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A variant affecting a putative miRNA target site in estrogen receptor (ESR) 1 is associated with breast cancer risk in premenopausal women

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MicroRNAs (miRNAs) negatively regulate expression of target transcripts by hybridization to complementary sites of their messenger RNA targets. Chen *et al.* have described several putative functional single nucleotide polymorphisms (SNPs) in miRNA target sites. Here, we selected 11 miRNA target site SNPs located in 3′ untranslated regions of genes involved in cancer and breast cancer to analyze their impact on breast cancer risk using a large familial study population. Whereas no association was observed for 10 SNPs, a significant association was revealed for the variant affecting a miRNA target site in the estrogen receptor (ESR) 1. Age stratification showed that the association was stronger in premenopausal women [C versus T: odds ratio (OR) = 0.60, confidence interval (CI) = 0.41–0.89, $P = 0.010$]. Furthermore, the effect was stronger in high-risk familial cases (C versus T: OR = 0.42, CI = 0.25–0.71, $P = 0.0009$). Clinical studies have shown that elimination of ESR1 significantly reduces breast cancer risk. Thus, therapies that inhibit ESR1 are used for breast cancer treatment. According to *in silico* analysis, ESR1_rs2747648 affects the binding capacity of miR-453, which is stronger when the C allele is present. In contrast, the T allele attenuates the binding of miR-453, which might lead to a reduced miRNA-mediated ESR1 repression, in consequence higher ESR1 protein levels and an increased breast cancer risk. Thus, the breast cancer protective effect observed for the C allele in premenopausal women is biologically reasonable. The analysis of large study populations in multicentre collaboration will be needed to verify the association and answer questions regarding the possible impact of this variant on therapeutic and clinical outcome.

Introduction

MicroRNAs (miRNAs) are small (~20–22 nt) non-coding RNAs that negatively regulate the expression of target transcripts by hybridization to imperfect complementary sites within the 3′ untranslated regions of their messenger RNA targets, resulting in either the cleavage of target messenger RNAs or repression of their translation (1).

It is estimated that in humans thousands of miRNAs are expressed and >500 miRNAs have been described yet. miRNAs are involved in different biological functions, including developmental pattern formation and embryogenesis, differentiation and organogenesis, growth control and cell death (2,3). Furthermore, miRNAs have shown to be involved in many types of cancers including breast cancer (4–11). About 50% of annotated human miRNAs are detected in regions of the genome known as fragile sites, that are associated with cancer (12). A global decrease in miRNA levels has been observed in human cancers, indicating that small RNAs may have an intrinsic function in tumor suppression (13). For instance, it has been observed that breast carcinomas have decreased expression levels of *miR-125b* compared with normal breast tissues, suggesting that downregulation of *miR-125b* affects the differentiation capability of cancer cells (14). A recent study has identified *miR-335* and *miR-126* as metastasis suppressor miRNAs in human breast cancer (15). Another recent study has revealed that miR-10b is highly expressed in metastatic breast cancer cells and positively regulates cell migration and invasion (16).

Chen *et al.* have predicted miRNA-binding sites throughout the genome (17). Using human SNP genotype data they have estimated that 30–50% of non-conserved miRNA-binding sites in human 3′ untranslated region are functional when miRNA and respective messenger RNA are coexpressed in the same tissue (17). They have shown that polymorphisms in predicted miRNA-binding sites are probably to be deleterious and might be good candidates for causal variants in complex diseases (17).

Recently, functional polymorphisms located in miRNA-binding sites of *SLITRK1* have shown to be associated with Tourette's syndrome (18). Furthermore, a mutation creating a potential illegimate miRNA target site in the myostatin gene has been shown to affect muscularity in sheep (19). Lately, it was demonstrated that aberrant allele frequencies of SNPs located in miRNA target sites are potentially associated with human cancers and Landi *et al.* have reported an association of SNPs in miRNA target sequences and colorectal cancer (20,21). In the present work, we investigated SNPs in miRNA target sites of cancer-relevant genes for its impact on breast cancer risk.

Breast cancer is the most frequent cancer among women in developed countries (22–24). Familial breast cancer constitutes 10% of total breast cancer (25,26). Among these, *BRCA1* and *BRCA2* mutations account for ~20%. Moreover *ATM*, *TP53* and *CHEK2* mutations or variants are responsible for a minor percentage of breast cancer. In addition, frequent low-risk breast cancer variants have been discovered by candidate gene and genome-wide association studies (27–30). However, the majority of familial breast cancer susceptibility variants still remain to be determined.

Based on the data of Chen *et al.* (17), we selected 11 putative functional SNPs in miRNA target sites located in genes related to cancer and breast cancer. These genes are assumed to be involved in cell-cycle control, apoptosis, carcinogen metabolism or DNA repair. Additionally, they are known to be somatically altered in early stages of breast cancer (Table I). We revealed that a variant in the ESR alpha gene (ESR1) affecting a putative miRNA-binding site of miR-453 was significantly associated with familial breast cancer risk, especially in premenopausal and high-risk women.

Table I. The respective genes: cyclin G2 (CCNG2), c-src tyrosine kinase (CSK), ESR1, met proto-oncogene (MET), nuclear receptor interacting protein 1A (NRIP1A), nuclear receptor interacting protein 1B (NRIP1B), p21 protein-activated kinase 6 (PAK6), p300/CBP-associated factor (PCAF), transforming growth factor alpha (TGFA), transforming growth factor beta receptor 1 (TGFBR1), tumor protein p53 inducible nuclear protein 1 (TP53INP1), the SNPs and the miRNAs, whose target sequence is probably affected, as well as the function of the respective genes and their relationship to cancer or breast cancer are shown

Gene	SNP	Gene function	Relation to cancer/breast cancer
CCNG2	rs4150094/hsa-miR-20	Cell-cycle inhibitory gene	Elevated cyclin G2 on breast cancer cell growth (31)
CSK	rs7085/hsa-miR-140	Cell growth and development gene	Overexpressed and highly activated in a wide variety of human cancer including breast cancer (32)
ESR1	rs2747648/hsa-miR-453 −181/−219	Nuclear hormone receptor involved in the regulation of eukaryotic gene expression	Reduction/inhibition of ESR1 significantly reduces breast cancer risk (33,34) and ESR1 inhibitors are used for breast cancer therapy (35,36)
MET	rs1621/hsa-miR199a	Receptor for hepatocyte growth factor and scatter factor	Amplification of MET is associated with various types of cancer (37–40)
NRIP1A	rs1056930/hsa-miR-27a	Modulates transcriptional repression and transcriptional activation	Overexpression of NRIP1 dose dependently inhibits estrogen-dependent reporter activity in human breast cancer cells (41,42)
NRIP1B	rs2822988/hsa-miR-219	Modulates transcriptional repression and transcriptional activation	Overexpression of NRIP1 dose dependently inhibits estrogen-dependent reporter activity in human breast cancer cells (41,42)
PAK6	rs2242119/hsa-miR-27a	p21-activated protein kinase, acts on apoptosis and is involved in the MAP kinase signaling pathway	PAK family may constitute a critical signaling module during tumor progression (43). PAK6 interacts with ESR1 (44)
PCAF	rs4858770/hsa-miR-205	Acts as a histone acetyltransferase to promote transcriptional activation	PCAF is induced by p53 in a panel of breast tumor cell lines (45)
TGFA	rs3771527/hsa-miR-23a	Promotes MAP kinase activity	Many types of human tumors may be driven by a TGF-a/EGFR autocrine loop (46). Expression of TGFA in breast cancer is correlated with poor prognosis (47)
TGFBR1	rs686/hsa-let-7b	Positive regulation of cell proliferation	TGFBR1 tumor-specific mutation in breast cancer (48–50)
TP53INP1	rs896849/hsa-miR-155	Promotes p53/TP53 phosphorylation	Decreased expression of TP53INP1 is involved in breast carcinoma progression (34)

MAP, mitogen-activated protein; PAK, p21 protein-activated kinase.

Materials and methods

Study population

Genotyping analyses were performed on genomic DNA of *BRCA1/2* mutation-negative index patients from 1223 German breast cancer families and 1495 unrelated German controls. Genomic DNA was extracted from the peripheral blood lymphocytes. All breast cancer cases were classified into six categories: (A1) families with two or more cases of breast cancer including at least two cases with onset under the age of 50 years; (A2) families with at least one male breast cancer case; (B) families with one or more cases of breast and at least one ovarian cancer; (C) families with two or more cases of breast cancer including one case diagnosed before the age of 50 years; (D) families with two or more cases of breast cancer diagnosed after the age of 50 years; (E) a single case of breast cancer with diagnosis before the age of 35 years. Thus, we accumulated familial cases and early onset cases, which are more probably to be due to a genetic cause, in our study population. The breast cancer cases comprised unrelated women that had been tested *BRCA1/2* mutation-negative by applying the denaturing high-performance liquid chromatography method on all exons, followed by direct sequencing of conspicuous exons. The blood samples were collected during the years 1997–2007 by seven centers of the German Consortium for Hereditary Breast and Ovarian Cancer (centers of Heidelberg, Würzburg, Cologne, Kiel, Düsseldorf, Berlin and Munich, see authors' affiliations). Index patients were first diagnosed with breast cancer and then referred to a family registry. All breast cancer patients gave an informed consent. The control population included healthy and unrelated female blood donors collected by the Institute of Transfusion Medicine and Immunology (Mannheim), sharing the ethnic background and sex with the breast cancer patients. The age distribution in the controls and cases was nearly identical (controls: mean age 45.6 years, median age 46 years; cases: mean age 45.1 years, median age 45 years). According to the German guidelines for blood donation, all blood donors were examined by a standard questionnaire and gave their informed consent. They were randomly selected during the years 2004–2007 for this study and no further inclusion criteria were applied during recruitment. The study was approved by the Ethics Committee of the University of Heidelberg (Heidelberg, Germany).

SNP selection

Among the polymorphisms in predicted miRNA-binding sites that might have a functional relevance in the study of Chen *et al.*, we selected 11 SNPs located in genes involved in cancer and breast cancer in order to investigate their impact on breast cancer risk: rs4150094 located in cyclin G2 (CCNG2); rs7085 located in c-src tyrosine kinase (CSK); rs2747648 located in estrogen receptor alpha (ESR1); rs1621 located in met proto-oncogene (MET); rs1056930 located in nuclear receptor interacting protein 1A (NRIP1A); rs2822988 located in nuclear receptor interacting protein 1B (NRIP1B); rs2242119 located in p21 protein-activated kinase 6 (PAK6); rs4858770 located in p300/CBP-associated factor (PCAF); rs3771527 located in transforming growth factor alpha (TGFA); rs868 located in transforming growth factor beta receptor 1 (TGFBR1) and rs896849 located in tumor protein p53 inducible nuclear protein 1 (TP53INP1).

In Table I, the respective gene, the SNP and the miRNA, whose target sequence is putatively affected, as well as the function of the respective gene are shown. The selected genes encode proteins shown to be involved in apoptosis, in cell-cycle control, carcinogen metabolism or DNA repair or are known to be somatically altered in early stages of breast cancer.

Genotyping

We performed a case–control study using genomic constitutional DNA of *BRCA1/2* mutation-negative female index patients and female unrelated German controls. The polymorphisms were investigated using TaqMan allelic discrimination. Primers and TaqMan probes were purchased from Applied Biosystems (Foster City, CA) and are listed in supplementary Table 1 (available at *Carcinogenesis* Online). Five nanograms of genomic DNA were used per assay. Polymerase chain reaction was done at 50°C for 2 min, 95°C for 10 min and 40–45× (92°C, 15 s and 60°C, 60 s). Samples were analyzed with the ABI Prism 7900HT detection system using SDS 1.2 software (Applied Biosystems). Genotyping call rates for all studies were >97%. The SNP assays were validated by regenotyping 5% of all samples. The concordance rate for the 11 SNPs varied from 99 to 100%.

Statistical analysis

Hardy–Weinberg equilibrium test was undertaken using the chi-square 'goodness-of-fit' test. Genotype-specific odds ratios (ORs), 95% confidence intervals (CIs) and *P*-values were computed by unconditional logistic regression using a tool offered by the Institute of Human Genetics, Technical University Munich, Munich, Germany (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl), and SAS version 9.1 (SAS Institute, Cary, NC). *P*-values were calculated using two-sided chi-square test. The power calculation was performed using the power and sample size calculation software PS version 2.1.31 (http://www.mc.vanderbilt.edu/prevmed/ps/index.htm). For example, we had an 80% power to detect an OR of 1.45 investigating a SNP with a minor allele frequency of 0.25 and an 80%

power to detect an OR of 1.56 investigating a SNP with a minor allele frequency of 0.10.

SNPs linked to ESR1_rs2747648 with $r^2 \geq 0.8$ and block definition were identified using Haploview version 3.32 (http://www.broad.mit.edu/mpg/haploview). Linked SNPs' breast cancer associations were further checked if they have being analyzed in the Cancer Genetic Markers of Susceptibility genome-wide association study (CGEMS) (http://caintegrator.nci.nih.gov/cgems/browseSetup.do).

Results

Among the SNPs in predicted miRNA-binding sites (17), we selected 11 SNPs to analyze their impact on breast cancer risk (Table I). A case–control study was performed using genomic constitutional DNA of female index patients of 1224 *BRCA1/2* mutation-negative breast cancer families and 1507 female unrelated German controls.

The genotyping results were in Hardy–Weinberg equilibrium in controls for all SNPs investigated. In cases, only for NRIP1A_rs1056930, a borderline significant aberration from Hardy–Weinberg equilibrium was observed ($P = 0.049$).

The 10 SNPs CCNG2_rs4150094, CSK_rs7085, MET_rs1621, NRIP1A_rs1056930, NRIP1B_rs2822988, PAK6_rs2242119, PCAF_rs4858770, TGFA_rs3771527, TGFBR1_rs868 and TP53INP1_rs896849 showed no significant differences in genotype distribution between cases and controls (Table II).

When stratifying for age <50 and ≥50 years, also no significant association was observed either analyzing these 10 SNPs (data not shown). Only TGFBR1_rs868 showed a borderline significant increased risk for heterozygous genotype carriers only in the ≥50 years of age group (OR = 1.25, 95% CI = 1.00–1.57, $P = 0.049$, data not shown) that is most probably a chance effect. Also when investigating only the high-risk cases including risk groups A1 and B, no significant associations were observed for these 10 SNPs (data not shown).

Regarding ESR1_rs2747648 located in estrogen receptor alpha, the C allele was less frequent in cases than in controls (C versus T: OR = 0.73, 95% CI = 0.54–0.97, $P = 0.029$; Table II). The protective effect of the C allele was stronger in homozygous than in heterozygous variant allele carriers indicating an allele dose-dependent association with breast cancer risk ($P_{trend} = 0.031$; Table II).

Table II. Genotype frequencies polymorphisms in breast cancer-related genes in the German study population, ORs with 95% CI and *P*-values

SNP	Genotypes	Cases	Controls	OR	95% CI	*P*
CCNG2_rs4150094, 3′ UTR, hsa-miR-20	AA (%)	1170 (95.6)	1447 (96.0)	1		
	AG (%)	53 (4.3)	60 (4.0)	1.10	0.75–1.59	0.646
	GG (%)	1 (0.1)	0 (0.0)	3.71	0.15–91.15	0.266
	A versus G			1.13	0.78–1.64	0.512
CSK_rs7085, 3′ UTR, hsa-miR-140	CC (%)	605 (50.1)	744 (50.3)	1		
	CT (%)	497 (41.2)	598 (40.5)	1.02	0.87–1.20	0.789
	TT (%)	105 (8.7)	136 (9.2)	0.95	0.72–1.25	0.713
	C versus T			0.99	088–1.12	0.908
ESR1_rs2747648, 3′ UTR, hsa-miR-453, −219/−181	TT (%)	1146 (93.9)	1373 (91.8)	1		
	CT (%)	72 (5.9)	117 (7.8)	0.74	0.54–0.99	0.049
	CC (%)	2 (0.2)	5 (0.33)	0.48	0.09–2.47	0.369
	C versus T			0.73	0.54–0.97	0.029
					[a]$P_{trend} = 0.031$	
MET_rs1621, 3′ UTR, hsa-miR-199a	AA (%)	500 (41.3)	604 (40.4)	1		
	AG (%)	570 (47.0)	691 (46.3)	0.99	0.85–1.17	0.966
	GG (%)	142 (11.7)	199 (13.3)	0.86	0.67–1.10	0.236
	A versus G			0.95	0.85–1.06	0.354
NRIP1A_rs1056930, 3′ UTR, hsa-miR-27a	GG (%)	520 (42.9)	635 (42.5)	1		
	AG (%)	523 (43.2)	669 (44.8)	0.95	0.81–1.12	0.576
	AA (%)	168 (13.9)	189 (12.7)	1.08	0.86–138	0.499
	G versus A			1.02	0.91–1.14	0.758
NRIP1B_rs2822988, 3′ UTR, hsa-miR-219	TT (%)	542 (44.9)	658 (44.3)	1		
	GT (%)	523 (43.3)	663 (44.7)	0.96	0.81–1.12	0.599
	GG (%)	143 (11.8)	163 (11.0)	1.06	0.83–137	0.623
	T versus G			1.02	0.894–1.13	0.899
PAK6_rs2242119, 3′ UTR, hsa-miR-27a	GG (%)	448 (37.0)	545 (36.8)	1		
	GT (%)	566 (46.7)	718 (48.4)	0.96	0.81–1.13	0.622
	TT (%)	197 (16.3)	219 (14.8)	1.10	0.87–1.37	0.441
	G versus T			1.03	0.92–1.15	0.635
PCAF_rs4858770, 3′ UTR, hsa-miR-205	CC (%)	849 (71.0)	1051 (70.1)	1		
	CT (%)	320 (26.8)	405 (27.0)	0.98	0.82–1.16	0.801
	TT (%)	26 (2.2)	43 (2.9)	0.75	0.46–1.23	0.250
	C versus T			0.94	081–1.09	0.419
TGFA_rs3771527, 3′ UTR, hsa-miR-23a	TT(%)	955 (78.9)	1173 (78.9)	1		
	AT(%)	245 (20.2)	302 (20.2)	0.99	0.82–1.20	0.723
	AA(%)	11 (0.9)	18 (1.2)	0.75	0.35–1.59	0.518
	T versus A			0.97	0.82–1.15	0.732
TGFBR1_rs868, 3′ UTR, hsa-let-7b	AA (%)	773 (63.9)	980 (65.8)	1		
	AG (%)	393 (32.5)	461 (31.0)	1.08	0.92–1.27	0.971
	GG (%)	44 (3.6)	48 (3.2)	1.16	0.76–1.77	0.455
	A versus G			1.08	0.94–1.23	0.277
TP53INP1_rs896849, 3′ UTR, hsa-miR-155	TT (%)	868 (71.6)	1018 (72.3)	1		
	CT (%)	313 (25.8)	354 (25.1)	1.04	0.87–1.24	0.354
	CC (%)	31 (2.6)	37 (2.6)	0.98	0.60–1.59	0.483
	T versus C			1.02	0.88–1.18	0.777

SNP, position of the SNP in the respective gene: 3′ untranslated region (UTR) and the miRNA, whose target sequence is probably affected is given.
[a]Chi-square test for trend.

In order to investigate if the association was stronger in premenopausal than in post-menopausal women, we stratified for age <50 and ≥50 years. Remarkably, the risk effect was higher in the younger age group <50 years (C versus T: OR = 0.60, CI = 0.41–0.89, P = 0.010; Table III). Again, an allele dose-dependent association was observed (P_{trend} = 0.012). In contrast, no associations were observed in the older age group ≥50 years (Table III).

Furthermore, the risk effect was stronger in the high-risk cases including risk groups A1 and B (C versus T: OR = 0.53, CI = 0.35–0.79, P = 0.002). Again, the risk effect was stronger in homozygous than in heterozygous variant allele carriers indicating an allele dose-dependent association with breast cancer risk (P_{trend} = 0.002; Table III). Stratification for age revealed that the risk effect was stronger in the age group <50 years of age (C versus T: OR = 0.42, CI = 0.25–0.71, P = 0.0009; P_{trend} = 0.001), whereas no effect was observed in the ≥50 years of age group.

Discussion

To investigate the influence of putative functional polymorphisms in miRNA-binding sites located in cancer-relevant genes on familial breast cancer risk, we performed a case–control study using a familial study population.

The strength of the present study is the large sample size of index cases of breast cancer families. We collected familial cases for our study because it has been shown that the use of familial cases in case–control studies significantly increases the power to detect rare alleles contributing to risk or protective effects in breast cancer (51). To eliminate all effects derived from mutations in the high-penetrance susceptibility genes *BRCA1* and *BRCA2* that account for ~25% of all familial breast cancer (28), only *BRCA1/2* mutation-negative familial breast cancer cases were included in our study.

Ten of eleven investigated SNPs did not show an association with breast cancer risk. We checked if these SNPs have been analyzed in the Cancer Genetic Markers of Susceptibility genome-wide association study (CGEMS) (http://caintegrator.nci.nih.gov/cgems/browseSetup.do). Only TP53IP1_rs896849 and PAK6_rs2242119 have been analyzed in CGEMS. Both did not show any trend for an association with breast cancer risk confirming our results.

The variant allele ESR1_rs2747648 located in the miRNA-binding site in the 3′ untranslated region of the ESR1 indicated an association with familial breast cancer risk in an allele dose-dependent manner (Table II). Stratification for age (≥50 years of age and <50 years of age) revealed that this risk effect was even stronger in the young age (premenopausal) group (C versus T allele: OR = 0.60, CI = 0.41–0.89, P = 0.010; P_{trend} = 0.012), whereas no significant risk effect was observed in the old age (post-menopausal) group (Table III).

The risk effect was stronger in high-risk cases including risk groups A1 and B (C versus T allele: OR = 0.53, CI = 0.35–0.79, P = 0.002) and occurred in an allele dose-dependent manner (P_{trend} = 0.002; Table III). In this high-risk group, the risk effect was also stronger in the <50 years of age group (C versus T: OR = 0.42, CI = 0.25–0.71, P = 0.0009; P_{trend} = 0.001), whereas no significant risk effect was observed in the old age (post-menopausal) group (Table III). The breast cancer categories A1 and B (see Materials and Methods) were chosen as high-risk categories since these categories are based on the most stringent family history inclusion criteria. Additionally, they have shown the highest *BRCA1/2* mutations frequencies in the German study population [37% for A1 and 52% for B (52)]. The observation of a higher risk effect in high-risk familial cases was in line with the higher power that can be expected when focusing on familial, genetically enriched cases (51,53) and case–control studies have reported an increase in risk effects when high-risk cases have been examined (54).

We intended to compare our results with findings from genome-wide studies. However, neither ESR1_rs2747648 itself nor any SNP in linkage disequilibrium of r^2 ≥0.8 with rs2747648 have been analyzed in CGEMS so far. Remarkably, there were no SNPs in linkage disequilibrium of r^2 ≥0.08 with rs2747648 on chromosome 6 at all. ESR1_rs2747648 is located exactly between two haplotype blocks. Thus, no indicator SNP or haplotype for ESR1_rs2747648 has already

Table III. Genotype frequencies of ESR1 polymorphism rs2747648 in the German study population according age stratification and high-risk families, ORs with 95% CI and P-values

SNP	Genotypes	Cases	Controls	OR	95% CI	P
Cases and controls ≥50						
ESR1_rs2747648	TT (%)	301 (93.2)	720 (92.5)	1		
	CT (%)	20 (6.2)	57 (7.3)	0.84	0.49–1.42	0.514
	CC (%)	2 (0.6)	1 (0.2)	4.78	0.43–52.96	0.159
		C versus T		0.98	0.60–1.59	0.931
Cases and controls <50						
ESR1_rs2747648	TT (%)	679 (94.2)	653 (91.1)	1		
	CT (%)	42 (5.8)	60 (8.3)	0.67	0.45–1.01	0.056
	CC (%)	0 (0.0)	4 (0.6)	0.11	0.006–1.99	0.042
		C versus T		0.60	0.41–0.89	0.010
					[a]P_{trend} = 0.012	
High-risk cases						
ESR1_rs2747648	TT (%)	625 (95.4)	1373 (91.8)	1		
	CT (%)	30 (4.6)	117 (7.8)	0.56	0.37–0.85	0.006
	CC (%)	0 (0.0)	5 (0.33)	0.20	0.01–3.62	0.132
		C versus T		0.53	0.35–0.79	0.002
					[a]P_{trend} = 0.002	
Cases high risk and controls <50						
ESR1_rs2747648	TT (%)	420 (95.9)	653 (91.1)	1		
	CT (%)	18 (4.1)	60 (8.3)	0.47	0.27–0.80	0.005
	CC (%)	0 (0.0)	4 (0.6)	0.17	0.01–3.22	0.109
		C versus T		0.42	0.25–0.71	0.0009
					[a]P_{trend} = 0.001	
Cases high risk and controls ≥50						
ESR1_rs2747648	TT (%)	205 (94.5)	720 (92.5)	1		
	CT (%)	12 (5.5)	57 (7.3)	0.74	0.39–1.40	0.355
	CC (%)	0 (0.0)	1 (0.2)	1.17	0.05–28.79	0.594
		C versus T		0.72	0.38–1.35	0.308

[a]Chi-square test for trend.

been analyzed in previous genome-wide or ESR1 SNP-based association studies (55–60).

The steroid estrogen is a strong risk factor for the initiation and progression of breast cancer (61–64). Its effect on the breast epithelium is primarily mediated by ESR1 (also known as ESR-alpha) (65). ESR1 is a member of the nuclear receptor family, a group of hormone-inducible transcription factors that activates gene expression by recruiting multiple coactivators. Clinical and animal studies have shown that reduction of estrogen or elimination of ESR1 significantly reduces breast cancer risk (33,34). Thus, therapies that inhibit estrogen synthesis or block ESR1 are used for breast cancer treatment (35).

The variant ESR1_rs2747648 affects the miRNA-binding site of miR-453, miR-181(b/d) and miR-219. Due to *in silico* analysis using miRanda (http://www.microrna.org/microrna/home.do), the variant ESR1_rs2747648 does not significantly effect the binding capacity of miR-219 and miR-181(b/d). However, the binding capacity of miR-453 is stronger when the C variant allele is present, enabling to bind the complementary G nucleotide of the miR-453 seed. In contrast, the T allele attenuates the binding of miR-453, which we hypothesize to lead to a reduced miRNA-mediated ESR1-repression, in consequence higher ESR1 protein levels and an increased breast cancer risk. Therefore, the breast cancer protective effect observed for the C allele is biologically reasonable. However, functional studies are necessary to test this hypothesis. Due to the fact that endogenous estrogen levels are high premenopausal and drop down post-menopausal, it is plausible that the risk effect of this variant can only be detected in premenopausal women.

Therapeutic agents that decrease the estrogen level or compete with ESR1 such as tamoxifen have contributed at least in part to the decrease in deaths from breast cancer in the past decades (36,66). Furthermore, tamoxifen have been implemented as chemopreventive strategy for premenopausal women at high risk for breast cancer (67). Nevertheless, many breast cancers either fail to respond or become resistant to these endocrine therapies. The genetic region of 6q25 containing ESR1 is lost in a significant number of breast tumors (28,68–72). In addition, somatic alterations of ESR1 have been identified in breast tumor biopsies (73–76). Yet, ~70 to 75% of breast cancers express ESR1, they are estrogen receptor positive (ER+). In contrast to breast cancer risk, where elimination or inhibition of ESR1 was shown to be protective (33,34), these hormone sensitive ER+ breast cancers have a better prognosis than ER− breast cancers. Only ER+ breast cancers respond to endocrine therapy like tamoxifen. It would be interesting to investigate if among hormone-sensitive, ER+ breast cancer patients, carriers of the ESR1_rs2747648 T allele benefit more from an endocrine therapy than C allele carriers and if this variant has an impact on the clinical outcome in general. Furthermore, it will be meaningful to examine if this variant influences the risk for breast cancer in post-menopausal women receiving a hormone replacement therapy. Unfortunately, we do not have data of hormone replacement therapy regarding the study population investigated here. As the breast cancer cases are index cases from breast cancer families, treatment with hormone replacement therapy will most probably be rare. ESR1_rs2747648 is a putative functional candidate SNP in a miRNA target site in a gene with a strong *a priori* biological relevance and probability to be involved in breast carcinogenesis. The protective effect of the C allele that is associated *in silico* with a stronger binding of miR-453, the allele dose dependency of this effect, the observation of the risk effect in the premenopausal group exhibiting high estrogen levels, the large familial study population and the stronger risk effect in the high-risk group of familial cases argue against a chance finding. However, large studies from multicentre collaboration will be needed to verify the association and to answer questions regarding the possible impact of this variant on therapeutic and clinical outcome.

Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

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