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Effect of NK cell receptor genetic variation on allogeneic stem cell transplantation outcome and in vitro NK cell cytotoxicity

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Abstract

Natural killer (NK) cells recognize malignant cells via their cell surface receptors and may kill them. Killer cell immunoglobulin-like receptors (KIR) genotypes of donors have been reported to adjust the risk of relapse after allogeneic stem cell transplantation (HSCT), particularly in patients with acute myeloid leukemia. To test whether non-KIR NK cell receptors have a similar effect, we screened 796 genetic polymorphisms in 14 non-KIR NK cell receptor genes for their associations with relapse and graft-versus-host disease (GVHD) after HSCT in 1,491 HSCT donors (from Finland, the UK, Spain, and Poland), divided into a discovery and replication cohort. Two polymorphisms flanking the gene CD226 (DNAM-1) and two flanking FCGR3A (CD16a) were associated with a nominally reduced risk for relapse and chronic GVHD, respectively. These associations could not be confirmed in the replication cohort of 446 HSCT donors from the same populations. The blood donor NK cells carrying these nominally protective genetic alleles had a higher in vitro killing activity than the noncarriers, potentially indicating functional effects. Taken together, these results show no robust effects of genetic variation in tested non-KIR NK cell receptors on the outcome of HSCT.

INTRODUCTION

Natural killer (NK) cells are cytotoxic immune cells that can kill malignant cells without specific antigen recognition or prior sensitization[1,2]. NK cell function is regulated by a complex network of cell receptor–ligand interactions, both inhibitory and activating, which may mask the effect of single factors. While killer cell immunoglobulin-like receptors (KIRs) appear to be amongst the key factors defining NK cell activity in allogeneic stem cell transplantation (HSCT), other NK cell receptors also regulate the activity[1,2]. These receptors include, for example, natural killer group 2, natural cytotoxicity receptors (NCRs), immunoglobulin-like transcripts, CD226, and 2B4 [3]. NK cells can detect and kill malignant cells, for example, with missing or disturbed human leukocyte antigen (HLA) class I expression[4].

NK cells are the first lymphocyte subset appearing after HSCT, their count reaching the normal levels in about three months post-transplant[5,6]. The beneficial effect of NK cell activation in HSCT was introduced by the studies of Ruggeri and co-workers[7]. They showed that acute myeloid leukemia (AML) patients with a specific HLA–KIR ligand mismatch with the donor were protected from relapse after HSCT. They furthermore showed that in the HLA–KIR ligand mismatched setting, NK cells were activated and killed the malignant cells[8]. The association between the KIR ligand mismatch and reduced risk for relapse has been found in many independent studies[9–11], however, not in a recent large study[12]. Cooley and coworkers[13,14], as well as many others[15–17], have shown that selecting the donors with KIR genetic haplotype B, that is, chromosomes containing a higher number of activating KIR gene types, improved relapse-free survival after HSCT. These studies have led to suggestions that it may be advantageous to select the HSCT donors according to their KIR genotypes for patients with AML. Whether the higher killing activity of the KIR haplotype B carriers can be shown in in vitro experiments is still unclear.

The relative importance of different NK cell receptors to the functional properties of NK cells or to the HSCT cell donor selection is currently not established. The receptors most likely act in a complex crosstalk. For example, NKp46 was reported to avert graft-versus-host disease (GVHD) by killing immature dendritic cells[18] and CD226 has been shown to be involved in the development of T-cell mediated GVHD in mice[19]. Hence, genetic variation in the non-KIR NK cell receptor genes could also be associated with the clinical outcomes of HSCT, particularly with relapse and GVHD. Indeed, for example, variation in the NKG2D gene has been reported to be associated with HSCT outcome[20] .

The ability to efficiently kill malignant cells makes NK cells an interesting candidate for novel cell therapies in settings other than HSCT[21]. In the past few decades, NK cell therapy has grown into an active field in cancer immunotherapy research. The sensitivity of the target cells to NK cell killing and therapeutic effects appear complex. The effects of cell donor genetics, such as the KIR genotype, are obvious[14]. On the other hand, Sheffer and coworkers[22] found that NK cell-sensitive targets typically had a mesenchymal cell type signature, with high H7-H6, low HLA-E, and low antigen presenting machinery expression levels. Hence, optimal selection criteria for cell therapy donors not only depend on the donor profile but also on the expected target cell types.

To better understand the role of various non-KIR NK cell receptors in HSCT and cell therapy, we investigated the association of 796 genetic polymorphisms in NK cell receptor genes with relapse and GVHD after HSCT in 1,491 donors from Finland, UK, Spain, and Poland. We focused on the donor NK receptor genotypes only, as we can expect that it primarily is the donor rather than the patient, whose NK cell killing activity is most important after HSCT. To find complementary functional evidence, we determined the effects of the associated genetic polymorphisms on the in vitro killing activity of NK cells isolated from genotyped blood donors. Although interesting association trends were found, we conclude that no statistically significant associations could be confirmed, and that there are no strong effects mediated by the non-KIR NK cell receptor gene polymorphisms.

RESULTS

Association of NK cell receptor gene polymorphisms with HSCT outcomes

An overview of the workflow is shown in Fig. 1. We analyzed the genetic association of 796 NK cell receptor gene polymorphisms (Supplementary Table S1 online) with acute GVHD, chronic GVHD, and relapse in 1,491 HSCT donors divided into a discovery (n = 1,045 donors) and replication (n = 446 donors) cohort. Only four polymorphisms provided evidence for statistical associations (Fig. 2A and 2C). All results of the association analyses can be found in the Supplementary material (Tables S2–S13) online.

In the discovery cohort, alleles rs3911730*C and rs8087187*A of genetic polymorphisms flanking (~ 250 kb telomeric) the CD226 gene were associated with relapse, with the betas of -0.73 (95% CI: -1.19 to -0.26, covariate-adjusted p-value = 0.002) and − 0.66 (95% CI: -1.12 to -0.19, covariate-adjusted p-value = 0.005), respectively. The alleles reduced the risk for relapse in the entire discovery cohort and in each subpopulation (Fig. 2A). Figure 3 shows the Kaplan-Meier analysis of relapse-free survival in relation to these two HSCT donor polymorphisms in the discovery cohort. Cox regression analyses (Supplementary material online) showed the hazard ratio of 0.6 (95% CI: 0.38 -0-95. p-value = 0.03) for the rs3911730*CA + *CC genotypes as compared to the *AA genotype, and the hazard ratio of 0.55 (95% CI: 0.35–0.88. p-value = 0.013) for the rs8087187*AC + *CC genotypes as compared to the *CC genotype. None of these associations, however, could be confirmed in the replication cohort, with the betas of 0.07 (95% CI: -0.73 to 0.87, covariate-adjusted p-value = 0.86) for rs3911730*C, and 0.07 (95% CI: -0.72 to -0.86, covariate-adjusted p-value = 0.86) for rs8087187*A, as well as statistically non-significant p-values in Kaplan-Meier and Cox regression analyses (Supplementary material online). None of these associations passed multiple testing correction.

Alleles rs11585450*G and rs1875763*C, of the polymorphisms flanking (~ 200 kb telomeric) the FCGR3A gene, were associated with a lower risk of chronic GVHD (limited, extensive, grade unknown vs. grade 0) with the beta of -0.34 (95% CI -0.56 to -0.12, covariate-adjusted p-value = 0.003 for both) in the discovery cohort. It is of note that in the Spanish discovery subpopulation, the beta was to the opposite direction. These associations could not be confirmed in the replication cohort (beta 0.02, 95% CI -0.35 to 0.39, covariate-adjusted p-value = 0.90 for rs11585450; beta 0.04, 95% CI -0.33 to 0.41, covariate-adjusted p-value = 0.82 for rs1875763) and failed to pass multiple testing correction in the discovery and replication cohorts (Supplementary material online).

As the NK cell effect may the strongest in AML, we included the disease group, AML/MDS versus the others, as a covariate in the association analyses. For these four polymorphisms the covariate was statistically non-significant in the discovery cohort (covariate-adjusted p-values 0.79 for rs3911730, 0.78 for rs8087187, 0.36 for rs11585450, 0.36 for rs1875763), showing no specific effects in this disease group.

Polymorphism selection with lasso regression

Lasso regression can perform variable selection by shrinking coefficient estimates to zero, excluding such variables from its models. We provided lasso with the covariates from the association analysis and NK cell receptor gene polymorphism dosages (for all 796 polymorphisms) for each of the six endpoints, constructing a model for each endpoint in the discovery and replication cohort. Lasso selected relevant variables from the clinical covariates and polymorphisms to be included in the models (Supplementary Tables S14–S25 online).

Of the polymorphisms with the association with relapse in the discovery cohort, rs3911730 was included in the lasso model for relapse in the discovery cohort. However, this polymorphism was not included in the lasso model for relapse in the replication cohort, thus failing to confirm the results of the discovery cohort. Both polymorphisms with an association with chronic GVHD (limited, extensive, grade unknown vs. grade 0), rs11585450 and rs1875763, were included in the lasso model for this endpoint in the discovery cohort but not in the model for the replication cohort.

Lasso did provide us with additional evidence for the polymorphism selection since three of the four polymorphisms were included in the lasso models. The last polymorphism not in the lasso model, rs8087187, is in such a strong linkage disequilibrium with rs3911730, that lasso likely excluded it from its model for marker being redundant. Based on all the analyses described above, we concluded to have sufficient support to test all the four polymorphisms with a nominal association with the outcome of HSCT (rs3911730, rs8087187, rs11585450, and rs1875763) for their effect on NK cell cytotoxicity in vitro.

Effect of NK cell receptor polymorphisms on in vitro NK cell cytotoxicity

Using NK cells from 13 genotyped blood donors of the Blood Service Biobank, we analyzed the effect of the four NK receptor gene polymorphisms, rs8087187, rs3911730, rs11585450, and rs1875763, on the NK cell killing activity in in vitro.

For the CD226 polymorphisms the genotype distributions of the blood donors were: two rs8087187*CA heterozygotes and 11 *AA homozygotes, and two rs3911730*AC heterozygotes and 11 *CC homozygotes. NK cells from blood donors with the rs8087187*CA and rs3911730*AC genotypes had an increased cytotoxicity (Fig. 2C), albeit with p-values failing to reach statistical significance (beta 17.96, 95% CI: -2.72 to 38.62, covariate-adjusted p-value = 0.22 for the heterozygotes; Table S26 online). Unfortunately, no homozygotes for the minor alleles were available.

Four blood donors were homozygous and four others heterozygous for FCGR3A rs1875763*C and rs11585450*G, the alleles showing the trend for reduced the risk for chronic GVHD in the HSCT discovery cohort (Fig. 2B). In the in vitro assay, the rs1875763*C and rs11585450*G homozygotes showed an increased cytotoxicity, the effect appeared recessive (Fig. 2D). The p-values for these genotypes were statistically non-significant (beta 17.11, 95% CI: -0.84 to 35.05, covariate-adjusted p-value = 0.22 for the homozygotes; Table S26 online).

DISCUSSION

As there is evidence that HSCTs with donors carrying KIR haplotype B result in a lower risk of AML relapse after HSCT[13,14], we analyzed the effect of genetic variation in other, non-KIR, NK cell surface receptors on the outcome of HSCT and additionally on the in vitro killing activity of NK cells. We have previously reported that in the Finnish population, partially overlapping with the present Finnish cohort, donor KIR haplotype B defined by KIR2DS2 and KIR2DL2 was associated with a lower risk for relapse[17]. Unfortunately, the genotyping arrays applied to the present samples did not contain sufficiently genetic markers for KIR gene imputation[23] and no sufficient DNA was available for a targeted, separate KIR typing. Therefore, we were not able to confirm the role of KIR genotype in the entire study cohort or to estimate its effect size as compared to those of other NK receptors. We focused on the effects of donor genotypes, as it can be assumed that the NK cell effects on the outcome of HSCT are predominantly mediated by donor NK cells. The number of HSCT donors was relatively high, 1,491, resulting in a sufficient power to detect the strongest, but not minor effects. The HSCTs were from four different populations and retrospective, both facts that may lead to heterogeneity and obvious limitations in available clinical data.

We found weak genetic associations between polymorphisms in the CD226 and FCGR3A genes and a reduced risk for relapse and chronic GVHD, respectively, in the discovery cohort but not in the replication cohort. The discrepancies may result from genuine subpopulation or cohort specific differences in HSCT protocols, or they may reflect false positive findings. As the year of transplantation was one of the covariates, the division into the discovery and replication cohorts according to the transplantation year should not explain the results. It is of note that the genome-wide association[24–28] or matching analyses have not pointed to the role of NK cell receptor gene polymorphisms. It was, nevertheless, of interest to find out that the putative GVHD and relapse protective alleles reported in the present study had effects on NK cell function in vitro. The associated alleles increased the NK cell killing activity, an effect clearly beneficial to the relapse risk. The results can be interpreted to give further support of the role of donor NK cells. However, our NK cell functional approach suffered from a sparse number of study subjects, particularly the number of samples with the minor alleles was low. The NK cells were obtained from apparently healthy blood donors that is both an advantage as they do not have disease or treatment-related heterogeneity, and a disadvantage as their state most likely do not reflect the HSCT setting. We also acknowledge the complex overall regulation of NK cell activity in which demonstration of single molecule effects is difficult.

The two NK receptors, CD226 and FCGR3A, with possible effects are related to the NK cell functions relevant to HSCT. CD226 encoding the DNAM-1 molecule[29] mediates immune cell adhesion and regulates NK cell cytotoxicity and is involved in T-cell mediated acute GVHD in mouse models[19]. FCGR3A encodes a low affinity Fc gamma receptor component which is a part of the triggering complex for NK cell cytolytic activity. Genetic variation in FCGR3A has been reported to be associated with antibody-dependent cytotoxicity of NK cells[30], potentially relevant in chronic GVHD, and the efficiency of cancer therapy[31]. The polymorphisms reported in the present study also change the expression levels of CD226 and FCGR3A according to the GTEx eQTL data in Ensembl (release 110). Nevertheless, it is too early, based on the present results only, to speculate how or even whether the expression level differences could affect the HSCT outcome. In conclusion, our results do not indicate strong and robust role in HSCT outcome for the non-KIR NK cell receptors studied in the present study. However, the present results do not exclude the possibilities of more complex interactions between the receptors, or receptors and ligands; their effects requires larger and more homogeneous cohorts.

MATERIALS AND METHODS

Study cohorts

The HSCT study population consisted of 1,491 HSCT donors from allogeneic HSCTs conducted in four countries: 765 from Finland (Helsinki and Turku University Hospitals), 352 from the UK (The Freeman Hospital, Newcastle Hospitals NHS Foundation Trust), 272 from Spain (IDIBGI Biobank), and 102 from Poland (Hematological departments of the Medical University of Gdansk, Maria Skłodowska-Curie Memorial Cancer Centre, and Institute of Oncology in Gliwice). The population demographics are described in Table 1.

Table 1
Demographics of the HSCT study populations.

		Finland		UK		Spain		Poland	
		Discovery	Replication	Discovery	Replication	Discovery	Replication	Discovery	Replication
Number of HSCT donors, n		572	193	237	115	179	93	57	45
HSCT time, years		1993–2011	2011–2018	1984–2011	2011–2017	2000–2009	2009–2014	2019–2021	2021–2022
	Missing, n (%)	17 (3)	8 (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Recipient age in years, median (range)		50 (18–70)	52 (20–69)	41 (19–65)	53 (20–72)	50 (18–69)	52 (20–72)	50 (20–73)	52 (20–73)
	Missing, n (%)[a]	0 (0)	0 (0)	0 (0)	0 (0)	2 (1)	1 (1)	0 (0)	1 (2)
Donor age in years, median (range)		44 (16–73)	39 (16–68)	37 (12–63)	34 (19–71)	46 (4–78)	51 (17–75)		
	Missing, n (%)[a]	278 (49)	0 (0)	6 (3)	7 (6)	47 (26)	11 (12)	57 (100)	45 (100)
Donor-recipient gender, n (%)	Male-male	175 (31)	76 (39)	94 (40)	52 (45)	60 (34)	31 (33)	22 (39)	17 (38)
	Male-female	143 (25)	49 (25)	60 (25)	34 (30)	42 (23)	20 (22)	10 (18)	10 (22)
	Female-male	117 (20)	29 (15)	47 (20)	12 (10)	42 (23)	25 (27)	14 (25)	8 (18)
	Female-female	120 (21)	39 (20)	35 (15)	16 (14)	35 (20)	17 (18)	11 (19)	10 (22)
	Missing	17 (3)	0 (0)	1 (0)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Stem cell source, n (%)	Peripheral blood	320 (56)	156 (81)	120 (51)	108 (94)	169 (94)	89 (96)	56 (98)	45 (100)
	Bone marrow	248 (43)	37 (19)	116 (49)	1 (1)	10 (6)	4 (4)	0 (0)	0 (0)
	Both	2 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)
	Missing	2 (0)	0 (0)	1 (0)	6 (5)	0 (0)	0 (0)	0 (0)	0 (0)
Donor type, n (%)	Sibling	470 (82)	76 (39)	145 (61)	27 (23)	179 (100)	93 (100)	37 (65)	28 (62)
	Register	102 (18)	112 (58)	92 (39)	88 (77)	0 (0)	0 (0)	1 (2)	1 (2)
	Haplo	0 (0)	5 (3)	0 (0)	0 (0)	0 (0)	0 (0)	19 (33)	16 (36)
	Missing	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Conditioning regimen, n (%)	Myeloablative	434 (76)	129 (67)	107 (45)	15 (13)	78 (44)	35 (38)	33 (58)	36 (80)
	Reduced intensity	136 (24)	53 (27)	130 (55)	100 (87)	95 (53)	58 (62)	23 (40)	8 (18)
	Other	2 (0)	11 (6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)
	Missing	0 (0)	0 (0)	0 (0)	0 (0)	6 (3)	0 (0)	1 (2)	0 (0)
aGVHD, n (%)	grade 0	374 (65)	92 (48)	85 (36)	32 (28)	122 (68)	55 (59)	32 (56)	29 (64)
	grade I-II	130 (23)	72 (37)	121 (51)	58 (50)	33 (18)	23 (25)	19 (33)	16 (36)
	grade III-IV	64 (11)	28 (15)	28 (12)	1 (1)	24 (13)	15 (16)	6 (11)	0 (0)
	Grade unknown	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Missing	4 (1)	0 (0)	3 (1)	24 (21)	0 (0)	0 (0)	0 (0)	0 (0)
cGVHD, n (%)	grade 0	253 (44)	89 (46)	81 (34)	20 (17)	81 (45)	34 (37)	23 (40)	22 (49)
	Yes, classification unknown	1 (0)	0 (0)	20 (8)	0 (0)	0 (0)	0 (0)	12 (21)	8 (18)
	Limited	105 (18)	28 (15)	56 (24)	32 (28)	16 (9)	8 (9)	0 (0)	0 (0)
	Extensive	171 (30)	68 (35)	46 (19)	29 (25)	41 (23)	15 (16)	0 (0)	0 (0)

		Finland		UK		Spain		Poland	
	Missing	42 (7)	8 (4)	34 (14)	34 (30)	41 (23)	36 (39)	22 (39)	15 (33)
Relapse, n (%)	Yes	202 (35)	66 (34)	80 (34)	23 (20)	52 (29)	16 (17)	9 (16)	4 (9)
	No	363 (63)	127 (66)	156 (66)	74 (64)	127 (71)	75 (81)	48 (84)	41 (91)
	Missing	7 (1)	0 (0)	1 (0)	18 (16)	0 (0)	2 (2)	0 (0)	0 (0)
Diagnosis, n (%)[b]	Acute myeloid leukemia	178 (31)	66 (34)	99 (42)	35 (30)	62 (35)	29 (31)	21 (37)	17 (38)
	Acute lymphoblastic leukemia	89 (16)	25 (13)	36 (15)	8 (7)	14 (8)	5 (5)	12 (21)	5 (11)
	Myelodysplastic syndrome	51 (9)	21 (11)	11 (5)	12 (10)	21 (12)	9 (10)	3 (5)	4 (9)
	Chronic myeloid leukemia	56 (10)	4 (2)	33 (14)	4 (3)	12 (7)	3 (3)	3 (5)	0 (0)
	Multiple myeloma	80 (14)	27 (14)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5 (11)
	Other	125 (22)	46 (24)	81 (34)	54 (47)	70 (39)	47 (51)	20 (35)	13 (29)

GVHD, graft-versus-host disease; aGVHD, acute GVHD; cGVHD, chronic GVHD

[a]Missing ages were imputed, see Materials and methods

[b]Five most frequent diagnoses are presented here

The HSCT study population was subdivided into a discovery cohort of 1,045 HSCT donors and a replication cohort of 446 HSCT donors, and the discovery cohort included the earliest 2/3 of HSCTs from each population (the year of HSCT was among the covariates in statistical analyses). The study participants gave an informed consent, or when no longer possible, the permit was granted by the VALVIRA, the National Supervisory Authority for Welfare and Health in Finland. All protocols including the collection of samples and use of these data were approved by the Ethical Review Boards of each collaborating hospital. The permit numbers are V/74832/2017 (Finland, VALVIRA), HUS/2152/2020 (Helsinki University Hospital, Finland), ETMK 78/2012 (Turku University Hospital, Finland), Biobank IDIBGI B.0000872 (Spanish HSCTs), 14/NE/1136 (the UK HSCTs), KB-561/2019 (Polish HSCTs). All protocols have been carried out according to relevant guidelines and regulation, such as the Declaration of Helsinki. In genome and statistical analyses, the SOPs of the R&D Department, the Finnish Red Cross Blood Service, were followed. For example, only the pseudonyms for patients and donors were available to the study group and sensitive data was handled only in data portals fulfilling the local regulations. No prisoners were recruited to donors.

In vitro NK cell killing activities were determined from 14 blood donors who gave an informed consent to donate the buffy coats from the standard blood donation bag for the present study, that has the ethical review board permit HUS/1845/2019 from the Helsinki University Ethical Review Board and the accepted project permit number 003-2019 from the Blood Service Biobank. Of them 13 had genotypes available in the Blood Service Biobank. The buffy coat samples were collected and handled according to the ethical permit HUS/1854/2019 and the biobank project permit 003-2019. The biobank collects samples only from the blood donors who have given the broad biobank consent according to the Finnish Biobank Act. The Biobank is supervised by the Finnish Medicinal Authority FIMEA.

Genotyping, lift-over, and genotype imputation

The HSCT donors were genotyped at the genome-wide level using the following arrays: Illumina Immunochip v1, Immunoarray v2, Illumina Global Screening Array v2 or v3, at the Finnish Institute of Molecular Medicine, Helsinki, Finland, or using an exome sequencing pipeline[32] at the McGill Genome Centre, Montreal, Canada. The genotyping data underwent a lift-over[33] to the human reference genome build GRCh38/hg38, followed by genome-wide SNP imputation. In the lift-over and imputation[34], the THL Biobank's SISu v3 reference panel was used for the Finnish samples, and for the rest, a reference panel of the European samples of the 1000Genomes project, provided with the protocols, was used. The blood donor genotypes were originally done by the FinnGen research project[35].

Association analysis

A set of 893 genetic polymorphisms, located flanking or in 14 NK cell receptor genes were studied for their associations with HSCT outcome. A complete list of the polymorphisms and genes is provided in the supplementary material (Supplementary Table S1 online). The genes were chosen for their reported role in NK cell function which could be assumed to regulate the NK cell killing activity. After imputation, 805 of the polymorphisms were present in the Finnish HSCT samples (genotypes included in the Finnish imputation reference panel), and 851 in the other HSCT samples

(genotypes included in the European reference panel). Of these, 796 polymorphisms were shared among all HSCT samples and included in the actual genetic association analyses. The workflow detailing all the analysis steps is depicted in Fig. 1.

Association analysis was conducted on the 796 NK receptor gene polymorphisms in the HSCT donors using acute GVHD (grade I-IV vs. grade 0, grade III-IV vs. grade 0), chronic GVHD (limited, extensive, grade unknown vs. grade 0; extensive vs. grade 0; extensive, grade unknown vs. grade 0), and relapse (yes vs. no) as endpoints. The HSCTs of nonmalignant disease were excluded from the analysis of relapse. The covariates included in the analyses were recipient and donor age, donor type (sibling, register, haplo identical), graft type, recipient–donor sex matching, conditioning regimen, disease (AML/myelodysplastic syndrome [MDS] or other), information on the occurrence of acute GVHD for the chronic GVHD endpoints, transplantation year, and the country of origin for the HSCTs. Missing donor and recipient ages were imputed with missForest the R package[36] using all the other covariates.

The genotype probability of the imputed NK cell receptor polymorphisms was used as the dosage information in the association analysis. The analysis was performed with Plink 2.0[37] with an additive model containing the parameters “covar-variance-standardize” to standardize quantitative covariates, and “ci 0.95” and “adjust” to compute the 95% confidence intervals and multiple testing-corrected p-values. First, all subpopulations were combined for the analysis, followed by investigating the effect sizes and their directions in the subpopulations separately. This was done both in the discovery and replication cohorts. The criteria for accepting an association for further analysis were, first, ranking the polymorphisms based on their consistency of association direction in the different subpopulations in the discovery cohort, and second, ranking the polymorphisms according to their p-values. Thus, in the discovery cohort, out of the polymorphisms that showed maximal consistency, the ones with the smallest p-values were selected.

Survival analysis

We performed survival analyses on relapse-free survival and overall survival for the two polymorphisms associated with relapse in the discovery cohort. Kaplan-Meier analysis and log-rank tests were used for this (ggsurvfit the R package version 0.3.1), and further, we performed Cox regression adjusting the analysis with covariates (survival the R package, version 3.5-7). The covariates were the country of origin, donor type, graft type, recipient and donor ages, donor-recipient sex matching, conditioning regimen type, diagnosis (AML/other), transplantation year, and HLA-match score.

NK cell receptor polymorphism selection with lasso

In addition to selecting NK cell receptor gene polymorphisms for the cytotoxicity analysis based on the results of the genetic association analysis, we used lasso regression analysis with glmnet the R package[38] for obtaining additional support for the selection. The same covariates and dosage information as in the association analysis were included in constructing the lasso models, and they were created separately for the six endpoints (acute GVHD (grade I-IV vs. grade 0, grade III-IV vs. grade 0), chronic GVHD (limited, extensive, grade unknown vs. grade 0; extensive vs. grade 0; extensive, grade unknown vs. grade 0), relapse (yes vs. no)) in the discovery and replication cohorts after which the variables included in the models were compared. The tuning parameter lambda was chosen with a ten-fold cross-validation for each model.

The best candidate NK receptor polymorphisms to assess for in the in vitro data were selected based on the HSCT association results and lasso models in the discovery cohorts. For the polymorphisms selected from the association analysis, we examined if lasso had selected them into its models for the respective endpoints.

NK cell cytotoxic activity in vitro **assays**

Human peripheral blood mononuclear cells were obtained from blood donors using Ficoll-Paque™ Plus (GE Healthcare Life Sciences) density gradient centrifugation, following the manufacturer's guidelines. To isolate NK cells, the samples were processed using the MicroBead technology (NK cell isolation kit, Miltenyi Biotec) and then cryopreserved for future analyses. After thawing, NK cells were allowed to rest overnight in the NK MACS expansion medium supplemented with cytokines and human AB serum. Luciferase-transduced K562-luc2 (ATCC® CCL¬243¬LUC2™) cells were used as the target cells. To assess the cytotoxic efficacy, NK cells and K562-luc2 + target cells were co-cultured at various effector: target ratios for 16–18 hours. Subsequently, the living target cells were quantified using the Victor Nivo® multimode plate reader (Perkin Elmer) after the addition of Luciferin reagent (ONE-Glo luciferase reagent, Promega, Madison, USA). The complete assay protocol will be described in a separate publication (Penna and coworkers, in preparation).

The NK cell in vitro cytotoxicity was analyzed using a linear mixed-effect model with a random intercept using the lme4 R package[39]. The models included the effector:target ratios, HLA-C and KIR genotypes, cytomegalovirus status, and the genotypes for the NK cell receptor gene polymorphisms selected with the association analysis and lasso regression on HSCT donors, as well as a group effect term to connect technical replicates and samples for the random intercept. The test was run for each genotype separately and significance was set at 0.05.

Declarations

Code availability

The code for association testing, lasso regression, and analyzing the in vitro data are publicly available in GitHub (https://github.com/FRCBS/NK_receptors).

Data availability

The code for association testing, lasso regression, and analyzing the in vitro data are publicly available in GitHub (https://github.com/FRCBS/NK_receptors/). Individual-level genotype data for HSCT donors are not publicly available due to restrictions set by the ethical permits but can be asked for relevant studies from the corresponding author: jukka.partanen@veripalvelu.fi. The blood donor data available can be inquired from the Blood Service Biobank at https://www.veripalvelu.fi/en/biobank/for-researchers/.

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AUTHOR CONTRIBUTIONS

JP conceived the study concept; JN, LP, JR, JP interpreted the results and drafted the manuscript; LP, JN, JR, K B-K, PL, SK, and KH collected the SNP candidates and planned their study pipeline; JN and JR analyzed the associations; LP, FJ, SK, and EK planned and performed NK cell assays; KK planned and LP collected blood donor biobank samples; JN, SK, US, M I-R, REC, DG, K B-K, PL, MB and SG provided the patient samples and their clinical details. All authors reviewed the manuscript.

ADDITIONAL INFORMATION

The authors declare no competing interests.

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Figures

Figure 1.

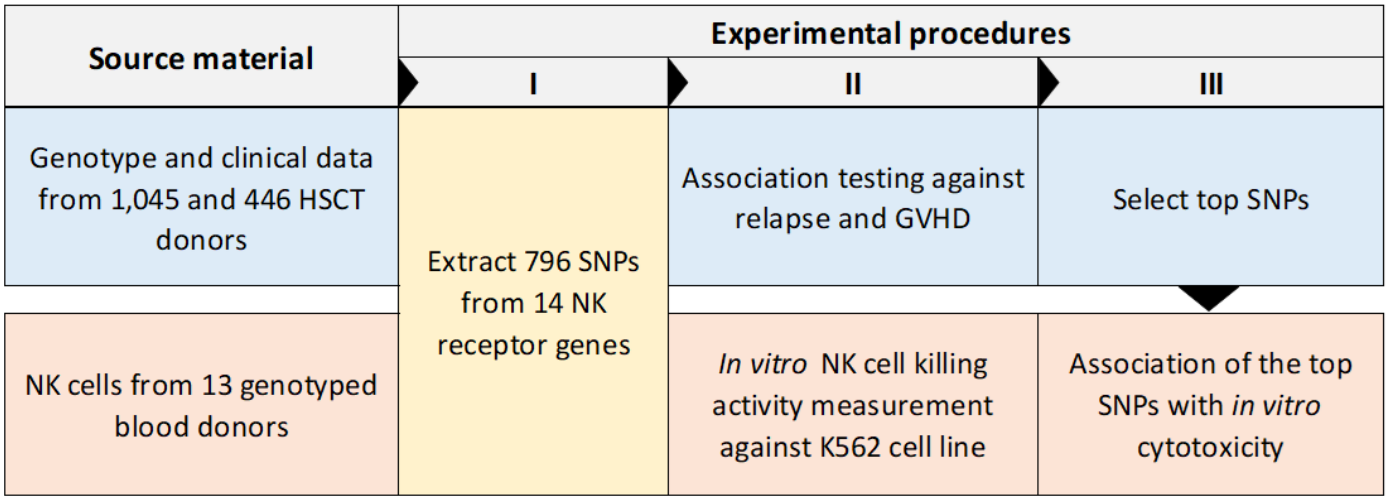

Figure 1

Overview of the workflow.

Figure 2.

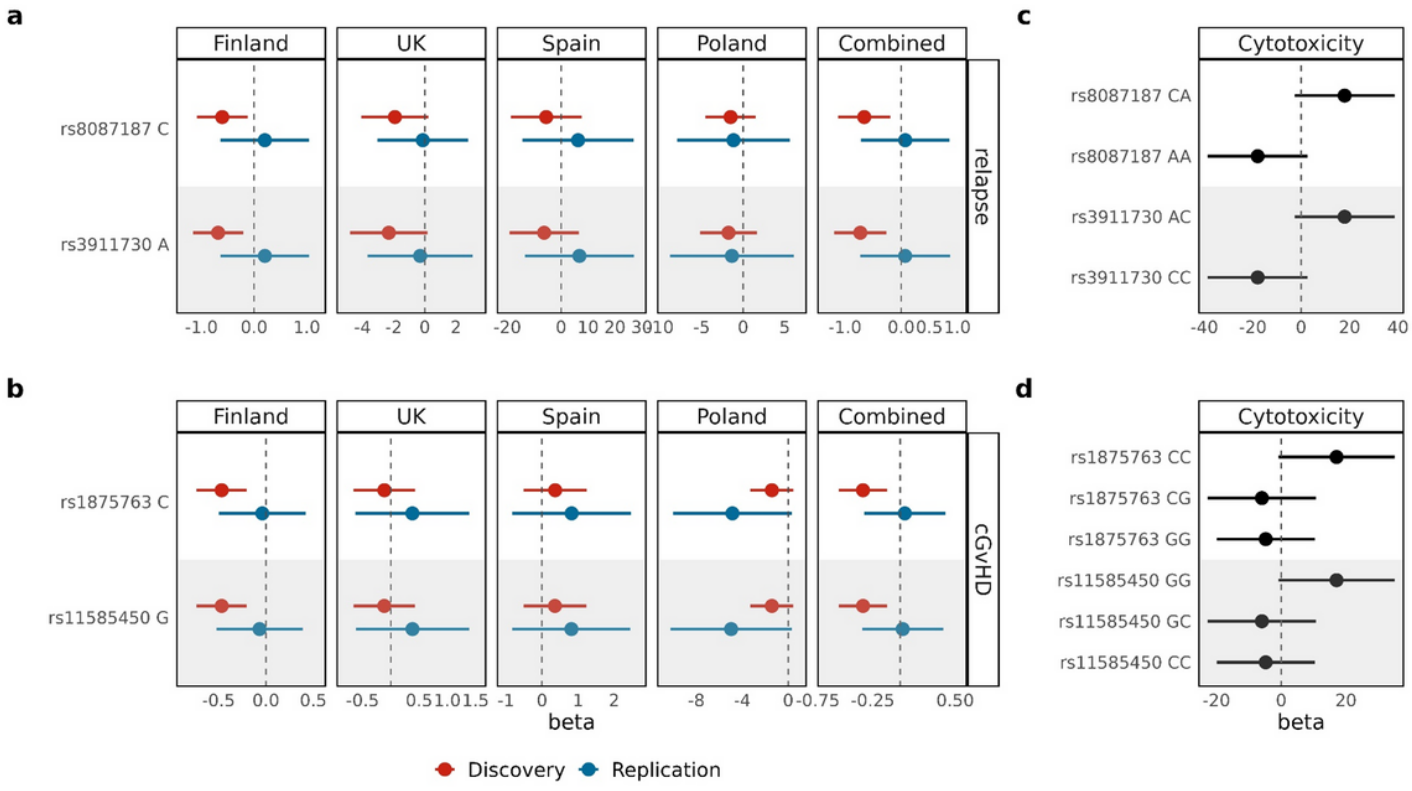

Figure 2

NK cell receptor polymorphisms associated with relapse and chronic GVHD, and their effect on NK cell cytotoxicity in vitro. A: Associations of CD226 genotypes with relapse, B: Associations of FCGR3A genotypes with chronic GvHD (limited, extensive, grade unknown vs. grade 0), C: NK cell

cytotoxicity levels in relation to the CD226 genotypes, D: NK cell cytotoxicity levels in relation to the FCGR3Agenotypes. The error bars show the confidence intervals (CI). cGvHD, chronic graft-versus-host disease.

Figure 3

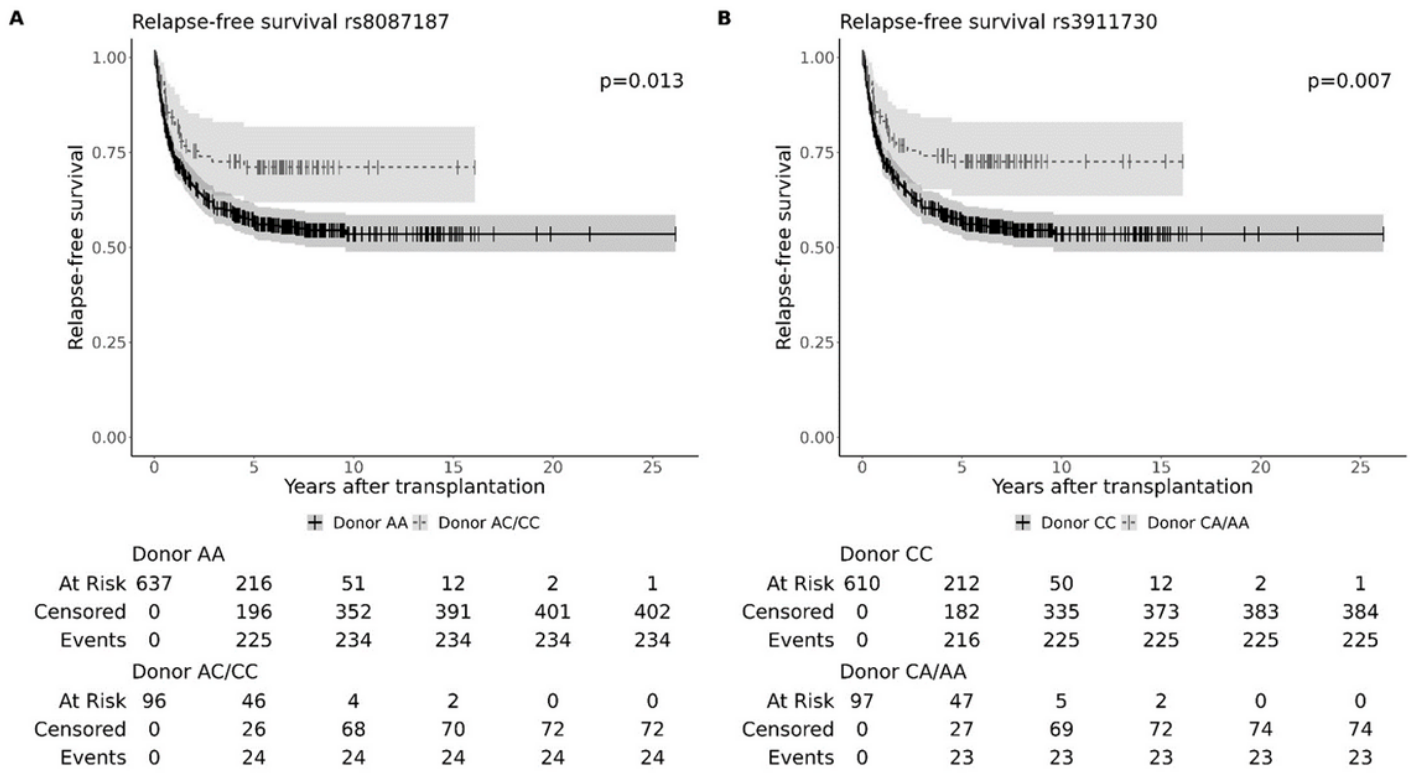

Figure 3

Kaplan-Meier analysis of relapse-free survival in the discovery cohort. Left panel: donor rs3911730*AA genotype compared to CC+AC genotypes. Right panel: donor rs8087187*CC genotype compared to AA+CA genotypes.

Supplementary Files

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- NihtilaNKreceptorsinHSCTsupplementaryTablesfinal.docx