

Supplemental Online Content

Lee KWC, Li MSC, Gai W, et al. Testing for *EGFR* variants in pleural and pericardial effusion cell-free DNA in patients with non–small cell lung cancer. *JAMA Oncol*. Published online December 29, 2022. doi:10.1001/jamaoncol.2022.6109

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eMethods

This supplemental material has been provided by the authors to give readers additional information about their work.

Supplementary Appendix

eTable 1. Demographic and Clinical Characteristic of the Patients		
Characteristics	Cohort 1, TKI-naïve (N=104)	Cohort 2, TKI-resistant (N=67)
Sex - no (%)		
Male	48 (46)	30 (45)
Female	56 (54)	37 (55)
Median age at diagnosis, years (range)	68 (36-91)	65 (47-91)
Ethnicity (%)		
Chinese	104 (100)	67 (100)
Smoking status (%)		
Never smoker	48 (46)	52 (78)
Ex- or current smoker	56 (54)	15 (22)
Stage, at recruitment (%)		
Stage IIIB/C	2 (2)	0
Stage IV		
M1a (intrathoracic)	36 (35)	12 (18)
M1b or M1c (extrathoracic)	54 (52)	35 (52)
Uncertain (stage IV)	12 (11)	20 (30)
PS, at recruitment (%)		
0-1	62 (60)	45 (67)
2	24 (23)	11 (16.5)
3-4	18 (17)	11 (16.5)
Site of drainage (%)		
Pleural effusion	99 (95)	66 (98)
Pericardial effusion	5 (5)	1 (2)
Tissue specimen (%)		
Pleural/pericardial fluid cytology cell block	67 (64)	54 (81)
Pleural biopsy	1 (1)	1 (1)
Lung biopsy	29 (28)	3 (5)
LN biopsy	4 (4)	0
Archival specimen	2 (2)	0
Others ^a	1 (1)	0
Tumour pathology data missing	0	9 (13)
Histologic subtype (%)		
Adenocarcinoma	90 (86)	66 (98)
Squamous cell carcinoma	4 (4)	0
Adenosquamous carcinoma	1 (1)	0
NSCLC not otherwise specified	9 (9)	1 (2)
Primary EGFR mutation (%)		
Exon19del	11 (11)	31 (46)
Exon 21 L858R mutation	27 (26)	35 (52)
Others ^b		1 (2)

Footnote a: one patient received a diagnosis of metastatic lung cancer from a colon biopsy

Footnote b: one patient had concurrent EGFR exon 18 G719X plus exon 20 S768I mutation and progressed on 2nd generation EGFR TKI

Abbreviations: TKI, tyrosine kinase inhibitor; PS, performance status; LN, lymph node; NSCLC, non-small cell lung cancer

eTable 2. Plasma-cfDNA ddPCR Assay Specificity, Sensitivity, PPV, NPV, and Concordance Compared With Tumor Tissue			
Sensitizing mutation (n=102)			
	Plasma+	Plasma-	
Tissue+	28	10	Sens=74% (95% CI 60-88)
Tissue-	2	62	Spec=97% (95% CI 93-100)
	PPV=93% (95% CI 84-100)	NPV=86% (95% CI 78-94)	Conc=88% ($\kappa=0.74$, $P<0.01$)
Exon19 deletion (n=102)			
	Plasma+	Plasma-	
Tissue+	6	5	Sens=55% (95% CI 25-84)
Tissue-	0	91	Spec=100% (95% CI 100-100)
	PPV=100% (95% CI 100-100)	NPV=95% (95% CI 90-99)	Conc=95% ($\kappa=0.68$, $P<0.01$)
Exon21 L858R (n=102)			
	Plasma+	Plasma-	
Tissue+	22	5	Sens=82% (95% CI 67-96)
Tissue-	2	73	Spec=97% (95% CI 94-100)
	PPV=92% (95% CI 81-100)	NPV=94% (95% CI 88-99)	Conc=93% ($\kappa=0.82$, $P<0.01$)
Exon20 T790M (n=39)			
	Plasma+	Plasma-	
Tissue+	7	7	Sens=50% (95% CI 24-76)
Tissue-	7	18	Spec=72% (95% CI 54-90)
	PPV=50% (95% CI 24-76)	NPV=72% (95% CI 54-90)	Conc=64% ($\kappa=0.22$, $P=0.17$)

eTable 3. Comparison of EGFR Detection by Plasma-cfDNA vs PE-cfDNA			
Sensitizing mutation	Plasma+	Plasma-	(n=104)
PE+	31	9	Concordance=91% ($\kappa=0.81$, $P<0.01$)
PE-	0	64	
Exon19del	Plasma+	Plasma-	(n=104)
PE+	6	4	Concordance=96% ($\kappa=0.73$, $P<0.01$)
PE-	0	94	
Exon21 L858R	Plasma+	Plasma-	(n=104)
PE+	25	5	Concordance=95% ($\kappa=0.88$, $P<0.01$)
PE-	0	74	
Exon20 T790M	Plasma+	Plasma-	(n=66)
PE+	20	14	Concordance=74% ($\kappa=0.49$, $P<0.01$).
PE-	3	29	

Abbreviations: EGFR, epidermal growth factor receptor; cfDNA, cell-free DNA; PE, pleural or pericardial effusion

eTable 4. Summary of Patients With Discrepant *EGFR* Testing Results in PE/Tissue in Cohort 1

Pt	Tumour Driver	Tumour T790M	PE		Plasma		TKI	TTD (months)
			Driver VAF	T790M VAF	Driver VAF	T790M VAF		
PW024 (pleural biopsy)	WT	WT	2.0% (L858R)	ND	0.3% (L858R)	ND	N	
PW121 (bronchial biopsy)	WT	WT	16.6% (L858R)	0.08%	0.6% (L858R)	ND	Osim	1.5
PW008	19del	WT	25.1%	2.2%	28.7%	ND	Y	12.3
PW028	L858R	WT	6.7%	2.8%	36.8%	ND	N	
PW060	WT	WT	ND	12.6%	ND	ND	N	
PW090	WT	WT	ND	0.06%	ND	0.5%	N	
PW102	L858R	WT	63.7%	0.15%	3.3%	0.4%	Y	1.1
PW147	19del	WT	6.4%	0.08%	16.8%	ND	Y	1.4 ^a
PY008	WT	WT	ND	0.06%	ND	ND	N	
PW038	19del	WT	ND	ND	ND	ND	N	

Footnote a: Pt PW147 experienced primary resistance to 1st gen TKI and switched to osimertinib 4 months ago with clinical response. He was continued on treatment till the day of data cutoff.

Abbreviations: EGFR, epidermal growth factor receptor; Pt, patient; Driver, driver mutation; PE, pleural or pericardial effusion; 19del, exon 19 deletion; L858R, exon 21 L858R mutation; WT, wild-type; M, mutant; ND, not detected; VAF, variant allele frequency; TKI, tyrosine kinase inhibitor; 1st or 2nd generation TKI; N, no; Y, yes; Osim, osimertinib; TTD, time-to-treatment discontinuation

eTable 5. Summary of Patients With Discrepant T790M Testing Results in Cohort 2

Pt	Tissue		PE		Plasma		Osim	TTD (m)
	Driver	T790M	Driver VAF	T790M VAF	Driver VAF	T790M VAF		
PW002	19del	WT	14.5%	2.5%	20.9%	8.9%	N	
PW023	19del	WT	16.8%	ND	4.0%	3.0%	Y	13.2
PW040 (pleural biopsy)	19del	WT	40.8%	11.5%	ND	ND	N	
PW064	L858R	M	29.5%	ND	ND	ND	Y	18.0
PW071 (lung biopsy)	L858R	WT	17.3%	1.7%	20.9%	3.4%	N	
PW085	19del	WT	95.2%	0.1%	84.9%	13.6%	Y	7.9
PW118	19del	WT	45.7%	4.5%	26.1%	0.2%	Y	1.1
PW119	L858R	WT	4.3%	0.6%	0.9%	ND	Y	11.3
PW123	L858R	WT	26.3%	0.8%	0.4%	ND	N	
PW129	L858R	M	21.0%	16.9%	ND	ND	Y	9.4
PW132	L858R	WT	21.9%	3.3%	ND	ND	Y	6.1 ^a
PW140	L858R	M	46.5%	6.3%	ND	ND	Y	9.4 ^a
PW160	L858R	WT	33.9%	0.3%	4.9%	1.6%	N	
PW167	L858R	M	87.2%	79.2%	ND	ND	Y	5.5 ^a
PW168	L858R	WT	48.5%	2.4%	22.8%	4.0%	N	
PY006	L858R	M	33.7%	2.9%	0.5%	ND	Y	1.1
PY007	L858R	M	41.8%	9.8%	0.7%	ND	Y	8.0
PY010 (lung biopsy)	19del	M	ND	ND	ND	ND	Y	23.7

Footnote a: Patients still continuing treatment at the time of data analysis.

Abbreviations: Pt, patient; Driver, driver mutation; PE, pleural or pericardial effusion; VAF, variant allele frequency; Osim, osimertinib; N, no; Y, yes; TTD, time-to-treatment discontinuation; 19del, exon 19 deletion; L858R, exon 21 L858R mutation; WT, wild-type; M, mutant; ND, not detected; CN, cytology negative; QI, cytology positive but inadequate cellularity for molecular testing

eTable 6. Summary of Patients With Cytology-Negative PE/Cytology-Positive PE but Inadequate Cellularity for *EGFR* Testing in Cohort 2

Pt	Tissue		PE		Plasma		Osim	TTD (m)
	Driver	T790M	Driver VAF	T790M VAF	Driver VAF	T790M VAF		
PW004	19del ^a	CN	ND	ND	56.4%	3.3%	Y	25.8
PW006	others ^b	CN	ND	0.94%	ND	0.1%	Y	0.4
PW019	L858R ^a	QI	4.4%	2.6%	ND	ND	N	
PW037	19del ^a	CN	2.7%	1.5%	34.6%	44.2%	Y	2.5
PW091	19del ^a	CN	5.9%	0.1%	ND	0.8%	Y	0.9
PW105	L858R ^a	QI	19.7%	13.4%	ND	ND	Y	17.8 ^c
PW111	19del ^a	QI	24.9%	12.5%	2.4%	0.8%	Y	9.2
PW130	19del ^a	QI	49.7%	24.4%	4.5%	2.2%	Y	0.4
PW146	L858R ^a	CN	3.0%	ND	65.2%	0.2%	N	

Footnote a: Driver mutation detected from treatment-naive specimen

Footnote b: Patient PW006 had *EGFR* G719X mutation

Footnote c: Patient receiving ongoing treatment at time of data analysis

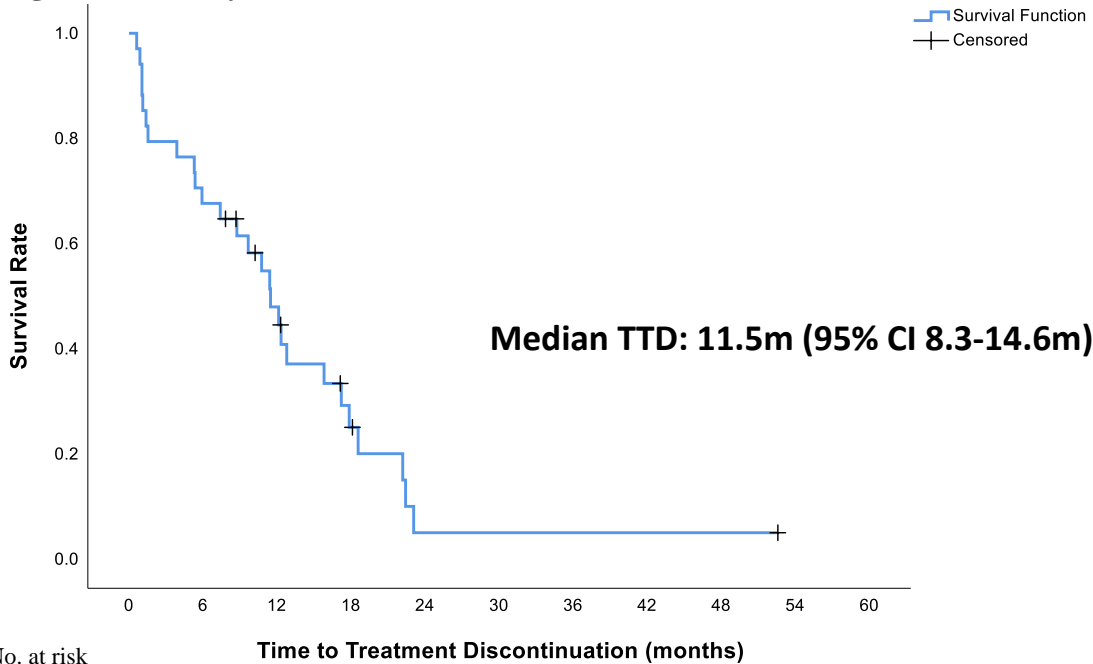
Abbreviations: Pt, patient; Driver, driver mutation; PE, pleural or pericardial effusion; VAF, variant allele frequency; Osim, osimertinib; N, no; Y, yes; TTD, time-to-treatment discontinuation; 19del, exon 19 deletion; L858R, exon 21 L858R mutation; ND, not detected; CN, cytology negative; QI, cytology positive but inadequate cellularity for *EGFR* testing

eTable 7. Summary of Patients With Pericardial Effusion

Pt	Cohort	Driver Mutation			T790M		
		Tissue	PE VAF	Plasma VAF	Tissue	PE VAF	Plasma VAF
PW039	1	WT	ND	ND	WT	ND	ND
PW048	1	L858R	0.85%	2.6%	WT	ND	ND
PW058	1	L858R	68.64%	ND	WT	ND	ND
PW104	1	WT	ND	ND	WT	ND	ND
PW113	1	L858R	20.34%	0.5%	WT	ND	ND
PW089	2	L858R	13.21%	0.5%	M	7.35%	0.63%

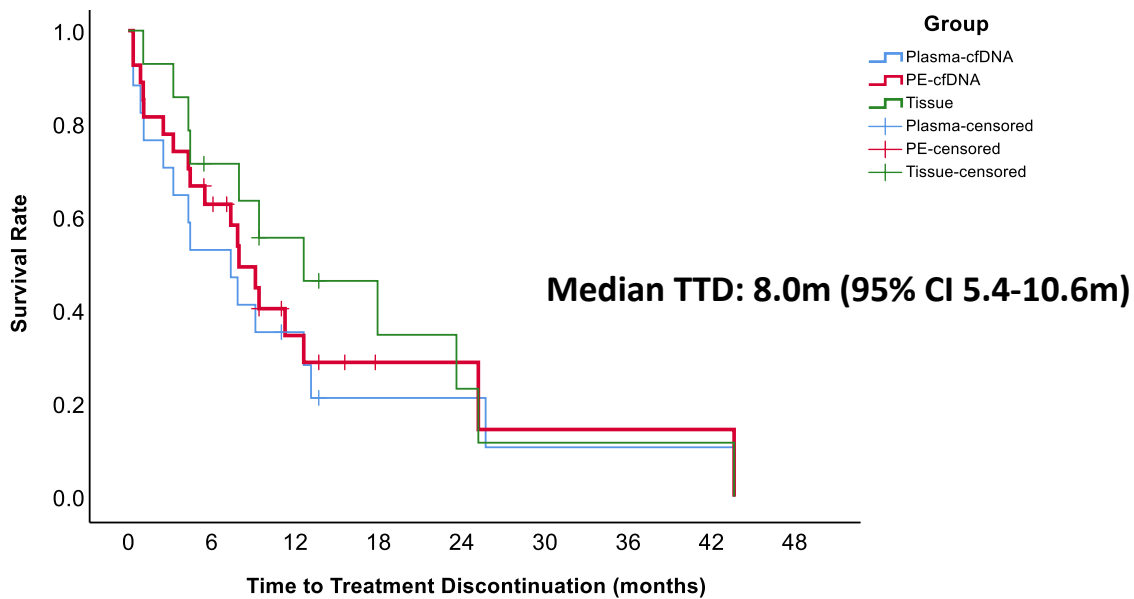
Abbreviations: Pt, patient; Driver, driver mutation; PE, pericardial effusion; L858R, exon 21 L858R mutation; WT, wild-type; M, mutant; ND, not detected; VAF, variant allele frequency;

eFigure 1. Efficacy of EGFR-TKI in *EGFR* Alteration Detected in PE-cfDNA



No. at risk
PE-cfDNA: 34 23 14 6 1 1 1 1 1

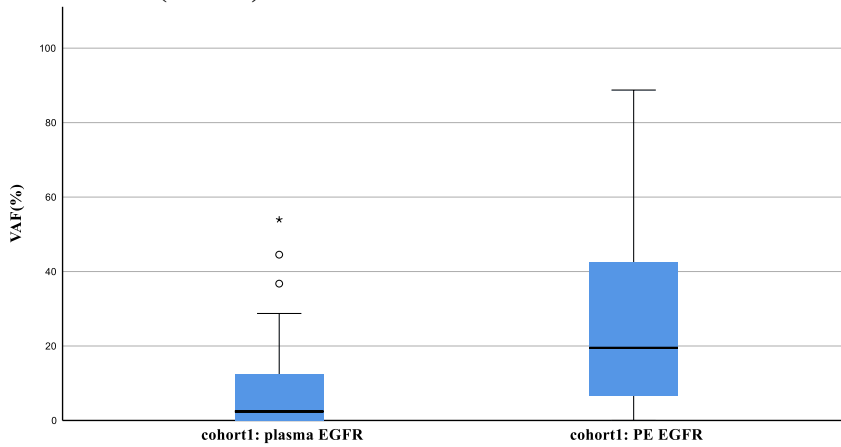
eFigure 2. Efficacy of Osimertinib in Patients with T790M Alteration Detected in PE-cfDNA, Plasma cfDNA and Tissue



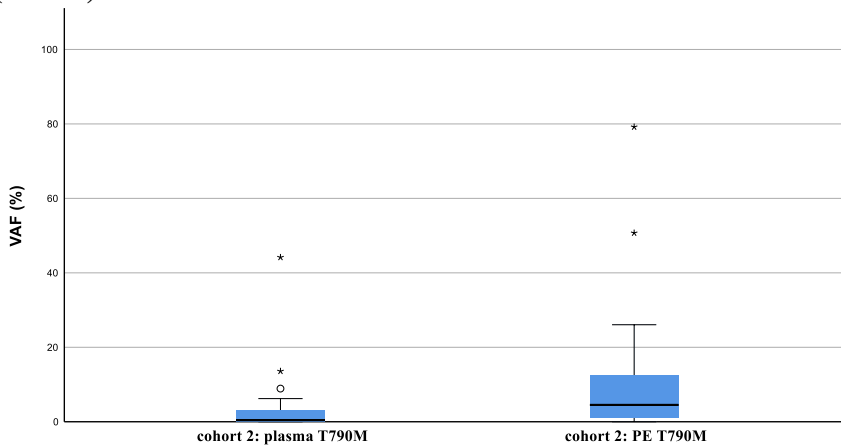
No. at risk
PE-cfDNA: 27 16 6 2 2 1 1 1
Plasma: 17 9 5 2 2 1 1 1
Tissue: 14 9 6 3 2 1 1 1

eFigure 2 denotes time-to-treatment discontinuation (TTD) of EGFR TKI in patients with sensitizing EGFR mutation detected in PE-cfDNA (n=34). 32 patients received first- or second- generation EGFR TKI while 2 patients received osimertinib. Figure 1(b) denotes TTD of osimertinib in patients with T790M mutation detected in PE-cfDNA (n=27), plasma (n=17) and tissue (n=14) respectively. The median TTD of patients with positive tumour and plasma T790M treated with osimertinib were 12.6 months (95% CI 1.3-24.0 months) and 7.4 months (95% CI 2.6-12.2 months) respectively. The difference in TTD between patients with different T790M positive samples were not statistically significant (tissue, p=0.47; plasma, p=0.61).

eFigure 3. Summary of VAF of Sensitizing *EGFR* Alteration Detected in PE-cfDNA vs Plasma in Cohort 1 (n = 38)



eFigure 4. Summary of VAF of T790M Alteration Detected in PD-cfDNA vs Plasma in Cohort 2 (n = 37)



In cohort 1, only patients with *EGFR* mutated tumour were included in this analysis; in cohort 2, only patients with either T790M positive plasma or PE-cfDNA were included in this analysis.

Abbreviations: VAF, variant allele frequency; PE, pleural or pericardial effusion; cfDNA, cell-free DNA

eMethods

Full study design

Between September 2016 and January 2021, patients with histologically confirmed non-small cell lung cancer (NSCLC) who presented with exudative or cytologically-proven malignant pleural or pericardial effusion (collectively referred hereinafter as PE) were eligible and prospectively enrolled at 2 major cancer centres (Prince of Wales Hospital (PWH), Pamela Youde Nethersole Eastern Hospital (PYNEH)) in Hong Kong.

Sample Collection, Delivery and Handling

10ml fresh pleural or pericardial fluid was collected after thoracentesis or pericardiocentesis was performed. For patients awaiting histological confirmation of a lung cancer diagnosis, PE samples collected in PWH were first sent to laboratory for centrifugation at $1,600 \times g$ (units of times gravity) for 10 minutes for cell removal, then frozen inside Eppendorf tubes at -80°C . When the diagnosis of NSCLC was confirmed, patients were approached for informed consent and blood sample collection. 10ml of venous blood was collected and sent to laboratory on the same day. Blood samples were centrifuged to remove cells at $1,600 \times g$ for 10 minutes, and then transferred to Eppendorf tubes for microcentrifugation at $16,000 \times g$ for 10 minutes. For patients with a known diagnosis of lung cancer during fluid collection, blood and PE samples were collected within the same working day for centrifugation. All the centrifuged samples were frozen at -80°C whilst awaiting testing.

In PYNEH, the logistic was identical except that unprocessed PE samples were either kept in plain bottles at refrigerated temperature ($2-8^{\circ}\text{C}$)¹ or in Streck tubes kept between $6-37^{\circ}\text{C}$ for up to one week before processing till the diagnosis of lung cancer was confirmed.

In both cohorts, 20ml PE samples were sent to the pathology lab of the corresponding hospital for cytological analysis. Epidermal growth factor receptor (EGFR) PCR testing was performed as per the treating physician's order if cytology revealed metastatic NSCLC. If cytology was negative or inadequate for molecular testing, other tissue specimens including cytological samples from repeat thoracentesis/pericardiocentesis, tissue biopsies, or archival specimens (for cohort 1) could be used as reference for tissue genotyping.

EGFR mutation testing by reference standard

Tumour cells were isolated from Formalin-fixed, paraffin-embedded (FFPE) tissue by microdissection. EGFR testing on tumour cells was carried out by the pathology lab of the corresponding cancer centre by cobas® EGFR Mutation Test v2 or the "Scorpion-ARMS" theascreen® EGFR Rotor-Gene Q (RGQ) PCR Kit as per local protocol. Both the cobas® EGFR Mutation Test v2 and the theascreen® EGFR Rotor-Gene Q (RGQ) PCR are FDA-approved companion diagnostics tests and are used in the participating cancer centres as diagnostic tools for EGFR mutations in NSCLC.

EGFR mutation testing by ddPCR

DNA was extracted from 1.6 mL PE and plasma samples with QIAamp DSP DNA Blood MiniKit (Qiagen) and eluted with $50\mu\text{L}$ H₂O. Each sample was tested in duplicate using the QX100/QX200 Droplet Digital™ PCR System (BioRad). The PCR mix for each reaction was set up by mixing $8\mu\text{L}$ of DNA sample, $10\mu\text{L}$ of ddPCR Supermix for Probes, $0.5\mu\text{L}$ of Uracil-DNA glycosylase, and the primer and probe mix in a reaction volume of $20\mu\text{L}$. The reaction mix was then subjected to a BioRad QX100/QX200 Droplet Generator for droplets generation according to the manufacturer's instructions. The droplets were transferred into a 96-well plate followed by thermal cycling on C1000 Touch Thermal Cycler (BioRad). After the PCR, the droplets readings were carried out by a BioRad QX100/QX200 reader and the results were analyzed using the QuantaSoft (version 1.7) software. The results of ddPCR test of PE-cfDNA and plasma-cfDNA were then compared with the conventional PCR results of their matched tissue specimens.

Treatment Analysis

Treatment efficacy was analyzed in patients who received EGFR tyrosine kinase inhibitor (TKI) in cohort 1, and in those who received osimertinib in cohort 2. Patients who were treated with cytotoxic chemotherapy, or started on TKI on an empirical basis (ie without proven activating EGFR mutation in cohort 1 or T790M mutation in cohort 2) were excluded from efficacy analysis. Treatment efficacy was assessed by time-to-treatment discontinuation (TTD), counted as the period from initiation of TKI treatment till permanent cessation. Survival data collection cut-off date was 19th July 2021. Kaplan-Meier method was used to summarize survival outcomes.

Reference

1. Boyanton BL, Crisan D. Sample Collection, Processing, and Storage for Molecular Genetic Testing. *Laboratory Hematology Practice*. Published online August 8, 2012:143-154. doi:10.1002/9781444398595.CH13

Eligibility Criteria

Inclusion Criteria

1. Age 18 years or older at time of informed consent.
2. Histologically confirmed metastatic NSCLC and exudative pleural effusion or cytologically-proven malignant pericardial effusion either prior to treatment with EGFR TKI or upon development of resistance to EGFR TKI.
3. Informed consent.

Exclusion Criteria

1. Malignancy other than NSCLC diagnosed within 5 years from diagnosis of NSCLC.
2. Other active malignancies.
3. Pregnancy.
4. Patients with any EGFR mutations on FFPE testing that contain only uncommon EGFR mutations (ie mutations other than EGFR exon 19 in-frame deletions, exon 21 L858R mutation, and exon 20 T790M mutation) are ineligible for cohort 1. Patients with compound mutations containing any of the above-mentioned common EGFR mutations will be included for analysis.
5. Prior exposure to third-generation EGFR TKI.
6. Patients with biopsies containing insufficient tissue for EGFR testing are ineligible for cohort 1.
7. Patients with bacterial culture-positive exudative pleural effusions.