[CANCER RESEARCH 56, 206–209, January 1, 1996]

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[1]

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ABSTRACT

Using a panel of 20 non-small cell lung cancer (NSCLC) cell lines established from previously untreated patients, we investigated the relationships between intrinsic chemoresistance (to four agents used commonly in the therapy of NSCLC) and *HER-2/neu* gene expression (which encodes glycoprotein p185^{neu}), *p53* gene mutations, and cell proliferation characteristics. Our results demonstrated that high p185^{neu} expression was correlated with chemoresistance, low S-phase fractions, and long doubling times. By contrast, cell lines expressing relatively low levels of p185^{neu} were relatively chemosensitive and had higher S-phase fractions and shorter doubling times. Although mutation of the *p53* gene was a common event in this panel of cell lines (present in 18 of 20 lines), there was no relationship between mutations at any specific codon and chemoresistance or cell proliferation characteristics. Multivariate analysis revealed that the level of p185^{neu} was the only independent predictor for chemoresistance to doxorubicin, etoposide, and probably cisplatin. Although intrinsic chemoresistance almost certainly is a multifactorial process, overexpression of p185^{neu} may be an important factor in the chemoresistance of NSCLC.

INTRODUCTION

The *HER-2/neu* gene, which encodes a M_r 185,000 transmembrane glycoprotein (p185^{neu}), is a membrane-bound receptor with tyrosine kinase activity (1). Activation (amplification and/or overexpression) of *HER-2/neu* has been detected in many types of human tumors. In lung cancer, overexpression of the *HER-2/neu* gene is encountered in a subgroup of NSCLCs[3] but not in small cell lung cancers. High expression of the gene is encountered more frequently in adenocarcinomas and has been linked to shortened survival (2, 3). Using a panel of human NSCLC cell lines as a model, we have demonstrated previously that overexpression of the *HER-2/neu* gene is associated closely with intrinsic multiple drug resistance (4). Recently, we have demonstrated that increased expression of p185^{neu} following transfection of the *HER-2/neu* gene into an NSCLC cell line expressing very low intrinsic levels of p185^{neu} enhances chemoresistance in the transfectant clones greatly, indicating that elevation of p185^{neu} may confer multiple drug resistance (5).

p53 is a nuclear DNA-binding protein with properties of a transcriptional activator. Following DNA damage, p53 acts as a checkpoint protein to suppress growth by inhibiting G_1 progression into the S-phase while the cell attempts to repair the damage or to promote apoptosis in cells that fail to repair (6, 7). Loss of p53 function has been reported to enhance cellular resistance to a variety of chemotherapeutic agents in certain experimental tumor models (6). Transfer of the wild-type *p53* gene into NCI-H358, a human NSCLC cell line with homozygous deletion of the entire *p53* gene, induced chemosensitivity (8).

In general, actively growing cells tend to be more susceptible to anticancer agents and *vice versa*. However, in breast cancer, although activation of the *HER-2/neu* gene and mutations of the *p53* gene have been linked to increased proliferation (9–11), the results of an association between these genetic aberrations and intrinsic chemoresistance are controversial (12–17). In this report, we investigated these relationships in a panel of NSCLC cell lines established from previously untreated patients.

MATERIALS AND METHODS

Cell Lines and Culture. We studied 20 NSCLC cell lines, which were established and characterized by one of us (A. F. G.) at the NCI-Navy Medical Oncology Branch (Division of Cancer Treatment, NCI, Bethesda, MD) from tumor specimens obtained from previously untreated patients (Table 1; Refs. 5 and 18): 11 adenocarcinomas, 3 adenosquamous carcinomas, 5 large cell carcinomas, and 1 squamous cell carcinoma. All of the cell lines were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum for at least 6 months before being tested.

Quantitative Measurement of the p185^{neu} Protein. Nearly confluent cells were scraped from flasks and centrifuged. The cell pellets were incubated with a Tris lysis buffer [10 mM Tris-HCl (pH 7.6), 1.5 mM EDTA, 10% glycerol, and 0.1% sodium azide], Dounce homogenized, and detergent extracted. Supernatants were collected, and protein values were determined. We used a commercial sandwich immunoassay for detection and quantitation of p185^{neu} (human neu assay kit; Oncogene Science Inc., Uniondale, NY.; Ref. 18) using the manufacturer's instructions. A standard curve was generated by using standard solutions. The concentration of p185^{neu} was determined by interpolation of the sample absorbance from the standard curve. Each experiment was performed in duplicate wells. Results of triplicate tests were expressed as human neu units/μg protein.

Population Doubling Times. Cell population doubling times were determined for cells in the log phase. Cells were detached with a trypsin:EDTA (1:250) solution, which was then neutralized, and the cells were resuspended in culture medium. Viable cell counts were determined by trypan blue exclusion using a hemocytometer. Cells were plated at a concentration of 5×10^4 in 3 ml culture medium in six-well (3-cm-diameter) cluster plates and fed every 3 days. The mean of hemocytometer counts of cells in replicate wells was determined daily or every other day for constructing growth curves and determining doubling times. The reported results are the means of three independently performed experiments.

S-Phase Fraction. S-phase fractions were determined by flow cytometry as described previously (19). In brief, single cell suspensions were stained with propidium iodide before being analyzed using a flow cytometer (Epics Profile; Coulter Electronics, Hialeah, FL) supplemented with a Cyonics (150-mW) argon ion laser (25 mW at 488 nm). Red fluorescence was detected through a 610-nm, long-pass absorbance filter (model 3802055; Coulter Electronics). The experiments were performed in triplicate, and the S-phase fractions were

Received 6/29/95; accepted 10/3/95.

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[1] This work was supported in part by National Science Council Grant NSC-82-0115-B075-130, by grants from the Institute of Biomedical Sciences, Academia Sinica, and by the Lung Cancer Foundation in memory of Dr. K. S. Lu.

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; NCI, National Cancer Institute; IC_{50}, 50% inhibitory concentration; EGF, epidermal growth factor.

determined with off-line multicycle software (Phoenix Flow Systems, Inc.). The coefficients of variation were less than 2%.

***In Vitro* Cytotoxicity Tests.** *In vitro* drug testing was performed using the tetrazolium dye colorimetric assay as described previously (5, 18). Four cytotoxic drugs, doxorubicin, cisplatin (both from Farmitalia Carlo Erba, Milan, Italy), etoposide (Bristol-Myers GmBH, Troisdorf, Germany), and melphalan (Sigma Chemical Co., St. Louis, MO) were tested. The drugs tested were dissolved in PBS at a concentration of 1 mM, and then all were diluted subsequently in culture medium. Each assay was done in four replicate wells, and the results, reported as IC_{50} values, were the means of three independently performed assays.

***p53* Gene Mutations in the Cell Lines Studied.** *p53* gene mutations of the 20 NCI cell lines had been studied previously by PCR-single-strand conformation polymorphism assays as reported by Mitsudomi *et al.* (20).

Data Analysis. The Spearman rank correlation was used for correlation analyses, and multivariate regression analyses were performed to determine the factor(s) that predict independently for drug resistance. Significance was assumed for $P < 0.05$.

RESULTS

The results of biological characteristics and drug sensitivity testing are listed in Tables 1 and 2, respectively.

Correlations between $p185^{neu}$ Expression and Doubling Time and S-Phase Fraction. There was a statistically significant correlation between population doubling time and S-phase fraction ($r = -0.618$; $P = 0.007$). The level of $p185^{neu}$ was correlated significantly with both doubling time and S-phase fraction ($r = 0.565$; $P = 0.014$; and $r = -0.48$; $P = 0.037$, respectively). These results indicate that slowly proliferating cell lines tend to express higher levels of $p185^{neu}$.

Relationships between Chemoresistance and $p185^{neu}$ Expression, Doubling Time, and S-Phase Fraction. Statistical analyses of the correlations between chemoresistance (expressed as IC_{50} values), doubling time, S-phase fraction, and $p185^{neu}$ levels are listed in Table 3. The IC_{50} values of doxorubicin, etoposide, cisplatin, and melphalan were correlated significantly with each other (all $P \leq 0.005$) and with the levels of $p185^{neu}$ (all $P < 0.004$). The IC_{50} values of doxorubicin and etoposide showed statistically significant correlations with population doubling time and S-phase fraction ($P = 0.038-0.0008$). However, the IC_{50} values of cisplatin and melphalan showed statistically significant correlations with population doubling time ($P = 0.013$ and 0.007, respectively), but not with S-phase fraction. Although the expression of the *p185*^{neu} gene, cell doubling time, and the S-phase fraction was correlated significantly with the chemosensitivities of the anticancer agents tested, multivariate analyses revealed that the level of $p185^{neu}$ was the only predictor for the chemoresistance to doxorubicin ($P = 0.0013$), etoposide ($P = 0.034$), and probably cisplatin (marginal significance, $P = 0.062$; Table 3).

Table 1 *Characteristics of cell lines*

Cell line	Cell type	Doubling time (h)	S-phase fraction (%)	$p185^{neu}$ (HNU/μg)[a]	*p53* mutation
NCI-H1155	LC	12.0 ± 0.4	40.9 ± 5.3	0.62 ± 0.05	Exon 8
NCI-H23	A	31.2 ± 1.4	40.2 ± 3.6	2.95 ± 0.12	Exon 7
NCI-H1299	LC	15.6 ± 0.4	44.3 ± 4.2	3.57 ± 0.29	Homozygous
NCI-H460	LC	14.4 ± 0.3	26.2 ± 4.2	4.81 ± 1.08	[b]
NCI-H226	S	50.4 ± 2.8	25.4 ± 1.5	6.25 ± 0.21	Exon 5
NCI-H810	LC	27.2 ± 3.0	31.7 ± 2.1	7.38 ± 1.15	Exon 8
NCI-H358	A	26.6 ± 1.4	30.2 ± 3.0	11.62 ± 1.65	Homozygous
NCI-H125	AS	37.6 ± 2.1	13.3 ± 2.4	12.65 ± 0.74	Exon 7
NCI-H838	AS	20.4 ± 0.9	27.4 ± 3.7	14.51 ± 0.94	[b]
NCI-H647	AS	36.2 ± 1.6	31.8 ± 3.1	16.16 ± 1.35	Intron 7
NCI-H441	A	35.0 ± 1.7	23.5 ± 0.7	19.05 ± 1.61	Exon 5
NCI-H1334	LC	38.4 ± 2.1	21.1 ± 0.5	19.91 ± 1.83	Exon 8
NCI-H820	A	44.8 ± 2.9	21.9 ± 1.6	26.34 ± 1.76	Exon 8
NCI-H1437	A	28.8 ± 1.2	32.8 ± 0.9	29.16 ± 5.04	Exon 8
NCI-H1404	A	63.6 ± 5.3	24.0 ± 3.4	33.80 ± 2.48	Exon 5
NCI-H322	A	29.6 ± 0.8	29.8 ± 0.5	35.75 ± 3.85	Exon 7
NCI-H676	A	35.8 ± 3.6	26.9 ± 2.8	39.41 ± 2.04	Exons 6 and 7
NCI-H1355	A	34.1 ± 1.0	23.8 ± 1.3	42.06 ± 2.31	Exon 8
NCI-H1435	A	48.0 ± 2.8	27.8 ± 2.4	53.70 ± 3.28	Exon 5
NCI-H522	A	45.0 ± 4.0	19.2 ± 2.7	73.94 ± 4.00	Exon 6

[a] HNU, human neu unit; A, adenocarcinoma; AS, adenosquamous cell carcinoma; LC, large cell carcinoma; S, squamous cell carcinoma.

[b] Found to have no mutation in the coding regions and contained wild-type *p53* function.

Table 2 *Results of drug sensitivity testings*[a]

Cell line	IC_{50} (μM)			
	Doxorubicin	Etoposide	Cisplatin	Melphalan
NCI-H1155	0.034 ± 0.002	0.44 ± 0.04	0.90 ± 0.20	5.71 ± 2.16
NCI-H23	0.020 ± 0.004	0.29 ± 0.06	2.09 ± 0.32	12.17 ± 2.95
NCI-H1299	0.032 ± 0.005	0.45 ± 0.02	2.08 ± 0.21	8.07 ± 1.72
NCI-H460	0.004 ± 0.001	0.095 ± 0.01	0.52 ± 0.04	6.14 ± 1.79
NCI-H226	0.102 ± 0.017	2.03 ± 0.25	5.05 ± 0.95	66.94 ± 8.25
NCI-H810	0.065 ± 0.019	1.11 ± 0.05	5.12 ± 0.77	7.96 ± 1.35
NCI-H358	0.051 ± 0.015	0.54 ± 0.14	1.16 ± 0.24	18.02 ± 2.49
NCI-H125	0.073 ± 0.017	1.31 ± 0.36	1.81 ± 0.77	12.34 ± 7.55
NCI-H838	0.127 ± 0.037	1.28 ± 0.24	3.86 ± 0.31	49.33 ± 3.75
NCI-H647	0.150 ± 0.047	4.87 ± 2.00	7.27 ± 1.14	48.17 ± 7.95
NCI-H441	0.126 ± 0.039	1.70 ± 0.67	3.38 ± 0.99	17.61 ± 4.04
NCI-H1334	0.275 ± 0.057	7.10 ± 0.47	5.15 ± 0.47	105.80 ± 9.24
NCI-H820	0.248 ± 0.018	5.53 ± 0.43	2.73 ± 0.49	41.99 ± 4.79
NCI-H1437	0.151 ± 0.024	4.64 ± 0.75	5.90 ± 1.61	38.88 ± 3.92
NCI-H1404	0.179 ± 0.031	10.74 ± 2.38	8.25 ± 2.25	27.05 ± 2.21
NCI-H322	0.165 ± 0.039	1.79 ± 0.54	2.85 ± 0.22	28.08 ± 12.14
NCI-H676	0.578 ± 0.099	57.60 ± 10.51	11.04 ± 2.14	27.02 ± 1.94
NCI-H1355	0.295 ± 0.037	6.39 ± 1.32	6.74 ± 1.29	117.70 ± 8.18
NCI-H1435	0.446 ± 0.123	18.21 ± 5.56	22.86 ± 2.36	106.89 ± 15.78
NCI-H522	0.323 ± 0.025	19.94 ± 1.88	3.53 ± 0.56	53.99 ± 8.83

[a] The results, reported as IC_{50} values, were the means of three independently performed assays (each assay was performed in four replicate wells).

Relationships between Chemoresistance, Doubling Time, S-Phase Fraction, and *p53* Gene Mutations. Mutations or homozygous deletions of the *p53* gene were present in 18 (90%) of 20 cell lines (Table 1). There were 4 cell lines with mutations at exon 5, H226, H441, H1404, and H1435; 2 cell lines with mutations at exon 6, H522, and H1334; 3 cell lines with mutations at exon 7, H23, H125, and H322; and 5 cell lines with mutations at exon 8, H810, H820, H1155, H1355, and 1437. One cell line, H647, had a mutation at intron 7, and one cell line, H676, had two mutation sites at exons 6 and 7. Two cell lines, H358 and H1299, had homozygous deletions of the gene. Only two lines, H460 and H838, had no mutations in the coding regions detected by the PCR/single-strand conformation polymorphism assay (20) and were found to have wild-type *p53* function.[4]

Although mutation of the *p53* gene was a common event, mutations at any specific exon of the *p53* gene were not related to chemoresistance, population doubling time, or S-phase fraction (Fig. 1).

DISCUSSION

Using a panel of NSCLC cell lines established from untreated patients as an experimental model, we demonstrated that cell lines expressing high levels of $p185^{neu}$ were relatively chemoresistant, had long population doubling times, and had low S-phase fractions. By contrast, actively proliferating cell lines tended to have lower levels of $p185^{neu}$ expression and were relatively chemosensitive. There seemed to be a trend between cell proliferation rate (expressed as population doubling time) and cytotoxicity to all four drugs tested. The findings that the cytotoxic effects of doxorubicin and etoposide but not those of cisplatin and melphalan was correlated significantly with the S-phase fractions are in accordance with the facts that doxorubicin and etoposide seem to exert maximum activity on cells in the S-phase,

[4] J-Y. Chen, unpublished data.

Table 3 *Correlations of doubling times, S-phase fraction, p185^{neu}, and drug sensitivities*[a]

	Doxorubicin		Etoposide		Cisplatin		Melphalan	
	Univariate *P*	Multivariate *P*	Univariate *P*	Multivariate *P*	Univariate *P*	Multivariate *P*	Univariate *P*	Multivariate *P*
Etoposide	0.0001 (0.962)							
Cisplatin	0.001 (0.756)		0.0005 (0.794)					
Melphalan	0.0009 (0.759)		0.0013 (0.735)		0.005 (0.644)			
Doubling time	0.0051 (0.642)	0.6333	0.0008 (0.771)	0.8457	0.0132 (0.568)	0.0895	0.0068 (0.621)	0.5185
S-Phase fraction	0.0377 (−0.477)	0.9605	0.0276 (−0.505)	0.7274	0.628 (−0.111)	0.1497	0.0645 (−0.424)	0.7355
p185^{neu}	0.0001 (0.935)	0.0013	0.0001 (0.886)	0.0341	0.0035 (0.669)	0.0623	0.0027 (0.689)	0.1469

[a] Twenty cell lines were tested on doxorubicin, etoposide, cisplatin, and melphalan. The cell doubling times, percentage of S-phase fractions, levels of p185^{neu}, and IC_{50} concentrations of the four drugs tested were determined. The Spearman rank correlation test was performed to analyze the univariate correlations, and the values in parentheses represent the Spearman rank correlation coefficients. Multivariate regression analyses were performed to determine which biological factor(s) may predict independently for drug resistance. Significance was assumed if $P < 0.05$.

whereas cisplatin and melphalan inhibit cells in G_1 or near the G_1-S boundary, as well as cells in the S-phase (21).

Mutations of the *p53* gene have been detected in 50–60% of NSCLC tumors and in a higher percentage of cell lines (20), and they may act as a negative prognostic factor (22, 23). Because of this high incidence, the multivariate analysis failed to detect a relationship between mutations and deletions and chemoresistance. Mutations of the *p53* gene, especially in exons 5 and 6, have been correlated with a high S-phase index in breast cancer cells (10, 11), whereas this association has not been demonstrated convincingly in NSCLC (24). We failed to find an association with mutations in specific exons and cell proliferation or chemoresistance.

In breast cancer, high expression of *HER-2/neu* is associated with increased cell proliferation (9), although its effect on the response to chemotherapy is controversial (12–15). In the present study, we demonstrated that intrinsic chemoresistance, cell-proliferating activity (cell doubling time and S-phase fraction), and *HER-2/neu* expression in NSCLC cells was correlated closely with each other. We found that the level of p185^{neu} was the only independent predictor for the chemoresistance to certain anticancer agents. These findings excluded the possibility that high levels of p185^{neu} might confer chemoresistance via down-regulation of cell-proliferating activity and reduction of the cell fraction in the sensitive phase of the cell cycle. The direct correlation between p185^{neu} and the chemoresistance is supported by the finding that elevation of the levels of p185^{neu} may induce chemoresistance in the *HER-2/neu*-transfected human NSCLC cells without significant alterations of their proliferating activity (5).

p185^{neu} is a member of the EGF receptor subgroup of the protein tyrosine kinase superfamily, which includes the EGF receptor and proteins encoded by the *HER-2/neu*, *HER-3*, and *HER-4* genes. The four receptors are generally coexpressed in various combinations and are found in a variety of tissues (25). EGF has been reported to enhance the radiosensitivity of human squamous cell carcinoma cells (26) and to enhance the sensitivity of human ovarian cancer cells to cisplatin (27). However, the potential biological significance of such DNA repair capability in EGF family receptor proteins, including p185^{neu}, remains to be determined.

Using our model system, we demonstrated previously that caffeine can enhance the cytotoxicities of a variety of DNA-damaging agents to a greater degree in high- than in low-p185^{neu}-expressing cell lines (18). The magnitude of enhancement of the chemosensitivities by caffeine was correlated with the level of p185^{neu}. Caffeine has been shown to override the drug-induced cell cycle arrest at checkpoints, in particular, G_2-M (28, 29), thus shortening the time for DNA repair, leading to enhanced cell killing (28, 29). Our finding, therefore, raised the possibility that high levels of p185^{neu} may enhance chemoresistance by potentiating DNA repair at the G_2-M checkpoint. Two recent reports have demonstrated that the chemosensitivity of high-p185^{neu}-expressing human ovarian and breast cancer cells to cisplatin can be enhanced by a p185^{neu}-specific antibody through the inhibition of DNA repair (30, 31). There are eight *Ras* mutants in this panel of cell lines (4). Although *Ras* gene mutations are an important negative prognostic factor, in our previous study, we failed to find that these genetic aberrations are related to chemoresistance (4).

Using a panel of NSCLC cell lines established from untreated patients, we demonstrated that slowly replicating NSCLC cells

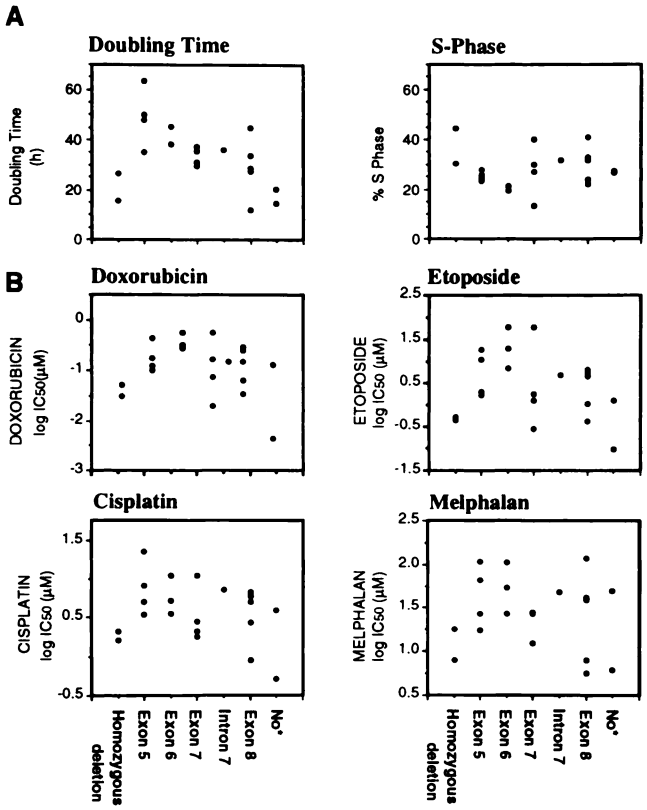

Fig. 1. Relations of the regions of *p53* mutations to doubling times and S-phase fractions (*A*) and chemoresistance to doxorubicin, etoposide, cisplatin, and melphalan (*B*). Each *dot* refers to one cell line. *No**, no mutation was detected in the coding regions of the *p53* gene and with wild-type *p53* function. The case distribution by the regions of *p53* mutations for every tested index was overlapping in a wide range.

express relatively high $p185^{neu}$ levels and are more chemoresistant. The level of $p185^{neu}$ is an independent predictor for the chemoresistance to doxorubicin, etoposide, and probably cisplatin. The role of *p53* mutations remains to be resolved, presumably in tumor systems having a lower mutational frequency. Although intrinsic chemoresistance almost certainly is a multifactorial process, overexpression of $p185^{neu}$ may be an important factor in the chemoresistance of NSCLC.

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