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Interrelationships between Cellular Nucleotide Excision Repair, Cisplatin Cytotoxicity, HER-2/*neu* Gene Expression, and Epidermal Growth Factor Receptor Level in Non-small Cell Lung Cancer Cells

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Nucleotide excision repair (NER) is a major repair mechanism for DNA lesions induced by cisplatin. Overexpressions of epidermal growth factor receptor (EGFR) and HER-2/*neu* have been reported to affect the sensitivity of certain human cancer cells to cisplatin, presumably by modification of DNA repair activity through interference with NER. Using an *in vitro* repair assay, we investigated NER activity of cisplatin-induced DNA lesions in a panel of 16 non-small cell lung cancer (NSCLC) cell lines. The interrelationships between NER activity, cisplatin sensitivity, HER-2/*neu* expression and EGFR level, were also analyzed. The results showed that high NER activity was closely correlated with cisplatin resistance and high levels of HER-2/*neu* expression ($P<0.05$). Analysis of the relationships between EGFR level and each of the other three parameters revealed no statistically significant correlations (all P values were >0.05 by Spearman rank correlation), but a trend of association (all the values of proportion of accordance were ≥62.5% by using a 2×2 contingency table). These results suggest that NER activity may play an important role in the cisplatin resistance of NSCLC cells and there may be an association between enhanced NER activity and high levels of p185^{neu} and probably EGFR in NSCLC cells. The finding that high levels of EGFR showed very little influence on the relationship between p185^{neu} and cisplatin resistance suggests that EGFR may be a less crucial factor in modulating the chemoresistance of NSCLC cells when compared with HER-2/*neu*.

Key words: Nucleotide excision repair — Cisplatin resistance — Epidermal growth factor receptor — HER-2/*neu* — Non-small cell lung cancer

The *erbB* gene family includes erbB-l (the gene encodes epidermal growth factor receptor, EGFR), *erbB-2* (also known as HER-2/*neu*), *erbB-3* and *erbB-4* genes. These four genes encode for receptor-type tyrosine-protein kinases and are usually co-expressed in various combinations in a variety of human tissues.[1, 2] Constitutive overexpression of *EGFR* gene and/or HER-2/*neu* gene is a frequent event in a variety of human tumors, including non-small cell lung cancer (NSCLC), but not small cell lung cancer.[3–15] In some types of tumors, aberrations of the genes may predict a poor prognosis and shorter survival.[7–15] In recent years, many studies have explored the effects on chemosensitivity resulting from altered expression and activation of the HER-2/*neu* receptor and the EGFR, indicating that alteration of the activation status of growth factor receptors not only can lead directly to the perturbation of growth regulation, but also may affect the sensitivity of cancer cells to chemotherapeutic agents.

We and others have linked a high level of HER-2/*neu* protein product, p185^{neu}, to chemoresistant phenotype in human NSCLC and breast cancer cell lines.[16–22] Tumor cell lines overexpressing p185^{neu} have been shown to be more resistant to cisplatin, taxol, etoposide, doxorubicin, melphalan, mitomycin-C, and carmustine.[16–20] A direct correlation between p185^{neu} and chemoresistance has been demonstrated by desensitizing HER-2/*neu*-transfected cancer cells to anticancer drugs through elevation of the levels of p185^{neu},[18–21] and sensitizing p185^{neu}-overexpressing cancer cells to anticancer drugs by inhibiting p185^{neu} tyrosine kinase activity[22, 23] or with p185^{neu}-specific antibodies.[24–27] While the mechanism by which p185^{neu} confers intrinsic chemoresistance remains unclear, several lines of evidence suggest that high-pl85^{neu}-expressing cancer cells may possess an enhanced DNA repair capacity.[25–29] However, the connection between HER-2/*neu* gene overexpression and intrinsic chemoresistance profiles in human cancers is controversial. Pegram *et al*. demonstrated that HER-2/*neu* overexpression was not sufficient to induce intrinsic, pleomorphic drug resistance *in vitro* and *in vivo*,

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and changes in chemosensitivity profiles resulting from HER-2/*neu* transfection observed *in vitro* were cell line-specific.[30]

The association between over-expression of EGFR and chemosensitivity, however, is less consistent. Activation and elevation of EGFR have been shown to sensitize human cancer cells of breast, ovary, head and neck, cervix, colon, pancreas and prostate, as well as NSCLC to several classes of anticancer agents, including cisplatin and carboplatin, 5-fluorouracil, melphalan, and taxo1.[31–33] The modulating effect of epidermal growth factor (EGF) on anticancer agents has been demonstrated to be dependent on the number of EGFR and associated with downregulation of a form of DNA repair or inhibition of this activity.[32, 33] In contrast, several reports have demonstrated that elevation of EGFR levels in human breast cancer cells might lead to increased chemoresistance to doxorubicin, vinblastine, and cisplatin, as well as 5-fluorouracil.[34] Reduction of receptor tyrosine kinase by EGF receptor-blocking antibodies resulted in enhanced antitumor activities of cisplatin and doxorubicin in human breast and squamous cancer cells *in vitro*, and in some cases *in vivo*, by a mechanism involving downregulation of DNA repair.[35–37]

The approximate 50% sequence homology between genes of $p185^{neu}$ and EGFR[38] suggests that the biological functions of these two genes may be related. After binding to the receptors erbB-3 and erbB-4, heregulin or *neu* differentiation factor can transactivate $p185^{neu}$ by inducing heterodimeric associations and cross-phosphorylation.[39, 40] Similarly, EGFR ligands (EGF, transforming growth factor-α, amphiregulin) can also transactivate $p185^{neu}$.[41, 42] The transactivation of $p185^{neu}$ by the EGFR is biologically relevant since it stoichiometrically alters $p185^{neu}$ phosphorylation, stimulates *in vitro* kinase activity,[41–44] and correlates with *in vivo* synergy in the transforming activity of these two receptors.[45] However, whether the constitutive co-expression status of these two *erbB* genes is related to the sensitivity of cancer cells to anticancer agents is not clear.

The above findings indicate that high levels of EGFR or $p185^{neu}$ may affect the sensitivity of cancer cells to several classes of anticancer agents including cisplatin, a commonly used DNA-damaging anticancer agent, in a variety of different experimental systems, presumably by modification of DNA repair activity. Although the mechanisms of cisplatin resistance are multifactorial, efficient repair of the affected DNA is thought to be one of the major causes. Cisplatin forms a variety of DNA adducts, the most prevalent of which is the 1,2-intrastrand crosslink (>90%).[46] Recent work revealed that 1,2-intrastrand crosslinks are repaired via the nucleotide excision repair (NER) pathway.[47] An augmented NER activity which may result from malignant transformation or progression, therefore, could play an important role in the cisplatin resistance of some types of human cancer.

Because the above observations suggest a connection between *erbB* genes and DNA repair, predictably involving NER, we were interested in the constitutive expression status of the *EGFR* and the HER-2/*neu* genes and its association with intrinsic chemoresistance and NER ability. Using a panel of 16 NSCLC cell lines, we applied an *in vitro* repair assay to investigate NER of cisplatin-induced DNA lesions and examined the relationships between NER activity and each of the three parameters, namely, cisplatin sensitivity, EGFR level and HER-2/*neu* expression. Our results in the present study demonstrate an association between cisplatin resistance and enhanced NER activity, overexpression of HER-2/*neu* gene, and probably EGFR level in the tested NSCLC cells. The results of the statistical analysis suggested that the correlation between the chemoresistance and constitutive expression of EGFR was less significant than the correlation between the chemoresistance and the expression of HER-2/*neu* in NSCLC.

MATERIALS AND METHODS

Cell lines and cell culture We studied the following 16 NSCLC cell lines, which were established and characterized in the NCI-Navy Medical Oncology Branch (Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) from tumor specimens obtained from previously untreated patients: ten adenocarcinomas (NCI-H23, H322, H358, H441, H522, H820, H838, H1355, H1435 and H1437); two adenosquamous carcinomas (H125 and H647); three large cell carcinomas (H460, H1155 and H1299), and one squamous cell carcinoma (H226).[16, 17, 29] All of the cell lines had been maintained in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum for >6 months before being tested.

***In vitro* chemosensitivity testing for cisplatin** The tetrazolium dye colorimetric assay was used to evaluate the chemosensitivity for cisplatin (Farmitalia Carlo Erba, Milano, Italy) of the cell lines. The optimal seeding density of individual cell lines was predetermined, as in our previous studies.[16, 17] Cisplatin was first dissolved in phosphate-buffered saline to 1 m*M*, and then diluted in culture medium to the desired concentrations. The duration of drug exposure was 96 h. The percentage of control absorbance was considered to represent the surviving fraction of cells and the IC_{50} values were defined as the concentrations of drug which produced 50% reduction in control absorbance. The experiments were performed in triplicate.

Nucleotide excision repair assay The *in vitro* nucleotide excision repair assay of Hansson and Wood[48] was adopted and has been detailed previously.[49] A cisplatin-damaged plasmid was used as the substrate for the repair proteins in whole-cell extracts to quantify the DNA resynthesis activity. The essentials of this assay are summarized below.

Plasmids The plasmid substrate for the repair assay was a 2959-bp pBS (pBluescript KS$^+$; Stratagene, La Jolla, CA) pretreated with cisplatin to induce DNA cross-links. Platination of pBS was performed according to the method of Hansson and Wood, using a drug-to-nucleotide molar ratio of 0.005. A 3738-bp undamaged plasmid, pHM14, was used as a negative control to determine the background noise due to nonspecific incorporation of radioactive [α-^{32}P]dCTP.

Cell extracts Whole cell extracts from each cell line were prepared according to the method of Manley[50] with minor modifications as described by Shivji *et al*.[51]

Repair reaction Cell extracts (100 mg) were incubated in a standard 50 μl reaction mixture containing 300 ng each of cisplatin-damaged pBS and nondamaged pHM in 45 m*M* Hepes-KOH (pH 7.8), 70 m*M* KCI, 7.4 m*M* $MgCl_2$, 0.9 m*M* dithiothreitol, 0.4 m*M* EDTA, 2 m*M* ATP, 40 m*M* phosphocreatine, 2.5 mg of creatine phosphokinase, 3.4% glycerol, 18 mg of bovine serum albumin, 20 μM each of dGTP and dTTP, 4 m*M* each of dATP and dCTP, and 2 mCi of [α-^{32}P]dCTP. The reaction was carried out at 30°C for 5 h before being terminated by the addition of 20 m*M* EDTA. Plasmid DNAs were purified, linearized by *Bam*HI, and subjected to electrophoresis. The DNA content in ethidium bromide-stained bands was quantified by densitometry using photographic negatives and compared to the standard. The incorporation of radioactive nucleotides was quantified with a Betascope (Betagen, Waltham, MA). DNA repair synthesis, expressed as specific incorporation of [α-^{32}P]dCTP, was calculated by subtraction of nonspecific incorporation by the nondamaged pHM control from the total incorporation by the cisplatin-damaged pBS substrate. The experiments were performed in triplicate.

Quantitative measurement of the gene expression Nearly confluent cells in the logarithmic growth phase were harvested, and 100 μg of protein from each sample was electrophoresed on 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gels. Detection of *EGFR* and HER-2/*neu* gene expression was carried out by standard western blotting analysis using specific antibodies. The primary monoclonal antibodies used for immunoblot analyses were b-3 (1:250) against p185^{neu} protein (Oncogene Science, Inc., Uniondale, NY) and anti-EGFR (1:250) recognizing EGFR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoblot analysis of EGFR was performed first. After stripping of the EGFR signals, the same blot was immunoblotted against anti-pl85^{neu}. Immunoblotting for β-actin was performed last after stripping of the p185^{neu} signals. The intensity of signals was quantitated by soft laser densitometry (Model SLR-2D/1D; Biomed, Fullerton, CA). The signal intensity of the cell line H23 was arbitrarily assigned a value of 1 unit, and the signals of the other cell lines were quantitated relatively to the signal

Table I. Summary of the IC_{50} Values of Cisplatin, NER Activity, and Levels of p185^{neu} and EGFR in the Tested NSCLC Cell Lines[a)]

Cell line	Cell type[b)]	Cisplatin IC_{50} (μM)	NER activity (dCMP, fmol)	p185^{neu} (relative units)	EGFR (relative units)
H1155	LC	1.75±0.06	54.7±9.3	0.47±0.25	0.75±0.30
H23	A	0.51±0.01	57.3±8.3	1.00	1.00
H226	S	5.00±0.17	62.0±13.5	1.87±0.46	6.28±0.65
H1299	LC	2.27±0.38	44.0±19.3	1.93±1.36	1.93±0.34
H460	LC	0.45±0.02	69.7±16.3	2.60±0.81	2.49±0.19
H125	AS	1.83±0.17	17.0±3.0	3.15±0.55	1.34±0.04
H358	A	1.10±0.34	84.3±14.5	4.35±1.71	1.46±0.19
H838	AS	2.18±0.23	37.1±19.2	6.82±2.31	1.95±0.08
H322	A	1.92±0.18	79.2±9.5	6.89±1.76	4.95±0.19
H1437	A	2.93±0.08	88.0±18.4	7.62±2.71	2.53±0.64
H1355	A	7.66±2.18	222.2±19.8	8.02±1.67	1.14±0.07
H441	A	3.74±1.11	40.0±10.8	9.66±1.23	2.47±0.42
H647	AS	5.63±0.52	129.0±15.7	9.86±2.49	4.61±0.55
H820	A	5.53±0.46	64.3±5.2	11.95±1.95	7.17±1.40
H1435	A	20.45±0.80	120.5±29.0	13.47±3.21	2.69±0.89
H522	A	5.27±0.64	93.3±10.2	14.97±2.47	1.30±0.35

a) The results are the means (± SE) of three independently performed assays.
b) A, adenocarcinoma; AS, adenosquamous cell carcinoma; LC, large cell carcinoma; S, squamous cell carcinoma.

intensity of H23. The protein levels were also normalized by densitometry of the β-actin signal. The experiments were performed in triplicate.

Data analysis The Spearman rank correlation was used for correlation analyses. The relationship of each pair of observed parameters was also examined by using a 2×2 contingency table, from which the value of proportion of accordance (POA) was calculated. According to the value (level) of each parameter, the 16 cell lines tested were divided into two equal groups, i.e., high- and low-level groups. POA=[(number of the cell lines which had high levels of both parameters A and B+number of the cell lines which had low levels of both parameters A and B)/16]×100%. For example, the POA value of NER activity vs. cisplatin chemosensitivity=(5+5)/16=62.5% (Fig. 3A). POA≥62.5% was arbitrarily chosen as a cut-off point for identification of an association between the two observed parameters.

RESULTS

Cisplatin sensitivity The chemosensitivity to cisplatin (expressed as IC_{50} value) was determined in 16 NSCLC cell lines and the results are shown in Table I. The IC_{50} value of the most resistant cell line H1435 (20.45 μM) was 45-fold higher than that of the most sensitive cell line H460 (0.45 μM).

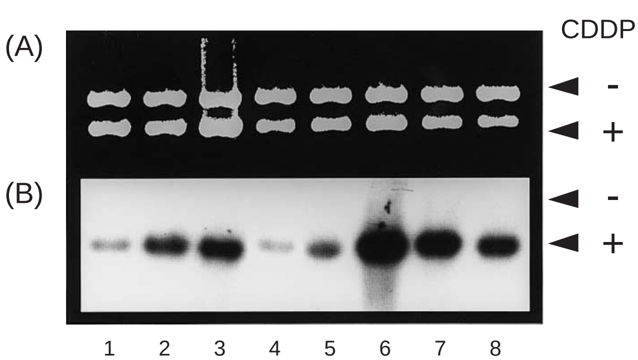

Fig. 1. Nucleotide excision repair of cisplatin-modified DNA with cell-free extracts. Cisplatin-damaged Bluescript plasmid (pBS) and a nondamaged pHM14 (pHM) control were incubated with whole-cell extracts (95 μg protein equivalvent) from each cell line to measure the specific incorporation of [α-^{32}P]dCTP into the damaged pBS. The band intensity was quantified with a Betascope. The repair activities, expressed as specific incorporation of [α-^{32}P]dCTP, are summarized in Table I. H125, lane 1; H460, lane 2; H647, lane 3; H838, lane 4; H1155, lane 5; H1355, lane 6; H1435, lane 7; and H1437, lane 8. (A) Ethidium bromide staining to verify DNA gel loading. (B) Autoradiographic results of (A) showing the incorporation of [^{32}P]dCTP into cisplatin-modified pBS. CDDP, cisplatin.

NER activity NER activity was determined by means of the *in vitro* NER assay. Fig. 1 demonstrates the NER activity of cisplatin-modified DNA with cell-free extracts from 8 of the 16 cell lines tested. Table I shows the mean value

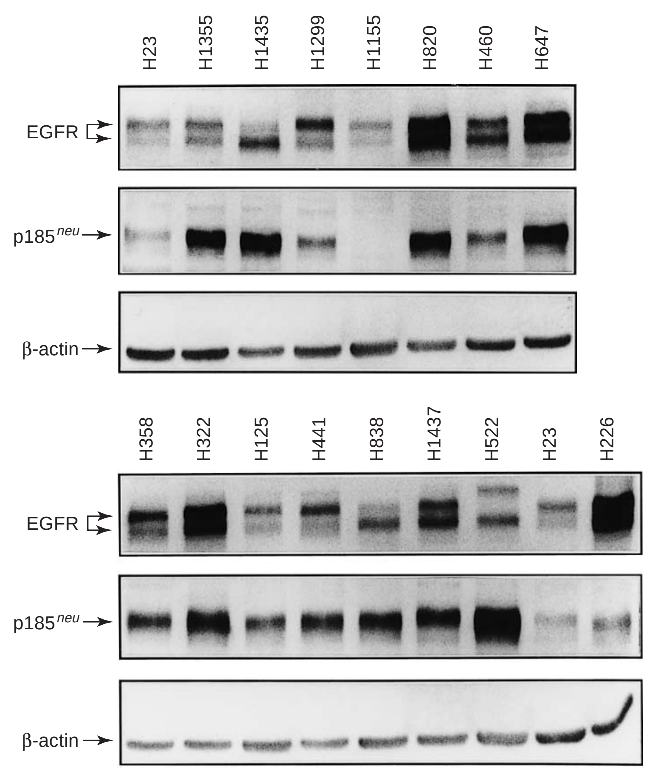

Fig. 2. A representative immunoblot analysis showing the expression of (a) EGFR, (b) HER-2/*neu*-encoded p185^{neu}, and (c) β-actin in the cell lysates of the 16 NSCLC cell lines examined. Cell extracts were prepared, and 100 μg of protein from each sample was electrophoresed and immunoblotted against the appropriate antibodies. Immunostaining for EGFR was performed first. After stripping of the EGFR signals, the blot was immunostained for p185^{neu}. Immunostaining for β-actin (43 kD) was performed last, using the same blot after stripping of the p185^{neu} signals. The intensity of the signals was quantitated with a soft laser densitometer (Model SLR-2D/1D; Biomed). The signal intensity of the cell line H23 was arbitrarily assigned a value of 1 unit, and the signals of the other cell lines were quantitated relatively to the signal intensity of H23. The EGFR and p185^{neu} protein levels were also normalized with respect to the β-actin signal. The levels of EGFR and p185^{neu} shown in Table I were the means of three independently performed analyses. The doublet signals of EGFR in different cell lines are most probably due to different mobility of the protein being differentially phosphorylated at serine and/or threonine residues during cell cycle progression. The signal intensity of the doublet bands is used to represent the constitutive level of EGFR expression. A similar but less marked pattern of structural modifications is also noted in p185^{neu}, as shown in this figure and previous reports.[29)]

of three experiments; the range of NER activity in this panel of 16 cell lines was from 17 to 222 fmol dCMP incorporation (H125 and H1355, respectively), with a 13-fold difference.

Constitutive levels of p185^{neu} and EGFR The mean values of three experiments in which either p185^{neu} or EGFR was quantitated by western blotting and densitometry were expressed as relative units (Table I). One example of three immunoblot analysis experiments is shown in Fig. 2 to illustrate the levels of EGFR and HER-2/*neu* expression of the entire panel of cell lines. Table I shows that the highest level of p185^{neu} in H522 (14.97) was 32-fold higher than the lowest level in H1155 (0.47) (Table I). In this panel of 16 cell lines, the level of HER-2/*neu* gene expression has been determined by northern blotting,[16] antibody-sandwich enzyme-linked immunosorbent assay (ELISA),[17, 29] and western blotting (the present study). The results obtained from the three different methods are closely correlated with each other (r=0.85–0.91 and $P \leq 0.001$ by the Spearman rank correlation test). There was a larger gap in the levels of EGFR expression (between 2.53 and 4.61). The highest level of EGFR in H820 (7.17) was 9.6-fold higher than the lowest level in H1155 (0.75) (Table I). Four cell lines, H647, H322, H226 and H820, expressed a level of EGFR>4.5 relative units (Table I).

Statistically significant correlation between each pair of cisplatin chemoresistance, NER activity, and p185^{neu} The Spearman rank correlation analysis showed that the correlations between the level of NER activity and the IC_{50} value of cisplatin (r=0.524, P=0.043), and the level of

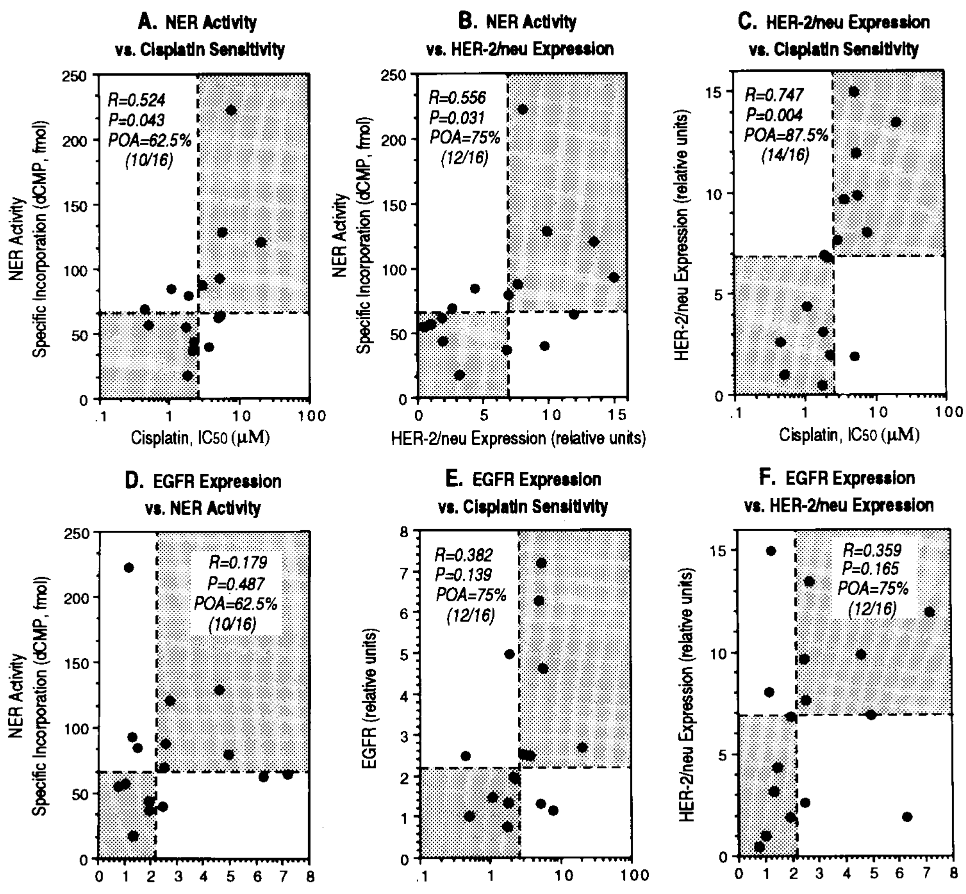

Fig. 3. Interrelationships between cisplatin sensitivity, NER activity, HER-2/*neu* gene expression and *EGFR* gene expression. *In vitro* cisplatin sensitivity assay was performed using the tetrazolium colorimetric assay. NER activity was determined by using an *in vitro* NER assay (see "Materials and Methods" and Fig. 1). Immunoblot analyses were performed to detect the expression of HER-2/*neu* gene and the *EGFR* gene. The intensity of the signals was quantitated with a soft laser densitometer (Model SLR-2D/1D; Biomed) (Fig. 2). The results were the means of three independently performed experiments. The R and P values calculated by using the Spearman rank correlation test and the values of POA (see "Data analysis") are labeled. The dashed line (horizontal or vertical) is applied to separate the cell lines in two equal groups (high- and low-level groups) according to the corresponding parameter (see "Data analysis").

p185^{neu} (r=0.556, P=0.031) were statistically significant: the greater the repair activity the higher the IC_{50} values and the higher the levels of p185^{neu} (Fig. 3, A and B, respectively). The present finding that there was a close correlation between the IC_{50} value of cisplatin and the levels of p185^{neu} (r=0.747, P=0.004; Fig. 3C) confirms our previous reports[16, 17] that the higher p185^{neu}-expressing NSCLC cells are more cisplatin-resistant. These correlations indicate that cisplatin-resistant NSCLC cell lines express a high level of p185^{neu} and have a greater NER activity. The data were also analyzed by the use of a 2×2 contingency table. The relationships between NER activity and the IC_{50} value, between NER activity and the level of p185^{neu}, and between p185^{neu} and the IC_{50} value showed POA values of 62.5%, 75.0%, and 87.5%, respectively (Fig. 3, A–C).

Relationships between EGFR expression and cisplatin chemoresistance, NER activity, and p185^{neu} Although Spearman rank correlation analyses did not show a significant correlation between the levels of EGFR expression and NER activity (r=0.18, P=0.49), cisplatin chemoresistance (r=0.38, P=0.14), and the HER-2/*neu* gene expression (r=0.36, P=0.16), the results of the 2×2 contingency table analysis showed that all the values of POA were ≥62.5% (62.5%, 75% and 75%, respectively) (Fig. 3, D–F). Therefore, it seemed that there was an association between the level of EGFR and each of the other three parameters (i.e., NER activity, cisplatin chemoresistance, and p185^{neu}). These results suggested that high EGFR-expressing NSCLC cells may be associated with high levels of NER activity and p185^{neu} expression and greater resistance to cisplatin.

Very little influence on the relationship between p185^{neu} and cisplatin chemoresistance by high levels of EGFR To assess whether co-expression status of EGFR and p185^{neu} would modulate the chemosensitivity constitutively, the 4 cell lines with EGFR levels>4.6 units were excluded from the 16 cell lines (there was a larger gap between 2.53 and 4.61 units in the levels of EGFR expression and the group mean value was 2.75, Table I) to see whether there was any change in the relationship between p185^{neu} and the IC_{50} values of cisplatin. As shown in Fig. 4, the regression line of the IC_{50} value of cisplatin vs. the level of p185^{neu} derived from the remaining 12 cell lines (dashed line; r=0.81, P=0.007) is very similar to and almost superimposable on that derived from the entire panel of 16 cell lines (solid line; r=0.75, P=0.004). These findings indicate that the relationship between the level of p185^{neu} and the chemosensitivity is very little influenced by even high levels of EGFR.

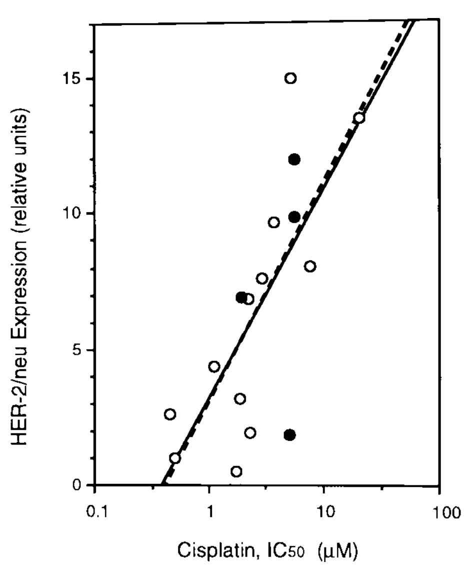

Fig. 4. Correlations of cisplatin sensitivity (expressed as IC_{50}) with p185^{neu} when the four highest EGFR expressors (EGFR levels>4.6 units, ●) were included (r=0.75, P=0.004) or excluded (r=0.81, P=0.007). The regression line derived from the entire panel of 16 cell lines (solid line) is very close to and almost superimposable on that derived from the 12 cell lines (○) with EGFR levels<2.53 units (dashed line).

DISCUSSION

Several reports have presented experimental evidence for direct involvement of DNA repair in HER-2/*neu* induced drug resistance.[25–27] Cancer cells that constitutively overexpressed HER-2/*neu* or had been engineered to overexpress human HER-2/*neu* were found to be resistant to anticancer drugs such as cisplatin, doxorubicin, etoposide, and taxol. They also showed an enhanced overall DNA repair capacity.[27, 52] In NSCLC, elevated HER-2/*neu* gene expression[16–18] and DNA repair[52] have been linked to intrinsic chemoresistance. Using a panel of cell lines, in the present study, we demonstrated a tight association between each pair of cisplatin resistance, NER activity, and HER-2/*neu* expression, suggesting that HER-2/*neu*-overexpressing NSCLC cells may have greater NER activity that leads to attenuation of the cytotoxicity of cisplatin. A high constitutive level of p185^{neu} is a predictor for intrinsic chemoresistance to DNA-damaging agents including cisplatin, doxorubicin, etoposide, carmustine, melphalan and mitomycin-C. The cytotoxic effects of these agents are closely correlated in NSCLC cells.[16] In addition to cisplatin, the sensitivity of the panel of cell lines to etoposide has been tested and showed a borderline association between the IC_{50} value of etoposide and NER activity (r=0.474 , P=0.066, data not shown; and IC_{50} etoposide vs. p185^{neu}, r=0.832, P=0.0013).[53] These findings suggest that elevated NER activity may be associated with intrinsic chemoresistance in NSCLC cells.

Although the effects of EGFR on chemosensitivity are less consistent and may be specific to particular tumor cell lines or cell types or to particular drugs, in the present study, we demonstrated a trend that a high constitutive level of EGFR may be related to cisplatin resistance and NER activity, indicating that activating the EGFR signal transduction pathway may induce chemoresistance rather than chemosensitivity in NSCLC cells. This result is in accordance with our previous finding that EGF can induce resistance to cisplatin and etoposide in most of the NSCLC cell lines tested (10/12) and this effect was accompanied and correlated with the inhibitory effect of EGF on cell proliferation.[53] While an inverse correlation between EGFR and HER-2/*neu* expression has been reported in bladder cancer tissues,[54] we found a positive but moderate association between the levels of these two *erbB* gene products in this study (POA value=75%). The finding that high levels of EGFR showed very little influence on the relationship between p185^{neu} and cisplatin resistance suggested that in the whole panel of tested cell lines the constitutive level of EGFR is a relatively less crucial biomarker for intrinsic chemoresistance when compared to HER-2/*neu*. Whether alteration of the activity of p185^{neu} or EGFR (elevated by gene transfection or blocked by specific antibodies) affects NER activity requires further investigation.

Evidence is mounting that the molecular basis for the increased susceptibility of cancer cells to anticancer drugs, and the development of treatment resistance, may originate from genetic lesions which alter apoptotic pathways. A pathway of apoptosis initiated by DNA damage is wild type p53-dependent and inactivation of p53 can lead to resistance to DNA-damaging agents (refs. 55–57 and others cited therein). Since our study demonstrated a close correlation of intrinsic multiple drug resistance to the expression of HER-2/*neu* gene in these 16 NSCLC cell lines in which mutation of *p53* gene was a common (87%) event,[16] it may be conjectured that the *erbB* genes function through a p53-independent pathway to modulate the apoptotic pathway, thus determining the fate of injured NSCLC cells. In breast cancer cells, Yu *et al.* recently reported that overexpression of p185^{neu} by transfection transcriptionally upregulates p21^{Cip1}, which associates with p34^{Cdc2}, inhibits taxol-mediated p34^{Cdc2} activation, delays cell entrance to the G2/M phase, and thereby inhibits taxol-induced apoptosis.[58] Moreover, *erbB* genes may associate with,[59] interact with or trigger some other factors to affect the status of chemoresistance.[27, 60, 61]

The effects of overexpressing EGFR and HER-2/*neu* on sensitivity of tumor cells to drugs seem to be intricate, involving some step(s) of an interconnected network of cell signaling pathways that determine malignant phenotypic characteristics of tumor cells, including metastatic potential as well as intrinsic chemoresistance. Current efforts to characterize the role of *erbB* gene products and their regulatory mechanisms will open opportunities to develop and to design specific inhibitors and strategies to overcome tumor metastasis and intrinsic chemoresistance. A clinical trial using Herceptin (a humanized monoclonal antibody directed against p185^{neu}) plus chemotherapeutic agents for treatment of metastatic breast cancer patients whose tumors overexpress p185^{neu} has demonstrated marked improvements in tumor response rate and remission time.[62] The success of Herceptin is a critical point in translational research, proving the paradigm that if we understand what are the genetic aberrations in human cancer, we can target them.

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REFERENCES

1) Parsons, J. T. and Parsons, S. J. Protein-tyrosine kinases, oncogenes, and cancer. *In* "Important Advances in Oncology," ed. V. T. DeVita, Jr., S. Hellman and S. A. Rosenberg, pp. 3–18 (1993). J. B. Lippincott Co., Philadelphia.
2) Hynes, N. E. and Stern, D. F. The biology of erbB-2/*neu*/HER-2 and its role in cancer. *Biochim. Biophys. Acta*, **1198**, 165–184 (1994).
3) Tateishi, M., Ishida, T., Mitsudomi, T., Kaneko, S. and Sugimachi, K. Immunohistochemical evidence of autocrine growth factors in adenocarcinoma of the human lung. *Cancer Res.*, **50**, 7077–7080 (1990).
4) Kameda, T., Yasui, W., Yoshida, K., Tsujino, T., Nakayama, H., Ito, M., Ito, H. and Tahara, E. Expression of ERBB2 in human gastric carcinomas: relationship between p185^{ERBB2} expression and the gene amplification. *Cancer Res.*, **50**, 8002–8009 (1990).
5) Schneider, P. M., Hung, M. C., Chiocca, S. M., Manning, J., Zhao, X., Fang, K. and Roth, J. A. Differential expression of the c-erbB-2 gene in human small cell and non-small cell lung cancer. *Cancer Res.*, **49**, 4968–4971 (1989).
6) Arteaga, C. L. Epidermal growth factor receptors and erbB-2 in human lung cancer. *In* "Lung Cancer: Principles and Practice," ed. H. J. Pass, J. B. Mitchell, D. H. Johnson

and A. T. Turrisi, pp. 99–106 (1996). Lippincott-Raven Publ., Philadelphia.

7) Maurizi, M., Scambia, G., Benedetti-Panici, P., Ferrandina, G., Almadori, G., Paludetti, G., De Vincenzo, R., Distefano, M., Brinchi, D., Cadoni, G. and Mancuso. S. EGF receptor expression in primary laryngeal cancer: correlation with clinicopathological features and prognostic significance. *Int. J. Cancer*, **52**, 862–866 (1992).
8) Neal, D. E., Marsh, C. and Bennett, M. K. Epidermal growth factor receptors in human bladder cancer: comparison of invasive and superficial tumors. *Lancet*, **i**, 366–368 (1985).
9) Sainsbury, J. R. C., Farndon, J. R., Needham, G. K., Malcolm, A. J. and Harris, A. L. Epidermal growth factor receptor status as predictor of early recurrence of and death from breast cancer. *Lancet*, **i**, 1398–1402 (1987).
10) Scambia, G., Benedetti-Panici, P., Ferrandina, G., Battaglia, F., Distefano, M., D'andrea, G., De Vincenzo, R., Maneschi, F., Ranelletti, F. O. and Mancuso, S. Significance of epidermal growth factor receptor expression in primary human endometrial cancer. *Int. J. Cancer*, **56**, 26–30 (1994).
11) Scambia, G., Benedetti-Panici, P., Distefano, M., Salerno, G., Romanini, M. E., Fagotti, A. and Mancuso, S. Epidermal growth factor, oestrogen and progesterone receptor expression in primary ovarian cancer: correlation with clinical outcome and response to chemotherapy. *Br. J. Cancer*, **72**, 361–366 (1995).
12) Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. and Press, M. Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. *Science*, **244**, 707–712 (1989).
13) Kern, J. A., Schwartz, D. A., Nordberg, J. E., Weiner, D. B., Greene, M. I., Torney, L. and Robinson, R. A. $p185^{neu}$ expression in human lung adenocarcinomas predicts shortened survival. *Cancer Res.*, **50**, 5184–5191 (1990).
14) Tateishi, M., Ishida, T., Mitsudomi, T., Kaneko, S. and Sugimachi, K. Prognostic value of c-erbB-2 protein expression in human lung adenocarcinoma and squamous cell carcinoma. *Eur. J. Cancer*, **27**, 1372–1375 (1991).
15) Allred, D. C., Clark, G. M., Tandon, A. K., Molina, R., Tormey, D. C., Osborne, C. K., Gilchrist, K. W., Mansour, E. G., Abeloff, M., Eudey, L. and McGuire, W. L. J. HER-2/*neu* in node-negative breast cancer: prognostic significance of overexpression influenced by the presence of *in situ* carcinoma. *J. Clin. Oncol.*, **10**, 599–605 (1992).
16) Tsai, C. M., Chang, K. T., Perng, R. P., Mitsudomi, T., Chen, M. H., Kadoyama, C. and Gazdar, A. F. Correlation of intrinsic chemoresistance of non-small cell lung cancer cell lines with HER-2/*neu* gene expression but not with ras gene mutations. *J. Natl. Cancer Inst.*, **85**, 897–901 (1993).
17) Tsai, C. M., Chang, K. T., Wu, L. H., Chen, J. Y., Gazdar, A. F., Mitsudomi, T., Chen, M. H. and Perng, R. P. Correlations between intrinsic chemoresistance and HER-2/*neu* gene expression, p53 gene mutations and cell proliferation characteristics in non-small cell lung cancer cell lines. *Cancer Res.*, **56**, 206–209 (1996).
18) Tsai, C. M., Yu, D., Chang, K. T., Wu, L. H., Perng, R. P., Ibrahim, N. K. and Hung, M. C. Enhanced chemoresistance by elevation of the levels of $p185^{neu}$ in the HER-2/*neu* transfected human lung cancer cells. *J. Natl. Cancer Inst.*, **87**, 682–684 (1995).
19) Yu, D., Liu, B., Tan, M., Li, J., Wang, S. S. and Hung, M. C. Overexpression of c-erbB-2/*neu* in breast cancer cells confers increased resistance to Taxol via mdr-l-independent mechanisms. *Oncogene*, **13**, 1359–1365 (1996).
20) Benz, C. C., Scott, G. K., Sarup, J. C., Johnson, R. M., Tripathy, D., Coronado, E., Shepard, H. M. and Osborne, C. K. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/*neu*. *Breast Cancer Res. Treat.*, **24**, 85–95 (1992).
21) Alaoui-Jamali, M. A., Paterson, J., Moustafa, A. I. and Yen, L. The role of ErbB-2 tyrosine kinase receptor in cellular intrinsic chemoresistance: mechanisms and implications. *Biochem. Cell Biol.*, **75**, 315–325 (1997).
22) Tsai, C. M., Levitzki, A., Wu, L. H., Chang, K. T., Cheng, C. C., Gazit, A. and Perng, R. P. Enhancement of chemosensitivity by tyrphostin AG825 in high $p185^{neu}$ expressing non-small cell lung cancer cells. *Cancer Res.*, **56**, 1068–1074 (1996).
23) Zhang, L. and Hung, M. C. Sensitization of HER-2/*neu*-overexpressing non-small cell lung cancer cells to chemotherapeutic drugs by tyrosine kinase inhibitor emodin. *Oncogene*, **12**, 571–576 (1996).
24) Hancock, M. C., Langton, B. C., Chan, T., Toy, P., Monahan, J. J., Mischak, R. P. and Shawver, L. K. A monoclonal antibody against the c-erbB-2 protein enhances the cytotoxicity of *cis*-diamminedichloroplatinum against human breast and ovarian tumor cell lines. *Cancer Res.*, **51**, 4575–4580 (1991).
25) Pietras, R. J., Fendly, B. M., Chazin, V. R., Pegrum, M. D., Howell, S. B. and Slamon, D. J. Antibody to HER-2/*neu* receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. *Oncogene*, **9**, 1829–1838 (1994).
26) Arteaga, C. L., Winnier, A. R., Poirier, M. C., Lopez-Larraza, D. M., Shawver, L. K., Hurd, S. D. and Stewart, S. J. p185c-erbB-2 signaling enhances cisplatin-induced cytotoxicity in human breast carcinoma cells: association between an oncogenic receptor tyrosine kinase and drug-induced DNA repair. *Cancer Res.*, **54**, 3758–3765 (1994).
27) Yen, L., Nie, Z. R., You, X. L., Richard, S., Langton-Webster, B. C. and Alaoui-Jamali, M. A. Regulation of cellular response to cisplatin-induced DNA damage and DNA repair in cells overexpressing p185(erbB-2) is dependent on the ras signaling pathway. *Oncogene*, **14**, 1827–1835 (1997).
28) Tsai, C. M., Perng, R. P., Chen, M. H., Jan, Y. H., Hung, M. C., Ku, T. Y. and Chang, K. T. Greater enhancement of chemosensitivity by caffeine in high $p185^{neu}$ expressing human non-small cell lung cancer cell lines. *J. Natl. Cancer Inst.*, **86**, 1018–1020 (1994).

29) Tsai, C. M., Chang, K. T., Chen, J. Y., Chen, Y. M., Chen, M. H. and Perng, R. P. The cytotoxic effects of gemcitabine-containing regimens against human non-small cell lung cancer cell lines which express different levels of p185^{neu}. *Cancer Res.*, **56**, 794–801 (1996).
30) Pegram, M. D., Finn, R. S., Arzoo, K., Beryt, M., Pietras, R. J. and Slamon, D. J. The effect of HER-2/*neu* overexpression on chemotherapeutic drug sensitivity in human breast and ovarian cancer cells. *Oncogene*, **15**, 537–547 (1997).
31) Christen, R. D., Hem, D. K., Porter, D. C., Andrews, P. A., MacLeod, C. L., Hafstrom, L. and Howell, S. B. Epidermal growth factor regulates the *in vitro* sensitivity of human ovarian carcinoma cells to cisplatin. *J. Clin. Invest.*, **86**, 1632–1640 (1990).
32) Kroning, R., Jones, J. A., Hom, D. K., Chuang, C. C., Sanga, R., Los, G., Howell, S. B. and Christen, R. D. Enhancement of drug sensitivity of human malignancies by epidermal growth factor. *Br. J. Cancer*, **72**, 615–619 (1995).
33) Dixit, M., Yang, J. L., Poirier, M. C., Price, J. O., Andrews, P. A. and Arteaga, C. L. Abrogation of cisplatin-induced programmed cell death in human breast cancer cells by epidermal growth factor antisense RNA. *J. Natl. Cancer Inst.*, **89**, 365–373 (1997).
34) Dickstein, B. M., Wosikowski, K. and Bates, S. E. Increased resistance to cytotoxic agents in ZR75B human breast cancer cells transfected with epidermal growth factor receptor. *Mol. Cell. Endocrinol.*, **110**, 205–211 (1995).
35) Fan, Z., Baselga, J., Masui, H. and Mendelsohn, J. Antitumor effect of anti-epidermal growth factor receptor monoclonal antibodies plus *cis*-diamminedichloraplatinum on well established A431 cell xenografts. *Cancer Res.*, **53**, 4637–4642 (1993).
36) Baselga, J., Norton, L., Masui, H., Pandiella, A., Coplan, K., Miller, W. H., Jr. and Mendelsohn, J. Antitumor effects of doxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies. *J. Natl. Cancer Inst.*, **85**, 1327–1333 (1993).
37) Aghajanian, C., Zhuo, Y., Ho, T. Y., Brown, C., Fan, Z., Baselga, J., Mendelsohn, J. and Spriggs, D. R. Anti-epidermal growth factor receptor monoclonal antibody treatment of A431 cells decreases cisplatin/DNA adduct repair in association with NF-KB induction [abstract]. *Proc. Am. Assoc. Cancer Res.*, **36**, 427 (1995).
38) Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T., Schlessinger, J., Francke, U., Levinson, A. and Ullrich, A. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. *Science*, **230**, 1132–1139 (1985).
39) Wen, D., Suggs, S. V., Karunagaran, D. and Liu, N. Structural and functional aspects of the multiplicity of *Neu* differentiation factors. *Mol. Cell. Biol.*, **14**, 1909–1919 (1994).
40) Peles, E. and Yarden, Y. *Neu* and its ligands: from an oncogene to neural factors. *BioEssays*, **15**, 815–824 (1994).
41) Stern, D. F. and Kamps, M. P. EGF-stimulated tyrosine phosphorylation of p185^{neu}: a potential model for receptor interactions. *EMBO J.*, **7**, 995–1001 (1988).
42) Johnson, G., Kannan, B., Shoyab, M. and Stromberg, K. Amphiregulin induces tyrosine phosphorylation of the epidermal growth factor receptor and p185^{erbB2}. *J. Biol. Chem.*, **268**, 2924–2931 (1993).
43) Goldman, R., Ben Levy, R., Peles, E. and Yarden, Y. Heterodimerization of the erbB-l and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. *Biochemistry*, **29**, 11024–11028 (1990).
44) Spivak-Kroizman, T., Rotin, D., Pinchasi, D., Ullrich, A., Schlessinger, J. and Lax, I. Heterodimerization of c-erbB2 with different epidermal growth factor receptor mutants elicits stimulatory or inhibitory responses. *J. Biol. Chem.*, **267**, 8056–8063 (1992).
45) Kokai, Y., Meyers, J. N., Wada, T., Brown, V. I., LeVea, C. M., Davis, J. G., Dobashi, K. and Greene, M. I. Synergistic interaction of pl85c-*neu* and the EGF receptor leads to transformation of rodent fibroblasts. *Cell*, **58**, 287–292 (1989).
46) Zamble, D. and Lippard, S. J. Cisplatin and DNA repair in cancer chemotherapy. *Trends Biochem. Sci.*, **20**, 435–439 (1995).
47) Huang, J. C., Zamble, D. B., Reardon, J. T., Lippard, S. J. and Sancar, A. HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease. *Proc. Natl. Acad. Sci. USA*, **91**, 10394–10398 (1994).
48) Hansson, J. and Wood, R. D. Repair synthesis by human cell extracts in DNA damaged by *cis*- and *trans*-diamminedichloroplatinum (II). *Nucleic Acids Res.*, **20**, 8073–8091 (1989).
49) Li, L., Liu, X., Glassman, A. B., Keating, M. J., Stros, M., Plunkett, W. and Yang, L. Y. Fludarabine triphosphate inhibits nucleotide excision repair of cisplatin-induced DNA adducts *in vitro*. *Cancer Res.*, **57**, 1487–1494 (1997).
50) Manley, J. L., Fire, A., Samuels, M. and Sharp, P. A. *In vitro* transcription: whole cell extract. *Methods Enzymol.*, **101**, 568–582 (1983).
51) Shivji, M. K. K., Podust, V. N., Hubscher, U. and Wood, R. D. Nucleotide excision repair DNA synthesis by DNA polymerase $\in$ in the presence of PCNA, RFC, and RPA. *Biochemistry*, **34**, 5011–5017 (1995).
52) Zeng-Rong, X., Paterson, J., Alpert, L., Tsao, M. S., Viallet, J. and Alaoui-Jamali, M. A. Elevated DNA repair capacity is associated with intrinsic resistance of lung cancer to chemotherapy. *Cancer Res.*, **55**, 4760–4764 (1995).
53) Tsai, C. M., Chang, K. T., Perng, R. P. and Chen, J. Y. Epidermal growth factor modulates chemosensitivity of non-small cell lung cancer cells through its effect on cell proliferation. *Proc. Am. Assoc. Cancer Res.*, **38**, 195 (1997).
54) Weidner, U., Peter, S., Strohmeyer, T., Hussnatter, R., Ackermann, R. and Sies, H. Inverse relationship of epider-

mal growth factor receptor and HER-2/*neu* gene expression in human renal cell carcinoma. *Cancer Res.*, **50**, 4504–4509 (1990).

55) Fisher, D. E. Apoptosis in cancer therapy: crossing the threshold. *Cell*, **78**, 539–542 (1994).
56) Liebermann, D. A., Hoffman, B. and Steinman, R. A. Molecular controls of growth arrest and apoptosis: p53-dependent and independent pathways. *Oncogene*, **11**, 199–210 (1995).
57) Vaux, D. L. and Strasser, A. The molecular biology of apoptosis. *Proc. Natl. Acad. Sci. USA*, **93**, 2239–2244 (1996).
58) Yu, D., Jing, T., Liu, B., Yao, J., Tan, M., McDonnell, T. J. and Hung, M. C. Overexpression of erbB2 blocks taxol-induced apoptosis by upregulation of $p21^{cip1}$, which inhibits $p34^{cdc2}$ kinases. *Mol. Cell*, **2**, 581–591 (1998).
59) Volm, M., Kastel, M., Mattern, J. and Efferth, T. Expression of resistance factors (P-glycoprotein, glutathione S-transferase-pi, and topoisomerase II) and their interrelationship to proto-oncogene products in renal cell carcinomas. *Cancer*, **71**, 3981–3987 (1993).
60) Yang, J. M., Sullivan, G. F. and Hait, W. N. Regulation of the function of P-glycoprotein by epidermal growth factor through phospholipase C. *Biochem. Pharmacol.*, **53**, 1597–1604 (1997).
61) Allen, G. C., Lubas, S., Wax, M. K. and Devore, R. F., III Epidermal growth factor regulates topoisomerase II activity and drug sensitivity in human KB cells. *Otolaryngol. Head Neck Surg.*, **114**, 785–792 (1996).
62) Slamon, D., Leyland-Jones, B., Shak, S., Paton, V., Bajamonde, A., Fleming, T., Fiermann, W., Wolter, J., Baselga, J. and Norton, L. Addition of Herceptin (humanized anti-HER2 antibody) to first line chemotherapy for HER2 overexpressing metastatic breast cancer markedly increases anticancer activity: a randomized, multinational controlled phase III trial. *Proc. Am. Soc. Clin. Oncol.*, **17**, 98a (1998).