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Analysis of Killer Cell Immunoglobulin-like Receptor Genes and Their HLA Ligands in Iranian Patients with Ankylosing Spondylitis

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ABSTRACT

Ankylosing Spondylitis (AS) is a chronic rheumatic disease which mainly involves the axial skeleton. It seems that non-HLA genes, as well as HLA-B27 gene, are linked to the etiology of the disease. Recently, it has been documented that KIRs and their HLA ligands are contributed to the Ankylosing Spondylitis. The aim of this study was to evaluate the KIR genes and their HLA ligands in Iranian AS patients and healthy individuals.

The present study includes 200 AS patient samples and 200 healthy control samples. KIR genotyping was performed using the polymerase chain reaction sequence-specific primer (PCR-SSP) method to type the presence or absence of the 16 KIR genes, 6 known specific HLA class I ligands and also, two pseudogenes.

Two KIR genes (KIR-2DL3 and KIR2DL5), and among the HLA ligands, two HLA ligands (HLA-C2^{Lys80} and HLA-B27) genes were significantly different between case and control groups. In addition, we found some interesting KIR/HLA compound genotypes, which were associated with AS susceptibility.

Our results suggest that the AS patients present more activating and less inhibitory KIR genes with combination of their HLA ligands than healthy controls. Once the balance of signal transduction between activating and inhibitory receptors is disturbed, the ability of NK cells to identify and lyse the targets in immune responses will be compromised. Accordingly, imbalance of activating and inhibitory KIR genes by up-regulating the activation and losing the inhibition of KIRs signaling or combination of both might be one of the important factors which underlying the pathogenesis of AS.

Keywords: Ankylosing spondylitis; HLA antigen; KIR receptor; Polymerase chain reaction

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INTRODUCTION

Ankylosing Spondylitis (AS) is a chronic and inflammatory rheumatologic disease which mainly

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affects the sacroiliac joints and spine, causing deformity of bones, which lead to disability of patients. Also, might involve the peripheral joints such as eyes, the skin, and the cardiovascular system. All these problems decrease the life's quality of the AS patients.[1,2] It usually affects individuals from the early age and most of the patients develop the first symptoms before their third decade of life and, a few of them present the symptoms in the fourth decade of life (the peak age of onset is at 15-35 years). Prevalence rate is 0.1-2% in different populations. Men are more often affected than women, with an approximate ratio 5:1.[3,4] It has been shown that the genetic and environmental factors play a major role in autoimmune diseases. AS is a rheumatologic disease which genetic contribution have a big part in it.[5-8] It is considered that AS is strongly associated with the human leukocyte antigen (*HLA*) *-B27* gene. But *HLA-B27* only makes up 16% of the total genetic risk of this disease. Hereby, this indicates that the genetic association may extend further the MHC class1 region.[9] Genome-wide linkage scans have implicated numerous non-MHC genomic regions, such as, 1p, 2p, 2q, 3p, 9q, 10q, 11p, 19q, and 16q.[10] Another studies showed that chromosomes 2q, 6p, 6q, 10q, 11q, 16q, 17q and 19q have a strong linkage with AS. Therefore, it seems that non-*HLA-B27* genes, both inside and outside the HLA, are related to the etiology of the disease.[11] Many genes have been shown to be involved in AS including; ERAP1 (Endoplasmic Reticulum Aminopeptidase 1), IL23R (Interleukin 23 Receptor), IL1(Interleukin1), CYP2D6 (Cytochrome P450 2D6), TLR4 (Toll-Like Receptor 4), ANKH (Human orthologue of the mouse progressive ankylosis gene) and KIRs.[12] In our past studies, we showed that the ERAP1 and IL23R are associated with the susceptibility to AS in Iranian population.[6,13] Killer cell immunoglobulin-like receptors (KIRs) regulate the activation and inhibition of NK cells by recognition of HLA class I molecules on target cells.[12] The main functions of NK cells are cytotoxicity and cytokine release. These functions are regulated by signals from a large group of activating, and inhibitory receptors. The number and type of inherited KIR genes in each individual are different.[14] Similar to MHC loci, KIR sequences are also extremely polymorphic. Killer cell immunoglobulin-like receptors (KIRs) bind to HLA class I molecules with different affinities and adjust the activation and inhibition of natural killer (NK) cells and $CD8^+$ T cells.[15] HLA-A binds to KIR3DL2, HLA-B to KIR3DL1, HLA-C to KIR2DL1/2/3, and HLA-G is recognized by KIR2DL4. The HLA-B allotypes have either the Bw4 or Bw6 epitope, but only the Bw4 binds to KIR3DL1. Also, KIR3DS1 and HLA-Bw4 seem to have a functional interaction. In addition, the HLA-C allotypes have either the C1 or C2 epitope, characterized by Ser77Asn80 and Asn77Lys80 respectively, which are shared by HLA-Cw alleles. The C1 and C2 epitopes are binding to KIR2DL2/3 and KIR2DL1 respectively.[14,16,17]

Generally, interaction between KIRs and class I HLA ligands, provide either activating or inhibitory signals to control the activity of NK cells, which are linked to the pathogenesis of various diseases. Recently, it has been shown that AS is contributed to KIR and HLA ligands in different ethnic groups.[18-21] We evaluated whether KIR genes and their HLA ligands are associated with AS in Iranian patients.

MATERIALS AND METHODS

Population Study

Patients were enrolled from the outpatient rheumatology clinic of Rheumatology Research Center, Shariati Hospital and Iran Ankylosing Spondylitis Society, Tehran, Iran. The population study includes 200 AS patients (156 men and 44 women) and 200 age- and sex-matched healthy control subject samples (152 men and 48 women). The mean ± SD age of patients and controls were 38.20±12 and 37.3±13 respectively. All participants in the study were from Iranian population and informed consent was obtained from all AS patients and healthy controls. All patients were seen by a qualified rheumatologist, and the diagnosis of AS had been confirmed. Disease severity and functional disabilities were deliberated through Bath Ankylosing Spondylitis Disease Activity Index (BASDAI),[22] Bath Ankylosing Spondylitis Functional Index (BASFI),[23] Bath Ankylosing Spondylitis Metrology Index (BASMI),[24] ASQOL (Ankylosing Spondylitis Quality Of Life) and NRSpain (Numeric Rating Scale of pain). Additionally, HLA-B27 circumstance for each AS patient and healthy control were evaluated. This study was approved by the Ethics Committee of Tehran University of Medical Sciences, Iran.

DNA Isolation

Five ml blood samples were taken from all the subjects. Genomic DNA was extracted from blood samples containing ethylenediamine tetra-acetic acid (EDTA) using standard phenol-chloroform method.[25] The purity and quantity of DNA samples were determined by NanoDrop (Thermo Scientific/2000C). All DNA samples were stored at -20ºc until genotyping.

KIR Genotyping

Here we present a polymerase chain reaction-sequence specific primer (PCR-SSP) method for KIR genotyping in all of the subjects. The PCR-SSP method was applied for KIR genotyping to type the presence or absence of the KIR and HLA loci, including; eighth inhibitory KIR genes (2DLI, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1,3DL2, and 3DL3), six activating KIR genes (2DS1, 2DS2, 2DS3, 2DS4, 2DS5, and 3DS1), two pseudogenes (2DP1, 3DP1) and six known specific HLA class I ligands (C1, C2, Bw4; $Bw4^{Ile80}$, $Bw4^{Thr80}$, A1 and B27). KIR genotyping was performed in a reaction volume of 10 µl and a pair of internal control primers, specific for non-polymorphic sequences of the growth hormone gene (GH), was included in each PCR reaction. The sequence of PCR primers which used for amplifying HLA and KIR genes is provided in Table 1.

Table 1. Oligonucleotide primer sets for KIR and HLA genotyping by PCR-SSP assay

No	Gene	Amplified alleles	Forward primer (5´–3´)	Reverse primer (5´–3´)	Size (bp)	Ref.
1	*KIR2DL1*	001–010	TTGGTCAGATGTCATGTTTGAA	TCCCTGCCAGGTCTTGCG	143	(42)
2	*KIR2DL2*	001–005	AAACCTTCTCTCTCAGCCCA	GCCCTGCAGAGAACCTACA	142	(42)
3	*KIR2DL3*	001–007	ACAAGACCCTCAGGAGGTGA	GCAGGAGACAACTTTGGATCA	160	(42)
4	*KIR2DL4*	001–012	TCAGGACAAGCCCTTCTGC	GACAGGGACCCCATCTTTC	130	(42)
5	*KIR2DL5A*	001,005	GCGTACGTCACCCTCCCG	ACTTCTAGGCCCATCACTCC	314	(43)
6	*KIR2DL5B*	002,004,006–009	CGTCACCCTCCCATGATGTA	ACTTCTAGGCCCATCACTCC	308	(43)
7	*KIR2DS1*	001–004,008	GTAGGCTCCCTGCAGGGA	ACAAGCAGTGGGTCACTTGAC	148	(44)
8	*KIR2DS2*	001–005	CTGCACAGAGAGGGGAAGTA	CAGAGGGTCACTGGGAGC	177	(43)
9	*KIR2DS3*	001–003	ACCTTGTCCTGCAGCTCCT	AGCATCTGTAGGTTCCTCCT	160	(42)
10	*KIR2DS4* (full)	001	CAGCTCCCGGAGCTCCTA	TGACGGAAACAAGCAGTGGA	224	(43)
11	*KIR2DS4* (var)	003,004,006,007,009	CTTGTCCTGCAGCTCCATC	TGACGGAAACAAGCAGTGGA	202	(43)
12	*KIR2DS5*	001,002,004–008	TGATGGGGTCTCCAAGGG	TCCAGAGGGTCACTGGGC	125	(44)
13	*KIR3DL1*	001–009,015–044,056,057	TGAGCACTTCTTTCTGCACAA	TAGGTCCCTGCAAGGGCAA	129	(43)
14	*KIR3DL2*	001–012,015–021	AAACCCTTCCTGTCTGCCC	TGGAAGATGGGAACGTGGC	134	(43)
15	*KIR3DL3*	001–0031	GCAATGTTGGTCAGATGTCAG	AGCCGACAACTCATAGGGTA	199	(42)
16	*KIR3DS1*	010–014,045–049,055	TCCATCGGTTCCATGATGCG	GACCACGATGTCCAGGGGA	111	(42)
17	*KIR2DP1*	001–003	ACATGTGATTCTTCGGTGTCAT	GTGAACCCCGACATCTGTAC	167	(43)
18	*KIR3DP1 (full)*	001,002,004	GGTGTGGTAGGAGCCTTAG	GAAAACGGTGTTTCGGAATAC	280	(42)
19	*KIR3DP1 (var)*	003,005,006	CGTCACCCTCCCATGATGTA	GAAAACGGTGTTTCGGAATAC	395	(42)
20	*HLA-C1* ^{Asn80}	Cw*010201–0113,0115 0119,0212,030201–0306,0308–0314,0316 0344,0346,0347,0411,0429,0611,070 101–0706,0708,0710–0748,0750-0753,080101–0809,0811–0815,120201–120305,1206–1208,1210–1220,140201–1403,1405–1409,1507,1521,160101–160103,160401,1606–1608,1610	GAGGTGCCCGCCCGGCGA	CGCGCAGGTTCCGCAGGC	332	(43)

Table 1. Continue

21	*HLA-C2* ^{Lys80}	Cw*0114,020201–0211,0213–0223,0307,0315,0345,04010101–040104,0403–0410,0412–0428,0430,0431,050101–0517,06020101–0610,0612-0616N,0707,0709,0749,0810,120401–1205,1209,1221,1404,150201–1506,1508–1520,1602,1609,1701–1704,1801–1803	GAGGTGCCCGCCCGGCGA	CGCGCAGTTTCCGCAGGT	332	(43)
22	*HLA-B-Bw4* ^{Thr80}	B*0802,1301–1304,1306–1308Q, 1310-1321,1536,1589,9515,1809,2701,3710,380201–3804,3808,3815,4047,44020101–4405,4407,4408,4410,4412–4417,4419N–4424,4426–4445,4447–4449,4451–4459,4704,4902,5309,5311–5313,,5607	GGAGCGAGGGGACCGCAG	GTAGTAGCGGAGCGCGGTG	344	(43)
23	*HLA-B-Bw4* ^{Ile80}	B*0736,0738,0803,1513,1516–151702,1523,1524,1567,1587,1595,2702,2730,380101–380102,38053807,3809–3814,3816,4013,4019,4406,4418,4425,4450,4818,4901,4903–4905,510101–5124,5126–5146,5148–5152,520101–5211,530101–5302,5304–5308,5310,5314,5412,5621,570101–5711,5713–5716,5801–5802,5804–5816,5901,5902	GAGCGAGGGGACCGCAG	GTAGTAGCGGAGCGCGATC	343	(43)
24	*HLA-A-Bw4*	A*0281,0287,2301–2317,2319Q,24020101–240302,2405–2411N,2413–2415,2417,2418,2420–2425,2427,2429–2443,2445N–2484N,250101–2506,2913,3107,3108,3110,3201–3215,6836,9212,9224,9229,9236	TGGCGCCCCGAACCCTCG	GCTCTGGTTGTAGTAGCGGA	456	(43)
			AACCCTCCTCCTGCTACTCTT	GCTCTGGTTGTAGTAGCGGA	446	(43)

Each reaction mixture contained 100 ng of genomic DNA, 0.1–1.5μM of each primer, 1μL of 10x PCR buffer, 1.5mM MgCl2 and 0.5U of Taq DNA polymerase. The PCR conditions were as follows: initial denaturation at 94°C for 2 min, 10 cycles of 94°C for 10 s, and 65°C for 60 s, followed by 20 cycles of 94°C for 10 s, 61°C for 50 s, and 72°C for 30 s. Amplification was carried out using the thermal cycler ABI 2720 (Applied Biosystems, Foster City, CA, USA). The amplification products were electrophoresed and separated using 2% agarose gel containing DNA safe Stain. Scanning of the gel by

ultraviolet (UV) light revealed the presence or absence of each KIR and HLA gene.

Statistical Analysis

Descriptive analysis of demographic and clinical characteristics of studied population was performed using IBM™ SPSS version 19. Genotype distributions were calculated based on geometrical series. Analyses on genotypes and alleles were conducted through χ^2 test or two-tailed Fisher's exact test, and the magnitude of risk associations are reported by odds ratios (OR) and confidence intervals (95% CI). Benjamini-Hochberg was applied to control the false discovery rate (FDR).[26]

RESULTS

Main Effects of KIR and HLA Genes on AS Susceptibility

We evaluated the presence/absence of 22 investigated KIR and HLA loci in AS patients and healthy controls. Association of KIR and HLA genes with the risk of AS are shown in Table 2. Two KIR genes including; KIR-2DL3 and 2DL5 were significantly different in cases and controls. According to our data, KIR-2DL3 showed a significant risk effect (*p*=0.005, OR=3.1, 95% CI=1.6-5.9) whereas, KIR-2DL5 showed a protective effect in AS patients (*p*=0.03, OR=0.4, 95% CI=0.2-0.8).

Table 2. Association of KIR and HLA genes with the risk of AS

Genes	Case (n=200)		Control (n=200)		*p* value	Adjusted -*p**	OR(95% CI)
	%	n	%	n			
Inhibitory KIR							
2DL1	97	194	99	198	0.15	0.32	0.3 (0.06-1.6)
2DL2	56.5	113	64.5	129	0.1	0.30	0.7 (0.5-1.06)
2DL3	**93**	**186**	**81**	**162**	**0.0003**	**0.005**	**3.1 (1.6-5.9)**
2DL4	100	200	100	200	-	-	-
2DL5	**82**	**164**	**91.5**	**183**	**0.005**	**0.03**	**0.4 (0.2-0.8)**
3DL1	95.5	191	92	184	0.14	0.32	1.8 (0.8-4.2)
3DL2	100	200	100	200	-	-	-
3DL3	100	200	100	200	-	-	-
Activating KIR							
2DS1	55.5	111	49	98	0.2	0.38	1.3(0.9-1.9)
2DS2	55	110	66	132	0.02	0.11	0.6 (0.4-0.9)
2DS3	35	70	42.5	85	0.12	0.32	0.7 (0.5-1.09)
2DS4	91	182	92.5	185	0.6	0.78	0.8 (0.4-1.6)
2DS5	43	86	34.5	69	0.08	0.28	1.4 (0.9-2.1)
3DS1	43.5	87	48	96	0.36	0.50	0.8 (0.56-1.23)
Pseudo KIR							
2DP1	100	200	100	200	-		
3DP1	100	200	100	200	-		
KIR HLA-Ligand							
HLA-C1Asn80	60.5	121	71.4	142.8	0.34	0.50	0.8 (0.5-1.2)
HLA-C2Lys80	**88**	**176**	**74.5**	**149**	**0.0005**	**0.005**	**2.5 (1.5-4.2)**
HLA-B-Bw4 Thr80	9	18	16.1	32.2	0.03	0.12	0.5 (0.3-0.9)
HLA-B-Bw4Ile80	60	120	55.2	110.4	0.3	0.48	1.2 (0.8-1.8)
HLA-Bw4-A1	40.5	81	45.3	90.6	0.3	0.48	0.8 (0.5-1.2)
HLA-B27	**74**	**148**	**2.1**	**4.2**	**<0.001**	**<0.001**	**139 (49-394)**

**p*-value≤0.05 was statistically significant (Benjamini Hochberg method).

Table 3. KIR genotypes in normal individuals and AS patients, eighteen distinct KIR genotypes were observed that differ from one other by the presence (shaded box) or absence (white box) of 16 KIR genes. The frequency of individuals who have the desired genotype was shown by number and percent (N %)

Genotype ID	KIR Genotype: Inhibitory KIR								Activating KIR						Pseudo gene		% Patients	% Control	*p*- value*	OR (CI)
	KIR2DL1	KIR2DL2	KIR2DL3	KIR2DL4	KIR2DL5	KIR3DL1	KIR3DL2	KIR3DL3	KIR2DS1	KIR2DS2	KIR2DS3	KIR2DS4	KIR2DS5	KIR3DS1	KIR2DP1	KIR3DP1				
KIR-1																	37(18.5)	37(18.5)	1	1 (0.6-1.6)
KIR-2																	21 (10.5)	1 (0.5)	**0.0001**	**23 (3.1-175)**
KIR-3																	7 (3.5)	17 (8.5)	0.03	0.4 (0.15-0.96)
KIR-4																	1 (0.5)	5 (2.5)	0.09	0.19 (0.02-1.7)
KIR-5																	4 (4.0)	1 (0.5)	0.17	4 (0.436.6)
KIR-6																	1 (0.5)	7 (3.5)	0.03	0.13 (0.01-1.1)
KIR-7																	7 (3.5)	16 (8.0)	0.053	0.4 (0.16-1.03)
KIR-8																	11 (6.5)	2 (1.0)	**0.01**	**5.7 (1.3-26)**
KIR-9																	1 (0.5)	4 (2.0)	0.17	0.2 (0.02-2.2)
KIR-10																	4 (2.0)	1 (0.5)	0.17	4 (0.4-36)
KIR-11																	0	6 (3.0)	**0.01**	**-**
KIR-12																	3 (1.5)	10 (5.0)	0.04	0.3 (0.07-1.07)
KIR-13																	9 (4.5)	6 (3.0)	0.4	1.5 (0.5-4.3)
KIR-14																	6 (3.0)	0	**0.01**	-
KIR-15																	10 (5.0)	18 (9.0)	0.1	0.5 (0.2-1.2)
KIR-16																	7 (3.5)	0	**0.007**	-
KIR-17																	1 (0.5)	4 (0.5)	0.17	0.2 (0.02-2.2)
KIR-18																	16 (4.0)	16 (4.0)	1	1 (0.5-2.05)

*p-value≤0.01 was statistically significant. The 0.01 is one type error rate for any contrast.

The HLA-C2[Lys80] and HLA-B27 were associated with the risk of AS (p=0.005, OR=2.5, 95% CI=1.5-4.2; and p<0.001, OR=139, 95% CI=49-394 respectively). HLA-B27 was the most important risk factor for susceptibility of AS.

Effects of Multiple-gene Variants of KIR and HLA on AS Susceptibility

In the evaluation of all KIR gene combinations (Table 3) the frequency of KIR-2, KIR-8, KIR-14, KIR-16 was higher in patients than in healthy controls (p=0.00001, p=0.01, p=0.01, p=0.007, respectively). Meanwhile, the frequency of KIR-11 was lower in patients than in healthy controls (p=0.01). In the evaluation of the inhibitory KIR gene combinations (Table 4) only the frequency of iKIR6 was lower in patients than in healthy controls (p=0.00008). Also, in the evaluation of the activating KIR gene combinations (Table 5) the frequency of aKIR-2, aKIR-6, aKIR-11 and aKIR-13 was higher in patients than in healthy controls (p=0.00002, p=0.01, p=0.013, p=0.002, respectively), and on the other hand, the frequency of aKIR-3, aKIR-5 and aKIR-9 was lower in patients than in healthy controls (p=0.002, p=0.003, and p=0.002, respectively). In the evaluation of the pseudogene combinations, there was no significant association

Table 4. Inhibitory Killer cell Immunoglobulin-like Receptors (iKIR) genotypes in normal individuals and AS patients, eight distinct iKIR genotypes were observed that differ from one other by the presence (shaded box) or absence (white box) of 8 inhibitory KIR genes. The frequency of individuals who have the desired genotype was shown by number and percent (N %).

Genotype ID	KIR								% Patients	% Control	*p*- value*	OR (CI)
	Inhibitory KIR											
	KIR2D 1	KIR2D 2	KIR2D 3	KIR2D 4	KIR2D 5	KIR3D 1	KIR3D 2	KIR3D 3				
iKIR-1									3 (1.5)	5 (2.5)	0.5	0.6 (0.1-2.5)
iKIR-2									4 (2.0)	1 (0.5)	0.17	4 (0.4-36)
iKIR-3									11 (5.5)	10 (5.0)	0.8	1.1 (0.4-2.6)
iKIR-4									62 (31.0)	44 (22.0)	0.04	1.6 (1-2.5)
iKIR-5									15 (7.5)	22 (11.0)	0.2	0.6 (0.3-1.3)
iKIR-6									5 (2.5)	26 (13.0)	**0.00008**	**0.2 (0.06-0.4)**
iKIR-7									21 (11.5)	23 (11.5)	0.7	0.9 (0.5-1.7)
iKIR-8									71 (35.5)	62 (31.0)	0.3	1.2 (0.8-1.8)

**p-value*≤0.0167 was statistically significant. The 0.0167 is one type error rate for any contrast.

Table 5. Activating Killer cell Immunoglobulin-like Receptors (aKIR) genotypes in normal individuals and AS patients, fifteen distinct aKIR genotypes were observed that differ from one other by the presence (shaded box) or absence (white box) of 6 activating KIR. The frequency of individuals who have the desired genotype was shown by number and percent (N %).

Genotype ID	Activating KIR						% Patients	% Control	*p*- value*	OR (CI)
	KIR2DS1	KIR2DS2	KIR2DS3	KIR2DS4	KIR2DS5	KIR3DS1				
aKIR-1							40 (20.0)	40 (20.0)	1	1 (0.6-1.6)
aKIR-2							22 (11.0)	2 (1.0)	**0.00002**	**12.2 (2.8-52.7)**
aKIR-3							9 (4.5)	26 (13.0)	**0.002**	**0.3 (0.14-0.69)**
aKIR-4							6 (3.0)	2 (1.0)	0.15	3 (0.6-15.3)
aKIR-5							8 (4.0)	24 (12.0)	**0.003**	**0.3 (0.1-0.6)**
aKIR-6							12 (6.0)	3 (1.5)	**0.01**	**4.1 (1.1-15)**
aKIR-7							8 (4.0)	6 (3.0)	0.5	1.3 (0.4-3.9)
aKIR-8							5 (2.5)	5 (2.5)	1	1 (0.2-3.5)
aKIR-9							3 (1.5)	16 (8.0)	**0.002**	**0.17 (0.05-0.6)**
aKIR-10							10 (5.0)	8 (4.0)	0.6	1.2 (0.5-3.2)
aKIR-11							6 (3.0)	0	**0.013**	-
aKIR-12							12 (6.0)	19 (9.5)	0.19	0.6 (0.3-1.3)
aKIR-13							9 (4.5)	0	**0.002**	-
aKIR-14							6 (3.0)	8 (4.0)	0.6	0.7 (0.25-2.1)
aKIR-15							19 (9.5)	22 (11.0)	0.6	0.8 (0.4-1.6)

**p*-value≤0.025 was statistically significant. The 0.025 is one type error rate for any contrast.

between AS patients and healthy subjects. In the evaluation of HLA gene combinations (Table 6) as we expected the frequency of HLA-2, HLA-5, HLA-7, HLA-9, HLA-15 ,which were negative for HLA-B27 but positive for other HLA genes, was lower in patients than in healthy controls (p=0.0002, p=0.0007, p=0.004, p=0.0007, p=0.001, respectively). Those variants which were positive for HLA-B27 (HLA-12, HLA-14, HLA-18, HLA-19, HLA-20, HLA-21, HLA-22, HLA-23) were significantly associated with AS (p=0.021, p=0.01, p=0.00006, p=0.00001, p=0.004, p=0.01, p=0.00003, p=0.000002, respectively).

Associations of KIR and HLA Genes with AS Clinical Features

We next investigated whether the KIR and HLA genes were correlated with disease severity and functional disabilities such as BASMI, BASFI, BASDAI, ASQOL and NRSpain with KIR and HLA genes in AS patients. There were no significant association between the mentioned clinical symptoms and KIR and HLA genes (Data not shown).

KIR/ HLA Compound Genotypes

In the assessment of KIR and HLA ligands combinations, there were some interesting results (Table7). The combination frequency of KIR2DL1^{+}/HLA-CW^{Lys+}, KIR2DL2^{-}/HLA-CW^{asp80-}, KIR2DL3^{+}/ HLA-CW^{asp80-}, KIR2DS1^{+}/HLA-CW^{Lys+}, KIR2DS2^{-}/HLA-CW^{asp80-} was higher in patients than in healthy controls (p=0.0009, p=0.01, p=0.0008, p=0.009, p=0.002 respectively). On the other hand,

Table 6. Human Leukocyte Antigen (HLA) genotypes in normal individuals and AS patients, twenty three distinct HLA genotypes were observed that differ from one other by the presence (shaded box) or absence (white box) of 6 HLA genes. The frequency of individuals who have the desired genotype was shown by number and percent (N %)

Genotypes	HLA-C1^{ASN}	HLA-C2^{LYS}	HLA-BW4^{Thr}	HLA-BW4^{ILe}	HLA-BW4	HLA-B27	Patients (N %)	Control (N %)	p-Value	OR (CI95%)
HLA-1		■					4 (2)	12 (6)	0.04	0.3 (0.1-1)
HLA-2	■	■					**2 (1)**	**18 (9)**	**0.0002**	**0.1 (0.02-0.4)**
HLA-3		■	■				2 (1)	5 (2.5)	0.2	0.4 (0.07-2)
HLA-4	■	■	■				2 (1)	7 (3.5)	0.09	0.3 (0.05-1.35)
HLA-5	■			■			**4 (2)**	**20 (10)**	**0.0007**	**0.2 (0.06-0.5)**
HLA-6		■		■			3 (1.5)	6 (3)	0.3	0.5 (0.1-1.9)
HLA-7	■	■		■			**11 (5.5)**	**28 (14)**	**0.004**	**0.3 (0.1-0.7)**
HLA-8	■				■		1 (0.5)	5 (2.5)	0.1	0.2 (0.02-1.7)
HLA-9		■			■		**2 (1)**	**16 (8)**	**0.0007**	**0.1 (0.02-0.5)**
HLA-10	■	■			■		5 (2.5)	10 (5)	0.2	0.4 (0.16-1.45)
HLA-11	■	■	■		■		0	4 (2)	0.04	-
HLA-12	■					■	5 (2.5)	0	**0.021**	-
HLA-13	■			■	■		16 (8)	20 (10)	0.4	0.7 (0.4-1.5)
HLA-14	■	■				■	**19 (9.5)**	**7 (3.5)**	**0.01**	**2.8 (1.1-7)**
HLA-15	■	■		■	■		**4 (2)**	**19 (9.5)**	**0.001**	**0.2 (0.06-0.58)**
HLA-16		■	■			■	5 (2.5)	3 (1.5)	0.5	1.6 (0.4-7)
HLA-17		■	■	■	■		1 (0.5)	3 (1.5)	0.3	0.3 (0.03-3.1)
HLA-18		■		■		■	**18 (9)**	**1 (0.5)**	**0.00006**	**19.6 (2.6-149)**
HLA-19	■	■		■		■	**21 (10.5)**	**1 (0.5)**	**0.00001**	**23.3 (3-175)**
HLA-20		■			■	■	**8 (4)**	**0**	**0.004**	**-**
HLA-21	■	■			■	■	**6 (3)**	**0**	**0.01**	**-**
HLA-22		■		■	■	■	**19 (9.5)**	**1 (0.5)**	**0.00003**	**20 (2.7-157)**
HLA-23	■	■		■	■	■	**25 (12.5)**	**1 (0.5)**	**0.000002**	**28.4 (3.8-211)**

*p-value≤0.025 was statistically significant. The 0.025 is one type error rate for any contrast.

Table 7. Association of KIR (receptor): HLA (ligand) combinations with AS

KIR(receptor):HLA(ligand)	*AS(n=200)*		*Control(n=200)*		*p-value**	**Odds Ratio**	*95% Confidence interval*	
	n	*%*	*n*	*%*			*Lower*	*Upper*
KIR2DL1 (+) **: HLA-CW^{Lys}** (-)	23	11.5	54	27	**0.00008**	0.35	0.2	0.6
KIR2DL1 (+) **: *HLA*-CW^{Lys}** (+)	171	85.5	144	72	**0.0009**	2.3	1.4	3.8
KIR2DL2 (-) **: *HLA*-CW^{asp80}** (-)	35	17.5	19	9.5	**0.01**	2.0	1.1	3.6
KIR2DL2 (+) **: *HLA*-CW^{asp80}** (+)	70	35	93	46.5	**0.01**	0.62	0.4	0.9
KIR2DL3 (+) **: *HLA*-CW^{asp80}** (-)	72	36	42	21	**0.0008**	2.1	1.3	3.3
KIR2DL3 (-) **: *HLA*-CW^{asp80}** (+)	8	4	25	12.5	**0.002**	0.3	0.12	0.66
KIR3DL1 (+) **: *HLA*-BW4^{Thr}** (+)	16	8	27	13.5	**0.07**	0.5	0.3	1.07
KIR2DS1 (-) **: *HLA*-CW^{Lys}** (-)	11	5.5	24	12	**0.02**	0.4	0.2	0.9
KIR2DS1 (+) **: *HLA*-CW^{Lys}** (-)	13	6.5	30	15	**0.006**	0.4	0.2	0.8
KIR2DS1 (+) **: *HLA*-CW^{Lys}** (+)	98	49	68	34	**0.002**	1.8	1.2	2.8
KIR2DS2 (-) **: *HLA*-CW^{asp80}** (-)	37	18.5	19	9.5	**0.009**	2.1	1.2	3.9
KIR2DS2 (+) **: *HLA*-CW^{asp80}** (+)	68	34	96	48	**0.004**	0.5	0.4	0.8

*Only significant associations have been shown

the combination frequency of KIR2DL1^{+}/HLA-CW^{Lys-}, KIR2DL2^{+}/HLA-CW^{asp80+}, KIR2DL3^{-}/HLA-CW^{asp80+}, KIR3DL1^{+}/HLA-BW4^{Thr+}, KIR2DS1^{-}/HLA-CW^{Lys-}, KIR2DS1^{+}/HLA-CW^{Lys-}, KIR2DS2^{+}/HLA-CW^{asp80+} was lower in patients than in healthy controls (p=0.00008, p=0.01, p=0.002, p=0.07, p=0.02, p=0.006, p=0.004 respectively).

DISCUSSION

NK cells are an important component of the innate immune system. These NK cells through cytokine production and cytotoxicity could fight against the abnormal cells. KIRs, present on NK cells, regulate the activities of NK cells through binding to HLA class I molecules and generate compound genotypes which modulate the activation and inhibition of NK cells. Studies have revealed that activating KIRs and compound KIR/HLA genotypes are related with the susceptibility of autoimmune disorders. In normal situation, there is a balance between the inhibition and activation signals of KIRs, but in the autoimmune disorders the increment of activation signals may initiate the immune responses.[27-29] It is well known, modifications of KIRs and their corresponding ligands expression strongly associated with the development and pathogenesis of autoimmune diseases.[21,30-33] We do not yet know the exact mechanisms that underlying the AS disease. Nevertheless, it is known that KIR and HLA genes variations and KIR/HLA combinations may have impact on the susceptibility of AS.[18,19,21]

In our study, we reported some interesting associations between KIR/HLA genes and AS in Iranian population (Table 2). We showed that the KIR2DL3 gene is strongly increased in AS patients than healthy controls, but previous studies in the United Kingdom[34] and the China[35] did not show significant association between KIR2DL3 gene and the susceptibility of AS. In addition, we figured out the KIR-2DL5 have a protective role in AS, but in previous studies, as we mentioned before, in United Kingdom and China did not show significant association. The

function of KIR is dependent on HLA molecules that expressed on target cells and it has been confirmed that HLA genes are associated with susceptibility of AS.[36-38] We found the presence of HLA-C2 ^{Lys80} and HLA-B27 which were associated with AS susceptibility (Table 2). It is reported that the HLA-Bw4 ligand, the presence of one or more -B-Bw4-^{Ile80}, -B-Bw4-^{Thr80} and -A-Bw4 epitopes, has a protective effect upon AS[21] and in some studies, absence of HLA-Bw4 alleles has been reported to associate with autoimmune diseases[39,40] but we could not find any association between these alleles with AS susceptibility.

In order to achieve a better combination view of KIR genes and their corresponding HLA ligands, we determined KIR and HLA genotypes (Table 3-6). There are plenty of combinations that significantly affect the disease risk.

In the evaluation of all KIR gene combinations (Table 3) the KIR2, KIR8, KIR14, KIR16 showed the significant risk effect on AS, which means these combinations increase the risk of AS disease. On the other hand, only KIR11 showed the protective effect and decrease risk of the disease. In the evaluation of the inhibitory KIR gene combinations (Table 4) only the iKIR6 decrease risk of the disease. In addition, in the evaluation of the activating KIR gene combinations (Table 5) the aKIR2, aKIR6, aKIR11 and aKIR13 increase the risk of AS disease, but, the aKIR3, aKIR5 and aKIR9 decrease risk of the disease. In the evaluation of HLA gene combinations (Table 6) as we expected HLA-2, HLA-5, HLA-7, HLA-9, HLA-15 which were negative for HLA-B27 but positive for other HLA genes reduced the AS susceptibility. Those variants which were positive for HLA-B27 (HLA-12, HLA-14, HLA-18, HLA-19, HLA-20, HLA-21, HLA-22, HLA-23) increased the AS susceptibility.

Interestingly, we observed that the specific KIR/HLA combinations can decrease or increase the AS susceptibility (Table 7).

In some studies and as well in our study, there are some confusing results about activating and inhibitory KIRs and their ligands. These conflicting results may be due to presence or absence of KIRs and their HLA ligands. As we know, KIRs are activated when they linked with specific ligands, so presence of inhibitory KIR without its specific ligand actually is un-functional. It is true in case of activating KIRs and their HLA ligands. More assessments are needed to evaluate the KIR and HLA ligand interactions. To achieve the better view of KIR and HLA genes complexity, more studies are needed to define the KIRs and HLA ligands involvement in natural immunity system and also, in different disease states.

Briefly, we documented that the presence of the specific stimulatory KIRs and the absence of certain inhibitory KIRs alone or in combination with their HLA ligands are associated with the pathogenesis of AS due to activation or inhibition of NK cells. Our results suggest that AS patients could present more functional activating KIR genes (activating KIRs with their HLA ligand) than healthy controls, the reverse is true in case of functional inhibitory KIR genes. Once the balance of signal transduction between activating and inhibiting receptors is disturbed, the ability of NK cells to identify and lyse the targets in immune responses will be compromised. Accordingly, imbalance of activating and inhibitory KIR genes by upregulate the activation and losing the inhibition of KIRs signaling or a combination of both might be the important factors underlying the pathogenesis of AS. Future studies should be done on genotyping, expression and function of KIR receptors and their HLA ligands in different populations to completely determine the association of KIR receptors and their HLA ligands in AS patients.

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