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Killer Cell Immunoglobulin like Receptors (KIR) Gene Variations in Rheumatic Fever and Rheumatic Heart Disease Patients from North India

Sarkar S[1#], Rastogi M[1#], Kumar R[2] and Chakraborti A[1*]

[1]*Department of Experimental Medicine and Biotechnology, Post Graduate Institute of Medical Education and Research, Chandigarh, India*

[2]*School of Public Health, Post Graduate Institute of Medical Education and Research, Chandigarh, India*

[#]*Both the authors contributed equally to this study*

[*]**Corresponding author:** Chakraborti A, Department of Experimental Medicine and Biotechnology, Post Graduate Institute of Medical Education and Research, Chandigarh- 160012, India, Tel: +91 172 2746277; Fax: +91 172 2746277; E-mail: superoxide14@gmail.com

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Abstract

The autoimmune sequelae of rheumatic fever (RF) and rheumatic heart disease (RHD) are due to untreated or partially treated pharyngitis caused by Group A streptococcus (GAS). RF/RHD usually affects the genetically susceptible individuals. KIR (Killer cell immunoglobulin like receptor) of NK (natural killer) cell has been documented in susceptibility to various autoimmune diseases. *KIR* is of activating, inhibitory, pseudo and framework types. In here, 29 patients (pharyngitis, RF and RHD) and controls were studied to establish the association of different *KIR* genes in development of RF/RHD. *KIR* genotyping in all the disease groups revealed that the frequency of activating *KIR2DS4A* and inhibitory *KIR2DL5B* were less in RHD compared to the control. On the other hand, frequency of activating *KIR2DS5A* was more in RHD than pharyngitis. A significant difference in the frequency of *KIR2DS2A* and *KIR3DL1B* were found in pharyngitis compared to control. Interestingly, the overall data revealed a marked decrease of activating genes in pharyngitis and an increase in RHD. However, in the study, the framework genes were comparatively conserved and pseudo genes did not show any significant change. Thus, the study suggests an association of KIR in the pathogenesis of RF/RHD by demonstrating the variations in specific genotypes. This can be correlated to the prolonged activation of NK cells which may be accountable for regulation of adaptive immune response and self-tolerance. Further study in large cohort may unveil more information regarding the role of KIR in the development of the disease.

Keywords: KIR; Natural killer cells; Group A streptococcus; Rheumatic fever; Rheumatic heart disease; Activating gene; Inhibitory gene

Introduction

Rheumatic fever (RF) and rheumatic heart disease (RHD) are the post infection autoimmune disease sequelae of pharyngitis caused by Group A streptococcus (GAS) or *Streptococcus pyogenes* [1]. The annual incidence of GAS infection reaches to 700 million worldwide. A total of 470000 RF cases are diagnosed with 233000 reported deaths due to complications of RHD [2]. The underlying mechanism of the development of RF/RHD is poorly understood. However, the proposed hypothesis in the development of RF/RHD is the autoimmune reaction due to molecular mimicry between different GAS and host proteins [3]. Host adaptive and innate immune systems actively participate in the regulation of autoimmune diseases and adjunct the progression of RF/RHD pathogenesis.

An important component of the innate immune system is NK (Natural killer) cells comprising 10-15% of total peripheral blood lymphocytes. NK cells are instrumental in playing a decisive role in immune activation and regulation [4]. Upon activation, NK cells trigger subsets of T-cells followed by release of cytokines and chemokines for the clearance of pathogens. During the initial stages of infection, NK cells secrete interferon-γ (IFN-γ), which triggers expression of MHC class I and class II on antigen presenting cells and initiates differentiation of CD4+ T cells to Th1 cells [5,6]. They sequentially produce IL-2 and IFN-γ, which induce the synthesis of cytokines and promote the proliferation of NK cells [7]. NK cells expresses KIR (killer cell immunoglobulin like receptor) on their surface and are divided into activating, inhibitory, pseudo and framework genes. KIR molecules target class-I human leukocyte antigen (HLA-1) during the time of NK cell inhibition. Continuous expression of inhibitory and activating genes interacts with HLA class-II molecule and subsequently generates signals to eliminate the cells lacking ligands for the KIR inhibitory receptor [8]. HLA class-II molecules, which have been found to be associated with RHD, are targeted by the KIR and subsequently regulate the function of NK cells [8,9].

The regulation of NK cells and its function depends on the balance between activating and inhibitory genes, which further help NK cells to initiate the effector functions like cytolytic activity [10]. The activation of NK cells may be explained by "missing self" and "induced self" hypothesis. Normally, cells expressing adequate MHC class-I proteins are not targeted by the NK cells. But, as per the "missing self" hypothesis, cells lacking MHC class-I proteins (either due to viral infection or cancer) are readily targeted and killed by the NK cells [11-13]. According to "induced self" hypothesis, stress condition created by malignant or virus infected cells produce signal like MICA/MICB and serve as ligand for the activating receptor NKG2D (natural killer group 2, member D) present on the surface of NK cells [14]. Unlike activating KIR receptors, which selectively interact with major histocompatibility complex (MHC) class I ligands, the inhibitory receptors of NK cells specifically target MHC class I ligands [15].

Rajagopalan et al. have demonstrated that peptides having low affinity to MHC class I show an antagonistic effect to the peptides having high affinity. Additionally, this antagonism is irrespective of KIR genotyping and frequency of activating or inhibitory receptors [16]. Framework genes are the haplotypes, which shows a conserved region in KIR genomic pattern [17]. The framework KIR receptors are thought to be consisted of haplotype A and B. Haplotype A *KIR* genes are considerably frequent in the Caucasian population, whereas, the haplotype B *KIR* genes comprises genotypes carrying more activating genes [18-21]. In contrast, pseudo *KIR* genes like 2DP1 cannot encode receptors due to the presence of aberrant reading frames [22]. *KIR* with different activating and inhibitory genes has been reported to be significantly associated with leukemia [23], autoimmune disease like type-I diabetes mellitus [24], uveitis [25], rheumatic diseases like systemic sclerosis [26], systemic lupus erythematosus [27,28], ankylosing spondylitis [29], rheumatoid arthritis [30] and autoimmune hepatitis [31]. In the present study, an attempt has been made to correlate the association of different KIR receptors with the progression of the RF/RHD pathogenesis.

Materials and Methods

Sample collection

Clinically diagnosed 5 to 15 year old pharyngitis and 5 to 65 year old RF/RHD patients (n=29), who visited the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, were included in the study. Blood samples from each disease group (pharyngitis, RF and RHD) as well as healthy individuals were collected after their written informed consents. All the RF/RHD patients were on prophylactic penicillin during the time of blood collection while, no penicillin prophylaxis were given to the patients of pharyngitis prior to the blood collection.

DNA isolation

Genomic DNA was isolated from whole blood by HiYieldTM Genomic DNA mini kit in accordance with the manufacturer's instructions (Real Biotech Corporation, Taiwan).

KIR genotyping

KIR is located on human chromosome 19q13.4 of the leukocyte receptor complex (LRC) and consists of 15 genes [32,33]. These genes can be classified into inhibitory genes (*2DL1, 2DL2, 2DL3, 2DL5*), activating (*2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DS1*), pseudo (*2DP1*), and framework genes (*2DL4, 3DL2, 3DL3*). 29 pairs of primers were used to amplify all the *KIR* genes. DRB1 was used as an internal control. PCR (polymerase chain reaction) primers and reaction conditions were followed as described previously [32].

Statistical analysis

Data were analyzed by Pearson's Chi-Square test and Fisher's exact test using SPSS software (Statistical Package for the Social Sciences), version 16, $p<0.05$ was considered as significant. On the basis of each *KIR* gene, the odds ratios between different *KIR* genes and groups were calculated for disease and control groups.

Results

To assess the role of KIR in RF/RHD pathogenesis, three disease groups- pharyngitis, RF and RHD were included in the study. *KIR* genotyping was done in all the disease groups and correlated with age and sex matched control. Different frequencies in terms of presence of specific *KIR* genes were observed in the study group (Table 1). Genotyping data when expressed statistically using linear-by-linear distribution, elicited that the frequency of activating *KIR2DS4A* ($p=0.035$) and inhibitory *KIR2DL5B* ($p=0.044$) were less in RHD compared to the control. *2DS5A*, another activating *KIR* was found to be more frequent ($p=0.014$) in RHD than pharyngitis. Besides, activating *KIR2DS2A* ($p=0.045$) was in increased frequency in pharyngitis compared to control. Activating gene *KIR3DL1B*, and a framework gene *KIR2DL4A* were found to be less frequent ($p=0.034$) in pharyngitis than the other groups. However, we did not find any significant association of pseudo genes in different groups. The framework genes were found to be conserved in all the study groups (Figure 1). The statistical odds revealed the possibility to develop each disease stage on the basis of a specific type of KIR genotype. Higher odd values of *3DL2A* (12.000 and 8.000 respectively in RHD and RF), *2DS5A* (6.750 in RHD) were observed compared to control (Table 2).

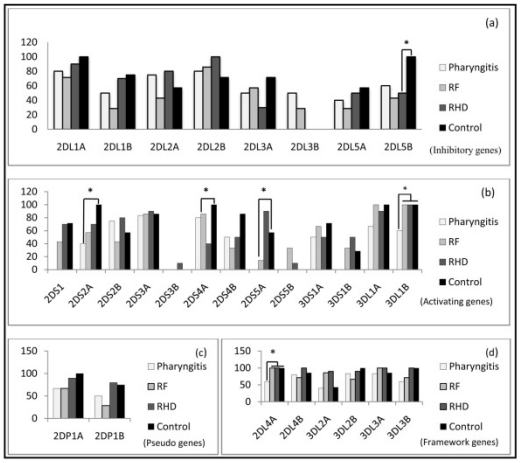

Figure 1: Bar diagram showing the frequency (%) of (a) inhibitory, (b) activating, (c) pseudo, and (d) framework KIR genes in different groups. The significant 'p' values are symbolized by *.

The findings showed that few *KIR* genes possessed 100% expression in the disease groups. RHD group possessed 100% frequency of inhibitory *KIR2DL2B*, activating *KIR3DL1B* and framework *KIR2DL4A*, *KIR2DL4B*, *KIR3DL3A*, *KIR3DL3B* genes. Similarly, RF patients exhibited 100% frequency of activating *KIR3DL1A*, *KIR3DL1B* and framework *2DL4A*, *KIR3DL3A*. On the other hand, several genes were not detectable in the disease groups. We observed that various activating *KIR* genes (*2DS1*, *2DS3B*, *2DS5A*, *2DS5B* and *3DS1B*) were absent in pharyngitis group. Besides, RHD group also showed absence of inhibitory *KIR2DL3B* and activating *KIR2DS3B*, *KIR2DS5B*. Activating *KIR2DS3B* was present only in RHD groups (10%) (Table 1 and Figure 1).

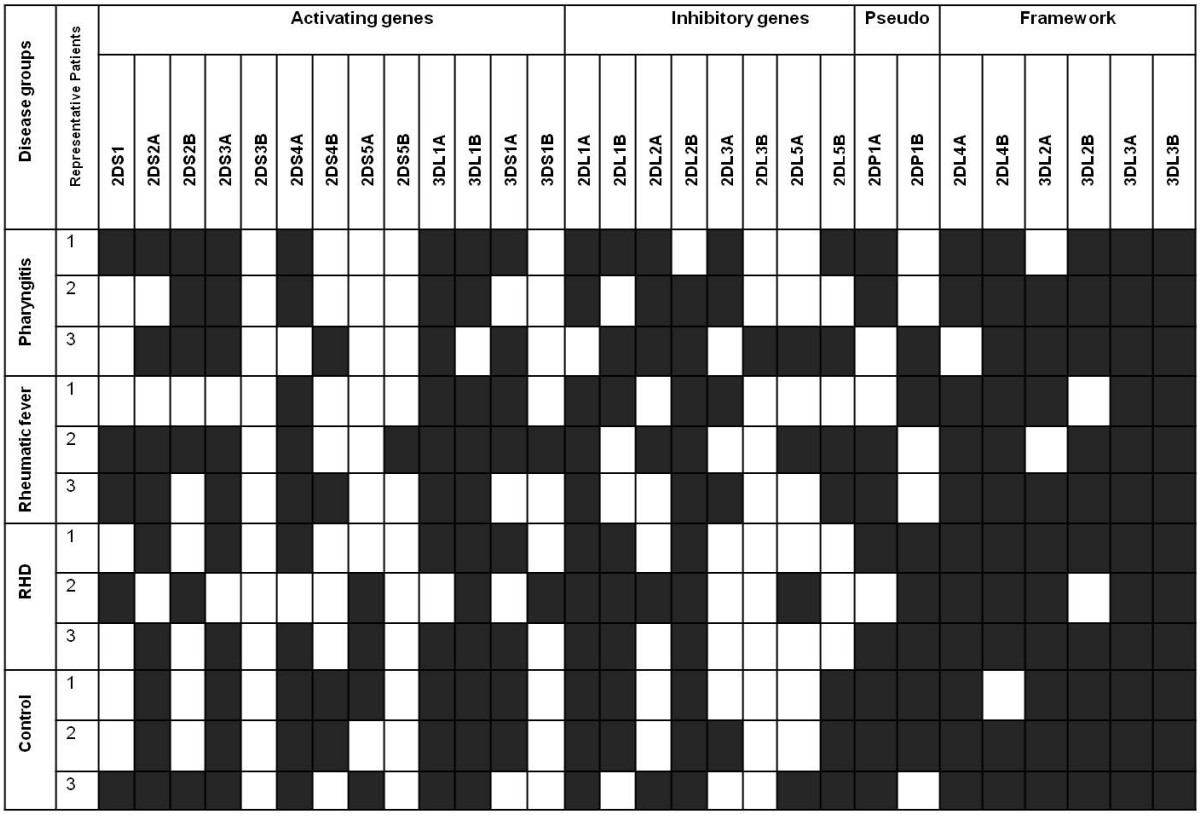

Figure 2: *KIR* gene frequencies of representative patients and controls. The presence or absence of a particular *KIR* is represented here respectively by shaded or white box.

The overall data revealed a marked decrease of activating genes in pharyngitis and an increase in RHD group (Table 2 and Figure 2). Interestingly, we also observed that frequency of pseudo genes were low in pharyngitis and RF when compared to RHD and control.

Discussion

NK cell, an important arm of innate immunity, acts as a bridge between the host innate and adaptive immune response during infection [34]. Regulation of NK cell function markedly alters the host adaptive immune response in different autoimmune disorders [23-31]. The putative functions of NK cell can be explained by the complex and discrete associations of their receptor molecules. NK cell expresses *KIR* on their surface, which plays a detrimental role in the trafficking of adaptive immune response by targeting different class of HLA ligands [8]. The extensive diversity of *KIR* genes among the different population as well as in the diseases has already been reported [34]. *KIR* gene variation is also observed in autoimmune diseases [23-31]. But, there are no such studies, which show the association of *KIR* genes in the RF/RHD pathogenesis. Hence, in the present study, we have reported the variation of different *KIR* genes in the early and late onset of the disease i.e. from pharyngitis to RF/RHD.

A wide diversity in *KIR* gene distribution was observed among the disease groups (Figure 1). Activating and inhibitory genes of *KIR* have the potential to differentially regulate the function of NK cells [10]. Short cytoplasmic domain of the activating *KIR* contains basic acids, which interact with the acidic or negatively charged amino acids of *DAP12* (12KDa) and provides signals to ITAM (immunoreceptor tyrosine based activation motif) to produce kinase enzymes. These enzymes activate the NK cells, which are cytotoxic to the target cells and release numerous proinflammatory and regulatory cytokines [IFN-γ, TNF-α, IL-1, IL-5, IL-10, IL-12 IL-13, IL-15, IL-18, granulocyte macrophage colony stimulating factor (GM-CSF)] and chemokines [macrophage inflammatory proteins (MIP-1α, MIP-1β), IL-8, and RANTES] [35]. Besides, the elevated level of cytokines like TNF-α, IFN-γ, IL-10, IL-6, IL-4 in RF/RHD is already reported [36,37].

One of the important mechanisms behind such elevation of cytokine secretion in RHD may be the activation of NK cells through activating *KIR*. In contrast, our study also reveals an overall increase in the frequency of activating *KIR* genes (*2DS1*, *2DS2*, *2DS3*, *3DS1B* and *2DS5A*) in RHD group compared to RF and pharyngitis (Table 2, Figure 1). We have observed a significant association of *2DS4A* in RF and *2DS5A* in RHD. It suggests that the prolonged activation of NK cells persists throughout the developmental stage of RHD (Table 1). Apart from these, the lower frequency of activating *KIR3DL1B* in pharyngitis suggests the poor involvement of NK cell in the initial stage of GAS infection. Our data is in the agreement with the study conducted by Rosaschino et al., where they have also found a significant diminution of NK cells number in school going children having pharyngitis [38]. However, a non-significant increase of activating *KIR2DS5B* has also been noticed.

Serial Number	*KIR* gene types	*KIR* genes	Disease Groups (%)			Control (%)	p-values between each groups
			Pharyngitis	RF	RHD	Healthy	
			1	2	3	4	
1	Inhibitory	*2DL1A*	80	71.4	90	100	NS
2		*2DL1B*	50	28.6	70	75	NS
3		*2DL2A*	75	42.9	80	57.1	NS
4		*2DL2B*	80	85.7	100	71.4	NS
5		*2DL3A*	50	57.1	30	71.4	NS
6		*2DL3B*	50	28.6	0	0	NS
7		*2DL5A*	40	28.6	50	57.1	NS
8		*2DL5B*	60	42.9	50	100	0.044* (between groups 3 and 4)
9	Activating	*2DS1*	0	42.9	70	71.4	NS
10		*2DS2A*	40	57.1	70	100	0.045* (between groups 1 and 4)
11		*2DS2B*	75	42.9	80	57.1	NS
12		*2DS3A*	83.3	85.7	90	85.7	NS
13		*2DS3B*	0	0	10	0	NS
14		*2DS4A*	80	85.7	40	100	0.035* (between groups 3 and 4)
15		*2DS4B*	50	33.3	50	85.7	NS
16		*2DS5A*	0	14.3	90	57.1	0.014* (between groups 2 and 4)
17		*2DS5B*	0	33.3	10	0	NS
18		*3DS1A*	50	66.7	50	71.4	NS
19		*3DS1B*	0	33.3	50	28.6	NS
20		*3DL1A*	66.7	100	90	100	NS
21		*3DL1B*	60	100	100	100	0.034*(between groups 1 and other groups)
22	Pseudo	*2DP1A*	66.7	66.7	90	100	NS
23		*2DP1B*	50	28.6	80	75	NS
24	Framework	*2DL4A*	60	100	100	100	0.034* (between group 1 and other groups)
25		*2DL4B*	80	71.4	100	85.7	NS
26		*3DL2A*	40	85.7	90	42.9	NS
27		*3DL2B*	83.3	66.7	90	100	NS
28		*3DL3A*	83.3	100	100	85.7	NS
29		*3DL3B*	60	71.4	100	100	NS
*Significant p-value; NS= Non Significant							

Table 1: Frequencies of *KIR* genes among the study groups.

The high odds ratios (more than 1.000) are indicative of the possibility to develop a particular stage of disease (i.e. pharyngitis or RF or RHD) on the basis of *KIR* genes distribution. In here, we have observed high odd ratios of activating *KIR* genes (*2DS2B, 2DS3A, 2DS5A* and *3DS1B*) in RHD group compared to RF and pharyngitis (Table 2).

This data suggests the activation of NK cells, which further results in the cytotoxicity during the late stage of the disease. Moreover, it has been reported that T cells with IL-2 secretion leads to the increased NK cell cytotoxicity, which ultimately causes the alteration in the heart valve due to fibrosis and hemodynamic changes [39].

KIR genes		Odd ratios in the disease groups against control ϕ		
		Pharyngitis	RF	RHD
Inhibitory gene	*2DL2A*	2.25	-	3
	2DL2B	1.6	2.4	-
Activating gene	*2DS2B*	2.25	-	3
	2DS3A	-	1	1.5
	2DS5A	-	-	6.75
	3DS1B	-	1.25	2.5
Pseudo gene	*2DP1B*	-	-	1.333
Framework gene	*3DL2A*	-	8	12
ϕThe *KIR* genes having lower odd ratios (<1.000) were exempted.				

Table 2: Odd values of selected *KIR* genes among the different disease groups.

Inhibitory *KIR* contains ITIM or immunoreceptor tyrosine based inhibitory motif. Tyrosine residues of ITIM are phosphorylated by kinases followed by dephosphorylation by SHP-1 (phosphatase like enzyme), which results in the inhibition of NK cell functions [40]. It has been shown that HLA class I acts as a ligand for the binding of different inhibitory KIR receptors. Inhibitory *KIR* genes target class-I HLA (i.e. HLA-A, HLA-B and HLA-C). Unlike HLA class-I, HLA class II is targeted by both activating and inhibitory KIR and induce signals for the elimination of target cell lacking ligands for inhibitory receptors [8]. Therefore, the interaction between receptor and ligands maintains the balance between different KIR and HLAs. In this study, we have observed a significant decrease in the frequency of inhibitory *KIR2DL5B* in all the disease groups compared to control. This data suggests that the NK cell inhibition is diminished due to the poor expression of its inhibitory receptor during the diseased condition. However, a non-significant increase in the frequency of inhibitory *KIR2DL2A* and *2DL2B* has been observed in RHD.

Our study, for the first time, unveils the wide diversity in *KIR* genotype associated with the pathogenesis of RF/RHD in north Indian population. It is evidenced from the study that a genetic balance between activating and inhibitory receptors of *KIR* is associated with the autoimmune regulation of RF/RHD, which may be due to the increased frequency of activating *KIR* genes or decreased frequency of inhibiting KIR genes or both. Moreover, activation of NK cells may induce self-tolerance and subsequently initiate the valvular vandalism in RHD pathogenesis.

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