Expression of Her-2/neu in Human Lung Cancer Cell Lines by Immunohistochemistry and Fluorescence in Situ Hybridization and its Relationship to in Vitro Cytotoxicity by Trastuzumab and Chemotherapeutic Agents

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ABSTRACT

Overexpression of the Her-2/neu oncogene and receptor protein was reported in ~20% of breast cancers and was associated with a poor prognosis. Her-2/neu expression was a predictor for response to trastuzumab, a monoclonal antibody that recognizes the Her-2/neu cell surface receptor. Data regarding the expression of Her-2/neu in lung cancer are far more limited, and there is little information regarding the influence of Her-2/neu expression and response to trastuzumab alone or in combination with chemotherapeutic agents. In this report we evaluated Her-2/neu gene expression by fluorescence in situ hybridization (FISH) and the cell surface expression of the Her-2/neu receptor by immunohistochemistry using the HercepTest and by FACS analysis in 31 lung cancer cell lines with 5 breast cancer cell lines as controls. By FACS, we found Her-2/neu overexpression (mean fluorescence intensity >8) in 2 of the 22 non-small cell lung cancer (NSCLC) cell lines (9%), none of 11 small cell lung cancer (SCLC) cell lines, and 4 of 5 breast cancer cell lines. A positive HercepTest (2+ or 3+) was found in 6 of 19 NSCLC cell lines (26%, 2+; 5%, 3+), 1 of 3 SCLC cell lines (33%), and 4 of 5 breast cancer cell lines (80%). One of 6 NSCLC cell lines examined (17%) had gene amplification with >32 copies of Her-2/neu/cell and had homogeneous staining regions. One NSCLC cell line had a maximum of 14 copies of Her-2/neu/cell, and 3 had modest increases in Her-2/neu gene copy number without gene amplification (maximum 5–8 copies/cell). None of the SCLC cell lines had more than a maximum of 4 copies/cell, whereas the 2 breast cancer cell lines had maximum Her-2/neu copy numbers of 80 and 5, respectively. Aneusomy rather than true amplification was the major cause of increased Her-2/neu expression in most of the NSCLC cell lines. There was a strong correlation when the results of fluorescence-activated cell sorter, HercepTest results, and FISH were compared in pairs. Furthermore, Trastuzumab produced a G1 cell cycle arrest and growth inhibition only in cell lines expressing Her-2/neu. The IC50 for growth inhibition was correlated with cell surface Her-2/neu expression. The combination of trastuzumab and chemotherapeutic agents produced more than additive growth inhibition in cell lines expressing Her-2/neu, but the level of additivity was not related to the amount of Her-2/neu expression. These data indicate that trastuzumab alone and in combination with chemotherapeutic agents should be tested in NSCLC patients and that Her-2/neu should be assessed by both immunohistochemistry and FISH methods in these studies to determine which test is the best predictor of outcome.

INTRODUCTION

Lung cancer is the leading cause of cancer death in the United States and the world. In the United States it is the leading cause of cancer death in both males and females and kills more individuals than breast, colorectal, and prostate cancers combined (1). The cure rate remains <15% despite some recent advances in chemotherapeutic agents (2). Recent advances in biology and molecular biology have led to the development of targeted therapies. Overexpression of dominant oncogenes attributed to gene amplification, increased chromosome copy number, transcription, and other means have been reported to occur frequently in lung cancer, although these changes generally occur late in tumor development (3). The erbB (Her) family of oncogenes is frequently overexpressed in lung cancer (4–12). The erbB gene family encodes for growth factor tyrosine kinase receptors that are felt to play a role in the autocrine growth of human lung cancers (13–17). The family consists of four receptors: erbB-1 (Her1), erbB-2 (Her-2/neu), erbB-3 (Her3), and erbB-4 (Her4). There are at least six ligands for these receptors. Binding of ligands to receptors causes heterodimerization and activation. In addition, Her-2/neu may be constitutively activated by mutation in cancer cells. The four receptors of this family are structurally similar with cysteine-rich domains, a membrane-spanning region, and an intracellular tyrosine kinase domain. Variable sequences in the cytoplasmic tail of each family member results in the recruitment and interaction of different second messengers and, thus, activation of downstream signal transduction pathways. Ligand binding to the extracellu-
lar domain initiates erbB receptor homo- and heterodimerization that expands the signaling diversity in this family of proteins. Signaling by receptor heterodimers may dominate over that of homodimers. Her2/neu has no known natural ligands and is the preferred heterodimeric partner for Her family ligand complexes.

Whereas the Her-2/neu proto-oncogene was originally identified as a dominant transforming oncogene produced as a result of point mutations, the gene (located on human chromosome 17) is frequently overexpressed by gene amplification rather than by mutation in human cancers (18–20). The degree of overexpression/amplification correlates with poor prognosis and also with lack of response to conventional therapy (4–12, 18–22). Her-2/neu protein overexpression is most often measured by IHC using one of several monoclonal antibodies, and gene expression is measured most often by FISH3 for clinical studies. In a recent study in breast cancer, FISH provided superior prognostic information (23). It is not known which test is the best predictor of response to targeted therapy.

The recognition of the role of the Her-2/neu signaling pathway in breast cancer led to the development of new treatment strategies designed to interfere with the pathway. One of the first approaches was to develop a monoclonal antibody to the Her-2/neu receptor that would block signal transduction and growth (24, 25). In preclinical models trastuzumab (Herceptin), a humanized monoclonal antibody, was shown to inhibit the growth of human breast cancers. This led to clinical trials where the antibody was shown to produce objective responses in a minority of Her-2/neu-positive patients (24). In combination with doxorubicin- or paclitaxel-based therapy, trastuzumab produced higher response rates and longer survival than either agent alone (25).

Studies of Her-2/neu in lung cancer have lagged behind those in breast cancer. Previous studies suggest that overexpression of Her-2/neu imparts a poor prognosis in NSCLC as it does in breast cancer and that overexpression occurs in about 20% of cases (4–12). However, there is little information about the role of trastuzumab in the treatment of lung cancer. Thus, the goals of this study were to determine the degree and molecular mechanism of Her-2/neu overexpression in a panel of lung cancer cell lines. Additionally, we sought to determine the effect of trastuzumab alone and in combination with cytotoxic chemotherapy agents on the growth of human lung cancer cell lines in vitro.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The NSCLC cell lines NCI-H322, NCI-H226, NCI-H441, NCI-H1703, NCI-H324, NCI-H2122, NCI-H125, NCI-H1334, NCI-H435, NCI-H157, NCI-H1264, NCI-H661, NCI-H520, and NCI-H460 were kindly provided by Drs. John Minna and Adi Gazdar (University of Texas Southwestern Medical School, Dallas, TX). The SCLC cell lines NCI-H345, NCI-H209, NCI-H187, NCI-H510, NCI-H146, NCI-H128, NCI-H82, NCI-H196, and NCI-N417 were also provided by Drs. John Minna and Adi Gazdar. The NSCLC line COLO699 was obtained from Dr. George Moore (Denver General, Denver, CO); the NSCLC line SW1573 was kindly provided by Dr. Hal Broxterman (Free University, Amsterdam, The Netherlands), and the NSCLC NE-18 was obtained from Dr. Karen Kelly (University of Colorado Health Science Center, Denver, CO). All of the above cell lines were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT) or in serum-free containing hydrocortisone, insulin, transferrin, estradiol, selenium (HITES) medium (26). The SCLC cell line SHP-77 was kindly provided by Dr. Aurelio Koros (University of Pittsburgh, Pittsburgh, PA) and maintained in RPMI 1640 with 10% FBS. The NSCLC lines A549, Calu-3, SKLU-1, and the breast cancer cell line SKBR3 were obtained from the American Type Culture Collection, Rockville, MD, and were maintained in RPMI 1640 supplemented with 10% FBS or MEM supplemented with 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% FBS. The breast cancer cell lines ZR75, MCF7, T47D, and MDA MB231 were obtained from Dr. Kathryn Horwitz (Univ. of Colorado Cancer Center) and were maintained in RPMI 1640 media supplemented with 10% FBS. All of the cell lines were grown in 5% CO2 at 37°C in incubators with 100% humidity.

Chemicals. Trastuzumab was kindly provided by Genentech, Inc., South San Francisco, CA. The gemcitabine was provided by Eli Lilly, Indianapolis, IN. The paclitaxel was kindly provided by Bristol Myers-Squibb, Princeton, NJ. The cisplatin was purchased from Sigma Chemical Co. (St. Louis, MO). Glaxo-Wellcome, Research Triangle Park, NC, kindly provided the vinorelbine.

MTT Growth Assay. Cell growth was assessed using a MTT assay (27). Briefly, 10,000 viable cells were plated in 100-μl 96-well plates (Corning, Ithaca, NY). After an overnight incubation, various cytotoxic chemotherapy agents and/or trastuzumab were added in varying concentrations to each of three replicate wells and incubated for 6 days. After 6 days, the tetrazolium salt was added at a concentration of 0.4 mg/ml to each well. The microtiter plates were incubated with the salt for 4 h at 37°C and then the medium was aspirated off leaving the dark blue formazan product in the bottom of the wells. The reduced MTT product was solubilized by adding 100 μl of 0.2 N HCl in 75% isopropanol to each well. Thorough mixing was done using a Tiertek multichannel pipetman (Flow Laboratorics). The absorbency of each well was measured using an automated plate reader (Molecular Devices, Sunnyvale, CA). For each concentration the mean value ± the SE was calculated. When cell lines were incubated with combinations of trastuzumab and chemotherapeutic agents, the combination effects were assessed with the isobologram method of Chou and Talalay (28).

Cell Cycle Distribution and Apoptosis. The effects of trastuzumab on cell cycle distribution and percentage of apoptotic cells was determined by flow cytometric and immunofluorescence assays. At 72–120 h after incubation with trastu-
zumab, 1 × 10^5 SHP-77, SKBR3, Calu-3, H157, and A549 cells were stained with 2.5% propidium iodide solution/0.3% saponin/0.001% RNase A in 10 mM EDTA. Stained nuclei were analyzed using a Coulter EPICS XL-MCL (Coulter Corp., Miami, FL) for the proportions of cells in the G1/G0, S, and G2-M phases of the cell cycle. Cell cycle studies were conducted twice as shown in the results. The resulting DNA distributions were analyzed using the ModFit LT Software (Verity House Software, Topsham, Maine; Ref. 29). The presence of apoptotic cells was confirmed by fluorescence microscopy.

**Immunofluorescence Staining.** For FACS analysis, cells (5 × 10^5) were incubated with the monoclonal antihuman HER-2/neu antibody c-erbB-2 Ab-2 (9G6.10; NeoMarkers, Fremont, CA) or the isotype matched control mineral oil plasma-cytoma-10 (Sigma Chemical Co.). The cells were counterstained with goat antimouse IgG1-FITC (Southern Bio-technology, Birmingham, AL). All of the staining was done on ice for 45 min followed by three washes in HBSS + 5% FBS. After staining, the cell fluorescence was measured by flow cytometry (Coulter EPICS-XL-MCL; Coulter Corp.). Using the Coulter software, the percentage of positive cells and their MFI were determined by comparison with the isotype matched control studied twice as shown in the results. The resulting DNA distributions were analyzed using the ModFit LT Software (Verity House Software, Topsham, Maine; Ref. 29). The presence of apoptotic cells was confirmed by fluorescence microscopy.

**Immunofluorescence Staining.** For FACS analysis, cells (5 × 10^5) were incubated with the monoclonal antihuman HER-2/neu antibody c-erbB-2 Ab-2 (9G6.10; NeoMarkers, Fremont, CA) or the isotype matched control mineral oil plasma-cytoma-10 (Sigma Chemical Co.). The cells were counterstained with goat antimouse IgG1-FITC (Southern Bio-technology, Birmingham, AL). All of the staining was done on ice for 45 min followed by three washes in HBSS + 5% FBS. After staining, the cell fluorescence was measured by flow cytometry (Coulter EPICS-XL-MCL; Coulter Corp.). Using the Coulter software, the percentage of positive cells and their MFI were determined by comparison with the isotype matched control stained cells. For IHC analysis the cell lines were centrifuged into a cell pellet and embedded in paraffin for immuno-histochemical staining with the HercepTest (Dako, Corp., Carpinteria, CA). Antigen retrieval was performed at 95°C in citrate buffer pH 6.0, 6.4 M sodium citrate dihydrate, 1.6 M citric acid monohydrate for 40 min. The slides were cooled at room temperature for 20 min and washed 3 × 3 min with Tris buffer pH 7.6, 0.15 M sodium chloride, 0.05 M Trizma HU. The slides were peroxidase block for 5 min and washed as above. The slides were incubated for 30 min with the primary antigen, followed by the secondary antigen (Visualization Reagent), and finally counter stained with hematoxylin. The slides were dehydrated, ethanol dehydration, a 30-s wash in 70% acetic acid, and ethanol dehydration. The PathVysion HER-2/neu DNA probe kit (Locus Specific Indicator LSI) Her-2/neu labeled in SpectrumOrange)/ chromsome enumerator probe (CEP) chromosome 17 labeled in SpectrumGreen from Vysis (Dowers Grove, IL) was applied to the slides according to the manufacturer’s directions, and the slides were incubated for 10 min at 80°C for codenaturation of probe and chromosomal DNA. Hybridization proceeded overnight at 37°C. After hybridization, the slides were washed in 50% formamide/2 × SSC/0.1% NP40 at 46°C. The chromatin was counterstained with 4’,6-diamidino-2-phenylinodole in Vectashield antifad (Vector, Burlingame, CA). A minimum of 200 nuclei were scored under a BX60 Olympus fluorescence microscope (Olympus, Boston, MA), using a triple band pass interference filter (blue/red/green) and a single band filter for blue, red, and green. Images were acquired using a cooled CCD camera (SenSys; Photometrics, Tucson, AZ) and merged using the SmartCapture software from Vysis.

**Statistical Comparisons.** Comparisons between the Her-2/neu protein expression as assessed by MFI in NSCLC versus SCLC, NSCLC versus breast cancer, and SCLC versus breast cancer cell lines were done using a Wilcoxon rank-sum test. Comparisons of the fraction of cells in the cell cycle distributions were performed using a χ^2 test. Correlation coefficients for comparisons of maximum Her-2/neu gene versus average Her-2/neu gene expression, Max Her-2/neu gene expression versus MFI, and IHC versus MFI were calculated as the Spearman correlation coefficient.

**RESULTS**

**Her-2/neu Expression.** The cell surface expression of Her-2/neu as assessed by FACS and IHC HercepTest is shown in Figs. 1 and 2 and summarized in Table 1B. By FACS analysis, 3 of 22 NSCLC cell lines (14%) had no cells expressing Her-2/neu. Twelve NSCLC cell lines (55%) had low Her-2/neu protein levels with a MFI of <4, 5 NSCLC lines (23%) had moderate expression (MFI between 4 and 8), and 2 NSCLC lines (9%) had high expression with a MFI >8. Only 1 of 11 SCLC cell lines (9%) expressed any Her-2/neu with a low MFI of 3.7. Four of 5 breast cancer cell lines (80%) expressed high levels of Her-2/neu with a MFI >8 (Fig. 1A). Among the lung cancer cell lines of various histology, the adenocarcinomas had the highest expression with an average MFI of 6.8 (range 0–36; median 3.8). The large cell carcinomas had an average MFI of 3.4 (range 2.4–4.7; median 3), and squamous carcinomas had an average MFI of 3.1 (range 0–4.3; median 3). SCLC cell lines had the lowest expression with only 1 cell line showing any expression (average MFI of 0.34). Breast cancer cell lines had an average MFI of 16. The differences in Her-2/neu expression between breast cancer and both NSCLC and SCLC cell lines were statistical significant (Wilcoxon rank-sum test; P < 0.001). The differences between the NSCLC cell types were not different. The NSCLC cell lines had significantly greater expression than the SCLC cell lines (P = 0.0002).

Cell surface protein expression was also assessed by IHC using the HercepTest in 19 of 22 NSCLC cell lines, 3 of the 11 SCLC cell lines, and 5 of the 5 breast cancer cell lines. Typical staining by IHC is shown in Fig. 2. Fig. 2D shows the 3+ staining observed in the SKBR3 breast cancer cell line with strong intensity in essentially all of the cells. One NSCLC cell line (5%) had 3+ staining by the HercepTest (Fig. 2C). Another 4 NSCLC cell lines (21%) had moderate 2+ expression with a large proportion of stained cells showing considerable cell surface intensity (Fig. 2B). The HercepTest 1+ staining, defined by weak cell surface intensity and many cells that had no staining, was present in 7 NSCLC cell lines (37%; Fig. 2A). There were 7 NSCLC cell lines that had no staining (0+; 37%), and none of these had a MFI >4. With the HercepTest, 2 breast cancer cell lines had 3+ staining, and 1 had 0+ staining.

The results comparing IHC to FACS are shown in Fig. 3A. There was an excellent correlation (r = 0.57; P = 0.002) between Her-2/neu cell surface expression assessed by IHC or
FACS. Using criteria of 0 or 1+ HercepTest and MFI ≤4 as negative and 2+ or 3+ HercepTest and MFI >6 as positive, 15 cell lines had no expression by either test, and 7 cell lines were positive by both methods. Five lung cancer cell lines had discordant results: a 2+/3+ HercepTest and negative MFI <4 in 3 cell lines and HercepTest 0/1+ with an MFI of 4–8 in 2 cell lines.

**FISH Analysis.** The degree and mechanism of Her-2/neu oncogene overexpression was assessed by FISH in 6 NSCLC, 2 SCLC, and 2 breast cancer cell lines. These cell lines were selected from those studied by both FACS and IHC to represent cell lines with a range of Her-2/neu protein expression from low to high. (summarized in Table 1B). Fig. 4 shows representative examples of the results in interphase (Fig. 4, A, B, E, and F) and metaphase (Fig. 4, C and D). Typical results in breast cancer (SKBR3) showed marked Her-2/neu gene amplification as shown by the massive amount of red (Her-2/neu) signals (Fig. 4A). The Calu-3 NSCLC lung cancer cell line also had gene amplification. The interphase nuclei displayed one to three large clusters of Her-2/neu (red signal) and two to six copies of...
Numerous small Her-2/neu signals (≈30) within each cluster indicate gene amplification but make detailed quantification of the copy number impossible. Metaphase analysis of the Calu-3 line showed that the amplification occurred in hsrs, as illustrated in Fig. 4C. Spectral karyotyping (Fig. 4D) identified three distinct types of derivative chromosome 17: der(17)t(12;17), der(17)t(2;17), and der(17)t(15;16;17). The first two were submetacentric chromo-

Table 1 Results of FACS, IHC, and FISH analysis of cell lines by histology

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<th>Histology</th>
<th># Tested</th>
<th>Mean%</th>
<th>Mean MFI</th>
<th>% MFI&gt;4</th>
<th># Tested</th>
<th>%2+/3+</th>
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<th>Average</th>
<th>Max</th>
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<td>Adeno</td>
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<td>33%</td>
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<td>80%</td>
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* FACS assay.
* IHC-HercepTest assay.
* FISH assay.
* ND, not done.
* Average, average no. Her-2 genes/cell.
* Max, maximum copies Her-2 gene/cell.
omes with clusters of Her-2/neu signal in hsr in the middle of the long arm. The third derivative was a large submetacentric chromosome with an apparently normal copy of Her-2/neu. A normal appearing copy of chromosome 17 was observed in few of the metaphases. In this lung adenocarcinoma cell line and in the breast cancer lines there was a good correlation between of the metaphases. In this lung adenocarcinoma cell line and in the breast cancer lines there was a good correlation between the gene amplification and marked protein overexpression.

Among the other NSCLC cell lines, hsr were not observed nor was there true gene amplification with gene:chromosome ratios of >1.1. However, 5 of the 6 NSCLC cell lines had increased Her-2/neu copy numbers and increased numbers of chromosome 17 with gene:chromosome ratios of 0.6:1.1. For example, the NCI-H332 adenocarcinoma cell line (Fig. 4E) had as many as 14 copies of Her-2/neu in some cells with an average of 6.5 copies of Her-2/neu/cell. There was an average of 5.7 copies of chromosome 17/cell (ratio, 1.14). The chromosome 17 content in this cell line represented 5–6 copies of a normal chromosome 17 and a der(6)t(6;17). The derivative chromosome displayed bright fluorescence signals in the metaphase and a patchy pattern of fluorescence in interphase nuclei suggesting duplication of the Her-2/neu gene. This cell line had moderate expression of Her-2/neu by FACS (MFI, 9.8) and IHC (2+). Thus, the level of Her-2/neu protein expression may be attributable to both a gain in chromosome and gene number, and the FISH gene expression results are concordant with the FACS and IHC protein expression results.

Three of 6 NSCLC cell lines examined by FISH had maximum Her-2/neu gene copy numbers of five to eight with average copy numbers of three to four and chromosome 17 numbers of three to 5.3. For example, the A549 adenocarcinoma (Fig. 4F) and the NCI-H460 (data not shown) large cell carcinoma cell lines had weak cell surface protein expression (MFI, 3.7 and 2.4, respectively) and showed balanced aneusomy by FISH. The A549 line was near triploid with an average of 3.7 copies of both chromosome 17 and the Her-2/neu gene/cell. NCI-H460 was hyperdiploid with trisomy for both chromosome 17 and Her-2/neu, whereas 1 of 200 cells had a clustered gene amplification. Unbalanced aneusomy was observed in some NSCLC lines. For example, the NCI-H1435 line had an average of 5.3 copies of chromosome 17/cell but only three copies of Her-2/neu (data not shown). The centromeric signal was found in four distinct types of chromosome, none of which resembled a normal chromosome 17. Other chromosomes carrying chromosome 17 material were a large metacentric and a medium-sized submetacentric chromosome detected in ~50% of the cells with one copy/cell. The most common was a small acrocentric chromosome probably del(17)(q11) usually present in more than two copies/cell. A large metacentric and a medium-sized submetacentric chromosome were detected in ~50% of the cells with one copy/cell. These three chromosomes carried no Her-2/neu sequences. The other derivative 17 was medium sized and metacentric with Her-2/neu sequences close to the centromere. In addition, two copies of a large submetacentric chromosome, der(14)t(8;6;14;17), also carried the Her-2/neu sequences in the distal region of the short arm. Although the ratio of Her-2/neu:chromosome 17 was <1, the gene copy number per cell matched the ploidy level of 3n, and the protein expression observed may have been attributable to the aneusomy. One NSCLC cell line had a maximum and average Her-2/neu copy number of two per cell.

Fig. 3 Correlation of various assessments of Her-2/neu cell surface expression by various methods and Her-2/neu gene copy number by histology □, Adeno; △, Squamous; ○, Large; ∇, SCLC; ▽, Breast. A, correlation between the cell surface Her-2/neu expression as assessed by IHC and by FACS with 27 cell lines that had both tests performed. There was an excellent correlation (r = 0.57; P = 0.002). The correlation was best at the highest and lowest values. B, correlation between the average and maximum number of copies of the Her-2/neu gene/cell. The correlation was excellent across all histologies (r = 0.91; P = 0.0003). C, correlation between the cell surface expression by FACS (MFI) and the maximum gene copy number as assessed by FISH in the 10 representative cell lines that had both analyses done. Once again, there was an excellent correlation (r = 0.76; P = 0.011). D, maximum copies of Her-2/neu gene by histology.
SCLC cell lines never had Her-2/neu gene amplification, and both cell lines examined had balanced aneusomy. For example, the NCI-H345 cell line was near triploid with an average of three copies of chromosome 17 per cell, each harboring one copy of Her-2/neu (data not shown). In the near diploid SHP-77, there were three copies of normal or derivative chromosome 17 per cell but only two copies of Her-2/neu, because one derivative was an isochromosome of the short arm (data not shown). The fact that no cell surface Her-2/neu expression was observed in these SCLC cell lines indicates that there are also abnormalities in gene transcription or translation or post-translational modification of the protein in SCLC cells.

There was an excellent correlation \( r = 0.91; P = 0.0003 \) between the maximum and average Her-2/neu gene copy number per cell (Fig. 3B). There was also an excellent correlation \( r = 0.76; P = 0.011 \) between the maximum gene copy number and the MFI cell surface expression (Fig. 3C). Both cell lines (SKBR3 and Calu-3) with marked gene amplification and copy numbers exceeding 32 had marked Her-2/neu protein overexpression with MFIs of 43 and 36, respectively, and 3+ HercepTest. One NSCLC cell line (NCI-H322) had a maximum gene copy number of 14 and an MFI of 9.8 with a 2+ HercepTest. Weak cell surface expression \( (\text{MFI} \leq 4 \text{ and HercepTest } 0 \text{ or } 1+) \) was observed in 7 lung cancer cell lines that all had maximum Her-2/neu gene copy numbers of less than eight. By cell type, the average maximum Her-2/neu gene copy number per cell was 23 for adenocarcinoma, 6 for large cell carcinoma, 4 for small cell carcinoma, 2 for squamous carcinoma, and 42 for breast cancer cell lines (Fig. 3D).

Effects of Trastuzumab on Cell Cycle Distribution. The effects of trastuzumab on the cell cycle distribution in the breast cancer cell line SKBR3; the NSCLC cell lines Calu-3, NCI-H322, A549, and NCI-H157; and the SCLC cell line SHP-77 are shown in Table 2. In the SKBR3 breast cancer cells, trastuzumab produced a statistically significant G1 cell cycle arrest with the percentage of cells in G1 increasing from 63% to 68% with a corresponding decrease in the fraction of cells in S phase from 25% to 20%. Trastuzumab also produced a G1 cell cycle arrest in the Her-2/neu-expressing NSCLC cell lines Calu-3 (MFI 36) and NCI-H322 (MFI 9.8) with an increase in G1 from 41% to 47% and 57% to 62% in the two lines, respectively. In the low expressing NSCLC line A549 (MFI 3.7), there was a smaller increase in the G1 fraction from 69% to 71%. There was no change in the cell cycle distribution in NCI-H157, which has low HER-2/neu expression (MFI 2.8) and SHP-77 cells, which lack Her-2/neu cell surface protein expression. No apoptosis was seen in any cell line after a 120-h exposure to trastuzumab.

Effects of Trastuzumab on Cell Growth. Varying concentrations of trastuzumab were added to cultures of cell lines, and the effect on cell growth was assessed in MTT assays in triplicate. Trastuzumab partially inhibited the growth of the markedly overexpressing breast cancer cell line SKBR-3 in a dose-dependent manner but had no effect on SCLC cell lines.
such as SHP-77, which lack Her-2/neu expression (Fig. 5). The growth of the breast cancer line SKBR-3 was inhibited by concentrations as low as 0.1 μM. Interestingly, even high concentrations failed to completely inhibit growth in this breast cancer line with marked gene amplification. The NSCLC cell line Calu-3 with high Her-2/neu expression was growth inhibited to a similar degree as SKBR-3. In contrast, higher concentrations of trastuzumab were required to inhibit the moderately Her-2/neu-expressing NSCLC cell lines NCI-H322 and A549. For example, trastuzumab concentrations of 10 μM were required to produce any growth inhibition of these cell lines, and only partial inhibition was observed at high concentrations (30 μM).

### Effects of Combinations of Trastuzumab and Chemotherapeutic Agents on Cell Growth

We used the isobologram CI method of Chou and Talalay (28) to quantitate the combined effects of trastuzumab with gemcitabine, vinorelbine, paclitaxel, and cisplatin (Table 3). Fig. 6 summarizes the results with trastuzumab and gemcitabine on four cell lines (SKBR-3 breast cancer, NCI-H322, A549 NSCLC, and SHP-77 SCLC). As shown in Fig. 6A, synergy (a CI <1) was observed on the overexpressing breast cancer cell line SKBR-3 at all of the concentrations of trastuzumab and gemcitabine. Synergy was observed with trastuzumab and gemcitabine on the NSCLC cell lines NCI-H322 (Fig. 6B) and A549 (Fig. 6C). In NCI-H322 (MFI, 9.8; IHC, 2+) the CI values were consistently <1 at all of the concentrations of both trastuzumab and gemcitabine, and the CIs were even lower than those observed with SKBR3 indicating strong synergy despite the fact that receptor expression was at a lower level. Surprisingly, synergy was as great in the moderately Her-2/neu-expressing NSCLC cell line A549 (MFI, 3.7; IHC, 1+) as in SKBR3 and H322. Trastuzumab failed to improve the growth inhibition of gemcitabine on the SCLC cell lines that do not express Her-2/neu as expected (Fig. 6D).

### Table 2 Effects of 10 μM trastuzumab (120-h exposure) on the cell cycle

<table>
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<tr>
<th>Cell line</th>
<th>MFI</th>
<th>%G0/G1 Exp1</th>
<th>%S Exp1</th>
<th>%G2/M Exp1</th>
<th>%G0/G1 Exp2</th>
<th>%S Exp2</th>
<th>%G2/M Exp2</th>
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<td>SKBR3</td>
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<td>63</td>
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<td>ND</td>
<td>ND</td>
<td>12</td>
<td>ND</td>
<td>47</td>
<td>25</td>
<td>ND</td>
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<tr>
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<td>68</td>
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<td>20</td>
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<td>18</td>
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<tr>
<td>10 μM</td>
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<td>47</td>
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<td>ND</td>
<td>ND</td>
<td>17</td>
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</tbody>
</table>

*a ND, not done.*
Table 3 summarizes the results of combinations of trastuzumab with gemcitabine, vinorelbine, paclitaxel, and cisplatin on the NSCLC cell lines A549, Calu-3, NCI-H322, and the breast cancer cell line SKBR3. Additive or greater combination effects were observed with all of the combinations. There were no obvious differences in the CI value based on the degree of cell surface expression for those cell lines that expressed Her-2/neu for any of the combinations. At drug concentrations in the IC30–70 range and trastuzumab concentrations of 4–10 μM, the average CI was 0.36 for Calu-3, 0.38 for NCI-H322, and 0.39 for A549 cells. It is quite interesting that marked synergy was observed even in A549 cells that do not have Her-2/neu gene amplification. Each of the chemotherapeutic agents had similar effectiveness when combined with trastuzumab. At these concentrations, the mean CI for the four drugs against the NSCLC cell lines were 0.26 for cisplatin, 0.55 for gemcitabine, 0.62 for paclitaxel, and 0.69 for vinorelbine.

**DISCUSSION**

The results of this study show that NSCLC cell lines often express moderate levels of cell surface Her-2/neu, whereas SCLC cell lines do not. The level of expression of cell surface Her-2/neu in the NSCLC cell lines is lower than in breast cancer cell lines, and the mechanism of overexpression is different. Whereas breast cancer cell lines overexpress Her-2/neu because of gene amplification in the majority of cases (17, 18), we found that gene amplification occurred infrequently in NSCLC cell lines (17%). Only the Calu-3 cell line had true gene amplification with a Her-2/neu gene:chromosome 17 ratio exceeding 2. One additional NSCLC cell line had a maximum Her-2/neu gene copy number of 14 with an average of 6.5. We found that the low to moderate cell surface expression of Her-2/neu in NSCLC is most often attributable to increased copy number from chromosome duplication and polysomy. For example, the...
cell line NCI-H322 has 6.5 copies of Her-2/neu and 5.7 copies of chromosome 17, and A549 has 3.7 copies of the gene and chromosome. In other studies the frequency and degree of Her-2/neu cell surface expression in lung cancer specimens (defined as 2+/3+ or 3+/3+) ranged from 13% to 54% and averaged 31% (2–12). In this study, 26% (5/19) of NSCLC cell lines were 2+ or 3+ by HercepTest, but only one was 3+. By FACS analysis 32% (7/22) had a MFI >4, but only 2 (9%) had an MFI >8. Total absence of Her-2/neu expression was uncommon in NSCLC cell lines, because only 3 had no cell surface expression by FACS, only 1 had a maximum gene copy number <4, and 7 had 0+ staining by HercepTest. The majority of NSCLC cell lines had mild to moderate Her-2/neu expression, 16 by all three methods including 77% by FACS, 67% by FISH, and 58% by HercepTest.

This communication is the first report with detailed comparisons of Her-2/neu expression by FACS, IHC, and FISH in lung cancer cell lines. There are several ways to quantitate Her-2/neu gene expression by FISH including the average or maximum copy number, the ratio of copy number:chromosome number, or the percentage of cells with unbalanced copy number. We found a good correlation of the results of each of these analyses in this study and in a study of samples from lung cancer patients (20). Among the 53 lung cancer patient samples evaluated by FISH in the companion study, we found few instances of gene amplification (5%) and many instances of increased copy number (20%) among 45 NSCLC patient tumors. There were no instances of gene amplification or copy number >4 in SCLC tumors, and no SCLC specimen had >1+ protein expression. In the present study of lung cancer cell lines and in breast cancer series in the literature, there was a good correlation between the FISH method of assessing Her-2/neu gene status and either the FACS or IHC method of assessing cell surface protein expression.

In literature studies of NSCLC, Her-2/neu expression has been assessed predominantly by IHC. The majority of studies found a direct relationship between prognosis and Her-2/neu expression with shorter survival in Her-2/neu+ cases. In some
reports, patients of which their tumors expressed Her-2/neu were less likely to respond to chemotherapy (22). In breast cancer, recent reports suggest that gene amplification is a superior method for assessing prognosis than protein expression by IHC (23). It needs to be determined whether increased copy number without amplification will be associated with a poor prognosis in NSCLC and whether FISH results will provide superior prognostic information compared with IHC.

There is little information in the literature regarding Her-2/neu gene or protein expression in NSCLC cells and the likelihood of response to trastuzumab alone or with chemotherapy. Both gene amplification and cell surface expression have been reported to predict response to trastuzumab alone or combined with doxorubicin or paclitaxel in breast cancer (19, 24). Recent evidence shows that gene amplification by FISH is the best predictor of response, and patients without gene amplification appear to have little benefit from trastuzumab therapy. This would imply that only a small fraction of NSCLC patients and no SCLC patients would benefit from trastuzumab therapy (19). Despite the fact that Her-2/neu overexpression and gene amplification occur less frequently in NSCLC than breast cancer, we found that marked synergistic growth inhibition occurred when standard cytotoxic chemotherapy was combined with trastuzumab in Her-2/neu-expressing NSCLC cell lines. We found a direct relationship between Her-2/neu expression and both cell cycle arrest and growth inhibition, but the synergy with chemotherapy was independent of the degree of expression in cell lines that expressed any level of cell surface Her-2/neu expression. Trastuzumab had no effect on cell cycle distribution, growth rate, or synergy with chemotherapy in nonexpressing cell lines. The results of this study predict that trastuzumab alone will produce partial objective responses in some NSCLC patients of which their tumors express Her-2/neu. Furthermore, the best responses will occur in the uncommon patient with marked HER-2/neu overexpression by gene amplification, because only Her-2/neu-expressing cell lines were growth inhibited, and the high expressing Calu-3 line was growth inhibited the most. The study results also suggest that synergy between trastuzumab and chemotherapeutic agents will exist in lung cancer as it does in breast cancer if the tumor expresses Her-2/neu, even if the degree of expression is low. Additionally, the results of this study suggest that among all of the agents tested here, the greatest synergy may exist between trastuzumab and gemcitabine. On the basis of this study, patients with overexpression of Her-2/neu should be considered for clinical trials. Randomized Phase III trials of chemotherapy alone versus chemotherapy plus trastuzumab in NSCLC patients with gene amplification would be most likely to determine the role for trastuzumab in NSCLC, but these studies will require ingroup participation because of the low frequency of gene amplification.

A number of clinical trials have been instituted in the first and second line therapy of advanced lung cancer. There are no randomized trials evaluating trastuzumab in NSCLC patients, but preliminary results from Phase II combination studies are just appearing in the literature (30). Randomized trials will be warranted if the response rates do not appear higher than those in historical series; the number of patients with 3+ Her-2/neu expression or gene amplification is very small, and these 3+ patients appear to do worse when treated with chemotherapy alone.

REFERENCES


Expression of Her-2/neu in Human Lung Cancer Cell Lines by Immunohistochemistry and Fluorescence in Situ Hybridization and its Relationship to in Vitro Cytotoxicity by Trastuzumab and Chemotherapeutic Agents

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