Landscape of acquired resistance to osimertinib in *EGFR*-mutant NSCLC and clinical validation of combined EGFR and RET inhibition with osimertinib and BLU-667 for acquired *RET* fusion.

Piotrowska Z\*1, Isozaki H\*1, Lennerz J2, Gainor JF1, Lennes IT1, Zhu VW3, Marcoux N1, Banwait M1, Digumarthy S4, Su W1, Yoda S1, Riley AK1, Nangia V1, Lin JJ1, Nagy R5, Lanman R5, Dias-Santagata D2, Mino-Kenudson M2, Iafrate AJ2, Heist RS1, Shaw AT1, Evans EK6, Clifford C6, Ou SI3, Wolf BB6, Hata AN1, Sequist LV1

**SUPPLEMENTAL DATA**

**SUPPLEMENTAL TABLE:**

Table S1. Gene Coverage of Assays Used in the Study.

|  |  |  |
| --- | --- | --- |
| Assay | # Patients Tested | Genes Covered/Details |
| MGH SNaPshot NGS-V1 | 5 | The gene targets covered by this assay are as follows (exons): AKT1 (3), ALK (22, 23, 25), APC (16), BRAF (11, 15), CDH1 (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16), CDKN2A (1, 2, 3), CTNNB1 (3), DDR2 (12, 13, 14, 15, 16, 17, 18), EGFR (7, 15, 18, 19, 20, 21), ERBB2 (10, 20), ESR1 (8), FBXW7 (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11), FGFR1 (4, 8, 15, 17), FGFR2 (7, 9, 12,14), FGFR3 (7, 8, 9, 14, 16), FOXL2 (1), GNA11 (5), GNAQ (4, 5), GNAS (6, 7, 8, 9), HRAS (2, 3), IDH1 (3, 4), IDH2 (4), KIT (8, 9, 11, 17), KRAS (2, 3, 4, 5), MAP2K1 (2, 3), MET(14, 16, 19, 21), NOTCH (25, 26, 34), NRAS (2, 3, 4, 5), PDGFRA (12, 14, 18, 23), PIK3CA (2, 5, 8, 10, 21), PIK3R1 (1, 2, 3, 4, 5, 6, 7, 8, 9, 10), PTEN (1,2, 3, 4, 5, 6, 7, 8, 9), RET (11, 16), ROS1 (38), SMAD4 (2, 3, 4, 5, 6, 7, 8, 9,10, 11, 12), SMO (9), STK11 (1, 2, 3, 4, 5, 6, 7, 8, 9), TP53 (1, 2, 3, 4, 5, 6,7, 8, 9, 10, 11), and VHL (1, 2, 3). |
| MGH SNaPshot NGS-V2 | 28 | The 91 gene targets covered by this test are as follows (exons): ABL1 (4-7), AKT1 (3,6), ALK (21-23,25), APC (16), ARID1A (1-20), ATM (1-63), ATRX (1-35), AURKA (2,5-8), BRAF (11,15), BRCA1 (2-23), BRCA2 (2-27), CCNE1 (3-8,10,12), CDH1 (1-16), CDK4 (2-7), CDKN2A (1-3), CIC (1-20), CSF1R (7,22), CTNNB1 (3), DAXX (1-8), DDR2 (12-18), DDX3X (1-17), EGFR (3,7,15,18-21), ERBB2 (8,10,19-21,24), ERBB3 (2-3,7-8), ERBB4 (3-4,6-9,15,23), ESR1 (8), EZH2 (16), FBXW7 (1-11), FGFR1 (4,7-8,13,15,17), FGFR2 (7,9,12,14), FGFR3 (7-9,14-16,18), FLT3 (11,14,16,20), FOXL2 (1), GNA11 (5), GNAQ (4-5), GNAS (6-9), H3F3A (2), HNF1A (3-4), HRAS (2-3), IDH1 (3-4), IDH2 (4), JAK2 (11,13-14,16,19), JAK3 (4,13,16), KDR (6-7,11,19,21,26-27,30), KEAP1 (2-6), KIT (2,8-11,13-15,17-18), KRAS (2-5), MAP2K1 (2,3,6-7), MAP3K1 (1-20), MDM2 (2-4,6,8,10), MEN1 (2-10), MET (2,11,14,16,19,21), MLH1 (12), MPL (10), MSH6 (1-10), MSI, MYC (1-3), MYCN (3), NF1 (1-58), NF2 (1-15), NOTCH1 (25-27,34), NPM1 (11),NRAS (2-5), PIK3CA (2,5,7-8,10,14,19,21), PIK3R1 (1-10), POLE (9-14), PTCH1 (1-23), PTEN (1-9), PTPN11 (3,13), RB1 (1-27), RET (10-11,13-16), RHOA (2-3), RNF43 (2-10), ROS1 (38), SDHB (1-8), SMAD2 (7), SMAD4 (2-12), SMARCA4 (3-36), SMARCB1 (2,4,5,9), SMO (3,5-6,9,11), SRC (14), STAG2 (3-34), STK11 (1-9), SUFU (1-12), TERT (1), TP53 (1-11), TP63 (1-14), TSC1 (3-23), TSC2 (2-42), TSHR (10), VHL (1-3). |
| MGH Solid Fusion Assay | 24 | TARGETS VALIDATED FOR CLINICAL REPORTING: ALK (19-22, intron 19), BRAF (7-12, 15), BRD4 (10, 11), EGFR (2-7 exon skipping/vIII variant, 7-9, 16, 20, 24, 25), EWSR1 (4-14), FGFR2 (2, 8-10, 17), MAML2 (2, 3), MET (exon 14 skipping), NRG1(1-3, 6), NUTM1 (3), RET (8-13), and ROS1 (31-37).ADDITIONAL TARGETED GENES (EXONS): AKT3 (1-3), AR (1-8), ARHGAP26 (2, 10-12), AXL (19,20), BRD3 (9-12), SF1 (5-9), CSF1R (7,11-13,22), ERG (2-11), ESR1 (3-6), ETV1 (3-13), ETV4 (2, 4-10), ETV5 (2, 3, 7-9), ETV6 (1-7), FGFR1 2,8-10, 17), FGFR3 (8-10, 17, intron 17), FGR (2), INSR (12-22), JAZF1 (2-4), MAST1 (7-9, 18-21), MAST2 (2, 3, 5, 6), MET (13, 15), MSMB (2-4), MUSK (7-9, 11-14), MYB (7-9, 11-16), NOTCH1 (2, 4, 26-31, internal exon 3-27 deletion), NOTCH2 (5-7, 26-28), NTRK1 (8,10-13), NTRK2 (11-17), NTRK3 (13-16), NUMBL (3), PDGFRA (7, exon 8 deletion, 10-14), PDGFRB (8-14), PIK3CA (2), PKN1 (10-13), PPARG (1-3), PRKCA (4-6), PRKCB (3), RAF1 (4-7, 9-12), RELA (3, 4), RSPO2 (1, 2), SPO3 (2), TERT (2), TFE3 (2-8), TFEB (1,2), THADA (24-31,36), and TMPRSS2 (1-6). |
| FoundationOne | 1 | See <https://assets.ctfassets.net/vhribv12lmne/6Rt6csmCPuaguuqmgi2iY8/>e3a9b0456ed71a55d2e4480374695d95/FoundationOne\_CDx.pdf |
| Guardant360 | 26 | See https://www.guardanthealth.com/medical-professionals/ |

**SUPPLEMENTAL FIGURES AND FIGURE LEGENDS** – see separate file.

**SUPPLEMENTAL METHODS**

***Cell Culture***

Cells were cultured in RPMI1640 (Life Technologies) supplemented with 10% FBS. MGH845-1 cells were additionally cultured in 150 nM osimertinib. All cells underwent routine authentication using short-tandem repeat profiling to confirm the identity of human cell lines and to rule out interspecies contamination. The cells were also routinely tested and verified to be free of mycoplasma contamination in 2018.

***Reagents and antibodies***

 Afatinib, osimertinib, cabozantinib, dabrafenib, LXH254 and trametinib were purchased from Selleck Chemicals and resuspended in DMSO. BLU-667 was provided by Blueprint Medicines. Phospho-EGFR (Y1068), EGFR, pBRAF (Ser445), RET, pAKT (Ser473), AKT, pERK1/2 (Thr202/Tyr204), ERK1/2 and Actin antibodies were purchased from Cell Signaling Technology. pRET (Y1062) antibody was from Abcam.

***Western blotting***

 Cells were treated with drugs in 6-well plates for 6 hours. Cell protein lysates were prepared as previously described.

***RT-PCR and sequencing***

 Total RNA from cell lines was extracted using an RNeasy Mini Kit (Qiagen). RNA (1 μg) was reverse transcribed using SuperScript™ II Reverse Transcriptase (invitrogen), according to the manufacturer’s instructions. *CCDC6-RET*, *PCBP2-BRAF*, *TBP*, and *EGFR* exon 20 were PCR amplified using the following primers: *CCDC6* exon 1 F– CGGACAGCGCCAGCG and *RET* exon 19 R– GCATTATTACAGTCCACCAGCG; (*PCBP2-BRAF* Primer 1) *PCBP2* exon 6 F– AGGTGGATGCAAGATCAAGG and *BRAF* exon 13 R– TAGCCAGTTGTGGCTTTGTG; (*PCBP2-BRAF* Primer 2) *PCBP2* exon 2 F– CGGTGTGATTGAAGGTGGAT and *BRAF* exon 18 R– ACAGGAAACGCACCATATCC; *TBP* F– CCCATGACTCCCATGACC and *TBP* R– TTTACAACCAAGATTCACTGTGG; *EGFR* exon 20 F–GAAGCCACACTGACGTGC and exon 20 R–CTCCTTATCTCCCCTCCCCG. The PCR products were confirmed by agarose gel electrophoresis. Following amplification, sanger sequencing performed at the CCIB DNA Core Facility at MGH.

***siRNA transfection***

 *BRAF* siRNAs (#1 HSS101092, #2 HSS101093) and Silencer negative control #1 siRNA were obtained from Ambion. MGH845-1 cells were reverse transfected with 30 nM siRNA and Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer’s instructions. Transfected cells were cultured at 37℃ for 48 hours before analysis.

***BLU667 + Osimertinib Treatment Protocol:***

# INclusion Criteria

* Signed informed consent document(s)
* Greater than or equal to 18 years of age
* ECOG performance status 0-2 (See Appendix A)
* Pathologically-confirmed, locally advanced or metastatic NSCLC
* *EGFR* mutation, as detected by local testing of tumor or circulating tumor nucleic acid in blood
* *RET*-fusion, as determined by local testing of tumor or circulating tumor nucleic acid in blood
* Patients must have adequate organ function, including the following laboratory values at the screening visit:
	+ Absolute neutrophil count (ANC) ≥1.0 x 10^9/L
	+ Platelets ≥ 75 x 10^9/L
	+ Hemoglobin (Hgb) ≥ 9.0 g/dL
	+ Calculated creatinine clearance ≥ 40 mL/min
	+ Total bilirubin < 1.5 x ULN
	+ Aspartate transaminase (AST) ≤ 3 x ULN, except for patients with liver metastases, who may only be included if AST ≤ 5 x ULN.
	+ Alanine transaminase (ALT) ≤ 3 x ULN, except for patients with liver metastases, who may only be included if AST ≤ 5 x ULN.

# Exclusion criteria

* Presence or history of interstitial lung disease or interstitial pneumonitis, including clinically significant radiation pneumonitis (i.e., affecting activities of daily living or requiring therapeutic intervention).
* Clinically significant, uncontrolled heart diseases.
	+ Unstable angina within 6 months prior to screening
	+ Myocardial infarction within 6 months prior to screening
	+ History of documented congestive heart failure (New York Heart Association functional classification III-IV)
	+ Uncontrolled hypertension defined by a Systolic Blood Pressure (SBP) ≥ 180 mm Hg and/or Diastolic Blood Pressure (DBP) ≥ 100 mm Hg, with or without anti- hypertensive medication. Initiation or adjustment of antihypertensive medication(s) is allowed prior to screening
	+ Any clinically important abnormalities in rhythm, conduction or morphology of resting ECG e.g. complete left bundle branch block, third degree heart block and second degree heart block not controlled by medication
	+ QTcF ≥ 470ms on the screening EKG. Patient has a history of prolonged QT syndrome or Torsades de pointes. Patient has a familial history of prolonged QT syndrome
* Patient received any anti-cancer therapy (including both systemic therapy and radiotherapy) within 14 days or 5 half-lives prior to the first dose of study drug, whichever is shorter. Prior osimertinib may be continued uninterrupted without a wash-out
* Patient is taking (and cannot cease) one of the prohibited concomitant medications listed below. If no specific wash-out period is listed in the table for a prohibited medication, the default wash-out period is 5 days or 5 half-lives, whichever is shorter
* Unable or unwilling to swallow tablets or capsules as per dosing schedule
* Patient has had a major surgical procedure within 14 days of the first dose of study drug (procedures such as central venous catheter placement, tumor needle biopsy, and feeding tube placement are not considered major surgical procedures).

**Treatment Schedule**

Treatment periods will be divided into 28-day treatment cycles. During the first cycle when the dose of BLU-667 is being escalated, there will be safety laboratory assessments every two weeks (plus an additional safety lab check on C1D8). Treatment will be continuous unless there is a delay. There will be a +/- 5-day visit window for assessments on D1 of each cycle. On Day 1 of all treatment cycles, the patient will first be assessed for continued treatment. These assessments will include:

* Physical exam to include vital signs, performance status
* Report of medications, both new and ongoing
* Review of any symptoms and health problems that have been experienced since starting the medication.
* Blood samples. Approximately 2 tablespoons for hematology and chemistries, including liver and kidney function tests.
* Review of drug diary and dispensation of new drug

Tumor assessments (using CT/MRI) will occur every 8-12 weeks from the first day of dosing until disease progression or drug discontinuation. Imaging studies should include sites of known or suspected disease and follow local institutional standards. All study treatments are outlined in the study calendar below.

**STUDY CALENDAR**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Protocol Activity** | **Screening (≤28 days)** | **Cycle 1****Day 1** | **Cycle 1 Day 8** | **Cycle 1****Day 15** | **Cycle 2****Day 1** | **Cycle 3+****Day 1** | **End of Treatment** |
| Informed consent | X |  |  |  |  |  |  |
| Registration |  | X |  |  |  |  |  |
| Tumor History | X |  |  |  |  |  |  |
| Medical History | X |  |  |  |  |  |  |
| Signs & Symptoms | X | X | X | X | X | X | X |
| Vitals, ECOG status | X | X | X | X | X | X | X |
| Physical Exam | X | X | X | X | X | X | X |
| Laboratory Studies | X | X | X | X | X | X | X |
| Adverse Events | X | X | X | X | X | X | X |
| Concomitant Medications | X | X | X | X | X | X | X |
| Tumor Assessment |  |  |  |  |  | X1 |  |

1Tumor assessment will occur between week 8 and 12 weeks after starting protocol therapy, and then every 8-12 weeks thereafter.