이학박사 학위논문

Heterogeneity of genetic changes associated with acquired crizotinib resistance in *Anaplastic lymphoma kinase*-rearranged lung cancer

Anaplastic lymphoma kinase 유전자가 재배열된 폐암에서 crizotnib 내성과 연관된 유전적 변화의 이질성(異質性)

2012년 08월

서울대학교 대학원
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김 소 연

A thesis of the Degree of Doctor of Philosophy

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이 논문을 이학박사 학위논문으로 제출함

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서울대학교 대학원

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by
Soyeon Kim
(Directed by Professor Dae Seog Heo, M.D., PhD)

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Approved by Thesis Committee:

Professor _____**Chairman**
Professor _____**Vice chairman**
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ABSTRACT

Purpose: *Anaplastic lymphoma kinase (ALK)*-rearranged lung cancers are markedly responsive to treatment with the *ALK* tyrosine kinase inhibitor crizotinib. Unfortunately, patients with this type of cancer can acquire resistance to crizotinib through several mechanisms, including *ALK* amplification and mutations at L1196M and G1269A. In this paper, we evaluated whether a specific gene mutation in *ALK* triggers resistance to crizotinib in *ALK*-rearranged lung cancer patients.

Material and Methods: Tumor samples were derived from seven ALK-positive NSCLC patients who showed acquired resistance to crizotinib and these were analyzed for ALK, EGFR, and K-ras mutations. In addition, available tumor tissues before crizotinib were retrieved for these molecular tests. *In vitro* cytotoxicity and *ALK* downstream signaling pathways were compared between *EML4-ALK*-positive (NCI-H3122) and crizotinib-resistant (SNU-2535) cell lines.

Results: The specimens of seven patients had evaluable material for secondary mutations that were reported previously. Three patients had developed either a G1269A or an L1196M mutation in the *ALK* tyrosine kinase domain, but no identifiable genetic mutation was found in the other four patients. One patient had both the L1196M and G1269A mutations. Interestingly, one patient who did not have any resistance mutation exhibited an EGFR L858R mutation in a post-crizotinib biopsy sample in addition to an ALK gene rearrangement, as determined by FISH analysis. We showed that

the L1196M and G1269A mutations inhibited crizotinib-mediated downregulation of ALK signaling and blocked cell growth inhibition in *EML4-ALK* L1196M- and G1269A-mutated cells. Furthermore, we developed crizotinib-resistant SNU-2535 cells from the pleural effusion of a patient who harbored a G1269A mutation. In addition, we found that crizotinib inhibited cell proliferation, induced apoptosis and inhibited signaling pathways, including Akt and ERK, in a wild-type *EML4-ALK*-positive cell line but not a crizotinib-resistant cell line.

Conclusions: Clinically, crizotinib is effective against *ALK*-rearranged lung cancer, but drug resistance is problematic. The current data demonstrate that the L1196M and G1269A mutations of the *ALK* tyrosine kinase domain are associated with resistance to crizotinib in some of *ALK*-rearranged lung cancer. However, a clear genetic mutation causing resistance to crizotinib has not been identified.

Keywords: ALK, EML4-ALK, Crizotinib, resistance mutation

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INTRODUCTION

Anaplastic lymphoma kinase (ALK) has been identified as the fusion partner of echinoderm microtubule-associated protein-like 4 (EML4) in the oncogene EML4-ALK resulting from a small inversion within the short arm of chromosome 2, inv(2) (p21p23), in non-small-cell lung cancer (NSCLC), and this fusion gene has been detected in 3.0-6.7% of NSCLC patients [3,4]. Chromosomal translocations involving ALK also occur in other cancers, including anaplastic large cell lymphomas and inflammatory myofibroblastic tumors. To date, variations in the break and fusion points of EML4 result in various isoforms of the fusion gene, but the complete intracellular portion of ALK, including the kinase domain, is preserved in all variants [4]. Anaplastic large cell lymphomas (ALCLs) and inflammatory myofibroblastic tumors (IMTs) have multiple fusion partners of ALK [5], and a recent study reported the presence of TFG-ALK [6] and the kinesin family member 5B (KIF5B)-ALK fusion in NSCLC [7,8]. Similar to EGFR mutations, EML4-ALK is more likely to be found in never-smokers and adenocarcinomas, although its occurrence is mutually exclusive with an EGFR mutation [9]. ALK is a receptor tyrosine kinase that belongs to the insulin receptor (IR) superfamily. The constitutive kinase activity of ALK fusion proteins leads to the cellular transformation and neoplasia of ALCL cells, and its inactivation results in a possible therapeutic approach for the treatment of ALCL [10]. The intact ALK kinase domain within EML4-ALK confers malignant transformation and oncogenic activity in vitro and in vivo, respectively [3,11,12]. A tyrosine kinase inhibitor (TKI) targeting

ALK, crizotinib (PF-02341066), which is produced by Pfizer, Inc., is currently in phase II and phase III clinical trials for advanced NSCLCs with ALK rearrangements, and early studies have demonstrated an objective response rate of 57% and a disease control rate of 87% at 8 weeks in patients with ALK-rearranged NSCLC [9,13,14]. Furthermore, the clinical efficacy of crizotinib has been observed in a patient with an ALK translocation IMT compared with no observed activity in another patient without the ALK translocation [15]. These results suggest that EML4-ALK is highly susceptible to ALK-targeted therapies and demonstrate the oncogenic properties of ALK. Although many patients derive significant clinical benefit from treatment, the development of drug resistance has limited the effect of crizotinib in NSCLC. The paradigm of acquired TKI resistance has been observed with EGFR mutation-targeted therapy in lung cancer patients. In some patients, resistance arises due to the acquisition of a secondary mutation in the gatekeeper region (T790M) within the EGFR [16,17]. Although the amino acid change does not impair the catalytic activity of EGFR, the mutation is predicted to block binding of either gefitinib or erlotinib to the EGFR ATP-binding domain [18,19]. Recently, Choi et al.[20] reported a secondary mutation within the EML4-ALK kinase domain in tumor cells isolated from a patient during the relapse phase of crizotinib treatment. In this patient, molecular analyses revealed that the resistant tumor cells harbored the C1156Y mutation and the gatekeeper mutation L1196M. These mutations occurred independently in distinct subclones of the resistant tumor. Moreover, a secondary mutation in ALK, F1174L, has been identified as one cause of crizotinib resistance in a patient

with an IMT who progressed while on crizotinib therapy [21]. Other studies have revealed that resistance might arise due to the acquisition of a secondary mutation within EML4-ALK. Secondary mutations, ALK amplification, KIT amplification, and autophosphorylation of EGFR were shown to be responsible for acquired resistance to crizotinib in ALK-translocated cancers [17,20-23]. Furthermore, because of the diversity of the clinically identified mutations to date, the degree of resistance to crizotinib is variable [24]. Secondary mutations may not represent the predominant mechanism of acquired crizotinib resistance; therefore, additional studies are needed to explain other mechanisms of resistance.

In this study, we used a crizotinib in a human NSCLC cell line that harbors the EML4-ALK fusion protein and in specimens from relapsed patients with ALK-rearranged lung cancer. We discovered two mutations in EML4-ALK in the relapsed patient specimens using RT-PCR and sequencing, as described in previous reports. We therefore examined whether a specific ALK mutation triggers resistance to crizotinib in ALK-rearranged lung cancer and clarified the underlying mechanisms of action.

MATERIALS AND METHODS

1. Characteristics of clinical samples

A 45-year-old male patient (SNUH #1) with no history of smoking was diagnosed with lung adenocarcinoma with liver metastasis in June 2009. Because the tumor did not harbor any EGFR mutations, the patient was treated with bevacizumab, gemcitabine, and cisplatin. After 4 cycles of conventional chemotherapy, his tumor progressed. We evaluated *EML4-ALK* translocation using fluorescence in situ hybridization (FISH) and immunohistochemistry, and an *EML4-ALK* translocation was found in his primary lung tumor specimen. The patient was enrolled in a clinical trial for crizotinib in February 2010. After 2 months of crizotinib, chest computed tomography (CT) scan revealed a partial response (Fig. 1A). However, after 6 months of treatment, the tumor had grown again. His CT scan showed new liver lesions, and he dropped out of the clinical trial. We hypothesized that the patient's resistance may have been due to an acquired secondary mutation in the *EML4-ALK* fusion gene that conferred resistance to crizotinib; therefore, we again obtained his pleural fluid with informed permission and sequenced the ALK tyrosine kinase domain in the second biopsy specimen. Since the relapse, the patient has been receiving various salvage therapies for advanced lung cancer.

A 56-year-old female (SNUH #4) with no history of smoking was diagnosed with lung adenocarcinoma with bone metastasis in October 2008. The patient was treated with two cycles of gemcitabine plus carboplatin and 7

cycles of second-line docetaxel. However, the tumor progressed, and her tumor tissue was found to harbor the *EML4-ALK* translocation. The patient was enrolled in a clinical trial for crizotinib in November 2009. She tolerated crizotinib well and exhibited a partial response. After 1 year of crizotinib, a chest CT scan revealed a new malignant pleural effusion (Fig. 1B). She dropped out of the clinical trial. We obtained her pleural effusion with informed permission when she developed acquired resistance to crizotinib.

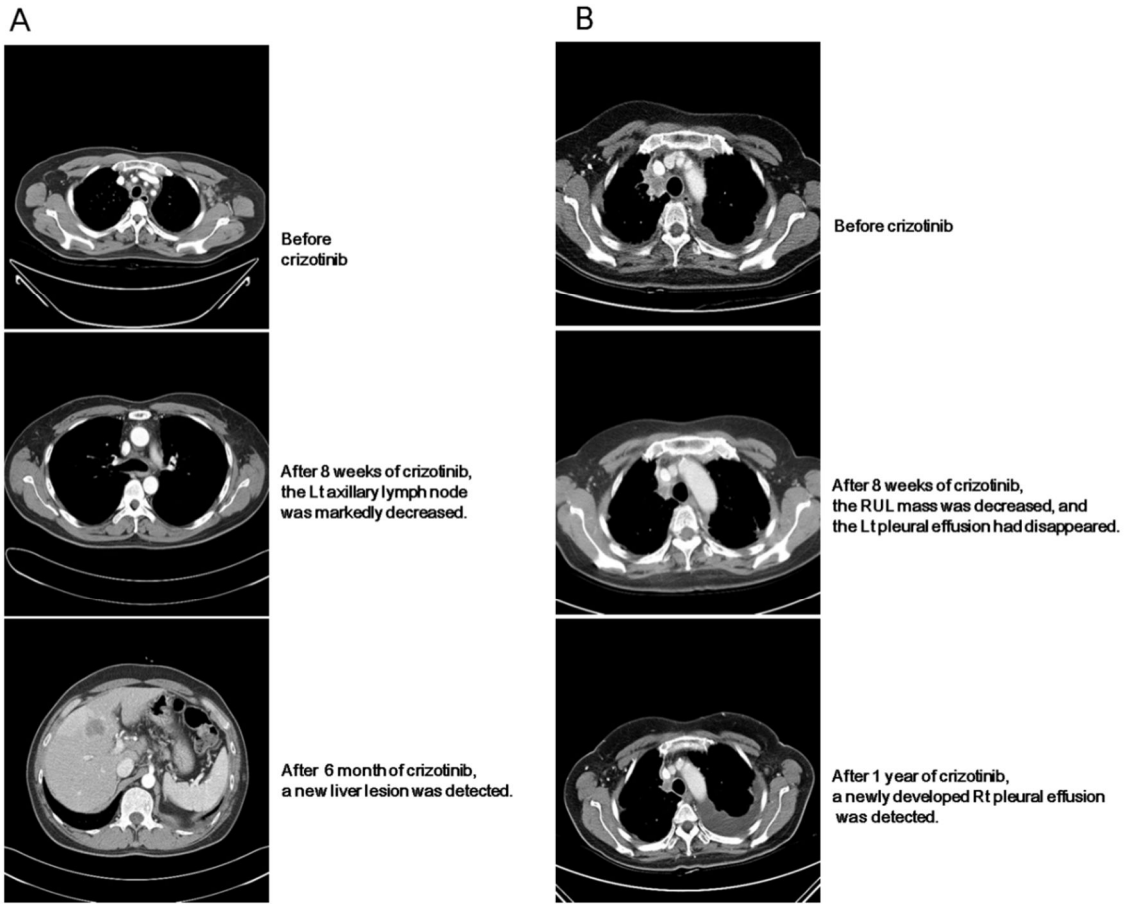


Figure 1. Characteristics of clinical samples

2. Cell lines and compounds

NCI-H3122 cells were kindly provided by Dr. Pasi A. Jänne (Dana-Farber Cancer Institute), and NCI-H2228 cells were purchased from ATCC (Manassas, VA). SNU-2535 cells were established at the Korean Cell Bank from the pleural effusion of patient (SNUH #4), who had developed acquired resistance to crizotinib. NCI-H3122, NCI-H2228 and SNU-2535 cells were maintained in RPMI 1640 medium with gentamicin (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GIBCO). The cell lines were incubated at 37°C in a humidified atmosphere of 5% CO_2. PF-02341066 (crizotinib) was kindly provided by Pfizer (Milwaukee, WI) and dissolved in dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO) for experiments.

3. Tissue procurement

Tumor specimens, including paraffin blocks, biopsies, and pleural effusions, were obtained through protocols approved by the Institutional Review Board of Seoul National University Hospital, and written informed consent was obtained from all donors. In total, 7 patients with acquired resistance to crizotinib were evaluated.

4. Isolation of genomic DNA and L1196M and G1269A mutation–specific PCR

Genomic DNA was isolated from cell pellets or tissues with an ALL-prep DNA/RNA micro kit (QIAGEN) according to the manufacturer`s protocol.

Exons of *ALK* were PCR amplified from genomic DNA using the High Fidelity plus PCR system (Roche, Indianapolis, IN) and sequenced bidirectionally by Sanger dideoxynucleotide sequencing with the primers for *ALK* (exons 23 and 25) described in supplementary table 1. Mutation-specific PCR primer sequences are provided in supplementary table 1. Direct sequence analysis was performed on an ABI 3730 DNA sequencer (Applied Biosystems, Carlsbad, CA). The reference sequence for *ALK* sequence comparison was NM_004304.4; for *EML4*, it was NM_019063.3. All of the mutations were confirmed at least twice from independent PCR isolates, and sequence tracing was reviewed in the reverse direction by visual inspection.

5. Reverse transcription polymerase chain reaction (RT–PCR)

Total RNA was isolated from the NSCLC cell lines with an RNA Mini kit (Invitrogen, Carlsbad, CA). Each sample was reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen). PCR amplification was performed with protocols with the specific primers [17,25,26]. Amplicons were analyzed on 1.0% agarose gels containing ethidium bromide (1 μg/ml), and visualization of the PCR products was performed under a UV transilluminator. The images were captured using the Gel Logic 200 imaging system (Kodak, Rochester, NY). The PCR products were purified using a PCR purification kit (Invitrogen) and a DNA concentration kit (ZYMO RESEARCH, Irvine, CA) and subsequently sequenced as described later.

6. Cloning of *EML4-ALK* expression constructs

EML4-ALK variant 1 cDNA from the H3122 cell line was amplified using primers. The PCR products were cloned into the TOPOTA vector (Invitrogen) and transformed into bacteria, and the *ALK* kinase domain (residues 1094-1396) was sequenced in individual clones. To generate *EML4-ALK* mutants, L1196M or G1269A mutations were introduced using site-directed mutagenesis (Agilent technologies, La jolla, CA) with mutant specific primers according to the manufacturer`s instruction. The PCR primers and conditions are listed in supplementary table 1. The sequencing products were analyzed with Sequencer software (Gene codes). The Basic Local Alignment and Search Tool was used against the BLAST database to determine the identity of unknown sequences. Retroviral infection and culture of Ba/F3 cells were done using previously described methods [3]. Polyclonal cell lines were established by puromycin selection and subsequently cultured in the absence of interleukin-3 (IL-3). Uninfected Ba/F3 cells were used as controls.

7. Cell proliferation and apoptosis assays

NSCLC cells were seeded at a density of 5,000 cells per well in 96-well plates, cultured in the presence of drugs or vehicle for 72 hours, and subjected to the CCK-8 colorimetric assay (Dojindo, Japan). The results were measured using an Eon™ Microplate Spectrophotometer (BioTek, Winoosk, VT) in at least duplicate samples according to the manufacturer's specifications [27]. The cells were collected and stained with Annexin V and 7 AAD for 15 minutes. Flow cytometry was performed using a FACSCalibur flow cytometer (Becton

Dickinson, Franklin Lakes, NJ).

8. Fluorescence in situ hybridization

FISH analysis using a commercially available ALK probe (Vysis LSI ALK dual-color, break-apart rearrangement probe; Abbott Molecular, Abbott Park, IL) was performed on FFPE tissue sections, cell pellets from pleural effusions or cell lines following the manufacturer's protocols [28].

8. EGFR and KRAS mutation analyses

EGFR and KRAS mutation analyses were performed on DNA extracted from NSCLC patient samples or cell lines as previously described [28].

10. Immunoblotting

The cells were resuspended in lysis buffer (Cell Signaling, Danvers, MA), incubated on ice for 10 min and centrifuged for 15 min at 4 ℃. Equal amounts of whole cell lysates were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was performed with NuPAGE 4–12% Bis-Tris gels (Invitrogen) according to the manufacturer's suggested procedures. The separated proteins were transferred to PVDF membranes and blocked with PBS containing 5% nonfat dry milk and 0.05% Tween 20 for 30 min. The membranes were incubated with primary antibodies overnight at 4°C and subsequently incubated with a secondary antibody for 1 hour at room temperature. Total ALK (#3333), phosphorylated ALK Tyr 1604 (#3341), total AKT (#4685), phosphorylated AKT (#4060S), total ERK p42/44 (#9102),

phosphorylated ERK (#9106), and GAPDH (#5174) antibodies were purchased from Cell Signaling Technology (Danvers, MA). The blots were washed, transferred to freshly prepared enhanced Lumi-Light Western Blotting Substrate (Roche, Indianapolis, IN), and subjected to imaging analysis with an LAS-3000 imaging system (Fuji Photo Film Co., Stamford, CT) according to the manufacturer's instructions.

11. Generation of NCI-H3122 crizotinib-resistant cells

NCI-H3122 cells were exposed to increasing concentrations of crizotinib beginning at 100 nM, the approximate IC_{50}, as described previously [1]. The drug concentrations were increased stepwise when cells returned to normal growth kinetics. The cells were monitored for stable resistance by testing them after growth in drug-free medium. DNA fingerprinting confirmed that the H3122CR1 cells were derived from the H3122 parental cells (data not shown).

Supplementary table 1. Primers for sequencing and PCR

Cloning	
V1-FL-F	CAA GAT GGA CGG TTT CGC CGG CAGTC
V1-FL-R	TCA GGG CCC AGG CTG GTT CAT GC
V3-FL-F	ACT CTG TCG GTC CGC TGA ATG AAG
V3-FL-R	CCA CGG TCT TAG GGA TCC CAA GG
Mutagenesis	
G1269A-F	GAA GAG TGG CCA AGA TTG CAG ACT TCG GGA TGG
G1269A-R	CCA TGG CGA AGT CTG CAA TCT TGG CCA CTC TTC
L1196M-F ¢	CCC GGT TCA TCC TGA TGG AGC TCA TGG CGG
L1196M-R ¢	CCG GGA TGA GCT CCA TCA GGA TGA ACC GGG
Sequencing	
ALK-TK-F	GTA CAA GCT GAG CAA GCT CCG CAC
ALK-TK-R	AGG CAC TTT CTC TTC CTC TTC CAC
RT-PCR	
GAPDH-F	CAT TGA CCT CAA CTA CAT GGT
GAPDH-R	TTG GCC AGG GGT GCT AAG CAG T
Exon 23-F	GTA ACT TTG TAT CCT GTT CCT CCC AG
Exon 23-R	CAC CCT GGG TTC CAT CGA GGA CTT G
Exon 25-F	CAC ACT GAA GTA TAC TAT ACT AAA G
Exon 25-R	GTA GAA AGT TGA CAG GGT ACC AGG AG
EML4-I13	AGG AGA GAA AGA GCT GCA GTG
ALK-I19	GCT CTG AAC CTT TCC ATC ATA CTT
V1genome-s	CCA CAC CTG GGA AAG GAC CTA AAG
V1genome-as	AGC TTG CTC AGC TTG TAC TCA GGG
Fusion-EML4-F *	GTC AGC TCT TGA GTC ACG AGT T
Fusion ALK-R *	ATC CAG TTC GTC CTG TTC AGA GC
alk-v1-s *	GTG CAG TGT TTA GCA TTC TTG GGG
KIF5B £	TAA GGA AAT GAC CAA CCA CCA G
TFG £	CGTTTATTGGATAGCTTGGAA
Mutation-specific PCR	
L1196M-sepecific-F	ATC CCT GCC CCG GTT CAT CCT GA
L1196M-sepecific-R	CTG CCC ACT CTT GCT CCT TCC ATC
G1269A-sepecific-F	CTG GAA GAG TGG CCA AGA TTG C
G1269A-sepecific-R	GTT GAC AGG GTA CCA GGA GAT G

*Takeuchi,K.,Y.L.Choi, et al. (2008) Clinical Cancer Research 14(20):6618-6624
£ Daisy Wing Sze Wong, Maria Pik Wong et al. (2011) Cancer
¢ Ryohei Katayama, Alice T. Shaw et al. (2011) PNAS 108(18):7535-40

RESULTS

The type of *ALK* translocation fusion transcript in patients with NSCLC

We identified 7 patients with *EML4-ALK*-positive NSCLC who developed clinical acquired resistance to crizotinib. One of these 7 tumors had an *EGFR* mutation (L858R); none harbored a *KRAS* mutation after crizotinib treatment (Table 1). The 7 patient specimens, the 2 *EML4-ALK*-positive cell lines (NCI-H3122 and NCI-H2228) and the resistant cell line (SNU-2535) established from a relapsed patient after crizotinib therapy were analyzed for the *EML4-ALK* fusion transcript using RT-PCR. PCR products were run on agarose gels, and the fragments representative of variants exon 13-exon 20 (432 bp) and exon 6-exon 20 (917 bp) were sequenced. We determined that 3 specimens and NCI-H3122 cells contained the *EML4-ALK* fusion gene variant 1, and 3 specimens and NCI-H2228 cells harbored the *EML4-ALK* fusion gene variant 3. In addition, we detected that *ALK* was fused with exon 15 of *KIF5B* in one patient specimen (SNUH #7; Fig. 2). We confirmed through sequencing analysis that the PCR products contained *EML4-ALK* variants 1 and 3 (Fig. 3).

To confirm that *EML4-ALK* was present in the sample that was extracted from *EML4-ALK*-positive NSCLC patients, we amplified the genomic fusion point between *EML4* and *ALK* using genomic DNA as the template. For long-range genomic PCR, we used PCR primers targeting *EML4* exon 13 or intron 13 and *ALK* exon 20 or intron 19. This approach led to the identification of an ~3 kb product from patients (SNUH #1 and #4) and an ~4 kb product from the NCI-H3122 cell line (Fig. 4A). The breakpoint in the patient sample (SNUH

#4) was located 2,738 bp downstream of *EML4* exon 13 and 228 bp upstream of *ALK* exon 20 (Fig. 4B). The breakpoint in patient (SNUH #1) was located 1,738 bp downstream of exon 13 and 1,205 bp upstream of exon 20 (Fig. 4C). These fusion points were distinct from those reported previously [3]. These data suggest that various isoforms of *EML4-ALK* fusion enes are present in NSCLC patients.

Table 1. Characteristics of the NSCLC patients from whom the specimens were derived.

Sample ID	Specimen	ALK translocation type	Pretreatment of crizotinib				Posttreatment of crizotinib			
			ALK	EGFR		KRAS	ALK	EGFR		KRAS
				FISH	SEQ			FISH	SEQ	
SNUH#1	Pleural effusion	E13;A20 (V_1)	Positive	WT	WT	NA	NA	NA	WT	WT
SNUH#2	Pleural effusion/ primary tumor	E6;A20 (V_{3b})	Positive	NA	NA	NA	Positive	N(D)	WT	WT
SNUH#3	Primary tumor	E13;A20 (V1)	Positive	WT(D)	WT	WT	Positive	N(D)	WT	WT
SNUH#4	Pleural effusion	E13;A20 (V_1)	Positive	NA	NA	NA	Positive	N(LP)	WT*	WT
SNUH#5	frozen tissue	E13;A20 (V_1)	Positive	N(LT)	WT	WT	Positive***	P**(HP)	Missense mut (L858R)	WT
SNUH#6	sputum/ Pleural effusion	E6;A20 ($V_{3a/b}$)	Positive	WT	WT	WT	Positive	N(D)	WT	WT
SNUH#7	frozen tissue/ Pleural effusion	KIF5B15;A20	Positive	WT	WT	NA	Positive	N(D)	WT	WT

N, negative; D, disomy; LT, low trisomy; LP, low polysomy; HP, high polysomy ; WT, wild type ;

*EGFR exon20 C2361G>A (Q787 polymorphism)

**EGFR/nucleus=5.6, EGFR/CEP=1.79

*** ALK positive (suspicious amplification)

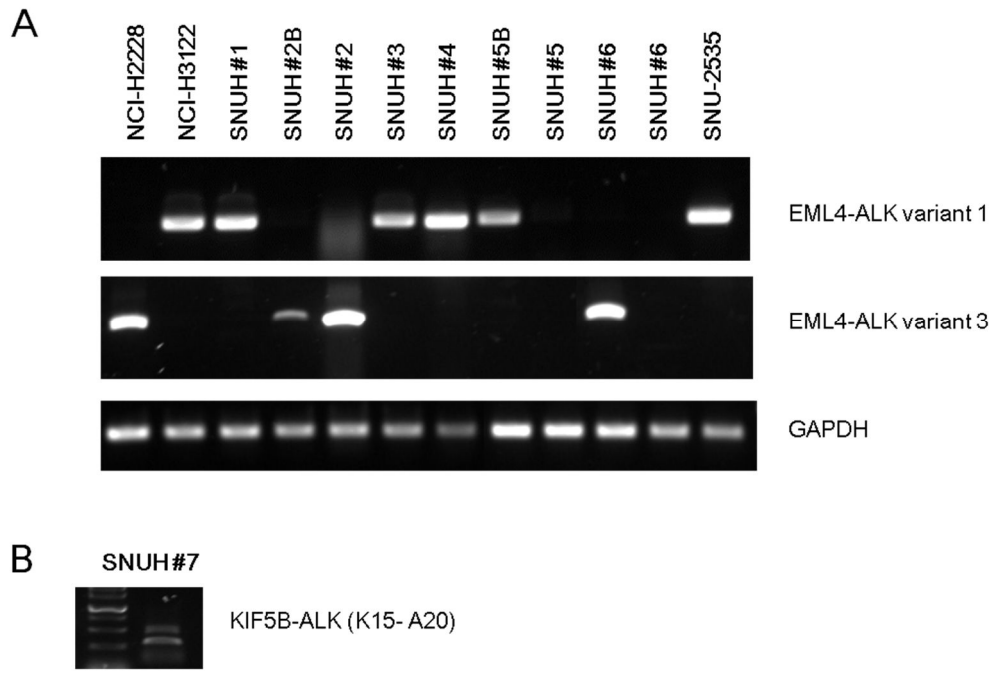


Figure 2. RT-PCR results for the detection of the EML4-ALK fusion transcript. (A) Virtual gel electrophoresis was performed on multiple RT-PCR products derived from 7 specimens and 3 cell lines. The 7 samples were known to harbor *EML4-ALK* variants 1 and 3 by multiplex RT-PCR. The exon 2 primer for *EML4* was expected to generate a PCR product of 917 bp with the exon 6-exon 20 fusion cDNA (variant 3), and the exon 13 primer was expected to generate products of 432 bp with exon 13-exon 20 cDNA (variant 1). (B) The exon 15 primer for *KIF5B* and exon 20 primer for *ALK* fusion transcripts in patient (SNUH# 7) are shown.

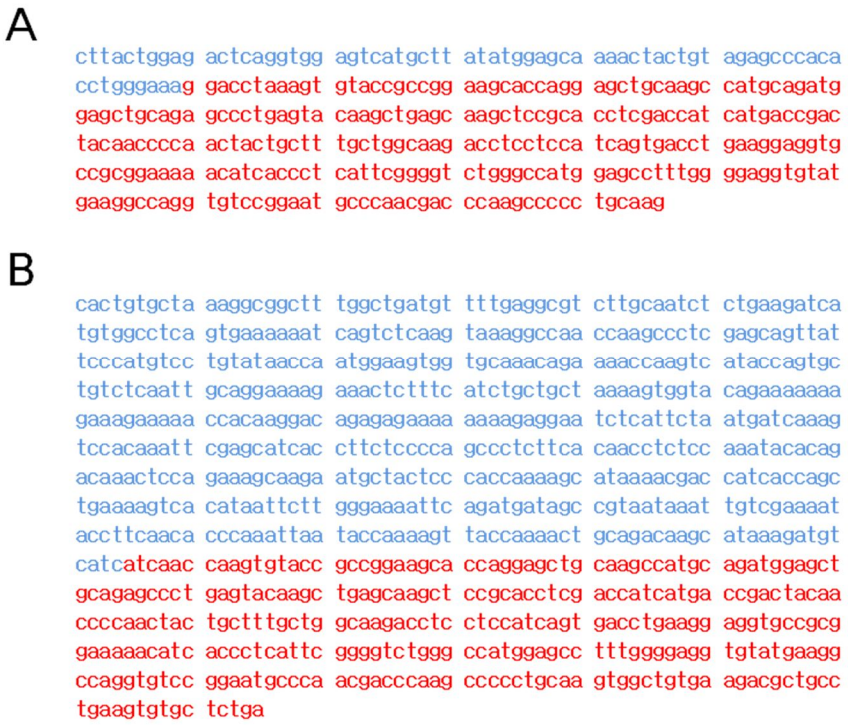


Figure 3. Sequence analysis for the detection of the *EML4-ALK* fusion transcript. The amino acid sequence of the PCR products of patients (SNUH #1 and #2) confirmed the presence of *EML4-ALK* fusion transcript variant 1 (A) and variant 3 (B). The residues corresponding to *EML4* or *ALK* are shown in blue and red, respectively.

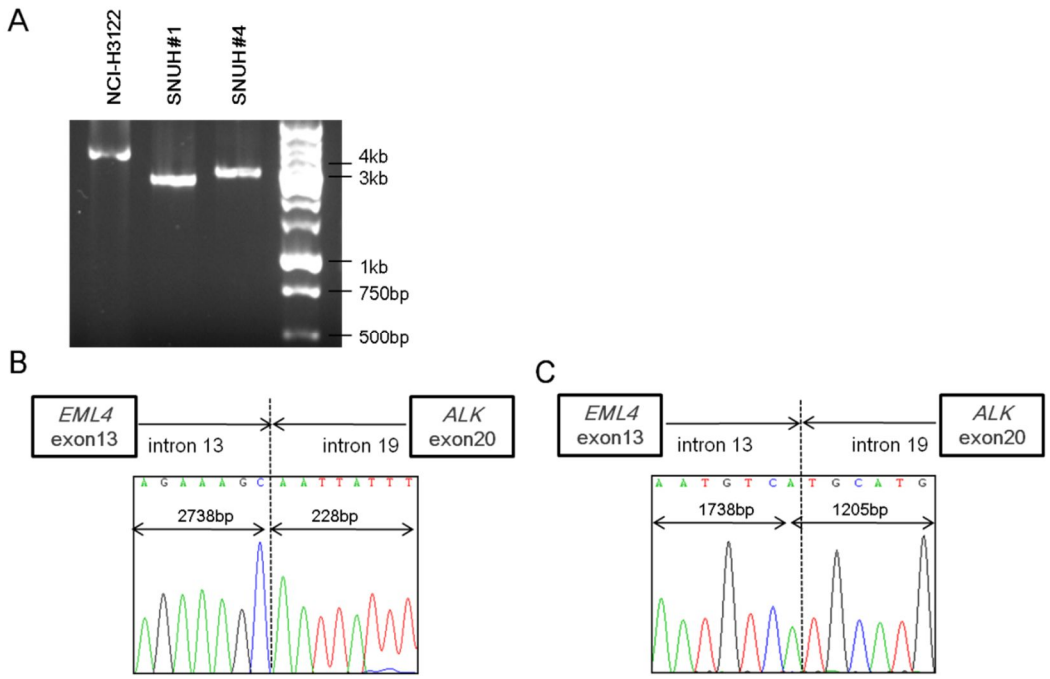


Figure 4. Screening representation of the genomic *EML4-ALK* fusion. The genomic structure of the EML4-ALK fusion was characterized in NSCLC tumor samples harboring transcript variant 1 (A). A 1kb DNA ladder was used as a marker. Sequencing of the PCR product from the genomic DNA of patients (SNUH #1 and #4) confirmed that *EML4* intron 13 is fused in frame with *ALK* intron 19 (B). The precise *EML4-ALK* breakpoint in the samples is indicated by the number of base pairs upstream and downstream of the representative *EML4* and *ALK* exons, respectively. The exact *EML4-ALK* breakpoint is indicated on the electropherogram by a dotted line.

The expression of *ALK* mutations in RNA and genomic DNA in lung cancer

Because resistance to tyrosine kinase inhibitors often results from acquired mutations within the kinase, we attempted to confirm the mutation of the *EML4-ALK* kinase domain by Sanger sequencing. We performed PCR with a forward primer targeted to *EML4* and a reverse primer targeted to *ALK* to amplify the fusion *EML4-ALK* cDNA and cloned the product into the TOPOTA vector. Sequencing of the *ALK* kinase domain from residues 1,094 to 1,396 demonstrated that one of the clinically progressing tumor samples contained G1269A and L1196M mutations, and the others contained only a G1269A or an L1196M mutation (Fig. 5A). We detected G→C and C→A substitutions at nucleotides 4,713 and 4,493 of *ALK* within *EML4-ALK* variant 1- or 3-positive samples. The 4,493 C→A substitution resulted in a leucine to methionine change within the *ALK* tyrosine kinase domain, corresponding to the L1196M gatekeeper mutation previously reported in a patient with acquired resistance to crizotinib [20]. The L1196M mutation of patient (SNUH #1) was detected at a low frequency (6.59%), and the mutation in patient (SNUH #6) was detected at a frequency of 27.8%. In addition, a G→C change at the position corresponding to nucleotide 4,713 of *EML4-ALK* cDNA was detected at frequencies of 68.75% (SNUH #4) and 28.5% (SNUH #1) in the patients' pleural effusion cDNA clones. However, we did not detect any other mutations in the TK domain, including the C1156Y substitution. To determine whether the ALK mutations had arisen de novo in the resistant specimens, we obtained pre-crizotinib treatment specimens from two patients

with resistance (SNUH #2, 5). We prepared total nucleic acids from each pre-crizotinib sample and sequenced the ALK TK domain by Sanger sequencing. None of the ALK mutations were discovered in the pre-crizotinib specimens. To corroborate these findings, we used genomic DNA sequencing to examine whether these mutations were present in genomic DNA. *ALK* exons 23 and 25 were amplified from genomic DNA using exon-specific primers. The PCR products were purified, and the individual products were sequenced. Notably, in the genomic DNA sequences, the peak corresponding to the mutation was approximately one-half of the wild-type peaks (Figs. 5B and 6). The 4,713 G→C nucleotide change results in a glycine→alanine change at the position corresponding to amino acid 1,269 of wild-type *ALK*. However, we could not detect the L1196M mutation in exon 23 amplified from genomic DNA in the sample from patient (SNUH #1). This problem may be due to contamination of the tumor specimen with noncancerous cells. Thus, to examine whether the G1269A and L1196M mutations were present at a low level in a subpopulation of patient specimens, we used a highly sensitive mutation-specific PCR assay [17] that can detect a mutation when it represents at least 1% of the mutated *ALK* alleles. Using this assay, we found both G1269A and L1196M in variant 1 *EML4-ALK* patient samples (SNUH #1 and 4) and the L1196M mutation in variants 1 and 3 (SNUH #1 and 6), which were detected by direct sequencing (Figs. 5C and 7). This result suggests that a fraction of the *EML4-ALK* fusion genes acquire the resistance mutation after crizotinib treatment.

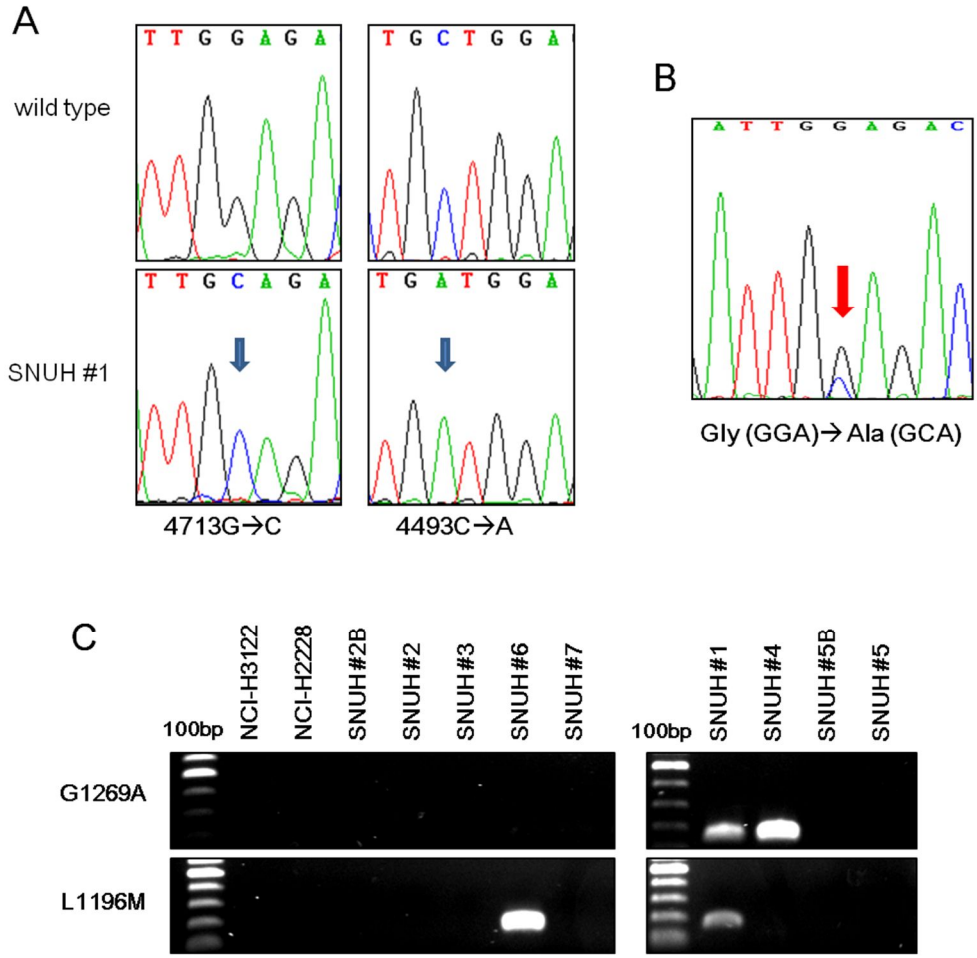


Figure 5. ALK mutations in relapsed patients after crizotinib. Electropherograms are presented for *EML4-ALK* variant 1 cDNA clones prepared from pleural effusion samples obtained from patients after relapse. The 4,713 G→C and 4,493 C→A mutations are present in the samples obtained after relapse (A). The sequencing chromatograms of the *ALK* G1269A (exon 25) mutation detected in the genomic DNA from patient (SNUH #1) are shown (B). Mutation-specific PCR assays (C). The 160 bp product amplified from genomic DNA after 30 PCR cycles is shown.

CCCAACTACTGCTTTGCTGGCAAGACCTCCTCCATCAGTGACCTGAAGGAGGTGCCGCGG
AAAAACATCACCCTCATTCGGGGTCTGGGCCATGGCGCCTTTGGGGAGGTGTATGAAGGC
CAGGTGTCCGGAATGCCCAACGACCCAAGCCCCCTGCAAGTGGCTGTGAAGACGCTGCCT
GAAGTGTGCTCTGAACAGGACGAACTGGATTTCCTCATGGAAGCCCTGATCATCAGCAAA
TTCAACCACCAGAACATTGTTCGCTGCATTGGGGTGAGCCTGCAATCCCTGCCCCGGTTC
ATCCTGATGGAGCTCATGGCGGGGGGAGACCTCAAGTCCTTCCTCCGAGAGACCCGCCCT
CGCCCGAGCCAGCCCTCCTCCCTGGCCATGCTGGACCTTCTGCACGTGGCTCGGGACATT
GCCTGTGGCTGTCAGTATTTGGAGGAAAACCACTTCATCCACCGAGACATTGCTGCCAGA
AACTGCCTCTTGACCTGTCCAGGCCCTGGAAGAGTGGCCAAGATTGCAGACTTCGGGATG
GCCCGAGACATCTACAGGGCGAGCTACTATAGAAAGGGAGGCTGTGCCATGCTGCCAGTT
AAGTGGATGCCCCCAGAGGCCTTCATGGAAGGAATATTCACTTCTAAAACAGACACATGG
TCCTTTGGAGTGCTGCTATGGGAAATCTTTTCTCTTGGATATATGCCATACCCCAGCAAA
AGCAACCAGGAAGTTCTGGAGTTTGTCACCAGTGGAGGCCGGATGGACCCACCCAAGAAC
TGCCCTGGGCCTGTATACCGGATAATGACTCAGTGCTGGCAACATCAGCCTGAAGACAGG
CCCAACTTTGCCATCATTTTGGAGAGGATTGAATACTGCACCCAGGACCCGGATGTAATC
AACACCGCT

PNYCFAGKTSSISDLKEVPRKNITLIRGLGHGAFGEVYEGQVSGMPNDPSPLQVAVKTLP
EVCSEQDELDFLMEALIISKFNHQNIVRCIGVSLQSLPRFILMELMAGGDLKSFLRETRP
RPSQPSSLAMLDLLHVARDIACGCQYLEENHFIHRDIAARNCLLTCPGPGRVAKIADFGM
ARDIYRASYYRKGGCAMLPVKWMPPEAFMEGIFTSKTDTWSFGVLLWEIFSLGYMPYPSK
SNQEVLEFVTSGGRMDPPKNCPGPVYRIMTQCWQHQPEDRPNFAIILERIEYCTQDPDVI
NTA

Figure 6. The amino acid sequence of G1269A and L1196M within the ALK kinase domain, which was the variant 1 putative EML4-ALK fusion protein cloned from a patient (SNUH# 1). The residues corresponding to the ALK kinase domain from amino acids 1,094 to 1,396 are shown. The residues corresponding to mutations L1196M (red) and G1269A (blue) are shown.

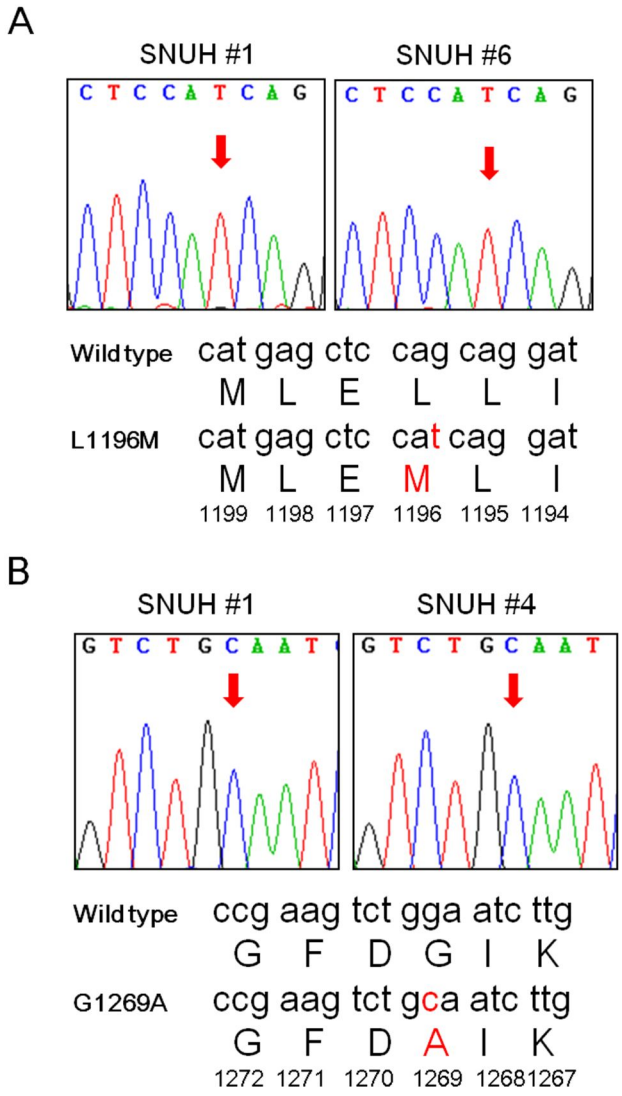


Figure 7. Sequencing of the mutation-specific PCR assay product. The L1196M and G1269A mutations in the genomic DNA of patients (SNUH #1, #4 and #6) are shown. The amino acid sequence of the *ALK* protein and the L1196M (A) and G1269A (B) mutation residues. The electropherograms of the two PCR products are shown in the reverse direction.

***ALK* mutations as resistance mechanisms to crizotinib**

To determine how the L1196M and G1269A mutations affect the *ALK* fusion proteins, we introduced the mutations into wild-type *EML4-ALK* variant 1 as described in the materials and methods. 293T cells were transiently transfected with cDNAs encoding *EML4-ALK* WT, L1196M, or G1269A for 48 hours. After treatment with crizotinib for 2 hours, the cell extracts were subjected to immunoblotting with an antibody against phospho-ALK and the downstream signaling pathways. The L1196M and G1269A mutants reduced the sensitivity of the wild type to crizotinib and diminished crizotinib-mediated inhibition of downstream AKT and ERK1/2 phosphorylation (Fig. 8B). Consistent with these findings, higher concentrations of crizotinib were required to inhibit ALK phosphorylation in the mutant cells compared with wild-type cells. To directly determine whether these mutations confer resistance to crizotinib, we engineered BaF3 cells to express EML4-ALK mutations and examined cell survival after treatment with crizotinib. As previously reported [17,22], we found that the G1269A and L1196M mutations conferred a high level of resistance to crizotinib (Fig. 8A).

We next examined the ability of crizotinib to inhibit the growth of 3 cancer cell lines known to harbor an *ALK* translocation and resistant cell lines from patients. We established a cell line, SNU-2535, from the pleural effusion of patient (SNUH #4), who harbored the G1269A mutation. DNA fingerprinting confirmed that the SNU-2535 cells were derived from patient (SNUH #4) (data not shown). We sequenced the *ALK* tyrosine kinase domain in SNU-2535 cells and detected the G1269A mutation (61.9%). In addition, the SNU-

2535 cells did not contain an *EGFR* or *KRAS* mutation, but retained the *ALK* gene rearrangement as determined by FISH analysis (Fig. 9B). We also generated crizotinib-resistant cell lines from NCI-H3122 cells. These resistant cells were designated H3122CR1 and maintained in 1 μM crizotinib. As previously reported, H3122CR1 cells harbor both the gatekeeper L1196M *EML4-ALK* mutation and amplification of the mutated *EML4-ALK* allele[17]. However, the generated H3122CR1 cells did not have a genetic alteration in *ALK* and an *EGFR* or *KRAS* mutation. SNU-2535 and H3122CR1 cells were more resistant to crizotinib compared with H3122 cells (IC50 = 4.099 and 6.011 μM, respectively; Fig. 10A). We evaluated ALK, AKT, and ERK phosphorylation in response to crizotinib in EML4-ALK-positive and crizotinib-resistant cell lines. SNU-2535 and H3122CR1 cells were minimally inhibited by downregulation of ALK signaling (Fig. 10B). Compared with SNU-2535 cells, in H3122 cells, the relative decrease in cell growth observed with crizotinib treatment correlated with increased apoptosis, as determined using FACS analysis (Fig. 10C). Collectively, these findings suggest that ALK phosphorylation and the phosphorylation of AKT and ERK were preserved at high doses of crizotinib in the SNU-2535 cells with the G1269A mutation compared with H3122 cells with wild-type *EML4-ALK*.

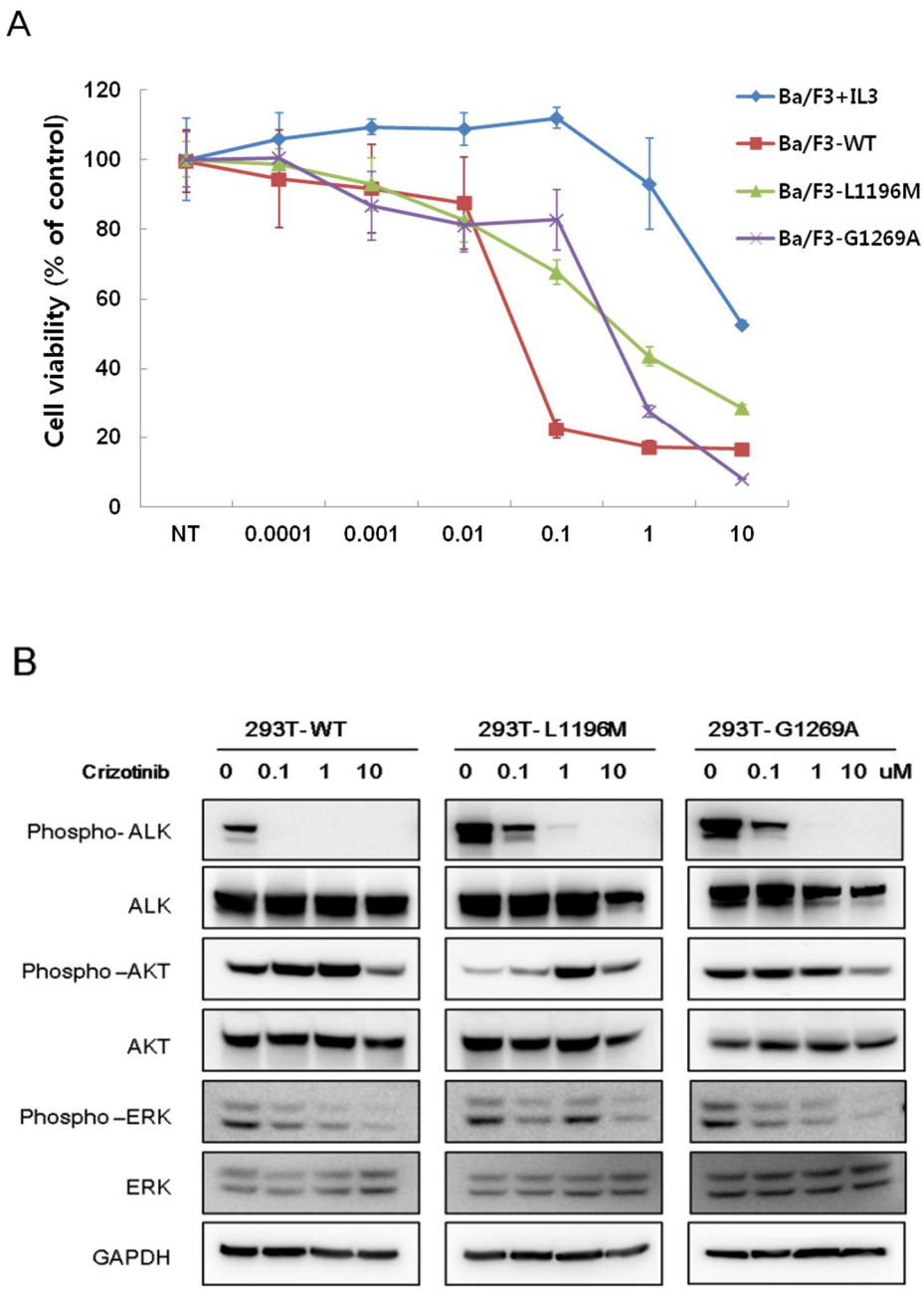


Figure 8. The effects of crizotinib against *EML4-ALK* expressing WT, L1196M or G1269A mutants. (A) BaF3 cells were transformed to express wild-type *EML4-ALK* or *EML4-ALK* harboring one of the two identified resistance mutations (L1196M or G1269A). The parental Ba/F3 cells (with

IL-3) or the *EML4-ALK*-expressing Ba/F3 cells (without IL-3) were treated with the indicated doses of crizotinib for 72 hours. Cell survival was measured using CCK-8. Each concentration was measured several times, and the average and SD are shown. The IC50 values were 14.874, 0.049, 0.216, and 0.482 μM in Ba/F3+IL3, Ba/F3-WT, Ba/F3-L1196M, and Ba/F3-G1269A cells, respectively. (B) 293T cells were transiently transfected with pc-DNA3.0-*EML4-ALK*. At 48 hours post-transfection, the cells were treated with the ALK inhibitor for 2 hours. The lysates were subjected to immunoblotting with antibodies specific for the indicated proteins.

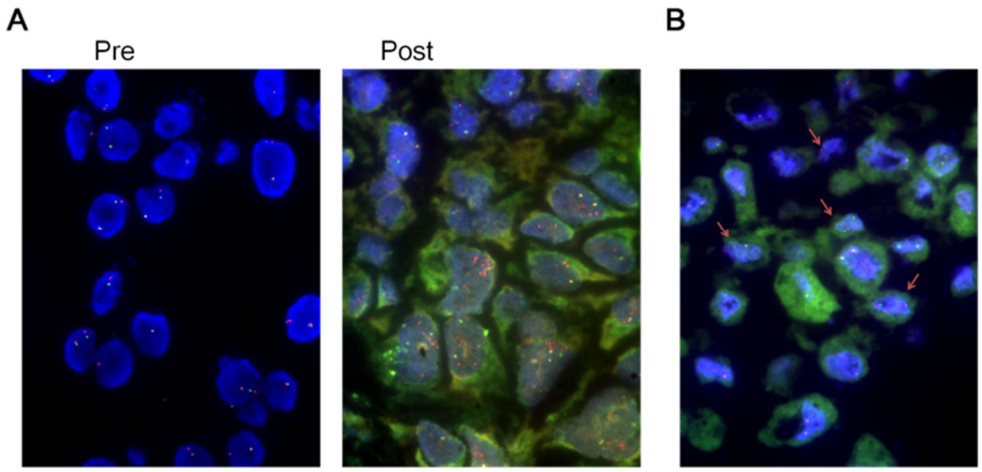
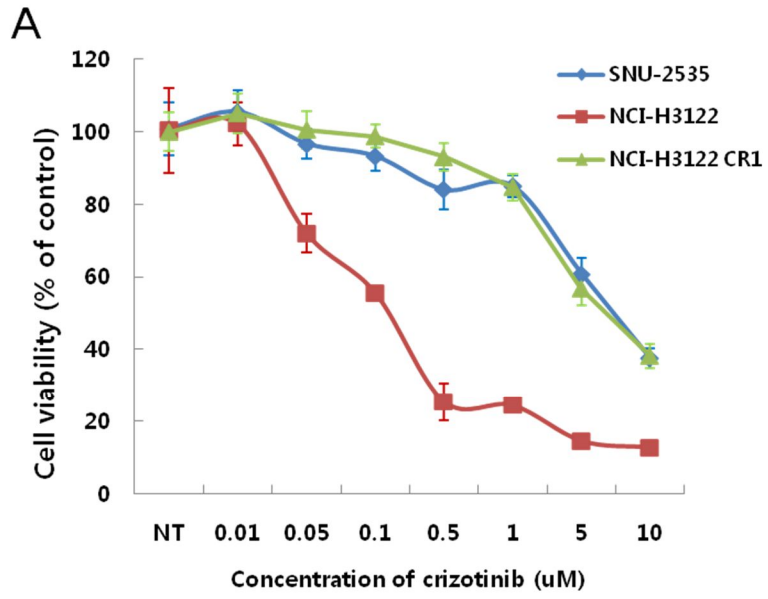
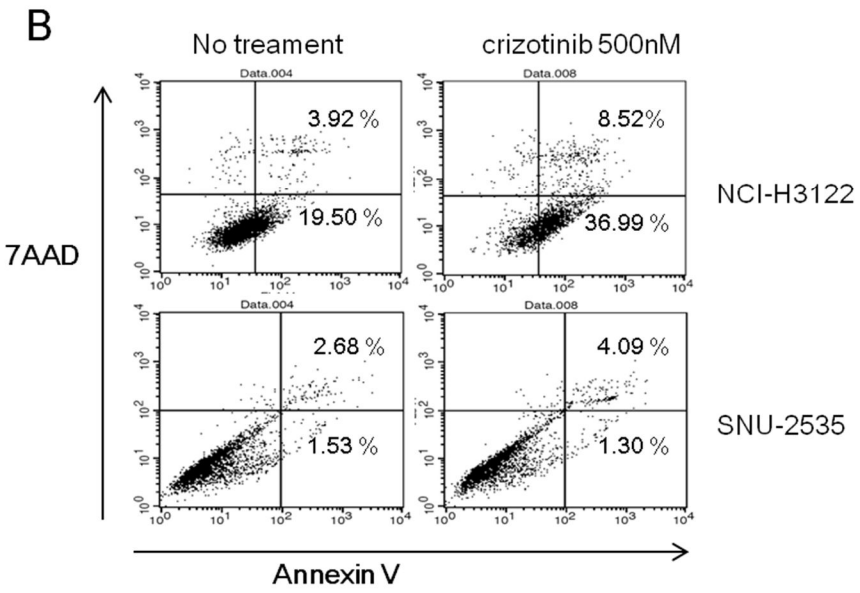


Figure 9. ALK fluorescence in situ hybridization (FISH) using a dual-color break-apart probe. (A) FISH analysis of patient #9 before crizotinib treatment (left) and after progression on crizotinib (right). (B) FISH analysis of SNU-2535 cells shows a gain of split green (5') and red (3') ALK signals for each tumor cell.


A
120
100
80
60
40
20
0
Cell viability (% of control)
SNU-2535
NCI-H3122
NCI-H3122 CR1
NT
0.01
0.05
0.1
0.5
1
5
10
Concentration of crizotinib (uM)


B
No treament
crizotinib 500nM
Data.004
Data.008
3.92 %
19.50 %
8.52%
36.99 %
2.68 %
1.53 %
4.09 %
1.30 %
NCI-H3122
SNU-2535
7AAD
Annexin V

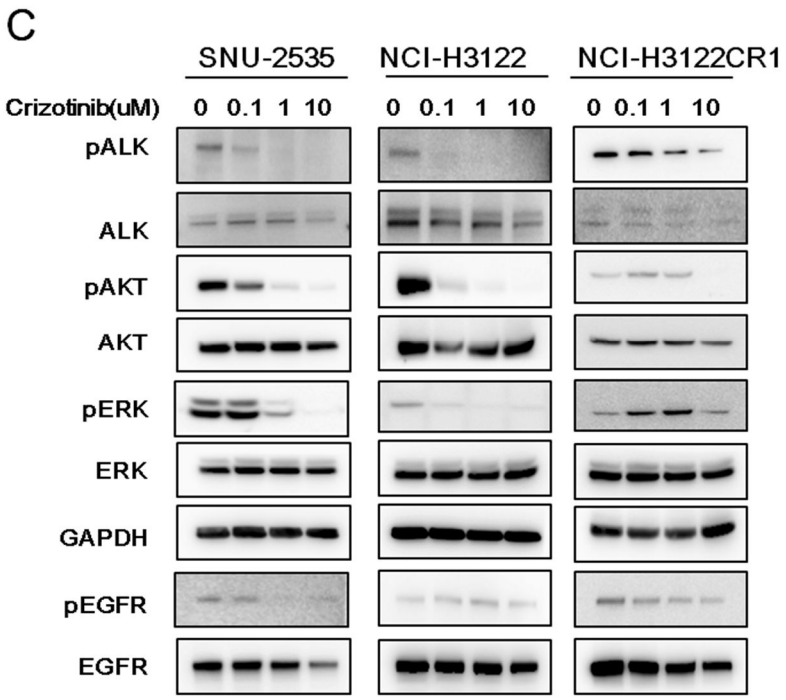


Figure 10. The potency of crizotinib in ALK mutant cell lines. NCI-H3122 (EML4-ALK variant 1 type), SNU-2535 (EML4-ALK mutant), and NCI-H3122CR1 (crizotinib-resistant) cells were treated with ALK tyrosine kinase inhibitor or vehicle for 72 hours. The data are presented as the percentage of viable cells compared with control (vehicle only) cells (A). Apoptosis is induced by crizotinib treatment (B). NCI-H3122 cells and SNU-2535 cells were treated with increasing concentrations of crizotinib for 24 hours. The cells were stained with Annexin V and propidium iodide and analyzed using FACS. Viable cells are defined as the AV/7AAD double-negative population. Apoptotic cells are defined as the sum of AV-positive, 7AAD-negative and AV/7AAD double-positive cell populations. H3122 and SNU-2535 cell lines were treated with crizotinib for 4 hours. The lysates were subjected to immunoblotting with antibodies specific for the indicated proteins (C).

DISCUSSION

ALK-rearranged lung cancers are a subset of cancers that are clinically sensitive to the crizotinib, which is being compared with standard chemotherapy [14]. However, a great deal remains to be understood about *EML4-ALK*. The identification of alternative strategies to treat lung cancers remains a clinical priority because acquired resistance to targeted ALK inhibition has appeared in previously reported cases [20]. Recently, most mechanisms represent one of two categories: the genetic alteration of the drug target or activation of bypass signaling [23,29]. Resistance mutations in the drug target markedly decrease the efficacy of the inhibitor against the target kinase [24, 25]. The most frequent resistance mutation represents the gatekeeper residue. Other mechanisms of acquired drug resistance involve gene amplification of the kinase target or activation of alternative signaling pathways [17,29,30]. EML4-ALK-positive NSCLC represents another tyrosine kinase-driven cancer that is highly responsive to tyrosine kinase inhibitor (TKI) therapy.

In this paper, we report results regarding the heterogeneity of resistance in NSCLC cases that progressed while on crizotinib therapy. We identified a mutation in relapsed patients who had been using crizotinib. One of the mutations identified was the gatekeeper L1196M substitution, which is analogous to T315I in ABL and T790M in EGFR;

the same L1196M mutation was detected in our patients with acquired resistance, as previously reported [17,22]. Notably, we identified another ALK kinase mutation, G1269A, which was reported [22] in two patients who possessed EML4-ALK variant 1, but we did not detect the C1156Y mutation, which has been previously reported [20]. As explained above, G1269 is flipped such that the carbonyl oxygen points toward the side chain of the conserved K1150 rather than making a conserved hydrogen bond with the histidine of the HRD motif [31]. These mutations were detected by direct sequencing and by the cloning and sequencing of individual clones in relapsed patient specimens after crizotinib. However, we could not detect the L1196M mutation in exon 23 amplified from genomic DNA in the sample from patient (SNUH #1). This problem may be due to contamination of the tumor specimen with noncancerous cells. Thus, to examine whether the G1269A and L1196M mutations were present at a low level in a subpopulation of patient specimens, we used a highly sensitive mutation-specific PCR assay [17] that can detect a mutation when it represents at least 1% of the mutated *ALK* alleles. We detected both the L1196M and G1269A mutations in one patient (SNUH #1), L1196M in one (SNUH #6) and G1269A in one (SNUH #4).

Interestingly, we did not detect any other mutation in 4 patients after resistance relapse to crizotinib. The observation that 3 out of 7 resistant

specimens had an ALK mutation suggests that resistance mutations in ALK-positive NSCLC will likely be a common mechanism of resistance to crizotinib. However, Doebele et al. have suggested that *ALK*-rearranged NSCLC with intrinsic or acquired resistance to crizotinib can occur through multiple different mechanisms [22]. Only one patient (SNUH #5) was *EGFR* positive, and the presence of an *EGFR* mutation (L858R) that was not present in the initial specimen was used to establish the *ALK*-positive diagnosis (Table 1 and Fig. 9). Furthermore, this patient was examined for the suspicious amplification of *ALK* in the post-treatment specimen compared with the pre-treatment specimen using FISH, but no *ALK* mutation was detected. Other studies have shown the coexistence of both an *EGFR* mutation or *KRAS* mutation and *ALK* gene rearrangement in the same tumor sample [22,32]. In this study, a specific potential resistance mechanism was identified in 4 of these cases (Fig.11).

In the present study, reduced cell growth inhibition by crizotinib was also observed in cells with the mutations, L1196M or G1269A, compared with the wild-type EML4-ALK cells. We further showed that resistance of crizotinib by mutation in tyrosine kinase resulted in significant inhibition of crizotinib-induced apoptosis in SNU-2535 cells, suggesting that ALK mutation mediated by inhibition of the ALK phosphorylation plays a pivotal role in crizotinib-induced

resistance in EML4-ALK lung cancer cells. These findings are consistent with the previous observation that ALK mutation contributes to ALK inhibitor induced cell growth inhibition in ALK mutation–expressing cells [20,22]. *In vitro* studies with transiently expression of L1196M or G1269A mutant *EML4-ALK* 293T cell lines show maintenance of ALK phosphorylation at higher doses of crizotinib compared with wild-type *EML4-ALK* expressing 293T cell line. Furthermore, we found that crizotinib prominently abrogated the phosphorylation of AKT and ERK in the *EML4-ALK*–positive lung cancer cell line NCI-H3122, consistent with previous results implicating activation of PI3K-AKT and MAPK signaling in malignant transformation by *EML4-ALK* [3]. In contrast, we found that crizotinib did not suppress ALK, ERK and AKT phosphorylation in the crizotinib-resistance lung cancer cell line H3122CR1 and SNU-2535.

To date, L1196M and G1269A are drug resistance mutations that have been found in relapsed patients after crizotinib therapy. We demonstrate that some patients without an ALK kinase domain mutation have heterogeneity in NSCLC with crizotinib resistance. Substantial information is known about variable resistance mutations among diverse, resistant patients. Therefore, the most effective therapeutic strategy for *ALK*-positive lung cancers may ultimately require targeting

of not only ALK resistance mutations but also emerging alternative pathways of resistance.

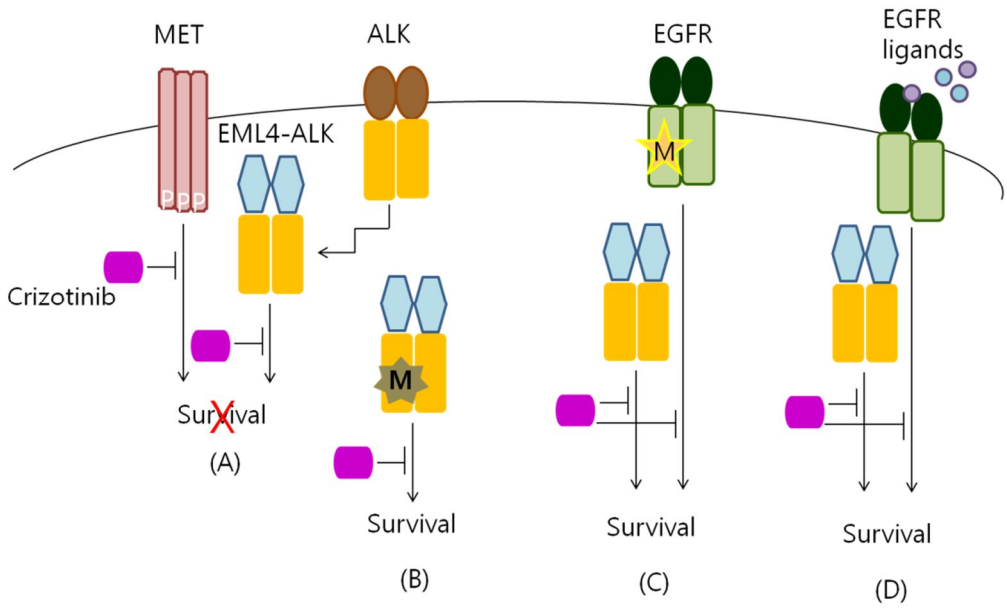


Figure 11. Mechanism of acquired resistance to ALK-TKI (modified from reviews of Takaaki Sasaki and Pasi A.Jänne [1,2]). (A) ALK-TKI inhibits phosphorylation of ALK and MET and the survival signal is shut down, leading to apoptosis of cells. (B) Secondary mutation (L1196M or G1269A etc.) prevents binding of ALK-TKI to ALK, resulting in cell survival. (C) Even when phosphorylation of ALK is inhibited by ALK-TKI, activation of the PI3K and MAPK pathway is maintained through an EGFR activating mutation (star). (D) EGFR ligands induce activation of the PI3K and MAPK pathway through EGFR; this activation is independent of ALK.

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국문 초록

서론: ALK 가 재배열된 폐암에서 ALK 의 tyrosine kinase 억제제인 crizotinib 이 임상실험에서 뛰어난 효과를 나타내었지만, 이내 crizotinib 에 대한 약제 내성을 나타내는 환자가 발생하였다. 약제내성의 원인으로는 ALK 가 과발현되어 있거나, tyrosine kinase 부위에 새로이 생긴 돌연변이가 알려져 있다. 본 논문에서는 ALK 가 재배열된 폐암환자에서 crizotinib 의 약제내성에 관여하는 유전자의 돌연변이에 대해서 확인하고자 한다.

방법: crizotinib 에 내성을 보인 7 명의 환자의 샘플을 이용해서 ALK 의 kinase domain 에서 나타나는 돌연변이 유무를 RT-PCR 과 sequencing 방법을 통해서 확인하였다. 또한, EGFR 과 KRAS 의 돌연변이 발생 유무도 추가적으로 확인하였다. *In vitro* 실험으로 ALK 가 재배열된 EML4-ALK 를 가지는 세포주 NCI-H3122 와 crizotinib 에 내성을 나타낸 환자에서 만든 세포주 SNU-2535 를 가지고 crizotinib 에 대한 항원항체반응(western blot)과 세포성장 실험등을 진행하였다.

결과: 기존에 보고된 L1196M 또는 G1269A 라는 ALK 의 이차적인 돌연변이를 7 명중에 3 명의 환자샘플에서 확인하였고 한 환자의 샘플에서는 두 개의 돌연변이가 함께 발현되고 있음을 확인 할 수

있었다. 반면에 나머지 4 명의 환자에서는 ALK kinase domain 에서 어떠한 돌연변이도 찾을 수 없었다. 흥미로운 사실은 돌연변이를 찾을 수 없었던 4 명의 환자 중 한 명의 환자샘플에서 EGFR-L858R 이라는 EGFR 의 신호전달을 활성화 시켜주는 돌연변이를 찾을 수 있었고, ALK 의 재배열이 여전히 유지 되고 있는 상황에서 이런 기작이 이루어지고 있음을 확인 할 수 있었다. 그래서 이런 L1196M 과 G1269A 돌연변이를 가지는 세포주를 만들기 위해서 retrovirus 를 이용하여 인위적으로 만든 후에, crizotinib 에 의한 약제효능에 어떤 영향을 끼치는지 확인해 보았다. 이들 돌연변이는 crizotinib 에 의해서 억제되는 ALK 의 신호전달과정을 방해하고 있었으며 종양 세포의 성장저해가 억제되고 있음을 확인할 수 있었다. 또한 환자샘플로부터 만들어낸 내성을 지닌 세포주 SNU-2535 에서 G1269A 가 존재함을 확인하였고, 이세포주에 crizotinib 을 주었을 때 세포성장 억제가 저해되고 Akt 와 ERK 와 같은 ALK 신호전달체들의 발현이 저해되고 있음을 확인 할 수 있었다. 이는 앞서서 찾아낸 돌연변이들이 crizotinib 에 내성을 나타내고 있음을 다시금 확인시켜주는 결과이다.

결론: 임상적으로, crizotinib 은 ALK 가 재배열 된 폐암에서 굉장한 효과를 보이는 약제이지만 내성이 발생되는 문제점을 가지고 있다. 본 논문에서 밝힌 L1196M 과 G1269A 돌연변이는 이런 폐암에서 crizotinib 의 내성에 관여하는 것을 확인 할 수 있었다. 하지만

crizotinib 에 대한 내성의 원인을 특정 유전자 돌연변이만으로는 모든 환자에서 설명할 수는 없었다. 이런 관찰의 결과들은 이후 환자에서 발생하는 crizotinib 의 약제 내성을 극복하는 치료법에 중요한 역할을 할 것이라고 생각된다.

주요어 : ALK, EML4-ALK, crizotinib, 내성 돌연변이
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