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Review

Oestrogen Receptor Mutants and Variants in Breast Cancer

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Oestrogen receptor (ER) status is the only biochemical predictive factor which is routinely measured in breast carcinomas. *ER* gene mutations can profoundly change the biochemical activity of the protein. If these occurred *in vivo*, they could be expected to affect breast cancer risk or phenotype, such as endocrine responsiveness. However, no mutations of significance have been described in breast carcinomas. In contrast, numerous variant forms of ER have been reported at the mRNA level. Most of these appear to be due to aberrant exon splicing which results in predicted protein products whose activities range from dominant positive to dominant negative. In some instances, these mRNA variants have also been demonstrated in normal tissue (breast and others). Their biological and clinical significance might be profound, but remain to be established because of a lack of evidence for their existence at the protein level. On the currently available data, routine analysis for ER mutants and variants is not justified. © **1997 Elsevier Science Ltd.**

Key words: oestrogen receptor mutants, oestrogen receptor variants, breast cancer

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INTRODUCTION

OESTROGEN RECEPTOR (ER) status has been measured in breast carcinomas for many years as an indicator of the likelihood of an individual patient's benefit from hormonal therapy and also as a guide to their general prognosis [1]. ER measurements have been and remain the most widely performed biochemical analysis on breast cancer tissues, and the majority of clinical trial reports include ER status as a potential covariate in their demographic data. The existence of abnormal forms of ER could be of major importance in the assessment of ER status, and thus could influence treatment choices for the individual and the interpretation of clinical trial data. Abnormal forms which lack function but are detectable by ER assays would generate false-positive results, and undetectable forms which have biological activity would lead to false-negative results. In addition to their impact on the assessment of ER status, the presence of mutant or variant ERs could be functionally important. For example, the presence of constitutively active receptor protein which does not require a ligand to propagate an oestrogenic signal might lead to resistance to endocrine therapy.

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In addition to the role ER mutants and variants may play in established disease, the possibility has been considered that malfunctioning ER mutants could lead to an increased risk of breast cancer, and this hypothesis has been subject to a number of investigations. This review seeks to summarise the data available to date, to provide an interpretation of the data and to assess the likely clinical importance of the mutants and variants which have been described so far.

WILD-TYPE OESTROGEN RECEPTOR STRUCTURE AND FUNCTION

The *ER* gene is located on the short arm of chromosome 6 between q24 and q27 and consists of 8 exons. A second *ER* gene has recently been discovered [2], but the physiological, pathological and pharmacological significance of it have yet to be established. The wild-type ER protein is approximately 65 kDa (kilodalton) and binds to oestradiol with high affinity [3]. ER belongs to the large superfamily of ligand-activated nuclear transcription factors [4, 5]. This family includes receptors for other steroid hormones, thyroid hormone receptor, retinoic acid receptor, vitamin D receptor and a large number of so-called 'orphan receptors' for which there is no identified ligand [6]. Sequence alignment of these receptors has led to the definition of six domains denoted by the letters A–F [7] (Figure 1). The functional activities of the various domains of the receptor

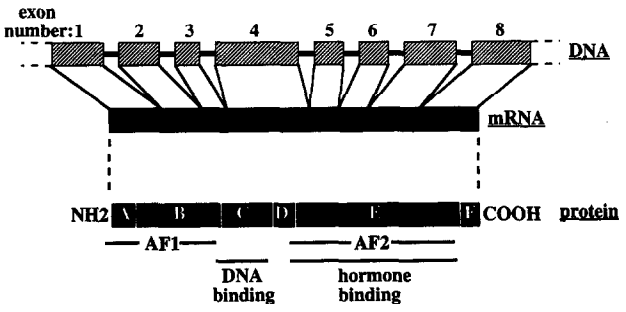

Figure 1. Domain and functional structure of *ER* gene and protein.

have been ascribed mainly by the use of deletion mutants [8]. The ligand-binding domain is largely composed of domain E, which is coded by parts of exon 4 through to much of exon 8. This region is thought to include a hydrophobic pocket which facilitates ligand binding [3, 9, 10]. The DNA binding domain includes two zinc fingers, which allow the protein to coordinate with specific oestrogen response elements on the upstream promoter region of oestrogen-dependent genes [9, 11, 12]. The domain is coded by exons 2 and 3, with one zinc finger being coded for by each of these exons. Two transcription activating functions have also been described (AF1 and AF2) [13, 14]. AF1 covers most of domain A and B and AF2 is largely coincident with the ligand-binding domain [8, 15, 16]. Nuclear localisation appears to be mediated by the nuclear localisation signal (residues 250 and 270) in region D, the so-called hinge region and is independent of ligand binding [17].

The binding of the receptor to its cognate ligand (oestradiol is the most potent) leads to a series of receptor activation steps. These include the dissociation of the receptor from heat shock binding proteins (including hsp 90) [18], the phosphorylation of a number of serine and tyrosine residues [19–22] and dimerisation [12]. The activated receptor complex binds to palindromic ERE sequences [12, 23] and activates transcription by forming protein–protein contacts between the AF1 and AF2 regions of ER and other components of the transcriptional machinery, including coactivator proteins such as SRC 1 [24]. The relative importance of the two AFs appears to vary markedly according to the promoter and cell type involved [25, 26]. Recombinant deletion mutants lacking the ligand-binding domain (containing AF2) are able to bind to EREs and activate transcription, thus indicating the constitutive activity of AF1 [8, 14, 27, 28]. However, the level of activity is markedly lower than that observed with activated wild-type receptors [27, 29]. In contrast, AF2 requires ligand to be present to achieve transcriptional activation, but with the degree of activation being dependent on the nature of the bound ligand [8, 13, 14, 27]. The mixed agonist activity of tamoxifen and such-like compounds may in part be due to their causation of ER dimerisation, with DNA–AF1 binding providing an agonist response, but with the drug antagonising the effect of oestrogen on AF2 [26].

Recently, it has become evident that differing levels of coactivators and corepressors may markedly impact on the phenotypic response levels to mixed agonists such as tamoxifen, and thus possibly explain the tissue-specific oestrogenic responses to these clinically valuable agents [24, 30]. This complex molecular pathway of oestrogen signalling indicates that the impact of ER variants and mutants may vary substantially between tissues and may be difficult to predict.

OESTROGEN RECEPTOR MUTANTS

The terms 'mutant' and 'variant' have been used in an inconsistent manner by different investigators and reviewers. For the purposes of this review, the description 'mutants' refers to changes within the DNA sequences of the *ER* gene. These may be at a germ-line or somatic level. The term 'variant' is used to describe those abnormalities which exist at the mRNA or protein level but which are not due to coding anomalies in DNA.

The search for germ-line mutations of *ER* has generally been to assess whether these might have an impact on the risk of developing breast cancer. The development of *ER* knock-out mice has shown that the complete absence of a functional *ER* gene leads to adult female mice with only vestigial ducts present at the nipples [31]. We are not aware of any reports of studies in which the impact of specific germline *ER* mutations have been assessed in transgenics. It is interesting to note that numerous mutations of the androgen receptor have been described with consequent androgen resistance syndromes [32], but an analogous syndrome of oestrogen resistance has been described only in one male [33].

A large number of recombinant receptor mutants have been constructed and indeed it is from site-directed mutation that the majority of our understanding of the molecular biology of ER, as described above, has been derived. Only those mutants which may have direct clinical relevance are discussed here. Of particular interest are those mutants which have been derived and characterised by Mahfoudi and associates [34]. They found that point mutagenesis between residues 538 and 552 in the mouse *ER* reduces oestrogen-dependent transcriptional activation without affecting hormone or DNA binding significantly. However, the pharmacological behaviour of oestrogen antagonists was dramatically altered by these mutants: both tamoxifen and the pure steroidal anti-oestrogen ICI 164384 behaved as strong agonists in HeLa cells expressing these mutants. Montano and associates [35] describe similarly changed responsiveness on introduction of the Leu 540 Gln mutation, but in addition to showing an agonist response to anti-oestrogens, this mutant shows an antagonist response to oestradiol. The presence of such AF2 mutations in breast carcinomas theoretically might account for the insensitivity of some breast cancers to tamoxifen treatment.

Therefore, it is of interest that Wolf and Jordan [36] reported that an MCF7 human breast cancer xenograft, which had acquired resistance to tamoxifen, was growth stimulated by tamoxifen and was found to contain an *ER* mutation in the ligand-binding domain. This encodes for a change of aspartate 351 to tyrosine and is therefore some distance from those mutants described by Mahfoudi and associates [34] and Montano and associates [35]. 80–90% of the total ER was described as mutant which presumably reflects a heterogeneous presence of cells containing the wild-type gene. Further studies showed that MDA-MB-231 cells transfected with the mutant *ER* responded to tamoxifen as an oestrogen [37]. Thus, there are data from both experimentally derived and spontaneous *ER* mutants which indicate that these aberrations could have consequences in terms of the endocrine sensitivity of breast cancer.

We have recently reported [38] our collaborative studies of *ER* mutations in leucocyte DNA from 143 patients with familial clustering of breast and/or ovarian cancer, and in DNA from 96 tumours of which 25 were tamoxifen resistant. CDGE (constant denaturant gel electrophoresis) or SSCP (single strand conformational polymorphism) analysis of PCR products from all 8 exons was followed by standard dideoxy sequencing of products with aberrant migration. 3 patients with a family history of cancer carried a Gly160Cys germ-line substitution in AF1. This was also present in 8 of 729 control leucocyte DNAs suggesting that this probably represented a polymorphism. Five third-base substitutions were detected in exons 1, 3, 4, 5 and 8 but all of these were also considered polymorphisms. None were associated with the hormone receptor status of the tumour or with tamoxifen resistance. Non-conservative somatic mutations were found in none of the 96 tumours examined.

Karnik and associates [39] investigated 20 tamoxifen-sensitive and 20 tamoxifen-resistant tumours using similar methodology. Four silent mutations were noted, and in one sensitive tumour, a Glu352Val mutant was found. Two mutant receptor sequences were noted in tamoxifen-resistant tumours, but their functionality was not established. Thus, consistent with our findings, the large majority of tamoxifen-resistant tumours did not contain *ER* mutations and this resistance was not determined by *ER* abnormalities at the DNA level.

The studies of Roodi and associates [40] were conducted from a different perspective. They investigated *ER* mutations in 118 ER-positive and 70 ER-negative tumours, asking whether mutations could explain the existence of ER negativity in breast cancer. They found no deletions or insertions and only two mis-sense mutations in a single ER-negative tumour. Five neutral polymorphisms were identified, none of which had any association with clinicopathological characteristics. However, a polymorphism at codon 325 was found to have a strong association with family history of breast cancer. The possible importance of this in the aetiology of breast cancer requires further study.

In contrast to these studies in which few mutants have been found in breast carcinomas, Witschke and associates [41] reported the existence of a 'superactive' *ER* mutant which was present in >30% of breast hyperplasias and in normal tissue associated with these lesions, but which was not present in normal tissue from non-diseased breasts. The lysine to arginine mutation in exon 4 was associated with a 200-fold increase in the proliferative response of MCF7 cells to oestradiol, yet the K_d of the mutant *ER* was similar to that of wild-type *ER*. Despite the apparent frequent occurrence of this in benign proliferative disease, this mutation has not been reported in breast carcinomas.

Thus, overall studies of germ-line mutations do not lend support to ER aberrations being a major influence on breast cancer risk. The few reported instances of *ER* mutants in breast carcinomas do not appear to be associated with hormone resistance. The frequency of mutants is probably not sufficient to warrant consideration when interpreting routine diagnostic data for decision-making on patient treatment. However, occasional anomalous presentation of individual cases might be explained by their occurrence.

ER VARIANTS

In contrast to the reports of only a small number of *ER* mutants, numerous variant mRNA forms have been described. These have a very frequent occurrence in breast carcinomas and, in some cases, have been described in normal breast tissue and other organs.

The majority of these are splice variants in which one or more exons are absent from the ER mRNA. However, we have also described two cryptic splice variants in which a novel splice junction occurs [42], and there are also descriptions of variants in which exon duplication [43] or non-ER sequences have been found in the transcript [44]. In the majority of these cases, where a length of message is missing, translation would be predicted to be out-of-frame; only two in-frame variants have been described in which exons 3 or 4 are deleted (Δ3 and Δ4) such that the predicted protein products would be deficient only in those regions. For those cases where translation is out-of-frame, a length of non-ER sequence is predicted prior to the occurrence of a stop codon which results in a truncated product. For the majority of products, no function has yet been described but the Δ5 variant has been found to be dominant positive in some model systems [45], and dominant negative activity has been suggested for both the Δ3 and Δ7 variants [46, 47].

A detailed description of each of these variants is not given in this review. Rather, the data resulting from the most widely studied varient, Δ5, are provided as illustrative of their possible importance. The reader is referred to these papers for a more complete listing [48, 49].

The Δ5 variant was discovered as a result of the development of a hypothetical explanation for the existence of ER-negative/PgR (progesterone receptor)-positive breast carcinomas [45]. This is a relatively uncommon phenotype and is unexpected since PgR is highly dependent on an oestrogenic signal for its expression [50]. While others had considered that this phenotype might be due to false-negative ER levels resulting from erroneous laboratory analysis, Fuqua and associates [45] developed the concept that this might be due to constitutively active ER variants which were undetected by conventional ligand-binding assays. They found that the Δ5 mRNA level was higher than the wild-type ER mRNA in 3 of 4 ER-negative/PgR-positive breast carcinomas. In contrast, in all five ER-positive/PgR-positive tumours examined, the variant was expressed at levels lower than wild-type receptor. Although this variant lacks only exon 5, this results in a predicted out-of-frame translation to yield a protein sequence containing exons [1–4] plus a unique non-oestrogen sequence of five amino acids after which a stop codon terminates translation (Figure 2). Thus, this would result in a truncated protein

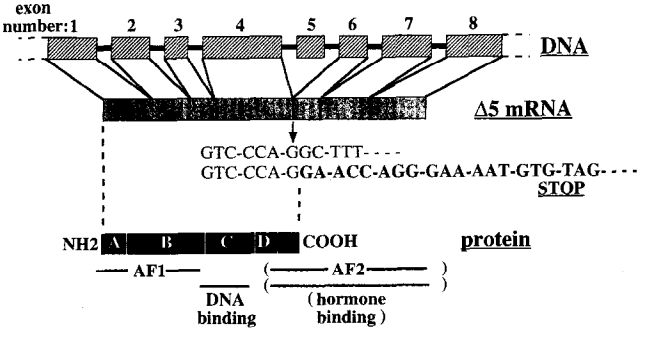

Figure 2. Structural consequences of the exon-5 deletion ER variant.

with a predicted molecular weight of 40 kDa, lacking all of the ligand-binding domain. However, far from being inactive, this variant when expressed in two yeast strains showed constitutive activation with 10–15% and 45% of the activity of the oestradiol-activated wild-type receptor [45, 51]. Additionally, when transfected into MDA-MB231 breast cancer cells, the variant activated an ERE-tk-luciferase plasmid with approximately 60% of the efficiency of the wild-type receptor [52]. As would be expected, this activity was independent of oestrogen ligand and was unaffected by oestrogen antagonists. Importantly, the Δ5 protein has been detected by immunoprecipitation of extracts from the ER-negative, weakly PgR-positive, BT20 breast cancer cell line (but not from MCF7 cells although they express the variant at the mRNA level) [51]. This is the only conclusive evidence of the existence of any deletion variant at the protein level in untransfected cells.

Of particular interest was the demonstration that MCF7 cells transfected with the Δ5 variant grew more rapidly than control plasmid-transfected cells and expressed the PgR levels to a much higher level than wild-type cells [53]. Additionally, variant-transfected cells were refractory to treatment with 4-hydroxytamoxifen, but the pure anti-oestrogen ICI 182780 suppressed growth in both control- and variant-transfected cells. The explanation for this dichotomy between the effects with the two anti-oestrogens is unclear, but a potential explanation is that the variant may dimerise with wild-type receptor and the latter is known to be markedly downregulated by ICI 182780 but not tamoxifen [54].

The clear implication of these data is that the overexpression of variant receptor could lead to tamoxifen resistance. Clinical data, however, do not support this being a significant mechanism for tamoxifen resistance in breast cancer patients. We examined 120 breast carcinoma homogenates by RT/PCR (reverse transcription/polymerase chain reaction) which yielded wild-type and variant product of differing size, and the data were expressed as a variant to wild-type ratio [55]. The median and interquartile ranges for this ratio were almost identical between 50 untreated controls and 70-tamoxifen resistant tumours (Figure 3). Thus, there was no indication for a substantial role for this variant in tamoxifen resistance in human breast cancer patients. The clinical data did, however, confirm that the variant was more commonly expressed in ER-negative tumours which expressed PgR or the other oestrogen-dependent protein pS2 (Figure 4). This, therefore, provides further circumstantial evidence that this variant might have a constitutively active stimulatory effect on PgR and pS2 expression.

In contrast to the data from Fuqua and associates [53], Rea and Parker [56] recently found that in three clones of Δ5 ER-transfected MCF7 cells, there was no difference in PgR/pS2 expression in comparison to wild-type (wt) cells. Additionally, the growth stimulatory effects of E2 and growth suppressive effects of tamoxifen and ICI 182780 were also unchanged. The explanation for this discordance between these results and those of Fuqua and associates [53] is unknown but might reflect differing MCF7 cell strains in the two laboratories.

The Δ5 band is the only form which has been convincingly demonstrated to have positive constitutive activity. Several other receptor forms have been identified in which receptor function is lost as a result of exon deletion and others in which dominant negative activity has been demonstrated. Dotzlaw and associates [57] identified ER variants which were essentially truncated either at exon 2 or exon 3 and both of these failed to activate transcription when cotransfected with a reporter vector into COS-1 cells. Therefore, it would appear that truncations of large portions of ER result in non-functional receptor, even if the DNA-binding region is intact. The transactivational function of the Δ4 ER variant in human embryocarcinoma and choriocarcinoma cells has also been found to be non-functional by

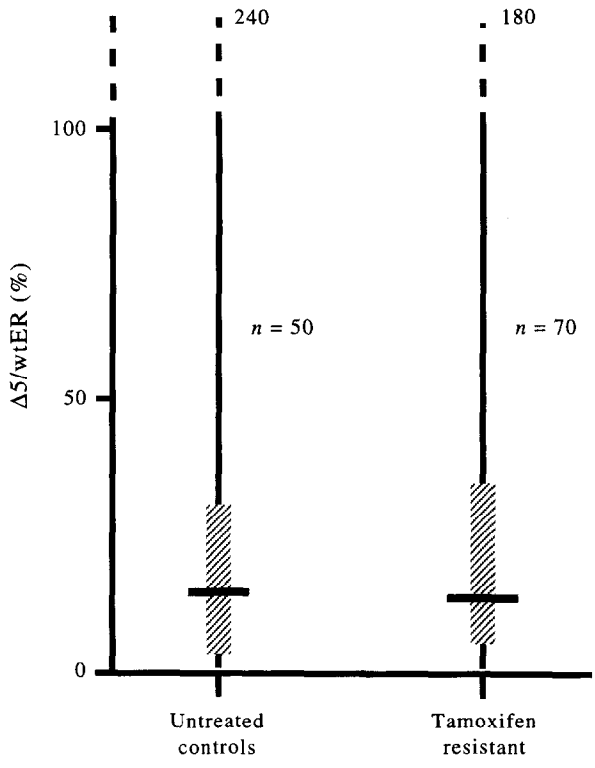

Figure 3. Δ5/wtER ratios in 50 untreated breast carcinomas compared with 70 tamoxifen-resistant breast carcinomas: narrow bars, range; wide hatched bars, interquartile range; horizontal line, median.

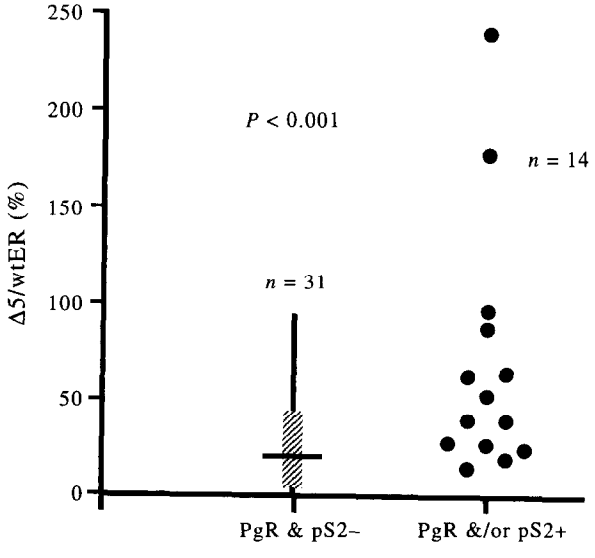

Figure 4. Δ5/wtER ratio in 31 ER-PgR-pS2 breast carcinomas (bars as in Figure 3) compared with 14 ER-PgR+ or pS2 + breast carcinomas (individual ratios).

Koehorst and associates [58], who found that it neither activated transcription nor interfered with wild-type receptor activity. Thus, while this variant contained both of the DNA-binding zinc fingers, unlike the Δ5 variant, no transactivating function appeared to persist.

The Δ3 variant is formed by an in-frame deletion which has a small effect on the predicted molecular weight, but lacks the critically important DNA-binding second zinc finger. Wang and Miksicek [46] found that this variant was transcriptionally inactive, but could repress the oestrogen-induced activity of an ERE-tk CAT promoter in HeLa cells. However, others were unable to confirm this [59]. The additional finding in the latter study that the level of expression of this variant was very low, suggests that this variant may not play a major role in ER-positive breast tumours.

In contrast, the ER variant lacking exon 7 which was also found to be transcriptionally inactive, downregulated the transactivational activity of wild-type ER when co-expressed in a yeast expression vector system [60]. Thus, this variant apparently operated in a dominant negative manner. The potential significance of this was supported by this variant being present in approximately half the ER-positive/PgR-negative tumours, suggesting that this might have an impact on the hormonal responsiveness of these tumours. If so, this variant should lead to reduced responsiveness of such ER-positive tumours to oestrogen.

It has been recognised by a number of groups that several variant messages may be expressed by a single tumour in addition to wild-type ER. Leygue and associates [61] have recently developed an approach to detecting the presence of each of these simultaneously in the same tumour. Their data indicate that certain variant profiles may be associated with established prognostic factors. However, these findings require confirmation in a much larger study.

VARIANTS IN NORMAL TISSUE

The majority of work on variants has concentrated on their expression in malignant tissues, but it has become apparent that many of these mRNA variants exist in normal tissues. Pfeffer and associates [49] recorded several exon deletion variants in a single normal breast specimen, and more recently Leygue and associates [62] reported variants deleted in exon 2 and exon 3, exons 2–3, exon 5 or exon 7 in all the normal breast samples examined. We have established that the Δ5 message is expressed alongside the wt receptor message in normal liver and endometrium as well as normal breast and breast cancer. However, our additional finding that the exon 4/7 cryptic splice variant was expressed in each of these same tissues except liver may be important in indicating that some degree of tissue specificity exists in the expression of certain variants [42].

DO ER mRNA VARIANTS HAVE PHYSIOLOGICAL OR PATHOLOGICAL SIGNIFICANCE?

At present, this fundamental question remains to be conclusively answered. The large number of variants (at a message level) and the lack of supporting data (at the protein level) might be indicative that their occurrence may be largely a laboratory artefact resulting from the extreme sensitivity of RT/PCR: possibly these message variants occur at very low level as 'background noise' in the normal process of *ER* gene transcription. However, this argument is difficult to sustain when it is recognised that some variants show tissue-specific expression [42]. Until recently, the probable significance of ER variants was also supported by the observation that the rest of this receptor superfamily rarely show variants: other than the ER variants, just one glucocorticoid receptor [63] and one androgen receptor [64] variant have been described. However, Leygue and associates [65] have now reported a number of progesterone receptor exon-deletion variants.

As to their potential role in normal tissues, it is now well established that firstly there are substantial between-tissue differential sensitivities to oestrogens, and secondly, that pharmacological agents exist, termed selective ER modulators (SERMS) [66] which have unexplained differential agonist and antagonist activity in different tissues. Hypothetically, if variants were expressed to a significant extent as proteins, they could exert a differential influence on tissue sensitivity and on specificity of pharmacological and physiological agents. A recent development has been the description of a 'raloxifene response element' on the promoter region of at least one oestrogen-sensitive gene [67]. ER interacts with this element via modulating proteins and, importantly in this context, does not require the conventional DNA-binding region to do so. Thus, variants of the Δ3 type might interact with this pathway, but not be able to bind to conventional EREs.

CONCLUSIONS AND SUMMARY

Point mutations in *ER* can have profound effects on protein function in model systems. However, their relevance to breast cancer appears to be slight, in terms of both risk of development and phenotype of established tumours. A large number of variant ERs exist at an mRNA level. The lack of data on expression at the protein level leaves only circumstantial evidence for their clinical importance. There are a number of data sets which are consistent with their involvement in the establishment of certain pathological tumour phenotypes, although not in the response of the tumour to tamoxifen. Theoretically, their differential expression could be important as an additional control on oestrogen/anti-oestrogen sensitivity. It does not appear likely that there will be any advantage to the measurement of any of these mutants or variants in routine clinical practice.

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