Estrogen receptor α gene *ESR1* amplification may predict endocrine therapy responsiveness in breast cancer patients

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Estrogen receptor (ER) α plays a crucial role in normal breast development and has also been linked to mammary carcinogenesis and clinical outcome in breast cancer patients. However, the molecular mechanisms controlling the expression of ERα are as yet not fully understood. Gene amplification is one of the important factors regulating protein expression. Recent studies on the amplification of the *ESR1* gene, which encodes ERα, have presented conflicting data. Using fluorescence *in situ* hybridization and real-time quantitative polymerase chain reaction analysis, we examined the *ESR1* status in a series of breast cancer tissues and analyzed its clinical importance. *ESR1* gene amplification and gain were found in 22.6 and 11.3% of samples, respectively, as determined by three-dimensional fluorescence *in situ* hybridization assay. Moreover, *ESR1* amplification and amplification plus gain were significantly negatively correlated with tumor size, number of positive lymph nodes, negative ERα, and positive human epidermal growth factor receptor 2 status. It has also been shown that *ESR1* amplification strongly correlates with higher expression levels of ER protein and that patients with *ESR1* amplification in their tumors apparently experience longer disease-free survival than those without. Our data suggest that *ESR1* amplification might prove to be helpful in selecting patients who may potentially benefit from endocrine therapy. (*Cancer Sci* 2009; 100: 1012–1017)

Estrogen receptor (ER) α signaling is known to be necessary for the growth and differentiation of normal breast epithelium, as well as for the initiation and progression of estrogen-dependent breast cancer. It was reported that the percentage of normal breast epithelial cells staining positive for ERα is generally low,[1] and that expression of the ERα protein fluctuates throughout the menstrual cycle.[2] On the other hand, significantly higher expression has been reported in premalignant breast disease,[3] and the level of ERα expression changes with progression of the disease in individual patients. Clinically, the ERα status of breast cancer patients is widely used both as an indicator for endocrine therapy responsiveness and also for prognosis prediction. However, despite its importance, the molecular mechanisms controlling the expression of ERα are not fully understood.

Nevertheless, it is known that ERα expression is regulated by numerous mechanisms. The silencing of ERα has been identified in breast tumor, including via a mutation within the open reading frame of the *ESR1* gene,[4] and via epigenetic changes such as DNA methylation of the promoter-proximal CpG island in the *ESR1* gene.[5,6] Moreover, it was recently reported that micro-ribonucleic acid (miRNA)-206[7,8] and miRNA-221/222[9] negatively regulate ERα at the post-transcriptional level. These abnormalities act alone or in combination to alter the functions or expression levels of ERα.

Gene amplification is one of the important factors regulating protein expression. Many copy number aberrations have been shown to recur across multiple cancer samples. These recurrent copy number aberrations frequently contain oncogenes and tumor-suppressor genes, and are associated with tumor progression, the clinical course of the cancer, or its responsiveness to therapy.[10] Knowledge of copy number aberration can have an immediate clinical use; for example, an effective diagnosis of the amplification or overexpression of human epidermal growth factor receptor (HER) 2/neu in breast tumors will allow such patients to benefit from treatment with the monoclonal antibody trastuzumab (Herceptin).[11] With respect to ERα, we previously described that loss of heterozygosity of the ER gene does not play an important role in the lack of ER function in breast cancer tissues.[12] On the other hand, some studies have reported amplification of *ESR1*.[13–20] For example, Holst and colleagues recently reported that more than 20% of breast cancers harbor genomic amplification of the *ESR1* gene. They also found that *ESR1* amplification is an indicator for defining a subtype of primary breast cancers that had particularly high ER expression levels and that this subtype might be optimally suited for hormonal therapy.[15] However, contradictory findings on the clinical importance of *ESR1* amplification for breast cancer patients have also been reported.[16–20]

Given the clinical importance of ERα and its gene *ESR1* in relation to ERα expression levels, we investigated the status of *ESR1* in a series of breast cancer tissues by means of fluorescence *in situ* hybridization (FISH) and real-time quantitative polymerase chain reaction (qPCR) analysis, in an attempt to elucidate the relationship between *ESR1* status and clinicopathological factors and prognosis of the patients.

Materials and Methods

Patients and breast cancer tissues. Primary invasive breast carcinoma specimens were obtained by surgical excision from 147 cases of patients at the Department of Breast and Endocrine Surgery, Kumamoto University Hospital (Kumamoto, Japan) between June 2001 and August 2006. FISH staining of *ESR1* status was from 133 available cases of paired tissues. These patients were from a consecutive series, and no exclusion criteria were applied. Informed consent was obtained from all patients before surgery. The Ethics Committee of Kumamoto University Graduate School of Medicine (Kumamoto, Japan) approved the study protocol.

The median age of the patients was 62 years (range, 30–86 years). Postoperative treatment was done in accordance with the recommendations of St Gallen's international consensus meeting for primary breast cancer.[21–23] On recurrence, patients

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with ERα-negative and progesterone receptor-(PgR) negative tumors were treated with cyclophosphamide + methotrexate + fluorouracile, fluorouracil + epirubicin + cyclophosphamide, and/or taxanes. Patients with hormone receptor-positive tumors and non-visceral metastases were treated with endocrine therapy, such as antiestrogens and aromatase inhibitors. Patients were followed postoperatively every 3 months for the first 5 years, and every 6 months thereafter. The median follow-up period was 40 months (range, 4–87 months).

Fluorescence *in situ* hybridization. Paraffin-embedded tissue blocks were sectioned at 4 μm thickness and mounted on slides. After deparaffinization and hydration using a commercial SPEC ESR1/CEN6 Dual Color Probe kit (ZytoVision, Bremerhaven, Germany), slides were processed according to the manufacturer's instructions. In brief, the tissue samples on the slides were dewaxed for 10 min in an incubator at 70°C, followed by xylene treatment for 10 min, twice. After rehydration in a series of graded ethanol solutions followed by rinsing in distilled water, the slides were treated with Pretreatment Solution Citric at 98°C for 15 min, and then washed twice with distilled water for 2 min.

The samples were treated with pepsin reagent for 15 min at 37°C, followed by washing with 2× saline–sodium citrate buffer for 5 min and water for 1 min. The slides were then dehydrated in a series of graded ethanol solutions and air dried.

Subsequently, the samples were treated with ESR1/CEN-6 Probe Mix (10 μL each) and then a coverslip was added. Finally, the edges of the hybridization area were sealed with rubber cement. For codenaturation of the probe and target DNA, slides were placed at 75°C for 10 min and then incubated at 37°C for 14–20 h with a Hybridizer instrument (Dako, Glostrup, Denmark).

After hybridization, the slides were washed and dehydrated. Anti-fading 4′,6-diamidino-2-phenylindole was applied.

Image analysis. Images were obtained with a fluorescence microscope (DP70; Olympus, Tokyo, Japan) equipped with ×100 UPlan Apo objective lens and image acquisition software (DP Manager; Olympus). For detection of small amplicons, image stacks containing three-dimensional datasets collected at 1.0–1.5-μm intervals through the *z*-axis were subjected to projections.

In normal cells, the CEN-6 DNA probe became bound to the centromere area of chromosome 6, and thus one to two red fluorescent signal spots were shown. The *ESR1* DNA probe became bound to the *ESR1* gene, and thus one to two green fluorescent signals were shown. We classified tissue samples with an ESR1/CEN-6 ratio greater than 1.0 but less than 2.0 as an *ESR1* gain, and those cases with an ESR1/CEN-6 ratio higher than 2 or with clusters of green signals as indicative of amplification of the *ESR1* gene.

Immunohistochemistry. Immunohistochemical staining was carried out as previously described,[24] on 4-μm thick tumor sections. Serial sections were prepared from selected blocks and float-mounted on adhesive-coated glass slides for ERα, PgR, or HER2 staining. Primary antibodies were monoclonal mouse antihuman ERα antibody (1D5; Dako) at 1:100 dilution, PgR antibody (636; DAKO) at 1:100 dilution, and rabbit antihuman c-erbB-2 oncoprotein antibody (Dako) at 1:200 dilution for HER2. The Envision system (EnVision labeled polymer, peroxidase; Dako) was used as the detection system for ERα, PgR, and HER2.

Immunohistochemical scoring. Immunostained slides were scored after the entire slide had been evaluated by light microscopy. The expression of ERα and PgR was scored by assigning a proportion score and an intensity score according to Allred's procedure.[25] In brief, a proportion score represented the estimated proportion of tumor cells staining positive, as follows: 0, none; 1, <1/100; 2, 1/100–1/10; 3, 1/10–1/3; 4, 1/3–2/3; and 5, >2/3. Any brown nuclear staining in invasive breast epithelium counted toward the proportion score. An intensity score represented the average intensity of the positive cells, as follows: 0, none; 1, weak; 2, intermediate; and 3, strong. The proportion and intensity scores were then added to obtain a total score, which could range from 0 to 8. Tumors with a score of 3 or more were considered to be positive for ERα expression. HER2 immunostaining was evaluated using the same method as used by the HercepTest (Dako). To determine the score of HER2 expression, the membrane staining pattern was estimated and scored on a scale of 0 to 3+. Tumors with scores of 2 or more were considered to be positive for HER2 overexpression.

***ESR1* gene amplification detection by real-time polymerase chain reaction.** Patient and control genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The concentration and purity of the genomic DNA preparations were measured and genomic DNA was stored at 4°C until use.

Probes and primers. We used the primers and probe for *ESR1* as reported by Holst *et al*.[15] The sequence of the primers and probe were: *ESR1* forward primer 5′-GCCAACGCGCAGGTCTA-3′ (562–578); *ESR1* reverse primer 5′-GCCGCAGCCTCAGA-3′ (623–610); and *ESR1* TaqMan probe FAM-CTCCCCTACGGC-CCC-NFQ. The polymerase chain reaction (PCR) product size for *ESR1* was 62 bp. The final optimized concentration for the *ESR1* TaqMan probe and each pair of primers was 250 and 900 nM, respectively. *RNase P* was chosen as a reference for gene dosage because of its single copy number. Primers and probe for *RNase P* were obtained from TaqMan endogenous control kits (4316844; Applied Biosystems).

Real-time qPCR. Real-time qPCR was carried out in a 96-well optical plate on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μL, adjusted with sterile water: 4.5 μL of DNA (~50 ng), 900 nM of each *ESR1* primer, 10 μL of Master Mix (2×), 250 nM of *ESR1* probe, and 1 μL of *RNase P* kit (20×). All reactions were carried out in triplicate. Thermal cycling conditions included an initial denaturation at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

Quantitative analysis. Calculation of the gene copy number was carried out using the comparative Ct method (ΔΔCt) that requires a healthy control sample (diploid) as a calibrator in all amplifications, and the *RNase P* gene was coamplified with the *ESR1* gene and served as an internal standard.

To use the ΔΔCt method, a validation experiment had to be run to show that the efficiencies of the target and the endogenous control amplifications were approximately equal. Then, we analyzed the samples and calibrated them in triplicate, and in parallel, for *ESR1* and *RNase P*. *ESR1* gene status were defined by the ratio of *ESR1* versus *RNase P* gene: >2.0 indicated amplification, and between 1.5 and 2.0 indicated a gain.

Statistical analysis. The χ^2-test for independence was adopted for statistical analysis of associations between *ESR1* gene status and clinicopathological factors. A disease-free survival curve was generated by the Kaplan–Meier method and verified by the log-rank (Mantel–Cox) test. Differences were considered significant when a *P*-value <0.05 was obtained.

Results

***ESR1* amplification and gain in relation to clinicopathological factors.** We analyzed the *ESR1* gene copy number ratio in 133 primary breast tumors (109 ER-positive tumors and 24 ER-negative tumors) by FISH. Of the 133 cases, 30 (22.6%) cases showed amplification for *ESR1*, and 15 (11.3%) demonstrated *ESR1* gene gain.

ESR1 amplification and amplification plus gain were significantly negatively correlated with tumor size ($P = 0.0025$ and $P = 0.0175$, respectively) and the number of positive lymph nodes ($P = 0.0238$

Table 1. *ESR1* amplification and gain in relation to clinicopathological factors

Clinicopathological factor	ESR1 FISH results				
	Analyzable (*n*)	Amplification (*n* (%))	*P*-value	Amplification + gain (*n* (%))	*P*
Total	133	30 (22.6)		45 (33.8)	
Age (years)					
<50	23	4 (17.4)	0.5146	5 (21.7)	0.1776
>50	110	26 (23.6)		40 (36.4)	
Tumor size (cm)					
<3.0	95	28 (29.5)	0.0025*	38 (40.0)	0.0175*
≥3.0	38	2 (5.3)		7 (18.4)	
No. positive lymph nodes					
0	82	23 (28.0)	0.0238*	33 (40.2)	0.0252*
≥1	50	6 (12.0)		11 (22.0)	
Histological grade					
1	74	14 (18.9)	0.2860	23 (31.1)	0.4437
2	28	10 (35.7)		13 (46.4)	
3	28	5 (17.9)		8 (28.6)	
Estrogen receptor					
Positive	109	30 (27.5)	0.0035*	45 (41.3)	0.0001*
Negative	24	0 (0.0)		0 (0.0)	
Progesterone receptor					
Positive	79	18 (22.8)	0.9392	31 (39.2)	0.1110
Negative	54	12 (22.2)		14 (25.9)	
HER2					
Negative	119	29 (24.4)	0.1446	44 (37.0)	0.0256*
Positive	13	1 (7.7)		1 (7.70)	

*$P < 0.05$ is considered as significant. HER, human epidermal growth factor receptor.

Table 2. Comparison of estrogen receptor (ER) amplification and expression

Expression	*ESR1* FISH (*n*)	ER immunohistochemistry results (Allred score)						
		0–2	3	4	5	6	7	8%
Normal	86	27.9	2.3	3.5	4.7	7.0	9.3	45.3
Gain	14	7.1	0	7.1	0	7.1	21.4	57.1
Amplified	30	0	0	0	0	0	10.0	90.0

FISH, fluorescence *in situ* hybridization.

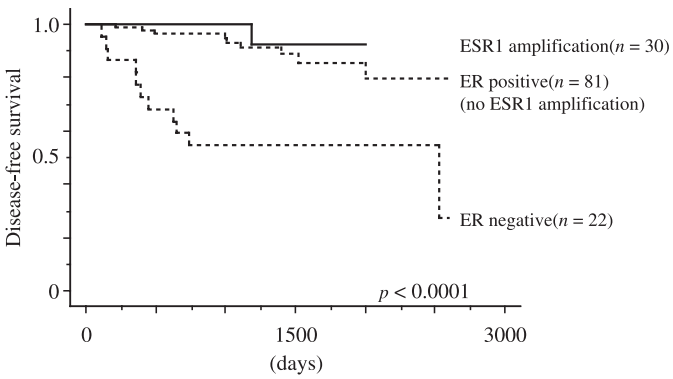

Fig. 1. Kaplan–Meier analysis of the effect on estrogen receptor (ER) status and *ESR1* amplification on disease-free survival for three groups of cancers: group 1, tumors with *ESR1* amplification; group 2, ER-positive tumors (Allred scores of 3–8) lacking *ESR1* amplification; and group 3, ER-negative tumors (Allred scores of 0–2).

and $P = 0.0252$, respectively), and positively correlated with ERα status ($P = 0.0035$ and $P = 0.0001$, respectively), whereas *ESR1* gene amplification plus gain was also significantly negatively correlated with HER2 status ($P = 0.0256$) (Table 1).

Correlation between *ESR1* amplification and ER expression. To investigate the influence of *ESR1* amplification on ER protein levels, we compared *ESR1* gene copy number to ER protein expression level by immunohistochemistry using Allred scoring (Table 2). Immunohistochemical analysis by Allred was successful in 130/133 (97.7%) breast cancer samples. There was a strong correlation between *ESR1* amplification and ERα expression. All tumors with *ESR1* amplification showed higher expression levels of ER protein (Allred scores of 7–8); a similar trend was seen with tumors demonstrating *ESR1* gain, with 78.5% of these samples showing strong ER expression (Allred scores of 7–8).

Patients with *ESR1* amplification in their tumors had better disease-free survival. We then examined whether *ESR1* amplification in primary breast tumors affected disease-free survival. In the analysis of disease-free survival, local recurrences and distant metastases were considered as an event. Among 21 cases of events, there were 16 cases of distant metastases and five of local recurrence.

For this analysis, we stratified the patients into three groups: group 1, tumors with *ESR1* amplification; group 2, ER-positive tumors (Allred scores of 3–8) lacking *ESR1* amplification; and group 3, ER-negative tumors (Allred scores of 0–2). It seems that patients with *ESR1* amplification in their tumors experienced longer disease-free survival than those without *ESR1* amplification, even though this difference did not reach statistical significance (Fig. 1).

We were unable to analyze the correlation between *ESR1* amplification and overall survival analysis because there were no cancer-related deaths in group 1.

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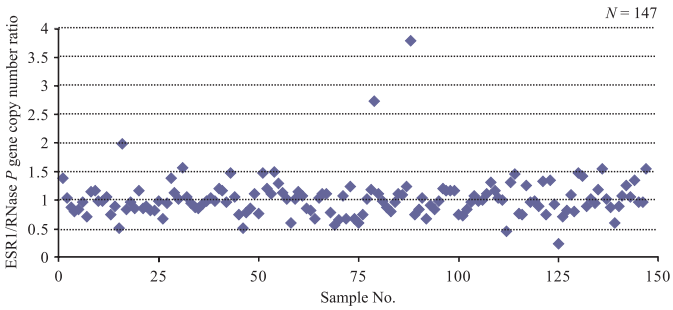

Fig. 2. Frequency distribution of *ESR1* amplification ratios in 147 breast cancers analyzed by real-time quantitative polymerase chain reaction. *ESR1* gene status was defined by the ratio of *ESR1* versus *RNase P*, as follows: >2.0 indicated amplification, between 1.5 and 2.0 indicated gain. The majority of cases had an amplification ratio <1.5, indicating normal copy number of *ESR1*. Two cases were considered to be amplified by real-time quantitative polymerase chain reaction.

***ESR1* amplification by real-time PCR.** The PCR amplification efficiency for the target gene and the reference gene were determined by constructing a standard curve. The amplification efficiency based on the slopes of the standard curves was 91% for *ESR1* and 93% for *RNase P*. Because the amplification efficiencies for both genes were approximately equivalent, it was possible to use the comparative Ct method (ΔΔCt) to determine the relative copy number of *ESR1*. Amplification plots for each triplicate showed near overlap, indicating that the assay was highly reproducible between replicates.

We observed amplification of *ESR1* in 2 out of 147 cases (1.4%); in both cases FISH analysis confirmed the amplification. Apart from these two, we found four gain cases, whereas the remainder had a normal ratio of *ESR1* to centromere 6 (Fig. 2).

We investigated the correlation between *ESR1* amplification by qPCR and ER status by immunohistochemistry, but no significant results were found (data not shown).

Discussion

In the present study, using FISH, we demonstrated that 30/133 (22.6%) breast cancer samples showed amplification and 15/133 (11.3%) showed gain of *ESR1*, which is consistent with the findings of Holst *et al*. that *ESR1* gene amplification was frequent in breast cancer.[15] Gene amplification, in general, occurs frequently in breast cancer. Multiple different oncogenes have been described previously as being amplified in breast cancer, including *HER2*, epidermal growth factor receptor, c-Myc gene (*MYC*), cyclin D1 gene (*CCND1*), and murine double minute 2 gene (*MDM2*).[26–30] However, there have been few reports about the frequency of *ESR1* gene amplification, even though increased ERα expression levels were found to be associated with breast cancer tumorigenesis and progression. The increased frequency of *ESR1* gene amplification reported by Holst *et al*. has prompted investigations about *ESR1* amplification. However, concerning the frequency of *ESR1* gene amplification in breast cancer, no consistent conclusions have been drawn.[16–20] Now, a variety of assays are available for detecting copy number variation (CNV), including Southern blotting, FISH, array-based comparative genomic hybridization (aCGH) and real-time qPCR. Southern blotting requires a large amount of genomic DNA and is costly in terms of reagents, work, and time. FISH is considered to be the most precise method for amplification detection,[31] as it is unaffected by the problem of tumor cell heterogeneity (see discussion below). aCGH is a powerful screening tool for detecting DNA copy changes and hence is used to identify cancer-associated chromosomal aberrations.[32] However, it is more expensive, time-consuming, and requires special equipment with software. Additionally, aCGH reflects the pattern of changes observed in the dominant clonal populations of a given tumor, which means that changes found in the less prevalent clonal populations are less likely to be identified with this technique.[10] The same applies to real-time qPCR. However, real-time qPCR offers several advantages, including speed, sensitivity, reproducibility, and low cost. Furthermore, it is informative due to a large dynamic range of quantification, and the need for very small amounts of starting material. Therefore, we analyzed DNA copy number ratios using real-time qPCR to confirm the FISH results. We observed, however, a discrepancy between FISH and real-time qPCR results. We found that only 2/147 (1.4%) samples showed amplification of the *ESR1* gene 30/133 (22.6%) using FISH. With regard to *ESR1* gene amplification, several studies,[16–20] have contested the frequency of the phenomenon as reported by Holst *et al*.[15] The findings from these other studies on *ESR1* amplification reported a frequency ranging from only 0 to 10% as detected by a variety of methods, including FISH, aCGH, and real-time qPCR.[16–20] The differences between FISH data obtained by various research groups could mainly result from whether manual or automated methodology was applied for scoring hybridization signals. It may also be possible that a proportion of low-level amplification was missed by automated scoring of FISH signals. Therefore, we carried out manual scoring by taking the image stacks containing three-dimensional data sets through the *z*-axis and analyzed the projection image in order to detect small-sized amplicons (Fig. 3). Analysis using three-dimensional FISH has proved to be a useful and reliable way to evaluate small nuclear components, such as chromatin arrangement and telomere clustering, as reported previously.[33–35] Most of the *ESR1*-amplified tumors had tiny confluent or small gene clusters of *ESR1* (3–10 copies), whereas only 6/30 (20%) cases had large clusters (>10 copies), as observed in the present study (Fig. 4). If we had not taken the image stacks into account as described above, the detection rate of *ESR1* amplification in our patient population would have been 18/133 (13.5%). On the other hand, it is likely that the inability of array-CGH and real-time qPCR to detect CNV was caused by breast tumor heterogeneity. This might have led the normal cells, or the tumor cells without CNV, to dilute the population of mutated tumor cells and thereby lower the average copy number of the population of

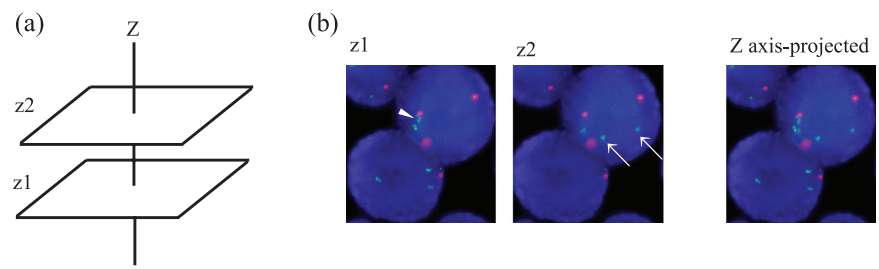

Fig. 3. Image restoration and analyses of three-dimensional images of the *ESR1* locus. (a) A series of images was taken through the *z*-axis. (b) Localization of the *ESR1* gene (green) and centromere 6 (red) is shown in the two fluorescence *in situ* hybridization images (z1, z2) and projected one. In a single-plane image, the *ESR1* gene could not be detected, as indicated by an arrowhead in z1 and by an arrow in z2, because of the small size of the amplicon.

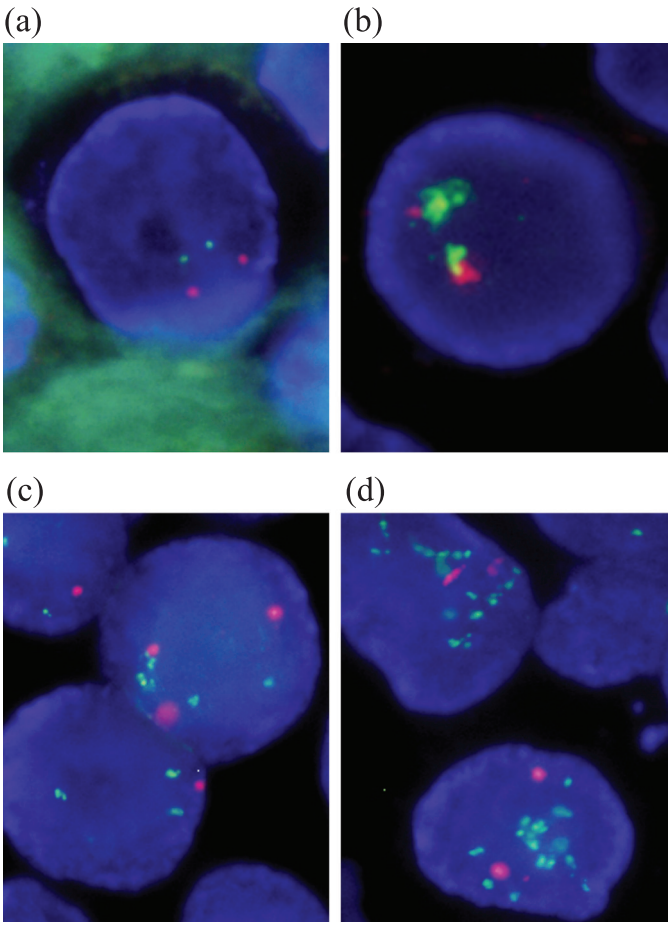

Fig. 4. Examples of fluorescence *in situ* hybridization findings in breast cancers with (a) normal *ESR1* copy number and (b–d) *ESR1* amplification. The *ESR1* gene probe is labeled in green, the centromere 6 reference probe in red. (a) Two copies of *ESR1* per nucleus. (b) Tiny confluent *ESR1* gene clusters. (c) Small cluster with three to nine clearly distinguishable *ESR1* gene copies. (d) Large clusters of more than 10 gene signals.

cells detected. Therefore, further studies using microdissected breast cancer tissue are urgently needed.

We observed that *ESR1* amplification was significantly correlated with small tumor size and negative lymph nodes. These data suggest that although *ESR1* amplification has a role in the proliferative mechanism of early stage breast cancer, it is not involved in tumor progression. Therefore, it is possible that breast cancer cells with *ESR1* amplification have a lower growth potential than those without amplification (which have acquired growth potential via other means), and that such tumor cells with *ESR1* amplification will gradually die or be outnumbered by the cells without amplification.

Although the expression status of ERα is a primary determinant in the hormone therapy of breast cancer, it can not reliably predict the responsiveness of the tumors to hormone therapy; not all ERα-positive breast cancers respond to hormone therapy. This discrepancy between ERα expression status and response to hormonal therapy probably derives from the different estrogen signaling pathway conditions.[36]

Therefore, a new diagnostic marker to screen patients who may potentially benefit from hormone therapy is required. Our results showed that *ESR1* amplification was significantly correlated with higher expression levels of ER protein and better disease-free survival. Considering that most patients with ERα-positive tumors will have adjuvant antiestrogen treatment, it is likely that *ESR1* amplification could be helpful in identifying a subgroup of individuals with breast cancer who would benefit most from the hormone therapy. Further studies in large cohorts of patients are needed to evaluate the predictive value of *ESR1* amplification for hormone therapy.

In conclusion, FISH assays appear to be highly sensitive and specific for detecting *ESR1* gene amplification, and obtaining images taken through the *z*-axis. Our present study has demonstrated *ESR1* gene amplification and gain in 22.6 and 11.3% of breast cancer samples, respectively, as determined by FISH assay. *ESR1* amplification and amplification plus gain were significant negatively correlated with tumor size, number of positive lymph nodes, and HER2 status, and positively correlated with ERα status. Moreover, it has been shown that *ESR1* amplification is strongly correlated with higher expression levels of ER protein and has possibilities as a predictive marker. Our data suggest that *ESR1* amplification might prove helpful in selecting patients who may potentially benefit from endocrine therapy.

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