Association of *HLA-G* 3'UTR 14-bp Ins/Del polymorphism with breast cancer among South Indian women

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ABSTRACT

Aim Human leucocyte antigen-G (HLA-G) and tumour necrosis factor-alpha (TNF-α) are potent immune mediators implicated in the pathogenesis of breast cancer. The polymorphisms in the 3′ untranslated region (3′UTR) of *HLA-G* and promoter region of *TNF-α* are well known to influence their expression levels and may consequently contribute to varied disease predisposition. Therefore, in the present study, we explored the effect of *HLA-G* 3′UTR (14-bp Ins/Del and +3142 C/G) and *TNF-α* promoter (–238 G/A and –308 G/A) polymorphisms on breast cancer risk among South Indian women.

Methods A total of 342 women (100 patients with breast cancer, 142 patients with benign breast disorder and 100 healthy women volunteers) were enrolled for this study. Genotyping of *HLA-G* and *TNF-α* polymorphisms were performed by direct PCR DNA amplification and amplification refractory mutation system PCR methods, respectively.

Results Significantly higher frequencies of *HLA-G* 14-bp Ins allele and Ins/+3142G haplotype were observed in patients with breast cancer than healthy controls (OR=1.56, P_c=0.036) and patients with benign breast disorder (OR=1.47, P_c=0.046). Similarly, subgroup analysis based on age at diagnosis (age≤50 years and >50 years) of breast cancer revealed higher frequencies of 14-bp Ins allele and Ins/+3142G haplotype in the patients of age >50 years than healthy controls (OR=1.77, P_c=0.03). Additionally, the extended haplotypes and multifactor dimensionality reduction analysis of the studied polymorphisms revealed significant contribution of *HLA-G* 14-bp Ins/Del polymorphism towards breast cancer risk.

Conclusion The findings of the present study suggest that the *HLA-G* 14-bp Ins/Del polymorphism could influence breast cancer pathogenesis among South Indian women.

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women across the globe with an alarmingly high mortality rate. Approximately, 1.67 million new cases of breast cancer were diagnosed in the year 2012, accounting for 25% of all cancers.[1] The aetiology of breast cancer is highly complex where multiple factors such as age, heredity, hormones and environment modulate the risk.[2] Although benign breast disorders commonly include all non-malignant breast tumours, some might possess significant premalignant potential.[3] Accumulating evidence indicates that the immune system displays both host-protective and tumour-promoting roles in cancer.[4] Evidently, immunosuppressive[5] and inflammatory[6] mediators have shown significant contribution towards breast cancer pathogenesis and progression.

Human leucocyte antigen-G (HLA-G) is a potent immunosuppressive and immunomodulatory non-classical HLA-class Ib protein encoded by the *HLA-G* located at chromosome 6p21 within the Class I MHC gene cluster.[7 8] Under normal physiological conditions, HLA-G is expressed only in certain fetal cells, adult immune privileged sites and precursor cells of erythroid and endothelial lineages, whereas induced expression has been reported in cancers, viral infections, transplantation, autoimmunity and inflammatory diseases.[9] Earlier studies have also documented high levels of HLA-G expression in breast cancer.[10 11] *HLA-G* is characterised by limited polymorphism and the magnitude of its expression depends on functional polymorphisms in the 3' untranslated region (3'UTR) and 5' upstream regulatory region.[12] Previous studies have reported that Ins allele of 14-bp Ins/Del polymorphism (*rs66554220*) and G allele of +3142 C/G polymorphism (*rs1063320*) in the 3'UTR of *HLA-G* are associated with low levels of HLA-G expression.[13 14] Mounting evidence suggests the role of these polymorphisms in predisposition to various cancers.[15] However, the reports on breast cancer are limited.

Tumour necrosis factor-alpha (TNF-α), a vital proinflammatory cytokine, has been implicated in inflammatory pathways that promote tumorogenesis.[16] The gene encoding TNF-α is located within the MHC locus at chromosome 6p21 and contains several single nucleotide polymorphisms (SNPs). In the promoter region of *TNF-α*, the polymorphisms with G to A transition at –238 (*rs361525*) and –308 (*rs1800629*) positions are well known to influence its expression.[17 18] Consequently, studies have reported the role of these *TNF-α* polymorphisms in various diseases, including breast cancer.[19]

Thus far, there are no studies reporting the association of *HLA-G* 14-bp Ins/Del and +3142 C/G polymorphisms with breast cancer risk among Indian populations while the reports on *TNF-α* –238 G/A and –308 G/A polymorphisms are scarce and inconsistent. Hence the present study focused to unveil the influence of these *HLA-G* and *TNF-α* polymorphisms on breast cancer risk among South Indian women.

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METHODOLOGY

Study subjects

The present case-control study is comprised of 100 patients with breast cancer and 142 patients with benign breast disorder, recruited from the Government Theni Medical College & Hospital (GTMCH). Further, 100 unrelated healthy women volunteers with no individual or familial history of breast malignancies were included in the control group. The work was approved by the ethics committees of Madurai Kamaraj University (MKU) and GTMCH. Blood samples (~3.5 mL) were collected from all the individuals after obtaining written informed consent.

DNA extraction and genotyping

DNA was extracted from the blood by salting out method,[20] which involves the dehydration and precipitation of the cellular proteins by using a saturated salt solution. Genotyping of *HLA-G* 14-bp Ins/Del and +3142 C/G polymorphisms was performed by direct PCR DNA amplification method, using specific primers and cycling conditions as described previously.[21] *TNF-α* –238 G/A and –308 G/A polymorphisms were genotyped by amplification refractory mutation system PCR method.[22] In this method, four different primers binding to the gene of interest were used to detect the presence or absence of normal and polymorphic alleles simultaneously in the same reaction.

Statistical analyses

Genotype data were analysed by Pearsons χ^2 test with Yates correction using Epi Info V.7 software (Centers for Disease control and prevention—http://www.cdc.gov/epiinfo). ORs at 95% CI were computed to assess the effect of each genotype, allele and haplotype of *HLA-G* and *TNF-α* polymorphisms on breast cancer risk. Hardy-Weinberg equilibrium (HWE), haplotype distribution and linkage disequilibrium (LD) between the SNPs were determined using Haploview V.4 software.[23] $P<0.05$ after correction was considered statistically significant. Further, the SNP-SNP interaction between *HLA-G and TNF-α* polymorphisms was predicted using multifactor dimensionality reduction (MDR) V.3 software.[24]

RESULTS

Genotype and allele frequencies of *HLA-G* and *TNF-α* polymorphisms

The genotype and allele frequencies of *HLA-G* 14-bp Ins/Del and +3142 C/G; *TNF-α* –238 G/A and –308 G/A polymorphisms were consistent with HWE in healthy controls, patients with benign breast disorder and patients with breast cancer ($p>0.05$) except for *HLA-G* +3142 C/G polymorphism in patients with benign breast disorder ($p=0.04$). The frequency of *HLA-G* 14-bp Ins allele was significantly higher among patients with breast cancer than healthy controls (OR (95% CI)=1.56 (1.05 to 2.31), $P_c=0.036$) and patients with benign breast disorder (OR (95% CI)=1.47 (1.02 to 2.12), $P_c=0.046$), while the Del allele frequency was significantly higher among healthy controls (OR (95% CI)=0.64 (0.43 to 0.95), $P_c=0.036$) and patients with benign breast disorder (OR (95% CI)=0.68 (0.47 to 0.98), $P_c=0.046$) than patients with breast cancer. There were no significant differences in the frequencies of genotypes and alleles of *HLA-G* +3142 C/G, *TNF-α* –238 G/A and –308 G/A polymorphisms between the study groups. Nevertheless, the frequency of *TNF-α* –308A allele was found to be higher among patients with benign breast disorder ($p<0.05$, $P_c=0.06$) than healthy controls (table 1).

HLA-G haplotypes and their LD

Pairwise nucleotide analysis of *HLA-G* polymorphisms generated three 14-bp/+3142 haplotypes namely Ins/+3142G, Del/+3142C, Del/+3142G. Frequency distribution of *HLA-G* haplotypes revealed significantly high frequency of Ins/+3142G haplotype among patients with breast cancer when compared with healthy controls (OR (95% CI)=1.56 (1.05 to 2.31), $P_c=0.036$) and patients with benign breast disorder (OR (95% CI)=1.47 (1.02 to 2.12), $P_c=0.046$) (table 2). The haplotype block analysis revealed a strong LD between the two *HLA-G* polymorphisms among patients with breast cancer (D'=1.0, logarithm of the odds (LOD)=14.87, $r^2=0.423$), patients with benign breast disorder (D'=1.0, LOD=19.15, $r^2=0.352$) and healthy controls (D'=1.0, LOD=11.28, $r^2=0.323$).

TNF-α haplotypes and their LD

Pairwise nucleotide analysis of *TNF-α* polymorphisms generated three *TNF-α* –238/–308 haplotypes namely –238 G/–308G, –238A/–308G and –238 G/–308A. Significant differences were not observed in the frequency distribution of *TNF-α* haplotypes between patients with breast cancer, patients with benign breast disorder and healthy controls (table 2). Haplotype block analysis of the *TNF-α* polymorphisms suggested a weak LD in patients with breast cancer (D'=1.0, LOD=0.22, $r^2=0.006$) and healthy controls (D'=1.0, LOD=0.18, $r^2=0.002$) and lack of LD in patients with benign breast disorder (D'=0.56, LOD=0.07, $r^2=0.003$).

Extended haplotypes of *HLA-G* & *TNF-α*

The haplotype analysis of *HLA-G* and *TNF-α* polymorphisms revealed the frequencies of various extended haplotypes. The extended haplotypes with considerable differences in their frequencies between the study groups are presented in table 2. The frequencies of Del/–308G (OR (95% CI)=0.59 (0.39 to 0.88), $P_c=0.012$), Del/–238G (OR (95% CI)=0.63 (0.42 to 0.93), $P_c=0.028$), Del/–238G/–308G (OR (95% CI)=0.58 (0.39 to 0.86), $P_c=0.0093$) extended haplotypes were significantly higher among healthy controls when compared with patients with breast cancer, whereas the frequency of Ins/+3142G/–308G (OR (95% CI)=1.48 (1.03 to 2.13), $P_c=0.043$) extended haplotype was significantly higher among patients with breast cancer than patients with benign breast disorder. Additionally, the frequencies of Ins/–308G and Ins/+3142G/–238G extended haplotypes were higher among patients with breast cancer when compared with healthy controls ($p<0.05$). However, the haplotype block analysis did not show the presence of LD between *HLA-G* and *TNF-α* polymorphisms in the study population (figure 1).

Subgroup analysis

The average (mean±SD) age at diagnosis of breast cancer in the study cohort was found to be 50.5±10.9 years. Subgroup analysis based on the age at diagnosis (age ≤50 years and >50 years) of breast cancer revealed significantly higher frequencies of *HLA-G* 14-bp Ins allele (OR (95% CI)=1.56 (1.05 to 2.31), $P_c=0.036$) and Ins/+3142G haplotype (OR (95% CI)=1.77 (1.08 to 2.88), $P_c=0.03$) among the patients with age >50 years when compared with healthy controls, whereas the prevalence of Del allele (OR (95% CI)=0.57 (0.35 to 0.92), $P_c=0.03$) was significantly higher among healthy controls. Additionally, the frequencies of Del/–238G

Table 1 Frequency distribution of *HLA-G* and *TNF-α* genotypes and alleles in healthy controls, patients with breast cancer and patients with benign breast disorder

	HC (n=100)	BC (n=100)	BBD (n=142)	HC versus BC		HC versus BBD		BBD versus BC	
	n (%)	n (%)	n (%)	OR (95% CI)	P_c	OR (95% CI)	P_c	OR (95% CI)	P_c
HLA-G 14-bp									
Ins/Ins	19 (19)	31 (31)	29 (20)	1.92 (0.99 to 3.68)	0.072	1.09 (0.57 to 2.08)	0.913	1.75 (0.97 to 3.15)	0.085
Ins/Del	48 (48)	46 (46)	68 (48)	0.92 (0.53 to 1.61)	0.887	1.0 (0.59 to 1.66)	0.910	0.92 (0.55 to 1.54)	0.874
Del/Del	33 (33)	23 (23)	45 (32)	0.61 (0.32 to 1.13)	0.156	0.94 (0.54 to 1.63)	0.940	0.64 (0.36 to 1.15)	0.182
Ins	86 (43)	108 (54)	126 (44)	1.56 (1.05 to 2.31)	**0.036**	1.06 (0.73 to 1.52)	0.837	1.47 (1.02 to 2.12)	**0.046**
Del	114 (57)	92 (46)	158 (56)	0.64 (0.43 to 0.95)	**0.036**	0.95 (0.66 to 1.36)	0.837	0.68 (0.47 to 0.98)	**0.046**
HLA-G +3142									
CC	10 (10)	7 (7)	19 (13)	0.68 (0.25 to 1.86)	0.612	1.39 (0.62 to 3.13)	0.551	0.49 (0.19 to 1.21)	0.171
GC	40 (40)	39 (39)	49 (35)	0.96 (0.54 to 1.69)	1.000	0.79 (0.46 to 1.34)	0.461	1.21 (0.71 to 2.06)	0.562
GG	50 (50)	54 (54)	74 (52)	1.17 (0.67 to 2.05)	0.671	1.09 (0.65 to 1.82)	0.847	1.08 (0.65 to 1.80)	0.874
C	60 (30)	53 (27)	87 (31)	0.84 (0.54 to 1.30)	0.505	1.03 (0.69 to 1.53)	0.961	0.82 (0.55 to 1.22)	0.376
G	140 (70)	147 (73)	197 (69)	1.19 (0.77 to 1.84)	0.505	0.97 (0.65 to 1.44)	0.961	1.23 (0.82 to 1.83)	0.376
TNF-α –238									
GG	85 (85)	82 (82)	115 (81)	0.8 (0.38 to 1.70)	0.703	0.75 (0.38 to 1.50)	0.522	1.07 (0.55 to 2.07)	0.975
GA	14 (14)	16 (16)	25 (18)	1.17 (0.54 to 2.55)	0.843	1.31 (0.64 to 2.67)	0.566	0.89 (0.45 to 1.77)	0.878
AA	1 (1)	2 (2)	2 (1)	2.02 (0.2 to 22.65)	1.000	1.41 (0.1 to 15.81)	0.759	1.43 (0.2 to 10.32)	0.876
G	184 (92)	180 (90)	255 (90)	0.78 (0.39 to 1.56)	0.600	0.76 (0.40 to 1.45)	0.505	1.02 (0.56 to 1.87)	0.938
A	16 (8)	20 (10)	29 (10)	1.28 (0.64 to 2.54)	0.600	1.31 (0.69 to 2.48)	0.505	0.98 (0.54 to 1.78)	0.938
TNF-α –308									
GG	95 (95)	90 (90)	125 (88)	0.47 (0.16 to 1.44)	0.283	0.39 (0.14 to 1.09)	0.103	1.22 (0.54 to 2.79)	0.785
GA	5 (5)	9 (9)	15 (11)	1.88 (0.61 to 5.82)	0.406	2.24 (0.79 to 6.39)	0.190	0.84 (0.35 to 1.99)	0.856
AA	0 (0)	1 (1)	2 (1)	–	–	–	–	0.71 (0.06 to 7.91)	0.759
G	195 (97)	189 (95)	265 (93)	0.44 (0.15 to 1.29)	0.202	0.36 (0.13 to 0.97)	0.060*	1.23 (0.57 to 2.65)	0.731
A	5 (3)	11 (5)	19 (7)	2.27 (0.77 to 6.66)	0.202	2.80 (1.03 to 7.62)	0.060*	0.81 (0.38 to 1.75)	0.731

All the significant P_c values are given in boldface.
*Uncorrected p<0.05.
BBD, patients with benign breast disorder; BC, patients with breast cancer; Del, deletion allele; HC, healthy controls; Ins, insertion allele; P_c, Yates' corrected p value.

(OR (95% CI)=0.54 (0.33 to 0.88), P_c=0.014), Del/–308G (OR (95% CI)=0.51 (0.31 to 0.83), P_c=0.0067), Del/–238G/–308G (OR (95% CI)=0.48 (0.29 to 0.80), P_c=0.0039) extended haplotypes were significantly higher among healthy controls while the frequencies of Ins/Ins genotype, Ins/–238G, Ins/–308G and Ins/+3142G/–308G extended haplotypes were higher among patients of age >50 years (p<0.05). On the other hand, there were no significant

Table 2 Frequency distribution of *HLA-G* and *TNF-α* haplotypes in healthy controls and patients with breast cancer

	HC (n=100)	BC (n=100)	BBD (n=142)	HC versus BC		HC versus BBD		BBD versus BC	
	n (%)	n (%)	n (%)	OR (95% CI)	P_c	OR (95% CI)	P_c	OR (95% CI)	P_c
HLA-G haplotype									
Ins/+3142G	86 (43)	108 (54)	126 (44)	1.56 (1.05 to 2.31)	**0.036**	1.06 (0.73 to 1.52)	0.837	1.47 (1.02 to 2.12)	**0.046**
Del/+3142C	60 (30)	53 (26)	87 (31)	0.84 (0.54 to 1.30)	0.505	1.03 (0.70 to 1.53)	0.961	0.82 (0.54 to 1.22)	0.376
Del/+3142G	54 (27)	39 (20)	71 (25)	0.65 (0.41 to 1.05)	0.098	0.9 (0.60 to 1.36)	0.697	0.73 (0.47 to 1.13)	0.189
TNF-α haplotype									
–238G/–308G	179 (90)	169 (85)	237 (83)	0.64 (0.35 to 1.16)	0.181	0.59 (0.34 to 1.02)	0.080	1.08 (0.66 to 1.77)	0.854
–238A/–308G	16 (8)	20 (10)	28 (10)	1.28 (0.64 to 2.54)	0.600	1.26 (0.66 to 2.39)	0.589	1.02 (0.55 to 1.86)	0.918
–238G/–308A	5 (2)	11 (5)	18 (6)	2.27 (0.77 to 6.66)	0.202	2.64 (0.96 to 7.23)	0.082	0.86 (0.39 to 1.86)	0.851
Extended haplotype									
Ins/+3142G/–238G	72 (36)	92 (46)	108 (38)	1.51 (1.01 to 2.26)	0.053*	1.09 (0.75 to 1.59)	0.719	1.38 (0.96 to 2.00)	0.097
Ins/+3142G/–308G	84 (42)	104 (52)	120 (42)	1.5 (1.01 to 2.22)	0.057*	1.01 (0.70 to 1.46)	0.969	1.48 (1.03 to 2.13)	**0.043**
Ins/–308G	84 (42)	104 (52)	120 (42)	1.5 (1.01 to 2.22)	0.057*	1.01 (0.70 to 1.46)	0.969	1.48 (1.03 to 2.13)	**0.043**
Del/–238G/–308G	109 (55)	82 (41)	137 (48)	0.58 (0.39 to 0.86)	**0.009**	0.78 (0.54 to 1.12)	0.206	0.75 (0.52 to 1.07)	0.138
Del/–238G	112 (56)	89 (44)	149 (52)	0.63 (0.42 to 0.93)	**0.028**	0.87 (0.60 to 1.25)	0.499	0.73 (0.50 to 1.05)	0.102
Del/–308G	111 (55)	85 (43)	145 (51)	0.59 (0.39 to 0.88)	**0.012**	0.84 (0.58 to 1.20)	0.383	0.71 (0.49 to 1.02)	0.078

All the significant P_c values are given in boldface.
*Uncorrected p<0.05.
BBD, patients with benign breast disorder; BC, patients with breast cancer; Del, deletion allele; HC, healthy controls; Ins, insertion allele; P_c, Yates' corrected p value.

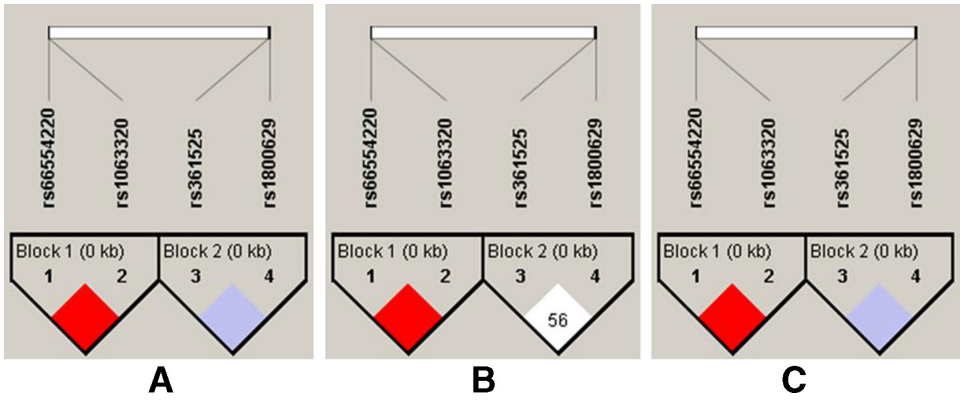

Figure 1 LD and haplotype blocks of *HLA-G* and *TNF-α* polymorphisms among (A) patients with breast cancer, (B) patients with benign breast disorder and (C) healthy controls. Each block indicates LD between the two consecutive polymorphisms. Block 1 represents the pairwise LD between *HLA-G* rs66554220 and rs1063320 and Block 2 between *TNF–α* rs361525 and rs1800629 polymorphisms. The standard (D'/LOD) LD scheme was selected in Haploview software and values inside each square denotes D'×100. LD, linkage disequilibrium.

differences in the frequency distribution of genotypes, alleles and haplotypes of *HLA-G* polymorphisms between the patients of age ≤50 years and healthy controls. Furthermore, the frequencies of genotypes, alleles and haplotypes of *TNF-α* polymorphisms did not vary significantly between the patient subgroups and healthy controls (tables 3 and 4).

SNP-SNP interaction analysis

In this study, the SNP-SNP interaction analysis performed using MDR has predicted four different models of breast cancer risk (table 5). Among them, the four-factor (rs66554220, rs1063320, rs361525, rs1800629) model was considered as the best interaction model with highest cross-validation count of 10/10 (100%) and maximum testing balanced accuracy of 0.625. In addition, the rs66554220 factor was the only single-factor model and is consistent among two, three and four-factor models of breast cancer risk. The interaction entropy graph revealed moderate synergistic effect of *HLA-G* +3142 C/G and *TNF-α* –238 G/A by removing an entropy of 0.29% (figure 2).

Table 3 Age-based subgroup analysis of *HLA-G* and *TNF-α* genotypes and alleles among patients with breast cancer

	HC (n=100)	HC versus BC age ≤50 years (n=51)			HC versus BC age >50 years (n=49)		
	n (%)	n (%)	OR (95% CI)	P_c	n (%)	OR (95% CI)	P_c
HLA-G 14-bp							
Ins/Ins	19 (19)	14 (27)	1.61 (0.73 to 3.56)	0.327	17 (35)	2.26 (1.05 to 4.90)	0.058*
Ins/Del	48 (48)	24 (47)	0.96 (0.49 to 1.89)	0.950	22 (45)	0.88 (0.44 to 1.75)	0.856
Del/Del	33 (33)	13 (25)	0.69 (0.33 to 1.48)	0.446	10 (20)	0.52 (0.23 to 1.17)	0.161
Ins	86 (43)	52 (51)	1.38 (0.85 to 2.22)	0.232	56 (57)	1.77 (1.08 to 2.88)	**0.030**
Del	114 (57)	50 (49)	0.73 (0.45 to 1.17)	0.232	42 (43)	0.57 (0.35 to 0.92)	**0.030**
HLA-G +3142							
CC	10 (10)	4 (8)	0.77 (0.23 to 2.57)	0.892	3 (6)	0.59 (0.15 to 2.24)	0.632
GC	40 (40)	22 (43)	1.14 (0.57 to 2.25)	0.845	17 (35)	0.8 (0.39 to 1.62)	0.655
GG	50 (50)	25 (49)	0.96 (0.49 to 1.88)	0.954	29 (59)	1.45 (0.73 to 2.89)	0.379
C	60 (30)	30 (29)	0.97 (0.58 to 1.64)	0.978	23 (23)	0.72 (0.41 to 1.25)	0.297
G	140 (70)	72 (71)	1.03 (0.61 to 1.73)	0.978	75 (77)	1.4 (0.80 to 2.44)	0.297
TNF-α –238							
GG	85 (85)	43 (84)	0.95 (0.37 to 2.41)	0.898	39 (80)	0.69 (0.28 to 1.67)	0.551
GA	14 (14)	7 (14)	0.98 (0.37 to 2.60)	0.839	9 (18)	1.38 (0.55 to 3.46)	0.651
AA	1 (1)	1 (2)	1.98 (0.1 to 32.32)	0.792	1 (2)	2.06 (1.3 to 33.69)	0.811
G	184 (92)	93 (91)	0.9 (0.38 to 2.11)	0.980	87 (89)	0.69 (0.31 to 1.54)	0.486
A	16 (8)	9 (9)	1.11 (0.47 to 2.61)	0.980	11 (11)	1.45 (0.65 to 3.27)	0.486
TNF-α –308							
GG	95 (95)	47 (92)	0.62 (0.16 to 2.41)	0.738	43 (88)	0.38 (0.11 to 1.30)	0.209
GA	5 (5)	4 (8)	1.62 (0.41 to 6.30)	0.738	5 (10)	2.16 (0.59 to 7.84)	0.399
AA	0 (0)	0 (0)	–	–	1 (2)	–	–
G	195 (97)	98 (96)	0.63 (0.16 to 2.39)	0.742	91 (93)	0.33 (0.10 to 1.08)	0.109
A	5 (3)	4 (4)	1.59 (0.42 to 6.06)	0.742	7 (7)	3.0 (0.93 to 9.71)	0.109

All the significant P_c values are given in boldface.
*Uncorrected p<0.05.
BC, patients with breast cancer; Del, deletion; HC, healthy controls; Ins, insertion; P_c, Yates' corrected p value.

Table 4 Age-based subgroup analysis of *HLA-G* and *TNF-α* haplotypes among patients with breast cancer

	HC (n=100)	HC versus BC age ≤50 years (n=51)			HC versus BC age >50 years (n=49)		
	n (%)	n (%)	OR (95% CI)	P_c	n (%)	OR (95% CI)	P_c
HLA-G haplotypes							
Ins/+3142G	86 (43)	52 (51)	1.38 (0.85 to 2.22)	0.232	56 (57)	1.77 (1.08 to 2.88)	**0.030**
Del/+3142C	60 (30)	30 (29)	0.97 (0.58 to 1.64)	0.978	23 (24)	0.72 (0.41 to 1.25)	0.297
Del/+3142G	54 (27)	20 (20)	0.66 (0.37 to 1.18)	0.204	19 (19)	0.65 (0.36 to 1.17)	0.196
TNF-α haplotypes							
–238G/–308G	179 (90)	89 (87)	0.8 (0.38 to 1.68)	0.696	80 (82)	0.52 (0.26 to 1.03)	0.087
–238A/–308G	16 (8)	9 (9)	1.11 (0.47 to 2.61)	0.980	11 (11)	1.45 (0.65 to 3.27)	0.486
–238G/–308A	5 (2)	4 (4)	1.59 (0.42 to 6.06)	0.742	7 (7)	3 (0.93 to 9.71)	0.109
Extended haplotypes							
Ins/+3142G/–308G	84 (42)	51 (50)	1.38 (0.85 to 2.23)	0.230	53 (54)	1.63 (0.99 to 2.65)	0.065*
Ins/–238G	72 (36)	45 (44)	1.4 (0.86 to 2.28)	0.213	47 (48)	1.64 (1.00 to 2.67)	0.064*
Ins/–308G	84 (42)	51 (50)	1.38 (0.85 to 2.23)	0.230	53 (54)	1.63 (0.99 to 2.65)	0.065*
Del/–238G/–308G	109 (55)	46 (45)	0.69 (0.42 to 1.11)	0.154	36 (37)	0.48 (0.29 to 0.79)	**0.006**
Del/–238G	112 (56)	48 (47)	0.69 (0.43 to 1.13)	0.177	40 (41)	0.54 (0.33 to 0.88)	**0.019**
Del/–308G	111 (55)	47 (46)	0.68 (0.42 to 1.11)	0.153	38 (39)	0.51 (0.31 to 0.83)	**0.009**

All the significant P_c values are given in boldface.
*Uncorrected p<0.05.
BC, patients with breast cancer; Del, deletion; HC, healthy controls; Ins, insertion; P_c, Yates' corrected p value.

DISCUSSION

Despite the advances in screening, diagnosis and treatment, the incidence and mortality rates of breast cancer are alarmingly high.[1] The latter might be due to delayed clinical presentation of the disease at extremely advanced stages,[25] which is difficult to treat to cure. There is accumulating evidence on various immune molecules as potential targets for therapeutic interventions.[26] HLA-G, a potent immunosuppressive molecule, is highly expressed in breast tumours and might be an effective biomarker for breast cancer.[27] In the present study, the genetic contribution of HLA-G and TNF-α towards breast cancer predisposition was evaluated.

The study suggests that *HLA-G* 3'UTR 14-bp Ins allele and Ins/+3142G haplotype are associated with increased breast cancer risk in the South Indian population. While Jeong *et al* reported that *HLA-G* 14-bp Ins allele is associated with reduced risk of breast cancer on the basis of tissue HLA-G expression.[11] A study on South-East Iranian population reported high prevalence of Del allele and Del/Del genotype among patients with breast cancer.[28] Contrastingly, in the present study, the Del allele prevalence is significantly high in healthy controls. The meta-analysis study on cancer risk reported significant association of 14-bp Ins/Del polymorphism with breast cancer.[29] In the Tunisian population,[30] there is no association of 14-bp Ins/Del polymorphism with breast cancer. Though the 14-bp Ins/Del polymorphism is not associated with breast cancer predisposition in Brazilian[31] and Iranian-Azeri[32] populations, it has been shown to contribute to shorter survival time and disease progression, respectively. In this study, the *HLA-G* +3142 C/G polymorphism is not associated with breast cancer development in the South Indian population. Yet, another study within the South Indian population has reported the association of *HLA-G* +3142 C/C genotype with risk of inflammatory rheumatic heart disease,[33] while in Tunisian population, +3142 G allele and GG genotype have shown protection to breast cancer.[30] Earlier studies have demonstrated

Table 5 Summary of *HLA-G* and *TNF-α* SNP-SNP interactions in breast cancer

Model	TBA	CVC	P value*
rs66554220	0.56	10/10	0.05
rs66554220, rs1800629	0.58	7/10	0.0144
rs66554220, rs1063320, rs361525	0.605	10/10	0.003
rs66554220, rs1063320, rs361525, rs1800629	0.625	10/10	**0.0004**

The P value for the model with highest TBA and CVC is given in boldface.
*χ^2 p value.
CVC, cross-validation consistency; SNP, single nucleotide polymorphism; TBA, testing balanced accuracy.

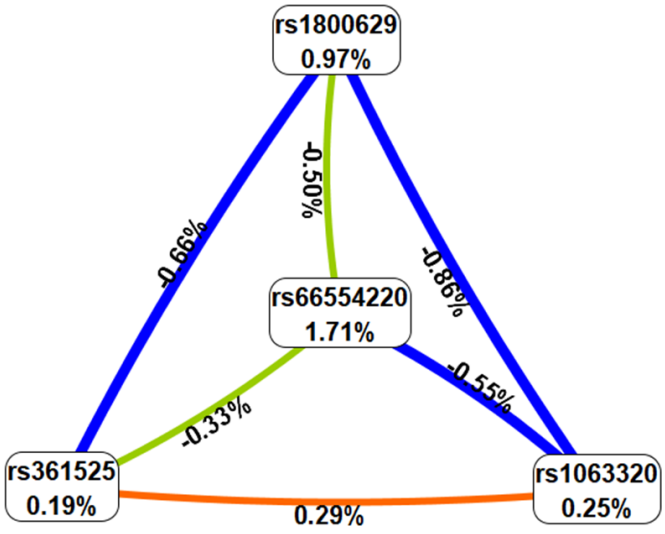

Figure 2 Interaction entropy graph to identify the SNP-SNP interaction of *HLA-G* and *TNF-α* by MDR analysis. The graph represents the percentage of entropy for independent factors (represented inside each box) and their interactions (represented on the line). The positive percentage of entropy denotes synergistic interaction while the negative percentage denotes redundancy. Here, a moderate degree of synergistic interaction exists between rs361525 and rs1063320 while all other interactions indicate moderate or highest degrees of redundancy. MDR, multifactor dimensionality reduction; SNP, single nucleotide polymorphism.

potential differences in sHLA-G and tissue HLA-G expression between breast cancer and benign breast disorder conditions.[34 35] In the present study, significant differences in the prevalence of alleles and haplotypes of *HLA-G* 14-bp Ins/Del polymorphism among patients with breast cancer and patients with benign breast disorder are reported. Additionally, the study also suggests the role of *HLA-G* 14-bp Ins/Del polymorphism in the delayed breast cancer onset by conferring increased risk to women over 50 years of age than the younger group. Nevertheless, similar investigation with an increased sample size is imperative to substantiate the findings.

In the present study, *TNF-α* –238 G/A and –308 G/A polymorphisms did not show a significant association with breast cancer risk. The current study is in concordance with the earlier reports on *TNF-α* –238 G/A polymorphism and breast cancer suggesting no significant association in North European[36] and Northeast Chinese Han[37] populations. Similarly, *TNF-α* –308 G/A polymorphism did not show association with breast cancer in North European,[36] Northeast Chinese Han,[37] Korean,[38] Italian,[39 40] Iranian,[41] Turkish,[42] USA and Polish[43] populations. Contrastingly, *TNF-α* –308 G/A polymorphism has been significantly associated with breast cancer in Tunisian[44 45] and North Indian[46] populations. In addition, Pooja *et al* have reported positive association of *TNF-α* –238 G/A and –308 G/A polymorphisms with breast cancer in the South Indian population.[47] However, the lack of association of *TNF-α* polymorphisms with breast cancer in the present study might be attributed to the low prevalence of *TNF-α* –238A and –308A alleles, which is commonly observed among Indians and other Asian populations.[48] The present study is also in line with the findings of Fang *et al* suggesting the absence of association between *TNF-α* –308 G/A polymorphism and breast cancer in Asian ethnicity.[49]

The variations in the association patterns of *HLA-G* and *TNF-α* polymorphisms with breast cancer between the studies might be due to geographical differences and clinical heterogeneity of breast cancer. Additional polymorphisms within the *HLA* region (predominantly those in the *HLA-G* or *TNF-α*) in strong LD with the studied polymorphisms might influence the association.[50–52]

There is accumulating evidence suggesting the significance of HLA-G mediated TNF-α regulation.[53–55] Furthermore, earlier findings on association of *TNF-α* polymorphisms with various class I and II *HLA* alleles[56] impelled us to assess the effect of extended haplotypes and SNP-SNP interaction of *TNF-α* and *HLA-G* in breast cancer. The study revealed significant impact of extended haplotypes on breast cancer risk; despite the absence of haplotype block between *HLA-G* and *TNF-α* polymorphisms. It is believed that when disease-related SNPs are outside haplotype blocks, SNP-SNP interactions can provide better information.[57] The SNP-SNP interaction analysis substantiate the present findings by suggesting a moderate synergistic interaction between *HLA-G* +3142 C/G and *TNF-α* –238 G/A polymorphisms and *HLA-G* 14-bp Ins/Del polymorphism as a single factor model influencing breast cancer risk.

Take home messages

- *HLA-G* 14-bp Ins allele and Ins/+3142G haplotype are associated with increased breast cancer risk in South Indian population.
- A moderate synergistic interaction is observed between *HLA-G* +3142 C/G and *TNF-α* –238 G/A polymorphisms.
- *HLA-G* 14-bp Ins/Del polymorphism could be a single factor influencing breast cancer risk as predicted by multifactor dimensionality reduction analysis.

CONCLUSION

The findings of the present study suggest that *HLA-G* 3'UTR 14-bp Ins/Del polymorphism could influence breast cancer pathogenesis in the South Indian population. Further prospective functional studies are warranted to substantiate the contribution of HLA-G and TNF-α in breast cancer.

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REFERENCES

1 Ferlay J, Soerjomataram I, Dikshit R, *et al*. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015;136:E359–86.
2 Mansfield CM. A review of the etiology of breast cancer. *J Natl Med Assoc* 1993;85:217–21.
3 Allred DC, Mohsin SK. Biological features of premalignant disease in the human breast. *J Mammary Gland Biol Neoplasia* 2000;5:351–64.
4 Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 2011;331:1565–70.
5 Elliott RL, Jiang XP, Phillips JT, *et al*. Human leukocyte antigen G expression in breast cancer: role in immunosuppression. *Cancer Biother Radiopharm* 2011;26:153–7.
6 Bhatelia K, Singh K, Singh R. Tlrs: linking inflammation and breast cancer. *Cell Signal* 2014;26:2350–7.
7 Geraghty DE, Koller BH, Orr HT. A human major histocompatibility complex class I gene that encodes a protein with a shortened cytoplasmic segment. *Proc Natl Acad Sci U S A* 1987;84:9145–9.
8 Tripathi P, Agrawal S. Non-Classical HLA-G antigen and its role in the cancer progression. *Cancer Invest* 2006;24:178–86.
9 Carosella ED, Favier B, Rouas-Freiss N, *et al*. Beyond the increasing complexity of the immunomodulatory HLA-G molecule. *Blood* 2008;111:4862–70.
10 Chen H-X, Lin A, Shen C-J, *et al*. Upregulation of human leukocyte antigen–G expression and its clinical significance in ductal breast cancer. *Hum Immunol* 2010;71:892–8.
11 Jeong S, Park S, Park B-W, *et al*. Human leukocyte antigen-G (HLA-G) polymorphism and expression in breast cancer patients. *PLoS One* 2014;9:e98284.
12 Hviid T, Rizzo R, Christiansen O, *et al*. HLA-G and IL-10 in serum in relation to HLA-G genotype and polymorphisms. *Immunogenetics* 2004;56:35–41.
13 Veit TD, Chies JAB. Tolerance versus immune response — microRNAs as important elements in the regulation of the HLA-G gene expression. *Transpl Immunol* 2009;20:229–31.
14 Svendsen SG, Hantash BM, Zhao L, *et al*. The expression and functional activity of membrane-bound human leukocyte antigen-G1 are influenced by the 3′-untranslated region. *Hum Immunol* 2013;74:818–27.

15 Dias FC, Castelli EC, Collares CVA, *et al*. The role of HLA-G molecule and HLA-G gene polymorphisms in tumors, viral hepatitis, and parasitic diseases. *Front Immunol* 2015;6:9.
16 Szlosarek PW, Balkwill FR. Tumour necrosis factor α: a potential target for the therapy of solid tumours. *Lancet Oncol* 2003;4:565–73.
17 Wilson AG, Symons JA, McDowell TL, *et al*. Effects of a polymorphism in the human tumor necrosis factor promoter on transcriptional activation. *Proc Natl Acad Sci U S A* 1997;94:3195–9.
18 Shih C-M, Lee Y-L, Chiou H-L, *et al*. Association of TNF-α polymorphism with susceptibility to and severity of non-small cell lung cancer. *Lung Cancer* 2006;52:15–20.
19 Qidwai T, Khan F. Tumour necrosis factor gene polymorphism and disease prevalence. *Scand J Immunol* 2011;74:522–47.
20 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16:1215.
21 Silva ID, Muniz YCN, Sousa MCPS, *et al*. HLA-G 3'UTR polymorphisms in high grade and invasive cervico-vaginal cancer. *Hum Immunol* 2013;74:452–8.
22 Milicic A, Lindheimer F, Laval S, *et al*. Interethnic studies of TNF polymorphisms confirm the likely presence of a second MHC susceptibility locus in ankylosing spondylitis. *Genes Immun* 2000;1:418–22.
23 Barrett JC, Fry B, Maller J, *et al*. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21:263–5.
24 Hahn LW, Ritchie MD, Moore JH. Multifactor dimensionality reduction software for detecting gene-gene and gene-environment interactions. *Bioinformatics* 2003;19:376–82.
25 Agarwal G, Pradeep PV, Aggarwal V, *et al*. Spectrum of breast cancer in Asian women. *World J Surg* 2007;31:1031–40.
26 Bateman AC. Molecules in cancer immunotherapy: benefits and side effects. *J Clin Pathol* 2019;72:20–4.
27 Zhang Y, Yu S, Han Y, *et al*. Human leukocyte antigen-G expression and polymorphisms promote cancer development and guide cancer diagnosis/treatment. *Oncol Lett* 2018;15:699–709.
28 Eskandari-Nasab E, Hashemi M, Hasani S-S, *et al*. Association between HLA-G 3'UTR 14-bp ins/del polymorphism and susceptibility to breast cancer. *Cancer Biomarkers* 2013;13:253–9.
29 Ge Y-Z, Ge Q, Li M-H, *et al*. Association between human leukocyte antigen-G 14-bp insertion/deletion polymorphism and cancer risk: a meta-analysis and systematic review. *Hum Immunol* 2014;75:827–32.
30 Zidi I, Dziri O, Zidi N, *et al*. Association of HLA-G +3142 C>G polymorphism and breast cancer in Tunisian population. *Immunol Res* 2016;64:961–8.
31 Ramos CS, Gonçalves AS, Marinho LC, *et al*. Analysis of HLA-G gene polymorphism and protein expression in invasive breast ductal carcinoma. *Hum Immunol* 2014;75:667–72.
32 Haghi M, Feizi MAH, Sadeghizadeh M, *et al*. 14-bp insertion/deletion polymorphism of the HLA-G gene in breast cancer among women from North Western Iran. *Asian Pacific Journal of Cancer Prevention* 2015;16:6155–8.
33 Poomarimuthu M, Elango S, Soundrapandian S, *et al*. "HLA-G 3'UTR gene polymorphisms and rheumatic heart disease: a familial study among South Indian population". *Pediatr Rheumatol* 2017;15:1–7.
34 Singer G, Rebmann V, Chen Y-C, *et al*. HLA-G is a potential tumor marker in malignant ascites. *Clin Cancer Res* 2003;9:4460–4.
35 He X, Dong D-dan, Yie S-mian, *et al*. HLA-G expression in human breast cancer: implications for diagnosis and prognosis, and effect on allocytotoxic lymphocyte response after hormone treatment in vitro. *Ann Surg Oncol* 2010;17:1459–69.
36 Azmy IAF, Balasubramanian SP, Wilson AG, *et al*. Role of tumour necrosis factor gene polymorphisms (-308 and -238) in breast cancer susceptibility and severity. *Breast Cancer Res* 2004;6:R395–400.
37 Xu F, Zhou G, Han S, *et al*. Association of TNF-α, TNFRSF1A and TNFRSF1B gene polymorphisms with the risk of sporadic breast cancer in northeast Chinese Han women. *PLoS One* 2014;9:e101138.
38 Park KS, Mok JW, Ko HE, *et al*. Polymorphisms of tumour necrosis factors A and B in breast cancer. *Eur J Immunogenet* 2002;29:7–10.
39 Giordani L, Bruzzi P, Lasalandra C,*et al*. Association of Breast Cancer and Polymorphisms of Interleukin-10 and Tumor Necrosis Factor- Genes. *Clin Chem* 2003;49:1664–7.
40 Scola L, Vaglica M, Crivello A, *et al*. Cytokine gene polymorphisms and breast cancer susceptibility. *Ann N Y Acad Sci* 2006;1089:104–9.
41 Kamali-Sarvestani E, Merat A, Talei A-R. Polymorphism in the genes of alpha and beta tumor necrosis factors (TNF-α and TNF-β) and gamma interferon (IFN-γ) among Iranian women with breast cancer. *Cancer Lett* 2005;223:113–9.
42 Gonullu G, Basturk B, Evrensel T, *et al*. Association of breast cancer and cytokine gene polymorphism in Turkish women. *Saudi Med J* 2007;28:1728–33.
43 Gaudet MM, Egan KM, Lissowska J, *et al*. Genetic variation in tumor necrosis factor and lymphotoxin-alpha (TNF–LTA) and breast cancer risk. *Hum Genet* 2007;121:483–90.
44 Chouchane L, Ahmed SB, Baccouche S, *et al*. Polymorphism in the tumor necrosis factor-? promotor region and in the heat shock protein 70 genes associated with malignant tumors. *Cancer* 1997;80:1489–96.
45 Mestiri S, Bouaouina N, Ahmed SB, *et al*. Genetic variation in the tumor necrosis factor-α promoter region and in the stress protein Hsp70-2: susceptibility and prognostic implications in breast carcinoma. *Cancer* 2001;91:672–8.
46 Kohaar I, Tiwari P, Kumar R, *et al*. Association of single nucleotide polymorphisms (SNPs) in TNF-LTA locus with breast cancer risk in Indian population. *Breast Cancer Res Treat* 2009;114:347–55.
47 Pooja S, Francis A, Bid HK, *et al*. Role of ethnic variations in TNF-α and TNF-β polymorphisms and risk of breast cancer in India. *Breast Cancer Res Treat* 2011;126:739–47.
48 Gupta R, Kapuria V, Das SN, *et al*. Single nucleotide polymorphisms in *TNF*-alpha, *TNFR2* gene and TNF-alpha production in Asian Indians. *Immunol Invest* 2009;38:240–54.
49 Fang F, Yao L, Yu XJ, *et al*. Tnfα –308 G/A polymorphism is associated with breast cancer risk: a meta-analysis involving 10,184 cases and 12,911 controls. *Breast Cancer Res Treat* 2010;122:267–71.
50 Hviid TVF, Christiansen OB. Linkage disequilibrium between human leukocyte antigen (HLA) class II and HLA-G—possible implications for human reproduction and autoimmune disease. *Hum Immunol* 2005;66:688–99.
51 Ovsyannikova IG, Vierkant RA, Pankratz VS, *et al*. Extended LTA, TNF, LST1 and HLA gene haplotypes and their association with rubella vaccine-induced immunity. *PLoS One* 2010;5:e11806.
52 Castelli EC, Mendes-Junior CT, Veiga-Castelli LC, *et al*. A comprehensive study of polymorphic sites along the HLA-G gene: implication for gene regulation and evolution. *Mol Biol Evol* 2011;28:3069–86.
53 Kapasi K, Albert SE, Yie S-M, *et al*. HLA-G has a concentration-dependent effect on the generation of an allo-CTL response. *Immunology* 2000;101:191–200.
54 Kanai T, Fujii T, Kozuma S, *et al*. Soluble HLA-G influences the release of cytokines from allogeneic peripheral blood mononuclear cells in culture. *Mol Hum Reprod* 2001;7:195–200.
55 Saurabh A, Chakraborty S, Kumar P, *et al*. Inhibiting HLA-G restores IFN-γ and TNF-α producing T cell in pleural tuberculosis. *Tuberculosis* 2018;109:69–79.
56 Wilson AG, de Vries N, Pociot F, *et al*. An allelic polymorphism within the human tumor necrosis factor alpha promoter region is strongly associated with HLA A1, B8, and DR3 alleles. *J Exp Med* 1993;177:557–60.
57 Hsieh A-R, Hsiao C-L, Chang S-W, *et al*. On the use of multifactor dimensionality reduction (MDR) and classification and regression tree (CART) to identify haplotype–haplotype interactions in genetic studies. *Genomics* 2011;97:77–85.