Heterogeneity of Genetic Changes Associated with Acquired Crizotinib Resistance in *ALK*-Rearranged Lung Cancer

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Background: Anaplastic lymphoma kinase (ALK)-rearranged non–small-cell lung cancer (NSCLC) is markedly sensitive to the ALK inhibitor crizotinib. However, acquired resistance to crizotinib is inevitable through several mechanisms. Therefore, this study was conducted to identify genetic alterations associated with crizotinib resistance.

Methods: Tumor samples were derived from seven ALK-positive NSCLC patients who showed acquired resistance to crizotinib, and these patients were analyzed for *ALK*, *EGFR*, and *KRAS* mutations and *ALK* and *EGFR* gene amplifications. In vitro cytotoxicity of crizotinib and *ALK* downstream signals were compared between crizotinib-naive and -resistant NSCLC cells.

Results: After a median duration of 6 months (range, 4–12 months), seven ALK-positive NSCLC patients developed acquired resistance to crizotinib. Three patients harbored secondary *ALK* mutations, including one patient with both mutations: L1196M (n = 2) and G1269A (n = 2). Of note, one patient displayed *ALK* gene copy number gain (4.1-fold increase compared with the pre-crizotinib specimen) and *EGFR* L858R mutation with high polysomy. The amphiregulin concentration was high in the supernatant fluid from five patients with malignant pleural effusion (116.4–18934.0 pg/ml). SNU-2535 cells derived from a patient who harbored the G1269 mutation were resistant to crizotinib treatment similar to H3122 CR1 cells. L1196M and G1269A mutant clones were less sensitive to crizotinib and ALK downstream signals were ineffectively suppressed in these clones.

Conclusions: Genetic changes associated with crizotinib resistance are heterogeneous in *ALK*-rearranged NSCLC patients who respond to crizotinib and subsequently develop resistance.

Key Words: Anaplastic lymphoma kinase, Non–small-cell lung cancer, Crizotinib, Acquired resistance, ALK mutation.

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Anaplastic lymphoma kinase (ALK) has been identified as the fusion partner of echinoderm microtubule-associated protein-like 4 (EML4) in non–small-cell lung cancer (NSCLC), and this fusion accounts for 3.0 to 6.7% of NSCLC patients.[1,2] Despite various fusion variants[2] and partners, including TRK-fused gene[3] and kinesin family member 5B (KIF5B),[4,5] the complete intracellular portion of ALK is preserved and oncogenic. In vitro and in vivo sensitivities to ALK tyrosine kinase inhibitors[6] were translated into clinical activities of crizotinib, a dual inhibitor of ALK and c-MET against ALK-positive NSCLC.[7] A recent phase I trial of crizotinib resulted in an objective response rate of 57%, a disease control rate of 87% at 8 weeks, and a median progression-free survival of 10 months.[7,8] In addition, a first-line phase III trial comparing crizotinib and pemetrexed plus cisplatin is currently ongoing in *ALK*-rearranged NSCLC patients (NCT01154140). Although *ALK*-rearranged NSCLC was shown to be highly susceptible to crizotinib treatment,[7] a fraction of tumors eventually became resistant to crizotinib.[9] In line with the T790M "gatekeeper" mutation in epidermal growth factor receptor (*EGFR*)-mutant NSCLC,[10] L1196M in the *ALK* gatekeeper site was identified in a Japanese patient who showed acquired resistance to crizotinib.[9] In addition, nongatekeeper mutations such as L1152R,[11] C1156Y,[9] and G1269A,[12] and alternative pathway activation, including ligand-dependent *EGFR* activation,[11] *EGFR* mutation,[12] or amplification,[13] were suggested to be resistance mechanisms in *ALK*-positive NSCLC treated with ALK inhibitors.

Here, we evaluated heterogeneous genetic changes associated with crizotinib resistance in seven *ALK*-rearranged NSCLC patients who initially responded and eventually showed resistance to crizotinib.

PATIENTS AND METHODS

Patients

Crizotinib at a dosage of 250 mg twice daily was administered to seven patients with *ALK*-rearranged NSCLC, of whom four were enrolled into phase I (NCT00585195) trial[7] and two were enrolled in phase II (NCT00932451) trial. All were diagnosed with stage IV NSCLC disease, and most patients received crizotinib as second-line treatment and beyond (Table 1). Response evaluation was based on the Response Evaluation Criteria in Solid Tumors version 1.0 per

protocol.[14] Tumor tissues were obtained in patients who failed crizotinib and provided informed consent. This study was approved by the Institutional Review Board of Seoul National University Hospital (H-1205-126-411).

Molecular Determinations of *ALK*, *EGFR*, and *KRAS* and Measurement of *EGFR* Ligand Levels

ALK fluorescence in situ hybridization (FISH) was analyzed using an *ALK* probe (Vysis LSI ALK dual-color, break-apart rearrangement probe; Abbott Molecular, Des Plaines, IL) in formalin-fixed and paraffin-embedded tissues or cell pellets derived from malignant pleural effusions. *ALK* FISH was considered positive when break-apart signals were observed in more than 15% of 50 or more analyzed cells.[7,15] In addition, the number of copies per cell of the *ALK* gene rearrangement was determined before and after crizotinib, and *ALK* gene amplification was defined as a greater than twofold increase of the mean of the rearranged gene per cell in the post-crizotinib specimen compared with the precrizotinib specimen.[12] *EGFR* amplification was evaluated using an *EGFR* probe (Vysis LSI EGFR; Abbott Molecular) and *EGFR* FISH positivity was determined according to the Colorado scoring criteria.[16] *EGFR* and *KRAS* mutations were analyzed using direct sequencing of extracted DNA from NSCLC patient tumors or cell lines.[17]

Amphiregulin and EGF levels were measured in the cell culture medium or supernatant fluid of pleural effusion using an enzyme-linked immunosorbent assay kit (Quantikine R&D Systems, Minneapolise, MN). All samples were run in at least duplicate. The concentrations of the EGFR ligands were determined by comparison with standard curves.

Cell Lines and Reagents

NCI-H3122 cells were provided by Pasi A. Jänne (Dana-Farber Cancer Institute, Boston, MA) and NCI-H2228 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). SNU-2535 cells were established at the Korean Cell Bank using pleural effusion in a patient who had developed acquired resistance to crizotinib (SNU4 patient, Table 1). 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 (GIBCO). The cell lines were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide. Crizotinib was kindly provided by Pfizer (Milwaukee, WI) and dissolved in dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO) for experiments. 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) and were used for immunoblotting. The blots were washed, transferred to freshly prepared enhanced Lumi-Light Western Blotting Substrate (Roche, Indianapolis, IN), and subjected to imaging analysis using an LAS-3000 imaging system (Fuji Photo Film Co., Stamford, CT).

Reverse Transcription–Polymerase Chain Reaction and Genotyping

Total RNA was isolated from the NSCLC cell lines using an RNA Mini kit (Invitrogen, Carlsbad, CA). Each sample was reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen). PCR amplification was performed using protocols with specific primers

TABLE 1. Clinical Characteristics of Patients with Acquired Resistance to Crizotinib

					Crizotinib outcomes					
Patient No.	**Age**	**Sex**	**Histology**	**Prior chemotherapy**	**Lines**	**Cycles**	**ORR**	**PFS, months**	**Specimens after resistance**	**OS, months**
SNU1	45	M	Adenocarcinoma	GP + bevacizumab × 4 GP × 6	2	7	PR	6	Pleural effusions	14
SNU2	51	F	Adenocarcinoma	Gefitinib × 1 Pemetrexed × 6 Navelbine × 6	5	8	PR	5	Pleural effusions, hepatic lesion	30
SNU3	64	M	Adenocarinoma	None	1	11	PR	9	Lung lesion	28
SNU4	57	F	Non-small cell carcinoma, NOS	GC × 2 Docetaxel × 7	3	13	PR	12	Pleural effusions (SNU-2535)	24
SNU5	47	F	Adenocarcinoma	Pemetrexed + cisplatin × 1 Irinotecan + cisplatin × 6	2	4	PR	4	Liver lesion	12
SNU6	36	F	Adenocarcinoma	Erlotinib × 1 Pemetrexed × 15 GN × 4	5	8	PR	6	Pleural effusions	29
SNU7	50	M	Adenocarcinoma	Paclitaxel + cisplatin × 2 Pemetrexed × 7 Gefitinib × 1	4	8	PR	6	Pleural effusion,hepatic lesion	25

GC, gemcitabine plus carboplatin; GN, gemcitabine plus navelbine; GP, gemcitabine plus cisplatin; ORR, overall response rate; OS, overall survival; PFS, progression-free survival from the first administration of crizotinib until the date of objective disease progression or death from any cause; PR, partial response.

(Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A387). 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. Genomic DNA was isolated from cell pellets or tissues using an ALL-prep DNA/RNA micro kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Exons of *ALK* were amplified from genomic DNA using the High Fidelity plus PCR system (Roche, Indianapolis, IN) and sequenced bi-directionally by Sanger dideoxynucleotide sequencing with the primers for *ALK* exons 23 and 25 (Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/JTO/A387). Mutation-specific PCR of L1196M and G1269A were performed with primer sequences. Direct sequence analysis was performed using an ABI 3730 DNA sequencer (Applied Biosystems, Carlsbad, CA). The reference sequences for *ALK* and *EML4* were NM_004304.4 and NM_019063.3, respectively. All mutations were confirmed at least twice from independent PCR isolates and sequence tracing was reviewed in the reverse direction by visual inspection.

Cloning of *EML4-ALK* Expression Constructs

EML4-ALK variant 1 cDNA from H3122 cells 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, the L1196M or G1269A mutation was introduced using site-directed mutagenesis (Agilent Technologies, La Jolla, CA) with mutant-specific primers according to the manufacturer's instructions. The PCR primers are listed in Supplementary Table 1 (Supplemental Digital Content 1, http://links.lww.com/JTO/A387). The sequencing products were analyzed using Sequencer software (Gene codes). The Basic Local Alignment and Search Tool (BLAST database) was used to determine the identification of unknown sequences. Retroviral infection and culture of Ba/F3 cells were performed using a previously described method.[1] Polyclonal cell lines were established by puromycin selection and subsequently cultured in the absence of interleukin-3. Uninfected Ba/F3 cells were used as controls.

Cell Proliferation and Apoptosis Assays

NSCLC cells at a density of 5000 cells per well in 96-well plates were cultured in the presence of crizotinib or vehicle for 72 hours, and cell proliferation was analyzed using the CCK-8 colorimetric assay (Dojindo, Tokyo, Japan). The absorbance was measured at 450 nm in an Eon Microplate Spectrophotometer (BioTek, Winoosk, VT) using at least duplicate samples. The NSCLC cells treated with crizotinib or vehicle were stained with Annexin V and 7 AAD, and then the cells were analyzed by flow cytometry using a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, NJ).

RESULTS

Patient Outcomes and the *ALK* Fusion Transcript Analysis

Treatment outcomes are described in Table 1. All patients achieved a partial response and subsequently experienced disease progression at a median time of 6 months (range, 4–12 months). Although most patients discontinued crizotinib at the time of documented progressive disease, SNU2 patients received crizotinib beyond disease progression owing to ongoing clinical benefit. *EML4-ALK* fusion transcripts were analyzed by reverse transcription–polymerase chain reaction and PCR fragments that represented variable truncations of *EML4* at exons 13 to 20 (432 base pairs) and 6 to 20 (917 base pairs) were sequenced. Variant 1 that fuses *EML4* exon 13 to *ALK* exon 20 (E13;A20) was identified in four patients and variant 3 (E6;A20) was observed in two patients (Table 2 and Fig. 1*A*). One patient harbored an *ALK* fusion at exon 20 with exon 15 of *KIF5B*.

ALK Mutations in Crizotinib-Resistant Tumors

The G→C and C→A substitutions at nucleotides 4713 (G1269A) and 4493 (L1196M) of *ALK* were identified and confirmed by Sanger sequencing. Of note, the SNU1 tumor harbored both L1196M (frequency, 6.59%) and G1269A (28.5%) mutations in separate clones (Fig. 1*B*). The SNU4 patient displayed the G1269A mutation at a frequency of 68.75%. In addition, the L1196M mutation was found in SNU6 tumoral cDNA clones (27.8%). These were confirmed using L1196M or G1269A mutation-specific PCR (Fig. 1*C*). However, the remaining four patients demonstrated no secondary mutation within the ALK kinase domain. SNU-2535 derived from an SNU4 patient harbored the G1269A mutation (61.9%).

ALK Mutations Confer Resistance to Crizotinib

293T cells were transiently transfected with cDNA encoding wild-type (WT) EML4-ALK, EML4-ALK L1196M, or EML4-ALK G1269A to evaluate resistant properties of these mutations. ALK phosphorylation was less decreased in 293T-L1196M or -G1269A cells than in 293T-WT cells after exposure to crizotinib in a dose-dependent manner (Fig. 2*A*). In addition, Ba/F3 cells that expressed secondary *ALK* mutations were resistant to crizotinib treatment compared with those expressing WT EML4-ALK (Fig. 2*B*). Apoptotic cells determined by Annexin V and 7-AAD were higher in H3122 cells than in SNU-2353 cells treated with crizotinib (Fig. 2*C*). H3122 CR1 cells that were generated by an increasing dose of crizotinib to 1 μM[13] and SNU-2535 cells were resistant to crizotinib compared with H3122 cells (half maximal inhibitory concentration [IC_{50}], 6.01 and 4.1 μM, respectively; Fig. 3*A*). Crizotinib treatment suppressed expression of phospho-ALK, -AKT, and -ERK in H3122 cells. However, crizotinib ineffectively suppressed phosphorylated proteins in SNU-2535 and H3122 CR1 cells (Fig. 3*B*). Apoptotic cells were reduced in SNU-2535 cells compared with those in H3122 cells after exposure to crizotinib (Fig. 3*C*).

TABLE 2. Heterogeneity of Genetic Changes Associated with Crizotinib Resistance in Seven Patients with ALK-Rearranged NSCLC

		Before crizotinib				After crizotinib				
No	ALK variants	ALK FISH	EGFR FISH	EGFR mutation	KRAS mutation	ALK FISH	ALK mutation	EGFR FISH	EGFR mutation	KRAS mutation
1	E13;A20	+	WT	–	ND	ND	L1196MG1269A	ND	–	–
2	E6;A20	+	ND	ND	ND	+	WT	WT[a]	–	–
3	E13;A20	+	WT[a]	–	–	+	WT	WT[a]	–	–
4	E13;A20	+	ND	ND	ND	+	G1269A	WT[b]	–	–
5	E13;A20	+	WT[b]	–	–	+[d]	WT	+[c]	+ (L858R)	–
6	E6;A20	+	WT	–	–	+	L1196M	WT[a]	–	–
7	KIF5B;A20	+	WT	–	–	+	WT	WT[a]	–	–

[a]EGFR disomy.
[b]EGFR low trisomy.
[c]EGFR high polysomy.
[d]ALK gene amplification.
ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization; ND, not done; NSCLC, non–small-cell lung cancer; WT, wild-type.

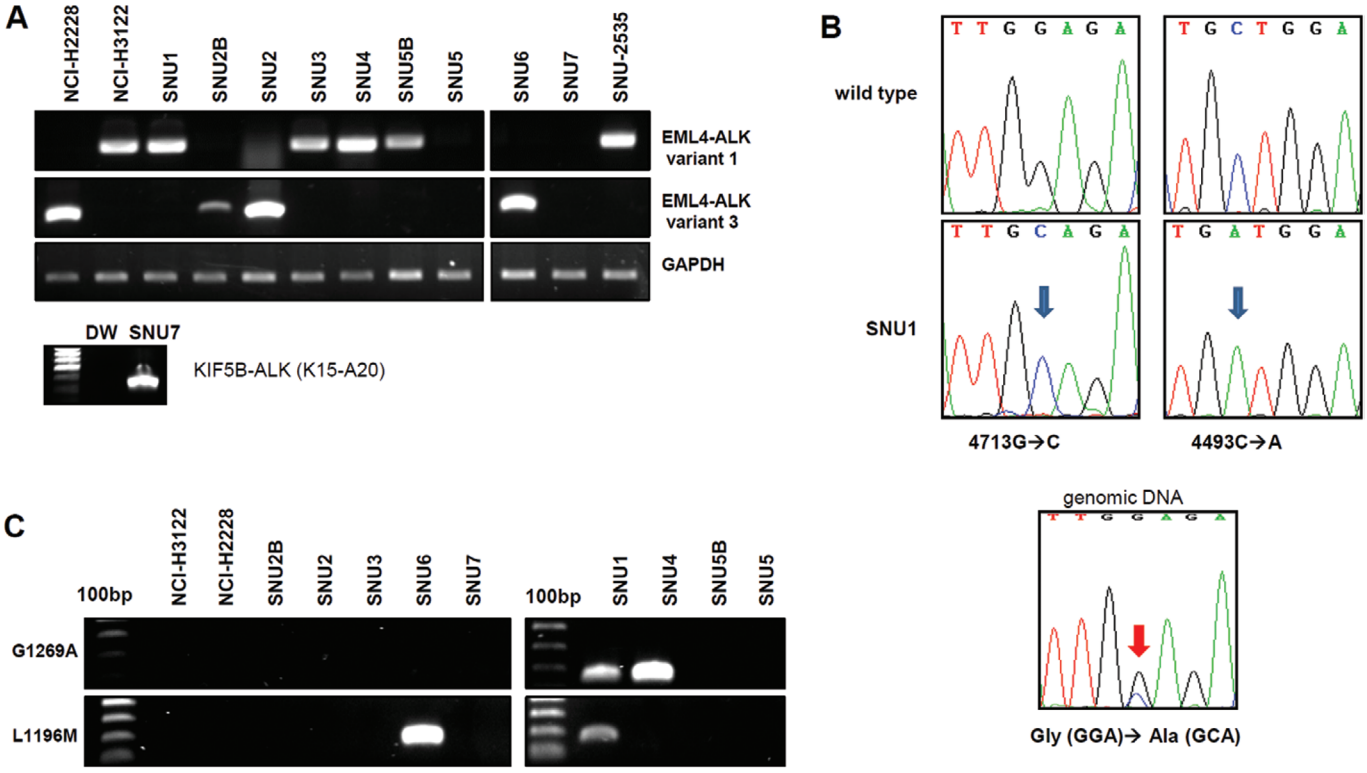

FIGURE 1. ALK fusion transcripts and ALK mutations. (A) Virtual gel electro-phoresis was performed on multiple RT-PCR products derived from patients' specimens ($n = 7$) and NSCLC cells ($n = 3$). The suffix "B" indicates tumor specimens before crizotinib treatment. Four and two patients harbored *EML4-ALK* variants 1 and 3, respectively. The *KIF5B-ALK* fusion transcript was detected in an SNU7 patient. (B) Electropherograms showed the 4713 G→C and 4493 C→A mutations in an SNU1 patient. (C) L1196M and G1269A were detected by mutation-specific PCR assays. ALK, anaplastic lymphoma kinase; NSCLC, non–small-cell lung cancer; RT-PCR, reverse transcription–polymerase chain reaction.

Heterogeneous Genetic Changes in Crizotinib-Resistant Tumors

Although patients with secondary *ALK* mutations showed no other genetic alteration, those without these mutations were tested for genetic alterations of alternative pathways (Table 2). Of note, the SNU5 patient had an *ALK* gene amplification (*ALK* gene copy number gain, 4.1-fold increase compared with the precrizotinib specimen; Fig. 4*A*) and L858R mutation in *EGFR* exon 21 with *EGFR* high polysomy (EGFR/CEP = 1.79; Fig. 4*B*). She carried a WT *EGFR* gene with disomy in the initial tumor tissue.

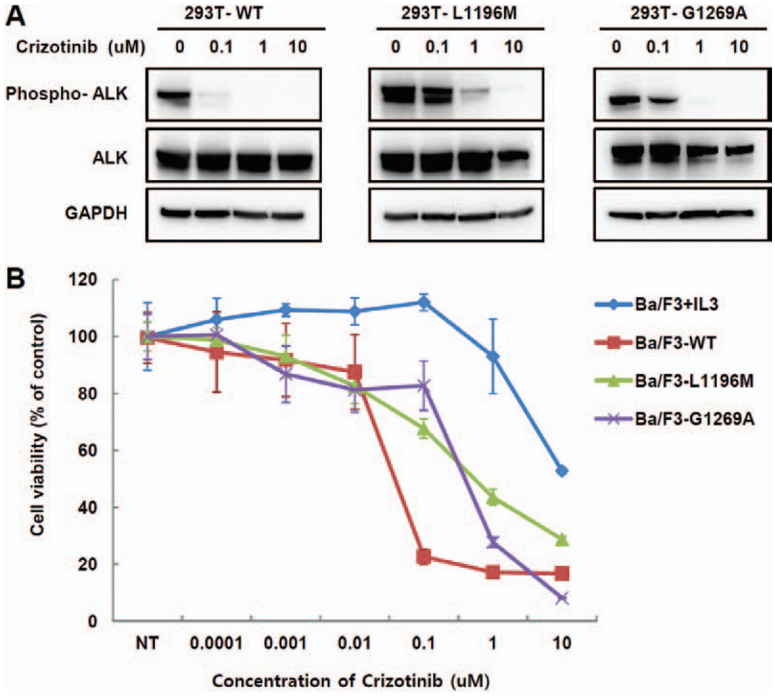

FIGURE 2. L1196M and G1269A mutant clones are resistant to crizotinib treatment. (A) 293T cells transiently transfected with *EML4-ALK* WT, L1196M, and G1269A constructs showed ineffective suppression of phospho-ALK in crizotinib-resistant clones. (B) Ba/F3 cells were transformed to express wild-type EML4-ALK or EML4-ALK harboring L1196M or G1269A mutations. The parental Ba/F3 cells with IL-3 or the EML4-ALK-expressing Ba/F3 cells without IL-3 were treated with crizotinib. Cell cytotoxicity was measured using CCK-8. The IC_{50} 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. ALK, anaplastic lymphoma kinase; IL, interleukin.

However, genetic alterations associated with acquired resistance to crizotinib were not found in three *ALK*-positive NSCLC patients. In addition, *ALK* or *EGFR* gene amplification and *EGFR* or *KRAS* mutation were not observed in SNU-2535 cells. *KRAS* gene mutation was not found in all tumor tissues.

The amphiregulin concentration was significantly high in supernatant fluid from SNU1 (1894.0 pg/ml) and SNU6 (1832.5 pg/ml) patients than the amphiregulin concentration in supernatant fluid from SNU2, 4, and 7 patients (116.4–460.0 pg/ml; Fig. 4*C*). Similarly, SNU1 and SNU6 patients demonstrated relatively high EGF concentrations (Fig. 4*D*). EGFR ligand levels were significantly high in SNU-2535 and H3122 CR1 cells than in H3122 cells.

DISCUSSION

Our study demonstrates that acquired resistance mechanisms are heterogeneous in *ALK*-rearranged NSCLC treated with crizotinib. Three of seven patients (43%) had secondary *ALK* mutations and one patient (14%) showed a high *ALK* gene copy number and activation of *EGFR* signals expressed by high *EGFR* polysomy and L858R in *EGFR* exon 21. In addition, five patients with malignant pleural effusions showed a high concentration of amphiregulin.

L1196M is a "gatekeeper" mutation in *ALK*, which is analogous to T315I in *ABL* and T790M in *EGFR*, and sterically interferes with crizotinib binding.[9] This confers a high level of resistance to crizotinib with an IC_{50} of 1000 nM or more and is identified even at the highest dose of crizotinib in in vitro mutagenesis screening.[7,18] Recently, six patients with *ALK*-rearranged NSCLC who received crizotinib harbored L1196M mutation after a median duration of 6 months (range, 4–28 months), including two patients in this study.[9,12,19] The insensitivity of L1196M clones to crizotinib and ineffective suppression of phospho-ALK was confirmed in our Ba/F3 and 293T systems, similar to previous studies.[9,11–13,19] Mutation at position G1269 in the DFG motif was identified by in vitro mutagenesis[18] and in two patients who showed acquired resistance to crizotinib.[12] G1269A mediated an intermediate resistance between C1156Y and L1196M,[12] a finding that was similarly observed in our analysis. Currently, four patients with *ALK*-positive NSCLC received crizotinib for a median duration of 9 months (range, 6–13 months) until disease progression with G1269A acquisition.[12] Of note, one patient had both L1196M and G1269A mutations that were confirmed by a highly sensitive mutation-specific PCR assay in different clones, findings similar to those in a case report,[9] suggesting heterogeneous tumor clones under a different pressure within the same patient. We found no *ALK* secondary mutation such as 1151Tins,[19] L1152R,[11] C1156Y,[9] F1174C,[20] G1202R,[19] D1203N,[20] or S1206Y[19] in our patients. However, because of unknown results of L1196M and G1269A in tumors before crizotinib, we do not know truly gained secondary *ALK* mutations in SNU1, 4, and 6 patients.

ALK copy number gain was observed in partially resistant H3122 cells, resulting in a step-wise evolution of acquired resistance to crizotinib.[13] This was confirmed in three patients, of whom one had a G1269A mutation,[12,19] and in our patient. Of note, our patient with *ALK* gene amplification harbored *EGFR* mutation (L858R) and *EGFR* high polysomy. She had no detectable *EGFR* mutation by direct sequencing before crizotinib and her tumor responded to crizotinib only for 4 months. Thus far, two patients have co-existent *ALK* dominant and nondominant mechanisms of resistance: one patient harbors G1202R mutation and *KIT* amplification,[19] whereas the other patient displayed *ALK* copy number gain

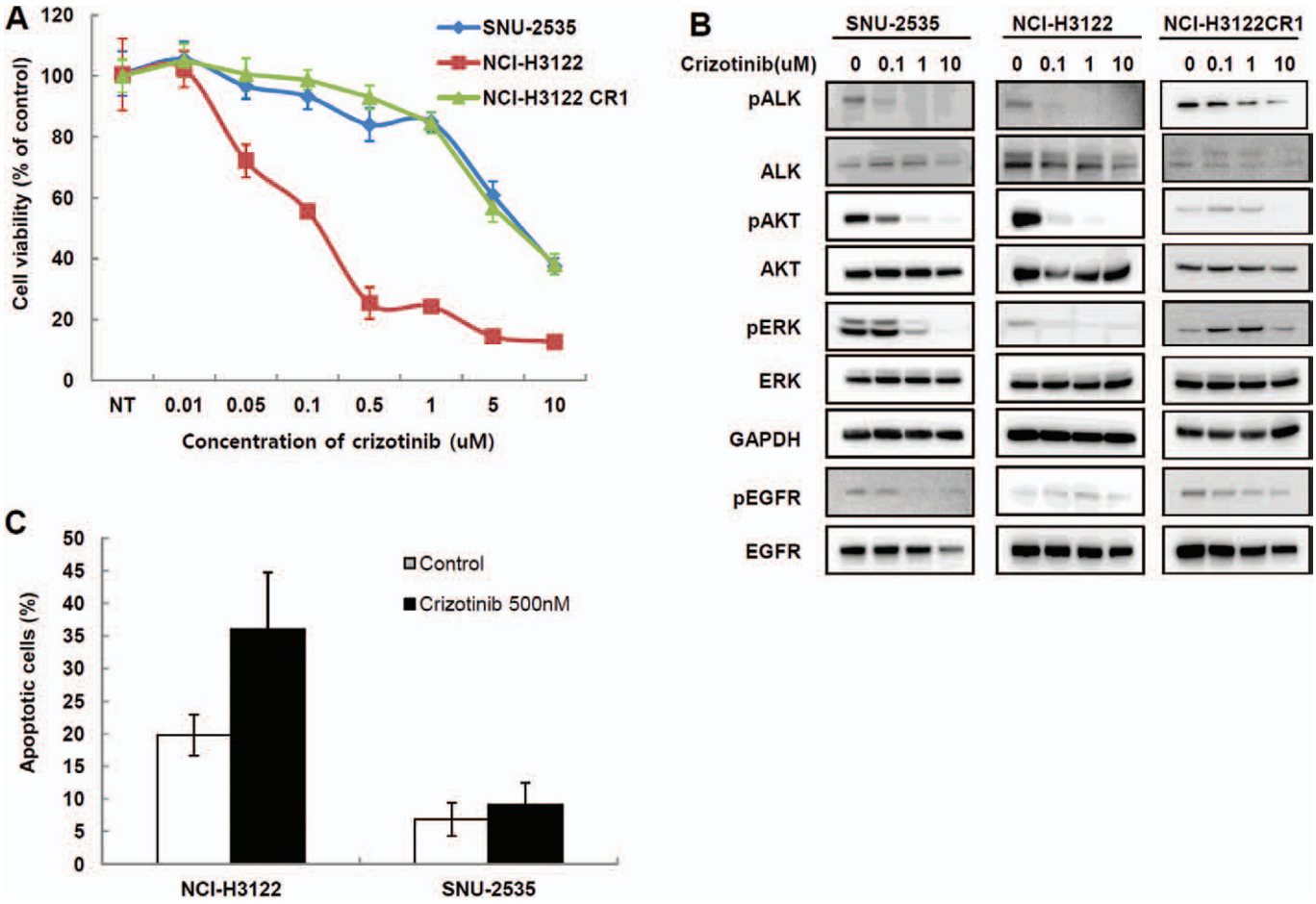

FIGURE 3. *ALK* mutant NSCLC cells are resistant to crizotinib treatment. (A) A cell viability assay was plotted for H3122, SNU-2535, and H3122 CR1 cells (crizotinib-resistant) treated with or without crizotinib for 72 hours. (B) Phospho-ALK downstream signals were less suppressed after treatment with crizotinib in SNU-2535 and H3122 CR1 cells than in H3122 cells. (C) Apoptosis induction was high in H3122 cells compared with that in SNU-2535 cells treated with crizotinib. ALK, anaplastic lymphoma kinase; NSCLC, non–small-cell lung cancer.

and *EGFR* mutation with high polysomy. These findings suggest heterogeneous resistance mechanisms associated with crizotinib treatment even in the same patient with *ALK*-rearranged NSCLC.

ALK nondominant mechanisms of resistance consist of second oncogene activation with partial *ALK* dependence and separate oncogene activation with *ALK* independence.[20] The former includes ligand-dependent EGFR[11] or KIT[19] activation; and the latter includes *KRAS* or *EGFR* mutation.[12] Of note, five patients with malignant pleural effusions at the time of crizotinib resistance had high amphiregulin concentration in supernatant fluid, suggesting ligand-dependent *EGFR* activation. However, unknown results of *EGFR* ligands in pre-crizotinib samples and *EGFR* phosphorylation in tumors might weaken the possible resistance mechanism associated with *EGFR* signals.

In conclusion, genetic changes associated with acquired crizotinib resistance are heterogeneous in *ALK*-rearranged NSCLC in our study. Despite a small number of patients and preexistent similar studies, it is hoped that demonstrated high concentrations of *EGFR* ligands in patients' samples and identified one patient with both *ALK* dominant and nondominant mechanisms of crizotinib resistance. Although we successfully identified *ALK* dominant and nondominant resistance mechanisms in four (57%) and one (14%) of seven patients, respectively, these were not observed in three patients. Therefore, a new technique such as next-generation sequencing is necessary to uncover a novel mechanism associated with crizotinib resistance. In addition, identification of resistant mechanisms might help to select a patient who benefits from new *ALK*-directed therapies: new agents such as an HSP90 inhibitor, or a combination of crizotinib and a new agent.

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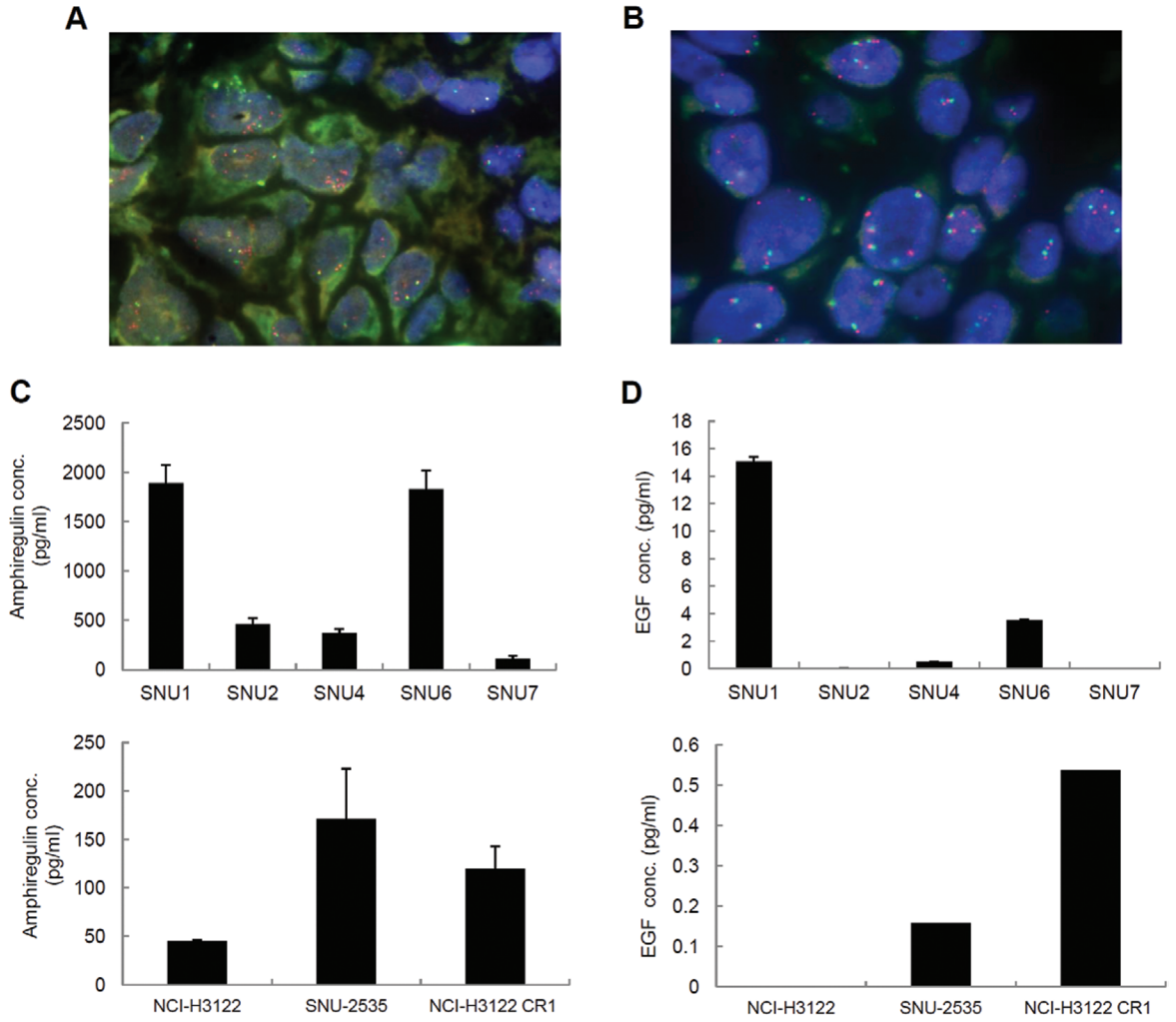

FIGURE 4. Heterogeneous genetic alterations in patients with acquired crizotinib resistance. (A) The SNU5 patient had an average *ALK* copy number gain of 8.5 in the post-crizotinib specimen compared with 2.0 in the pre-crizotinib specimen. (B) *EGFR* high polysomy was observed in the SNU5 patient in the post-crizotinib sample. (C) Amphiregulin and (D) EGF concentrations in patients and NSCLC cells. NSCLC, non–small-cell lung cancer.

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