Association of soluble HLA-G and HLA-G 14bp ins /del Polymorphism in some Iraqi patients with Breast Cancer

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Abstract

Human leukocyte antigen-G is known to be implicated in a tumor-driven immune escape mechanism in malignancies. The main objective of the study is to evaluate the HLA-G 14-bp polymorphism in the 3`-untranslated region of the HLA-G gene in that associated with the susceptibility to breast cancer patients. This study has been done on 60 BC patients & 30 Benign tumor and30 control groups. Estimation of sHLA-G markers was assayed by using ELISA technique. Thus, polymorphism of HLAG HLA-G 14-bp were detected by PCR methods. The plasma levels of HLA-G shows a significant elevation in BC patients compared to benign breast tumour and controls (32.79±0.9*vs.* 13.84 ±0.30 and 12.72 ±0.45 ng/ml, $P \leq 0.05$). There was a significant difference between newly diagnosed breast cancer patients and healthy control groups in homozygous genotype HLA-G 14-bp del/del genotyping (66.7% vs. 36.7%; OR=3.45; P<0.01). While, no significant differences in the homozygous genotypes Ins/ins frequency were observed between patients with breast cancer (6.6%) and the healthy control group (0.0%) (OR=3.45, P>0.001). There was a significant increase frequency of heterozygous genotype Ins/del in controls compared to patients (63.3%vs.26.7%). No significant difference was found between the patients and control groups at HLA-G 14bp insertion and deletion allelic frequency**.**

Keywords: *Human leucocyte antigen-G, Gene polymorphism, Breast cancer*

Introduction

Breast cancer is most commonly found malignancy in women around the world, & almost 1.7 million new cases have been diagnosed in 2012, representing approx.. (12%) of the new cancer cases & (25%) of all women cancers[(1)]. The increased CEA is correlated with metastatic disease in breast cancer. The pre-operative CEA measurements was shown to be related to pathological stages & it is stages dependent. The levels of circulating CEA in BC patients directly depends upon the sizes of metastatic & the primary tumor. CEA in BC patients is replaced by other markers with high specificity like CA- 15-3[(2)]. The tumor marker is used to diagnose and monitor the clinical course of the breast cancer, and this marker can also be found in benign breast tumors[(3)(4)(5)].The HLA-G gene can be an excellent candidate gene for the disease susceptibility, since, given its immunomodulatory function, can act as a protective molecule in inflammatory responses[(6)]. The human leukocyte antigen-G (HLA-G) belongs to class I non-classical HLA gene family & is located on chromosome 6p21[(7)]. HLA-G gene encodes 7 isoforms by alternative splicing of the primary transcript, including 4 membrane-bound (HLA-G1, -G2, -G3, & -G4) as well as 3 soluble isoforms (HLA-G5, -G6, & -G7) [(8)]. HLA-G plays an important role in suppression of the immune responses and participate in the long-term immune tolerance or escape. HLA-G expression may be induced in many diseases such as cancers[(9)(10).] HLA-G alleles relatively restrict polymorphisms & low sequence variations in many populations[(11)(12).] To HLA-G gene, 47 alleles were assigned, primarily in the exons 2, 3, & 4. The diversity of the promoter and the 3-untranslated region (UTR) of HLA-G gene controlled the HLAG protein expression[(13).] HLA-G gene also has the absence or presence of a 14 bp at the 3`-UTR) [(14)(15)]. A 14 bp

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ins/del polymorphism in exon 8 in the 3´UTR of HLA-G was found to be associated with the stability and splicing patterns of *HLA-G* mRNA isoforms. In addition, HLAG polymorphisms were investigated in several cancers types and were regarded as predictive markers and risk factors for cancers[16].

Patient & Method

This study has been performed on 60 untreated patients with breast cancers, who were diagnosed by oncologist consultant doctors at the oncology teaching hospital baghdad / Iraq and 30 healthy control groups with age range 40-60 years (Mean:50.73±1.60) from March to June 2019 for diagnosis and treatment. All of them had no malignancy other than BC, recurrent breast cancer cases and un treated with chemotherapy or radiotherapy or hormone therapy were also excluded. In addition, 30 Benign and 30 apparently healthy women were also included in this study; matched patients with mean (46.90±2.58; 48.93±3.00). Five ml venous blood was collected from each patients and controls. Blood samples were divided into two aliquots. The first aliquot was transferred into an EDTA tube and stored at -20 ºC until assayed for HLA-G 14bp ins/del polymorphisms. The second aliquot was to transfer to EDTA tube and then centrifuge samples for 10 min at 5000 rpm and then collect the plasma to assayed sHLA-G.

Mesurment of sHLA-G in the sera of the studied groups

The serum sHLA-G levels were estimated by sHLA-G Enzyme Linked Immunosorbent Assay (ELISA) kit (Elabscience, USA).

The amplification of exon 8 of HLA-G 14bp in/del gene using PCR.

DNA was extracted from EDTA blood samples of the BC patients & the healthy controls using the commercial Geneaid method kit (Geneaied Biotech. Ltd, Taiwan). The genotype of HLA-G 14bp ins/del was detected by PCR with specific primer(forword 5'-TCA CCC CTC ACT GTG ACT GAT A - 3' and reverse 5'-GCA CAA AGA GGA GTC AGG GTT - 3'). Five µL of Master Mix was used in a 25 µL reactions mixture with 1 µL for each primer and 1.5 µL DNA, then complete volume by add 16.5 µL. The PCR Steps composed of of initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 40 seconds, annealing at 50°C for 45 seconds, extension -1 at 72°C for 40 seconds and extension- 2 at 72°C for 7 minutes. The PCR product have been observed under the UV on a 2% agaroses gel (Promega Company, USA) stained by 3 µl red safe stains. The size targeted fragment size was compared with 5 µl of universal DNA ladder fragments (KAPA™, Universal LadderKK6302, USA).

Statistical Analysis

The IBM SPSS version 25 computer program was used to calculate the mean, standard error and the probability by using student t-test, ANOVA table and Duncan test. Also, For allele & genotyping frequencies, the online Hardy-Weinberg calculator was applied to calculate the variation significance. The differences were significant if $P<0.05$.

Results

The serum HLA-G level was significantly elevated in BC patients in comparison to benign breast tumour and control groups (32.79±0.9*vs.* 13.84 ±0.30 and 12.72 ±0.45 ng/ml, $P\leq0.05$). Thus no significant variation was pointed between benign tumor group & the healthy controls (13.84 ± 0.30 and 12.72 ±0.45 ng/ml, $P> 0.05$) (Table 1).

Table 1: Plasma level of sHLA-G in untreated BC patients and control group.

Groups		Plasma sHLA-G level (ng/ml)- Mean± SE
Patients	Breast cancer	32.79 ± 0.9 A
Contol	Benign tumor	13.84 ± 0.30 B
	Healthy	12.72 ± 0.45 B

Duncan test, Similar letters: No significant variation ($p > 0.05$) between the means. Different letters:Significant variation ($p \leq 0.05$) between means.

Genotyping of HLA-G 14bp in/del polymorphisms in exon 8 at the 3′UTR regions were performed by PCR methods. The HLA-G 14 bp alleles & genotypes frequency in breast cancer patient group & the healthy control are shown in the table (2). The allele & genotyping frequency of HLA-G 14bp polymorphisms have been examined as for Hardy-Weinberg equilibrium

(HWE).

The current HLA-G Ins/del findings detected 2 alleles (Ins & del), which were corresponding to 3 genotypes (Ins/ins, Ins/del & del/del). No significant differences were shown between the expected and observed genotype frequency among SLE patients and the control group.

Comparing between controls & patients revealed a significant difference between the BC patients and healthy control groups in homozygous genotype HLA-G 14-bp del/del genotyping (66.7% vs. 36.7%; OR=3.45; P<0.01). While, no significant differences in the homozygous genotypes Ins/ins frequency were observed between patients with breast cancer (6.6%) and the healthy control group(0.0%) (OR=3.45, P=0.279). In addition, there was a significant increase frequency of heterozygous genotype Ins/del in controls compared to patients (63.3%vs.26.75) (Table 2).

In contrast, del allele exhibited a non-significant increased frequency in the patient group in comparison with the control group (80% vs. 68%). Ins allele showed a non-significant elevation in healthy control groups compared to breast cancer patients (32% vs. 20%) (Table 2).

Table (2): Allele Frequencies and HLA-G-14bp genotype among BC patients and controls.

Genotyping	Breast cancer (60)				A healthy control group (30)				p-value
	Observed frequency		Expected frequency		Observed frequency		Expected frequency		
	No.	%	No.	%	No.	%		%	
Del/Del	40	66.7	38.40	64.0	11	36.7	14.01	46.96	0.01
Ins/ins	4	6.6	2.40	4.0	0	0.0	3.01	10.03	0.297
Ins/del	16	26.7	19.20	32.0	19	63.3	12.98	43.28	1.2×10^{-3}
Total	60	100.0	60.0	100.0	30	100.0	30.0	100	
P-HWE	0.196(NS)				0 .011(NS)				
Allele Frequency									
Ins	20				32			0.076	
Del	80				48			0.01	

OR: Odd ratio, ***P***: Fischer's exact probability (two-tailed), ***P*-HWE**: the probability of Hardy-Weinberg Equilibrium.

Discussion

serum levels of CEA and CA15-3 in patients with breast cancer are higher than the healthy control group.

Circulating levels of sHLA-G were significantly increased in BC patients in comparison with the controls, which is close to a formerly data reported by Khattab and Jeong *et al*. who stated that serum sHLA-G was significantly higher in BC group than that of the controls[17][18]. We found that serum sHLA-G levels were significantly increased in the breast cancer group, which was in agreement with results of Rebman *et al* [19] who showed a significant high serum sHLA-G level in patients who suffered from BC. This result strongly hypothesized that sHLA-G could be utilized as tumour markers in the sera of BC cancer patients for the detection or treatment monitoring. Despite the usefulness of sHLA-G might be proven to help identifying malignant *vs* benign clinical cases, there are lways many challenges ahead.

Several studies demonstrated that the HLA-G molecules are relatively highly expressed in different tumour types like hematologic malignancies (acute leukemia, lymphomas), primary solid (melanomas, neck & head, urogenital, gastrointestinal, lung and breast cancers) as well as metastases [20][16]. This mechanism can be used by tumors to escape from immune surveillance[21]. Other studies showed that HLA-G expression is affected by 14-bp ins/del polymorphisms in the 3′ UTR[22]. In 3' untranslated region (3' UTR) of HLA-G gene, an insertion/deletion polymorphism of 14 bp was found to affect the stability of mRNA. The effect of this polymorphism in disease susceptibility is controversial. In Iraqi people, no report on HLA-G polymorphisms regarding BC has been found. In the present study, HLA-G Ins/Del polymorphism & risk of Iraqi women with breast cancer patients have been studied, there was a significant increased frequency of del/del genotype as well as of del allele in breast cancer patients compared to the controls, while Ins/del genotype as well as Ins allele showed a non-significantly decreased frequency in the controls when compared with the patients. The present results were in agreement with Al Omar and Mansour, (2019) who showed a high significant increase between the14-bp Del allele & occurrence of breast cancer. Females with homozygous genotype Del/Del were 2.5-fold more probably to develop breast cancer than the non homozygous[23]. Our results affirm the important influence of HLA-G 14-bp Ins/Del polymorphism on BC occurrence in the Saudi Arabia population and agreed with those stated formerly by[23] and agreed with the Tunisian results which confirmed the elevation of del allele frequencies among the patients in comparison with the control group and conferred a risk to BC development (52% Vs 45%). An Iranian study showed elevated Del allele & del/del genotype frequiencies in BC women in comparison with the control group[24]. These results illustrated the potential role played by the 14-bp polymorphisms in BC history development owing to its role in HLA-G alternative splicing & in the stability of RNA[25][18].

Our results did not agree with Ge *et, al.* & Ramos *et al*. when they stated that the HLA-G 14-bp Ins/Del polymorphisms were correlated with BC and all cancer risks among Asian people[26] and Brazilian population[27]. In this state, several studies revealed that the 14-bp Ins/Del polymorphisms played a key role in development of different diseases such as various cancers types including the Non Hodgkin lymphomas[28], hepatocellular carcinoma[29]and esophagal carcinoma. These discrepancies in the results between studies may be associated with the genetic variations between various ethnic people investigated.

Conclusions

These results showed an association between breast cancer susceptibility and HLA-G 14-bp Del/del as a potential genetic risk factor in the progression of the disease. While HLA-G 14-bp Ins/del was considered as a protective factor against breast cancer.

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

Conflict of Interest: Non

Funding: Self-funding

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