Establishment and characterization of a triple negative basal-like breast cancer cell line with multi-drug resistance

SERGEI BOICHUK, AIGUL GALEMBIKOVA, ALEXANDR SITENKOV, RAMIL KHUSNUTDINOV,
PAVEL DUNAEV, ELENA VALEEVA and NATALIA USOLOVA

Department of Pathology, Kazan State Medical University, Kazan 420012, Russia

Abstract. Triple-negative breast carcinoma (TNBC) is one of the most aggressive subtypes of breast cancer and is associated with an unfavorable prognosis. The management of TNBC is currently based on the use of classical cytotoxic drugs, i.e., anthracyclines and/or microtubule-binding agents (TBAs). However, conventional chemotherapy is not always effective in these tumors and a systemic relapse is often observed, potentially due to the development of multi-drug resistance (MDR). Therefore, an improved understanding of MDR mechanisms may improve the therapeutic strategies for TNBC. In the present study, a paclitaxel-resistant (TxR) breast cancer cell subline of HCC1806 TNBC cells was established and characterized. The resistance index of this subline was calculated according to the IC_{50} of HCC1806-TxR relative to the parental HCC1806 cells (16.86-fold). TxR-cells also exhibited cross-resistance to vinblastin, doxorubicin and etoposide (~14-, ~4- and ~3-fold, respectively). As assessed with reverse transcription-quantitative polymerase chain reaction, TxR-resistant cells exhibited the upregulated expression of a number of multidrug resistance-associated genes, including MDR-1, MRP-1, -5, -6 and YB-1. The TxR cells also exhibited an increased expression of MDR-related proteins including MDR1 and MRP-1, which led to a substantial increase (5.4-fold) of the paclitaxel efflux from TxR-cells. In addition, the pro-apoptotic protein Fas was downregulated, whereas the anti-apoptotic Bcl-2 was upregulated, in TxR-cells. This may explain why a reduced extent of apoptosis was observed when TxR cells were exposed to TBAs and topoisomerase type II inhibitors, relative to the parental HCC1806 cells. Thus, the HCC1806-TxR cell line may serve as an appropriate model for the analysis of chemoresistance mechanisms in TNBCs, and for the investigation of novel anticancer agents for overcoming MDR-mediated mechanisms in TNBC.

Introduction

A total of 12-25% of all cases of breast cancer are characterized by the lack of expression of the estrogen receptor and progesterone receptor in addition to the absence of the overexpression of human epidermal growth factor receptor-2 (HER-2), and are therefore classified as triple-negative breast cancer (TNBC) (1). TNBC is amongst the most aggressive subtypes of breast cancer, which is unresponsive to hormonal therapy and HER-2-targeted antibody therapy (2). Thus, TNBC is characterized by poor overall survival time, predominantly due to the unavailability of strategies for specific therapy. Cytotoxic chemotherapy is currently the main therapeutic option for TNBC (2), including anthracyclines (for example doxorubicin) and/or microtubule-binding agents (TBAs; such as taxans). The molecular mechanism of action for TBAs is associated with cell cycle arrest, predominantly at G2/M; apoptosis is induced as a consequence of mitotic exit failure or 'mitotic catastrophe' (3-5). A number of TBAs are in broad use in the clinical treatment of TNBC. Although certain patients initially respond to these classical cytotoxic drugs, patients exhibit high rates of systemic relapse during early stages and decreased overall survival times following metastasis (6,7). This may be due to drug resistance development arising from the overexpression of drug-efflux pumps, including the multi-drug resistance (MDR)-associated transporter P-glycoprotein (MDR-1) or proteins from the MDR-associated protein (MRP) family, collectively members of the ATP-binding cassette (ABC) transporter superfamily (8,9).

Furthermore, non-target-specific chemotherapy is toxic, and the use of anthracyclines and TBAs is limited by multiple side-effects, including cardiotoxicity, myelo- and immuno-suppression, neutropenia, mucositis and fluid retention; the drugs also exhibit low bioavailability. Thus, there is an urgent requirement for the development of novel, safe, and effective therapeutic options for TNBC. To achieve this goal, appropriate models for the analysis of the mechanisms to bypass MDR in TNBC are required.

In the present study, a paclitaxel-resistant (TxR) HCC1806-derived breast cancer cell subline that harbored a MDR phenotype, with cross-resistance to anthracyclines and TBAs, was

Correspondence to: Professor Sergei Boichuk, Department of Pathology, Kazan State Medical University, 49 Butlerov Street, Kazan 420012, Russia
E-mail: boichuksergei@mail.ru

established and characterized. This HCC1806-TxR TNBC subline may serve as an appropriate model for analyzing the chemoresistance mechanisms of TNBC, and to allow the study of potential novel methods for bypassing MDR.

Materials and methods

Chemical compounds. Doxorubicin, paclitaxel, vinblastine and cisplatin were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany); etoposide was obtained from Calbiochem (EMD Millipore, Billerica, MA, USA).

Antibodies. The primary antibodies used for western blotting and flow cytometry were as follows: anti-ABC subfamily G member 2 (ABCG-2; cat. no. 42078P; Cell Signaling Technology Inc., Danvers, MA, USA), Alexa-488-conjugated anti-phosphorylated histone subunit H3 S10 (pH3 S10; cat. no. 9708S; Cell Signaling Technology Inc., Danvers, MA, USA), cleaved caspase-3 (cat. no. 9661S; Cell Signaling Technology, Inc.), anti-PARP (cat. no. 436400; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), MDR-1 (cat. no. sc-55510) MRP-1 (cat. no. sc-18835) and β-actin (cat. no. sc-8432; Santa Cruz Biotechnology, Dallas, TX, USA); HRP-conjugated secondary antibodies [anti-mouse immunoglobulin (Ig)G, cat. no. sc-2005; anti-rabbit IgG, cat. no. sc-2004] for western blotting were purchased from Santa Cruz Biotechnology, Inc.

Cell lines and culture conditions. MES-SA/Dx5 Cell Lysate was obtained from Santa Cruz Biotechnology (cat. no. sc-2284). The human basal-like TNBC cell line, HCC1806, was purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 medium supplemented (Paneco, Moscow, Russia) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. To establish a TxR TNBC subline, HCC1806 cells were initially incubated in 0.5 nM paclitaxel for 4 weeks. When stable growth was achieved, the paclitaxel concentration was doubled every 4-5 weeks over 8 months to a maximum dose of 100 μM. Prior to experimental use, TxR-cells were maintained in paclitaxel-free culture medium as previously described, and passaged at least 3 times. All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in an incubator (Lamsystems, Miass, Russia) throughout the present study.

Intracellular drug concentration. HCC1806 and HCC1806-TxR cells were exposed to 100 nM paclitaxel for 2 h. Cells were rinsed twice with ice-cold PBS, harvested into centrifuge tubes, centrifuged at 250 x g for 5 min at room temperature and collected for solid-phase extraction high performance liquid chromatography (HPLC; LC-20 Prominence; Shimadzu Corporation, Kyoto, Japan). The detection wavelength was 227 nm and the limit of detection for paclitaxel was 5.0 ng. Experimental conditions were as follows: mobile phase; water and acetonitrile in a ratio of 40:60; Ascentis® C18 column, 4.6x250 mm; guard cartridge, Ascentis® C18 Supelguard with 5 μm particle size, 20x4 mm; isocratic mode (flow rate, 1 ml/min; temperature, 40°C;

pressure, 35 bar; Sigma-Aldrich). Paclitaxel (Sigma-Aldrich) was used as the internal standard. The method of absolute calibration was used for the quantitation of paclitaxel. The intracellular concentration of paclitaxel was defined as the total amount of paclitaxel/10^6 cultured cells.

Cellular survival assay. Exponentially growing cells were seeded ($3.2x10^4$ cells/well) into the 96-well flat-bottomed plates (Corning Incorporated, Corning, NY, USA) and incubated for 24 h at 37°C with RPMI-1640 culture medium. The cells were then cultured for 24 or 48 h at a range of concentrations of chemotherapeutic agents, including paclitaxel (1 nM-10 μM), doxorubicin (0, 03125-8 μg/ml), vinblastine (1 nM-10 μM), cisplatin (0, 03125-80 μM) or etoposide (2, 5-640 μM). All the aforementioned agents were previously diluted in dimethyl sulfoxide (DMSO). DMSO was used as a negative control in untreated cells. MTS reagent (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay; Promega Corporation, Madison, WI, USA) was added at 2 mg/ml to assess the relative number of viable cells. The MTS and cells were incubated for ≥1 h and the production of formazan, dissolved using DMSO, was assessed by the relative absorbance at 492 nm on a MultiScan FC plate reader (Thermo Fisher Scientific, Inc.). The resulting IC_{50} values were defined as the compound concentration required to inhibit cellular growth by 50% after 48 h of post treatment. The data was normalized to the DMSO control group.

Real-time monitoring of cell proliferation using an i-CELLigence system. An *in vitro* growth curve characterization of HCC1806 parental and TxR cells cultured in the presence of paclitaxel was performed using an iCELLigence system (ACEA Biosciences, Inc., San Diego, CA, USA). Cells ($5x10^4$) were seeded in electronic microtiter plates (E-Plate; Roche Diagnostics, Basel, Switzerland) and incubated for 24 h to obtain the growth baseline reading. Then cells were treated with 10, 50 or 100 nM paclitaxel in triplicate, and cell index (CI) measurements were obtained, with a signal detected every 30 min until the end of the experiment (72 h). Normalized CI values were calculated with RTCA iCelligence software version 1.1.1 (Roche Diagnostics).

Western blot analysis. For western blot analysis, whole-cell extracts were prepared by scraping HCC1806 parental and TxR cells, or MES-SA control cells, into radioimmuno-precipitation buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, supplemented with protease and phosphatase inhibitors). The cellular lysates were incubated for 1 h at 4°C and then clarified by centrifugation for 30 min at 14,000 x g at 4°C. Protein concentrations were measured by the Bradford assay. Samples containing 30 μg of protein were resolved on 4-12% Bis-Tris or 3-8% Tris-acetate NuPAGE gels (Invitrogen; Thermo Fisher Scientific, Inc.), transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA), probed with primary (1:1,000 and incubated overnight at 4°C), and secondary antibodies (1:1,000 and incubated for 1 h at room temperature) and visualized with enhanced chemiluminescence (Western Lightning Plus-ECL reagent, PerkinElmer, Inc., Waltham, MA, USA). The MES-SA cells acted as a positive control for ABC protein expression.

Table I. Primers for quantitative polymerase chain reaction.

Gene	Forward	Reverse
GAPDH	GACCACAGTCCATGCCATCA	TCCACCACCCTGTTGCTGTA
P-glycoprotein	ATGCTCTGGCCTTCTGGATGGGA	ATGGCGATCCTCTGCTTCTGCCCAC
MRP-1	GCATGA TCCCTGAAGACGA	TAGAGCTGGCCCTTGTACTC
MRP-2	TAGAGCTGGCCCTTGTACTC	TCAACTTCCCAGACATCCTC
MRP-3	CGCCTGTTTTTCTGGTGGTT	TCCCCCAGTCACAAAGATG
MRP-4	GCTGAGAATGACGCACAGAA	TCCCAGCAAGGCACGATATT
MRP-5	GTCCTGGGTATAGAAGTGTG	CAGAAGATCCACACAACCCT
MRP-6	TTGGATTCGCCCTCATAGTC	TCTTTTGGTCTCAGTGGCCT
MRP-7	CTCCCACTGGATCTCTCAGC	TCGCATACACGGTGAGGTAG
Bcl-2	ATGTCCAGCCAGCTGCACCTGAC	GCAGAGTCTTCAGAGACAGCCAGG
Bcl-2 associated X	GCTTCAGGGTTTCATCCAGG	AAAGTAGGAGAGGAGGCCGT
Fas	CAGGCTAACCCCACTCTATG	TGGGGGTGCATTAGGCCATT
Caspase-8	ACTTCAGACACCAGGCAGGGCT	GCCCCTGCATCCAAGTGTGTTC
Y-box binding protein 1	GACTGCCATAGAGAATAACCCCAG	CTCTCTAGGCTGTTTTGGGCGAGGA

All primers are presented in the 5'-3' direction. MRP, multidrug resistance-associated protein.

Cell cycle analysis. For flow cytometry cell cycle analysis, the cells were treated with paclitaxel at 10 or 1,000 nM for 24-48 h and trypsinized. Subsequent to centrifuging at 300 x g for 5 min at room temperature, the cells were washed in PBS, fixed in 4% paraformaldehyde and permeabilized with ice-cold 90% methanol. The washed cells were stained with Alexa-488-conjugated anti-pH3 S10 (1:500 and incubated for 1 h at room temperature in the dark), were then counterstained with propidium iodide (30 min at room temperature in the dark) (Sigma-Aldrich) and analyzed by fluorescence-activated cell sorting on a FC500 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). Cells were counted and analyzed using the Kaluza software version 1.3 (Beckman Coulter, Inc.).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). At 24 h after plating $1x10^6$ HCC1806 or HCC1806-TxR cells, total RNA was extracted using TRIzol reagent (cat. no. BC032; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and resuspended in diethyl pyrocarbonate-treated H_2O. RNA was reverse transcribed to cDNA using the Moloney murine leukemia virus reverse transcriptase kit (Evrogen JSC, Moscow, Russia) according to the manufacturer's protocol (cat. no. SK021), and subjected to qPCR. A total of 1 μl template cDNA was used in the qPCR reaction, with 5X qPCRmix-HS SYBR (Evrogen JSC) and 10 mM of each forward and reverse primer (Table I). qPCR was performed with the CFX96 Real-Time detection system (Bio-Rad Laboratories, Inc.), according to the manufacturer's protocol. Thermal cycling conditions were as follows: 3 min at 95℃, 45 cycles (15 sec at 95℃, 10 sec at 56℃, 30 sec at 72℃) and a final extension step of 5 min at 72℃. Each sample was processed in parallel with assays for GAPDH and the absolute levels of each mRNA were normalized relative to GAPDH. The $2^{-\Delta\Delta Cq}$ method (10) was then used to calculate relative gene expression.

Statistical analysis. All experiments were repeated a minimum of 3 times. The results are presented as the mean ± standard deviation for each group. Statistical analyses (Student's t-test, Mann-Whitney U test) were performed using Statistical software program version 7.0 (S.A. Glantz, McGraw Hill Education, NY, USA) and GraphPad Prism version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Establishment of taxol-resistant HCC1806 breast cancer cell line. The HCC1806-TxR cell line was established following a continuous treatment with a gradually increasing concentration of paclitaxel, from 0.5-100 nM. Approximately 8 months and 55 passages were required to develop HCC1806 cells with stable drug resistance. It was then demonstrated that HCC1806-TxR cells were less sensitive to paclitaxel treatment when compared with parental HCC1806 cells; the paclitaxel drug resistance index was 16.86 (Table II). In addition to resistance to paclitaxel, HCC1806-TxR cells exhibited cross-resistance to vinblastine, doxorubicin and etoposide (drug resistance index, 14.65, 4.36 and 3.18, respectively), whereas there was no identified cross-resistance to cisplatin. Paclitaxel resistance in HCC1806-TxR cells remained stable following 1 month of culturing without paclitaxel and following storage at -80℃ for 6 months (data not shown).

Alterations in cellular morphology and growth kinetics. The morphology of the TxR cells was distinct from the parental cells. HCC1806-TxR cells exhibited an enlarged and oval-shaped morphology, and an increased nucleus/cytoplasm ratio when compared with the parental cells (Fig. 1A). The resistant cells were consistent in size and shape in monolayer proliferation, and their growth kinetics were different from those of the parental cells (Fig. 1B). When cells were cultured

Table II. IC$_{50}$ values for HCC1806 and HCC1806-TxR cells.

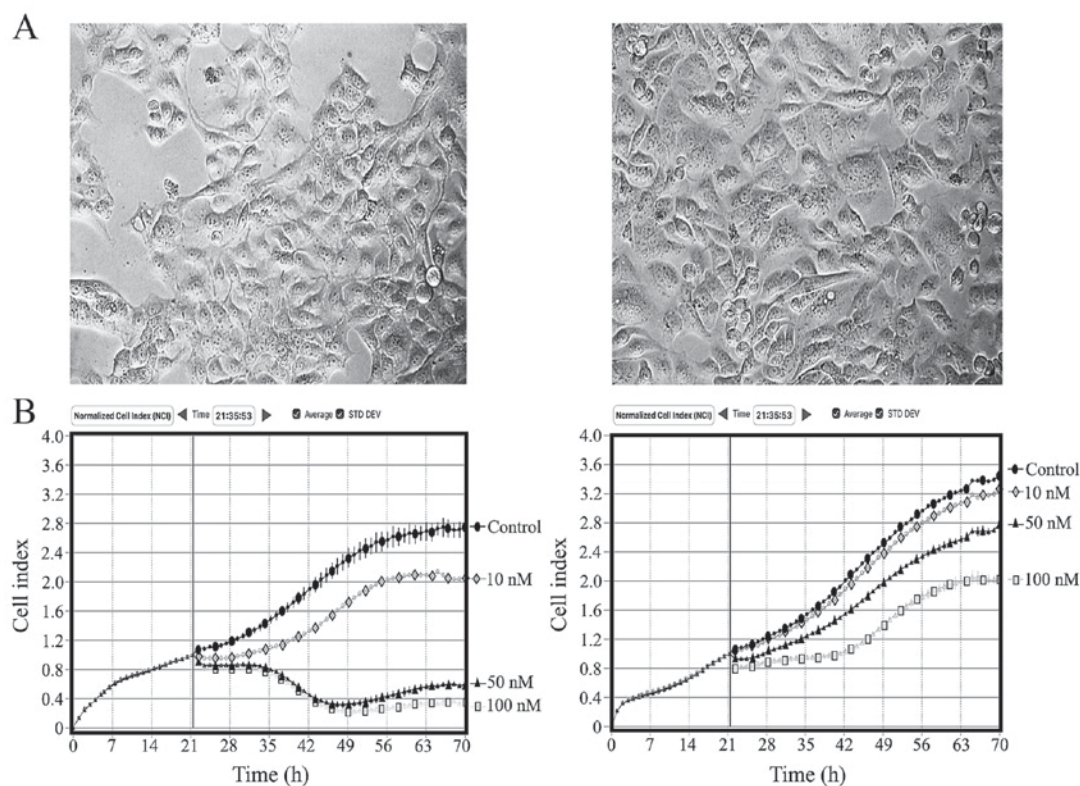| Drug	HCC1806 (%)	HCC1806-TxR (%)	Fold change
Paclitaxel, nM	4.68±0.44 (9.39)	78.9±3.8 (4.8)	16.86
Vinblastine, nM	0.2±0.04 (20)	2.93±0.32 (10.92)	14.65
Doxorubicin, μg/ml	0.53±0.03 (5.6)	2.31±0.23 (9.9)	4.36
Etoposide, mM	72.94±9.64 (10.6)	231.91±15.73 (3.1)	3.18
Cisplatin, μM	10.27±1.09 (13.2)	10.19±0.32 (6.8)	1.01

TxR, taxol resistant.

Figure 1. Differences in morphology and growth between HCC1806 and HCC1806-TxR cells. (A) The establishment of a drug-resistant phenotype in HCC1806 cells was associated with alterations to cell morphology. HCC1806-TxR cells (left) exhibited an increasingly oval shape, an enlarged size and a increased nucleus/cytoplasm ratio compared with the parental HCC1806 cells (right). (B) Growth kinetics of HCC1806 and HCC1806-TxR cells treated with a range of paclitaxel concentrations. The normal growth of the TxR subline was similar to the HCC1806 parental line; however, when treated with paclitaxel, the relative growth was greater in the TxR cells. TxR, taxol resistant.

with paclitaxel, a difference in growth kinetics between the TxR and parental HCC1806 cells was also detected (Fig. 1B), which was consistent with the MTS-based cytotoxicity data (Table II).

Paclitaxel induced cell accumulation in M-phase and apoptosis in parental, but not TxR, HCC1806 cells. As the major mechanism for the action of paclitaxel is the inhibition of tubulin depolymerization to induce apoptosis due to 'mitotic catastrophe', the ability of paclitaxel to induce the accumulation of cells in M-phase in parental vs. paclitaxel-resistant HCC1806 cells was compared. pH3 S10 staining was combined with propidium iodide DNA staining to count the number of mitotic cells by flow cytometry. An increase of the pH3 S10-positive (i.e., mitotic) HCC1806 cells was observed subsequent to 10 or

1,000 nM paclitaxel exposure (Fig. 2). In contrast, the number of mitotic paclitaxel-treated HCC1806-TxR cells was significantly less when compared with parental cells (P<0.001 for 10 and 1,000 nM; Fig. 2).

To assess the ability of paclitaxel to induce apoptosis in parental vs. TxR HCC1806 cells, the expression of cleaved caspase-3 and PARP, specific markers for apoptosis, was observed. An increase in the cleavage of PARP and caspase-3 was detected in paclitaxel-treated HCC1806 cells, reflecting the ability of paclitaxel to induce apoptosis in HCC1806 parental cells (Fig. 3A). In contrast, relatively low cleavage of PARP and caspase-3 were observed in paclitaxel-treated HCC1806-TxR cells (Fig. 3B), thereby confirming the cytotoxicity MTS-based data by indicating the resistance of the HCC1806-TxR subline to paclitaxel.
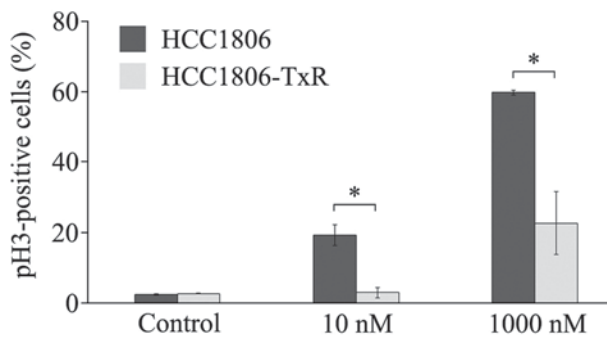
Figure 2. Relative M-phase distribution of HCC1806 and HCC1806-TxR cells following exposure to PTX. The number of mitotic cells following PTX exposure for 48 h, as determined with pH3 staining in flow cytometry. Values are means ± standard deviation (n=3). *P<0.001. TxR, taxol resistant; PTX, paclitaxel; pH3, phosphorylated histone subunit 3.
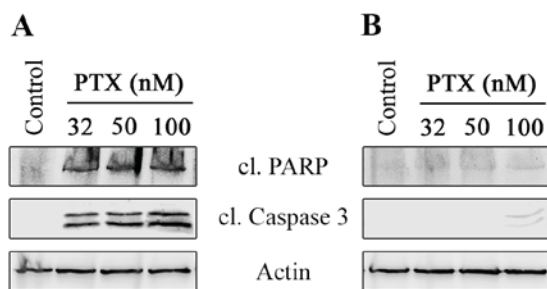

Figure 3. PTX induces the apoptosis of HCC1806 parental cells, and not HCC1806-TxR cells. Western blot analysis of (A) HCC1806 and (B) HCC1806-TxR cells for cleaved forms of PARP and caspase-3, markers of apoptotic cell death, following the treatment with dimethyl sulfoxide or PTX for 48 h. β-actin was used as a loading control. PTX, paclitaxel; TxR, taxol resistant; PARP, poly(ADP-ribose) polymerase; cl., cleaved.

The expression of chemoresistance-associated genes is altered in HCC1806-TxR cells. As the resistance of patient tumors to chemotherapeutic agents is highly dependent on the activities of the transporters that efflux drugs from tumor cells, the expression of the MDR-1, MRP-1 and ABCG2 ABC transporter proteins in HCC1806-TxR cells was assessed. Western blot analysis revealed the overexpression of the MDR-1 and MRP-1 proteins in HCC1806-TxR cells when compared with the parental HCC1806 cells (Fig. 4). However, the expression level of another protein associated with the MDR phenotype, ABCG2, was unchanged between the parental and TxR lines.

Since a potential mechanism for MDR-1 overexpression in paclitaxel-resistant cells is the induction by Y-box-binding protein 1 (YB-1), which binds to a cis-acting element of the MDR-1 promoter to increase MDR-1 mRNA expression (11), YB-1 mRNA levels were compared between parental HCC1806 cells and the TxR subline. There was an increase (3.6-fold) of YB-1 mRNA expression in TxR-cells when compared with the parental HCC1806 cells (Table III). Similarly, a number of MRP mRNAs were observed to be overexpressed in HCC1806-TxR cells when compared with the parental HCC1806 cells (Table III). In TxR cells, the levels of MRP-1, -5, and -6 were elevated to 2.3, 15.7 and 5.7-fold, respectively. As paclitaxel-induced cell death is associated with the activation of apoptosis mechanisms, the expression of the apoptosis-related genes, Bcl-2 associated X (Bax), Bcl-2,
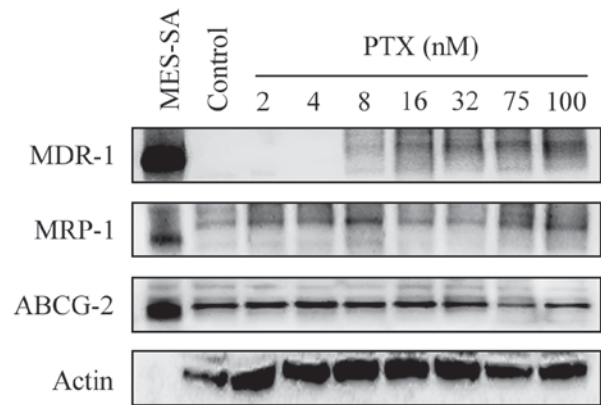

Figure 4. Expression of MDR-associated proteins in HCC1806-TxR cells. The expression of MDR-1 and MRP-1 increased in a dose-dependent manner in paclitaxel-treated HCC1806-TxR cells, whereas the expression of ABCG-2 remained unchanged. The lysate of MES-SA cells was used as a positive control for the expression of the MDR-associated proteins; β-actin was used as a loading control. MDR, multidrug resistance; TxR, taxol resistant; MDR-1, P-glycoprotein; MRP-1, multidrug resistance-associated protein-1; ABCG-2, ABC subfamily G member 2.

caspase-8 and Fas, were compared in parental vs. TxR cells. The expression of Bax and caspase-8 mRNA were unchanged in resistant cells, but a trend towards the downregulation of Fas was observed when compared with parental cells. In contrast, Bcl-2 expression was upregulated in the TxR subline (Table III).

Intracellular drug concentration. As cellular mechanisms for drug resistance include a decline of intracellular drug concentrations as a result of increased efflux by overexpressed ABC proteins, and considering the overexpression of MDR-1 and MRP-related genes in the TxR cells, the intracellular paclitaxel concentrations in HCC1806 cells were compared with HCC1806-TxR cells. As presented in Fig. 5, at 2 h of paclitaxel exposure, HCC1806-TxR-cells contained ~540 ng paclitaxel, which was much lower than in the parental HCC1806 cells (2,930 ng; P<0.05).

Discussion

In the present study, a paclitaxel-resistant basal-like TNBC cell line was established from the parental HCC1806 cell line. The paclitaxel IC$_{50}$ value for HCC1806-TxR cells was ~17-fold higher when compared with parental HCC1806 cells. Of note, the paclitaxel resistance remained stable after 1 month of culturing without paclitaxel and following storage at -80˚C for 6 months. HCC1806-TxR cells exhibited alterations to cellular morphology (for example enlarged, oval-shaped) when compared with parental cells. Additionally, increased growth was observed in HCC1806-TxR cells exposed to paclitaxel compared with the parental HCC1806 cells.

Paclitaxel is a commonly used chemotherapeutic drug for the treatment of patients with a range of types of cancer, including breast, ovarian and lung cancer (12-14). The molecular mechanism for the action of paclitaxel is its ability to bind microtubules and induce their stabilization, thus preventing their disassembly during mitosis (3,5). Therefore, in paclitaxel-treated cells, microtubules become locked in a

Table III. Alterations in the expression level of chemoresistance and apoptosis associated genes in HCC1806-TxR cells relative to HCC1806 cells, as determined by reverse transcription-quantitative polymerase chain reaction.

Gene	Fold change
P-glycoprotein	1.73
MRP-1	2.3
MRP-2	1.33
MRP-3	0.19
MRP-4	1.28
MRP-5	15.74
MRP-6	5.76
MRP-7	1.51
Bcl-2	1.65
Bcl-2 associated X	1.12
Fas	0.63
Caspase-8	0.98
Y-box binding protein 1	3.59

TxR, taxol resistant; MRP, multidrug resistance-associated protein.
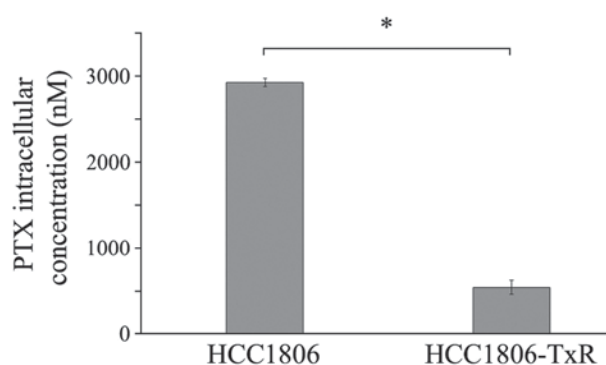

Figure 5. The intracellular PTX concentration following a 2 h exposure to 100 nM PTX. The concentration of PTX was measured by solid-phase extraction high performance liquid chromatography. PTX, paclitaxel. *P<0.001.

polymerized state, which leads to G2/M arrest, induces the accumulation of cells in M-phase and leads to cell death via apoptosis (15-18). Thus, a substantial decrease of mitotic and apoptotic cell amount in TxR-subline after paclitaxel exposure was expected. Indeed, a substantial decrease of pH3 S10-positive (i.e., mitotic) cells (Fig. 2) and a low level of PARP and caspase-3 cleavage of in paclitaxel-treated HCC1806-TxR cells was observed when compared with parental HCC1806 cells (Fig. 3B). This data was consistent with the cytotoxicity MTS-based data, indicating the resistance of the HCC1806-TxR subline to paclitaxel.

Additionally, HCC1806-TxR exhibited a cross-resistance to other types of chemotherapeutic agents, including vinblastine, and etoposide and doxorubicin topoisomerase type II inhibitors. The resistance indexes for the drugs indicated above were 14.65, 4.36 and 3.18, respectively. This data is consistent with previous study, indicating that cancer cells in culture becoming resistant to a single drug may become resistant to a class of drugs with a similar mechanism of action as well (19). In addition, cancer cells may acquire a cross-resistance to structurally and mechanistically unrelated drugs; this phenomenon is known as MDR. For example, McDonald *et al* previously demonstrated that docetaxel-resistant MDA-MB and MCF-7 breast cancer cells developed a cross-resistance to paclitaxel and vincristine (20), concordant to the findings of the present study. Multiple reports have indicated that resistant sublines commonly express MDR-1 and MRP proteins (9,21,22). Clinical studies also report the development of cross-resistance to multiple anticancer agents following the initial success of chemotherapy (23,24), which may also be due to the overexpression of MDR-1 and MRPs.

Thus, the molecular mechanism of cross-resistance of HCC1806-TxR to vinblastine and topoisomerase type II inhibitors may be due to the increased expression of ABC transporters, important mediators of drug efflux. An increased expression of MDR-1 and MRP-1 proteins in HCC1806-TxR cells was observed (Fig. 4), which may lead to the increased efflux of paclitaxel and other types of chemotherapeutic drugs (for example alkylating agents, topoisomerase type II inhibitors), resulting in MDR (Fig. 5).

In order to investigate the genes involved in paclitaxel resistance, the gene expression profile between HCC1806 and HCC1806-TxR cells was compared. MDR-1, MRP-1, -5 and -6 were upregulated in the HCC1806-TxR subline, suggesting that these factors served a function in the observed MDR phenotype. The increased activity of ABC transporters in the HCC1806-TxR subline was additionally confirmed by a marked (5.4-fold) decrease in the intracellular paclitaxel content in TxR-cells at 2 h of paclitaxel exposure when compared with parental HCC1806 cells (Fig. 5), thus indicating an increased efflux of chemotherapeutic drugs in the HCC1806-TxR subline. Furthermore, differences in expression of genes associated with apoptosis between the parental and TxR-cells, including Bcl-2 and Fas, were also detected. This data may also explain the low expression of apoptotic markers (cleaved PARP and caspase-3) in TxR cells subsequent to paclitaxel exposure when compared with parental HCC1806 cells (Fig. 3).

In conclusion, a HCC1806 subline was established with high resistance to paclitaxel, which also harbored a cross-resistance to diverse types of chemotherapeutic agents, including those with similar and different modes of action to paclitaxel. The MDR phenotype was associated with MDR-1, MRP-1, -5 and -6 overexpression, thus providing an effective efflux of anticancer drugs from HCC1806-TxR cancer cells. In addition, the TxR breast cancer subline also resisted apoptosis subsequent to the exposure to paclitaxel. This may be due to the downregulation of pro-apoptotic proteins (Fas) and the upregulation of anti-apoptotic proteins (Bcl-2). Paclitaxel resistance remained stable subsequent to 1 month of culturing without paclitaxel and following prolonged storage at -80˚C. Taken together, the established MDR in a HCC1806-derived breast cancer subline may be appropriate for *in vitro* screenings of novel anticancer agents for overcoming MDR-mediated mechanisms in TNBC.

Acknowledgements

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