Supplementary materials and methods

Cell culture

All culture media and reagents were from Gibco (Invitrogen, Carlsbad, CA, USA). Cells used for experiments were tested for absence of *Mycoplasma* by Real-Time PCR and passaged for fewer than four months after resuscitation. A549 were cultured in DMEM containing 10% fetal calf serum (FCS), NCI-H2228, NCI-H441, HCC78, NCI-H1993 and NCI-H1299 in RPMI containing 10% FCS, Calu-1 and SK-LU-1 in MEM containing 10% FCS and supplemented with 1x MEM non-essential amino acids and 1 mM sodium pyruvate, and HBE4-E6/E7 in Keratinocyte-SFM supplemented with 0.2 ng/ml EGF and 25 µg/ml bovine pituitary extract.

Sequences of FHIT small interfering RNA

Three FHIT specific sequences were selected in the coding sequence of *FHIT* (GeneBank accession number: NM_002012) to generate 21-nucleotide sense and 21-nucleotide antisense strands of the type (19N) TT (N, any nucleotide). The selected 19-nt sequences were as follow: FHIT si1, 5'-CAUCUCAUCAAGCCCUCUG-3'; FHIT si2, 5'-GGAAGGCUGGAGACUUUCA-3' and FHIT si3 5'-GGAAGGACUUUCCUGCCUCU-3' (Eurogentec, Seraing, Belgium). Three corresponding scrambled duplexes which do not recognize any sequence in the human genome were used as controls.

Sequences of FHIT gRNA

FHIT CRISPR/Cas9 KO plasmid (h) (sc-404220, Santa Cruz Biotechnology) is a pool of 3 different gRNA plasmids. Sequences of gRNA were as follows: gRNA A, sense: AGGTCATGGAAGCGCTCCAC; gRNA B, sense: AACAAATCGGCCACTTCATC and gRNA C, sense: TTTCCTATTCACAAGAGCGA.

Immunohistochemistry

Immunohistochemistry for FHIT, pHER2 and HER2 amplification was done on serial paraffin tissue sections. For FHIT and pHER2 detection, after antigen retrieval in Target Retrieval Solution, pH9 (Dako, Glostrup, Denmark) and endogenous peroxidase inhibition in Bloxall Blocking Solution (Vector laboratories, Burlingame, CA), subsequent steps were performed with the ImmPress HRP Reagent kit peroxidase anti-Rabbit IgG or anti-Mouse IgG (Vector laboratories), and HRP activity was revealed with Immpact NovaRed peroxidase substrate kit (Vector laboratories). Immunohistochemistry for detection of HER2 amplification was performed with the Ventana anti-HER2/neu (4B5) rabbit monoclonal primary antibody (cat n° 790-4493, Roche diagnostics GmbH, Mannheim, Germany) using a Ventana Benchmark XT autostainer (Roche diagnostics GmbH) according to the manufacturer's instructions. Detection of vimentin expression on companion paraffin tumour sections for primary culture cells was also done on the Ventana Benchmark XT autostainer with the anti-vimentin mouse monoclonal V9 antibody (Dako).

Antibodies

Antibodies used for immunohistochemistry (IHC), immunofluorescence (IF) and western blotting (WB) were as follows: rabbit polyclonal antibodies to FHIT (IHC: 1:400 and WB: 1:300; ab15287, Abcam, Cambridge, UK), total ERK (tERK) (WB: 1:1000, C-16, Santa Cruz Biotechnology, Santa Cruz, CA) and phospho-HER2 (pHER2) (Tyr1248) (IHC: 1:400, IF: 1:250 and WB: 1:1000; cat n° 06-229, Millipore, Merck KGaA, Darmstadt, Germany); rabbit monoclonal antibody to FHIT (IF: 1:250; cat n° 14434-R104, Sino Biological, Beijing, China); mouse monoclonal antibodies to total HER2 (IF: 1:40 and WB: 1:500; NCL-L-CB11, Leica Biosystems, Newcastle, UK), phospho-ERK (pERK) (Tyr204) (WB: 1:1000, E-4, Santa Cruz Biotechnology), E-cadherin (IF: 1:100 and WB: 1:2500, clone 36, BD Biosciences, San Jose, CA), vimentin (IF: 1:200; WB: 1:10000, clone V9, Dako, Glostrup, Denmark) and GAPDH (WB: 1:75000; clone 6C5, Chemicon, Millipore, Billerica, MA, USA).

Modified Boyden chamber invasion assay

The *in vitro* invasive properties of cells were assessed using a modified Boyden chamber assay. An aliquot of 10⁵ cells in serum-free medium was placed in the upper compartment of the invasion chamber (BD BioCoat Matrigel Invasion Chamber, BD Biosciences, Bedford, MA, USA). The lower compartment was filled with medium containing 10% FCS. The chambers were incubated for 17 to 24 hours at 37°C. The filters were then fixed in methanol and stained with hematoxylin. Quantification of the invasion assay was performed by counting the number of cells at the lower surface of the filters (23 fields at 400-fold magnification).

3D spheroid invasion model

Spheroids were prepared with the hanging drop method in a 5% methylcellulose solution. Spheroids were then seeded in a 1.2 mg/ml type I collagen gel. Timelapse videomicroscopy was done under a Zeiss Axio Observer.Z1 microscope (Carl Zeiss MicroImaging GlbH, Germany) equipped with an environmental chamber maintained at 37°C and 5% CO2 and an ORCA Flash4.0 v2 camera (Hamamatsu Photonics, Hamamatsu city, Japan) and driven by Metamorph software (Universal Imaging corporation, West Chester Molecular devices, Downingtown, PA). Phase-contrast images at a 10-fold magnification of spheroids were successively recorded every 30 min during 72 h. Dispersion of spheroids into collagen gel was analysed by a plugin developed in our laboratory for ImageJ software (http://imageJ.nih.gov/ij/index.html).

MTT assay

For cell line analysis, cells were seeded in 12-well plates at concentration of 30,000 cells per well. Twenty-four hours after plating, cells were treated with vehicle or HER2 inhibitor The

number of viable cells was evaluated by MTT assay. Briefly, after treatment, cells were incubated with 1mg/ml MTT reagent (Sigma-Aldrich, Saint Louis, MO) during 2 hours. Cells were then lysed with propan-2-ol and absorbance of each well was measured at 560 nm using a microplate reader. Experiments were performed with three replicates for each treatment group. For primary tumour cell analysis, cells were plated in type IV collagen-coated 48 well plates at concentration of 10,000 cells per well. The number of viable cells was evaluated before and after treatment by MTT assay as described above. IC50 were calculated from individual log dose-response curves fitted by Prism version 5.0 (GraphPad Software, La Jolla, CA).

HER2 exon 20 sequencing

DNA was extracted from cell pellets with the EZ1 DNA tissue kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. HER2 exon 20 region was amplified by end-point PCR with forward 5'-GCCATGGCTGTGGGTTTGTGATGG-3' and reverse 5'-ATCCTAGCCCCTTGTGGACATAGG-3' primers using the AmpliTaq Gold Fast PCR Master Mix (ThermoFisher Scientific, Foster City, CA). After purification with the NucleoFast PCR clean-up kit (Macherey-Nagel), amplicons were subjected to the BigDye Terminator v3.1 Cycle Sequencing kit (ThermoFisher Scientific) and again purified on Sephadex G50 resin. Sequencing was performed with the 3730x1 DNA Analyzer (ThermoFisher Scientific) and sequences were analysed with the SeqScape 2.5.0 software (ThermoFisher Scientific)).

CISH

Cell pellets were prepared as cytoblocks (ThermoFisher Scientific). HER2 amplification detection was performed with the ZytoDot 2C SPEC ERBB2/CEN 17 probe kit (Zytovision, Bremerhaven, Germany) according to the manufacturer's instructions.



Figure S1: Analysis of FHIT expression level of A549 CRISPR/Cas9 clones. Western blot analysis of FHIT level in A549 CRISPR/Cas9 control and KO FHIT clones. GAPDH served as loading control.



Figure S2: HER2 activation is not correlated to HER2 overexpression/amplification in NSCLC. Immunohistochemistry analysis showing ampliHER2 and pHER2 staining on serial sections of two SCC cases and one ADC case (scale bar = $220 \,\mu$ m). Abbreviations: ampliHER2, amplified HER2; pHER2, phospho-HER2.



Figure S3: Analysis of HER2 status of lung tumour cell lines. A. Screening of HER2 exon 20 mutation by DNA sequencing. The positive control harbours an insertion c. 2324_2325 ins12, p. A775_G776 ins YVMA. A549 are shown as representative of negative cell lines. **B.** Screening of HER2 amplification by CISH. A copy of a HER2 gene appears as a green dot and a copy of a chromosome 17 centromeric region as a red dot. The positive control shows a high level HER2 amplification. A549 are shown as representative of negative cell lines (scale bar = 8 µm).



Figure S4: Characterization of A549 CRISPR/Cas9 transfectants used in *in vivo* **tumour xenograft model**. Western blot analysis of FHIT, pHER2 and tHER2 levels in A549 CRISPR/Cas9 KO FHIT and control transfectants inoculated s.c. into the flanks of nude mice for the irbinitinib experiment (**A**) and the trastuzumab experiment (**B**). Levels of GAPDH served as loading controls. Abbreviations: pHER2, phospho-HER2; tHER2, total HER2.

Description of the population studied by immunohistochemistry.

Variables	n=45
Age (years)	63.3 ± 7.5
Sex	
Male	36 (80.0%)
Female	9 (20.0%)
Histological type	
Adenocarcinomas	22 (48.9%)
Squamous cell carcinomas	23 (51.1%)
Differentiation status	
Well	13 (28.9%)
Mild	11 (24.4%)
Poor	21 (46.7%)
Tumour size	
T1	14 (31.1%)
T2	22 (48.9%)
T3	8 (17.8%)
T4	1 (2.2%)
Lymph node metastases	
NO	29 (64.5%)
N1	11 (24.4%)
N2	5 (11.1%)
Tumour stage	
IA	12 (26.7%)
IB	12 (26.7%)
IIA	8 (17.8%)
IIB	3 (6.6%)
IIIA	10 (22.2%)
FHIT loss score	
Score 0	9 (20.0%)
Score 1	7 (15.6%)
Score 2	3 (6.7%)
Score 3	6 (13.3%)
Score 4	20 (44.4%)
pHER2 score	
Score 0	17 (37.8%)
Score 1	10 (22.2%)
Score 2	9 (20.0%)
Score 3	3 (6.7%)
Score 4	6 (13.3%)
tHER2 score	
Score 0	40 (88.9%)
Score 1+	2 (4.45%)
Score 2+	1 (2.2%)
Score 3+	2 (4.45%)

Description of the population studied by western blotting.

Variables	n=48
Age (years)	64.1 ± 7.3
Sex	
Male	41 (85.4%)
Female	7 (14.6%)
Histological type	
Adenocarcinomas	25 (52.1%)
Squamous cell carcinomas	23 (47.9%)
Differentiation status	
Well	13 (27.1%)
Mild	18 (37.5%)
Poor	17 (35.4%)
Tumour size	
T1	6 (12.5%)
T2	32 (66.7%)
T3	10 (20.8%)
Lymph node metastases	
NO	24 (50.0%)
N1	18 (37.5%)
N2	5 (10.4%)
N3	1 (2.1%)
Tumour stage	
IA	5 (10.4%)
IB	12 (25.0%)
IIA	12 (25.0%)
IIB	7 (14.6%)
IIIA	11 (22.9%)
IIIB	1 (2.1%)

Description of the population studied by MTT assay.

Variables	n=22		
Age (years)	66.8 ± 9.6		
Sex			
Male	12 (54.5%)		
Female	10 (45.5%)		
Histological type			
Adenocarcinomas	15 (68.2%)		
Squamous cell carcinomas	5 (22.7%)		
Pleomorphic carcinomas	2 (9.1%)		
Differentiation status			
Well	7 (31.8%)		
Mild	3 (13.7%)		
Poor	12 (54.5%)		
Tumour size			
T1	2 (9.1%)		
T2	13 (59.1%)		
Τ3	5 (22.7%)		
T4	2 (9.1%)		
Lymph node metastases			
NO	14 (63.6%)		
N1	5 (22.7%)		
N2	3 (13.7%)		
Tumour stage			
IA	2 (9.1%)		
IB	5 (22.7%)		
IIA	2 (9.1%)		
IIB	7 (31.8%)		
IIIA	5 (22.7%)		
IIIB	1 (4.6%)		
Vimentin			
Negative	10 (45.5%)		
Positive	12 (54.5%)		

Results of HER2 mutation and amplification screening in lung tumour cell lines.

Cell line	HER2 exon 20 mutation	HER2 amplification
		(HER2:CEN-17 ratio)
HBE4-E6/E7	No	No (0.98)
H2228	No	No (1.00)
H441	No	No (0.94)
HCC78	No	No (1.57)
A549	No	No (1.07)
H1993	No	No (1.11)
H1299	No	No (0.95)
Calu-1	No	No (1.03)
SK-LU-1	No	No (0.99)

Association between FHIT^{low}/pHER2^{high} phenotype and vimentin positivity in primary tumour cells from NSCLC patients.

Variable	FHIT ^{low} /pHER2 ^{high}	Others	OR	р
	(n = 10)	(n = 12)	[95% CI]	
Vimentin positive	8 (66.7%)	4 (33.3%)	8.000	0.0427
(n = 12)			[1.126 – 56.82]	
Vimentin negative	2 (20.0%)	8 (80.0%)		
(n = 10)				