KIR Gene Content Does Not Contribute to Susceptibility to Graves' Disease

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ABSTRACT

Background: Killer cell immunoglobulin-like receptors (KIR) are expressed on NK cells and a subset of T cells. The variable KIR receptors along with their ligands, HLA class I, influence risk for autoimmune and malignant diseases. **Objective:** To investigate the *KIR* gene profiles in relation to susceptibility to Graves' disease in patients with ophthalmopathy. **Methods:** *KIR* genes profiles were analyzed in 90 patients presenting Graves' disease with ophthalmopathy representing upper eyelid retraction, swelling, redness, conjunctivitis, and bulging eyes and were compared with the KIR gene profiles of 112 healthy controls. The presence and absence of 11 variable *KIR* genes were characterized using a gene-specific PCR typing system. **Results:** There was no significant difference in the distribution of *KIR* gene profiles between patients and controls. **Conclusion:** Our data show that none of the *KIR* genotypes contribute in susceptibility to Graves' disease; although the role of HLA ligand remains to be characterized.

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INTRODUCTION

Graves' disease (GD) is an organ specific autoimmune disease and the most common endocrine disorder in humans. The disease is caused by autoantibodies that promote thyrotoxicosis by mimicking the role of TSH and activate the TSH receptor. The antibodies stimulate follicular hypertrophy and hyperplasia, leading to thyroid enlargement and augment thyroid hormone production (1). The etiology of the immune reaction to thyroid tissue is unknown, however, based on epidemiological evidence, the interaction between the susceptible genes and environmental factors increase GD risk (2). Genetic predisposition likely plays a role in breaking the tolerance to self-antigens and induces the autoimmune response (3). Furthermore, in the first stage of the disease, gene susceptibility alone, or combined with environmental toxins such as drugs or smoking, in addition to infection lead to thyrocyte apoptosis(4-6). This primes the cascade of immune reactions causing self-antigen presentation through dendritic cells (DC) in the regional lymph nodes and a Th1 response that eventually results in the development of the autoimmune disease (7). In the second stage, the thyroid cells and DCs present auto-antigens to T CD4+ cells which differentiate into Th1, Th2, Th17 and Treg cells (8). Furthermore, the recruited cytotoxic T lymphocytes and natural killer cells in the thyroid gland induce apoptosis. Thus, the innate response is linked to thyroid destruction and dysfunction.

Natural killer (NK) cells are effector cells of innate immunity that help initiate adaptive immunity through interaction with dendritic cells (9). The NK function is regulated through the interaction between the NK cell receptors and HLA class I on the target cells. This engagement generally generates inhibitory signals that stop NK cells from lysing the target cells. Furthermore, the surface activating receptors on the NK cells regulate the effector function. A subtle balance between the signals transduced from activating and inhibitory receptors determine the NK cell function (10). Additionally, NK cells produce cytokines as regulatory components in both innate and adaptive immune responses. They can influence adaptive immune cells, such as T cells and DCs, through production of pro-inflammatory cytokines and/or cell-cell contact (11). Conversely, the effector response must be regulated to prevent the autoimmune response against self cells. NK cells appear to be endowed with potential dual functions and thus can be considered as double-edged swords (12,13).

Killer cell immunoglobulin-like receptors (KIR), a compact cluster of genes on chromosome 19q13.4, are expressed on NK cells and a subset of T cells, such as the CD8+ cytotoxic T lymphocytes (CTL) with memory phenotypes and $CD4^{+}CD28^{null}$ T cells (14-16). The *KIR* gene family is comprised of 14 receptors of which KIR3DL1, 3DL2, 3DL3, 2DL1, 2DL2, 2DL3 and 2DL5 are involved in the inhibition of NK cell response; KIR3DS1, 2DS1, 2DS2, 2DS3, 2DS4, and 2DS5 trigger activation of NK cell response; and KIR2DL4 mediates both NK cell inhibitory and activating functions (17,18). The inhibitory receptors engage with their corresponding specific HLA ligands (motifs) of HLA-A (A3/A11),-B (Bw4) and -C (C1/C2) molecules (17). However, the ligands for several KIRs have not yet been identified.

Individual variability in the *KIR* gene family depends on variation in the gene number and sequence polymorphism (19), which may provide individual genetic basis of susceptibility to autoimmune conditions (20). Consistent with this notion, specific *KIR* genotypes have been associated with a wide range of autoimmune diseases. Increasing evidence indicates a greater role of activating *KIR* genotypes in some autoimmune

diseases, such as the presence of *KIR2DS2* which is associated with type I diabetes, systemic lupus erythematous (SLE), and systemic sclerosis (21-23). Moreover, the presence of *KIR3DS1* seems to play an essential role in developing ankylosing spondylitis (AS, 24). Possession of more activating and less inhibitory *KIR* genes has been reported to be associated with birdshot chorioretinopathy (BCR), Vogt-Koyanagi-Harada (VKH) disease and HLA-B27-associated acute anterior uveitis (AAU) (25). Thus, the present study aims to analyze the contribution of inhibitory and activating *KIR* genes in GD risk. For this reason, we characterized *KIR* genes in 90 GD patients and 112 healthy controls.

MATERIALS AND METHODS

Study Population. Genomic DNA samples from 90 GD patients (mean age: 37.58 ± 13.68 years; 65.5% female and 34.5% male) and 112 healthy controls from the southern part of Iran (Fars province) were included in this study. The patients were recruited at the Motahari Polyclinic Center, Shiraz University of Medical Sciences. All patients met the criteria for GD diagnosis according to the clinical and laboratory criteria of Grave's disease (26). The GD clinical phenotype was confirmed based on the expertise of the referring GD specialist. The inclusion criterion for the study was having developed Graves' ophthalmopathy (GO). All the GD patients developed GO which was defined as class III or higher in the American Thyroid association mnemonic NOSPECS scheme (27). The controls were age, sex and ethnicity matched to the patients. The study was reviewed and approved by the Medical Research Ethics Committee of Shiraz University of Medical Sciences.

Genotyping. DNA was extracted from peripheral blood samples using a QIAamp blood kit (Qiagen, Hilden, Germany). The quality and quantity of DNA was determined by UV spectrophotometry, and the concentration was adjusted to 100 ng/μL**.** DNA samples were typed for the presence and absence of 11 *KIR* genes by using a gene-specific polymerase chain reaction (PCR) typing system as described elsewhere (28). Unusual and unique genotypes were confirmed by duplex SSP-PCR method (29,30) and compared with worldwide human genotype data from the "http://www.allelefrequencies.net" database (31).

Different primer sets were used to type each *KIR* gene (primers provided by Dr. Rajalingam). The PCR reaction (15 μl volume) consisted of a final concentration of 1X PCR buffer II (10 mM Tris-HCl and 50 mM KCl), 200 mM of each dNTP, 1 mM of each forward and reverse primer , 3 mM of MgCl2 and 0.5 units of AmpliTaq (CinnaGen, Iran). The thermal cycling conditions used for the PCR amplification was 95°C for 3 min followed by five cycles of 94°C for 20 s, 65°C for 20 s, and 72°C for 90 s; then 32 cycles of 94°C for 20 s, 61°C for 20 s, and 72°C for 90 s, finished with 10 min extension at 72°C. Following amplification, 15 μl of the products was electrophoresed on 2% agarose gels and visualized by a UV transilluminator. To check the specificity and accuracy of the assay, a panel of 20 UCLA KIR exchange reference DNA samples (provided by Dr. Rajalingam) was used and our genotyping data revealed 100% consistency with the reference DNA according to the UCLA KIR Exchange Summary (http://www.hla.ucla.edu/cellDNA/Cell/summaryKir.htm).

Statistical Analysis. The percentage of individuals carrying each *KIR* gene and the genotype was determined by direct counting (individuals positive for the gene/genotype divided by the individuals tested per population x 100). We evaluated AA genotypes with *KIR2DL1-2DL3-3DL1-2DS4* and Bx genotypes with any other combination of *KIR* loci. Furthermore, the frequency of AA and Bx (BB+AB) genotypes was predicated as we described elsewhere (32). Differences in the distribution of *KIR* genes and genotypes was estimated by a two-tailed Fisher Exact probability (p) test with $p<0.05$ considered as statistically significant.

RESULTS

In this study, we analyzed the frequency of eleven *KIR* genes in 90 Graves' patients compared to 112 healthy controls. We did not find any significant difference in the frequency of inhibitory and activating *KIR* genes between patients and controls. In fact, patients and controls displayed similar frequencies of inhibitory and activating *KIR* genes (Figure 1).

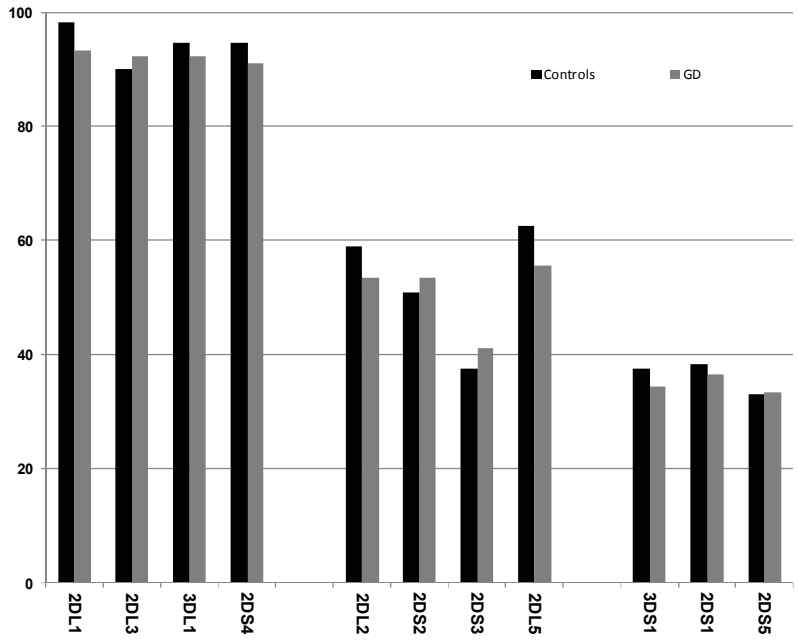

Figure 1. The killer cell immunoglobulin-like receptors in patients with Graves' disease (GD) and controls. The frequencies of *KIR* genes in GD compared to healthy controls. No significant difference in *KIR* gene frequency was observed between patients and controls.

Based on the presence and absence of *KIR* genes, 41 distinct *KIR* genotypes were identified in the 202 individuals analyzed in our study. The overall differences between controls and patients in the distribution of *KIR* genotypes were not statistically significant. Graves' patients exhibited only 23 out of 41 genotypes. Thirteen of these previously reported in worldwide genotype data (Gonzalez-Galarza et al., 2011) were unique to the patient group (15.4%). Ten genotypes were shared between patients and controls (84.1% vs. 74.8%, Figure 2).

Number	Genotypes	*KIR* gene content											Controls	GD
		Centromeric half						Telomeric half						
		2DS2	*2DL2*	*2DL3*	*2DL5*	*2DS3*	*2DL1*	*3DL1*	*3DS1*	*2DS5*	*2DS1*	*2DS4*	(n=112)	(n=90)
1	AA			■			■	■				■	24.1	33.3
2	Bx	■	■	■	■	■	■	■				■	12.5	13.3
3		■	■	■	■	■	■	■	■	■	■	■	4.4	12.2
4		■	■	■			■	■				■	9.8	7.7
5				■	■		■	■	■	■	■	■	11.6	5.5
6		■	■		■	■	■	■				■	5.3	4.4
7		■	■	■	■	■	■		■	■	■		0.9	3.3
8		■	■	■	■		■	■	■	■	■	■	3.5	2.2
9				■	■	■	■	■	■	■	■	■	1.8	1.1
10		■	■	■				■				■	0.9	1.1
11				■	■		■		■	■	■		0	2.2
12		■	■	■	■	■	■	■		■	■	■	0	1.1
13		■	■	■	■	■	■	■	■		■	■	0	1.1
14		■	■	■	■	■	■	■			■	■	0	1.1
15		■	■	■	■		■		■	■	■		0	1.1
16		■	■	■	■			■	■	■	■	■	0	1.1
17				■				■				■	0	1.1
18		■	■		■	■		■				■	0	1.1
19				■	■		■	■	■		■	■	0	1.1
20				■	■	■	■	■	■	■	■		0	1.1
21		■	■		■			■	■	■	■	■	0	1.1
22		■	■					■				■	0	1.1
23				■	■	■	■		■	■	■		0	1.1
24			■	■	■		■	■	■	■	■	■	3.5	0
25		■	■	■	■	■	■	■	■			■	2.6	0
26		■	■	■	■		■	■				■	2.6	0
27				■	■	■	■	■	■		■	■	1.8	0
28		■	■		■	■	■		■	■	■		1.8	0
29		■	■	■	■		■	■		■	■	■	1.7	0
30			■	■	■		■	■	■		■	■	0.9	0
31			■	■	■	■	■	■	■	■	■	■	0.9	0
32			■	■			■	■				■	0.9	0
33			■	■	■	■	■	■	■			■	0.9	0
34				■		■	■	■				■	0.9	0
35				■	■		■	■		■	■	■	0.9	0
36		■	■	■	■	■	■		■		■		0.9	0
37		■	■	■	■	■	■		■		■		0.9	0
38		■	■	■	■	■	■				■		0.9	0
39		■	■		■	■	■	■	■	■	■	■	0.9	0
40			■				■	■				■	0.9	0
41		■	■		■			■		■	■	■	0.9	0
Number of Genotypes													28	23

Figure 2. *KIR* gene profiles in Graves' patients. Forty-one genotypes were observed that differed from each other by the presence (shaded box) and absence (white box) of 11 *KIR* genes. The frequency of each genotype is presented in percentage and the overall difference in the distribution of *KIR* genotypes between Graves' patients and healthy controls was not statistically significant. Data for *2DL4, 3DL2, 3DL3, 3DP1, and 2DP1* was not presented as they were present in most or all of the individuals.

Our analysis of the difference between A and Bx genotypes revealed that the frequency of the AA genotype was slightly increased in Graves' disease patients in comparison to controls (33.3% vs. 24.1%), even if it did not reach a significant difference. Conversely, Bx genotypes (AB and BB) decreased in the controls compared to the patients (66.6% vs. 75.9%).

DISCUSSION

In the present study no significant difference was found between inhibitory and activating *KIR* genes in patients and controls. Graves' disease is an autoimmune disorder in which the immune tolerance is broken. Several epidemiological studies revealed the role of NK cell receptors, especially the KIR receptors, in autoimmune diseases. All patients displayed Graves' ophthalmopathy (GO), an inflammatory disorder of the orbit (33) with infiltration of immune cells such as CD4+ and CD8+ T cells, B cells and macrophages, to the orbit (34). Up to now, no clear evidence has been reported the role of NK cells in the GO inflammatory reaction. Our data suggests that the *KIR* genes do not contribute to the immune-pathogenesis of GD with ophthalmopathy. Furthermore, the impairment of Treg may actually assist the effector T cells to halt the progression of the disease (3). The GD immunepathogenesis pathway clearly demonstrates a significant role for T cells in disease progression in the late phase, especially the memory $CD4^{+}CD28^{-}$ KIR^{+} T cells that produce large amounts of IFN-γ (35). However, this study was unable to find any specific *KIR* genes that play a role in the autoimmune process.

Although, the direct role of NK cell receptors in GD pathogenesis is unknown, Wenzel et al. reported that NK cell activity significantly decreased in GD, and the functional impairment did not correlate with serum levels of thyroxine, the presence or severity of ophthalmopathy, or titers of serum thyroid antibodies (36). Also, Solerte et al reported a deficiency in NK cell cytotoxicity and cytokine secretion in GD patients. Furthermore, they suggested that the defect might be determined by the expansion of T and B cell immune compartments playing a pathological role in autoimmune thyroid disease (37). Thus, NK cells seem to exert immunomodulatory control over other immune cells. Therefore, distinct NK cell receptors rather than *KIR* receptors appear to have a role in breaking tolerance.

From epidemiological studies, the difference between A and Bx genotypes was associated with susceptibility to different diseases (38). Our analysis of the difference between A and Bx genotypes revealed that the frequency of the AA genotype was slightly increased in Graves' disease patients in comparison to controls while Bx genotypes decreased in the controls compared to the patients. Thus, a specific contribution of *KIR* gene profiles was not observed in the study.

There is increasing evidence that KIR receptors and their HLA ligands play a major role in autoimmune susceptibility. Moreover, a majority of disease associations indicate a role of activating KIR receptors. Activating KIR2DS2 has been reportedly implicated in inflammation associated with autoimmune diseases such as systemic sclerosis, rheumatoid vacuities, type I diabetes and systemic lupus erythematous (21,23). Moreover, the increase of activating KIR3DS1 and KIR2DS1 has been seen in ankylosing spondylitis (24,39). On the contrary, our study reveals no significant contribution of KIR receptors in susceptibility to GD. As mentioned above, Graves'

disease is categorized by the formation of pathogenic autoantibodies against thyroid peroxidase (TPO), thyroglobulin (Tg), and the TSH receptor (TSHR). These data lead us to conclude that the KIR gene content does not involve in susceptibility or protection in Graves' disease, while the role of KIR-HLA interaction is an issue that requires being clarified.

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