HLA-G 14-bp Insertion /Deletion (In/Del) Polymorphism in Breast Cancer Iraqi Arabian Women

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Abstract

Background: Breast cancer is a group of diseases in which the breast cells change and divide uncontrolled, typically resulting in a lump or mass. Human leukocyte antigen –G (HLA-G) gene is a none –classic major histocompatibility complex (MHC) class I molecule that is highly expressed in cancer pathologies and is one of the strategies used by tumor cells to escape immune surveillance. A14-bp insertion/deletion (In/Del) polymorphism in exon 8 of the 3` untranslated region (3`- UTR) of the HLA-G gene has been suggested to be associated with HLA-G mRNA stability and the expression of HLA-G. **The present study** aimed to the genotyping of the 14-bp In/Del in the HLA-G gene and its relation with clinical characteristics in Iraqi Arab breast cancer women.

Materials and methods : Sixty women affected with breast cancer were enrolled in this study, in addition to 40 age –sex and ethnically matched healthy individuals participated as the control group, genotyping was performed using conventional polymerase chain reaction (PCR) and electrophoresis assays with specific primers.

Results: The results of current study revealed that the prevalence of HLA-G 14-bp homozygous insertion genotype (ins/ins) was higher in breast cancer patients (26.7%) , while in control group (12.5%) (OR = 2.55, 95%CI=0.86-7.54 , P= 0.088). The frequency of the ins allele was higher in breast cancer patients (51.7%), while in the control group (40 %), but no significant differences were observed in the distribution of alleles and genotypes between the patients and control groups. Moreover, we evaluated the possible correlation of the HLA-G 14-bp In/Del genotypes and clinical characteristics of the patients, but no statistically significant correlation was found, except for histologic grade and tumor type under ($P \leq 0.05$).

Conclusion: The result revealed the most frequencies polymorphism is the homozygous insertion genotype (ins/ins) of the HLA-G 14-bp and its allele in Iraqi Arabian breast cancer women. No significant association between this polymorphic site and breast cancer although the data revealed a significant relation in genotype and allele frequency of 14-bp polymorphism between grade I, II, and III of breast cancer patients Larger case-control study including other polymorphic sites of the HLA-G gene is necessary to substantiate the importance of HLA-G polymorphisms and linkage disequilibrium in breast cancer risk.

Keyword: *Breast cancer, HLA-G, 14-bp insertion/deletion, polymorphism, Iraq*

Introduction

Breast cancer is the second most common cancer in the world and the most common cancer to be diagnosed in women (24.2%(, about one in 4 of all new cancer cases diagnosed in women worldwide are breast cancer [(1)] . In 2018 an estimation of 2.1 million new breast cancer were diagnosed constituting (11.6%) of all cancer cases in women and 627.000 (6.6%) breast cancer death worldwide [(2)]. In Iraq, breast cancer is considered the most common cancer [(3)]. It ranks the first among the commonest malignancies among all the population, there were 6206 cases in 2018 considered 6094 females and 112 males, the percentage of total constitute around 19.70 % with a rate of 16.3 for every 100000 populations [(4)].

Human leukocyte antigen- G (HLA-G) belongs to the family of non-classical HLA class I genes, located within the MHC region at the chromosomal region on the short arm of human chromosome 6 (6p21.3) [5] . This gene was firstly described by Geraghty and colleagues in 1987 [6]. Encompasses at least four membrane-bound and three soluble isoforms [7] . HLA-G is characterized by its restricted tissue distribution/expression, low rate of polymorphism, limited protein variability, short cytoplasmic tail, alternative splicing generating several membranes bound and soluble isoforms, modulation of an immune response (immune tolerance), and immunosuppressive properties [8, 5] . HLA-G act as a negative regulator of the human immune response by several mechanisms, including the inhibition of the cytotoxic effects of T lymphocytes and natural killer (NK) cells, as well as the prevention of antigen recognition and anti-proliferative responses of CD4+ T cells [9] . HLA-G can be inhibiting the proliferation of T and B cells, and cytotoxic lymphocyte (CTL), the phagocytic activity of neutrophils, and the function of dendritic cell (DC), via ILT2 and ILT4 signalling [10] .

HLA-G expression initially was observed in extra villous cytotrophoblasts and is considered to play an important role in the maintenance of fetal-maternal immune tolerance [7] . Later found it can be switched on in various pathological conditions such as cancers, viral infections, organ transplantation, and autoimmune and inflammatory diseases [11]. HLA-G may play a pivotal role in the occurrence and progression of malignant tumors [8]. Increased HLA-G expression has been observed and reported in different tumor types, including breast cancer [12]. Therefore, the role of HLA-G in malignancies has gained considerable clinical interest in the possibility of exploiting it as a molecular biomarker and a therapeutic target [13]. The 14-bp insertion /deletion (ins/del) polymorphism in exon 8 of the 3` untranslated region ( 3`UTR) of HLA-G is the most widely studied polymorphism. It has been demonstrated that the HLA-G 3`UTR 14- bp ins/del variation implicates the stability and isoform splicing patterns of HLA-G mRNA [8]. The HLA-G 14 bp ins/del polymorphism has been previously evaluated in several malignancies, like lymphocytic leukemia and osteosarcoma [14, 15]. In the present study, we assessed the frequency of the HLA-G 14-bp insertion/deletion polymorphism and its relation with clinical characteristics in Iraqi Arabian breast cancer women.

Materials and Methods

Patients

Sixty Iraqi Arabian breast cancer women with a mean age (50.6 ±10.4) were enrolled in this study between May 2019 and February 2020. The age range was (30-71) years. In addition to forty apparently healthy individuals were participated as a control group with a mean age (46.9 ± 9.8), the age range was (30-64) years, matched patients for ethnicity (Iraqi Arabic), The diagnosis was done under consultant medical staff at the Oncology Teaching Hospital of the Medical City, Baghdad, according to the clinical mammographic, histological findings. Patients were early detected. None of the patients received chemotherapy or radiotherapy or treatment with mastectomy before blood collection. Blood samples were collected in EDTA containing tubes from all participated individuals. All examined women were residence in a different area of Baghdad and another governorate. The informed consent and agreement to give blood samples were obtained from all participants in this case-control study. The present study was approved by the ethics committee at the Ministry of Health in Iraq.

Genotyping

The Genomic DNA was extracted from EDTA blood samples using ReliaPrep™ Blood gDNA Miniprep System (Promega Corporation, USA) and after assessing purity and concentration, it was subjected to PCR amplification. The following primers were used for the HLA-G gene: GE14HLA-G Forward: 5`-GTGATGGGCTGTTTAAAGTGTCACC-3`) and RHG4 Reverse: 5`-GGAAGGAATGCAGTTCAGCATGA-3` [16]. The PCR reaction was performed in a final volume of 25 µl, which included 12.5 µl GoTaq green Master mix, 1 µl of each primer (10 µM), 3 µl of DNA sample, and 7.5 µl nuclease-free water. The PCR conditions were initial denaturation at 95 °C for 5 min (one cycle), followed by 30 cycles of denaturation at 95 °C (30 sec), annealing at 64°C (30 sec) and extension at 72°C (30 sec), followed by a final extension at 72°C for 7 min. The PCR products were analysed for HLA-G bp ins/del depending on the absence or presence of the 14bp allele band in agarose

(2%) gel electrophoresis which was stained with ethidium bromide. In case del/del homozygous genotype (-14bp/-14bp), one band of DNA fragment length 210bp has appeared, while insertion of the homozygous genotype (+14bp/+14bp), also one band appeared, it was 224 bp, the appearance of two bands (210bp and 224bp), indicate to the heterozygous genotype -14bp/+14bp. Figure (1).

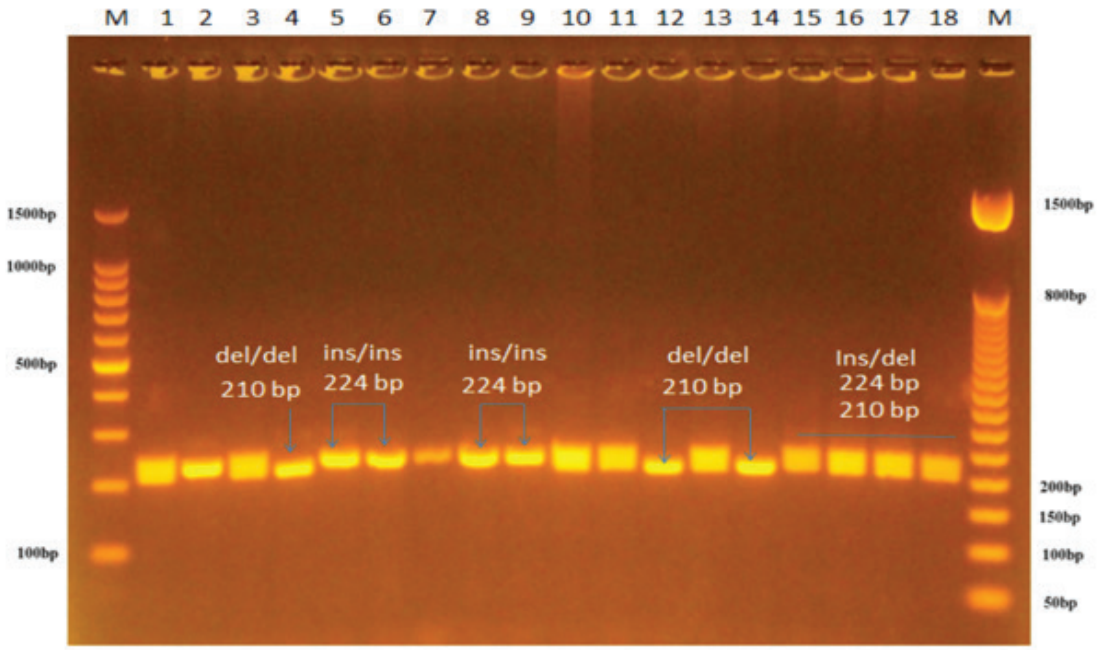

Figure 1: Detection of 14 base pair (bp) In/Del polymorphisms on agarose gel (2%) stained with ethidium bromide. Lane M: DNA markers, Lane 1,3,10,11,13,15,16,17.18 : Heterozygous (ins/del) , Lane 2,5,6,8,9, : Homozygous (ins/ins) , Lane 4,12,14 : Homozygous (del/del).

Statistical Analysis

The data were examined for normality, homogeneity and normal distribution by using the IBM SPSS version 26.0 computer program was used to calculate the allele and genotyping frequencies [17]. Pearson's chi-square test at $p \leq 0.05$ used to calculate the probability. A Pearson's correlation used to determine the relationship between the studied parameters. For genotyping and alleles frequencies, the odd ratio (OR) and 95% confidence interval (CI) calculated by WinPepi version 11.65 [18]. Online Hardy-Weinberg calculator [19] used for genotyping and alleles frequencies calculations.

Results

Patients clinical characteristics

The clinic-pathological characteristics of the patients are summarized in Table (1). Moreover as shown in Table (1), we examined association of the HLA-G 14-bp In/Del genotypes and other clinical characteristics of the patients, but no statistically significant correlation was identified ($P \leq 0.05$), with the exception of histological grade and tumor type.

Table 1: Correlation between HLA-G 14-bp In/Del polymorphism genotypes and clinico-pathological characteristics

Characteristics	Patients no,(%)	Genotypes/14-bp In/Del polymorphism			P. value
		del/del	ins/del	ins/ins	
Tumor type					
Invasive ductal carcinoma (IDC)	55 (91.7)	10	30	15	0.006*
Invasive lobular carcinoma(ILC)	5 (8.3)	4	0	1	
Histological grade					
Grade I	1(1.7)	1	0	0	
Grade II	46 (76.7)	10	27	9	0.034*
Grade III	13(21.7)	3	3	7	
Tumor stage					
Stage I	7 (11.7)	2	3	2	
Stage II	26 (43.3)	6	15	5	0.157
Stage III	18 (30)	3	6	9	
Stage IV	9 (15)	3	6	0	
Tumor Size(cm)					
≤2cm	14 (23.3)	6	5	3	0.141
>2cm	46 (76.7)	8	25	13	
Lymph Node Metastasis					
Positive	37 (61.7)	9	15	13	0.113
Negative	23 (38.3)	5	15	3	
Estrogen receptor					
Positive	48 (80)	12	25	11	0.415
Negative	12 (20)	2	5	5	
Progesterone receptor					
Positive	45 (75)	11	24	10	0.401
Negative	15 (25)	3	6	6	
HER-2/neu receptor					
Positive	14 (23.3)	2	8	4	0.653
Negative	46 (76.7)	12	22	12	

Polymorphism of the 14bp insertion /deletion (In/Del) in the HLA-G gene

In this case-control study, we examined the distribution of alleles and genotypes of the most commonly studied HLA-G 14-bp In/Del polymorphism located in exon 8. Sixty women with breast cancer and 40 healthy women without a history of breast cancer were enrolled in the present study. The PCR products showed three bands of different DNA fragments length it were (210 and 224) bp for del/del and ins/ins genotypes respectively beside (210 and 224) bp for heterozygous genotype (ins/del), Figure (1). The current study, showed, no significant difference was found at allelic and genotype levels between the breast cancer patients and control groups, although there is a slightly higher allele and genotype frequency of HLA-G 14-bp insertion (ins/ins) than 14-bp deletion (del/del) in breast cancer patients. The frequency of the allelic variant and genotype of studied polymorphism in breast cancer patients and healthy women are reported in Table (2).

Table 2: Genotype distribution and allele frequencies of HLA-G 14-bp In/Del gene polymorphism in breast cancer patients and control women

Genotype / HLA-G14-bp In/ Del Polymorphism	Patients no.= 60,(%)	Control no.= 40,(%)	χ^2	P.value	Odds Ratio (OR)	Confidence Interval 95% (CI)
del/del	14 (23.3)	13 (32.5)	1.023	0.312	0.63	0.26-1.53
ins/del	30 (50.0)	22 (55.0)	0.240	0.624	0.82	0.37-1.81
ins/ins	16 (26.7)	5 (12.5)	2.903	0.088	2.55	0.86-7.54
Allele frequency						
del	58 (48.3)	48 (60.0)	2.623	0.105	0.62	0.35-1.10
ins	62 (51.7)	32 (40.0)	2.623	0.105	1.60	0.91-2.83

It was found that the HLA-G 14-bp insertion allele (ins) frequency was higher in patients (51.7%) versus controls (40 %), while 14-bp deletion allele (del) frequency was lower in patients (48.3%) versus controls (60 %). Similarly, the homozygous insertion genotype (ins/ins) was higher among patients (26.7%) versus controls (12.5%), the homozygous deletion genotype (del/del) was higher in controls (32.5%) versus patients (23.3%) , and the heterozygous insertion-deletion genotype (ins/del) was slightly higher in controls (55%) versus patients (50%) . In our population, the 14-bp genotype distribution proportions agreed with expected Hardy- Weinberg equilibrium both in patients (P= 0.993) and in the control (P=0.356) groups as shown in Table (3).

Table 3: Allele and genotype frequencies and Hardy-Weinberg equilibrium of HLA-G 14bp In/Del gene polymorphism in breast cancer patients and controls no: Number, If < 0.05 not consistent with HWE (Hardy-Weinberg Equilibrium).

Genotype/HLA-G14bp ins/del polymorphism			del/del	ins/del	ins/ins	HWE P≤	Alleles	
							del	ins
Breast cancer Patients Total no. = 60	Observed	no.	14	30	16	0.993 NS	58	62
		%	23.3	50.0	26.7		48.3	51.7
	Expected	no.	14.0	30.0	16.0			
		%	23.3	30.0	16.0			
Controls Total no. = 40	Observed	no.	13	22	5	0.356 NS	48	32
		%	32.5	55.0	12.5		60.0	40.0
	Expected	no.	14.4	19.2	6.4			
		%	36	48	16			

The frequency of homozygous ins/ins genotype was higher in patients of grade III (53.8%) while the frequency of homozygous del/del genotype and heterozygous ins/del genotype was lower in patients of grade III (23.1%) for each. Concerning the relation of 14-bp allele frequency with histological grade and stage, it was found that the 14-bp insertion allele frequency was higher in patients of grade III (65.4%). Therefore, our result revealed significant differences in genotype and allele frequency of 14-bp polymorphism between grade I, II, and III of breast cancer patients (P=0.034). Current results showed higher frequency of homozygous insertion genotype in patients with stage III 50% and found that the 14-bp insertion allele frequency was higher in patients of stage III (66.7%) while 14-bp deletion allele frequency was lower in stage III (33.3 %). Therefore, our result revealed no significant differences at both allele and genotype of 14-bp ins/del polymorphism depend on stage of breast cancer patients (P= 0.157). As shown in table (4)

Table 4: Genotype distribution and allele frequencies of HLA-G 14bp In/Del gene polymorphism depend on grade and stage of breast cancer patients

Genotype/ HLA-G14-bp In/Del polymorphism	Grade Total no. = 60			Stage Total no. = 60			
	Grade I no =1,(%)	Grade II no =46,(%)	Grade III no=13,(%)	Stage I no=7,(%)	Stage II no =26,(%)	StageIII no =18,(%)	StageIV no=9,(%)
del/del	1 (100)	10 (21.7)	3 (23.1)	2 (28.6)	6 (23.1)	3 (16.7)	3 (33.3)
ins/del	0 (0.0)	27 (58.7)	3 (23.1)	3 (42.9)	15 (57.7)	6 (33.3)	6 (66.7)
ins/ins	0 (0.0)	9 (19.6)	7 (53.8)	2 (28.6)	5 (19.2)	9 (50.0)	0 (0.0)
P-value	0.034*			0.157 NS			
Allele frequency							
del	2 (100)	47 (51.1)	9 (34.6)	7 (50.0)	27 (51.9)	12 (33.3)	12 (66.7)
ins	0 (0.0)	45 (48.9)	17 (65.4)	7 (50.0)	25 (48.1)	24 (66.7)	6 (33.3)

no: Number,*Significant differences between percentage using Person Chi-square (χ^2 test) at 0.05 level

Discussion

The immune system can play a dual role in breast cancer. Immune response can promote tumorigenesis through inflammatory pathways suppressing adaptive immunity, or it can prevent tumor formation through active immune surveillance, Consistent with this concept,some breast cancer patients display clear evidence of immune suppression, they have lower absolute numbers of peripheral blood lymphocytes [20] . Tumour-infiltrating T lymphocytes (TILs) and mature DCs have been correlated with lymph node involvement and tumor grade, and the presence of DCs is associated with shorter disease-free and overall survival [21] . In this context of decreased immune response, it has been suggested that HLA-G plays a role in immune suppression in the tumor microenvironment, because HLA-G exerts an overall negative immune function inhibiting the activity of NK cells, CTLs and antigen-presenting cells (APCs), all of which are essential to the development of a cytotoxic anti-tumor immune response [12] .

This study was aimed to investigate the HLA-G 14-bp genotypes in Iraqi Arabian women suffering from breast cancer as HLA-G contribution to the suppression of immune responses. The breast cancer patients and control group were representative random samples of different ages from the populations. The result showed that all HLA-G 14-bp genotypes were consistent with the Hardy-Weinberg expectations in both patients and the control group. Our results for the distribution the 14-bp ins/del polymorphism was found in 50 % of patients compared to 55% of control group, no significant differences depend on the 14-bp ins/del polymorphism between the patients and control groups. However, for allele frequency , the 14-bp insertion was more frequent 62 (51.7%) for in women with breast cancer than 32 (40) in the control group, and inversely related to the 14-bp deletion. Although there is a slightly higher allele frequency of HLA-G 14-bp ins 62 (51.7%) than 14-bp del 58 (48.3%) in the breast, no significant difference

was found at allelic and genotype levels between the case and control groups. The genotype 14bp insertion/ insertion was more frequent (26.7 %) in women with breast cancer than 12.5% in the control group ,while the distribution of the del/del genotype was (23.3% vs. 32.5%) in patients and control groups (odd ratio =2.55, P= 0.088 , confidence interval was very wide CI- 0.86-7.54), therefor a large samples for the two selected groups are needed to confirm this association. HLA-G 14-bp polymorphisms showed no association with clinical parameters with the exception of histological grade and tumor type. Our results agree with meta-analysis data that was obtained by Jiang *et al*., [8], it revealed that the HLA–G 14–bp ins/ins genotype and (ins) allele were associated with the total cancer risk of breast cancer and oesophageal cancer.

A recently published Iraqi study referred to a significant difference (P<0.01) between breast cancer patients and control groups in HLA-G 14-bp del/del genotyping (66.7% vs. 36.7%), while no significant differences (P>0.001) in the genotypes ins/ins frequency (6.6% and 0.0%) in patients and control groups respectively. They found a significant increase in the frequency of heterozygous genotype ins/del in controls compared to patients (63.3%vs.26.7%). Concerning the allelic frequency, also no significant difference was found between the patients and control groups at HLA-G 14bp ins/del [22]. The present results were disagree with AL-Omar and Mansour [23] who showed a high significant increase between the 14-bp del allele and occurrence of breast cancer, also our results disagree with the Tunisian results which confirmed the elevation of deletion allele frequencies among the patients in comparison with the control group and conferred a risk to breast cancer development (52% Vs 45%) [24]. These discrepancies in the results may be associated with the genetic variations between various ethnic people investigated, geographic climate, daily lifestyle, ethnic diversity and dietary habits [8].

Our results showed an association between breast cancer susceptibility and HLA-G 14-bp ins/ins, it may be consider as a potential genetic risk factor in the progression of the breast cancer in proposed genetically individuals. Although our data excluded a significant association between this polymorphic site and breast cancer, a larger case-control study including other polymorphic sites of the HLA-G gene is necessary to substantiate the importance of HLA-G polymorphisms and linkage disequilibrium in breast cancer risk. This finding can highlight this gene as candidate to future more studies to establish the exact role of HLA-G in progression steps of breast cancer.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: None

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