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The association between functional *HLA-G* 14 bp insertion/deletion and +3142 C>G polymorphisms and susceptibility to multiple sclerosis.

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Highlights

- A significant increased frequency of the +3142 G allele was found in MS patients compared to HD
- The 14 bp DEL / +3142 G haplotype frequency was significantly increased in MS patients compared to HD
- An association was detected between the lower age of disease onset (ADO) in patients with the +3142 C/C genotype.
- The +3142 C>G, but not the 14 bp INS/DEL, polymorphism may constitute a genetic susceptibility factor to MS in the Tunisian population.

Abstract

We aimed to investigate two main polymorphisms in the 3' untranslated region (3'UTR) of the *HLA-G* gene [14 bp insertion/deletion (INS/DEL) and +3142 C>G] and to assess their impact on the soluble HLA-G (sHLA-G) production in patients with multiple sclerosis (MS). This study included 60 patients with relasping-remitting (RR) MS and 112 healthy donors (HD). Mutations were identified by PCR and PCR–RFLP, and serum sHLA-G quantification was performed by ELISA. For the 14 bp INS/DEL polymorphism, variants frequencies were similar in patients and controls, whereas a significant increased frequency of the +3142 G allele was found in MS patients compared to HD (63.4% *vs* 52.3%, p=0.04; OR=1.58, 95%CI=1.003–2.48). In addition, an association was found between MS susceptibility and the haplotypes regrouping both studied polymorphisms. Indeed, the 14 bp DEL / +3142 G haplotype frequency was significantly increased in MS patients compared to HD (20.8% *vs*

12.5%, p=0.04, OR=1.84). On the other hand, no associations were detected between both polymorphhisms and clinical parameters, except the lower age of disease onset (ADO) in patients with the +3142 C/C genotype. Moreover, our study doesn't show any significant variation of sHLA-G serum levels between patients and controls. Our findings showed that the +3142 C>G, but not the 14 bp INS/DEL, polymorphism may constitute a genetic susceptibility factor to MS in the Tunisian population. However, no association was found between the two polymorphisms and sHLA-G serum levels.

Abbreviations:

ADO: age of disease onset

bp: base pairs

CI: confidence interval

CNS: central nervous system

CSF: cerebrospinal fluids

EDSS: Expanded Disability Status Scale

HD: healthy volunteer blood donors

HLA: human leukocyte antigen

HWE: Hardy-Weinberg equilibrium

IL: Interleukin

INS/DEL: insertion/deletion

LD: linkage disequilibrium

MHC: major histocompatibility complex

MRI: magnetic resonance imaging

MS: Multiple sclerosis

OR: Odds ratio

PCR: polymerase chain reaction

RR-MS: Relapsing remitting Multiple sclerosis

sHLA-G: soluble HLA-G

Tregs: T regulatory cells

SNP: single nucleotide polymorphism

UTR: untranslated region

vs: *versus*

Keywords: HLA-G antigens, Genetic polymorphism, Haplotypes, Multiple sclerosis, Tunisia.

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating and neurodegenerative disease of the central nervous system (CNS) with a multi-factorial etiology involving an interplay of both environmental [1] and genetic factors [2, 3]. Whole genome studies indicate that the major histocompatibility complex (MHC), notably the gene encoding human leucocyte antigen G (HLA-G), represents the major MS susceptibility locus [4, 5].

HLA-G is a non classical HLA class I molecule with a limited tissue distribution in non pathological conditions. It is characterized by its low polymorphism, and by the presence of both membrane-bound (HLA-G1, -G2, -G3, and -G4) and soluble (HLA-G5, -G6, and -G7) isoforms generated by the alternative mRNA splicing of the gene transcript [6, 7]. Published data support the role of HLA-G molecules in the modulation of MS disease activity via the

interaction with its specific inhibitory receptors: ILT-2 (LILRB1/CD85j), ILT-4 (LILRB2/CD85d), CD8 and KIR2DL4 (CD158d) expressed by immune cells in the central nervous system [8, 9].

Under physiological conditions the cellular expression of HLA-G is very limited [10, 11]. This expression was shown to be increased in several pathological conditions such as tumors [12-15], lymphoproliferative disorders [16], allograft transplant acceptance [17, 18] and several inflammatory diseases [19, 20].

The *HLA-G* gene expression level is controlled by several polymorphisms in the promoter or the 3' untranslated region (3' UTR). Those polymorphisms may act by modifying the affinity of gene targeted sequences for transcriptional or post-transcriptional factors [21, 22]. The 14 bp insertion/deletion (INS/DEL) polymorphism (rs66554220) at the position +2960 in the exon 8 of the 3'UTR was shown to influence mRNA stability and expression [22-24]. The presence of the 14 bp insertion (INS) affects mRNA stability and splicing which causes a lower HLA-G protein production [23-25]. Whereas the deletion allele (DEL) stabilizes the mRNA with a consequent higher HLA-G expression [25, 26]. The +3142 bpC>G polymorphism (rs1063320) is also located in the 3' UTR of the *HLA-G* gene. This location is a site targeted by specific miRNAs. The binding of the +3142 G allele to three miRNAs (miR-148a, miR-148b and miR-152) is predicted to be more stable than the binding of the +3142 C allele, resulting in a lower protein production [27, 28]. The association of these polymorphisms with the level of *HLA-G* expression has been reported in several inflammatory and autoimmune diseases [28, 29]. Several studies have found a peculiar or reduced expression of both *HLA-G* mRNA and protein in auto-immune diseases [30- 33] and other inflammatory pathological conditions [34] such as inflammatory bowel disease [35, 36], myositic lesions [37], psoriatic lesions on skin [38], rheumatoid arthritis [39], celiac disease [40] and Behçet disease [19, 41, 42] but findings remain controversial. HLA-G, with its

immunosuppressive activity, could play an important role in the immune regulatory process of MS. In fact, few published studies tried to investigate the possible association between *HLA-G* polymorphism and MS [43-46] and results were not conclusive.

The soluble isoforms of HLA-G are thought to play an important role in the pathogenesis of several diseases [19, 31], notably MS [20, 45, 46]. Previous studies have reported elevated serum and cerebrospinal fluid (CSF) levels of the sHLA-G molecule in MS [20, 47]. Thus, the evaluation of serum sHLA-G levels as a biomarker for MS may be of interest [48].

Tunisia, together with North Africa, is considered as a low prevalence zone for multiple sclerosis [49]. Consequently, only very few studies have taken interest in this disorder in this area. And, to the best of our knowledge, no published studies have investigated the *HLA-G* 3'UTR 14 bp INS/DEL and the *HLA-G* +3142 C>G polymorphisms and their association with serum levels of sHLA-G in Tunisian or even African or Arab patients with MS.

The aim of this study was to investigate the two main polymorphisms in the 3'-UTR of the *HLA-G* gene, 14 bp INS/DEL and +3142 C>G, and to assess their impact on the level of production of sHLA-G in Tunisian patients with MS.

2. Materials and Methods

2.1. Subjects

This case–control study enrolled 60 Tunisian (38 females, 22 males; mean age 37.7 ± 6.4 years) unrelated patients affected by definite MS according to the classification of McDonald [50] and followed at the Department of Neurology, Fattouma Bourguiba Hospital, Monastir, Tunisia, during the period from 2001 to 2013. All patients had relapsing-remitting (RR) course in agreement with the criteria of Lublin [51]. Disease disability was assessed in

all RR-MS patients at the time of sample collection using Kurtzke's Expanded Disability Status Scale (EDSS) [52] (mean at entry: 2,9 ± 1,4, range from 1 to 8). The duration of the disease was expressed in months (mean at entry: 65.6 ± 101.8, range from 12 to 180). At entry, none of the patients had fever or other symptoms or signs of acute infections. Clinical assessment and blood sampling were performed during routine clinics, with written informed consent and local ethical board approval. None of MS female patients was pregnant before entering the study. Unrelated 112 sex and age matched healthy subjects (62 females, 50 males; mean age=35.4 ± 6.9 years) who had donated blood at the Regional Center for Blood Transfusion, Monastir, Tunisia, were used as controls.

2.2. HLA-G polymorphism typing

Patients' and controls' genomic DNA was extracted from peripheral blood cells by the spin column technique using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The *HLA-G* 14 bp INS/DEL polymorphism in exon 8 of the *HLA-G* gene was determined by polymerase chain reaction (PCR) and performed as previously described [53]. Briefly, 100 ng of genomic DNA were added to a final volume of 25 µL, with final concentrations as follows: PCR buffer 1X; 1.5 mM $MgCl_2$; 0.4 mM of each dNTP; 1 unit of *Taq*-polymerase and 10 pmol of each primer (GE14HLAG-5'- GTGATGGGCTGTTTAAAGTGTCACC-3', HRG4-5'-GGAAGGAATGCAGTTCAGCATGA-3'). Thermocycling conditions were as follows: initial denaturation for 2 min at 94°C, followed by 30 cycles at 94°C for 30 s, annealing at 64°C for 60 s, extension at 72°C and a final extension at 72°C for 10 min. The amplified products were analyzed by electrophoresis on a 3% agarose gel containing ethidium bromide and visualized under ultraviolet light. The 14 bp insertion allele (INS) amplification yielded a

224 bp fragment whereas the deletion allele (DEL) amplification yielded a fragment of 210 bp.

The *HLA-G* +3142 C<G polymorphism was determined by restriction fragment length polymorphism PCR (RFLP-PCR). Briefly, 100 ng of genomic DNA were added to a final volume of 25 μL, with final concentrations as follows: PCR buffer 1X; 2.0 mM $MgCl_2$; 0.2 mM of each dNTP; 1.0 unit of *Taq*-polymerase and 10 pmol of each primer (GMIRNAF-5'-CATGCTGAACTGCATTCCTTCC-3', GMIRNAR-5' CTGGTGGGACAAGGTTCTACTG-3') [31]. Thermocycling conditions were: 94◦C for 5 min; 32 cycles of 94◦C for 30 s, 65.5◦C for 30 s and 72◦C for 60 s followed by a final extension step at 72◦C for 5 min. The amplified PCR products were cleaved with 3U of BaeGI (New England Biolabs Inc., Ipswich, MA) at 37◦C for three hours, according to manufacturer's instructions. RFLP products were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide and visualized under ultraviolet light. The C allele yielded an intact fragment of 406 bp while the G allele yielded fragments of 316 and 90 bp.

2.3. sHLA-G Enzyme-Linked Immunosorbent Assay (ELISA)

sHLA-G levels in serum samples from 51 patients with MS and 51 HD, were determined in triplicate as previously reported [20] using as capture antibody [the MoAb MEM-G9 (Exbio, Praha, Czech Republic)] which recognizes the HLA-G molecule, in β2-microglobulin associated form. As detecting antibody, an anti-β2-microglobulin MoAb conjugated with HRP was used (DakoCytomation, Rødovre, Denmark). The intra-assay coefficient of variation (CV) was 1.4% and the inter-assay CV was 4.0%. The limit of sensitivity was 1.0 ng/ml.

2.4. Statistical analysis

Allele frequencies of HLA-G were tested for the Hardy–Weinberg equilibrium (HWE) for both patients and control groups using the $\chi 2$ test. The same test (or Fisher's exact test, when appropriate) was applied to compare the frequencies of alleles, genotypes and haplotypes between the MS and control groups. P-value was considered to be statistically significant when <0.05. Odds ratios (OR) were calculated according to the Woolf's formula and are given with 95% confidence interval (95%CI).

Pairwise linkage disequilibrium (LD) was estimated by squared allele frequency correlation (r^2) using Arlequin software. The minimum haplotype frequency was set at 1 %. To estimate haplotype frequencies, the expectation-maximization (EM) algorithm was considered using the same software, considering epsilon value of e^{-7}, maximum interaction of 5,000 and standard deviation through 5,000 bootstraps.

All quantitative results are expressed as the mean ± standard deviation. The normality of each variable was checked using the Kolmogorov-Smirnov test. As normality of data distribution was rejected in several variables, continuous variables were compared using Kruskal-Wallis and Mann Whitney U tests, and correlations were assessed by the Spearman rank correlation coefficient. P-value < 0.05 was considered to be statistically significant. Analyses were done with the SPSS® software.

3. Results

3.1. HLA-G 14 bp INS/DEL and +3142 C>G polymorphism typing

We analysed 60 Tunisian RR-MS patients and 112 Tunisian control subjects for two 3'UTR *HLA-G* polymorphic sites: 14 bp INS/DEL and +3142 C>G. Both studied polymorphisms were in HWE in MS patients and controls (p > 0.05). Allele and genotypic frequencies of the two polymorphisms for RR-MS patients and controls are reported in Table 1.

Regarding the *HLA-G* 14 bp INS/DEL polymorphism, the investigation of allelic and genotypic frequencies showed no statistically significant differences between patients and controls (Table 1). Furthermore, we noted that the INS/DEL genotype was the most frequent in both RR-MS patients and controls (50% and 48.2%, respectively).

Concerning the +3142 C>G polymrphism, we found that the G allele was significantly more frequent in the RR-MS group in comparison with the control group (63.4% *vs* 52.3%, p=0.04; OR=1.58, 95%CI=1.003–2.48) (Table 1). Moreover, the determination of the genotypic frequencies of this polymorphism showed that C/G + G/G genotypes were presented at higher frequencies than the C/C genotype in MS patients as compared to controls (91.7% *vs* 73.2%, p=0.004, OR=4.02, 95%IC=1.47-11.01). Hence the C/C genotype frequency is decreased in patients group compared to controls group (p=0.004; OR=0.25, 95%CI=0.08-0.73) (Table 1).

3.2. Haplotypes and combined genotypes analysis

The frequencies of different haplotypes (14 bp DEL / +3142 C; 14 bp DEL / +3142 G; 14 bp INS / +3142 G and 14 bp INS / +3142 C) are presented in Table 2. The *HLA-G* 14 bp DEL / +3142 G haplotype was more frequent in RR-MS patients in comparison with controls (20.8% *vs* 12.5%, p=0.04; OR=1.84, 95%CI=0.98-3.45). No linkage disequilibrium was found between both studied polymorphisms of *HLA-G* (r^2=0.004).

The comparison of combined genotypes obtained from these two *HLA-G* polymorphisms showed that the 14 bp DEL/DEL / +3142 C/G combined genotype was significantly increased in RR-MS patients compared to controls (18.35% *vs* 6.2%, p=0.013; OR=3.37, 95%CI=1.12-10.34) while the 14 bp DEL/DEL / +3142 C/C combined genotype

was significantly decreased in RR-MS patients compared to controls (5% *vs* 19.7%, p=0.009; OR=0.22, 95%CI=0.05-0.81) (Table 3).

3.3. HLA-G polymorphisms association with clinical parameters

The data related to the assessment of *HLA-G* polymorphisms association with clinical parameters are summarized in tables 4 and 5. Our study showed a significant association between the +3142 C>G polymorphism and age of disease onset (ADO) in patients with RR-MS. The mean ADO for patients with the +3142 C/C genotype was significantly lower (28.00±2.12 years) than the mean ADO for patients with the +3142 C/G or the +3142 G/G genotypes (32.91±10.60 years and 32.33±11.44 years, respectively) (p=0.011) (Table 4). Moreover, no associations were found between *HLA-G* polymorphisms and disease duration or MRI activity and no correlation was retrieved between these polymorphisms and the EDSS score (Tables 4 and 5).

3.4. sHLA-G serum levels association with clinical parameters and HLA-G polymorphisms

The mean serum level of sHLA-G was almost the same in RR-MS patients and controls (21.17±4.35 ng/mL *vs* 22.65±3.11 ng/mL, p=0.58) with no significant variation between males and females of both groups (21.17±4.35 ng/mL *vs* 22.65±3.11 ng/mL, p=0.35) (Table 6). Furthermore, in the RR-MS patients group, no association was found between serum levels of sHLA-G and MRI activity (20.53±4.34 ng/mL *vs* 21.85±4.33 ng/mL, p=0.28) and no correlation was observed between serum levels of sHLA-G and EDSS scores (r=0.01 , p=0.92).

Moreover, our results showed that, for both patients and controls, the mean levels of serum sHLA-G does not vary with the genotypic variants of the two studied polymorphisms (Table 6)

4. Discussion

Since the HLA-G molecule, with its tolerogenic proprieties, is currently believed to play a certain role in the modulation of MS autoimmunity, [8, 54-56] several studies have focused on the possible link between *HLA-G* genetic polymorphisms and susceptibility to MS, and whether the HLA-G production is affected by those polymorphisms [43-46, 57]. To the best of our knowledge, there are no available data regarding this issue for Tunisian patients with MS.

In the present study we investigated two main polymorphisms in the 3'UTR of the *HLA-G* gene (14 bp INS/DEL and +3142 C>G). These two polymorphisms are presumed to affect the level of HLA-G protein production.

Our results showed that only the +3142 C>G polymorphism frequencies vary significantly between patients and controls. The lack of association between the 14 bp INS/DEL polymorphism and MS, noted in our series, was also reported by Kroner et al. who studied three polymorphic sites in the *HLA-G* gene including the 14 bp INS/DEL polymorphism and found no association between any of these three polymorphisms and MS in a German population [43]. Similar results were also reported in Iranian [45] and Polish [44] patients with RR-MS. Moreover, a meta-analysis carried out by Lee et al. [30] to assess the association between the 14 bp INS/DEL polymorphism and autoimmune diseases, concluded that this polymorphism is not associated with MS, rheumatoid arthritis and Crohn's disease, but associated with several other autoimmune diseases such as systemic lupus erythematosus, suggesting that this polymorphism has disease-dependent functionality. The *HLA-G* 14 bp INS/DEL polymorphism may be not a common genetic factor for various autoimmune diseases, and different pathogenic mechanisms might be involved in the development of polygenic autoimmune diseases [30].

Concerning the +3142 C>G polymorphism, our study showed that the +3142 G allele frequency was increased while the +3142 C/C genotype frequency was decreased in RR-MS patients comparing to controls. This result is in agreement with that found by Rizzo et al., who reported a significant decreased frequency of *HLA-G* +3142 C/C (frequency = 8.7%) in Italian MS patients [46].

Thus, the G allele seems to be a susceptibility factor to MS development (OR=1.58, 95%CI=1.003–2.48), while the C/C genotype seems to be associated with protection against the development of this disease (OR=0.25, 95%CI=0.08-0.73). The +3142 C>G polymorphism might affect disease susceptibility by its influence on post-transcriptional regulation of HLA-G expression, as suggested by Tan et al. in patients with asthma [28]. Moreover, in our series, we noted that the 14 bp DEL / +3142 G haplotype could also be a susceptibility factor to MS (p=0.04, OR=1.84, 95%IC=0.98-3.45). Besides, the 14 bp DEL/DEL / +3142 C/G combined genotype seems to be also a susceptibility factor to MS (OR=3.37, 95%CI=1.12-10.34), whereas the 14 bp DEL/DEL / +3142 C/C combined genotype could be associated with protection in our series (OR 0.22, 95 % CI=0.05–0.81). This shows that susceptibility to MS may be not related to a single allele but may depend on the combination of several polymorphic sites forming haplotypes of susceptibility. Indeed, Wisniewski and coauthors when evaluating the -725C>G>T, -716T>G and 14 bp INS/DEL polymorphisms in MS, have found that the -725G/-716T/14 bp DEL haplotype may constitute a susceptibility factor to this disease in the Polish population [44].

On the other hand, the investigation of the impact of *HLA-G* gene polymorphisms on clinical parameters shows that the +3142 C>G, but not the 14 bp INS/DEL polymorphism may influence the initiation of the disease. Indeed, MS patients with +3142 C/C genotype appear to be more prone to have early disease onset comparing to patients with +3142 C/G and G/G genotypes. Our findings are contrasting with those of Wisniewski and co-authors

who reported that MS patients with 14 bp INS/INS and 14 bp INS/DEL genotypes are more susceptible to have early disease onset comparing to patients with 14 bp DEL/DEL genotype [44]. Moreover, our study showed that the studied polymorphisms have no effect on disease activity (MRI activity) or disease severity (EDSS score). This agrees in part with the results reported by Kroner et al. and Mohammadi et al. [43, 45] who found no association between *HLA-G* gene polymorphism and severity of the disease.

In the present study, the assessment of serum levels of the sHLA-G molecule did not lead to any significant difference between MS patients and HD. These findings are in disagree with those found by Muhammadi et al. who reported a significant increase of sHLA-G serum levels in plasma of RR-MS Iranian patients compared to healthy controls [45]. In our series, no associations were found between serum sHLA-G concentrations and ADO, disease activity or disease severity. Our results are in agreement with those of Rizzo et al. who reported a similar serum sHLA-G levels in MRI active and inactive patients [46]. But, interestingly the authors found a significant increase of the concentrations of this molecule in CSF of MRI active patients. Thus, the stable sHLA-G levels observed in serum of both patients with active and inactive disease may not exclude an increased production of this molecule in other inflammatory compartments such as CSF. The findings of Fainardi and coauthors corroborate this hypothesis. In fact, they reported reciprocal fluctuations in CSF and serum concentrations of sHLA-G in clinical and MRI active MS, since serum levels were decreased in patients without clinical disease activity, whereas CSF levels were increased in those with MRI inactive disease [20, 47, 58]. Furthermore, it has been demonstrated that, in MS patients, HLA-G expression is upregulated on CSF monocytes and on microglia, macrophages and endothelial cells located in demyelinating areas [8]. Altogether, these observations delineate the role of sHLAG in the pathogenesis of MS. This immunoregulatory molecule may act

together with other cellular and molecular immunosuppressive effectors, notably the regulatory T cells (Tregs) and IL-10, to control disease activity [59, 60].

On the other hand, our study did not find any association between serum sHLA-G concentrations and *HLA-G* polymorphisms, suggesting that the both studied *HLA-G* polymorphisms have no impact on the level of production of sHLA-G in Tunisian patients with MS. In contrast, Mohammadi et al. found in their study that the 14 bp deletion polymorphism is associated with an increase of sHLA-G plasma levels in Iranian patients with MS [45]. Furthermore, Rizzo et al. showed that the highest serum sHLA-G levels were observed in MS patients with the 14 bp DEL/DEL / +3142 C/C combined genotype while the lowest serum sHLA-G concentration were found in patients with the 14 bp INS/INS / +3142 G/G combined genotype, suggesting that, in MS, the release of sHLA-G molecules could depend not only on local microenvironment, represented by the presence or absence of inflammatory activity, but may also be controlled by the two main polymorphisms at the 3'UTR of the *HLA-G* gene [46].

Taken together, our findings suggest that the *HLA-G* 14 bp INS/DEL and +3142 C>G polymorphisms does not influence, alone, the expression level of the *HLA-G* gene. This means that other polymorphic sites may be involved in the regulation of the expression of this gene. In fact, it was reported that the *HLA-G* 3'UTR +3187 A/G polymorphism, which is in linkage disequilibrium with these polymorphisms, affects the production levels of sHLA-G [61]. Thus, it cannot be excluded that all these polymorphic sites may work together to regulate *HLA-G* expression in MS. Nevertheless, the discrepancy between those results and ours could be due to sample sizes, the ethnic groups and methods used in different studies.

The main limit of this study is the small sample size of patients with MS that could weaken the strength of statistical tests. Therefore, a more extensive research including a larger number

of Tunisian RR-MS patients is needed to better clarify the interrelationships between the 14 bp INS/DEL and +3142 C>G polymorphisms, sHLAG serum levels and MS.

In conclusion, our study showed that the *HLA-G* +3142 C>G polymorphism seems to be associated with susceptibility to MS. Indeed, the +3142 G and the 14 bp DEL/DEL / +3142 C/G combined genotype may constitute genetic risk factors for MS, whereas the +3142 C/C genotype may constitute a protection factor. Moreover, MS patients with the +3142 C/C genotype have lower age of disease onset. However, genetic variations of the *HLA-G* 14 bp INS/ DEL and +3142 C>G polymorphisms seemingly does not impact sHLA-G serum levels in patients with MS.

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Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Table 1: Comparison of allelic and genotypic frequencies between Tunisian RR-MS patients and control subjects.

	RR-MS patients		Control subjects			
	N	Frequency (%)	N	Frequency (%)	*p*-value	OR (CI 95%)
14 bp INS/DEL*	**60**		**112**			
Allele						
14 bp INS	56	46.7	108	48.3	0.87	1
14 bp DEL	64	53.3	116	51.7		
Genotype						
14 bp INS/INS	13	21.7	27	24.1	0.86	
14 bp INS/DEL	30	50.0	54	48.2	0.94	
14 bp DEL/DEL	17	28.3	31	27.7	0.93	
+3142 C>G	**60**		**112**			
Allele						
G	76	63.4	117	52.3		
C	44	36.6	107	47.7	0.04**	1.58 (1.003-2.48)
Genotype						
GG	21	35	35	31.3	0.61	

CG	34	56.7	47	42	0.06	
CC	5	8.3	30	26.7	0.004	0.25 (0.08-0.73)
					0.004***	4.02 (1.47-11.01)

*14 bp INS/DEL: 14 base pairs deletion/insertion.

** G allele *vs* C allele

***GG+CG vs CC

RR-MS: Relapsing Remitting Multiple Sclerosis; OR: odds ratio; CI: confidence interval; N: number.

Comparisons were made by the Fisher's exact test or the chi-squared test.

Table 2: Comparison of haplotype frequencies between RR-MS patients and control subjects.

	RR-MS cases		Control subjects			
	N	Frequency (%)	N	Frequency (%)	*p*-value	OR (CI 95%)
Subjects	**60**		**112**			
Haplotypes	**120**		**224**			
DEL/C	39	32.5	88	39.3	0.21	
DEL/G	25	20.8	28	12.5	0.04	1.84 (0.98-3.45)
INS/G	51	42.5	89	39.7	0.61	
INS/C	5	4.2	19	8.5	0.13	

DEL : 14 base pairs deletion.; INS : 14 base pairs insertion; C: +3142 C; G: +3142 G.

RR-MS: Relapsing Remitting Multiple Sclerosis; OR: odds ratio; CI: confidence interval; N: number.

Comparisons were made by the Fisher's exact test or the chi-squared test.

Table 3: Comparison of combined genotype frequencies between Tunisian RR-MS patients and control subjects.

	RR-MS cases		Control subjects			
	N	Frequency (%)	N	Frequency (%)	*p*-value	OR (CI 95%)
Genotypes	**60**		**112**			
DEL/DEL-C/C	3	5	22	19.7	0.009	0.22 (0.05-0.81)
DEL/DEL-C/G	11	18.35	7	6.2	0.013	3.37 (1.12-10.34)
DEL/DEL-G/G	3	5	2	2	0.34	
DEL/INS-C/C	2	3.3	8	7.1	0.49	
DEL/INS-C/G	21	35	31	27.7	0.31	
DEL/INS-G/G	7	11.7	15	13.3	0.74	
INS/INS-G/G	11	18.35	18	16	0.7	
INS/INS-C/G	2	3.3	9	8	0.33	

DEL : 14 base pairs deletion.; INS : 14 base pairs insertion; C: +3142 C; G: +3142 G.

RR-MS: Relapsing Remitting Multiple Sclerosis; OR: odds ratio; CI: confidence interval; N: number.

Comparisons were made by the Fisher's exact test or the chi-squared test.

Table 4: Genotypic frequencies of 14 bp *HLA-G* polymorphism in 60 RR-MS patients grouped according to: age of disease onset, disease duration, EDSS score and MRI activity.

	INS/INS	**INS/DEL**	**DEL/DEL**	***p*-value**
Age of disease onset (years)	30.76±12.37	32.56±10.07	33.00±10.11	*0.74
Disease duration (years)	3.61±3.15	5.76±4.12	6.05±3.86	**0.305
EDSS score	2.15±1.14	3.16±1.68	2.76±1.88	**0.55
MRI activity				
MRI active RR-MS	4 (15.38%)	15 (57.69%)	7 (26.92%)	0.05
MRI inactive RR-MS	9 (26.47%)	15 (44.11%)	10 (29.41%)	

Del : deletion, Ins : insertion

RR-MS: Relapsing Remitting Multiple Sclerosis;

EDSS : Expanded Disability Status Scale; MRI : Magnetic Resonance Imaging.

Data showed by n (%) or Mean±SD. Comparisons were made by the Fisher's exact test or the chi-squared test (χ^2) for qualitative variables.

* *p* values were obtained with t-test.

***p* values were obtained with Mann Whitney U test.

Table 5: Genotypic frequencies of the *HLA-G* +3142 C>G polymorphism in 60 RR-MS patients grouped according to: age of disease onset, disease duration, EDSS score and MRI activity.

	G/G	G/C	C/C	*p*-value
Age of disease onset (years)	32.33±11.44	32.91±10.60	28.00±2.12	*0.011
Disease duration (years)	5.9±3.78	4.61±3.63	8.4±5.27	**0.102
EDSS score	2.48±1.56	2.94±1.53	3.8±2.68	**0.373
MRI activity				
MRI active RR-MS	6 (23.07%)	17 (65.38%)	3 (11.53%)	0.21
MRI inactive RR-MS	15 (44.11%)	17 (50. %)	2 (5.88%)	

RR-MS: Relapsing Remitting Multiple Sclerosis;

EDSS : Expanded Disability Status Scale; MRI : Magnetic Resonance Imaging.

Data showed by n (%) or Mean±SD. Comparisons were made by the Fisher's exact test or the chi-squared test (χ^2) for qualitative variables.

* *p* values were obtained with t-test.

***p* values were obtained with Mann Whitney U test.

Table 6: Serum sHLA-G levels in RR-MS group and Healthy controls according to demographic, clinical characteristics and disease activity.

	RR-MS group		Healthy group	
Variables	**sHLA-G**	***p*-value**	**sHLA-G**	***p*-value**
Sex				
Male	23.48±2.77		22.43±3.20	
Female	21.91±3.56	0.06*	23.22±3.08	0.61*
***HLA-G* 14 bp INS/DEL genotype**				
INS/INS	22.31±3.58	0.503*	21.74±3.52	0.98*
INS/DEL	20.50±4.82		22.66±2.92	
DEL/DEL	21.34±4.17		22.63±3.56	
***HLA-G* +3142 C>G genotype**				
G/G	21.84±3.48	0.314*		0.51*
G/C	20.27±5.10		22.79±3.24	
C/C	22.90±2.89		21.23±1.19	
Age of disease onset (correlation)	0.09	0.49**		
Disease duration (correlation)	0.1	0.48**		
EDSS score (correlation)	0.01	0.92**		

MRI activity				
MRI active MS	20.53±4.34	0.28		
MRI inactive MS	21.85±4.33			

14bp : 14 base pairs; DEL : deletion; INS : insertion. ;

RR-MS: Relapsing Remitting Multiple Sclerosis;

EDSS : Expanded Disability Status Scale ; MRI : Magnetic Resonance Imaging;

*Anova test

**Pearson correlation test