

ORIGINAL ARTICLE

HER2/*neu* Oncogene and Sensitivity to the DNA-Interactive Drug Doxorubicin

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ABSTRACT Breast tumor cells overexpressing the proto-oncogene HER2/*neu* are known to be less responsive to certain DNA-binding chemotherapeutic agents. The current study specifically investigates the correlation between chemosensitivity to the DNA-binding drug doxorubicin and cellular HER2/*neu* protein levels in a panel of eight breast cancer cell lines (HS-578, BT-474, MDA-MB-453, MDA-MB-231, MDA-MB-175, MCF-7, ZR-75-1 and T47D). The IC₅₀ (the drug concentration required to inhibit cell growth by 50%) values for the cell lines were determined by the sulforhodamine B assay. IC₅₀ values were correlated with HER2/*neu* protein levels determined by Western blotting. An almost linear relationship between IC₅₀ and HER2/*neu* protein level for seven cell lines ($p = 0.02, r^2 = 0.680$) was found, with protein levels increasing as resistance increased. The findings suggest that overexpression of HER2/*neu* correlates with increased resistance to doxorubicin in seven of eight breast cancer cell lines studied. The observation that, in one cell line (MDA-MB-175), doxorubicin IC₅₀ did not correlate with HER2/*neu* levels, suggests that in these cells, an as-of-yet unidentified factor contributes to resistance. If the observed correlation, which was present in seven of eight cell lines, is confirmed in a larger sample size, increased HER2/*neu* levels may be implemented as a predictor of breast tumor sensitivity to doxorubicin.

INTRODUCTION

Breast tumors are characterized by a variety of cellular disorders, including mutations and overexpression of signal transduction proteins. Notably, the mutation and overexpression of the proto-oncogene HER2/*neu* has been observed in 15 to 35% of breast tumors (1-6). Many other malignancies, including ovarian, gastric and kidney tumors have also exhibited overexpression of the HER2/*neu* protein (1,7,8). These observations have generated attempts to use anti-HER2/*neu* antibodies to specifically deliver chemotherapeutic drugs to tumors (9).

The HER2/*neu* (c-erbB-2) proto-oncogene is located on chromosome 17q21 and encodes a 185 kD

transmembrane phosphoglycoprotein, which consists of an intracellular kinase and a cysteine-rich extracellular receptor domain (10). HER2/*neu* belongs to a family of proteins, which, like the epidermal growth factor receptor, regulate cell growth as receptor tyrosine kinases (11-13).

The oncogenic potential of the HER2/*neu* protein can emerge in two ways. First, the proto-oncogene may be altered by a single point mutation that replaces valine 664 with a glutamic acid residue (12,14). A structurally altered gene product is generated that confers transforming potential, enhanced tyrosine kinase activity and ultimately enhanced cell proliferation (12). Second, gene transfer experiments have demonstrated that amplification of the normal HER2/*neu* gene product alone is sufficient to induce cell transformation and oncogenic potential (15), while antisense oligonucleotides specific for HER2/*neu* cause a reduction in cell growth (16).

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The mechanism of enhanced proliferation is believed to be increased ligand-induced receptor-receptor interactions or overthreshold ligand-induced stimulation in cells overexpressing *HER2/neu* (12,17,18). Additionally, poor patient prognosis and aggressive tumor growth are associated with overexpression of *HER2/neu* in several tumor types (2,19-21). Further, studies have suggested that this oncogene may be involved in tumor resistance to chemotherapeutic drugs (20-22).

In the past 20 years, many antineoplastic agents have been isolated and synthesized. Of these, doxorubicin (DOX; Adriamycin) has emerged as the most utilized antitumor drug worldwide (23,24). Clinically, this anthracycline is used to treat many solid tumors, such as carcinomas of the breast, bladder, endometrium, lung, ovaries, stomach and thyroid, as well as sarcomas of bone and soft tissue, pediatric solid tumors and lymphoid tumors (24).

The cytotoxicity of DOX is mediated via its capacity to covalently bind double stranded DNA. This subsequently induces protein-linked double strand DNA breaks through the action of topoisomerase II α (24). Topoisomerase II α controls DNA supercoiling by cleaving and reannealing double stranded DNA. Intercalators such as DOX interfere with topoisomerase II by stabilizing the cleavable complex, which converts the enzyme into a cellular poison (25-27). The importance of topoisomerase II in the antineoplastic mechanism of DOX has been highlighted by studies demonstrating that decreases in topoisomerase II expression leads to resistance to DOX (28,29).

Other mechanisms of anthracyclines include inhibition of DNA synthesis and RNA polymerases, and formation of free radicals (24,30). Further, free radical formation has also been implicated as a mechanism by which DOX kills human breast cancer cell line MCF-7 *in vitro* (31,32).

Unfortunately, the use of DOX in cancer chemotherapy is frequently plagued by tumor resistance, which, in some cases, is mediated by the human multidrug resistance-associated protein Pgp (33,34). However, not all resistance can be explained by this mechanism. As previously mentioned, *HER2/neu* has also been implicated in DOX resistance (20-22). Evidence, such as increased sensitivity to DOX and other antineoplastic agents following reduction in *HER2/neu* activity by treatment with anti-*HER2/neu* antibody (35,36) and antisense oligonucleotides (37) and resistance to DOX correlating with *HER2/neu* expression in cancer cell lines (23,24,38,39), suggest that *HER2/neu* is related to DOX resistance. Furthermore, there is preliminary evidence that drug resistance linked to overexpression

of the *HER2/neu* is a consequence of its role in DNA repair (40,41).

However, the relevant studies do not entirely agree with the hypothesis that *HER2/neu* expression correlates with chemoresistance. Namely, Pedram et al. demonstrated that *HER2/neu* overexpression was not sufficient to produce drug resistance in breast and ovarian cancer cell lines (42). Also, basic evidence suggests that increased *HER2/neu* activation leads to increases in topoisomerase II expression (43) and reduced gene copies of *HER2/neu* are linked to decreased gene copies of topoisomerase II (44). These studies suggest that increased *HER2/neu* would lead to increased topoisomerase II α , which would increase the antineoplastic activity of DOX. Finally, clinical evidence from Muss et al. demonstrates that patients who overexpress *HER2/neu* are more likely to benefit from high doses of DOX (45). Due to the conflicting evidence suggesting that *HER2/neu* might not be involved in drug resistance, studies designed to further define the role of *HER2/neu* in tumor response to chemotherapeutic agents are in demand.

This work examines the correlation between the expression of *HER2/neu* and breast tumor cell response to DOX in a panel of eight breast cancer cell lines and finds that, in seven of the eight cell lines, there is a positive correlation of *HER2/neu* expression to DOX resistance.

MATERIALS AND METHODS

Cell Culture

Breast cancer cell lines (ATCC) (HS-578, BT-474, MDA-MB-453, MDA-MB-231, MDA-MB-175, MCF-7, ZR-75-1, T47D) were grown in RPMI 1640 (Gibco; Burlington, ON) complete medium containing penicillin streptomycin (2.5 mL/500mL RPMI), L-glutamine (5mL/500 mL RPMI) and fetal bovine serum (FBS) (10%) (Gibco). Incubation of cell lines was at 37°C in a 5% CO₂ atmosphere. The growth medium was changed biweekly and plates approaching 100% confluence were split into new plates by trypsinization.

Determination of the Sensitivity of the Breast Cell Lines to Doxorubicin

Exponentially growing cell monolayers were incubated with serially diluted concentrations of DOX (Sigma, Oakville, ON) (0.1nM to 800nM in RPMI complete medium), and incubated for five days in 96-well plates. Cytotoxicity was evaluated by the sulforhodamine B assay. Briefly, the cells were fixed by the addition of 50 μ l of cold trichloroacetic acid (TCA) (50%) at 4°C for one hour. The wells were washed four times with water and stained with sulforhodamine B

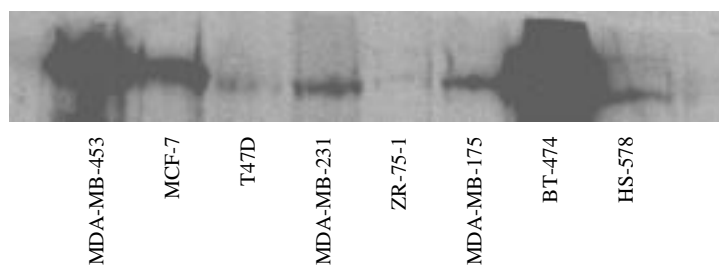


Figure 1. HER2/*neu* expression in breast cancer cell lines from Western blotting.

(0.4%) (Sigma) dissolved in 1% acetic acid. The plates were air-dried and the resulting colored residue dissolved in 200 μ l of Tris base (10mM). The optical density (OD) of each well was measured at 540nm with a microplate reader (Model 3550, BioRad; Mississauga, ON). The results were calculated from at least two independent experiments run in triplicate. Additionally, the IC₅₀ value was calculated from each independent experiment. The graphs, therefore, represent the average IC₅₀ for the indicated cell line.

Determination of HER2/*neu* Levels in the Breast Tumor Cell Lines

Exponentially growing cells were collected in cold PBS and centrifuged to separate a pellet that was then washed with hypotonic buffer. Cells were lysed by sonication (3 x 10 seconds) and the pellet (nuclei) separated from the supernatant (cytosol) by centrifugation (14 000 rpm). The supernatant was removed and the pellet was resuspended in hypotonic buffer containing 0.1% SDS which was kept on ice for 10 minutes. The protein concentrations of each cell line were measured against a prepared standard curve based on the absorbances of nine serial dilutions at 595 nM, using the method of Bradford (46).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as outlined (47). The samples were subjected to 8% SDS-PAGE gels, and finally transferred to nitrocellulose membranes. The membranes were rinsed in Tris buffered saline (TBS) and Tween 20 (T) and blocked to prevent antibody binding to non-specific sites with a solution of TBS+T+1% bovine serum albumin. To detect HER2/*neu*, the primary antibody (Cedar Lane; Hornby, ON) (mouse anti-human) was incubated with membranes overnight at 4°C (concentration 1:5000). To detect HER2/*neu* labeled proteins, an anti-mouse antibody (Cedar Lane) (concentration 1:3000) was incubated with membranes for 30 minutes at room temperature. Enhanced chemoluminescence (Amersham; Oakville, ON) was used to identify immuno-tagged proteins that were then visualized by exposure on Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY).

Correlation Between Breast Tumor Cell Sensitivity to Doxorubicin with Levels of HER2/*neu*

Levels of HER2/*neu* previously identified by a single Western blot were quantified by BioImage (Visage Electrophoresis Gel Analysis System; Ann Arbor, MI) densitometric scan and expressed in relative OD units. Statistical significance was assumed for $p < 0.05$ using Mann-Whitney rank sum test. All statistical analyses were performed with the GraphPad Prism (San Diego, CA) software package. OD units indicate relative levels of HER2/*neu*.

RESULTS

Quantification of HER2/*neu*

The relative levels of HER2/*neu* in the eight cell lines were quantified by Western blotting (Figure 1, Table 1). BT-474 and MDA-MB-453 expressed similar amounts of the protein. The levels of HER2/*neu* expressed in these two cell lines were approximately 2-fold greater than in MDA-MB-175, 1.7-fold greater in MCF-7, 5-fold greater than in both HS-578 and MDA-231, 10-fold greater than T47D, and 26.7-fold greater than ZR-75-1. This indicates that the levels of HER2/*neu* was quite variable within the cell population, a distribution pattern that is critical for the validity of the correlation.

Chemosensitivity

The IC₅₀ for DOX was determined in each of the eight tumor cell lines (Table 1, Figure 2A and 2B). The

Table 1. Doxorubicin IC₅₀ and HER2/*neu* levels for breast cancer cell lines.

Breast Cancer Cell Lines	IC ₅₀ DOX (nM)	HER2/ <i>neu</i> Level (OD)
T47D	2.706	0.154
MDA-MB-231	3.560	0.357
MCF-7	4.160	0.978
HS-578	6.508	0.351
ZR-75-1	6.690	0.059
MDA-MB-453	22.340	1.661
BT-474	64.600	1.691
MDA-MB-175	121.100	0.850

IC₅₀: Inhibitory concentration; OD: Optical density

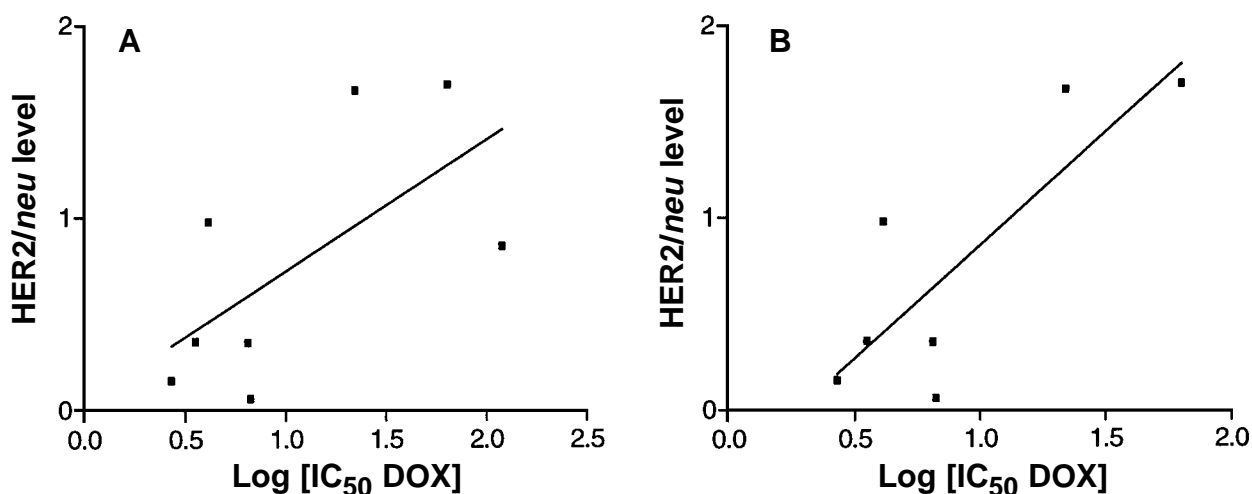


Figure 3. Correlation between HER2/*neu* protein levels and IC₅₀ for doxorubicin. **A:** Includes a panel of eight breast cancer cell lines (HS-578, BT-474, MDA-MB-453, MDA-MB-231, MDA-MB-174, MCF-7, ZR-75-1 and T47D) ($p = 0.08$, $r^2 = 0.42$). **B:** Includes a panel of seven breast cancer cell lines (as in A, not including MDA-MB-175) ($p = 0.02$, $r^2 = 0.68$).

The exact mechanism that links overexpression of HER2/*neu* to DOX resistance is not clearly understood. It has been shown that altered drug accumulation or detoxification is not involved in HER2/*neu* mediated resistance (40). DNA repair, as well as dysregulation of cell cycle checkpoint and apoptotic mechanism seem to be responsible for the chemoresistance induced by HER2/*neu* overexpression, however, it remains to be determined which HER2/*neu* signaling cascade initiates these mechanisms (40).

In this study, one of the cell lines, MDA-MB-175, which expressed low levels of HER2/*neu*, showed resistance to DOX. The value for this cell line significantly deviated from the correlation established between the seven other cell lines. The current authors believe that this cell line may express another mechanism of resistance, most probably the P-glycoprotein-mediated mechanism (*mdr-1*). It has been shown that HER2/*neu* overexpression can lead to intrinsic drug resistance independent of *mdr-1* in MDA-MB-435 cells (51). That is, it is possible that MDA-MB-175 is a case wherein the *mdr-1* mechanism of chemoresistance dominates that of HER2/*neu*, and represents the central mode of resistance. The levels of P-glycoprotein (*mdr-1*) in MDA-MB-175 have not yet been reported. HER2/*neu*, however plays a prognostic role that is independent of its involvement in multi-drug resistance (*mdr-1*) (52).

The correlation reported in the current study establishes the value of HER2/*neu* levels in predicting chemosensitivity to doxorubicin. A firm correlation may then be applied in the clinic to design more effective chemotherapy regimens for solid breast tumors. The correlation may be further strengthened by

extension to more cell lines. Carefully controlled experiments may begin to demonstrate whether overexpressed HER2/*neu* confers resistance alone or by a mechanism dependent on other factors, a finding that has applications to treatment and to patient prognosis.

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