Non-classical human leukocyte antigen class I in Tunisian children with autism

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Abstract

Autism spectrum disorders (ASD) are one of the most common childhood morbidities characterized by deficits in communication and social skills. Increasing evidence has suggested associations between immune genes located in the human leukocyte antigen (HLA) complex and etiology of autism.

In this study, we investigated whether the non-classical class I HLA-G, -E, and -F polymorphisms are associated with genetic predisposition to autism in Tunisia. We aimed to find a correlation between HLA-G genotypes and soluble HLA-G (sHLA-G) levels. We have analyzed the HLA-G, -E, and -F genotypes of 15 autistic children and their parents. DNA typing of HLA class I genes was performed using PCR-SSP and PCR-RFLP methods. Also, we evaluated the serum levels of HLA-G (1 and 5) by a validated ELISA technique in autistic probands and their parents.

No association was found between any polymorphism and autism in the study subjects. Additionally, we found no correlation between sHLA-G1 and sHLA-G5 and autism. Also, no significant difference in sHLA-G testing in parents and offspring was found. However, parents carrying [GG] genotype presented a higher sHLA-G levels than those carrying ([CC]+[GC]) genotypes (p = 0.037).

From this preliminary study, we conclude that the investigated polymorphisms of HLA-G, -E, and -F genes did not lead to autism susceptibility in Tunisian children. However, the CGTIGA haplotype was found to be associated with the disease.

Key words: *polymorphism, autism, HLA-G, HLA-E, HLA-F.*

Introduction

Autism spectrum disorders (ASD) are neurodevelopmental conditions characterized by qualitative impairments of social interaction and repetitive behavior, with restricted interests diagnosed before the age of 3 [1]. The etiology of ASD is not fully understood. Although several studies have focused on the etiology of this disease, none of them have provided clear answers about the real cause of ASD [2, 3].

Several immune abnormalities have been detected in children with ASD [1, 4]. Indeed, neurobiological studies in ASD highlighted pathways involved in neural development, synapse plasticity, structural brain abnormalities, neurological processes, cognition, and behavior [3, 4]. In fact, HLA molecules, which are expressed on the surface of all nucleated cells, binds two types of receptors [5]. Firstly, the HLA molecules interact with T cell receptors (TCR) presented on the T lymphocytes, stimulating the activation of adaptive responses [6]. Secondly, HLA molecules link to the natural killer (NK) cells via the killer immunoglobulin-like receptor (KIR), leading to a reduced NK cell activity [5]. Classical HLA-C and non-classical HLA-G molecules, binding maternal KIRs, are highly expressed in the fetal trophoblast region. In particular, the HLA-G is responsible for the compliance of mothers to tolerate genetically different fetal tissues during pregnancy [5]. Recently in 2013, Patterson *et al.* have shown that maternal and prenatal infections increased the risk of developing autism in offspring. Moreover, a prenatal infection can affect brain development of fetus in uterus [7]. Furthermore, non-classical HLA class Ib genes are formed during embryonic growth and are expressed in both the ovocyte and blastocyst stages [8].

Genetic polymorphisms of the major histocompatibility complex (MHC) genes coding for HLA molecules have been reported to be associated with ASD development [3]. The classical HLA class Ia (HLA-A, HLA-B, HLA-Cw, and HLA-DR) polymorphisms were systematically typed in ASD children and their relatives; both stratification and haplotype-based analysis were performed in order to take into account their potential linkage disequilibrium (LD) and co-segregation [3]. However, there is a limited number of studies investigating the relationship between the non-classical HLA class Ib (HLA-F, HLA-G, and HLA-E) and the etiology of autism. Therefore, this present study aimed to report the allele frequencies of six polymorphisms located in non-classical Ib HLA genes (HLA-F*01:02, HLA-F*01:03, HLA-F*01:04, 14pb Ins/Del, +3142C>G, and HLA-E*01:01/01:03) and investigate their potential association with ASD in autistic probands from Tunisian families. Also, a measurement of soluble levels of HLA-G in serum was performed in order to examine the potential correlation with HLA-G genetic polymorphisms.

Material and methods

Study population

Fifteen families with one child having a diagnosis of ASD were enrolled in the study, between January and December 2004, for a total of 45 individuals. Diagnoses were performed at the Department of Child and Adolescent Psychiatry, Hédi Chaker University Hospital, Sfax, Tunisia, based on the international classification of diseases 10th edition (ICD-10) [9]. In ICD-10, ASD include Asperger's disorder, childhood disintegrative disorder, and mental retardation.

All individuals were born in Tunisia and were of Tunisian descent. Thirteen males and two females (mean age, 4.5 ±2 years) were ASD children, and their score at the child autistic rating scale (CARS) was ≥ 30 [10]. ASD children underwent examinations of mental status and neurological and neuropsychological evaluations by neurologists and psychiatrists from the "ERAHMA" association and Hédi Chaker University Hospital, Sfax, Tunisia. None of the autistic subjects presented an identifiable cause of their disease. Thirty individuals were parents.

Informed parental consent approved by the committee of medical ethics (Hédi Chaker Hospital Ethics Committee, Sfax, Tunisia) was obtained from all participants and legal guardians prior to inclusion into the study. The work has been carried out in accordance with the Declaration of Helsinki for experiments in humans.

Genotyping

Genomic DNA were extracted from EDTA anticoagulated peripheral blood samples (5 ml), using the standard phenol–chloroform method [11], and frozen at –20°C until use.

For the determination of the four following polymorphisms: HLA-E*01:01/01:03 A>G and HLA-F *(01:02 T>C, 01:03 C>T, and 01:04 A>C) variants alleles, the protocols of polymerase chain reaction with sequence specific primers (PCR-SSP) described by Lauterbach *et al.* and Pan *et al.* [12, 13], respectively, were used. PCR amplification was carried out in a 25µl volume, containing 10µl master mix (Qiagen), 1 pmol or 1.2 pmol of each specific primer, 1 pmol of each internal control primer, 100 ng genomic DNA, and 0.25µl of Taq DNA polymerase (Boiron GmbH., Germany). Nine and three sets of amplification primers were designed based on the published sequences (http://hla.alleles.org/data/txt/f_gen.txt), respectively, for HLA-F and HLA-E isoforms. Each PCR included specific primers pair and an internal control primers pair (that amplify a 439-bp fragment in the hormone growth factor gene) [14].

To identify the HLA-G +3142 C>G polymorphism, a PCR approach was used, followed by restriction enzyme analysis with 1µl of BaeGI (500 U/µl) (New England Biolabs, USA) with 10 µL of the amplification product in a final volume of 50 µl, as previously described by Graebin *et al.* [15]. The digestion was carried out overnight at 37°C and checked on 3% agarose gel.

The 14-bp Insertion/ Deletion (Ins/Del) polymorphism genotyping was performed by PCR for exon 8-specific primers according to the conditions previously described by Shankarkumar *et al.* [16]. Two PCR products were generated: 224-bp and 210-bp bands, respectively corresponding to ins and del. The PCR product was visualized and interpreted by two different observers. The sizing of PCR products was performed using 50 pb DNA ladder.

All PCR reactions were done by using a GenAmp PCR system 9700 thermocycler (Applied Biosystems, Fisher Scientific, USA).

Evaluation of soluble HLA-G serum levels

The sera were obtained from all participants and stored at -80°C for later analytical analysis. Soluble HLA-G (sHLA-G) levels (both secreted HLA-G5 and shed HLA-G1) were measured by a validated enzyme-linked immunosorbent assay (ELISA) method, using anti-HLA-G MEM-G/9 or 5A6G7 (Exbio, Prague, Czech Republic) as capture antibody, and anti-β2-microglobulin antibody conjugated with peroxidase (Dako) as secondary antibody [17]. The concentration of sHLA-G was estimated by comparing the absorbance at 450 nm after subtraction of the blank control absorbance values, with a standard curve. sHLA-G levels were normalized for flushing volume and total protein content (sHLA-G, ng/ml/total flushing sample, ml/total proteins, ng/ml). The standard curve was made by a serial dilution of the standard supernatants of HLA-G/721.221. The limit of sensitivity was 1 ng/ml. All samples were run in duplicate.

Allele frequencies

The allele frequencies of the studied HLA-E and HLA-F polymorphisms in other populations were extracted from the 1000 Genomes Project database, available from http://www.1000genomes.org/, while for the HLA-G polymorphisms, the allele frequencies of other ethnic populations were extracted from the study of Sabbagh *et al.* [18].

Statistical analysis

Data were analyzed using PLINK v1.07 software, a free open-source whole genome association analysis toolset (http://zzz.bwh.harvard.edu/plink/). The χ^2 test was used to assess differences in allele frequencies between Tunisians and other populations, and to test the deviation from the Hardy-Weinberg equilibrium (HWE), which is a theory stating that the genetic variation in a population will remain constant from one generation to the next in the absence of disturbing factors. The genotypic, allelic, and haplotypic frequencies were calculated by PLINK v1.07 software. Continuous variables were analyzed using the Mann-Whitney U test and expressed as mean ± standard error of the mean (SEM). The transmission disequilibrium test (TDT) [19] was used to assess allelic association and performed using PLINK v1.07 software. TDT simultaneously measures linkage and association by comparing the allele transmitted and the one that is not transmitted from a heterozygous parent to the child.

Haploview software 4.2 was used to visualize the structure of pair-wise linkage disequilibrium (LD) between the polymorphism in terms of Lewontin's D' and the squared correlation coefficient between allele frequencies (r_c) expressed as a function of D' [20]. Haplotype blocks were defined using the "solid spine" incorporated in the haploview program. All SNPs with minor allele frequencies < 0.01% were excluded, and the minimum haplotype frequency was set at 1%. The confidence interval for strong LD was set between 0.7 and 0.98.

Results

Alleles and genotypes frequencies

HLA-G allele frequencies

The allelic frequency of the14bp-Del allele was 0.41. This frequency is close to the frequency of the minor allele in the African population (0.41) (Table 1).

For the polymorphism +3142C>G, the frequency of allele C was 0.48 in the Tunisian population. This frequency is near to the percentage found in European (Tuscan, Italy) (TSI), 0.45) and African population (Luhya from Webuye, Kenya (LWK), 0.47), but different from Asiatic population, except for the Han Chinese from South China (CHS) (Table 1).

HLA-E allele frequencies

The frequency of allele HLA-E*01:01 was 0.45. This frequency is close to the percentage found in Latino-American population (people of Mexican ancestry from Los Angeles (0.45)), but higher than African population and lower than European (Table 1).

HLA-F allele frequencies

Only the HLA-F*01:03 was polymorphic in Tunisian population, with an allelic frequency equaling to 0.25 for the C allele. This frequency was different from those found in Asian and Latino American populations (Table 1). The HLA-F*01:02 and the HLA-F*01:04 provided one genotype [CC], with a similar frequency in comparison with African population, except for the Luhya from Webuye, Kenya, and different from European population for the HLA-F*01:04 allele (Table 1).

Association analysis with autism

All genotype distributions of the studied non-classical HLA-class Ib polymorphisms were in accordance with the HWE ($p > 0.05$). As shown in Table 2, no association was found between the six studied markers and autism ($p > 0.05$). Twelve non-redundant haplotypes (representing a combination of HLA-F*01:02, HLA-F*01:03, HLA-F*01:04, 14pb Ins/Del, HLA-G +3142C>G, and HLA-E*01:01/01:03) inferred in the Tunisian population, as presented in Table 3. In fact, the two most frequent haplotypes were CGCICG and CGTDGA, with a frequency of 0.17 and 0.16, respectively. Only the haplotype CGTIGA was reported as associated with autism ($p = 0.042$).

Linkage disequilibrium

We derived $|D'|$ values to assess linkage disequilibrium (LD) between alleles of the 4 polymorphic variants (14bp-Ins/Del HLA-G, +3142C>G HLA-G, HLA-E*01:01/01:03, and HLA-F*01:03). The LD plot showed a correlation between 14bp-Ins/Del and +3142C>G polymorphisms of HLA-G gene. Based on pairwise analysis of these neighboring SNPs, we reported a weak LD ($r_c = 0.22$). However, no blocks of LD could be detected with any of the methods described in haploview 4.2 (Fig. 1).

Soluble HLA-G dosage

The results showed high prevalence of HLA-G5 levels in offspring compared to the parents (positivity percentage = 27.22% vs. 17.39%, respectively), although this did not reach statistical significance (mean ±SEM: 0.68 ±0.46 vs. 0.80 ±0.6 ng/ml, respectively; $p = 0.50$). Concerning the levels of sHLA-G and sHLA-G1, no difference was reported between parents and offspring ($p = 0.62$ and $p = 0.85$, respectively).

Table 1. Allelic frequencies of the polymorphisms studied in Tunisian and other populations

Parameter	HLA-F			HLA-G		HLA-E
Nucleotide sequence variation	HLA-F*01:02	HLA-F*01:03	HLA-F*01:04	14bp Ins/Del	+3142C>G	HLA-E*01:01/01:03
Nucleotide change	T/C	C/T	A/C	Ins/Del	C/G	A/G
Ancestral allele	C	T	C	Ins	G	G
Minor allele in Tunisian population	T	C	A	Del	C	G
Minor allele frequency (MAF) in Tunisian population (*n* = 45)	T (**0**)	C (**0.25**)	A (**0**)	Del (**0.41**)	C (**0.48**)	G (**0.45**)
Africa						
GUI (*n* = 60)	NA	NA	NA	0.63	0.33	NA
SER (*n* = 239)	NA	NA	NA	0.48	0.24	NA
TOR (*n* = 30)	NA	NA	NA	0.35	0.43	NA
YRI (*n* = 88)	0	0.26	0	0.36	0.35	0.34
YAN (*n* = 175)	NA	NA	NA	0.30	0.40	NA
LWK (*n* = 97)	0.015	0.11	0.01	0.41	0.47	0.46
ASW (*n* = 61)	0.008	0.12	0	0.45	0.39	0.53
Europe						
POR (*n* = 60)	NA	NA	NA	0.52	0.38	
IBS (*n* = 14)	0	0.11	0	0.43	0.50	0.5
TSI (*n* = 98)	0	0.19	0.02	0.40	0.47	0.41
CEU (*n* = 85)	0	0.21	0.03	0.35	0.56	0.39
GBR (*n* = 89)	0	0.13	0.01	0.43	0.51	0.38
FIN (*n* = 93)	0	0.33	0.02	0.28	0.66	0.48
Asia						
JPT (*n* = 89)	0	0.01	0	0.25	0.25	0.70
CHB (*n* = 97)	0	0.05	0	0.39	0.34	0.58
CHS (*n* = 100)	0	0.015	0	0.29	0.45	0.64
America						
SEB (*n* = 155)	NA	NA	NA	0.41	0.46	NA
NEB (*n* = 128)	NA	NA	NA	0.38	0.45	NA
CLM (*n* = 60)	0.008	0.16	0	0.33	0.44	0.49
MXL (*n* = 66)	0.008	0.08	0	0.43	0.41	0.45
PUR (*n* = 55)	0	0.19	0	0.31	0.50	0.55

ASW – people of African ancestry from the Southwestern United States, CEU – Utah residents with Northern and Western European ancestry, CHB – Han Chinese from Beijing, CHS – Han Chinese from South China, CLM – Colombians from Medellin, Colombia, FIN – Finnish from Finland, GBR – British from England and Scotland, GUI – natives of Guinea-Bissau, IBS – Iberian populations from Spain, Ins – insertion, JPT – Japanese from Tokyo, Japan, LWK – Luhya from Webuye, Kenya, MXL – people of Mexican ancestry from Los Angeles, California, USA, n – number of individuals, NA – not available, NEB – Northeastern Brazilians from Recife, Pernambuco, Brazil, POR – Portuguese, PUR – Puerto Ricans from Puerto Rico, SEB – Southeastern Brazilians from Ribeirao Preto, Sao Paulo, Brazil, SER – Serer from Niakhar, Senegal, TOR – Tori from Tori-Bossito, Benin, TSI – from Tuscany, Italy, YAN – Yansi from Bandundu, Democratic Republic of the Congo, YRI – Yoruba from Ibadan, Nigeria. The ancestral allele state was inferred from dbSNP (Build.142). Minor allele frequency (MAF) in Tunisian population is provided by this current study.
The allele frequencies of the HLA-E and HLA-F polymorphisms were extracted from the 1000 Genomes Project database, available from (http://www.1000genomes. org/). The allele frequencies of the HLA-G polymorphisms were extracted from the study of Sabbagh et al. [18]

Table 2. Association analysis between polymorphisms and autism

CHR	Nucleotide sequence variation	Position	χ^2	OR	95% CI	*p*-value
6	HLA-F*01:02	29723501	NA	NA	NA	NA
6	HLA-F*01:04	29723805	NA	NA	NA	NA
6	HLA-F*01:03	29725234	0.111	1.25	0.3357-4.655	0.7389
6	14bp Ins/Del	29830804	0.0667	1.143	0.4144-3.152	0.7963
6	+3142C>G	29830972	0.05263	1.111	0.4515-2.734	0.8185
6	HLA-E*01:01/01:03	30490287	1.316	1.714	0.6749-4.354	0.2513

CHR – chromosome, OR – TDT odds ratio, 95% CI – confidence interval for TDT odds ratio, χ^2 – TDT χ^2 statistic, NA – not available
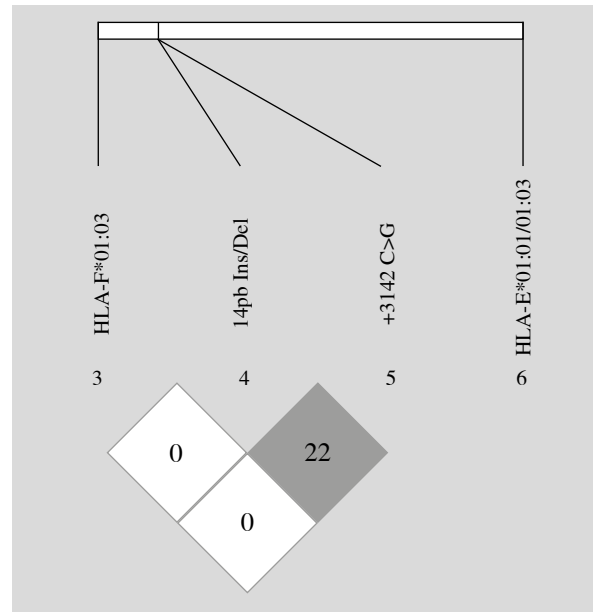
Table 3. Haplotypes and association with autism

Haplotype	Frequency	T	U	CHISQ	*p*-value
CGCDCG	0.0317	0.5	0.5	0	1
CGTDCG	0.0617	1.03	0.036	0.9316	0.334
CGCICG	0.0509	0.72	2	0.602	0.437
CGTICG	0.1758	6.86	3.60	1.013	0.314
CGTDGG	0.0806	1.88	0.85	0.3823	0.536
CGTICA	0.0491	0	0	NA	NA
CGCICA	0.0401	1.02	1.25	0.02375	0.877
CGTICA	**0.1229**	2.85	3.6	0.08563	0.769
CGTDGA	0.079	1.75	1.244	0.08754	0.767
CGTDGA	**0.1636**	3.8	4.36	0.0351	0.851
CGCIGA	0.0481	2	0	2	0.157
CGTIGA	0.0962	0.53	5.53	4.116	**0.042**

T – transmitted minor allele count, U – un-transmitted, CHISQ – χ^2 test, TDT – χ^2 statistic

The LD reported in Figure 1 was the r^2.

Fig. 1. Linkage disequilibrium plot for HLA-G, HLA-E, and HLA-F

Various differences in total sHLA-G levels among parents and children were observed when HLA-G genetics were considered. Interestingly, children with Ins allele had more enhanced sHLA-G levels and those with Del allele presented low sHLA-G without significance (Table 4). Indeed, parents with two ancestral alleles 14bp Ins (Ins/Ins) had a lower sHLA-G levels than those with the genotype Del/Del and Ins/Del. Interestingly, the offspring homozygous for 14bp Ins allele exhibited enhanced sHLA-G concentrations (Table 4).

Furthermore, referring to +3142C>G polymorphism, parents with G allele showed the highest levels of sHLA-G. The difference between sHLA-G in +3142C>G genotype ([GG] vs. [CG] + [CC]) was statistically significant ($p = 0.037$). On the other hand, offspring had similar sHLA-G levels for all genotypes, without statistically distinct differences ((([GG] vs. ([CG] + [CC])); $p = 0.80$).

The effect of haplotypes on sHLA-G levels in serum was also studied. The concomitant presence of Del and G alleles was enhanced with sHLA-G levels in parents (2.01 ±0 vs. 0.80 ±0.61 ng/ml). However, offspring with Ins/G haplotypes had the highest sHLA-G levels (3.31 ±1.66 vs. 1.27 ±0.57 ng/ml). Despite this fact, no significant p-value was reported ($p = 0.566$ and $p = 0.197$, respectively) (data not shown).

Discussion

The autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impaired social interaction, restricted communications skills, and unusual repetitive behavior [4]. The underlying causes of autism are still unknown; nevertheless, both genetic and environmental causes are believed to contribute to the risk of autism [21]. Various clues suggest that a family history (like autoimmune disease), immunogenetics, epigenetic, and internal and external environmental factors are the potential trig-

gers for this disease [22]. In fact, a possible autoimmune component was suggested as factor in this etiology [23]. Many studies reported an association between autism and autoimmune thyroiditis or hypothyroidism [24], rheumatic fever [25], rheumatoid arthritis, celiac disease ulcerative colitis, and psoriasis [26, 27].

Maternal infection and exposure to toxic compounds could alter the peripheral immune system, which can occur during fetal development and lead to long-term epigenetic changes in child [28]. The high-rate of autoimmune diseases in mothers of autistic children suggests that the autoimmune response, which takes place in the expectant mothers, adversely affects the developing fetus in utero [22]. In fact, the inflammatory process has been associated with autism, and a high level of inflammatory cytokines (IL-6, IFN-γ, TNF-α, and IL-8) was reported [22, 29]. A significant increase in the autoantibodies was found in the sera of autistic mothers [22, 30, 31]. Also, a raised number of B lymphocytes was observed against a decreased rate in T lymphocytes within autistic patients [22, 32]. Although the origins of abnormal immune activity in autism are still unknown, it was hypothesized that the mechanism might involve HLA genes and their products [23]. The importance of HLA in autoimmune conditions has been known for many years. In fact, many studies have shown some associations of classical MHC class I, II, and III alleles and autism [28, 33-36]. In particular, the widely admitted major susceptibility allele for rheumatoid arthritis (an endophenotype described in autistic patients) now is HLA-DRB1*04 loci.

Even though an evidence of association between the HLA system and autism was reported, no studies were carried out on the HLA class Ib. In fact, the non-classical Ib major histocompatibility complex includes three genes: HLA-G, HLA-E, and HLA-F. The generated molecules are characterized by tolerogenic functions and interact not only with NK cells, but also with T lymphocyte subsets and other immune cells, playing a major role in fetus acceptation [37, 38]. These HLA-Ib genes are characterized by low allelic polymorphisms [28, 37]. For the first time, we investigated the implication of the six popular polymorphisms of these genes within 15 Tunisian autistic families.

Concerning HLA-G gene, two polymorphisms +3241C>G and 14bp Ins/Del were examined, both located in the region of 3'UTR of the exon 8 of the gene. These polymorphic sites play a pivotal role in the regulation of HLA-G expression, on the one hand through modifying HLA-G mRNA stability [39], and on the other hand by the binding of specific microRNAs [40]. Controversial results have been reported among the association of HLA-G gene in different pathologies because of missing variables including ethnicity and genetic background information [18, 40]. In the present study, we reported the allelic frequency of +3241C>G and 14bp Ins/Del in Tunisian population. Furthermore, our frequencies are close to African population

Table 4. Total sHLA-G levels according to 14bp-Ins/Del and +3142C>G alleles and genotypes

Parameter	sHLA-G (ng/ml) in parents	sHLA-G (ng/ml) in offspring
	Mean ±SEM	
14bp Ins/Del		
Alleles		
Ins	0.72 ±0.25	1.90 ±0.64
Del	1.81 ±0.91	0.72 ±0.55
p	0.870	0.168
Genotypes		
Del/Del	2.63 ±2.63	0 ±0
Del/Ins	0.98 ±0.56	1.21 ±0.89
Ins/Ins	0.55 ±0.36	2.59 ±1.44
p[*]	0.45	0.26
+3142C>G		
Alleles		
C	0.42 ±0.22	1.82 ±0.59
G	1.78 ±0.71	1.43 ±0.66
p	0.157	0.878
Genotypes		
CC	0.6150 ±0.6150	1.405 ±1.405
CG	0.2950 ±0.2640	1.205 ±0.8933
GG	3.053 ±1.795	1.657 ±1.657
p[**]	**0.037**	**0.8**

bp – base pairs, Del – deletion, Ins – insertion, p – p-value of Mann-Whitney test, SEM – standard error of mean, [*] Del/Ins + Del/Del vs. Ins/Ins, [**] CC + CG vs. GG

and the same observation was reported for the +3142C>G polymorphism. However, these frequencies differ from other populations, especially the Asiatic one [18, 40]. No strong linkage disequilibrium was observed between 14bp Ins/Del and +3142C>G. This result was in discordance to those found in Europe, but in accordance within African, American, and Asian populations [18, 40]. Moreover, the two most frequent haplotypes were CGCICG and CGTD-GA, with a frequency of 0.17 and 0.16, respectively. These haplotypes differ at the polymorphic sites within HLA-F (only one SNP) HLA-G and HLA-E genes. This phenomenon, known as "Yin-Yang haplotypes" has been reported by Zhang *et al.* [41] for many other genes in the human genome and seems to be a general characteristic of eukaryotic genomes. An association with autism was reported for the haplotype CGTIGA. Despite this fact, neither an allelic nor a genotypic association was found in a response to the interaction between the studied SNPs.

HLA-G gene is characterized by seven possible isoforms that are generated by alternative splicing of the

primary transcript of HLA-G. Four are membrane-bound (HLA-G1, -G2, -G3, and -G4), and three are soluble iso-forms (HLA-G5, -G6, and -G7). A supplemental soluble form of HLA-G is the shedding HLA-G1 generated after metalloproteinase' cleavage of the HLA-G1. We failed to identify any association between sHLA-G levels in parents with one or two 14bp-Del.

Additionally, we reported high sHLA-G in parents with GG genotype. This preliminary result is contrary to several studies highlighting that G allele is related to low sHLA-G expression because the +3142G allele is predicted to bind three miRNAs (miR-148a, -148b, and -152) [39, 42-44].

In autistics, we reported some haplotypes with increased sHLA-G, including firstly GG Ins/Ins, CC Ins/Ins, and CG Ins/Del. Based on the findings of Xu *et al.* who demonstrated that G/14bp-Ins haplotype is increased in human papillomavirus (HPV18) infected patient, we suggest a possible interference in sHLA-G levels in autistics [45]. The discrepancy between previous studies and ours' may be due to ethnicity and sample size.

In the present study, for the HLA-F gene, three essential polymorphisms were studied: HLA-F*01:02 (C to T substitution at position 38 in exon 1), HLA-F*01:03 (C to A mutation at position 212 in exon 2), and HLA-F*01:04 alleles (T to C substitution at position 814 in exon 4) [46]. The HLA-F*01:02 and the HLA-F*01:04 alleles were non-polymorphic like in other populations in the world. The HLA-F*01:03 has the same allele frequency as European or African populations. Similar finding was also observed for the HLA-E*01:03.

Despite the absence of notable difference in alleles frequencies in Tunisian population compared to other populations in the world, our work still is the first to study HLA-G, -E, and -F polymorphisms and their eventual association with autism in Tunisian population. Our result reveals an association at the haplotypic level with CGTI-GA haplotype. Nevertheless, no correlation between HLA-Ib (HLA-G, HLA-E, and HLA-F) and autism in Tunisian autistic families was found. Therefore, larger samples are still needed to clearly establish the association of HLA-Ib polymorphisms in autism. Our results suggest a potential association between haplotypes and sHLA-G levels in autistics.

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