paraffin-embedded (FFPE) tissue, BB cytology, and pleural effusion cytology samples from patients with NSCLC.

materials and methods

This was an observational study using control DNA admixtures and clinical samples. Patients provided written informed consent for samples to be used in research. The study was conducted as a collaborative research of AstraZeneca KK with National Cancer Center Hospital East (NCCHE) and Hyogo Cancer Center (HCC) after protocol approval by each Institutional Review Board and was conducted in accordance with ethical guidelines for epidemiological studies.

samples and DNA extraction

DNA admixtures

Four types of mutant plasmids were prepared including the *EGFR* mutation L858R, T790M, G719S, and E746-A750 deletion (nt del 2234-2249) in the Blue Heron pUC plasmid by Invitrogen Inc. (Tokyo, Japan). The sequence inserted into each plasmid corresponded with the longest sequence requirements spanning the exons across all of the methods to be evaluated, from −300 to +220 bp for exon 18 (for G719S) and from −200 to +200 bp for exons 19, 20, and 21 (for E746-A750 deletion, T790M, and L858R, respectively). Admixtures were prepared at Saitama Medical University Hospital. The plasmid preparations (5.4 ng/μl) were diluted with water and whole-human genomic DNA (12.5 ng/μl) (Promega Inc., Madison, WI) to prepare an admixture containing a 1 : 1 ratio (confirmed by Sanger sequencing) of copies of mutated and wild-type *EGFR* (5.4 fg/μl plasmid DNA, 10 ng/μl genomic DNA; referred to here as a 100% admixture). The 100% admixture solution was then diluted with genomic DNA to provide DNA solutions simulating those isolated from a clinical sample containing *EGFR*-mutated and wild-type cells at ratios of 50 : 50 (50% admixture), 25 : 75 (25%), 10 : 90 (10%), 5 : 95 (5%), 2 : 98 (2%), and 1 : 99 (1%). The samples were divided into aliquots for each laboratory, randomized and assigned an identification code, and 20 μl of each sample sent to the laboratories for mutation testing in a blinded manner. Ten wild-type control samples (from a single stock of genomic DNA) (10 ng/μl) were also distributed for testing.

formalin-fixed paraffin-embedded samples

In total, 120 FFPE NSCLC samples collected at NCCHE ($n = 100$) and HCC ($n = 20$) between December 2005 and October 2009 were used. Twelve consecutive sections (5-μm thickness), prepared by Sanritsu Co. Ltd (Tokyo, Japan) from each FFPE tissue block, were allocated as follows: sections 1 and 12, hematoxylin–eosin (H&E) staining; sections 2 and 7, PCR direct sequencing; sections 3 and 8, Cycleave PCR; sections 4 and 9, PCR-Invader; sections 5 and 10, PNA-LNA PCR clamp; sections 6 and 11, Scorpion ARMS. Samples were randomly assigned an identification code by Sanritsu Co. Ltd, with separate identification codes for the samples for PCR direct sequencing and Cycleave PCR (as they were to be analyzed by the same laboratory). A table of corresponding randomized identification codes was retained by AstraZeneca KK until analysis. H&E-stained sections (Sanritsu Co. Ltd) were reviewed by a single pathologist at NCCHE for histological type, tumor cell content, and tumor dimension in a blinded manner. DNA was extracted at each testing laboratory using their own standard operating procedures (SOPs), all of which utilized the QIAamp kit (QIAGEN Japan, Tokyo, Japan) (see supplemental Methods, available at *Annals of Oncology* online).

bronchofiberscopic brushing cytology samples

Thirty BB cytology samples (with matched FFPE samples available) obtained at NCCHE ($n = 10$) and HCC ($n = 20$) between 2006 and 2009 were used. Samples were collected by exfoliative cytodiagnosis brushing or curette washing in saline solution, without anticoagulant, and stored frozen. The BB cytology samples were randomized and assigned an identification code. The presence of tumor cells and histological type were confirmed by a pathologist at each center. DNA was extracted (QIAamp DNA Mini kit, QIAGEN Japan) at Kinki University of Medicine (Department of Genome Biology) and divided into 22 μl aliquots for analysis by the testing laboratories (direct sequencing was excluded due to the small amount of DNA anticipated, and for Scorpion ARMS, if the DNA concentration was <1 ng/μl, only exon 19 deletions, L858R, and T790M mutations were analyzed—see supplemental Methods, available at *Annals of Oncology* online).

pleural effusion cytology samples

Pleural effusion cytology samples were provided by NCCHE. Twenty pleural effusion cytology samples were collected from patients diagnosed with NSCLC (adenocarcinoma) between February 2009 and February 2010 and confirmed by a pathologist to contain tumor cells. Samples were frozen within 10 and 30 min of sampling and stored at −80°C. Frozen samples were thawed at 37°C and refrozen rapidly three times to disrupt the cells and ensure an even distribution and then divided into five equal aliquots that were sent to each of the testing laboratories. Samples were randomly assigned an identification code as for the FFPE samples. DNA was extracted at each laboratory using their own SOPs, all of which were based on the use of the QIAamp kit (see supplemental Methods, available at *Annals of Oncology* online).

EGFR mutation analysis

Samples were analyzed using five different *EGFR* mutation tests carried out by four different testing laboratories: PCR-Invader [4, 12] by BML Inc. (Tokyo, Japan); PNA-LNA PCR clamp [5] by Mitsubishi Chemical Medience Corp. (Tokyo, Japan); PCR direct sequencing (with the exception of the BB cytology samples, due to the anticipated tumor DNA yield based on published evidence regarding the detection limit of this method [13]) [6] by SRL Inc. (Tokyo, Japan), Cycleave PCR [7] also by SRL Inc., and Scorpion ARMS [14, 15] by Genzyme Analytical Services (Los Angeles, CA). Scorpion ARMS analysis employed the DxS *EGFR* Mutation Test Kit for research use only [QIAGEN Manchester (formerly DxS Ltd), UK] and was carried out according to the manufacturer's instructions with modifications described in the supplemental Methods (available at *Annals of Oncology* online). The other methods were carried out using each of the laboratories' experimental set up, with data analysis and quality control completed according to their own specific protocols (further details in the supplemental Methods, available at *Annals of Oncology* online). Samples were defined as mutation negative where sufficient material was present to generate a result but the presence of a mutation was not observed within the detection limit of the assay. The *EGFR* mutations detected by each *EGFR* mutation test are shown in supplemental Table S1 (available at *Annals of Oncology* online).

Analysis data (positive, negative, not detected, mutation type) and any supplemental information (e.g. failure of PCR amplification) were reported to AstraZeneca KK (Osaka, Japan).

statistical analysis

The correct determination rates (whether or not the positive/negative *EGFR* mutation assessment result was correct) and sensitivity (lowest percentage DNA admixture detected) by *EGFR* mutation type were assessed using DNA admixture samples for each *EGFR* mutation test.

The success and positive rates of each *EGFR* mutation test were determined using FFPE, and BB and pleural effusion cytology samples. The success rate was defined as the proportion of samples successfully analyzed

where it was possible to determine the mutation status. Samples were classified as unsuccessful where it was not possible to determine the mutation status, the PCR amplification failed, or if values of samples exceeded the cut-off value of Scorpion ARMS. The positive rate was defined as the number of samples analyzed as positive by each method as a proportion of the number of samples successfully analyzed. False-positive and false-negative rates were not determined, as no reference or 'gold standard' has been defined for *EGFR* mutation analysis.

The concordance rates and Cohen's kappa coefficients were determined between different methods of detection and between FFPE versus BB cytology sample types for mutation types commonly detectable by all assessed methods. Cohen's kappa coefficient was calculated as: kappa = (Po−Pe)/(1−Pe), where Po is the observed concordance rate and Pe is the expected probability of chance agreement.

results

patient samples

In total, 116 FFPE samples were evaluable for analysis, as four samples were confirmed not to contain NSCLC cells. The majority of samples were of adenocarcinoma histology and had a tumor cell content of at least 50%. Both tissue and tumor dimensions were ≤19 mm in most samples.

Of the 30 BB cytology samples (24 adenocarcinoma, four squamous, one adenosquamous, one large cell), one sample was excluded from the analysis because its matching FFPE sample was not judged as NSCLC. The samples were taken at a mean of 39 days (range 20–70 days) before operation and the mean DNA concentration was 8.73 ng/μl (range 0.2–40.3 ng/μl). All 20 pleural effusion cytology samples were assessable for analysis. Volumes of pleural effusion cytology samples used for each test method were 0.7–0.8 ml.

comparability of five *EGFR* mutation tests

DNA admixtures

PCR-Invader, PNA-LNA PCR clamp, Cycleave PCR, and Scorpion ARMS methods detected each of the *EGFR* mutation types L858R, T790M, G719S, and the in-frame deletion E746-A750 type I at ratios ranging from 1% to 50% of mutant/wild-type allele. PCR direct sequencing detected all types of mutations in samples containing 5%–50% of plasmid DNA but could not detect any of the mutations in the 1% mutant DNA admixture, nor exon 19 deletion or L858R in the 2% mutant DNA admixture. There were no false positives in wild-type samples.

formalin-fixed paraffin-embedded samples

Success rates of all five *EGFR* mutation tests were over 90% in FFPE samples (Table 1). Concordance rates between any two methods ranged from 85.3% to 99.1% including samples unsuccessfully analyzed and from 94.3% to 100% excluding samples unsuccessfully analyzed (supplemental Table S2, available at *Annals of Oncology* online). The rate of type 1 discordance (different mutations detected between the methods) was 3.4% (4/116 samples) and the rate of type 2 discordance (mismatch of mutation status between the methods) was 6.9% (8/116 samples) in FFPE samples (supplemental Table S3, available at *Annals of Oncology* online).

Unsuccessful rates of mutation analyses and discordance rates by tumor/sample characteristics for FFPE samples are shown in supplemental Figure S1 (available at *Annals of Oncology* online). Higher unsuccessful rates were associated with histological subtype [squamous cell carcinoma, 4/7 (57.1%)], older sample age [year of surgery 2006, 9/10 (90.0%)], and larger tumor dimension [20–29 mm, 3/15

Table 1. Success rate and *EGFR* mutation status determined by different *EGFR* mutation tests in FFPE, BB, and pleural effusion samples

Sample type and method	No. of samples successfully analyzed (%)	No. of mutation-positive samples (%)[a]	No. of mutation-negative samples (%)[a]
FFPE samples ($n = 116$)			
Scorpion ARMS	115 (99.1)	65 (56.5)	50 (43.5)
PCR-Invader	116 (100.0)	65 (56.0)	51 (44.0)
PNA-LNA PCR clamp	106 (91.4)	61 (57.5)	45 (42.5)
PCR direct sequencing	110 (94.8)	64 (58.2)	46 (41.8)
Cycleave PCR	116 (100.0)	63 (54.3)	53 (45.7)
BB cytology samples ($n = 29$)			
Scorpion ARMS	29 (100.0)	15 (51.7)	14 (48.3)
PCR-Invader	29 (100.0)	17 (58.6)	12 (41.4)
PNA-LNA PCR clamp	29 (100.0)	17 (58.6)	12 (41.4)
Cycleave PCR	29 (100.0)	16 (55.2)	13 (44.8)
Pleural effusion cytology samples ($n = 20$)			
Scorpion ARMS	20 (100.0)	11 (55.0)	9 (45.0)
PCR-Invader	20 (100.0)	11 (55.0)	9 (45.0)
PNA-LNA PCR clamp	20 (100.0)	10 (50.0)	10 (50.0)
PCR direct sequencing	20 (100.0)	11 (55.0)	9 (45.0)
Cycleave PCR	20 (100.0)	11 (55.0)	9 (45.0)

[a]Percentage calculated based on the number of samples successfully analyzed; *EGFR* mutation status was determined before the study and samples were selected to allow for an ~1 : 1 ratio of mutation-positive : mutation-negative samples.

ARMS, Amplification Refractory Mutation System; BB, bronchofiberscopic brushing; FFPE, formalin-fixed paraffin-embedded; PNA-LNA, peptide nucleic acid-locked nucleic acid.

(20.0%)] (supplemental Figure S1, available at *Annals of Oncology* online). Discordance rates tended to be higher in samples with low tumor cell content [0–20%, 2/10 (20.0%); 30–40%, 3/10 (30%)], smaller tumor dimension [0–9 mm, 11/53 (20.8%)], smaller tissue dimension [0–9 mm, 8/33 (24.2%)], and older sample age [year of surgery 2006, 2/10 (20.0%)] (supplemental Figure S1, available at *Annals of Oncology* online).

Concordance rates between five methods for the two major mutation types in FFPE samples were 81.8% (18/22) for exon 19 deletions and 87.2% (34/39) for L858R.

PCR direct sequencing identified rare mutations in six patients that were not detected by any other methods [V689L and E690V, E709V, V834L, I706T D770_N771 (insSVD), H773_V774(insPH)].

bronchofiberscopic brushing cytology samples

Success rates of the four *EGFR* mutation tests utilized for analysis of BB cytology samples were 100% (Table 1) and concordance rates between two methods ranged from 93.1% to 100% (supplemental Table S2, available at *Annals of Oncology* online). Discordances between two methods were found in two (6.9%) samples (supplemental Table S4, available at *Annals of Oncology* online). Both were type 2 discordances (mismatch of mutation status between the methods): in one sample, G719C detected by PCR-Invader and PNA-LNA PCR clamp was assessed as negative by Cycleave PCR (G719X not analyzed by Scorpion ARMS due to insufficient sample). In the remaining sample, L858R detected by PCR-Invader, PNA-LNA PCR clamp, and Cycleave PCR was assessed as negative by Scorpion ARMS.

Concordance rates between analysis of BB cytology and FFPE samples ranged from 65.5% to 96.6% including samples unsuccessfully analyzed and 93.1%–96.6% excluding samples unsuccessfully analyzed (supplemental Table S5, available at *Annals of Oncology* online). Discordances in analysis of BB cytology samples versus FFPE samples by the same detection method (excluding discordances due to unsuccessful mutation analysis of FFPE samples) were observed in three (10.3%) samples (supplemental Table S4, available at *Annals of Oncology* online); all were type 2 discordances (mismatch of mutation status between the methods).

pleural effusion cytology samples

Success rates of all five *EGFR* mutation tests were 100% (Table 1) and concordance rates between two methods ranged from 85.0% to 100% in the pleural effusion samples (supplemental Table S2, available at *Annals of Oncology* online). Discordances between five methods were found in three (15.0%) samples (supplemental Table S6, available at *Annals of Oncology* online). All were type 2 discordances (mismatch of mutation status between the methods): in one sample, an exon 19 deletion was detected by all methods except PCR direct sequencing; in another, an exon 19 deletion was only detected by Scorpion ARMS, Cycleave PCR, and PCR direct sequencing; and in the third sample, L858R was only detected by PCR-Invader. In one of the other 17 samples, PCR direct sequencing detected an additional mutation [exon 18 deletion (E709_T710>D)], which the other methods were not designed to assess.

discussion

Analysis of the control DNA admixture samples showed that the *EGFR* mutation tests had comparable sensitivity, with the exception of direct sequencing. The sensitivity of direct sequencing, although higher than expected and reported elsewhere [15], was lower than the other techniques.

The results of this study showed that all five *EGFR* mutation tests had comparable success rates (over 90%) in FFPE samples. These were consistently high success rates given that the fixation of some of the samples was not ideal (e.g. long fixation times). The success rates of direct sequencing were higher than anticipated based on previous studies of clinical samples. For example, in a recent study, ARMS and direct sequencing were used to detect known *EGFR* mutations in NSCLC FFPE samples, and ARMS was found to be a more sensitive and robust technique [13]. However, it should be recognized that even when utilizing the same technologies, differences in reagents, DNA quality, software, and crucially, primer design and amplicon size have a huge influence on direct sequencing success rates and mutation detection potential. Our results show that the processes implemented by the laboratory in this study are highly optimized for the detection of *EGFR* mutations from tumor DNA using direct sequencing. As the testing laboratories also carried out the DNA extraction (with the exception of BB cytology samples), the slight differences in DNA extraction and processes across the different laboratories could also impact on the overall performance of the test methods.

All the FFPE samples were examined by a pathologist and generally found to be of high quality and tumor content. The numbers of samples with different tumor/sample characteristics were too low to make any definitive conclusions regarding unsuccessful and discordance rates by these characteristics. However, sample unsuccessful rates appear to be associated with squamous cell carcinoma, older samples, and samples with long tumor dimension, all of which can make it difficult to extract DNA. In addition, discordance rates appeared higher in older samples or samples of low tumor cell content, short tumor dimension or short tissue dimension, where the quantities of DNA are small.

Concordance rates were generally over 85% (>94%, excluding samples unsuccessfully analyzed) between any two *EGFR* mutation tests in FFPE samples. The lowest concordance rates between the five methods were in comparison with the PNA-LNA PCR clamp method. As the PNA-LNA PCR clamp method also had a higher unsuccessful rate than the other methods, concordance rates were lower in comparison with other methods when including samples unsuccessfully analyzed. However, all kappa statistics were ≥ 0.70, indicating a high concordance of analysis results. Concordance rates for the two major *EGFR* mutation types, exon 19 deletions and L858R, across the five mutation tests in FFPE samples were also high (81.8% and 87.2%, respectively), illustrating the suitability of all

five methods for *EGFR* mutation analysis in clinical studies and diagnostic applications. However, as the concordance rates were not 100% for any one method, we would advocate the selection of a single method for consistent use during a clinical study. With regard to daily practice, the decision to select and adopt a particular technology is at the discretion of individual laboratories and may be influenced by the diagnostic environment in which they reside. Selection factors may include technical expertise of operators, cost, test status (*in vitro* diagnostic versus laboratory-developed test), or availability of instrumentation.

Several factors may have contributed to the discordances between the *EGFR* mutation tests. These factors may have included differences in sensitivity and specificity, different DNA extraction procedures between laboratories, variation in tumor cell content within and across samples, and tumor heterogeneity within an FFPE block [10, 11, 16].

The performance of all five *EGFR* mutation tests was comparable in the analysis of both BB and pleural effusion cytology samples, with 100% success rates. BB cytology samples showed high concordance rates (>93%, excluding samples unsuccessfully analyzed) between pairs of *EGFR* mutation tests and versus FFPE samples by each detection method. Using the PNA-LNA PCR clamp method, analysis of BB cytology samples was successful where the matched FFPE sample failed analysis. Some mutations were detected in cytology samples of low DNA concentrations where matching FFPE samples were assessed as mutation negative. This result suggests that cytology samples can be useful in mutation analysis when tissue samples cannot be used, are in a small quantity, or degradation of FFPE samples is suspected. Pleural effusion cytology samples may be particularly suitable for analysis as they can be obtained easily, non-invasively and repeatedly, and generally contain plenty of cancer cells, relative to other sample types.

To our knowledge, this is the first high-quality comparison study of *EGFR* mutation tests in both FFPE and cytology samples. The results of the current study indicate that cytology-derived DNA is a suitable alternative to FFPE samples for the analysis of *EGFR* mutations and may be useful when FFPE samples are unavailable for molecular analysis. Other studies have also shown that ARMS can be used to detect *EGFR* mutations in cytology samples from transbronchial needle aspirates [17] or pleural effusion [18] and that this technique appeared to be more sensitive than direct sequencing in this sample type. Other methods for *EGFR* mutation testing, including pyrosequencing [19] and high-resolution melting analysis [20], also exist.

In summary, the performance of all five *EGFR* mutation tests was comparable in the analysis of FFPE and cytology samples. Where *EGFR* mutation tests and standard operating procedures are used in a reliable robust way, with trained operators, in a well-developed diagnostic setting, comparable results are obtained across mutation tests and sample types. FFPE specimens are currently the sample of choice for determining *EGFR* mutation status [11]. However, the ability to use cytology samples allows additional patients to be tested for *EGFR* mutations, and therefore, more appropriate treatment of their disease.

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disclosure

KG has received honoraria from Ono Pharmaceutical and Chugai Pharmaceutical and fees for consultancy/advisory boards from Ono Pharmaceutical. MS has received honoraria from Chugai Pharmaceutical and AstraZeneca. KN has received research grants from Daiichi Sankyo, Chugai Pharmaceutical, AstraZeneca, Glaxo SmithKline, and Solasia Pharma KK, research support from Chugai Pharmaceutical, and honoraria from Kyowa Hakko Kirin, Sumitomo Bakelite, Taiho Pharmaceutical, and Qiagen. KH has received patent fees from Mitsubishi Chemical Medience. TM has received honoraria from AstraZeneca and Chugai Pharmaceutical. JW, ED, RM, and TT are employees of AstraZeneca and hold stock in AstraZeneca. GI has declared no conflicts of interest.

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Prophylactic cranial irradiation in small-cell lung cancer: Findings from a North Central Cancer Treatment Group Pooled Analysis

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Background: This pooled analysis evaluated the outcomes of prophylactic cranial irradiation (PCI) in 739 small-cell lung cancer (SCLC patients with stable disease (SD) or better following chemotherapy ± thoracic radiation therapy (TRT) to examine the potential advantage of PCI in a wider spectrum of patients than generally participate in PCI trials.
Patients and methods: Three hundred eighteen patients with extensive SCLC (ESCLC) and 421 patients with limited SCLC (LSCLC) participated in four phase II or III trials. Four hundred fifty-nine patients received PCI (30 Gy/15 or 25 Gy/10) and 280 did not. Survival and adverse events (AEs) were compared.
Results: PCI patients survived significantly longer than non-PCI patients {hazard ratio [HR] = 0.61 [95% confidence interval (CI): 0.52–0.72]; $P < 0.0001$}. The 1- and 3-year survival rates were 56% and 18% for PCI patients versus 32% and 5% for non-PCI patients. PCI was still significant after adjusting for age, performance status, gender, stage, complete response, and number of metastatic sites (HR = 0.82, $P = 0.04$). PCI patients had significantly more grade 3+ AEs (64%) compared with non-PCI patients (50%) ($P = 0.0004$). AEs associated with PCI included alopecia and lethargy. Dose fractionation could be compared only for LSCLC patients and 25 Gy/10 was associated with significantly better survival compared with 30 Gy/15 (HR = 0.67, $P = 0.018$).
Conclusions: PCI was associated with a significant survival benefit for both ESCLC and LSCLC patients who had SD or a better response to chemotherapy ± TRT. Dose fractionation appears important. PCI was associated with an increase in overall and specific grade 3+ AE rates.
Key words: PCI, prophylactic cranial irradiation, radiotherapy, small-cell lung cancer, survival

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Clinical Cancer Research

Activation of HER Family Signaling as a Mechanism of Acquired Resistance to ALK Inhibitors in EML4-ALK –Positive Non–Small Cell Lung Cancer

Junko Tanizaki, Isamu Okamoto, Takafumi Okabe, et al.

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Cancer Therapy: Preclinical

Clinical Cancer Research

Activation of HER Family Signaling as a Mechanism of Acquired Resistance to ALK Inhibitors in EML4-ALK–Positive Non–Small Cell Lung Cancer

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Abstract

Purpose: Anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitors (TKI) such as crizotinib show marked efficacy in patients with non–small cell lung cancer positive for the echinoderm microtubule-associated protein–like 4 (EML4)–ALK fusion protein. However, acquired resistance to these agents has already been described in treated patients, and the mechanisms of such resistance remain largely unknown.

Experimental Design: We established lines of EML4-ALK–positive H3122 lung cancer cells that are resistant to the ALK inhibitor TAE684 (H3122/TR cells) and investigated their resistance mechanism with the use of immunoblot analysis, ELISA, reverse transcription and real-time PCR analysis, and an annexin V binding assay. We isolated EML4-ALK–positive lung cancer cells (K-3) from a patient who developed resistance to crizotinib and investigated their characteristics.

Results: The expression of EML4-ALK was reduced at the transcriptional level, whereas phosphorylation of epidermal growth factor receptor (EGFR), HER2, and HER3 was upregulated, in H3122/TR cells compared with those in H3122 cells. This activation of HER family proteins was accompanied by increased secretion of EGF. Treatment with an EGFR-TKI induced apoptosis in H3122/TR cells, but not in H3122 cells. The TAE684-induced inhibition of extracellular signal–regulated kinase (ERK) and STAT3 phosphorylation observed in parental cells was prevented by exposure of these cells to exogenous EGF, resulting in a reduced sensitivity of cell growth to TAE684. K-3 cells also manifested HER family activation accompanied by increased EGF secretion.

Conclusions: EGF-mediated activation of HER family signaling is associated with ALK-TKI resistance in lung cancer positive for EML4-ALK. *Clin Cancer Res; 18(22); 6219–26. ©2012 AACR.*

Introduction

Lung cancer is the leading cause of cancer death worldwide. Fusion of the echinoderm microtubule-associated protein–like 4 gene (*EML4*) with the anaplastic lymphoma kinase gene (*ALK*), which results in the production of a fusion protein (EML4-ALK), occurs in 5% to 10% of cases of non–small cell lung cancer (NSCLC; refs. 1–3). This transforming fusion leads to activation of downstream signaling molecules, and inhibition of ALK signaling has shown marked antitumor effects in NSCLC positive for EML4-ALK in both preclinical and clinical studies (4). Despite their initial response, however, individuals with EML4-ALK–positive NSCLC treated with ALK inhibitors eventually acquire resistance to these drugs (5), and the molecular mechanisms responsible for such resistance remain largely uncharacterized. With the use of an *in vitro* cell model and cells newly derived from a patient with acquired resistance to the ALK inhibitor crizotinib, we have now uncovered a previously unknown mechanism of such resistance.

Materials and Methods

Cell culture and reagents

The H3122 human NSCLC cell line was obtained as previously described (6), and H1299 human NSCLC cells were obtained from American Type Culture Collection. The cells were maintained under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma), which contains no biologic ligands including EGF, supplemented with 10% FBS. TAE684, crizotinib, and BIBW2992 were obtained from ShangHai Biochempartner. Paclitaxel was from Sigma, and adriamycin was from Wako. Recombinant

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Translational Relevance

ALK-targeted therapy shows marked clinical efficacy in lung cancer patients with the *EML4-ALK* fusion gene. Such patients eventually develop resistance to ALK tyrosine kinase inhibitors (TKI), however, and the mechanisms of such resistance remain largely unknown. We have now established ALK-TKI–resistant lines of *EML4-ALK*–positive H3122 cells and shown that activation of the epidermal growth factor receptor (EGFR) and the related proteins HER2 and HER3 by autocrine EGF stimulation is associated with such resistance. Furthermore, we established K-3 cells from a specimen of *EML4-ALK*–positive lung cancer obtained from a patient who developed ALK-TKI resistance. These cells also manifested EGF-dependent activation of HER family proteins. Our observations have thus uncovered a previously unknown mechanism of ALK-TKI resistance in *EML4-ALK*–positive lung cancer and they may provide a basis for circumvention of such resistance.

human epidermal growth factor (EGF) was from R&D Systems.

Generation of TAE684-resistant H3122 cells

H3122 cells were initially exposed to 0.01 μmol/L TAE684. They were then isolated by limiting dilution, maintained, and passaged normally with gradually increasing doses of TAE684, up to a maximum of 1 μmol/L. The established resistant cell lines, designated H3122/TR1 and H3122/TR2, were maintained in medium containing the maximal dose of TAE684 (1 μmol/L) to maintain selective pressure for TAE684 resistance.

Growth inhibition assay *in vitro*

Cell viability was assessed with an MTT assay as previously described (7).

Annexin V binding assay

Binding of annexin V to cells was measured with the use of an Annexin-V-FLUOS Staining Kit (Roche), as previously described (7).

Immunoblot analysis

Immunoblot analysis was conducted as previously described (7). Rabbit polyclonal antibodies to phosphorylated human ALK (pY1608), to ALK, to the phosphorylated EGF receptor (pY1068), to phosphorylated HER2 (pY1221), to phosphorylated HER3 (pY1289), to phosphorylated AKT, to AKT, to phosphorylated STAT3, and to STAT3 were obtained from Cell Signaling Technology; those to HER3, to extracellular signal-regulated kinase (ERK), and to phosphorylated ERK were from Santa Cruz Biotechnology; those to HER2 were from Millipore; and those to β-actin were from Sigma. Mouse monoclonal antibodies to the EGF receptor (EGFR) were obtained from Invitrogen.

Reverse transcription and real-time PCR analysis

Total RNA was extracted from cells with the use of an RNeasy Mini Kit (Qiagen) and was subjected to reverse transcription (RT) with the use of a SuperScript Preamplification System (Invitrogen Life Technologies). The resulting cDNA was then subjected to real-time PCR analysis with the use of a One Step SYBR PrimeScript RT-PCR Kit (Takara Bio) and ABI PRISM 7900HT system (Applied Biosystems). The PCR primers (forward and reverse, respectively) included those for EML4-ALK (5′-GTGCAGTGTTTAGCATTCTTGGGG-3′, 5′-TCTTGCCAGCAAAGCAGTAGTTGG-3′), EGF (5′-TGCAACTGTGTTGTTGGCTACATC-3′, 5′-TGGTTGACCCCCATTCTTGAG-3′), TGF-α (5′-TCAGTTCTGCTTCCATGCAACC-3′, 5′-TTTCTGAGTGGCAGCAAGCG-3′), and amphiregulin (5′-AGAGTTGAACAGGTAGTTAAGCCCC-3′, 5′-GTCGAAGTTTCTTTCGTTCCTCAG-3′). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal standard.

ELISA for ligands

The concentrations of EGF, TGF-α, and amphiregulin in conditioned medium were determined as previously described (8), with the use of a Human Quantikine ELISA Kit (R&D Systems).

Isolation of crizotinib-resistant cells from a clinical specimen

K-3 cells were established at Kinki University Faculty of Medicine from the pleural effusion of a patient with EML4-ALK–positive NSCLC who developed resistance to crizotinib. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. The clinical specimen was obtained from Kinki University Hospital for study with the approval of the Institutional Review Board, and the patient provided written informed consent.

Results

Generation of ALK inhibitor–resistant lines from EML4-ALK–positive H3122 cells

The NSCLC cell line H3122 expresses EML4-ALK and is highly sensitive to the ALK TKI TAE684. We generated TAE684-resistant H3122 cell lines (H3122/TR1 and H3122/TR2) by exposing the parental cells to increasing

Table 1. IC_{50} values of TAE684 and crizotinib for inhibition of the growth of H3122 and H3122/TR cell lines

	IC50, μmol/L	
Cell line	**TAE684**	**Crizotinib**
H3122	0.053	0.15
H3122/TR1	1.034	2.26
H3122/TR2	1.100	1.51

NOTE: Data are means of triplicates from representative experiments that were repeated a total of 3 times.

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concentrations of TAE684. Short tandem repeat analysis of H3122/TR cells confirmed their H3122 origin (data not shown). The IC_{50} of TAE684 for inhibition of the growth of the parental H3122 cells was 0.053 μmol/L, whereas those for the H3122/TR cell lines as well as for EML4-ALK–negative H1299 cells were all more than 1 μmol/L (Table 1, Fig. 1A). The H3122/TR cells were also resistant to crizotinib, another ALK-targeted TKI, but not to other drugs including paclitaxel and adriamycin (Table 1, Fig. 1A, and data not shown). TAE684 also induced a marked increase in the level of apoptosis in H3122 cells, whereas it had no such effect in H3122/TR cells (Fig. 1B). Collectively, these data suggested that H3122/TR cells had acquired resistance specific to ALK inhibitors.

Activation of alternative signaling pathways in H3122/TR cells

We next investigated the mechanism responsible for resistance to ALK-TKIs in H3122/TR cells. Immunoblot analysis revealed that the abundance of EML4-ALK was reduced in both H3122/TR cell lines compared with that in H3122 cells (Fig. 2A). Quantitative RT-PCR analysis showed that the amount of EML4-ALK mRNA was also reduced markedly in H3122/TR cells relative to that in the parental cells (Supplementary Fig. 1), suggesting that the downregulation of EML4-ALK expression is mediated at the transcriptional level. We did not detect any secondary mutations of ALK in H3122/TR cells (data not shown), with such mutations having previously been shown to give rise to ALK-TKI resistance (5, 9, 10). TAE684 inhibited the phosphorylation of ERK and STAT3 in H3122 cells, but had no such effects in H3122/TR cells (Fig. 2A). Given that signaling via ERK and STAT3 is maintained in H3122/TR cells in the presence of TAE684, we investigated whether alternative upstream pathways are activated in these cells. We found that the level of EGFR phosphorylation was increased in the resistant cells compared with the parental cells, although the abundance of EGFR was decreased in the former cells (Fig. 2B). Phosphorylation of the EGFR-related proteins HER2 and HER3 was also increased in both H3122/TR cell lines (Fig. 2B). To investigate the mechanism responsible for activation of these receptor tyrosine kinases in H3122/TR cells, we examined the expression of ligands for HER family proteins, including EGF, TGF-α, and amphiregulin. Quantitative RT-PCR analysis revealed that the amount of EGF mRNA, but not that of TGF-α or amphiregulin mRNAs, was greatly increased in H3122/TR cells compared with that in parental H3122 cells (Fig. 2C). Consistent with these results, ELISA showed that secretion of EGF, but not that of TGF-α or amphiregulin, was increased in H3122/TR cells (Fig. 2D). We next examined the effect of an EGFR-TKI on apoptosis in the parental and resistant cell lines. Treatment with BIBW2992, an irreversible TKI for EGFR, induced apoptosis in H3122/TR cells, but not in H3122 cells (Fig. 2E). Furthermore, the extent of apoptosis induced by the combination of TAE684 and BIBW2992 in H3122/TR cells

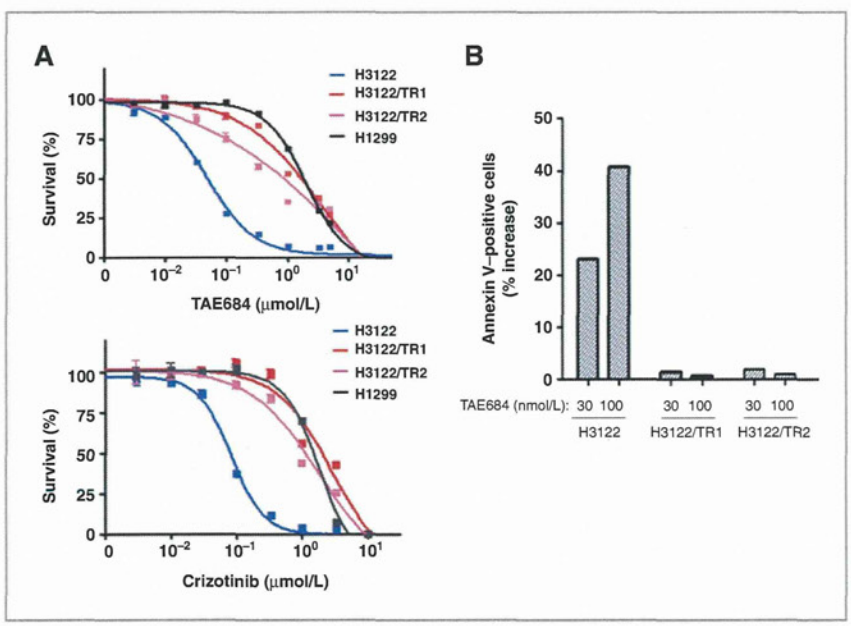

Figure 1. Effects of ALK kinase inhibitors on cell proliferation and apoptosis in H3122, H3122/TR1, H3122/TR2, and H1299 cells. A, cells were cultured for 72 hours in complete medium containing various concentrations of TAE684 (upper panel) or crizotinib (lower panel), after which cell viability was assessed. Data are means of triplicates from representative experiments and are expressed as percentage survival. B, cells were incubated for 72 hours with or without TAE684 (30 or 100 nmol/L), after which the number of apoptotic cells was determined by staining with annexin V and propidium iodide followed by flow cytometry. Data are means of triplicates from representative experiments.

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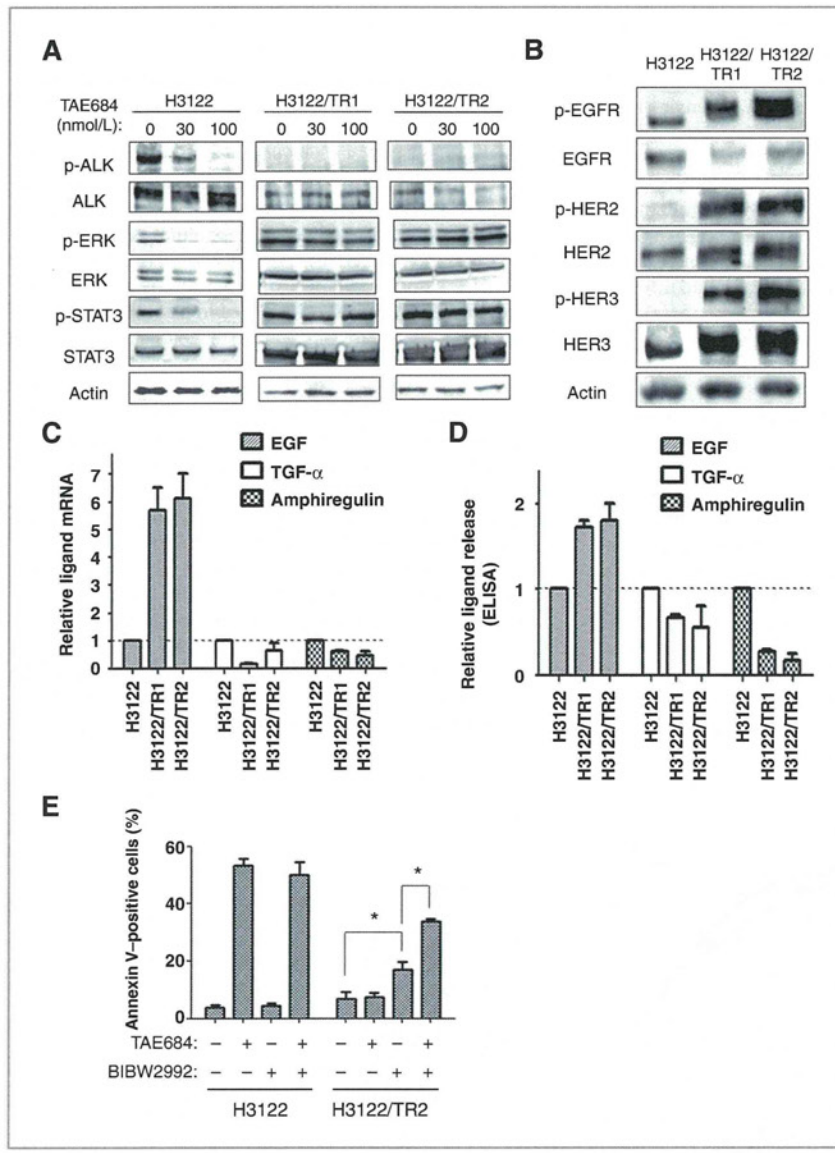

Figure 2. Activation of alternative signaling pathways in H3122/TR cells. A, H3122 or H3122/TR cells were incubated with the indicated concentrations of TAE684 for 6 hours, after which cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of ALK, ERK, or STAT3 as well as with those to β-actin (loading control). B, cells were deprived of serum for 24 hours, lysed, and subjected to immunoblot analysis with antibodies to phosphorylated or total forms of EGFR, HER2, or HER3. C, total RNA extracted from the indicated cell lines was subjected to RT and real-time PCR analysis of EGF, TGF-α, and amphiregulin mRNAs. Data were normalized by the amount of GAPDH mRNA and then expressed relative to the corresponding value for H3122 cells, and they are means ± SE from three independent experiments. D, cells were cultured overnight in medium containing 10% FBS and then incubated for 24 hours in serum-free medium, after which the latter culture supernatants were collected and assayed for EGF, TGF-α, and amphiregulin with an ELISA. Data are expressed relative to the corresponding value for H3122 cells and are means ± SE from three independent experiments. E, cells were incubated for 48 hours in the absence or presence of TAE684 (100 nmol/L) or BIBW2992 (200 nmol/L), as indicated, after which the proportion of apoptotic cells was determined by staining with annexin V and propidium iodide followed by flow cytometry. Data are means ± SE from 3 independent experiments. *, $P < 0.05$ (Student's t test) for the indicated comparisons.

was significantly greater than that induced by BIBW2992 alone (Fig. 2E). Together, these observations thus indicated that EGFR, HER2, and HER3 are activated in H3122/TR cells, that this activation is accompanied by increased secretion of EGF, and that such HER family activation contributes, at least in part, to the survival of H3122/TR cells in the presence of TAE684.

EGF activates HER family signaling and induces TAE684 resistance in parental H3122 cells

To investigate whether EGF secretion plays a role in ALK-TKI resistance, we examined the effect of exogenous EGF on ALK-TKI sensitivity in H3122 cells. We found that exposure to EGF induces resistance to TAE684 in these cells (Fig. 3A), supporting the notion that increased secretion of this ligand can lead to the development of ALK-TKI resistance. TAE684 inhibited EML4-ALK phosphorylation in H3122 cells in the absence or presence of EGF stimulation, whereas it failed to inhibit downstream signaling including the phosphorylation of ERK and STAT3 in the presence of EGF (Fig. 3B). EGF increased the levels of EGFR, HER2, and HER3 phosphorylation in H3122 cells (Fig. 3C). These data suggested that EGF-induced activation of EGFR, HER2, and HER3 might underlie resistance to ALK inhibitors in H3122/TR cells.

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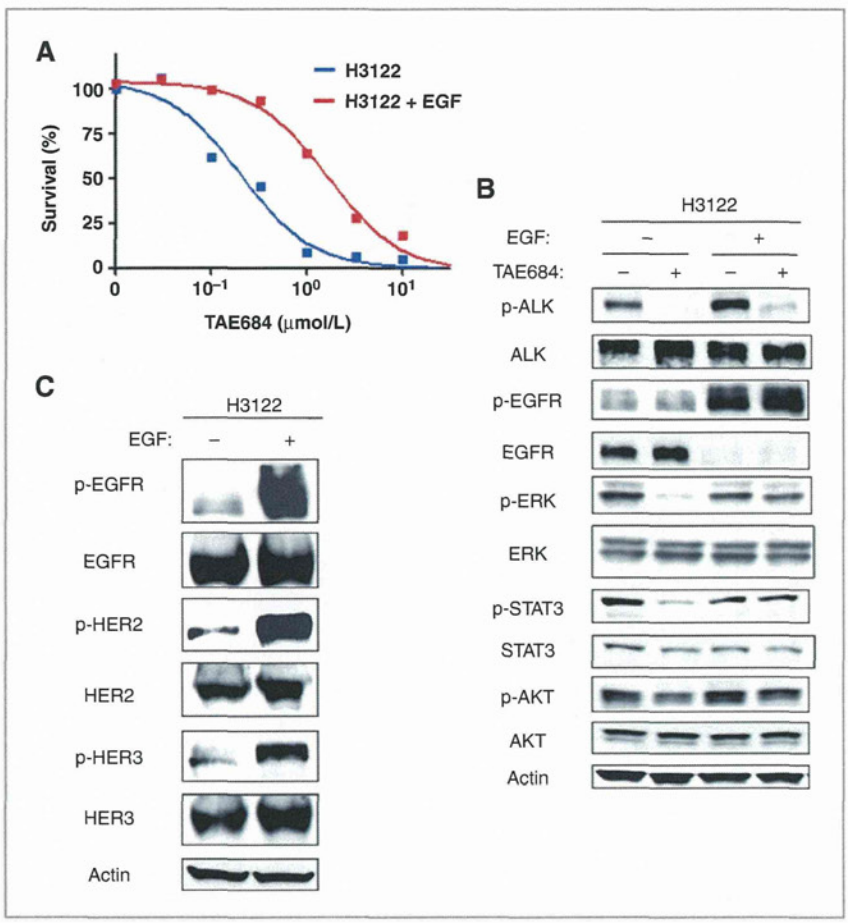

Figure 3. Effects of EGF stimulation in H3122 cells. A, cells were treated for 72 hours with various concentrations of TAE684 in the absence or presence of EGF (100 ng/mL), after which cell viability was assessed. Data are means of triplicates from representative experiments and are expressed as percentage survival. B, cells were incubated with or without EGF (100 ng/mL) for 15 min and then in the additional absence or presence of TAE684 (100 nmol/L) for 6 hours. Cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins. C, H3122 cells were incubated with or without EGF (100 ng/mL) for 6 hours, after which cell lysates were prepared and subjected to immunoblot analysis.

Isolation of drug-sensitive revertants from ALK-TKI–resistant cells

We cultured H3122/TR1 cells continuously in the absence of TAE684 for more than 2 months and thereby obtained H3122/TR revertant (H3122/TR rev) cells that had regained sensitivity to TAE684 (Fig. 4A). RT-PCR analysis and ELISA revealed that both the amount of EGF mRNA and the extent of EGF secretion in H3122/TR rev cells had been restored to levels similar to those in parental H3122 cells (Fig. 4B). This reduced level of EGF expression in H3122/TR rev cells compared with that in H3122/TR1 cells was accompanied by downregulation of EGFR, HER2, and HER3 phosphorylation as well as by upregulation of both the abundance and phosphorylation of EML4-ALK (Fig. 4C). These data suggested that downregulation of EML4-ALK expression and activation of HER family proteins, and the associated shift in signal transduction from the ALK signaling pathway to HER family pathways, are responsible for the acquired resistance to ALK inhibitors in H3122/TR cells.

Establishment of an ALK-TKI–resistant cell line from a clinical specimen

We established a cell line, designated K-3, from the pleural effusion of a patient with EML4-ALK–positive lung cancer that had developed clinical resistance to crizotinib after treatment for 6 months. The K-3 cells were found to be resistant to both crizotinib and TAE684, with IC_{50} values of 3.25 and 5.96 μmol/L, respectively (Fig. 5A). We did not detect any secondary mutations of ALK in K-3 cells. On the other hand, similar to the results obtained with H3122/TR cells, the abundance of EGF mRNA and the extent of EGF secretion were both markedly increased in K-3 cells compared with those in H3122 cells (Fig. 5B). Furthermore, immunoblot analysis showed that the level of EML4-ALK phosphorylation was decreased in K-3 cells, whereas that of EGFR, HER2, and HER3 phosphorylation was increased (Fig. 5C). These results thus showed concordance between an *in vitro* model and clinical experience of ALK-TKI resistance, further supporting the association of such resistance with EGF-dependent HER family activation.

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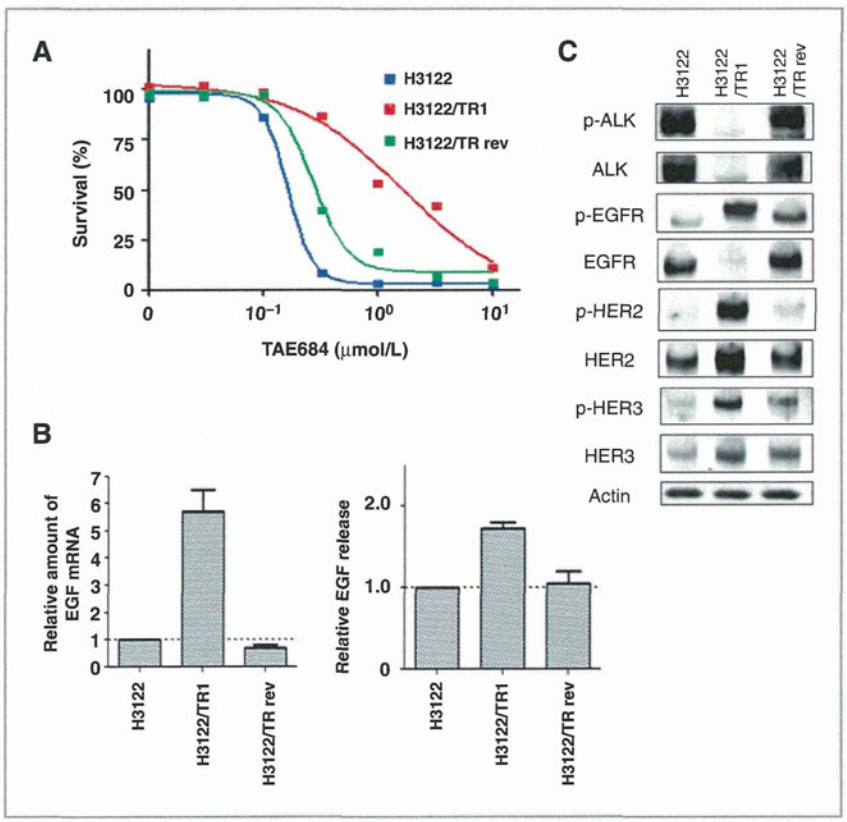

Figure 4. Characterization of H3122/TR revertant cells. A, H3122, H3122/TR1, or H3122/TR rev cells were cultured for 72 hours in complete medium containing various concentrations of TAE684, after which cell viability was assessed. Data are means of triplicates from representative experiments and are expressed as percentage survival. B, total RNA extracted from the indicated cell lines was subjected to RT and real-time PCR analysis of EGF mRNA as in Fig. 2C (left). Culture supernatants were also assayed for EGF by ELISA as in Fig. 2D (right). All data are expressed relative to the corresponding value for H3122 cells and are means ± SE from 3 independent experiments. C, cells were deprived of serum for 24 hours, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins.

Discussion

EML4-ALK has been identified in 5% to 10% of NSCLC cases, and ALK-TKIs show marked antitumor effects in such tumors (3, 11, 12). However, acquired resistance to ALK inhibitors has already been found to limit the therapeutic potential of these agents (5, 10, 13), with investigation of the underlying mechanisms of such resistance thus being warranted. In the present study, we generated ALK-TKI–resistant (H3122/TR) lines from EML4-ALK–positive H3122 cells by exposing the latter sensitive cells to the ALK inhibitor TAE684, which is a more potent and selective inhibitor of ALK than is crizotinib. The H3122/TR cells were found not to harbor secondary mutations of ALK, which have previously been shown to underlie ALK-TKI resistance (5, 10, 13). Instead, we found that H3122/TR cells manifest increased activation of EGFR, HER2, and HER3 mediated by EGF as well as a reduced level of EML4-ALK activation. Such EGF-induced activation of HER family proteins was associated with sustained downstream signaling in the presence of TAE684, indicative of a shift in survival dependency from the ALK signaling pathway to HER family pathways in the ALK-TKI–resistant cells. Indeed, we found that the combination of an ALK inhibitor and an EGFR inhibitor–induced apoptosis in H3122/TR cells, further supporting the notion that the EGFR signaling pathway contributes to survival in these cells. The clinical relevance of this resistance mechanism was supported by the observation that K-3 cells, which were isolated from a specimen of EML4-ALK–positive NSCLC that developed resistance to the ALK-TKI crizotinib *in situ*, also manifested EGF-mediated HER family activation. Consistent with our results, amphiregulin-mediated EGFR activation was recently shown to be associated with resistance to ALK inhibitors (9, 14). Collectively, these data suggest that ligand-activated HER family signaling gives rise to ALK-TKI resistance. We and others have shown that ligand-mediated signaling pathway activation accompanied by upregulation of ligand mRNA is associated with resistance to molecularly targeted therapy in several cancer models (9, 15). In the present study, we found that the abundance of EGF mRNA is increased in H3122/TR cells. Given that the *EGF* gene contains consensus binding sequences for many transcription factors, including NF-κB, AP-1, AP-2, AP-3, and SP1 (16), such factors may play a role in the upregulation of EGF expression observed in our study.

In the present study, we found that, in the absence of ALK inhibition, activation of EGFR, HER2, and HER3 in H3122/TR cells becomes downregulated in association with a reversion to TAE684 sensitivity. Consistent with

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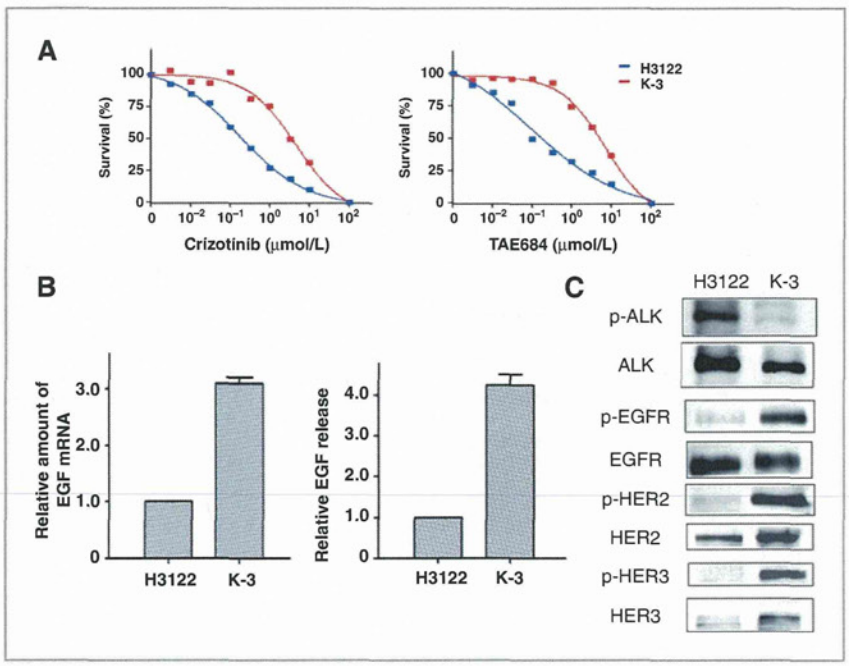

Figure 5. Characterization of K-3 cells established from a clinical specimen of EML4-ALK–positive NSCLC with acquired resistance to crizotinib. A, K-3 or H3122 cells were cultured for 72 hours in complete medium containing various concentrations of TAE684 or crizotinib, after which cell viability was assessed. Data are means of triplicates from representative experiments and are expressed as percentage survival. B, total RNA extracted from the indicated cells was subjected to RT and real-time PCR analysis of EGF mRNA as in Fig. 2C (left). Culture supernatants were also assayed for EGF by ELISA as in Fig. 2D (right). All data are expressed relative to the corresponding value for H3122 cells, and they are means ± SE from 3 independent experiments. C, cells were deprived of serum for 24 hours, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins.

these results, termination of treatment with an EGFR inhibitor after the development of drug resistance in EGFR mutation–positive NSCLC resulted in the loss of the resistance-associated mutation (T790M) and restoration of tumor sensitivity to treatment with EGFR inhibitors in both preclinical and clinical settings (17–19). Our observations now suggest that the development of resistance to ALK inhibitors is potentially reversible. They provide a rationale for temporary cessation of treatment after the development of ALK-TKI resistance in patients with EML4-ALK–positive lung cancer to allow the tumor to regain drug sensitivity. Clinical evaluation of such an approach may thus be warranted.

In conclusion, with the use of *in vitro* cell lines and cells isolated from a patient, we have uncovered a previously unidentified mechanism of acquired ALK-TKI resistance in NSCLC positive for EML4-ALK. Our data add to the complexity of drug resistance mechanisms, further investigation of which is required if they are to be understood and conquered.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Tanizaki, I. Okamoto, T. Okabe, K. Takezawa, K. Nakagawa
Development of methodology: J. Tanizaki, T. Okabe, H. Kaneda, K. Nishio
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Tanizaki, I. Okamoto, T. Okabe, K. Sakai, K. Tanaka, H. Hayashi, K. Kuwata, H. Yamaguchi, E. Hatashita
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Tanizaki, I. Okamoto, K. Sakai, K. Kuwata, H. Yamaguchi, E. Hatashita, K. Nakagawa
Writing, review, and/or revision of the manuscript: J. Tanizaki, I. Okamoto, H. Kaneda, K. Nakagawa
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Tanizaki, K. Sakai, H. Hayashi, K. Kuwata, H. Yamaguchi, E. Hatashita
Study supervision: I. Okamoto, K. Nakagawa

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A phase I/II study of carboplatin plus gemcitabine for elderly patients with advanced non-small cell lung cancer: West Japan Thoracic Oncology Group Trial (WJTOG) 2905

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ABSTRACT

Introduction: Monotherapy with a third generation anticancer agent has been regarded as the standard therapy for elderly patients with advanced non-small-cell lung cancer (NSCLC). However, it is unclear whether elderly patients with a good performance status can tolerate platinum-doublet chemotherapy like younger patients.
Methods: A combination phase I/II study was conducted in chemo-naive elderly patients with NSCLC to establish the toxicity and maximum tolerated dose (MTD) and to investigate the antitumor activity of carboplatin (CBDCA) plus gemcitabine (GEM). GEM was infused on days 1 and 8, and CBDCA on day 1 every 3 weeks.
Results: Seventy-five patients were enrolled. The most frequent toxicities were hematological, especially thrombocytopenia. Three of three patients experienced a dose-limiting toxicity at dose level 3: 1000 mg/m^2 GEM with AUC 5 CBDCA (MTD), and one of seven patients at level 2a: 1000 mg/m^2 GEM with AUC 4 CBDCA (recommended dose). In the phase II study, the overall response rate was 22.2% and the median overall survival time was 14.2 months.
Conclusions: Although the recommended dosage is restricted to a lower level compared to younger patients, combination therapy using CBDCA with GEM is tolerable and promising for elderly patients with advanced NSCLC.

1. Introduction

Lung cancer is the most common cause of cancer-related death all over the world, and the number of elderly patients with lung cancer patients has increased in recent years. Nearly 50% of patients with advanced non-small cell lung cancer (NSCLC) are 70 years old or older [1]. Platinum-based combination chemotherapy is the standard treatment for younger patients with advanced NSCLC, who have good performance status (PS). Even elderly patients, if they have a good performance status, may tolerate aggressive treatment. However, many elderly patients cannot tolerate standard platinum-based chemotherapy as compared to their young counterparts, because of reduced organ function and concomitant morbidities. Therefore, some clinical trials have been performed to establish a standard chemotherapy regimen for elderly people with advanced NSCLC. The Elderly Lung Cancer Vinorelbine Italian Study (ELVIS) trial showed that chemotherapy with vinorelbine (VNB) alone was significantly more efficacious than only best supportive care in terms of overall survival and quality of life [2]. Furthermore, in two randomized controlled trials, gemcitabine alone or docetaxel alone was also considered a useful therapy [3,4]. These studies suggested single-agent chemotherapy was the appropriate

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approach to elderly patients by practice, however, as these studies included about 20% of patients with PS 2, we should interpret these results with caution. Recently, many efforts have been made to identify the elderly patients who can and those who cannot tolerate platinum-based doublet chemotherapy. Among them, a comprehensive geriatric assessment (CGA) that evaluates functional status, nutrition status, comorbidities, cognitive status, and socioeconomic status, has been used to assess the tolerability of elderly patients to cancer chemotherapy. Although this method has been used in clinical trials involving elderly patients with lung cancer, no consensus has been reached as yet. At present, in spite of many efforts, PS remains as the most important predictor of tolerability to chemotherapy in elderly patients. Until now, only a few phase II studies of CDDP-based chemotherapy were conducted in selected (good PS) elderly patients with advanced NSCLC [5–7]. Most of those studies found that CDDP-based chemotherapy was active and induced acceptable toxicities. However, those studies used CDDP with modified schedule and/or attenuated doses to reduce toxicities, such as renal, neural, and gastrointestinal toxicities. Considering this background, substitution of CBDCA-based chemotherapy for CDDP-based chemotherapy has drawn attention. Therefore, we planned to perform a phase I/II study of gemcitabine in combination with carboplatin for the elderly with advanced NSCLC but with a good performance status of 0–1 (PS 0–1). The purposes of this study are to determine the recommended dose, examine the feasibility of this combination chemotherapy in the phase I study, and assess the antitumor activity in the phase II study.

2. Patients and methods

2.1. Patient eligibility

Patients with histologically or cytologically confirmed NSCLC who had received no previous chemotherapy were eligible. The eligibility criteria were as follows: (1) stage IV or stage III lung cancer not amenable to surgery and curative radiotherapy; (2) measurable lesions; (3) age 70 years or older; (4) Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0–1; (5) adequate organ function [absolute neutrophil count (ANC) $\geq$ 2000/μL, platelet count $\geq$ 100,000/μL, hemoglobin count $\geq$ 9.5 g/dL, serum total bilirubin $\leq$ 1.5 mg/dL, serum transaminase $\leq$ 100 IU/L, serum creatinine $\leq$ 1.5 mg/dL, $SpO_2 \geq 90\%$]. Surgery and prior radiotherapy except for the primary tumors and evaluable lesions were allowed. At least two weeks had to have passed after completion of the previous radiotherapy. The exclusion criteria were as follows: (1) pulmonary fibrosis or interstitial pneumonitis with symptoms or apparent abnormalities on the chest X-ray; (2) massive pleural effusion, pericardial effusion, or ascites; (3) symptomatic brain metastases; (4) active concurrent malignancies; (5) patients who had received a bone marrow transplantation or peripheral blood stem cell transplantation; (6) severe drug allergies; (7) severe infections requiring antibiotics or severe comorbidities such as gastrointestinal bleeding and heart disease; (8) poorly controlled diabetes. This study was approved by the Institutional Review Board at each institute, and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

2.2. Pretreatment and follow-up studies

Prior to entry, complete history-taking and physical examination were performed regarding age, height, weight, performance status, histologic diagnosis, location of primary tumor, evaluable lesions and non-evaluable lesions, tumor stage, details of previous treatment, and presence of complications. The pretreatment laboratory investigations included complete blood cell count with differential WBC, platelet count, serum electrolytes, biochemical profile, chest X-ray, and radiographic imaging of chest, abdomen, brain, and bone by computed tomography scan, magnetic resonance imaging, and scintigraphy. After initiation of therapy, blood count and biochemical profile were repeated weekly. Chest X-ray was repeated once a week or once per two weeks. Lesions were measured every month. Toxicity was evaluated in accordance with the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. Tumor responses were assessed using the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines [8]. Survival estimation was performed using the Kaplan–Meier method [9].

2.3. Drug administration and dose escalation

The treatment schedule included gemcitabine given intravenously as a 30-min infusion on days 1 and 8, and carboplatin given intravenously over 60–90 min after completion of gemcitabine infusion on day 1 every 3 weeks. All patients were allowed to receive antiemetics with dexamethasone, metoclopramide, or 5HT3 antagonists at the discretion of the physician in charge. G-CSF prophylaxis was not allowed. Doses of gemcitabine on day 8 were given if the ANC was $\geq$1500/μL, platelet count $\geq$ 100,000/μL, serum total bilirubin $\leq$ 1.5 mg/dL, serum transaminases $\leq$ 100 IU/L, no fever elevation with infection, and the other non-hematological toxicities were less than grade 2. The subsequent courses were delayed if any of the following parameters were not met: PS 0–1, ANC $\geq$ 2000/μL, platelet count $\geq$ 100,000/μL, serum transaminases $\leq$ 100 IU/L, serum total bilirubin $\leq$ 1.5 mg/dL, serum creatinine $\leq$ 1.5 mg/dL, no fever elevation with infection, or the other non-hematological toxicities were less than grade 2.

If dose-limiting toxicities (DLTs) occurred, the patient was withdrawn from the study in principle, but when an antitumor effect could be expected, treatment continuation was possible by reducing the dose. Specifically, when DLTs occurred in level 2a, we reduced the dose to level 1. When DLTs occurred in level 2b, if level 2a had been tolerable and level 3 intolerable, we reduced the dose to level 2a. When DLTs also occurred in level 2b, if level 2a had been intolerable, we reduced the dose to level 1. When DLTs occurred in level 3, we reduced the dose to level 2a. At the initial dose level, dose reduction was not permitted and when it was required, the patient was withdrawn from this study.

The starting dose of gemcitabine was 800 mg/m^2 and the targeted AUC of carboplatin was 4 (level 1); the subsequent dose levels were as follows: level 2a; gemcitabine 1000 mg/m^2 and CBDCA AUC 4; level 2b, gemcitabine 800 mg/m^2 and CBDCA AUC 5; level 3, gemcitabine 1000 mg/m^2 and CBDCA AUC 5. When DLTs did not occur in level 1, we moved on to level 2a. When DLTs did not occur in level 2a, we moved on to level 3. If level 2a had not been tolerable, we moved on to level 2b. If level 3 had not been tolerable, we also moved back to level 2b. Intra-patient dose escalation was not allowed. At least three patients were treated at each dose level, and three additional patients were entered at the same dose level if DLT was observed in one or two of the first three patients. MTD was defined as the dose level at which all three of the first three patients, or three of any six patients, experienced DLT. The definitions of DLT were as follows: (1) grade 4 neutropenia for more than seven days; (2) grade 3 febrile neutropenia; (3) grade 4 thrombocytopenia or need for platelet transfusions; (4) grade 3 non-hematological toxicities; (5) delayed administration of the subsequent course by more than 2 weeks or delayed administration of gemcitabine on day 8 by more than one week.

Table 1
Patients' characteristics.

	Phase I part	Phase II part
No. of patients	25	55
Age, years		
Median	76	76
Range	72–83	71–86
Sex		
Male/female	15/10	37/18
Performance status		
0/1/2	8/16/1	20/35/0
Histology		
Adeno	15	40
Squamous cell	9	11
Large	0	1
Others	1	3
Stage		
II/IIIA/IIIB	0/2/10	1/3/11
IV	13	40
Previous therapy		
Surgery	1	7
Radiation	3	6

2.4. Statistical analysis

The primary endpoint of the phase II study was an objective response by RECIST. Secondary endpoints included progression free survival (PFS) and overall survival. This study assumed that a response rate of 35% would indicate usefulness, while a rate of 20% would be the lower limit of interest. With $\alpha = 0.1$ and $\beta = 0.1$, we required patient addition to become 50 patients in total at the recommended dose level. Survival time was calculated using the Kaplan–Meier method [9].

3. Results

3.1. Patient characteristics

Patients were registered from 14 institutions members of the West Japan Thoracic Oncology Group (WJTOG). Between July 2005 and December 2007, seventy-five patients were enrolled (phase I study; dose level – number of patients: 1–7, 2a–7, 2b–9, 3–3, phase II study; 2a–49, plus seven of level 2a of the phase I study to give a total of 56 patients). One patient at level 2b of the phase I study was excluded from the assessment of toxicity and efficacy as he could not receive this treatment due to persistent thrombocytopenia. In the phase II study, one patient was also excluded from the assessment of toxicity and efficacy because of registration error. Furthermore, another patient in the phase II study who was determined to have stage II NSCLC after registration, was excluded from the evaluation of efficacy, but assessed for toxicity. Therefore, the characteristics of 25 assessable patients in the phase I study and 55 in the phase II study are shown in Table 1. The median age was 76 (range 72–83) in the phase I study, and 76 (range 71–86) in the phase II study, respectively. Four patients of the phase I study and 13 of the phase II study received previous radiation and/or surgery.

3.2. Toxicities encountered in the phase I study

The median number of courses for the 25 assessable patients was 3.0 (range 1–9). The major treatment-related toxicities after all courses are listed in Table 2. The most frequent toxicities were hematological. More than grade 3 thrombocytopenia occurred in 13 of 25 patients (52%), and four patients (16%) received platelet transfusions (one patient at level 2a, two at level 2b, and one at level 3); and, one patient at level 2b presented hemosputum. More than grade 3 leukopenia, neutropenia, and anemia occurred in 52%, 60%, and 40% of the patients, respectively. Non-hematological toxicities were generally mild. One patient at level 2a experienced grade 3 pneumonitis. There was no case of treatment-related death. Four patients underwent dose reduction after the first course owing to treatment-related adverse events (two patients at level 2b, two at level 3). The median length of the delay before starting the subsequent course was 25 days (21–41 days). Among 83 courses, 58% of courses proceeded to the next course without delay, as is stipulated in the protocol.

Table 2
Treatment-related adverse events in the phase I part ($n = 25$) and in the phase II part ($n = 55$).

	Phase I part Grade				Phase II part Grade			
	1	2	3	4	1	2	3	4
Leucopenia	3	5	12	1	8	17	24	2
Neutropenia	0	3	9	6	0	10	18	16
Anemia	1	9	8	2	8	28	8	0
Thrombocytopenia	6	6	9	4	15	13	18	6
Nausea	11	4	0	0	19	6	0	0
Vomiting	3	5	0	0	4	3	0	0
Appetite loss	13	5	1	0	22	8	1	1
Fatigue	8	1	1	0	21	6	1	1
Constipation	4	3	0	0	12	1	0	0
AST	11	2	1	0	25	2	0	0
ALT	9	4	0	0	17	3	1	0
Pneumonitis	0	0	1	0	0	0	1	0

3.3. MTD, DLTs and response to treatment in the phase I study

At level 1, one patient suffered from grade 3 appetite loss, which was considered a DLT. As six other patients at level 1 did not experience any DLTs, the dose was escalated to the next level. At level 2a, one patient experienced grade 4 thrombocytopenia on day 14 and received platelet transfusions. Moreover, the same patient developed dyspnea on day 22, and radiographic images showed pneumonitis of grade 3. Six other patients at level 2a did not experience any DLTs. At level 3, all three enrolled patients had DLTs, which consisted of grade 3 neutropenic fever, grade 4 thrombocytopenia requiring platelet transfusions, and delayed administration of gemcitabine on day 8 by more than one week due to hematological toxicities. According to the protocol criteria, the dose level was lowered to level 2b. At level 2b, three of eight assessable patients experienced DLTs, grade 3 dyspnea which was unrelated to pneumonitis, delayed administration of gemcitabine on day 8 by more than one week due to hematological toxicities, and grade 4 thrombocytopenia with hemosputum on day 14 requiring platelet transfusions. The protocol criteria indicated that dose level 3 was intolerable, but level 1, 2a, and 2b were considered as tolerable. As a result after considering toxicities, the Independent Data Monitoring Committee decided to choose level 2a (gemcitabine 1000 mg/m^2, CBDCA AUC 4) as recommended dose (Table 3).

As for efficacy in the phase I study, there were five partial responses among 25 assessable patients, with an overall response rate of 20% (Table 4).

3.4. Toxicities in the phase II study

The median number of courses was 3.0 (range 1–6). The major treatment-related toxicities after all courses are listed in Table 2. The most frequent toxicities were hematological as well as those encountered in the phase I study. More than grade 3 thrombocytopenia occurred in 24 of 55 assessable patients (44%), and four patients (16%) received platelet transfusions. No patient had severe hemorrhagic complications. More than grade 3 leukopenia, neutropenia, and anemia occurred in 47%, 62%, and 15% of the patients, respectively. Non-hematological toxicities were generally mild, the

Table 3
Dose limiting toxicities (DLTs) in the phase I part.

Dose level	CBDCA AUC	GEM mg/m^2	No. of pts	No. of pts with DLTs	DLTs
1	4	800	7	1	Grade 3 appetite loss
2a (RD)	4	1000	7	1	Grade 4 thrombocytopenia + grade 3 pnuemonitis
3 (MTD)	5	1000	3	3	Delay in administration of GEM on day 8 due to hematological toxicities Grade 4 thrombocytopenia Grade 3 neutropenic fever
2b	5	800	8	3	Grade 3 dyspnea without pnuemonitis Delay in administration of GEM on day 8 due to hematological toxicities Grade 4 thrombocytopenia

CBDCA, carboplatin; GEM, gemcitabine; pts, patients; AUC, area under the blood concentration time curve; RD, recommneded dose; MTD, maximum tolerated dose.

Table 4
Tumor response in the phase I part and in the phase II part.

	No. of pts	CR (%)	PR (%)	SD (%)	PD (%)	NE (%)
Phase I part						
Level 1	7	0 (0)	2 (28.6)	2 (28.6)	2 (28.6)	1 (14.3)
Level 2a	7	0 (0)	1 (14.3)	3 (42.9)	2 (28.6)	1 (14.3)
Level 2b	8	0 (0)	1 (12.5)	5 (62.5)	2 (25.0)	0 (0)
Level 3	3	0 (0)	1 (33.3)	2 (66.7)	0 (0)	0 (0)
Total (phase I part)	25	0 (0)	5 (20.0)	12 (48.0)	6 (24.0)	2 (8.0)
Phase II part	54	0 (0)	12 (22.2)	28 (51.9)	8 (14.8)	6 (11.1)

Objective response rate in the recommended dose: 22.2% (95% CI: 11.1–33.3). CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; pts, patients.

same as in the phase I study. There was no case of pneumonitis in the additional 48 patients. Twelve patients required dose reduction due to primarily hematological toxicities. The median length of the delay before starting the subsequent course was 27 days (21–46 days).

3.5. *Efficacy in the phase II study*

Among 54 evaluable patients, there were 12 partial responses, for an overall response rate of 22.2% (95% confidence interval (CI); 11.1–33.3). And disease control rate of 74.5% was observed in 28 patients having stable disease (Table 4). The median progression free survival time and the median overall survival time were 4.3 months (95% CI; 4.2–5.5), and 14.2 months (10.3–18.2), respectively (Fig. 1A and B). The 1-year survival rate was 59.8%.

4. Discussion

Monotherapy with a third generation anticancer agent such as vinorelbine or gemcitabine has been regarded as the standard therapy for elderly patients with advanced non-small-cell lung cancer (NSCLC) on the basis of results of several randomized phase III studies that included patients with PS 0–2 [2–4]. However, it has been recently proposed that elderly patients with PS 0–1 whose general condition is relatively well can tolerate platinum-doublet chemotherapy like younger patients. Thus, this phase I/II study was conducted in which elderly patients with advanced NSCLC were treated with the combination of CBDCA and gemcitabine that is one of the standard treatments for younger patients with NSCLC.

On the basis of the results from the phase I part of the study, dose level 2a was selected as the recommended dosage in the phase II part: specifically, CBDCA AUC 4 on day 1 plus gemcitabine 1000 mg/m^2 on days 1 and 8 of a 3-week cycle. These doses were distinctly lower than the standard doses for younger patients, that is, CBCDA AUC 5 to 5.5 plus gemcitabine 1000–1200 mg/m^2 [10–12]. Dose level 3 in the present study, which was the same with the standard doses for younger patients, was found to be intolerable since DLTs were observed in three of three patients at this dose level. Although the present study enrolled selective elderly patients with PS 0–1, the obtained results were different from those in younger patients. In fact, Maestu et al. treated elderly patients with advanced NSCLC with CBDCA AUC 4 on day 1 plus gemcitabine 1250 mg/m^2 on days 1 and 8 of a 3-week cycle in their phase II study [13]. In recent years, many clinical studies have set no upper age limit and enrolled any patients as long as their performance status (PS) is preferable. However, the results of the present study suggest necessity of clinical studies specific in elderly patients. Frequency

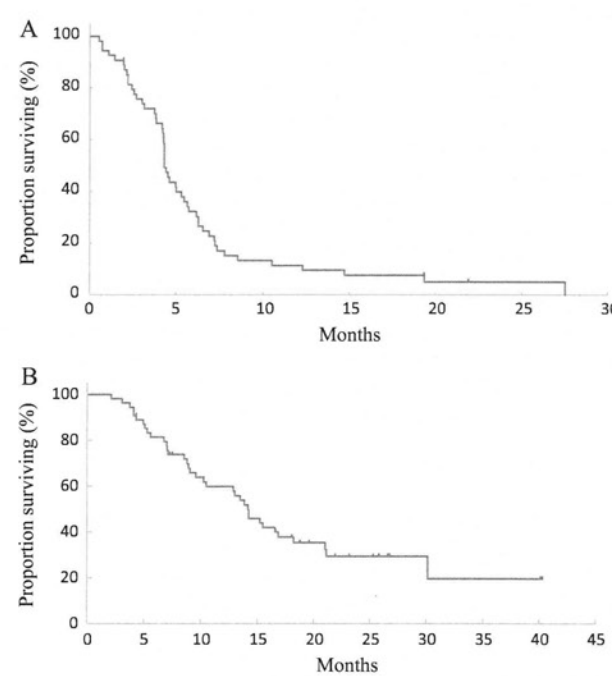

Fig. 1. (A) Kaplan–Meier curve for progression-free survival, (B) Kaplan–Meier curve for overall survival.

and severity of toxicity noted in elderly patients that were different from those in younger patients as expected also warrant conduct of dose finding and feasibility studies in elderly patients.

The DLT of the CBDCA/gemcitabine combination used in the present study was hematotoxicity mainly involving thrombocytopenia. Three randomized phase III studies have been conducted to evaluate the CBDCA/gemcitabine combination in younger patients with advanced NSCLC [10–12]. Rudd et al. compared this combination to MIP (mitomycin, ifosfamide and cisplatin) and reported that grade 3/4 thrombocytopenia and neutropenia occurred in 24% and 34% of the patients, respectively [10]. Zatloukal et al. compared this combination to the combination of cisplatin plus gemcitabine and reported that grade 3/4 thrombocytopenia and neutropenia occurred in 22.6% and 30.3% of the patients, respectively [11]. In the three-arm comparative phase III study of Treat et al. that included the CBDCA/paclitaxel and paclitaxel/gemcitabine combinations in addition to the CBDCA/gemcitabine combination, the reported incidences of grade 3/4 thrombocytopenia and neutropenia were 64.6% and 39%, respectively. The observed higher incidence of thrombocytopenia in the third studies compared to the other studies might be attributable to the dose of CBDCA AUC 5.5 used in the third study [12]. Nonetheless, no bleeding events were reported in any of these studies. The incidences of grade 3/4 thrombocytopenia and neutropenia in the present study were 44% and 62%, respectively. The incidence of neutropenia was slightly higher than the above mentioned three studies [10–12], while the incidence of thrombocytopenia was comparable. No bleeding complications were reported in the present study. At the recommended doses of CBDCA and gemcitabine selected and used in the present study, thrombocytopenia observed in elderly patients was not so serious and was tolerable.

In the phase II part of the present study, the response rate as the primary endpoint was 22.2% (95% CI, 11.1–33.3%) with the median PFS of 4.3 months and median overall survival of 14.2 months. Since the lower confidence limit of the response rate was below the predefined margin of 20%, this combination therapy is probably not considered to be definitely effective. However, the disease control rate of 74.5% and median overall survival of 14.2 months are considerably promising results. Recently, it came to be known that mutations in the tyrosine kinase domain of the *epidermal growth factor receptor* (*EGFR*) gene are associated with significant responses of EGFR tyrosine kinase inhibitors (TKIs) and increased progression free survival [14,15]. These mutations are significantly more frequent in Japanese subjects, female patients, patients with adenocarcinoma, or nonsmokers [16,17]. Therefore, treatment with EGFR TKIs after progression might have contributed to improvement of overall survival in patients with particular adenocarcinoma. However, at the time when this study was conducted, measurement of EGFR mutations was not commercially available in Japan and the association between the effects of EGFR TKIs and EGFR mutations was still unknown, although gefitinib and erlotinib were already approved. Therefore, we have no information about the frequency of EGFR mutations in this study.

At all events, it is reasonable to initiate a randomized phase III study to compare the CBDCA/gemcitabine combination to monotherapy with a third generation anticancer agent in elderly patients with advanced NSCLC with good PS.

Recently, results of two randomized studies which compared platinum-based combination chemotherapy with single-agent chemotherapy were reported with contradicting conclusion [18,19]. The French study group performed a randomized phase III study comparing the combination of CBDCA and paclitaxel to monotherapy with a third generation anticancer agent in elderly patients with advanced NSCLC. Their results indicated that the combination therapy was associated with severer toxicity but led to a statistically significant improvement in the survival [18]. On the other hand, Japanese intergroup trial also reported results of a randomized phase III study comparing the combination of weekly CDDP and docetaxel with tri-weekly docetaxel alone in elderly patients with advanced NSCLC at the 2011 ASCO Annual Meeting [19]. Unlike the French study, this study could not show the superiority of the CDDP-based combination chemotherapy, although the possibility that divided doses of CDDP lowered the effects could not be denied. From these results, the CBDCA-based chemotherapy would become a standard treatment in selective elderly patients and our challenge for the future is to determine whether PS is the single factor required for selection of patients who can tolerate CBDCA-based chemotherapy or any other factors should be also evaluated. Individualized selection of therapy in elderly patients will also become very important in the near future.

In conclusion, although the recommended dosage is restricted to a lower level compared to younger patients, the combination therapy of CBDCA and gemcitabine is tolerable in elderly patients with advanced NSCLC and the observed favorable disease control rate and overall survival suggest that this combination therapy is effective in this patient group.

Conflict of interest statement

Dr. Kurata has received honoraria from Eli Lilly; the others have no conflict of interest.

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