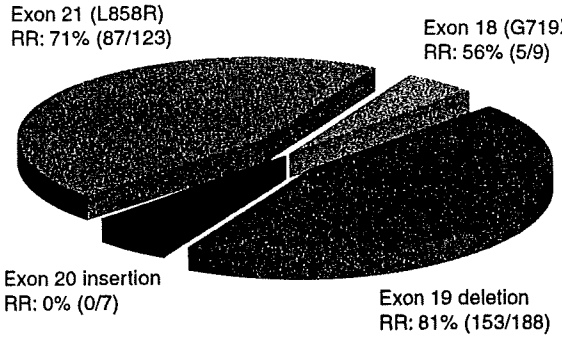

Figure 2. Distribution of EGF receptor mutations and response rates to EGF receptor tyrosine kinase inhibitors.
RR: Response rate.

from patients who had developed resistance to EGFR TKIs. In some specimens, *MET* amplification can occur concurrently with T790M.

EGFR mutation & amplification

There is increasing evidence that *EGFR* mutations and high *EGFR* gene copy number are associated with higher response rates to TKIs and longer survival. Both mutation and amplification of EGFR in lung cancers have been reported in association with clinical responses to TKIs. The *EGFR* locus can undergo both mutation and amplification. Yatabe *et al.* examined the topographical distribution of amplification in three microdissected portions each of 48 individual lung cancers with confirmed mutations [97]. Gene amplification was found in 11 lung cancers. Strikingly, nine of the cancers showed heterogeneous distribution, and amplification was associated with higher histologic grade or invasive growth. They also examined 17 precursor lesions and 21 *in situ* lung adenocarcinomas and found that only one *in situ* carcinoma harbored gene amplification. Taken together, their results show that mutation occurs early in the development of lung adenocarcinoma, and that amplification may be acquired in association with tumor progression. In general, tumors with EGFR mutations tend to have gene amplification. Mutation and amplification are probably both important in determining EGFR 0TKI sensitivity. The FISH scoring system, generated by the Colorado group, stratifies results into six groups by number of copies of the *EGFR* gene and frequency of tumor cells in the sample. These groups include disomy, low trisomy, high trisomy, low polysomy, high polysomy and gene amplification, with high polysomy or gene amplification being considered FISH positive [98,99]. However, the role of high polysomy is unclear.

KRAS mutation

Activating mutation of the *KRAS* gene was one of the earliest discoveries of genetic alterations in lung cancer known as a poor prognostic indicator. It was reported that the occurrence of *EGFR* and *KRAS* mutations are strictly mutually exclusive [100,101]. This finding can be explained by the fact that the KRAS–MAPK pathway is one of the downstream signaling pathways of EGFR. *KRAS* mutations predominantly occur in Caucasian patients with a history of smoking. Pao *et al.* reported that lung cancers with *KRAS* mutations are resistant to EGFR TKIs [102].

Postmarketing surveillance

It was shown that erlotinib, another EGFR TKI, extended the median survival time in the BR.21 trial [8]. In the BR.21 study, patients with NSCLC, after failure of first- or second-line chemotherapy, were randomized to receive erlotinib 150 mg/day or placebo (2:1, respectively). Statistically significant differences were observed for OS (6.7 vs 4.7 months; HR: 0.70; $p < 0.001$) and PFS (2.2 vs 1.8 months; HR: 0.61; $p < 0.001$) in favor of erlotinib. These results led to regulatory approval of erlotinib for NSCLC refractory to chemotherapy. However, gefitinib failed to prolong survival in comparison with placebo in the overall population in the ISEL study, possibly due to the refractory, difficult-to-treat nature of the population [12]. Based on the lack of improvement in survival in response to gefitinib, the FDA has restricted the labeling of gefitinib. Both gefitinib and erlotinib are currently available and are used to treat patients with advanced or metastatic NSCLC in the second- or third-line setting or, sometimes, in the first-line setting for selected patients. Most patients treated with these agents, however, had progressive disease even after showing an initial dramatic response. Among the mechanism of acquired resistance to EGFR TKIs, T790M secondary mutation or amplification of the *MET* oncogene was reported frequently [89,95,96]. However, other secondary mutations have also been reported. Of note, unlike T790M secondary mutation, some mutations, such as E884K or L747S mutations, may result in different sensitivities to gefitinib and erlotinib, resulting in different tumor responses to these two agents. Choong *et al.* reported a case of erlotinib-refractory adenocarcinoma with leptomeningeal metastases that had a L858R+ E884K somatic mutation of the *EGFR* [103]. Gefitinib responded to erlotinib-refractory lung cancer, showing a differential response between erlotinib and gefitinib that was mediated by the *EGFR* mutation E884K. On the other hand, Costa *et al.* reported a case of differential response to erlotinib in *EGFR*-mutated lung cancers with acquired resistance to gefitinib carrying the L747S secondary mutation [104]. Therefore, although half of patients could overcome the resistant T790M secondary mutation by empirical use of irreversible new EGFR TKIs [90], identification of the mechanism of acquired resistance in each patient could guide the proper use of these two different EGFR TKIs.

Safety & tolerability

Compared with conventional chemotherapeutic agents, gefitinib produces relatively few severe side effects, such as hematotoxicity. Gefitinib is generally well tolerated, even in elderly patients or patients with poor performance status. The principal side effects of gefitinib are skin rash, acniform changes of the skin, diarrhea, nausea, vomiting and anorexia. Diarrhea was actually the dose-limiting toxicity in Phase I studies. Most toxicities

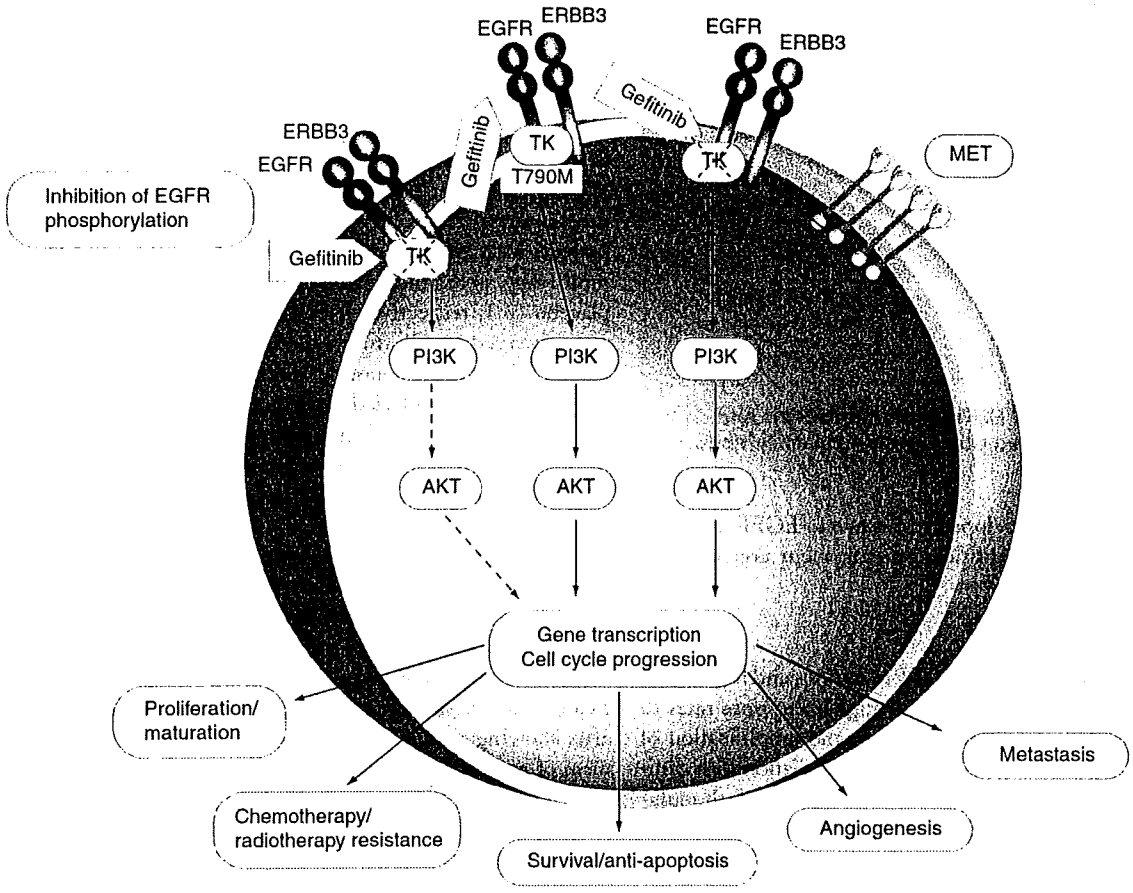

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Figure 3. Mechanism of action of gefitinib signal-transduction blockage through EGFR TK and mechanisms of acquired resistance to gefitinib. When gefitinib is administered, EGFR TK is specifically inhibited and the survival signal is blocked leading to apoptosis of cancer cells. When a secondary threonine-to-methionine mutation at codon 790 of the *EGFR* gene (T790M) is acquired, T790M prevents gefitinib from binding EGFR TK. Alternatively, when *MET* is activated by amplification, ERBB3 is phosphorylated by *MET*. Even when EGFR TK is inhibited by gefitinib, activation of the PI3K/AKT pathway is maintained through ERBB3 phosphorylation [113]. EGFR: EGF receptor; TK: Tyrosine kinase.

are common toxicity criteria grade 1 or 2. Interstitial lung disease has been observed in patients receiving gefitinib [105,106]. Worldwide, the incidence of interstitial lung disease is approximately 1% (2% in the Japanese postmarketing experience and ~0.3% in a US expanded-access program), with approximately a third of the cases being fatal. Retrospective studies on the incidence of interstitial lung disease (ILD) and prospective studies involving 3000 subjects were conducted in Japan. The risk factors of ILD have been identified as male gender, prior history of smoking and pre-existing ILD. In addition, a case–cohort study that involved the identification of cohorts among patients receiving treatment for NSCLC to determine their relative risks was conducted [107]. For this study, 4423 subjects were included in the analysis as a cohort. Among them, 122 patients were identified with ILD. The results suggest that, regardless of patients' background, administration of gefitinib carries a 3.23-fold risk of ILD compared with conventional chemotherapeutic agents. The risk factors for ILD incidence do not apply to women, adenocarcinoma patients or nonsmokers – patient groups who are more likely to benefit from gefitinib treatment. In clinical practice, it may be possible to use such risk factors as a reference for selecting appropriate patients for gefitinib treatment to reduce the incidence of ILD. Interestingly, the issue of ILD in patients with NSCLC, after gefitinib or other treatments, appears to be a problem largely limited to Japan. From the AstraZeneca Global Drug Safety Database, the reporting rate of ILD-type events in patients receiving treatment with gefitinib was only 0.23% worldwide, excluding Japan, based on more than 275,000 patients worldwide estimated to have been exposed to gefitinib. Even for neighboring countries, the pattern differs from Japan: the rate for East Asian countries, including Korea and Taiwan, but excluding Japan, was 0.17%.

The reasons for this difference in incidence of ILD between Japan and other countries remain unclear, but may relate to both constitutional and environmental factors specific to Japan or Japanese patients.

Regulatory affairs

Gefitinib is approved in 36 countries worldwide for the treatment of NSCLC (Box 1). Gefitinib was approved for clinical use in Japan on 5 July 2002, ahead of many countries in the world. It was approved by the FDA on 5 May 2003 and, subsequently, by several other countries. However, in the wake of the aforementioned ISEL trials, which indicated the failure to improve survival time with gefitinib in comparison with placebo, an application for approval for gefitinib to the EMEA was withdrawn on 4 January 2005, and the FDA has restricted the labeling of gefitinib. However, an application for approval for gefitinib was subsequently submitted to the EMEA in May 2008 following reporting of the INTEREST trial.

Conclusion

Gefitinib is generally well tolerated, has encouraging efficacy and quality of life benefits and offers hope for patients with advanced lung cancer. Gefitinib is effective as a first-, second- or third-line treatment option for advanced NSCLC. Despite the failure of combining TKIs with chemotherapy in several large Phase III clinical trials, sequential dosing regimens of gefitinib with chemotherapy is still a viable clinical research paradigm (WJTOG0203). In addition, recent results of a randomized Phase III study (IPASS) have shown an improved PFS in the gefitinib arm, indicating the possibility of gefitinib as a first-line therapy in selected patients. As a second-line therapy, gefitinib has been shown to be equivalent to docetaxel in terms of OS, with less toxicity and improved quality of life. There is some evidence that *EGFR* mutations and high *EGFR* gene copy number are associated with higher response rates and longer survival, although this is not always the case, as highlighted by the results of the INTEREST study. In the near future, treatments may be selected based on the results of *EGFR* and *KRAS* mutation status, *EGFR* copy number or, possibly, the type of histology (adenocarcinoma). Ongoing prospective trials in which patients with *EGFR* mutations are randomized to chemotherapy or EGFR TKI should help to determine the importance of mutation testing in selecting therapy for subsets of patients with lung cancer. In summary, gefitinib has provided an important alternative approach for palliation of previously treated advanced disease NSCLC patients, and it is likely that there will be increasing use of first-line gefitinib in subgroups of NSCLC patients based on their clinical and molecular characteristics.

Expert commentary

The use of the TKIs gefitinib and erlotinib grew substantially as agents for second- and third-line therapies, replacing a proportion of injectable chemotherapy agents. Although gefitinib has provided an important alternative approach for palliation of previously treated advanced NSCLC patients and is currently not approved for first-line use, it is likely that there will be increasing use of first-line gefitinib in subgroups of NSCLC patients based on their clinical and molecular characteristics. In prior studies, the predictive factors of gefitinib response were female gender, never-smoking status and adenocarcinoma histology. Indeed, before the emerging understanding of EGFR mutations, these factors were important references for physicians in choosing susceptible patients to gefitinib treatment. Grouping patients into best, intermediate and worst categories with respect to potential benefit from gefitinib has practical implications. Based on currently available information, an example of one of the best groups might include Asian women who have never smoked and have adenocarcinoma. An intermediate group might

Box 1. Countries where gefitinib is approved for use.

- Japan
- Australia
- USA
- Argentina
- Singapore
- South Korea
- Taiwan
- Malaysia
- Mexico
- Philippines
- Canada
- Curacao
- Dominican Republic
- Nicaragua
- Hong Kong
- Israel
- New Zealand
- Honduras
- Guatemala
- Thailand
- United Arab Emirates
- Switzerland
- Indonesia
- India
- Peru
- El Salvador
- Bahrain
- Panama
- Venezuela
- Chile
- Serbia/Montenegro
- Uruguay
- Qatar
- Russia
- China
- Sri Lanka

comprise smokers with adenocarcinoma, and the worst group might consist of male smokers with squamous cell carcinoma. However, clinicians are also faced with the question of whether gefitinib treatment is worthwhile in specific patient subgroups based on their clinical characteristics. It has been reported that gefitinib was more effective in never-smokers than smokers, but it is important to note that the risk of death was reduced even in smokers subsets [17,108]. Thus, at this point, it does not seem that patients should be excluded from gefitinib treatment based solely on clinical considerations. Perhaps, more importantly, we need to gather more information regarding the benefit of chemotherapy versus gefitinib in specific patient populations. The observation of higher response rates with gefitinib in selected groups of patients, as well as the disappointing results with simultaneous chemotherapy and gefitinib in unselected patients, led lung cancer researchers to study the potential predictive value of molecular profiles in patients treated with gefitinib. There is increasing evidence that *EGFR* mutations and high *EGFR* gene copy number are associated with higher response rates and longer survival. By contrast, *KRAS* mutations may predict the worst outcomes on gefitinib. Determining the optimum way to select patients for future therapy seems to be a key factor in improving results for individual lung cancer patients.

Five-year view

Gefitinib was the most commonly prescribed EGFR TKI, and still is in Japan and Asia, but the use of gefitinib as a proportion of all second-line therapies declined rapidly during the period of observation after findings from clinical studies suggested that it did not improve survival and after the subsequent FDA labeling change. On the other hand, erlotinib prescriptions increased substantially. However, sequential dosing regimens of gefitinib with chemotherapy is a viable clinical research paradigm [17], and recent results of a randomized Phase III study (IPASS) have demonstrated improved PFS in the gefitinib arm, indicating the possibility of gefitinib as the first-line therapy in selected patients. In addition, gefitinib has been shown to be equivalent to docetaxel in terms of overall survival with less toxicity and improved quality of life in the second-line therapy (INTEREST). Future research of gefitinib will include potential synergistic effects with chemotherapy using an intermittent combination in selected patients or *EGFR*-mutated patients. In addition, it is possible that, in the next 5 years, gefitinib may have a role in early-stage NSCLC as postoperative adjuvant therapy or neoadjuvant therapy. Currently, allowing for test availability and differing preferences, oncologists use mutational analysis to help them choose among possible treatments and to guide the most rational order that these therapies should be administered for individual patients. The *EGFR* mutation appears to be the most sensitive predictor of response to gefitinib. With the advances in sensitive and specific examination for the detection of *EGFR* mutation, such as high-resolution melting analysis, scorpion arms or mutant-enriched PCR, it is now possible to identify the status of *EGFR* mutation in patients, as long as histological samples are available [81,109–111]. Recently, Maheswaran *et al.* have reported the detection of mutations in EGFR of circulating lung cancer cells [112]. Molecular analysis of circulating tumor cells from the blood may offer the possibility of monitoring changes in epithelial tumor genotypes during the course of treatment. In the near future, treatments will be selected based on the results of *EGFR* and *KRAS* mutation status, *EGFR* copy number or possibly histology (adenocarcinoma vs nonadenocarcinoma). As we now know, however, resistance to gefitinib in patients with the *EGFR* mutation develop eventually. In 50% of these cases, the resistance was due to a second-site point mutation (T790M), 20% was due to *MET* gene amplification and the remainder due to unknown causes. Evaluation of the combination of gefitinib with other targeting agents, such as those that inhibit molecules in the same signalling pathway or angiogenesis inhibitors, may potentially enhance clinical outcome and reduce the emergence of resistance.

Key issues

- Gefitinib has encouraging efficacy, is generally well tolerated and has quality-of-life benefits.
- In prior studies, the predictive factors of gefitinib response were female gender, never-smoking status and adenocarcinoma histology.
- From a clinician's perspective, it would be useful to categorize patients into the best, intermediate, and worst EGF receptor (EGFR)-tyrosine kinase inhibitor treatment-outcome groups. Based on currently available information, an example of one of the best groups might include Asian women who have never smoked and have adenocarcinoma. An intermediate group might comprise of smokers with adenocarcinoma, and the worst group might consist of male smokers with squamous cell carcinoma.
- Sequential dosing regimens of gefitinib with chemotherapy is a viable clinical research paradigm, and recent results of a randomized Phase III study (IPASS) have showed improved progression-free survival in the gefitinib arm, indicating the possibility of gefitinib as the first-line therapy in selected patients. In addition, gefitinib has been shown to be equivalent to docetaxel in terms of overall survival with less toxicity and improved quality of life in second-line therapy (INTEREST).
- Currently, the treatments (cytotoxic chemotherapy vs gefitinib) are selected based on the results of *EGFR* and *KRAS* gene mutation status, *EGFR* gene copy number or, possibly, the type of histology (adenocarcinoma).
- Among those, EGFR mutation appears to be most sensitive predictor of response to gefitinib. However, resistance to gefitinib develops eventually. In 50% of these cases, the resistance was due to a second-site point mutation (T790M), 20% *MET* gene amplification and the remainder unknown causes.
- Evaluation of the combination of gefitinib with other targeting agents may potentially enhance clinical outcome and reduce the emergence of resistance.

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No writing assistance was utilized in the production of this manuscript.

Information resources

- US Food and Drug Administration www.fda.gov/default.htm
- Medicine Net www.medicinenet.com/gefitinib/index.htm
- National Cancer Institute – Clinical trials www.cancer.gov/clinicaltrials
- AstraZeneca Pharmaceuticals information resource www.iressa.com

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- •• of considerable interest

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ORIGINAL ARTICLE

Relationship of mRNA expressions of RanBP2 and topoisomerase II isoforms to cytotoxicity of amrubicin in human lung cancer cell lines

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Abstract
Purpose RanBP2 is a small ubiquitin-like modifier ligase for DNA topoisomerase II (TopoII) and plays a role in maintaining chromosome stability by recruiting TopoII to centromeres during mitosis. Engineered-mice with low amounts of RanBP2 have been reported to form lung adenocarcinomas. Furthermore, in the murine embryonic fibroblasts, formation of chromatin bridges in anaphase, a distinctive feature of cells with impaired DNA decatenation by chemical inhibition of TopoII, has been reported. In this study, we tested whether the association between mRNA expression of the RanBP2 gene and chemosensitivity of a TopoII inhibitor, amrubicin could be seen.
Methods Using a panel of 20 lung cancer cell lines, the mRNA expression levels of the RanBP2, TopoII-alpha and TopoII-beta genes were examined by quantitative real-time reverse transcription PCR. The in vitro cytotoxicity of amrubicin was assessed using a tetrazolium-based colorimetric assay (MTT assay).
Results Although RanBP2 mRNA expression was infrequently downregulated in human lung cancer cell lines, significantly higher RanBP2 transcripts were observed in small cell lung cancer than non-small cell lung cancer. There were no correlations between chemosensitivity of amrubicin and mRNA expression levels of the RanBP2, TopoII-alpha and TopoII-beta genes.
Conclusions Our in vitro results suggest that mRNA expressions of RanBP2 and TopoII isoforms are unlikely to be a predictive biomarker for the sensitivity to amrubicin.

Keywords SUMO ligase · Topoisomerase II inhibitor · Predictive biomarker · Chromosomal instability · Lung cancer

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Introduction

Lung cancer is a leading cause of cancer mortality in the United States and in Japan [13, 29]. Lung cancer has two main types: small cell lung cancer (SCLC) and non-SCLC (NSCLC). The major histological subtypes of NSCLC include adenocarcinoma, squamous carcinoma and large cell carcinoma. About 15% of lung cancers are SCLC. SCLC spreads rapidly and widely forming additional large tumors in lymph nodes, bones, adrenal glands, liver and brain. Because of its aggressive nature the overall survival of SCLC is worse than that of NSCLC and only 5–10% at 5 years. Survival of patients with either SCLC or NSCLC is strongly correlated with the stage of disease. For patients with advanced tumors, the prognosis is dismal because the available treatment regimens such as chemotherapy and radiation therapy are essentially palliative and primarily serve only to prolong survival. In fact, combination chemotherapy with etoposide plus cisplatin or irinotecan plus cisplatin for extensive-stage (ES) SCLC as well as the common first-line platinum-based combination regimens for advanced NSCLC only produced a median survival time of about 1 year [18, 19, 23]. Thus, new treatment approaches are clearly required.

Amrubicin, developed and approved in Japan for the treatment of SCLC and NSCLC, is a totally synthetic

anthracycline anticancer agent and a potent TopoII inhibitor [14]. Amrubicin monotherapy with 45 mg/(m^2 day) for 3 consecutive days by intravenous administration produced response rates of 75.8 and 27.9% for previously untreated patients with ES-SCLC and advanced NSCLC, respectively. A phase II study of the combination of 60 mg/m^2 cisplatin and 40 mg/(m^2 day) amrubicin for 3 days has been reported to show response rate of 87.8%, the MST of 13.6 months and 1-year survival rate of 56.1% against ES-SCLC. Based on this result, Japan Clinical Oncology Group (JCOG) is conducting a randomized phase III study to compare the combinations of cisplatin plus amrubicin and cisplatin plus irinotecan for previously untreated ES-SCLC.

To improve clinical outcomes in advanced lung cancer, clinical integration of molecular biomarkers that predict responses to chemotherapeutic agents may be indispensable [16]. Recently, RanBP2 has been reported to act as a small ubiquitin-like modifier (SUMO) ligase for DNA TopoII and play an important role in targeting TopoII to centromeres during mitosis and in maintaining chromosome stability [5]. Embryonic fibroblasts derived from the engineered mutant mice with low expression of RanBP2 have been reported to show formation of chromatin bridges in anaphase, a distinctive feature of cells with impaired DNA decatenation by chemical inhibition of TopoII [4], suggesting that low expression of RanBP2 may have an analogous effect of TopoII inhibitors. In addition, RanBP2 has a tumor suppressor function since these mutant mice succumbed to a range of cancers, primarily lung carcinomas, and were also susceptible to chemically-induced tumorigenesis. Based on these observations, we hypothesized that RanBP2 expression might be involved in chemosensitivity of a TopoII inhibitor, amrubicin.

The identification of molecular biomarkers with the potential to predict treatment outcomes is essential for individualizing the most beneficial chemotherapy. As one of the multiple approaches to establishing predictive biomarkers, we evaluated whether there would be associations between mRNA expression of the RanBP2 gene as well as the TopoII-alpha and beta genes and chemosensitivity to amrubicin using human lung cancer cell lines.

Materials and methods

Cell lines and drug

Fifteen NSCLC and five SCLC cell lines used were described previously [24]. These cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Amrubicin was kindly provided by Sumitomo pharmaceuticals Company, Osaka, Japan.

Cytotoxicity assay

Cytotoxicity was evaluated using an MTT assay as described previously [11]. Suspensions of exponentially growing cells were dispensed into wells of 96-well tissue-culture plates. After incubation at 37°C for 24 h, the solutions of amrubicin at various concentrations were added, and then incubated for 3 days. The effects of treatment were expressed as percent growth inhibition using untreated cells as the uninhibited control and assessed by IC50 (drug concentrations inducing a 50% reduction of cell survival) which was calculated from dose–response curves.

RNA preparation and RT-PCR amplification

Total RNA was extracted and further purified as described previously [24]. The RNAs were stored at −80°C until use. Total RNA (50 ng) extracted from each cell line was subjected to one-step real-time reverse transcriptase-PCR (RT-PCR) for absolute quantitating mRNA levels of the RanBP2, TopoII-alpha, TopoII-beta and beta-actin genes as described previously. The PCR primers used were as follows.

RanBP2-S:	5′-CAATGGAAATGGGGAAGACTTT-3′
-AS:	5′-CATCACTTCAGTCCCACCTGTA-3′
TopoII-alpha-S:	5′-GGTGTGGAACTAGAAGGCCTAA-3′
-AS:	5′-TGAATCAGACCAGGGATTTCTC-3′
TopoII-beta-S:	5′-TTTTTCACCATCATTTGGTCTG-3′
-AS:	5′-GGGCTTAGGGACTGTATCTGAA-3′
Beta actin-S:	5′-TTCTACAATGAGCTGCGTGTG-3′
-AS:	5′-CAGCCTGGATAGCAACGTACA-3′

Linear regression analysis of standard-curves demonstrated a strong correlation for all the genes ($R^2 > 0.99$). The relative gene expression levels were normalized with a house keeping gene, beta-actin.

Western blot analysis

Western blot analysis was done as described previously [11], using the following primary antibodies: anti-RanBP2 (ab2938, Abcam), anti-TopoII-alpha (ab45175, Abcam), anti-TopoII-beta (ab58442, Abcam) and anti-actin (A2268, Sigma-Aldrich) antibodies.

Statistical analyses

The strength of the association between the expression levels of RanBP2, TopoII-alpha and TopoII-beta and chemosensitivity data was calculated by either Pearson's correlation coefficient or linear regression analysis. Correlations were considered significant at $P < 0.05$. For comparison of IC50 values of amrubicin and each gene expression level among histological subtypes, we employed one-way

analysis of variance (ANOVA) followed by Bonferroni post-test. All analysis was performed with the use of Stat View software version 5.0.

Results

Chemosensitivity of amrubicin was examined using 20 human lung cancer cell lines including 15 NSCLC cells and 5 SCLC cells. Cytotoxicity following a 72 h continuous exposure of amrubicin was measured by MTT assay. The IC 50 value of amrubicin in SK-LC-3 was about 9 μM, while the IC 50 values in the other cell lines were less than 1 μM as shown in Table 1. There was no significant difference between histological types (Fig. 1).

The mRNA quantifications of the RanBP2, TopoII-alpha, TopoII-beta genes were carried out in real-time PCR and the expression levels were normalized with beta-actin as an internal control (Table 1). Among 20 cell lines tested, the level of RanBP2 mRNA expression in an H460 cell line was about 20-fold lower than those in non-tumorous lung tissues obtained from two patients with lung cancer. There were statistically significant differences in the RanBP2 expression between SCLC and the other histological subtypes ($p < 0.05$) (Fig. 2a). We checked RanBP2 protein expression in two lung cancer cell lines, SK-LC-2 and H460, representing high and low expression of the RanBP2 gene, and found similar mRNA and protein expression patterns (Fig. 2d). We also found statistically higher expression levels of TopoII-alpha in SCLC and adenocarcinoma cell lines compared with those in normal lung tissues,

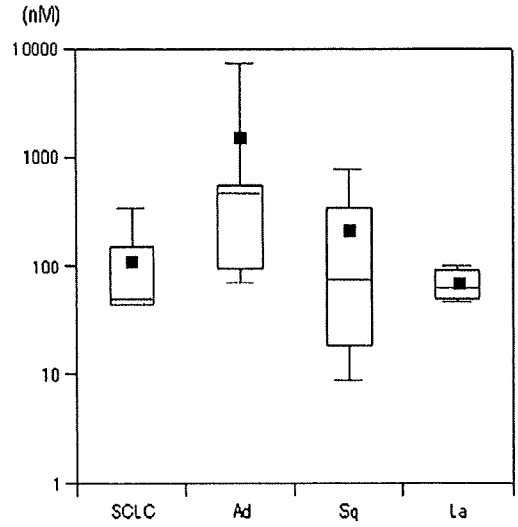

Fig. 1 IC50 values of Amrubicin in lung cancer cell lines. *Box plots* show relationships between IC50 values of Amrubicin and the four histological subtypes of lung cancer. The *horizontal line* within each *box* represents the median value and the closed box shows the mean value, respectively

Table 1 IC50 values for amrubicin and relative mRNA expression for RanBP2, TopoII alpha and TopoII beta in lung cancer cell lines

Cell line	Histology	Amrubicin (μM)	RanBP2	TopoIIa	TopoIIb
ACC-LC-94	Ad	0.0668	0.621	1.324	1.174
ACC-LC-319	Ad	0.579	1.108	1.682	1.437
SK-LC-3	Ad	8.99	2.634	2.009	1.899
A549	Ad	0.131	1.191	1.553	1.665
SK-LU-1	Ad	0.492	1.307	2.930	1.479
VMRC-LCD	Ad	0.0835	4.134	2.660	2.942
RERF-LC-MT	Ad	0.469	0.661	0.8719	0.753
Calu1	Sq	0.203	1.280	2.173	1.750
SK-MES-1	Sq	0.0768	1.160	0.883	0.807
PC-1	Sq	0.009	1.937	1.739	2.888
RERF-LC-A	Sq	0.0222	0.717	1.454	1.036
PC-10	Sq	0.77	0.713	1.049	1.170
NCI-H460	La	0.101	0.043	1.518	2.098
Calu6	La	0.0469	1.467	1.116	1.828
SK-LC-6	La	0.0632	2.362	2.508	4.383
ACC-LC-48	SCLC	0.0512	1.957	1.672	2.044
ACC-LC-49	SCLC	0.0866	2.592	1.975	3.523
ACC-LC-80	SCLC	0.0459	3.953	1.361	1.993
ACC-LC-172	SCLC	0.0439	2.387	4.450	4.207
SK-LC-2	SCLC	0.337	3.662	3.510	4.264
NL 1	Normal lung	NA	1.006	0.158	2.447
NL 2	Normal lung	NA	0.913	0.179	1.937

NL 1 and NL 2: non-tumorous lung tissues obtained from two patients with lung cancer.
Ad adenocarcinoma,
La large cell carcinoma,
SCLC small cell lung cancer,
Sq squamous cell carcinoma.
NA not available

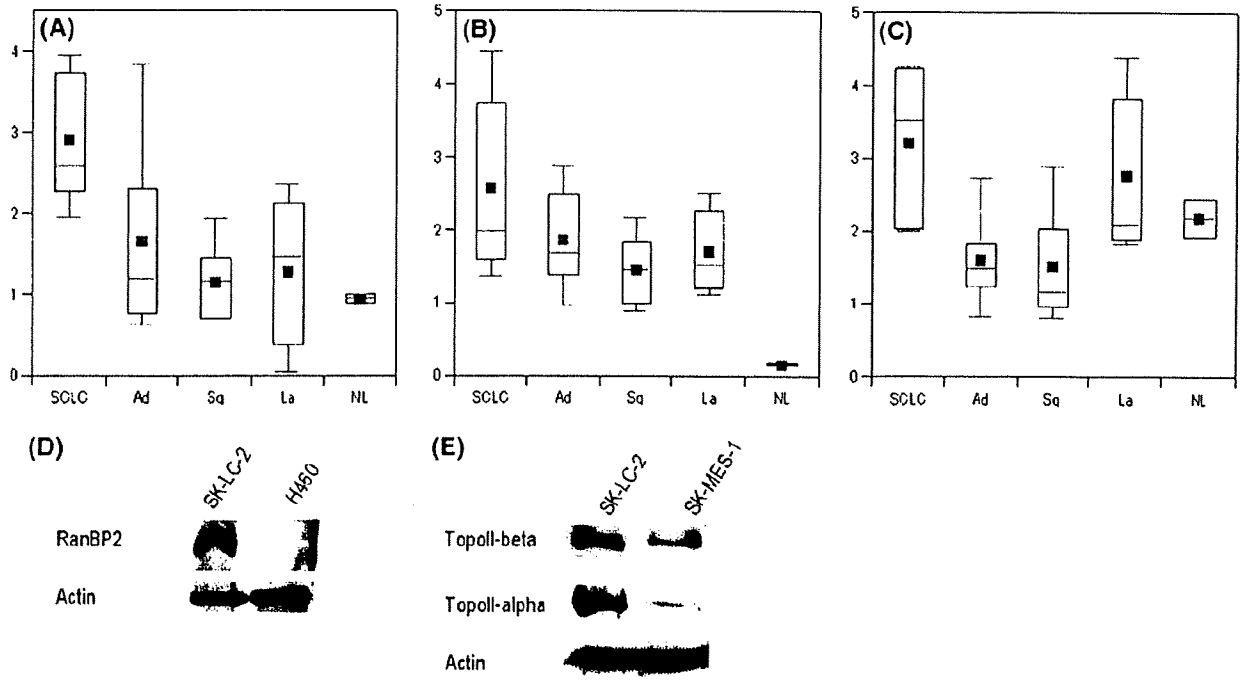

Fig. 2 Relative mRNA expression for (a) RanBP2, (b) TopoII-alpha and (c) TopoII-beta among histological subtypes and normal lung tissues, and protein expression for (d) RanBP2 and (e) TopoII isoforms in representative cell lines. a RanBP2 mRNA expression in SCLC was higher than those in the other histological subtypes of lung cancer. b TopoII-alpha mRNA expression levels in lung cancer cell lines were relatively higher compared to those in normal lung tissues. c The expression levels of TopoII-beta in lung cancer cell lines were similar to those in normal lung tissues. d, e Western blot analyses for RanBP2 and TopoII isoforms in two lung cancer cell lines representing high and low expression, respectively. The expression patterns of protein and mRNA were not different

although there were no significant differences in TopoII-alpha mRNA expression levels among four histological subtypes of lung cancer (Fig. 2b). On the other hand, the expression levels of TopoII-beta in lung cancer cell lines were similar to those of normal lung tissues, although relatively higher expression levels were observed in SCLC and large cell carcinoma (Fig. 2c). In addition, we checked TopoII-alpha and TopoII-beta protein expressions in two lung cancer cell lines, SK-LC-2 and SK-MES-1, representing high and low expression of the two TopoII isoforms, and found that protein expression patterns of these genes were not different with mRNA expression patterns (Fig. 2e).

There were weak but significant positive correlations between RanBP2 and TopoII-alpha mRNA expressions, between RanBP2 and TopoII-beta mRNA expressions and between TopoII-alpha and TopoII-beta mRNA expressions among 20 lung cancer cell lines ($r = 0.532$; $P < 0.05$, Fig. 3a and $r = 0.623$; $P < 0.05$, Fig. 3b, $r = 0.647$; $P < 0.01$, Fig. 3c, respectively). Chemosensitivity data were analyzed in relation to the mRNA expression levels of the RanBP2, TopoII-alpha, TopoII-beta genes using linear regression analysis. No significant associations were observed between the IC50 values of amrubicin and the mRNA expression levels of RanBP2 (Fig. 4a), TopoII-alpha (Fig. 4b) and TopoII-beta (Fig. 4c) among 20 cell lines.

Discussion

RanBP2 has been reported to be involved in both nucleocytoplasmic transport and mitosis and also act as a SUMO ligase for DNA TopoII and play a role in maintaining chromosome stability by recruiting TopoII to centromeres during mitosis [5]. In addition, RanBP2 hypomorphic mice are particularly sensitive to spontaneous and carcinogen-induced lung tumors, indicating that RanBP2 might play a potential tumor suppressor role in human lung cancer. Two previous studies reported that RanBP2 mRNA expression levels are substantially reduced in human non-SCLC [2, 8]. However, the present study showed that RanBP2 transcript levels were infrequently downregulated in human lung cancer cell lines compared with normal lung tissues, although there were statistically significant differences in the RanBP2 expression between SCLC and NSCLC. Consistent with our results, several lines of evidence from publicly available human gene expression data of the Oncomine

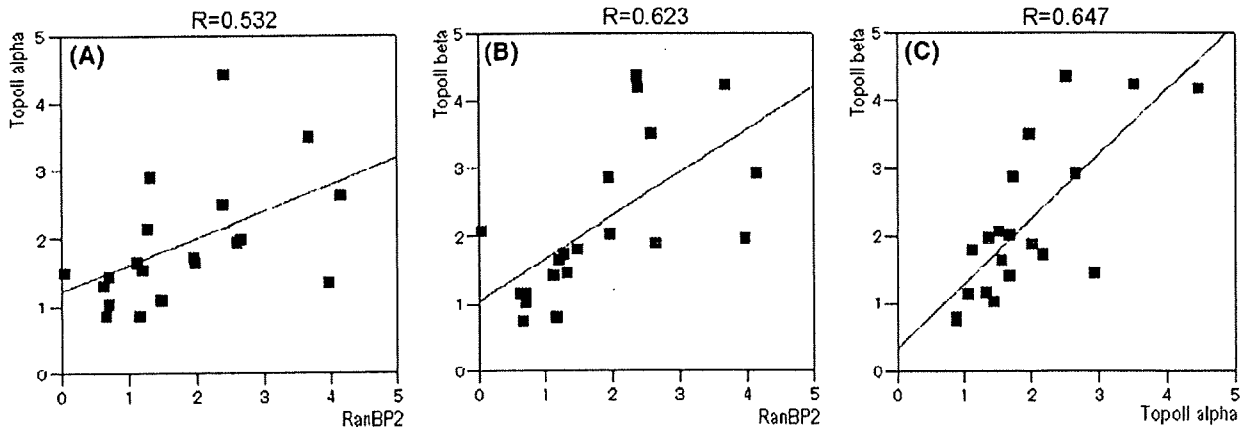

Fig. 3 Correlations between a RanBP2 and TopoII alpha mRNA expression, b RanBP2 and TopoII beta mRNA expression and c TopoII alpha and TopoII beta mRNA expression in lung cancer cell lines

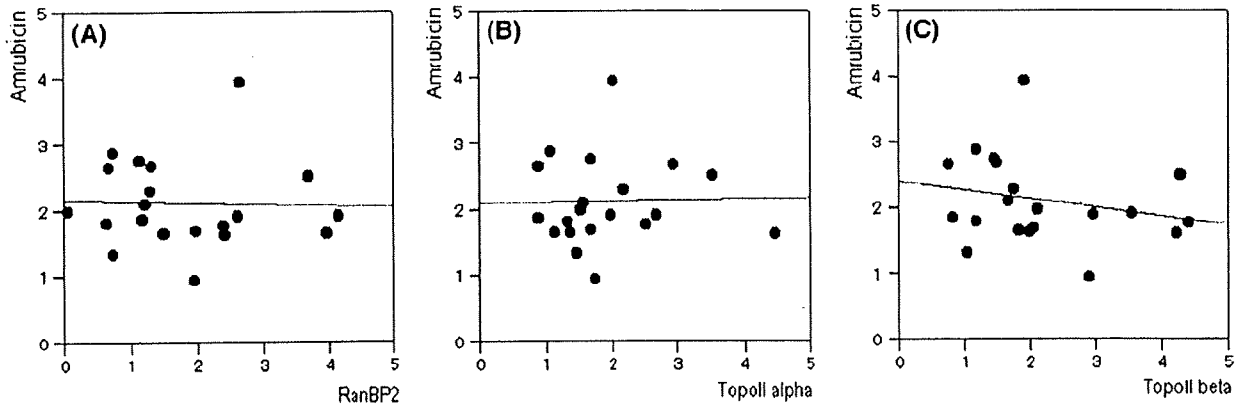

Fig. 4 Associations between relative mRNA expression for (a) RanBP2, (b) TopoII alpha and (c) TopoII beta and chemosensitivity of Amrubicin Log(IC50 in nM)

database (http://www.oncomine.com) and GEO profiles (http://www.ncbi.nlm.nih.gov/geo/) reported that RanBP2 mRNA expression levels are not reduced in NSCLC compared with normal lung tissues [3, 22, 26–28, 30]. In addition, there is a microarray study showing that RanBP2 expression levels are similar to those of our data in four overlapping lung cancer cell lines [9]. The concordance and discordance between our findings and previous works might be caused by the difference between cell lines and resected human lung tumors as well as the different experimental conditions used. Thus, further studies are warranted to establish the role of RanBP2 as a tumor suppressor gene in human lung carcinogenesis.

In RanBP2 hypomorphic murine embryonic fibroblasts (MEFs), formation of chromatin bridges in anaphase, a distinctive feature of cells with impaired DNA decatenation by mutation or chemical inhibition of TopoII-alpha [4], was observed, while spindle structure, kinetochore–microtubule interactions, and localization of kinetochore and spindle assembly checkpoint proteins appeared normal [5]. Therefore, the low expression of RanBP2 may have an analogous effect of TopoII inhibitors, although the inhibitors are able to cause an inevitable consequence of DNA damage at high doses [4, 21]. Then, we speculated that there might be an association between RanBP2 mRNA expression and chemosensitivity of a TopoII inhibitor, amrubicin and tested whether we could see it using human lung cancer cell lines. However, we did not find any associations, suggesting that cytotoxicity of amrubicin might come mainly from DNA damage response induced at high doses and that formation of chromatin bridges in anaphase caused by low expression of the RanBP2 gene might not have additional effects on amrubicin-induced DNA damage response.

The two isozymes, TopoII-alpha and TopoII-beta function to unknot and decatenate covalently closed circles of DNA, although functional differences of these isozymes

and their differential spliced variants as well as precise role of their homodimerization and heterodimerization are unknown [20, 21]. There are several lines of evidence indicating a close relationship between TopoII-alpha levels and drug sensitivity in cell lines made resistant to TopoII inhibitors [7, 17, 25], cell lines with reduced expression of TopoII [1] and a VP-16-resistant breast cancer cell line infected with adenovirus containing TopoII-alpha [32]. Another study has shown the relationship between TopoII expression and multidrug sensitivity including TopoII inhibitors using eight human lung cancer cell lines [10]. There is also some evidence that TopoII-beta may be related with resistance to TopoII inhibitors [6, 15]. However, we did not find any association between expression levels of TopoII isoforms and chemosensitivity of amrubicin. Consistent with our results, a previous report of unselected human lung cancer cell lines also showed no clear association between TopoII-alpha protein expression and in vitro sensitivity to TopoII inhibitors [31]. Another study also failed to show importance of the enzyme using a panel of cell lines [12]. Although the behavior of cell lines in vitro may differ from the in vivo situation, and depend on the experimental conditions, these contradictory findings may require further investigation.

Amrubicin is highly active and one of the most potent anticancer drugs against SCLC and NSCLC [14]. Among the toxicities, hematologic adverse events such as leukopenia and thrombocytopenia are frequent and dose-limiting factors. Although identification of molecular biomarkers with the potential to predict treatment outcomes is essential to eliminate the use of any ineffective agents and to avoid toxic side effects [16], the cellular response to amrubicin is still poorly understood. To predict drug response in lung cancer patients, integrated analyses such as array-based mRNA expression profile, epigenome profiles, proteome analysis would be needed.

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Efficacy of Erlotinib for Brain and Leptomeningeal Metastases in Patients with Lung Adenocarcinoma Who Showed Initial Good Response to Gefitinib

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Introduction: The efficacy of high-dose (1250 mg/d) gefitinib for the treatment of leptomeningeal metastasis in a patient with lung cancer harboring a mutation in the epidermal growth factor receptor (EGFR) gene was previously reported. We speculate that erlotinib, instead of high dose of gefitinib, may be also effective for the treatment of central nervous system (CNS) lesions, as trough serum concentration of erlotinib is nine times higher than that of gefitinib.

Patients and Methods: Patients with lung cancer in whom CNS lesions developed after an initial good response to gefitinib for extra CNS lesions were enrolled in the study. Tumor response, performance status, neurologic symptoms, and survival were retrospectively evaluated.

Results: All seven patients had *EGFR* mutations in their primary tumors except one patient. The median interval between gefitinib withdrawal and erlotinib administration was 5 days. Three patients showed partial response, three had stable disease, and one had progressive disease. Performance status and symptoms improved in five patients. The overall survival from the initiation of erlotinib treatment ranged from 15 to 530 days (median, 88 days).

Conclusions: Erlotinib was a reasonable option for the treatment of CNS diseases that appeared after a good initial response of extra CNS disease to gefitinib.

Key Words: Lung cancer, Brain metastasis, EGFR-TKI, BBB, CNS.

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Tyrosine kinase inhibitors (TKIs) of the epidermal growth factor receptor (EGFR) have been widely used for the treatment of patients with non-small cell lung cancer (NSCLC). Somatic activating mutations of the tyrosine kinase domain of the *EGFR* gene are highly associated with sensitivity of NSCLC to EGFR TKIs.[1–4] Nevertheless, the disease in the majority of these patients eventually progresses, despite an initial dramatic response to treatment, after a median of about 10 months.[5,6] The central nervous system (CNS), e.g., the brain or the leptomeninges, is a common site for metastasis of NSCLC. Patients with CNS metastasis in general suffer from deterioration of performance status (PS) and therefore do not have a long-survival time. Although the recent advent of radiosurgery techniques confers better local control of brain metastases, currently there is no efficient method of treatment for leptomeningeal metastases.

High-dose gefitinib (1250 mg/d) was reportedly effective for the treatment of leptomeningeal metastasis in a patient with lung cancer harboring an *EGFR* mutation.[7] In this study, the gefitinib concentration in the cerebrospinal fluid (CSF) was 6.2 nM at a dose of 500 mg daily, whereas it was 39 nM at a dose of 1250 mg daily, with a serum concentration of 3730 nM. On the other hand, the median IC_{50} value of cell lines that carry an activating mutation of the *EGFR* gene is 90 nM.[8] This difference in concentration between the serum and the CSF is thought to be associated with the blood–brain barrier (BBB).

Erlotinib is also an anilinoquinazoline compound that specifically inhibits EGFR tyrosine kinase, similarly to the action of gefitinib. Its dose was set at 150 mg daily, which equals to the maximum tolerated dose (MTD) of this drug. Trough serum concentration of erlotinib (administered at 150 mg/d) is 3.5 μM that is nine times higher than that of gefitinib (0.4 μM) administered at the usual dose of 250 mg/d,[9–13] approximately one third of the MTD of gefitinib (700 mg/d).

Prompted by these observations, we speculated that erlotinib, instead of a high dose of gefitinib, may also be effective for the treatment of CNS lesions in patients with NSCLC harboring *EGFR* mutations who showed an initial good response to gefitinib. We report the response of brain and leptomeningeal metastases to erlotinib in seven of these patients.

PATIENTS AND METHODS

Patients

The records of 43 patients with NSCLC that was pathologically diagnosed and treated with erlotinib at our institution between April 2005 and September 2008 were retrospectively reviewed in this study. We identified those who had been treated with erlotinib for CNS lesions that developed after an initial good response of their extra CNS lesions to gefitinib. This study was approved by the institutional review board of the Aichi Cancer Center Hospital, and written informed consent for genetic analysis was obtained for each patient at the time of diagnosis or operation.

Treatment and Response Evaluation

Medical records, serum carcinoembryonic antigen (CEA) levels, chest radiograph, chest–abdominal computed tomography scan, brain magnetic resonance imaging (MRI), and ^{18}F-fluorodeoxyglucose positron emission tomography (PET) were retrospectively reviewed. Erlotinib of 150 mg daily were administered to the patients until progressive disease. They all had previously received 250 mg gefitinib daily. Treatment response was evaluated according to the RECIST. Because of retrospective nature of this study, strict application of RECIST was impossible. Nevertheless, we defined tumor response when the long axis of the target lesion shrank by more than 30%.

Mutational Analysis

We extracted RNA or DNA from tumor samples and analyzed *EGFR* mutations as previously reported.[5,14] Briefly, we performed direct sequencing of the product of the reverse transcription polymerase chain reaction of exons 18 to 21 of the *EGFR* gene.

RESULTS

We identified seven patients who met our criteria. Patient characteristics and clinical courses are summarized in Table 1. There were five women and two men, and their ages ranged from 58 to 81 years (median, 61 years). We confirmed the presence of *EGFR* mutations in the primary tumors of all patients, with the exception of one patient, for whom a tumor specimen was not available. Four patients had a deletion mutation in exon 19, and two had a point mutation in exon 21 (L858R). Six patients had been locally pretreated with whole brain radiation therapy or radiosurgery, before disease progression in CNS.

Disease outside of the CNS was initially controlled by gefitinib monotherapy in all seven patients. The median duration of gefitinib administration was 310 days (range, 113–1211 days), and all patients showed progressive disease in their CNS; four patients exhibited disease progression in the CNS, and the other three patients developed new symptomatic brain or leptomeningeal metastases associated with deterioration of PS. Disease outside of CNS had been under

TABLE 1. Clinical Characteristics of Patients

Case	Age/ Sex	Histology	EGFR Mutation	Initial Metastatic Sites	Initial Response of Extra CNS Lesions to G	TTF to G, d	CNS Disease After G	Neurological Symptoms
1	81/M	Adeno	X19del	Brain, bone	CR	275	PD (new LMM)	Dysmnesia, gait disorder
2	63/F	Adeno	X19del	Lung, skin, Med LN	PR	516	PD (new brain)	Consciousness disorder
3	58/F	Adeno	L858R	Brain, lung	SD	113	PD (new LMM)	Headache, postural disorder
4	60/F	Adeno	X19del	Brain, bone	SD	1211	PD (new LMM)	Syncope, polyopia
5	64/M	Adeno	NA	Brain, lung	PR	192	PD	Consciousness disorder, gait disorder
6	60/F	Adeno	L858R	Brain, bone	NA	242	PD	Dysmnesia, gait disorder
7	61/F	Adeno	X19del	Bone, Med LN	CR	382	PD (new LMM)	Headache, vomiting

G, gefitinib; E, erlotinib; Med LN, mediastinal lymph nodes; TTF, time to treatment failure; CR, complete response; PR, partial response; SD, stable disease; PD, progression disease; NA, not available; LMM, leptmeningeal metastasis.

TABLE 2. Erlotinib Treatment in Patients with CNS Involvement

Case	Interval Between G and E, d	Response of CNS Lesions to E	Change of PS	Metastasis-Related Neurological Symptoms	CEA Level, ng/ml	Interval Between E Start and Death, d	Adverse Effects
1	35	PR	4 → 4	Improved	43.6 → 11.5	178	Rash, FN
2	2	SD	3 → 1	Improved	451.0 → 7.1	247	Rash
3	47	SD	4 → 3	Improved	67.0 → 47.6	60	—
4	5	PR	1 → 1	Improved	3429.5 → 1294.5	530	Rash, diarrhea
5	2	PR	3 → 2	Improved	NA	88	Rash
6	8	SD	4 → 4	Progress	17.4 → 9.5	15	—
7	1	NA	3 → 4	Progress	136.7 → 110.8	23	—

G, gefitinib; E, erlotinib; CNS, central nervous system; PS, performance status; CEA, carcinoembryonic antigen; PR, partial response; SD, stable disease; NA, not available; FN, febrile neutropenia.

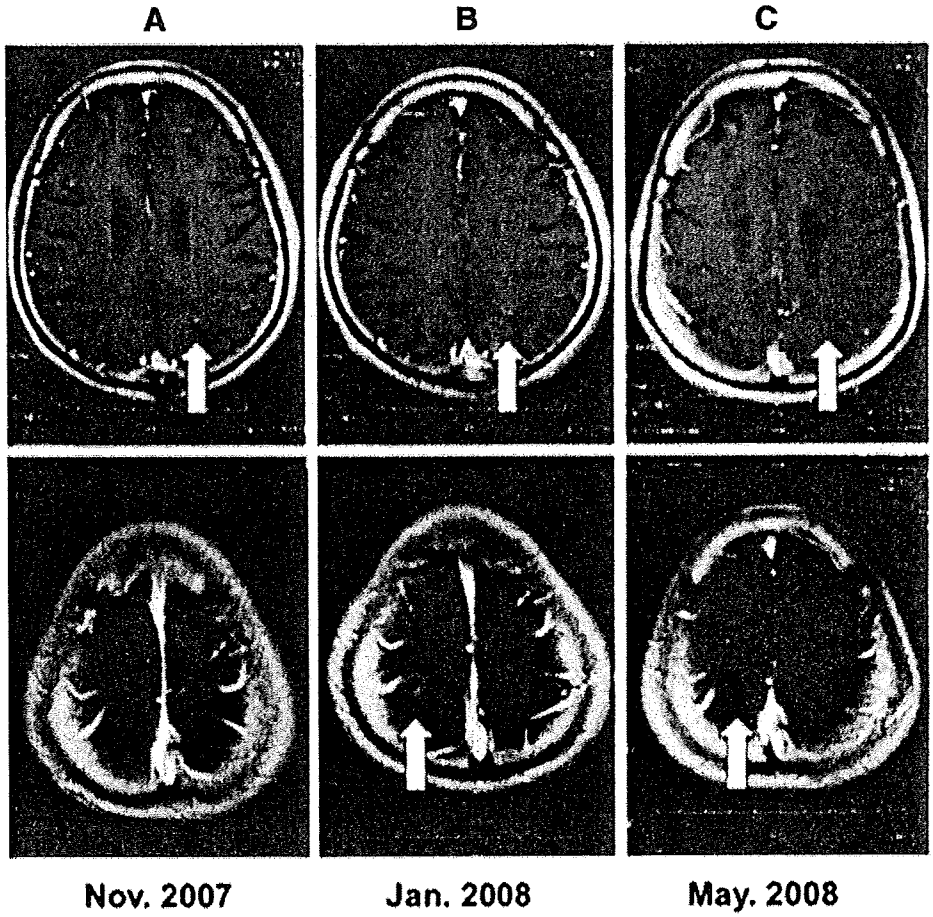

FIGURE 1. Contrast-enhanced T1-weighted magnetic resonance imaging (MRI) of the brain of patient 1. *A*, MRI study performed 8 months after initiation of gefitinib. A small nodule was in the occipital lobe (arrow), but no lesions were recognized in parietal lobe. *B*, The occipital lesion increased in size, and a new lesion appeared in parietal lobe in January 2008. *C*, The brain metastases shrunk 4 months after the initiation of erlotinib therapy.

good control in all patients during gefitinib therapy. Gefitinib was replaced with erlotinib without interposition of other drugs. The duration of drug holiday ranged from 1 to 47 days (median, 5 days). The PS in most patients at the initial erlotinib administration was 3 or 4.

Using RECIST, we found that three patients showed partial response, three patients remained stable disease, and diagnostic imaging was not available for one patient (Table 2). PS and metastasis-related neurologic symptoms improved in five patients, whereas the remaining two patients had disease progression. We confirmed that the CEA levels were reduced in six patients after erlotinib administration, with the exception of one patient, for whom information on CEA level was not available.

EGFR mutation analysis performed in a CSF sample from the patient 7 before erlotinib treatment revealed a point mutation in exon 20 (T790M), which is regarded as a resistant mutation[15,16] in addition to an exon 19 deletion mutation. Her disease progressed rapidly, even after replacement of gefitinib with erlotinib, and she died 23 days after the drug switch.

Case Report of Patient 1

The patient was an 81-year-old man who underwent left upper lobectomy in August 2006. The tissue sample of his primary tumor carried a deletion mutation in exon 19 of the *EGFR* gene. Nevertheless, his serum CEA level was 13.9 ng/ml in March 2007. Although he was asymptomatic, his brain MRI and PET scan revealed multiple metastases in the brain and bone. We treated him with 250 mg of gefitinib daily because he was elderly and had an *EGFR* mutation. The serum CEA level had decreased to 6.3 ng/ml in May 2007. We discontinued gefitinib treatment because of headache and general fatigue at December 25, 2007. Although the PET and computed tomography scans revealed remarkable improvement of bone metastasis, the brain MRI revealed the presence of new brain metastases (Figure 1*A*, *B*) and new leptomeningeal metastasis, and the serum CEA level increased to 43.6 ng/ml in January 2008. Dysmnesia and gait disorder became apparent, which escalated him to PS 4. Because of a difficulty in swallowing, enteral nutrient and a daily dose of 150 mg of erlotinib dissolved in water were administered via a nasogastric tube from January 30. His dysmnesia improved within 1 month after the initiation of erlotinib treatment. MRI revealed a remarkable improvement of brain metastasis in May 2008 (Figure 1*C*). His serum CEA level decreased to 11.3 ng/ml in June 2008. He continued to take erlotinib for 178 days until he died of pulmonary lymphangiosis on July 28, 2008.

DISCUSSION

In this study, we showed that erlotinib elicited tumor responses and improvement of PS in three of seven patients

who developed CNS lesions after an initial good response of extra CNS lesions to gefitinib. Neurologic symptoms and serum CEA level improved in five of seven and six of six patients, respectively. In addition, brain MRI revealed partial response in three patients.

Gefitinib and erlotinib are similar anilinoquinazoline compounds. Although it seems that erlotinib has a slightly broader spectrum of kinase inhibition than gefitinib,[17] they are essentially EGFR-specific TKIs. The most prominent difference between these two drugs is the dose setting. Although the approved daily dose of erlotinib (i.e., 150 mg/d) is equal to the MTD, the daily dose of gefitinib was set at 250 mg/d, because response and survival were not different between 250 and 500 mg of gefitinib in two phase II trials.[18,19] This difference of dose setting is reflected in the differences observed in their serum concentration. The C_{max} and area under the curve were 2120 ng/ml and 38,420 ng/h/ml for a dose of erlotinib of 150 mg daily,[12] and 307 and 5041 ng/h/ml for a dose of gefitinib of 225 mg daily,[13] respectively. The administration of 700 mg of gefitinib resulted in Cmax and area under the curve of 2146 ng/ml and 36.077 ng/h/ml,[13] respectively. Nevertheless, several reports revealed an unsatisfactory disease control by erlotinib after gefitinib failure, with response rates ranging from 9.5 to 14%.[20,21] This can be explained by the fact that the 2 common mechanisms of acquired resistance to EGFR-TKI, i.e., T790M secondary mutation and *MET* gene amplification, are both refractory to gefitinib and to erlotinib.[22]

Animal studies revealed that the delivery of gefitinib to the CNS of normal mice is hindered by the BBB.[23] It is possible that gefitinib may not have free access to the brain in human,[24] as another small, low-molecular weight TKI, imatinib, is shown to have limited brain penetration.[25] Hence, the CSF concentration of gefitinib is usually much lower than that observed in the serum.[23] Although there are several reports that gefitinib is effective for the treatment of brain metastases of several tumors,[24,26–31] these observations are thought to be dependent on a combination of the degree of disruption of the BBB caused by tumor invasion[32] and a sensitivity of cancer cells to the drug. Thus, dose escalation is thought to be a reasonable strategy to circumvent the EGFR-TKI-sensitive tumor cells that are present in the CNS.

In this study, we used 150 mg of erlotinib instead of 1250 mg of gefitinib. Even after a very short interval, erlotinib conferred appreciable and meaningful clinical responses, which included the improvement of the level of consciousness. While preparing this manuscript, Yi et al.[33] reported that treatment of elrotinib or an increased dose of gefitinib is an effective therapeutic option for selected patient with NSCLC and leptomenigeal metastasis. This response can be explained as follows; tumor cells in the CNS had not previously been exposed to gefitinib and, therefore, did not need to develop a resistance mechanism, thus remaining sensitive to erlotinib which traversed the BBB because of its relatively higher serum concentration compared with that of gefitinib. In the case reported by Jackman et al., the tumors of the lung, liver, and intestine had a T790M mutation, in addition to the exon 19 deletion, while the T790M mutation was not detected in the postmortem CNS tumor specimens. Relatively short survival of patient 1 despite that the CNS lesions responded well to erlotinib can be interpreted as follows; gefitinib had reached to the extra CNS lesions and acquired resistance to TKI such as T790M had already developed. Therefore, even erlotinib could not control the extra CNS lesions despite improvement of brain metastasis.

In patient 7, tumor cells of the CSF had a T790M mutation after gefitinib treatment and before erlotinib administration. Her disease progressed rapidly, even after the switch to erlotinib treatment, and the patient died 23 days after the initiation of erlotinib administration, as could be expected. In this case, we speculate that gefitinib could reach the CNS, where it was able to control tumor cells for a while initially. Nevertheless, the resistant clone carrying the T790M mutation eventually developed during the 382 days of gefitinib administration.

In conclusion, we report the effectiveness of erlotinib for the treatment of CNS lesions after gefitinib failure. This situation is relatively common in Japan, because there was an interval of over 5 years between the approval of gefitinib and erlotinib in our country.

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