World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i3.359

World J Gastroenterol 2010 January 21; 16(3): 359-364
ISSN 1007-9327 (print)

BRIEF ARTICLE

NOD2, *IL23R* and *ATG16L1* polymorphisms in Lithuanian patients with inflammatory bowel disease

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Supported by The German Ministry of Education and Research through the National Genome Research Network
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Received: September 25, 2009 Revised: November 14, 2009
Accepted: November 21, 2009
Published online: January 21, 2010

Abstract

AIM: To investigate the frequency of *NOD2*, *IL23R* and *ATG16L1* genetic variants in a case-control panel for inflammatory bowel disease (IBD) from Lithuania.

METHODS: One hundred and eighty unrelated IBD patients [57 Crohn's disease (CD) and 123 ulcerative colitis (UC)] and 186 healthy controls were genotyped for the following known genetic susceptibility variants: *NOD2* - Arg702Trp (rs2066844), Gly908Arg (rs2066845) and Leu1007insC (rs2066847), as well as *IL23R* - Arg381Gln (rs11209026) and *ATG16L1* - Thr300Ala (rs2241880).

RESULTS: The effect that carriership of at least one *NOD2* risk allele predisposes to CD was replicated in the Lithuanian population (41.1% CD *vs* 16.9% controls, $P = 2 \times 10^{-4}$, OR = 3.48, 95% CI: 1.81-6.72). In the allelic single marker analysis, Leu1007insC was strongly associated with CD (21.4% CD *vs* 4.7% controls, $P = 3.687 \times 10^{-8}$, OR = 5.54, 95% CI: 2.85-10.75). Neither the other two *NOD2* variants, nor the known variants in *IL23R* and *ATG16L1* were found to be risk factors for CD, UC or IBD. However, our relatively small study population was underpowered to demonstrate such weak to moderate disease associations.

CONCLUSION: The results support a strong association between CD susceptibility and the Leu1007insC variant in *NOD2* in the Lithuanian study population.

Key words: *NOD2*; *IL23R*; *ATG16L1*; Single nucleotide polymorphisms; Crohn's disease; Ulcerative colitis; Lithuania

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Sventoraityte J, Zvirbliene A, Franke A, Kwiatkowski R, Kiudelis G, Kupcinskas L, Schreiber S. *NOD2, IL23R* and *ATG16L1* polymorphisms in Lithuanian patients with inflammatory bowel disease. *World J Gastroenterol* 2010; 16(3): 359-364 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i3/359.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i3.359

INTRODUCTION

The inflammatory bowel diseases (IBD) refer to two

clinically defined conditions, ulcerative colitis (UC) and Crohn's disease (CD) that represent major burdens of morbidity in Western countries, with prevalence rates in North America and Europe ranging from 21 to 246 per 100 000 inhabitants for UC and 8 to 214 per 100 000 inhabitants for CD[1]. Although the exact aetiology of IBD remains unclear, accumulating data suggests that IBD occurs from the combined effects of genetic predisposition and environmental factors[2].

Linkage, candidate gene, targeted association mapping and genome-wide association studies have identified many common variants associated with IBD and have rapidly expanded our fundamental knowledge of complex disease biology. The first and most consistently replicated genetic susceptibility variants, were found in the *NOD2* gene[3-5], attributed to the recognition of bacterial products, along with several other genetic loci coding for cytokines involved in acquired immune responses (*IL23R*[6]) and genes related to the autophagy pathway (*ATG16L1*[7]).

Given the heterogeneity in allele frequencies reported for the genetic factors involved in the pathogenesis of IBD in different European populations[8], we aimed to perform the first genetic study of IBD in a low-incidence population[9,10] of North-Eastern Europe - Lithuania. We examined the frequencies of the previously described variants in the *NOD2, IL23R* and *ATG16L1* genes in a Lithuanian IBD study population.

MATERIALS AND METHODS

Patients

The study included 57 unrelated patients with CD, 123 with UC and 186 healthy, age- and gender-matched controls. All study participants were of Caucasian ethnicity. The recruitment of the study individuals was performed at the Department of Gastroenterology, Kaunas University of Medicine Hospital during the period from 2003 to 2006. Written informed consent from all participants and approval of the Kaunas Regional Biomedical Research Ethics Committee (Protocol No. 84/2003) was obtained. The diagnosis of either CD or UC was based on standard clinical, endoscopic, radiological and histological criteria[11]. Patients' demographic and phenotypic details are summarized in Table 1. The clinical characteristics provided in the table are given according to the Montreal classification[12].

Genotyping

Genomic DNA was isolated from EDTA peripheral blood using the Invisorb Blood Giga Kit from Invitek (Berlin, Germany). The three *NOD2* variants - Arg702Trp (rs2066844), Gly908Arg (rs2066845) and Leu1007insC (rs2066847), and the *IL23R* variant Arg381Gln (rs11209026) were genotyped using Applied Biosystem's (Foster City, CA, USA) allele-specific TaqMan™ or TaqMan-MGB assays (Table 2); *ATG16L1* variant Thr300Ala (rs2241880) detection was performed using a pre-designed TaqMan® single nucleotide polymorphism (SNP) genotyping assay (ID C_9095577_20). Genotyping was performed on an automated platform using the TaqMan® (Applied Biosystems, Foster City, CA, USA) technique as previously described[13]. All genotyped markers had a call rate greater than 95% in case and healthy control samples.

Table 1 Summary of clinical and demographic characteristics of the IBD patients *n* (%)

Characteristics	CD	UC
Gender (male/female)	27/30	68/55
Age (years ± SD)	40.5 ± 14.9	45.4 ± 16.4
Age at diagnosis (years ± SD)	31.7 ± 16.6	34.3 ± 14.7
Familial IBD	0	0
Surgery treatment	15 (26.3)	3 (2.4)
Disease extension in UC		
Proctitis	-	26 (21.1)
Left-sided colitis	-	61 (49.5)
Extended colitis	-	36 (29.3)
Disease localization in CD		
Terminal ileum, L1	17 (29.8)	-
Colon, L2	16 (28.1)	-
Ileocolon, L3	23 (40.3)	-
Upper GI, L4	1 (1.8)	-
Disease Behavior in CD		
Non-stricturing, non-penetrating, B1	41 (71.9)	-
Stricturing, B2	5 (8.8)	-
Penetrating, B3	11 (19.3)	-
Perianal disease, B4	-	-
Extraintestinal manifestations		
Joints	6 (10.5)	13 (10.6)
Cutaneus	3 (5.3)	4 (3.3)
Ocular	1 (1.8)	0
Hepatobiliary	0	2 (1.6)

IBD: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis.

Statistical analysis

Each SNP was checked for conformance with Hardy-Weinberg equilibrium in the control group using Fisher's exact test ($P_{HWE} > 0.01$). Single-marker association analyses between cases and controls were performed using χ^2 statistics or Fisher's exact genotypic test. The significance level of the tests for considering *P*-values as significant was set to < 0.05. Data were evaluated using the web interface SISA[14]. Carriership of mutated alleles in case and control populations was estimated by direct counting.

The population attributable risk percentage (PAR%) was calculated as the attributable risk percentage (AR%) multiplied by the proportion of exposed cases, where AR% was estimated from the odds ratio (OR), assuming that the exposure of the control population to the disease-associated variant reflects the true prevalence of the variant in the general population[15].

RESULTS

All five SNPs were successfully genotyped in our North-Eastern European IBD case-control panel comprising 57 CD and 123 UC patients from Lithuania. The distribution of genotypes within the control group was

Table 2 TaqMan® primer and probe sequences of *NOD2* and *IL23R* assays

Marker	Primers	Probes
NOD2		
rs2066844	5'-TTCCTGGCAGGGCTGTTGTC	TET-CCTGCTC**T**TGGCGCCAGGCC
	5'-AGTGGAAGTGCTTGCGGAGG	FAM-CCTGCTC**C**GGCGCCAGGC
rs2066845	5'-ACTCACTGACACTGTCTGTTGACTCT	TET-TTCAGATTCTGG**C**GCAACAGAGTGGGT
	5'-AGCCACCTCAAGCTCTGGTG	FAM-TTTTCAGATTCTGG**G**GCAACAGAGTGGGT
rs2066847	5'-CCAGGTTGTCCAATAACTGCATC	VIC-TGCAGG**C**CCCTTG
	5'-CCTTACCAGACTTCCAGGATGGT	FAM-CTGCAG**G**CCCTTG
IL23R		
rs11209026	5'-CGTCTTTGCTGTTATGTTGTCAATTCTT	VIC-CAGATCATTCC**A**AACTG
	5'-AGAAAACAGAAATTCTGCAAAAACCTACC	FAM-ACAGATCATTCC**G**AACTG

The examined alleles are highlighted by bold underlined typing.

Table 3 Association statistics for the *NOD2*, *ATG16L1* and *IL23R* variants in the Lithuanian IBD population

Gene marker	Minor allele	Controls (n = 186)			CD (n = 56)				UC (n = 123)			
		GT (11/12/22)	MAF	P_{HWE}	GT (11/12/22)	MAF	P_{CCA}	OR (95% CI)	GT (11/12/22)	MAF	P_{CCA}	OR (95% CI)
NOD2												
rs2066844	T	0/9/171	0.025	> 0.99	0/2/54	0.018	> 0.99	0.71 (0.15-3.33)	0/10/113	0.041	0.278	1.65 (0.66-4.13)
rs2066845	C	1/7/169	0.025	0.099	0/3/53	0.027	> 0.99	1.06 (0.28-3.97)	0/1/121	0.004	0.055	0.16 (0.02-1.25)
rs2066847	insC	2/13/166	0.047	0.048	4/16/36	0.214	3.687×10^{-8}[a]	5.54 (2.85-10.75)	1/8/114	0.041	0.711	0.86 (0.39-1.91)
ATG16L1												
rs2241880	G	44/89/53	0.476	0.560	16/28/11	0.546	0.199	1.32 (0.86-2.03)	33/61/25	0.534	0.164	1.26 (0.91-1.75)
IL23R												
rs11209026	A	3/16/167	0.059	0.017	0/4/52	0.036	0.335	0.59 (0.20-1.75)	0/11/109	0.045	0.477	0.76 (0.36-1.61)

Minor allele frequencies (MAF), genotype counts (GT; 11 = homozygous for minor allele; 12 = heterozygous for common allele; 22 = homozygote for common allele), allelic test P values ([a]$P_{CCA} < 0.05$), and odds ratios (OR, shown for the minor allele) with 95% confidence intervals (CI) are depicted for both the CD and UC case-control population.

consistent with Hardy-Weinberg equilibrium (Table 3). For each of the variants studied, the risk of carrying the variant was compared between the CD, UC and healthy controls groups. The genotype and minor allele frequencies are presented in Table 3.

As expected, none of the studied individuals were carriers of all three *NOD2* risk alleles. However, two CD patients were determined as compound heterozygotes. The combined allele carriership in the group of patients with CD was much higher than in controls (41.1% *vs* 16.9%) and resulted in significant association ($P = 2 \times 10^{-4}$, OR = 3.48, 95% CI: 1.81-6.72) whereas no significant difference was observed between UC patients and controls. The PAR% in CD patients was 29.5% for possession of one or more *NOD2* variant alleles at any of the three sites.

In the allelic single marker analysis of the *NOD2* variants, a significant association was detected only between CD and Leu1007insC. For this variant, both the allelic and genotypic tests revealed P-values < 10^{-4} (OR_{allele} = 5.54, 95% CI: 2.85-10.75; $OR_{carriership}$ = 6.12, 95% CI: 2.88-13.15), resulting from the increased minor allele frequency (MAF) in cases (21.4%) *vs* controls (4.7%). In the UC group, the risk allele frequency of 4.1% was almost identical with the frequency detected in the controls. The frequencies of the other two *NOD2* variants: Arg702Trp and Gly908Arg were low in both controls and IBD patients groups and were not statistically significant.

The allele frequencies distribution for the *IL23R* and *ATG16L1* disease associated variants were almost identical between cases and controls and did not demonstrate significant differences.

DISCUSSION

This is the first report on the prevalence of the previously defined *NOD2*, *ATG16L1* and *IL23R* disease associated variants in an IBD case-control sample from Lithuania. Baltic countries still observe low IBD incidence rates, especially for CD in their populations. In Estonia (1993-1998) the incidence rate of CD was reported to be 1.4 per 100 000 inhabitants[16]; and in Lithuania (2006) - 2.0 per 100 000 inhabitants[9,10]. Therefore, analysis of the genetic contribution to disease susceptibility in this region was of great interest.

Since 2001, following the identification of *NOD2* as the first gene conferring susceptibility to CD[3-5] a significant number of studies have replicated the association of the Arg702Trp, Gly908Arg and Leu1007insC variants with the development of CD in populations of Caucasian origin from Europe and North America[17]. However, significant heterogeneity in the frequencies of these variants has been observed not only between ethnically divergent populations[18,19], but also within Europe[17].

Our study results add to this pattern. The carriage of at least one *NOD2* variant was highest in the CD patients group (41.1%) compared to the control group (16.9%) and resulted in the OR = 3.48 (95% CI: 1.81-6.72). These data are in concordance with previously reported rates of 30%-50% in CD and 7%-20% in controls from other European regions[17]. The Leu1007insC variant was responsible for the major contribution of *NOD2* to disease susceptibility in the Lithuanian CD population (MAF = 21.4%, OR = 5.54, 95% CI: 2.89-10.75). These data are consistent with previous reports from Central Europe and North America (MAF = 6.6%-16%)[17] and contrast markedly with studies performed in Northern Europe, where carriage rates of Leu1007insC and other *NOD2* variants are relatively low, i.e. the carriage of at least one *NOD2* variant varies from 2.8% to 22%[20,21]. However, we were not able to confirm the association between Arg702Trp, Gly908Arg and IBD susceptibility in our study group. These findings are in contrast with previous reports from Southern and Central European populations, where a positive association between Arg702Trp, Gly908Arg and CD was detected. The reported alleles frequency rates in these European countries vary from 6.7% to 12.5% for Arg-702Trp, from 3.3% to 6.1% for Gly908Arg, respectively, in CD patients and from 3.5% to 6.9% and from 0.6% to 3.0%, respectively, in controls[17].

Moreover, the PAR%, an indication of the contribution of a mutation to the disease in a specific area, was 29.5% in the present study and contrasts with the other Northern European populations reporting lowest PAR% (range: 1.88%-11%)[20,21]. The PAR% measured in the Central European populations and North America was around 30%[3,5,17]. Therefore, the results of our study indicate that CD in Lithuania has a strong genetic background that is related partially to *NOD2* susceptibility variants. Interestingly, the relatively high carriership frequency of any of the three *NOD2* alleles in the healthy controls (16.9%) in our study is in contrast with data of low CD incidence in Lithuania[9]. This indicates the importance of environmental factors (e.g. diet, lifestyle) in disease development.

The first two genome wide association studies identified genetic alterations within *IL23R*[6] a component of the adaptive immune system - and *ATG16L1*[7] - a protein involved in autophagic processes - to be associated with IBD and CD. These findings broadened the understanding of the different pathways that are involved in IBD susceptibility and/or pathogenesis. In addition, the *IL23R* and *ATG16L1* findings were confirmed in large independent Caucasian samples[22-34]. A study in Japan[35] failed to replicate these results, supporting the previously reported distinct ethnic difference of the genetic background of CD. Upon analysis of the Lithuanian IBD population we were not able to confirm any of these findings. We were just able to observe trends for possible associations with *ATG16L1* risk allele. The frequencies and contributable risk of the *ATG16L1* G allele reported in our study (55% CD and 48% controls, OR: 1.32) were similar to the published studies performed in Germany (59% CD and 52% controls, OR: 1.35)[22], UK (57% CD and 51% controls, OR: 1.30)[23], Hungary (58% CD and 50% controls, OR: 1.39)[26] and pooled study of German, Dutch and Hungarian cohorts (57% CD and 51% controls, OR: 1.32)[24]. The allele frequency distribution of the protective *IL23R* variant in our control samples (5.9%) was similar to previous reports in Caucasian populations (approx. 6%), whereas the allele frequencies in our both IBD cases groups were higher (3.6% CD and 4.5% UC) compared to the results of other studies (1.87%-2.85% CD and 1.9%-2.68% UC)[26-34].

It must be noted that our relatively small study population was underpowered to demonstrate such weak to moderate disease associations. The panel had a power of 80% to detect an odds ratio of 1.8 or higher at the 5% significance level, assuming a frequency of the disease-associated allele of at least 30% in the controls. Therefore, larger-sized case-control panels will be needed in order to further evaluate the importance of the herein tested loci.

In summary, our study provides clear evidence that the *NOD2* Leu1007insC variant increases susceptibility to CD in the Lithuanian study population, whereas associations of *IL23R* and *ATG16L1* variants with any of the distinct IBD subtypes need to be further evaluated in larger North-Eastern European IBD sample collections.

ACKNOWLEDGMENTS

The authors wish to thank all patients, families and physicians for their cooperation. We also thank the technical staff at the Institute for Clinical Molecular Biology (Kiel, Germany) for their expert help. The popgen biobank received infrastructure support through the DFG excellence cluster "Inflammation at Interfaces".

COMMENTS

Background

Numerous genome-wide and linkage studies have identified and replicated significant association between inflammatory bowel disease (IBD) development and polymorphisms of genes attributed to recognition of bacterial products (*CARD15*), adaptive immune responses (*IL23R*), and autophagosome pathways (*ATG16L1*). However, there has been reported a heterogeneity in allele frequencies of genetic factors involved in the pathogenesis of IBD in different European populations. The genetic association with IBD susceptibility has never been investigated in Lithuania previously.

Research frontiers

The research was performed to obtain data about the frequency of *NOD2, IL23R* and *ATG16L1* genetic variants in a case-control study group for IBD from Lithuania.

Innovations and breakthroughs

The results of the authors' study indicate that Crohn's disease (CD) in Lithuania has a strong genetic background that relates partially to *NOD2* susceptibility variants, especially Leu1007insC. The relatively high carriership frequency of any of the three *NOD2* alleles in the healthy controls (16.9%) in this study is in contrast with the data of low CD incidence in Lithuania. This indicates the importance of environmental factors (e.g. diet, lifestyle) in disease development.

Applications

This is one of the first studies investigating the genetic association with IBD in a North-Eastern European country. The results of this study confirm that the heterogeneity of variants might be observed within Europe and will further help to understand the role of interplay between genetic and environmental factors in the development of complex diseases. Future studies in larger study groups

and further analysis of the biological functions of the identified variants are required to understand their role in determining the risk of CD and ulcerative colitis in ethnically divergent populations.

Terminology

NOD2 is a member of the NACHT-LRR receptor (NLR) protein family, which is known to be involved in recognition of microbial structures. *ATG16L1* encodes a protein which is part of a larger family of proteins that are required for the intracellular degradation system - autophagy process. *IL23R* encodes a protein which is a subunit of the receptor for IL23A/IL23 and participates in JAK-STAT3 signaling pathway.

Peer review

The authors concluded that the *NOD2* Leu1007insC variant increases susceptibility to CD in the Lithuanian study population, whereas associations of *IL23R* and *ATG16L1* variants with any of the distinct IBD subtypes need to be further evaluated in larger Eastern European IBD sample collections. The study was conducted with good design and convincing analysis, and the manuscript has been well written and solid conclusions have been drawn.

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