bax expression was detected both in ER-positive and -negative cells, the expression of antiapoptotic molecules Bcl-2, Akt and phospho-Akt (pAkt) were revealed at much higher level in ER(+) KPL-1 cells as compared to ER(-) KPL-4 cells.

KPL-4) treated with various doses of paclitaxel. Although proapoptotic molecule Bax expression was detected both in ER-positive and -negative cells, the expression of anti-apoptotic molecules Bcl-2, Akt and phospho-Akt (pAkt) were revealed at much higher level in ER(+) KPL-1 cells as compared to ER(-) KPL-4 cells (Fig. 4). This pattern of the gene expression in the cells treated with paclitaxel suggested that the Bcl-2 might have a role in the resistance of ER(+) cells to paclitaxel.

Effects of estradiol (E2) on expression of Bcl-2, Bax and Bid mRNA. To determine the effects of estradiol (E2) on the expression of Bcl-2, Bax and Bid mRNA in ER(+) (ZR-75-1, T47D, MCF7 and KPL-1) and ER(-) (KPL-4, ZR-75-30) cell lines, we performed RT-PCR in the conditions induced by E2. E2 (100 nM) induced the Bcl-2 mRNA levels in ER-positive cell lines in a time-dependent manner, while there was no detectable expression of Bcl-2 in ER(-) KPL-4 cells. On the other hand, there was no significant difference

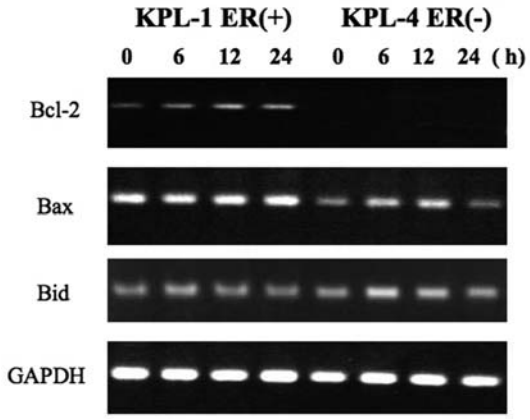


Figure 5. Effects of estradiol (E2) on the expression of Bcl-2, Bax and Bid mRNA in ER(+) and ER(-) cell lines. E2 induced Bcl-2 expression in ER-positive cell lines in a time-dependent manner, while there was no detectable Bcl-2 in ER(-) KPL-4 cells. On the other hand, no significant difference was detected for Bax and Bid mRNA levels before or after E2 treatment either in ER(+) or ER(-) cells.

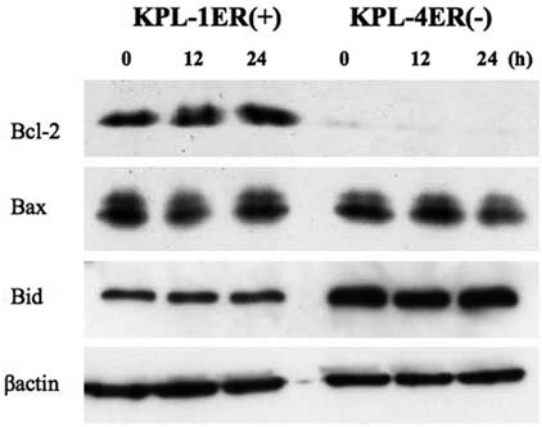


Figure 6. Effects of estradiol (E2) on the expression of Bcl-2, Bax and Bid proteins in the ER-positive and -negative cell lines. An increase in Bcl-2 protein was detected in ER(+) KPL-1 cells, whereas no Bcl-2 expression was detected in ER(-) KPL-4 cells. On the other hand, no significant difference in Bax and Bid protein expression was detected either in ER(+) KPL-1 or and ER(-) KPL-4 cells.

in Bax and Bid mRNA levels before or after E2 treatment either in ER(+) or ER(-) cells (Fig. 5).

Effects of estradiol (E2) on expression of Bcl-2, Bax and Bid proteins. To determine the effects of estradiol (E2) (100 nM) on the expression of Bcl-2, Bax and Bid protein in the ER-positive and -negative cell lines, Western blotting was performed. 0, 12 and 24 h after treatment with E2, an increase in Bcl-2 protein was detected in ER(+) KPL-1 cells, whereas no Bcl-2 expression was detected in ER(-) KPL-4 cells similar to the mRNA expression (Fig. 6). On the other hand, no significant difference in Bax or Bid protein expression was detected either in ER(+) KPL-1 or ER(-) KPL-4 cells similar to mRNA expression pattern.

Expression of Bcl-2 after treatment with 5-aza-2'-deoxycytidine in ER(-) KPL-4 cells. When ER(-) KPL-4 cells were treated with 5-aza-2'-deoxycytidine for 96 h and then induced by estradiol, a restoration of Bcl-2 expression was detected (Fig. 7). These data suggested a link between ER and anti-apoptotic molecule Bcl-2 expression, which may explain the resistance to paclitaxel in ER(+) cells.

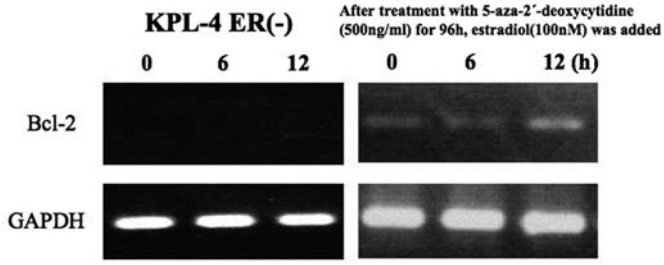


Figure 7. Expression of Bcl-2 after treatment with 5-aza-2'-deoxycytidine in ER(-) KPL-4 cells. When ER(-) KPL-4 cells were treated with 5-aza-2'-deoxycytidine for 96 h and then induced by estradiol, a restoration of Bcl-2 expression was detected.

ER expression in parent MDA-MB-231 and its ER-stably transfected cell lines. We examined ER expression in MDA-MB-231 and its wild-type and mutant ER stably transfected cells (S30 and JM6, respectively). Immunohistochemistry showed no expression of ER in MDA-MB231 cells, while its stable transfectants, S30 and JM6, demonstrated ER protein (Fig. 8a). The cell lines were furthermore subjected to paclitaxel treatment at various doses and the chemosensitivity was measured. While the parent ER(-) MDA-MB-231 cells were sensitive to paclitaxel treatment, its ER stable transfectant S30 and JM6 cells revealed resistance to the drug at all doses (Fig. 8b).

To identify the protein expression patterns of MDA-MB-231 and its stable transfectants, PowerBlot microarray system was used. This system can detect the relative levels of 40 different proteins in two groups of cell lines. The Power Blot analysis revealed a clear-cut difference in the expression of some proteins. The proapoptotic protein Bid was decreased in ER stable transfectants from 4- to 28-fold as compared to the ER(-) parent cells, whereas Cox-2 and NF-κB p65 proteins were enhanced from 2 to 10 times as compared to the control parent cells (data not shown). Increase or decrease in the protein expression of some other molecules such as PI3-kinase (4-fold decrease) and EGFR (3-fold increase) in JM6 cells as compared to MDA-MB-231 cells, suggesting that some signaling pathways in wild-type and mutant ER stable cells might be different (data not shown).

In light of the data obtained from PowerBlot analysis, we examined the Bid protein expression by Western blotting. Western blot analysis showed a decrease of Bid in S30 cells and almost total loss in JM6 cells, suggesting that the paclitaxel sensitivity in MDA-MB-231 cells, but resistance in its ER-stable cells, could be due to regulation of Bcl-2 family proteins, anti-apoptotic molecules, Bcl-2 as well as Bcl-x, and pro-apoptotic molecules, Bax and Bid (Fig. 9).

Discussion

ER is a member of the nuclear receptor superfamily of transcription factors. ER activation of gene expression is dependent on ligand, cellular, and gene promoter context and

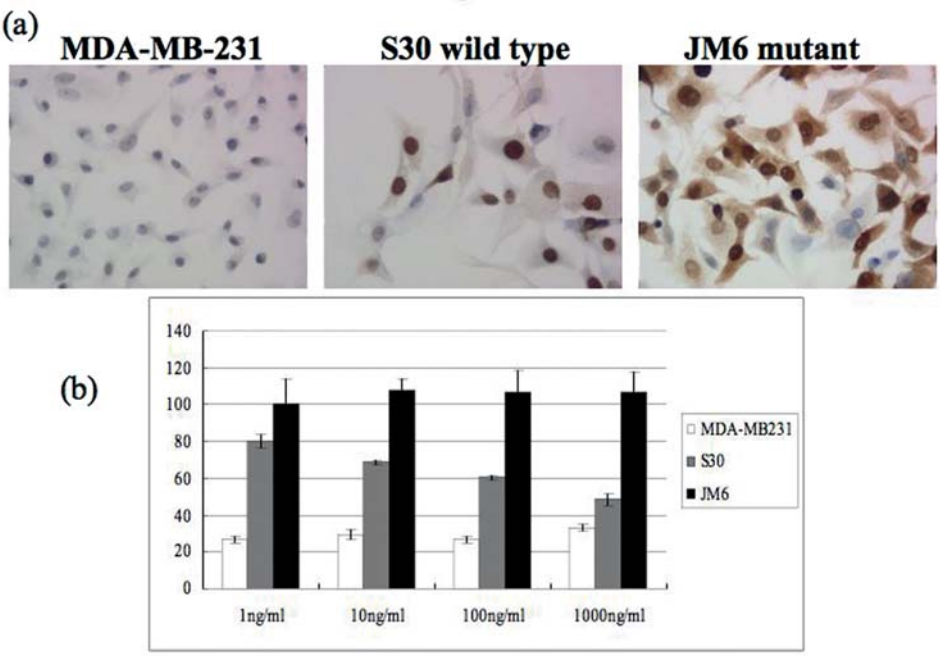


Figure 8. ER expression in parent MDA-MB-231 and its ER-stably transfected S30 and JM6 cell lines. Immunohistochemistry showed no expression of ER in MDA-MB231 cells, while its stable transfectants, S30 and JM6, demonstrated ER protein (a). MTT assay after paclitaxel treatment in various doses showed chemosensitivity of the parent ER(-) MDA-MB-231 cells but resistance of its ER stable transfectants S30 and JM6 cells in all doses (b).

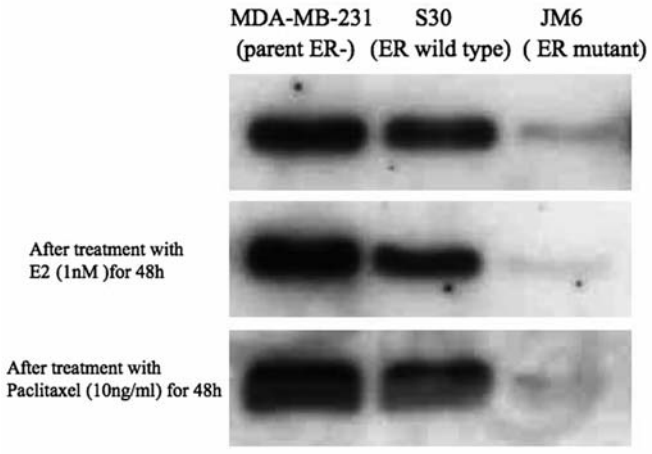


Figure 9. Bid protein expression by Western blotting based on the data obtained from PowerBlot analysis. The analysis showed a decrease of Bid in S30 cells and almost total loss in JM6 cells, suggesting that paclitaxel sensitivity in MDA-MB-231 cells but resistance in its stable transfectants.

receptor isoform. ER is a critical growth-regulatory gene in breast cancer, and its expression status is tightly linked to the prognosis and treatment outcome of breast cancer patients.

Paclitaxel is a drug used in treatment of cancer. The cytotoxicity of paclitaxel is linked to both inhibition of cell proliferation and apoptosis. Our results indicated that ER-negative breast cancer cell lines were more sensitive to paclitaxel than ER-positive breast cancer cell lines. The molecular consequences of paclitaxel exposure and the resultant induction of DNA fragmentation and apoptotic cell death have been documented in breast and ovarian cancers (1,10,11). We have demonstrated that paclitaxel, a chemotherapeutic agent that is widely used in the clinical treatment of breast cancer, induced apoptosis in KPL-4 breast cancer cells after 36 h of treatment and detected dose-dependent increased activity of caspase-3. Paclitaxel has been shown to trigger cell death primarily via caspase-independent routes in the non-small cell lung cancer cell line (12). Okano and Rustgi (13) recently reported that physiologically relevant concentrations of paclitaxel caused cell death via both caspase-dependent and -independent pathways in human esophageal squamous cancer cells.

The expression of anti-apoptotic molecules Bcl-2, Akt and phospho-Akt (pAkt) were revealed at much higher level and the expression of proapoptotic molecule Bid was revealed at lower level in ER(+) KPL-1 cells as compared to ER(-) KPL-4 cells. A direct interaction between tubulin and several pro-apoptotic and anti-apoptotic members of the Bcl-2 family has been demonstrated by effects on the assembly of microtubules from pure rat brain tubulin (14).

Estrogens stimulate growth of hormone-responsive breast cancer cells through complex and still incompletely characterized mechanisms. We have demonstrated that estradiol increased Bcl-2 mRNA level and proteins in ER-positive cell lines. However, no significant changes in Bax mRNA level and proteins in breast cancer cell lines. These results are in agreement with a previous report where estrogen up-regulated the anti-apoptotic Bcl-2 gene, whereas Bax level was not affected by E2 in breast cancer cell line (4,5,15-17).

In breast cancer cell lines or tumor specimens, altered expression of host genes has been associated with aberrant DNA methylation, including BRCA1, RARß, 14-3-3, Wilms tumor 1, p16, uPA, estrogen receptor, E-cadherin, maspin, and others (8,18-20). DNAs from human ER-negative breast cancer cell lines have been shown to be highly methylated

at the CpG Island of ER gene as determined by Southern blot analysis (21). Upon treatment with the demethylating agent 5-aza-2'-deoxycytidine, the ER gene CpG Island was demethylated resulting in re-expression of a functional ER (22). We showed that 5-aza-2'-deoxycytidine induced ER gene expression in ER-negative breast cancer cell lines, KPL-4 and ZR-75-30.

After ER gene expression in KPL-4 was activated by 5-aza-2'-deoxycytidine, E2 induced time-dependent up-regulation of Bcl-2 mRNA. In breast carcinomas, Bcl-2 has been shown to associate with ER status (23). Thus, Bcl-2 may be regulated through the interaction of estrogen with ER.

In conclusion, our results demonstrate that ER expression may influence chemosensitivity to paclitaxel through anti-apoptotic gene regulation. Regulation of the pathway may be crucial to increase the efficacy of taxanes in ER-positive breast cancer.

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