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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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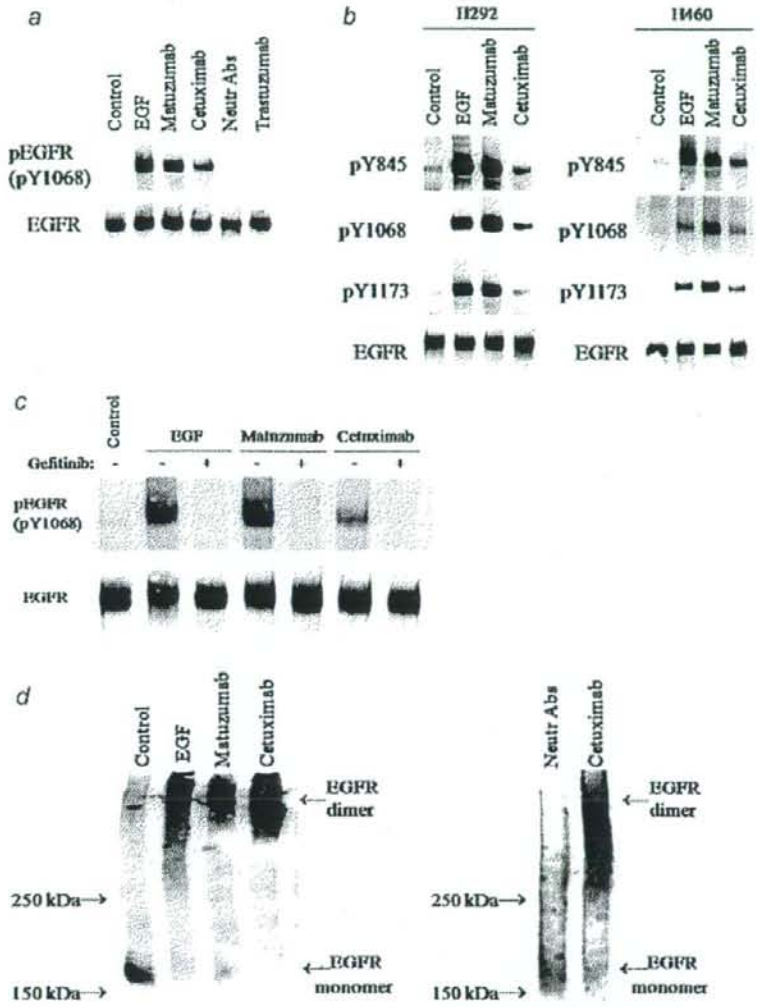

FIGURE 1 – EGFR phosphorylation induced by matuzumab or cetuximab as a result of receptor dimerization and activation of the receptor tyrosine kinase. (*a*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence (Control) or presence of matuzumab (200 nM), cetuximab (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 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.

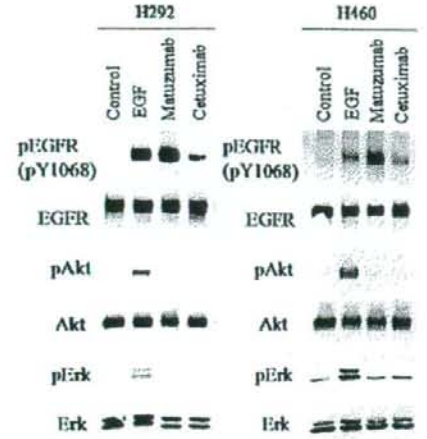

FIGURE 2 – Failure of matuzumab or cetuximab to activate Akt or Erk. H292 or H460 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies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

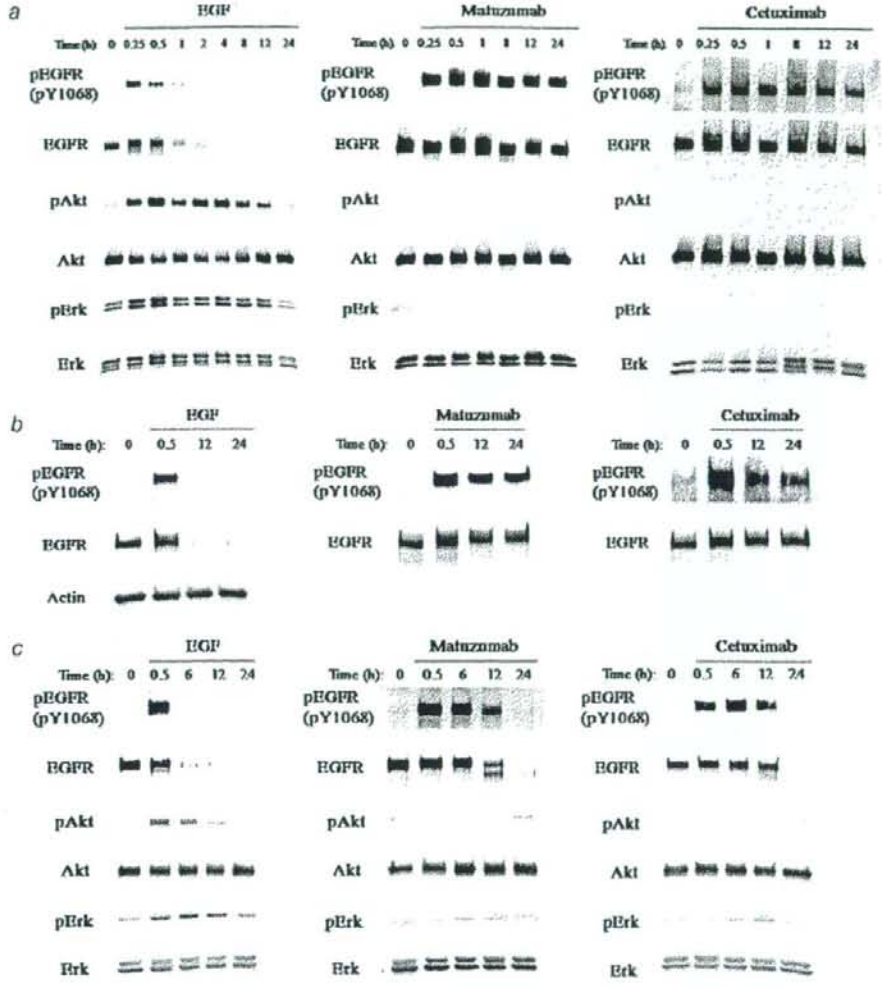

FIGURE 3 – Lack of EGFR turnover in cells treated with matuzumab or cetuximab. (*a*) H292 cells were deprived of serum overnight and then incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM), respectively. Cell lysates were subjected to immunoblot 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 theY1068-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

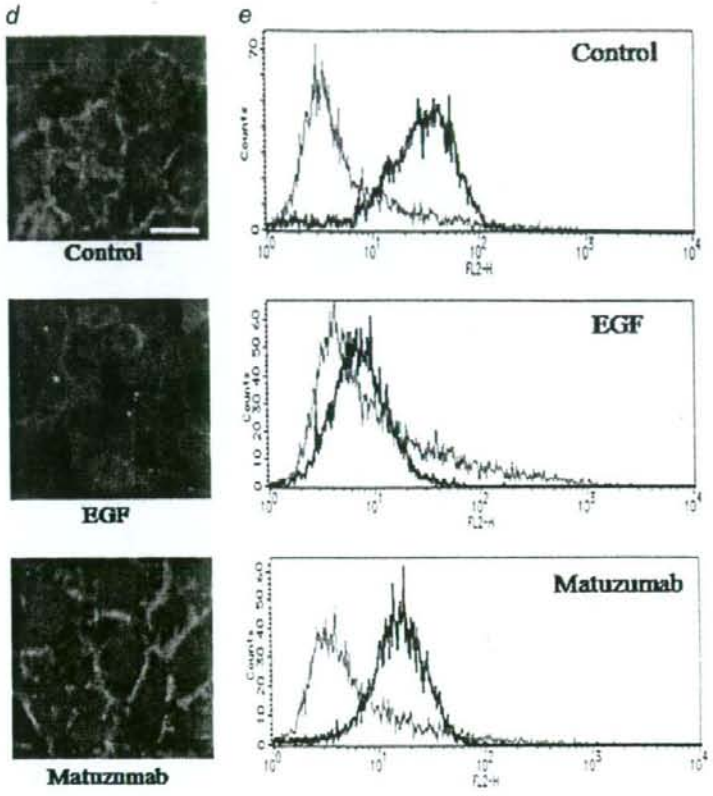

FIGURE 3 – CONTINUED

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 I – 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 I).[31] We treated serum-deprived cells of the 3 NSCLC lines with

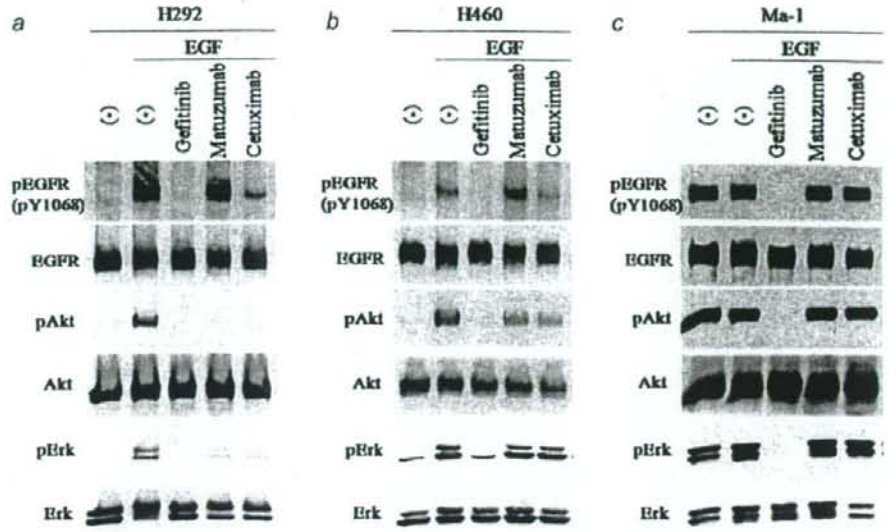

FIGURE 4 – Effects of matuzumab and cetuximab on EGF-induced EGFR signaling. H292 (*a*), H460 (*b*) and Ma-1 (*c*) cells were deprived of serum overnight and then incubated first for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or gefitinib (10 μM) and then for an additional 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.

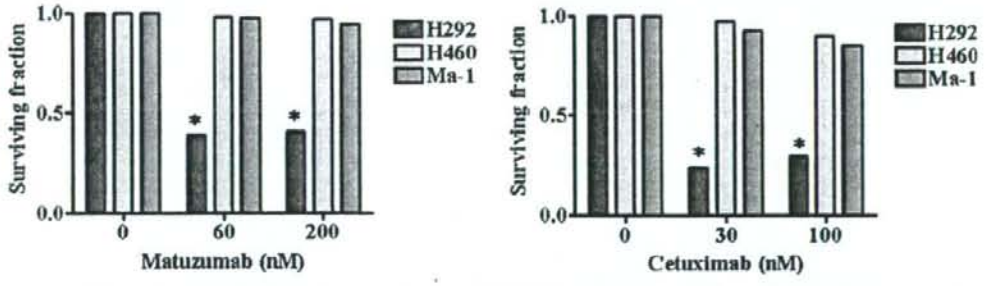

FIGURE 5 – Effects of matuzumab and cetuximab on cell survival. H292, H460 or Ma-1 cells were plated at a density of 200 cells per 25-cm^2 flask in triplicate and cultured for 7 days in the presence of the indicated concentrations of matuzumab or cetuximab. They were then incubated with medium alone 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).

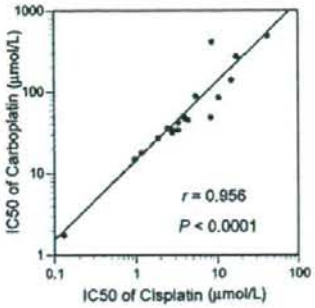

Figure 1 Correlation between chemosensitivities to cisplatin and carboplatin.

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.

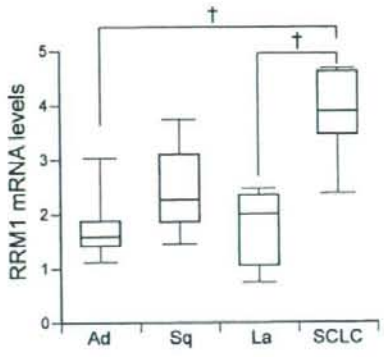

Figure 2 Predominant mRNA expression of the RRM1 gene in SCLC cell lines compared with NSCLC cell lines. Box plots show relationships between RRM1 mRNA expression and the four histological types of lung cancer. The line within each box indicates the median value. †$P < 0.005$ by ANOVA with Bonferroni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

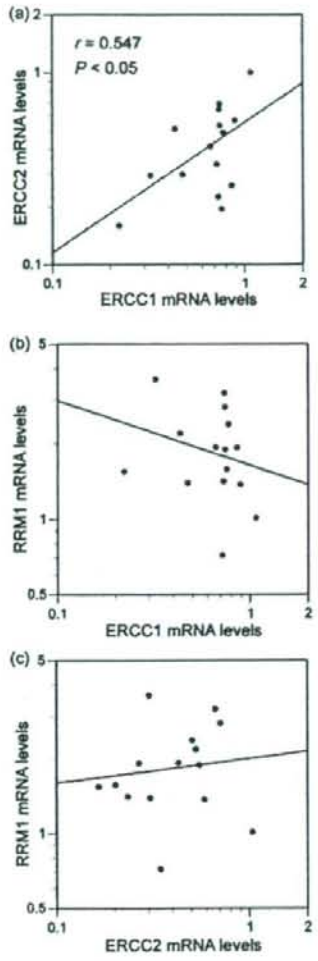

Figure 3 Correlations between (a) ERCC1 and ERCC2 mRNA expression, (b) ERCC1 and RRM1 mRNA expression and (c) ERCC2 and RRM1 mRNA expression.

analyses from clinical trials, in which the expression of biomarkers in transbronchial and percutaneous lung biopsy samples is evaluated. Thus, as one of many approaches to integrating molecular analysis with individualized chemotherapy, the *in vitro* associations between mRNA expression of the ERCC1, ERCC2 and RRM1 genes and chemosensitivity to platinum agents and gemcitabine was assessed. However, the behaviour of cell lines adapted to grow *in vitro* may differ from the *in vivo* situation, and laboratory findings may not always accurately model the clinical situation.

RRM1 expression is reported to be associated with the response to gemcitabine *in vitro*.[23] Increased RRM1 expression has been reported in two gemcitabine-resistant NSCLC cell lines. In addition, upregulation of RRM1 has been reported in different gemcitabine-resistant cell lines,[24-26] and in a murine colon cancer model.[27] Reduced RRM1 expression has also been reported to be associated with increased sensitivity to gemcitabine in the human NSCLC H23 cell line using transfection and knockdown techniques.[7] Low levels of RRM1 expression are associated with poor survival among patients with resected NSCLC.[28] Association of increased RRM1 expression with resistance to gemcitabine was also reported in the setting of preoperative NSCLC, as well as in advanced NSCLC. In a prospective induction phase II clinical trial of chemotherapy with platinum and gemcitabine RRM1 mRNA expression was correlated with tumour response.[29] However, in the present study there was no correlation between RRM1 mRNA expression and chemosensitivity to gemcitabine, cisplatin or carboplatin. Possible explanations for the differences between this study and other *in vitro* studies are the use of tissues from different sources and the use of different assay systems, such as overexpression and/or knockdown techniques for molecular biomarkers in a limited number of cell lines. The discrepancy between this study and *in vivo* studies might be explained by possible technical limitations such as the quality of mRNA extracted from the small samples obtained by lung biopsy and the specificity of the antibody used.

The association between ERCC1 and chemosensitivity to cisplatin has been evaluated in many *in vitro* and *in vivo* studies. Increased expression of ERCC1 was associated with cisplatin resistance in ovarian cancer cells.[30] Transfection of the ERCC1 gene into an ERCC1-deficient Chinese hamster ovary (CHO) cell line conferred DNA adduct repair capability and cisplatin resistance.[31] In a human colon cancer cell line with mismatch repair deficiency, ERCC1 antisense RNA abrogated the synergistic cytotoxicity of gemcitabine and cisplatin *in vitro*.[32] The association between ERCC1 mRNA expression and chemoresponsiveness to cisplatin has been observed in primary gastric cancer and in ovarian cancer.[33-35] In the present study, there was no association between ERCC1 mRNA expression and chemoresponsiveness to either cisplatin or gemcitabine. The lack of association between ERCC1 mRNA expression and chemoresponsiveness to cisplatin is consistent with a previous *in vivo* study, of mRNA from formalin-fixed paraffin-embedded primary tumour specimens from patients with advanced NSCLC before treatment with cisplatin and gemcitabine. However, low ERCC1 mRNA expression was associated with longer survival and a trend towards a higher response rate.[16] A recent study also reported no association between ERCC1 mRNA expression and chemoresponsiveness or survival in patients with advanced NSCLC treated with platinum-based chemotherapy.[36]

ERCC1 mRNA expression in formalin-fixed paraffin-embedded tumour specimens obtained by bronchoscopic fine needle aspiration biopsy[15] is a prognostic factor in patients with resected NSCLC,[37] and patients with advanced NSCLC treated with

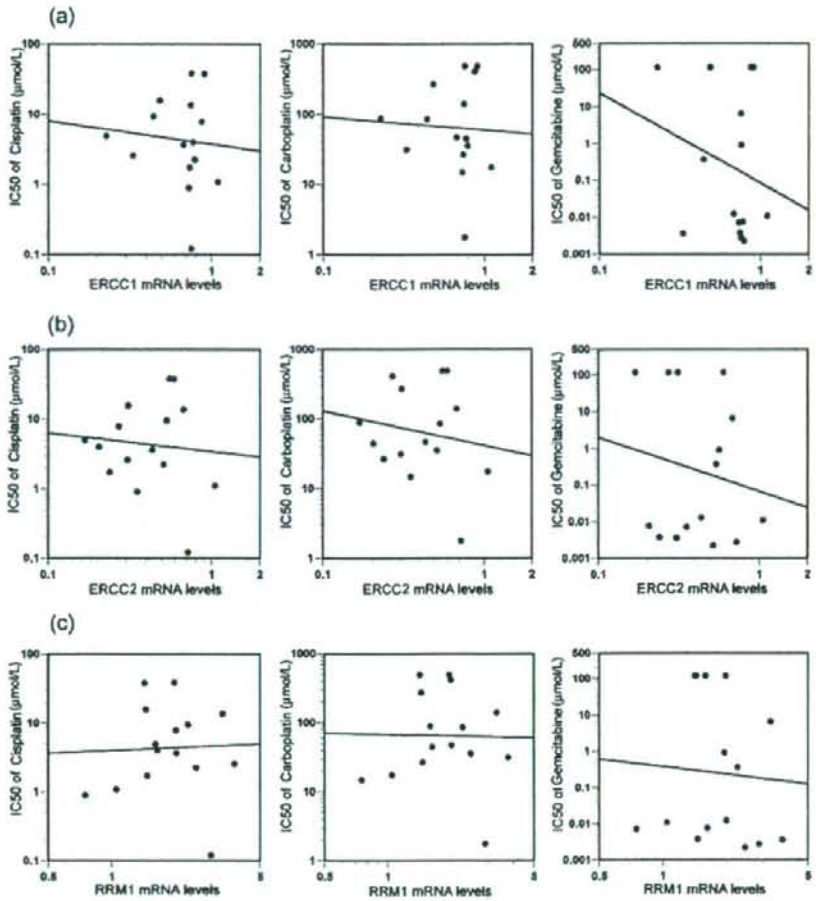

Figure 4 Associations between mRNA expression for (a) ERCC1, (b) ERCC2 and (c) RRM1 and chemosensitivities to cisplatin, carboplatin and gemcitabine.

cisplatin and gemcitabine. Furthermore, ERCC1 protein, as measured by immunohistochemical scoring, is a determinant of survival after surgical treatment of early stage NSCLC. ERCC1 protein is a prognostic factor for clinical outcome and a predictive biomarker for cisplatin-based adjuvant chemotherapy in patients with completely resected ERCC1-negative NSCLC,[8] although a problem with the specificity of the anti-ERCC1 mAb 8F1 has been reported.[22] Thus, further studies are needed to establish the role of ERCC1 in NSCLC.

The ERCC2 gene codes for a DNA helicase, which is a member of the multi-step NER pathway. The Asp312Asn polymorphism, resulting from a G/A substitution in exon 10 of the ERCC2 gene has been highly conserved through evolution, and has been reported to be a prognostic factor in patients with advanced NSCLC treated with cisplatin.[38] In addition, an *in vitro* study showed that ERCC2 overexpression leads to cisplatin resistance in a glioma cell line,[39] suggesting that expression of the ERCC2 gene may be associated with chemosensitivity to cisplatin in lung cancer cells. However, the present study failed to show associations with sensitivity to platinum agents and gemcitabine. Therefore, ERCC2 also needs further evaluation in lung cancer.

Five SCLC cell lines were included to determine whether the associations between ERCC1, ERCC2 and RRM1 mRNA expression and chemosensitivity to platinum agents and gemcitabine reported for NSCLC could be extended to SCLC. Platinum agents are key drugs and gemcitabine has modest activity in the treatment of SCLC with response rates of 11.9–13%.[40,41] However, the present study failed to show any associations. These findings are supported by a previous study, in which gene expression and the growth

inhibitory activities of various anticancer agents were similar for 19 NSCLC and 10 SCLC cell lines.[42]

There have been no *in vitro* studies examining the association between RRM1, ERCC1 or ERCC2 and chemosensitivity to platinum agents and gemcitabine, except for studies using overexpression and/or knockdown techniques. Although this *in vitro* study did not show associations in a panel of lung cancer cell lines, definitive conclusions cannot be drawn from the data, because only a limited number of cell lines were used. Exploration of the relationship between drug response phenotype and tumour genome mRNA expression profile, using cell line panels and/or tumour tissues together with cDNA and oligonucleotide arrays, would be a promising approach in the search for predictive biomarkers.[43,44] Finally, in order to validate pharmacogenetic or pharmacoproteomic candidates for lung cancer in clinical settings, further careful and more comprehensive studies using multiple approaches are warranted.

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clinical studies

Multicentre prospective phase II trial of gefitinib for advanced non-small cell lung cancer with epidermal growth factor receptor mutations: results of the West Japan Thoracic Oncology Group trial (WJTOG0403)

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The purpose of this study was to evaluate the efficacy of gefitinib and the feasibility of screening for epidermal growth factor receptor (*EGFR*) mutations among select patients with advanced non-small cell lung cancer (NSCLC). Stage IIIB/IV NSCLC, chemotherapy-naive patients or patients with recurrences after up to two prior chemotherapy regimens were eligible. Direct sequencing using DNA from tumour specimens was performed by a central laboratory to detect *EGFR* mutations. Patients harbouring *EGFR* mutations received gefitinib. The primary study objective was response; the secondary objectives were toxicity, overall survival (OS), progression-free survival (PFS), 1-year survival (1Y-S) and the disease control rate (DCR). Between March 2005 and January 2006, 118 patients were recruited from 15 institutions and were screened for *EGFR* mutations, which were detected in 32 patients – 28 of whom were enrolled in the present study. The overall response rate was 75%, the DCR was 96% and the median PFS was 11.5 months. The median OS has not yet been reached, and the 1Y-S was 79%. Thus, gefitinib chemotherapy in patients with advanced NSCLC harbouring *EGFR* mutations was highly effective. This trial documents the feasibility of performing a multicentre phase II study using a central typing laboratory, demonstrating the benefit to patients of selecting gefitinib treatment based on their *EGFR* mutation status.
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Keywords: epidermal growth factor receptor (*EGFR*) mutation; gefitinib; non-small cell lung cancer (NSCLC); multicentre prospective phase II; central laboratory

Gefitinib, a tyrosine kinase inhibitor (TKI), is an orally active small molecule that functions as a selective epidermal growth factor receptor (*EGFR*) inhibitor (Ranson *et al*, 2002). Two phase II trials (Fukuoka *et al*, 2003; Kris *et al*, 2003) for previously treated non-small cell lung cancer (NSCLC) (IDEAL-1 and -2, respectively) have documented favourable objective responses in 14–18% of patients. However, in a phase III trial (Thatcher *et al*, 2005), no survival benefit of gefitinib was observed when compared with best-supportive care (BSC) for previously treated NSCLC. In contrast, we have seen a significant survival benefit of erlotinib compared with BSC as a salvage therapy (BR21); erlotinib is also an *EGFR*-TKI and its chemical structure, which is based on quinazoline, is quite similar to that of gefitinib (Shepherd *et al*, 2005). Although we do not know whether differences between gefitinib and erlotinib were responsible for these different outcomes, appropriate patient selection to identify good responders is likely crucial for revealing the clinical benefits of the *EGFR*-TKI family.

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Patient subset analyses of these randomised phase III trials or retrospective trials (Kaneda *et al*, 2004; Miller *et al*, 2004) clearly show the existence of populations that are more likely to respond to gefitinib and erlotinib, including women, patients with adenocarcinoma (especially with bronchial alveolar carcinoma (BAC)), nonsmokers and Asian patients (compared with Caucasians). Somatic mutations in specific regions of exons 18, 19 and 21 of the ATP-binding domain of *EGFR* have recently been shown to have strong associations with sensitivity to gefitinib or erlotinib (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004). Consistent with these findings, the frequencies of these *EGFR* mutations were higher in women, patients with adenocarcinoma, nonsmokers and Asians, all of whom are among the more frequent responders, as mentioned above (Shigematsu *et al*, 2005). There are two characteristic types of *EGFR* mutations. One is the presence of in-frame deletions, including the amino acids at codons 746–750 in exon 19, and the other is an amino-acid substitution at codon 858 (L858R) in exon 21. Recent analyses (Bell *et al*, 2005) of phase II and III trials for *EGFR*-TKI, in which patients were not selected based on their mutation status, have suggested that *EGFR* mutations are correlated with response to therapy but are not correlated with overall survival (OS). Furthermore, *EGFR* gene amplification/copy number (Cappuzzo *et al*, 2005; Hirsch *et al*, 2005) or overexpression (Hirsch *et al*, 2003) has been shown to be a more useful prognostic marker of response to gefitinib treatment. Patient selection according to *EGFR* mutation status may yield a superior survival rate by excluding patients who are unlikely to respond to gefitinib treatment. However, other populations that might obtain a clinical benefit from gefitinib treatment, even in the absence of *EGFR* mutation, may exist.

Three Japanese groups (Asahina *et al*, 2006; Inoue *et al*, 2006; Yoshida *et al*, 2007) have reported prospective phase II studies of gefitinib for advanced-stage NSCLC that were designed to consider the *EGFR* mutation status of the patients. All of these studies have reported a high response rate and extended progression-free survival (PFS) period, compared with historical controls. However, all of these studies had a relatively short observation period, making the data preliminary. Moreover, the original sample size was calculated after patient selection, and a critical consideration of the suitability of the assay used to detect the mutations (which was performed using small paraffin-embedded specimens obtained from bronchoscopic biopsies), and the estimated *EGFR*-positive rate were lacking. Additionally, all the trials were conducted at single institutions located in one small area of Japan. Thus, the published data may not be representative of the situation found in general clinical practice throughout Japan and therefore may not directly translate to the general feasibility of gefitinib treatment in Japan.

In view of this situation, we performed a multicentre prospective phase II trial of gefitinib for advanced NSCLC harbouring *EGFR* mutations. We prospectively registered patients from 15 different institutes in Japan at the beginning of *EGFR* mutation screening using a central database. Whether or not tissue was available from a bronchoscopic biopsy or surgery was not an inclusion criterion. All the clinical samples from the registered patients were delivered to a central laboratory that then determined the *EGFR* mutation status or the histological BAC features. The analysis of the survival data was based on a minimum observation period of at least 15 months from the time of entry of the last patient.

MATERIALS AND METHODS

Eligibility criteria

Eligible patients had histologically confirmed stage III NSCLC for which thoracic irradiation was not indicated or were stage IV. Chemotherapy-naive patients or those who had previously received up to two prior chemotherapy regimens, including those performed in an adjuvant setting, were eligible. Other eligibility criteria included an age $\geqslant$20 years, measurable disease, the availability of sufficient amounts of tumour specimen for *EGFR* mutation analysis, an Eastern Cooperative Oncology Group performance status of 0–2, adequate organ function (WBC$\leqslant$3000 μl^{-1}, platelets$\geqslant$75 000 μl^{-1}, AST and ALT$\leqslant$100 IU l^{-1}, serum creatinine$\leqslant$twice the upper limit of the reference range; $P_{aO2}\geqslant$60 mm Hg). The exclusion criteria included pulmonary fibrosis, the presence of symptomatic brain metastasis, active concomitant malignancy, severe heart disease, active gastrointestinal bleeding and continuous diarrhoea. All the patients signed a written informed consent form. Approval of this study and the gene analyses were obtained from the Institutional Review Board and the Ethics Committee of each hospital.

EGFR gene analysis

Tumour specimens were obtained using bronchial fiberscope or surgical procedures. The specimens were fixed with formalin and embedded in paraffin. Four slices (4–5 μm) from the embedded block were sent to a central laboratory (Mitsubishi Chemical Safety Institute Ltd., Ibaraki, Japan) for genetic analysis. Most of the tumour specimens were available prior to the registration of this study. Genomic DNA was isolated from specimens using QIAamp Micro kits (QIAGEN KK, Tokyo, Japan). The *EGFR* mutations in exons 18, 19 and 21, as previously reported (Lynch *et al*, 2004; Paez *et al*, 2004), were determined using polymerase chain reaction (PCR) amplification and intron–exon boundary primers according to the published method. An *EGFR* registrant mutation in exon 20, which was reported by Pao *et al* (2005) was also examined using PCR and the previously reported primers. Polymerase chain reaction was performed using a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), and the PCR products were confirmed using a Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA), then sequenced directly using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 3100 (Applied Biosystems). All sequencing reactions were performed in both forward and reverse directions and were analysed using the Basic Local Alignment Search Tool (BLAST); all the electropherograms were reanalysed by visual inspection to check for mutations. The presence of an *EGFR* mutation was confirmed using at least three independent PCR.

All sequence data were sent from the central laboratory to Kinki University. A principle investigator then confirmed whether or not the *EGFR* mutation status was positive, and the results were sent to the West Japan Thoracic Oncology Group (WJTOG) data centre. The data centre then informed each participating centre of the results of the genetic analysis and requested that the eligibility criteria of the patients be rechecked to insure that only *EGFR*-positive subjects were registered in the trial. Each tumour was categorised according to histology by a pulmonary pathologist (JF). The percentage of area exhibiting a BAC pattern was also examined to determine the WHO pathological category.

Treatment plan

Gefitinib (250 mg day^{-1}) was administered once daily. Treatment was continued uninterrupted until disease progression or intolerable toxicity (grade 4 nonhaematological toxicities, any incidents of interstitial pneumonia or a treatment delay of more than 2 weeks because of adverse effects). Gefitinib administration was delayed if the patient's leukocyte and platelet counts were lower than 1500 and 5000 μl^{-1}, respectively, and was withheld until these counts had recovered. Gefitinib administration was also delayed if grade 3 or greater nonhaematological toxicities without nausea, vomiting or alopecia occurred and was withheld until recovery to grade 2.

Routine clinical and laboratory assessments and chest X-ray assessments were performed weekly or biweekly, where possible; CT examinations of the target lesion were performed every month, and magnetic resonance imaging of the whole brain and a bone scan were performed every 3 months. The objective responses of the patients were evaluated every month using the Response Evaluation Criteria in Solid Tumours (RECIST) guidelines (Therasse *et al*, 2000). Tumour response was centrally evaluated by independent reviewers at an extramural conference and was performed for the intent-to-treat population. All adverse effects that occurred during gefitinib treatment were reported, and the severity of the effects was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0.

Statistical analyses

The primary end point of this study was the response rate. A one-stage design using the binominal probability was used to determine the sample size. Assuming that a response rate of 50% would indicate potential usefulness, whereas a rate of 25% would be the lower limit of interest, and with $\alpha = 0.10$ (two side) and $\beta = 0.20$, the estimated accrual number was 23 patients. Estimating that the *EGFR*-positive rate would be about 20%, the screening number required to accrue 23 *EGFR*-positive patients was 115. After assuming an inevaluability rate of $<10\%$, the final required screening number was 125.

The secondary end points of this study were toxicity, OS, PFS, 1-year survival (1Y-S) and the disease control rate (DCR). Survival analyses were conducted on the intent-to-treat population using follow-up data available as of 30 April 2007. The survival curves were estimated using Kaplan–Meier plots.

RESULTS

Patient characteristics

Between March 2005 and January 2006, 118 patients were prospectively screened from 15 institutions; 117 of them underwent *EGFR* mutation analysis (tumour tissue was not available for one patient). The median time required for the *EGFR* mutation analysis was 12 days (range: 7–28 days). Among the 117 patients, *EGFR* mutations were detected in 32 patients (27%), 14 of whom had a deletion in or near E746-A750 (including one del E746-T751 ins A, two del L747-T751 and one del L747-T753 ins S) in exon 19. A further 17 had L858R, and one had a L861Q point mutation in exon 21 (Table 1).

Tissue samples from 17 patients (53%) were obtained by transbronchial biopsy. The *EGFR* detection rates for the surgical specimens and the bronchoscopic biopsy specimens were similar (30 *vs* 25%). The *EGFR* mutations were significantly more frequent in women ($P \leqslant 0.02$), in patients with adenocarcinoma ($P = 0.001$) and in people who had never smoked ($P < 0.001$) (Table 2). Finally, 28 patients (14 with deletions in exons 19 and 14 with point mutations in exon 21) were actually registered and received treatment with gefitinib, whereas four patients were dropped from the study as they became ineligible because of tumour progression during the time required for the mutation analysis.

Patient characteristics are listed in Table 3. In the initial screening, there were 56 female patients (48%), 97 patients (83%) with adenocarcinoma and 53 (45%) who had never smoked. The frequency of these characteristics was higher among the patients with *EGFR* mutations who were actually registered; namely, 18 patients (64%) were women, 27 (96%) had adenocarcinoma and 19 (68%) had never smoked. The median age of the 28 actually registered patients was 68 years; 24 patients (86%) had a good performance status (0–1), 22 (79%) had stage IV diseases and 17 (61%) were chemotherapy naive. Thoracic irradiation was contraindicated in one patient with stage IIIA disease because of the large irradiation field that would have been required. All five patients with stage IIIB diseases had malignant effusions. Four patients had received adjuvant therapies; five had received platinum doublets or a combination of gemcitabine and vinorelbine as their first-line therapy. Two patients had received two regimens of platinum doublets followed by docetaxel or pemetrexed. One patient had received local radiation for pain control.

Table 1 Type of *EGFR* mutations ($n = 32$)

Characteristics	No. of patients	%
Exon 18	0	0
Exon 19	14	44
del E746-A750	10	32
del E746-T751 ins A	1	3
del L747-T751	2	6
del L747-T753 ins S	1	3
Exon 21	18	56
L858R	17	53
L861Q	1	3

EGFR = epidermal growth factor receptor.

Table 2 Relationship between patient characteristics and *EGFR* mutation status

	EGFR mutation positive ($n = 32$)		*EGFR* mutation negative ($n = 85$)		
Characteristics	**No. of Patients**	**%**	**No. of Patients**	**%**	***P***
Sex					
Male	11	34	50	59	
Female	21	66	35	41	<0.02
Histology					
Adenocarcinoma	31	97	66	78	
Nonadenocarcinoma	1	3	19	22	$=0.001$
Smoking status					
Never	21	66	31	36	
Current/former	11	34	54	64	<0.001

EGFR = epidermal growth factor receptor.

Response and survival

The objective tumour responses are listed in Table 4. The overall response rate and DCR were 75% (95% CI: 57.6–91.0%) and 96% (95% CI: 87.0–96.4%), respectively. Five out of ten male patients (50%), six out of nine smokers (67%) and five out of eight male smokers with adenocarcinoma (63%) achieved a PR. One female nonsmoker with squamous cell carcinoma also achieved a PR. Among the registered patients with *EGFR* mutations, the response rate was no different between current/former smokers and those who had never smoked (67 *vs* 79%) or between chemotherapy-naive and postchemotherapy patients (77 *vs* 73%). Female and patients with a mutational deletion in exon 19 tended to have a higher response rate than male (89 *vs* 50%) and patients with a missense mutation in exon 21 (86 *vs* 64%), respectively.

The median follow-up time was 18.6 months (range: 13.8–23.4 months). The median PFS time was 11.5 months (95% CI: 7.3 months to -) (Figure 1A). The median OS has not yet been reached, and the 1Y-S was 79% (95% CI: 63.4–93.8%) (Figure 1B).