Estrogen Receptor Alpha Gene Amplification Is an Independent Predictor of Long-Term Outcome in Postmenopausal Patients with Endocrine-Responsive Early Breast Cancer

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ABSTRACT

Purpose: Estrogen receptor (ER) expression is a prognostic parameter in breast cancer, and a prerequisite for the use of endocrine therapy. In ER^+ early breast cancer, however, no receptor-associated biomarker exists that identifies patients with a particularly favorable outcome. We have investigated the value of *ESR1* amplification in predicting the long-term clinical outcome in tamoxifen-treated postmenopausal women with endocrine-responsive breast cancer.

Experimental Design: 394 patients who had been randomized into the tamoxifen-only arm of the prospective randomized ABCSG-06 trial of adjuvant endocrine therapy with available formalin-fixed, paraffin-embedded tumor tissue were included in this analysis. IHC ERα expression was evaluated both locally and in a central lab using the Allred score, while *ESR1* gene amplification was evaluated by FISH analysis using the *ESR1/CEP6* ratio indicating focal copy number alterations.

Results: Focal *ESR1* copy-number elevations (amplifications) were detected in 187 of 394 (47%) tumor specimens, and were associated with a favorable outcome: After a median follow-up of 10 years, women with intratumoral focal *ESR1* amplification had a significantly longer distant recurrence-free survival [adjusted HR, 0.48; 95% confidence interval (CI), 0.26–0.91; $P = 0.02$] and breast cancer–specific survival (adjusted HR 0.47; 95% CI, 0.27–0.80; $P = 0.01$) as compared with women without *ESR1* amplification. IHC ERα protein expression, evaluated by Allred score, correlated significantly with focal *ESR1* amplification ($P < 0.0001$; χ^2 test), but was not prognostic by itself.

Conclusions: Focal *ESR1* amplification is an independent and powerful predictor for long-term distant recurrence-free and breast cancer–specific survival in postmenopausal women with endocrine-responsive early-stage breast cancer who received tamoxifen for 5 years.

Introduction

The use of validated gene expression assays can help oncologists to assess recurrence risks in endocrine-treated hormone-receptor positive (HR^+), human epidermal growth factor 2–negative ($HER2^-$) early-stage breast cancer, and to identify patients who are likely to benefit from the addition of chemotherapy to standard endocrine therapy. Most of the newer assays use algorithms that are based on the expression of several proliferation- and estrogen-associated genes, and mainly rely on the prognostic usage of genes arbitrarily identified by nonhierarchical clustering (1).

Disappointingly, however, despite decades of research, there is still no single ERα-associated biomarker that can be used to identify subgroups of HR^+ early breast cancer patients with a particularly good or poor outcome.

Amplification (increased copy number) of the *ESR1* gene encoding ERα has been reported in up to about 30% of early breast cancers depending on detection and scoring methods (2–10). Some studies have suggested that *ESR1* amplification as detected by FISH could identify a subset of cancers that might respond particularly well to anti-ER treatment (2, 3), but others linked *ESR1* amplification to therapy resistance rather (5, 7, 11).

It is likely that the compilation of patient cohorts and different detection methods, as well as variable definitions of *ESR1* amplification, have contributed to these discrepant findings (7–9). In order to investigate whether *ESR1* amplification is associated with long-term outcome in endocrine-responsive early breast cancer, we have therefore analyzed breast cancer samples from the prospective clinical ABCSG-6 trial for *ESR1* copy-number changes using simple scoring criteria and a commercially available *ESR1* probe.

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Translational Relevance

Estrogen receptor (ER) expression is a favorable prognostic parameter in breast cancer, and a predictor for response to endocrine therapy. Within the subgroup of ER$^+$ tumors, however, no receptor-associated biomarker exists which identifies patients with a particularly good long-term outcome. By using tumor tissues from tamoxifen-treated postmenopausal women with endocrine-responsive, early breast cancer, who were randomized into the prospective ABCSG-06 phase III study, we have investigated the value of *ESR1* amplification in predicting the long-term clinical outcome. We found that focal *ESR1* amplification is an independent and powerful predictor for long-term distant recurrence-free and breast cancer–specific survival in postmenopausal women with endocrine-responsive early-stage breast cancer who receive adjuvant endocrine therapy. These results may guide therapeutic strategies in patients with ER$^+$/HER2$^-$ early breast cancer and contribute to our understanding of appropriate patient selection.

Materials and Methods

This study is part of the ABCSG translational research program (abcsg.research). 537 women included in this study had been randomized into the prospectively randomized adjuvant endocrine trial ABCSG-6 between 1990 and 1995, and had received 5 years of adjuvant tamoxifen (12). Approximately 50% of patients participating in that trial were subsequently rerandomized to receive 3 years of extended anastrozole versus no further treatment (ABCSG-6a; ref. 13). For the purpose of the main analysis, these patients were censored at the time of their follow-up when they were entered into ABCSG-6a to ensure treatment homogeneity of this study (i.e., none of the patients in this analysis had been exposed to any endocrine treatment other than 5 years of tamoxifen). Notably, none of the HER2-positive patients had received HER2-directed therapy. Eventually, 394 patients who received tamoxifen monotherapy and had tumor blocks available were included in this study. In an additional exploratory analysis, we investigated the long-term outcome of 254 patients who had not experienced a recurrence by the end of 5 years of adjuvant tamoxifen, and who had subsequently been re-randomized to receive either 3 additional years of anastrozole or no further treatment as part of the ABCSG6a extension study. Trial design, inclusion criteria and the main clinical results of these trials have been previously reported (12, 13). Formalin-fixed, paraffin-embedded (FFPE) tumor blocks were collected from participating centers at the time of surgery and were stored at room temperature. Ethical Approval was obtained from Institutional Review Boards. Written informed consent forms were obtained from every patient prior to participation in the described trial.

ER IHC

Freshly cut 4-μm tissue sections were used for ERα IHC analysis as previously described (2). IHC detection of ERα protein was performed using the antibody NCL-L-ER-6F11 (Novocastra). In brief, slides were deparaffinized and subjected to antigen retrieval in a pressure cooker at 120°C for 12 minutes in citrate buffer at pH 6. The primary antibody NCL-L-ER-6F11 (Novocastra) was prediluted 1:1,000 and incubated overnight at 4°C. The Vectastain ABC Elite system was used for detection of antibody binding. IHC scoring was performed according to the Allred score (14, 15). In brief, ERα staining intensity was recorded on a 4-tiered scale (0–3) and the percentage of ERα-positive tumor cells on a 5-tiered (1–5) scale. Addition of both parameters resulted in an 8-tiered score, and a score of >2 was considered positive.

FISH

Large FFPE sections were treated using the ZytoLightSPEC ESR1/CEN 6 Dual Color Probe Kit (Zytovision) according to the manufacturer's instructions with minor modifications: Slides were heated overnight at 58°C before deparaffinization. Probe hybridization time at 37°C was extended to 48–72 hours.

Evaluation of *ESR1* amplification status

A pathologist marked areas for FISH scoring on a consecutive hematoxylin and eosin–stained reference slide. All slides were centrally analyzed by an experienced scientist. Tumor regions with clearly detectable FISH signals for both *ESR1* and *CEP6* were selected for analysis. In case of tumor heterogeneity of elevated *ESR1/ CEP6* signal ratios, the areas with the highest ratios were chosen. In 10 to 20 representative tumor cell nuclei showing distinguishable FISH signals, the number of *ESR1* and *CEP6* signals was determined. The average number of *ESR1* and centromere 6 (*CEP6*) signals were used to calculate the *ESR1/CEP6* copy-number ratio indicating focal copy number increase. High-level *ESR1* amplification was defined as an *ESR1/CEP6* ratio ≥2.0, and elevated *ESR1/CEP6* copy-number ratios from ≥1.3 to <2 were considered as low-level amplification (gains; refs. 6, 16, 17). Tumors with an *ESR1/CEP6* ratio of <1.3 were classified as "not amplified." For statistical analysis, tumors with *ESR1* low- and high-level amplification (see representative examples in **Fig. 1**) were combined into one group of *ESR1* amplification ("increased *ESR1* copy number") in the predefined analysis plan.

Statistical analysis

The primary endpoints of the statistical analyses were distant recurrence-free survival (DRFS) and breast cancer–specific survival (BCSS). DRFS was defined as the interval between the date of surgery and the first evidence of relapse at any distant site. Because of the median age of 65 at trial initiation and the long-term follow-up, patients were censored if they had, in the absence of breast cancer recurrence, died from confirmed reasons unrelated to their malignancy. Baseline data, dichotomized according to *ESR1* amplification status (increased versus normal copy numbers), were compared in univariate analyses using the χ^2 test and in a multiple logistic model. Survival rates were estimated using the Kaplan–Meier method. The prognostic value of *ESR1* amplification status (including low- and high-level amplifications in separate or combined analysis) was studied using univariate and multivariable Cox models. Interaction terms between *ESR1* copy number and HER2 were assessed in Cox models. All *P* values are shown as the results of two-sided tests. A *P* value of ≤0.05 was considered statistically significant. All statistical analyses were performed using SPSS software version 15.0 (SPSS, Inc.)

Data availability statement

Data were generated by the authors. The dataset used is not publicly available as it may contain information that would compromise patient consent. Please contact the corresponding author for more information and to request access to these data.

Results

Prevalence of focal *ESR1* amplification

Of the 996 female patients with early breast cancer who were randomized into the tamoxifen-only arm in ABCSG 6, FFPE tumor samples were available in 537. Of these, *ESR1* gene and centromere 6

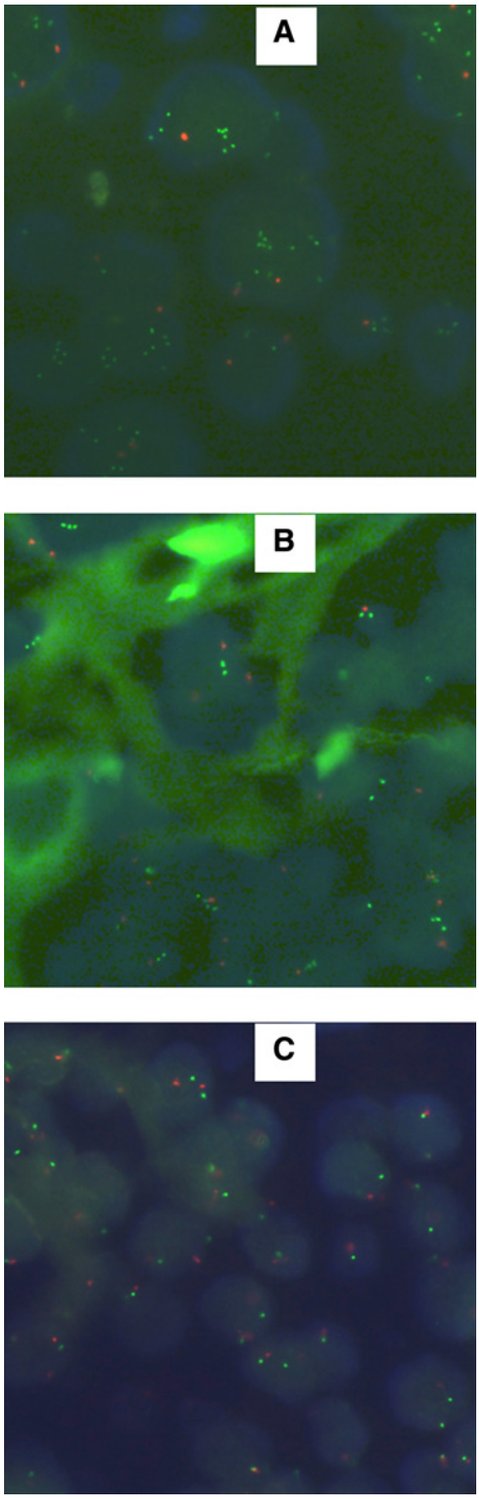

Figure 1.
Representative examples of *ESR1* status in breast carcinomas as determined by FISH. **A,** High-level *ESR1* amplification (4–10 copies per nucleus). **B,** Low-level *ESR1* amplification (1–4 gene copies per nucleus. 28–30 *ESR1* signals to 22 *CEP6* signals shown altogether). **C,** Normal, copy number not increased. The red signals correspond to centromere.

copy numbers were assessable in 394 cases (**Fig. 2**). Focal low-level (9%) and high-level (38%) amplifications were detected in 187 of 394 interpretable cancers (47%). Eight of 394 (2%) samples exhibited a ratio of <1, while the vast majority (272/394; 69%) ESR1/CEP6 ratios ranged from 1.0 to 2.0. (**Fig. 3**). Because there was no statistically significant difference in survival between tumors with *ESR1* low- and high-level amplifications (HR for relapse, 4.93; 95% CI, 0.66–36.64, $P = 0.12$; HR for death, 2.49; 95% CI, 0.58–10.62, $P = 0.22$), we combined these tumors into one group of tumors with *ESR1* amplification (increased copy number) for further analysis. *ESR1* amplification was significantly linked to patient age >60 ($P = 0.006$) but were unrelated to breast cancer tumor size, tumor grade, or nodal status. These data are summarized in **Table 1**.

Association between *ESR1* amplification and ERα protein expression

Increased *ESR1* copy numbers (amplification) were significantly correlated with ERα protein expression both, when measured locally in participating trial centers using the Remmele Score ($P = 0.001$, χ^2 test), and when measured centrally using the Allred score ($P < 0.0001$, χ^2 test). With one exception (4%) out of 24 samples, we did not observe low- or high-level *ESR1* amplifications in confirmed ERα-negative tumors (i.e., Allred score of 0–2), while *ESR1* amplification was found in 127 of 206 (62%) tumors with an Allred score of 7–8 (**Table 2**).

Prognostic relevance

Median follow-up of the patients was 10 years. Tumor size, nodal status, and *ESR1* amplification were significantly associated with DRFS in univariate analyses (**Table 3**).

Likewise, tumor size, nodal status, tumor grade, and *ESR1* amplification were significantly associated with BCSS in univariate analysis (**Table 3**). The independent effect of *ESR1* amplification on DRFS and BCSS was assessed by multivariable Cox proportional Hazard models adjusted for age, tumor size, nodal status, tumor grade, and HER2. In these multivariable analyses, *ESR1* amplification remained significantly associated with prolonged DRFS (adjusted HR for relapse, 0.48; 95% CI, 0.26–0.91; $P = 0.02$) and improved breast cancer–specific survival (adjusted HR for death, 0.47; 95% CI, 0.27–0.80; $P = 0.01$) when compared with women with tumors exhibiting normal *ESR1* copy numbers. Within the group of *ESR1*-amplified tumors, however, we did not observe an association between the level of amplification and outcome for both, DDFS (adjusted HR, 0.88; 95% CI, 0.70–1.13; $P = 0.317$) and BCSS (adjusted HR, 0.89; 95% CI, 0.72–1.46; $P = 0.89$)

In contrast, assessment of ERα protein by Allred score did not allow for discrimination between DRFS and BCSS in tamoxifen-treated women (adjusted HR for relapse, 0.86; 95% CI, 0.59–1.25; $P = 0.43$ and adjusted HR for death, 0.85; 95% CI, 0.58–1.23; $P = 0.38$; **Fig. 4A–D**).

No significant interaction was observed between *ESR1* amplification and HER2 with respect to DRFS ($P_{\text{interaction}} = 0.63$) and BCSS ($P_{\text{interaction}} = 0.82$). We then investigated possible associations between *ESR1* amplification and DRFS and BCSS in relation to the patient's nodal status, and found that *ESR1* amplification was associated with an improved DRFS and BCSS in nodal-positive tumors, while this correlation was absent in nodal-negative tumors (**Fig. 5A–D**).

We also performed an additional outcome analysis in patients who had not experienced a recurrence by the end of 5 years of adjuvant tamoxifen had been rerandomized to receive either 3 additional years of anastrozole ($n = 125$; 51%) or no further ($n = 120$; 49%) treatment. We did not find a significant interaction between AI intake and *ESR1*

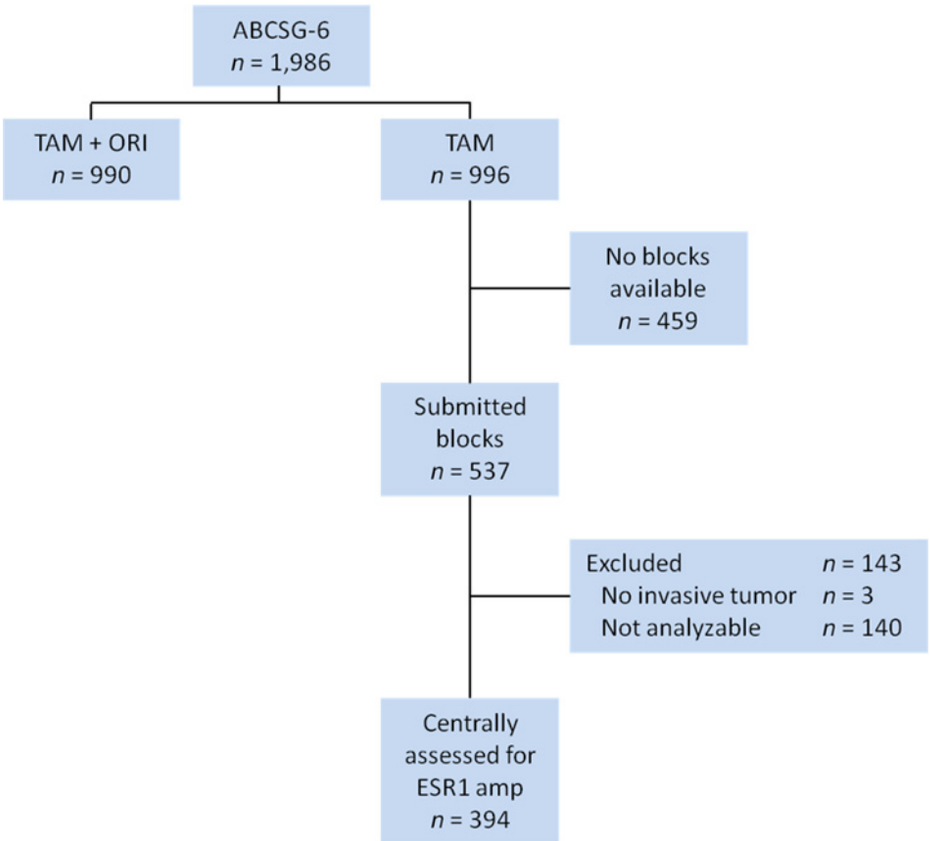

Figure 2.
Description of the process of tumor block and patient selection.

amplification. Furthermore, both DRFS and BCSS were not associated with *ESR1* amplification in either the anastrozole-treated, or the untreated patient population (data not shown).

Discussion

Qualitative ERα expression identifies endocrine-responsive tumors, and is both an established prognostic parameter and a predictor for response to endocrine therapy in early breast cancer. A recent meta-analysis of 19 studies including 30,754 patients, however, found no clear evidence for a correlation between higher quantitative ERα and better disease outcome in patients with stage 1–3 breast cancer (18). These results are in line with our observation which also demonstrated that semiquantitative ERα protein expression using the Allred score was unable to predict the long-term outcome.

Furthermore, *in vitro* studies show that mutations in the hotspot ligand-binding domain of the ERα gene *ESR1* confer ligand-independent activity and relative resistance to tamoxifen and fulvestrant, but these mutations develop under the selective pressure of endocrine treatments, and are infrequent in untreated early ER$^+$ breast cancers (19, 20).

Consequently, whereas *ESR1* mutations in circulating DNA are commonly detected in metastatic disease, they are rarely detectable at the initial diagnosis or during adjuvant treatment, thus limiting their utility to predict long-term outcome in endocrine-treated early breast cancer (21).

In this study, we retrospectively analyzed breast cancer tissues from the prospective adjuvant ABCSG-6 trial using a commercial *ESR1* FISH probe and simple scoring criteria to detect cancers with elevated *ESR1* gene copy numbers.

Using FISH, we detected focal *ESR1* amplifications (copy-number elevations) in 47% of 394 hormone receptor–positive breast cancers. This is somewhat higher than the combined fraction of *ESR1* amplifications and gains in previous studies performed by us (36%) or others (34%; refs. 2, 3). However, using a different FISH assay and an *ESR1/CEP6* ratio of 1.3 as cutoff for *ESR1* gain, a recent study by Laenkholm and colleagues has reported comparable rates of *ESR1* copy-number elevations in 42% of breast cancer cases (16). Of note, we selected ERα-

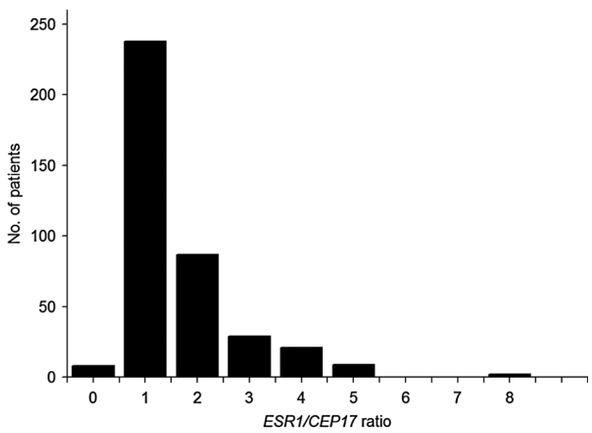

Figure 3.
Histogram detailing the levels of ESR1 amplification in the study population in whole-digit steps.

Table 1. Patient and tumor characteristics.

Characteristic	Tamoxifen arm ABCSG-6 $n = 996$	Patients with tumor block $n = 537$	Patients evaluable $n = 394$	No ESR1 amplification $n = 207$	ESR1 amplification $n = 187$	*P*
Age						
Median, years	64.8	65.2	65.3	66.5	63.0	
Range, years	43.7–80.7	43.7–80.7	43.7–80.7	43.7–80.7	48.4–79.9	
≤60 years	315 (32%)	165 (31%)	126 (32%)	79 (38%)	47 (25%)	0.006
>60 years	681 (68%)	372 (69%)	268 (68%)	128 (62%)	140 (75%)	
Tumor size						
≤2 cm	577 (58%)	296 (55%)	224 (57%)	112 (54%)	112 (60%)	0.29
>2 cm–≤5 cm	390 (39%)	229 (43%)	162 (41%)	92 (44%)	70 (37%)	
>5 cm	29 (3%)	12 (2%)	8 (2%)	3 (1%)	5 (3%)	
Nodal status						
Negative	617 (62%)	320 (60%)	235 (60%)	118 (57%)	117 (63%)	0.69
1–3 positive nodes	255 (26%)	145 (27%)	104 (26%)	57 (28%)	47 (25%)	
4–10 positive nodes	92 (9%)	51 (10%)	39 (10%)	23 (11%)	16 (9%)	
>10 positive nodes	32 (3%)	21 (4%)	16 (4%)	9 (4%)	7 (4%)	
Tumor grade						
G1	147 (15%)	90 (17%)	71 (18%)	39 (19%)	32 (17%)	0.11
G2	567 (57%)	308 (57%)	234 (59%)	130 (63%)	104 (56%)	
G3	217 (22%)	109 (20%)	89 (23%)	38 (18%)	51 (27%)	
Unknown	65 (7%)	30 (6%)	—	—	—	
Estrogen receptor						
Negative	25 (3%)	8 (2%)	6 (2%)	4 (2%)	2 (1%)	0.001
Low	203 (20%)	115 (21%)	86 (22%)	56 (27%)	30 (16%)	
Medium	377 (38%)	213 (40%)	157 (40%)	90 (44%)	67 (36%)	
High	361 (36%)	193 (36%)	145 (37%)	57 (28%)	88 (47%)	
Unknown	30 (3%)	8 (2%)	—	—	—	
Progesterone receptor						
Negative	208 (21%)	122 (23%)	91 (23%)	49 (24%)	42 (23%)	0.94
Low	212 (21%)	114 (21%)	88 (22%)	44 (21%)	44 (24%)	
Medium	279 (28%)	153 (29%)	115 (29%)	62 (30%)	53 (28%)	
High	264 (27%)	138 (26%)	100 (25%)	52 (25%)	48 (26%)	
Unknown	33 (3%)	10 (2%)	—	—	—	
ER (Allred) $n = 392$						
0–2	—	—	24 (6%)	23 (11%)	1 (1%)	<0.001
3–4	—	—	37 (9%)	24 (12%)	13 (7%)	
5–6	—	—	125 (32%)	80 (39%)	45 (24%)	
7–8	—	—	206 (53%)	79 (38%)	127 (68%)	
HER2 $n = 327$						
Negative	—	—	289 (88%)	154 (90%)	135 (87%)	0.49
Positive	—	—	38 (12%)	18 (11%)	20 (13%)	

positive tumors in our current study (94% ER positive by IHC), while there were only 77% ER-positive tumors in our previous study and 82% in the study by Tomita and colleagues (2, 3). The higher fraction of cancers with increased *ESR1* copy numbers in our current study therefore also reflects the known close association between ERα expression and *ESR1* copy-number alterations (2).

Because the initial description of *ESR1* amplification in breast cancer, there has been considerable discrepancy regarding the reported prevalence and the clinical relevance of *ESR1* amplification in early breast cancer (8). Several groups have independently detected *ESR1* copy-number elevations in breast cancer samples in more than 20% by using FISH technology (2–4, 8, 16, 22). These results were, however, challenged by studies in which predominantly alternative techniques were employed: Quantitative PCR (qPCR), multiplex-ligation dependent probe amplification (MLPA), comparative genomic hybridization (CGH), and single nucleotide polymorphism (SNP) microarrays yielded considerably lower amplification rates of *ESR1* amplification of <10% and did not demonstrate a prognostic role for *ESR1* gene copy changes (6, 8, 23–27).

Table 2. Correlation Allred score and *ESR1* amplification status.

Allred score	No amplification	Amplification
0–2	96%	4%
3–4	65%	35%
5–6	64%	36%
7–8	38%	62%
Total	53%	47%

Although these discrepancies could be the consequence of patient selection in individual studies, it is obvious that significant percentages of these differences could in part be caused by the applied technology: Nonmorphological methods such as qPCR, MLPA, SNP microarrays, or Southern blotting are particularly sensitive to contamination with nonmalignant breast tissue and to tumor heterogeneity in the copy-number status, which could conceal amplifications of low copy-

Table 3. Cox proportional hazard models for DRFS and BCSS.

	DRFS				BCSS			
	Univariate models		Multivariable model		Univariate models		Multivariable model	
Variables	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age	0.98 (0.94–1.01)	0.13	0.98 (0.95–1.02)	0.36	0.99 (0.96–1.02)	0.43	1.00 (0.96–1.04)	0.98
Tumor size		0.003		0.30		0.002		0.97
pT2 vs. pT1	2.44 (1.46–4.09)	0.001	1.41 (0.77–2.58)	0.27	2.47 (1.46–4.17)	0.001	1.08 (0.56–2.07)	0.83
pT3 vs. pT1	1.35 (0.18–10.00)	0.77	0.41 (0.05–3.43)	0.41	3.05 (0.72–12.97)	0.13	0.98 (0.19–5.19)	0.98
Nodal status		<0.0001		<0.0001		<0.0001		<0.0001
1–3 vs. 0 nodes	1.64 (0.85–3.17)	0.14	1.17 (0.56–2.43)	0.68	1.61 (0.82–3.15)	0.16	1.25 (0.58–2.70)	0.57
4–10 vs. 0 nodes	6.24 (3.25–11.95)	<0.0001	5.31 (2.59–10.87)	<0.0001	5.84 (2.97–11.48)	<0.0001	4.48 (2.00–10.00)	0.0003
>10 vs. 0 nodes	7.81 (3.58–17.04)	<0.0001	11.16 (4.21–29.60)	<0.0001	9.45 (4.55–19.62)	<0.0001	10.27 (3.63–29.08)	<0.0001
Tumor grade		0.15		0.50		0.03		0.64
G2 vs. G1	1.84 (0.82–4.13)	0.14	1.71 (0.70–4.16)	0.24	1.59 (0.70–3.59)	0.27	1.38 (0.56–3.41)	0.49
G3 vs. G1	2.39 (0.99–5.72)	0.05	1.56 (0.54–4.50)	0.42	2.76 (1.18–6.45)	0.02	1.67 (0.58–4.82)	0.34
HER2	1.86 (0.91–3.82)	0.09	1.93 (0.92–4.07)	0.08	2.59 (1.32–5.09)	0.006	2.88 (1.39–5.98)	0.004
ESR1 amp/gain	0.53 (0.31–0.89)	0.02	0.48 (0.26–0.91)	0.02	0.56 (0.34–0.95)	0.03	0.47 (0.27–0.80)	0.01

number level and consequently result in a considerable underestimation of *ESR1* amplification rates (8, 9, 28).

Another factor that might have led to an underestimation of *ESR1* amplification rates in some studies is the choice of improper cut-off levels for calling amplification. By setting the cut-off levels too high, copy-number elevations of low level would be considered unamplified, which has already been shown to have impact for several other genes, although it is still unclear how low gain rates translate into clinically relevant clinical outcomes (6, 8, 9).

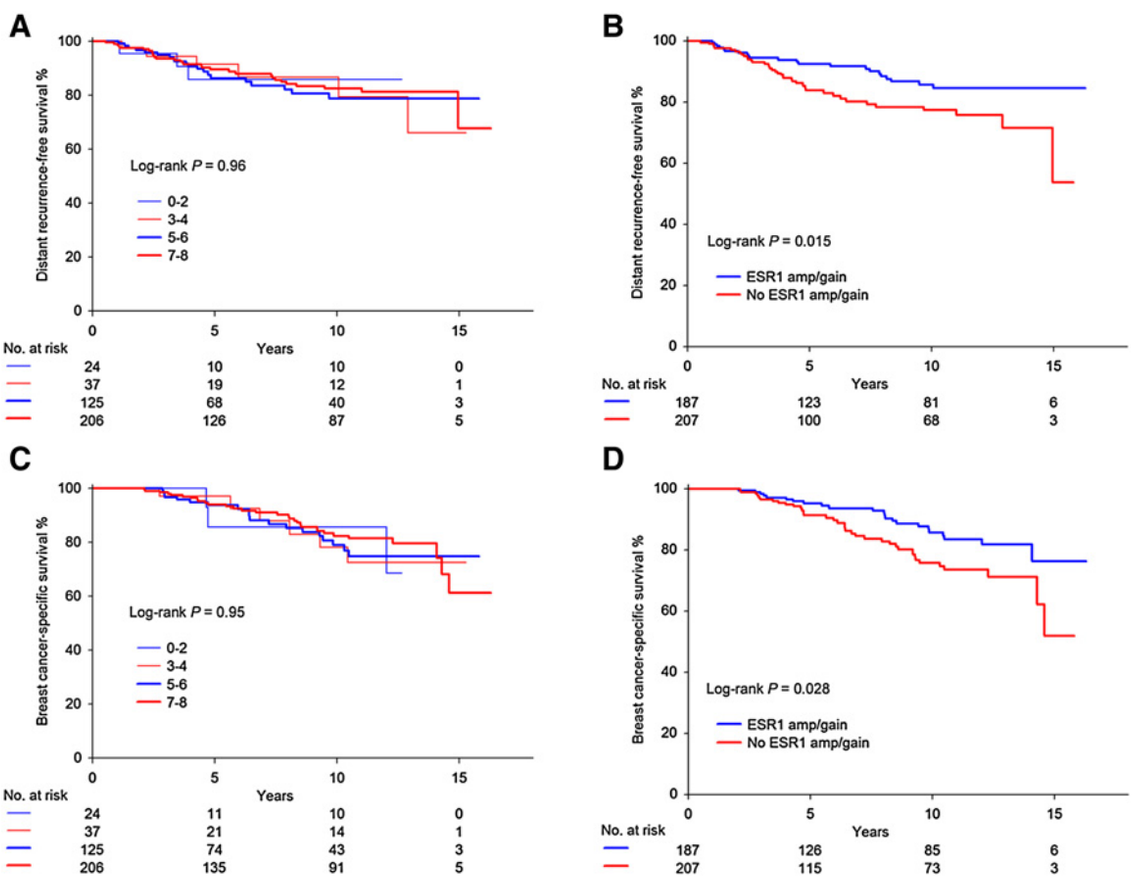

Figure 4.

DRFS in patients treated with tamoxifen according to ERα expression by Allred score (**A**), and *ESR1* amplification status (**B**), and BCSS in patients treated with tamoxifen according to ERα expression by Allred score (**C**), and *ESR1* amplification status (**D**).

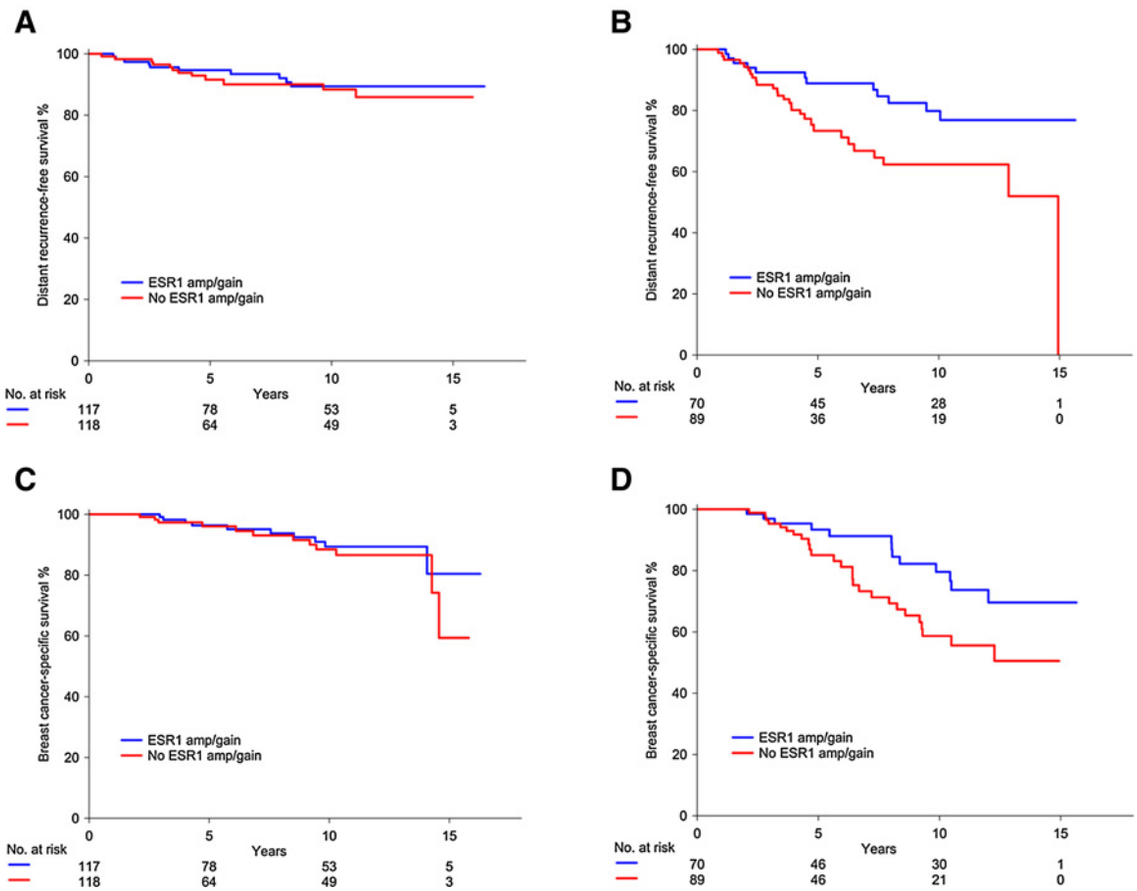

Figure 5.
DRFS in patients treated with tamoxifen in nodal-negative (**A**) and in nodal-positive (**B**) patients. BCSS in patients treated with tamoxifen in nodal-negative (**C**) and in nodal-positive (**D**) patients.

Our findings are supported by an earlier report describing a prolonged survival of women whose tumors exhibited *ESR1* amplification among the 175 patients who had undergone tamoxifen treatment (2). They are also in line with findings by Babyshkina and colleagues who recently found that in tamoxifen-treated luminal tumors, a significant decrease in *ESR1* mRNA expression levels and a heterogeneous ERα protein expression pattern were both associated with a significantly shorter PFS (29).

Our results are, however, contradictory to a retrospective case–control study of 91 patients in whom *ESR1* amplifications were significantly more common in primary breast carcinomas which recurred within the first 4 years after diagnosis (11). Furthermore, in a subset of the BIG 1–98 trial, *ESR1* amplification status alone did not predict for DFS after 5 years of endocrine therapy, but was prognostic in combination with the *HER2* amplification status (5). In both studies, however, only tumors with an *ESR1/CEP-6* ratio of ≥2 (i.e., high-level amplification) were considered positive and low-level *ESR1* amplification (copy number gains) were not included. It is well possible that the prognostic value of the *ESR1/CEP-6* ratio assessment in these trials was simply compromised by selection of an inadequate cut-off value.

The mechanism by which *ESR1* amplification renders an endocrine-responsive tamoxifen-treated tumor less aggressive remains unclear. One hypothesis, however, suggests that the amplification a particular gene might be driven by the tumors' addiction to a pathway in which the protein product of the respective gene is involved (30). Such a mechanism has been suggested in two independent studies that observed focal *ESR1* amplifications of low-level copy-number change in long-term estrogen-deprived (LTED) MCF7 breast cancer cell lines. Yet another experimental study showed that breast-cancer-derived xenografts respond to estrogen treatment of tumor cells that harbor *ESR1* amplification (31, 32). Further evidence comes from a phase II study which evaluated antiestrogen treatment, and found focal *ESR1* amplification in response to endocrine deprivation by (33). Taken together, these functional studies provide strong evidence for the potential clinical relevance of *ESR1* amplification as a mechanism of ERα pathway regulation.

In summary, we have detected focal *ESR1* amplification (copy-number increase) in 47% of ERα-positive and endocrine-responsive early stage breast cancers from postmenopausal women who had been enrolled in the ABCSG-6 trial. In this well-defined and meticulously documented prospective clinical trial patient population receiving 5 years of tamoxifen therapy, we demonstrate that *ESR1* amplification status is a predictor for long-term DRFS. In contrast to most multigenomic assays, whose reported prognostic value is limited to DFS and DDFS, we here demonstrate that the

ESR1 copy-number ratio is also predictive of survival (34, 35). An analysis of *ESR1/CEP17* ratios in patients included in prospectively designed studies with aromatase inhibitors will help to further establish the prognostic role of this easily available biomarker in endocrine-responsive early breast cancer.

Authors' Disclosures

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Authors' Contributions

C.F. Singer: Data curation, formal analysis, supervision, investigation, methodology, writing–original draft, writing–review and editing. **F. Holst:** Investigation, methodology, writing–original draft, writing–review and editing. **S. Steurer:** Data curation, investigation, writing–review and editing. **E. Burandt:** Data curation, investigation, writing–review and editing. **S. Lax:** Investigation, writing–review and editing. **R. Jakesz:** Data curation, supervision, investigation, writing–review and editing. **M. Rudas:** Supervision, investigation, writing–review and editing. **H. Stöger:** Data curation, supervision, investigation, writing–review and editing. **R. Greil:** Data curation, supervision, investigation, writing–review and editing. **G. Sauter:** Data curation, investigation, writing–review and editing. **M. Filipits:** Formal analysis, investigation, methodology, writing–original draft, writing–review and editing. **R. Simon:** Supervision, investigation, writing–review and editing. **M. Gnant:** Data curation, supervision, investigation, methodology, writing–original draft, writing–review and editing.

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