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Analytical performance of the cobas EGFR mutation assay for Japanese non-small-cell lung cancer

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ABSTRACT

Introduction: Clinical outcomes in non-small-cell lung cancer (NSCLC) patients with epidermal growth factor receptor (EGFR) mutations have been reported to be correlated with the use of EGFR-tyrosine kinase inhibitors (EGFR-TKIs). Therefore, it is essential to confirm the presence of EGFR mutations using highly sensitive testing methods. In this study, we compared the performance of the cobas® EGFR Mutation Test (cobas EGFR assay) and the *therascreen*® EGFR RGQ PCR Kit (*therascreen* EGFR assay) for use as an *in vitro* diagnostic (IVD) product.

Methods: We extracted DNA from 150 formalin-fixed, paraffin-embedded tissue samples from 150 patients diagnosed with NSCLC, and performed a comparative study of the cobas EGFR and *therascreen* EGFR assay methods. All discordant results were re-analyzed by direct sequencing.

Results: The concordance rate between the cobas EGFR assay and the *therascreen* EGFR assay was 98.0% (145/148). EGFR mutations were detected at a frequency of 40.9% (61/149) in NSCLC specimens using the cobas EGFR assay and 40.2% (60/149) using the *therascreen* EGFR assay. Three discrepant results were found in this study. Two double mutations were detected by the cobas EGFR assay but only one in the *therascreen* EGFR assay. No invalid results resulted from sample analysis by the cobas EGFR assay.

Conclusions: Our results show a high concordance rate (98.0%) of cobas EGFR assay with an existing IVD product, the *therascreen* EGFR assay. Since they are IVD diagnostic products, both assays proved to be simple, validated methods in detecting the most common, clinically significant EGFR mutations and proved to be helpful for appropriate treatment guidance for NSCLC patients.

1. Introduction

Non-small-cell lung cancer (NSCLC) patients frequently have activating EGFR mutations and respond well to treatment with small molecule EGFR-tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib [1–4]. Both the American Society of Clinical Oncology and the Japan Lung Cancer Society recommend EGFR mutation testing in patients being considered for EGFR-TKI treatment as a first-line therapy [5]. Similar guidelines recommending testing for *EGFR* mutations were established by the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology [6]. Patients' *EGFR* mutation status prior to the commencement of treatment impacts outcomes and, as a result, EGFR testing has been developed as a companion diagnostic; this relationship between therapeutic and diagnostic agents contributes to personalized healthcare. Recently, it was reported that about half of patients who are initially sensitive to EGFR-TKIs may acquire resistance to EGFR-TKIs [7] following a period of therapy, mainly as a result of the appearance of *EGFR* mutations associated with resistance to treatment, such as T790M. Indeed, a recent study suggested that the T790M mutation may be present in a small proportion of tumor cells prior to treatment, with the proportion of mutant alleles increasing gradually during treatment [8]. Similar findings were observed for exon 20 insertions; that they are usually associated with primary or de novo resistance to EGFR-TKI therapy [9]. Thus, it is important to re-assess *EGFR* mutation status during treatment to determine the most appropriate treatment regimens for patients.

A number of PCR-based techniques are used in the clinic for the assessment of *EGFR* mutations. In Japan, the "Scorpion-ARMS" *therascreen*® EGFR Rotor-Gene Q (RGQ) PCR Kit (*therascreen* EGFR

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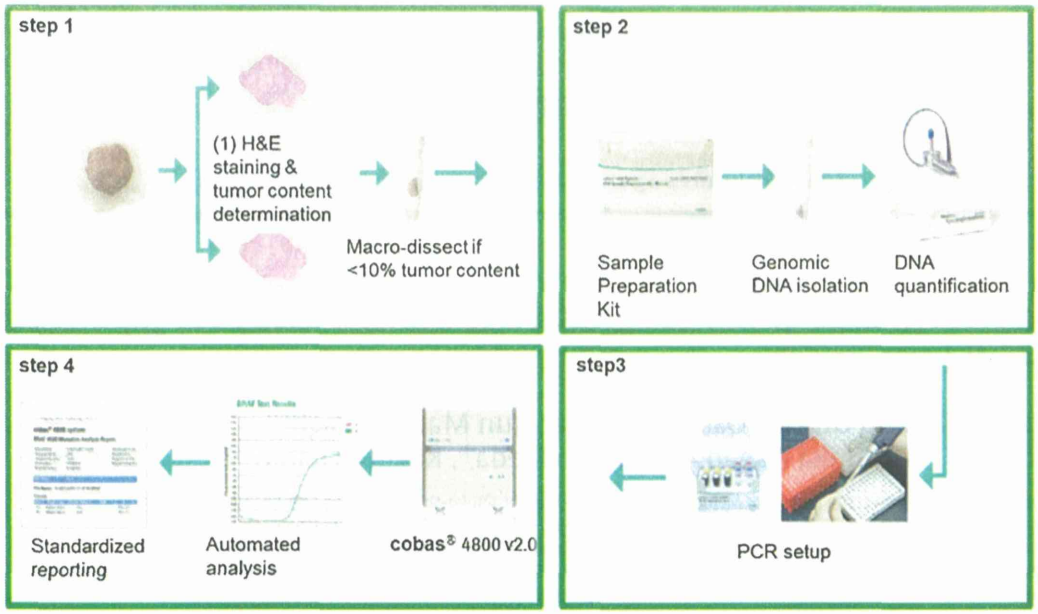

Fig. 1. Assay flow for the cobas EGFR mutation assay. The assay is composed of four steps. Step 1: 5-μm sections are prepared from FFPE tissue. One section is used for H&E staining to assess tumor content and the other section is used for DNA isolation. Step 2: Genomic DNA is isolated using the cobas® DNA Sample Preparation Kit. Step 3: DNA is mixed with reagents after quantification. Step 4: DNA is amplified using the cobas z 480 system. Results are automatically reported.

assay; Qiagen, Hilden, Germany) is the only available *in vitro* diagnostic (IVD) test.

In this study, we compared the performance of the cobas EGFR assay and the *therascreen* EGFR assay using formalin-fixed, paraffin-embedded (FFPE) tissue specimens from NSCLC patients.

2. Materials and methods

2.1. Tissue samples

A series of archived 150 FFPE tissue samples which was surgically resected from 150 Japanese patients diagnosed with NSCLC, collected between March 2011 and December 2012, was obtained from Tokyo Medical University (Tokyo, Japan) and Funabashi Medical Hospital (Funabashi, Japan). All patients enrolled in the study provided informed consent for the use of resected tissue. The study was approved by the ethics committee of each participating institute and conducted according to Institutional Review Board guidelines.

2.2. cobas EGFR Mutation Test

The cobas EGFR assay is an allele-specific real-time PCR system (Figure, Supplemental Digital Content 1; Fig. 1) that qualitatively measures the amplification of DNA to identify 41 mutations in exons 18–21 of the *EGFR* gene from 50 ng of DNA derived from human FFPE NSCLC tissues (Table, Supplemental Digital Content 2). Within each reaction mixture, exon 28 was amplified as an internal control. DNA isolation, amplification/detection, and result reporting can be performed in less than 8 h with up to 30 specimens processed simultaneously. The cobas EGFR assay has fully automated results reporting.

2.3. Specimen preparation for cobas EGFR assay

Two FFPE tissue sections of 5 μm thickness were prepared for this assay. One was used for DNA extraction and the other was used to confirm the presence of tumor content by hematoxylin and eosin (H&E) staining, which was performed by a pathologist. Any specimen containing <10% tumor content by area was macrodissected.

2.4. DNA extraction

FFPE tissue specimens were deparaffinized and then DNA extraction was performed according to the standard procedure described in the cobas® DNA Sample Preparation Kit (Roche Molecular Systems, Inc., USA) package insert. Briefly, the sample was incubated for 1 h at 56 °C and then for additional one hour at 90 °C in the presence of a protease and chaotropic lysis/binding buffer that causes the release of nucleic acids but protects released genomic DNA from degradation by DNase. The amount of genomic DNA was spectrophotometrically determined and adjusted to a fixed concentration of 2 ng/μL.

2.5. PCR amplification and detection

A total of 150 ng of DNA is required for the cobas EGFR assay. Target DNA was amplified and detected using the cobas® z480 analyzer (Roche Molecular Systems Inc.) according to the instructions for the cobas® EGFR Mutation Test, which measures the fluorescence generated by specific PCR products. All results were automatically performed by cobas® 4800 software.

2.6. therascreen® EGFR RGQ PCR Kit

The *therascreen* assay is a real time-PCR assay that combines the Amplification Refractory Mutation System (ARMS) and Scorpions fluorescent primer/probe system. It can detect 29 somatic mutations in exons 18–21 of *EGFR*. A maximum of 7 results can be obtained from one run. The *therascreen* EGFR assay was performed according to the manufacturer's guidelines (Qiagen). Briefly, DNA was isolated from FFPE tissue samples and the total sample DNA assessed by amplifying a region of exon 2 from *EGFR* by PCR. Next, the DNA samples were tested for the presence or absence of *EGFR* mutations by real-time PCR using a Scorpion probe and primers specific for wild type and mutant *EGFR* DNA. The difference

Table 1
Clinical characteristics of the patients providing surgically resected FFPE samples in NSCLC.

	N = 149
Gender	
Male	75
Female	74
Age	
Younger than 65 years	42
Older than 65 years	107
Histology	
Adenocarcinoma or adeno-squamous cell carcinoma (Ad)	126
Squamous cell carcinoma (Sq)	17
Large cell carcinoma (la)	2
Other	4
Smoking history	
Smoker	18
Ever smoker	73
Never smoker	56
ND	2

ND, not determined; *N*, number.

between the mutation assay cycle threshold (C_T) and control assay C_T from the same sample was used to calculate sample ΔC_T values. Samples designated mutation positive if the ΔC_T was less than the cutoff ΔC_T value.

2.7. Sanger sequencing

DNA samples obtained from specimens that were discordant between cobas EGFR and *therascreen* EGFR assays were amplified using the following site-specific primers: Exon 18 Forward, 5′-TGGAGCCTCTTACACCCAGT-3′, Reverse, 5′-ACAGCTTGCAAGGACTCTGG-3′; Exon 19 Forward, 5′-TCTGGATCCCAGAAGGTGAG-3′, Reverse, 5′-CAGCTGCCAGACATGAGAAA-3′; Exon 20 Forward, 5′-CATTCATGCGTCTTCACCTG-3′, Reverse, 5′-GTCTTTGTGTTCCCGGACAT-3′; Exon 21 Forward, 5′-GATCTGTCCCTCACAGCAGGGTC-3′, Reverse, 5′-GGCTGACCTAAAGCCACCTCC-3′. The fragments were subcloned into the Zero Blunt TOPO vector (Zero Blunt TOPO PCR Cloning Kit; Life Technologies, USA). Direct sequencing was performed with 100 colonies from one specimen by ABI3100 Genetic Analyzer (ABI) using the BigDye® Terminators v3.1 Cycle Sequencing Kit (Life Technologies). One mutation detected in 100 results was classed as "Mutation Detected" in this study. This assay required 1 μg of genomic DNA from specimens. Sanger sequencing was performed with the specimen that resulted double mutation (L858R and M790M) from cobas EGFR assay but single mutation (L858R) from *therascreen* EGFR assay at Mitsubishi Chemical Medicine Corporation followed by daily routine. The sequencing result was used as Golden standard.

3. Results

3.1. Study population

A series of 150 FFPE tissue samples from patients diagnosed with NSCLC was examined. One specimen was excluded owing to a lack of a completed consent form, leaving 149 samples available for analysis. The clinical and pathological characteristics of the patients providing the evaluable specimens are summarized in Table 1.

3.2. EGFR mutation types

EGFR mutations were identified in 63 NSCLC specimens (42.3%) using the cobas EGFR assay and 61 specimens (40.9%) using the *therascreen* EGFR assay (Table, Supplementary Digital Content 4). Exon 19 deletions (Ex19del) and a point mutation (L858R) accounted for 90.5% (57/63) and 93.4% (57/61) of all mutations identified using the cobas EGFR assay and *therascreen* EGFR assay, respectively (Table, Supplementary Digital Content 3). This confirms the findings of a previous study [10], which found that Ex19del and L858R mutations accounted for 90% of NSCLC *EGFR* activating mutations. The exon 20 insert mutation (Ex20Ins) was not observed in any of the samples tested in this study. A T790M point mutation was detected by the cobas EGFR assay (0.68%) but not by the *therascreen* EGFR assay.

Table 2
Methods correlation between mutation findings using the cobas EGFR and *therascreen* EGFR assays.

		therascreen			Total
		MD	MND	Invalid	
cobas	MD	59	2	0	61
	MND	1	86	1	88
	Invalid	0	0	0	0
	Total	60	88	1	149

MD, mutation detected; MND, mutation not detected.

Table 3
Detailed concordant rate between cobas EGFR and *therascreen* EGFR assays.

Mutation	MD concordance	MND concordance	Total concordance
G719X	100% (3/3)	100% (145/145)	100% (148/148)
exon19del	95.7% (22/23)	100% (125/125)	99.3% (146/147)
S768I	100% (1/1)	99.3% (146/147)	99.3% (147/148)
T790M	–	99.3% (147/148)	99.3% (147/148)
exon20ins	–	100% (148/148)	100% (148/148)
L858R	100% (34/34)	99.1% (113/114)	99.3% (147/148)

Del, deletion; Ins, insertion; MD, mutation detected; MND, mutation not detected.

3.3. Invalid test rate

Mutation analysis of exons 18–21 of the *EGFR* gene was successfully performed in all 149 specimens (100%) using the cobas EGFR assay. In contrast, in experiments using the *therascreen* EGFR assay, two test specimens initially gave invalid test results. In those cases, DNA was extracted from new FFPE tissue samples. However, because one sample gave an invalid result again, this case was excluded from the analysis, resulting in an invalid rate of 0.68% (1/148) for the *therascreen* EGFR assay (Table 2). In addition, one invalid control occurred in the *therascreen* EGFR assay (data not shown).

3.4. Method correlation agreement analysis

The correlation rate between cobas EGFR assay and *therascreen* EGFR assay was 98.0%. Of the 149 evaluable samples tested, only three discordants between the two EGFR mutation assays were observed (Table 3).

3.5. Re-analysis of discordants by direct sequencing

Test specimens that gave discordant results between the cobas EGFR and *therascreen* EGFR assays were retested using direct sequencing from sub-cloned samples (Table 4). A discordant MND by *therascreen* EGFR assay was observed by direct sequencing to be an L858R point mutation, confirming the MD result assessed by cobas EGFR assay. In addition, an Ex19del mutation identified as MD by *therascreen* EGFR assay was shown to be MND by direct sequencing, again confirming the cobas EGFR assay result. The cobas EGFR assay identified one case with a double mutation, L858R and T790M

Table 4
Re-analysis of discordants by direct sequencing.

	therascreen		Cobas		Sequencing (reanalysis)
Sample 1	MND	–	MD	L858R	MD
Sample 2	MD	EX19Del	MND	–	MND
Sample 3	MND	–	MD	S768I	MND
Sample 4	MD	L858R	MD	L858R,T790M	L858R

Del, deletion; Ins, insertion; MD, mutation detected; MND, mutation not detected.

Table 5
Re-analysis: combined *therascreen* EGFR assay and Sanger sequencing for resolution of discordant results.

		therascreen and/or Sanger sequencing	
		MD	MND
cobas	MD	60	1
	MND	0	87

MD, mutation detected; MND, mutation not detected.

(Table 4). However, only the L858R mutation was identified by the *therascreen* EGFR assay and only the T790M mutation was detected by direct sequencing. We then performed a re-analysis using a combination of the *therascreen* EGFR assay and Sanger sequencing for resolution of the discordant results (Table 5). This demonstrated an MD concordance rate of 100% (60/60), an MND concordance rate of 98.9% (87/88) and a total concordance rate of 99.3% (147/148) between the tests.

4. Discussion

The overall correlation results of the cobas EGFR assay, an existing EGFR mutation screening method (the *therascreen* EGFR assay) plus direct sequencing was 99.3% (147/148) (Table 5). It also indicated that the cobas assay is at least as robust method to detect the most common clinically significant EGFR mutations as the existing *therascreen* EGFR assay.

Although we identified 3 discordant results among 149 (2.0%) specimens in this study, retesting by direct sanger sequencing confirmed that two of the three discordant results were in fact correctly called by the cobas EGFR assay. Although both assays share similar characteristics in terms of amplification methods and detection principles, the slight differences (e.g. probe and primer construction) between the two of them, influenced their sensitivities to the mutations. Also, the remaining discordant result analysis highlighted the importance of the purity of the extracted DNA for the PCR amplification. In fact, an Ex 20 S768I mutation identified as Mutation Detected (MD) by the cobas EGFR assay but not the *therascreen* EGFR assay, was not detected by direct sequencing, either. In this case, direct sequencing failed more than two times to detect the EGFR gene when using the extracted DNA from the QIAmp DNA FFPE Tissue extraction kit (Qiagen) suggesting that the quality of the DNA was not adequate for the testing (data not shown). This potential difference in DNA quality might be the reason why we have experienced discordant results in some cases.

One T790M mutation was detected together with L858R by the cobas EGFR assay in this study. As there is known heterogeneity with regard to the T790M mutation within tumor cells, it is difficult to mention that the extracted DNA was completely the same, even if we used serial sections. However the raw data from the cobas system showed high enough signals to robustly detect the mutation (data not shown). According to the package insert, cobas EGFR needs at least 3.13 ng DNA which includes 5% mutated DNA to detect the mutation. Therefore it appears that the cobas test might be more sensitive than the *therascreen* test because, according to the *therascreen* package insert, it needs 7.02% mutated DNA within the input DNA [11,12].

About half of the patients who are initially sensitive to EGFR-TKIs may acquire resistance to EGFR-TKIs [7] following a period of therapy, mainly because of the selection for the cells with the T790M mutation in EGFR. In addition, the correlation between the presence of intrinsic T790M mutations and patient outcomes has been shown [8], and is probably related to the slow growth of tumors bearing the T790M mutation. Thus, it is important to re-assess EGFR mutation status during treatment in order to determine the most appropriate treatment regimens for patients.

For IVD products, it is important to have rapid and simple testing. The cobas EGFR assay has two advantages over the *therascreen* assay in this regard. One is that the process consists of easily performed and stable methods. Additionally, it takes only 8 h to go from tumor specimen to results using the semi-automated system. Thus, patients assessed using the cobas EGFR assay can begin the most appropriate treatment within a shorter time period. The other advantage is that only a very small amount of DNA (150 ng) is required to detect the tumor mutation status using the cobas EGFR assay. Moreover, it confirms the accuracy of the results by co-amplification of an internal control (i.e. exon 28). One of the issues associated with detecting EGFR mutations in advanced NSCLC patients is not obtaining a sufficient quantity of specimen to confirm the presence/absence of several biomarkers. It is important to be able to perform tests using just a small amount of DNA; thus, the cobas EGFR assay is suitable and reliable for the detection of targeted common EGFR mutations. In this study, we had high concordance with surgically resected specimens which had enough tumors. However, at clinical practice, minimal invisible samples such as pleural effusion or bronchial wash would be used from advanced NSCLC patients having difficulty of collecting tissue. To access this difficulty, even if the samples are small enough, at least confirming the amount of tumor cells by pathologist is required to have appropriate test result. Under the condition, it might be able to provide reliable result even if using either FFPE samples or cytology samples. It is important to accumulate the data with cytology samples which makes improvement of suitable testing for advanced NSCLC patients in the future.

5. Conclusion

In the near future, more mutations that can serve as predictive markers for molecular-targeted treatments will be discovered, and mutation detection tests will play an increasingly important role in the clinical setting. The benefits of treatment will be maximized only if used together with clinically validated and accurate companion diagnostics. The cobas system offers the possibility of detecting additional mutations, not only mutations of EGFR. The combination of the cobas system with molecular-targeted treatments represents an important tool for physicians, supporting their efforts to effectively treat tumors.

Conflict of interest

Hideharu Kimura, Tatsuo Ohira, Osamu Uchida, Jun Matsubayashi, Shinichirou Shimizu, Toshitaka Nagao, Kazuto Nishio, Norihiko Ikeda was funded by Roche Diagnostics K.K. (Tokyo, Japan). There was no other financial support for the investigators.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2013.12.012.

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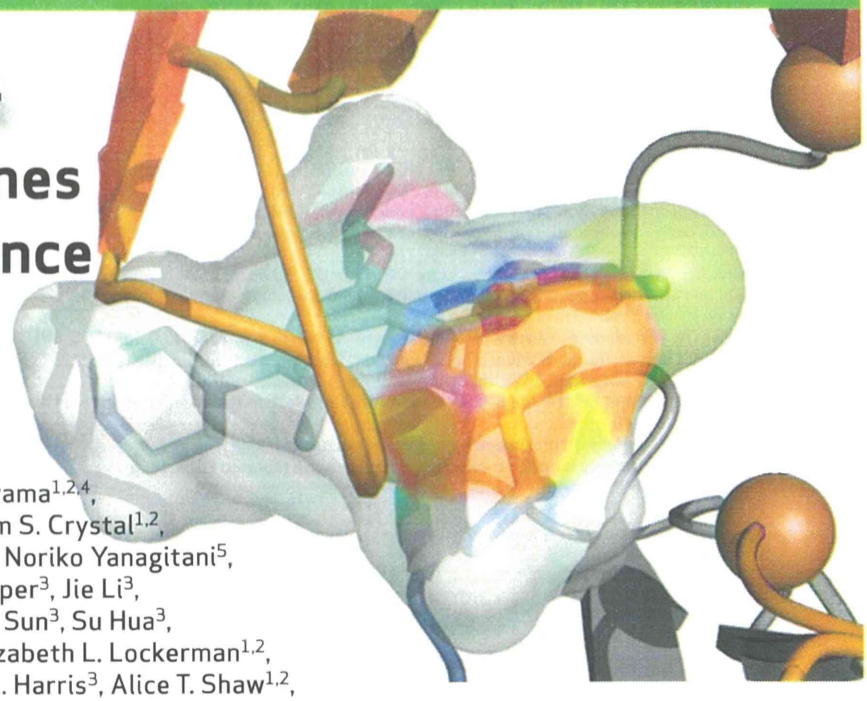
RESEARCH ARTICLE

The ALK Inhibitor Ceritinib Overcomes Crizotinib Resistance in Non-Small Cell Lung Cancer



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ABSTRACT Non-small cell lung cancers (NSCLC) harboring anaplastic lymphoma kinase (*ALK*) gene rearrangements invariably develop resistance to the ALK tyrosine kinase inhibitor (TKI) crizotinib. Herein, we report the first preclinical evaluation of the next-generation ALK TKI, ceritinib (LDK378), in the setting of crizotinib resistance. An interrogation of *in vitro* and *in vivo* models of acquired resistance to crizotinib, including cell lines established from biopsies of patients with crizotinib-resistant NSCLC, revealed that ceritinib potently overcomes crizotinib-resistant mutations. In particular, ceritinib effectively inhibits ALK harboring L1196M, G1269A, I1171T, and S1206Y mutations, and a cocrystal structure of ceritinib bound to ALK provides structural bases for this increased potency. However, we observed that ceritinib did not overcome two crizotinib-resistant ALK mutations, G1202R and F1174C, and one of these mutations was identified in 5 of 11 biopsies from patients with acquired resistance to ceritinib. Altogether, our results demonstrate that ceritinib can overcome crizotinib resistance, consistent with clinical data showing marked efficacy of ceritinib in patients with crizotinib-resistant disease.

SIGNIFICANCE: The second-generation ALK inhibitor ceritinib can overcome several crizotinib-resistant mutations and is potent against several *in vitro* and *in vivo* laboratory models of acquired resistance to crizotinib. These findings provide the molecular basis for the marked clinical activity of ceritinib in patients with *ALK*-positive NSCLC with crizotinib-resistant disease. *Cancer Discov; 4(6); 662–73. ©2014 AACR.*

See related commentary by Ramalingam and Khuri, p. 634.

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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INTRODUCTION

Chromosomal rearrangements of anaplastic lymphoma kinase (*ALK*) are detected in 3% to 7% of non–small cell lung cancers (NSCLC; refs. 1, 2). These rearrangements result in constitutively active ALK fusion proteins with potent transforming activity (2, 3). Lung cancers with *ALK* rearrangements are highly sensitive to ALK tyrosine kinase inhibition, underscoring the notion that such cancers are addicted to ALK kinase activity. On the basis of early-phase studies, the multitargeted tyrosine kinase inhibitor (TKI) crizotinib was approved by the FDA in 2011 to treat patients with advanced NSCLC harboring *ALK* rearrangements (1). However, despite a high response rate of 60% in *ALK*-rearranged NSCLC, most patients develop resistance to crizotinib, typically within 1 to 2 years.

Studies of *ALK*-rearranged lung cancers with acquired resistance to crizotinib have identified *ALK* fusion gene amplification and secondary ALK tyrosine kinase (TK) domain mutations in about one third of cases (4–6). To date, seven different acquired resistance mutations have been identified among crizotinib-resistant patients. The most frequently identified secondary mutations are L1196M and G1269A. In addition to these mutations, the 1151T-ins, L1152R, C1156Y, G1202R, and S1206Y mutations have also been detected in crizotinib-resistant cancers (4, 6–10). In approximately one third of crizotinib-resistant tumors, there is evidence of activation of bypass signaling tracts such as EGFR or c-KIT (6, 9). In the remaining one third of crizotinib-resistant tumors, the resistance mechanisms remain to be identified.

Next-generation ALK inhibitors with improved potency and selectivity compared with crizotinib have been developed to overcome crizotinib resistance in the clinic. We previously evaluated the ability of several ALK TKIs (TAE684, AP26113, ASP3026, and CH5424802) to inhibit ALK activity in models harboring different *ALK* secondary mutations (6, 11). These studies revealed variable sensitivity to these ALK inhibitors depending on the specific resistance mutation present. For example, the gatekeeper L1196M mutation was sensitive to TAE684, AP26113, and ASP3026, whereas 1151T-ins conferred resistance to all next-generation ALK TKIs. Ceritinib is an ATP-competitive, potent, and selective next-generation ALK inhibitor (12). The kinase selectivity has been tested in a cellular proliferation assay against 16 different kinases, and aside from ALK, no inhibition below 100 nmol/L was observed (12). In the phase I study of ceritinib in *ALK*-positive NSCLC, marked antitumor activity has been observed in both crizotinib-relapsed and crizotinib-naïve patients (13, 14). On the basis of this impressive clinical activity, ceritinib received FDA approval on April 29, 2014.

Herein, we present the first report examining the activity of ceritinib in preclinical models of *ALK*-positive lung cancer with acquired resistance to crizotinib, as well as an early biologic insight into mechanisms of resistance to ceritinib arising in patients.

Table 1. Ceritinib is a potent ALK inhibitor

	GI_{50} (nmol/L)		
	Crizotinib	Ceritinib	Fold
ALK enzymatic assay	3	0.15	20
H2228	107	3.8	28
H3122	245	6.3	39

NOTE: GI_{50} values for *in vitro* ALK enzymatic assay or H3122 and H2228 cell survival assay for crizotinib and ceritinib are shown.

RESULTS

Ceritinib Exhibits Potent Activity in Crizotinib-Naïve *ALK*-Positive NSCLC Models

In vitro enzymatic studies revealed that ceritinib was approximately 20-fold more potent against ALK than crizotinib (Table 1). Similarly, ceritinib was more potent than crizotinib against two *ALK*-rearranged lung cancer cell lines, H3122 and H2228 (Fig. 1A and B, Table 1). Accordingly, ceritinib led to suppression of ALK phosphorylation as well as the downstream PI3K–AKT, MEK–ERK, and mTOR signaling pathways at lower doses than crizotinib (Fig. 1C and D).

To further assess the cellular specificity of ceritinib, we determined the GI_{50} (concentration needed to reduce the growth of treated cells to half that of untreated cells) of ceritinib against a panel of tumor cell lines bearing different oncogenic drivers. Whereas ceritinib was potent against the two lung cancer cell lines with *ALK* rearrangements, it was not potent against NSCLC or breast cancer cell lines driven by KRAS, EGFR, PI3K, or HER2, with GI_{50}s >1 μmol/L (Supplementary Fig. S1A).

We next compared the efficacy of ceritinib and crizotinib *in vivo* using treatment-naïve H2228 xenograft models (Fig. 1E). Tumor-bearing animals were treated with either high-dose crizotinib (100 mg/kg) or ceritinib (25 mg/kg or 50 mg/kg) once daily for 14 days. Both crizotinib (100 mg/kg) and ceritinib (25 and 50 mg/kg) were well tolerated in this study (Supplementary Fig. S1B). As expected, marked tumor regression was observed in all groups during the treatment. After treatment was stopped, the animals were monitored for tumor progression. Although recurrent tumors were detected within 11 days of drug withdrawal in mice treated with crizotinib, mice treated with ceritinib at 50 mg/kg remained in complete remission with no discernible tumor growth for 4 months. In the mice treated with ceritinib at 25 mg/kg, tumor regrowth was observed in 4 of 8 animals after 1 month, whereas complete remission was maintained in the other 4 animals for 4 months. Thus, ceritinib had more durable antitumor activity than crizotinib, even after the drugs were discontinued. It is also worth noting that the exposure of crizotinib at 100 mg/kg is approximately 3-fold to 5-fold greater than the exposures achieved at the human maximum tolerated dose (MTD; 250 mg, twice a day; ref. 15) and that ceritinib at 25 to 50 mg/kg is predicted to be achievable at

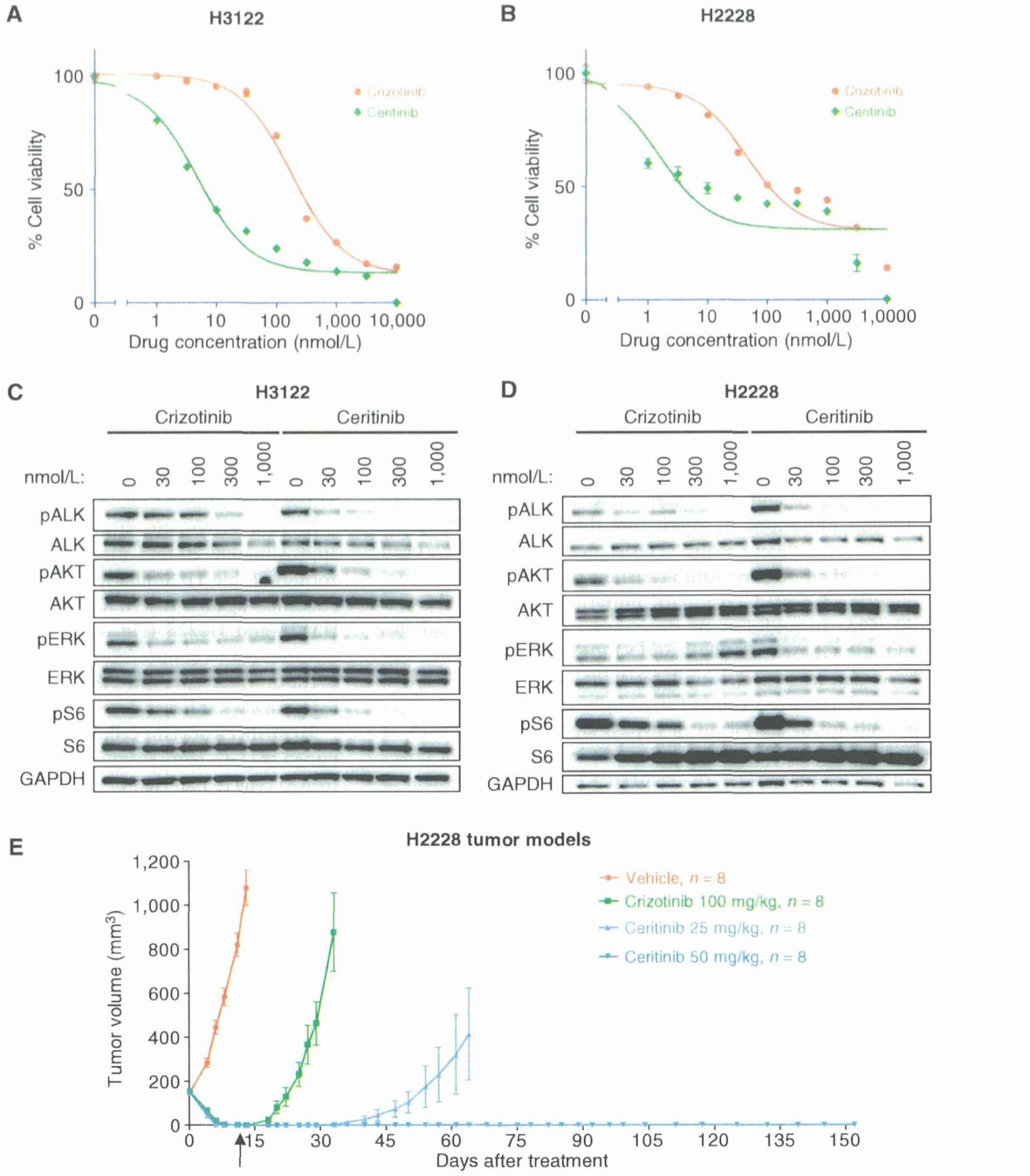

Figure 1. Ceritinib is a potent ALK inhibitor in crizotinib-naïve models. **A** and **B,** cell survival assay of H3122 (**A**) and H2228 (**B**) cells treated with the indicated doses of crizotinib or ceritinib for 72 hours. Cell survival was assayed by CellTiter-Glo. **C** and **D,** H3122 (**C**) and H2228 (**D**) cells were treated with the indicated concentrations of crizotinib or ceritinib for 6 hours. Lysates were probed with antibodies directed against the specified proteins. **E,** SCID beige bearing H2228 cells were administered crizotinib or ceritinib orally once daily for 14 days. The arrow indicates when treatments were stopped, and tumor growth was monitored in animals up to 4 months. Tumor volumes, mean ± SD ($n = 8$). p, phosphorylated.

the human MTD (750 mg every day). We also evaluated the efficacy of ceritinib in a primary explant model derived from a crizotinib-naïve NSCLC tumor MGH006 (6). Treatment of these mice with 25 mg/kg ceritinib also led to tumor regressions (Supplementary Fig. S1C). Altogether, these data demonstrate that ceritinib is potent against crizotinib-naïve *ALK*-rearranged cell lines and tumor models *in vivo* and *in vitro*.

Ceritinib Is Active against Patient-Derived Cell Lines from Crizotinib-Resistant Cancers with and without Resistant Mutations

To investigate the activity of ceritinib against crizotinib-resistant mutations, we used crizotinib-resistant cell line models harboring the two most common EML4–ALK

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Cell line	ALK mutation	GI_{50} (nmol/L) Crizotinib	GI_{50} (nmol/L) Ceritinib	Fold
H3122 CR1	L1196M	2884	230	13
MGH021-4	G1269A	500	80	6
MGH045	L1196M	891	25	36

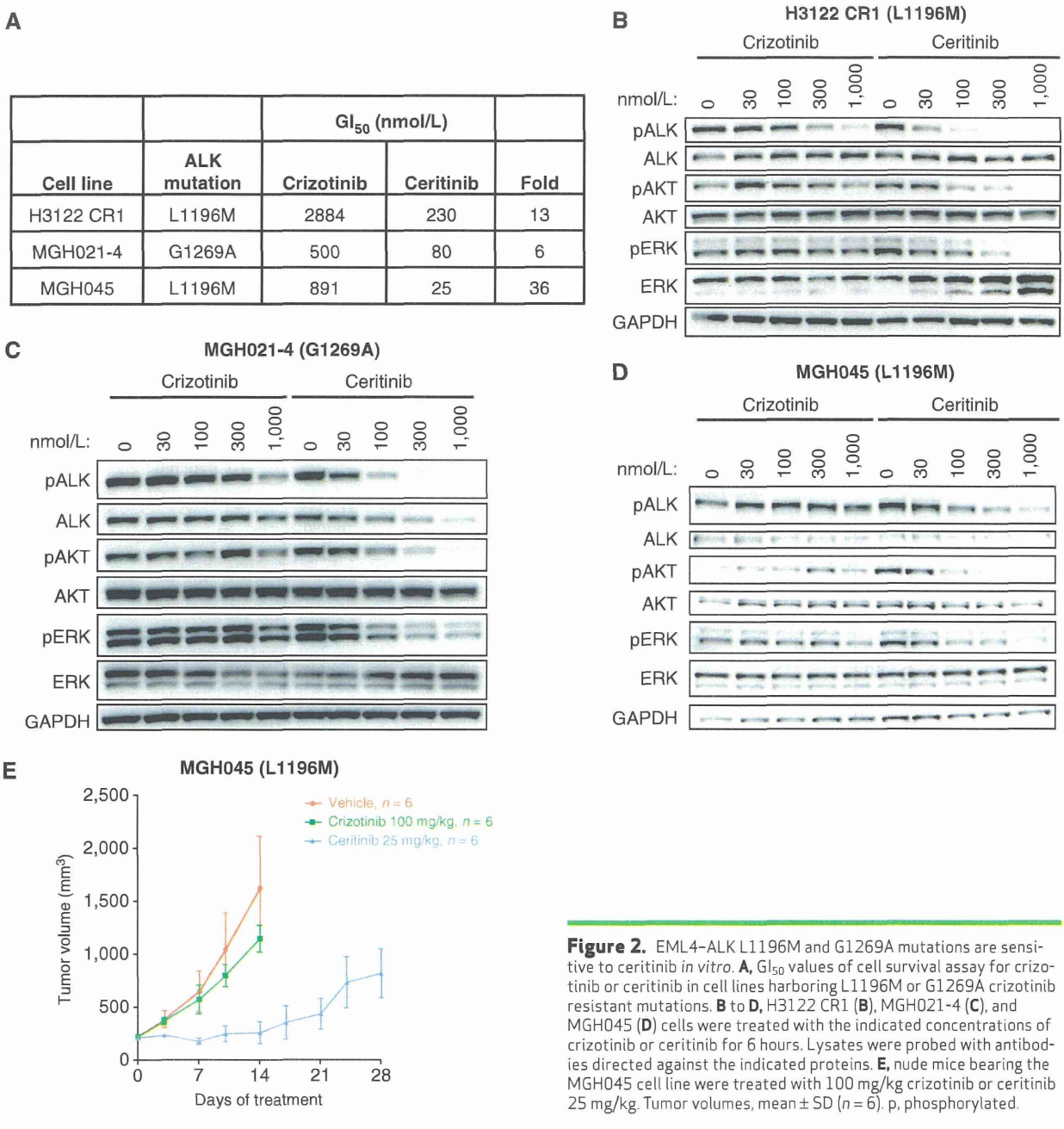

Figure 2. EML4–ALK L1196M and G1269A mutations are sensitive to ceritinib *in vitro*. **A,** GI_{50} values of cell survival assay for crizotinib or ceritinib in cell lines harboring L1196M or G1269A crizotinib resistant mutations. **B** to **D,** H3122 CR1 (**B**), MGH021-4 (**C**), and MGH045 (**D**) cells were treated with the indicated concentrations of crizotinib or ceritinib for 6 hours. Lysates were probed with antibodies directed against the indicated proteins. **E,** nude mice bearing the MGH045 cell line were treated with 100 mg/kg crizotinib or ceritinib 25 mg/kg. Tumor volumes, mean ± SD ($n = 6$). p, phosphorylated.

mutations, L1196M and G1269A. We have previously described the H3122 CR1 crizotinib-resistant cell line, which developed resistance *in vitro* by chronic exposure to crizotinib. This cell line harbors both the L1196M *EML4–ALK* gatekeeper mutation and amplification of the *EML4–ALK* allele (11). In addition, we also examined two novel cell lines established from biopsies of patients whose *ALK*-rearranged lung cancers had become resistant to crizotinib in the clinic. These two patient-derived resistant lines, MGH045 and MGH021-4, harbor the L1196M and G1269A mutations, respectively. The MGH021-4 line is a clonal cell line established from MGH021, a tumor harboring both 1151T-ins and G1269A mutations; MGH021-4 cells harbor only the G1269A mutation (5). This clone, therefore, represents an early generation of the patient-derived cell line. The GI_{50} values of ceritinib against all of these resistant cell lines were decreased 6-fold to 36-fold compared with crizotinib (Fig. 2A and Supplementary Fig. S2A–S2C). Accordingly, phosphorylation of ALK and downstream ERK and AKT were more effectively suppressed by lower doses of ceritinib compared with crizotinib (Fig. 2B–D).

To further assess the activity of ceritinib against crizotinib-resistant *ALK*-positive tumors *in vivo*, we examined the efficacy of ceritinib against xenografts derived from MGH045 cells that harbor the L1196M resistance mutation. As shown in Fig. 2E, treatment of MGH045 tumor-bearing mice with

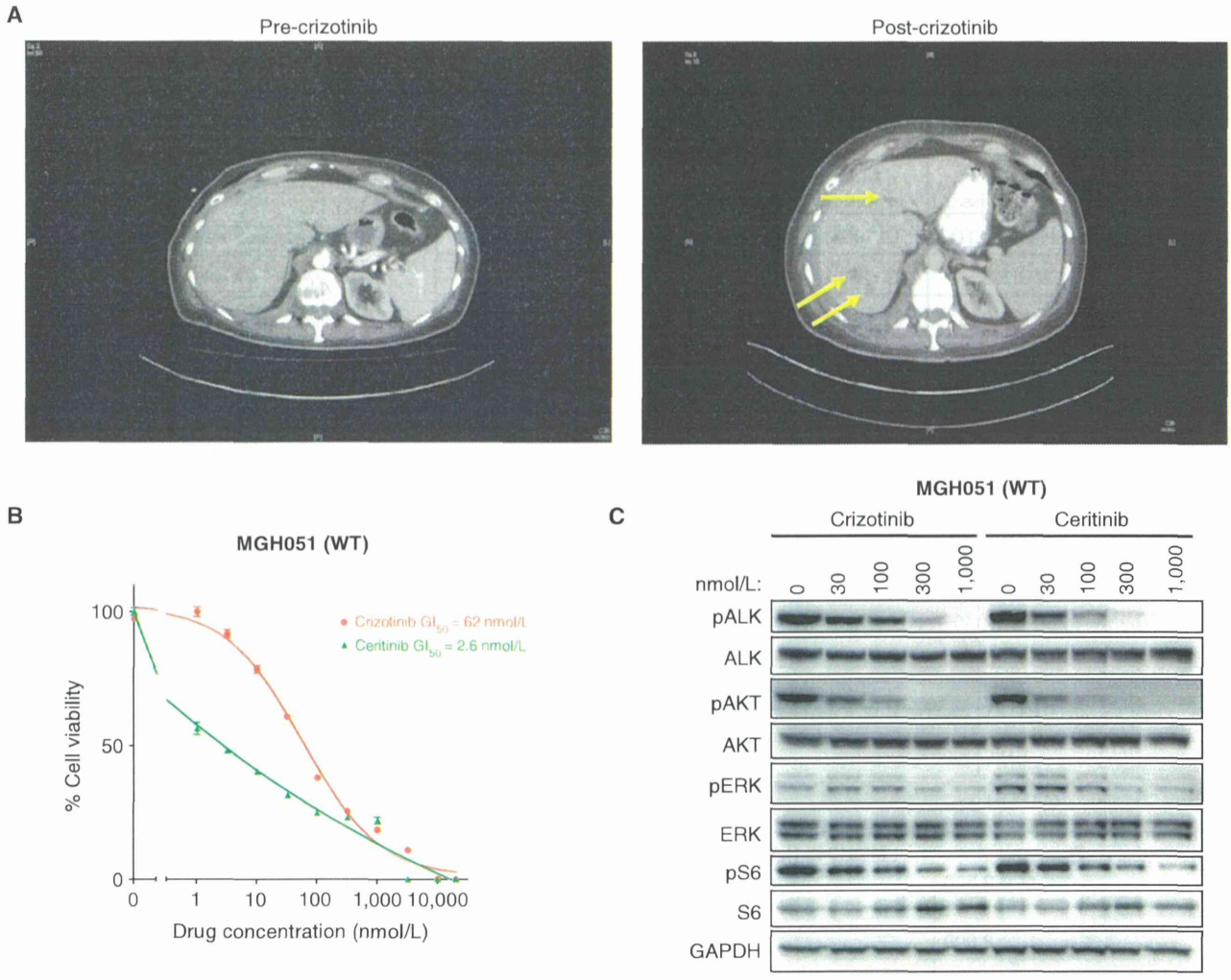

Figure 3. Ceritinib is active in ALK wild-type (WT) crizotinib-resistant cell line. **A,** abdominal computed tomography (CT) images of patient MGH051 before treatment with crizotinib and after 11 weeks of crizotinib. Several new hepatic metastases (yellow arrows) were detectable after crizotinib treatment consistent with disease progression. A repeat biopsy of a hepatic metastasis was performed within 2 weeks of crizotinib discontinuation. **B,** MGH051 cells were treated with the indicated doses of crizotinib or ceritinib for 7 days. After the incubation, the cell survival was assayed by CellTiter-Glo. **C,** MGH051 cells were treated with the indicated concentrations of crizotinib or ceritinib for 24 hours. Lysates were probed with antibodies directed against the indicated proteins.

low-dose ceritinib (25 mg/kg) was more effective than with high-dose crizotinib in controlling tumor growth. These data demonstrate that ceritinib is active against cancers derived from patients with acquired resistance to crizotinib and is more potent than crizotinib against *ALK*-rearranged cancers harboring the L1196M and G1269A resistance mutations.

The ongoing clinical trial of ceritinib demonstrates that crizotinib-resistant *ALK*-positive tumors, including tumors without *ALK* mutation or gene amplification, are responsive to ceritinib treatment (13). This raises the possibility that many of these resistant tumors may develop because of inadequate target suppression. We investigated the efficacy of crizotinib and ceritinib against a crizotinib-resistant *ALK*-positive cell line without *ALK* resistance mutations, MGH051. As shown in Fig. 3A, this cell line was derived from a biopsy of a liver lesion that developed in a patient on crizotinib. Assessment of the biopsy sample revealed no *ALK* mutations or gene amplification. The cell line derived from the biopsy also did not harbor any *ALK* resistance mutations. This resistant cell line was highly sensitive to ceritinib *in vitro*, and, surprisingly, the MGH051 cell line was also sensitive to crizotinib (Fig. 3B). Accordingly, phosphorylation of ALK and downstream AKT and ERK was efficiently suppressed by crizotinib and ceritinib (Fig. 3C). These data suggest that cancers with acquired resistance to crizotinib without *ALK*-resistant mutations may remain sensitive to ALK inhibition (please see "Discussion").

Assessment of Ceritinib Activity against a Panel of *ALK* Mutations

To systematically assess the potency of ceritinib against *ALK* resistance mutations, we used Ba/F3 cells engineered