A role for estrogen-receptor variants in endocrine resistance

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ABSTRACT

We have isolated an estrogen-receptor (ER)-variant mRNA from ER-negative/progesterone receptor (PgR)-positive human breast cancers. This variant lacks exon 5 of the hormone-binding domain, resulting in a truncated receptor protein which is unable to bind estrogen. In vitro experiments show that the exon 5 variant possesses constitutive ER activity. For example, when transfected into ER-negative MDA-MB-231 breast cancer cells, it stimulates an estrogen-responsive element (ERE)-dependent reporter system in the presence or absence of exogenous hormone. Proliferation is unaffected by the antiestrogen tamoxifen, which usually inhibits the growth of MCF-7 cells, suggesting that the exon 5 variant may be important clinically in drug resistance. Preliminary studies have found expression of the exon 5 variant transcript in ER-positive breast cancers, but these studies need to be expanded to detect the protein in tumors. It is also possible that ER variants which influence cell proliferation may be involved in carcinogenesis. In this context, we have identified a novel ER variant in a preliminary survey of hyperplastic lesions which are thought to be precursors of invasive breast cancer. This variant possesses a single base-pair alteration in the hormone-binding domain, and exhibits enhanced stimulation of an ERE-reporter system when cotransfected into MDA-MB-231 breast cancer cells. We are currently evaluating its prevalence in premalignant breast lesions, and its ability to influence cell proliferation.

INTRODUCTION

It has been appreciated for a long time that there is a relationship between the steroid hormone estrogen and breast carcinoma growth, and successful therapeutic strategies have been designed targeting this growth pathway. There is also a large number of studies which demonstrate that the estrogen receptor (ER) is the most important factor in predicting response to such antiestrogen therapies. Most studies show that patients with advanced breast cancer whose tumors express ERs have a 70-80% probability of response, compared with less than 10% in those patients whose tumors do not contain the receptor (McGuire 1978). A clinical paradox is that when patients fail to respond to antiestrogen therapies they usually continue to express ERs. Therefore endocrine resistance, at least in the majority of breast cancer patients, is not due to a simple loss of the ER (Hull et al. 1983).

It has been suggested that there are a number of potential mechanisms which may be associated with clinical endocrine resistance. These mechanisms include: (1) overexpression of growth factors, such as fibroblast growth factor-4 (McLeskey et al. 1993), c-erbB-2 (Benz et al. 1993), and transforming growth factor-β (Butta et al. 1992), (2) reduced intratumoral drug concentration (Osborne et al. 1991, Johnston et al. 1993), (3) antiestrogen metabolism (Osborne et al. 1992), and (4) the appropriation of antiestrogens from the ER by excessive antiestrogen-binding sites in some breast tumors (Pavlik et al. 1992). We have hypothesized that alterations in the ER might be one mechanism of antiestrogen resistance, reasoning that there might be altered ERs present that are constitutive stimulators of breast-tumor proliferation. We will review one such altered
ER, cloned from breast cancers, which when over-expressed in vitro confers resistance to antiestrogen treatment. We will also present a model suggesting that altered ER expression may be an early event in breast cancer evolution contributing to inappropriate breast cell proliferation and carcinogenesis.

STRATEGY TO ISOLATE ALTERED ERs FROM TUMORS

Many studies published over the last few years have greatly enhanced our knowledge of the structure and function of the ER, and this has further helped us to understand the possible relationship between specific ER alterations and certain breast cancer clinical characteristics, such as response to hormonal therapies. The pioneering work of Kumar et al. (1986, 1987) revealed that the ER contains a hormone-independent activation domain (termed activation factor-1 or AF-1) at the amino terminus, and a hormone-dependent transactivation domain (AF-2) at the carboxy terminus, in addition to a DNA-binding and a hormone-binding region. We rationalized that an ER which had suffered a deletion of AF-2 might be present in breast tumors. This would leave only the AF-1 domain functional, and this has been shown to have varying activities in different cells. In HeLa cells, AF-1 exhibits only 5% of the transactivation activity of wild-type receptor; in chicken embryo fibroblast cells it has about half (Tora et al. 1989). Now it is also appreciated that ER transactivational activity is determined both by the cell type and by the promoter used. Recently, McDonnell and coworkers (Tzukerman et al. 1994), have shown that AF-1 or AF-2 function depends strongly on the estrogen-responsive promoter context: both functions are required on some promoters, but they can act independently on other promoters.

We have utilized the known structure of these functional domains to guide us in our search for ER alterations in primary human breast tumors. To increase the likelihood of isolating such an ER variant, we began our search with specimens from patients who were ER negative, as assayed by classical ligand-binding assay, but nevertheless progesterone receptor (PgR) positive. We reasoned that we might detect alterations or truncations within the AF-2 domain in these patients such that the ER might be unable to bind hormone, and thus appear ER negative by ligand-binding assay, but would still be capable of independently stimulating estrogen-responsive genes such as that for the PgR.

![Figure 1](image.png)

**Figure 1** Transient transactivation assay of ER activity in MDA-MB-231 cells. Cells were transfected with 10ng of the pcDNAI vector containing either wild-type ER (WTER), or the exon 5 ER-deletion variant (Ex 5), 1µg of ERE-tk-luciferase DNA, and 100ng of a CMV-β-galactosidase reporter plasmid for standardization of transfection efficiency. Transfections were accomplished with the aid of Lipofectamine reagent (Gibco-BRL, Grand Island, NY, USA) for 6 h. DNA was removed, and after an additional 12 h in estrogen deprived media, 10⁻¹⁰M estradiol was added for 18 h, and luciferase activity was then determined. Luciferase activity was corrected for β-galactosidase values, and data are expressed as relative luciferase activity.

THE EXON 5 ER-DELETION VARIANT IS A CONSTITUTIVELY ACTIVE RECEPTOR IN BREAST CANCER CELLS

We isolated such an ER variant (Fuqua et al. 1991), lacking exon 5 of the hormone-binding domain, from ER-negative PgR-positive breast tumors. The precise deletion of this exon results in an interruption in translation after codon 370. Thus, a major portion of the hormone-binding domain is missing from the variant, leaving the AF-1 domain intact. We then
utilized a yeast ER-transactivation assay to demonstrate that the exon 5 ER-deletion variant was capable of stimulating the transcriptional activity of a consensus estrogen-responsive element (ERE) in the absence of hormone. However, the activity of this variant was only about 10-15% of that of wild-type ER in a yeast reporter system (Fuqua et al. 1991).

Since we know that the transactivation activity of AF-1 is dependent on cell type, and because we wanted to determine the ultimate relevance of the exon 5 ER-deletion variant in clinical breast cancer, we have now analyzed the activity of the variant in ER-negative MDA-MB-231 breast cancer cells (Fig. 1). These cells were transiently cotransfected with CMV-driven wild-type ER or exon 5 ER-deletion variant plasmids, together with an ERE-tk-luciferase reporter plasmid (Tzukerman et al. 1994). In this assay, estrogen is absolutely required for wild-type ER activity. In contrast, the exon 5 ER-deletion variant is active in the absence of exogenous hormone, and this activity is about two-thirds of that exhibited by wild-type ER. This result further underscores the importance of cellular context in the transactivational response of AF-1, and suggests that the exon 5 ER-deletion variant may be a potent dominantly acting receptor in human breast cancer cells.

We have also found the exon 5 variant to be abundant in ER-positive, PgR-positive tumors (Zhang et al. 1993). In these tumors, the exon 5 ER-deletion variant was found to be invariably coexpressed along with wild-type ER. In fact, the variant was often expressed in excess of wild-type

![Figure 2](image-url) Figure 2 Monolayer growth of MCF-7 cells transfected with the exon 5 ER-deletion variant (EX5 Tf) or with the DNA vector alone (Con Tf), treated with estradiol (E), tamoxifen (T), or 4-hydroxytamoxifen (OHT). MCF-7 cells and transfecants were harvested in PBS containing 1mM EDTA, pelleted, washed with PBS, resuspended in phenol red-free medium containing 10% charcoal-stripped fetal calf serum, and plated at 15,000 cells per well in 12-well plates. After 24 h, the 'day 1' plates were harvested and counted and the remaining plates were switched to media containing compounds or ethanol vehicle alone (maximum ethanol concentration in media was 0.1%). Left panel: growth of MCF-7 cells transfected with the pCDNAI control plasmid, and grown in control media, C (■), or media containing 10^{-5}M estradiol (○), 10^{-6}M tamoxifen (▲), or 10^{-6}M 4-hydroxytamoxifen (●). Right panel: growth of MCF-7 cells transfected with exon 5 ER-deletion variant plasmid, and grown in control medium (■), or medium containing 10^{-9}M estradiol (○), 10^{-6}M tamoxifen (▲), or 10^{-5}M 4-hydroxytamoxifen (●). Data are presented as means±S.E.M.
ER RNA in ER-positive tumors. Therefore, we hypothesized that the activity of the variant might be functionally predominant in those tumors where it was found to be expressed at high levels. Furthermore, its expression in the majority of ER-positive tumors suggests that it may also participate in breast cancer development. Perhaps as was shown for v-erbA, a mutant form of the thyroid hormone type α receptor, the deletion of the hormone-binding domain contributes to its oncogenic hormone-independent activity (Zenke et al. 1990). Thus we were interested in studying whether an ER variant, such as the truncated exon 5 variant, might function as an oncogenic isoform of the receptor.

To address these questions, we have now stably transfected the exon 5 ER-deletion variant into MCF-7 cells which express abundant wild-type ER (Fig. 2). This has been accomplished using either an inducible promoter system (Fuqua & Wolf 1995), or the efficient CMV promoter expression vector pcDNAI (InVitrogen, San Diego, CA, USA). By means of the inducible promoter we found that expression of the exon 5 ER-deletion variant resulted in an increase in hormone-independent growth in soft agar (results not shown). But the important clinical question is whether expression of this variant affects the growth of cells when they are challenged with antiestrogens, since the variant is without the majority of the domain required for antiestrogen binding. We would predict that cells which express this variant might be resistant to the mitogenic stimulus of estrogens, and to the inhibitory effect of antiestrogens. Therefore we tested the growth of our transfectants in cell culture; the anchorage-dependent growth in vitro of the CMV-exon 5 ER-deletion variant-transfected MCF-7 cells is shown in Figure 2, right-hand panel. For comparison, MCF-7 cells stably transfected with the pcDNAI vector alone are also shown, in the panel to the left. As expected, the growth of the control-transfected cells was stimulated by estrogen, and inhibited by the nonsteroidal antiestrogens tamoxifen and 4-hydroxytamoxifen. In contrast, the growth of the exon 5-transfected cells was unaffected by administration of either tamoxifen or 4-hydroxytamoxifen. These results suggest that cells which express the exon 5 ER-deletion variant, even when wild-type ER is coexpressed, are resistant to nonsteroidal antiestrogens. It is tempting to speculate that expression of truncated ERs, such as the exon 5 ER-deletion variant, may be responsible for some forms of endocrine resistance, but this conclusion obviously awaits confirmation in large clinical trials. We are therefore evaluating its expression in a variety of clinical specimens, focusing upon its role in breast tumor patients with known tamoxifen responses.

![Figure 3 Comparison of RPA and RT/PCR assays for semiquantitation of exon 5 ER-deletion variant (Ex5) and wild-type ER (WT) RNA levels in three samples (labeled 1, 2, and 3) from patients who were sensitive to tamoxifen treatment. 30µg of RNA was evaluated by RPA according to the method of Zhang et al. (1993). The protected fragments representing levels of the Ex5 or WT RNA are indicated. Semiquantitative RT/PCR was performed as described by Fuqua et al. (1990), using primers to the ER bordering exon 5; primers to a control gene, actin (Act.) were also included.](image)

We first began these studies using the RNase-protection assay (RPA), which we had earlier used to verify that alternatively spliced forms of the ER were truly present in breast tumors, and were not just artifacts of sensitive polymerase chain reaction (PCR) amplification (Fuqua et al. 1991, 1992). RPA performed on RNA from three breast cancer patients who responded to tamoxifen treatment is shown in Figure 3, left-hand panel. An exon 5 ER-deletion RPA probe was used which generates bands specific for either exon 5, or wild-type mRNA. In these three patients, the RPA showed that exon 5 ER message exceeded that of wild-type ER. However, if a semi-quantitative reverse transcriptase (RT)/PCR assay was used to simultaneously measure the relative
levels of the variant to wild-type ER (shown in the panel on the right of Fig. 3), then wild-type ER was seen to predominate in these same three patients. The reason for this discrepancy is unknown at present, but it does suggest that one must view with caution results obtained with RNA-based semiquantitative assays. We are currently concentrating our efforts at generating an antibody specific for the exon 5 ER-deletion variant, so that clinical correlative studies can be properly conducted measuring the predicted protein product of this RNA variant.

ALTERED ER ISOLATED FROM EARLY BREAST DISEASE

We are also examining whether ER variants are present in some of the earliest stages of breast carcinogenesis. As with the model proposed for the evolution of colon carcinomas, there is epidemiological evidence that breast epithelial neoplasms may progress along a continuum from normal to hyperplasia, to dysplasia, and finally to overt neoplasia. As one progresses along this continuum, there is a concordant increase in the risk of subsequent development of invasive breast cancer. As shown in the early studies of Dupont & Page (1985), there is a twofold increase in risk for hyperplasias, a fourfold increase in risk for dysplasias, and a tenfold increase in risk for carcinoma in situ. In contrast to this epidemiological evidence for the evolution of breast cancer, there is still a paucity of molecular or genetic evidence in support of this model.

We have hypothesized that either inappropriate expression of wild-type ER, or expression of ER variants, may drive abnormal proliferation in premalignant breast lesions, providing the environment for further genetic alterations leading to cancer. Changes in the ER may be one of the first events in the evolution of breast epithelial neoplasms. Thus far, however, we have not detected overexpression of the exon 5 ER-deletion variant in breast hyperplasias, and its expression may be specifically associated with later events in breast cancer progression. Since many of the variants isolated to date, such as the exon 5 ER-deletion variant, represent alternatively spliced forms of wild-type ER, there may be tumor-specific RNA-splicing mechanisms which account for the overexpression of ER variants that we have observed in invasive breast cancer.

However, we have recently identified an ER variant in hyperplastic breast lesions with a single base-pair alteration (C Wiltschke & SAW Fuqua, unpublished observations). This variant contains a Lys to Arg change at the beginning of the hormone-binding domain. Interestingly, this variant exhibits greatly increased activity in transactivation assays in which an ERE-tk-luciferase reporter construct is used in MDA-MB-231 cells. We hypothesize that its expression may be associated with enhanced proliferation, and we are currently exploring its role in the early evolution of breast cancer.

CONCLUSION

There were reports of variant forms of the ER almost as soon as the sequence of the wild-type receptor was published. First, work with laboratory-generated receptors containing point mutations or large deletions provided many insights into the function of various regions of the receptor. More recently, several naturally occurring variants have been described, some of which have altered functions as would be predicted from studies of their artificially generated counterparts. In particular, we have discovered two variants which appear to be associated with either invasive breast cancer or premalignant breast disease. One of these variants, an ER translated from an mRNA lacking sequence corresponding to exon 5, is capable of conferring resistance to nonsteroidal antiestrogens when it is transfected into MCF-7 human breast cancer cells. However, further studies of material obtained from patient biopsies will be required before any definitive conclusions can be drawn about the relevance of the exon 5 ER-deletion variant in clinical breast cancer.

Some speculation seems reasonable, however, based on the ubiquitous nature of the exon 5 deletion variant in breast tumors. It is possible that expression of certain variant ERs confers a growth advantage to breast cancer cells and that, as conditions surrounding the cell change (exposure to antiestrogens, etc.), different variant ER isoforms may be expressed to provide a growth advantage, or maintain a growth stimulus, for evolving tumor cell clones.
Alternatively, variant ERs may be normal components of the transcriptional machinery of a mammary epithelial cell, and may be involved in regulating genes in response to different signals. Variant forms of the ER may actually regulate completely different genes from those regulated by the wild-type ER, depending on the promoter context. Again, many studies must be completed before any solid conclusions can be drawn as to the role of ER variants in the biology of either normal or malignant mammary epithelium.

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