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Original Article

Killer cell immunoglobulin-like receptor gene cluster predisposes to susceptibility to B-cell acute lymphoblastic leukemia in Chinese children

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Abstract: B-cell acute lymphoblastic leukemia (B-ALL) is the most common malignancy in children. Killer cell immunoglobulin-like receptors (KIRs) are mainly expressed on natural killer (NK) cells and regulate killing of cancer cells. To investigate the possible association of *KIR* genes with B-ALL in Chinese children, we used polymerase chain reaction with sequence-specific primers (PCR-SSP) to determine the *KIR* genotypes of 137 B-ALL patients and 288 healthy children of Chinese Han origin. Herein we report no significant difference in the carrying frequency of individual *KIR* genes and haplotypes between patients and controls; however, individuals carrying *C4Tx* genotypes were more frequent in the B-ALL group compared with healthy controls (11.7% vs. 5.9%, *P*=0.038). In addition, the centromeric *KIR* gene cluster, *KIR2DS2-2DL2-2DS3-2DL5*, was significantly increased in the B-ALL group compared with healthy controls (13.9% vs. 7.3%, *P*=0.030). These data suggest that the *C4Tx* genotype and centromeric *KIR* gene cluster (KIR2DS2-2DL2-2DS3-2DL5) might predispose to susceptibility to B-ALL in Chinese children.

Keywords: Killer cell immunoglobulin-like receptor, gene cluster, B-cell acute lymphoblastic leukemia, genotype

Introduction

B-cell acute lymphoblastic leukemia (B-ALL) is the most common childhood malignancy and accounts for approximately 80% of all pediatric ALL cases. The causes of B-ALL have not been established. With the development of molecular biology, genetic and immune factors are known to be important in the pathogenesis of ALL [1]. NK cells, which are essential components of the innate immune system, have become the focus of research [2].

NK cells play an important role in defending against tumor cells [2]. The development and function of NK cells is governed by a dynamic balance between inhibiting and activating cell surface receptors, including killer cell immunoglobulin-like receptors (KIRs) [2, 3]. *KIR* genes are located on chromosome 19q13.4 and form part of the leukocyte receptor complex (LRC). To date, the family of *KIR* genes compromise 16 loci, including 2 pseudogenes (*KIR2DP1* and *KIR3DP1*) and 4 framework *KIR* genes (*KIR-2DL4*, *KIR3DL2*, *KIR3DL3*, and *KIR3DP1*). Fourteen functional and highly homologous *KIR* genes encode the key receptors that trigger activation (*2DS1-5* and *3DS1*), inhibition (*2DL1-3*, *2DL5*, and *3DL1-3*), or activation and inhibition (*2DL4*) of NK cells [4]. Furthermore, based on the presence of one or more activating *KIR* genes and linkage disequilibrium between *KIR* loci, *KIR* haplotypes can be classified into two basic groups (A and B) [5]. 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 a variable gene content, comprising at least one of the activating *KIR* genes (except *KIR2DS4*) and inhibitory genes (*KIR2DL5* and *KIR2DL2*). According to genetic studies [5], distinct populations have a different distribution of *KIR* haplotypes. For example, group A haplotypes are overrepresented in northeast Asians, while the A and B haplotypes are equally distrib-

uted in Caucasians. Given the distinct combination of maternal and paternal *KIR* haplotypes and the multiallelic diversity of *KIR* genes, it is likely that individuals have quite different *KIR* genotypes. Consequently, the inherited diversity of *KIR* genes within and across populations may influence immunity and susceptibility to disease.

Several association studies have suggested a possible association between *KIR* genes and the development of certain forms of leukemia [6-10]. Recently, several studies have analyzed the association between *KIR*s genes/genotypes and childhood B-ALL in Western countries [11, 12]; however, the studies had conflicting results. In an effort to address genetic factors that might contribute to the susceptibility to childhood B-ALL, we performed a case-control study and evaluated the *KIR* genes/genotypes with the aim of investigating the association between *KIR* genes/genotypes and B-ALL in Chinese children.

Materials and methods

Patients and controls

We performed a case-control study at the West China Second University Hospital. One hundred thirty-seven unrelated children diagnosed with B-ALL (89 boys and 48 girls; mean ± S.D.: 6.15 y ± 4.11 y) were enrolled from the Pediatric Hematology/Oncology Department between September 2011 and July 2013. There were 288 gender- and age-matched unrelated controls (164 boys and 124 girls; mean ± S.D.: 6.27 y ± 3.81 y) from the same hospital who underwent health examinations during the same time period. B-ALL patients were diagnosed according to established criteria that included morphology, immunophenotyping, cytogenetics, and molecular biology (MICM), and treated based on therapeutic guidelines (Chinese Children Leukemia Guideline [CCLG-ALL 2008]). Control subjects and their relatives had no history of malignant diseases or autoimmune disorders. The study was approved by the Institutional Ethics Committee of West China Second University Hospital and informed consents were obtained from parents or guardians.

KIR genotyping

Genomic DNA was extracted from peripheral blood samples with a DNA extractor kit (RelaxGene Blood DNA System; Tiangen Biotech Co., LTD., Beijing, China) according to the manufacturer's instructions. The quality and quantity of the extracted DNA samples were determined by UV spectrophotometry. DNA samples in sterile water were stored at -20°C for further studies.

Genotyping of the KIR was performed to detect the presence or absence of 16 KIR loci (*KIR2DL1-5*, *2DS1-5*, *3DL1-3*, *3DS1*, *2DP1*, and *3DP1*) using PCR amplification with sequence-specific primers (PCR-SSP), as described previously [13] with minor modifications [14]. We amplified 100-200 ng of DNA in a 12.5-ul PCR mixture containing 6.25 μl of 2×PCR buffer (2×Taq PCR MasterMix, KT201; Tiangen Biotech Co., LTD.) between 4 pm and 10 pm for each specific primer. All amplifications were performed in a programmable PCR thermal cycler (Bio-Rad C1000 Touch Thermal Cycler; Richmond, CA, USA). The reaction products were run on 2.5%-3% agarose gels for electrophoresis to determine if the genes were present. The gel was visualized using a UV light source and the gel was photographed for a permanent record. The appearance of the amplicon band of the expected molecular weight was considered to indicate the presence of the gene in the genomic DNA sample. Each PCR experiment included one negative and one positive control.

Prediction of haplotypes from genotypes

The *KIR* gene content was used to determine *KIR* genotypes and haplotypes, as previously detailed [15]. Haplotypes 1 and 2 were referred to haplotype A; all of the other haplotypes were designated as haplotype B. We assigned each child to one of three genotypes (AA, BB, and AB). The AB and BB genotypes were previously referred to as *KIR* genotype Bx [16].

Classification of genotypes on the basis of centromeric and telomeric gene clusters

Based on linkage disequilibrium, the *KIR* cluster is subdivided into two regions [17]. One-cluster comprises the *KIR2DS2-2DL2-2DS3-2DL5* genes and is located at the centromeric half of the *KIR* gene complex, whereas another cluster comprises the *KIR3DS1-2DL5-2DS1-2DS5* genes and is located at the telomeric half of the complex. For simplicity, Ashouri et al. [18] refer to these clusters as C4 and T4 (C represents centromeric, T represents telomeric, and

Table 1. Comparison of carrying *KIR* genes, genotypes, and gene clusters in B-ALL and controls

Types	B-ALL (n=137)		Control subjects (n=288)		OR (95% CI)	*P*
	%F	(N)	%F	(N)		
A haplotype-associated KIR genes						
2DL1	100	(137)	99.7	(287)	--	--
2DL3	97.8	(134)	97.9	(282)	--	--
3DL1	95.6	(131)	98.3	(283)	--	--
2DS4*	76.6	(105)	69.8	(201)	1.420 (0.889-2.270)	0.142
B haplotype-associated KIR genes						
2DL2	28.5	(39)	22.9	(66)	1.339 (0.844-2.124)	0.215
2DL5	38.0	(52)	33.7	(97)	1.205 (0.789-1.839)	0.388
2DS2	28.5	(39)	22.9	(66)	1.339 (0.844-2.124)	0.215
2DS3	19.0	(26)	14.2	(41)	1.411 (0.822-2.421)	0.210
2DS5	22.6	(31)	22.2	(64)	1.024 (0.629-1.666)	0.925
3DS1	31.4	(43)	29.5	(85)	1.092 (0.703-1.698)	0.694
2DS1	32.8	(45)	29.5	(85)	1.168 (0.754-1.809)	0.486
KIR genotypes and gene clusters						
AA genotypes	54.7	(75)	56.6	(163)	0.928 (0.616-1.397)	0.719
BB genotypes	13.9	(19)	8.3	(24)	1.771 (0.934-3.358)	0.077
AB genotypes	31.4	(43)	35.1	(101)	0.847 (0.548-1.308)	0.453
C4Tx genotypes	11.7	(16)	5.9	(17)	2.108 (1.031-4.311)	0.038
CxT4 genotypes	18.2	(25)	17.7	(51)	1.037 (0.611-1.760)	0.892
CxTx genotypes	13.1	(18)	18.4	(53)	0.671 (0.376-1.196)	0.174
C4T4 genotypes	2.2	(3)	1.4	(4)	1.590 (0.351-7.202)	0.686
C4 gene-cluster	13.9	(19)	7.3	(21)	2.047 (1.061-3.50)	0.030
T4 gene-cluster	20.4	(28)	19.1	(55)	1.088 (0.654-1.810)	0.745

Carrying frequency (%F) of each gene is defined as the number of individuals carrying the gene (N) divided by the number of individuals studied (*n*) in the given group. The *p* values are shown in bold, indicating significant (<0.05) differences. *KIR2DS4 was considered absent when the PCR amplified only the 22-bp deleted amplicon.

4 indicates the number of genes). On the basis of the presence and absence of C4 and T4 clusters, the Bx genotypes were further divided into the following four subsets: C4Tx (presence of C4 and absence of T4); CxT4 (absence of C4 and presence of T4); C4T4 (presence of both C4 and T4); and CxTx (absence of both C4 and T4).

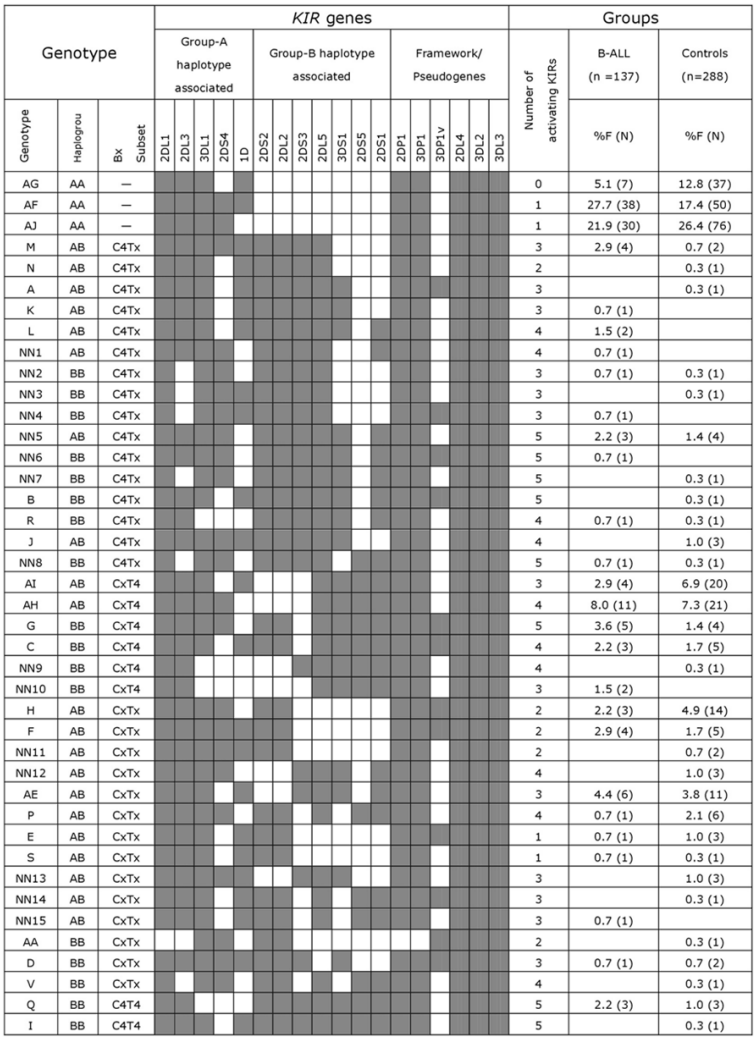
Statistical analysis

The observed carrying frequency of each *KIR* gene was calculated as the percentage of positive numbers among all specimens. The frequencies of genotypes and gene clusters were calculated as the percentage of the numbers of AA, AB, and BB genotypes and gene clusters among all specimens. Pearson's Chi-squared test or Fisher's exact test were used to analyze the differences between B-ALL patients and controls in the frequencies of carrying *KIR* genes, genotypes, and gene clusters. We also assessed the combined odds ratios (ORs) and corresponding 95% confidence intervals (CIs) for each variable. All data analyses were performed with SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA). A *P*-value <0.05 was considered significant.

Results

No difference between B-ALL patients and healthy controls in carrying frequencies of individual KIR genes

To better understand the impact of the underlying biology, 16 *KIR* genes were assessed in Chinese children with B-ALL (n=137) and healthy controls (n=288). A univariate comparison of *KIR* carrying frequencies between the B-ALL patients and healthy controls was performed (**Table 1**). As expected, *KIR3DL2*, *3DL3*,



Genotype			*KIR* genes																			Groups		
			Group-A haplotype associated					Group-B haplotype associated							Framework/ Pseudogenes							Number of activating KIRs	B-ALL (n =137)	Controls (n=288)
Genotype	Haplogrou	Bx Subset	2DL1	2DL3	3DL1	2DS4	1D	2DS2	2DL2	2DS3	2DL5	3DS1	2DS5	2DS1	2DP1	3DP1	3DP1v	2DL4	3DL2	3DL3			%F (N)	%F (N)
AG	AA	—																			0	5.1 (7)	12.8 (37)
AF	AA	—																			1	27.7 (38)	17.4 (50)
AJ	AA	—																			1	21.9 (30)	26.4 (76)
M	AB	C4Tx																			3	2.9 (4)	0.7 (2)
N	AB	C4Tx																			2		0.3 (1)
A	AB	C4Tx																			3		0.3 (1)
K	AB	C4Tx																			3	0.7 (1)	
L	AB	C4Tx																			4	1.5 (2)	
NN1	AB	C4Tx																			4	0.7 (1)	
NN2	BB	C4Tx																			3	0.7 (1)	0.3 (1)
NN3	BB	C4Tx																			3		0.3 (1)
NN4	BB	C4Tx																			3	0.7 (1)	
NN5	AB	C4Tx																			5	2.2 (3)	1.4 (4)
NN6	BB	C4Tx																			5	0.7 (1)	
NN7	BB	C4Tx																			5		0.3 (1)
B	BB	C4Tx																			5		0.3 (1)
R	BB	C4Tx																			4	0.7 (1)	0.3 (1)
J	AB	C4Tx																			4		1.0 (3)
NN8	BB	C4Tx																			5	0.7 (1)	0.3 (1)
AI	AB	CxT4																			3	2.9 (4)	6.9 (20)
AH	AB	CxT4																			4	8.0 (11)	7.3 (21)
G	BB	CxT4																			5	3.6 (5)	1.4 (4)
C	BB	CxT4																			4	2.2 (3)	1.7 (5)
NN9	BB	CxT4																			4		0.3 (1)
NN10	BB	CxT4																			3	1.5 (2)	
H	AB	CxTx																			2	2.2 (3)	4.9 (14)
F	AB	CxTx																			2	2.9 (4)	1.7 (5)
NN11	AB	CxTx																			2		0.7 (2)
NN12	AB	CxTx																			4		1.0 (3)
AE	AB	CxTx																			3	4.4 (6)	3.8 (11)
P	AB	CxTx																			4	0.7 (1)	2.1 (6)
E	AB	CxTx																			1	0.7 (1)	1.0 (3)
S	AB	CxTx																			1	0.7 (1)	0.3 (1)
NN13	AB	CxTx																			3		1.0 (3)
NN14	AB	CxTx																			3		0.3 (1)
NN15	AB	CxTx																			3	0.7 (1)	
AA	BB	CxTx																			2		0.3 (1)
D	BB	CxTx																			3	0.7 (1)	0.7 (2)
V	BB	CxTx																			4		0.3 (1)
Q	BB	C4T4																			5	2.2 (3)	1.0 (3)
I	BB	C4T4																			5		0.3 (1)

Figure 1. *KIR* gene content diversity of B-ALL and controls. Within 288 unrelated individuals representing Chinese Han populations, 41 genotypes that differed by the presence (*shaded box*) and absence (*white box*) of 16 *KIR* genes were observed. The frequency of each genotype is calculated as the percentage frequency (%F) of the number of individuals carrying the genotype (*N*) among the number of individuals studied (*n*) in the given group.

3DP1, *2DP1*, and *2DL4* were present in almost every sample (data not shown). In addition, the frequency of *KIR2DL1*, *KIR2DL3*, and *KIR3DL1* genes within the A haplotype were >90% in both groups. Conversely, marked variation was apparent in the genes associated with the B haplotype. Compared with healthy controls, the B-ALL group exhibited an increased presence of B haplotype centromeric genes (*KIR2DL2*, *KIR2DS2*, and *KIR2DL5*) as well as *KIR2DS3* (**Table 1**). The carrying frequencies for all of the KIR genes were not statistically different between children with B-ALL and the healthy controls (**Table 1**).

KIR gene content diversity in B-ALL patients and healthy controls

Within the study panel of 137 B-ALL Chinese children and 288 healthy controls, we identified 41 distinct *KIR* gene content profiles (genotypes) carrying a different number and combination of 16 *KIR* genes (**Figure 1**) that comprised 27 genotypes in the B-ALL group and 34 genotypes in the healthy controls. Fifteen genotypes designated as NNx were not reported by Hsu et al. [15]. The dominant genotypes (AF, AG, and AJ) comprised the homozygous combination of A haplotypes. The combined frequencies in the 2 groups (54.7% and 56.6%, respectively) were comparable to those reported in the Chinese population [19]. Conversely, the BB genotypes were more frequently represented in B-ALL patients than controls but did not reach statistical significance (13.9% vs. 8.3%, P=0.077). The C4Tx genotype was significantly increased in the B-ALL patients compared with the healthy controls (11.7% vs. 5.9%, P=0.038). Consequently, the frequency of the C4 gene cluster was significantly increased in B-ALL patients compared with the healthy controls (13.9% vs. 7.3%, P=0.030). Conversely, there was no significant difference in the frequency of the telomeric *KIR* gene cluster (*KIR3DS1-2DL5-2DS1-2DS5*) between the 2 groups (20.4% vs. 19.1%, P>0.05; **Table 1**).

Discussion

We report herein the first case-control association study to show an impact of *KIR* gene clusters on childhood B-ALL. Indeed, this is one of the largest studies focusing on the association between *KIR*-gene clusters and childhood

B-ALL. Our analysis provides the first evidence of an association between the C4Tx genotype and the centromeric *KIR* gene cluster (*KIR2DS2-2DL2-2DS3-2DL5*) and susceptibility of Chinese children to B-ALL.

In previous case-control studies involving *KIR*s, small sample sizes, age-of-onset effects, different types of leukemia, and heterogeneity of the populations made it impossible for researchers to draw definitive conclusions [12]. Several previous studies focused on adult leukemia patients [6-8, 20]. Specifically, one group [20] has shown the involvement of an inhibitory *KIR-HLA* gene in susceptibility to chronic lymphoid (n=31) and myeloid leukemia (n=48) in a Belgian population, but did not demonstrate an association between KIR-HLA ligand interactions and acute lymphoid leukemia. In addition, Verheyden et al. [6] reported that *KIR2DS2*, a gene characteristic of the C4 *KIR* gene cluster, had a significantly higher frequency among patients with various types of leukemia (ALL [n=8]). The data support the possibility that *KIR2DS2* increases the risk of leukemia [6]. In another study, Giebel et al. [7] demonstrated a protective role for *KIR2DS4* in the regulation of NK cell-mediated immunosurveillance against chronic myeloid leukemia (n=31) in patients of Polish and German origin, but no associations with AML (n=38) and ALL (n=21) were evident. Yet another study [8] showed that the frequency of *KIR2DS4* in Chinese patients with chronic myeloid leukemia (n=135) was higher than in healthy controls, but a significant protective association existed between *KIR2DS3* and ALL (n=61).

B-ALL is the most frequent form of leukemia affecting children. Almalte et al. [11] performed a case-control study in Canadian children of French origin by studying the frequency of all 6 activating *KIR* genes (n=100 [B-ALL] and n=245 [controls]). Almalte et al. [11] found that harboring activating *KIR* genes is associated with a reduced risk for developing B-ALL in these children, and the higher number of activating *KIR* genes the lower the risk for developing B-ALL. Conversely, Babor et al. [12] reported no association between individual *KIR* genes and childhood B-ALL with relatively larger samples of B-ALL patients of European origin (n=185). Another study [21] found that the *KIR* A/A genotype frequency was significantly increased in children with ALL, which was in agreement with the Almalte et al. study [11]. One study [22] showed that *KIR* A homozygosity offered protection from adult leukemia in Chinese southern Han. NK cells from *KIR* A homozygous individuals were strongly cytotoxic to leukemic cells and the incidence of activating *KIR*s increased in childhood ALL cases compared to controls in northern Indians [23]. In the present study, 16 *KIR* genes, including activating and inhibiting *KIR* genes and *KIR* pseudogenes, were typed by PCR-SSP in 137 B-ALL Chinese Han children and compared with 288 healthy controls. The analysis enabled the identification of extended *KIR* genotypes. We observed that none of the individual *KIR* genes, whether activating or inhibiting *KIR* genes, tended to increase or reduce the risk for developing this type of leukemia ($P>0.05$). Our results were quite different from the report by Almalte et al. [11], but consistent with the results of Babor and colleagues [12], suggesting no association between individual *KIR* genes and childhood B-ALL. Due to the strong similarity and linkage disequilibrium between *KIR* genes it may be necessary to assess extended *KIR* genotypes to provide an important plausibility control [12]. Moreover, given the historic patient sample collections in the Almalte et al. study [11], high-quality sample collections are quite demanding for increasing amplification efficiency.

It is noteworthy that we are the first group to study the impact of *KIR* gene clusters on the susceptibility to childhood B-ALL in Chinese children. The B-ALL group had an increased frequency of the C4Tx genotype compared to healthy controls (**Table 1**). The *KIR* centromeric genecluster [*KIR2DS2-2DL2-2DS3-2DL5(C4)*] was significantly increased in the B-ALL group. Whatever the mechanism, the increased C4 gene cluster frequency in the B-ALL group suggested that the prevalence of B centromeric genes is a risk factor. As hypothesized, specific *KIR* genes or combinations of activating and inhibiting *KIR* genes may participate in the pathogenesis of leukemia, potentially explaining the remarkably different susceptibility to B-ALL in Chinese children.

The ligands and functions of activating KIRs are not documented as well as inhibiting KIRs. Thus, the mechanism involved in the regulation

of NK cell functions by activating KIRs is unclear. In addition, distinct expression characteristics of KIR, down-regulation of natural cytotoxicity receptors, and the lack of expression of ligands for activation receptors may contribute to disease progression [2, 24]. In fact, the cumulative evidence suggests that the interaction of KIR receptors and HLA-I ligands plays an important role in triggering an immune response against leukemia [25]. The leukemic cells can down-regulate the expression of HLA class I molecules, and in some situations, show a complete loss of a HLA class I allele [26]. Not all inhibitory signals mediated by the various KIRs are of equal weight [27]. Our findings shed light on the biologic implications of *KIR* gene associations in terms of the potential balance between inhibitory and activating effects. Perhaps all of the above factors would lead to one of only two outcomes (activation or inhibition of effector cells), a characteristic that is entirely predictable of a multigenic, functionally closely related, highly polymorphic family of genes.

In conclusion, we report here the first case-control association study to show the impact of *KIR* gene clusters on childhood B-ALL. Our results provide the first evidence for an association between childhood B-ALL and certain *KIR* genotypes and gene clusters. The C4Tx genotype, which encoding the C4 *KIR* gene cluster (KIR2DS2-2DL2-2DS3-2DL5), might predispose to susceptibility to B-ALL in Chinese Han children. In addition, we recommend further investigation of the expression and function of *KIR* genes, HLA ligands, and the expression of HLA class I molecules on leukemic cells to better understand the underlying mechanism(s) of possible NK cell dysfunction in B-ALL.

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Disclosure of conflict of interest

None.

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