

Supplementary Materials: Post-surgery circulating tumor cells and *AXL* overexpression as new poor prognostic biomarkers in resected lung adenocarcinoma

Supplementary Text 1:

Clinical criteria:

Complete resection was defined as demonstrating cancer-free surgical margins, both grossly and histologically. Preoperative evaluation included history, physical examination, and laboratory and radiographic studies including PET CT scan. The follow-up schedule was the usually performed for resected NSCLC, consisting in a first clinical visit one month after surgery, then one every 3 months in the first year after resection and finally one every 6 months from the second to the fifth year. At least one chest CT scan was performed every 6 months and a PET-CT study per year. Local recurrence was defined as that occurring at the following sites: ipsilateral lung, bronchial stump or staple line, and regional lymph node (subcarinal, periesophageal, ipsilateral or contralateral mediastinum, supraclavicular, or hilar lymph nodes). Distant recurrence included metastases in the contralateral lung, liver, adrenal glands, brain, bone, or other locations. Patterns of recurrence were determined by clinical assessment, radiographical test and supplementary data from bronchoscopy, biopsy, and PET SUV.

Supplementary Text 2:

Isolation and characterization of CTCs

Paired epithelial samples slides were incubated with primary Goat Anti-EGFR (Santa Cruz biotech) and secondary Donkey anti-Goat IgG (H+L) Cross-Adsorbed Alexa Fluor 350 (Invitrogen). Finally, slides were mounted with SlowFade™ Antifade Kit (Invitrogen) and visualized under fluorescence microscope (Zeiss Epifluorescence Microscopy Zeiss Axio Imager A.1).

Baseline elutes resulted from the epithelial CTC isolation were subjected to a second immunomagnetic selection with primary Mouse Anti-Vimentin (FITC) (Santa Cruz biotech® sc-6260). They were incubated with Goat Anti-EGFR (Santa Cruz biotech® sc-31157) antibodies and subsequently with secondary Anti-FITC Microbeads (Miltenyi Biotec 130-048-701) and Donkey anti-Goat IgG (H+L) Cross-Adsorbed Alexa Fluor 555 (Invitrogen A-21432) antibodies. Finally, samples were eluted through a new magnetic column, spun down on a new slide and mounted with VECTASHIELD mounting medium with DAPI (Vector Labs) to be visualized under fluorescence microscope.

Table S1. qRT-PCR primers sequence.

Gene	Primer sequence
<i>AXL</i>	F: CAATGGGGACTACTACCGCC
	R: GAAGGACCACACATCGCTCT
<i>MET</i>	F: CTGACTTGCTGAGAGGAGGC
	R: GGTTCATCTTCGGTGCCAG
<i>IL6R</i>	F: TCAGTGTACCTGGCAAGAC
	R: GGAGGTCCTTGACCATCCAT
<i>GAPDH</i>	F: ATCACCATCTTCCAGGAGCGAGA
	R: CATGGTTCACACCCATGACGAACA
β 2M	F: TGCTGTCTCCATGTTTGATGTATCT
	R: TCTCTGCTCCCCACCTCTAAGT

Table S2. EGFR Expression in CTCs.

	NSCLC		ADC		SCC	
CTC (+) patients	EGFR+	EGFR-	EGFR+	EGFR-	EGFR+	EGFR-
CTC1 (+) (n = 40)	25 (62.5%)	15 (37.5%)	12 (80%)	3 (20%)	13 (52%)	12 (48%)
EMT CTC1 (+) (n = 11)	11 (100%)	0 (0%)	3 (100%)	0 (0%)	8 (100%)	0 (0%)
CTC2 (+) (n = 27)	10 (37%)	17 (63%)	3 (25%)	9 (75%)	7 (46.7%)	8 (53.3%)
CTC3 (+) (n = 13)	6 (46.2%)	7 (53.8%)	3 (42.9%)	4 (57.1%)	3 (50%)	3 (50%)
Number of CTCs						
CTC1 (+) (n = 207)	117 (56.5%)	90 (43.5%)	31 (51.7%)	30 (48.3%)	86 (61.4%)	60 (38.6%)
EMT CTC1 (+) (n = 16)	16 (100%)	0 (%)	3 (100%)	0 (0%)	13 (100%)	0 (0%)
CTC2 (+) (n = 39)	18 (46.2%)	21 (53.8%)	5 (29.4%)	12 (70.6%)	13 (59.1%)	9 (40.9%)
CTC3 (+) (n = 22)	15 (68.2%)	7 (31.8%)	5 (55.6%)	4 (44.4%)	10 (76.9%)	3 (23.1%)

Table 3. Univariate and multivariate Cox proportional hazards regression analysis for relapse-free survival.

Characteristics	NSCLC						ADC						SCC					
	Univariate			Multivariate			Univariate			Multivariate			Univariate			Multivariate		
	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>
Age	1.00	0.97-1.03	0.927				1.00	0.95-1.04	0.847				1.00	0.95-1.06	0.989			
Gender																		
Men	0.88	0.35-2.24	0.788				1.36	0.46-4.01	0.583				0.81	0.11-6.1	0.840			
Women	1.00						1.00						1.00					
Histological type																		
ADC	0.80	0.44-1.47	0.473															
SCC	1.00																	
Stage																		
I	1.00		0.004				1.00		0.046				1.00		0.087			
II	1.70	0.83-3.49	0.146				1.33	0.49-3.56	0.577				2.30	0.78-6.88	0.135			
III	3.75	1.71-8.24	0.001				3.53	1.28-9.78	0.015				4.04	1.56-14.14	0.029			
Size (cm)																		
>4cm	2.66	1.43-4.94	0.002	2.65	1.38-5.10	0.004	1.55	0.67-3.60	0.307				7.16	2.07-24.7	0.002	6.77	1.94-23.56	0.003
≤4cm	1.00			1.00			1.00						1.00			1.00		
PET (SUVmax)																		
>9.4	1.19	0.64-2.21	0.590				1.23	0.5-3.05	0.658				1.47	0.53-4.07	0.463			
≤9.4	1.00						1.00						1.00					
N status																		
N0	1.00						1.00		0.143				1.00		0.094	1.00		0.192
N1	2.85	1.36-5.95	0.006				2.76	0.96-7.94	0.06				3.22	1.16-9.27	0.031	2.72	0.92-7.98	0.070
N2	1.87	0.72-4.90	0.200				1.93	0.55-6.80	0.307				1.71	0.38-7.65	0.486	1.26	0.28-5.84	0.764
Surgical approach																		
Thoracotomy	1.70	0.88-3.27	0.113				1.47	0.64-3.36	0.361				2.68	0.78-9.21	0.117			
VATS	1.00						1.00						1.00					
Resection type																		
Pneumonectomy	1.56	0.75-3.27	0.236				3.72	1.02-13.63	0.047	4.23	1.13-15.8	0.032	1.42	0.54-3.73	0.482			
Lobectomy	1.00						1.00			1.00			1.00					
Adjuvant chemotherapy																		
Yes	1.69	0.92-3.10	0.088				1.73	0.77-3.93	0.188				1.56	0.63-3.84	0.335			
No	1.00						1.00						1.00					
Adjuvant radiotherapy																		
Yes	2.92	1.14-7.46	0.025				3.29	0.96-11.33	0.059				2.58	0.59-11.22	0.206			
No	1.00						1.00						1.00					
CTC1 (number)	0.96	0.86-1.06	0.416				0.96	0.82-1.11	0.553				0.94	0.76-1.16	0.566			
CTC1																		

Presence	0.98	0.53-1.81	0.953				1.44	0.62-3.35	0.395			0.74	0.3-1.82	0.511	
Absence	1.00						1.00					1.00			
EGFR+ CTC1															
Presence	0.89	0.45-1.78	0.744				1.25	0.51-3.04	0.625			0.66	0.22-1.98	0.454	
Absence	1.00						1.00					1.00			
CTC2 (number)	1.12	0.97-1.29	0.120				1.13	0.97-1.32	0.113			1.13	0.79-1.62	0.513	
CTC2															
Presence	1.61	0.86-3.03	0.139				2.40	1.01-5.49	0.046	2.51	1.07-5.87	0.034	1.18	0.45-3.12	0.733
Absence	1.00						1.00			1.00			1.00		
EGFR+ CTC2															
Presence	0.57	0.18-1.84	0.348				1.98	0.46-8.56	0.361				0.27	0.03-1.99	0.197
Absence	1.00						1.00						1.00		
CTC3 (number)	1.04	0.87-1.24	0.657				1.02	0.79-1.32	0.885				1.09	0.86-1.38	0.497
CTC3															
Presence	1.75	0.78-3.91	0.174				1.60	0.51-5.0	0.418				2.02	0.64-6.34	0.231
Absence	1.00						1.00						1.00		
EGFR+ CTC3															
Presence	1.44	0.36-5.79	0.606				1.86	0.26-13.51	0.540				1.32	0.18-9.53	0.786
Absence	1.00						1.00						1.00		
EMT CTC1															
Presence	0.51	0.15-1.74	0.285				2.29	0.46-11.35	0.312				0.19	0.24-1.45	0.108
Absence	1.00						1.00						1.00		
AXL															
High	0.74	0.39-1.39	0.346				2.65	1.03-6.81	0.044				0.52	0.17-1.57	0.243
Low	1.00						1.00						1.00		
IL6R															
High	1.35	0.68-2.67	0.398				1.70	0.67-4.32	0.264				2.48	0.97-6.30	0.057
Low	1.00						1.00						1.00		
MET															
High	0.65	0.34-1.25	0.200				1.78	0.73-4.34	0.204				0.50	0.16-1.51	0.216
Low	1.00						1.00						1.00		
GAPDH															
High	3.34	1.30-8.55	0.012	2.78	1.07-7.18	0.035	8.31	1.11-62.03	0.039				0.21	0.03-1.57	0.129
Low	1.00			1.00			1.00						1.00		
miR-21															
High	1.24	0.65-2.38	0.520				0.40	0.91-1.74	0.220				0.21	0.03-1.57	0.129
Low	1.00						1.00						1.00		
miR-222															
High	1.54	0.79-2.98	0.198				2.07	0.80-5.38	0.135				3.10	1.05-9.20	0.041
Low	1.00						1.00						1.00		
miR-24															
High	1.63	0.83-3.23	0.158				1.65	0.69-3.94	0.260				2.40	0.86-6.66	0.093
Low	1.00						1.00						1.00		

miR-30c											
High	0.53	0.27-1.02	0.056		1.43	0.59-3.46	0.428		0.29	0.10-0.81	0.018
Low	1.00				1.00				1.00		
miR155											
High	0.68	0.32-1.45	0.324		1.68	0.70-4.00	0.243		0.58	0.21-1.60	0.291
Low	1.00				1.00				1.00		

HR: hazard ratio; CI: confidence interval; *p*: *p*-value; N: node; VATS: video-assisted thoracic surgery.

Table S4. Univariate and multivariate Cox proportional hazards regression analysis for overall survival.

Characteristics	NSCLC						ADC						SCC					
	Univariate			Multivariate			Univariate			Multivariate			Univariate			Multivariate		
	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>	HR	95% CI	<i>p</i>
Age	1.02	0.98-1.06	0.449				1.03	0.97-1.09	0.385				1.01	0.95-1.06	0.872			
Gender																		
Men	1.81	0.55-5.89	0.327				1.36	0.39-4.74	0.630				21.81	0.0-1.9x10 ⁵	0.506			
Women	1.00						1.00						1.00					
Histological type																		
ADC	1.05	0.55-2.00	0.882															
SCC	1.00																	
Relapse																		
Yes	6.10	2.77-13.41	<0.001	3.70	1.61-8.54	0.002	5.07	1.62-19.44	0.007	15.0	1.04-216.2	0.047	6.91	2.43-19.66	<0.001	6.42	2.17-19.04	0.001
No	1.00			1.00			1.00			1.00			1.00			1.00		
Stage																		
I	1.00						1.00		0.036				1.00		0.028			
II	1.79	0.80-4.01	0.154				1.81	0.58-5.63	0.303				1.77	0.56-5.58	0.331			
III	4.47	1.99-10.03	<0.001				4.46	1.42-13.97	0.010				4.60	1.45-15.58	0.01			
Size (cm)																		
>4cm	3.23	1.64-6.37	0.001	3.40	1.56-7.41	0.002	3.54	1.34-9.02	0.008				3.19	1.14-8.90	0.027			
≤4cm	1.00			1.00			1.00						1.00					
PET (SUVmax)																		
>9.4	1.57	0.80-3.10	0.189				2.56	0.96-6.84	0.061	6.4	0.96-42.5	0.055	1.05	0.4-2.75	0.928			
≤9.4	1.00						1.00			1.00			1.00					
N status																		
N0	1.00		0.001	1.00		0.02	1.00		0.007				1.00		0.019	1.00		0.075
N1	3.75	1.73-8.12	0.001	2.46	1.05-5.76	0.038	5.83	1.93-17.55	0.002				2.83	0.88-9.09	0.081	1.37	0.41-4.60	0.609
N2	2.96	1.25-7.02	0.014	3.30	1.23-7.50	0.016	1.63	0.34-7.49	0.528				4.28	1.46-12.58	0.008	3.54	1.19-10.59	0.024
Surgical approach																		
Thoracotomy	1.56	0.78-3.11	0.205				1.94	0.75-5	0.172				1.25	0.45-3.46	0.674			
VATS	1.00						1.00						1.00					
Resection type																		
Pneumonectomy	2.49	1.23-5.05	0.011				5.67	1.51-21.33	0.010				2.20	0.88-5.46	0.091			
Lobectomy	1.00						1.00						1.00					
Adjuvant chemotherapy																		
Yes	1.73	0.91-3.29	0.098				1.58	0.63-3.98	0.334				1.87	1.75-4.53	0.186			
No	1.00						1.00						1.00					

Adjuvant radiotherapy															
Yes	2.00	0.70-5.70	0.194				2.07	0.46-9.2	0.341			1.93	0.44-8.41	0.383	
No	1.00						1.00					1.00			
CTC1 (number)	0.94	0.82-1.09	0.421				0.94	0.76-1.17	0.577			0.94	0.78-1.14	0.527	
CTC1															
Presence	0.95	0.49-1.83	0.870				1.45	0.56-3.74	0.448			0.65	0.26-1.61	0.347	
Absence	1.00						1.00					1.00			
EGFR+ CTC1															
Presence	1.02	0.49-2.10	0.968				1.56	0.59-4.18	0.372			0.67	0.22-2.02	0.476	
Absence	1.00						1.00					1.00			
CTC2 (number)	1.12	0.97-1.31	0.132				1.14	0.96-1.35	0.134			1.10	0.76-1.59	0.617	
CTC2															
Presence	1.47	0.75-2.89	0.264				1.72	0.66-4.45	0.264			1.22	0.46-3.22	0.685	
Absence	1.00						1.00					1.00			
EGFR+ CTC2															
Presence	0.42	0.10-1.76	0.240				0.77	0.10-5.83	0.804			0.28	0.04-2.11	0.217	
Absence	1.00						1.00					1.00			
CTC3 (number)	0.95	0.76-1.19	0.643				0.99	0.76-1.29	0.943			0.90	0.60-1.34	0.595	
CTC3															
Presence	1.61	0.64-4.02	0.312				3.12	0.91-10.76	0.072	10.8	1.54-76.4	0.017	0.84	0.19-3.73	0.816
Absence	1.00						1.00			1.00			1.00		
EGFR+ CTC3															
Presence	1.11	0.22-5.54	0.897				1.41	0.20-10.19	0.731				0.71	0.04-11.79	0.809
Absence	1.00						1.00						1.00		
EMT CTC1															
Presence	0.55	1.60-1.91	0.347				1.65	0.29-9.21	0.571				0.21	0.03-1.65	0.212
Absence	1.00						1.00						1.00		
AXL															
High	2.13	0.82-5.52	0.121				3.80	1.44-9.99	0.007	15.7	1.63-150.7	0.017	0.47	0.17-1.32	0.150
Low	1.00						1.00			1.00			1.00		
IL6R															
High	1.31	0.64-2.69	0.458				2.02	0.76-5.32	0.157				0.42	0.00-40.56	0.366
Low	1.00						1.00						1.00		
MET															
High	1.81	0.91-3.62	0.091	3.09	1.47-6.52	0.003	3.03	1.17-7.87	0.023				1.86	0.74-4.72	0.189
Low	1.00			1.00			1.00						1.00		
GAPDH															
High	2.33	1.12-4.85	0.024				32.41	0.32-3311.44	0.141				2.23	0.88-5.67	0.092
Low	1.00						1.00						1.00		
miR-21															
High	0.30	0.07-1.25	0.099				0.24	0.03-1.84	0.170				1.48	0.55-3.99	0.437
Low	1.00						1.00						1.00		

miR-222											
High	1.42	0.72-2.82	0.315		2.73	0.89-8.39	0.079		0.55	0.20-1.53	0.253
Low	1.00				1.00				1.00		
miR-24											
High	1.88	0.91-3.92	0.090		2.91	0.95-8.98	0.063		0.48	0.17-1.32	0.154
Low	1.00				1.00				1.00		
miR-30c											
High	0.21	0.03-1.56	0.129		1.33	0.51-3.47	0.555		0.39	0.13-1.21	0.102
Low	1.00				1.00				1.00		
miR155											
High	0.39	0.12-1.29	0.123		0.51	0.12-2.27	0.380		0.27	0.04-2.08	0.209
Low	1.00				1.00				1.00		

HR: hazard ratio; CI: confidence interval; *p*: *p*-value; N: node; VATS: video-assisted thoracic surgery.