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Estrogen receptor alpha polymorphisms: correlation with clinicopathological parameters in breast cancer

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Polymorphisms in estrogen receptor alpha gene (ESR1) have been previously associated with breast cancer risk; however, the results were not fully consistent. Our purpose was to study interactions between common genotypes in ESR1, breast cancer risk and tumor phenotypes. 6 ESR1 single nucleotide polymorphisms (SNPs) were genotyped in 103 breast cancer patients and 90 controls using hybridization probes; the genotypes were correlated with known prognostic factors for breast cancer and 5 years-follow up data. To assess estrogen and progesterone receptors (ER, PR) and HER2/neu expressions, immunohistochemistry was performed. Our results showed that rs3798577 was significantly associated with the risk of breast cancer, the common allele C conferring susceptibility (p-trend=$4x10^{-5}$); rs3798577 was also correlated with PR expression (p=0.01), but not with ER expression; rs2228480 (p=0.047) and rs1801132 (p=0.02) were associated with the age at diagnosis; rs1801132 was correlated with hypercholesterolemia (p=0.003) and increased BMI (body mass index) (p=0.01); rs2234693 showed a low significant association (p=0.042) with the tumor grade; rs3798577 was correlated with disease-free survival (p=0.05), allele C conferring increased risk for relapses, but it reached not statistical significance after adjustments. In conclusions, we identified four genotypes significantly correlated with either the risk or some tumor characteristics, suggesting that the main selection criteria of the investigated SNPs (frequency and the position in modulating domains of the gene) are pertinent instruments for establish correlations between the gene structure and the tumor phenotype.

Key words: estrogen receptor alpha gene, single nucleotide polymorphisms, breast cancer

Breast cancer is the second leading cause of cancer deaths in women today (after lung cancer) and the most common cancer among women, excluding nonmelanoma skin cancers. According to the American Cancer Society, about 1.3 million women will be diagnosed with breast cancer annually worldwide and approximately 465,000 will die from this disease. The lifetime probability of developing breast cancer in developed countries is about 4.8% (the probability is about 13% for any type of cancer), respectively about 1.8% in developing countries. It is estimated that 192,370 women will be diagnosed and 40,170 women will die of breast cancer in 2009 [1].

Malignant transformation occurs through the accumulation of mutations in genes regulating cell division, apoptosis, invasiveness or metastasis. To date, six genes were associated with high risk of breast cancer (BRCA1, BRCA2, TP53, PTEN, STK11 and CDH1) and four genes were associated with modest risk (PALB2, BRIP1, ATM and CHEK2). Also, there are some low penetrance genes which can be associated with an increased risk, such as: ESR1, CASP8, FGRF2, TOX3, MAP3K1, LSP1, 8q24, etc. [2, 3, 4]. However, more than 50% of the genetic predisposition remains unexplained and recently more emphasis has been placed on single-nucleotide polymorphisms [5, 6]. At present, it is widely accepted that breast cancer is a complex disease determined by the combined effect of several or even many genetic variants and environmental factors [7]. Notably, none significant breast cancer susceptible SNPs was found in ATM, BRCA1, BRCA2, CHEK2, TP53, genes that are known to be associated with increased breast cancer risk [8].

There are strong evidences that the level of ESR1 transcription and interactions of ERα with cofactors influence the carcinogenesis and estrogens represent risk factors for endocrine-related cancers such as breast, ovarian and endometrium malignant tumors. Estrogen mediates its effects by interacting

with specific nuclear receptors – ERα and ERβ –, which are ligand-activated transcription factors and now are serving as a basis for many therapeutic interventions. The two ERs are encoded by separate genes, ESR1 and ESR2 located on chromosomes 6q 25.1 and 14q 23-24.1, respectively. ERs are localized in the nucleus and cytoplasm of the cell and only a minor fraction is localized in the cell plasma membrane. In the absence of ligands, ERs stably bind the heat-shock proteins (Hsps90, 70, 56) which keep the receptors in an active state until high-affinity ligand binds to ERs and induces their dissociation, then dimerization and translocation to the nucleus – in the classical "genomic pathway". In this genomic mechanism, the complex between estrogen and ERs binds in the promoter-proximal and promoter-distal estrogen-responsive enhancer elements (EREs) of estrogen-responsive genes. This binding can be direct or indirect through protein-protein interactions with activator protein 1 (AP-1) or SP1 or FOXA1 sites in the promoter region of estrogen-responsive genes. At this level, the regulation of gene transcription is based on the estrogen-ERs complex – dependent recruitment of both specific coactivators and corepressors and the basal transcription machinery. This "genomic" mechanism occurs in hours. In contrast, estrogen can act more quickly (seconds or minutes) via "nongenomic" mechanism, either through the ER located in or adjacent to the plasma membrane, or through other non-ER plasma membrane associated estrogen-binding proteins, resulting in cellular responses, such as increased levels of calcium or NO (nitride oxide) and activation of kinases [9–12].

The aim of our study was to genotype ESR1 gene polymorphisms in breast cancers in order to search for associations between these polymorphisms and the risk of breast cancer. On the other hand, we correlated the allele susceptibility with clinical and morphopathological parameters like stage, tumor size, nodal and metastasis status, histopathological type and grade, hormone receptors (ER and PR), HER2/neu overexpression, menopausal status, obesity, hypercholesterolemia, type II diabetes mellitus and disease-free survival.

Patients and methods

*SNPs selection***.** After searching in public SNP databases, such as dbSNP http://www.ncbi.nlm.gov/SNP/ and available literature, we choose six SNPs. The selection criteria were:1) >10 % frequencies in the Caucasian population; 2) position in the gene or in the functional domains of the ERα protein and possible functional relevance; because the non-synonymous SNPs are rare and non-validated in the Caucasian population we selected silent SNPs located in coding or modulating regions of ESR1; 3) the results from the previous genetic studies. For SNP prioritization, we accessed the SNP function prediction webs FASTSNP [13] and PUPASUITE [14]. The position of the selected SNPs is represented in Fig.1.

Patients and healthy controls. Hundred and three unrelated Caucasian patients with breast cancer were included into the current study. Our control group consisted in 90 healthy

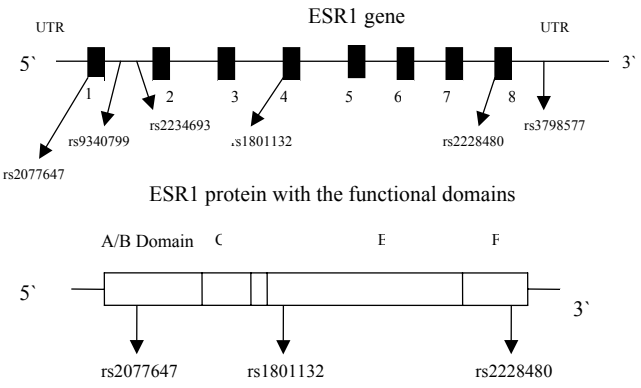

Figure 1. The position of investigated SNP on the gene, respectively on the ESR1protein. The exons are represented with black boxes.

women without family history of any type of cancers (first-and second-degree relatives). Clinical, histopathological characteristics and follow-up data were taken from the files of the Oncology Departments of our University and are resumed in *table 3*. Written informed consent for DNA analysis was obtained from all subjects and the Ethics Committee of our University approved the study.

*SNP genotyping***.** Genomic DNA was isolated from 52 paraffin-embedded breast cancer tissue samples (adjacent non-cancerous cells) using RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion) and from 51 frozen cancer tissues using QiAmp DNA minikit (Qiagen) according to the manufacturer protocol. In healthy subjects, the genomic DNA was extracted from whole blood collected on K_3EDTA using QiAmp DNA Blood kit. For SNP genotyping, we used hybridization probes. Two pairs of primers and probes were designed by TIB MOLBIOL GmbH, Berlin. The sequences of primers and probes that we used are shown in Tab.1. All PCRs were performed in 0.2 ml thin-walled PCR tubes with 25 µl reactions mix on a Swift Maxi Instrument (ESCO) using 30 ng of genomic DNA, with the forward and the reverse primers at 0.5 µM each and the probes at 0.3 µM each. PCR conditions were specific for each polymorphism and are resumed in Tab.2. The amplification cycles were followed by a melting cycle conducted in capillary tubes using LightCycler1.5 (Roche Applied Science) in which DNA was denatured at 95°C for 30s, cooled to 30°C using a rate of 1°C/s and held for 120s. Temperature was raised to 75°C with a transition rate of 0.1°C/s. Fluorescence was continuously monitored during the melt. Melting curves were converted into negative derivative curves of fluorescence with respect to temperature (dF/dT) by the LC Data Analysis software. All analyses were performed with background correction and color compensation. As a negative control, the template DNA was replaced with PCR-grade water.

Pathological diagnosis and grading were done on hematoxylin-eosin stained slides and were based on the standard recommendations by WHO and Elston and Ellis modified Scarff-Bloom-Richardson grading system [15]. In order to establish the

Table 1. The sequences of primers and probes

Polymorphisms and target sequences (The minor allele is displayed second)	Sequences	Tm °C
rs2234693 Target sequence: TCCAAATGTCCCAGC[C/T]GTTTTATGCTTTGTC	Forward primer: TgCTCAgTCTCTACATgTTCCT	52,7
	Reverse primer: TCCAgggTTATgTggCAAT	54,8
	Sensor: TgTCCCAgCCgTTTTATgC--FL	57,3
	Anchor:LC640-TTgTCTCTgTTTCCCAgAgACCCTgAg--PH	66,5
rs9340799 Target sequence: ATATTTTTCTTTCAC[A/G]TTTTC TGGTTTATTT	Forward primer: AgACTTAATgTTTTTgCAggAAT	53,1
	Reverse primer: CAAAATgAAATTAgCTggTTTCT	53,4
	Sensor: CAACTCCAgACCACACTCAgg-FL	57,3
	Anchor:LC640-TCTgggAAACAgAGACAAAgCATAAAACAgC--PH	66,5
rs2077647 Target sequence: CATCCCGGTAGGG[T/C]CTACGAAACCACACC	Reverse primer: CAAAATgAAATTAgCTggTTTCT	53,4
	Sensor: CAACTCCAgACCACACTCAgg-FL	57,3
	Anchor:LC640-TCTgggAAACAgAGACAAAgCATAAAACAgC--PH	66,5
	Anchor:LC640-CCCTACTgCATCAgATCCAAgggAACg-PH	68,0
rs1801132 Target sequence: GGATGCTGAGCCCCC[C/G]ATACTCTATTCCGAG	Forward primer:ACCTgTgTTTTCAgggATACgA	57,0
	Reverse primer:gCTgCgCTTCgCATTCTTAC	59,6
	Sensor:gCTgAgCCCCCCATACTCTA-FL	57,0
	Anchor:LC640-CCgAgTATgATCCTACCAgACCCTTCA-PH	63,4
rs2228480 Target sequence: GGGTTTCCCTGCCAC[G/A]GTCTGAGAGCTCCCT	Forward primer: CTgTgTCTTCCCACCTACAg	52,8
	Reverse primer: gggTAAAATgCAgCAgggATT	58,8
	Sensor: TCCCTgCCACAgTCTgAgAgC--FL	61,5
	Anchor: LC640-CCCTggCTCCCACACggTTCAg--PH	69,3
rs3798577 Target sequence: GGAGCTGAACAGTAC[T/ C] TGT-GCAGGATTGTTG	Forward primer: CCTgAACTTgCAgTAAggTCA	54,7
	Reverse primer: CCACCCTgAgCAAgTCT	51,9
	Sensor: gAACAgTACCTgTgCAggATT--FL	51,4
	Anchor: LC640-TTgTggCTACTAgAgAACAAgAgggAA-PH	61,1

Table 2. PCR conditions for each polymorphism

Polymorphism	Denaturation		Annealing		Extension	
	T°C	Time (s)	T°C	Time (s)	T°C	Time (s)
rs2234693	95	30	52	30	70	40
rs9340799	95	30	52	30	70	40
rs2077647	95	30	57	30	70	40
rs1801132	95	30	55	30	70	40
rs2228480	95	30	52	30	70	40
rs3798577	95	30	52	30	70	40

tumor phenotypes, the expression of ER, PR and HER2/neu were immunohistochemically assessed, using standardized automated procedures. The detailed methodology is presented in another paper [16]. For ER and PR, samples were considered positive when at least 10% of nuclei were immunoreactive, independently of the intensity of the immunostain. For the HER2/neu overexpression, scores zero and +1 were considered negative and scores +2 and +3 were considered positive [17].

*Data analysis***.** Hardy-Weinberg equilibrium (HWE) was calculated for each SNP in each control group and samples separately, using Pearson's chi-square test. This step was performed also for patients in order to determine whether there was any departure from HWE because such a finding can suggest that the marker is linked to a susceptible or protective allele. Chi-square test was used to calculate the differences in the allele frequencies between cases and controls. For association analyses between single loci polymorphisms and breast cancers, we performed logistic regression and Armitage`s trend-test using the HWE and association test calculator (http://ihg2.helmholtz-muenchen.de/cgi-bin). Considering the polymorphic alleles as "risk allele", we calculated odds ratios (OR) and 95% confidence intervals (CI). LD (linkage disequilibrium) between all possible pairs of loci, separately for controls and cases, was estimated with Genepop software package (version 1.2) (http://genepop.curtin.edu.au/) which uses the Markov chain method to estimate the exact *p* value and Fisher exact test [18]. CubeX software (http://www.oege.org/software/cubex/) was accessed to calculate D` (standardized linkage disequilibrium coefficient) and r^2 (correlation coefficient) [19].

Disease-free survival time was calculated as the time from cancer diagnosis to recurrences or death, censoring at the date of last contact (Tab.3). The Kaplan-Meier method was used to compute 5-year survival rates. Categorical data were compared with the use of the chi-square test. For comparisons between groups of categorical variables we used logistic regression. Odds ratios (OR) and their 95% confidence intervals (95% CIs) were calculated. We applied the Cox regression model

Table 3. Disease-free survival by demographics and known prognostic factors for breast cancer

Characteristic	Cases	5-years relapses	5-years disease-free survival	Cox regression		
				P value	HR	95% CI
Age at diagnosis (Median=53)						
<40	7	2	5			
40-49	26	7	17			
50-59	36	8	21	0.36	1.20	0.80-1.81
≥60	33	12	18			
Unknown	1	0	0			
Menopause						
Yes	72	21	40			
No	30	8	21	0.59	0.80	0.35-1.81
Unknown	1	1	0			
Histopathological type						
Invasive ductal carcinoma	66	23	35	0.068	0.43	0.17-1.06
Other types*	37	6	26			
Grade						
1	9	2	4			
2	57	13	37	0.14	1.60	0.85-3.00
3	37	14	20			
Tumor size						
<5cm	76	15	49	***0.003***	3.06	1.47-6.35
≥5cm	27	14	12			
Lymph node metastasis						
Present	66	24	33	***0.025***	0.33	0.12-0.87
Absent	37	5	28			
Distant metastasis						
Present	8	5	2			
Absent	94	23	59	***0.001***	0.29	0.14-0.59
Unknown	1	4	0			
TNM stage						
I-IIA	40	7	28			
IIB-IIIA	50	13	29	***0.003***	2.30	1.33-3.96
IIIB-IV	13	9	4			
ER status						
Positive	58	13	38			
Negative	40	14	21	0.37	1.36	0.68-2.69
Unknown	5	2	2			
PR status						
Positive	54	13	35			
Negative	44	14	24	0.77	1.10	0.56-2.14
Unknown	5	2	2			
HER2/neu						
Overexpression (+2,+3)	27	13	12			
Negative (0,+1)	62	9	43	***0.002***	0.50	0.32-0.77
Unknown	14	7	6			
BMI						
>25 kg/m^2	19	6	13			
≤25 kg/m^2	32	10	22	0.79	1.05	0.69-1.59
Unknown	52	13	26			
Hypercholesterolemia						
>200 mg/dl	21	7	14			
≤200 mg/dl	31	9	22	0.95	1.01	0.66-1.54
Unknown	51	13	25			
Diabetes mellitus type II						
Present	9	4	5			
Absent	43	12	31	0.89	0.97	0.66-1.43
Unknown	51	13	25			
Risk category**						
Low(<26)	22	4	18			
Intermediate (26-50)	25	7	18	0.38	1.38	0.75-2.69
High (>50)	56	19	37			

*Other types of carcinomas (37): Lobular: 10, Medullary: 8, Papillary:2, Apocrine carcinoma:2, Undifferentiated: 4, Mucinous:4, Inflammatory:5, Adenoid cystic: 1.
**Prespecified relapse risk score at 10 years generated using a computerized clinicopathological prognostic model based on age, comorbidities, ER, tumor grade, tumor size and lymph node status (http://www.adjuvantonline.com, version 8.0).

Table 4: Association between genotyped polymorphisms in ESR1 and breast cancer (breast cancers versus controls)

SNP	Tests for deviation from Hardy-Weinberg equilibrium (HWE)		Tests for association (C.I.: 95% confidence interval)				
	Controls (90)	**Breast cancer**	allele freq. difference	heterozygous	homozygous	Recessive model	Armitage's trend test
			Risk allele 2				
rs3798577	n11=30 (28.65) n12=41 (43.69) n22=18 (16.65) p=0.56	n11=8 (13.65) n12=59 (47.69) n22=36 (41.65) p=0.016	**[1]<->[2]** OR=2.29 C.I=[1.52-3.45]chi2=15.90 p=0.00007	**[11]<->[12]** OR=5.39 CI.=[2.24-12.95] chi2=15.87 p=0.00007	**[11+]<->[22]** OR=7.50 CI=[2.86-19.65] chi2=18.60 p=0.00002	**[11]<->[12+22]** OR=6.03 CI=[2.59-14.05] chi2=20.24 p=6.834e-06	**common odds ratio** OR=2.62 chi2=16.72 p=0.00004
rs2228480	n11=62 (62.57) n12=21 (19.85) n22=1 (1.57) p=0.59	n11=61 (64.49) n12=41 (34.02) n22=1 (4.49) p=0.03	OR=1.66 C.I.=[0.95-2.89] chi2=3.29 p=0.06)	OR=1.98 C.I.=[1.05-3.74] chi2=4.56 p=0.03	OR=1.01 C.I.=[0.06-16.61] chi2=0.0 p=0.99	OR=1.94 C.I.=[1.03-3.62] chi2=4.37 p=0.03	OR=1.68 chi2=3.82 p=0.050
rs2077647	n11=34 (35.13) n12=40 (37.73) n22=9 (10.13) p=0.58	n11=39 (41.02) n12=52 (47.96) n22=12 (14.02) p=0.39	OR=1.08 C.I.=[0.71-1.66] chi2=0.15 p=0.69	OR=1.13 C.I.=[0.61-2.10] chi2=0.16 p=0.69	OR=1.16 C.I.=[0.43-3.09] chi2=0.09 p=0.76	OR=1.14C. I.=[0.63-2.05] chi2=0.19 p=0.66	OR=1.08 chi2=0.16 p=0.68
rs1801132	n11=65 (66.50) n12=23 (19.99) n22=0 (1.50) p=0.16	n11=83 (83.07) n12=19 (18.86) n22=1 (1.07) p=0.9	OR=0.75 C.I.=[0.40-1.41] chi2=0.77 p=0.38	OR=0.64 C.I.=[0.32-1.28] chi2=1.55 p=0.21	OR=2.35 C.I.=[0.09-58.71] chi2=0.78 p=0.37	OR=0.68 C.I.=[0.34-1.34] chi2=1.23 p=0.26	OR=0.87 chi2=0.84 p=0.36
rs9340799	n11=38 (40.67) n12=45 (39.66) n22=7 (9.67) p=0.2	n11=44 (49.42) n12=54 (43.16) n22=4 (9.42) p=0.01	OR=0.89 C.I.=[0.58-1.37] chi2=0.25 p=0.61	OR=1.03 C.I.=[0.57-1.86] chi2=0.01 p=0.90	OR=0.49 C.I.=[0.13-1.81] chi2=1.16 p=0.28	OR=0.96 C.I.=[0.54-1.71] chi2=0.02 p=0.89	OR=0.82 chi2=0.31 p=0.57
rs2234693	n11=37 (34.84) n12=38 (42.31) n22=15 (12.84) p=0.33	n11=32 (41.19) n12=65 (46.62) n22=4 (13.19) p=0.0007	OR=0.93 C.I.=[0.61-1.41] chi2=0.11 p=0.74	OR=1.98 C.I.=[1.06-3.67] chi2=4.70 p=0.03	OR=0.31 C.I.=[0.09-1.02] chi2=3.95 p=0.04	OR=1.5 C.I.=[0.83-2.72] chi2=1.83 p=0.17	OR=0.8 chi2=0.13 p=0.72

Legend: rs3798577: T= allele 1; ***rs2228480:*** G =allele 1; ***rs2077647:*** T=allele 1; ***rs1801132:*** C =allele 1; ***rs2234693:***C=allele 1; ***rs9340799:*** A=allele 1.
The following equations correspond to risk allele 2: Odds ratio (allele freq. difference) Case_a2 * Control_a1) / (Case_a1 * Control_a2); Odds ratio (heterozygous) Case_12 * Control_11) / (Case_11 * Control_12);Odds ratio (homozygous) Case_22 * Control_11) / (Case_11 * Control_22);Odds ratio (allele positivity) (Case_12+Case_22) * Control_11) / (Case_11 * (Control_12+Control_22));Common odds ratioCase_12*Control_11/N01 + Case_22*Control_12/N12 + 4*(Case_22*Control_11/N02))/
(Case_11*Control_12/N01 + Case_12*Control_22/N12 + 4*(Case_22*Control_11*Case_11*Control_22)**0.5/N02)
p = p value (Pearson)

to evaluate the effect of covariates on overall survival. Hazard ratios (HR) and their 95% confidence intervals (95% CIs) were calculated. The P values for all hypothesis tests were two-sided, and we set statistical significance at P<0.05. All analyses were conducted with Stata 9.2 (Statacorp, Texas, USA). In order to create homogenous groups regarding the usual known prognostic parameters, we classified the patients in relapse risk categories using a computerized system called Adjuvant! Online Standard Version 8.0 available at http://www.adjuvantonline.com [20].

Results

ESR1 genotyping results and association tests. The genotyping success rate for the six selected SNPs was between 93.33% and 99.03%. Among our controls, the allele and genotypes frequencies are presented in Tab. 4 and there were not significant differences from the previous findings in Caucasian population [21]. The observed genotype frequencies showed that all six genotyped SNPs were in HWE proportions in the control groups and the p values are presented in table 4. In

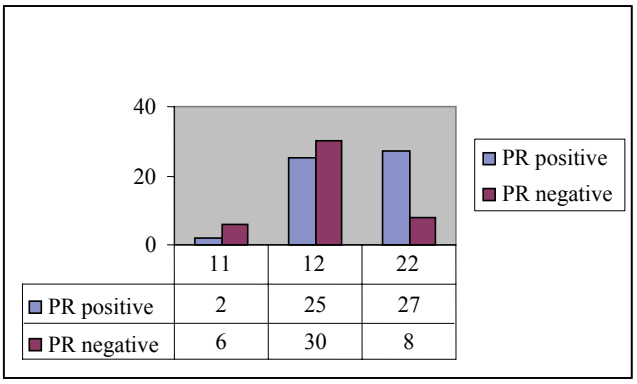

Fig.2. Rs3798577 genotypes and PR expression

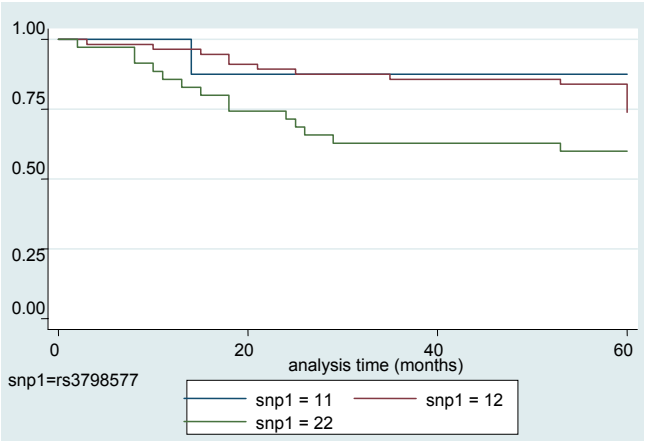

Fig.3. Kaplan-Meier survival estimates, by ESR1 rs3798577

the cases group, SNP rs2228480 (*p*=0.03), rs3798577 (p=0.01), rs2234693 (*p*=0.00007) and rs9340799 (p=0.01) did not respect Hardy-Weinberg proportions.

Associations with breast cancer (cases versus controls). Table 4 presents the genotypic and allelic frequencies within the controls and breast cancer patients, indicating that for rs3798577 the frequency of the common allele 1(T) is significantly lower ($p=7x10^{-5}$) and the heterozygous CT ($p=7x10^{-5}$) and homozygous for the polymorphic allele C($p=2x10^{-5}$) represent susceptible genotypes (p-trend=$4x10^{-5}$). For rs2228480, it was a trend for allele 1 (G) to be less represented (p=0.06) and the heterozygous (GA) were significantly more represented in cases (p=0.03). For rs2234693, the majority of breast cancer cases were heterozygous (p=0.03).

*Linkage disequilibrium analysis***.** Across all populations, we estimated linkage disequilibrium for each pair of loci using two different softwares, CubeX software for pair-wise values D` and r^2 and Genepop software to estimate the *p* value and Fisher exact test. Within controls, using CubeX, only the pairs between SNPs 1,2,3 and 4 were in low LD (D'=1.0 and r^2 range between 0.68 and 0.307); rs2234693 and rs9340799 were in LD each other but not with the other genotyped SNPs. For the breast cancer cases, only rs2234693&rs9340799 pair was in medium LD (D`=1, r^2=0.76). Exploring the haplotype frequencies for the pairs which were in LD, we did not find statistically evident susceptible haplotypes although, the haplotype T-A of rs2234693&rs9340799 (f_{11}=0.63) and G-C of rs2228480&rs3798577 (f_{21}=0.448) showed a trend to be higher represented (p=0.09 respectively p=0.06) in patients.

Associations between disease-free survival and usual clinical and histopathological parameters. Performing Cox regression analysis in order to evaluate the associations between disease-free survival and available clinical and pathological characteristics, we observed statistical significant correlations with the tumor size (p=0.003), the lymph node status (p=0.025), distant metastasis (p=0.001), TNM stage (p=0.003) and HER2/neu expression (p=0.002). Although we observed correlations, the results did not attaint statistical significance for age at diagnosis, menopausal status, histopathological type, grade, ER/PR status, BMI and hypercholesterolemia. These results are presented in Tab. 3.

*Associations between genotypes, tumor phenotypes and disease-free survival***.** To check the relationships between SNPs genotypes and prognostic tumor phenotypes we performed Pearson chi test and score test for trend of odds. From the genotyped SNPs, four SNPs showed significant associations, and namely, rs3798577 showed a correlation with the PR expression (p=0.01) (Fig.2), but not with ER expression (p=0.17); rs2228480 showed a marginally association with the age at diagnosis (p=0.047), women possessing the common variant being older at diagnosis; rs1801132 showed the same trend as rs2228480 regarding the age at diagnosis (genotype 11 associated with onset of cancer after 50 years, p=0.024); genotype 12 (CG) of rs1801132 was associated with hypercholesterolemia (p=0.003) and increased BMI (0.01). Comparison between genotypes and grade showed only for rs2234693 a low significant association (p=0.042), genotype 12 being more common in high grade tumors. Performing Kaplan-Meier survival analysis, only rs3798577 was correlated with survival (p=0.05) (Fig.3), allele C conferring increased risk of relapses, but it did not reach statistical significance after adjustments for known prognosis factors and the difference in survival could be attributable to the association of this SNP with PR status. In order to have homogenous groups of patients, for a better relevance of our correlations between genotypes and disease-free survival, we classified the patients into three 10 years-relapse risk categories (22 low, 25 intermediate and 56 high) accessing Adjuvant! Online version 8.0. This computerized system is developed based on the information from the Surveillance, Epidemiology, and End Results (SEER) database and the results of various individual clinical trials as well as the published literature and uses age, comorbidities, estrogen receptor status, tumor grade, tumor size and lymph node status as covariates. None of the genotyped SNPs showed

Table 5. Genotypes correlations with the clinical and histhopathological parameters

Characteristic	Score test for trend of odds (*p*)					
	rs3798577	rs2228480	rs2077647	rs1801132	rs9340799	rs2234693
Age at diagnosis	0.14	**0.047**	0.57	**0.025**	0.33	0.12
Menopause	0.51	0.95	0.79	0.46	0.34	0.20
Histopathological type	0.91	0.67	0.54	0.74	0.40	0.47
Grade	0.97	0.1	0.59	0.40	0.08	**0.04**
Tumor size	0.21	0.94	0.65	0.44	0.19	0.31
Lymph node metastasis	0.98	0.31	0.30	0.13	0.15	0.13
Distant metastasis	0.10	1.00	0.20	0.90	0.27	0.26
TNM stage	0.57	0.84	0.43	0.16	0.25	0.19
ER status	0.17	0.34	0.93	0.69	0.14	0.13
PR status	**0.01**	0.26	0.76	0.54	0.21	0.24
HER2/neu	0.61	0.87	0.74	0.45	0.89	0.86
BMI	0.49	0.91	0.31	**0.01**	0.61	0.74
Hypercholesterolemia	0.23	0.94	0.24	**0.003**	0.39	0.28
Diabetes mellitus type II	0.55	0.18	0.92	0.53	0.30	0.74
Disease-free 5-years survival	0.07	0.71	0.50	0.07	0.61	0.44
Relapse risk category at 10 years	0.78	0.76	0.42	0.32	0.48	0.52

Table 6. Interactions between SNPs and breast cancer evolution (Cox regression - Breslow method for ties)

SNP_ evolution	HR	SE	Z	P	95%CI
rs3798577	1.07	0.04	1.95	**0.05**	0.99-1.15
rs2228480	0.94	0.19	-0.29	0.77	0.62-1.41
rs2077647	1.04	0.05	0.84	0.40	0.94-1.15
rs1801132	1.14	0.10	1.47	0.14	0.95-1.35
rs9340799	1.07	0.09	0.87	0.38	0.90-1.27
rs2234693	1.10	0.08	1.29	0.19	0.95-1.28

a significant correlation regarding the disease free survival when correlated with these categories of risk. These results are presented in Tab. 5 and 6.

Discussions

The present study uses unrelated breast cancer patients of Caucasian ancestries from Western part of Romania in order to identify common variants associated with breast cancer risk on one hand and tumor clinicopathological characteristics, on the other hand. Six ESR1 SNPs were selected and genotyped and the results were compared with tumor known prognosis phenotypes and follow up data. Polymorphisms in estrogen receptor alpha (ESR1) have been previously associated with breast cancer risk, clinical and demographic characteristics but, however, the results were not fully consistent and need further genotyping in breast cancer cohorts for which long-term follow-up data are available.

The most widely studied polymorphisms of ESR1 are *PvuII (T397C) (rs2234693)* and *xbaI (A351G) (rs9340799)* located in intron 1. They are separated by 50 base pairs and are in linkage disequilibrium. *Xba*I and *pvu*II polymorphisms were previously associated with breast and prostate cancer and also with bone mineral density, age at menopause, spontaneous abortions, HRT, colon and urotelial cancers, cardiovascular and Alzheimer's diseases, hepatitis B and the risk for hepatocarcinoma [2, 22–23]. It was suggested that the polymorphic allele T was associated with increased levels of androstendione [24]. Possible functional mechanisms attributed to these polymorphisms include changes in ERα gene expression by altering the binding of transcriptional factors and influence on alternative splicing of ERα gene. The first intron in a gene, like the promoter, usually contains a larger number of regulatory sequences than other introns. However, the results are still conflicting and the molecular mechanism by which these polymorphisms influence receptor activity are still unclear. It was noted that the T→C transition is associated with the loss of the PvuII restriction site, results in a potential binding site for *myb* transcription factors that, in the presence of *b-myb*, is capable of augmenting in vitro the transcription of a downstream reporter construct 10-fold [25]. Thus, the presence of polymorphic allele might amplify ERα transcription. An alternative explanation is that the two polymorphisms in intron 1 may be in linkage disequilibrium with causal synonymous polymorphisms elsewhere in the ERα or another gene. In this regard, it has been established that intron 1 polymorphisms are in linkage disequilibrium with the upstream TA and GT repeats polymorphism in the promoter of ESR1, which were associated with microsatellite instability [26]. Searching in FASTSNP SNP function prediction web, we found that rs9340799 (*xba*I) is an intronic enhancer, representing a binding site for the helix-loop-helix transcription factor Th1/E47 (G

allele) and rs2234693 (*pvu*II) serves as transcription binding sites for v-myb (C allele) and SRY (T allele).

In our cases, we did not observe differences for *Xba*I, but for *Pvu*II, the heterozygous were higher represented (p=0.03). In accordance with the foregoing publications [22, 27–29], the two SNPs were in LD (D`=1, r^2=0.76). The haplotype T-A was more frequently represented in cancers cases, although the difference was not statistically significant (p=0.06). For breast cancers, regarding these markers, the results from literature are divergent, some publications found no significant risk [27, 30–32], other found risk only for haplotypes [33] and another publications found an increased risk for the polymorphic alleles [34–35] or contrariwise for the wild type [36]. In our cases, rs2234693 showed a low significant association (p=0.042) with the tumor grade, genotype 12 being more common in high-grade tumors. We did not find further significant associations between these SNPs and other tumor characteristics.

Rs2077647 (C/T) is a silent polymorphism located in exon 1 (S10S). This location corresponds to the A/B structural domain, respectively TAF1 functional domain (ligand independent transactivation domain) and can be activated via the non-genomic pathway through compounds like AMPc, dopamine, growth factors like IGF and EGF, resulting in activation of the kinases pathways (MAPK/p38, PLC/PKC, JAK/STAT). The effects that are mediated by this mechanism are induced quickly (seconds or minutes) and regulate numerous cellular processes from proliferation and apoptosis to differentiate function of target cells. The A/B domain contains a co-regulator domain that binds co-activators or co-repressors to the ER, with an important role for the modulation of ER transcription.

In accordance with other studies on Caucasian population [37–38] our results did not show significant differences over the control group for this marker. In Taiwanese population, Hsiao [39] found an increased risk for breast cancer in the presence of allele T.

Rs 1801132 is a synonymous, CG SNP in codon 325 (325Pro) of exon 4 of ESR1, located in the hormone binding domain, and more precisely in the structural domain E from the carboxiterminal region of the protein, corresponding to the functional domain AF-2 (ligand-dependent transactivation). This region is related to the receptor dimerization, chaperone binding and recruitment of coregulators. Searching in SNP function prediction webs PUPASUITE and FASTSNP we found that rs1801132 represents a target for the exonic splicing enhancers sc35 and sf2 (arginine serine-rich splicing factors) that interact with small nucleolar RNA and are required for the first step in the splicing reaction and spliceosome assembly. According to FASTSNP prediction report, the C allele of rs1801132 disrupts also the binding site for GATA-1 and GATA-2 transcription factor. In the literature, the results were divergent: some studies found the G allele protective [38] while other studies found increased risk with G allele [40–42]; other studies found no risk [43–44] or found risk only when included this polymorphisms in haplotypes [33, 45–46]. In our study, rs1801132 showed a trend to be associated with the age at diagnosis (genotype 11 associated with onset of cancer after 50 years, p=0.024), hypercholesterolemia (p=0.003) and increased BMI (p=0.01).

Rs2228480 (**G/A**) (594Thr) is a silent polymorphism located in exon 8 of ESR1, within the F structural domain, respectively the functional domain TAF-2 (ligand-dependent transactivation). The functionality of this SNP is not known yet, but it seems to recruit coregulators. Yang et al. suggest that the C-terminal amino acids of ERalpha (the F domain) are critical for attenuation of E2 induced receptor dimerization and transcriptional activity; the F-domain mutants showed increased receptor dimerization [47]. They also observed enhanced interaction of F domain mutants with p160 family coactivator SRC1. Accessing PUPASUITE web we observed that the polymorphic allele (A) disrupts the binding sites for the SR proteins SC35, SF2 and SRp40 (nucleolar, serine-rich protein). Overexpression of SRp40 was found during mouse mammary tumorigenesis; aberrant increases in SC35 have been associated with the cancer phenotype and SF2/ASF has been recently identified as a proto-oncogene [48–50].

In our cases of breast cancer, the heterozygous frequency was significantly higher than in controls. This SNP was in linkage disequilibrium with rs3798577 and the haplotype C-G showed a low association with the risk of breast cancer (f_{21}=0.448, p=0.06). The previous studies associated the minor allele A of rs2228480 with an increased risk of breast cancer because of early exposure to estrogen (early onset of menarche) [38, 41, 51]. In line with these studies, we observed an association between this SNP and the age at diagnosis (p=0.047), women possessing the common variant being older at diagnosis.

Rs3798577(T/C) polymorphism is located in the 3'-UTR of ESR1. Although its functionality is not known yet, taken in account that 3'-UTR region is associated with the preferred target for microRNAs and splicing factors, it seems to modulate the ERα expression. Using PUPASUITE or FASTSNP web, we were not able to find modulating factors that could include in their target this polymorphism. Rs2228480 and rs3798577 were associated with survival and risk to develop distant metastasis (risk alleles A and C); G allele for rs2228480 was previously associated with an increased risk for relapse [37]. In accordance with the aforementioned publication, in our patients, the C allele was strongly associated with the risk of breast cancer (p=0.0007). We observed also that the strength of linkage disequilibrium between the two SNPs in the 3' region of ESR1 (rs2228480 and rs3798577) is low (D`=0.471, p=0.03), despite them being separated by just over 1 kilobase. On the other hand, this SNP was correlated with survival (p=0.05), allele C conferring an increased risk for relapses. However, it reached not statistical significance after adjustments for known prognostic factors; the difference in survival could be attributable to the association between this polymorphism with PR status (p=0.01).

In summary, we investigated six SNPs in the ESR1 gene and identified four genotypes significantly correlated either with the risk of breast cancer or some clinicopathological characteristics, suggesting that the main selection criteria of the investigated SNPs (frequency and the position in modulating domains of the gene) are pertinent instruments for establish correlations between the gene structure and the tumor phenotype. In order to understand the implications of risk variants on tumor biology and the importance of SNPs screening for prevention strategies, further genotyping should be performed.

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