Research Paper

Study of Human Leukocyte Antigen-G gene 14-bp Ins/Del and Codon 93 (CAC/CAT) Polymorphisms Association with Breast Cancer Susceptibility

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Human leukocyte antigen-G is a non-classic human leukocyte antigen I molecule. This protein is expressed aberrantly in numerous forms of malignant diseases like glioblastoma multiforme, ovarian cancer, lymphoblastic and breast cancer. Human leukocyte antigen-G polymorphisms association with breast cancer has been extensively studied. One amongst the foremost studied variants was human leukocyte antigen-G 14-base pair insertion/deletion polymorphism. Nevertheless, results are contradictory or inconclusive. We aim to investigate two human leukocyte antigen-G polymorphisms, 14-base pair insertion/deletion 3' untranslated region and for the first time and codon 93 (CAC/CAT) polymorphisms potentially associated with breast cancer risk in the Tunisian population. This study involved 151 breast cancer patients and 187 healthy controls. Genotyping of the human leukocyte antigen-G codon 93 (CAC/CAT) and 14- base pair insertion/deletion variants was performed respectively by amplification-refractory mutation system and polymerase chain reaction. The analysis of genotype and allele frequencies revealed that human leukocyte antigen-G 14-base pair insertion/deletion polymorphism was associated with breast cancer susceptibility in the Tunisian population (Odds ratio=0.53, 95 % confidence interval=0.33-0.85, p=0.0022) without correlation with clinical parameters. We also observed an association of the human leukocyte antigen-G codon 93 (CAC/CAT) polymorphisms with breast cancer. In terms of analysis of the combined 14-base pair insertion/deletion and codon 93 CT genotype, we detected that the CC insertion/insertion and CC insertion/deletion genotype decrease the risk of breast cancer development (Odds ratio=0.2083; p=0.0097; Odds ratio=0.3571; p=0.0053, respectively). In addition, the haplotype frequency distribution analysis disclosed that the C insertion haplotype has a protective effect against breast cancer development (Odds ratio=0.4749; 95 % confidence interval=0.4425-0.9277; p=0.0153). Taken together, female's homozygosity for human leukocyte antigen-G 14-base pair insertion and human leukocyte antigen-G codon 93 (CAC) alleles may protect against breast cancer susceptibility.

Key words: Breast cancer, human leukocyte antigen-G codon 93 (CAC), 14-base pair insertion/deletion polymorphisms, amplification-refractory mutation system, polymerase chain reaction

Breast Cancer (BC) is considered as the most common malignancy in women worldwide with 2.1 million cases in 2018. BC, which refers to breast carcinoma, is responsible for a high mortality[1]. Although BC is considered a multifactorial neoplasm, many challenges remain to clearly identify the factors involved in metastasis.

Metastasis is the spread of primary tumor cells to surrounding tissues of the origin or to more distant tissues, referred to as secondary sites[2]. While circulating in the blood, tumor cells have the ability to escape immune cell attacks by modulating their membrane-bound proteins such as the tolerant molecule Human Leukocyte Antigen-G (HLA-G)[3-6].

HLA-G is an unconventional HLA class I molecule described for its ability to modulate the immune

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response. Its main function is to inhibit Natural Killer (NK) cell activity and to promote immune tolerance[7-10]. HLA-G expression is not restricted to the cell membrane, however, a soluble fraction in body fluids is also detected[11]. The main soluble isoforms of HLA-G are HLA-G1, resulting from membrane-bound isoform shedding and HLA-G5, which is a secreted isoform[12]. HLA-G is a vital molecule in tumor escape in many cancers. Indeed, various types of malignant diseases, such as glioblastoma, ovarian cancer, malignant melanoma, lung cancer, colorectal cancer and BC are characterized by a high expression of HLA-G[13-18]. The presence of an elevated proportion of HLA-G positive tumor cells (pre or post-treatment) has been correlated with response to chemotherapy and overall survival[16,19-22].

HLA-G expression in tumor lesions is beneath the control of genetic background. Indeed, several polymorphisms of HLA-G alleles are associated with differential expression patterns[23]. One amongst the foremost studied variants was HLA-G 14-base pair (bp) Insertion/Deletion (Ins/Del) polymorphism located in exon 8[24,25]. This latter polymorphism association with BC and clinical parameters has been studied in several populations. However, the results are contradictory or inconclusive[23,24]. In this context, solely two registered studies have interested in this correlation between the HLA-G variant and BC in Tunisia, but they are discordant[26,27].

HLA-G coding sequence counts 75 polymorphisms, some of them are silent variants such as the codon 93 in exon 3[7]. However, this variant happens with high frequency compared to others[28]. Till date, this polymorphism has been poorly studied and no such work has been reported in BC.

In this work, we investigated two HLA-G polymorphisms; the HLA-G 14-bp Ins/Del 3' Untranslated Region (3'-UTR) polymorphism and for the first time the codon 93 polymorphism (CAC/CAT) potential association with BC risk in the Tunisian population.

MATERIALS AND METHODS

The study subjects:

This study concerned 151 patients and 187 healthy controls. Patient samples were collected between February 2014 and July 2016 at the oncology department of the Fatouma Bourguiba University Hospital Center in Monastir, Tunisia. The controls are volunteers recruited for this study with no family history of BC. Controls are matched to cases according to their geographic and ethnic origin. All BC cases are confirmed by histopathology, in addition to mammography. This study has been approved by the CHU Fatouma Bourguiba Ethics Committee and was conducted in accordance with the Declaration of Helsinki II. Each participant signed informed consent.

Deoxyribonucleic Acid (DNA) extraction and HLA-G genotyping:

Genomic DNA was extracted by the "salting-out" method and the final DNA concentration was 10 ng/ml. HLA-G rs66554220 was performed by Polymerase Chain Reaction (PCR) as described by Hviid *et al.*[29], using the following primers, forward primer: 5'-GTGATGGC TGTTTAAAGTGTCACC-3' and reverse primer: 5'-GGAAGGAATGCAGTTCAGCATGA-3'. In a PCR reaction volume of 10 µl, we added 10 pg of genomic DNA, 10 pmol of each primer 5 µl of REDTaq® ReadyMix™ PCR reaction mix. The DNA was amplified as follows: Denaturation step at 95° for 5 min, then 35 cycles of 30 s at 95°, annealed at 60° for 30 s and extended at 72° for 30 s, with a final extension step for 5 min.

The PCR product was separated by electrophoresis on a 3 % agarose gel stained with ethidium bromide. Two bands were visualized i.e. the 224 bp band for the Ins allele and the 210 bp band for the Del allele. Two blind lectures were performed on the gel. Genotyping of the HLA-G 93 CAC/CAT codon was performed by PCR-Amplification-Refractory Mutation System (ARMS) as described by Matte *et al.*[30] with minor modifications. Briefly, 10 µl of PCR mixture containing 100 pg of genomic DNA, 5 to 10 pmol of each primer and 5 µl of ThermoFisher Scientific's REDTaq® PCR reaction mixture ReadyMix™. DNA was amplified as follows-denaturation step at 95° for 5 min, then 35 cycles of 60 s at 95°, annealed at 60° for 60 s and extended at 72° for 90 s, with a final extension step for 10 min. The following primers were used: P1: 5'-AGT CTC CGG GTC TGG GAT CCA CCC GAGG-3'; P2: 5'-TGA CCG AGG GGG TGG GGG GCC AGG TTC TGAC-3'; P3: 5'-CCC AGG TCG CAG CCA ATC ATC CAC TGG AGG CTA-3'; P4: 5'-TGG TAC CCG CGC GCT GCA GCA TCT CCT TCC-3'.

The PCR product was separated by electrophoresis on a 2 % agarose gel stained with ethidium bromide.

Three bands were visualized, Control: 435 bp; allele C: 303 bp and allele T: 191 bp. Two blind lectures on the gel were performed.

Statistical analysis:

Data analysis was performed using Statistical Package for the Social Sciences (SPSS) 16 software package and by GraphPad prism. Inter-group significance assessment was done using the two-step Fisher exact test. Hardy-Weinberg equilibrium was tested for both BC patients and controls using the Chi square (χ^2) test. Haplotype and genotype analysis was assessed by SNPStats software. The Odds Ratio (OR) was calculated for each risk factor and was given with its 95 % Confidence Interval (CI). Differences in protein expression and correlation to alleles and genotypes were assessed by Mann-Whitney test. Pearson's test was performed for non-continuous variables. The p-value less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

This study involved 151 patients and 187 healthy controls. Key characteristics of the two populations are presented in Table 1. No statistical significance was recorded in Body Mass Index (BMI) between BC subjects and controls (p=1000). Nevertheless, a significant difference between the two populations was observed in terms of age, breastfeeding, menopausal status and oral contraceptive use (p=0.0149; 0.0001 and 0.0148 respectively). In fact, the BC rate among postmenopausal cases was 152.6 *vs.* 19.7 among premenopausal cases per 100 000 population worldwide in 2018[1].

The genotypes and allele frequencies of the 14-bp Ins/del polymorphisms were presented in Table 2. Both cohort were in Hardy-Weinberg equilibrium (p=0.44; p=0.14 for patients and controls, respectively).

Considering the Del/Del genotype as a reference (OR=1.00), we have seen significantly lower frequencies of Ins/Del (0.46 *vs.* 0.55; p=0.0022) and Ins/Ins (0.08 *vs.* 0.16; p=0.0022) variants in BC *vs.* controls with an OR of 0.53 and 0.31 respectively. Similar results were observed in co-dominant and recessive inheritance models, suggesting that the Ins/Del and Ins/Ins genotypes could be protective factors in the development of BC.

There was also a significant decrease in the Ins allele frequency in patients compared to the control, 8.2 % and 16.6 % respectively (OR=0.45, 95 % CI=0.22-0.92, p=0.022). In parallel, we observed significant difference in the frequency of the Del allele in patients compared to control (68 % and 56 % respectively) resulting in an OR of 1.722 (95 % CI 1.247-2.377; p=0.009). We also observed high heterozygosity in the controls.

To investigate the possible correlation between Ins/Del variants and cancer progression, we stratified genotypes and allele frequencies with clinical parameters of BC patients. The results presented in Table 3 showed no association between 14-bp Ins/Del polymorphism and histological grade, nodal status and distant metastasis. In addition, there were no significant differences between the later polymorphism among BC population with respect to Progesterone Receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER2) expression status.

Table 4 lists the genotype and alleles frequencies of HLA-G codon 93 polymorphism (CAC/CAT) distribution between BC and control women. We observed higher frequencies of the CAC/CAT (0.42 *vs.* 0.36) and CAT/CAT (0.14 *vs.* 0.08) genotypes in BC *vs.* control women. CAC/CAT and CAT/CAT variants increase susceptibility to BC in dominant tested inheritance models (OR=1.61, 95 % CI=1.03-2.51, p=0.036) and could be a risk factor in the development of BC. We also observed a significant association between CAT allele frequency and BC risk (OR=1.519, 95 % CI=1.081-2.134, p=0.0191).

Also, we did not observe any statistical association with clinical characteristics (data not shown). Both cohorts are in Hardy-Weinberg equilibrium (p=0.46 and p=0.44 for patients and controls respectively).

In terms of analysis of the combined 14-bp Ins/Del and codon 93 CAC/CAT genotypes, we detected that the CC Ins/Del (OR=0.3571; 95 % CI=0.3613-0.8373; p=0.0053) and CC Ins/Ins (OR=0.2083; 95 % CI=0.1485-0.9055; p=0.0097) genotypes were associated to lower risk of developing BC. No correlation with clinical parameters was recorded (data not presented) (Table 5).

The analysis of the haplotypes frequencies distribution (Table 6) revealed a decrease in the frequency of CIns haplotypes in BC patients compared to controls, 17.34 % and 32.33 % respectively. The CIns haplotype may have a protective effect against BC (OR=0.4749; 95 % CI=0.4425-0.9277; p=0.0153). Considering the CIns haplotype as reference (OR=1.00), in contrast the CDel haplotype

was associated to BC risk (OR=2.106; 95 % CI=1.180-3.759; p=0.0153). We also observed a non-significant increase in the frequencies of TDel and TIns haplotypes in BC subjects compared to controls (21.11 % and 14.15 % respectively). The calculated D-value was 0.036, indicating low linkage disequilibrium between the two polymorphisms.

BC is the commonest malignancy among women worldwide with high rate of mortality[1]. In the blood, tumor cells can escape immune cell attacks by modulating their membrane-bound proteins such as the tolerant molecule HLA-G[3-6]. HLA-G gene polymorphisms association with susceptibility to BC have been the scope of several studies, in particular the 14-bp Ins/Del variant located in exon 8[24,25]. In this context, two teams investigated the possible involvement of this variant with the BC susceptibility within the Tunisian population. Nevertheless, the results are discordant[1,7]. In the present work, we studied two HLA-G polymorphisms, the 14-bp HLA-G 14-bp Ins/Del 3'-UTR polymorphism and for the first time, the codon 93 (CAC/CAT) polymorphisms possible association with BC in the Tunisian population.

In this study, we obtained a significant variation in the frequencies of the Del and Ins alleles between the two cohorts. An increase in the Del allele frequency in patients was recorded. In addition, we found that the 14-bp Del/Ins and Ins/Ins HLA-G 3'-UTR variants decreased susceptibility to BC (OR=0.53; p=0.0022 and OR=0.45; p=0.02, respectively). This is in agreement with the study by Ouni *et al.* where they reported that the frequencies of the Del allele and the Del/Del genotype are more prevalent in BC patients than in controls and associated with BC risk[26]. They also reported that the Ins allele may has a protective effect against BC development in the Tunisian population. Similar results are described in other populations[31-33] and in meta-analysis studies[23,24]. However, Zidi *et al.* described that the HLA-G 3'-UTR 14-bp Ins/Del polymorphism was not associated with BC[27]. This discrepancy may be due to the relatively small number of genotyped subjects (187) compared to our study (328). Indeed, Li *et al.* reported a significant association with a high risk of BC only in a large sample size under the dominant inheritance model (Del/Del *vs.* Ins/Del+Ins/Ins)[34] as we have reported.

TABLE 1: STUDY PARTICIPANT CHARACTERISTICS

Parameters	Patients (151)	Controls (187)	p
Age (y)	49.7±12	36.0±7.8	<0.001
BMI (kg/m^2)	28.76±5.5	28.06±7.05	1
Menarche (y)	12.61±1.3	12.1±1.0	1
Breast feeding	100 (75)	187 (100)	<0.001
Menopausal status	62 (41)	22 (12)	<0.0001
Oral contraception users	21 (14)	11 (6)	0.0148

Note: BMI: Body Mass Index

TABLE 2: ASSOCIATION OF HLA-G 14-BP INS/DEL POLYMORPHISM AND BC RISK

HLA-G polymorphisms	Controls (n=187)	Patients (n=146)	OR	95 % CI	p
14-bp Ins/Del					
Codominant					
Del/Del	62 (34.2 %)	66 (45.2 %)	1		
Del/Ins	100 (55.2 %)	68 (46.6 %)	0.64	0.4-1.02	0.13
Ins/Ins	19 (10.5 %)	12 (8.2 %)	0.59	0.27-1.32	
Dominant					
Del/Del	62 (34.2 %)	66 (45.2 %)	1		
Del/Ins-Ins/Ins	119 (65.8 %)	80 (54.8 %)	0.63	0.40-0.99	0.044*
Recessive					
Del/Del-Del/Ins	162 (89.5 %)	134 (91.8 %)	1		0.48

Ins/Ins	19 (10.5 %)	12 (8.2 %)	0.76	0.36-1.63	
Allele					
Del	224 (62 %)	200 (68 %)	1		
Ins	138 (38 %)	92 (32 %)	0.7467	0.5392-1.034	0.458

Note: Del: Deletion; Ins: Insertion; OR: Odds Ratio; CI: Confidence Interval and *p<0.05

TABLE 3: 14-BP INS/DEL POLYMORPHISM AND BC CLINICAL PARAMETERS ASSOCIATION

Parameters	Number of valid cases	Pearson chi-square (Asymptotic significance 2-sided)
Histological_grade_SBR	140	2.700 (0.845)
Nodal_status	125	3.146 (0.790)
Distant_metastasis	125	0.737 (0.865)
PR	135	0.956 (0.812)
HER2 expression	135	1.507 (0.681)

Note: SBR: Scarff-Bloom-Richardson; PR: Progesterone Receptor and HER2: Human Epidermal Growth Factor Receptor 2

TABLE 4: ASSOCIATION OF HLA-G CODON 93 (CT) POLYMORPHISMS AND BC RISK

HLA-G polymorphisms codon 93	Controls (n=187)	Patients (n=151)	OR	95 % CI	p
Genotypes					
C/C	103 (61 %)	76 (53.1 %)	1		
C/T	66 (39 %)	67 (46.9 %)	1.38	0.88-2.16	0.17
Alleles					
CAC	272 (80 %)	219 (77 %)	1		
CAT	66 (20 %)	67 (23 %)	1.261	0.8590-1.851	0.2409

Note: OR: Odds Ratio; CI: Confidence Interval

TABLE 5: GENOTYPES INTERACTION ON BC RISK

14-bp Ins/Del	Codon 93	Controls (n=163)	Patients (n=140)	OR	95 % CI	p
Del/Del	CC	40 (24.539 %)	44 (31.428%)	1		
Del/Ins	CC	52 (31.901 %)	26 (18.571 %)	0.4545	0.2405-0.8590	0.0175*
Ins/Ins	CC	10 (6.134 %)	5 (3.571 %)	0.4545	0.1431-1.444	0.2623
Del/Del	CT	18 (11.042 %)	19 (13.571 %)	0.9596	0.4425-2.081	1
Del/Ins	CT	35 (21.472 %)	39 (27.857 %)	1.013	0.5417-1.894	1
Ins/Ins	CT	8 (4.907 %)	7 (5 %)	0.7955	0.2644-2.393	0.7823

Note: Ins: Insertion; Del: Deletion; OR: Odds Ratio, CI: Confidence Interval and *p<0.05

TABLE 6: HAPLOTYPE ASSOCIATION WITH BC

14-bp Ins/Del	Codon 93	Controls Frequency (%)	Patients Frequency (%)	OR	95 % CI	p
Del	C	52.17	59.51	1		
Ins	C	28.25	17.2	0.54	0.33-0.86	0.0098*
Ins	T	9.93	14.32	1.26	0.67-2.36	0.47
Del	T	9.65	8.98	0.93	0.44-1.97	0.84

Note: OR: Odds Ratio, CI: Confidence Interval and *p<0.05

In an opposite context, some studies, although 14-bp Del/Ins is not correlated with BC risk, nevertheless associated with this polymorphism with poor patient outcomes[33,35,36]. It is increasingly evident that soluble HLA-G (sHLA-G) was linked to cancer risk[37-39]. Indeed, sHLA-G promotes the escape of cancer cells from immune effector cells[40]. Several studies correlate 14-Ins/Del haplotypes with the HLA-G transcript level[25,41-43]. They propose that the presence of Ins sequence is always associated with low messenger Ribonucleic Acid (mRNA) levels. Interestingly, mRNAs with the 14-bp sequences are further processed, eliminating the 92 base regions containing the 14-bp sequence and the sites of the +3003 T/C and +3010 G/C polymorphisms[44]. This region carries multiple mRNA binding sites, which explains why short mRNAs are more stable. Overall, this may clarify the Ins 14-bp allele protective effect, reported in this study.

Stratification of alleles and genotype frequencies with clinical parameters shows no correlation. These results are concordant with prior studies analysing the 14-bp Ins/Del polymorphism in the Tunisian population[26,27]; the same results have been reported in other populations as well[32,45].

Previous study has reported that polymorphism of the 93 CAC/CAT codon of exon 3 of HLA-G was associated with preeclampsia and was in linkage disequilibrium with the 14-bp Ins/Del alleles[46]. Till date, this polymorphism in cancer, has been poorly studied and no such work has been reported in BC. Nevertheless, Rebmann *et al.* have detected that the CAC allele (G*01013) expresses less sHLA-G than the CAT allele (G*01012), which may be associated with BC and poor outcomes among BC patients[47]. In addition, we have seen a significant of the 93 CAT codon of HLA-G with BC risk (p=0.0191). The results showed that the CAC/CAT and CAT/CAT variants increase susceptibility to BC. Further studies are needed to confirm 93 CAC/CAT codon associations with BC.

The analysis of the haplotypes frequencies distribution showed that the CAC Ins haplotype has a protective effect against BC risk (OR=0.4749; 95 % CI= 0.4425-0.9277; p=0.0153). In contrast, we demonstrated that the CAC Del haplotype was associated to the BC risk. This is a tie in with prior teams finding, that the CAC allele (G*01013) expresses less sHLA-G than the CAT allele (G*01012) and haplotypes with 14-bp Ins sequences are associated with lower mRNA level as well as Ins/Ins genotype[25,41,47,48]. Similar results were described in Tunisian population[27]. Curiously, the CIns haplotype has also been attributed to be of low frequency in women suffering from recurrent miscarriages and seems to be a protective allele (OR=0.2621, 95 % CI=0.1093-0.6287, p=0.023)[49].

In addition, genotype combination analysis showed that CC Ins/Del and CC Ins/Ins genotypes decreased the susceptibility of developing BC (OR=0.3571; p=0.0053; OR=0.2083, p=0.0097, respectively), which can be attributed to the presence of the CIns allele.

Summarizing, in this study, we reported that the HLA-G 14-bp Ins/Del and 93 CAC/CAT codon polymorphisms are associated to BC risk in the Tunisian population. No association with clinical parameters was detected. Our results suggest that 14-bp Ins allele and homozygosity for codon 93 (CAC) polymorphism may protect against BC susceptibility.

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Conflict of interests:

The authors declared no conflict of interest.

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