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ABNORMAL ESTROGEN RECEPTOR IN CLINICAL BREAST CANCER

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Summary—We have discovered a number of estrogen receptor variants in clinical breast cancer tissues. We have base-pair insertions, transitions, and deletions of exons 3, 5 and 7. Using a transactivation assay we have discovered receptors with outlaw function consisting of both dominant-positive receptors which are transcriptionally active in the absence of estrogen, and dominant-negative receptors which are transcriptionally inactive themselves but prevent normal estrogen receptor function.

INTRODUCTION

Estrogen receptor (ER) is an excellent marker of differentiation. It predicts improved disease-free survival in breast cancer and, most important, predicts the likelihood of benefit from tamoxifen therapy. But there are still many key issues regarding ER to be considered. First, why are some breast tumors ER-negative? And second, why do some ER-positive tumors behave as if they are ER-negative (e.g. fail antiestrogen therapy), and some ER-negative tumors behave as if they are ER-positive [e.g. synthesize progesterone receptor (PgR)]?

With respect to the loss of functional ER, there are a number of possibilities that need to be examined. We could have a genomic deletion of the gene itself. We could have mutations or rearrangements of the gene. We could have a down-regulation of transcription at the promoter level. We could have methylation within the coding domain or the promoter region. And finally, we could have an altered message such as that which occurs with alternative splicing. We must also consider *aberrant* function, in other words, outlaw receptors. We should consider particularly the possibility of a dominant-positive ER, i.e. a variant receptor that is active even in the absence of estrogen. We should also consider the possibility of a dominant-negative ER, i.e. a variant receptor which is not only inactive, but prevents the function of normal ER. We will first briefly review published studies from other laboratories on ER DNA, RNA and abnormal function, and then turn to ER variant studies ongoing in San Antonio.

PUBLISHED STUDIES ON ER VARIATION

Considering ER DNA studies, Koh *et al.* [1] looked at 34 breast cancer patients by Southern hybridization analysis and did not find any evidence for ER amplification or rearrangement, while Nembrot *et al.* [2] reported evidence for a 1.6 to 3-fold amplification in 6 of 14 cases. Falette *et al.* [3] looked at methylation of the ER gene by Southern analysis and found different methylation patterns in normal breast and adjacent tumor tissue, and in ER-positive and ER-negative tumors, but there was no difference in receptor expression as a function of methylation. Castagnoli *et al.* [4] found a PvuII RFLP in the ER gene of 14 of 20 males. Hill *et al.* [5] studied this same RFLP and found that it correlated with ER expression in 188 breast cancer patients. However, Parl *et al.* [6] found the PvuII RFLP to be correlated with age but not ER expression in a smaller number of breast cancer patients. In a follow up study, he also located the PvuII RFLP within intron 1; this time no correlation with either age or ER expression was seen in 260 breast cancer patients [7]. And finally, Wanless *et al.* [8] described a HindIII RFLP in the ER gene in a small percentage of breast cancer patients, which correlated with PgR expression.

Turning to ER mRNA, Bartlett-Lee *et al.* [9] found a good correlation between ER mRNA,

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protein, and ligand binding. Rio *et al.* [10], by Northern analysis, found no gross structural alterations in ER message. Piva *et al.* [11] found that ER mRNA correlated with ER protein. Henry *et al.* [12] found that ER mRNA assays were more sensitive than ligand binding, and May *et al.* [13] studied the ratio of ER protein to mRNA and found that a high ratio correlated with the risk of relapse. The first RNA variant described was by Garcia *et al.* [14] who used an RNase protection assay and found in 8 of 66 ER-positive tumors a nucleotide mismatch in the B coding region which correlated with low ligand binding. She subsequently found that the mismatch corresponded to a C to T transition at nucleotide 257, resulting in an alanine to valine substitution which removed a BbvI restriction site [15]. In a rather surprising turn of events, Lehrer *et al.* [16] found that 50% of breast cancer patients with the B variant had spontaneous abortions compared to only 10% of patients with wild-type ER, and Schachter and co-workers [17] reported that spontaneous abortions occur only in the B variant ER-positive breast cancer patients and not in the ER-negative or non-breast cancer patients. The variant was also found (in heterozygous form) in about 12% of genomic DNA's, apparently unrelated to ER status of breast tumors or to the presence of breast cancer at all [18]. No explanation for these findings is yet available.

Murphy and Dotzlaw [19] using Northern hybridization analyses of breast tumor RNA found a number of smaller size ER mRNA variants resulting from deletions of the hormone binding domain. They prepared a cDNA library from one of these breast cancer biopsies and found 84 unique amino acids introduced at the exon 3 intron boundary (amino acid 253) that were L-1 repetitive sequences [20]. These sequences were followed by a stop codon resulting in a truncated 37 kDa protein. More recently, Murphy and co-workers [21] reported an ER variant with an insertion of 6 unique amino acids at the exon 2 intron boundary (amino acid 214), finally followed by a stop codon for a total of 220 amino acids. Benz and co-workers [22] using ER gel-retardation assays found that some ER-positive tumors either did not bind or bound only weakly to a synthetic estrogen response element (ERE). This decrease in binding was associated with a 50 kDa variant dimer or a 50/67 kDa heterodimer of wild type plus variant.

Concerning abnormal function, one can consider the situation of active ER in the absence of estrogen. Zava *et al.* [23] was one of the first to speculate about the possibility of biologically active ER without estrogen. Horwitz and co-workers [24–26] suggested that permanently activated ER might explain the high persistent levels of PgR in T47D tissue culture cells. Sluyser [27] brought a different focus to the problem and hypothesized that mutated or truncated ER without estrogen might be able to act as an oncogene and stimulate breast cancer growth. We will see such a variant from our own laboratory below.

Thus, in the past few years there has been a lot of activity devoted to discovering abnormal ER in clinical breast cancer, and trying to determine whether these receptors are associated with altered function. We would now like to summarize in more detail our studies in San Antonio.

SCREENING FOR ER VARIANTS

Since it is not feasible to sequence the whole ER in both normal and tumor tissue from every breast cancer patient, we decided to develop more selective screening strategies to search for abnormal ER in clinical experiments. We chose to produce polymerase chain reaction (PCR) amplified cDNA [28] from known functional domains of the ER message [29, 30], and to use direct sequencing, chemical mismatch cleavage (CMC) [31], and single stranded conformational polymorphism analyses (SSCP) [32] to detect ER RNA variants. Finally, we also used gel-retardation assays to detect ER DNA binding variants.

We first examined ER-negative/PgR-positive tumors, reasoning that some tumors might have a variant ER lacking ligand binding but possessing transcriptional activating capability. We used oligonucleotide primers to PCR amplify exons 4, 5 and 6 from the ligand binding region of the ER mRNA [28]. This normally results in a cDNA of length 438 bp. We indeed found this fragment in many of these ER−/PgR+ tumors, though it was seldom present in ER−/PgR− tumors [33]. But in some tumors we also found a 300 bp PCR fragment, which upon sequencing revealed a precise deletion of exon 5. In order to be sure that this finding was not the result of PCR artifacts, we performed an RNAse protection assay using total RNA isolated from the tumors, which demonstrated directly that the

exon 5 deletion indeed exists in clinical breast cancer. The variant is found in ER+ tumors as well, but the ratio of the variant to wild-type ER is 2 to 3-fold higher in some of the apparently ER−/PgR+ tumors. About half of the ER−/PgR+ tumors overexpressed this variant.

We have used gel-retardation assays [28] to screen for binding of possible variant receptors to a synthetic ERE. Figure 1 is an example of a gel-retardation assay, showing an extract from a receptor-positive tumor retarding the migration of the ERE. We show that the complex does indeed contain ER by further upshifting the complex with antibodies which bind the ER. The results for antibodies D75, H222, and the combination of D75 and H222 are shown. For negative control purposes, we use B39, which is an antibody to PgR. A receptor-negative tumor showed no retention of ERE in the gel. Using this assay we have identified an ER with a 3′ truncation—that is, it binds ERE and is upshifted by ER antibodies except those with target epitopes in the C-terminal region (Fuqua, manuscript in preparation). This

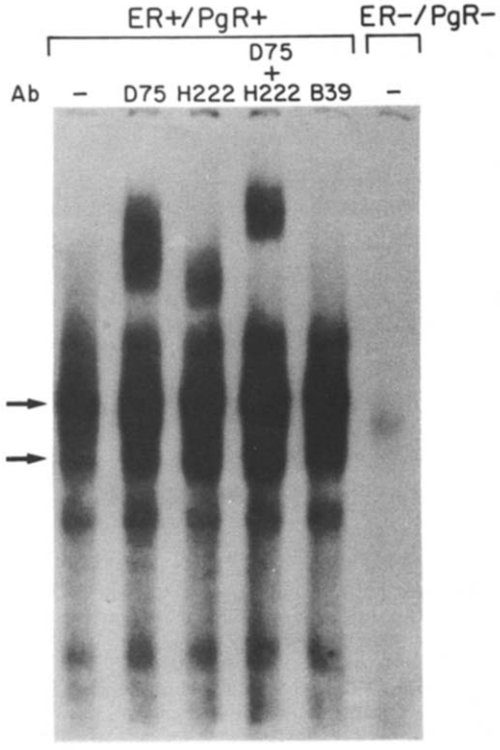

Fig. 1. ERE gel-retardation assay using protein extracts from ER+/PgR+ and ER−/PgR− breast tumors. Specific binding of ER to its ERE is denoted with arrows. ER-specific antibodies D75 and H222 were used to confirm the specificity of the complex, and the PgR-specific B39 antibody was used as a negative control.

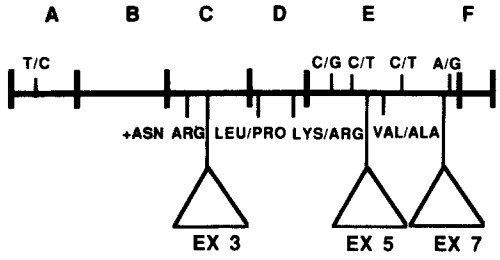

Fig. 2. Schematic diagram of the location of some of the ER RNA variants detected within functional domains of the ER.

receptor was cloned and sequenced, and we found that exon 7 was precisely deleted in this tumor.

Figure 2 is a diagram summarizing some of the variants we have found by these techniques in San Antonio to date. We have found deletions of exons 3, 5 and 7, an asparagine + arginine insertion in the DNA binding domain, and several single amino acid substitutions in domains D and E. There are also many silent base pair substitutions throughout the gene.

FUNCTION OF VARIANT RECEPTORS

For ascertaining possible altered function of some of these variant receptors, we established a yeast expression vector function assay [27]. Either wild-type or variant ER is inserted into a plasmid under the control of a metallothionein promoter. Another plasmid containing the ERE controlling a β-galactosidase (β-gal) reporter gene is also transformed into the same yeast cell. This system thus tests the transcriptional activating ability of wild type and variant ER. In a typical experiment where wild type ER is inserted under estrogen-free conditions (Table 1), there is no β-gal synthesis. Adding copper to increase the absolute amount of receptor made does not result in any β-gal synthesis, but the addition of estrogen dramatically increases synthesis. Therefore, this system is exquisitely estrogen dependent. We also see an example of an exon 3 deletion variant, which is completely inactive in this assay, as might have

Table 1 Yeast transactivation assay for ER variant function

	β-gal activity			
Treatment	Wild type ER	Exon 3 del	Exon 5 del	Exon 7 del
Control	0	0	400	0
+Cu	0	0	800	0
+E	2700	0	800	0

The ER gene, wild type or variant, is present under a Cu-inducible metallothionein promoter, while ER activity is detected by induction of β-gal under control of an ERE.

been expected since it has no DNA binding domain.

We then used this system to examine possible outlaw receptors, those with abnormal function either dominant-positive (transcriptionally active in the absence of estrogen), or dominant-negative (transcriptionally inactive but preventing function of *normal* ER). The exon 5 deletion that we first described had been cloned from an ER-negative, PgR-positive tumor, so that a dominant-positive variant was suspected. Indeed, in the yeast expression system, there was appreciable β-gal synthesis in the absence of estrogen which was increased when the level of the variant receptor protein was increased by copper, but was not affected by estradiol addition. Such a receptor would have the potential of stimulating breast tumor growth in the absence of estrogen.

Table 1 also shows an example of a dominant-negative receptor, the 3′truncated exon 7 deletion discovered in our gel-retardation studies. In this assay the exon 7 deletion causes no β-gal synthesis under control, copper stimulated, or estrogen stimulated conditions. Therefore, it is transcriptionally inactive. But we also questioned whether it could prevent the function of *normal* ER, i.e. be dominant-negative. Figure 3 therefore illustrates a yeast system assay where wild-type and variant are both present in the same cell, the wild type under a constitutive promoter and the variant under an inducible metallothionein promoter. The variant can thus be progressively turned on by copper. The top curve represents wild type ER by itself, and the bottom curve, barely seen, is the exon 7 ER deletion variant by itself. The middle curve represents variant and wild type ER together. It can be seen that as the copper concentration is increased and the variant ER is progressively induced, the variant interferes with the wild-type receptor's ability to induce β-gal at the ERE. It is noteworthy that even at the highest copper concentration Western blot analysis revealed no more than equivalent levels of wild type ER and variant ER. Thus, this is a very potent dominant-negative variant.

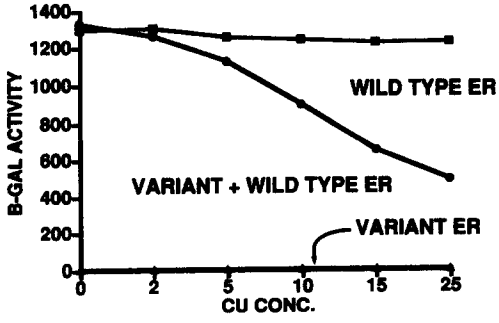

Fig. 3. Yeast transactivation results showing the amount of β-gal activity as a function of copper concentration (μM) in the presence of estrogen. The exon 7 deletion ER variant and wild type ER were introduced into yeast cells either alone or simultaneously and β-gal levels determined.

CONCLUSION

In summary, we have used the screening techniques of CMC and SSCP of selected PCR fragments, and also gel-retardation assays, to discover a number of ER variants in clinical breast cancer tissues. We have found base pair insertions, transitions, and deletions, and deletions of exons 3, 5 and 7. Using a yeast transactivation assay we have discovered receptors with outlaw function consisting of both dominant-positive receptors which were transcriptionally active in the absence of estrogen, and dominant-negative receptors which are transcriptionally inactive themselves, but prevented normal ER function. Future study should focus in particular on such dominant-positive and dominant-negative variants and their possible clinical significance.

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REFERENCES

1. Koh E. H., Ro J., Wildrick D. M., Hortobagyi G. N. and Blick M.: Analysis of the estrogen receptor gene structure in human breast cancer. *Anticancer Res.* **9** (1989) 1841–1846.
2. Nembrot M., Quintana B. and Mordoh J.: Estrogen receptor amplification is found in some estrogen receptor-positive human breast tumors. *Biochem. Biophys. Res. Commun.* **166** (1990) 601–607.
3. Falette N. S., Fuqua S. A. W., Chamness G. C., Cheah M. S., Greene G. L. and McGuire W. L.: Estrogen receptor gene methylation in human breast tumors. *Cancer Res.* **50.** (1990) 3974–3978.
4. Castagnoli A., Maestri I., Bernardi F. and Del Senno L.: PvuII RFLP inside the human estrogen receptor gene. *Nucleic Acids Res.* **15** (1987) 866.
5. Hill S. M., Fuqua S. A. W., Chamness G. C., Greene G. L. and McGuire W. L.: Estrogen receptor expression

in human breast cancer associated with an estrogen receptor gene restriction fragment length polymorphism. *Cancer Res.* **49** (1989) 145–148.
6. Parl F. F., Cavener D. R. and Dupont W. D.: Genomic DNA analysis of the estrogen receptor gene in breast cancer. *Breast Cancer Res. Treat.* **14** (1989) 57–64.
7. Yaich L. E., Dupont W. D., Cavener D. R. and Parl F. F.: The estrogen receptor PVU II restriction fragment length polymorphism is not correlated with estrogen receptor content or patient age in 260 breast cancers. The Endocrine Society, Washington DC (1991) p. 175 (Abstr).
8. Wanless C., Barker S., Puddefoot J. R., Panahy C., Goode A. W., Vinson G. P. and Phillips I. R.: Somatic change in the estrogen receptor gene associated with altered expression of the progesterone receptor. *Anticancer Res.* **11** (1991) 139–142.
9. Barrett-Lee P. J., Travers M. T., McClelland R. A., Luqmani Y. and Coombes R. C.: Characterization of estrogen receptor messenger RNA in human breast cancer. *Cancer Res.* **47** (1987) 6653–6659.
10. Rio M. C., Bellocq J. P., Gairard B., Rasmussen U. B., Krust A., Koehl C., Camderoli H., Schiff V., Renaud R. and Chambon P.: Specific expression of the pS2 gene in subclasses of breast cancers in comparison with expression of the estrogen and progesterone receptors and the oncogene ERBB2. *Proc Natn Acad. Sci. U.S.A.* **84** (1987) 9243–9247.
11. Piva R., Bianchini E., Kumar V. L., Chambon P. and Del Senno L.: Estrogen induced increase of estrogen receptor RNA in human breast cancer cells. *Biochem. Biophys. Res. Commun.* **155** (1988) 943–949.
12. Henry J. A., Nicholson S., Farndon J. R., Westley B. R. and May F. E. B.: Measurement of oestrogen receptor mRNA levels in human breast tumours *Br. J. Cancer* **58** (1988) 600–605.
13. May E., Mouriesse H., May-Levin F , Contesso G. and Delarue J.-C.: A new approach allowing an early prognosis in breast cancer: the ratio of estrogen receptor (ER) ligand binding activity to the ER-specific mRNA level. *Oncogene* **4** (1989) 1037–1042.
14. Garcia T., Lehrer S., Bloomer W. D. and Schachter B.: A variant estrogen receptor messenger ribonucleic acid is associated with reduced levels of estrogen binding in human mammary tumors *Molec. Endocr.* **2** (1988) 785–791.
15. Garcia T., Sanchez M., Cox J. L., Shaw P A., Ross J. B. A., Lehrer S. and Schachter B.: Identification of a variant form of the human estrogen receptor with an amino acid replacement. *Nucleic Acids Res.* **17** (1989) 8364.
16. Lehrer S., Sanchez M., Song K. K., Dalton J., Levine E., Savoretti P., Thung S. and Schachter B.: Oestrogen receptor B-region polymorphism and spontaneous abortion in women with breast cancer. *Lancet* **335** (1990) 622–624.
17. Lehrer S., Schmutzler R., Rabin J., Chaparro C., Levine E., Thung S. N. and Schachter B.: A variant human estrogen receptor (ER) gene is associated with a history of spontaneous abortion in women with ER positive breast cancer, but not in women with ER negative breast cancer or women without breast cancer. The Endocrine Society, Washington DC (1991) p. 38 (Abstr).
18. Schmutzler R. K., Sanchez M., Lehrer S., Chaparro C. A., Phillips C., Rabin J. and Schachter B.: Incidence of an estrogen receptor polymorphism in breast cancer patients. *Breast Cancer Res. Treat.* **19** (1991) 113–120.
19. Murphy L. C. and Dotzlaw H.: Variant estrogen receptor mRNA species detected in human breast cancer biopsy samples. *Molec. Endocr.* **3** (1989) 687–693.
20. Dotzlaw H. and Murphy C.: Cloning and sequencing of a variant sized estrogen receptor (ER) mRNA detected in some human breast cancer biopsies. *13th A. San Antonio Breast Cancer Symp.* San Antonio, TX (1990). *Breast Cancer Res. Treat.* **16** (1990) 147 (Abstr.).
21. Dotzlaw H., Alkhalaf M. and Murphy L. C.: Multiple estrogen receptor (ER) like mRNAs in human breast cancer biopsies. The Endocrine Society, Washington DC (1991) p. 173 (Abstr.).
22. Scott G. K., Kushner P., Vigne J.-L. and Benz C. C.: Truncated forms of DNA-binding estrogen receptors in human breast cancer. *Clin. Res.* **38** (1991) 311 (Abstr.).
23. Zava D. T., Chamness G. C., Horwitz K. B. and McGuire W. L.: Human breast cancer: biologically active estrogen receptor in the absence of estrogen? *Science* **196** (1977) 663–664.
24. Horwitz K. B.: Is a functional estrogen receptor always required for progesterone receptor induction in breast cancer? *J. Steroid Biochem.* **15** (1981) 209–217.
25. Horwitz K. B., Mockus M. B. and Lessey B. A.: Variant T47D human breast cancer cells with high progesterone-receptor levels despite estrogen and antiestrogen resistance. *Cell* **28** (1982) 633–642.
26. Graham II M. L., Krett N. L., Miller L. A., Leslie K. K., Gordon D. F., Wood W. M., Wei L. L. and Horwitz K. B.: T47D $_{CO}$ cells, genetically unstable and containing estrogen receptor mutations, are a model for the progression of breast cancers to hormone resistance. *Cancer Res.* **50** (1990) 6208–6217.
27. Sluyser M.: Oncogenes homologous to steroid receptors? *Nature* **315** (1985) 546. (Letter to the Editor).
28. Fuqua S. A. W., Falette N. F. and McGuire W. L.: Sensitive detection of estrogen receptor RNA by polymerase chain reaction assay. *J. Natn. Cancer Inst.* **82** (1990) 858–861.
29. Kumar V., Green S., Stack G., Berry M., Jin J.-R. and Chambon P.: Functional domains of the human estrogen receptor. *Cell* **51** (1987) 941–951.
30. Kumar V. and Chambon P.: The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* **55** (1988) 145–156.
31. Cotton R. G. H., Rodrigues N. R. and Campbell R. D.: Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. *Proc. Natn. Acad. Sci. U.S.A.* **85** (1988) 4397–4401.
32. Orita M , Iwahana H., Kanazawa H., Hayashi K. and Sekiya T.: Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natn. Acad. Sci. U.S.A.* **86** (1989) 2766–2770
33. Fuqua S. A. W., Fitzgerald S. D., Chamness G. C., Tandon A. K., McDonnell D. P., Nawaz Z., O'Malley B. W. and McGuire W. L.: Variant human breast tumor estrogen receptor with constitutive transcriptional activity. *Cancer Res.* **51** (1991) 105–109.