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Associations of killer cell immunoglobulin-like receptor genes with rheumatoid arthritis

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Abstract. *Objective:* Rheumatoid Arthritis (RA) is an autoimmune and chronic inflammatory disease of unknown etiology. Killer cell immunoglobulin-like receptors are expressed on the surface of natural killer cells and CD28*null* T-cells, both present in synovial membrane of RA. Therefore we evaluated the associations of *KIR* genes with RA.
Methods: 16 *KIR* genes were genotyped in 100 healthy subjects (HS) and 100 RA patients from Western Mexico using PCR-SSP. Differences in *KIR* genotypes and gene frequencies were assessed using the X^2 test.
Results: Gene frequency of *KIR2DL3* was lower in RA than in HS ($p = 0.0019$), whereas *KIR2DL2* and *KIR2DS2* were higher in RA than HS ($p = 0.0004$ and $p = 0.0487$, respectively). In addition were identified 38 genotypes (from G1-G38) in both studied groups, and the genotype frequencies of G1, G6 and G14 showed significant differences ($p = 0.0001$, $p = 0.0208$ and $p = 0.0300$, respectively).
Conclusions: The presence of *KIR2DL2*, *KIR2DS2* and absence of *KIR2DL3* are associated with RA. Moreover, two genotypes BX are associated with RA. These results suggest that *KIRs* can be involved in RA susceptibility.

Keywords: *KIR*, killer cell immunoglobulin-like receptor, CD158, natural killer cell receptors, rheumatoid arthritis, autoimmune disease

1. Introduction

Rheumatoid Arthritis (RA) is a chronic inflammatory autoimmune disease, affecting about 1% of the world population [1]. RA is considered a clinical syndrome spanning several disease subsets, where the main clinical manifestation is the formation of proliferative pannus which triggers persistent synovitis, damage and the loss of function in the diarthrodial joints [1,2].

The etiologic factors to RA vary from one population to another, in which the initiator agents and the environment acting on genetically susceptible subjects [3,4]. The genetic contribution to RA susceptibility has been estimated to present about 60% [4], where certain alleles of *HLA-DRB1* gene have been considered the major genetic risk factor [5]. However, the etiologic fraction of *HLA-DRB1 locus* accounts for only 30% of genetic susceptibility. This indicates that different genes to those of *HLA-DR* are also involved in the susceptibility to disease [4,5].

The *killer cell immunoglobulin-like receptors* gene cluster has generated great interest due to its complex genetic variability which cause a high degree of KIR heterogeneity between individuals, besides that has been previously implicated in inflammatory and autoimmune conditions [6]. The *KIR* gene cluster spans approximately 150 kb in the leukocyte receptor complex located on chromosome 19q13.4 [7].

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The KIRs (CD158 family) are a group of genetically variable membrane proteins involved in immune function of the natural killer cells and CD28*null* T-cells, both present in synovial membrane of RA [8]. Reduced blood CD56*dim* NK cells activity has been shown to occur in patients with RA, on the other hand CD56*bright* NK cells have been shown to accumulate in the synovial fluid producing large quantities of IFN-γ contributing to the inflammatory environment characteristic of the disease [9].

CD28*null* T-cells are expanded in RA, these cells produce large amounts of IFN-γ, and express granzyme B and perforin, giving them the capability to lyses target cells and cause damage to tissue [8,10]. They tend to preferentially and even exclusively display KIR activating receptors as KIR2DS2 which could play role as co-stimulatory molecule. KIR2DS2 T-cell clones are frequently observed on RA patients with vascular complications [8,10].

The effector activity of these cells is determined by the activator/inhibitor balance of KIR receptors and their interaction with HLA class I. The major ligands for inhibitory KIRs are HLA-C molecules [7]. There are many allelic variants of *HLA-C* but in terms of KIR recognition these can be reduced in two groups: HLA-C1 molecules (characterized by asparagine at position 80) are recognized by inhibitory KIRs: KIR2DL2 and KIR2DL3, whereas HLA-C2 molecules (characterized by lysine at position 80) are recognized by KIR2DL1 [7, 11]. The KIR2DS2, KIR2DS3 and KIR2DS1 are activating KIRs and almost identical to the inhibitory KIRs counterpart in sequence but have a lower affinity for HLA-C, suggesting that these class I molecules may not be the major ligands of activating KIRs [7,12].

The *KIR* genotypes exhibit differences at two levels of variability: the structural level (*KIR* gene content, presence/absence) and the allelic level (between alleles of *KIR* genes contained) [6]. Nomenclature [13] for *KIR* genotypes include: AA genotype (predominantly inhibitor) in which must be absent genes: *KIR2DL2, KIR2DL5, KIR3DS1, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5;* and BX genotype (predominantly activator) when at least one of them is present. Thus the major functional distinction between the two groups of genotypes AA and BX is the number of activating *KIRs* genes [13].

Expression analysis of *KIR* genes reveals that different clones of NK cell and CD28*null* T-cells in a particular individual express different combinations of *KIRs* encoded within their genome [7,14]. The KIR molecules have been considered potentially important in susceptibility to infection and polygenic disorders such as autoimmunity [12].

Associations of *KIR* genes: *KIR2DL2, KIR2DS2, KIR3DS1* and *KIR2DS4* with RA have been demonstrated in Caucasian [10], Polish [15] and Taiwanese [16] populations. Here, we examined whether *KIR* genes could be associated with RA in the western Mexico population.

2. Subjects, materials and methods

2.1. Subjects

A total of 100 RA patients classified by a physician rheumatologist according to the American College of Rheumatology and European League Against Rheumatism (ACR-EULAR) criteria [17] were enrolled in the study. The RA patients were recruited from the Rheumatology Department of The Hospital General de Occidente, Jalisco, Mexico. As a controls group, we recruited 100 healthy subjects (HS). The RA patients and HS were born in western Mexico comprising the states of: Jalisco, Nayarit, Colima, Aguascalientes, Guanajuato, Michoacan and Zacatecas, as well as similar genetic background and a family history of Mexican ancestors, at least back to the third generation. Informed consent was obtained from both study groups. The committee of biosecurity and ethics of Centro Universitario de Ciencias de la Salud of Universidad de Guadalajara approved the present study in compliance with The Code of Ethics of the World Medical Association (Declaration of Helsinki, Korea 2008).

2.2. DNA extraction

Genomic DNA was isolated from peripheral blood leucocytes using procedures described previously [18]. Briefly, it comprised precipitation with NaCl saturated salts from leukocytes contained in 5 mL of venous blood with ethylene diamine tetra-acetic acid as an anticoagulant. DNA was then precipitated in absolute ethanol, dissolved in distilled and sterile water and store at -20°C until analysis.

2.3. KIR genotyping by PCR-SSP

PCR-SSP (Polymerase Chain Reaction with specific-sequence primers) was performed in a total reac-

Table 1
Demographic and clinical characteristics of RA patients and HS

Characteristics	RA $n = 100$	HS $n = 100$
Demographic		
Age, years (range)	48 ± 14 (18–83)	43 ± 11 (18–74)
Sex (F/M)	89/11	89/11
Clinical		
Anti-CCP positive (> 5 U/mL)	87%	0%
Family history of autoimmunity	57%	18%
Disease duration, years (range)	9.0 ± 8.9 (0.3–40)	NA

Values represent the mean, minimum and maximum range; some of them represent percentage; RA rheumatoid arthritis; Family history of autoimmunity: Family related autoimmune disease of first and second degree of consanguinity; Anti-CCP: Anti-Cyclic Citrullinated Peptide antibodies; NA: Not Applicable.

tion volume of 20 μL, consisting of 4 μL of DNA, 10 μL of BioMix Red 10X (Bioline®) and volume rest of primers solution. For the amplification were used specific-sequence primers [19], genotyping allow to identify presence or absence (by direct count) of 16 *KIR* genes in each subject and the identification of the allele *KIR2DS4*001*. All amplified products were resolved on a 3.0% agarose gel in buffer TBE 1X Ultrapure (Invitrogen®), stained with solution Syber Safe (Invitrogen®), 30 minutes in dark conditions and photographed with Molecular Imaging Software V5.X (Kodak®).

2.4. Statistical analysis

Associations with specific *KIR* genes, *KIR* genotypes and gene combinations were tested using the *chi* squared (X^2) test considered Yates correction when necessary and were statistically significant when $p < 0.05$. For this purpose we used the Epidat V3.1 statistical software.

3. Results

3.1. Subjects characteristics

The main demographic and clinical characteristics of RA patients were: 89 female, 11 male; mean age 48 ± 14, range: 18–83 years; 87% with Cyclic Citrullinated Peptide antibodies (anti-CCP) positive and average disease duration of 9.0 years, range 0.3–40. All patients were classified according to ACR-EULAR 2010 criteria, and were treated with disease modifying antirheumatic drugs (DMARDs) and/or non-steroidal anti-inflammatory drugs (NSAIDs). The main characteristics of HS were: 89 female, 11 male; mean age 43 ± 11 years, range 18–74; and 0% with CCP antibodies positive. Full details of both groups of study are shown in Table 1.

3.2. Gene frequencies

The genes frequencies of *KIR2DL3, KIR2DL2* and *KIR2DS2* show significantly differences. The *KIR2DL3* gene was distributed less frequently in RA ($p = 0.0019$, $OR = 0.213$); whereas the gene frequencies of *KIR2DL2* and *KIR2DS2* were significantly higher in RA patients ($p = 0.0004$, $OR = 2.819$ and $p = 0.0487$, $OR = 1.750$, respectively).

The frame genes: *KIR2DL4*, *KIR3DL2, KIR3DL3* and *KIR3DP1* were virtually present in 100% of RA patients and HS. *KIR2DP1* pseudogene was present in 97% of RA patients and 98% of HS. The rest of gene frequencies: *KIR3DL1, KIR2DL1, KIR2DS4, KIR2DL5, KIR3DS1, KIR2DS1* and *KIR2DS3* were different in both groups without show significant differences. Full details of all gene frequencies are shown in Table 2.

3.3. AA and BX genotypes groups frequencies

KIR genotypes were classified as AA (predominantly inhibitor) and BX (predominantly activator). The frequency of AA group was decreased in RA compared with HS ($p = 0.0171$, $OR = 0.461$), whereas BX group was significantly increased in RA patients ($p = 0.0171$, $OR = 2.168$) (see Table 3).

3.4. Genotype frequencies

We analyzed the genes present in our study population and we found 38 genotypes (named as G1 to G38) identified and classified according with the Allele Frequency Database Net (ID number) [13]. Genotype frequencies of G6 and G14 in RA patients were significantly increased ($p = 0.0208$, $OR = 2.796$ and $p = 0.0300$, $OR = 6.887$, respectively). In contrast the genotype G1 frequency was increased significantly

Table 2
Frequency of 16 *KIR* genes in RA patients and HS

KIR gene	RA ($n = 100$) %	HS ($n = 100$) %	*p*	*OR*	95% CI
3DL1	98	93	NS		
2DL1	96	99	NS		
2DL3	79	**95**	**0.0019**	0.213	0.079–0.569
2DS4	96	91	NS		
2DL2	**70**	45	**0.0004**	2.819	1.582–5.026
2DL5	47	55	NS		
3DS1	37	44	NS		
2DS1	41	44	NS		
2DS2	**55**	41	**0.0487**	1.750	1.001–3.054
2DS3	19	21	NS		
2DS5	36	42	NS		
2DL4	100	100	NS		
3DL2	100	99	NS		
3DL3	100	100	NS		
2DP1	97	98	NS		
3DP1	100	100	NS		

Significant differences between RA patients and HS are in bold; CI: Confidence Interval; NS: Not Significant.

Table 3
Frequency of *KIR* genotypes groups AA and BX in RA patients and HS

KIR Genotypes	RA ($n = 100$) %	HS ($n = 100$) %	*p*	*OR*	95% CI
Group AA	19	**34**	**0.0171**	0.461	0.242–0.877
Group BX	**81**	66	**0.0171**	2.168	1.140–4.124

Significant differences between RA patients and controls are in bold; CI: Confidence Interval.

in HS ($p = 0.0001$, $OR = 0.248$). It also identified four new genotypes: G35, G36, G37 and G38, not yet registered by allele Frequency Database Net [13], two in HS and two in RA patients. The representation of the 38 genotypes and their frequencies are shown in Table 4.

4. Discussion

This study allowed us to identify genes and genotypes *KIR* associated with RA, which suggest them as genetic factors of protection or susceptibility to disease. Moreover, our results suggest that *KIR2DL2*, *KIR2DL3* and *KIR2DS2* genes have immunological implications, they share the same set of ligands HLA-C1 and the KIR expressing cells: CD28*null* T-cells and NK cells are present in patients with RA [8,9,14]. Below we discuss the possible immunological involvement of these genetic factors associated with RA.

Gene *KIR2DL3* has frequency significantly decreased in RA, this suggests that the absence of this gene may be associated with susceptibility to develop RA. This gene encodes a receptor inhibitor, KIR2DL3, of intracellular signaling which contributes the blockade of cytotoxic function [7,20] and secretion of cytokines such as IFN-γ important in RA autoreactivity [20,21]. In this context, *KIR2DL3* gene could play a protector role against disease.

The *KIR2DS2* gene also showed association with RA, suggesting it as a susceptibility factor to disease. Codes for the receptor KIR2DS2 associated noncovalently with the adapter molecule DAP-12 (which contains domains ITAM) [12], which allows it to activate intracellular signaling in NK cell. Moreover, can act as a co-stimulatory molecule in CD28*null* T-cells increasing activation by T cell receptor (TCR) [14]. These two functions of the receptor trigger increased IFN-γ secretion and release of granzyme and perforin, contributing to the destruction joints in RA patients [10]. This possibly represents the immune impact of *KIR2DS2* in the disease.

The *KIR2DL2* gene frequency was higher in RA patients, suggesting it may have a role as a susceptibility factor to disease. *KIR2DL2* encodes a receptor inhibitor with stronger affinity for ligand HLA-C1 that KIR2DL3 [22]. Its role as inhibitor of intracellular signaling could be blocked in RA patients by two hypothetical mechanisms: the first reported in the study by Fadda et al. [22] where differences in the sequence of peptides contents in HLA class I molecules can regulate the activity of NK cell antagonizing inhibition

Table 4
The 38 *KIR* genotypes identified and their frequency in RA patients and HS

Genotype			*KIR* genes																	RA	HS			
Group	G	ID*	*3DL1*	*2DL1*	*2DL3*	*2DS4*	*2DL2*	*2DL5*	*3DS1*	*2DS1*	*2DS2*	*2DS3*	*2DS5*	*2DL4*	*3DL2*	*3DL3*	*2DP1*	*3DP1*	n=100 %	n=100 %	*p*	*OR*	95% CI
AA	G1	1	■	■	■	■								■	■	■	■	■	11	**34**	**0.0001**	0.248	0.118 - 0.519
AA	G2	180	■	■		■								■	■	■	■	■	7	0	NS		
AA	G3	195	■	■	■									■	■	■	■	■	1	0	NS		
BX	G4	2	■	■	■	■		■	■	■			■	■	■	■	■	■	8	9	NS		
BX	G5	3	■	■	■	■		■	■	■				■	■	■	■	■	9	9	NS		
BX	G6	4	■	■	■	■	■				■			■	■	■	■	■	**18**	7	**0.0208**	2.796	1.138 - 6.866
BX	G7	5	■	■	■	■	■	■			■	■		■	■	■	■	■	6	7	NS		
BX	G8	6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	3	6	NS		
BX	G9	7	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	2	0	NS		
BX	G10	8	■	■	■	■		■	■	■		■	■	■	■	■	■	■	0	2	NS		
BX	G11	9	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	2	1	NS		
BX	G12	12	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	0	1	NS		
BX	G13	18	■	■	■	■	■	■	■	■			■	■	■	■	■	■	5	2	NS		
BX	G14	19	■	■	■	■	■							■	■	■	■	■	**9**	1	**0.0300**	6.887	1.203 - 39.45
BX	G15	27	■	■	■	■		■	■		■	■	■	■	■	■	■	■	0	1	NS		
BX	G16	33	■	■	■	■		■	■	■				■	■	■	■	■	0	1	NS		
BX	G17	44	■	■	■	■	■				■	■	■	■	■	■	■	■	0	1	NS		
BX	G18	46	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	0	1	NS		
BX	G19	68		■	■		■	■	■	■	■	■	■	■	■	■	■	■	0	1	NS		
BX	G20	69		■	■			■	■	■			■	■	■	■	■	■	1	5	NS		
BX	G21	70		■	■		■	■	■	■	■	■	■	■	■	■	■	■	0	1	NS		
BX	G22	71	■	■		■	■	■			■	■		■	■	■	■	■	2	1	NS		
BX	G23	72	■			■	■				■	■		■	■	■		■	3	1	NS		
BX	G24	73	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	2	2	NS		
BX	G25	80	■	■	■		■	■	■	■	■		■	■	■	■	■	■	0	1	NS		
BX	G26	90	■	■		■	■	■	■	■	■	■		■	■	■	■	■	2	1	NS		
BX	G27	118	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	1	0	NS		
BX	G28	192	■	■	■	■					■	■		■	■	■	■	■	1	0	NS		
BX	G29	200	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	0	1	NS		
BX	G30	243		■			■	■	■	■	■	■	■	■	■	■	■	■	1	0	NS		
BX	G31	269	■	■	■	■					■	■	■	■	■	■	■	■	1	0	NS		
BX	G32	344	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	1	0	NS		
BX	G33	394	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	2	0	NS		
BX	G34	413	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		0	1	NS		
BX	G35	NAS1	■	■	■		■	■	■		■	■	■	■	■	■	■	■	1	0	NS		
BX	G36	NAS2	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	1	0	NS		
BX	G37	NAS3	■	■	■		■	■	■	■	■		■	■	■	■	■	■	0	1	NS		
BX	G38	NAS4	■	■	■	■		■	■	■	■	■	■	■		■	■	■	0	1	NS		

Significant differences between RA patients and controls are in bold; Black box = *locus* detected; CI: Confidence Interval; NS: Not significant; G:Genotypes identified in this study; ID: ID assigned by the Allele Frequency Net Database; NAS: Not yet Assigned ID.

mediated by KIR2DL2 favoring activation by its counterpart KIR2DS2; and the second reported by Matsui et al. [23] where antibodies to KIR2DL2 are found in 30% of RA patients and probably are involved in the breakdown of self tolerance.

The evidence found in our study of the genes *KIR2DS2* and *KIR2DL2* as possible genetic susceptibility factors: is also seen in the western European and Polish populations studied by Yen et al. for *KIR2DS2* [10]; and in the study of Majorczyk et al. [15], where both genes *KIR2DS2* and *KIR2DL2* were found associated with RA in patients with extra-joins manifestations. Conversely in Caucasian population from northern Ireland, Middleton et al. not found association of these genes *KIR* with RA [24].

On the other hand, when we analyzed by group genotypes AA or BX, the frequency of the BX group was increased in patients with RA compared with HS. These BX genotypes are those containing activators genes (as *KIR2DS2*) which could contribute significantly to the intracellular signaling (dependent ligands HLA class I) [7,25] and activate cytotoxic function and secretion of cytokines [12,20] and thus favors to the pathology.

However, when we analyzed the individual genotypes, the G6 genotype (BX) showed association with RA, suggesting it as a susceptibility factor. This genotype contains *KIR2DL2/2DS2* genes, which were reported by McGeough et al. [26], more frequently in patients who respond to therapy with anti-TNF-α. In our study did not consider the treatment, making it possible to focus future studies into the response to treatment in patients with this genotype.

The frequency of the genotype G14 in RA patients was augmented. This suggests the genotype G14 as a susceptibility factor to disease. It is interesting to highlight that in RA patients with genotype G14, the only activator gene is *KIR2DS4*, it could be play an important role in the immunopathogenesis of RA. Yen et al. [16] found association of *KIR2DS4* gene with RA patients in Taiwanese population; however we not found association of frequency of *KIR2DS4* with RA. On other hand, has been reported that function inhibitory of KIR2L2 is blocked by autoantibodies [22,23] in RA patients. This situation could be present in patients with genotype G14, favoring the activation by *KIR2DS4* gene.

The frequency of genotype G1 was increased significantly in HS and this genotype present *KIR2DL3* gene. This gene show association as protection factor to RA, this fact appears to be only when *KIR2DS2* and *KIR2DL2* are absent (genes associated with RA), like in this genotype G1, but when *KIR2DL3* is present in the same genotype with *KIR2DL2* (G14) or both *KIR2DL2* and *KIR2DS2* (G6) genes, seem to have no effect.

We analyzed the genotypes considering the absence or presence of genes that had shown significant differences: *KIR2DL3/2DL2/2DS2*. Interestingly there are two genotypes (G2 and G33) with none of these genes, corresponding to 9 individuals in the RA group while in HS we found none ($p = 0.0095$, $OR = 20.87$, 95% CI = 1.198–363.6). It is noteworthy that only 27 individuals have been reported for 8 genotypes, with this feature, in different populations around the world [13], but none of these individuals are patients with RA. So it would be important to investigate whether this feature is unique to our population and can be used as a genetic marker for susceptibility to this disease.

We can conclude that this study of *KIR* genes, allowed suggest genetics factors for susceptibility and protection against RA in population of western of Mexico. This work provides a rationale to realize genetics and functional studies, in which it would be interesting to do an analysis on subgroups of patients with RA since particular clinical manifestation of RA may have different genetic background with respect to *KIR* genotype.

Conflicts of interest

The authors have declared no conflicts of interest.

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