SUPPLEMENTARY MATERIAL AND METHODS:

NSCLC resistant cell lines

HCC4006 and HCC827 EGFR mutant cell lines were exposed to increasing concentrations of EGFR TKIs over 6 months in a manner similar to previously reported (1). For EGFR TKI-resistant HCC827 cells, clones were used as described previously (1). All resistant cells are able to proliferate normally in the presence of 10 μmol/L EGFR TKIs. Upon confirming resistance, cells were cultured without drugs and their resistance to TKI was examined periodically.

STR assay

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen). DNA samples were quantified using NanoDrop ND-2000 and Qubit with DNA measurement solution (Thermo Fisher Scientific) to ensure quality. Samples were submitted to the University of Illinois at Chicago Center for Genomic Research-DNA services Facility for STR profiling. Results were compared to known STR profiles for NSCLC cell lines available on the ATCC website.

	HCC827		ERC4		HCC827ΔNT		HCC827ΔCDH1		HCC827Flag-V5		HCC827 EDN1	
TH01	6	6	6	6	6	6	6	6	6	6	6	6
D21S11	31	31	31	31	31	31	31	31	31	31	31	31
D5S818	12	12	12	12	12	12	12	12	12	12	12	12
D13S317	9	9	9	9	9	9	9	9	9	9	9	9
D7S820	11	12	11	12	11	12	11	12	11	12	11	12
D16S539	12	12	12	12	12	12	12	12	12	12	12	12
CSF1PO	11	11	11	11	11	11	11	11	11	11	11	11
AMEL	X	X	X	X	X	X	X	X	X	X	X	X
vWA	18	18	18	18	18	18	18	18	18	18	18	18
TPOX	8	8	8	8	8	8	8	8	8	8	8	8

	HCC4006		HCC4006Ge-R		HCC4006O-R		HCC4006Ge-R shNT		HCC4006Ge-R shEDN1	
TH01	7	7	7	7	7	7	7	7	7	7
D21S11	31	31	31	31	31	31	31	31	31	31
D5S818	12	12	12	12	12	12	12	12	12	12
D13S317	11	12	11	12	11	12	11	12	11	12
D7S820	9	12	9	12	9	12	9	12	9	12
D16S539	11	12	12	12	12	12	12	12	12	12
CSF1PO	10	10	10	10	10	10	10	10	10	10
AMEL	X	X	X	X	X	X	X	X	X	X
vWA	16	17	16	17	16	17	16	17	16	17
TPOX	8	9	8	9	8	9	8	9	8	9

As we show previously in HCC4006Ge-R cells (1), D16S539 STR on chromosome 11 was missing in HCC4006O-R cells. HCC4006Ge-R shNT and shEDN1 cell lines derived from HCC4006Ge-R cells present the same profile. This result suggests that HCC4006 cell line is heterogeneous and contains two discrete subpopulations of cells with different STR profiles.

List of antibodies

Target	Application	Vendor	Catalog Number
α-Smooth muscle actin	IHC	Agilent/Dako	IS61130-2
ß-actin	WB	CST	4967
CD31	IHC	CST	77699
CD44	IHC	CST	3578
E-cadherin (24E10)	WB/IHC	CST	3195
EGF Receptor (D38B1) XP	WB/IHC	CST	4267
GAPDH	WB	abcam	ab8245
Ki67	IHC	Agilent/Dako	IR62661-2
N-cadherin (D4R1H) XP	WB/IHC	CST	9102
Phospho-EGFR (Tyr1068) (D7A5) XP	WB/IHC	CST	3777
Vimentin (D21H3) XP	WB	CST	2741
Vinculin (E1E9V) XP	WB	CST	13901
Anti-Mouse IgG, HRP-linked	WB	CST	7076
Anti-Rabbit IgG, HRP-linked	WB	CST	7074

Abbreviations: WB: Western blot, IHC: Immunohistochemistry, CST: Cell Signaling Technology.

Luminex assay

Millipore Milliplex Human Angiogenesis/Growth Factor Magnetic Bead Panel-Cancer Multiplex (10-Plex, HAGP1MAG-12K) Assay kit was used for the discovery of EDN1 and VEGF-A that are differentially expressed in epithelial and mesenchymal cells. For additional measurements, EDN1 and VEGF Luminex duplex assay kits were purchased from R&D Systems (Luminex Human Magnetic Assay (1-Plex LXSAHM-01 kit). NSCLC cells were seeded in 60mm dishes and grown to 70% confluency on the day of assay. Cells were treated with gefitinib 100 nmol/L, LY2157299 1 μmol/L or TGFβ1 10 ng/ml for 72 hours when necessary and supernatant was harvested. Cell debris was removed by centrifugation and resulting supernatants were subject to Luminex assay. Several aliquots were made and snap-frozen in LN2 and kept in -80^{o}C freezer until use. The volume of supernatant and the amount of protein upon cell lysis were recorded at the time of the harvest to normalize the target quantity.

VEGF and EDN1 Quantikine ELISA

Briefly, 0,1x10^6 cells were plated in 6 well plates and left 24 hours to adhere. After 24 hours media was replaced with 2 mL of either 21% or 1% 0_2 overnight conditioned media and incubated at the specified conditions for 72 hours in a Xvivo X2-052814-1-01 hood (BioSpherix). Media was collected and flash frozen in LN2 and cells were counted. ELISA assay was performed as indicated by the manufacturer. VEGF and EDN1 secretion levels were normalized to cell number.

cDNA/shRNA constructs and lentiviral infection

cDNAs were cloned into pLX304 lentiviral expression vectors (Addgene). pLKO.1 Lentivirus coding for cDNA were packaged in 293LTV cells (Cell Biolabs) and transduced to the target cells as previously described (1).

pLX304 was a gift from David Root (Addgene plasmid # 25890; http://n2t.net/addgene:25890; RRID: Addgene_25890) (1). cDNA for EDN1 (HsCD00005385) was obtained from DNASU, Arizona State University Biodesign Institute, and were cloned to pLX304 vector.

shRNA lentiviral vector construct designed by the RNAi Consortium was used as described previously (1). shRNA knockdown and viral transduction were performed as reported previously (1).Using TransIT-LT1 transfection reagent (Mirus), the 293LTV cell line (Cell Biolabs) was transfected with pLKO.1 constructs and packaging plasmids, pCMV-ΔR8.2dvpr and pCMV-VSV-G. Lentiviral supernatants were collected and quality of viral supernatant was routinely tested with Lenti-X Go Stix (Clontech). The supernatants were applied to target cells. Following puromycin selection, cell viability assays were performed 7 days post-infection.

Target	RNAi Consortium Number	Sequence
Non-Target	N/A	5' – GCGCGATAGCGCTAATAATTT – 3'
CDH1	TRCN0000039664	5' – AATGCCATCGTTGTTCACTGG - 3'
EDN1	TRCN0000003847	5' – GCAGTTAGTGAGAGGAAGAAA – 3'

Histology and immunohistochemistry

All immunohistochemistry procedures were performed in the Department of Pathology at Hospital Quirónsalud (Valencia, Spain). Tumors were sectioned on slides with 5 µm thickness. Then paraffin sections were deparaffinized in xylene and rehydrated in a graded alcohol series and boiled with citrate buffer pH 6. Sections were incubated with primary antibodies specific for CDH1, CD44, CDH2, VIM, pEGFR, CD31, αSMA and ki67. For antibody detection, Envision detection system peroxidase/DAB (Dako) was used. The procedure was

performed on a Dako Autostainer Link 48. Image acquisition and analyses were performed with a Leica DMD108 microscope.

Vessel density measurement

CD31 immunohistochemical staining were analyzed by our board-certified pathologists. The two most vascularized areas within the tumor samples ("hot spots") were chosen at low magnification (x40) and vessels were counted in a higher magnification (x200, field: 0.16mm^2). Microvessel density (MVD) average was calculated evaluating four independent samples per group.

Murine and drug treatments studies

Tumor size assessment and short-term gefitinib treatment effect studies

Approximately 5×10^6 total cells were injected subcutaneously in the flanks of NOD *scid* gamma (NSG) mice. The size of the engrafted tumors was measured with digital calipers and grown until 100 mm^3. The tumors were then randomized and mice were treated (oral gavage) daily for 6 days either with vehicle or gefitinib (50 mg/kg).

Bosentan and gefitinib combination treatments studies

Briefly, 5×10^6 HCC4006 tumor cells were engrafted on Nu/Nu mice and their size was measured with digital calipers. The engrafted tumors were randomized and mice were treated daily either with vehicle (2% DMSO, 30% PEG-400, 2% Tween-80), bosentan (200 mg/kg), gefitinib (20 mg/kg) or the combination of gefitinib and bosentan by oral gavage.

Drug treatments

To measure intratumoral concentration of gefitinib, one bolus of gefitinib (20 mg/kg, oral gavage) was given one-hour prior the tumor samples were harvested. In the appropriate group, a simultaneous intratumoral injection of rEDN1 (5 ng in 25 µl) or ambrisentan (320 µg/kg) was administered.

The effect on blood flow was measured with laser-Doppler after intratumoral injection of bosentan (100 mg/kg) and/or intratumoral injection of rEDN1 (5 ng in 25 µl).

Mass Spectrometry

The concentration of gefitinib has been measured using UPLC separation in an Acquity UPLC system (Waters, UK) equipped with an Acquity UPLC HSS C18 (2.1 × 100 mm; Waters) column. The MS analysis was performed using a Waters Xevo TQ-S mass spectrometer (Waters) equipped with an ESI source working in the multiple reaction monitoring (MRM).

Detection of EGFR T790M with ddPCR

Droplet digital PCR (ddPCR) was performed at the Biomarkers and Precision Medicine Unit (Instituto de Investigación Sanitaria La Fe), as previously described (2).

REFERENCES

1. Soucheray M, Capelletti M, Pulido I, Kuang Y, Paweletz CP, Becker JH*, et al.* Intratumoral Heterogeneity in EGFR-Mutant NSCLC Results in Divergent Resistance Mechanisms in Response to EGFR Tyrosine Kinase Inhibition. Cancer research **2015**;75:4372-83
2. Oxnard GR, Paweletz CP, Kuang Y, Mach SL, O'Connell A, Messineo MM*, et al.* Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. Clin Cancer Res **2014**;20:1698-705