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Single nucleotide polymorphisms (SNPs) in the estrogen receptor gene and breast cancer susceptibility

Elizabeth L. Schubert[a,*], Ming K. Lee[a], Beth Newman[b], Mary-Claire King[a]

[a]*Division of Medical Genetics University of Washington, Box 357720, Seattle, WA 98195-7720, USA*
[b]*School of Public Health and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA*

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Abstract

In order to evaluate the role of inherited variation in the estrogen receptor (ESR1) gene in human breast cancer, we determined intronic sequences flanking each ESR1 exon; identified multiple SNPs and length polymorphisms in the ESR1 coding sequence, splice junctions and regulatory regions; and genotyped families at high risk of breast cancer and population-based breast cancer patients and controls. Of 10 polymorphic sites in ESR1, four are synonymous SNPs, two are nonsynonymous SNPs and four are length polymorphisms; five are novel. No ESR1 polymorphisms were associated with breast cancer, either in the high-risk families or the case-control study. We therefore conclude that inherited genetic variation is not a mechanism by which the estrogen receptor is commonly involved in breast cancer development. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Breast cancer is a major public health issue, with some 180,000 women being diagnosed annually in the United States alone, leading to some 46,000 deaths [1]. It has been estimated that 5–10% of breast cancers arise from an inherited predisposition to the disease [2,3] with approximately 85% of these cases due to the breast cancer susceptibility loci BRCA1 and BRCA2. Perhaps 15–20% of families with inherited breast cancer may lack mutations in either BRCA1 or BRCA2 [4,5]. The identification and characterization of other breast cancer susceptibility genes would yield new insight into breast tumorigenesis and potentially provide targets for development of new therapeutics in the future. Hypothetically, some of these genes might be less penetrant than BRCA1 or BRCA2 and therefore influence breast cancer risk among women with no family history of the disease.

The estrogen receptor (ESR1) is a particularly good candidate for an inherited breast cancer susceptibility gene due to its biological role in normal breast cell development and function. The estrogen receptor protein is a critical component of hormonal regulation of breast tissue, which is underscored by its presence being a positive prognostic indicator for hormone therapy in breast tumors (reviewed in Ref. [6]).

Mice genetically engineered to lack a functional ESR1 gene [7,8] are viable and the heterozygotes have no obvious defects. However, both homozygous null males and females are sterile and have low skeletal bone density. Homozygous females also display minimal mammary duct development. A human male homozygous for a protein truncating mutation in ESR1 has been described [8,9] with the phenotype of bone dismorphism, low bone density and estrogen insensitivity. Female members of this patient's family were heterozygous for the same mutation without obvious clinical effects. Both the knockout mice and the homozygous null human patient indicate that lack of estrogen receptor activity is compatible with growth to adulthood and that heterozygotes have no obvious developmental phenotype.

* Corresponding author. Tel.: + 1-206-616-4294; fax: + 1-206-616-4295.
E-mail address: schubert@u.washington.edu (E.L. Schubert).

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Table 1
SSCA primers used in analysis of the ESR1 coding and promoter regions. Four sets of primers were used in the promoter screen, thirteen in the genomic region. Three of the seventeen primer sets were previously described [33]. Primers used for direct sequencing of the enhancer region are as published [38]. Primers for the coding region flank the exons and allow for analysis of the adjacent splice junctions

ESR1 exon	Genomic sequence	Forward primer	Reverse primer	Size of amplicon	ESR1 coding sequence amplified[a]
5′ UTR	X68051	TGTCACCAACAAAGGGTAACAGTC	GGCTCTTGAGACCAGTACAAAAAAG	206	
		AATCCCTGCCATTCCACG	CACTAGCCAGAAAGGTAAGTTGC	239	
	X62462	as above	as above	239	
		TCTATCCAGCAGCGACGACAAG	AAAAAGAGCACAGCCCGAGG	352	
		TCGTCCTGGGAGCTGCACTTG	TCGTTCCCTTGGATCTGATGC	279	
1	X03635	CCACGGACCATGACCATGAC	TGCTGCTGTCCAGGTACA	151	284–434
		AACCGTCCGCAGCTCAAGATCC	TCGTTCTCCAGGTAGTAGGGCAC	325	371–697
		CAGGTCTACGGTCAGACC	GCGGCGCGGGCGCGCG	291	503–744
2	AF123494	TCAGGATAAAGTGGATCTGCTG	GGCTCTTAGGATCTGCTCATAGG	336	745–935
3	AF123495	TGAGGAAGTATTAGGAAAACAGCGG	TTCCAATGGGTAGAGCCAGCAC	291	936–1051
4	AF123496	GCTCACCTGTGCTTGAAAGTATTTC	GGCTGTTCTTCTTAGAGCGTTTG	281	1052–1208
		TCCACCTGTGTTTTCAGGGGATAC	AAGGGTCTGGTAGGATCATACTCGG	267	1052–1301
		AGTATGATCCTACCAGACCCTTCAG	CACATCTCCTGTAACCTTGAAAC	240	1281–1388
5	AF123497	GTCAGTTCAAATCCCTGTTGC	TGATCGTAAAGAACATGCTACTCC	272	1389–1527
6	AF123498	ACCCTTTCATGTCTTGTGGAA	GAGTGGGTAGATCGTATCTGGTTG	291	1528–1661
7	AF123499	CATCCCATGAACACTCTGGGTC	GGGGGCATGTTTTCTTTATGTC	313	1662–1845
8	AF123500	TGCTAAGTGTCTTTGGAGTTCCTC	AAGTGGCTTTGGTCCGTCTC	256	1846–1995
		CTGTGTCTTCCCACCT	AGGGATTATCTGAACCGTGT	290	1846–2078

[a] based on Genbank M12674.

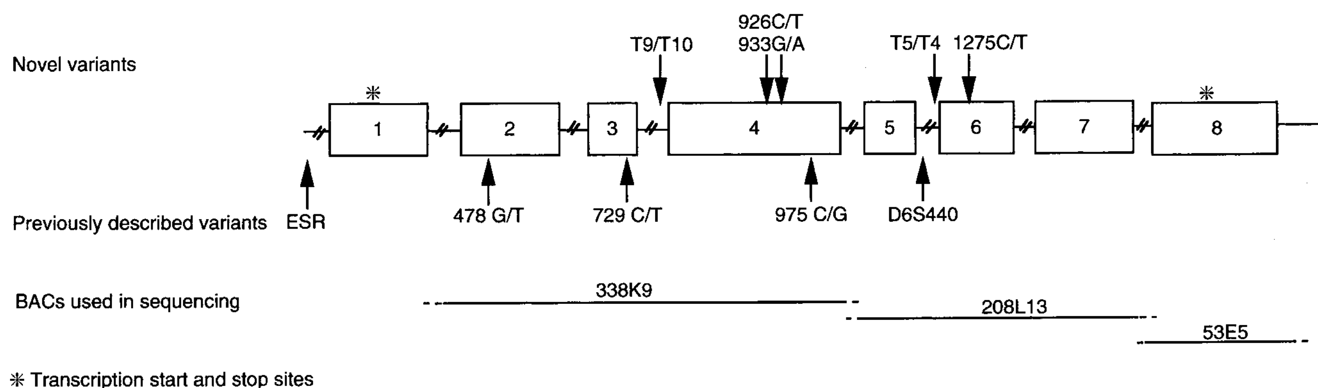

Fig. 1. Estrogen receptor structure and variants. The figure graphically depicts exons 1–8 of the genomic structure of the ESR1 with the variants detected during the course of this study, as listed in Table 2. Variants listed above the graphic are those which are novel to this study, while those listed below were previously described. The exons are drawn to scale, however intronic regions are truncated to maximize the use of space. Underneath the figure are bars that depict the BAC clone inserts used in generating the primary sequence data.

Several studies have suggested that genetic alteration of the ESR1 locus might be involved in breast tumorigenesis. The genetic region of 6q25 containing ESR1 is lost in a significant number of breast tumors [10–15] and the tumorigenic phenotype may be rescued by microcell mediated transfer of this segment back into tumor cells [10,16]. Interestingly, this same genomic region has also been implicated in several other tumor types, including ovarian cancer [17,18], malignant mesothelioma [19], salivary gland carcinoma [20] and non-Hodgkin lymphoma [21]. Somatic alteration of ESR1 has been identified in breast tumor biopsies [22–25].

Several studies have reported that particular inherited variants of the ESR1 locus are more common among breast cancer patients than controls [26–28]. Three previously described variants in the ESR1 coding region have been associated with an increased risk of breast cancer: 478G > T (Gly160Cys) [27], 908A > G (Lys303Arg) [25] and 975C > G (silent) [28,29]. Also, the ESR1 region was coinherited with breast cancer in a high-risk family [30].

Genetic screening of the ESR1 locus has been hampered by a lack of published genomic sequence information, which is essential to screen the gene and splice junctions thoroughly, although the complete cDNA [31] and short sequences flanking each exon have been published [32,33]. We therefore determined substantial genomic sequence surrounding each ESR1 exon using a modified end-rescue approach. Once intronic sequence information was known, primers were designed to amplify and screen each exon and flanking splice junctions, as well as the promoter region. Probands and relatives from high-risk breast cancer families were screened for inherited alterations in ESR1. Three SNPs were also genotyped in breast cancer patients and controls from the population-based Carolina Breast Cancer Study (CBCS).

Several novel variants of the ESR1 were detected and the marker D6S440 was localized to ESR1 intron 5, thereby precisely locating ESR1 on genomic maps. No variant, either novel or previously described, was associated with breast cancer in the families or the case-control series.

2. Materials and methods

2.1. Subjects

Probands from 25 families with four or more cases of breast cancer, diagnosed at any age, were evaluated for germline mutations in the estrogen receptor locus, ESR1. Also included were probands from another six families with ovarian cancer or male breast cancer as well as female breast cancer. These 31 probands were screened completely for variants in the ESR1 locus using the primers listed in Table 1 and the methods outlined below. No families in this series have positive LOD scores for linkage to either BRCA1 or BRCA2 nor any detected mutation in BRCA1 or BRCA2. In addition, 139 probands from other families with multiple cases of breast cancer were analyzed for ESR1 variants 478 G > T and 975 C > G. Families in this study are predominately Caucasian living in the United States. All participants in the project provided written informed consent prior to donating blood samples and were given genetic counseling as part of

their enrollment procedure. The project as a whole, including counseling protocol and consent forms, has been approved by the University of Washington Institutional Review Board.

A series of cases and controls from the Carolina Breast Cancer Study (CBCS: [34,35]) were analyzed for two previously described variants which alter the ESR1 coding sequence; 478 G > T and 908 A > G. The CBCS is a population-based, case-control study of breast cancer in women residents of a 24-county area of central and eastern North Carolina. Cases included women diagnosed with primary, incident breast cancer during 1993–1996 and between the ages of 20 and 74 years. As a consequence of the sampling design, the CBCS case group contains roughly equal numbers of African-American and white patients and patients diagnosed before and after the age of 50. Controls were frequency matched to cases by age and race. Protocols for interviewing participants, obtaining blood samples and analyzing germline DNA were approved by the University of North Carolina at Chapel Hill Medical School IRB.

2.2. Modified end rescue to sequence genomic regions surrounding each exon

Exon structure for the ESR1 gene had been previously described [32,33]. A modified BAC end-rescue approach (based on Ref. [36]) was used to sequence the genomic regions surrounding each exon. We used previously described PCR primers [33] to amplify 5′ and 3′ regions of the genomic locus to isolate BAC clones (Research Genetics) that contained ESR1. Three BAC clones were isolated (338K9, 208L13 and 53E5) which contained overlapping inserts covering the entire ESR1 genomic region (Fig. 1). The BAC end-rescue protocol consists of digesting the BAC DNA with a common-cutting restriction enzyme (such as Rsa1) and then ligation of the fragments to a vectorette of known sequence. An initial round of PCR is performed on the ligated fragments using one primer within the exon of interest and one within the vectorette. This serves to increase the copy number of fragments in the template DNA which contain the region between the primer in the exon and the restriction enzyme site within the adjacent intron where the vectorette was ligated. This initial PCR product was then sequenced with fluorescent terminator chemistry (dRhodamine, ABI) using a primer within the exon to generate sequence from the exon 'outwards' into the adjacent intron. The resulting sequences were compiled using the program SeqHelp [37] into contigs consisting of each exon and the flanking intronic regions. Sequence of each exon and the flanking intronic regions have been submitted to Genebank and are described in Table 1.

2.3. Single strand conformation analysis (SSCA) and sequencing

PCR primers for amplification of individual exons of ESR1 were designed to evaluate both coding sequences and splice junctions. A total of 13 primer pairs were used to amplify the coding region of ESR1; another four primer sets were used to screen the promoter region (see Table 1). Three of the 17 primer sets in Table 1 were previously described [33], the rest were developed in the course of this study. A putative ESR1 enhancer region was screened by direct sequencing using previously described primers [38]. PCR template was gDNA isolated from peripheral blood. PCR for SSCA was carried out in 25-μl volumes containing 25 ng gDNA; 1 × PCR buffer (Boehringer Mannheim); 200 μM dATP, dGTP and dTTP (Boehringer Mannheim); 10 μM dCTP (Boehringer Mannheim); 25 pmol each primer (GibcoBRL); 0.5 μCi ^{32}P-dCTP (NEN DuPont); and 1.25 U Taq DNA polymerase (Boehringer Mannheim). Amplified samples were diluted 1:1 in formamide buffer (98% formamide, 10 mM EDTA, pH 8, 0.05% Bromophenol blue, 0.05% Xylene cyanol), held at 95°C for 5 min, then cooled quickly to 4° and held on ice for 5 min. For each sample, 5 μl was loaded onto an SSCA gel (MDE: FMC Bioproducts) and run at 5 W constant power for 12–17 h, depending on the size of the fragment, in 0.6× Tris–borate EDTA buffer (TBE). Gels were dried on a vacuum gel dryer and exposed to film for 12–24 h. Fragments that showed a variant by SSCA or restriction enzyme assay (for the variant 478 G > T) were reamplified and sequenced using florescent terminator chemistry (dRhodamine, ABI).

2.4. Screening for known ESR1 variants

We screened for the variant 478 G > T (Gly160Cys: 27) in 139 samples from breast cancer probands as well as in 105 breast cancer cases and 151 controls from the CBCS. PCR amplification was based on the primers listed in Table 1, followed by digestion with MvaI; the variant eliminates a MvaI site from the wild-type sequence. Probands from 133 breast cancer families were screened by SSCA analysis as described above for three previously reported ESR1 variants: 729 C > T [27], 908 A > G [25] and 975 C > G [28,29].

Breast cancer cases and controls from the CBCS were screened for the variant 478 G > T by PCR followed by restriction enzyme assay and for the variant 908 A > G by SSCA analysis of exon 4 as described above.

Table 2
All variants detected during screen of the ESR1 locus. Table 2 lists all of the variants detected during the ESR1 genomic screen reported here. A total of 10 variants were detected, 5 of which are novel and 5 of which have been previously described. Frequencies are listed of the seven ESR1 region variants that were detected at appreciable frequency in probands of breast cancer families

Variant	Locale	Codon	Effect	Frequency[a]			
1 (−1355) $(TA)_n$	intron 1A	–	17 alleles (ESR)				
478 G > T	exon 2	160	Gly > Cys	G	0.98	T	0.02
729 C > T	exon 3	243	silent	C	0.97	T	0.03
760 (−17)Δ T	intron 3	–	none	T9	0.58	T10	0.42
926 C > T	exon 4	309	Ser > Phe	C	1.00		
933 G > A	exon 4	311	silent	G	1.00		
975 C > G	exon 4	325	silent	C	0.79	G	0.21
1235 (+224) $(CA)_n$	intron 5	–	9 alleles (D6S440)				
1236 (−33) Δ T	intron 5	–	none	T5	0.91	T4	0.09
1275 C > T	exon 6	425	silent	C	0.98	T	0.02

[a] In probands of breast cancer families.

3. Results

Genomic structure and position of ESR1 are indicated in Fig. 1 and Table 1. ESR1 comprises eight coding exons [32] and multiple alternate splice forms in the 5′ UTR [39,40]. Primers indicated in Table 1 were designed to amplify genomic DNA encompassing the coding regions, flanking splice sites and all known 5′ UTR splice variants of ESR1.

Ten polymorphisms in the ESR1 coding sequence and introns were observed in this study (Table 2 and Fig. 1). These include two amino acid substitutions, one previously reported and the other novel and detected only in African-Americans, four silent single nucleotide polymorphisms, two length polymorphisms in intronic poly-T sequences and two dinuclotide-repeat polymorphisms. The previously described variant 908 A > G was not observed in either the family probands or CBCS samples. The repeat polymorphism ESR in the promoter region was previously described [41] and the other repeat polymorphism, D6S440 [42], was newly localized to ESR1 intron 5. No additional variants were detected in the ESR1 promoter. Frequencies of alleles at ESR1 polymorphic sites detected during this study are indicated in Table 2.

Three amino acid substitutions in ESR1 occurred in study subjects. Two of 139 familial breast cancer patients were heterozygous for the nonsynonymous SNP 478 T (160 Cys), however 478 T was not co-inherited with breast cancer in either family. In the CBCS, the frequency of 478 T was zero in Caucasian cases, 0.02 in Caucasian controls and zero among African-American cases and controls. None of 133 familial patients carried the missense variant 309 Phe (926 T). In the CBCS, the frequency of 309 Phe (926 T) was 0.01 among African-American cases, 0.003 among African-American controls and zero among Caucasian cases and controls. 309 Phe (926 T) is in complete linkage disequilibrium with the nearby silent variant 933 A. The previously reported amino acid substitution 303 Arg (908 G) was not detected in any familial breast cancer cases, CBCS cases or CBCS controls.

Association of breast cancer with three synonymous SNPs in the ESR1 coding sequence and two intronic polymorphisms was evaluated in high-risk breast cancer families. Silent variants 729 T, 760 (−17) del T and 1236 (−33) del T occurred in probands of three, four and four families, respectively, but in no family was the rare allele inherited with breast cancer.

In order to test whether the silent variant 975 G was associated with familial breast cancer, we genotyped probands of 133 families with multiple cases of breast cancer. Frequency of the 975 G allele in probands was 0.21. For families with probands carrying the G allele, other female relatives were genotyped. Among relatives with breast cancer, frequency of 975 G was 0.28; among unaffected female relatives, frequency of 975 G was 0.24. Hence, 975 G was not associated with familial breast cancer in this series ($P = 0.18$).

4. Discussion

No inherited variant of ESR1 was associated with breast cancer in the high-risk families or cases and controls in our series. Based on sequence conservation and known ESR1 functional domains, two of three nonsynonymous SNPs may represent nonconservative changes. The ESR1 variant 478 G > T (Gly160Cys) has been previously associated with inherited breast cancer [27]. We detected this variant in two probands of breast cancer families as well as in three controls from the CBCS and did not find evidence for its association with breast cancer. Amino acid Gly 160 is located near the end of the ESR1 B region, between the transactivation and DNA binding domains. This amino acid is conserved between human and sheep but

not with other species (chicken, finch, mouse, rat, *Xenopus* or catfish), indicating that this residue may not be vital to ESR1 protein activity. The substitution of a nonpolar residue such as glycine for another nonpolar reside such as cysteine is not likely to substantially alter protein folding in that area.

The novel variant 926 C > T (Ser309Phe) was detected in two African-American breast cancer cases and in one African-American control from the CBCS series. This variant changes an amino acid conserved through tetrapods and may alter ESR1 protein function. The substitution of an uncharged polar residue such as serine for a nonpolar residue such as phenylalanine may change the polarity of that region of the protein and may alter its conformation. Serine 309 is located in the E domain of ESR1, in a region that has been shown to be involved in hormone binding [43].

The previously reported estradiol-sensitive variant 908 A > G (Lys303Arg: 25) was not detected in any of 314 individuals screened in our study, suggesting that this is not a common allele in the US population. Amino acid 303 is the first residue within the E domain, in the hormone-binding region. This amino acid is conserved among tetrapods, underscoring its importance to ESR1 protein function.

Four synonymous SNPs were detected during this study, two of which are novel: none are associated with inherited breast cancer in our series. 729 C > T has been previously described [27], in our series 729 T was observed with a frequency of 0.03. The novel variant 933 A is only detected in linkage disequilibrium with the nearby nonsynonymous SNP 926T described above. The silent variant 975 C > G in codon 325 has been suggested to be more frequent in breast cancer cases than in controls [28] and in breast cancer patients with family history [29] although these findings have not been confirmed [44]. The SNP 975 G was fairly frequent in our series, as described in Table 2 and was not co-inherited with breast cancer in families. The previously undescribed silent variant 1275 C > T was detected in a single family proband and did not segregate with disease in that family.

This study was designed to investigate the possibility of inherited susceptibility to breast cancer arising from genomic ESR1 variants and did not detect an association between inherited variation at ESR1 and breast cancer incidence. Previous studies focusing on ESR1 genomic structure have not detected a difference in frequency of loss of heterozygosity (LOH) of the region in tumors which are ER positive versus those which are ER negative [12,45]. The combined lack of LOH and inherited loss of function ESR1 mutations indicate that ESR1 is not responsible for loss of ER function in tumors in the manner of a classical, inherited tumor suppressor gene. Variation in ESR1 protein in tumors may therefore be due to somatic mutations or epigenetic effects during tumorigenesis.

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