A low microRNA-630 expression confers resistance to tyrosine kinase inhibitors in EGFR-mutated lung adenocarcinomas via miR-630/YAP1/ERK feedback loop

Wu et al.,

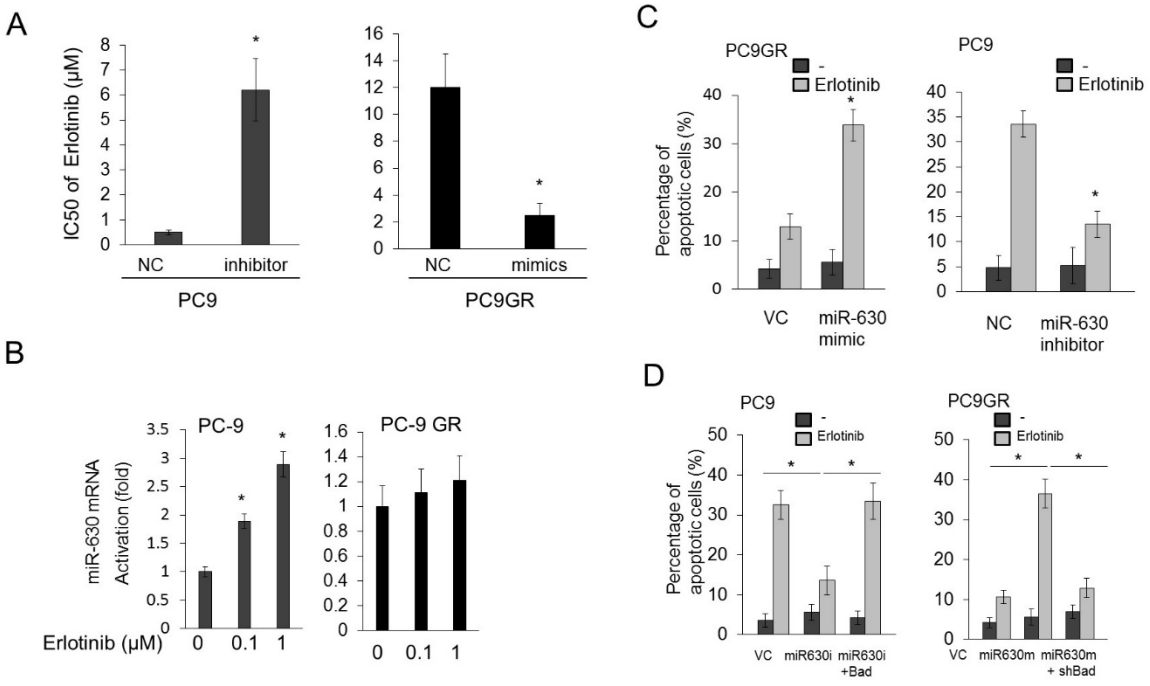

Figure S1. MiR-630 expression levels are associated with erlotinib resistance in EGFR-mutated lung adenocarcinoma cells. (A) A miR-630 inhibitor was transfected into PC9 cells. MiR-630 mimics were transfected into low miR-630 expressing PC9GR cells. After 24 h, the cells were treated with four concentrations of erlotinib to calculate the IC50 values. NC: nonspecific inhibitor/ mimic control. P value was calculated by the Student's *t*-test. The significant differences in experimental groups were compared to NC (*P < 0.05). (B) PC9 and PC9GR cells were treated with two concentrations of Erlotinib for 5 h and then miR-630 expression of these cells was evaluated by real-

time PCR. P value was calculated by the Student's *t*-test. The significant differences in experimental groups were compared to vehicle treatment (*P < 0.05). (C) PC9 and PC9GR cells were transfected with miR-630 inhibitors and mimics for 24 h. These cells were treated with 0.1% DMSO or 10 μM erlotinib for 24 h and then subjected to annexin-V and PI staining, followed by flow cytometry analysis. P value was calculated by the Student's *t*-test. The significant differences in experimental groups were compared to NC (*P < 0.05) during different treatment. (D) PC9 cells were transfected with indicated combination of miR-630 inhibitor and Bad overexpression plasmids for 24h. PC9GR cells were transfected with the indicated combination of miR-630 mimics and shBad for 24h. These cells were treated with 0.1% DMSO or 10 μM of erlotinib for 24 h and were subjected to annexin-V and PI staining, followed by a flow cytometry analysis. The percentage of apoptotic cells in the annexin V+/PI- population plus annexin-V+/PI+ is summarized. P value was calculated by the Student's *t*-test. The significant differences in experimental groups were compared to indicated treatment (*P < 0.05).

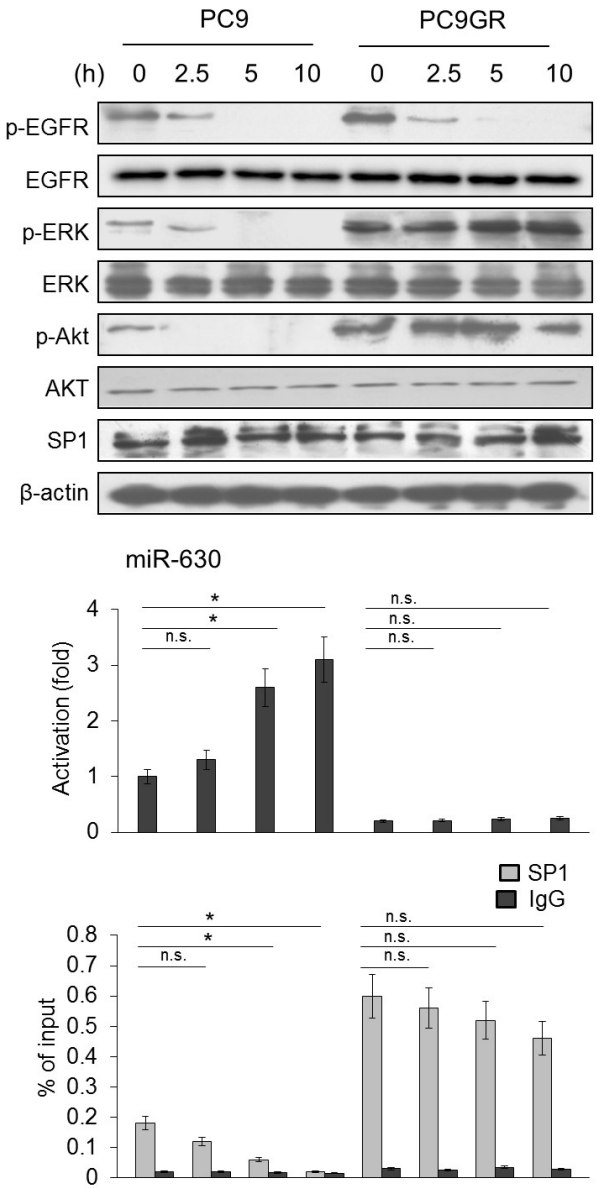

Figure S2. Time-course experiments was performed to examine the SP1 expression and the recruitment of SP1 to miR-630 promoter between PC and PC9GR cells. PC9 and PC9GR cells were treated with 1μ M of gefitinib for 0~10 h and then the cell lysates were evaluated for expression of p-EGFR, p-AKT, total AKT, p-ERK, total ERK, EGFR, and β-actin by western blotting. MiR-630 expression of these cells was

evaluated by real-time PCR. The DNA binding activity of SP1 onto the miR-630 promoter was evaluated by ChIP. P value was calculated by the Student's *t*-test. The significant differences in experimental groups were compared to indicated treatment (*$P < 0.05$).

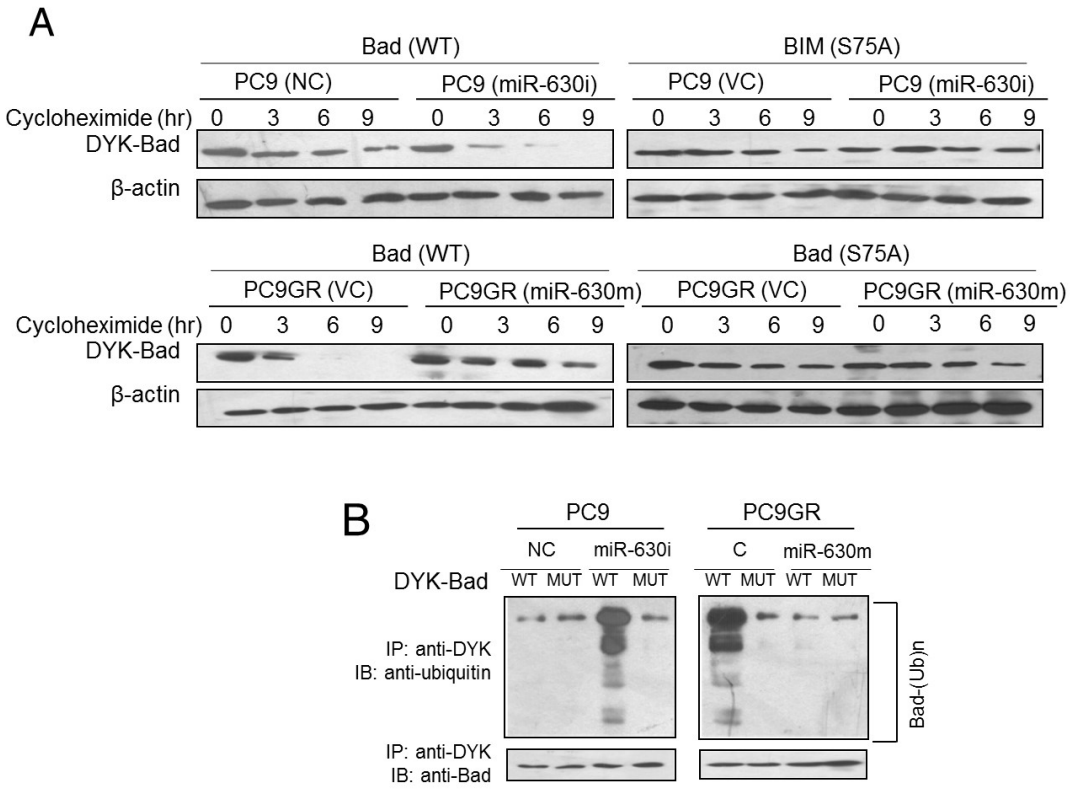

Figure S3. Bad phosphorylation at Serine-75 could be responsible for Bad degradation by miR-630 loss. (A) PC9 cells were transfected with the indicated combinations of miR-630 inhibitor, Bad-WT or mutant Bad (Bad-S75A) for 24 h. PC9GR cells were transfected with the indicated combinations of miR-630 mimic , Bad-WT or Bad-S75A for 24 h. Consequent, these cells were incubated with cycloheximide (20 μg/ml) at the indicated time points and then these cells were harvested and lysed to analyze Bad levels by an anti-DYK antibody. (B) PC9 cells were transfected with the indicated combinations of miR-630 inhibitor, Bad-WT or Bad-S75A for 24 h. PC9GR cells were transfected with the indicated combinations of miR-630 mimic , Bad-WT or Bad-S75A for 24 h. Consequent, these cells were treated with

or without 10 μM MG132. After MG132 treatment for 5 h, the cells lysates were immunoprecipitated with anti-DYK-conjugated beads and then immunoprecipitates were separated by SDS-PAGE for the evaluating ubiquitin pattern by a western blotting.

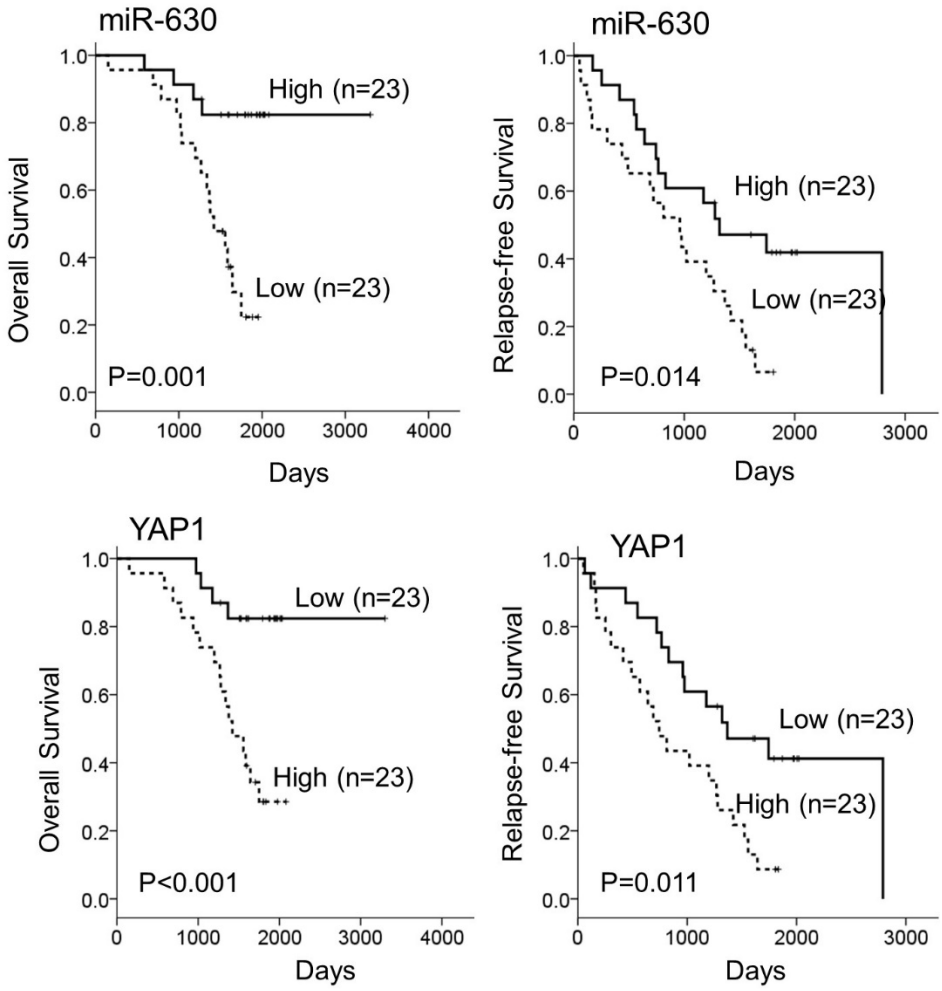

Figure S4. MiR-630 and YAP1 expression could be associated with prognosis in lung cancer patients. Low miR-630 and high YAP1 expression are associated with an unfavorable overall survival (OS) and relapse-free survival (RFS) in lung cancer patients.

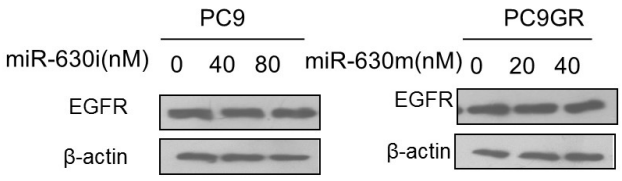

Figure S5. No change in EGFR expression by miR-630 manipulation was observed in PC9GR and PC9 cells. Two dose of miR-630 inhibitor were transfected into PC9 cells. Two dose of miR-630 mimics were transfected into low PC9GR cells. After 48 h, the cells lysates were evaluated for the expression of EGFR by western blotting.

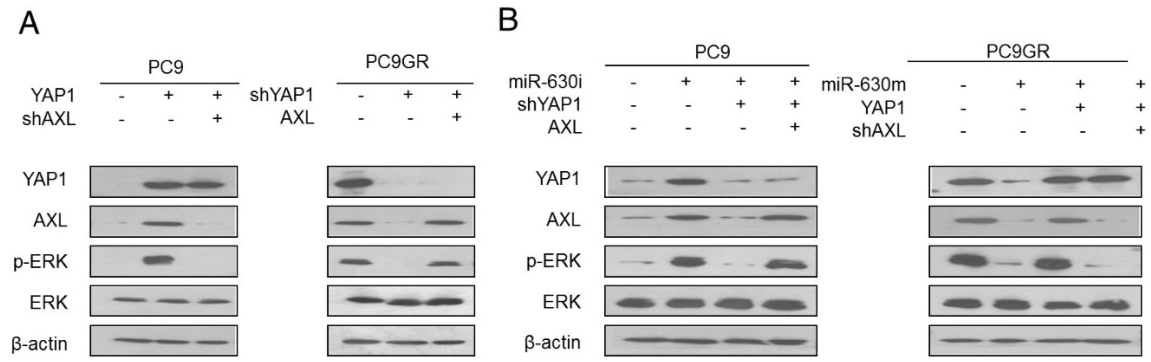

Figure S6. Activation of YAP1/ERK axis via the AXL signaling may play a key role in low miR-630-mediated TKI resistance in lung cancer. (A) PC9 cells were transfected with the indicated combination of YAP1 overexpression plasmids and shAXL for 48h. PC9GR cells were transfected with the indicated combination of shYAP1 and AXL overexpression plasmids for 48h. The cells lysates were evaluated for expression of YAP1, AXL, p-ERK, total ERK and β-actin by western blotting. (B) PC9 cells were transfected with the indicated combination of miR-630 inhibitor (miR-630i), shYAP1 and AXL overexpression plasmids for 48h. PC9GR cells were transfected with the indicated combination of miR-630 mimic (miR-630m), YAP1 overexpression plasmids and shAXL for 48h. The cells lysates were evaluated for expression of YAP1, AXL, p-ERK, total ERK and β-actin by western blotting.

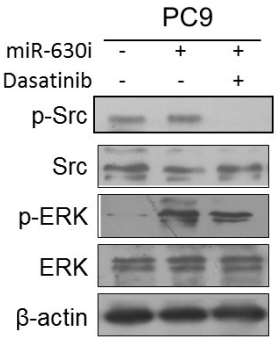

Figure S7. The involvement of Src signaling pathway in low miR-630-mediated TKI resistance did not observe in EGFR-mutated lung adenocarcinoma cells. PC9GR and PC9 cells were transfected with miR-630 inhibitor for 24 h, followed by treatment with or without Dasatinib (10 μM) for 5 h. and then the cell lysates were evaluated for expression of p-Src, total Src, p-ERK, total ERK, EGFR, and β-actin by western blotting.

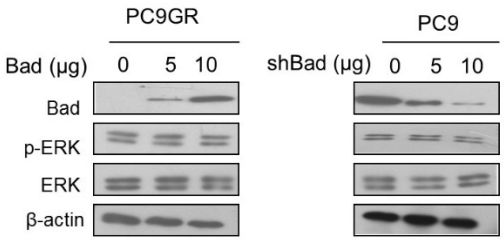

Figure S8. No change in ERK signaling by Bad manipulation were observed in PC9GR and PC9 cells. Two dose of shBad plasmids were transfected into PC9 cells. Two dose of Bad-overexpression plasmids were transfected into low PC9GR cells. After 48 h, the cells lysates were evaluated for the expression of Bad, p-ERK, and total ERK by western blotting.

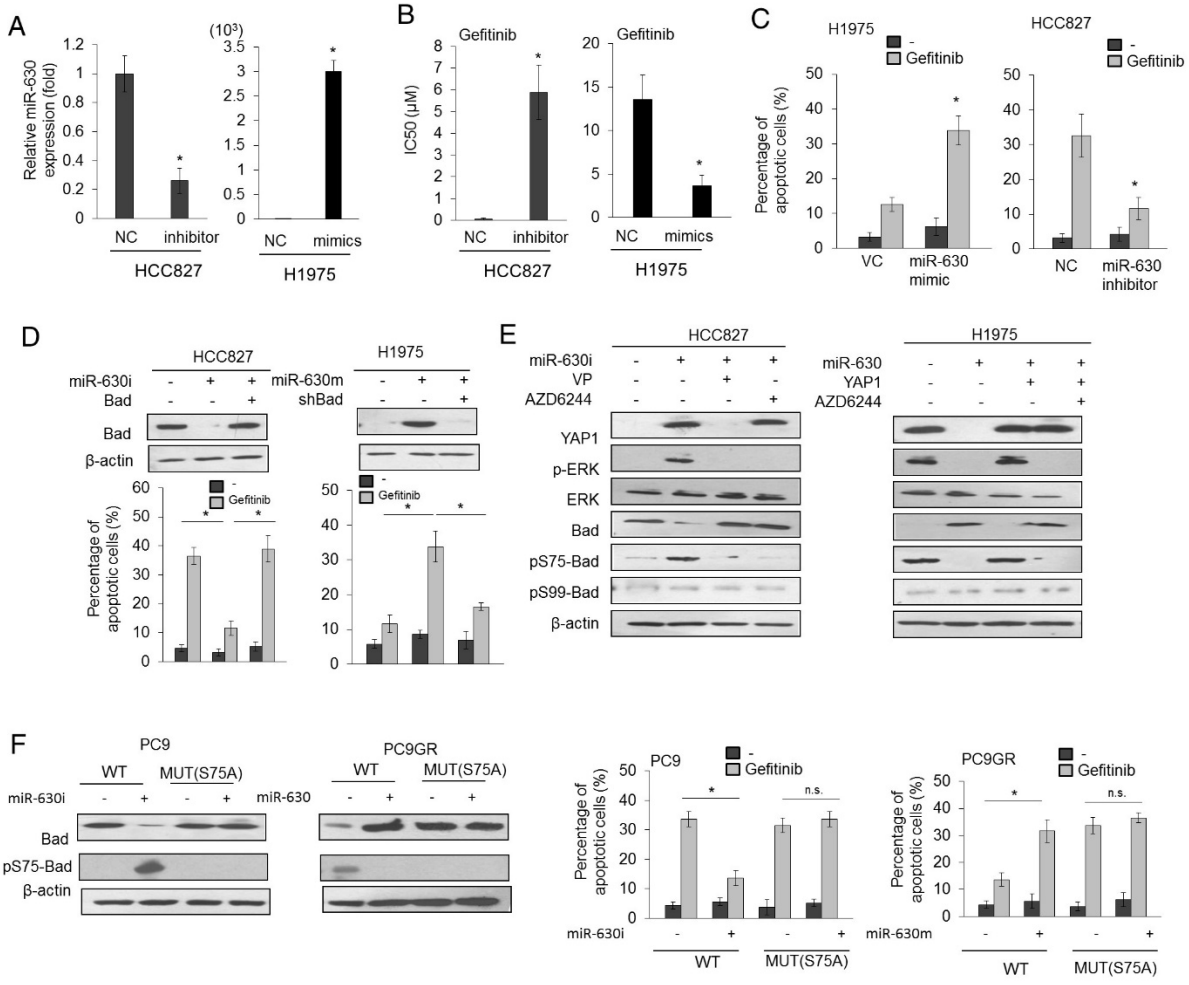

Figure S9. Low miR-630 may confer TKI resistance in lung adenocarcinoma (HCC827 and H1975 cells) via the miR-630/YAP1/ERK feedback loop. (A) (B) A miR-630 inhibitor was transfected into HCC827 cells. MiR-630 mimics were transfected into low miR-630 expressing H1975 cells. After 24 h, the cells were treated with four concentrations of gefitinib to calculate the IC50 values. NC: nonspecific shRNA control. VC: Vector control. (C) HCC827 and H1975 cells were transfected with miR-630 inhibitors and mimics for 24 h. These cells were treated with 0.1% DMSO or 10 μM gefitinib for 24 h and then subjected to annexin-V and PI staining,

followed by flow cytometry analysis. (D) YAP1-overexpressing HCC827 and H1975 cells were treated with 10 μM AZD6244 for 5 h. The cell lysates were evaluated for the expression of YAP1, p-ERK, total ERK and β-actin by western blotting. MiR-630 expression of these cells were evaluated by real-time PCR. (E) HCC827 cells were transfected with indicated combination of miR-630 inhibitor and Bad overexpression plasmids for 24h. H1975 cells were transfected with the indicated combination of miR-630 mimics and shBad for 24h. These cells were treated with 0.1% DMSO or 10 μM of gefitinib for 24 h and were subjected to annexin-V and PI staining, followed by a flow cytometry analysis. (F) HCC827 cells transfected with miR-630 inhibitors were treated with 5μM verteporfin (VP) or 10 μM AZD6244 for 5 h. MiR-630-overexpressing H1975 cells were treated with 10 μM AZD6244 for 5 h or transfected with YAP1 overexpression plasmid for 48h. The cell lysates were evaluated for expression of Bad, pS75-Bad, pS99-Bad, p-ERK, total ERK and β-actin by western blotting. (G) HCC827 cells were transfected with indicated combinations of miR-630 inhibitor, wildtype (WT), and S75A mutant (Mut) Bad overexpression plasmids for 24h. H1975 cells were transfected with the indicated combination of miR-630 mimics, wildtype (WT), and S75A mutant (Mut) Bad overexpression plasmids for 24h. These cells were treated with 0.1% DMSO or 10 μM of gefitinib for 24 h and were subjected to annexin-V and PI staining, followed by flow cytometry analysis. P value was

calculated by the Student's *t*-test. The significant differences in experimental groups were compared to vehicle or indicated treatment (*P < 0.05).

Table S1. EGFR mutation status (Exon 19~21) in 46 lung adenocarcinoma patients.

Patients' No.	EGFR mutation status	Patients' No.	EGFR mutation status
P1	Exon 19 del	P37	Exon21 L858R
P2	Exon 21 L858R	P38	Exon18 G719X
P3	Exon21 L858R	P39	Exon21 L858R
P4	Exon 19 del	P40	Exon 19 del
P5	Exon21 L858R	P41	Exon 19 del
P6	Exon21 L858R	P42	Exon 19 del
P7	Exon21 L858R	P43	Exon 19 del
P8	Exon21 L858R	P44	Exon 19 del
P9	Exon 19 del	P45	Exon21 L858R
P10	Exon 19 del	P46	Exon21 L858R
P11	Exon 19 del		
P12	Exon 20 insertion		
P13	Exon 19 del		
P14	Exon 19 del		
P15	Exon21 L858R		
P16	Exon21 L858R		
P17	Exon 19 del		
P18	Exon21 L858R		
P19	Exon21 L858R		
P20	Exon18 G719X		
P21	Exon21 L858R		
P22	Exon21 L858R		
P23	Exon 19 del		
P24	Exon 19 del		
P25	Exon 19 del		
P26	Exon 19 del		
P27	Exon 20 insertion		
P28	Exon 19 del		
P29	Exon 19 del		
P30	Exon 19 del		
P31	Exon21 L858R		
P32	Exon21 L858R		
P33	Exon 19 del		
P34	Exon 19 del		
P35	Exon 19 del		
P36	Exon21 L858R		

Table S2. Cox regression analysis for the prognostic value of miR-630 and YAP1 mRNA levels on OS and RFS in lung adenocarcinoma patients.

	OS				RFS			
Variables	Patient No.	Adjusted HR*	95%CI	P	Patient No.	Adjusted HR*	95%CI	P
miR-630								
High	23	1			23	1		
Low	23	6.71	1.97–22.9	0.002	23	2.91	1.27-6.69	0.012
YAP1								
Low	23	1			23	1		
High	23	6.31	1.99-20.1	0.002	23	2.74	1.31-5.70	0.007

OS: overall survival; HR: Hazard ratio; RFS: relapse free survival.

*HR for all cases was adjusted by age, gender, smoking status and stage.