Effect of *NOD2/CARD15* variants in T-cell depleted allogeneic stem cell transplantation

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Background and Objectives. Three single nucleotide polymorphisms (SNP) in the *NOD2/CARD15* gene have been associated with the incidence and the severity of acute graft-versus-host disease (GVHD) following allogeneic stem cell transplantation (SCT). We hypothesized that the clinical effect of SNP in *NOD2/CARD15* might be different in patients submitted to T-cell-depleted allogeneic SCT, in which donor T cells, the main effectors of GVHD, are eliminated.

Design and Methods. SNP 8, 12 and 13 in *NOD2/CARD15* were studied using a Taqman protocol in 85 patients undergoing HLA-identical, T-cell-depleted SCT and in 71 of their sibling donors.

Results. *NOD2/CARD15* variants were present in nine (11%) patients and six (8%) donors. The incidences of acute GVHD and chronic GVHD were not associated with either the donors' or recipients' *NOD2/CARD15* variants. In contrast, these genetic variants were associated with a lower disease-free survival (17% vs. 48%, p=0.03). Death due to pulmonary infection was more frequent in the group of patients with *NOD2/CARD15* variants. In the multivariate analysis, only *NOD2/CARD15* variants (RR 2.3, p=0.04) and older age (RR 2.2; p=0.04) were independent prognostic factors for disease-free survival.

Interpretation and Conclusions. *NOD2/CARD15* variants have a deleterious effect on clinical outcome in T-cell-depleted allogeneic SCT, which is independent of GVHD. These results supports the hypothesis that the detrimental effect of *NOD2/CARD15* variants in such a transplant setting might be produced by an alteration of the innate immune system more than by activation of the adaptive immune system.

Key words: *NOD2/CARD15*, polymorphisms, stem cell transplantation.

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Gastrointestinal mucosal damage and subsequent bacterial translocation seem to play a pivotal role in the initiation and maintenance of the inflammatory status that leads to graft-versus-host disease (GVHD).[1] In this sense, gut decontamination has been shown to protect against GVHD in animal models[2] and also in the clinical setting.[3] The *NOD2/CARD15* gene is involved in the innate immune response against bacterial infections in the gastrointestinal tract; it has recently been shown that variants of this gene are associated with a higher incidence of acute GVHD and transplant-related mortality after allogeneic stem cell transplantation.[4,5] Nod2 is a cytosolic protein belonging to the nucleotide-binding oligomerization domain (NOD) family. It is involved in the maintenance of commensal and gastrointestinal mucosal homeostasis;[6] it recognizes muramyl dipeptide (MDP), a component of peptidoglycan present in the cell wall of Gram-positive and Gram-negative bacteria. Upon ligand binding, Nod2 oligomerizes and activates the nuclear factor κB (NF-κB) signaling pathway inducing the transcription of several genes encoding proinflammatory cytokines.[7] Nod2 is expressed mainly in monocytes, macrophages, dendritic cells and Paneth cells.[8] Nod2 contains three distinct functional domains: multiple carboxy-terminal leucine-rich repeats, a centrally nucleotide binding domain and two amino-terminal caspase recruitment domains. Three common single nucleotide polymorphisms (SNP) in the leucine-rich repeats domain - SNP8 (R702W), SNP12 (G908R) and SNP13 (L1007finsC) - have been previously associated with an increased risk of Crohn's disease.[8] It has been suggested that these variants might be associated with an abnormal inflammatory response against normal bacterial flora.[9] These *NOD2/CARD15* variants have also been associated with the incidence of grades III-IV acute GVHD.[4] Holler *et al.* suggested that such an association was due to an increased release of cytokines for recipients with the polymorphisms, causing a stimulation of recipient dendritic cells and donor T cells.[4] We hypothesized that an abnormal inflammatory response associated

with these three *NOD2/CARD15* variants might influence clinical outcome independently of donor T-cell stimulation. This effect, not directly linked to GVHD, might be detectable in transplants in which donor T cells have been eliminated. On this background, we analyzed the clinical relevance of *NOD2/CARD15* variants in 85 patients undergoing T-cell depleted allogeneic-SCT.

Design and Methods

Patients

One hundred patients underwent myeloablative, T-cell-depleted allogeneic SCT from HLA-matched siblings in our Unit between March 1995 and May 2004; stored DNA from 85 patients and 71 of their donors was available at the time of the design of the present study. The main clinical characteristics of the patients are summarized in Table 1. Conditioning was based on cytoxan 120 mg/kg and total body irradiation 1300 cGy administered in four fractions, and was performed according to standard protocols. Patients with acute leukemia in first complete remission, myelodysplasia with a low or intermediate International Prognostic Scoring System score and chronic myeloid leukemia in first chronic phase were considered in early stage. Ciprofloxacin 500 mg/12h and fluconazole 400 mg/24h were used as gut decontamination. The local Ethic Committee approved the study, and all patients and donors gave written informed consent to genetic analysis of their biological samples.

Table 1. Clinical characteristics.

N. 85	
Age at transplant, years (range)	42 (22-59)
Sex, n. female (%)	44 (52)
Underlying disease	
Acute leukemia, n. (%)	38 (45)
Myeloproliferative disorder, n. (%)	17 (20)
Lymphoma/Myeloma, n. (%)	13 (15)
Myelodysplasia/Aplasia, n. (%)	8 (9)
Chronic lymphocytic leukemia, n. (%)	9 (11)
Advanced stage, n. (%)	32 (38)
Source, peripheral blood, n. (%)	81 (95)
Conditioning regimen	
CyTBI	74 (88)
BuCy	5 (6)
Other	5 (6)
N. $CD34^+$ cells infused	4.8 (1.3-14.2)

N: number; CyTBI: cyclophosphamide and total body irradiation; BuCy: busulphan and cyclophosphamide.

Allelic discrimination of the NOD2/CARD15 variants

Genomic DNA from whole blood samples from patients and donors was isolated using a QIAmp DNA Blood Mini Kit (QIAgen, Germany) following the manufacturer's instructions. Allelic variants at SNP8 (R702W, rs2066844), SNP12 (G908R, rs2066845) and SNP13 (L1007finsC, rs2066847) of the *NOD2/CARD15* gene were genotyped using the TaqMan® SNP Genotyping Assay (PE Applied Biosystems, Foster City, CA, USA). The specific primers and FAM™- and VIC®-dye labeled probes used have been previously described[10] and were designed by the Applied Biosystems Assay-on-Demand service. Reactions were performed in a 96-well plate in a final volume of 15 μL. For each individual reaction 7.5 μL of TaqMan® 2X Universal PCR Master Mix (PE Applied Biosystems), 400 nM of each forward and reverse primer, 250 nM of each labeled probe and 50 ng of genomic DNA template were mixed. Polymerase chain reaction (PCR) conditions were: 95°C for 10 minutes, followed by 40 two-step cycles of denaturation (92°C for 15 seconds) and annealing/extension (60°C for 1 minute). After the PCR run the released fluoresence was measured by the ABI 7500 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). Control samples of each genotype, which were confirmed by sequencing, were included in each run.

Statistical analysis

Individuals heterozygous for at least one of the three *NOD2/CARD15* SNP analyzed (SNP8, SNP12 and SNP13) were considered *variant*, while individuals lacking these SNP were considered *wild type*. Four major end-points were considered, incidence of grades II-IV acute GVHD, chronic GVHD, disease-free-survival (defined as patients alive at last contact and without clinical evidence of relapse), and non-relapse mortality. Probabilities of acute and chronic GVHD were calculated by the cumulative incidence method (marginal probability), which accommodates competing risks. Graft failure, relapse, or death without GVHD were considered as competing risks. The cumulative incidence among the different groups was statistically compared by Gray's method. For disease-free survival and non-relapse mortality, actuarial curves were obtained by the Kaplan-Meier method and statistically compared using the log-rank test. *p* values were two-sided, and a significance level of α=0.05 was used. The following variables were included in the univariate analysis: age of patients and donors older or younger than the median, sex of patients and donors, donor female into recipient male, cytomegalovirus serology of patients and donors, source of stem cells, conditioning regimen and phase of the disease. All prognostic variables in the univariate analysis with a *p* value ≤0.2 were included for the multivariate analysis to eliminate the redundancy among highly correlated characteristics, each of which may be individually significant. This analysis was performed

Table 2. Allele frequencies.

	Patients and donors (N=156)	*NCBI database*
SNP 8		
Allele C	0.929	0.981
Allele T	0.070	0.019
SNP 12		
Allele G	0.987	0.991
Allele C	0.013	0.009
SNP 13		
Wild type	0.981	0.995
Frameshift mutation	0.019	0.005

Table 3. *NOD2/CARD15* genotypes.

	Patients n. (%)	*Donors n. (%)*
WT/WT*	76 (89)	65 (92)
SNP8/WT	6 (7)	4 (5)
SNP12/WT	2 (3)	0 (0)
SNP13/WT	0 (0)	2 (3)
SNP8/WT-SNP13/WT	1 (1)	0 (0)

**WT: wild type for SNP 8, 12 and 13.*

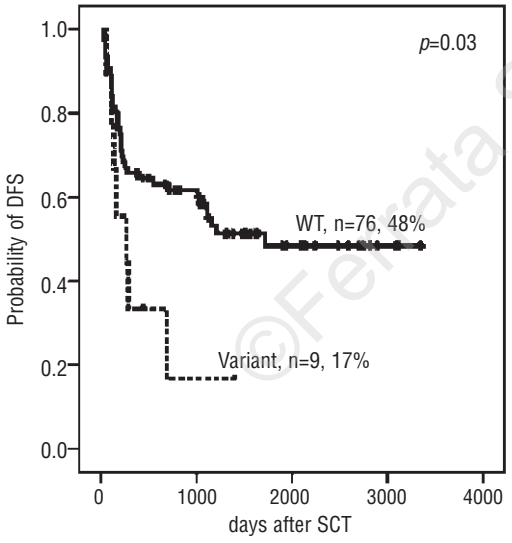

Figure 1. Disease-free survival (DFS) according to *NOD2/CARD15* genotype. *Variant* signifies heterozygotes for SNP8, SNP12 or SNP13 *NOD2/CARD15*. *WT* indicates patients with wild type *NOD2/CARD15*. (48% vs. 17%, *p*=0.03).

using Fine and Gray's method for acute and chronic GVHD and the stepwise proportional hazard Cox's regression model for disease-free-survival. The proportional hazard assumption was graphically checked for each co-variate before performing the regression analysis. Statistical studies were performed by means of Cran R software (*cmpr* package) and SPSS software (12.0 Chicago, IL, USA).

Results

Frequencies of the variants

NOD2/CARD15 allele frequencies in the 156 individuals (patients and donors) were not different from those present in the NCBI database (*http://www.ncbi.nlm.nih.gov/SNP*) (Table 2) and in the normal population of similar age from our geographical area.[11] Nine (11%) patients and six (8%) donors were heterozygous for any of the three variants analyzed; one patient was a compound heterozygote for the SNP8 and SNP13; no patients were homozygous for any of the variants studied. Detailed genotype frequencies for both patients and donors are given in Table 3. There were no differences in pre-transplant characteristics according to *NOD2/CARD15* genotype (*data not shown*).

Incidence of and risk factors for acute and chronic GVHD

The cumulative incidence of grades II-IV acute GVHD was 10% (CI 95%: 6-21). No single factor was associated with grade II-IV acute GVHD. Of the 85 patients studied, 78 (91%) survived more than 100 days after stem cell infusion and were included in the assessment of chronic GVHD. The cumulative incidence of overall chronic GVHD was 27% (CI 95%: 16-37). The only factor associated with an increased incidence of chronic GVHD was previous acute GVHD (56% vs 16%, *p*=0.04).

Incidence of and risk factors for disease-free survival and non-relapse mortality

The median follow-up of the surviving patients was 57 months (range 12-111) and that of the patients who died after the transplant was 12 months (range 0.7-76). The actuarial probability of disease-free survival was 45% (CI 95%: 33%-57%). Factors associated with a lower disease-free survival were the presence of *NOD2/CARD15* variants in the recipient (17% vs. 48%, *p*=0.03) (Figure 1), age older than the median (34% vs. 65%, *p*=0.01), and positive cytomegalovirus serology in the recipient (39% vs. 64%, *p*=0.06). In the multivariate analysis only *NOD2/CARD15* genotype (RR 2.3, CI 95%: 1.02-5.4, *p*=0.04) and older age (RR 2.2, CI 95%: 1.05-4.7, *p*=0.04) remained statistically significant. Four patients with *NOD2/CARD15* variants died in remission, the cause being respiratory failure due to pneumonia in all of them (100%). In contrast, among the 17 patients with wild type *NOD2/CARD15* who died in remission, the cause of death was pneumonia in only 10 (58%). Relapse rate did not differ according to donor or recipient *NOD2/CARD15* genotype (*data not shown*). The actuarial probability of non-relapse mortality was

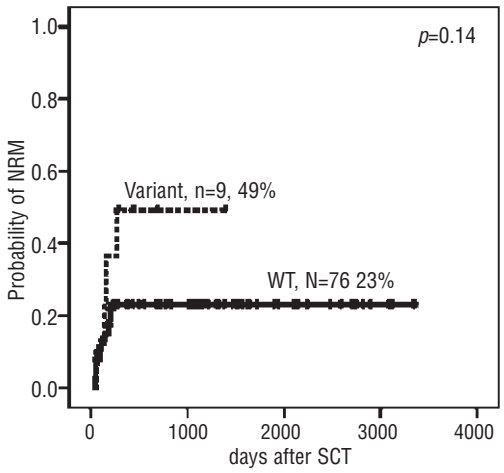

Figure 2. Non-relapse mortality (NRM) according to *NOD2/CARD15* genotype. *Variant* signifies heterozygotes for SNP8, SNP12 or SNP13 *NOD2/CARD15*. *WT* indicates patients wild type *NOD2/CARD15* (49% vs. 23%, *p*=0.14).

26% (CI 95%: 16%-35%). The only factor associated with an increased non-relapse mortality was the patient's age being older than 38 years (33% vs. 11%, *p*=0.03); a trend towards a higher non-relapse mortality was found in cytomegalovorus-positive patients (32% vs. 10%, *p*=0.07) and in patients with *NOD2/CARD15* variants (49% vs. 23%, *p*=0.14) (Figure 2). In the multivariate analysis no single factor remained statistically significant.

Discussion

In this study we showed that patients with variant alleles of *NOD2/CARD15* undergoing T-cell-depleted allogeneic SCT had a lower disease-free survival than that of patients with the wild-type genotype. Indeed, this genetic marker was one of the two most important independent prognostic factors for poor disease-free survival in the multivariate analysis. This detrimental effect was not due to an increase in the incidence and severity of GVHD, as previously reported for unmanipulated allogeneic SCT,[4,5] since this complication was not associated with the presence of variant alleles of *NOD2/CARD15* in either the donor or the recipient. The observed effect is not surprising considering the physiological role of the Nod2 protein and the functional change due to variant alleles. Nod2 is a cytosolic protein involved in monocyte intracellular recognition of peptidoglycan of bacterial walls, leading to the induction of inflammatory responses by activation of *NF-κB*[12]. In addition, NF-κB activation promotes the secretion of pro-inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β.[13,14] Polymorphisms of *NOD2/CARD15* alter the physiological response to infections through two mechanisms: a deficient response to infections by the non-specific innate and the adaptive immune response.

It has been considered that polymorphisms in *NOD2/CARD15* are associated with a diminished NF-κB response, resulting in a possible defect in the defense against bacteria.[12] More recently, a direct antibacterial activity for Nod2 in epithelial intestinal cells has been proposed.[15] In a study by Hisamatsu *et al*. mice with polymorphisms in *NOD2/CARD15* did not control intraluminal bacterial infection as efficiently as mice with the wild-type gene. These findings suggest that *NOD2/CARD15* has a crucial role in recognizing the presence of bacteria at the epithelial barrier and is necessary to initiate an immune response. Thus, *NOD2/CARD15* polymorphisms might facilitate bacterial proliferation and a higher incidence of sepsis.[16] This diminished control of infections could be of particular relevance in patients submitted to T-cell-depleted allogeneic SCT, in which the risk of infections is higher, due to the slower adaptive immune reconstitution than in patients submitted to T-cell-depleted allogeneic SCT.[17] In fact, in our series the most important cause of death in patients with *NOD2/CARD15* variants in clinical remission after T-cell-depleted allogeneic SCT was pulmonary infections. Interestingly, Nod2 is expressed in bronchial epithelium and is involved in the intracellular recognition of pneumococci, the major cause of community-acquired pneumonia;[18] moreover, *NOD2/CARD15* variants have been associated with bronchiolitis obliterans syndrome after allogeneic SCT.[19]

A second effect of variant alleles of *NOD2/CARD15* on clinical outcome may be associated with a deregulation of the innate immune system, with impaired tolerance against bacterial infections and with an abnormal excess of the inflammatory NF-κB response, similarly to what has been proposed to occur in Crohn's disease.[8] A number of studies have shown that family members of *NOD2/CARD15* might have a role in down-regulating the NF-κB pathway[20] and that the most common *NOD2/CARD15* variant in humans might result in a gain-of-function.[21] In animal models it was found that *NOD2/CARD15* variants led to a heightened inflammatory response to bacteria, whereas mice without the *NOD2/CARD15* variant had a normal response. The mutant mice produced higher levels of pro-inflammatory cytokines, particularly mature interleukin-1β. As a result, one third of the mutant mice developed severe colonic inflammation and died, whereas none of the mice with the normal *NOD2/CARD15* genotype was affected.[7] These data would support the concept that increased mortality was due not only to GVHD but also to monocyte-macrophage dysfunction leading to pulmonary complications.[4] In line with this, in our series there was an important proportion of deaths due to pulmonary infection in patients in clinical remission who

carried the *NOD2/CARD15* variants. Besides its role in the non-specific innate immune system, Nod2 generates signals for the adaptive immune response.[14,22] Such a response is generated upon activation of Nod2 by residual products of Gram-positive and Gram-negative bacteria, which induces secretion of interleukin-6 and interleukin-12, leading to the expression of co-stimulatory molecules on dendritic cells, a crucial step in initiating alloimmune reactions. This cytokine release also promotes the differentiation of naïve T cells into effector T cells.[14,22] These events would explain the strong association of *NOD2/CARD15* variants with the incidence and severity of GVHD found by Holler *et al.*,[4] which was associated with a higher cumulative incidence of transplant-related mortality.

In conclusion, our results support the hypothesis that *NOD2/CARD15* polymorphisms influence the clinical outcome of allogeneic SCT recipients in different ways depending on the type of transplant. In those patients submitted to T-cell-depleted SCT the detrimental effect is possibly due to an altered response of the innate immune system rather than to activation of the adaptive immune system. Although with a different perspective from that reported by others[2,4] the data presented here support the important beneficial effects of gut decontamination for the success of allogeneic SCT, especially in patients with *NOD2/CARD15* variants. These results also highlight the importance of detecting genetic differences between donors and recipients not only in HLA-associated genes but also in other genes[23] with a potential impact on clinical outcome.

MG performed the statistical analysis and wrote the material and methods and results sections. AU design the study, wrote the discussions and contributed to the rest of the paper. JA performed the experiments (sequencing) and contributed to the matherial and methods. FF, CM, MR AG, and CT contributed to the acquision of data. JR, SP and AN, performed the experiments. EC, MM and EM critically revised the manuscript. JY contributed to the design of the study, wrote the introduction and reviewed the manuscript.

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