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PROSPECTS

Estrogen Receptor Mutations in Breast Cancer

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Abstract It is fairly well accepted that the presence of estrogen receptor (ER) identifies those breast cancer patients with a lower risk of relapse and better overall survival [Clark and McGuire, 1988], and the measurement of ER has become a standard assay in the clinical management of breast cancer. Receptor status also provides a guideline for those tumors which may be responsive to hormonal intervention [McGuire 1978; Osborne et al., 1980; Rose et al., 1985]. But only about half of ER-positive patients will respond to the various hormonal therapies available, and of those who do initially respond, most will eventually develop hormonally unresponsive disease following a period of treatment even though ER is often still present. Loss of ER from initially ER-positive tumors biopsied again at a later date has been estimated at only 19% [Gross et al., 1984]. Obviously the simple measurement of ER presence by ligand-binding assays does not provide us with an adequate estimate of the functional state of the receptor.

In 1985 Sluyser and Mester hypothesized that the loss of hormone dependence of certain breast tumors may be due to the presence of mutated or truncated steroid receptors that activate transcription even in the absence of hormone [Sluyser and Mester, 1985]. Based on the recent identification of several ER sequence variants in human breast cancer cell lines and tumor specimens, we would now like to propose that some of these identified mutations play a role in receptor dysfunction in vivo, and will review those ER mutations which may prove to be important in breast cancer progression.

Key words: steroid hormone receptors, breast tumors, RNA splicing

RECEPTOR STRUCTURE/FUNCTION STUDIES

During the past decade ERs have been cloned from several species [Walter et al., 1985; Maxwell et al., 1987; White et al., 1987], allowing extensive structure/function analyses. Based upon amino acid sequence similarity of the cloned cDNAs, these proteins can each be divided into six conserved domains, A–F [Kumar et al., 1987]. These functional domains are responsible for transcriptional activation and repression, nuclear localization, DNA binding, and hormone binding of the receptor. Figure 1 shows the various domain-associated functions that we will refer to when describing the ER variants that have been identified in breast cancer.

The least well-conserved region is the N-terminal A/B region, which contains a constitutive activation function that activates transcription of estrogen-responsive genes in a cell-type-specific manner [Tora et al., 1989; Bocquel et al., 1989]. The next region, amino acid domain C, is highly conserved, and contains 20 invariant residues, including 9 cysteines, which fold into 2 zinc-finger DNA binding motifs [Evans, 1988, and references therein]. This DNA-binding domain is responsible for the recognition of specific enhancer sequences found in hormone-responsive genes, generally referred to as hormone response elements (HREs). Each receptor recognizes its own response element, so that extracellular hormonal signals are transduced via the receptor to specific target genes. Discrimination between different HREs is determined by three amino acids at the base of the first zinc finger [Umesono and Evans, 1989], and single base pair substitutions can produce receptors that recognize different HREs [Mader et al., 1989]. Thus, sequence mutations within this region could have important functional consequences. The C-terminal extremity of the DNA-binding domain of the ER also contains a nuclear localization sequence (NLS) important for the nuclear location of the receptor [Chambraud et al., 1990].

The DNA-binding domain C is separated from the large hydrophobic E domain by a hinge of

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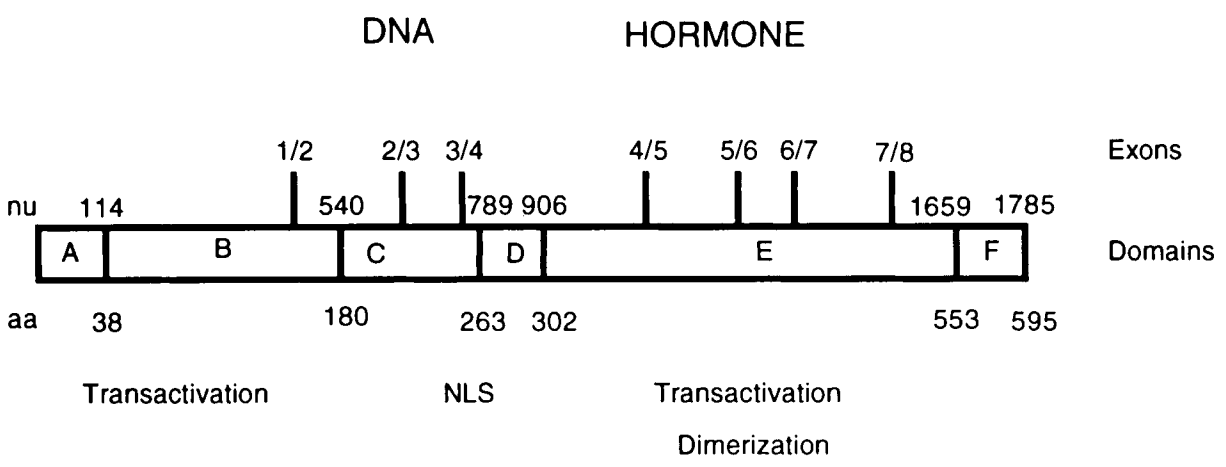

Fig. 1. Shown are the locations of the six functional domains of the estrogen receptor (A–F) and the eight exon/intron borders. The amino acid (aa) and nucleotide (nu) sites of the six domains are shown below and above, respectively. Regions involved in transactivation, dimerization, and nuclear localization (NLS) are also indicated. Data taken from Kumar et al. [1987] and Ponglikitmongkol et al. [1988].

variable length and amino acid composition (the D region). Several in vitro substitutions within this single domain have had little effect on ER function [Kumar et al., 1987]; however, this result must be interpreted with caution because one report suggests that a single mutation within this region inactivates the related v-erbA nuclear receptor [Damm et al., 1987].

The E domain is a complex region containing the hormone binding site and a region required for stable dimerization of the receptor [Kumar and Chambon, 1988; Fawell et al., 1990]. In addition, this region contains a transcriptional activation function which is hormone inducible, and which synergizes with the A/B region transactivating activity depending on target gene promoter context [Tora et al., 1989; Berry et al., 1990]. There has been a recent report that the ER may have transcriptional activator and repressor function in the absence of hormone [Tzukerman et al., 1990], but this has been disputed by others [Berry et al., 1990]. What is apparent from all of these studies, however, is that the E region is very complex, and mutations within this region of the receptor could have profound consequences on receptor function. As discussed below, preliminary results with ER mRNA variants found in breast tumors also suggest this to be the case. The significance of the F domain is unknown at present.

DOMAIN-ASSOCIATED MUTATIONS WITHIN THE ER OF HUMAN BREAST CANCERS

The A/B Domain

Many investigators have utilized the functional domains of the ER as a basis for their search for mutations. Garcia et al. [1988] prepared probes to small regions across domains A–F, and used RNase protection assays to screen 71 tumors for mutations or deletions in ER mRNA. They identified a C to T transition at nucleotide 257 which changes alanine 86 to a valine within the B domain of the ER [Garcia et al., 1989]. Interestingly, the presence of this ER variant in ER-positive patients correlated with low levels of estrogen binding [Garcia et al., 1988]. The authors enlarged their study population without observing a difference in the frequency of this B variant in ER-positive as compared to ER-negative tumors, and concluded that the variant may not be related to breast cancer development [Schmutzler et al., 1991]. Unfortunately, this study did not report whether the ER-positive patients with the B variant again demonstrated low ER levels. The small number of specimens so far studied, and the low frequency of the B variant make it difficult to access the functional significance of low ER protein levels and the associated low estrogen binding in B variant, ER-positive patients. This variant may also be associated with spontaneous abortion in women with breast cancer [Lehrer et al., 1990], but once again confirmation of this interesting result awaits a larger study. An analysis of whether this change within the B domain confers altered ER transcriptional capacity could also lend support to its clinical significance.

The DNA-Binding C Domain

ER function depends on its binding to specific estrogen response elements (EREs) usually located within the promoter region of estrogen-

responsive genes. Gel-retardation assays have proven to be a sensitive assay for measuring this ERE-DNA binding capacity using even relatively impure ER preparations [Kumar and Cambon, 1988]. Scott et al. [1991], using gel-retardation assays, have reported that approximately two-thirds of breast tumors exhibiting high ER content ($\geq$ 100 fmol/mg protein) retain DNA-binding ability, while the majority of tumors with either low or intermediate ER levels (20–99 fmol/mg) have lost this activity. Furthermore, there was a significant association between ERE-DNA binding and progesterone receptor (PgR) content. Thus the presence of intact DNA binding correlated with standard predictors of hormone responsiveness, high ER, and positive PgR content. Many of the ER-positive tumors also expressed a truncated, non-DNA-binding form of the receptor, perhaps arising from posttranslational mechanisms. These authors further suggest that the loss or truncation of ERE-DNA binding ER could be a new prognostic factor for determining antiestrogen resistance. A simple assay such as gel retardation could indeed be applied in a clinical laboratory for the measurement of functional ER; however, assessment of the clinical utility of the assay awaits determination of hormone response data on the patients in the original study, and of course confirmation by other groups.

Murphy and Dotzlaw [1989] have taken another approach to identify mutant ER in human biopsies. They found variant 4.5, 3.8, and 2.5 kb ER mRNA species using Northern hybridization analyses. These smaller variant mRNAs appeared to be missing some or all of the E and F domains of the receptor when Northern blots were hybridized with domain-specific probes. A cDNA library was prepared from a tumor containing these smaller messages, and two abnormal ER clones were isolated that detected the 3.8 and 4.5 kb ER mRNAs [Dotzlaw and Murphy, 1990; Dotzlaw et al., 1991]. One clone, clone 24, is identical to wild-type ER up to the exon 3/intron border. At this point (amino acid 253), the sequence diverges and 83 unique amino acids are included before a stop codon is reached. This clone thus contains both of the DNA-binding zinc fingers, but is completely missing the hormone-binding domain of the receptor. Previous in vitro mutagenesis data suggest that ER constructs lacking the hormone-binding domain might be constitutively active as transcriptional inducers of estrogen-responsive genes [Kumar et al., 1987]. However, this variant is a nonfunctional receptor in COS cells. The second clone isolated from this tumor-specific cDNA library, clone 5, has only been partially characterized. It is missing exon 2, and like clone 24, also diverges from wild-type ER sequence at the exon 3/intron border. It has not been reported whether this variant is a functional receptor.

To isolate the 2.5 kb variant ER mRNA that was identified, a second cDNA library was prepared from a pool of tumors which only expressed this mRNA species [Dotzlaw et al., 1991]. The cDNA isolated from this library, clone 4, was identical in sequence to wild-type ER until the exon 2/intron border, at which point the sequence diverged. The 3′ sequence is related to the LINE-1 family of repeated DNA sequences. It is interesting to note that all of these variant ERs diverge at exon/intron borders, as will be seen also for several variants isolated by others [Graham et al., 1990; Wang et al., 1991; Fuqua et al., 1991a, 1992; McGuire et al., 1991]. In vitro functional analyses with clone 4 suggest that this variant is nonfunctional. It may require in vivo studies to address the clinical significance of these reported variants.

Another aspect of studies searching for variant ERs in human breast tumors is that wild-type ER sequences are often present along with the variant species [Murphy and Dotzlaw, 1989; Graham et al., 1990; Fuqua et al., 1991b, 1992]. This is not surprising; breast cancer heterogeneity has been appreciated clinically for some time. Graham et al. [1990] postulated that one mechanism for this heterogeneity, and possibly for the development of hormone resistance, is the evolution of mutant ERs by genetically unstable cell subpopulations within tumors. Biological evidence of this is suggested by their analysis of clonal cell lines generated from an estrogen-unresponsive T47D breast cancer cell line. They have observed the emergence of aneuploid T47D sublines that coevolve with hormonal resistance. Furthermore, several different variant ERs as well as wild-type ER have been sequenced from these cells. Of note is that these variants usually involve changes at exon/intron borders, again suggestive of splicing errors, and include changes within both the DNA and hormone binding domains [Graham et al., 1990]. It is unknown whether these variant receptors are functional, or whether similar variants have been detected in human tumor specimens.

Wang and Miksicek [1991] have also reported the isolation of two C domain mRNA variants from T47D breast cancer cells. These variants

had lost either exon 2 or 3, and neither was able to function as transcriptional inducers of an estrogen-responsive gene construct. However, the exon 3 deletion variant *inhibited* estrogen-dependent transcriptional activation by *wild-type* ER in a dominant-negative fashion. We similarly have isolated exon 3 deletion variant ERs from primary human tumor specimens, though our in vitro studies suggest that this variant does not function in a dominant-negative manner when coexpressed with wild-type ER. These disparate results could reflect the different in vitro systems utilized to determine variant dysfunction, and emphasize the need for in vivo studies to confirm any in vitro results.

We have sequenced polymerase chain reaction (PCR)-amplified cDNA from the DNA binding domain of 10 ER-positive tumors, and all but one contained the wild-type ER sequence (Fuqua et al., 1991a; McGuire 1991). This one tumor contained a 6 bp insertion within the DNA binding domain at the exon 2/intron border, which results in the in-frame introduction of 2 amino acids (asparagine + arginine). This 6 bp ER variant is reduced in transcriptional activity as compared to wild-type ER, and appears to be expressed in a variety of tissues. Preliminary results suggest that it may inhibit normal ER function [unpublished results]. It will be interesting to determine the genomic structure of this variant; it may represent a small alternative exon of the ER gene.

ER VARIANTS WITHIN THE HORMONE-BINDING E DOMAIN ASSOCIATED WITH DISCORDANT RECEPTOR PHENOTYPES

ER-Negative/PgR-Positive Phenotype

We have adopted several screening techniques to identify ER mutations in breast tumors [McGuire et al., 1991]. Our strategy was to focus upon the discordant receptor phenotypes, ER-negative/PgR-positive and ER-positive/PgR-negative, to increase our likelihood of detecting sequence alterations. We were the first to report the existence in an ER-negative/PgR-positive tumor of an ER truncated within the hormone binding domain which can activate transcription in the absence of hormone [Fuqua et al., 1991b]. This variant ER is completely missing exon 5 of the E domain, which results in a truncated out-of-frame protein of approximately 40,000 daltons due to the introduction of a stop codon ER codon 366. This variant may also result from alternative splicing, since genomic PCR amplification from tumors and sequence analysis of the exon 4 border into the intervening intron sequence has failed to detect mutations in the 5′ donor splice site [unpublished results]. Sequence analysis of the 3′ acceptor site will be required in order to definitively answer whether the exon 5 deletion variant is indeed the result of alternative splicing.

To confirm that the variant was not an artifact of PCR amplification and to evaluate its level of expression in tumors, we have performed RNase protection assays using the ER exon 5 deletion variant as probe. Both the variant and wild-type ER RNAs were detected in tumors [Fuqua et al., 1991b], though the ratio of the variant as compared to the wild-type ER message was two- to three-fold higher in some apparently ER-negative as compared to ER-positive tumors. We hypothesize that since the variant generates a transcriptionally active, dominant-positive receptor, it may account for PgR expression in some ER-negative/PgR-positive breast tumors. Furthermore, the exon 5 variant may be clinically relevant as a factor in hormonally unresponsive breast cancer. When the variant is stably overexpressed in ER-positive MCF-7 cells, these cells become unresponsive to the growth inhibitory effects of several hormonal therapies, such as treatment with antiestrogens and high dose progestins [unpublished results]. Questions remaining to be answered are whether the exon 5 deletion variant confers resistance to these agents in vivo, and whether it is frequently expressed in antiestrogen-resistant tumors; these experiments are currently under way.

ER-Positive/PgR-Negative Phenotype

We hypothesized that ER-positive/PgR-negative tumors could contain a variant ER unable to function as a transcriptional inducer of PgR expression, and we employed gel-retardation assays to screen for such a variant. Our results, in contrast to others [Scott et al., 1991; Foster et al., 1991], suggested that the majority of such tumors have ERs capable of binding to ERE DNA [Fuqua et al., 1992]. We did, however, identify a 3′ truncated ER with this assay which is unable to function as a transcriptional inducer of estrogen-responsive genes. This variant appears to be another alternative splicing transcript which is precisely missing exon 7 within the E domain. When this variant was cointroduced into yeast cells at equivalent levels with wild-type ER, the activity of wild-type receptor was reduced by approximately half. The exon 7 variant, at least in this assay, thus appears as a

potent dominant-negative inhibitor of ER function. This variant has also been detected in T47D cells; however, it does not appear to have an effect on wild-type ER when coexpressed in a twentyfold excess in transient transfection assays [Wang and Miksicek, 1991]. Once again, this discrepancy in results with apparently identical ER variants must reflect the different assay systems used to evaluate its function.

We also compared the level of expression of the exon 7 deletion variant to wild-type ER in a series of breast tumors. The variant was significantly overexpressed in some of the ER-positive/PgR-negative tumors, and we would like to suggest that its overexpression could interfere with normal ER function, and perhaps be responsible for the PgR-negative status of certain ER-positive tumors. Future studies are directed at assessing its presence in a larger series of tumors with clinical follow-up. Such a variant could render a tumor functionally receptor-negative and hormone-independent in clinical course.

SUMMARY

It is interesting that so many of the variants thus far identified represent transcriptional splicing errors. Breast tumors may suffer from general splicing defects, different splicing factor activities, or alternatively, a derangement of regulated splicing pathways. The high frequency with which dysfunctional ER variants have been reported in both breast tumors and established cell lines suggests that they may play a role in the progression of breast cancer. It is obvious from this review that much work is required to determine whether these variants play a role in hormonally unresponsive disease. Undoubtedly, as work progresses on the in vivo dysfunctioning of these variants, a clearer understanding of their ultimate clinical significance will be achieved.

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