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Polymorphisms in the promoter region of *ESR2* gene and breast cancer susceptibility

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ABSTRACT

Genetic variations like single nucleotide polymorphisms (SNPs) in genes involved in estrogen biosynthesis, metabolism and signal transduction have been suggested to affect breast cancer susceptibility. In this study we tested the hypothesis that polymorphisms in the promoter of *ESR2* gene may be associated with increased risk for breast cancer. We analyzed three SNPs in the promoter region of human *ESR2* gene by means of allele-specific tetra-primer PCR. A total of 318 sporadic breast cancer cases and 318 age-matched controls were included in the study. With regard to homozygous genotypes, women with sporadic breast cancer more frequently carried the CC genotype of *ESR2* promoter SNP rs2987983 (OR 1.99, $p = 0.005$). Calculation of allele positivity demonstrated that presence of T allele of this SNP was more frequent in healthy women. Our data suggest that a SNP in the promoter region of *ESR2* gene might be able to affect breast cancer risk. These results further support the emerging hypothesis that ERβ is an important factor in breast cancer development.

1. Introduction

Breast cancer is the most frequently diagnosed cancer and the second-most common cause of cancer related death in women. Estrogens are pivotal in the growth and development of both normal and neoplastic mammary tissues, and mediate most of their action via ligand-dependent transcription factors, the estrogen receptors (ER). Despite the fact that ER expression is an important prognostic and predicitve factor in breast cancer [1], the situation seems to be far more complex. Identification of the second estrogen receptor gene *ESR2* and its gene product, ERβ [2] has led to a re-evaluation of estrogen action in target tissues such as breast tumors. ERβ is expressed both in normal and neoplastic human breast tissue [3–4], but its role in either is not fully understood. In animal studies, while ERα has been shown to be essential for normal mammary gland development, ERβ effects are more subtle, with roles in terminal differentiation [5] and modulation of ERα activity being described [6–8]. In contrast to ERα, published data suggest that ERβ expression declines during breast tumorigenesis [3,9]. This down-regulation of ERβ in breast cancer compared with normal breast tissue suggests a role for ERβ as a tumor suppressor [10]. Nevertheless, ERβ expression in breast tumors varies widely [11], [4] and attempts to correlate ERβ with various biomarkers have resulted in varied, often contradictory conclusions [12]. This might also be due to differential detection of variant non-ligand binding ERβ proteins which have been detected in breast tissues [13–14] and which code for proteins exerting functions distinct from that of the full-length ERβ 1 protein [8,15].

Single nucleotide polymorphisms (SNPs) are the most frequent sequence variations in the human genome. SNPs located in exon regions may alter protein function, whereas SNPs in the gene promoter can modify gene expression levels [16–22]. Polymorphisms in genes involved in estrogen biosynthesis, metabolism and signal transduction have been suggested to play a role in breast cancer risk [23–31]. In the last years, a multitude of SNPs both in *ESR1* and *ESR2* gene have been identified and different genotype–phenotype association studies have been published examining the significance of randomly chosen SNPs in different hormone-dependent diseases [18,32–35]. In this study, for the first time three SNPs were selected which are located in the promoter region of *ESR2* gene and which thus could be able to modify expression level of this receptor. We compared allele frequencies of these SNPs in 318 healthy women and 318 women with sporadic breast cancer.

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2. Patients and methods

2.1. Patients

Blood samples from 318 Caucasian women with sporadic breast cancer and such as many age-matched Caucasian women not having any malignancy were included in this study. The median age of the patients collective and the control group was 55.6 and 54.9 years, respectively (age at diagnosis for case patients and age at inclusion for control subjects). The histopathological characteristics of the patients are shown in Table 1. Samples of breast cancer case participants were provided by the Institute of Pathology, University of Regensburg, in an anonymous and randomized manner. Included were Caucasian female sporadic breast cancer patients with available information on grading, tumor size, nodal status, and receptor status of ERα, PR and HER2 from 2002 to 2007. Control subjects were selected from the same geographic origin as the cases, the Oberpfalz area of Regensburg, Bavaria, Germany. Inclusion criterion for the control subjects was the absence of known malignancies. The study was approved by the Ethical Committee of the University of Regensburg.

2.2. SNP analysis

Three SNPs in the 0N promoter region of *ESR2* gene were identified using the Internet sites www.genecards.org and http://www.ncbi.nlm.nih.gov/SNP. The basis for SNP selection was their location in the 5′ region directly adjacent to the transcription start site of *ESR2* gene. Single nucleotide polymorphism rs2987983 (C/T) is located at position 63833406 of chromosome 14, rs3020450 (A/G) is located at position 63838055 and SNP rs3020449 (A/G) (formerly rs8004842) is located at position 63843145 of chromosome 14 (Fig. 1).

Genomic DNA was isolated from 100 μl EDTA-blood after addition of 300 μl lysis buffer (1%, v/v TritonX, 0.32 M Sucrose, 0.01 M Tris (pH 7.5) and 5 mM $MgCl_2$) and two-fold centrifugation (13,000 × *g*) for 30 s. Pellet was resuspended in 50 μl PCR buffer (GoTaq buffer, Promega, Madison, USA) containing 0.5% Tween 20 and 10 mAnson units proteinase K (Merck, Darmstadt, Germany) followed by incubation at 50 °C over night and finally heat inactivation of the enzyme for 10 min at 95 °C. The genomic DNA-containing lysate was subjected to a tetra-primer ARMS PCR approach [36] allowing allele-specific amplification using the PCR primers listed in Table 2 (synthesized at Metabion, Martinsried, Germany). For this purpose, to 100 ng of genomic DNA, 2 μl of 5 × GoTaq buffer, 0.2 μl of dNTP Mix (10 mM) (Fermentas, St. Leon-Rot, Germany), 0.2 μl of each PCR primer (10 μM) and 0.5 units GoTaq polymerase (Promega, Madison, USA) was added and PCR reaction was carried out using a T1 thermocycler (Biometra, Germany). PCR program was 10 min 94 °C followed by 38 PCR cycles of 94 °C (30 s), 56 °C (30 s) and 72 °C (60 s), followed by a final extension for 5 min step at 72 °C. PCR products were analyzed by 1.5% agarose gelelectrophoresis. Allele-

Table 1
Histopathological characteristics and receptor status of breast cancer cases included in this study (*n* = 318).

Characteristic	Patients numbers				
Tumor size	pT1 174	pT2 113	pT3 9	pT4 19	pTx 3
Histological grade	G1 36	G2 169	G3 113	Gx -	
Nodal status	N0 189	N1–3 118	Nx 11		
ERα status	neg. 67	pos. 238	ERx 13		
PR status	neg. 125	pos. 178	PRx 15		
HER2 status	neg. 203	pos. 45	HER2x 70		

The median age of patients was 55.6 years (range 24–82 years).

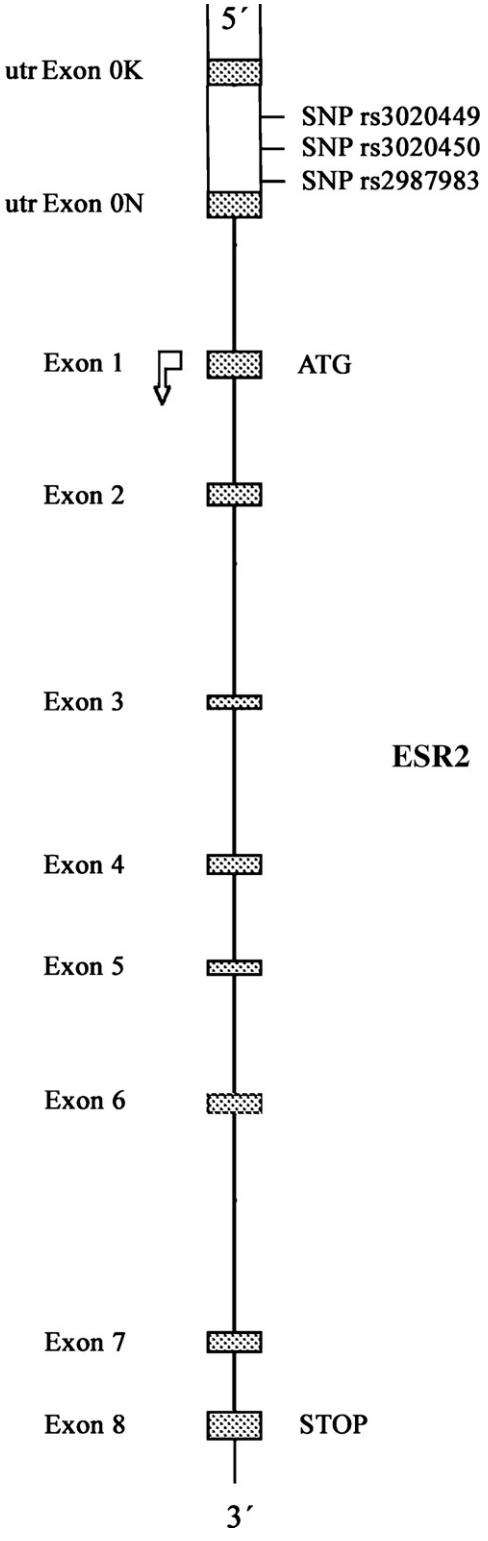

Fig. 1. The single nucleotide polymorphisms analyzed in this study are located in the 5′ region of estrogen receptor β gene (*ESR2*), namely in the promoter region of untranslated exon 0N.

Table 2
PCR primers used for *ESR2* gene SNP analysis.

polymorphism	Primer	Sequence
rs2987983	IP1	TCACAATTCAGGTAGAATTGGAATAATAAC
	IP2	CCTGGTTTAATGCAGAGTGGAGATGA
	0P1	ATTGTAGGATATTTTGGAGACAGGCAG
	OP2	TTATTATACAAGGAAACCTCACTGCAGG
rs3020449	IP1	GCATTGTCCTTTTTACATATTGTTAGGGTA
	IP2	AATTCTCAAGGAAATTTTAGCAAAGCC
	OP1	TAGATTTTGTCAAACACTTTTGGTGGAT
	OP2	CCAAATGATTAAGGAGAAATAACAGCAG
rs3020450	IP1	TAGTTTCCTTGTGTTCTCTGTTCTCTACG
	IP2	GGGAGAAGAGAGCCCAGGATTTCGAT
	OP1	CAACTAGGAAGTGTTTGTGCTGAAAACC
	OP2	GTCTCTTCTGAATTACACAGGTGCATGG

specific PCR product sizes for SNP rs2987983 were 180/258 bp (C/T), 156/212 bp (A/G) for rs3020450 and 231/193 bp (A/G) for SNP rs3020449. Genotyping success rate was 98.6 %. As a quality control for genotyping, in each PCR run three previously characterized DNA samples representing the heterozygous and the two homozygous genotypes were analyzed in addition to the unknown samples.

2.3. Statistical analysis

Deviation from the Hardy–Weinberg equilibrium was estimated by the Fisher's exact test and the χ^2 test, and all values were subjected to one-way ANOVA to achieve homogeneity of variance. Statistical tests for association (C.I.: 95% confidence interval) and for significance were carried out using SPSS for Windows 8.0 (SPSS, Inc., Chicago, IL). $P < 0.05$ was considered statistically significant. After tests for deviation from Hardy–Weinberg equilibrium were conducted, allele frequency, allele positivity and genotype frequencies were determined. Odds ratio (OR) was calculated using the more frequent homozygous genotypes as reference group.

3. Results

3.1. Genotype analysis

After genotyping of 318 breast cancer patients and such as many women without any malignancy, we observed a higher frequency of homozygous CC genotype of *ESR2* promoter SNP rs2987983 in women with breast cancer (0.19 vs. 0.11). Genotype–phenotype association suggested that CC genotype of rs2987983 could be a risk factor for breast cancer development (OR 1.99 [95% C.I.: 1.23–3.23], $p = 0.005$), whereas TT genotpye was more frequent in the control group and thus is suggested to have protective effects (Table 3). Frequency of the heterozygous genotype did not significantly differ between both groups. In this study, no significant differences between healthy women and women with breast cancer were found for the homozygous/heterozygous frequencies of SNP rs3020450 and rs3020449.

3.2. Allele frequency

Analysis of allele frequencies of the three *ESR2* SNPs in 318 breast cancer patients and such as many controls revealed that women with breast cancer less frequently carried the T allele of SNP rs2987983 (0.59 vs. 0.65, $p = 0.024$). Presence of C allele was significantly more frequent in breast cancer patients (Table 3). In contrast, no significant difference in the frequency of SNPs rs3020450 and rs3020449 was detected between healthy women and women with breast cancer.

3.3. Allele positivity

Phenotpye–genotype association analyzing the allele positivity of three *ESR2* promoter SNPs revealed that T allele positivity of SNP rs2987983 was less frequent among breast cancer patients than among healthy women (0.81 vs. 0.88, $p = 0.014$) (Table 3). Breast cancer patients more often exhibited C-positive alleles of this SNP. No significant differences between healthy women and women with breast cancer could be found in allele positivity analysis of SNPs rs3020450 and rs3020449.

3.4. Association with breast cancer characteristics

Last we compared the frequencies of SNP genotypes between breast cancer subgroups with different tumor characteristics. The respective subgroup size allowed statistical analysis of nodal status (node positive, $n = 118$ vs. node negative, $n = 189$), tumor size (pT1, $n = 174$ vs. pT2, $n = 113$) and histological grading (G2, $n = 169$ vs. G3, $n = 113$). Significant differences between the compared subgroups were observed in none of these tests (data not shown). Since interactions between ERβ and ERα are present in breast cancer cells, we also compared the SNP genotypes between the ERα-positive and ERα-negative subgroup ($n = 238$ vs. $n = 67$, respectively). Neither in this analysis nor in association with PR- or HER2-status, we observed a different allele or genotype frequency of the both SNPs tested between both groups (data not shown).

Table 3
Test for phenotype–genotype association. After tests for deviation from Hardy–Weinberg equilibrium were conducted, allele frequency, allele positivity and genotype frequencies were determined. Odds ratio (OR) was calculated using the more frequent homozygous genotypes as reference group. (Controls: $n = 318$; cases: $n = 318$).

SNP	Allele frequency		Allele positivity		Genotype frequency		
rs2987983	T	C	T	C	TT	CC	CT
Controls	0.65	0.35	0.88	0.58	0.43	0.11	0.46
Cases	0.59	0.41	0.81	0.63	0.37	0.19	0.44
p	0.024	0.024	0.014	0.167		0.005	0.53
OR					CC: 1.99	[95% C.I.: 1.23–3.23]	
					CT: 1.11	[95% C.I.: 0.79–1.56]	
rs3020449	A	G	A	G	AA	GG	AG
Controls	0.57	0.43	0.81	0.81	0.33	0.18	0.49
Cases	0.57	0.43	0.67	0.67	0.33	0.18	0.49
p	1.00	1.00	1.00	1.00	1.00	1.00	1.00
OR						1.00	1.00
rs3020450	A	G	A	G	AA	GG	AG
Controls	0.33	0.67	0.55	0.89	0.10	0.45	0.45
Cases	0.33	0.67	0.55	0.89	0.10	0.45	0.45
p	1.00	1.00	1.00	1.00	1.00	1.00	1.00
OR					1.00		1.00

4. Discussion

Single nucleotide polymorphisms (SNPs) in genes of the steroid hormone metabolism or signaling are potential factors which could affect cancer risk in hormone-dependent tissues [37]. Since discovery of *ESR2* gene and its product ERβ in 1996, several groups have characterized its unique expression profile, but few have examined possible associations of *ESR2* polymorphisms with breast cancer risk. In previous studies, randomly chosen *ESR2* SNPs were demonstrated to be weakly associated with endometrial cancer, anorexia nervosa, bulimia, ovulatory dysfunction and bone mineral density [38–41].

To date there are three studies examining the association of *ESR2* SNPs and breast cancer risk [31,42,43]. In a recent study, six randomly chosen *ESR2* SNPs were examined in a Chinese population and a weak association of *ESR2* SNP (C33390G) with breast cancer risk was demonstrated and was shown to be dependent on endogenous estrogen levels [31]. These results could not be verified in two studies on Caucasian populations. A study on *ESR2* SNPs in 219 sporadic breast cancer patients and 248 controls from Finland showed no difference in the allele distribution of the six studied polymorphisms between the breast cancer and control groups [42]. In another study analyzing the three randomly chosen *ESR2* SNPs rs1256049, rs4986938 and rs928554 in 400 familial and 323 breast cancer cases, no significant association for any of the single polymorphisms studied was found, but only haplotype analysis suggested one haplotype associated with increased risk in sporadic breast cancer patients [43].

Given that in *ESR2* gene, no non-synonymous exon SNPs exist which would lead to an altered amino acid sequence of ERβ protein, in this study we decided to specifically focus on a second type of polymorphisms with potential functional significance, SNPs in the promoter region of *ESR2* gene (Fig. 1). The promoter region of *ESR2* gene is complex and consists of several tissue-specific promoters and different 5′-untranslated exons. It is only beginning to be described and thus we hypothesized that SNPs located in this region could be able to affect binding of enhancer or repressor proteins regulating *ESR2* gene transcription. Altered ERβ protein levels could then modulate estrogen effects on breast cancer development. SNPs in the promoter regions of various genes previously have been associated to the risk for several diseases [44–45].

The results of the genotype–phenotype association study we performed for the three *ESR2* promoter SNPs clearly suggested that the homozygous CC genotype of SNP rs2987983 could be a risk factor for breast cancer development. Supporting this association, analysis of allele frequency revealed a higher C allele frequency of this polymorphism in women with breast cancer. Furthermore, association with allele positivity suggested that mere presence of C allele confers an increased breast cancer risk.

This report is the first one analyzing the association of *ESR2* promoter SNPs rs2987983 and rs3020449 with breast cancer risk. However, one of these SNPs, rs2987983, recently was analyzed in a prostate cancer study, in which it was described to be a risk factor for prostate cancer development [46]. The same SNP recently also was reported to be a risk factor for hypospadias [47]. Polymorphism rs3020450 earlier was studied in the Cancer Genetic Markers of Susceptibility (CGEMS) project by means of the Illumina HumanHap550 assay, genotyping 1140 breast cancer cases and an equivalent number of controls. Our results are in line with the data of the CGEMS project, demonstrating no association between rs3020450 and breast cancer risk [48].

Generally, the function of ERβ and its splice variants in breast cancer is only beginning to be characterized. Its prognostic significance and relevance as a predictive factor for outcome of endocrine therapies are extensively studied, however they still remain unclear. Primarily from results of in vitro studies, ERβ is suggested to be a tumor suppressor in mammary epithelial cells by ligand- and ERα-dependent or—independent regulation of cell cycle, apoptosis, migration or telomerase stability [49–53].

To date, no functional studies on the *ESR2* promoter polymorphisms analyzed in this report have been performed. Thus, it remains unclear whether the association with breast cancer risk which we observed for CC-genotypes of SNP rs2987983 is mediated by altered ERβ receptor levels. The general low odds ratios reveal that the effect of these gene polymorphisms are low, as expected from a complex etiology. However, our data suggest that a SNP in the promoter region of *ESR2* gene is able to affect breast cancer risk. These data support the emerging hypothesis that ERβ is an important factor in breast cancer development and encourage further efforts to examine the significance of *ESR2* SNPs in combination with other gene polymorphisms known to affect breast cancer risk.

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