The Impact of *CASP8* rs10931936 and rs1045485 Polymorphisms as well as the Haplotypes on Breast Cancer Risk: A Case-Control Study

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Abstract

To investigate the association of rs1045485 and rs10931936 in caspase 8 (*CASP8*) and their haplotypes with molecular profile as well as breast cancer in Iran, 287 breast cancer patients and 490 healthy women were genotype using the amplification refractory mutation system and polymerase chain reaction restriction fragment length polymorphism. Results indicated a protective effect for CC genotype of rs1045485 and the decrease risk of breast cancer for C-C haplotype of rs10931936-rs104548 in *CASP8*.

Introduction: Single nucleotide polymorphisms account for most genetic predispositions to breast cancer in the general population. Because of the lack of studies concerning the 2 common polymorphisms in caspase 8 (*CASP8*), namely rs104548 and rs10931936 in Iranian population, we evaluated the association of these 2 polymorphisms and their haplotypes with breast cancer and molecular profile. **Materials and Method:** Blood samples were collected from 287 breast cancer patients and 490 controls. Genotyping of rs1045485 and rs10931936 was conducted using an amplification refractory mutation system and polymerase chain reaction restriction fragment length polymorphism, respectively. PHASE version 2 (Matthew Stephens) was used to estimate the frequencies of haplotypes. Statistical analysis was performed using SPSS 16.0 (SPSS Inc). **Results:** Although hormone receptors and the molecular profile did not indicate any significant association with different genotypes ($P > .05$), patients with CC genotype of rs1045485 were more likely to have HER2-positive breast cancer than those with GG genotype (odds ratio [OR], 2.93; 95% confidence interval [CI], 1.0 4-8.26). In addition, CC genotype of D302H was associated with a decreased risk of breast cancer to 48% (OR, 0.52; 95% CI, 0.30-0.90) whereas no significant association was found between rs10931936 and breast cancer. Haplotype analysis indicated C-C haplotype is associated with the decreased risk of breast cancer (OR, 0.69; 95% CI, 0.52-0.91). **Conclusion:** Our data showed a protective effect for CC genotype of rs1045485 variant and C-C haplotype of rs10931936-rs104548 in *CASP8* in association with the decrease risk of breast cancer whereas rs10931936 showed no significant association. *CASP8* rs1045485 polymorphism might be a candidate genetic marker to evaluate risk of breast cancer. However, further larger studies can confirm such findings.

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Introduction

Being the most prevalent malignancy in women globally, breast cancer is characterized as the main cause of cancer death among women in low-income countries.[1] Despite the higher incidence of breast cancer in developed regions, the survival rates of the disease is disappointingly lower in less developed countries.[2] Detecting the

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disease in the late stages stemming from the dearth of early detection strategies is the likely rationale for the high rates of breast cancer mortality in less developed regions.[3]

As a multifactorial disease genetic and nongenetic factors such as age of menarche, taking oral contraceptives, and body mass index (BMI) take part in the etiology of breast cancer.[4] Regarding the genetic susceptibility, the mutations in high as well as moderate penetrance genes such as *BRCA1*, *BRCA2* (BRCA1, BRCA2 DNA repair associated), *ATM* (ATM serine/threonine kinase), and *PALB2* (partner and localizer of BRCA2) account for only 25% of all hereditary breast cancer cases aggregated in families whereas low penetrance single nucleotide polymorphisms (SNPs) are responsible for most sporadic breast cancer in the general population.[5,6]

Many SNPs associated with cancer susceptibility have been identified through carefully conducted genome-wide association studies (GWAS).[7] The contribution of 2 GWAS confirmed SNPs in the caspase 8 (*CASP8*) gene as one of the key initiators of the apoptosis pathway, namely rs1045485 (D302H) and rs10931936, to breast cancer risk, and have been studied in various ethnicities with controversial results.[8-10] For instance, although MacPherson et al[10] reported a significant association between D302H and breast cancer risk in the United Kingdom population, the locus in a Chinese population was not even polymorphic.[11] Although the aspartate to histidine change at D302H theoretically could affect the auto processing of caspase 8 and consequently its molecular interactions, the effect is yet to be ratified in functional studies. Moreover, rs10931936, which is an intronic variant in *CASP8* has been verified in European as well as Japanese populations as a risky SNP among other risky genetic variants.[12,13] According to the significance of validating the GWAS results in each individual population and because of the lack of such studies regarding rs1045485 (D302H) as well as rs10931936 and their haplotypes in association with breast cancer in Iran, the current study was conducted as a case-control study to appraise such association in Iranian population, to our knowledge, for the first time. Additionally, we tested the hypothesis concerning whether or not there is an association between age at diagnosis, age of menarche, and BMI as breast cancer risk factors as well as tumor hormonal receptor status with the 2 previously mentioned variants.

Materials and Methods

Study Population

The study was approved by the Ethical Committee of Mashhad University of Medical Sciences. Blood samples were collected from breast cancer patients and healthy controls in sterile ethylene diamine tetra acetic acid (EDTA) tubes for DNA extraction. Two hundred eighty-seven patients, who were referred to Omid Hospital, Mashhad University of Medical Sciences, and 490 healthy controls who were referred to clinicians for screening, were included in this study. Written informed consent was obtained from all participants. A questionnaire was used to collect demographic information including age at the time of diagnosis and history of screening and clinical information such as tumor characteristics, type of breast cancer, and age at menarche. The World Health Organization classification was used to classify the tumor types of the breast. They were classified as invasive breast carcinoma (ductal carcinoma) or invasive lobular carcinoma.[14] Additionally, the third group consisted of all other types of breast cancer, which was defined as the rare types of tumor according to Cancer Research UK.[15] Moreover, HER2 status was evaluated using the American Society of Clinical Oncology recommendation for HER2 testing in breast cancer.[16]

Blood Collection and DNA Extraction

We collected 5 mL of peripheral blood in EDTA tubes for DNA extraction using the salting out method. Quality of the extracted DNA was confirmed using electrophoresis on 1% agarose gel. Also, DNA concentration was carried out using the Epoch Microplate Spectrophotometer (BioTek Instruments Inc, Winooski, VT) at a wavelength of 260 nm and 280 nm and stored at −20°C with the concentration of 100 ng/μL.

Genotyping

Genotyping of the rs10931936 and rs1045485 polymorphisms was conducted using the polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) and the amplification refractory mutation system (ARMS), respectively.

We performed ARMS-PCR for rs10931936 in a 10-μL reaction volume containing 4 μL Taq 2x master mix (Ampliqon), 1 μL of each outer primer, and 1.5 μL of each inner primer (10 μM) and 1 μL (100 ng) genomic DNA. The primers used for detection of rs10931936 alleles were as follows: outer forward: 5' TTG AAA AAG AGT CGA GGT AAT TGA C 3'; outer reverse: 5' AGG TTT TCT TCA GTC TCT CTG TGT TCA G 3'; inner forward: 5' AAG CCT ATA CAA TCC TCT GAT TCA TAC ATC 3'; and inner reverse: 5' AAG AAT AGT TGC TGG CTC TAT GAG ATG A 3' to produce products with the size of 382, 246, and 194 base pair (bp). The PCR condition was as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 15 seconds, 63°C for 20 seconds, 72°C for 20 seconds, and 72°C for 5 minutes as the final extension step. The PCR products were separated using electrophoresis in 1.5% agarose gel.

Polymerase chain reaction amplifications for rs1045485 were performed using a forward primer: 5' CAT TTT TGA GAT CAA GCC CCG C 3' and a reverse primer: 5' CCC TTG TCT CCA TGG GAG AGG A 3'.[17] The final volume for each reaction was 15 μL containing 7 μL Taq 2x master mix (Ampliqon), 2 μL (200 ng) genomic DNA, 1.5 μL of each primer (10 μM), and 3 μL water. The PCR condition for the 132 bp PCR product was as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 15 seconds, 62°C for 15 seconds, 72°C for 15 seconds, and 72°C for 5 minutes as the final extension step in a Veriti 96-well PCR Thermal Cycler (Applied Biosystems). The PCR product was then digested by *BstuI* restriction enzyme (New England Biolabs) and 1 X NEBuffer 3.1 at 37°C to form 2 fragments of 112 bp and 20 bp. Agarose gel (3.5%; UltraPure Agarose, Invitrogen) was performed to separate products of RFLP-PCR.

Statistical Analysis

Statistical analysis was performed using SPSS version 16.0 (SPSS Inc). The allele frequencies were tested for deviation of the Hardy–Weinberg equilibrium by using the Pearson χ^2 distribution with 1 degree of freedom. The associations between the risk of breast cancer and its risk factors and genotypes were assessed using binary logistic regression. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for measured risk factors. Logistic

Table 1 Results of Univariate Logistic Regression Analysis on the Association of Demographic Characteristics Between Patients With Breast Cancer and the Healthy Group

Characteristics	Breast Cancer Group	Healthy Group	*P*	OR (95% CI)
Age, Y	47.39 ± 11.00	43.97 ± 11.42	.00007	1.03 (1.01-1.04)
Age at Marriage	20.15 ± 5.61	20.43 ± 4.44	.46818	0.99 (0.96-1.02)
Age at Menarche	12.84 ± 2.21	13.23 ± 1.56	.00938	0.89 (0.91-0.97)
Age at First Gestation	21.54 ± 5.21	22.52 ± 4.54	.01493	0.96 (0.93-0.99)
BMI	27.64 ± 5.38	25.54 ± 4.34	7.6632E-8	1.10 (1.06-1.13)
History of Screening				
Negative	199 (90%)	376 (80.5%)		
Positive	22 (10%)	191 (19.5%)	.00197	0.46 (0.28-0.75)

Data are presented as mean ± SD or n (%) except where otherwise noted.
Abbreviation: BMI = body mass index.

regression was also used to assess the associations of hormone-related risk factors with genetic models in cases. *P* values < .05 were considered as significant. Multivariate logistic regression analysis was used to assess the variables that were independently associated with the risk of breast cancer according to the baseline model. A backward logistic regression (LR) model was performed to choose variables for multivariable analysis. For this purpose, covariates with $P < .05$ in the univariate analysis were entered into the multivariable analysis.

Haplotype frequency distributions were deduced from genotype data and compared between cases and controls using the PHASE software module version 2 (Matthew Stephens).[18,19] Linkage disequilibrium (LD) was calculated using the 2LD program version 1.00 (Jing Hua Zhao).[20] ORs and 95% CIs were calculated to estimate the degree of the association between haplotypes and the risk of breast cancer. *P* values < .05 were considered as significant.

Table 2 Frequency Distribution of Tumor Characteristics of Patients With Breast Cancer

Characteristic	n (%)
Tumor Subtype	
Invasive ductal carcinoma	207 (72.1%)
Invasive lobular carcinoma	4 (1.4%)
Rare types of breast cancer	15 (5.2%)
Unreported	61 (21.3%)
ER	
Negative	62 (21.6%)
Positive	225 (78.4%)
PR	
Negative	73 (25.4%)
Positive	213 (74.2%)
Unreported	1 (0.3%)
HER2	
Negative	170 (59.2%)
Positive	84 (39.3%)
Equivocal	26 (9.1%)
Unreported	7 (2.4%)
Molecular Category	
ER and/or PR^{+} and $HER2^{+/-}$	231 (80.5%)
ER and PR^{-} and $HER2^{+}$	28 (9.8%)
$ER/PR/HER2^{-}$	28 (9.8%)
Stage	
I	39 (13.6%)
II	125 (43.6%)
III	91 (31.7%)
VI	20 (7%)
Unreported	12 (4.2%)

Abbreviations: ER = estrogen receptor; PR = progesterone receptor.

Results

Characteristics of the Study Population

The demographic characteristics of the 2 groups and clinical features of the disease are summarized in Tables 1 and 2. A total of 287 women diagnosed with breast cancer and 490 healthy women were included in this study, with a mean age of 47.39 ± 11.00 and 43.97 ± 11.42 years, respectively. There was a significant difference in age between the 2 groups ($P < .01$). The result indicated that the age of marriage was not different between cases and controls. However, the age of menarche was significantly different between patients and healthy people because this factor was lower in cases compared with the control group ($P = .009$). The age of healthy women at the time of first gestation compared with breast cancer patients was slightly higher with a decreased risk of breast cancer (OR, 0.96; 95% CI, 0.93-0.99; $P = .01$).

A significant difference was observed in the mean BMI ($P < .01$). It was higher in breast cancer patients (27.64 ± 5.38) than in healthy controls (25.54 ± 4.34).

Positive history of breast cancer screening (history of self-examination, ultrasonography, and mammography) was higher in the control group (191 individuals, 19.5%) than for patients (22 individuals, 10%; $P < .05$).

Of 287 breast tumors, 72.1% of them (207 patients) were invasive ductal carcinoma. A large proportion of cases (231 patients, 80.5%) were hormone receptor-positive/HER2-negative. Triple negative breast cancer was observed in 9.8% of patients (28 individuals). Further investigation showed that the most of the patients (164 patients, 57/2%) belonged to the early stage (stage I and II) group.

Table 3 Distribution of Genotypes and Allele Frequency of rs10931936 and rs1045485 Polymorphisms in Patients With Breast Cancer and Controls

Genetic Model	Genotype	Breast Cancer Group (n = 287), n (%)	Healthy Group (n = 490), n (%)	*P*	OR (95% CI)
rs10931936	CC	151 (52.6)	276 (56.3)		
	CT	111 (38.7)	184 (37.6)	.534020	1.10 (0.81-1.50)
	TT	25 (8.7)	30 (6.1)	.145518	1.52 (0.86-2.68)
Dominant model	TT+CT	136 (47.4)	214 (43.7)		
	CC	151 (52.6)	276 (56.3)	.315487	0.86 (0.43-1.15)
Recessive	TT	25 (8.7)	30 (6.1)		
	CC+CT	262 (91.3)	460 (93.9)	.176630	0.68 (0.39-1.19)
Multiplicative model	C	413 (71.9)	736 (75.1)		
	T	161 (28.1)	244 (24.9)	.172269	1.18 (0.93-1.48)
Additive model	CT	111 (81.6)	184 (86)		
	TT	25 (18.4)	30 (14)	.275494	1.38 (0.77-2.47)
Codominant	CC+TT	176 (61.3)	306 (62.4)		
	CT	111 (38.7)	184 (38.6)	.755163	1.05 (0.78-1.42)
rs1045485	GG	208 (72.5)	307 (62.7)		
	GC	60 (20.9)	129 (26.3)	.036881	0.69 (0.48-0.98)
	CC	19 (6.6)	54 (11)	.019909	0.52 (0.30-0.90)
Recessive model	GG	208 (72.5)	307 (62.7)		
	CC+GC	79 (27.5)	183 (37.3)	.005348	0.64 (0.46-0.88)
Dominant model	GG+GC	268 (93.4)	436 (89)		
	CC	19 (6.6)	54 (11)	.044621	0.57 (0.33-0.99)
Multiplicative model	G	476 (82.9)	743 (75.8)		
	C	98 (17.1)	237 (24.2)	.001056	0.65 (0.50-0.84)
Additive model	GG	208 (77.6)	307 (70.4)		
	GC	60 (22.4)	129 (29.6)	.036881	0.69 (0.48-0.98)
Codominant	GG+CC	227 (79.1)	361 (73.7)		
	GC	60 (20.9)	129 (26.3)	.089800	0.74 (0.52-1.05)

Association Between the 2 Polymorphisms and the Risk of Breast Cancer

A total of 287 breast cancer patients and 490 healthy control subjects were successfully genotyped for rs10931936 and rs1045485 polymorphisms.

The distribution of rs10931936 polymorphism followed Hardy–Weinberg equilibrium in the 2 groups. However, the genotype frequencies of rs1045485 did not conform to the Hardy–Weinberg equilibrium in breast cancer and healthy groups (see Supplemental Tables 1-4 in the online version). To verify the results of the genotyping, 10% of the samples were randomly regenotyped for rs1045485 and the results were consistent with the previously genotyped samples.

The genotype and allele frequencies of the rs10931936 and rs1045485 variants and their association with breast cancer risk are shown in Table 3. No significant association was explored between the *CASP8* rs10931936 polymorphism and breast cancer in any of the genetics models. Moreover, the allelic frequencies were not significantly different between breast cancer cases and control subjects for this SNP. Also, we did not find any association for rs10931936 after adjustment.

Genotype frequencies of rs1045485 were 72.5%, 20.9%, and 6.6% for GG, GC, and CC genotypes in breast cancer patients, respectively. CC and GC genotypes showed significantly higher frequencies in healthy controls than in patients ($P = .03$ and $P = .01$, respectively). CC genotype was associated with the decreased risk of breast cancer to 48% (OR, 0.52; 95% CI, 0.30-0.90). The multiplicative model indicated that the distribution of C allele is higher in controls than in cases. The C allele was associated with the decreased risk of breast cancer to approximately 35% (OR, 0.65; 95% CI, 0.50-0.84; $P = .001$). Also, the 2 groups in either recessive (GG vs. GC+CC) or dominant (GG+GC vs. CC) models were significantly different ($P = .005$ and $P = .04$, respectively). The frequency of GC genotype in the additive model (GG vs. GC) was found to be significantly higher in healthy subjects than in patients (OR, 0.65; 95% CI, 0.48-0.98; $P = .3$). However, there was no association between case and control groups in a codominant model (GG+CC vs. GC; $P = .08$). After adjustment for age, BMI, and history of screening, at the final step of the backward LR model (see Supplemental Tables 5-8 in the online version), rs1045485 still was significantly associated with breast cancer.

Association of rs10931936 and rs1045485 Haplotypes and Risk of Breast Cancer

Haplotype frequencies were investigated in the patient and control groups. Results appeared for all 4 expected haplotypes in our analysis. In this combination, the most frequent for breast cancer patients and controls was C-G (56.6% and 53.9%, respectively) and

Table 4 Association of rs10931936 and rs1045485 Haplotypes and Risk of Breast Cancer

Haplotype rs10931936-rs1045485	Breast Cancer Group (n = 287)	Healthy Group (n = 490)	*P*	OR (95% CI)
C-G	325 (56.6%)	325 (56.6%)		
C-C	88 (15.3%)	208 (21.2%)	**.01**	0.69 (0.52-0.91)
T-G	151 (26.3%)	215 (21.9%)	.30	1.14 (0.89-1.47)
T-C	10 (1.7%)	29 (3%)	.12	0.56 (0.27-1.17)
D' Coefficient = 0.239668				
Diplotypes				
C-G/C-G	108 (37.6%)	163 (33.3%)		
C-G/C-C	30 (10.5%)	81 (16.5%)	**.02**	0.559 (0.34-91)
C-G/T-G	79 (27.5%)	121 (24.7%)	.94	0.985 (0.68-1.43)
C-C/C-C	13 (4.5%)	32 (6.5%)	.16	0.613 (0.31-1.22)
C-C/T-G	28 (9.8%)	42 (8.6%)	.98	1.006 (0.59-1.72)
C-C/T-C	4 (1.4%)	21 (4.3%)	**.03**	0.287 (0.10-0.86)
T-G/T-G	21 (7.3%)	23 (4.7%)	.33	1.378 (0.73-2.61)
T-G/T-C	2 (0.7%)	6 (1.2%)	.41	0.503 (10.10-2.54)
T-C/T-C	2 (0.7%)	1 (0.2%)	.37	3.019 (0.27-33.70)

Data are presented as n (%) except where otherwise noted.
The values in bold represent statistically significant values.

the rare type was T-C (1.7% and 3%, respectively). A significant difference was observed in C-C haplotypes between groups ($P = .01$). Also, C-G/C-C and C-C/T-C diplotypes were associated with the decreased risk of breast cancer. The results are shown in Table 4.

Association Between the 2 Polymorphisms and Breast Cancer Risk Factors and Tumor Receptor Status

The association between *CASP8* genotypes of rs10931936 and rs1045485 and breast cancer risk factors including age at diagnosis, age at menarche, and BMI was considered in breast cancer patients. Any association between these factors and genotypes was not explored ($P > .05$). The results are shown in Table 5.

Statistical analyses of estrogen receptor, progesterone receptor, and receptor-based molecular category in association with different genotypes of rs10931936 and rs1045485 variants did not indicate any significant result ($P > .05$). However, in a comparison of CC genotype of rs1045485 with GG in association with HER2 status suggested that patients with the rare homozygous genotype were more likely to have HER2-positive breast cancer than those with the common homozygous one (OR, 2.93; 95% CI, 1.0 4-8.26). Moreover, the same result was observed in the dominant model (OR, 2.79;

Table 5 Results of Univariate Logistic Regression Analysis on the Association of rs10931936 and rs1045485 Polymorphisms and Demographic Factors in Breast Cancer Patients

Characteristic	rs10931936				rs1045485			
Genotype	**CC**	**CT**	**TT**	***P***	**GG**	**GC**	**CC**	***P***
Age at Diagnosis (as a Continuous Variable)	47.56 ± 11.32	47.10 ± 10.49	47.56 ± 11.54	.945876	47.44 ± 10.87	47.64 ± 11.46	46.00 ± 11.39	.852841
Age at Menarche	12.84 ± 2.21	12.88 ± 1.93	12.68 ± 3.38	.938929	12.74 ± 2.45	13.22 ± 1.40	12.58 ± 1.16	.385894
BMI (as a Continuous Variable)	27.28 ± 4.98	28.18 ± 5.76	27.48 ± 6.03	.454311	27.29 ± 5.40	28.19 ± 4.74	30.30 ± 4.81	.094386
Age at Diagnosis (as a Categorical Variable)								
Age at diagnosis younger than 40 years	36 (24.2)	28 (26.4)	6 (24)		53 (26)	14 (24.1)	3 (16.7)	
Age at diagnosis 40 years or older	113 (75.8)	78 (73.6)	19 (76)	.912804	151 (74)	44 (75.9)	15 (83.3)	.672257
BMI (as a Categorical Variable)								
BMI <25	42 (32.1)	24 (25.3)	7 (31.8)		58 (31.5)	14 (28)	1 (7.1)	
BMI ≥25	89 (67.9)	71 (74.7)	15 (68.2)	.524372	126 (68.5)	36 (72)	13 (92.9)	.150712

Data are presented as mean ± SD or number (%).
Abbreviation: BMI = body mass index.

95% CI, 1.00-7.79). There was also a weak correlation between rs1045485 and HER2 status ($r^2 = 0.152$; $P < .05$). The results are shown in Supplemental Tables 9-14 in the online version.

Further analysis showed no significant difference in genotype frequencies (in all genetic models) between stage categories (early stage vs. late stage). The results are shown in Supplemental Table 15 in the online version.

Discussion

In the current study we evaluated the association of 2 common polymorphisms (rs10931936 and rs1045485) in *CASP8,* previously identified in GWAS, with the risk of breast cancer in an Iranian population. To date, to our knowledge, these SNPs have not been studied in our population. We found a statistically significant association between rs1045485 and the risk of breast cancer in a group of Iranian women with clinically confirmed breast cancer. Indeed, the C allele of rs1045485 showed a protective effect for breast cancer risk in our population, which is consistent with previous studies. The contribution of apoptosis disruption to different cancers including breast cancer has been well established in multiple studies. Different players take part in the development of the apoptosis pathway among which caspase 8 is a key initiator that triggers the external route of the programmed cell death. Because of the importance of evaluating the genetic predisposition to breast cancer in the general population, many studies in various regions have focused particularly on the association of capase-8 common polymorphisms with breast cancer risk.

As reported for other study populations, there is mixed evidence regarding the association of *CASP8* rs1045485 (D302H) with the risk of breast cancer. Compared with the study conducted by MacPherson et al, who first reported a 42% protective effect of the CC genotype of rs1045485 in the United Kingdom population, we observed approximately the similar result of 48% protection for that particular genotype in our population.[10] Similarly Frank et al suggested that patients encoding the C allele of the mentioned variant were associated with the decreased risk of breast cancer in a group of German women with a family history of breast cancer although the results were statistically nonsignificant.[9] Also, in a larger study by the Breast Cancer Association Consortium, Cox et al reported the same protective effect of the CC genotype in association with breast cancer to 26% in a group of patients with European ancestry.[8] However, this variant is very rare (heterozygosity < 0.01) in Asian individuals according to a study in a Japanese population.[13] These findings comply with the fact that fundamental differences in the genetic architecture of various ethnicities result in the variety of important phenotypic characteristics of the disease such as age at onset, tumor histopathologic features, and response to treatments in each individual population. However, the adjustment for the risk factors of breast cancer included in our study, namely age at diagnosis, age at menarche, and BMI, dispelled the concerns of the confounding effect of these factors on the observed association.

Despite the GWAS reported association of rs10931936 with breast cancer,[21] all other replication studies including this study showed no significant association.[12,13]

Haplotypes, as a combination of different alleles at the same chromosome, can increase the power of association studies rather than single loci analysis.[22] According to the results of haplotype analysis, the C-C haplotype was observed in controls more than in cases with a significant difference. The results showed the C allele of rs10931936 is more frequent in healthy people. However, the protective effect of this allele might be masked by the sample size of the tested groups. Also, the C allele of rs1045485 was identified as a protective variant. This result supports the fact that the C-C haplotype might be defined as a protective marker for breast cancer. Similarly, a combination of haplotypes revealed the decreased risk of breast cancer in relationship with C-G/C-C and C-C/T-C diplotypes. On the basis of our data, CC and CT genotypes of rs10931936 as well as CC and CG genotypes of rs1045485 are related to a decline in the susceptibility to breast cancer.

The analysis of the association between rs1045485 and clinicopathological characteristics indicated that breast cancer patients with the CC genotype were more likely to have HER2-positive breast cancer compared with individuals carrying the GG genotype. This association might be a result of the effect of CASP8 protein on HER2 cleavage and activation of the apoptosis pathway.[23] Although it has been observed that the expression of *CASP8* mRNA is lower in tumor tissue than normal breast tissue,[24] the effect of *CASP8* D302H on mRNA and protein expression in breast tissue has not been evaluated. According to our finding regarding association of CC genotypes with breast cancer risk as well as HER2 status, this variant might influence the expression of *CASP8* in mRNA or protein level. Furthermore, activity of CASP8 protein and its interactions with other proteins might be influenced by the change of aspartic acid to histidine. Accordingly, in the relationship between rs1045485 and HER2 status, this variant might be useful for application in personalized medicine, therapeutic intervention, and breast cancer genetic screening.

Because the association studies concerning *CASP8* SNPs have not been done before in our population, we designed this pilot study to estimate the association in our population. Undoubtedly, larger sample sizes must be included in the future studies from which more study power expandable to the whole population would be obtained. Finding novel genetic markers would shed light on the early detection of the disease in women with higher risk for developing breast cancer. Moreover, regarding the lack of functional studies on the rs1045485 variant, which is an aspartic to histidine substitution, studies concerning this issue would further elucidate the story behind such a genetic association. Finally, although the lack of association between rs10931936 and breast cancer was in accordance with the results from studies in other ethnicities, there are few reports about this variant, which suggests future analyses in different ethnicities with acceptable sample sizes.

Conclusion

The current study on D302H in *CASP8* revealed a protective effect for the homozygote form of the rare allele in its association with breast cancer. Similarly, the C-C haplotype of rs10931936 and rs1045485 was identified to have a protective influence. Besides,

rs10931936 failed to show any association with either breast cancer or its risk factors, which is consistent with few previous studies in other populations.

Clinical Practice Points

- To investigate the association of rs1045485 and rs10931936 and their haplotypes with molecular profile as well as breast cancer in Iran, 287 breast cancer patients and 490 healthy women were genotyped using ARMS and PCR-RFLP.
- Results indicated a protective effect for the CC genotype of rs1045485 and a decreased risk of breast cancer for the C-C haplotype of rs10931936-rs104548 in *CASP8*.
- The analysis of the association between rs1045485 and clinicopathological characteristics indicated that breast cancer patients with the CC genotype were more likely to have HER2-positive breast cancer compared with individuals carrying the GG genotype.
- Future studies with larger sample sizes to identify novel genetic markers would shed light on the early detection of the disease in women with a higher risk for developing breast cancer.

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Disclosure

The authors have stated that they have no conflicts of interest.

Supplemental Data

Supplemental figures accompanying this article can be found in the online version at https://doi.org/10.1016/j.clbc.2019.02.011.

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Supplemental Table 1 Hardy–Weinberg Equilibrium of rs1045485 for Breast Cancer Patients

	Genotype				Allele	
Genotype	11	12	22	Total	1	2
Observed	208	60	19	287	0.829268293	0.170731707
Observed Frequencies	0.724738676	0.209059233	0.066202091	1		
Expected	197.3658537	81.26829268	8.365853659	287		
Expected Frequencies	0.687685901	0.283164783	0.029149316	1		
χ^2	0.5729718	5.566011771	13.51745716			
P	0.449080413	0.018312425	0.000236354			
Overall χ^2	19.65644073	Overall *P*	5.39086E-05			

Supplemental Table 2 Hardy–Weinberg Equilibrium of rs1045485 for Healthy Controls

	Genotype				Allele	
Genotype	11	12	22	Total	1	2
Observed	307	129	54	490	0.758163265	0.241836735
Observed Frequencies	0.626530612	0.263265306	0.110204082	1		
Expected	281.6576531	179.6846939	28.65765306	490		
Expected Frequencies	0.574811537	0.366703457	0.058485006	1		
χ^2	2.280195625	14.29692278	22.41057727			
P	0.131035345	0.00015612	2.20158E-06			
Overall χ^2	38.98769568	Overall *P*	3.42E-09			

Supplemental Table 3 Hardy–Weinberg Equilibrium of rs10931936 for Breast Cancer Patients

	Genotype				Allele	
Genotype	11	12	22	Total	1	2
Observed	151	111	25	287	0.719512195	0.280487805
Observed Frequencies	0.526132404	0.386759582	0.087108014	1		
Expected	148.5792683	115.8414634	22.57926829	287		
Expected Frequencies	0.517697799	0.403628792	0.078673409	1		
χ^2	0.039439836	0.202343507	0.259527542			
P	0.842579837	0.652835847	0.610444926			
Overall χ^2	0.501310885	Overall *P*	0.778290491			

Supplemental Table 4 Hardy–Weinberg Equilibrium of rs10931936 for Healthy Controls

	Genotypes				Alleles	
Genotype	11	12	22	Total	1	2
Observed	276	184	30	490	0.751020408	0.248979592
Observed Frequencies	0.563265306	0.375510204	0.06122449	1		
Expected	276.3755102	183.2489796	30.3755102	490		
Expected Frequencies	0.564031653	0.373977509	0.061990837	1		
χ^2	0.000510204	0.003077952	0.004642158			
P	0.981979157	0.955756622	0.945679456			
Overall χ^2	0.008230314	Overall *P*	0.995893298			

Supplemental Table 5 Results of Correlation Analysis Between Demographic Variables

	Age	Age at First Gestation	Age at First Menstruation	BMI	Patient History in Screening
Age					
Pearson correlation	1	−0.187[a]	0.008	0.236[a]	0.202[a]
Significance (2-tailed)		0.000	0.837	0.000	0.000
n	770	618	708	715	685
Age at First Gestation					
Pearson correlation	−0.187[a]	1	0.028	−0.231[a]	0.124[a]
Significance (2-tailed)	0.000		0.494	0.000	0.003
n	618	622	588	573	570
Age at First Menstruation					
Pearson correlation	0.008	0.028	1	−0.080[b]	0.053
Significance (2-tailed)	0.837	0.494		0.039	0.176
n	708	588	710	661	653
BMI					
Pearson correlation	0.236[a]	−0.231[a]	−0.080[b]	1	−0.009
Significance (2-tailed)	0.000	0.000	0.039		0.816
n	715	573	661	716	638
Patient History in Screening					
Pearson correlation	0.202[a]	0.124[a]	0.053	−0.009	1
Significance (2-tailed)	0.000	0.003	0.176	0.816	
n	685	570	653	638	688

Abbreviation: BMI = body mass index.
[a]Correlation is significant at the .05 level (2-tailed).
[b]Correlation is significant at the .01 level (2-tailed).

Supplemental Table 6 Results of Backward LR to Choose Appropriate Variables for Multivariable Logistic Regression Variables in the Equation

	Significance	Exp(B)	95% CI for Exp(B)	
			Lower	Upper
Step 1[a]				
Age	.077	1.017	0.998	1.037
Age at first gestation	.694	0.991	0.950	1.035
Age at menarche	.360	0.947	0.843	1.064
BMI	.002	1.076	1.028	1.126
History of screening (1)	.004	0.436	0.249	0.766
Constant	.041	0.078		
Step 2[a]				
Age	.061	1.018	0.999	1.037
Age at menarche	.357	0.947	0.843	1.064
BMI	.001	1.077	1.029	1.127
History of screening (1)	.003	0.429	0.246	0.749
Constant	.009	0.061		
Step 3[a]				
Age	.074	1.017	0.998	1.036
BMI	.001	1.080	1.033	1.129
History of screening (1)	.003	0.425	0.243	0.741
Constant	.000	0.029		

Abbreviations: BMI = body mass index; LR = logistic regression.
[a]Variable(s) entered in step 1: age, age at first gestation, age at first menses, BMI, history of screening.

Supplemental Table 7 Multivariate Logistic Regression Analysis for rs1045485

	Significance	Exp(B)	95% CI for Exp(B)	
			Lower	Upper
Age	.001	1.028	1.011	1.045
BMI	.000	1.074	1.032	1.118
History of Screening (1)	.000	0.381	0.221	0.655
rs1045485	.001			
rs1045485 (1)	.022	0.603	0.390	0.930
rs1045485 (2)	.002	0.304	0.142	0.649

Abbreviation: BMI = body mass index.

Supplemental Table 8 Multivariate Logistic Regression Analysis for rs10931936

	Significance	Exp(B)	95% CI for Exp(B)	
			Lower	Upper
Age	.001	1.028	1.012	1.045
BMI	.001	1.070	1.028	1.113
History of Screening(1)	.000	0.383	0.223	0.657
rs10931936	.805			
rs10931936 (1)	.511	1.131	0.784	1.631
rs10931936 (2)	.896	1.052	0.495	2.235

Abbreviation: BMI = body mass index.

Supplemental Table 9 Results of Univariate Logistic Regression Analysis of the Association of rs1045485 and rs10931936 Polymorphisms and Estrogen Receptor

	Receptor Status	Estrogen Receptor			
Genetic Model	Genotypes	Negative, n (%)	Positive, n (%)	OR (95% CI)	*P*
rs1045485	GG	46 (74.2)	162 (72)		
	GC	12 (19.4)	48 (21.3)	1.14 (0.56-2.32)	.726049
	CC	4 (6.5)	15 (6.7)	1.07 (0.34-3.37)	.914801
Dominant model	CC	4 (6.5)	15 (6.7)		
	GC+GG	58 (93.5)	210 (93.3)	1.04 (0.33-3.24)	.951917
Recessive model	GG	16 (25.8)	63 (28)		
	GC+CC	46 (74.2)	162 (72)	1.12 (0.59-2.12)	.732133
rs10931936	CC	35 (56.5)	116 (51.6)		
	CT	21 (33.9)	90 (40)	1.29 (0.71-2.37)	.40657
	TT	6 (9.7)	19 (8.4)	0.96 (0.35-2.58)	.928315
Dominant model	CC	35 (56.5)	116 (51.6)		
	CT+TT	27 (43.5)	109 (48.4)	1.22 (0.69-2.15)	.494561
Recessive model	TT	6 (9.7)	19 8.4)		
	CC+CT	56 (90.3)	206 (91.6)	0.86 (0.33-2.26)	.760673

Supplemental Table 10 Results of Univariate Logistic Regression Analysis of the Association of rs1045485 and rs10931936 Polymorphisms and Progesterone Receptor

	Receptor Status	Progesterone Receptor			
Genetic Model	Genotypes	Negative, n (%)	Positive, n (%)	OR (95% CI)	*P*
rs1045485	GG	54 (74)	154 (72.3)		
	GC	13 (17.8)	46 (21.6)	1.24 (0.62-2.47)	.539599
	CC	6 (8.2)	13 (6.1)	0.76 (0.28-2.10)	.595983
Dominant model	CC	6 (8.2)	13 (6.1)		
	GC+GG	67 (91.8)	200 (93.9)	0.73 (0.27-1.99)	.532484
Recessive model	GG	54 (74)	154 (72.3)		
	GC+CC	19 (26)	59 (27.7)	0.92 (0.50-1.68)	.78194
rs10931936	CC	41 (56.2)	109 (51.2)		
	CT	25 (34.2)	86 (40.4)	1.29 (0.73-2.29)	.377296
	TT	7 (9.6)	18 (8.5)	0.97 (0.38-2.49)	.944856
Dominant model	CC	41 (56.2)	109 (51.2)		
	CT+TT	32 (43.8)	104 (48.8)	1.22 (0.72-2.09)	.461542
Recessive model	TT	7 (9.6)	18 (8.5)		
	CC+CT	66 (90.4)	195 (91.5)	0.87 (0.35-2.18)	.766479

Supplemental Table 11 Results of Univariate Logistic Regression Analysis of the Association of rs1045485 and rs10931936 Polymorphisms and HER2 Receptor

Genetic Model	Receptor Status	HER2 Receptor			
	Genotype	Negative, n (%)	Positive, n (%)	OR (95% CI)	*P*
rs1045485	GG	130 (76.5)	57 (67.9)		
	GC	33 (19.4)	18 (21.4)	1.24 (0.65-2.39)	.51241
	CC	7 (4.1)	9 (10.7)	2.93 (1.04-8.26)	**.041754**
Dominant model	CC	7 (4.1)	9 (10.7)		
	GC+GG	163 (95.9)	75 (89.3)	2.79 (1.00-7.79)	**.049402**
Recessive model	GG	130 (76.5)	57 (67.9)		
	GC+CC	40 (23.5)	27 (32.1)	1.54 (0.86-2.75)	.144172
rs10931936	CC	89 (52.4)	40 (47.6)		
	CT	63 (37.1)	39 (46.4)	1.38 (0.80-2.38)	.250851
	TT	18 (10.6)	5 (6)	0.62 (0.21-1.78)	.373049
Dominant model	CC	89 (52.4)	40 (47.6)		
	CT+TT	81 (47.5)	44 (52.4)	1.21 (0.72-2.04)	.477934
Recessive model	TT	18 (10.6)	5 (6)		
	CC+CT	152 (89.4)	79 (94)	0.53 (0.19-1.49)	.232032

The values in bold represent statistically significant values.

Supplemental Table 12 Results of Univariate Logistic Regression Analysis on the Association of rs1045485 and rs10931936 Polymorphisms and Receptor Status

Genetic Model	Receptor Status	Receptor Status				
	Genotype	TNBC, n (%)	$HER2^+$, n (%)	ER/PR^+, n (%)	OR (95% CI)	*P*
rs1045485	GG	167 (72.3)	19 (67.9)	22 (78.6)		
	GC	49 (21.2)	2 (25)	4 (14.3)	0.87 (0.54-1.40)	.56904
	CC	15 (6.5)	2 (7.1)	2 (7.1)	1.03 (0.50-2.11)	.933469
Dominant model	CC	15 (6.5)	2 (7.1)	2 (7.1)		
	GC+GG	216 (93.5)	26 (9.2.9)	26 (9.2.9)	1.06 (0.52-2.16)	.869498
Recessive model	GG	167 (72.3)	19 (67.9)	22 (78.6)		
	GC+CC	64 (27.7)	9 (32.1)	6 (21.4)	0.91 (0.60-1.39)	.658601
rs10931936	CC	121 (52.4)	14 (50)	16 (57.1)		
	CT	91 (39.4)	11 (39.3)	9 (32.1)	0.89 (0.60-1.33)	.580909
	TT	19 (8.2)	3 (10.7)	3 (10.7)	1.13 (0.61-2.10)	.694974
Dominant model	CC	121 (52.4)	14 (50)	16 (57.1)		
	CT+TT	110 (47.6)	14 (50)	12 (42.9)	0.94 (0.65-1.36)	.736609
Recessive model	TT	19 (8.9)	3 (10.7)	3 (10.7)		
	CC+CT	212 (91.8)	25 (89.3)	25 (89.3)	1.18 (0.65-2.15)	.579693

Abbreviations: ER = estrogen receptor; PR = progesterone receptor; TNBC = triple-negative breast cancer.

Supplemental Table 13 Correlation Analysis of rs1045485 and HER2 Status

	rs485code	HER2 Status
rs485code		
Pearson correlation	1	0.152[a]
Significance (2-tailed)		.011
n	777	280
HER2 Status		
Pearson correlation	0.152[a]	1
Significance (2-tailed)	.011	
n	280	280

[a]Correlation is significant at the .05 level (2-tailed).

Supplemental Table 14 Correlation Analysis of Dominant Model of rs1045485 and HER2 Status

	HER2 Status	485 Dominant
HER2 Status		
Pearson correlation	1	−0.124[a]
Significance (2-tailed)		.038
n	280	280
485 Dominant		
Pearson correlation	−0.124[a]	1
Significance (2-tailed)	.038	
n	280	777

[a]Correlation is significant at the .05 level (2-tailed).

Supplemental Table 15 Results of Univariate Logistic Regression Analysis of the Association of rs1045485 and rs10931936 Polymorphisms and Stage

Genetic Model	Receptor Status	Stage			
	Genotype	Early, n (%)	Late, n (%)	OR (95% CI)	*P*
rs1045485	GG	118 (72)	80 (72.1)		
	GC	37 (22.6)	22 (19.8)	0.88 (0.48-1.60)	.667758
	CC	9 (5.4)	9 (8.1)	1.47 (0.56-3.88)	.43063
Dominant model	CC	9 (5.5)	9 (8.1)		
	GC+GG	155 (94.5)	102 (91.2)	0.66 (0.25-1.71)	.391509
Recessive model	GG	118 (72)	80 (72.1)		
	GC+CC	46 (28)	31 (27.9)	1.01 (0.59-1.72)	.982528
rs10931936	CC	86 (52.4)	56 (50.5)		
	CT	64 (39)	46 (41.4)	1.10 (0.66-1.83)	.702499
	TT	14 (8.5)	9 (8.1)	0.99 (0.40-2.43)	.977759
Dominant model	CC	86 (52.4)	56 (50.5)		
	CT+TT	78 (47.6)	55 (49.5)	1.08 (0.67-1.75)	.746139
Recessive model	TT	14 (8.5)	9 (8.1)		
	CC+CT	150 (91.5)	102 (91.9)	1.06 (0.44-2.54)	.899801