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ER-α36, a novel isoform of ER-α66, is commonly over-expressed in apocrine and adenoid cystic carcinomas of the breast

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ABSTRACT

Background ER-α36 is a novel 36 kDa isoform of the full-length oestrogen receptor alpha (ER-α66). ER-α36 primarily localises to the cytoplasm and the plasma membrane, and responds to membrane-initiated oestrogen and antioestrogen signalling pathways.
Aim To examine the expression of ER-α36 in apocrine and adenoid cystic carcinoma of the breast, both of which are consistently ER-α66 negative and currently lack effective targeted therapeutic options.
Methods 19 pure apocrine carcinomas (17 invasive and two in-situ carcinomas) and 11 adenoid cystic carcinomas of the breast were evaluated for ER-α36 expression, along with expressions of ER-α66, progesterone receptor (PR) and androgen receptor (AR) using immunohistochemical methods.
Results All pure apocrine carcinomas showed a characteristic steroid receptor expression profile (ER-α66 and PR negative, AR strongly positive). ER-α36 expression was detected in 18/19 pure apocrine carcinomas (94.7%, 95% CI 75.1 to 98.7) in predominantly membranous and cytoplasmic distribution. When positive, pure apocrine carcinomas uniformly (100% of cells) expressed ER-α36. All adenoid cystic carcinomas were uniformly negative for all three classic steroid receptors, but ER-α36 was detected in 8/11 cases (72.7%, 95% CI 42.8 to 90) with the similar sub-cellular pattern of expression as in the pure apocrine carcinomas. When positive, adenoid cystic carcinomas expressed ER-α36 in the majority of cells (average 76%).
Conclusion ER-α36, a novel isoform of ER-α66, is frequently over-expressed in apocrine and adenoid cystic carcinomas of the breast. These results indicate a potential for a novel targeted treatment in these cancers.

INTRODUCTION

Oestrogen receptor alpha (ER-α) expression in breast cancer is the most important predictor of antioestrogen therapy.[1] Approximately 75% of all breast carcinomas are ER-α positive, which is a good predictor of the treatment response.[2–4] Current immunohistochemical methods use antibodies against the full-length receptor protein (ER-α66, see figure 1), but there are at least three different isoforms of ER-α66 described in humans. Two isoforms have been detected in breast cancers.[5 6] These are ER-α46 and the novel isoform, ER-α36 which we recently identified and cloned.[7 8] They are generated through multiple promoter usage or alternative splicing.[5] Although most steroid hormone receptors primarily localise in the nuclei, additional oestrogen receptors have been reported to exist in the cytoplasm and on the plasma membrane.[3]

The novel 36 kDa isoform ER-α36 lacks the transcriptional activation domains found in ER-α66. The DNA-binding domain, dimerisation, partial ligand-binding domains as well as three myristoylation sites near the N-terminus are retained in ER-α36.[7 8] While ER-α66 is predominantly detected in the cell nucleus, ER-α36 mainly localises to the cytoplasm and on the plasma membrane.[7 8] ER-α36 has been shown to transduce the membrane-initiated steroid signalling (MISS) cascade, and function as a dominant-negative effector of oestrogen-dependent and independent transactivation mediated by ER-α66.[7] We have recently demonstrated the expression of ER-α36 in both ER-α66 positive (MCF-7) and negative breast cancer cell lines (MDA-MB-231), as well as in a proportion of invasive breast carcinomas of no special type.[8 9]

In this study, we focused on adenoid cystic carcinoma (ACC) and pure apocrine carcinomas (PAC), two special types of breast carcinomas that are characteristically negative for ER-α66,[10–12] and showed that the majority of them express ER-α36 protein.

MATERIALS AND METHODS

Breast tissue samples

Formalin-fixed, paraffin-embedded tissues (11 adenoid cystic carcinomas and 19 apocrine carcinomas) were selected from the files of the Departments of Pathology at Creighton University Medical Center (Omaha, Nebraska, USA), the Institute of Oncology Ljubljana (Slovenia), Thomas Jefferson University Hospital (Philadelphia, Pennsylvania, USA) and Kansas University Medical Center (Kansas City, Kansas, USA), after the approval of the Institutional Review Board of Creighton University School of Medicine. None of the 30 patients included in this study had received neoadjuvant therapy.

Immunohistochemistry

Immunohistochemical assays for ER-α66 (clone 6F11, Ventana Medical Systems, Tucson, Arizona, USA), progesterone receptor (PR; clone 16, Ventana Medical Systems), androgen receptor (AR; clone AR441, DakoCytomation, Carpinteria, California, USA), epidermal growth factor receptor (EGFR;

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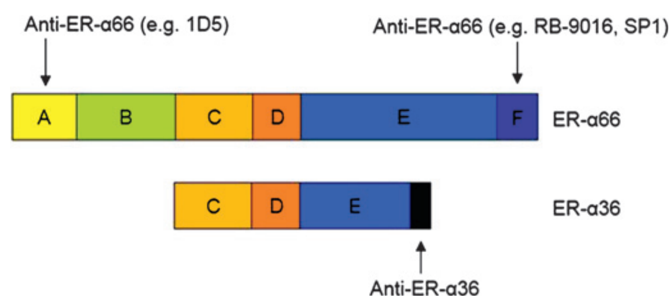

Figure 1 Domain structures of oestrogen receptor (ER)-α66 and ER-α36. Arrows indicate the specific antibodies and their corresponding epitopes.

(DAKO EGFR PharmDX diagnostic kit; DakoCytomation) and Her-2/neu (Clone CB11, Ventana Medical Systems) expression were previously performed as a part of clinical evaluation. The expression of ER-α66, PR and AR (the percentage of cells with the nuclear staining), and EGFR and Her-2/neu (the extent and the intensity of the membranous staining) were measured on an automated cellular imaging system (ACIS, ChromaVision Medical Systems, San Juan Capistrano, California, USA). The results of ER-α66, PR, AR, EGFR and Her-2/neu expression for both cohorts have recently been reported.[11 12]

Antibody used for the detection of ER-α36 was custom-made by Alpha Diagnostic International (San Antonio, Texas, USA); it was raised against the 20 unique amino acids at the C-terminal of ER-α36.[8 9] Immunohistochemical staining with this ER-α36 specific antibody was performed using the rabbit ImmunoCruz Staining System (Santa Cruz, sc-2051, California, USA) according to the manufacturer's instructions. Briefly, slides with 5 μm thick formalin-fixed paraffin-embedded tissue sections were deparaffinised and hydrated in sequential treatment of xylene, ethanol and water. Citrate buffer (0.01 M citric acid, pH 8.0) was used to retrieve epitopes in a pressure cooker. Endogenous peroxidase activity was quenched with 3% H_2O_2 before anti-ER-α36 (at 1:100 dilution) antibody was applied. Biotinylated secondary antibody and streptavidin-horseradish peroxidase were added subsequently, and 3,3′-diaminobenzidine tetrahydrochloride was used as substrate for chromogenic visualisation before counterstaining with haematoxylin. The extent and cellular distribution of staining was evaluated by two investigators on a double-headed microscope. The subcellular localisation and percentage of positive cells was recorded.

For ERα-36 expression, only the membranous/cytoplasmic pattern of staining was considered specific.[8 9] Weak (1+) intensity was defined as faint and incomplete membrane positivity. Moderate (2+) intensity and strong (3+) staining were both varying degrees of circumferential staining of membranes and the cytoplasm. The tumour was considered positive if a proportion of stained cells exceeded 1% at any intensity.[9]

Statistical analysis

To access the differences between proportions, two-sided Fisher's exact tests were used; α was set at a level of 0.05. The kappa statistic was applied to determine the consistency among the tests and 95% CIs are given around the estimates. Statistical analyses were carried out using SPSS V.17.0.

RESULTS

Patient characteristics

The mean age was 62 years (range 53–71 years) for patients diagnosed with ACC and 74 years for PAC (range 63–90 years).

Special breast cancer subtype characterisation

All PACs (including two in-situ carcinomas) were characteristically ER-α66 negative, PR negative and AR positive.[11] Her-2/neu over-expression (3+) was observed in 10/19 cases (52.6%, 95% CI 31.53 to 72.80); EGFR expression was observed in 17/19 (89.5%, 95% CI 68.30 to 96.79). Of the two apocrine carcinomas in-situ, one over-expressed Her-2/neu (score 3+) and the other was positive for EGFR (score 3+).

All ACC samples exhibited a characteristic triple-negative breast cancer profile (ER-α66 negative, PR negative, Her-2/neu negative). EGFR was detected in 7/11 (63.6%, 95% CI 34.89 to 84.83) ACCs, as reported recently.[12]

Table 1 summarises the immunohistochemical characteristics of these two cancer types.

ER-α36 protein expression in PAC and ACC

ER-α36 protein was detected in 18/19 PACs (94.7%, 95% CI 75.13 to 98.77) and in 8/11 cases of ACC (72.7%, 95% CI 42.81 to 90.08). Moderate to strong membranous and cytoplasmic expression (figure 2C,D) was seen in both cancer types. The average percentage of ER-α36 positive cells was 100% for PAC and 76.2% for ACC. Nuclear staining was absent in all but one case of PAC, which exhibited both nuclear and cytoplasmic/membrane distribution of the ER-α36.

We also analysed our cases for the co-expression of ER-α36 with EGFR and Her-2/neu proteins because of reported interactions between membrane steroid receptors and growth factors with tyrosine kinase activity.[13] Co-expression of ER-α36 and EGFR was observed in 16 cases of PAC and 7 cases of ACC. Although co-expression of ER-α36 and EGFR in both types of breast carcinomas was statistically significant (p=0.018), the measure of agreement was only marginally convincing (κ=0.52, 95% CI 0.12 to 0.93) (table 2). Co-expression of ER-α36 and Her-2/neu however was not found to have a significant relationship (p=1.00).

Adjacent normal breast tissue (ductal epithelium) was negative or showed reduced expression of ER-α36 in comparison to expression in the malignant epithelium.

DISCUSSION

ER-α66 expression is routinely evaluated by immunohistochemistry in all breast cancers since it has been shown to play a pivotal role[2–4] in patient treatment and outcome. Approximately 75% of all breast carcinomas are regarded as ER-α66 positive, and are thus amenable to targeted therapy with antioestrogens.[2–4]

The *ER-α* gene (*ESR1*) is composed of six functional domains encoded by eight exons that commonly produce a 66.2 kDa protein (ER-α66).[5] Numerous ER-α mRNA transcript isoforms of

Table 1 Immunohistochemical characteristics of 30 breast samples

Tumour type	ER-α66 [95% CI]	PR [95% CI]	Her-2/neu* [95% CI]	EGFR† [95% CI]	ER-α36 [95% CI]
PAC	0/19 (0%) [0.13 to 16.84]	0/19 (0%) [0.13 to 16.84]	10/19 (52.6%) [31.53 to 72.80]	17/19 (89.5%) [68.30 to 96.79]	18/19 (94.7%) [75.13 to 98.77]
ACC	0/11 (0%) [0.21 to 26.46]	0/11 (0%) [0.21 to 26.46]	0/11 (0%) [0.21 to 26.46]	7/11 (63.6%) [34.89 to 84.83]	8/11 (72.7%) [42.81 to 90.08]

*Her-2/neu protein positivity defined as 3+ score by immunohistochemistry.
†EGFR protein positivity defined as a membranous staining if a proportion of stained cells exceeded 1% at any intensity.
ER, oestrogen receptor; PR, progesterone receptor; EGFR, epidermal growth factor receptor; PAC, pure apocrine carcinoma; ACC, adenoid cystic carcinoma.

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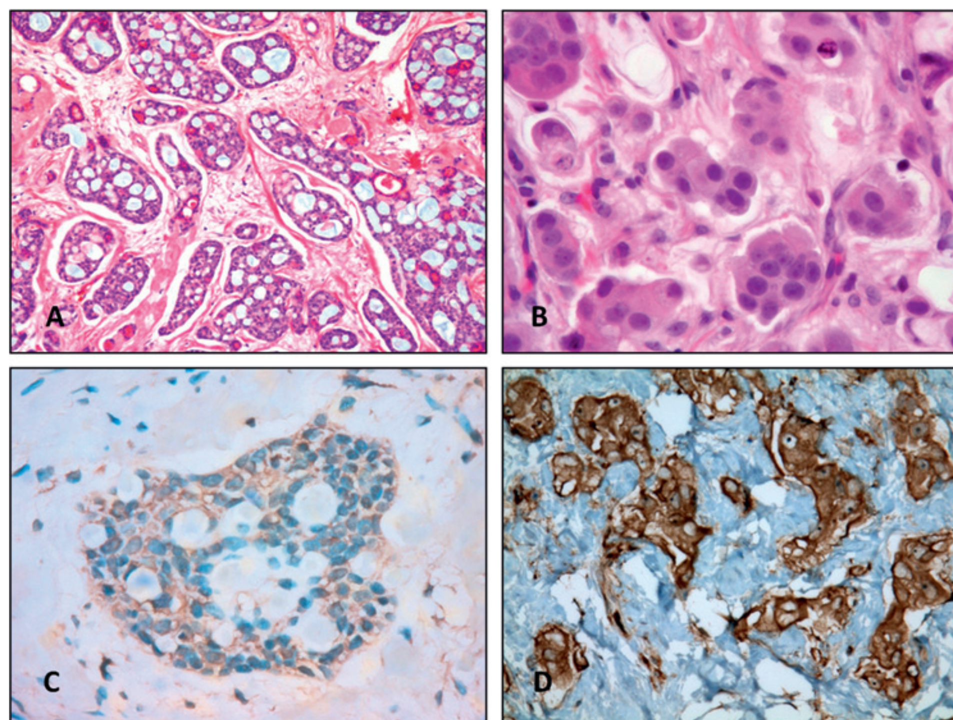

Figure 2 Representative H&E slides of: (A) a case of adenoid cystic carcinoma of the breast; (B) a pure apocrine carcinoma of the breast; (C) immunohistochemical staining of the tissue samples for ER-α36, demonstrating strong membrane and cytoplasmic staining in an adenoid cystic carcinoma; (D) a pure apocrine carcinoma of the breast.

the gene have been described,[6] and are generated through alternative splicing or promoter usage.[5] Several isoforms of the ER-α protein have been isolated in humans, including ER-α36, recently identified and cloned by our group.[7–9] This novel ER-α isoform is a product of a transcript initiated from a previously unidentified promoter in the first intron of the *ER-α66* gene and is identical to the ER-α66 encoded by exons 2–6 of the *ER-α66* gene, resulting in a smaller, 36 kDa protein (ER-α36).[7 8] In the experimental cell models of breast cancer, it served as a membrane-based oestrogen receptor mediating MISS and as a dominant-negative modulator of ER-α66 mediated transcription activity.[7] We have previously reported that ER-α36 is frequently detected in invasive breast carcinomas of no special type,[9] and that it may also play a role in prognosis.[14]

Our current study showed that ER-α36 was also frequently expressed in pure apocrine carcinoma and adenoid cystic carcinomas, which are characteristically negative for the full length ER-α66.[10–12]

ER-α36 was over-expressed in the neoplastic cells in comparison to adjacent normal ductal epithelia, emphasising its role in tumour progression. It was almost exclusively expressed on the plasma membrane and in the cytoplasm; nuclear staining was observed in only one case of PAC. These results are in accordance with our recently published experimental and clinical investigations in the breast cancer cell lines and human breast cancers, where ER-α36 expression was localised primarily to the plasma membrane (50%) and the cytoplasm (40%), with little or no nuclear staining (up to 10%).[7–9] Subcellular localisation of ER-α36 is in sharp contrast to the nuclear localisation of the full-length ER-α66. Nuclear expression of ER-α66 is considered a diagnostic hallmark for 'ER-positive' tumours, although functionally active extranuclear pools of ER-α66 exist.[4 15] Cytoplasmic ER-α66 expression can be observed in breast cancer cells after long-term treatment with tamoxifen, coinciding with resistance to the drug.[16] Shi *et al* indicated the importance of ER-α36 in development of endocrine resistance in a subgroup of invasive breast carcinomas that exhibit co-expression of ER-α66 and ER-α36.[14]

Identification of the ER-α36 isoform in PAC explains reported discrepancies between mRNA detection and negative ER protein expression. Bratthauer *et al* reported that ER mRNA was detectable in all cases of apocrine carcinomas despite a complete absence of ER protein using the commercially available immunohistochemical assay against ER-α66.[17] The authors used RT-PCR amplification of ER mRNA using primers that covered the first and second exon of the *ER-α66* messenger RNA, and concluded that the immunohistochemical absence of ER was not a consequence of an abnormal transcript but did not elaborate. We hypothesise that detectable mRNA in apocrine carcinomas might well be one of the alternatively spliced isoforms of ER-α including currently studied ER-α36. ER-α36 protein could not have been detected due to the lack of antibody specificity; the ER-α66 antibody used in their study (clone CC4-5, Novocastra Laboratories) recognises the N-terminal of the ER-α66 which is not shared between the full-length and ER-α36 isoform (see figure 1).[18] Other commonly used commercial antibodies (eg, 1D5, SP1, RB-9016) recognise the epitopes located on either the N- or C-terminal of the *ER-α* gene which are not represented in either ER-α36 or ER-α46 isoforms.[5]

Functional importance of ER-α36 is reportedly related to the non-genomic (non-classic) ER activities, among which activation of the mitogen-activated protein kinase (MAPK/ERK) signalling pathway plays a major role.[8] The MAPK/ERK signalling pathway is activated in response to both oestrogens

Table 2 Relationship between oestrogen receptor (ER)-α36 and epidermal growth factor receptor (EGFR) expression

Variable		EGFR Negative	EGFR Positive	Total
ER-α36	Negative	3	1	4
	Positive	3	23	26
Total		6	24	30

p=0.018, κ=0.52 (95% CI 0.12 to 0.93).

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Take-home messages

- Adenoid cystic carcinoma and pure apocrine carcinoma are two rare, special types of breast cancer characteristically negative for oestrogen receptor-α66 (ER-α66).
- The novel 36 kDa isoform of ER-α66 (ER-α36) lacks the transcriptional activation domains of ER-α66 and predominantly localises to the cytoplasm and on the cell membrane. It mediates non-classic (non-genomic) oestrogen signalling.
- ER-α36 is commonly over-expressed in adenoid cystic carcinoma and pure apocrine carcinoma of the breast, which may indicate a potential for novel treatment strategies in these cancers.

(eg, 17β-oestradiol) and antioestrogens (eg, tamoxifen) which might be of particular importance for ER-α66 negative breast carcinomas since this subgroup might still respond to antioestrogen based therapy.[8] Previous experiments demonstrated that the antioestrogens induce a stronger and a more prolonged activation of the MAPK/ERK signalling pathway than the oestrogens.[8]

Our study also revealed a significant relationship between ER-α36 and EGFR expression, indicating a close interaction of the two membrane-initiated signalling.[13 19] Although Her-2/neu can also be actively involved in membrane-initiated steroid signalling, we could not identify a significant correlation between ER-α36 and Her-2/neu in our study.

In summary, membranous ER-α36 is over-expressed in breast tumours showing a complete absence of the full-length ER-α66 protein, indicating a potential for targeting the non-genomic growth signalling.

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Competing interests None declared.

Ethics approval This study was conducted with the approval of the Institutional Review Board of Creighton University School of Medicine.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. **Katzenellenbogen BS,** Frasor J. Therapeutic targeting in the estrogen receptor hormonal pathway. *Semin Oncol* 2004;**31**:28—38.
2. **Nadji M,** Gomez-Fernandez C, Ganjei-Azar P, *et al*. Immunohistochemistry of estrogen and progesterone receptors reconsidered: experience with 5,993 breast cancers. *Am J Clin Pathol* 2005;**123**:21—7.
3. **Levin ER,** Pietras RJ. Estrogen receptors outside the nucleus in breast cancer. *Breast Cancer Res Treat* 2008;**108**:351—61.
4. **Allred DC.** Issues and updates: evaluating estrogen receptor-α, progesterone receptor, and HER2 in breast cancer. *Mod Pathol* 2010;**23**:S52—9.
5. **Bretschneider N.** *Regulation of estrogen responsive genes by the human Estrogen receptor alpha (ERα)*. Heidelberg: Combined Faculties for the Natural Sciences and for Mathematics of the Ruperto Carola University of Heidelberg, 2007 (dissertation).
6. **Hirata S,** Shoda T, Kato J, *et al*. Isoform/variant mRNAs for sex steroid hormone receptors in humans. *Trends Endocrinol Metab* 2003;**14**:124—9.
7. **Wang ZY,** Zhang XT, Shen P, *et al*. Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66. *Biochem Biophys Res Commun* 2005;**336**:1023—7.
8. **Wang ZY,** Zhang XT, Shen P, *et al*. A variant of estrogen receptor-alpha, hER-alpha36: transduction of estrogen- and antiestrogen-dependent membrane-initiated mitogenic signaling. *Proc Natl Acad Sci USA* 2006;**103**:9063—8.
9. **Lee LM,** Cao J, Deng H, *et al*. ER-alpha36, a novel variant of ER-alpha, is expressed in ER-positive and -negative human breast carcinomas. *Anticancer Res* 2008;**28**:479—83.
10. **Weigelt B,** Horlings HM, Kreike B, *et al*. Refinement of breast cancer classification by molecular characterization of histological special types. *J Pathol* 2008;**216**:141—50.
11. **Vranic S,** Tawfik O, Palazzo J, *et al*. EGFR and HER-2/neu expression in invasive apocrine carcinoma of the breast. *Mod Pathol* 2010;**23**:644—53.
12. **Vranic S,** Frkovic-Grazio S, Lamovec J, *et al*. Adenoid cystic carcinomas of the breast have low topo IIα expression but frequently overexpress EGFR protein without EGFR gene amplification. *Hum Pathol* 2010;**41**:1617—23.
13. **Kampa M,** Pelekanou V, Castanas E, *et al*. Membrane-initiated steroid action in breast and prostate cancer. *Steroids* 2008;**73**:953—60.
14. **Shi L,** Dong B, Li Z, *et al*. Expression of ER-{alpha}36, a novel variant of estrogen receptor {alpha}, and resistance to tamoxifen treatment in breast cancer. *J Clin Oncol* 2009;**27**:3423—9.
15. **Fowler AM,** Santen RJ, Allred DC. "Dwarf" estrogen receptor in breast cancer and resistance to tamoxifen. *J Clin Oncol* 2009;**27**:3413—15.
16. **Fan P,** Wang JP, Santen RJ, *et al*. Long-term treatment with tamoxifen facilitates translocation of estrogen receptor alpha out of the nucleus and enhances its interaction with EGFR in MCF-7 breast cancer cells. *Cancer Res* 2007;**67**:1352—60.
17. **Bratthauer GL,** Lininger RA, Man YG, *et al*. Androgen and estrogen receptor mRNA status in apocrine carcinomas. *Diagn Mol Pathol* 2002;**11**:113—18.
18. **Tavassoli FA,** Purcell CA, Bratthauer GL, *et al*. Androgen receptor expression along with loss of bcl-2, ER, and PR expression in benign and malignant apocrine lesions of the breast. Implications for therapy. *Breast J* 1996;**2**:261—9.
19. **Zhang XT,** Kang LG, Ding L. A positive feedback loop of ER-α36/EGFR promotes malignant growth of ER-negative breast cancer cells. *Oncogene* Published Online First: 11 October 2010. doi:10.1038/onc.2010.458.

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