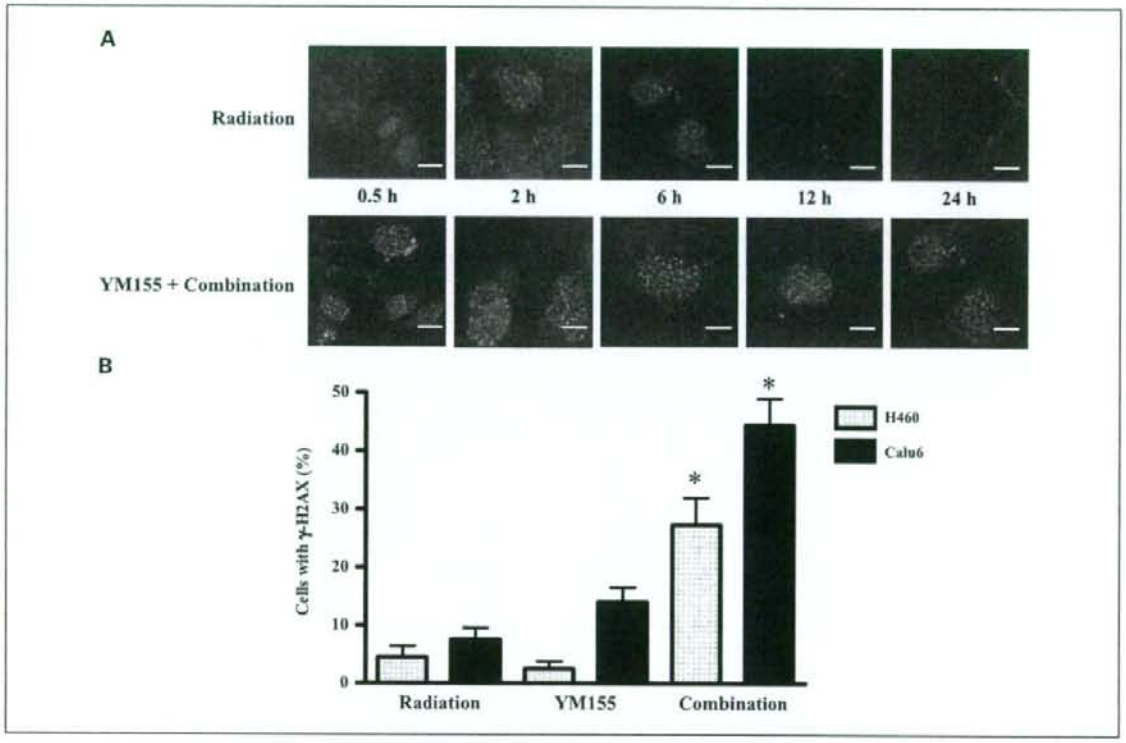

Fig. 4. Effect of YM155 on the radiation-induced formation of γ-H2AX foci in NSCLC cells. *A*, H460 cells were incubated with vehicle (0.1% DMSO) or 50 nmol/L YM155 for 48 h and then exposed to 3 Gy of γ-radiation. After incubation for the indicated times in drug-free medium, the cells were fixed and subjected to immunofluorescence staining for γ-H2AX (green fluorescence). Scale bar, 10 μm. *B*, H460 or Calu6 cells were incubated with vehicle or YM155 and then exposed (or not) to γ-radiation as in *A*. They were fixed at 24 h after irradiation and the percentage of cells containing γ-H2AX foci was determined. Columns represent means from three independent experiments; bars represent SD. $^*P < 0.05$ versus the corresponding value for radiation or YM155 alone.

mitogen-activated protein kinases, and cyclin-dependent kinases, have been shown to suppress survivin expression by targeting various signaling pathways, these drugs inhibit survivin expression nonspecifically (15–17, 19, 32). Gene therapy strategies based on small interfering RNA or other antisense oligonucleotides are specific for survivin, but the effective delivery of these molecules remains a challenge for the transition to the clinic (33). YM155 is a small-molecule agent that specifically inhibits survivin expression in various types of cancer cell lines *in vitro* (14). In addition, YM155 has been shown both to distribute preferentially to tumor tissues rather than to plasma as well as to exert pronounced antitumor activity in tumor xenograft models *in vivo* (14). The use of YM155 as a single agent in phase I clinical trials did not reveal significant toxicity (34). Although phase II studies of YM155 use as a single agent for certain types of cancer are currently under way, the effects of YM155 in combination with radiation have not been reported. We now show that YM155 increased the sensitivity of tumor cells to radiation *in vitro* and *in vivo*.

Clonogenic survival analysis, the most reliable approach for assessing the ability of genotoxic agents to induce cell death (35), revealed that YM155 markedly potentiated the decrease in NSCLC cell survival induced by γ-radiation. Given that induction of apoptosis is a key mechanism of cytotoxicity for most antitumor agents, including γ-radiation, defects in apoptotic signaling may underlie resistance to such agents (36). Radiation-sensitive tumors undergo radiation-induced apoptosis *in vitro* more readily than do radiation-resistant tumors (37–40). Treatment with caspase inhibitors has been shown to protect tumor cells against radiation-induced apoptosis and to increase their radioresistance (21, 41, 42), suggesting that radiation-induced apoptosis is caspase-dependent and that caspases contribute to radiosensitivity. The antiapoptotic activity of survivin is mostly attributable to inhibition of the activation of downstream effectors of apoptosis such as caspase-3 and caspase-7 (25). We have now shown that radiosensitization of NSCLC cells by YM155 was associated with increases both in the activity of caspase-3 and in the proportion of apoptotic cells. Our findings thus suggest that YM155 sensitized tumor cells to radiation at least in part by enhancing radiation-induced apoptosis.

We examined further the mechanism by which YM155 induces radiosensitization. Survivin is essential for the proper execution of mitosis and cell division, with disruption of survivin expression resulting in cell division defects that can lead to polyploidy and the formation of multinucleated cells (43, 44). Although treatment with 50 nmol/L YM155 for 48 hours inhibited survivin expression in NSCLC cells, it

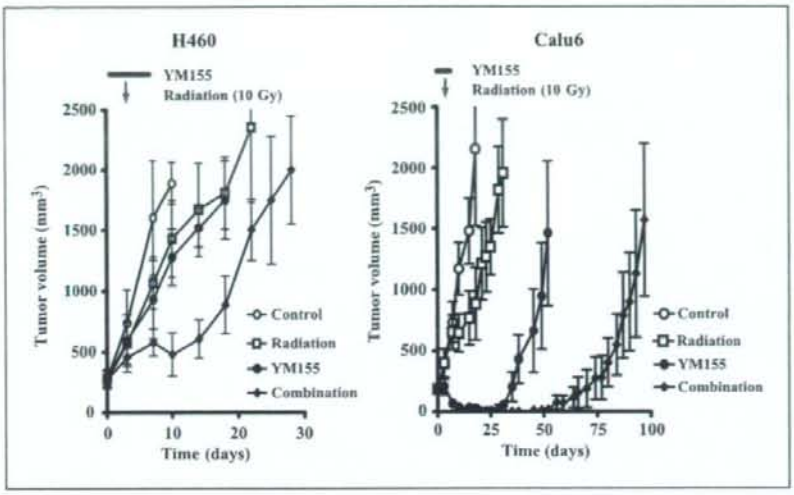

Fig. 5. Effect of YM155 on the growth of H460 or Calu6 tumors in mice subjected to single-dose radiotherapy. Cells were injected into the right hind limb of nude mice and allowed to grow. The mice were divided into four treatment groups: control, radiation alone, YM155 alone, or the combination of YM155 and radiation. YM155 (5 mg/kg) or vehicle was administered by continuous infusion over 7 d, and mice in the radiation groups were subjected to γ-irradiation with a single dose of 10 Gy on day 3 of drug treatment. Tumor volume was measured at the indicated times after the onset of treatment. Points, means from eight mice per group; bars, SE.

did not induce polyploidy (data not shown), suggesting that YM155-induced radiosensitization in the present study was not attributable to cell division defects caused by survivin depletion. Survivin was previously suggested to enhance tumor cell survival after radiation exposure through regulation of DSB repair (21). We therefore investigated the effect of YM155 on the repair of radiation-induced DSBs by immunofluorescence imaging of γ-H2AX foci. H2AX is a histone that is phosphorylated by ataxia telangiectasia mutated and DNA-dependent protein kinase in response to the generation of DSBs (45, 46). This reaction occurs rapidly, with half-maximal amounts of γ-H2AX generated within 1 minute and maximal amounts within 10 minutes (47), and a linear relation has been shown between the number of γ-H2AX foci and that of DSBs (48). The number of γ-H2AX foci is thus a sensitive and specific indicator of the existence of DSBs, with a decrease in this number reflecting DSB repair. We found that YM155 inhibited the repair of radiation-induced DSBs in NSCLC cells. If left unrepaired, DSBs can result in chromosome loss or cell death; agents that inhibit such repair thus increase the sensitivity of cells to ionizing radiation (49, 50). Our results therefore suggest that inhibition of DSB repair by YM155 contributes to the radiosensitization induced by this drug. Given that suppression of survivin expression impairs the repair of radiation-induced DNA damage (9, 21), our results further suggest that inhibition of DNA repair by YM155 is attributable to down-regulation of survivin expression.

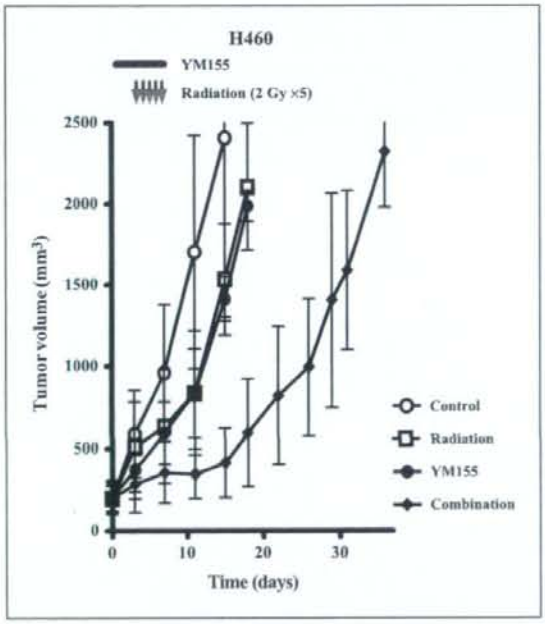

Fig. 6. Effect of YM155 on the growth of H460 tumors in mice subjected to fractionated radiotherapy. H460 cells were injected into the right hind limb of nude mice and allowed to grow. The mice were divided into four treatment groups: control, radiation alone, YM155 alone, or the combination of YM155 and radiation. YM155 (5 mg/kg) or vehicle was administered by continuous infusion over 7 d, and mice in the radiation groups were subjected to γ-irradiation with a daily dose of 2 Gy on days 3 to 7 of drug treatment. Tumor volume was measured at the indicated times after the onset of treatment. Points represent means from eight mice per group; bars represent SE.

The antitumor activity of YM155 has previously been shown to be time-dependent, with continuous infusion of the drug resulting in greater antitumor activity and less systemic toxicity compared with bolus injection in tumor xenograft models *in vivo* (14). Ongoing clinical trials of YM155 are thus being done with the drug administered on a continuous schedule. We also administered YM155 by continuous infusion in our *in vivo* experiments. The combination of YM155 with single-dose radiotherapy resulted in a marked increase in tumor growth delay compared with that apparent with either radiation or YM155 alone, indicating that YM155 enhanced the antitumor effect of ionizing radiation *in vivo*. Given that standard radiation therapy in the clinic is delivered according to a fractionated schedule, we also examined whether YM155 enhanced the tumor response to clinically relevant fractionated doses (2 Gy) of radiation. Indeed, YM155 was also effective in

enhancing the tumor response to such fractionated radiation. The enhancement factor with fractionated radiation (3.0) was similar to that observed with single-dose radiation (3.3) for H460 tumor xenografts.

Resistance to cytotoxic drugs and radiation is a major limiting factor in the treatment of cancer patients. Cross-resistance has been noted between radiotherapy and chemotherapy and has been attributed to defects in apoptosis signaling or to an enhanced capacity for DNA repair (51, 52). Our findings provide evidence that YM155 may break radioresistance by promoting apoptosis and inhibiting DNA repair. Previous studies have shown that suppression of survivin expression increases the sensitivity of tumor cells to chemotherapy(18, 53). It will therefore be of interest to determine whether YM155 also sensitizes tumor cells to chemotherapy.

In conclusion, we have shown that YM155 sensitizes NSCLC cells to radiation both *in vitro* and in animal models *in vivo*. The radiosensitization induced by YM155 seems to be attributable to the promotion of caspase-mediated apoptosis and inhibition of the repair of radiation-induced DNA damage. Our preclinical results provide a rationale for future clinical investigation of the therapeutic efficacy of YM155 in combination with radiotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Pharmacokinetic Analysis of Carboplatin and Etoposide in a Small Cell Lung Cancer Patient Undergoing Hemodialysis

Ken Takezawa, MD, Isamu Okamoto, MD, PhD, Masahiro Fukuoka, MD, PhD, and Kazuhiko Nakagawa, MD, PhD

Cancer chemotherapy is not well established for patients on hemodialysis (HD). A 77-year-old man on HD presented with small cell lung cancer. He was treated with the combination of carboplatin and etoposide while the pharmacokinetics of the drugs were monitored. The patient showed a response with manageable toxicity and remained progression free for at least 8 months. The area under the concentration-time curve for each antitumor agent in the patient was within the therapeutic range achieved in individuals with normal renal function. Carboplatin and etoposide chemotherapy combined with HD thus allowed the drugs to achieve an appropriate area under the concentration-time curve and sufficient efficacy in a small cell lung cancer patient with chronic renal failure.

Key Words: Small cell lung cancer, Hemodialysis, Pharmacokinetics, Chemotherapy.

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The prognosis of patients with chronic renal failure has improved as a result of progress in hemodialysis (HD), and opportunities to treat malignant tumors that develop in such HD patients are increasing. However, little is known of the safety or efficacy of chemotherapy for malignant tumors in HD patients. We analyzed the pharmacokinetics of combination chemotherapy with carboplatin (CBDCA) and etoposide in a patient with small cell lung cancer (SCLC) undergoing HD.

CASE REPORT

A 77-year-old man with chronic renal failure due to diabetic nephropathy presented with a mass in the left hilar area in March 2007. The general condition of the patient, who had undergone HD, three times a week, was fair, with symptoms such as cough, weight loss, and fever being absent. His Eastern Cooperative Oncology Group performance status was 1. Computed tomography of the chest revealed a 45/33 mm mass in the lower left lobe as well as interstitial pneumonia in the lower left and lower right lobes. Histopathologic analysis of a transbronchial biopsy specimen revealed SCLC. No distant metastasis was detected on systemic examinations, and the patient was diagnosed with limited-stage SCLC. Laboratory testing revealed blood urea nitrogen and creatinine levels of 101 and 8.6 mg/dl, respectively. Other examined laboratory parameters were within normal limits, but subsequent evaluation of serum tumor markers revealed an increased level (18.2 ng/ml) of neuron-specific enolase, which is not affected by renal function.[1]

Radiotherapy was not appropriate for the patient because of his bilateral interstitial pneumonia. Given his good performance status and after obtaining informed consent, we treated the patient with the combination of CBDCA and etoposide (Figure 1). On day 1 of the treatment cycle, the patient received an intravenous injection of etoposide (50 mg/m^2) over 60 minutes followed by an intravenous injection of CBDCA (250–275 mg/m^2) also over 60 minutes. HD was initiated 60 minutes after completion of CBDCA administration and was performed for 4 hours. On day 3, etoposide (50 mg/m^2) was administered over 60 minutes and HD was performed for 4 hours beginning 2 hours after completion of etoposide injection. The doses of CBDCA and etoposide as well as the timing of HD were based on previous studies.[2–4] The treatment was well tolerated. Nonhematologic toxicities such as nausea, vomiting, and fatigue were not observed. The patient also did not experience neutoropenia or thrombocytopenia (Nadir neutrophil and platelet counts during 3 cycles of chemotherapy were 2200/μl and 15.5 $\times$ 10^4/μl, respectively). Prophylactic administration of granulocyte colony-stimulating was not carried out. After three cycles of chemotherapy, each separated by an interval of 3 weeks, the tumor had decreased in size and the serum neuron-specific enolase level had decreased to within normal limits (6.3 ng/ml). The patient remained progression free 8 months after the initiation of treatment.

Pharmacokinetic analysis of CBDCA and etoposide was performed for the first and third courses of chemotherapy. Serial blood samples were collected 0, 1, 2, 3, 4, 5, 6, 24, 37, 41, 42, 49, 53, and 54 hours after completion of CBDCA administration as well as 0, 2, 3, 4, 5, 6, 7, 25, 48, 50, 52, 54, 55, and 73 hours after completion of the first etoposide administration. Each blood sample was analyzed for free

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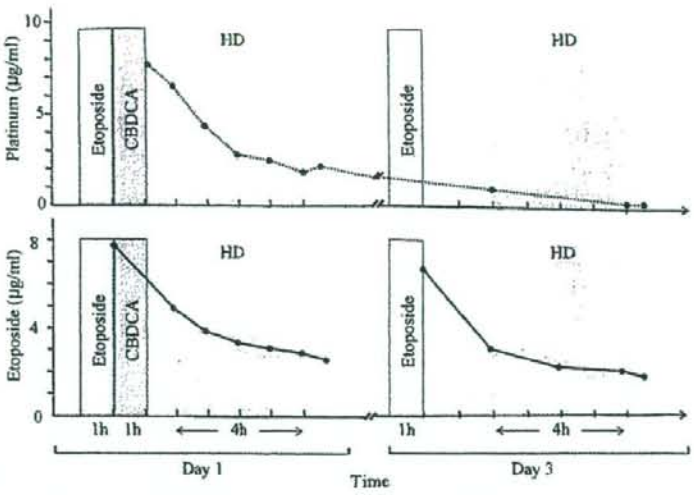

FIGURE 1. Chemotherapy and hemodialysis schedule as well as the plasma concentrations of free platinum and etoposide for the proband. Data are for the first of three cycles of chemotherapy. HD, hemodialysis; CBDCA, carboplatin.

platinum and etoposide (Figure 1) as described previously.[5] In the first cycle, the area under the concentration-time curve (AUC) was 4.10 minutes mg/ml for free platinum and 4401 and 3612 minutes μg/ml for etoposide on days 1 and 3, respectively. In the third course of chemotherapy, for which the CBDCA dose was increased from 250 to 275 mg/m^2, the AUC of free platinum was 4.16 minutes mg/ml. The maximal concentration and half-life of free platinum were 7.7 μg/ml and 2.51 hours in the first cycle and 9.4 μg/ml and 1.93 hours in the third cycle.

DISCUSSION

Many lung cancer patients undergoing HD as a result of impaired renal function may be "undertreated" because chemotherapy regimens are not well established for such individuals. The lack of pharmacokinetic data for most cytotoxic agents in HD patients makes it difficult to administer chemotherapy effectively. Given his old age, bilateral interstitial pneumonia, and renal dysfunction, the present patient might have been considered too high a risk for chemotherapy and recommended to receive best supportive care. However, taking into account the sensitivity of SCLC to platinum combination chemotherapy, we treated him with CBDCA and etoposide while monitoring the pharmacokinetics of these antitumor agents.

CBDCA is a less emetic and less nephrotoxic analog of cisplatin and is preferred over cisplatin for use in patients with renal insufficiency. The desired AUC for CBDCA can be individualized with the use of Calvert's formula on the basis of individual renal function.[6] In previous studies of CBDCA-based chemotherapy in patients undergoing HD, a CBDCA dose of 100 to 150 mg/body was chosen according to this formula, with the glomerular filtration rate set to zero because of the absence of renal function (Table 1).[7–10] In these studies, HD was performed 16 to 24 hours after completion of CBDCA administration, resulting in an AUC of 4.43 to 6.9 minutes mg/ml. More recently, administration of a relatively high dose (300 mg/m^2) of CBDCA with initiation of HD 0.5 to 1.5 hours after completion of drug injection has been shown to be feasible and effective in lung cancer patients undergoing HD.[2–4] However, the AUC of CBDCA in these latter studies was not determined. In the present study, we found that a CBDCA dose of 250 to 275 mg/m^2 administered completely 1 hour before HD gave rise to an AUC for free platinum of 4.10 to 4.16 minutes mg/ml, a therapeutic blood level, consistent with the antitumor efficacy observed

TABLE 1. Previous Studies of Carboplatin-Based Chemotherapy in Cancer Patients on Hemodialysis

	Disease	No. of Patients	Carboplatin Dose	Interval Between Carboplatin Infusion and Hemodialysis (h)	AUC (min mg/ml)
Watanabe et al.[7]	Ovarian cancer	1	125 mg	16	4.43
Jeyabalan et al.[8]	Ovarian cancer	1	125 mg	24	N.D
Chatelut et al.[9]	Ovarian cancer	1	150 mg	24	6.06–6.70
Motzer et al.[10]	Germ cell tumor	2	100 mg/m^2	24	6.7–6.9
Inoue et al.[2]	SCLC	3	300 mg/m^2	1	N.D
Yanagawa et al.[3]	NSCLC/epipharynx ca	2	300 mg/m^2	0.5	N.D
Haraguchi et al.[4]	SCLC	1	300 mg/m^2	1.5	N.D

N.D, not determined; NSCLC, non-small cell lung cancer.

in the previous studies[2–4]. Our presented study supports that relatively high dose administration of CBDCA with initiation of HD 1 hour after drug injection would be an alternative strategy for patients with HD -dependent renal insufficiency.

Etoposide is active against various types of malignant tumors, but its membrane permeability in HD remains unclear. The AUC range for etoposide in 13 patients with normal renal function treated with this drug at a dose of 100 mg/m^2 was previously shown to be 2291 to 6832 minutes μg/ml (Ref. [11]). The present patient was treated with etoposide at 50 mg/m^2 on days 1 and 3, with HD being initiated 2 hours after completion of the drug injection. The AUC of etoposide was 3612 to 4401 minutes μg/ ml, values that are within the range achieved in patients with normal renal function. Indeed, the combination chemotherapy in the proband induced a tumor response that persisted for at least 8 months. Administration of etoposide at 100 mg/m^2 on days 1, 3, and 5 in combination with cisplatin at 80 mg/m^2 was shown to be acceptable in 4 lung cancer patients with renal dysfunction.[12] In the previous study, HD was performed soon after drug administration, resulting in an AUC for etoposide of 4800 to 6204 minutes μg/ml. Data from the previous studies and our present patient thus indicate that etoposide can be administered safely in HD patients.

The present case shows that CBDCA and etoposide chemotherapy combined with HD resulted in AUCs for these drugs within the therapeutic range in a SCLC patient with chronic renal failure. Although further studies are needed, our findings suggest that this regimen of combination chemotherapy can be administered to lung cancer patients with renal insufficiency.

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Association of epidermal growth factor receptor (*EGFR*) gene mutations with *EGFR* amplification in advanced non-small cell lung cancer

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Somatic mutations in the epidermal growth factor receptor (*EGFR*) gene are associated with the response to *EGFR* tyrosine kinase inhibitors in patients with non-small cell lung cancer (NSCLC). Increased *EGFR* copy number has also been associated with sensitivity to these drugs. However, given that it is often difficult to obtain sufficient amounts of tumor tissue for genetic analysis from patients with advanced NSCLC, the relationship between these two types of *EGFR* alterations has remained unclear. We have now evaluated *EGFR* mutation status both by direct sequencing and with a high-sensitivity assay, the Scorpion-amplification-refractory mutation system, and have determined *EGFR* copy number by fluorescence *in situ* hybridization (FISH) analysis in paired tumor specimens obtained from 100 consecutive patients with advanced NSCLC treated with chemotherapy. *EGFR* mutations or FISH positivity (*EGFR* amplification or high polysomy) were apparent in 18% (18/100) and 32% (32/100) of patients, respectively. The Scorpion-amplification-refractory mutation system was more sensitive than direct sequencing for the detection of *EGFR* mutations. Furthermore, *EGFR* mutations were associated with *EGFR* amplification ($P = 0.009$) but not with FISH positivity ($P = 0.266$). Our results therefore suggest the existence of a significant association between *EGFR* mutation and *EGFR* amplification in patients with advanced NSCLC. (*Cancer Sci* 2008; 99: 2455–2460)

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase of the ErbB family and has been implicated in the proliferation and survival of cancer cells. Aberrant expression of *EGFR* has been detected in many human epithelial malignancies, including non-small cell lung cancer (NSCLC).[1,2] This receptor has therefore been identified as a promising target for anticancer therapy, and several agents have been synthesized that inhibit its tyrosine kinase activity. *EGFR* tyrosine kinase inhibitors (TKI) have been evaluated most extensively in individuals with NSCLC, and they have had a substantial impact on the treatment of this disease by offering additional therapeutic options for patients with advanced NSCLC.[3–6]

Somatic mutations in the tyrosine kinase domain of *EGFR* have been detected in a subset of NSCLC patients who respond to EGFR TKI[7–9] and have been shown to be closely associated with sensitivity to these drugs.[10–14] Indeed, we and others have prospectively demonstrated a high response rate to EGFR TKI therapy in NSCLC patients with *EGFR* mutations.[15–21] An increased copy number of the *EGFR* gene, as revealed by fluorescence *in situ* hybridization (FISH), has also emerged as an effective molecular marker of EGFR TKI sensitivity in NSCLC.[22–24] We previously showed that *EGFR* mutation and *EGFR* amplification are associated in human NSCLC cell lines and that endogenous EGFR expressed in such cell lines positive for both of these *EGFR* alterations are activated constitutively.[25] However, the relationship between *EGFR* mutation and FISH positivity for *EGFR*, which reflects gene amplification or high polysomy, has remained unclear.[22–24,26,27] Indeed, only a few studies have evaluated the relationship between mutation and gene copy number for *EGFR* because of the difficulty in obtaining tumor samples suitable for genetic analysis from individuals with advanced NSCLC. We previously showed that the Scorpion-amplification-refractory mutation system (ARMS) is a sensitive technique for the detection of *EGFR* mutations in tumor specimens such as pleural effusion fluid or tissue obtained by transbronchial needle aspiration.[28–30] In the present study, we evaluated *EGFR* mutation status in small tumor specimens from patients with advanced NSCLC both by direct sequencing and by Scorpion-ARMS and compared the sensitivity of these methods for the detection of *EGFR* mutations. Furthermore, we determined *EGFR* copy number by FISH analysis in paired tumor specimens and examined its relationship to *EGFR* mutation.

Materials and Methods

Patients. The present retrospective study recruited consecutive patients with advanced NSCLC who received chemotherapy at Kinki University Hospital between January 2003 and December 2005. Patients eligible for the study had histologically confirmed stage III or IV NSCLC that was not curable by surgical resection or radiotherapy, irrespective of the presence of measurable lesions or good performance status (PS). Patients with recurrence after surgical resection were excluded. Complete clinical information and tissue blocks suitable for genetic analysis were available for 100 patients. We examined the relationship between *EGFR* mutation and *EGFR* copy number as well as the influence of these *EGFR* alterations on clinical outcome. Tumor response was assessed by computed tomography and evaluated according to the Response Evaluation Criteria in Solid Tumors.[31] Survival was calculated from the date of initiation of chemotherapy either to the date of death from any cause or to the date of last contact. Some patients had been receiving EGFR TKI treatment before the demonstration in 2004 that mutations in *EGFR* confer increased sensitivity to these drugs. Moreover, many patients had already died before the initiation of our genetic analysis, preventing us from obtaining informed consent. The institutional review board

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therefore approved our study protocol with the conditions that samples would be processed anonymously and analyzed only for somatic mutations (not for germline mutations) and that the study would be disclosed publicly, according to the Ethical Guidelines for Human Genome Research published by the Ministry of Education, Culture, Sports, Science, and Technology, the Ministry of Health, Labor, and Welfare, and the Ministry of Economy, Trade, and Industry of Japan. The present study also conforms to the provisions of the Declaration of Helsinki.

Identification of *EGFR* mutations. The tumor specimens were fixed with formalin and embedded in paraffin. DNA was extracted with the use of a QIAamp Micro kit (Qiagen K.K., Tokyo, Japan) from tumor tissue derived either by macrodissection or by laser-capture microdissection carried out to enrich tumor cells. Polymerase chain reaction-based direct sequencing of exons 18–21 and ARMS with designed 'Scorpion' primers were applied for the allele-specific detection of *EGFR* mutations. Only the following previously described mutations[7,8] were classified as mutations in the present study: G719X in exon 18, deletion of E746 to A750 or of neighboring residues in exon 19, as well as L858R and L861Q in exon 21. Patients were regarded as *EGFR* mutation positive if a mutation in *EGFR* was detected either by direct sequencing or by ARMS. All mutations were confirmed by analysis of at least two independent amplification products.

Determination of *EGFR* copy number. *EGFR* copy number was determined by FISH analysis with the use of dual-color DNA probes (LSI *EGFR* SpectrumOrange/CEP 7 SpectrumGreen; Vysis, Downers Grove, IL, USA). The tumor specimens were classified into six categories on the basis of the FISH results, as described previously.[22] Those with high polysomy (≥4 copies of *EGFR* in ≥40% of cells) or gene amplification (presence of a tight *EGFR* gene cluster and a ratio of *EGFR* to chromosome 7 of ≥2 or ≥15 copies of *EGFR* per cell in ≥10% of cells analyzed) were considered FISH positive, with those in the remaining categories being considered FISH negative.

Statistical analysis. The relationships among *EGFR* status, clinical characteristics, and tumor response to EGFR TKI were analyzed with Fisher's exact test as appropriate. Survival curves were constructed by the Kaplan–Meier method, and the differences in survival between patient subgroups were compared by the log-rank test. The impact of various factors on survival was evaluated by univariate and multivariate analysis according to the Cox regression model. A P-value <0.05 was considered statistically significant. All statistical analysis was carried out with StatView software (SAS Institute, Cary, NC, USA).

Results

Patient characteristics. Between January 2003 and December 2005, a total of 125 consecutive patients diagnosed histologically with advanced NSCLC underwent chemotherapy at Kinki University Hospital. Tissue specimens from 100 patients were assessable for both *EGFR* mutation and *EGFR* copy number. Of these specimens, 72 were obtained by bronchoscopic biopsy, 15 by percutaneous needle biopsy (12 from lung, two from bone, and one from lymph node), six by thoracoscopic biopsy, and seven by surgery for diagnosis or palliative therapy. The clinical characteristics of these 100 patients are shown in Table 1. Most of the patients were male (64%) and had a history of smoking (67%), and adenocarcinoma was the most prevalent tumor histology (61%). Most patients (83%) also had a good Eastern Cooperative Oncology Group PS (0 or 1), and 63% received second-line or subsequent rounds of chemotherapy. Fifty-three patients (53%) were treated with EGFR TKI. Seventy patients (70%) had died by the time of genetic analysis, with the median follow-up time for the 30 survivors being 14.6 months.

***EGFR* alterations in non-small cell lung cancer.** Patients were analyzed for *EGFR* mutations by direct sequencing of exons 18 through 21 and by Scorpion-ARMS (Table 2). *EGFR* mutations, consisting of in-frame deletions in exon 19 ($n = 5$) and point mutations in exon 21 ($n = 13$), were detected in 18 patients (18%). Eight *EGFR* mutations were detected by direct sequencing and 16 mutations were detected by Scorpion-ARMS. Ten of the 16 mutations detected by Scorpion-ARMS were not identified by direct sequencing. However, two of the deletions in exon 19 (E746_S752 and E746_T751) that were detected by direct sequencing were not identified by Scorpion-ARMS, given that the Scorpion primers were designed only for detection of the E746_A750 deletion in exon 19. *EGFR* mutations were significantly more frequent in tumors of women than in those of men (33 vs 9%), in adenocarcinomas than in tumors with other histologies (28 vs 3%), and in never-smokers than in smokers (42 vs 6%) (Fig. 1a). One of the 18 *EGFR* mutations was detected in a squamous cell carcinoma. Determination of *EGFR* copy number by FISH analysis revealed gene amplification in six patients and high polysomy in 26 patients, with 32 patients thus being classified as FISH positive (Table 3). In contrast to *EGFR* mutation, FISH

Table 1. Characteristics of patients with advanced non-small cell lung cancer ($n = 100$)

Characteristic	Subset	No. patients
Sex	Male	64
	Female	36
Smoking history	Never-smoker	33
	Smoker	67
Tumor histology	Adenocarcinoma	61
	Other	39
Eastern Cooperative Oncology Group performance status	0	24
	1	59
	≥2	17
No. chemotherapies	1	37
	≥2	63

Table 2. Detection of epidermal growth factor receptor (*EGFR*) mutations by direct sequencing or amplification-refractory mutation system (ARMS) ($n = 100$)

Site	Mutation	Direct sequencing	ARMS	Direct sequencing or ARMS
Exon 19	15-bp deletion	1	3	3
	16-bp deletion	1	0	1
	19-bp deletion	1	0	1
Exon 21	L858R	5	13	13
Total		8 (8%)	16 (16%)	18 (18%)

Table 3. Determination of epidermal growth factor receptor gene copy number by fluorescence *in situ* hybridization (FISH) analysis ($n = 100$)

FISH status	Finding	No. patients
Positive	Gene amplification	6
	High polysomy	26
	Total	32
Negative	Low polysomy	35
	High trisomy	2
	Low trisomy	26
	Disomy	5
	Total	68

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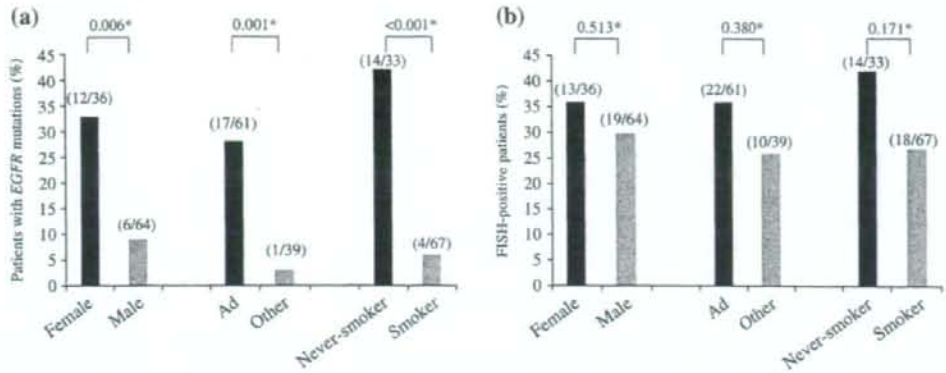

Fig. 1. Sex, tumor histology, and smoking status of patients with advanced non-small cell lung cancer and with either (a) epidermal growth factor receptor (*EGFR*) mutations or (b) a high *EGFR* copy number. Ad, adenocarcinoma. *P-values were determined by Fisher's exact test.

Table 4. Relationship between epidermal growth factor receptor (*EGFR*) mutation and either fluorescence *in situ* hybridization (FISH) status of *EGFR* amplification

Mutation status	FISH status		Gene amplification	
	Positive	Negative	Positive	Negative
Positive ($n = 18$)	8	10	4	14
Negative ($n = 82$)	24	58	2	80
P-value*		0.266		0.009

*Determined by Fisher's exact test.

positivity was not associated with sex, tumor histology, or smoking status (Fig. 1b). Although no relationship was apparent between *EGFR* mutation and FISH positivity (gene amplification or high polysomy), *EGFR* mutation and *EGFR* amplification were significantly associated (Table 4). The clinicopathological and genetic features of patients with *EGFR* mutations are shown in Table 5.

Overall survival. For the total patient population, the median overall survival was 12.3 months, with a 1-year survival rate of 51.7%. Univariate analysis revealed that overall survival was significantly longer in women, never-smokers, patients with a favorable PS, and those with *EGFR* mutations (Table 6; Fig. 2a). In contrast, no difference in overall survival was apparent between FISH-positive and FISH-negative patients (Table 6; Fig. 2b). We also carried out multivariate analysis to identify factors that contribute to overall survival, with covariates including clinicopathological and genetic factors (sex, smoking history, tumor histology, PS, *EGFR* mutation status, FISH status). Female sex and favorable PS were found to be independent prognostic factors (Table 6).

Responsiveness to epidermal growth factor receptor tyrosine kinase inhibitor treatment. Of the 53 patients treated with EGFR TKI, 40 individuals were assessable for objective response. Whereas the rate of response to EGFR TKI treatment for patients with *EGFR* mutations was significantly higher than that for those without such mutations (71.4 vs 11.5%, $P < 0.001$), there was no significant association between FISH status and responsiveness

Table 5. Clinicopathological and genetic features of patients with epidermal growth factor receptor (*EGFR*) mutations

No.	Age (years)	Sex	Smoking status	Histology	Response to EGFR TKI	Type of *EGFR* mutation		*EGFR* copy number
						Sequencing	ARMS	
1	72	F	Never	Ad	PR		L858R	Low trisomy
2	58	F	Never	Ad	PR	L858R	L858R	Gene amplification
3	81	F	Never	Ad	SD	L858R	L858R	High polysomy
4	72	F	Never	Ad	NE		L858R	Gene amplification
5	48	M	Smoker	Ad	SD		L858R	Low trisomy
6	67	F	Never	Ad	SD		L858R	Low trisomy
7	59	F	Never	Ad	PR		L858R	High polysomy
8	78	M	Smoker	Ad			L858R	High trisomy
9	71	F	Never	Ad	PR		L858R	Low polysomy
10	82	F	Never	Ad	PR	L858R	L858R	Low trisomy
11	67	F	Never	Ad		L858R	L858R	High polysomy
12	87	F	Never	Sq	PR	L858R	L858R	Low polysomy
13	78	M	Never	Ad			L858R	Gene amplification
14	56	F	Never	Ad	PR		(E746_A750)del	Low polysomy
15	63	M	Never	Ad	PD	(E746_A750)del	(E746_A750)del	Gene amplification
16	63	M	Smoker	Ad	PR		(E746_A750)del	Low polysomy
17	61	M	Smoker	Ad	PR	(E746_S752)del insV		Low trisomy
18	73	F	Never	Ad	PR	(E746_T751)del insS		High polysomy

Ad, adenocarcinoma; ARMS, amplification-refractory mutation system; NE, not evaluated; PD, progressive disease; PR, partial response; SD, stable disease; Sq, squamous cell carcinoma; TKI, tyrosine kinase inhibitor.

Table 6. Univariate and multivariate analyses of prognostic factors for overall survival

Factor	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Sex (female/male)	0.54	0.32–0.91	**0.021**	0.55	0.32–0.93	**0.025**
Smoking history (never-smoker/smoker)	0.50	0.30–0.85	**0.011**			
Histology (adenocarcinoma/other)	0.64	0.39–1.05	0.077	0.68	0.40–1.14	0.141
ECOG PS (0/≥1)	0.44	0.24–0.79	**0.006**	0.48	0.29–0.86	**0.019**
EGFR mutation status (positive/negative)	0.52	0.28–0.97	**0.039**			
FISH status (positive/negative)	1.36	0.82–2.23	0.231	1.49	0.88–2.50	0.130

CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; EGFR, epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization; HR, hazard ratio; PS, performance status. Multivariate analysis was carried out using the stepwise method (include, <0.05; exclude, >0.2). Significant *P*-values are shown in bold.

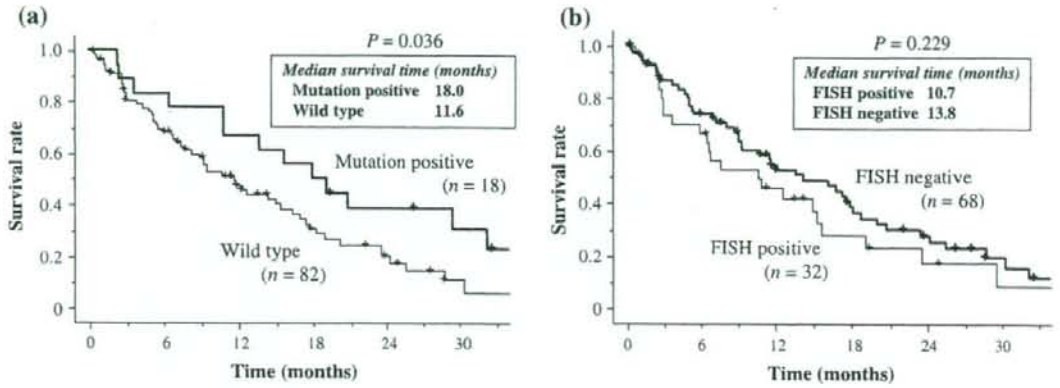

Fig. 2. Kaplan–Meier plots of overall survival in patients with advanced non-small cell lung cancer and either (a) with or without epidermal growth factor receptor (*EGFR*) mutations or (b) with or without a high *EGFR* copy number. FISH, fluorescence *in situ* hybridization.

to EGFR TKI (44.4 vs 29.0% for FISH-positive vs FISH-negative patients, respectively, $P = 0.437$).

Discussion

We have analyzed both *EGFR* mutation and *EGFR* copy number in paired tumor specimens as well as the relationship between these two types of *EGFR* alterations in advanced NSCLC. We used two methods to detect *EGFR* mutations, direct sequencing and Scorpion-ARMS, which identified eight and 16 mutations, respectively. Direct sequencing failed to detect 10 of the 16 mutations identified by Scorpion-ARMS. Of the 10 patients with *EGFR* mutations detected by Scorpion-ARMS alone, seven were assessable for an objective response to EGFR TKI, with five exhibiting a partial response and two having stable disease. Consistent with previous observations,[28–30] our data thus indicate that Scorpion-ARMS is more sensitive than direct sequencing for detection of the two major types of *EGFR* mutation that reflect responsiveness to EGFR TKI. It should be noted, however, that most polymerase chain reaction-based systems for mutation analysis, including Scorpion-ARMS, are able to detect only known *EGFR* mutations targeted by the designed primers. Indeed, two minor variants of deletion mutation in exon 19 were not identified by Scorpion-ARMS in the present study. Given the exclusion of recurrence after surgical resection in our study, most tumor specimens analyzed were obtained either by transbronchial lung biopsy or by percutaneous needle lung biopsy. The amount of tumor tissue obtained by these procedures is limited, but our results suggest that it is sufficient both for histopathological analysis and for the detection of *EGFR* mutations by Scorpion-ARMS in patients with advanced NSCLC.

Scorpion-ARMS identified three E746_A750 deletion mutations in exon 19 and 13 L858R point mutations in exon 21 in the present study. The frequency of the E746_A750 mutation detected by Scorpion-ARMS thus appeared low compared with that of the L858R mutation. Previous studies have shown that the incidence of the E746_A750 deletion is approximately the same as that of the L858R mutation.[10,12] The sensitivity of Scorpion-ARMS for detection of the E746_A750 deletion is equivalent to that for detection of the L858R point mutation. The low frequency of the E746_A750 deletion mutation in the present study is thus likely due to the small number of samples.

Previous studies have revealed a higher prevalence of *EGFR* mutations in East Asians than in Caucasians.[4,10–12,20,22,24,26,27,32–36] The prevalence of *EGFR* mutations in our Japanese cohort was low (18%) compared with values determined previously for East Asian populations. Given that most previous studies examined only individuals treated with EGFR TKI, patient selection based on clinical predictors might have led to an increase in the proportion of subjects with adenocarcinoma histology, a factor known to be associated with *EGFR* mutations. In contrast, our study was carried out with consecutive cases irrespective of EGFR TKI treatment. The relatively low proportion of patients with adenocarcinoma histology (61%) in our cohort is therefore consistent with the low prevalence of *EGFR* mutations. However, the FISH positivity of 32% in our study is similar to that in previous studies that adopted the same criteria, with values ranging from 31 to 48%.[22–24,26,27] Consistent with previous

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results,[1,7–9,12] *EGFR* mutations were significantly more frequent among women, never-smokers, and patients with adenocarcinoma in the present study. In contrast, neither *EGFR* amplification (analysis not shown) nor FISH positivity was associated with any such clinicopathological factor in our study, although the relationship between *EGFR* amplification and never-smoking status approached statistical significance ($P = 0.090$).

The relationship between *EGFR* mutation and FISH positivity (gene amplification or high polysomy) in NSCLC patients has remained unclear.[22–24,26,27] In the present study, we have demonstrated a significant relationship between *EGFR* mutation and *EGFR* amplification, but not between *EGFR* mutation and FISH positivity, in tumor specimens from patients with advanced NSCLC. *EGFR* mutant alleles were previously found to be amplified selectively, resulting in a high *EGFR* copy number, as detected by quantitative real-time polymerase chain reaction analysis.[12] *EGFR* amplification has also been shown to be acquired during invasive growth of lung adenocarcinoma with *EGFR* mutations.[37] Furthermore, recent studies have found that an increase in *EGFR* copy number is a relatively late event in NSCLC pathogenesis[38] and that *EGFR* mutation precedes *EGFR* amplification but not necessarily high polysomy.[37,39] These observations thus support the existence of a close association between *EGFR* mutation and *EGFR* amplification. We previously showed that *EGFR* mutation was significantly associated with *EGFR* amplification in human NSCLC cell lines and that endogenous EGFR expressed in such cell lines that manifested both of these *EGFR* alterations were activated constitutively as a result of ligand-independent dimerization.[25] However, the biological consequences of high polysomy for *EGFR* have not been elucidated. We did not find any cut-off value of high polysomy that was associated with *EGFR* mutation. We therefore propose that *EGFR* amplification, but not high polysomy, plays a key role in the pathogenesis of NSCLC and correlates with *EGFR* mutation.

We sought to determine whether *EGFR* mutation or *EGFR* copy number might affect overall survival of NSCLC patients. Previous studies of EGFR TKI have suggested that *EGFR* mutation is a favorable prognostic indicator for patients with NSCLC.[35,36] We also found that the survival time of patients with *EGFR* mutations was longer than that of those without them (18.0 vs 11.6 months, $P = 0.036$) in the univariate analysis. However, interpretation of this result requires that the effect of EGFR TKI on survival be taken into account, given that 83% (15/18) of patients with *EGFR* mutations were treated with EGFR TKI compared with only 46% (38/82) of those without such mutations. Indeed, analysis of survival after initiation of EGFR TKI treatment as a second-line or subsequent therapy revealed a survival time of 15.6 months for mutation-positive patients vs 6.0 months for mutation-negative patients in our study. It was therefore not possible to determine the prognostic significance of *EGFR* mutation for NSCLC patients. To clarify whether *EGFR* mutation is a predictor of sensitivity to EGFR TKI or a prognostic indicator for NSCLC patients, we are currently carrying out a phase III randomized study comparing platinum-based chemotherapy with gefitinib in chemotherapy-naive NSCLC patients with *EGFR* mutations. Patients with FISH-positive tumors tended to have a shorter survival time than did those with FISH-negative tumors (10.7 vs 13.8 months), although this difference was not statistically significant. This result is consistent with previous observations indicative of an association between high *EGFR* copy number and poor prognosis for certain malignancies, including NSCLC.[1,40]

In conclusion, we have analyzed both *EGFR* mutation and *EGFR* copy number in paired tumor specimens from patients with advanced NSCLC. We found that Scorpion-ARMS is more sensitive than direct sequencing for detection of *EGFR* mutations in small tumor specimens. Furthermore, we showed that *EGFR* mutation was significantly associated with *EGFR* amplification but not with FISH positivity. These observations warrant confirmation in further studies as well as exploration of the biological mechanisms of the relationship between *EGFR* mutation and *EGFR* amplification. The effects of *EGFR* mutation and *EGFR* copy number on clinical outcome in individuals with advanced NSCLC also warrant investigation in a prospective study.

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The anti-EGFR monoclonal antibody blocks cisplatin-induced activation of EGFR signaling mediated by HB-EGF

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Abstract **Cisplatin is a key agent in combination chemotherapy for various types of solid tumor. We now show that cisplatin activates signaling by the epidermal growth factor receptor (EGFR) by inducing cleavage of heparin-binding epidermal growth factor-like growth factor (HB-EGF). Matuzumab, a monoclonal antibody to EGFR, inhibited cisplatin-induced EGFR signaling, likely through competition with the soluble form of HB-EGF for binding to EGFR. Matuzumab enhanced the antitumor effect of cisplatin in nude mice harboring human non-small cell lung cancer xenografts. Our findings shed light on the mechanism by which monoclonal antibodies to EGFR might augment the efficacy of cisplatin.**

Keywords: EGF receptor; Heparin-binding EGF-like growth factor; Matuzumab; Cisplatin; Non-small cell lung cancer

1. Introduction

Cisplatin is a key component of combination chemotherapy for various types of solid tumor, but its effectiveness is limited by the development of chemoresistance [1]. Several nonphysiological stimuli that induce cellular stress, such as hyperosmolarity, wounding, UV or γ-radiation, reactive oxygen species, and chemotherapeutic agents, trigger activation of the epidermal growth factor receptor (EGFR) [2–11]. Ligand binding to EGFR induces receptor dimerization and activation of the receptor kinase, triggering intracellular signaling pathways such as those mediated by the protein kinases Akt or extracellular signal-regulated kinase (Erk), which play fundamental roles in the control of numerous cellular processes such as growth, proliferation, and survival [12–18]. EGFR signaling pathways activated by cellular stressors are thus of clinical interest because of their potential role in tumor resistance to chemotherapy [2–11]. The effects of cisplatin on EGFR signaling pathways have remained unclear, but the potential role of these pathways in cisplatin resistance makes it important to examine whether EGFR inhibitors might enhance the antitumor effects of this drug [8,9].

We have now examined the molecular mechanism of cisplatin-induced activation of EGFR and the effects of this drug on downstream signaling pathways. We also examined the effects of matuzumab (EMD72000, humanized mouse immunoglobulin G1), a monoclonal antibody (mAb) to EGFR [19], on cisplatin-dependent EGFR signaling. Finally, the antitumor effect of matuzumab combined with cisplatin was evaluated in order to provide insight into the mechanism by which anti-EGFR mAbs might augment the efficacy of cisplatin.

2. Materials and methods

2.1. Cell culture and reagents

The human non-small cell lung cancer (NSCLC) cell lines NCI-H292 (H292), NCI-H460 (H460), and A549 were obtained and cultured as previously described [20]. Matuzumab and gefitinib were also obtained as previously described [19]; GM6001 was from Calbiochem (La Jolla, CA); cisplatin, CRM197, and epidermal growth factor (EGF) were from Sigma (St. Louis, MO); and heparin-binding EGF-like growth factor (HB-EGF) was from R&D Systems (Minneapolis, MN).

2.2. Immunoblot analysis

Immunoblot analysis was performed as described previously [20]. Primary antibodies to the Tyr^{845}-phosphorylated form of EGFR, to EGFR, to phosphorylated Erk, to Erk, to phosphorylated Akt, and to Akt as well as horseradish peroxidase (HRP)-conjugated goat antibodies to mouse or rabbit immunoglobulin G were obtained as described previously [20]. Primary antibodies to the intracellular COOH-terminal domain of HB-EGF and HRP-conjugated donkey antibodies to goat immunoglobulin G were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Assessment of tumor growth inhibition in vivo

Tumor cells (2×10^6) were injected subcutaneously into the flank of 7-week-old female athymic nude mice. The mice were divided into four treatment groups of seven or eight animals: those treated over 2 weeks by intraperitoneal injection of vehicle, matuzumab (0.05 mg, twice per week), cisplatin (6 mg/kg of body weight, twice per week), or both matuzumab and cisplatin. Treatment was initiated when tumors in each group achieved an average volume of 200 mm^3, with tumor volume being determined twice weekly for 41 days after the onset of treatment from caliper measurement of tumor length (L) and width (W) according to the formula $LW^2/2$.

2.4. Ki67 index

Tumors were removed from some animals 14 days after treatment initiation and were stained with a mouse mAb to human Ki67 (clone MIB-1; Dako, Carpinteria, CA), as previously described [21]. The

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Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; mAb, monoclonal antibody; NSCLC, non-small cell lung cancer; HB-EGF, heparin-binding EGF-like growth factor; HRP, horseradish peroxidase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

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Ki67 index was determined as the percentage of Ki67-positive cells by scoring at least 300 tumor cells in each of 10 well-preserved fields of each tumor at a magnification of ×200 (CX41 light microscope; Olympus, Tokyo, Japan).

2.5. TUNEL staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) analysis of tumor sections was performed as described previously [22]. The number of apoptotic cells in each of 10 fields (×200) per tumor was determined with a light microscope (CX41, Olympus).

2.6. Statistical analysis

Quantitative data are presented as means ± S.D. and were compared among groups by one-way analysis of variance followed by Tukey's multiple comparison test. A P value of <0.05 was considered

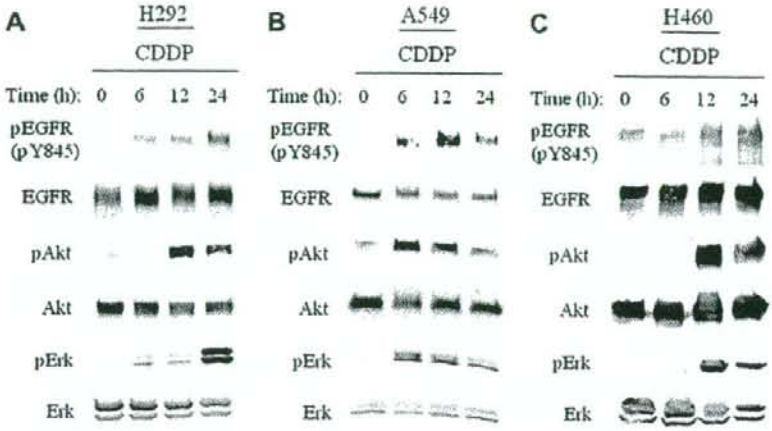

Fig. 1. Cisplatin-induced activation of EGFR and of downstream signaling pathways mediated by Akt or Erk. Serum-deprived H292 (A), A549 (B), or H460 (C) cells were incubated for the indicated times in the absence or presence of cisplatin (CDDP, 100 μM). Cell lysates were then subjected to immunoblot analysis with antibodies to the Tyr^{845}-phosphorylated form of EGFR (pEGFR), to phosphorylated Akt, or to phosphorylated Erk as well as with antibodies to total forms of these proteins.

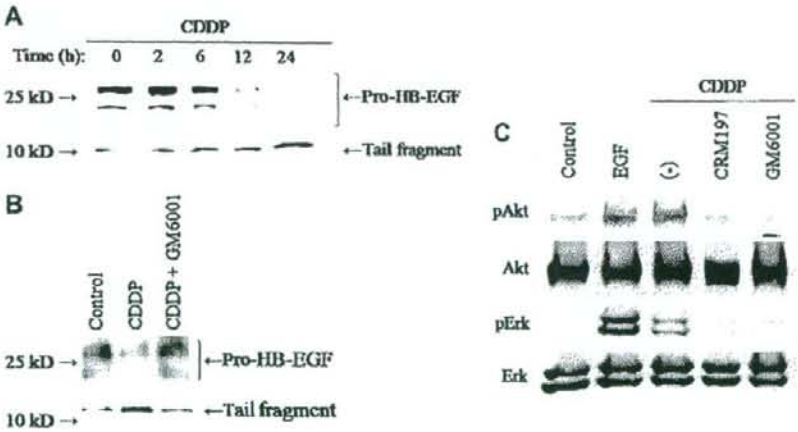

Fig. 2. Cisplatin-induced HB-EGF cleavage and its role in activation of EGFR signaling pathways by cisplatin. (A) Serum-deprived H292 cells were incubated for the indicated times in the presence of cisplatin (100 μM). Cell lysates were then subjected to immunoblot analysis with antibodies to the intracellular COOH-terminal domain of HB-EGF. The positions of molecular size standards (left) as well as of bands corresponding to pro-HB-EGF and to the cleaved tail fragment (right) are indicated. (B) Serum-deprived H292 cells were incubated alone (control) or with cisplatin (100 μM) in the absence or presence of GM6001 (10 μM) for 12 h. Cell lysates were then subjected to immunoblot analysis as in (A). (C) Serum-deprived H292 cells were incubated with EGF (100 ng/ml) for 15 min as a positive control or with cisplatin (100 μM) in the absence or presence of GM6001 (10 μM) or CRM197 (10 μg/ml) for 12 h. Cell lysates were then subjected to immunoblot analysis with antibodies to phosphorylated or total forms of Akt or Erk.

statistically significant. Statistical analysis was performed with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

3. Results and discussion

3.1. Cisplatin activates EGFR as well as downstream Akt and Erk signaling pathways

Cellular stress induced by several chemotherapeutic agents or γ-radiation triggers the activation of EGFR signaling pathways, with this effect being thought to play an important role in resistance to chemotherapy or radiotherapy [6–11]. We examined the effects of cisplatin on EGFR and downstream signaling pathways mediated by Akt or Erk in human NSCLC cell lines (H292, A549, H460). Cisplatin induced the phosphorylation of EGFR, Akt, and Erk in a time-dependent manner, without affecting the total amounts of these proteins, in all three cell lines (Fig. 1). These results thus showed that cisplatin activates EGFR and downstream signaling pathways mediated by Akt or Erk.

3.2. Cisplatin activates EGFR signaling pathways by inducing the cleavage of HB-EGF

HB-EGF is a membrane-bound EGFR ligand that activates EGFR after its release from the membrane in response to cellular stress [3,5,23–25]. To determine whether HB-EGF contributes to cisplatin-induced EGFR signaling, we examined the possible effect of cisplatin on cleavage of the membrane-bound pro-form of HB-EGF in H292 cells. Cisplatin induced a time-dependent decrease in the amount of pro-HB-EGF and a consequent increase in the amount of a COOH-terminal fragment of this protein referred to as the "tail fragment" (Fig. 2A). These effects of cisplatin were inhibited by GM6001 (Fig. 2B), a potent inhibitor of matrix metalloproteinases responsible for HB-EGF cleavage [23,24], suggesting that cisplatin induces metalloproteinase-mediated cleavage of the ectodomain of HB-EGF and its release from the cell sur-

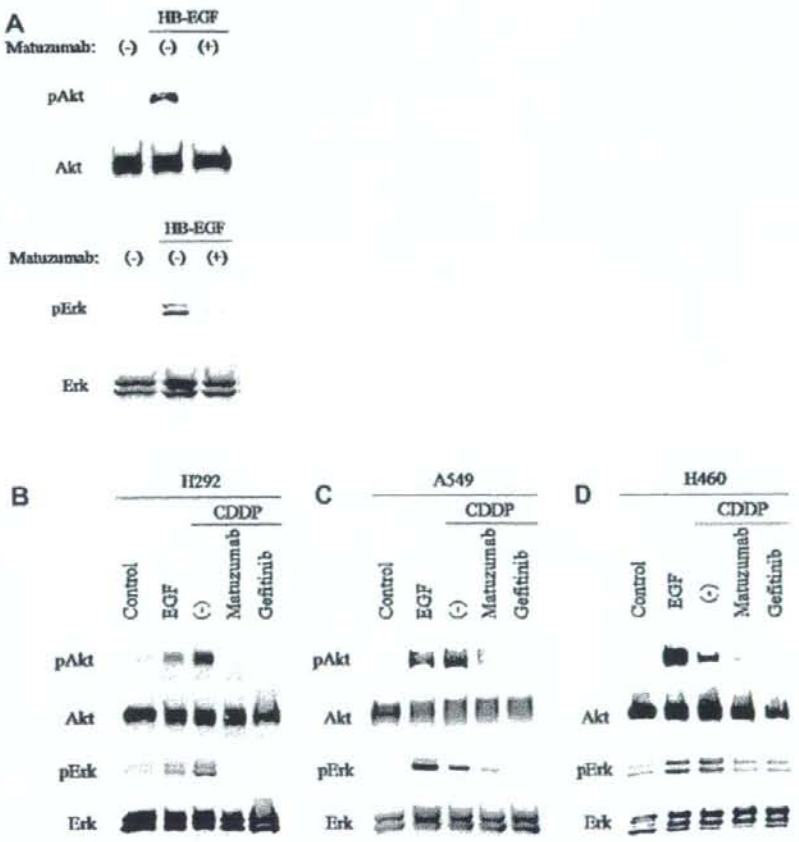

Fig. 3. Inhibition by matuzumab of EGFR signaling induced by HB-EGF or by cisplatin. (A) Serum-deprived H292 cells were incubated first for 2 h in the absence or presence of matuzumab (200 nM) and then for 15 min in the additional absence or presence of HB-EGF (10 ng/ml). Cell lysates were then subjected to immunoblot analysis with antibodies to phosphorylated or total forms of Akt or Erk. (B–D) Serum-deprived H292 (B), A549 (C), or H460 (D) cells were incubated with EGF (100 ng/ml) for 15 min as a positive control or with cisplatin (100 μM) in the absence or presence of matuzumab (200 nM) or gefitinib (10 μM) for 12 h. Cell lysates were then subjected to immunoblot analysis as in (A).

face. GM6001 also blocked the activation of Akt and Erk by cisplatin (Fig. 2C), implicating HB-EGF cleavage in cisplatin-induced EGFR signaling. To explore further whether cisplatin-induced EGFR signaling is dependent on HB-EGF activity, we examined the effect of CRM197, a nontoxic mutant form of diphtheria toxin that binds specifically to and neutralizes HB-EGF, which has also been identified as a diphtheria toxin receptor [26]. CRM197 completely inhibited the activation of Akt and Erk by cisplatin (Fig. 2C), suggesting that cisplatin promotes EGFR signaling by inducing the cleavage of HB-EGF. Consistent with this notion, the time course of cisplatin-induced activation of EGFR signaling (Fig. 1A) was similar to that of cisplatin-induced release of HB-EGF from the cell surface (Fig. 2A).

Cisplatin has previously been shown to increase the amount of HB-EGF mRNA in various types of cancer cells [7], and expression of the HB-EGF gene was found to be increased in cisplatin-resistant cancer [27]. The chemotherapeutic drugs SN38, doxorubicin, and imatinib also induce EGFR signaling and subsequent chemoresistance through metalloproteinase-dependent cleavage of HB-EGF [7,10]. It is possible that EGFR signaling resulting from metalloproteinase-mediated cleavage of HB-EGF represents a common mechanism of cellular resistance to various chemotherapeutic agents.

3.3. Effects of matuzumab on cisplatin-induced EGFR signaling

The clinical efficacy of treatment with anti-EGFR mAbs has been thought to be due to their prevention of ligand binding to EGFR [28,29]. We hypothesized that anti-EGFR mAbs might inhibit cisplatin-induced EGFR signaling by blocking the binding of the released ectodomain of HB-EGF to EGFR. To test whether anti-EGFR mAbs inhibit EGFR signaling induced by HB-EGF, we examined the effects of the humanized anti-EGFR mAb matuzumab. Matuzumab indeed prevented the activation of Akt and Erk by HB-EGF (Fig. 3A), indicating that this mAb inhibits HB-EGF–dependent EGFR signaling. We next examined the effect of matuzumab on cisplatin-induced EGFR signal transduction. The activation of EGFR downstream signaling by cisplatin was abolished by gefitinib in H292, A549, and H460 cells (Fig. 3B–D), suggesting that cisplatin-induced EGFR signaling requires the tyrosine kinase activity of EGFR. Matuzumab also markedly inhibited

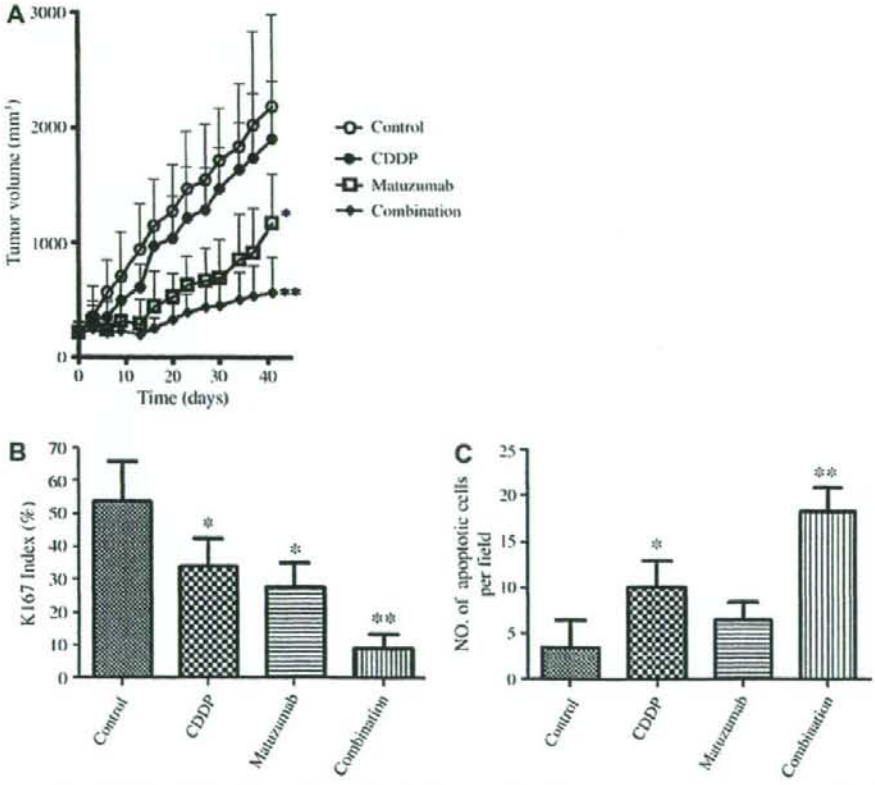

Fig. 4. Enhancement by matuzumab of the antitumor effect of cisplatin in vivo. (A) Nude mice harboring H292 tumor xenografts (200 mm^3) were treated with a single intraperitoneal dose of matuzumab (0.05 mg) or cisplatin (6 mg/kg), with both agents, or with vehicle (control) twice a week for 14 days. Tumor volume was determined at the indicated times after the onset of treatment. (B) The Ki67 index was determined from sections of H292 tumor xenografts 14 days after the initiation of treatment as in (A). (C) Quantitation by TUNEL staining of the number of apoptotic cells per field (×200) in H292 tumor xenografts 14 days after the initiation of treatment as in (A). Data in (A–C) are means ± S.D. $^*P < 0.05$ versus control; $^{**}P < 0.05$ versus control or each agent alone.

cisplatin-induced EGFR signaling in all three cell lines (Fig. 3B–D). These results thus suggested that matuzumab blocks cisplatin-induced EGFR signaling through inhibition of HB-EGF-dependent activation of EGFR.

Matuzumab exerts its antitumor effect both by competition with EGF for binding to EGFR and by blockade of the EGFR turnover that is important for activation of downstream signaling pathways mediated by Akt or Erk [19,28,29]. The soluble form of HB-EGF includes the EGF-like domain, a common structure in members of the EGF family of proteins that consists of 40–45 amino acids and contains six cysteine residues, but it binds not only to EGFR but also to ErbB4, whereas EGF binds specifically to EGFR [23–25]. The corresponding binding site of EGFR or the ligand function of HB-EGF may therefore differ from those for EGF. Nevertheless, we have now shown that matuzumab also inhibits the activation of EGFR signaling by both HB-EGF and cisplatin.

3.4. Matuzumab enhances the antitumor action of cisplatin in H292 xenografts

If cisplatin-induced EGFR signaling plays an important role in the development of cisplatin resistance, matuzumab might be expected to enhance the antitumor effect of cisplatin by inhibiting such signaling. We therefore determined the efficacy of combined treatment with matuzumab and cisplatin in nude mice with solid tumors formed by H292 cells injected into the flank. Combination therapy with matuzumab and cisplatin inhibited tumor growth to a significantly greater extent than did treatment with matuzumab or cisplatin alone (Fig. 4A).

Tumors treated with the combination of matuzumab and cisplatin also manifested both a significantly smaller Ki67 index (Fig. 4B), a marker of cell proliferation, and a significantly greater proportion of apoptotic cells (Fig. 4C), compared with tumors treated with either agent alone. Matuzumab alone or in combination with cytotoxic agents was previously shown to inhibit Akt or Erk phosphorylation in human tissue samples or human xenografts in nude mice [30–34]. The combination of matuzumab and cisplatin likely reduced the Ki67 index in the present study because matuzumab blocked the cisplatin-induced activation of Erk, which is important for cancer cell proliferation as a component of the Ras-MEK-Erk signaling pathway [17,18]. The increase in the number of apoptotic cells in tumors treated with both matuzumab and cisplatin likely resulted from inhibition by matuzumab of the cisplatin-induced activation of Akt, which contributes to antiapoptotic signaling through several pathways [15,16]. Our data thus indicate that matuzumab enhanced the antitumor effect of cisplatin, with the combination treatment inhibiting tumor cell proliferation and inducing apoptosis to a greater extent than treatment with either agent alone. Our data showing that gefitinib also blocked cisplatin-induced activation of Akt and Erk may explain the previous observation that the growth-inhibitory action of cisplatin in A549 tumors was increased fourfold in combination with gefitinib [35]. Our findings suggest the importance of EGFR signaling in the development of chemoresistance to cisplatin, and they provide insight into the mechanism by which anti-EGFR mAbs might augment the efficacy of cisplatin. Clinical studies of the therapeutic efficacy of matuzumab combined with cisplatin are thus warranted.

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Phase I Dose-escalation and Pharmacokinetic Trial of Lapatinib (GW572016), a Selective Oral Dual Inhibitor of ErbB-1 and -2 Tyrosine Kinases, in Japanese Patients with Solid Tumors

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Objective: The Phase I dose-escalation study was conducted to evaluate the safety and pharmacokinetics of lapatinib (GW572016), a dual ErbB-1 and -2 inhibitor, in Japanese patients with solid tumors that generally express ErbB-1 and/or overexpress ErbB-2.
Methods: Patients received oral lapatinib once daily until disease progression or in an event of unacceptable toxicity.
Results: Twenty-four patients received lapatinib at dose levels of 900, 1200, 1600 and 1800 mg/day; six subjects enrolled to each dose level. The majority of drug-related adverse events was mild (Grade 1–2); the most common events were diarrhea (16 of 24; 67%), rash (13 of 24; 54%) and dry skin (8 of 24; 33%). No Grade 4 adverse event was observed. There were four Grade 3 drug-related adverse events in three patients (i.e. two events of diarrhea at 1600 and 1800 mg/day each and γ-glutamyl transpeptidase increase at 1800 mg/day). The maximum tolerated dose was 1800 mg/day. The pharmacokinetic profile of lapatinib in Japanese patients was comparable to that of western subjects.
Conclusions: Lapatinib was well tolerated at doses of 900–1600 mg/day in Japanese solid tumor patients. Overall, our findings were similar to those of overseas studies.

Key words: ErbB-1 – ErbB-2 – lapatinib – phase I – tyrosine kinase inhibitor

INTRODUCTION

Dysregulation of the human epidermal growth factor (ErbB) family of cell surface receptors has been noted in several solid tumors. Binding of extracellular ligand to ErbB receptors activates multiple intracellular signaling pathways that can promote tumor growth through processes, such as cell proliferation, differentiation and inhibition of apoptosis. ErbB-1 and ErbB-2 are implicated in the pathogenesis of several cancers (1), and their overexpression in epithelial tumors—including those of the lung, breast, head and neck, colon, stomach, ovary and prostate—often correlates with poor prognosis (2,3).

ErbB receptors present two rational targets for inhibition: blockade of the extracellular ligand-binding domain by monoclonal antibodies and inhibition of the intracellular tyrosine kinase domain by small molecules (4). Several anticancer agents target specific ErbB isoforms. For example, the small molecule tyrosine kinase inhibitors gefitinib (Iressa®) and erlotinib (Tarceva®) and the monoclonal antibody cetuximab (Erbitux®) all target ErbB-1 (5–7), and thus, they are indicated for the treatment of non-small cell lung cancer (NSCLC) and colorectal cancer (8,9). Furthermore, a monoclonal antibody directed against ErbB-2 (trastuzumab, Herceptin®) has been approved for patients with ErbB-2-overexpressing breast cancer (10). Sensitivity to some of these agents is strongly associated with the expression levels of ErbB-1 and -2 (2,3).

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