

Estrogen receptor alpha (*ESR1*) gene amplification is frequent in breast cancer

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Using an Affymetrix 10K SNP array to screen for gene copy number changes in breast cancer, we detected a single-gene amplification of the *ESR1* gene, which encodes estrogen receptor alpha, at 6q25. A subsequent tissue microarray analysis of more than 2,000 clinical breast cancer samples showed *ESR1* amplification in 20.6% of breast cancers. Ninety-nine percent of tumors with *ESR1* amplification showed estrogen receptor protein overexpression, compared with 66.6% cancers without *ESR1* amplification ($P < 0.0001$). In 175 women who had received adjuvant tamoxifen monotherapy, survival was significantly longer for women with cancer with *ESR1* amplification than for women with estrogen receptor-expressing cancers without *ESR1* amplification ($P = 0.023$). Notably, we also found *ESR1* amplification in benign and precancerous breast diseases, suggesting that *ESR1* amplification may be a common mechanism in proliferative breast disease and a very early genetic alteration in a large subset of breast cancers.

Breast cancer is the leading malignancy in women, accounting for more than 350,000 deaths per year worldwide¹. Several molecular pathways are known to have a role in breast cancer development and progression. Perhaps the most important pathway involves estrogen receptor alpha protein. The causal relationship between estrogen receptor expression and cellular responsiveness to estrogens and antiestrogens has been extensively studied in cell lines², animal

models³ and humans⁴ and makes estrogen receptor one of the most important therapeutic targets in breast cancer⁵. More than two-thirds of breast cancers show estrogen receptor expression at the time of diagnosis⁶, and immunohistochemical detection of estrogen receptor expression is routinely used in making decisions on hormonal therapy (anti-estrogen receptor) for breast cancer⁷. Current anti-estrogen receptor treatment strategies include blocking by selective modulators (such as tamoxifen and raloxifene), destabilization and degradation of estrogen receptor by selective downregulators (such as fulvestrant) and disruption of estrogen synthesis (aromatase inhibitors, such as anastrozole, letrozole or exemestane), any of which alone can result in a substantial decrease of tumor growth in about 30%–50% of estrogen receptor-positive patients⁸.

Increasing gene copy number (amplification) is a major mechanism for cancer cells to boost the expression of gene products that provide them with a growth or survival advantage. Numerous genes have been

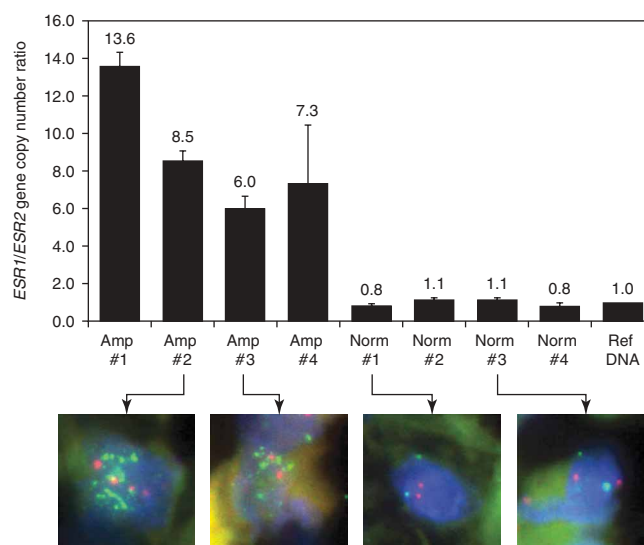


Figure 1 qPCR-based determination of *ESR1* gene copy numbers in tumors with *ESR1* amplification ('Amp #1' through 'Amp #4') and without amplification ('Norm #1' through 'Norm #4') selected according to FISH analysis. DNA from lymphocytes ('Ref DNA') was included as a calibrator for a normal *ESR1/ESR2* gene copy number ratio. FISH images show cell nuclei (blue) from selected cases hybridized with probes directed against *ESR1* (green, RP11-450E24) and centromere 6 (red). *ESR1* amplification is seen as clusters of green signals ('Amp #2') or as a ratio of green to red signals that is >2 ('Amp #3'). Error bars represent s.e.m. from triplicate analysis.

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Received 12 December 2006; accepted 1 March 2007; published online 8 April 2007; doi:10.1038/ng2006

Table 1 Relationship between *ESR1* copy number changes and invasive breast cancer phenotype

		<i>ESR1</i> FISH results				Estrogen receptor immunohistochemistry results			
		On array (<i>n</i>)	Analyzable (<i>n</i>)	Amplification	Gain	<i>P</i>	Analyzable (<i>N</i>)	Positive ^a	<i>P</i>
All cancers		2,197	1,739	20.6%	15.3%		2,018	76.6%	
Histology	Ductal carcinoma	1,552	1,207	21.5%	16.0%		1,429	77.1%	
	Lobular carcinoma	312	207	19.3%	13.8%		275	87.6%	
	Mucinous carcinoma	69	37	35.6%	24.4%	0.0337 ^b	61	93.4%	<0.0001 ^b
	Medullary carcinoma	58	48	2.0%	2.0%	<0.0001 ^b	52	17.3%	<0.0001 ^b
	Tubular carcinoma	56	42	18.6%	14.0%		48	89.6%	
	Cribiform carcinoma	65	55	29.8%	12.3%		56	91.1%	
	Papillary carcinoma	30	27	19.2%	15.4%		28	67.9%	
	Others ^a	79	56	4.9%	14.8%		69	34.8%	
pT stage	pT1	820	578	22.3%	15.6%	0.7295	716	80.4%	0.0020
	pT2	1,023	811	19.7%	14.8%		948	73.2%	
	pT3	124	92	18.8%	13.5%		114	72.8%	
	pT4	242	189	17.8%	16.8%		229	80.3%	
Nodal stage	pN0	950	711	22.3%	14.4%	0.0422	849	78.1%	0.1765
	pN1	793	608	16.5%	16.5%		726	75.6%	
	pN2	121	90	14.8%	12.5%		113	70.8%	
BRE grade	G1	545	421	25.6%	15.2%	<0.0001	522	92.9%	<0.0001
	G2	844	685	21.6%	18.5%		833	86.3%	
	G3	655	571	15.0%	11.7%		661	51.9%	

^aAccording to Allred score. ^bVersus ductal carcinoma.

found amplified in a fraction of breast cancers, including *ERBB2* at 17q21 (ref. 9). The example of *ERBB2* and the success of trastuzumab (Herceptin) for treatment of Her2-amplified or Her2-overexpressing breast cancers also shows that amplified genes may be particularly suited as therapeutic targets¹⁰.

To investigate the clinical relevance of *ESR1* amplification, we used FISH to analyze a tissue microarray (TMA) containing more than 2,000 breast cancers¹¹, using an *ESR1*-specific probe prepared from BAC RP11-450E24. We scored FISH results manually. This method is optimal for analysis of tissue sections and results in high interlaboratory agreement^{12,13} and clinically robust data^{14–17}. Based on the generally accepted scoring system used for *ERBB2* amplification evaluation in US Food and Drug Administration (FDA)-approved test kits (PathVysion), we considered *ESR1* amplification to be present if there were at least twice as many signals for *ESR1* as for centromere 6. Because exact signal number counts are difficult in cases of intrachromosomal amplification, tumors with tight signal clusters were also rated as amplified, independent of their *ESR1*–centromere 6 ratio. We classified tissue samples with an *ESR1*/centromere 6 ratio greater than 1.0 but less than 2.0 as having ‘*ESR1* gains’. All other tissues (*ESR1*/centromere 6 ratio ≤ 1.0) were considered normal. According to these criteria, we found *ESR1* amplification in 358/1,739 (20.6%) analyzable tissue samples. *ESR1* copy number gains were present in another 266 (15.3%) tumors. To validate gene amplification by an independent method, we designed a quantitative PCR (qPCR) assay to compare DNA copy numbers of *ESR1* and *ESR2* in four tumor samples with *ESR1* amplifications and four tumor samples without amplification (as determined by FISH). We selected *ESR2* as a reference gene because it showed normal gene copy numbers in all eight tumors, as determined by FISH (data not shown). We included a commercially available reference DNA in the experiment as a calibrator for normal *ESR1* and *ESR2* gene copy numbers. qPCR analysis confirmed the presence or absence of *ESR1* amplification in all eight tumor samples. In amplified samples, we measured 6- to 14-fold

increases in gene copy numbers (corresponding to 12–26 copies of the *ESR1* gene; Fig. 1).

Using the same breast cancer TMA and the same definition of amplification, we have previously found amplifications of *CCND1* in 20.7% of the tumors, *ERBB2* in 17.8%, *MDM2* in 6.1%, *MYC* in 5.1% and *EGFR* in 0.8% (ref. 18). *ESR1* amplification was unrelated to the presence of other amplifications, except for a weak association with *CCND1*; we found *ESR1* amplification in 77/324 (23.7%) tumors with *CCND1* amplification but in only 233/1,240 (18.8%) tumors without *CCND1* amplification ($P = 0.05$, Supplementary Table 1 online). This was expected, as previous studies had already demonstrated that *CCND1* amplification occurred preferentially in estrogen receptor-positive cancers¹⁹.

Most amplified tumors showed a clustered arrangement of additional *ESR1* copies, indicating intrachromosomal amplification (homogeneously staining regions-type amplification). *ESR1*

Table 2 Prevalence of *ESR1* copy number changes in normal and premalignant breast tissues

Histology	On array (<i>n</i>)	Analyzable (<i>n</i>)	<i>ESR1</i> FISH result	
			Amplification (%)	Gain (%)
Normal breast tissue	50	21	0.0	0.0
Fibrocystic disease	22	13	0.0	7.7
Apocrine metaplasia	14	4	0.0	0.0
Usual ductal hyperplasia	27	12	8.3	25.0
Atypical ductal hyperplasia	5	1	0.0	100.0
Sclerosing adenosis	15	8	0.0	0.0
Papilloma	31	22	36.4	4.5
DCIS	62	40	35.0	7.5
LCIS (lobular neoplasia)	10	3	33.3	33.3

Table 3 Comparison of estrogen receptor amplification and expression

	<i>ESR1</i> FISH results	Estrogen receptor immunohistochemistry results (Allred score)			
		<i>n</i>	0–2 (%)	3–4 (%)	5–6 (%)
Normal	1,056	33.3	8.2	14.1	44.3
Gain	255	2.4	1.6	7.5	88.6
Amplified	341	0.6	0.3	4.7	94.4

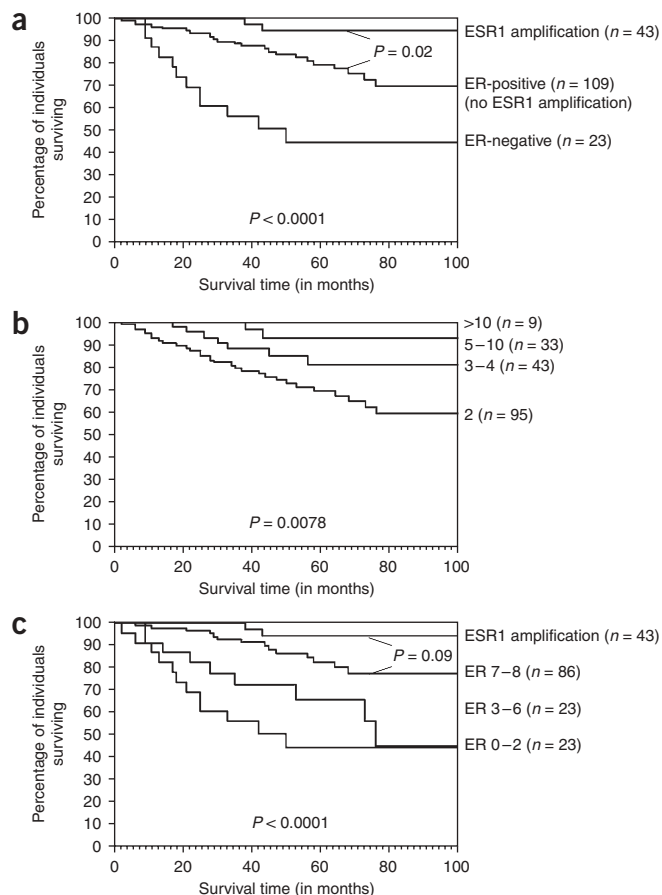
alterations were significantly associated with low-grade tumors ($P < 0.0001$) and absence of lymph node metastases ($P < 0.05$) (Table 1). Medullary cancers, which are characterized by a high-grade phenotype and which belong to the basal type of breast cancer, had a particularly low fraction of amplified tumors (2.0%), whereas mucinous cancers that are mostly low grade had particularly high rates of *ESR1* amplifications (35.6%). Because of the association of *ESR1* amplification with low-malignancy cancer phenotypes, we expanded our analysis to a series of nonmalignant and preneoplastic tissues (Table 2). Most notably, we saw a high frequency of *ESR1* amplification in benign papillomas (8/22, 36%), but amplification was also present in usual ductal hyperplasias (1/12, 8.3%). These findings would be consistent with a very early, if not initializing, role for estrogen receptor amplification for a subset of breast cancers. Thus far, gene amplification has never been demonstrated in benign breast lesions. The data suggest that *ESR1* amplification is not a hallmark for invasive breast cancer but may represent a key mechanism for various types of proliferative breast disease. As gene amplification is thought to be nonreversible, it is tempting to speculate that estrogen receptor amplification may constitute a decisive mechanism for initiation of neoplastic (clonal) breast disease. Therefore, it seems possible that the presence of *ESR1* amplification may be indicative of proliferative breast disease with increased potential for malignant transformation. This hypothesis would be consistent with previous immunohistochemical studies suggesting a possible link between high estrogen receptor expression in benign proliferative breast disease and an increased risk for breast cancer development^{20,21}. Thus, it is possible that *ESR1* amplification status represents a useful prognostic marker in individuals with benign-appearing proliferative breast lesions.

To investigate the impact of *ESR1* amplification on estrogen receptor protein levels, we next compared *ESR1* gene copy numbers to estrogen receptor protein expression levels by immunohistochemistry. Immunohistochemical analysis was successful in 2,018/2,197 (92%) breast cancers (Table 1). All immunohistochemical staining was scored on the same day by one pathologist (G.S.) to minimize potential variations between observers. As expected, *ESR1* amplification was tightly linked to estrogen receptor protein expression ($P < 0.0001$, Table 3). Virtually all tumors with increased *ESR1* gene copy numbers (amplifications and gains) had high expression of estrogen

Figure 2 Impact of *ESR1* amplification (defined as an *ESR1*/centromere 6 copy number ratio ≥ 2.0) and expression on prognosis in affected individuals who received tamoxifen monotherapy. (a) Impact of estrogen receptor status and *ESR1* amplification on survival for three groups of cancers: estrogen receptor (ER)-negative (Allred scores of 0–2), estrogen receptor-positive status (Allred scores of 3–8, with no *ESR1* amplification) and *ESR1*-amplified. (b) Impact of *ESR1* copy number on survival. (c) Immunohistochemistry results by Allred scores versus *ESR1* amplification.

receptor protein. Among 341 breast cancers with *ESR1* amplification, 339 (99%) had detectable estrogen receptor expression. The vast majority of these tumors (94%) had the highest possible estrogen receptor scores (7–8) according to Allred²². This was also true for tumors showing *ESR1* gains: 89% of these samples showed strong estrogen receptor expression (Allred scores of 7–8). However, the data also showed that *ESR1* amplifications or gains were not the sole reason for high estrogen receptor expression. Almost half (46%) of strong estrogen receptor expressers (Allred score of 7–8) did not have any *ESR1* gene copy number alterations.

The availability of 175 breast cancers from affected individuals who had undergone adjuvant anti-estrogen receptor monotherapy with tamoxifen allowed us to investigate the possible impact of *ESR1* amplification on response to anti-hormonal therapy. For this analysis, we first stratified the individuals into three groups: (i) estrogen receptor-negative cancers (Allred scores of 0–2), (ii) estrogen receptor-positive cancers (Allred scores of 3–8) lacking *ESR1* amplification and (iii) cancers with *ESR1* amplification. Despite the small size of this cohort, the retrospective nature of data collection and the potentially variable duration of tamoxifen therapy, there was a difference in survival between individuals with tumors with *ESR1* amplification versus those with estrogen receptor-positive tumors (as determined by immunohistochemistry) without *ESR1* amplification (Allred scores of 3–8, $P = 0.02$, Fig. 2a). To exclude that the observed prognostic difference was caused by classical prognostic factors of breast cancer, we performed a multivariate analysis (Cox proportional hazards) with estrogen receptor and progesterone receptor expression, tumor grade and pT and pN categories as variables. We found that the prognostic



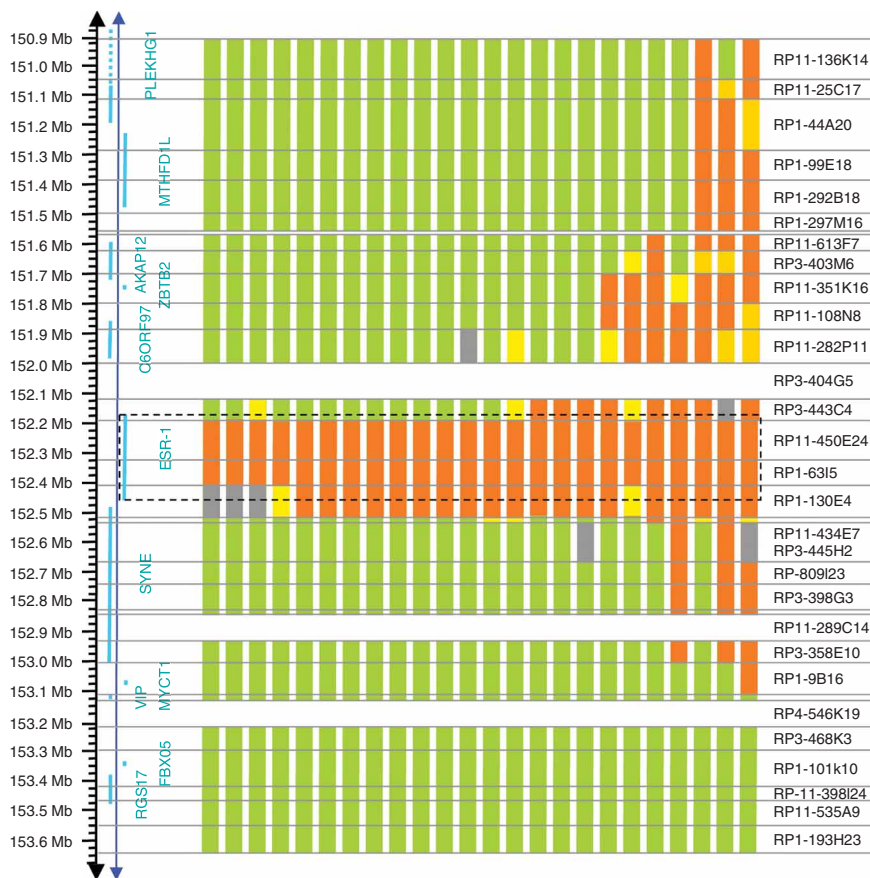


Figure 3 Size and position of the *ESR1* amplicon in 24 breast cancer samples. Genomic localization is given as distance in Mb from the p-telomere of chromosome 6 (according to National Center for Biotechnology Information (NCBI) Mapview build 3.6). Loci of genes are indicated in blue. Bold bars represent FISH findings in 24 breast cancers using probes corresponding to the indicated BAC clones (gray letters). Green indicates normal copy number; yellow, gain; red, amplification; gray, analysis failed. Gaps indicate positions of clones that were not available for analysis.

a 6q25.1 amplification (among 30 other amplicons) containing *ESR1* in 3 of 31 analyzed breast cancers²⁴. To a large extent, the difficulties detecting the 6q25.1 amplicon seem to be caused by its generally small size, which makes it difficult to identify even with high-resolution CGH arrays. To precisely determine the size and localization of the amplicon, we conducted additional FISH analyses in 24 *ESR1*-amplified breast cancers using 31 probes covering a ~2.7-Mb genomic region from approximately 1 Mb centromeric to 1 Mb telomeric of the *ESR1* gene locus (Fig. 3). We analyzed all but four small regions (RP1-236H13, RP3-404G5, RP11-133I21 and RP1-200K18; BAC clones were not available for these regions). FISH mapping confirmed that the amplicon is usually small, spanning approximately 600 kb (RP3-

443C3–RP1-130E4) and including *ESR1* and a 100–200 kb upstream region that does not encode any genes in the majority of cases. We were not able to confirm amplification of a 16-kb fragment, which includes exon 1 of *ESR1* (part of RP3-443C3), in all cases. This may be due to this FISH probe's lack of sensitivity to detect amplifications potentially covering only a small part of the probed sequence. However, we confirmed the presence of amplification in exon 1 by qPCR in four of these tumors (Fig. 1).

Our study, based on a 'fishing expedition', demonstrates that one of the most frequently analyzed and best-characterized genes in breast cancer (with more than 30,000 PubMed entries as of November 2006) is a common subject of gene amplification. The availability of large, well-characterized TMAs allowed us to study the epidemiology of *ESR1* amplifications extensively. Although we analyzed only one small tissue sample (with a diameter of 0.6 mm) per tumor, we detected *ESR1* amplification in >20% of breast cancers. The marked association between *ESR1* amplification and positive estrogen receptor immunohistochemistry (99% of our tumors with amplification are estrogen receptor positive by immunohistochemistry) or estrogen receptor expression levels not only validates our experimental approach but also demonstrates the strong functional importance of *ESR1* gene amplification. We did not detect mutations in *ESR1* in four amplified and four nonamplified cancers (data not shown), arguing against a major role for *ESR1* sequence alterations for estrogen receptor protein overexpression in amplified cancers.

In summary, our data suggest that *ESR1* amplification is a frequent event in proliferating breast disease and breast cancer. *ESR1* amplification might be instrumental in defining a subtype of primary breast

value of *ESR1* amplification in tamoxifen-treated individuals was independent of these factors (Supplementary Table 2 online). Response to tamoxifen was also dependent on the absolute *ESR1* copy number (Fig. 2b). However, combinations of *ESR1* and other markers, including amplification of *CCND1*, *ERBB2*, *EGFR*, *MDM2* and *MYC*, did not show any impact on prognosis or response to tamoxifen treatment (data not shown). To evaluate a potential bias caused by the high fraction of strongly estrogen receptor-positive tumors (>90%) in the group with *ESR1* amplification group, we repeated the analysis, separating estrogen receptor-positive tumors into groups according to Allred score (Fig. 2c). This small subset of cancers retained a trend toward superior prognosis of tumors with *ESR1* amplification as compared with tumors without amplification but with highest estrogen receptor expression (Allred scores of 7–8), but the difference was not statistically significant ($P = 0.09$). Altogether, these data strongly suggest that *ESR1* amplification may identify a subgroup of breast cancers with high estrogen receptor expression as being particularly likely to respond to anti-estrogen therapy. We found this result slightly unexpected, because an opposite mechanism is well known to occur in prostate cancer. In this tumor type, amplification of the androgen receptor does not occur in untreated primary tumors but develops in about 20%–30% of individuals undergoing antihormonal therapy and causes resistance to additional antihormonal therapy²³.

Given the critical role of estrogen receptor expression for breast cancer therapy and the importance of gene amplification for drug target overexpression, we find it remarkable that the relevance of *ESR1* amplification was not discovered until now. Only one matrix comparative genomic hybridization (CGH) study had previously described

cancers, and perhaps other proliferative breast diseases, that have particularly high estrogen receptor expression and that might be optimally suited for hormonal therapy.

METHODS

Tissues. We analyzed an existing breast prognosis TMA containing more than 2,000 breast tissues¹¹. The pathohistological and clinical data from these tissues are summarized in **Table 1**. The type of adjuvant treatment was known for 420 affected individuals. A subset of 261 affected individuals received hormonal treatment with tamoxifen (the standard length of treatment was 5 years, but detailed information was not available). In addition, we analyzed a TMA containing 50 normal breast samples and 186 non-neoplastic and premalignant breast lesions, including 62 ductal carcinoma *in situ*, 10 LCIS (lobular neoplasias), 14 apocrine metaplasias, 27 usual ductal hyperplasias, 5 atypical ductal hyperplasias, 22 cases of fibrocystic disease, 31 papillomas and 15 cases of sclerosing adenosis.

FISH. TMA sections were treated according to the Paraffin Pretreatment Reagent Kit protocol (Vysis) before hybridization. For the breast cancer TMA study, FISH was performed with a digoxigenin-labeled BAC probe (BAC RP11-450E24, RZPD) containing the *ESR1* gene and a Spectrum Orange-labeled chromosome 6 centromeric probe (CEP6) as a reference (purchased from Vysis). Hybridization and post-hybridization washes were according to the Vysis LSI procedure. Probe visualization using fluorescent isothiocyanate (FITC)-conjugated sheep anti-digoxigenin (Roche Diagnostics) was as described²⁵. Slides were counterstained with 125 ng ml⁻¹ 4',6-diamino-2-phenylindole in an antifade solution. Hybridization and post-hybridization washes were according to the Vysis LSI procedure. Slides were then counterstained with 125 ng ml⁻¹ 4',6-diamino-2-phenylindole in an antifade solution. All BACs used for the amplicon mapping study are listed in **Figure 3**.

Immunohistochemistry. Immunohistochemical detection of estrogen receptor alpha protein was performed using antibody NCL-L-ER-6F11 (Novocastra). In brief, slides were deparaffinized and incubated in a pressure cooker at 120 °C for 12 min in pH 6 citrate buffer (Retrievit 6 #BS-1006-00, BioGenex). After blocking of endogenous peroxidase, prediluted (1:1,000) primary antibody was applied, and the slides were incubated overnight at 4 °C. The Vectastain ABC Elite system was used for detection of antibody binding. Immunohistochemistry was scored according to the Allred scale²². In brief, estrogen receptor staining intensity was recorded on a four-step scale (0–3) and the fraction of estrogen receptor positive tumor cells on a five-step scale (1–5). Combination of both parameters resulted in an eight-step score, where all samples with score >2 are regarded as estrogen receptor positive.

qPCR for *ESR1* amplification detection. qPCR was performed in triplicate using combinations of primer pairs and TaqMan probes targeting genomic sequences in the *ESR1* (exon 1) and *ESR2* (exon 5) genes. The sequences of PCR primers and hybridization probes are listed in **Supplementary Table 3** online. The PCR program included a 10-min denaturation at 94 °C followed by 40 cycles of 15 s at 72 °C and 1 min at 60 °C. The nonamplified *ESR2* gene served as an internal control for the normalization of *ESR1* PCR products. Relative quantification results were calculated according to the $\Delta\Delta C_t$ method.

***ESR1* sequence analysis.** Four breast cancers showing *ESR1* amplification and four breast cancers with normal copy numbers (same as for qPCR analysis) were selected for *ESR1* mutation analysis. We deparaffinized the formalin-fixed tissues and extracted DNA according to Qiagen's protocols. All *ESR1* exons (1–8) were amplified by PCR and sequenced directly using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Primers used for PCR and sequencing are listed in **Supplementary Table 3**. Sequencing products were analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Statistics. Contingency table analysis and χ^2 tests were used to study the relationship between histological tumor type, grade, stage and gene amplification. Survival curves were plotted according to Kaplan-Meier. A log-rank test was applied to examine the relationship between gene amplifications and

patient survival. Cox regression analysis was performed to test for interdependencies between analyzed variables and their relationship to patient survival.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank the University Medical Center Hamburg Eppendorf for providing an outstanding environment for research. We are grateful to S. Schnöger, A. Blaszczyk-Wewer, G. Rieck, M. Sachs, S. Schmidt, M. Härtling and M. Mirlacher for technical support.

AUTHOR CONTRIBUTIONS

F.H., P.S. and C.R. produced the *ESR1* FISH probes, performed FISH analysis and contributed to the writing of the paper; Z.J. contributed to FISH analysis; M.W. and F.H. performed amplicon mapping; F.H. and O.H. performed qPCR analysis; A.L. and L.T. performed estrogen receptor immunohistochemistry analysis; F.J. contributed to data analysis and interpretation; K.A.-K. and G.S. selected tissues and performed histological diagnosis and R.S. and G.S. designed the study and contributed to the writing of the paper.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/naturegenetics.

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