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PRECLINICAL STUDY

Amplification of *ESR1* may predict resistance to adjuvant tamoxifen in postmenopausal patients with hormone receptor positive breast cancer

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Abstract The estrogen receptor (ER) is the target of tamoxifen, but endocrine therapies do not benefit all patients with ER positive tumors. We therefore hypothesized that copy number changes in the *ESR1* gene, encoding ER, confer resistance. Within a consecutive series of ER positive, postmenopausal patients allocated to 5 years tamoxifen, we identified 61 patients with recurrence less than 4 years and 48 patients without recurrence at least 7 years after initiation of adjuvant tamoxifen. Archival tissue containing primary tumor was collected from 97 patients (89%). Tumor samples were analyzed for *ESR1* copy number changes using FISH with a probe covering the *ESR1* gene at 6q25 and a reference probe covering the centromere of chromosome 6. The assay was validated in a material of 120 normal breast samples. FISH analysis for *ESR1* was successful in 91 patients (94%). Amplification (ratio *ESR1*/CEN-6 $\geq$ 2.0) was observed in 11 of 50 (22%) patients with early recurrence, compared to two of 41 (5%) patients without recurrence. The difference is statistically significant ($P = 0.033$). In both groups, two patients with *ESR1* deletion (ratio *ESR1*/CEN-6 < 0.8) were identified. *ESR1* amplification was significantly associated with poor disease-free survival ($P = 0.0054$) and overall survival ($P = 0.0004$). This pilot study supports our hypothesis that *ESR1* amplification is associated with a poorer outcome following adjuvant treatment with tamoxifen in ER positive early breast cancer. This study also revealed the existence of *ESR1* deletions. The prognostic and predictive impact of *ESR1* copy number changes needs further exploration in clinical trials.

Keywords *ESR1* · Estrogen receptor · Gene amplification · Tamoxifen · Endocrine therapy · Gene aberrations · Breast cancer

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Introduction

The estrogen receptor (ER) is the earliest established and most widely used biomarker in breast cancer, and it is recommended that the tumor ER status be determined in all breast cancer patients [1, 2]. ER has both a prognostic and a predictive value. ER status is a prognostic factor, but shifts from a positive prognostic factor initially to a slightly negative prognostic indicator 5–6 years after diagnosis [3]. ER is the target of tamoxifen. The efficacy of adjuvant endocrine therapies is associated with the presence of ER in the primary tumor [4], and patients with ER negative breast cancer are unlikely to benefit from tamoxifen. Unfortunately, endocrine therapies do not benefit all

patients with ER positive tumors and in a search for explanations for this, many features of ER have been studied in the past [5], including ERα and ERβ protein, ER mRNA, and numerous ER cofactors, e.g., AIB1 [6, 7]. Array techniques have provided information on ER target genes and response elements [8]. Also studies investigating polymorphisms [9] and mutations within the *ESR1* gene that codes for ERα have been reported.

Over a 17-year period, gene amplification of the *ESR1* gene, encoding the ER, has been the focus of very few studies [10–14], although gene amplification is the major mechanism behind the cancer-related changes of many oncogenes [15], including *ERBB2* (*HER2*) [16], *TOP2A* [17], *CCND1* [18], and *MYC* [19]. The first *ESR1* gene studies were based on Southern blotting technique and showed contradictory results [10, 11]. With more sensitive techniques, *ESR1* was included in the list of possible genes located in loci that frequently undergo amplification or deletions in breast cancer [20]. Recently, the frequency of *ESR1* amplifications was established [13, 14] to be approximately 20% among 2,000 breast cancer specimens analyzed by FISH (fluorescence in situ hybridization) technique, and *ESR1* amplification was reported to be associated with good response to adjuvant tamoxifen treatment. The initial *ESR1* FISH study [13] has led to much debate about the frequency of *ESR1* amplification in breast cancer and the implication of the methodological variation for the estimation of the frequency [21–26], but direct comparison of *ESR1* FISH with quantitative PCR [14] showed the superiority of the FISH method for detection of these low level amplifications.

This study [27] was initiated before publication of the study by Holst et al. [13], and our hypothesis does not take their result into account. We hypothesized that copy number changes of the *ESR1* gene confer resistance to tamoxifen because amplification is an abnormal status and normal ER protein expression (ER positive status) is requisite for response to tamoxifen. We designed a study to analyze the frequency of abnormal copy number of *ESR1* according to outcome in breast cancer patients having adjuvant treatment with tamoxifen.

Materials and methods

Patient samples

Tissue blocks were collected from the archives at the department of Pathology at Herlev and Roskilde Hospitals. From a nationwide, previously described cohort of 1,115 Danish postmenopausal patients allocated to tamoxifen 20 mg daily for 5 years following radical surgery from 1995 to 2006 for early hormone receptor positive breast cancer [28], we extracted a subgroup whose tumor blocks were located at one of the two participating pathology departments and who had a recurrence less than 4 years (61 patients) or were without recurrence at least 7 years after initiation of adjuvant tamoxifen (48 patients). Later revision of the patient data led to exclusion of one patient who had recurrence after 5 years from the early recurrence group and two patients where the tissue block did not contain tumor tissue; one from each recurrence group. All tumors were hormone receptor positive with more than 10% stained cells. All samples except two were analyzed on tissue microarrays (TMAs). Two samples were analyzed on whole sections. The TMAs were constructed by a technologist supervised by a pathologist (EB). An HE staining was available to identify areas of invasive breast tumor. The biomarker study was conducted according to the REMARK recommendations [29].

Collection of tissue samples from normal breasts

For assay validation we analyzed *ESR1* gene copy numbers in 120 samples collected consecutively and retrospectively at the department of Pathology at Herlev University Hospital from breast reduction operations with no identified malignancy. Areas of normal breast glands suitable for TMA construction were identified by a pathologist (EB). TMAs were constructed using Advanced Tissue Arrayer, ATA-100 (Chemicon International, Temecula, CA, USA) from formalin-fixed, paraffin-embedded normal breast tissue. Duplicate 2.0-mm tissue cores from each donor block were re-embedded into recipient paraffin block containing 40 tissue cores.

Collection of tissue samples from ER negative tumors

To validate the observation of *ESR1* deletions we identified nine ER negative breast tumors from the tissue archives at Dako A/S which contains anonymous tissue blocks from various types of cancer. Information on some tumor characteristics was available, but patient characteristics including outcome were unknown.

FISH analysis

Samples from tumor tissue and normal tissue were analyzed for *ESR1* copy number changes using FISH with a probe covering the *ESR1* gene at 6q25 and a reference probe covering the centromere of chromosome 6. The RP11-450E24 and RP11-54K4 BAC clones (Fig. 1) that make up the *ESR1* probe applied in this study covers all coding sequence of the *ESR1* gene. Nearly 30% of the *ESR1* probe covers up- and downstream flanking sequences

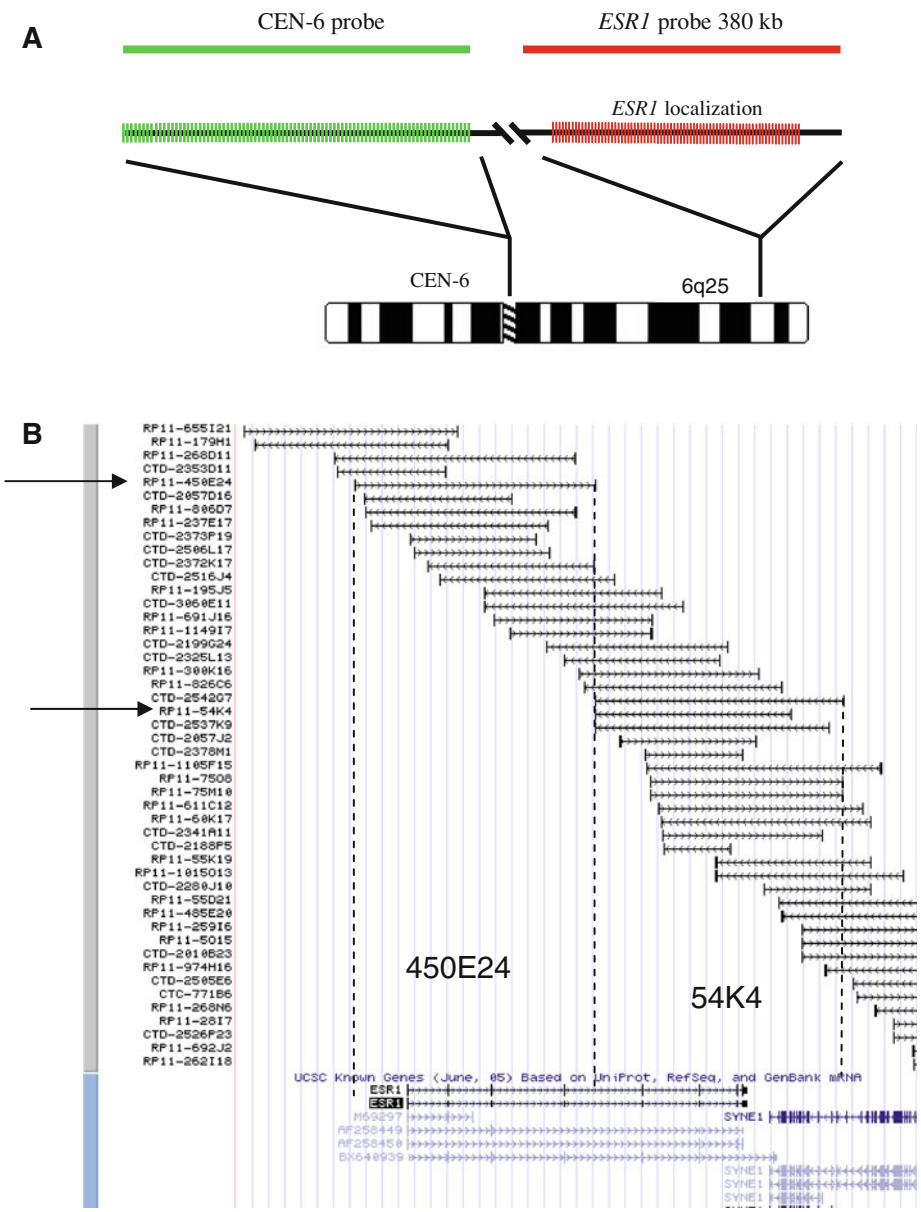

Fig. 1 Localization and coverage of the *ESR1*/CEN-6 probe mixture (**a**). The *ESR1* probe covered the entire *ESR1* gene and flanking regions and consisted of 2 BAC clones (BAC RP11-450E24 and RP11-54K4) (**b**) localized using the UCSC Genome Browser on Human March 2006 Assembly

including approximately 4% of the neighboring *SYNE1* gene. However, as the *SYNE1* overlapping part make up less than 6% of the *ESR1* probe, separate amplification or deletion of *SYNE1* would not be detectable by use of the *ESR1* probe. The *ESR1* targeted BAC clones were labeled with Texas Red fluorochrome by Nick translation. The centromere 6 targeted references probe was based on a mixture of PNA (peptide nucleic acid) oligoes labeled with fluorescein (FITC). A mixture of alu PNA oligoes were used as blocking agent. The FISH protocol described below is optimized for a DNA/PNA mixture. Further details are described elsewhere [30]. Prior to FISH analysis, the slides were deparaffinized twice in Xylene for 5 min followed by rinse in 96 and 70% ethanol (each twice for 2 min). FISH was performed with Dako Histology FISH accessory kit (K5599, Dako A/S, Glostrup, Denmark) according to the manufacturer's instructions. Briefly the method consists of pretreatment for 10 min at 95–99°C in pretreatment solution followed by pepsin treatment for 10 min at room temperature (K5599, vial 2, RTU, pH 2.0). After denaturation at 82°C for 5 min and overnight hybridization at 45°C, excess of probe is removed by stringent wash at 65°C for 10 min. After dehydration in ethanol, the slides are mounted in antifade with DAPI (K5599, vial 5). Figure 2 illustrates the appearance of *ESR1* amplification using a Lieca DM microscope equipped with a Lieca Image System and filters for Texas Red, FITC, and DAPI. The ratio was calculated as the number of signals for the gene probe divided by the number of signals for the centromere 6 reference probe. Cases were scored as *ESR1* FISH

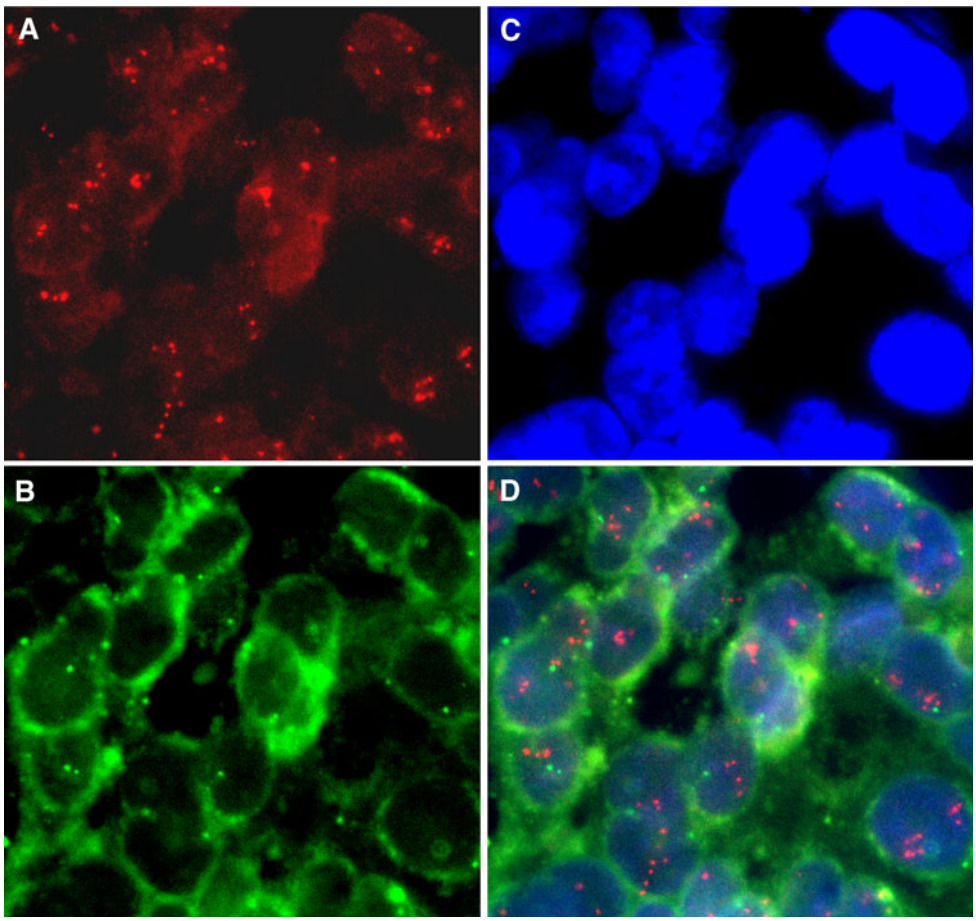

Fig. 2 *ESR1* amplification in a breast cancer sample. In the individual filters, the red *ESR1* signals (**a**), the green centromere 6 signals (**b**) and the DAPI counterstaining (**c**) is seen. **d** shows the combined picture of the individual filters

amplified when the ratio was ≥2.0 and deleted when the ratio was <0.8.

Slide evaluation

The slides were evaluated according to the *TOP2A* FISH scoring guidelines (from Dako K5333 USA package insert, 1st edition 2008.01.18, approved by the American Food and Drug Administration (FDA)). These guidelines specifically allow detection of both amplifications and deletions compared to the HER2 guidelines [31] that focus on amplifications. For a nucleus to be included in the scoring, the HER2 guidelines require that it contains signals of both colors. The TOP2A guidelines only require the presence of a green reference signal for a nucleus to be included in the scoring. The reason for this difference is that the truncated nuclei in cut sections of a deleted, diploid tissue sample will either contain one or zero red gene signals, and omission of all nuclei with zero red signals will prevent detection of most deleted cases. A previously conducted pilot study [32] showed that two different counting methods gave identical results: Either the signals were counted in 60 nuclei or sufficient nuclei were included until a total of 60 red gene probe signals were counted along with the green reference probe signals in the same nuclei. An average of 35 nuclei was counted. The latter method has the advantage that the highest number of cells will be counted in the deleted and normal cases, while the lowest number of cells will be counted in highly amplified cases. Highly amplified cases are often obvious to identify just by looking in the microscope, but are more time demanding to evaluate if 60 nuclei have to be scored.

Statistical analysis

The DBCG Data Center collected and analyzed the data. Associations between *ESR1* status and other clinicopathological characteristics were analyzed by Chi-square or Fischer's exact test. *P*-values are two-tailed. Disease-free survival (DFS) was defined as the duration of survival without invasive loco-regional recurrence, distant metastasis, contralateral breast cancer, second primary non-breast invasive cancer, or death irrespective of cause. DFS was analyzed unadjusted using the log-rank test. For multivariate analysis the Cox proportional hazards regression model was applied to assess the adjusted hazard ratio (HR) of DFS by *ESR1* status, and to explore interactions. Factors included in the multivariate analyses were age, tumor size

(0–2, >2–5, >5 cm), nodal status (0–3, 4–9, >9 positive), histological type and grade (ductal grade I, ductal grade II, ductal grade III, unknowns, missing or other histological types). Statistical analyses were done with the SAS 8.2 program package.

Ethics

All data used and presented were collected and analyzed by the DBCG registry. The biological substudy was conducted according to the Helsinki declaration and approved by the Scientific Ethical Committee of the Capital Region (1H-D-2007-0034, June 19th 2007).

Results

Archival tissue from primary tumor was retrieved from 97 of the 109 patients (89%). FISH analysis for *ESR1* was successful in 91 of the 97 patients (94%). Patient characteristics are seen in Table 1. Patients in the early recurrence group had more positive lymph nodes ($P < 0.001$) and more tumors with higher grade ($P = 0.04$) compared to patients in the non-recurrence group. The distribution of *ESR1*/CEN-6 ratios in patients without recurrence for 7 or more years compared to patients experiencing recurrence within the first 4 years is seen in Fig. 3a. Ratios were found in the range 0.50–3.13. Overall 14% of the patients had amplification of the *ESR1* gene. All amplifications were in the low level range with a ratio from 2.00 to 3.13. Nine amplifications were found in diploid tumors (<2 average references signals per nucleus) and four in triploid tumors (<3 average reference signals per nucleus). If no reference probe had been included most of the amplifications would have been missed as only four of the samples had an average of five or more *ESR1* gene signals/nucleus. In addition, we observed *ESR1* deletion in four tumors, two in each recurrence group. The deletions were in the range 0.50–0.76. Three deletions were in diploid tumors and one in a triploid tumor. If no reference

Table 1 Patient characteristics correlated with recurrence groups

Parameter		All patients N = 91	Patients without recurrence more than 7 years N = 41	Patients with recurrence <4 years N = 50	Test for no difference between groups *P*-value
Age	Mean		61.7	61.2	0.76
	(range)		(48–72)	(50–76)	
	SD		7.1	6.9	
Age groups	18–59 years	35	15	20	0.47
	60–69 years	42	18	24	
	>70 years	14	8	6	
Tumor size	0–20 mm	35	18	17	0.48
	>20 mm	54	22	32	
Histological type	Inv Ductal	75	34	41	0.71
	Inv lobular	15	7	8	
	Inv other	1	0	1	
Grade (only for ductal type)	I	20	16	4	0.04
	II	40	15	25	
	III	14	2	12	
	Unknown	3	1	2	
	Missing	14	7	7	
HR status	Pos	87	38	49	0.32
	Neg/unknown	4	3	1	
Nodal status	Pos	85	39	46	0.69
	Neg	6	2	4	
No. of positive lymph nodes	0	6	2	4	<0.001
	1–3	49	31	18	
	>4	36	8	28	
No. of lymph nodes analyzed	1–4	1	1	0	0.83
	5–9	12	5	7	
	>10	78	35	43	

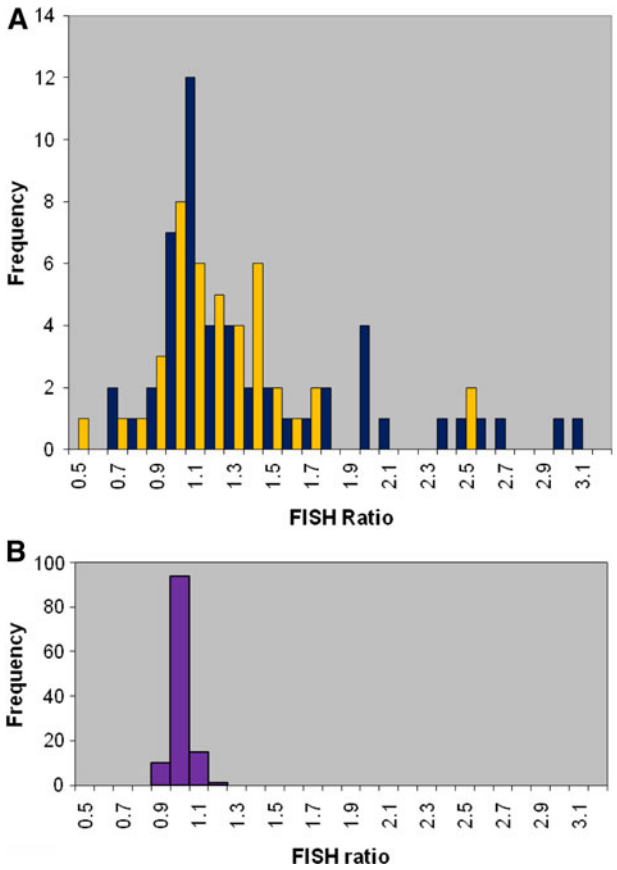

Fig. 3 Distribution of *ESR1*/CEN-6 ratios in **a** 50 patients with early recurrence (*blue*) and 41 patients recurrence free for at least 7 years (*yellow*) and the distribution of *ESR1*/CEN-6 ratios **b** in 120 samples from normal breast tissue (*purple*)

probe had been included only three deletions would have been detected.

Amplification was observed in 11 of 50 (22%) patients with recurrence within the first 4 years of tamoxifen treatment, compared to two of 41 (5%) patients who were free of recurrence at least 7 years after initiation of tamoxifen treatment (Table 2). The difference is statistically significant ($P = 0.033$; Fishers exact test). Compared to patients with *ESR1* normal tumors, patients with *ESR1* amplifications had a significant shorter disease-free ($P = 0.0054$) and overall survival ($P = 0.0004$) as illustrated in the Kaplan–Meyer plots of Fig. 4. *ESR1* status is shown according to patient characteristics in Table 3, and Cox proportional hazards regression analysis of disease-free survival was carried out according to *ESR1* status and patient characteristics. The risk of recurrence was significantly increased in the presence of *ESR1* amplification (HR 2.8; $P = 0.0048$), more than four positive lymph nodes (HR 3.0; $P = 0.0002$) and high tumor grade (HR 3.1; $P = 0.0043$). In a corresponding Cox analysis of overall survival *ESR1* amplification (HR 3.8; $P = 0.0009$), more than four positive lymph nodes (HR 3.2; $P = 0.0013$) and high tumor grade (HR 2.3; $P = 0.0321$) were identified as independent prognostic factors.

For assay validation we analyzed *ESR1* gene copy numbers in 120 normal breast samples (Figs. 3b and 5) and found neither amplifications nor deletions. In each sample 60 nuclei were evaluated. An average of 1.78 red signals representing the *ESR1* genes was counted and 1.69 green signals representing the centromeric reference sequence. The *ESR1*/CEN-6 ratio varied from 0.96 to 1.29 with a mean of 1.06. The ratios were distributed normally (Fig. 5) with a standard deviation of 0.04.

To validate the observation of deletions, we performed *ESR1* FISH on nine ER negative, but otherwise anonymous tissue samples. All nine samples had ratios below 1.00 in the range from 0.61 to 0.96 (data not shown), and six of the samples had *ESR1* deletion, indicating that the frequency of *ESR1* deletion may be higher in an ER negative patient series.

Discussion

The ER is encoded by the *ESR1* gene localized on chromosome 6q25.1, and copy number changes of *ESR1* have only been the subject of a very limited number of studies in contrast to the intensive study of the ER protein and its cofactors [5]. This study reports on the existence of both *ESR1* amplifications and deletions. The frequency of amplification varies from 5% in a group of patients without recurrence to 22% in a group of patients with recurrence within the first 4 years after initiation of treatment. We found amplification in *ESR1* to be a strong independent prognostic marker of poor survival in postmenopausal patients allocated to 5 years of tamoxifen following radical surgery for early hormone receptor positive breast cancer. The amplifications were in the low level range compared to *ERBB2* (*HER2*) amplifications, and this may be the

Table 2 Distribution of tumors with *ESR1* amplification and deletion among patients with early recurrence and patients with late or no recurrence

	ESR1 amplification	*ESR1* normal	*ESR1* deletion	Total
Patients with recurrence within 4 years	11 (22%)	37 (74%)	2 (4%)	50
Patients without recurrence for 7 years	2 (5%)	37 (90%)	2 (5%)	41
Total	13 (14%)	74 (81%)	4 (5%)	91

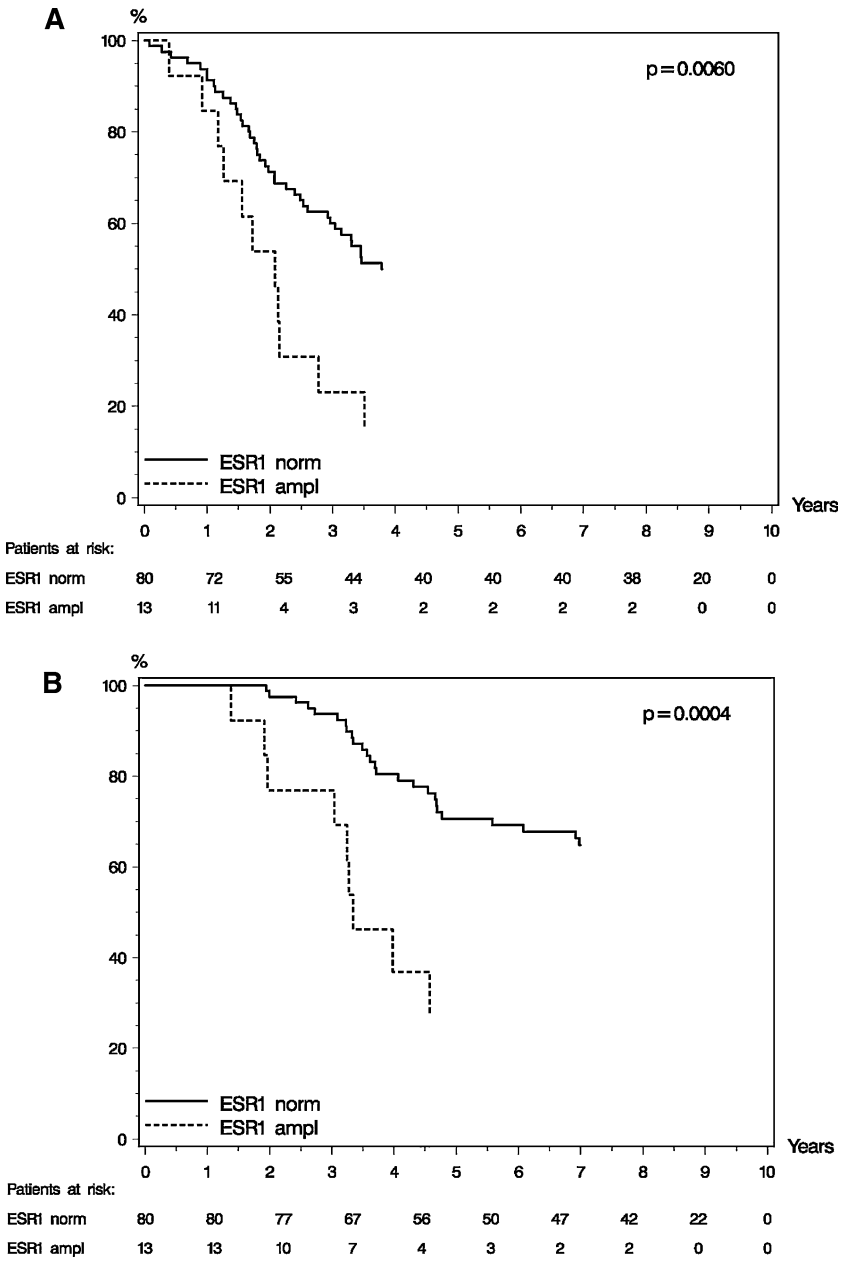

Fig. 4 Recurrence-free survival (**a**) and overall survival (**b**) of patients with and without *ESR1* amplifications

explanation for the infrequent report of *ESR1* amplifications. However, all amplifications were well outside the normal range established in 120 normal breast samples.

Genetic alterations including copy number changes are the major cancer causing mechanism [15], but amplification of *ESR1* has been the focus in few studies [10–13]. Regulation of the ER has primarily been studied at the protein and mRNA level, and many studies have been devoted to ER as a transcription factor, e.g., ER regulation of other genes [8]. However, if the *ESR1* gene itself is abnormal, in structure or copy number, it may not be capable of regulating other genes normally. In a study using Southern hybridization, no major structural rearrangements of *ESR1* were identified in a series of 188 primary breast cancers [9] and subsequent studies using related techniques for the study of polymorphisms, e.g., restriction fragment length polymorphism (RFLP) and loss of heterozygosity (LOH), have confirmed that structural changes within the *ESR1* gene are uncommon in breast cancers and do not explain differences in protein expression and ER status [11, 33–35].

The first *ESR1* gene amplification studies were based on Southern blotting technique and showed contradictory results [10, 11]. In accordance with our observations, a study from 1990 using Southern hybridization reported low level amplification ranging from 1.6- to 3-fold in 6 of 14 ER positive tumors, while no amplification could be detected among eight ER negative tumors included in this

Table 3 Patient characteristics correlated to *ESR1* status

Parameter		All patients N = 91	Patients without *ESR1* amplification N = 78	Patient with *ESR1* amplification N = 13	Test for no difference between groups *P*-value
Age (cont)	Mean		61.0	63.8	0.17
	(range)		(48–74)	(50–76)	
	SD		6.6	8.8	
Age (group)	18–59 years	35	31	4	0.26
	60–69 years	42	37	5	
	>70 years	14	10	4	
Tumor size	0–20 mm	35	29	6	0.54
	>20 mm	54	47	7	
Histological type	Inv ductal/inv other	76	64	12	0.45
	Inv lobular	15	14	1	
Grade (only for ductal type)	I + unknown	37	33	4	0.55
	II + III	54	45	9	
HR status	Pos	87	75	12	0.47
	Neg/unknown	4	3	1	
Nodal status	Pos	85	74	11	0.20
	Neg	6	4	2	
No. of positive lymph nodes	0	6	4	2	0.80
	1–3	49	44	5	
	>4	35	30	6	
No. of lymph nodes analyzed	1–4	1	1	0	0.12
	5–9	12	12	0	
	>10	78	65	13	

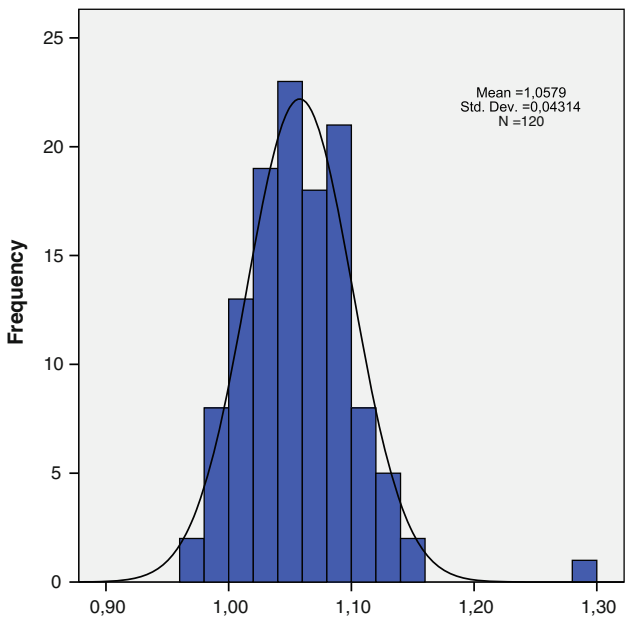

Fig. 5 The distribution of *ESR1*/CEN-6 ratios in 120 samples from normal breast tissue

study [10]. The frequency of amplification could not be confirmed in a study from 1992 [11] as only a single low level amplification among 29 ER positive tumors was reported, and the existence of *ESR1* amplifications has not historically been widely appreciated [13, 21]. However, the sensitivity of the Southern hybridization method may not allow detection of low level amplifications and deletions. With more sensitive techniques, *ESR1* was included in the list of possible genes located in loci that frequently undergo amplification or deletions in breast cancer [20].

Two recent FISH studies [13, 14] report the frequency of *ESR1* amplifications to be in the range 20–23%. Our study supports the existence of *ESR1* amplifications varying from 5% in the group of patients with relatively good prognosis to 22% in the poor prognosis group of patients, and amplifications were significantly associated with poor outcome following treatment with Tamoxifen. In contrast to the two previous studies, we exclusively analyzed the prognostic implications of *ESR1* aberrations within an ER positive cohort, and the exclusion of ER negative patients partly explains the differences observed between studies.

All patients in this study were ER positive with more than 10% stained cells as demanded for receiving adjuvant endocrine treatment. We have deliberately focused on the additive role of *ESR1* amplifications in a patient cohort selected for treatment by ER immunohistochemistry and have not tried to compare ER protein level with *ESR1* gene status. Both the studies of Holst et al. [13] and Tomita et al.

[14] show that although all patients with *ESR1* amplification have high protein expression (Allred score 7–8), only approximately half of the patients with high protein expression have amplifications. Thus, high protein expression apparently is not always the result of amplification, as in, e.g., HER2 protein overexpression. This lack of consistence might be one of the reasons for our finding in this study, that amplification is a predictor of resistance, with amplification reflecting gene disturbance with loss of normal function.

The initial *ESR1* FISH study [13] has led to much debate about the frequency of *ESR1* amplification in breast cancer and correspondences from four groups report on frequencies of only 0–10% using a variety of methods, including comparative genomic hybridization (GCH), FISH, CISH, and quantitative PCR [21–26]. Some of the methodological debate has been resolved by the most recent study [14] that confirms the data by Holst et al. [13], but also compares the *ESR1* FISH data directly with quantitative PCR data from the same patient material. The study found 22.6% *ESR1* amplifications using FISH compared to 1.4% *ESR1* amplifications using q-PCR, thus providing an explanation for the reported discrepancy among the other studies [22–25] and demonstrating the diminished sensitivity of the PCR-based detection of amplifications. Using FISH in an ER positive patient population we found amplification of *ESR1* in 14% but the frequency depends on the selected study samples. According to the DBCG Registry 80.8% of breast cancers were ER positive during the study period, corresponding to an estimated frequency of about 11% in the total population [28]. The discrepancies mimic early days of HER2 diagnostics, when different patient populations, methods, scoring guidelines, and cut-off levels led to enormous debate until firm guidelines were established [31]. *ESR1* amplifications seem to be at a lower level than *HER2* and will thus be more difficult to detect as demonstrated by Tomita and coworkers [14]. Therefore, conflicting results must be expected when different methods are used [10–14, 22–26] and the majority of platforms may not detect the *ESR1* amplifications [21].

The 13 amplified tumors of this study had an average of 3–7 *ESR1* signals per nucleus while Holst et al. [13] reports a higher average number of signals. Using their definition of "*ESR1* gain" (*ESR1*/CEN-6 ratio > 1.0), the majority of our cases ($68 = 73\%$) would have been classified as having gain. Our assay validation showed no copy number variation among 120 normal breast samples. It is worth noting that the majority of normal samples would have been scored as having "*ESR1* gain" using the criteria of *ESR1* ratio > 1.0 from Holst et al. [13]. One possible explanation for this difference is the use of different signal counting guidelines, especially for two signals not separated by the diameter of a signal. In line with HER2 scoring guidelines [31], we consider such a "duplet" signal as only one signal created by chromatin folding. Although Holst et al. [26] reports high intra-observer reproducibility, no intra-laboratory reproducibility studies have been performed, which could have revealed differences of signal counting instructions. Our assay included a reference probe to estimate the ploidy level of the samples. If only the red gene signals had been used in the assay, as in a single color FISH assay, most of the low level amplifications would have been missed and only four amplifications have an average of more than five gene signals/nucleus. Thus, using the cut-off criteria of Reis-Filho et al. [25, 36] would have reduced our frequency of amplifications to 4%.

In this patient series, we also identified four tumors with *ESR1* deletion, two in each outcome group, and confirmed the existence of deletions by analysis of nine ER negative breast tumor samples. This is the first identification of *ESR1* deletions in patient samples by FISH technique, but an in vitro study [37] using simultaneous fluorescence immunophenotyping and FISH detected *ESR1* deletions in four of six breast cancer cell lines. However, as no correlation to ER expression could be found, this solitary study concluded that *ESR1* deletions are not the major cause of absent or reduced ER expression in breast cancer cell lines. Although the *ESR1* deletions may not correlate with ER protein expression, the observation on patient samples deserves further analysis.

In summary, our data support that *ESR1* amplification is a frequent feature of ER positive tumors [13, 14]. We observe *ESR1* gene amplification in 14% of the tumors, ranging from 5% in patients with superior outcome to 22% in patients with inferior outcome on adjuvant treatment with tamoxifen. The *ESR1* amplifications are significantly associated with poor disease-free and overall survival, and in this respect our data disagree with recent *ESR1* FISH observations [13, 14]. The clinical utility of *ESR1* copy number changes deserves more attention until the exact importance for patient diagnostics has been established.

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