PREFACE

Endocrine oncology is a broad subject that would be difficult, if not impossible, to cover adequately in a single book. Cancers of endocrine tissues, such as breast and prostate, are very important from a public health point of view because of their increasing prevalence; they have also been the focus of intensive research, which has expanded dramatically in recent years. In order to keep this book to a manageable size and still have it be useful to the reader with an interest in this subject, I decided to focus primarily on the endocrinology of cancers of the breast, prostate, endometrium, and ovary. As a result, there is very little information in this book on the molecular genetics of endocrine cancers, and such information is available in other excellent books.

Despite the great advances in our understanding of the genetics of endocrine cancers, important and controversial issues relating to the endocrinology and cell biology of malignancies of endocrine tissues remain to be resolved, and I have tried to cover these issues in detail in *Endocrine Oncology*. For example, while it has been known for many years that steroid hormones, particularly estradiol, influence breast cancer development and progression, many issues remain to be resolved regarding the true role of estradiol in breast cancer progression. Indeed, it is still not clear how to predict response of breast cancer patients with estrogen receptor-positive disease to antiestrogen therapy. Of further importance to the field is the relatively limited understanding, still, of how steroid hormones function to regulate normal mammary gland homeostasis in humans. For that reason, the first six chapters of this book focus on that specific area of research, and the first three chapters focus primarily on the role of estrogen and progesterone receptors in normal mammary gland function. The recent observations that estrogens and progestins signal normal mammary epithelial cell proliferation via paracrine mechanisms to neighboring cells, which are steroid hormone receptor-negative, are exciting and may help to shed light on many aspects of human breast carcinogenesis. These findings are relevant to the question of how many pathways or precursor cells are able to give rise to human breast cancers that either express or do not express steroid hormone receptors, and this important topic is the subject of Chapter 4. As is discussed in Chapter 5, expression of certain growth factor receptors can modify the expression of steroid hormone receptors, which in turn can influence breast cancer progression. These receptors may also influence the response of steroid hormone receptor-positive breast cancer cells to antiestrogens. A detailed discussion of factors that influence response to antiestrogens is presented in Chapter 6. Thus, the first part of this book attempts to cover several important and intertwined issues in ways that may help to clarify the important issues that remain to be resolved in the field.

As is evident from Chapters 7 and 8, steroids are not the only hormones important in breast cancer development. Prolactin, which is clearly important in rodent models of breast carcinogenesis, may play a similar role in human breast cancer development. In addition, peptide hormones such as chorionic gonadotropin may play important roles in modifying breast cancer progression.

With a similar approach, the second part of the book examines the role of steroid hormones in prostate cancer development and treatment. In many ways, breast and pros-

tate cancers are parallel diseases in that they are both influenced by steroid hormones, both give rise to what is initially hormone receptor-positive disease that responds to endocrine therapy, and both eventually progresses to a hormone-independent state. These issues are discussed in Chapters 13 through 15, which also demonstrates that, while there are many parallels between breast and prostate cancers, there are many distinguishing features as well.

The next two chapters focus on epithelial ovarian cancer and endometrial cancer. Once again, the emphasis of these chapters is on the endocrinology of these diseases. Since the pathogenesis of endometrial cancer appears to be influenced by certain antiestrogens that are used in breast cancer therapy, the issue of how estrogens affect different target tissues is critically important to our understanding of disease progression and the use of antiestrogen therapy.

Having focused on the role of hormones in the development of breast, prostate, ovarian, and endometrial cancer in the first 13 chapters of the book, the next four chapters present an in-depth discussion of the role of growth factors in endocrine neoplasia. A wealth of data in the literature points to an intimate interaction between hormones and growth factors in mediating normal tissue homeostasis and in pathological processes involving endocrine tissues. In particular, members of the epidermal growth factor family, the insulin-like growth factors and their binding proteins, and the fibroblast growth factors have all been implicated in the progression of endocrine neoplasia. Clearly, a book that focuses on endocrine aspects of cancers of endocrine tissues would be incomplete without a detailed discussion of the role of growth factors in the progression of these diseases.

It has recently become clear that the ability of steroid hormone receptors to influence gene expression is modified by the repertoire of transcriptional co-activators and co-repressors present in target cells. Furthermore, some of the genes that code for these proteins may function as oncogenes in breast and other cancers. It is also now known that hormones can directly affect the expression of proteins that modify the cell death response of epithelial cells under both physiologic and pathologic conditions. Finally, while it is well known that inactivation of tumor suppressor genes is important in cancer progression, endocrine tissues such as breast and ovary seem to have their own special suppressor genes, *BRCA1* and *BRCA2*. Thus, these three subjects, which are of particular importance to the development of endocrine malignancies, are covered in the final three chapters of this book.

As I mentioned at the outset, no book on endocrine oncology can be complete, since this subject encompasses a vast area of clinical medicine and cancer biology research. It was my intention, and it is my hope, that in developing this book, some of the most important issues relating to the endocrinology and cell biology of endocrine neoplasia have been appropriately identified and thoroughly discussed. It is also my hope that the readers of this book learn as much as I did from the outstanding contributions made by the authors, to whom I am greatly indebted for their hard work and dedication.

Stephen P. Ethier, PhD

2 Multiple Facets of Estrogen Receptor in Human Breast Cancer

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Contents

INTRODUCTION

Estrogen is a major regulator of mammary gland development and function, and affects the growth and progression of mammary cancers *(1,2)*. In particular, the growth responsiveness of breast cancer (BC) cells to estrogen is the basic rationale for the efficacy of the so-called endocrine therapies, such as antiestrogens. Estrogens mediate their action via the estrogen receptor (ER), which belongs to the steroid/thyroid/retinoid receptor gene superfamily *(3)*. The protein products of this family are intracellular, ligand-activated transcription factors regulating the expression of several gene products, which ultimately elicit a target tissue-specific response *(4)*. Indeed, ER, together with progesterone receptor (PR), expression in human breast tumors, are important prognostic indicators, as well as markers of responsiveness to endocrine therapies *(5,6)*. However, although the majority of human BCs are thought to be initially hormone-responsive, it is well appreciated that alterations in responsiveness to estrogen occurs during breast tumorigenesis. During BC progression, some ER-positive BCs are *de novo* resistant to endocrine therapies, and of those that originally respond to antiestrogens, many develop resistance. This progression from hormonal dependence to independence is a significant clinical problem,

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because it limits the useful of the relatively nontoxic endocrine therapies, and is associated with a more aggressive disease phenotype *(7)*. This occurs despite the continued expression of ER, and often PR *(8,9)*. The ER is pivotal in estrogen and antiestrogen action in any target cell, but the nature of the ER is clearly multifaceted.

Until recently, it was thought that only one ER gene existed. However, a novel ER, now referred to as ERβ, has recently been cloned and characterized *(10,11)*. Moreover, it has recently been shown that ERβ mRNA is expressed in both normal and neoplastic human breast tissue *(12–14)*. This suggests that ERβ may have a role in estrogen action in both normal and neoplastic human breast tissue. Furthermore, it has now become apparent that several variant mRNA species of both the classical ERα and ERβ can be expressed in human breast tissues, and may therefore have roles in estrogen and antiestrogen signal transduction *(13,15–18)*. The current data suggest that an evaluation of estrogen interaction with human breast tissue needs to include ERα, ERβ, and any variant forms of these receptors that may be expressed. The following chapter focuses on the multifaceted nature of the ER in human breast tissues.

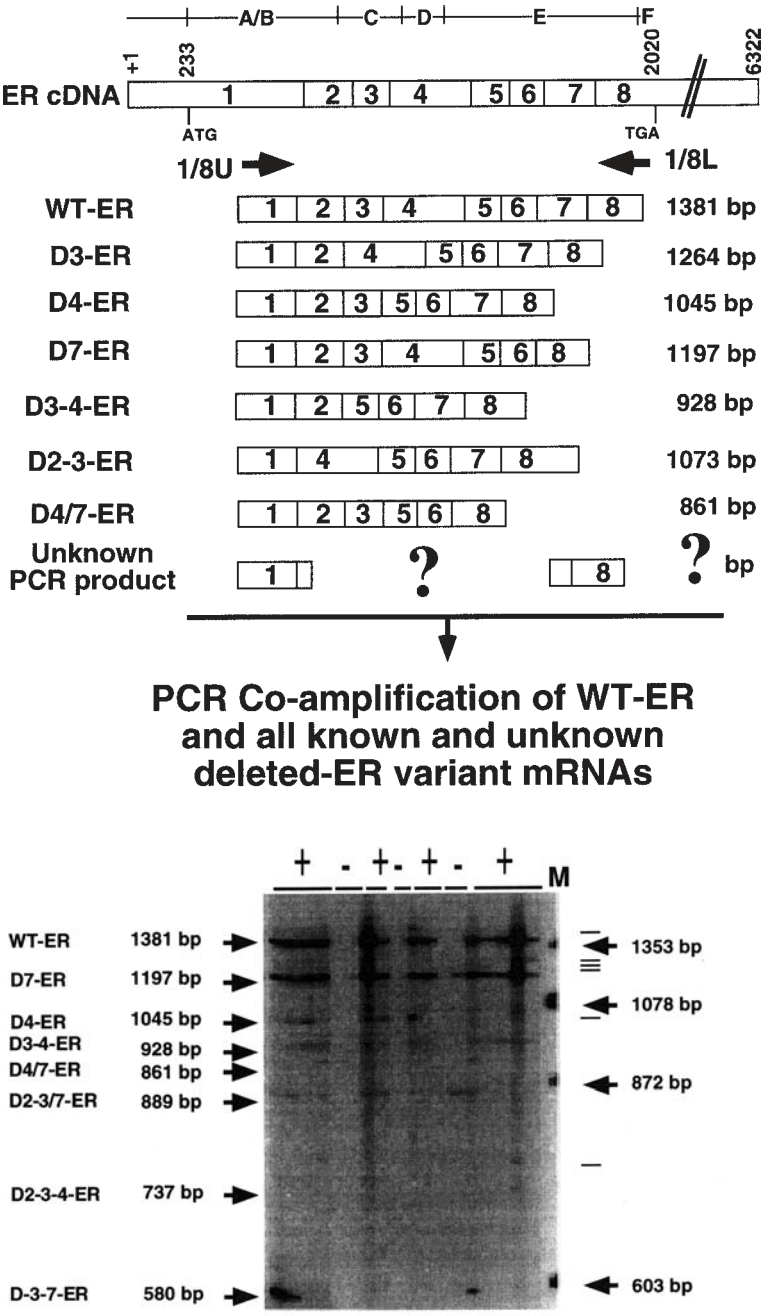
ERα AND ITS VARIANTS

Identification of ERα Variant mRNAs in Human Breast Tissues

A large body of data has accumulated supporting the existence of ERα variants *(19,20)*. The majority of the data supporting the expression of ERα variants has been at the mRNA level. Two main structural patterns of ERα variant mRNAs have been consistently identified: the truncated ERα mRNAs *(21)* and the exon-deleted ERα mRNAs *(22)*. The truncated ERα mRNAs were originally identified, by Northern blot analysis, as fairly abundant smaller-sized mRNA species in some human BC biopsy samples *(23)*. The cDNAs of several truncated ERα mRNAs have been cloned and found to contain authentic polyadenylation signals followed by poly(A) tails. The exon-deleted ERα mRNAs have been identified mostly from reverse transcription polymerase chain reaction (RT-PCR) products, using targeted primers.

Multiple ERα variant mRNAs are often detected in individual tumor specimens. In order to determine the relative frequency and pattern of variant expression in a particular sample, an RT-PCR approach was developed that allowed the simultaneous detection of all deleted ERα variant mRNAs containing the primer annealing sites in exons 1 and 8, at levels that represent their initial relative representation in the RNA extract. Since truncated transcripts do not have exon 8 sequences, they will not be measured by this technique. Examples of the results obtained are shown (Fig. 1), and serve to illustrate that

Fig. 1. Top panel. Schematic representation of WT ERα (WT-ER) cDNA and primers allowing co-amplification of most of the described exon-deleted ERα variants. ERα cDNA contains eight different exons coding for a protein divided into structural and functional domains (A–F). Region A/B of the receptor is implicated in transactivating function (AF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another transactivating function (AF-2). 1/8U and 1/8L primers allow amplification of 1381-bp fragment corresponding to WT ERα mRNA. Co-amplification of all possible exon-deleted or -inserted variants, which contain exon 1 and 8 sequences, can occur. Amplification of the previously described ERα variant mRNAs deleted in exon 3 (D3-ER), exon 4 (D4-ER), exon 7 (D7-ER), both exons 3 and 4 (D3–4-ER), exons 2 and 3 (D2–3-ER), exons 4 and exon 7 (D4/7-ER), would generate 1264-, 1045-, 1197-, 928-, 1073-, and 861-bp fragments, respectively. **Bottom panel.** Co-amplification of WT ERα and deleted variant mRNAs in breast tumor samples. Total RNA extracted from ER-positive (+) and ER-negative (–) breast tumors was reverse-transcribed and PCR-amplified, as described *(24)*, using 1/8U



and 1/8L primers. Radioactive PCR products were separated on a 3.5% acrylamide gel, and visualized by autoradiography. Bands reproducibly obtained within the set of tumors studied, and which migrated at 1381, 1197, 1045, 928, 889, 861, 737, and 580 bp, were identified as corresponding to WT-ER mRNA and variant mRNAs deleted in exon 7 (D7-ER), exon 4 (D4-ER), both exons 3 and 4 (D3–4-ER), exons 2, 3, and 7 (D2–3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2, 3, and 4 (D2–3–4-ER), and within exon 3 to within exon 7 (D-3–7-ER), respectively. PCR products indicated by dashes (-), barely detectable within the tumor population, i.e., present in less than or equal to three particular tumors, have not yet been identified. M, Molecular weight marker (phi174, Gibco-BRL, Grand Island, NY). Adapted with permission from ref. *24*.

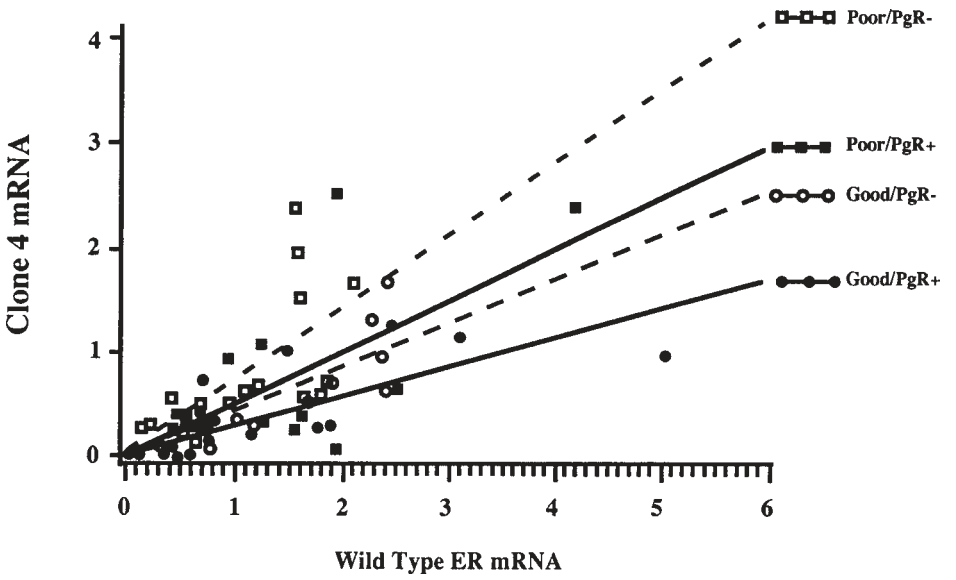


Fig. 2. Linear regression analysis of the relationship between the clone-4-truncated ERα mRNA and the WT ERα mRNA in the various groups. Closed circles represent the good prognosis/ER-positive–PR-positive group; open circles represent the good prognosis/ER-positive–PR-negative group; closed squares represent the poor prognosis/ER-positive–PR-negative group; open squares represent the poor prognosis/ER-positive–PR-negative group. Good vs Poor, $P = 0.0004$; PR-negative vs PR-positive. $P = 0.011$. Reproduced with permission from ref. *25*.

a complex pattern of exon-deleted variant ERα transcripts are expressed in any one tumor, that the pattern and relative frequency of detection of ERα variant mRNAs may vary between tumors, and that, in some cases, the relative frequency of detection of individual ERα variant mRNAs may be correlated with known prognostic markers *(24)*.

An example of such a correlation is shown in Fig. 2 *(25)*. The expression of the truncated clone-4 ERα variant mRNA was measured relative to the wild-type (WT) ERα mRNA in a group of breast tumors. The relative expression of the clone-4 variant was significantly increased in those tumors with characteristics of poor prognosis, compared to those tumors with good prognostic characteristics, i.e., clone-4 expression was higher in large tumors with high S-phase fraction, and from patients with nodal involvement, compared to small tumors with low S-phase fraction from patients without nodal involvement. Also, in this group, the relative expression of clone-4 was significantly higher in PR-negative tumors vs PR-positive tumors, suggesting a correlation of increased truncated variant expression and markers of endocrine resistance.

Data support the possibility that ERα variant proteins exist, and that their pattern and frequency are different from different individuals. In some cases, the expression of single ERα variant mRNA species was correlated with known markers of prognosis and endocrine sensitivity. This, in turn, suggested the hypothesis that altered expression of ERα variants may be a mechanism associated with progression to hormone independence.

Putative Biological Significance of ERα Variant mRNAs

Expression of ERα Variant mRNAs in Normal and Neoplastic Human Breast Tissue

Most studies investigating ERα variant mRNAs have used human BC tissues or cell lines *(19)*. However, it is now known that both truncated and exon-deleted ERα variant

mRNAs can be detected in other tissues, including normal tissues *(19)*. In particular, ERα variant mRNAs have been identified in normal human breast tissue and cells *(26–29)*. Therefore, ERα variant mRNAs are not tumor-specific, are not found in the complete absence of the WT ERα mRNA, and are probably generated by alternative splicing mechanisms.

These observations raised the question of whether the expression of ERα variant mRNAs is altered during breast tumorigenesis and/or progression. When the level of expression of individual variant ERα mRNAs was measured relative to the level of the WT ER transcript, differences between normal and breast tumor tissues were found. The relative expression of clone-4-truncated ERα variant mRNA and the exon-5-deleted ERα variant mRNA, but not the exon-7-deleted ERα variant mRNA, was significantly increased in breast tumors, compared to normal breast tissues obtained from both reduction mammoplasties and normal tissues adjacent to breast tumors *(26,27)*. Preliminary data suggests that this is also true for samples of ER-positive breast tumors and their matched, adjacent normal tissues *(29a)*; there is also evidence suggesting that an exon-3-deleted ERα variant mRNA is decreased in BCs, compared to normal human breast epithelium *(29)*. Because this ERα variant mRNA encodes a protein that can inhibit WT ERα transcriptional activity *(30)* and causes growth suppression when stably overexpressed in ER-positive MCF-7 human BC cells *(29)*, it was concluded that the exon-3-deleted ERα variant may function to attenuate estrogenic effects in normal mammary epithelium. This function is markedly reduced via decreased exon-3-deleted ERα expression during breast tumorigenesis. In preliminary studies of ER-positive human breast tumor samples and their matched adjacent normal tissues, a statistically significant decreased relative expression of the exon-3-deleted ERα mRNA in the tumor, compared to the normal breast tissues, was noted *(29a)*.

The available data provide evidence for an extensive and complex pattern of alternative splicing associated with the ERα gene, which may be altered during breast tumorigenesis.

Specificity of ERα Splice Variants in Human Breast Tumors

It is unlikely that the mechanisms generating alternatively spliced forms of ERα result from a generalized deregulation of splicing processes within breast tumors, since similar variants for the glucocorticoid receptor *(16,28)*, the retinoic acid receptors-α and -γ *(28)*, and vitamin D_3 receptor *(16)* have not been found in breast tumor tissues. However, similar splice variants of PR (*see* subheading Expression of Other Steroid Hormone Receptors, below) were found in both normal and neoplastic breast tissues *(31,32)*.

Expression of ERα Variant mRNAs During BC Progression

As described above, the relative expression of at least one ERα variant mRNA, i.e., clone-4-truncated ERα mRNA, is significantly higher in primary breast tumors with characteristics of poor prognosis (including the presence of concurrent lymph node metastases), compared to primary tumors with good prognostic markers (including lack of concurrent lymph node metastases) *(25)*. An increased relative expression of exon-5-deleted ERα mRNA has been found in locoregional BC relapse tissue (in the same breast as the original primary tumor, but no lymph node metastases) obtained from patients following a median disease-free interval of 15 mo, compared to both the corresponding primary breast tumor *(33)* and the primary breast tumor tissue of patients who did not relapse during this period. Although the difference did not reach statistical significance,

these same authors reported a trend toward higher relative expression of exon-5-deleted ERα mRNA in primary tumors of women who relapsed, compared to primary tumors of those that did not relapse. Together, these data suggest that, in addition to altered expression of ERα variant mRNA, which occurs during breast tumorigenesis, further changes in ERα variant expression may occur during BC progression. However, another study *(34)* has recently found no significant differences in the relative expression of clone-4-truncated, exon-5-deleted, and exon-7-deleted ERα mRNAs, between a series of primary breast tumors and their matched concurrent lymph node metastasis, suggesting that altered expression of ERα variant mRNAs probably occurs prior to the acquisition of the ability to metastasize, and therefore may be a marker of future metastatic potential. This hypothesis remains to be tested.

Expression of ERα Variant mRNAs and Endocrine Resistance

The hypothesis that altered forms of ERα may be a mechanism associated with endocrine resistance has been suggested for some time. Moreover, the identification of ERα variant mRNAs in human breast biopsy samples *(23,35,36)* provided good preliminary data for the hypothesis. In addition, preliminary functional data of the recombinant exon-5-deleted ERα protein suggested that it possessed constitutive, hormone-independent transcriptional activity that was about 15% that of the WT ER *(36)*. The data using a yeast expression system were also consistent with the correlation of relatively high levels of exon-5-deleted ERα mRNA in several human BC biopsy samples classified as ER-negative and PR-positive and/or pS2-positive *(36–38)*. It was also found that the exon-5-deleted ERα mRNA was often co-expressed at relatively high levels with the WT ERα in many human BC that were ER-positive *(38)*. It has been observed that transiently expressed exon-5-deleted ERα has an inhibitory effect on endogenously expressed WT ERα in MCF-7 human BC cells *(39)*, although it does not decrease the WT activity to the same extent as hydroxytamoxifen. In contrast, in human osteosarcoma cells, exon-5-deleted ERα was shown to have little effect alone, but significantly enhanced estrogen-stimulated gene expression by transiently co-expressed WT ERα *(40)*. The limitations of transient expression analysis were addressed by two groups who stably overexpressed the exon-5-deleted ERα in MCF-7 human BC cells *(41,42)*. However, different phenotypes were obtained by the two groups. No effect of the recombinant exon-5-deleted ERα on growth or estrogen/antiestrogen activity in MCF-7 cells was found in one study *(41)*; in the other study *(42)*, the overexpression of recombinant exon-5-deleted ERα in MCF-7 cells was associated with estrogen-independent and antiestrogen-resistant growth. The reasons for the differences between the two studies are unclear, but may be the result of different MCF-7 variants, or changes that could have occurred in the transfectants in addition to transgene expression. The transgene in the Rea and Parker study *(41)* was episomally maintained; in the study by Fuqua et al. *(42)*, the transgene was presumably integrated into the host chromosomes in a random fashion.

Several laboratories have developed cell culture models of estrogen independence and antiestrogen resistance. Variable results have been obtained when the association of altered ERα variant mRNA expression with estrogen/antiestrogen responsiveness was investigated. An increased relative expression of an exon-3 + 4-deleted ERα variant mRNA was found in an estrogen-independent MCF-7 cell line (T5-PRF) derived by long-term growth in estrogen-depleted medium *(43,44)*. However, this cell line was still sensitive to antiestrogens *(43)*. Although one cell line that was tamoxifen (TAM)-resistant had

differential expression of an exon-2-deleted ERα and an exon-5-deleted ERα mRNA, compared to the parental cell line *(45)*, other independently derived antiestrogen-resistant clones showed no major differences in the expression of ERα variant mRNAs *(46,47)*.

Investigation of ERα splice variants, using clinical tissue samples, has also led to variable conclusions. The relative expression of the clone-4-truncated ERα variant mRNA was significantly increased in primary breast tumors with characteristics of poor prognosis, compared to tumors with good prognostic characteristics *(25)*. Similarly, the relative expression of clone 4 was significantly higher in PR-negative vs PR-positive tumors, suggesting a correlation of increased truncated variant expression and markers of endocrine resistance *(25)*. Furthermore, an increased frequency of detection of ERα variant mRNAs deleted in exons 2–4 and 3–7 was associated with high tumor grade, but an increased detection of an exon-4-deleted ERα variant mRNA was associated with low tumor grade *(24)*. The presence of exon-5-deleted ERα mRNA was found in one study *(39)* to be associated with increased disease-free survival. However, no difference in the relative expression of an exon-5-deleted ERα variant mRNA was found between all TAM-resistant tumors and primary control breast tumors *(37)*, although, in the subgroup of TAM-resistant tumors that were ER-positive/pS2-positive, the relative expression of the exon-5-deleted ERα was significantly greater than the control TAM-sensitive group.

Although increased expression of any one ERα variant does not correlate with TAM resistance of BCs overall, its association with, and therefore possible involvement in, endocrine resistance in some tumors cannot be excluded. Moreover, the presence of multiple types of ERα variant mRNAs in any one tumor or normal tissue sample has been well documented *(24,28)*, but no data have been published in which total ERα splice variant expression has been analyzed in relationship to endocrine resistance and prognosis. Although mutations have been found in the ERα gene in human breast tumors, they are rare and are not more frequent in TAM-resistant tumors *(48)*.

Identification of ERα Variant Proteins

The detection of proteins that correspond to ERα variant mRNAs remains an important issue. It is relevant, therefore, to understand the structure of these proteins. The predicted proteins of some of the most frequently detected ERα variant transcripts are shown schematically in Fig. 3. All of the variant transcripts would encode ERα proteins missing some structural/functional domains of the WT ERα. Although the ERα variant transcripts encode several different types of protein, there are some common themes that emerge. A common feature of these putative proteins is the universal presence of the A/B region, which is known to contain the cell and promoter specific AF-1 function. Exon-4-deleted and exon-3 + 4-deleted ERα mRNAs are in frame and encode proteins that do not bind ligand. However, the majority of the most abundantly expressed variant transcripts, i.e, exon-7-deleted, an exon-4 + 7-deleted, and the clone-4-truncated ERα mRNAs, encode proteins that are C-terminally truncated, and cannot bind ligand. Thus, a common feature of these variants is the inability to bind ligand. The results obtained, in which recombinant techniques were used to measure the function of individual ERα variants in vitro, are variable, and often depend on co-expression of the WT receptor. It is difficult to make general conclusions, but many recombinant ERα variant proteins have been observed to modulate the activity of the WT receptor. However, the relevance of the relative levels of expression of WT and variant ERα proteins that are achieved under the experimental conditions used is unclear, because limited data have been published on the

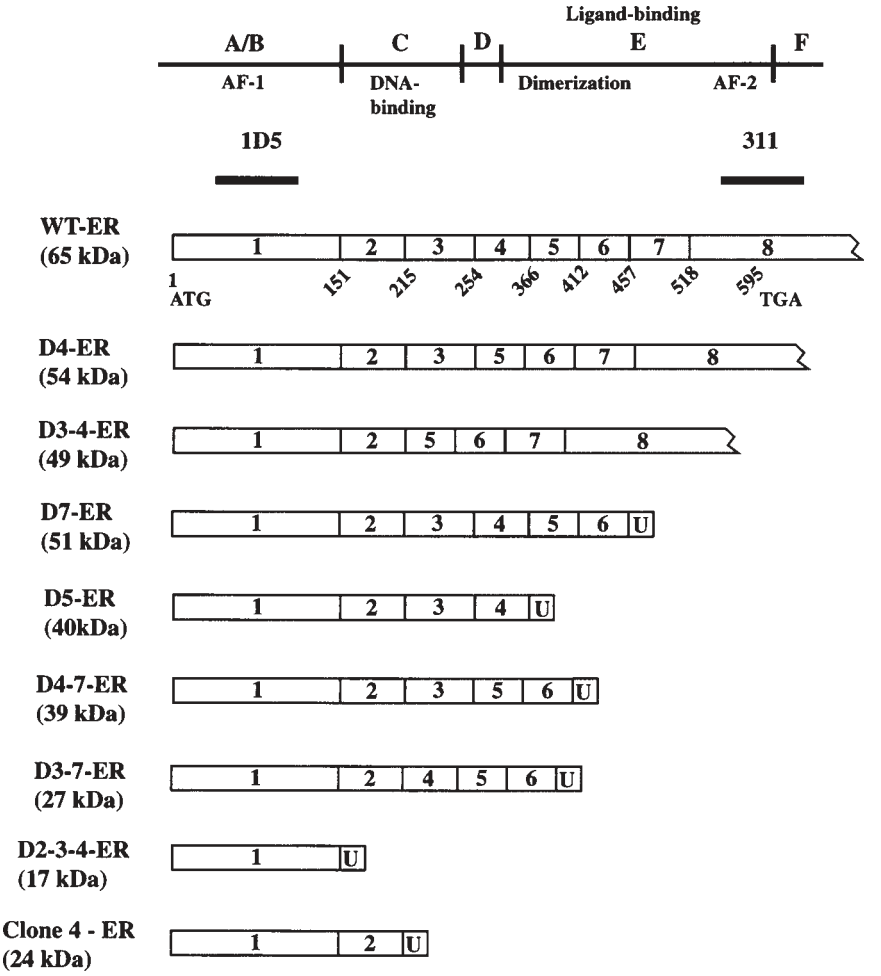


Fig. 3. Schematic representation of the ERα variant proteins predicted to be encoded by ERα variant mRNAs. Identical sequence is depicted by numbered exons. U, amino acid sequence unrelated to WT human ERα amino acid sequence. U sequences are unique to any particular variant. The position of N- and C-terminal epitopes, recognized by 1D5 and AER311 Abs, respectively, are indicated.

detection of ERα variant proteins encoded by known ERα variant mRNAs in tissues or cells in vivo.

From a different perspective, the prediction that the majority of ERα variant proteins are C-terminally truncated has implications for the determination of clinical ER status. Early detection, and changes in clinical practice, have resulted in smaller amounts of breast tumor tissue being available for assay. For this and other reasons, the use of immunohistochemistry (IHC) methods to assess ER status is becoming more common. Therefore, depending on the antibodies (Abs) used, the presence of C-terminally truncated ERα variant proteins could theoretically influence determination of ER status of the tumor sample. The authors have tested this experimentally, by transiently transfecting WT ERα and clone-4-truncated ERα expression vectors into Cos-1 cells, and determining ER status of the cells, using Abs either to the N-terminus of the ERα (Fig. 3, 1D5, Dako) or Abs to the C-terminus (Fig. 3, AER311, Neomarkers). Preliminary data, using

different combinations of WT ERα and variant ERα expression vectors transfected into Cos-1 cells, indicate that the signals (expressed as H-scores, which take into account the intensity of staining and the number of positively staining cells) obtained with the N-terminal and C-terminal Abs, become increasing discrepant (N-terminal > C-terminal signal) with increasing variant expression, presumably because of increased ERα-like proteins containing the N-terminal region, but not the C-terminal region. These preliminary data suggest that increased expression of C-terminally truncated ERα variant proteins could interfere with the IHC determination of ER status.

This possibility was investigated in human breast tumor tissues *(49)*. A series of breast tumors was assayed for ERα, using the set of Abs described above, and the H-scores from each Ab were compared for each tumor. The tumors fell into two distinct groups: one in which the H-scores obtained with each Ab were consistent and not significantly different from each other; and another group, in which the H-scores obtained with each Ab were inconsistent and significantly different from each other. Further, in all but one case, the H-score was higher for the N-terminal Ab, compared to the C-terminal Ab *(50)*. In preliminary experiments using a subset of the original tumor set, the authors found similar results, using another set of N-terminal and C-terminal ERα Abs. Together with the previous experimental data, one interpretation of the tumor data would be that the discrepant tumors had higher levels of C-terminally truncated ERα-like proteins.

To address the hypothesis that the C-terminally truncated ERα-like proteins could correspond to proteins encoded by ERα variant transcripts, the authors compared expression of ERα variant mRNAs in the consistent and inconsistent tumors. The results show a significantly higher relative expression and detection of ERα variant mRNAs that would encode C-terminally truncated proteins in the inconsistent vs the consistent tumors *(50)*. These results suggest that, irrespective of function, the expression of significant amounts of C-terminally truncated ERα variant proteins could interfere with the IHC determination of ER status, which, in turn, might underlie some of the inconsistencies between ER status and clinical response to endocrine therapy. These data are consistent with the hypothesis that ERα variant mRNAs may be stably translated in vivo. However, such data are indirect, and other mechanisms, e.g., altered epitope detection, increased proteolytic activity, and so on, may underlie the discrepant ERα H-scores found in some human breast tumors.

More recently, data published from several independent groups support the detection of ERα-like proteins in cell lines and tissues in vivo, which could correspond to those predicted to be encoded by previously identified ERα variant mRNAs. The presence of an exon-5-deleted ERα protein was demonstrated immunohistochemically in some human breast tumors, using a monoclonal Ab specific to the predicted unique C-terminal amino acids of the exon-5-deleted ERα protein *(39)*. However, although there was a correlation between IHC detection and presence or absence of exon-5-deleted ERα mRNA determined by RT-PCR, the group was unable to detect any similar protein by Western blotting, suggesting either very low levels, compared to WT ERα, or differential stability of the variant protein relative to the WT ERα during the extraction procedure. In addition, an ERα-like protein, consistent with that predicted to be encoded by the exon-5-deleted ER mRNA, is expressed in some BT 20 human BC cell lines, as determined by Western blot analysis *(51)*. Western blotting of ovarian tissue has identified both a 65-kDa WT ERα protein and a 53-kDa protein recognized by ERα Abs to epitopes in the N-terminus and C-terminus of the WT protein, but not with an Ab recognizing an epitope encoded

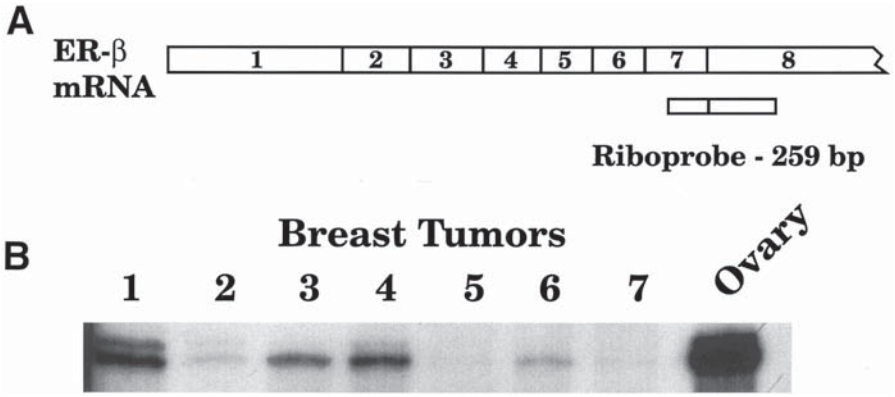


Fig. 4. Detection of ERβ mRNA in human breast tumors by RNase protection assay. (**A**) Schematic representation of hERβ mRNA showing various exon sequences, and identifying the riboprobe position and size of the expected protected fragment (259 bp). (**B**) Total RNA was isolated from seven breast tumor samples, and 25 μg was used in an RNase protection assay, as previously described *(21)*. Ovarian RNA was used as a positive control.

by exon 4 *(52)*. These results correlated with the presence of both WT and exon-4-deleted ERα mRNAs in these tissues, and suggested that the 53-kDa protein was derived from the exon-4-deleted ERα mRNA.

More recently, a 61-kDa ERα-like protein and a more abundant 65-kDa WT ERα protein were identified in MCF-7 cells *(29)*. The 61-kDa protein is thought to be encoded by an exon-3-deleted ERα mRNA expressed at low levels in these cells, and its co-migration, both before and after dephosphorylation with the recombinant exon-3-deleted ERα protein, when expressed at higher levels after stable transgene expression in another MCF-7 clone, was thought to strongly suggest its identity with the recombinant exon-3-deleted ERα protein.

There is accumulating evidence suggesting that variant ERα proteins, which correspond to those predicted to be encoded by some of the ERα variant mRNAs, can be detected by conventional technologies in clinical specimens.

ERβ AND ITS VARIANTS

Identification of ERβ mRNA in Human Breast Tissues

With the discovery of ERβ, which had properties similar to, yet distinct from, ERα *(10, 11,53,54)*, and can interact with the ERα *(55,56)*, it became important to know whether ERβ was expressed in human breast tumors, and, if so, what role it plays in estrogen/antiestrogen action.

The authors have detected the presence of ERβ mRNA, both by RT-PCR *(12,14)* and by RNase protection assay (Fig. 4; *14*), in some human BC biopsy samples and some human BC cell lines. *In situ* hybridization analysis suggested that expression of ERβ mRNA could be detected in the BC cells of a human BC biopsy sample *(14)*. Using an RT-PCR approach to analyze both ERβ and ERα mRNA expression in a range of breast tumors *(12)*, the following was observed: There was no correlation between ERβ expression and ERα expression in breast tumors; in some cases, both ERβ and ERα mRNA were expressed in the same tumor; in those tumors in which both ER mRNAs were expressed,

the relative expression appeared to vary widely among tumors. Furthermore, ERβ mRNA can be detected in normal human breast tissues by RT-PCR *(13)* and RNase protection assay *(14)*. Although there are no data reporting the expression of ERβ protein(s) in human breast tissues as yet, the available information suggest that ERβ may be expressed in both normal and neoplastic human breast tissues, and may have a role in these tissues.

Expression of ERβ mRNA During Breast Tumorigenesis

The demonstration of ERβ mRNA expression in both human breast tumors and normal human breast tissue suggests that the well-documented role of estrogen in breast tumorigenesis *(1,57)* may involve both receptors. Using a multiplex RT-PCR approach, it has been shown that the ERα:ERβ ratio in a small group of ER-positive human breast tumors was significantly higher than the ratio in their adjacent normal breast tissues *(58)*. The increase in ERα:ERβ ratio in breast tumors was primarily the result of a significant upregulation of ERα mRNA in all ER-positive tumors, in conjunction with a lower ERβ mRNA expression in the tumor, compared to the normal compartment in some, but not all, ER-positive cases. Preliminary data suggest that the level of ERβ mRNA in breast tumors may be correlated with the degree of inflammation (unpublished data). Because *in situ* hybridization data suggest that expression of ERβ mRNA could be detected in the cancer cells of a human BC biopsy sample *(14)*, and that human lymphocytes in lymph nodes can also express ERβ mRNA *(14)*, it is possible that the cell type contributing to the expression of ERβ mRNA may be heterogeneous, depending on the tumor characteristics. If the RNA studies reflect the protein levels of the two ERs, results to date provide evidence to suggest that the role of ERα- and ERβ-driven pathways, and/or their interaction, probably changes during breast tumorigenesis.

Identification of ERβ Variant mRNAs in Human Breast Tissues

The presence of multiple ERα variant mRNAs in both normal and neoplastic human breast tissues has led to the question of the expression of ERβ variant mRNAs. Several ERβ variant mRNAs have been detected. The authors have identified an exon-5 + 6-deleted ERβ mRNA in human breast tumors *(59)*. This transcript is in-frame, and would be expected to encode an ERβ-like protein deleted of 91 amino acids within the hormone binding domain. A human ERβ variant mRNA, deleted in exon 5, was identified in MDA-MB231 human BC cells and in some human breast tumor specimens *(18)*. Although that group was unable to detect an exon-5-deleted ERβ mRNA in normal human breast tissue, the authors have detected both exon-5-deleted ERβ mRNA and an exon-6-deleted ERβ mRNA, as well as an an exon-5 + 6-deleted ERβ mRNA, in normal human breast tissue samples *(13)*, and in some human breast tumors. The exon-5-deleted ERβ mRNA and the exon-6-deleted ERβ mRNA are out-of-frame and predicted to encode C-terminally truncated ERβ-like proteins, which would not bind ligand.

More recently, several exon-8-deleted human ERβ mRNAs have been identified *(17)* from a human testis cDNA library, and by RT-PCR from the human BC cell line MDA-MB435. These variants have been named human ERβ2–5. It should be noted that human ERβ2 is not the equivalent of the ERβ variant mRNA with an in-frame insertion of 54 nucleotides between exons 5 and 6 identified in rodent tissues *(13,60,61)*, and also named ERβ2. The authors have been unable to detect an equivalent of the rodent ERβ2 mRNA in any normal or neoplastic human tissue so far studied *(13)*.

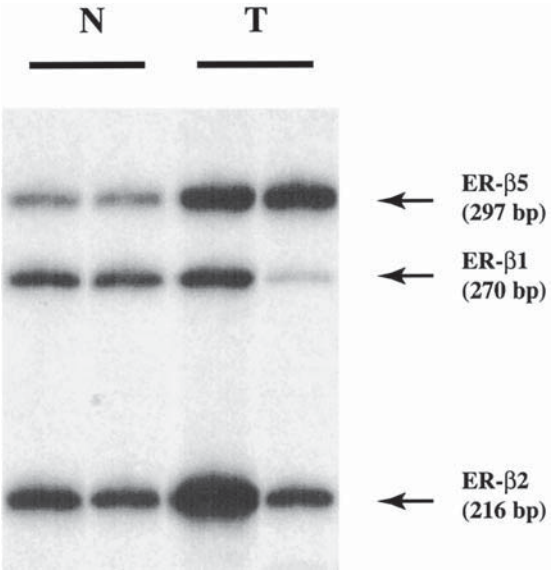


Fig. 5. RT-triple primer PCR analysis *(26)* of the relative expression of human ERβ1, human ERβ5, and human ERβ2 mRNAs in normal (N) and breast tumor (T) tissue samples.

Several of the human ERβ variants deleted in exon 8, specifically hERβ2 and hERβ5, can be detected in normal human mammary gland and in several human BC cell lines *(17)*. The predominant type of hERβ exon-8-deleted mRNA present varies among the different cell lines. The authors have confirmed the presence of the hERβ2 and the hERβ5 variant mRNAs in several normal human breast tissue samples from both reduction mammoplasties and normal tissue adjacent to breast tumors (Fig. 5; unpublished data). Moreover, the authors have identified both hERβ2 and the hERβ5 variant mRNAs in several human breast tumor samples (Fig. 5; unpublished data). Using a semiquantitative RT-triple primer PCR approach *(26)*, which simultaneously measures the relative expression of the WT hERβ1 and the two variant hERβ2 and hERβ5 mRNAs, it appears that, in most, but not all, cases, the level of the variant mRNA species exceeds that of the WT hERβ1 (Fig. 5; unpublished data) in both normal and neoplastic human breast tissues. The known sequence of all human ERβ-like transcripts is shown schematically in Fig. 6; also shown in this figure are the proteins predicted to be encoded by these variant hERβ mRNAs. All the hERβ variant mRNAs identified to date are predicted to encode proteins that are altered in the C-terminus in some fashion, and are unlikely to bind ligand *(62)*. However, published data *(17)* suggest that some of these variant receptors can form homo- or heterodimers among themselves and with WT hERβ and hERα, and may preferentially inhibit hERα DNA-binding transcriptional activity *(62)*.

Putative Role of ERβ and Its Variants in Breast Cancer

Transient transfection studies have provided data which suggest that ERβ1, i.e., the WT ERβ, can only mediate an antagonist response when bound to TAM-like agents, in contrast to the TAM-bound WT ERα, which can mediate either an antagonist or agonist activity on a basal promoter linked to a classical estrogen response element *(53,63)*. This suggests the possibility that altered relative expression of the two ERs may underlie

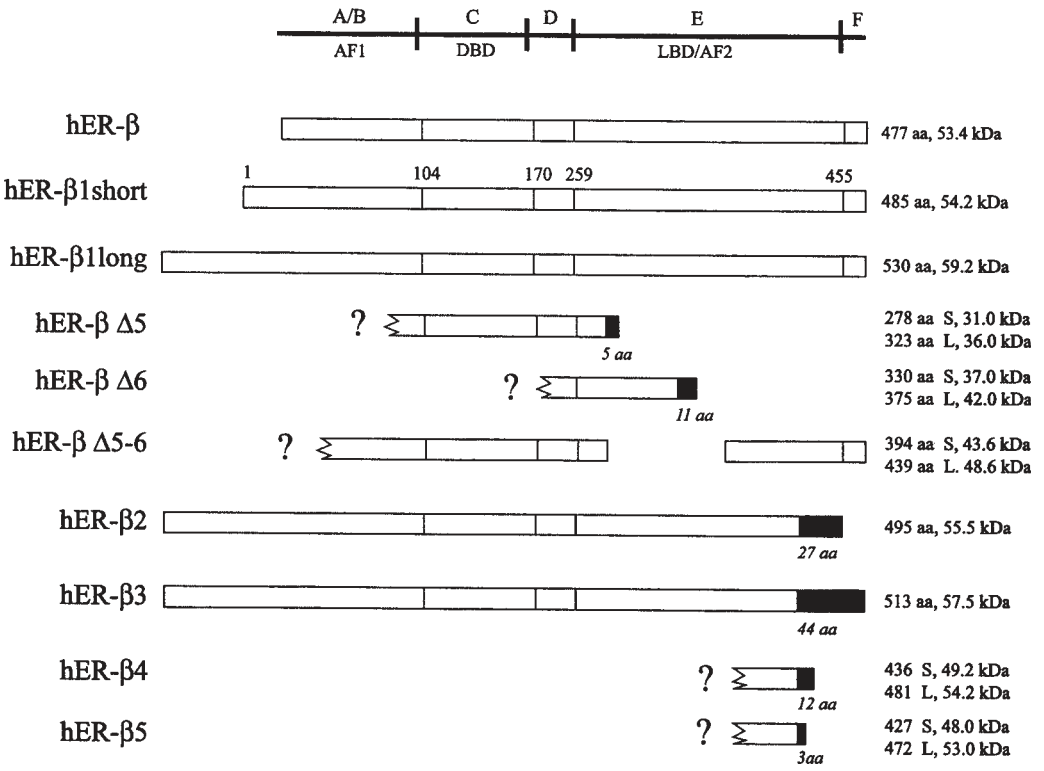


Fig. 6. Human ERβ isoforms. All hERβ isoforms are aligned. White boxes indicate identity of amino acid between sequences. Amino acid positions of the different structural domains are indicated for the hERβ1 short *(14)*, which contains eight extra N-terminal amino acids, compared to the first hERβ described *(10)*. hERβ1 long (Genbank AF051427) contains 45 additional N-terminal amino acids. hERβ1Δ5 *(13,18)*, hERβ1Δ6 *(13)*, hERβ2 (Genbank AF051428, AB006589cx), hERβ3 (Genbank AF060555), hERβ4 (Genbank AF061054), and hERβ5 (Genbank AF061055) are truncated, and contain different C-terminal amino acids (black boxes). hERβΔ5–6 *(13)* (Genbank AF074599) is missing 91 amino acids within the LBD/AF-2 domain. For each receptor, the length (aa) and the calculated molecular mass (kDa), when known or corresponding to the short (S) or the long (L) forms of the putative proteins, are given. Broken boxes and question marks indicate that flanking amino acid sequences are unknown.

altered responses to antiestrogens, and could be a mechanism of altered responsiveness to antiestrogens in human BC. The activity of the estrogen-bound ERβ1 on activating protein 1 (AP-1)-containing promoters is inhibitory, in contrast to that of estrogen-bound ERα, which stimulates transcription *(54)*. Furthermore, antiestrogens of all types demonstrated marked transcriptional activity through ERβ1 on promoters that contained AP-1 sites *(54)*. A nonligand-binding hERβ variant protein, encoded by the variant hERβ2 (also named hERβcx), can heterodimerize with ERβ1, but preferentially heterodimerizes with ERα, and shows a dominant-negative activity only against ERα-mediated transactivation *(17,62)*. It is possible, therefore, that ERβ1 and its variants could have a direct regulatory role on ERα activity. Since the authors have observed an increased ratio of ERα:ERβ mRNA in human breast tumors, compared to their adjacent matched normal tissues, which primarily results from increased expression of ERα mRNA in the breast tumor component *(58)*, it is possible that this may translate into unregulated ERα activity and unregulated growth responses mediated through ERα.

However, there are several issues that must be addressed before anyone can begin to develop rational pathophysiologically relevant hypotheses regarding the role of ERβ and/or its variants in human breast tissues. First, it is not yet known whether ERβ and ERα are expressed together in the same breast cells, or separately in different normal or neoplastic cell populations. Second, studies so far have only measured mRNA levels. No studies of ERβ protein expression in human breast have been published to date. Therefore, the pathophysiological relevance of the relative levels of ERβ and ERα expression achieved in transient expression studies, and the resulting functional outcome, are unknown. Third, some in vitro studies have been done using an N-terminally truncated ERβ1 *(64)*, and the functional impact of this is also unknown.

EXPRESSION OF OTHER STEROID HORMONE RECEPTORS AND THEIR VARIANTS IN HUMAN BC

The observation that the PR gene showed a complex pattern of alternative splicing similar to, although not as extensive as, that of ERα, led to the further characterization of PR variants *(16,31,32)*. Two commonly expressed variant transcripts identified in human breast tumors and normal human breast tissue were cloned and sequenced. Variant PR mRNAs with either a precise deletion of exon 6 or exon 4 were identified in most breast tumors examined. PR transcripts deleted in exon 2, exons 3 + 6, or exons 5 + 6, were also found in a few breast tumors *(31,32)*. The exon-6-deleted transcript was the most abundant and frequently expressed PR variant mRNA in the human breast tumors examined, and specific PCR primers were designed to determine the expression of this transcript, relative to the WT PR, using RT-PCR analysis *(27)*. Altered expression of ERα variant mRNAs was observed previously between normal and neoplastic breast tissue; therefore, it was of interest to determine if exon-6-deleted PR mRNA expression was altered during breast tumorigenesis. Using an approach similar to that described previously *(27)*, the relative expression of the exon-6-deleted variant PR mRNA to the WT PR mRNA was examined in 10 normal reduction mammoplasty samples and 17 breast tumors. The relative expression of the exon-6-deleted PR variant to the WT PR mRNA was found to be significantly lower ($P < 0.01$) in normal breast tissues (median = 4.8%) than in breast tumors (median = 13.9%) (unpublished data).

The exon-2-deleted PR mRNA encodes a C-terminally truncated PR-like protein without a DNA or a ligand-binding domain *(32)*. The exon-4-deleted PR mRNA is in-frame, but encodes a protein deleted in exon 4 sequences, missing a nuclear localization signal, and the recombinant protein representing exon-4-deleted PR-A did not bind DNA and had little effect on WT PR-A function *(32)*. Exon-6-deleted PR variant mRNA is out-of-frame and encodes a C-terminally truncated PR-like protein lacking the hormone-binding domain, and the exon-5 + 6-deleted PR variant mRNA is in-frame, but encodes a protein deleted in exon 5 + 6 sequences of the hormone-binding domain *(32)*. Richter et al. *(32)* have demonstrated that recombinant proteins, representing the exon-6-deleted PR-A and the exon-5 + 6-deleted PR-A are dominant-negative transcriptional inhibitors of both the WT PR-A and PR-B *(32)*. It is possible, therefore, that the presence of PR variant proteins encoded by the identified PR variant mRNAs could modify WT PR activity and influence responses to endocrine therapies. Small, variant PR-like proteins have been identified by Western blotting in some breast tumors *(32,65,66)*, which correspond in size to some of the proteins predicted to be encoded by some of the exon-deleted PR mRNAs. However,

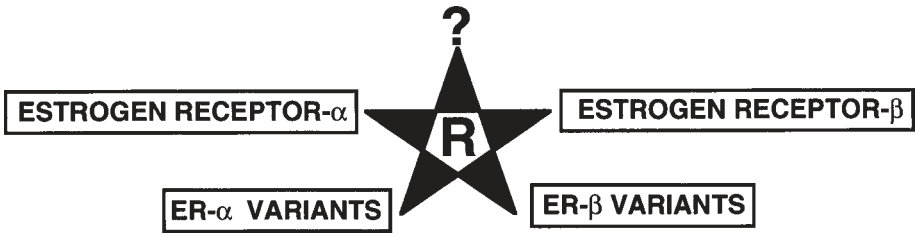


Fig. 7. Schematic representation of the known and unknown (?) multiple facets of the estrogen receptor (R).

some data *(66)* suggest that the presence and abundance of PR variant mRNAs may not correlate with the detection of these smaller-sized PR immunoreactive species in human breast tumors.

The measurement of PR is an important tool in clinical decision-making with respect to prognosis and treatment of human BC. Furthermore, the level of PR expression provides important clinical information *(67)*. As the use of enzyme-linked immunosorbent assays and IHC assays for PR detection increases, it is likely that variant PR expression will interfere with these assays, whatever their function. PR Ab (AB-52 Ab) used in such assays detect epitopes in the N-terminal region of the WT molecule, which is shared by truncated PR-like molecules. If any or all of the deleted PR variant mRNAs so far identified are translated into stable proteins, they will be co-detected with the WT PR in such assays. Presence of PR variants may also be a factor contributing to discrepancies between biochemical measurement and immunological detection of PR. Indeed, the potential for ERα variant expression to interfere with the IHC assessment of ER status has been documented *(49,50,68)*.

CONCLUSIONS AND CONTROVERSIES

The multifaceted nature of the ER is suggested by the expression of ERα mRNA, ERβ mRNA, and their variant mRNAs in both normal and neoplastic human breast tissues (Fig. 7). There is a large body of molecular data that support at least the potential for the multifaceted nature of the ER, and therefore estrogen/antiestrogen signaling in both normal and neoplastic human breast tissues. Alterations in the relative expression of several ER-like mRNAs have been shown to occur during breast tumorigenesis, and the relative frequency of detection and expression of individual ER-like mRNAs can be correlated with different prognostic characteristics in BC. This, in turn, suggests a possible role in breast tumorigenesis and possibly hormonal progression in BC. However, there are still major gaps that need to be filled before there can be a clear idea of the pathophysiological and functional relevance of the experimental results so far in hand. Unequivocal data are required to support the in vivo detection of variant ERα, variant ERβ, and WT ERβ proteins, which correspond to the variant ERα, variant ERβ, and WT ERβ mRNA species, respectively. There is a need to experimentally determine putative function, using expression levels that reflect pathophysiological levels of expression. There is a need to know if the two WT ERs and/or their variants are co-expressed in the same cells within heterogeneous normal and neoplastic breast tissues. Further, given the detection of multiple forms of variant ER-like species in any one breast tissue sample, the limitations in interpreting data from experimental systems, in which only one variant species is considered in the presence or absence of WT protein, needs to be understood.

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