Establishment of A Novel Paclitaxel–Resistant Triple–Negative Breast Cancer Cell Line

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Received 30 July 2022 ● Revised 26 October 2022 ● Accepted 26 October 2022 ● Published online 29 December 2022

Abstract:
Objective: Triple–negative breast cancer (TNBC) is one of the most aggressive subtypes of breast cancer, and is associated with poor prognosis. Paclitaxel, a microtubule–binding agent, and chemotherapeutic agent, has been widely used in the management of TNBC. However, acquired chemoresistance is one of the main obstacles leading to ineffective TNBC treatment. Therefore, this study aimed to establish and characterize paclitaxel–resistant triple–negative breast cancer cell lines for investigating the molecular mechanisms of drug resistance.

Material and Methods: Hs578T cancer cell lines were used to develop paclitaxel–resistant (Hs578T–PR) cells, via repeated exposure to increasing concentrations of paclitaxel in a stepwise manner. Drug response and growth curves were then measured by Sulforhodamine B assay. Changes in cell morphology were examined by microscopy. Furthermore, expression of mRNA, related to molecular mechanisms in drug–resistant cells, was identified by using real–time polymerase chain reaction (qPCR).

Results: The Hs578T–PR cell lines were successfully established in just 4 months. They were highly resistant to paclitaxel, with their IC50 and resistance index at 72.8±3.08 nM and 7.4–folds, respectively, compared to the parental cells. For identifying molecular mechanisms underlining paclitaxel resistance, using the RT–qPCR analysis, it was found that Hs578T–PR cells exhibited kinesin family member 3C overexpression. Furthermore, the pro–apoptotic protein BAX was down–regulated; whereas the anti–apoptotic Bcl–2 was up–regulated in Hs578T–PR cells.

Conclusion: Novel, highly paclitaxel–resistant (Hs578T–PR) cell lines established in this study could represent a useful model for identifying the molecular mechanisms of chemoresistance, and for evaluating the efficacy of novel anti–cancer drugs to overcome chemoresistance in TNBC.
Keywords: cell line, paclitaxel resistance, TNBC, triple-negative breast cancer

Introduction

Worldwide, breast cancer is the most commonly diagnosed type of cancer in women as well as the leading cause of cancer-related deaths. In term of diagnosis, breast cancer is often classified into four subtypes; which is dependant on the molecular expression of cell surface receptors: normal-like, luminal (estrogen receptor–positive), human epidermal growth factor receptor 2– (HER2–) enriched and basal-like. Tumors that lack expression of all three receptors are denoted as: triple–negative breast cancer (TNBC), which has a high incidence–to–mortality ratio (approximately 50%). Its prominent features are the lack of estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2). This in turn limits possible treatment options.

Material and Methods

Cell line and culture conditions

Human breast cancer cell line Hs578T was purchased from the American Type Culture Collection (ATCC). The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Himedia). The condition for cell culture was with 10% fetal bovine serum (FBS; Gibco), 1% Penicillin Streptomycin (Pen Strep; Gibco), and 1% GlutaMAX™-I (Gibco), at 37°C in a humidified atmosphere of 5% CO2 within an incubator.

Establishment of drug–resistant cell line

Drug–resistant cells were established from Hs578T cells by exposing cancer cells to increasing concentrations of paclitaxel (Fresenius Kabi Oncology Ltd., India) in a stepwise manner (ranging from IC10–IC50 concentrations). In each round of resistant selection, cancer cells were continuously exposed to the drug concentration for 7 days. The media was then changed and replaced with a drug–free media until cell recovery was at 100% confluency, or when having doubling time at the same rate as the parental
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The cells were seeded in 96-well culture plates, as 5,000 cells/well in 100 µL completed DMEM media. They were then incubated at 37°C overnight, before adding paclitaxel in varying concentrations. After being incubated for a further 3 days, the cells were washed with PBS, fixed with 10% trichloroacetic acid for 1 hr, and then washed again with PBS. After this, 1% SRB solution was added to the 50 µL/wells for staining. The samples were incubated for 30 min at room temperature and rinsed three times with 1% acetic acid afterward. The culture plates were then dried at room temperature overnight. The stained samples were further dissolved in 50 µL of 10 mM Tris–base solution, and the absorbance was measured at 510 nm. An inhibitory concentration of 50% (IC50) was determined by using Graphpad prism.

**Sulforhodamine B (SRB) assay**

The cells were seeded in 96-well culture plates, as 5,000 cells/well in 100 µL completed DMEM media. They were then incubated at 37°C overnight, before adding paclitaxel in varying concentrations. After being incubated for a further 3 days, the cells were washed with PBS, fixed with 10% trichloroacetic acid for 1 hr, and then washed again with PBS. After this, 1% SRB solution was added to the 50 µL/wells for staining. The samples were incubated for 30 min at room temperature and rinsed three times with 1% acetic acid afterward. The culture plates were then dried at room temperature overnight. The stained samples were further dissolved in 50 µL of 10 mM Tris–base solution, and the absorbance was measured at 510 nm. An inhibitory concentration of 50% (IC50) was determined by using Graphpad prism.

**Real–time Polymerase Chain Reaction (qPCR)**

The total RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Then, 1 µg of the total cellular RNA for each of the conditions was used to generate complementary DNA, using Superscript III reverse transcriptase and oligo–dT primers (Invitrogen, Paisley, UK). qPCR, was conducted using HOT FIREPol EvaGreen qPCR Mix Plus (no ROX). The amplification was performed on a CFX Connect real–time system (Bio-Rad, Hercules, CA, USA). The results were normalized using the L19 mRNA expression as a reference gene. All the qPCR experiments were repeated at least three times; as independent experiments, and the relative expressions were shown as average±S.D. (n=3). Statistical significance was determined by Student's t-test (significant, *p-value<0.05, **p-value<0.01, ***p-value<0.001). Primer sequences are described in Table 1.

**Determination of apoptotic index**

The apoptotic index was analyzed by DAPI (4′–6-diamidino–2-phenylindole) staining. Cells (2×10⁵ cells) were exposed to the paclitaxel 10 nM for 24 hr. The cells were then fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X–100 and incubated with DAPI. The apoptotic index was scored by counting the cells with condensed chromatin and fragmented under a Lionheart FX Automated Microscope.

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**Table 1** List of primer sequences used in this study

<table>
<thead>
<tr>
<th>Genes</th>
<th>5′-Forward-3’</th>
<th>5′-Reverse-3’</th>
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<tbody>
<tr>
<td>KIF3C</td>
<td>AACTCCAAAGCTCAGCTACCTACCT</td>
<td>CAGGAAACCTTGGAGGAATAG</td>
</tr>
<tr>
<td>BCL2</td>
<td>CTGGAGTGGTGGGGTGCA</td>
<td>GGGCGTACAGTCCAAA</td>
</tr>
<tr>
<td>BAX</td>
<td>ACCAAGAAGCTGAGGGTGCA</td>
<td>CGGTGAAAGTTGCGGTGCA</td>
</tr>
</tbody>
</table>

KIF3C=kinesin family member 3C, BAX=Bcl-2 associated x-protein, Bcl-2=B-cell lymphoma 2
Statistics
All experiments were repeated at least three times; as independent experiments, and the data are presented as average ± S.D. (n=3). Statistical significance was determined by Student’s t-test (significant, *p-value<0.05, **p-value<0.01, ***p-value<0.001).

Results
Establishment of the paclitaxel-resistant Hs578T cell line
Firstly, to develop a paclitaxel-resistant Hs578T cell line, intermittent exposure of parental Hs578T TNBC cells was used, by increasing concentrations of paclitaxel, utilizing a time-stepwise escalation and consisting of concentrations of IC10 to IC50 over 4 months. This was to generate an in vitro paclitaxel-resistant cell line. After 7 days of exposure to the given drug dosage, morphological alterations of the cells were found. Higher concentrations of paclitaxel led to an increasing, enlarged cell size compared to that of the parental cells: as illustrated in Figure 1A. As shown in Figure 1B, Hs578T-PR revealed a significant increase in diameter, approximately 158.61 mm; whereas, Hs578T was 142.78 mm. In addition, it was also found that Hs578T-PR cells exhibited slowed, slower growth compared to the parental Hs578T cells for 72.79 h and 82.01 hr, respectively: as shown in Figure 1C.

To determine the level of drug resistance, the surviving, resistant cells were compared to the parental sensitive cells using SRB assay. Briefly, Parental Hs578T and Hs578-PR cells were treated with varying paclitaxel concentrations (0, 0.2, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25.0, 50.0, and 100.0 nM) for 72 hours. The IC50 (the drug concentration causing 50% growth inhibition) and Resistance Index (RI index) were then calculated. As shown in Figure 2, the Hs578T-PR cell line is highly resistant to paclitaxel, with an IC50 of 72.8±3.08 nM. The paclitaxel resistance value of Hs578T-PR cells was 7.4-fold higher than that of the parent cells.

The expression of chemoresistance-associated genes was altered in the Hs578T-PR cells
In addition to the RI index, developed chemoresistant cells were then characterized to clearly understand the entire changes of cellular expression within the developed cell lines. This was achieved by identifying related mechanisms using specific paclitaxel-resistant molecules; such as kinesin family member 3C (KIF3C), B-cell lymphoma 2 (Bcl-2), and Bcl-2 associated x-protein (BAX) and RT-qPCR analysis was used to examine the results of this investigation. As shown in Figure 3; KIF3C, Bcl-2, and BAX expression was found in both Hs578T and Hs578T-PR cells. KIF3C/L19, Bcl-2/L19, and BAX/L19 ratios were then calculated. When compared to the Hs578T cells, the results showed that KIF3C mRNA expression had increased in the Hs578T-PR cells (Figure 3A). In terms of apoptosis-related genes, it was found that Hs578T-PR exhibited lower levels of pro-apoptosis BAX expression (Figure 3B). Conversely, Hs578T-PR cell line overexpressed anti-apoptosis Bcl-2 (Figure 3C). Consistent with apoptotic phenotype analysis using DAPI staining, the results showed that parental Hs578T cell line has more apoptosis cells than Hs578T-PR cell line after being treated with paclitaxel 10 nM for 24 hr: as shown in Figure 4.

Discussion
Chemotherapy resistance is the main cause of unsuccessful cancer treatment. To fully comprehend the mechanism of chemoresistance as well as to overcome chemo-resistance in TNBC, the most crucial step is to generate drug-resistant cancer cell lines. In this study, paclitaxel-resistant Hs578T cell lines were established. This was achieved by increasing the concentrations of
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Figure 1  Morphological Changes of an established Hs578T–PR cell line (A). Light micrographs of Hs578T cells after gradually being treated with increasing concentrations of paclitaxel; ranging from 0.5–10 nM. (B) Measurements of the cell sizes revealed a significant increase in diameter in the Hs578T–PR cells. (C) Doubling time of sensitive and resistant cell lines. Graphs represent the average of three independent experiments±S.D. (n=3). Statistical significance was determined by Student’s t-test (significant, *p-value<0.05).
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Figure 2 Drug response curve of Hs578T and HS578T-PR cells to paclitaxel. SRB assay was performed after treating the cells with varying concentrations of paclitaxel for 72 hr. The percentage of cell survival, as a function of drug concentrations, was plotted and IC50 were calculated. The graphs were generated from GraphPad Prism 8.

paclitaxel (IC10–IC50), via a time–stepwise escalation to acquired drug–resistance for 4 months. Our findings demonstrated that the established Hs578T-PR cells were able to demonstrate tolerance to paclitaxel 7.4 folds when compared with parental cells. To our knowledge, this is the first report on the establishment of Hs578T-paclitaxel resistance cell lines; with both a faster establishment time and higher resistance. This is compared to previous reports, in that paclitaxel–resistant MDA–MB–231 cells were established in 10 months, with a 3.8-fold RI index; whereas, 5-fluorouracil–resistant MDA–MB–231 cells were generated in 8 months, with a 5.5-fold RI index. Paclitaxel functions by binding to tubulin and inhibiting the disassembly of microtubules; thereby, resulting in the inhibition of cell division. KIF3C plays a significant role in the formation, maintenance, and remodeling of the bipolar mitotic spindle. Additionally, overexpression of KIF3C has been shown to be associated with taxane resistance in MDA–MB–231. For identifying the molecular mechanism underlining paclitaxel resistance in this new cell line, using RT–qPCR analysis, it was found that Hs578T-PR cells also exhibited KIF3C overexpression. In addition, the pro–apoptotic protein BAX was down–regulated; whereas the anti–apoptotic Bcl–2 was up–regulated in Hs578T–PR cells.

Consistent with previous studies, MCF–7 paclitaxel–resistance has been shown to down–regulate BAX; whereas, Bcl2 (B–cell lymphoma 2) was up–regulated. Interestingly, MDR is another key molecular mechanism related to chemoresistance; including Pgp (P–glycoprotein), which functions as a drug efflux pump. One limitation of this present study is that only three specific gene expressions were investigated in paclitaxel–resistant TNBC cells. Hence,
**Figure 3** mRNA expression of key molecular mechanisms related to paclitaxel resistance (A) KIF3C, (B) BAX2, and (C) Bcl–2 were determined by RT–qPCR. The bar graphs represent the average of three independent experiments ± S.D. (n=3). Statistical significance was determined by Student’s t-test (significant, *p*-value<0.05)
Figure 4 Apoptotic phenotype analysis of Hs578T and Hs578T-PR cells after being treated with concentrations of paclitaxel 10 nM for 24hr were detected by DAPI staining. The bar graphs represent the average of three independent experiments±S.D. (n=3). Statistical significance was determined by Student’s t-test (significant, *p-value<0.05)
further studies are required to deeply characterize these new, resistant cells. This would include better identification of the molecular mechanisms related to paclitaxel resistance, using transcriptomic analysis, growth kinetics and cell cycle analysis as well as the investigation of the potential of cross-resistance to a broad spectrum of chemotherapeutic agents.

Conclusion

A newly paclitaxel resistance Hs578T–PR cell line was successfully established within a period of only 4 months; with a remarkably high Resistance Index: approximately 7.4-fold compared to parental cells. This Hs578T–PR cell line will represent clinically relevant tools to investigate molecular mechanisms related to chemoresistance, and for evaluating the efficacy of novel anti-cancer drugs to overcome chemoresistance in TNBC.

Acknowledgement

This study was supported by grants from the Graduate School, Prince of Songkla University. Thanaporn Khunpitak would like to thank the Graduate Scholarship Faculty of Medicine, Prince of Songkla University for supporting her master’s degree study.

Conflict of interest

There are no potential conflicts of interest to declare.

References