Letter to the Editor

Novel Association of Killer Cell Immunoglobulin-like Receptor Genes with EBV-infectious Diseases in Children*

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Killer cell immunoglobulin-like receptors (KIRs) which are mainly expressed on natural killer (NK) cells are implicated in many virus infections. However, it is unclear whether or not KIRs are associated with susceptibility to Epstein-Barr virus (EBV) infection related diseases. Therefore, the purpose of our study was to investigate possible correlation between polymorphisms of *KIR* genes and infectious mononucleosis (IM)/EBV-associated hemophagocytic lymphohistiocytosis (EBV-HLH). The polymorphisms of *KIR* genes were detected by polymerase chain reaction with sequence-specific primers (PCR-SSP). The results would contribute to clarify the association of KIRs with EBV induced diseases, and provide new insights into the role of NK cells and innate immune response against viral infections and/or subsequent progression.

Epstein-Barr virus (EBV) belongs to human herpes virus. EBV-infected individuals primarily exhibit subclinical infections or self-limiting acute infectious mononucleosis (IM), while minority of these individuals are presented with fatal EBV-associated hemophagocytic lymphohistiocytosis (EBV-HLH). It is well known that natural killer (NK) cells play a pivotal role in immunological response to EBV infections. The cytotoxicity and production of cytokines, which belong to antiviral activities of NK cells, are regulated by the balance between activating and inhibitory receptors on cell surface.

Killer cell immunoglobulin-like receptors (KIRs) are a highly polymorphic and homologous multigene family of receptors and are mainly expressed on NK cells. *KIR* genes are mapped on chromosome 19q13.4 within the 150kb leukocyte receptor complex (LRC). To date, 14 functional *KIR* genes have been described, encoding key receptors that trigger activation (KIR2DS1-5, KIR3DS1), inhibition (KIR2DL1-3, KIR2DL5, KIR3DL1-3), or both (KIR2DL4) of NK cells respectively. Furthermore, based on the presence of a single or multiple activating *KIR* genes and linkage disequilibrium between *KIR* loci, *KIR* haplotypes can be classified into two basic groups, A and B[1]. Briefly, in addition to the framework *KIR* genes, group A haplotypes have a relatively fixed content including *KIR2DL1*, *KIR2DL3*, *KIR2DS4*, and *KIR3DL1*. In contrast, group B haplotypes have variable gene contents comprising at least one of the activating *KIR* genes except *KIR2DS4* and the inhibitory genes *KIR2DL5* and *KIR2DL2*. Given the distinct combination of maternal and paternal *KIR* haplotypes, it is reasonable to imagine that individuals have quite different *KIR* genotypes. Consequently, the inherited diversity of *KIR* genes within and across populations may influence people's immunity and susceptibility to diseases.

Accumulated researches have reported the relationship between highly polymorphic *KIR* genes and virus infections. However, in IM/EBV-HLH individuals, the precise pathogenesis and the clinical diversity of EBV associated diseases are not fully clarified. The present study was aimed to investigate whether polymorphisms of KIRs would be associated with genetic resistance or susceptibility to EBV-infectious diseases in Chinese children.

A case-control study was performed in West China Second University Hospital of Sichuan University. Two hundred and twenty patients with EBV infection were enrolled from the hospital between September 2009 and April 2013, including 157 IM patients and 63 EBV-HLH patients. IM patients were diagnosed based on clinical presentations and positive serum IgM antibody for EBV viral capsid antigens (VCA) and/or plasma EBV

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DNA copies. And EBV-HLH patients were diagnosed according to the guidelines HLH-2004 from the Histo-cytology Society plus positive EBV-VCA-IgM and/or plasma EBV DNA copies (Supplement Table 1, see the website www.besjournal.com). Two hundred and eighty eight sex- and age-matched unrelated controls who did health examination were enrolled from the same hospital at the same time. This study was approved by the Ethics Committee of the West China Second University Hospital of Sichuan University, and informed consents were obtained from parents or guardians.

Genomic DNA were extracted from peripheral blood samples with DNA extractor kit (TIANGEN BIOTECH). The quality and quantity of extracted DNA samples were determined by UV spectrophotometry. Genotyping of KIRs were performed to detect the presence or absence of *KIR* loci (*KIR2DL1-5*, *KIR1D*, *KIR2DS1-5*, *KIR3DL1-3*, *KIR3DS1*, *KIR2DP1*, *KIR3DP1*, *KIR3DP1v*), using polymerase chain reaction (PCR) with published sequence-specific primers (PCR-SSP)[2-3]. 2 μL genomic DNA, 6.25 μL 2×PCR buffer and 2 μL of each specific primer were mixed in the total of 12.5 μL. PCR products were amplified by the programmable PCR thermal cycler (BIO-RAD) according to the reference[2]. The reaction products were run on 2.5%-3% agarose gels for electrophoresis and the gel was visualized using a UV light source and photographed for a permanent record. Each PCR included negative and positive control.

Then the *KIR* gene content was used to infer *KIR* genotypes and haplotypes as previously described[3-4]. Genotypes for the centromeric (*Cen*) and telomeric (*Tel*) parts of the *KIR* locus were assigned according to the presence or absence of one or more *B haplotype*-defining *KIR* genes (Supplement Table 2, see the website www.besjournal.com). The observed carrier frequency of each *KIR* genes (*pf*, *%*) was determined by direct counting. Genotype frequency=n1/N, haplotype frequency=n2/2N and N was the total number of individuals tested.

Statistical analysis was conducted using Pearson's Chi-squared test or two-tailed Fisher's exact test to analyze the differences between IM/EBV-HLH patients and controls in the frequencies of carrying *KIR* genes, genotypes and haplotypes. All data were analysed by SPSS version 19.0 software (SPSS Inc., USA). A *P*-value less than 0.05 was considered statistically significant.

The observed frequencies of *KIR2DS1*, *KIR2DS5*, *KIR3DS1*, and *KIR2DL5* were significantly higher in EBV-HLH patients than those in healthy controls (*P*=0.034, 0.004, 0.002, and 0.002, respectively). Meanwhile, the observed frequencies of *KIR2DS2, KIR2DS4, KIR2DL2,* and *KIR2DL5* were significantly higher in IM patients than those in healthy controls (*P*=0.005, 0.009, 0.005, and 0.013 respectively) (Table 1). These results indicate that the carriers of *KIR2DS1, KIR2DS5, KIR3DS1*, and *KIR2DL5* might be more susceptible to EBV-HLH, meanwhile *KIR2DS2, KIR2DS4*, *KIR2DL2*, and *KIR2DL5* might be related to the increased risk for IM.

The increased frequencies of inhibitory *KIR2DL5* in both EBV-HLH group and IM patients indicate that *KIR2DL5* might be related to EBV infection. The mechanism of KIR-mediated inhibition of NK cells involves recruitment of Src homology 2-containing protein tyrosine phosphatase-1 (SHP-1) and SHP-2 to two phosphorylated cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). KIR2DL5 has two ITIMs and can recruit both SHP-1 and SHP-2. It can inhibit cytotoxicity of NK cells mainly via SHP-2[5]. Therefore, *KIR2DL5* carriers are more susceptible to IM and EBV-HLH, which might be in part attributed to the decreased lysis of EBV-infected cells by NK cells.

The observed frequencies of *KIR3DS1* in EBV-HLH patients was significantly increased as compared with IM patients (*P*=0.033) (Table 1). Our findings suggest that activating *KIR3DS1* might be a risk factor for EBV-infected patients to progress to HLH. KIR3DS1 plays a specialized role in activating NK cells. KIR3DS1 can trigger both cytotoxicity and IFN-gamma production[6], meanwhile IFN-gamma was found extremely elevated in HLH patients[7] and xenograft models of chronic active EBV infection (CAEBV) and EBV-HLH[8]. Therefore, the increased influence of KIR3DS1 might result in higher activating capacity of NK cells and increased inflammatory response in EBV-HLH individuals, which might also lead to NK cell/CD8 lymphocyte exhaustion/depletion and make multiple organ susceptible to NK cell attack[9].

We detected 21 kinds of *KIR* haplotypes. The frequency of haplotype 1 was significantly lower while the frequencies of haplotype 4 and 8 were significantly higher in IM patients than those in healthy controls (*P*=0.001, 0.016, and 0.000, respectively). Meanwhile, the frequency of haplotype 2 was significantly lower in EBV-HLH patients than those in IM patients (*P*=0.035) and normal controls (*P*=0.022), otherwise the frequency of haplotype 5 was significantly higher in EBV-HLH

patients than those in IM patients (*P*=0.046) and healthy controls (*P*=0.001) (Table 2).

The frequency of *Cen-A/A* was significantly lower (*P*=0.005) while the frequency of *Cen-A/B* was significantly higher in IM patients than those in healthy controls (*P*=0.016). Meanwhile, the frequency of *Tel-A/A* was significantly higher in healthy controls than those in IM patients and EBV-HLH patients (*P*=0.043 and *P*=0.001, respectively), while the frequency of *Tel-A/B* was significantly higher in EBV-HLH patients than that in IM group (*P*=0.004) (Table 3).

As well known, only a small part of EBV infected individuals progress to life-threatening EBV-HLH. EBV-HLH is characterized by high cytokinemia due to excessive activation of NK cells and T lymphocytes. Paladino et al. reported that increased frequencies of activating NK receptors were associated with higher natural killer cytotoxicity in individuals who had worse progression of HCV infection and liver injury[10]. Similarly, We found statistically increased frequencies of *Cen-A/B* and *Tel-A/B* in IM and/or EBV-HLH patients, which contained more activating *KIR* genes (B haplotype genes *KIR2DS1*, *KIR2DS2*, and *KIR3DS1*), and consequently *Tel-A/B* (*KIR3DS1* and *KIR2DS4* with *KIR3DS1* and/or *KIR2DS1*) positive EBV-infected individuals might be more likely to develop into HLH. Otherwise, individuals having A haplotype genes in the centromeric and telomeric regions of both *KIR* haplotypes (*Cen-A/A* and *Tel-A/A* genotypes) were associated with a decrease in the incidence of EBV-infectious diseases. The results of our study showed that more activating KIRs expressed on NK cells might produce excessive activation of NK cells which might be associated with high cytokinemia of EBV-HLH.

In summary, our findings indicate that *KIR2DL5*, hyplotype 4 and 8, genotype *Cen-A/B* may be associated with susceptibility to IM, while *KIR2DL5*, haplotype 5 and genotype *Tel-A/B* may be associated with susceptibility to EBV-HLH. The *KIR3DS1* may increase risk for EBV-infected individual to develop to

Table 1. Comparison of Observed *KIR* gene Frequencies between EBV-HLH, IM, and Controls

Genes	EBV-HLH (*n*=63) (%)	IM (*n*=157) (%)	Normal (*n*=288) (%)	*P* Values		
				EBV-HLH *vs.* Normal	IM *vs.* Normal	EBV-HLH *vs.* IM
KIR activating genes						
2DS1	27 (42.9)	54 (34.4)	84 (29.2)	**0.034**	0.255	0.239
2DS2	20 (31.7)	55 (35.0)	65 (22.6)	0.124	**0.005**	0.642
2DS3	14 (22.2)	32 (20.1)	40 (13.9)	0.097	0.073	0.762
2DS4	45 (71.4)	127 (80.9)	200 (69.4)	0.756	**0.009**	0.124
1D	32 (50.8)	70 (44.6)	151 (52.4)	0.814	0.114	0.404
2DS5	25 (39.7)	43 (27.4)	64 (22.2)	**0.004**	0.223	0.074
3DS1	31 (49.2)	53 (33.8)	84 (29.2)	**0.002**	0.316	**0.033**
KIR inhibitor genes						
2DL1	63 (100)	156 (99.4)	287 (99.7)	1.000	1.000	1.000
2DL2	20 (31.7)	55 (35.0)	65 (22.6)	0.124	**0.005**	0.642
2DL3	63 (100)	151 (96.2)	283 (98.3)	0.590	0.207	0.186
2DL5	34 (54.0)	71 (45.2)	96 (33.3)	**0.002**	**0.013**	0.240
3DL1	60 (95.2)	154 (98.1)	283 (98.3)	0.158	1.000	0.357
Framework Genes/Pseudogenes						
2DL4	63 (100)	157 (100)	288 (100)	1.000	1.000	1.000
3DL2	63 (100)	157 (100)	288 (100)	1.000	1.000	1.000
3DL3	63 (100)	157 (100)	288 (100)	1.000	1.000	1.000
2DP1	63 (100)	157 (100)	287 (99.7)	1.000	1.000	1.000
3DP1	63 (100)	156 (99.4)	287 (99.7)	1.000	1.000	1.000
3DP1v	8 (12.7)	16 (10.2)	37 (12.8)	0.974	0.408	0.590

Note. The *P* values are shown in bold when they indicate significant (<0.05) differences.

HLH. On the contrary, the haplotype 1, 2 and genotype *Cen-A/A* and *Tel-A/A* may act as protective factors for susceptibility to IM or EBV-HLH. This would help to explain why the individuals are predisposed to be susceptible to EBV-HLH or IM and also to distinguish the factors that have effect on prognosis. Further research on the relationship of activating KIRs and their ligands and hypercytokinemia of EBV-HLH will be required to explore the potential of KIRs as a targeted therapy for EBV-infected diseases.

Table 2. Comparison of *KIR* Haplotype Frequencies in IM, EBV-HLH, and Controls

Haplo-type	*N* (%F)			*P* Values		
	EBV-HLH (*n*=126)	IM (*n*=314)	Normal (*n*=576)	EBV-HLH *vs.* Normal	IM *vs.* Normal	EBV-HLH *vs.* IM
1	27 (21.4)	63 (20.1)	173 (30.0)	0.053	**0.001**	0.748
2	42 (33.3)	139 (44.3)	256 (44.4)	**0.022**	1.000	**0.035**
3	3 (2.4)	1 (0.3)	11 (1.9)	0.725	0.066	0.073
4	10 (7.9)	26 (8.3)	25 (4.3)	0.093	**0.016**	0.905
5	25 (19.8)	39 (12.4)	55 (9.5)	**0.001**	0.183	**0.046**
6	2 (1.6)	5 (1.6)	17 (3.0)	0.551	0.212	1.000
7	2 (1.6)	1 (0.3)	3 (0.5)	0.221	1.000	0.198
8	3 (2.4)	16 (5.1)	5 (0.9)	0.159	**0.000**	0.205
9	1 (0.8)	4 (1.3)	8 (1.4)	1.000	1.000	1.000
10	0	0	1 (0.2)	-	-	-
11	0	4 (1.3)	0	-	-	-
12	0	0	2 (0.3)	-	-	-
13	0	1 (0.3)	1 (0.2)	-	1.000	-
14	0	1 (0.3)	0	-	-	-
15	0	2 (0.6)	1 (0.2)	-	0.285	-
16	2 (1.6)	1 (0.3)	5 (0.9)	0.615	0.671	0.198
17	3 (2.4)	4 (1.3)	8 (1.4)	0.426	1.000	0.414
19	2 (1.6)	3 (1.0)	2 (0.3)	0.150	0.352	0.627
20	0	1 (0.3)	0	-	-	-
21	4 (3.2)	3 (1.3)	3 (0.5)	**0.022**	0.431	0.107

Note. The *P* values are shown in bold when they indicate significant (<0.05) differences.

Table 3. Analysis the Frequencies of *Cen* and *Tel* genotypes in EBV-HLH, IM, and Normal Cohorts

Genotypes	EBV-HLH (*n*=63) (%)	IM (*n*=157) (%)	Normal (*n*=288) (%)	*P* Values		
				EBV-HLH *vs.* Normal	IM *vs.* Normal	EBV-HLH *vs.* IM
Cen-A/A	43 (68)	102 (65)	223 (77)	0.144	**0.005**	0.753
Cen-A/B	20 (32)	49 (31)	60 (21)	0.069	**0.016**	1.000
Cen-B/B	0	6 (4)	5 (2)	0.590	0.207	0.186
Tel-A/A	31 (49)	97 (62)	206 (72)	**0.001**	**0.043**	0.098
Tel-A/B	29 (46)	55 (35)	77 (26)	**0.004**	0.082	0.167
Tel-B/B	3 (5)	5 (3)	5 (2)	0.158	0.333	0.692

Note. The *P* values are shown in bold when they indicate significant (<0.05) differences.

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