Osimertinib resistance landscape Le et al 2018

Supplementary Material and Methods:

Sequencing methods:

Molecular Diagnostic Laboratory test (MDL): MD Anderson MDL is a tissue molecular profiling method, which was completed using CLIA-certified next generation sequencing assays performed by our institution's Molecular Diagnostic Laboratory. For limited tissue sample, molecular testing was with a NGS based analysis for the detection of mutations in hotspot regions of 50 genes. In April 2016, an expanded NGS based analysis of 134 unique genes for the detection of somatic mutations in coding sequences of 128 genes and selected copy number variations (amplifications) in 49 genes.

Biodesix GeneStrat: Genestrat is a laboratory test service that determines the presence of somatic genetic variants in circulating nucleic acids (DNA and RNA) from the plasma of patients with cancer using ddPCR (droplet digital polymerase chain reaction) . In the ddPCR process, a patient sample is dispersed in an emulsion so that individual nucleic acid molecules are isolated. After amplification, nucleic acids are quantified by counting the emulsion that contains PCR end-product, or positive reactions. Detection of variants occurs relative to a background of at least 300 copies of wild type or a control gene. GeneStrat is a genomic approach to detection of insertion, deletions and point mutations, as well as fusion products.

Pyrosequencing: this EGFR mutation testing through pyrosequencing technology tests for targeting mutations on four EGFR exons: 18, 19, 20, and 21. Paraffin embedded tissue is sectioned and deparaffinized. The tumor area in the section is outlined by a board-certified pathologist. The tumor area is microdissected and used for DNA extraction. The sequences to be analyzed are amplified using PCR and then sequenced using pyrosequencing. The sequences to be analyzed

for each exon are as following: DSCTCCGGTGC for exon 18, codon 719, using a dispensing order of CATGTCACTCGTG; TATCAAGGAATTAAGAGAAGCAACATCTCCGAAAG for exon 19 deletion, using a dispensing order of CTATCACTGTCAGCTCGATCGTCATCGTCACGC; CAKCGTGG and ATCAYGCAG for exon 20, codon 786, 790, and insertion, using a dispensing order of GCAGTACGTGTCGTGTACGTGACCACACTG and GATCATCTG respectively; CKGGCCAAACDGCTGGGT for exon 21, codon 858, using a dispensing order of ACGTGTCACATGTC. The reagents and instrument are from Qiagen. The pyrosequencer is a Pyromark MD 96. The test is validated in the Molecular Diagnostic Laboratory at Moffitt Cancer Center, this assay demonstrated a sensitivity of 5%, meaning is there is 5% or more tumor cells in the specimen, it will be detected. This test covers over 95% of known mutations at EGFR with a specificity of 95% during validation against the results from a reference laboratory. The test performance characteristics were determined by the Molecular Diagnostic Laboratory at Moffitt Cancer Center. This type of test has not been approved by the US Food and Drug Administration. However, such approval is not required for clinical implementation, and test results have been shown to be clinically useful. This laboratory is CAP accredited and CLIA certified to perform high complexity testing.

Moffitt trusite: Moffitt trusite uses targeted next-generation sequencing to analyze coding regions of the most inclusive annotated RefSeq transcript for each of the targeted genes. Target enrichment was performed using amplicon based targeted capture using Trusight Tumor Panel (Illumina). Sequencing of enriched libraries was performed in multiplex on the Illumina MiSeq using the paired-end, 150 base-pair configuration. For single base-pair substitutions, this test has a sensitivity of 98.95% for variant allele frequencies of 3-5% and a sensitivity of 100% for variant allele frequencies >5%. Specificity and positive predictive value are 100% for substitutions with

a variant allele frequency >3%. Cutoff criteria were set such that a minimum variant allele frequency of 3% and a depth of 1000x were required to call single nucleotide variants.For insertions and deletions, an evaluation of this gene panel identified 22 of 22 insertions and deletions.Cutoff criteria were set such that a minimum variant allele frequency of 10% and a depth of 2000x were required to call insertions and deletions.Note that it is possible that pathogenic variants may not be reported by one or more of the tools because of the parameters used. However, tool parameters were optimized to maximize specificity and sensitivity.

Germline tests and definition of germline EGFR T790M mutation

In our cohort, germline tests were NOT performed directly for any of the genetic profiling platforms. For tissue samples using MD Anderson Molecular Diagnostic Laboratory (MDL) test and Foundation One, no germline control blood was required, therefore, the report only reflects the tumor sample genetic alterations. For liquid biopsies using Guardant360, the germline mutation is not directly tested, but inferred by allelic frequency. As Guardant Health state in their website, https://portal.guardanthealth.com/myguardant/resources, the germline mutations' allelic frequency is near 50%, whereas most of the somatic tumor mutations are below 0.5%. In our cohort, the germline T790M cases were inferred from very high allelic frequency (near 50%).

Cell lines, western blotting and MTS assay

Cell lines (H1975, HCC827, HCC4006, and Ba/F3) were maintained in 10% fetal bovine serum (FBS; Sigma) RPMI medium. Erlotinib, osimertinib and propranolol were obtained from the institutional pharmacy at the University of Texas MD Anderson Cancer Center. Norepinephrine

(Sigma-Aldrich). The rest of the compounds including topetinib were obtained from Selleck Chemicals.

The procedure for western blotting was previously described [Nillson et al 2017]. The following antibodies were used for Western blotting: pEGFR (Cell Signaling), EGFR (Santa Cruz Biotechnology Inc.), p-ERK (Cell Signaling), E-cadherin (Cell Signaling), Vimentin (Cell Signaling), c-Met (Cell Signaling), Vinculin (1:10,000; Sigma-Aldrich), and b-actin (1:10,000; Sigma-Aldrich).

Cells (2000 cells per well in 96-well plates) were treated with increasing concentrations of inhibitors. After 5 days, MTS (3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium, inner salt) assays were performed. To evaluate the effect of norepinephrine (NE) on EGFR TKI resistance, we seeded cells in 24-well plates (40,000 cells per well) and treated them with NE for 24 hours, and then, osimertinib was added to the culture medium. After 5 days, cell viability was measured by MTS assay. propanolol (1 mM) were added 1 hour before the addition of NE.

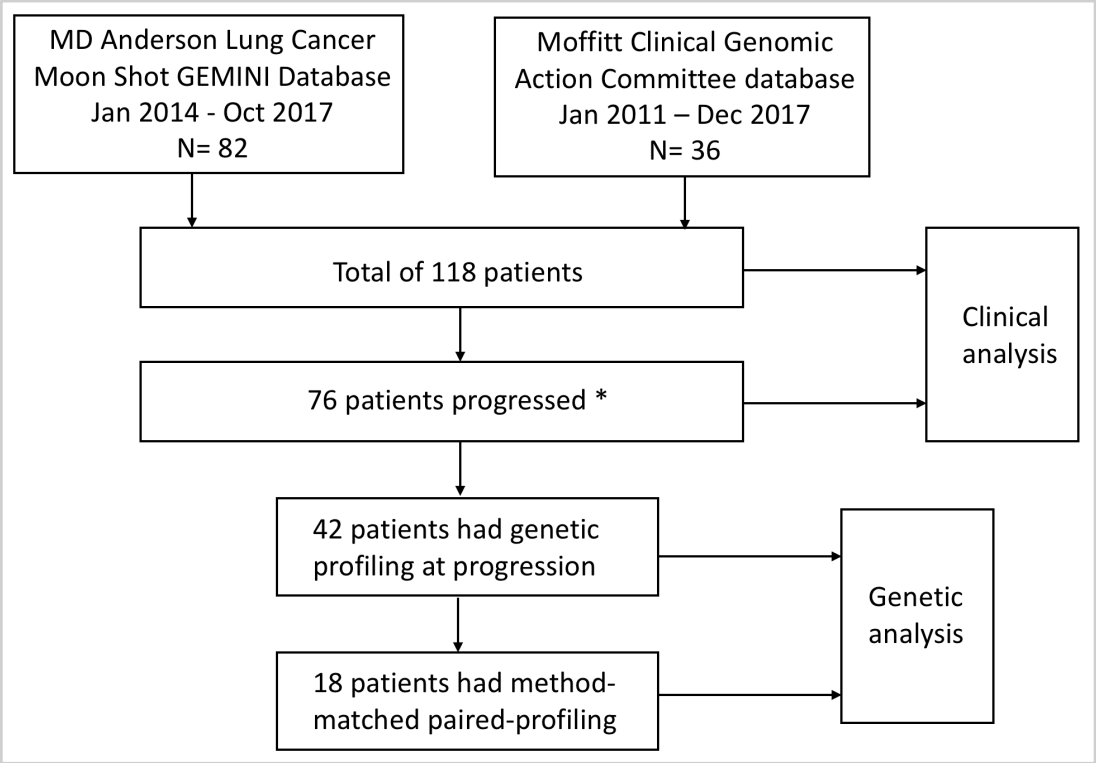
T790M, TP53, cell cycle gene status analysis

Each of the 42 cases with genetic profiling at progression on osimertinib was evaluated for T790M, TP53 and cell cycle gene status. T790M was classified as loss vs. preserved. TP53 was classied as mutant vs. wildtype. In cases where TP53 was present in the pre-treatment test but not in the post-treatment test, the status was deemed wildtype. In the cases where TP53 mutations were present both in the pre- and post-treatment tests, the status was deemed mutant. For cell cycle genes, CDK4, CDK6, CCND1 and CCNE1 amplifications, as well as CNKN2A loss were

used for cell cycle gene alteration analysis. Similar to TP53 status analysis, alterations were defined solely based on post-treatment status.

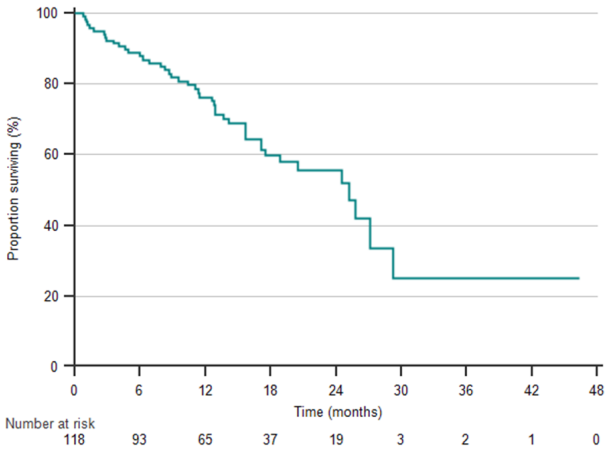
Supplementary Table and Figure Legends

Supple. Figure 1. Consort diagram for the retrospective cohort from MD Anderson Cancer Center and Moffit Cancer Center

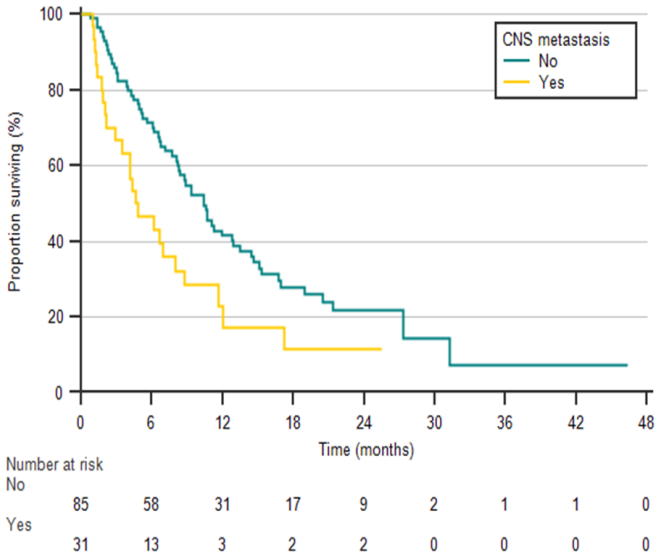


* cut-off MDA Feb 2018 and Moffitt Jan 2018

Supple. Figure 2. Kaplan-Meier estimate of median overall survival since starting of osimertinib (OS 1) was 25.2 months (95% CI, 17.5 to 29.2).

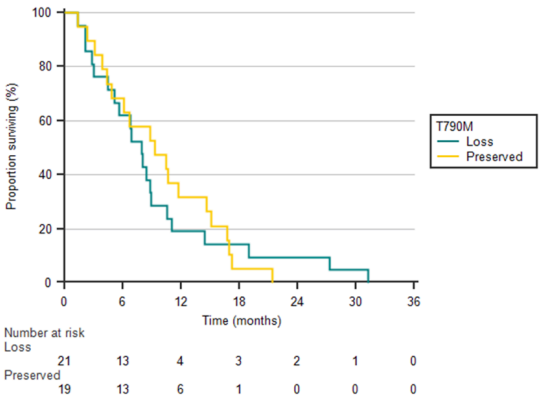


Supple. Figure 3. Kaplan-Meier estimate of PFS1 inpatients without and with CNS metastases 10.4 months versus 4.6 months (HR 1.9, 95% CI 1.2-3.0, log-rank p=0.01).



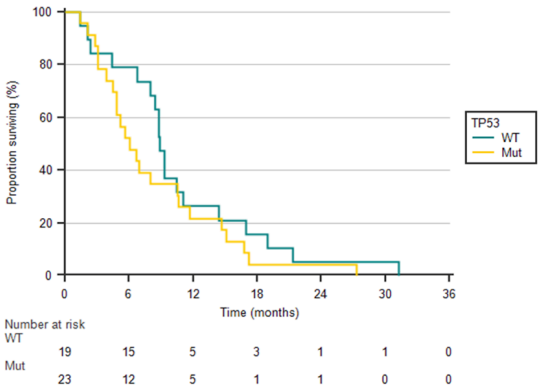
Supple Figure 4. Multivariate analysis of PFS1 based on T790M and TP53 alteration status. (A) Kaplan-Meier estimates of survival for T790M-preserved versus T790M-loss patients. Two germline T790M cases were excluded from this analysis. (B) Kaplan-Meier estimates of survival for TP53 mutated (MUT) versus wild-type (WT) patients.

A



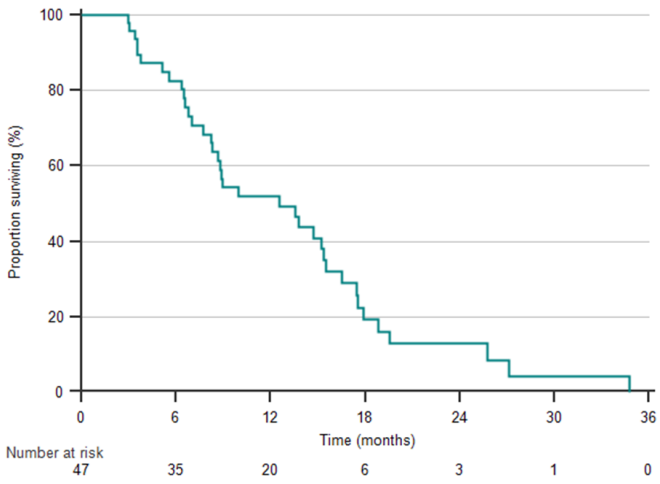
PFS1 T790M Preserved vs Loss 9.3 months vs 8.0 months (HR 0.9, 95% CI 0.5 – 1.8, log-rank p=0.84)

B

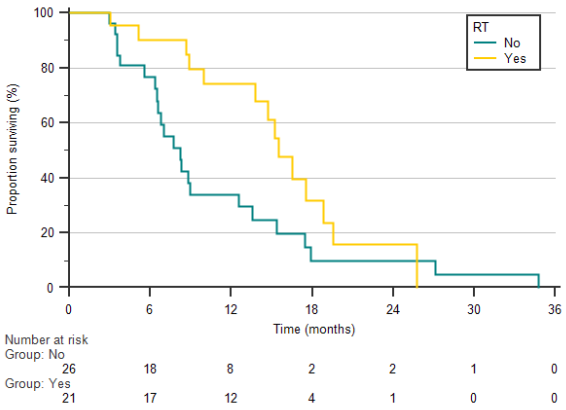


PFS1 TP53 WT vs Mut 9.3 months vs 8.0 months (HR 1.4, 95% CI 0.8 – 2.6, log-rank p=0.27)

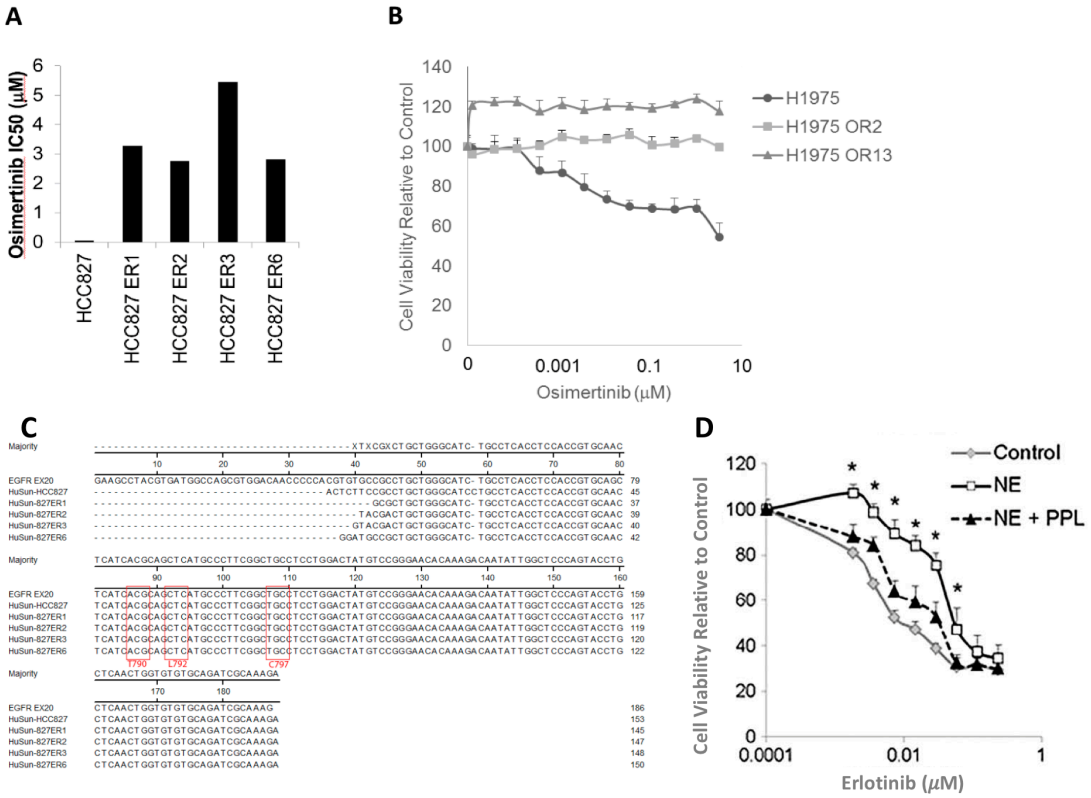
Supple. Figure 5. Kaplan-Meier estimates of PFS2 was estimated at 12.6 months (95% CI 8.3-15.5 months).



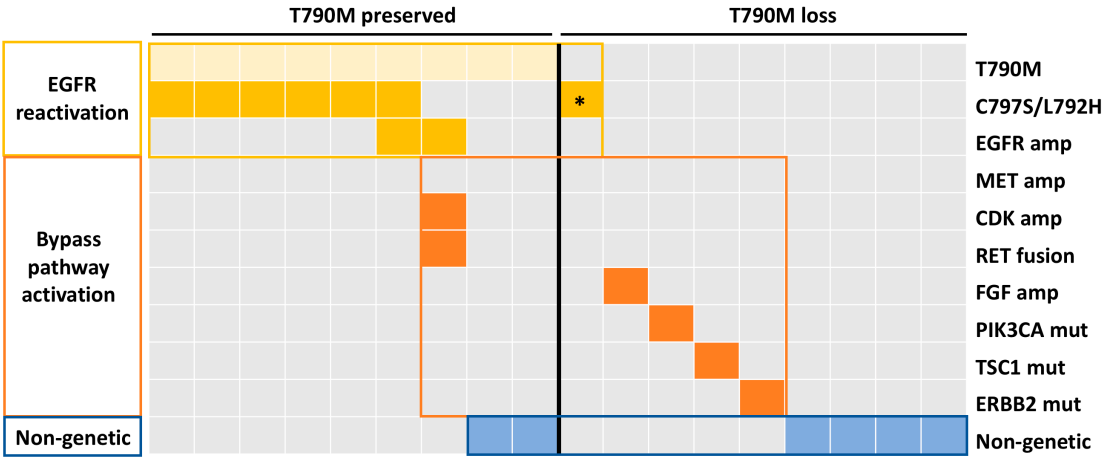
Supple. Figure 6. Kaplan-Meier estimates of PFS2 for patients who received radiation vs. not received radiation while continued on osimertinib after first progression. Radiation group vs. no radiation 15.5 vs 8.2 months; HR 0.5; 95% CI 0.3 to 1.0, log-rank *p*=0.05



Supple. Figure 7. (A) Erlotinib-resistant HCC827 cells (HCC827-ER) were also resistant to osimertinib. IC50 of four different erlotinib-resistant HCC827 cell clones were shown. All greater than 2uM. (B). H1975 OR2 and OR13 cells are resistant to osimertinib compared to H1975 parental cells. (C). Sequencing results for EGFR exon 20 region of HCC827, HCC827 ER1, ER2, ER3 and ER6 clones. (D). HCC827 cells were treated with propranolol (PPL) before norepinephrine (NE) stimulation (* indicates $p \leq 0.05$). After 24 hours, cells were treated with erlotinib for 5 days. Cell viability was evaluated by MTS assay (adapted from Nilsson et al 2017 Sci Transl Med (25)).



Supple. Fig. 8. Acquired resistance mechanisms in platform-matched 18 cases. Each column represents a patient case and each row represents a genetic alteration.



* EGFR V834L

Supple. Table 1. Radiation sites for patients who received local consolidative radiotherapy (LCT) while continuing on osimertinib.

Genetic ID	MDACC/MCC ID	Site of progression	Organs	Number of lesions radiated	Comments
	MDA1	Brain	Brain	One	GammKnife
3	MDA14	Right lower lobe	Lung	Two	
23	MDA18	L3 spine	Bone	One	
25	MDA24	Left iliac	Bone	One	
26	MDA26	Right hilum	Lymph nodes	One	
	MDA27	Left hilum	Lymph nodes	Two	
11	MDA28	Brain	Brain	>15	WBRT
	MDA41	Right lower lobe + right hilum + mediastinal lymph nodes	Lung + Lymph nodes	Three	
37	MDA42	Left pelvic bone	Bone	One	
31	MDA45	Right 4th rib + left iliac	Bone	Two	
36	MDA54	T1-T4 + T11-L1 spine	Bone	Two	
	MDA60	Right upper lobe	Lung	One	
9	MDA61	C6-7 spine + right upper lobe	Lung + Bone	Two	
6	MDA64	Right lower lobe + right hilum	Lung + Lymph nodes	Two	
	MDA67	Right upper lobe	Lung	One	
	MDA71	L1-L3 spine	Bone	One	
34	MDA74	Right upper lobe	Lung	One	
41	MCC27	Left acetabulum	Bone	One	
42	MCC56	Mediastinal lymph node (aortopulmonary window)	Lymph nodes	One	
39	MCC15	Left upper lobe	Lung	One	
15	MCC16	Right hepatic lobe lesion	Liver	One	

Supple. Table 2. Genetic profiling in 42 patient who progressed on osimertinib treatment

(please see as a separate file)

Supple. Table 3. Six patients received osimertinib as the first line treatment. All had T790M mutations at initial diagnosis with 4 cases were deemed to have germline EGFR T790M mutation.

ID	EGFR status	Platform	T790M Germline	Best response	Progressed	Acquired mutations
A25	G719A T790M	Guardant360	Yes (AF 52%)	Stable disease	Yes	None
A46	H773R T790M	Tissue MDL	Called as germline due to high AF	Stable disease	Yes	TP53 P83fs
A48	T790M de novo	Guardant360	Yes (AF 50%)	Response	No	N/A
A58	L858R and T790M	Tissue MDL	Called as germline due to high AF	Progression	Yes	Not tested
A02	Del19 T790M de novo	Tissue MDL	Uncertain	Response	No	N/A
A38	L858R T790M de novo	Tissue MDL	Uncertain	Progression	Yes	Not tested

Supple. Table 4. The genetic tests' timing relationship to clinical progressions (PFS1 and PFS2) for the 42 patients with pre- and post-osimertinib genetic data.

Genetic ID	Pre-osimertinib tests		Clinical dates			Post-osimertinib tests	
	Platform	Date Performed	Date osi start	Date PFS1	Date PFS2	Platform	Date Performed
1	MDL	10/2015	4/2016	6/2016	NA	GH	6/2016
2	clinical note		09/2014	12/2015	NA	MDL	10/2016
						GH	9/2016
3	MDL	9/2014	11/2015	09/2016	06/2017	MDL	10/2016
4	PCR	9/2015	10/2016	01/2017	04/2017	GH	4/2017
	Foundation	9/2016				Foundation	5/2017
5	GH	3/2016	05/2016	06/2016	08/2016	GH	8/2016
6	MDL	5/2012	09/2014	01/2016	04/2016	MDL	1/2016
7	MDL	2/2012	10/2015	07/2017	01/2018	GH	7/2017
	GH	8/2015					
8	MDL	11/2012	07/2015	09/2016	12/2016	GH	11/2016
	MDL	5/2014					
9	clinical note		05/2016	10/2016	07/2017	GH	9/2017
						Foundation	9/2017
10	MDL	4/2014	12/2015	08/2016	NA	MDL	8/2016
	MDL	12/2015					
11	MDL	11/2014	06/2016	03/2017	09/2017	MDL	4/2017
	MDL	5/2016					
GM20	PCR	2/2016	01/2016	06/2016	NA	MDL	6/2017
GM21	MDL	10/2014	01/2017	10/2017	NA	GH	10/2017
22	MDL	07/2013	06/2016	05/2017	NA	MDL	5/2017
	PCR	01/2015					
	GH	5/2016					
23	GH	8/2016	01/2017	03/2017	10/2017	GH	10/2017
	MDL	10/2016					
24	clinical note		01/2015	08/2017	NA	GH	1/2017
25	MDL	9/2014	06/2016	02/2017	09/2017	GH	9/2017
	MDL	5/2016					
26	MDL	12/2014	08/2016	11/2016	05/2017	GH	6/2017
	MDL	7/2016					
27	GH	01/2016	02/2016	12/2016	NA	MDL	1/2017
28	GH	11/2015	02/2017	08/2017	10/2017	GH	8/2017

	MDL	12/2015					
	GH	11/2016					
	MDL	12/2016					
29	MDL	5/2016	07/2016	01/2017	07/2017	GH	1/2017
						GH	7/2017
30	Guardant	01/2016	03/2016	05/2017	NA	MDL	5/2017
31	MDL	12/2015	05/2017	08/2017	10/2017	MDL	8/2017
	PCR	4/2017					
32	Foundation	8/2016	12/2016	09/2017	NA	Foundation	9/2017
33	MDL	02/2015	08/2016	01/2017	NA	MDL	1/2017
34	MDL	3/2014	12/2015	06/2017	01/2018	MDL	7/2017
35	MDL	12/2014	06/2016	10/13/2016	12/2016	GH	12/2016
	GH	4/2016				MDL	1/2017
36	GH	9/2016	07/2017	09/2017	10/2017	MDL	9/2017
	PCR	7/2017					
37	GH	1/2017	01/2017	10/2017	NA	GH	10/2017
38	clinical note	7/1905	09/2014	12/2016	07/2017	GH	12/2016
39	GH	12/15	1/16	9/2016	5/2017	GH	9/2016
40	Biodesix	10/16	12/16	6/17	8/17	GH	7/17
41	GH	1/17	2/17	11/17	NA	GH	11/17
42	Foundation	8/17	9/17	11/17	NA	GH	11/17
12	Pyrosequencing	9/13	6/15	11/16	NA	Foundation	11/16
13	GH	2/16	2/16	12/16	NA	Biodesix	12/16
14	Moffitt Trusite	9/15	11/15	3/17	NA	Foundation	3/17
15	GH	3/16	5/16	11/16	8/17	Guardant	9/16 and 7/17
16	Pyrosequencing	3/16	3/16	2/17	4/17	Foundation	4/17
17	GH	7/16	8/16	2/17	NA	Foundation ACT	1/17
18	GH	8/15	8/15	12/15	NA	GH	12/15
19	GH	3/16	5/16	9/16	NA	GH	9/16

Supple. Table 5. Tertiary mutations in EGFR gene

Pt ID	Sensitizing mut	T790M	Tertiary mut	Other EGFR mut	CADD score
2	del19	preserved	L792H		30 (L792H)
3	del19	preserved	C797S		24.7 (C797S)
6	del19	preserved	C797S		
7	del19	preserved	C797S		
9	L858R	preserved	C797S	H870R [a]	23 (H870R)
10	L858R	preserved	L792H	V843I [b]	28.1 (V843I)
11	del19	preserved	C797S		
12	del19	preserved	C797S		
14	del19	preserved	C797S		
16	L858R	preserved	C620W [a]	Rearrangement [a]	26.7 (C620W)
17	del19	preserved	C797G		27.9 (C797G)
18	del19	preserved	C797S		
20		germline T790M	H773R [b]		23.3
21		germline T790M	G719S [b]		26.3
26	del19	lost	P596L [a]		27.1
29	L858R	lost	L718Q [a]		25.8
32	del19	lost	G724S [a]		28.5
34	L858R	lost	V834L [a]		33

a indicates acquired mutation; b indicates pre-existing mutation prior to starting osimertinib.

Supple. Table 6. HCC827 parental and HCC827 ER2 cells' sensitivity to multiple small molecule inhibitors.

Drug (IC50 uM)	HCC827	HCC827 ER2
Erlotinib	0.02	>10
Osimertinib	0.02	13.6
Afatinib	0.01	8.99
Crizotinib (MET inhibitor)	>10	0
Trametinib (MEK inhibitor)	0.35	0.000039
Abemaciclib (CDK4/6 inhibitor)	1.55	0.82
Palbociclib (CDK4/6 inhibitor)	4.08	15
Dinaciclib (pan-CDK inhibitor)	0.008	0.41
Dabrafenib (BRAF inhibitor)	>10	>10
Vemurafenib (BRAF inhibitor)	>10	>10

Supple Table 7. Platform-matched pre- and post-osimertinib genetic profiling in 18 patients.

(please see as a separate file)