

Polymorphisms in RAD51 and their relation with breast cancer in Saudi females

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Abstract: The present study aimed at investigating the relationship between rs1801320 (G>C), rs1801321 (G>T), and rs2619681 (C>T) *RAD51* gene polymorphisms and the risk of breast cancer development in Saudi females. The genotypes were analyzed using TaqMan genotyping assay and polymerase chain reaction-restriction fragment length polymorphism. The genotype and allele frequencies were computed using chi-square or Fisher's exact test (two-tailed) by SPSS 21 software. The results showed that rs1801321G>T GG genotype and G allele frequency were strongly ($P<0.0001$) related to an elevated risk of breast cancer, while the mutant T allele appeared to provide protection against breast cancer development as observed from the significantly lower ($P<0.0001$) frequencies of the TT and GT genotypes in cancer patients compared to the healthy controls. The variant rs1801320G>C showed no significant differences in the frequencies of the genotypes and alleles in the patients and the control groups. The CC genotype and C allele frequency of rs2619681 (C>T) variant were significantly ($P=0.012$) higher in cancer patients, whereas the T allele showed a protective effect against cancer development. The frequencies of the three single-nucleotide polymorphisms did not differ in cancer patients with different tumor grades and human epidermal growth factor receptor 2 status (+ or -). However, the genotype frequency of rs1801320 (135G>C) differed in the patients with estrogen receptor (ER)+ and ER-, where CC genotype showed a significantly higher prevalence in the females with ER- who were suffering from breast cancer. In addition, the frequency of C allele of rs2619681 (C>T) was also significantly higher in the breast cancer patients who were ER+ and progesterone receptor (PR)+ compared to those with ER- and PR-. In the Saudi females, rs1801320 did not show an association with risk of breast cancer. Taken together, the results suggest that RAD51 rs1801321 polymorphism may be involved in the etiology of breast cancer in the Saudi females; however, further studies are necessary to confirm this relation.

Keywords: RAD51, breast cancer, Saudi Arabia, single nucleotide polymorphism

Introduction

DNA damage may be caused by ultraviolet and ionizing radiations, environmental chemical agents, and reactive oxidative stresses resulting from various metabolic processes occurring normally in the body.¹ A variety of mutagens in the environment also cause DNA damage, DNA adducts formation, and breaks in DNA strand.² If left uncorrected, damages to DNA accumulate in cells and may lead to dysregulation of the cell cycle, autonomous and uncontrolled growth and development of such invasive mechanisms, which finally lead to the development of cancer.³ Several mechanisms have been developed by mammalian cells, specific for each type of damage, to repair the damaged DNA, in an attempt to maintain the integrity of the genome. Moreover, the genes responsible for the gene products involved in the repair mechanisms are contemplated as possible cancer susceptibility genes.^{4,5} The proper functioning of the DNA repair mechanisms is essential as this is a means to preserve genomic stability

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and functionality. Several studies have reported that highly penetrant mutations in these genes are possible causative factors in some of the familial cancer syndromes, while a number of low-penetrant single-nucleotide polymorphisms (SNPs) may predispose to the development of different forms of cancers, including breast cancer.⁶⁻⁸

Several molecular epidemiological studies have been performed in an attempt to evaluate the relation between polymorphic sites in the different DNA repair genes and different types of cancer risk in diverse populations.^{9,10} Among the genes, *RAD51* has been considered as a possible candidate due to its central involvement in the repair of double-strand DNA breaks by facilitating homologous pairing and strand exchanges in recombinatory structures.¹¹⁻¹³ The *RAD51* gene, a highly conserved gene found in most eukaryotes, from yeast to humans, encodes for the RAD51 protein. The *RAD51* family members are homologous to the bacterial Rec A and yeast *RAD51*. The RAD51 protein is involved in homologous recombination and interacts directly with X-ray repair cross-complementing proteins 2 and 3 (XRCC2, XRCC3), Breast cancer genes 1 and 2 (BRCA1, BRCA2), and so on to form a complex that is essential during double-strand break repair and DNA cross-links (especially, XRCC2 and XRCC3) and hence plays an essential role in the maintenance of chromosome stability. Several studies have reported that the RAD51 protein exhibits reduced expression in breast cancers, both sporadic and familial, and based on these findings, it was hypothesized that the genetic mutations and polymorphisms within the *RAD51* gene may contribute to the differences observed between individuals with regard to susceptibility to breast cancer tumorigenesis.^{2,8,14,15} Several population-based studies have been conducted, but, interestingly, results from different studies present contradictory results in different populations.¹⁵⁻¹⁹

To the best of our knowledge, since no studies have been reported on the association between SNPs in *RAD51* and susceptibility to breast cancer development in Saudi females, we initiated this study on three SNPs in *RAD51* [rs1801320 (G>C), rs1801321 (G>T), and rs2619681 (C>T)] in a group of Saudi breast cancer patients and compared the results with normal healthy controls. This article presents our findings and shows a close relation between the susceptibility to breast cancer development and some of the polymorphic sites in *RAD51*.

Subjects and methods

The study population

The Saudi population investigated during this study comprised 96 females (median age =48 years) suffering from

breast cancer who attended the outpatient clinics of the clinical coinvestigators (HAK) at King Fahad Medical City Hospital, Riyadh, Saudi Arabia, and 96 age- and sex-matched normal healthy controls who also attended King Fahad Medical City Hospital for minor illnesses and were recruited following diagnostic exclusion of cancer or history of cancer and cancer-related diseases. The patient and control populations were from Saudi Arabian ethnicity, and the majority (86/96) had a sporadic onset. Physical examination was carried out, and demographic data, age at diagnosis, tumor grade, and receptor status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) were recorded. The Institutional Review Board of King Khalid Hospital approved the study (IRB No. 15-089E); written informed consent was obtained from all individuals included in the study.

DNA extraction protocol

Approximately 3 mL blood samples were collected by venipuncture in vacutainer tubes containing ethylenediaminetetraacetic acid from all subjects enrolled in the study and were stored at 4°C until required for DNA extraction. Genomic DNA was extracted using QIAmp DNA Blood Mini Kit (Qiagen N.V., Venlo, the Netherlands), and the manufacturer's instructions were followed. After extraction of the DNA, each sample was subjected to purification, and the DNA was spectrophotometrically quantitated using the NanoDrop 8000 (Thermo Fisher Scientific, Waltham, MA, USA). Purity was determined using the standard A_{260}/A_{280} and A_{260}/A_{230} ratios, and the DNA was stored until required for genotyping.

Genotyping

Three SNPs in *RAD51* were selected and genotyped. Two SNPs [rs1801321 (G>T) and rs2619681 (C>T)] were analyzed using TaqMan allelic discrimination assay and one, rs1801320 (G>C), was analyzed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) (designed using PRIMER3). RFLP primers were validated by sequencing the PCR products. For TaqMan genotyping assay, the primers and probe mixture were purchased directly through the assays-on-demand service of Thermo Fisher Scientific. During analysis, 20 ng of DNA per reaction was used with 5.6 µL of 2× Universal Master Mix and 200 nM primers (Thermo Fisher Scientific). All genotypes were determined by endpoint reading on an ABI 7500 real-time PCR instrument using the Sequence Detection Software (Thermo Fisher Scientific). Negative controls were

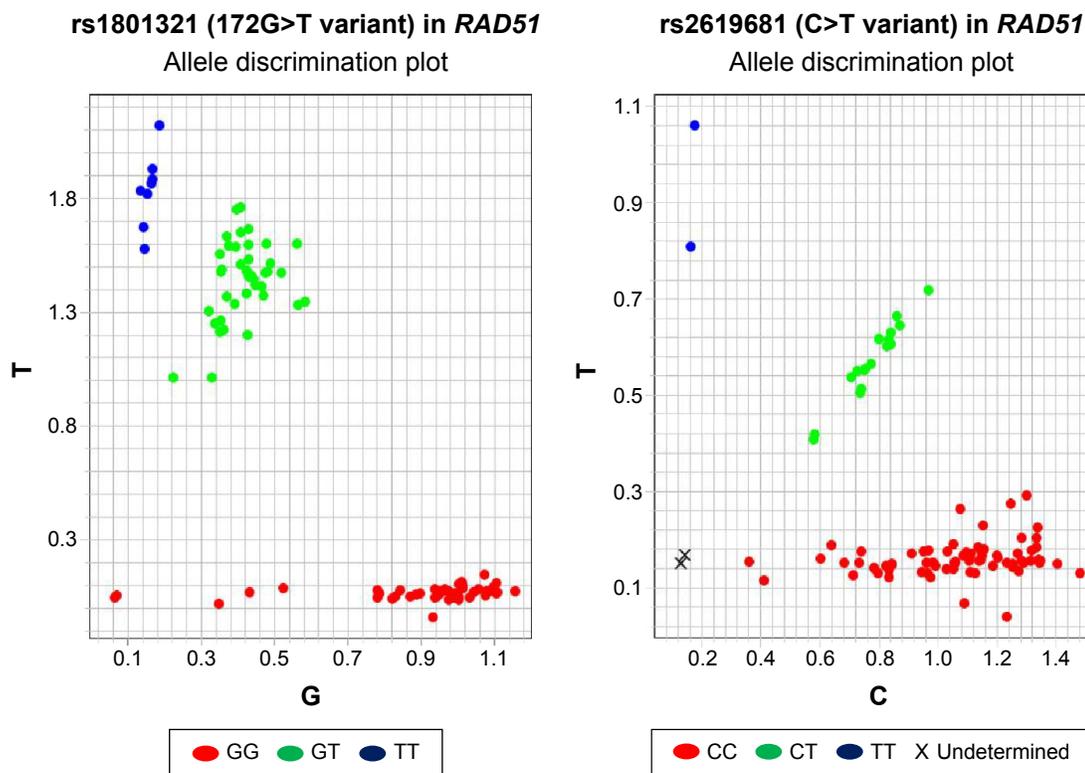


Figure 1 Detection of the rs1801321 (G>T) and rs2619681 (C>T) genotypes in *RAD51* using TaqMan genotyping assay.

used in RFLP and TaqMan assay experiments to reduce experimental errors. Figure 1 presents the results of genotyping using the TaqMan genotyping kit.

For rs1801320 (135G>C) genotyping, the primers used and the fragments obtained after restriction of the PCR product with restriction endonuclease BstNI are presented in Table 1. A total of 2.5 μ L of 10 \times PCR buffer (Qiagen), 2 μ L of 2.5 mM deoxyribonucleotide triphosphate (dNTPs) (Promega Corporation, Fitchburg, WI, USA), 1 μ L from each of primers F and R (5 pmol), 2 μ L of DNA (20 ng/ μ L), and 0.2 μ L of hot start Taq polymerase (Qiagen) were mixed, and the volume was made up to 25 μ L by deionized RNase- and DNase-free water. Cycling parameters presented in Qiagen handbook were followed: initial activation was carried out at 95°C for 15 minutes, followed by 35 cycles of denaturation at

95°C for 40 seconds, annealing at 61.8°C for 40 seconds, and elongation at 72°C for 40 seconds and then the final elongation step at 72°C for 10 minutes. After amplification, the PCR product was subjected to agarose gel electrophoresis to confirm that the amplification had taken place and a 600 bp fragment was obtained. The PCR products were digested with BstNI for 2 hours at 60°C, and the fragments obtained were subjected to electrophoresis in 3% agarose gel. Ethidium bromide was incorporated in the gel for visualization of the DNA fragments under ultraviolet light. For each genotype of the tested SNP, the sizes of the fragments obtained are presented in Table 1. Figure 2 presents an electrophoretogram of the fragments following agarose gel electrophoresis.

As a quality control measure for verification of genotyping procedure for rs1901320 using PCR-RFLP, 5% of the

Table 1 Primers used for amplification of *RAD51* SNP (rs1801320, G>C) and fragments obtained after restriction with BstNI for the three genotypes

RAD51 (rs1801320)	5'-3' primer sequences	Tm	Product size (bp)	RE used	Size (bp) of the fragments obtained after RE treatment
RAD51 F	AAGGGAAGAGGGCAGTCTGT	61.8°C	600	BstNI	GG: 136, 176, 161, 127 bp
RAD51 R	CACAAGTGGACCTCAGTCT				GC: 136/127, 337, 176, 161 bp
					CC: 136, 337, 127 bp

Abbreviations: SNP, single-nucleotide polymorphism; RE, restriction endonuclease; Tm, melting temperature.

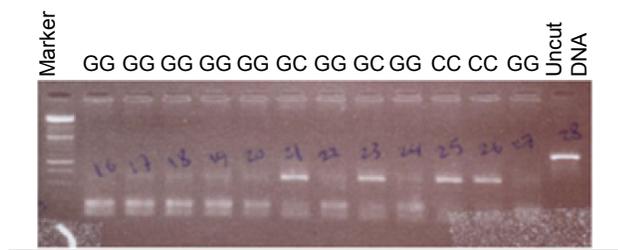


Figure 2 Agarose gel electrophoresis of rs1801320 in *RAD51* 135G>C variant after digestion with BstNI (the genotype is shown at the top of the electrophoretogram).

samples genotyped were randomly selected and subjected to sequencing analysis. The results of the three genotypes are presented in Figure 3 and were reproducible without any discrepancies.

Statistical analysis

Each sample was assigned its genotype. The number of each genotype was obtained manually, and the frequencies of the genotypes and alleles were manually computed. Any deviation from Hardy–Weinberg equilibrium was checked using the website <http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>. The frequencies of the genotypes and alleles in the cases and controls were calculated using the chi-square test and unadjusted odds ratios (ORs), and 95% confidence intervals (CIs) were calculated by Fisher's exact test (two-tailed). Statistical analysis was carried out using SPSS 21 for Windows. A P -value of ≤ 0.05 was considered statistically significant.

Results

Table 2 presents the demographic data and receptor status of the breast cancer patients investigated during this study. The frequency of each genotype and its alleles was calculated in the total patients group and in the patients grouped according to the age of diagnosis, tumor status, and receptor status. The genotype and allele frequencies of the three SNPs investigated in the breast cancer patients and the controls are presented in Table 3. For rs1801321G>T, the majority of breast cancer patients were homozygous for the wild-type G allele and a strong relation was observed between rs1801321 polymorphism and breast cancer susceptibility. The GG genotype and G allele were strongly associated with an elevated risk of breast cancer in the Saudi population with $P < 0.0001$. The mutant T, on the other hand, seems to provide protection against breast cancer development since the frequency of the TT and GT genotype was significantly lower in the patients compared to the controls ($P < 0.0001$). For the rs1801320 G>C, no significant differences were found in the genotype and allele frequencies for the C and G alleles in breast cancer patients and normal controls. For rs2619681 (C>T), the homozygous CC genotype occurred at a significantly higher frequency in the breast cancer patients ($P < 0.05$). When the patients were compared to the control group, the allele frequency showed statistically significant difference ($P < 0.05$) and the OR for the T allele was 0.487. Since the frequency of the T allele was higher in the control group, it showed a protective effect against cancer development.

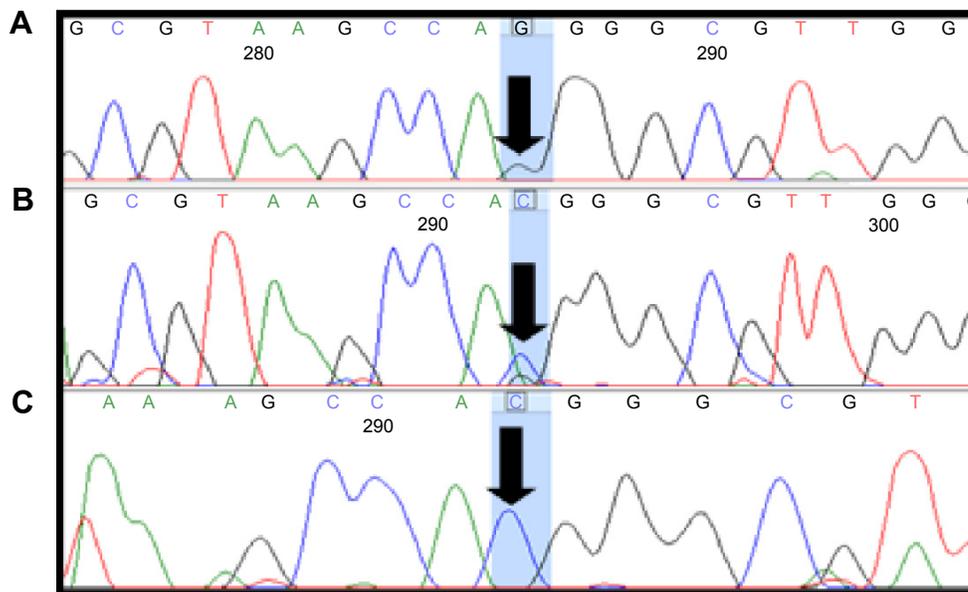


Figure 3 Sequence of the (A) wild-type allele (G), (B) heterozygous allele (GC), and (C) homozygous mutant allele (C) of rs1801320 in *RAD51* (135G>C variant).

Table 2 Demographic data and receptor status of the patients with breast cancer

Patients with cancer	Average age (years)	Tumor grade (number of patients)	ER status (number of patients)	PR status (number of patients)	HER2 status (number of patients)
96	<40=27	I=1	ER+ =55	PR+ =56	HER2+ =41
	>40=71	II=49	ER- =45	PR- =44	HER2- =57
		III=37			
		IV=1			

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

The cancer patients were grouped on the basis of the age of diagnosis, tumor stage, and the ER, PR, and HER2 statuses. The genotype frequencies were calculated, the allele frequencies were obtained from the different groups, and the results were compared. Table 4 summarizes the results obtained. There was no significant difference in the genotype and allele frequencies of the three SNPs in patients who had an early onset of cancer compared to those with a late onset. The frequencies of the three SNPs also did not differ in patients with different tumor grades and HER2 status (+ or -). However, the frequency of rs1801320 (135G>C) differed in the ER+ and ER-, where the CC genotype was significantly higher in the ER- breast cancer females. Finally, the allele

frequency of C allele of rs2619681 C>T was significantly higher in the ER+ and PR+ breast cancer patients compared to that of the ER- and PR- patients. The results of rs2619681 C>T in the PR+ and ER+ breast cancer patients in comparison to those of the PR- and ER- breast cancer patients are presented in Table 5.

Discussion

Research conducted over the last few decades has made it apparent that the pathways involved in the repair of DNA are continuously monitoring chromosomes in an attempt to correct damages caused by endogenous mutagens and exogenous agents, such as ultraviolet light or cigarette smoke.

Table 3 Genotype and allele frequencies of studied SNPs in breast cancer patients and controls

Genotype	Cases	Controls	OR	95% CI	χ^2	P-value
RAD51 rs1801321 G>T						
Genotype frequency						
GG	48 (50%)	0	Ref			
GT	40 (41.666%)	45 (46.875%)	0.009	0.001–0.154	38.41	<0.0001
TT	8 (8.333%)	51 (53.125%)	0.002	0.000–0.030	79.28	<0.0001
GT + TT	48 (50%)	96 (100%)	0.005	0.000–0.086	64.00	<0.0001
Allele frequency						
G	136 (0.708)	45 (0.234)	Ref	0.080–0.199	86.54	<0.0001
T	56 (0.291)	147 (0.765)	0.126			
RAD51 rs1801320 G>C						
Genotype frequency						
GG	59 (59%)	60 (60%)	Ref			
GC	31 (31%)	31 (31%)	1.017	0.550–1.879	0.00	0.957
CC	10 (10%)	9 (9%)	1.130	0.429–2.980	0.06	0.804
GC + CC	41 (41%)	40 (40%)	1.042	0.593–1.833	0.02	0.885
Allele frequency						
G	149 (0.745)	151 (0.755)	Ref	0.671–1.659	0.05	0.817
C	51 (0.255)	49 (0.245)	1.055			
RAD51 rs2619681 C>T						
Genotype frequency						
CC	75 (79.787%)	63 (66.315%)	Ref			
CT	17 (18.085%)	25 (26.315%)	0.571	0.283–1.152	2.48	0.11533
TT	2 (2.127%)	7 (7.368%)	0.240	0.048–1.197	3.50	0.06152
CT + TT	19 (20.21%)	32 (33.68%)	0.499	0.258–0.964	4.35	0.03696
Allele frequency						
C	167 (0.888)	151 (0.794)	Ref	0.274–0.865	6.19	0.01281
T	21 (0.111)	39 (0.205)	0.487			

Abbreviations: SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval; Ref, reference.

Table 6 Frequency of rs1801321 172G>T in RAD51 in different types of cancer in different populations

Cancer types	Population	No of patients studied	Effect	References
<i>RAD51</i> rs1801321 (172G>T)				
Ovarian cancer	Poland	Patients =210	No association	39
Ovarian cancer	Polish	Patients =120, controls =120	No association	40
Breast cancer	Caucasian	Patients =289, controls =548	No association	41
Colorectal cancer	Poland	Patients =320, controls =320	No association	42
Endometrial cancer	Polish	Patients =240, controls =240	No association	43

rs1801320, a G to C transversion at the position +135, and rs1801321, a G to T transversion at the position 172. Both these sites are located in the 5' untranslated region and are believed to have functional relevance. They are shown to affect stability of mRNA or translational efficiency and lead to altered level and function of the polypeptide product (*RAD51* protein), thus influencing the DNA repair capacity to some extent.^{31,32} Reduced expression of the *RAD51* protein is reported in both sporadic and familial breast cancer patients, and based on these findings, it has been hypothesized that the genetic variation within the *RAD51* gene may contribute to carcinogenesis.^{7,8,17,19} It is suggested that these findings also explain observed interindividual differences with regard to breast cancer susceptibility.

Extensive molecular epidemiological studies on the frequency of these polymorphisms in cancer patients and healthy controls in different populations have revealed contradictory findings. With regard to both rs1801320 135G>C and rs1801321 172G>T, studies have shown a close association between this SNP and risk of different cancers. A few of the studies are summarized in Tables 6 and 7. In breast cancer patients, the studies also revealed contradictory results and showed both association and no association.^{7,8,18} This led to meta-analyses of the previous studies by different groups

of researchers, and again, there were contradictions.¹⁹ These differences to some extent were related to the type of breast cancer, ie, sporadic or familial, and were due to different *BRCA1* and 2 statuses of the patients. Since results of the previous studies were inconclusive, a recent extensive meta-analysis was conducted to determine the associations between *RAD51* gene polymorphisms (rs1801320 135G>C and rs1801321 172G>T) and risk of cancer.³³ This meta-analysis was performed on 54 published case-control studies. Among them, 42 studies with 19,142 cases and 20,363 controls were for rs1801320 135G>C polymorphism and 12 studies with 6,646 cases and 6,783 controls for rs1801321 172G>T polymorphism. The overall results showed that for 135G>C (rs1801320) polymorphism, the pooled results indicate significantly increased risk for overall cancers, especially breast cancer, while for rs1801321 172G>T (rs1801321) polymorphism, this meta-analysis showed a decreased cancer risk in head and neck cancers.

Despite the conclusive results of the comprehensive meta-analyses, our study presents another contradictory finding in Saudi breast cancer patients. For rs1801320 135G>C, no association was observed between the genotype and allele frequencies in the breast cancer patients and the normal controls. When the breast cancer patients were grouped

Table 7 Frequency of rs1801320 135G>C in RAD51 in different types of cancer in different populations

Cancer types	Population	No of patients studied	Effect	References
Larynx cancer	Polish	Patients =253	Association	44
Breast cancer	Polish	Patients =790	Association	45
Endometrial cancer	Polish	Patients =230	Association	46
Sporadic breast cancer	Polish	Patients =700, controls =708	Association	47
Ovarian cancer	Poland	Patients =210	Association	39
Breast cancer	Serbian	Patients (<i>BRCA-</i>) with history of cancer =48, patients without history of cancer =107, control group =114	Association	48
Esophageal cancer	Chinese	Patients =123, controls =61	Association	7
Ovarian cancer	Polish	Patients =120, controls =120	Association	40
Breast cancer	Iranian	Patients =294, controls =315	Association	8
Endometrial cancer	Polish	Patients =240, controls =240	Association	43
Acute myeloid leukemia	Chinese	Patients =806, controls =704	Association	49
Colorectal cancer	Poland	Patients =320, controls =320	Association	42

Abbreviation: *BRCA*, breast cancer.

according to the age of diagnosis, tumor grade, and ER, PR, and HER2 statuses of the patients, and the frequency of rs1801320 was compared in the two groups, no significant differences were identified except in those with ER+ and ER– phenotypes, where the homozygous CC genotype occurred at a higher frequency in the individuals who were ER– and the difference compared to the results in ER+ was statistically significant. On the other hand, our results in Saudis show a highly significant association of rs1801321 172G>T with breast cancer, where the G allele was highly predisposing to breast cancer risk and almost 50% of the breast cancer patients had the GG genotype, 41.7% had the GT genotype, and only 8.3% had the TT genotype. On the other hand, none of the control group had the GG genotype, 53.13% had the TT genotype, and 46.88% had the GT genotype. The T allele was highly protective (OR =0.126; 95% CI =0.080–0.199; $P<0.0001$).

The results of this study suggest a relation between RAD51 and susceptibility to develop breast cancer. Interestingly, a polymorphism, a G>T transversion in the 5' untranslated region, which probably affects the stability of mRNA of RAD51, leading to an increase in the rate of its posttranscriptional expression and hence an increase in the level of RAD51 protein decreases the risk of breast cancer. This well known affect is an interaction between several proteins, including BRCA1 and BRCA2, which are the two key players during homologous recombination. Hence, *RAD51* functions as a tumor suppressor gene. This hypothesis needs confirmation, and further detailed investigations are required to work out the mechanism.

As far as rs1801320 was concerned, the results in Saudis are in agreement with the results reported from USA, Poland, and other populations,^{6,17,34–38} and the results of the meta-analysis that did not find any association between this SNP and breast cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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