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Original Article

Potential Role of Zinc Finger 365 *rs10822013* and *rs10995190* in Mammographic Density, Sporadic Breast Cancer Risk, and Prognosis

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What's Known

- Zinc Finger protein 365 (*ZNF365*) is one of the prominent loci confirmed in pooled and Genome Wide Association Studies (GWAS) analysis. The association of *rs10822013* and *rs10995190* polymorphisms with the risk of breast cancer in European and East-Asian countries has been confirmed. Moreover, this polymorphism is associated with the mammographic dense area and percent density.

What's New

- The present study illustrated the prognostic role of *rs10822013* and *rs10995190* in breast cancer in a group of the Iranian population.
- The C-A haplotype of *rs10822013-rs10995190* was associated with breast density. According to Kaplan-Meier plots, the AG genotype of *rs10995190* was significantly associated with overall survival in our examined population.

Abstract

Background: Despite suggesting many genetic risk markers as the outcome of Genome-wide association studies (GWAS) for breast cancer, replicating the results in different populations has remained the main issue. In this regard, this study assessed the association of two variations in Zinc Finger 365 (*ZNF365)* in an Iranian population.

Methods: In a case-control study conducted at Mashhad University of Medical Sciences, Mashhad, Iran, between 2017 and 2020, *ZNF365-rs10822013* and *rs10995190* were genotyped using Allele-Specific PCR (AS-PCR). Breast density was assessed using mammography images. PHASE software module version 2 and SPSS version 16.0 were used for haplotype and statistical analyses. Quantitative and qualitative variables were compared between groups using independent *t* tests and Chi square tests, respectively. Binary logistic regression analysis was performed to calculate odds ratios. Multivariate analysis was then undertaken for the baseline variables, with a $P<0.05$ in the univariate analysis. The survival analysis was performed using the Kaplan-Meier method and the log-rank test.

Results: In this survey, 732 females, including 342 breast cancer patients and 390 healthy subjects, were enrolled. *rs10822013*-T allele (P=0.014), *rs10995190*-G allele (P=0.003), and TG haplotype (P=0.002) were significantly associated with the increased risk of breast cancer. Moreover, *rs10995190*-GG genotype (P=0.042) and C-G haplotype (P=0.019) revealed a significant association with better overall survival. However, considered polymorphisms and their haplotypes indicated no association with breast density and clinical features of breast cancer.

Conclusion: *ZNF365* variants might be a potential risk marker of breast cancer in the Iranian population. The interaction between alleles in haplotypes may modulate the amount of the risk conferred by these variants. Further studies on different ethnic groups can validate these results.

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Introduction

Breast cancer is the most prevalent malignancy in women in developed and developing countries.[1] While developed areas indicate a greater incidence the age of diagnosis is younger,[2-4] and

the mortality rate is higher in developing regions.[1] It might be due to the diagnosis at the late stages because of the lack of early diagnostic tests in such areas.[5] While breast cancer has an unknown etiology, there is abundant evidence supporting that genetic variations play a central role in the pathogenesis and progression of the disease. Genetic polymorphisms are critical elements of the differences in breast cancer susceptibility among individuals.[6] However, there is diversity in the distribution of alleles among populations, and various loci represent different risk rates.[3, 7]

Genome-wide association studies (GWAS) have recognized several loci containing common variants that influence breast cancer risk and prognosis confirmed in different ethnicities.[8, 9] Zinc Finger protein 365 (*ZNF365*) is one of the striking loci confirmed in meta-analysis and GWAS analysis.[10-12] *ZNF365* encodes a Zinc Finger protein with several isoforms showing different expression patterns. It acts as a cell cycle regulator, and the lack of its function in the cell causes abnormal recombination and aneuploidy.[13] According to The Human Protein Atlas, RNA expression has been observed in normal breast tissue and MCF7, as a metastatic breast adenocarcinoma cell line. Evaluating breast tumors revealed a lower survival rate associated with a higher expression of the *ZNF365*. On the other hand, another report has represented the lower expression of *ZNF365* in triple-negative breast cancer than others and a better survival associated with its higher expression level.[13, 14] Therefore, data on the importance of *ZNF365* in breast cancer development and prognosis is controversial.

Extensive studies have presented the significant effects of *ZNF365* variants on chromosome 10q21.2 rejoin on breast cancer risk.[11, 15, 16] Genotyping analyses have confirmed the association of *rs10822013* and *rs10995190* polymorphisms with the risk of breast cancer in European and East-Asian populations.[11] *rs10995190* has also been confirmed as a breast cancer susceptibility locus in patients carrying *BRCA2* mutations.[17] Besides, this polymorphism is associated with the mammographic dense area and percent density.[18] Although these variations are located in intronic places, these may confer their function via altering gene expression by affecting the binding of transcription factors to DNA or changing splice sites.[19, 20] According to this information, *ZNF365* may have a potential role in mammographic density, breast cancer development, and prognosis. However, there is no study concerning the association of the two common polymorphisms of this gene in the Iranian population. Therefore, this study was conducted to evaluate the association of *rs10822013* and *rs10995190* with breast density, cancer risk, and prognosis in a group of the Iranian population.

Patients and Methods

The study was approved by the Ethical Committee of Mashhad University of Medical Sciences (Ethical approval number: IR.MUMS.fm.REC.1394.472), and written informed consent was obtained from all participants.

Study Population

The current study was performed at Mashhad University of Medical Sciences, Mashhad, Iran. Sampling was done between 2017 and 2020 based on the convenience sampling method.

The control group included healthy subjects aged between 18-65 years old, referred to clinicians for breast cancer screening, and clinical breast exams (and, in some participants, mammography) confirmed their health. All patients referred to Omid Hospital (Mashhad University of Medical Sciences, Mashhad, Iran) whose breast cancer was confirmed histopathologically, were included in the study without restricting age or histological type. Participants were excluded from the study in case of a positive history of hereditary cancers in the family after considering pedigrees.

Demographic data such as age at diagnosis, weight, height, history of lactation, history of abortion, physical activity, history of screening, age at menarche and menopause, and age at first pregnancy were collected using a data gathering form. Pathologic, mammographic, and clinical data were extracted from the patient's clinical records. Standard protocols were utilized to categorize the clinical features.[21, 22] Moreover, the mammographic density report was under Breast Imaging-Reporting and Data System (BI-RADS) classifications.[23]

Blood Collection, DNA Extraction, and Genotyping

After obtaining informed consent, 4 mL of peripheral blood sample was collected in K2-EDTA tubes (VACUETTE® TUBE 4 mL K2E K2EDTA, Greiner Bio-One International, Kremsmünster, Austria). Genomic DNA was extracted using the salting-out method.[24] DNA concentration and purity were measured with the Epoch™ Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). The quality of DNA samples was evaluated by 1% agarose gel electrophoresis. Then, samples were stored at -80 °C until being used for analysis.

Genotype analysis of *ZNF365* polymorphisms was performed using Allele-Specific PCR based on allele-specific primers. A PCR reaction tube was prepared to detect each allele in a 10 µL total volume, using 1 µL of DNA (200 ng), 5 µL Taq PreMix Master Mix (Parstous, Mashhad, Iran), 1µL of one of the forward primers (10 µM), 1 µL of common reverse primer (10 µM), and 2 µL nuclease-free water. Primers were designed by Web-based Allele-Specific PCR (WASP) online software (National Biobank of Thailand, Thailand)[20] as follows:

rs10822013 Forward C: 5 CCAGATGGCACAAGAAAATAC 3 (an amplicon with a size of 189 bp)
rs10822013 Forward T: 5 ACCAGATGGCACAAGAAAATGT 3 (an amplicon with a size of 190 bp)
rs10822013 Common Reverse: 5 ATCACCTGGCTGACATGACA 3
rs10995190 Forward G: 5 GTTGTGTCCAAGTGCATATTTAG 3 (an amplicon with a size of 194 bp)
rs10995190 Forward A: 5 GTTGTGTCCAAGTGCATATTGAA 3 (an amplicon with a size of 194 bp)
rs10995190 Common Reverse: 5 TTGCTAGCAACAATGAGGGGTG 3

PCR conditions included 10 min of initial denaturation at 95 °C. Then, 35 cycles were done, including denaturation at 95 °C for 15 sec, annealing at 56 °C for *rs10995190*-G and 58 °C for *rs10995190*-A and *rs10822013* for 15 sec, and extension at 72 °C for 15 sec. The 10 min final extension was performed at 72 °C. Amplification was performed in a Veriti 96 well PCR Thermal Cycler (Applied Biosystems, Foster City, California, United States). Finally, all the samples underwent electrophoresis with 2% agarose gel (figure 1). 10% of samples were randomly re-genotyped to confirm the genotyping data.

Statistical Analysis

The Hardy-Weinberg Equilibrium (HWE) assumption was assessed in the case and control samples using the Chi square test. Descriptive statistics were determined for all variables, including mean±SD, frequency, and percentage. Normal distribution was assessed using the Kolmogorov–Smirnov statistic. Quantitative and qualitative variables were compared between groups using independent *t* tests and Chi Square test, respectively. Binary logistic regression analysis was performed to assess the association between SNPs and the risk of breast cancer and calculate odds ratios. Multivariate analysis was then undertaken for the baseline variables, with a P<0.05 found in the univariate analysis.

The period from breast cancer diagnosis based on the first pathology result until death due to cancer or the end of the study date was defined as overall survival (January 31, 2021). The designed survival curves using the Kaplan-Meier method and the log-rank test were used to estimate differences between the groups. Statistical significance was set at P<0.05. Data were analyzed using SPSS version 16.0 (IBM, USA).

The distribution of haplotypes was assumed using the PHASE program version 2, a program implementing the method for reconstructing haplotypes from population data.[25, 26] Diplotyping was also done using PHASE haplotype outputs. Linkage disequilibrium (LD) was calculated by the 2LD program version 1.00.[27] Using SPSS version 16.0, odds ratios and 95% CI were calculated to estimate the degree of the association between haplotypes and the risk of breast cancer. A statistical P value less than 0.05 was considered significant.

Results

Characteristics of the Study Population

The current study collected 342 breast cancer samples and 390 healthy subjects. The characteristics of the study population are

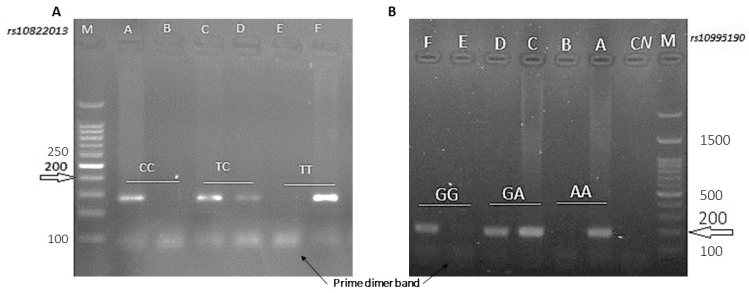

Figure 1: Agarose gel electrophoresis (2%) shows various genotypes of *rs10822013* and *rs10995190* polymorphisms as follows. A: Represent *rs10822013*, lane A, C, and E; Polymerase chain reaction (PCR) product size for allele A (189 bp), lane B, D, and F; PCR product size for allele T (190 bp), lane M; Marker (DNA ladder 50 bp). B: Represent *rs10995190*, lane A, C, and E; PCR product size for allele C (194 bp), lane B, D, and F; PCR product size for allele G (194 bp), lane M; marker (DNA ladder 100 bp), lane CN; negative control.

summarized in table 1. There was no significant difference in the mean age, menarche age, menopause age, and the history of abortion and lactation between cases and controls. However, the age at first pregnancy varied significantly. Evaluation of menopause status indicated that a significantly higher percentage of the patients belonged to the post-menopause group. History of screening (clinical breast exam, mammography, MRI) and BMI were significantly different between cases and controls. Evaluation of breast density indicated higher areas of dense breasts (BI-RADS 3 & 4 or C & D) in breast cancer patients with a significant difference between groups.

Tumor features of breast cancer patients are summarized in table 2. According to the World Health Organization (WHO) classification for the types of breast cancer tumors in 2012,[21] invasive ductal carcinoma (IDC), with 85.9% of all identified types of tumors, indicated the highest frequency. According to the pathologic subtyping, most patients were hormone receptor-positive. Evaluating HER2 status by IHC was performed based on the American Society of Clinical Oncology (ASCO) recommendation for Her2 IHC testing.[22] The results showed that the majority of patients were HER2$^-$.

Association of Breast Cancer Risk, Breast Density, and Clinical Characteristics of the Tumor with Genotypes, Haplotypes, and Diplotypes

Allele and genotype frequencies are summarized in table 3. All genotypic frequencies of *rs10822013* were in Hardy–Weinberg equilibrium in the healthy controls (P=0.89). The T allele, as the risky allele, was significantly higher in the patients than in the controls. The TT genotype, as a risky genotype, indicated significantly higher frequency in cases than in the control group. Evaluation of genetic models revealed that the distribution of the TT genotype compared with C allele carriers (TC+CC) in the recessive model was significantly different between breast cancer and control people.

The genotype frequencies of *rs10995190* followed the Hardy–Weinberg equilibrium in controls (P=0.90). There was a significant difference in allele frequency between patients and healthy individuals. GG genotype was the most frequent genotype in breast cancer, with a higher frequency in cases than in controls. Furthermore, the dominant model indicated a significant difference between the groups, as the GG+GA genotypes were higher in patients than in controls. A significant difference was also observed between groups in the recessive model.

Further investigation indicated no significant difference in the distribution of genotypes and alleles of *ZNF365 rs10822013* and *rs10995190* between dense and non-dense breasts and tumor characteristics.

Table 1: The characteristics and cancer risk factors in healthy controls and breast cancer patients

Characteristic		Breast cancer	Control	OR (95% CI)	P value
Age		47.02±10.68	45.41±11.60	1.01 (1.00-1.03)	0.052
Age	Age of diagnosis<40	83 (24.5%)	137 (35.2%)		
	Age of diagnosis≥40	256 (75.5%)	252 (64.8%)	1.67 (1.21-2.32)	0.002
Age of menarche		13.07±1.64	13.27±1.62	1.08 (0.98-1.19)	0.107
Age of menopause[a]		47.24±5.77	48.19±5.31	1.03 (0.98-1.08)	0.215
Age of the first gestation		21.29±5.04	22.4±4.57	1.06 (1.02-1.10)	0.001
Menopause status	Pri & pre-menopause	192 (59.1%)	283 (73.9%)		
	Post-menopause	133 (40.9%)	100 (26.1%)	1.96 (1.43-2.69)	<0.001
History of lactation	Negative	16 (5.4%)	10 (3.3%)		
	Positive	283 (94.6%)	289 (96.7%)	0.61 (0.27-1.37)	0.233
History of abortion	Negative	188 (64.8%)	202 (68.7%)		
	Positive	102 (35.2%)	92 (31.3%)	1.19 (0.84-1.68)	0.320
History of screening	Negative	250 (89.6%)	295 (79.7%)		
	Positive	29 (10.4%)	75 (20.3%)	0.46 (0.29-0.72)	0.001
Body Mass Index		27.61±5.08	25.56±4.39	1.10 (1.06-1.13)	<0.001
Body Mass Index	BMI<25	93 (28.8%)	181 (48.7%)		
	BMI≥25	230 (71.2%)	191 (51.3%)	2.34 (1.71-3.21)	<0.001
Physical activity	Negative	91 (39.7%)	33 (11.5%)		
	Positive	138 (60.3%)	253 (88.5%)	0.20 (0.13-0.31)	<0.001
Density[b]	Non-dense (A-B)	58 (43.0%)	71 (68.9%)		
	Dense (C-D)	77 (57.0%)	32 (31.1%)	2.91 (1.71-4.96)	<0.001

[a]The age of the last menstrual cycle in individuals with natural menopause. [b]Density has been categorized based on BI-RADS classification. Data are presented as mean±SD or n (%). Quantitative and qualitative variables were compared between groups using independent *t* tests and Chi square test, respectively. Binary logistic regression analysis was used to calculate odds ratios. A statistical P value less than 0.05 was considered significant.

Table 2: Distribution of tumor characteristics of breast cancer cases

Characteristics		N (%)
Tumor subtype	Invasive Ductal Carcinoma	267 (78.1)
	Precursor lesions	13 (3.8)
	Invasive Lobular Carcinoma	9 (2.6)
	Others	22 (6.4)
	Unreported	31 (9.1)
Grade	Low (I & II)	198 (57.9)
	High (III)	70 (20.5)
	Unreported	74 (21.6)
Tumor size	T1 & T2	225 (65.8)
	T3 & T4	62 (18.1)
	Unreported	55 (16.1)
Lymph node	Negative	112 (32.7)
	Positive	168 (49.2)
	Unreported	62 (18.1)
Metastasis	Negative	266 (77.8)
	Positive	16 (4.7)
	Unreported	60 (17.5)
Stage	Early (I & II)	175 (51.2)
	Late (III & IV)	103 (30.1)
	Unreported	64 (18.7)
ER status	Negative	75 (21.9)
	Positive	235 (68.7)
	Unreported	32 (9.4)
PR status	Negative	87 (25.4)
	Positive	223 (65.2)
	Unreported	32 (9.4)
HER2	Negative	199 (58.2)
	Positive	82 (24.0)
	Equivocal	21 (6.1)
	Unreported	40 (11.7)
Receptor status	luminal A & B	241 (70.5)
	HER2 overexpression	31 (9.1)
	TNBC	34 (9.9)
	Unreported	36 (10.5)

*Data is according to the immunochemistry (IHC) test. Data are presented as number (%). ER: Estrogen Receptor; PR: Progesterone Receptor; HER2: Human Epidermal Growth Factor Receptor 2; TNBC: Triple-negative breast cancer.

The frequencies of haplotypes and diplotypes are shown in table 4. The most frequent haplotype was T-G, with a significant difference between groups. The C-A haplotype was also significantly different between patients and healthy groups.

PHASE software identified nine diplotypes according to genotype data. Four diplotypes, including T-G/C-A, C-G/T-G, T-G/T-G, and T-G/T-A, had frequencies over 10%. The comparison of diplotype distribution between breast cancer cases and healthy controls indicated that the frequency of T-G/T-G diplotype was significantly different between cases and controls.

Evaluation of dense and non-dense breasts indicated a significant difference in the C-A haplotype. However, there was no association between haplotypes/diplotypes and clinical features of breast cancer, including grade, stage, hormone receptors, and HER2 status.

Survival Analysis in Association with Genotypes, Haplotypes, and Diplotypes

Univariate Cox regression analysis indicated the stage (Late vs. Early) (P=0.007, HR=3.17, 95% CI [1.36-7.38]), ER status (Positive vs. Negative) (P<0.001, HR=0.19 95% CI [0.08-0.41]) and PR status (Positive vs. Negative) (P=0.002, HR=0.29, 95% CI [0.13-0.64]) were associated with overall survival. Results of multivariate analysis demonstrated that the stage (P=0.024, HR=2.67, 95% CI [1.14-6.29]) of the disease and ER status (P=0.001, HR=0.26, 95% CI [0.11-0.60]) were independently and significantly associated with overall survival. Therefore, the survival analysis of polymorphisms, haplotypes, and diplotypes was adjusted for pathologic features.

According to Kaplan-Meier plots, the AG genotype of *rs10995190* was significantly associated with overall survival. Patients carrying *rs10995190* AG compared with GG

Table 3: Distribution of genotypes and alleles of *ZNF365 rs10822013* and *rs10995190* polymorphisms in breast cancer/healthy and dense/non-dense groups

Genetic model	Genotype	Breast cancer	Control	OR (95%CI)	P value
rs10822013	CC	45 (13.2%)	68 (17.4%)	Reference	
	TC	157 (45.9%)	195 (50.0%)	1.22 (0.79-1.87)	0.373
	TT	140 (40.9%)	127 (32.6%)	1.67 (1.07-2.60)	0.025
Dominant	CC	45 (31.3%)	68 (17.4%)	Reference	
	TC+TT	297 (86.8%)	322 (82.6%)	1.39 (0.93-1.10)	0.111
Recessive	CC+TC	202 (59.1%)	263 (67.4%)	Reference	
	TT	140 (40.9%)	127 (32.6%)	1.44 (1.06-2.94)	0.019
Multiplicative	C	247 (36.1%)	331 (42.4%)	Reference	
	T	437 (63.9%)	449 (57.6%)	1.30 (1.06-1.61)	0.014
rs10995190	AA	18 (5.3%)	42 (10.8%)	Reference	
	GA	144 (42.1%)	178 (45.6%)	1.89 (1.04-3.42)	0.036
	GG	180 (52.6%)	170 (43.6%)	2.47 (1.37-4.46)	0.003
Dominant	AA	18 (5.3%)	42 (10.8%)	Reference	
	GA+GG	324 (94.7%)	348 (89.2%)	2.17 (1.23-3.85)	0.008
Recessive	AA+GA	162 (47.4%)	220 (56.4%)	Reference	
	GG	180 (52.6%)	170 (43.6%)	1.44 (1.07-1.93)	0.015
Multiplicative	A	180 (26.3%)	262 (33.6%)	Reference	
	G	504 (73.7%)	518 (66.4%)	1.42 (1.13-1.78)	0.003
		Non-dense breast (129)	Dense breast (109)		
rs10822013	CC	21 (16.3%)	13 (11.9%)	Reference	
	TC	67 (51.9%)	48 (44.0%)	1.16 (0.53-2.54)	0.715
	TT	41 (31.8%)	48 (44.0%)	1.89 (0.84-4.24)	0.122
Dominant	CC	21 (16.3%)	13 (11.9%)	Reference	
	TC+TT	108 (83.7%)	96 (88.1%)	1.44 (0.68-3.02)	0.341
Recessive	CC+TC	88 (68.2%)	61 (56.0%)	Reference	
	TT	41 (31.8%)	48 (44.0%)	1.69 (0.99-2.87)	0.052
Multiplicative	C	109 (42.2%)	74 (33.9%)	Reference	
	T	149 (57.8%)	144 (66.1%)	1.42 (0.98-2.07)	0.064
rs10995190	AA	11 (8.5%)	8 (7.3%)	Reference	
	GA	56 (43.4%)	41 (37.6%)	1.01 (0.37-2.72)	0.990
	GG	62 (48.1%)	60 (55.0%)	1.33 (0.50-3.54)	0.567
Dominant	AA	11 (8.5%)	8 (7.3%)	Reference	
	GA+GG	118 (91.5%)	101 (92.7%)	1.18 (0.46-3.04)	0.736
Recessive	AA+GA	67 (51.9%)	49 (45.0%)	Reference	
	GG	62 (48.1%)	60 (55.0%)	0.76 (0.45-1.26)	0.283
Multiplicative	A	78 (30.2%)	57 (26.1%)	Reference	
	G	180 (69.8%)	161 (73.9%)	0.82 (0.55-1.22)	0.325

Data are presented as n (%). Genotypes and alleles were compared between groups using Chi square. Binary logistic regression analysis was used to calculate odds ratios. A statistical P value less than 0.05 was considered significant.

genotype ones were significantly associated with overall survival. This result was also observed in the recessive model (GG v. AG+AA). However, after adjustment for stage and ER status, the GG genotype was significantly related to better overall survival in the recessive model (P=0.042, HR=0.37 95% CI [0.14-0.97]). Moreover, *rs10822013* revealed no association with survival both before and after adjustment. The results are shown in figure 2.

Analysis of Kaplan–Meier curves for haplotypes of *rs10822013* and *rs10995190* polymorphisms demonstrated that the C-G haplotype carrying the protective allele of *rs10822013* (C) and risky allele of *rs10995190* (G) was significantly associated with better overall survival. This observation was not changed after adjustment of stage and ER status (P=0.019. HR=0.09, 95% CI [0.01-0.67]). The results are shown in figure 3.

The survival plots designed by the Kaplan-Meier test displayed that breast cancer patients with C-G/T-G diplotype had better overall survival (P=0.030). However, this result was not confirmed after adjustment for stage and ER status. The results are shown in figure 4.

Discussion

In the present study, the role of *ZNF365 rs10822013* and *rs10995190* polymorphisms was evaluated in the mammographic density, breast

Table 4: Association of *ZNF365 rs10822013* and *rs10995190* haplotypes and diplotypes with breast density and the risk of breast cancer

Variables		Breast cancer	Control	OR (95% CI)	P value
Haplotype *rs10822013-rs10995190*	C-G	144 (21.1%)	170 (21.8%)	0.96 (0.75-1.23)	0.730
	T-G	360 (52.6%)	348 (44.6%)	1.38 (1.12-1.70)	0.002
	C-A	103 (15.1%)	161 (20.6%)	0.68 (0.52-0.90)	0.006
	T-A	77 (11.3%)	101 (12.9%)	0.85 (0.62-1.17)	0.323
Diplotype	(T-G/C-A)	77 (22.5%)	92 (23.6%)	0.94 (0.67-1.33)	0.731
	(C-G/T-G)	74 (21.6%)	82 (21.0%)	1.04 (0.73-1.48)	0.840
	(T-G/T-G)	79 (23.1%)	55 (14.2%)	1.83 (1.25-2.68)	0.002
	(T-G/T-A)	51 (14.9%)	64 (16.4%)	1.12 (0.75-1.67)	0.579
Variables		**Non-dense breast**	**Dense breast**	**OR (95% CI)**	**P value**
Haplotype *rs10822013-rs10995190*	C-G	56 (21.7%)	45 (20.6%)	0.94 (0.60-1.46)	0.777
	T-G	124 (48.1%)	116 (53.2%)	1.23 (0.86-1.76)	0.263
	C-A	53 (20.5%)	29 (13.3%)	0.59 (0.36-0.97	0.038
	T-A	25 (9.7%)	28 (12.8%)	1.37 (0.77-2.43)	0.277
Diplotype	(T-G/C-A)	29 (22.5%)	18 (16.5%)	0.68 (0.35-1.31)	0.251
	(C-G/T-G)	33 (25.6%)	25 (22.9%)	0.87 (0.48-1.57)	0.636
	(TG/T-G)	22 (17.1%)	28 (25.7%)	1.68 (0.90-3.15)	0.105
	(T-G/T-A)	18 (14.0%)	17 (15.6%)	1.14 (0.56-2.34)	0.722

Data are presented as numbers (percentage, %). Haplotypes and diplotypes were compared between groups using Chi-Square. Binary logistic regression analysis was used to calculate odds ratios. A statistical P value less than 0.05 was considered significant

cancer risk, and prognosis in an Iranian population for the first time. Our results represented that the *rs10995190*-G and the *rs10822013*-T alleles were significantly associated with the increased risk of breast cancer. Moreover, haplotype and diplotype analyses were significantly associated with density values, breast cancer risk, and overall survival.

Considering *rs10822013* in the Iranian population revealed a significantly higher frequency of the T allele in breast cancer patients than in healthy people, with an effect size of 30% as a risky allele. The *rs10822013-T* allele distribution has been reported as 18% in Africans and almost 50% in Asians, Europeans, and Americans. However, the frequency varies between 11% and 55% in different sources.[28-31] The TT genotype of *rs10822013*, compared with TC+CC genotypes, increased the risk of breast cancer by up to 44%. Based on a GWAS, *rs10822013* presents a 10% risk of breast cancer in East-Asian women.[11] Furthermore, it was associated with the risk of breast cancer in the Han Chinese population[30] but not in the Singapore Chinese population.[29] Controversial results may arise from the sample size and a highly different allele frequency of *rs10822013* between populations, conferring breast cancer susceptibility in some ethnicities but not others.

The *rs10995190*-G allele frequency was 66.4% in the present study. ALelle FREquency Database (ALFRED) database has reported the frequency of this allele between 57% in Europe to 100% in Africa, Asia, and other continents. Despite the variation in allele frequency between different ethnicities, the G allele is the most frequent in all populations.[30, 32] Our data indicated an increased risk of breast cancer of about 29% for the *rs10995190*-G allele in the multiplicative model (G vs. A) and 79% in the dominant model (GG+GA vs. AA). According to previous studies, despite the lack of association in Asia, *rs10995190* was associated with breast cancer in the European population.[11, 33] However, further assessment is needed in different ethnicities to confirm or reject this hypothesis of whether *rs10995190* is precisely associated with breast cancer. Besides its association with breast cancer risk, some evidence confirmed the impact of *rs10995190* on breast density, and the A allele decreases mammographic density by up to 18% even after excluding breast cancer cases or adjusting for case-control status.[18] Moreover, another study reported the association between mammographic density measurements and *rs10995190.*[34] The lack of relevance in our study may be due to insufficient sample size or a different genetic basis in the study population compared to others. As a result, breast density may be a confounding factor in assessing the association of *rs10995190* with breast cancer. Therefore, evaluating the susceptibility rate of breast cancer in relationship with *rs10995190* needs adjustment for breast density. On the other hand, this variant is not related to the impact of menopausal hormone therapy on mammographic density.[35] Inconsistent with our findings, a previous study in the Han Chinese population reported an association between *rs10822013* and ER status.[30] Thus, the influence

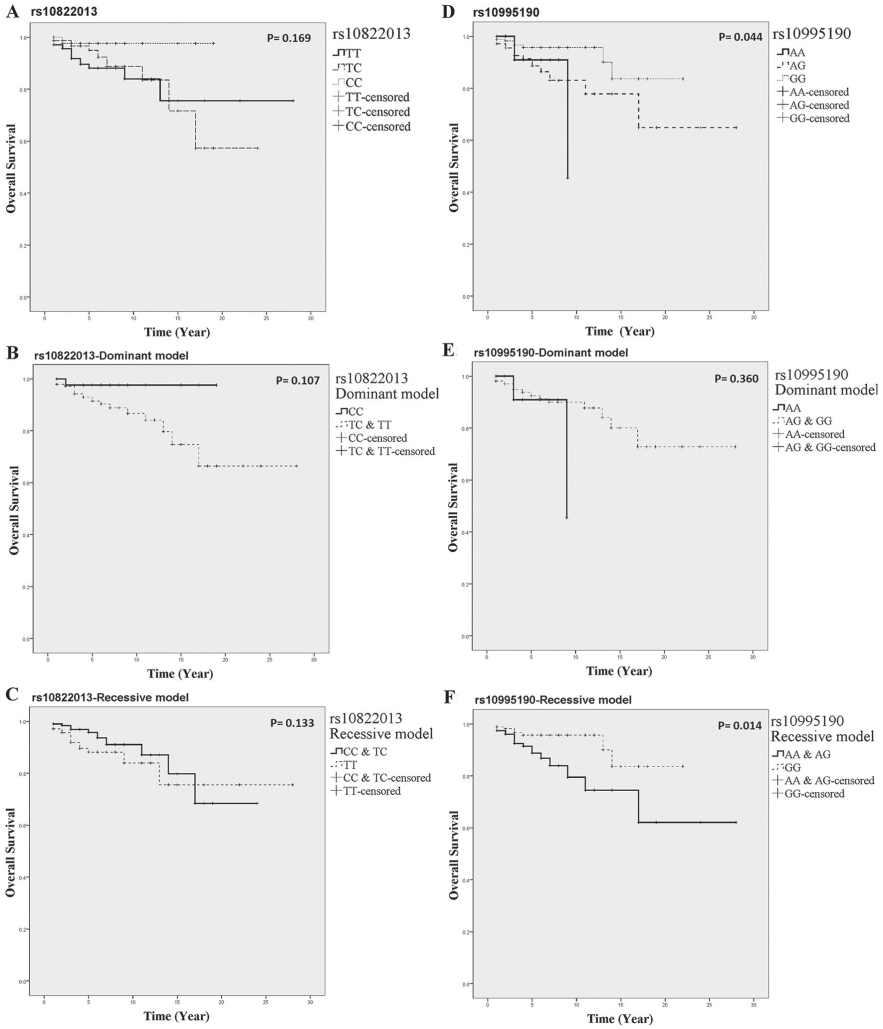

Figure 2: Plots indicate the association of *ZNF365 rs10822013* and *rs10995190* polymorphisms with overall survival. A: Plot for *rs10822013* genotypes with no difference in overall survival (P=0.169). B: Plot for *rs10822013* using the dominant model (TC+TT vs. CC) with no difference in overall survival (P=0.107). C: Plot for *rs10822013* using the recessive model (TT vs. TC+CC) with no difference in overall survival (P=0.133). D: Plot for *rs10995190* genotypes with a significant difference in overall survival (P=0.044). Patients carrying the *rs10995190* AG genotype tended to have lower survival than those carrying the GG genotype, with a hazard ratio (HR) of 2.68 and 95% CI (1.14-6.28). E: Plot for *rs10995190* using the dominant model (GG+AG vs. AA) with no difference in overall survival (P=0.360). F: Plot for *rs10995190* using the recessive model (GG vs. AG+AA) with a difference in overall survival (P=0.014). Patients carrying the *rs10995190* GG genotype tended to have higher survival than those carrying AG+AA genotypes, with a hazard ratio (HR) of 0.36 and 95% CI (0.16-0.84). A statistical P value less than 0.05 was considered significant.

of *rs10995190* on breast density and cancer may happen via hormonal and non-hormonal pathways, including estrogen hormone and developmental signaling. Although its higher expression causes better survival,[13] no analysis reports the involvement of *ZNF365* pathways in cancer.

To the best of our knowledge, the present study was the first report evaluating the haplotype patterns of the *ZNF365* common variations. Identified haplotypes potentially involve one or more susceptibility alleles, and recognizing their action approach will aid individual-level risk prediction. According to the results, the considered variants are not observed most often together in our population, because these SNPs are not in tight LD (D' coefficient=0.04, P=0.177). Further analysis indicated that the T-G haplotype (*rs10822013-rs10995190*), with a higher frequency in breast cancer patients, was a risky haplotype that could increase the risk of the disease by up to 44%. It was along with the impact of T and G alleles on the risk of breast cancer. Conversely, a haplotype containing C and A alleles (C-A haplotype) had a protective effect of up to 32%. Moreover, the C-A haplotype of *rs10822013-rs10995190* was associated with breast density.

Evaluation of breast cancer prognosis revealed the association of *rs10995190*-GG genotype and C-G haplotype of *rs10822013-rs10995190* with better overall survival. While the *rs10995190*-G

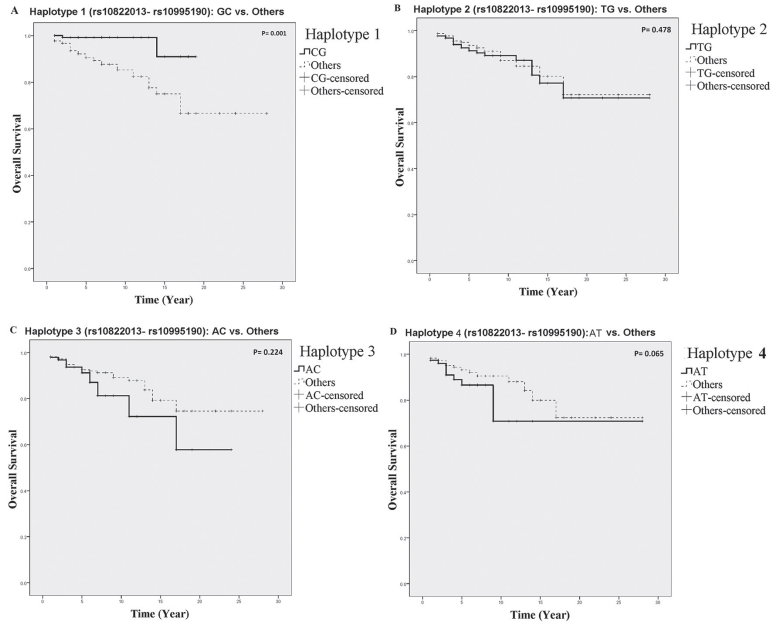

Figure 3: Plots indicate the association of *ZNF365 rs10822013* and *rs10995190* haplotypes with overall survival. A: Plot for *rs10822013-rs10995190* CG haplotype compared with other haplotypes with a difference in overall survival (P=0.001). Patients carrying the *rs10822013-rs10995190* CG haplotype tended to have higher survival than those carrying other haplotypes, with a hazard ratio (HR) of 0.14 and 95% CI (0.03-0.57). B: Plot for *rs10822013-rs10995190* TG haplotype compared with other haplotypes with no difference in overall survival (P=0.478). C: Plot for *rs10822013-rs10995190* AC haplotype compared with other haplotypes with no difference in overall survival (P=0.224). D: Plot for *rs10822013-rs10995190* AT haplotype compared with other haplotypes with no difference in overall survival (P=0.065). A statistical P value less than 0.05 was considered significant.

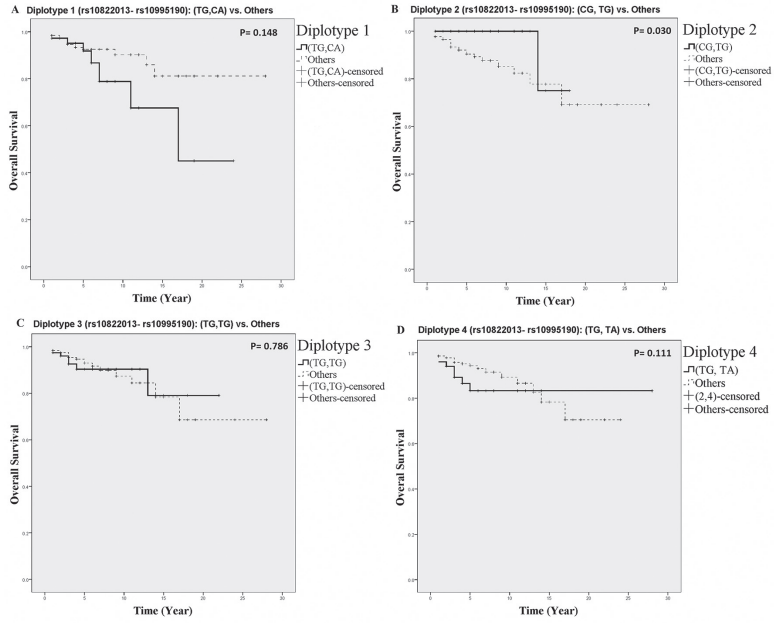

Figure 4: Plots indicate the association of *ZNF365 rs10822013* and *rs10995190* dipolotypes with overall survival. A: Plot for *rs10822013-rs10995190* (TG-CA) diplotype compared with other diplotype with no difference in overall survival (P=0.149). B: Plot for *rs10822013-rs10995190* (CG-TG) diplotype compared with other diplotype with a difference in overall survival (P=0.030). However, cox regression analysis did not confirm this finding, P=0.062, hazard ratio (HR) 0.15, and 95% CI (0.02-1.10). C: Plot for *rs10822013-rs10995190* (TG-TG) diplotype compared with other diplotype with no difference in overall survival (P=0.786). D: Plot for *rs10822013-rs10995190* (TG-TA) diplotype compared with other diplotype with no difference in overall survival (P=0.111). A statistical P value less than 0.05 was considered significant.

allele and GG genotype were associated with the increased risk of breast cancer, genotypes and haplotypes carrying the G allele could cause a better prognosis. This contradictory role may be influenced by different pathways that *ZNF365* is involved in and the role of other contributors and modulator variants. In this regard, triple-negative breast cancer, a poor prognosis type of tumor, has been associated with a decreased level of *ZNF365* expression.[13] Analysis of the expression

profile in association with *rs10995190* may help clear the dual role of the GG genotype as a hazard generator of breast cancer development and increasing survival.

Conclusion

The present study introduced the potential risk of *rs10822013* and *rs10995190* in breast cancer. Furthermore, it provided some evidence of involving *ZNF365* haplotypes in mammographic density. However, the genetic background of diverse ethnicities influences the distribution of alleles. Therefore, it may modify the association of the variants with different disease characteristics. Since accumulating data support the role of these variants in breast cancer, further studies are required to investigate the association of *ZNF365* variations with the risk of breast cancer and clinical features of the disease. Expression and functional *in silico*/*in vitro* analyses are also essential for investigating the role of *ZNF365* intronic and deep intronic variations on the stability and activity of *ZNF365* protein and other potential non-coding RNAs in the pathogenesis of breast cancer and survival. A better understanding of the mechanism of carcinogenesis will help pave the path toward personalized medicine for patients with breast cancer and the implementation of breast cancer prevention.

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Authors' Contribution

SR.Gh: Laboratory work, revision; L.H: Laboratory work, drafting; S.AKh: Data collection, revision; MR.N: Laboratory work, drafting; M.A: Laboratory work, revision; Sh.E: Data collection, revision; A.KhSh: Data collection, laboratory work, revision; F.HSh: Data collection, revision; A.P: Study design, statistical analysis, drafting; F.A: Study design, data collection, statistical analysis, drafting; All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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