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NK-KIR Gene Repertoire and Outcome of Patients with Acute Myeloid Leukemia after Allogeneic Hematopoietic Cell Transplantation from Unrelated Donors

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Abstract: In this retrospective study, the influence of donor and recipient KIR-gene content and their respective ligands including clinical parameters as potential confounding variables on the outcome of 150 acute myeloid leukemia (AML) patients undergoing allogenic hematopoietic cell transplantation (HCT) from unrelated donors was systematically investigated. There was no significant influence of KIR ligand mismatching and of donor/recipient KIR haplotype combinations on overall survival (OS), disease free survival (DSF), non-relapse mortality (NRM) and relapse. Isolated effects of KIR haplotypes, were detected for acute, chronic Graft versus Host Disease (aGvHD and cGvHD) as well as for the cumulative incidence of non-relapse mortality and relapse. The incidence of non-relapse mortality was evaluated in donor and recipient pairs harbouring KIR AA homozygosity (AA/Bx: p=0.038, HR=0.73, 95% CI=0.35-1.46 and AA/AA: p=0.043, HR=0.64, 95% CI 0.53-1-17). Our data suggest that KIR genotyping may be useful in patients in whom several HLA-identical unrelated donors can be identified but is probably not necessary for the primary donor selection algorithm.

Keywords: KIR, stem cell transplantation, GvL.

1. INTRODUCTION

Natural killer (NK) cell effector function plays a pivotal role in cytotoxicity against tumor cells and control of tumor growth [1]. In allogeneic hematopoietic cell transplantation, NK cells may contribute to graft-versus-leukemia (GvL) effects [2-4]. NK cells are regulated by surface-expressed activating and inhibitory killer-cell-immunoglobulin-like receptors (KIR). The latter interact with human leukocyte antigen class I molecules (HLA) [5, 6]. To date 14 functional KIR genes and two pseudogenes have been identified [7] . Inhibitory KIRs interact mainly with HLA-C group 1 or group 2 molecule [8, 9].

Until now, several different models have been put forward to account for the influence of KIR-ligands on HCT. i) In the ligand model, the absence of specific HLA ligands in the recipient for donor inhibitory KIR, enhances NK cell activity and supports GvL effects and is based on HLA-C ligand status (ligand-ligand model or "missing-self" theory). ii) In a second model, receptor-ligand model, based on the setting that donor's KIR genotype includes at least one inhibitory KIR gene that has no cognate ligand in the recipient, three different subgroups can be defined in this model: (a) the donor possesses 2DL2/2DL3 and the patients lacks HLA-C ligand groups 1: (b) the donor is positive for 2DL1 and the patients lacks HLA-C group 2 or (c) the donor is positive for 3DL1 and the patients lacks Bw4. The impact of KIR-ligand incompatibility on the outcome of HCT remains controversial. Several reports have suggested that a KIR-epitope mismatch is associated with improved OS and decreased relapse incidence [10-13], whereas some studies published the detrimental effect of the incompatibility of KIR-epitopes incompatibilities [14-20]. iii) The third model takes into consideration of the patient's and donor KIR genotype [20-22], or number of donor's activating KIR [18, 16].

2. MATERIAL AND METHODS

Hematopoietic Stem Cell Transplantation

The aim of this retrospective study was to systematically analyse the contribution of KIR genes and their respective ligands on the outcome of AML patients undergoing allogenic HCT from unrelated donors including clinical parameters as potential confounding variables.

This retrospective study included a total of 150 patients with acute myeloid leukemia (AML) who had received allogeneic HCT at the Transplant Center of the Department of Medicine I of the University Hospital Dresden between 1998 and 2006. The patient's characteristics are summarized in Table **1**.

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In 139 cases, patients were transplanted with G-CSF-stimulated peripheral blood stem cells (PBSC) while bone marrow (BM) was used in 11 cases. In vivo T-cell depletion was applied in 29 cases. The conditioning regimen was standard myeloablative and consisted mainly of total body irradiation (TBI) (12Gy) and cyclophosphamide (120 mg/kg) or busulfan (14-16 mg/kg) and fludarabine. Reduced intensity protocols, i.e. TBI ≤ 6 Gy, busulfan ≤ 8 mg/kg or cyclophosphamide ≤ 60 mg/kg were used in 7 transplantations. For the prophylaxis and treatment of graft-versus-host disease cyclosporine A, tacrolimus, mycophenolatmofetil, everolimus and methotrexate was used.

Table 1. Patients Characteristic

Patient Characteristic	Data
Number of patient	150
Sex	
Male	82
Female	68
Median age, years (range)	53 (18-76)
Mean of follow-up	23.4 (0.7-87.9) months
Diagnosis	
AML	150
Transplantation status	
Complete remission 1/Complete remission 2	81
Refractory/Relapse	53
HLA compatibility	
Mismatch	25
Match	125
Stem cell source	
PBSC	139
Bone marrow	11
Conditioning Regimen	
Standard	143
Reduced Intensity	7
T-cell Depletion	29
KIR- receptor-ligand compatibility matched	68
mismatched	82
Donor/Recipient KIR haplotype	
AA/AA	15
AA/Bx	32
Bx/AA	24
Bx/Bx	79
Recipient/donor HLA-C ligand status	
C1C1/C1C1	53
C1C1/C1C2	2
C1C2/C1C1	3
C1C2/C1C2	72
C2C2/C1C2	1
C2C2/C2C2	19

Genotyping for KIR and HLA-C Ligand

KIR Genotyping

The presence and the absence of 14 KIR genes in donor and recipient including KIR2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, 3DL3, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DS1, and two pseudogenes, 2DP1 and 3DP1 were analysed by sequence-specific-primer polymerase chain reaction (SSP-PCR) from archived DNA samples using whole genome amplification as previously described [22].

HLA-C ligand Genotyping

KIR haplotype were assigned according to McQueen and coworkers [21]. The distinction of HLA-C ligand groups 1 (N80) and 2 (K80) was also performed by SSP-PCR and HLA-C ligand status was assigned following the protocol of Frohn and coworkers [23].

Statistical Analysis

The following outcomes parameters were investigated in this study: overall survival (OS), disease- free survival (DFS), cumulative incidence of relapse and non-relapse mortality, incidence of acute and chronic GvHD.

For the later analysis, death from other causes than AML and relapse were regarded as competing risk, the same was true for death and the incidence of aGvHD and cGvHD. Whereas OS and DFS was assessed by Kaplan-Meier survival analysis and compared by using log-rank tests, non-relapse mortality and incidence of relapse and aGvHD were analyzed by cumulative incidence estimation. The incidence of cGvHD was calculated using crosstabulation (Chi-Square). Acute and chronic GvHD were diagnosed and graded using established criteria [24, 25]. aGvHD defines development of grade 1-4 GvHD during the first 100 days after transplantation. cGvHD was defined as GvHD occurring more than 100 days post-transplantation. McNemar's test was used for pairwise comparison of KIR gene frequencies in patients and donors. In addition, clinical variables, i.e. remission status, transplant source, transplant manipulation, conditioning regimen, sex-status, CMV-status and HLA match were considered as potential confounding variables and their contribution were also assessed. All statistical procedures were carried out as implemented in SPSS, version 16 (SPSS Inc., Chicago, IL, USA). In this report any p-value < 0.05 is regarded as statistically significant. No attempt was made to adjust the p values in this study.

3. RESULTS

Influence of Clinical Variables on the HSCT Outcome

Univariate analysis revealed that HLA matching contributed significantly to a better outcome. For OS, 44.29 vs.

Table 2. Pairwise Comparison of KIR gene Frequencies in Patients and Donors

	Donor/Recipient					
KIR Genes	**(+/+)**	**(-/-)**	**(+/-)**	**(-/+)**	**Chi-Square**	**p-value**
2DL1	142	0	7	0		
2DL2	50	31	27	41	2.485	0.115
2DL3	118	5	11	15	0.346	0.556
2DL4	45	39	33	32	0.000	1.00
2DS1	48	40	30	31	0.000	1.00
2DS2	52	34	26	37	1.587	0.208
2DS3	20	75	24	30	0.463	0.496
2DS5	19	73	34	23	1.754	0.185
3DS1	29	52	37	31	0.368	0.544

Table 3. Univariate Analysis

Independent Variables	Cohort Size	Outcome Parameter	Association	Data	Statistical Test	p-value
Clinical variables						
HLA-matching	125/150	OS	improved	44.29 vs. 18.87 months	KM	0.006
	125/150	DFS	improved	44.46 vs. 17.90 months	KM	0.008
KIR and KIR ligands						
Donor/recipient (KIR haplotype)						
Bx/Bx	79/150	aGvHD	high incidence	72.37 vs 54.29	CI	0.01
	79/150	cGvHD	decreased		CT	0.04
AA/AA	15/150	cGvHD	increased		CT	0.007
	15/150	mortality	high incidence	53.33 vs 24.16	CI	0.04
AA/Bx	32/150	mortality	high incidence	40.32 vs 23.18	CI	0.03
Recipient/donor (HLA-C ligand) status						
C2C2/C2C2	19/150	aGvHD	high incidence	38.89 vs 67.19	CI	0.03
Number of donor's inhibitory KIR genes						
donor> recipient (2 genes)	10/150	aGvHD	increased		CT	0.04
Number of donor's activating KIR genes						
donor > recipient (3 genes)	15/150	aGvHD	increased		CT	0.03
donor > recipient (4 genes)	9/150	aGvHD	increased		CT	0.04
Influence of single KIR genes						
R2DL3	133/150	DFS	improved		KM	0.03

CI: Cumulative incidence estimation, CT: Cross tabulation (Chi-square), KM: Kaplan-Meier analysis

Table 4. Multivariate Analysis

	Overall Survival (OS)			
	n	**Harzard Ratio (HR)**	**95% Confidence Interval (CI)**	**p-value**
HLA-matching				
HLA-matched	125			

Table 4. contd…

	Overall Survival (OS)			
	n	**Harzard Ratio (HR)**	**95% Confidence Interval (CI)**	**p-value**
HLA-mismatched	25	0.503	0.311-0.812	0.005
Condition of regimen				
standard	143			
reduced intensity	7	0.638	0.243-1.680	0.363
Transplantation status				
CR1/CR2	81			
non-CR	53	2.352	1.489-3.716	<0.001
Stem cell source				
PBSC	139			
bone marrow	11	1.336	0.586-3.045	0.491
Patient CMV status				
Positive	87			
negative	63	1.089	0.689-1.722	0.716
Donor/reciepient KIR haplotype				
AA/AA	15	1.517	0.660-3.488	0.326
AA/Bx	32	0.725	0.359-1.462	0.369
Bx/Bx	79	0.646	0.355-1.179	0.155
Donor/recipeint HLA-C ligand status				
C2C2/C2C2	19			
non-C2C2/C2C2	131	1.002	0.479-2.096	0.996
KIR-receptor ligand compatibility				
matched	68			
mismatched	62	1.090	0.689-1.723	0.731

18.87 month survival (p=0.006) and for DFS 44.46 vs. 17.90 month survival (p=0.008) was noted (Table **3**).

Multivariate analysis showed a significant influence of HLA compatibility (HLA-matched vs mismatched: p=0.005, HR=0.503, 95% CI=0.311-0.812) and of transplantation status (CR vs non-CR: p<0.001, HR=2.352, 95% CI=1.489-3.716) on OS (Table **4**).

KIR Gene Content and KIR Ligand Comparison.

KIR Gene Content

Comparison of KIR gene frequencies revealed no significant differences in frequencies between donors and recipients as analyzed by pairwise comparison (Table **2**).

Recipient and Donor's HLA-C Ligand Status

An incidence of aGvHD (p=0.03) for donor/recipient involving C2C2/C2C2 was noted.

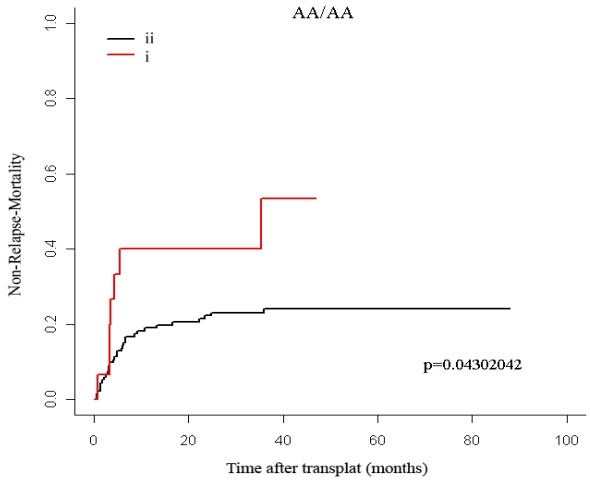
KIR Matching Analysis

KIR Ligand-Ligand Model

Based on HLA-B and -C analysis transplants were considered KIR-ligand mismatched when the recipient lacks KIR ligands present in the donor. There was no HLA-Bw4 ligand mismatch in GvH direction observed. For HLA-C-encoded ligands, a mismatch in GvH direction was observed in 3 cases.

KIR Receptor-Ligand Model

KIR ligand mismatch was seen in 54.6% (82/150) of the cases on the receptor-ligand model. In the univariate and multivariate analyses KIR-ligand incompatibility revealed no significant impact on any end-points investigated in this study.



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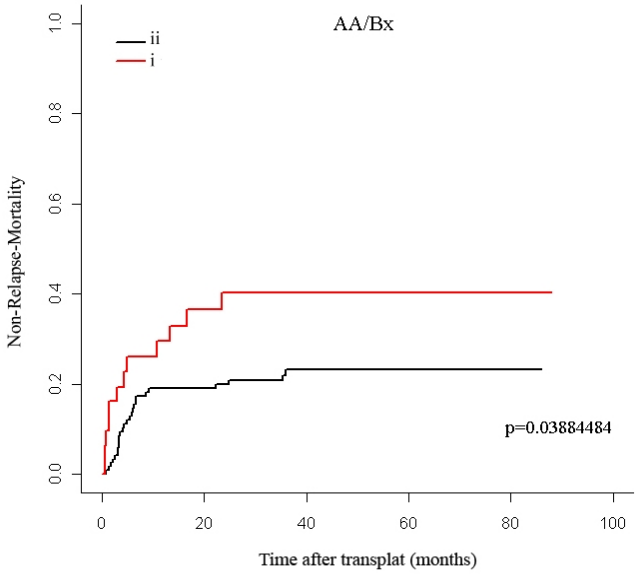

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Fig. (1). Cumulative incidence estimation of non-relapse-mortality in donor involving KIR AA haplotype. (**1A**) Donor and recipient carrying KIR AA/AA haplotype: (i) donor and recipient having AA/AA KIR haplotype (n=15/150). (ii) donor and recipient with non AA/AA KIR haplotype (n=135/150). (**1B**) Donor and patient having KIR AA/Bx haplotype: (i) donor and recipient classified to AA/Bx KIR haplotype (n=32/150). (ii) donor and patient with non AA/Bx (n=118/150).

KIR haplotype Analysis

The influence of donor and recipient KIR genotype on OS, DFS, a/cGvHD, NRM and relapse was analyzed for the following combinations of donor/recipient genotype AA/AA, AA/Bx, Bx/AA and Bx/Bx. There was no association of donor/recipient KIR genotype on OS and DFS. Analyses with regard to NRM: the donors carrying KIR AA Haplotype was associated with non-relapse mortality (AA/Bx: p=0.03, HR=0.73, 95% CI=0.35-1.46 and AA/AA: p=0.04, HR=0.64, 95% CI=0.35-1.17) (Fig. **1**). Analyses with regard to acute and chronic GvHD: high incidence of aGvHD (p=0.012, HR=0.646, 95% CI=0.055-1.179) was noted for donors and patients with Bx/Bx combination. The occurrence of cGvHD (p=0.007) was observed in recipient and donor involving AA/AA combination.

Quantitative Distribution of Inhibitory and Activating KIR Receptors

When the distribution of inhibitory and activating genes in donors and recipients was compared, no significant heterogeneity was seen. An increased risk of aGvHD (p=0.04) and the number of donor and recipient inhibitory genes was

observed when donors had two inhibitory KIR genes in excess of the recipient. There was an increased occurrence of aGvHD and the number of activating genes noted when donors had three (p=0.03) or four (p=0.04) activating KIR genes in excess of the patient.

KIR2DL3 in recipient was the single gene that significantly effect DFS (p=0.03).

4. DISCUSSION AND CONCLUSION

Our results indicated no significant impact of the recipient KIR HLA-C ligand status on OS, DFS, NRM and relapse while a high incidence of aGvHD (p=0.03) for donor/recipient involving C2C2/C2C2 was noted. Several studies described inferior OS and DFS in the presence of HLA-C2 in recipient [10, 26, 27]. This may be related to the reconstitution of NK cells after HCT as the C1-component NK cell (KIR2DL2/3) is expressed earlier and at higher frequency compared to the C2- component NK cell (KIR2DL1) [28]. Lastly, an inferior OS (p=0.01) and a higher rate of relapse (p=0.04) in recipients being C1C2 heterozygous was seen in comparison to C1 or C2 homozygous patients [27].

In contrast to the ligand-ligand model which only includes the HLA typing, both the KIR gene content and the HLA typing are considered in the receptor-ligand model. Although several studies suggested that KIR-ligand incompatibility should be considered for donor selection in HCT, the role of KIR-ligand mismatching in HCT is still controversial. The reasons for the discrepancy in the results of KIR-ligand mismatching on the outcome of HCT in literature may be related with the small sized and heterogeneous cohorts analysed. Furthermore, in some studies KIR typing was not performed, only HLA typing result (ligand-ligand model) was used to predict KIR epitope incompatibility. As KIR genes are located on chromosome 19q13.4 and segregate independently from HLA genes on chromosome 6. Therefore, assumptions, based on HLA typing do not reliably predict, KIR ligand incompatibility because of the putative lack of the corresponding gene in donor. This fact was supported by recent work of Leung and coworkers. The prediction of the risk of relapse was more accurate using the receptor-ligand model than with the ligand-ligand model [29].

Furthermore, Witt and co-worker suggested that the impact of NK-alloreactivity is strongly dependent on transplant protocol. Infusion of a very high dose of hematopoietic stem cells and *ex-vivo* T-cell depletion are potential prerequisites for higher NK cytotoxicity [30]. In regard to the transplant protocol, Giebel and coworkers have reported a benefit of alloreactivity of NK when antihymocyte globulin (ATG) *in vivo* T-cell depletion of donor and recipient was performed. However, this was not confirmed in other studies [14-16].

Several studies have indicated an influence of KIR gene and haplotype distribution on the outcome of HCT. Our results indicated that transplantation using donor having KIR AA haplotype leads to increase NRM (Fig. **1**). The incidence of aGvHD, but decreased cGvHD was noted for donors and recipients carrying Bx/Bx KIR haplotype. Cooley and coworkers reported that transplantation from donors carrying at least one B haplotype was associated with a significantly improved relapse free survival and overall survival compared to transplantation of grafts from type A haplotype donors [31]. Another study of Cooley indicated a significantly reduced relapse incidence and thus improved DSF in AML but not ALL patients transplanted from donors having 2 or more B gene-content motifs [32]. Another study has also suggested a benefit for A haplotype homozygous recipients receiving grafts from donors having at least one B haplotype [33]. This is in the line with our findings. Of course, the larger size of the cohort reported by Cooley increases its statistical power compared to the current analysis. In addition, we describe an increased incidence of aGvHD in donors and recipient pairs carrying KIR B heterozygosity. In contrast to the aforementioned study, the poorest survival due to aGvHD was noted in haplotype B heterozygous donor/haplotype A homozygous recipient (p=0.005) [21].

Lastly, when investigating the distribution of inhibitory and activating genes in donor and recipient, we found an association of aGvHD with the number of activating KIR genes in the respective donor. A high number of donor activating KIR genes (3 and 4 genes more than recipient) is a risk factor for the occurrence of aGvHD (p=0.03 and 0.04, respectively). Comparable results have been reported by other authors [18]. A high relapse rate in the presence of a high number of donor activating KIR genes (activating KIR ≥ 4 versus activating KIR ≤ 3, p=0.002) and a low number of donor activating genes associated with reduced disease free survival. Furthermore a higher rate of relapse was found in the presence of KIR B haplotype in the donor being homozygous (BB) or heterozygous (AB) compared to donors having an A haplotype [16]. These observations were consistent with the finding of Schellekens and her group who reported a high relapse rate may occur when patients have more activating KIR genes [34]. The presence of activating genes in the donor leads to enhanced alloreactivity of donor-derived NK cells. This could theoretically support GvL effects and thereby improve OS. On the other hand the immune reconstitution could be impaired and the risk of GvHD may be increased. This is in agreement with our results as the most favorable OS, but increased incidence of aGvHD was observed in donors and recipients having the Bx haplotype which implies a higher number of activating KIR in the donor.

Several studies have described a benefit for single KIR genes on HST outcome [20, 21, 34]. In our study recipient 2DL3 significantly improved DFS. A reduced risk of developing severe fibrosis after liver transplantation in presence of recipient KIR 2DL3 was published by de Arias and coworker [35]. In contrast to our study the absence of recipient 2DL3 and 2DL1 was reported to be associated with improved OS [34]. The underlying mechanism for the influence of patient KIR2DL3 on HCT outcome is still unknown. However, the influence of individual KIR genes on outcome of HCT should be carefully interpreted, as KIRs are prone to high linkage disequilibrium.

Strategies for donor selection incorporating KIR genotypes are still under development due to the uncertain underlying mechanism and the partly conflicting results reported for KIR-ligand mismatches. The absence of a ligand for the corresponding inhibitory KIRs in the recipient triggers NK-

alloreactivity. However, KIR2DS1 and KIR2DS2 have been reported to interact with HLA-C group 2 and group 1 respectively [36, 37]. When both inhibitory and activating KIRs interact with the cognate ligand, alloreactivity is determined by the balance of the signal. Most ligands of activating KIR have been not identified, thus it is difficult to extrapolate these algorithms. Our results suggest that KIR genotyping may provide additional information which could be utilized in selected cases for donor/recipient selection. Overall, however, controversies still prevail arguing against routine KIR typing of donor and recipient before allogeneic HCT.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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