ER-α36, a Novel Variant of ER-α, is Expressed in ER-positive and -negative Human Breast Carcinomas

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Abstract. *Background: The status of estrogen receptor-alpha (ER-α) expression is one of the most important diagnostic and prognostic factors of breast cancer. ER-α is a 66-kDa, ligand-induced transcription factor, characteristically detected in the cell nucleus by immunohistochemistry (IHC) in breast cancer specimens. Recently, we identified and cloned a 36-kDa novel variant of ER-α, ER-α36, which lacks both transactivation domains and functions as a dominant-negative effector of transactivation activities of the full-length ER-α (ER-α66) and ER-β. ER-α36 primarily localizes to the cytoplasm and plasma membrane, and responds to both estrogens and antiestrogens by transducing membrane-initiated signaling cascades, stimulating proliferation and possibly contributing to a more aggressive phenotype in breast carcinomas. ER-α36 is expressed in established ER-positive and -negative breast cancer cell lines. However, its expression and localization in breast cancer specimens have not been evaluated. As ER-α36 may play important roles in breast cancer tumorigenesis, it is of clinical importance to examine the expression pattern of ER-α36, in addition to that of ER-α66, for more comprehensive molecular profiling of breast carcinomas. Patients and Methods: Thirty-one breast cancer patient tissues were evaluated for ER-α36 and ER-α66 protein expression status by IHC and six additional patient tissue samples were analyzed by Western blot analysis using antibodies specific to ER-α66 or ER-α36. Results: Our experiments reveal a cytoplasmic and plasma-membrane-associated expression pattern of ER-α36 in both ER-α66-positive and -negative breast cancer samples. Furthermore, ER-α36 expression appears to be associated with decreasing nuclear and/or cytoplasmic ER-α66 expression, suggesting its potential use as a diagnostic and prognostic marker. Conclusion: ER-α36 is a novel isoform of ER-α, frequently expressed in ER-α66-negative cancers, whose detection may provide additional information for better diagnosis and prognosis.*

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Estrogen receptor-alpha (ER-α) expression profiling in breast cancer patients is one of the most important determinants of a cancer patient susceptibility to endocrine (anti-estrogen) therapy (1). ER-α expression is currently assessed by immunohistochemical methods (IHC) using commercial ER-α antibodies. Approximately 70% of breast cancer cases are ER-α- or (ER)-positive, and the remaining (30%), ER-negative (2). In general, patients with ER-positive cancers respond favorably to anti-estrogens such as tamoxifen while ER-negative patients do not (3). However, up to thirty percent of ER-positive patients are unresponsive to anti-estrogen therapy, while some ER-negative breast cancer patients do respond favorably to tamoxifen treatment (4-5). The contradiction to the conventional theory may be explained by potential differential expression of ER variants which may not be detected by the commercial ER antibodies against ER-α.

ER-α36, a 36 kDa protein, is a variant of the full-length 66 kDa ER-α (ER-α66), which lacks ligand-dependent and -independent transactivation domains (AF-1 and AF-2) but retains portions of ligand- and DNA-binding domains (6, 7). Unlike ER-α66, which is often detected in the cell nucleus, ER-α36 mainly localizes in the cytoplasm and plasma membrane (7). As a result, ER-α36 transduces membrane-initiated estrogen signaling cascades and functions as a dominant-negative effector of estrogen-dependent and independent transactivation induced by ER-α66 (7). ER-α36 expression was detected in both ER-α66-positive and -negative breast cancer cell lines (7). Here, we examined thirty-one cases of breast carcinoma tissues for the potential

differential ER-α66 and ER-α36 expression patterns by immunohistochemistry and six cases by Western blot analysis, using antibodies specific to ER-α66 or ER-α36.

Patients and Methods

Cancer tissue samples. Formalin-fixed, paraffin-embedded blocks of breast cancer tissues (31 cases, from 2003 to 2005) were randomly selected and provided by the Department of Pathology of the Run Run Shaw Hospital, Hangzhou, P.R. China, after approval of the Institutional Review Board. Cancer tissues from these patients were all in stages II, III and IV of breast cancer. Snap- frozen breast cancer tissues from six additional patients were randomly selected from samples collected by the Department of Pathology of the Run Run Shaw Hospital and were used in Western blot analysis. None of the thirty-seven patients had received anti-estrogen therapy prior to surgery and no follow-up data were available.

Immunohistochemistry assay. Immunohistochemistry assay for ER-α66 and ER-α36 expression was performed on the paraffin-embedded tissue sections using the peroxidase labeled streptavidin-biotin method and a commercially available ER-α66 antibody (RDI-ERECPNabrx; Research Diagnostics Inc., Concord, MA, USA) that recognizes the *N*-terminal of ER-α66. ER-α36-specific antibody was custom-made by Alpha Diagnostic International (San Antonio, TX, USA) against the 20 unique amino acids at the *C*-terminal of ER-α36 (7).

Immunohistochemical staining was performed using UltraSensitive™ S-P kits (Maixin-Bio, P.R. China) according to the manufacturer's instructions, with specific antibodies against ER-α66 or ER-α36 as primary antibodies. Briefly, slides with 5 μm-thick tissue sections were deparaffinized and hydrated in sequential treatment of xylene, ethanol and water. Citrate buffer (0.01 M citric acid, pH 6.0) was used to retrieve antigens in a heated pressure cooker. Endogenous peroxidase activity was quenched with 3% H_2O_2 before anti-ER-α66 (at 1:300) or anti-ER-α36 (at 1:100) antibody was applied. Biotinylated secondary antibody and streptavidin-horseradish peroxidase were added subsequently, and 3,3'-diaminobenzidine tetrahydrochloride was used as substrate for chromogenic visualization before counterstaining with hematoxylin. The extent and cellular distribution of staining was evaluated by two investigators on a double-headed microscope. ER expression was defined as positive if more than 10% of tumor cells stained positive.

Western blot analysis. Frozen cancer tissues were homogenized in T-PER tissue protein extract reagent (Pierce, Rockford, IL, USA). The protein concentrations of the lysates were measured and proteins were denatured by boiling in a gel-loading buffer and separated by SDS/PAGE in 10% gels. Protein from a normal mammary gland was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). Equal amounts of proteins were analyzed by Western blot analysis as described elsewhere (7), and the same blot was stripped and reprobed with an anti-actin antibody to ensure equal loading.

Statistical analysis. The SURVEYFREQ procedure of the SAS statistical package (SAS, Cary, NC, USA) was used to calculate the frequencies, 95% confidence intervals and statistical significance of potential differences in the frequencies of the four phenotypes in terms of ER-α66 and ER-α36 expression in all 37 patients (31 specimens for immunohistochemistry assay and 6 tissue samples for Western blot analysis).

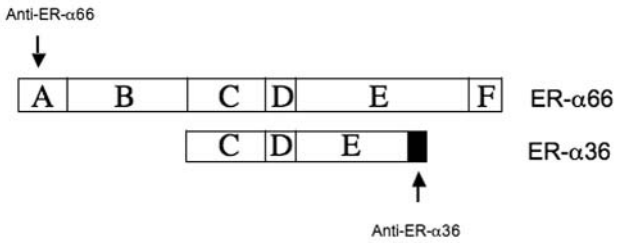

Figure 1. *Schematic illustration of the protein structures of estrogen receptors ER-α66 and ER-α36. The specific antibodies used and their epitopes are also indicated.*

Results

ER-α36 is expressed in both ER-α66-positive and -negative breast cancer patients. To determine the expression pattern of ER-α36 in breast cancer tissues, immunohistochemistry (IHC) assays were performed on tissue samples from 31 breast cancer patients using an ER-α36-specific antibody raised against the 20 unique amino acids at the *C*-terminal of ER-α36 (Figure 1). The expression patterns of ER-α66 in the same tissue samples were examined with an ER-α66-specific antibody that recognizes the *N*-terminal of the ER-α66 (Figure 1). The results from our IHC assays showed that ER-α36 was expressed in both ER-α66-positive and -negative breast cancer cases (Table I), consistent with our previous finding using established breast cancer cell lines (7). All the four possible phenotypes (ER-α66 alone, ER-α36 alone, ER-α66 and ER-α36, and absence of both) were present among breast cancer patients. The frequencies of these four phenotypes are not significantly different ($p=0.595$). The current clinical evaluation of ER status only checks the expression profile of ER-α66, not ER-α36, which may underestimate the true ER status of the tumors.

Western blot analysis of breast cancer tissue lysates from the six randomly selected patients showed co-expression of ER-α66 and ER-α36 in three out of six cases (Figure 2). Expression of ER-α36 alone was observed in two out of six cases, while only one case of breast cancer expressed neither of the receptors (Figure 2). These data further indicated that a subset of breast carcinomas lacked ER-α66 expression (*i.e.* classified as ER-negative breast cancer) but in fact expressed ER-α36. The IHC assay also confirmed that ER-α66 was not detected in the two specimens that highly expressed ER-α36 (Figure 3).

Table I also shows the frequencies and 95% confidence intervals (95% C.I.) of the four phenotypes when results from the IHC assays and Western blot analyses were combined (total thirty-seven patients). Again, there was no significant difference in the frequencies among the four phenotypes ($p=0.526$); the frequency of ER-positive cases was

Table I. *Frequency of ER-α66 and ER-α36 expression in 37 breast cancer cases.*

ER Expression	Number	% (95% C.I.)	Clinical ER-α status % (95% C.I.)	Comprehensive ER-α status % (95% C.I.)
ER-α66	7	19 (6-32)	51 (34-68) ER-positive	70 (55-86) ER-positive*
ER-α66 & ER-α36	12	32 (17-48)		
ER-α36	7	19 (6-32)	49 (32-66) ER-negative	
None	11	30 (14-45)		30 (14-45) ER-negative

C.I., confidence interval; *$p<0.02$ compared to ER-negative. Clinical ER-α status is evaluated based on ER-α66 expression alone. Comprehensive ER-α status is evaluated based on expression of both ER-α66 and ER-α36.

significantly higher than that of ER-negative cases ($p=0.014$). IHC further confirmed the existence of breast cancer tissues that express abundant amounts of ER-α36 (Figure 3 A, C) but little or no ER-α66 expression (Figure 3B, D), which would be considered clinically ER-negative in usual practice.

ER-α36 localizes to the cytoplasm and plasma membrane. ER-α66 is a ligand-activated transcription factor, thus it is firmly established that ER-α66 localizes to the cell nucleus and, as such, pathologists usually only score nuclear staining of ER as a positive signal in breast cancer tissues prepared for IHC. Our previous molecular studies revealed a different localization pattern of ER-α36, primarily in the cytoplasm and plasma membrane of cells (7). We further examined whether this localization pattern of ER-α36 would be confirmed in breast cancer tissues. IHC assays of the thirty-one breast cancer tissues confirm our previous studies with established breast cancer cell lines in that ER-α36 is primarily expressed in the cytoplasm and plasma membrane with little or no nuclear staining (Figure 4C, D). Surprisingly, a weak cytoplasmic expression of ER-α66 was observed frequently in ER-α36-expressing breast cancer tissues (Figure 4B).

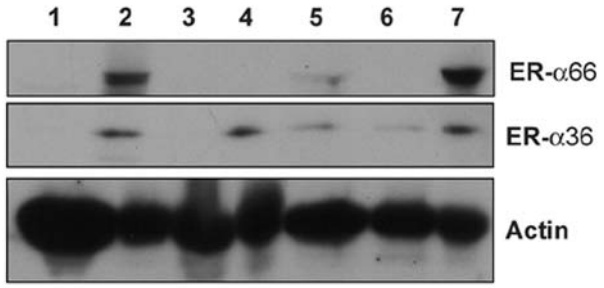

Figure 2. *Western blot analysis of the expression of ER-α66 and ER-α36 in human breast cancer samples with anti-ER-α66 or anti-ER-α36 specific antibodies. Lane 1: normal mammary gland; Lane 2: infiltrating ductal carcinoma; Lane 3; infiltrating ductal carcinoma; Lane 4: Invasive ductal carcinoma; Lane 5: infiltrating lobular carcinoma; Lane 6: infiltrating lobular carcinoma; Lane 7: Ductal carcinoma in situ. The same blot was stripped and reprobed with an anti-actin antibody.*

Discussion

Results from this study demonstrated that ER-α36 is expressed in breast cancer tissues of subsets of ER (ER-α66)-positive and -negative patients, consistent with findings from our previous experiments using established breast cancer cell lines (7). All four possible phenotypes in terms of expression of ER-α66 and ER-α36 were present with similar frequencies among the breast cancer patients. Many breast cancer patients that are clinically ER-negative in the currently medical practice (determined by lack of nuclear ER-α66 expression) may express ER-α36 although some are truly negative for both ER-α66 and ER-α36. Thus, our data reveal the potential shortcomings of routine clinical ER status evaluation based on ER-α66 expression alone and offer new information potentially useful for treatment of "ER-negative" breast cancer patients. However, it is currently unclear whether patients with breast cancer positive for both ER-α36 and ER-α66, or ER-α36 alone, would respond to endocrine therapy more favorably than patients with breast cancer positive for ER-α66 alone.

In this report, we also confirmed in breast cancer patients' tissue that ER-α36 localizes to the plasma membrane and cytoplasm with little or no nuclear staining, which is consistent with results from our previous report using established breast cancer cell lines (7). A surprising new finding is that the cytoplasmic expression of ER-α66 was frequently observed in ER-α36-positive breast cancer tissues while in breast cancer tissues positive for ER-α66 alone, it is primarily expressed in the cell nucleus. A similar phenomenon of cytoplasmic ER-α66 expression was observed in breast cancer cells with long-term treatment of tamoxifen eventually leading to drug-resistance (8). These findings suggest that the cytoplasmic expression of ER-α66, which is often neglected in the clinical evaluation of ER status, may be involved in the mechanism of tamoxifen resistance in breast cancer. It may be important to evaluate ER-α36 expression in breast cancer tissues from patients who are undergoing tamoxifen therapy. The finding also suggests a possible regulatory interaction between the two

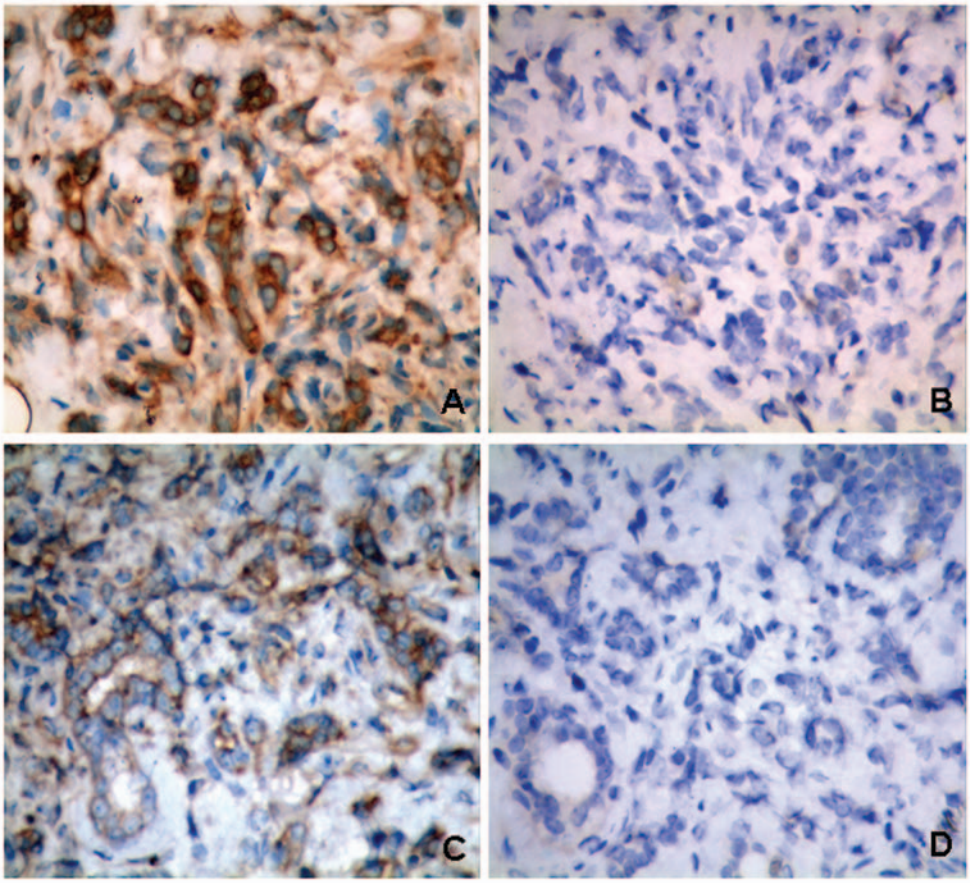

Figure 3. *Immunohistochemistry demonstrating ER-α36 and ER-α66 expression in two breast cancer cases. A & B, Tissue from one patient showing strong, cytoplasmic and membrane expression of ER-α36 (A) but little or no ER-α66 (B) expression. C & D. Tissue from another patient showing distinct membrane expression of ER-α36 (C) but no characteristic ER-α66 nuclear expression (D) (all at x400 magnification).*

isoforms of ER in which ER-α36 may facilitate translocation of ER-α66 from the nucleus to the cytoplasm, or simply retain ER-α66 in the cytoplasm.

Previously, Leclercq's group reported cytoplasmic localization of iodinated estradiol- labeled ER isoforms that are devoid of amino-terminal domains in human breast carcinomas (9). They also observed electrophoresed estrogen receptor bands with variable intensity and different molecular weights of 66, 50, 37 and 28 kDa in almost all cytosol from primary breast cancer (10). Although smaller molecular weight ER isoforms that localized in the cytoplasm in these studies were considered degradation products, in light of our previous and current studies, those proteins may have been the true, functional variants of ER. Furthermore, sequential transplantations of MXT mouse mammary tumors in nude mice resulted in an apparent passage of a 35 kDa ER from the nucleus to the cytoplasm with a concomitant progressive loss of the original ER, ER-α66, suggesting the relevance of the 35 kDa ER isoform expression in aggressive breast tumors (11). As ER-α36 has been implicated in membrane-initiated mitogenic estrogen signaling and is thought to mediate estrogen-stimulated proliferation (7), it is tempting to speculate that breast cancer patients with both ER-α66 and ER-α36 expression may represent the breast cancer population that is in transition to a more malignant phenotype or an advanced stage of breast cancer progression. On the other hand, breast cancer tissues only expressing ER-α36 may represent the subset of patients that are diagnosed as ER-negative, but could still respond to mitogenic estrogen signaling and anti-estrogen treatment. Further investigations to determine the therapeutic and prognostic implications of ER-α36 expression in breast cancer in association with patients' clinical course and outcome will be critical for improvements in overall breast cancer care and management.

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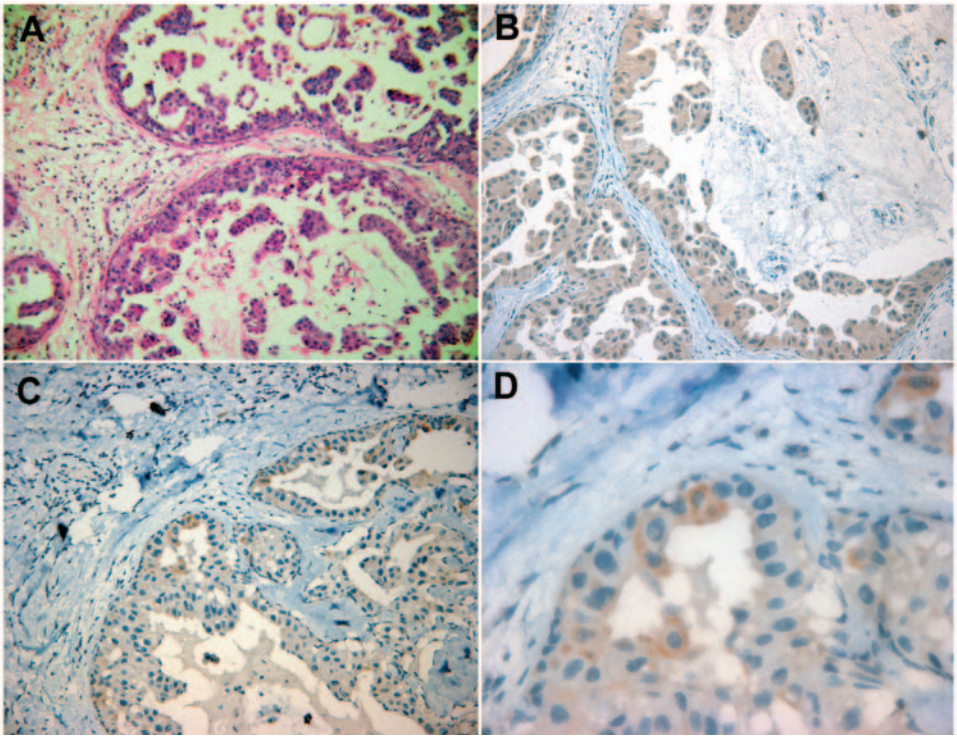

Figure 4. *Immunohistochemical results of human breast carcinoma tissue, showing different localization patterns of ER-α66 and ER-α36 in breast carcinoma cells from the same patient. A, H&E at x200. B, IHC using an ER-α66-specific antibody, showing diffuse and weak cytoplasmic staining of breast carcinoma cells with little nuclear staining at x200. C & D, IHC using an ER-α36 specific antibody at x200 and at x600, respectively, revealing the cytoplasmic and membrane expression pattern of ER-α36 in breast carcinoma cells.*

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