Human Immunology 80 (2019) 163–168

Contents lists available at ScienceDirect

Human Immunology

journal homepage: www.elsevier.com/locate/humimm

Bronchiolitis obliterans after allogeneic hematopoietic stem cell transplantation: The effect of *NOD2/CARD15* mutations in a Tunisian population

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A B S T R A C T

Bronchiolitis obliterans (BO) is a serious lung complication that can develop after allogenic stem cell transplantation. It has been suggested that single nucleotide polymorphisms (SNPs) that affect the NOD2/CARD15 gene impair its function and result in an uncontrolled innate immune response in the recipient, thereby leading to BO.

One hundred eighty-one donor-recipient pairs were analyzed for the association between NOD2 gene variants (SNP8 [Arg702Trp], SNP12 [Gly908Arg], and SNP13 [Leu1007fsinsC]) and the occurrence of BO. Ten patients (2.8%) developed this complication. The incidence of BO increases in recipient variant patient group from 4.7% to 23% in donor Wild-type group in SNP8 ($p < 0.001$). The incidence rose to 19% when the recipient carried the SNP12 variant ($p < 0.001$) in the Tunisian population. Analyses demonstrated that recipient NOD2CARD15 variants (SNP8 and SNP12) present a greater risk in developing BO than recipients without mutation. Our study demonstrated that NOD2/CARD15 typing may be useful in identifying patients at high risk for BO.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is currently the treatment of choice for certain hematological malignancies (mainly acute leukemias), but it also is the choice for benign hematological diseases, such as bone marrow suppression, hemoglobinopathies, immune deficiency, and several metabolic diseases [1]. Certain factors influencing post-allograft development are now well known, including the age of the recipient, and the type and status of the disease during the transplant. Ever since the first transplants were performed, most patients have been clinically observed to exhibit an immunological reaction of the donor towards the recipient, a condition that is called graft-versus-host disease (GVH) [2]. This immune reaction can cause major complications after allografting that are potentially lethal, but it is also generally associated with an anti-tumor response that is referred to as the graft-versus-leukemia (GvL) effect [2].

Evaluations of allogeneic bone marrow transplantations remain a difficult exercise in daily clinical practice. For a given patient, it is necessary to estimate, in the most objective manner possible, the risk that is associated with the procedure on one hand, and the risk that is incurred during the natural progression of the disease on the other [3].

A further complication of HSCT is the development of bronchiolitis obliterans (BO), which is considered a form of GVH [4]. It is characterized visually as an obstructive ventilatory disorder and histologically as a fibrotic inflammatory process that results in constriction of small airways and their obliteration. Due to airway obstruction, patients develop hyperinflation with areas of atelectasis (partial or complete lung collapse), a lack of mobilization of bronchial secretions, and the development of bronchiectasis (permanent airway enlargement) and pulmonary fibrosis [5]. The term 'BO syndrome' has been proposed as a means of identifying patients who develop a progressive and irreversible decline in FEV1 (forced expiratory volume in 1 s) during pulmonary function tests. When the value of FEV1 is < 75% of FVC (Forced Vital Capacity), then the subject is considered to be affected by BO [6,7].

The pathogenesis of BO is not fully understood. Some explanations assume that it is secondary to lung injury that is attributable to conditioning, viz., the period of preparing the patient for HSCT using either chemotherapy alone or chemotherapy combined with radiotherapy [8]. As has been previously predicted, the incidence of BO would be lower after non-myeloablative conditioning compared to myeloablative conditioning [9]. Another hypothesis is that bronchiolitis obliterans develops as lesions that are secondary to an infectious process [10]. The last possible mechanism is an alloreactive immune response, in which

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https://doi.org/10.1016/j.humimm.2018.12.005
Received 17 September 2018; Received in revised form 8 December 2018; Accepted 11 December 2018
Available online 12 December 2018

donor T-cells attack epithelial cells of the recipient's bronchioles, which leads to an uncontrolled inflammatory reaction that is followed by BO. With regard to the last mechanism, we have been interested in the specific receptors of innate immunity PRRs (Pattern-Recognition Receptors) that recognize PAMPS (Pathogen-Associated Molecular Patterns) [11,12].

The NOD2 (Nucleotide-binding Oligomerization Domain-containing protein 2) belongs to a family of proteins that are involved in the innate immune response of the host to bacterial agents [13]. Innate immunity allows both the recognition of relatively invariant microbial structures and an immediate response by the body, as opposed to the adaptive or acquired immune response [5].

The *NOD2/CARD15* gene (Nucleotide-binding Oligomerization Domain-containing protein 2 Caspase Recruitment Domain-Containing Protein 15) encodes an intra-cytoplasmic receptor of the NLR (NOD-like Receptors) family that is involved in the recognition of bacterial muramyl dipeptide (MDP) [14]. The NOD2 protein has the characteristic structure of NLR proteins: an N-terminal CARD (Caspase Recruitment Domain); a NACHT or NOD (Nucleotide-binding and Oligomerization Domain); and a leucine-rich LRR (Leucine Rich C-terminal domain Repeat) [15]. LRR recognition of the MDP antibacterial motif allows the activation of NOD2, which triggers a signaling cascade in nuclear factor κB (NFkB) pathways and MAP kinases, in synergistic interaction with the signal that is triggered by TLR2 (Toll-like receptor 2). The regulation of transcription leads to the production of cytokines and other molecules that are involved in antimicrobial defense [16].

Variants of the aforementioned gene alter the function of NOD2. These mutations in the LRR region are single nucleotide polymorphisms (SNPs), which were initially detected in Crohn's disease [17]. These polymorphisms are responsible for a modification of the inflammatory response and anti-infectious defense. They have also been implicated in certain complications following HSCT, such as GVHD and BO [2,3]. Three genetic variants of NOD2 have been reported to be associated with BO. These represent 80% of the mutations in this gene. Two are non-conservative missense mutations (R702W and G908R), while the third is a shifting mutation (L1007fsinsC), which generates a truncated 32-amino acid protein [18].

We studied the effect of genes coding for the molecules of innate immunity on post-transplant development of polymorphisms in a population of Tunisian allografted patients. Among the many examples that have been previously described, we elected to analyze the SNP8, SNP12, SNP13 polymorphisms of the *NOD2/CARD15* gene [19]. The objective of this study is to determine the effects of these polymorphisms in both the donor and recipient during post-transplant development on the occurrence of BO.

2. Materials and methods

2.1. Ethical considerations

This study was conducted according to protocols that were recommended and approved by the Committee of Medical Ethics of Tunisia.

2.2. Patient selection and data collection

The cohort contained 362 subjects: 181 recipients and their 181 respective donors of HSCTs. Recipients were subjected to HLA-matched hematopoietic stem cell transplantation (HSCT) protocols at the National Bone Marrow Transplantation Centre from 2010 to 2013; all clinical data were collected from the same institution [20].

2.3. Sampling DNA

Blood samples were collected in EDTA (Ethylenediamine tetraacetic acid) anticoagulant at the National Blood Transfusion Centre of Tunis, from all individuals prior to HSCT [21]. Genomic DNA of recipients and their respective sibling donors of HSCTs were prepared from whole blood using the QIamp DNA Blood Mini Kit (Qiagen, Leiden, The Netherlands), and stored until analysis at the National Blood Transfusion Centre.

Table 1
Primers for NOD2/CARD15 PCR-SSP genotyping.

SNPs	Sequence 5′-3′	Product size
SNP 8	SNP8*C TGAGAAGGCCCTGCTCC SNP8*T TGAGAAGGCCCTGCTCT Rv AGAGTTGTAGTCCAGCTGCAG	316 bp
SNP12	SNP12*G GGCCTTTTCAGATTCTGGG SNP12*C GGCCTTTTCAGATTCTGGC Rv GACATTTCCAAGTCACCCAG	281 bp
SNP13	SNP13W CCCTCCTGCAGGCCCT SNP13*Cins CCCTCCTGCAGGCCCC Rv AACCGCAGAAGGTCTGATC	412 bp

Abbreviations: Bp, Base Pairs; W, Wild Type; SNP, Single Nucleotide Polymorphism; Rv, reverse primer.

2.4. BO grading

Diagnosis of BO is defined according to standard clinical criteria [6,7]. All patients who were included in the study had undergone spirometry prior to HSTx.

2.5. Genotyping assay using sequence-specific primers (PCR-SSP)

The presence of *NOD2/CARD15* variants (SNPs) was detected by using sequence-specific primer PCR (polymorphism chain reaction) reactions (PCR-SSP) (Table 1). Three SNPs were selected for this study: SNP8 (+2104C > T [rs17860491], SNP12 + 2722G > C [rs17860492], and SNP13 3020insC [rs17860493]) [22].

We performed PCR amplification with a Gen Amp PCR system (2700, Perkin Elmer, Norwalk, CT, USA). All PCR reactions were performed in a total volume of 10 µL reaction medium containing 2×10^{-4} M of each dNTP (nucleoside triphosphate), 1×5.10^{-7} M of forward and reverse NOD2/CARD15-specific primers, 0.7×10^{-7} M of forward and reverse internal control primers, 1.5×10^{-3} M $MgCl_2$, 1×10^{-7} L × 5 Green buffer (Promega, Madison, WI, USA). PCR reactions were performed using 100 ng of genomic DNA and 0.5 U Taq polymerase (Go Taq DNA Polymerase, Promega). The amplification protocol was 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, elongation at 72 °C for 30 s, and 10 min at 72 °C for final elongation. The PCR products were run on 1% agarose gel and visualized with UV illumination and photo documented (UVItec Ltd., Cambridge, UK) [20,21].

We verified the specificity of primers before sequence alignment with the Primer-BLAST tool, which is available on the website of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

2.6. Statistical analysis

In this study, individuals that were heterozygous for at least one of the three SNPs were considered variants, while individuals lacking these SNPs were considered wild types. We analyzed each SNP separately. Observed genotype frequencies were compared with Hardy-Weinberg expectation using Chi-square statistics.

A case-control cohort study was carried out. The cases were identified as the patients who had developed BO, while the controls were the other patients who did not develop BO.

For overall survival (OS) in patients who have *NOD2/CARD15* variants versus those that do not, actuarial curves were calculated using

the Kaplan-Meier method and compared using log rank tests. To complement survival rates, the cumulative incidence method was employed to estimate the probability of failure (mortality) by maximum-likelihood, and implemented using NCSS 12 software (NCSS, Kaysville, UT). The presence or absence of NOD2 variants in recipients were treated as pre-transplant grouping factor and were not considered as a competing risk.

The association between *NOD2* variants and the occurrence of BO was evaluated using Chi-square tests. Odds-ratios for bivariate and multivariate logistic regression models were used to analyze the risk factors and the probabilities of BO.

Binary logistic regression was used to compare groups, where the response of patients with BO was coded 1, and those not exhibiting the disease, 0. The response variable was regressed against several predictors, which were retained in the model. From the equations, we then estimated the probability of BO occurrence as decimal fractions bounded by 0 and 1. Further, logistic regression isolated the effects of each predictor, which allowed us to identify residual effects of a given explanatory variable (sex, GVHD, conditioning, the presence of the SNPs) on the variable of interest (Occurrence of BO), once the effects of the other predictors are controlled for. Except for the constant and the residuals, each term in the equation for the logistic regression model that was used is a product of a regression coefficient and a variable. In choosing this additive form, we assumed that the 'effect' of an individual variable on the response variable is measured by the magnitude of its beta-coefficient, and that this 'effect' is independent of the other variables and coefficients. While the independent variables may still affect one another, but this does not preclude the assumption that the effect of a predictor on the response (dependent) variable is unaffected by the other predictors. In order to utilize the information that was provided by genotyping of the three selected SNPs, we also created dummy variables for those groups. Dummy coding assigned a value of 1 to the variant individuals for each SNP and a value of 0 to the wild-type subjects [23].

3. Results

3.1. General genotyping analysis

The molecular approach that was used has permitted typing of the 3 selected SNPs of the NOD2 gene with high sensitivity. The molecular assay showed the presence of all possible genotypes for the SNP12.

NOD2/CARD15 variants occurred with a frequency of 25.4% (46/181) in recipients and 20.4% (37/181) in donors who were typed in our study. Homozygous and heterozygous variants were observed in this cohort. Frequencies were 11.60% for recipients (R) and 8.83% for donors (D) for SNP 8, 11.60% (R) and 9.39% (D) for SNP12, and 2.2% (R) and 1.6% (D) for SNP13. In 21 pairs (11.6%), only recipients had SNP8 and SNP12; in 4 pairs (2.2%), only recipients had SNP13 (Table 2).

In 17 pairs (9.3%), mutations occurred in donors only for SNP8; 14 pairs (7.3%) for SNP12; 4 pairs (2.2%) for SNP13, and in 3 pairs (1.6%), both donors and recipients exhibited mutations. In this consecutive cohort of patients, recipient- and donor-specific characteristics, together with transplant-specific procedures, were equally distributed between patient/donor pairs with and without variants (Table 3).

Table 2
Percentage of 3 common variants of *NOD2/CARD15* in donors and recipients.

NOD2/CARD15variants	Donors	Recipients
G908R SNP8	8.83%	11.60%
R702W SNP12	9.39%	11.60%
1007fs SNP13	1.6%	2.2%

Table 3
Clinical characteristics of the patient/donor pairs.

Characteristics	Number of patients
Average patient age (years)	22
Patient sex	
Male	103
Female	78
Diagnoses	
Chronic myeloid leukemia CML	18
Acute myelogenous leukemia AML	67
Acute lymphoblastic leukemia ALL	53
Fanconi anemia FA	09
Aplasic anemia AA	18
Multiple myeloma MM	09
Thalassemia THL	07
Conditioning	
BU + CY busulphan and cyclophosphamide	115
VP16 + TBI vepesid and total body irradiation	66
GVHD prophylaxis	
cyclosporine and methotrexate	172
cyclosporine	9

All patients had undergone HLA-matched HSCT at the National Bone Marrow Transplantation Centre of Tunis.

Table 4
Analysis of association between the 3 SNPs of NOD2 gene and the occurrence of BO in recipients.

	p	OR	95% C.I.	
			Lower	Upper
SNP8 CT	0.000	10.000	2.748	36.392
SNP12 GC	0.003	5.852	1.577	21.716
SNP13 WC	0.652	0.980	0.966	0.995

Abbreviations: OR, odds ratio; 95% C.I., confidence interval.

3.2. Case-control study for BO: single marker association analysis

We performed X^2 analysis on the three genotyped SNPs using the case-control analysis method. Results are shown in Table 4.

We found a significant association between the SNP8 CT genotype, SNP12 GC genotype and the occurrence of BO. Further, we determined the prevalence of BO: Ten patients (2.8%) developed this complication, with a median time to diagnosis of 81 days (range, 21–240 days) after allogeneic SCT (Table 5). The association of *NOD2/CARD15* variants with the occurrence of BO demonstrated a significant effect on OS for the group of recipients. In fact, OS decreases from 78% to 17% ($p < 0.001$) (Fig. 1) when the recipient carried a *NOD2/CARD15* variant.

Table 5
Clinical characteristics of transplant recipients who developed BO.

Patients	Time (days)	GVHD	Conditioning	Diagnosis	FEV1	FEV1pre-HST
H1	60	+	BU + CY	CML	62%	> 80%
H2	240	+	BU + CY	CML	52%	> 80%
H3	72	+	BU + CY	AML	44%	> 80%
B	30	+	VP 16 + TBI	AML	30%	95%
K	120	+	VP 16 + TBI	ALL	42%	89%
M1	135	+	VP 16 + TBI	ALL	55%	> 80%
O	45	+	BU + CY	ALL	40%	> 80%
Z	21	+	BU + CY	AML	72%	> 80%
H4	240	+	BU + CY	AML	66%	100%
M2	90	+	BU + CY	AML	74%	102%

Abbreviations: FEV1, forced expiratory volume in 1 s, relative percentage (versus pre-HST × FEV1); Time, time (days) to diagnosis; See Table 2 for explanations of diagnosis acronyms; FEV1pre-HST, baseline FEV1 prior to HST.

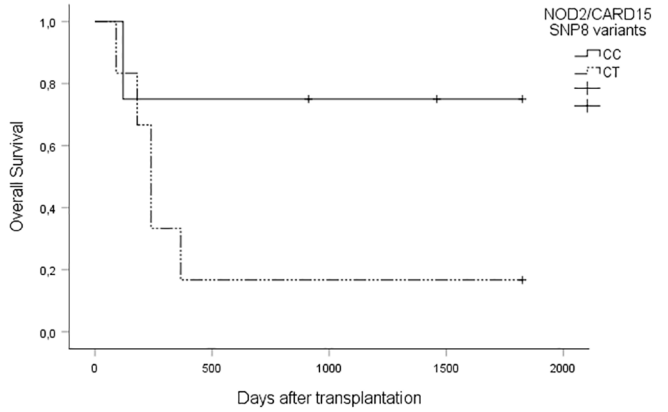

Fig. 1. Overall survival according to the number of NOD2/CARD15 variants in patients versus wild-type.

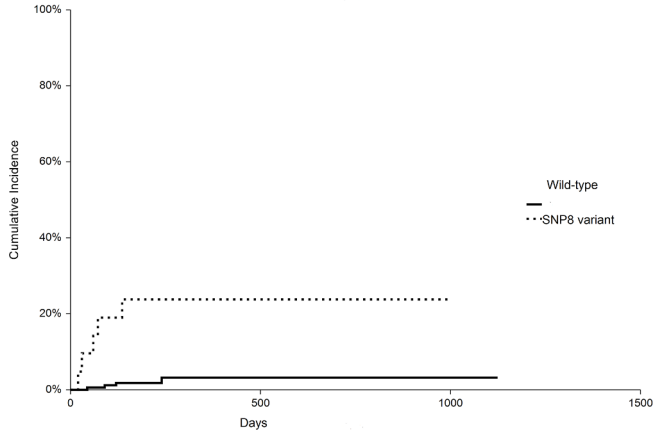

Fig. 2. Cumulative incidence of BO after HSCT in wild- type recipient group and variant SNP8 recipient group. Individuals who are homozygous CC for the SNP8 are considered wild-type and individuals who are heterozygous CT for the SNP8 are considered variants.

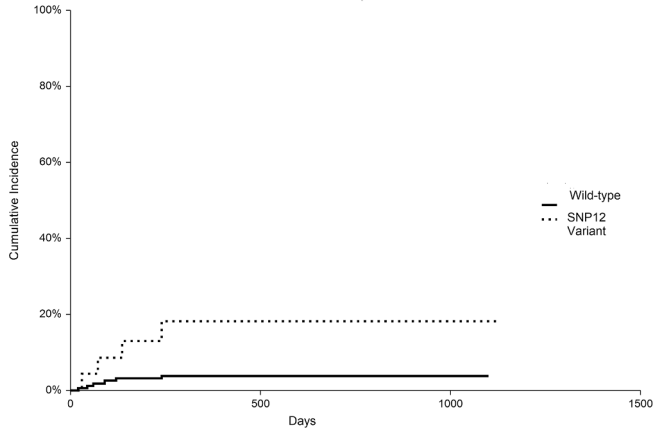

Fig. 3. Cumulative incidence of BO after HSCT in wild-type recipient group and variant SNP12 recipient group. Individuals who are homozygous GG for the SNP12 are considered wild-type and individuals who are heterozygous GC for the SNP12 are considered variants.

We also compared the pathophysiologic relevance of recipient *NOD2/CARD15* variants for BO development by determining cumulative incidence in the cases of absence or presence of the mutated allele in recipients. The incidence of BO increased from 4.7% in the wild-type recipient group (did not carry a *NOD2* SNP8 variant) to 23%, when the recipient carried SNP8 variant ($p < 0.001$) (Fig. 2). Incidence rose to 19%, when the recipient carried the SNP12 variant ($p < 0.001$) (Fig. 3).

Table 6
Parameters of multiple regression model of risk factors for BO.

	p	OR	95% C.I.	
			Lower	Upper
Sex	0.833	1.234	0.174	8.733
Conditioning	0.458	2.011	0.318	12.722
SNP12 = GC	0.414	3.027	0.212	43.213
SNP13 = WC	0.999	0	0	–
SNP8 = CT	0.004	18.437	2.602	130.63
GVHD	0.02	12.716	1.502	107.624

Abbreviations: OR, estimated odds ratio.
Risk factors are sex mismatch with donor and recipient; conditioning: conditioning with TBI; SNP12GC: recipient carrying GC genotype vs recipient not carrying this genotype; SNP13WC: recipient carrying WC genotype vs recipient not carrying this genotype; SNP8CT: recipient carrying CT genotype vs recipient not carrying this genotype; GVHD: patient who developed GVHD and BO vs patient with only BO.

3.3. Multivariate analysis of risk factors for BO

Given that the SNP8 and SNP12 variants are associated with the occurrence of BO, we decided to investigate their effect in multivariate regression including other risk factors (GVHD, sex mismatch and conditioning). This model showed that BO is affected by the presence of SNP8 (CT genotype) and the presence of the GVHD complication in recipients ($p < 0.001$). In fact, the risk of developing BO in a SNP8 variant recipient increased 18-fold relative to a wild-type recipient ($p < 0.001$) (Table 6).

4. Discussion

In this study, we examined the association between polymorphisms of the *NOD2/CARD15* gene and the occurrence of bronchiolitis obliterans. For this, we selected three major polymorphisms of the gene that are associated with this complication: Gly908Arg, Arg702Trp, and a mutation resulting from an insertion into the reading frame Leu1007fsinsC [24]. These mutations, which are located within or near the ligand recognition domain, confer a “loss of function” phenotype on the patient by preventing the activation of the NFκB pathway by MDP and, therefore, the elimination of bacteria. The production of pro-inflammatory cytokines IL-6 (Interleukin 6) and TNFa (Tumor necrosis factor) by mononuclear cells that are isolated from peripheral blood after stimulation with MDP is impaired in BO patients with the Leu1007fsinsC variant [25]. The importance of the presence of a SNP 8 polymorphism of NOD2/CARD15 in the recipient has been demonstrated; in our cohort of allografts, there is indeed an effect of this polymorphism on post-treatment complications in terms of increased risk of BO in our population [26].

These results are consistent with those published by Hildebrandt et al. [19]. The localization of the variant (on the recipient or donor cells) seems to be of importance, since several studies have shown that the existence of mutations in the recipient is associated with an increased risk for the occurrence of BO. The hypotheses that explain these results are based upon the pathophysiology of BO and certain findings in experimental models [27]. Most published studies of NOD2 functions describe the mechanisms that are associated with the control of infection by microorganisms. The existence of variants in the recipient suggests a decrease in the immune response that is triggered by the presence of bacteria in contact with pulmonary epithelial cells and,

consequently, an alteration of antimicrobial defenses with a potential increase in translocations that may explain morbidity and increased mortality [22].

In the presence of a polymorphism in the donor, it is conceivable that there is an alteration in the immune response that is derived from the donor cells and a decrease in Th1 (lymphocyte T helper 1) responses that are mediated by the NFκB pathway. However, Th1 polarization is the basis of LT (lymphocyte T) activation. This alteration of Th 1 responses may contribute, therefore, to the decrease in the allogeneic graft-versus-host reaction (GVHD) [28].

Another hypothesis from Ditschkowski et al. [29] is that NOD2 is a negative regulator of TLR2 and that its loss of function causes an exacerbation of the pro-inflammatory response, which is initiated by TLR2 in DC (dendritic cells) and pulmonary macrophages. Yet, the prevalence of BO and the frequencies of *NOD2/CARD15* in the Tunisian transplant population were comparable to those reported by other studies [29].

Note that in the study of the Hildebrandt et al. [25], the incidence of BO was 2.6% and the cohort contained 427 (donor/recipient) pairs. These results were most similar to our own. Ditschkowski et al. [29] describe the overall incidence of obliterative bronchiolitis, which increased from 2.1% to 22% when a mutation of NOD2 occurred in the recipient. In our population, the cumulative incidence of BO is 4.7% in recipients without variants, but these increased to 23% in the presence of a SNP 8 polymorphism of NOD2 in the recipient and rose to 19% in the presence of SNP12. The analyses for the remaining polymorphism (SNP13) are not significant. Indeed, the data were insufficient to make any definitive conclusion regarding SNP 13, which occurred less frequently in our study. Another pulmonary disease, viz., sarcoidosis, has been the subject of several studies, which have attempted to understand the effect of *NOD2/CARD15* variants, Sarcoidosis is an inflammatory disease of unknown cause. The diagnosis of this disease is based upon the presence of immune granulomas in the tissues. These granulomas are clusters of inflammatory cells that can disrupt the normal structure and functioning of the different tissues and organs in which they are mostly located, i.e., the lungs and the lymphatic system [30]. The presence of *NOD2/CARD15* variants was associated with pulmonary sarcoidosis phenotypes. This association offers an intriguing insight into a dysregulated response to bacteria. In fact, Sato et al. [31] suggested that the dysfunction of this gene, which plays an important role in the activation of T lymphocytes, could influence the normal trafficking of T lymphocytes to the lung, thereby leading to parenchymal abnormalities that result in fibrosis of the lungs. It was also suggested that the presence of NOD2 variants contributes to an impaired response to bacteria, regardless of whether these species are commensal or pathogenic, with an abnormal T-cell activation in patients with sarcoidosis [31].

In summary, our data are consistent with results that were obtained in previous studies showing that polymorphisms of the NOD2 gene in the recipient are significantly associated with the incidence of BO after hematopoietic stem cell transplantation (HSCT). This discovery may help identify patients who are at risk for developing BO using *NOD2/CARD15* typing. The prevalence of BO and *NOD2/CARD15* frequencies in the Tunisian allograft population was comparable to those reported by other studies [25].

Finally, we released a retrospective study, which is limited by the reduced study group size and the lack of information about patients. Nevertheless, we were able to find a significant relationship between NOD2 polymorphisms and the occurrence of BO.

5. Conclusion

In conclusion, we analyzed 3 single nucleotide polymorphisms (SNPs) in the gene coding for NOD2 to determine whether these genetic polymorphisms were associated with the development of BO or not. Ten patients with BO and 171 control individuals (without BO) were included. BO patients heterozygous CT for SNP8, and those who were heterozygous GC for SNP12 carried significantly more risk alleles for developing this complication than did the wild-type control group.

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