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Review Article

Alterations and Polymorphisms of the Estrogen Receptor Gene in Breast Cancer

HIROTAKA IWASE AND SHUNZO KOBAYASHI

The existence of hormone-independent tumors is a substantial problem for the present endocrine treatment of breast cancers. Recently, numerous variant estrogen receptors (ERs) at the mRNA level have been detected with base pair insertions, transitions, and deletions, as well as alternative splicing, yielding deletion of exon 3, 5, or 7. It has been shown that the loss of hormone dependence in breast tumors is partly due to the presence of mutated or truncated ERs that can activate the transcription of an estrogen-regulatable gene in the absence of estrogen. The mechanism of the loss of hormone dependency is, however, still very complex. Thus, further work assessing the correlation between clinical behavior and ER variants is required to determine whether these variants play a role in hormone-resistant disease. Additionally, a possible linkage to the ER gene has been found in some breast cancer families, suggesting that either the ER gene itself or an adjacent gene may be breast cancer susceptibility genes.

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Key words: Estrogen receptor gene, Mutation, Polymorphism, Hormone resistance, Breast cancer susceptibility

Human breast cancer is a typical hormone dependent tumor, and various endocrine treatments have been employed in advanced or recurrent cases. These treatments have also been performed as a part of postoperative adjuvant therapy. In the 1960's, Jensen and Jacobson[1] reported the accumulation of tritiated estrogen in the rat uterus. Their findings that the binding of estradiol to the cytoplasmic receptor was the initial and necessary step for hormone accumulation in the nucleus, led to the "two step mechanism" theory. The discovery of estrogen-binding protein, estrogen receptor (ER), provided a better understanding of hormonal influence on the development and clinical behavior of breast cancer. The measurement of ER in cancer tissues is now an important procedure used to discriminate between the hormone dependent and independent tumors in order to determine whether endocrine treatments should be administered[2].

The ER is a 66 kDa nuclear protein and a member of the steroid hormone receptor superfamily[3]. The ER has six conserved domains: A/B domain, an amino-terminal transcriptional activation domain; C domain, a central DNA-binding domain that contains two zinc-binding fingers; D domain, a hinge region; E domain, a hormone-binding domain required for stable dimerization of the receptor; and F region, a domain whose function is still unknown at present (Fig 1). It also contains sequences for dimerization in association with heat-shock proteins, and a nuclear localization signal[4-6]. In the presence of hormone, ligand binding causes receptor dissociation from an inactive complex containing heat-shock proteins (HSP90)[6]; this dissociation allows subsequent tight nuclear binding to the estrogen-responsive element (ERE) and gene activation. In the estrogen responsive MCF-7 breast cancer cell line, expression and secretion of TGF-α, insulin-like growth factor-I, platelet-derived growth

Department of Surgery II, Nagoya City University Medical School. Reprint requests to Hirotaka Iwase, Department of Surgery II, Nagoya City University Medical School, Kawasumi 1, Mizuho-ku, Nagoya 467, Japan.

Abbreviations:
ER, Estrogen receptor; PgR, Progesterone receptor; kb, Kilobase; kDa, Kilodalton; SSCP, Single strand conformation polymorphism; PCR, Polymerase chain reaction; RT, Reverse transcription

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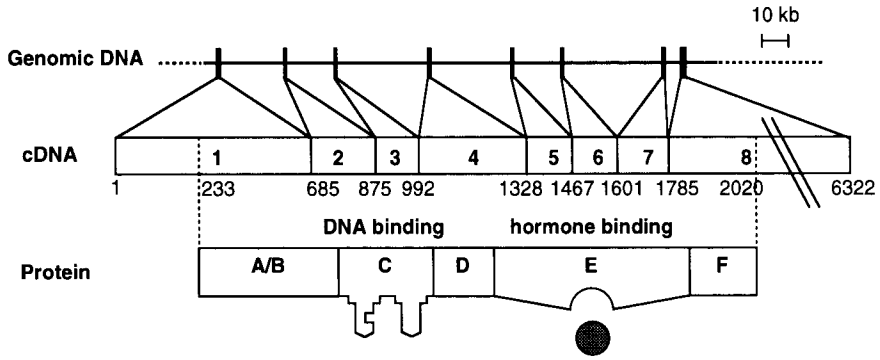

Fig 1. Structure of ER protein, cDNA and genomic DNA. The ER protein has conserved domains: A/B domain, an amino-terminal transcriptional activation domain; C domain, a central DNA-binding domain that contains two zinc-binding fingers; D domain, a hinge region; E domain, a hormone-binding domain required for stable dimerization of the receptor; and F region, a domain of unknown function at present. Its total size including introns is over 140 kb and consists of 8 exons and its cDNA defines a sequence of 6322 nucleotides and includes a 1788 nucleotide coding region which is flanked by untranslated sequences of 232 nucleotides and 4303 nucleotides at its 5′ and 3′ ends, respectively.

factor, TGF-β, and cathepsin D protease are stimulated by estrogen, leading to the hypothesis that TGF-α has autocrine function mediating estrogen-induced cell proliferation[4,5].

The ER gene was cloned and sequenced in 1986 by Chambon's group[7]. It is located on chromosome 6q25.1[8]. Its total size including introns is over 140 kb and consists of 8 exons, and its cDNA defines a sequence of 6322 nucleotides and includes a 1788 nucleotide coding region which is flanked by untranslated sequences of 232 nucleotides and 4303 nucleotides at its 5′ and 3′ ends, respectively[9,10] (Fig 1).

It is well known that the proliferation of tumor cells depends on estrogen in the early stages of human breast cancer. Subsequently, the cancer cells may acquire new proliferative pathways as a result of multiple genetic alterations. This then enables some tumor cells to bypass estrogen dependent proliferation[11]. However, the mechanisms underlying loss of estrogen responsiveness in breast cancers are not well understood. Sluyser hypothesized that the loss of hormone dependence in breast tumors may be partly due to the presence of mutated or truncated steroid receptors that activate transcription even in the absence of hormone[12]. The importance of the ER in breast cancer is underscored by Zuppan's[13] findings of a possible linkage to the ER gene in late onset breast cancer families using three RFLP markers. Moreover, Andersen[14] observed that ER alleles having *Xba* I restriction site were significantly more frequent in patients with breast cancer than in normal controls. These reports suggest that either the ER gene itself or an adjacent gene may be one of breast cancer susceptibility genes.

In this review, the association of ER gene alterations with loss of hormone dependence as well as the relationship between ER gene polymorphisms and breast cancer susceptibility are discussed.

Estrogen Receptor Gene Alterations in Human Breast Cancer

1) At the DNA Level: Kou *et al*[15] found no evidence for amplification or alterations of the ER gene at the DNA level in 34 breast cancer patients by Southern hybridization analysis. Additionally, our group[16] and Watts *et al*[17] reported similar results following Southern blot analysis of the ER gene in other cohorts of breast cancer patients (Fig 2, a). In contrast, Nembrot *et al*[18] have demonstrated that, in some breast cancer patients (6 of 14), there was a 1.6- to 3-fold amplification of the ER gene.

Roodi *et al*[19] searched for mutations in 188 breast cancer patients by single strand conformation polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis, and DNA sequencing. They reported that in the majority of primary breast cancers, the ER-negative phenotype was due to deficient ER expression at the transcriptional or post-transcriptional level, and was

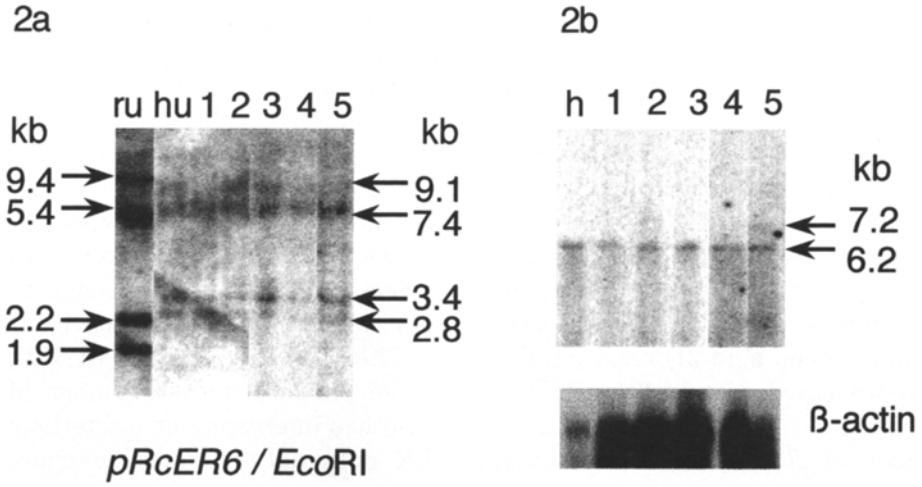

Fig 2. a, Southern blot analysis of the samples from rat uterus (lane 1), human uterus (lane 2) and breast cancer tissues (lane 3-4) using rat ER cDNA, pRcER6/*Eco*RI (probe/enzyme). Four bands (9.1, 7.4, 3.4 and 2.8 kb) in the human samples and the identical bands (9.4, 5.4, 2.2 and 1.9 kb) in rat sample were seen. Neither rearrangement nor amplification were seen. b, Northern blot analysis of RNAs from rat uterus (lane 1) and breast cancer tissues (lane 2-6). Major bands of the 6.2 kb were seen and a larger 7.2 kb band was seen in case 5. The 7.2 kb sized band might be caused by alternative splicing.

not the result of mutations in the coding region of the ER gene. In our studies[20], there were neither germline nor somatic mutations in the ER gene in ER-negative and PgR-positive breast cancers as assessed by SSCP analysis and DNA sequencing. Furthermore, we[21] did not find a role for loss of heterozygosity (LOH) of the ER gene in the lack of ER function in breast cancer tissues (Fig 3). Mutation of one allele and loss or replacement of a chromosomal segment containing the other allele was not accompanied by changes in ER expression. Thus, ER alterations did not appear to occur at the DNA level but as is discussed below, may be detectable at the messenger RNA (mRNA) level.

2) At the mRNA Level: At the mRNA level, various variant ER messages have been detected, and there are many reviews concerning the relationship between variant ERs and loss of hormone dependence[12,22-24].

Several groups have found a good correlation between levels of ER mRNA analyzed by northern blotting and protein with estrogen binding[25-27]. However, May *et al*[28] studied the ratio of ER protein to mRNA and found that a high ratio correlated with an increased risk of relapse. Additionally, Rennie *et al*[29] have reported that although 64% of in ER-positive tumors had the normal 6.5 kb ER mRNA, 9% had additional smaller ER mRNA, 27% had variant forms, and 25% had no ER mRNA signal. In our studies[16], ER expression did not correlate well with ER mRNA levels by northern blot analysis using the rat ER-cDNA[30] as a probe. Only one of 45 breast cancer specimens expressed an aberrant mRNA which may be caused by a abnormal splicing (Fig 2, b).

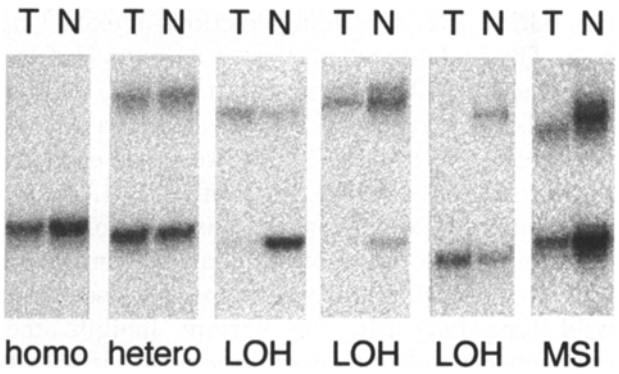

Fig 3. Examples of loss of heterozygosity (LOH) using microsatellite marker (*ESR*). Lane 1 shows constitutional homozygosity, lane 2 shows normal diploid genotype, lane 3-5 show LOH and lane 6 shows microsatellite instability. T and N indicate, respectively, tumor and lymphocyte genomic DNA from the same patient.

Murphy and Dotzlaw[31] found a number of smaller size ER mRNA variants in breast tumors, which resulted from deletions of the hormone

binding domain. They prepared a cDNA library from one of these breast cancer biopsies and found that 84 unique amino acids introduced at the exon 3 intron boundary (amino acid 253) were the long interspersed repetitive LINE-1 sequences. These sequences were followed by a stop codon resulting in a truncated 37-kDa protein detected in only one of 61 breast tumors. More recently, they reported an ER variant with an insertion of 6 unique amino acids at the exon 2 intron boundary (amino acid 21) resulting in a 220 amino acid truncated protein (24-kDa), expressed more commonly than 37-kDa protein[32]. Additionally, Scott *et al*[33] found that decreased ERE binding in some tumors was associated with a 50-kDa variant ER dimer or a 50/97-kDa heterodimer containing wild type and variant ER components.

McGuire and Fuqua *et al* used the screening techniques of chemical mismatch cleavage and single stranded conformational polymorphism (SSCP) and have found base pair insertions, transitions and deletions as well as alternative splicing in ER mRNAs, yielding deletions of exon 3, 5, or 7. The biologic properties of an exon-3 deletion mutant (Δ3ER) generated from T47D cells have also been reported[34]. This variant has an in-frame deletion of exon 3, the exon that encodes the second zinc-binding finger of the DNA-binding domain. Expression of this variant in a reporter system does not stimulate an estrogen-regulated reporter; however, if co-expressed with wild type (wt) ER, the variant inhibits the estrogen-dependent transcriptional activity of wt ER[35].

In a transient transfection system in yeast, the variant ER that lacks exon 5 (Δ5ER), which encodes part of the hormone binding domain, is constitutively active and promotes transcription of an estrogen-responsive reporter construct in the absence of hormone. Transfection of this variant receptor into MCF-7 cells, an ER-positive, estrogen-responsive breast cancer cell line, stimulates colony formation and progesterone receptor (PgR) levels in a hormone independent manner[36]. The Δ5ER encodes a dominant-positive receptor, if both wild-type and variant ER mRNAs are expressed. Overexpression of this variant can result in an estrogen-independent phenotype[37]. However, Daffada *et al*[38] assessed the level of Δ5ER mRNA relative to wild type ER mRNA (%Δ5/wt) in 70 tamoxifen-resistant and 50 primary breast carcinomas using reverse transcription/polymerase chain reaction (RT-PCR), and concluded that Δ5ER mRNA is unlikely to be responsible for tamoxifen resistance in most breast cancers. Additionally, Zhang *et al*[39] demonstrated that the Δ5ER is not specific to ER-negative tumors, but was also found in 19 of 20 ER-positive tumors often in excess of wt ER levels. These two studies suggest that Δ5ER does not contribute to ER negativity in tumors.

The exon-7 deleted ER (Δ7ER), which lacks a large portion of the hormone-binding domain, also has interesting characteristics. This mutant ER does not activate the transcription of an estrogen-regulatable gene; however, when co-expressed with wt ER, it suppresses the transcriptional activity of normal ER. Thus, expression of the Δ7ER with wt ER in breast cancer cells would result in an ER-positive but being resistant to hormonal therapy.

There are some other reports concerning variant ER mRNAs. Karnik *et al*[40] found a 42-bp replacement in exon 6 as well as a single base pair deletion in exon 6 in two tamoxifen-resistant metastatic tumors but not in the primary tumor. The remaining 18 of 20 tamoxifen-resistant tumors did not contain mutations in any of the 8 exons of the ER cDNA. Karnik *et al* have suggested that mutations in the ER occur at a low frequency and do not account for most estrogen-independent, tamoxifen-resistant breast tumors. Hill *et al* showed that the ER-negative human breast cancer cell line BT-20 expresses an ER with an abnormally low molecular weight, 40 kDa. These findings collectively support the hypothesis that variant ERs exist *in vivo* and can affect the hormone responsiveness of breast tumors[41]. Table 1 shows the characteristic of the loss of hormone dependence and variant ERs.

3) Variant ERs in Normal Mammary Tissue and Other Tumors: Leygue *et al* analyzed ER mRNAs in normal human mammary tissue and reported that several ER variant mRNAs are present in normal human breast tissue, but that the level of expression of some of these variants may be lower in normal tissue than in tumor tissue[42]. These results suggest that the mechanisms generating ER variant mRNAs exist in normal breast tissue and may be deregulated in breast cancer tissues. Although ER is expressed in diverse human tissues and tumors, such as meningiomas, lung cancers and thyroid cancers[43], the role of

Table 1. mRNA Variants of the Estrogen Receptor Gene and Their Function

	Function	Protein	RNA
Murphy (1989)[31]	Negative	37 kDa ER	84 amino acids insertion of exon 3 intron boundary
Dotzlaw (1992)[32]	Negative	ER truncated at 220 (24 kDa ER)	6 amino acids insertion of exon 2 intron boundary
Scott (1991)[33]	Decreased ERE binding	50 kDa ER	–
Wang (1991)[34] Fuqua (1992)[35]	Dominant negative	61.2 kDa ER	Exon 3 deletion
Fuqua (1993)[36]	Dominant positive	ER truncated at 371	Exon 5 deletion
Castle (1995)[37]	Dominant negative	ER truncated at 466	Exon 7 deletion

ERE, Estorgen responsive element.

hormone dependence in these tissues has not been clarified. Villa *et al* have reported that 14 patients (7 males and 7 females) with hepatocellular carcinoma (HCC) expressed normal and variant ER transcripts in cirrhotic tissue but only variant transcripts in the tumor[44]. They concluded that variant ER transcripts are translated into truncated receptors which constitutively activate transcription, thus favoring deregulated proliferation in the liver. Moreover, Koehorst *et al*[45] have reported that two types of variant ERs (Δ7ER and Δ4ER) were found in meningiomas, which expressed high levels of PgR and low levels of ER. Hirata *et al*[46] reported that both Δ7ER and Δ5ER variants are expressed in normal uterine endometrium as well as in endometrial cancers. Thus, ER variants may also play physiologic and/or pathologic roles in other tissues and tumors.

4) Other Factors Influencing ER Function: DNA methylation is known to be involved in eukaryotic gene control, and can effect development and tumorigenesis. The ER gene was found to be methylated in placental tissues, but normal breast tissues exhibit a different methylation pattern, as assessed by *Hpa* II and *Msp* I restriction enzyme digests[47]. In addition, specific sites in the hormone-binding domain of the ER gene were observed to be differently methylated in different human breast tumor specimens. Thus, DNA methylation may be an additional molecular measure of the genetic heterogeneity in breast cancer[47]. However, Watts *et al*[17] have reported that although the methylation of the ER gene varied between tumors, but the degree of methylation did not correlate with levels of receptor protein expression.

Other proteins can also influence ER function. Doucas *et al*[48] have demonstrated that overexpression of c-*fos* or c-*jun* in MCF-7 cells suppresses estrogen-induced transcription of an estrogen-responsive reporter gene. Feavers *et al*[49] reported two nonhistone proteins (NHP-1 and NHP-2), which bind an ERE with high affinity, enhance the binding of the estrogen-ER complex to an ERE. Similar factors, which stimulate binding of receptors to their cognate DNA response elements, have also been shown for PgR[50] and thyroid hormone receptors[51]. In addition, Shiba *et al*[52] reported that calpain, a calcium-activated neural protease and a thiol protease regulated by Ca^{2+}, which was involved in mammary malignant transformation, affected ER function in breast cancer tissues.

Estrogen Receptor Gene and Breast Cancer Susceptibility

1) ER Gene and Familial Breast Cancer: Some tumors are known to be inherited in specific families. Genetic analysis of these families has led to the mapping of genes implicated in retinoblastoma, Wilms tumor, multiple endocrine neoplasia (MEN), Li-Fraumeni syndrome and some types of colon cancer[53]. The incidence and mortality rate of breast cancer is recently increasing in Japan and Western countries, and the life-risk of breast cancer is thought to be 1 in 10 in Western countries and 1 in 50 in Japan[54]. Although hereditary breast cancer accounts for 5% to 10% of all breast cancer patients in Western countries[55], the percentage of those patients in Japan is not clear.

Recently, linkage analyses of early-onset familial breast and ovarian cancer have focussed

on one of the breast cancer susceptibility candidate genes located on chromosome 17q12-21, *BRCA1*[56]. In October 1994, Miki *et al*[57] identified a strong candidate for the *BRCA1* gene alteration by positional cloning methods. Probable predisposing mutations were detected in five of eight kindreds thought to segregate *BRCA1* susceptibility alleles. The mutations include an 11-bp deletion, a 1-bp insertion, a stop codon, a missense substitution, and an inferred regulatory mutation. The *BRCA1* protein contains a zinc finger domain in its amino-terminal region, but is otherwise unrelated to any previously described proteins, and its function is still not understood, especially in sporadic breast cancer. Wooster *et al*[58] performed a genomic linkage search in 15 high-risk breast cancer families that were unlinked to the *BRCA1* locus on 17q21. This analysis uncovered a second breast cancer susceptibility locus, *BRCA2*, located in a 6-cM interval (between *D13S289* and *D13S267*) on chromosome 13q12-q13. They also reported identification of a gene from this region in which they detected 6 different germline mutations in breast cancer families. The role of both *BRCA1* and *BRCA2* in the carcinogenesis and dissemination of sporadic breast cancer need to be clarified.

In 1991, Zuppan[13] found a possible linkage (1.85 Lod score) to the ER gene in one extended family with eight patients with late onset breast cancer using three RFLP markers, *Xba* I, *Sac* I, and *Hin*dIII, and proposed that the ER gene may also be a breast cancer susceptibility candidate gene. Additionally, we demonstrated a possible linkage to the ER gene in four Japanese breast cancer families, using a microsatellite marker (*ESR*) of the ER gene and four markers (*D17S250, D17S846, D17S855, D17S579*) in the *BRCA1* region[59]. In two of the four families, the affected women shared an allele in the ER gene, but did not share *BRCA1* allele types.

These results suggested that a subgroup of familial breast cancer patients have inherited mutations in the ER gene. However, mutations in the ER gene have been proposed to be lethal because ER protein is essential for endocrine homeostasis. Recently, though Smith *et al*[60] reported that a 28 year-old man with a history of continued linear growth into adulthood had a mutated ER gene at the germline level, with a cytosine-to-thymine transition at codon 157 of both alleles, resulting in a premature stop codon. They concluded that disruption of the ER in humans need not be lethal. Furthermore, Wooster *et al*[61] have reported a germline mutation in the androgen receptor gene in a rare male breast cancer family. Thus, there is a possibility that some germline mutations of the ER gene which result in breast cancer susceptibility have not yet been detected.

2) Association of ER Gene Polymorphisms with ER Function and Breast Cancer Susceptibility: Although mutations in the ER gene on the germline level have been thought to be quite rare, there are many reports with respect to polymorphism of the ER gene and their associations with ER function and breast cancer susceptibility.

Hill *et al*[41] found that a *Pvu* II RFLP in the ER gene is correlated with ER expression in 188 breast cancer patients. Wanless *et al*[62] have described a *Hin*dIII RFLP in the ER gene in a small percentage of breast cancer patients, which also correlated with PgR expression. However, Yaich *et al* reported that the *Pvu* II RFLP was located within intron 1, 0.4 kb upstream of exon 2, and did not correlate with either age or ER expression in 257 breast cancer patients[63].

Garcia *et al*[64] used an RNase protection assay and found a nucleotide mismatch in the B-coding region that correlated with low ligand binding activity in 8 of 66 ER-positive tumors. They subsequently found that the mismatch correlated to a C to T transition at nucleotide 257, resulting in an alanine to valine substitution which removes a *Bbv* I restriction site. Lehrer *et al*[65] 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 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[66]. Berkowitz *et al*[67] concluded that this polymorphism appears to be a marker for breast cancer risk only among the subgroups who have had a history of repeated abortions.

Andersen[14] reported that the ER gene or a gene closely linked to it is involved in the development of at least a subset of breast carcinomas. He observed that ER alleles having *Xba* I restriction site were significantly more frequent in breast cancer patients than in non-cancer controls and alleles with the *Pvu* II restriction site were more frequent in patients with PgR-negative primary tumors than in patients with PgR-positive pri-

mary tumors. Lehrer *et al*[68] also reported that breast cancer patients with a familial history had lower dissociations of the ER and PgR in tumor tissues than those patients without a familial history. These reports further suggested that some ER haplotypes may be related to breast cancer susceptibility.

In 1995, Roodi *et al*[19] identified 6 polymorphic sites in the ER gene and determined the allele frequencies of each haplotype in 188 breast cancer cases. One polymorphism in codon 325 was strongly associated with a family history of breast cancer. Additionally, we independently found the same sequence polymorphism in codon 325 by screening genomic DNA mutations in ER-negative/PgR-positive breast cancers[20]. Interestingly, in our series, the sequence variant in codon 325 was observed more frequently in breast cancer patients than in noncancer control cases[69] (Table 2). Since codon 325 is located in the hormone binding domain, this polymorphic site which appears to correlate with breast cancer susceptibility may affect ER function. Alternatively, this polymorphism may be in linkage disequilibrium with a coding or undetectable regulatory mutation. Further investigations need to be performed to assess the relationships between polymorphisms of the ER gene and breast cancer susceptibility. Table 3 shows characteristics of the polymorphisms of the ER gene.

Conclusions

In ER-positive and hormone-independent tumors, mutant ERs may coexist with wt ER. Additionally, the mutant receptor may be constitutively active and no longer responsive to hormone. In ER-negative and hormone-independent tumors, a complete loss of ER as well as the constitutive activation by a mutant ER, which might not be detected by protein analysis, may occur. Further work is necessary to establish the association between clinical behavior and molecular changes in the ER to determine whether these variants actually play a role in hormonally-unresponsive breast cancers.

If wt ER were transfected into breast cancer

Table 2. Estrogen and Progesterone Receptor Phenotyes and the Allele Frequency in Codon 325 of the Estrogen Receptor Gene (n=174)

		ER (−)		ER (+)		Total	Control
		PgR (−)	PgR (+)	PgR (−)	PgR (+)		
Allele	wt/wt	22	12	19	52	105	22
	wt/vt	19	9	14	18	60	8
	vt/vt	4	2	2	1	9	0
frequency of variant		0.300	0.283	0.257	0.141	0.279[a]	0.133[a]
95% confidence limit		0.18-0.47	0.14-0.46	0.16-0.46	0.15-0.39	0.21-0.36	0.07-0.24

[a] $p=0.057$ (chi-squared test).
wt, Wild type; vt, Variant type.

Table 3. Characteristics of Polymorphisms of the Estrogen Receptor Gene

	Variation type	Probe/Enzyme pairs	Max hetero	Characteristics
Hill (1989)	PT	POR8/*Puv* II	−	correlate ER expression[41]
Zuppan (1989) Andersen (1994)	RS	M72/*Xba* I	0.449	− breast cancer susceptibility[14]
Garcia (1989) Lehrer (1990)	PT	PCR product/*Bbv* I	0.177	low ligand binding correlate spontaneous abortion[66]
Senno (1992) Iwase (1995)	$(TA)_n$ repeat	PCR	0.917	− LOH analysis correlate with age and histological grade[21]
Roodi (1995) Iwase (1996)	PT	PCR product/*Hinf* I	0.14	correlation with family history[19] breast cancer susceptibility[20]

PT, Point variation, RS, Restriction site; Max hetero, Maximum heterozygosity index.

cells with ER negative or into cells dominantly occupied by a variant ER, the tumor might become hormonally responsive. Sluyser[70] found that cells transfected with wt ER are growth-inhibited by estradiol. This inhibition was observed both in fibroblasts that normally did not express ER and in breast cancer cells which had become hormone-independent. He emphasized that these tumor cells underwent hormone-induced apoptosis, resembling the glucocorticoid-induced apoptosis of leukemic cells. New strategies for treating hormone-independent breast cancer may develop from these findings in the future.

Additionally, the relationship between some variants of the ER gene and breast cancer susceptibility may exist in a selective high risk group of breast cancer patients as well as normal women. ER variant may also result in a general tumorigenesis in familial breast cancer patients.

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