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study of ceritinib, five of 19 crizotinib-resistant tumors harbored resistance mutations at residues 1196, 1269, and 1206, with one tumor harboring both G1269A and 1151T-ins. The patients harboring these resistance mutations all exhibited significant tumor shrinkage (13).

Importantly, as has been observed in the clinic, ceritinib showed potent efficacy *in vitro* and *in vivo* against a crizotinib-resistant tumor that did not harbor an *ALK* resistance mutation or gene amplification (Fig. 3B). Interestingly, the patient-derived cell line also retained sensitivity to crizotinib *in vitro*, demonstrating that these cells are still sensitive to ALK inhibition. One potential explanation for this finding is that, in the clinic, crizotinib fails to achieve tumor levels that completely inhibit ALK, and that tumor cells can survive through modest input from activation of bypass tracks such as EGFR. However, these cells remain sensitive to complete ALK inhibition. In the setting of a more potent ALK inhibitor, ALK is inhibited fully, abrogating the functional role of bypass tracks and leading to the elimination of tumor cells. It is also possible that this patient relapsed on crizotinib because of poor adherence to therapy or due to a stromal contribution. Similar findings were also observed in the H2228 xenograft model that developed resistance to crizotinib *in vivo*, did not develop an *ALK* mutation, and was sensitive to ceritinib (Fig. 5A). These findings may explain, at least in part, the finding that ceritinib is highly active in crizotinib-resistant cancers with or without *ALK* resistance mutations.

The initial interrogation of ceritinib-resistant patient biopsies supports the notion that ceritinib is able to effectively suppress many crizotinib-resistant mutations, but the G1202R and F1174V/C mutants are resistant to ceritinib. It is noteworthy that in two cases, the crizotinib-resistant mutations, S1206Y and G1269A, were no longer observed in the ceritinib-resistant biopsies in which the G1202R mutations were observed (Fig. 6A). This suggests that predominant clones with the S1206Y and G1269A mutations were suppressed by ceritinib, whereas much more rare clones with G1202R mutations were selected by ceritinib. These findings give further support to the notion that there are multiple populations of resistant clones whose emergence is dependent on the selective pressure applied.

Altogether, our *in vitro* and *in vivo* data, including cell line models established from crizotinib-resistant patient samples, demonstrate that the next-generation ALK inhibitor ceritinib is active against most crizotinib-resistant tumors. This is consistent with the marked clinical activity of ceritinib in patients with *ALK*-positive NSCLC who progressed on crizotinib. As resistance to ceritinib has already been observed in the clinic, future studies will need to identify mechanisms of resistance to ceritinib other than mutations in the G1202 and F1174 residues to maximize the clinical benefit afforded by next-generation ALK-targeted therapies.

## METHODS

### Cell Lines and Reagents

All human lung cancer samples were obtained from patients with informed consent at the Massachusetts General Hospital (MGH) and the Japanese foundation for Cancer Research (JFCR), and all procedures were conducted under an Institutional Review Board (IRB)-approved protocol. Cells in pleural effusion were collected by centrifugation at 440 × *g* for 10 minutes. After red blood cells were lysed with the Red Blood Cell Lysis Solution (BioLegend), cells were grown in ACL-4 (Invitrogen) supplemented with 1% FBS or RPMI-1640 supplemented with 10% FBS and 1× Antibiotic-Antimycotic. After the cells started growing stably, clonal cell lines were also established.

H3122, H2228, A549, H460, H1299, HCC827, and H522 cell lines were provided by the Center for Molecular Therapeutics (CMT) at Massachusetts General Hospital (Boston, MA), which performs routine cell line authentication testing by single-nucleotide polymorphism and short-tandem repeat analysis. BT-474, SKBR3, and the *ALK*-positive patient-derived cell lines used in this study are from the Engelman laboratory (Boston, MA) and have been previously tested for mutation status to confirm their authenticity. A549, H460, H1299, HCC827, H522, SKBR3, H2228, H3122, H3122 CR1, and MGH021-4 cell lines were cultured in RPMI-1640 supplemented with 10% FBS. For survival assays, H2228 were cultured in 1% FBS. The MGH045 cell line was cultured in ACL-4 supplemented with 1% FBS, and MGH051 and BT-474 were cultured in DMEM supplemented with 10% FBS.

Mouse myeloma Ba/F3 cells were cultured in DMEM supplemented with 10% FBS with (parental) or without (EML4–ALK) IL3 (0.5 ng/mL). cDNAs encoding *EML4–ALK* variant1 or *EML4–ALK* variant3 containing different point mutations were cloned into retroviral expression vectors, and virus was produced as previously described (11). After retroviral infection, Ba/F3 cells were selected in puromycin (0.5 μg/mL) for 2 weeks. IL3 was withdrawn from the culture medium for more than 2 weeks before experiments.

Crizotinib was purchased from ChemieTek, and ceritinib was provided by Novartis. Both were dissolved in DMSO for *in vitro* experiments. Ceritinib was formulated in 0.5% methyl cellulose/0.5% Tween 80 and crizotinib in 0.1 N HCl or 0.5% methyl cellulose/0.5% Tween 80 for *in vivo* studies.

### Western Blot Analysis

A total of $5 \times 10^5$ cells were treated in 6-well plates for 6 hours with the indicated drugs. Cell protein lysates were prepared as previously described (6, 11). Phospho-ERK (T202/Y204), ERK, S6, phospho-S6, phospho-AKT (S473 and T308), AKT, phospho-ALK (Y1282/1283), and ALK antibodies were obtained from Cell Signaling Technology. GAPDH was purchased from Millipore.

### Survival Assays

Cells (2,000 or 5,000) were plated in triplicate into 96-well plates. Seventy-two hours (48 hours for Ba/F3 cells and 7 days for MGH051) after drug treatments, cells were incubated with a CellTiter-Glo assay reagent (Promega) for 15 minutes, and luminescence was measured with a Centro LB 960 Microplate Luminometer (Berthold Technologies).

### In Vivo Efficacy Study of Ceritinib

SCID beige mice for crizotinib-resistant H2228 xenograft tumor models, nude mice for MGH006 primary explants and MGH045 cells were randomized into groups of 5, 6, or 8 mice with an average tumor volume of approximately 150 $mm^3$ and received crizotinib or ceritinib daily treatments by oral gavage as indicated in each study. Tumor volumes were determined by using caliper measurements and calculated with the formula (length × width × height)/2.

### In Vitro Enzymatic Assay

An enzymatic assay for the recombinant ALK kinase domain (1066–1459) was conducted using the Caliper mobility shift methodology, using fluorescently labeled peptides as kinase substrates. The

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Caliper assay was performed at 30°C for 60 minutes in a total volume of 9 μL. The reaction was terminated by the addition of 16 μL of stop solution [100 mmol/L HEPES, 5% (v/v) DMSO, 0.1% (v/v) coating reagent, 10 mmol/L EDTA, 0.015% (v/v) Brij 35]. After termination of the reactions, the plates were transferred into the Caliper LabChip 3000 workstation for analysis.

### *Analysis of ALK/Ceritinib and ALK/Crizotinib Costructures*

The ALK/ceritinib costructure was determined by the soaking of 2 mmol/L ceritinib into apo crystals grown in 0.2 mol/L sodium acetate trihydrate/20% PEG3350 using protein expressed and purified as previously described (18). The ALK/ceritinib final model determined to 2.0 Å (PDB 4MKC on hold) was superimposed with the coordinates of the ALK/crizotinib costructure (PDB 2XP2) for analyses.

### *Patient Sample Analyses*

The patients with ALK-positive NSCLC with acquired ceritinib resistance underwent biopsy of their resistant tumors between January 2011 and September 2013. Standard histopathology was performed to confirm the diagnosis of malignancy as previously described (6). The electronic medical record was reviewed retrospectively to obtain clinical information under an IRB-approved protocol. This study was approved by the IRB of MGH or the Cancer Institute Hospital of JFCR.

## Disclosure of Potential Conflicts of Interest

M. Nishio has received honoraria from the speakers' bureaus of Pfizer and Chugia Pharmaceutical Co., Ltd. A.T. Shaw is a consultant/advisory board member of Novartis, Pfizer, and ARIAD. J.A. Engelman has received commercial research grants from Novartis and Sanofi-Aventis, and is a consultant/advisory board member of Novartis, Sanofi-Aventis, Chugia Pharmaceutical Co., Ltd., and Ventana Medical Systems, Inc. No potential conflicts of interest were disclosed by the other authors.

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