Predicting survival using clinical risk scores and non-HLA immunogenetics

running title: Predicting HSCT survival

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Conflict of Interest

The authors declare no conflict of interest.

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Abstract

Previous studies of non-histocompatibility leukocyte antigen (HLA) gene single nucleotide polymorphisms (SNPs) on subgroups of patients undergoing allogeneic haematopoietic stem cell transplantation (HSCT) revealed an association with transplant outcome. This study further evaluated the association of non-HLA polymorphisms with overall survival in a cohort of 762 HSCT patients using data on 26 polymorphisms in 16 non-HLA genes. When viewed in addition to an already established clinical risk score (EBMT-score), three polymorphisms: rs8177374 in the gene for MyD88-adapter-like (MAL) (p=0.026), rs9340799 in the estrogen receptor gene (ESR) (p=0.003) and rs1800795 in interleukin 6 (IL-6) (p=0.007) were found to be associated with reduced overall survival, while the haplo-genotype (ACC/ACC) in interleukin 10 (IL-10) was protective (p=0.02). The addition of these non-HLA polymorphisms in a Cox regression model alongside the EBMT-score improved discrimination between risk groups and increased the level of prediction compared to the EBMT-score alone (gain in prediction capability for EBMT-genetic-score 10.8%). Results also demonstrated how changes in clinical practice through time have altered the effects of non-HLA analysis.

The study illustrates the significance of non-HLA genotyping prior to HSCT and the importance of further investigation into non-HLA gene polymorphisms in risk prediction.

Introduction

Haematopoietic stem cell transplantation (HSCT) is the major curative therapy for disorders of the blood and immune system. However, the rate of survival in patients with HSCT has remained at 40-60% for the last two decades, owing to post-transplant complications including infection, graft-versus-host disease (GvHD), and relapse.

Five relevant clinical factors influencing transplantation success in patients with haematological disorders, including chronic myeloid leukaemia (CML) and acute lymphocytic and acute myeloid leukaemia (ALL and AML), have been identified by the European Group for Blood and Marrow Transplantation (EBMT). These risk factors (EBMT-factors) are patient age, sibling donor/matched unrelated donor (MUD), patient-donor gender combination, stage of disease, and time from diagnosis to transplant. A clinical risk score (EMBT-score) utilising the EBMT-factors was proposed in order to aid the prediction and prevention of post-transplant complications.[1-4]

Previous genetic association studies have suggested that, besides HLA genes, non-HLA genes may play an important role in transplant outcome. To date, these studies with single nucleotide polymorphisms (SNPs) have used small subsets of patients.[5-9] Although genome-wide association studies (GWAS) have been performed,[10] no SNP genotypes have been clearly identified so far that could be used to predict outcome.

In this study, we assessed the association of candidate polymorphisms with overall survival using a large cohort of patients undergoing HSCT. The goal was to identify non-HLA SNPs with an impact on overall survival when viewed in addition to the already established EBMT-score. Prediction capability was also evaluated. Since changes in transplant protocols can affect transplant outcome, the study also took into account date of transplant.

Methods

Patients

A total of 762 patients with malignant haematological diseases, having complete data on the EBMT-factors[1,3,4] and with known date of death or last contact, were included in the study. These patients were transplanted between November 1983 and December 2005 at seven European transplant centres. The patients and donors gave informed consent to participate in the study in accordance with the Declaration of Helsinki and EBMT guidelines. The protocol was approved by the Local Research Ethics Committee at the coordinating centre (Newcastle upon Tyne, UK). Follow-up time was between 7 months and 20 years, with a median of 5-6 years. Patient and donor characteristics are presented in Table 1. Overall, death occurred in 399 patients (52%). Causes of death were relapse (41%), GvHD (18%), infection (18%), multiple organ failure (5%), acute respiratory distress syndrome (4%), veno-occlusive disease (2%), interstitial pneumonitis (1%), and others (11%). The majority of the cohort after the year 2000 had high-resolution tissue typing for HLA Class I A,B,C, and Class II DP,DQ, and DR.

Candidate non-HLA Polymorphisms

DNA was prepared from archived frozen peripheral blood mononuclear cells. Genotyping was outsourced to Kbioscience (http://www.kbioscience.co.uk) who used fluorescence-based competitive PCR technology (KASPar) and designed the assays for the SNPs based on the DNA sequence (50 bases) either side of the SNP. Genotypes were available for 743 patient-donor pairs on the following genes: CD14, CD91, C3, ESR1, GCR, HSP70-hom, IFNG, IL1RN, IL4, IL6, IL10, IL12B, IL13, LOX1, MAL, MDR1, NOD2, TNF, TNFRSF1B, and VDR (Table 2). Candidate SNPs were selected according to findings on smaller patient cohorts by our

coordinating centre in Newcastle (ESR1,[11] IFNG,[12] IL1RN,[13] IL4,[14] IL6,[15,16] IL10,[17] IL13,[18] TNF,[19] TNFRSF1B,[20] and VDR[21]). SNPs were also selected according to findings by other groups in HSCT (MAL,[22] MDR1,[23,24,25] and NOD2[26]), and according to previous disease association studies in autoimmune (CD14,[27] GCR,[28,29] HSP70-hom,[30] and IL12B[31]) or inflammatory disease (and recently found to be de-regulated in a rat model for GvHD:[32] C3,[33] LOX1,[34] and CD91[35]).

Statistical analysis

Clinical differences in patients treated up to and after year 2000 were assessed using Fisher's exact test. This division in time was chosen as transplant protocols were changed at that time with the introduction of Imatinib and a subsequent increase in survival rate.[2]

Biallelic SNPs were considered under the additive, dominant, and recessive modes of inheritance[10] (Supplementary Section A). In the models, each SNP was used with the mode demonstrating the strongest association with survival.

The genes HSP70-hom, IL12B, and MDR1 and GCR-haplotype were excluded from the analysis due to missing genotypes (missingness>33%). A total of 26 polymorphisms from 16 genes were available for analysis (Table 2).

A power of 80% in the cohort with 52% deaths was achieved for SNPs in an additive mode for sample sizes n≥300, minor allele frequency (MAF)>15%, and an expected hazard ratio HR≥1.50. In a dominant mode, this power was achieved for n≥400. In a recessive mode, this was attained for n≥500, MAF≥25%, and HR≥2.00. A few SNPs with a MAF<10% (i.e. IL6 rs1800796, LOX1 rs11053646, and the three SNPs in NOD2) had a very low power at all settings; even so, we retained these SNPs for further analysis.

Association of risk score for overall survival

The EBMT risk score (EBMT-score) was derived by a summation procedure of the EBMT-factors.[1,3,4] The EBMT-score was subsequently implemented in analyses on an ordinal scale [low to high risk 0-7]. The additional effect of each individual polymorphism was evaluated using separate Cox regression models. The likelihood ratio test (LRT) was applied to compare a Cox regression model including the EBMT-score and one polymorphism to a model including only the EBMT-score; a nominal p-value of 0.05 was used. Analyses were performed for the cohort as a whole as well as for several subgroups.

Using stepwise selection, we built an additional Cox regression model to establish whether multiple non-HLA polymorphisms improved the model when added in with the EBMT-score (α=0.05 for variable entry and α=0.10 for variable removal[36]). All available polymorphisms were chosen as candidates in this procedure. Hazard ratios (HRs) and 95% confidence intervals (95% CI) were reported.

A new risk score (EBMT-genetic-score) was developed using this last Cox regression model. The score was derived by summing up the clinical EBMT-score and the genetic score points given for the polymorphisms. The latter were obtained by dividing the respective regression coefficients by the coefficient of the EBMT-score and rounding to the nearest integer[37] (Supplementary Section B).

Assessment of prediction

Two statistical approaches were used to assess the risk score prediction capability: the concordance index (C-index)[3,4,38-40] and the R-square measure of the gain in prediction (R^2).[41] The C-index measures the agreement between risk score and observed survival time. A higher risk score should correspond to a shorter observed survival time. A C-index=0.5

implies that a risk score has no predictive discrimination, whereas C-index=1 implies maximum predictive discrimination. The U-statistic[40] was also used to test whether the EBMT-genetic-score was better than the EBMT-score as regards agreement with observed survival time. In addition, R^2 was used to quantify the improvement in prediction[41] of the new EBMT-genetic-score over the EBMT-score. This measure is a combination of the 0.632 bootstrap estimate of prediction error[42] and the explained variation using Schoenfeld residuals[43-44] (see Supplementary Section C). Larger values of R^2 (>0%) mean that the EBMT-genetic-score correctly predicts outcome more often than the EBMT-score. R^2=0% means that both scores have equivalent predictive ability and R^2=100% means that the EBMT-genetic-score has perfect predictive ability (i.e. the predicted and the actual outcomes always agree).

Results

Clinical characteristics

Sixty percent of the patients underwent HSCT after the year 2000. There was evidence that, after 2000, transplants involved more patients and donors over 40 years of age, more HLA-matched unrelated donors, more lymphoma, more donor cells from peripheral blood, more later stage disease, more T-cell depletion, and more reduced-intensity conditioning (RIC) (Table 1).

There was no significant centre effect on overall survival (likelihood ratio test, P value= 0.49).

Association of the EBMT risk score and single polymorphisms with overall survival

The EBMT-score[1,3,4] was significantly associated with overall survival (HR=1.16, 95% CI=1.09-1.24, p<0.001).

The top ten candidate polymorphisms associated with overall survival in the whole cohort, while controlling for EBMT-score, are given in Table 3. The IL10 haplotype in donors demonstrated the lowest p-value and the presence of ACC/ACC was protective (HR=0.48, 95% CI=0.29-0.80, LRT p-value=0.002). This haplotype was also the only polymorphism significantly associated with survival in all subgroups (Supplementary Table 1). Nine of the ten polymorphisms were also highest ranked amongst patients without T-cell depletion and with myeloablative conditioning regimens. IL10 haplotype, IL10 rs1800896(G), IL4 rs2243250(T), and IL6 rs1800797(A) in donors, ESR1 rs2234693(C) and GCR rs33388(T) in patients were observed in over 50% of the assessed clinical subgroups.

Association and prediction of multiple polymorphisms with overall survival

After stepwise selection, the final model contained the EBMT-score and four selected polymorphisms (n=419 due to missing genotypes). The presence of haplo-genotype ACC/ACC of IL10 in donors was protective against patient death (HR=0.49, 95% CI=0.26-0.89, p-value=0.020). The risk of death increased with an increased number of T alleles in MAL rs8177374 in patients (additive, HR=1.34, 95% CI=1.04-1.74, p-value=0.026), with the presence of allele G in ESR1 rs9340799 in patients (dominant, HR=1.52, 95% CI=1.15-2.01, p-value=0.003), and with the increased number of C alleles in IL6 rs1800795 in donors (additive, HR=1.29, 95% CI=1.07-1.55, p-value=0.007) (Table 4, Supplementary Figure 1).

Three out of four of these polymorphisms were significantly associated with death due to relapse. In addition, MAL rs8177374 was associated with death due to GvHD and IL6 rs1800795 was associated with death due to infection (Supplementary Table 2).

When the multivariate Cox regression model (Table 4) was compared to a model containing the EBMT-score alone, the estimated R^2 for gain in prediction indicated a 5.1% gain in prediction ability by adding the four polymorphisms (separately) to the EBMT-score.

Comparing EBMT-genetic-score and EBMT-score

The new EBMT-genetic-score, derived through summing the individual risk score values (Supplementary Section B), ranged from 1 to 15. The scores were grouped into five distinct categories according to the observed groupings of the Kaplan-Meier survival curves (results not shown). The Cox regression model, with five ordered categories of the EBMT-genetic-score, revealed increasing hazard ratios with increasing EBMT-genetic-score (n=419, Table 5). The risk values of the EBMT-genetic-score displayed a clearer separation of the survival curves when compared to values of the EBMT-score (Supplementary Figure 2).

Kaplan-Meier curves for the EBMT-genetic-score were plotted to establish if the EBMT-genetic score is appropriate for the clinical subgroups: disease diagnosis, sibling donor/matched unrelated donor, T-cell depletion, conditioning regimen, and year of transplantation. The plots consistently demonstrated that higher risk scores corresponded to lower survival (Supplementary Figure 3).

For the whole cohort, the higher EBMT-genetic-score corresponded to shorter observed survival times compared to the EBMT-score (U-statistic p-value<0.001, Table 6). This also proved to be the case in subgroups of patients transplanted before and after 2000. Estimation of the gain in prediction ability indicated that there was benefit in utilising the single EBMT-genetic-score (R^2=10.8%, Table 6) over the previous model containing the EBMT-score and four separate polymorphisms (R^2=5.1%).

Kaplan Meier survival curves for EBMT-score and EBMT-genetic-score before and after 2000 (n=419) appear in Figures 1a-1d. Compared to the EBMT-score, the EBMT-genetic-score better discriminates the survival curves and a higher score consistently corresponds to a lower survival probability. It was also apparent that, when using the EBMT-genetic-score, those treated after 2000 had improved chances of survival in comparison with those treated before 2000 (Figures 1c, 1d). For those with the lowest scores (1-6), 3-year survival was 85% and 95% for patients treated before and after 2000, respectively; for those with the highest scores (13-15), 1-year survival was 15% and 42% for patients treated before and after 2000, respectively.

Discussion

The aim of this work was to study the effect of non-HLA polymorphisms on overall survival of HSCT patients in addition to the EBMT-score. In our study, the IL10 promoter haplo-genotype ACC/ACC in donors was one of the most important polymorphisms associated with improved overall survival. IL10 is an important cytokine in the regulation of the immune response. However, it can have a stimulatory effect on B cells, increasing MHC class II expression and antibody production. IL10 haplotypes have been shown to correlate with IL10 protein production,[45] with the GCC haplotype being associated with the highest IL10 production.

The ATA/ACC genotype has been identified as a protective factor for overall survival in CML patients with sibling donors.[9] SNP rs1800872(A) and haplotype ACC in patients have been shown to demonstrate a strong association with severe acute GvHD (aGvHD III-IV) in patients with matched related donors.[7,10] In our whole cohort, the IL10 promoter haplo-genotype ACC/ACC in donors proved to be significantly associated with increased overall survival,

whereas SNP rs1800872(A) in patients revealed only borderline association. The ACC/ACC genotype in the donor is associated with intermediate production of IL10 and the ACC haplotype has been shown to be protective in aspergillosis.[46] The AA genotype of IL10 rs1800872 in the patient is associated with a decreased risk of aGvHD[47] and an increased risk of non-relapse mortality; this was confirmed in follow-up GWAS studies.[10,48] In addition, the presence of the GG genotype of IL10 rs1800896 in the patient was found to be associated with the risk of chronic GvHD (cGvHD).[49]

Furthermore, we discovered that MAL rs8177374(T) in patients is associated with reduced overall survival whereas parallel smaller studies have reported that the presence of the T allele in donors resulted in less cGvHD and a reduction in transplant-related mortality. In addition, the presence of the T allele in patients was associated with an increased risk of relapse.[50] Our study revealed a strong association between the presence of the T allele and relapse and an increased risk of death in patients who relapsed. Moreover, the T allele was strongly associated with GvHD. The T allele is regarded in the literature as the inflammatory allele, and T-heterozygous individuals have increased protection from infection. Interestingly, another study revealed that patients transplanted from donors with the T allele have a lower incidence of fungal infections, aGvHD, and improved overall survival.[22] MAL protein was originally identified in intermediate and late stages of T-lymphocyte differentiation[51] and the MAL mRNA expression was also found to be related with differentiation in urothelial cells, neuronal cells,[52,53] and oesophageal epithelium.[54] The MAL-A variant containing all four exons is abundantly expressed in peripheral blood lymphocytes[55] and positively expressed in the gastrointestinal tract, respiratory tract, and haematopoietic system. MAL is important in the innate immune response; it is involved in

279 Toll-like receptor signalling,[56] so could be important in the development of aGVHD in the
280 recipient.
281 Our study also indicated that ESR1 rs9340799(G) in patients is associated with reduced
282 overall survival. The G allele has been previously reported as associated with reduced overall
283 survival and increased risk of aGvHD in patients with HLA-matched siblings.[11] ESR1 is thought
284 to inhibit IL6 production.[57]
285 Furthermore, we found IL6 rs1800795(C) in donors to be associated with reduced overall
286 survival. The G allele in patients and/or donors has been reported by GWAS studies as a risk
287 factor for both aGvHD and cGvHD in patients with HLA-matched related[10,21] and unrelated[10]
288 donors. In our study, there was evidence of an increased risk of aGvHD in patients
289 transplanted from donors with the C allele present. Among these patients, those
290 transplanted before 2000 had evidence of reduced survival compared to those transplanted
291 later (Supplementary Figure 4a), possibly as a result of a higher rate of standard
292 myeloablative conditioning before 2000.
293 The G allele of this SNP is reported to correlate with higher serum IL6 levels in systemic-
294 onset juvenile chronic arthritis.[58] However, the CC genotype has been associated with higher
295 levels of IL6 in polymyalgia rheumatica.[59] It could therefore be that increased levels of IL6 in
296 our cohort exacerbated the inflammatory milieu, leading to increased transplant-related
297 complications such as infection and poorer survival.
298 We have also demonstrated that polymorphisms modelled via Cox regression, either in a
299 joint model with the EBMT-score or combined with the latter as a single EBMT-genetic-score
300 factor, contribute to a better discrimination of the risk groups (C-index) and increase the
301 prediction of survival (R^2) compared to the EBMT-score alone. Changes in HSCT clinical
302 protocols during 2000 greatly improved patient survival. The consideration of an EBMT-

genetic-score highlighted an improvement in survival, especially for those at higher risk of death. More transplants from MUDs were performed after 2000 and it could be argued that the improvement in survival of patients with high-risk non-HLA genotypes is due to the improved quality of HLA matching after 2000. However, no difference in survival was evident between siblings or MUD patients transplanted after 2000 (Supplementary Figure 4b).

In addition, a recent review has revealed that other pre-transplant clinical factors (e.g., CMV status and Karnofsky performance score) also play a role in survival and could be used alongside EBMT-score[4].

In conclusion, we hypothesise that implementing risk scores for pre-transplant risk assessment from clinical and genetic factors enhances the prediction of overall survival for patients undergoing HSCT. The potential of considering non-HLA polymorphisms in pre-transplant risk assessment is evident with the promising results for polymorphisms in genes IL10, MAL, ESR1 and IL6. Further investigations into pre-transplant risk assessment could also include other potential predictors such as mRNA and microRNA expression.[60]

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary information is available at Bone Marrow Transplantation's website.

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Figure 1 Kaplan-Meier curves of patients undergoing HSCT up to and after the year 2000 (n=419) for 5 years follow-up a) EBMT-scores before 2000, b) EBMT-scores after 2000, c) EBMT-genetic-scores before 2000 and d) EBMT-genetic-scores after 2000.

Supplementary Figure 1 Kaplan-Meier survival curves (for 5years follow-up) of patients undergoing HSCT for each polymorphism appearing in the Cox regression model (n=419) a) IL10 haplotype – donor b) MAL rs8177374– patient c) ESR1 rs9340799 - patient d) IL6 rs1800795-donor.

Supplementary Figure 2 Kaplan-Meier survival curves (for 5 years follow-up) of patients undergoing HSCT a) EBMT-score (n=743), b) EBMT-genetic-score (n=419).

Supplementary Figure 3 Kaplan-Meier survival curves (for 5 years follow-up) of patients undergoing HSCT according to the EBMT-genetic-score, by various clinical subgroups.

Supplementary Figure 4 Kaplan-Meier survival curves (for 5 years follow-up) of patients undergoing HSCT according to year of transplant, by type of donor

Supplementary Table 1 Top ten polymorphisms from the whole cohort that remained as top polymorphisms in the subgroup analyses for association with overall survival.

Supplementary Table 2 P-values for the association of selected polymorphisms with different causes of death (n=419), using a Cox regression model including a single polymorphism and the EBMT-score.

Supplementary Sections A, B, C – Additional information describing: a) the method of coding the biallelic SNPs (additive, dominant and recessive), b) an example of risk score calculation c) derivation of the R-square measure of the gain in prediction.