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Brief report: *EGFR* L858M/L861Q cis mutations confer selective sensitivity to afatinib

Jamie A. Saxon^{a,b}, Lynette M. Sholl^c, and Pasi A. Jänne^{a,b,d,e}

^aLowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, Massachusetts, USA 02215

^bDepartment of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, Massachusetts, USA 02215

^cDepartment of Pathology, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, Massachusetts, USA 02115

^dDepartment of Medicine, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, Massachusetts, USA 02115

^eBelfer Center for Applied Cancer Science, Dana-Farber Cancer Institute, 360 Longwood Avenue, Boston, Massachusetts, USA 02115

Abstract

Introduction—Tyrosine kinase inhibitors (TKIs) have been developed to treat patients with epidermal growth factor receptor (*EGFR*)-mutant lung cancers. However, the therapeutic efficacy of TKIs in patients with uncommon *EGFR* mutations remains unclear.

Methods—Next-generation sequencing was performed on a patient's lung adenocarcinoma tumor sample, revealing rare combined in cis (on the same allele) *EGFR* mutations. Stable Ba/F3 and NIH-3T3 cell lines harboring the mutations were established to investigate the effect of first, second, and third generation *EGFR* TKIs on cell proliferation by MTS assay and *EGFR* phosphorylation by Western blotting.

Results—*EGFR* L858M/L861Q mutations in cis were detected in a non-small cell lung cancer patient's tumor. The patient demonstrated primary resistance to erlotinib and was subsequently treated with afatinib, which caused tumor regression. In *in vitro* studies, first and third generation

Corresponding author: Pasi A. Jänne, MD, PhD, Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, LC4114, Boston, MA 02215, Phone: (617) 632-6076, Fax: (617) 582-7683, Pasi_janne@dfci.harvard.edu.

Conflicts of interest:

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TKIs exhibited a decreased capacity to prevent EGFR phosphorylation and inhibit cell proliferation in *EGFR* L858M/L861Q cells compared to cells harboring the common *EGFR* L858R point mutation. In contrast, afatinib treatment reduced proliferation and inhibited EGFR phosphorylation in L858M/L861Q and L858R mutant cells at similar concentrations.

Conclusions—Afatinib may be a beneficial therapeutic option for a subset of lung cancer patients with rare *EGFR* mutations in their tumors. Understanding how uncommon mutations affect protein structure and TKI binding will be important for identifying effective targeted therapies for these patients.

Keywords

NSCLC; EGFR mutation; afatinib; targeted therapy

INTRODUCTION

Epidermal growth factor receptor (*EGFR*) mutations are reported in approximately 10–30% of non-small cell lung cancers (NSCLCs) worldwide.^{1–3} These mutations occur in the tyrosine kinase domain and result in ligand-independent activation of the receptor and signaling through downstream pro-survival and anti-apoptotic pathways. The most common *EGFR* mutations are in-frame deletions in exon 19 and the L858R point mutation in exon 21.³ Most patients whose tumors harbor these classic mutations have significant radiographic and clinical responses to the first generation reversible ATP-competitive EGFR tyrosine kinase inhibitors (TKIs) erlotinib and gefitinib and the second generation irreversible TKI afatinib.^{1,4,5}

However, less common mutations are found in approximately 10–15% of *EGFR*-mutant lung cancers,^{3,6} and the relevance of these mutations as clinical biomarkers for targeted therapy has not been clearly established. Here, we describe the identification of novel *EGFR* L858M/L861Q mutations in cis (on the same allele) in a NSCLC patient that confer decreased sensitivity to first and third generation EGFR TKIs while retaining responsiveness to afatinib. Our findings suggest that afatinib may be a beneficial therapeutic option for patients with rare *EGFR* mutations with primary resistance to first-generation EGFR inhibitors.

MATERIALS AND METHODS

Patient information

The patient, a 62-year-old Caucasian female with < 1 pack year smoking history, presented with symptoms of shortness of breath that were initially felt to be panic attacks. Two months later, her exertional shortness of breath worsened. A chest X-ray was obtained and identified a diffuse infiltrative process predominately in the upper lobes. A chest CT identified irregular interlobular septal thickening predominately in the upper lobes along with nodularity in a subpleural distribution. Given the diffuse nature of the process, a video assisted thoracoscopic surgical wedge resection from the right upper and middle lobes was performed. Pathologic evaluation demonstrated extensive involvement by a poorly differentiated adenocarcinoma. Immunohistochemistry demonstrated the tumor cells to be

positive for cytokeratin 7, CEA, TTF1 with focal cells also staining for mucin. The patient's tumor specimen was sent for targeted next generation sequencing (NGS) to help guide treatment.

DNA extraction, NGS, and data analysis

Targeted sequencing was performed using a next generation sequencing assay ("OncoPanel") to detect single nucleotide variants, small insertion-deletion mutations, copy number variations, and structural variants in tumor DNA extracted from fresh, frozen or formalin-fixed paraffin-embedded samples containing at least 20% tumor cells.⁷⁻⁹ The assay covers the complete exonic sequences of 300 cancer genes and 113 introns across 35 genes for genomic rearrangements.

Cell lines and reagents

Ba/F3 cells were maintained in RPMI-1640 (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco), and NIH-3T3 cells were maintained in DMEM (Gibco) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Generation of *EGFR* L858R Ba/F3 and *EGFR* L858R NIH-3T3 cells was previously described.^{10,11} Gefitinib, afatinib, and osimertinib were purchased from Selleck Chemicals, and stock solutions of drugs were prepared in DMSO.

EGFR L858M/L861Q mutant construct and retroviral infection

The L858M and L861Q point mutations were introduced into human *EGFR* cDNA by site-directed mutagenesis using the Quick Change Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol with the following primers: 5'-ttccgacccagctgtttggccatcccaaatctgtg-3' and 5'-cacagatttgggatggccaacagctgggtgcggaa-3'. Successful generation of the construct was confirmed by DNA sequencing. The construct was shuttled into the retroviral vector JP1540 using the Agilent Cre Recombinase kit (Agilent). Ba/F3 and NIH-3T3 cells were infected with retrovirus according to standard protocols.¹² Stably-transfected cell lines were obtained by puromycin selection (2 µg/mL; Sigma-Aldrich) for NIH-3T3 cells and by puromycin selection and IL-3 withdrawal for Ba/F3 cells. *EGFR* expression was verified by Western blotting, and pooled stable cells were used for experiments.

Cell proliferation assays

Cell growth/growth inhibition was assessed by MTS assay. 3000 cells were seeded per well in 96 well plates and exposed to drug (3.3 nM to 10 µM) for 72 hours. All experimental points included 6 to 12 wells, and data were averaged from three independent experiments. Data were displayed graphically using GraphPad Prism (GraphPad Software, Inc.), and the curves were fitted using a nonlinear regression model with sigmoidal dose response.

Western blotting analysis

EGFR L858R and *EGFR* L858M/L861Q NIH-3T3 cells were plated at 1×10^6 cells per 10-cm plate and treated with the indicated concentrations of inhibitors for 6 hours the following day. After 6 hours of treatment, cells were washed with PBS and lysed with NP40 buffer

(Calbiochem) supplemented with Complete Mini protease inhibitor and PhosSTOP phosphatase inhibitors (Roche). Lysates were separated by SDS-PAGE gel, transferred to nitrocellulose membranes, and probed the following antibodies: phospho-EGFR(Y1068) (3777), total EGFR (2232) (Cell Signaling), and tubulin (T9026) (Sigma Aldrich). Densitometry was measured using ImageJ. Samples were normalized to tubulin before determining the ratio of phospho-EGFR in treated versus untreated samples.

RESULTS

Targeted NGS of the patient's tumor revealed two *EGFR* mutations, L858M (in 30% of 190 reads) and L861Q (in 28% of 175 reads). These two mutations were in cis with one another (Fig. 1). Based on these findings, the patient was initiated on erlotinib therapy at 150 mg once daily 4 months after initial presentation (Fig. 2). At the time of erlotinib initiation, the patient was using supplemental oxygen (2–3 liters/minute). After 1 month of treatment, her erlotinib dose had to be reduced to 100 mg once daily due to skin toxicity. Repeat staging 2 months after initiation of erlotinib demonstrated stable disease (Fig. 2). However, after 4 months of erlotinib, her shortness of breath and oxygen requirement (3–4 liters/minute) had worsened. She was started on combination chemotherapy with carboplatin and pemetrexed and received 4 cycles of treatment. Repeat imaging demonstrated a minor response. The patient still required supplemental oxygen (3 liters/minute) and had substantial shortness of breath. After 4 months of chemotherapy, and following the development of worsening shortness of breath, she was switched to afatinib 40 mg once daily. The patient noted improvement in her breathing with just a few days of treatment. Following 2 weeks of afatinib therapy, she no longer required supplemental oxygen and her shortness of breath had markedly improved. Repeated imaging after 2 months of afatinib demonstrated a dramatic radiographic response (Fig. 2). She has remained on afatinib, with grade 1 diarrhea as her only side effect, for 10 months and continues treatment as of the writing of this report.

To understand whether the patient's resistance to erlotinib and sensitivity to afatinib could be explained by the unique *EGFR* L858M/L861Q mutations, the effect of the L858M/L861Q mutations on drug sensitivity was examined *in vitro*. Stable *EGFR* L858M/L861Q Ba/F3 cells were generated, and cell viability/proliferation after treatment with EGFR TKIs was measured by MTS assay. Compared to *EGFR* L858R mutant cells, L858M/L861Q cells demonstrated reduced sensitivity to gefitinib treatment (L858R IC₅₀ 28.3 nM vs. L858M/L861Q IC₅₀ 169.1 nM) (Fig. 3A, B), but the response to afatinib was similar for both cell lines (L858R IC₅₀ 1.5 nM vs. L858M/L861Q IC₅₀ 1.8 nM). In addition, L858M/L861Q cells exhibited decreased sensitivity to osimertinib, a third generation irreversible and mutant-specific EGFR inhibitor (L858R IC₅₀ 8.0 nM vs. L858M/L861Q IC₅₀ 28.8 nM) (Fig. 3A, B). Using stable *EGFR* L858M/L861Q NIH-3T3 cells, we examined the capacity of gefitinib, afatinib, and osimertinib to prevent EGFR phosphorylation by Western blotting. Gefitinib and osimertinib inhibited EGFR L858M/L861Q phosphorylation less potently than EGFR L858R (Fig. 3C), while afatinib treatment significantly reduced EGFR L858M/L861Q phosphorylation even at low concentrations.

DISCUSSION

In the era of precision medicine, genotyping of *EGFR* has become a standard practice for NSCLC, but the identification of a rare mutation does not necessarily indicate a clear course of action for therapy. This is the first report of a patient with *EGFR*-mutant lung cancer with primary resistance to erlotinib but responsiveness to afatinib, and our *in vitro* findings indicate that this therapeutic response was the result of rare *EGFR* L858M/L861Q cis mutations in the patient's tumor. According to the COSMIC database (v.78), *EGFR* L858M/L861Q cis mutations have been reported in three other lung cancer patients, although no data is available on the treatment or outcome of these patients.^{13–15} Previous reports suggest that patients with *EGFR* L861Q mutations have a response rate of 50–60% to gefitinib and erlotinib, responding less favorably than patients with the more common L858R point mutation or exon 19 deletion.^{6,16} Similarly, our findings suggest that the potency of first generation inhibitors to prevent EGFR signaling is reduced in the setting of the L858M/L861Q mutation. Additionally, our results indicate that treatment with the mutant-specific inhibitor osimertinib may not sufficiently inhibit EGFR L858M/L861Q signaling, suggesting that afatinib may be the most beneficial first line therapy for a subset of patients with rare *EGFR* mutations. Together, these data suggest that the various EGFR inhibitors may have distinct capabilities to inhibit signaling of specific EGFR mutants. Additional studies to understand how uncommon mutations affect EGFR protein structure and TKI binding as well as studies to measure the potency of TKIs in the setting of rare mutations will provide valuable therapeutic insight for NSCLC patients with uncommon *EGFR* mutations in their tumors.

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ABBREVIATIONS

CT	Computed tomography
EGFR	Epidermal growth factor receptor
NGS	Next-generation sequencing
NSCLC	Non-small cell lung cancer
TKI	Tyrosine kinase inhibitor

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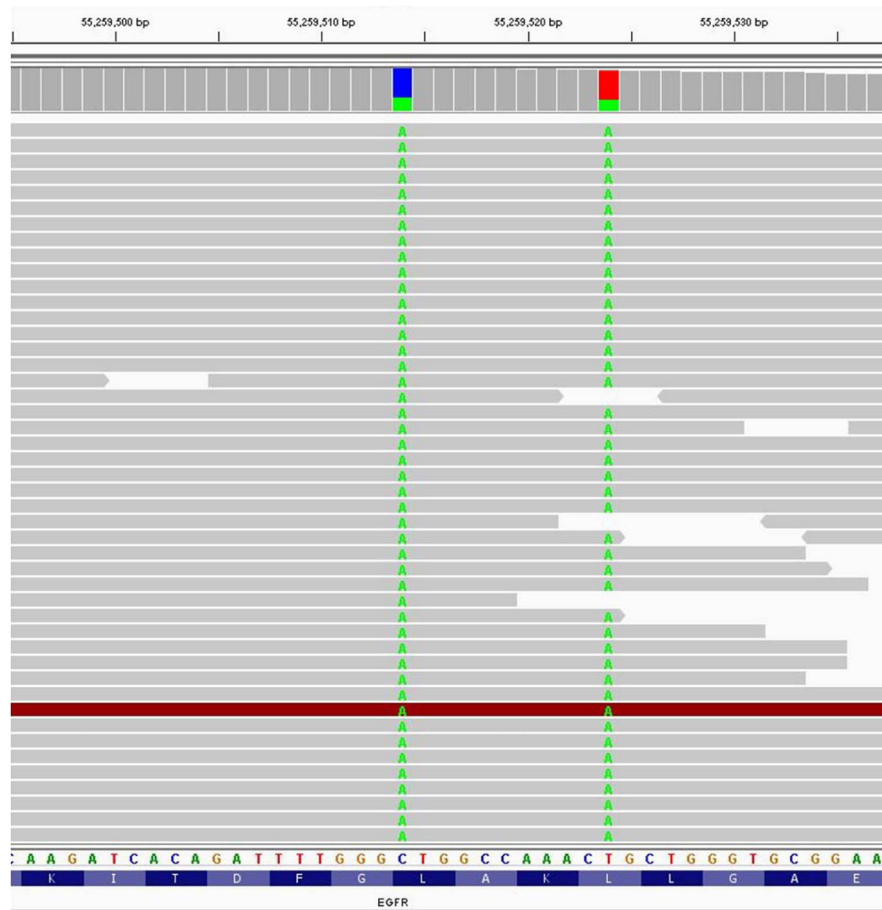


Figure 1. Identification of *EGFR* L858M and L861Q mutations in cis from lung cancer tumor biopsy

Alignment of NGS sequencing reads from the patient's tumor reveals the 2572C>A and 2582T>A nucleotide substitutions resulting in L858M and L861Q missense mutations in exon 21 of *EGFR*. These substitutions co-occurred in the same reads, indicating that the mutations are in cis.

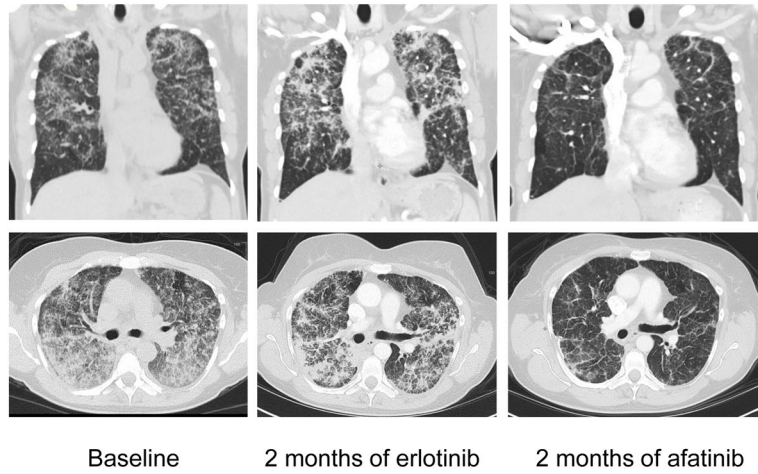


Figure 2. Computed tomography (CT) scans of a NSCLC patient exhibiting primary resistance to erlotinib and subsequent response to afatinib

Coronal and transverse CT scan images at baseline before treatment, after two months of erlotinib treatment, and after two months of afatinib treatment.

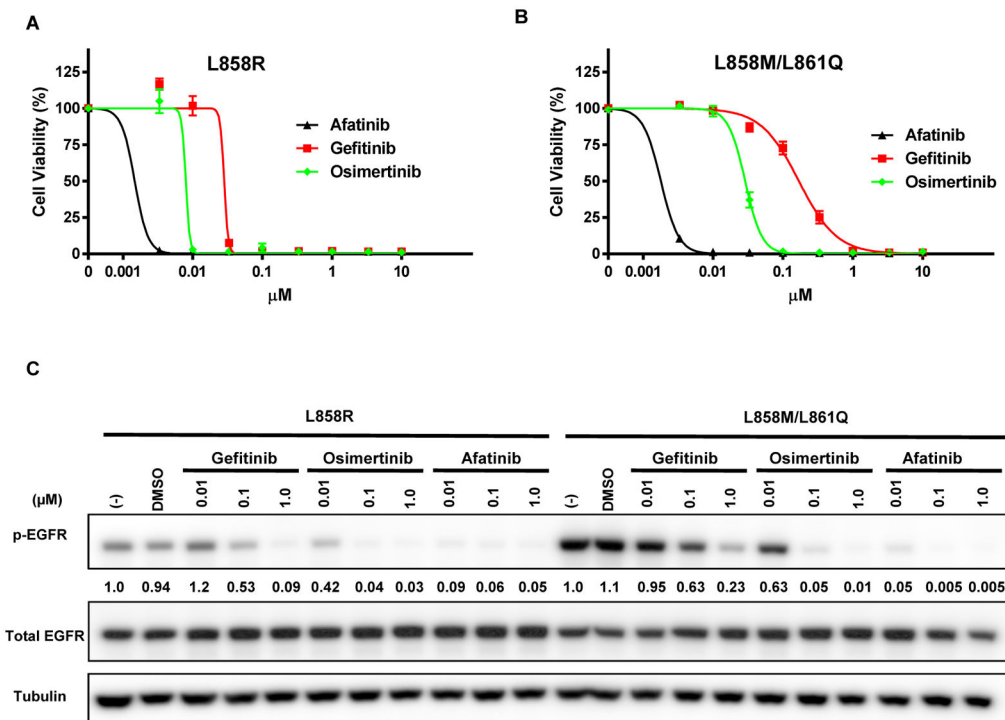


Figure 3. *In vitro* studies demonstrate reduced sensitivity of EGFR L858M/L861Q to gefitinib and osimertinib compared to EGFR L858R

Viability of (A) *EGFR* L858R and (B) *EGFR* L858M/L861Q Ba/F3 cells after 72 hours of treatment with gefitinib, osimertinib, and afatinib, detected by MTS assay. Error bars represent standard error of the mean. (C) Immunoblotting of lysates from *EGFR* L858R and *EGFR* L858M/L861Q NIH-3T3 cells treated with the indicated concentrations of gefitinib, osimertinib, or afatinib. The ratio of phospho-EGFR in treated vs. untreated samples was quantified by densitometry for each cell line.