A Novel Acquired Exon 20 EGFR M766Q Mutation in Lung Adenocarcinoma Mediates Osimertinib Resistance but is Sensitive to Neratinib and Poziotinib

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ABSTRACT

Introduction: Osimertinib is an effective third-generation tyrosine kinase inhibitor (TKI) for EGFR-mutant lung cancers. However, treatment for patients with acquired resistance to osimertinib remains challenging. We characterized a novel EGFR mutation in exon 20 that was acquired while on osimertinib.

Methods: A 79-year-old woman had disease progression during third-line treatment with osimertinib for an EGFR L858R/T790M–mutant lung cancer. Sequencing of circulating cell-free DNA showed EGFR L858R, an acquired novel EGFR M766Q mutation in exon 20, and no evidence of EGFR T790M. Homology modeling was performed to investigate the effects of M766Q on binding to osimertinib. L858R and L858R/M766Q mutations were retrovirally introduced into Ba/F3 and NIH/3T3 cells and evaluated for sensitivity to first-generation (erlotinib), second-generation (afatinib, neratinib, and poziotinib), and third-generation TKIs (osimertinib) by cell viability and colony-formation assays. EGFR-mediated signaling pathways were interrogated by western blotting.

Results: Modeling suggested that EGFR M766Q could disrupt osimertinib binding. L858R/M766Q double-mutant cells were 12-fold more resistant to osimertinib, and more than 250-fold more resistant to erlotinib and afatinib, as compared to L858R-mutant cells. In contrast, double-mutant cells remained sensitive to neratinib and poziotinib at clinically relevant doses (concentration that inhibits 50%, 4.3 and 1.3 nM, respectively). This was corroborated by the effects of the TKIs on colony formation and EGFR signaling.

Conclusions: Acquisition of EGFR M766Q exon 20 mutation is a novel mechanism of acquired resistance to osimertinib. EGFR-mutant lung cancers with an acquired EGFR M766Q
mutation in the setting of osimertinib resistance may be sensitive to neratinib and poziotinib.

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Keywords: Lung adenocarcinoma; EGFR; Osimertinib resistance; Neratinib; Poziotinib

Introduction

Osimertinib is a third-generation EGFR-tyrosine kinase inhibitor (TKI) with high efficacy in patients who have NSCLC harboring the EGFR T790M-resistance mutation.1 Most EGFR T790M-mutant patients ultimately progress on osimertinib treatment, and up to half of those patients show loss of T790M and many of them acquire other EGFR alterations.2,3 Treatment for these patients remains challenging. Because osimertinib is increasingly being used as frontline therapy for EGFR-mutant lung cancers, we expect exon 20 resistance mutations will become more common. We describe a novel EGFR M766Q exon 20 mutation with retention of L858R, but loss of T790M, in a patient with lung adenocarcinoma who progressed on third-line osimertinib therapy. Our findings suggest that neratinib or poziotinib may be a beneficial therapeutic option for patients whose recurrence after osimertinib therapy lacks T790M, but harbors rare EGFR mutations at or near M766.

Materials and Methods

Patient and Clinical Sample Collection

Sequencing data and clinical history were obtained through an institutional review board–approved protocol at the Rutgers Cancer Institute of New Jersey. As part of routine care on progression of disease, plasma was obtained and sent for sequencing (Guardant Health, Redwood City, CA).

Homology Modeling

We used the Swiss-Model automated homology modeling server to extract the structural information from the template structure (PDB ID:4zau), model mutated sidechains using a backbone-dependent sidechain rotamer library, and resolve unfavorable interactions or steric clashes.4 We did not model EGFR S306L because there was no structural precedent, and based on its location in EGFR, we predicted that it is unlikely to affect the kinase catalytic domain.

Experimental Assays

NIH/3T3 (American Type Culture Collection, Manassas, VA) and interleukin 3–dependent Ba/F3 (DSMZ German collection) cells were obtained in 2018 and maintained, infected, and selected as described.5 Cells were confirmed negative for mycoplasma.6 Inhibitors were purchased from Selleck Chemicals. Plasmids were a gift from Matthew Meyerson (Addgene, Watertown, MA; #11012/#32072). Mutations were introduced using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). MTS assays, soft agar colony-forming assays and western blotting were performed following standard methods. Concentration that inhibits 50% (IC50) values were calculated using GraphPad Prism 5.0. Significance was determined by a two-tailed t test. Anti-EGFR (#2232), P-EGFR (#3777), ERK1/2 (#4695), P-ERK1/2 (#9101), and P-AKT (#4060) antibodies were from Cell Signaling Technology (Danvers, MA). Anti-AKT (#5298) and vinculin (#73614) antibodies were from Santa Cruz Biotechnology (Dallas, TX).

Results

Case Report

A 79-year-old never-smoker female presented with a right upper lobe mass and underwent a right upper lobectomy in November 2007. Pathology revealed a 5-cm well-differentiated adenocarcinoma with areas of bronchoalveolar growth pattern, no pleural or lymphovascular invasion, and 0 of 7 involved lymph nodes (T2N0M0). In August 2008, she had multiple thyroid nodules and underwent a right partial thyroidectomy, which revealed well-differentiated adenocarcinoma, similar to her lung adenocarcinoma, confirming metastatic disease. Subsequently, genomic testing of the lung mass revealed an EGFR L858R mutation, and she was started on erlotinib in May 2009. Her dose fluctuated between 150 mg and 100 mg daily due to side effects including diarrhea, sores on scalp, alopecia, and curling of her eyelashes and toenails. A repeat lung biopsy in May 2014 after radiographic disease progression again showed adenocarcinoma. Genomic testing identified EGFR L858R and T790M mutations. After seeking consultation elsewhere, she entered a clinical trial with rociletinib, but experienced adverse events including hyperglycemia and thrombocytopenia, and displayed evidence of disease progression in September 2016. She was started on osimertinib, 40 mg daily in September 2016, and she had stable disease and clinical benefit.

In August 2017, imaging studies showed disease progression in the lung and possible new osseous metastases. She then presented to the Rutgers Cancer Institute of New Jersey. Our review confirmed the progressive disease, but the patient refused a biopsy. A blood sample was sent for circulating cell-free tumor DNA sequencing, which revealed mutant
allele frequencies: \( EGFR \) L858R (0.6%), \( TP53 \) V203M (0.2%), and variants of uncertain clinical significance including \( EGFR \) S306L (3.3%) and M766Q (0.2%). \( EGFR \) S306L was reported in one other lung adenocarcinoma in cBioportal (www.cbioportal.com), although the clinical implications and biological effects of this mutation are unknown. Mutations in \( EGFR \) at M766 have not been reported in cBioportal; however, an \( EGFR \) M766T mutation was reported to be activating. Based on the sequencing report, we recommended re-starting erlotinib. Her preference was to continue osimertinib and not to pursue other treatments. She died in January 2019 due to complications of progressive disease.

**Homology Modeling of \( EGFR \) Double-Mutant Structure**

In the osimertinib co-crystal structure with wild-type \( EGFR \) (PDB ID:4zau), residue M766 stabilizes position T790 with favorable noncovalent contact of .44 nm (Fig. 1). In the double mutant model, Q766 comes within .37 nm of T790, too close for legitimate van der Waals interaction (Fig. 1B). Q766 appears to push T790 forward into the inhibitor binding site, thereby weakening osimertinib binding.

**Response of Cells Expressing \( EGFR \) L858R/M766Q to \( EGFR \)-TKIs**

We generated Ba/F3 cells stably expressing \( EGFR \) L858R alone or in cis with M766Q, and \( EGFR \) L858R/ T790M/C797S–expressing Ba/F3 cells to serve as a control for osimertinib resistance. All mutations induced interleukin-3–independence (Supplementary Fig. 1), and mutant proteins were expressed at equivalent levels (Supplementary Fig. 2). Ba/F3 cells expressing \( EGFR \) L858R/M766Q were more than 10-fold more resistant to osimertinib compared to cells expressing \( EGFR \) L858R (IC\(_{50}\): 50.62 nM versus 4.20 nM) (Fig. 2A; Table 1), consistent with other reported osimertinib-resistance mutations, including \( EGFR \) L858R/L718V and hotspot mutations near C797. Patients who lose T790M upon osimertinib resistance may display sensitivity to earlier generation TKIs such as erlotinib or afatinib. However, Ba/F3 cells expressing L858R/M766Q were resistant to both erlotinib (IC\(_{50}\): 4450 nM versus 16.5 nM) and afatinib (IC\(_{50}\): 46.4 nM versus 0.16 nM), compared to the single \( EGFR \) L858R mutation (Figs. 2B and C; Table 1).

Neratinib is a dual HER2/EGFR irreversible inhibitor with generally low activity in clinical trials potentially because of dose-limiting diarrhea. However, responses were seen in patients who have NSCLC with the \( EGFR \) G719X and other exon 18 mutations. \( EGFR \) L858R/M766Q and \( EGFR \) L858R displayed similar sensitivity to neratinib at low concentrations (IC\(_{50}\): 4.32 nM and 3.42 nM, respectively) (Fig. 2D, Table 1).

Poziotinib is an irreversible pan-\( EGFR \) TKI that successfully inhibits exon 20 \( EGFR/HER2 \) insertion mutations and is currently in phase II clinical trials for \( EGFR \) exon 20 insertion mutations in NSCLC. Exon 20 insertions sterically hinder the binding of osimertinib, but models have shown that the smaller size and unique shape of poziotinib enables better binding of the compound to the drug.
binding pocket, allowing it to overcome structural changes induced by exon 20 insertions.13 Poziotinib successfully inhibited the growth of \( \text{EGFR} \text{L858R/M766Q}\) expressing cells at clinically achievable concentrations (IC\(_{50}\): 1.33 nM) (Fig. 2, Table 1).

Similar to the MTS assays, NIH/3T3 cells expressing \( \text{EGFR} \text{L858R/M766Q}\) were significantly less sensitive to osimertinib, erlotinib, and afatinib in soft agar colony-forming assays than cells expressing \( \text{EGFR} \text{L858R}\) (Figs. 3A and B), whereas the single and double mutant-

| Table 1. IC\(_{50}\) Values of Ba/F3 Cells Expressing Mutant EGFR Constructs for Various Generations of EGFR-TKIs (nM) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Osimertinib     | Erlotinib       | Afatinib        | Neratinib       | Poziotinib      |
| \( \text{EGFR L858R} \) | 4.20            | 16.50           | 0.16            | 3.42            | 0.15            |
| \( \text{EGFR L858R/M766Q} \) | 50.62          | 4450            | 46.40           | 4.32            | 1.33            |
| \( \text{EGFR L858R/T790M/C797S} \) | 1630           | 7400            | 1500            | 1830            | 5300            |

IC\(_{50}\), concentration that inhibits 50%; TKI, tyrosine kinase inhibitor.
expressing cells were similarly sensitive to neratinib and poziotinib (Figs. 3A and B).

EGFR L858R-expressing Ba/F3 cells showed inhibited EGFR and downstream pathways (AKT and ERK1/2) in a dose-dependent manner caused by osimertinib, erlotinib, and afatinib, but the EGFR L858R/M766Q double-mutant cells displayed decreased inhibition (Figs. 4A-C). In contrast, neratinib and poziotinib inhibited phosphorylation of EGFR, AKT, and ERK1/2 at similar concentrations.

Figure 3. EGFR L858R/M766Q reduces the inhibition of osimertinib, erlotinib and afatinib, but not neratinib or poziotinib, on colony formation. NIH/3T3 cells (1 × 10⁵) expressing EGFR L858R or L858R/M766Q were seeded in agar with or without tyrosine kinase inhibitor (TKI). (A) Quantification of colony formation rate, relative to the rate of formation without drug (4X objective), from three replicates each and two independent experiments; Asterisks indicate significant difference between cells expressing EGFR L858R versus L858R/M766Q (*p < 0.05, **p < 0.01, and ***p < 0.00001). (B) Representative images (original magnification ×10) of colonies with or without TKI after 3 weeks; Scale bar indicates 0.5 mm.
between double- and single-mutant cells (Figs. 2D and E).

**Discussion**

*EGFR* mutation testing is standard-of-practice for patients who have advanced-stage NSCLC with evidence of adenocarcinoma histology. Osimertinib is commonly used to treat patients who develop the *EGFR* T790M resistance mutation, and is also increasingly used in the first-line setting. The best therapeutic course of action in patients whose tumors progress after an initial response to osimertinib is not clear. This is the first report of a patient whose lung cancer recurred after osimertinib therapy with an acquired *EGFR* L858R/M766Q mutation.

Collectively, our data confirmed that *EGFR* L858R/M766Q is capable of causing clinical resistance to osimertinib. This novel exon 20 mutation also mediated resistance against first- and second-generation TKIs commonly administered to patients who have lost a detectable *EGFR* T790M mutation after treatment with osimertinib. EGFR activity is regulated by repositioning of the αC-helix, which rotates into the adenosine triphosphate–site in the active state, and rotates outwards in the inactive state. Exon 20 insertional mutations are

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**Figure 4.** The *EGFR* M766Q mutation reduces the inhibition of phosphorylation of EGFR and downstream pro-growth proteins by osimertinib, erlotinib, and afatinib. Transduced Ba/F3 cells were plated at $1 \times 10^6$ cells/mL at hour 0. At hour 2, the drug or DMSO matching the concentration of the highest drug plate was added to the media for 4 hours. (A, B, C) *EGFR* L858R/M766Q exhibits increased levels of phosphorylated pro-growth proteins, including P-EGFR, P-AKT, and P-ERK1/2, as compared to *EGFR* L858R, when challenged with osimertinib (A), erlotinib, (B) and afatinib (C). (D, E) The levels of inhibited phosphorylation of *EGFR* L858R, *EGFR* L858R/M766Q, and downstream proteins are equivalent when challenged with neratinib (D) or poziotinib (E).
believed to cause conformational transitions which decrease the binding affinity of adenosine triphosphate–site TKIs. Although we did not rule out some effect of EGFR S306L, the modeling and cell-based data support that EGFR M766Q was likely a driving factor behind the osimertinib resistance observed in our patient. We also cannot rule out that the cells harboring EGFR L858R and M766Q were initially present, possibly subclonally, at diagnosis, and then were selected for and expanded during treatment with osimertinib. The presence of pre-existing resistant subclones of other TKI resistance mutations such as T790M has been shown and may be a significant source of acquired resistance to targeted therapy.

EGFR L858R/M766Q–expressing cells responded to neratinib and poziotinib similarly to EGFR L858R–expressing cells, at clinically achievable concentrations. Although neratinib was approved by the U.S. Food and Drug Administration for certain HER2+ breast cancer patients, it is not approved for NSCLC due to dose-limiting toxicities. Use of prophylactic loperamide has been reported to reduce incidence and severity of diarrhea and improve tolerance to neratinib. Poziotinib is another potential treatment candidate for patients who progress on osimertinib with exon 20 mutations, or insertions at M766 or in the αC-helix, although it also has significant side effects. Additional analysis of large-scale clinical data can provide insight into how frequently this mutation is encountered. The work presented here suggests that neratinib and poziotinib should be evaluated in the clinic for this novel mechanism of acquired resistance to osimertinib in EGFR-mutant lung cancer.

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Supplementary Data
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