Acquired $\text{MET}^{\text{D1228V}}$ Mutation and Resistance to MET Inhibition in Lung Cancer

Magda Bahcall¹, Taeho Sim²,³, Cloud P. Paweletz⁴, Jyoti D. Patel⁵, Ryan S. Alden², Yanan Kuang⁴, Adrian G. Sacher¹, Nam Doo Kim⁶, Christine A. Lydon¹, Mark M. Awad¹,⁷, Michael T. Jaklitsch⁸, Lynette M. Sholl⁹, Pasi A. Jänne⁴,⁷, and Geoffrey R. Oxnard¹,⁷

ABSTRACT

Amplified and/or mutated MET can act as both a primary oncogenic driver and as a promoter of tyrosine kinase inhibitor (TKI) resistance in non–small cell lung cancer (NSCLC). However, the landscape of MET-specific targeting agents remains underdeveloped, and understanding of mechanisms of resistance to MET TKIs is limited. Here, we present a case of a patient with lung adenocarcinoma harboring both a mutation in $\text{EGFR}$ and an amplification of MET, who after progression on erlotinib responded dramatically to combined MET and EGFR inhibition with savolitinib and osimertinib. When resistance developed to this combination, a new MET kinase domain mutation, D1228V, was detected. Our in vitro findings demonstrate that MET D1228V induces resistance to type I MET TKIs through impaired drug binding, while sensitivity to type II MET TKIs is maintained. Based on these findings, the patient was treated with erlotinib combined with cabozantinib, a type II MET inhibitor, and exhibited a response.

SIGNIFICANCE: With several structurally distinct MET inhibitors undergoing development for treatment of NSCLC, it is critical to identify mechanism-based therapies for drug resistance. We demonstrate that an acquired MET D1228V mutation mediates resistance to type I, but not type II, MET inhibitors, having therapeutic implications for the clinical use of sequential MET inhibitors.

INTRODUCTION

The majority of patients with advanced non–small cell lung cancer (NSCLC) harboring mutations in the $\text{EGFR}$ gene can initially be successfully treated with EGFR tyrosine kinase inhibitors (TKI); however, the emergence of resistance to these agents is inevitable in most patients (1). Several mechanisms of resistance have now been clinically recognized, the most common being the $\text{EGFR}^{\text{T790M}}$ mutation, which can be successfully treated with third-generation EGFR TKIs like osimertinib (2). MET amplification, which can occur as an acquired or de novo resistance mechanism, is a second well-established resistance mechanism known to bypass EGFR...
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inhibition and impart resistance to EGFR TKIs, and as such requires a therapeutic strategy directed at both EGFR and MET (3). Indeed, patients with such a signature of TKI resistance have benefited from various off-label combinations of EGFR TKIs and the MET inhibitor crizotinib (4).

The MET oncogene has long been a candidate for drug development in NSCLC, but with modest success. Notably, multiple phase III trials of agents aiming to target MET signaling have recently failed (5–7). However, the identification of oncogenic MET alterations in subsets of lung cancer has again led to increased interest in targeting this oncoprotein. Three scenarios now exist in which oncogenic MET alterations have been described as potentially targetable—de novo amplification of MET (8), exon 14 skipping mutations in MET (9), and acquired amplification of MET after resistance develops to EGFR TKI (3).

Multiple MET TKIs are in development for advanced NSCLC, but much of the clinical data on drug activity are preliminary. Like many other TKIs, MET inhibitors fall into two functionally distinct classes: type I inhibitors which preferentially bind the active conformation of MET, such as crizotinib, and type II inhibitors which bind the inactive conformation, such as cabozaatinib (10). Type II inhibitors often inhibit a broader array of kinase targets, potentially resulting in more off-target side effects. Although the availability of multiple MET inhibitors lends itself to sequential targeted therapies, specific molecular factors affecting drug sensitivity and resistance have not been well characterized.

Here, we describe a patient with metastatic NSCLC with MET-mediated resistance to EGFR TKI who responded to treatment with a type I MET inhibitor, savolitinib (11), given in combination with a third-generation EGFR inhibitor, osimertinib (2). The patient then developed acquired resistance mediated by a novel MET kinase domain mutation.

RESULTS

Case Report

The patient was a 30-year-old female never-smoker who presented with dyspnea and was found to have a large left upper lobe mass and mediastinal adenopathy. Bronchoscopic biopsy demonstrated lung adenocarcinoma positive for an EGFR exon 19 deletion mutation. Due to the extent of locally advanced disease and the presence of a potentially targetable genotype, she was initiated on induction afatinib prior to definitive therapy. Surprisingly, scans after 4 weeks demonstrated mild growth of the lung mass. She was switched to induction chemotherapy with cisplatin and pemetrexed and had a radiographic response, which was then followed by concurrent chemoradiation. Unfortunately, she developed progression as she was completing radiotherapy, with a growing left cervical lymph node. This was biopsied and demonstrated recurrent lung adenocarcinoma. Imaging further demonstrated new bone metastases. She received a brief course of erlotinib, but her disease continued to progress (Fig. 1A).

Targeted next-generation sequencing (NGS) of the patient’s biopsy at recurrence confirmed the presence of the known EGFR exon 19 deletion and additionally identified high-level MET amplification, potentially explaining her drug resistance (Supplementary Fig. S1A); no other resistance mechanisms, including EGFR T790M, were detected. The patient then enrolled in a phase I clinical trial (NCT02143466) combining the MET TKI savolitinib (800 mg once daily) with the mutant-selective EGFR TKI osimertinib (80 mg once daily). She experienced a dramatic clinical response, with near-complete resolution of progressive cervical lymphadenopathy after 4 weeks of treatment (Fig. 1B). However, after 36 weeks on therapy, the patient developed a new growing pulmonary nodule consistent with objective disease progression (Fig. 1C). The lung nodule was removed surgically in an attempt at achieving local control of her progressive disease, but the patient had further disease progression in the lung and was removed from study therapy.

Tumor Sequencing Identifies a Secondary MET Kinase Domain Mutation

In search of genomic changes mediating resistance to combined EGFR and MET inhibition, we performed targeted NGS of the biopsy obtained following the development of resistance to savolitinib and osimertinib. Tumor NGS confirmed the high-level MET amplification and the EGFR exon 19 deletion, which had both been present on prior tumor NGS (Supplementary Fig. S1A and S1B). In addition, tumor NGS found 8 variants present at >5% allelic fraction (AF), 6 of which were also detected in a biopsy specimen from before treatment with savolitinib and osimertinib (Supplementary Table S1). Of the two acquired variants detected, one affected the MET kinase domain, D1228V (3683A>T), and was selected as a candidate variant for explaining drug resistance (Supplementary Fig. S1C).

Serial Plasma Genotyping Detects an Acquired MET Mutation as Resistance Develops

We performed serial genotyping of plasma cell-free DNA (cfDNA) collected during the treatment period using droplet digital PCR (ddPCR). Plasma ddPCR detected high levels of the EGFR exon 19 deletion prior to therapy, and rapid reduction of these levels after therapy was started, with levels becoming undetectable after 12 weeks of treatment (Fig. 1D). At the time of disease progression (36 weeks), we could again detect the EGFR exon 19 deletion. We developed a new ddPCR assay for MET D1228V and found that this mutation increased concurrently with the progression of the EGFR exon 19 deletion in plasma. Levels of mutant EGFR and MET in plasma continued to increase as the patient progressed further on postprogression therapy (Fig. 1C and D).

Protein Modeling Predicts Differences in Drug Binding between Type I and Type II MET Inhibitors

We developed models of the wild-type (WT) and mutant MET protein to study drug binding. Savolitinib, just as with other type I MET TKIs, binds to the MET kinase in its active conformation, whereas crizotinib, and type II inhibitors which bind the inactive conformation, such as cabozantinib (10). Type II inhibitors often inhibit a broader array of kinase targets, potentially resulting in more off-target side effects. Although the availability of multiple MET inhibitors lends itself to sequential targeted therapies, specific molecular factors affecting drug sensitivity and resistance have not been well characterized.

Here, we describe a patient with metastatic NSCLC with MET-mediated resistance to EGFR TKI who responded to treatment with a type I MET inhibitor, savolitinib (11), given in combination with a third-generation EGFR inhibitor, osimertinib (2). The patient then developed acquired resistance mediated by a novel MET kinase domain mutation.

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the MET kinase to cabozantinib, a type II inhibitor, which binds MET in its inactive conformation. The binding to wild-type MET does not rely on the availability of Tyr1230 (Fig. 2C), and drug binding is unaffected by the D1228V mutation (Fig. 2D).

**In Vitro Studies Demonstrate That MET<sub>D1228V</sub> Induces Resistance to Type I but Not Type II MET Inhibitors**

To validate our protein modeling studies, we performed a signaling analysis in 293T cells engineered to express either full-length wild-type MET or full-length MET<sub>D1228V</sub>. We found that both type I and II MET inhibitors are able to inhibit autophosphorylation of wild-type MET. However, the MET<sub>D1228V</sub> mutation rendered all type I MET inhibitors ineffective, as evidenced by the failure of savolitinib, crizotinib, and INC280 to inhibit MET phosphorylation (Fig. 3A). In contrast, the type II MET inhibitors cabozantinib, MGCD265, and merestinib (LY2801653) were able to effectively suppress MET phosphorylation in the presence of the D1228V mutation.

To further validate our biochemical findings, we introduced the oncogenic TPR–MET fusion (TPR–MET<sup>WT</sup>) or TPR–MET harboring the D1228V mutation (TPR–MET<sup>D1228V</sup>) into Ba/F3 cells or the EGFR-mutant EGFR TKI-sensitive PC9 cell line, and analyzed the ability of savolitinib alone, cabozantinib alone, or either agent in combination with gefitinib to inhibit viability and/or downstream effectors of the MET and EGFR signaling axes (Fig. 3B–D). The TPR–MET<sup>WT</sup> Ba/F3 cells were highly sensitive to both savolitinib and cabozantinib in the dose-escalated MTS viability assay (Fig. 3B). In contrast, the TPR–MET<sup>D1228V</sup> Ba/F3 cells failed to respond to savolitinib but maintained sensitivity to cabozantinib (Fig. 3B). In addition, the introduction of the wild-type or the D1228V-mutant TPR–MET fusion into PC9 cells (Supplementary Fig. S2) imparted resistance to gefitinib in both cases as expected (Fig. 3C), but the D1228V mutant showed a differential response to the combination of gefitinib and savolitinib, as compared with the wild-type. Although the TPR–MET<sup>WT</sup> PC9 cells responded to gefitinib in combination with either savolitinib or cabozantinib, the D1228V mutant was sensitive only to the gefitinib and cabozantinib combination (Fig. 3C). A Western blot analysis of the EGFR and MET signaling in the TPR–MET bearing PC9 cell derivatives further supported our cell viability studies. In the TPR–MET<sup>WT</sup> PC9 cells, phosphorylation of both EGFR and MET along with the MAPK/ERK pathway were completely inhibited following exposure to either the gefitinib/cabozantinib or gefitinib/savolitinib treatments (Fig. 3D).
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**DISCUSSION**

The MET oncogene represents an exciting therapeutic target in advanced NSCLC, with at least six MET TKIs currently in clinical development across the three different settings where genomic alterations suggest dependence upon oncogenic MET (Supplementary Table S2): de novo MET amplification, MET exon 14 skipping mutations, and MET amplification in resistance to EGFR TKIs. At least eight different combinations of MET and EGFR TKIs are being investigated in the context of EGFR TKI resistance. Our finding of a MET kinase domain mutation that leads to resistance to type I but not type II TKIs can have more off-target activity and treatment-to-type II KIT inhibitors (imatinib), secondary KIT mutations, including the gatekeeper mutation T315I (14, 15). Conversely, in gastrointestinal stromal tumors resistant to type II TKI inhibitors (imatinib), secondary KIT mutations can interfere with type II inhibitor binding while maintaining sensitivity to type I inhibitors (dasatinib, crenolanib; refs. 16, 17). Although type II TKIs can have more off-target activity and treatment-
Figure 3. **MET**<sup>D1228V</sup> causes resistance to type I **MET** inhibitors, whereas sensitivity to type II **MET** inhibitors is maintained. **A**, **MET**<sup>D1228V</sup> reduces the ability of type I **MET** kinase inhibitors (black) to dephosphorylate **MET**, but does not affect the activity of type II **MET** kinase inhibitors (blue). **B**, MTS growth inhibition assay of TPR–**MET**<sup>WT</sup> or TPR–**MET**<sup>D1228V</sup> transformed Ba/F3 cells exposed to dose-escalated savolitinib or cabozantinib shows differential sensitivity of TPR–**MET**<sup>D1228V</sup>. **C**, MTS growth inhibition assay of TPR–**MET**<sup>WT</sup> or TPR–**MET**<sup>D1228V</sup> transduced PC9 cells exposed to dose-escalated gefitinib alone or in combination with savolitinib or cabozantinib shows differential sensitivity of TPR–**MET**<sup>D1228V</sup> PC9 to the **MET** inhibitors. **D**, TPR–**MET**<sup>D1228V</sup> reduces the ability of savolitinib to dephosphorylate TPR–**MET** and inhibit downstream signaling in combination with gefitinib, but does not affect the activity of cabozantinib in combination with gefitinib. **E**, As predicted, the cancer exhibited a response after 4 weeks of cabozantinib combined with erlotinib. gef., gefitinib; sav., savolitinib; cab., cabozantinib.

related side effects, the alternate spectrum of activity may prove to be valuable for the management of drug resistance.

The emergence of the **MET**<sup>D1228V</sup> mutation following savolitinib treatment suggests effective on-target pressure against the **MET** kinase. Another mutation at this residue, D1228N, has also been recently reported in a patient with **MET**-driven NSCLC and acquired resistance to crizotinib, suggesting that this mechanism of resistance is likely to become a recurring clinical problem (18). The development of secondary kinase domain mutations, as has been seen for targeted therapies against EGFR, ALK, and ROS1 (19–21), often marks effective and specific inhibition of an oncogenic target. Indeed, the absence of such an on-target resistance mechanism can raise concern over effective target inhibition. For example, one study performed plasma cfDNA genotyping on 19 patients with acquired resistance to osimertinib; of 15 with detectable
tumor DNA, 6 (40%) were positive for an EGFR^C797S^ mutation, which impairs covalent binding of osimertinib to the EGFR protein (22). A more recent study performed plasma cfDNA genotyping of 43 patients with acquired resistance to rociletinib, another third-generation EGFR TKI; of 40 with detectable tumor DNA, only 1 (2.5%) was positive for EGFR^C797S^, perhaps indicating less-effective on-target inhibition by this drug (23). Given that the commercially available multikinase MET TKIs, crizotinib and cabozantinib, received regulatory approval as agents targeting other oncoproteins, our finding of a secondary MET resistance mutation supports MET inhibition as the etiology of the dramatic response seen in cases like the one we present. Moreover, Asp1228 is highly conserved among receptor tyrosine kinases and may be a mutation hotspot in MET and other tyrosine kinases, as evidenced by prior reports of mutations at this residue (Table 1), warranting closer scrutiny when evaluating TKI resistance.

The development of MET inhibitors in advanced NSCLC has been challenging, with recent negative phase III trials for onartuzumab, a monoclonal antibody targeting MET (5), and tivantinib, a putative oral MET inhibitor (6, 24). Yet there is a surging interest in MET inhibition in NSCLC due both to the identification of oncogenic splice-site mutations near exon 14 of MET in approximately 3% of NSCLC (9) and to the regulatory approval of osimertinib for T790M-positive resistance to EGFR TKI (2), which elevates interest in treatments for other resistance mechanisms like MET amplification (1). With such a range of MET inhibitors in active development, we are hopeful that an improved understanding of their activity and mechanisms of resistance will lead to more treatment options for the emerging populations of MET-driven lung cancers.

**METHODS**

**Patient**

The patient provided written informed consent for treatment on trial NCT02143466. She additionally consented to a correlative protocol allowing genomic analysis of tumor and plasma specimens. Both studies were approved by the Dana-Farber Cancer Institute Institutional Review Board and conducted in accordance with the Declaration of Helsinki.

**Tumor and Plasma Analysis**

Targeted NGS was performed at the Center for Advanced Molecular Diagnostics at Brigham and Women’s Hospital using a 282-gene panel, as described previously (9). Genotyping of plasma cfDNA was performed at the Belfer Center for Applied Cancer Science at the Dana-Farber Cancer Institute using ddPCR. The development of the ddPCR assay for EGFR mutations has been described previously (25); a new ddPCR assay was developed and optimized for detection of MET^D1228V^ (see Supplementary Methods).

**Protein Modeling**

Based on the co-crystal structures of the MET kinase domain in complex with PF-04254644 (type I kinase inhibitor) or foretinib (type II kinase inhibitor), molecular docking studies of savolitinib and cabozantinib on the MET kinase domain (wild-type and D1228V mutant) were carried out using the GLIDE module. Molecular dynamics simulations (500 ns isothermal and isobaric simulation) of the complexes of the MET kinase domain (wild-type and D1228V mutant) were carried out using the Desmond software package (see Supplementary Methods).

**Antibodies and Compounds**

Antibodies against pMET (Tyr-1234; sc-101736; 1:400) and HSP90 (SC-7947; 1:400) were purchased from Santa Cruz Biotechnology; MET

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*Red, aspartate corresponding to residue D1228 in MET; green, tyrosine corresponding to Y1230 in MET; blue, recurring DFG motif; purple, additional tyrosines in the activation loop.

*Published as D1246X mutations based on an alternate numbering system.
(D1C2; 1:1,000), pEGFR (Ty-1068; #2777, 1:1,000), EGFR (D38B1; 1:1,000), pERK1/2 (197G2; 1:1,000), ERK1/2 (D13.14.4E; 1:1,000), pAKT (Ser-473, D9E; 1:1,000), AKT (#9272; 1:1,000) and anti-rabbit IgG-HRP (1:5,000) from Cell Signaling Technology; α-tubulin (1:5,000) from Sigma Aldrich; and anti-mouse IgG-HRP (1:5,000) from GE Life Sciences. Savolitinib was kindly provided by AstraZeneca; crizotinib, cabozantinib, INCB28060, and MGCD-265 were purchased from Selleckchem; and merestinib was purchased from MedChem Express.

Expression Vectors

Full-length human MET, transcript variant 2, cDNA (NM_000245.2) was amplified from a banked tumor specimen with an unrelated genetic alteration, and the TPR–MET fusion was amplified from the pBABE-puro TPR–MET plasmid created by the laboratory of Dr. Robert Weinberg (Addgene plasmid #10902). Both amplicons were subcloned into pDNR-dual (BD Biosciences) via the HindIII and XHOI restriction sites as described previously (26). The MET

\[
\text{Glu}^{1202}\text{Val} \]

mutation was introduced using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) and the following mutagenic primers: Forward: 5'-gtgatgtgtgggtcgcagctgtatgtataagactattxt-3'; Reverse: 5'-taattgctttccttagctgctgctagataaattacg-3'. All constructs were confirmed by DNA sequencing. Constructs were shuttled into the lentiviral expression vector JP1698 or JP1722 using the BD Creator System (BD Biosciences).

Cell Lines

293T and PC9 cells were purchased from the ATCC (in 2009) and Sigma Aldrich (in 2014), respectively. Ba/F3 cells were a generous gift from the laboratory of Dr. David Weinstock (in 2014). The 293T cells were cultured in DMEM, supplemented with 10% FBS, streptomycin, and penicillin. PC9 and transformed Ba/F3 cells were maintained in RPMI-1640 supplemented with 10% FBS, streptomycin, and penicillin. The human 293T and PC9 cell lines were authenticated in August 2016 using the Promega GenePrint 10 System at the RTSF Genomics Core at Michigan State University. Ba/F3 cells were not authenticated, because their short tandem repeat profile has not been made publicly available.

For transient MET overexpression, 5 × 10^5 293T cells were transfected with 1 μg DNA and 6 μL FuGENE HD (Promega) in Opti-MEM media (Gibco). Media were replaced 16 hours after transfection with complete DMEM. Seventy-two hours after transfection, cells were treated with inhibitors for 5 hours and subsequently lysed for Western blotting.

Viral Transductions

For stable TPR–MET expression, PC9 and Ba/F3 cells were transduced with lentivirus according to standard procedures (27). Briefly, 293T cells were transfected with the lentiviral construct in combination with the packaging plasmids VSVG and Δ8.2 (Addgene) using FuGENE HD Transfection Reagent (Promega) as per the manufacturer’s protocol. Viral supernatants were harvested 48 hours after transfection, filtered, added to preplated cells along with 10 μg/mL polybrene, and centrifuged for 1 hour at 2,100 rpm. Cells with successful lentiviral integration were selected with 15 μg/mL blasticidin.

Viability Assays

Stable TPR–MET-driven and IL3-independent Ba/F3 cells were treated with dose-escalated savolitinib or cabozantinib over the course of 72 hours. TPR–MET-transduced PC9 cells were treated with dose-escalated gefitinib alone or in combination with 1 μmol/L savolitinib or 1 μmol/L cabozantinib over the course of 72 hours. Growth and inhibition of growth were assessed by MTS assay according to previously established methods (24). All experimental points were set up in 6 to 12 wells, and all presented data are representative of several replicates.

Drug Treatments and Western Blotting

TPR–MET PC9 derivatives were treated with each inhibitor alone or in combination with gefitinib, each at 1 μmol/L, for 6 hours. Cell lysis, Western blotting, and immunoblotting were done as described previously (28). Blots were developed on Amersham Imager 600 (GE Healthcare Life Sciences).

Disclosure of Potential Conflicts of Interest

M.M. Awad is a consultant/advisor board member for Boehringer Ingelheim, Clavis Oncology, and Pfizer. P.A. Jänne reports receiving a commercial research grant from AstraZeneca and is a consultant/advisor board member for Ariad Pharmaceuticals, AstraZeneca, Boehringer Ingelheim, Chugai Pharmaceuticals, Ignyta, LabCorp, LOXO Oncology, Merrimack Pharmaceuticals, Pfizer, and Roche/Genentech. G.R. Oxnard has received honoraria from AstraZeneca, Boehringer Ingelheim, and Chugai, and is a consultant/advisor board member for Ariad, Astra-Zeneca, Boehringer Ingelheim, Clavis, and Genentech. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


Grant Support

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