Pergamon

0960-0760(94)E0054-O

J. Steroid Biochem. Molec. Biol. Vol. 49, No. 4–6, pp. 327–331, 1994

0960-0760/94 $7.00 + 0.00

Estrogen Receptors: New Perspectives in Breast Cancer Management

E. Petrangeli,[1]* C. Lubrano,[2] F. Ortolani,[3] L. Ravenna,[4] A. Vacca,[3] S. Sciacchitano,[3] L. Frati[3] and A. Gulino[4]

[1]Institute of Biomedical Technology, C.N.R., Rome, [2]V Clinica Medica, [3]Department of Experimental Medicine, University "La Sapienza", Rome and [4]Department of Experimental Medicine, University of L'Aquila, L'Aquila, Italy

The imbalance between proliferative and differentiative estrogenic effect, caused by quantitative and qualitative alteration of the estrogen receptor (ER) expression, may play a determinant role in mammary neoplastic transformation. Our studies demonstrate that ER levels are significantly higher in human mammary neoplastic tissues when compared to perineoplastic tissues and that increased ER expression is associated with ER gene hypomethylation. During progressive multifactorial carcinogenesis, ER overexpression may represent an early step in neoplastic transformation. In fact, high levels of ER represent good markers of differentiation and can predict the likelihood of benefiting from anti-estrogen therapy. Nevertheless, about 35% of ER-positive breast cancers are resistant to endocrine therapy and 10% of ER-negative tumors behave as hormone-sensitive tumors. Recent studies on ER mRNA variants, which naturally occur in human breast tumors, demonstrated mutations, deletions and alternative splicings, yielding deletions of exons 3, 4, 5 and 7. ER variants exhibited altered functions or changed the responsiveness to hormonal therapy. Analysis of these variants could be a useful parameter to better predict tumor responsiveness to anti-estrogen therapy. Recently, a regain of hormonal responsiveness by ER-negative breast cancer cells has been reported following ER gene transfection. However, estradiol treatment inhibits rather than stimulates cell growth as well as the metastatic and invasive potential of the ER gene transduced cells. Transfer of the ER gene may be considered as a new therapeutic approach in the management of hormone-independent breast cancer.

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Estrogens regulate mammary cell growth and differentiation through interaction with a specific receptor (ER) which binds to estrogen responsive DNA sequences and regulates the expression of proteins involved in mitogenic or differentiative processes. An appropriate ER expression is required to control the physiological estrogen responsiveness of target tissue. There is evidence that demonstrates that an abnormal ER expression often occurs in breast cancer, suggesting that an altered ER may play a determinant role in pathological proliferation, neoplastic transformation and progression.

Proceedings of the XVI Meeting of the International Study Group for Steroid Hormones, Vienna, Austria, 28 Nov.–1 Dec. 1993.
*Correspondence to E. Petrangeli.

ER OVEREXPRESSION IN BREAST CANCER

Few studies on non-malignant breast ER have been performed compared to the many reports done on breast cancer [1–5]. Peterson *et al.* [6] evaluated ER frequency and distribution using immunohistochemistry in 18 biopsy specimens removed from patients undergoing mammoplasty and found that stromal cells were ER-negative and only 7% of epithelial cells were positively stained, while 40–70% of ER stained cells were present in breast carcinoma [7, 8].

Fabris *et al.* [9] performed an immunocytochemical analysis of ER on 150 samples including proliferative and non-proliferative breast disease, atypical hyperplasia and non-invasive *in situ* carcinoma as well as on 150 cases of breast cancer. They found that normal

breast structures generally displayed a higher proportion of negative cells, with positive cells showing a degree of staining intensity lower than neoplastic cells. In benign breast disease, ER expression was heterogenous with low staining intensity. In contrast, in atypical hyperplasia and *in situ* carcinoma high ER expression was observed also displaying a homogenous distribution. In the latter case the enhanced and homogenous ER expression could modify the proliferative capacity leading to autonomous growth. Breast cancer exhibited high staining intensity in positive cells, although with an heterogenous staining pattern, probably due to cancer clonal heterogeneity. Ricketts *et al.* [10] investigated 143 fine needle aspirate and 40 biopsies from women with benign breast lump and measured their ER levels using enzyme immunoassay. ER levels ranged from 0 to 37 fmol/mg protein (mean: 4); these levels were significantly lower with respect to the values measured in 126 women with breast cancer (range: 0–139, mean: 37 fmol/mg protein). Following evaluation of ER and progesterone receptors (PR) by immunocytochemistry, they found that only 16% of the samples were ER-positive, setting a cutoff of 50% stained epithelial cells, based on response to endocrine therapy. They described significantly higher ER-positivity in the follicular stage than in the luteal stage of the cycle. PR staining was stronger than ER and 26% of the samples exhibited PR-positive cells. PR-positivity significantly correlated with ER status and showed a tendency to increase with body mass index. Patients with a family history of breast cancer showed significantly higher PR-positivity.

We studied steroid receptor expression and ER gene methylation in 37 neoplastic and in 35 perineoplastic tissues from patients with primary breast cancer. ER and PR were measured in the cytosol (c) and in nuclear extracts (n) by enzyme immunoassay, both in cancer and in non-malignant tissues taken from the same patients and compared with ER gene methylation. The cytosolic and nuclear localization of steroid receptors corresponded to a functional evaluation, nuclear steroid receptors meaning the activated form, dissociated from other protein complexes, able to bind to DNA hormone-responsive element. The mean values of cytosolic and nuclear steroid receptors were significantly higher in the neoplastic samples (Fig. 1). To assess steroid receptor positivity, we selected as threshold values, 15 fmol/mg cytosolic protein and 50 fmol/mg DNA for cytosolic and nuclear steroid receptors, respectively, which corresponded to the objective responses to endocrine therapy. However, this therapy-based criterion was not applied to non-neoplastic breast tissues. Because of the large incidence of very low levels of ERc and PRc in perineoplastic tissues, we selected 5 fmol/mg cytosolic protein as a cut-off, corresponding approximately to median values for these parameters. Although there was a significant linear positive correlation between cytosolic and nuclear steroid receptor levels ($P = 0.0001$ for ER and $P = 0.0001$ for PR), we found discordance between ERc and ERn incidence in 26% of perineoplastic and in 8% of neoplastic tissues. The discordance between PRc and PRn incidence was 31% in perineoplastic and 19% in neoplastic tissues. The lower discordance in neoplastic tissues was due to the frequent steroid receptor overexpression which occurred in the latter samples. For clinical evaluation, we considered as positive all samples which displayed at least one of either cytosolic or nuclear steroid receptors. The overall steroid receptor incidence in neoplastic and perineoplastic breast tissues is shown in Table 1. Only the incidence of ER was lower in perineoplastic tissues, and the phenotype ER^-PR^+, usually not found in this group of breast cancer, occurred in 14.3% of perineoplastic tissue (Table 2). Thus, in the typical hormone-dependent non-malignant breast tissue, we found lower ER and PR levels and ER incidence, while PR

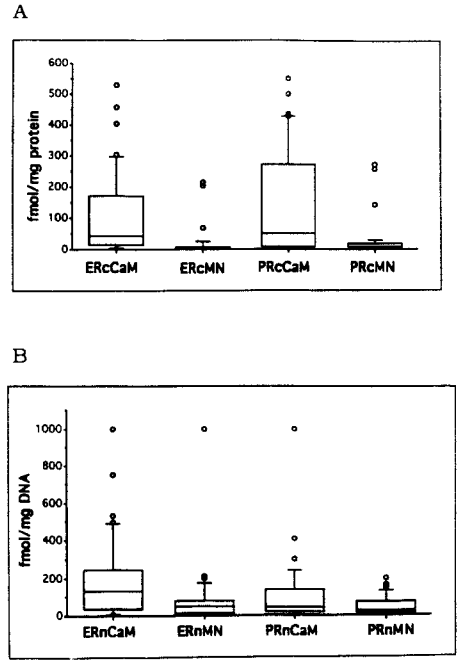

Fig. 1. Box plots for cytosolic (A) and nuclear (B) steroid receptors in neoplastic (CaM) and perineoplastic (MN) tissues. Boxes comprise levels from 25th to 75th percentile. Horizontal bars indicate the median value. The *t*-test showed significantly higher values of ERc ($P = 0.0003$), PRc ($P = 0.0001$), ERn ($P = 0.0063$), and PRn ($P = 0.0231$) in neoplastic tissues.

Table 1. Comparison of steroid receptors status in neoplastic and perineoplastic tissues

	Cancer	Perineoplastic tissues
ER^+	28/37 (75.68%)	20/35 (57.14%)
PR^+	24/37 (64.86%)	24/35 (68.57%)

Table 2. Incidence of paradoxal ER/PR phenotype in neoplastic and perineoplastic tissues

Receptor phenotype	Cancer	Perineoplastic tissues
ER^+PR^-	4/37 (10.8%)	1/35 (2.9%)
ER^-PR^+	0/37 (0%)	5/35 (14.3%)

incidence was comparable to that of malignant tissue. This suggests that lower ER levels are sufficient to promote PR expression in non-malignant tissue. We evaluated ER gene methylation patterns in neoplastic and non-malignant samples using Southern blot analysis. The differences between MspI and HpaII enzymatic restriction patterns led to an overall estimation of ER gene methylation pattern. The ER gene was hypomethylated more frequently in the neoplastic, 70%, as compared to 25% in perineoplastic tissues. The methylation status in both was inversely correlated to ERc and ERn levels as well as to PRc and PRn (Fig. 2), confirming that ER expression up-regulates PR levels.

In conclusion, we found a predominant phenotype in non-malignant breast tissue characterized by a hypermethylated ER gene with low or undetectable steroid receptor expression. Cell proliferation of less aggressive hormone-dependent breast cancer appears to be linked to ER gene hypomethylation and steroid receptors overexpression, with a good likelihood of benefit from tamoxifen therapy. During neoplastic progression, more aggressive malignant phenotype is characterized by negative steroid receptors and hormone-resistance.

Considering the low steroid receptor levels in normal breast tissue, it appears that high ER levels are linked to neoplastic breast tissues. In this regard, there are many key issues to be considered. First, can we consider non-malignant breast tissues with ER gene hypomethylation and ER overexpression as high risk samples? And secondly, what is the role of ER overexpression in breast neoplastic transformation? Is the ER overexpression an epiphenomenon of neoplastic loss of cell growth control, with dysfunction of the mechanisms that regulate ER expression? Or, can overexpressed ER be considered as an activated oncogene or co-oncogene? Finally, is it possible to control pathological proliferation by restoring physiological regulation of ER?

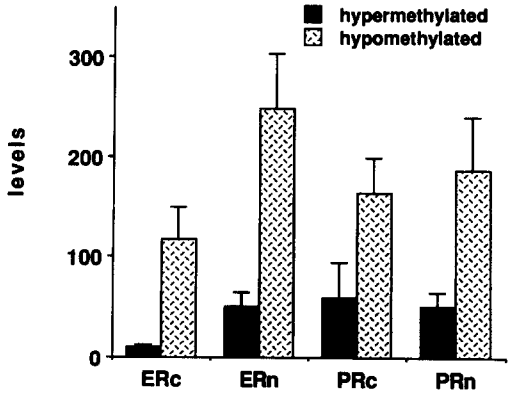

Fig. 2. Comparison of steroid receptor levels (mean values + SE) on ER gene hypermethylated and hypomethylated subgroups. Cytosolic and nuclear steroid receptors are expressed respectively as fmol/mg protein and fmol/mg DNA. ER gene hypomethylated samples expressed significantly higher levels of ERc ($P = 0.001$), ERn ($P = 0.0007$), PRc ($P = 0.0224$), and PRn ($P = 0.0111$).

ER VARIANTS IN BREAST CANCER

Following the cloning of ER cDNA [11, 12], several studies have been performed to screen for the presence of structural abnormalities of ER in breast cancer.

Several authors [13, 14] have reported restriction fragment length polymorphisms (RFLP) within the ER gene, describing PvuII RFLP, with controversial data regarding the correlation with ER and PR expression. Subsequent studies [15] demonstrated that the sequences responsible for PvuII RFLP are located within intron 1 of the ER gene. Wanless *et al.* [16] reported an HindIII RFLP that appeared to be correlated with increased PR expression.

We analyzed the restriction pattern of the ER gene in 50 samples of neoplastic and perineoplastic breast tissues. Twenty micrograms of extracted DNA were digested with restriction enzymes, separated on 0.8% agarose gels, transferred to Gene Screen Plus filters and hybridized with an ER probe. An 1.3 kb EcoRI fragment of the plasmid pOR3 [11], kindly provided by Dr P. Chambon, was used as a specific probe. We observed the appearance of an extra 8.6-kb band and a parallel decrease of the hybridization intensity of the 4.8-kb band in 60% of both neoplastic and perineoplastic tumors (Fig. 3). This restriction variant pattern was also observed in 5 out of 23 meningiomas, but not in the other brain tumors, or in peripheral blood lymphocytes obtained from healthy volunteers [17]. Thus, it does not seem to be due to physiologically occurring EcoRI RFLP. Hybridization with several ER cDNA fragments, spanning the different ER domains, showed that the altered restriction pattern affected the genomic region coding for the DNA binding domain [17]. Since the appearance of the extra 8.6-kb variant band was associated with a decrease in the hybridization intensity of the 4.8-kb invariant band, the altered genomic restriction pattern could arise from the involvement of only one allele or from cellular heterogeneity. The distribution of the ER gene EcoRI variant appeared to be frequently associated with slightly higher steroid receptor levels (Fig. 4).

The functional meaning of ER gene RFLPs remains unclear. In some cases they could be related to intronic sequences regulating ER expression.

Recent studies, concerning naturally occurring ERmRNA variants, demonstrated the presence of

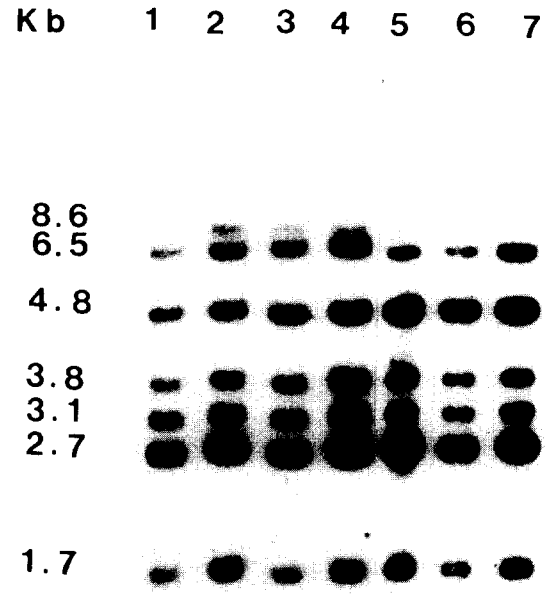

Fig. 3. Southern blot analysis of genomic DNA extracted from perineoplastic breast tissues (lanes 1, 3 and 5), primary breast cancers (lanes 2, 4 and 6) and peripheral blood lymphocytes of healthy volunteers (lane 7). 10 μg of DNA were digested with EcoRI, electrophoresed on a 0.8% agarose gel, and transferred to nitrocellulose filters. The blots were hybridized with a ^{32}P-labeled 1.3 kb EcoRI fragment of the plasmid pO3. The size of the EcoRI bands is indicated in kilobases.

mutations, deletions and alternative splicing, yielding deletions of exon 3, 4, 5 and 7 (reviewed in [18]) in breast tumors. ER variants can exhibit altered functions or change the responsiveness to hormonal therapy. Jiang *et al.* [19] found that a point mutation leading to the substitution of Val for Gly at codon 400 in the ligand binding domain of ER, resulted in the enhanced estrogenic activity of 4-hydroxytamoxifen in

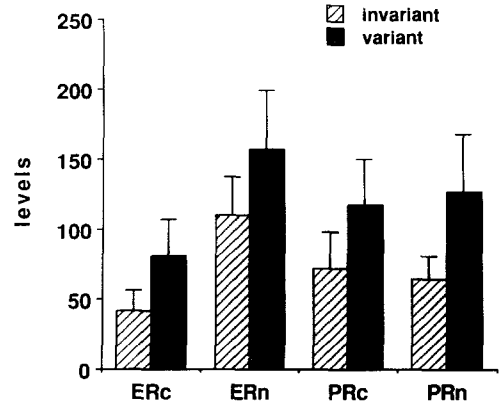

Fig. 4. Comparison of steroid receptor levels (mean values + SE) in subgroups with invariant and variant EcoRI genomic patterns of ER. Cytosolic and nuclear steroid receptors are expressed respectively as fmol/mg protein and fmol/mg DNA. The *t*-test did not show a significant difference of ERc ($P = 0.0897$), ERn ($P = 0.1714$), PRc ($P = 0.1381$) and PRn levels ($P = 0.0839$) in the two subgroups.

stable ER transfectants of the breast cancer cell line. An ER mRNA variant, lacking exon 5, was able to activate constitutively the estrogen-dependent transcription [20]. In contrast, the exon 7 deleted ER variant, abundant in some ER$^+$PR$^-$ breast tumors [21] and the exon 3 deleted ER, expressed in the T47D cell line [22], acted as dominant negative regulators of wild type ER. The expression of the exon 4 deleted ER mRNA variant in MCF7 and ZR75-1 has been described recently [23], although its function was not investigated. Scott *et al.* [24] showed that in 67% of ER-positive tumors expressing a 50 kDa ER variant, the receptor either did not bind or bound weakly to estrogen-responsive element sequences. The identification of ER variants could be a useful parameter to identify some form of tumor responsiveness to anti-estrogen therapy. In fact, approx. 35% of ER-positive breast cancers are resistant to endocrine therapy and 10% of ER-negative tumors behave as hormone-sensitive tumors [25, 26]. The presence of dysfunctional ER could explain unexpected responses to endocrine therapy. Puntiform mutations may interfere with anti-estrogen action. Tumors expressing deleted constitutively activated ER can activate estrogen-responsive gene expression, although expressing low ER levels. The presence of high levels of negative dominant ER may have a role in the hormone-dependence escaping, determining the occurrence of different mechanisms to induce cell growth.

FUTURE PERSPECTIVES FOR GENE THERAPY

Yiang *et al.* [27] and Garcia *et al.* [28] showed that ER-negative breast cells transfected with ER cDNA regain hormonal sensitivity. However, the responsiveness to hormonal treatment was different from ER-positive breast cancer. In fact, it has been reported that the administration of pure anti-estrogen alone did not induce any reduction of cell growth and invasive potential of the cell, while such effects were determined by 17-β-estradiol treatment. This paradoxic effect of 17-β-estradiol was blocked by simultaneous administration of anti-estrogens. These data appear to contradict previous observations suggesting a possible oncogenic role of ER overexpression and may be related to a different role of ER in different stages of malignant progression. Increased expression of ER may have a pathogenetic role in the early phase of neoplastic transformation, while the absence of detectable levels of ER in the more advanced stages of neoplastic progression may be considered as an epiphenomenon related to tumor de-differentiation. The reactivation or transfer of the ER gene may be considered as a new therapeutic approach to limit growth and invasiveness in the more aggressive ER-negative tumors. Other experiments are required to define the final goal of hormonal gene therapy in breast cancer. It would be particularly beneficial to restore, not only the

presence of ER, but also the physiological control of the expression and the activity of the ER gene.

Acknowledgements—We thank Stacie Punturieri for English revision. pOR3 was kindly provided by P. Chambon. This work is supported by a grant from Progetto Finalizzato ACRO of CNR.

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