Inhibitory Killer Cell Immunoglobulin-Like Receptor KIR3DL1 in Combination with HLA-B Bw4^{iso} Protect against Ankylosing Spondylitis

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ABSTRACT

Background: The HLA class I molecules serve as ligands for both T cell receptors and killer cell immunoglobulin-like receptors (KIRs). **Objective:** We investigated the HLA-C and HLA-Bw4 alleles as well as KIRs expression on CD56 positive lymphocytes to evaluate whether these genes and molecules could influence Ankylosing spondylitis (AS) susceptibility, alone or in combination. **Methods:** We typed 40 AS patients and 40 normal controls for HLA-C asn^{80} (group 1) and HLA-C lys^{80} (group 2), HLA-B Bw4^{thero}, HLA-B Bw4^{iso} and HLA-A Bw4 alleles by PCR-SSP method. We also assessed the expression of KIR2DL1/2DS1, KIR2DL2/2DL3, KIR3DL1 and KIR2DS4 by flow cytometry. The Pearson chi-square or Fisher exact test was performed for statistical analysis. **Results:** The frequency of HLA-B Bw4^{iso} but not HLA-B Bw4^{thero} and HLA-A Bw4, ligand for the inhibitory KIR3DL1, was significantly reduced in AS patients as compared with controls ($p<0.01$). No significant differences were observed in gene carrier frequencies of HLA-C group 1 and 2 between AS and controls. Although no differences were found in the expression of KIR receptors between AS and normal subjects, we found that expression of KIR3DL1 in the presence of HLA Bw4-B^{iso} gene was reduced in patients with AS compared to healthy controls ($p<0.009$). **Conclusion:** We conclude that HLA-B Bw4^{iso}, the ligand of inhibitory KIR3DL1, with and without the expression of KIR3DL1 might be involved in protection against AS. Our results suggest that besides the HLA and KIR genotype, expression levels of KIRs may be involved in the pathogenesis of AS disease

Keywords: Ankylosing Spondylitis, HLA, KIR3DL1, CD56

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INTRODUCTION

Ankylosing spondylitis (AS) is a progressive, weakening disease that affects approximately 0.9% of individuals worldwide. The precise mechanisms underlying the initiation and progression of AS is still unclear (1). Genome-wide scan and association studies have demonstrated that in addition to HLA-B27, non-B27 genes, either within or outside the HLA, are involved in the etiology of the disease (2). The genetic evidences together with animal models suggest that HLA-B27 has some unique characteristics that can result in the promotion of the development of inflammation. However, it seems that additional genes outside the HLA region are also involved in AS (3, 4).
MHC class I molecules are recognized by receptors on several different types of immune cells. It has been known that T-cell receptors (TCRs) on cytotoxic T CD8 can recognize MHC class I molecules. More recently, it has been reported that there are additional MHC class I receptors in the killer immunoglobulin like receptor (KIR) and leukocyte Ig receptor (LIR) families that are expressed on many types of leukocytes, including T cells, NK cells, NKT cells, monocytes, macrophages and dendritic cells (5-8). KIRs are monomeric receptors possessing high allelic polymorphism. UP to now fourteen distinct KIR receptors have been identified in humans. KIRs contain two or three extra cellular immunoglobulin-like domains which bind to relevant ligands. Based on the length of the cytoplasmic tail, there are two kinds of KIR receptors. This receptor family can be subdivided functionally according to the length of their cytoplasmic tail, long-tailed KIRs (KIR2DL or KIR3DL) and short-tailed KIRs (KIR2DS or KIR3DS (9). Long or short cytoplasmic tails of KIR receptors involve in signal transduction. Long tails include immune-receptor tyrosine-based inhibitory motifs (ITAM), which trigger inhibitory signals. The receptors with a short tail are associated with immune-receptor tyrosine-based activation motifs (ITAM), which trigger activating signals upon binding to a relevant ligand. After interaction with HLA class I ligands, KIR genes produce inhibitory or activating signals to regulate the activation of NK and T cells. This activity contributes to the pathogenesis of diverse kinds of diseases.
There is a considerable variation in gene content and allelic polymorphism between activating and inhibitory receptors. Similarly, HLA-C alleles are also highly polymorphic. For example, Dimorphisms in the HLA-Cα chain that are characterized by Ser-77/Asn-80 and Asn-77/Lys-80 define serologically distinct HLA-C groups termed HLA-C group 1 and HLA-C group 2, respectively (10,11). KIR3DL1 binds to allotypes with the Bw4 motif (HLA-Bw4), a serologically defined public epitope in α1 domain (residues 77-83) that presents on 40% of the HLA-B allotypes and certain HLA-A molecules (HLA-A23,24,25,32). The dimorphic position 80 among the HLA-B Bw4-containing allotypes ($Bw4^{Isoluecine80}$ and $Bw4^{Threonine80}$) affects its interaction with KIR3DL1 subtypes, where HLA-B $Bw4^{Ile80}$ generally exhibits stronger inhibition through KIR3DL1 compared to HLA-B $Bw4^{Thr80}$.
NK cells play a key role in regulating autoimmune responses (12,13). The NK-inhibitory receptors mostly include the KIR family that mainly recognizes MHC class I molecules (14,15). Decreased numbers and impaired function of peripheral blood NK cells in patients with autoimmune diseases and alterations in KIR repertoire expression on NK cells and on T cells have been associated with autoimmune diseases such as Behcet's disease, type I diabetes and psoriasis (16-18). There is no reported study on KIRs gene expression in AS patients so far.

CD56 or neural cell adhesion molecules are expressed on the surface of neurons, glia, skeletal muscle and natural killer cells. They have a role in cell-cell adhesion and in homing into inflammation areas. CD56 bearing activated cells are able to migrate to sites of inflammation via intercellular adhesion molecules. Moreover, CD56 expressing cells have been described as a subset of regulatory T cells involved in immuno-surveillance and anti-tumor responses (9,14). As the ultimate function of killer cells is controlled by the expressed KIRs, the aim of this study was to investigate the presence of KIR molecules on the CD56 cells with and without corresponding HLA ligand genes. Moreover, we aimed to identify if the expression of KIR receptors alone or in combination with corresponding HLA ligands could influence the susceptibility to AS disease.

PATIENTS AND METHODS

Subjects. The study included 40 patients (75% male; mean age 36 years) diagnosed as having AS according to the modified New York criteria. The severity of the disease was measured by the Bath Ankylosing Spondylitis Functional Index (19) and the Bath Ankylosing Spondylitis Activity Index (20). Laboratory tests in these patients were consistent with a seronegative disease. Rheumatoid factor was negative and serum immunoglobulins and complements were within normal values. All patients received similar treatment with non-steroidal anti-inflammatory and/or cyclooxigenase-2 inhibitors. 40 normal subjects (72% male; mean age 32 years) who had no history of autoimmune diseases, infectious diseases, malignancies, or immunosuppressive therapy were considered as controls. All patients and normal subjects were HLA-B27 positive in HLA typing. The study was approved by the Institutional Review Board of Iran University of Medical Sciences. Written informed consent was obtained from all participants.
Monoclonal Antibodies (mAbs). The mAbs used in this study were FITC-conjugated anti-CD56, PE-conjugated anti-CD158a/h (KIR2DL1/2DS1), PE-conjugated anti-CD 158b (KIR2DL2/2DL3), PE-conjugated anti-CD158 e (KIR3DL1), PE-conjugated anti-CD158i (KIR2DS4) and PE-or FITC-conjugated mouse isotype controls. All antibodies were purchased from CALTAG laboratories, CA.
Cell Preparation and KIR Phenotyping. The frequency of KIR-expressing $CD56^+$ lymphocytes were assessed by flow cytometry. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by Lymphoprep (Nyegaard, Oslo, Norway) gradient centrifugation. PBMCs were incubated in a culture dish in a humidified 5% CO2, 95% air atmosphere at 37°C for 60 minutes. After incubation, nonadherent cells were collected. These cell suspensions were washed twice in phosphate-buffered saline (PBS). Surface phenotyping was carried out using a two-color immunofluorescence staining technique with isotype-specific mouse anti-human antibody conjugated with FITC and/or PE. Stained cells were suspended in 0.5 mL of PBS and analyzed by flow cytometry. Lymphocyte subsets were identified by gating analysis. Negative controls for each experiment were performed with FITC- and PE-labeled mouse immunoglobulin-G and unlabeled samples. The percentages of KIR/CD56 positive cells obtained from flow cytometric dot plots were analyzed for each experiment.
HLA Typing. DNA was extracted from peripheral blood and PCR-SSP method was done using EPITOP-TYPE kit (BAG health care, Germany) according to recommended instructions.

Statistical Analysis. Data are expressed as percentages. All data were collected in a computer database and analyzed. The Pearson chi-square or Fishers exact test was performed to assess the association of KIR surface antigens and HLA genes with AS disease. All statistical tests were considered as significant if p was less than 0.05.

RESULTS

In this study we typed the gene expression of KIRs 2DS4, 3DL1, 2DL2/2DL3, and 2DL12DS1 on the surface of CD56 positive lymphocytes as well as HLA-C and HLA-BW4 alleles. Using PCR technique, we genotyped the HLA-C group 1 (ligands for KIR 2DL2/2DL3), HLA-C group 2 (ligands for KIRs 2DL1/2DS1 and 2DS4) and HLA BW4 alleles which are ligands for KIR3DL1. All data obtained from flow cytometry of KIR receptors and PCR HLA typing is represented in graphs number 1 to 3.

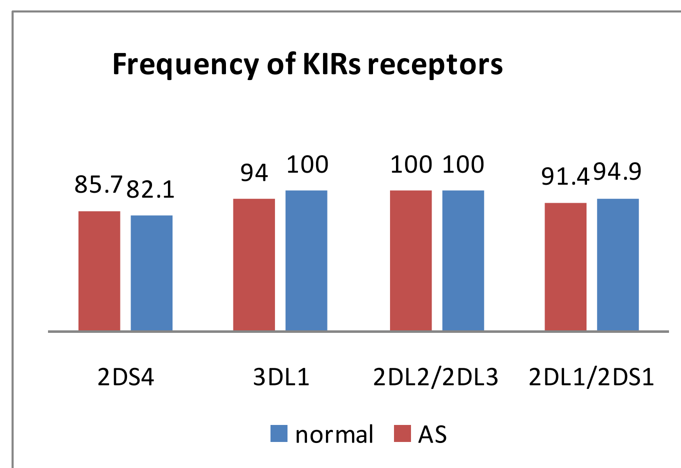

Figure 1. The comparison of KIR receptors frequencies between AS and normal controls (40 subjects for each group). Data are presented as percentages. P values were determined using two-tailed Fisher's exact test. No significant differences were found between two groups (p> 0.05).

As shown in Figure 1, we found no significant differences in the expression of these KIRs between AS patients and normal controls.
The comparison of HLA gene frequencies in AS and controls are represented in Figure 2. The results indicated a significant decrease in HLA $BW4^{iso}$ (p=0.01) and a non-significant decrease in HLA A-BW4 (p=0.08) in AS patients.

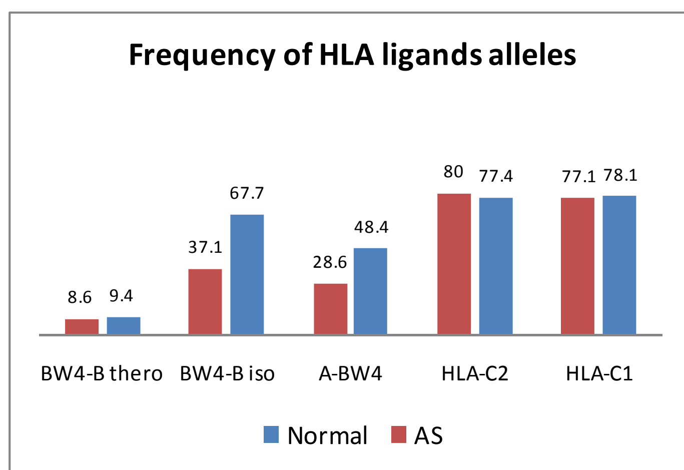

Figure 2. The comparison of HLA ligand gene frequencies between AS and healthy controls (40 subjects for each group). A significant decrease in HLA-Bw4 B^{iso} is shown in AS patients ($p < 0.01$).

Furthermore, the analysis of KIR-HLA combinations showed that the percentages of KIR 3DL1/HLA-$BW4^{iso}$ in AS was significantly lower ($p<0.009$) than those in normals (Figure 3).

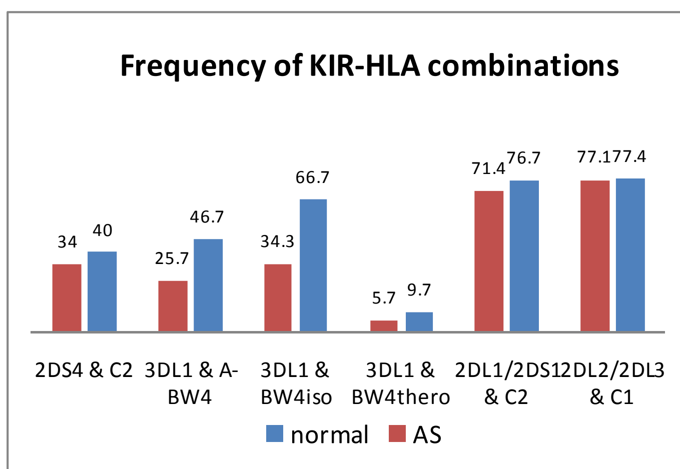

Figure 3. The comparison of KIR-HLA combination frequencies between AS and healthy controls (40 subjects for each group). A significant decrease in combination of KIR3DL1 and HLA-Bw4 B^{iso} is shown in AS patients compared to controls ($p< 0.009$).

DISCUSSION

The genetic variability of KIRs and/or HLA ligands has been studied in AS and several autoimmune diseases. A number of associations have been identified between risks of autoimmune disease and proposed activating KIR or KIR/HLA genotypes accompanied by a lack of inhibition (10,21). The majority of these studies is purely genetic studies and can not show the exact effect of variation in expression of KIRs on the pathogenesis of the disease. In addition, alleles of some of KIR genes may not be expressed or may have lower binding affinities than others (22,23). Therefore, in the present study besides the HLA genes, expression of KIR receptors was also evaluated instead of KIR genes. Application of mAbs used in this study is for the identification and enumeration of KIR positive cells separated PBMCs from and phenotypic analysis of NK cells after cell separation. In this study, because of some instrumental limitations we failed to exclude the CD3 positive cells from the experiments. Therefore, in addition to NK, subsets of CD3+/KIR+ activated T cells might be included within the examined cells. Considering that there is no evidence of expression of CD56 on the surface of NKT cells, we calculated the frequencies of KIR receptors on the surface of CD56 $^{+}$ lymphocytes including NK and activated T cells with and without corresponding HLA ligands.
Although Dian et al. reported the correlation of KIR genotypes with AS, we found no alteration of KIR expressions in AS compared to normals. This would be attributed to the inconsistency in KIR genotypes and gene expressions.
In the case of HLA ligands, it has been reported that alteration in the expression of these ligands are associated with autoimmune diseases (6,7). Moreover, Jiao reported that HLA-C alleles were more frequent in individuals with AS than in normals (10). Here, we showed no changes either in HLA-C group 1 or in HLA-C group 2 alleles, presumably due to the small sample size in our study. However, in the case of HLA-BW4, in accordance with a recently reported study (24), we found that HLA Bw4-B^{iso} subtype which is a ligand for inhibitory KIR-3DL1 was less common in AS subjects than in normals. KIR3DL1 is a polymorphic, inhibitory NK cell receptor specific for the Bw4 epitope carried by HLA-A and HLA-B allotype subsets. It has been reported that polymorphism within the KIR3DL1 receptor has functional consequences in terms of recognition of the target by NK cells (25,26). Bw4 molecule is used by the inhibitory receptor KIR 3DL1 as a ligand. Polymorphism at sites throughout HLA class I molecule can influence the interaction of the Bw4 epitope with KIR3DL1. This influence is probably mediated by changes in the peptides bound, which alter the conformation of the Bw4 epitope (27). As any effect of KIR on disease susceptibility might depend on the presence of putative HLA ligands within an individual, distinct combinations of KIR/HLA may have a combined effect on AS susceptibility (24). Accordingly, we indicated that individuals negative for both inhibitory KIR3DL1 receptor and HLA Bw4-B^{iso} allele were more common in AS than in control subjects. We suggest that this KIR-HLA combination might contribute to the pathogenesis of AS by influencing NK cell activity. Indeed, although we found no change of KIR3DL1 expression frequency alone in AS compared to controls, we demonstrated that the lack of KIR3DL1-HLA Bw4-B^{iso} combination was more common in AS patients. Therefore, we suggest that reduced HLA Bw4-B^{iso} genotype with and without its inhibitory receptor KIR 3DL1, may influence the inhibitory effect of NK cytotoxicity leading to continued injury in AS. In other words, as the KIR3DL is an inhibitory receptor upon interaction with HLA Bw4 on tar

get cells, reduced frequency of KIR3DL1-HLA Bw4-B^{iso} combination might result in autoimmune damage via direct lysis of normal cells. However, against other reports indicating the role of activation KIRs in AS and other autoimmune diseases (24,28), we failed to detect a high percentage of activated KIR-2DS4 expressing subjects in AS patients. Taken together, we hypothesized that the reduction of inhibitory KIR 3DL1 expressing cells in combination with the lack of its ligand HLA Bw4-B^{iso} may influence AS disease susceptibility. However, because of small sample sizes, our findings in this preliminary study are not complete. Hence, to determine the precise role of KIR/HLA and also the correlations between their genotypes and gene expressions in AS patients, further comparative studies using large sample sizes are recommended.

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