ORIGINAL RESEARCH ARTICLE

Study of HLA-G gene 14-bp ins/del and codon 93 (CAC/CAT) polymorphisms association with spontaneous abortion in a Tunisian population

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Abstract

In the recent years, spontaneous abortion (SA) frequency increased awareness about medical and social-economic related problems. Human leukocyte antigen-G (HLA-G) is a non-classic HLA I molecule initially described in trophoblastic tissue in foeto-maternal interface. HLA-G polymorphism association with SA has been extensively studied. One amongst the foremost studied variants was HLA-G 14-bp Insertion (Ins)/Deletion (Del) polymorphism. Nevertheless, such a study was not performed in Tunisian population yet. We aim to investigate two HLA-G polymorphisms, 14-bp Ins/Del 3'UTR, and for the first time, codon 93 (CAC/CAT) polymorphisms association with SA risk in the Tunisian population. The results revealed HLA-G 14-bp Ins/Del polymorphism and CIns haplotype association with SA. No association of the HLA-G codon 93 (CAC/CAT) polymorphism to SA was observed. Considering both polymorphism together, female's homozygosity for 14-bp Ins/Del and for codon 93 (CAC/CAT) polymorphism may increase the risk of SA susceptibility. *(Afr J Reprod Health 2022; 26[1]: 103-109).*

Keywords: Polymorphism, spontaneous abortion, HLA-G, genotype, foetus, haplotypes

Résumé

Au cours des dernières années, la fréquence des avortements spontanés (AS) a accru la sensibilisation aux problèmes médicaux et socio-économiques. L'antigène G leucocytaire humain (HLA-G) est une molécule HLA I non classique initialement décrite dans le tissu trophoblastique à l'interface fœto-maternelle. L'association du polymorphisme HLA-G avec SA a été largement étudiée. L'une des variantes les plus étudiées était le polymorphisme HLA-G 14-bp Insertion (Ins)/Deletion (Del). Néanmoins, une telle étude n'a pas encore été réalisée dans la population tunisienne. Nous visons à étudier deux polymorphismes HLA-G, 14-bp Ins/Del 3'UTR, et pour la première fois, les polymorphismes du codon 93 (CAC/CAT) associés au risque SA dans la population tunisienne. Les résultats ont révélé le polymorphisme Ins/Del HLA-G 14-bp et l'association de l'haplotype CIns avec SA. Aucune association du polymorphisme du codon HLA-G 93 (CAC/CAT) à SA n'a été observée. Considérant les deux polymorphismes ensemble, l'homozygotie de la femme pour le polymorphisme Ins/Del de 14 pb et pour le codon 93 (CAC/CAT) peut augmenter le risque de sensibilité à l'AS. *(Afr J Reprod Health 2022; 26[1]: 103-109).*

Mots-clés: Polymorphisme, avortement spontané, HLA-G, génotype, fœtus, haplotypes

Introduction

In the past few years, abortion frequency increased awareness about medical and social-economic related problems. In fact 2-5% of couples suffer from abortion[1]. Several clinical studies focus on abortions causes**.** Abortion causes can be attributed to alterations in endocrine, hematology and immune system, crucial for fetal development and pregnancy success[2]. The hypothesis that immunological factors might be implicated in SA was proven[2]. The immunological interaction between mother and fetus is governed by two major phenomenon: fetal antigen presentation and recognition and maternal immune system reaction to these antigens. It is already known that gestation success depend on immunological recognition of pregnancy[3].

In contrast to HLA-A and -B Class I genes that are down regulated in human feto-maternal barrier, HLA-G Class Ib, are highly expressed[4]. HLA-G is a non classical HLA class I antigen that is thought to bind to the CD8 T-cell receptor and is involved in interaction with NK cells (Natural Killer Cells) and antigen-presenting cells[5]. HLA-G was initially described in trophoblastic tissue in

foeto-maternal interface. Seven isoforms of HLA-G are characterized: 4 membranous isoforms (HLA-G1, -G2, -G3 and -G4) and 3 soluble isoforms (HLA-G5, -G6, -G7)[6]. HLA-G is implicated in immune-tolerance with the inhibition of immunity cells such as NK, LT CD4+, LT CD8+ and APC and switch of cytokines production from Th1 profile to Th2 profile[7].

It was demonstrated that HLA-G isoforms expressed on the surface of transfected cells were able to inhibit the activity of cytotoxic NK cells and T cells via their interaction with inhibitory receptors KIR2DL4 and / or ILT-2 which strengthens the immune-tolerance [8]. It has also been studied that HLA-G is expressed in some transplanted patients suggesting that it regulates the allogenic response[9-11]. Any alteration of HLA-G structure or/and expression at the foeto-maternal barrier affects the allogenic foetus tolerance leading to pregnancy loss[11].

Since the discovery of HLA-G role in the induction of maternal-fetal tolerance, HLA and specially HLA-G polymorphisms association with SA has been extensively studied[12,13]. One amongst the foremost studied variants was HLA-G 14-bp Insertion/Deletion polymorphism. Nevertheless, the proposed results are contradictory and even inconclusive. In addition, such a study was never performed in Tunisian population before.

HLA-G coding sequence counts 75 polymorphisms, some of them are silent variants such as the codon 93 in exon 3[14]. However, this variant happens with high frequency compared to others[15]. Till date, this polymorphism has been poorly studied in pregnancy complication and no such work has been reported in SA.

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

Methods

The study subjects

Two Hundred and Forty Three (243) Tunisian women from the Fatouma Bourguiba, University Hospital Center of Monastir in Tunisia participated in our study in accordance with the Helsinki Declaration (as revised in 1983).

Table 1: The baseline characteristics of patients and controls

	Cases (62)	**Controls (181)**
Age (yr)	27.7 ± 8.1	27± 8.0
BMI (kg/m)	28.7± 5.1	26.8± 4.9
Menarche (yr)	12.5± 1.4	12.1± 1.0
Smokers	6.2%	6.6%
Menstrual historic: regular	60.9%	60.2%
irregular	39.1%	36.8%
Oral contraception users	26.56%	14.9%

Local ethic committees approved the study protocol and written informed consent was obtained from all subjects. Case group consists of 62 SA patients with two or more spontaneous abortions before 20 weeks of gestation. 181 healthy women without any history of abortion (control group) having at least one normal birth were recruited between 9 and 12 weeks gestation. None of the patients was suffering from any other syndrome or disease.
The baseline characteristics of two groups is summarized below.

DNA extraction and HLA-G genotyping

Genomic DNA was extracted by the salting method and the final DNA concentration was 10ng/ml. HLA-G rs66554220 was performed by PCR as described by Hviid *et al*[16], using the following primers: forward primer: 5'-GTGATGGCTGTTTAAAGTGTCACC-3'; reverse primer 5'-GGAAGGAATGCAGTTCAGCATGA-3'. In a PCR reaction volume of 10 µl, we added 10pg of genomic DNA, 10pmol of each primer 5 µl of REDTaq® ReadyMix™ PCR reaction mix. The DNA was amplified as follows: denaturation step at 95°C for 5 min, then 35 cycles of 30 s at 95° C, annealed at 60° for 30 s, and extended at 72° C for 30 S with a final extension step for 5 mins.

The PCR product was separated by electrophoresis on a 3% agarose gel stained with ethidium bromide. Two bands were visualized: the 224 bp band for the insertion allele and the 210 bp band for the deletion allele. Two blind lectures were performed on the gel. Genotyping of the HLA-G 93 CAC/CAT codon was performed by PCR-ARMS as described by Matte C *et al*[17] 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°C, annealed at 60° for 60 s, and extended at 72° C for 90 S, with a final extension step for 10 min. The following primers were used:
P1-5'-
AGTCTCCGGGTCTGGGATCCACCCGAGG-
3 .'
P2-5 '-
TGACCGAGGGGGTGGGGGGCCAGGTTCTG
AC-3'
P3-5'-
CCCAGGTCGCAGCCAATCATCCACTGGAG
GCTA-3'
P4- 5'-
TGGTACCCGCGCGCTGCAGCATCTCCTTCC
-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 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 $\chi 2$ 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 was assessed by Mann-Whitney test. Pearson's test was performed for non-continuous variables. A P-value less than 0.05 was considered statistically significant.

Results

This study involves 62 patients and 181 healthy controls. The genotypes and alleles frequencies distribution of 14bp- Ins/del polymorphisms are presented in Table 2. The result analysis shows that, HLA-G 14-bp Ins/Ins genotype frequency is higher in women with SA in comparison to healthy ones, 30.6%, and 16.6% respectively. The results show that HLA-G 14-bp Ins/Ins variant increases the susceptibility to SA, (OR = 2.22, 95%CI = 1.14-4.33, P=0.021). In contrast, the Del/Del genotypes is a protective factor against SA risk (OR = 0.4025, 95%CI = 0.1742-0.9300, P=0.0367). We also obtain a significant increase of Ins allele frequency in patients compared to control 55% and 44% respectively (OR = 1.533, 95%CI = 1.017-2.0310, P=0.0475). In contrast, the Del allele frequency is significantly lower in patients compared to control 45% and 56% respectively, (OR = 0.6523, 95%CI = 0.4328-0.9830, P=0.0475). The genotype frequencies were in Hardy-Weinberg equilibrium for both cohorts (P=0.9 and 0.13).

In Table 3, we summarize the genotype and alleles frequencies distribution of codon 93 (CAC/CAT) polymorphism of HLA-G. Results shows non-significant differences in the genotypes and in the allele frequencies between patients and controls. The genotypes frequencies are in Hardy-Weinberg equilibrium for both cohort (P=0.36 and P=0.44 for patients and controls respectively).

In terms of 14-bp Ins/Del and codon 93 CT, combined genotype analysis reveals a decrease in CTDelDel and CC DelIns genotypes frequencies in SA patients; 4.83% vs 10.58% and 22.58% vs 28.82%, compared to controls respectively but remain statistically non-significant (P=.733 and P=0.5298). Interestingly we recorded a significant increase of the CC InsIns genotype frequency among SA patients, 20.96% vs 9.41% in controls (OR=3.482; 95%CI= 1.158 – 10.48; P=0.0317). The double homozygote genotype seems to be a risk factor to develop SA in Women (Table 4).

The Haplotype frequencies distribution analysis shown in Table 5 reveals a decrease of CDel haplotype frequency between controls and SA patients with 41.22% and 27.75% respectively. The CDel haplotype could be a protector factor against SA risk (OR=0.4741; 95%CI=0.2453-0.9163; P= 0.0320). We also note a significant increase of the haplotypes CIns frequency in SA subject compared to controls, 44.83% and 32.33% respectively. The haplotype CIns may be associated to SA risk (OR=2.06; 95%CI=1.18-3.61; P= 0.012).
In addition, this study shows a non-significant variations in the frequencies of TDel and TIns haplotypes in SA subjects compared to controls (P=0.16 and P=0.61, respectively). The calculated D' value was 0.096 indicating a very low linkage disequilibrium between the two polymorphisms.

Table 2: Association of HLA-G 14-bp Ins/Del polymorphism and SA risk

HLA-G polymorphisms	Controls n=181	Patients n= 62	OR	95% CI	P
14-bp Ins/Del					
Codominant					
Del/Del	51 (28.2%)	13 (21%)	1		
Del/Ins	100 (55.2%)	30 (48.4%)	1.18	0.57-2.45	0.064
Ins/Ins	30 (16.6%)	19 (30.6%)	2.48	1.08-5.74	
Dominant					
Del/Del	51 (28.2%)	13 (21%)	1		
Del/Ins-Ins/Ins	130 (71.8%)	49 (79%)	1.48	0.74-2.95	0.26
Recessive					
Del/Del-Del/Ins	151 (83.4%)	43 (69.3%)	1		
Ins/Ins	30 (16.6%)	19 (30.6%)	2.22	1.14-4.33	0.021*
Allele					
Del	202 (56%)	56 (45%)	1		
Ins	160 (44%)	68 (55%)	1.533	1.017-2.310	0.0475*

*=P<0.05

Table 3: Association of HLA-G codon 93 CT polymorphism and SA risk

HLA-G polymorphisms	Controls n=174	Patients n= 62	OR	95% CI	P
Codon 93 Codominant					
C/C	96 (55.2%)	34 (56.8%)	1		
C/T	64 (36.8%)	22 (35.5%)	0.97	0.52-1.81	
T/T	14 (8.1%)	6 (9.7%)	1.21	0.43-3.40	0.92
Dominant					
C/C	96 (55.2%)	34 (54.8%)	1		
C/T-T/T	78 (44.8%)	28 (45.2%)	1.01	0.57-1.82	0.96
Recessive					
C/C-C/T	160 (92%)	56 (90.3%)	1		0.70
T/T	14 (8 %)	6 (9.7%)	1.22	0.45-3.34	
Allele					
C	256 (74%)	90 (73%)	1		
T	92 (26%)	34 (27%)	1.051	0.662-1.667	0.8144

Table 4: Genotypes interaction on SA risk

14-bp Ins/Del	Codon 93	Controls n=170	Patients n= 62	OR	95% CI	P
Del/Del	CC	30 (17.64%)	7 (11.29%)	1		
Del/Ins	CC	49 (28.82%)	14 (22.58%)	1.224	0.4438-3.379	0.8021
Ins/Ins	CC	16 (9.41%)	13 (20.96%)	3.482	1.158-10.48	0.0317*
Del/Del	CT	18 (10.58%)	3 (4.83%)	0.7143	0.1636-3.118	0.7333
Del/Ins	CT	33 (19.41%)	13 (20.96%)	1.688	0.5945-4.794	0.4399
Ins/Ins	CT	10 (5.88%)	6 (9.67%)	2.571	0.6976-9.479	0.1767
Del/Del	TT	3 (1.76%)	3 (4.83%)	4.286	0.7085-25.92	0.1270
Del/Ins	TT	10 (5.88%)	3 (4.83%)	1.286	0.2782-5.941	0.7068
Ins/Ins	TT	1 (0.588%)	0			

*=P<0.05

Table 5: Haplotype association with SA

14-bp Ins/Del	Codon 93	Controls Frequency %	Patients Frequency %	OR	95% CI	P
Del	C	41.22	27.75	1		
Ins	C	32.33	44.83	2.06	1.18 -3.61	0.012*
Ins	T	11.87	10.01	1.24	0.54 – 2.87	0.61
Del	T	14.58	17.41	1.72	0.81– 3.62	0.16

*=P<0.05

Discussion

In this work we investigated two HLA-G polymorphisms; the HLA-G 14-bp Insertion/Deletion 3'UTR polymorphism, and for the first time the codon 93 polymorphism (CAC/CAT) potential association with SA risk in the Tunisian population. We obtained a significant variation in the frequencies of the Del and Ins alleles between the two cohorts. An increase in the Ins allele and a decrease of Del allele frequencies in patients was recorded. We also found that the HLA-G 3'UTR 14-bp Del/Del variants decreased susceptibility to SA in the Codominant inheritance model (OR = 0.4025, 95%CI = 0.1742-0.9300, P=0.0367). In contrast, the HLA-G 3'UTR 14-bp Ins/Ins variants in pregnant women was associated to High risk of SA (OR = 2.22, 95%CI = 1.14-4.33, P=0.021). Our results are in agreement with various studies reporting the association of the genotype HLA-G 14-bp InsIns genotype with SA[13,18-20]. Nevertheless, we have divergent results to other teams describing no association of HLA-G Ins/Del polymorphism with SA[21-23].

HLA-G is not only an exclusive cell membrane Immunoglobulin but its soluble fraction is also detected[24]. The main soluble isoforms are HLA-G1 resulting from membrane isoform shedding and HLA-G5 which is a secreted isoform[25]. Soluble HLA-G concentration was reported to be critical for embryo implantation and pregnancy success[13]. Several studies reported that there is an association between 14bp-Ins/Del haplotypes and the HLA-G transcription level (26-29). They advance that the presence of the 14bp sequence is always associated with low mRNA levels. In fact, multiple miRNAs such miR-93, miR-2110, miR-508-5p, miR-616, miR-331-5p, miR-589 and miR-513b targeted the 14-bp fragment region[30]. This can explain the HLA-G mRNA low-level association to the 14bp sequence. Interestingly, the transcript carrying with the 14bp sequences are further processed leading to the elimination of the 92 base region containing the 14bp sequence and the sites of the +3003 T/C and +3010 G/C polymorphisms[30]. Overall, this may clarify the Ins 14-bp allele effect reported in this study. In this context Zidi et al have reported that low levels of HLA-G1 and HLA-G5 in Tunisian women experienced multiple abortions[31].

The same authors describe sHLA-G high level in individuals with Del/Del or Del/Ins genotypes among Tunisian population[32]. Sine Hylenius *et al* has previously 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[33]. Till date this polymorphism has been poorly studied and not such work has been reported in SA. Results have shown that there is no association of the polymorphism of the 93 CAC/CAT codon of HLA-G with SA either for the alleles or even for the genotype frequencies. Further studies are required to confirm 93 CAC/CAT codon implication In SA among Tunisian Population.

The analysis of the haplotype frequency distribution showed that the CIns haplotype increases the SA Risk (OR=2.06; 95%CI=1.18-3.61; P= 0.012). This is supported by prior teams findings that the CAC allele (G*01013) expresses less sHLA-G than the CAT allele (G*01012) and Haplotypes with 14bp Ins sequences are associated with lower mRNA level as well as Ins/Ins genotype[27,28,34,35]. Also, the CIns haplotype has been attributed to be of low frequency in women suffering from recurrent miscarriages[1].

In addition, genotype combination analysis showed that CCInsIns increased the susceptibility of developing SA (OR=3.482; 95% CI=1.158-10.48 P=0.0317) which can be attributed to the presence of the CIns allele.

Conclusion

This study reported that the HLA-G 14-bp Ins/Del polymorphism was related to SA risk in the Tunisian population. Moreover, no role of the 93 CAC/CAT codon of HLA-G was observed. Our results suggest that homozygosity for 14-bp Ins/Del and codon 93 (CAC/CAT) polymorphism may increase the risk of SA among Tunisian Population.

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Contribution of authors

Dr. Sana Belkahla conceived, designed the study, do the experiment work and prepared the manuscript, Insha Nahvi collect and analysed data and Hena Khan help in manuscript preparation.

Conflict of interest

Authors declare no conflicts of interest and approved the manuscript.

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