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NOD2 polymorphisms in clinical phenotypes of common variable immunodeficiency disorders

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Summary

Common variable immunodeficiency disorders (CVIDs) are a heterogeneous group of diseases characterized by hypogammaglobulinaemia and consequent susceptibility to infection. CVID patients commonly develop a variety of additional manifestations for which the causative factors are not fully understood. Two such manifestations are granulomatous disease and enteropathy. Because the ability to predict complications would aid clinical management, we continue to search for possible disease modifier genes. NOD2 acts a microbial sensor and is involved in proinflammatory signalling. Particular mutations of the *NOD2* gene are associated with Crohn's disease including gly908arg, leu1007finsc and arg702trp polymorphisms. We hypothesized that *NOD2* polymorphisms may be a disease modifier gene towards an enteropathic or granulomatous phenotype within CVIDs. Sequence-specific primers returned genotypes for 285 CVID patients from centres across the United Kingdom and Europe. We present the frequencies of the different phenotypes of patients within our international cohort. Arg702trp polymorphisms were significantly less frequent than wild-type (WT) ($P = 0{\cdot}038$) among international CVID patients with splenomegaly. Gly908arg polymorphisms were more prevalent than WT in UK patients with autoimmune disorders ($P = 0{\cdot}049$) or enteropathy ($P = 0{\cdot}049$). *NOD2* polymorphisms were not more prevalent than WT in CVID patients with clinical phenotypes of granulomata. UK allele frequencies of 0·014, 0·056 and 0·026 were found for gly908arg, arg702trp and leu1007finsc *NOD2* polymorphisms, respectively. These do not differ significantly from UK immunocompetent controls confirming, as expected, that in addition these *NOD2* polymorphisms do not confer susceptibility to CVIDs *per se*.

Keywords: antibody deficiency, common variable immunodeficiency, disease susceptibility/resistance/polymorphisms, immunodeficiency – primary, immunogenetics

Introduction

Common variable immunodeficiency disorders (CVIDs) are the most common forms of clinically significant primary immune deficiencies in humans, with a prevalence estimated at minimum to be one in 30 000 [1–3]. CVIDs are characterized by markedly reduced levels of serum immunoglobulins and subsequent increased susceptibility to infection [4]. CVIDs remain a diagnosis of exclusion and patient cohorts demonstrate highly variable phenotypes [5]. Patients usually present with increased susceptibility to infections, especially encapsulated bacteria, affecting mainly the upper and lower respiratory tract and the gastrointestinal tract [6]. CVID patients are more susceptible to autoimmune diseases, unexplained enteropathy, polyclonal lymphoproliferation and granulomata for which the causative factors (genetic and/or environmental) are not fully understood. The genes involved in the susceptibility of these polygenic diseases remain to be defined [3,7].

Granulomatous disease occurs in 8–22% of CVID patients, depending on the patient cohort, with large variations in the prevalence of this complication between countries [5]. The cause(s) of these non-caseating granulomatous infiltrations is unknown, although recurrent and persistent

infections, dysregulated T cell function or macrophage activation have been suggested [8]. The early phase in the development of granulomata is characterized by the accumulation and activation of T helper type 1 (Th1)-type lymphocytes, involving proinflammatory cytokines, namely tumour necrosis factor (TNF) [9], interleukin (IL)-1β [10], IL-2 [11], IL-6, IL-8 [12] and interferon (IFN)-γ [13]. Although the histology of CVID enteropathy is similar to that of coeliac disease, namely villous blunting, intraepithelial T cells in association with epithelial apoptosis, these findings persist despite adherence to a gluten-free diet.

Genes that affect the production of these cytokines could act as potential disease modifier genes for the granulomatous complications of CVIDs [1]. The finding that Nod-like receptors (NLRs) are involved in the granulomata of Crohn's disease and sarcoidosis led to the hypothesis that polymorphisms in these genes might also act as disease modifiers in the varying clinical phenotypes seen in patients with CVIDs.

Nucleotide-binding oligomerization domain (NOD)2, also known as caspase recruitment domain (CARD) 15, is a 163319 base pair gene comprised of 12 exons (GenBank Accession number AJ303140, http://www.ncbi.nlm.nih.gov). It maps to the IBD1 locus in the pericentromeric region of chromosome 16 [14] and encodes a protein composed of two NH2-terminal CARDs, a nucleotide-binding domain (NBD) and 10 COOH-terminal leucine-rich repeats (LRRs). It has been shown to be expressed in macrophages, neutrophils and dendritic cells, as well as Paneth cells of the small intestine [15]and bronchial cells in the lungs [16].

NOD2 acts as a cytosolic pathogen recognition protein to activate the cell or induce apoptosis [17]. *NOD2* polymorphisms have been shown to be associated with Crohn's disease, a granulomatous condition of the digestive tract [18–21]. The most prevalent associated *NOD2* polymorphisms are leu1007fsinsc (SNP13 in exon 11), gly908arg (SNP12 in exon 8) and arg702trp (SNP8 in exon 4) found in the LRR region of the gene and representing 31%, 18% and 32%, respectively, of the total Crohn's disease-associated polymorphisms [19]. The remaining 19% of polymorphisms come from 27 additional rare variants considered as disease-causing mutations, also located primarily in the LRR region of the gene. *NOD2* polymorphisms are strong predictors of ileal disease, but are not associated with perianal or colonic disease [22]. Relative risk is greatest for the leu1007fsinsc variant [22]; however, *NOD2* polymorphisms are neither necessary nor sufficient for the development of Crohn's disease.

NOD2 polymorphisms are also associated with other chronic inflammatory disorders, including Blau syndrome (BS) and early-onset sarcoidosis (EOS). EOS and inheritable BS share the characteristic clinical features of juvenile-onset systemic granulomatous syndrome that mainly affects skin, joints and eyes. Patients with CVIDs are sometimes misdiagnosed as having sarcoidosis, although sarcoidosis is not readily confused clinically with CVID provided that serum immunoglobulin measurements are taken and infection history taken. *NOD2* polymorphisms related to BS and EOS affect the central nucleotide binding domain of *NOD2*, rather than the C-terminal portion associated with Crohn's disease [23,24]. Other conditions associated with *NOD2* mutations include psoriatic arthritis [25] and graft-*versus*-host disease [26].

Drewe and Powell reported an expanding phenotype of inflammatory diseases associated with *NOD2* mutations and in particular a case with CVID [27]. Evidence is presented that disorders of the innate immune axis are of interest in CVIDs whether through disease-causing or disease-altering mechanisms [28]. We hypothesize that *NOD2* polymorphisms, focusing upon those associated with Crohn's disease, might contribute to the granulomatous and/or enteropathic CVID phenotypes. We investigated the frequency of the most common *NOD2* polymorphisms associated with Crohn's disease in a cohort of CVID patients to see if there is any association with granulomata or gut involvement, in the hope that this might provide a prognostic marker for these complications in addition to suggesting a disease modification mechanism.

Methods

Patient cohort

A total of 299 unselected CVID patients were recruited from: the John Radcliffe Hospital, Oxford (75), the Royal Free Hospital, London (99), Queens Medical Centre, Nottingham (20), Northern General Hospital, Sheffield (24), Path Links, Scunthorpe (6), St Anne University Hospital, Brno, Czech Republic (33) and Medical School Hannover, Hannover, Germany (42). Ethical approval was obtained from the South West Research Ethics Committee (MREC/04/6/18) for the UK samples and from institutional review boards in the international centres. Samples were processed blind.

A clinical data sheet was completed by the managing clinicians for all patients included in the study and validated independently to ensure that recruited subjects fulfilled European Society for Immunodeficiencies (ESID) diagnostic criteria [4] and correct identification of complications including bronchiectasis (radiologically confirmed), autoimmunity (with details of site), granuloma (location and method of diagnosis i.e. biopsy proven), lymphoid interstitial pneumonitis (LIP) (and method of diagnosis i.e. radiological or histological), splenomegaly (confirmed radiologically >11 cm on ultrasound or palpable) and enteropathy (confirmed by histology).

Amplification of gene region using polymerase chain reaction (PCR)

Primers for the exon regions of interest were purchased from Sigma-Genosys (Haverhill, Suffolk, UK) (Table 1). Primers were made up to final concentrations as detailed in Table 1 with a 796 base pair control amplicon derived from a con-

Table 1. NOD2 primer mixes for sequence specific primer polymerase chain reaction (PCR).

Polymorphism	NCBI reference	SNP position	Sense primer 5′–3′	Sense conc. μg/ml	Anti-sense primer 5′–3′	AS conc. μg/ml	Amplicon size
Arg702trp	rs2066844	2104C	TGAGAAGGCCCTGCTCC	20	AGAGTTGTAGTCCAGCTGCAG	10	278
		2104T	CTGAGAAGGCCCTGCTCT	10		10	
Gly908trg	rs2066845	2722G	GGCCTTTTCAGATTCTGGG	2.5	GACATTTCCAAGTCACCCAG	5	242
		2722C	GGCCTTTTCAGATTCTGGC	20		20	
Leu1007finsc	rs2066847	3020	CCCTCCTGCAGGCCCT	10	AACCGCAGAAGGTCTGATC	10	377
		3030Ci	CCCTCCTGCAGGCCCC	10		10	

NCBI: National Centre for Biotechnology Information; SNP: single nucleotide polymorphism.

served region of DRB1 so that a positive control was run within each reaction. Three μl of this primer mix was added to appropriate pre-oiled [10 μl mineral oil (Sigma-Aldrich Company Ltd, Dorset, UK)] wells of a 96-well PCR plate (Corning Incorporated, Kennebunk, ME, USA). Biotaq polymerase (Bioline, London, UK) was used at a concentration of 1 μl *Taq* in 208 μl TDMH (TDMH is our in-house name for the PCR buffer used; it is derived from Tris-buffer, dNTPs, Magnesium and H_2O) (kindly donated by the tissue typing laboratory, Churchill Hospital, Oxford). Patient genomic DNA purified from peripheral blood was added to this Taq/TDMH mixture at a ratio of 1:23 (up to 1:8 in the event of PCR failure) and 5 μl of this mixture was then added appropriately to the 96-well PCR plate to make a reaction volume of 18 μl. PCR amplification was performed using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Molecular grade water (Sigma) in place of primers as a negative control was run with each sample.

Agarose gel electrophoresis

Synthesis of appropriately sized products was confirmed by running on agarose gel containing $3{\cdot}3 \times 10^{-4}$ g/l ethidium bromide (Sigma) alongside a DNA ladder (Sigma). Gels were viewed under ultraviolet light and photographed (Transluminator UVP, Upland, CA, USA).

Genetic sequencing

A random representation of each genotype was sequenced using an Applied Biosystems 3730 DNA Analyser with Sequencing Analyser version 5·2 software (Applied Biosystems) for genotype confirmation.

Calculation of allele frequency and statistical analysis

Allele frequency was calculated using the formula below:

$$\text{Allele frequency} = [(\text{heterozygotes} \times 1) + (\text{homozygotes} \times 2)] / [2 \times \text{population (n)}]$$

Fisher's exact test (GraphPad Prism® version 4) was used to compare allele frequencies. *P*-values < 0·05 were considered as significant.

Results

Complications among international cohort of CVID patients

Frequencies of complications within our CVID cohort are given in Fig. 1 and Table 2. Details of complications requested from managing clinicians (see Materials and methods) were validated from the information given and if necessary confirmed by independent review. The clinical data were finally 99·3% complete. Subsequent to validation, eight patients were unselected as they did not meet CVID diagnostic criteria.

Allele frequency of *NOD2* polymorphisms in CVID

Optimized protocols were used to genotype our cohort of 291 confirmed CVID patients for gly908Arg, arg702trp and leu1007finsc polymorphisms of the *NOD2* gene. Thirty-six heterozygous (four gly908arg, 21 arg702trp and 11 leu1007fins) polymorphisms, one homozygous gly908arg

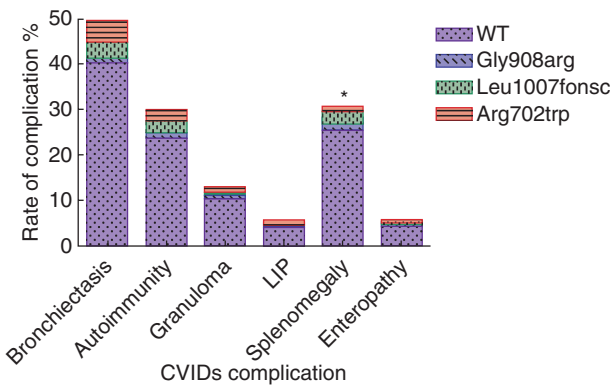

Fig. 1. Frequency of disease complications and *NOD2* polymorphisms among 285 international common variable immunodeficiency (CVID) patients. *Arg702trp polymorphisms are significantly ($P = 0{\cdot}038$) reduced within CVIDs with splenomegaly. Gly908arg polymorphisms appear more frequent among CVIDs with enteropathy; however, this has not reached statistical significance here ($P = 0{\cdot}072$).

Table 2. Prevalence of complications and NOD2 polymorphisms among 285 international common variable immunodeficiency (CVID) patients.

	Complication					
NOD2 genotype	Bronchiectasis	Autoimmunity	Granuloma	LIP	Splenomegaly	Enteropathy
Gly908Arg	4	4	2	1	4	2
Leu1007finsC	10	7	1	0	8	1
Arg702Trp	14	7	3	2	3	0
Total with at least one mutation	26*	17*	6	3	14*	3
WT	112	64	30	13	71	13
Total	138	81	36	16	85	16

*Patients within these groups had multiple *NOD2* polymorphisms. LIP: lymphoid interstitial pneumonitis; WT: wild-type.

and one homozygous arg702trp polymorphism were found among our UK cohort giving gly908arg, arg702trp and leu1007finsc allele frequencies of 0·014, 0·056 and 0·026, respectively. Six samples were unusable, providing 285 results for statistical analysis. No significant difference was found when comparing the UK CVIDs from our cohort to published data on NOD2 allele frequencies in 1876 UK control subjects (0·011, 0·047 and 0·027, respectively) [29].

NOD2 polymorphisms and disease complications in CVID

Fisher's exact tests were conducted for *NOD2* WT *versus* the three Crohn's disease associated polymorphisms for each CVID disease complication group defined (Fig. 1). A trend towards a gly908arg polymorphism conferring possible susceptibility towards autoimmunity, granuloma, LIP, splenomegaly and enteropathy is seen; however, these differences were not significant as defined. The most significant finding observed within the international cohort was a possible protective role of an arg702trp polymorphism from splenomegaly ($P = 0{\cdot}038$, relative risk RR = 0·374). Interestingly, among the UK CVIDs cohort with a gly908arg polymorphism ($n = 5$), a significantly increased frequency of autoimmune disease ($P = 0{\cdot}049$, RR = 2·400) and enteropathy ($P = 0{\cdot}049$, RR = 5·771) are seen in comparison to the WT CVIDs. Autoimmune disease and enteropathy were prevalent in the UK cohort at 34·1 and 7·8%, respectively. No other significant associations were found within the UK subset of the international cohort or the international cohort as a whole between *NOD2* genotype and disease complication phenotype.

Discussion

We investigated the presence of those polymorphisms found to be associated with Crohn's disease, namely gly908arg, arg702trp and leu1007finsc in the DNA of 285 CVID patients. These *NOD2* polymorphisms were examined as potential disease modifiers within CVIDs. Autoimmune disease and enteropathy were found to be more prevalent in those UK patients with a gly908arg polymorphism. Interestingly, autoimmunity and enteropathy were not found to be associated significantly with each other in a previous European study of CVIDs [5]. Furthermore, the enteropathy of CVID is resistant to gluten withdrawal and so distinct from coeliac disease.

Granuloma and autoimmune disease are associated independently with splenomegaly [5]. As splenomegaly was shown to be less prevalent in the international cohort in CVID patients with arg702trp polymorphisms, this may protect from granuloma development. Enteropathy is associated with splenomegaly, although only weakly [5], suggesting that these may be independent gene associations with each phenotype.

No significant association was seen between *NOD2* polymorphisms and other disease complications investigated (bronchiectasis, granulomatous disease, lymphoid interstitial pneumonitis). Because invasive investigations are required for histological diagnosis, variable thresholds of individual clinicians for requesting these investigations may have affected ascertainment, especially as this can be missed if not affecting a crucial anatomical site along with the variability in prevalence of complications between countries [5] and individual physicians' awareness for taking biopsies. As expected, no significant differences in allele frequency were found between CVID patients and geographically matched controls.

Crohn's disease is characterized by a dysregulated mucosal immune response [28]. The precise mechanisms by which *NOD2* polymorphisms contribute to this dysregulated mucosal immunity are still under speculation [30]. Proposals include defective regulation of responses to commensal and/or pathogenic bacteria [31–33] or via irregular secretion of α-defensins by Paneth cells in response to *NOD2* stimulation [34]. Impaired IL-17 secretion following *NOD2* stimulation in Crohn's disease patients may contribute to impaired bacterial clearance [35].

A key proposed role of *NOD2* is as a negative regulator of IL-12 stimulated through Toll-like receptor (TLR)-2 [36]. Presence of a Crohn's disease-like polymorphism increases TLR-2-mediated activation of nuclear factor (NF)-kB, resulting in increased production of IL-12 [37]. This leads to a Th1 skew, which is proposed as important in the pathogenesis of Crohn's disease. We hypothesized that this might also be important in the pathogenesis of the T cell infiltra-

tion of the epithelium and the interstium in unexplained enteropathy or of T cell activation in granulomata in CVIDs. The expanding pool of interest in the role of the innate immune system in CVIDs has also been supported recently by findings of abnormalities in the TLR-7/-9 pathways [38]. Further functional assays are required to investigate these hypotheses and the interplay of intracellular *NOD* receptors with TLRs in the recognition of bacteria which are the major pathogens in antibody deficiencies [39].

The polymorphisms investigated in this study account for 81% of the NOD2 polymorphisms found to be associated with Crohn's disease, but this leaves 19% not yet investigated here [19]. It should also be noted that genetic susceptibility to Crohn's disease is not limited to chromosome 16 and NOD2. Numerous genes have been found to be associated with inflammatory bowel diseases, and genomewide scans have identified additional susceptibility loci [40–44]. Further investigation into the other domains is essential alongside stimulation studies before a role of NOD2 as a disease modifier towards a granulomatous phenotype in CVIDs can be excluded. The importance of environmental factors on the development of complications and the pathogenesis of disease must also continue to be considered, and there are clearly multiple susceptibility factors and disease modifiers for Crohn's and other granulomatous disorders.

Conclusions

From this study it is seen that these major NOD2 polymorphisms are not associated with a granulomata in patients with CVIDs and are unlikely to act as disease-modifying polymorphisms, although there may be a protective role of arg908trp from splenomegaly within international CVID patients and a role of gly908arg polymorphisms towards enteropathic complications in UK CVIDs. Association of gly908arg with autoimmune disease within our UK cohort was also demonstrated and provides a hypothesis for further studies of patients with these complications.

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Disclosure

The authors have no conflicts of interest.

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