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Report

Estrogen receptor beta (ESR2) polymorphisms in familial and sporadic breast cancer

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Summary

Estrogen is involved in both normal mammary development and in breast carcinogenesis. A family history of disease and exposure to estrogen are major risk factors for developing breast cancer. Estrogen exerts its biological effects through binding to the estrogen receptors, estrogen receptor alpha (ESR1) and the more recently discovered estrogen receptor beta (ESR2). Genetic variation in genes involved in estrogen biosynthesis, metabolism and signal transduction have been suggested to play a role in breast cancer risk. We therefore tested the hypothesis that common genetic variants of the ESR2 gene may be associated with increased risk for breast cancer and this risk may vary between breast cancer groups. We investigated three common ESR2 polymorphisms, rs1256049 (G1082A), rs4986938 (G1730A) and rs928554 (Cx + 56 A → G) for association to breast cancer risk. A total of 723 breast cancer cases and 480 controls were included in the study. Of the breast cancer cases, 323 were sporadic and 400 were familial, the familial cases were further divided into familial high-risk and familial low-risk breast cancer cases. We found no overall statistically significant association for any of the single polymorphisms studied. Haplotype analysis suggested one haplotype associated with increased risk in sporadic breast cancer patients (OR = 3.0, $p = 0.03$). Further analysis is needed to elucidate the role of estrogen receptor beta in breast cancer susceptibility.

Introduction

Breast cancer is a multifactorial disease involving a complex interplay between genetic and environmental factors. Since the discovery of BRCA1 and BRCA2 [1,2] in the early 1990's intensive efforts to discover further breast cancer susceptibility genes has largely been unsuccessful. The failure to identify novel high penetrant genes points to the existence of moderate penetrant mutations in as yet unidentified genes which confer an increased breast cancer risk but also a number of low penetrant genes which combine to increase women's risk [3]. It is likely that the risk varies between these rare moderate penetrant genes and the accumulation of several low penetrant common alleles. This polygenic model of breast cancer susceptibility has focused on association studies of single nucleotide polymorphisms (SNPs) in candidate genes to identify such low penetrant genes. Genes involved in hormone biosynthesis and metabolism are suitable candidates as possible breast cancer susceptibility genes as increased hormonal exposure is a risk factor for breast cancer [4].

Estrogen plays a central role in the normal development of the mammary gland but is also involved in breast cancer progression. Epidemiological studies have shown that exposure to estrogen is a risk factor for breast cancer, with prolonged or increased exposure increasing the risk whereas reduced exposure has a protective effect [4]. Estrogen exerts its biological effects through the estrogen receptors, estrogen receptor alpha (ESR1) [5,6] and the more recently discovered estrogen receptor beta (ESR2) [7,8]. ESR1 and ESR2 belong to the nuclear receptor superfamily of ligand-inducible transcription factors. They possess high sequence similarity in their DNA- and ligand-binding domains but diverge at the transcriptional activation domain, indicating they may target distinct sets of genes [7]. As is the case with ESR1, there also exists several different ESR2 isoforms resulting from alternative splicing of wild type ESR2 [9–11]. The estrogen receptors form both homodimers and heterodimers and different ESR2 isoforms have been suggested to have a regulatory role in ESR1 activity [11,12]. Several studies have shown that

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ESR2 is expressed in normal breast tissues and breast cancers [13–16]. It has also been shown that ESR2 is downregulated in breast carcinogenesis [15–17]

In order to determine whether genetic polymorphisms in the estrogen receptors have any effect on breast cancer risk a number of association studies have been carried out. The majority of studies to date have focused on the association between polymorphisms in the ESR1 gene and breast cancer risk, however, results from these studies are inconsistent [18]. There are currently three published reports investigating the association of polymorphisms in ESR2 and breast cancer risk. These studies investigated sporadic and incident breast cancer cases from Finland, China and USA respectively [19–21]. Two of these studies have found an association between ESR2 polymorphisms and breast cancer risk [20,21].

In the present study we chose to analyze three common single nucleotide polymorphisms (SNPs) in the ESR2 gene; rs1256049 (G1082A), rs4986938 (G1730A) and rs928554 (Cx + 56 A → G). These SNPs have been evaluated previously in relation to different phenotypes and found to have minor allele frequencies above 1% [22,23]. We investigated these SNPs for association with breast cancer risk in 723 breast cancer cases. Of these 723 breast cancer cases 323 were sporadic, while the remainder had a hereditary component. Of the familial group, 212 were high-risk cases and 188 were low-risk cases. This is the first study to investigate SNPs in ESR2 for association to both sporadic and familial breast cancer.

Materials and methods

Study subjects

Blood samples were collected from a total of 323 patients with sporadic breast cancer and 400 patients with familial breast cancer. The sporadic patients and 141 of the familial cases were collected as a population based breast cancer cohort at the Clinic of Oncology at Södersjukhuset and Karolinska University Hospital, Stockholm, Sweden while the remaining 259 familial cases were collected at the Department of Clinical Genetics, Karolinska University Hospital, Stockholm, Sweden. The 400 familial cases were divided into two groups, namely cases from high-risk families (n = 212) and cases from low risk families (n = 188). Families were divided into high-risk and low-risk groups based on pedigree analysis, where families with multiple affected family members and an apparent dominant mode of inheritance are termed familial high-risk, while those families with two affected women and an unclear mode of inheritance are termed familial low-risk. Our subclassification of families into two groups is consistent with empirical risk estimates, where high-risk families have a 3–5 fold increased risk of developing breast cancer while low-risk families have a 2–3 fold increased risk [24,25]. For all samples from Södersjukhuset and Karolinska University Hospital, a family history of breast cancer was obtained and all samples were screened for mutations in BRCA1 exon 11, which accounts for the majority of Swedish mutations [26]. All familial cases from Karolinska Hospital proceeded through genetic counseling and those who met the criteria for BRCA1/2 screening were screened negative for mutations while the remaining samples did not fulfill the criteria necessary for BRCA1 or BRCA2 testing. Controls were 480 blood donors from the Stockholm region of Sweden. The institutional ethical committee of the hospitals involved approved this study.

DNA amplification/polymerase chain reaction

DNA was extracted from peripheral blood lymphocytes of all cases and controls by the standard Phenol/Chloroform method. Three polymorphisms in ESR2 were studied, a G → A change at position 1082 in exon 5 (rs1256049), a G → A change at position 1730 in the 3′UTR of exon 8 (rs4986938) and a G → A change at Cx + 56 (rs928554), which is located 56 bases 3′ of the coding part of ESR2 Cx exon 9.

Prior to genotyping of the three polymorphisms, the region surrounding each polymorphism was amplified by PCR. Primers for amplification and the sequences surrounding each polymorphism are shown in Table 1. Pyrosequencing technology was used to genotype both rs1256049 and rs4986938, with the forward primers being biotinylated and reverse primers used for sequencing. RFLP analysis was used to genotype rs928554, using primers as previously described by Nilsson et al. [23].

PCR amplifications were carried out in 50 μl reaction mixture containing 100 ng genomic DNA, 20 pmol of each primer, 100 μM dNTPs, 1× PCR buffer II (Applied Biosystems, Foster City, California), 1.5 mM $MgCl_2$ and 1.25 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, California). PCR conditions were 9 min 30 s at 95 °C; 6 cycles of 30 s at 95 °C, 1 min at 66 °C with −1 °C/cycle and 1 min at 72 °C; followed by a further 44 cycles of 30 s at 95 °C, 1 min at 60 °C and 1 min at 72 °C; with a final 7 min at 72 °C.

Pyrosequencing

Following PCR amplification the rs1256049 and rs4986938 SNPs were investigated by Pyrosequencing, briefly described below. Biotinylated PCR templates were immobilized on streptavidin-coated paramagnetic Sepharose beads in Binding Buffer (5 mM Tris–HCL (pH 7.6), 1 M NaCl, 0.5 mM EDTA, 0.05% Tween 20) by incubation for 5 min in constant agitation at 1400 rpm. After immobilization, the bead-template complexes were submerged in 70% alcohol and denatured in 0.5 M NaOH followed by washing in 1× Wash Buffer (20 mM Tris–Acetate, 5 mM $MgAc_2$, pH 7.6) and the beads were then added to 45 μl of Annealing

Table 1. Primer sequences, amplicon sizes and the investigated polymorphic sequence for each of the three investigated polymorphisms

SNP ID	Forward Primer	Reverse Primer	Size	Interrogated Sequence 5′–3′
rs1256049	Biotin-5′-GTTCTGAGCCGAGGTCGTAGTGCTT-3′	5′-CACCTCCATCCAACAGCTCTCCAAG-3′	325 bp	CCTGTTCGACCAAGT[G/A]CGGCTCTTGGAGAGC
rs4986938	Biotin-5′-TGCTGGAGATGCTGAATGCCACGTGCTTC-3′	5′-TCCTGACACACTGGAGTTCACGCTTCAG-3′	191 bp	CCCACAGAGTCACA[G/A]GCTGAAGCGTGAACT
rs928554	5′-CTCTACTTAAGGGCAGAAAAGGCCTCTC-3′	5′-GTTGGATTGATAATAGAAAGGAAGGTG-3′	265 bp	GTGTGGTCAGCTGTG[A/G]CTGCCAACAGATGCA

Buffer (1× Annealing buffer, 10 mM Tris–Acetate, pH 7.6) containing 15 pmol of the sequencing primer (Table 1). Annealing took place at 80 °C for 2 min followed by cooling to room temperature. Real-time pyrosequencing was carried out in an automated 96-well pyrosequencer using PSQ SNP96MA enzymes and substrates (Pyrosequencing AB, Uppsala, Sweden). Pyrogram readouts were converted to numerical values for peak heights, using a software module designed for this purpose (Pyrosequencing AB, Uppsala, Sweden). Genotypes were analyzed manually by visual inspection of each pyrogram by two independent researchers. Results were validated by repeat analyses of at least 100 control subjects and 100 cases.

Restriction fragment length polymorphism (RFLP)

The rs928554 SNP was evaluated using restriction enzyme digestion instead of pyrosequencing due to the sub-optimal sequence surrounding this variant. The G → A change generates a restriction site for the enzyme Tsp509I. The 265 bp amplified PCR products was digested at 65° for 1 h and separated on a 4.5% agarose gel. Samples homozygous for the G allele demonstrated 2 bands upon digestion, one band of 187 bp and a second of 78 bp. Heterozygote samples produced 4 bands of size, 178, 149, 78 and 38 bp. The homozygous variant samples produced 3 bands of size 149 bp, 78 bp and 38 bp.

Statistical evaluation

Genotypic and allelic data for each of the three polymorphisms were compared between the cases and controls using chi-square analysis. Odds ratios with 95% confidence intervals were calculated using wt/wt genotype as reference genotype and comparing wt/var and var/var to this reference. All three polymorphisms were tested for adherence to Hardy-Weinberg equilibrium (HWE) in both cases and controls.

Linkage disequilibrium and haplotype analysis

Estimation of linkage disequilibrium (LD) and haplotype analysis was carried out using both the Haploview v3.1.1 1 program [27] and the UNPHASED program [28]. The UNPHASED program was run through the GLUE interface at http://www.rfcgr.mrc.ac.uk/. Genotype and marker data were loaded in linkage format files into the Haploview v 3.1.1 program for estimation of LD in the region and generation of inferred haplotypes. The default algorithm used by the program is based on the work of Gabriel et al. [29], where 95% confidence bounds on D′ are generated and each comparison is called strong LD, inconclusive or strong recombination. A block is generated if 95% of informative comparisons are in strong LD.

Association analysis of inferred haplotypes was also carried out using the COCAPHASE program within the

UNPHASED package. This program uses standard unconditional logistic regression identical to the model-free method of T5 of EHPLUS and the log-linear modeling. The EM algorithm is used to obtain haplotype frequency estimates.

Results

Genotyping

Genotypes were scored for the following three SNPs; rs1256049 (G1082A), rs4986938 (G1730A) and rs928554 (Cx + 56) in 89%, 92% and 95% of subjects respectively. Results were validated by repeat analyses of at least 100 control subjects and 100 cases for each SNP, with genotype results being replicated with 100% accuracy. The rs1256049 polymorphism was the least common in our study group, with the frequency of the minor allele being 5% in the control population and this differed very little in the patient group. Both the rs4986938 and rs928554 polymorphisms were common with variant allele frequencies of 36% and 43% respectively in the control group. The genotype distributions of each SNP were determined to be in Hardy-Weinberg equilibrium in both cases and controls.

Association analysis

We tested each polymorphism for association with breast cancer risk in each of the three breast cancer subgroups; sporadic breast cancer, high-risk breast cancer and low-risk breast cancer, and the results are presented in Table 2. There was no overall statistically significant difference in the genotype distribution of any of the three individual SNPs. The GA genotype of rs4986938 SNP showed a suggestively protective effect on breast cancer risk in the low-risk patients (OR = 0.72, 95% CI; 0.50–1.05). While the variant G allele of the rs928554 polymorphism demonstrated a marginally significant difference between low-risk cases and controls and appeared to be associated with an elevated risk of breast cancer (OR = 1.6 95% CI; 0.98–2.87).

Haplotype analysis

Due to the strong amount of linkage disequilibrium (LD) known to present in the ESR2 gene we also investigated if these three polymorphisms were in linkage disequilibrium and if there were any common haplotypes associated with disease. All three SNPs were shown to be in strong LD, with D′ values ranging from 0.86 to 1.00 (data not shown). The three SNPs were included in haplotype analysis and association of constructed haplotypes was tested by comparing the frequencies of the inferred haplotypes in each of the breast cancer groups to the frequencies in the control group. Seven different haplotypes were identified in our study material, and results of haplotype associations are presented in Table 3. Haplotype analysis was carried with the common allele of each polymorphism labeled as 1 and the minor allele labeled 2. Haplotypes were considered rare if they had a frequency less than 0.005% and so the 2-1-2 (A-G-G) and 2-2-1 (A-A-A) haplotypes are rare in the three breast cancer groups.

There was a modestly significant difference in the frequency of the 1-2-2 (G-A-G) haplotype in sporadic breast cancer cases compared to controls subjects. The 1-2-2 haplotype was suggested to be associated with an increased risk of breast cancer in this group and has an OR = 3.0 (p = 0.03). It was also apparent that having the contrary 2-1-1 (A-G-A) haplotype was associated with a decreased risk of sporadic breast cancer, as this haplotype had an OR = 0.4 (p = 0.03). These haplotypes exhibited the same tendency in the low-risk patient group, however due to the smaller sample size the results were not statistically significant. This trend was not seen in the high-risk patients.

Discussion

In the present study we have identified a common ESR2 haplotype that is suggested to be associated with increased risk of sporadic breast cancer. Since the discovery of estrogen receptor beta (ESR2) in 1996 there have been several studies examining its expression profile and characterizing the different ESR2 splice variants [30–35]. A number of studies have investigated the association of ESR2 polymorphisms with different phenotypes, such as anorexia nervosa, bulimia, ovulatory dysfunction, bone mineral density and endometrial cancer [22,36–38]. There are to date three studies examining the association of ESR2 variants and breast cancer risk [19–21]. The first published study of ESR2 polymorphisms and breast cancer risk examined 219 sporadic breast cancer patients from Finland [19]. This study investigated six polymorphisms, including rs1256049 (G1082A) and rs4986938 (G1730A), the genotype frequencies were similar to those found here in our study and they did not find any statistically significant associations between sporadic breast cancer and the six polymorphisms investigated. In a large study conducted in Shanghai consisting of 1459 incident cases of breast cancer, Zheng et al. investigated 5 common variants in ESR2 and found no overall statistically significant differences between the allele frequencies of cases and controls [20]. However, when taking only postmenopausal women into account they found a statistically significant difference in allele frequencies for the intron 5 SNP C14206T (p = 0.03) and also a marginally significant result for the exon 7 synonymous SNP C33390 (p = 0.07). Having found an association in post-menopausal women the investigators looked at women who had prolonged estrogen exposure due to a greater number of menstrual years. The results strengthened the association between C33390 and breast

Table 2. Genotype and allele distribution for rs1256049, rs4986938 and rs928554 SNPs in a set of sporadic, low-risk and high-risk breast cancers and population controls

	Genotypes	Sproradic breast cancer			Familial low-risk breast cancer			Familial high-risk breast cancer			Controls
		n (%)	OR (95% CI)[a]	*p*-value[b]	*n* (%)	OR (95% CI) [a]	*p*-value[b]	*n*(%)	OR (95% CI) [a]	*p*-value[b]	*n*(%)
rs1256049	G/G	292 (0.93)	1	0.195	171 (0.93)	1	0.358	165 (0.90)	1	0.797	356 (0.89)
	G/A	22 (0.07)	0.65 (0.38–1.12)		13 (0.07)	0.66 (0.35–1.26)		17 (0.09)	0.90 (0.49–1.62)		41 (0.10)
	A/A	0	0.40 (0.02–9.94)		0	0.69 (0.03–17.1)		1 (0.01)	2.16 (0.13–34.7)		1 (0.01)
	G	610 (0.97)	0.63 (0.37–1.07)	**0.084**	355 (0.96)	0.64 (0.34–1.21)	0.166	347 (0.95)	0.96 (0.55–1.67)	0.882	753 (0.95)
	A	22 (0.03)			13 (0.04)			19 (0.05)			43 (0.05)
rs4986938	G/G	127 (0.41)	1	0.431	90 (0.48)		0.243	81 (0.41)	1	0.982	175 (0.42)
	G/A	153 (0.49)	1.10 (0.81–1.50)		71 (0.38)	0.73 (0.50–1.05)		91 (0.46)	1.03 (0.72–1.48)		190 (0.45)
	A/A	32 (0.11)	0.81 (0.50–1.31)		25 (0.13)	0.87 (0.51–1.48)		26 (0.13)	1.00 (0.58–1.71)		56 (0.13)
	G	409 (0.65)	0.96 (0.77–1.19)	0.693	251 (0.67)	0.86 (0.67–1.17)	0.260	253 (0.64)	1.01 (0.79–1.30)	0.933	540 (0.64)
	A	219 (0.35)			121 (0.33)			143 (0.36)			302 (0.36)
rs928554	A/A	92 (0.29)	1	0.787	44 (0.24)	1	0.153	57 (0.28)	1	0.660	140 (0.31)
	A/G	166 (0.53)	1.12 (0.80–1.55)		96 (0.53)	1.33 (0.88–2.02)		108 (0.53)	1.16 (0.79–1.70)		229 (0.51)
	G/G	54 (0.17)	1.03 (0.67–1.59)		41 (0.23)	1.63 (0.98–2.71)		40 (0.20)	1.23 (0.75–2.00)		80 (0.18)
	A	352 (0.56)	1.03 (0.83–1.26)	0.807	184 (0.51)	1.26 (0.99–1.61)	0.059	222 (0.54)	1.11 (0.88–1.40)	0.392	509 (0.57)
	G	276 (0.44)			178 (0.49)			188 (0.46)			389 (0.43)

[a]Odds ratios were calculated using homozygous wild-type as reference.
[b]*p* values were calculated according to χ^2 test.
n = number of cases, OR = Odds Ratio, CI = Confidence interval.

Table 3. Haplotype association results, comparison of haplotype frequencies in breast cancer cases and controls. Haplotypes are represented by numerical values, where 1 represents the common allele and 2 the minor allele. 1-1-1 is the reference haplotype

Haplotypes	Sporadic breast cancer					Familial low-risk breast cancer					Familial high-risk breast cancer				
	Cases (%)	Controls	OR	χ^2	p-value[a]	Cases (%)	Cotrols	OR	χ^2	p-value[a]	Cases	Control	OR	χ^2	p-value[a]
1-1-1	20.4	16.4	1	2.887	0.089	15.5	16.4	1	0.180	0.672	12.9%	16.4%	1	2.117	0.146
1-1-2	41.3	42.9	0.772	0.169	0.681	48.3	42.9	1.189	2.927	0.087	44.9%	42.9%	1.327	0.351	0.554
1-2-1	32.9	34.4	0.764	0.199	0.656	31.1	34.4	0.954	1.055	0.304	36.5%	34.4%	1.344	0.405	0.525
1-2-2*	2.0	0.5	3.034	4.222	0.039	1.36	0.5	2.697	1.588	0.208	0.5%	0.5%	1.236	0.020	0.887
2-1-1	3.2	5.7	0.451	4.536	0.033	3.4	5.6	0.631	2.655	0.103	5.1%	5.6%	1.142	0.142	0.707
2-1-2*	0.2	0.2	0.957	0.0002	0.987	0.2	0.08	2.1	0.842	0.359	0.2%	0.2%	1.236	0.085	0.771
2-2-1*						0.2	0.08	2.1	2.066	0.151	1.04e-11	1.07e-11	1.236	0.226	0.635

* Denotes rare haplotypes with a frequency of < 0.005.
[a]p values were calculated according to χ^2 test.

cancer risk, women with the risk genotypes, CG or GG, having a RR of 2.41 (95% CI, 1.19–4.86, $p = 0.01$). The authors also investigated the risk of endogenous estrogen exposure and the risk genotype and observed the highest risk in those individuals who had both the risk genotype and high hormone levels. A recent study has evaluated the association between ESR1, ESR2 and progesterone receptor (PGR) SNPs and haplotypes and breast cancer risk in 1006 breast cancer cases [21]. This study included the three SNPs investigated in the present study and found no statistically significant association of these ESR2 SNPs. No genotype or allele frequency data is given for the ESR2 SNPs for comparison with our data, however upon haplotype analysis 5 haplotypes were found to be significantly associated with an increased risk for breast cancer in the Ashkenazi Jewish population and these haplotypes include the 3 SNPs from this study as part of a larger haplotype block. In comparison to the present study the associated haplotypes contained the 1-1-1 (G-G-A) haplotype constructed from the 3 SNPs included in this study.

Our study is the first to investigate different breast cancer sub-groups with respect to association to ESR2 variants. We have analyzed 723 breast cancer cases, of which 323 were sporadic in nature and a further 400 had a family history of disease. We have further divided the familial cases into familial high- and familial low-risk breast cancer families, where high-risk families are defined as having at least three first-degree relatives, or two first degree and one second degree relative with breast cancer and low-risk families have two cases of breast cancer in first or second degree relatives. We found no statistically significant associations when examining any of the three SNPs individually, and as these common variants represent a silent SNP and two 3′UTR SNPs it would be difficult to assign them with any causative function. Therefore it was of interest for us to investigate the haplotypes formed by these common SNPs and to determine if any common haplotypes were associated with breast cancer risk. Our analysis suggested an association of one haplotype, haplotype 1-2-2 (G-A-G), with an increased risk of sporadic breast cancer although the p-values are not highly significant. Individuals with this haplotpye had an OR of 3.0 ($p = 0.03$). The contrary haplotype, haplotype 2-1-1 (A-G-A), was suggested to have a protective effect, reducing the risk of breast cancer in this group, OR = 0.4 ($p = 0.03$). As was the case with the individual SNPs, no difference was found in haplotype frequencies of the high-risk cases compared to control subjects.

The results of this study indicate that ESR2 may play a role in breast cancer susceptibility. As with all association studies examining multiple variants the risk of Type I errors should be considered. However, it is difficult to adjust the p-values for multiple testing in this study because these three sequence variants are not independent due to the strong LD in the region. The control subjects used in this study were population based blood donors collected in the Stockholm region of Sweden. It has been pointed out that matching for sex may not be relevant unless the variant causes early sex-specific mortality [39,40]. If there was a sex-related effect on mortality this should show a deviation from Hardy-Weinberg equilibrium (HWE) and in this study all genotypes were in HWE indicating the results were not influenced by gender specific differences in allele frequencies.

We have identified an ESR2 haplotype that is suggested to be associated with an increased risk of breast cancer. Our results imply that although the SNPs examined in this study do not play a causal role in the etiology of breast cancer, the ESR2 gene may be involved in breast cancer susceptibility. Due to the large amount of linkage disequilibrium across the ESR2 gene it is possible that these SNPs are in LD with an as yet unidentified causal variant within of close to the ESR2 gene. Therefore it will be worthwhile to investigate this locus in more detail in an attempt to identify novel breast cancer susceptibility alleles and further understand the complex etiology of breast carcinogenesis.

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