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ORIGINAL ARTICLE

No impact of *NOD2/CARD15* on outcome after SCT

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Recent studies have pointed towards an association between certain single nucleotide polymorphisms (SNPs) in the *NOD2/CARD15* gene, and negative outcome of Allo-SCT. In this study, 198 patients and their corresponding donors were analyzed retrospectively for the occurrence of *NOD2/CARD15* mutations to evaluate the impact on clinical results after Allo-SCT. In all, 7.6% of the patients and 11% of the donors were heterozygous for one of three SNPs 8, 12 or 13. Contrary to earlier findings, we found no significant impact on incidence of acute GVHD or TRM following Allo-SCT. These differences in results could be due to a lower mutation frequency in the studied population and/or a lower overall incidence of severe GVHD. On the basis of these findings we conclude that a consideration to *NOD2/CARD15* mutation status is not pertinent when selecting a donor for Allo-SCT at our centre.
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Introduction

NOD2/CARD15 was initially identified as the first gene linked to increased susceptibility for Crohn's disease.[1,2] It is expressed in paneth cells in the intestinal mucosa and myeloid-derived cells such as monocytes/macrophages, neutrophils and DCs. It encodes for a cytoplasmic protein that has been shown to function as a sensor for the cell wall component muramyl dipeptide, which is present in both gram-positive and gram-negative bacteria. *NOD2/CARD15* partakes in activation of the first-line epithelial defence in the intestine, and regulation of inflammatory response through intracellular pathways involving nuclear factor κB and IκB kinase.[3]

Recent studies have pointed towards an association between certain single nucleotide polymorphisms (SNPs) in the *NOD2/CARD15* gene, and an increased incidence of severe acute GVHD, intestinal GVHD and TRM following Allo-SCT.[4–6] This increased risk was correlated with occurrence of the gene variants in recipients, as well as in donors, even though the results have been somewhat contradictory. However, the negative impact on Allo-SCT outcome was invariably strongest when the mutations occurred in both patients and donors.

In this report, we describe a retrospective analysis of the impact of *NOD2/CARD15* SNPs 8, 12 and 13 on the incidence of acute GVHD and TRM after Allo-SCT, in a consecutive group of patients who have undergone myeloablative Allo-SCT at our centre between 1996 and 2006.

Materials and methods

Patients and donors

Between January 1996 and February 2006, 455 patients with haematological malignancies underwent SCT at the Centre for Allogeneic Stem Cell Transplantation, Karolinska University Hospital. All children below the age of 15 years (88 patients) were excluded, as were all patients who had received reduced-intensity conditioning prior to transplantation (119 patients). Of the remaining 248 individuals, stored DNA samples were available from 198 recipients and their corresponding donors. Table 1 summarizes patient and donor characteristics. For details concerning the transplantation procedure and supportive care refer to previously published work by Svahn *et al.*[7] All recipients and donors were typed for HLA class I and II by allele level PCR sequence-specific primers.[8] Aside from patients with HLA-identical related donors ($n=80$), and unrelated donors matched for HLA-A, HLA-B, DRβ1 ($n=101$), 17 patients with subtype mismatch donors were included. Diagnosis for acute GVHD was based on clinical signs, and/or histopathological findings in biopsies from skin, liver and the gastrointestinal tract. Grading was performed based on established criteria.[9]

Antibacterial prophylaxis

During the pancytopenic stage, all patients were prescribed prophylactic therapy with ciprofloxacin 500 mg postoperatively twice daily until ANC $>0.50\times10^9$/l. Ten patients were not able to follow this treatment for a median of

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Table 1 Patient characteristics

Criteria	*Data*
No. of patients	198
Diagnosis	
ALL	46
AML	79
CML	56
CLL	5
MDS/MPS	8
Lymphoma	4
Females/males	84/114
Median age (years) (range)	
Patients	37 (15–61)
Donors	37 (11–66)
Disease stage	
Low risk/high risk[a]	118/80
Donors	
Females/males	87/111
HLA-identical siblings	78
HLA-identical parent	2
Matched unrelated	101
Subtype MM unrelated	17
Conditioning	
CY + TBI	117
CY + BU	81
+ ATG	121
GVHD prophylaxis	
CsA + MTX	189
CsA + Prednisolone	6
CsA + MMF	2
FK + MMF	1
Stem cell source	
Peripheral blood	112
Bone marrow	86

Abbreviations: ATG = antithymocyte globulin; FK = tacrolimus; MDS = myelodysplastic syndromes; MM = mismatch; MMF = mycophenolate mofetil; MPS = myeloproliferative syndromes.
[a]Low risk = first complete remission or first chronic phase.

3 days (range 1–6) due to nausea and/or oral mucositis. Trimethoprim-sulphamethoxazole was used as prophylaxis against pneumocystis carinii infection during the first 6 months after engraftment.

Typing for NOD2/CARD15 SNPs

DNA was isolated from EDTA blood samples collected before transplant. Typing for *NOD2/CARD15* SNPs 8, 12 and 13 was done using a TaqMan real-time PCR protocol as described.[10] Probes and primers were synthesized by CyberGene AB (Huddinge, Sweden). Reporter dyes FAM and TET were used, in combination with quencher dye TAMRA, to discriminate SNP alleles. Final concentrations in the reaction mix were as follows: 300 nM of each forward and reverse primers, 150 nM of each FAM and TET TaqMan probes and 1–10 ng/μl of DNA sample. Amplification and detection were done using ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analysis was performed in December 2006. The incidence of GVHD and TRM was estimated nonparametrically. Patients were censored at the time of death, relapse or last follow-up. Relapse and non-relapse mortality are competing events. Their incidence rates were therefore estimated using a nonparametric estimator of cumulative incidence curves. Predictive analyses for GVHD and TRM were based on the proportional hazard model for subdistribution of competing risk. Univariate and multivariate analyses were then performed using Gray's test and the proportional subdistribution hazard regression model of Fine and Gray. A stepwise backward procedure was used to construct a set of independent predictors for each end- point. All predictors with a *P*-value below 0.10 were considered, and sequentially removed if the *P*-value in the multiple model was above 0.05. All tests were two-sided. The type I error rate was fixed at 0.05 for factors potentially associated with time-to-event outcomes. Analyses were performed using the cmprsk package (developed by Gray, June 2001), Splus 6.2 software and Statistica software. The Mann–Whitney *U*-test was used to compare continuous variables and the χ^2 method was used to compare the distribution of categorical variables.

Results and discussion

Mutation frequencies

In the analyzed group, 15 of the recipients (7.6%) and 23 of the donors (12%) were heterozygous for one of the three SNPs. One donor carried two different mutations. In three of the patient–donor pairs (1.5%) mutated gene variants occurred in both individuals. We found no cases of homozygosity. Thus, *NOD2/CARD15* mutations were found in 35 of the preformed transplants (18%).

As indicated earlier by Hampe *et al*., the prevalence of SNPs in the Scandinavian population seems to be lower compared to other geographic populations studied.[4–6,10] The prevalence in the donor group is, however, similar to previously documented results, which correlates with the fact that a large part of the cases consisted of unrelated transplantations and that the main part of these donors originate from central Europe.

The scarcity of simultaneous mutations in recipient and donor could also in part be explained by the lower ratio of HLA-identical sibling donors in this study; the probability of siblings sharing the same *NOD2/CARD15* genotype is about 50%, if one of the parents is heterozygous.

Incidence of severe aGVHD and TRM

Eighty-eight patients died at a median time of 6.8 (0.25–110) months after Allo-SCT. Median follow-up for surviving patients was 78 (6–126) months. The overall cumulative incidence of acute GVHD III–IV was 14%, which correlates well with previous results from our centre.[11]

In univariate analysis for acute GVHD II–IV, we included factors such as *NOD2* mutation, homecare, age, donor type, cell dose, high/low risk, stem cell source and

viral serostatus. Significant factors for increased risk of acute GVHD II–IV were high CD34 dose ($P=0.02$) and hospitalization as compared to homecare during the pancytopenic phase after Allo-SCT ($P=0.02$). In multivariate analysis only the latter was significantly associated with severe acute GVHD II–IV (odds ratio 3.4, confidence interval 1.2–9.3, $P=0.02$).

For TRM, however, a CMV infection ($P=0.03$) and the occurrence of acute GVHD II–IV ($P<0.001$) were significant risk factors in univariate analysis. In multivariate analysis, only acute GVHD II–IV showed statistical significance (odds ratio 8.9, confidence interval 4.3–18.7, $P<0.001$).

Thus, contrary to earlier results, we found that the occurrence of *NOD2/CARD15* gene variants, in either recipients or donors, had no significant impact on the incidence of acute GVHD or TRM following Allo-SCT (Figure 1). There was also no significant difference in overall survival and relapse-free survival (Figure 2). There may be several possible causes for these divergent findings.

First, the frequencies of mutations in this population are significantly lower compared to those found in previous studies. We found only three cases with coinciding mutations in donor and recipient, which is the setting that has been shown to have the strongest impact on Allo-SCT outcome.

Also, there is a lower overall incidence of severe acute GVHD in this study, which in turn could reflect differences in the preventive immunosuppressive therapy. Moreover, at our centre, treatment for acute GVHD is initiated at grade I, which might avert a progression to severe acute GVHD in many cases.[12]

T-cell depletion is known to be a factor that can greatly influence the incidence of acute GVHD after SCT. It was shown in a recent study that T-cell depletion could reduce or cancel the impact of *NOD2/CARD15* mutations on acute GVHD.[13] A majority of the patients in this study (61%) had undergone *in vivo* T-cell depletion with antithymocyte globulin and this might be an additional explanation to the lack of significant findings. However, we were not able to study this effect specifically since antithymocyte globulin was used in all the unrelated donor transplantations (118 of 118), and in very few of the related transplantations (3 of 80). Consequently, any additional impact of T-cell depletion would be impossible to distinguish from differences between related and unrelated SCT.

The possibility of discrepancy in the occurrence of acute GVHD among different ethnic populations has been discussed. Supporting this notion is a study from the Center for International Blood and Marrow Transplant Research (CIBMTR), which showed that the Japanese and Swedish populations had a lower probability of acute GVHD.[14] However, a study performed at our centre showed no significant difference in acute GVHD in HLA-identical sibling transplants in Scandinavian and non-Scandinavian patients.[15] Thus, the lower incidence of acute GVHD in our patients is most likely not due to mutations in *NOD2/CARD15* or other genes that occur in low frequencies, but rather to the immunosuppressive prevention and treatment.

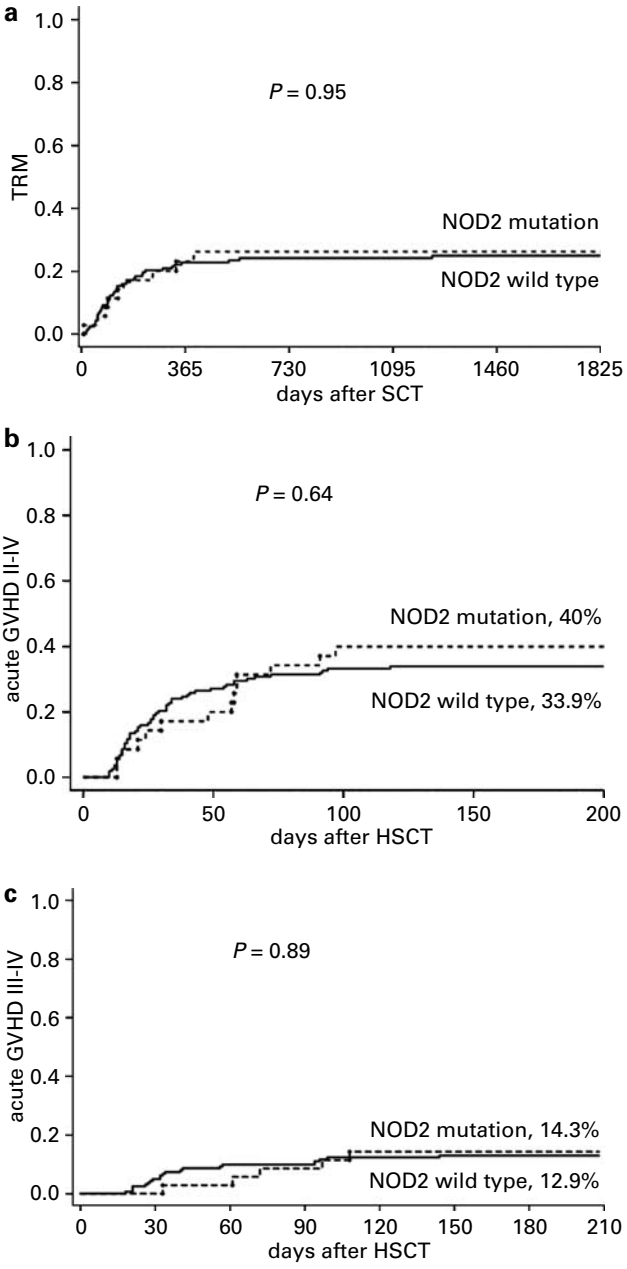

Figure 1 Incidence of TRM and severe acute GVHD in relation to mutations in the NOD2 gene. There were no significant difference in incidence of TRM (**a**), acute GVHD grade II–IV (**b**), or acute GVHD grade III–IV (**c**) between the group were at least one mutated SNP occurred in the donor and/or recipient (*NOD2* mutation) and the one with wild-type single nucleotide polymorphism (*NOD2* wild type).

The antibiotic spectrum of GI decontamination therapy has been proposed as one possible cause to the variation of the impact that *NOD2/CARD15* mutations have on Allo-SCT outcome in different populations. Prophylaxis that covers both gram-negative and gram-positive bacteria has been shown to cancel the negative effects of a *NOD2/CARD15* mutation.[5] However, all patients in this study received only gram-negative prophylaxis.

On the basis of these findings we conclude that a consideration of *NOD2/CARD15* mutation status is not

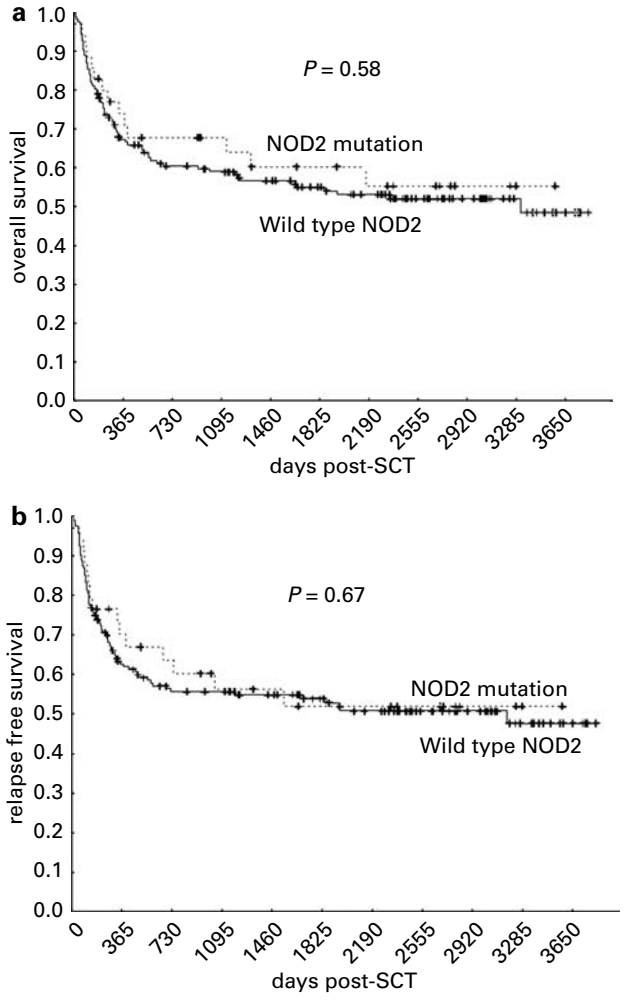

Figure 2 Overall survival and relapse-free survival in relation to mutations in the *NOD2* gene. There was no significant difference in overall survival (**a**) and relapse-free survival (**b**) between the group with at least one mutated single nucleotide polymorphism in the donor and/or recipient and the one with wild-type *NOD2*.

pertinent when selecting a donor for Allo-SCT at our centre. However, it still remains crucial that more centres evaluate their patient populations for the impact of *NOD2/CARD15* mutations, to identify additional centre-specific factors, and thus shed further light on the clinical significance of a dysfunctional NOD2 system on the course of events after Allo-SCT.

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