Data S1.

Extra methods information

Tissue microarrays. Fixative used, including concentration and duration: 10% buffered formalin. Minimum 24 h of fixation. Thickness of sections: 2 µm. Stain used, including temperature and duration: As per IHC detection kit (BOND Polymer Refine Detection, DS9800, Leica). Type of microscope used: *light*

Immunohistochemistry (IHC)-tumor infiltrating lymphocytes

Fixative used, including concentration and duration: 10% buffered formalin. Minimum 24 h of fixation. Resin: Paraffin. Antigen retrieval step - heating temperature, washing reagent and rehydration in descending alcohol series: All the IHC steps were performed on BOND Max (Leica). Blocking reagent and percentage, temperature and duration: As per IHC detection kit (BOND Polymer Refine Detection, DS9800, Leica. Primary antibody dilution, catalogue number, and temperature and duration of incubation/secondary antibody dilution, catalogue number, supplier, conjugate, and temperature and duration of incubation: As per IHC detection kit (BOND Polymer Refine Detection, DS9800, Leica Microsystems, Inc.).

Fluorescence in situ hybridisation (FISH). Fixative used, including concentration and duration: 10% buffered formalin. Minimum 24 h of fixation. Resin: Paraffin. Thickness of section: 5 µm. The reagents of the ZytoLight SPEC ERBB2/TOP2A/CEN 17 Triple Color Probe (Zytovision, GmbH) and ZytoLight FISH-Tissue Implementation kit (catalog no. Z-2028-5/-20) were employed.

The ZytoLight SPEC ERBB2/TOP2A/CEN 17 Triple Color Probe:

- ZyGreen (excitation 503 nm/emission 528 nm)-labeled polynucleotides (~10 ng/µl), which target sequences mapping in 17q12-q21.1* (chr17:37,572,531-38,181,308) harboring the ERBB2 gene region (Fig. 1).
- ZyOrange (excitation 547 nm/emission 572 nm) labeled polynucleotides (~4.5 ng/µl), which target sequences mapping in 17q21.1-q21.2* (chr17:38,323,741-38,818,030) harboring the TOP2A gene region (Fig. 1).
- ZyGold (excitation 532 nm/emission 553 nm) (~12 ng/µl), which target sequences mapping in 17p11.1-q11.1 specific for the alpha satellite centromeric region D17Z1 of chromosome 17.
- DAPI: Excitation 358 nm/emission 461 nm.

Specimen pretreatment (dewaxing, proteolysis) according to the instructions for use of the ZytoLight FISH-Tissue Implementation kit.

Steps:

1. Incubation of slides for 10 min at 70˚C (e.g., on a hot plate).
2. Wash twice for 10 min in xylene.
3. Incubate in 100, 100, 90 and 70% ethanol, each for 5 min.
4. Wash twice for 2 min in deionized or distilled water.
5. Incubation for 15 min in pre-warmed Heat Pretreatment Solution Citric (PT1) at 98˚C.
6. Transfer slides immediately to deionized or distilled water, wash twice for 2 min and drain off or blot off the water.
7. Add pepsin solution to the specimens and incubate for 11-12 min at 37˚C in a humidified atmosphere (this is the modification from the proposed protocol).

8. Wash for 5 min in Wash Buffer SSC (WB1)
9. Wash for 1 min in deionized or distilled water
10. Dehydration: In 70, 90 and 100% ethanol, each for 1 min
11. Air dry sections.

Denaturation and hybridization

1. Add 10 µl of the ZytoLight FISH Probe onto each pretreated specimen.
2. Cover specimens with a 22x22 mm coverslip and seal using rubber cement (e.g., Fixogum Rubber Cement).
3. Place the slides on a hot plate or hybridizer and denature specimens for 10 min at 75˚C.
4. Transfer the slides to a humidified chamber and hybridize overnight at 37˚C (e.g., in a hybridization oven; Dako; Agilent Technologies, Inc.).

Day 2

1) Preparation of 1X Wash Buffer A: Dilute 1 part 25X Wash Buffer A (WB2) with 24 parts deionized or distilled water. Fill three staining jars with the 1X Wash Buffer A and pre-warm it to 37˚C.

2) DAPI/DuraTect-Solution (MT7): Bring to room temperature before use.

Post-hybridization and detection:

1. Remove the rubber cement.
2. Remove the coverslip by submerging in 1X Wash Buffer A at 37˚C for 2 min.
3. Wash using 1X Wash Buffer A twice for 5 min at 37˚C.
4. Incubation of slides in 70, 90 and 100% ethanol, for 1 min.
5. Air dry the samples.
6. Add 25 µl DAPI/DuraTect-Solution (MT7) onto the slides.
7. Avoiding trapped bubbles, cover the samples with a coverslip (24x60 mm).
7. Incubate in the dark for 15 min.
8. Store the slides in the dark at 2-8˚C

Detection of FISH: Nikon 80i fluorescence microscope with a motorized 4 slide stage, equipped with Plan Apo x100/1.4 oil objective lens (Nikon), an appropriate four filter set [DAPI, doublePath FIRC/TRITC, ZyGreen, ZyOrange, ZyYellow (all from Chroma Technology Corp)] and an ultrasensitive black and white camera (QImaging). As a source of fluorescence illumination, the X-cite 120 (EXFO Photonic Solutions Inc.) equipped with a long-life 120-watt metal halide short arc lamp was used. The system was controlled by the cytogenetic software XCyto-Gen (Alphelys). For all probes, sequential, digital images were captured by a stack motor for the DAPI (1 or 2 planes at 0.5 µm), ZyGreen (5 planes at 1.0 µm), ZyOrange (5 planes at 1.0 µm) and ZyYellow (5 planes at 1.0 µm). The resulting images were reconstructed with blue, yellow, green and orange pseudo-colors.

Figure S1. Histograms of the distributions of the examined markers based on the normalized expression of mRNA encoding. AREG, amphiregulin; BTC, betacellulin; EGF, epidermal growth factor; EREG, epiregulin; HBEGF, heparin Binding EGF like growth factor; IGFBP4, insulin-like growth factor binding protein 4; NRG1, neuregulin 1; RARA, retinoic acid receptor α; TGFA, transforming growth factor α; TGFB1, transforming growth factor β1; THRA, thyroid hormone receptor α.

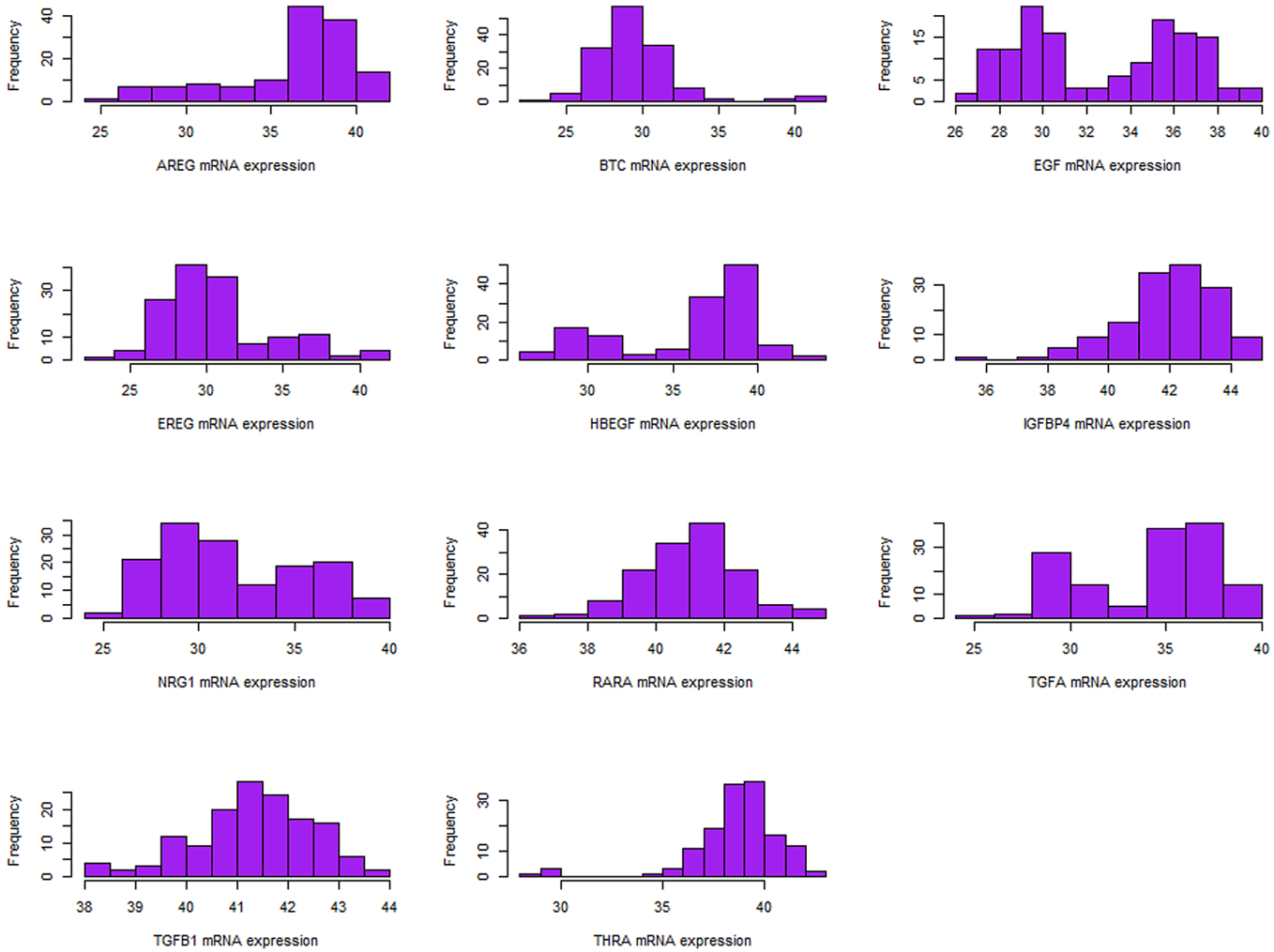

Table SI. IHC protocols.

IHC Abs	Clone/catalog no.	Manufacturer	Staining protocol
ER	6F11/NCL-L-ER-6F11	Novocastra; Leica Microsystems, Ltd.	Antigen retrieval, 20' ER1; antibody dilution, 1:70; 20'-RT, Bond Refine Detection kit protocol
PgR	1A6/NCL-L-PGR	Novocastra; Leica Microsystems, Ltd.	Antigen retrieval, 20' ER1; antibody dilution, 1:70; 20'-RT, Bond Refine Detection kit protocol
HER2	Polyclonal/A0485	Dako; Agilent Technologies, Inc.	Antigen retrieval, 20' ER1; Antibody dilution, 1:500; 30'-RT, Bond Refine Detection kit protocol
Ki-67	MIB-1/M7240	Dako; Agilent Technologies, Inc.	Antigen retrieval, 20' ER2; Antibody dilution, 1:70; 20'-RT, Bond Refine Detection kit protocol
p-mTOR (Ser2448)	49F9/2976	Cell Signaling Technology, Inc.	Antigen retrieval, 20' ER1; Antibody dilution, 1:30; 20'-RT, Bond Refine Detection kit protocol
PTEN	6H2.1/M3627	Dako; Agilent Technologies, Inc.	Antigen retrieval, 20' ER2; Antibody dilution, 1:300; 60'-RT, Bond Refine Detection kit protocol

ER1, citric acid, pH6; ER2, EDTA, pH 9; ', minutes; RT, room temperature; IHC, immunohistochemistry; Abs, antibodies; ER, estrogen receptor; PgR, progesterone receptor; p-, phosphorylated.

Table SII. Distribution of the markers of interest according to HER2 status in the entire cohort.

Marker parameters	HER2 status (by central assessment)		
	Negative	Positive	P-value
AREG			
n	44	91	0.096
Mean	36.02	37.04	
Median	36.93	37.87	
Std	3.75	3.44	
Min	27.58	25.29	
Max	41.43	42.14	
BTC			
n	46	96	0.435
Mean	29.29	29.07	
Median	29.12	29.04	
Std	2.01	1.88	
Min	23.02	24.38	
Max	34.57	34.05	
EGF			
n	47	97	0.138
Mean	32.12	33.15	
Median	30.65	33.78	
Std	3.66	3.60	
Min	27.62	26.93	
Max	38.25	39.63	
EREG			
n	47	94	0.424
Mean	29.78	30.27	
Median	29.19	29.61	

Std	3.08	3.06	
Min	23.02	24.31	
Max	37.82	37.21	
HBEGF			
n	45	91	0.045[a]
Mean	35.08	36.31	
Median	36.87	37.95	
Std	4.26	3.90	
Min	27.62	27.15	
Max	42.87	40.96	
IGFBP4			
n	45	93	0.517
Mean	41.88	41.93	
Median	42.26	42.02	
Std	2.02	1.34	
Min	35.54	38.88	
Max	44.94	44.53	
NRG1			
n	47	95	0.772
Mean	31.72	31.72	
Median	30.19	30.37	
Std	4.04	3.44	
Min	25.29	24.67	
Max	39.22	38.32	
RARA			
n	45	95	0.455
Mean	40.78	41.09	
Median	41.02	41.13	

Std	1.58	1.37	
Min	37.10	36.73	
Max	43.40	44.47	
TGFA			
n	46	92	0.104
Mean	33.36	34.31	
Median	35.08	35.47	
Std	3.70	3.53	
Min	27.62	24.67	
Max	39.11	39.94	
TGFB1			
n	47	98	0.002[a]
Mean	40.90	41.50	
Median	40.83	41.63	
Std	1.17	1.11	
Min	38.08	38.03	
Max	43.11	43.70	
THRA			
n	46	93	0.002[a]
Mean	38.11	38.98	
Median	38.61	39.32	
Std	1.91	2.28	
Min	29.62	28.64	
Max	41.38	42.91	

[a]P<0.05. AREG, amphiregulin; BTC, betacellulin; EGF, epidermal growth factor; EREG, epiregulin; HBEGF, heparin binding EGF like growth factor; IGFBP4, insulin like growth factor binding protein 4; NRG1, neuregulin 1; RARA, retinoic acid receptor α; TGFA, transforming growth factor α; TGFB1, transforming growth factor β1; THRA, thyroid hormone receptor α; HER2, human epidermal growth factor receptor 2; N, number; Std, standard deviation.

Table SIII. Distribution of the markers of interest according to ER/PgR status in HER2-positive patients.

Marker	ER/PgR status		P-value
	Negative	Positive	
AREG	37.83 (27.15-41.00)	37.94 (25.29-42.14)	0.565
BTC	29.06 (24.38-33.89)	29.02 (25.29-34.05)	0.740
EGF	33.52 (26.93-39.16)	34.88 (26.93-39.63)	0.228
EREG	29.98 (24.31-37.11)	29.31 (25.27-37.21)	0.628
HBEGF	37.78 (28.74-40.96)	38.03 (27.15-40.44)	0.643
IGFBP4	41.52 (38.88-43.71)	42.38 (38.91-44.53)	0.007[a]
NRG1	30.40 (27.18-37.93)	30.30 (24.67-38.32)	0.711
RARA	40.67 (36.73-43.38)	41.31 (39.00-44.47)	0.028[a]
TGFA	35.48 (28.55-39.94)	35.46 (24.67-38.60)	0.875
TGFB1	41.28 (38.03-43.70)	41.80 (38.39-43.63)	0.078
THRA	39.01 (29.42-41.91)	39.36 (28.64-42.91)	0.438

Data are presented as median (min-max). [a]$P<0.05$. ER, estrogen receptor; PgR, progesterone receptor. AREG, amphiregulin; BTC, betacellulin; EGF, epidermal growth factor; EREG, epiregulin; HBEGF, heparin binding EGF like growth factor; IGFBP4, insulin like growth factor binding protein 4; NRG1, neuregulin 1; RARA, retinoic acid receptor α; TGFA, transforming growth factor α; TGFB1, transforming growth factor β1; THRA, thyroid hormone receptor α.

Table SIV. Distribution of the markers of interest according to disease presentation status in the entire cohort.

Marker	Disease presentation status		P-value
	R-MBC	*de novo* MBC	
AREG	37.63 (27.58-42.14)	37.63 (25.29-41.83)	0.499
BTC	29.09 (24.67-34.57)	29.11 (23.02-34.05)	0.864
EGF	30.90 (26.93-39.63)	34.28 (26.93-38.13)	0.366
EREG	29.44 (24.31-37.82)	29.39 (23.02-37.11)	0.842
HBEGF	37.03 (27.22-42.87)	37.76 (27.15-40.96)	0.546
IGFBP4	41.82 (35.54-44.94)	42.28 (38.45-44.34)	0.210
NRG1	30.30 (25.29-39.22)	30.33 (24.67-38.65)	0.407
RARA	41.02 (37.10-44.47)	41.12 (36.73-44.47)	0.997
TGFA	34.73 (27.52-39.94)	35.57 (24.67-38.91)	0.101
TGFB1	41.31 (38.03-43.63)	41.39 (38.08-43.70)	0.218
THRA	39.11 (28.64-42.91)	38.88 (35.33-42.53)	0.691

Data are presented as median (min-max). $P<0.05$. R-MBC, relapsed metastatic breast cancer. AREG, amphiregulin; BTC, betacellulin; EGF, epidermal growth factor; EREG, epiregulin; HBEGF, heparin binding EGF like growth factor; IGFBP4, insulin like growth factor binding protein 4; NRG1, neuregulin 1; RARA, retinoic acid receptor α; TGFA, transforming growth factor α; TGFB1, transforming growth factor β1; THRA, thyroid hormone receptor α.

Table SV. Associations of markers of interest with selected clinicopathological parameters.

A, EGF					
Clinicopathological parameter	n	mRNA expression[a]			P-value
		Total	Low	High	
Visceral metastases	141				0.044
No		44 (31.2)	28 (38.9)	16 (23.2)	
Yes		97 (68.8)	44 (61.1)	53 (76.8)	
B, IGFBP4					
Clinicopathological parameter	n	mRNA expression[a]			P-value
		Total	Low	High	
TILs	122	10.0 (1.00,80.0)	10.0 (1.00,80.0)	5.0 (1.00,78.0)	0.029
Histological grade	132				0.009
I-II		47 (35.6)	16 (24.6)	31 (46.3)	
III		85 (64.4)	49 (75.4)	36 (53.7)	
C, TGFB1					
Clinicopathological parameter	n	mRNA expression[a]			P-value
		Total	Low	High	
PTEN	136				<0.001
Loss		70 (51.5)	46 (66.7)	24 (35.8)	
No loss		66 (48.5)	23 (33.3)	43 (64.2)	
D, NRG1					
Clinicopathological parameter	n	mRNA expression[a]			P-value

		Total	Low	High	
Age at trastuzumab initiation, years	142	55.4 (28.9,85.9)	57.8 (28.9,82.7)	52.8 (30.9,85.9)	0.035
≤50		46 (32.4)	16 (22.5)	30 (42.3)	0.012
>50		96 (67.6)	55 (77.5)	41 (57.7)	
Bone metastases	139				0.048
No		79 (56.8)	45 (65.2)	34 (48.6)	
Yes		60 (43.2)	24 (34.8)	36 (51.4)	
PTEN	134				0.039
Loss		68 (50.7)	41 (59.4)	27 (41.5)	
No loss		66 (49.3)	28 (40.6)	38 (58.5)	
E, RARA					
Clinicopathological parameter	n	mRNA expression[a]			P-value
		Total	Low	High	
PTEN	131				0.028
Loss		67 (51.1)	39 (60.9)	28 (41.8)	
No loss		64 (48.9)	25 (39.1)	39 (58.2)	
F, THRA					
Clinicopathological parameter	n	mRNA expression[a]			P-value
		Total	Low	High	
PTEN	130				0.005
Loss		68 (52.3)	43 (64.2)	25 (39.7)	
No loss		62 (47.7)	24 (35.8)	38 (60.3)	
G, TGFA					

Clinicopathological parameter	n	mRNA expression[a]			P-value
		Total	Low	High	
Age at trastuzumab initiation, years	138	55.6 (28.9,85.9)	57.7 (28.9,85.9)	52.8 (30.9,83.7)	0.021
≤50		45 (32.6)	16 (23.2)	29 (42.0)	0.018
>50		93 (67.4)	53 (76.8)	40 (58.0)	
Menopausal status	137				0.019
Postmenopausal		99 (72.3)	56 (81.2)	43 (63.2)	
Premenopausal		38 (27.7)	13 (18.8)	25 (36.8)	

[a]Median value as a cut-off. Values presented as median (min, max) or n (%). EGF, epidermal growth factor; EREG, epiregulin; IGFBP4, insulin like growth factor binding protein 4; NRG1, neuregulin 1; RARA, retinoic acid receptor α; TGFA, transforming growth factor α; TGFB1, transforming growth factor β1; THRA, thyroid hormone receptor α. PTEN, phosphatase and tensin homolog.