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Highlights

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Killer cell immunoglobulin-like receptor genes in Spanish multiple sclerosis patients

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- The frequency of the *KIR2DL5* and *KIR3DS1* genes were increased in MS patients.
- The carriage of the *KIR2DL1* gene was associated with a higher progression index.
- The HLA-Bw4 motif protects against MS.
- Non significant associations were found between KIR genes and response to IFN-β.

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Killer cell immunoglobulin-like receptor genes in Spanish multiple sclerosis patients

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ABSTRACT

Killer cell immunoglobulin-like receptors (KIRs) are regulators of cytolytic activity of natural killer and certain T cells through interactions with human leukocyte antigen (HLA) class I ligands. KIRs have been shown to contribute to the pathogenesis of several autoimmune diseases, but their role in multiple sclerosis (MS) is still unclear. Here we determined the influence of KIR genes and their HLA class I ligands on susceptibility to MS and on the response to interferon-beta treatment in a Spanish population. KIR and HLA genotyping were performed in 200 MS patients and 200 controls. Significantly higher frequencies were found for *KIR2DL5* and *KIR3DS1* genes in MS patients and the carriage of the *KIR2DL1* gene was associated with a higher progression index. Moreover, the frequency of the *HLA-Bw4* motif was significantly reduced in MS patients. The *KIR2DL1* and *HLA-C2* matches were more frequent in MS patients, whereas the *KIR3DL1* and *HLA-Bw4* matches were more frequent in healthy controls. Nevertheless, non significant associations were found between all the KIR genes and therapeutic response to interferon-beta. Our results confirm that the carriage of *HLA-Bw4* is a protective factor in MS and suggest that *KIR2DL5* and *KIR3DS1* may have a predisposing role in the disease.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory immune-mediated disease of the central nervous system. Susceptibility to MS is determined by many genes, each exerting a relatively moderate effect on the overall disease predisposition (Oksenberg et al., 2008) and interplaying with as yet unidentified environmental factors (Compston and Coles, 2002; Willer and Ebers, 2000). The human leukocyte antigen (HLA) class II allele DRB1*1501 is the best established factor associated with MS susceptibility, although recently some HLA class I alleles (Bergamaschi et al., 2010) and few other genes outside the HLA region (Oksenberg and Baranzini, 2010; Hoffjan and Akkad, 2010) have been identified as influencing MS susceptibility. Interferon beta (IFN-β) is one of the most widely used first line treatment in MS, but a high percentage of patients fail to respond optimally and a reliable biomarker is needed in clinical practice (Rio et al., 2009).

Many studies suggest that natural killer (NK) cells might play a role in the regulation of MS (Takahashi et al., 2004; Hao et al., 2010). NK cells activity is the result of a delicate balance between activating and inhibitory signals, delivered by cell surface receptors belonging to several families, being the killer cell immunoglobulin-like receptors (KIRs) one of the most important.

KIRs are a family of receptors which present wide genetic variability. They are expressed on the cell surface of NK cells and on some subsets of T lymphocytes. KIRs regulate the inhibition and activation of cell responses through recognition of polymorphic motifs on HLA class I molecules (*HLA-A*03* and *HLA-A*11* alleles, and alleles with the HLA-Bw4, HLA-C1 or HLA-C2 motifs) on target cells. KIRs have two or three extracellular immunoglobulin domains, a transmembrane region and an intracellular domain. The latter differentiates the inhibitory from the activating receptors, so inhibitory KIR molecules (KIR2DL or KIR3DL) bear long cytoplasmic tails whereas activating

KIRs (KIR2DS or KIR3DS) present short cytoplasmic tails (Lanier, 1998), with the exception of KIR2DL4, which, though bearing a long tail, may have both inhibitory and activating capacities (Kulkarni et al., 2008).

Ligation of activating KIRs leads to the enhancement of cytotoxic functions of NK and T cells and costimulation of T cells,

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favouring the proliferative response when the T receptor complex stimulation is suboptimal (Snyder et al., 2004). Therefore, activating KIRs could be a risk factor for autoimmune diseases such as MS. In turn, ligation of inhibitory KIRs inhibits cellular activation, leading to a downregulation of proinflammatory Th1 cytokine production, inhibition of apoptosis and reduction of cell cytotoxic events (Vivier and Anfossi, 2004), acting as a brake on autoimmune responses.

Individuals differ both in number and in combinations of KIR genes in their genotype. In general, two groups of haplotypes can be distinguished, with a distribution that varies between ethnic populations (Uhrberg, 2005). The A haplotypes encode mainly inhibitory receptors with only 1–2 activating KIRs (*KIR2DS4* and/or *KIR2DL4*) (Purdy and Campbell, 2009). In contrast, B haplotypes present an important variation in gene content and have 2–6 activating KIR genes. The framework genes (*KIR3DL2*, *KIR3DL3* and *KIR2DL4*) are present in both groups.

Due to the fact that KIR and HLA genes present a great diversity and segregate independently, the KIR–HLA combinations can be completely different between unrelated individuals and these differences can be up to 75% between family members regardless of HLA identity (Leung et al., 2005). Therefore, an individual may inherit both the KIRs and their corresponding HLA ligands or just one of them.

The association between KIR genes and autoimmune diseases has been widely studied. In particular, the *KIR2DS2* gene has been reported as a risk factor for rheumatoid arthritis (Yen et al., 2001), lupus erythematosus and scleroderma (Pellett et al., 2007).

In a Norwegian cohort, Lorentzen et al. (2009) found that the frequency of the *HLA-Bw4* ligand was decreased in MS patients, and hypothesized that this reduction might exert a protective role in MS. On the other hand, in an Italian cohort, Fusco et al. (2010) found that the frequency of the activating *KIR2DS1* gene was lower in MS patients.

Our aim was to determine the gene content of KIRs and their known HLA ligands in Spanish MS patients in order to confirm the results obtained in other Caucasian populations, and to evaluate whether KIR receptors could be a measurable biomarker to predict IFN-β responsiveness.

2. Materials and methods

2.1. Study subjects

A total of 200 Spanish Caucasian patients with clinically definite MS according to the McDonald criteria (McDonald et al., 2001) were recruited through the Multiple Sclerosis unit of Carlos Haya Regional University Hospital in ~~Malaga~~, Spain. As controls, 200 age-, sex- and ethnicity-matched healthy unrelated subjects were obtained from the Transfusion Centre Blood Bank of Málaga.

The clinical characteristics of the MS patients enrolled in the study are given in Table 1. A total of 137 MS patients with at least 2 years of IFN-β treatment were selected to evaluate KIR gene frequencies between responders and non-responders to this immunomodulatory therapy. The criteria to classify patients as non-responders to IFN-β was the presence of any relapse or an increase in the expanded disability status scale (EDSS) score of at least one point, confirmed at 6 months (Rio et al., 2009).

Written informed consent was obtained from the patients and controls under protocols approved by the Institutional Research Ethics Committees of Carlos Haya Hospital and the Transfusion Centre of ~~Malaga~~, as well as the local Authorities.

Table 1
Clinical characteristics of the 200 MS patients.

Sex: female/male ratio	140/60 (70% *vs.* 30%)
Age (years)	42.30 ± 10.73 (18–70)
Mean age at onset (years)	29.46 ± 10.52 (8–61)
Clinical form at onset (%)	
Bouts	98.5
Progressive	1.5
Clinical form at present (%)	
Relapsing remitting	80.5
Secondary progressive	18.0
Progressive relapsing	1.0
Primary progressive	0.5
Mean disease duration (years)	12.87 ± 8.82 (2–42)
EDSS score at present	2.52 ± 1.85 (0–9)
Progression index (EDSS score at present/disease duration)	0.26 ± 0.25
Current treatment (%)	
Rebif® (IFNbeta 1a)	26.0
Avonex® (IFNbeta 1a)	25.0
Betaferon® (IFNbeta 1b)	17.5
Copaxone® (Glatiramer acetate)	23.5
Tysabri® (Natalizumab)	7.0
Cyclophosphamide	1.0
Response to IFN-beta treatment	
Responders	58 (42.3%)
Non-responders	79 (57.7%)

Quantitative data are presented as mean ± standard deviation (minimum–maximum).
EDSS: expanded disability status scale.

2.2. DNA isolation

For each subject, genomic DNA was isolated from peripheral blood samples collected in ethylene diamine tetraacetic acid using a commercial modification of the salting-out method (QIAamp 96 DNA Blood Kit, Qiagen, Hilden, Germany).

2.3. KIR genotyping

KIR genotyping was performed by polymerase chain reaction-sequence specific primers (PCR-SSP) as described previously (Gomez-Lozano and Vilches, 2002) in all the recruited subjects for the following KIR genes: *2DL1*, *2DL2*, *2DL3*, *2DL4*, *2DL5*, *3DL1*, *3DL2*, *3DL3*, *2DS1*, *2DS2*, *2DS3*, *2DS4*, *2DS5* and *3DS1*.

2.4. HLA genotyping

HLA genotyping was carried out in all the recruited subjects. *HLA-B* and *HLA-C* genotyping was performed to a consistent two digit resolution level by PCR followed by sequence-specific oligonucleotide probe hybridization (INNO-LiPA HLA-B Update Plus and INNO-LiPA HLA-C; Innogenetics, Belgium). Genotyping for the *HLA A*03* and *A*11* alleles was done by PCR-SSP as previously described (Bunce et al., 1995).

2.5. KIR ligands

Ligand groups were defined as follows: KIRs 2DL1, 2DS1 and 2DS4 recognize the HLA-C2 epitope (Katz et al., 2001) (Asn^{77} and Lys^{80}; present in *HLA-Cw*02*, **04*, **05*, **06*, **15*, **1602*, **17* and **18* alleles). KIRs 2DL2, 2DL3, 2DS2 and 2DS3 recognize the HLA-C1 epitope (Kunert et al., 2007) (Ser^{77} and Asn^{80}; present in *HLA-Cw*01*, **03*, **07*, **08*, **12*, **14* and **1601* alleles). The ligand for KIR3DL1 and 3DS1 is the HLA-Bw4 motif (Martin et al., 2007) (specified by five variable amino acids spanning positions 77–83, and found in

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Table 2
KIR genes carrier frequencies in MS patients and controls.

KIR genes	MS patients (%)	Controls (%)	p_{uc}	OR (95% CI)
Inhibitory				
2DL1	86.5	85.5	0.773	1.087 (0.617–1.912)
2DL2	57.0	54.5	0.614	1.107 (0.746–1.642)
2DL3	84.0	83.5	0.892	1.037 (0.610–1.765)
2DL4	100.0	100.0		
2DL5	59.5	46.0	**0.006**	**1.725 (1.160–2.563)**
3DL1	93.0	93.5	0.842	0.924 (0.423–2.018)
3DL2	100.0	100.0		
3DL3	100.0	100.0		
Activating				
2DS1	52.0	45.0	0.161	1.324 (0.894–1.962)
2DS2	55.0	53.5	0.763	1.062 (0.717–1.574)
2DS3	35.5	28.0	0.107	1.415 (0.927–2.161)
2DS4	89.0	93.0	0.162	0.609 (0.302–1.227)
2DS5	38.0	32.0	0.208	1.302 (0.863–1.967)
3DS1	46.0	35.5	**0.032**	**1.548 (1.036–2.312)**

OR: odds ratio with 95% confidence interval (CI).
p_{uc}: p values non corrected for multiple testing.

approximately 40% of the known HLA-B allotypes), whereas the ligands for KIR3DL2 are the *HLA-A*03* and **11* alleles.

2.6. Statistical analysis

All statistical analyses were performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). Carrier frequencies of KIR genes and HLA alleles were compared between MS patients and healthy controls by Pearson χ^2 test or Fisher's exact test, estimating odds ratios (OR) with 95% confidence intervals (CI). Comparisons between KIR genes or HLA alleles and clinical characteristics of MS patients were performed either by Pearson χ^2 or by Fisher's exact test for the categorical variables and by Mann–Whitney or Kruskal–Wallis tests for the quantitative variables. Results were considered significant at a p value <0.05. To avoid false-positive results due to multiple testing we applied the Bonferroni correction that is robust against positive dependence. p Values non corrected for multiple testing are indicated as p_{uc}.

Multivariate forward logistic regression analyses were also performed to estimate the OR with 95% CI. These included the significant interactions in the previous univariate analyses as independent variables and the status of having MS as a dependent variable. The Wald test and an inclusion $p < 0.05$ and exclusion $p < 0.10$ were used.

3. Results

3.1. KIR gene frequencies

The frequencies of KIR genes in our cohort (Table 2) were similar to the frequencies other authors have reported in a Spanish population (Ordonez et al., 2009). The frequencies of the *KIR2DL5* and *KIR3DS1* genes were significantly higher in MS patients than in controls (59.5% *vs.* 46%, $p_{uc} = 0.006$ and 46% *vs.* 35.5%, $p_{uc} = 0.032$, respectively), though statistical significance was lost after Bonferroni correction. The *KIR2DL5* gene has been shown to be in linkage disequilibrium with activating KIR genes (Du et al., 2008). To establish whether this was the cause of the differential distribution of this KIR gene between MS patients and controls, we selected all those subjects carrying the *KIR2DL5* gene and compared the activating KIR gene content between MS patients and controls, finding no differences in the distribution of the activating KIR genes (data not shown).

3.2. Number of activating/inhibitory KIR genes

We examined whether the number of activating and inhibitory KIR genes per phenotype may influence disease susceptibility through a gene dosage effect. MS patients had a higher number of activating KIR genes than controls, with a slightly higher proportion of MS patients carrying at least three activating KIR genes compared to controls (57% *vs.* 48%, $p_{uc} = 0.072$). Concerning the number of inhibitory KIR genes, most of the control subjects had six inhibitory KIR genes, whereas the majority of MS patients had seven, though the differences were not significant.

3.3. KIRs and clinical variables

There were no significant differences in the frequency of any KIR gene between males and females, either in the controls or in the MS patients. Patients carrying the *KIR2DS5* gene had a lower age at onset of the disease (27.84 *vs.* 30.48 years, $p_{uc} = 0.024$). On the other hand, MS patients carrying the *KIR2DL1* gene had a higher age at onset (29.95 *vs.* 26.41 years, $p_{uc} = 0.006$) and also presented a higher progression index compared with MS patients not carrying this gene (0.27 *vs.* 0.17, $p_{uc} = 0.013$).

3.4. KIR genotypes

Based on the presence or the absence of A and/or B haplotypes, we identified the subjects as either having an A/A or a B/– genotype. The B/– genotype was the most commonly observed in both groups of subjects, but non significant differences were observed in the distribution of these genotypes between MS patients and controls (data not shown).

3.5. HLA ligand distribution

All the samples tested for KIR genes were genotyped to determine the frequencies of the HLA alleles with KIR-binding motifs (Table 3): *HLA-C* (*C1* and *C2* epitope group alleles), *HLA-A* (*A*03* and *A*11* alleles) and *HLA-B* (alleles with the *Bw4* motif). The frequency of the *HLA-C1/C2* heterozygous subjects was higher among MS patients than controls (56.0% *vs.* 45.5%, $p_{uc} = 0.035$), as was the frequency of the subjects lacking the HLA-Bw4 motif (Bw6/Bw6) (45% *vs.* 32.5%, $p_{uc} = 0.010$).

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Table 3
Genotype frequencies of the HLA alleles with KIR-binding motifs in MS patients and controls.

HLA genotypes	MS patients (%)	Controls (%)	p_{uc}	OR (95% CI)
C1/C1	24.5	32.0	0.095	0.690 (0.445–1.069)
C1/C2	56.0	45.5	0.035	1.524 (1.028–2.261)
C2/C2	19.5	22.5	0.461	0.834 (0.515–1.351)
A3+/A11+	3.0	2.5	0.759	1.206 (0.362–4.018)
A3+/A11−	16.0	17.5	0.687	0.898 (0.531–1.518)
A3−/A11+	14.0	10.5	0.285	1.388 (0.759–2.537)
A3−/A11−	67.0	69.5	0.591	0.891 (0.585–1.358)
Bw4/Bw4	16.0	22.5	0.099	0.656 (0.397–1.085)
Bw4/Bw6	39.0	45.0	0.224	0.781 (0.525–1.163)
Bw6/Bw6	45.0	32.5	0.010	1.699 (1.132–2.551)
Bw4 carriers	55.0	67.5	0.010	0.588 (0.392–0.884)
Bw4 noncarriers	45.0	32.5		

OR: odds ratio with 95% confidence interval (CI).
p_{uc}: p values non corrected for multiple testing.

3.6. *KIR–KIR ligand interactions*

Finally, matches and mismatches of the KIR–HLA interactions were assessed (Table 4). The interaction of *KIR2DL1* with its *HLA-C2* ligand (KIR+/HLA+) was again more frequent in MS patients (66.5% *vs.* 55.5%, p_{uc} = 0.015), conferring a risk for MS (OR = 1.797; 95% CI = 1.120–2.884). Interactions of *KIR3DL1* with *HLA-Bw4*, however, were less frequent in MS patients than in controls (50.5% *vs.* 63.5%, p_{uc} = 0.007), having a protective effect on disease susceptibility (OR = 0.561; 95% CI = 0.368–0.856). On the other hand, in the absence of *KIR3DS1*, carrying the *HLA-Bw4* motif conferred a lower risk for MS (p_{uc} = 0.002; OR = 0.433; 95% CI = 0.254–0.739).

The multivariate logistic regression analyses demonstrated a significant positive association of the interaction *KIR3DS1:HLA-Bw4* with MS [OR (95% CI): 2.068 (1.231–3.475), p_{uc} = 0.006], strong enough to counteract the protective effect of the carriage of *HLA-Bw4* [OR (95% CI) = 0.437 (0.275–0.693), p_{uc} = 0.0004] (adjusting for the Bw4, KIR3DS1 and KIR3DS1:Bw4 variables). On the other hand, the interaction *KIR3DL1:HLA-Bw4* exerted a synergic protective effect [OR (95% CI):0.586 (0.393–0.875), p_{uc} = 0.009] with that afforded by the carriage of *HLA-Bw4* alone (adjusting for the Bw4, KIR3DL1 and KIR3DL1:Bw4 variables).

3.7. *KIRs and IFN-β response*

No significant associations were found between KIR genes and therapeutic response to IFN-β, though the *KIR2DS5* gene showed a slightly higher gene carrier frequency in non-reponders to IFN-β therapy compared to responders, but not reaching statistical significance [46.6% *vs.* 30.4%, p_{uc} = 0.053; OR (95% CI) = 0.501 (0.248–1.013)]. Additionally, similar frequencies of HLA genotypes were found between responders and non responders to IFN-β treatment (data not shown).

4. Discussion

In the pathogenesis of MS, an important role is exerted by T and NK lymphocytes and the activity of these cells is regulated by interactions of KIR receptors with HLA-class I ligands on target cells. Even though these receptors have been widely studied in autoimmune diseases, just two works have studied the role of the KIR genes and of their HLA ligands in MS, with different results (Lorentzen et al., 2009; Fusco et al., 2010).

We have evaluated a possible association of KIRs in MS susceptibility in a Spanish population. Our results suggest that the *KIR2DL5* and *KIR3DS1* genes confer a slight susceptibility to MS, and reveal that the differential distribution of *KIR2DL5* between MS patients and controls was not due to linkage disequilibrium with activating KIR genes. Lorentzen et al. found a lower *KIR2DL1* and a higher *KIR2DS4* and *KIR3DL1* carrier frequency among MS patients compared to controls (Lorentzen et al., 2009). These slight divergences with Lorentzen's study are related to differences in KIR gene carrier frequencies between MS patients and controls, although none of these associations was robust enough to survive corrections for multiple comparisons in both studies. These discrepancies might be due to our smaller sample size, or might be indicating that KIR genes exert a relatively modest effect on the overall MS predisposition, and are merely reflecting differences in genetic backgrounds.

Meanwhile, Fusco et al. found a lower frequency of *KIR2DS1* and *KIR2DL5* as well as a higher frequency of *KIR2DS4* in MS patients (Fusco et al., 2010), attributing the relationship with *KIR2DL5* to the linkage with *KIR2DS1*.

KIR2DL5 encodes for a recently described transmembrane receptor, gathering a combination of genetic, structural and functional features which are unique among KIRs. KIR2DL5 is characterized by a longer cytoplasmic domain providing this receptor with a higher inhibitory power (Yusa et al., 2004). Our data suggest that KIR2DL5 may be involved in the pathogenesis of MS, but its exact role in the inhibition of cellular activation in MS is yet to be determined.

The *KIR3DS1* gene, however, encodes for a short cytoplasmic tail receptor which promotes cell cytotoxicity. Interaction of KIR3DS1 with HLA-Bw4 on target cells is able to elicit the activation of NK cells and/or T cells, and, in turn, lead to autoimmune damage by direct lysis of normal cells or recruitment of other immune cells (Martin et al., 2002).

The fact that in our MS patient population, one activating and one inhibitory KIR gene are increased would seem contradictory. Nevertheless, it is necessary to bear in mind that NK cells have different roles within the immune system: they are able to cause direct lysis of the target cells and they also play an important role in immune regulation, as has been documented in MS (Takahashi et al., 2004). Thus, a KIR receptor could have different functions depending on the immune context. It is noteworthy that Jiao et al. (2008) also found that the frequencies of the *KIR3DS1* and *KIR2DL5* genes were increased in ankylosing spondylitis patients, indicating that these KIRs could exert an important role in autoimmune diseases.

Many studies indicate that KIR receptors could act in a synergic way, generating a cellular response which is the result of the predominance of activating or inhibitory receptors (Lanier, 2005). This fact may influence MS susceptibility through a gene dosage effect, as higher numbers of activating and/or inhibitory KIR genes might facilitate a higher number of effective KIR–HLA interactions, and

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Table 4
Interactions between specific KIR genes and HLA class I ligands in receptor–ligand pairs in controls and MS patients.

KIR/HLA	MS patients (%)	Controls (%)	p_{uc}	OR (95% CI)
2DL1+/C2+	66.5	55.5	0.015	1.797 (1.120–2.884)
2DL1+/C2−	20.0	30.0		
2DL1−/C2+	9.0	12.5	0.084	0.320 (0.085–1.303)
2DL1−/C2−	4.5	2.0		
2DL2+/C1+	45.5	42.0	0.616	1.178 (0.621–2.231)
2DL2+/C1−	11.5	12.5		
2DL2−/C1+	35.0	35.5	0.577	1.232 (0.591–2.572)
2DL2−/C1−	8.0	10.0		
2DL3+/C1+	68.5	65.0	0.399	1.258 (0.737–2.146)
2DL3+/C1−	15.5	18.5		
2DL3−/C1+	12.0	12.5	0.943	0.960 (0.310–2.968)
2DL3−/C1−	4.0	4.0		
3DL1+/Bw4+	50.5	63.5	**0.007**	**0.561 (0.368–0.856)**
3DL1+/Bw4−	42.5	30.0		
3DL1−/Bw4+	4.5	4.0	0.883	1.125 (0.236–5.371)
3DL1−/Bw4−	2.5	2.5		
3DL2+/A3,A11+	33.0	30.5	0.591	1.122 (0.736–1.710)
3DL2+/A3,A11−	67.0	69.5		
3DL2−/A3,A11+	0	0		
3DL2−/A3,A11−	0	0		
2DS1+/C2+	38.5	29.5	0.198	1.498 (0.808–2.778)
2DS1+/C2−	13.5	15.5		
2DS1−/C2+	37.0	38.5	0.252	1.442 (0.770–2.698)
2DS1−/C2−	11.0	16.5		
2DS2+/C1+	45.0	41.0	0.346	1.372 (0.709–2.654)
2DS2+/C1−	10.0	12.5		
2DS2−/C1+	35.5	36.5	0.948	1.024 (0.504–2.078)
2DS2−/C1−	9.5	10.0		
2DS3+/C1+	30.5	24.0	0.974	1.017 (0.373–2.774)
2DS3+/C1−	5.0	4.0		
2DS3−/C1+	50.0	53.5	0.536	1.192 (0.683–2.082)
2DS3−/C1−	14.5	18.5		
2DS4+/C2+	68.0	63.5	0.084	1.504 (0.946–2.392)
2DS4+/C2−	21.0	29.5		
2DS4−/C2+	7.5	4.5	0.809	1.190 (0.289–4.897)
2DS4−/C2−	3.5	2.5		
3DS1+/Bw4+	27.5	22.0	0.777	0.912 (0.483–1.721)
3DS1+/Bw4−	18.5	13.5		
3DS1−/Bw4+	27.5	45.5	0.002	0.433 (0.254–0.739)
3DS1−/Bw4−	26.5	19.0		
Bw4+/3DS1+	27.5	22.0	**0.006**	**2.068 (1.231–3.475)**
Bw4+/3DS1−	27.5	45.5		

OR: odds ratio with 95% confidence interval (CI).
p_{uc}: p values non corrected for multiple testing.

might diminish the necessary threshold for cell activation or inhibition. In our study, although a higher proportion of MS patients than controls carried at least three activating *KIR* genes, these differences did not reach statistical significance. This higher number of activating KIR genes could deregulate the balance between activating and inhibitory signals in MS patients, and maintain a prolonged activation state of the immune system. This would then become a risk factor for the development of the disease. Patients also present more activating *KIR* genes in other autoimmune diseases, such as ankylosing spondylitis (Jiao et al., 2008) or type I diabetes (van der Slik et al., 2003).

The clinical manifestations in our patients seemed to be associated with certain KIR genes. Patients carrying the *KIR2DS5* gene had a lower age at onset, indicating that even though this gene does not predispose to the disease, it may contribute to an earlier appearance of clinical symptoms. Moreover, patients carrying the *KIR2DL1* gene presented a higher progression index (current EDSS/disease duration), having a shorter disease duration to reach the same disability. These KIRs are related to a more aggressive form of the disease. These results concerning the influence of KIR genes in clinical MS manifestations need to be validated with further studies, as KIR genes related to disease progression in other diseases depend on epistatic interactions between KIR loci and HLA-class I loci (Martin et al., 2007). Gender differences in MS susceptibility could not be explained by a differential KIR gene distribution between males and females.

None of these associations was robust enough to withstand the correction for multiple testing, indicating that the size of our cohort was not large enough to obtain strong p values. These results therefore need to be confirmed in a larger cohort.

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Focusing on the KIR ligands, the higher frequency of the heterozygous genotype *HLA-C1/C2* in MS patients found in our study may have functional implications. The higher diversity of the HLA-C ligands in MS patients may guarantee the matches between KIRs and ligands and, therefore, the redundancy in functional interactions between KIR and HLA molecules. The absence of the HLA-Bw4 motif (Bw6/Bw6) was more frequent in our MS patients, in agreement with data reported by the two previous studies in MS (Lorentzen et al., 2009; Fusco et al., 2010). The HLA Bw6 group shows a slight linkage disequilibrium with the HLA class II MS predisposing alleles *DRB1*15* and *DRB1*03*. But after stratifying for *HLA-DRB1* alleles conferring an increase (*DRB1*1501* and **03*) or a decrease (*DRB1*01*) in MS risk, the lack of *HLA-Bw4* was still more frequent in MS patients, confirming that the HLA-Bw4 motif itself is a protective factor for MS (Lorentzen et al., 2009).

The interactions between *KIR* genes and cognate ligands may exert a strong influence on the overall responsiveness of NK, or T cells expressing these receptors, and may have the potential to influence both the innate and the adaptive immune responses (Purdy and Campbell, 2009). Since both KIR and HLA ligands are encoded by highly polymorphic genes, it is feasible that susceptibility to MS may be influenced by the coordinate inheritance of certain KIR/HLA gene combinations under epistatic influences.

In our study, inheritance of the *KIR2DL1–HLA-C2* pair was more frequent in MS patients than in controls, in disagreement with the findings reported by Lorentzen et al. (2009) who found that carriers of both *KIR2DL1* and *HLA-C2* had a lower risk of MS than those who only carried *HLA-C2*. It is difficult to predict how this interaction may impact on MS susceptibility but the interaction between the KIR2DL1 receptor and its HLA-C2 ligand has been reported to be more powerful than interactions between KIR2DL2 and KIR2DL3 with HLA-C1 (Fan et al., 2001). Furthermore, for homozygous *KIR2DL1* gene subjects, the inheritance of the *HLA-C2* ligand induces those NK cells expressing KIR2DL1 on their surface to double (Yawata et al., 2006). The higher matching frequencies for *KIR2DL1* and *HLA-C2* genes in our MS patients would lead to an increased expression of KIR2DL1 on the surface of certain T and NK cells and to the generation of more effective molecular interactions between both surface molecules, suppressing activation of NK and T cells. These results have to be interpreted with caution, because the outcome on cell activation would probably depend more on the final imbalance between activating and inhibitory signals than on a single specific interaction.

The KIR3DL1:HLA-Bw4 interaction leads to an inhibitory signal, limiting the activation of the NK or T cell. Specific *HLA-Bw4* and *KIR3DL1* matches were less frequent in MS patients than in controls, principally due to the lower frequency of the *Bw4* motif among MS patients, as the *KIR3DL1* frequency was similar between patients and controls (93% *vs.* 93.5%, respectively) and the logistic regression analysis provided support for a protective effect of the *HLA-Bw4:KIR3DL1* interaction in MS. The role of the KIR3DL1 receptor in MS is far from being understood, but it has recently been reported that this KIR receptor can negatively regulate the Treg function in a mouse model of type 1 diabetes, turning this receptor into an important target for the treatment of autoimmune diseases (Qin et al., 2011).

The carriage of the *KIR3DS1* gene conferred a higher risk of MS. In the absence of *KIR3DS1*, the presence of *HLA-Bw4* exerted a protective role in MS. However, the multivariate logistic regression analysis suggests that in the joint inheritance of *HLA-Bw4* and *KIR3DS1* genes, the effect of this KIR gene is strong enough to counteract the protective role of the HLA-Bw4 ligand and the conjunction of these KIR–HLA genes doubles the risk for MS, suggesting an epistatic interaction between them.

The novelty of our work is that it is the first to address the carriage of KIR genes as a biomarker to predict IFN-β responsiveness. We found no association between KIR genes or HLA genotypes and the therapeutic response, indicating that KIR genes are not good markers to predict the clinical response to IFN-β treatment. However, there was a trend for an association between *KIR2DS5* gene carrier frequency and poor response to IFN-β therapy. Recently, IFN-β treatment has been reported to reduce the KIR surface expression on CD8+ T cells in MS patients (Martinez-Rodriguez et al., 2010), so it would be worth analysing the KIR surface expression between responders and non-responders to IFN-β in order to assess its value as a predictive marker of clinical response to this immunomodulatory therapy.

In conclusion, we have studied here in a Spanish MS patient cohort the contribution of the KIR–HLA system in susceptibility to MS. In particular, we have corroborated that the *HLA-Bw4* motif is a protective factor for this disease. We also found a possible association of the *KIR3DS1* and *KIR2DL5* genes in susceptibility to MS and an association of the *KIR2DL1* and *KIR2DS5* genes with a higher MS severity. Discrepancies in the KIR genes associated with MS in the different reports mean further studies with larger sample sizes are necessary to clarify the role of KIR–HLA interactions in MS susceptibility.

Conflict of interest statement

The authors declare the absence of any conflict of interest.

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