NSCLC. Several mechanisms are believed to be responsible for intrinsic and acquired resistance to EGFR-TKIs, including secondary *EGFR* T790M and minor mutations, *MET* amplification, and activation of the MET/HGF axis, acquiring an epithelial to mesenchymal transition (EMT) signature, and transformation from NSCLC into small cell lung cancer (SCLC) [8-13]. More recently, AXL kinase activation, loss of the EGFR-mutant allele, and emergence of cancer-stem cell (CSC)-like properties have been reported as possible mechanisms of resistance [14-16]. However, it is likely that additional mechanisms remain to be identified.

In this review, we focus on the NSCLCs harboring *EGFR*-activating mutations, and we summarize the mechanisms of drug sensitivity and resistance to EGFR-TKIs. We also describe some possible molecularly targeted strategies for further improving the outcomes of NSCLC patients with *EGFR*-activating mutations.

## *EGFR*-activating Mutations in NSCLC

EGFR (ErbB1) is a member of the ErbB transmembrane receptor family, which includes ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4). These receptors have similar structures and consist of three domains: an extracellular domain, a transmembrane domain, and an intracellular domain. The extracellular domain has a ligand-biding region, and several ligands including EGF bind here. The ligand binding causes receptor homo- or hetero-dimerization between EGFR and other ErbB family members at the cell surface, followed by internalization of the dimerized receptor. The receptor dimerization results in autophosphorylation of the intracellular EGFR tyrosine kinase domain. Subsequently, the phosphorylated tyrosine kinase stimulates an intracellular signal transduction cascade through several downstream pathways (including the Ras-Raf-MEK-ERK, PI3K-AKT-mTOR, and JAK-STAT3 pathways), leading to cell proliferation and apoptosis (Fig. 1) [17, 18].

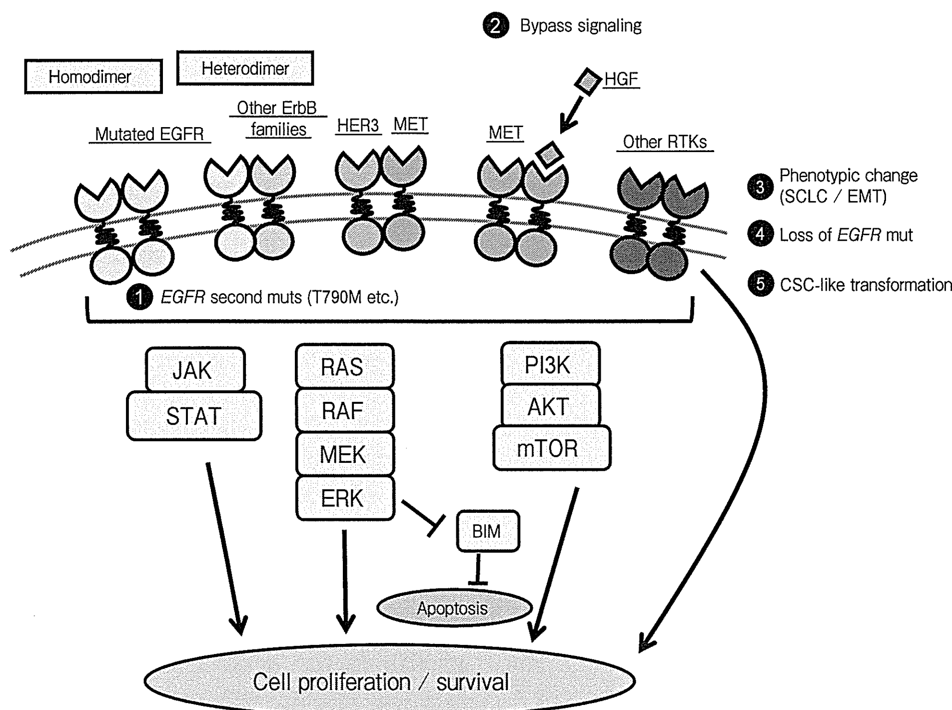


Fig. 1 Signaling pathways and mechanisms of acquired resistance to EGFR-TKIs in *EGFR*-mutated NSCLC. (1) *EGFR* T790M mutations and other less common mutations. (2) Kinase switches and bypass signaling mechanisms. (3-5) Other possible mechanisms related to acquired EGFR-TKI resistance. mut, mutation; RTK, receptor tyrosine kinase; SCLC, small-cell lung cancer; EMT, epithelial to mesenchymal transition; CSC, cancer-stem cell.

When a mutation occurs in exons that encode the EGFR tyrosine kinase protein (*i.e.*, exons 18–21), EGFR is activated ligand-independently, leading to carcinogenesis [2, 3]. About 80%–90% of these *EGFR* mutations are either short in-frame deletions in exon 19 or point mutations that result in a substitution of arginine for leucine at codon 858 (L858R) in exon 21 [19]. Approximately 3% of the mutations occur at codon 719, resulting in the substitution of glycine to cysteine, alanine or serine (G719X) in exon 18. Another approx. 3% are in-frame insertion mutations in exon 20 [19]. These *EGFR*-activating mutations are most common in patients with adenocarcinoma histology, women, never-smokers, and individuals of Asian ethnicity; approx. 40% of lung adenocarcinoma patients in Japan have an *EGFR* mutation [20–22]. *EGFR* mutations have also been detected in normal small bronchial and bronchiolar epithelium obtained from sites adjacent to tumors, suggesting that the EGFR mutations are early events in the pathogenesis of lung adenocarcinomas [23, 24].

Cancer cells with mutant *EGFR* are physiologically dependent on the continued activity of specifically activated or overexpressed oncogenes for the maintenance of their malignant phenotype, in a phenomenon called 'oncogene addiction' [25]. This addiction, at the same time, results in a greater sensitivity to small-molecule inhibitors that target the kinase domain of EGFR. In first-line treatment, EGFR inhibitors showed approx. 75% response rate in patients with typical *EGFR* mutations. Randomized trials have also demonstrated improved PFS for *EGFR*-mutant patients receiving EGFR-TKIs compared to chemotherapy [4–7].

## Molecular Mechanisms in Resistance to EGFR-TKI

***Primary resistance to EGFR-TKIs.*** There are some cancer cell populations that exhibit intrinsic resistance to EGFR-TKIs although they have *EGFR*-activating mutations. Multiple clinical trials have shown a disease control rate of approx. 90% for patients with *EGFR* mutations, suggesting that 10% of the patients harboring *EGFR* mutations are intrinsically resistant to EGFR-TKIs [4–7]. Some molecular mechanisms of this primary resistance have been uncovered in recent research.

**1. EGFR-TKI-resistant mutations.** It has been shown that the most prevalent *EGFR* exon 20 insertion mutation, which accounts for up to 4% of all *EGFR* mutations, is resistant to reversible (gefitinib and erlotinib) and irreversible (neratinib, afatinib, and dacomitinib) EGFR-TKIs in preclinical models and clinical samples [26, 27]. Another mutation that contributes to primary TKI resistance is T790M, a point mutation that results in the substitution of methionine for threonine at codon 790 in exon 20. They show TKI resistance through steric hindrance to EGFR-TKIs in crystal structure analyses or by increased affinity for adenosine triphosphate (ATP) [8, 28]. T790M has been identified as a minor clone in treatment-naïve tumor specimens with *EGFR*-activating mutations [29–31]. Su *et al.* reported that T790M was detected in 2.8% by direct sequencing, 25.2% by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), and 34.2% by next-generation sequencing (NGS) in TKI-naïve NSCLC tumors harboring *EGFR*-activating mutations [31].

**2. EGFR signal-related alteration.** Some EGFR signal-related gene alterations have been reported to contribute to primary EGFR-TKI resistance. It has been reported that *EGFR* mutations and *PIK3CA* mutations could co-occur and result in EGFR-TKI resistance [32–34]. *PIK3CA* mutations have also been shown to be acquired after EGFR-TKI treatment and to induce acquired TKI resistance [13]. Loss of phosphatase and tensin homolog (PTEN) similarly contributes to primary resistance to EGFR-TKIs [34, 35]. The pro-apoptotic protein BIM is known to be a mediator of TKI-induced apoptosis, and it is upregulated in some *EGFR* mutant cancer cells [36]. The inhibition and downregulation of BIM expression promoted intrinsic resistance to EGFR-TKIs in a preclinical model and clinical samples [37]. A recent report suggests that a genetic polymorphism in *BIM* results in alternative splicing and altered BIM function, which may contribute to intrinsic TKI resistance [38].

**3. Non-EGFR signal-related alteration.** Hepatocyte growth factor (HGF), a ligand of MET receptor tyrosine kinase, was reported to induce the EGFR-TKI resistance of cancer cells harboring *EGFR* mutations by restoring the PI3K-AKT signaling pathway via the phosphorylation of MET [12]. HGF was overexpressed in approx. 30% primary resistant

NSCLC harboring *EGFR* mutations, suggesting the activation of the MET signal pathway through HGF stimulation might be associated with primary TKI resistance.

***Acquired resistance to EGFR-TKIs.*** All patients with *EGFR* mutations who initially respond to the first-generation EGFR-TKIs gefitinib or erlotinib ultimately develop acquired resistance to EGFR-TKIs over time (median 6-12 months). Acquired resistance to EGFR-TKIs is strongly associated with patient mortality, and thus further investigations of the mechanisms of acquired resistance to EGFR-TKIs are of great importance.

**1. EGFR T790M "gatekeeper" mutation and other less common mutations.** The most common mutation associated with acquired resistance to EGFR-TKIs is *EGFR* T790M, a secondary point mutation in exon 20 [8, 9]. T790M is associated with over 50% of adenocarcinoma cases with acquired resistance [13, 39]. *EGFR* T790M is analogous to the *ABL* T315I, *KIT* T670I, and *ALK* L1196M "gatekeeper" mutations observed in imatinib-resistant chronic myelogenous leukemia, gastrointestinal stromal tumors (GISTs), and crizotinib-resistant NSCLCs, respectively [40-42]. Interestingly, among patients with acquired resistance to EGFR-TKIs, although the molecular basis is unclear, the presence of T790M is associated with a favorable prognosis relative to acquired resistance via other processes [43]. Other less common mutations associated with EGFR-TKI resistance include *EGFR* D761Y (in TKI-naïve and acquired-resistant tumors) [30, 44], L747S [45], and T854A [46].

**2. "Kinase switch" and bypass signaling mechanisms.** Acquired resistance to EGFR-TKIs can develop through a "kinase switch" mechanism. One major bypass signaling is the MET, the receptor of HGF. *MET* amplification was observed in 5%-20% of tumor samples with acquired resistance to EGFR-TKIs [10, 11, 13, 47]. The cancer cells with *MET* amplification undergo a kinase switch through an ErbB3-mediated activation of downstream PI3K-AKT signaling that bypasses the inhibited EGFR [10, 11]. Other bypass signaling tracts through *HER2* amplification [48], *CRKL* amplification [49], *MAPK1* amplification [50], *PIK3CA* mutations [13], and *BRAF* mutations [51] have been described as possible mechanisms of acquired EGFR-TKI resistance. Moreover, in several preclinical models, the loss of IGF binding proteins (IGFBPs) with the subsequent activation of IGF1R signaling [52], FGFR1 activation through FGF2 autocrine [53], increased FAS expression and NF$\kappa$B pathway activation [54], and upregulation of integrin beta1 [55] caused by EGFR-TKI treatment have also been reported to result in acquired EGFR-TKI resistance.

**3. Phenotypic change: small-cell transformation and EMT.** Examinations of re-biopsied samples revealed that phenotypic changes could occur and be responsible for acquired resistance after EGFR-TKI treatment. Some studies observed the transformation from NSCLC to small-cell lung cancer (SCLC) after EGFR-TKI treatment [13, 56]. These tumors maintained the *EGFR*-activating mutation with the expression of neuroendocrine markers and, surprisingly, they responded to conventional chemotherapy for SCLC.

Another well-known phenotypic change related to acquired resistance involves EMT. EMT is a phenomenon in which cells with epithelial phenotypes acquire mesenchymal characteristics, and EMT plays an important role in cancer metastasis and drug resistance. In preclinical models and clinical samples, EMT features were observed after the acquisition of resistance to EGFR-TKIs [13, 16, 57-59]. The activation of several pathways including the TGF-$\beta$ -IL-6 [60], Slug [61], Notch-1 [62], and PDGFR [63] pathways were reported to be associated with EMT and EGFR-TKI resistance. Possible mechanisms such as Axl upregulation [14] and MED12 downregulation [64] were reported as key molecules in EMT-related EGFR-TKI resistance. In addition, we reported the relation between epigenetic alteration and EGFR-TKI treatment [16]. We showed that the CpG island hypermethylation-associated silencing of the miR-200 family in acquired resistance to EGFR-TKI cells with EMT features.

**4. Loss of activating mutation.** We and another group reported that the loss of the activated *EGFR* mutant allele could result in acquired EGFR-TKI resistance [15, 16]. We established an EGFR-TKI-resistant cell line using the *EGFR*-mutated and -amplified cell line HCC827 under exposure to a high concentration of gefitinib, and the results revealed that the cells showed a progressive decrease in the *EGFR*-mutated and -amplified allele through the course of

passages. We confirmed in clinical samples obtained from before and after EGFR-TKI failure that the *EGFR* 19del mutation had disappeared in recurrent tumors [16].

**5. Stem-cell like transformation and other mechanisms.** We have established many cell lines with acquired EGFR-TKI resistance under different cell culture conditions, and these cell lines showed that the manner of drug exposure could influence the mechanism of their acquired resistance [16]. In general, drug-resistant cell lines were established under a stepwise escalation of concentration. However, we established the EGFR-TKI-resistant cell lines under an initially high concentration of EGFR-TKI (but similar to the plasma concentration after an oral administration of EGFR-TKIs). As a result, some established resistant cells under a high concentration of EGFR-TKI showed CSC-like features with EMT features (including CSC-related marker upregulation), increased side-population, and self-renewal capability (Fig. 2). The cells showed extremely high drug resistance to not only multiple EGFR-TKIs but also conventional chemotherapeutic agents.

CSCs have been attracting interest as a source of cancer cells, and the significance of stem cell-like properties in lung cancer has been investigated in both basic and clinical research [65-67]. Many of the relationships between CSCs and EGFR-TKI resistance remain unclear and the biological meaning of CSC-related markers such as ALDH1A1, ABC-transporters ABCB1 and ABCG2, and CD44 is unknown. Further research is needed to obtain additional clarification.

## Overcoming Molecular Mechanisms of Resistance to EGFR-TKIs

The first-generation EGFR-TKIs gefitinib and erlotinib have been used as first-line or second-line therapy for advanced *EGFR*-mutant NSCLCs, although sequential drug resistance has been inevitable. Many investigators have attempted to delay or overcome this resistance through preclinical examinations and clinical trials, and some promising strategies have been reported.

***Beyond progressive disease (PD) strategies and the re-challenge of TKIs.*** The repetitive use of EGFR-TKIs in *EGFR*-mutant patients with acquired resistance to gefitinib or erlotinib might be clinically beneficial in select patients. Several reports have demonstrated that patients who acquire resistance could re-respond to EGFR-TKIs after a drug

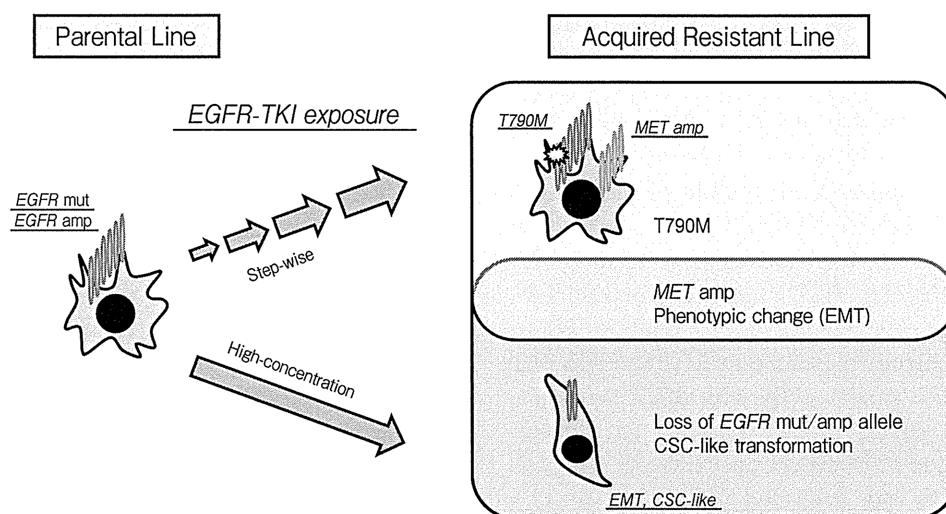


Fig. 2 Preclinical model of acquired resistance to EGFR-TKIs. The drug exposure method could affect the acquisition mechanisms of EGFR-TKI resistance. Under a conventional step-wise concentration of EGFR-TKI exposure, cancer cells with the *EGFR* T790M mutant or *MET* amplification were observed. In contrast, under a high (but similar to the plasma) concentration of EGFR-TKI exposure, cancer cells with wild-type *EGFR* or cancer stem cell (CSC)-like feature appeared. mut, mutation; amp, amplification; EMT, epithelial to mesenchymal transition; CSC, cancer-stem cell.

holiday [68, 69]. A prospective trial is being conducted to test whether an EGFR-TKI in addition to chemotherapy beyond progression is better than chemotherapy alone at the time of resistance (NCT01544179).

***Next-generation kinase inhibitors and the blockade of bypass signaling.*** To delay or overcome EGFR-TKI resistance, second- and third-generation EGFR-TKIs which are more potent than first-generation TKIs and could affect other receptors/pathways are being developed. Second-generation irreversible EGFR-TKIs such as afatinib (BIBW-2992) and dacomitinib (PF-299804) are ATP mimetics that covalently bind to the Cys-797 of EGFR, and they are reported to be able to inhibit T790M in cis to *EGFR* activating mutation at lower concentrations than first-generation TKIs in preclinical models. In addition, third-generation EGFR inhibitors such as WZ-4002, CO-1686, and AZD-9291 have been developed as EGFR inhibitors specifically selected to target *EGFR* mutations with T790M [70-72]. Several prospective clinical trials evaluating these drugs are currently ongoing. At the same time, T790M status is becoming important to predict patient response. Therefore, an examination of the T790M status in addition to *EGFR*-activating mutation before and during EGFR-TKI treatment is important. It will also be necessary to establish methods to repeatedly quantitate the T790M population using noninvasive techniques such as a circulating DNA analysis (the so-called "liquid-biopsy").

Other approaches to overcome resistance are combination treatment with TKIs and other conventional chemotherapies, antibodies, and immunotherapies. The combination of both irreversible EGFR-TKI BIBW-2992 and the EGFR-specific antibody cetuximab was reported to induce the dramatic shrinkage of erlotinib-resistant tumors harboring the T790M mutation, because together BIBW-2992 and cetuximab efficiently depleted both phosphorylated and total EGFR [73]. Such strategies like this method blocking both the intracellular and the extracellular domains of the EGFR, a so-called "vertical blockade", might be an additional strategy to effectively overcome EGFR-TKI resistance.

As mentioned above, acquired EGFR-TKI resistance can develop through kinase switches and alternative bypass signal activations. The blockade of each activated signal consonant with individual resistant cells could contribute to the delay and overcoming of acquired resistance. In this sense, the most promising strategy in preclinical modes may be the dual use of MET and EGFR-TKIs in cells with *MET* amplification [10, 74].

***Novel agents against EGFR-TKI resistance: epigenetic drugs, immuno-gene therapy, and others.*** It has been reported that epigenetic alterations are a key determinant in the maintenance of cancer cells, especially with high-level resistance to cytotoxic therapy and potent tumorigenic capacity [75]. Among these epigenetic alterations, DNA methylation and chromatin deacetylation are the most fundamental alterations. Whereas genetic alterations are usually fixed in the genome, epigenetic alterations are potentially reversible, offering a therapeutic opportunity. Histone deacetylate (HDAC) is an enzyme that regulates chromatin remodeling and is crucial in the epigenetic regulation of various genes. In preclinical studies, HDAC inhibitors such as trichostatin A and vorinostat (SAHA) showed an anti-tumor effect in EGFR-TKI resistant cells due to *BIM* polymorphism [76] and CSC-like features [16].

Heat shock protein (HSP) 90 inhibitors may also overcome EGFR-TKI resistance. A number of signaling molecules in the EGFR pathway are processed for activation and degradation by the HSP family of enzymes. Because the increased expression of these HSP clients mediates resistance to EGFR inhibitor therapy, HSP90 inhibitors represent a promising class of agents [77-79]. In addition, we found that the proteasome inhibitor bortezomib had an anti-tumor effect in both parental and acquired EGFR-TKI-resistant cells harboring T790M, *MET* amplification, and CSC-like features in a preclinical model [16].

In a recent preclinical study, we demonstrated that gene therapy using REIC/Dkk-3-expressing adenovirus vector (Ad-REIC) showed a potent anti-tumor effect in many NSCLC cells, even after they harbored acquired resistance to EGFR-TKIs [80]. A clinical trial to test the anti-tumor effect of Ad-REIC against NSCLC showing resistance to conventional drugs is in preparation. This new type of therapeutic strategy that may not target EGFR or other oncogene pathways could be a breakthrough to overcome EGFR-TKI resistance.

## Conclusions

We provided an overview of drug resistance mechanisms in EGFR-TKI treatment and presented some possible strategies to overcome EGFR-TKI resistance. Both cancer cell autonomous mechanisms and the tumor microenvironment could contribute to primary and acquired EGFR-TKI resistance.

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# A New Human Lung Adenocarcinoma Cell Line Harboring the *EML4-ALK* Fusion Gene

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**Objective:** The *echinoderm microtubule associated protein-like 4 (EML4)-anaplastic lymphoma kinase (ALK)* fusion gene was identified in patients with non-small cell lung cancer. To the best of our knowledge, there are only three cell lines harboring the *EML4-ALK* fusion gene, which have contributed to the development of therapeutic strategies. Therefore, we tried to establish a new lung cancer cell line harboring *EML4-ALK*.
**Methods:** A 61-year-old Japanese female presented with chest discomfort. She was diagnosed with left lung adenocarcinoma with T4N3M1 Stage IV. Although she was treated with chemotherapy, her disease progressed with massive pleural effusion. Because the *EML4-ALK* rearrangement was found in a biopsied specimen using fluorescence *in situ* hybridization, she was treated with crizotinib. She did well for 3 months.
**Results:** Tumor cells were obtained from the malignant pleural effusion before treatment with crizotinib. Cells continued to proliferate substantially for several weeks. The cell line was designated ABC-11. The EML4-ALK fusion protein and genes were identified in ABC-11 cells using fluorescence *in situ* hybridization and immunohistochemistry, respectively. ABC-11 cells were sensitive to crizotinib and next-generation *ALK* inhibitors (ceritinib and AP26113), as determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Phosphorylated ALK protein and its downstream signaling were suppressed by treatment with crizotinib in western blotting. Furthermore, we could transplant ABC-11 cells subcutaneously into BALB/c nu/nu mice.
**Conclusions:** We successfully established a new lung adenocarcinoma cell line harboring the *EML4-ALK* fusion gene. This cell line could contribute to future research of *EML4-ALK*-positive lung cancer both *in vivo* and *in vitro*.

*Key words: lung cancer – EML4-ALK – crizotinib*

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## INTRODUCTION

The discovery in 2007 of the *echinoderm microtubule associated protein-like 4* (*EML4*)-*anaplastic lymphoma kinase* (*ALK*) fusion gene in non-small cell lung cancer (NSCLC) (1) highlighted the importance of ALK tyrosine kinase inhibitors (TKIs). A first generation ALK-TKI, crizotinib, which was initially formulated as a c-MET inhibitor, caused dramatic responses in patients with *EML4-ALK*-positive tumors in early clinical trials (2). The US Food and Drug Administration approved crizotinib (Xalkori®, Pfizer Inc., NY, USA) <4 years after the discovery of the fusion gene. Subsequently, crizotinib has resulted in superior progression-free survival compared with platinum-based chemotherapy (3).

Clinical samples have been employed in most ALK studies, including those assessing fusion gene variants, fusion genes with a positive ratio in fluorescence *in situ* hybridization (FISH), and mechanisms of resistance to crizotinib (4–7). The results of these studies suggest that ALK-positive lung cancers are heterogeneous. Therefore, ALK-positive lung cancers should be assessed in several different ways. To the best of our knowledge, only three cell lines harboring the *EML4-ALK* fusion gene have been reported: H2228 (*EML4-ALK* variant 3a/b E6; A20), H3122 and DFCI032 (*EML4-ALK* variant 1 E13; A20) (5). Therefore, additional ALK-positive cell lines are needed as tools for basic research to develop novel therapeutic strategies for this disease. In this study, we established a new cell line derived from a patient with NSCLC harboring the *EML4-ALK* fusion gene. This cell line could be useful for investigating ALK-positive lung cancer.

## PATIENTS AND METHODS

### Patient

A 61-year-old Japanese female never-smoker presented with chest discomfort. She was diagnosed with left lung

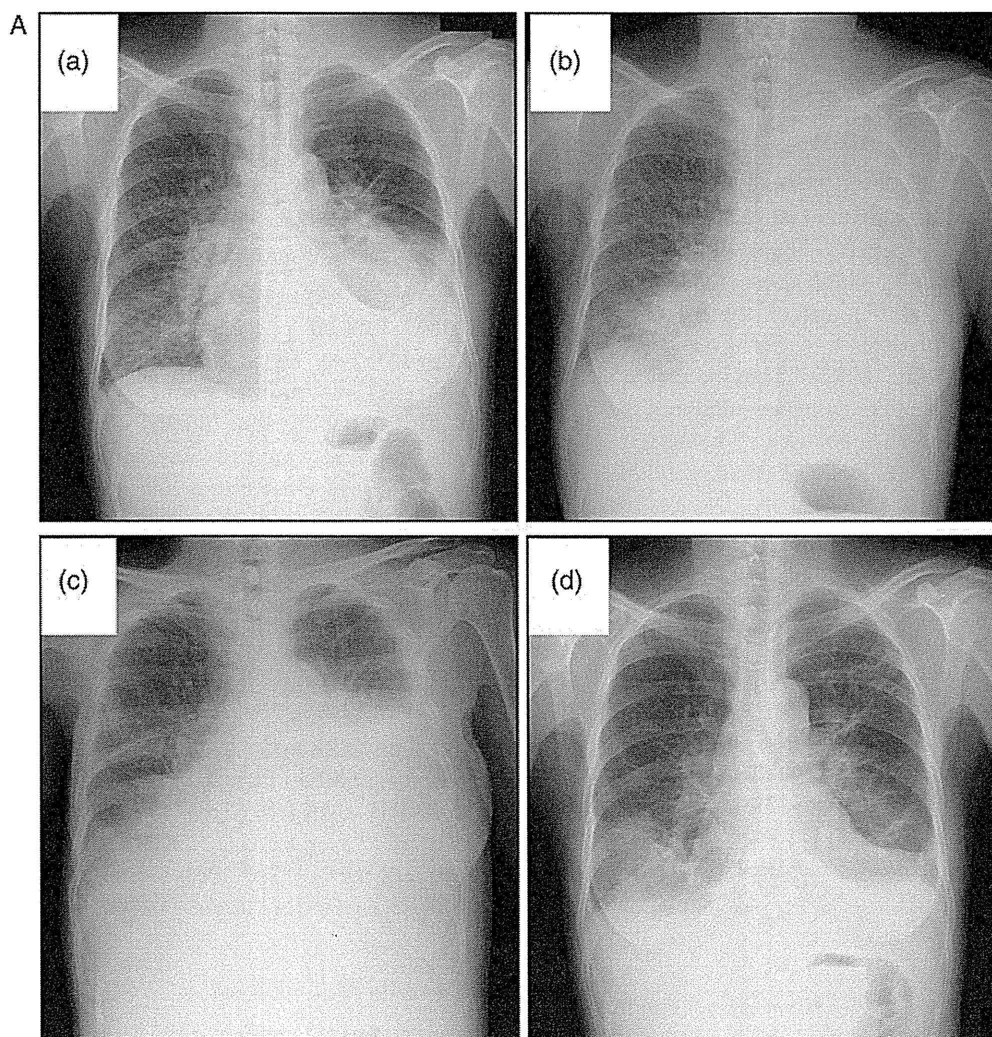


**Figure 1.** Crizotinib showed remarkable response in a patient with non-small cell lung cancer harboring echinoderm microtubule associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK) fusion gene. (A) the chest X-ray images at pre-crizotinib treatment (a) and at Days 3, 6 and 24 after the beginning of treatment with crizotinib (b–d). Left pleural effusion had been markedly decreased.

adenocarcinoma with T4N3M1 Stage IV. Her Eastern Cooperative Oncology Group performance status (PS) was one. Because her tumor cells did not harbor any epidermal growth factor receptor (EGFR) mutations, she was treated with chemotherapy consisting of carboplatin and pemetrexed. During this first-line chemotherapy, an *EML4-ALK* rearrangement in the tumor was detected using FISH. The tumor progressed after three cycles of chemotherapy. Subsequently, she received docetaxel as a second-line therapy; however, her disease progressed rapidly. Her PS deteriorated to two, and her left pleural effusion was drained to relieve her respiratory condition. She was then treated with 250 mg crizotinib twice per day. During the few days after beginning the treatment, the pleural effusion increased on chest radiography. Her respiratory distress then improved gradually, and the pleural effusion was reduced further at Day 24 (Fig. 1A). However, the tumor regrew with massive pleural effusion 3 months after crizotinib treatment. Crizotinib was discontinued, and she received best supportive care.

### Establishing the ALK-positive Lung Cancer Cell Line

Pleural effusion was drained to control the patient's respiratory condition before crizotinib treatment was started. Numerous tumor clusters were observed cytologically in the effusion. Mononuclear cells were isolated from the malignant effusion using the Ficoll-Hypaque method and were washed twice with RPMI 1640 medium (8). The cells were suspended in a Petri dish in ACL-4, which is serum-free medium described by Gazdar *et al.* (9), and cultured for 2 months. Subsequently, the culture media were changed to RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and the cells were cultured at 37°C in a humidified atmosphere with 5% $CO_2$. The cells began to grow within a week and proliferated consistently thereafter. The cell line was designated ABC-11.

### Cell Lines and ALK Inhibitors

The lung adenocarcinoma cell lines, H2228 (*EML4-ALK* variant 3a/b E6; A20) and A549 (KRAS G12S) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). PC-9 (EGFR del E746_A750) was purchased from Immuno-Biological Laboratories (Takasaki, Gunma, Japan). Crizotinib and AP26113 were purchased from Selleck Chemical (Houston, TX, USA). Ceritinib was purchased from Chemietek (Indianapolis, IN, USA).

### Mycoplasma Testing

ABC-11, H2228, A549 and PC-9 cells were not contaminated with mycoplasma, confirmed using the luminescent MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland) and a compact luminometer Gene Light GL-200A (Microtec, Chiba, Japan), following the manufacturer's instructions.

### Next-generation Sequencing

Genomic DNAs were extracted from cell line using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). The genomic DNAs were enriched using GeneRead DNAseq Targeted Panels V2 (Human Lung Cancer Panel) (Qiagen) and sequenced using Miseq (illumine, San Diego, CA, USA). The data were analyzed using Illumine VariantStudio (illumina).

### Drug Sensitivity Assay

Drug sensitivities were determined using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (10). Cells were seeded on 96-well plates at a density of 3000 cells per well and exposed continuously to each drug for 96 h. Following treatment, cells were incubated at 37°C with MTT reagent for 4 h, and the absorbance at 570 nm was measured using a 680 Microplate Reader (Bio-Rad, Hercules, CA, USA). The absorbance values were expressed as a ratio of treated to untreated cells. The concentration required to inhibit the growth of tumor cells by 50% ($IC_{50}$) was used to evaluate the effect of the drug. Assays were performed in triplicate, and the mean and standard error (SE) were calculated.

### Immunoblotting

Cells were lysed using radioimmunoprecipitation assay buffer (1% Triton X-100, 0.1% sodium dodecyl sulphate, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM glycerol-phosphate, 10 mM NaF and 1 mM Na-orthovanadate) containing protease inhibitor tablets (Roche, Tokyo, Japan). Proteins were separated by electrophoresis on polyacrylamide gels and transferred to nitrocellulose membranes. Subsequently, the membranes were incubated with the appropriate antibodies overnight at 4°C. The bands were then detected using ECL Plus (GE Healthcare, Fairfield, CT, USA) and imaged using the LAS-4000 (Fujifilm, Tokyo, Japan).

### Antibodies

Rabbit antisera against phospho-ALK (Tyr1604), STAT3, phospho-STAT3 (Tyr705) (D3A7), Akt, phospho-Akt (pSer473), ERK1/2, phospho-ERK1/2 (pT202/pY204) and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). Polyclonal antibodies against ALK were purchased from Invitrogen (Carlsbad, CA, USA).

### Xenograft Mouse Model

Female BALB/c nu/nu mice at 7 weeks of age were purchased from Japan Charles River Co. (Yokohama, Japan). Cells ($2 \times 10^6$) were injected subcutaneously into the backs of the mice.

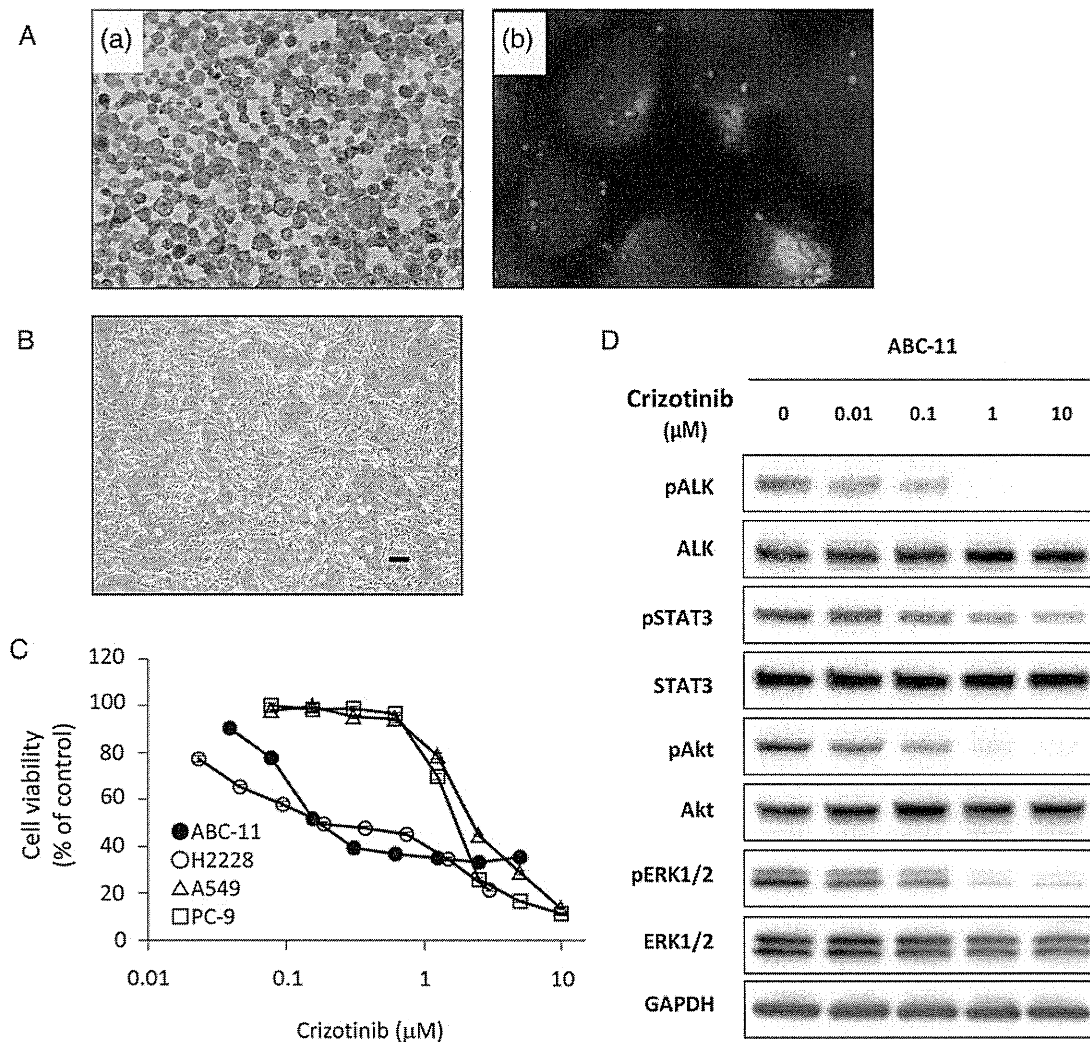


**Figure 2.** Characteristics of a new *EML4-ALK*-positive cell line, ABC-11. (A) Identification of EML4-ALK in ABC-11 cells. (a) Immunohistochemistry of ALK. ABC-11 cells have abundant ALK protein. (b) Fluorescent *in situ* hybridization analysis of *ALK* gene (red, ALK3′; green, ALK5′). ABC-11 cells harbor *EML4-ALK* fusion gene. (B) Morphologic observation. Cells were seeded ($1 \times 10^6$ per dish) and were cultured in medium. Microscopic image was taken after 6 days. Scale bar, 100 μm. (C) Sensitivity to crizotinib. Cells (ABC-11 and H2228 were 3000 cells; A549 and PC-9 were 2500 cells) were seeded per well on 96 well plates and were treated with various concentrations of crizotinib for 96 h. The viable cells were assessed as described in Patients and Methods. Points, mean values of triplicate cultures; bars, standard error. (D) Effects of crizotinib on ALK and its downstream signaling. Cells were incubated with various concentrations of crizotinib for 4 h. Lysates were analyzed by immunoblotting. Crizotinib suppressed pALK, pSTAT3, pAkt and pERK1/2.

## RESULTS

### Characteristics of ABC-11

The EML4-ALK fusion protein and gene were identified in ABC-11 cells by immunohistochemistry (anti-ALK antibody (5A4) was purchased from Abcam (Cambridge, UK)) and FISH, respectively (SRL, Tokyo, Japan) (Fig. 2A). *EML4-ALK* was observed; specifically, exons 20–29 of ALK were fused to exons 1–6b of EML4, variant 3b, as determined using multiplex reverse transcription-PCR and exon array analyses (Mitsubishi Chemical Medience, Tokyo, Japan) (data not shown) (11). *EGFR*, *KRAS*, *LKB1*, *TP53* and *p16INK4A* mutations, which had been reported in NSCLC so far, were

**Table 1.** $IC_{50}$ values of next-generation anaplastic lymphoma kinase-tyrosine kinase inhibitors (ALK-TKIs)

| | $IC_{50}$ (μM) | |
|---|---|---|
| | Ceritinib | AP26113 |
| ABC-11 | 0.13 ± 0.052 | 0.073 ± 0.0097 |
| H2228 | 0.84 ± 0.021 | 0.19 ± 0.016 |
| PC-9 | 2.19 ± 0.21 | 0.25 ± 0.0039 |

$IC_{50,}$ 50% inhibitory values of ALK TKIs in ABC-11, H2228 and PC-9 cells.

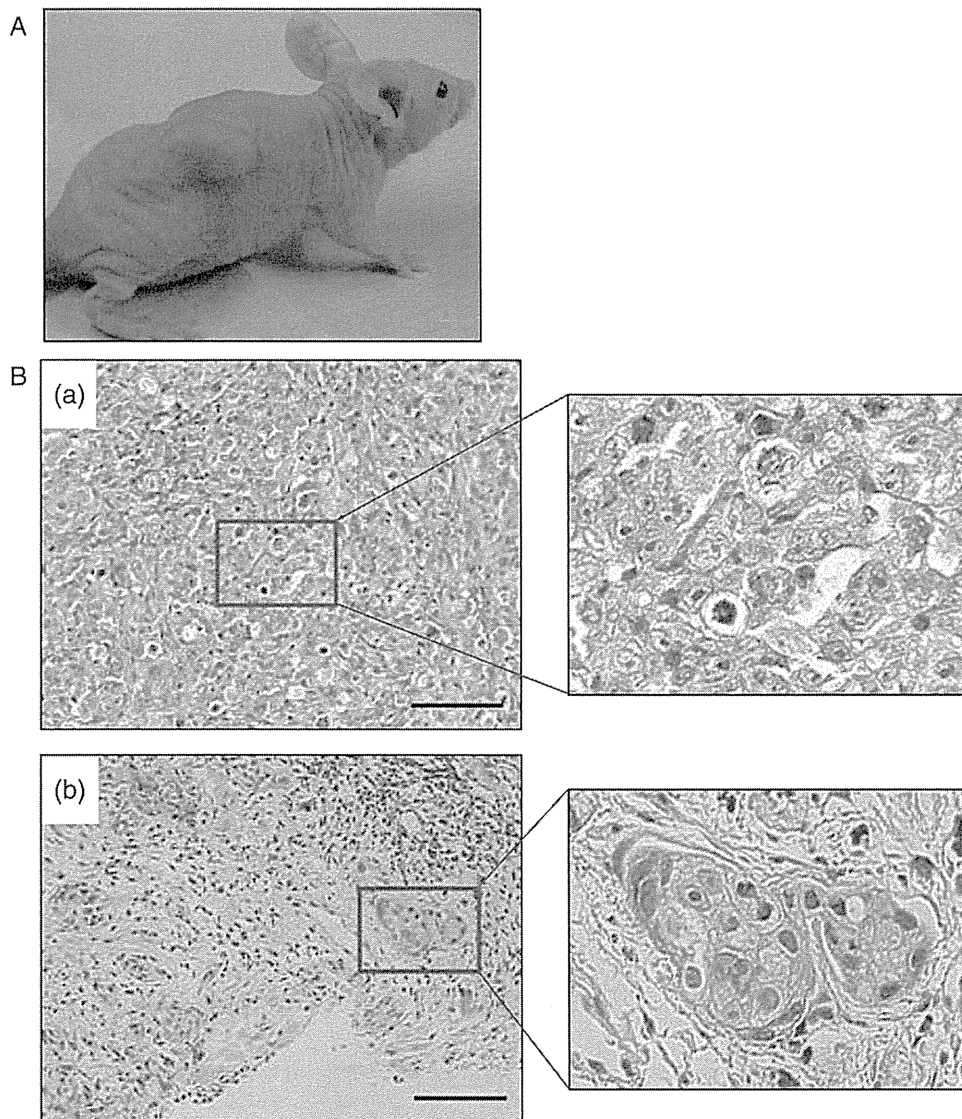


**Figure 3.** Establishment of xenograft mouse bearing ABC-11. (A) A xenograft mouse. Subcutaneous tumor was observed at 20 days after injection of ABC-11 cells. (B) Light microscopic image of tumor tissues derived from a xenograft mouse and the patient. Samples were subjected to histological examination using hematoxylin-eosin staining. (a) Tumor from xenograft mouse was harvested at 20 days after injection of ABC-11 cells. (b) Primary tumor was obtained at diagnosis. Scale bar, 100 μm. Both right small panels show high-power field.

not observed in ABC-11 cells according to next-generation sequencing.

Microscopic images of ABC-11 cells revealed that they exhibited an amoeboid form (Fig. 2B). The $IC_{50}$ value (mean $\pm$ SE) of crizotinib in ABC-11 cells was 0.17 $\pm$ 0.018 μm, which was similar to H2228 cells (0.18 $\pm$ 0.066 μm) (Fig. 2C). ABC-11 cells were also sensitive to next-generation ALK-TKIs, ceritinib and AP26113 (Table 1). The levels of pALK and its downstream proteins (pSTAT3, pAkt and pERK1/2) were suppressed in the presence of crizotinib (Fig. 2D).

ABC-11 cells that had been injected into the backs of the mice grew steadily. Figure 3A shows the tumors at 20 days after injection. The tumor of the xenograft (Fig. 3a) was harvested and compared with the primary lesion of the patient (Fig. 3b). Both samples were determined to be adenocarcinomas.

## DISCUSSION

We established a new lung adenocarcinoma cell line harboring the *EML4-ALK* fusion gene from the pleural effusion of an ALK-positive lung cancer patient. We also successfully established a mouse model bearing ABC-11 cells xenografts. The cell line was sensitive to crizotinib as well as H2228 cells. Recently, next-generation ALK-TKIs, including ceritinib (Novartis), alectinib (Chugai) and AP26113 (Ariad), have

been developed (12). The effectiveness of such novel compounds should be assessed using multiple cell lines including ABC-11 cells.

The tumor in the patient initially responded very well to crizotinib; however, it progressed within 3 months of the initial crizotinib treatment (Supplementary data, Figure S1). This suggests that ABC-11 cells might possess the ability to acquire resistance to crizotinib. To date, mechanisms underlying crizotinib resistance, such as *ALK* amplification, *ALK* secondary mutations (L1197M, C1156Y, G1202R, S1206Y, 1151Tins or G1269A), and the activation of alternative receptor tyrosine kinases (*KIT* amplification, *EGFR* mutation or *KRAS* mutation) have been reported using preclinical models and clinical samples (4–6, 13–16). Therefore, the ABC-11 cell line will be a useful tool to investigate acquired resistance to crizotinib. We are currently planning to establish an ALK-TKI-resistant ABC-11 cell line.

In conclusion, we established a new cell line derived from a patient with lung adenocarcinoma harboring an ALK fusion gene. This cell line could contribute to future studies of *EML4-ALK*-positive lung cancer both *in vivo* and *in vitro*.

## Supplementary Data

Supplementary data are available at http://www.jjco.oxfordjournals.org.

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## Conflict of interest statement

Katsuyuki Kiura received honoraria from Chugai pharmaceutical Co., Ltd, Pfizer Inc., Japan and Novartis Pharma K.K. Nagio Takigawa received honoraria from Pfizer Inc., Japan. Katsuyuki Hotta received honoraria from Chugai pharmaceutical Co., Ltd.

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ORIGINAL ARTICLE

# Extended sleeve lobectomy after induction chemoradiotherapy for non-small cell lung cancer

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**Abstract**
*Purpose* Extended sleeve lobectomy is a challenging surgery. While induction chemoradiotherapy (ChRT) followed by surgery is one of the therapeutic strategies used for locally advanced non-small cell lung cancer (NSCLC), ChRT can impair the anastomotic healing potential. We herein present our experience with cases who underwent an extended sleeve lobectomy after induction ChRT.
*Methods* The medical records of patients who underwent a surgery for NSCLC after ChRT were reviewed.
*Results* Between December 2007 and January 2013, nine patients underwent an extended sleeve lobectomy; the left lingular division and lower lobe in four patients, the right upper lobe and trachea in one patient, the carina and trachea in one patient, the right middle and lower lobes in one patient, the right upper and middle lobes and carina in one patient and the right upper lobe and superior segment of the lower lobe in one patient. While no postoperative 90-day deaths occurred, one case developed a bronchopleural fistula on postoperative day (POD) 25 and one case developed a bronchovascular fistula on POD 163. No cases of local recurrence developed.
*Conclusions* Our experience suggests that an extended sleeve lobectomy after induction ChRT is feasible, but careful patient selection and perioperative management are mandatory.

**Keywords** Sleeve lobectomy · Lung cancer · Chemoradiotherapy · Induction therapy

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## Introduction

Sleeve lobectomy is an established surgical procedure for avoiding a pneumonectomy [1]. Previous studies have shown an equivalent cure rate and superior quality of life for a sleeve lobectomy compared with a pneumonectomy [2–5]. Okada et al. [6] reported the usefulness of an extended sleeve lobectomy, which is a more challenging procedure. Indeed, the resection of the bronchus during such a procedure can cause several difficulties, such as an increase in the tension at the site of the anastomosis and mismatches in the size of the bronchial orifices.

Induction chemoradiotherapy (ChRT) followed by surgery is one of the available therapeutic strategies for locally advanced non-small cell lung cancer (NSCLC). However, the effects of induction therapy, especially that of radiotherapy, can impair the anastomotic healing potential of the bronchial stump or the anastomosis in an anatomical pulmonary resection [7, 8]. We previously reported the feasibility of performing a sleeve lobectomy after induction ChRT, especially when the blood supply of the pulmonary artery to the spared lobe was preserved [9]. Among these cases, there were five patients who underwent an extended sleeve lobectomy. In the present study, we added four cases with an extended sleeve lobectomy after induction ChRT to focus on this procedure and its clinical outcomes.

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## Materials and methods

### Patients

The medical records of patients with NSCLC who had undergone surgery after ChRT were reviewed. The International Association of the Study of Lung Cancer TNM staging system for NSCLC was used for staging [10]. The clinical disease stage was determined with an image analysis, including enhanced chest CT scans, 18-fluoro-2-deoxyglucose positron emission tomography-CT scans, enhanced brain magnetic resonance imaging and bronchoscopy [11].

### Induction therapy

Induction ChRT was performed in eight cases using docetaxel and cisplatin with concurrent thoracic radiation, as described previously [12]. For one patient who had a synchronous head and neck tumor, 5-fluorouracil and nedaplatin were used for chemotherapy. Radiotherapy was started on the first day of chemotherapy using a linear accelerator (6–10 megavolts). A total radiation dose of 46 or 40 Gy with a conventional fractionation (2 Gy/day) was planned. The details of the radiation field were as described in a previous study [12]. Following induction ChRT, the tumor response and patient condition was evaluated, and patients without progressive disease and in good general condition underwent surgery.

### Evaluations

The Eastern Cooperative Oncology Group criteria (with some modifications) were used to assess the tumor radiological response, which was classified as a complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD) [12, 13]. The follow-up procedure was as previously reported [14]. The overall survival (OS) and the disease-free survival (DFS) were calculated from the date of initialing induction therapy until the date of death or of the last follow-up for OS and until confirmed disease recurrence or death for the DFS.

## Results

### Patient characteristics

We performed nine extended sleeve lobectomies between November 2007 and January 2013. The clinicopathological characteristics of the patients are shown in Table 1. The median age of the seven males and two females was 60 years (range 50–73 years). The histological subtype was squamous cell carcinoma in six patients and adenocarcinoma in three patients. The disease stage was clinical stage (c-stage) IIIA in six patients, c-stage IIIB in one patient and c-stage IIA in two patients. The radiation dose was 46 Gy in seven patients and 40 Gy in two patients. The radiological response was a PR in five patients (56 %) and SD in four patients (44 %). The median time from the end of induction therapy until pulmonary resection was 38 days.

A complete resection was performed for all of the patients with a negative bronchial margin as determined by frozen sections. A posterolateral thoracotomy was applied for eight patients, and a trap door approach was applied to one patient with a Pancoast-type tumor. A sleeve bronchial resection was performed on the left lingular division and lower lobe in four patients (Cases 4, 5, 8 and 9), the right upper lobe in two patients (Cases 2 and 3), the right middle and lower lobes (Case 1), the right upper and middle lobe (Case 6) and the right upper lobe and superior segment of the lower lobe (S6) in one patient (Case 7).

**Table 1** The characteristics of the patients who underwent sleeve resection after induction chemoradiotherapy

| Case | Sex | Age | Histology | TNM | Stage | RT dose (Gy) | Extended resection | Additional resection | Complication | Coverage |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 60 | SQ | T2aN1M0 | IIA | 40 | RMLL | | BPF | PFP |
| 2[a] | M | 54 | AD | T4N2M0 | IIIB | 40 | RUL + Trachea | Clavicular vessels, Ribs | | GO |
| 3 | M | 58 | SQ | T3N2M0 | IIIA | 46 | RUL + Carina | | | GO |
| 4 | F | 50 | AD | T2aN2M0 | IIIA | 46 | LLDLL | | | GO |
| 5 | M | 64 | AD | T2aN2M0 | IIIA | 46 | LLDLL | | | GO |
| 6 | M | 51 | SQ | T4N1M0 | IIIA | 46 | RUML + Carina | | | PFP |
| 7 | F | 64 | SQ | T3N1M0 | IIIA | 46 | RUL + S6 | Tangential PA resection | BVF | GO |
| 8 | M | 73 | SQ | T2aN1M0 | IIA | 46 | LLDLL | | | GO |
| 9 | M | 67 | SQ | T2aN2M0 | IIIA | 46 | LLDLL | | | GO |

*SQ* squamous cell carcinoma, *AD* adenocarcinoma, *RUL* right upper lobe, *RUML* right upper and middle bilobectomy, *RMLL* right middle and lower lobes, *RLL* right lower lobe, *LLDLL* left lingular division and lower lobe, *BPF* bronchopleural fistula, *BVF* bronchovascular fistula, *PFP* pericardial fat pad, *GO* greater omentum

[a] Case 2 was a Pancoast-type tumor which required combined resection of the clavicular artery and vein, tracheal wall and 1st–4th ribs

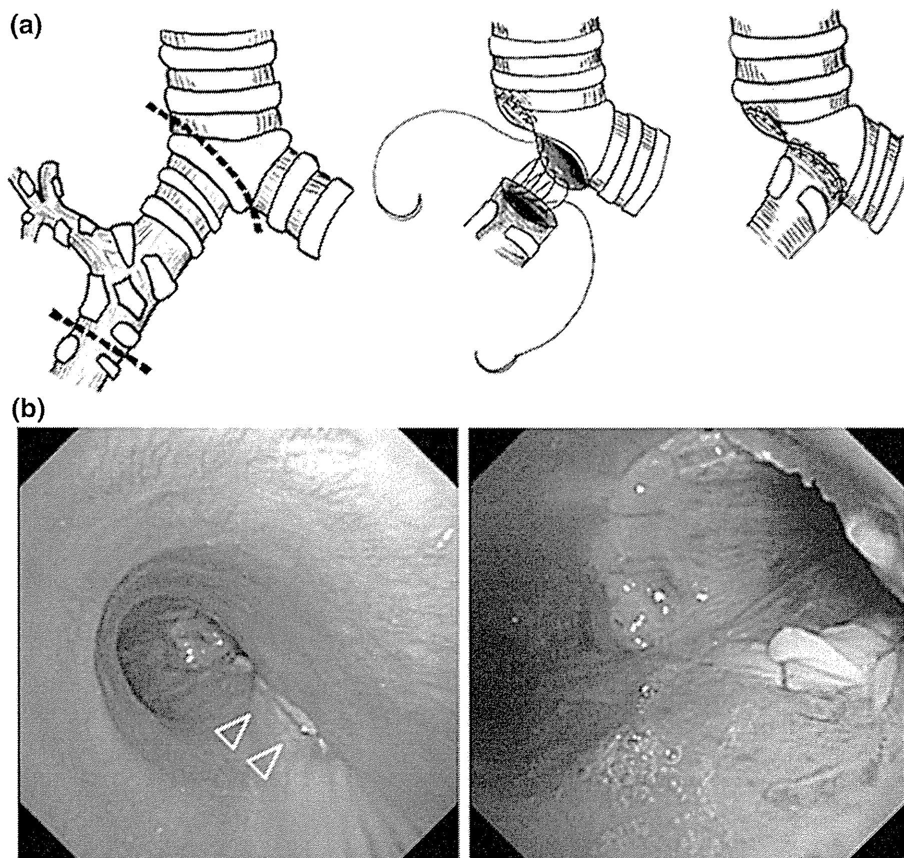


**Fig. 1** Images of Case 3. **a** A schematic diagram of the procedure. The proximal side of the resection line included the right tracheal wall and carina. The membranous and cartilaginous portions of the right side trachea were sutured to control the orifice size for subsequent anastomosis with the right intermedius bronchus. **b** The results of the bronchoscopic examination on postoperative day 30 for Case 3. The *open arrowheads* indicate the suture line of the membranous and cartilaginous portions of the right side trachea

The major structures that were additionally resected are shown in Table 1. It is important to note that the carina and trachea in Case 3 and the carina in Case 6 were extensively resected due to tumor invasion. The tracheal wall was extensively resected because of extranodal invasion of the right lower paratracheal lymph node in Case 2. With regard to Case 7, a tumor arising from the right posterior segmental bronchus of the upper lobe (B2) had invaded the right main and intermedius bronchus. Bronchial biopsy specimens obtained during a bronchoscopy after induction ChRT revealed tumor cells at the bifurcation of the right middle and lower lobar bronchi, and at the superior segmental bronchus of the lower lobe (B6). The middle lobar and basal segmental bronchi were intact.

Surgery and outcome

With regard to the surgical procedures in our cases, a complete lymph node dissection of the ipsilateral superior mediastinum and subcarina was routinely performed. For patients with tumor originating from the lower lobe, the station 8 and 9 lymph nodes were also dissected. A bronchial anastomosis was performed with running sutures using 4-0 PDS II (Ethicon, Somerville, NJ) for the bottom portion of the anastomosis. Interrupted sutures using 4-0 PDS II were placed on the other portion. The bronchial suture line was wrapped with a pericardial fat pad or omental pedicled flap with prophylactic intent. The details of the prophylactic wrapping for each case are shown in Table 1.

As a particular aspect of each procedure, in cases that required a tracheal or carinal resection with the resection of the right upper lobe (Cases 2, 3) or the right upper and middle lobes (Case 6), suture closure between the membranous and the cartilaginous portions of the proximal airway was first performed to adjust the orifice size for the subsequent anastomosis (Fig. 1a). Subsequently, anastomosis of the proximal and distal orifice was performed as described above. The postoperative bronchoscopy findings for Case 3 are shown in Fig. 1b.

In Case 7, an extended sleeve lobectomy of the right upper lobe and superior segment of the lower lobe (S6) was performed (Fig. 2a). Reconstruction was achieved by recreating a new orifice using the medial third of the circumference of the basal segmental and middle lobar bronchi. This double-barreled lumen was fashioned, and the anastomosis of the right main bronchus to the newly created bronchial lumen was performed in a telescopic manner. The postoperative bronchoscopy findings for Case 7 performed on postoperative day (POD) 69 are shown in Fig. 2b. While the site of bronchial anastomosis was covered with white

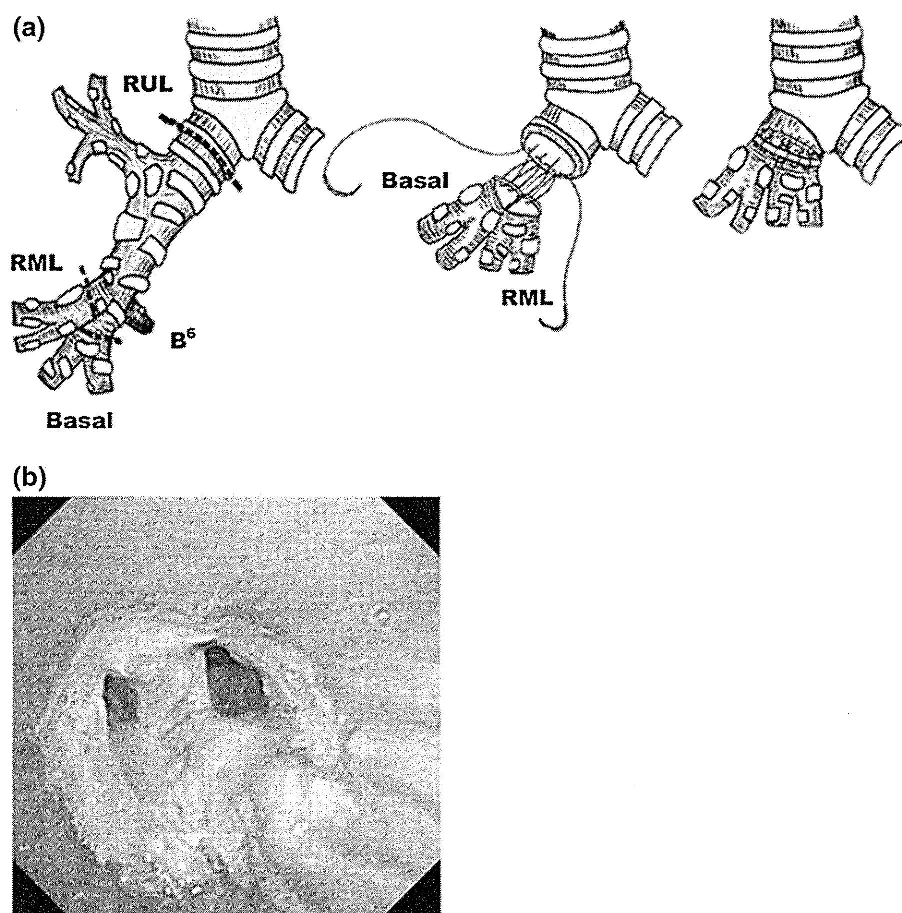


**Fig. 2** Images of Case 7. **a** A schematic diagram of the procedure. A reconstruction was achieved by first recreating a double-barreled lumen using the basal segmental and middle lobar bronchi. This double-barreled lumen was anastomosed to the right main bronchus. The *broken lines* indicate the resected line of the bronchi. **b** The results of a bronchoscopic examination performed on postoperative day 69. The site of bronchial anastomosis was covered with white granulation

granulation, no other signs to suggest ischemia or other problems were observed.

A right middle and lower lobectomy was performed for Case 1, and an anastomosis between the right main and right upper lobar bronchi was performed. With regard to the sleeve bronchial resection of the left lingular division and lower lobe, we previously reported Cases 4 and 5, in which we described the procedure for adjusting the mismatches in the proximal and distal bronchial stumps [15]. Cases 8 and 9 were newly added.

While no postoperative 90-day deaths occurred in this series, critical bronchial complications developed in two cases. A bronchopleural fistula occurred on POD 25 in one case that underwent a right middle and lower lobectomy (Case 1). Completion pneumonectomy was successfully performed. In another case (Case 7), the patient underwent a right upper and superior segmental sleeve resection and was discharged from the hospital on POD 28. During the follow-up in our outpatient clinic, a fatal bronchovascular fistula was assumed to have occurred on POD 163, when she was transferred to an emergency hospital and died. Except for these two cases, there were no cases of bronchial complications, including stenosis or life-threatening complications.

The pre- and postoperative pulmonary function data were available for seven cases (Cases 1, 3, 4, 5, 6, 8 and 9). The preoperative first–second forced expiratory volume before surgery was $2.52 \pm 0.58$ L (mean $\pm$ standard deviation), while that after surgery was $1.80 \pm 0.66$ L, indicating an acceptable preservation of the pulmonary function.

### Response and survival

The radiological response was a PR and SD in four patients each. A complete pathological response was obtained in two (22 %) patients. At the time of the data analysis in January 2014, disease recurrence had developed in two patients as distant metastases, and none of the patients had experienced locoregional recurrence. The two-year OS and DFS rates were 66.7 and 55.6 %, respectively (Fig. 3). No local recurrences have been observed in the present series.

## Discussion

Based on the disease extent, some lung cancer cases require an extended sleeve lobectomy, rather than a typical sleeve lobectomy, to avoid a pneumonectomy. The anastomosis

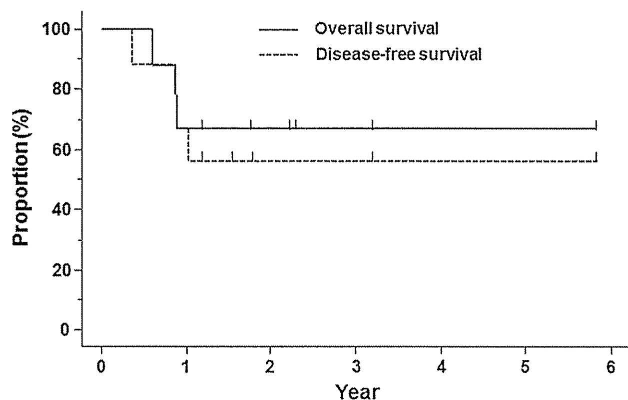


**Fig. 3** The survival curves of patients who underwent an extended sleeve lobectomy after induction chemoradiotherapy

of the spared lung to the proximal bronchus is one of the critical points of an extended sleeve lobectomy. The wider resection of the bronchus causes significant mismatch of the proximal and distal bronchial orifice sizes. Some of our procedures were intended to ameliorate the size mismatch, simplifying the anastomosis. In cases with tracheal or carinal resections (Cases 2, 3, 6), suture closure between the membranous and cartilaginous portions of the proximal airway can control the newly created orifice size on the proximal side of the anastomosis. In Case 7, a sleeve resection of the right upper, middle and superior segments of the lower lobe might have been an alternative option, as one of the authors (S.M.) had described in a previous report [15]. However, from the viewpoint of the anastomosis, the anastomosis of a double-barreled bronchus created using the basal segmental and middle lobar bronchi with a larger orifice size was easier to anastomose to the right main bronchus than the basal segmental bronchus. Concerning the situation observed in Case 7, tumors arising from the central portion of the upper lobe (B2) sometimes invade the intermedius bronchus and involve the B6 extensively on the distal side. However, considering the anatomical location of the upper lobar bronchus, the middle lobar bronchus and the B6, the involvement of the middle lobar bronchus is less likely than the involvement of the B6, suggesting that the situation presented here is not particularly rare. While Case 7 ultimately resulted in a late-onset fatal bronchovascular fistula, our procedure may still be applicable for similar cases, but careful perioperative management is crucial.

In Cases 4, 5, 8 and 9, the anastomosis between the left main and superior divisional stumps was performed using two different procedures, as mentioned in our previous report [16, 17]: creating a “wine cup stoma” of the superior divisional bronchial stump edged by the partially excised walls of the upper lobar and the lingular divisional bronchi (Procedure #1), and reefing of the membranous portion of the left main bronchus with adjusting stitches (Procedure #2). We performed Procedure #1 for Cases 5, 8, and 9 and Procedure #2 for Case 4.

While the survival and pulmonary function after surgery were acceptable, the feasibility of extended sleeve lobectomy after ChRT remains a concern, because ChRT, especially radiotherapy, is known to be a possible risk factor for bronchial complications [18, 19]. Even among the small number of patients in our study, severe bronchial complications occurred in 22 % of our series. As reported previously, the pulmonary arterial branch to the right upper lobe had been sacrificed in Case 1, and this was considered to be a risk factor for an anastomotic complication. In Case 7, two possible factors might have been responsible for the bronchovascular fistula other than the complexity of the surgical procedure. One was postoperative pneumonia that developed on POD 2 caused by an extended spectrum beta lactamase-producing strain of *Escherichia coli* that had also caused pneumonia in this patient during the induction ChRT prior to surgery. Another was the presence of diabetes mellitus, for which the patient was receiving treatment. These factors have previously been reported as risk factors for a bronchopleural fistula after lung surgery [20, 21].

It is difficult to analyze the risk factors for serious complications of extended sleeve lobectomy after induction ChRT because of the variety of disease extents and procedures. Furthermore, our sample size is not large enough to investigate the risk factors and to compare the feasibility of extended sleeve lobectomy with conventional sleeve lobectomy or pneumonectomy after induction ChRT.

Although our experience is limited, there are two issues that we consider important in terms of the indications and follow-up for our treatment. First, the indications for complicated bronchoplasty should be carefully considered for patients even with minor complications, including a recent history of pneumonia. Second, surgeons should positively suspect anastomotic failure in patients with the risk factors for bronchial complications mentioned above to avoid further critical complications, such as a bronchovascular fistula. A completion pneumonectomy without delay may be necessary for this situation. Careful management during the perioperative period, as well as technical experience, is mandatory, especially when surgeons perform a challenging operation.

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