Brief Report

LYMPHOID NEOPLASIA

KIR ligand C2 is associated with increased susceptibility to childhood ALL and confers an elevated risk for late relapse

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Key Points

- C2 confers increased susceptibility to childhood B-ALL.
- C2 is associated with increased risk of late relapse in childhood B-ALL.

A role for *HLA class I* polymorphism in childhood acute lymphoblastic leukemia (ALL) has been suggested for many years, but unambiguous associations have not been found. Here, we show that the *HLA-C*–encoded supertypic epitope C2, which constitutes a high-affinity ligand for the inhibitory natural killer (NK) cell receptor KIR2DL1, is significantly increased in ALL patients (n = 320; *P* = .005). Stratification for ethnicity and disease subtype revealed a strong association of *C2* with B-ALL in German cases (*P* = .0004). The effect was independent of *KIR2DS1* and *KIR2DL1* allelic polymorphism and copy number. Analysis of clinical outcome revealed a higher incidence of late relapse (>2.5 years) with increasing number of *C2* alleles (*P* = .014). Our data establish C2 as novel risk factor and homozygosity for C1 as protective for childhood B-ALL supporting a model in which NK cells are involved in immunosurveillance of pediatric B-ALL via interaction of KIR with HLA-C ligands. (*Blood*. 2014;124(14):2248-2251)

Introduction

Natural killer (NK) cells serve important functions in immunosurveillance of hematologic malignancies. Inhibitory killer cell immunoglobulin-like receptors (KIR) recognizing the *HLA-C*–encoded ligands C1 and C2 are central to this process. C2 is recognized by the inhibitory KIR2DL1 with high affinity and specificity as well as by the stimulatory KIR2DS1 receptor. KIR2DL2/3 interact with C1 ligands with less affinity and specificity, ie, KIR2DL2 and to a lesser extent KIR2DL3 show some cross-reactivity with C2 ligands.[1] In the setting of hematologic stem cell transplantation, the presence of C2 ligands was already identified as risk factor in patients with myeloid leukemia.[2-4] In lymphoblastic leukemia, recent studies suggest that KIR or KIR ligands may play a role.[5,6] Here, we asked the question if the *C1/C2* dimorphism constitutes a susceptibility locus for childhood acute lymphoblastic leukemia (ALL).

Study design

Patients and controls

We analyzed a cohort of 320 pediatric ALL cases treated at the University Hospital Düsseldorf between 1988 and 2013. Patient characteristics and inclusion and exclusion criteria are given in supplemental Table 1 and supplemental Figure 1, available on the *Blood* Web site. The healthy control cohort was taken from the Düsseldorf Bone Marrow Registry as part of a prospective *HLA* class I typing project involving randomly selected donors (n = 1515). Donors did not undergo any specific medical examination or selection according to health status, were not related to any of the patients, and were not previously selected for stem cell donation for an unrelated leukemic patient. German ethnicity was assigned to donors with first and last name of the German-speaking area and residency in Germany.

KIR and *HLA* genotyping

KIR genotyping was performed by polymerase chain reaction with sequence-specific primers (PCR-SSP) as previously described.[7] A total of 10% of samples were randomly retyped by an independent *KIR* typing method with full concordance.[8] Subtyping for *KIR2DL1* alleles (*KIR2DL1*001-004*) was done as previously described.[9] Additional SSPs were used for *KIR2DL1*004* (forward-CATCTTCTTCCAGGTAACCCCC, reverse-TTTTGTTGGAGCACCAGCA). *KIR2DL1* copy-number variation was analyzed by SYBR green–based real-time PCR (StepOnePlus PCR system; Applied Biosystems). Results were normalized to *KIR3DL2*, which served as internal 2-copy control gene (gene frequency in whites of 99.7%).[10] *HLA* class I typing was performed by Luminex technology (One Λ). Confirmatory typing for *C1/C2* epitopes was performed by PCR-SSP as previously described.[11]

Statistics

Two-tailed Fisher's exact test was used to compare categorized groups. Cumulative incidences of relapse and late relapse (defined as relapse occurring ≥30 months from remission[12]) were analyzed after adjusting for the competing risk of nonrelapse mortality. Interactions of different covariates on the analytical end point relapse were evaluated by stepwise proportional hazards general

Submitted May 2, 2014; accepted August 15, 2014. Prepublished online as *Blood* First Edition paper, August 27, 2014; DOI 10.1182/blood-2014-05-572065.

F.B. and A.R.M. contributed equally to this study.

The online version of the article contains a data supplement.

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Table 1. Association of *KIR* and *HLA* class I ligands with incidence of childhood ALL

	Cases		Controls			
	n	%	n	%	OR (95% CI)	*P*†
ALL						
C1/C1	98	30.6	592	39.1	0.69 (0.53-0.89)	**.0051**
C1/C2	161	50.3	695	45.9	1.19 (0.94-1.52)	.1563
C2/C2	61	19.1	228	15.0	1.33 (0.97-1.82)	.0764
C1 alleles‡	357	55.8	1879	62.0	0.77 (0.65-0.92)	**.0037**
C2 alleles	283	44.2	1151	38.0	1.29 (1.09-1.54)	
B-ALL, German						
C1/C1	57	27.9	420	41.2	0.54 (0.39-0.75)	**.0004**
C1/C2	110	53.9	463	45.4	1.44 (1.07-1.95)	.0312
C2/C2	37	18.1	137	13.4	1.44 (0.97-2.15)	.0987
C1 alleles‡	224	54.9	1303	63.8	0.69 (0.56-0.85)	**.0008**
C2 alleles	184	45.1	737	36.2	1.45 (1.17-1.80)	
Bw4/Bw4	15	11.0	139	13.7	0.78 (0.44-1.37)	.4243
Bw4/Bw6	68	50.0	481	47.6	1.10 (0.77-1.58)	.6478
Bw6/Bw6	53	39.0	391	38.7	1.01 (0.70-1.46)	1.0000
***KIR2DL1*§**						
0	9	6.3	16	2.7	2.45 (1.06-5.67)	.0393
1	42	29.4	172	28.7	1.04 (0.69-1.55)	.9182
2	91	63.6	409	68.2	0.82 (0.56-1.20)	.3216
3	1	0.7	3	0.5	1.40 (0.15-13.5)	.5756
**001/*002*	85	41.1	357	35.7	1.25 (0.92-1.70)	.1542
**003*	89	43.0	452	45.2	0.91 (0.68-1.23)	.5912
**004*	32	15.5	186	18.6	0.80 (0.53-1.20)	.3212
Other	1	0.5	4	0.4	1.21 (0.13-10.86)	1.0000
***KIR2DS1*‖**						
0	91	61.9	376	62.7	0.97 (0.67-1.40)	.9243
1	50	34.0	197	32.8	1.05 (0.72-1.54)	.8450
2	6	4.1	27	4.5	0.90 (0.37-2.23)	1.0000

†*P* values were calculated by Fisher's exact test, ORs are denoted with 95% CI, and significant *P* values (after correction for multiple testing according to the Bonferroni method) are highlighted in bold.

‡Allele frequency = (*x*/*n*)/2 (where *x* is the number of alleles and *n* the number of individuals).

§*KIR2DL1* copy number (from 0-3 gene copies) and subtyping (indicated alleles) was performed for all cases and controls having *C2*. *KIR2DL1*001* and **002* encode identical mature proteins and were not differentiated by our typing method.

‖*KIR2DS1* copy number (from 0-2 gene copies) was performed for all cases and controls having *C2*.

linear model (PHGLM) analysis using Cox regression models analyzing time to relapse with nonrelapse mortality as competing risk. Differences between time-to-relapse distributions were compared by a log-rank test.

Results and discussion

In order to examine the influence of *HLA-C*–encoded KIR ligands, a cohort of pediatric ALL patients (n = 320) was stratified into 3 groups according to the *C1/C2* dimorphism. As shown in Table 1, the frequency of patients with 2 *C1* alleles was significantly decreased, whereas the corresponding carrier frequency for *C2* was significantly increased in the ALL cohort ($P = .0051$). Furthermore, allele frequencies were significantly biased toward *C2* in ALL patients ($P = .0037$). This observation was true for B-cell ALL (B-ALL) (n = 273), whereas in T-cell ALL (T-ALL) (n = 43), the distribution was similar to controls (data not shown). Because *C1* and *C2* frequencies vary greatly between different ethnicities even within Europe, further analysis was restricted to B-ALL patients of German origin, which constituted the largest disease- and ethnically matched subgroup in our patient cohort (n = 204). *C2* allele frequencies of the German control cohort (n = 1020, 36.2%) were comparable to those from populations of the same area retrieved from a public database,[13] confirming that the control cohort indeed is representative for German ethnicity (supplemental Figure 2). Similar to the previous analysis comprising all cases, homozygosity for *C1* was significantly decreased in B-ALL patients of German ethnicity (Table 1). Of note, no significant difference was seen for *C1* carrier frequency (*C1/C1* + *C1/C2*) between cases and controls (81.9% vs 86.6%). In contrast, *C2* carrier frequency (*C1/C2* + *C2/C2*) was significantly increased in the German B-ALL cohort (72.1% vs 58.8%, $P = .0004$). Together with the fact that the odds ratios (ORs) are similar for *C1/C2* and *C2/C2* individuals (OR = 1.44) in B-ALL German cases (Table 1), this clearly suggests that presence of *C2* constitutes a risk factor in B-ALL, whereas homozygosity for *C1* confers an equally strong protective effect (Table 1).

It was next examined if frequencies of C2-specific *KIR* genes *KIR2DL1* and *KIR2DS1* were also affected. In our previous study of B-ALL patients (cases included in the present larger cohort), no significant deviations from control values were seen for any *KIR* gene.[14] However, epistatic interaction of the C2-specific *KIR2DL1* gene with C2 might be difficult to detect given the high carrier frequency of *KIR2DL1* in whites (>95%).[10,15] We thus attempted to detect a possible epistatic interaction by analyzing allelic and copy-number variation (CNV). *KIR2DL1* CNV of German B-ALL cases was comparable to German controls except cases with 0 copies, which were found more frequently in B-ALL patients (Table 1 and supplemental Table 2). However, following adjustment for multiple testing, significance was lost. We also considered allelic variation of *KIR2DL1* as possible contributing factor. Analysis of allele-specific expression in NK cells of healthy controls by flow cytometry revealed a significantly decreased expression of KIR2DL1*004, an allele that was previously shown to have impaired signal-transduction capacity (supplemental Table 3).[16] However, PCR-SSP subtyping for the most common alleles (covering >98% of *KIR2DL1* allotypes in whites)[15] including *KIR2DL1*004* did not reveal significant differences (Table 1 and supplemental Table 2). Similarly, no significant changes in frequency and CNV were found for the C2-specific stimulatory *KIR2DS1* (Table 1 and supplemental Table 2). Of note, *KIR2DS1* exhibits only minor allelic polymorphism (*2DS1*002* present in >97% of whites).[15] Thus, our thorough analysis of KIR2DL1 polymorphism did not reveal any overt epistatic interaction with C2 in B-ALL.

We next asked if *HLA-C*–encoded KIR ligands would also impact clinical outcome. Indeed, B-ALL patients homozygous for *C2* exhibited a nonsignificant increase in relapse frequency (Figure 1A). Importantly, time-dependent analysis revealed that *C1/C1* patients exhibited a very low frequency of late relapse and that the number of *C2* alleles was significantly associated with increasing risk for late relapse (Figure 1B). Distribution of known confounding factors (age, sex, initial leukocyte count, and immunophenotype) was comparable among the 3 groups (supplemental Table 1). Multivariate analysis including the above-mentioned confounding factors and considering the number of C2 ligands as covariate confirmed a significantly increased risk for relapse in B-ALL with C2 ligands ($P = .013$; OR = 2.32; 95% confidence interval [CI], 1.18-4.57). A significant association of C2 ligands with relapse was also seen when National Cancer Institute criteria for pediatric high-risk leukemia (white blood cell count ≥50 000/μL and/or age <1 year or >10 years) were applied for multivariate analysis ($P = .046$; OR = 1.21; 95% CI, 1.00-1.46). We next evaluated if the C2 effect was sensitive to the changes in

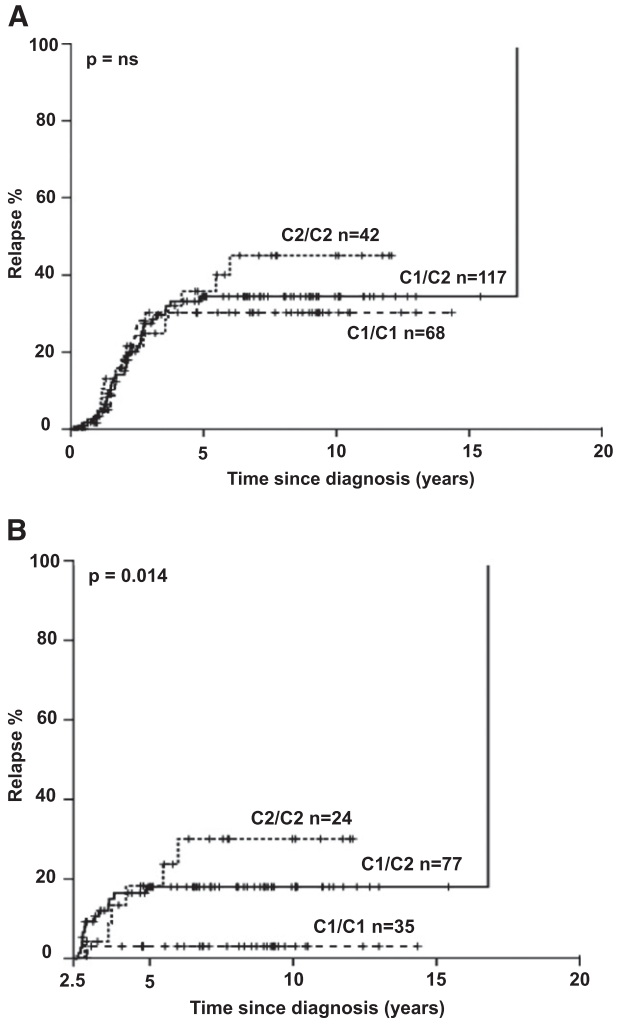

Figure 1. Increased frequency of late relapse in B-ALL patients with *HLA-C2*. Kaplan Meier analysis was performed for all B-ALL patients with available clinical and follow-up information. Patients not achieving remission during induction chemotherapy and patients undergoing hematopoietic stem cell transplantation without prior relapse were excluded from this analysis. Cases were divided into 3 groups based on *C1/C1*, *C1/C2*, and *C2/C2* genotypes. (A) Kaplan-Meier estimates are shown for all patients for the full observation time starting at the time of diagnosis. (B) Only patients who achieved complete remission, finished standard treatment, and remained event free for at least 2.5 years were analyzed (qualifying for late relapse ≥30 months after diagnosis). *P* values were calculated by 2-sided log-rank test; A: $P = .391$).

treatment protocols that were implemented in the last 2 decades. Splitting the cases into an "early" (1988-2003) and a "late" cohort (2004-2010) of similar size revealed a stable C2 effect, suggesting that evolution of treatment regimen did not confound analyses (supplemental Figure 3). Notably, no significant association with leukemia-free survival and overall survival was found (supplemental Figure 4).

The present study shows that HLA-C2 ligands not only are associated with increased susceptibility to B-ALL but also constitute a risk factor for late relapse. These observations are compatible with a model in which C2 ligands impair NK cell–mediated surveillance for leukemic cells at both the stage of leukemia initiation and in remission following completion of chemotherapy. Mechanistically, the inhibitory KIR2DL1 binds to C2 with higher affinity than KIR2DL2/3 to C1 and KIR2DL2 to C2. The exceptional specificity and affinity of the KIR2DL1-C2 interaction might lead to stronger inhibition of NK cells, which could compromise the efficiency of leukemia surveillance. The fact that we could not detect a major influence of *KIR2DL1* allelic variation or CNV is not necessarily at odds with this affinity model. Firstly, *KIR* CNV is associated with increased expression frequency but not with increased mean expression on the cell surface and does not majorly influence target cell recognition.[17] Thus, CNV effects on leukemia recognition might be quite subtle. Secondly, significant allele-specific expression differences were restricted to KIR2DL1*004, which is a relatively rare variant in whites. Our study might thus be underpowered to detect a significant allele-specific effect.

Notably, we did not find any association of the KIR ligand Bw4 with B-ALL (Table 1). It is currently unclear how these results compare with a recent study describing an association with Bw4 in non-Hispanic white ALL patients.[6] Notably, in both studies, high-affinity inhibitory KIR ligands seem to be associated with susceptibility to pediatric ALL.

This is the first study of childhood B-ALL reporting an association of KIR ligands with clinical outcome in a nontransplant setting. If confirmed in independent studies, these observations might have relevance for adjustment of therapy (eg, intensity, duration) to the increased risk of late relapse in B-ALL patients with C2 ligands, particularly those with homozygous C2 ligands.

Acknowledgments

The authors thank all parents who gave their consent to use the biological material from their minors and all volunteering adult blood donors.

This work was supported by the Deutsche Krebshilfe e.V. (M.U., R.M., and A.B.) (project 110351), the Forschungskommission of the Medical Faculty of the Heinrich-Heine-Universität Düsseldorf (F.B.), and the Deutsche Forschungsgemeinschaft (M.U.) (UH 91/7-1).

Authorship

Contribution: F.B. and A.R.M. contributed equally to this work; F.B., A.R.M., and M.U. designed the project, performed the experiments, and wrote the paper; N.S. performed the experiments; J.C.F. and J.E. contributed samples and performed statistical analysis; O.C. and A.M. performed analyses and revised the manuscript; A.B. and R.M. contributed clinical samples, treated patients according to the current therapy protocol, and critically revised the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

1. Parham P, Norman PJ, Abi-Rached L, Guethlein LA. Human-specific evolution of killer cell immunoglobulin-like receptor recognition of major histocompatibility complex class I molecules. *Philos Trans R Soc Lond B Biol Sci.* 2012; 367(1590):800-811.
2. Giebel S, Locatelli F, Wojnar J, et al. Homozygosity for human leucocyte antigen-C ligands of KIR2DL1 is associated with increased risk of relapse after human leucocyte antigen-C-matched unrelated donor haematopoietic stem cell transplantation. *Br J Haematol.* 2005;131(4): 483-486.
3. Hsu KC, Gooley T, Malkki M, et al; International Histocompatibility Working Group. KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy. *Biol Blood Marrow Transplant.* 2006; 12(8):828-836.
4. Fischer JC, Ottinger H, Ferencik S, et al. Relevance of C1 and C2 epitopes for hemopoietic stem cell transplantation: role for sequential acquisition of HLA-C-specific inhibitory killer Ig-like receptor. *J Immunol.* 2007;178(6):3918-3923.
5. Almalte Z, Samarani S, Iannello A, et al. Novel associations between activating killer-cell immunoglobulin-like receptor genes and childhood leukemia. *Blood.* 2011;118(5): 1323-1328.
6. de Smith AJ, Walsh KM, Ladner MB, et al. The role of KIR genes and their cognate HLA class I ligands in childhood acute lymphoblastic leukemia. *Blood.* 2014;123(16):2497-2503.
7. Vilches C, Castaño J, Gómez-Lozano N, Estefanía E. Facilitation of KIR genotyping by a PCR-SSP method that amplifies short DNA fragments. *Tissue Antigens.* 2007;70(5):415-422.
8. Uhrberg M, Parham P, Wernet P. Definition of gene content for nine common group B haplotypes of the Caucasoid population: KIR haplotypes contain between seven and eleven KIR genes. *Immunogenetics.* 2002;54(4): 221-229.
9. Shilling HG, Guethlein LA, Cheng NW, et al. Allelic polymorphism synergizes with variable gene content to individualize human KIR genotype. *J Immunol.* 2002;168(5):2307-2315.
10. Jiang W, Johnson C, Jayaraman J, et al. Copy number variation leads to considerable diversity for B but not A haplotypes of the human KIR genes encoding NK cell receptors. *Genome Res.* 2012;22(10):1845-1854.
11. Frohn C, Schlenke P, Ebel B, Dannenberg C, Bein G, Kirchner H. DNA typing for natural killer cell inhibiting HLA-Cw groups NK1 and NK2 by PCR-SSP. *J Immunol Methods.* 1998;218(1-2): 155-160.
12. Borgmann A, von Stackelberg A, Hartmann R, et al; Berlin-Frankfurt-Münster Relapse Study Group. Unrelated donor stem cell transplantation compared with chemotherapy for children with acute lymphoblastic leukemia in a second remission: a matched-pair analysis. *Blood.* 2003; 101(10):3835-3839.
13. Gonzalez-Galarza FF, Christmas S, Middleton D, Jones AR. Allele frequency net: a database and online repository for immune gene frequencies in worldwide populations. *Nucleic Acids Res.* 2011; 39(Database issue):D913-D919.
14. Babor F, Manser A, Schönberg K, et al. Lack of association between KIR genes and acute lymphoblastic leukemia in children. *Blood.* 2012; 120(13):2770-2772, author reply 2772.
15. Vierra-Green C, Roe D, Hou L, et al. Allele-level haplotype frequencies and pairwise linkage disequilibrium for 14 KIR loci in 506 European-American individuals. *PLoS ONE.* 2012;7(11): e47491.
16. Bari R, Rujkijyanont P, Sullivan E, et al. Effect of donor KIR2DL1 allelic polymorphism on the outcome of pediatric allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol.* 2013; 31(30):3782-3790.
17. Béziat V, Traherne JA, Liu LL, et al. Influence of KIR gene copy number on natural killer cell education. *Blood.* 2013;121(23):4703-4707.