involved 23 patients and was conducted using doses in the range of 0.15–1.35 mg/m$^2$. The major hematological toxicity was neutropenia (all patients = grade 3). Nonhematological toxicities included anorexia, malaise, nausea, and alopecia. The maximum tolerated dose (MTD) was not determined. One patient with sarcoma showed partial response (PR). Three patients with non-small-cell lung cancer (NSCLC) showed a >50% tumor reduction; however, this did not satisfy the criteria for PR, as the duration of the response was short.[10]

The present study, a phase I repeated-dose administration study of TZT-1027, was conducted according to a schedule consisting of weekly administration of the drug for 3 weeks followed by a 4-week observation period.

## Patients and Methods

**Study design.** The present study, an open-label, dose-escalating phase I study, was conducted in Japanese patients with solid tumors to determine the MTD, identify the recommended dose for phase II studies, and assess the pharmacokinetic profile of TZT-1027. The study and the written consent form were approved by the Institutional Review Board. All patients provided informed consent before study entry. The study was conducted in accordance with the Good Clinical Practice Guidelines and the Declaration of Helsinki.

**Patient eligibility.** Patients with histologically or cytologically confirmed solid tumors that were refractory to standard therapy or for which no effective therapy was available were eligible to participate in the present study. Other inclusion criteria included: no prior chemotherapy or radiotherapy within 4 weeks of study entry (within 2 weeks of study entry in the case of hormone drugs and antimetabolites); age ≥15 years and ≤75 years; Eastern Cooperative Oncology Group (ECOG) performance status ≤2; life expectancy at least 3 months; adequate bone marrow function with hemoglobin ≥9.5 g/dL, white blood cell (WBC) count 4000–12 000/mm$^3$, and platelet count ≥100 000/mm$^3$; normal hepatic function with serum bilirubin ≤1.5 mg/dL and serum aspartate aminotransferase and alanine aminotransferase ≤2.0 times the upper limit of the respective normal ranges; and adequate renal function with serum creatinine ≤ the upper limit of the respective normal range. All patients signed a written informed-consent form. Exclusion criteria included the presence of symptomatic brain metastases or pulmonary fibrosis, history of severe cardiac disorder (including severe atrial or ventricular arrhythmia or heart block), and pregnancy.

**Treatment and dose escalation.** TZT-1027 was given intravenously over 60 min in 250 mL saline. TZT-1027 was administered three times at weekly intervals (days 1, 8, and 15). The 4-week period after the third administration was designated as the observation period. The second and third administrations were conducted after confirmation of a WBC of 3000/mm$^3$ or more and neutrophil count of 1500/mm$^3$ or more. When these parameters did not meet the above-described criteria, the administration was delayed until they met the criteria; if, however, the criteria were not met even after 2 weeks of the final administration, the drug administration was discontinued altogether. If tumor regression was recognized and the patients recovered from adverse events by 4 weeks after the third administration (on day 15), re-administration at the same dose was allowed. Patients in whom the three weekly administrations of TZT-1027 failed for reasons other than dose-limiting toxicity (DLT) were replaced.

The starting dose was 0.3 mg/m$^2$, and the dose was increased up to 2.1 mg/m$^2$ (Table 1). The total dose of the three sessions (0.3 mg/m$^2$ × 3) was lower than 1.05 mg/m$^2$, which was lower than the 1.35 mg/m$^2$ used in the single-dose study. The safety of the maximum dose used (i.e. 1.35 mg/m$^2$) was confirmed in the single-dose phase I study carried out prior to the present study in Japan. According to the dose-escalation plan shown in Table 1, the dose was increased gradually to the maximum allowable dose (MAD). MAD was defined as the dose at which grade 3 or more severe hematotoxicity or grade 2 or more severe cardiac, hepatic, renal, or pulmonary toxicity appeared in two-thirds of patients. The MAD was reached at a dose of 1.5 mg/m$^2$; however, it was judged that estimation of the MTD is required for estimation of the recommended dose for phase II studies. Under approval by the Efficacy Safety Assessment Committee, the dose could be increased according to the protocol.

**Table 1. Number of TZT-1027 administrations**

| Dose of TZT-1027 (mg/m$^2$) | Number of patients | Number of administrations | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| 0.30 | 3 | 0 | 0 | 3 |
| 0.45 | 4 | 0 | 0 | 4 |
| 0.60 | 3 | 0 | 0 | 3 |
| 0.75 | 3 | 0 | 0 | 3 |
| 0.90 | 3 | 0 | 0 | 3 |
| 1.05 | 4 | 1 | 0 | 3 |
| 1.20 | 3 | 0 | 0 | 3 |
| 1.35 | 3 | 0 | 0 | 3 |
| 1.50 | 3 | 0 | 0 | 3[†] |
| 1.65 | 3 | 0 | 1 | 2 |
| 1.80 | 4 | 1 | 0 | 3 |
| 2.10 | 4 | 2 | 1 | 1 |
| Total | 40 | 4 | 2 | 34 |

[†]One patient had five administrations.

Maximum tolerated dose was defined as the minimum dose at which DLT appeared in at least two-thirds of the patients, and the recommended dose was defined as one dose level lower than the MTD. DLT was defined as follows: (i) grade 4 neutropenia; (ii) grade 4 leukopenia; (iii) grade 4 thrombocytopenia; and (iv) grade 3/4 non-hematological toxicity, excluding nausea and vomiting. When grade 4 leukopenia was confirmed, administration of granulocyte colony stimulating factor (G-CSF) was allowed. When grade 4 thrombocytopenia appeared, platelet transfusion was allowed.

Toxicity was assessed using the Adverse Drug Reaction Criteria of the Japan Society for Cancer Therapy.[11] The criteria are approximately similar to the Common Toxicity Criteria adopted by the National Cancer Institution in the USA.

**Treatment assessment.** Baseline assessment, including a complete medical history, physical examination, vital signs, ECOG performance status, blood counts, serum biochemistry, and urinalysis, was conducted to assess patient eligibility and had to be completed 5 days before the start of treatment.

During the TZT-1027 administration period and the subsequent 4-week observation period, routine biochemistry, hematology, and urinalysis were carried out weekly. Electrocardiograms were recorded before the first administration and after the third administration of TZT-1027, and at the end of the observation period. The left ventricular ejection fraction was assessed before TZT-1027 administration, after the third administration of the drug, and 2 weeks into the observation period. Chest X-rays were obtained at least twice: before the start of treatment and at the end of the observation period. Imaging examinations, including computed tomography, were obtained as necessary for evaluating the antitumor effects of the drug. Tumor response was evaluated according to Criteria for the Evaluation of Direct Effects of Solid Cancer Chemotherapy of the Japan Society for Cancer Therapy.[12]

**Pharmacokinetic sampling, assay, and analysis.** The pharmacokinetic profile of TZT-1027 was evaluated after the first and third administration. Blood samples were collected immediately

before the drip infusion, at the end of the drip infusion, and 3, 6, and 24 h after the drip infusion. All blood samples were centrifuged immediately after sampling at 2 000 g for 10 min at 4°C, and the plasma samples were stored at −20°C until analysis. Plasma concentrations were determined using the liquid chromatography–mass spectrometry method.

Pharmacokinetic analysis of data from individual plasma samples was made using standard model-independent (non-compartmental) methods. The following pharmacokinetic parameters were calculated: area under the curve (AUC), maximum concentration ($C_{max}$), half-life ($T_{1/2}$), mean residence time, and total clearance.

## Results

**Patient characteristics.** The demographic characteristics of the patients are shown in Table 2. Forty patients (28 men and 12 women) with a median age of 60 years were enrolled in the present study. The most frequently encountered tumor type was NSCLC.

All patients were included in the assessment of safety. The patients in whom TZT-1027 could be administered only once or twice for reasons other than DLT were considered to be unevaluable for DLT and replacement patients were added for administration of the same dose. TZT-1027 could be administered three times in 34 patients.

The drug was administered only twice in two patients; administration was discontinued because of DLT in one of these patients (1.65 mg/m$^2$), and because of increased tumor size in the other patient (2.1 mg/m$^2$). Drug administration was discontinued after the first administration in four patients because of DLT in two of these patients (2.1 mg/m$^2$) and lack of fulfillment of the hematological criteria for further drug administration (neutrophil count <1500/mm$^3$ or WBC count <3000/mm$^3$) in the remaining two patients at 1.05 and 1.8 mg/m$^2$, respectively.

**Table 2. Patient characteristics**

| Characteristic | *n* |
|---|---|
| Patients | 40 |
| Sex | |
| Male | 28 |
| Female | 12 |
| Median age (years) | 60 (range 25–74) |
| Performance status | |
| 0 | 16 |
| 1 | 18 |
| 2 | 6 |
| Tumor type | |
| Lung | 17 |
| Soft tissue | 4 |
| Esophagus | 3 |
| Pancreas | 2 |
| Colorectum | 2 |
| Thymoma | 2 |
| Mesothelioma | 2 |
| Stomach | 1 |
| Breast | 1 |
| Carcinoid | 1 |
| Bile duct | 1 |
| Rectum | 1 |
| Duodenum | 1 |
| Pharynx | 1 |
| Mediastinum | 1 |
| Previous treatment | |
| Chemotherapy | 30 |
| Radiotherapy | 3 |
| Surgery | 2 |
| Combination | 5 |

**Dose-limiting toxicity.** As shown in Table 1, 12 different doses of TZT-1027, ranging from 0.3 to 2.1 mg/m$^2$, were administered. Three to four patients were treated at each dose.

Dose-limiting toxicity appeared in two patients at 2.1 mg/m$^2$. One was a 59-year-old man with malignant mediastinal tumor who developed grade 4 neutropenia/leukopenia, grade 3 myalgia, and grade 4 constipation. He had received chest radiotherapy as pretreatment. On day 4 after drug administration, he developed grade 3 myalgia. On day 5 after drug administration, ileus appeared. On day 8 he developed grade 4 leukopenia (700/mm$^3$) and grade 4 neutropenia (272/mm$^3$). On days 9–12, G-CSF was administered, with improvement of the leukopenia and neutropenia. The myalgia and ileus subsided on days 11 and 20, respectively. The other patient was a 73-year-old male patient with NSCLC who developed grade 3 constipation and grade 4 neutropenia. He had received chest radiotherapy and docetaxel administration as pretreatment. On day 8 after the drug administration, grade 4 neutropenia was detected. On day 9, grade 3 constipation occurred. On days 8–12, G-CSF was administered, with improvement of the neutropenia. The constipation also subsided on day 16.

As DLT appeared in two-thirds of the patients at 2.1 mg/m$^2$, the dose was determined to be the MTD. At 1.8 mg/m$^2$, which was one dose level lower than 2.1 mg/m$^2$, no patients were noted with DLT, and the toxicity was also within the tolerated range. Based on these observations, this dose was judged as the recommended dose for phase II studies. DLT in other patients included grade 4 neutropenia, which occurred in one patient after three administrations of TZT-1027 at 1.5 mg/m$^2$, and in one patient after two administrations of TZT-1027 at 1.65 mg/m$^2$. None of the patients developed febrile neutropenia. There were no treatment-related deaths.

**Hematological toxicities.** Neutropenia was the major DLT of TZT-1027. Hematological toxicities as a function of the total numbers of patients and courses of TZT-1027 are shown in Table 3. Grade 4 neutropenia was observed at doses of 1.5 mg/m$^2$. The severity grade of neutropenia tended to increase with increased dose. G-CSF was used in only two patients who developed DLT at 2.1 mg/m$^2$, whereas the neutrophil count improved spontaneously in the other patients. Both anemia and thrombocytopenia were relatively mild. There was only one event of grade 3 thrombocytopenia at a dose of 2.1 mg/m$^2$.

**Nonhematological toxicities.** Table 4 shows the overall drug-related non-hematological toxicities observed. The common non-hematological toxicities were malaise, nausea, vomiting, and constipation. The most frequently observed toxicity was malaise, and phlebitis was rare in the present study. The DLT were grade 3/4 constipation and grade 3 myalgia at a dose of 2.1 mg/m$^2$. Concerning the myalgia, grade 2 myalgia was recorded in another patient at 2.1 mg/m$^2$. Three patients developed peripheral neuropathy, grade 1 at 1.35 and 1.65 mg/m$^2$, and grade 2 at 2.1 mg/m$^2$. There were no cases of cardiovascular toxicity.

**Pharmacokinetic studies.** The pharmacokinetics of TZT-1027 were assessed in all patients at the first administration and in 34 patients at the third administration. The pharmacokinetic parameters determined during the first and third administrations of TZT-1027 are shown in Table 5. The maximum plasma TZT-1027 concentration occurred at the end of the 1-h infusion. The plasma concentrations during the third administration were almost the same as those during the first administration. No evidence of accumulation was observed during the third administration.

The $C_{max}$ and AUC tended to increase with the dose, whereas the changes in $T_{1/2}$ did not show any dose-dependent tendency (Table 5; Fig. 2). The correlation between pharmacokinetics (AUC and $C_{max}$) and hematological toxicity (decrease in the percentage neutrophil count from baseline) showed that the



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Table 3. Hematological toxicities

| Dose (mg/m²) | No.patients | Leucopenia | | | | Neutropenia | | | | Hemoglobin decreased | | | Thrombocytopenia | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Grade | | | | Grade | | | | Grade | | | Grade | | | |
| | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 1 | 2 | 3 | 4 |
| 0.30 | 3 | 1 | | | | | 1 | | | | | | | | | |
| 0.45 | 4 | 1 | | | | 1 | | | | 1 | | 1 | | | | |
| 0.60 | 3 | 1 | 1 | | | | 2 | | | 1 | 1 | | | | | |
| 0.75 | 3 | 1 | 1 | | | | | 1 | | | 1 | | | | | |
| 0.90 | 3 | 3 | | | | 1 | 1 | | | | 1 | | | | | |
| 1.05 | 4 | 2 | 1 | | | | 1 | 1 | | 1 | 1 | | | | | |
| 1.20 | 3 | | 2 | 1 | | | 2 | 1 | | | 3 | | 1 | | | |
| 1.35 | 3 | | 2 | 1 | | | 2 | 1 | | | 2 | 1 | | | | |
| 1.50 | 3 | 1 | 1 | 1 | | | 1 | 1 | 1 | 1 | | | | | | |
| 1.65 | 3 | 1 | 1 | 1 | | | 1 | | 1 | | 1 | | | | | |
| 1.80 | 4 | | 3 | 1 | | 1 | 1 | 2 | | | 1 | 1 | 1 | | | |
| 2.10 | 4 | | | 2 | 1 | | | 1 | 2 | | 1 | | | | 1 | |
| Total | 40 | 11 | 12 | 7 | 1 | 3 | 12 | 8 | 4 | 4 | 12 | 3 | 2 | 0 | 1 | 0 |

Table 4. Nonhematological toxicities reported most frequently (>5%)

| Dose (mg/m²) | No. patients | Malaise | | | | Nausea/vomiting | | | Alopecia | | | Constipation | | | | Phlebitis | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Grade | | | | Grade | | | Grade | | | Grade | | | | Grade | | |
| | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 4 | 2 | 3 | 4 |
| 0.30 | 3 | | | | | | | | 1 | | | | | | | | | |
| 0.45 | 4 | 1 | | | | | | | 1 | | | | | | | | | |
| 0.60 | 3 | | | | | | | | 1 | | | | | | | | | |
| 0.75 | 3 | 1 | | | | 1 | | | | | | 1 | | | | | | |
| 0.90 | 3 | | | | | 2 | | | | | | | | | | | | |
| 1.05 | 4 | 2 | | | | 2 | | | 1 | | | | | | | | | |
| 1.20 | 3 | 1 | | | | | | | 1 | | | | | | | | | |
| 1.35 | 3 | 1 | 1 | | | | 1 | | | | | | | | | | | |
| 1.50 | 3 | 1 | | | | 1 | | | | | | | | | | 2 | | |
| 1.65 | 3 | 2 | | | | 1 | | | 1 | | | | | | | | | |
| 1.80 | 4 | | | | | | 1 | | 1 | | | | | | | 1 | | |
| 2.10 | 4 | 3 | | | | | | | 1 | | | | | 1 | 1 | | | |
| Total | 40 | 12 | 1 | 0 | 0 | 7 | 2 | 0 | 8 | 0 | 0 | 1 | 0 | 1 | 1 | 3 | 0 | 0 |

Table 5. Pharmacokinetic parameters of TZT-1027 at the first administration

| Dose (mg/m²) | No. patients | $C_{max}$ (ng/mL) Mean (CV%) | AUC (ng h/mL) Mean (CV%) | $Cl_{tot}$ (l/h/m²) Mean (CV%) | $T_{1/2}$ (h) Mean (CV%) | MRT (h) Mean (CV%) |
|---|---|---|---|---|---|---|
| 0.30 | 3 | 21.3 (24.4) | 49.1 (24.3) | 6.4 (27.0) | 3.4 (7.6) | 2.4 (16.0) |
| 0.45 | 4 | 44.3 (71.7) | 125.4 (86.0) | 6.9 (93.8) | 3.7 (21.8) | 3.2 (35.5) |
| 0.60 | 3 | 46.6 (43.0) | 132.1 (65.5) | 5.8 (50.3) | 4.1 (20.4) | 3.1 (26.2) |
| 0.75 | 3 | 52.2 (57.7) | 153.0 (77.6) | 7.2 (66.0) | 3.9 (31.2) | 3.1 (26.1) |
| 0.90 | 3 | 80.5 (46.5) | 209.6 (60.0) | 5.4 (52.3) | 3.3 (32.5) | 2.4 (24.6) |
| 1.05 | 4 | 123.9 (19.3) | 401.1 (37.5) | 2.9 (30.1) | 5.8 (44.8) | 4.6 (59.3) |
| 1.20 | 3 | 103.2 (40.8) | 276.7 (57.4) | 5.4 (54.3) | 3.9 (47.7) | 2.8 (40.9) |
| 1.35 | 3 | 112.4 (22.0) | 325.2 (17.7) | 4.3 (19.1) | 4.8 (15.4) | 3.1 (4.8) |
| 1.50 | 3 | 219.1 (27.2) | 652.9 (28.3) | 2.5 (33.9) | 5.6 (25.2) | 3.6 (16.6) |
| 1.65 | 3 | 177.3 (38.9) | 527.7 (30.2) | 3.3 (27.5) | 5.1 (22.1) | 3.5 (27.8) |
| 1.80 | 4 | 233.6 (34.9) | 731.2 (45.8) | 2.8 (40.1) | 5.4 (16.0) | 3.7 (28.7) |
| 2.10 | 4 | 246.5 (36.3) | 991.8 (50.8) | 2.5 (37.8) | 7.8 (28.2) | 6.9 (41.5) |

AUC, area under the curve; $C_{max}$, maximum concentration; $Cl_{tot}$, total clearance; MRT, mean residence time; $T_{1/2}$, half-life.

neutrophil count tended to decrease as AUC and $C_{max}$ increased ($r = 0.58$ and 0.58, respectively).

**Response evaluation.** The antitumor activity was assessed in all patients, with 16 patients showing no change. One patient with invasive thymoma who had previously received the cisplatin, vincristine, doxorubicin plus etoposide regimen, gemcitabine plus vinorelbine, and thoracic radiation at 40 Gy showed PR at 1.5 mg/m². Although administration of TZT-1027 was discontinued after the fifth administration (see Discussion) in this patient due to DLT (grade 4 neutropenia), the duration of PR was 183 days.

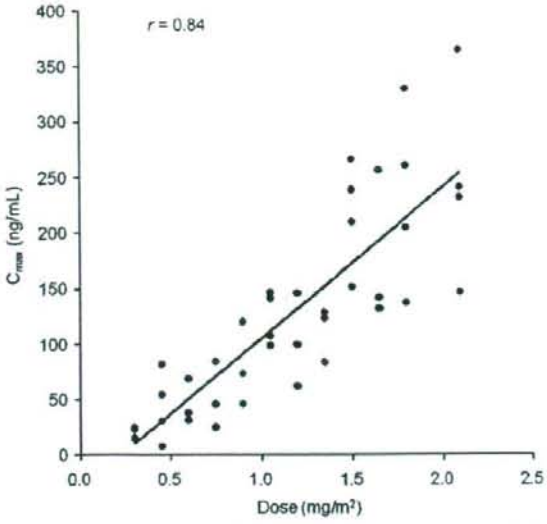


**Fig. 2.** Correlation between dose and maximum concentration ($C_{max}$) at the first administration.

## Discussion

Cellular tubulin is a well-established target for anticancer agents. Although currently available antitubulin agents, including the taxanes and vinca alkaloids, are highly effective anticancer agents, their clinical usefulness is limited due to their high rates of intrinsic or acquired resistance and systemic toxicities. Thus, it is important to develop newer agents targeting the tubulin and microtubule system that may be effective against tumors resistant to the existing anticancer agents and having an improved toxicity profile. A number of potent cytotoxic compounds have been discovered over the past decade, and candidate anticancer agents originating from marine life have been examined in human clinical trials. Of these compounds, dolastatin 10 and dolastatin 15 have been evaluated extensively in clinical studies. Cemadotin, an analog of dolastatin 15, was evaluated in phase I studies by several administration schedules and was found to cause neutropenia as a major DLT, apart from cardiac toxicity and hypertension.[13] Tasidotin, another dolastatin 15 analog, was also found to be associated with the DLT of neutropenia, ileus, and elevated transaminase levels.[14,15] Phase I studies of dolastatin 10 revealed that the drug caused neutropenia as a DLT.[16,17]

TZT-1027 was developed with the goal of obtaining the potent antitumor activity of the parent compound, but with reduced toxicity. In mice, intravenous injection of TZT-1027 showed efficacy equivalent to or greater than that of dolastatin 10. At the beginning of the present study, there were only data from a single-dose study in humans. Thus, the present study was the first repeated-dose phase I study conducted in humans. For this reason, TZT-1027 was, as a rule, administered three times at weekly intervals. The administration period was followed by a 4-week period of observation with the aim of confirming recovery of the patients from any toxicity. The administration was judged to be beneficial in the patients in whom no serious toxicity was noted and tumor regression was recognized after the three administrations. The drug was allowed to be continued even after the 4-week observation period only in the above patients. Because one patient with invasive thymoma experienced 70% tumor regression during the 4-week observation period, it was administered twice more until the patient developed the DLT of grade 4 neutropenia. This patient showed tumor regression by approximately 80% at the maximum, which confirmed PR.

The most frequently encountered DLT was grade 4 neutropenia, which either resolved spontaneously without treatment or could be reversed by administration of G-CSF. Other DLT included grade 4 leukopenia, grade 3 myalgia, and grade 3 and 4 constipation. However, peripheral neurological disturbance was mild, and it was considered that the toxicity of this antitubular agent resembled that of the vinca alkaloids rather than that of the taxanes. With regard to the pharmacokinetics, the AUC and $C_{max}$ increased with the dose. It was considered from the blood concentrations of the drug after the first and third administrations that the drug did not show accumulation.

On the basis of the results of the present study, some repeated-dose phase I studies were conducted after the present study. In the Netherlands, a repeated-dose study on days 1 and 8 of a 3-week course was conducted in patients with solid tumors. The dose of TZT-1027 was escalated to 2.7 mg/m$^2$, which produced neutropenia and infusion arm pain as DLT. The recommended dose of TZT-1027 for phase II studies was determined to be 2.4 mg/m$^2$.[18] In Japan also, a phase I study was conducted with the drug administered on days 1 and 8 of a 3-week course. Eighteen patients were enrolled in the study. Neutropenia was the principal DLT. Phlebitis was the most frequently encountered non-hematological toxicity. The recommended dose was determined to be 1.5 mg/m$^2$. This recommended dose was lower than that determined in the phase I study in the Netherlands.[19]

The recommended dose determined in the present study was 1.8 mg/m$^2$, which is also lower than that determined in the Netherlands study. The results of the pharmacokinetic parameters of TZT-1027 were similar between the present study and the Netherlands study. Therefore, it might be difficult to explain the difference in the recommended dose from the point of view of only pharmacokinetics. The possible difference might be the severity of bone marrow toxicity. In the present study, three of four patients at 2.1 mg/m$^2$ and one of four patients at 1.8 mg/m$^2$ could not receive TZT-1027 administration on day 8 on schedule. In a phase II study of TZT-1027 carried out in patients with treated soft-tissue sarcoma in the USA,[19] the dose used was 2.4 mg/m$^2$. Dose reduction to 1.8 mg/m$^2$ was required in approximately 40% of the patients, suggesting that 2.4 mg/m$^2$ may be a slightly high dose for patients who have received chemotherapy.

Some reports have shown that TZT-1027 exerts both considerable vascular effects and a direct cytotoxic effect, resulting in its strong antitumor activity,[20,21] and that TZT-1027 enhances the antitumor effect of ionizing radiation.[22] Clinical development of TZT-1027 in the future may include systemic treatment as a new anticancer drug with antiangiogenesis effects, and simultaneous combined use of the drug with radiation as a radiation sensitizer.

In conclusion, in the present study the MTD and recommended dose of TZT-1027, a synthetic analog of the natural marine product dolastatin 10, were determined to be 2.1 and 1.8 mg/m$^2$, respectively, for Japanese patients with advanced solid tumors, with the drug administered on days 1, 8, and 15. TZT-1027 showed less neurotoxicity than other tubulin inhibitors. These results suggest that TZT-1027 might be a promising new tubulin polymerization inhibitor that is generally well tolerated when administered on a weekly dosing schedule.

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# Randomized phase II study of two different schedules of gemcitabine and oral TS-1 in chemo-naïve patients with advanced non-small cell lung cancer (NSCLC).

**Sub-category:** Metastatic Lung Cancer
**Category:** Lung Cancer--Metastatic Lung Cancer
**Meeting:** 2008 ASCO Annual Meeting

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**Citation:** *J Clin Oncol* 26: 2008 (May 20 suppl; abstr 8103)
**Author(s):** M. Satouchi, Y. Kotani, N. Katakami, T. Shimada, Y. Urata, S. Yoshimura, Y. Funada, A. Hata, M. Ando, S. Negoro

**Abstract:** **Background:** TS-1, a novel oral fluorouracil derivative, has been shown to have anti-tumor activity with relatively mild adverse effects, and it is used in the treatment of NSCLC in Japan. The combination of gemcitabine (GEM) and 5-FU demonstrates a marked synergistic cytotoxic effect in a sequence-dependent manner in *in vitro* assay. This study was conducted in order to evaluate the efficacy and safety and to compare dosing schedules of gemcitabine combined with TS-1 in chemo-naïve NSCLC patients (pts). **Methods:** Pts with chemo-naive stage IIIB/IV NSCLC, an ECOG-PS of 0 or 1, and normal renal, liver, and bone marrow functions were randomized into 1 of 2 treatment arms. Oral TS-1 was administered daily from day 1 to 14, and GEM was given on days 1 and 8 (Arm A) or days 8 and 15 (Arm B). This cycle was repeated every 21 days. **Results:** A total of 80 pts were entered and 79 pts, treated in this trial. Randomization was well balanced across patient characteristics except for cell type (adenocarcinoma/squamous cell carcinoma = 37/4 (Arm A), 27/10 (Arm B)). Grade 3/4 hematological toxicities were neutropenia (54%), febrile neutropenia (9%), thrombocytopenia (11%) and anemia (4%). The hematological toxicity profiles did not differ very much between the two arms. Grade 3 pneumonitis was observed in 2 pts (3%). The response rate was 23.1% (95% confidence interval [CI]=11.1-39.3%) in Arm A and 30.6% (95% CI=16.3-46.1%), Arm B. Median time-to- progression (TTP) in Arm A was 4.1 months (95% CI=2.8-5.5) and Arm B, 5.4 months (95% CI=3.8-6.3) (p=0.75). Median survival time in Arm A was 15.7 months (95% CI=8.6-23.3) and Arm B, 22.4 months (95% CI=11.5-unknown) (p=0.32). **Conclusions:** The combination of GEM and TS-1 was determined to be feasible and effective for advanced NSCLC, and these results, particularly the favorable MST of Arm B, warrant further investigation of the Arm B dosing schedule for this combination for NSCLC.

Abstract Disclosures

**Associated Presentation(s):**

1. **Randomized phase II study of two different schedules of gemcitabine and oral TS-1 in chemo-naïve patients with advanced non-small cell lung cancer (NSCLC).**
   Meeting: 2008 ASCO Annual Meeting
   Presenter: Miyako Satouchi, MD
   Session: Lung Cancer — Metastatic (General Poster Session)
   
   

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1. **FLEX: A randomized, multicenter, phase III study of cetuximab in combination with cisplatin/vinorelbine (CV) versus CV alone in the first-line treatment of patients with advanced non-small cell lung cancer (NSCLC).**
   Meeting: 2008 ASCO Annual Meeting Abstract No: 3 First Author: R. Pirker
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1. **Randomized phase II study of two different schedules of gemcitabine and oral TS-1 in chemo-naïve patients with advanced non-small cell lung cancer (NSCLC).**
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   Category: Lung Cancer--Metastatic Lung Cancer - Metastatic Lung Cancer
2. **Randomized phase III study of platinum-doublet chemotherapy followed by gefitinib versus continued platinum-doublet chemotherapy in patients (pts) with advanced non-small cell lung cancer (NSCLC): Results of West Japan Thoracic Oncology Group trial (WJTOG.**

Meeting: 2008 ASCO Annual Meeting Abstract No: LBA8012 First Author: T. Hida
Category: Lung Cancer--Metastatic Lung Cancer - Metastatic Lung Cancer

3. **Randomized, phase III study of mitomycin/vindesine/cisplatin (MVP) versus weekly irinotecan/carboplatin (IC) or weekly paclitaxel/carboplatin (PC) with concurrent thoracic radiotherapy (TRT) for unresectable stage III non-small cell lung cancer (NSCLC).**

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1. **Randomized phase II study of two different schedules of gemcitabine and oral TS-1 in chemo-naïve patients with advanced non-small cell lung cancer (NSCLC).**

   Meeting: 2008 ASCO Annual Meeting
   Presenter: Miyako Satouchi, MD
   Session: Lung Cancer — Metastatic (General Poster Session)

   
   

2. **Randomized Phase II Study Of Docetaxel (doc) Plus Cisplatin (cddp) Versus Doc Plus Irinotecan In Advanced Non-small Cell Lung Cancer (nsclc); A West Japan Thoracic Oncology Group (wjtog) Study**

   Meeting: 2001 ASCO Annual Meeting
   Presenter: Miyako Satouchi, MD, PhD
   Session: Small Cell and Non-Small Cell Lung Cancer (General Poster)

   
   

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*Int. J. Cancer:* **122,** 1530–1538 (2008)


# Matuzumab and cetuximab activate the epidermal growth factor receptor but fail to trigger downstream signaling by Akt or Erk

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**Molecular inhibition of the epidermal growth factor receptor (EGFR) is a promising anticancer strategy, and monoclonal antibodies (mAbs) to EGFR are undergoing extensive evaluation in preclinical and clinical trials. However, the effects of anti-EGFR mAbs on EGFR signaling have remained unclear. We have now examined the effects of 2 anti-EGFR mAbs, matuzumab (EMD72000) and cetuximab (Erbitux), both of which are currently under assessment for treatment of various cancers, on EGFR signal transduction and cell survival in nonsmall cell lung cancer cell lines. Similar to EGF, matuzumab and cetuximab each induced phosphorylation of EGFR at several tyrosine phosphorylation sites as a result of receptor dimerization and activation of the receptor tyrosine kinase. In contrast to the effects of EGF, however, EGFR activation induced by these antibodies was not accompanied by receptor turnover or by activation of downstream signaling pathways that are mediated by Akt and Erk and are important for regulation of cell proliferation and survival. In addition, clonogenic survival assays revealed that matuzumab and cetuximab reduced the survival rate of H292 cells, in which they also inhibited the EGF-induced activation of Akt and Erk. Although we have examined only a few cell lines, our results indicate that the antitumor effects of matuzumab and cetuximab depend on inhibition of EGFR downstream signaling mediated by Akt or Erk rather than on inhibition of EGFR itself.**


**Key words:** EGF receptor; signal transduction; matuzumab; cetuximab; nonsmall cell lung cancer

The epidermal growth factor receptor (EGFR, also known as ErbB1), a member of the ErbB family of receptor tyrosine kinases, is a 170-kDa plasma membrane glycoprotein composed of an extracellular ligand binding domain, a transmembrane region and an intracellular tyrosine kinase domain with a regulatory COOH-terminal segment.[1] Binding of ligand to EGFR induces receptor dimerization, activation of the receptor kinase and autophosphorylation of specific tyrosine residues within the COOH-terminal region of the protein.[1] These events trigger intracellular signaling pathways that promote cell proliferation and survival.[2,3]

EGFR is frequently overexpressed in many types of human malignancy, with the extent of overexpression being negatively correlated with prognosis.[4,5] Recognition of the role of EGFR in carcinogenesis has prompted the development of EGFR-targeted therapies that include both small-molecule tyrosine kinase inhibitors (TKIs) that target the intracellular tyrosine kinase domain and monoclonal antibodies (mAbs) that target the extracellular domain.[6–8] Among EGFR-TKIs, gefitinib and erlotinib have been extensively evaluated in nonsmall cell lung cancer (NSCLC), and sensitivity to these drugs has been correlated with the presence of somatic mutations in the EGFR kinase domain or with EGFR gene (*EGFR*) amplification.[9–16] Among anti-EGFR mAbs, cetuximab (Erbitux), a chimeric mouse-human antibody of the immunoglobulin (Ig) G1 subclass, has proved efficacious in the treatment of irinotecan-refractory colon cancer[17] and was recently approved by the U.S. Food and Drug Administration for the treatment of patients with head and neck squamous cell carcinoma.[18] Several clinical studies of anti-EGFR mAbs such as matuzumab (EMD72000, humanized IgG1) and cetuximab are ongoing for other types of cancer including NSCLC.[19–24] Anti-EGFR mAbs bind to the extracellular ligand binding domain of the receptor and are thereby thought to block ligand binding.[18,25] The antitumor effects of these mAbs are thus thought to be attributable to inhibition of EGFR signaling as well as to other mechanisms such as antibody-dependent cellular cytotoxicity.[18,26] However, the detailed effects of anti-EGFR mAbs on EGFR signaling have remained unclear.[27–30]

We have now examined in detail the effects on EGFR signal transduction of 2 anti-EGFR mAbs, matuzumab and cetuximab, both of which are used clinically, to provide insight into the mechanisms of their antitumor effects.

## Material and methods

### *Cell culture and reagents*

The human NSCLC cell lines NCI-H292 (H292), NCI-H460 (H460) and Ma-1 were obtained as previously described[31] and were cultured under a humidified atmosphere of 5% $CO_2$ at 37°C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. Matuzumab and cetuximab were kindly provided by Merck KGaA (Darmstadt, Germany) and Bristol Myers (New York, NY), respectively; gefitinib was obtained from AstraZeneca (Macclesfield, UK); and trastuzumab (Herceptin; Genentech, South San Francisco, CA) was obtained from Chugai (Tokyo, Japan). Neutralizing antibodies to EGFR (clone LA1) were obtained from Upstate Biotechnology (Lake Placid, NY).

### *Immunoblot analysis*

Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis on a 7.5% gel, and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific sites, the membrane was incubated consecutively with primary and secondary antibodies, and immune complexes were detected with the use of enhanced chemiluminescence reagents, as described previously.[31] Primary antibodies to the specific intracellular phosphorylation sites of EGFR (pY845, pY1068 or pY1173), to Erk, to phospho-Akt and to Akt were obtained from Cell Signaling Technology (Beverly, MA); those to the extracellular domain of EGFR (clone 31G7) were from Zymed (South San Francisco, CA); those to the intracellular domain of EGFR (EGFR 1005) and to phospho-Erk were from Santa Cruz Biotechnology (Santa Cruz, CA); and those to β-actin (loading control) were from Sigma. Horseradish peroxidase-conjugated goat antibodies to mouse or rabbit IgG were obtained from Amersham Biosciences (Little Chalfont, UK).

### *Chemical cross-linking assay*

Cells were incubated first with 1 mM bis(sulfosuccinimidyl) suberate ($BS^3$; Pierce, Rockford, IL) for 20 min at 4°C and then with

*Abbreviations:* EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; mAb, monoclonal antibody; NSCLC, nonsmall cell lung cancer; Ig, immunoglobulin; $BS^3$, bis(sulfosuccinimidyl) suberate; PE, R-phycoerythrin; PI3K, phosphoinositide 3-kinase.

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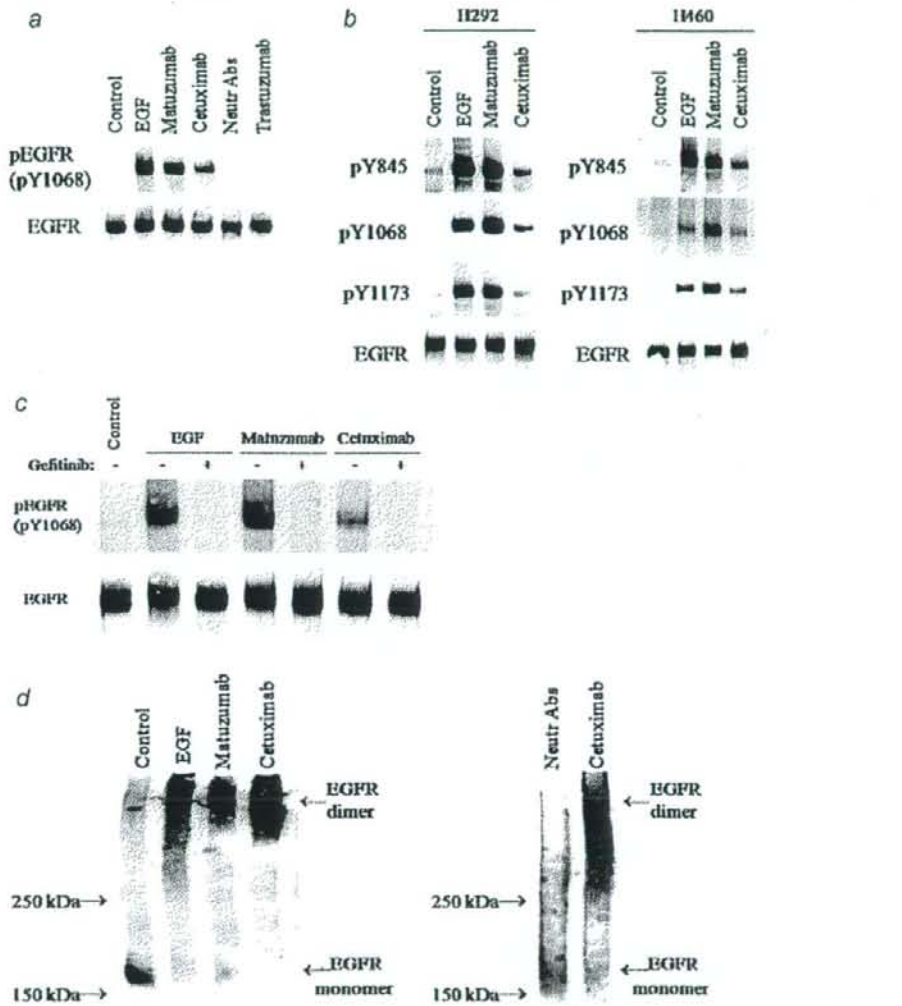


FIGURE 1 – EGFR phosphorylation induced by matuzumab or cetuximab as a result of receptor dimerization and activation of the receptor tyrosine kinase. (*a*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence (Control) or presence of matuzumab (200 nM), cetuximab (100 nM), neutralizing antibodies to EGFR (80 nM), trastuzumab (50 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR (pY1068) and to total EGFR (the extracellular domain). (*b*) H292 or H460 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y845-, Y1068- or Y1173-phosphorylated forms of EGFR and to total EGFR (the extracellular domain). (*c*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM), EGF (100 ng/ml) or gefitinib (10 μM), as indicated. Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR and to total EGFR (the extracellular domain). (*d*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM), neutralizing antibodies to EGFR (80 nM) or EGF (100 ng/ml). The cells were then washed and exposed to the chemical cross-linker $BS^3$ after which cell lysates were subjected to immunoblot analysis with antibodies to EGFR (the intracellular domain). The positions of EGFR monomers and dimers as well as of molecular size standards are indicated.

250 mM glycine for 5 min at 4°C to terminate the cross-linking reaction, as described previously.[31] Cell lysates were resolved by SDS-polyacrylamide gel electrophoresis on a 4% gel and subjected to immunoblot analysis with rabbit polyclonal antibodies to the intracellular domain of EGFR (EGFR 1005).

*Immunofluorescence analysis*

Cells were grown to 50% confluence in 2-well Lab-Tec Chamber Slides (Nunc, Naperville, IL), deprived of serum overnight, and then incubated with 200 nM matuzumab or EGF (100 ng/ml) for 4 hr at 37°C. They were fixed with 4% paraformaldehyde for

30 min at 4°C, permeabilized with 0.1% Triton X-100 for 10 min, and exposed to 5% nonfat dried milk for 1 hr at room temperature. The cells were stained with rabbit polyclonal antibodies to the intracellular domain of EGFR (EGFR 1005) for 1 hr at room temperature and then incubated for an additional 45 min with Alexa 488-labeled goat antibodies to rabbit IgG (Molecular Probes, Eugene, OR). Cell nuclei were counterstained for 5 min at room temperature with 4′,6-diamidino-2-phenylindole (Sigma) at 2 μg/ml. The chamber slides were mounted in fluorescence mounting medium (DakoCytomation, Hamburg, Germany), and fluorescence signals were visualized with a fluorescence microscope (Eclipse E800; Nikon, Kawasaki, Japan). Negative controls (secondary antibodies alone) did not yield any substantial background staining.

*Flow cytometry*

Cells were deprived of serum overnight and then incubated with 200 nM matuzumab or EGF (100 ng/ml) for 4 hr at 37°C. They were isolated by exposure to trypsin, and aliquots of $\sim 1.0 \times 10^6$ cells were incubated for 2 hr at 4°C either with an R-phycoerythrin (PE)-conjugated mouse mAb to EGFR (clone EGFR.1; Becton Dickinson, San Jose, CA), which does not interfere with the binding of EGF to EGFR,[32] or with a PE-conjugated isotype-matched control mAb (Becton Dickinson). The cells were then examined by flow cytometry (FACScalibur, Becton Dickinson) to detect the intensity of EGFR staining at the cell surface.

*Clonogenic assay*

Cells were plated in triplicate at a density of 200 per 25-$cm^2$ flask containing 10 ml of medium and were cultured for 7 days in the presence of the indicated concentrations of matuzumab or cetuximab. They were then incubated in medium alone for 7 days at 37°C, fixed with methanol:acetic acid (10:1, v/v), and stained with crystal violet. Colonies containing >50 cells were counted for calculation of the surviving fraction as follows: (mean number of colonies)/(number of inoculated cells × plating efficiency). Plating efficiency was defined as the mean number of colonies divided by the number of inoculated cells for untreated controls.

## Results

*Matuzumab and cetuximab induce EGFR phosphorylation in a manner dependent on the receptor tyrosine kinase activity*

With the use of immunoblot analysis, we first examined the effects of the anti-EGFR mAbs matuzumab and cetuximab on EGFR phosphorylation in human NSCLC H292 cells, which express wild-type EGFR. Incubation of the serum-deprived cells for 15 min with EGF, matuzumab or cetuximab-induced phosphorylation of EGFR on tyrosine-1068 (Y1068), whereas treatment of the cells with neutralizing antibodies to EGFR or with trastuzumab, a mAb specific for HER2 (ErbB2), had no such effect (Fig. 1*a*). Furthermore, like EGF, matuzumab and cetuximab each induced phosphorylation of EGFR on Y845, Y1068 and Y1173 in H292 and H460 cells (Fig. 1*b*), the latter of which are also human NSCLC cells that express wild-type EGFR.

To determine whether the antibody-induced phosphorylation of EGFR requires the kinase activity of the receptor, we examined the effect of gefitinib, a specific EGFR-TKI. H292 cells were deprived of serum and then exposed to matuzumab, cetuximab or EGF for 15 min in the absence or presence of gefitinib. EGFR phosphorylation on Y1068 induced by EGF, matuzumab or cetuximab was completely blocked by gefitinib (Fig. 1*c*). These findings thus indicated that, like EGF, matuzumab and cetuximab each induce EGFR phosphorylation by activating the tyrosine kinase of the receptor.

*Matuzumab and cetuximab induce EGFR dimerization*

Ligand-dependent EGFR dimerization is responsible for activation of the receptor tyrosine kinase.[33,34] To examine whether

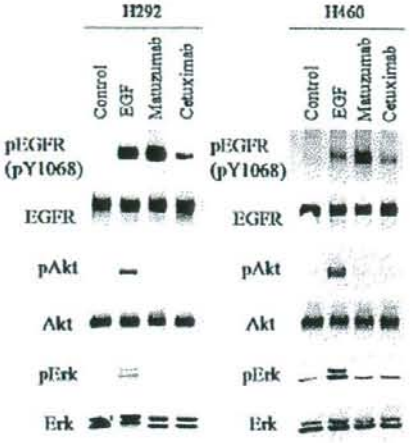


FIGURE 2 – Failure of matuzumab or cetuximab to activate Akt or Erk. H292 or H460 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR, to phosphorylated Akt and to phosphorylated Erk as well as with antibodies to total EGFR (the extracellular domain), Akt or Erk.

matuzumab or cetuximab induces EGFR dimerization, we incubated serum-deprived H292 cells with the mAbs for 15 min and then exposed the cells to the chemical cross-linker $BS^3$. Immunoblot analysis of cell lysates with antibodies to the intracellular domain of EGFR revealed that matuzumab and cetuximab each induced EGFR dimerization to an extent similar to that observed with EGF, whereas only the monomeric form of the receptor was detected in control cells or in cells treated with neutralizing antibodies to EGFR (Fig. 1*d*). These data thus suggested that matuzumab and cetuximab activate EGFR through induction of receptor dimerization.

*Matuzumab and cetuximab fail to induce signaling downstream of EGFR*

EGFR signaling is transduced by 2 main pathways mediated by phosphoinositide 3-kinase (PI3K) and Akt and by Ras, Raf and Erk.[35,36] To determine whether EGFR phosphorylation induced by matuzumab or cetuximab is accompanied by activation of these pathways, we examined the levels of phosphorylated (activated) Akt and Erk in H292 and H460 cells treated with these antibodies for 15 min after serum deprivation. In contrast to the effects of EGF, neither matuzumab nor cetuximab induced the phosphorylation of Akt or Erk in H292 or H460 cells (Fig. 2). These results thus indicated that matuzumab and cetuximab induce EGFR activation but fail to activate the downstream Akt and Erk signaling pathways.

*Matuzumab and cetuximab do not induce EGFR downregulation*

Endocytic trafficking of EGFR is important for full activation of Erk and PI3K.[37] To examine further the defect in signaling downstream of EGFR activation by matuzumab or cetuximab, we determined the effects of these mAbs on receptor turnover. H292 or H460 cells were deprived of serum and then cultured with EGF, matuzumab or cetuximab for various times up to 24 hr, after which the levels of phosphorylated and total EGFR, Akt and Erk were measured. In both H292 and H460 cells treated with EGF, the amount of total EGFR decreased in a time-dependent manner

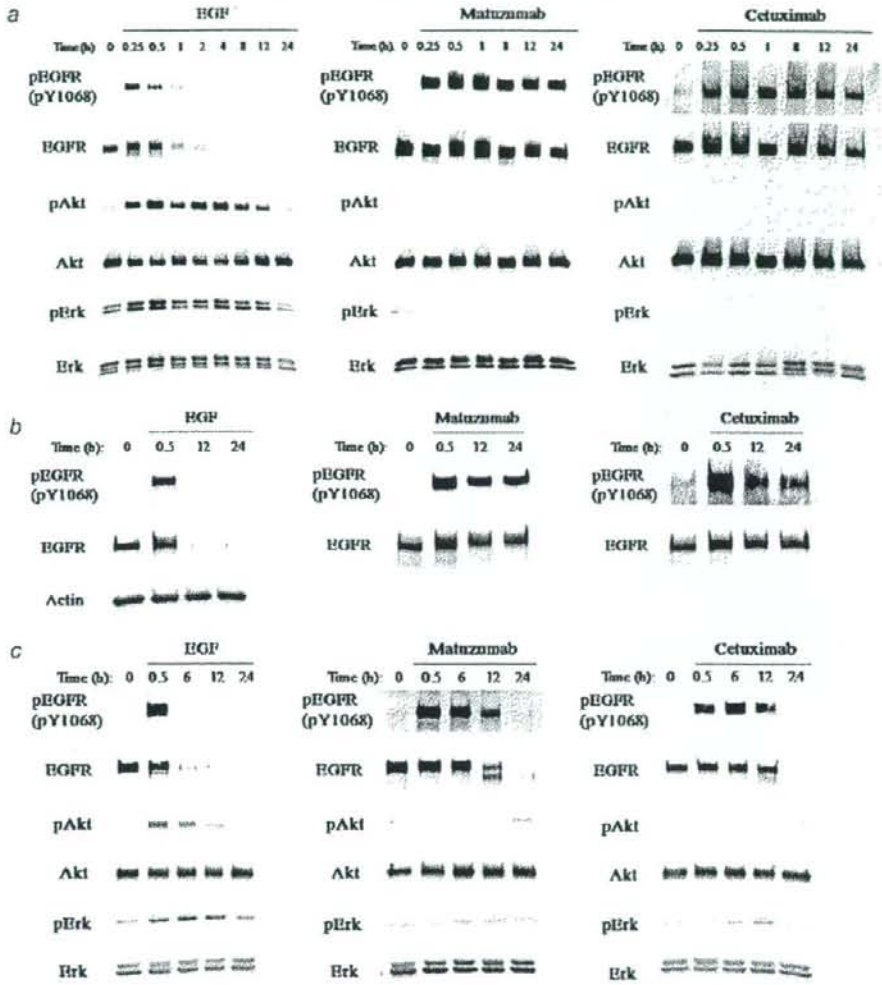


FIGURE 3 – Lack of EGFR turnover in cells treated with matuzumab or cetuximab. (*a*) H292 cells were deprived of serum overnight and then incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM), respectively. Cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (pY1068), Akt or Erk as well as with those to total EGFR (the extracellular domain), Akt or Erk. (*b*) H292 cells deprived of serum overnight were incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM). Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR, to total EGFR (the intracellular domain) or to β-actin (loading control). (*c*) H460 cells deprived of serum overnight were incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM), after which cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (pY1068), Akt or Erk as well as with those to total EGFR (the intracellular domain), Akt or Erk. (*d*) H292 cells plated on chamber slides were deprived of serum overnight and then incubated for 4 hr in the absence or presence of matuzumab (200 nM) or EGF (100 ng/ml). The cells were fixed, permeabilized, and stained with antibodies to EGFR and Alexa 488-labeled secondary antibodies (green). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). Fluorescence signals were visualized with a fluorescence microscope, and the merged images are shown. Scale bar, 20 μm. (*e*) H292 cells were deprived of serum overnight and then incubated for 4 hr in the absence or presence of matuzumab (200 nM) or EGF (100 ng/ml). The cells were stained with either a PE-conjugated mAb to EGFR (right peaks) or a PE-labeled isotype-matched mAb (left peaks) and analyzed by flow cytometry. Representative histograms of relative cell number versus PE fluorescence are shown.

(Figs. 3*a*–3*c*), an effect that has been shown to be the result of receptor internalization and degradation.[30,38] In parallel with this EGFR downregulation, the extent of EGF-induced tyrosine phosphorylation of EGFR also decreased and was virtually undetectable by 4–6 hr (Figs. 3*a*–3*c*). The phosphorylation of Akt and Erk induced by EGF persisted for at least 12 hr but had declined by 24 hr in both cell lines (Figs. 3*a* and 3*c*). In contrast, the levels of phosphorylated and total EGFR in H292 cells treated with

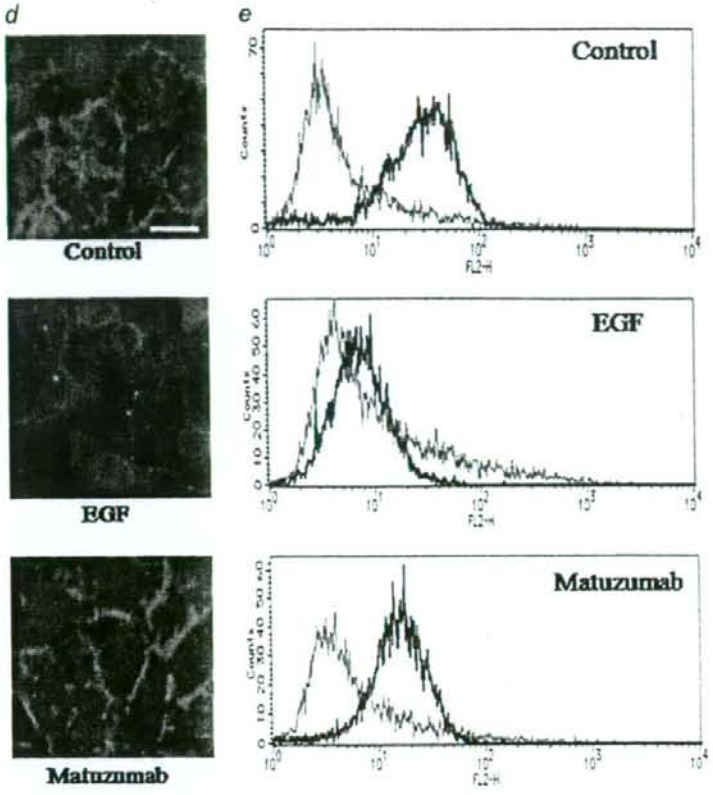


FIGURE 3 – CONTINUED

matuzumab or cetuximab for 24 hr were similar to those apparent after exposure to the antibodies for only 15 or 30 min (Figs. 3*a* and 3*b*). A marked delay in EGFR turnover was also apparent in H460 cells treated with matuzumab or cetuximab (Fig. 3*c*), although EGFR dephosphorylation and downregulation had occurred by 24 hr. Neither matuzumab nor cetuximab induced the activation of Akt or Erk or affected the total amounts of these proteins over a period of 24 hr in either cell line (Figs. 3*a* and 3*c*). We eliminated the possibility that the antibodies to the extracellular domain of EGFR used for the immunoblot analysis shown in Figure 3*a* bind only to the unoccupied form of EGFR (as a result of competition with EGF, matuzumab or cetuximab) by performing the immunoblot analysis shown in Figures 3*b* and 3*c* with antibodies to the intracellular domain of EGFR. These results thus suggested that downregulation of EGFR is impaired in cells treated with matuzumab or cetuximab, likely explaining the failure of these antibodies to activate downstream signaling by Akt and Erk.

To confirm that the inability of the anti-EGFR mAbs to induce EGFR downregulation is attributable to a failure to induce internalization-dependent receptor degradation, we treated serum-deprived H292 cells with matuzumab or EGF for 4 hr and then examined the expression of EGFR by immunofluorescence analysis (Fig. 3*d*) or flow cytometry (Fig. 3*e*). Whereas EGFR was localized at the cell surface in control cells, treatment with EGF resulted in internalization and a decrease in the fluorescence intensity of EGFR. In contrast, EGFR remained at the surface of cells treated with matuzumab. These data suggested that, in contrast to EGF-EGFR complexes, antibody-EGFR complexes remain at the cell surface and do not undergo internalization and degradation.

TABLE 1 – CHARACTERISTICS OF NSCLC CELL LINES

| Cell line | *EGFR* mutation | *EGFR* copy number |
|---|---|---|
| H292 | Wild type | Polysomy |
| H460 | Wild type | Monosomy |
| Ma-1 | del E746-A750 | Gene amplification |

*Effects of matuzumab and cetuximab on EGF-induced signaling and cell survival*

We next determined whether matuzumab or cetuximab inhibits ligand-dependent EGFR signal transduction. To examine also whether the effects of these antibodies are dependent on *EGFR* status, we studied 3 human NSCLC cell lines: 2 cell lines (H292, H460) that possess wild-type *EGFR* alleles and 1 (Ma-1) with an *EGFR* mutation in exon 19 that results in deletion of the residues E746–A750. Our recent fluorescence in situ hybridization analysis[31] revealed that *EGFR* copy number is increased (polysomy) in H292 cells and that H460 cells exhibit monosomy for *EGFR*. Ma-1 cells were also found to manifest *EGFR* amplification (Table 1).[31] We treated serum-deprived cells of the 3 NSCLC lines with

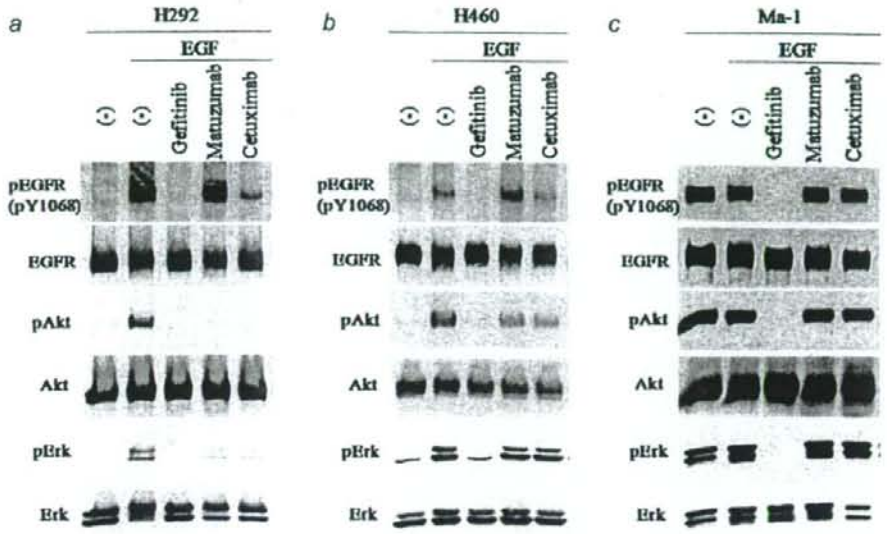


FIGURE 4 – Effects of matuzumab and cetuximab on EGF-induced EGFR signaling. H292 (*a*), H460 (*b*) and Ma-1 (*c*) cells were deprived of serum overnight and then incubated first for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or gefitinib (10 μM) and then for an additional 15 min in the additional absence or presence of EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (pY1068), Akt or Erk as well as with those to total EGFR (the extracellular domain), Akt or Erk.


FIGURE 5 – Effects of matuzumab and cetuximab on cell survival. H292, H460 or Ma-1 cells were plated at a density of 200 cells per 25-cm$^2$ flask in triplicate and cultured for 7 days in the presence of the indicated concentrations of matuzumab or cetuximab. They were then incubated with medium alone for 7 days before determination of the number of colonies containing >50 cells for calculation of the surviving fraction. Data are means of triplicates from a representative experiment. *$p < 0.001$ *versus* the corresponding value for cells not exposed to mAb (Student's *t*-test).

matuzumab, cetuximab or gefitinib for 15 min and then stimulated them with EGF for 15 min. Gefitinib prevented the phosphorylation of EGFR, Akt, and Erk induced by EGF in H292 (Fig. 4*a*) and H460 (Fig. 4*b*) cells. The level of EGFR phosphorylation in EGF-treated H292 or H460 cells was not substantially affected by matuzumab or cetuximab, likely because these antibodies also induce EGFR phosphorylation. However, whereas matuzumab and cetuximab did not substantially affect EGF-dependent phosphorylation of Akt or Erk in H460 cells, they markedly inhibited these effects of EGF in H292 cells. As we showed previously,[31] EGFR, Akt, and Erk are constitutively activated in the *EGFR* mutant cell line Ma-1 cell (Fig. 4*c*). Furthermore, whereas gefitinib blocked the phosphorylation of each of these 3 proteins in Ma-1 cells, matuzumab and cetuximab did not.

Finally, we performed a clonogenic assay to determine whether cell survival is affected by the differences in EGF-dependent signaling among H292, H460 and Ma-1 cells after treatment with matuzumab or cetuximab (Fig. 5). Matuzumab and cetuximab each induced a marked reduction in the survival rate of H292 cells, consistent with the inhibition of EGF-dependent EGFR downstream signaling by these antibodies in these cells. In contrast, neither mAb affected the survival of H460 or Ma-1 cells, consistent with the lack of inhibition of EGF-dependent or constitutive EGFR downstream signaling by matuzumab or cetuximab in these cell lines. These results suggested that the effects of matuzumab and cetuximab on EGF-dependent or constitutive EGFR downstream signaling are correlated with their effects on cell survival in NSCLC cell lines.

## Discussion

The effectiveness of treatment with anti-EGFR mAbs has been thought to be based on prevention of ligand binding to EGFR and consequent inhibition of EGFR activation.[18,25,26] Matuzumab and cetuximab have recently been developed as EGFR-inhibitory mAbs for clinical use.[17–22–25] A structural study revealed that cetuximab binds to the extracellular ligand binding domain (domain III) of EGFR,[25] and matuzumab is also thought to bind to domain III on the basis of its observed competition with EGFR ligands.[18] We have now shown that matuzumab and cetuximab induced phosphorylation of EGFR at several sites, including Y845, Y1068 and Y1173. These findings are consistent with previous observations that mAb 225, the mouse mAb equivalent to cetuximab, is able to induce EGFR dimerization and activation.[38,39] Cetuximab was also recently shown to induce phosphorylation of EGFR in head and neck squamous cell carcinoma cell lines[29] as well as in NSCLC cell lines including H292.[40] These in vitro results appear to contradict observations that matuzumab and cetuximab inhibit EGFR phosphorylation in vivo.[28,41,42] This apparent discrepancy may be due to the more complex cellular environment in vivo, including the presence of stromal cells that interact with tumor cells. We have also now shown that gefitinib, a specific EGFR-TKI, completely blocked EGFR phosphorylation induced by matuzumab or cetuximab, confirming that this effect of the antibodies is dependent on the intrinsic tyrosine kinase activity of EGFR. Furthermore, our cross-linking analysis showed that matuzumab as well as cetuximab activated EGFR through induction of receptor dimerization. Although recent structural analysis has revealed that cetuximab restricts the range of the extended conformation of EGFR that is required for ligand-induced receptor dimerization,[25] matuzumab and cetuximab likely induce EGFR dimerization in a manner dependent on their immunologically bivalent binding capacities, as was previously shown for mAb 225.[39] We found that neutralizing antibodies to EGFR did not activate EGFR, even though they also recognize the external domain of EGFR and compete with EGFR ligands for receptor binding.[43] The neutralizing antibodies did not induce EGFR dimerization, however, likely accounting for their inability to activate EGFR. This difference in the ability to induce EGFR dimerization between matuzumab and cetuximab on the one hand and the neutralizing antibodies on the other might be due to differences in the corresponding binding sites on EGFR.

To examine the mechanism by which matuzumab and cetuximab exert antitumor effects despite their induction of EGFR activation, we investigated the effects of antibody-induced EGFR activation on EGFR downstream signal transduction. We found that EGFR activation induced by matuzumab or cetuximab was not accompanied by activation of downstream signaling pathways mediated by Akt and Erk, both of which play an important role in regulation of cell proliferation and survival.[35,36] Moreover, we found that the antibody-EGFR complexes were not removed from the plasma membrane, in contrast to the rapid receptor turnover induced by EGF. In response to ligand binding, the ligand-EGFR complex is rapidly internalized and then either recycled back to the cell surface or proteolytically degraded.[44–46] The internalized EGFR interacts with various signaling proteins that are important for sustained activation of the major signaling pathways mediated by PI3K-Akt and Erk.[44,47] The activity of the PI3K-Akt and Erk pathways is thus greatly reduced in cells that are defective in internalization of ligand-EGFR complexes as a result of their expression of a mutant form of dynamin.[37] Furthermore, expression in glioblastoma cells of an EGFR chimeric protein that does not undergo internalization resulted both in a reduction in the extent of EGFR-dependent activation of Akt and Erk as well as in inhibition of tumor growth.[48] These observations thus suggest that inhibition of EGFR turnover by matuzumab or cetuximab is likely responsible for the failure of these mAbs to activate Akt and Erk.

We examined the effects of matuzumab and cetuximab on EGF-dependent EGFR signaling and on cell survival in 3 NSCLC cell lines of differing *EGFR* status. The inhibition of EGF-dependent activation of Akt and Erk by these antibodies appeared related to the inhibition of clonogenic cell survival in the 3 cell lines. With regard to NSCLC cell lines harboring wild-type *EGFR* alleles, matuzumab and cetuximab markedly inhibited EGF-dependent phosphorylation of Akt and Erk in H292 cells but not in H460 cells. Both antibodies inhibited cell survival in H292 cells but not in H460 cells. These results suggest that the antitumor effects of matuzumab and cetuximab depend on inhibition of EGFR downstream signaling such as that mediated by Akt and Erk rather than on inhibition of EGFR itself. Our present data are consistent with previous observations that cetuximab did not inhibit EGFR phosphorylation completely even in cells sensitive to this antibody.[27,30] It is possible that the difference in sensitivity to matuzumab and cetuximab between the 2 cell lines expressing wild-type EGFR in the present study is due to the difference in gene copy number, given that we found an increase in *EGFR* copy number in H292 cells compared with that in H460 cells.[31] A previous clinical study showed that *EGFR* copy number correlated with the response to cetuximab treatment in individuals with colorectal cancer.[49] *EGFR* copy number was not determined by fluorescence in situ hybridization in previous clinical studies of NSCLC patients treated with matuzumab or cetuximab.[19,22–24] Several clinical studies of the therapeutic efficacy of anti-EGFR antibodies in NSCLC patients are underway, and investigation of the potential of molecular markers including *EGFR* copy number to predict clinical response is warranted. Matuzumab and cetuximab failed to inhibit both activation of Akt and Erk and clonogenic cell survival in Ma-1 cells, which express a mutant form of EGFR that shows an increased sensitivity to EGFR-TKIs such as gefitinib and erlotinib.[9–16] We recently showed that cells expressing EGFR mutants exhibit constitutive, ligand-independent receptor dimerization and activation,[31] likely explaining the lack of effect of matuzumab or cetuximab on EGFR signaling or cell survival in such cells. However, previous studies showed that cetuximab exerted an antitumor effect in a cell line with an *EGFR* mutation, whereas several other cell lines with *EGFR* mutations were resistant to cetuximab.[27,30] Our results are consistent with clinical observations showing that the presence of an *EGFR* mutation is not a major determinant of a positive response to cetuximab in individuals with NSCLC or colorectal cancer.[22,50,51]

In conclusion, we have shown that EGFR turnover is impaired in cells treated with the anti-EGFR mAbs matuzumab or cetuximab, resulting in inhibition of EGFR downstream signaling. Although our study is limited by the small number of cell lines analyzed, our findings provide important insight into the mechanisms by which anti-EGFR mAbs exert their antitumor effects, and they suggest that it may be possible to predict the therapeutic efficacy of such mAbs by assessment of EGFR signal transduction.

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ORIGINAL ARTICLE

# mRNA expression of RRM1, ERCC1 and ERCC2 is not associated with chemosensitivity to cisplatin, carboplatin and gemcitabine in human lung cancer cell lines

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**mRNA expression of RRM1, ERCC1 and ERCC2 is not associated with chemosensitivity to cisplatin, carboplatin and gemcitabine in human lung cancer cell lines**
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**Background and objective:** Expression of genes involved in DNA repair and/or DNA synthesis, including ribonucleotide reductase M1 (RRM1) and excision repair cross-complementation 1 (ERCC1) has been reported to be associated with chemosensitivity to platinum agents and gemcitabine. The aim of this study was to test whether similar associations would be seen between mRNA expression for the RRM1, ERCC1 and ERCC2 genes and *in vitro* chemosensitivity in lung cancer.

**Methods:** Using a panel of 20 lung cancer cell lines, including 15 NSCLC and 5 small cell lung cancers (SCLC), the mRNA expression levels for the RRM1, ERCC1 and ERCC2 genes were examined by quantitative real-time reverse transcription PCR. The *in vitro* cytotoxicity of cisplatin, carboplatin and gemcitabine was assessed using a tetrazolium-based colorimetric assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay).

**Results:** Significantly, higher RRM1 mRNA expression was found in SCLC compared with NSCLC. However, there were no correlations between mRNA expression of the ERCC1, ERCC2 and RRM1 genes and chemosensitivity to cisplatin, carboplatin or gemcitabine.

**Conclusions:** These *in vitro* results suggest that further studies are needed to evaluate the expression of the RRM1, ERCC1 and ERCC2 genes as predictive biomarkers for sensitivity to platinum agents and gemcitabine.

**Key words:** chemosensitivity, DNA repair, DNA synthesis, lung cancer, predictive biomarker.

## INTRODUCTION

Lung cancer is a leading cause of cancer deaths both in Japan and the USA.[1,2] Despite advances in the molecular biology, diagnosis and treatment of non-small cell lung cancer (NSCLC), which accounts for about 85% of all lung cancers, the improvement in long-term survival has only been marginal.[3] The best prospects of a cure are offered by surgical removal of early stage lung cancer, followed by concurrent chemoradiotherapy for locally advanced lung cancer. Chemotherapy for advanced lung cancer offers mild benefits in improvement of quality of life and increased survival time.

The common first-line chemotherapeutic regimens for advanced NSCLC are platinum-based combinations. The combinations of cisplatin or carboplatin with another cytotoxic agent such as paclitaxel, docetaxel, gemcitabine, vinorelbine or irinotecan produce similar response rates of about 30–40% and a median survival time of about 1 year.[4,5] To improve clinical outcomes in advanced NSCLC, clinical integration of molecular biomarkers that predict

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responses to chemotherapeutic or molecularly targeted agents, leading ultimately to individualized chemotherapy, may be important. Despite intensive studies, however, only mutations of the epidermal growth factor receptor (EGFR) gene have been validated as correlating with the clinical efficacy of EGFR tyrosine kinase inhibitors.[6]

Recently, expression of genes involved in DNA repair and/or DNA synthesis have been reported to be associated with chemosensitivity to platinum agents and gemcitabine, as well as clinical outcomes in patients with surgically resected early stage NSCLC.[7–9] Excision repair cross-complementation 1 (ERCC1) is one of the key enzymes in the nucleotide excision repair (NER) pathway.[10] Platinum agents such as cisplatin and carboplatin induce monoadducts and intrastrand or interstrand cross-linking of DNA.[10] The removal of adducts from genomic DNA is mediated by the NER pathway, in which ERCC1 forms a heterodimer with the xeroderma pigmentosum group F (XPF) protein and excises the nucleotide segment containing the adducts in coordination with XPG. ERCC2/XPD is also a component of the NER mechanism.[11] Enhanced gene expression in the NER pathway has been thought to be a major cause of resistance to cisplatin and other DNA-damaging chemotherapeutic agents. Ribonucleotide reductase M1 (RRM1) is involved in DNA synthesis, catalysing the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides, which is the molecular target of gemcitabine.[12] Earlier work had suggested that patients with low levels of tumour RRM1 mRNA expression had improved survival compared with those with high RRM1 mRNA expression levels, when treated with gemcitabine.[11] Therefore, analysis of the expression of these genes could be useful in the development of predictive biomarkers for NSCLC.

The identification of molecular biomarkers with the potential to predict treatment outcomes is essential for triaging patients to the most beneficial therapy. As one of the multiple approaches to establishing robust predictive biomarkers, we evaluated whether there would be associations between mRNA expression of the ERCC1, ERCC2 and RRM1 genes and *in vitro* chemosensitivity to cisplatin, carboplatin and gemcitabine.

## METHODS

### Cell lines

Fifteen NSCLC and five small cell lung cancer (SCLC) cell lines were used. Two NSCLC and 4 SCLC sell lines, with the prefix ACC-LC-, were established in our laboratories at Aichi Cancer Center. These cell lines were derived from lymph node metastases (–80, –94), pleural effusions (–49, –319) or pericardial effusions (–48, –172). NCI-H460 and A549 were purchased from the American Type Culture Collection (Manassas, VA, USA). PC-1 and PC-10 were generously provided by Dr Y. Hayata (Tokyo Medical University, Tokyo, Japan). The remaining 10 cell lines (VMRC-LCD, RERF-LC-MT, -AI, Calu1, Calu6, SK-MES-1, SK-Lu-1 and SK-LC-2, –3 and –6) were generous gifts from Dr Old and Dr M. Akiyama. All cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum.

### Drugs

Gemcitabine (Gemzar) was provided by Eli Lilly, Kobe, Japan. Cisplatin and carboplatin were provided by Bristol-Myers Squibb, Tokyo, Japan.

### Cytotoxicity assay

Exponentially growing cells were harvested and resuspended at a final concentration of $1–20 \times 10^4$ cells/mL in fresh medium. Cell suspensions (100 μL) were dispensed into 96-well tissue culture plates and after 24 h at 37°C, various concentrations of the anticancer agents were added and incubated for 3 days. Cytotoxicity was evaluated by complete dose–response curves in the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT assay) as described previously.[13] The per cent cytotoxicity was calculated as: % cytotoxicity = {1-[Optical Density (OD) treated]/(OD control)} × 100. Each experiment was repeated at least three times. The cytotoxic effect of each treatment was assessed as the IC50 (drug concentration inducing a 50% reduction in cell survival in comparison with the control untreated cells), which was calculated from the dose–response curves.

### RNA preparation

Cells were lysed with 1 mL of Isogen (Nippongene, Toyama, Japan) and total RNA was extracted according to the manufacturer's protocol, with the addition of glycogen to facilitate RNA precipitation. The RNA was further purified and treated with DNase (RNeasy kit, Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, and stored at –80°C until use.

### Reverse transcriptase-PCR amplification

Total RNA (50 ng) extracted from each cell line was subjected to one-step real-time reverse transcriptase (RT)-PCR for absolute quantitation of the mRNA levels of the ERCC1, ERCC2, RRM1 and β-actin genes, using the Applied Biosystems 7500F PCR system (Applied Biosystems, Foster City, CA, USA). The assays were performed in 20 μL reaction mixtures, using a One-step SYBR PrimeScript RT-PCR kit (TAKARA, Ohtsu, Japan) according to the manufacturer's protocol. The sequences of the primers are shown in Table 1. The RT-PCR condition was an initial incubation at 42°C for 5 min followed by 10-s incubation at 95°C, then 40 cycles at 95°C (5 s), 60°C (34 s). Linear regression analysis of standard curves demonstrated a strong correlation for all genes ($r^2 > 0.98$). The

**Table 1** The primer sequences and PCR reaction conditions

| | Forward primer sequence | Reverse primer sequence |
|---|---|---|
| ERCC1 | CTCAAGGAGCTGGCTAAGATGT | CATAGGCCTTGTAGGTCTCCAG |
| ERCC2 | CTGGAGGTGACCAAACTCATCTA | CCTGCTTCTCATAGAAGTTGAGC |
| RRM1 | CGCTAGAGCGGTCTTATTTGTT | TTGCTGCATCAATGTCTTCTTT |
| β-actin | TTCTACAATGAGCTGCGTGTG | CAGCCTGGATAGCAACGTACA |

ERCC1, excision repair cross-complementation 1; ERCC2, excision repair cross-complementation 2; RRM1, ribonucleotide reductase M1.

**Table 2** IC50 values for cisplatin, carboplatin and gemcitabine in lung cancer cell lines

| Cell line | Histology | Cisplatin (μmol/L) | Carboplatin (μmol/L) | Gemcitabine (μmol/L) |
|---|---|---|---|---|
| ACC-LC-94 | Ad | 1.14 | 18.4 | 0.0119 |
| ACC-LC-319 | Ad | 16.5 | 284 | >128 |
| SK-LC-3 | Ad | 39.7 | 512 | >128 |
| A549 | Ad | 4.22 | 47 | 0.00821 |
| SK-Lu-1 | Ad | 40.2 | 512 | 1 |
| VMRC-LCD | Ad | 14.3 | 147 | 7.17 |
| RERF-LC-MT | Ad | 5.21 | 92.9 | >128 |
| Calu1 | Sq | 9.96 | 89.9 | 0.398 |
| SK-MES-1 | Sq | 1.81 | 28.1 | 0.00411 |
| PC-1 | Sq | 0.127 | 1.84 | 0.00303 |
| RERF-LC-AI | Sq | 2.69 | 33 | 0.00394 |
| PC-10 | Sq | 8.23 | 430 | >128 |
| NCI-H460 | La | 3.83 | 49.4 | 0.0135 |
| Calu6 | La | 0.939 | 15.5 | 0.00778 |
| SK-LC-6 | La | 2.35 | 37.3 | 0.00244 |
| ACC-LC-48 | SCLC | 3.2 | 35.8 | 0.0191 |
| ACC-LC-49 | SCLC | 3.71 | 52.8 | 1 |
| ACC-LC-80 | SCLC | 3.18 | 43.7 | 0.0344 |
| ACC-LC-172 | SCLC | 2.78 | 35.2 | 0.0125 |
| SK-LC-2 | SCLC | 7.91 | 50.9 | >128 |

Ad, adenocarcinoma; La, large cell lung cancer; SCLC, small cell lung cancer; Sq, squamous cell lung cancer.

relative gene expression levels were normalized to those of the house keeping gene, β-actin.

### Statistical analysis

The strength of the association between the expression of ERCC1, ERCC2 and RRM1 and chemosensitivity of the cell lines was calculated using either Pearson's correlation coefficient or linear regression analysis. Correlations were considered significant at $P < 0.05$. One-way analysis of variance (ANOVA) followed by the Bonferroni post-test was used for comparison of RRM1 expression levels among the different cell lines. All analyses was performed using Stat View version 5.0 software.

## RESULTS

Chemosensitivities to cisplatin, carboplatin and gemcitabine were examined in 20 human lung cancer cell lines, including 15 NSCLC and 5 SCLC cell lines. Cytotoxicity was measured by the MTT assay following 72 h of continuous exposure to the drugs. The IC50 values for these agents on each cell line are shown in Table 2. The IC50 values of gemcitabine for ACC-LC-319, SK-LC-3, RERF-LC-MT and PC-10 and SK-LC-2 were greater than 128 μmol/L, which was above the clinically achievable plasma concentration. There were statistically significant positive correlations between the cytotoxicities of cisplatin and carboplatin among the 15 NSCLC cell lines ($r = 0.966$; $P < 0.0001$), as well as for all 20 lung cancer cell lines, including the 5 SCLC cell lines ($r = 0.956$; $P < 0.0001$), suggesting that these agents induced similar cytotoxic effects in lung cancer cells (Fig. 1). There was a relatively weak but statistically significant correlation between the cytotoxicity of gemcitabine and that of cisplatin or carboplatin among the 15 NSCLC cell lines ($r = 0.715$; $P < 0.001$ for cisplatin, $r = 0.792$; $P < 0.001$ for carboplatin), as well as for all 20 lung cancer cell lines ($r = 0.701$; $P < 0.001$ for cisplatin, $r = 0.733$; $P < 0.001$ for carboplatin, data not shown).

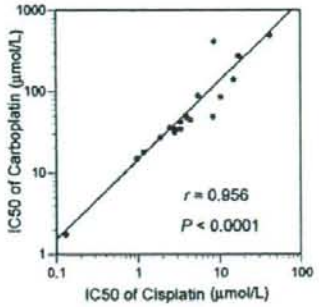


**Figure 1** Correlation between chemosensitivities to cisplatin and carboplatin.

**Table 3** Relative mRNA expression for ERCC1, ERCC2 and RRM1 in lung cancer cell lines

| Cell line | RRM1 | ERCC1 | ERCC2 |
|---|---|---|---|
| ACC-LC-94 | 1.046 | 1.090 | 1.045 |
| ACC-LC-319 | 1.438 | 0.480 | 0.307 |
| SK-LC-3 | 1.416 | 0.899 | 0.588 |
| A549 | 1.628 | 0.767 | 0.203 |
| SK-Lu-1 | 1.956 | 0.751 | 0.553 |
| VMRC-LCD | 3.291 | 0.744 | 0.671 |
| RERF-LC-MT | 1.593 | 0.225 | 0.167 |
| Calu1 | 2.268 | 0.438 | 0.531 |
| SK-MES-1 | 1.459 | 0.735 | 0.236 |
| PC-1 | 2.889 | 0.749 | 0.713 |
| RERF-LC-AI | 3.739 | 0.327 | 0.303 |
| PC-10 | 1.993 | 0.864 | 0.269 |
| NCI-H460 | 2.002 | 0.671 | 0.431 |
| Calu6 | 0.745 | 0.725 | 0.348 |
| SK-LC-6 | 2.47 | 0.782 | 0.508 |
| ACC-LC-48 | 2.388 | 0.414 | 0.257 |
| ACC-LC-49 | 4.602 | 0.670 | 0.455 |
| ACC-LC-80 | 3.826 | 1.080 | 0.435 |
| ACC-LC-172 | 3.896 | 0.472 | 0.841 |
| SK-LC-2 | 4.688 | 3.402 | 1.906 |

ERCC1, excision repair cross-complementation 1; ERCC2, excision repair cross-complementation 2; RRM1, ribonucleotide reductase M1.

Expression of mRNA for the ERCC1, ERCC2 and RRM1 genes was quantified by real-time PCR and normalized to β-actin mRNA expression (Table 3). mRNA expression for RRM1 was higher in SCLC cell lines compared with NSCLC cell lines. There were statistically significant differences in RRM1 expression between SCLC and adenocarcinoma, and between SCLC and large cell carcinoma (Fig. 2). There was also a statistically significant correlation between ERCC1 mRNA expression and ERCC2 mRNA expression among the 15 NSCLC cell lines ($r = 0.547$; $P < 0.05$, Fig. 3a), as well as for all 20 lung cancer cell lines ($r = 0.666$; $P < 0.005$, data not shown). However, there were no associations between RRM1 mRNA expression and either ERCC1 mRNA (Fig. 3b) or ERCC2 mRNA (Fig. 3c) expression in these cell lines.

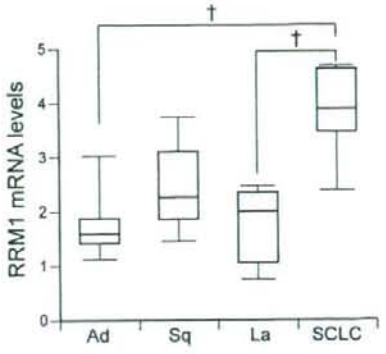


**Figure 2** Predominant mRNA expression of the RRM1 gene in SCLC cell lines compared with NSCLC cell lines. Box plots show relationships between RRM1 mRNA expression and the four histological types of lung cancer. The line within each box indicates the median value. †$P < 0.005$ by ANOVA with Bonferroni correction.

The chemosensitivity data were analysed in relation to mRNA expression of the ERCC1, ERCC2 and RRM1 genes using linear regression analysis. No significant associations were observed between the IC50 values of cisplatin, carboplatin and gemcitabine and mRNA expression for ERCC1 (Fig. 4a), ERCC2 (Fig. 4b) or RRM1 (Fig. 4c) among the 15 NSCLC cell lines. Similar results were obtained for all 20 lung cancer cell lines, including the five SCLC cell lines (data not shown).

## DISCUSSION

Better and more accurate definition of the biological characteristics of the tumour is considered important for improving clinical outcome in advanced NSCLC especially in predicting response to combination chemotherapy.[14] Several reports have been published on the molecular and/or immunohistochemical analysis of molecules involved in DNA repair and/or DNA synthesis, using transbronchial and percutaneous biopsy samples from locally advanced or metastatic NSCLC.[7,11,15–17] However, there are several problems associated with mRNA and/or protein expression analyses using small tissue samples obtained by lung biopsy,[18,19] including the considerable intratumour heterogeneity, mRNA fragmentation, inevitable contamination with normal fibroblasts, the fixation procedure and storage conditions.[20] As mRNA extracted from formalin-fixed paraffin-embedded tissues is considerably fragmented, quantitative RT-PCR often yields unsatisfactory results.[21] In addition, problems with the specificity of the antibodies used for immunohistochemical analyses have been reported.[22] These limitations may result in misleading molecular