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Is there any Association of NOD2/CARD15 Gene Polymorphism with Tunisian Pemphigus Foliaceus?

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Abstract

Background: Knowledge about the pathogenesis of Pemphigus Foliaceus (PF), an autoimmune disorder of the skin natural barrier, is still modest. Genetic susceptibility factors have been widely studied, while environmental factors remain still ambiguous. NOD2/CARD15 gene encodes an intracytoplasmic receptor involved in recognition of microbial components and NF-κB inflammatory signaling pathway. Three common CD-associated variations (R702W, G908R and 1007fs) cause a "loss of function" of the molecule and lead to a chronic inflammation in the intestinal epithelial barrier. The aim of this study is to analyze NOD2/CARD15 gene polymorphisms in Tunisian endemic PF.

Methods and Results:A case-control study including 79 PF patients and 160 controls was conducted using PCR-RFLP and direct sequencing. Our results showed that the three SNPs are not polymorphic in both patients and controls (allelic frequencies were 0.63% vs 1.25%, 0.63% vs 1.87% and 0% vs 0.62%, respectively). There was no association of mutant alleles with the disease.

Conclusions: Our results suggest that the three common variants of NOD2/CARD15 gene are not involved in susceptibility to Tunisian PF. The alteration of the molecule's functionality caused by these mutations seems to not interfere with the development of the disease.

Keywords: NOD2; CARD15; Single nucleotide polymorphism; Restriction fragment length polymorphism; Pemphigus; Tunisia

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Introduction

"Pattern Recognition Receptors" (PRRs) are innate immunity receptors involved in anti-infectious defense of the organism [1]. There are several classes of PRRs: the "Toll-Like-Receptors" (TLRs), the "Retinoic acid inducible gene-I-Like Receptors" (RLRs), the "Nod-Like Receptors" (NLRs) and the "C-type Lectin-Receptors" (CLRs) [2]. NOD2/CARD15 (nucleotide binding oligomerisation domain 2/ caspase-recruitment domain containing protein 15) is an intracytoplasmic receptor from the NLRs family. It is composed of three parts: two N-terminal domains of recruitment and activation of caspases (CARD), a Nucleotide-Binding Domain (NBD) in the middle and a C-terminal domain composed of Leucin-Rich-Repeats (LRRs). This receptor is expressed mainly in monocytes/macrophages, dendritic and epithelial cells and is upregulated by pro-inflammatory stimuli [3]. NOD2 receptor is involved in the recognition of bacterial components through its LRRs, leading to NF-κB inflammatory signaling pathway [3,4]. The NOD2/CARD15 gene is located in the peri-centromeric region of chromosome 16 (16q12) and belongs to the super-family of Ced4 apoptosis regulators [5-7]. Three main mutations, within or near the LRRs domain of the receptor, are commonly associated with Crohn's Disease (CD), an inflammatory disorder of the intestinal epithelium: two "missense mutations" (R702W, G908R) and a "frameshift" mutation generating a stop codon and leading to a truncated protein (L1007fsinsC) [3]. The impaired function of the receptor caused by these mutated variants in intestinal phagocytic and epithelial cells results in abnormal function of the intestinal barrier leading to increased bacterial invasion and inflammation of the intestine. It has been also demonstrated that Nod2 wild-type (unlike mutated variants) could induce a more efficient bacterial clearance and adaptive immune response by mediating autophagy. Furthermore, it could activate antiviral innate immune response after viral ssRNA recognition [3]. Indeed, the human microbiome, which behavior is affected by host susceptibility factors, seems to play a major role in the pathogenesis of inflammatory disorders located in epithelial surfaces like the gut (CD) and the skin [2]. Pemphigus Foliaceus (PF), an endemic disease in rural Tunisian regions, is

Table 1: Position, genetic/amino-acid variation and size of NOD2/CARD15 SNPs.

Gene	SNPs	Rs Code	Position	Genetic Variation	Amino-acid Variation	Size (pb)
NOD2/CARD15	R702W	rs2066844	Exon 4	C/T	R702W	185
	G908R	rs2066845	Exon 8	G/C	G908R	163
	L1007fs	rs2066847	Exon 11	Insertion of a « C » base	L1007fs	151

Table 2: Primers and enzymes used for NOD2/CARD15 SNPs genotyping.

Gene	SNPs	Rs Code	Primers	Restriction enzyme
NOD2/CARD15	R702W	rs2066844	F : 5'-AGATCACAGCAGCCTTCCTG-3'	MspI
			R: 5'-CACGCTCTTGGCCTCACC-3'	
	G908R	rs2066845	F : 5'-CTCTTTTGGCCTTTTCAGATTCTG-3'	HhaI
			R : 5'-CAGCTCCTCCCTCTTCACCT-3'	
	L1007fs	rs2066847	F : 5'-GGCAGAAGCCCTCCTGCAGGGCC-3'	ApaI
			R: 5'-CCTCAAAATTCTGCCATTCC-3'	

an autoimmune blistering disorder of the skin epithelial barrier [7,8]. Pathogenesis of PF involves a complex interaction of multiple factors (genetic, immunological, environmental, and infectious factors) [7-9]. The possible implication of various infectious agents ("black fly", Onchocerciasis, Leishmaniasis, Staphylococcus aureus toxin, Herpes virus....) in the etiopathogenesis of pemphigus has been reported in different studies [10-12]. PRRs (including NODs) are described to play an important role in the infectious defense in the skin natural barrier [2,13]. In this study, we analyzed the role of the 3 common CD-associated NOD2 variants (R702W, G908R and L1007fsinsC), which encode a receptor for microbial components with impaired function, in the susceptibility to Tunisian endemic PF.

Patients and Methods

We conducted a case-control study which included 79 South-Tunisian unrelated patients with PF (department of Dermatology, Hedi Chaker University Hospital of Sfax, Tunisia) and 160 healthy controls not suffering from any autoimmune nor inflammatory disease). All patients had clinical, histological and immunological criteria of PF. The sex ratio was 9/1; the average age was 33.5 years. Controls were matched to patients in age (± 5 years), sex and geographical origin. All patients and healthy controls gave informed consent to participate in the study. We chose to analyze 3 tag-SNPs (rs2066844 (R702W), rs2066845 (G908R), rs2066847 (1007fs)) of the NOD2/CARD15 gene located in the peri-centromeric region of chromosome 16 (16q12) (Table1). The 3 mutations are located inside or close to the C-terminal domain of NOD2 protein: «LRR domain», which is involved in recognition of bacterial wall component (muramyl dipeptide).

Blood samples were collected in EDTA-anticoagulated tubes and DNA was extracted using standard methods. All SNPs were genotyped by conventional polymerase chain reaction/restriction fragment length polymorphism analysis (PCR-RFLP) (Table 1). Primers were designed using primer3 software (http://primer3.ut.ee/) and restriction enzymes were selected using the NEBcutter software (http://nc2.neb.com/NEBcutter2/) (Table 2). Digestion products were electrophoresed through 3% agarose gel and scored following ethidium-bromide staining. The accuracy of the genotyping was confirmed by direct sequencing of each SNP.

The data were analysed with Hardy-Weinberg equilibrium tests. Statistical analysis was carried out using SHEsis program (http://analysis.bio-x.cn) and SPSS 16.0 software. P values <0.05 were considered statistically significant. Allele and genotype frequencies were calculated and associations with susceptibility to PF were tested by calculating Odds Ratios (OR) with asymptotic 95% Confidence Intervals (CIs).

Results

The results of the allelic and genotypic frequencies distribution of the studied NOD2/ CARD15 SNPs (R702W, G908R and L1007fs) in patients and controls are summarized in Table 3. The distribution of R702W, G908R, and L1007fs genotypes were in accordance with the Hardy-Weinberg equilibrium test results (p >0.05). Minor of all the three polymorphisms was consistent with that reported in the HapMap database. In our control group, the heterozygous mutations were found in 4 controls (2.5%) for each of the R702W and G908R SNPs, and in 2 controls (1.25%) for the L1007fs SNP. The G908R homozygous mutation was found in only 1 individual (0.62%) of the 160 controls. No other homozygous mutations were found in this group.

The heterozygous mutation frequencies of R702W, G908R and L1007fs SNPs in PF patients were equal or lower than those found in the control group (1.26%, 1.26% and 0%, respectively). No homozygous genotypes were found in patients. No significant differences in genotypic and allelic frequencies of the 3 SNPs were detected among the two groups (p values >0.05). Despite the small number of heterozygote patients (1 patient with R702W-CT and 1 patient with G908R-GC), we performed stratified analyzes for rs2066844 (R702W) and rs2066845 (G908R) genotypes in PF patients according to gender, age, disease severity (based on involved surface body area and Activity Score) and need for immunosuppressive therapy or plasmapheresis. No statistical differences were observed between allelic/genotypic frequencies and patients' stratifications (data not shown). None of the individuals (patients/controls) presenting the NOD2/ CARD15 mutations had clinical features of an inflammatory bowel disorder.

Discussion

In PF pathogenesis, genetic susceptibility factors have been widely studied, however involvement of environmental factors remains misunderstood. In this data, we studied the possible role of variation (tag SNPs) in a gene encoding an innate receptor (NOD2/CARD15) involved in the recognition of microbial components in the development of endemic Tunisian PF. To the best of our

Table 3: Allelic and genotypic frequencies of NOD2 gene variants in Tunisian patients with pemphigus and healthy controls.

SNP	Genotypic frequencies n(%)				Allelic frequencies %	
rs2066844 (R702W)		CC	CT	TT	C	T
	Controls (n =160)	156 (97.50)	4 (2.50)	0	98.75	1.25
	Patients (n =79)	78 (98,73)	1 (1.26)	0	99.37	0.63
	p	0.522			0.524	
	χ^2	0.408			0.404	
(G908R) rs2066845		GG	GC	CC	G	C
	Controls (n =160)	155 (96.87)	4 (2.50)	1 (0.62)	98.13	1.87
	Patients (n =79)	78 (98.73)	1 (1.26)	0	99.37	0.63
	p	0.777			0.614	
	χ^2	0.502			0.254	
rs2066847 (L1007fs)		WT/WT	WT/MT	MT/MT	WT	MT
	Controls (n =160)	158 (98.75)	2 (1.25)	0	99.38	0.62
	Patients (n =79)	79 (100)	0	0	100	0
	p	0.315			0.316	
	χ^2	1.008			1.004	

WT: Wild Type; MT: Mutated Type; P: Probability

knowledge, the possibility of NOD2/CARD15 gene involvement in PF pathogenesis has not been studied previously. Several arguments have led us to suspect that these gene variants could be involved in susceptibility to PF. (i) Environmental factors, including bacterial infections, have been implicated in the pathogenesis of PF [10]. (ii) Recently, we have demonstrated that the expression of some TLRs (TLR2, TLR3 and TLR4) is significantly up-regulated in the epidermis of Tunisian PF patients [14], (iii) Several functional studies showed that various skin cells (keratinocytes and Langerhans cell) express the NOD2 receptor and are involved via this receptor in the immune defense of the skin natural barrier [3,13,15]. (iv) Finally, genetic association of different SNPs of genes encoding NOD2 receptor or related molecules (especially the 3 common CD-associated NOD2 variants) with diseases involving skin lesions and/or diseases with auto-inflammatory or autoimmune pathogenesis (atopic dermatitis, psoriatic arthritis, BlauSyndrom /Early onset of sarcoidosis...) were largely described [8,16-18]. NOD2/CARD15 gene mutations could therefore lead to an inability of the immune system to control bacterial infection and to the development of chronic inflammation [2], which may cause various dysimmune disorders such as PF.

Our results showed that these 3 SNPs are not polymorphic in the general Tunisian population (minor allele frequency <5%). These findings are relatively consistent with those described in two previous Tunisian studies and in two other North African studies [19-24]. Thus, these polymorphisms constitute rare mutations in healthy Tunisian population.

The common association of NOD2 mutations with CD and Inflammatory Bowel Disease [19,20] has been widely, but not universally [21] replicated, especially in North African populations [9,22-26]. Despite the similarities in etio-pathogenesis between CD and PF (two disorders of natural epithelial barriers, role of the microbiome in the disease development, expression of PRRs, especially NODs by intestinal and skin cells and their role in infectious defense) [3], our analysis provides evidence of no genetic association between Tunisian endemic PF development and variation in NOD2 gene. The higher prevalences of the 3 CD-associated variations found in phenotypic healthy controls compared to PF patients could be explained by the need of complex interactions (gene–gene or gene–environment) for the development and the clinical expression of the disease.

Conclusion

In summary, our results suggest that, despite genetic and proteomic arguments, the three common variants of NOD2/CARD15 genes are not involved in susceptibility to Tunisian PF. We conclude that the genetic variants analyzed do not alter the functionality of the molecules in a way that would interfere with the development of the disease. Genetic association studies of other pattern recognition receptors in PF are necessary to confirm the importance of environmental factors in the pathogenesis of this autoimmune disease.

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