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ORIGINAL ARTICLE

Prediction of NK Cell Licensing Level in Selection of Hematopoietic Stem Cell Donor, Initial Results

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Abstract Natural killer (NK) cell licensing status depends on clonal expression of inhibitory killer cell immunoglobulin-like receptors (iKIR) and short term HLA environment. Licensed NK cells are more efficient in tumor killing than unlicensed NK cells. Cognate KIR–HLA pairs in hematopoietic stem cell transplant (HSCT) donor and recipient are decisive for the possible change in the NK cell licensing status after HSCT. We assessed clinical outcomes in 297 patients with lymphoproliferative or myeloproliferative malignancies, or myelodysplastic syndrome in a model with upward licensing, downward resetting, and unchanged licensing genetics status after T cell replate HSCT from unrelated donors. We found extremely low (0%) relapse/progression incidence (RI), and better (59%) event-free survival (EFS) in recipients with upward licensing status and highly increased RI (37.5%), and reduced EFS (8%) among patients with the downward resetting status of repopulated donor NK cells after HSCT, as compared with unchanged NK cell licensing (RI 23%, EFS 47%). These trends were confirmed in adjusted multivariable models (for RI $p = 6.66E{-}09$, OR = 1.47, 95% CI 1.29–1.66 and for EFS $p = 3.79E{-}13$, OR = 1.67, 95% CI 1.50–1.84). Differences in the incidence of acute graft versus host disease (GvHD 62, 69, and 47%) and chronic GvHD (24, 44, and 15%, respectively) in three groups were insignificant. It would be rationale the preferential selection of the donors with upward licensing over downward resetting inhibitory KIR:HLA constellation and inclusion of the KIR genotyping in the donor selection algorithm for malignant patients. Further studies using enlarged cohorts of patients with more homogenous diagnosis are essential to reliably verify these preliminary data.

Keywords NK cell licensing · Hematopoietic stem cell transplantation · Unrelated donors · Bone marrow donor selection · Hematopoietic malignancy · Relapse

Introduction

Natural killer (NK) cells are involved in clearance of cancer cells in certain types of cancers via natural cytotoxicity (Re et al. 2006). As a biological function, the natural cytotoxicity is triggered by the cellular expression and engagement of NK cell receptors to target cell ligands rather than direct genomic content of any of their genes. Upon receptor engagement, NK cells integrate multiple signals from c-type lectin-like (e.g., NKG2), NK cell, and killer cell immunoglobulin-like receptors (KIRs), including inhibitory and activating receptors (Long et al. 2013; Nowak et al. 2015). Among NK cell receptors, inhibitory KIRs (iKIRs) are unique by their group specific ligation to ubiquitously expressed HLA class I ligands from C1, C2, Bw4 and/or A3/11 groups thus forming cognate KIR:HLA pairs (e.g., KIR2DL2/3:C1, KIR2DL1:C2, KIR3DL1:Bw4 and/or KIR3DL2:A3/11). In humans, the KIRs and HLA

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show extended polymorphism although their genes segregate independently of each other. As a consequence, every person expresses an individual pattern of cognate KIR:HLA pairs.

In allogeneic hematopoietic stem cell transplantation (HSCT), the expression of iKIRs on NK cells led to the discovery of NK cell alloreactivity. The event-free survival and relapse were superior in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) patients when transplanted from donors lacking the ligand for the corresponding iKIR (Giebel et al. 2003; Kawase et al. 2009; Oevermann et al. 2014; Ruggeri et al. 2002, 2007). Surprisingly, the negative effects of this kind of "missing ligand" recognition on survival and relapse have been also described, sometimes by the same research teams producing confusion on how to select better bone marrow donors or treat patients more efficiently (Brunstein et al. 2009; Davies et al. 2002). Yet, another research group observed no significant differences in cumulative incidences of relapse and non-relapse mortality in patients transplanted with KIR-ligand matched and mismatched donors but they observed a significantly lower risk of relapse with a KIR haplotype B donor (Michaelis et al. 2014). Together, these data suggest the important role of factors additional to KIR-ligand mismatch in NK cell tumor killing capacity. Among these factors, inhibitory receptor-ligand pairing and/or NK cell licensing variations may come into question.

To acquire the capacity to kill efficiently a target cell with low MHC class I expression, NK cells have to be educated by the detection of host MHC class I molecules by their cognate inhibitory receptors (a process also referred to as education or licensing) (Kim et al. 2005). Although generated by inhibitory KIRs, the NK cell licensing highly increases the NK cell reactivity against tumor in animal and human models (Anfossi et al. 2006; Kim et al. 2005). Furthermore, the education process requires the MHC class I to be expressed on all or most cells, or hyporesponsiveness is dominantly induced (Orr and Lanier 2010). Moreover, the NK cells reset their licensing status and lose their responsiveness when exposed to an MHC class I deprived environment (Joncker et al. 2010). The reverse, upward NK cell licensing is also possible and it takes only a few days in new MHC class I environment to change NK cell licensing level (Elliott et al. 2010). Fast reversible functional profile of NK cell licensing aroused our curiosity as to how often these changes in the genetics KIR:HLA licensing constellation of NK cells become effective in allogeneic transplant setting, and how these changes affect the frequency of relapse/progression and event-free survival in transplanted malignant patients. Our objective was to assess the potential differences in anticancer immunosurveillance when donors' NK cells were genetically assigned to upward licensed or downward reset groups in malignant recipient's HLA environment after HSCT as compared with unchanged licensing status.

Materials and Methods

All patients ($N = 297$) with myeloproliferative or lymphoproliferative malignant diseases had received T cell-replate HSCT between 2002 and 2010 from HLA-matched or mismatched unrelated donors including 10, 9 or 8 allele match out of the possible 10 alleles at HLA-A, -B, -C, -DRB1 and -DQB1 loci. Clinical records were, retrospectively, collected according to European Group for Blood and Marrow Transplantation (EBMT) guidelines by transplant physicians from nine Polish transplant centers (see "Acknowledgements").

Patient diagnosis and other characteristic distributions in total cohort and three compared groups are detailed in Table 1. Written informed consent was obtained from all patients. The study was approved by the Institutional Bioethics Committee of the Institute of Hematology and Transfusion Medicine, Warsaw and was performed in accordance with the Declaration of Helsinki.

KIR genotyping was performed on genomic DNA from unrelated HSC donors with the use of polymerase-chain-reaction amplification with sequence-specific primers. KIR genotyping kit (Inno-Train Diagnostik, Kronberg, Germany) was used according to the manufacturer's protocol, with minor modifications as detailed previously (Nowak et al. 2012). Sixteen blind samples were KIR-retyped for quality control and the results were consistent with primary data. Alleles of *HLA-A, -B,* and *-C* loci were assigned to four iKIR-ligating groups (C1, C2, Bw4, and A3/11), according to 77-83 amino acid residue sequence of the HLA class I heavy chain (Dorak 2014; Graef et al. 2009; Robinson et al. 2013). *HLA-A* alleles with Bw4 epitope were incorporated into Bw4 iKIR-ligand group (Foley et al. 2008). Subsequently cognate *iKIR:HLA* gene pairs were assigned according to the classification described by Long and Rajagopalan (2000) and Graef et al. (2009), who described ligation capacity of these receptors and ligands. Generally, four following *iKIR:HLA* cognate gene pair types were assigned in donors in different combinations: *KIR2DL2:C1* and/or *KIR2DL3:C1* (KIR2DL2/3:C1), *KIR2DL1:C2*, *KIR3DL1:Bw4* and *KIR3DL2:A3/11*, further called the licensing pairs.

In final study model, all donor–recipient pairs were assigned to one of the three groups predictive for NK cell licensing level, including: (1) downward resetting (licensing *iKIR:HLA* gene pair present in donor and cognate *HLA* ligand allele negative in recipient), (2) unchanged

Table 1 Patient characteristics and their distributions depending on predicted post HSCT NK cell licensing status

Patient characteristics at HSCT	Total	Predicted post HSCT NK cell licensing status		
	N (%)	Unchanged	Downward	Upward
Number	297 (100)	268 (90.2)	13 (4.4)	16 (5.4)
Age			$p = 0.46$	$p = 0.23$
Patient's age, years (>Median, %)	32 (2–60)	181 (68)	11 (85)	12 (75)
Diagnosis			$p = 0.71$	$p = 0.75$
AML	136 (46)	122 (46)	7 (54)	7 (44)
MDS	15 (5)	13 (5)	0 (0)	2 (13)
ALL	75 (25)	68 (25)	2 (15)	5 (31)
CML/CLL	59 (20)	52 (19)	4 (31)	3 (19)
Lymphoma	9 (3)	9 (3)	0 (0)	0 (0)
MM	7 (2)	7 (3)	0 (0)	0 (0)
Risk phase[a]			$p = 0.55$	**$p = 0.012$**
Low	160 (54)	146 (54)	8 (62)	6 (38)
Intermediate	52 (18)	44 (16)	1 (8)	7 (44)
High	58 (20)	53 (20)	4 (31)	1 (6)
Unknown	27 (9)	25 (9)	0 (0)	2 (13)
Donor ethnicity			$p = 0.20$	$p = 0.12$
Polish registry	62 (21)	60 (22)	1 (8)	1 (6)
External	229 (77)	202 (75)	12 (92)	15 (94)
Not specified	6 (2)	6 (2)	0 (0)	0 (0)
Graft source			$p = 0.45$	$p = 0.21$
PBSC	212 (71)	188 (70)	10 (77)	14 (88)
BM	72 (24)	68 (25)	2 (15)	2 (13)
Not specified	13 (4)	12 (4)	1 (8)	0 (0)
Conditioning regimen			$p = 0.98$	$p = 0.14$
Myeloablative	183 (62)	163 (61)	7 (54)	13 (81)
Reduced intensity	102 (34)	95 (35)	4 (31)	3 (19)
Unknown	12 (4)	10 (4)	2 (15)	0 (0)
Graft manipulation				
T cell replate	297 (100)	268 (100)	13 (100)	16 (100)
Immunosuppression			$p = 0.44$	$p = 0.49$
Calcineurin inhibitors[b]	242 (81)	217 (81)	10 (77)	15 (94)
Metothrexate	126 (42)	119 (44)	4 (31)	3 (19)
ATG[c]	250 (84)	225 (84)	11 (85)	14 (88)
Steroids	6 (2)	5 (2)	1 (8)	0 (0)
Graft failure			$p = 1.00$	$p = 1.00$
Graft failure	8 (3)	7 (3)	1 (8)	0 (0)
Final status			**$p = 0.016$**	$p = 0.11$
Alive in complete remission	130 (44)	122 (46)	1 (8)	7 (44)
Relapsed	60 (20)	56 (46)	4 (31)	0 (0)
Dead for non-relapse causes	82 (28)	69 (26)	7 (54)	6 (38)
Unknown	25 (8)	21 (8)	1 (8)	3 (19)
Graft versus host disease			$p = 0.36$	$p = 0.67$
Acute	146 (49)	127 (47)	8 (62)	11 (69)
Chronic	74 (25)	65 (24)	2 (15)	7 (44)
Unknown	90 (30)	83 (31)	5 (38)	2 (13)
HLA match			**$p < 0.00001$**	**$p < 0.00001$**
10/10	201 (68)	201 (75)	0 (0)	0 (0)

Table 1 continued

Patient characteristics at HSCT	Total	Predicted post HSCT NK cell licensing status		
	N (%)	Unchanged	Downward	Upward
9/10	77 (26)	55 (21)	9 (69)	13 (81)
8/10	19 (6)	12 (4)	4 (31)	3 (19)

The distributions in downward resetting and upward licensing groups were compared with unchanged licensing group using χ^2 test of independence. *p* values of χ^2 test are shown in upper rows

p values <0.05 are marked in bold

[a] Low risk phase, first complete remission (CR1) or chronic phase (CP1); Intermediate risk phase, CR2 or CP2; High risk phase, CR3-4, CP3-4, accelerated phase, relapse, progression or primary induction failure phase at the time of transplantation

[b] Calcineurin inhibitors, Cyclosporin A (CsA) or Tacrolimus

[c] *ATG* anti-thymocyte globulin, *AML* acute myeloid leukemia, *MDS* myelodysplastic syndrome, *ALL* acute lymphoblastic leukemia, *CML* chronic myeloid leukemia, *CLL* chronic lymphocyte leukemia, *Lymphoma* non-Hodgkin or Hodgkin lymphoma, *MM* multiple myeloma, *PBSC* peripheral blood stem cells, *BM* bone marrow

licensing, and (3) upward licensing (*iKIR* gene but not cognate *HLA* ligand allele present in donor and cognate *HLA* ligand allele present in recipient). Due to low level of HLA class I mismatch, the downward and upward licensing constellations did not overlapped in donor–recipient pairs from this study group.

Descriptive statistics, such as number and median for continuous variable and percentage for categorical variables were provided in Table 1. The distributions of patient's characteristics in groups of changed licensing genetics status were compared to unchanged licensing group using χ^2 test and *p* values are shown in upper rows in Table 1. For univariate analysis of the associations with outcome measures, the univariate Cox proportional hazard regression model was fitted to calculate the unadjusted *p*, hazard ratios (HR), and 95% confidence intervals (95% CI). The primary variables included the diagnosis, risk phase, patient's age and gender, time from diagnosis to HSCT, donor ethnicity, immunotherapy with anti-thymocyte globulin, immunotherapy with calcineurin inhibitors, conditioning regimen with myeloablation versus reduced intensity, graft source including peripheral blood stem cells (PBSC) as compared with bone marrow and HLA mismatch level with 0, 1 or 2 allele mismatch out of ten alleles tested in HLA-A, -B, -C, -DRB1, and -DQB1 loci. The risk phase is graded according to the following scale: (1) low risk phase, first complete remission (CR1) or chronic phase (CP1); (2) intermediate risk phase, CR2 or CP2; (3) high risk phase, CR3-4, CP3-4, accelerated phase, relapse, progression or primary induction failure phase at the time of transplantation.

Kaplan–Meier method and Cox regression models were used to examine the hazard ratios of failure for time-to-event outcomes of HSCT with *iKIR:HLA* licensing gene constellation change in recipients including times to relapse/progression, event-free survival (EFS), and acute and chronic graft versus host disease (aGvHD, cGvHD). Non-relapse mortality (NRM) was regarded as a competing risk for purposes of estimating the relapse/progression. Competing risks for relapse/progression and NRM were estimated using Gray's test. For multivariate analysis the stepwise linear regression model was used with backward elimination. The analysis started with full model including the variables with *p* value less than 0.15 in univariate analysis. The procedure was automatically stopped when no variable in the new original model could be removed and all the next best candidate could not be retained in the new original model. Then, the new original model was our selected model. Statistics were estimated and Kaplan–Meier survival analyses were made using Statistica 12.5 package (StatSoft, Tulsa, USA) or R version 3.2.1 (R Foundation for Statistical Computing, Vienna, Austria) (Scrucca et al. 2007) and *p* levels <0.05 were considered statistically significant.

Results and Discussion

In donors' genome, a different number of *iKIR:HLA* cognate gene pairs were encoded and could potentially change their licensing constellation in recipients. The frequencies of cognate *iKIR:HLA* pairs were similar in donors and recipients (see, Supplementary Table 1). Of the 297 patients studied, 29 (10%) of the donor–recipient pairs had changed NK cell licensing genetics status, including 13 (4.4%) with downward resetting and 16 (5.4%) with upward licensing gene pair constellation at transplantation (Table 1). Among 94 donor–recipient pairs with HLA-ligand mismatch, the unchanged, downward resetting and upward licensing *HLA-iKIR* gene pair states were in 65 (69%), 13 (14%), and 16 (17%) patients, respectively. This distribution suggests that HLA class I mismatch may result

in functionally different NK cell licensing settings in considerable proportions of HSCT recipients. The distribution of patients' characteristics (Table 1) were similar in study groups except the risk phase at transplantation in upward licensing group, where patients transplanted in intermediate phase were more frequent and those transplanted in low and high risk phases were less frequent than in unchanged licensing status group. As expected, HLA mismatch level distributions were significantly different in groups with downward resetting and upward licensing as compared with unchanged licensing group.

In univariate analysis (Table 2), the NK cell licensing status was significantly associated with relapse/progression ($p = 0.0026$, HR = 4.76, 95% CI 1.72–13.13) and EFS ($p = 0.0031$, HR = 2.32, 95% CI 1.32–4.06). Among independent variables, the risk phase was associated with relapse/progression ($p = 0.020$, HR = 1.43, 95% CI 1.05–1.93), EFS ($p = 0.0000021$, HR = 1.60, 95% CI 1.31–1.94) and NRM ($p = 0.00052$, HR = 1.59, 95% CI 1.22–2.07), and treatment with calcineurin inhibitors was associated with NRM ($p = 0.045$, HR = 1.90, 05% CI 1.01–3.54).

Table 2 Univariate association analysis of time-dependent outcome measures with independent variables including predicted post HSCT NK cell licensing status

Variable	*p*	HR	95% CI
Relapse/progression			
Risk phase	0.020	1.43	1.05–1.93
ATG	0.15	0.58	0.27–1.21
Conditioning regimen	0.060	1.77	0.97–3.19
NK cell licensing status	0.0026	4.76	1.72–13.13
EFS			
Diagnosis	0.053	1.08	0.99–1.15
Risk phase	0.0000	1.60	1.31–1.94
NK cell licensing status	0.0031	2.32	1.32–4.06
NRM			
Risk phase	0.0005	1.59	1.22–2.07
Calcineurin inhibitors	0.045	1.90	1.01–3.54
Conditioning regimen	0.12	0.68	0.41–1.10
PBSC (versus BM)	0.062	1.80	0.97–3.31
NK cell licensing status	0.21	1.64	0.75–3.57
Acute GvHD			
ATG	0.13	3.45	0.45–25.9
NK cell licensing status	0.92	0.71	0.00–47.8
Chronic GvHD			
HLA mismatch level	0.0010	2.10	1.34–3.26
NK cell licensing status	0.017	0.35	0.14–0.82

Shown are pairwise associations with p values <0.15 in Cox proportional hazard regression model

One hundred and forty six (49%) patients developed aGvHD, and 74 (25%) developed cGvHD. Cumulative incidences of aGvHD at day 100 in downward resetting, upward licensing, and unchanged licensing groups were relatively high (62, 69, and 47%, respectively) and were not associated with predicted NK cell licensing status ($p = 0.92$, HR = 0.71, 95% CI 0.00–47.8). The incidences of cGvHD (24, 15, and 44%, respectively) were significantly different in three strata ($p = 0.017$, HR = 0.35, 95% CI 0.14–0.82) in univariate analysis, accompanied by significant association of cGvHD with the level of HLA mismatch ($p = 0.0010$, HR = 2.10, 95% CI 1.34–3.26).

Sixty-one (21%) patients experienced relapse or progression of malignant disease after transplantation, and 82 (28%) experienced non-relapse mortality. Estimated 2-year cumulative incidence of relapse/progression in downward resetting group was 37.5% compared with 0% in upward licensing group and 23% in unchanged licensing group ($p = 0.0026$, HR = 4.76, 95% CI 1.72–13.13; Fig. 1a), indicating the relapse/progression effect is detrimental in downward resetting and favorable in upward licensing group as compared with unchanged licensing group. To adjust the potential influence of HLA mismatch level, we estimated the 2-year cumulative incidence of relapse/progression when three study groups were restricted to HLA class I mismatched donor–recipient pairs ($N = 83$). In HLA class I mismatched donor–recipient pairs, the distribution of 2-year relapse/progression remained similar to that in total cohort (37.5, 0, and 23% for downward resetting, upward licensing, and unchanged licensing group, respectively; $p = 0.0081$, HR = 4.62, 95% CI 1.49–14.34; Fig. 1b), suggesting that those mismatched HLA not involved in NK cell licensing are neutral for relapse/progression of malignant diseases in patients given T cell replate HSCT.

The 2-year EFS in patients transplanted from HLA mismatched donors with downward resetting, upward licensing, and unchanged licensing gene constellations were 8, 59, and 47%, respectively (HR = 1.83; $p = 0.020$; 95% CI 1.10–3.04; Fig. 2a).

In patients who received HLA mismatched transplant, the estimated 2-year cumulative NRM was 82% in downward resetting group and 48% in upward licensing group compared with 35% in unchanged licensing group. This trend was statistically insignificant ($p = 0.16$, HR = 1.65, 95% CI 0.82–3.33; Fig. 2b). The cumulative incidences with competing risk analysis confirmed statistically significant trend for relapse/progression (Gray's test, $p = 0.025$) and not significant trend for NRM ($p = 0.15$) in three study groups.

In multivariate analysis (Table 3), the NK cell licensing status remained the independent prognostic factor for relapse/progression ($p = 6.66E{-}09$, OR = 1.47, 95% CI

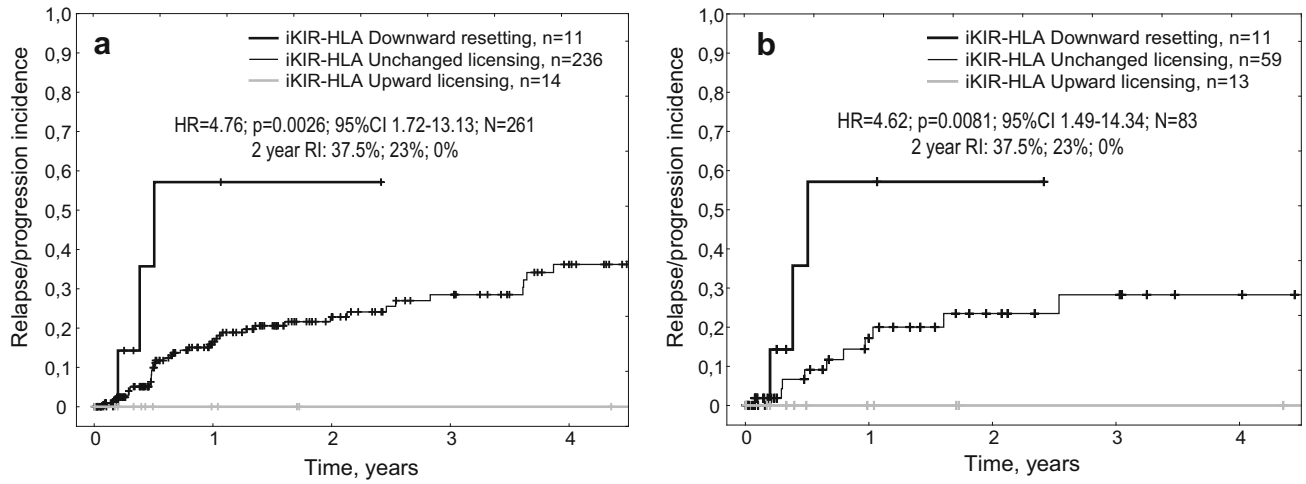

Fig. 1 Cumulative incidence of relapse/progression in total cohort of malignant HSCT recipients (**a**) and in HLA mismatched transplants subgroup (**b**) according to downward resetting, unchanged and upward licensing prediction of NK cell licensing level. *Vertical bars* assign censored cases

1.29–1.66) and EFS ($p = 3.79E{-}13$, OR = 1.67, 95% CI 1.50–1.84). Higher level of HLA mismatch was also associated with better EFS ($p = 9.52E{-}07$, OR = 1.29, 95% CI 1.16–1.41). For the NRM analysis, the predicted NK cell licensing status was excluded from multivariate model, whereas treatment with calcineurin inhibitors ($p = 4.20E{-}13$, OR = 1.65, 95% CI 1.45–1.87) and PBSC as graft source ($p = 0.013$, OR = 1.17, 95% CI 1.03–1.32) remained in final model.

Together, the adjusted multivariate models confirmed the NK cell licensing gene pair status being an independent prognostic factor for relapse/progression and EFS. Acute and chronic GvHD were not associated with NK cell licensing status, however, HLA mismatch level remained independent prognostic factor for cGvHD ($p = 0.025$, OR = 1.15, 95% CI 1.01–1.30), suggesting independence of the GvHD and post transplant NK cell licensing gene pair constellation.

Our results suggest that HLA class I KIR ligand allele and inhibitory receptor (iKIR) gene mismatch between unrelated donor and recipient can induce NK cell licensing variations of opposite direction. Both, downward resetting and additional upward licensing constellations of those genes are possible in considerable proportions of cases, depending on donor encoded KIR, and cognate HLA allele and recipient's mismatch in cognate HLA class I. We demonstrate that these gene constellations have functional impact in allogeneic HSCT from unrelated donor in malignant patients. The downward resetting genetics constellation results in greatly increased relapse/progression, and adverse EFS and the upward licensing constellation improve these outcomes, as compared to unchanged licensing. The influence of donor KIR and HLA ligand genotypes has been largely investigated focusing specifically on one or the other genetics system, often disregarding functional iKIR:HLA cognate pairs and showing some discrepant outcomes (Brunstein et al. 2009; Davies et al. 2002; Giebel et al. 2003; Kawase et al. 2009; Oevermann et al. 2014; Ruggeri et al. 2002, 2007). Of importance, inhibitory signal from iKIR:HLA engagement is only delivered to NK cell if activating pathways are triggered (Farag et al. 2002). Inhibitory signal interrupts the activation pathways at an early stage thus protecting healthy self cells with low or absent expression of stress-induced activating ligands. Inhibitory KIRs function as a protective mechanism for normal autologous cells, provided iKIR engagement by self HLA class I cognate ligands (Farag et al. 2002). On the contrary, malignant or otherwise transformed unhealthy cells strongly express different induced activating ligands. The engagement of these ligands by activating NK cell receptors shifts the signal to strong activation that, in transformed cells, overcomes the self iKIR:HLA inhibition and triggers efficient NK cell killing of those cells. Cancer cell biology dictates, however, that in many cancers the "low HLA" clones are selected via immunoescape. This clonal evolution is inconsistent with currently accepted hypothesis of "missing ligand" recognition where NK cells with expressed iKIR are more cytolytic to cancer cells deprived from cognate HLA. In contrast to studies with one genetics system (KIR or HLA ligand), the study of KIR and HLA systems together can systematically uncover certain licensing constellations depending on receptor–ligand pairs. NK cell licensing depends on simultaneous expression of iKIRs by NK cells and cognate HLA class I ligands in the environment, thus stimulating internal capacity of NK cells to kill tumors (Kim et al. 2005, 2008). The dynamics of acquiring and resetting the NK cell licensing

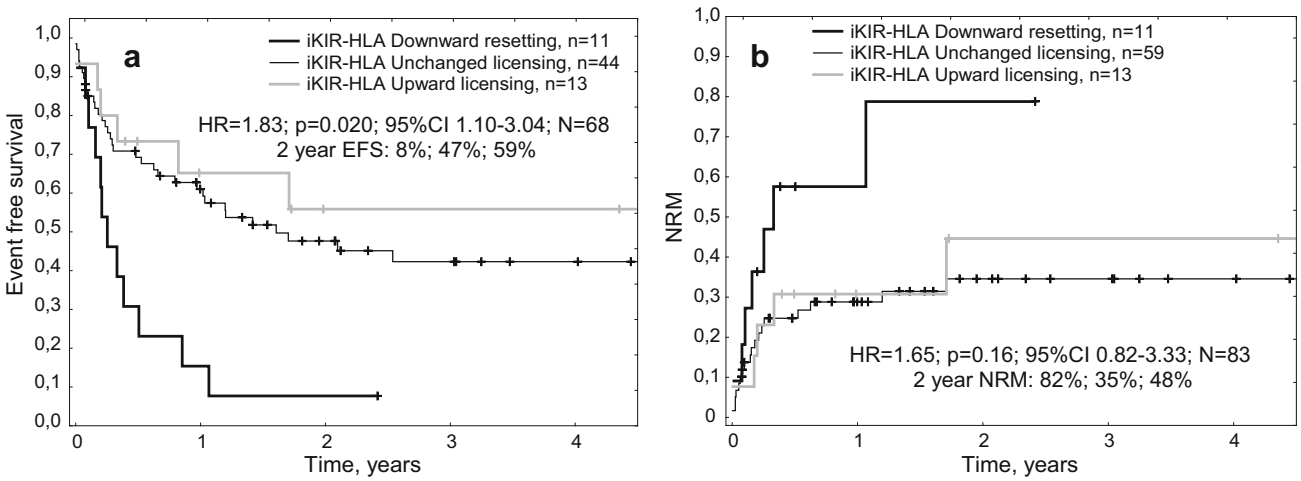

Fig. 2 Kaplan–Meier estimate of event-free survival (**a**) and non-relapse mortality (**b**) according to downward resetting, unchanged and upward licensing prediction of NK cell licensing level in HLA mismatched subgroup ($N = 83$). *Vertical bars* assign censored cases

Table 3 Multivariate analysis of time-dependent outcome measures with covariates and predicted post HSCT NK cell licensing status in HSCT patients

Variable	*F*	*p*	OR	95% CI
Relapse/progression				
NK cell licensing status	20.05	6.66E−09	1.47	1.29–1.66
EFS				
NK cell licensing status	33.21	3.79E−13	1.67	1.50–1.84
HLA mismatch level	25.21	9.52E−07	1.29	1.16–1.41
NRM				
Calcineurin inhibitors	29.55	4.20E−13	1.65	1.45–1.87
PBSC (versus BM)	6.22	1.33E−02	1.17	1.03–1.32
Acute GvHD				
ATG	8.61	3.58E−03	0.85	0.76–0.94
Chronic GvHD				
HLA mismatch level	5.03	2.58E−02	1.15	1.01–1.30

Shown are best models in stepwise regression linear model with backward elimination

F denotes test value

in a short time after the passing to the new HLA environment (Elliott et al. 2010; Joncker et al. 2010) promotes the occurrence of this phenomenon in allogeneic HSCT from HLA or KIR mismatched donor.

In our previous study, the transplanted malignant patients with missing HLA ligand, the one cognate to donor's iKIR:HLA pair showed a significantly worse outcome (Nowak et al. 2014) and in another study, donor–recipient KIR pairings with some HLA ligand groups conferred better control of leukemic relapse after haploidentical transplantation in AML and ALL patients (Zhao et al. 2015). These studies suggest that both effects are possible depending either on downward resetting or upward licensing receptor-ligand gene constellation between donor's NK cells and new HLA environment in recipient after HSCT.

Our study has several weaknesses. The study cohort, especially group with changed NK cell licensing gene constellation was small and heterogeneous in terms of risk phase at transplantation and the diagnosis. The expression of functional gene pairs was putative (based on ubiquitous expression of HLA class I and stochastic expression of iKIRs in healthy individuals) and NK cell killing capacity was not confirmed in vitro. Considering weaknesses, these results should be interpreted with caution.

In conclusion, although the number of patients analyzed was limited, this study shows that in malignant patients' given T cell-replate unrelated HSCT an

important role exists for NK cell licensing. We found significant advantage for malignant patients given the transplant from donors whose NK cells potentially acquired additional NK cell licensing in new HLA environment. In this cohort, equally probable was a significant aggravation of the relapse or progression in patients transplanted from donors whose NK cells potentially downward reset their licensing level in recipient's HLA environment. Therefore, it would be rationale the preferential selection of the donors with upward licensing over downward resetting iKIR:HLA constellation and inclusion of the KIR genotyping in the donor selection algorithm for malignant patients. Further studies using enlarged cohorts of patients with more homogenous diagnosis are essential to reliably verify these preliminary data.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest in connection with the authorship.

References

Anfossi N, André P, Guia S et al (2006) Human NK cell education by inhibitory receptors for MHC class I. Immunity 25:331–342

Brunstein CG, Wagner JE, Weisdorf DJ et al (2009) Negative effect of KIR alloreactivity in recipients of umbilical cord blood transplant depends on transplantation conditioning intensity. Blood 113:5628–5634

Davies SM, Ruggieri L, DeFor T et al (2002) Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. Killer immunoglobulin-like receptor. Blood 100:3825–3827

Dorak MT (2014) Major histocompatibility complex, http://www.dorak.info.hla. Accessed 21 Jan 2014

Elliott JM, Wahle JA, Yokoyama WM (2010) MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. J Exp Med 207:2073–2079

Farag SS, Fehniger TA, Ruggeri L et al (2002) Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. Blood 100:1935–1947

Foley BA, De Santis D, Van Beelen E et al (2008) The reactivity of Bw4+HLA-B and HLA-A alleles with KIR3DL1: implications for patient and donor suitability for haploidentical stem cell transplantations. Blood 112:435–443

Giebel S, Locatelli F, Lamparelli T et al (2003) Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. Blood 102:814–819

Graef T, Moesta AK, Norman PJ et al (2009) KIR2DS4 is a product of gene conversion with KIR3DL2 that introduced specificity for HLA-A*11 while diminishing avidity for HLA-C. J Exp Med 206:2557–2572

Joncker NT, Shifrin N, Delebecque F et al (2010) Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. J Exp Med 207:2065–2072

Kawase T, Matsuo K, Kashiwase K et al (2009) HLA mismatch combinations associated with decreased risk of relapse: implications for the molecular mechanism. Blood 113:2851–2858

Kim S, Poursine-Laurent J, Truscott SM et al (2005) Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature 436:709–713

Kim S, Sunwoo JB, Yang L et al (2008) HLA alleles determine differences in human natural killer cell responsiveness and potency. Proc Natl Acad Sci USA 105:3053–3058

Long EO, Rajagopalan S (2000) HLA class I recognition by killer cell Ig-like receptors. Semin Immunol 12:101–108

Long EO, Kim HS, Liu D et al (2013) Controlling natural killer cell responses: integration of signals for activation and inhibition. Annu Rev Immunol 31:227–258

Michaelis SU, Mezger M, Bornhäuser M et al (2014) KIR haplotype B donors but not KIR-ligand mismatch result in a reduced incidence of relapse after haploidentical transplantation using reduced intensity conditioning and CD3/CD19-depleted grafts. Ann Hematol 93:1579–1586

Nowak J, Mika-Witkowska R, Graczyk-Pol E (2012) Genetic methods of HLA typing. In: Witt M, Dawidowska M, Szczepanski T (eds) Molecular aspects of hematologic malignancies. Springer, Berlin, Heidelberg, pp 325–339

Nowak J, Kościńska K, Mika-Witkowska R et al (2014) Donor NK cell licensing in control of malignancy in hematopoietic stem cell transplant recipients. Am J Hematol 89:E176–E183

Nowak J, Kościńska K, Mika-Witkowska R et al (2015) Role of donor activating KIR-HLA ligand-mediated NK cell education status in control of malignancy in hematopoietic cell transplant recipients. Biol Blood Marrow Transplant 21:829–839

Oevermann L, Michaelis SU, Mezger M et al (2014) KIR B haplotype donors confer a reduced risk for relapse after haploidentical transplantation in children with ALL. Blood 124:2744–2747

Orr MT, Lanier LL (2010) Natural killer cell education and tolerance. Cell 142:847–856

Re F, Staudacher C, Zamai L, Vecchio V, Bregni M (2006) Killer cell Ig-like receptors ligand-mismatched, alloreactive natural killer cells lyse primary solid tumors. Cancer 107:640–648

Robinson J, Halliwell JA, McWilliam H et al (2013) The IMGT/HLA database. Nucleic Acids Res 41:D1222–D1227

Ruggeri L, Capanni M, Urbani E et al (2002) Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 295:2097–2100

Ruggeri L, Mancusi A, Capanni M et al (2007) Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. Blood 110:433–440

Scrucca L, Santucci A, Aversa F (2007) Competing risk analysis using R: an easy guide for clinicians. Bone Marrow Transplant 40:381–387

Zhao XY, Chang YJ, Zhao XS et al (2015) Recipient expression of ligands for donor inhibitory KIRs enhances NK-cell function to control leukemic relapse after haploidentical transplantation. Eur J Immunol 45:2396–2408