BIM and mTOR expression levels predict outcome to erlotinib in EGFR-mutant non-small-cell lung cancer

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Supplementary Material

Supplementary Table 1. Patient characteristics of the 19 patients included in the validation cohort

Clinical parameter	N(%)
Sex	
Female	14 (73.7)
Male	5 (26.3)
Age group	
<65 years	11 (77.9)
>= 65 years	8 (42.1)
Smoking status	
Never smoker	15 (78.9)
Former smoker	3 (15.8)
Current Smoker	1 (5.3)
ECOG PS	
0-1	12 (63.2)
2	7 (36.8)
Histologic Diagnosis	
Adenocarcinoma	19 (100.0)
Clinical Stage	
IIIA	1 (5.3)
IIIB	2 (10.5)
IV	16 (84.2)
Bone metastasis	
Yes	9 (47.4)
No	10 (52.6)
Brain metastasis	
Yes	7 (36.8)
No	12 (63.2)
Other metastasis	
Yes	12 (63.2)
No	7 (36.8)
Type of EGFR mutation	
del19	12 (63.2)
L858R	7 (36.8)
Type of EGFR TKI	
Erlotinib	13 (68.4)
Gefitinib	5 (26.3)
Afatinib	1 (5.3)
Response	
Complete response	2 (10.5)
Partial response	10 (52.6)
Stable disease	7 (36.8)

Supplementary Table 2: Univariate analyses of progression-free and overall survival in 57 patients from the EURTAC trial included in the present study.

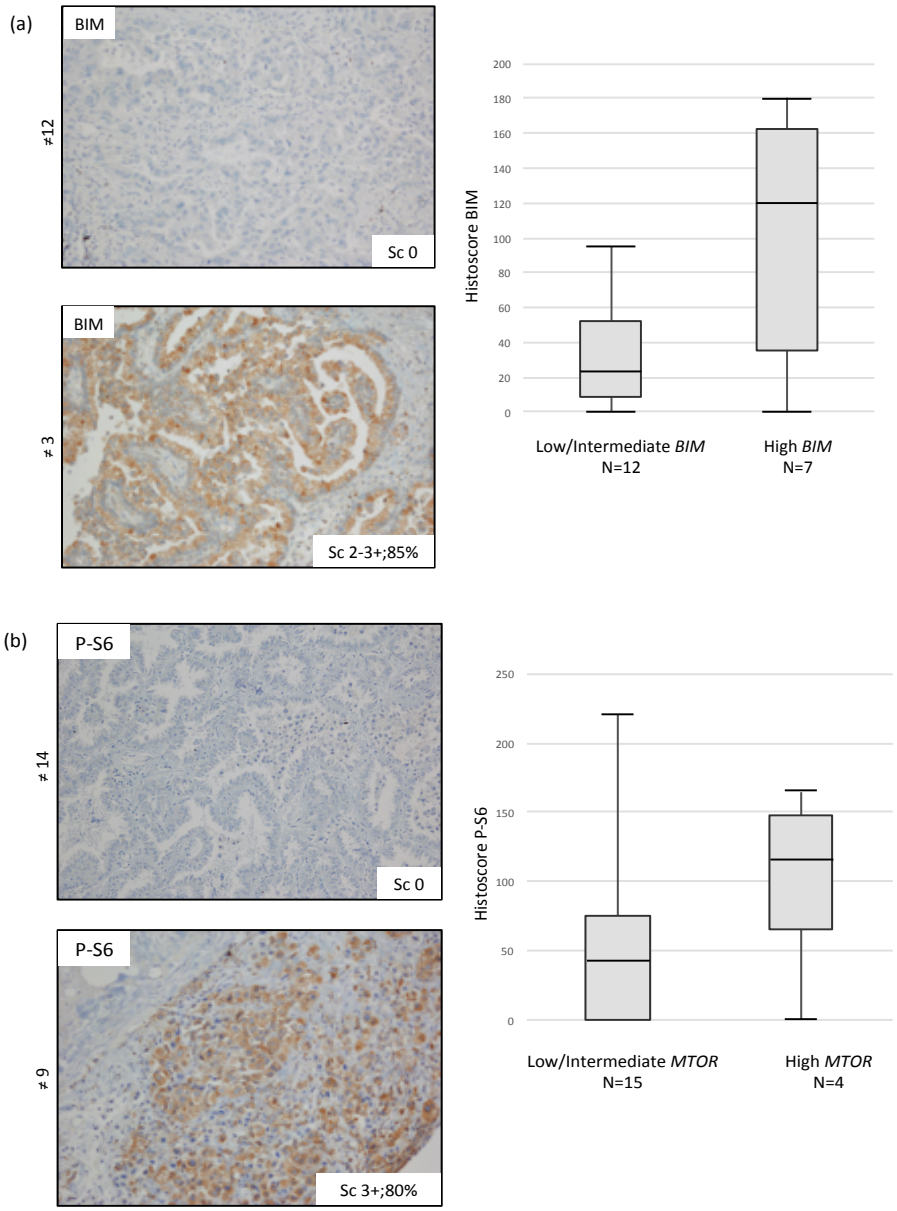
	PFS		OS	
Variable	HR (95%CI)	P	HR (95%CI)	P
Gender				
Female	1.00	-	1.00	
Male	1.14 (0.60, 2.18)	.6859	1.20 (0.63, 2.28)	.5773
Smoking history				
Current smoker	1.00		1.00	
Never smoked	1.10 (0.33, 3.68)	.8737	0.39 (0.15, 1.05)	.1791
Former smoker	1.05 (0.30, 3.68)	.9391	0.46 (0.17, 1.31	.1472
ECOG performance status				
1,2	1.00		1.00	
0	0.67 (0.35, 1.27)	.2156	0.78 (0.41, 1.49)	.4554
Treatment				
Chemotherapy	1.00		1.00	
Erlotinib	0.48 (0.25, 0.93)	.0265	1.28 (0.69, 2.35)	.4321
Bone metastasis				
No	1.00		1.00	
Yes	1.50 (0.77, 2.92)	.2298	1.19 (0.60, 2.38)	.6178
Brain metastasis				
No	1.00		1.00	
Yes	2.31 (0.89, 5.97)	.0749	2.66 (1.10, 6.43)	.0293
Type of EGFR mutation				
del19	1.00		1.00	
L858R	0.69(0.36, 1.30)	.2467	0.87(0.46, 1.64)	.6659
***BIM* expression**				
Low/intermediate	1.00		1.00	
High	0.40 (0.20, 0.80)	.0095	0.39 (0.19, 0.82)	.0124

Supplementary Table 3. Primers and probes used for gene expression analyses

GENES	REFSEQ		PRIMERS	PROBES
β-actin	NM_001101.3	F	5´ TGAGCGCGGCTACAGCTT 3´	6FAM 5´ACCACCACGGCCGAGCGG 3´ TAMRA
		R	5´ TCCTTAATGTCACGCACGATTT 3´	
MTOR	NM_004958	F	5´ AGGCCGCATTGTCTCTATCAA 3´	6FAM 5´ TGCAATCCAGCTGTTTG 3´ MGB
		R	5´GCAGTAAATGCAGGTAGTCATCCA 3´	
DGKA	NM_001345	F	5´ CCCAGTGATTTTGCCCAGC 3´	6FAM 5´ AATACTCCACCAAAAAG 3´ MGB
		R	5´ CCATCCTCGAAGAGCTTTAGGA3´	
PDE4A	NM_001111307	F	5´ GGAGACCATGCAGACCTATCG 3´	6FAM 5´ TGGCCTCGCACAAGT 3´ MGB
		R	5´ GCTCACGGTTCAACATCCTTTT3´	
PDE4D	NM_ 001104631	F	5´ ATCATCCTGGTGTGTCCAATCA 3´	6FAM 5´ TCTGATCAATACAAACTCT 3´ MGB
		R	5´ GGAATCATTGTACATCAAGGCAAGT3´	

Supplementary Figures:

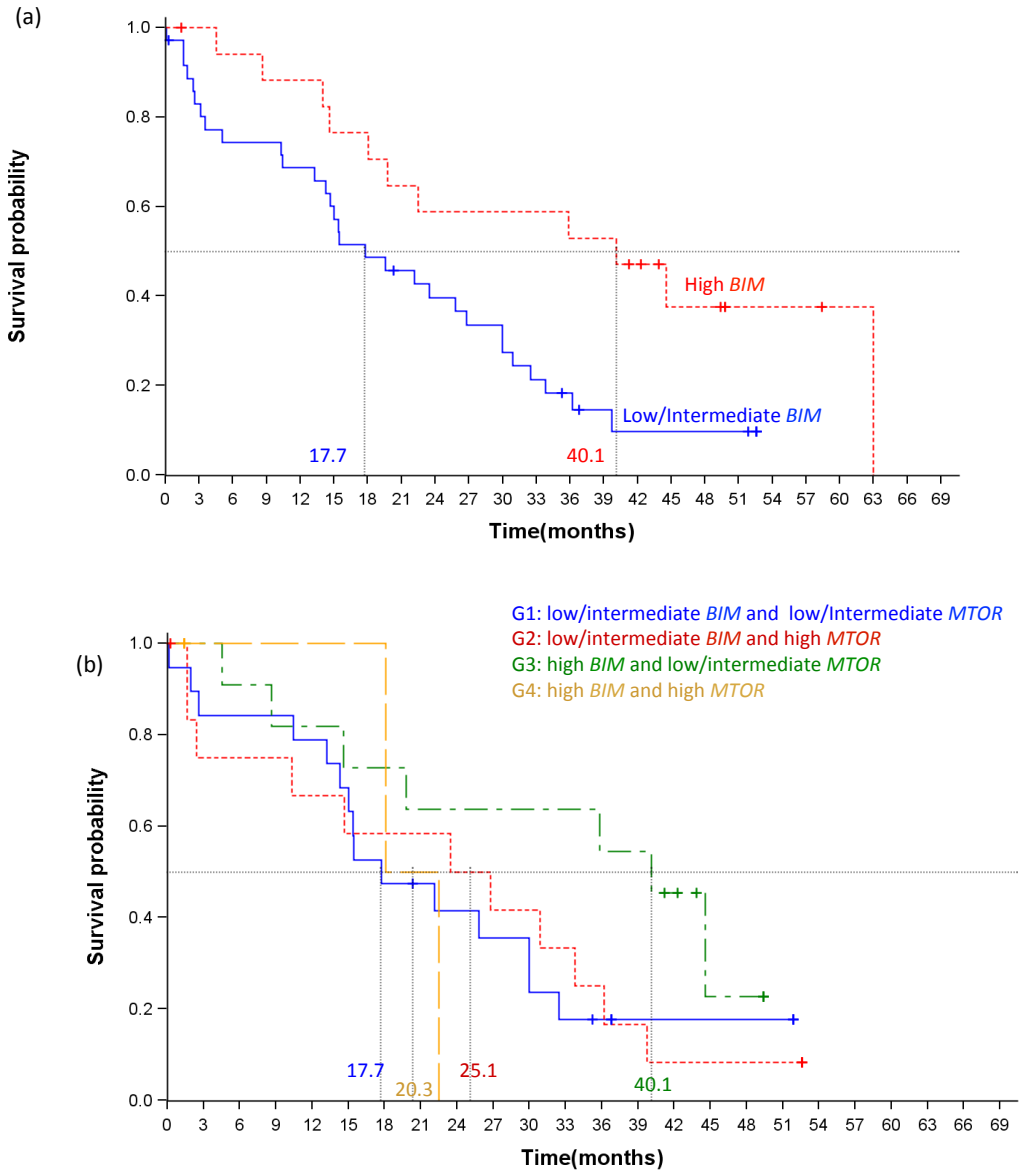
Supplementary Figure 1:



Examples of immunohistochemical analysis and correlation between mRNA and protein expression. (a). Left: Representative cases of BIM protein expression. BIM staining was considered positive when either strong (3+) or moderate (+2) cytoplasmic staining was observed (scale bar 100µm). Representative IHC staining images for a negative case (score 0; Sc 0) and a positive case (score 2-3+ in 85% of the cells; Sc 2-3+). Right: The correlation between BIM protein and mRNA

expression is presented by box- and -whisker plots. Medians, interquartile, maximum and minimum are shown. Though not statistically significant a trend for a positive correlation was found between *BIM* mRNA and protein expression (Wilcoxon test two-side *P* value =.1161). **(b).** Representative cases of P-S6 protein expression with anti-p-S6 (Ser240/244). P-S6 staining was considered positive when only strong (3+) cytoplasmic staining was observed (scale bar 100μm). Representative IHC staining images for a negative case (score 0; Sc 0) and a positive case (score 3+ in 80% of the cells; Sc 3+). Right: The correlation between P-S6 protein and *MTOR* mRNA expression is presented by box- and -whisker plots. Medians, interquartile, maximum and minimum are shown. Though not statistically significant a trend for a positive correlation was found between *MTOR* mRNA and P-S6 protein expression (Wilcoxon test two-side *P* value = .4048).

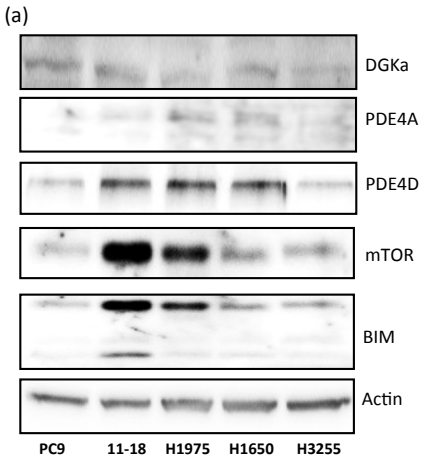
Supplementary Figure 2:

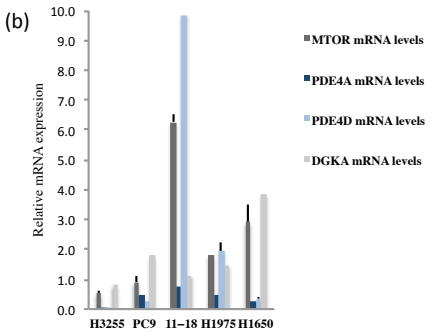


Overall survival, according to *BIM* and *MTOR* mRNA expression levels for the training cohort of patients form the EURTAC study. (a). Overall survival according to *BIM* mRNA levels for all the 57 *EGFR*-mutant NSCLC patients included in the present analysis. OS was 40.1 months (95%CI 14.6-63.0) for the 36 patients with high *BIM* (red line) and 17.7 months (95%CI 13.2-26.8) for the 18 patients with low/intermediate *BIM* mRNA expression (blue line); *P*=.010. **(b)**. Overall survival by *BIM* and *MTOR* mRNA levels for the 46 *EGFR*-mutant NSCLC patients of the

training cohort in whom both *BIM* and *MTOR* mRNA evaluation was possible. Median OS was 17.7 (95%CI 13.3-30), for 19 patients (G1) with low/intermediate both *BIM* and *MTOR* and 25.1 months (95%CI 1.6-36.2) for the 13 patients (G2) with low/intermediate *BIM* and high *MTOR*. Median OS was 40.1 months (95%CI 8.6-NR) for 11 patients (G3) with high *BIM* and low/intermediate *MTOR* and 20.3 months (95%CI 18.1-22.5) for three patients (G4) with high both *BIM* and *MTOR*; *P*=.2497. Note Supplementary Figure 2: *BIM* expression levels were divided into high (>2.96) and low (<1.83) or intermediate (1.83-2.96). *MTOR* expression levels were divided into high (>1.97) and low (<0.91) or intermediate (0.91-1.97).

Supplementary Figure 3:





EGFR-mutant cell lines	*MTOR*	*PDE4A*
H3255	0.55	0.08
PC9	0.91	0.49
11–18	6.28	0.77
H1975	1.81	0.48
H1650	2.91	0.26

EGFR-mutant cell lines	*PDE4D*	*DGKA*
H3255	0.06	0.81
PC9	0.24	1.78
11–18	9.86	1.13
H1975	1.95	1.47
H1650	0.36	3.82

Correlation of MTOR expression with the expression of DGKA and PDE4. (a). mTOR and DGKa, PDE4A and PDE4D expression in *EGFR*-mutant lung adenocarcinoma cell lines. Cell lysates were collected and probed with anti-mTOR and anti-DGKa, anti-PDE4A anti-PDE4D antibodies. Actin was used as the loading control. The protein levels of PDE4D and mTOR are similarly increased in 11-18, H1975 and H1650 cells. **(b).** *MTOR*, *DGKA*, *PDE4A and PDE4D* mRNA expression in *EGFR*-mutant lung adenocarcinoma cell lines by qRT-PCR normalized to β-actin. Correlation between the four biomarkers was assessed using Pearson´s correlation analysis. There was a significant positive correlation between *MTOR* and *PDE4D* mRNA expression (r=0.92, *P*=.0244). Pearson correlation coefficients (r) of 0.75 (P=.1418) and 0.05 (P=.09413) were found between *MTOR* and *PDE4A* and *MTOR* and *DGKA,* respectively. Values are the mean± standard deviation of triplicate experiments. Error bars indicate the standard deviation.