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First-Line Gefitinib in Patients Aged 75 or Older With Advanced Non–Small Cell Lung Cancer Harboring Epidermal Growth Factor Receptor Mutations

NEJ 003 Study

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Introduction: Recent studies have demonstrated that first-line treatment with gefitinib, an epidermal growth factor receptor (EFGR)–targeted tyrosine kinase inhibitor, is significantly superior to standard chemotherapy for advanced non–small-cell lung cancer (NSCLC) harboring EGFR sensitive mutations. Meanwhile, the efficacy of gefitinib therapy among elderly populations diagnosed with EGFR-mutated NSCLC has not yet been elucidated. The purpose of this study was to investigate the efficacy and feasibility of gefitinib for chemotherapy-naive patients aged 75 or older with NSCLC harboring EGFR mutations; generally, these patients have no indication for treatment with platinum doublets.

Methods: Chemotherapy-naive patients aged 75 years or older with performance status 0 to 1 and advanced NSCLC harboring EGFR mutations, as determined by the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp method, were enrolled. The enrolled patients received 250 mg/day of gefitinib orally.

Results: Between January 2008 and May 2009, 31 patients were enrolled, all of whom were eligible. The median age was 80 (range, 75–87) years. Twenty-five patients (81%) were women, and 30 patients (97%) had adenocarcinoma. The overall response rate was 74% (95% confidence interval, 58%–91%), and the disease control rate was 90%. The median progression-free survival was 12.3 months. The common adverse events were rash, diarrhea, and liver dysfunction. One treatment-related death because of interstitial lung disease occurred.

Conclusions: This is the first study that verified safety and efficacy of first-line treatment with gefitinib in elderly patients having advanced NSCLC with EGFR mutation. Considering its strong antitumor activity and mild toxicity, first-line gefitinib may be preferable to standard chemotherapy for this population.

Key Words: Non–small cell lung cancer, Epidermal growth factor receptor mutation, Gefitinib

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Non–small-cell lung cancer (NSCLC), which accounts for 80% of lung cancer, remains the major cause of cancer-related death in both Western and Asian countries. With prolongation of life expectancy, both the incidence and mortality of lung cancer in the elderly are rising. In Japan, 48 500 individuals aged 70 years or older were estimated to die of lung cancer in 2009[1]; moreover, the ratio of elderly patients dying from lung cancer increased from 57% in 1989 to 72% in 2009. Treatment strategy in elderly patients with lung cancer has, thus, become an important issue.

About half of the newly diagnosed NSCLC patients have advanced disease, with no indication for local therapy such as surgery and radiotherapy. Chemotherapy for the elderly shows similar efficacy to that observed in younger

patients. However, it is generally more toxic, in terms of both incidence and severity, because of age-related weakening of organ function.[2] Consequently, standard chemotherapy for elderly NSCLC patients, especially those aged 75 years or older, is performed as monotherapy with vinorelbine, gemcitabine, or docetaxel instead of platinum doublets, which are the standard for younger patients.[3–7] Although a recent phase III study suggested that the platinum doublet of monthly carboplatin and weekly paclitaxel may be superior to the gemcitabine or vinorelbine monotherapy in the elderly population, the treatment-related death rate of the doublet group was determined to be 7%.[8] Thus, investigation into safer and more effective treatments for elderly NSCLC patients is required.

Gefitinib, an orally administered tyrosine kinase inhibitor (TKI) of the epidermal growth factor receptor (EGFR), is a key molecularly targeted drug used for the treatment of advanced NSCLC. In May 2004, seminal studies showed that the presence of somatic mutations in the kinase domain of EGFR strongly correlated with increased responsiveness to EGFR TKIs in patients with NSCLC.[9,10] Before this observation, it had been known that subgroups of NSCLC patients, including those of Asian race, female sex, nonsmoking status, and having adenocarcinoma, displayed significant responses to gefitinib.[11,12] These subgroups turned out to have a high incidence of EGFR mutations.[13] Recently, two phase III studies comparing gefitinib treatment with chemotherapy in chemo-naive patients selected on the basis of EGFR mutations were reported from Japan.[14,15] These studies revealed the superiority of gefitinib treatment over standard chemotherapy by demonstrating that first-line gefitinib administration doubled progression-free survival (PFS) as compared with standard chemotherapy. One of two studies we conducted, namely the NEJ002 study, demonstrated that treatment with gefitinib provided patients with a better quality of life as compared with chemotherapy.[16] The eligibility criteria in these studies was limited to patients aged 75 years or younger, as the treatments with platinum doublets were considered to be inappropriate for more elderly populations because of increased toxicity. Moreover, it has been reported in Japan that this more elderly group of patients develop interstitial lung disease (ILD) frequently when treated with gefitinib.[17] In previous studies, we demonstrated that patient selection by EGFR mutation can dramatically improve the risk–benefit balance of gefitinib treatment; however, no study thus far has investigated the efficacy and feasibility of first-line gefitinib treatment in elderly NSCLC patients with EGFR mutation. Thus, the current phase II study was conducted.

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METHODS

Patient Selection

This multicentric phase II study was approved by the institutional review board of each participating institute. The main eligibility criterion was to select chemotherapy-naive patients with NSCLC harboring sensitive EGFR mutations. Namely, patients with exon 19 deletions, L858R, L861Q, G719A, or G719S were included, but those with a resistant T790M mutation were excluded. Patients who were 75 years of age or older with Eastern Cooperative Oncology Group performance status (PS) 0 to 2 were also deemed eligible. Other eligibility requirements were stage IIIB to IV or postoperative recurrent NSCLC, presence of a measurable lesion according to the Response Evaluation Criteria in Solid Tumors (RECIST), adequate organ function including liver function (aspartate transaminase and alanine aminotransferase ≤100 U/liter, total bilirubin <2.0 mg/dL), and written informed consent.

EGFR Mutation

Cytological or histological specimens were examined for EGFR mutation by the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PCR) clamp method.[18] Briefly, genomic DNA fragments containing mutation hot spots of the EGFR gene were amplified via PCR in the presence of a peptide nucleic acid clamp primer synthesized from a peptide nucleic acid with a wild-type sequence. This method leads to preferential amplification of the mutant sequence, which is then detected by a fluorescent primer that incorporates locked nucleic acids to increase the specificity. As a result, the mutant EGFR sequence is detected in specimens that contain 100 to 1000 excess copies of wild-type EGFR sequence. The sensitivity and specificity of the peptide nucleic acid-locked nucleic acid PCR clamp method are 97% and 100%, respectively.

Drug Administration

Gefitinib was administered orally once a day at a dose of 250 mg. Patients continued to receive gefitinib until progression of disease, occurrence of intolerable severe toxicity, or withdrawal of consent. When severe toxicity was observed, patients were allowed to receive a reduced dose of gefitinib in accordance with the protocol.

Treatment Assessment

Complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) were determined based on RECIST version 1.0. The primary end point of this study was overall objective response rate (ORR), which was the rate of patients with CR + PR; secondary end points were PFS, overall survival (OS), and toxicities. Computer

tomography (CT) scans were taken every month until CR or PR was observed. CR and PR required confirmation via reassessment no earlier than 4 weeks after the first assessment meeting the criteria for response. After the confirmation, CT scans were taken every other month until PD was observed. The CT films of all patients were extramurally reviewed for confirmation of response. PFS was defined as the time from the date of randomization to the first observation of disease progression or death. OS was defined as the time from the date of randomization to the date of death or the most recent follow-up. Toxicities were evaluated according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 3.0.

Statistical Consideration

Sample size was determined using the data as follows. Response rates greater than 70% had been previously observed in nonage-restricted patients with EGFR-mutated NSCLC.[15] Meanwhile, clinical studies with elderly patients that investigated the efficacy of first-line chemotherapies in Japan showed ORR of 28% to 55%.[7,19] Thus, we assumed that an ORR of more than 55% was clinically useful, whereas an ORR of less than 30% was not clinically useful. With $\alpha = 0.05$ and $\beta = 0.1$, the number of patients required was 27. Allowing 10% loss in follow-up, a total of 30 patients were planned for enrollment.

All enrolled patients were evaluated for efficacy of received regimen. All patients treated with gefitinib, even for a short period of time, were entered into safety analysis.

RESULTS

Patient Characteristics

Between January 2008 and May 2009, a total of 31 patients were enrolled. Baseline characteristics are described in Table 1. The median age at the time of enrollment was 80.3 years (range, 75–89 years); 52% of the patients were over the age of 80. Of the 31 patients enrolled, 25 (81%) were women and 2 (6%) had a PS of 2. Histological types were all adenocarcinoma except for one adenosquamous carcinoma. There were 7 patients (23%) with stage IIIB, 22 (71%) with stage IV, and 2 (6%) with postoperative recurrence.

Efficacy

The ORR was 74.2% (95% confidence interval [CI], 57.9%–90.5%); one patient had CR, and 22 patients had PR. Five of the remaining 8 patients (16.1%) had SD, with the resulting disease control rate (CR + PR + SD) reaching 90.3% (Table 2). This result attained the primary end point by a wide margin. The median follow-up period at the time of analysis was 27.5 months. Of all 31 patients enrolled, 15 (48.3%) were alive and free from progression for at least 6 months. The median PFS was 12.1 months (Fig. 1*A*), the 1-year OS was 83.9% (95% CI, 70.2%–97.6%), and 2-year OS was 58.1% (95% CI, 45.2%–70.9%). At the data cutoff point (December 2010), 13 patients (41.9%) had died, and the median OS was 33.8 months (Fig. 1*B*).

TABLE 1. Character

	N = 31	(%)
Sex		
Women	6	19
Men	25	81
Age		
Mean (SD)	80.3	(4.1)
Range	75–89	
Smoking status		
Nonsmoker	23	74
Smoker	8	26
Performance status		
0	16	55
1	13	39
2	2	6
Stage		
IIIB	7	23
IV	22	71
Postop	2	6
Histology		
Adenocarcinoma	30	97
Adenosquamous	1	3

Safety and Toxicity

Toxicity data for all 31 patients are presented in Table 3. Nine patients (29%) had a grade 3 adverse event (AE); 1 had a grade 5 AE ILD, and died of respiratory failure. The most common hematologic AE was elevation of transaminases; grade 3 to 4 elevation occurred in three patients (19%). The most common nonhematologic AEs were rash in 21 patients (71%), diarrhea in 10 patients (32%), and appetite loss in 9 patients (29%). Dose reduction was seen in 14 patients (45%). Incidence and severity of AEs were acceptable and comparable with previous reports.[13–15]

Treatment After Progression of Disease

Patient management after the protocol treatment was retrospectively investigated. Any treatment was allowed after confirmation of PD. Gefitinib was continued in 10 of 20 patients confirmed to have PD. Three patients were treated with monotherapies of cytotoxic agents, including vinorelbine, gemcitabine, or docetaxel, and one patient was given

TABLE 2. Response Rate of Treatment With Gefitinib

Response	N = 31	(%)
CR	1	3
PR	22	71
Stable disease	5	16
Progressive disease	3	10
Overall response rate (CR + PR)	23	74
95% confidence interval		(57.9–90.5)

CR, complete response; PR, partial response.

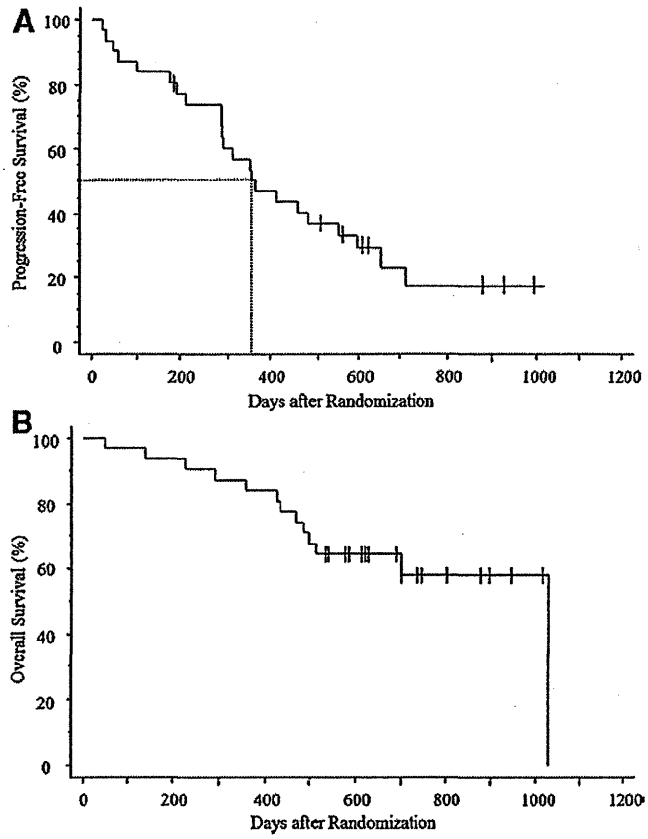

FIGURE 1. Progression-free survival and overall survival. Kaplan–Meier curves for progression-free survival are shown for the progression-free survival population (*A*), and Kaplan–Meier curves for overall survival are shown in (*B*). In (*A*) and (*B*), tick marks indicate patients for whom data were censored.

TABLE 3. Safety—Hematologic and Nonhematologic Toxicity

	NCI-CTC Grade					Grade
	1	2	3	4	5	3–4 (%)
Hematologic adverse events						
Leukocytopenia	2	1	0	0	0	0
Neutropenia	0	1	0	0	0	0
Anemia	6	4	0	0	0	0
Thrombocytopenia	2	1	0	0	0	0
AST/ALT	7	2	6	0	0	19
T-Bil	3	1	0	0	0	0
Creatinine	5	1	0	0	0	0
Hyperkalemia	7	0	0	0	0	0
Nonhematologic adverse events						
Pneumonitis	0	0	0	0	1[a]	3
Rash	12	10	1	0		3
Nail change	4	2	0	0		0
Stomatitis	3	0	0	0		0
Alopecia	3	0	0	0		0
Appetite loss	7	2	1	0		3
Nausea/vomiting	1	0	0	0		0
Diarrhea	9	2	1	0		3
Constipation	2	0	0	0		0
Fatigue	4	1	0	0		0

NCI-CTC, National Cancer Institute Common. Terminology Criteria; AST, androgen suppression therapy; ALT, alanine aminotransferase; T-Bil, total bilirubin.
[a]Treatmant-related death.

erlotinib. No patient was treated with platinum doublets. Six patients did not receive any second-line treatment.

DISCUSSION

This is the first study targeting elderly patients with EGFR-mutated NSCLC. In this study, gefitinib displayed remarkable efficacy without increased toxicity.

We have previously reported a single-arm phase II study in which gefitinib was administered to frail patients with poor PS or elderly patients who were unfit to undergo treatment with cytotoxic agents.[20] In that study, the patients enrolled were 20 to 74 years old with a PS of 3 to 4, 75 to 79 years old with a PS of 2 to 4, and aged 80 years or older (super-elderly) with a PS of 1 to 4. Patients older than 74 years of age accounted for 39% of the total enrolled patients but, nevertheless, OS was 17.8 months (Table 4). The current study strengthened the conclusion of the previous one and provided more information with respect to the efficacy of gefitinib in elderly NSCLC patients with EGFR mutation.

We defined elderly patients as those who were 75 years old and older. Many studies and subgroup analyses were performed by considering elderly cases as 70 years of age or older, especially in Western countries. We have regarded patients aged 70 to 75 years as being treatable with platinum-based chemotherapy. In fact, patients in this age group were enrolled in the NEJ002 study and were able to withstand treatment with platinum doublet. Accordingly, we excluded this group of patients from enrollment in the present study. Considering the aging of population structures and the increased longevity in Japan, we thought that the candidate selection for this study was reasonable.

Currently, in elderly patients, single-agent chemotherapy with a third generation agent (vinorelbine, gemcitabine, or taxanes) is the recommended approach according to the American Society of Clinical Oncology guidelines.[2–7] Gefitinib, which is considered minimally toxic, is often selected for the treatment of advanced NSCLC in elderly patients. Crino et al. performed a randomized phase II study (Gefitinib Versus Vinorelbine in Chemotherapy-Naïve Elderly Patients With Advanced Non-Small-Cell Lung Cancer [INVITE]) of gefitinib versus vinorelbine treatment in 196 chemotherapy-naive unselected elderly patients.[21] There were no statistical differences between gefitinib and vinorelbine in terms of PFS, OS, and ORR. Their study showed obviously lower efficacy of gefitinib in nonselected patients, as compared with the results shown from our study of EGFR-mutated patients.[22–24] These differences in effectiveness among studies highlight the importance of selection of patients by EGFR mutation analysis when administrating gefitinib. Furthermore, in another study of gefitinib treatment in Japanese patients aged

TABLE 4. Pivotal Clinical Trials of Cytotoxic Agents or EGFR-TKIs in Elder Patients With NSCLC and Recent Trials of Gefitinib in Patients selected by EGFR Mutation

Trial	Treatment	*n*	ORR (%)	PFS (mo)	MST (mo)	*p* Value
Cytotoxic agent in unselected elder patients						
ELVIS[3]	VNR	76	19.7		6.4	0.04
	BSC	78	—		4.8	
MILES[5]	VNR + GEM	232	21	4.1	6.9	NS
	GEM	233	16	4.4	6.5	
	VNR	233	18	4.4	8.3	
WJTOG9904[7]	DTX	89	22.7	5.5	14.3	$p = 0.138$
	VNR	91	9.9	3.1	9.9	
EGFR-TKI in unselected elder patients						
Ebi N.[25]	Gefitinib	49	25	4	10	—
Crino L.[21]	Gefitinib	97	3.1	2.7	5.9	NS
	VNR	99	5.1	2.9	8.0	
Jackman D. M.[27]	Erlotinib	80	10	3.5	10.9	—
Chen Y. M.[28]	Erlotinib	57	22.8	4.6	11.7	$p = 0.70$
	VNR	56	8.9	2.5	9.3	
EGFR-TKI in selected younger patients						
WJTOG3405[14]	Gefitinib	86	62.1	9.2	Immature	$p < 0.001$ (PFS)
	CDDP + DTX	86	32.2	6.3		
NEJ002[15]	Gefitinib	114	73.7	10.8	30.5	$p < 0.001$ (PFS)
	CBDCA + PTX	110	30.7	5.4	23.6	
EGFR-TKI in selected elder patients (current study)						
NEJ003	Gefitinib	31	74.2	12.1	33.8	—

ELVIS, Elderly Lung Cancer Vinorelbine Italian Study; MILES, Multicenter Italian Lung Cancer in the Elderly Study; ORR, overall response rate; PFS, progression-free survival; MST, median survival time; VNR, vinorelbine; BSC, best supportive care; NS, not significant; GEM, gemcitabine; DTX, docetaxel; EGFR-TKI, epidermal growth factor receptor–tyrosine kinase inhibitors; CDDP, cisplatin; CBDCA, carboplatin; PTX, paclitaxel.

75 or older, which included about 40% of the patients who were examined for EGFR mutations and 14% of the patients with EGFR-mutated tumors, the response rate was only 25%.[25] Meanwhile, there have been a few studies of treatment for elderly unselected patients with erlotinib, which is supposed to be more toxic than gefitinib as the administered dose was set near the maximum tolerance dose.[26,27] The response rates in these studies were 10% or less, which were similar to those from the gefitinib studies conducted in Western populations (Table 4). In the other Asian study, erlotinib was compared with vinorelbine treatment in patients aged 70 or older.[28] That study demonstrated that erlotinib yielded a higher response rate and PFS than vinorelbine. The percentage of mutation-positive patients was 30% of those who were examined for EGFR mutations in the erlotinib group. This high proportion might have contributed to the better results of the erlotinib group. The treatment of unselected NSCLC patients with erlotinib was also as ineffective as with gefitinib. Efficacy results in patients selected by EGFR mutation in the current study were substantially superior to those observed in the studies of gefitinib or erlotinib with unselected cases. Surprisingly, the median PFS and 2 year-survival rate here were comparable with results obtained in NEJ002 (12.3 versus 10.8 months, 58% versus 61%, respectively) despite the limited enrollment of an elderly population in this study. These two studies, namely NEJ002 and NEJ003, have very similar backgrounds as they were performed during almost the same time period at identical institutions. It was suggested that gefitinib displayed similar efficacy in elderly patients when compared with their younger counterparts (Table 4). Although the current phase II study could not verify whether gefitinib prolonged PFS in elderly patients in comparison with younger patients, gefitinib might still prove to be the most suitable agent for elderly patients with EGFR-mutated NSCLC.

Elderly patients generally have more comorbidities and lower organ function than younger patients. Treatment-related toxicity in the elderly is a more significant issue than for younger patients. A subgroup analysis of BR.21 showed that elderly patients treated with erlotinib displayed similar efficacy with respect to survival and quality of life as their younger counterparts but experienced greater toxicity.[27] In the current study, toxicity was generally mild and predictable. Rash, diarrhea, and elevation of transaminase were observed frequently, similar to other studies with EGFR-TKIs. The single case of treatment-related death that occurred in our study was because of ILD, although this condition was not found in other patients. The frequency of ILD in the current study was comparable with that previously reported in Japan. Unfortunately, this patient did not respond to treatment with a large dose of corticosteroid, which is generally used for such conditions.[17,29] Advanced age and smoking, preexisting ILD, and poor performance status have been reported as risk factors for ILD during treatment with gefitinib.[17] Elderly patients treated with EGFR-TKIs should be monitored with further caution for ILD. On the whole, gefitinib was found to be a well-tolerated therapy for elderly patients with mutated NSCLC.

In conclusion, first-line gefitinib treatment is highly effective with acceptable toxicity for elderly patients with advanced NSCLC harboring EGFR mutations. Together with our previous studies (NEJ001, NEJ002), gefitinib is shown to be an ideal therapy for all types of NSCLC patients with EGFR mutation.

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Clinical Cancer Research

Predictive Biomarkers and Personalized Medicine

A Prospective PCR-Based Screening for the *EML4-ALK* Oncogene in Non–Small Cell Lung Cancer

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Abstract

Purpose: *EML4-ALK* is a lung cancer oncogene, and ALK inhibitors show marked therapeutic efficacy for tumors harboring this fusion gene. It remains unsettled, however, how the fusion gene should be detected in specimens other than formalin-fixed, paraffin-embedded tissue. We here tested whether reverse transcription PCR (RT-PCR)-based detection of *EML4-ALK* is a sensitive and reliable approach.

Experimental Design: We developed a multiplex RT-PCR system to capture *ALK* fusion transcripts and applied this technique to our prospective, nationwide cohort of non–small cell lung cancer (NSCLC) in Japan.

Results: During February to December 2009, we collected 916 specimens from 853 patients, quality filtering of which yielded 808 specimens of primary NSCLC from 754 individuals. Screening for *EML4-ALK* and *KIF5B-ALK* with our RT-PCR system identified *EML4-ALK* transcripts in 36 samples (4.46%) from 32 individuals (4.24%). The RT-PCR products were detected in specimens including bronchial washing fluid ($n = 11$), tumor biopsy ($n = 8$), resected tumor ($n = 7$), pleural effusion ($n = 5$), sputum ($n = 4$), and metastatic lymph node ($n = 1$). The results of RT-PCR were concordant with those of sensitive immunohistochemistry with ALK antibodies.

Conclusions: Multiplex RT-PCR was confirmed to be a reliable technique for detection of *ALK* fusion transcripts. We propose that diagnostic tools for *EML4-ALK* should be selected in a manner dependent on the available specimen types. FISH and sensitive immunohistochemistry should be applied to formalin-fixed, paraffin-embedded tissue, but multiplex RT-PCR is appropriate for other specimen types. *Clin Cancer Res; 1–8. ©2012 AACR.*

Introduction

An oncogenic fusion between the echinoderm microtubule-associated protein–like 4 gene (*EML4*) and the anaplastic lymphoma kinase gene (*ALK*) was discovered by functional screening with a non–small cell lung cancer (NSCLC) specimen (1). *EML4* and *ALK* are located within a short distance (~12 Mbp) of each other on the short arm of human chromosome 2, and a small inversion involving the 2 loci is responsible for generation of the *EML4-ALK* fusion in lung cancer. The EML4-ALK tyrosine kinase undergoes constitutive dimerization through a coiled-coil domain within EML4, resulting in kinase activation and conferring potent transforming ability (2, 3). Transgenic mice expressing *EML4-ALK* in lung alveolar cells develop multiple adenocarcinoma nodules soon after birth, but treatment with an ALK inhibitor results in the rapid clearance of such nodules, confirming the addiction of EML4-ALK–positive tumors to the kinase activity of the fusion protein (4). The therapeutic efficacy of ALK inhibitors has been confirmed in other transgenic mice expressing EML4-ALK (5).

Several ALK inhibitors have already entered clinical trials or are under preclinical development (6–10). Marked therapeutic efficacy of one such compound, crizotinib, has been described in patients with NSCLCs positive for *EML4-ALK*, with an overall response rate of 57% (7), and crizotinib was recently approved as a therapeutic drug by the U.S. Food

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

The nucleotide sequence of the novel *EML4-ALK* variant cDNA from patient J-#189 has been deposited in the DDBJ/EMBL/GenBank databases under the accession number AB663645.

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Translational Relevance

The recent approval of an ALK inhibitor by the U.S. Food and Drug Administration has rendered urgent the development of a diagnostic scheme for tumors harboring *ALK* fusion genes. Whereas FISH is effective for analysis of formalin-fixed, paraffin-embedded (FFPE) tissue, how to test other types of specimen remains unsettled. We conducted a prospective, nationwide screening for *EML4-ALK*– or *KIF5B-ALK*–positive lung carcinomas in Japan with the use of a newly developed multiplex reverse transcription (RT)-PCR system. Various subtypes of *EML4-ALK* cDNA were identified in 36 of 808 specimens with adequate RNA quality. The RT-PCR results were concordant with those of immunohistochemistry, and *EML4-ALK* PCR products were detected in independent specimens from the same individuals. As far as we are aware, our study represents the first prospective RT-PCR–based screening for *EML4-ALK*, and it shows that multiplex RT-PCR is reliable for detection of the fusion gene in non-FFPE specimens.

and Drug Administration within a remarkably short period after target discovery (3, 11).

The failure of crizotinib treatment in individuals without oncogenic *ALK* fusions (12) and an adverse effect of treatment with gefitinib on the prognosis of patients with NSCLCs who do not harbor mutations of the EGF receptor (*EGFR*) gene (13) both suggest that ALK inhibitors should be administered only to patients positive for oncogenic ALK proteins. FISH-based detection of *ALK* rearrangements has proved to be of diagnostic use in the trials with crizotinib (7). Furthermore, detection of ALK proteins by sensitive immunohistochemistry (IHC) has been described (14, 15), and one such immunohistochemical screening approach resulted in the identification of another oncogenic ALK fusion, KIF5B-ALK (14). However, a substantial proportion of patients attending clinics are diagnosed with lung cancer on the basis of pathologic analysis of bronchial lavage fluid, pleural effusion, or sputum. Given that these specimens are not always suitable for the preparation of formalin-fixed, paraffin-embedded (FFPE) tissue required for FISH or IHC, individuals who are diagnosed solely by analysis of such specimens cannot receive EML4-ALK tests. To allow the sensitive detection of *EML4-ALK* and *KIF5B-ALK* in such specimens, we have now developed a multiplex reverse transcription (RT)-PCR system that captures the 2 *ALK* fusions, and we have tested its reliability as a diagnostic tool in our large-scale prospective cohort.

Materials and Methods

Prospective collection of NSCLC specimens

During February to December of 2009, we collected a total of 916 lung cancer specimens from 853 independent patients through our multicenter, nationwide networks in Japan. All specimens but resected tumors were mixed with RLT buffer (Qiagen) immediately after sampling, a step that markedly inhibits RNA degradation for up to 3 days at room temperature (data not shown). Resected tumor samples were snap-frozen and stored at −80°C until extraction of RNA and DNA. Portions of the samples were sent to Jichi Medical University (Tochigi, Japan) for multiplex RT-PCR analysis of *EML4-ALK* and *KIF5B-ALK* fusions and to Saitama Medical University (Saitama, Japan) for peptide nucleic acid–locked nucleic acid (PNA-LNA) PCR clamp analysis of *EGFR* mutations (16). All specimens were confirmed by pathologic analysis to contain malignant cells. More than half of the specimens were collected through the North-East Japan Study Group network according to the NEJ004 protocol. The study was approved by the Institutional Review Board of each participating center, and written informed consent was obtained from each study subject. All statistical analysis was conducted with 2-sided tests, and a $P < 0.05$ was considered statistically significant.

Clinicopathologic features of EML4-ALK–positive NSCLC

The clinicopathologic features of patients with *EML4-ALK*–positive or -negative tumors in our cohort are summarized in Table 1 and Supplementary Table S1. Consistent with previous observations, *EML4-ALK*–positive patients were significantly younger than those without *EML4-ALK* ($P < 0.001$, Student' t test) and were enriched in never or light smokers ($P < 0.001$, Fisher exact test). Our data also indicated that *EML4-ALK*–positive tumors are more likely to occur in women than in men ($P < 0.001$, Fisher exact test). In the present cohort, *EML4-ALK* was detected only in lung adenocarcinoma ($P < 0.001$, Fisher exact test), for which the fusion-positive rate was 6.11%.

A total of 718 specimens were screened for *EGFR* mutations, with such mutations being detected in 171 cases (23.8%). Whereas most *EML4-ALK*–positive tumors did not harbor *EGFR* mutations ($P = 0.002$, Fisher exact test), we did detect one tumor doubly positive in this regard. *EML4-ALK* and *EGFR* mutations are largely mutually exclusive (17, 18), but, importantly, such exclusiveness may not be absolute (19). Given that the presence of *EML4-ALK* and *EGFR* mutations in our doubly positive patient was examined with cells isolated from bronchial washing fluid, which was the only available specimen for molecular analysis in this individual, we were not able to determine whether there was a genuinely double-positive tumor in the lung or there were multiple independent tumors each positive for *EML4-ALK* or mutated *EGFR*.

We also attempted to examine the mutation status of *KRAS* among our 32 cases positive for *EML4-ALK*. We were able to sequence *KRAS* cDNAs for 26 of these patients, none of whom showed *KRAS* alterations (data not shown), confirming the mutual exclusivity of *EML4-ALK* and *KRAS* mutations (17, 20, 21).

Quality assessment of samples

Complementary DNA prepared from the specimens was first subjected to RT-PCR analysis with primers (5′-

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Table 1. Characteristics of subjects positive for *EML4-ALK* by the RT-PCR diagnostic system.

Identification number	Sex/ age, y	Pathologic classification	Specimen type	*EML4-ALK* variant	Smoking history (pack-years)	TNM classification	Clinical stage	iAEP	*EGFR* mutation	*KRAS* mutation
J-#1	M/27	Adenocarcinoma	Sputum (2 different time points)	E13;A20	0	cT4N3M1	4	+	–	–
J-#4	F/39	Adenocarcinoma	Metastatic lymph node	E20;A20	NA	cTxN3M1	4	+	–	–
J-#7	M/74	Adenocarcinoma	Bronchial washing fluid	E13;A20	50	cT4N3M1	4	ND	–	–
J-#12	F/56	Adenocarcinoma	Resected tumor	E13;A20	0	cT1N0M0	1A	+	–	ND
J-#53	M/48	Adenocarcinoma	Tumor biopsy/sputum	E13;A20	0	cT3N2M1	4	+	–	–
J-#88	F/37	Adenocarcinoma	Pleural effusion	E13;A20	0	cT4N3M1	4	ND	–	–
J-#127	F/49	Adenocarcinoma	Tumor biopsy	E6a/b;A20	0.9	cT1N2M1	4	+	–	–
J-#189	F/37	Adenocarcinoma	Resected tumor	E14::ins2; ins56A20	0	cT2N1M1	4	+	–	–
J-#210	F/37	Adenocarcinoma	Resected tumor	E13;A20	0	cT4N2M1	4	ND	–	–
J-#215	F/61	Adenocarcinoma	Sputum	E13;A20	82	cT4N2M1	4	ND	–	–
J-#330	M/72	Adenocarcinoma	Pleural effusion/resected tumor (2 different regions)	E13;A20	0	cT4N1M1	4	+	–	–
J-#350	F/53	Adenocarcinoma	Pleural effusion	E13;A20	0	cT4N2M0	3B	ND	–	–
J-#378	F/78	Adenocarcinoma	Resected tumor	E13;A20	0	cT1N0M0	1A	ND	–	–
J-#385	F/80	Adenocarcinoma	Pleural effusion	E6a/b;A20	0	cT4N3M1	4	ND	–	–
J-#391	F/55	Adenocarcinoma	Tumor biopsy	E13;A20	16.5	cT2N2M1	4	+	–	ND
J-#392	F/38	Adenocarcinoma	Tumor biopsy	E13;A20	34	cT4N2M0	3B	+	–	ND
J-#393	F/42	Adenocarcinoma	Tumor biopsy	E13;A20	0	cT4N3M1	4	–	–	ND
J-#409	F/35	Adenocarcinoma	Tumor biopsy	E13;A20	0	cT4N0M0	3B	+	–	–
J-#422	M/69	Adenocarcinoma	Tumor biopsy	E6a/b;A20	0	cT2N2M0	3A	ND	–	–
J-#450	F/30	Adenocarcinoma	Bronchial washing fluid	E6a/b;A20	0	cT4N2M1	4	+	–	–
J-#530	F/55	Adenocarcinoma	Bronchial washing fluid	E13;A20	0	cT1N1M1	4	+	+	ND
J-#646	F/36	Adenocarcinoma	Bronchial washing fluid	E6a/b;A20	0	cT2N3M0	3B	ND	–	–
J-#657	F/62	Adenocarcinoma	Bronchial washing fluid	E13;A20	15	cT4N2M0	3B	ND	–	–
J-#759	F/32	Adenocarcinoma	Resected tumor	E13;A20	12	cT1N0M0	1A	ND	–	–
J-#771	M/32	Adenocarcinoma	Tumor biopsy	E6a/b;A20	15	cT1N3M1	3B	ND	–	–
J-#817	M/33	Adenocarcinoma	Pleural effusion	E13;A20	0	cT2N1M1	4	ND	–	–
J-#848	M/57	Adenocarcinoma	Bronchial washing fluid	E18;E20	0	cT4N2M0	3B	ND	–	–
J-#887	F/32	Adenocarcinoma	Bronchial washing fluid	E6a/b;A20	0	cTxN3M1	4	ND	–	ND
J-#927	M/36	Adenocarcinoma	Bronchial washing fluid	E6a/b;A20	30	cT4N3M1	4	–	–	–
J-#928	F/71	Adenocarcinoma	Bronchial washing fluid	E6a/b;A20	0	cT4N3M1	4	ND	–	–
J-#996	M/52	Adenocarcinoma	Bronchial washing fluid	E6a/b;A20	0	cT3N3M0	3B	ND	–	–
J-#1001	F/32	Adenocarcinoma	Bronchial washing fluid	E13;A20	6.5	cT2N2M1	4	+	–	–

Abbreviations: F, female; M, male; NA, not available; ND, not determined.

CTGTGGAGGCTGAACTGGATC-3′ and 5′-TCATCAACAAGCTCCACGGTG-3′) specific for the human ribonuclease P (RNase P) gene (GenBank accession number NM_005837). Given that we previously showed that the abundance of RNase P mRNA is similar to that of *EML4-ALK* mRNA in NSCLCs (data not shown), we used the successful amplification of RT-PCR products for RNase P as a threshold for selection of specimens for further analysis. Exclusion of small cell lung cancer specimens and filtering on the basis of RNase P mRNA abundance resulted in the isolation of 808 specimens of primary NSCLCs obtained from 754 individuals.

As shown in Supplementary Fig. S1, bronchial washing fluid, including bronchoalveolar lavage fluid and washing fluid for the brush, needle, forceps, and other implements used in bronchoscopy, constituted 66.3% of the 808 eligible samples, with the remaining specimens including pleural effusion (12.8%); surgically resected tumor (7.05%); sputum (4.33%); tumor biopsy tissue including that obtained by transbronchial lung biopsy and transbronchial needle aspiration (3.71%); peripheral blood (3.71%); cardiac effusion, spinal fluid, or ascites (1.36%); and metastatic lesions of NSCLCs (0.74%).

Multiplex RT-PCR analysis of EML4-ALK and KIF5B-ALK

Each specimen (with the exception of resected tumors) was mixed with an equal volume of RLT buffer at the Institute at which it was harvested. The resulting mixture was sent to Jichi Medical University, where DNA and RNA were extracted with the use of an automated BioRobot EZ1 workstation (Qiagen). The isolated RNA was subjected to RT with a ReverTra Ace qPCR RT kit (Toyobo), and the resulting cDNA was subjected to PCR for 50 cycles of incubation at 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute with AmpliTAQ Gold DNA polymerase (Applied Biosystems) and with 2 μmol/L of each of the following

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primers: F-1, 5′-GCTTTCCCCGCAAGATGGACGG-3′; F-2, 5′-TACCAGTGCTGTCTCAATTGCAGG-3′; F-3, 5′-GTGCAGTGTTTAGCATTCTTGGGG-3′; F-4, 5′-AGCTACATCACACACCTTGACTGG-3′; F-5, 5′-TCAAGCACATCTCAAGAGCAAGTG-3′; F-6, 5′-ATCCTGCGGAACACTATTCAGTGG-3′; F-7, 5′-GACAGTTGGAGGAATCTGTCGATG-3′; F-8, 5′-CAGCTGAGAGAGTGAAAGCTTTGG-3′; and R-1, 5′-TCTTGCCAGCAAAGCAGTAGTTGG-3′. All PCR products were subjected to Sanger sequencing to confirm the presence of *EML4-ALK* or *KIF5B-ALK* cDNA.

Results

Multiplex RT-PCR system

In addition to the original *EML4-ALK* fusion cDNA in which exon 13 of *EML4* is fused to exon 20 of *ALK* in an in-frame manner (designated the E13;A20 variant by analogy with karyotype nomenclature; see http://atlasgeneticsoncology.org/Tumors/inv2p21p23NSCCLungID5667.html), 14 different variants of *EML4-ALK* have been described (1, 14, 21–27). Seven exons of *EML4* are theoretically capable of in-frame fusion with exon 20 of *ALK* (Fig. 1A), and all but the E1;A20 variant would be expected to produce an oncogenic EML4-ALK protein, given that the coiled-coil domain encoded by exon 2 is required for constitutive dimerization of EML4-ALK. In addition, 6 different exons of *KIF5B* are theoretically capable of in-frame fusion with exon 20 of *ALK* (Fig. 1A).

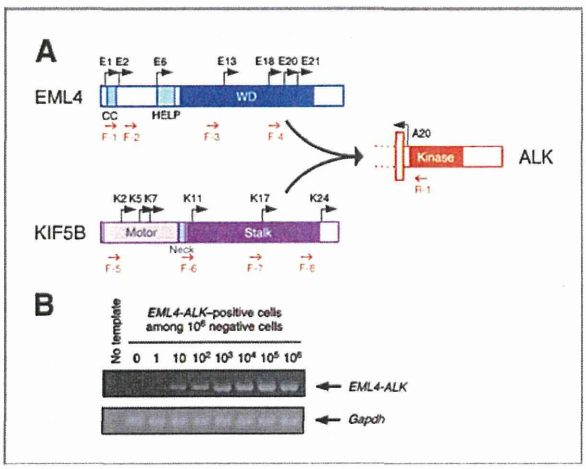

Figure 1. Multiplex RT-PCR system for detection of *EML4-ALK* and *KIF5B-ALK*. A, schematic representation of the structure of EML4, KIF5B, and ALK proteins. The positions of exons (E for *EML4* and K for *KIF5B*) theoretically capable of fusing in-frame to exon 20 (A20) of *ALK* are indicated by arrows. The positions of 8 forward primers (F-1 to F-8) and 1 reverse primer (R-1) for PCR are also indicated below the corresponding proteins. EML4 contains a coiled-coil domain (CC), a hydrophobic EMAP-like protein domain (HELP), and WD repeats (WD). KIF5B consists of an amino-terminal ATP-dependent motor domain, a neck region, and a stalk region. B, various numbers (0 to 1×10^6) of *EML4-ALK* (E13;A20)–positive BA/F3 cells (1) were mixed with a fixed number (1×10^6) of *EML4-ALK*–negative BA/F3 cells, and each mixture was analyzed with our multiplex RT-PCR system. A cDNA for mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was also amplified by PCR as an internal control with the primers 5′-TGTGTCCGTCGTGGATCTGA-3′ and 5′-CCTGCTTCACCACCTTCTTGA-3′.

To detect any such *EML4-ALK* or *KIF5B-ALK* fusion mRNAs, we developed a multiplex RT-PCR system. We had previously screened our archive of frozen tumors by RT-PCR analysis with 2 forward primers targeted to *EML4* and 1 reverse primer targeted to *ALK* (24), but such PCR conditions resulted in the amplification of products as large as ~1,300 bp for some variants. In this prospective study, we were faced with the analysis of a large number of samples with different levels of RNA quality. If the size of PCR products varied substantially among different *EML4-ALK* or *KIF5B-ALK* variants, some variants with large PCR products might not be amplified efficiently from specimens with low RNA quality. To be able to diagnose all possible fusions even with such samples, we therefore designed 4 forward primers for each of *EML4* and *KIF5B* so that the size variation among all possible RT-PCR products is minimal (Fig. 1A). This new multiplex system faithfully detected all known fusion variants from *EML4-ALK*–positive specimens in our previous archive of NSCLCs (data not shown).

To examine the sensitivity of our RT-PCR system, we mixed *EML4-ALK*–expressing BA/F3 cells (0 to 1×10^6) with *EML4-ALK*–negative cells (1×10^6) and then subjected them to RT-PCR analysis. A fusion cDNA was readily identified even with 10 positive cells (0.001%) among 1×10^6 negative cells (Fig. 1B), showing the high sensitivity of the RT-PCR system.

To confirm the potential of our RT-PCR–based system, we compared it with a sensitive immunohistochemical approach and with FISH for the diagnosis of our archive of surgically resected and freshly frozen tumors with high RNA quality. Fifteen NSCLC specimens that previously stained positive by our sensitive immunohistochemical approach, which is based on an intercalated antibody-enhanced polymer (iAEP) method (14), were analyzed by RT-PCR and FISH together with 96 iAEP-negative specimens in a blinded manner. RT-PCR analysis of all these specimens ($n = 111$) yielded a diagnosis identical to that obtained with the iAEP method ($P = 7.3 \times 10^{-19}$, Fisher exact test; data not shown). Analysis of the same sample set by a split FISH assay with Vysis probes (Abbott Laboratories) revealed that all of the iAEP-positive cases showed a rearranged *ALK* locus, whereas one iAEP-negative sample gave a discordant result (negative by iAEP and RT-PCR but positive by FISH; Supplementary Fig. S2). The reason for this discrepant result remains unclear, but the multiple signals obtained with the 3′-*ALK* probe in the FISH analysis are indicative of amplification of the *ALK* gene or its adjacent region. Despite this discrepancy, the RT-PCR and iAEP data were highly concordant with the FISH results ($P = 1.2 \times 10^{-17}$, Fisher exact test). Compared with the iAEP method, therefore, both the sensitivity and specificity of our RT-PCR system were 100%. In comparison with the Vysis FISH, the sensitivity and specificity of RT-PCR were 93.8% and 100%, respectively.

Detection of EML4-ALK

Screening of the 808 eligible specimens with our multiplex RT-PCR system identified positive products in 36 samples (4.46%) obtained from 32 different individuals

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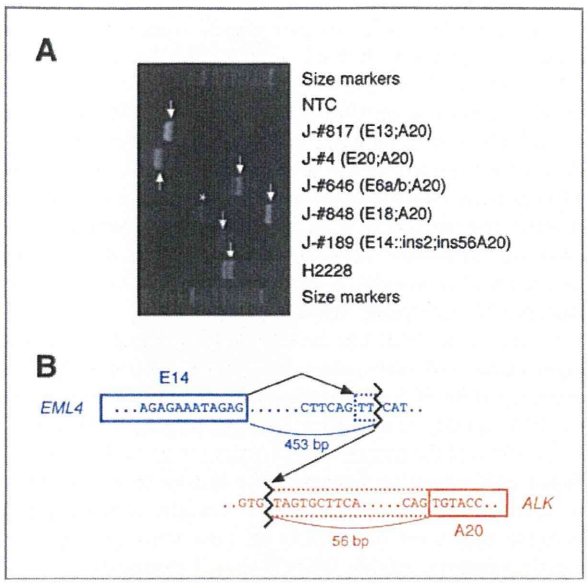

Figure 2. Multiplex RT-PCR detection of *EML4-ALK*–positive NSCLCs. A, RT-PCR products for each of the *EML4-ALK* variants identified in our cohort were separated by agarose gel electrophoresis. RT-PCR products spanning the *EML4-ALK* fusion points are indicated by arrows; the asterisk indicates a nonspecific product. An NSCLC cell line, H2228, harboring the E6a/b;A20 variant of *EML4-ALK* was used as a positive control for the PCR reaction. Size markers include a 50-bp DNA ladder (Invitrogen). NTC, no-template control. B, genomic structure of the fusion point for a novel variant of *EML4-ALK*. Nucleotide sequencing of the genomic PCR and RT-PCR products from patient J-#189 revealed that exon 14 of *EML4* (blue) was spliced to a TT sequence adjacent to the genomic ligation point, with transcription continuing in an in-frame manner into intron 19 and exon 20 of *ALK* (red).

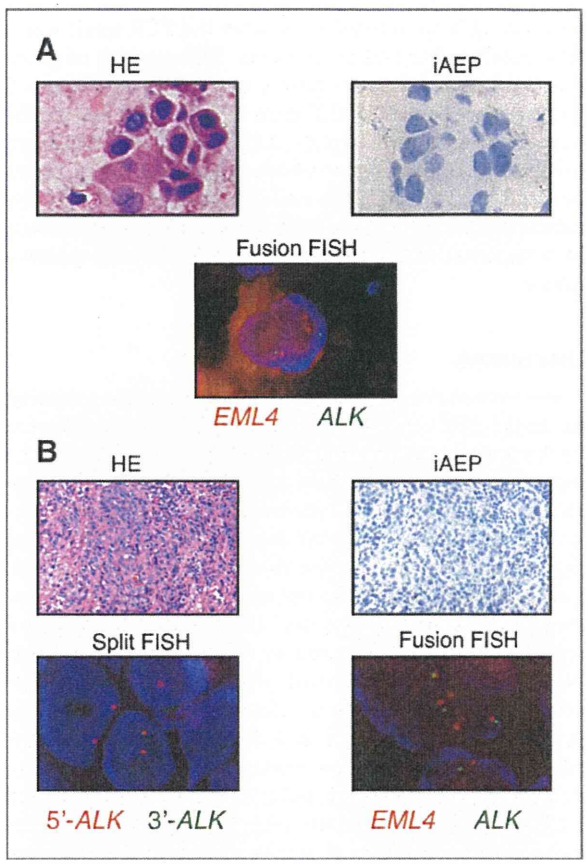

Figure 3. Specimens positive for *EML4-ALK* by RT-PCR but negative by iAEP-based IHC and by FISH. Sections of tumor biopsy specimens for J-#393 tumor (A) and J-#927 (B) were stained with hematoxylin-eosin (HE), subjected to immunohistochemical analysis by the iAEP method, and examined by split or fusion FISH. The color of fluorescence for the probes in each hybridization is indicated below the FISH images. Nuclei are stained blue with 4′,6-diamidino-2-phenylindole (DAPI).

(4.24%; Table 1, Fig. 2A). Nucleotide sequencing of each PCR product identified 19 cases positive for the E13;A20 variant, 10 cases for E6a/b;A20, a single case each for E18;A20, E20;A20, and a novel variant. *EML4-ALK* was detected in a wide range of specimens including bronchial washing fluid ($n = 11$), tumor biopsy ($n = 8$), resected tumor ($n = 7$), pleural effusion ($n = 5$), sputum ($n = 4$), and metastatic lymph node ($n = 1$). We did not detect any *KIF5B-ALK* cDNAs, confirming the rarity of this fusion gene.

Importantly, an E13;A20 product was consistently identified in both of the sputa obtained at different time points from patient J-#1. Likewise, an E13;A20 product was detected in both the tumor biopsy and sputum from patient J-#53 as well as in the pleural effusion and 2 resected tumor specimens from patient J-#330, supporting the reliability of our RT-PCR approach.

Sequence determination for the RT-PCR product from patient J-#189 revealed that exon 14 of *EML4* was fused to exon 20 of *ALK* with an intervening sequence. Genomic PCR analysis of the J-#189 specimen with a forward primer targeted to exon 14 of *EML4* and a reverse primer targeted to exon 20 of *ALK* yielded a specific product, nucleotide sequencing of which revealed that a position 453 bp downstream of *EML4* exon 14 was ligated to a position 56 bp upstream of *ALK* exon 20 (Fig. 2B). In the transcript of this fusion gene, exon 14 of *EML4* is thus spliced to a TT sequence that is located within *EML4* intron 14 and which is directly ligated to intron 19 of *ALK*. This splicing event results in an in-frame fusion between the mRNA sequences derived from *EML4* and *ALK*. Furthermore, a full-length cDNA for this variant, here designated E14::ins2;ins56A20, was isolated by RT-PCR analysis (Supplementary Fig. S3), and the potent transforming ability of the encoded protein was confirmed with an *in vitro* focus formation assay (Supplementary Fig. S4).

Comparison between multiplex RT-PCR and sensitive IHC

Finally, we applied the iAEP method to the *EML4-ALK*–positive cases for which FFPE specimens were also available ($n = 15$). All but 2 cases (J-#393 and J-#927) manifested clear immunoreactivity with antibodies to ALK (Table 1). FISH analysis of these 2 specimens also failed to detect the *EML4-ALK* rearrangements (Fig. 3). Given that genomic DNA was not available for the tumor of patient J-#393, we

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were not able to determine whether the PCR result was a false-positive. For J-#927, however, PCR analysis of genomic DNA with a forward primer targeted to *EML4* exon 6 and a reverse primer to *ALK* exon 20 resulted in the amplification of an approximately 8.8-kbp genomic fragment, nucleotide sequencing of which revealed a fusion event between intron 6 of *EML4* and intron 19 of *ALK* (Supplementary Fig. S5). Isolation of the genomic fusion point thus indicates that J-#927 indeed harbors an *EML4-ALK*–positive tumor.

Discussion

We have conducted a large-scale, prospective screening for *EML4-ALK* with an RT-PCR–based approach. Whereas RNA extraction and cDNA synthesis add extra labor to the diagnostic procedure, certain introns of *EML4* are too large (intron 6 spans >16 kbp, for instance) for reliable amplification by genomic PCR. We therefore adopted RT-PCR as the method for our prospective screening. Specific PCR products were successfully isolated from different types of specimen, even from sputum (J-#1, J-#53, J-#215) and washing fluid of a tumor biopsy needle (J-#530). Multiple positive results obtained with different specimens of the same individuals further reinforce the reliability of our multiplex RT-PCR system as a diagnostic tool for *EML4-ALK*–positive tumors. Importantly, a subset of *EML4-ALK*–positive individuals diagnosed in the present study entered a clinical trial for crizotinib, and the response rate of the evaluable patients ($n = 9$) was 100% with this drug, again verifying the accuracy of our RT-PCR–based diagnosis.

The frequency of *EML4-ALK* in our cohort was 4.24% for all NSCLC cases and 6.11% for lung adenocarcinoma, values similar to those obtained in previous studies (20, 21). However, our prevalence data might be overestimates because the knowledge of mutual exclusiveness for *EML4-ALK* and *EGFR* mutations may have affected patient selection for our specimen collection. Indeed, *EGFR* mutation frequency among our cohort (23.8%) is slightly lower than that (30.9%) determined in a previous large-scale screening in Japan (28).

The clinicopathologic features of patients with *EML4-ALK*–positive tumors determined in the present study are also in agreement with those previously described, with a bias toward a young age, adenocarcinoma histology, and never or light smoking. Whereas a previous large-scale screening for *EML4-ALK* based on FISH did not detect a sex preference for the fusion gene (7), our cohort revealed a significant female preference. Such a sex difference was evident even among individuals below 40 years of age ($P = 0.03$, Fisher' exact test) and among those with an adenocarcinoma histology ($P = 0.005$, Fisher' exact test). Further large-scale studies are warranted to determine whether this uneven sex distribution of *EML4-ALK* is related to particular clinicopathologic features or ethnic groups.

Given that *EML4-ALK* and *EGFR* mutations are almost mutually exclusive and that the fusion gene is enriched in lung adenocarcinoma with an early onset, it should prove to be clinically beneficial to pay special attention to such subsets of patients. Indeed, *EML4-ALK* was detected in 27.7% of *EGFR* mutation–negative adenocarcinomas in individuals of younger than 50 years and in 50.0% of those in individuals of younger than 40 years in our cohort. Given the marked efficacy of ALK inhibitors in patients with *EML4-ALK*–positive NSCLCs (7), however, physicians should not dismiss the diagnosis in other subsets of patients. For example, *EML4-ALK* was even detected in an 80-year-old woman and in another woman with an intense smoking history (82 pack-years; Table 1).

Multiplex RT-PCR has both advantages and disadvantages compared with other techniques. Importantly, the accuracy of RT-PCR–based diagnosis depends markedly on the RNA quality of specimens. In our cohort, for instance, 71 (7.75%) of the initial 916 specimens were excluded from *EML4-ALK* screening because of a failure to obtain PCR products for RNase P (the other 37 samples were excluded because they were not NSCLCs). Low RNA quality thus clearly hampers reliable RT-PCR–based diagnosis.

Also, as expected, there was a large variation in the PCR cycle number required for successful amplification among specimens. In our cohort, 50 cycles of PCR allowed detection of PCR products for all positive cases, but such extensive amplification may also generate nonspecific products (as shown in Fig. 2A). Further optimization of primer sequences or combinations may minimize the generation of such byproducts. Furthermore, whereas our system should be able to capture all in-frame fusions of *ALK* to *EML4* or *KIF5B*, it is not capable in its present form of detecting *ALK* fusions to other partners, such as *KLC1-ALK*, which was recently shown to be present infrequently in NSCLCs (29).

On the other hand, RT-PCR can be readily applied to specimens such as sputum, bronchial washing fluid, or pleural effusion that may not be suitable for preparation of FFPE samples. Whereas the latter 2 specimen types can be used for the preparation of cell blocks suitable for analysis by FISH or IHC, this procedure may not be as widely adopted in the clinic as is FISH or IHC. More importantly, it is difficult to generate cell blocks or FFPE samples from sputum. Our current prospective screening identified 4 *EML4-ALK*–positive sputa of 35 samples (Table 1, Supplementary Fig. S1), showing that sputum is a suitable specimen for RT-PCR analysis. Indeed, sputum was the only available specimen from patient J-#215 both for the diagnosis of NSCLCs and for the detection of *EML4-ALK*. If RT-PCR had not been applied to this patient's sputum, we would not have been able to identify her tumor as positive for *EML4-ALK*, and she would not have had the chance to receive treatment with an ALK inhibitor in Japan.

Furthermore, PCR-based detection of *EML4-ALK* should have a higher analytic sensitivity compared with IHC or FISH (Fig. 1B). Even with sputum obtained from a patient with chronic bronchitis, RT-PCR was able to readily detect *EML4-ALK* at a concentration of 10 positive cells/mL (1). Thus, provided that RNA is not substantially degraded, RT-PCR–based diagnosis is expected to have a strong advantage

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with regard to the detection of low numbers of *EML4-ALK*–positive cells.

Ideally, every NSCLC case should be examined for the presence of *EML4-ALK*, with a sensitive and accurate diagnostic strategy for the oncogenic fusion being essential for the adoption of ALK inhibitors in the clinic. Given the reliable detection of *EML4-ALK* mRNA by multiplex RT-PCR shown in the present study, we propose the following scheme for the comprehensive diagnosis of *EML4-ALK*–positive NSCLCs. For sputum, bronchial lavage fluid, pleural effusion, or other specimens that may not be suitable for the preparation of FFPE tissue, multiplex RT-PCR should be applied to detect *ALK* fusion mRNAs. In contrast, given that FFPE specimens usually have fragmented RNA, they should be subjected to FISH and to sensitive immunohistochemical analysis such as that described previously (14, 15). Furthermore, FISH or IHC can be applied to cell blocks prepared from some non-FFPE specimens. No single technique is therefore able to detect *EML4-ALK* in all types of specimen, and appropriate tests should be chosen on the basis of the specimens available for a given patient.

Disclosure of Potential Conflicts of Interest

H. Mano is the CEO of CureGene Co., Ltd.; has commercial research grant from Illumina, Inc. and Astellas Pharma Inc.; has ownership interest (including patents); and is on the consultant/advisory board of Chugai Pharmaceutical, Astellas Pharma Inc., and Daiichi Sankyo Co., Ltd. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Soda, K. Isobe, A. Inoue, S. Oizumi, Y. Fujita, A. Gemma, Y. Yamashita, K. Takeuchi, H. Miyazawa, T. Tanaka, K. Hagiwara
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Soda, T. Ueno, H. Mano
Writing, review, and/or revision of the manuscript: S. Oizumi, A. Gemma, K. Hagiwara, H. Mano
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Maemondo, K. Takeuchi, K. Hagiwara
Study supervision: H. Mano

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Case report

Miliary brain metastases in 2 cases with advanced non-small cell lung cancer harboring *EGFR* mutation during gefitinib treatment

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ABSTRACT

Here we report 2 cases of non-small cell lung cancer (NSCLC) with sensitive epidermal growth factor receptor (EGFR) gene mutation that developed miliary brain metastases characterized by dementia and disorientation during gefitinib therapy. One patient's therapy was switched from gefitinib to chemotherapy followed by whole brain radiotherapy (WBRT), which resulted in disease progression with coma. Gefitinib reinitiation improved the patient's symptoms. The other patient continued gefitinib during WBRT and achieved complete remission of the miliary metastases and lived 18 months longer. These results suggest that gefitinib concomitant with WBRT is an optional strategy for the treatment of patients with EGFR-mutated NSCLC with miliary metastases to prevent disease flare.

1. Introduction

As compared to standard chemotherapy, gefitinib, which is a tyrosine kinase inhibitor of epidermal growth factor receptor (EGFR-TKI), has demonstrated novel efficacy in patients with advanced non-small cell lung cancer (NSCLC) and a sensitive EGFR mutation [1]. Unfortunately, many patients who experience marked improvement following gefitinib therapy eventually face disease progression due to acquired resistance. The central nervous system (CNS) is a common site of relapse during EGFR-TKI treatment; however, the treatment strategy for such a situation remains unresolved [2]. The present study reports 2 characteristic cases of miliary brain metastasis under a gefitinib treatment regimen that were successfully managed using whole brain radiotherapy (WBRT) and concomitant gefitinib use.

1.1. *Case 1*

A 37-year-old Japanese woman visited our hospital presenting with weight loss and exertional dyspnea. Chest radiography showed bilateral diffuse granular opacity of the lungs and a mass in the right lower lung field (Fig. 1A). Transbronchial biopsy revealed lung adenocarcinoma (stage: cT4N3M1 [PUL, OSS], stage IV; Eastern Cooperative Oncology Group performance status: 4) with deletion of exon 19 of the *EGFR* gene. Gefitinib was administered as first-line treatment, resulting in almost complete response for 8 months. Despite the primary lesion and

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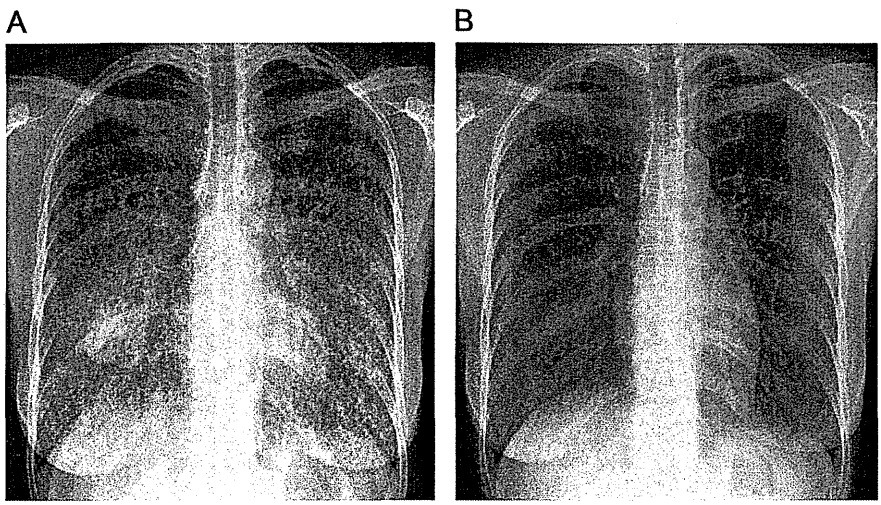

Fig. 1 – Case 1: Chest radiography. A: Bilateral diffuse granular opacity in the lungs and a mass in the right lower lung field. B: Marked improvement in primary lesion and miliary lung metastases, with disease progression to brain and bone metastases.

multiple lung metastases being well controlled (Fig. 1B), further metastases were observed in the brain and bones. Gefitinib therapy was then terminated and a second line chemotherapy with carboplatin and paclitaxel was initiated.

Seven days later, the patient complained of disorientation and difficulty in writing. Her dementia progressed to coma accompanied by mutism within several days of admission. Electroencephalography revealed generalized slowing in the delta and theta ranges. Laboratory results were unremarkable and her serum tested negative for the anti-Hu antibody. Findings of brain magnetic resonance imaging (MRI) with gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA) were unchanged from those of earlier examination (Fig. 2A). Lumbar puncture revealed atypical cells in the cerebrospinal fluid without the typical clinical features of meningitis carcinomatosa.

The patient was given WBRT (30 Gy/10 fractions) on day 16 after chemotherapy with carboplatin and paclitaxel to treat the brain metastases. Despite gradual improvement in consciousness with this treatment, 1 month after WBRT therapy, MRI scanning still showed enhanced miliary nodules in the brain (Fig. 2B); therefore, gefitinib was reinitiated via nasogastric tube on day 43 after chemotherapy. Her family hoped that the gefitinib therapy would improve her condition despite the treatment's possible side effects and ineffectiveness. One month later, the patient's condition improved dramatically and she was again able to speak and eat unaided; subsequent MRI revealed decreased brain metastases (Fig. 2C). She was discharged and spent 10 months at home under gefitinib treatment. The patient's memory disturbances gradually improved and she began to enjoy theatrical performances again. Her overall survival time after diagnosis was 1 year and 8 months.

1.2. *Case 2*

A 64-year-old Japanese woman with dry cough was referred to our hospital because of an abnormality detected on chest radiography by her family doctor. Subsequent chest computed tomography showed a mass in her right lower lung lobe with right pleural effusion and evidence of carcinomatous lymphangitis. Cytological examination of a transbronchial specimen demonstrated lung adenocarcinoma (stage: cT4N0M1 [PUL], stage IV; ECOG PS: 1), with deleted exon 19 of the EGFR gene. She was treated using gefitinib as first-line treatment, which led to a partial response lasting 7 months; however, both T2-weighted and Gd-DTPA-enhanced brain MRI performed as a periodic examination revealed numerous enhanced miliary metastases in the cerebral cortex and basal ganglia unaccompanied by clinical symptoms (Fig. 3A). Because no disease progression was observed in the other lesions, gefitinib therapy was continued and WBRT (36 Gy/12 fractions) was delivered concurrently. Subsequent brain MRI showed complete regression of the multiple brain metastases (Fig. 3B). Gefitinib treatment was maintained for 18 more months. The patient's overall survival time after diagnosis was 2 years and 5 months.

2. Discussion

Miliary brain metastasis, which is a rare form of CNS relapse, is characterized by perivascularly distributed diffuse miliary nodules unaccompanied by intraparenchymal invasion. Madow and Alpers first reported a case of carcinomatous encephalitis with these pathological features in 1951 [3]. The incidence of metastatic brain carcinomas was estimated at 3.8%, and lung adenocarcinoma was identified as the most common primary lesion [4,5]. Clinical symptoms included dementia and disorientation with rare progression to coma. Although establishing a diagnosis using imaging studies is sometimes difficult even in cases accompanied by dementia, MRI with Gd-DTPA is the most effective means of identifying miliary brain metastases [6]. Reports have described miliary brain metastases in some autopsy cases without imaging study abnormalities, indicating that antemortem diagnosis is difficult even after the onset of dementia. Our 2 cases here indicate that the treatment of miliary brain metastases

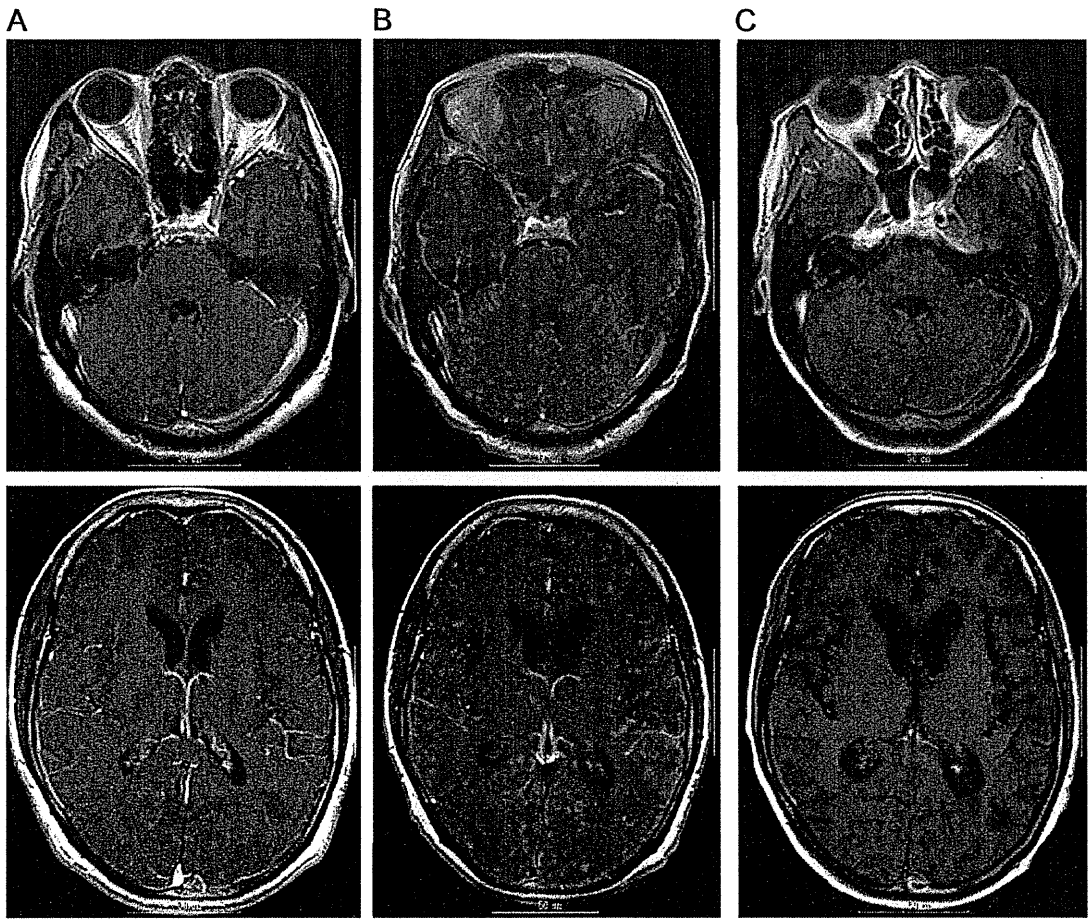

Fig. 2 – Case 1: Brain magnetic resonance imaging (MRI). A: With gadolinium-diethylenetriamine pentaacetic acid-enhanced MRI at 2 levels, the pons (upper) and basal ganglia (lower) showing no significant change after the patient became comatose. B: Multiple enhanced miliary nodules 1 month after radiation therapy. C: Decrease in brain metastatic lesions 2 months after radiation therapy.

without symptoms may be beneficial given this discrepancy between clinical symptoms and imaging findings.

Adenocarcinoma with a miliary pattern of lung metastases has recently been suggested as a distinct subtype that is primarily found in patients who have never smoked and is related to the exon 19 deletion EGFR mutation type [7]. Moreover, Sekine et al. reported a radiographic feature of miliary brain metastases in NSCLC with exon 19 deletion [8]. The 2 patients in the present study also showed adenocarcinoma with exon 19 deletion and miliary brain metastases. Because few reports have suggested an appropriate strategy for the management of such miliary brain metastases, further investigations are needed.

MRI scanning in both cases here showed the typical findings of miliary brain metastases suggested in earlier reports, but the 2 clinical courses were very different. In case 1, the patient demonstrated severe symptoms relating to a CNS disorder–dementia–that had progressed to coma with mutism. In contrast, in case 2, the patient was asymptomatic, and the brain metastases were detected by periodic MRI. Because the progression of CNS metastases quite often compromises a patient's quality of life, periodic MRI examinations may be beneficial for patients, although appropriate patient selection should be investigated further in terms of a cost–benefit balance. Regarding the treatment regimen, patients with miliary brain metastases arising from EGFR-mutated NSCLC might benefit from combined treatment with WBRT and gefitinib as illustrated in case 2, in which the patient's condition was maintained using the same strategy for over 18 months after the onset of miliary brain metastases.

Disease flare after discontinuation of EGFR-TKI has recently been a concern among patients with EGFR-mutated NSCLC [9]. The clinical course in case 1 was considered a typical pattern of disease flare after gefitinib discontinuation, and it successfully improved with readministration. With regard to toxicity, Ma et al. reported that the toxicities that were frequently observed during combined treatment using WBRT and gefitinib for NSCLC patients with brain metastasis included rash (86%), diarrhea (43%), nausea, vomiting, headache, and fatigue, most of which were mild and manageable [10]. Our patients also experienced rash, appetite loss, and

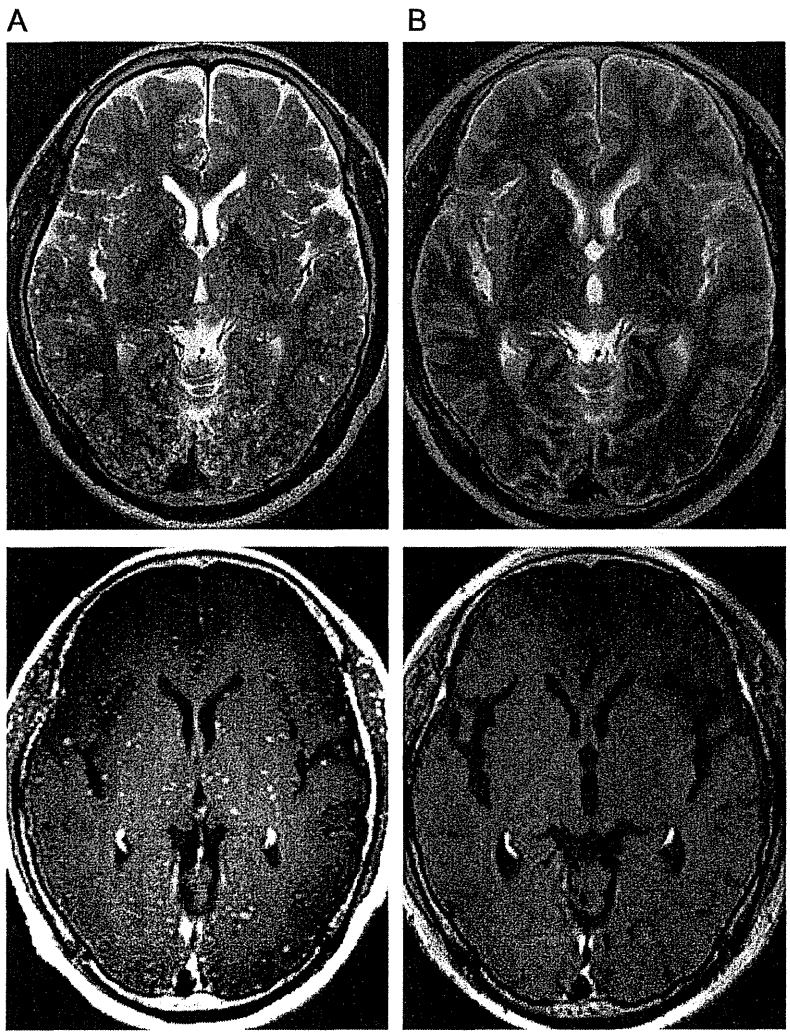

Fig. 3 – Case 2: Brain magnetic resonance imaging (MRI). A: Prior to radiotherapy, T2-weighted MRI scan (upper) showing multiple, small, high-intensity lesions in the cerebral cortex and basal ganglia. MRI enhanced with gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA) (lower) showing multiple miliary enhancing lesions at the same site. B: Nine months after radiation therapy, both T2-weighted (upper) and Gd-DTPA-enhanced MRI scans (lower) showed virtually complete disappearance of the metastatic lesions.

headache, although these toxicities were quite mild. In terms of efficacy and safety, this combination treatment merits further investigation.

Miliary brain metastases in EGFR-mutated NSCLC patients during treatment with EGFR-TKI must be considered a possibility. For a patient with such miliary brain metastases, combination treatment with WBRT and EGFR-TKI may be effective even in the presence of progressively disturbed consciousness.

Conflict of interest

Toshihiro Nukiwa received lecture fees from Boehringer Ingelheim.

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