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Brief report

NOD2 genetic variants and sarcoidosis-associated uveitis☆

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ABSTRACT

Purpose: Identifying genetic risk factors for developing sarcoidosis-associated uveitis could provide insights into its pathogenesis which is poorly understood.
We determine if variants in *NOD2* confer an increased risk of developing uveitis in adults with sarcoidosis.
Methods: In this genetic case-control study, 51 total subjects were enrolled: 39 patients diagnosed with sarcoid-related uveitis and 12 patients with systemic sarcoidosis without ocular involvement as controls. Sanger sequencing of the eleven exons of the *NOD2* gene was performed on DNA obtained from whole blood. Sanger sequencing data were aligned against the *NOD2* NCBI-RefSeq reference sequence to identify novel mutations in uveitis patients. For common variants, allele frequencies in cases versus controls were compared using the chi-square test.
Results: There were no significant differences in *NOD2* common variant allele frequencies between sarcoidosis patients with and without uveitis, and none of the pathogenic *NOD2* mutations associated with Blau syndrome were found in this cohort. However, four rare, non-synonymous variants were identified in four patients with ocular sarcoidosis and none of the controls. Variants rs149071116, rs35285618, and 16:g.50745164T > C have never been previously reported to be associated with any disease and may be pathogenic. The fourth variant, rs2066845, is associated with Crohn's disease and psoriatic arthritis.
Conclusions: Despite the phenotypic overlap between sarcoidosis and Blau syndrome, none of the established pathogenic *NOD2* variants were present in adults with sarcoidosis. However, four novel, rare, non-synonymous variants were identified in four cases with ocular sarcoidosis. Further investigation is needed to explore the potential clinical significance of these polymorphisms.

1. Introduction

NOD2 mutations have been shown to be responsible for three granulomatous inflammatory diseases: Crohn's disease, *NOD2*-associated autoinflammatory disease and Blau syndrome [1]. Blau syndrome is a systemic Mendelian disease whose diagnostic triad includes uveitis, granulomatous dermatitis, and arthritis [1], and its phenotype overlaps significantly with early-onset sarcoidosis. Because of the similarity between sarcoidosis and Blau syndrome, there is a strong rationale to investigate *NOD2* variation in patients with adult-onset sarcoidosis, particularly those who develop uveitis as this is a characteristic of both disorders. One previous study examined *NOD2* variants in 13 patients with sarcoidosis-associated uveitis [2]. The investigators did not find any of the disease-causing variants associated with Blau syndrome in sarcoidosis patients. The purpose of this study is to investigate *NOD2* variants, including those that have been newly discovered since the previous publication, in a larger sample size of sarcoidosis patients with and without uveitis to determine if there are previously reported mutations or novel mutations in *NOD2* that predispose to ocular involvement.

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2. Materials and methods

Patients with sarcoidosis were identified from the Ocular Immunology and Uveitis Clinics of the Massachusetts Eye and Ear Infirmary (MEEI). This study was approved by the MEEI Institutional Review Board/Human Studies Committee. Controls were defined as patients with systemic sarcoidosis but no ocular involvement. Cases were defined as having sarcoidosis-related uveitis. Systemic sarcoidosis was defined based on definitive or presumed sarcoidosis by American Thoracic Society (ATS) and the World Association for Sarcoidosis and Other Granulomatous Disorders (WASOG) statement criteria [3]. With this criteria, diagnosis is established by either: 1) histological confirmation with evidence of disease involvement in either the thorax or two more other organ systems; or 2) characteristic chest radiographs (bilateral hilar lymphadenopathy) plus erythema nodosum or without diagnosis of another explanatory condition after a minimum of 2 years follow up. Presence or absence of uveitis was determined by clinical examination by a fellowship-trained uveitis specialist. Classification of ophthalmic involvement was based on the criteria set forth by the International Workshop on Ocular Sarcoidosis (IWOS) [4]. A diagnosis of definitive ocular sarcoidosis was made in the presence of a compatible uveitis with a confirmatory biopsy. Presumed ocular sarcoidosis was defined by a compatible uveitis supported by bilateral hilar lymphadenopathy seen on chest imaging without a biopsy.

Sanger sequencing of the eleven exons of the *NOD2* gene were performed on DNA extracted from the participants' blood. Sanger sequencing data were aligned against the *NOD2* NCBI-RefSeq reference sequence. DNASTAR SeqMan Pro software was used for quality control filtering, variant calling and analysis. The variants detected in our patients were cross-referenced against *NOD2* variants that have been previously associated with Blau syndrome, sarcoidosis or Crohn's disease [1]. They were also annotated to identify novel rare, non-synonymous mutations in *NOD2* for potential functional significance using Human BP Codon Resource (HBCR). HBCR uses predicted sequence changes based on Ensembl gene models, and pathogenicity data extracted from a curated database, PolyPhen, Sorting Intolerant From Tolerant (SIFT), the database of Non-Synonymous Functional Prediction (dbNSFP) resource, and Combined Annotation Dependent Depletion (CADD) scores. HBCR also annotates variants with allele frequencies in multiple reference populations.

Baseline demographic and clinical characteristics between cases and controls were compared using the *t*-test for continuous variables and the chi-square test for categorical variables. For common NOD2 variants, allele frequencies between cases and control were compared using the chi-square test. All statistical analyses were performed in Stata 12.1 (College Station, Texas).

3. Results

51 subjects were enrolled, including 39 patients diagnosed with sarcoid-related uveitis as cases and 12 patients with systemic sarcoidosis without ocular involvement as controls. Table 1 summarizes the clinical characteristics of the patients. The gender, age, race and duration of sarcoidosis were not statistically different between cases and controls ($p > 0.05$).

In our patients with adult-onset sarcoidosis, we did not identify any of the single amino acid mutations in *NOD2* that have been associated with Blau syndrome [1,5]. Table 2 shows all of the polymorphisms in *NOD2* identified by sequencing in our patients. In comparing with the previous publication on *NOD2* variants in ocular sarcoidosis, there were six variants that led to protein changes that overlapped between the two studies. Four of them were common variants (rs2067085, rs2066842, rs2066843, rs1861759) and we did not find that they were associated with uveitis to a statistically significant degree. The fifth was a rare variant associated with Crohn's disease (rs2066844, MAF = 0.03). In our cohort, none of the patients with this variant had a history of Crohn's disease. The sixth (rs5743291) was a rare variant associated with early onset sarcoidosis, but the frequency of this allele was not significantly different between those with and without uveitis [minor allele frequencies (MAFs) = 0.067 and 0.068 respectively].

We identified four rare, non-synonymous variants that were present in only one case each. Two, rs149071116 and novel variant NM_022162.1:c.1342T > C (NOD2:p.Ser448Pro), have never been previously associated with any disease. SIFT, a sequence homology-based tool that sorts intolerant from tolerant amino acid substitutions and predicts whether an amino acid substitution will have a phenotypic effect, predicted both variants to be damaging. Polymorphism rs149071116 was identified in a 34-year-old Asian man with anterior uveitis in the right eye and panuveitis and macular edema in the left eye. The patient with variant NM_022162.1:c.1342T > C (NOD2::p.Ser448Pro) was a 36-year-old Caucasian man with panuveitis, choroiditis, periphlebitis and papillitis. He required methotrexate in addition to his prednisone to control his ocular inflammation and also had renal and pulmonary involvement during course of his disease. The third polymorphism, rs35285618, is a low frequency single nucleotide protein (SNP) (MAF = 0.013) located six amino acids downstream of the R702W variant that is associated with Crohn's disease in African Americans [6]. It was predicted to be "possibly damaging" by PolyPhen but "tolerated" by SIFT. It was found in a 40-year old Hispanic man of European descent with bilateral panuveitis, lung, skin, and parotid gland involvement. The fourth polymorphism, rs2066845, is also a low frequency SNP that is associated with Crohn's disease and psoriatic arthritis [7–9]. The patient with this variant was a 64-year-

Table 1
Demographic and clinical characteristics of sarcoidosis patients.

		Sarcoidosis with uveitis (n = 39)	Sarcoidosis without uveitis (n = 12)	P value[a]
Age in years, mean (SD)		56.6 (15.1)	54.2 (6.8)	0.58
Gender	M	11	5	0.48
	F	28	7	
Duration of sarcoidosis in years, mean (SD)		9.1 (7.5)	8.3 (10.3)	0.72
Ethnicity	CA	28	10	0.27
	AA	10	1	
	HA	1	1	

SD = standard deviation, M = male, F = female, CA = Caucasian American, AA = African American, HA = Hispanic American.
[a] Continuous variables compared with the *t*-test and categorical variables compared with the chi-square test.

Table 2
NOD2 variants identified in sarcoidosis patients.

NOD2 polymorphisms identified in patients with sarcoidosis						Variant allele frequency			
Variants in genomic HGVS format with chromosome: Position	SNP rs ID number	Mutation type	Nucleotide change	Peptide change	Prior associations with disease	Minor allele frequency from 1000 genomes	Sarcoidosis with uveitis N = 39 (allele frequency)	Sarcoidosis without uveits N = 12 (allele frequency)	P Value
Common variants (minor allele frequency ≥ 0.05)									
16:g.50731096 G > A	rs2076752	5′ UTR	47G > A NM_022162.1:c.-59G > A by VEP	V16	None	A = 0.14	19 (0.24)	6 (0.18)	0.59
16:g.50733374 G > T	rs2076753	Intronic	74-25G > T	NA	None	T = 0.11	16 (0.20)	5 (0.18)	0.53
16:g.50733859 C > G	rs2067085	Syn	534C > G	S178S	Crohn's disease [10]	G = 0.24	26 (0.33)	6 (0.18)	0.20
16:g.50744624 C > T	rs2066842	Non-syn	802C > T	P268S	None	T = 0.10	13 (0.17)	4 (0.13)	0.50
16:g.50745199 C > T	rs2066843	Syn	1377C > T	R459R	Crohn's disease [11,12]	T = 0.1076	15 (0.19)	5 (0.18)	0.60
16:g.50745583 T > G	rs1861759	Syn	1761T > G	R587R	None	G = 0.2161	28 (0.36)	7 (0.22)	0.27
16:g.50759547 A > T	rs1077861	Intron	2966 + 64A > T	NA	Chronic obstructive pulmonary disease [13]	T = 0.33	39 (0.5)	11 (0.46)	0.47
Rare variants (minor allele frequency < 0.05)									
16:g.50733808 C > T	NA	Syn	483C > T	F161F	None	T = 0.0026	1 (0.01)	0 (0)	NA
16:g.**50741800**[a] **C > T**	**rs149071116**	**Non-syn**	**575C > T**	**A192V**	**None**	**NA**	**1 (0.01)**	**0 (0)**	**NA**
16:g.50744638 C > T	rs35090774	Syn	816C > T	S272S	None	T = 0.0042	1 (0.01)	0 (0)	NA
16:g.**50745164**[a] **T > C**	**NA**	**Non-syn**	**1342T > C**	**S448P**	**None**	**NA**	**1 (0.01)**	**0 (0)**	**NA**
16:g.50745275 C > T	rs5743274	Syn	1453C > T	L485L	None	T = 0.01	1 (0.01)	0 (0)	NA
16:g.50745655 C > T	rs61736932	Syn	1833C > T	A611A	Crohn's disease, Ulcerative colitis [14]	T = 0.0026	1 (0.01)	0 (0)	NA
16:g.50745926 C > T	rs2066844	Non-syn	2104C > T	R702W	Crohn's disease [7]	T = 0.01	2 (0.02)	1 (0.04)	NA
16:g.**50745945**[a] **G > A**	**rs35285618**	**Non-syn**	**2123G > A**	**R708H**	**Crohn's disease** [6]	**A = 0.005**	**1 (0.01)**	**0 (0)**	**NA**
16:g.50745996 C > G	rs5743278	Non-syn	2174C > G	725G	None	G = 0.018	1 (0.02)	1 (0.09)	NA
16:g.**50756540**[a] **G > C**	**rs2066845**	**Non-syn**	**2722G > C**	**G908R**	**Crohn's disease,** [7,8] **Psoriatic arthritis** [9]	**C = 0.004**	**1 (0.01)**	**0 (0)**	**NA**
16:g.50757276 G > A	rs5743291	Non-syn	2863G > A	V955I	Crohn's disease [15]	A = 0.03	6 (0.06)	4 (0.18)	0.50

Bold: Rare, non-synonymous variants that were only in cases.
SNP = Single nucleotide polymorphism, 5′ UTR = 5′ untranslated region, Syn = Synonymous, Non-syn = Non-synonymous, NA = Not available.
[a] Rare, non-synonymous variants those were only present in cases.

old woman with history of anterior uveitis, good response to medication and a good visual acuity outcome.

4. Discussion

We identified 18 polymorphisms in 51 patients with sarcoidosis, which expands from prior work performed by Martin et al. [2] Our study similarly found that *NOD2* disease-associated genetic variants and common *NOD2* variants are not associated with uveitis in adult-onset sarcoidosis patients. Our sample size, albeit modest, was larger than the previous study. Our study was also performed at a time when the annotation of the human genome is more complete, and we are better able to characterize the frequency and potential pathogenicity of variants, particularly rare variants.

With this enhanced annotation of the human genome, we did identify four rare polymorphisms that lead to protein changes and were found only in patients with uveitis. The identification of these variants in only those with ocular manifestations is not sufficient to establish pathogenicity, particularly given their low MAF and the limited number of controls without uveitis included in this study. The potential pathogenicity needs to be further explored with *in vitro* experiments. Given the significant overlap in phenotype between Blau syndrome and sarcoidosis and the important role of *NOD2* variation in a variety of inflammatory diseases, exploration of *NOD2* variants in a larger cohort could also help to assess whether these or other rare variants may contribute to the development of uveitis in a subset of sarcoidosis patients. Including sarcoidosis uveitis patients without pulmonary involvement in such a cohort may be particularly revealing as they may be closer phenotypically to Blau syndrome patients in whom pulmonary involvement is not common.

5. Conclusions

Despite the phenotypic overlap between early onset sarcoidosis and Blau syndrome, none of the Blau syndrome-associated *NOD2* variants were present in adults with sarcoidosis. However, four rare, non-synonymous variants were identified in four cases with ocular sarcoidosis. Additional investigation with larger cohorts is needed to explore the potential clinical significance of these variants.

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