Determination of Survivin as a critical mediator for cross-resistance to Paclitaxel and Herceptin in breast cancer

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Abstract

A common problem in the clinical treatment of breast cancer has become the resistance of tumor cells to chemotherapeutic agents. Two types of such agents are the monoclonal antibody trastuzumab (Herceptin) and the antitumor drug paclitaxel (Taxol). The mechanisms of resistance of the two drugs have been extensively researched. Here, we investigate the possibility of cross-resistance between Herceptin and Taxol, thought to be possibly mediated by the protein Survivin. Survivin production was displayed to have a role in mediation of cross-resistance to Taxol and Herceptin. Thus, Survivin provides a future possible target for anti-cancer therapy.

Introduction

Resistance to these drugs has been encountered as both primary and acquired, and increase in the prevalence of acquired resistance will necessitate the exploration of resistance mechanisms for the possibility of positive clinical outcomes.[1,2] Understanding the mechanisms that govern the resistance to these two anti-tumor therapies will be paramount in the further development of new anti-tumor therapies.

Herceptin is a monoclonal antibody that targets the growth factor receptor erbB2 and is used for treating breast cancers that over-express the erbB2 receptor. The antibody binds to the extracellular domain of the erbB2 receptor and prevents the activation of the intracellular tyrosine kinase.[3] Herceptin-resistant breast cancer cells are thought to have developed their resistant phenotype through activation of the phosphotidylinositol 3-kinase (PI3K)/Akt signaling pathway.[2] We have recently reported that this downstream signaling is a result of heterotrimerization of three receptor tyrosine kinases (RTKs), erbB2, erbB3, and Insulin-like Growth Factor-I Receptor (IGF-IR).[2] When associated together, these three RTKs activate one another, overriding the inhibitory effects of Herceptin.[2] When these RTKs are activated, they upregulate several downstream signaling pathways such as the PI3K/Akt, Src, and MAPK signaling within the cell that are shown to inhibit apoptosis. The most significant of the three will be the signaling of PI3K/Akt which has been demonstrated to be a result of interactions between the erbB2 and erbB3 receptors.[4]

Interactions between erbB2 and erbB3 have also been demonstrated in breast cancer cells resistant to a different antitumor drug known as Taxol.[2] Taxol works by hyper stabilizing microtubules so that cells are unable to function during metaphase of mitosis and are forced toward apoptosis.[5] Our recent studies reveal that the paclitaxel resistance-induced by erbB2/erbB3 receptors is due to the upregulation of a protein called Survivin, a member of the IAP (inhibitor of apoptosis) family, via PI3K/Akt signaling-dependent mechanism.[2] However, the precise mechanism by which the erbB2/erbB3/PI3K/Akt signaling upregulates Survivin expression is unknown. It has also been suggested by some studies that activation of IGF-IR signaling may increase Survivin levels through a process of protein translation-induced by activation of Akt.[6]

The Herceptin-resistant cells have shown activation of PI3K/Akt and IGF-IR signaling.[3] Both pathways are able to increase the production of Survivin in breast cancer cells permitting resistance to Taxol.[2,6] Thus, it is conceivable to hypothesize that Herceptin-resistant breast cancer cells may exhibit resistant phenotype in response to taxol, and Survivin may be a critical mediator of this cross-resistance. We will evaluate the responsiveness of Herceptin-sensitive and -resistant breast cancer cells to Taxol-induced growth inhibition and/or apoptosis.

Experimental design and methods:
Cells and cell cultures

Human breast cancer cell lines SKBR3 and BT474 were maintained in DMEM/F-12 (1:1) medium (Sigma) containing 10% fetal bovine serum (Sigma). Both cell lines and the Herceptin-resistant sublines were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO_2 and were split twice a week.

Cell proliferation assay

The CellTiter AQ Non-Radioactive Cell Proliferation kit (Promega) was used to determine cell viability. Cells plated onto 96-well plates for 24 h were then grown in a control medium or the same medium containing varying concentrations of Taxol and incubated for another 72 h. After reading at 490 nm with a micro-plate reader, the percentages of surviving cells from each group relative to controls, defined as 100%, were determined by reduction of MTS.

Quantification of apoptosis

An apoptosis ELISA kit (Roche Diagnostics) was used to measure quantitatively cytoplasmichistone-associated DNA fragments as we previously reported.[3,5]

Western blot analysis

Cell lysates were boiled in SDS-sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose (Bio-Rad), and probed with primary antibody. After the blots were incubated with horseradish peroxidase-labeled secondary antibody (Jackson ImmunoResearch), the signals were detected using the enhanced chemiluminescence reagents (Amersham Life Science).

Production of lentivirus containing specific shRNA

The lentiviral expression vector pLKO.1-ConshRNA or pLKO.1-SurshRNA and lentivirus packaging plasmids pCMV-VSVG and PCMV-ΔA.9 were co-transfected into virus packaging cell line 293T using FuGene6 (Roche). After 24 h, the culture media was replaced with fresh medium. The virus in conditioned medium was then harvested in 3 consecutive days, filtered with low-protein binding filters (Millex-HV, 0.45-mm polyvinylidene difluoride; Millipore Corp.) and stored at -80°C freezer.

Gene silencing with the lentivirus encoding specific shRNA

Before infection, the ConshRNA or SurshRNA lentivirus-containing media (5mL) were thawed completely at room temperature. Another 5 mL of fresh medium containing polybrene (8 μg/mL) was added into the virus-containing media which was used to replace the culture media of interested cells. After 24 h, the virus-infected cells were selected by puromycin (1 μg/mL) for 48 h and subjected to required assays.

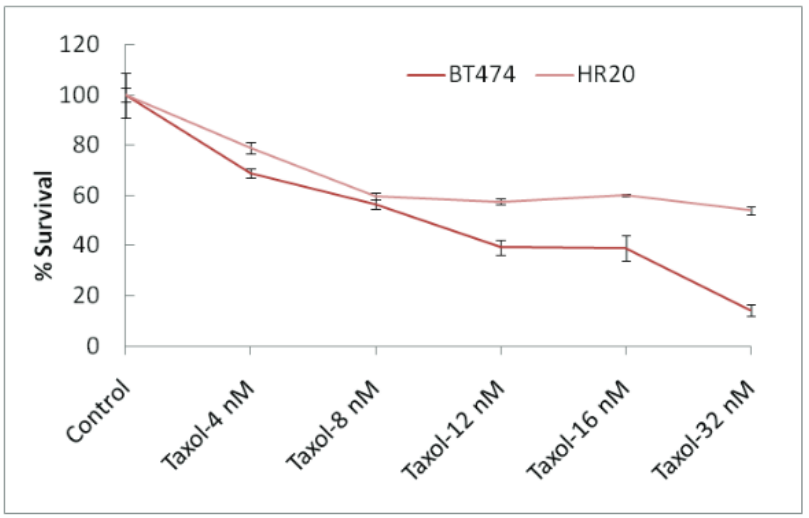

Figure 1. MTS assay analysis of BT474 and Herceptin resistant sub-line HR20. This analysis was compiled from data from multiple trials.

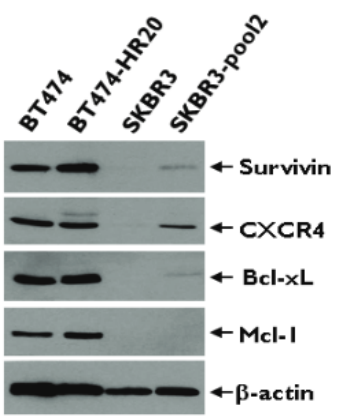

Figure 2. Western blot analysis of all four cell lines and presence of various proteins.

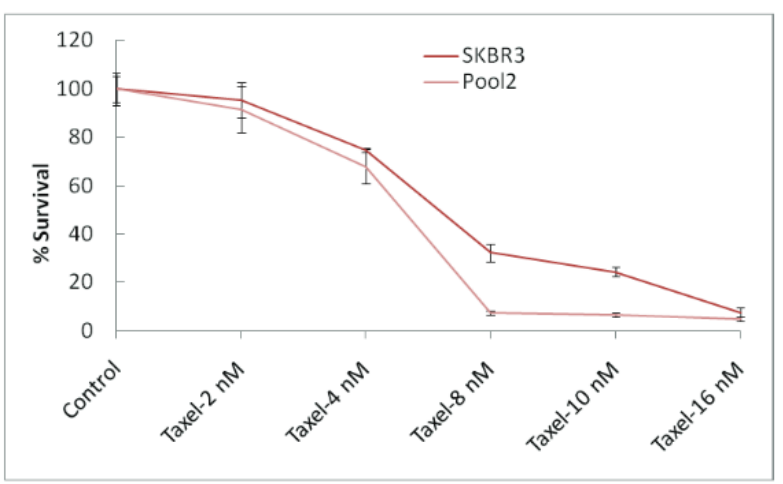

Figure 3. MTS assay analysis of SKBR3 and Herceptin resistant sub-line Pool2. This analysis was compiled from data from multiple trials.

Statistical analyses

Statistical analysis of all experimental data was performed using a two-sided student's t test. Significance was set at $P < 0.05$.

Results and Discussion:

To investigate the likelihood of cross-resistance between Herceptin resistant cells and resistance to Taxol, both cell lines and herceptin resistant cell sub-lines were plated in cultures of increasing concentrations of Taxol. Only one of the cell line pairs, BT474 & HR20, demonstrated significantly positive results. In MTS proliferation assay tests, HR20 presented with comparable survival rates at a dose of 4 nM Taxol and higher survival rates than BT474 at doses 8, 12, 16 and 32 nM Taxol. This is demonstrated in Figure 1. This data suggests that there is a high possibility of a link between resistance to Herceptin and an induced resistance to Taxol, but research with other cell lines is required to confirm this finding. However, the results of tests on the SKBR3 cell line and Herceptin resistant subline did not display positive results. Pool2 demonstrated comparable survival rates at doses of 2 and 4 nM Taxol and lower survival rates at doses of 8, 10 and 16 nM Taxol as is seen in Figure 3. It is very possible that Survivin does not play a role in Taxol resistance in this cell line.

Western blot analysis of all four cell lines revealed that HR20 had higher Survivin production than BT474 and SKBR3 and Pool2 had similar Survivin production. This is shown in Figure 2.

Due to the negative results with SKBR3 and Pool2 indicated by Figure 3 and the apparent difference of Survivin production in SKBR3 and Pool2 indicated in Figure 2, gene silencing was only carried out on the HR20 subline and one trial of Apoptosis ELISA analysis for this cell line was developed and can be seen in Figure 4.

The MTS assay, apoptosis ELISA, and western blot analysis on the Survivin silenced cell lines are all ongoing strategies to strengthen the hypothesis.

Although strategies to strengthen this hypothesis have not been completed and are currently ongoing, there is data to support the conclusion that Survivin is a critical mediator of the cross-resistance between Herceptin and Taxol. This is possibly the first instance of cross-resistance mediated by the same mechanism between two different types of therapy, monoclonal antibody therapy and chemotherapy. A reproducible link may be able to change the way antitumor therapy is approached. Positive results could also lead to new antitumor therapies that would target

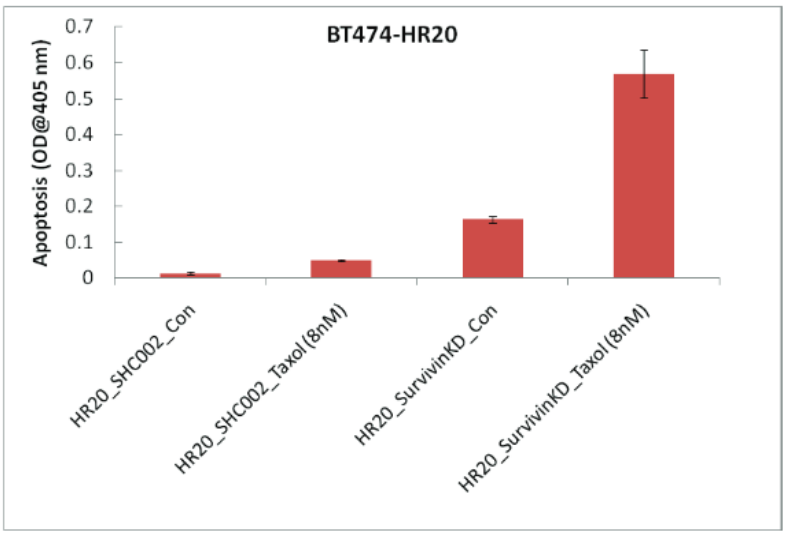

Figure 4. Apoptosis ELISA of the BT474-HR20 sub-line and a Survivin gene silenced counterpart.

the production of Survivin. Combining therapies that target Survivin and erbB2 or microtubules might allow physicians to bypass acquired resistance and to continue treating patients. Survivin production in Herceptin resistant cells would also indicate that patients with primary or acquired resistance to Herceptin have acquired resistance to Taxol as well. Investigation of Herceptin resistant cells being resistant to drugs similar in form and function to Taxol and of Taxol resistant cells being resistant to Herceptin is necessary.

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SET/MYND lysine methyltransferases regulate gene transcription and protein activity

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Introduction

From regulated gene expression to mitosis, chromatin acts as a structurally flexible repository of the genome.[1] In this manifestation, an entire chromosome is sequentially compacted through a series of highly ordered packaging while distinct regions of DNA are selectively made accessible to transcriptional complexes.[2,3] Thus, chromatin maintains a dynamic architecture that allows approximately 2 m of DNA to be condensed in the nucleus while retaining a remarkable degree of functionality.[4,5] At its foundation, chromatin consists of a succession of nucleosomes, the basic structural units,[6] consisting of 146 base pairs of DNA, wrapped 1.7 times around an octamer of core histones and separated by a linker region of approximately 50 base pairs. The primary histones involved in the assembly of a nucleosome are histones H2A, H2B, H3 and H4. Histone tails interact with the poly-anionic backbone of the core DNA, marginally contributing to nucleosomal stability.[7] Therefore, regulation of chromatin structure and transcription is often mediated through post-translational modifications that alter specific residues along these tails.[8] These modifications can affect the accessibility of nuclear factors to DNA or induce the recruitment of such factors to transcriptional or chromatin assembly pathways.[9,10]

Histone tail alterations encompass the greatest range of variation in epigenetic regulation, encompassing more than 50 known sites of modification.[11,12] Histones are subject to several forms of post-translational modification, including methylation, citrullination, acetylation, phosphorylation, SUMOylation and ADP-ribosylation.[13] These modifications impart biological consequences by acting as marks for the specific recruitment of regulatory complexes and affecting the structure of the nucleosome. Acting in concert, the combination of different histone modifications is thought to constitute a "histone code" that is interpreted in the form of specific nuclear events.[14,15] Although the interplay among various histone modifications is still largely nebulous, a paradigm is rapidly emerging whereby methylation, acetylation, or phosphorylation at independent sites work in tandem with other such modifications to convey unique biological consequences.[16] Such crosstalk has already been clearly demon-