Supplementary

Table S1 Patient characteristics of 18 cases harboring *EGFR* major mutation, whose blood sample collected before treatment with afatinib

Characteristics	Ex19del (n=10)	L858R (n=8)	P value
Age (years)	66.5 [45–77]	69.0 [39–77]	0.56
Gender			0.15
Male	8 (80.0)	3 (37.5)	
Female	2 (20.0)	5 (62.5)	
Tumor histology			>0.99
Adenocarcinoma	9 (90.0)	7 (87.5)	
Other	1 (10.0)	1 (12.5)	
ECOG PS			0.73
0	1 (10.0)	1 (12.5)	
1	7 (70.0)	7 (87.5)	
2	2 (20.0)	0 (0.0)	
Smoking			>0.99
Current or former	5 (50.0)	4 (50.0)	
Never	5 (50.0)	4 (50.0)	
Brain metastases			0.05
Yes	8 (80.0)	2 (25.0)	
No	2 (20.0)	6 (75.0)	
Liver metastases			0.48
Yes	2 (20.0)	0 (0.0)	
No	8 (80.0)	8 (100.0)	
Bone metastases			>0.99
Yes	6 (60.0)	4 (50.0)	
No	4 (40.0)	4 (50.0)	

Data are presented as median [range] or n (%). *EGFR*, epidermal growth factor receptor; Ex19del, exon 19 deletion; ECOG PS, Eastern Cooperative Oncology Group performance status.

https://dx.doi.org/10.21037/tlcr-24-471

Table S2 HR and P value calculated from PFS for each genetic mutation in 22 cases whose plasma samples were collected before treatment with afatinib

Gene	N	PFS (months)	Univariate analysis		
			HR	95% CI	P
KMT2D					
Negative	14	7.57	1		
Positive	8	12.9	0.71	0.29-1.75	0.46
LRP1B					
Negative	14	7.68	1		
Positive	8	13.3	0.77	0.31-1.88	0.56
NF1					
Negative	14	9.12	1		
Positive	8	9.30	0.97	0.40-2.36	0.94
NOTCH1					
Negative	16	7.68	1		
Positive	6	16.0	0.51	0.19-1.40	0.19
SETD2					
Negative	16	11.5	1		
Positive	6	5.38	1.13	0.43-2.97	0.80
TP53					
Negative	16	11.5	1		
Positive	6	7.68	1.07	0.41-2.81	0.89
ADGRB3					
Negative	17	7.97	1		
Positive	5	12.8	0.99	0.36-2.74	0.98
APC					
Negative	17	10.3	1		
Positive	5	5.83	0.97	0.35-2.70	0.95
ATM					
Negative	17	7.40	1		
Positive	5	15.3	0.66	0.34-1.87	0.44
NTRK1					
Negative	17	7.97	1		
Positive	5	12.8	0.68	0.24-1.90	0.46
RBM10					
Negative	17	7.97	1		
Positive	5	12.8	0.65	0.23-1.84	0.42

Table S2 (*continued*)

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Table S2 (*continued*)

Gene	N	PFS (months)	Univariate analysis		
			HR	95% CI	P
AKT1					
Negative	18	7.28	1		
Positive	4	16.2	0.35	0.11-1.10	0.07
ALK					
Negative	18	8.83	1		
Positive	4	12.5	0.57	0.18-1.75	0.32
CREBBP					
Negative	18	7.68	1		
Positive	4	14.9	0.54	0.78-1.67	0.29
DDR2					
Negative	18	9.12	1		
Positive	4	11.3	0.72	0.24-2.23	0.57
ERBB2					
Negative	18	9.12	1		
Positive	4	9.47	1.13	0.37-3.39	0.83
HRAS					
Negative	18	9.12	1		
Positive	4	8.97	1.42	0.46-4.32	0.54
MGA					
Negative	18	9.12	1		
Positive	4	11.4	0.63	0.20-1.96	0.42
MLH1					
Negative	18	9.12	1		
Positive	4	8.97	0.98	0.32-2.99	0.97
PDGFRA					
Negative	18	7.68	1		
Positive	4	16.2	0.47	0.15-1.48	0.20
PKHD1					
Negative	18	7.68	1		
Positive	4	14.9	0.54	0.18-1.67	0.29
PTPRD					
Negative	18	7.68	1		
Positive	4	16.0	0.55	0.18-1.70	0.30

Table S2 (*continued*)

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Table S2 (*continued*)

Gene	N	PFS (months)	Univariate analysis		
			HR	95% CI	P
ROS1					
Negative	18	7.68	1		
Positive	4	14.9	0.54	0.18-1.67	0.29
RUNX1T1					
Negative	18	7.28	1		
Positive	4	16.2	0.41	0.13-1.26	0.12
STK11					
Negative	18	7.68	1		
Positive	4	15.5	0.51	0.16-1.58	0.24
TSC1					
Negative	18	7.68	1		
Positive	4	14.9	0.53	0.17-1.64	0.27
ERBB4					
Negative	19	10.3	1		
Positive	3	4.83	0.96	0.27-3.45	0.95
FGFR2					
Negative	19	7.97	1		
Positive	3	12.8	0.70	0.20-2.44	0.58
KIT					
Negative	19	7.40	1		
Positive	3	17.0	0.36	0.10-1.27	0.11
MET					
Negative	19	10.3	1		
Positive	3	4.83	0.96	0.27-3.45	0.95
PIK3CG					
Negative	19	10.3	1		
Positive	3	7.40	1.17	0.34-4.08	0.80
PIK3R1					
Negative	19	7.97	1		
Positive	3	12.8	0.89	0.26-3.08	0.86
RET					
Negative	19	7.97	1		
Positive	3	16.7	0.56	0.16-2.00	0.37

Table S2 (*continued*)

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Table S2 (*continued*)

Gene	N	PFS (months)	Univariate analysis		
			HR	95% CI	P
TNFAIP3					
Negative	19	7.97	1		
Positive	3	12.8	0.87	0.25-3.01	0.83
ARID1A					
Negative	20	9.12	1		
Positive	2	11.4	0.73	0.17-3.21	0.68
BRAF					
Negative	20	9.12	1		
Positive	2	9.97	1.56	0.34-7.02	0.57
CTNNB1					
Negative	20	11.5	1		
Positive	2	3.75	9.41	1.54-57.5	0.015
EPHA5					
Negative	20	9.12	1		
Positive	2	11.4	0.73	0.17-3.21	0.68
FGFR1					
Negative	20	9.12	1		
Positive	2	10.3	1.00	0.23-4.43	1.00
FGFR3					
Negative	20	11.5	1		
Positive	2	4.38	5.15	0.99-26.9	0.052
KEAP1					
Negative	20	7.68	1		
Positive	2	16.9	0.41	0.09-1.86	0.25
MYC					
Negative	20	7.68	1		
Positive	2	19.6	0.30	0.07-1.40	0.13
PIK3CA					
Negative	20	9.12	1		
Positive	2	10.8	0.91	0.21-4.02	0.91
RASSF1					
Negative	20	9.12	1		
Positive	2	10.9	0.77	0.17-3.41	0.73

Table S2 (*continued*)

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Table S2 (*continued*)

Gene	N	PFS (months)	Univariate analysis		
			HR	95% CI	P
RB1					
Negative	20	11.5	1		
Positive	2	5.38	2.99	0.60-14.9	0.18
SMARCA4					
Negative	20	9.12	1		
Positive	2	14.0	0.58	0.13-2.61	0.48
AMER1					
Negative	21	7.97	1		
Positive	1	34.4	<0.01	0-Inf	1.00
BAI3					
Negative	21	7.97	1		
Positive	1	22.1	0.31	0.04-2.45	0.27
CDKN2A					
Negative	21	10.3	1		
Positive	1	4.83	3.71	0.43-31.8	0.23
CDKN2B					
Negative	21	7.97	1		
Positive	1	22.1	0.31	0.04-2.45	0.27
FBXW7					
Negative	21	10.3	1		
Positive	1	4.83	3.71	0.43-31.8	0.23
FHIT					
Negative	21	10.3	1		
Positive	1	3.37	20.5	1.28-327	0.03
GRM8					
Negative	21	10.3	1		
Positive	1	4.83	3.71	0.43-31.8	0.23
KDR					
Negative	21	7.97	1		
Positive	1	22.1	0.31	0.04-2.45	0.27
MAP2K1					
Negative	21	7.97	1		
Positive	1	17.0	0.41	0.05-3.18	0.40

Table S2 (*continued*)

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Table S2 (*continued*)

Gene	N	PFS (months)	Univariate analysis		
			HR	95% CI	P
NRAS					
Negative	21	7.97	1		
Positive	1	17.0	0.41	0.05-3.18	0.40
NTRK2					
Negative	21	7.97	1		
Positive	1	17.0	0.41	0.05-3.18	0.40
NTRK3					
Negative	21	7.97	1		
Positive	1	22.1	0.31	0.04-2.45	0.27
PIK3R2					
Negative	21	10.3	1		
Positive	1	5.83	2.48	0.30-20.2	0.40
RARB					
Negative	21	7.97	1		
Positive	1	13.1	1.03	0.13-7.95	0.97
U2AF1					
Negative	21	7.97	1		
Positive	1	17.0	0.41	0.05-3.18	0.40

HR, hazard ratio; PFS, progression-free survival; CI, confidence interval.

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Table S3 Patient characteristics of 40 cases whose blood sample collected at the acquisition of afatinib resistance

Characteristics	Data
Age (years)	68 [39–85]
Gender	
Male	17 (42.5)
Female	23 (57.5)
Tumor histology	
Adenocarcinoma	38 (95.0)
Adenosquamous carcinoma	2 (5.0)
EGFR type	
Ex19del	22 (55.0)
L858R	11 (27.5)
Other[†]	7 (17.5)
ECOG PS	
0	11 (27.5)
1	25 (62.5)
2	3 (7.5)
Smoking	
Current or former	21 (52.5)
Never	19 (47.5)

Data are presented as median [range] or n (%). [†], G719X n=3, G719X+L861Q n=1, G719X+S768I n=2, L861Q n=1. *EGFR*, epidermal growth factor receptor; Ex19del, exon 19 deletion; ECOG PS, Eastern Cooperative Oncology Group performance status.

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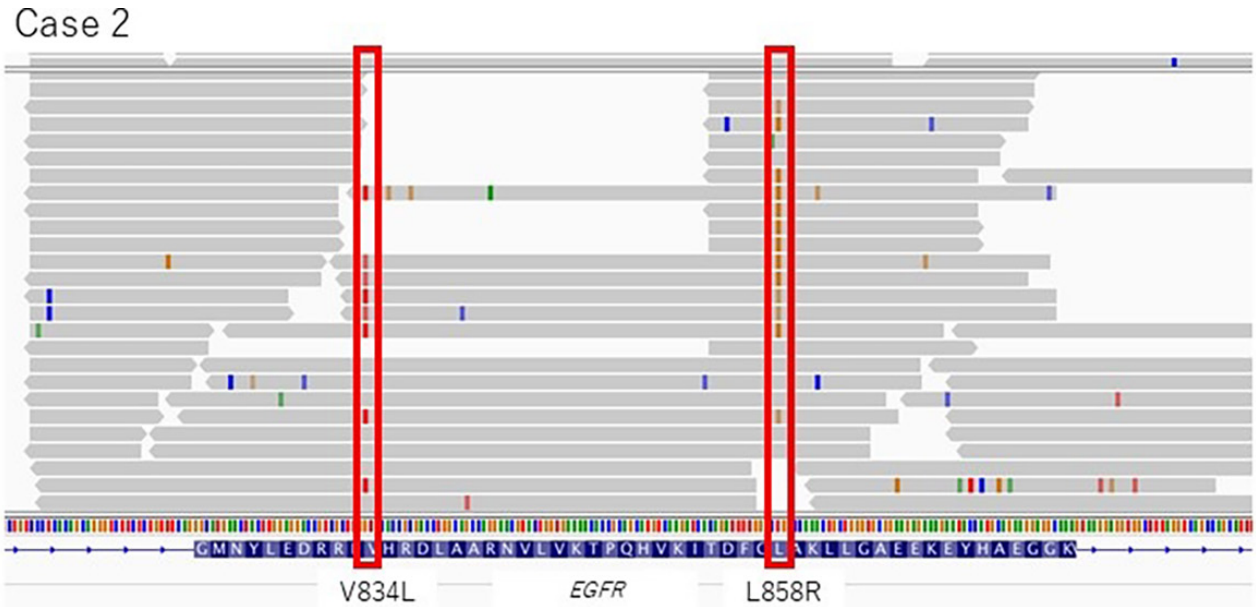

Figure S1 Sequence track in IGV of cfDNA with *EGFR*-L858R+V834L. The representative data of case 2 is shown. *EGFR*, epidermal growth factor receptor; IGV, Integrative Genomics Viewer; cfDNA, cell-free DNA.

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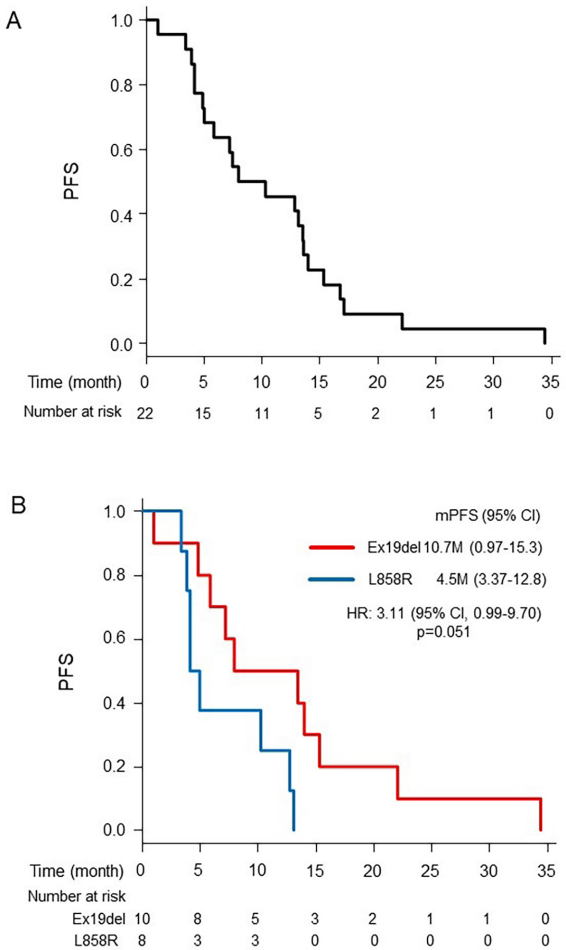

Figure S2 PFS of afatinib treatment in 22 cases whose paired plasma specimens were obtained before and at the acquisition of afatinib resistance. (A) PFS of afatinib treatment in all cases. (B) PFS stratified by types of *EGFR* mutations (*EGFR*-Ex19del *vs.* *EGFR*-L858R). PFS, progression-free survival; mPFS, median progression-free survival; CI, confidence interval; Ex19del, exon 19 deletion; M, months; HR, hazard ratio; *EGFR*, epidermal growth factor receptor.

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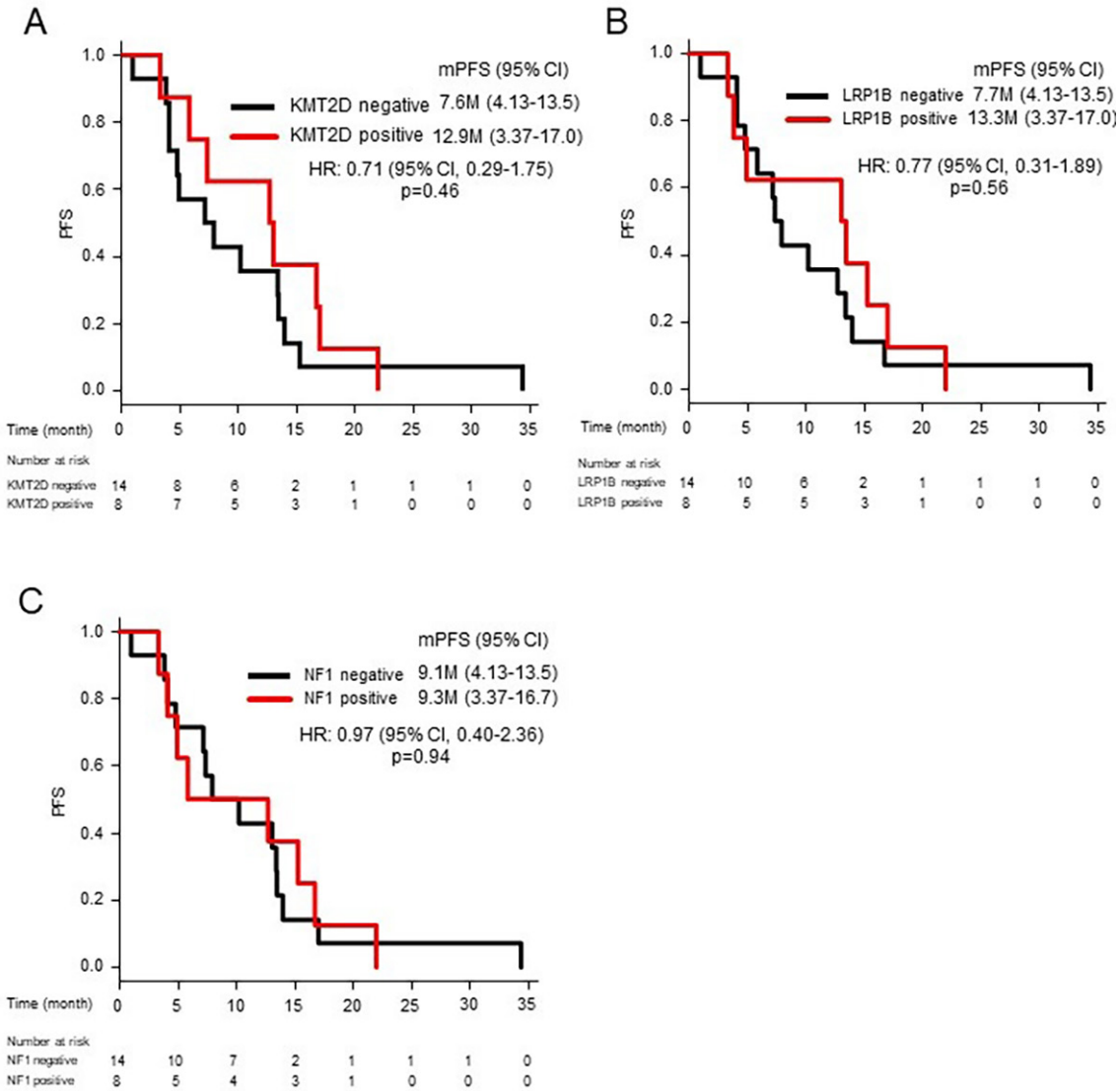

Figure S3 PFS of afatinib treatment stratified by mutations in (A) *KMT2D*, (B) *LRP1B*, and (C) *NF1*, in 22 cases whose paired specimens were obtained before and at the acquisition of afatinib resistance. PFS, progression-free survival; mPFS, median progression-free survival; CI, confidence interval; M, months; HR, hazard ratio.

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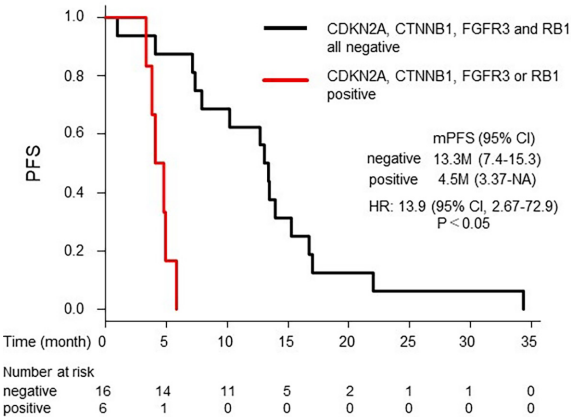

Figure S4 PFS of afatinib treatment in six patients with mutations in any gene, such as *CDKN2A*, *CTNNB1*, *FGFR3*, and *RB1*, in their cfDNA had significantly shorter PFS with afatinib, compared with that of 16 patients without these gene mutations. PFS, progression-free survival; mPFS, median progression-free survival; CI, confidence interval; M, months; NA, not available; HR, hazard ratio; cfDNA, cell-free DNA.

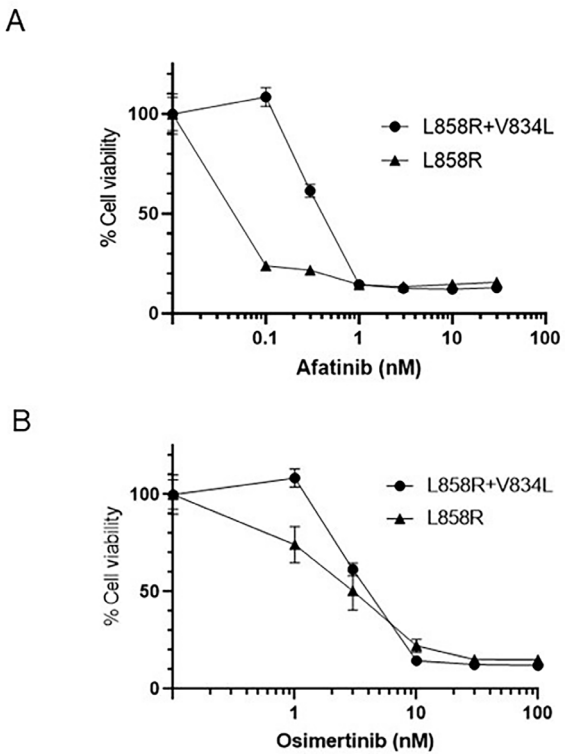

Figure S5 Sensitivity of Ba/F3 cells transfected with *EGFR*-L858R+V834 to higher concentrations of EGFR-TKIs. Ba/F3 cells were transfected with *EGFR*-L858R, and *EGFR*-L858R+V834L in cis. The cells were incubated with (A) afatinib or (B) osimertinib. The cell viability was determined by CCK-8 methods. Means ± standard deviations of triplicate culture are shown. *EGFR*, epidermal growth factor receptor; TKIs, tyrosine kinase inhibitors; CCK-8, Cell Counting Kit-8.

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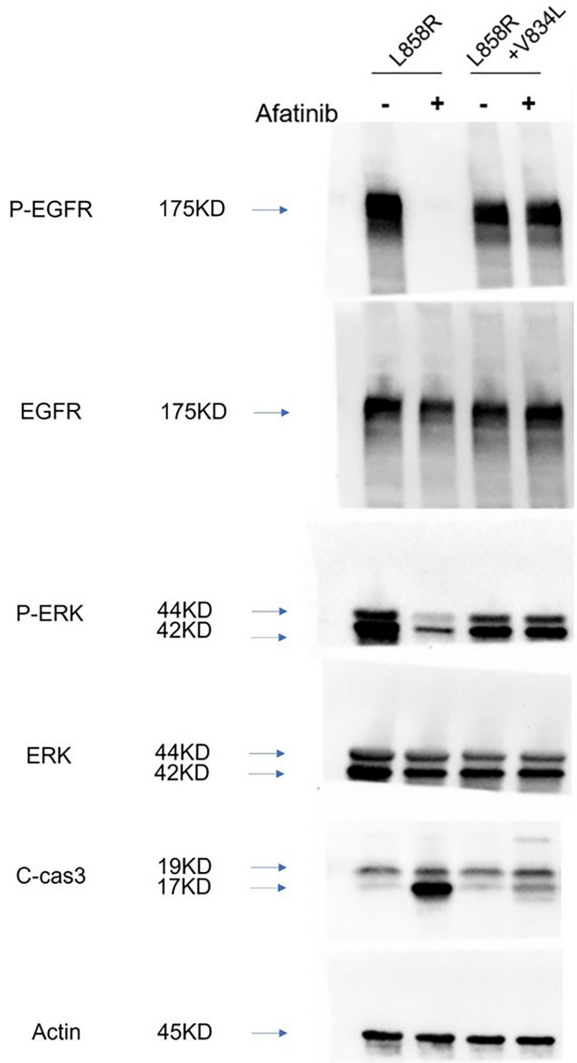

Figure S6 Ba/F3 cells transfected with *EGFR*-L858R or *EGFR*-L858R+V834L in cis were treated with afatinib (0.3 nM) for 24 h. The expression of indicated proteins was determined by western blotting. *EGFR*, epidermal growth factor receptor; p-, phosphorylated-; ERK, extracellular signal-related kinase; cas3, caspase-3.

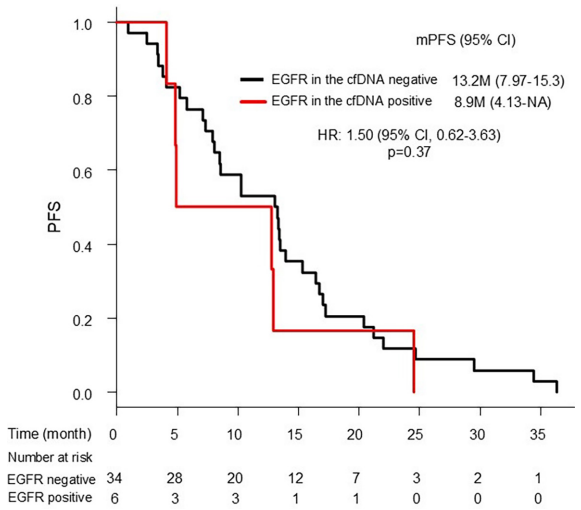

Figure S7 PFS of six patients in which driver *EGFR* mutations were detected in cfDNA at afatinib resistance and the 34 cases in which driver *EGFR* mutations were not detected in the cfDNA. PFS, progression-free survival; mPFS, median progression-free survival; CI, confidence interval; *EGFR*, epidermal growth factor receptor; M, months; NA, not available; HR, hazard ratio; cfDNA, cell-free DNA.

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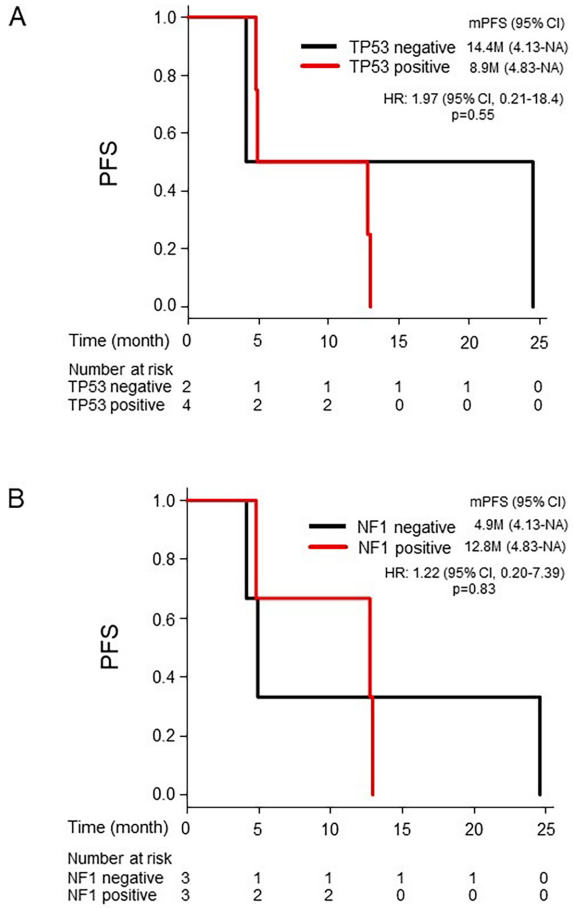

Figure S8 PFS stratified by known EGFR-TKI resistance associated gene mutations in six cases with whom the driver *EGFR* mutation was detected in cfDNA obtained at the acquisition of afatinib resistance. (A) *TP53*. (B) *NF1*. PFS, progression-free survival; mPFS, median progression-free survival; CI, confidence interval; M, months; NA, not available; HR, hazard ratio; *EGFR*, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; cfDNA, cell-free DNA.

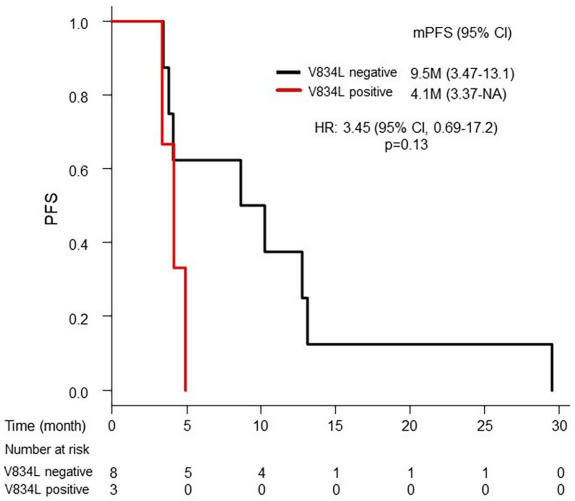

Figure S9 PFS of afatinib treatment in patients with *EGFR*-L858R stratified by detection of *EGFR*-V834L in cfDNA either before afatinib treatment or at the acquisition of afatinib resistance. PFS, progression-free survival; mPFS, median progression-free survival; CI, confidence interval; M, months; NA, not available; HR, hazard ratio; *EGFR*, epidermal growth factor receptor; cfDNA, cell-free DNA.

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